PROJECT TITLE: ROLE OF GLUCOSE, ACETATE AND PLASMA IN THE MAINTENANCE OF MITOCHONDRIAL FUNCTION, ENERGY METABOLISM AND CELL INTEGRITY DURING PLATELET STORAGE IN ADDITIVE SOLUTIONS

BY

CHRISTINE V. SAUNDERS

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Cardiff University School of Medicine
Cochrane Medical Education Centre
Heath Park
Cardiff CF14 4YU

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JOURNAL PUBLICATIONS (Reproduced at end of thesis)

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TABLE OF ABBREVIATIONS

12*S*-HETE	12*S*- hydroxyeicosatetraenoic acid
AC	Adenylyl cyclase
ACD	Acid citrate dextrose
ACE	Accumulated centrifugal effect
ADP	Adenosine diphosphate
AEC	Adenylate energy charge
AM	Acetoxymethyl
AMP	Adenosine monophosphate
Apaf-1	Apoptotic protease-activating factor 1
AS	Additive solution
ATP	Adenosine triphosphate
β-TG	β-thromboglobulin
BC-PC	Buffy coat-derived platelet concentrate
BEST	Biomedical Excellence for Safer Transfusion
BPL	Bio Products Laboratory
BSA	Bovine serum albumin
CalDAG-GEFI	Ca^{2+} and diacylglycerol regulated guanine nucleotide exchange factor 1
cAMP	cyclic AMP
CARD	Caspase recruitment-domain
CAT	Calibrated automated thrombography
CCCP	carbonyl cyanide 3-chlorophenylhydrazone
CCI	Corrected count increment
CE	Council of Europe
CPD	Citrate phosphate dextrose
DAG	Diacylglycerol
DCFH-DA	2′-7′-dichlorodihydrofluoroscein diacetate
DEHP	Di-2-ethylhexyl phthalate
DISC	Death-inducing signalling complex
DMPE	di-14:0-phosphatidylethanolamine

DMS	Demarcation membrane system
DMSO	Dimethyl sulphoxide
DTS	Dense tubular system
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ESC	Extent of shape change
FADD	Fas-associated death domain
FFA	Free fatty acids
FITC	Fluorescein isothiocyanate
Gla	γ-carboxyglutamic acid
GSH	Reduced glutathione
GSSG	Oxidised glutathione
HPLC	High-pressure liquid chromatography
HSR	Hypotonic shock response
IAP	Inhibitor of apoptosis
IMS	Intermembrane space
IP_3	Inositol 1,4,5-trisphosphate
IP_3R	Inositol 1,4,5-trisphosphate receptor
LDH	Lactate dehydrogenase
MFI	Mean/median fluorescence intensity
$\Delta\Psi m$	Mitochondrial membrane potential
MOMP	Mitochondrial outer membrane permeabilisation
MPV	Mean platelet volume
MS	Mass spectrometry
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NHTR	Non-haemolytic transfusion reaction
NTA	Nanoparticle tracking analysis
OCR	Oxygen consumption rate
OCS	Open canalicular system
PAR	Protease-activated receptor
PAS	Platelet additive solution
PBS	Phosphate buffered saline

PC	Platelet concentrate
PE	Phosphatidylethanolamine
PE	Phycoerythrin
PEP	Phosphoenolpyruvate
PF4	Platelet factor 4
PI3K	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PPP	Platelet poor plasma
PRP	Platelet-rich plasma
PS	Phosphatidylserine
PSGL-1	P-selectin glycoprotein ligand 1
PSL	Platelet storage lesion
PTP	Permeability transition pore
PVC	Polyvinyl chloride
R/G	Red/green (fluorescence ratio)
RIAM	Rap1-interacting adaptor molecule
ROS	Reactive oxygen species
SAS	Standard additive solution
sCD62P	Soluble CD62P
SOCE	Store-operated calcium entry
SOCE	Store-operated calcium entry
SOD	Superoxide dismutase
STIM1	Stromal interaction molecule 1
TCA cycle	Tricarboxylic acid cycle
TF	Tissue factor
TMEM16F	Transmembrane protein 16F
TMRM	tetramethylrhodamine methyl ester
TxA_2	Thromboxane A_2
UHW	University Hospital of Wales
VASP	Vasodilator-stimulated phosphoprotein
vWF	von Willebrand factor
vWFR	von Willebrand factor receptor
WBS	Welsh Blood Service

SUMMARY

PROJECT TITLE: ROLE OF GLUCOSE, ACETATE AND PLASMA IN THE MAINTENANCE OF MITOCHONDRIAL FUNCTION, ENERGY METABOLISM AND CELL INTEGRITY DURING PLATELET STORAGE IN ADDITIVE SOLUTIONS

A potential benefit of the use of artificial media for the suspension of platelets as concentrates is a reduction of the morphological, functional and metabolic changes observed in platelets during storage and collectively referred to as the platelet storage lesion (PSL). A better understanding of the nature of the PSL may suggest strategies for manipulation of the storage environment to improve platelet viability and efficacy post-transfusion. In this context, two principal considerations formed the basis for the study:

- The hypothesis that apoptosis is a central mechanism responsible for the changes observed in the PSL.
- The investigation of this hypothesis within the applied setting of improving the storage environment of platelet concentrates.

The study investigated the role on the PSL of plasma protein (in the form of albumin), acetate and glucose in leucoreduced platelet concentrates suspended in a medium with minimal plasma. A 14-day storage study on platelet concentrates in either plasma or a 70:30 ratio of a commercial additive solution (SSP+™) and plasma provided an overview of platelet *in vitro* characteristics under standard storage conditions. The work led to targeted investigations into the nature of the cell death mechanism in platelet concentrates.

Results suggested that in storage media with adequate energy stores, a Bcl-2 protein-mediated mechanism of cell death was viable, though possibly storage-time dependent and limited by pre-existing levels of anti-apoptotic Bcl-2 proteins in the platelets. Further studies would be required to determine if this mechanism is akin to caspase-dependent apoptosis. In media lacking glucose, a mechanism more reminiscent of necrosis was observed, associated with decreased ATP levels, accelerated mitochondrial dysfunction, elevated intracellular free calcium and culminating in platelet disruption.

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1. INTRODUCTION

Platelets are the smallest of the cellular elements in the circulation. Their role in the maintenance of haemostasis has been recognised since the late 19th century (Coller, 2007). More recently, their involvement in inflammation and white cell recruitment has been identified (for recent reviews see Morrell et al., 2007, Li et al., 2011, Semple et al., 2011). To place the thesis in context, this introduction begins by providing an abridged description of platelet structure, formation and function in the normal individual. The role of platelets in transfusion medicine forms the second section of the introduction; including current methods of platelet concentrate (PC) preparation and the challenges faced by transfusion centres in ensuring adequate stocks of this component are maintained. The interest in the use of additive solutions for platelet storage will be introduced at this stage, in concert with the changes undergone by platelets during storage – collectively referred to as the platelet storage lesion (PSL). An overview of the apoptotic process is included to provide a foundation for subsequent discussion on the central hypothesis of the thesis, which states that apoptosis has a principal role in the development of the progressive changes observed in platelets during storage.

STRUCTURE OF THE RESTING PLATELET

Platelets are anucleate, discoid cells with a diameter ranging between 2 to 5 µm and a mean cell volume between 6 - 10 fL (White, 2007). The cytoskeleton of the platelet is comprised of tubulin polymers as well as spectrin tetramers interconnected by long actin filaments. Spectrin molecules form a dense membrane skeleton adjacent to the cytoplasmic side of the plasma membrane (Fox et al., 1988) (figure 1.1). Spectrin self-assembles into twisted strands 200 nm in length that form heterodimers of αβ subunits that associate in a head to tail alignment to form tetramers interconnected at their termini by the barbed ends of actin filaments capped by adducing proteins (Barkalow et al., 2003).

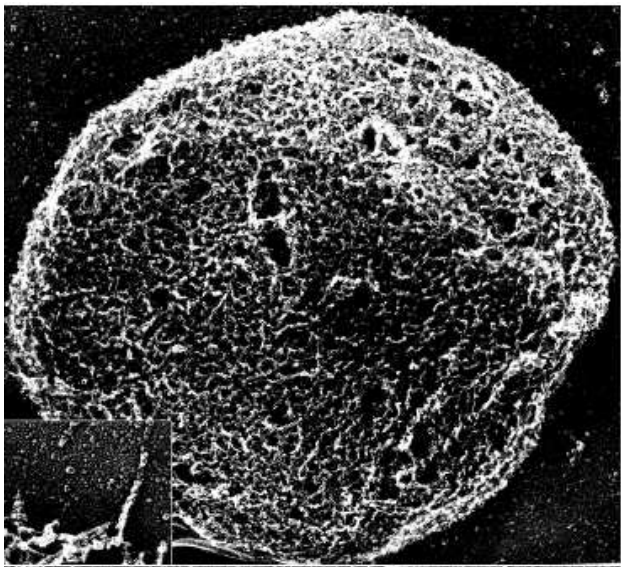


Figure 1.1: Membrane skeleton of the resting platelet with holes to the open canalicular system also evident (see text) (Hartwig, 2006) (Reproduced with permission, © Elsevier Inc)

Actin comprises the most abundant protein in platelets, with 30 – 40% of the approximately 2 million copies in the platelet forming linear polymers (F-actin) cross-linked by other proteins, such as filamin and α-actinin, to form a stable, supporting structure (Fox, 1993). Actin filaments extend from the space-filling network in the centre of the platelet towards the periphery, where their orientation changes to allow the filaments to run parallel to the membrane and bind to the spectrin membrane skeleton via adducin links (Hartwig and DeSisto, 1991, Hartwig, 2006). Of particular interest is the binding of filamin to the cytoplasmic tail of the GP1bα subunit of the von Willebrand factor receptor (vWFR) on the platelet surface, a linkage that emerges between the dense meshwork of spectrin strands and helps to constrain movement in the latter (Hartwig, 2007). The high concentration of vWFR on the platelet surface (25000 copies per platelet) also aids the stability of the surface skeleton with respect to the underlying actin framework (Hartwig et al., 1999). Patients with Bernard-Soulier syndrome lack this filamin-GPIbα linkage and present with abnormally large and fragile platelets (Nurden, 2007). The discoid shape of the resting platelet is principally maintained by a coiled microtubule positioned adjacent to the plasma membrane. Observations suggest it is a single contiguous structure approximately 100 µm in length and formed into 8 to 12 coils to fit within the circumference of the platelet (figure 1.2).

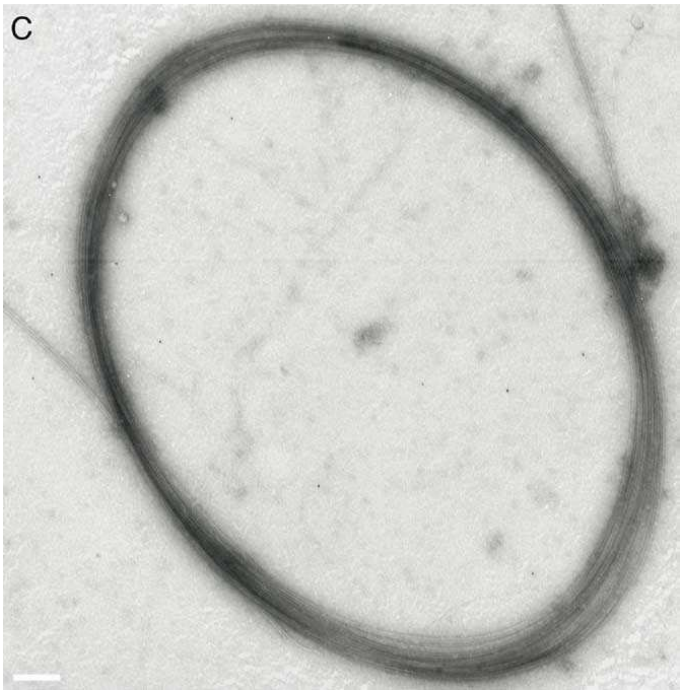


Figure 1.2: Microtubule ring of resting platelet (scale bar equals 0.1µm) (Hartwig, 2006)(Reproduced with permission, © Elsevier Inc)

The platelet exterior is demarcated by a relatively thick glycocalyx comprising glycoproteins intimately involved in platelet adhesion, activation and aggregation. The surface appearance of platelets under scanning electron microscopy appears fairly convoluted, with openings to the open canalicular system (OCS) appearing as small pits. The latter is morphologically similar to the plasma membrane and forms an internal continuation of this membrane. The actin filaments not associated with the cytoskeletal framework described above form a cytoplasmic skeleton which provides a scaffold for the suspension of intracellular organelles.

Platelets contain two main types of secretory organelles, termed α-granules and dense granules. Approximately 40 to 80 α-granules with an approximate diameter of 500 nm are present in normal platelets, containing a variety of proteins including vonWillebrand factor (vWF), CD62P (P-selectin), coagulation factors V and VIII, fibrinogen, integrins $\alpha_{IIb}\beta_3$ and $\alpha_5\beta_3$, and various chemokines such as β-thromboglobulin (β-TG), RANTES and platelet factor 4 (PF4) (Reed, 2007, Blair and Flaumenhaft, 2009). Normally separate from each other, the fusion of α-granules in a proportion of the platelet population may be one of the earliest morphological indications of a storage lesion (White and Clawson, 1980). Dense granules are smaller and fewer in number, with only

4 to 8 normally present per platelet. They are known to contain high concentrations of adenine nucleotides (including ATP and ADP), serotonin, pyrophosphate, calcium and magnesium (McNicol and Israels, 1999). In addition, platelets contain a minimal number of lysosomes, though their function in platelets is not yet clear. Numerous particles of glycogen are also present in the platelet cytoplasm, often in dense concentrations, though again their function in the platelet remains to be fully determined (White, 2007). Mitochondria in platelets are relatively few in number, but are important in maintaining the energy requirements of the cell as well as having an increasingly recognised role in the cell's "decision" to proceed to clearance through a mechanism of programmed cell death.

Platelets contain a complex and characteristic network of internal membranes comprised of two distinct systems not found in other blood cells. The OCS is derived from invaginations of the plasma membrane and greatly enhances the surface area exposed to the external environment (Escolar et al., 1989). It can serve as a major conduit for the uptake of compounds from the external environment as well as the rapid expulsion of substances from secretory organelles following platelet activation. The OCS also provides a source of conveniently accessed membrane required during the formation of structures such as pseudopodia following platelet adhesion to an injured area (White, 2007). The dense tubular system (DTS) is comprised of residual smooth endoplasmic reticulum (ER) originating from rough ER in the megakaryocyte. It is involved in the synthesis of prostaglandins and thromboxane A_2 and is believed to comprise the principal store of intracellular calcium in the platelet (Ebbeling et al., 1992).

PLATELET FORMATION

Platelets originate from megakaryocyte precursor cells, which are in turn derived from myeloid progenitor cells. Uniquely among haemopoietic precursor cells, megakaryocytes undergo successive cycles of DNA replication without dividing; a process referred to as endomitosis and constituting cycles of interrupted mitosis. Up to six cycles of DNA replication have been observed, though a final diploid number of 16N is more common (Italiano and Hartwig, 2007). Following endomitosis, the megakaryocyte undergoes a process of maturation in the bone marrow which includes

the formation of the secretory granules and the development of the complex demarcation membrane system (DMS) characteristic of the mature megakaryocyte (Schulze et al., 2006). Both α-granules and dense granules mature within the megakaryocyte, possibly via multivesicular bodies which form an intermediate stage and may serve to sequester the characteristic proteins and compounds into their appropriate secretory body. Some of the proteins associated with α-granules are synthesised endogenously, including β-thromboglobulin and vonWillebrand factor. Others, such as fibrinogen, are subsequently obtained from the megakaryocyte environment via receptor-mediated endocytosis (Handagama et al., 1987). Additional proteins important for platelet function are also enclosed within granules at this stage. The DMS is an extensive network that dominates the cytoplasm of the mature megakaryocyte. Its most likely role is thought to be the provision of membrane during the platelet production process (Mahaut-Smith et al., 2003, Italiano and Hartwig, 2007). Approximately 10^{11} platelets are produced daily, based on each megakaryocyte able to generate 10^3 platelets and a rate of megakaryocyte generation of 10^8 per day (Hartwig, 2003).

There have been a number of models posited to explain platelet formation. The currently favoured model involves the formation of proplatelets – long, cytoplasmic extensions of the megakaryocyte characterised by terminal swellings approximately the same size of mature platelets (Becker and De Bruyn, 1976, Radley and Haller, 1982). The formation of proplatelets involves the initial spreading of the megakaryocyte followed by the dissociation of the cortical cytoplasm at one end of the cell. The breakdown of the megakaryocyte proceeds from this point, with the cytoplasmic material increasingly condensed into pseudopodia which further develop into thin cytoplasmic connections only 2 to 4 nm in diameter, tipped with the swollen proplatelets. The process ends approximately 10 hours after initiation with a rapid "retraction" of the cytoplasmic connections that frees the proplatelets and discards the remnants of the megakaryocyte nucleus (Italiano et al., 1999, Bluteau et al., 2009) (figure 1.3). Techniques allowing proplatelet formation in mice to be studied as a dynamic process *in vivo* suggest that megakaryocytes remain in close contact with bone marrow sinusoids, with extensions protruding into microvessels from which fragments resembling proplatelets are released into the vasculature and mature to produce platelets. The authors additionally suggest that the process is likely to be aided by blood

flow-induced shear stress (Junt et al., 2007). *In vitro* evidence on isolated proplatelets has provided initial confirmatory evidence that platelet formation is potentiated by shear and may proceed through intermediate stages termed “preplatelets” that may help in regulating platelet size (Thon et al., 2010).

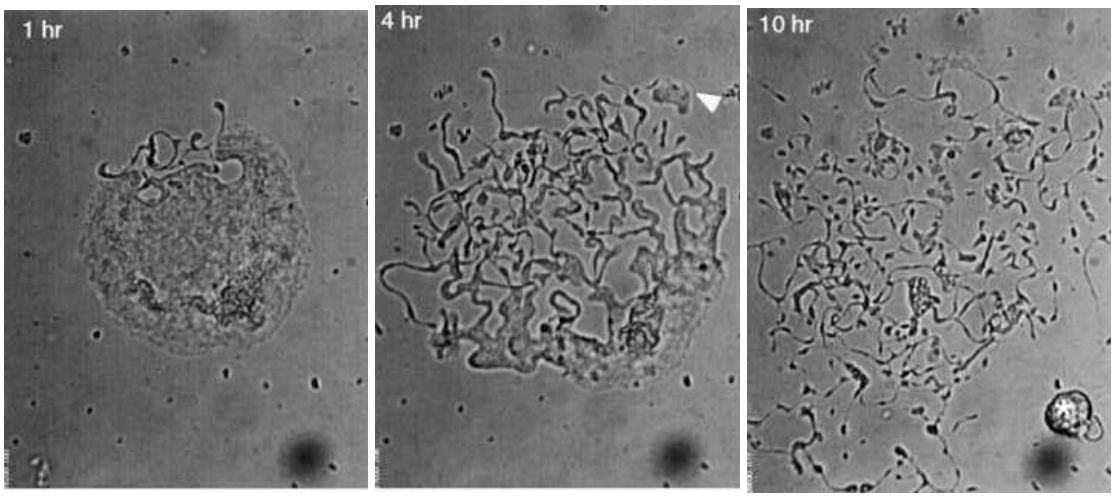


Figure 1.3: Megakaryocyte (murine) forming proplatelets in vitro (Italiano et al., 1999) (Reproduction permitted with accreditation, © The Rockefeller University Press)

The tubulin and actin polymers involved in shape maintenance in the mature discoid platelet also play a vital role in the transformation of megakaryocytes into proplatelets (Patel et al., 2005b). Microtubules composed of dimers of αβ-tubulin initially extend from the centre of the immature megakaryocyte to the periphery. With the development of pseudopodia, the microtubules consolidate into bundles below the plasma membrane of these structures and progressively thin within the cytoplasmic connections to form coils composed of 5 to 10 microtubules around the terminal proplatelets, consistent with the microtubule coil noted in mature platelets. Microtubules are also involved in the migration of organelles and granules from the cell body of the megakaryocyte to the proplatelet (Richardson et al., 2005, Italiano and Hartwig, 2007). Figure 1.4 provides a summary of platelet formation based on this favoured model.

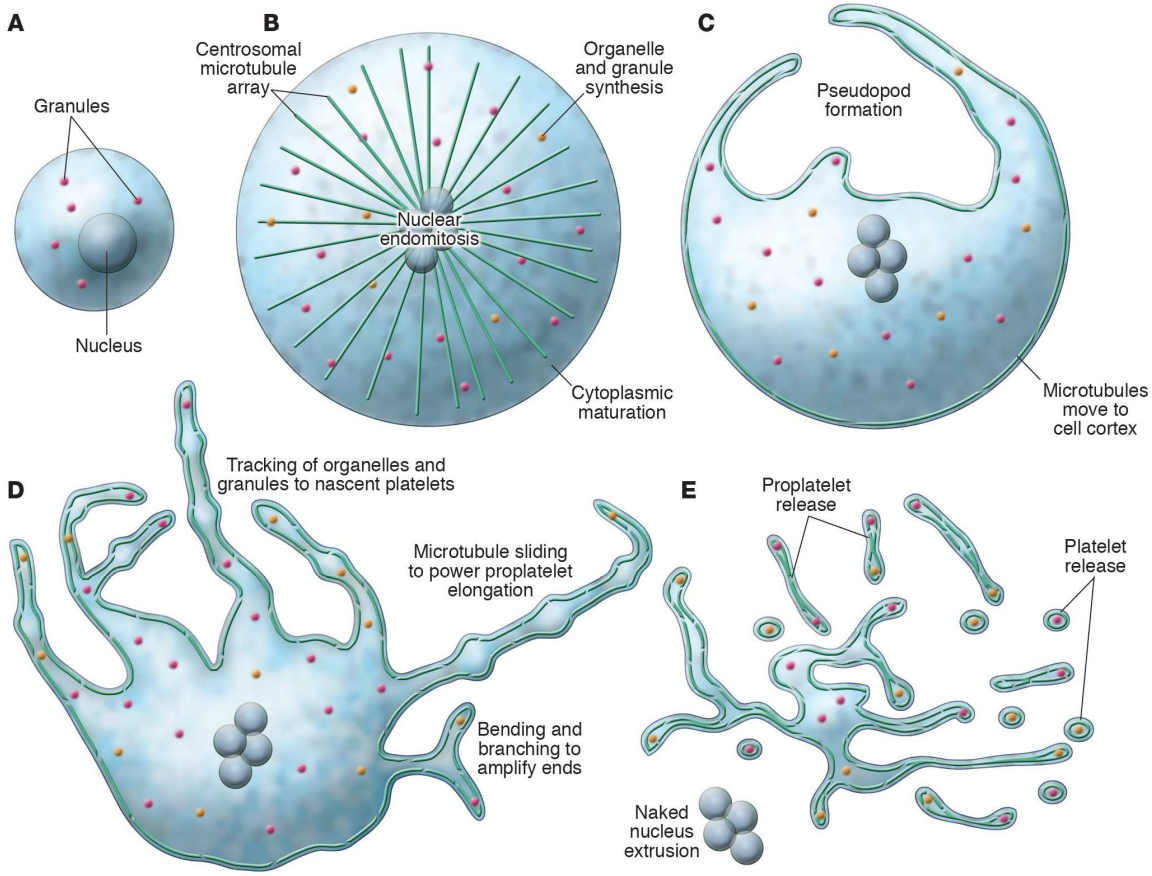


Figure 1.4: Summary of platelet biogenesis from megakaryocytes by the formation of proplatelets (Patel et al., 2005a)(Reproduced with permission, © American Society for Clinical Investigation)

The dissociation of megakaryocytes into proplatelets displays morphological similarities to apoptosis, including disruption of cytoskeletal proteins, DNA fragmentation, condensation of nuclear and plasma membranes, blebbing, cell shrinkage and the formation of fragmentary bodies (Kaluzhny et al., 2002). The comparison is supported by the presence in mature megakaryocytes of caspases – proteases known to be central players in the execution of apoptosis - and the absence of anti-apoptotic members of the Bcl-2 family of proteins, as well as the inhibition of proplatelet formation by the addition of the pan-caspase inhibitor z-VAD-fmk (De Botton et al., 2002, Clarke et al., 2003). Mature platelets reside in the circulation for approximately ten days. In view of this limited lifespan, it is interesting to speculate whether programmed cell death plays a role in the clearance of platelets from the circulation, and whether such a concept can be extended to explain the limited time platelets can currently be stored as concentrates.

APOPTOSIS

Apoptosis was first invoked to describe a morphologically distinct form of cell death originally characterised by chromatin and nuclear condensation followed by fragmentation of the cell into membrane-bound apoptotic bodies able to be removed by phagocytes in a controlled process (Kerr et al., 1972). This was contrasted with the cellular disruption seen with necrosis, leading to the release of intracellular contents into the general environment and the onset of inflammation (Perrotta et al., 2003). Further, apoptosis is generally considered to be an energy-dependent process, with marked reduction in ATP levels having been described as a "switch" promoting the onset of a necrotic cell death (Leist et al., 1997, Eguchi et al., 1997, Nicotera and Melino, 2004). Morphological changes reminiscent to those described in apoptotic cells were noted in platelets exposed to various agonists as well as in platelets stored in culture or as platelet concentrates (reviewed by Leytin and Freedman, 2003). As nuclear involvement was found not to be a requirement for apoptosis to occur, the idea that a similar mechanism may be at least partially responsible for the adverse changes observed in platelets stored as concentrates was suggested (Mignotte and Vayssiere, 1998, Brown et al., 2000, Li et al., 2000).

Central to the description of many of the processes linked to apoptosis is the role of a family of proteases called caspases that are able to specifically cleave proteins after aspartic acid residues, with specificity conferred by the four amino acids on the NH_2-terminal side of the cleavage site (Thornberry and Lazebnik, 1998). Initiator caspases such as caspase-8 and -9 act as mediators and control the activation of effector caspases such as caspase-3, which actually execute the demise of the cell by disrupting cellular structures and inactivating proteins that inhibit apoptosis (Snyder and Kuter, 2000, Taylor et al., 2008). Two principal pathways are currently invoked to describe the progression of apoptosis: the extrinsic pathway (also referred to as the death receptor pathway) and the intrinsic (or mitochondrial) pathway (figure 1.5).

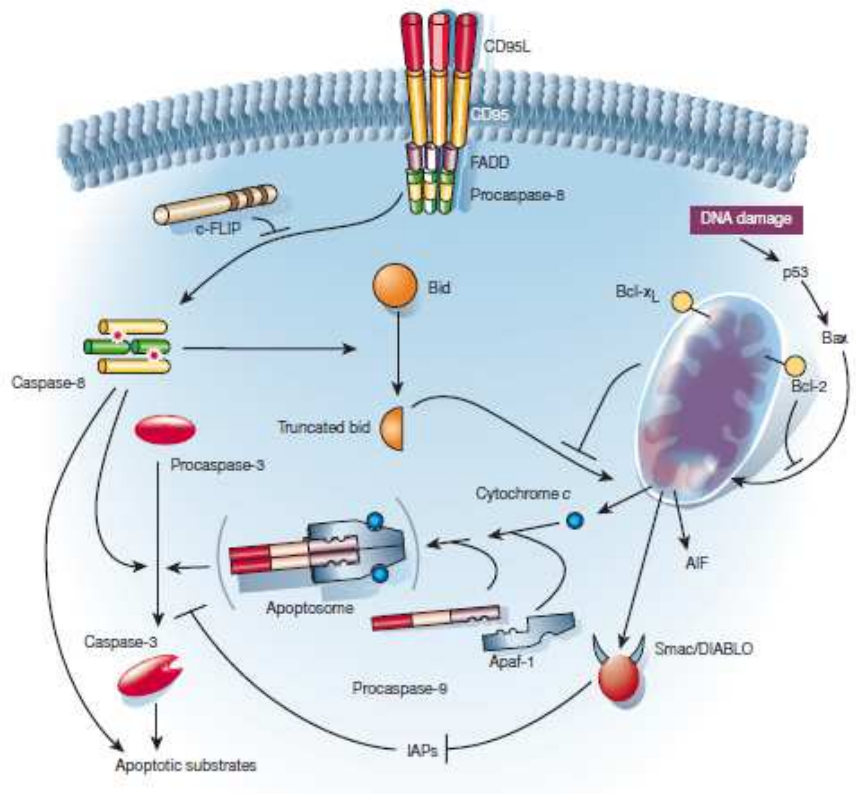


Figure 1.5: Principal components of the extrinsic and intrinsic pathways of apoptosis (see text for details) (Hengartner, 2000) (Reproduced with permission, © Nature Publishing Group)

The extrinsic pathway is initiated by the activation of various death receptors by their associated ligands, including CD95. Activation of these receptors leads to the assembly of the death-inducing signalling complex (DISC) which recruits multiple copies of procaspase-8 to the complex via the Fas-associated death domain protein (FADD). The proximity of the procaspase-8 molecules results in their activation which subsequently leads to the activation of the effector caspases (Green, 2011, Kroemer et al., 2007, Hengartner, 2000, Walczak and Sprick, 2001). Evidence for the involvement of this pathway in platelet death has not been conclusively established (Leytin, 2012).

As the alternative name of the intrinsic pathway implies, mitochondria play a central role, with various intracellular signals converging on the mitochondria and promoting permeabilisation of the mitochondrial membrane. This results in the release of pro-apoptotic factors from the mitochondrial intermembrane space (IMS), including cytochrome c. This constitutes the principal pathway in vertebrates (Green and

Kroemer, 2004) and is the mechanism of apoptosis alluded to and investigated in this thesis by virtue of the selection of assays adopted.

Intrinsic (Mitochondrial) Pathway of Apoptosis

Cytochrome c is a principal component of the mitochondrial respiratory chain and considered a key factor in many models of apoptosis. It is primarily found in association with the phospholipid cardiolipin in the space between the inner and outer mitochondrial membrane, either in a loosely bound conformation mediated by electrostatic interactions or as a tightly bound form. Only the soluble form of cytochrome c is released into the cytosol, requiring first the disassociation of the protein from cardiolipin followed by release into the cytosol (Ott et al., 2002). Once in the cytosol, cytochrome c binds to apoptotic protease activating factor-1 (Apaf-1), promoting its binding to dATP/ATP which in turn leads to the formation of a multimeric complex referred to as the apoptosome (Bao and Shi, 2006) (figure 1.6). Apaf-1 also incorporates a caspase-recruitment domain (CARD) which is exposed following oligomerisation, recruiting procaspase-9 and assisting its autoactivation to caspase-9 (Bratton and Salvesen, 2010). This effector caspase is subsequently able to cleave and activate the zymogen of the executioner caspase, caspase-3 (Zou et al., 1999, Wang, 2001). Concomitant with the release of cytochrome c is the release of compounds such as Smac/DIABLO which interfere with the action of inhibitors of apoptosis (IAPs) – a family of proteins able to inhibit active caspases (Du et al., 2000, Verhagen et al., 2000, Bratton and Salvesen, 2010).

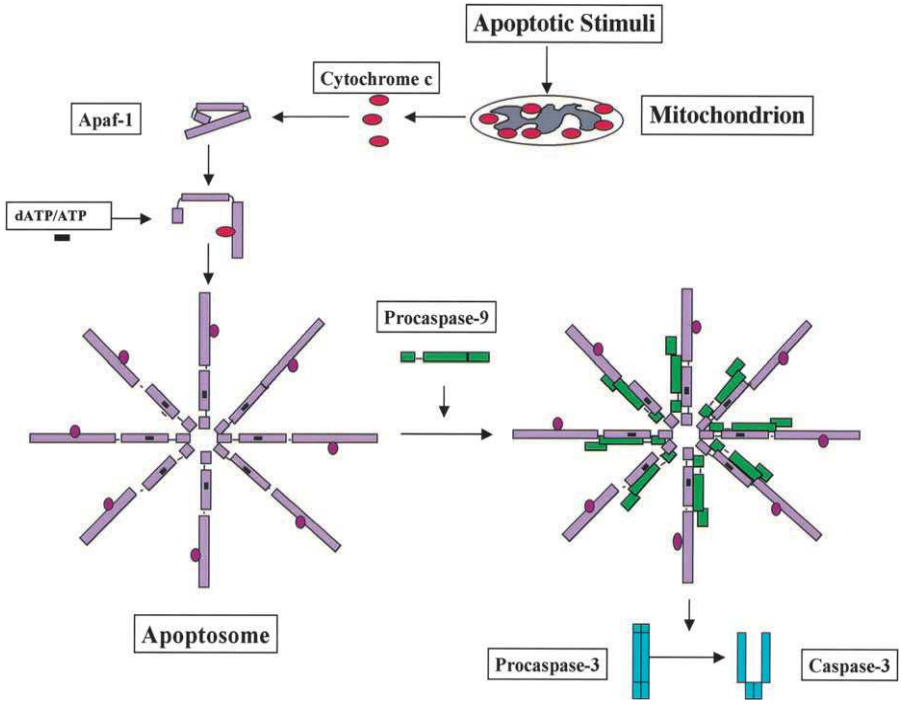


Figure1.6: Activation of caspase-3 by the apoptosome following release of cytochrome c into the cytosolic space (Wang, 2001) (Reproduced with permission, © Cold Spring Harbor Laboratory Press)

Permeabilisation of the outer mitochondrial membrane would seem to be a pre-requirement for the release of pro-apoptotic factors normally residing within these organelles. One suggested control mechanism involves the Bcl-2 family of proteins, which contains both pro- and anti-apoptotic members (Green and Kroemer, 2004). Mitochondrial outer membrane permeabilisation (MOMP) regulated by the pro-apoptotic members Bak or Bax has even been suggested to be a "point of no return" (Dewson and Kluck, 2009). Bak is found in association with the mitochondrial outer membrane in normal cells, whereas Bax translocates to the membrane from the cytosol following activation. Pro-apoptotic Bak or Bax may disrupt the membrane via mitochondrial pores formed by the oligomerisation of the proteins or via disruption of the phospholipid layers (Finkel, 2001, Dewson and Kluck, 2009). Alternatively, it has been suggested that Bax interacts with components of the permeability transition pore (PTP) – a complex of proteins linking the inner and outer mitochondrial membranes. Questions remain over the importance of this structure in apoptosis, though the pseudopathological conditions that promote pore opening such as elevated cytosolic calcium levels, low ATP concentrations and oxidative stress, may point to a role in other forms of cell death (Kroemer and Reed, 2000, Orrenius et al., 2003, Halestrap,

2006, Crompton, 1999, Duchen, 1999). The anti-apoptotic members Bcl-2 and Bcl-X_L block the oligomerisation of Bax and Bak, thus preventing the release of compounds such as cytochrome c. In addition, they may influence mitochondrial physiology more directly by maintaining the $\Delta\Psi m$ and the exchange of adenine nucleotides (Desagher and Martinou, 2000). A third class of BCl-2 family proteins is termed the BH3-only proteins and appear to control the activity of previously mentioned members of the family.

PLATELET FUNCTION

Platelets play a vital role in the formation of a haemostatic plug at sites of vascular injury, thus helping to arrest bleeding and promote wound healing. Two principal processes enable this function; the initial adhesion of platelets to the damaged subendothelium and the subsequent aggregation of platelets to one another to form the primary haemostatic plug (Wei et al., 2009). In addition, activated platelets provide a catalytic surface for the activity of the coagulation factors (Galan et al., 2000), leading to the stabilisation of the primary plug with fibrin.

Platelet Adhesion to the Vessel Wall

The initial interaction of platelets with an injured vessel wall is mediated via platelet receptors to the newly exposed subendothelium. The glycoproteins GPIbα and GPVI have been identified as playing an important role in the initial tethering of the platelet to the injured area. A more stable contact is subsequently accomplished following the activation of the integrins $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ (Brass et al., 2007).

GPIbα and its associated subunit, GPIbβ, form a noncovalent complex with GPIX and GPV, with approximately 25 000 to 50 000 copies of the complex expressed on the surface of the platelet (Kroll et al., 1996). Although GPIbα has been shown to bind to various ligands, its association with the A1 domain of vWF is the best codified in terms of the platelet's role in haemostasis (Wei et al., 2009). vWF is an adhesive, multimeric glycoprotein found in the α-granules of platelets as well as the Weibel-Palade bodies of endothelial cells (Sadler, 2009). The majority of vWF from the endothelial cells is secreted into the extravascular space and binds to collagen exposed on the breached

vessel wall (Ruggeri, 2003, Furie and Furie, 2008). On initial release from the Weibel-Palade bodies, vWF is composed of very large prothrombotic multimers of up to 20000 kDa (Andrews et al., 2007). On release, these ultra-large multimers are cleaved into smaller and less adhesive units by the metalloproteinase ADAMTS-13 (Padilla et al., 2004). vWF is also found in plasma at concentrations of approximately 2×10^{-8} mol/L (Goto et al., 1998), but inappropriate binding of this soluble fraction to the GPIbα expressed on the platelet surface is avoided by the requirement of a stimulus to confer either vWF or its receptor to an active conformation (Andrews et al., 2007, Lenting et al., 2010). The conditions of high shear experienced within arterioles, with shear rates between 1000 to 10000 per second, may provide such a trigger (Jackson et al., 2009). At these high shear rates, the capture of platelets to the vessel wall appears to principally involve the binding of GPIbα to vWF. This initial contact occurs quickly but also has a high dissociation rate and is insufficient by itself to secure the platelets to the site of injury. Instead, the effect is to slow the platelets and promote contact with the vessel wall, with the discoid shape of the platelet resulting in a hesitant sliding action which increases the surface area in contact with the vessel wall (Jackson, 2007). The recent discovery of membrane tethers – long strands of lipid membrane extruded from platelets under high flow conditions - may aid this process by reducing the strain on the primary adhesive bonds (Dopheide et al., 2002) (figure 1.7).

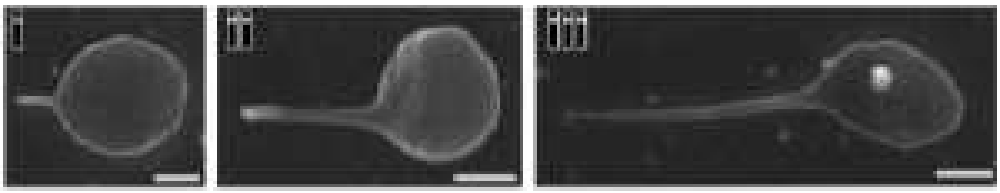


Figure 1.7: Scanning electron micrographs showing the formation of platelet tethers on a surface of immobilized vWF (Scale bar equals 1 μm) (Dopheide et al., 2002) (Reproduced with permission, © The American Society of Hematology)

GP1b/IX/V is also able to bind to collagen indirectly via the A3 domain of vWF. However, there is increasing evidence that the principal platelet receptor for collagen is GPVI and the initial translocation phase may aid in promoting the binding of GPVI receptors on the platelet surface to exposed fibrillar collagen on the damaged endothelium. Although the affinity of this interaction is again relatively weak, it serves to activate and re-configure the integrins $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ to a high-affinity state, with

GPIb/IX/V and GPVI possibly functioning synergistically in this regard (Nieswandt and Watson, 2003, Andrews et al., 2007). The integrin $\alpha_2\beta_1$ (also referred to as GPIa/IIa) was the first collagen receptor identified (Santoro, 1986) and is currently ascribed a supporting role in platelet adhesion by reinforcing various "outside-in" signalling events (Varga-Szabo et al., 2008).

Platelet Aggregation

The release of soluble agonists from initially activated platelets following adhesion to the exposed endothelium serves to amplify the activation response through the binding of ADP, thromboxane A_2 and thrombin to their respective G-protein coupled receptors, serving to maintain $\alpha_{IIb}\beta_3$ in a high-affinity configuration (Wei et al., 2009). Associated with the binding of platelet receptors to their respective ligands on the subendothelium are transient increases in the concentration of cytoplasmic calcium; a potent second messenger in a variety of platelet interactions. Binding of GPIbα to VWF results in transient elevations of calcium unrelated to extracellular levels of the ion (Nesbitt et al., 2002) and enhanced by the binding of ADP to its $P2Y_1$ receptor, leading to platelet shape change and activation of the integrin $\alpha_{II\beta}\beta_3$ (Jin et al., 1998). Binding of ADP to its second P2-type receptor – $P2Y_{12}$ – is also believed to contribute to $\alpha_{II\beta}\beta_3$ activation (Leon et al., 2004). The more stable binding associated with integrin $\alpha_{IIb}\beta_3$ to vWF and consequent thrombus formation results in more prolonged increases in intracellular free calcium levels (Savage and Ruggeri, 2007).

The integrin $\alpha_{IIb}\beta_3$ (also known as GPIIb/IIIa) is critically involved in the formation of platelet-platelet aggregation and clot formation. It is the most abundant glycoprotein on the platelet surface, with approximately 60 000 to 80 000 copies per platelet (Wagner et al., 1996). In addition, there is an intracellular pool of the integrin located in the α-granules available to enhance surface expression following activation (Shattil et al., 1998, Wagner et al., 1996). The high-affinity conformation of $\alpha_{IIb}\beta_3$ is able to bind to various ligands. Binding to fibrinogen and vWF directly promote platelet aggregation, whilst binding to fibrin, fibronectin, thrombospondin and vitronectin may help to mediate platelet adhesion and aggregation (Plow et al., 2007). The interaction between the integrin $\alpha_{IIb}\beta_3$ and fibrinogen has been found to be the dominant mechanism for

platelet aggregation at lower shear rates below 1000 s^{-1}, characteristic of veins and the larger vessels of the arterial circulation. Free-flowing platelets are able to bind directly to fibrinogen on the surface of damaged vessels under these conditions with the previously described binding of GPIbα to VWF playing a lesser role (Jackson, 2007). Binding of activated platelets to one another via fibrinogen and its receptors forms stable contacts that promote thrombus growth (Goto et al., 1998) (figure 1.8).

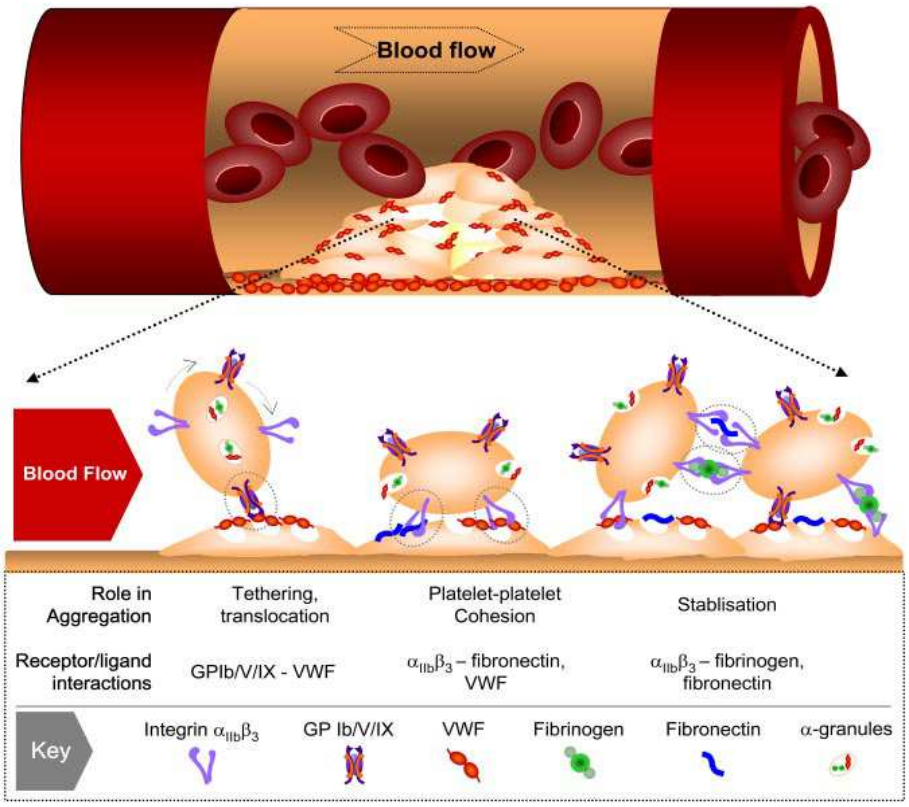


Figure 1.8: Receptor-ligand interactions between platelets and the endothelial surface under conditions of high shear, leading to initial tethering and translocation of the platelets prior to platelet-platelet interactions and stabilisation of the platelet plug (Jackson, 2007) (Reproduced with permission, © The American Society of Hematology)

Although the binding of GPIbα to vWF and of integrin $\alpha_{IIb}\beta_3$ to fibrinogen have been found to dominate at different conditions of shear flow in perfusion experiments, the formation of a thrombus *in vivo* will likely result in microenvironments that will require the various mechanisms that promote platelet adhesion and aggregation to operate synergistically to create a stable haemostatic plug. This is supported by experiments on knockout mice which have shown that both vWF and fibrinogen are required for stable thrombus growth (Ni et al., 2000).

Platelet Involvement in the Coagulation Response

The creation of a haemostatic plug serves to localise the subsequent coagulation response at the site of injury. Spatial confinement of the reactions involved in coagulation would aid the activity of the various enzymes and enhance the formation of a stable fibrin clot. Anionic phospholipids, principally phosphatidylserine (PS), on the platelet surface are believed to serve this purpose (Bevers et al., 1982, Krailadsiri et al., 2001). PS serves to anchor coagulation proteins containing domains rich in γ-carboxyglutamic acid (Gla) residues formed by carboxylation of glutamic acids in a vitamin K-dependent manner (Vermeer, 1990, Lemmon, 2008). The Gla domain is present in prothrombin as well as factors VII, IX and X (Zwaal et al., 1998). Binding to membrane-bound PS requires Ca^{2+} ions which are thought to coordinate the required structural modification of the Gla domain as well as anchoring the protein to the bound lipid (Huang et al., 2003, Ohkubo and Tajkhorshid, 2008).

The distribution of phospholipids on the platelet membrane of the resting platelet is asymmetric, with cholinephospholipids (phosphatidylcholine and sphingomyelin) preferentially expressed on the outer leaflet of the plasma membrane and aminiphospholipids, principally phosphatidylserine and phosphatidylethanolamine (PE), mostly confined to the inner leaflet (Vance, 2008). The maintenance of this asymmetry is thought to be dependent on the activity of two ATP-dependent processes. The dominant of the two processes involves the transport of PS and PE from the outer to the inner leaflet by a translocase specific to the transport of aminophospholipids, with one molecule of ATP estimated to be hydrolysed per phospholipid transported (Beleznay et al., 1993). A less-specific ‘floppase’ transports both choline- and aminophospholipids from the inner to the outer leaflet. As the half-times for floppase activity are on the order of 1.5 hours compared with approximately 5 – 10 minutes for the aminophospholipid translocase, the cumulative result is an asymmetric distribution of PS and PE (Connor et al., 1992). ATP depletion or the inactivation of the aminophospholipid translocase is not sufficient to substantially disrupt lipid asymmetry in isolation due to the slow transbilayer movement of lipids (Bevers and Williamson, 2010). Rapid loss of asymmetry occurs through the activity of a bi-directional lipid scramblase, which requires sustained micromolar concentrations of intracellular calcium evident as a result of platelet activation or programmed cell death (Bevers et al., 1995).

Unlike the translocase and floppase, the function of the scramblase is not energy-dependent, though prolonged ATP depletion may decrease its activity (Martin et al., 1995). Interestingly, the increase in intracellular calcium also inhibits the activity of the aminophospholipid translocase, thus ensuring that the loss of asymmetry is not corrected (Bevers et al., 1999) (figure 1.9).

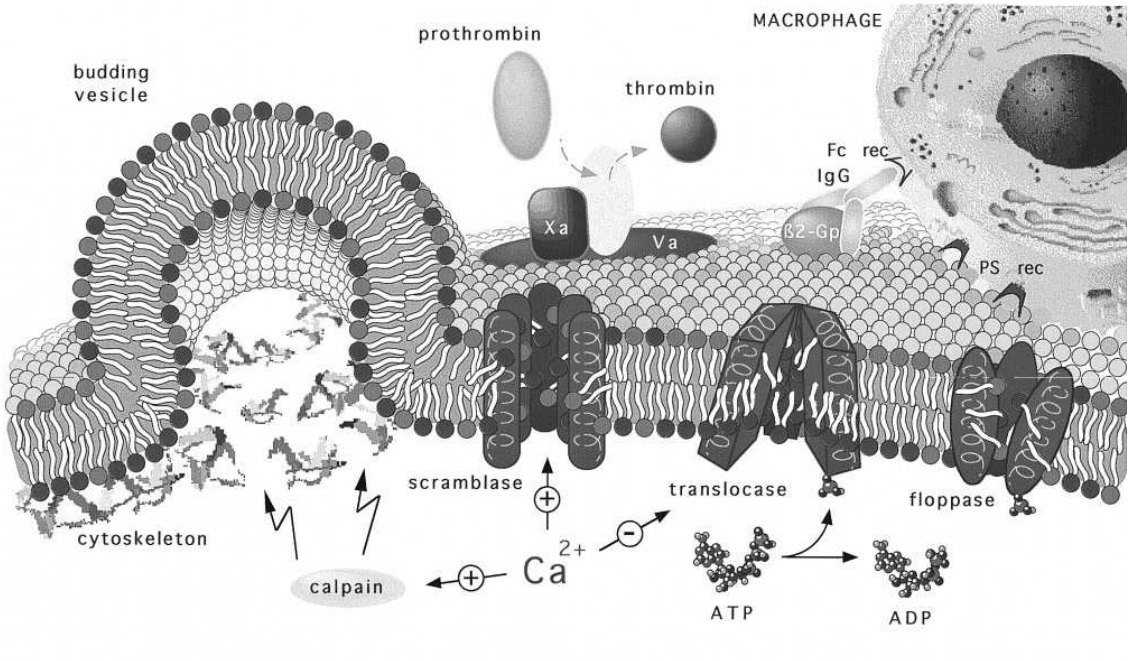


Figure 1.9: Model of mechanisms involved in membrane phospholipid asymmetry. Also alluded to is the importance of PS in promoting coagulation and clearance of apoptotic cells by phagocytes. (Zwaal and Schroit, 1997) (Reproduced with permission, © The American Society of Hematology)

Briefly, current understanding posits that the coagulation response is initiated when injury to the vessel wall exposes subendothelial tissue factor (TF) (Weiss et al., 1989). Circulating factor VIIa binds to the exposed TF forming a complex that proceeds to activate factors IX and X to their active forms (Mackman, 2009). The factor Xa formed by this process combines with factor Va on the exposed endothelium to generate a limited quantity of thrombin. It serves to activate factor VIII by dissociating it from circulating vWF and allowing it to form a complex with FIXa on the surface of activated platelets. This tenase complex further recruits factor Xa and promotes its association with factor Va to form a prothrombinase complex that stimulates a burst of thrombin formation from its prothrombinase precursor (Monroe and Hoffman, 2006, Gale, 2011) (figure 1.10).

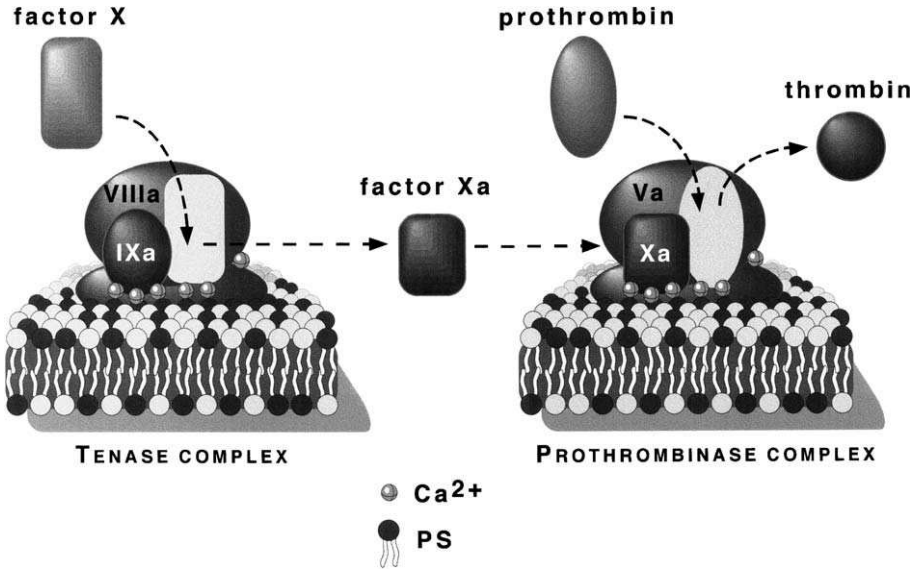


Figure 1.10: Schematic of tenase and prothrombinase complexes in association with membrane phospholipids (Zwaal et al., 2004) (Reproduced with permission, © Elsevier B.V.)

Thrombin is a potent platelet agonist which promotes further platelet activation via G-protein-coupled protease-activated receptors (PARs) on the platelet surface (Jardin et al., 2007, Jackson et al., 2003). PAR-1 and PAR-4 are both specifically cleaved by thrombin, with the suggestion that PAR-1 mediates platelet activation by low amounts of thrombin while more sustained activation involves PAR-4 and requires higher concentrations of thrombin (Kahn et al., 1999). Agonist-stimulated platelet activation is initiated by hydrolysis of phosphatidylinositiol 4,5-bisphosphate (PIP_2) by phospholipase C into the second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). As well as accelerating platelet activation, thrombin is responsible for stabilising the haemostatic plug by converting fibrinogen to fibrin (Furie and Furie, 2008, Mann et al., 2003). IP_3 binds to its receptor on the DTS, promoting the release of Ca^{2+} from intracellular stores. The decrease in Ca^{2+} releases the DTS-located transmembrane protein stromal interaction molecule 1 (STIM1), allowing it to translocate to the platelet surface and regulate the opening of the calcium channel Orai1, resulting in an influx of extracellular calcium in a mechanism referred to as store-operated calcium entry (SOCE) (Varga-Szabo et al., 2009). Increased concentrations of Ca^{2+} and DAG activate "Ca^{2+} and diacylglycerol regulated guanine nucleotide exchange factor 1" (CalDAG-GEFI) (Crittenden et al., 2004, Bergmeier and Stefanini, 2009),

which leads to the interaction of Rap1b, Rap1-interacting adaptor molecule (RIAM) and talin 1. The interaction leads to the exposure on talin of an integrin binding site which disrupts the connection between the α and β subunits of the integrin αIIbβ3, altering the configuration and allowing for the subsequent binding to fibrinogen and vWF (Petrich et al., 2007) (figure 1.11).

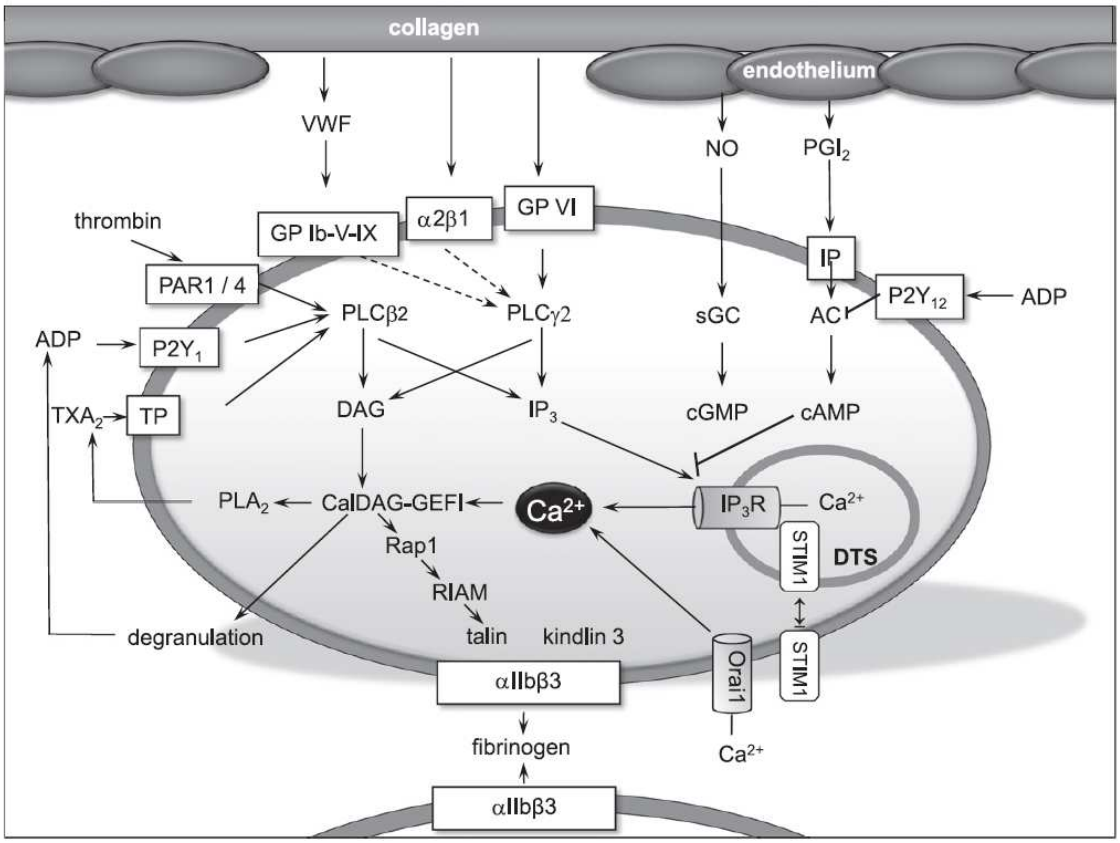


Figure 1.11: Interaction of the principal platelet receptors with the various effectors associated with platelet adhesion and aggregation (for details see text) (Broos et al., 2012) (Reproduced with permission, © Elsevier B.V.)

The TF/VIIa complex is able to assemble and function adequately in the absence of anionic phospholipids (Bach et al., 1986). However, the assembly and activity of the tenase and prothrombinase complexes is highly dependent on the surface exposure of PS – a fact illustrated in the rare congenital bleeding disorder known as Scott syndrome in which the inability to express PS on platelet activation leads to a failure to bind factors Va and VIIIa to the platelet surface, with consequent failure to effectively generate thrombin (Nurden and Nurden, 2007). First recognised in lymphocytes, surface expression of PS is also associated with cells entering apoptosis and is believed to act as a recognition signal for cell clearance by phagocytes (Fadok et al., 1992, Bevers and

Williamson, 2010). A study on B lymphocytes from a Scott patient detected normal PS exposure in cells stimulated to undergo apoptosis but failed to detect a similar change in cells exposed to a calcium ionophore, suggesting that two distinct pathways of PS exposure may be operating in haemopoietic cells (Williamson et al., 2001, Zwaal et al., 2004). A recent study investigated this concept in platelets using mice with variable expression of the pro-apoptotic Bcl-2 family members Bak and Bax. $Bak^{-/-}Bax^{-/-}$ platelets exposed to the apoptosis-inducing BH3 mimetic ABT737 failed to express PS on the surface, whereas exposure to either a calcium ionophore or CRP/thrombin resulted in normal levels of PS exposure. These results confirmed the hypothesis that expression of PS on the platelet surface follows two distinct pathways depending on whether the platelets are subjected to a physiologic agonist or an apoptotic process (Schoenwaelder et al., 2009, Bevers and Williamson, 2010). Finally, in addition to its procoagulant function, PS also serves to contain the extent of thrombin formation by directing the action of the protein C pathway, which degrades the FVIIIa and FVa associated to the PS-expressing lipid surface (Zwaal and Schroit, 1997).

Although the haemostatic role of platelets has been considered their primary function, there is an increasing appreciation of their contribution to inflammation through their recruitment of leucocytes to sites of vascular injury and the release or expression of various mediators associated with inflammatory conditions (McNicol and Israels, 2008, Zarbock et al., 2007).

PLATELETS IN CLINICAL PRACTICE

Platelet concentrates are generally administered to treat thrombocytopenia, which constitutes a common complication of critical illness (Arnold et al., 2006, Tinmouth and Freedman, 2003). Platelet transfusion constitutes the principal replacement therapy to combat active bleeding exacerbated by a low platelet count in conditions such as sepsis and other causes of disseminated intravascular coagulation, disease-induced and iatrogenic bone marrow failure and gynaecological emergencies such as preeclampsia Increasingly, platelet concentrates have been used prophylactically to maintain platelet counts, particularly in cancer patients undergoing chemotherapy and in recipients of stem cell or bone marrow transplants (Perrotta and Snyder, 2007, Levi et al., 2009,

British Committee for Standards in and Blood Transfusion Task Force, 2003). A platelet concentration of 10×10^9/L is often recommended as a transfusion trigger in these stable patients, with the aim of raising the platelet count by at least 20×10^9/L per single adult dose (Williamson, 2004).

PLATELET CONCENTRATES

Techniques for the separation of platelets from whole blood and their administration as a concentrate in order to increase the platelet number without increasing the fluid load to the recipient have been described since the 1950s (Minor and Burnett, 1952). The development of gas-permeable plastic containers greatly facilitated the production of PC and promoted their routine use (Perrotta and Snyder, 2007).

Storage Packs

A critical development stemmed from the recognition that platelets require an environment that allows adequate exchange of oxygen and carbon dioxide. First-generation plastic containers were composed of polyvinyl chloride (PVC) rendered pliable by use of the plasticizer di-2-ethylhexyl phthalate (DEHP) (Murphy et al., 1982). The lifetime of platelets in PVC containers was limited to 3 days and even within this short period, pH levels in many of the units had decreased below 6.0 by end of storage; a condition known to be harmful and lead to loss of viability (Murphy et al., 1970). Additionally, there were concerns about possible harmful effects caused by the leaching of plasticizer into the component (Jaeger and Rubin, 1972) which led to the development of containers comprised of polyethylene instead of PVC. *In vitro* studies with these packs found that pH was better maintained, with results correlating with a reduced rate of lactate production. Polyethylene is approximately twice as permeable to oxygen as standard PVC and it was suggested that this increased rate of oxygen availability was responsible for the improved pH levels (Murphy and Gardner, 1975). Studies under anoxic conditions had previously shown that an inability to maintain oxidative metabolism will increase the cell's dependency on anaerobic metabolism in an attempt to preserve energy levels, leading to an increase in the rate of lactate production – a process referred to as the Pasteur effect (Guppy et al., 1995).

The polyethylene storage packs were found to be too fragile for practical component manufacture. However, the accepted requirement to maintain appropriate gas exchange led to the development of second generation containers using plastics such as polyolefin that were sufficiently robust for routine use and retained the required permeability to maintain adequate gas exchange (Murphy et al., 1982). Further work suggested that other physical characteristics of the storage pack, including the thickness of the plastic film and the size of the pack, also played a role in maintaining platelet viability. Together, these parameters serve to define the oxygen diffusion capacity of the container – a more useful measure of the pack's ability to maintain platelet viability than oxygen permeability alone (Wallvik and Akerblom, 1990). Thus, thinner plastics can help to compensate for lower oxygen permeability (Murphy and Gardner, 1975) and a modest increase in pack size can aid gaseous exchange by maximising the surface area for diffusion (Kakaiya and Katz, 1984, de Wildt-Eggen et al., 1998). Since platelets have a fixed demand for oxygen at a set temperature, the measure of oxygen diffusion capacity can also aid in defining the maximum platelet yield that a storage pack can support (Wallvik and Akerblom, 1990). Associated with the ability to allow oxygen into the packs is the ability for carbon dioxide to diffuse to the outside, since a build-up of CO_2 would lead to the formation of carbonic acid and contribute to a decrease in pH over time.

Sterilisation

Bacterial contamination of the platelet component is a complication of PC storage with potentially fatal consequences. Estimates of the prevalence of bacterial contamination in platelet components range from 1 in 2000 to 1 in 3000 units, with the risk of a severe transfusion reaction of approximately 1 in 50000, significantly exceeding the risk of receiving a unit contaminated with one of the viruses routinely screened for by transfusion services in the UK (Blajchman et al., 2004). Numerous procedures have been introduced to reduce the risk of bacteraemia in PC; including rigorous arm cleaning before venupuncture, the diversion of the first 20-30 mL of blood to prevent the skin plug removed by the needle from entering the main pack, and monitoring of units for bacterial activity over the storage period or before transfusion of the component (Pietersz, 2009). A vital component of these efforts is the provision by pack manufacturers of a closed collection and storage system with guaranteed sterility. Three

principal methods of sterilisation have been adopted: steam sterilisation, γ-irradiation and use of ethylene oxide. Some authors have suggested that the method of sterilisation can affect the characteristics of platelets under storage, with steam sterilisation resulting in higher levels of platelet activation and lactate production compared with ethylene oxide (van der Meer and Pietersz, 2007).

Agitation

Studies have shown that storing platelets under static conditions results in a relatively rapid decrease in pH, lower aggregation responses and increased platelet activation; as measured by supernatant levels of secretory granule contents such as platelet factor 4 and β-thromboglobulin (Mitchell et al., 1994, Snyder et al., 1983). Since such studies have been performed using storage packs with adequate permeability for oxygen, it is the lack of mixing that is generally attributed to be responsible for the relatively poor storage characteristics (Wallvik and Akerblom, 1990). The mode of agitation has also been found to impact on platelet viability, with horizontal flat-bed agitators widely adopted over rotational mixers (Holme et al., 1978). Periods of interrupted agitation are likely to occur as PCs are transported to hospitals or temporarily stored in small facilities with limited resources. Studies have determined that storage under static conditions for up to 24 hours at any time point during the storage period does not lead to significant platelet damage as measured by various *in vitro* assays (Van Der Meer et al., 2007, Hunter et al., 2001). Interruption of agitation for longer periods had a more significant effect on pH levels, with longer static periods and higher platelet concentrations resulting in lower pH values (Dumont et al., 2007).

Temperature

Current guidelines for the storage of PC require the units to be stored at 22°C ± 2°C. The risk of a PC developing a clinically relevant dose of bacteria over the storage period would be decreased if PC could be stored at temperatures of 2 – 6°C typical of red cell components. Bacterial contamination in platelet components is estimated to be 50 times more prevalent than in red cells (Kuehnert et al., 2001), with the higher storage temperature a significant factor of this increased risk. Experiments investigating storage of platelets at 4°C using metabolically suppressed platelets - in which energy stores for the platelets were depleted by initial storage at 37°C in a glucose-free, antimycin A-

containing buffer - suggest that cold storage at 4°C seems to stabilise metabolic activity and does not lead to increased expression of markers associated with platelet activation, such as CD62P and CD63. In addition, response in functional assays such as TRAP-induced aggregation was improved compared with platelets stored at ambient temperatures (Badlou et al., 2005, Cauwenberghs et al., 2007). Morphological changes do occur during cold storage that are similar to the changes following platelet activation and have been termed cold-induced activation. Such changes appear to be the result of F-actin fragmentation and microtubule depolymerisation. Changes in the viscosity of the platelet membrane have also been noted in animal studies. The effect is the loss of the normal discoid shape and the appearance of platelets with a spherocytic morphology. It is unclear whether such a change in shape would by itself pose a problem for the transfusion of these platelets, as animal studies have suggested spherocytic platelets are not preferentially cleared from the circulation (Hoffmeister et al., 2003a). However, early studies showed that platelets stored at 4°C were rapidly cleared from the circulation following transfusion (Josefsson, 2007). More recent investigations have measured a down-regulation in the expression of CD42b, which may be related to the mechanism for clearance of chilled platelets (Sandgren et al., 2007). Chilling irreversibly clusters the GPIb/IX/V complex on the platelet surface. Hepatic macrophages expressing the integrin $\alpha_M\beta_2$ are capable of detecting the clustered GPIbα through binding of the lectin domain of the integrin to the β-GlcNAc residues of the glycoprotein's N-linked glycans. Recognition leads to the phagocytosis of the chilled platelets and removal from the circulation, with approximately two thirds of platelets chilled before infusion estimated to be quickly cleared by this mechanism (Hoffmeister et al., 2003a). Initally encouraging results suggested masking of the GlcNAc residues by enzymatic galactosylation may preclude the premature clearing of chilled platelets (Hoffmeister et al., 2003b, Babic et al., 2007). However, the results from the *in vitro* studies did not translate to better retention of chilled platelets in a phase 1 clinical trial (Wandall et al., 2008), and rapid clearance from the circulation remains the most intractable problem in adopting cold storage for platelet concentrates.

On the opposite side of the temperature scale, storing platelets at physiological temperatures of 37°C is not contemplated, as the *in vitro* characteristics of platelet ageing are accelerated. These include an increased rate of ATP utilisation and a

doubling of oxygen consumption compared to platelets stored at 22°C, suggesting an increase in metabolism (Holme and Heaton, 1995). A recent study by Bertino et. al. (2003) also demonstrated accelerated decreases in pH and platelet concentration along with increases in lactate dehydrogenase levels. This last study also suggested that programmed cell death may be promoted by storage at 37°C (Bertino et al., 2003).

Current Methods of PC Preparation

Current methodologies for the preparation of platelet concentrates are based on three approaches (Tynngard, 2009):

- Single donor PCs collected by apheresis technology; an automated process by which platelets are separated from whole blood by centrifugation or elution, with the red cells returned to the donor. Depending on the donor, between one and three adult dose PCs may be collected during the approximately 90 minute procedure, with 4 to 5 litres of whole blood being processed (Perrotta and Snyder, 2007).
- Platelet concentrates prepared from platelet rich plasma (PRP-PC), in which PRP is separated from whole blood by a gentle centrifugation step. The PRP is separated from the red cells into a satellite pack which is then subjected to a "hard" centrifugation step. The excess plasma is extracted into a separate pack and the resulting platelet pellet gently re-suspended in the remaining 50 – 70 mL of plasma to generate a concentrate. Four to six of these units may be pooled to provide a single adult dose. Pooling traditionally occurred shortly before transfusion due to concerns that storage of pooled concentrates may increase the risk of bacterial growth and degrade the quality of the component (Sweeney et al., 2004). Recent studies, however, have provided robust evidence that pooling does not adversely affect the platelets. Preparation of PRP-PC remains the preferred method for PC production in the United States.
- Platelet concentrates prepared from a pool of 4-6 whole blood-derived buffy coats (BC-PC). An initial hard centrifugation step separates the buffy coat, containing the white cells and platelets, from the bulk of the red cells and plasma. The buffy coats may be stored overnight without agitation at 22 ± 2°C before being pooled (though some organisations prefer to store the whole blood overnight, using butanediol plates to cool and maintain the whole blood at approximately 22°C for up to 24 hours) (Murphy, 2005, Pietersz, 2011, Dijkstra-Tiekstra et al., 2011). Between four

to six buffy coats are aseptically pooled using an autologous unit of plasma (or an artificial suspending medium). Gentle centrifugation of the buffy coat pool separates the platelets as a concentrate from the red cells and a significant fraction of the white cells, with removal of the remaining white cells achieved through an integral filter leading to the PC storage pack. This is the method currently adopted in Europe and Canada (figure 1.12). A detailed description of the preparation of PC from pooled buffy coats is provided in chapter 2 (Methods).

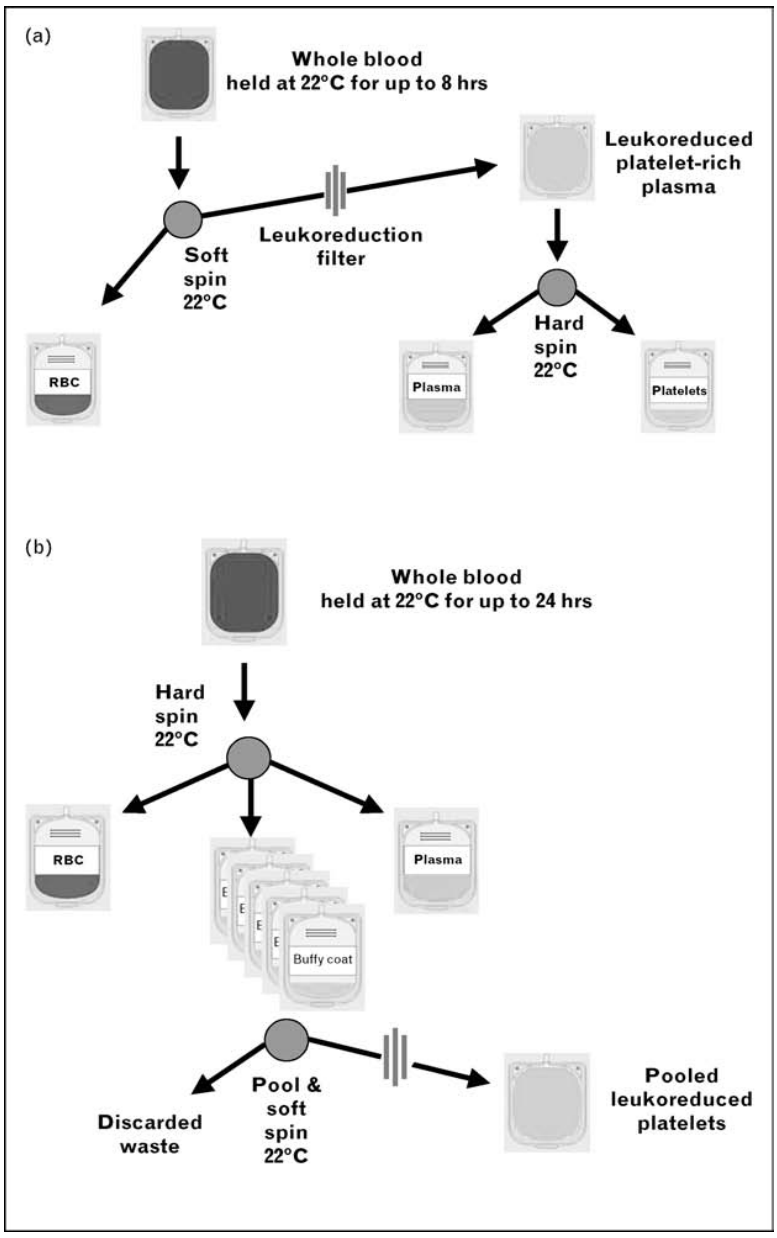


Figure1.12: Schematic of whole blood-derived platelet concentrates (a) from platelet-rich plasma (b) from pooled buffy coats (Vassallo and Murphy, 2006) (Reprinted with permission, © Lippincott Williams & Wilkins)

Improvements in the preparation and storage of PCs have allowed the extension of the storage period from an initial limit of three days to five days. The ability to store PCs for longer periods has long been desired, as it would ease the logistical pressures on transfusion services to maintain the supply, especially as mandatory blood grouping and virology testing requirements significantly impinge on a shelf life limited to five days. In the 1980s, the US Food and Drug Administration was sufficiently confident in the ability of storage packs to retain adequate platelet viability over seven days that they approved the adoption of this extended storage period. Concerns over an increase in cases of bacterial contamination forced regulatory authorities to reverse the decision (Braine et al., 1986, Heal et al., 1986, Slichter, 2004). However, the introduction of approved measures to monitor the sterility of platelet concentrates has allowed the limited re-introduction of 7-day storage periods. An associated development is the introduction of pathogen inactivation technology. In PC, currently CE marked pathogen inactivation methods rely on the targeting of nucleic acids with compounds which subsequently photoactivate on exposure to ultraviolet wavelenghts, causing irreversible damage to the molecules and preventing the proliferation of viruses, bacteria and white cells (Prowse, 2012).

A further consideration is the impact of an extended storage period on *in vitro* platelet characteristics as well as platelet function and recovery following transfusion. Changes in platelet morphology, functional responses, metabolic parameters and markers of senescence have been noted in relation to the processing and storage of PC. Collectively, these changes have been termed the platelet storage lesion (PSL). Numerous studies have suggested that prolonging the storage period is related to the increased expression of the PSL, leading to the requirement of a better understanding of this phenomenon if further improvements in the provision of PCs to hospitals are to be forthcoming (for recent reviews see Maurer-Spurej and Chipperfield, 2007, Shrivastava, 2009, Ohto et al., 2009, Thon et al., 2008). The following section describes the nature of the storage lesion in association with some of the assays adopted for its investigation.

PLATELET STORAGE LESION

Platelet Morphology

Platelet activation results in a transformation from the normal discoid morphology of the resting platelet to a more spherical form with spiny projections (Maurer-Spurej and Chipperfield, 2007). Similar morphological changes have been observed in platelets stored as concentrates and have been linked to reduced platelet recoveries in early studies, leading to the suggestion that the maintenance of a discoid form is a prerequisite for platelet survival (see Goodrich et al. for references Goodrich et al., 2006). A scoring system was developed to provide a semi-quantitative measure of platelet morphology as viewed under oil-phase microscopy (1000× magnification), with platelets categorised as discs, spheres, dendritic forms or ballooned forms. Each form is given a score from 4 to 0 and the percentage of each type multiplied by the score (Kunicki et al., 1975, Murphy et al., 1994). Recovery and survival of platelets in radiolabelled re-infusion studies were found to correlate with the morphology score in these early studies. Reviews of subsequent studies have shown a 20-45% decrease in the morphology score between fresh and 5-day old platelets (Murphy et al., 1994, Cardigan and Williamson, 2003). A change in morphology with storage has also been suggested from monitoring the "swirling" of platelets in a storage pack. A population of discoid platelets will scatter incident light in numerous directions due to the varying orientation of the platelets in relation to the light as the unit is gently rocked. The appearance is that of a turbulent fog when the unit is held up to a light source. Spherical platelets have a uniform shape and will not produce this effect (Brecher and Hay, 2004, Bertolini et al., 2000). However, murine studies have shown that discoid platelets may also be removed rapidly from the circulation and both murine and primate platelets activated *in vitro* with thrombin and displaying a non-discoid morphology are able to circulate normally. Such work has led some to suggest that shape change is not related to platelet clearance in the circulation (Hoffmeister et al., 2003a, Italiano et al., 2003). In addition, evidence from a study using a rabbit model suggested that the morphological score is not related to the haemostatic efficiency of transfused platelets (Rothwell et al., 2000).

Associated with the measure of platelet morphology is the measure of platelet size, generally described by the mean platelet volume (MPV). MPV *in vivo* appears to be maintained independent of platelet age. An increase in MPV is evident in

thrombocytopenic patients, possibly related to a compensatory thrombopoiesis, with the suggestion that younger platelets are larger and functionally more responsive (Thompson et al., 1984, Albanyan et al., 2009). During storage, an increase in MPV is often evident, arguably related to swelling of the platelet population and a disc-to-sphere transformation. Some authors have suggested caution in the interpretation of MPV results, as platelet lysis or the formation of microparticles may lead to a relative decrease in this measure (Fijnheer et al., 1989). Indeed, studies often report a decrease in platelet concentration with duration of storage, the most likely explanation being the breakdown of relatively fragile, possibly older, platelets (Hornsey et al., 2008). Studies have also noted that the processing methodology can have a relative impact on MPV. A lower MPV has been noted in apheresis-derived platelet concentrates compared with BC-PC, possibly due to preferential collection of smaller platelets by the apheresis technology (Albanyan et al., 2009).

Platelet Activation

The monitoring of platelet activation as a component of the PSL has included the measurement of markers associated with secretory granule release, the formation of microparticles and conformational changes in membrane glycoproteins such as the integrin $\alpha_{II\beta}\beta_3$, as well as the morphological changes already described (Heijnen et al., 1999, Kulkarni et al., 2000). Assays may be performed on non-stimulated platelets from PCs to determine the spontaneous activation caused by processing and storage, as well as agonist-stimulated platelets to determine the residual capacity for platelets to respond to physiological agonists (Vetlesen et al., 2007). As a marker of α-granule release, surface expression of CD62P has generally been found to increase with storage, with the concentration of thrombin required to achieve an equivalent level of expression also increasing with longer storage periods (Leytin et al., 2008). The latter observation suggests a depletion of the α-granule contents with storage, though alternative explanations may involve shedding of the PAR-1 receptor or disruption of the signalling process (Reiter et al., 2003, Jilma-Stohlawetz et al., 2008). CD62P binds to the P-selectin glycoprotein ligand-1 (PSGL-1) receptor principally found on the myeloid lineage of leucocytes, leading to the suggestion that an increased expression of CD62P in activated platelets could be responsible for their accelerated clearance from the circulation following transfusion (Perseghin et al., 2004, Gutensohn et al., 2002).

However, studies investigating the relationship between surface CD62P and platelet recovery post-transfusion have yielded inconsistent results (Holme et al., 1997, Dumont et al., 2002, Rinder and Smith, 2003), possibly reflecting the lack of standardisation in methodology which may vary with respect to sample preparation as well as flow cytometric settings. A 1992 study by Triulzi et al concluded that there was no correlation between CD62P expression and corrected count increments (r^2=0.34) (Triulzi et al., 1992). Proteolytic cleavage of CD62P from the platelet surface has been found to yield a 100 kDa soluble fragment which has been suggested by some authors to constitute a better indicator of platelet activation during storage than surface expression of the protein (Michelson et al., 1996, Berger et al., 1998). Expression of CD62P has been reported to correlate well with other markers of degranulation such as β-thromboglobulin, RANTES and platelet factor 4 - all constituents of α-granules and all reported as increasing significantly with storage (Shanwell et al., 2003, Cardigan et al., 2003). As with CD62P expression, correlation of these markers with platelet recovery has been poor (Murphy et al., 1994, Snyder, 1992). Dense granule release has been investigated via the expression of CD63 (Sandgren et al., 2008). The use of this marker alongside CD62P in studies of cerebral ischaemia have found continued expression of CD63 with none of the degradation evident with CD62P (Marquardt et al., 2002), leading to the suggestion that it may form a better marker of *in vitro* platelet activation than CD62P (Sandgren et al., 2007).

Flow cytometric assays have also been employed to investigate changes to membrane glycoproteins during storage. Surface expression of CD42b (the GPIbα component of the GPIb-IX-V complex) has been found to decrease moderately with storage, though levels remain at approximately 90% of starting levels (Diedrich et al., 2008, Cardigan et al., 2008). The decrease may be related to redistribution of the glycoprotein into the open canalicular system and although a possible consequence would be a decrease in adhesive function, no such effect has been measured under flow conditions (Turner et al., 2005, Sandgren et al., 2008).

Platelet-derived microparticles are small (40 nm – 1 μm) fragments expressing platelet-specific antigens that have been found to increase in number with storage of PC (Niewland and Sturk, 2007). The increased formation of microparticles is associated

with platelet activation, with increased formation noted after stimulation with physiological agonists as well as the calcium ionophore A23187, exposure to high shear and in clinical situations associated with platelet activation (Heijnen et al., 1999). There is some hesitation in employing microparticle formation as a marker for monitoring the PSL, as their small size is at the limit of the resolution possible by flow cytometry (Maurer-Spurej and Chipperfield, 2007). Indeed, transmission electron microscopy studies have implied that there are two distinct populations of microparticles with apparent diameters of 0.4 – 0.6 μm and 0.08 – 0.2 μm (Sandberg et al., 1985). The latter population would likely be lost in the background "noise" of the instrument (Bode et al., 1991).

Platelet Metabolism

Platelets under standard storage conditions employ both aerobic and anaerobic metabolism to maintain their energy stores. Extracellular pH of PCs is employed by transfusion services as an ubiquitous measure of the effectiveness of processing and storage methods to maintain the metabolic activity of platelets, as levels below 6.2 have been associated with poor performance in other *in vitro* parameters (Murphy et al., 1994, Rinder and Smith, 2003). The decrease in pH traditionally reported in PC suspended in plasma is attributed to the accumulation of lactic acid due to continued glycolysis, even in the presence of adequate oxygen (Murphy and Gardner, 1975), with a pH of 6.0 associated with a lactate concentration of approximately 30 mmol/L (Gulliksson, 2003). The concentration of hydrogen ions will be buffered by the presence of bicarbonate in the plasma, which in PC suspended in 100% plasma is sufficient to buffer lactate levels up to approximately 20 - 25 mmol/L (Murphy, 2002, Kilkson et al., 1984). This has led to the suggestion that lactate concentration would be a better predictor of *in vivo* recovery than pH (Goodrich et al., 2006). Rates of lactate production and glucose consumption have also been adopted to monitor the PSL. Aerobic metabolism has been indirectly monitored in PC through the measurement of partial pressures of oxygen and carbon dioxide, with increases in pO_2 and concurrent decreases in pCO_2 interpreted as a decline in oxidative metabolism (Holme et al., 1987). Conversely, lower pO_2 levels are indicative of increased oxygen consumption (Picker et al., 2009). Some care is required in the interpretation of these parameters with current gas-permeable storage packs, as the pressures also relate to the equilibration of O_2 and

pCO_2 in the pack relative to atmospheric pressures (Zhang et al., 2008a). An additional parameter that may be useful in this context is a calculated rate of oxygen consumption that includes the gas permeability of the plastic as well as PC and atmospheric partial pressures of oxygen (Kilkson et al., 1984).

Losses of ATP and ADP associated with PC storage have been reported in the literature (Rao et al., 1993), though levels of ATP are most often presented in publications. Starting levels of ATP in PC are somewhat variable depending on the methodology used to perform the measurements (Girotti et al., 1989) but there seems to be a growing consensus that ATP levels below 4.0 µmol/10^{11} platelets (or approximately 70% of day 1 values) are related to reduced *in vivo* recoveries below 50% (Cardigan et al., 2005). Early studies also noted that ATP levels were only observed to decrease after glucose concentrations decreased below 50% of starting levels (Savage, 1982). A further measure to assess the energy potential of platelets in PCs is the adenylate energy charge (AEC), regarded as indicative of the overall energy status of the cell. It requires the measurement of ATP, ADP and AMP levels, and is calculated as follows: AEC = (2ATP + ADP)/2(ATP + ADP + AMP) (Rao et al., 1981, Verhoeven et al., 1985).

Platelet Function and Viability

One of the few *in vitro* assays adopted to monitor the PSL that has been found to correlate with platelet recovery post-transfusion is the extent of shape change (ESC) (r=0.71), with levels below 15% (or less than 60% of day 1 values) correlating with less than 50% recovery (Holme et al., 1998, Diedrich et al., 2008). The assay is a measure of the change in light transmission of a platelet sample in response to the addition of ADP (VandenBroeke et al., 2004). Significant correlation also exists between the ESC assay and morphology scores (r=0.93) (Holme et al., 1998) and this may have led to the common interpretation of this assay as an indicator of platelet shape change in response to the addition of a weak agonist. This explanation has been recently challenged, with the suggestion that the decrease in light transmission in Born-type aggregometers is caused by the formation of microaggregates rather than a disc to sphere transformation. Two lines of evidence have been used to support this interpretation: i) the observation that platelets at 37°C appeared as smooth discs when at rest, with no change in morphology when exposed to stirring at 1000 rpm even though light transmission under

these conditions increased abruptly, ii) platelets that had already been observed to have undergone a change in shape proceeded to show a decrease in light transmission on the addition of 0.5 iu/mL thrombin (Maurer-Spurej and Devine, 2001). In addition, the authors suggest that microaggregation is distinct from normal aggregation, since platelets from patients with Glanzmann's thrombasthenia, which lack the integrin $\alpha_{IIb}\beta_3$ required for complete platelet aggregation, continue to show the initial decrease in light transmission.

An assay often performed in conjunction with the ESC is the hypotonic shock response (HSR), which measures the relative recovery of platelets to a hypotonic insult. The addition of water results in swelling of the platelets with gradual extrusion of the water by viable platelets, probably via an ATP-dependent expulsion of K^+ and Cl^- ions (Okada et al., 2001). This change can be photometrically measured, since the refractive index of the platelets decreases with the uptake of water, leading to an increase in light transmission which gradually declines again as the water is extruded (Holme, 1998). The HSR has shown reasonable correlation with *in vivo* platelet recovery (r=0.57) (Holme et al., 1998), with recovery levels below 50% associated with HSR values below 70-75% (Cardigan et al., 2005) or less than 60% (Diedrich et al., 2008) for PC suspended in 100% plasma.

Determining the ability of platelets stored as PC to function normally after transfusion is a major goal of the various assays employed to study the PSL, and the capacity of stored platelets to form aggregates *in vitro* in response to various physiological agonists would seem a logical approach to accomplish this. Traditional platelet aggregometers measure light transmission through a platelet sample. Aggregation would be expected to result in an increase in light transmission as the formation of large particles enhanced the unimpeded transmission of light (Born, 1962). The rate of aggregate formation and the maximum response are the parameters generally quoted (Tynngard, 2009). Platelets stored for 5 days under standard conditions have been shown to have a poor aggregation response to single agonists such as ADP, epinephrine and collagen (Murphy et al., 1994), with confirmatory results from studies which found a reduced aggregation response in platelets stored as PC compared to platelets retained as whole blood (Shapira et al., 2000, Jansen et al., 2004). The response to stronger agonists such as

thrombin or paired weaker agonists has been reported as better maintained (Murphy et al., 1994). The latter may be a better indicator of performance *in vivo* since activation by single weak agonists does not reflect conditions in the circulation (Cardigan and Williamson, 2003). Another factor to consider in the interpretation of aggregation response is the observation that a reduced response can recover after transfusion (Miyaji et al., 2004). In addition, studies with platelets stored at 4°C have concluded that platelet aggregation response does not correlate with *in vivo* platelet recovery and survival, as the chilled platelets have an improved aggregation response but are rapidly cleared from the circulation (Kattlove et al., 1972). Interestingly, work with PCs suspended in additive solutions or washed platelets have resulted in a decreased or absent aggregation response on addition of ADP (Schoenfeld et al., 2004). A decrease in ADP receptor responsiveness due to the lack of a permissive factor present in plasma has been suggested as a possible explanation (Keuren et al., 2006). Platelet adhesion to glass slides coated with collagen has also been observed to decrease substantially with increased platelet storage; though, in contrast to the previous observation, the presence of plasma did not seem to impact on the results (Brown et al., 2000). As an assay for the determination of platelet function after transfusion, standard aggregometry suffers from the disadvantage that reactions are restricted to a low shear environment which does not reflect conditions *in vivo*. A variety of techniques and instruments have been introduced to address this limitation. One approach is to measure the adhesion and/or aggregation of platelets to a subendothelial surface/matrix or an agonist-coated membrane under controlled shear conditions (Morrison et al., 2007, Tanaka, 2006, Rand et al., 2003). A second approach measures the elastic properties of a forming blood clot. Application of these techniques to the study of the storage lesion appears to be limited, though studies measuring the strength of clot retraction have discerned little or no change after five days of storage (Tynngard et al., 2008, Reid et al., 1998).

Mechanisms of Cell Death

Associated with the maintenance of ATP levels in storage may be the functional capacity of the platelets' mitochondria to maintain the electron transport chain across the inner mitochondrial membrane (Newmeyer and Ferguson-Miller, 2003). The deliberate uncoupling of oxidative phosphorylation in platelets through the addition of the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP) has been

reported to result in a marked reduction in platelet recovery post-transfusion in a mouse model (Kile, 2009). The experimental approach has been proposed as a model for the PSL, since the disruption of oxidative phosphorylation has been associated with platelet shape change, disruption of membrane phospholipid asymmetry, microparticle formation, surface expression of CD62P and loss of membrane GPIbα (Bergmeier et al., 2003). The impact on the mitochondrial inner membrane can be discerned by measurements of the mitochondrial membrane potential, commonly performed by measuring the uptake of cationic dyes into the mitochondria. Published data on the behaviour of the mitochondrial membrane potential in PC over the storage period vary, with some studies suggesting there is little or no change whilst others report significant decreases (Perrotta et al., 2003, Li et al., 2005b). This discrepancy extends to the response of this parameter to the addition of calcium ionophore (Perrotta et al., 2003, Albanyan et al., 2009). Alternative approaches to the measurement of inner mitochondrial membrane permeabilisation, such as loading of the platelets with calcein which does not normally cross this barrier, may help to resolve some of these questions (Kroemer and Reed, 2000). Although the physiological role of mitochondria has traditionally been confined to the generation of ATP, an increasing body of work has linked mitochondria with events leading to programmed cell death (Jackson and Schoenwaelder, 2010). A fall in mitochondrial membrane potential has been described as an early event during apoptosis, with a collapse of the potential associated with irreversible cell death (Hortelano et al., 1999). Investigation of apoptosis in relation to the PSL is not as well established as the aforementioned parameters. However, changes to the expression of anionic phospholipids on the platelet surface over the storage period, as measured by the binding of fluorescently-labelled annexin V, have been reported in multiple studies. Increased binding is often interpreted as increased expression of phosphatidylserine and thus as a marker of platelet activation and procoagulant activity (Brown et al., 2000); with progressive exposure indicative of apoptosis (Perez-Pujol et al., 2005). PS on the cell surface is also considered to be a marker for the phagocytic removal of cells (Fadok et al., 1992), though it is unclear if this forms a substantive mechanism for the clearance of platelets after transfusion (Bergmeier et al., 2003).

With increasing interest on the possible role of apoptosis in the PSL, investigators are adopting a wider range of assays targeting specific aspects of the process. These include methods for measuring caspase activity (Bertino et al., 2003, Perrotta et al., 2003), the release of intermembrane space proteins such as cytochrome c and the role of Bcl-2 family members (Leytin et al., 2006); with approaches that manipulate the platelet environment with the use of inhibitors further contributing to the understanding of the mechanisms involved in the PSL (Dasgupta et al., 2010, Lopez et al., 2007).

Platelet Recovery and Survival

In vivo measures to assess the efficacy of transfusing platelet components generally involve platelet recovery and survival studies - in which autologous platelets radiolabelled with chromium-51 or indium-111 are re-infused into healthy volunteers - or calculations of the platelet increment following a transfusion. Recent scrutiny of recovery and survival methods has led to the publication of guidelines and protocols for the sample preparation and analysis of results from the Biomedical Excellence for Safer Transfusion to aid in standardisation of techniques (The Biomedical Excellence for Safer Transfusion, 2006). Limits of 66% and 50% as a proportion of values from fresh units have been suggested as acceptable for recovery and survival measures, respectively (Murphy, 2004), and appear to be generally accepted (AuBuchon and Snyder, 2006). Significant variability in some of the required factors remains, such as estimates of blood volume and differences in the uptake of transfused platelets by the recipient's spleen (Holme, 2008, Aster, 1966). Additionally, the response by thrombocytopenic patients is likely to differ from that in healthy volunteers, with an apparent shortening in mean platelet lifespan observed in patients with platelet counts below 90×10^9/L (Hanson and Slichter, 1985, de Wildt-Eggen and Gulliksson, 2003). Corrected count increment (CCI) is the standard by which transfusion efficacy is clinically assessed, though parameters such as post-transfusion count and presence or absence of bleeding are often used (Arnold et al., 2006). Assuming all transfused platelets are viable, the expected increment should be 20×10^9/L (Holme, 2008). In practice, increments between $10 – 15 \times 10^9$/L are more commonly seen, with increments decreasing by 35 – 50% in day 7 platelet concentrates compared with fresh units (Murphy, 2002). Levels below 7.5×10^9/L have been regarded as constituting a failed transfusion (Norfolk et al., 1998). In addition, the relationship between recovery and

survival studies and the CCI remains to be established (van der Meer et al., 2010). The above investigations also provide little information on the ability of transfused platelets to perform their haemostatic function; a factor which may be related to the lack of correlation between *in vivo* studies and many of the assays used to assess the PSL. Nevertheless, with these limitations in mind, the *in vitro* characterisation of platelets stored as concentrates remains an important aspect in the determination of PC quality, not least because of the range of *in vitro* assays able to be applied to the study of the increasingly complex physiological role of platelets.

ADDITIVE SOLUTIONS

Reports on the use of artificial media for the suspension of platelets as concentrates have been evident in the literature since the 1980s (Rock et al., 1985). Interest has continued due to a number of actual and purported benefits, including:

- Increasing the volume of plasma available for fractionation. This provided the initial impetus for the development of additive solutions, and though not currently a consideration in the UK it remains a practical goal in many countries.
- Reducing the occurrence of non-haemolytic transfusion reactions (NHTRs). The frequency of reactions has been reported as between 20-30%, though moderate to severe reactions were only noted in 2.2% of PC transfusion episodes in a relatively recent study (Schoenfeld et al., 2004, Enright et al., 2003). Leucoreduction has reduced the incidence of NHTRs caused by the release of white cell-associated cytokines such as interleukin-6 and tumour necrosis factor α (Muylle and Peetermans, 1994). Plasma proteins have generally been implicated in allergic reactions. The washing of platelets with saline or Plasmalyte-A has been reported to reduce the incidence of reactions, as has the removal of the majority of the plasma component (Vo et al., 2001, Heddle et al., 1999, Azuma et al., 2009). Studies comparing the incidence of allergic reactions after transfusion of PCs stored in additive solution compared to plasma have also observed a reduction (de Wildt-Eggen et al., 2000). Finally, removing the plasma would facilitate the transfusion of ABO incompatible PC transfusions (Ringwald et al., 2006).
- A consideration is the requirement for low plasma concentrations in some pathogen inactivation treatments (Williamson, 2004).

– Of particular relevance to this study is the possibility that optimising the storage media may reduce the adverse consequences of the PSL, leading to a more standardised component with an extended shelf life (Holme, 1998, Sandgren et al., 2010).

There are presently four additive solutions CE marked and approved for use in Europe (Cardigan, 2008). These are PAS-II, Composol and PAS-IIIM; marketed as T-Sol™ (Fenwal; Mont Saint Guibert, Belgium), Composol®PS (Fresenius Kabi; Bad Homburg, Germany) and SSP+™ (MacoPharma; Mouvaux, France), respectively. In addition, PAS-III (marketed as Intersol; Baxter Healthcare, IL) is approved for use with a pathogen reduction system (Knutson et al., 2000) and is the only solution licensed for use in the USA (Alhumaidan and Sweeney, 2012). The composition of the four solutions is provided in table 1.1.

Table 1.1: Composition of CE marked platelet additive solutions

	Composition (mmol/L)			
	T-Sol™[1]	Intersol[3][4]	Composol®PS[2]	SSP+™[2]
Sodium chloride	115.5	77.3	90.0	69.0
Sodium acetate	30.0	32.5	27.0	32.5
Trisodium citrate	10.0	10.8	10.9	10.8
Potassium chloride	-		5.0	5.0
Magnesium chloride.$6H_2O$	-		1.5	1.5
Sodium gluconate	-		23.0	-
Sodium dihydrogen phosphate	-	6.3	-	6.7
Disodium hydrogen phosphate	-	21.5	-	21.5
pH	7.2	7.2	7.0	7.2

[1]: (Hornsey et al., 2006). [2]: (Zhang et al., 2008a). [3]:(Sandgren et al., 2010). [4]:(Knutson et al., 2000)

Components of Additive Solutions

Gulliksson has proposed that four elements are requisite for a platelet storage media, referred to as “cornerstones”: citrate, acetate, phosphate and glucose (Gulliksson, 2000).

Indeed, some of the early studies considering the prospects of developing synthetic media for platelet storage were based on red cell anticoagulants such as CPD (citrate phosphate dextrose). Reports suggested adequate 5-day storage of BC-PC with suspensions of 40% CPD plasma and 60% saline (Gulliksson et al., 1992).

Citrate

Citrate concentrations below 8 mmol/L have been associated with clotting due to activation of coagulation mechanisms and the formation of fibrin (Prowse et al., 1987). Conversely, citrate concentrations between 14-26 mmol/L were associated with increased generation of lactate compared with the lower concentration (Gulliksson, 1993), suggesting low concentrations of citrate should be adopted to limit lactate production and the concomitant drop in pH. Such studies led to the compromise value of approximately 10 mmol/L employed in most additive solutions (Murphy, 1999).

Phosphate

Phosphate has been postulated to have two seemingly contradictory effects during platelet storage, acting as a buffer whilst also promoting glycolysis and the production of lactate (Ringwald et al., 2006). The stimulation of glycolysis is likely due to the requirement for phosphate in the conversion of glyceraldehydes-3-phosphate to 3-phosphoglycerate in the second stage of the process (Gulliksson et al., 2000). A study by Gullikson et al compared PC collected in acid citrate dextrose (ACD) anticoagulant with PAS-II against PC collected in CPD with PAS-II and observed significantly lower lactate production in the former (Gulliksson et al., 1997). Further studies comparing PC in PAS-II with PC in PAS-III (PAS-II with added phosphate) (Hornsey et al., 2006) confirmed the reduction in glycolysis was most likely to be due to the presence of phosphate (Gulliksson et al., 2000). Despite the increased glycolysis, a phosphate concentration of 10 mmol/L has been found to stabilise pH in storage media with minimal plasma carry-over (Shimizu and Murphy, 1993). The effect of phosphate on pH may not be a simple linear relation, since no difference in pH was found by increasing the phosphate concentration from 26 to 40 mmol/L (Dekkers et al., 2007). As an intricate component of adenine nucleotides, the presence of phosphate in the storage media may be expected to impact on the concentrations of ATP and ADP. Lower levels of adenine nucleotides have indeed been observed with PC suspended in media lacking

phosphate (Gulliksson et al., 1997, Gulliksson et al., 2000). This may be a particular consideration for media used in conjunction with ACD anticoagulant, which unlike CPD lacks phosphate, and continues to be the preferred anticoagulant for apheresis-collected PCs due to the ease of platelet re-suspension (Flatow and Freireich, 1966). Composol®PS contains gluconate instead of phosphate, with the aim of retaining the buffering capacity of the medium without the increase in lactate production.

In phosphate-containing media such as SSP$^{+™}$, the inclusion of magnesium and potassium has been shown to reduce glycolysis as well as platelet activation, with better maintenance of pH and HSR (de Wildt-Eggen et al., 2002). Gullikson et al have also suggested that the presence of K^+ and Mg^{2+} may allow the reduction of the residual plasma volume in AS-containing storage media from the current requirement of 30% to 20% (Gulliksson et al., 2002). The mechanism responsible for the suppression of glycolysis is unclear and may be indirect (Zhang et al., 2008b). Reduced activation has been demonstrated by lower CD62P expression and lower levels of secretory granule contents such as RANTES, PF4 and β-TG (Shanwell et al., 2003). As well as reduced CD62P expression, increased concentrations of Mg^{2+} decreased ADP-induced aggregation as well as the binding of fibrinogen to ADP-activated platelets (Gulliksson et al., 2003). It is possible that Mg^{2+} may inhibit aggregation by altering membrane fluidity or by influencing the formation of cAMP (Sheu et al., 2002). Potassium also plays a role in maintaining the platelet membrane potential (Ishikawa and Sasakawa, 1987); in addition, a decrease in cytosolic concentrations has been suggested to play a role in apoptosis by stimulating cytochrome c release and thus promoting the formation of the apoptosome (Gogvadze et al., 2004).

Acetate

Acetate has been included in synthetic media to act as an oxidisable fuel and its presence has been observed to decrease anaerobic metabolism and increase oxygen consumption (Holme, 1992). In human plasma, acetate is present at very low levels, with concentrations between 0.05 – 0.25 mmol/L (Skutches et al., 1979). It is metabolised via the tricarboxylic acid (TCA) cycle from where it undergoes further oxidation to CO_2 and H_2O (figure 1.13). When added exogenously to PCs it is rapidly taken up by platelets, with reports that almost 50% is converted to CO_2 within ten minutes (Holme, 1992). Studies with $^{14}CO_2$-labelled acetate confirmed this rapid

turnover, with consumption rates calculated as 0.5 mmol/day/10^{10}plts and 90% of the metabolised acetate converted to CO_2. Acetate also appeared to be used preferentially by aerobic metabolism, with all the oxygen consumed able to be accounted for by the metabolism of acetate (Guppy et al., 1990). The ease with which acetate is metabolised to acetyl CoA may actually inhibit the oxidation of other potential fuels such as fatty acids, since acetyl CoA suppresses the β-oxidation of free fatty acids (Murphy, 1999). A further benefit of incorporating acetate in the storage media is the removal of a H^+ ion, given that acetate is metabolised in its acid form. This results in a buffering action against the decrease in pH produced by glycolysis.

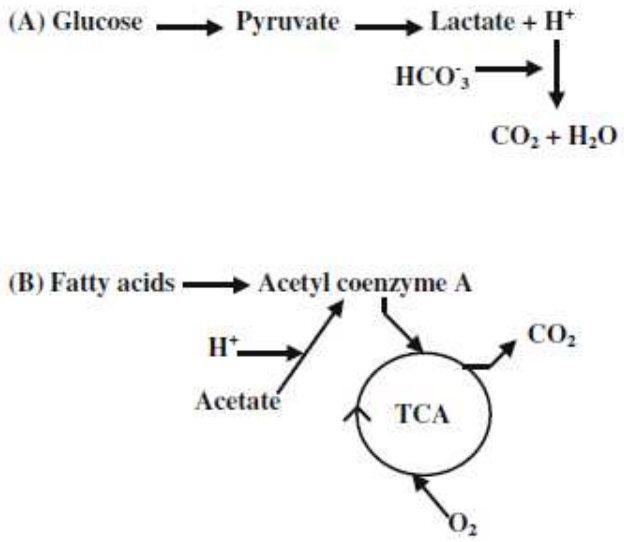


Figure 1.13: Simplified schematic of metabolic pathways. (A) – glycolysis (B) – tricarboxylic acid (TCA) cycle (Tynngard, 2009) (Reproduced with permission, © Elsevier Ltd)

The presence of acetate may also have a more direct action against the generation of lactate since acetyl CoA combines with oxaloacetate to form citrate, which then proceeds through to the TCA cycle. Citrate and ATP both inhibit phosphofructokinase, one of the rate-limiting enzymes of the glycolytic cycle, thus reducing lactate formation (Murphy, 1999). It has been postulated that, theoretically, the presence of acetate could provide unlimited buffering capacity for PC, as the rate of acetate consumption matches the rate of lactate production at 22°C (Murphy, 2002). In practice, studies have measured reductions in bicarbonate consumption of nearly 60% in PRP-PC suspended in plasma with exogenously added acetate compared with controls (Murphy, 1995).

However, despite its apparent role as an oxidative fuel, storage studies suggest that the addition of acetate does not aid in the maintenance of ATP levels during storage (Holme, 1992).

Glucose

The final component of Gullikson's required quartet is glucose. Metabolism of glucose is able to proceed via either glycolysis or through the TCA cycle to complete oxidation. A study by Kilkson et al investigated the relationship between glucose consumption, lactate production and oxygen consumption in platelet concentrates and concluded that if glucose was the sole source of oxidisable fuel, the ratio of glucose consumption to lactate production should be 0.66. The measured value was 0.52 - very close to that expected from the stoichiometry of glucose consumption by glycolysis. The results thus suggested that a fuel source other than glucose was primarily responsible for oxidative metabolism (Kilkson et al., 1984). The study also concluded that approximately 85% of energy generation in PC is obtained from aerobic metabolism and only 15% from glycolysis. Measurements of $^{14}CO_2$ originating from radiolabelled glucose concluded that only 1.4% of the glucose was entering the TCA cycle, corroborating Kilkson's earlier work (Guppy et al., 1990). These studies led to the question of whether glucose could be omitted from the storage media, thus negating the pH drop caused by glycolytic lactate production (Murphy et al., 1991). There were also practical advantages in that heat sterilisation methods caused the caramelisation of glucose at the neutral or alkaline pH suitable for PC storage (Ringwald et al., 2006). However, studies omitting glucose have resulted in lower *in vivo* recoveries and poorer *in vitro* characteristics, including lower ATP levels during storage and accelerated platelet lysis; leading various authors to conclude that glucose may be necessary throughout the storage period (Gulliksson, 2001, Li et al., 2005a, Zhang et al., 2008a). As seen in table 1.1, residual plasma provides the only source of glucose in currently accepted formulations.

Requirement for Plasma

Guidelines for the use of additive solutions for PC storage currently require the incorporation of approximately 30% of autologous plasma in the suspension media. The requirement is based on results from various storage studies that have concluded residual plasma is needed to maintain platelet quality *in vitro* (Klinger et al., 1996,

Keuren et al., 2006), though the proportion of plasma may depend on the particular additive solution used (Cardigan and Williamson, 2003). A 30% carryover of plasma will provide approximately 7.5 mmol/L of glucose to an adult dose PC with an approximate rate of consumption of 1.0 mmol/L per day (Murphy, 1999). Thus, there should be sufficient glucose to maintain stores for the nominal 5-day storage period and possibly 7 days. A further consideration is the buffering capacity provided by the bicarbonate in the plasma, which converts the free H^+ ion produced by glycolysis into CO_2 and H_2O. The former is subsequently able to diffuse through the gas-permeable storage packs, thus stabilising the extracellular pH (Tynngard, 2009). Plasma may also provide a further source of fuel for aerobic metabolism in the form of free fatty acids (FFA) (Holmsen, 1981, Cesar et al., 1987). The concentration of FFA increases in plasma with storage due to the hydrolysis of plasma triglycerides, though there remains some ambiguity as to whether plasma carryover would supply a sufficient concentration to constitute the primary fuel source (Kilkson et al., 1984, Gulliksson, 2000, Ringwald et al., 2006). Studies with minimal plasma carryover (<2%) have reported decreased HSR and discoid shape which could not be attributed to metabolic changes and may instead point to plasma factors that aid in the maintenance of membrane integrity (Holme, 1992, Gulliksson, 2001).

AIMS OF THE THESIS

Two principal considerations formed the basis of this study:

- The hypothesis that apoptosis is a central mechanism responsible for the changes collectively referred to as the platelet storage lesion
- The investigation of this hypothesis within the applied setting of improving the storage environment of platelet concentrates to enhance the efficacy of platelets following transfusion.

In this context, the thesis aimed to investigate the occurrence of an apoptosis-like process during PC storage in relation to more familiar parameters of the storage lesion and the effect of different storage conditions. To simplify the environment in which the platelets were stored, leucoreduced BC-PC suspended in a synthetic medium with minimal plasma carryover were selected as the system for study. This allowed for the controlled manipulation of the storage medium whilst minimising the possible impact

on results of white cell metabolism and the complex composition of plasma. The role of plasma proteins (in the form of albumin), acetate and glucose were selected for investigation. The rationale for selecting these constituents for investigation was:

- Albumin: The requirement for 30% plasma carryover to reduce the expression of the storage lesion may be related to the presence of plasma survival factors, with a putative role in maintaining membrane integrity and reducing apoptosis-related measures such as caspase 3 activation and changes in membrane permeability (Brown et al., 2000, Perrotta et al., 2003). The addition of albumin from a commercially available human source was thus hypothesized to delay the onset of markers associated with cell death and platelet disruption.
- Acetate: The buffering capacity of acetate may be the principal reason for its consideration as one of the essential constituents of additive solutions. However, its role as an oxidisable fuel may be more ambiguous, with studies reporting an increase in oxygen consumption but an inability to maintain ATP levels. Since apoptosis is classically described as an ATP-dependent process, the presence of acetate in the storage medium may be expected to promote expression of the markers of cell death, despite a decrease in lactate formation and associated fall in pH.
- Glucose: The impact, if any, of glucose on the apoptosis markers selected for the study may aid in determining whether the requirement for glucose throughout the storage period apparent in various studies is related to inhibition of an apoptosis-like process (Li et al., 2005a).

An initial extended storage study on BC-PC suspended in either plasma or a 70:30 ratio of SSP+™ and plasma provided an overview of the *in vitro* characteristics of platelets stored as concentrates under standard conditions.

Subsequent to this series of experiments, the opportunity to collaborate with colleagues at the School of Medicine, University Hospital of Wales, allowed for further investigations aimed at exploring the central hypothesis more directly. These studies were based on the ability of platelets from Scott Syndrome patients to express PS on their surface in response to a pro-apoptotic BH3 mimetic (ABT-737). An increasing expression of aminophospholipids on the surface of Scott platelets during storage, comparable to the response from normal platelets, would suggest that a mechanism akin

to apoptosis plays a role in the PSL. The investigations were subsequently extended to study the expression of aminophospholipids during storage in relation to thrombin generation. Finally, the possibility that a Bcl-2 protein-mediated mechanism of cell death may be viable in stored platelets was investigated. An acknowledged weakness of the thesis is the lack of platelet functional assays among the suite of methods adopted, and the option remains to undertake future studies to further investigate the results in relation to platelet function.

There is an increasing appreciation that the PSL involves most if not all physiological platelet functions (Cauwenberghs et al., 2007). In particular, the concept that platelets may undergo an apoptosis-like process during storage may contribute significantly to the understanding of the PSL and suggest strategies for possible manipulation of the platelet storage environment to further improve platelet viability and efficacy post-transfusion.

2. METHODS

EXPERIMENTAL DESIGN

Storage characteristics of platelets stored as concentrates over an extended storage period: comparison of PC suspended in 100% autologous plasma versus PC suspended in a 70:30 SSP+™/plasma medium

A minimum of 10 units from each of the two processes were required for the study, with sample size being derived from the current UK guidelines for performing an initial evaluation of a novel blood component (James, 2005). A non-paired design was adopted to reflect the standard method of processing. Units were stored for 14 days, with samples aseptically removed for testing on days 1, 2, 3, 6, 8, 10 and 14 - day 1 being the day of PC preparation. The range of assays undertaken aimed to investigate aspects of platelet metabolism, viability, morphology, activation and apoptosis-like activity. Details of unit preparation and assay methodology are provided in the following sections. For ease of reference, the assays are listed below:

Unit characteristics

Unit volume

Platelet concentration

Platelet yield

End of storage sterility and leucodepletion

Platelet Metabolism

pH (22°C or 37°C; dependent on storage medium)

pO_2 (37°C)

pCO_2 (37°C)

Bicarbonate levels (37°C)

Glucose levels and rate of consumption

Lactate levels and rate of production

Oxygen consumption rate (22°C)

ATP and ADP levels

Viability and morphology measures

Mean platelet volume

Swirling

Extent of shape change

Hypotonic shock response

Platelet activation

Surface expression of CD62P (% positive and median fluorescence intensity)

Soluble CD62P concentration

Apoptosis-like activity

Annexin V binding to platelet surface

Mitochondrial membrane potential

Storage characteristics of platelets suspended in standard additive solution: Investigation of the impact of albumin, glucose and acetate on the platelet storage lesion

The standard additive solution (SAS) provided the necessary electrolytes and formed the basis of the storage medium. The impact on the platelet storage lesion of the addition of albumin, glucose and acetate to the additive solution was investigated in three separate studies, termed simply Albumin Study, Glucose Study and Acetate Study. Each experiment within a study required the preparation of four buffy coat-derived PCs suspended in SAS. The influence of donor variability on platelet storage characteristics was minimised by pooling the four platelet concentrates and re-splitting them into four now identical units. “Optimal” concentrations of the two reagents not being investigated were also added to the medium (though as a result of results obtained in the albumin study, albumin was not added to units in the glucose and acetate studies). Four different concentrations of the reagent of interest were added to each of the four units. Samples were removed aseptically for testing on days 2, 3, 6, 8 and 10; day 1 being the day of PC preparation and day 0 the day of whole blood collection.

In addition to the tests listed above, two more assays designed to determine if an apoptosis-like mechanism was associated with the PSL were adopted for this series of studies. These were:

Intracellular calcium levels

Identification and quantitation of phospholipids on the platelet surface (in collaboration with the University Hospital of Wales)

Finally, an additional experiment was performed to determine the impact on platelet storage characteristics of the processing method employed to create the units for the additive study. In this experiment, three platelet concentrates were prepared by the same method but re-suspended in autologous plasma instead of additive solution.

STATISTICAL ANALYSIS

Comparison of PC suspended in 100% autologous plasma versus PC suspended in a 70:30 SSP+™/plasma medium

Normality was not met by some of the data groups (using the Kolmogorov-Smirnov test for normality), therefore summary statistics are presented as the median and range in the tables and median and interquartile range (25^{th} and 75^{th} percentiles) in the graphs. Differences between groups were analysed by the Mann-Whitney Rank Sum test, with a p value <0.05 regarded as statistically significant.

Investigation of the impact of albumin, glucose and acetate on the platelet storage lesion

Due to the small number of replicate experiments (n=3) in the albumin study, statistical comparisons between the test groups were not undertaken and the results from all three replicates are provided in the text. Graphical representations of the results are restricted to displaying the mean of the three results only.

For the glucose and acetate studies, all results are presented as mean ± standard deviation, with statistical comparisons undertaken by a one way ANOVA. A p-value below 0.01 was considered statistically significant. Subsequent multiple comparisons were performed by the Holm-Sidak method, with an overall significance level of 0.05. All graphs and statistical analysis were generated with SigmaPlot version 10.0 (Systat Software Inc., Chicago, IL).

ETHICAL CONSIDERATIONS

Whole blood collections were obtained from volunteer donors in the South Wales region by established blood collection teams of the Welsh Blood Service. The buffy coats were surplus to the requirements of the blood service and were destined to be discarded; thus, there was no impact on the service provided to local hospitals. Buffy coats were initially identified simply by a donation number, with no donor information appended to the units. The platelet concentrates processed from the buffy coats were assigned a test number unrelated to the donation number. Donor anonymity was thus ensured throughout the studies. All components were discarded following testing according to standard operating procedures in place at the Welsh Blood Service. The study was reviewed and approved by the Welsh Blood Service management. The South East Wales Research Ethics Committee was approached and stated that the proposed study raised no ethical issues and did not require an ethics submission (March 2006 – Appendix 1).

PREPARATION OF PLATELET CONCENTRATE UNITS

PLATELET CONCENTRATES IN 100% AUTOLOGOUS PLASMA

Background

Platelet concentrates were prepared using the method employed at the Welsh Blood Service for the routine processing of buffy coat-derived platelet concentrates. The buffy coats were derived from the processing of whole blood donations collected into bottom and top packs (RCB436CU2; Pall Medical, Ascoli, Italy). The whole blood was separated into its components within 8 hours of collection, with the buffy coats stored overnight at 22 ± 2°C without agitation. Four buffy coats are required for the preparation of one adult dose platelet concentrate.

Method

- Connect four ABO-specific buffy coats by means of a TSCD II sterile tubing welder (Terumo Corporation, Tokyo, Japan). Sterile connect an ELX storage pack (Pall Medical, Portsmouth, UK) to the last buffy coat and a plasma unit from one of the four whole blood collections to the first buffy coat.

- Pool the buffy coats together into the last pack, using the plasma to "flush" any remaining material from the buffy coat packs.
- Centrifuge the pooled buffy coat at 900 *g* (based on a 210 mm average radius of rotation) for an accumulated centrifugal effect (ACE) of 1.27×10^7 at 22°C (Sorvall RC12BP; Thermo Fisher Scientific, Loughborough, UK).
- Express the platelet concentrate through the integral Autostop white cell reduction filter (Pall Medical, Portsmouth, UK) using an Optipress II blood processor (Fenwal Europe, Mont-Saint-Guibert, Belgium).

PLATELET CONCENTRATES IN 70% SSP+™ and 30% PLASMA

Background

Commercially available additive solutions licensed in Europe for the production of platelet concentrates require approximately 30% of autologous plasma to be retained in the unit (Sandgren et al., 2010). The commercial additive solution selected was SSP+™ (MacoPharma UK Ltd, Twickenham, UK). The selection was based on *in vitro* studies that suggest platelets are adequately maintained in a SSP+™/plasma suspending medium for at least 7 days, with results comparing favourably with those from other commercial additive solutions (Ringwald et al., 2006, Gulliksson et al., 2002, Hornsey et al., 2006).

Development of Method

A previous study performed at the Welsh Blood Service determined the optimal centrifugation settings for the preparation of platelet concentrates suspended in a medium of approximately 70% plasma and 30% additive solution (Owen H., BSc project). The study added 280 mL of additive solution (T-Sol; Baxter Healthcare Corp., Deerfield, IL) to four pooled buffy coats. To confirm that this would be a suitable volume of SSP+™ to use, data from a study performed at the Welsh Blood Service in 2003 that measured the volume and haematocrit of the dry buffy coats used for the preparation of PCs was reviewed. The mean volume of the buffy coats was 56.3 mL and the mean haematocrit was 0.448 (n=68). The calculated mean volume for plasma in the buffy coats would thus be 31.1 mL. For platelet concentrates comprised of four buffy coats, the total volume of plasma would be 124.3 mL. For 70% of the unit to be

composed of additive solution, 291.2 mL of SSP+™ would need to be added to the pooled buffy coats. The addition of 280 mL of SSP+™ would thus result in a SSP+™ to plasma ratio of approximately 69% to 31%. This was judged acceptable and allowed for the use of the pre-established centrifugation settings.

Method

The method outlined above for the preparation of PCs in 100% plasma was adopted for the preparation of units in 70% additive solution, with the exception that 280 mL of SSP+™ was used to pool the buffy coats. SSP+™ was purchased from MacoPharma in 300 mL packs and the correct volume to be added determined by weight. In addition, due to the lower specific gravity of the suspending medium, the centrifugation setting used for the processing of the pooled buffy coat was a gentler 550 *g* for 4 minutes 30 seconds at 22°C. Leucodepletion of the packs was performed as for the units suspended in 100% plasma.

All units were stored at 22 ± 2°C on a flatbed agitator (Helmer, Noblesville, IN) under constant agitation.

PLATELET CONCENTRATES SUSPENDED IN 100% ADDITIVE SOLUTION

Background

The series of experiments investigating the characteristics of platelets stored solely in additive solution aimed to control the suspending medium in order to determine the impact of various reagents on platelet survival and function *in vitro*. Two principles thus guided the preparation of units; first, the removal of as much plasma as possible and secondly, the successful leucodepletion of the units. As a suspending medium, plasma provides a complex environment for the platelets. Similarly, as white cells degrade during storage they release substances such as cytokines into the extracellular environment which are known to adversely affect platelet survival (Bordin et al., 1994). The presence of either plasma or white cells would introduce a significant variable and would make it more difficult to attribute any changes in platelet characteristics during storage to the presence or absence of the reagent under investigation.

The formulation of the standard additive solution used as the basis of the suspending media was provided by Pall Medical's R&D Group in Covina, California. Its constituents are as follows:

Sodium chloride	110 mmol/L
Potassium chloride	5 mmol/L
Magnesium chloride hexahydrate	3 mmol/L
Citric acid	2.5 mmol/L
Tribasic sodium citrate dihydrate	7.5 mmol/L
Sodium dihydrogen phosphate monohydrate	4 mmol/L

Total volume: 400 mL

Development of Procedure

Standard Additive Solution

The preparation of the standard additive solution was outsourced to Stockport Pharmaceuticals (Stockport, UK). The company provided the required formulation as a single batch with a long shelf life, thus negating another potential source of variability in the results. The solution was transferred from the glass bottles in which it had been supplied to the plastic packs using a transfer needle to directly connect the glass bottle to a spare plasma storage pack.

The procedure was performed in a Biological Safety Cabinet Class II (Bioquell, Andover, UK). A port on the empty plasma pack was opened and a sampling site coupler with a needle injection site (Baxter Healthcare Ltd, Newbury, UK) was inserted. The bottle was supported upside down by a retort stand and the plasma pack secured below it with a second clamp. A sterile 16G transfer needle (Baxa Ltd, Bracknell, UK) was used to pierce the sampling site coupler and the pack manoeuvred to allow the other end of the needle to pierce the cap on the bottle. A sterile venting pin (Baxa Ltd, Bracknell, UK) was inserted into the same cap to allow air displacement to push the solution into the plasma pack (figure 2.1). The surfaces of the sample site coupler and the bottle cap were cleaned with pre-injection swabs (SSL International PLC, Durham, UK), whilst clamps and the operator's hands were sprayed with a chlorhexidone/ethanol

spray (Hydrex Hard Surface Spray, Adams Heathcare, Leeds, UK). Preliminary trials of the method confirmed that the solution remained sterile during the transfer.

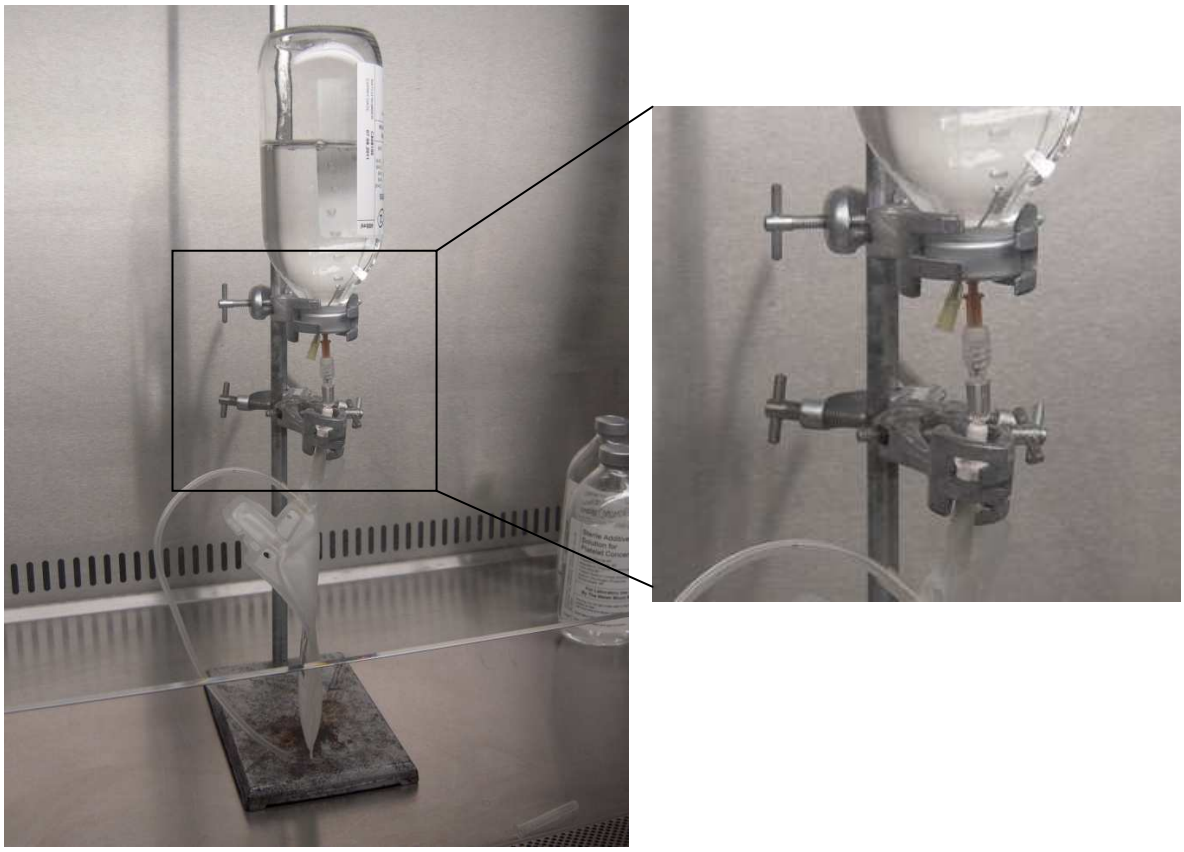


Figure 2.1: Procedure for transferring SAS from manufacturer's bottle to labile plasma pack

The pH of the standard additive solution was 4.9, which would be too acidic even during the processing stage of platelet concentrate production. The pH of the solution was raised by the addition of sodium phosphate dibasic (Na_2HPO_4), with the aim of achieving a pH of 7.2 (as is the case in the commercial solution SSP+™). Subsequent trials suggested a concentration of 55 mmol/L of Na_2HPO_4 would be required to raise the pH of the SAS to approximately 7.2. To confirm whether concerns about the low pH of the additive solution would translate to low pH levels in actual components, four platelet components were individually prepared. Two units were suspended in unaltered SAS and the remaining two in SAS with 55 mmol/L Na_2HPO_4 to compensate for the low pH. The pH_{22^oC} levels for the former two units were 6.0 and 6.1, whilst for the compensated units levels were 6.7 and 7.1. As pH levels below 6.2 have been associated with loss of platelet viability (Murphy et al., 1994, Gulliksson, 2001), it was decided to

modify the composition of the SAS at the time of platelet preparation with the addition of Na_2HPO_4.

Processing of Platelet Concentrates Suspended in Additive Solution

Preparation of reagents

Albumin

Zenalb®20 (Bio Product Laboratory, Elstree, UK) is a 20% (w/v) solution routinely issued by UK transfusion centres to hospitals and was selected as a cost effective source of human albumin. The normal concentration of albumin in the circulation is 3.3 – 5.2 g/dL (Boldt, 2010). Adopting 4.0 g/dL as the optimal concentration; a unit volume of 300 mL would require the addition of 12 g of albumin, equating to a volume of 60 mL of Zenalb®20. For the albumin study, one of the four replicate packs would thus contain 60 mL albumin, one unit would contain half this concentration (30 mL) and a third unit would contain twice this concentration (120 mL). The fourth unit would be suspended in SAS only. (All four units would also contain defined concentrations of acetate, glucose and sodium bicarbonate – see below). A method for aseptically replacing the correct volume of SAS with the required volume of albumin was developed:

- Transfer the contents of three 100 mL Zenalb®20 bottles into a single empty plasma storage pack, using the same procedure described above for the transfer of SAS from glass bottles to a plastic storage pack.
- Sterile connect a waste pack to three of the packs containing 400 mL of SAS. Remove the volume of SAS to be replaced by albumin, estimating the volume by weight.
- Sterile connect the pack containing albumin to each of the three packs in turn and transfer the required volume of albumin.

The contents of the modified SAS packs are now ready to re-suspend the platelets following their hard centrifugation.

Unit identification:

The four units in each experiment were identified by a test number as follows:

ALB-000-nn (no albumin added)

ALB-030-nn (30 mL albumin added; equivalent to 2 g/dL)

ALB-060-nn (60 mL albumin added; equivalent to 4 g/dL)

ALB-120-nn (120 mL albumin added: equivalent to 8 g/dL)

(In each case, nn refers to a unique test number)

Glucose

Glucose was purchased as dextrose monohydrate (Sigma-Aldrich, Dorset, UK: code D9559, molecular weight = 198.17). The initial study establishing the storage characteristics of platelet concentrates suspended in 100% autologous plasma (study 1; chapter 3) found the mean concentration of glucose in these units to be 15.2 mmol/L at the beginning of storage. The "optimal" concentration in SAS-suspended units was thus chosen to be 15 mmol/L. This concentration was also expected to last for the entire storage period, as studies have shown that glucose utilisation rarely exceeds 1 mmol/L per day (Murphy, 1999). Aiming for a final unit volume of 315 mL and the addition of glucose in a 5 mL volume:

$$C_i V_{i =} C_f V_f$$

$$C_i \times 0.005 = 15 \times 10^{-3} \times 0.315$$

$$C_i = 0.945 \text{ mmol/L}$$

Therefore, 0.9364 g of dextrose monohydrate was dissolved in 5 mL of distilled water to yield the optimal concentration.

Unit identification:

The four units in each experiment were identified by as follows:

GLU-000-nn (no glucose added)

GLU-075-nn (final concentration of 7.5 mmol/L)

GLU-150-nn (final concentration of 15 mmol/L)

GLU-300-nn (final concentration of 30 mmol/L)

(In each case, nn refers to a unique test number)

Acetate

Acetate was sourced as sodium acetate trihydrate (Sigma-Aldrich, Dorset, UK: code S1304, molecular weight = 136.08). Exogenously added acetate has been reported to be metabolised at approximately 0.5 mmol/L per day per 10^{12} platelets (Murphy, 1995). In a later publication, the same author recommended that the optimal concentration of acetate should be at least 2 mmol/L for each day of projected storage (Murphy, 1999). As the maximum storage period originally envisaged for this series of experiments was 14 days, this would require a concentration of 28 mmol/L acetate to be added to the storage media, corresponding well with the concentration of 32.5 mmol/L acetate present in the commercial additive solution, SSP+™. Aiming for a final unit volume of 315 mL and the addition of acetate in a 5 mL volume:

$$C_i V_{i=} C_f V_f$$

$$C_i \times 0.005 = 28 \times 10^{-3} \times 0.315$$

$$C_i = 1.764 \text{ mmol/L}$$

Therefore, 1.2002 g of sodium acetate trihydrate was dissolved in 5 mL of distilled water to yield the "optimal" concentration.

Unit identification:

The four units in each experiment were identified by as follows:

ACE-000-nn (no acetate added)

ACE-014-nn (final concentration of 14 mmol/L)

ACE-028-nn (final concentration of 28 mmo/L)

ACE-056-nn (final concentration of 56 mmol/L)

(In each case, nn refers to a unique test number)

Sodium bicarbonate

All units were provided with the same concentration of sodium bicarbonate (Sigma-Aldrich; Dorset, UK: code S1554, molecular weight = 84.01) for increased buffering capacity. The concentration was derived from a variety of sources. First, the normal concentration of bicarbonate in adult humans is approximately 25 mmol/L (Kumar and Clark, 2009). In addition, a number of studies evaluating the storage characteristics of platelet concentrates suspended in plasma have measured mean bicarbonate levels at the start of storage between 15 – 19 mmol/L (Gulliksson, 2000, Sweeney et al., 2006,

Wagner et al., 2008, Hornsey et al., 2008). Finally, Pall Medical's R&D Group recommended the final concentration of sodium bicarbonate should be 20 mmol/L (S. Holme, personal communication) and this value was adopted for all additive solution studies. Aiming for a final unit volume of 315 mL and the addition of acetate in a 7.5 mL volume, the final concentration of acetate required was:

$$C_i V_{i=} C_f V_f$$

$$C_i \times 0.0075 = 20 \times 10^{-3} \times 0.315$$

$$C_i = 0.840 \text{ mmol/L}$$

Therefore, 0.5293 g of sodium bicarbonate was dissolved in 7.5 mL of distilled water to yield the optimal concentration. (The slightly higher volume of distilled water compared to glucose and acetate was necessary to fully dissolve the sodium bicarbonate).

Preparation of Units

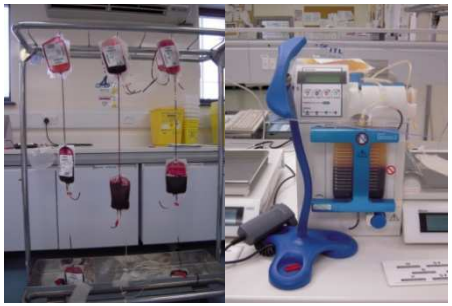
Blood services occasionally receive requests to provide platelet concentrates with minimal plasma levels for individual patients. The protocol developed by the Welsh Blood Service for such an eventuality was modified for the processing of platelet concentrates in SAS and is described in detail below. This method was not initially preferred because it requires that the platelets experience a hard centrifugation step without the expected cushioning provided by the other cellular components, likely resulting in higher initial levels of platelet activation. However, the requirement for leucodepleted units dictated the adoption of this harsher processing methodology.

- Four ABO-specific platelet concentrates were prepared from buffy coats held overnight without agitation at 22 ± 2°C by the method previously described in connection with the preparation of PC in 100% plasma.
- The four platelet concentrates were sterile connected to a 2 litre transfer pack (Terumo Europe, Leuven, Belgium) and the contents gently mixed.
- The pooled concentrates were split by weight into the four ELX packs, resulting in four identical units. A waste pack was sterile connected to each of the four PCs.
- The four PCs were centrifuged at 2400 *g* for 10 minutes at 22°C to pellet the platelets (calculated from an average radius of rotation for the rotor of 210 mm).

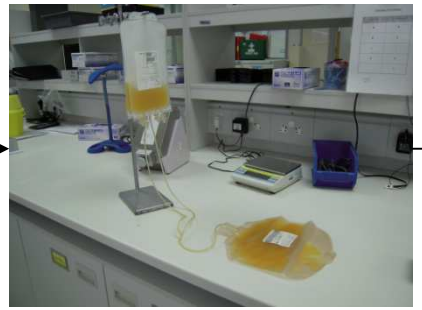
- The majority of the plasma was removed into the waste pack by gravity, with care exercised to minimise the loss of platelets.
- Packs containing SAS were sterile connected to the ELX packs and the platelets re-suspended in 300 mL of SAS as soon as possible by gentle manual mixing.
- The required concentrations of glucose, sodium bicarbonate and acetate were added to the units aseptically in a Class II biological safety cabinet using sterile syringes and 0.2 µm syringe filters (Pall Life Sciences, Farlington, UK).
- Units were stored on a flatbed agitator (Helmer, Noblesville, IN) at 22 ± 2°C under constant agitation.

Figure 2.2 provides a diagrammatic summary of the processing method.

Figure 2.2: PROCESSING OF PLATELET CONCENTRATES SUSPENDED IN ADDITIVE SOLUTION



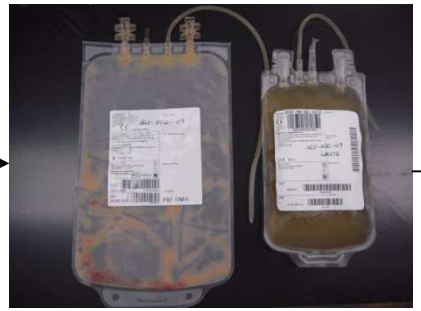
4 ABO-specific PC processed in plasma, including leucodepletion



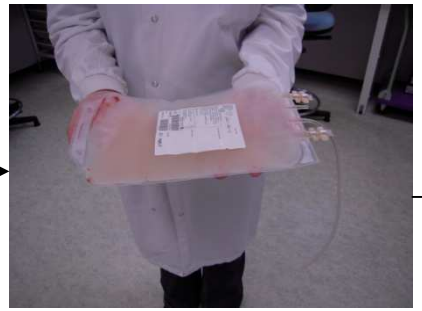
4 PC pooled into 2 L transfer pack

Pooled PC re-split into four identical units

Centrifuge at 2400 *g* for 10 minutes at 22°C to pellet platelets



Removal of waste plasma by gravity



Re-suspension of platelets in SAS by gentle manual mixing. (Albumin is added to required units at this stage)

Addition of required concentrations of glucose, acetate and sodium bicarbonate injected into units via 0.2 µm syringe filter. Storage of units at 22°C with constant agitation

Determination of Residual Plasma

The residual plasma after PC preparation was calculated by volume in seven units from the glucose study as follows:

- Calculate the volume of the PC pool before centrifugation.
- Calculate the volume of the PC pool after centrifugation and after removal of the majority of the plasma.
- Calculate the volume of the platelets (based on the MPV) and subtract from the above values.
- Calculate the percentage of plasma retained.

A mean percentage of 4.46 ± 1.11% of plasma was retained by the method adopted.

SAMPLING FROM UNITS

The units were gently mixed and the tail stripped three times to obtain a representative sample without unduly damaging the platelets through excessive stripping. A sampling pouch (MacoPharma, Mouvaux, France) was aseptically connected to the tail on the platelet concentrate by means of a TSCD II sterile tubing welder (Terumo Corporation, Tokyo, Japan) and approximately 15 mL transferred from the unit. The sample was subsequently dispensed into a 30 mL plastic universal container (Sterilin Ltd, Caerphilly, UK) from which all further samples for testing on the day were obtained.

DESCRIPTIVE PARAMETERS

UNIT VOLUME

Unit volume was calculated by subtracting the tare weight of the ELX storage pack (36.6 g) from the gross weight and dividing the result by the specific gravity of the suspending medium. The specific gravities for the various suspending media were as follows:

100% plasma	1.03 (Dumont, 2003)
70% plasma/30% SSP+	1.01 (Sandgren, 2008)
100% SAS	1.00 (as water)

PLATELET CONCENTRATION AND MEAN PLATELET VOLUME

A sample of approximately 500 – 1000 µL was transferred into a vacutainer containing K_2EDTA as an anticoagulant (Greiner Bio-One GmbH, Kremsmünster, Austria) and the vial allowed to mix for at least 30 minutes before analysis. Platelet concentration ($\times 10^9$/L) and mean platelet volume (MPV) were obtained with a Pentra 80 haematology analyser (HoribaABX Diagnostics, Montpellier, France). The platelet yield was calculated by multiplying the platelet concentration by the unit volume in litres.

RESIDUAL WHITE CELL COUNTING

Confirmation that the units had been successfully leucodepleted was obtained via a flow cytometric assay used routinely at the Welsh Blood Service for the quality monitoring of the white cell reduction processes. The method employs a kit (DNA-Prep; Beckman Coulter, Milton Keynes, UK) that includes propidium iodide (PI) as a nuclear stain. Fluorospheres with a known concentration allows absolute counting to calculate the white cell concentration per µL. Each sample was acquired for three minutes at high flow rate on an FC500 flow cytometer (Beckman Coulter, Miami, FL). Previous validation of the assay determined a limit of quantitation of 0.2 WBC/unit.

CONFIRMATION OF END OF STORAGE STERILITY

At the end of storage period, approximately 20 mL from the unit was transferred to an aseptically connected sampling pouch (MacoPharma, Mouvaux, France). The sample was equally divided between an aerobic and anaerobic culture bottle (Biomérieux Inc, Durham, NC) in a Class II biological safety cabinet (Bioquell, Andover, UK). The bottles were incubated at 36°C for 7 days in a bacterial monitoring system (BacTAlert 3D, Organon Teknika Corp., Durham, NC). The units were retained throughout the incubation period in order to allow for the confirmation of any positive results. As most of the assays would be adversely affected by the presence of bacteria, results from any units confirmed to be contaminated would be rendered void.

MEASUREMENT OF pH AND METABOLITES

Background

Levels of metabolites were measured with an ABL705 biochemistry analyser (Radiometer Ltd, Copenhagen, Denmark). The instrument is regularly calibrated, with performance monitored by quality control material. Measurements are performed by the instrument at 37°C.

The oxygen consumption rate (OCR) is a measure of oxygen utilisation that takes into account the platelet concentration and the permeability characteristics of the storage pack (Kilkson et al., 1984), and was adopted as a complementary measure to the extracellular partial pressure of oxygen.

Practical Considerations

Extracellular pH was reported at 22°C for the extended storage study with PCs suspended in plasma or SSP+™ to reflect the storage temperature and the temperature recommended for the reporting of quality monitoring in the UK Guidelines (James, 2005). The temperature correction provided by the instrument was adopted for the pH measurements (Severinghaus, 1966), though there is some uncertainty regarding the accuracy of this conversion factor when applied to PCs suspended in a medium other than plasma (Cardigan et al., 2008). Due to the minimal volumes of plasma in PC stored in SAS, pH was reported at 37°C for these studies (Ringwald et al., 2012). Blood gases were reported at 37°C to conform to most of the existing literature. By contrast, the OCR was calculated using partial pressures of oxygen at 22 °C – the targeted storage temperature of the components. The temperature correction calculation for pO_2 provided by the biochemistry analyser was regarded as unsuitable for this application since it contained a factor for a measured or assumed value of oxyhaemoglobin, assuming the use of a whole blood sample. Thus, the correction factor provided by Kilkson et al was adopted, requiring the multiplication of the measured pO_2 at 37°C by a factor of 0.856 (Kilkson et al., 1984).

Due to the labile nature of blood gases, in particular pO_2, measurements were acquired as soon as possible after sampling and in all cases within two minutes of sampling, with syringes capped during transfer to the instrument.

Calculation of oxygen consumption rate

Based on the following calculation:

$$C(O_2) \times \frac{platelet\ content}{10^9} = K(O_2) \times \frac{\Delta PO_2}{760}$$

(Kilkson et al., 1984)

where:

$C(O_2)$ is the oxygen consumption rate (nmol/min/10^9 plts)

$K(O_2)$ is the capacity for O_2 transport by the container

ΔPO_2 is the partial pressure of oxygen in the atmosphere minus the partial pressure of oxygen in the concentrate

The O_2 transmission rate for the ELX storage pack is 2201 mL m^{-2} day^{-1} (Pall Medical, Covina, CA; personal correspondence). Rearranging the above equation and using kPa as the unit for pressure:

$$C(O_2) = 2201 \times \frac{\Delta PO_2 / 101.325}{Plt\ concentration\ (10^9)}$$

where:

101.325 = atmospheric pressure (kPa) (Barry and Chorley, 2003)

21.3 = partial pressure of O_2 in the atmosphere (kPa) (21%) - used to calculate ΔPO_2

Calculation of Bicarbonate Levels

Bicarbonate levels were calculated based on the relationship between pH, $[CO_2]$ and $[HCO_3^-]$ described by the Henderson-Hasselbalch equation:

$$pH = pK + \log \frac{[HCO_3^-]}{[CO_2]}$$

The first dissociation constant for carbonic acid (K_{a1}) in plasma at 37°C was employed, with a pK_a value of 6.1 (Wooten, 2003). A value for the Henry constant of 0.034 (Sander) was used to relate the concentration of CO_2 to its measured partial pressure. The concentration of bicarbonate was thus calculated as:

$$[HCO_3^-] = pCO_2 \times 10^{(pH-6.1)} \times 0.034$$

(with the partial pressure of oxygen converted from kPa to mmHg by multiplying the former by the conversion factor of 7.500638 (ABL705 Reference Manual)).

MEASUREMENT OF ATP AND ADP

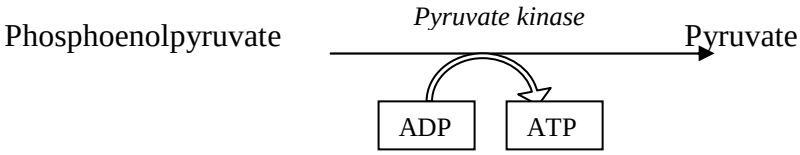
Background

The assay was developed by the Coagulation Laboratory at the University Hospital of Wales (UHW), Cardiff and modified for use with a Lucy 1 microplate luminometer (Anthos, Salzburg, Austria). The principle behind the assay is the ATP-dependent oxidation of luciferin (D-LH_2):

$$\text{D-}LH_2 + ATP + O_2 \xrightarrow{\textit{Luciferase}} AMP + PP + \text{D-L} + CO_2 + LIGHT$$

(Holme and Peck, 1998)

The firefly luciferase-catalysed reaction generates light in the visible range (540-600 nm), the intensity of which can be related to the concentration of ATP by means of a standard line. Levels of ADP in the sample are measured indirectly in a separate step by first converting ADP to ATP. This utilises the final reaction of the glycolytic pathway in which phosphoenolpyruvate (PEP) is converted to pyruvate, irreversibly transferring a phosphoryl group from PEP to ADP in the process:



(*Courtesy of University Hospital of Wales, Cardiff)*

The "native" level of ATP measured in the first step of the assay is subtracted from the "total" ATP level obtained in the PEP conversion to pyruvate to yield the concentration of ADP.

Development of the Assay

Platelet concentrate samples from the buffy coat-derived process have a platelet concentration approximately three times as high as the concentration in whole blood from a normal individual. As the ATP extraction method used is based on the latter, the PC samples were diluted 1 in 3 with double-centrifuged autologous plasma, resulting in

concentrations of approximately 250 to 300 × 10^9/L. Following the extraction of ATP by ethanol, the samples were stored below -70°C before analysis.

Samples were initially prepared in 12 x 75 mm plastic test tubes and pre-diluted 1 in 10 in a Tris-EDTA assay buffer. The ATP standard incorporated in the kit was similarly diluted. The same volume of sample and ATP standard was used in order to simplify the calculation.

Method

Microplates

Chromalux HB high binding, high reflectivity white polystyrene microplates suitable for luminescence applications were purchased from Dynex Technologies (Jencons Scientific Ltd, Leighton Buzzard, UK)

Reagents

ATP Kit SL (Biothema Luminescent Assays, Handen Sweden. Distributor: Labtech International, Ringmer, UK: code LAB-144-041).

Kit comprises:

ATP SL reagent (containing D-luciferin, luciferase, Mg^{2+}, inorganic pyrophosphate and bovine serum albumin)

ATP standard at a concentration of 10^{-5} mol/L ATP

Tris-EDTA buffer

Phosphoenolpyruvate (PEP) (Sigma-Aldrich, Dorset, UK: code P7002)

Pyruvate kinase from rabbit muscle, Type VII (Sigma-Aldrich, Dorset, UK: code P7768).

Bovine serum albumin (BSA) (Sigma-Aldrich, Dorset, UK: code A7030).

Magnesium acetate tetrahydrate (Merck, Darmstadt, Germany: code A712419 628)

Potassium acetate (Sigma-Aldrich, Dorset, UK: code 236497)

Tris (hydroxymethyl) amine (VWR International, Poole, UK: code 103154M)

Acetic acid, glacial (Fisher Scientific, Loughborough, UK: code A/0406/PB08)

Ethylenediamintetraacetic acid (EDTA) disodium salt (VWR International, Poole, UK: code 100933T)

Triton X-100 (Sigma-Aldrich, Dorset, UK: code T9284)

Ethanol, absolute (Sigma-Aldrich, Dorset, UK: code 02880)

Preparation of Reagents

Disodium EDTA: 0.1 mol/L solution in 0.9% NaCl.

Triton X-100: 10% (v:v) in 0.9% NaCl (Viscous fluid: pipette slowly and allow to dissolve overnight at ambient temperature).

Magnesium acetate: 0.1 mol/L solution in distilled water.

Potassium acetate: 0.1 mol/L solution in distilled water.

Assay buffer (Tris-EDTA buffer)

- Dissolve 1.2114 g of Tris (0.1 mol/L) and 0.0744 g of EDTA (0.002 mol/L) in 100 mL of distilled water
- Adjust pH to 7.75 with glacial acetic acid.
- Prepare on the day of assay and discard any excess.

BSA buffer

- To 10 mL of assay buffer add 0.01 g of BSA
- Prepare on the day of assay and discard any excess.

PEP stock solution; 100 µM

- Add 0.0117 g to 500 µL of distilled water.
- Prepare on the day of assay and discard any excess

PEP working solution

- Prepare just before use as follows:

100 µM PEP stock solution	50 µL
Magnesium acetate, 0.1 M	1.250 mL
Potassium acetate, 0.1 M	0.750 mL

Pyruvate kinase

- Prepare a 1 in 10 dilution using BSA buffer (10 µL required per sample).

ATP-SL reagent

- Add the entire contents of the diluent vial to the lyophilised reagent. Protect from light. (On the advice of colleagues from UHW, the reconstituted reagent was stored for a maximum of three days at 2 – 8°C).

ATP Standard

- Prepare a 1 in 10 dilution in Tris-EDTA buffer to a total volume of 10 mL. Protect from light. Discard excess.

ATP Extraction

- Prepare a 1 in 3 dilution of the platelet concentrate in double-centrifuged autologous plasma to yield an approximate platelet concentration of 300×10^9/L.
- Pipette 500 µL of diluted sample into two labelled cryovials (one vial to be retained as a spare).
- Add 50 µL of 0.1 mmol/L EDTA and 50 µL of 10% Triton X-100.
- Vortex for a few seconds and add 500 µL of absolute ethanol.
- Vortex for a few seconds. Incubate the vials for 15 to 30 minutes at 2 – 8°C before storing below -70°C.

Assay

- Thaw samples at 37°C in a waterbath.
- Centrifuge at 2000 *g* for 15 minutes at 4°C. Decant the supernatant into a labelled tube.
- Prepare 1 in 10 dilution of the supernatant, using assay buffer as the diluent.
- Prepare two tubes per sample and a pair of blanks as follows:

	Tube A	Tube B	Blank	Blank + PK
Pre-diluted sample	10 µL	10 µL	/	/
Assay buffer	400 µL	400 µL	410 µL	410 µL
PEP working solution	40 µL	40 µL	40 µL	40 µL
Pyruvate kinase	/	10 µL	/	10 µL

- Incubate for 30 minutes at ambient temperature in the dark.
- Dispense 230 µL of "Blank" in position A1 of a microplate and 230 µL of "Blank + PK" in position A2.

- Dispense 230 µL of "tube A" for the first sample into B1 and 230 µL of the corresponding "tube B" in position B2.
- Follow the pattern with any remaining samples. Load the plate into the luminometer.

General Operation of the Luminometer

Set the operating temperature of the instrument to 25°C. The input line from dispenser 1 is secured in the ATP-SL reagent whilst the input line from dispenser 2 is secured in the ATP standard. Prime the Hamilton dispenser three times and insert the output lines from both dispensers into their corresponding apertures in the luminometer. Dispenser 1 is programmed to dispense 25 µL of ATP-SL reagent on an initial measurement run, with dispenser 2 dispensing 5 µL of ATP standard on the following run. Sixty points are measured over a one minute period, with the peak intensity of light emitted comprising the reportable value. Four peak measurements are obtained per sample:

A1: No pyruvate kinase added

A2: No pyruvate kinase added; ATP standard added

B1: Pyruvate kinase added

B2: Pyruvate kinase added; ATP standard added

Calculation:

ATP standard is provided in the kit at a concentration of 10^{-5} mol/L of ATP. The final volume dispensed into the microplate following sample preparation is 255 µL + 5 µL of ATP standard. Therefore, the concentration of standard in the microplate well is:

$$255/5 = 51$$

$$10^{-5}/51 = 1.96 \times 10^{-7} \text{ mol/L}$$

Calculation of the blank:

$$\text{Blank} = \frac{Blank1}{(Blank2 - Blank1)} \times 1.96$$

ATP concentration ($\times 10^{-7}$):

$$\left(\frac{A1}{(A2 - A1)} \times 1.96 \right) - Blank$$

ATP (µmol/10^{11}platelets):

$$ATP \times 10^{-7} \times 10 = ATP \times 10^{-6} \equiv ATP\ \mu mol/L$$

Convert platelet concentration from 10^9/L to 10^{11}/L

Divide ATP concentration (µmol/L) by platelet concentration (x 10^{11} platelets)

Total ATP concentration as measured by the addition of pyruvate kinase in tube B is obtained as above using the appropriate peak measurements from tube B and the blank containing pyruvate kinase.

ADP concentration (µmol/10^{11}platelets):

Total ATP – ATP

HYPOTONIC SHOCK RESPONSE

Background

The hypotonic shock response (HSR) assesses the ability of platelets to recover from the addition of a hypotonic solution to the suspending medium. Metabolically viable platelets with intact membranes are able to extrude the excess water, in conjunction with K^+ and Cl^- ions, in an ATP dependent process (VandenBroeke et al., 2004, Okada et al., 2001). The osmotic swelling caused by the uptake of water by the platelets results in a lowering of the refractive index of the platelets and a related increase in light transmission. The effect is reversed as healthy platelets resume their normal volume. This change in light transmission can be measured with a platelet aggregometer. The initial decrease in transmission following the addition of a volume of distilled water is measured. After a four minute incubation at 37°C with constant stirring the light transmission is measured again and the percentage recovery to the hypotonic shock is calculated as follows (see figure 2.3):

$$HSR\ recovery\ (\%) = \frac{Y' \times 100}{Y - PBS}$$

(modified from Holme, 1998)

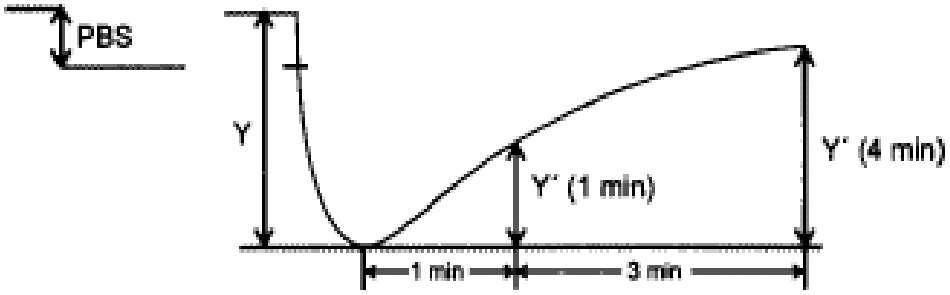


Figure 2.3: Diagram illustrating the expected chart output for the hypotonic shock response (Holme et al., 1998) (Reproduced with permission, © John Wiley and Sons)

where,

Y = Initial decrease in light transmission

Y' = Light transmission after 4 minutes (Light transmission after 1 minute not used)

PBS = Light transmission through sample containing PBS instead of water (to compensate for the effect of diluting the sample)

Development of the Assay

The HSR assay in use at the Pall Medical Blood Processing R&D Group in the United States was adopted for this study following a visit to that laboratory in 2006. The method used an SPA-2000 aggregometer (Chrono-Log, Havertown, PA) which performs the calculations automatically; however, a chart recorder is also needed to provide a visual record of the reaction and allow the operator to determine if the kinetics of the response are acceptable.

Some points to note include:

- The assay was performed in duplicate, and if the HSR recoveries varied by more than 10%, the assay was repeated to provide a third result. The mean of the two closest values was reported as the percentage recovery.
- Autologous platelet-poor plasma was used as the sample diluent, following published recommendations for its use in preference to AB plasma or additive solution (VandenBroeke et al., 2004).

Method

Reagents

Phosphate buffered saline (PBS), pH 7.4 (Sigma-Aldrich, Dorset, UK: code P-4417)

HEPES (VWR International Ltd, Lutterworth, UK: code 441485H)

Distilled water

Preparation of reagents

Phosphate buffered saline:

Dissolve 1 tablet of PBS in 200 mL of distilled water. Store at 2 – 8°C for up to 6 months – discard if there are visible signs of contamination.

HEPES buffer (1 mol/L):

Dissolve 2.383 g of HEPES in 10 mL of distilled water. Store at 2 – 8°C for up to 6 months – discard if there are visible signs of contamination.

Preparation of platelet sample and platelet- poor plasma (PPP)

HEPES buffered platelet poor plasma (PPP):

Double centrifuge approximately 12 mL of autologous plasma at 2000 *g* for 10 minutes at 22°C. Dispense 10 mL into a universal and add 150 μL of HEPES buffer (final concentration 15 mmol/L).

Platelet concentrate sample:

Require 7 mL of sample with a platelet concentration of 300×10^9/L; therefore, in 7 mL, aiming to contain 2100×10^6 platelets. Example:

PC with a concentration of 1264×10^6/mL platelets

2100/1264 = 1.661 mL of PC sample required in

7.000 – 1.661 = 5.339 mL of HEPES-buffered PPP

Assay

- Switch on SPA-2000 aggregometer. Ensure temperature is 37 ± 1°C before use. Place toggle switch on HSR.
- Warm distilled water and PBS in a 37°C waterbath.
- Pipette 500 μL of HEPES-buffered PPP into a microcuvette containing a stir bar and place in the well marked “PPP”.
- Pipette 500 μL of pre-warmed, diluted platelet concentrate sample into each of four microcuvettes containing stir bars. Place in the 37°C incubation wells.

- Press STEP and insert one of the samples into the "PRP" well following the instrument's directions. Press STEP again.
- When the instrument prompts for the addition of PBS, firmly inject 250 µL of pre-warmed PBS into the sample and press STEP once immediately.
- When the instrument prompts for a new sample, remove the PBS-diluted sample and insert a second PC sample into the "PRP" test well and press STEP once.
- When the instrument prompts for the addition of water, firmly inject 250 µL of pre-warmed distilled water into the sample microcuvette and press STEP once.
- The aggregometer will automatically calculate and print the %HSR after a four minute recovery period.
- Repeat the procedure to obtain a duplicate result. Ensure the results are within 10% of each other. If not, repeat the assay.
- Report the mean of the replicate results.

EXTENT OF SHAPE CHANGE

Background

Addition of ADP to a suspension of platelets unable to aggregate due to the presence of EDTA results in a decrease in light transmission through the sample which can be measured with a platelet aggregometer. This photometric effect is generally attributed to a disc to sphere transformation by healthy platelets in response to the weak agonist. Platelets that have already changed to a spherical form as a result of insult, including prolonged storage, cannot undergo further shape change (VandenBroeke et al., 2004). The percentage decrease in light transmission is thus related to the proportion of platelets with a normal discoid morphology in the original sample and provides a more objective measure of platelet morphology than light microscopy (Murphy et al., 1994). The derivation of the calculation for the extent of shape change is as follows (Holme et al., 1998):

where,

X = % transmission of platelet poor plasma (PPP)

Y = % transmission of platelet rich plasma (PRP)

A = maximum decrease following shape change

Conversion of the percentage of transmission to extinction is provided by

$$E = -\log(T\%/100)$$

Therefore,

$$E_{PPP} = -\log(X/100)$$

$$E_{PRP} = -\log(Y/100)$$

$$E_{\text{shape change}} = -\log[(Y-A(X-Y))/100]$$

$$ESC = (E_{\text{shape change}} - E_{PRP})100\%/(E_{PRP} - E_{PPP})$$

Development of the Assay

The extent of shape change (ESC) assay used the ChronoLog SPA-2000 aggregometer. The %ESC was calculated automatically by the instrument, with a chart recorder also providing a graphical representation of the reaction.

Method

Reagents

Phosphate buffered saline (PBS), pH 7.4 (Sigma-Aldrich, Dorset, UK: code P-4417)

Adenosine diphosphate (ADP) (Sigma-Aldrich, Dorset, UK: code 01905)

Ethylenediaminetetraacetic acid (EDTA) disodium salt (VWR International Ltd, Lutterworth, UK: code 443882G)

HEPES (VWR International Ltd, Lutterworth, UK: code 441485H)

Preparation of reagents

Phosphate buffered saline:

Dissolve 1 tablet of PBS in 200 mL of distilled water. Store at 2 – 8°C for up to 6 months – discard if there are visible signs of contamination.

ADP (1 mmol/L):

Dissolve 0.0043 g ADP in 10 mL of PBS. Pipette 200 µl aliquots into labelled cryovials and store below -60°C for up to 6 months

EDTA (0.1 mol/L):

Dissolve 3.722 g of EDTA in 100 mL of distilled water. Store at 2 – 8°C for up to 6 months – discard if there are visible signs of contamination.

HEPES buffer (1 mol/L):

Dissolve 2.383 g of HEPES in 10 mL of distilled water. Store at 2 – 8°C for up to 6 months – discard if there are visible signs of contamination.

Preparation of platelet sample and platelet- poor plasma (as for HSR assay)

The same sample prepared for the HSR assay can be used for the ESC assay.

Assay

- Switch on SPA-2000 aggregometer. Ensure temperature is 37 ± 1°C before use. Place toggle switch on ESC.
- Pipette 500 μL of HEPES-buffered PPP into a microcuvette containing a stir bar and place in the well marked "PPP".
- Pipette 500 μL of pre-warmed, diluted platelet concentrate sample into each of two microcuvettes containing stir bars. Place in the 37°C incubation wells.
- Press STEP and insert one of the samples into the "PRP" well following the instrument's directions. Press STEP again.
- When the instrument prompts for the addition of EDTA, firmly inject 10 μL of EDTA into the sample and press STEP once immediately.
- When the instrument prompts for the addition of ADP, firmly inject 10 μL of ADP into the sample microcuvette and press STEP once immediately.
- The aggregometer will automatically calculate and print the %ESC.
- Repeat the procedure to obtain a duplicate result. Ensure the results are within 5% of each other. If not, repeat the assay and report the mean of the two closest results.

SURFACE CD62P EXPRESSION AND ANNEXIN V BINDING

Background

CD62P is an adhesion molecule stored in the α-granules of platelets and expressed on the platelet surface following activation (Berger et al., 1998). An anti-CD62P monoclonal antibody conjugated to a fluorescent dye allows for the identification and quantitation by flow cytometry.

Annexin V is a glycoprotein with a high binding affinity for negatively charged phospholipids in the presence of calcium ions (Picker et al., 2009). These lipids include phosphatidylserine (PS) and phosphatidylethanolamine (PE) which are normally preferentially found on the inner leaflet of the platelet membrane. Failure of the calcium and ATP-dependent processes that maintain this asymmetric distribution results in translocation of the phospholipids to the outer leaflet. Addition of exogenous annexin V conjugated to a fluorescent dye is able to bind to the negatively charged phospholipids on the platelet surface and allow for the monitoring of this phenomenon throughout storage (Dachary-Prigent et al., 1993).

Development of the Assay

The assay combines the measurement of the surface expression of CD62P and annexin V binding and was initially developed by the Welsh Blood Service in collaboration with Beckman Coulter as part of an MSc project investigating the effect of bacterial contamination on platelet activation (Pearce N, MSc project). The assay uses annexin V bound to fluorescein isothiocyanate (FITC) provided as a kit that also includes a concentrated buffer solution incorporating Ca^{2+} ions. Anti-CD62P bound to phycoerythrin (PE) was purchased separately. Fluorescence was detected on the FL1 and FL2 detectors, respectively, of an FC500 flow cytometer (Beckman Coulter, Miami, FL). Platelets were identified on their scatter characteristics using the automatic gating function provided by the FC500 to avoid operator variability. Although the option to use the largest gate was selected (excluding 0.11% of the population), it is acknowledged that by using this approach, platelet fragments and platelets with abnormal morphology which could express surface CD62P and/or aminophospholipids, and may thus be of biological importance, could be excluded from the analysis. Though this limitation was expected to be more evident in samples from older units, the software proved flexible enough to deal with the change in morphology occurring in the majority of the population, as illustrated in figure 2.4.

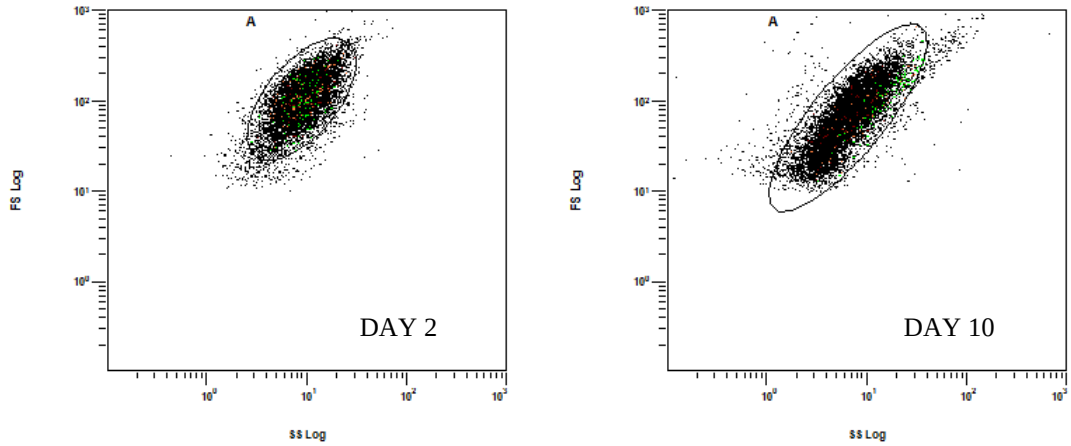


Figure 2.4: Scatter characteristics of events from glucose study. Example taken from unit with no added glucose and comparing the scatter characteristics on day 2 compared to day 10. Discriminator is set at 2 on forward scatter

A separate tube using anti-CD61 conjugated to PC7 was used to verify that the population identified on scatter comprised predominantly of platelets. (A separate tube was employed as a verifier as it was suspected that the CD61 antibody was interfering with the binding of the anti-CD62P and resulting in a lower estimation of surface CD62P. A similar approach was recently described by Albanyan et. al (Albanyan et al., 2009). In addition to determining the percentage of platelets expressing CD62P and binding to annexin V, the assay provides a measure of the degree of surface expression per platelet by means of the mean or median fluorescence intensity. The mean fluorescence intensity was used for the binding of annexin V as there was a clear separation between negative and positive events. By contrast, the expression of surface CD62P was not clearly defined and thus the median fluorescence intensity was preferred (figure 2.5). The regions used to identify positive events were positioned for each individual assay by means of isotypic controls, with the region set to include 1% of negative events.

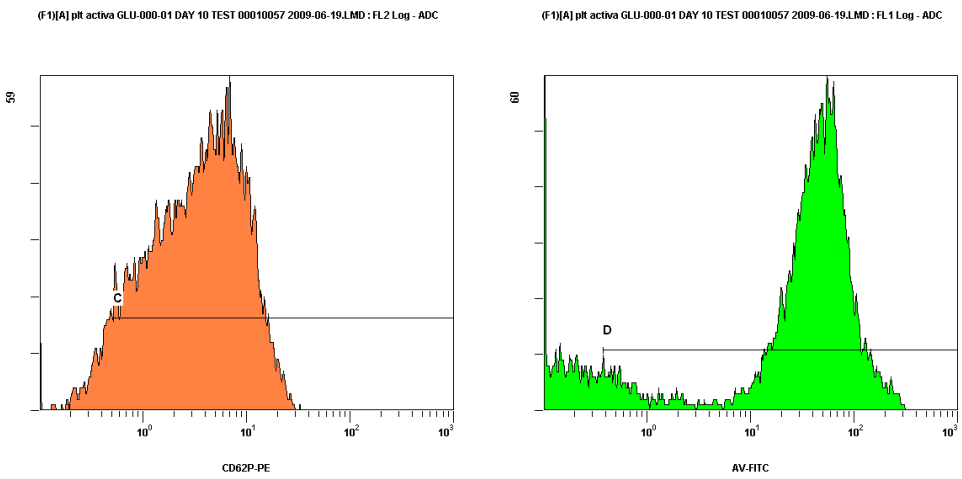


Figure 2.5: Comparison of single parameter histograms illustrating surface expression of CD62P and annexin V binding on day 10 platelets (glucose study)

During the initial development phase, the final volts and gains chosen for the assay were transferred to a separate protocol. Fluorospheres (Flow-Set; Beckman Coulter, Milton Keynes, UK) acquired through this protocol were used to set targets identifying where the Flow-Set fluorospheres were expected to fall with subsequent applications of the protocol. Performing the Flow-Set protocol prior to the assay thus allowed confirmation that events will fall within the expected regions. The samples were not fixed; therefore, the assay was performed as quickly as possible to minimise any extraneous activation of the platelets.

Assay

Reagents

Annexin V-FITC kit (Beckman Coulter, Milton Keynes, UK: code IM3546)

Annexin V buffer (10x) (Becton Dickinson, Oxford, UK: code 556454)

CD61-PC7 (Beckman Coulter, Milton Keynes, UK: code IM3716)

IgG1-PC7 (Beckman Coulter, Milton Keynes, UK: code 737662)

CD62P-PE (Beckman Coulter, Milton Keynes, UK: code IM1759U)

IgG1 (Mouse)-PE (Beckman Coulter, Milton Keynes, UK: code A07796)

IgG1 (Mouse)-FITC (Beckman Coulter, Milton Keynes, UK: code A07795)

Flow-Set fluorospheres (Beckman Coulter, Milton Keynes, UK: code 6607007)

PC7 Setup kit (Beckman Coulter, Milton Keynes, UK: code 737664)

Preparation of Reagents

Annexin V Buffer (1x):

Dilute 1 volume of 10x annexin V buffer with 9 volumes of distilled water. (In the procedure this is simply referred to as AV buffer.)

Preparation of Samples

The final platelet concentration required for the assay is approximately 5×10^6 platelets. The sample volumes required are listed in Table 2.1:

Table 2.1: Required volumes to provide a platelet concentration of 5×10^6 platelets

Platelet concentration ($\times 10^9$/L)	Volume of platelet concentrate required (µL)
1000	5.00
900	5.55
800	6.25
700	7.14

Samples with a platelet concentration greater than 1000×10^9/L would require a preliminary dilution.

Procedure

- Seven test tubes are required:

  1 – Dilution of sample for verifier tube
  2 – Controls preparation
  3 – Verifier preparation
  4 – Test preparation
  5 – Controls
  6 – Verifier
  7 - Test

- Pipette the required volume of platelet concentrate to the bottom of tubes 1, 2 and 4.
- Make up the volume of tube 1 to 25 µL with AV buffer and transfer 5 µL to the bottom of tube 3.
- Pipette 20 µL of IgG1-FITC, 20 µL of IgG1-PE and 10 µL of IgG1-PC7 to the bottom of tube 2.
- Pipette 10 µL of CD61-PC7 to the bottom of tube 3.
- Pipette 20 µL of CD62P-PE to the bottom of tube 4.

- Gently mix all three preparation tubes and incubate in the dark for 15 minutes at ambient temperature.
- Dispense sufficient AV buffer to increase the volume of tube 4 to 100 µL. Transfer 10 µL to the bottom of tube 7 (test). Add 90 µL of AV buffer followed by 1 µL of annexin V-FITC. Incubate in the dark for 15 minutes at ambient temperature.
- Dispense sufficient AV buffer to increase the volume of tube 2 to 100 µL. Mix and transfer 10 µL to the bottom of tube 5 (controls) and add 491 µL of AV buffer.
- Pipette 5 µL of AV buffer to tube 3. Mix and transfer 10 µL to the bottom of tube 6 (verifier) followed by 491 µL of AV buffer.
  Tubes 5 and 6 can remain with tube 7 until the last has finished incubating.
- After the second incubation, add 400 µL of AV buffer to tube 7.
- Acquire immediately on the flow cytometer.

SOLUBLE CD62P LEVELS

Background

A solid phase enzyme-linked immunosorbent assay (ELISA) was selected for the measurement of soluble CD62P (sCD62P) in the supernatant of platelet concentrates. The 96-well plates are pre-coated with a monoclonal antibody specific for sCD62P. The intensity of the colour reaction is proportional to the level of sCD62P in the sample, with levels quantified against a line generated by a series of standard provided in the kit by the manufacturer.

Development of the Assay

The kit was used according to manufacturer's instructions. The sensitivity of the assay is quoted by the manufacturer as typically less than 0.5 ng/mL.

Assay

Reagents

Human soluble P-selectin/CD62P immunoassay (R&D Systems Europe; Abingdon, UK; code BBE6)

[includes sCS62P 96 well microplate, sCD62P standards, sCD62P control, sCD62P conjugate, conjugate diluent, sample diluent, wash buffer concentrate, substrate and stop solution]

Preparation of Standards/Samples

Standards and Control:

Reconstitute all standards with 1 mL of distilled water – no further dilution required.

Reconstitute the control with 500 µL of distilled water. Subsequently, dilute the reconstituted control 1 in 20 with sample diluent.

Samples:

Thaw frozen samples in 37°C waterbath and prepare 1 in 20 dilutions with sample diluent.

Procedure

[All standards, samples and controls are assayed in duplicate and the mean of the two results reported]

- Add 100 µL of standards, samples and control to each well on the microplate
- Add 100 µL of conjugate to each well. Cover and incubate the plate for one hour at ambient temperature.
- Aspirate and wash the plate three times with diluted wash buffer. Blot the plate dry after the final wash to remove any excess fluid.
- Add 100 µL of substrate to each well. Incubate the plate for 15 minutes at ambient temperature.
- Stop the colour reaction with 100 µL of acid stop solution.
- Measure the optical density of each well on a Spectra II microplate reader (Tecan UK Ltd, Reading, UK), using 450 nm as the primary wavelength with a correction filter set to 620 nm.

The software automatically calculates the concentration of soluble CD62P based on the standard line.

COMPOSITION OF EXTERNALISED PHOSPHOLIPIDS ON THE PLATELET MEMBRANE AS MEASURED BY MASS SPECTROMETRY

Background

The School of Medicine at Cardiff University has developed a method for detecting and quantifying specific aminophospholipids externalised on the platelet membrane using mass spectrometry (MS). The procedure involves labelling of the externally expressed

phospholipids with a cell-impermeable biotinylation reagent - sulfo-NHS-biotin – with L-lysine used to quench any excess biotin. Lipids are extracted using a solvent mixture of acetic acid, isopropanol and hexane, with the lipids suspended in the hexane layers. The hexane layers are dried under nitrogen flow and analysed for PE- or PS-esterified 12*S*-hydroxyeicosatetraenoic acids (12*S*-HETEs) on MS/MS spectra (Thomas et al., 2010, Zhang et al., 2002) at the apex of peaks obtained during liquid chromatography separation. 12-HETE is an eicosanoid derived from arachidonic acid by the action of 12-lipoxygenase, with the esterified form of 12*S*-HETE generally retained on the cell membrane. Four 12*S*-HETE-PE structures and one 12*S*-HETE-PS were identified based on the mass/charge ratio of characteristic daughter ions (figures 2.6 and 2.7). Internal standards, in the form of 10 ng of 12(S)-HETE-d_8 and 10 ng of di-14:0-phosphatidylethanolamine (DMPE), were included in 1 mL of sample containing 2×10^8 platelets per mL to allow for the quantitation of phospholipids on the platelet surface.

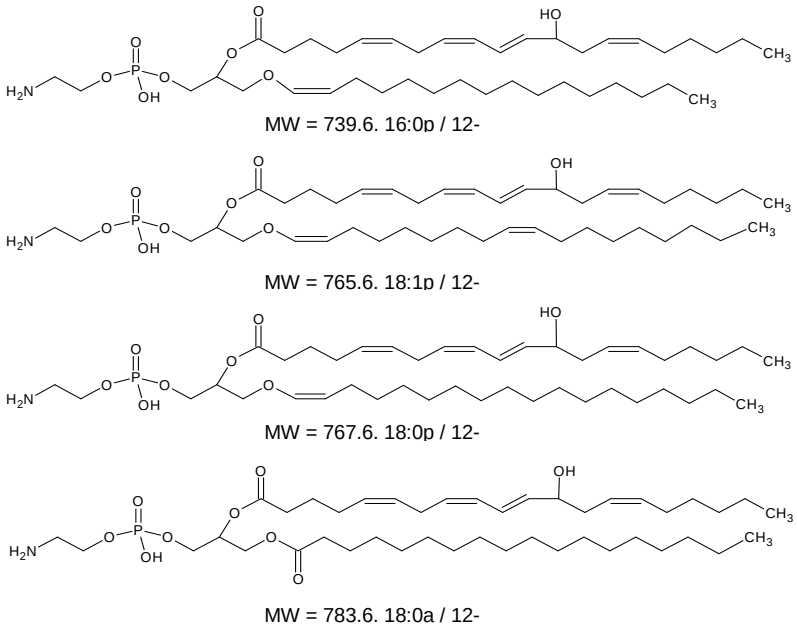


Figure 2.6: Structures of four 12-HETE-PE present in platelets (courtesy of C Thomas; School of Medicine, Cardiff University)

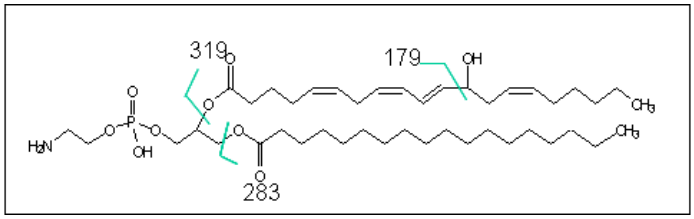


Figure 2.7: Example of fragmentation pattern for 12-HETE-PE (courtesy of C Thomas; School of Medicine, Cardiff University)

Development of the Assay

Total lipid extraction and separation of the hexane layers was performed at the Welsh Blood Service following the method provided by the School of Medicine. The drying of the hexane layers under nitrogen flow was performed at the School of Medicine, University Hospital of Wales. Sample preparation and storage was undertaken in 10 mL borosilicate glass tubes (Fisher Scientific UK Ltd, Loughborough, UK).

Assay

Reagents

Acetic acid (HPLC grade) (Fisher Scientific, Loughborough, UK: code A/0406/PB08)

Propan-2-ol (HPLC grade) (Fisher Scientific, Loughborough, UK: code P/7508/17

Hexane (HPLC grade) (Fisher Scientific, Loughborough, UK: code H/0406/17)

Sodium chloride (VWR International Ltd, Lutterworth, UK: code 102414J)

Sodium bicarbonate (Sigma-Aldrich, Dorset, UK: code S1554)

Potassium chloride (Sigma-Aldrich, Dorset, UK: code P3911)

Disodium hydrogen phosphate (Sigma-Aldrich, Dorset, UK: code 56814)

Magnesium chloride (Sigma-Aldrich, Dorset, UK: code M2670)

HEPES (VWR International Ltd, Lutterworth, UK: code 441485H)

D+-Glucose (Sigma-Aldrich, Dorset, UK: code G8270)

12(S)-HETE-d8 (Cayman Chemical, Ann Arbor, MI : code 334570)

DMPE (Axxora UK Ltd, Nottingham, UK : code ALX-300-037)

Tin (II) chloride (Sigma-Aldrich, Dorset, UK: code 204722-10G)

L-lysine (Sigma-Aldrich, Dorset, UK: code L5501-5G)

EZ-Link Sulfo-NHS-Biotin (Fisher Scientific, Loughborough, UK: code 21217)

Chloroform (Sigma-Aldrich, Dorset, UK: code C2432)

Methanol (HPLC grade) (Fisher Scientific, Loughborough, UK: code M/4056/15)

Reagent Preparation

TYRODE'S BUFFER (in 200 mL distilled water)

Sodium chloride (NaCl)	134 mmol/L	1.5662 g
Sodium bicarbonate ($NaHCO_3$)	12 mmol/L	0.2016 g
Potassium chloride (KCl)	2.9 mmol/L	0.0432 g
Disodium hydrogen phosphate (Na_2HPO_4)	0.34 mmol/L	0.0097 g
Magnesium chloride ($MgCl_2$)	1 mmol/L	0.0407 g
HEPES	10 mmol/L	0.4766 g
Glucose	5 mmol/L	0.1802 g

Final pH: 7.4 (adjust with 2.5 mol/L NaOH).

STORAGE: Store at 2 – 8°C for 1 month

ACID-CITRATE-DEXTROSE (ACD) (in 50 mL distilled water)

Trisodium citrate	85 mmol/L	1.2499 g
Citric acid	65 mmol/L	0.6243 g
Glucose	100 mmol/L	0.9008 g

STORAGE: Store at 2 – 8°C for 1 month

TYRODE'S + ACD

Combine Tyrode's buffer and ACD in a 9:1 (v:v) ratio prior to use.

SOLVENT MIXTURE

[1M acetic acid/prpan-2-ol/hexane at 2:20:30, v/v/v)

Acetic acid 1M	4 mL
Propan-2-ol	40 mL
Hexane	60 mL

STORAGE: Store at ambient temperature for 3 months.

TIN II CHLORIDE ($SnCl_2$), 100 mmol/L

Dissolve 0.0190 g of $SnCl_2$ in 1 mL absolute ethanol.

STORAGE: Store at 2 – 8°C for 3 months.

DMPE *(10 μL required per sample - Purchased as 50 mg white powder)*

Dissolve 1 mg (0.0010 g) in 1 mL chloroform to yield stock concentration of 1×10^3 ng/μL. Dispense approximately 20 μL into glass microvials and store below -70°C. When required, thaw a vial of the stock solution and dilute 1 μL with 600 μL chloroform and 400 μL methanol to yield concentration of 1 ng/μL.

[Final dilution is a chloroform:methanol ratio of 4:1 (v:v)]

STORAGE: Store vials of stock solution below -70°C for 6 months. Do not re-freeze thawed vials.

12(S)-HETE-d8 *(Purchased as 25 μg in 250 μL acetonitrile)*

Dilute 5 μL of 12(S)-HETE-d8 in 395 μL of chloroform plus 100 μL of methanol to yield a final concentration of 1 ng/μL.

STORAGE: Reagent as bought – 2 years at -20°C. Working solution – discard on day of the assay

L-LYSINE *(50 mmol/L)*

Dissolve 0.0366 g in 5 mL of distilled water.

STORAGE: Store at 2 – 8°C for 3 months.

Lipid Extraction

- Centrifuge 1 mL of platelet concentrate at 900 *g* for 10 minutes at 22°C.
- Discard the supernatant and re-suspend the pellet in approximately 2 mL of Tyrode's/ACD buffer. (Re-suspension may require gentle mixing with a disposable pipette).
- Centrifuge the re-suspended platelets at 800 *g* for 10 minutes at 22°C. Discard the supernatant and re-suspend the pellet in Tyrode's/ACD buffer to a platelet concentration of 2×10^8 platelets per mL.
- Pipette 1 mL of the platelet suspension into a labelled 10 mL glass vial and add 0.0015 g of sulfo-NHS-biotin. Incubate for 10 minutes at ambient temperature.
- Quench any excess biotin by adding 10 μL of 50 mmol/L L-lysine and incubate for 10 minutes at ambient temperature.
- Add 10 μL of 100 mmol/L tin (II) chloride to reduce the hydroperoxide to their corresponding stable alcohols and incubate for 10 minutes at ambient temperature.

- Add 2.5 mL of solvent mixture to extract the lipids.
- Add 10 μL of 12-HETE-d8 followed by 10 μL of DMPE as standards. Vortex and add 2.5 mL of hexane.
- Vortex and centrifuge at 360 *g* for 5 minutes at 4°C.
- Recover the hexane (upper) layer containing the extracted lipids into a labelled 10 mL borosilicate glass vial.
- Add an equal volume of hexane to the remaining aqueous solution, vortex and re-centrifuge at 360 *g* for 5 minutes at 4°C.
- Combine the hexane layers and store below -70°C until transport in dry ice can be arranged to the School of Medicine.

MITOCHONDRIAL MEMBRANE POTENTIAL

Background

Changes in mitochondrial membrane potential ($\Delta\Psi m$) were detected flow cytometrically using the fluorescent cationic dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolylcarbocyanine iodide). JC-1 exists as a monomer at low concentrations, with an emission maximum of 529 nm. At higher concentrations it forms red-fluorescing J-aggregates with an emission maximum of 590 nm.

The membrane potential determines the concentration of JC-1 in the mitochondrial matrix, with passage of the dye through the inner mitochondrial membrane eased with higher (more negative) potentials. The resulting accumulation of JC-1 promotes the formation of J-aggregates. Depolarisation of the membrane results in a decrease in the concentration of JC-1 in the matrix, favouring the retention of the green-fluorescing monomeric configuration. A loss of membrane potential during platelet storage will thus be evident as a decrease in the red-green fluorescence ratio (Verhoeven et al., 2005, Li et al., 2005b).

Development of Assay

The adoption of a platelet concentration of 30×10^9/L and an incubation of 20 minutes at 37°C stemmed from the optimal conditions suggested by Verhoeven et.al (Verhoeven et al., 2005). T-Sol™ platelet additive solution (116 mmol/L sodium chloride, 10 mmol/L sodium citrate, 30 mmol/L sodium acetate, pH 7.2) was used as the sample

diluent. Precedent for the use of an additive solution in this context was set by Verhoeven et.al with the use of Composol-PS (Fresenius Kabi, Bad Homburg, Germany). JC-1 was purchased in powder form (5 mg). After consultation with the manufacturer, it was decided to prepare 5 mmol/L stock solutions of the dye in anhydrous dimethyl sulphoxide (DMSO) and store the aliquots below -30°C until required.

Repeatability of the assay was determined using ten individually prepared samples from a six-day old apheresis platelet concentrate, yielding a coefficient of variation of 2.41%. Baseline levels for the assay obtained from 27 one-day old apheresis platelet concentrates resulted in a range for the red/green fluorescence ratio of 4.49 – 6.32. Investigations into the robustness of the assay concentrated on the impact of a delay in the preparation of the samples, as this would determine the timing of the assay alongside the other planned tests. It was determined that the fluorescence ratio remained stable for at least 40 minutes after sampling from the unit, allowing potentially more labile assays to be prepared first.

Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) is a protonophore that acts as a mitochondrial membrane disruptor and uncoupler of oxidative phosphorylation. The addition of CCCP was thus expected to act as a positive process control (Hortelano et al., 1999)(Hortelano, 1999, 28). CCCP was added at a concentration of 5 µmol/L. Platelet populations were identified based on scatter characteristics, with a stop on 50000 events. Green fluorescence was detected on FL1, with the FL2 detector used to identify the red-fluorescing J-aggregates.

Method

Reagents

CCCP (Sigma-Aldrich, Dorset, UK: code C2759)

JC-1 (Invitrogen, Paisley, UK: code: T3168)

DMSO, anhydrous (Sigma-Aldrich, Dorset, UK: code D8418)

T-Sol™ platelet additive solution (Baxter, Norfolk, UK: code RDB7846)

Preparation of Reagents

0.1M CCCP stock solution:

- Prepare a 0.1 M stock solution (0.0205 g of CCCP to 997 µL of DMSO)
- Dispense into labelled cryovials as 20 µL aliquots and store below -30°C

5 mM CCCP (working solution):

- Add 380 µL of DMSO to thawed vial of CCCP stock solution

5 mM JC-1 stock solution:

- Dispense 1.534 mL of DMSO into the 5 mg vial of JC-1.
- Once dissolved, dispense into labelled cryovials in 25 µL aliquots

100 µM JC-1 (working solution):

- Add 1.225 mL of DMSO to thawed vial of JC-1 stock solution

Procedure

- Obtain a platelet count from the unit sample
- Dilute with T-Sol to a concentration of 30×10^9/L (at least 2 mL required per assay) Two plastic test tubes are required per assay, one serving as the test and a second serving as a control
- Dispense 5 µL of 100 µM JC-1 to the bottom of both test tubes
- Dispense 1 µL of 5 mM CCCP to the bottom of the control tube
- Dispense 995 µL of the diluted sample to both tubes
- Cap tubes and incubate in a 37°C waterbath for 20 minutes (protect from light)
- Acquire on the flow cytometer.

INTRACELLULAR FREE CALCIUM

Background

A flow cytometric assay using two fluorescence indicators was developed for the measurement of intracellular free calcium. Fluo-4 is a derivative of fluo-3 in which two chlorine atoms are substituted by fluorine, resulting in improved excitation from the standard 488 nm argon laser used in the FC500 flow cytometer. Emission maximum is 516 nm, with binding of calcium ions resulting in increased fluorescence. In contrast, Fura Red exhibits decreased fluorescence on binding to calcium, with an emission maximum of approximately 675 nm. The separation in emission maxima, coupled with the differing response of the dyes to varying calcium concentration, allows for the

adoption of a ratiometric analysis, with both dyes combined in a single assay. Obtaining a ratio from two separate dyes minimises the effect of various artefacts unrelated to changes in calcium concentration. These include the impact of cell size and shape as well as variations in the loading and subsequent leakage of fluoresecent indicators (Dustin, 2000).

The common fluorescent indicators for Ca^{2+} are polycarboxylate anions that cannot cross the lipid bilayer of the plasma membrane. The uptake and retention of the dyes is facilitated by the use of the acetoxymethyl (AM) ester of each dye. The AM group masks the negative charge of the carboxyl groups allowing the ester form to pass through the cell membrane. Once inside the cell, nonspecific esterases cleave the masking groups, allowing the carboxyl groups to bind to calcium ions and resulting in a charged form of the dye that is more easily retained inside the cell (Takahashi et al., 1999). A complication of the use of AM esters is their low solubilities. This can be improved with the addition of a mild, non-ionic surfactant such as Pluronic® F-127 (Kao, 1994).

Development of the Assay

A modified Tyrode's buffer (137 mmol/L NaCl, 2.8 mmol/L KCl, 1 mmol/L $MgCl_2$, 12 mmol/L $NaHCO_3$, 0.4 mmol/L Na_2HPO_4, 10 mmol/L HEPES, 5.5 mmol/L glucose, 0.35% BSA (w/v)) was used as the sample diluent (do Ceu Monteiro et al., 1999). Bovine serum albumin was added to the medium on the basis that serum proteins such as BSA can improve the loading efficiency (Kao, 1994). The net effect was to accentuate the difference between the two emission fluorescences.

Dyes were purchased in 50 µg volumes and reconstituted in anhydrous DMSO to a concentration of 1 mmol/L to provide a stock solution. A working solution was prepared on the day of the assay by adding equal volumes of Pluronic® F-127. Any remaining stock solution was stored below -30°C. Any remaining working solution was discarded at the end of the day, since Pluronic® F-127 can decrease the stability of AM esters. (The manufacturer recommended that AM esters be used at a final concentration between 1 – 10 µmol/L). Little difference in fluorescence intensity was evident within this range and a final concentration of 5 µmol/L was adopted for both dyes.

Incubation times of fifteen and thirty minutes were evaluated. The impact on Fluo-4 was minimal, but a marked increase in fluorescence intensity was noted for Fura Red with the longer incubation period. As fluorescence is expected to decrease upon calcium binding with Fura Red, a higher starting point would be advantageous and an incubation period of thirty minutes was adopted for the assay. Compartmentalisation refers to the uptake of dye by organelles within the cell, some of which may contain high levels of calcium and thus potentially contribute to the fluorescence signal. As some operators have reported increased compartmentalisation with incubation temperatures of 37°C compared to ambient temperature, the latter was adopted (Takahashi et al., 1999, Dustin, 2000).

Cytosettings were initially established using individual dyes and subsequently optimised using a combined protocol. Final settings were:

FL1 (Fluo-4 detector): 550 volts

FL4 (Fura Red detector): 675 volts

Compensation: FL4-FL1 = 1.8 FL1-FL4 = 5.0

The calcium ionophore A23187 was employed as a positive control, with a final concentration of 20 µmol/L. A final concentration of 5 x 10^6 platelets was initially adopted based on the concentration used for the CD62P/Annexin V-binding assay and found to yield acceptable results. Platelets were identified based on their scatter characteristics, with a stop on 10000 platelet events.

Method

Reagents

Fura Red (Invitrogen, Paisley, UK: code F-0321)

Fluo-4 (Invitrogen, Paisley, UK: code F-14201)

A23187 free acid calcium ionophore (Invitrogen, Paisley, UK: code A-1493)

DMSO, anhydrous (Sigma-Aldrich, Dorset, UK: code D8418)

Pluronic® F-127 (20%) in DMSO (Invitrogen, Paisley, UK: code P3000MP)

Preparation of Reagents

Modified Tyrode's buffer (200 mL):

Sodium chloride	137 mmol/L	1.6013 g

Potassium chloride	2.8 mmol/L	0.0418 g
Magnesium chloride	1 mmol/L	0.0407 g
Sodium hydrogen carbonate	12 mmol/L	0.2016 g
Disodium hydrogen phosphate	0.4 mmol/L	0.0114 g
HEPES	10 mmol/L	0.4766 g
Glucose	5.5 mmol/L	0.1982 g
Bovine serum albumin	0.35% (w/v)	0.7000 g
Final pH (7.4; adjusted with NaOH)		

Filter through a 0.2 µm syringe filter in a Class II biological safety cabinet and store at 2–8°C for 4 weeks. Check visually for contamination before use.

Fluo-4 (stock solution):

- To 50 µg vial add 45.6 µL of anhydrous DMSO

Fura Red (stock solution):

- To 50 µg vial add 45.9 µL of anhydrous DMSO

Working solutions of fluorescent dyes:

- To 13 µL of the reconstituted dye add 13µL of Pluronic® F-127 to yield a working solution of 0.5 mmol/L

Calcium ionophore (A23187):

- Reconstitute the ionophore in anhydrous DMSO to create a stock solution of 1 mg per mL (2 mmol/L)
- Aliquot into labelled cryovials and store below -30°C.

Procedure

- Two test tubes are required per assay – one labelled “test” and the second labelled “control”.
- Pipette 5 µL of Fluo-4 and Fura Red working solutions into each tube.
- Pipette 490 µL of the diluted platelet sample into both tubes. Vortex for a few seconds, cap and incubate in the dark for 30 minutes at ambient temperature.
- After the incubation period, add 5 µL of 2 mmol/L A23187 to the control tube. Vortex for a few seconds.

- To both tubes add 1 mL of modified Tyrode,s buffer and acquire immediately on the flow cytometer.

INVESTIGATION OF PLATELET AGEING DURING STORAGE IN SCOTT SYNDROME PATIENT

Background

Platelets from Scott Syndrome patients do not express PS on their surface in response to physiological agonists such as collagen/thrombin. However, it was not known whether they would show an increased expression in response to pro-apototic agonists such as the BH3-mimetic ABT-737. Scott platelets thus potentially provided a model for investigating whether the aminophospholipid translocation noted during storage in normal platelets is caused by a cell death process that may be attributable to apoptosis. If Scott syndrome platelets expressed aminophospholipids in response to ABT-737 an increased expression of aminophospholipids in Scott platelets with storage would be suggestive of apoptosis as a causative mechanism in the PSL rather than activation through a scramblase dependent mechanism.

Development of the Assay

The expression of aminophospholipid on the platelet surface was measured flow cytometrically as the percentage of platelets binding annexin V, using the method described above. The volume of whole blood that could be obtained was restricted to 7 x 5 mL vacutainer vials. A method was thus developed to transfer the platelet-rich plasma (PRP) from the gently-centrifuged tubes to a neonatal PC storage pack. This required the vials to be opened. Preliminary trials of the method were thus undertaken with samples from normal volunteers to ensure that the sterility of the units could be maintained.

Informed consent for this study was obtained by the University Hospital of Wales after review by The South East Wales Ethics Committee.

Method – Unit Preparation

- Seven 5 mL 9NC coagulation sodium citrate 3.2% vacutainers (Greiner Bio-one, Kremsmünster, Austria) were used to collect approximately 30 mL of peripheral

whole blood at the University Hospital of Wales from the patient and two controls. The samples were subsequently transported to the Welsh Blood Service with minimal delay.

- Samples were centrifuged at 800 *g* for 5 minutes at 22°C.
- The vacutainers were opened inside a Class II microbiology safety cabinet and the PRP transferred by syringe and 19G needle into Baxter PL2410 neonatal platelet storage packs via a sterile sample site coupler (Baxter Healthcare Ltd, Newbury, UK). An isopropyl alcohol/chlorhexidine swab was used on the surface of the coupler both before and after needle insertion. Approximately 3 mL of PRP was obtained from each vacutainer for a total sample volume of approximately 15 mL.
- The neonatal packs were stored at 22 ± 2°C with constant horizontal agitation. (The packs are designed to hold between 40 and 70 mL of concentrated platelets. A volume of 15 mL appeared to be too low to allow for adequate mixing of the contents. Units were thus periodically mixed manually throughout the day).
- Samples were taken on days 1, 2, 5, 7 and 9, with day 0 being the day of sample collection. Approximately 150 µL was aseptically removed from the units via a sample site coupler using a 1 mL syringe with 19G needle and dispensed into a 12 × 75 mm plastic tube. (All sampling was performed in a Class II safety cabinet).
- Flow cytometric analysis was performed immediately after sampling.

THROMBIN GENERATION IN SCOTT PLATELETS DURING STORAGE

An extension of the study on platelet ageing was performed by colleagues at UHW investigating the functional capacity of stored platelets in a Scott patient. As phosphatidylserine provides a procoagulant surface for the activities of the tenase and prothrombinase complexes in the normal coagulation response, the question arose as to whether an increase in aminophospholipid expression on the surface of Scott platelets would correlate with an increase in thrombin generation and microvesicle formation. The annexin V binding assay was adopted by the team at the School of Medicine, as was the unit preparation method described above. The thrombin generation assay was performed at UHW by calibrated automated thrombography (CAT).

CHAPTER 3. COMPARISON OF *IN VITRO* STORAGE CHARACTERISTICS OF PLATELET CONCENTRATES STORED IN 100% AUTOLOGOUS PLASMA VERSUS A MEDIUM COMPRISED OF 70% SSP+™/30% PLASMA

INTRODUCTION

Despite the continued interest in the use of additive solutions for the storage of PCs, there have been relatively few studies comparing the characteristics of such units with PCs suspended in 100% autologous plasma beyond five days of storage (Cardigan et al., 2008). A study was therefore undertaken to expand on the current body of published data and provide a comparative data for subsequent experiments. Study 1 thus compared the storage characteristics of buffy coat-derived pooled platelets suspended as concentrates in 100% autologous plasma (the current standard storage conditions) with similarly prepared PC suspended in a medium comprising 70% SSP+™ and 30% plasma over a period of 14 days, including parameters associated with mechanisms of cell death. The frequency of testing resulted in significant volume loss and contributed to median platelet yields below the UK Guidelines' specification by the end of storage (226×10^9/unit and 198×10^9/unit in 100% plasma and 70% SSP+™ units, respectively). It is unclear how this volume loss may have affected results, limiting this study to a comparative investigation.

Data are replicates of 13 experiments for 100% plasma and 12 experiments for SSP+. Summary statistics are presented as the median and range in the tables and median and interquartile range (25^{th} and 75^{th} percentiles) in the graphs. Differences between groups were analysed by the Mann-Whitney Rank Sum test, with a p value <0.05 regarded as statistically significant. Comparisons within test groups over time were performed against baseline (day 1) results by the Wilcoxon Signed Rank test, with statistical significance assigned to p values <0.01.

RESULTS

Platelet Yield and Volume

The median platelet yields for units in plasma and 70% SSP+™ on day 1 were 320 × 10^9/unit and 301 × 10^9/unit, respectively, with no statistically significant difference between the two processes (p=0.165). Corresponding median unit volumes were 315.5 mL and 317.1 mL (p=0.849). All units met the UK Guidelines' specification requiring a platelet yield ≥ 240 × 10^9/unit (James, 2005). All units were successfully leucoreduced (WBC counts < 1 × 10^6/unit) and remained sterile at end of storage.

Viability and Morphology

Platelet concentration

There was a gradual decrease in platelet concentration in both study groups over the 14-day storage period, with median end of storage values 86.3% and 81.9% of day 1 values in plasma and SSP+™ units, respectively (figure 3.1). Compared to baseline day 1 values, the decrease was statistically significant in units in 100% plasma on days 10 and 14 (p<0.001) and in units suspended in 70% SSP+™ from day 6 (p=0.003 to <0.001). Lower median values were noted in the units suspended in 70% SSP+™ throughout the storage period, though a statistically significant difference was not evident until day 14 (p=0.023) (table 3.1).

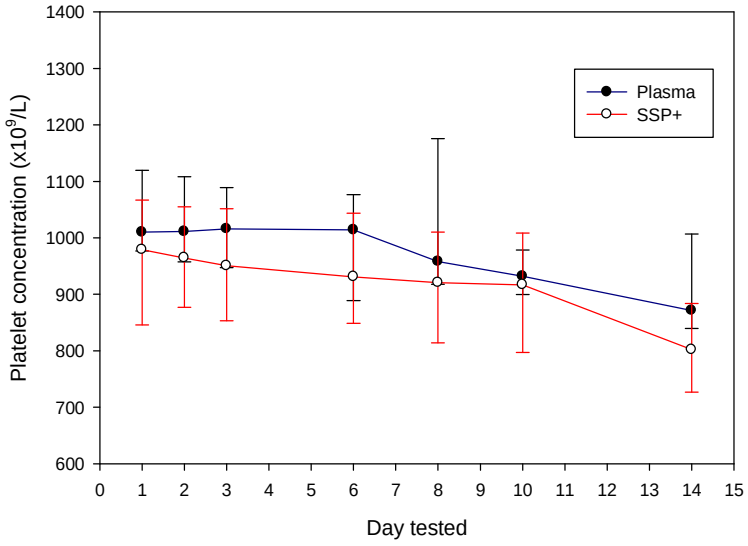


Figure 3.1: Platelet concentration in PC stored in 100% plasma vs PC stored in 70% SSP+ ™ (median ± interquartile range)

Table 3.1: Summary results for platelet concentration – PC in 100% plasma vs 70% SSP+™

Day Tested	Platelet concentration ($\times 10^9$/L)				
	100% Plasma (n=13)		70% SSP+™ (n=12)		
	Median	Range	Median	Range	p value
1	1010	865-1323	979	814-1246	0.142
2	1011^{NS}	833-1290	965^{NS}	817-1254	0.221
3	1016^{NS}	867-1345	951^{NS}	801-1235	0.242
6	1014^{NS}	818-1290	931*	772-1213	0.355
8	958^{NS}	813-1356	921*	767-1171	0.068
10	932*	807-1271	917*	734-1082	0.289
14	872*	768-1218	802*	685-931	0.023

*: Statistically significant difference ($p<0.01$) when compared against day 1 values
^{NS}: No statistically significant difference when compared against day 1 values

Swirling

Swirling remained strong (score of 3) throughout the 14 days of storage in all units suspended in 70% SSP+™. In the units suspended in 100% plasma, eight of the thirteen units showed reduced swirling with a score of 2 by day 10, decreasing further to scores of 1 or 0 by day 14 in eleven of the units.

Mean platelet volume

Mean platelet volumes were lower in the platelets suspended in 70% SSP+™, with median values of 7.9 fL compared to 9.1 fL on day 1 for platelets in 100% plasma. This difference was retained throughout storage and, with the exception of day 3 ($p=0.079$), was statistically significant at all time points ($p=0.035$ to <0.001) (table 3.2). However, the variation associated with the plasma results were a source of concern (figure 3.2). An inconsistency was identified between the last four results and those preceding them. The median on day 1 for the first nine units was 9.2 fL compared with 7.8 fL for the last four units tested, with a similar difference retained throughout the storage period. There was a two month gap between the two groups of results, but no clear indication of what could have caused the discrepancy. There were no changes to unit preparation and the analyser performance was routinely monitored with daily quality control material. Statistically significant differences were obtained from day 6 in 100% plasma units ($p=0.002$ to <0.001) when comparing results to baseline day 1 values. By contrast,

significant differences were obtained on days 2 (p=0.004), 3 and 14 (p=0.002 for both) in units in 70% SSP+™ (table 3.2).

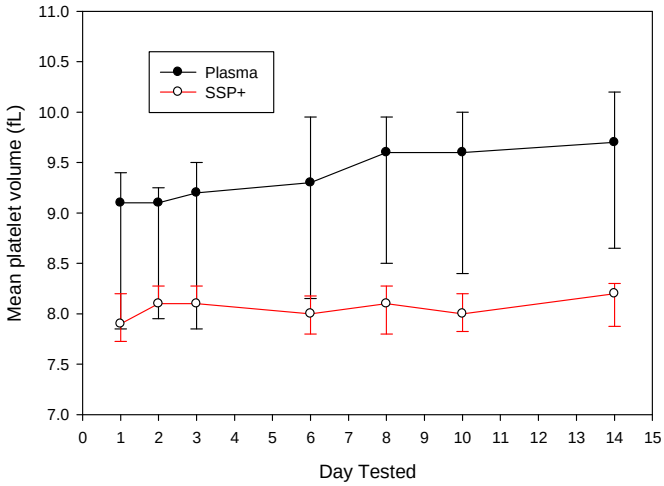


Figure 3.2: Mean platelet volume in PC stored in 100% plasma vs PC stored in 70% SSP+™ (median ± interquartile range)

Table 3.2: Summary results for mean platelet volume – PC in 100% plasma vs 70% SSP+™

Day Tested	Mean platelet volume (fL)				
	100% Plasma (n=13)		70% SSP+™ (n=12)		
	Median	Range	Median	Range	p value
1	9.1	7.7-10.2	7.9*	7.4-8.3	0.014
2	9.1^{NS}	7.6-10.1	8.1*	7.4-8.4	0.035
3	9.2^{NS}	7.2-10.2	8.1*	7.7-8.4	0.079
6	9.3*	7.7-10.8	8.0^{NS}	7.6-8.6	0.004
8	9.6*	8.1-10.6	8.1^{NS}	7.6-8.6	<0.001
10	9.6*	8.1-10.8	8.0^{NS}	7.6-8.4	<0.001
14	9.7*	8.4-10.4	8.2*	7.6-8.7	<0.001

*: Statistically significant difference (p<0.01) when compared against day 1 values
NS: No statistically significant difference when compared against day 1 values

Hypotonic shock response and extent of shape change

The response to hypotonic shock was retained close to day 1 values until after day 3, at which point the response was found to decrease with further storage in both test groups (figure 3.3).This became statistically significant in both test groups from day 6 (p=0.003 to <0.001) (table 3.3). A convincing difference (p<0.001) between the results for the two groups was not evident until day 14, when the median for units in plasma was 30.5% compared to 40.0% for units in SSP+™ (table 3.3). A similar pattern was evident

for the extent of shape change assay (figure 3.4), with median levels starting at 35.2% and 35.1% for plasma and SSP+™ units respectively (p=0.435); decreasing by day 14 to 9.8% and 14.2% (p<0.001). As with HSR, the decrease was statistically significant from day 6 to the end of storage (p<0.001) (table 3.4).

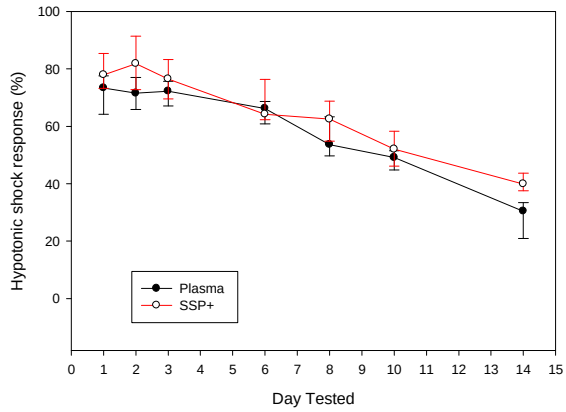


Figure 3.3: Hypotonic shock response in PC stored in 100% plasma vs PC stored in 70% SSP+™ (median ± interquartile range)

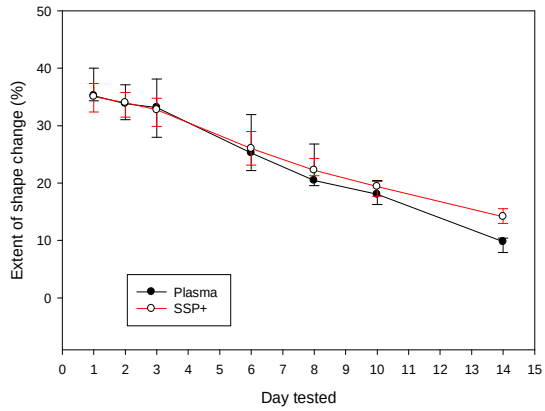


Figure 3.4: Extent of shape change in PC stored in 100% plasma vs PC stored in 70% SSP+™ (median ± interquartile range)

Table 3.3: Summary results for hypotonic shock response – PC in 100% plasma vs 70% SSP+™

Day Tested	Hypotonic shock response (%)				
	100% Plasma (n=12)		70% SSP+™ (n=12)		
	Median	Range	Median	Range	p value
1	73.4	53.0-87.4	78.0	68.5-92.2	0.089
2	71.5^{NS}	51.5-88.2	81.8^{NS}	68.8-93.7 (n=8)	0.034
3	72.3^{NS}	53.3-80.0	76.5^{NS}	64.6-92.8	0.184
6	66.3*	45.8-74.5	64.2*	55.9-78.0	0.583
8	53.7*	42.0-65.1	62.5*	49.5-78.0	0.043
10	49.2*	31.4-63.1	52.0*	39.8-61.9	0.273
14	30.5*	2.8-42.8	40.0*	36.8-53.3	<0.001

*: Statistically significant difference (p<0.01) when compared against day 1 values
^{NS}: No statistically significant difference when compared against day 1 values

Table 3.4: Summary results for extent of shape change – PC in 100% plasma vs 70% SSP+™

Day Tested	Extent of shape change (%)				
	100% Plasma (n=12)		70% SSP+™ (n=12)		
	Median	Range	Median	Range	p value
1	35.2	23.5-43.3	35.1	31.4-39.2	0.435
2	33.9^{NS}	30.3-39.8	34.0^{NS}	30.7-39.3 (n=8)	1.000
3	33.2^{NS}	25.2-38.8	32.8^{NS}	28.2-39.1	0.977
6	25.3*	21.4-33.5	26.0*	21.6-30.4	0.817
8	20.5*	18.4-28.5	22.3*	18.8-27.3	0.564
10	18.1*	14.9-23.1	19.4*	14.9-22.6	0.470
14	9.8*	1.5-13.7	14.2*	10.6-18.8	<0.001

*: Statistically significant difference ($p<0.01$) when compared against day 1 values
^{NS}: No statistically significant difference when compared against day 1 values

Platelet Metabolism

Glucose and Lactate Levels

Median glucose levels in plasma and SSP+™ units on day 1 were 15.2 mmol/L and 4.6 mmol/L, respectively, reflecting the lack of glucose in the SSP+™ additive solution and resulting in statistically significant differences ($p<0.001$) between the two test groups throughout storage (table 3.5). Glucose levels declined gradually to a minimum of 3.7 mmol/L by day 14 in plasma units, whereas stores were depleted by day 10 in units suspended in 70% SSP+™ (figure 3.5). A statistically significant difference from baseline day 1 levels was present by day 2 in units in 100% plasma and by day 3 in units suspended in 70% SSP+™ ($p<0.001$ in all cases) (table 3.5).

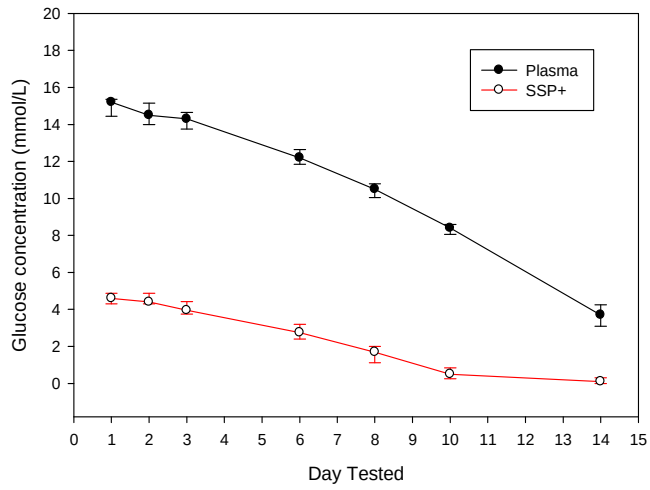


Figure 3.5: Glucose concentration in PC stored in 100% plasma vs PC stored in 70% SSP+™ (median ± interquartile range)

Table 3.5: Summary results for glucose concentration – PC in 100% plasma vs 70% SSP+™

Day Tested	Glucose concentration (mmol/L)				
	100% Plasma (n=13)		70% SSP+™ (n=12)		
	Median	Range	Median	Range	p value
1	15.2	14.0-17.2	4.6	4.2-5.4	<0.001
2	14.5*	13.5-16.4	4.4^{NS}	3.8-5.2	<0.001
3	14.3*	12.9-16.2	4.0*	3.4-4.8	<0.001
6	12.2*	11.1-14.7	2.8*	1.9-3.3	<0.001
8	10.5*	9.1-13.1	1.7*	0.7-2.4	<0.001
10	8.4*	7.3-11.7	0.5*	0.0-1.4	<0.001
14	3.7*	2.1-7.9	0.1*	0.0-0.3	<0.001

*: Statistically significant difference ($p<0.01$) when compared against day 1 values
^{NS}: No statistically significant difference when compared against day 1 values

The decrease in glucose concentration was reflected in a concomitant increase in lactate levels, with median levels at the start of the storage period of 8.3 mmol/L increasing to 25 mmol/L by day 14 in PC suspended in 100% plasma (table 3.6). In contrast, levels in units suspended in 70% SSP+™ plateau at a median of 14.6 mmol/L on day 10, coincident with the depletion of glucose stores in these units (figure 3.6). As with glucose, statistically significant differences ($p<0.01$) were found by day 2 and by day 3 in 100% plasma units and 70% SSP+™ units, respectively.

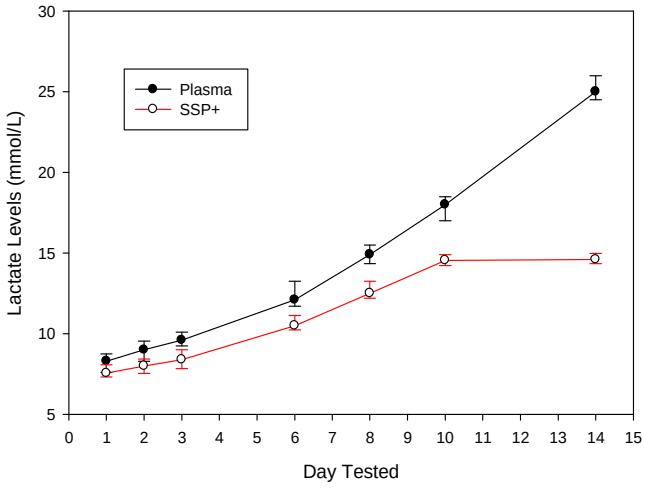


Figure 3.6: Lactate concentration in PC stored in 100% plasma vs PC stored in 70% SSP+™ (median ± interquartile range)

Table 3.6: Summary results for lactate concentration – PC in 100% plasma vs 70% SSP+™

Day Tested	Lactate concentration (mmol/L)				
	100% Plasma (n=13)		70% SSP+™ (n=12)		
	Median	Range	Median	Range	p value
1	8.3	7.1-10.6	7.6	6.9-8.7	0.036
2	9.0*	7.9-11.6	8.0^{NS}	7.1-9.0	0.002
3	9.6*	8.6-12.2	8.4*	7.6-9.6	<0.001
6	12.1*	11.1-16	10.5*	9.8-12.3	<0.001
8	14.9*	13.4-18	12.5*	10.7-14.3	<0.001
10	18*	16-21	14.6*	13.1-16	<0.001
14	25*	21-32	14.6*	13.9-16	<0.001

*: Statistically significant difference ($p<0.01$) when compared against day 1 values
^{NS}: No statistically significant difference when compared against day 1 values

Rates of glucose consumption increased throughout the storage period in units stored in 100% plasma, partly as a reflection of the loss of platelets with storage evidenced by the decrease in platelet concentration, resulting in a statistically significant difference ($p<0.001$) compared to baseline levels beyond day 6 (table 3.7). Glucose consumption also increased with storage in PC suspended in 70% SSP+™ until day 10. Beyond this point, the depletion of glucose in these units resulted in a steep decline in glucose consumption (figure 3.7). Statistically significant differences ($p<0.001$) compared to baseline levels of consumption were only evident between days 6 and 10 in these units. Glucose consumption in the SSP+™ test group was lower than in the plasma units, with statistically significant differences between the two groups at all time points ($p=0.011$ to <0.001) (table 3.7).

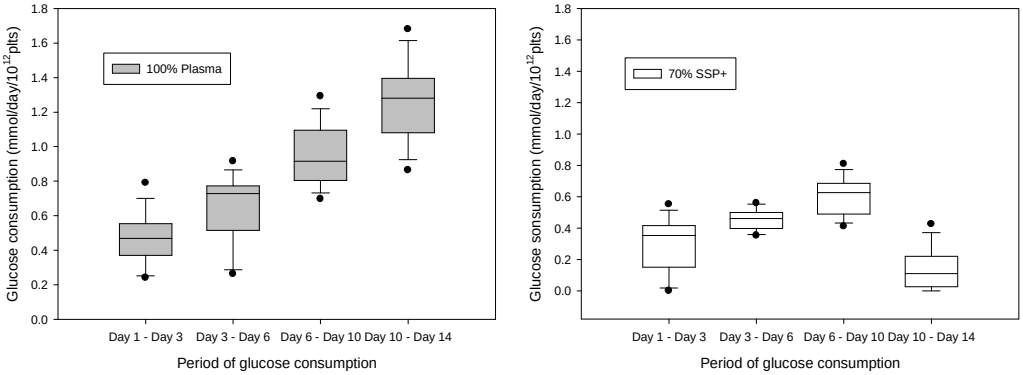


Figure 3.7: Glucose consumption rates in PC stored in 100% plasma vs PC stored in 70% SSP+™ (plot shows median, 25th, 75th, 10th and 90th percentiles, with other values individually plotted)

Table 3.7: Summary results for glucose consumption – PC in 100% plasma vs 70% SSP+™

Period of glucose consumption	Glucose consumption (mmol/day/10^{12}plts)				
	100% Plasma (n=13)		70% SSP+™ (n=12)		
	Median	Range	Median	Range	p value
Day 1 - 3	0.47	0.24-0.79	0.35	0.00-0.55	0.011
Day 3 - 6	0.73^{NS}	0.26-0.92	0.46^{NS}	0.35-0.56	0.008
Day 6 -10	0.92*	0.70-1.29	0.63*	0.41-0.81	<0.001
Day 10 -14	1.28*	0.86-1.68	0.11^{NS}	0.00-0.43	<0.001

*: Statistically significant difference ($p<0.01$) when compared against day 1 values
^{NS}: No statistically significant difference when compared against day 1 values

The pattern of lactate production mirrored that observed with glucose consumption, with lactate production ceasing with the depletion of glucose stores (figure 3.8).

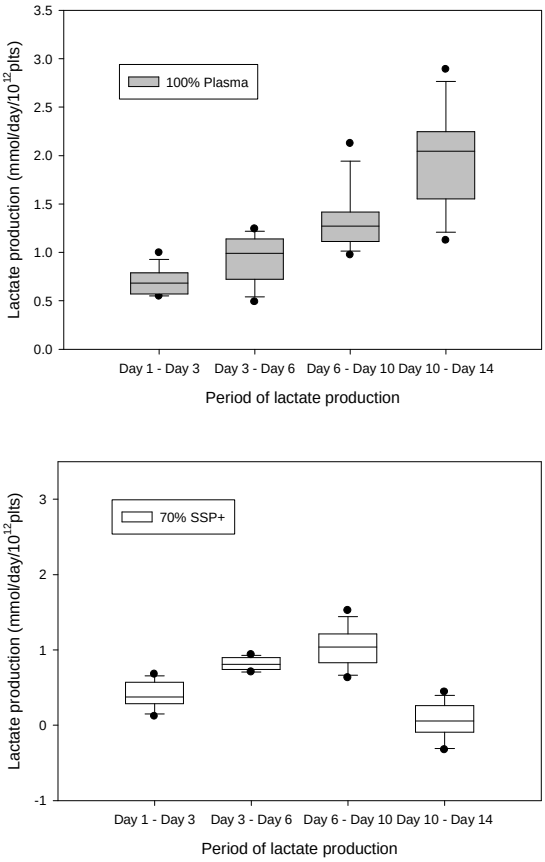


Figure 3.8: Lactate production in PC stored in 100% plasma vs PC stored in 70% SSP+™ (plot shows median, 25th, 75th, 10th and 90th percentiles, with other values individually plotted)

Table 3.8: Summary results for lactate production – PC in 100% plasma vs 70% SSP+™

Period of lactate production	Lactate production (mmol/day/10^{12}plts)				
	100% Plasma (n=13)		70% SSP+™ (n=12)		
	Median	Range	Median	Range	p value
Day 1 - 3	0.68	0.55-0.99	0.38	0.12-0.68	0.011
Day 3 - 6	0.99*	0.49-1.24	0.81*	0.71-0.94	0.008
Day 6 -10	1.27*	0.97-2.12	1.04*	0.63-1.52	<0.001
Day 10 -14	2.04*	1.12-2.89	0.06^{NS}	-0.33-0.44	<0.001

*: Statistically significant difference (p<0.01) when compared against day 1 values
NS: No statistically significant difference when compared against day 1 values

pH $_{(22°C)}$

$pH_{22°C}$ values were similar in both test groups on day 1, with median values of 7.070 and 7.064 in 100% plasma and SSP+™ units, respectively (p=0.231) (table 3.9). The pattern subsequently deviated, with units in plasma showing an increase in pH until day 3 followed by a decline through to day 14 which showed a minimum level for the median of 6.613 (range: 6.521 – 6.870), resulting in statistically significant differences (p<0.001) compared with baseline day 1 values at all time points except day 10 (p=0.787) (table 3.9) . Units in 70% SSP+™ showed a more gradual increase to day 6, with a more pronounced rise after day 10 following the depletion of glucose stores in these units, resulting in a day 14 median value of 7.295 (figure 3.9). In these units, statistically significant differences (p<0.001) compared to day 1 values were evident throughout storage.

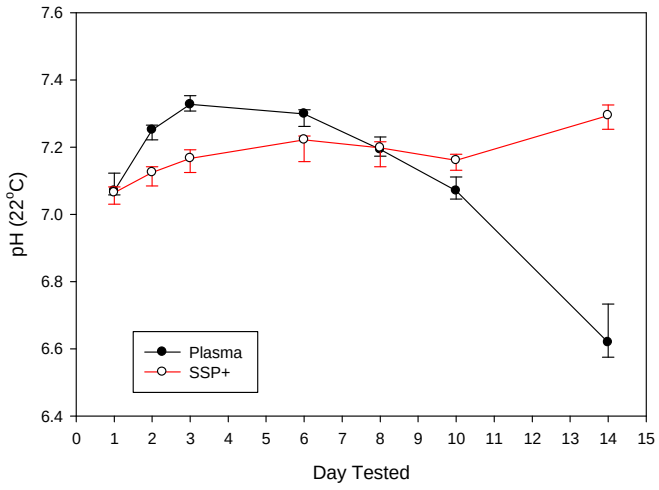


Figure 3.9: $pH_{22°C}$ levels in PC stored in 100% plasma vs PC stored in 70% SSP+ ™ (median ± interquartile range)

Table 3.9: Summary results for $pH_{22°C}$ – PC in 100% plasma vs 70% SSP+ ™

Day Tested	pH (22°C)				
	100% Plasma (n=13)		70% SSP+™ (n=12)		
	Median	Range	Median	Range	p value
1	7.070	7.014-7.129	7.065	7.010-7.114	0.231
2	7.251*	7.176-7.314	7.125*	7.056-7.158	<0.001
3	7.327*	7.274-7.382	7.167*	7.092-7.202	<0.001
6	7.299*	7.219-7.366	7.222*	7.107-7.257	<0.001
8	7.193*	7.092-7.282	7.198*	7.071-7.241	0.913
10	7.070^{NS}	6.914-7.159	7.161*	7.092-7.220	<0.001
14	6.613*	6.521-6.870	7.295*	7.162-7.346	<0.001

*: Statistically significant difference (p<0.01) when compared against day 1 values
^{NS}: No statistically significant difference when compared against day 1 values

Bicarbonate Levels

Bicarbonate levels in plasma units decreased gradually from a median on day 1 of 18.46 mmol/L to 1.59 mmol/L by day 14. Levels in SSP+™ units, in contrast, were relatively more stable, with starting levels of 7.59 mmol/L decreasing to 4.09 mmol/L by the end of storage, constituting 53.9% of day 1 levels compared to 8.6% of day 1 values in 100% plasma units. Statistically significant differences compared with baseline day 1 levels were evident in plasma units at all time-points, but after day 3 in SSP+™ units ($p<0.001$ in all cases) (table 3.10). The pattern reflected the more stable pH values observed with SSP+™ units (figures 3.10 and 3.9). Statistically significant differences were noted throughout storage between the two test groups ($p<0.001$ at all time points).

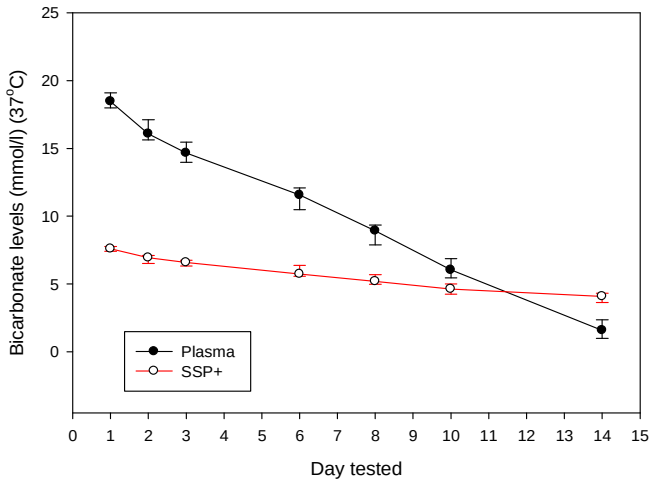


Figure 3.10: Bicarbonate levels in PC stored in 100% plasma vs PC stored in 70% SSP+™ (median ± interquartile range)

Table 3.10: Summary results for bicarbonate levels – PC in 100% plasma vs 70% SSP+™

Day Tested	Bicarbonate levels (37°C) (mmol/L)				
	100% Plasma (n=13)		70% SSP+™ (n=12)		
	Median	Range	Median	Range	p value
1	18.46	16.62-20.50	7.59	5.69-7.91	<0.001
2	16.08*	14.91-18.08	6.93^{NS}	6.29-8.13	<0.001
3	14.66*	13.70-16.46	6.59^{NS}	6.01-7.68	<0.001
6	11.56*	9.82-13.21	5.73*	5.17-6.58	<0.001
8	8.93*	7.13-10.36	5.20*	4.53-6.11	<0.001
10	6.03*	4.45-7.79	4.63*	3.85-5.56	<0.001
14	1.59*	0.43-4.09	4.09*	3.11-5.16	<0.001

*: Statistically significant difference ($p<0.01$) when compared against day 1 values
^{NS}: No statistically significant difference when compared against day 1 values

Blood Gases

Median $pO_{2(37°C)}$ levels increased steadily throughout the period of storage in both test groups, from median day 2 levels of 12.7 and 14.0 kPa in plasma and 70% SSP+™ units, respectively, to a maximum of 18.4 and 18.8 kPa by day 14 (figure 3.11). Statistically significant differences between the two groups were evident only on day 1 ($p=0.027$). This was reflected in the pairwise comparison with baseline day 1 values, with statistically significant differences ($p<0.001$) in plasma units at all time-points but no significant differences on days 2 and 3 in 70% SSP+™ units (table 3.11). $pCO_{2(37°C)}$ differed significantly between the two groups from day 1 ($p<0.001$ at all time points), with median levels decreasing from 11.4 to 2.79 kPa in units suspended in 100% plasma compared to 4.85 to 1.58 kPa for units in 70% SSP+™ (day 1 to day 14) (figure 3.12). The decrease in pCO_2 in 100% plasma units between days 1 and 3 coincided with the increase in pH observed in these units during the same period, with statistically significant differences ($p=0.009$ to <0.001) compared to day 1 values at all time-points in both test groups (table 3.12).

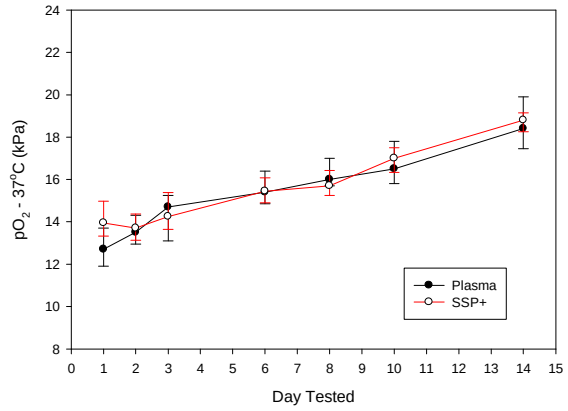


Figure 3.11: pO_2 levels in PC stored in 100% plasma vs PC stored in 70% SSP+™ (median ± interquartile range)

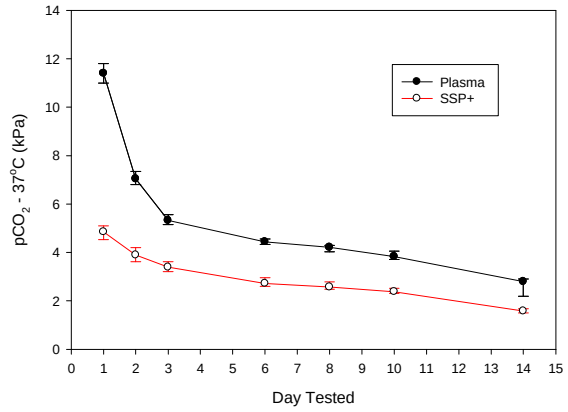


Figure 3.12: pCO_2 levels in PC stored in 100% plasma vs PC stored in 70% SSP+™ (median ± interquartile range)

Table 3.11: Summary results for pO_2 – PC in 100% plasma vs 70% SSP+™

Day Tested	pO_2 (37°C) (kPa)				
	100% Plasma (n=13)		70% SSP+™ (n=12)		
	Median	Range	Median	Range	p value
1	12.7	10.5-14.6	14.0	10.5-15.6	0.027
2	13.5*	10.6-15.1	13.7^{NS}	12.4-15.8	0.586
3	14.7*	12.4-15.8	14.3^{NS}	12.6-16.1	0.913
6	15.4*	14.0-17.7	15.5*	13.7-16.5	0.785
8	16.0*	14.1-17.9	15.7*	14.7-17.7	0.275
10	16.5*	15.5-18.7	17.0*	15.2-18.0	0.723
14	18.4*	16.0-22.5	18.8*	17.2-19.8	0.913

*: Statistically significant difference ($p<0.01$) when compared against day 1 values
^{NS}: No statistically significant difference when compared against day 1 values

Table 3.12: Summary results for pCO_2 – PC in 100% plasma vs 70% SSP+™

Day Tested	pCO_2 (37°C) (kPa)				
	100% Plasma (n=13)		70% SSP+™ (n=12)		
	Median	Range	Median	Range	p value
1	11.4	9.8-12.9	4.85	3.45-5.18	<0.001
2	7.04*	6.34-7.49	3.89*	3.53-4.33	<0.001
3	5.32*	4.96-5.71	3.39*	3.13-3.75	<0.001
6	4.44*	4.03-4.66	2.72*	2.42-3.08	<0.001
8	4.21*	3.67-4.57	2.57*	2.31-2.87	<0.001
10	3.83*	2.97-4.16	2.38*	2.09-2.58	<0.001
14	2.79*	1.38-3.32	1.58*	1.47-1.89	<0.001

*: Statistically significant difference ($p<0.01$) when compared against day 1 values
^{NS}: No statistically significant difference when compared against day 1 values

Oxygen consumption rates were similar, with levels in both test groups gradually decreasing after day 3 (figure 3.13). Statistically significant differences ($p<0.01$) compared to baseline day 1 levels were present after days 2 and 3 in plasma units and units in 70% SSP+™, respectively. Although statistically significant differences between the two groups were evident on days 6 ($p=0.041$), day 8 ($p=0.010$) and day 14 ($p=0.040$) (table 3.13), the differences were not convincing and may be related to the higher platelet concentrations measured in the units suspended in 100% plasma (figure 3.1).

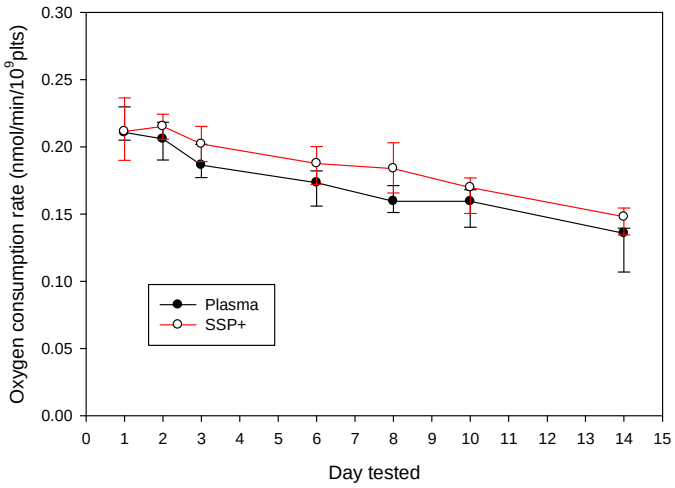


Figure 3.13: Oxygen consumption rate (22°C) for PC stored in 100% plasma vs PC stored in 70% SSP+™ (median ± interquartile range)

Table 3.13: Summary results for oxygen consumption rate (22°C) – PC in 100% plasma vs 70% SSP+™

Day Tested	Oxygen consumption rate (22°C) – (nmol/min/10^9plts)				
	100% Plasma (n=13)		70% SSP+™ (n=12)		
	Median	Range	Median	Range	p value
1	0.211	0.182-0.270	0.211	0.169-0.247	0.683
2	0.206^{NS}	0.184-0.241	0.215^{NS}	0.173-0.231	0.242
3	0.186*	0.163-0.226	0.202^{NS}	0.183-0.227	0.053
6	0.173*	0.153-0.195	0.188*	0.165-0.214	0.041
8	0.159*	0.126-0.178	0.184*	0.151-0.218	0.010
10	0.159*	0.118-0.189	0.170*	0.142-0.181	0.165
14	0.136*	0.048-0.182	0.148*	0.122-0.160	0.040

*: Statistically significant difference ($p<0.01$) when compared against day 1 values
^{NS}: No statistically significant difference when compared against day 1 values

ATP and ADP Levels

ATP levels decreased in plasma units from a median of 7.37 µmol/10^{11}plts on day 1 to 4.01 µmol/10^{11}plts on day 14 in units suspended in 100% plasma. Levels in units with SSP+™ were not significantly different until day 14 (p=0.023), with a higher median of 4.845 µmol/10^{11}plts at this time point compared to plasma units (table 3.14). End of storage levels in 100% plasma units were thus 54% of day 1 values and 61% of starting values for units in SSP+™ units. An initial increase in ATP levels was found in both test groups; a pattern previously observed in some published studies (figure 3.14) (Wallvik and Akerblom, 1983, Sandgren et al., 2010). Despite the similar pattern of ATP levels with storage, a statistically significant difference (p<0.01) compared to baseline day 1 values was only obtained on day 14 in SSP+™ units compared with days 2, 3, 6 and 14 in plasma units (table 3.14).

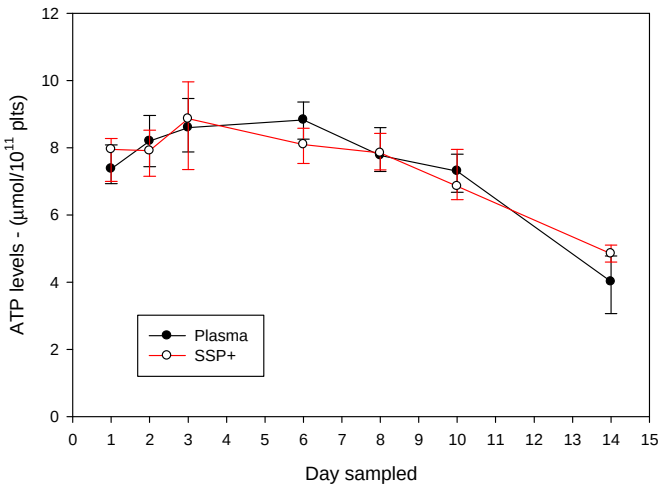


Figure 3.14: ATP levels for PC stored in 100% plasma vs PC stored in 70% SSP+™ (median ± interquartile range)

Table 3.14: Summary results for ATP levels – PC in 100% plasma vs 70% SSP+™

Day Tested	ATP levels – (µmol/10^{11}plts)				
	100% Plasma (n=13)		70% SSP+™ (n=12)		
	Median	Range	Median	Range	p value
1	7.37	5.68-8.69	7.95	6.38-8.98	0.399
2	8.20*	6.40-9.58	7.91^{NS}	5.96-10.36	0.497
3	8.60*	7.58-9.89	8.86^{NS}	6.37-10.88	0.935
6	8.83*	6.83-9.81	8.09^{NS}	7.24-9.74	0.087
8	7.77^{NS}	6.53-9.62	7.84^{NS}	6.33-9.44	0.892
10	7.30^{NS}	5.77-9.07	6.85^{NS}	5.85-9.10	0.497
14	4.01*	1.95-5.32	4.85*	3.69-5.75	0.023

*: Statistically significant difference (p<0.01) when compared against day 1 values
^{NS}: No statistically significant difference when compared against day 1 values

ADP levels also decreased with storage, from a median on day 1 of 5.99 µmol/10^{11}plts for 100% plasma units to 1.66 µmol/10^{11}plts by day 14. Respective levels in SSP+™ units were 5.70 and 2.55 µmol/10^{11}plts, with a statistically significant difference only evident at end of storage ($p<0.001$). In both test groups, statistically significant differences ($p<0.001$) compared to baseline day 1 levels were obtained after day 6 (table 3.15). Although the error bars indicate a greater degree of variation in the results compared with the ATP data, the overall pattern indicates a decrease in ADP levels unrelated to the levels of ATP in both test groups (figure 3.15).

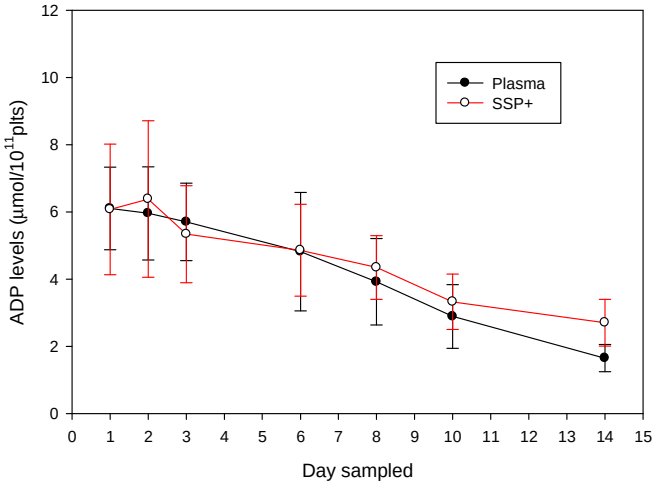


Figure 3.15: ADP levels for PC stored in 100% plasma vs PC stored in 70% SSP+™ (median ± interquartile range)

Table 3.15: Summary results for ADP levels – PC in 100% plasma vs 70% SSP+™

Day Tested	ADP levels – (µmol/10^{11}plts)				
	100% Plasma (n=13)		70% SSP+™ (n=12)		
	Median	Range	Median	Range	p value
1	5.99	4.03-8.80	5.70	3.53-10.32	0.765
2	5.74^{NS}	3.48-9.02	6.34^{NS}	3.49-10.66	0.807
3	5.70^{NS}	3.12-8.02	5.62^{NS}	2.98-8.18	0.644
6	4.31^{NS}	2.45-7.82	4.55^{NS}	2.69-7.13	0.978
8	3.78*	2.06-6.76	4.37*	2.88-6.02	0.221
10	3.02*	1.69-4.75	3.58*	1.60-4.25	0.183
14	1.66*	1.03-2.34	2.55*	1.62-4.26	<0.001

*: Statistically significant difference ($p<0.01$) when compared against day 1 values
^{NS}: No statistically significant difference when compared against day 1 values

Platelet Activation

CD62P – Surface Expression and Soluble Concentration

Surface expression of CD62P is reported as percentage positive expression and median fluorescence intensity. The percentage of platelets expressing CD62P in 100% plasma units increased from median levels of 53.5% on day 1 to 89.5% on day 14, with corresponding expressions of 55.7% and 72.7% in SSP+™ units. In both groups, the percentage positive expression decreased before increasing again in the latter half of the storage period, though a delay was evident in the pattern provided by the SSP+™ units compared with the units in 100% plasma (figure 3.16). A comparison with baseline day 1 levels showed statistically significant differences (p=0.009 to <0.001) at all time-points except day 6 in plasma units. The shallower decrease in expression in SSP+™ in the first half of the storage period resulted in significant differences compared to day 1 only on days 6 and 14 (p<0.001) (table 3.16). Statistically significant differences between the groups were noted at various points during storage (p=0.002 on day 2, p=0.011 on day 3 and finally p <0.001 on day 14) (table 3.16).

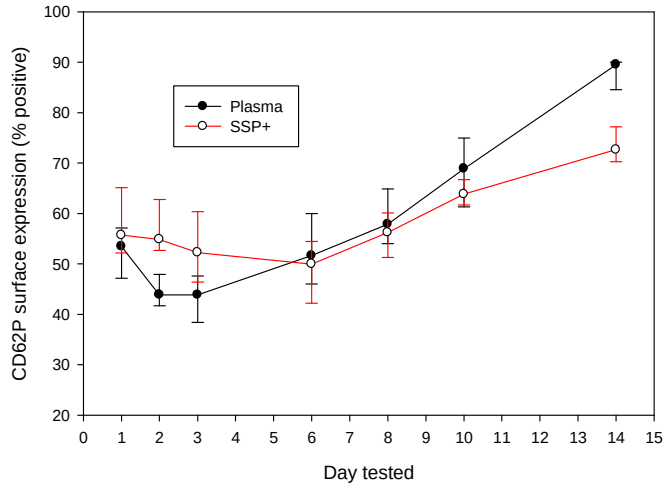


Figure 3.16: Surface CD62P expression (% positive) for PC stored in 100% plasma vs PC stored in 70% SSP+ ™ (median ± interquartile range)

Table 3.16: Summary results for surface expression of CD62P (% positive) – PC in 100% plasma vs 70% SSP+™

Day Tested	Surface expression of CD62P – (% positive)				
	100% Plasma (n=13)		70% SSP+™ (n=12)		
	Median	Range	Median	Range	p value
1	53.45	38.06-67.20	55.71	46.93-69.33	0.221
2	43.87*	29.54-64.36	54.84^{NS}	47.03-71.23	0.002
3	43.86*	27.59-59.16	52.27^{NS}	39.36-62.98	0.011
6	51.64^{NS}	37.61-63.42	49.99*	38.89-57.98	0.312
8	57.86*	49.84-71.59	56.22^{NS}	39.78-61.84	0.201
10	68.90*	58.17-78.18	63.84^{NS}	57.43-73.75	0.202
14	89.51*	80.17-94.68	72.65*	67.11-82.11	<0.001

*: Statistically significant difference ($p<0.01$) when compared against day 1 values
^{NS}: No statistically significant difference when compared against day 1 values

Median fluorescence intensity was similar between the two test groups until day 10, with no statistically significant differences from days 1 to 8 (table 3.17). In both test groups, a slight decrease in median fluorescence intensity was observed from day 1 to day 6. Statistically significant differences compared to day 1 levels were noted on days 2 (p=0.006) and 3 (p=0.003) in 100% plasma units. In both test groups significant differences were also obtained on day 14 (p=0.007 in SSP+™ and <0.001 in 100% plasma) (table 3.17). Beyond day 10 MFI increased at a markedly greater rate in 100% plasma units, resulting in statistically significant differences between the groups on days 10 and 14 (p=0.046 and <0.001, respectively); with median values at end of storage of 2.88 and 1.75 in 100% plasma and SSP+ (figure 3.17).

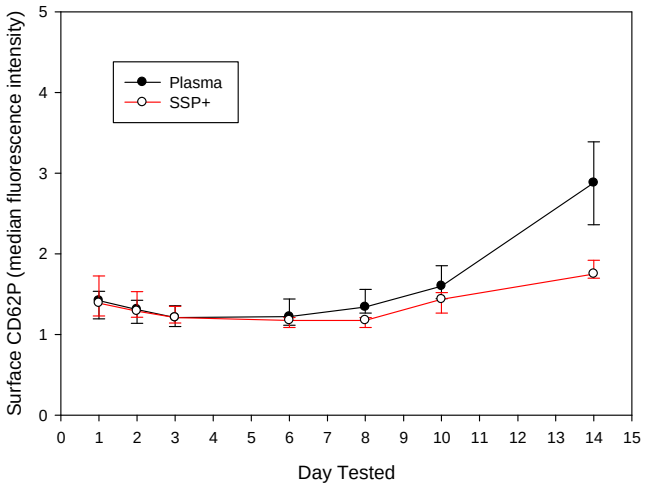


Figure 3.17: Surface CD62P expression (MFI) for PC stored in 100% plasma vs PC stored in 70% SSP+™ (median ± interquartile range)

Table 3.17: Summary results for surface expression of CD62P (median fluorescence intensity) – PC in 100% plasma vs 70% SSP+™

Day Tested	Surface expression of CD62P – (median fluorescence intensity)				
	100% Plasma (n=13)		70% SSP+™ (n=12)		
	Median	Range	Median	Range	p value
1	1.42	1.05-2.24	1.39	1.05-1.99	0.683
2	1.31*	1.00-1.85	1.29^{NS}	1.01-1.82	0.605
3	1.21*	0.93-1.66	1.21^{NS}	1.07-1.76	0.764
6	1.22^{NS}	1.06-1.65	1.18^{NS}	1.05-1.43	0.174
8	1.34^{NS}	1.12-2.01	1.31^{NS}	1.12-1.61	0.157
10	1.60^{NS}	1.31-2.14	1.44^{NS}	1.14-1.74	0.046
14	2.88*	2.30-4.72	1.75*	1.62-2.49	<0.001

*: Statistically significant difference (p<0.01) when compared against day 1 values
^{NS}: No statistically significant difference when compared against day 1 values

Soluble CD62P levels were significantly lower in units suspended in 70% SSP+™ throughout the storage period due to the lower volume of plasma in the suspending medium (p=0.001 to <0.001) (figure 3.18). Starting levels were 89.7 and 52.0 ng/mL for units in plasma and SSP+™ respectively, increasing at a constant rate with storage to median levels on day 14 of 160.3 and 134.8 ng/mL (table 3.18). Statistically significant differences compared to baseline day 1 levels were obtained at all time points in both test groups with the exception of day 2 in 100% plasma units (p<0.001 in all cases) (table 3.18).

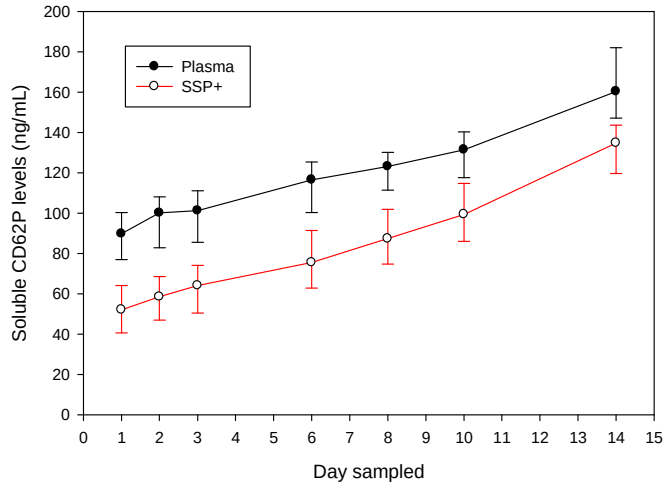


Figure 3.18: Soluble CD62P levels for PC stored in 100% plasma vs PC stored in 70% SSP+™ (median ± interquartile range)

Table 3.18: Summary results for soluble CD62P levels – PC in 100% plasma vs 70% SSP+™

Day Tested	Soluble CD62P levels (ng/mL)				
	100% Plasma (n=11)		70% SSP+™ (n=12)		
	Median	Range	Median	Range	p value
1	89.72	66.78-139.29	52.03	25.02-73.77	<0.001
2	100.11[NS]	69.77-141.52	58.48*	26.74-84.03	<0.001
3	101.24*	80.93-140.18	64.07*	28.46-87.97	<0.001
6	116.49*	96.08-167.43	75.51*	40.61-100.08	<0.001
8	123.13*	101.88-193.52	87.35*	45.20-108.47	<0.001
10	131.42*	115.79-206.55	99.40*	55.00-121.77	<0.001
14	160.33*	137.98-241.45	134.77*	75.10-157.17	0.001

*: Statistically significant difference (p<0.01) when compared against day 1 values
[NS]: No statistically significant difference when compared against day 1 values

Markers of Cell Death

Annexin V Binding

Annexin V binding to the platelet surface, measured as percentage positive expression, increased gradually in both test groups until day 8, with the 100% plasma units displaying a proportionately higher rate of aminophospholipid expression leading to statistically significant differences on days 3 to 8 (p=0.041 to <0.001) (table 3.19). Beyond day 8, the rate of annexin V binding increased in both test groups, resulting in median values on day 14 of 30.07% and 29.07% in 100% plasma and SSP+™ units, respectively (p=0.532) (figure 3.19). A comparison with baseline day 1 levels showed statistically significant differences (p<0.001 in all cases) after day 3 in both test groups (table 3.19).

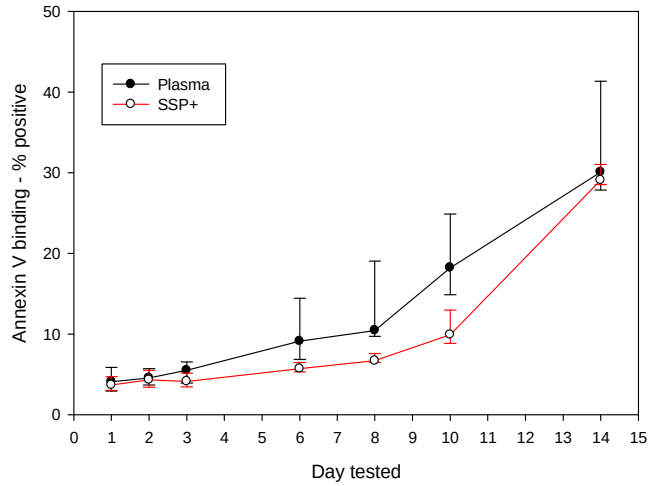


Figure 3.19: Annexin V binding (% positive) for PC stored in 100% plasma vs PC stored in 70% SSP+™ (median ± interquartile range)

Table 3.19: Summary results for annexin V binding (% positive) – PC in 100% plasma vs 70% SSP+™

Day Tested	Annexin V binding (% positive)				
	100% Plasma (n=13)		70% SSP+™ (n=12)		
	Median	Range	Median	Range	p value
1	4.10	2.63-15.56	3.67	2.42-6.81	0.568
2	4.55^{NS}	2.82-14.86	4.31^{NS}	2.33-6.49	0.463
3	5.49^{NS}	3.28-13.18	4.14^{NS}	3.24-6.34	0.041
6	9.11*	6.35-17.44	5.70*	3.76-10.05	<0.001
8	10.44*	8.17-20.91	6.69*	4.99-9.14	<0.001
10	18.21*	9.04-31.71	9.91*	7.83-15.88	0.001
14	30.07*	23.75-46.47	29.07*	21.46-35.48	0.532

*: Statistically significant difference (p<0.01) when compared against day 1 values
^{NS}: No statistically significant difference when compared against day 1 values

When analysed as mean fluorescence intensity, annexin V binding was found to be significantly lower throughout storage in the 100% plasma units (p=002 to <0.001) (figure 3.20). Median day 1 results of 24.7 and 31.6 for plasma and SSP+™ units, respectively, decreased to 11.2 and 21.8 by day 10; subsequently increasing to corresponding median values of 12.4 and 29.5 on day 14. A comparison with baseline day 1 values showed statistically significant differences (p=0.003 to <0.001) after day 3 in 100% plasma units. A similar pattern was obtained with 70% SSP+™ units, with the exception of day 14 where the increase in MFI after day 10 resulted in similar values to day 1 (p=0.413) (table 3.20).

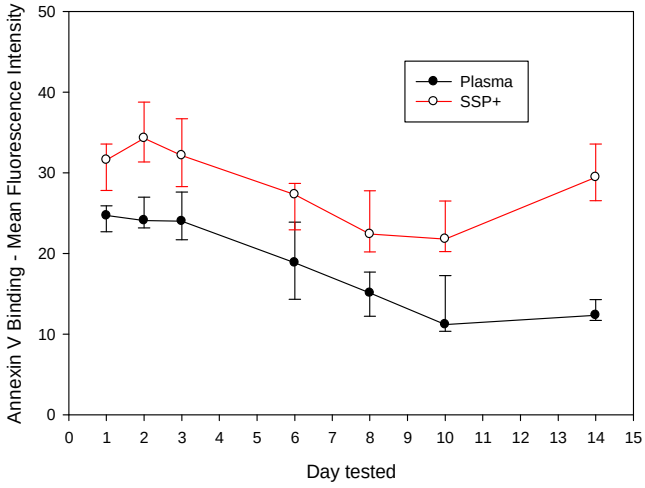


Figure 3.20: Annexin V binding expressed as mean fluorescence intensity for PC stored in 100% plasma vs PC stored in 70% SSP+™ (median ± interquartile range)

Table 3.20: Summary results for annexin V binding (mean fluorescence intensity) – PC in 100% plasma vs 70% SSP+™

Day Tested	Annexin V binding (mean fluorescence intensity)				
	100% Plasma (n=13)		70% SSP+™ (n=12)		
	Median	Range	Median	Range	p value
1	24.7	18.2-27.7	31.6	25.6-38.5	<0.001
2	24.1^{NS}	20.2-27.6	34.3^{NS}	26.7-44.5	<0.001
3	24.0^{NS}	19.3-33.3	32.2^{NS}	25.6-45.2	<0.001
6	18.9*	12.2-27.0	27.3*	19.4-34.2	0.002
8	15.1*	8.3-21.8	22.4*	18.4-30.0	<0.001
10	11.2*	8.0-19.0	21.8*	12.4-37.8	<0.001
14	12.4*	11.1-15.8	29.5^{NS}	23.2-35.2	<0.001

*: Statistically significant difference (p<0.01) when compared against day 1 values
^{NS}: No statistically significant difference when compared against day 1 values

Mitochondrial Membrane Potential

Mitochondrial membrane potential, analysed as the ratio of red to green fluorescence for the JC-1 dye, decreased in both groups with storage (figure 3.21). Median values of 5.44 and 6.75 on day 1 in plasma and SSP+™ units respectively (p=0.009) decreased to corresponding levels of 3.74 and 3.43 by end of storage. A comparison against baseline day 1 levels showed statistically significant differences in both test groups after day 2 (p=0.007 to <0.001). Statistically significant differences between the two groups were evident at a number of time points, but were interspersed with non-statistically significant changes on days 6 (p=0.121) and 10 (p=0.849) (table 3.21).

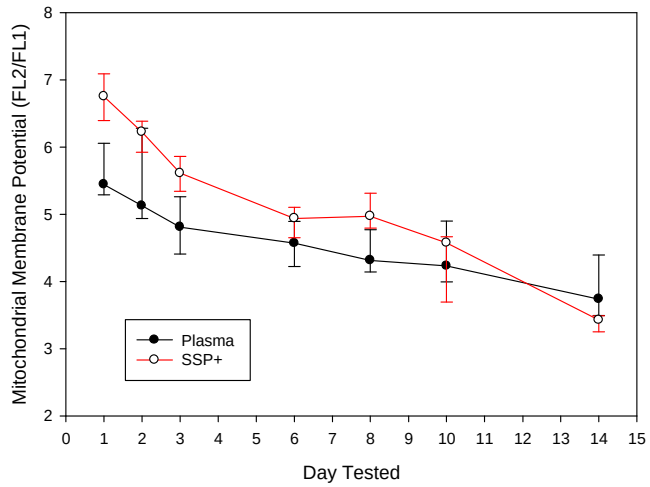


Figure 3.21: Mitochondrial membrane potential (FL2/FL1JC-1 ratio) for PC stored in 100% plasma vs PC stored in 70% SSP+™ (median ± interquartile range)

Table 3.21: Summary results for annexin V binding (mean fluorescence intensity) – PC in 100% plasma vs 70% SSP+™

Day Tested	Mitochondrial membrane potential (FL2/FL1 JC-1 ratio)				
	100% Plasma (n=13)		70% SSP+™ (n=12)		
	Median	Range	Median	Range	p value
1	5.44	4.49-7.48	6.75	5.88-7.23	0.009
2	5.13^{NS}	4.77-7.44	6.22^{NS}	5.82-6.63	0.036
3	4.81*	4.16-6.58	5.61*	4.94-6.43	0.005
6	4.57*	3.67-6.11	4.94*	3.45-5.57	0.121
8	4.32*	3.97-5.99	4.97*	4.35-5.47	0.008
10	4.23*	3.73-5.57	4.57*	3.31-5.02	0.849
14	3.74*	3.30-5.18	3.43*	3.04-3.69	0.003

*: Statistically significant difference (p<0.01) when compared against day 1 values
^{NS}: No statistically significant difference when compared against day 1 values

DISCUSSION

The platelets suspended in a medium containing 70% SSP+™ performed at least as well as platelets in 100% autologous plasma for up to 10 days of storage. From this point, the exhaustion of glucose in these units may lead to accelerated deterioration of platelet viability which may not be reflected by the standard quality monitoring parameters of pH and swirling. The increase in the percentage positive expression of annexin V binding and decrease in mitochondrial membrane potential suggest an apoptosis-like process may be involved in the platelet storage lesion, with mitochondrial changes preceding an increase in the percentage of platelets expressing annexin V. However, more targeted studies would be required to define the time course of events and their relation to platelet death, with the expectation that the studies to follow employing additive solutions manipulated to isolate the role of particular components of the solution may offer further insight into this process. From the perspective of clinical transfusion practice, although the assessment of platelet *in vitro* characteristics is a logical starting point for the investigation of platelet viability beyond 7 days of storage, the questions of haemostatic function as well as platelet recovery and survival following transfusion would need to be addressed prior to any consideration of extending the storage period of PCs beyond seven days.

CHAPTER 4. IMPACT ON PLATELET *IN VITRO* STORAGE CHARACTERISTICS OF THE INCLUSION OF ALBUMIN TO ADDITIVE SOLUTIONS

INTRODUCTION

In Europe, the approved use of platelet additive solutions for the storage of platelet concentrates requires the retention of approximately 30% of autologous plasma in the suspending medium, though some studies suggest a reduction of the plasma fraction to 20% is sufficient to retain acceptable platelet function and viability for 7 days (Gulliksson et al., 2003, Sweeney et al., 2006). Similar restrictions apply to the only platelet additive solution approved for use by the US Food and Drug Administration – PAS-III (marketed as InterSol™; Fenwal Inc., Lake Zurich, IL) – which requires the carry-over of 65% of autologous plasma (www.fda.gov; accessed on 08/09/2012). The question remains as to why this contribution by plasma should be necessary. One hypothesis is that plasma contains survival factors that retard the progressive deterioration of platelets stored as concentrates (Brown et al., 2000), with a possible candidate in the form of plasma proteins such as albumin which have been postulated to play a role in the maintenance of platelet membrane integrity (Holme, 1992). Other potentially beneficial roles for albumin include the scavenging of reactive oxygen species (Nicholson et al., 2000) and a possible role in the delay of apoptosis, with the latter having been reported in cultured endothelial cells (Zoellner et al., 1996). The aim of the study was thus to determine whether human albumin (in the form of a commercially available therapeutic formulation) would delay the onset of the platelet storage lesion, with emphasis on markers indicative of an apoptosis-like process.

Statistical comparisons between the test groups were not undertaken due to the small number of replicates and the results from all three experiments are provided in the text, (with the exception of unit platelet yield and volume). Graphical representations of the results are restricted to displaying the mean only.

The four test groups are:

ALB-000-nn (no albumin added)

ALB-030-nn (30 mL albumin added; equivalent to 2 g/dL)

ALB-060-nn (60 mL albumin added; equivalent to 4 g/dL)

ALB-120-nn (120 mL albumin added: equivalent to 8 g/dL)

(In each case, nn refers to a unique test number)

RESULTS

Platelet Yield and Volume

The mean platelet yield and volume for the four test groups are presented in table 4.1. Although the small numbers in each group preclude the use of a statistical comparison, all the units met the specification for platelet yield of $\geq 240 \times 10^9$ plts/unit set out in the UK Guidelines for Transfusion Services (James, 2005). The unit volumes also confirm that the units in all four test groups were consistent at the start of the storage period. All pools had residual white cell counts $<1\times10^6$/unit and all units were confirmed negative for bacterial contamination at the end of storage.

Table 4.1: Platelet yield and unit volume prior to sampling

Test Group	Platelet Yield ($\times10^9$/unit)	Volume (mL)
ALB-000-nn	303 ± 29	337.5 ± 5.0
ALB-030-nn	304 ± 17	341.8 ± 7.6
ALB-060-nn	307 ± 16	342.4 ± 4.1
ALB-120-nn	301 ± 22	338.6 ± 11.2

Data presented as mean ± SD (n=3)

Function and Morphology

Platelet Concentration

Platelet concentration in all four test groups was very similar on days 2 and 3. Subsequent to this, the platelet concentration in the three albumin-containing groups declined with storage in a dose-dependent manner, with units containing the highest levels of albumin showing the most marked decline. By the end of storage on day 10, mean concentrations in units containing 30 mL, 60 mL and 120 mL added albumin were 360×10^9/L, 260×10^9/L and 169×10^9/L, respectively (table 4.2). In contrast, units in

the test group without albumin showed a minimal decrease towards the end of storage, with mean day 10 levels of 840 × 10^9/L corresponding to 93.6% of the mean platelet concentration on day 2 (897 × 10^9/L) (figure 4.1). A similar pattern of sustained platelet concentration throughout storage was present in units re-constituted with plasma.

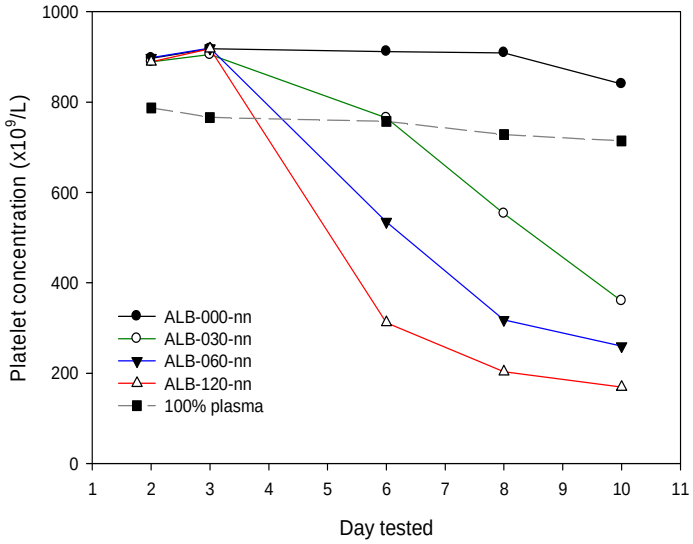


Figure 4.1: Platelet concentration (albumin study; mean, n=3)

Table 4.2: Platelet concentration in albumin study

TEST NUMBER	Platelet concentration (×10^9/L)				
	DAY 2	DAY 3	DAY 6	DAY 8	DAY 10
ALB-000-01	807	897	894	890	824
ALB-000-09	951	952	949	940	853
ALB-000-17	933	904	893	898	844
Mean	897	918	912	909	840
ALB-030-02	817	829	800	612	432
ALB-030-10	942	958	786	533	382
ALB-030-18	909	928	710	515	267
Mean	889	905	765	553	360
ALB-060-03	834	878	611	402	227
ALB-060-11	930	967	562	312	368
ALB-060-19	931	911	433	239	185
Mean	898	919	535	318	260
ALB-120-04	784	843	350	225	238
ALB-120-12	960	988	344	233	185
ALB-120-20	924	923	241	151	83
Mean	889	918	312	203	169

Mean Platelet Volume

As with the platelet concentration, the mean platelet volume between the four test groups was similar on days 2 and 3. Day 6 saw a marked increase in MPV in all the units containing albumin, with a suggestion that the highest MPVs were present in the

group with the highest concentration of albumin (figure 4.2). The rate of increase in MPV declined between days 6 and 10, with mean values on day 10 of 10.0, 10.1 and 10.4 fL in the groups containing 30 mL, 60 mL and 120 mL added albumin, respectively. This may be due to the lysis of platelets – a conclusion corroborated by the sharp decrease in platelet concentration in these units. Mean platelet volume also increased after day 3 in the test group lacking albumin, but the increase was not as marked and the mean MPV on day 10 peaked at 9.0 fL (table 4.3).

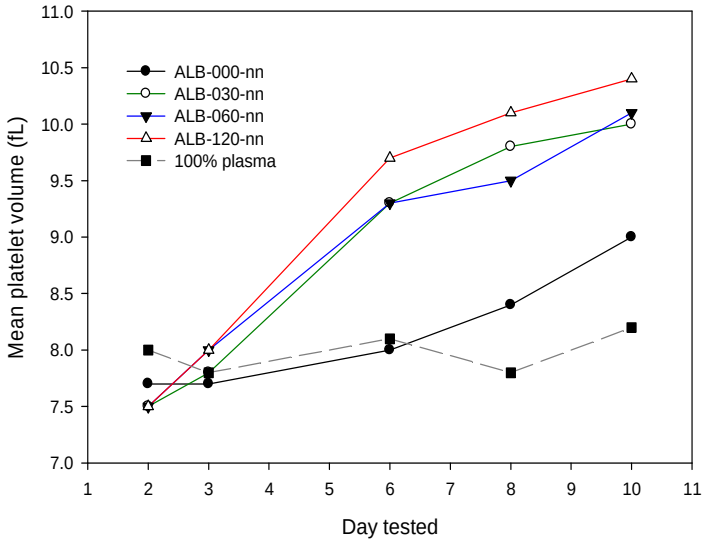


Figure 4.2: Mean platelet volume (albumin study; mean, n=3)

Table 4.3: Mean platelet volume in albumin study

TEST NUMBER	Mean platelet volume (fL)				
	DAY 2	DAY 3	DAY 6	DAY 8	DAY 10
ALB-000-01	7.6	7.7	8.1	8.6	9.1
ALB-000-09	7.7	7.7	7.9	8.1	8.6
ALB-000-17	7.8	7.8	7.9	8.4	9.2
Mean	7.7	7.7	8.0	8.4	9.0
ALB-030-02	7.3	7.7	9.0	9.7	9.7
ALB-030-10	7.4	7.6	9.6	9.7	10.1
ALB-030-18	7.8	8.2	9.2	10.0	10.2
Mean	7.5	7.8	9.3	9.8	10.0
ALB-060-03	7.3	7.8	9.3	9.9	10.1
ALB-060-11	7.4	7.9	9.3	9.1	10.1
ALB-060-19	7.8	8.2	9.3	9.6	10.2
Mean	7.5	8.0	9.3	9.5	10.1
ALB-120-04	7.3	7.7	10.0	10.1	10.2
ALB-120-12	7.7	7.9	9.7	9.7	10.6
ALB-120-20	7.6	8.3	9.5	10.6	10.4
Mean	7.5	8.0	9.7	10.1	10.4

Swirling

Swirling remained moderate throughout the storage period in the test group lacking albumin, with all units having a score of 2. In the three test groups containing albumin, swirling appeared stronger in days 2 and 3, with most units assigned a score of 3. However, on day 6, units in these test groups scored either 1 or 0 and by day 8, swirling was judged to be absent in all units (table 4.4). No distinction was evident between the three test groups with added albumin.

Table 4.4: Swirling in albumin study

TEST NUMBER	Swirling				
	DAY 2	DAY 3	DAY 6	DAY 8	DAY 10
ALB-000-01	2	2	2	2	2
ALB-000-09	2	2	2	2	2
ALB-000-17	2	2	2	3	2
ALB-030-02	2	3	1	0	0
ALB-030-10	3	3	1	0	0
ALB-030-18	3	3	1	0	0
ALB-060-03	3	3	1	0	0
ALB-060-11	3	3	1	0	0
ALB-060-19	3	3	1	0	0
ALB-120-04	3	3	1	0	0
ALB-120-12	3	3	1	0	0
ALB-120-20	3	3	0	0	0

Hypotonic Shock Response and Extent of Shape Change

The summary results for the hypotonic shock response and the extent of shape change are presented in tables 4.5 and 4.6. Values for both the HSR and ESC parameters were similar in all four test groups on days 2 and 3. Subsequent to this, results for the three test groups containing albumin were unreliable, based on the pattern of the graph output. Figure 4.3 illustrates the difficulties encountered, with representative HSR and ESC results on days 2 and 10 for one of the units with 30 mL added albumin. By contrast, the graphs from the test group lacking albumin were judged to be acceptable, showing a gradual decrease in both parameters with storage. Figures 4.4 and 4.5 compare the ESC and HSR results between the test group lacking albumin and units re-constituted in 100% plasma. In both cases, responses steadily decline with storage. The graphs also clearly display lower values for both parameters in the units suspended in additive solution compared with units re-suspended in autologous plasma, even though

sample dilutions were prepared with autologous plasma in both cases. Lower values for these assays have been reported in PCs suspended in a medium of additive solution and plasma (ratios of 70:30 and 80:20) compared to PCs in 100% plasma (Gulliksson et al., 2003, Sandgren et al., 2008) and are likely related to the performance of these assays in a suspending medium other than plasma, as suggested by Sandgren et al (Sandgren et al., 2008), possibly in combination with the harsh unit processing methodology.

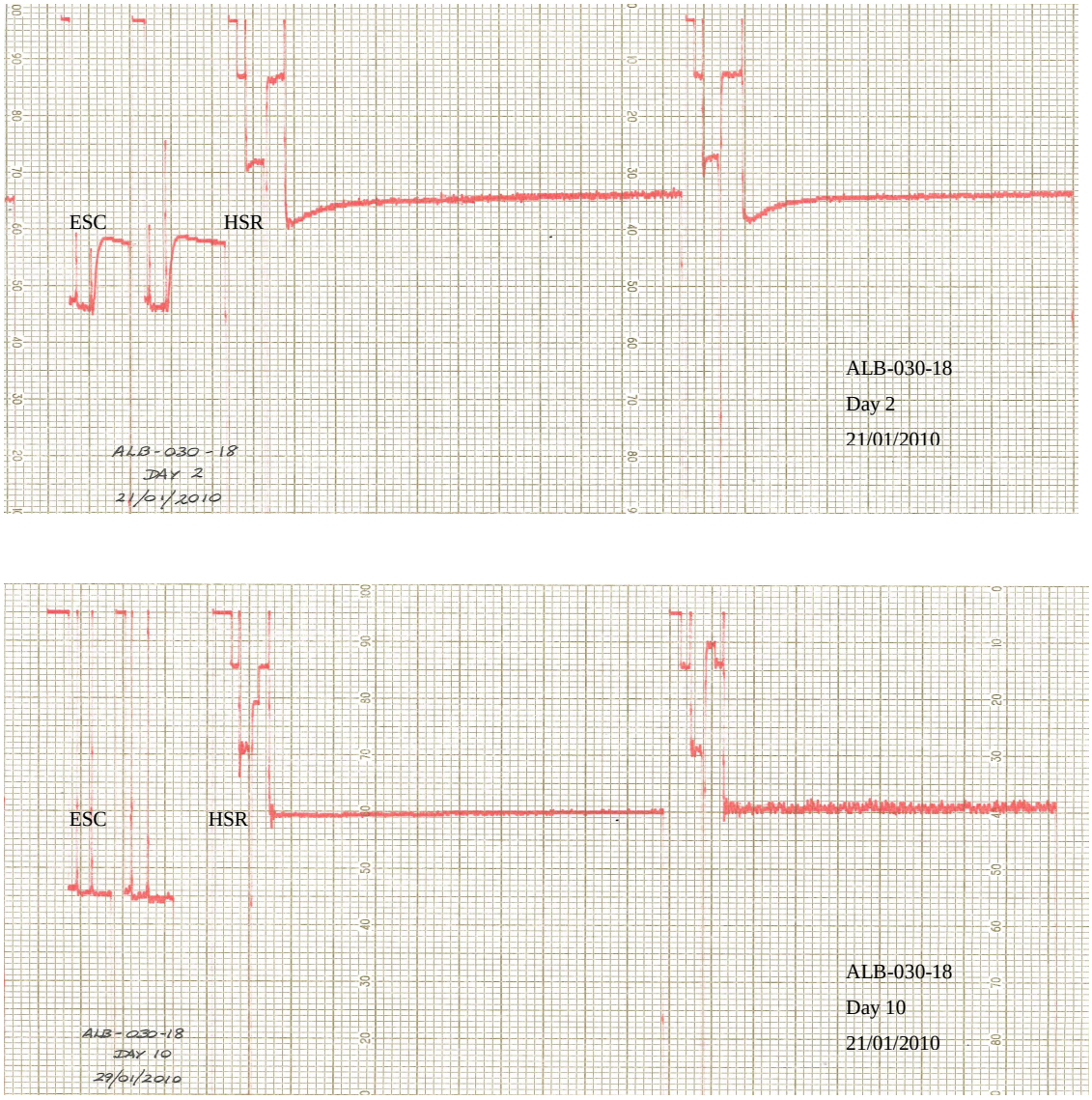


Figure 4.3: ESC and HSR outputs for ALB-030-18 on days 2 and 10 (all results in duplicate) illustrating the difficulties of interpreting results after day 3 in the test groups containing albumin.

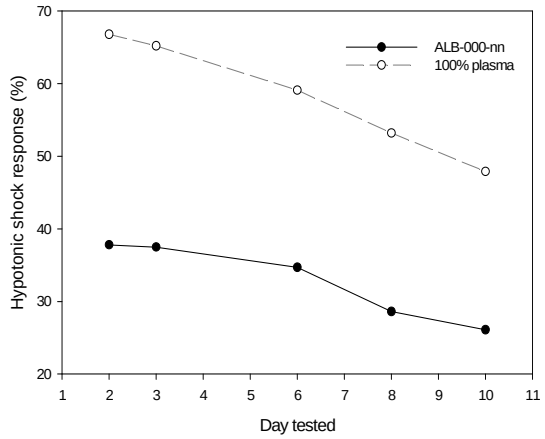


Figure 4.4: Hypotonic shock response (albumin study; mean, n=3)

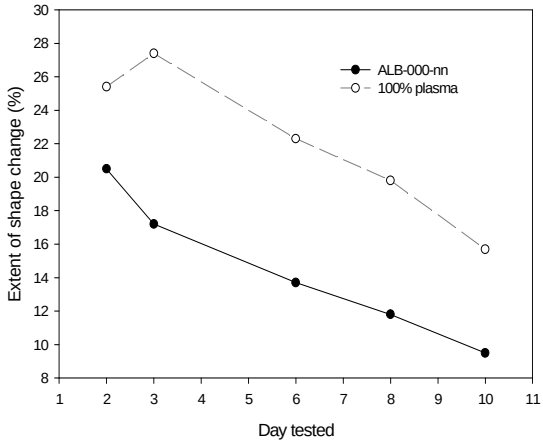


Figure 4.5: Extent of shape change (albumin study; mean, n=3 except for day 2 in 100% plasma, where n=2)

Table 4.5: Hypotonic shock response in albumin study

TEST NUMBER	Hypotonic shock response (%)				
	DAY 2	DAY 3	DAY 6	DAY 8	DAY 10
ALB-000-01	44.6	45.0	31.7	29.3	30.0
ALB-000-09	37.5	28.2	23.1	29.9	23.5
ALB-000-17	31.4	39.2	49.2	26.7	24.8
Mean	37.8	37.5	34.7	28.6	26.1
ALB-030-02	37.1	49.3	NM	NM	NM
ALB-030-10	32.5	28.7	NM	NM	NM
ALB-030-18	38.2	32.5	NM	NM	NM
Mean	35.9	36.8	N/A	N/A	N/A
ALB-060-03	32.3	36.9	NM	NM	NM
ALB-060-11	35.2	25.3	NM	NM	NM
ALB-060-19	32.9	33.4	NM	NM	NM
Mean	33.5	31.9	N/A	N/A	N/A
ALB-120-04	40.3	42.0	NM	NM	NM
ALB-120-12	37.1	36.0	NM	NM	NM
ALB-120-20	40.8	39.6	NM	NM	NM
Mean	39.4	39.2	N/A	N/A	N/A

NM: Not measured (graph not accepted) *N/A: Not applicable*

Table 4.6: Extent of shape change in albumin study

TEST NUMBER	Extent of shape change (%)				
	DAY 2	DAY 3	DAY 6	DAY 8	DAY 10
ALB-000-01	20.7	18.0	14.1	10.7	8.2
ALB-000-09	21.1	15.0	13.0	13.4	11.6
ALB-000-17	19.8	18.6	13.9	11.3	8.7
Mean	20.5	17.2	13.7	11.8	9.5
ALB-030-02	17.5	20.0	NM	NM	NM
ALB-030-10	19.0	15.2	NM	NM	NM
ALB-030-18	20.0	18.7	2.3	NM	NM
Mean	18.8	18.0	N/A	N/A	N/A
ALB-060-03	18.1	19.1	NM	NM	NM
ALB-060-11	19.8	17.4	NM	NM	NM
ALB-060-19	19.2	16.7	NM	NM	NM
Mean	19.0	17.7	N/A	N/A	N/A
ALB-120-04	20.0	18.1	NM	NM	NM
ALB-120-12	12.8	16.3	NM	NM	NM
ALB-120-20	20.5	18.9	NM	NM	NM
Mean	17.8	17.8	N/A	N/A	N/A

NM: Not measured (graph not accepted) *N/A: Not applicable*

Platelet Metabolism

pH (37°C)

From day 2, $pH_{(37°C)}$ levels were markedly lower in the units containing albumin, with a dose-dependent effect apparent from day 2. $pH_{(37°C)}$ levels reached a minimum on day 6 in the albumin-containing test groups, with mean levels of 6.730, 6.699 and 6.648 in groups containing 30 mL, 60 mL and 120 mL of albumin, respectively. After day 6, levels marginally increased (figure 4.6). Contrary to the pattern noted in the other three test groups, units without albumin showed a gradual decrease in $pH_{(37°C)}$ throughout storage, but due to the higher levels at the start of the storage period, levels in all three units in this test group remained above 6.9 on day 10 (table 4.7). A similar pattern was present in units re-constituted in plasma, with the exception of a modest increase at the beginning of the storage period.

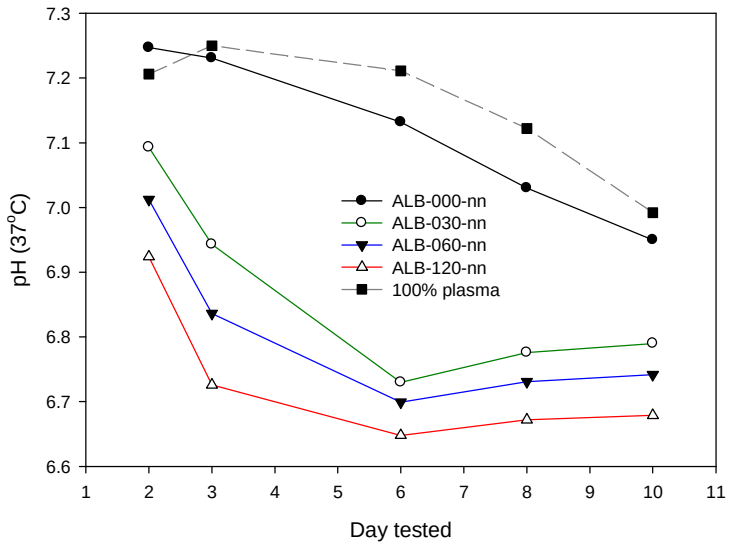


Figure 4.6: pH (37°C) (albumin study; mean, n=3)

Table 4.7: pH (37°C) in albumin study

TEST NUMBER	pH (37°C)				
	DAY 2	DAY 3	DAY 6	DAY 8	DAY 10
ALB-000-01	7.227	7.214	7.114	7.001	6.944
ALB-000-09	7.241	7.218	7.115	7.020	6.968
ALB-000-17	7.273	7.262	7.167	7.068	6.937
Mean	7.247	7.231	7.132	7.030	6.950
ALB-030-02	7.123	7.014	6.705	6.763	6.779
ALB-030-10	7.078	6.921	6.734	6.777	6.792
ALB-030-18	7.079	6.895	6.752	6.788	6.799
Mean	7.093	6.943	6.730	6.776	6.790
ALB-060-03	7.036	6.884	6.693	6.729	6.743
ALB-060-11	7.002	6.828	6.686	6.721	6.731
ALB-060-19	6.998	6.797	6.717	6.744	6.752
Mean	7.012	6.836	6.699	6.731	6.742
ALB-120-04	6.968	6.801	6.609	6.632	6.641
ALB-120-12	6.927	6.719	6.623	6.648	6.655
ALB-120-20	6.877	6.657	6.713	6.736	6.740
Mean	6.924	6.726	6.648	6.672	6.679

Glucose and Lactate

The glucose levels decreased in the units containing albumin, with all the exogenous glucose consumed by day 6 in all three groups. The decrease was more gradual in the units without added albumin, with all glucose depleted by day 10 (figure 4.7). The concentration of glucose added was 15 mmol/L and the fall in glucose, in the absence of albumin, was similar to that seen in other experiments where glucose was also depleted at day 10 (figure 5.6). The pattern was mirrored by lactate concentrations, with levels reaching a plateau in test groups containing albumin at approximately 24 mmol/L on day 6, coincident with the depletion of glucose (figure 4.8). Lactate levels in units lacking albumin continued to increase throughout storage, to a mean level of 19 mmol/L on day 10 (table 4.9).

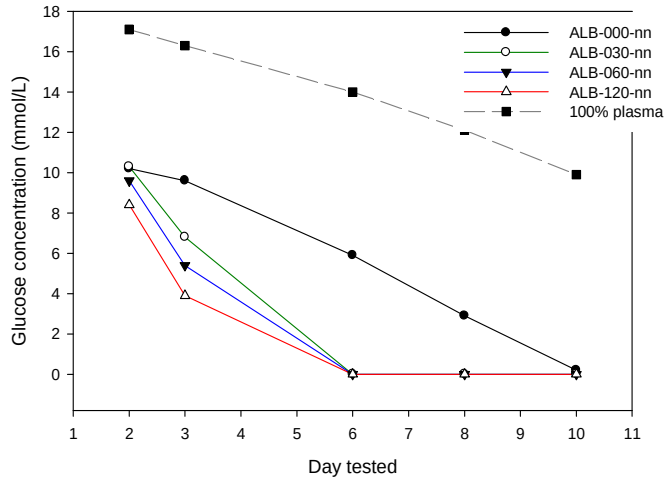


Figure 4.7: Glucose levels (albumin study; mean, n=3)

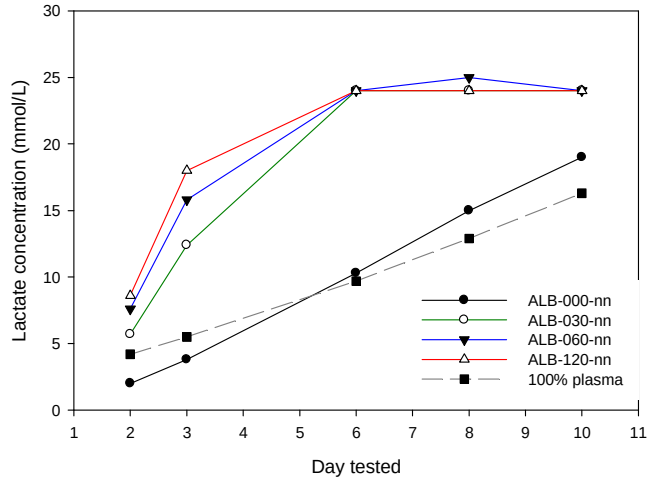


Figure 4.8: Lactate levels (albumin study; mean, n=3)

Table 4.8: Glucose levels in albumin study

TEST NUMBER	Glucose concentration (mmol/L)				
	DAY 2	DAY 3	DAY 6	DAY 8	DAY 10
ALB-000-01	8.9	8.6	5.3	2.3	0.0
ALB-000-09	9.4	8.7	4.9	2.1	0.0
ALB-000-17	12.3	11.4	7.5	4.3	0.8
Mean	10.2	9.6	5.9	2.9	0.3
ALB-030-02	10.7	8.2	0.0	0.0	0.0
ALB-030-10	10.5	6.4	0.0	0.0	0.0
ALB-030-18	9.8	5.7	0.0	0.0	0.0
Mean	10.3	6.8	0.0	0.0	0.0
ALB-060-03	9.7	6.3	0.0	0.0	0.0
ALB-060-11	9.8	5.4	0.0	0.0	0.0
ALB-060-19	9.3	4.5	0.0	0.0	0.0
Mean	9.6	5.4	0.0	0.0	0.0
ALB-120-04	9.2	5.9	0.0	0.0	0.0
ALB-120-12	9.6	4.3	0.0	0.0	0.0
ALB-120-20	6.4	1.4	0.0	0.0	0.0
Mean	8.4	3.9	0.0	0.0	0.0

Table 4.9: Lactate levels in albumin study

TEST NUMBER	Lactate concentration (mmol/L)				
	DAY 2	DAY 3	DAY 6	DAY 8	DAY 10
ALB-000-01	1.8	3.5	10.0	15	19
ALB-000-09	2.3	4.0	10.2	16	20
ALB-000-17	2.0	3.9	10.7	15	19
Mean	2.0	3.8	10.3	15	19
ALB-030-02	4.8	10.3	26	26	25
ALB-030-10	6.1	12.8	25	25	26
ALB-030-18	6.2	14.1	22	22	21
Mean	5.7	12.4	24	24	24
ALB-060-03	6.9	14.4	26	26	25
ALB-060-11	7.8	16	25	26	26
ALB-060-19	8.0	17	22	22	21
Mean	7.6	15.8	24	25	24
ALB-120-04	7.5	17	27	26	26
ALB-120-12	8.9	18	25	26	27
ALB-120-20	9.3	18	19	19	19
Mean	8.6	18	24	24	24

Glucose consumption initially increased in the test group lacking albumin from a mean rate of 0.69 mmol/L/day/10^{12}plts between days 2 and 3 to 1.65 mmol/L/day/10^{12}plts between days 6 and 8. Beyond day 8, a slight decrease was observed to a mean rate of 1.51 mmol/L/day/10^{12}plts (table 4.10). This pattern was replicated in the lactate production rates for the same units, with a maximum mean rate of 2.76 mmol/L/day/10^{12}plts between days 6 to 8, and was similar to the rates in plasma-reconstituted units (figures 4.9 and 4.10). In the test groups containing albumin, initial rates of glucose consumption and lactate production were markedly higher than in the units lacking albumin, with mean rates between days 2 and 3 in units with 30 ml added albumin of 3.94 mmol/L/day/10^{12}plts and 7.45 mmol/L/day/10^{12}plts, respectively. Rates sharply declined as the stores of exogenous glucose were consumed, with no further lactate production beyond day 6 coincident with the depletion of glucose stores.

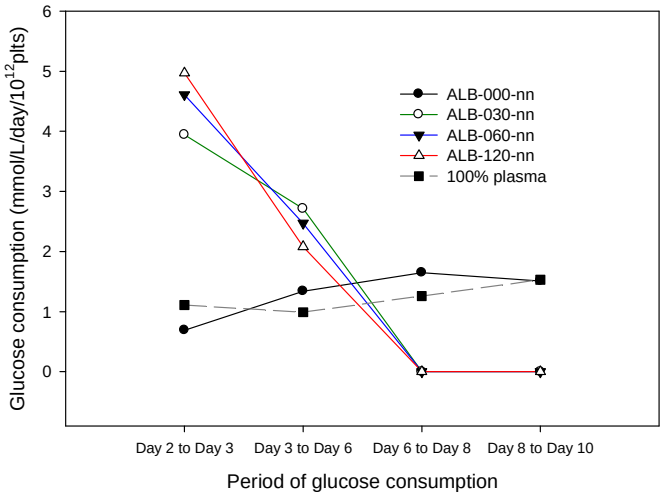


Figure 4.9: Glucose consumption (albumin study; mean, n=3)

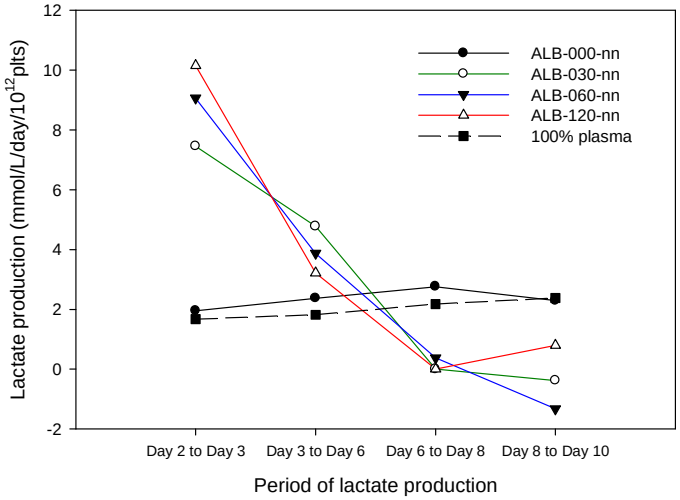


Figure 4.10: Lactate production (albumin study; mean, n=3)

Table 4.10: Glucose consumption in albumin study

TEST NUMBER	Glucose consumption (mmol/L/day/10^{12}plts)			
	DAY 2-3	DAY 3-6	DAY 6-8	DAY 8-10
ALB-000-01	0.35	1.23	1.68	1.34
ALB-000-09	0.74	1.33	1.48	1.17
ALB-000-17	0.98	1.45	1.79	2.01
Mean	0.69	1.34	1.65	1.51
ALB-030-02	3.04	3.36	0.00	0.00
ALB-030-10	4.32	2.45	0.00	0.00
ALB-030-18	4.46	2.32	0.00	0.00
Mean	3.94	2.71	0.00	0.00
ALB-060-03	3.97	2.82	0.00	0.00
ALB-060-11	4.64	2.35	0.00	0.00
ALB-060-19	5.21	2.23	0.00	0.00
Mean	4.61	2.47	0.00	0.00
ALB-120-04	4.06	3.30	0.00	0.00
ALB-120-12	5.44	2.15	0.00	0.00
ALB-120-20	5.41	0.80	0.00	0.00
Mean	4.97	2.08	0.00	0.00

Table 4.11: Lactate production in albumin study

TEST NUMBER	Lactate production (mmol/L/day/10^{12}plts)			
	DAY 2-3	DAY 3-6	DAY 6-8	DAY 8-10
ALB-000-01	2.00	2.42	2.80	2.33
ALB-000-09	1.79	2.17	3.07	2.23
ALB-000-17	2.07	2.52	2.40	2.30
Mean	1.95	2.37	2.76	2.29
ALB-030-02	6.68	6.43	0.00	-0.96
ALB-030-10	7.05	4.66	0.00	1.09
ALB-030-18	8.60	3.22	0.00	-1.28
Mean	7.45	4.77	0.00	-0.38
ALB-060-03	8.76	5.19	0.00	-1.59
ALB-060-11	8.65	3.92	1.14	0.00
ALB-060-19	9.77	2.48	0.00	-2.36
Mean	9.06	3.87	0.38	-1.32
ALB-120-04	11.68	5.59	-1.74	0.00
ALB-120-12	9.34	3.50	1.73	2.39
ALB-120-20	9.42	0.57	0.00	0.00
Mean	10.15	3.22	0.00	0.80

Bicarbonate

The concentration of bicarbonate mirrored the pattern observed with glucose, with levels decreasing more rapidly in units with added albumin until day 6 (figure 4.11). By contrast, bicarbonate levels steadily decreased in units without added albumin from a similar mean concentration on day 1 of 14.05 mmol/L to 5.13 mmol/L by day 10 (table 4.12).

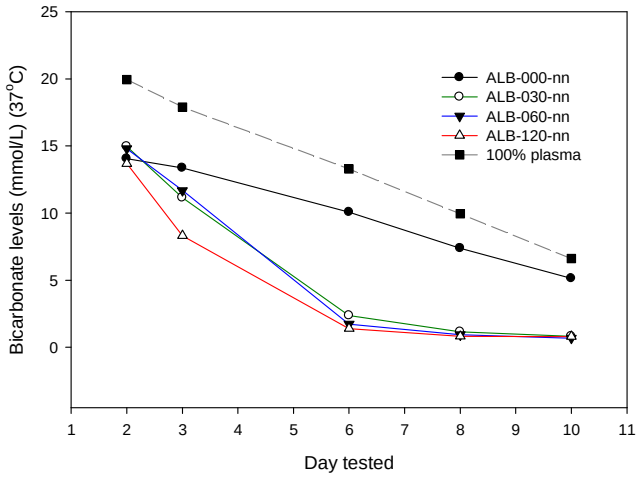


Figure 4.11: Bicarbonate levels (37°C)
(albumin study; mean, n=3)

Table 4.12: Bicarbonate levels (37°C) in albumin study

TEST NUMBER	Bicarbonate concentration (mmol/L)				
	DAY 2	DAY 3	DAY 6	DAY 8	DAY 10
ALB-000-01	11.99	12.04	9.24	6.56	4.95
ALB-000-09	13.58	12.78	9.74	7.13	5.14
ALB-000-17	16.56	15.26	11.22	8.32	5.29
Mean	14.05	13.36	10.07	7.33	5.13
ALB-030-02	14.95	12.07	3.01	1.29	1.16
ALB-030-10	15.13	11.10	2.17	1.25	0.75
ALB-030-18	14.80	10.28	1.90	0.88	0.56
Mean	14.96	11.15	2.36	1.14	0.82
ALB-060-03	14.92	10.61	1.97	1.13	0.91
ALB-060-11	14.59	9.94	1.78	0.92	0.65
ALB-060-19	14.90	9.46	1.45	0.75	0.48
Mean	14.80	10.00	1.73	0.93	0.68
ALB-120-04	14.09	9.54	1.40	0.87	1.08
ALB-120-12	15.19	8.92	1.53	0.82	0.79
ALB-120-20	11.80	6.46	1.27	0.78	0.53
Mean	13.69	8.31	1.40	0.82	0.80

Blood Gases

The partial pressures of oxygen and carbon dioxide in the albumin-containing test groups showed markedly different patterns over the ten days of storage compared with non-albumin units (figures 4.12 and 4.13). $pO_{2(37°C)}$ levels were higher in all three albumin-containing test groups throughout the storage period with a suggestion of a dose-dependent effect on days 2 and 3, with mean $pO_{2(37°C)}$ of 21.1 kPa in units with 120 mL albumin compared with 18.6 kPa in units with 30 ml added albumin. By day 6, levels plateau at maximum mean values of approximately 24-25 kPa, suggesting an equilibration of pO_2 with atmospheric partial pressure of oxygen. In non-albumin units, $pO_{2(37°C)}$ levels increased steadily after day 3 (mean $pO_{2(37°C)}$ of 13.7 kPa), reaching a mean value on day 10 of 17.3 kPa (table 4.13). $pCO_{2(37°C)}$ levels were also higher at the start of storage in albumin-containing units, with a similar suggestion of a dose-dependent effect. However, between days 3 and 6, levels steeply declined, with mean values below 1 kPa by day 8 in all three test groups with added albumin (figure 4.13). By contrast, $pCO_{2(37°C)}$ levels in non-albumin-containing units decreased gradually from mean values of 3.91 kPa on day 2 to 2.84 kPa by day 10 (table 4.14).

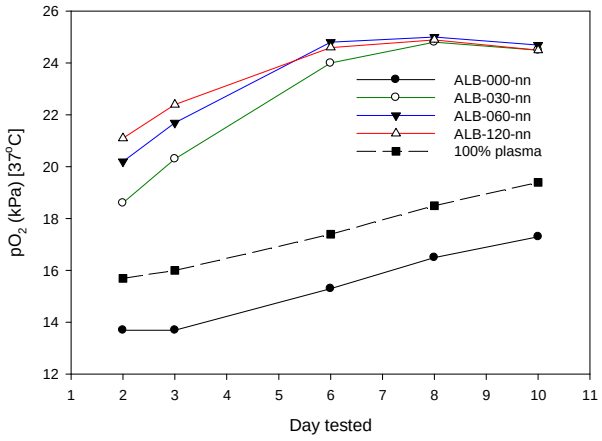


Figure 4.12: pO_2 levels (37°C) (albumin study; mean, n=3)

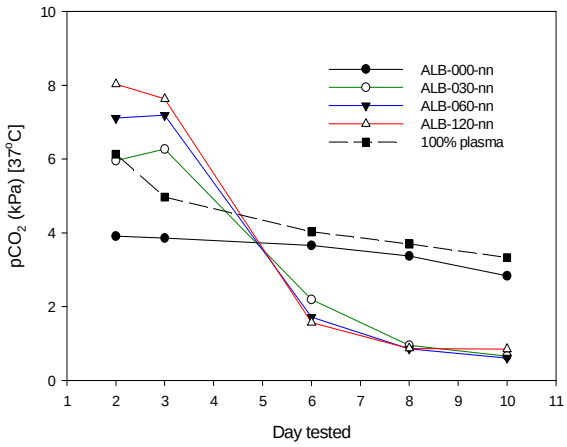


Figure 4.13: pCO_2 levels (37°C) (albumin study; mean, n=3)

Table 4.13: Partial pressure of oxygen in albumin study

TEST NUMBER	pO_2 (37°C) (kPa)				
	DAY 2	DAY 3	DAY 6	DAY 8	DAY 10
ALB-000-01	14.6	14.2	15.3	16.0	17.6
ALB-000-09	13.0	13.0	14.8	16.2	16.8
ALB-000-17	13.6	13.8	15.7	17.3	17.6
Mean	13.7	13.7	15.3	16.5	17.3
ALB-030-02	16.9	18.7	22.8	24.2	24.6
ALB-030-10	18.1	20.4	24.5	24.9	24.8
ALB-030-18	20.8	21.7	24.8	25.3	24.2
Mean	18.6	20.3	24.0	24.8	24.5
ALB-060-03	18.8	20.7	24.3	24.7	24.9
ALB-060-11	19.7	21.6	24.8	25.0	24.8
ALB-060-19	22.0	22.7	25.2	25.2	24.5
Mean	20.2	21.7	24.8	25.0	24.7
ALB-120-04	20.2	21.8	24.2	24.7	24.7
ALB-120-12	21.0	22.6	24.7	24.8	24.7
ALB-120-20	22.0	22.8	25.0	25.2	24.2
Mean	21.1	22.4	24.6	24.9	24.5

Table 4.14: Partial pressure of carbon dioxide in albumin study

TEST NUMBER	pCO_2 (37°C) (kPa)				
	DAY 2	DAY 3	DAY 6	DAY 8	DAY 10
ALB-000-01	3.51	3.63	3.51	3.23	2.78
ALB-000-09	3.85	3.82	3.69	3.36	2.73
ALB-000-17	4.36	4.12	3.77	3.51	3.02
Mean	3.91	3.86	3.66	3.37	2.84
ALB-030-02	5.56	5.77	2.93	1.10	0.95
ALB-030-10	6.24	6.57	1.98	1.03	0.60
ALB-030-18	6.09	6.46	1.66	0.71	0.44
Mean	5.96	6.27	2.19	0.95	0.66
ALB-060-03	6.78	6.84	1.97	1.04	0.81
ALB-060-11	7.17	7.29	1.81	0.86	0.60
ALB-060-19	7.39	7.45	1.37	0.67	0.42
Mean	7.11	7.19	1.72	0.86	0.61
ALB-120-04	7.49	7.45	1.70	1.00	1.22
ALB-120-12	8.87	8.41	1.80	0.91	0.86
ALB-120-20	7.73	7.02	1.21	0.71	0.48
Mean	8.03	7.63	1.57	0.87	0.85

Oxygen consumption rates were relatively stable in the test group lacking albumin, with mean values of 0.17 nmol/min/10^9plts on day 10 compared with 0.23 nmol/min/10^9plts on day 2, with a similar pattern observed from units re-constituted in plasma (figure 4.14). Rates were lower throughout the storage period in units containing albumin, with mean values on day 2 ranging between 0.08 nmol/min/10^9plts in units with 120 mL albumin to 0.13 nmol/min/10^9plts in units with 30 ml albumin (table 4.15). Oxygen consumption had essentially ceased in units containing albumin after day 6 of storage (figure 4.14).

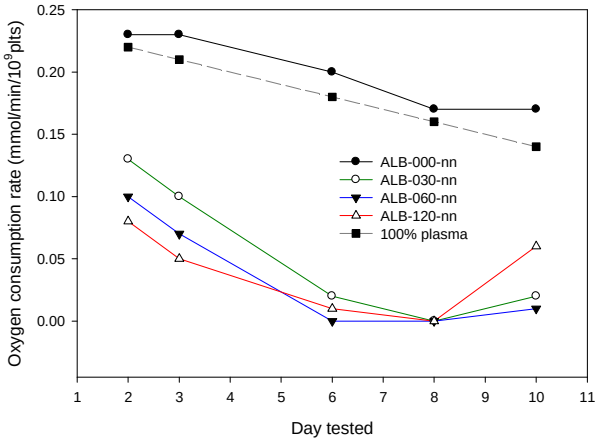


Figure 4.14: Oxygen consumption rates (22°C) in albumin study (mean, n=3)

Table 4.15: Oxygen consumption rate in albumin study (22°C)

TEST NUMBER	Oxygen consumption rate (nmol/min/10^9plts)				
	DAY 2	DAY 3	DAY 6	DAY 8	DAY 10
ALB-000-01	0.24	0.22	0.20	0.19	0.16
ALB-000-09	0.23	0.23	0.20	0.17	0.18
ALB-000-17	0.22	0.23	0.19	0.16	0.16
Mean	0.23	0.23	0.20	0.17	0.17
ALB-030-02	0.18	0.14	0.05	0.02	0.01
ALB-030-10	0.13	0.09	0.01	0.00	0.00
ALB-030-18	0.08	0.06	0.00	-0.02	0.05
Mean	0.13	0.10	0.02	0.00	0.02
ALB-060-03	0.14	0.09	0.02	0.01	0.00
ALB-060-11	0.10	0.06	0.00	-0.01	0.00
ALB-060-19	0.06	0.04	-0.01	-0.02	0.04
Mean	0.10	0.07	0.00	-0.01	0.01
ALB-120-04	0.11	0.07	0.04	0.02	0.01
ALB-120-12	0.08	0.04	0.01	0.01	0.02
ALB-120-20	0.05	0.04	-0.01	-0.04	0.15[#]
Mean	0.08	0.05	0.01	-0.01	0.06

[#]: Outlier value related to low platelet concentration

ATP and ADP Levels

ATP levels moderately declined with storage in units lacking albumin, decreasing from mean levels of 5.56 µmol/10^{11}plts on day 2 to 3.44 µmol/10^{11}plts by the end of storage, corresponding to 62% of day 2 levels. A similar gradual decrease in ATP was noted from days 2 to 3 in the albumin test groups, with all units maintaining ATP levels above 4 µmol/10^{11}plts. Beyond this time point, ATP levels decreased steeply in all test groups containing albumin (figure 4.15). By day 6, mean ATP levels were 1.05, 0.49 and 0.43 µmol/10^{11}plts in units with 30 mL, 60 mL and 120 mL added albumin, respectively, and continued to decline to corresponding mean values at end of storage of 0.30, 0.16 and 0.19 µmol/10^{11}plts (table 4.16).

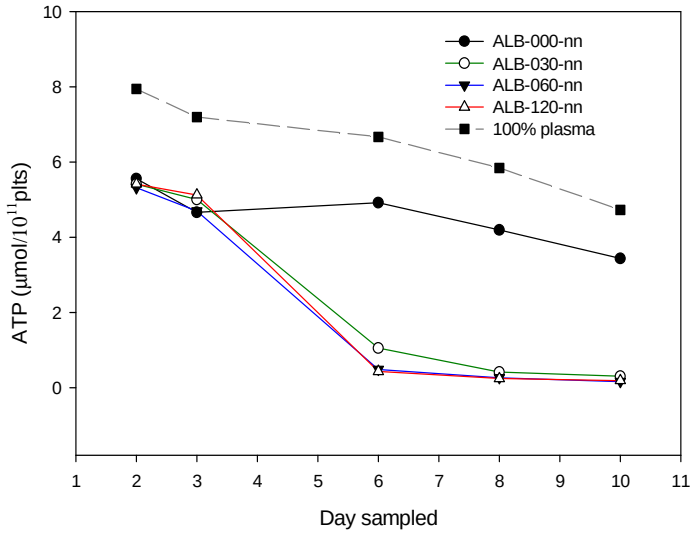


Figure 4.15: ATP levels (albumin study; mean, n=3)

Table 4.16: Adenosine triphosphate levels in albumin study

TEST NUMBER	ATP (µmol/10^{11}plts)				
	DAY 2	DAY 3	DAY 6	DAY 8	DAY 10
ALB-000-01	5.67	4.84	4.92	4.23	3.62
ALB-000-09	6.49	4.88	6.20	4.62	3.09
ALB-000-17	4.51	4.26	3.64	3.73	3.59
Mean	5.56	4.66	4.92	4.19	3.43
ALB-030-02	5.37	4.87	1.56	0.52	0.40
ALB-030-10	5.87	5.26	0.94	0.40	0.28
ALB-030-18	5.00	4.89	0.65	0.34	0.22
Mean	5.41	5.01	1.05	0.42	0.30
ALB-060-03	5.66	4.53	0.55	0.28	0.19
ALB-060-11	5.88	5.05	0.54	0.27	0.21
ALB-060-19	4.42	4.51	0.37	0.25	0.10
Mean	5.32	4.70	0.49	0.26	0.16
ALB-120-04	5.33	5.07	0.55	0.25	0.19
ALB-120-12	5.91	5.01	0.45	0.28	0.21
ALB-120-20	5.01	5.30	0.28	0.20	0.17
Mean	5.42	5.13	0.43	0.24	0.19

ADP levels decreased with storage in all test groups, with similar day 2 levels ranging from mean levels of 3.40 µmol/10^{11}plts in units with no albumin to 4.13 µmol/10^{11}plts in units with 30 ml albumin (table 4.17). In the test groups with added albumin, mean levels had decreased by day 10 to 0.40, 0.41 and 0.52 µmol/10^{11}plts in units with 120 mL, 60 mL and 30 mL albumin, respectively; equating to approximately 11.5% of levels on day 2. Although ADP levels also decreased with storage in units lacking albumin, levels remained at 49% of day 2 levels by end of storage (mean of 1.66 µmol/10^{11}plts) (figure 4.16).

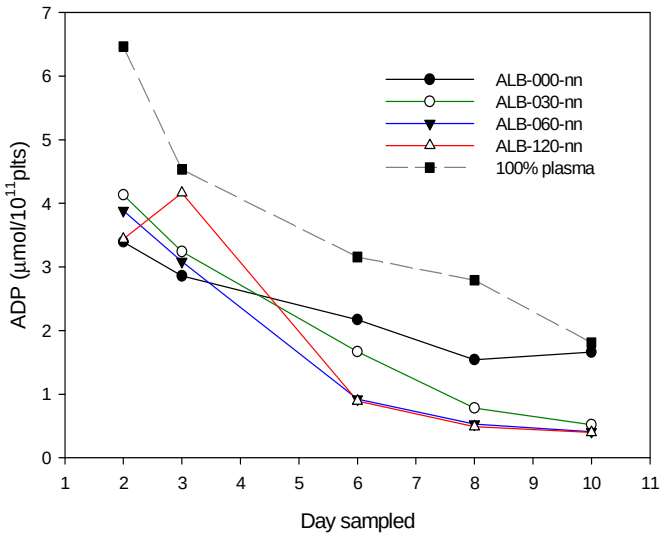


Figure 4.16: ADP levels (albumin study; mean, n=3; except for ALB-030-nn day 3; ALB-120-nn days 2 and 3 where n=2)

Table 4.17: Adenosine diphosphate levels in albumin study

TEST NUMBER	ADP (µmol/10^{11}plts)				
	DAY 2	DAY 3	DAY 6	DAY 8	DAY 10
ALB-000-01	3.31	1.91	2.09	1.99	1.79
ALB-000-09	3.04	3.35	2.78	1.06	2.18
ALB-000-17	3.84	3.32	1.65	1.59	1.01
Mean	3.40	2.86	2.17	1.54	1.66
ALB-030-02	3.54	3.43	2.00	1.01	0.59
ALB-030-10	4.45	3.05	1.68	0.75	0.64
ALB-030-18	4.41	10.07*	1.32	0.59	0.34
Mean	4.13	3.24#	1.67	0.78	0.52
ALB-060-03	2.90	2.13	1.31	0.76	0.52
ALB-060-11	3.96	3.72	0.89	0.51	0.45
ALB-060-19	4.81	3.42	0.58	0.33	0.26
Mean	3.89	3.09	0.92	0.53	0.41
ALB-120-04	2.81	3.77	1.17	0.59	0.45
ALB-120-12	4.07	4.55	0.83	0.51	0.40
ALB-120-20	14.86*	11.57*	0.67	0.38	0.35
Mean	3.44#	4.16#	0.89	0.49	0.40

*: Results considered outliers and not used in mean calculation or graph
#: Mean n=2

Platelet Activation

Surface Expression and Soluble Levels of CD62P

The percentage of platelets expressing CD62P on day 2 ranged from mean levels of 76.81% in the test group with no albumin to 84.13% in units with 30 mL albumin. Percent positive expression remained relatively stable in the test group with no albumin, increasing marginally to a mean value of 85.49% at end of storage (table 4.18). In units containing albumin, the suggestion of a dose-dependent response during the early storage period was evident from day 6 (figure 4.17). Mean values on day 6 in these three test groups were 92.65, 89.77 and 78.12% in units with 30 mL, 60 mL and 120 mL added albumin, respectively. CD62P expression decreased from this time point in all three groups in a dose-dependent pattern, resulting in mean day 10 values of 81.52, 62.01 and 48.70% in units with 30 mL, 60 mL and 120 mL albumin, respectively.

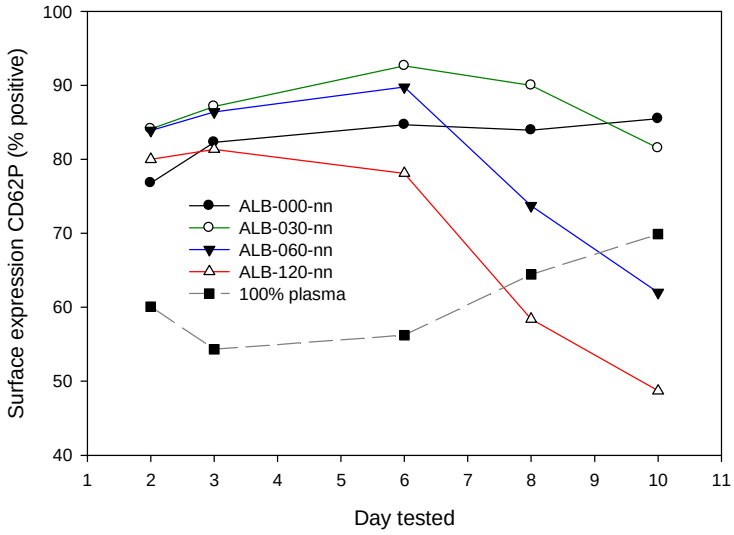


Figure 4.17: Percent positive expression of CD62P (albumin study; mean, n=3)

Table 4.18: Surface expression of CD62P in albumin study (percent positive expression)

TEST NUMBER	Surface CD62P expression (% positive)				
	DAY 2	DAY 3	DAY 6	DAY 8	DAY 10
ALB-000-01	77.95	82.96	85.36	85.23	87.22
ALB-000-09	73.27	80.08	82.77	83.12	84.98
ALB-000-17	79.22	83.89	85.93	83.46	84.27
Mean	76.81	82.31	84.69	83.94	85.49
ALB-030-02	85.06	89.08	92.73	94.60	86.71
ALB-030-10	83.17	87.05	94.07	90.25	83.62
ALB-030-18	84.16	85.39	91.16	85.20	74.22
Mean	84.13	87.17	92.65	90.02	81.52
ALB-060-03	84.89	88.18	92.14	82.94	66.74
ALB-060-11	82.56	85.19	90.35	75.48	63.70
ALB-060-19	84.24	85.87	86.83	62.78	55.58
Mean	83.90	86.41	89.77	73.73	62.01
ALB-120-04	82.19	83.28	82.19	78.23	46.41
ALB-120-12	79.10	81.73	75.93	53.28	39.30
ALB-120-20	78.72	79.06	76.23	43.68	60.38
Mean	80.00	80.36	78.12	58.40	48.70

Surface CD62P expression reported as the median fluorescence intensity increased initially in units lacking albumin up to day 6 (mean MFI of 3.70) before showing a slight decrease during the remainder of the storage period, resulting in a mean value on day 10 of 3.37 (table 4.19). In units with 120 mL albumin, MFI decreased progressively with storage from a mean value on day 2 to of 2.24 to a mean day 10 value of 1.44. Units with 30 mL and 60 mL albumin showed a mean increase in MFI values between days 2 to 3 which subsequently decreased to values similar to units with 120 mL albumin by day 10 of storage (figure 4.18).

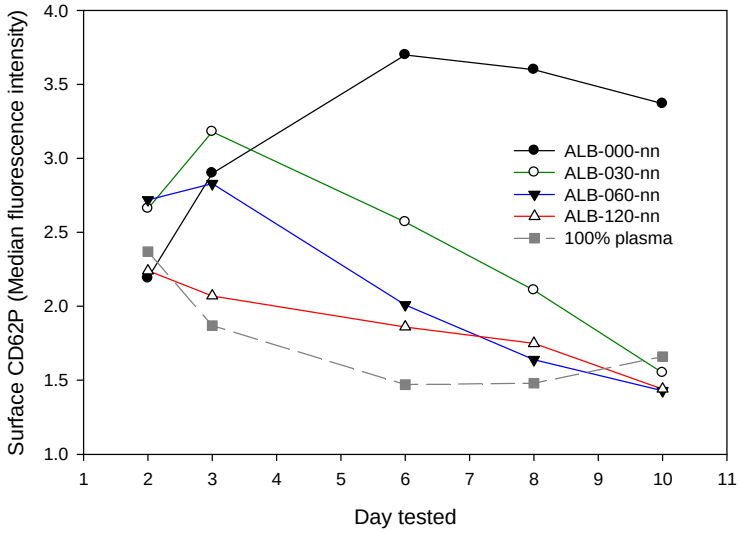


Figure 4.18: Median fluorescence intensity of CD62P (albumin study; mean, n=3)

Table 4.19: Surface expression of CD62P in albumin study (median fluorescence intensity)

TEST NUMBER	Surface CD62P expression (median fluorescence intensity)				
	DAY 2	DAY 3	DAY 6	DAY 8	DAY 10
ALB-000-01	2.38	3.21	4.02	4.02	3.64
ALB-000-09	2.12	2.73	3.64	3.54	3.36
ALB-000-17	2.07	2.75	3.45	3.24	3.12
Mean	2.19	2.90	3.70	3.60	3.37
ALB-030-02	2.91	3.61	2.96	2.71	1.92
ALB-030-10	2.61	3.12	2.66	2.03	1.49
ALB-030-18	2.45	2.80	2.08	1.58	1.25
Mean	2.66	3.18	2.57	2.11	1.55
ALB-060-03	3.10	3.27	2.38	1.84	1.49
ALB-060-11	2.56	2.75	1.94	1.69	1.68
ALB-060-19	2.49	2.47	1.71	1.40	1.11
Mean	2.72	2.83	2.01	1.64	1.43
ALB-120-04	2.52	2.34	1.94	1.52	1.43
ALB-120-12	2.20	2.05	2.07	2.01	1.90
ALB-120-20	2.01	1.82	1.56	1.73	1.00
Mean	2.24	2.07	1.86	1.75	1.44

Soluble CD62P levels on day 2 were similar in all four test groups, with mean levels ranging from 35.94 ng/mL in units with no added albumin to 37.67 ng/mL in units with 30 mL albumin (table 4.20). Levels increased in all test groups after day 3, though the rate of increase was higher in the albumin-containing units. By day 10, mean values in units lacking added albumin were 101.83 ng/mL, compared to 210.92 ng/mL and 200.80 ng/mL in units with 30 mL and 60 mL albumin, respectively. Units with the highest concentration of albumin showed a more modest increase to a mean day 10 level of 162.18 ng/mL. The dose-dependent pattern of surface CD62P expression most clearly illustrated by the percent positive expression was not as evident with the soluble marker.

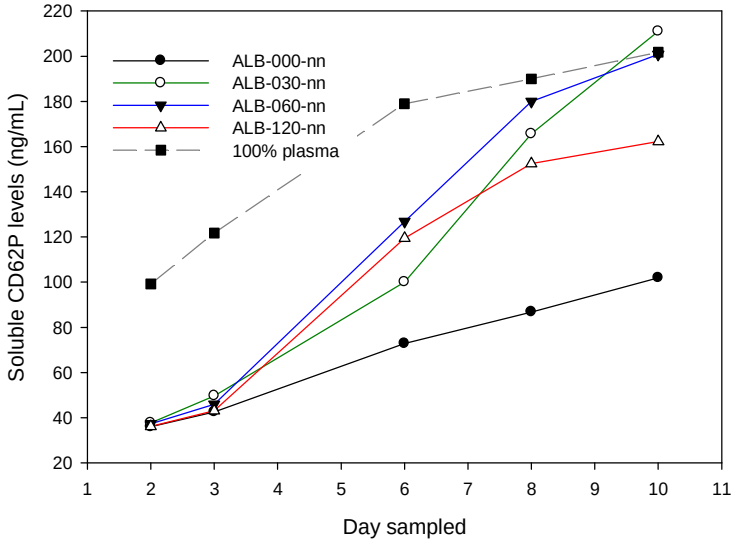


Figure 4.19: Soluble CD62P levels (albumin study; mean, n=3)

Table 4.20: Soluble CD62P levels in albumin study

TEST NUMBER	Soluble CD62P levels (ng/mL)				
	DAY 2	DAY 3	DAY 6	DAY 8	DAY 10
ALB-000-01	35.40	38.75	76.95	86.55	101.45
ALB-000-09	28.55	35.91	60.58	77.10	99.95
ALB-000-17	43.87	52.76	80.78	96.69	104.10
Mean	35.94	42.47	72.77	86.78	101.83
ALB-030-02	43.10	57.61	86.55	134.82	212.71
ALB-030-10	28.12	40.53	101.59	179.46	219.88
ALB-030-18	41.79	50.75	112.04	182.46	200.18
Mean	37.67	49.63	100.06	165.58	210.92
ALB-060-03	42.36	49.02	110.39	184.40	227.13
ALB-060-11	30.43	42.69	121.73	178.42	185.87
ALB-060-19	38.98	45.91	148.20	176.87	189.38
Mean	37.26	45.87	126.77	179.90	200.80
ALB-120-04	37.98	45.85	99.09	133.87	153.73
ALB-120-12	32.45	39.37	123.08	158.53	172.20
ALB-120-20	37.82	43.87	136.09	164.84	160.62
Mean	36.08	43.03	119.42	152.41	162.18

Indicators of Cell Death

Annexin V Binding

The percentage of platelets binding annexin V in all four test groups was similar at the start of storage, with mean values on days 2 and 3 of approximately 11 to 12%. After day 3, there was a significant increase in the percentage positive expression in all three albumin-containing groups, with levels exceeding 90% by day 6 in units with 60 mL and 120 mL added albumin (figures 4.20 and 4.21). By contrast, the test group with no added albumin showed only a modest increase in the percent positive expression by day 8 (mean value 17.04%), after which there was a moderately enhanced increase to a mean level of 30.5% by the end of storage (table 4.21).

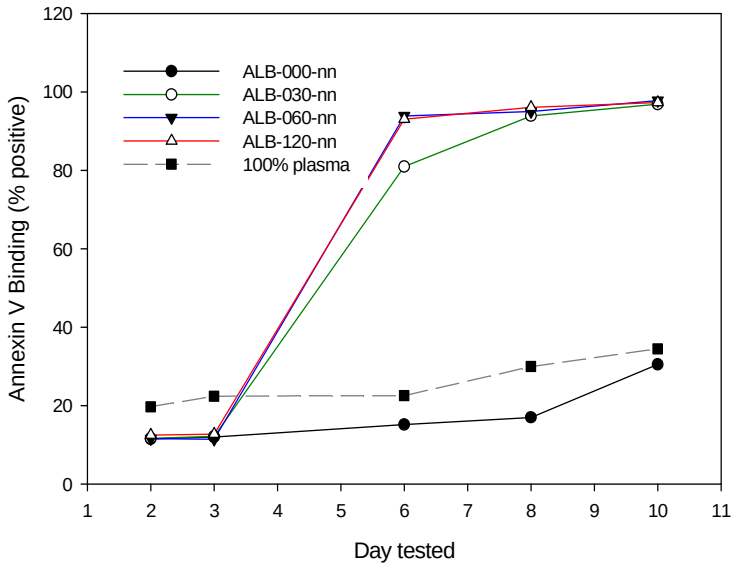


Figure 4.20: Annexin V binding expressed as percentage positive expression (albumin study; mean, n=3)

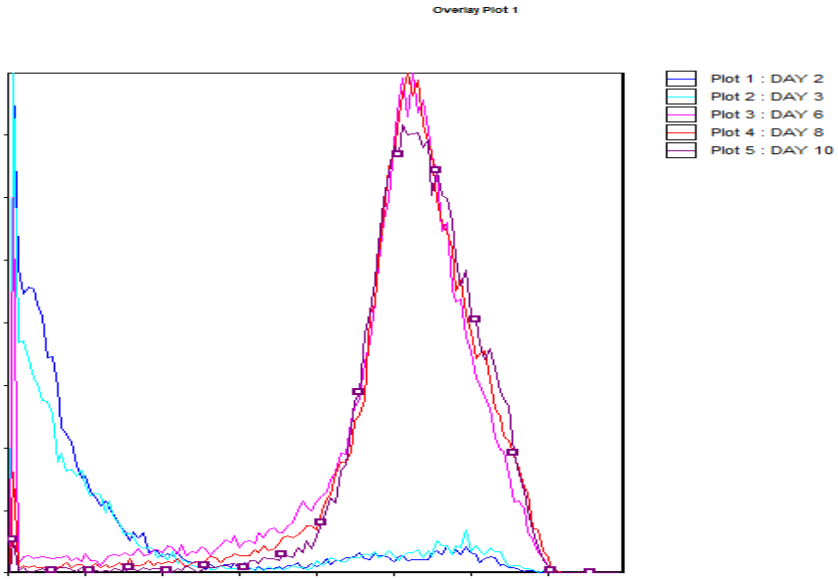


Figure 4.21: Annexin V binding expressed as percentage positive expression (albumin study; representative sample with 60 mL added albumin), illustrating the marked increase in the percentage of platelets expressing aminophospholipids on their surface by day 6 in units with added albumin

Table 4.21: Annexin V binding in albumin study (percent positive expression)

TEST NUMBER	Annexin V binding (% positive expression)				
	DAY 2	DAY 3	DAY 6	DAY 8	DAY 10
ALB-000-01	7.50	8.11	11.02	11.73	30.20
ALB-000-09	7.60	7.94	12.65	16.04	35.23
ALB-000-17	19.48	19.93	21.91	23.35	26.12
Mean	11.53	11.99	15.19	17.04	30.52
ALB-030-02	7.30	9.36	63.54	92.48	94.14
ALB-030-10	9.10	10.11	90.10	93.86	98.09
ALB-030-18	18.62	16.96	89.31	95.38	98.34
Mean	11.67	12.14	80.98	93.91	96.86
ALB-060-03	8.44	7.45	93.50	94.84	96.88
ALB-060-11	8.80	11.45	94.32	96.19	98.13
ALB-060-19	17.61	15.54	93.82	94.09	98.54
Mean	11.62	11.48	93.88	95.04	97.85
ALB-120-04	8.30	7.22	92.04	96.51	96.57
ALB-120-12	13.04	15.79	92.72	95.88	97.36
ALB-120-20	16.16	15.21	94.54	95.82	98.07
Mean	12.50	12.74	93.10	96.07	97.33

Annexin V binding expressed as the mean fluorescence intensity did not result in the marked differences between the albumin and non-albumin-containing test groups described above for the percent positive expression, though the test group lacking added albumin showed a generally lower MFI and a more stable pattern throughout storage (figure 4.22). Mean MFI expression increased modestly in these units from 35.6 on day 2 to 41.7 by end of storage. The test groups containing albumin showed an increase in MFI from days 2 to 6, beyond which MFI stabilised or moderately decreased. Units with 120 mL added albumin displayed the most marked increase in MFI, with a mean value of 29.2 on day 3 increasing to a maximum of 60.8 by day 6 before decreasing to a mean of 55.0 by end of storage.

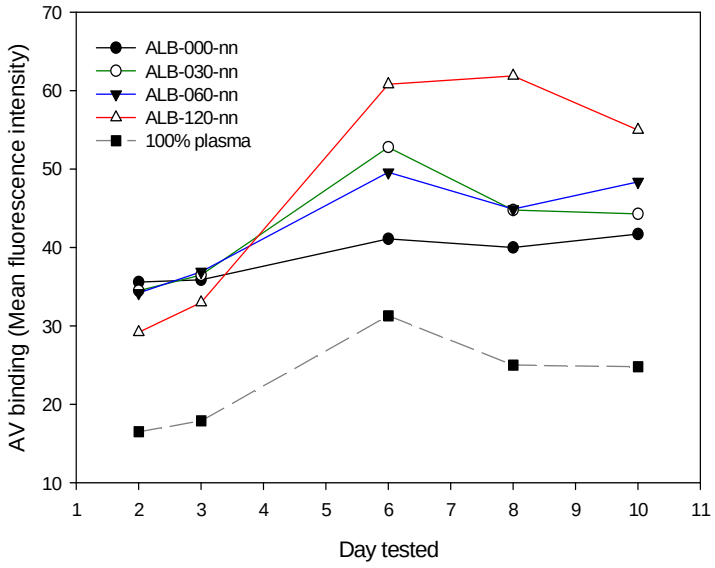


Figure 4.22: Annexin V binding expressed as mean fluorescence intensity (albumin study; mean, n=3)

Table 4.22: Annexin V binding in albumin study (mean fluorescence intensity)

TEST NUMBER	Annexin V binding (mean fluorescence intensity)				
	DAY 2	DAY 3	DAY 6	DAY 8	DAY 10
ALB-000-01	38.6	33.3	45.0	39.4	44.2
ALB-000-09	32.8	36.4	38.9	41.5	42.1
ALB-000-17	35.5	37.9	39.5	39.1	38.9
Mean	35.6	35.9	41.1	40.0	41.7
ALB-030-02	36.2	34.3	57.5	45.2	43.2
ALB-030-10	36.2	38.2	52.8	46.6	49.5
ALB-030-18	31.0	36.9	48.2	42.5	40.2
Mean	34.5	36.5	52.8	44.8	44.3
ALB-060-03	37.6	38.1	48.9	44.5	43.9
ALB-060-11	34.7	35.3	48.2	54.1	59.5
ALB-060-19	30.3	37.4	51.6	36.0	41.9
Mean	34.2	36.9	49.6	44.9	48.4
ALB-120-04	28.4	31.2	56.3	58.5	46.5
ALB-120-12	26.6	30.4	61.1	69.0	62.9
ALB-120-20	32.7	37.5	64.9	58.3	55.5
Mean	29.2	33.0	60.8	61.9	55.0

Figure 4.23 illustrates the four species of PE-esterified 12*S*-HETEs detected by the mass spectrometric assay. The assay identifies PE- or PS-esterified 12*S*-HETEs that normally remain associated with the plasma membrane, with the various forms identified by the mass/charge ratio of characteristic daughter ions.

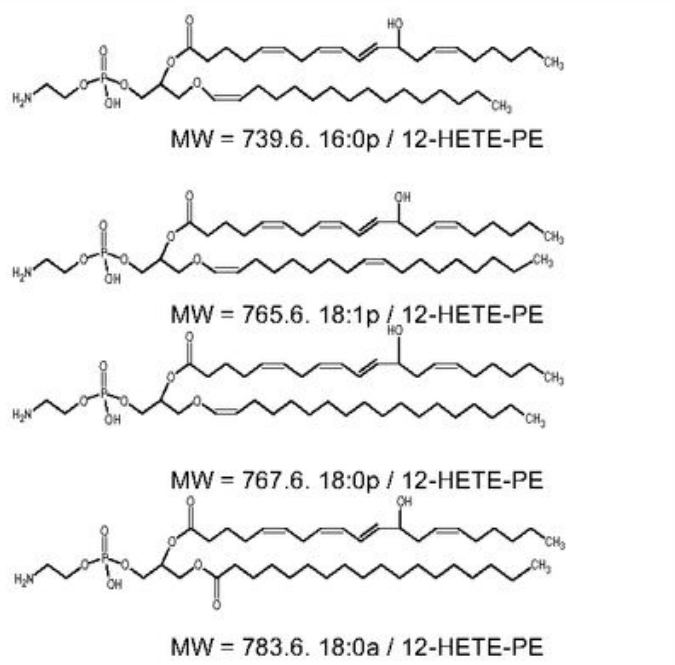


Figure 4.23: Four PE-esterified isoforms of 12S-HETE. (Biotinylation of the sample adds 226.3 amu to the mass.Ionisation for mass spectrometric measurements reduces the amu by 1 due to the loss of a hydrogen ion. In addition,the values for the isoforms presented in the graphs represent the un-oxidised substrate lipid. This accounts for the molecular weight differences quoted above and the values presented on the graphs) (O'Donnell and Murphy, 2012).

Figure 4.24 shows the concentrations of the four phosphatidylethanolamine and one phosphatidylserine aminophospholipids known to be expressed on the surface of platelets following activation. Results for the biotinylated samples of one representative experiment are reported as the ratio of analyte to an internal standard. On days 2 and 3, the expression of the four PE phospholipids remained relatively low in all four test groups. Between days 3 and 6, the expression of PE in the three albumin-containing units increased markedly and generally remained at this elevated level for the remainder of the storage period. In contrast, the relatively low levels of expressed PE in the unit lacking albumin were retained throughout the ten days of storage, reflecting the relatively low percent positive results obtained with the annexin V binding assay. With PS, the pattern suggests a progressive increase in concentration with storage. Although the levels of PS in the unit lacking albumin remained below those of the albumin-containing units after day 3, levels of PS continued to increase with storage in this unit as well.

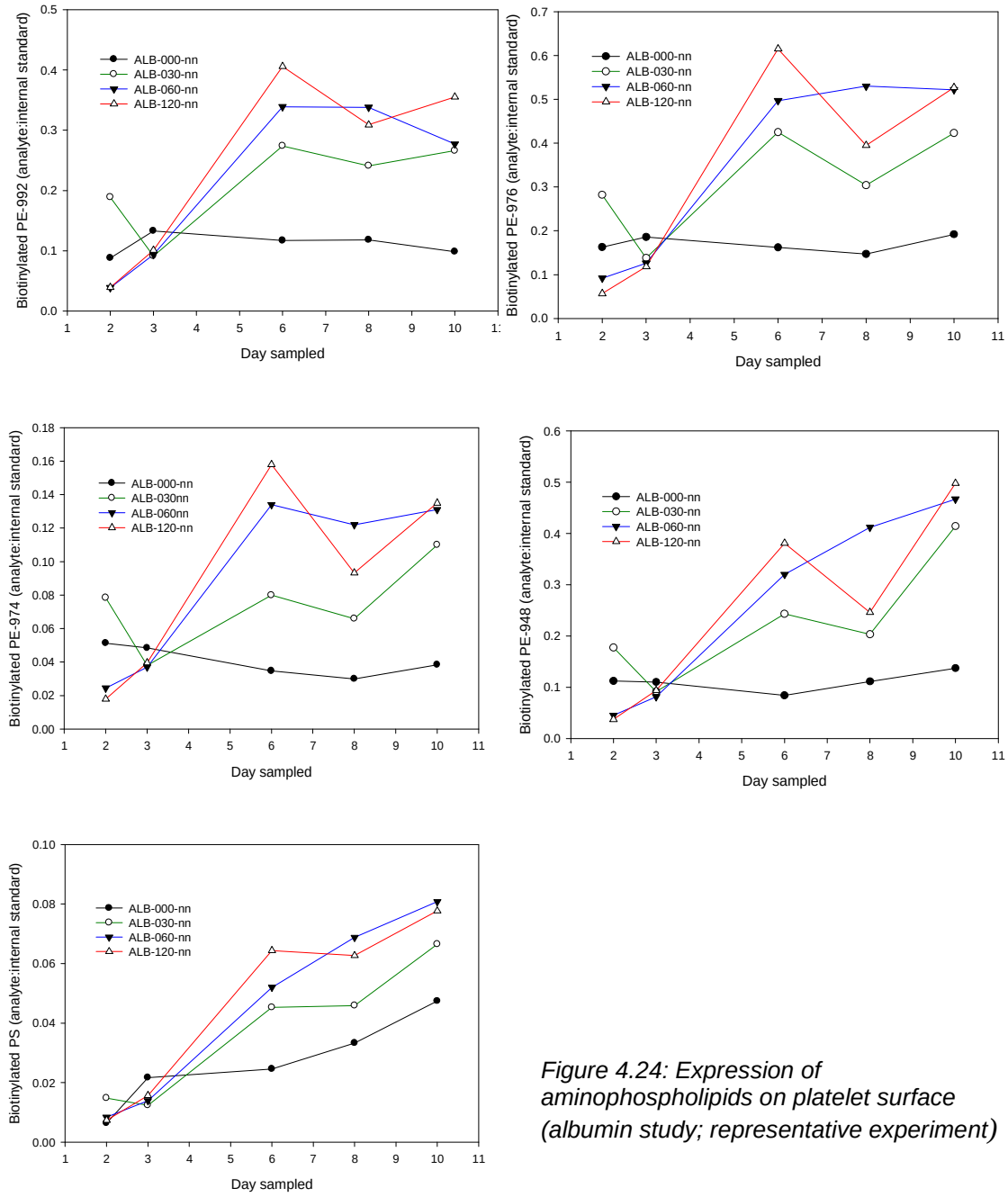


Figure 4.24: Expression of aminophospholipids on platelet surface (albumin study; representative experiment)

Mitochondrial Membrane Potential

Day 2 levels of mitochondrial membrane potential were similar for all four test groups, with mean red/green fluorescence ratios of 8.5 to 9.0 and a decrease evident in all four groups from day 2. The rate of decline was markedly increased with the addition of albumin - all three of these test groups attaining a minimal ratio of 2.7 by day 6 (considered as the limit of the assay). A mean ratio of 3.3 was noted in the non-albumin containing test group by end of storage (table 4.23). This decrease in potential with storage, suggested by the decrease in the fluorescence ratio, was not evident with the

units re-constituted in plasma, which showed a relatively minor decrease in the ratio with storage (figure 4.25).

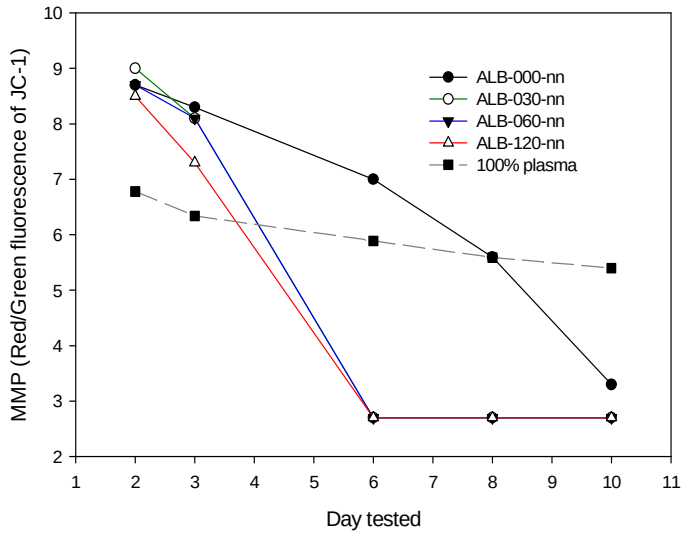


Figure 4.25: Mitochondrial membrane potential expressed as fluorescence of red/green fluorescence with JC-1 (albumin study; mean, n=3)

Table 4.23: Mitochondrial membrane potential in albumin study

TEST NUMBER	MMP (median red/green JC-1 fluorescence)				
	DAY 2	DAY 3	DAY 6	DAY 8	DAY 10
ALB-000-01	9.1	8.9	7.6	6.6	3.1
ALB-000-09	9.2	8.5	7.6	6.3	3.2
ALB-000-17	7.9	7.6	5.9	3.9	3.7
Mean	8.7	8.3	7.0	5.6	3.3
ALB-030-02	9.1	7.9	2.8	2.7	2.7
ALB-030-10	9.3	8.8	2.7	2.7	2.7
ALB-030-18	8.7	7.4	2.7	2.7	2.7
Mean	9.0	8.1	2.7	2.7	2.7
ALB-060-03	9.1	8.5	2.7	2.7	2.7
ALB-060-11	9.3	8.4	2.7	2.7	2.7
ALB-060-19	7.8	7.6	2.7	2.7	2.7
Mean	8.7	8.1	2.7	2.7	2.7
ALB-120-04	9.3	8.0	2.7	2.8	2.7
ALB-120-12	8.3	7.3	2.7	2.7	2.7
ALB-120-20	7.8	6.7	2.7	2.7	2.7
Mean	8.5	7.3	2.7	2.7	2.7

Intracellular free calcium

Levels of intracellular free calcium were minimal in all four test groups on days 2 and 3, with mean Fluo-4/FuraRed ratios of 0.23 to 0.27 in units with 120 mL and 30 mL albumin, respectively. (By comparison, mean ratios in control samples prepared with the calcium ionophore A23187 were 9.60 and 10.47 in the same test groups). Ratios in units without added albumin remained low throughout storage, increasing marginally between days 8 and 10 to a mean of 0.39 and mirroring the results obtained with units re-constituted in plasma (figure 4.26). In units with added albumin, ratios increased after day 3 in a dose-dependent manner, with the highest concentration of albumin resulting in the highest levels of free calcium. Mean ratios in units with 120 mL albumin reached a maximum on day 8 of 8.75, before decreasing to 6.40 on day 10. Units with 60 ml albumin followed a similar pattern, attaining a lower mean ratio of 6.60 on day 8. Units with 30 mL albumin attained a maximum mean ratio of 5.65 on day 10 (table 4.24).

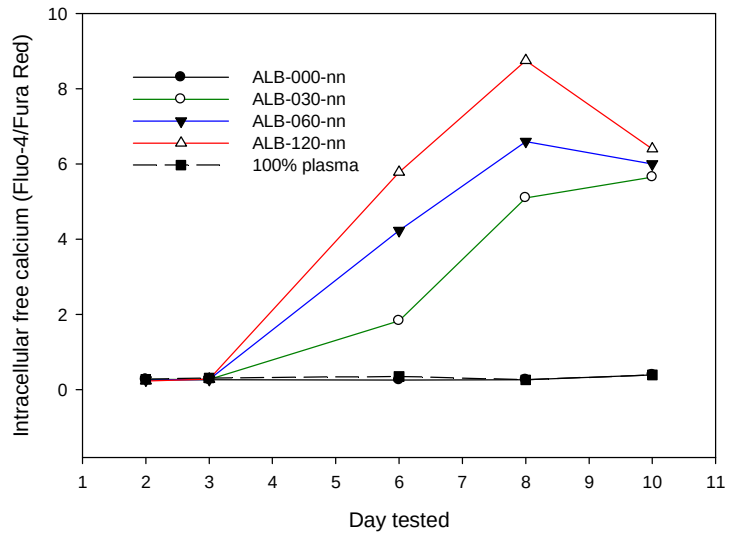


Figure 4.26: Intracellular free calcium expressed as fluorescence of fluo-4/ FuraRed fluorescence (albumin study; mean, n=3)

Table 4.24: Intracellular free calcium in albumin study

TEST NUMBER	Intracellular free calcium (Fluo-4/FuraRed)				
	DAY 2	DAY 3	DAY 6	DAY 8	DAY 10
ALB-000-01	0.19	0.23	0.20	0.20	0.29
ALB-000-09	0.32	0.28	0.29	0.31	0.49
ALB-000-17	0.27	0.28	0.27	0.27	0.40
Mean	0.26	0.26	0.25	0.26	0.39
ALB-030-02	0.22	0.23	0.68	3.80	4.20
ALB-030-10	0.33	0.29	2.09	5.86	7.78
ALB-030-18	0.27	0.28	2.72	5.63	4.98
Mean	0.27	0.27	1.83	5.10	5.65
ALB-060-03	0.23	0.22	3.30	7.40	4.40
ALB-060-11	0.28	0.29	4.44	6.70	7.55
ALB-060-19	0.24	0.30	4.95	5.71	6.05
Mean	0.25	0.27	4.23	6.60	6.00
ALB-120-04	0.20	0.21	5.10	6.20	5.30
ALB-120-12	0.29	0.36	6.30	7.76	7.60
ALB-120-20	0.21	0.26	5.93	12.30	6.31
Mean	0.23	0.28	5.78	8.75	6.40

DISCUSSION

The study investigated the impact on platelet *in vitro* characteristics of adding exogenous albumin to an artificial storage medium, with the inclusion of albumin expected to confer a beneficial effect on platelet viability over the course of the ten-day storage period. The hypothesis was not upheld by the results, with the presence of albumin in the storage medium having a detrimental effect on all the parameters measured. Early indications were evident in the measures for metabolism. Relatively high pCO_2 levels in albumin-containing units were already evident on day 2, suggestive of a burst of oxidative metabolism during this early time period which may already have been declining before this first measurement, since the oxygen partial pressure was found to be significantly higher than in non-albumin containing units by day 2. Rapid rates of glucose consumption between days 2 and 3 culminated in the depletion of glucose stores in all albumin-containing units by day 6. This coincided with a

precipitous decline in ATP levels in albumin-containing units between days 3 and 6. Between days 3 and 6 there was a marked fall in platelet viability in the study groups containing albumin which was consistently reproduced by all the parameters tested. Annexin V binding and intracellular calcium levels did not increase in any of the test groups until after day 3, with calcium levels responding more directly to the varying concentrations of albumin in the suspending media. Mitochondrial membrane potential was already declining at the beginning of the storage period. The gradual decline in MMP in the non-albumin test group contrasted with the relatively static nature of annexin V binding and calcium levels in the same units and suggests that changes in MMP pre-empt the increase in intracellular calcium and phospholipid changes related to apoptosis and may not be related to the latter. A suggestion of a concentration-dependent phenomenon was provided by certain of the parameters; in particular pH, platelet concentration, surface CD62P expression, annexin V binding and intracellular calcium levels, although statistical comparison was not performed due to insufficient replicates.

The addition of human albumin to the additive solution resulted in a clear degradation of platelet viability in excess of changes associated with the platelet storage lesion in more conventional media. The human albumin solution used was a 20% solution of commercially available human albumin (Zenalb®20, Bio Products Laboratory, Elstree, UK). This product is infused into humans without reported adverse effects on platelet number. In order to establish whether the changes observed were specific to this formulation of albumin or generic to albumin products, further studies were undertaken with a different commercially available solution, as well as with fatty acid-free lyophilised albumin from human serum.

SUPPLEMENTARY INVESTIGATIONS – ALBUMIN STUDY

PLATELET CONCENTRATE PREPARATION WITH ALTERNATIVE ALBUMIN SOLUTION

A supplementary experiment was performed using a 20% human albumin solution from Baxter Healthcare. A limited number of parameters were investigated, concentrating on the markers of cell death. The same protocol was used to prepare and store the units, with sampling occurring at the same time points. The results are presented below in table and graph format, with the corresponding graph from the units manufactured with the BPL formulation reproduced to scale alongside for ease of comparison.

RESULTS

Platelet Concentration

Table 4.25: Platelet Concentration in units prepared with Baxter albumin solution

TEST NUMBER	Platelet Concentration (x10^9/L)				
	DAY 2	DAY 3	DAY 6	DAY 8	DAY 10
ALB-000-21	903	832	856	871	938
ALB-030-22	830	819	818	785	757
ALB-060-23	863	877	876	745	772
ALB-120-24	796	799	752	714	711

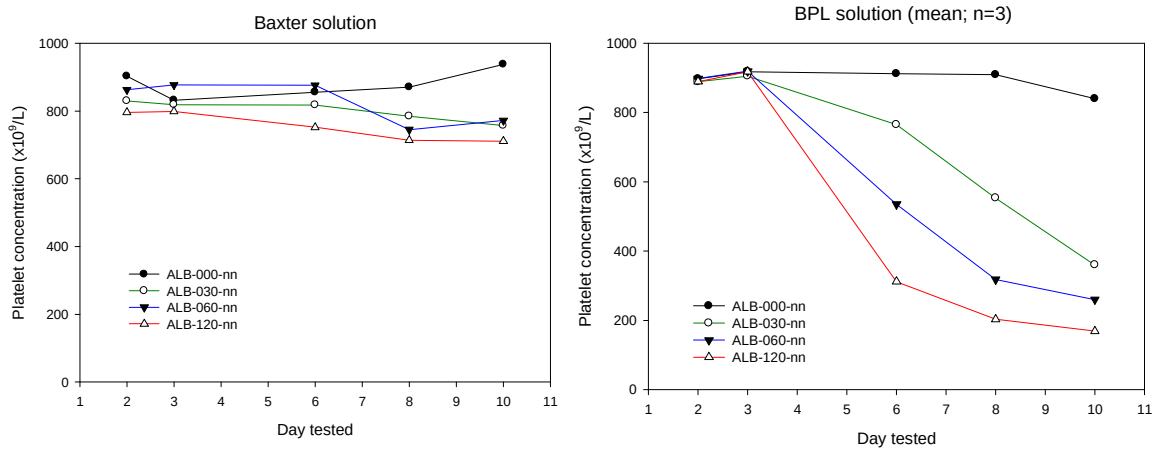


Figure 4.27: Comparison of platelet concentration in units suspended in Baxter's albumin solution versus BPL's Zenalb® 20

Lactate Levels

Table 4.26: Lactate levels in units prepared with Baxter albumin solution

TEST NUMBER	Lactate concentration (mmol/L)				
	DAY 2	DAY 3	DAY 6	DAY 8	DAY 10
ALB-000-21	2.2	3.9	10.1	14.7	20.0
ALB-030-22	2.4	4.2	10.3	15.0	17.0
ALB-060-23	2.7	4.7	11.0	16.0	21.0
ALB-120-24	2.5	4.2	9.7	13.6	18.0

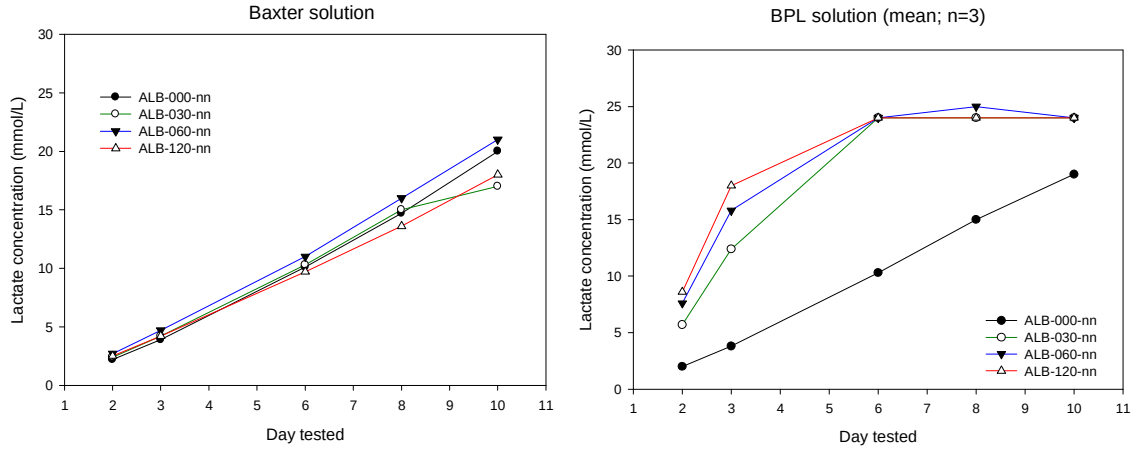


Figure 4.28: Comparison of lactate levels in units suspended in Baxter's albumin solution versus BPL's Zenalb® 20

Mitochondrial Membrane Potential

Table 4.27: MMP in units prepared with Baxter albumin solution

TEST NUMBER	Mitochondrial Membrane Potential (Median R/G)				
	DAY 2	DAY 3	DAY 6	DAY 8	DAY 10
ALB-000-21	8.58	8.66	3.86	5.63	4.23
ALB-030-22	8.90	8.43	7.50	7.17	3.28
ALB-060-23	8.51	8.21	7.78	7.17	5.85
ALB-120-24	7.50	7.50	7.56	6.98	6.27

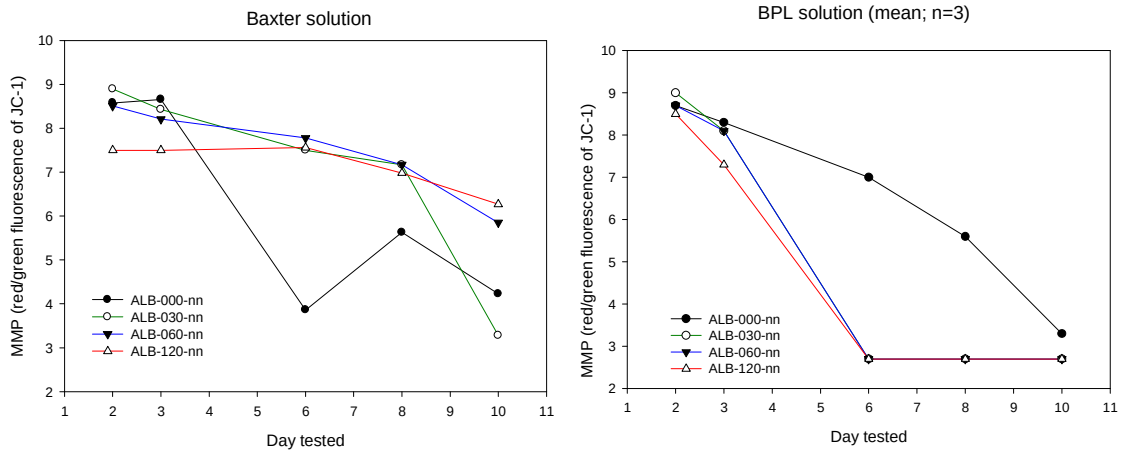


Figure 4.29: Comparison of JC-1 red/green fluorescence ratio in units suspended in Baxter's albumin solution versus BPL's Zenalb® 20

Annexin V Binding

Table 4.28: Annexin V binding in units prepared with Baxter albumin solution

TEST NUMBER	Annexin V Binding (% Positive)				
	DAY 2	DAY 3	DAY 6	DAY 8	DAY 10
ALB-000-21	10.58	11.21	13.13	13.28	18.58
ALB-030-22	9.77	10.66	13.33	13.35	38.04
ALB-060-23	8.53	10.36	10.97	13.51	17.89
ALB-120-24	14.4	9.22	16.31	15.96	19.91

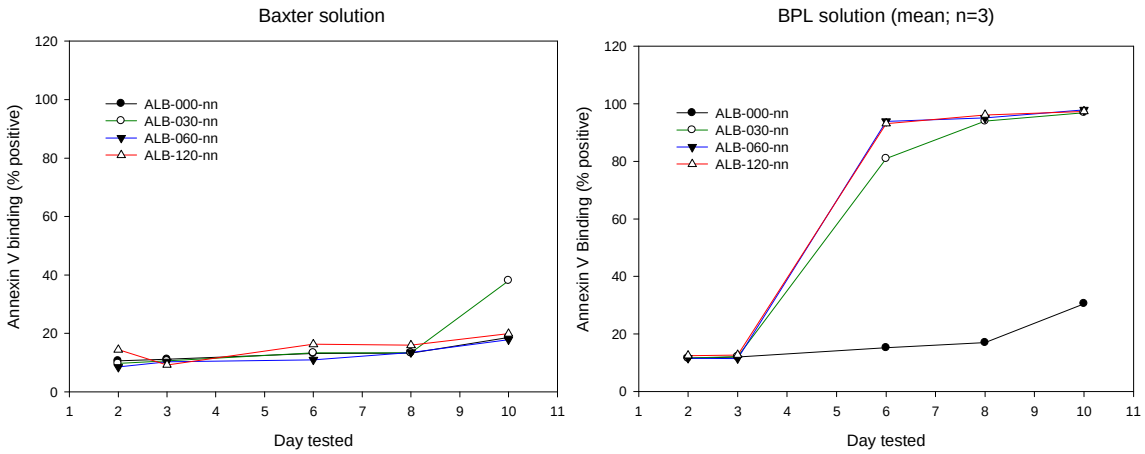


Figure 4.30: Comparison of annexin V binding (% positive expression) in units suspended in Baxter's albumin solution versus BPL's Zenalb® 20

Intracellular Free Calcium

Table 4.29: Intracellular free calcium in units prepared with Baxter albumin solution

TEST NUMBER	Ca (Fluo-4/Fura Red)				
	DAY 2	DAY 3	DAY 6	DAY 8	DAY 10
ALB-000-21	0.29	0.30	0.25	0.22	0.25
ALB-030-22	0.33	0.35	0.32	0.25	0.49
ALB-060-23	0.31	0.27	0.27	0.25	0.25
ALB-120-24	0.47	0.26	0.31	0.24	0.37

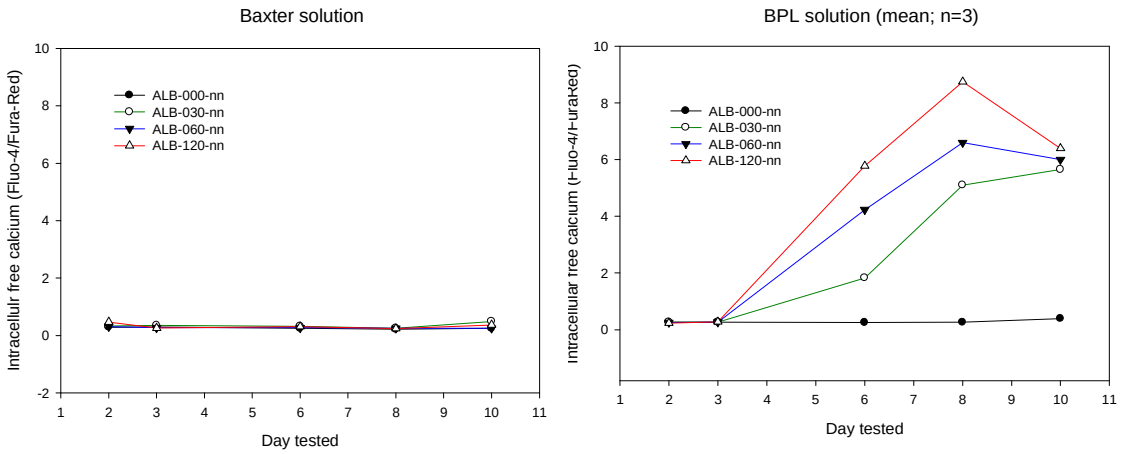


Figure 4.31: Comparison of intracellular free calcium levels in units suspended in Baxter's albumin solution versus BPL's Zenalb® 20

DISCUSSION

The disruption of the platelets evident after day 3 in the units prepared with the Zenalb®20 solution was not evident in the experiment utilising the formulation from Baxter. Platelet concentration was maintained relatively constant throughout the ten-day storage period, without the dose-dependent decrease noted in the BPL units and attributed to platelet lysis. The increased rate of metabolism evident in the same units from the beginning of the storage period – here depicted solely by lactate levels – was also not observed in the Baxter experiment. Finally, the platelets prepared and stored with the Baxter albumin solution did not display the marked changes in the markers of cell death after day 3 that were seen in units stored with the BPL solution.

Although storage of platelets in the Baxter formulation did not result in the adverse changes noted with the BPL formulation, there was also no suggestion that storage in an

additive solution containing albumin resulted in any benefits compared to the unit lacking albumin, though it would be premature to reach any firm conclusions from a single experiment.

PC PREPARATION WITH POWDERED HUMAN SERUM ALBUMIN

A further experiment was undertaken using a powdered form of fatty acid-free (≤0.007%) albumin derived from human serum (Sigma-Aldrich, Dorset, UK: code A3782). By minimising the presence of components other than albumin, the aim was to directly determine whether albumin was the causative agent of the deleterious effects on the *in vitro* platelet characteristics. As before, four ABO blood group-specific PCs were pooled and re-split into four identical units, followed by hard centrifugation to pellet the platelets and remove the majority of the suspending plasma. Two of the units were re-suspended in SAS into which 3 g of powdered albumin had been dissolved, whilst one unit was re-suspended with a similar volume of SAS containing 15 mL of Zenalb® 20 albumin solution (calculated to contain 3 g of albumin). This concentration of albumin was half the lowest concentration used in the initial experiments. A lower concentration was chosen due to the marked effects noted over the ten day storage period in the latter. The fourth unit broke in the centrifuge, negating the opportunity to include a negative control planned to consist of a PC suspended in SAS lacking albumin.

As above, the corresponding graphs from the units manufactured with the BPL formulation are reproduced to scale for ease of comparison.

RESULTS

Platelet Concentration

Table 4.30: Platelet concentration for units in HSA study

TEST NUMBER	Platelet Concentration (x10^9/L)				
	DAY 2	DAY 3	DAY 6	DAY 8	DAY 10
ALB-HS3-005	832	786	770	751	664
ALB-HS3-006	833	818	793	725	693
ALB-Z15-007	766	809	743	598	477

ALB-HS3-nnn: unit with human serum album
ALB-Z15-nnn: unit with 15 mL Zenalb® 20 albumin solution

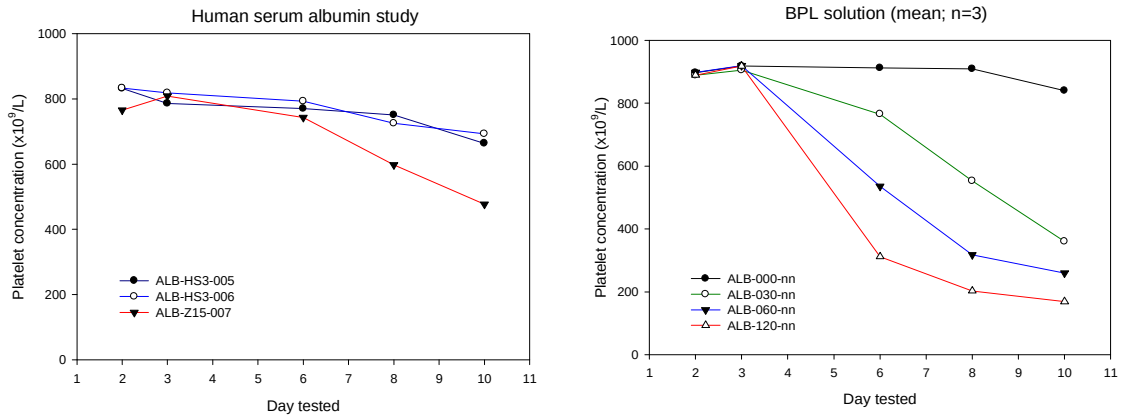


Figure 4.32: Comparison of platelet concentration in units suspended in powdered human serum albumin versus BPL's Zenalb® 20 (ALB-Z15-007).

Lactate Levels

Table 4.31: Lactate levels for units in HSA study

TEST NUMBER	Lactate (mmol/L)				
	DAY 2	DAY 3	DAY 6	DAY 8	DAY 10
ALB-HS3-005	2.4	4.6	11.1	17	23
ALB-HS3-006	2.6	4.6	10.8	17	21
ALB-Z15-007	5.7	12.7	27	27	27

ALB-HS3-nnn: unit with human serum album
ALB-Z15-nnn: unit with 15 mL Zenalb® 20 albumin solution

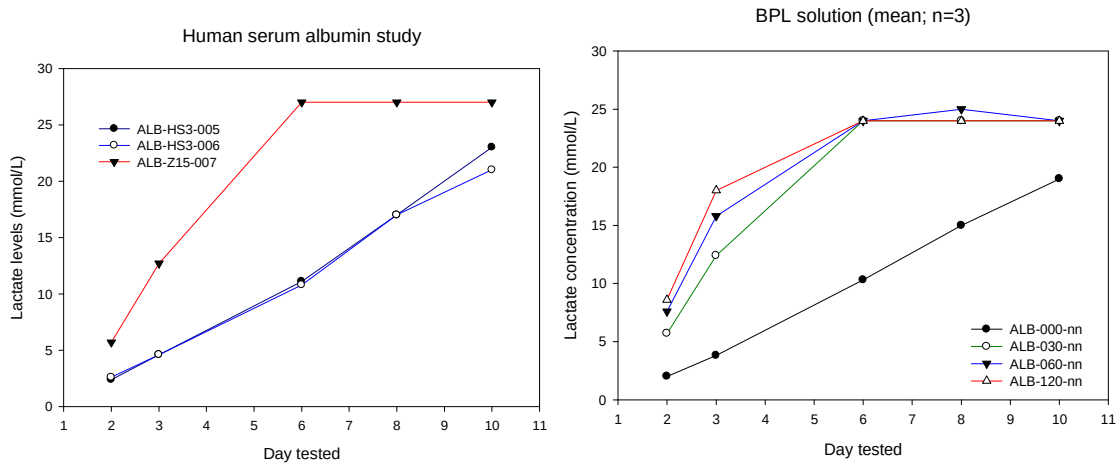


Figure 4.33: Comparison of lactate levels in units suspended in powdered human serum albumin versus BPL's Zenalb® 20 (ALB-Z15-007)

Mitochondrial Membrane Potential

Table 4.32: MMP for units in human serum albumin study

TEST NUMBER	Mitochondrial Membrane Potential (Median R/G)				
	DAY 2	DAY 3	DAY 6	DAY 8	DAY 10
ALB-HS3-005	10.6	9.8	7.8	5.9	5.2
ALB-HS3-006	6.9	9.6	8.6	6.9	3.1
ALB-Z15-007	9.7	8.8	2.8	2.8	2.7

ALB-HS3-nnn: unit with human serum album
ALB-Z15-nnn: unit with 15 ml Zenalb® 20 albumin solution

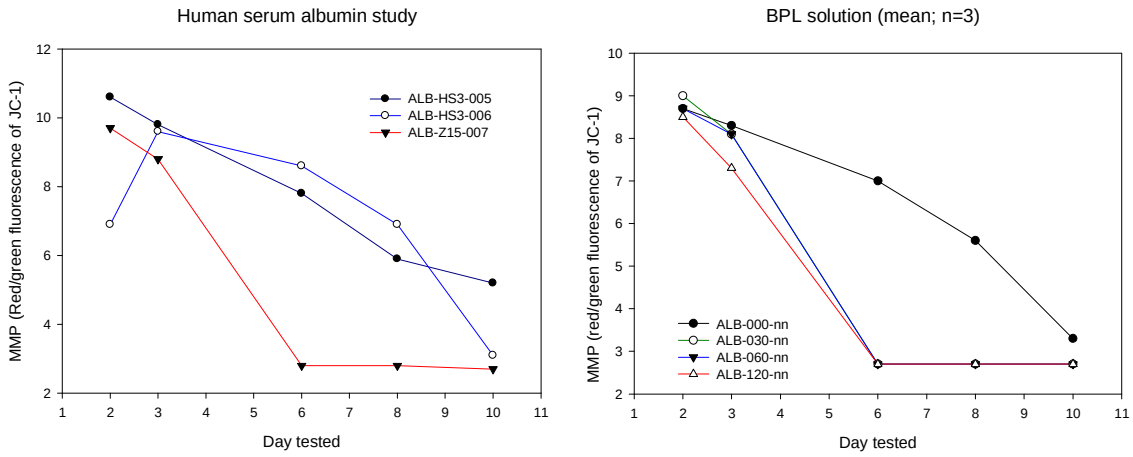


Figure 4.34: Comparison of JC-1 red/green fluorescence ratio in units suspended in powdered human serum albumin versus BPL's Zenalb® 20 (ALB-Z15-007)

Annexin V Binding

Table 4.33: Annexin V binding (human serum albumin study)

TEST NUMBER	Annexin V Binding (% Positive)				
	DAY 2	DAY 3	DAY 6	DAY 8	DAY 10
ALB-HS3-005	9.12	9.80	11.93	17.43	19.51
ALB-HS3-006	10.03	7.69	12.27	13.63	38.15
ALB-Z15-007	9.63	11.64	61.33	91.88	94.79

ALB-HS3-nnn: unit with human serum album
ALB-Z15-nnn: unit with 15 ml Zenalb® 20 albumin solution

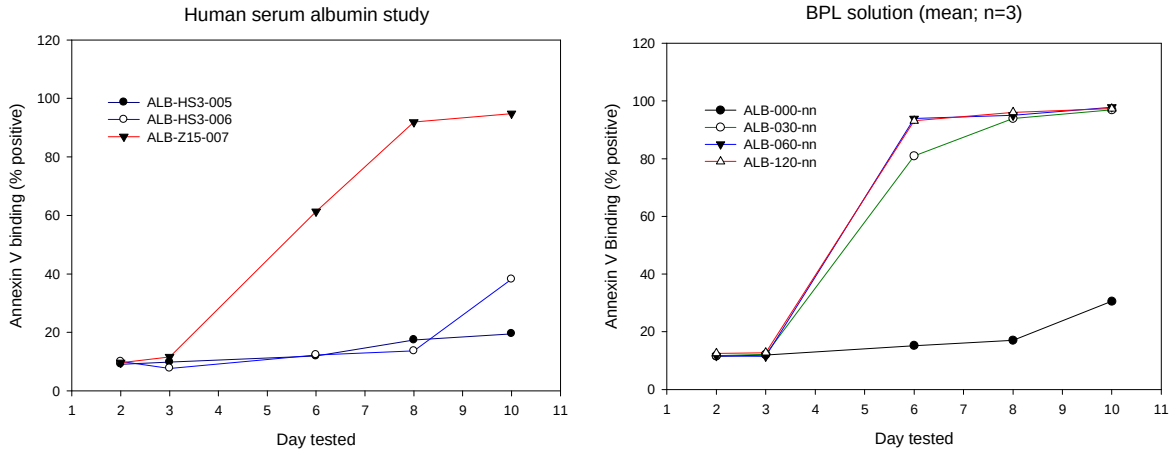


Figure 4.35: Comparison of annexin V binding in units suspended in powdered human serum albumin versus BPL's Zenalb® 20 (ALB-Z15-007)

Intracellular free calcium

Table 4.34: Intracellular free calcium (human serum albumin study)

TEST NUMBER	Intracellular free calcium (Fluo-4/Fura Red)				
	DAY 2	DAY 3	DAY 6	DAY 8	DAY 10
ALB-HS3-005	0.32	0.32	0.35	0.38	0.41
ALB-HS3-006	0.28	0.40	0.37	0.35	0.45
ALB-Z15-007	0.27	0.44	1.04	4.22	7.0

ALB-HS3-nnn: unit with human serum album
ALB-Z15-nnn: unit with 15 ml Zenalb® 20 albumin solution

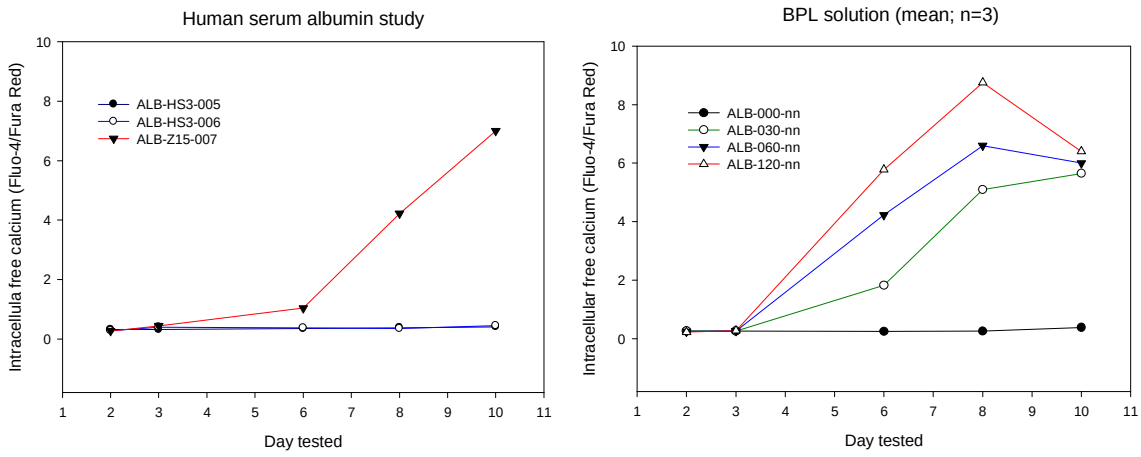


Figure 4.36: Comparison of intracellular free calcium in units suspended in powdered human serum albumin versus BPL's Zenalb® 20 (ALB-Z15-007)

DISCUSSION

The supplementary experiments confirmed that albumin *per se* did not cause the accelerated deterioration of platelets during the ten day storage period observed with Zenalb® 20 albumin solution and confirmed that the effect of Zenalb® 20 albumin was reproducible. The generation of lactate in the unit containing Zenalb® 20 occurred at the same accelerated rate as observed in the original experiments, despite the lower initial concentration of albumin solution. The lactate level reached a plateau of 27 mmol/L by day 6. At the same time point, lactate levels in units with the powdered albumin were 11.1 and 10.8 mmol/L. Among the parameters reflecting apoptotic changes, the two units incorporating the lyophilysed serum albumin provided results similar to those obtained in the original experiments in units lacking albumin or re-suspended in autologous plasma. By contrast, unit ALB-Z15-007, containing 15 mL of Zenalb® 20,

displayed accelerated disruption of the mitochondrial membrane potential as well as increased binding of annexin V and higher levels of intracellular free calcium. Interestingly, the lower concentration of albumin used in this experiment provided the suggestion of a time course for these changes. Mitochondrial membrane potential already attained minimal levels by day 6, with no clear difference between the rate of potential loss and Zenalb® 20 concentration. The suggestion of a concentration-dependent effect for annexin V binding in the initial study was reinforced with the results from ALB-Z15-007, whilst the clear influence of Zenalb® 20 concentration on intracellular calcium was also confirmed by this supplementary study. Thus, full disruption of the mitochondrial membrane potential occurred by day 6, followed by nearly maximal annexin V binding (% positive expression) by day 8 and intracellular free calcium levels similar to the maximal levels attained in the initial study by day 10 – the degree of platelet disruption being indicative of a toxic process in the model studied.

CHAPTER 5. IMPACT ON PLATELET *IN VITRO* STORAGE CHARACTERISTICS OF THE INCLUSION OF GLUCOSE TO ADDITIVE SOLUTIONS

INTRODUCTION

Commercial additive solutions currently available in Europe and North America do not include glucose due to manufacturing difficulties associated with its incorporation. In Japan, studies have been undertaken with an additive solution (M-Sol) derived from the mixing of commercial solutions pre-approved for clinical use and containing 15 mmol/L glucose, with encouraging results (Azuma et al., 2009, Hirayama et al., 2010). Debate has continued over the past 20 years regarding the benefits of including glucose, with some authors suggesting it is necessary for maintaining platelet viability *in vitro* (Gulliksson, 2000), despite metabolic studies which show it is not a principal substrate for oxidative metabolism (Kilkson et al., 1984, Guppy et al., 1990) and concern regarding its role in lactate production and associated lowering of extracellular pH. It is possible that glucose is required for the maintenance of other mechanisms related to platelet function and viability (Murphy, 1999, Li et al., 2005a). It is in this context that this study sought to investigate the impact of glucose on the platelet storage lesion, with emphasis on platelet activation and markers of cell death.

All results are presented as mean ± standard deviation of 5 replicate experiments, with statistical comparisons undertaken by a one way ANOVA. A p-value below 0.01 was considered statistically significant. Subsequent multiple comparisons were performed by the Holm-Sidak method, with an overall significance level of 0.05. The four test groups are:

GLU-000-nn (no glucose added)

GLU-075-nn (final concentration of 7.5 mmol/L)

GLU-150-nn (final concentration of 15 mmol/L)

GLU-300-nn (final concentration of 30 mmol/L)

(In each case, nn refers to a unique test number)

The graphs also show the median ± range of 3 replicate experiments performed with PC manufactured by the same method but re-suspended in 100% autologous plasma, in order to provide a visual estimation of the possible impact of the harsh processing on the platelet *in vitro* characteristics.

RESULTS

Platelet Yield and Volume

The mean platelet yield and volume at the start of the storage period for the four test groups are presented in table 5.1. All the units met the specification for platelet yield of $\geq 240 \times 10^9$ plts/unit set out in the UK Guidelines for Transfusion Services (James, 2005), with no statistically significant differences between the four test groups ($p=0.939$). The unit volumes also confirm that the preparation of the units in all four test groups was consistent ($p=0.711$). All units were successfully leucodepleted (residual WBC counts $<1\times10^6$/unit), with bacterial cultures at the end of storage proving negative.

Table 5.1: Platelet yield and unit volume prior to sampling

Test Group	Platelet Yield ($\times10^9$/unit)	Volume (mL)
GLU-000-nn	296.6 ± 21.6	337.8 ± 4.9
GLU-075-nn	295.3 ± 15.3	340.3 ± 3.9
GLU-150-nn	302.4 ± 20.8	340.0 ± 3.0
GLU-300-nn	298.6 ± 17.0	341.2 ± 6.1

Data are presented as mean ± SD (n=5)

Function and Morphology

Platelet Concentration

Platelet concentration was similar in all four test groups until day 6 of storage ($p=0.720$ on day 6). Beyond this time point, there was a clear decline in platelet concentration in the group lacking additional glucose in relation to the other three test groups, with statistically significant differences present on days 8 and 10 ($p<0.001$ in both cases) (table 5.2) (figure 5.1). The mean platelet concentration on day 10 in units with no added glucose was 63% of the starting concentration, compared to 96% for units with 30 mmol/L of added glucose ($549 \pm 34 \times10^9$/L versus $838 \pm 26 \times10^9$/L) (table 5.3). The difference between the starting concentrations in the four test groups and units reconstituted in 100% plasma may be due to variability during the processing of the

components, as a different operator was involved in the packing and centrifugation of the units in plasma.

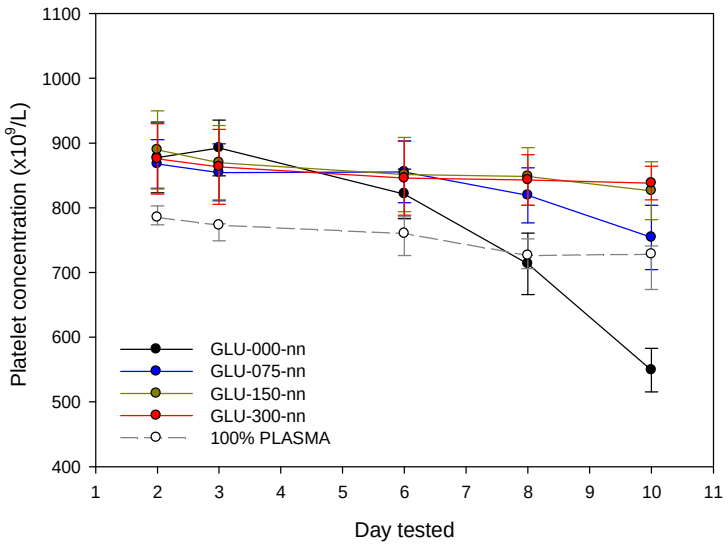


Figure 5.1: Platelet concentration (glucose study; mean ± SD; n=5; 100% plasma n=3)

Table 5.2: Statistical comparison between test groups for platelet concentration - glucose study

Comparison Between Groups					
	Day 2	Day 3	Day 6	Day 8	Day 10
One Way ANOVA	0.930	0.686	0.720	<0.001	<0.001
Multiple Pairwise Comparison (Holm-Sidak method)					
Comparison			Day 8	Day 10	
GLU-300 vs. GLU-000			<0.001 (0.002)	<0.001 (0.002)	
GLU-150 vs. GLU-000			<0.001 (0.002)	<0.001 (0.002)	
GLU-075 vs. GLU-000			0.001 (0.003)	<0.001 (0.003)	
GLU-300 vs. GLU-075			NS	NS	
GLU-300 vs. GLU-150			NS	NS	
GLU-150 vs. GLU-075			NS	NS	

Statistical significance: Unadjusted P value (critical Level) NS = Not significant

Table 5.3: Summary results for platelet concentration - glucose study

Final Concentration of Added Glucose	Platelet Concentration (x10⁹/L)				
	Day 2	Day 3	Day 6	Day 8	Day 10
0 mmol/L	878 ± 55	892 ± 43	821 ± 38	713 ± 47	549 ± 34
7.5 mmol/L	868 ± 38	855 ± 44	855 ± 48	819 ± 42	754 ± 50
15 mmol/L	889 ± 61	870 ± 57	851 ± 57	848 ± 45	826 ± 45
30 mmol/L	876 ± 54	863 ± 58	846 ± 57	843 ± 39	838 ± 26

Mean ± SD (n=5)

Mean Platelet Volume

The most pronounced increase in mean platelet volume over the period of storage was noted with units lacking additional glucose (figure 5.2). Mean MPV on day 10 in these units was 9.5 ± 0.3 fL compared with 8.7 ± 0.6 fL in units containing 30 mmol/L of glucose (table 5.5). However, these differences were not found to be statistically significant between any of the test groups at any time point (table 5.4).

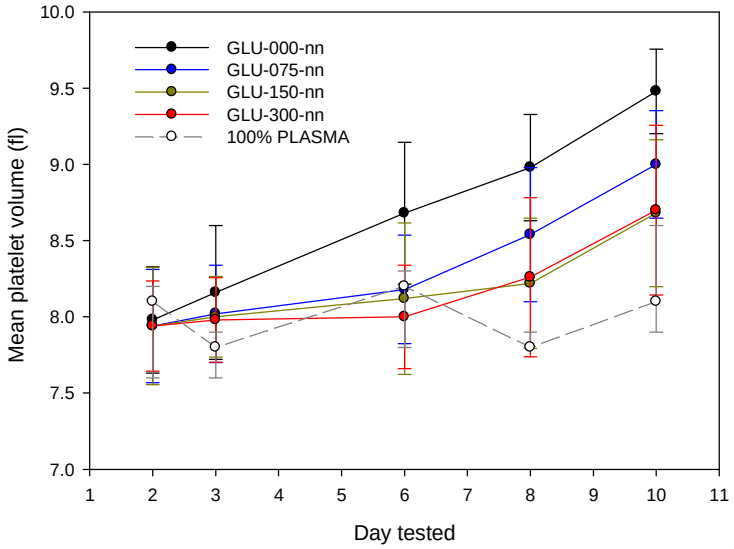


Figure 5.2: Mean platelet volume (glucose study; mean ± SD, n=5; 100% plasma n=3)

Table 5.4: Statistical comparison between test groups for mean platelet volume – glucose study

Comparison Between Groups					
	Day 2	Day 3	Day 6	Day 8	Day 10
One Way ANOVA	0.997	0.824	0.093	0.052	0.033

Table 5.5: Summary results for mean platelet volume - glucose study

Final Concentration of Added Glucose	Mean platelet volume (fL)				
	Day 2	Day 3	Day 6	Day 8	Day 10
0 mmol/L	8.0 ± 0.3	8.2 ± 0.4	8.7 ± 0.5	9.0 ± 0.3	9. 5 ± 0.3
7.5 mmol/L	7.9 ± 0.4	8.0 ± 0.3	8.2 ± 0.4	8.5 ± 0.4	9.0 ± 0.4
15 mmol/L	7.9 ± 0.4	8.0 ± 0.3	8.1 ± 0.5	8.2 ± 0.4	8.7 ± 0.5
30 mmol/L	7.9 ± 0.3	8.0 ± 0.3	8.0 ± 0.3	8.3 ± 0.5	8.7 ± 0.6

Mean ± SD (n=5)

Swirling

Swirling was not as distinct as in units suspended in plasma, with only one unit with 30 mmol/l added glucose having a score of 3 (day 10). However, even within these constraints, swirling was weaker from the start of the storage period in units lacking glucose and reported as absent in these units by day 10 (table 5.6). In units with 15 and 30 mmol/L of added glucose swirling was maintained with a score of 2 throughout storage, whilst in the test group with 7.5 mmol/L of added glucose, a decrease from a score of 2 to 1 was observed in four of the five units by day 10.

Table 5.6: Swirling - glucose study

Final Concentration of Added Glucose (mmol/l)	TEST NUMBER	Swirling				
		DAY 2	DAY 3	DAY 6	DAY 8	DAY 10
0 mmol/l	GLU-000-01	1	2	1	0	0
	GLU-000-05	2	1	1	1	0
	GLU-000-09	1	1	1	1	0
	GLU-000-13	1	1	1	0	0
	GLU-000-17	1	1	1	0	0
7.5 mmol/l	GLU-075-02	1	2	2	2	1
	GLU-075-06	2	2	2	2	1
	GLU-075-10	2	2	2	2	2
	GLU-075-14	2	2	2	2	1
	GLU-075-18	1	2	2	1	1
15 mmol/l	GLU-150-03	1	2	2	2	2
	GLU-150-07	2	2	2	2	2
	GLU-150-11	2	2	2	2	2
	GLU-150-15	2	2	2	2	2
	GLU-150-19	1	2	2	2	2
30 mmol/l	GLU-300-04	1	2	2	2	2
	GLU-300-08	2	2	2	2	2
	GLU-300-12	2	2	2	2	2
	GLU-300-16	2	2	2	2	3
	GLU-300-20	1	2	2	2	2

Hypotonic Shock Response and Extent of Shape Change

The hypotonic shock response was depressed in all four test groups compared to units re-suspended in plasma, with day 2 levels of approximately 20-30% compared to 67% for the latter (figure 5.3). It is possible that this is an artefact of the assay associated with platelet concentrates suspended in additive solution, despite autologous plasma being utilised as the sample diluent. The resulting graphs were at the limit of what was considered possible to interpret, based on the chart recordings of the responses. Despite this, mean levels resulted in a decrease in the HSR over the course of the storage period with all four test groups. There was also the suggestion of a dose-dependent response, with units lacking glucose having the lowest HSR values (Table 5.8); though a statistically significant difference between this group and the test groups containing glucose was only observed on day 8 ($p<0.001$) (table 5.7).

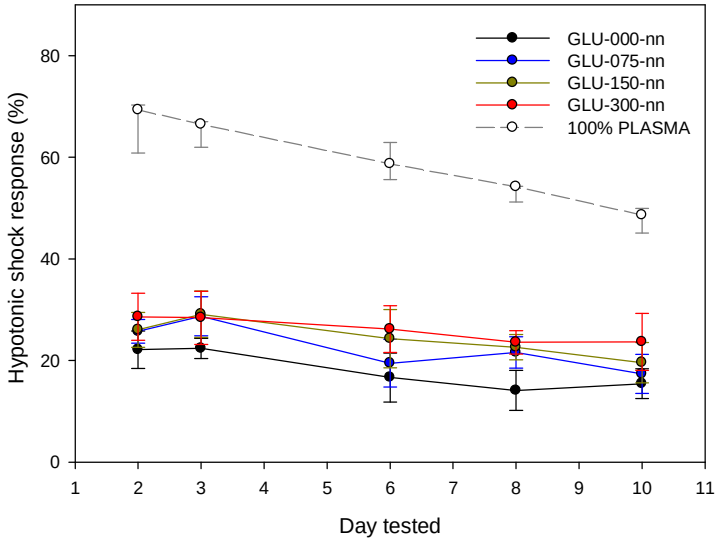


Figure 5.3: Hypotonic shock response (glucose study; mean ± SD, n=5; 100% plasma n=3)

Table 5.7: Statistical comparison between test groups of hypotonic shock response – glucose study

Comparison Between Groups					
	Day 2	Day 3	Day 6	Day 8	Day 10
One Way ANOVA	0.079	0.058	0.030	<0.001	0.038
Multiple Pairwise Comparison (Holm-Sidak method)					
Comparison	Day 8				
GLU-300 vs. GLU-000	<0.001 (0.009)				
GLU-150 vs. GLU-000	<0.001 (0.010)				
GLU-075 vs. GLU-000	0.001 (0.013)				
GLU-300 vs. GLU-075	NS				
GLU-300 vs. GLU-150	NS				
GLU-150 vs. GLU-075	NS				

Statistical significance: Unadjusted P value (critical Level) NS = Not significant

Table 5.8: Summary results for hypotonic shock response - glucose study

Final Concentration of Added Glucose	Hypotonic shock response (%)				
	Day 2	Day 3	Day 6	Day 8	Day 10
0 mmol/L	22.1 ± 3.7	22.4 ± 2.0	16.6 ± 4.8	14.1 ± 3. 9	15.4 ± 2.9
7.5 mmol/L	25.7 ± 2.3	28.7 ± 3.9	19.4 ± 4.7	21.6 ± 3.1	17.3 ± 3.9
15 mmol/L	26.0 ± 3.4	29.1 ± 4.5	24.3 ± 5.8	22.6 ± 2.5	19.6 ± 3.9
30 mmol/L	28.6 ± 4.6	28.4 ± 5.2	26.2 ± 4.6	23.6 ± 2.3	23.7 ± 5.6

Mean ± SD (n=5)

Extent of shape change showed a similar pattern between the test groups to that described for the hypotonic shock response, with units lacking glucose having the lowest responses, and a gradual decline evident in all groups with increased storage (figure 5.4). ESC decreased in units lacking glucose from day 2 values of 11.8 ± 4.3% to 3.0 ± 1.6% by day 10. Results for units with 30 mmol/L glucose at the same time points were 13.0 ± 6.4% (p=0.974) and 9.8 ± 1.5% (p=0.002), respectively (table 5.10). Dose-dependency between the three test groups containing added glucose was not observed with this parameter, with no statistically significant differences in evidence at any point during storage (table 5.9). A depressed response compared to units re-suspended in plasma was again observed.

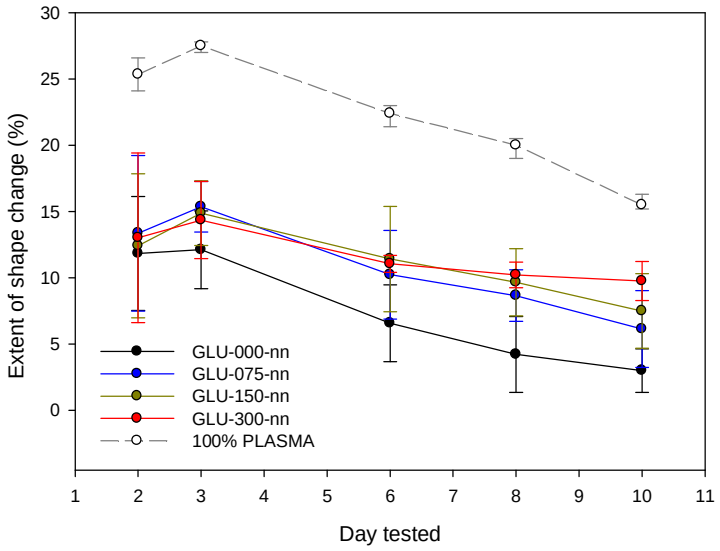


Figure 5.4: Extent of shape change (glucose study; mean ± SD, n=5; 100% plasma n=3)

Table 5.9: Statistical comparison between test groups for extent of shape change – glucose study

Comparison Between Groups					
	Day 2	Day 3	Day 6	Day 8	Day 10
One Way ANOVA	0.974	0.242	0.077	0.002	0.002
Multiple Pairwise Comparison (Holm-Sidak method)					
Comparison			Day 8		Day 10
GLU-300 vs. GLU-000			0.001 (0.009)		<0.001 (0.009)
GLU-150 vs. GLU-000			0.001 (0.010)		0.007 (0.010)
GLU-075 vs. GLU-000			0.006 (0.013)		NS
GLU-300 vs. GLU-075			NS		NS
GLU-300 vs. GLU-150			NS		NS
GLU-150 vs. GLU-075			NS		NS

Statistical significance: Unadjusted P value (critical Level) NS = Not significant

Table 5.10: Summary results for extent of shape change - glucose study

Final Concentration of Added Glucose	Extent of shape change (%)				
	Day 2	Day 3	Day 6	Day 8	Day 10
0 mmol/L	11.8 ± 4.3	12.1 ± 2.9	6.6 ± 2.9	4.2 ± 2.8	3.0 ± 1.6
7.5 mmol/L	13.4 ± 5.9	15.4 ± 1.9	10.2 ± 3.3	8.7 ± 2.0	6.1 ± 2. 9
15 mmol/L	12.4 ± 5.4	14.9 ± 2.4	11.4 ± 4.0	9.7 ± 2.5	7.5 ± 2. 8
30 mmol/L	13.0 ± 6.4	14.4 ± 2.9	11.1 ± 0.6	10.2 ± 1.0	9.8 ± 1 .5

Mean ± SD (n=5)

Platelet Metabolism

Extracellular $pH_{37°C}$

Day 2 levels of $pH_{37°C}$ were similar in all four test groups, with mean levels of approximately 7.45 (p=0.233). The units lacking glucose showed a steady increase in $pH_{37°C}$ with storage, resulting in a mean level on day 10 of 7.952 ± 0.035 (table 5.12). In contrast, $pH_{37°C}$ levels gradually decreased in all the units containing glucose up to day 6, with the rate of decrease closely matched in the three test groups (figure 5.5). By this point, glucose stores in units with starting glucose levels of 7.5 mmol/L had been depleted (Figure 5.6) and $pH_{37°C}$ in this test group increased, with the slope of the line similar to the slope generated with the test group lacking glucose. The rate of decrease in units with 15 mmol/L and 30 mmol/L of glucose remained similar up to day 8, when the rate of decrease fell in the former group as glucose levels neared zero (figure 5.5). This is reflected in the statistical comparison, with significant differences evident on days 8 and 10 between all the groups with the exception of the units with 15 mmol/L and 30 mmol/L of added glucose (table 5.11). Despite the decrease over time, all glucose-containing units retained $pH_{37°C}$ levels above 6.95 throughout the ten-day storage period (table 5.12).

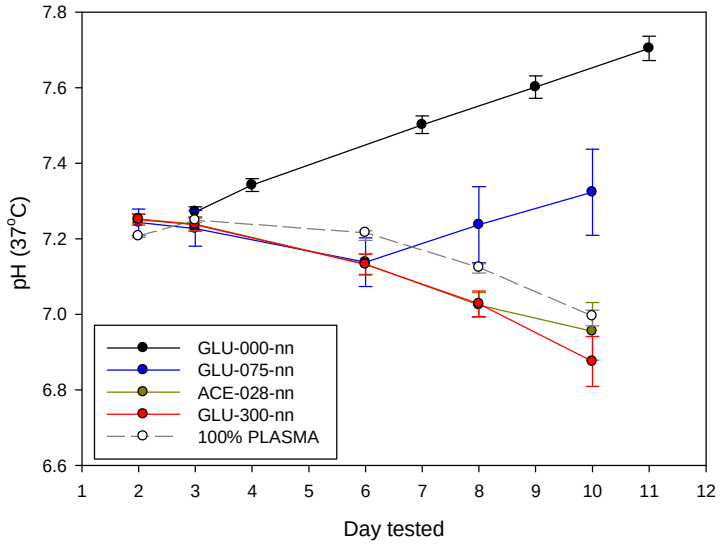


Figure 5.5: $pH_{37°C}$ levels (glucose study; mean ± SD, n=5; 100% plasma n=3)

Table 5.11: Statistical comparison between test groups for pH (37°C) – glucose Study

Comparison Between Groups					
	Day 2	Day 3	Day 6	Day 8	Day 10
One Way ANOVA	0.225	<0.001	<0.001	<0.001	<0.001
Multiple Pairwise Comparison (Holm-Sidak method)					
Comparison	Day 3	Day 6	Day 8	Day 10	
GLU-300 vs. GLU-000	<0.001 (0.010)	<0.001 (0.009)	<0.001 (0.010)	<0.001 (0.009)	
GLU-150 vs. GLU-000	<0.001 (0.013)	<0.001 (0.010)	<0.001 (0.009)	<0.001 (0.010)	
GLU-075 vs. GLU-000	<0.001 (0.009)	<0.001 (0.013)	<0.001 (0.013)	<0.001 (0.017)	
GLU-300 vs. GLU-075	NS	NS	<0.001 (0.025)	<0.001 (0.013)	
GLU-300 vs. GLU-150	NS	NS	NS	NS	
GLU-150 vs. GLU-075	NS	NS	<0.001 (0.017)	<0.001 (0.025)	

Statistical significance: Unadjusted P value (critical Level) NS = Not significant

Table 5.12: pH (37°C) levels in glucose study

Final Concentration of Added Glucose	pH (37°C)				
	Day 2	Day 3	Day 6	Day 8	Day 10
0 mmol/L	7.271 ± 0.013	7.342 ± 0.017	7.502 ± 0.024	7.602 ± 0.029	7.704 ± 0.033
7.5 mmol/L	7.243 ± 0.035	7.228 ± 0.048	7.138 ± 0.065	7.237 ± 0.101	7.323 ± 0.114
15 mmol/L	7.253 ± 0.011	7.239 ± 0.018	7.133 ± 0.027	7.025 ± 0.032	6.955 ± 0.077
30 mmol/L	7.251 ± 0.015	7.238 ± 0.017	7.132 ± 0.028	7.028 ± 0.034	6.875 ± 0.066

Mean ± SD (n=5)

Glucose and Lactate

Glucose levels decreased with storage at a similar rate in all three test groups containing exogenous glucose until stores were depleted, which occurred on day 6 for units with 7.5 mmol/L of added glucose and day 10 for units with 15 mmol/L of glucose (figure 5.6). Units with the highest concentration of added glucose at 30 mmol/L maintained glucose levels of 9.2 ± 0.5 mmol/L by day 10 (table 5.14); similar to levels in units re-suspended in plasma (mean of 9.9 mmol/L). The statistical comparison reflected the gradual consumption of glucose with storage, with statistically significant differences evident at all time points but restricted by day 10 to differences between the units with 30 mmol/L added glucose and the other three test groups ($p<0.001$) (table 5.13).

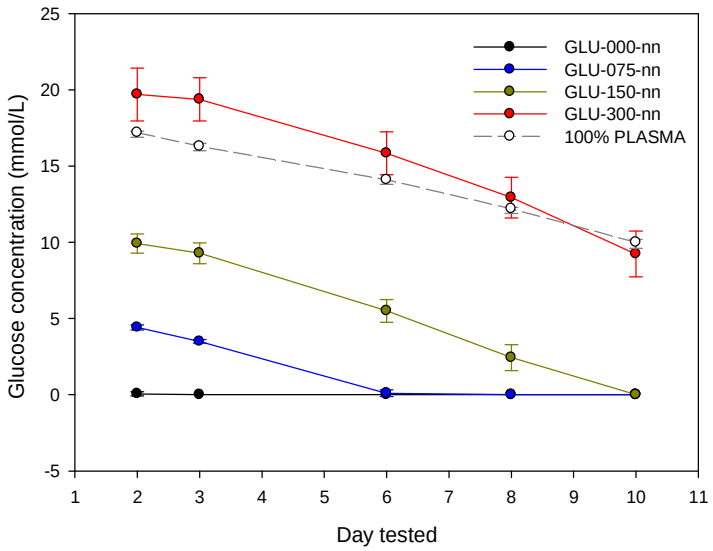


Figure 5.6: Glucose levels (glucose study; mean ± SD, n=5; 100% plasma n=3)

Table 5.13: Statistical comparison between test groups for glucose concentration – glucose study

Comparison Between Groups					
	Day 2	Day 3	Day 6	Day 8	Day 10
One Way ANOVA	<0.001	<0.001	<0.001	<0.001	<0.001
Multiple Pairwise Comparison (Holm-Sidak method)					
Comparison	Day 2	Day 3	Day 6	Day 8	Day 10
GLU-300 vs. GLU-000	<0.001 (0.009)	<0.001 (0.009)	<0.001 (0.009)	<0.001 (0.010)	<0.001 (0.013)
GLU-150 vs. GLU-000	<0.001 (0.013)	<0.001 (0.017)	<0.001 (0.017)	<0.001 (0.025)	NS
GLU-075 vs. GLU-000	<0.001 (0.050)	<0.001 (0.050)	NS	NS	NS
GLU-300 vs. GLU-075	<0.001 (0.010)	<0.001 (0.010)	<0.001 (0.010)	<0.001 (0.009)	<0.001 (0.010)
GLU-300 vs. GLU-150	<0.001 (0.017)	<0.001 (0.013)	<0.001 (0.013)	<0.001 (0.013)	<0.001 (0.009)
GLU-150 vs. GLU-075	<0.001 (0.025)	<0.001 (0.025)	<0.001 (0.025)	<0.001 (0.017)	NS

Statistical significance: Unadjusted P value (critical Level) NS = Not significant

Table 5.14: Summary results for glucose concentrations - glucose study

Final Concentration of Added Glucose	Glucose concentration (mmol/L)				
	Day 2	Day 3	Day 6	Day 8	Day 10
0 mmol/L	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0. 0 ± 0.0
7.5 mmol/L	4.4 ± 0.2	3.5 ± 0.1	0.1 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
15 mmol/L	9.9 ± 0.6	9.3 ± 0.7	5.5 ± 0.8	2.4 ± 0.8	0.0 ± 0.0
30 mmol/L	19.7 ± 1.7	19.4 ± 1.4	15.8 ± 1.4	12.9 ± 1.3	9.2 ± 1 .5

Mean ± SD (n=5)

The calculated glucose consumption takes into account the platelet concentration over the 10-day storage period. As suggested by the levels of glucose in the units, rates of consumption were independent of the starting concentration of glucose, with no statistically significant differences between the test groups between days 2 and 3 (p=0.412) and differences thereafter confined to confirming the presence or absence of glucose (table 5.15). Though independent of starting levels of added glucose, the rate of glucose consumption was noted to increase with storage (figure 5.7; table 5.16).

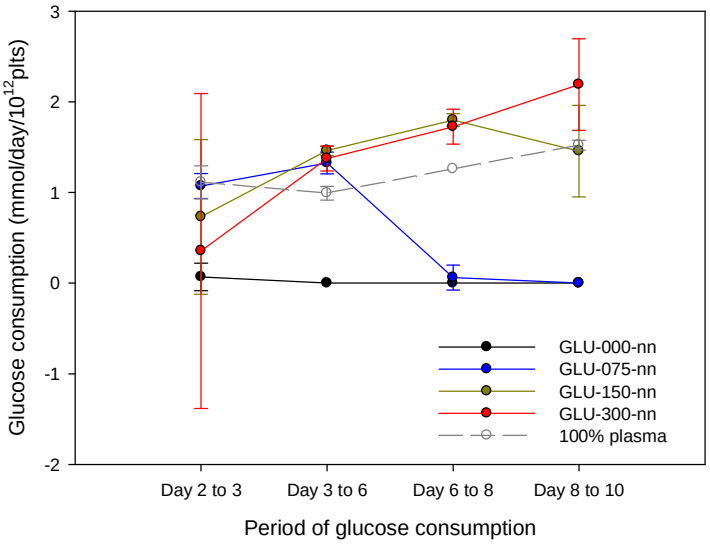


Figure 5.7: Glucose consumption (glucose study; mean ± SD, n=5; 100% plasma n=3)

Table 5.15: Statistical comparison between test groups for glucose consumption – glucose study

Comparison Between Groups				
	Day 2-3	Day 3-6	Day 6-8	Day 8-10
One Way ANOVA	0.413	<0.001	<0.001	<0.001
Multiple Pairwise Comparison (Holm-Sidak method)				
Comparison	Day 3-6		Day 6-8	Day 8-10
GLU-300 vs. GLU-000	<0.001 (0.002)		<0.001 (0.003)	<0.001 (0.002)
GLU-150 vs. GLU-000	<0.001 (0.002)		<0.001 (0.002)	<0.001 (0.003)
GLU-075 vs. GLU-000	<0.001 (0.003)		NS	NS
GLU-300 vs. GLU-075	NS		<0.001 (0.003)	<0.001 (0.002)
GLU-300 vs. GLU-150	NS		NS	NS
GLU-150 vs. GLU-075	NS		<0.001 (0.002)	<0.001 (0.003)

Statistical significance: Unadjusted P value (critical Level) NS = Not significant

Table 5.16: Summary results for glucose consumption - glucose study

Final Concentration of Added Glucose	Glucose consumption (mmol/day/10^{12} plts)			
	Day 2-3	Day 3-6	Day 6-8	Day 8-10
0 mmol/L	0.07 ± 0.15	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
7.5 mmol/L	1.07 ± 0.14	1.33 ± 0.12	0.06 ± 0.14	0.00 ± 0.00
15 mmol/L	0.73 ± 0.85	1.46 ± 0.06	1.80 ± 0.07	1.46 ± 0.50
30 mmol/L	0.35 ± 1.74	1.38 ± 0.13	1.73 ± 0.19	2.19 ± 0.51

Mean ± SD (n=5)

Lactate levels on day 2 were similar in all four test groups, despite the different starting concentrations of glucose (p=0.060). Mean values ranged from 2.0 ± 0.3 mmol/L in units lacking glucose to 2.5 ± 0.3 mmol/L in units with 30 mmol/L added glucose (table 5.18). Lactate levels in the former are likely to be due to residual levels due to the lack of a washing stage in the processing method of the units. As expected, no further lactate was produced in this test group. In the remaining three groups, lactate levels were affected by the glucose concentrations over the storage period, with levels reaching a plateau following the depletion of glucose (figure 5.8). This was best illustrated in units with 7.5 mmol/L added glucose, in which glucose stores were consumed by day 6 and levels of lactate were retained at approximately 10 mmol/L for the remainder of the storage period. Statistically significant differences were evident between all the test groups by day 10 (p<0.001) (table 5.17).

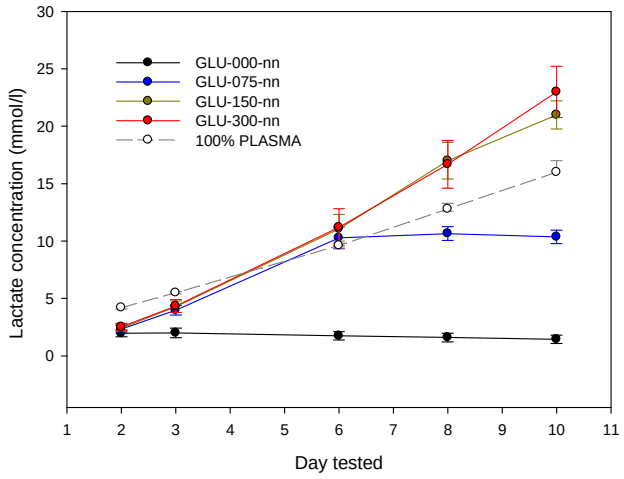


Figure 5.8: Lactate levels (glucose study; mean ± SD, n=5; 100% plasma n=3)

Table 5.17: Statistical comparison between test groups for lactate concentrations – glucose study

Comparison Between Groups					
	Day 2	Day 3	Day 6	Day 8	Day 10
One Way ANOVA	0.060	<0.001	<0.001	<0.001	<0.001
Multiple Pairwise Comparison (Holm-Sidak method)					
Comparison	Day 3	Day 6	Day 8	Day 10	
GLU-300 vs. GLU-000	<0.001 (0.009)	<0.001 (0.009)	<0.001 (0.010)	<0.001 (0.009)	
GLU-150 vs. GLU-000	<0.001 (0.010)	<0.001 (0.010)	<0.001 (0.009)	<0.001 (0.010)	
GLU-075 vs. GLU-000	<0.001 (0.013)	<0.001 (0.013)	<0.001 (0.013)	<0.001 (0.025)	
GLU-300 vs. GLU-075	NS	NS	<0.001 (0.025)	<0.001 (0.013)	
GLU-300 vs. GLU-150	NS	NS	NS	0.029 (0.050)	
GLU-150 vs. GLU-075	NS	NS	<0.001 (0.017)	<0.001 (0.017)	

Statistical significance: Unadjusted P value (critical Level) NS = Not significant

Table 5.18: Summary results for lactate concentration - glucose study

Final Concentration of Added Glucose	Lactate concentration (mmol/L)				
	Day 2	Day 3	Day 6	Day 8	Day 10
0 mmol/L	2.0 ± 0.3	2.0 ± 0.4	1.7 ± 0.4	1.6 ± 0.4	1. 4 ± 0.4
7.5 mmol/L	2.3 ± 0.3	4.0 ± 0.4	10.3 ± 0.9	10.7 ± 0.6	10.4 ± 0. 6
15 mmol/L	2.4 ± 0.3	4.3 ± 0.5	11.1 ± 1.3	17 ± 1.6	21 ± 1.2
30 mmol/L	2.5 ± 0.3	4.3 ± 0.6	11.2 ± 1.6	17 ± 2.1	23 ± 2.2

Mean ± SD (n=5)

The pattern for rates of lactate production over the storage period mirrored those of glucose consumption, with the rates of both increasing with storage until glucose stores were depleted (figures 5.7 and 5.9). Statistically significant differences were observed at all time-points ($p<0.001$); the pairwise comparison confirming the visual interpretation of the results (table 5.19).

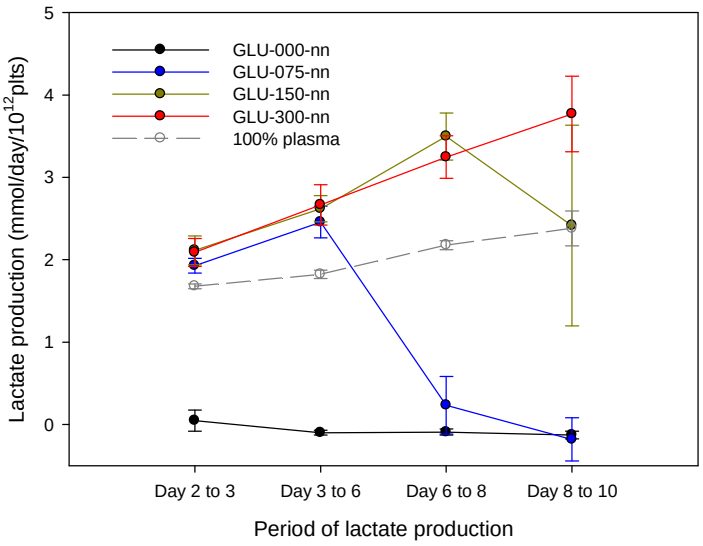


Figure 5.9: Lactate production (glucose study; mean ± SD, n=5; 100% plasma n=3)

Table 5.19: Statistical comparison between test groups for lactate production – glucose study

Comparison Between Groups				
	Day 2-3	Day 3-6	Day 6-8	Day 8-10
One Way ANOVA	<0.001	<0.001	<0.001	<0.001
Multiple Pairwise Comparison (Holm-Sidak method)				
Comparison	Day 2-3	Day 3-6	Day 6-8	Day 8-10
GLU-300 vs. GLU-000	<0.001 (0.002)	<0.001 (0.002)	<0.001 (0.002)	<0.001 (0.002)
GLU-150 vs. GLU-000	<0.001 (0.002)	<0.001 (0.002)	<0.001 (0.002)	<0.001 (0.003)
GLU-075 vs. GLU-000	<0.001 (0.003)	<0.001 (0.003)	NS	NS
GLU-300 vs. GLU-075	NS	NS	<0.001 (0.003)	<0.001 (0.002)
GLU-300 vs. GLU-150	NS	NS	NS	NS
GLU-150 vs. GLU-075	NS	NS	<0.001 (0.003)	<0.001 (0.003)

Statistical significance: Unadjusted P value (critical Level) NS = Not significant

Table 5.20: Lactate production in glucose study

Final Concentration of Added Glucose	Lactate production (mmol/day/10^{12} plts)			
	Day 2-3	Day 3-6	Day 6-8	Day 8-10
0 mmol/L	0.05 ± 0.13	-0.10 ± 0.03	-0.09 ± 0.04	-0.13 ± 0.05
7.5 mmol/L	1.93 ± 0.09	2.46 ± 0.19	0.23 ± 0.35	-0.18 ± 0.26
15 mmol/L	2.11 ± 0.18	2.62 ± 0.16	3.50 ± 0.29	2.41 ± 1.22
30 mmol/L	2.09 ± 0.17	2.66 ± 0.25	3.24 ± 0.26	3.77 ± 0.46

Mean ± SD (n=5)

Bicarbonate Levels

Bicarbonate levels in the group lacking added glucose remained stable throughout the ten days of storage at approximately 16 mmol/L (figure 5.10). In units with added glucose, levels decreased at a similar rate in all test groups until glucose stores were depleted. This was best observed in units with 7.5 mmol/L added glucose; with the depletion of glucose on day 6, the rate of bicarbonate loss decreased so that the mean concentration on day 10 (8.73 ± 2.28 mmo/l) was 85% of that measured on day 6 (10.23 ± 2.19 mmol/L). By comparison, the mean bicarbonate level on day 10 in units with 30 mmol/L added glucose was 47% of the mean on day 6 (4.90 ± 0.73 versus 10.42 ± 0.58 mmol/L, respectively) (table 5.22). Statistically significant differences on days 6 and 8 between the group with 30 mmol/L added glucose and the other test groups were confined to the test group with no added glucose. By day 10, a statistically significant difference was also evident with the units containing 7.5 mmol/L added glucose ($p<0.001$ in all cases) (table 5.21).

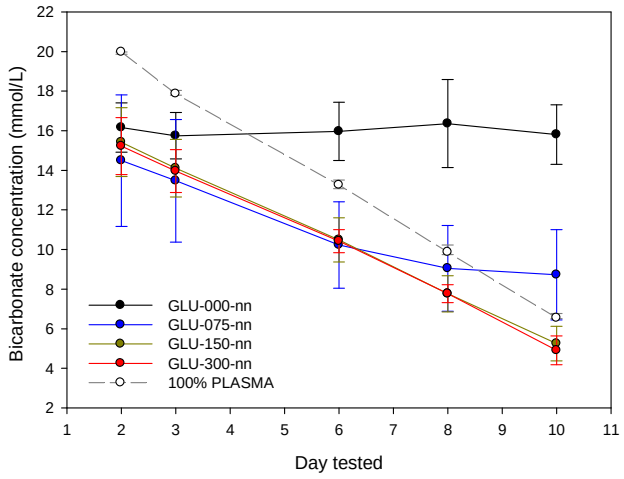


Figure 5.10: Bicarbonate levels (37°C) (glucose study; mean ± SD, n=5; 100% plasma n=3)

Table 5.21: Statistical comparison between test groups for bicarbonate levels – glucose study

Comparison Between Groups					
	Day 2	Day 3	Day 6	Day 8	Day 10
One Way ANOVA	0.665	0.285	<0.001	<0.001	<0.001
Multiple Pairwise Comparison (Holm-Sidak method)					
Comparison	Day 6		Day 8		Day 10
GLU-300 vs. GLU-000	<0.001 (0.010)		<0.001 (0.009)		<0.001 (0.009)
GLU-150 vs. GLU-000	<0.001 (0.013)		<0.001 (0.010)		<0.001 (0.010)
GLU-075 vs. GLU-000	<0.001 (0.009)		<0.001 (0.013)		<0.001 (0.013)
GLU-300 vs. GLU-075	NS		NS		0.001 (0.017)
GLU-300 vs. GLU-150	NS		NS		NS
GLU-150 vs. GLU-075	NS		NS		0.002 (0.025)

Statistical significance: Unadjusted P value (critical Level) NS = Not significant

Table 5.22: Summary results for bicarbonate levels - glucose study

Final Concentration of Added Glucose	Bicarbonate concentration (mmol/L) (37°C)				
	Day 2	Day 3	Day 6	Day 8	Day 10
0 mmol/L	16.16 ±1.25	15.75 ± 1.16	15.96 ± 1.47	16.36 ± 2.22	15.81 ± 1.50
7.5 mmol/L	14.49 ± 3.32	13.47 ± 3.09	10.23 ± 2.19	9.05 ± 2.17	8.73 ± 2.28
15 mmol/L	15.43 ± 1.74	14.10 ± 1.46	10.49 ± 1.12	7.77 ± 0.91	5.25 ± 0.87
30 mmol/L	15.22 ± 1.44	13.97 ± 1.08	10.42 ± 0.58	7.76 ± 0.45	4.90 ± 0.73

Mean ± SD (n=5)

Blood Gases

The partial pressure of oxygen increased with storage, with the three test groups containing glucose generating similar results throughout the storage period and finishing with mean levels of approximately 18 kPa by end of storage (figure 5.11). No statistically significant differences were evident between these three test groups at any point in the storage period (table 5.23). The test group lacking glucose showed a steeper increase in pO_2 after the initial three days of storage, with a mean day 10 partial pressure of 20.0 ± 0.5 kPa (p=0.006) (table 5.24).

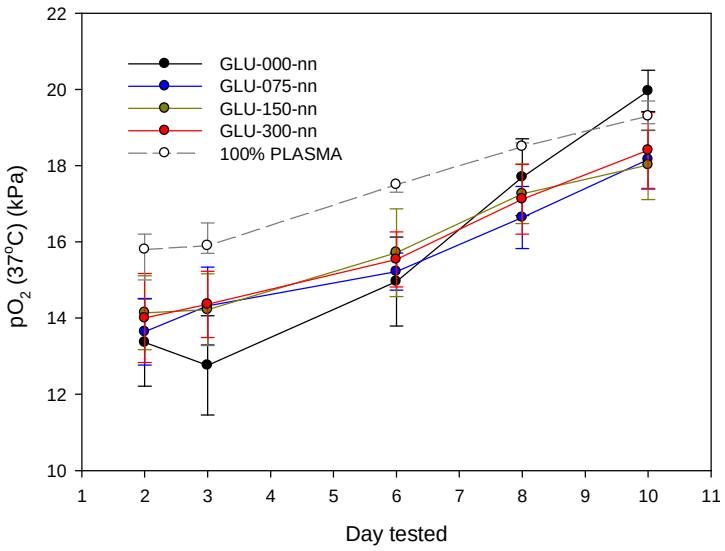


Figure 5.11: Partial pressure of oxygen
(glucose study; mean ± SD, n=5; 100% plasma n=3)

Table 5.23: Statistical comparison between test groups for partial pressure of oxygen – glucose study

Comparison Between Groups					
	Day 2	Day 3	Day 6	Day 8	Day 10
One Way ANOVA	0.643	0.079	0.587	0.336	0.006
Multiple Pairwise Comparison (Holm-Sidak method)					
Comparison	Day 10				
GLU-300 vs. GLU-000	0.009 (0.013)				
GLU-150 vs. GLU-000	0.002 (0.009)				
GLU-075 vs. GLU-000	0.003 (0.010)				
GLU-300 vs. GLU-075	NS				
GLU-300 vs. GLU-150	NS				
GLU-150 vs. GLU-075	NS				

Statistical significance: Unadjusted P value (critical Level) NS = Not significant

Table 5.24: Summary results for partial pressure of oxygen - glucose study

Final Concentration of Added Glucose	Partial pressure of oxygen (kPa) (37°C)				
	Day 2	Day 3	Day 6	Day 8	Day 10
0 mmol/L	13.4 ± 1.1	12.8 ± 1.3	15.0 ± 1.2	17.7 ± 1. 0	20.0 ± 0.5
7.5 mmol/L	13.6 ± 0.9	14.3 ± 1.0	15.2 ± 0.5	16.6 ± 0.8	18.2 ± 0.8
15 mmol/L	14.1 ± 1.0	14.2 ± 0.9	15.7 ± 1.2	17.3 ± 0.8	18.0 ± 0.9
30 mmol/L	14.0 ± 1.2	14.4 ± 0.9	15.5 ± 0.7	17.1 ± 0.9	18.4 ± 1.0

Mean ± SD (n=5)

The partial pressures of carbon dioxide over the ten days of storage reflected the pattern of glucose and bicarbonate metabolism more closely than pO_2 (figure 5.12). In units lacking added glucose, pCO_2 decreased steadily with storage from day 2 pressures of 4.27 ± 0.25 kPa to a mean of 1.54 ± 0.09 kPa on day 10. By contrast, from similar day 2 values (p=0.829), partial pressures in units with 30 mmol/L added glucose had decreased to a mean of 3.20 ± 0.08 kPa by day 10 (table 5.26). Statistically significant differences on day 6 were limited to differences between the units with no added glucose and units with glucose. By day 10, significant differences were evident between all the test groups (p<0.001) (table 5.25)

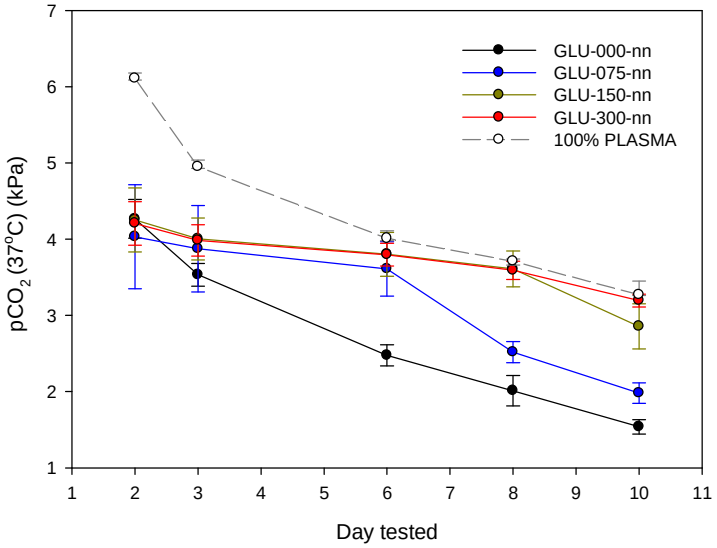


Figure 5.12: Partial pressure of carbon dioxide (glucose study; mean ± SD, n=5; 100% plasma n=3)

Table 5.25: Statistical comparison between test groups for partial pressure of carbon dioxide – glucose study

Comparison Between Groups					
	Day 2	Day 3	Day 6	Day 8	Day 10
One Way ANOVA	0.829	0.144	<0.001	<0.001	<0.001
Multiple Pairwise Comparison (Holm-Sidak method)					
Comparison	Day 6		Day 8		Day 10
GLU-300 vs. GLU-000	<0.001 (0.010)		<0.001 (0.010)		<0.001 (0.009)
GLU-150 vs. GLU-000	<0.001 (0.009)		<0.001 (0.009)		<0.001 (0.010)
GLU-075 vs. GLU-000	<0.001 (0.013)		<0.001 (0.025)		0.001 (0.025)
GLU-300 vs. GLU-075	NS		<0.001 (0.017)		<0.001 (0.013)
GLU-300 vs. GLU-150	NS		NS		0.007 (0.050)
GLU-150 vs. GLU-075	NS		<0.001 (0.013)		<0.001 (0.017)

Statistical significance: Unadjusted P value (critical Level) NS = Not significant

Table 5.26: Summary results for partial pressure of carbon dioxide - glucose study

Final Concentration of Added Glucose	Partial pressure of carbon dioxide (kPa) (37°C)				
	Day 2	Day 3	Day 6	Day 8	Day 10
0 mmol/L	4.27 ± 0.25	3.53 ± 0.15	2.48 ± 0.14	2.01 ± 0.20	1.54 ± 0.09
7.5 mmol/L	4.03 ± 0.68	3.87 ± 0.57	3.61 ± 0.36	2.52 ± 0.14	1.98 ± 0.14
15 mmol/L	4.25 ± 0.42	4.00 ± 0.27	3.80 ± 0.29	3.61 ± 0.24	2.86 ± 0.30
30 mmol/L	4.21 ± 0.29	3.98 ± 0.21	3.80 ± 0.15	3.59 ± 0.12	3.20 ± 0.08

Mean ± SD (n=5)

Oxygen consumption rates decreased similarly with storage in all test groups, with starting levels of approximately 0.23 nmol/min/10^9plts decreasing to mean levels of approximately 0.16 nmol/min/10^9plts by end of storage (figure 5.13; table 5.28). No statistically significant differences were noted between the test groups at any time point (table 5.27). Thus, the concentration of glucose, or even its absence, did not appear to impact on oxygen consumption rate. The gradual decrease in consumption reflects the gradual accumulation of oxygen in the units with storage. With a continued decrease in oxygen consumption, the partial pressure of oxygen would be expected to continue to increase until levels equilibrated with the atmospheric partial pressure.

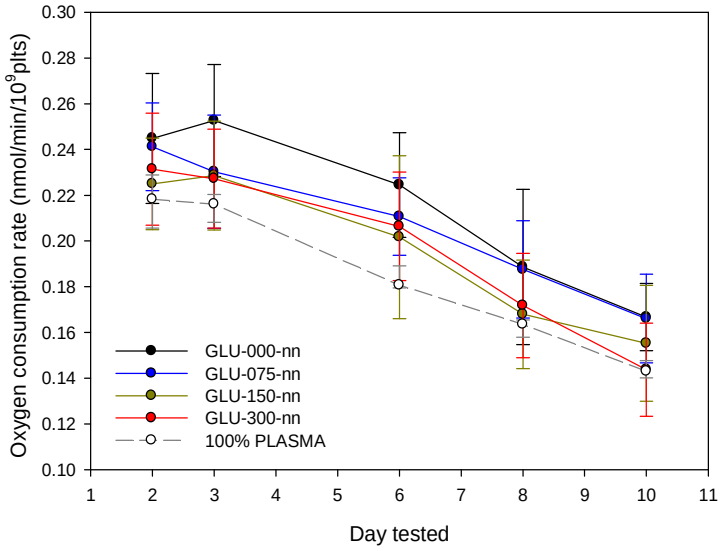


Figure 5.13: Oxygen consumption rate (glucose study; mean ± SD, n=5; 100% plasma n=3)

Table 5.27: Statistical comparison between test groups for oxygen consumption rate – glucose study

Comparison Between Groups					
	Day 2	Day 3	Day 6	Day 8	Day 10
One Way ANOVA	0.533	0.313	0.548	0.490	0.273

Table 5.28: Summary results for oxygen consumption rates - glucose study

Final Concentration of Added Glucose	Oxygen consumption rate (nmol/min/10^9plts) (22°C)				
	Day 2	Day 3	Day 6	Day 8	Day 10
0 mmol/L	0.24 ± 0.03	0.25 ± 0.02	0.22 ± 0.02	0.19 ± 0.03	0.17 ± 0.01
7.5 mmol/L	0.24 ± 0.02	0.23 ± 0.02	0.21 ± 0.02	0.19 ± 0.02	0.17 ± 0.02
15 mmol/L	0.22 ± 0.02	0.23 ± 0.02	0.20 ± 0.04	0.17 ± 0.02	0.16 ± 0.03
30 mmol/L	0.23 ± 0.02	0.23 ± 0.02	0.21 ± 0.02	0.17 ± 0.02	0.14 ± 0.02

Mean ± SD (n=5)

ATP and ADP Levels

Levels of ATP were similar at the start of the storage period in the three test groups containing glucose, with mean values on day 2 of approximately 5.4 µmol/10^{11}plts. Mean levels in the test group lacking glucose were lower at 4.38 ± 0.56 µmol/10^{11}plts, though this difference did not achieve statistical significance (p=0.016) (table 5.30). ATP levels decreased steadily with storage in units lacking glucose to a mean of 1.09 ± 0.09 µmol/10^{11}plts by day 10 (table 5.30). The rate of decline was lower in the other three test groups until glucose stores were depleted, at which point ATP levels were observed to decrease at an accelerated rate that more closely resembled that seen in units lacking added glucose (figure 5.14). Day 10 ATP levels were thus relatively well maintained in units with 30 mmol/L added glucose, with a mean of 4.18 ± 0.64 µmol/10^{11}plts. This constituted 78% of the day 2 levels compared with only 25% of day 2 levels in the test group lacking added glucose. Statistically significant differences were evident between all the test groups by day 10 (p<0.001) (table 5.29).

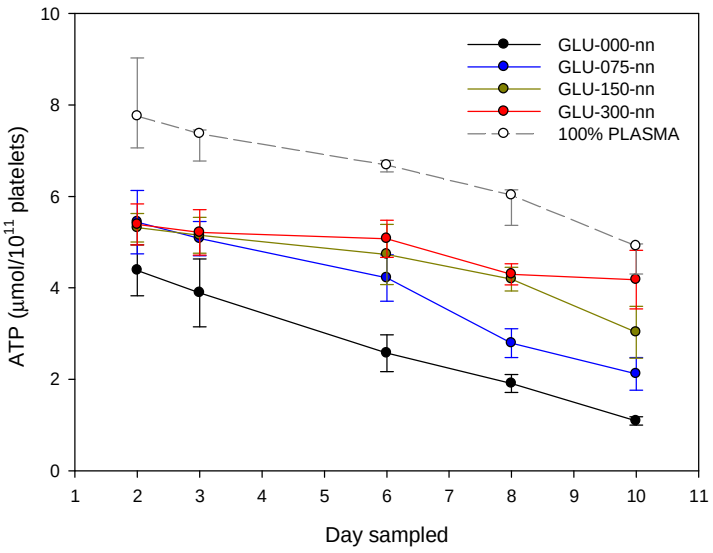


Figure 5.14: ATP Levels (glucose study; mean ± SD, n=5; 100% plasma n=3)

Table 5.29: Statistical comparison between test groups for ATP levels – glucose study

Comparison Between Groups					
	Day 2	Day 3	Day 6	Day 8	Day 10
One Way ANOVA	0.016	0.003	<0.001	<0.001	<0.001
Multiple Pairwise Comparison (Holm-Sidak method)					
Comparison	Day 3	Day 6	Day 8	Day 10	
GLU-300 vs. GLU-000	0.001 (0.009)	<0.001 (0.009)	<0.001 (0.009)	<0.001 (0.009)	
GLU-150 vs. GLU-000	0.002 (0.010)	<0.001 (0.010)	<0.001 (0.010)	<0.001 (0.013)	
GLU-075 vs. GLU-000	0.002 (0.013)	<0.001 (0.013)	<0.001 (0.025)	0.003 (0.025)	
GLU-300 vs. GLU-075	NS	0.016 (0.017)	<0.001 (0.013)	<0.001 (0.010)	
GLU-300 vs. GLU-150	NS	NS	NS	0.001 (0.017)	
GLU-150 vs. GLU-075	NS	NS	<0.001 (0.017)	0.007 (0.050)	

Statistical significance: Unadjusted P value (critical Level) NS = Not significant

Table 5.30: Summary results for ATP levels - glucose study

Final Concentration of Added Glucose	ATP Levels (µmol/10^{11}plts)				
	Day 2	Day 3	Day 6	Day 8	Day 10
0 mmol/L	4.38 ± 0.56	3.89 ± 0.74	2.57 ± 0.40	1.91 ± 0.20	1.09 ± 0.09
7.5 mmol/L	5.44 ± 0.69	5.08 ± 0.38	4.21 ± 0.51	2.79 ± 0.31	2.12 ± 0.36
15 mmol/L	5.31 ± 0.31	5.15 ± 0.39	4.73 ± 0.66	4.19 ± 0.26	3.03 ± 0.57
30 mmol/L	5.39 ± 0.45	5.21 ± 0.50	5.07 ± 0.41	4.30 ± 0.23	4.18 ± 0.64

Mean ± SD (n=5)

ADP levels decreased with storage in all the test groups. Although mean levels were lower in the test group lacking added glucose, no statistically significant differences were noted between any of the test groups at any of the time points (table 5.31). In all test groups, levels on day 10 were between 20- 30% of levels on day 2 (table 5.32). Unlike ATP, loss of ADP appeared unrelated to glucose levels and other metabolic parameters (figure 5.15).

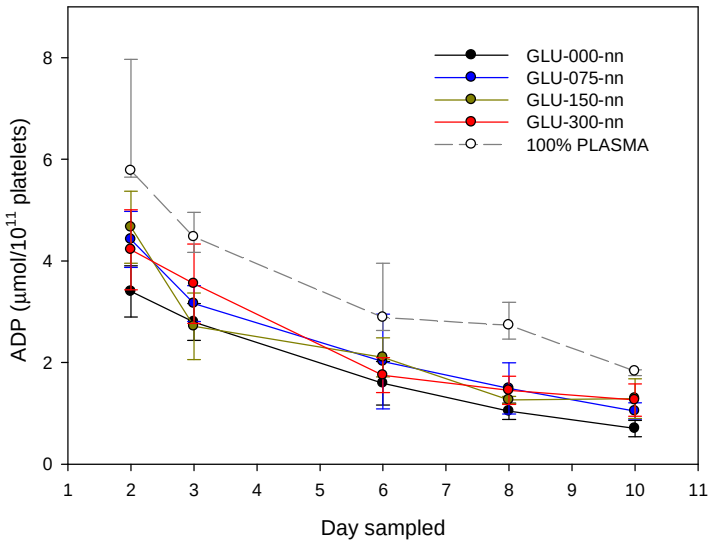


Figure 5.15: ADP Levels (glucose study; mean ± SD, n=5; 100% plasma n=3)

T*able 5.31: Statistical comparison for ADP levels – glucose study*

Comparison Between Groups					
	Day 2	Day 3	Day 6	Day 8	Day 10
One Way ANOVA	0.037	0.121	0.488	0.114	0.014

Table 5.32: Summary results for ADP - glucose study

Final Concentration of Added Glucose	ADP Levels (µmol/10^{11}plts)				
	Day 2	Day 3	Day 6	Day 8	Day 10
0 mmol/L	3.40 ± 0.50	2.79 ± 0.36	1.59 ± 0.43	1.04 ± 0.16	0.70 ± 0.16
7.5 mmol/L	4.42 ± 0.55	3.16 ± 0.35	2.02 ± 0.93	1.49 ± 0.51	1.04 ± 0.16
15 mmol/L	4.66 ± 0.71	2.71 ± 0.66	2.10 ± 0.39	1.26 ± 0.07	1.29 ± 0.39
30 mmol/L	4.22 ± 0.79	3.55 ± 0.78	1.75 ± 0.34	1.45 ± 0.28	1.26 ± 0.32

Mean ± SD (n=5)

Platelet Activation

Surface Expression and Soluble Levels of CD62P

Surface expression of CD62P resulted in similar percentage positive expression in all four test groups on day 2, with mean levels of approximately 80% (p=0.826) (table 5.34); markedly higher than the mean of 60.08% obtained from units re-suspended in plasma, despite the use of the same processing method. Percentage positive expression increased between days 2 and 3 in all four test groups, after which the expression remained relatively stable in the three test groups containing glucose (figure 5.16). The test group lacking glucose continued to increase in percent positive expression until day 6, resulting in a mean level of 94.04 ± 0.97 % and a statistically significant difference against the test groups with added glucose (p<0.001) (table 5.33). Beyond this time point, a gradual decrease was observed, resulting in levels at the end of storage similar to those obtained with the test groups containing exogenous glucose (p=0.577).

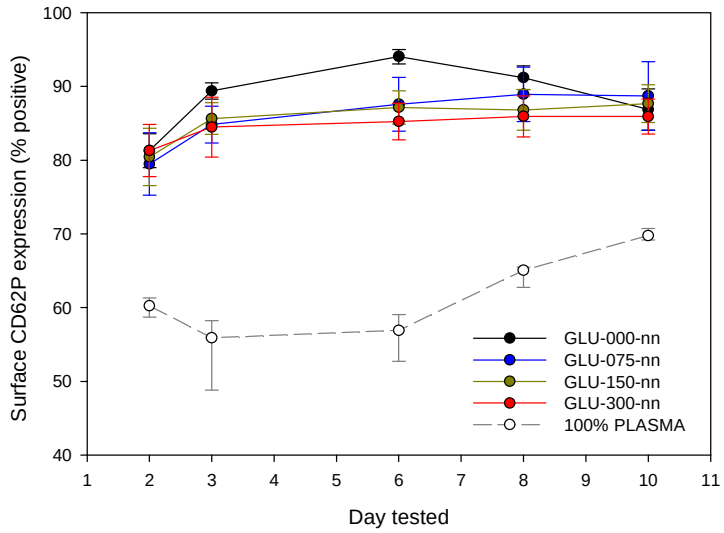


Figure 5.16: Percent positive expression of surface CD62P (glucose study; mean ± SD, n=5; 100% plasma n=3)

Table 5.33: Statistical comparison between test groups for CD62P surface expression (% positive) – glucose study

Comparison Between Groups					
	Day 2	Day 3	Day 6	Day 8	Day 10
One Way ANOVA	0.826	0.039	<0.001	0.041	0.577
Multiple Pairwise Comparison (Holm-Sidak method)					
Comparison	Day 6				
GLU-300 vs. GLU-000	<0.001 (0.009)				
GLU-150 vs. GLU-000	0.001 (0.010)				
GLU-075 vs. GLU-000	0.001 (0.013)				
GLU-300 vs. GLU-075	NS				
GLU-300 vs. GLU-150	NS				
GLU-150 vs. GLU-075	NS				

Statistical significance: Unadjusted P value (critical Level) NS = Not significant

Table 5:34: Summary results for percent positive expression of CD62P - glucose study

Final Concentration of Added Glucose	Surface expression of CD62P (% positive)				
	Day 2	Day 3	Day 6	Day 8	Day 10
0 mmol/L	81.29 ± 2.30	89.39 ± 1.11	94.04 ± 0.97	91.18 ± 1.62	86.87 ± 2.80
7.5 mmol/L	79.47 ± 4.23	84.83 ± 2.51	87.60 ± 3.65	88.93 ± 3.69	88.71 ± 4.63
15 mmol/L	80.43 ± 3.88	85.65 ± 2.16	87.15 ± 2.27	86.80 ± 2.75	87.67 ± 2.56
30 mmol/L	81.30 ± 3.53	84.47 ± 4.07	85.25 ± 2.49	85.93 ± 2.80	85.91 ± 2.37

Mean ± SD (n=5)

Surface CD62P median fluorescence intensity was higher on days 6 and 8 in the test group lacking glucose, with mean levels of 4.25 ± 0.33 and 4.32 ± 0.76, respectively (table 5.36). However, this was not a statistically significant difference compared with the units with added glucose (p=0.031 on day 6 and p=0.055 on day 8) (table 5.35). MFI increased in all four test groups (from mean day 2 levels between 2.33 ± 0.12 and 2.49 ± 0.21 in units lacking glucose and units with 30 mmol/L added glucose (p=0.544)), until days 6 – 8; beyond which a gradual decrease or stabilisation in MFI was evident. As with percent positive expression, MFI results for surface CD62P were markedly lower in the units re-suspended in autologous plasma (figure 5.17).

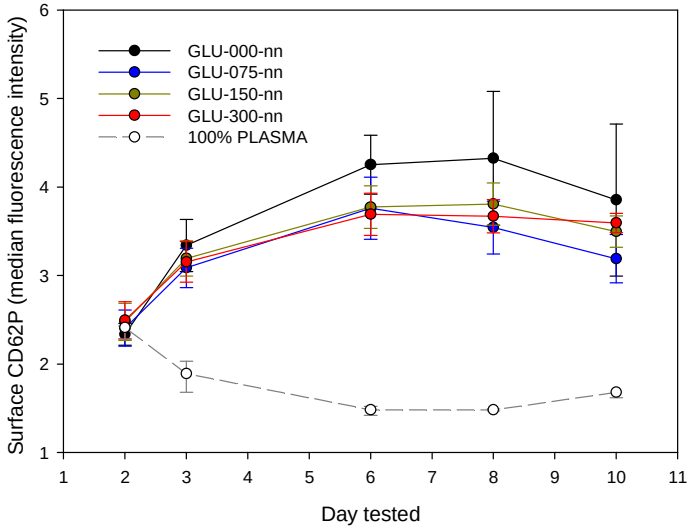


Figure 5.17: Surface CD62P expressed as median fluorescence intensity (glucose study; mean ± SD, n=5; 100% plasma n=3)

Table 5.35: Statistical comparison for CD62P surface expression (MFI) – glucose study

Comparison Between Groups					
	Day 2	Day 3	Day 6	Day 8	Day 10
One Way ANOVA	0.544	0.414	0.031	0.055	0.196

Table 5.36: Summary results for median fluorescence intensity of surface CD62P - glucose study

Final Concentration of Added Glucose	Surface expression of CD62P (median fluorescence intensity)				
	Day 2	Day 3	Day 6	Day 8	Day 10
0 mmol/L	2.33 ± 0.12	3.34 ± 0.30	4.25 ± 0.33	4.32 ± 0.76	3.85 ± 0.86
7.5 mmol/L	2.40 ± 0.20	3.09 ± 0.22	3.76 ± 0.35	3.54 ± 0.30	3.19 ± 0.27
15 mmol/L	2.48 ± 0.21	3.19 ± 0.20	3.77 ± 0.24	3.81 ± 0.24	3.50 ± 0.18
30 mmol/L	2.49 ± 0.21	3.15 ± 0.23	3.69 ± 0.24	3.67 ± 0.19	3.59 ± 0.11

Mean ± SD (n=5)

Levels of soluble CD62P increased with storage in all test groups from similar starting levels of approximately 35 to 45 ng/mL (p=0.345) (table 5.38). Levels increased at a faster rate in units lacking added glucose, leading to a statistically significant difference between these units and units with added glucose by day 6 (p<0.001). Beyond day 6, soluble CD62P increase at a faster rate in both units lacking added glucose and units with 7.5 mmol/L, resulting in a statistically significant difference (p<0.001) between these test groups and units with 15 mmol/L and 30 mmol/L added glucose (table 5.37) (figure 5.18). Mean soluble CD62P levels on day 10 were 268.27 ± 23.46 ng/mL in units lacking glucose and 92.30 ± 12.31 ng/mL in units with 30 mmol/L.

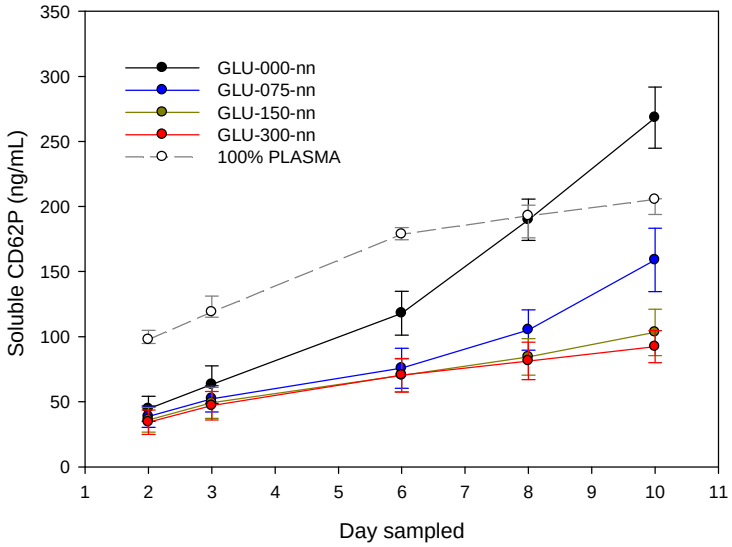


Figure 5.18: Soluble CD62P (glucose study; mean ± SD, n=5; 100% plasma n=3)

Table 5.37: Statistical comparison between test groups for soluble CD62P levels – glucose study

Comparison Between Groups					
	Day 2	Day 3	Day 6	Day 8	Day 10
One Way ANOVA	0.345	0.189	<0.001	<0.001	<0.001
Multiple Pairwise Comparison (Holm-Sidak method)					
Comparison	Day 6		Day 8		Day 10
GLU-300 vs. GLU-000	<0.001 (0.010)		<0.001 (0.009)		<0.001 (0.009)
GLU-150 vs. GLU-000	<0.001 (0.009)		<0.001 (0.010)		<0.001 (0.010)
GLU-075 vs. GLU-000	<0.001 (0.013)		<0.001 (0.013)		<0.001 (0.013)
GLU-300 vs. GLU-075	NS		NS		<0.001 (0.017)
GLU-300 vs. GLU-150	NS		NS		NS
GLU-150 vs. GLU-075	NS		NS		<0.001 (0.025)

Statistical significance: Unadjusted P value (critical Level) NS = Not significant

Table 5.38: Summary results for soluble CD62P levels - glucose study

Final Concentration of Added Glucose	Soluble CD62P (ng/mL)				
	Day 2	Day 3	Day 6	Day 8	Day 10
0 mmol/L	44.58 ± 9.66	63.14 ± 14.43	117.99 ± 16.92	189.96 ± 15.88	268.27 ± 23.46
7.5 mmol/L	38.53 ± 8.20	52.28 ± 10.12	75.70 ± 15.39	105.12 ± 15.44	158.88 ± 24.30
15 mmol/L	36.11 ± 9.47	49.23 ± 11.95	70.25 ± 12.74	84.37 ± 13.94	103.36 ± 17.84
30 mmol/L	34.34 ± 9.25	46.98 ± 10.89	70.37 ± 12.94	81.34 ± 14.42	92.30 ± 12.31

Mean ± SD (n=5)

Markers of Cell Death

Surface Expression of Aminophospholipids

Annexin V binding to the platelet surface, measured as percentage positive expression, was similar in all four test groups at the start of storage. Day 2 levels in units lacking glucose (13.53 ± 3.60 %) were higher than in test groups with added glucose (9.42 ± 2.96 % in 30 mmol/L added glucose), but this difference was not found to be statistically significant (p=0.094) (table 5.39). Annexin V binding continued to increase in units lacking glucose throughout storage, with mean levels on day 10 of 66.31 ± 4.88 %. Annexin V binding in the test groups with added glucose also increased with storage, with the rate of increase accelerating in units with 7.5 mmol/L added glucose around day 6 and in units with 15 mmol/L after day 8 (figure 5.19). In contrast, no such accelerated rise in annexin V binding was observed in units with 30 mmol/L added glucose, with day 10 percent positive binding remaining at relatively low levels (15.73 ± 3.60 %) (table 5.40). Statistically significant differences were evident between all the test groups by end of storage (p<0.001).

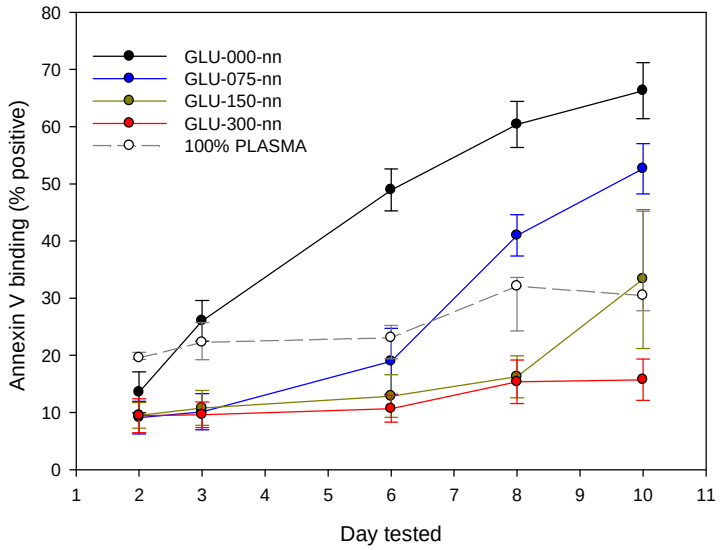


Figure 5.19: Percent positive expression of annexin V binding (glucose study; mean ±SD, n=5; 100%plasma n=3)

Table 5.39: Statistical comparison between test groups for Annexin V binding (% positive expression) – glucose study

Comparison Between Groups					
	Day 2	Day 3	Day 6	Day 8	Day 10
One Way ANOVA	0.094	<0.001	<0.001	<0.001	<0.001
Multiple Pairwise Comparison (Holm-Sidak method)					
Comparison	Day 3	Day 6	Day 8	Day 10	
GLU-300 vs. GLU-000	<0.001 (0.009)	<0.001 (0.009)	<0.001 (0.009)	<0.001 (0.009)	
GLU-150 vs. GLU-000	<0.001 (0.013)	<0.001 (0.010)	<0.001 (0.010)	<0.001 (0.013)	
GLU-075 vs. GLU-000	<0.001 (0.010)	<0.001 (0.013)	<0.001 (0.025)	0.008 (0.050)	
GLU-300 vs. GLU-075	NS	0.005 (0.017)	<0.001 (0.013)	<0.001 (0.010)	
GLU-300 vs. GLU-150	NS	NS	NS	0.001 (0.025)	
GLU-150 vs. GLU-075	NS	NS	<0.001 (0.017)	0.001 (0.017)	

Statistical significance: Unadjusted P value (critical Level) NS = Not significant

Table 5.40: Summary results for Annexin V binding (% positive expression) - glucosestudy

Final Concentration of Added Glucose	Annexin V binding (% positive)				
	Day 2	Day 3	Day 6	Day 8	Day 10
0 mmol/L	13.53 ± 3.60	26.02 ± 3.57	48.94 ± 3.66	60.40 ± 4.04	66.31 ± 4.88
7.5 mmol/L	9.11 ± 2.86	10.13 ± 3.16	18.95 ± 5.76	40.98 ± 3.60	52.65 ± 4.40
15 mmol/L	9.49 ± 2.22	10.79 ± 3.04	12.87 ± 3.72	16.25 ± 3.68	33.35 ± 12.15
30 mmol/L	9.42 ± 2.96	9.60 ± 2.24	10.67 ± 2.35	15.35 ± 3.80	15.73 ± 3.60

Mean ± SD (n=5)

Mean fluorescence intensity (MFI) was higher throughout storage in the test group lacking glucose (figure 5.20). This resulted in statistically significant differences on day 3, 6 and 8 between units lacking glucose and at least two of the other test groups ($p<0.001$, $p=0.009$ and $p=0.001$, respectively) (table 5.41). However, no association between glucose concentration in the medium and annexin V binding MFI was seen. MFI was retained at relatively stable levels in all test groups to the end of storage, with no statistically significant difference between any of the test groups by day 10 ($p=0.107$) (table 5.41).

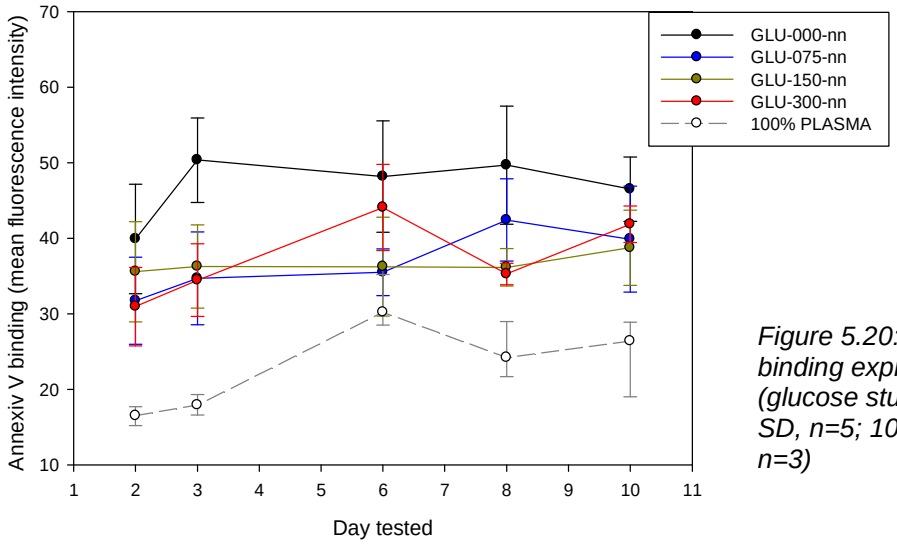


Figure 5.20: Annexin V binding expressed as MFI (glucose study; mean ± SD, n=5; 100%plasma n=3)

Table 5.41: Statistical comparison for Annexin V binding (MFI) – glucose study

Comparison Between Groups					
	Day 2	Day 3	Day 6	Day 8	Day 10
One Way ANOVA	0.134	<0.001	0.009	0.001	0.107
Multiple Pairwise Comparison (Holm-Sidak method)					
Comparison	Day 3		Day 6	Day 8	
GLU-300 vs. GLU-000	<0.001 (0.009)		NS	<0.001 (0.009)	
GLU-150 vs. GLU-000	0.001 (0.013)		0.006 (0.010)	0.001 (0.010)	
GLU-075 vs. GLU-000	<0.001 (0.010)		0.004 (0.009)	NS	
GLU-300 vs. GLU-075	NS		NS	NS	
GLU-300 vs. GLU-150	NS		NS	NS	
GLU-150 vs. GLU-075	NS		NS	NS	

Statistical significance: Unadjusted P value (critical Level) NS = Not significant

Table 5.42: Summary results for Annexin V binding expressed as MFI - glucose study

Final Concentration of Added Glucose	Annexin V binding (mean fluorescence intensity)				
	Day 2	Day 3	Day 6	Day 8	Day 10
0 mmol/L	39.9 ± 7.3	50.4 ± 5.6	48.2 ± 7.4	49.7 ± 7.8	46.5 ± 4.3
7.5 mmo/L	31.7 ± 5.8	34.7 ± 6.1	35.5 ± 3.1	42.4 ± 5.5	39.9 ± 7.0
15 mmol/L	35.6 ± 6.6	36.3 ± 5.5	36.2 ± 6.6	36.2 ± 2.5	38.7 ± 5.0
30 mmol/L	31.0 ± 5.2	34.5 ± 4.8	44.1 ± 5.7	35.3 ± 1.4	41.9 ± 2.4

Mean ± SD (n=5)

The concentrations of the four phosphatidylethanolamine (PE) species are shown in figure 5.21. Levels of all four forms of PE increased with storage, with the expressions in the unit lacking glucose consistently higher than in the units containing added glucose. A suggestion of a dose-dependent pattern in the three units containing glucose may be evident in that the expression of all PE forms was lower in the unit with 30 mmol/L added glucose. However, it would be premature to infer a relationship between the extent of PE expression and glucose concentration from one result. Initial determination of surface phospholipid exposure by mass spectrometry generated results for phosphatidylserine that did not confirm the pattern obtained by flow cytometric annexin V binding (percent positive results) in that a decrease in expression with storage was noted in all four test groups and no clear difference in results between the different concentrations of glucose was seen. To confirm whether these results were

reproducible, a further set of mass spectrometry measurements were undertaken and it is the results from this second experiment that are reproduced here. The expression of phosphatidylserine obtained by mass spectrometry showed a decrease in expression with storage, noted in all four test groups, and no clear difference in results between the different concentrations of glucose – a marked deviation from the pattern obtained by annexin V binding. A similar set of results was obtained with both experiments, suggesting that the discrepant results are not necessarily due to an error in the preparation of the samples for mass spectrometry.

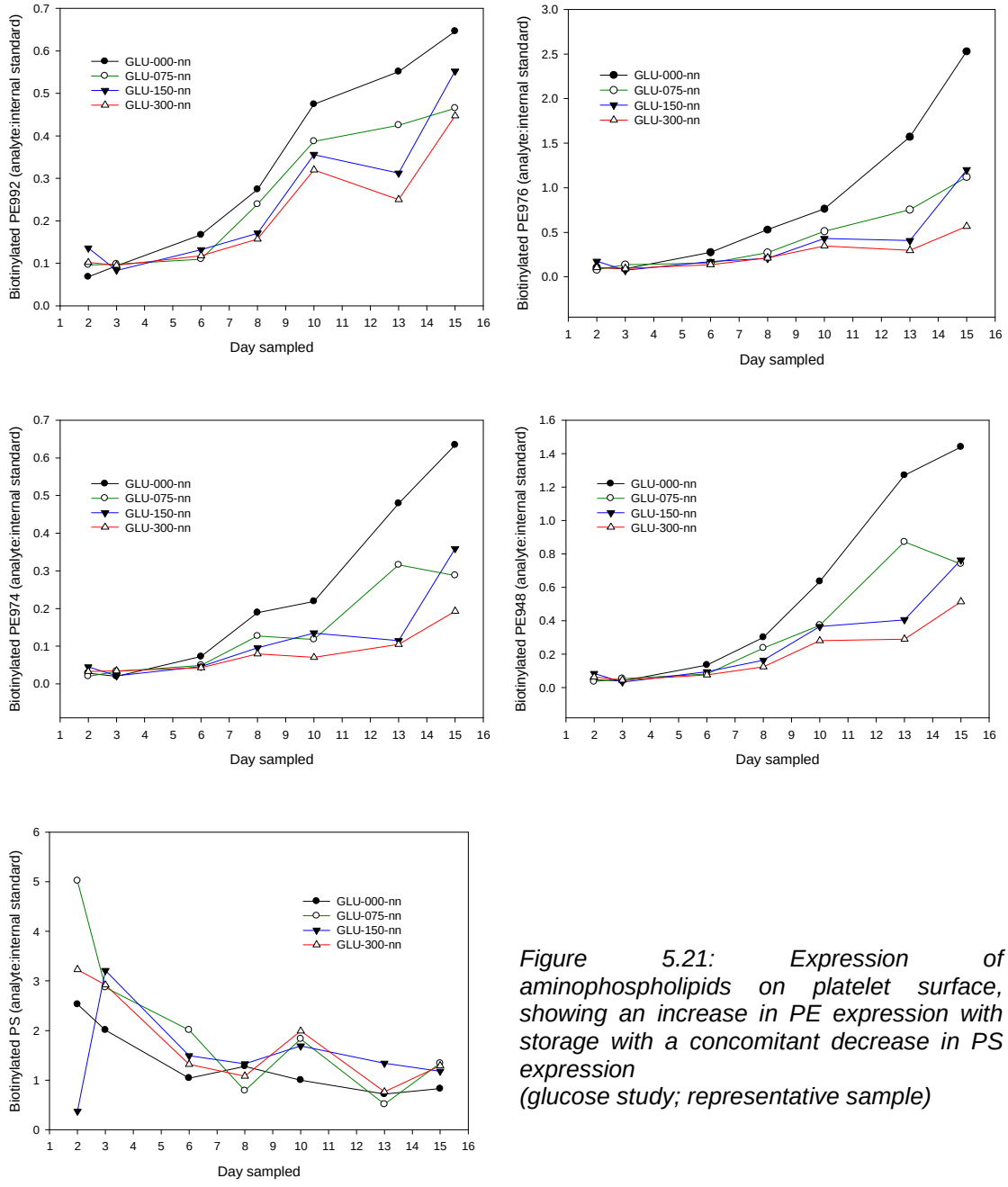


Figure 5.21: Expression of aminophospholipids on platelet surface, showing an increase in PE expression with storage with a concomitant decrease in PS expression
(glucose study; representative sample)

Mitochondrial Membrane Potential

Mitochondrial membrane potential on day 2 showed similar activity in all three test groups containing glucose, with mean ratios between 8.66 ± 1.23 for units with 7.5 mmol/L to 9.03 ± 1.01 in units with 30 mmol/L. By contrast, units lacking glucose had significantly lower mean ratios of 5.98 ± 1.15 (p=0.004) (table 5.43). Levels decreased with storage in all four test groups. In units without added glucose, MMP ratios stabilised by day 6 at approximately 3 (2.95 ± 0.10 on day 10); close to the assay limit of 2.7 suggested by the albumin-containing units in study 2. Figure 5.22 suggests an accelerated disruption of the MMP occurred with lower concentrations of added glucose, with mean day 8 ratios of 3.30 ± 0.16 and 5.65 ± 1.76 in units with 7.5 mmol/L and 30mmol/L added glucose, respectively (p=0.006) (table 5.44).

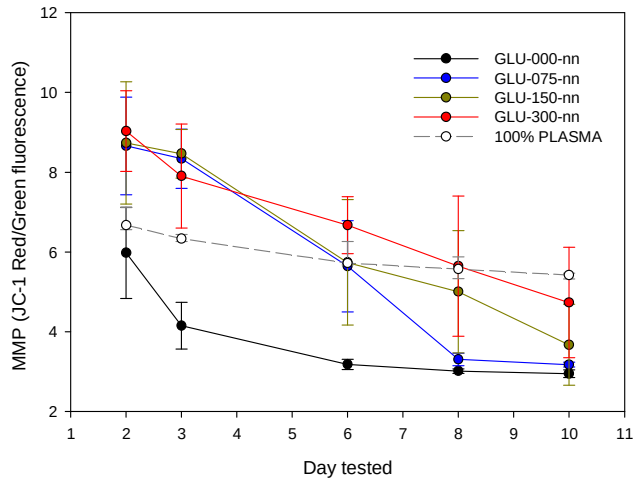


Figure 5.22: Mitochondrial membrane potential (red/green ratio) (glucose study; mean ± SD, n=5; 100% plasma n=3)

Table 5.43: Statistical comparison for MMP (JC-1 Red/Green Fluorescence) – glucose study

Comparison Between Groups					
	Day 2	Day 3	Day 6	Day 8	Day 10
One Way ANOVA	0.004	<0.001	<0.001	0.006	0.022
Multiple Pairwise Comparison (Holm-Sidak method)					
Comparison	Day 2	Day 3	Day 6	Day 8	
GLU-300 vs. GLU-000	0.001 (0.009)	<0.001 (0.013)	<0.001 (0.009)	0.003 (0.009)	
GLU-150 vs. GLU-000	0.003 (0.010)	<0.001 (0.009)	0.001 (0.010)	NS	
GLU-075 vs. GLU-000	0.004 (0.013)	<0.001 (0.010)	0.002 (0.013)	NS	
GLU-300 vs. GLU-075	NS	NS	NS	0.006 (0.010)	
GLU-300 vs. GLU-150	NS	NS	NS	NS	
GLU-150 vs. GLU-075	NS	NS	NS	NS	

Statistical significance: Unadjusted P value (critical Level) NS = Not significant

Table 5.44: Summary results for mitochondrial membrane potential - glucose study

Final Concentration of Added Glucose	Mitochondrial membrane potential (JC-1 Red/Green MFI)				
	Day 2	Day 3	Day 6	Day 8	Day 10
0 mmol/L	5.98 ± 1.15	4.15 ± 0.59	3.18 ± 0.13	3.01 ± 0.06	2.95 ± 0.10
7.5 mmol/L	8.66 ± 1.23	8.34 ± 0.74	5.64 ± 1.14	3.30 ± 0.16	3.17 ± 0.06
15 mmol/L	8.73 ± 1.53	8.46 ± 0.61	5.74 ± 1.57	5.00 ± 1.53	3.67 ± 1.02
30 mmol/L	9.03 ± 1.01	7.90 ± 1.30	6.67 ± 0.71	5.65 ± 1.76	4.73 ± 1.39

Mean ± SD; n=5

Intracellular free calcium

Intracellular free calcium levels were similar on days 2 and 3, with no statistically significant differences evident between the four test groups (p=0.745 on day 2 and p=0.929 on day 3) (table 5.45). Beyond this time point levels deviated (figure 5.23). They remained stable in units with 30 mmol/L throughout the storage period. In units with 15 mmol/L added glucose, levels remained similarly stable until day 8, increasing to a mean ratio of 0.46 ± 0.17 on day 10 from a mean of 0.34 ± 0.05 on day 8. A similar increase was observed earlier in the storage period in units with 7.5 mmol/L added glucose, with day 8 levels increasing to a mean of 0.52 ± 0.9 on day 8 from a mean ratio of 0.35 ± 0.05 on day 6 (table 5.46). Levels in this last test group continued to increase, with no statistically significant differences observed between this test group and units lacking added glucose on either day 8 or 10 (table 5.45). Of note is that the numerical values for the ratios of Fluo-4/Fura-Red observed in this study were markedly lower than the levels observed with the addition of the calcium ionophore A23187 (14.95 ± 3.41 in units with no added glucose on day 10) and suggest that the release of calcium into the cytosol was relatively low.

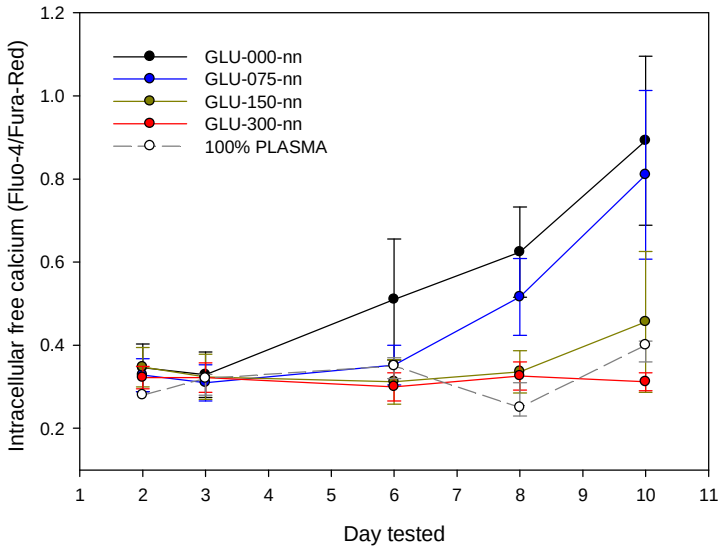


Figure 5.23: Intracellular free calcium
(glucose study; mean ± SD, n=5; 100% plasma n=3)

Table 5.45: Statistical comparison for intracellular free calcium – glucose study

Comparison Between Groups					
	Day 2	Day 3	Day 6	Day 8	Day 10
One Way ANOVA	0.745	0.929	0.003	<0.001	<0.001
Multiple Pairwise Comparison (Holm-Sidak method)					
Comparison	Day 6		Day 8		Day 10
GLU-300 vs. GLU-000	0.001 (0.009)		<0.001 (0.009)		<0.001 (0.009)
GLU-150 vs. GLU-000	0.002 (0.010)		<0.001 (0.010)		0.001 (0.013)
GLU-075 vs. GLU-000	0.008 (0.013)		NS		NS
GLU-300 vs. GLU-075	NS		0.001 (0.013)		<0.001 (0.010)
GLU-300 vs. GLU-150	NS		NS		NS
GLU-150 vs. GLU-075	NS		0.002 (0.017)		0.004 (0.017)

Statistical significance: Unadjusted P value (critical Level) NS = Not significant

Table 5.46: Summary results for intracellular free calcium - glucose study

Final Concentration of Added Glucose	Intracellular free calcium (Fluo-4/FuraRed)				
	Day 2	Day 3	Day 6	Day 8	Day 10
0 mmol/L	0.35 ± 0.06	0.33 ± 0.05	0.51 ± 0.15	0.62 ± 0.11	0.89 ± 0.20
7.5 mmol/L	0.33 ± 0.04	0.31 ± 0.04	0.35 ± 0.05	0.52 ± 0.09	0.81 ± 0.20
15 mmol/L	0.35 ± 0.05	0.32 ± 0.05	0.31 ± 0.05	0.34 ± 0.05	0.46 ± 0.17
30 mmol/L	0.32 ± 0.03	0.32 ± 0.04	0.30 ± 0.03	0.33 ± 0.03	0.31 ± 0.02

Mean ±SD; n=5

A more detailed examination of the flow cytometry histograms revealed a distinct sub-population of platelets was responsible for the overall increase in the Fluo-4/FuraRed ratios, and that this sub-population failed to respond to the addition of A23187 (figure 5.24).

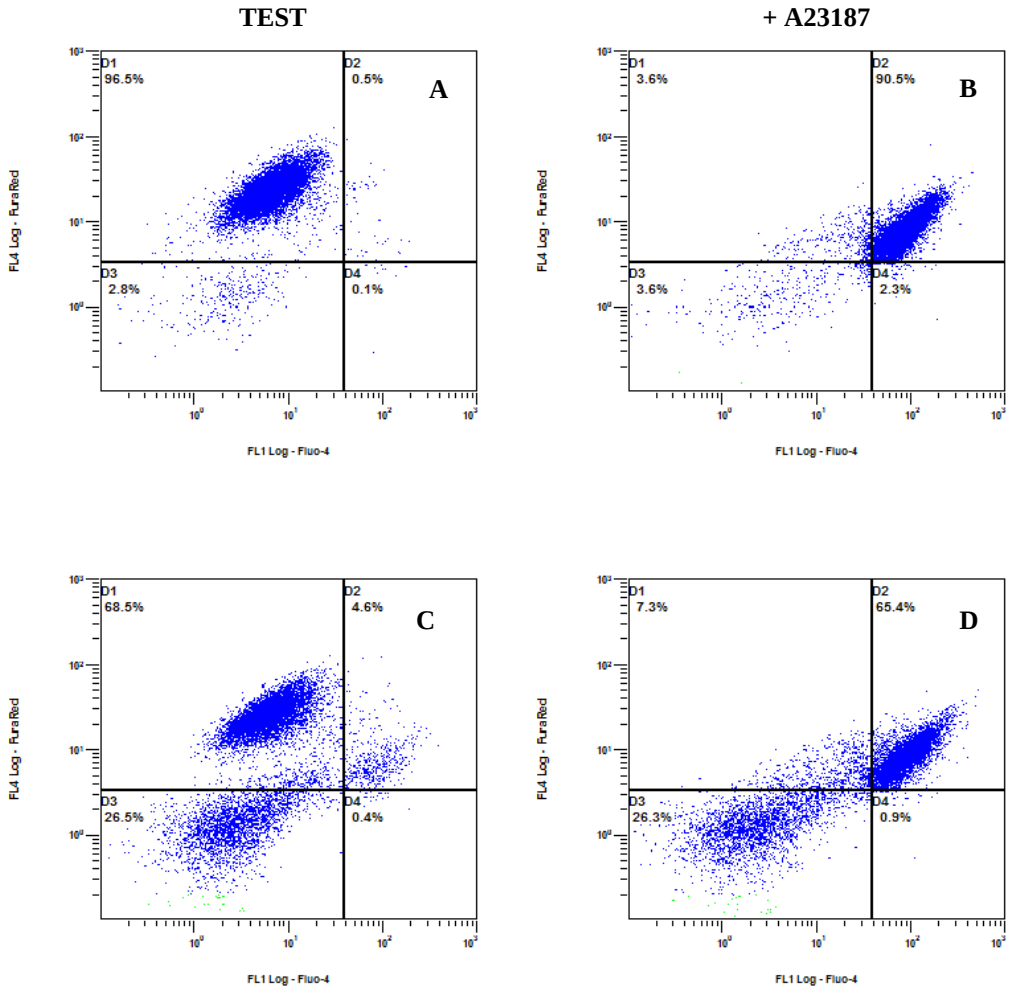


Figure 5.24: Staining characteristics of platelets incubated with Fluo-4 and FuraRed in a representative unit with no added glucose. A) Day 2 platelets showing minimal uptake of the dyes compared with B) which displays the response following the addition of 5 µL of 2 mM A23187. C + D) Histograms of platelets from the same unit on day 10 showing a sub-population of platelets in D3 with substantially reduced fluorescence from FuraRed and a lack of response to the addition of the calcium ionophore.

DISCUSSION

The depletion of glucose coincided with an increase in annexin V binding (% positive), as well as a decrease in ATP and platelet concentration (the last indicating platelet disruption), with a statistically significant loss of platelets with ATP levels below 2 µmol/10^{11}plts ($p<0.001$). The observed increase in soluble CD62P followed this timeline and may reflect platelet disruption in addition to shedding of surface of CD62P. The aminophospholipid translocase believed to be responsible for preferentially maintaining PE and PS on the inner leaflet of the plasma membrane is ATP-dependent (Zwaal and Schroit, 1997). Thus, a loss of translocase activity as ATP levels declined could at least partly explain the increase in phospholipid expression with storage. The steep rise in annexin V binding as glucose stores are depleted was coincident with increased levels of intracellular free calcium, though quantitatively the increases may be small and may not attain the micromolar concentrations that have been reported to accelerate the adverse effect on the translocase (Zwaal and Schroit, 1997). The increase in intracellular free calcium may also be directly related to the decline in ATP, since calcium-regulatory channels such as the sarco/endoplasmic Ca^{2+} ATPase (SERCA) and the plasma membrane Ca^{2+} ATPases (PMCA) are ATP-dependent (Varga-Szabo et al., 2009).

The synthesis of ATP is a critical role of mitochondria, driven by the electron transport chain on the inner mitochondrial membrane. Disruption of the proton motive force would result in depolarisation of the membrane potential, regarded in some studies as an early event in apoptosis (Mignotte and Vayssiere, 1998). The mitochondrial membrane potential ($\Delta\Psi m$) was observed to decline from the start of the storage period in all units, indicating impaired function of the organelle with storage, irrespective of the glucose concentration. In addition, however, there were significantly lower levels in units lacking glucose. The results suggest a relationship between the presence of glucose and improved mitochondrial function (though care is required in the interpretation of the results due to the relatively high variability between replicates).

The observations seemed consistent with the proposal that a process akin to apoptosis was a central component of the platelet storage lesion. However, the loss of ATP and cellular disruption coincident with the exhaustion of glucose stores is not consistentwith

the classical description of apoptosis, since this is an energy dependent process (Kung et al., 2011). A possible scenario is that platelets stored as concentrates in conditions where glucose is absent become progressively depleted in ATP, leading to the surface expression of aminophospholipids in a non-energy dependent process more reminiscent of necrosis.

HSR and ESC values in SAS-suspended PCs were low compared with PCs re-suspended in 100% plasma, as well as values reported in the literature for platelets stored in additive solutions containing potassium and magnesium (Gulliksson et al., 2002, VandenBroeke et al., 2004). A possible explanation may lie in the harsh PC processing method in combination with the low residual plasma levels, which may have activated the platelets prior to storage. A further factor may be the lack of calcium in the SAS, with Wagner et al reporting lower ESC and higher surface CD62P expression in platelets suspended in M-Sol without Ca^{2+}; an observation that concurs with the results in the present study (Wagner et al., 2010). The relatively high phosphate concentration may have also promoted glycolysis and may explain the relatively high glucose consumption rates compared to published results (Cardigan et al., 2008, Gulliksson et al., 2003).

CHAPTER 6. IMPACT ON PLATELET *IN VITRO* STORAGE CHARACTERISTICS OF THE INCLUSION OF ACETATE TO ADDITIVE SOLUTIONS

INTRODUCTION

Acetate is one of four reagents regarded by some authors as a beneficial, or even essential, component of platelet additive solutions (de Wildt-Eggen and Gulliksson, 2003). It is believed to function as a substrate for aerobic metabolism, and has been found to decrease the requirement for glucose in the suspending medium (Ringwald et al., 2006). It is also expected to act as a buffer since its entry into the tricarboxylic acid cycle requires a hydrogen ion (Murphy, 1999), thus resulting in the maintenance of a higher extracellular pH. In addition, it is known to reduce the production of lactate, possibly due to an inhibitory effect on phosphofructokinase – a rate-limiting enzyme in glycolysis (Murphy, 1995). The majority of publications on the impact of acetate on platelet characteristics have concentrated on the metabolic effects, with less emphasis placed on its role on other aspects of the platelet storage lesion. This study thus aimed to determine the impact of different concentrations of acetate on platelet activation and indicators of cell death.

All results are presented as mean ± standard deviation of 5 separate experiments, with statistical comparisons undertaken by a one way ANOVA. A p-value below 0.01 was considered statistically significant. Subsequent multiple comparisons were performed by the Holm-Sidak method, with an overall significance level of 0.05. The four test groups are:

ACE-000-nn (no acetate added)

ACE-014-nn (final concentration of 14 mmol/L)

ACE-028-nn (final concentration of 28 mmol/L)

ACE-056-nn (final concentration of 56 mmol/L)

(In each case, nn refers to a unique test number)

The graphs also show the median ± range of 3 replicate experiments performed with PC manufactured by the same method but re-suspended in 100% autologous plasma to provide a visual estimation of the possible impact of the harsh processing on the platelet *in vitro* characteristics.

RESULTS

Platelet Yield and Volume

The mean platelet yield and volume is reported in table 6.1, with all units meeting the UK Guidelines' specification for platelet yield ≥240 × 10^9 per unit (James, 2005). Volumes were comparable to those obtained in studies 2 and 3. No statistically significant differences were evident between any of the groups for either platelet yield (p=0.988) or volume (p=0.952). All units were successfully leucedepleted (residual WBC count <1×10^6/unit) and were confirmed negative for bacterial contamination at the end of storage.

Table 6.1: Platelet yield and unit volume prior to sampling

Treatment Group	Platelet Yield (×10^9/unit)	Volume (mL)
ACE-000-nn	286.2 ± 23.5	340.6 ± 3.1
ACE-014-nn	283.5 ± 23.1	340.2 ± 6.5
ACE-028-nn	282.1 ± 24.2	341.5 ± 4.7
ACE-056-nn	281.3 ± 20.7	340.1 ± 1.7

Data are presented as mean ± SD (n=5)

Function and Morphology

Platelet Concentration and Mean Platelet Volume

Platelet concentration decreased in all treatment groups with storage (figure 6.1). There was no statistically significant difference between groups at any time point (table 6.2). The loss of platelets was similar in all four test groups, with concentrations at the end of the storage period being 93.2 – 95.2% of day 2 levels (table 6.3). This loss was comparable to the decrease noted in conventionally manufactured platelet concentrates in plasma (92.8%) reported in chapter 3.

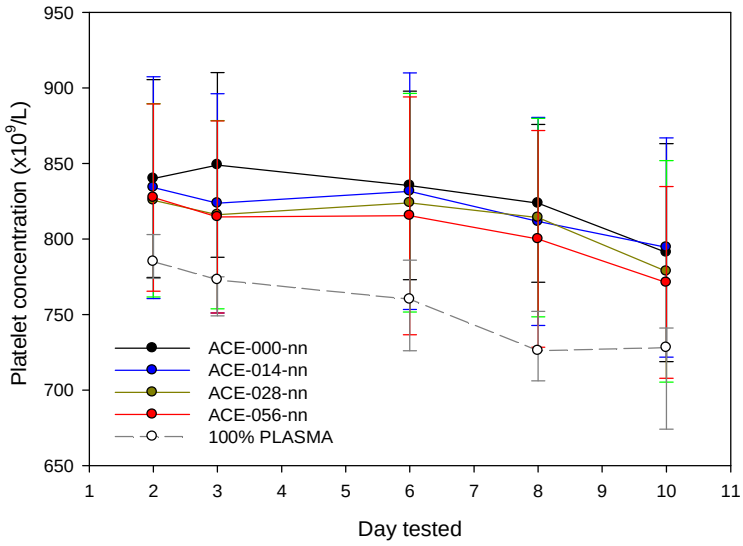


Figure 6.1: Platelet concentration (acetate study; mean, n=5; 100% plasma n=3)

Table 6.2: Statistical comparison for platelet concentration - acetate study

Comparison Between Groups (One Way ANOVA)					
	Day 2	Day 3	Day 6	Day 8	Day 10
One Way ANOVA	0.985	0.824	0.974	0.952	0.949

Table 6.3: Summary results for platelet concentration - acetate study

Final Concentration of Added Acetate	Platelet concentration (x10^9/L)				
	Day 2	Day 3	Day 6	Day 8	Day 10
0 mmol/L	840 ± 65	849 ± 61	835 ± 62	824 ± 52	791 ± 72
14 mmol/L	834 ± 73	846 ± 75	832 ± 78	812 ± 69	794 ± 73
28 mmol/L	826 ± 64	816 ± 62	824 ± 72	814 ± 66	779 ± 73
56 mmol/L	827 ± 62	815 ± 64	815 ± 79	800 ± 72	771 ± 63

Mean ± SD (n=5)

Day 2 values for mean platelet volume ranged from a mean of 7.9 ± 0.2 fL in units with no added acetate to 8.1 ± 0.2 fL in units with 56 mmol/L added acetate (p=0.444) (Table 6.5). MPV increased with storage in all test groups. The increase was greater in units with no added acetate and units with the highest concentration of acetate (56 mmol/L), with statistically significant differences between these units and the remaining two test groups on day 8 (p<0.001) (table 6.4). It should be noted, however, that the mean differences in MPV on day 10 between the test groups was a maximum of 0.5 fL but this was not associated with platelet disruption, as shown by the lack of significant differences in the platelet concentration.

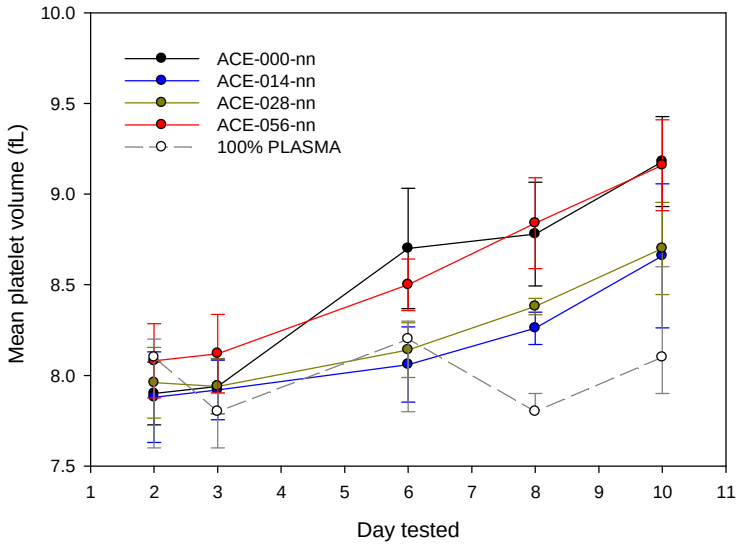


Figure 6.2: Mean platelet volume (acetate study; mean, n=5; 100% plasma n=3)

Table 6.4: Statistical comparison for mean platelet volume - acetate study

Comparison Between Groups					
	Day 2	Day 3	Day 6	Day 8	Day 10
One Way ANOVA	0.444	0.261	<0.001	<0.001	0.016
Multiple Pairwise Comparison (Holm-Sidak method)					
Comparison			Day 6		Day 8
ACE-056 vs. ACE-000			NS		NS
ACE-028 vs. ACE-000			0.001(0.010)		0.005 (0.017)
ACE-014 vs. ACE-000			<0.001(0.009)		<0.001 (0.010)
ACE-056 vs. ACE-014			0.006(0.013)		<0.001 (0.009)
ACE-056 vs. ACE-028			NS		0.002 (0.013)
ACE-028 vs. ACE-014			NS		NS

Statistical significance: Unadjusted P value (critical Level) NS = Not significant

Table 6.5: Summary results for mean platelet volume - acetate study

Final Concentration of Added Acetate	Mean platelet volume (fL)				
	Day 2	Day 3	Day 6	Day 8	Day 10
0 mmol/L	7.9 ± 0.2	7.9 ± 0.2	8.7 ± 0.3	8.8 ± 0.3	9. 2 ± 0.2
14 mmol/L	7.9 ± 0.2	7.9 ± 0.2	8.1 ± 0.2	8.3 ± 0.1	8.7 ± 0.4
28 mmol/L	8.0 ± 0.2	7.9 ± 0.2	8.1 ± 0.2	8.4 ± 0.0	8.7 ± 0.3
56 mmol/L	8.1 ± 0.2	8.1 ± 0.2	8.5 ± 0.1	8.8 ± 0.3	9.2 ± 0.3

Mean ± SD (n=5)

Swirling

Swirling at the end of the storage period was moderate to strong in all test groups. In units with no added acetate and 14 mmol/L acetate, swirling was at least moderate throughout the storage period. In units with 28 mmol/L and 56 mmol/L acetate, weak swirling was seen in some of the units on day 2 but improved over the storage period (table 6.6).

Table 6.6: Swirling - acetate study (individual results)

Final Concentration of Added Acetate (mmol/l)	TEST NUMBER	Swirling				
		DAY 2	DAY 3	DAY 6	DAY 8	DAY 10
0 mmol/l	ACE-000-01	3	2	3	3	3
	ACE-000-05	2	3	3	3	3
	ACE-000-09	2	2	3	3	3
	ACE-000-13	2	2	2	2	2
	ACE-000-17	2	3	2	2	2
14 mmol/l	ACE-014-02	2	2	3	3	3
	ACE-014-06	2	3	3	3	3
	ACE-014-10	2	2	3	3	3
	ACE-014-14	2	2	2	2	2
	ACE-014-18	2	2	2	2	2
28 mmol/l	ACE-028-03	2	2	2	3	3
	ACE-028-07	2	2	3	3	3
	ACE-028-11	2	2	3	3	3
	ACE-028-15	1	2	2	2	2
	ACE-028-19	1	1	2	2	2
56 mmol/l	ACE-056-04	2	2	2	3	3
	ACE-056-08	2	2	3	3	3
	ACE-056-12	1	1	2	3	3
	ACE-056-16	1	2	2	2	2
	ACE-056-20	1	1	2	2	2

Hypotonic Shock Response and Extent of Shape Change

Statistically significant differences in hypotonic shock responses were observed on day 2 between all the test groups with the exception of units with no added acetate and units with 14 mmol/L acetate ($p<0.001$). Mean day 2 levels in units with no acetate were 45.4 ± 4.7% compared to 16.5 ± 5.7% in units with 56 mmol/L acetate ($p<0.001$) (table 6.7). HSR in the latter test group remained stable throughout the storage period. The chart recordings from such low responses were virtually flat and suggested the limits of this assay were being reached. However, the responses in the test group with 28 mmol/L acetate were also observed to be fairly stable, reducing from a mean of 27.7 ± 9.2% on day 2 to 23.1 ± 3.2% on day 10 (83% of day 2 values) (table 6.8). By contrast, the responses in units with no acetate decreased to 63% of day 2 levels by end of storage (45.4 ± 4.7% to 28.4 ± 3.8% by day 10). The pattern suggests a dose-dependent effect of acetate on the hypotonic shock response occurred early during the storage period, prior to the onset of measurements (figure 6.3).

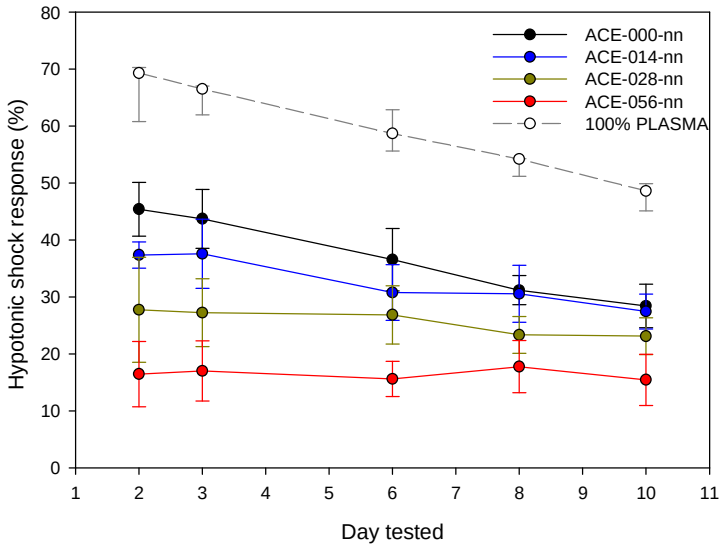


Figure 6.3: Hypotonic shock response (acetate study; mean ± SD, n=5; 100% plasma n=3)

Table 6.7: Statistical comparison of hypotonic shock response - acetate study

Comparison Between Groups					
	Day 2	Day 3	Day 6	Day 8	Day 10
One Way ANOVA	<0.001	<0.001	<0.001	<0.001	<0.001
Multiple Pairwise Comparison (Holm-Sidak method)					
Comparison	Day 2	Day 3	Day 6	Day 8	Day 10
ACE-056 vs. ACE-000	<0.001 (0.009)	<0.001 (0.009)	<0.001 (0.009)	<0.001 (0.009)	<0.001 (0.009)
ACE-028 vs. ACE-000	<0.001 (0.013)	<0.001 (0.013)	0.005 (0.017)	0.007 (0.013)	NS
ACE-014 vs. ACE-000	NS	NS	NS	NS	NS
ACE-056 vs. ACE-014	<0.001 (0.010)	<0.001 (0.010)	<0.001 (0.010)	<0.001 (0.010)	<0.001 (0.010)
ACE-056 vs. ACE-028	0.009 (0.017)	0.011 (0.025)	0.002 (0.013)	NS	0.005 (0.013)
ACE-028 vs. ACE-014	0.023 (0.025)	0.010 (0.017)	NS	0.012 (0.017)	NS

Statistical significance: Unadjusted P value (critical level) NS = Not significant

Table 6.8: Summary results for hypotonic shock response - acetate study

Final Concentration of Added Acetate	Hypotonic shock response (%)				
	Day 2	Day 3	Day 6	Day 8	Day 10
0 mmol/L	45.4 ± 4.7	43.7 ± 5.2	36.5 ± 5.5	31.2 ± 2. 6	28.4 ± 3.8
14 mmol/L	37.4 ± 2.3	37.6 ± 6.1	30.8 ± 4.9	30.5 ± 5.0	27.4 ± 3.1
28 mmol/L	27.7 ± 9.2	27.3 ± 5.9	26.9 ± 5.1	23.3 ± 3.3	23.1 ± 3.2
56 mmol/L	16.5 ± 5.7	17.0 ± 5.3	15.6 ± 3.1	17.8 ± 4.6	15.5 ± 4.5

Mean ± SD (n=5)

The same general pattern noted for hypotonic shock response was observed with extent of shape change (figure 6.4), although statistically significant differences were only present on day 3 between units with 56 mmol/L acetate and units with either 14 mmol/L acetate or no acetate (p=0.002) (table 6.9). Mean day 3 results in units with no added acetate were 19.1 ± 3.1% compared with 10.2 ± 4.7% for units with 56 mmol/L. The response decreased by day 10 to a mean of 11.8 ± 1.7% and 8.0 ± 2.5, respectively (p=0.041) (table 6.10).

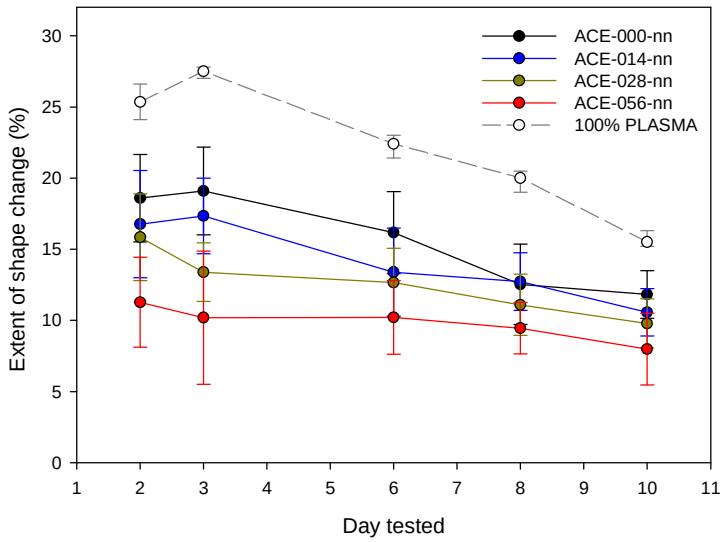


Figure 6.4: Extent of shape change (acetate study; mean ± SD, n=5; 100% plasma n=3)

Table 6.9: Statistical comparison for extent of shape change - acetate study

Comparison Between Groups					
	Day 2	Day 3	Day 6	Day 8	Day 10
One Way ANOVA	0.018	0.002	0.027	0.115	0.041
Multiple Pairwise Comparison (Holm-Sidak method)					
Comparison			Day 3		
ACE-056 vs. ACE-000			<0.001 (0.009)		
ACE-028 vs. ACE-000			NS		
ACE-014 vs. ACE-000			NS		
ACE-056 vs. ACE-014			0.003 (0.010)		
ACE-056 vs. ACE-028			NS		
ACE-028 vs. ACE-014			NS		

Statistical significance: Unadjusted P value (critical level) NS = Not significant

Table 6.10: Summary results for extent of shape change - acetate study

Final Concentration of Added Acetate	Extent of shape change (%)				
	Day 2	Day 3	Day 6	Day 8	Day 10
0 mmol/L	18.6 ± 3.1	19.1 ± 3.1	16.2 ± 2.9	12.5 ± 2. 8	11.8 ± 1.7
14 mmol/L	16.8 ± 3.8	17.3 ± 2.7	13.4 ± 3.1	12.7 ± 2.0	10.6 ± 1.7
28 mmol/L	15.8 ± 3.1	13.4 ± 2.1	12.7 ± 2.4	11.1 ± 2.2	9.8 ± 1 .7
56 mmol/L	11.3 ± 3.2	10.2 ± 4.7	10.2 ± 2.6	9.5 ± 1.8	8.0 ± 2. 5

Mean ± SD (n=5)

Platelet Metabolism

Extracellular pH (37°C)

$pH_{(37°C)}$ levels in all test groups were similar on day 2 (p=0.198), with mean levels ranging between 7.252 ± 0.011 and 7.275 ± 0.015 in units with 0 mmol/L and 28 mmol/L acetate, respectively (table 6.12). Levels subsequently decreased with storage in all test groups, with the rate of decrease significantly greater in the test group lacking acetate (figure 6.5). Statistically significant differences were present between these units and units with added acetate on days 3 (p=0.002), 6 and 8 (p<0.001 for both days), with $pH_{37°C}$ in units lacking acetate reaching a minimum level of 6.817 ± 0.050 on day 8. Figure 6.5 suggests that in units with added acetate, $pH_{37°C}$ decreased more rapidly in units with 56 mmol/L acetate compared with the remaining two test groups, with statistically significant differences between units with 56 mmol/L and 28 mmol/L acetate on day 8 (mean levels of 6.999 ± 0.042 and 7.080 ± 0.026, respectively; p<0.001) and between units with 56 mmol/L and 14 mmol/L acetate on day 10 (mean levels of 6.902 ± 0.061 and 6.994 ± 0.028, respectively; p=0.001) (table 6.11). The increase in $pH_{37°C}$ in units lacking acetate observed after day 8 may be attributable to the depletion of glucose stores in these units (figure 6.6).

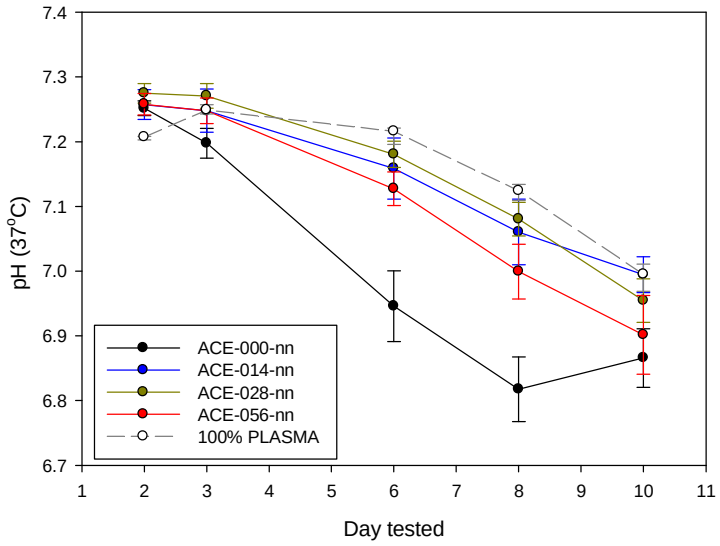


Figure 6.5: pH (37°C) (acetate study; mean ± SD, n=5; 100% plasma n=3)

Table 6.11: Statistical comparison for pH (37°C) - acetate study

Comparison Between Groups					
	Day 2	Day 3	Day 6	Day 8	Day 10
One Way ANOVA	0.198	0.002	<0.001	<0.001	0.001
Multiple Pairwise Comparison (Holm-Sidak method)					
Comparison	Day 3	Day 6	Day 8	Day 10	
ACE-056 vs. ACE-000	0.005 (0.013)	<0.001 (0.013)	<0.001 (0.013)	NS	
ACE-028 vs. ACE-000	<0.001 (0.009)	<0.001 (0.009)	<0.001 (0.009)	0.005 (0.013)	
ACE-014 vs. ACE-000	0.005 (0.010)	<0.001 (0.010)	<0.001 (0.010)	<0.001 (0.009)	
ACE-056 vs. ACE-014	NS	NS	NS	0.004 (0.010)	
ACE-056 vs. ACE-028	NS	NS	0.010 (0.017)	NS	
ACE-028 vs. ACE-014	NS	NS	NS	NS	

Statistical significance: Unadjusted P value (critical level) NS = Not significant

Table 6.12: Summary results for pH (37°C) - acetate study

Final Concentration of Added Acetate	pH (37°C)				
	Day 2	Day 3	Day 6	Day 8	Day 10
0 mmol/L	7.252 ± 0.011	7.198 ± 0.023	6.946 ± 0.055	6.817 ± 0.050	6.866 ± 0.045
14 mmol/L	7.257 ± 0.023	7.248 ± 0.033	7.159 ± 0.047	7.060 ± 0.051	6.994 ± 0.028
28 mmol/L	7.275 ± 0.015	7.271 ± 0.019	7.180 ± 0.020	7.080 ± 0.026	6.955 ± 0.034
56 mmol/L	7.258 ± 0.017	7.248 ± 0.020	7.127 ± 0.026	6.999 ± 0.042	6.902 ± 0.061

Mean ± SD (n=5)

Glucose and Lactate Levels

Glucose levels decreased at approximately the same rate in all units containing acetate, with no statistically significant differences between these three test groups at any point during storage (table 6.13). Levels on day 2 ranged from 10.5 ± 1.4 mmol/L in units with 14 mmol/L acetate to 12.1 ± 0.7 mmol/L in units with 28 mmol/L acetate. By the end of storage, glucose stores had been essentially depleted in units with 56 mmol/L acetate (0.1 ± 0.3 mmol/L). In units with 14 mmol/L and 28 mmol/L acetate, day 10 glucose levels were 0.9 ± 1.2 and 1.4 ± 0.8 mmol/L, respectively (table 6.14). In the units lacking acetate glucose was metabolised at a faster rate, with stores depleted by day 8 and a statistically significant difference between these units and the three test groups with added acetate ($p<0.001$) (table 6.13).

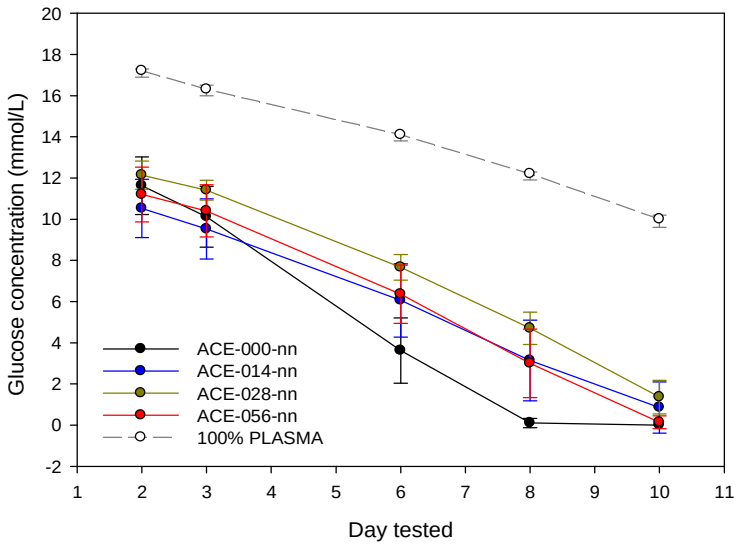


Figure 6.6: Glucose levels (acetate study; mean ± SD, n=5; 100% plasma n=3))

Table 6.13: Statistical comparison for glucose concentration - acetate study

Comparison Between Groups					
	Day 2	Day 3	Day 6	Day 8	Day 10
One Way ANOVA	0.250	0.155	0.003	<0.001	0.040
Multiple Pairwise Comparison (Holm-Sidak method)					
Comparison		Day 6		Day 8	
ACE-056 vs. ACE-000		0.008 (0.010)		0.004 (0.013)	
ACE-028 vs. ACE-000		<0.001 (0.009)		<0.001 (0.009)	
ACE-014 vs. ACE-000		NS		0.003 (0.010)	
ACE-056 vs. ACE-014		NS		NS	
ACE-056 vs. ACE-028		NS		NS	
ACE-028 vs. ACE-014		NS		NS	

Statistical significance: Unadjusted P value (critical level) NS = Not significant

Table 6.14: Summary results for glucose concentration - acetate study

Final Concentration of Added Acetate	Glucose concentration (mmol/L)				
	Day 2	Day 3	Day 6	Day 8	Day 10
0 mmol/L	11.6 ± 1.4	10.1 ± 1.5	3.6 ± 1.6	0.1 ± 0.2	0.0 ± 0.0
14 mmol/L	10.5 ± 1.4	9.5 ± 1.5	6.1 ± 1.8	3.1 ± 2.0	0.9 ± 1.2
28 mmol/L	12.1 ± 0.7	11.4 ± 0.5	7.7 ± 0.6	4.7 ± 0.8	1.4 ± 0.8
56 mmol/L	11.2 ± 1.3	10.4 ± 1.3	6.4 ± 1.4	3.0 ± 1.7	0.1 ± 0.3

Mean ± SD (n=5)

The pattern of glucose utilisation described above was mirrored in the levels of lactate, which increased at a faster rate in the test group lacking acetate. Lactate levels in these units reached a maximum of 22 ± 3 mmol/L on day 8, before decreasing marginally after the depletion of glucose (figure 6.7). In the three test groups with added acetate, lactate levels increased throughout storage, with no statistically significant differences at any time point (table 6.15). In contrast, statistically significant differences were observed between units with no exogenous acetate and the three test groups with added acetate on days 2 to 8 ($p<0.001$ to 0.001). By day 10 the cessation of lactate production in the former due to the depletion of glucose resulted in similar values for lactate concentration in all test groups ($p=0.541$) (table 6.15).

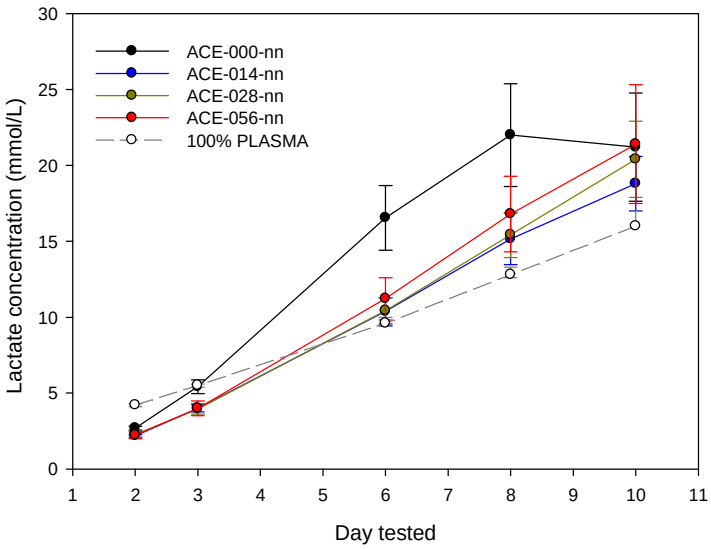


Figure 6.7: Lactate levels (acetate study; mean ± SD, n=5; 100% plasma n=3)

Table 6.15: Statistical comparison for lactate concentration - acetate study

Comparison Between Groups					
	Day 2	Day 3	Day 6	Day 8	Day 10
One Way ANOVA	<0.001	<0.001	<0.001	0.001	0.541
Multiple Pairwise Comparison (Holm-Sidak method)					
Comparison	Day 2	Day 3	Day 6	Day 8	
ACE-056 vs. ACE-000	<0.001 (0.010)	<0.001 (0.010)	<0.001 (0.013)	0.003 (0.009)	
ACE-028 vs. ACE-000	0.001 (0.013)	<0.001 (0.009)	<0.001 (0.010)	<0.001 (0.010)	
ACE-014 vs. ACE-000	<0.001 (0.009)	<0.001 (0.013)	<0.001 (0.009)	<0.001 (0.009)	
ACE-056 vs. ACE-014	NS	NS	NS	NS	
ACE-056 vs. ACE-028	NS	NS	NS	NS	
ACE-028 vs. ACE-014	NS	NS	NS	NS	

Statistical significance: Unadjusted P value (critical level) NS = Not significant

Table 6.16: Summary results for lactate concentration - acetate study

Final Concentration of Added Acetate	Lactate concentration (mmol/L)				
	Day 2	Day 3	Day 6	Day 8	Day 10
0 mmol/L	2.7 ± 0.1	5.4 ± 0.5	16.5 ± 2.1	22 ± 3	21 ± 4
14 mmol/L	2.2 ± 0.1	4.0 ± 0.2	10.4 ± 0.9	15 ± 2	19 ± 2
28 mmol/L	2.3 ± 0.2	4.0 ± 0.3	10.4 ± 0.9	15 ± 1	20 ± 3
56 mmol/L	2.2 ± 0.2	4.0 ± 0.5	11.2 ± 1.4	17 ± 2	21 ± 4

Mean ± SD (n=5)

Glucose consumption and lactate production confirm that glucose stores were depleted more rapidly in units lacking acetate (figures 6.8 and 6.9). Rates in these units reached a maximum between days 3 to 6 (2.57 ± 0.14 mmol/day/10^{12}plts for glucose consumption; 4.39 ± 0.42 mmol/day/10^{12}plts for lactate production) (tables 6.18 and 6.20). Beyond day 6 rates decreased, with minimal production of lactate between days 8 and 10 coinciding with the depletion of glucose stores. Statistically significant differences in glucose consumption between units lacking acetate and the three test groups with added acetate were observed between days 2 to 3, 3 to 6 and between days 8 to 10 ($p<0.001$ in all cases) (table 6.17). Similar differences were found in lactate production (table 6.21). No significant differences were evident between the three test groups containing exogenous acetate.

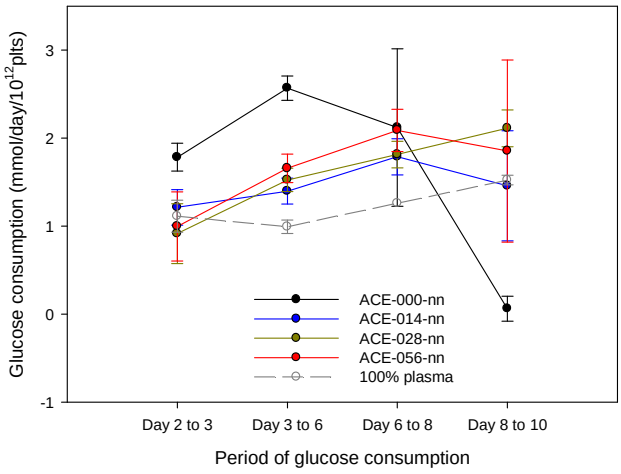


Figure 6.8: Glucose consumption (acetate study; mean ± SD, n=5; 100% plasma n=3)

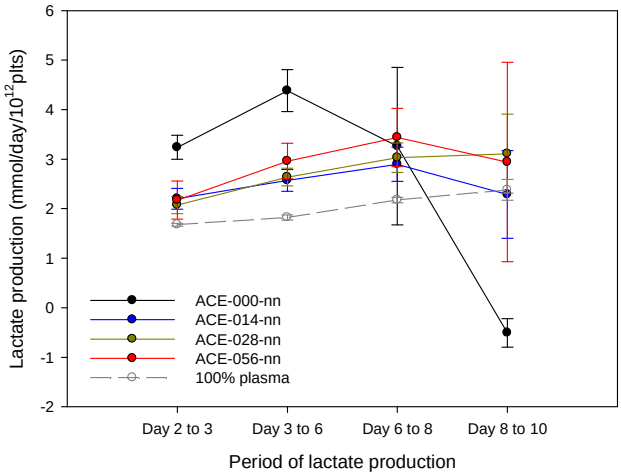


Figure 6.9: Lactate production (acetate study; mean ± SD, n=5; 100% plasma n=3)

Table 6.17: Statistical comparison for glucose consumption - acetate study

Comparison Between Groups				
	Day 2-3	Day 3-6	Day 6-8	Day 8-10
One Way ANOVA	<0.001	<0.001	0.580	<0.001
Multiple Pairwise Comparison (Holm-Sidak method)				
Comparison	Day 2-3	Day 3-6	Day 8-10	
ACE-056 vs. ACE-000	<0.001 (0.002)	<0.001 (0.003)	<0.001 (0.002)	
ACE-028 vs. ACE-000	<0.001 (0.002)	<0.001 (0.002)	<0.001 (0.002)	
ACE-014 vs. ACE-000	NS	<0.001 (0.002)	NS	
ACE-056 vs. ACE-014	NS	NS	NS	
ACE-056 vs. ACE-028	NS	NS	NS	
ACE-028 vs. ACE-014	NS	NS	NS	

Statistical significance: Unadjusted P value (critical level) NS = Not significant

Table 6.18: Summary results for glucose consumption - acetate study

Final Concentration of Added Acetate	Glucose consumption (mmol/day/10^{12} plts)			
	Day 2-3	Day 3-6	Day 6-8	Day 8-10
0 mmol/L	1.78 ± 0.16	2.57 ± 0.14	2.12 ± 0.89	0.06 ± 0.14
14 mmol/L	1.21 ± 0.20	1.40 ± 0.15	1.79 ± 0.21	1.46 ± 0.63
28 mmol/L	0.92 ± 0.34	1.53 ± 0.13	1.81 ± 0.15	2.11 ± 0.21
56 mmol/L	1.00 ± 0.39	1.66 ± 0.16	2.09 ± 0.24	1.85 ± 1.03

Mean ± SD (n=5)

Table 6.19: Statistical comparison for lactate production - acetate study

Comparison Between Groups				
	Day 2-3	Day 3-6	Day 6-8	Day 8-10
One Way ANOVA	<0.001	<0.001	0.772	<0.001
Multiple Pairwise Comparison (Holm-Sidak method)				
Comparison	Day 2-3	Day 3-6	Day 8-10	
ACE-056 vs. ACE-000	<0.001 (0.002)	<0.001 (0.003)	<0.001 (0.002)	
ACE-028 vs. ACE-000	<0.001 (0.002)	<0.001 (0.002)	<0.001 (0.002)	
ACE-014 vs. ACE-000	<0.001 (0.003)	<0.001 (0.002)	0.002 (0.003)	
ACE-056 vs. ACE-014	NS	NS	NS	
ACE-056 vs. ACE-028	NS	NS	NS	
ACE-028 vs. ACE-014	NS	NS	NS	

Statistical significance: Unadjusted P value (critical level) NS = Not significant

Table 6.20: Summary results for lactate production – acetate study

Final Concentration of Added Acetate	Lactate production (mmol/day/10^{12} plts)			
	Day 2-3	Day 3-6	Day 6-8	Day 8-10
0 mmol/L	3.24 ± 0.24	4.39 ± 0.42	3.26 ± 1.59	-0.61 ± 0.29
14 mmol/L	2.20 ± 0.21	2.57 ± 0.22	2.90 ± 0.34	2.29 ± 0.89
28 mmol/L	2.07 ± 0.18	2.64 ± 0.18	3.03 ± 0.30	3.11 ± 0.80
56 mmol/L	2.18 ± 0.38	2.96 ± 0.36	3.44 ± 0.59	2.94 ± 2.01

Mean ± SD (n=5)

Bicarbonate Levels

Bicarbonate levels on day 2 ranged from 14.83 ± 1.77 mmol/L in units with 14 mmol/L acetate to 16.22 ± 0.38 mmol/L in units with 28 mmol/L acetate (table 6.22), with no statistically significant differences between any of the test groups (p=0.315) on days 2 and 3 (p=0.308). Beyond day 3, bicarbonate levels decreased at a faster rate in units with no added acetate, with statistically significant differences between these units and the test groups with acetate at all remaining time points (p<0.001 in all cases) (figure 6.10), reflecting the more acute consumption of glucose in the test group lacking acetate. Bicarbonate levels in units with no exogenous acetate reached a minimum on day 10 of 2.63 ± 0.24 mmol/L compared to 5.79 ± 0.91 mmol/L in units with 28 mmol/L acetate (table 6.22). There was a suggestion that bicarbonate was more rapidly consumed in units with 56 mmol/L compared with the other two test groups containing acetate, with statistically significant differences on day 10 against the test groups with 14 and 28 mmol/L added acetate (table 6.21).

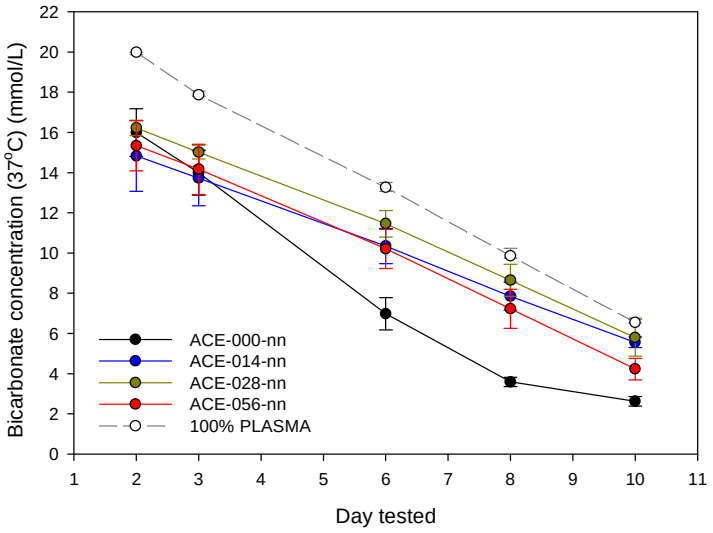


Figure 6.10: Bicarbonate concentration (37°C) (acetate study; mean ± SD, n=5; 100% plasma n=3)

Table 6.21: Statistical comparison for bicarbonate concentration - acetate study

Comparison Between Groups					
	Day 2	Day 3	Day 6	Day 8	Day 10
One Way ANOVA	0.315	0.308	<0.001	<0.001	<0.001
Multiple Pairwise Comparison (Holm-Sidak method)					
Comparison	Day 6		Day 8		Day 10
ACE-056 vs. ACE-000	<0.001 (0.013)		<0.001 (0.013)		<0.001 (0.013)
ACE-028 vs. ACE-000	<0.001 (0.009)		<0.001 (0.009)		<0.001 (0.009)
ACE-014 vs. ACE-000	<0.001 (0.010)		<0.001 (0.010)		<0.001 (0.010)
ACE-056 vs. ACE-014	NS		NS		0.002 (0.025)
ACE-056 vs. ACE-028	NS		0.007 (0.017)		<0.001 (0.017)
ACE-028 vs. ACE-014	NS		NS		NS

Statistical significance: Unadjusted P value (critical level) NS = Not significant

Table 6.22: Summary results for bicarbonate concentration - acetate study

Final Concentration of Added Acetate	Bicarbonate concentration (mmol/L)				
	Day 2	Day 3	Day 6	Day 8	Day 10
0 mmol/L	15.99 ± 1.19	14.00 ± 1.12	6.98 ± 0.80	3.59 ± 0.23	2.63 ± 0.24
14 mmol/L	14.83 ± 1.77	13.72 ± 1.36	10.35 ± 0.87	7.85 ± 0.69	5.56 ± 0.26
28 mmol/L	16.22 ± 0.38	15.02 ± 0.34	11.46 ± 0.66	8.65 ± 0.79	5.79 ± 0.91
56 mmol/L	15.34 ± 1.25	14.17 ± 1.25	10.21 ± 0.97	7.23 ± 0.97	4.23 ± 0.53

Mean ± SD (n=5)

Blood Gases

Partial pressures of oxygen increased steadily in units with added acetate throughout the storage period, from day 2 pressures of approximately 13.5 kPa to day 10 pressures ranging from 17.5 ± 1.5 kPa in units with 14 mmol/L acetate to 19.3 ± 1.6 kPa in units with 56 mmol/L acetate (table 6.24). However, no statistically significant differences were evident between any of these three test groups at any time point (table 6.23). Day 2 pO_2 mean values in units lacking acetate were higher than in the test groups containing acetate (16.2 ± 1.4 kPa compared with 13.5 kPa), though this did not result in a statistically significant difference (p=0.042). Partial pressures continued to increase and reached a maximum on day 6 (19.3 ± 1.0 kPa), with statistically significant differences evident between units with no added acetate and all three test groups containing acetate (p=0.002) (table 6.23, figure 6.11). By the end of storage, no statistically significant differences were observed between any of the test groups (p=0.116).

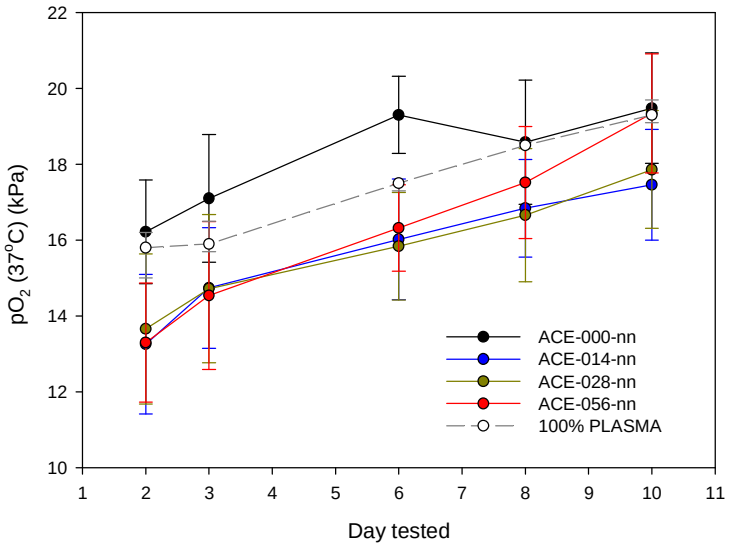


Figure 6.11: pO_2 (37°C) (acetate study; mean ± SD , n=5; 100% plasma n=3)

Table 6.23: Statistical comparison for pO_2 - acetate study

Comparison Between Groups					
	Day 2	Day 3	Day 6	Day 8	Day 10
One Way ANOVA	0.042	0.117	0.002	0.234	0.116
Multiple Pairwise Comparison (Holm-Sidak method)					
Comparison	Day 6				
ACE-056 vs. ACE-000	0.002 (0.013)				
ACE-028 vs. ACE-000	<0.001 (0.009)				
ACE-014 vs. ACE-000	0.001 (0.010)				
ACE-056 vs. ACE-014	NS				
ACE-056 vs. ACE-028	NS				
ACE-028 vs. ACE-014	NS				

Statistical significance: Unadjusted P value (critical level) NS = Not significant

Table 6.24: Summary results for pO_2 (37°C) - acetate study

Final Concentration of Added Acetate	pO_2 (37°C) (kPa)				
	Day 2	Day 3	Day 6	Day 8	Day 10
0 mmol/L	16.2 ± 1.4	17.1 ± 1.7	19.3 ± 1.0	18.6 ± 1. 6	19.5 ± 1.5
14 mmol/L	13.3 ± 1.8	14.7 ± 1.6	16.0 ± 1.6	16.8 ± 1.3	17.5 ± 1.5
28 mmol/L	13.7 ± 2.0	14.7 ± 2.0	15.8 ± 1.4	16.7 ± 1.8	17.9 ± 1.6
56 mmol/L	13.3 ± 1.6	14.5 ± 1.9	16.3 ± 1.1	17.5 ± 1.5	19.3 ± 1.6

Mean ± SD (n=5)

In units containing added acetate, pCO_2 decreased gradually with storage from day 2 levels between 4.03 ± 0.27 kPa to 4.25 ± 0.13 kPa in units with 14 and 28 mmol/L acetate, respectively, to end of storage values between 2.66 ± 0.58 kPa and 3.16 ± 0.35 kPa in units with 56 and 28 mmol/L acetate, respectively (table 6.26). There were no statistically significant differences between any of the groups with acetate at any time during storage (table 6.25). Units with no added acetate had starting pCO_2 values similar to the other three test groups (p=0.115). Beyond day 6, pCO_2 decreased faster compared to the other test groups, resulting in day 10 values of 1.77 ± 0.18 kPa and statistically significant differences compared to units containing acetate (p<0.001) (table 6.25) (figure 6.12). This decrease in pCO_2 coincided with the decrease in pO_2 between days 6 and 8 and the decrease in the rate of glucose consumption.

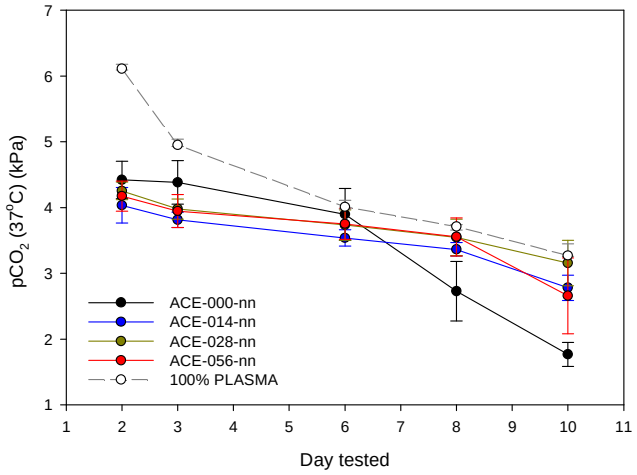


Figure 6.12: pCO_2 (37°C) (acetate study; mean ± SD, n=5; 100% plasma n=3)

Table 6.25: Statistical comparison for pCO_2 - acetate study

Comparison Between Groups					
	Day 2	Day 3	Day 6	Day 8	Day 10
One Way ANOVA	0.115	0.007	0.238	0.002	<0.001
Multiple Pairwise Comparison (Holm-Sidak method)					
Comparison	Day 3		Day 8		Day 10
ACE-056 vs. ACE-000	0.009 (0.010)		<0.001 (0.009)		0.001 (0.013)
ACE-028 vs. ACE-000	NS		<0.001 (0.010)		<0.001 (0.009)
ACE-014 vs. ACE-000	0.001 (0.009)		0.005 (0.013)		<0.001 (0.010)
ACE-056 vs. ACE-014	NS		NS		NS
ACE-056 vs. ACE-028	NS		NS		NS
ACE-028 vs. ACE-014	NS		NS		NS

Statistical significance: Unadjusted P value (critical level) NS = Not significant

Table 6.26: Summary results for pCO_2 (37°C) - acetate study

Final Concentration of Added Acetate	pCO_2 (37°C) (kPa)				
	Day 2	Day 3	Day 6	Day 8	Day 10
0 mmol/L	4.42 ± 0.28	4.38 ± 0.33	3.90 ± 0.39	2.73 ± 0.45	1.77 ± 0.18
14 mmol/L	4.03 ± 0.27	3.81 ± 0.12	3.54 ± 0.12	3.36 ± 0.10	2.78 ± 0.19
28 mmol/L	4.25 ± 0.13	3.98 ± 0.15	3.73 ± 0.23	3.54 ± 0.28	3.16 ± 0.35
56 mmol/L	4.18 ± 0.23	3.95 ± 0.25	3.75 ± 0.24	3.55 ± 0.29	2.66 ± 0.58

Mean ± SD (n=5)

Oxygen consumption correlated with the results for partial pressures of oxygen, with the significantly lower rates in the treatment group lacking acetate corresponding to the increased retention of oxygen in these units (figure 6.13). Statistically significant differences between units lacking acetate and the three test groups with acetate were obtained on days 2 ($p=0.009$) and day 6 ($p<0.001$), with mean rates at these time points of 0.19 ± 0.02 and 0.12 ± 0.02 mmol/min/10^9plts, respectively, for the test group lacking acetate (table 6.28). By day 8, there were no statistically significant differences between any of the four test groups ($p=0.081$) (table 6.27).

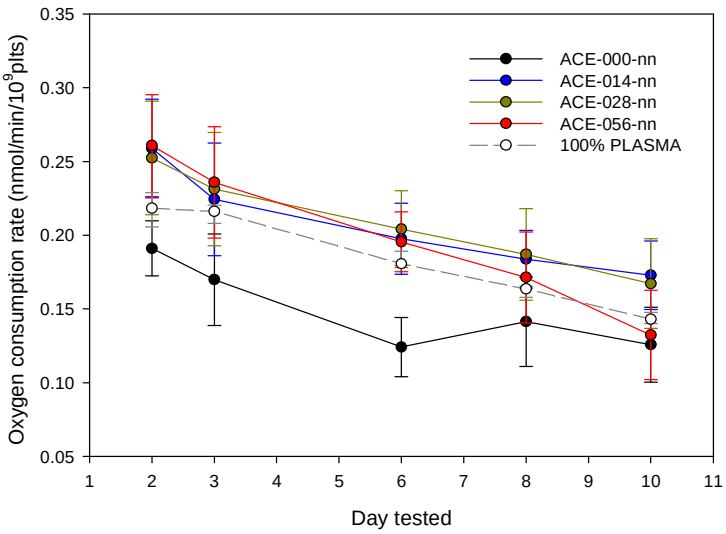


Figure 6.13: Oxygen consumption rate (acetate study; mean ± SD, n=5; 100% plasma n=3)

Table 6.27: Statistical comparison for oxygen consumption rate - acetate study

Comparison Between Groups					
	Day 2	Day 3	Day 6	Day 8	Day 10
One Way ANOVA	0.009	0.039	<0.001	0.081	0.032
Multiple Pairwise Comparison (Holm-Sidak method)					
Comparison		Day 2		Day 6	
ACE-056 vs. ACE-000		<0.001 (0.009)		<0.001 (0.013)	
ACE-028 vs. ACE-000		<0.001 (0.013)		<0.001 (0.009)	
ACE-014 vs. ACE-000		<0.001 (0.010)		<0.001 (0.010)	
ACE-056 vs. ACE-014		NS		NS	
ACE-056 vs. ACE-028		NS		NS	
ACE-028 vs. ACE-014		NS		NS	

Statistical significance: Unadjusted P value (critical level) NS = Not significant

Table 6.28: Summary results for oxygen consumption rate - acetate study

Final Concentration of Added Acetate	Oxygen consumption rate (nmol/min/10^9plts)				
	Day 2	Day 3	Day 6	Day 8	Day 10
0 mmol/L	0.19 ± 0.02	0.17 ± 0.03	0.12 ± 0.02	0.14 ± 0.03	0.13 ± 0.03
14 mmol/L	0.26 ± 0.03	0.22 ± 0.04	0.20 ± 0.02	0.18 ± 0.02	0.17 ± 0.02
28 mmol/L	0.25 ± 0.04	0.23 ± 0.04	0.20 ± 0.03	0.19 ± 0.03	0.17 ± 0.03
56 mmol/L	0.26 ± 0.03	0.24 ± 0.04	0.20 ± 0.02	0.17 ± 0.03	0.13 ± 0.03

Mean ± SD (n=5)

ATP and ADP Levels

Day 2 levels of ATP ranged from 5.22 ± 0.41 µmol/10^{11}plts in units with 56 mmol/L acetate to 5.48 ± 0.65 µmol/10^{11}plts in units lacking exogenous acetate, with no statistically significant differences between any of the test groups (p=0.876). In units lacking acetate, ATP increased to maximal levels on day 6 of 6.91 ± 0.68 µmol/10^{11}plts, with statistically significant differences between these units and the three test groups containing acetate (p<0.001) (table 6.29). Levels in this test group subsequently declined by day 10 to 5.15 ± 1.54 µmol/10^{11}plts (table 6.30), with a statistically significant difference only evident against units with 56 mmol/L acetate (p=0.006). ATP levels in units with 56 mmol/L acetate by day 10 had decreased to 2.65 ± 0.94 µmol/10^{11}plts compared to 4.02 ± 0.3 and 4.00 ± 0.22 µmol/10^{11}plts in units with 28 and 14 mmol/L acetate, respectively; though this difference was not statistically significant due to the relatively large error bars in the test group with 56 mmol/L (figure 6.14). By the end of storage, mean levels of ATP in units lacking acetate were 94% of day 2 levels. By contrast, mean levels in units with 56 mmol/L added acetate were 51% of levels on day 2 (table 6.30).

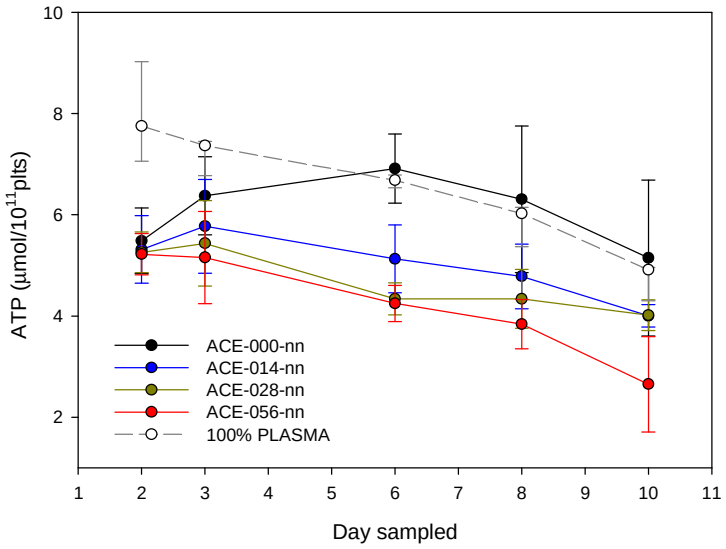


Figure 6.14: ATP levels (acetate study; mean ± SD, n=5; 100% plasma n=3)

Table 6.29: Statistical comparison of ATP levels between test groups - acetate study

Comparison Between Groups					
	Day 2	Day 3	Day 6	Day 8	Day 10
One Way ANOVA	0.876	0.182	<0.001	0.003	0.006
Multiple Pairwise Comparison (Holm-Sidak method)					
Comparison	Day 6		Day 8	Day 10	
ACE-056 vs. ACE-000	<0.001 (0.009)		<0.001 (0.009)	<0.001 (0.009)	
ACE-028 vs. ACE-000	<0.001 (0.010)		0.003 (0.010)	NS	
ACE-014 vs. ACE-000	<0.001 (0.013)		NS	NS	
ACE-056 vs. ACE-014	NS		NS	NS	
ACE-056 vs. ACE-028	NS		NS	NS	
ACE-028 vs. ACE-014	NS		NS	NS	

Statistical significance: Unadjusted P value (critical level) NS = Not significant

Table 6.30: Summary results for ATP levels - acetate study

Final Concentration of Added Acetate	ATP (µmol/10^{11}plts)				
	Day 2	Day 3	Day 6	Day 8	Day 10
0 mmol/L	5.48 ± 0.65	6.37 ± 0.77	6.91 ± 0.68	6.31 ± 1.45	5.15 ± 1.54
14 mmol/L	5.32 ± 0.67	5.77 ± 0.93	5.13 ± 0.67	4.78 ± 0.64	4.00 ± 0.22
28 mmol/L	5.26 ± 0.40	5.44 ± 0.84	4.34 ± 0.32	4.34 ± 0.58	4.02 ± 0.30
56 mmol/L	5.22 ± 0.41	5.15 ± 0.91	4.25 ± 0.36	3.84 ± 0.49	2.65 ± 0.94

Mean ± SD (n=5)

Mean day 2 levels of ADP ranged from 3.43 ± 0.90 to 3.97 ± 0.70 µmol/10^{11}plts in units with 14 and 56 mmol/L acetate, respectively. As in the previous studies, ADP decreased with storage in all test groups (figure 6.15). The only statistically significant difference was found between units with 56 mmol/L and units with no added acetate on day 6 (p=0.004) (table 6.31). (The subsequent increase in ADP in the former may be due to a discrepant set of results for day 8, since ADP would not be expected to increase following a decrease.) By day 10, mean levels in units with 56 mmol/L acetate and units lacking acetate had decreased to 1.19 ± 0.28 and 1.38 ± 0.55 µmol/10^{11}plts, respectively; corresponding to 30% and 40% of day 2 levels.

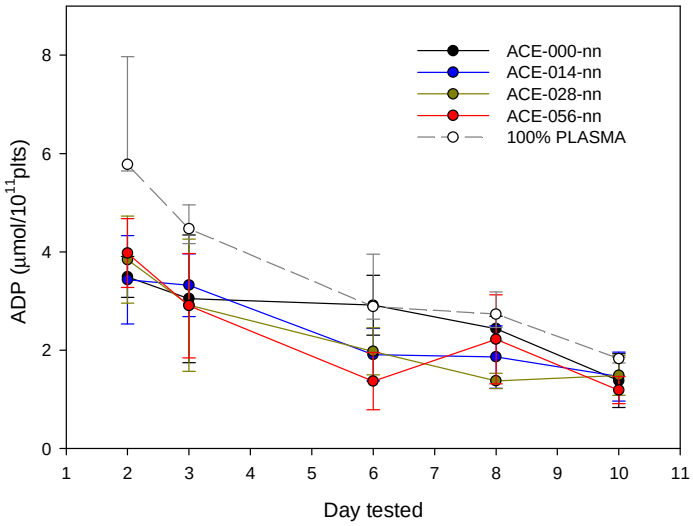


Figure 6.15: ADP levels (acetate study; mean ± SD, n=5; 100% plasma n=3)

Table 6.31: Statistical comparison for ADP levels - acetate study

Comparison Between Groups					
	Day 2	Day 3	Day 6	Day 8	Day 10
One Way ANOVA	0.612	0.929	0.004	0.049	0.712
Multiple Pairwise Comparison (Holm-Sidak method)					
Comparison	Day 6				
ACE-056 vs. ACE-000	<0.001 (0.009)				
ACE-028 vs. ACE-000	NS				
ACE-014 vs. ACE-000	NS				
ACE-056 vs. ACE-014	NS				
ACE-056 vs. ACE-028	NS				
ACE-028 vs. ACE-014	NS				

Statistical significance: Unadjusted P value (critical level) NS = Not significant

Table 6.32: Summary results for ADP levels - acetate study

Final Concentration of Added Acetate	ADP (µmol/10^{11}plts)				
	Day 2	Day 3	Day 6	Day 8	Day 10
0 mmol/L	3.49 ± 0.42	3.05 ± 1.30	2.92 ± 0.61	2.44 ± 0.25	1.38 ± 0.55
14 mmol/L	3.43 ± 0.90	3.32 ± 0.64	1.91 ± 0.53	1.86 ± 0.63	1.46 ± 0.50
28 mmol/L	3.84 ± 0.89	2.92 ± 1.34	1.98 ± 0.48	1.38 ± 0.15	1.48 ± 0.40
56 mmol/L	3.97 ± 0.70	2.90 ± 1.06	1.37 ± 0.58	2.22 ± 0.91	1.19 ± 0.28

Mean ± SD (n=5)

Platelet Activation

Surface Expression and Soluble Levels of CD62P

The expression of CD62P on the platelet surface suggested that platelet activation increased with higher concentrations of acetate early during the storage period, though on day 2 this did not reach statistical significance (p=0.019) (table 6.33). The percentage of positive expression increased in all four groups between days 2 and 3, with mean values on day 3 of 74.37 ± 3.34% in units with no added acetate compared to 87.03 ± 2.46% in units with 56 mmol/L acetate (p<0.001) (table 6.34). The percent positive expression in the four test groups after this time point increased only marginally to mean day 10 levels of 75.67 ± 4.48 % and 89.68 ± 2.01 % in units with no acetate and 56 mmol/L acetate, respectively (p<0.001) (figure 6.16).

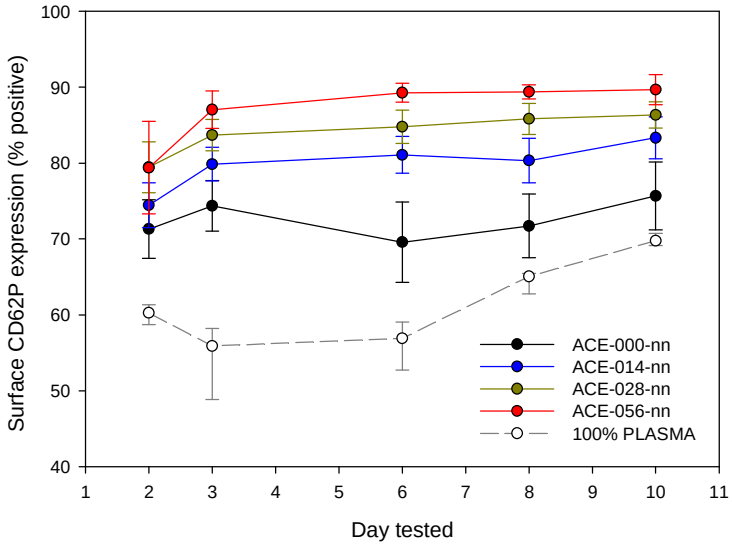


Figure 6.16: Percent positive expression of surface CD62P (acetate study; mean ± SD, n=5; 100% plasma n=3)

Table 6.33: Statistical comparison of surface CD62P percent positive expression - acetate study

Comparison Between Groups					
	Day 2	Day 3	Day 6	Day 8	Day 10
One Way ANOVA	0.019	<0.001	<0.001	<0.001	<0.001
Multiple Pairwise Comparison (Holm-Sidak method)					
Comparison	Day 3	Day 6	Day 8	Day 10	
ACE-056 vs. ACE-000	<0.001 (0.009)	<0.001 (0.009)	<.0001 (0.009)	<0.001 (0.009)	
ACE-028 vs. ACE-000	<0.001 (0.010)	<0.001 (0.010)	<0.001 (0.010)	<0.001 (0.010)	
ACE-014 vs. ACE-000	0.004 (0.017)	<0.001 (0.013)	<0.001 (0.017)	<0.001 (0.013)	
ACE-056 vs. ACE-014	<0.001 (0.013)	<0.001 (0.017)	<0.001 (0.013)	0.004 (0.017)	
ACE-056 vs. ACE-028	NS	NS	NS	NS	
ACE-028 vs. ACE-014	NS	NS	0.007 (0.025)	NS	

Statistical significance: Unadjusted P value (critical level) NS = Not significant

Table 6.34: Summary results for surface CD62P percent positive expression - acetate study

Final Concentration of Added Acetate	Surface CD62P expression (% positive)				
	Day 2	Day 3	Day 6	Day 8	Day 10
0 mmol/L	71.30 ± 3.85	74.37 ± 3.34	69.57 ± 5.29	71.71 ± 4.21	75.67 ± 4.48
14 mmol/L	74.46 ± 2.97	79.86 ± 2.21	81.08 ± 2.43	80.32 ± 2.93	83.33 ± 2.76
28 mmol/L	79.44 ± 3.36	83.68 ± 2.07	84.79 ± 2.20	85.81 ± 2.03	86.33 ± 1.72
56 mmol/L	79.39 ± 6.08	87.03 ± 2.46	89.27 ± 1.26	89.38 ± 0.92	89.68 ± 2.01

Mean ± SD (n=5)

In units containing acetate, surface CD62P median fluorescence intensity increased from days 2 to 6, after which MFI values remained relatively stable or, in the test group with 56 mmol/L acetate, declined till the end of storage (figure 6.17). A statistically significant difference was observed between units with 56 mmol/L acetate and units with 14 mmol/L acetate from days 2 to 6 ($p=001$ to $p< 0.001$). By day 10, the decrease in MFI in the former resulted in similar mean MFI values of 3.38 ± 0.26 and 3.27 ± 0.22, respectively (table 6.36). Mean day 2 values in units with no added acetate (1.87 ± 0.17) were significantly different to values noted in units with 56 mmol/L (2.54 ± 0.18) and 28 mmol/L (2.27 ± 0.21) ($p<0.001$) (table 6.35). In contrast to units with acetate, MFI values in units with no acetate were retained at relatively low levels after an initial increase between days 2 and 3, with a mean MFI on day 10 of 1.89 ± 0.20 compared with a day 2 value of 1.87 ± 0.17 (table 6.36). The pattern seen with these units was similar to that observed with units re-suspended in plasma and markedly different to that observed in units with added acetate (figure 6.17).

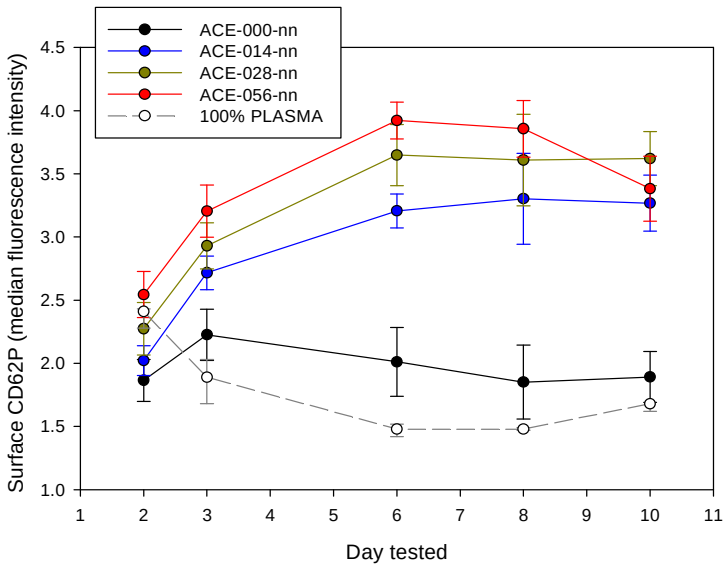


Figure 6.17: Median fluorescence intensity of surface CD62P (acetate study; mean ± SD, n=5; 100% plasma n=3)

Table 6.35: Statistical comparison of surface CD62P (median fluorescence intensity) - acetate study

Comparison Between Groups					
	Day 2	Day 3	Day 6	Day 8	Day 10
One Way ANOVA	<0.001	<0.001	<0.001	<0.001	<0.001
Multiple Pairwise Comparison (Holm-Sidak method)					
Comparison	Day 2	Day 3	Day 6	Day 8	Day 10
ACE-056 vs. ACE-000	<0.001 (0.009)	<0.001 (0.009)	<0.001 (0.009)	<0.001 (0.009)	<0.001 (0.010)
ACE-028 vs. ACE-000	0.002 (0.013)	<0.001 (0.010)	<0.001 (0.010)	<0.001 (0.010)	<0.001 (0.009)
ACE-014 vs. ACE-000	NS	<0.001 (0.013)	<0.001 (0.013)	<0.001 (0.013)	<0.001 (0.013)
ACE-056 vs. ACE-014	<0.001 (0.010)	0.001 (0.017)	<0.001 (0.017)	NS	NS
ACE-056 vs. ACE-028	NS	NS	NS	NS	NS
ACE-028 vs. ACE-014	NS	NS	0.004 (0.025)	NS	NS

Statistical significance: Unadjusted P value (critical level) NS = Not significant

Table 6.36: Summary results for surface CD62P median fluorescence intensity - acetate study

Final Concentration of Added Acetate	Surface CD62P expression (median fluorescence intensity)				
	Day 2	Day 3	Day 6	Day 8	Day 10
0 mmol/L	1.87 ± 0.17	2.23 ± 0.20	2.01 ± 0.27	1.85 ± 0.29	1.89 ± 0.20
14 mmol/L	2.02 ± 0.12	2.72 ± 0.13	3.21 ± 0.13	3.30 ± 0.36	3.27 ± 0.22
28 mmol/L	2.27 ± 0.21	2.93 ± 0.18	3.65 ± 0.24	3.61 ± 0.36	3.62 ± 0.21
56 mmol/L	2.54 ± 0.18	3.20 ± 0.21	3.92 ± 0.15	3.86 ± 0.22	3.38 ± 0.26

Mean ± SD (n=5)

Levels of soluble CD62P on days 2 and 3 were similar in all test groups, with no statistically significant differences (p=0.997 and 0.534, respectively) (table 6.37). Levels were observed to increase with storage in all test groups (figure 6.18). After day 3, levels were higher in units containing acetate (all concentrations) compared with the test group lacking acetate, with statistically significant differences on days 6 and 8 (p<0.001 in both cases). Although the pattern remained evident on day 10, the results were no longer significantly different (p=0.014). Mean levels in units lacking acetate increased from 36.62 ± 5.39 ng/mL on day 2 to 88.59 ± 9.34 ng/mL on day 10, an increase of 242%. Comparative levels in units with 56 mmol/L acetate were 36.80 ± 6.74 ng/mL and 113.53 ± 8.05 ng/mL; an increase of 309% (table 6.38). As noted previously, the comparatively high concentrations of soluble CD62P in the units re-suspended in plasma are derived from the relatively higher fraction of this protein already present in the suspending medium.

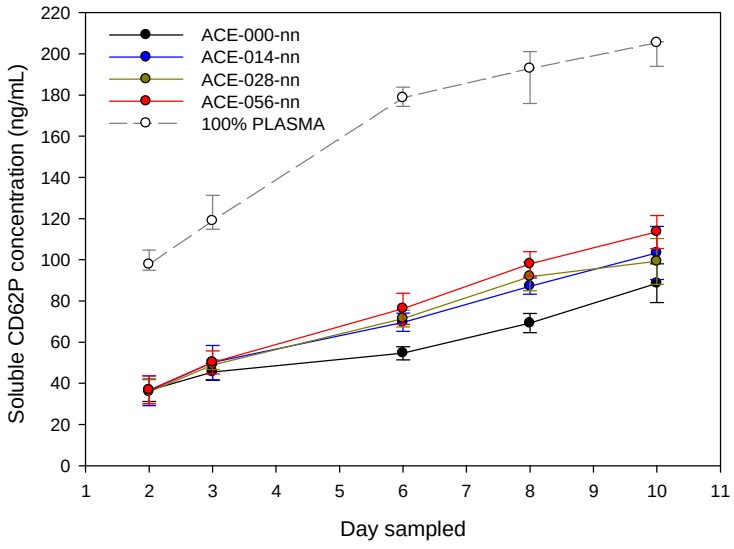


Figure 6.18: Soluble CD62P levels (acetate study; mean ± SD, n=5; 100% plasma n=3)

Table 6.37: Statistical comparison of soluble CD62P levels - acetate study

Comparison Between Groups					
	Day 2	Day 3	Day 6	Day 8	Day 10
One Way ANOVA	0.997	0.534	<0.001	<0.001	0.014
Multiple Pairwise Comparison (Holm-Sidak method)					
Comparison		Day 6		Day 8	
ACE-056 vs. ACE-000		<0.001 (0.009)		<0.001 (0.009)	
ACE-028 vs. ACE-000		<0.001 (0.010)		<0.001 (0.010)	
ACE-014 vs. ACE-000		<0.001 (0.013)		<0.001 (0.013)	
ACE-056 vs. ACE-014		NS		0.007 (0.017)	
ACE-056 vs. ACE-028		NS		NS	
ACE-028 vs. ACE-014		NS		NS	

Statistical significance: Unadjusted P value (critical level) NS = Not significant

Table 6.38: Summary results for soluble CD62P - acetate study

Final Concentration of Added Acetate	Soluble CD62P (ng/mL)				
	Day 2	Day 3	Day 6	Day 8	Day 10
0 mmol/L	36.62 ± 5.39	45.57 ± 4.16	54.60 ± 3.18	69.22 ± 4.66	88.59 ± 9.34
14 mmol/L	36.44 ± 7.27	50.08 ± 8.27	69.61 ± 4.43	87.23 ± 3.98	103.30 ± 12.95
28 mmol/L	35.92 ± 5.76	48.98 ± 2.33	71.42 ± 4.09	91.87 ± 6.94	99.20 ± 11.04
56 mmol/L	36.80 ± 6.74	50.13 ± 5.51	76.21 ± 7.55	97.97 ± 5.91	113.53 ± 8.05

Mean ± SD (n=5)

Markers of Cell Death

Expression of Aminophospholipids

The percentage of platelets binding annexin V was similar in all four test groups at the start of the storage period, with mean values on day 2 between 10.24 ± 0.99 % in units with 56 mmol/L acetate and 11.50 ± 4.40 % in units with 28 mmol/L acetate, with no statistically significant differences between any of the test groups (p=0.939) (table 6.39). The percentage positive expression increased modestly with storage in units with 14 and 28 mmol/L acetate, with mean day 10 levels of 23.95 ± 5.00 and 19.36 ± 5.22 %, respectively (figure 6.19). By contrast, annexin V binding increased after day 6 in units with no added acetate and after day 8 in units with 56 mmol/L acetate, resulting in a statistically significant difference between the latter test group and units with 14 and 28 mmol/L acetate by day 10 (p=0.001) (table 6.39). Mean day 10 values for units without acetate and units with 56 mmol/L acetate were 34.54 ± 6.07 % and 46.89 ± 15.84 %, respectively (table 6.40).

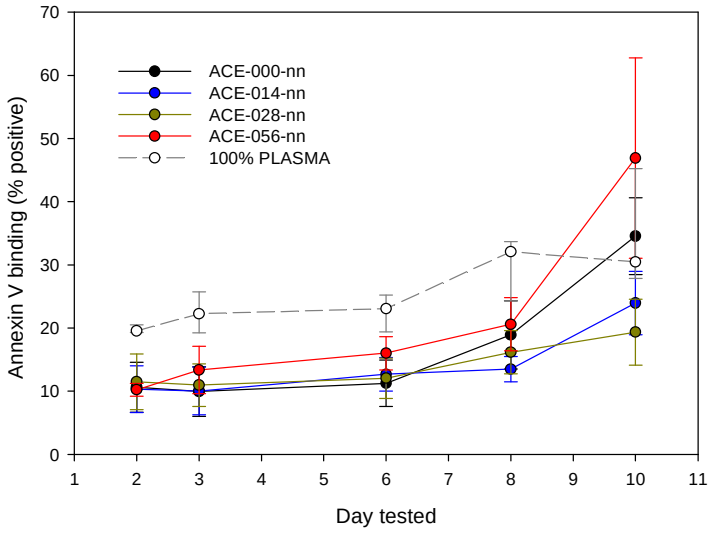


Figure 6.19: Annexin V binding as percent positive expression (acetate study; mean ± SD, n=5; 100% plasma n=3)

Table 6.39: Statistical comparison of annexin V binding (% positive) - acetate study

Comparison Between Groups					
	Day 2	Day 3	Day 6	Day 8	Day 10
One Way ANOVA	0.939	0.456	0.111	0.056	0.001
Multiple Pairwise Comparison (Holm-Sidak method)					
Comparison	Day 10				
ACE-056 vs. ACE-000	NS				
ACE-028 vs. ACE-000	NS				
ACE-014 vs. ACE-000	NS				
ACE-056 vs. ACE-014	0.001 (0.010)				
ACE-056 vs. ACE-028	<0.001 (0.009)				
ACE-028 vs. ACE-014	NS				

Statistical significance: Unadjusted P value (critical level) NS = Not significant

Table 6.40: Summary results for annexin V binding (% positive) - acetate study

Final Concentration of Added Acetate	Annexin V binding (% positive)				
	Day 2	Day 3	Day 6	Day 8	Day 10
0 mmol/L	10.64 ± 3.93	9.97 ± 3.93	11.25 ± 3.67	18.91 ± 5.41	34.54 ± 6.07
14 mmol/L	10.33 ± 3.70	10.05 ± 3.78	12.69 ± 2.65	13.50 ± 2.02	23.95 ± 5.00
28 mmol/L	11.50 ± 4.40	10.97 ± 3.36	12.05 ± 3.17	16.17 ± 3.42	19.36 ± 5.22
56 mmol/L	10.24 ± 0.99	13.37 ± 3.75	16.03 ± 2.64	20.60 ± 4.21	46.89 ± 15.84

Mean ± SD (n=5)

Annexin V binding measured as mean fluorescence intensity remained fairly stable throughout the ten days of storage in units lacking acetate and in the test group with 14 mmol/L acetate (figure 6.20). In units with 28 mmol/L acetate there was a slight rise, with day 2 values of 29.7 ± 5.2 increasing to 36.2 ± 3.1 by day 10 (table 6.42). Figure 6.20 shows mean MFI values in units with 56 mmol/L were higher from day 2 compared to the other test groups. However, statistically significant differences were not evident until day 10 ($p<0.001$) (table 6.41), with a mean value in this test group of 47.3 ± 8.7 at the end of storage.

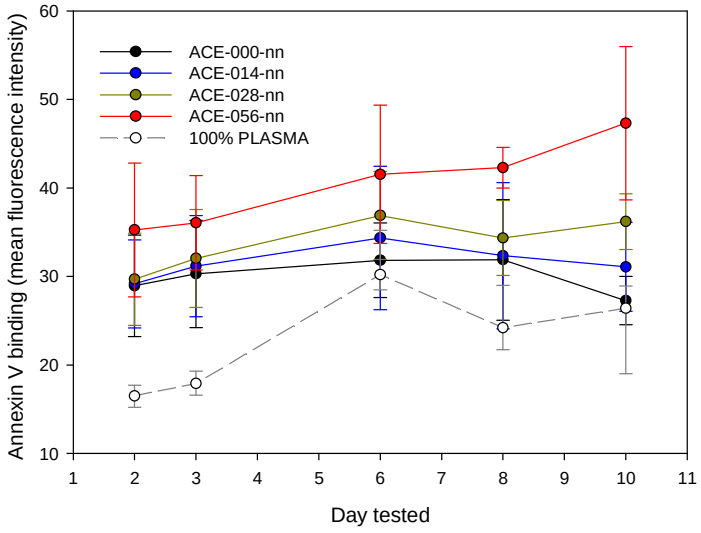


Figure 6.20: Annexin V binding as mean fluorescence intensity (acetate study; mean ± SD, n=5; 100% plasma n=3)

Table 6.41: Statistical comparison of annexin V binding (mean fluorescence intensity) - acetate study

Comparison Between Groups					
	Day 2	Day 3	Day 6	Day 8	Day 10
One Way ANOVA	0.315	0.412	0.151	0.044	<0.001
Multiple Pairwise Comparison (Holm-Sidak method)					
Comparison	Day 10				
ACE-056 vs. ACE-000	<0.001 (0.009)				
ACE-028 vs. ACE-000	NS				
ACE-014 vs. ACE-000	NS				
ACE-056 vs. ACE-014	<0.001 (0.010)				
ACE-056 vs. ACE-028	0.005 (0.013)				
ACE-028 vs. ACE-014	NS				

Statistical significance: Unadjusted P value (critical level) NS = Not significant

Table 6.42: Summary results for annexin V binding (mean fluorescence intensity) - acetate study

Final Concentration of Added Acetate	Annexin V binding (mean fluorescence intensity)				
	Day 2	Day 3	Day 6	Day 8	Day 10
0 mmol/L	28.9 ± 5.7	30.3 ± 6.1	31.8 ± 4.2	31.9 ± 6.8	27.3 ± 2.7
14 mmol/L	29.2 ± 5.0	31.2 ± 5.7	34.4 ± 8.1	32.3 ± 8.3	31.1 ± 5.0
28 mmol/L	29.7 ± 5.2	32.0 ± 5.5	36.9 ± 5.0	34.3 ± 4.2	36.2 ± 3.1
56 mmol/L	35.3 ± 7.6	36.1 ± 5.3	41.5 ± 7.8	42.3 ± 2.3	47.3 ± 8.7

Mean ± SD (n=5)

The expression of aminophospholipds as quantified by mass spectrometry is presented graphically in figure 6.21 for a representative sample. The four forms of PE as well as PS displayed a consistent pattern of relatively low and unvarying expression between days 2 and 8. There was increased expression of all forms of PE and PS after day 8 in all four test groups. The flow cytometric assay also showed an accelerated increase in the percent positive expression of annexin V binding towards the end of storage; though this was more marked in units lacking acetate and units with 56 mmol/L acetate (figure 6.19). There was no clear relationship between the concentration of acetate and the expression of either PE or PS measured by mass spectrometry.

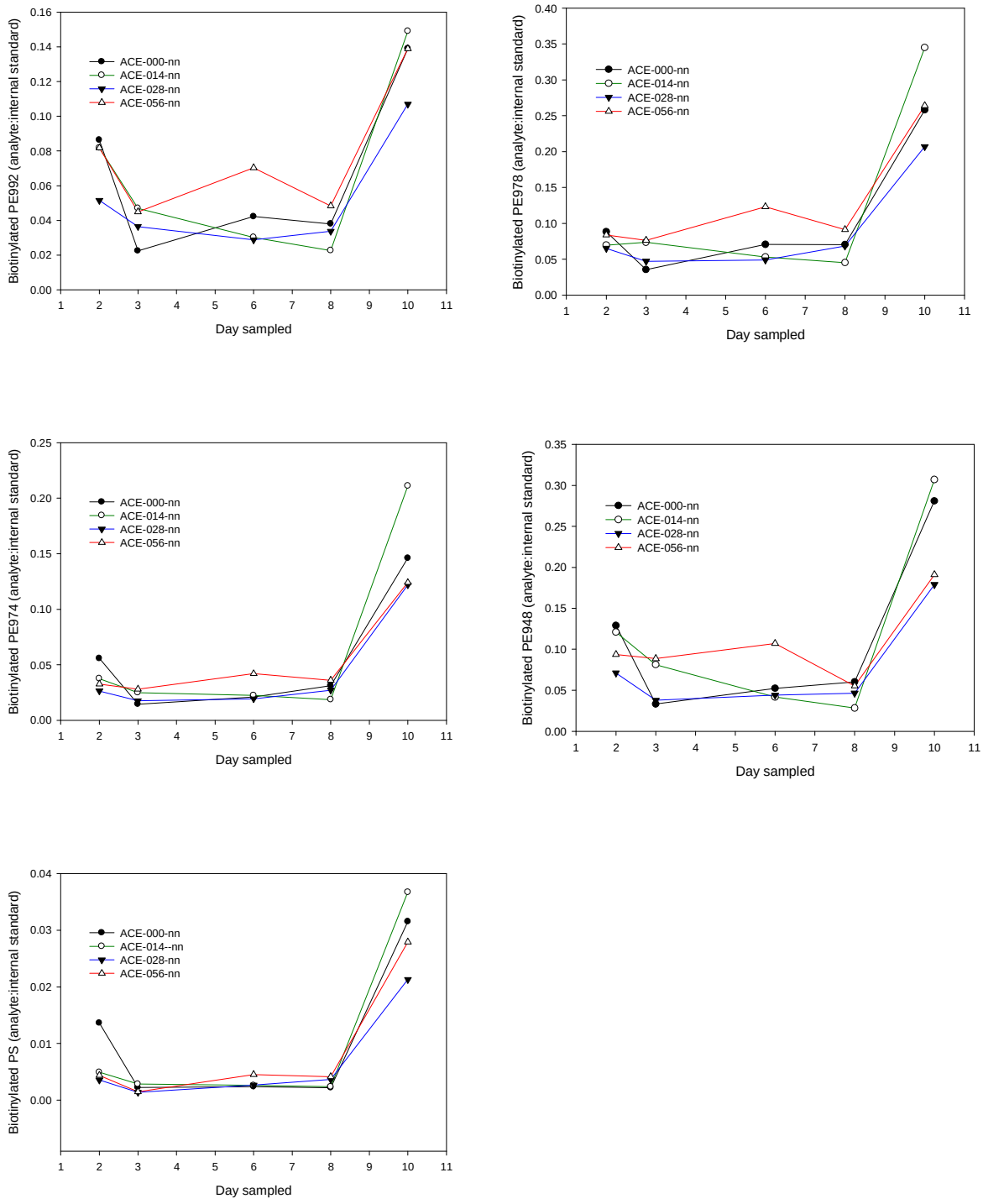


Figure 6.21: Expression of aminophospholipids on platelet surface measured by mass spectrometry, showing an increase in aminophospholipid expression after day 8 (acetate study; representative sample)

Mitochondrial membrane potential decreased steadily throughout the storage period in all test groups (figure 6.22). Ratios of red/green JC-1 fluorescence in units with 28 mmol/L acetate decreased from 8.26 ± 1.20 on day 2 to 4.50 ± 0.56 on day 10; a decrease of 46%. Percentage decreases in MMP ratios from days 2 to 10 were 65%, 57% and 64% in units with no acetate, 14 mmol/L and 56 mmol/L acetate, respectively (table 6.44). The decrease noted in the four treatment groups was not as evident in units re-constituted in plasma, where day 10 fluorescence ratios were 80% of day 2 values (figure 6.22). Statistically significant differences were evident between some of the test groups on days 6, 8 and 10 (table 6.43). However, no clear pattern is evident between MMP and the starting concentrations of acetate from this data.

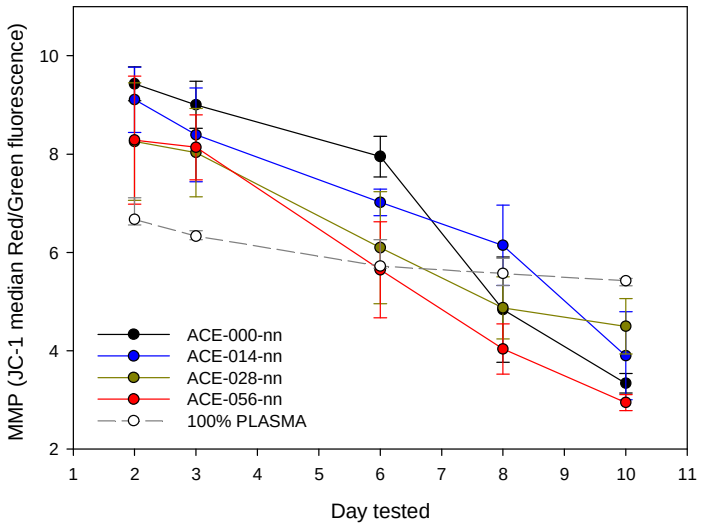


Figure 6.22: Mitochondrial membrane potential measured as JC-1 median red/green fluorescence (acetate study; mean ± SD, n=5; 100% plasma n=3)

Table 6.43: Statistical comparison of mitochondrial membrane potential (JC-1 median red/green fluorescence) between test groups - acetate study

Comparison Between Groups					
	Day 2	Day 3	Day 6	Day 8	Day 10
One Way ANOVA	0.172	0.232	0.001	0.006	0.002
Multiple Pairwise Comparison (Holm-Sidak method)					
Comparison	Day 6		Day 8	Day 10	
ACE-056 vs. ACE-000	<0.001 (0.009)		NS	NS	
ACE-028 vs. ACE-000	0.002 (0.010)		NS	0.004 (0.010)	
ACE-014 vs. ACE-000	NS		NS	NS	
ACE-056 vs. ACE-014	NS		0.001 (0.009)	NS	
ACE-056 vs. ACE-028	NS		NS	<0.001 (0.009)	
ACE-028 vs. ACE-014	NS		NS	No	

Statistical significance: Unadjusted P value (critical level) NS = Not significant

Table 6.44: Summary results for mitochondrial membrane potential (median red/green JC-1 fluorescence) - acetate study

Final Concentration of Added Acetate	Annexin V binding (mean fluorescence intensity)				
	Day 2	Day 3	Day 6	Day 8	Day 10
0 mmol/L	9.43 ± 0.35	9.00 ± 0.48	7.95 ± 0.41	4.84 ± 1.07	3.34 ± 0.20
14 mmol/L	9.11 ± 0.66	8.39 ± 0.95	7.02 ± 0.27	6.14 ± 0.82	3.90 ± 0.90
28 mmol/L	8.26 ± 1.20	8.03 ± 0.90	6.10 ± 1.14	4.87 ± 0.63	4.50 ± 0.56
56 mmol/L	8.29 ± 1.30	8.14 ± 0.66	5.65 ± 0.98	4.04 ± 0.51	2.95 ± 0.16

Mean ± SD (n=5)

Intracellular Free Calcium

Levels of intracellular free calcium on day 2 were comparable to those observed in units re-suspended in plasma, with day 2 ratios between 0.24 ± 0.01 (no added acetate) and 0.29 ± 0.02 (56 mmol/L acetate) (table 6.46). Statistically significant differences were noted at this time point between units without acetate and units with 56 mmol/L and 28 mmol/L acetate (p=0.002). However, there were no statistically significant differences on days 3, 6 and 8 between any of the test groups (table 6.45). In units with 14 mmol/L and 28 mmol/L acetate, intracellular free calcium remained stable throughout the storage period (figure 6.23). In units with no added acetate, there was marked increase in the dye ratio between days 8 and 10, from a mean of 0.31 ± 0.11 to 0.55 ± 0.09. A similar increase is apparent in units with 56 mmol/L acetate. However, there is one clear outlier in the results from these units on day 10. Without this result the mean ratio would be 0.40 ± 0.11 – higher than the mean values in units with 14 mmol/L and 28 mmol/L acetate but not generating a statistically significant difference. Exclusion of this outlier resulted in statistically significant differences on day 10 between units lacking acetate and units with either 14 mmol/L and 28 mmol/L acetate (p<0.001) (table 6.45). (The flow cytometry histograms from this outlier did not indicate a clear problem with this sample; therefore it was decided not to reject the result from the analysis.)

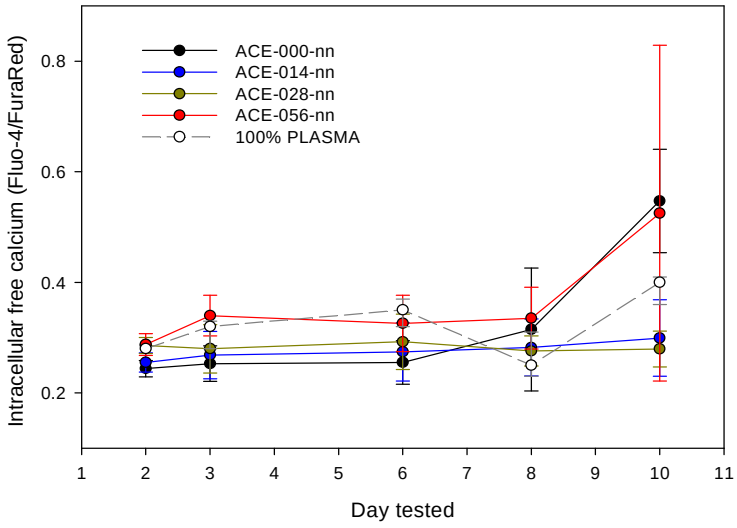


Figure 6.23: Intracellular free calcium
(acetate study; mean ± SD , n=5; 100% plasma n=3)

Table 6.45: Statistical comparison of intracellular free calcium (Fluo-4/FuraRed) - acetate study

Comparison Between Groups					
	Day 2	Day 3	Day 6	Day 8	Day 10
One Way ANOVA	0.002	0.015	0.167	0.501	0.030 (<0.001)
Multiple Pairwise Comparison (Holm-Sidak method)					
Comparison	Day 2		Day 10		
ACE-056 vs. ACE-000	0.001 (0.002)		NS		
ACE-028 vs. ACE-000	0.001 (0.002)		<0.001 (0.002)		
ACE-014 vs. ACE-000	NS		<0.001 (0.002)		
ACE-056 vs. ACE-014	NS		NS		
ACE-056 vs. ACE-028	NS		NS		
ACE-028 vs. ACE-014	NS		NS		

Statistical significance: Unadjusted P value (critical level) NS = Not significant
In red: alternative analysis without outlier from 56 mmol/L acetate test group (day 10)

Table 6.46: Summary results for intracellular free calcium (Fluo-4/FuraRed) - acetate study

Final Concentration of Added Acetate	Intracellular free calcium (Fluo-4/FuraRed fluorescence)				
	Day 2	Day 3	Day 6	Day 8	Day 10
0 mmol/L	0.24 ± 0.01	0.25 ± 0.03	0.26 ± 0.04	0.31 ± 0.11	0.55 ± 0.09
14 mmol/L	0.26 ± 0.02 (n=4)[#]	0.27 ± 0.04	0.27 ± 0.05	0.28 ± 0.05	0.30 ± 0.07
28 mmol/L	0.29 ± 0.01	0.28 ± 0.04	0.29 ± 0.05	0.28 ± 0.03	0.28 ± 0.03
56 mmol/L	0.29 ± 0.02	0.34 ± 0.04	0.33 ±0.05	0.34 ± 0.06	0.53 ± 0.30 (0.40 ± 0.11)

Mean ± SD (n=5)
In red: result minus outlier from 56 mmol/L acetate test group (day 10)
[#]: One outlier removed from analysis – wide Fluo-4 peak; result not accepted

DISCUSSION

Based on published studies, the addition of acetate to the storage media would be expected to increase oxygen consumption, decrease lactate production and delay the consumption of glucose (Hornsey et al., 2006). The combined effects would be a stabilisation of pH at a higher value than if acetate were absent. This study confirmed the above expectations in the test group lacking acetate. The test groups containing acetate did not yield the hypothesised direct relationship between an increase in acetate concentration and an enhancement of the above effects, with no clear dose-dependency

with these parameters. The improved maintenance of ATP levels that would be expected with the addition of increasing concentrations of a metabolic substrate such as acetate was also absent - an observation suggested by some earlier studies (Holme, 1992) which may be related to the metabolism of acetate into acetyl coA, a reaction that requires the consumption of an ATP molecule (Knowles et al., 1974).

The inclusion of acetate appeared to have an adverse effect on the parameters of the hypotonic shock response and the extent of shape change. This may be related to the inability to retain ATP levels as effectively in acetate-containing units. Interestingly, the difference between the treatment groups are evident from day 2 of storage, which may suggest a more immediate impact on platelet function and morphology with the presence of acetate. A similar early effect with increasing concentrations of acetate was evident with the surface expression of CD62P, implying that acetate at the concentrations used in this study had an adverse effect on platelet function and activation despite its expected benefit on platelet metabolism. The addition of acetate in the storage medium appeared to have a relatively neutral effect on the indicators of cell death, although there is a suggestion (not always translating to statistical significance) that units with the highest concentrations of acetate experienced a greater degree of cell death. The increase in annexin V binding and intracellular free calcium in the units lacking acetate after day 8 may be related to the exhaustion of glucose in these units rather than a direct consequence of the lack of exogenous acetate. In summary, the results from many of the parameters tested were ambiguous and did not reflect a clear benefit for the inclusion of acetate beyond the retention of a higher pH.

CHAPTER 7.

INVESTIGATION OF THE ROLE OF TMEM-16F IN PLATELET PHOSPOLIPID EXPRESSION UNDER STORAGE CONDITIONS

INTRODUCTION

Stimulation of platelets by agonists such as thrombin and or collagen leads to a rapid increase in intra-cellular calcium and activation of phospholipid scramblase, resulting in the translocation of PS to the external leaflet of the platelet membrane (Zwaal et al., 2005, Bevers et al., 1982). This process is markedly impaired in patients with Scott syndrome, a rare bleeding disorder where patients have a deficiency of TMEM-16F (Suzuki et al., 2010, Castoldi et al., 2011). In order to establish whether TMEM-16F was involved in the expression of PS following in vitro aging of platelets, investigations were performed on the platelets from a patient with Scott syndrome. Investigation of the Scott platelets is undertaken on a protocol agreed by the South East Wales Ethics Committee and not requiring full ethical approval. A similar pattern of annexin V binding in Scott syndrome and control platelets would imply that the membrane transport processes responsible for the expression of PS during in vitro aging were intact in Scott Syndrome platelets and not related to phospholipid scramblase. To confirm that apoptotic mechanisms were intact in Scott syndrome and could potentially be implicated in the increased annexin V positivity, the effect of activating apoptosis in Scott and control platelets was investigated with ABT-737. ABT-737 promotes apoptosis indirectly by binding to anti-apoptotic members of the Bcl-2 family, including Bcl-2 and Bcl-X_L; thus mimicking the action of sensitizing BH3-only proteins (Oltersdorf et al., 2005).

A comparative storage study measuring annexin V binding in Scott and control platelets over an 8-day storage period was undertaken at the WBS. The University Hospital of Wales adopted the annexin V binding assay previously described and expanded on this study by investigating the potential for thrombin generation in stored Scott platelet. Repeat experiments on the Scott syndrome platelets were not possible because it was not possible to take multiple samples from the patient.

METHODS

Sample collection – storage study

Peripheral whole blood samples (7 × 5 mL coagulation sodium citrate 3.2% vacutainers) were obtained from two normal controls and a Scott Syndrome patient. Samples were centrifuged at 800 *g* for 10 minutes at 22°C to obtain PRP. The PRP was transferred by syringe and 19G needle into Baxter PL2410 neonatal platelet storage packs. Approximately 3 mL of PRP was obtained from each vacutainer, resulting in a total volume of approximately 15 mL. Neonatal packs were stored on a horizontal agitator at 22 ± 2°C, with an additional gentle manual mix provided at irregular intervals throughout the storage period. Samples for testing were taken on days 0 (day of preparation), 1, 4, 6 and 8 by means of a sterile 19G needle and syringe.

Response of Scott Platelets to ABT-737 BH3 Mimetic - Experimental Design

Samples from two normal controls and the Scott patient were obtained as above. Samples were centrifuged at 140 *g* for 15 minutes at 22°C to obtain PRP which was subsequently adjusted with autologous platelet poor plasma to provide a platelet count of 150 × 10^9/L. Samples were treated as follows:

- TIME 0 – measurements performed as soon as possible
- CRP – addition of collagen-related peptide; a strong platelet agonist that mimics the structure of collagen (Polanowska-Grabowska, 2003).
- NO ABT-737 – No reagents added; 4 hour incubation at ambient temperature
- ABT-737 POS – 20 µM ABT-737 added; 4 hour incubation at ambient temperature
- ABT-737 NEG – 20 µM A-779024.0 added (less active enantiomer of ABT-737) 4 hour incubation at ambient temperature

(CRP was provided by the Oxford Haemophilia and Thrombosis Centre. ABT-737 and its enantiomer were provided by Abbot Pharmaceuticals).

Thrombin Generation

Thrombin generation studies were performed in PRP (150 × 10^9/L) at UHW by Calibrated Automated Thrombography (CAT). Briefly, the use of a slow-reacting fluorogenic substrate allows the generation of thrombin to be continuously measured, with the thrombin concentration calculated based on the activity of a parallel sample

containing a thrombin calibrator (Hemker et al., 2003). Thrombin generation was initiated with 0.15 pM tissue factor (diluted innovin).

RESULTS

Annexin V Binding to the Platelet Surface

The platelet concentration and mean platelet volume on day 0 (the day of collection and unit preparation) are presented in Table 7.1, with comparatively similar values obtained from all three samples.

Table 7.1: Platelet Concentration and Mean Platelet Volume

DAY TESTED	Platelet Concentration ($x10^9$/L)			Mean Platelet Volume (fL)		
	Scott Patient	CONTROL 1	CONTROL 2	Scott Patient	CONTROL 1	CONTROL 2
0	178	193	208	7.4	6.4	7.0

The annexin V binding results are expressed both as percent positive expression and mean fluorescence intensity (Table 7.2). Although a statistical comparison is not possible, the percent positive expression was similar between the Scott syndrome platelets and the normal controls (figure 7.1). Values at the end of the storage period were high compared with results from adult-dose PC suspended in plasma at the same time point (chapter 3). This is likely to be due to the small sample volume of approximately 15 mL being stored in a neonatal pack designed to hold between 40 to 70 mL, resulting in the platelets being unable to agitate adequately. Marked variation in the results for mean fluorescence intensity was present between all three samples and the only observation from this data is that all three samples showed an increase in MFI after day 2 of storage (figure 7.2).

It was not possible to repeat the experiments on *in vitro* ageing with Scott platelets in our laboratory because of limitations on the amount of blood that could be donated by the patient. However, a similar study on the same patient performed in the laboratory of Professor Bevers showed that over a three-day period, annexin V positivity increased similarly compared to control platelets (data submitted).

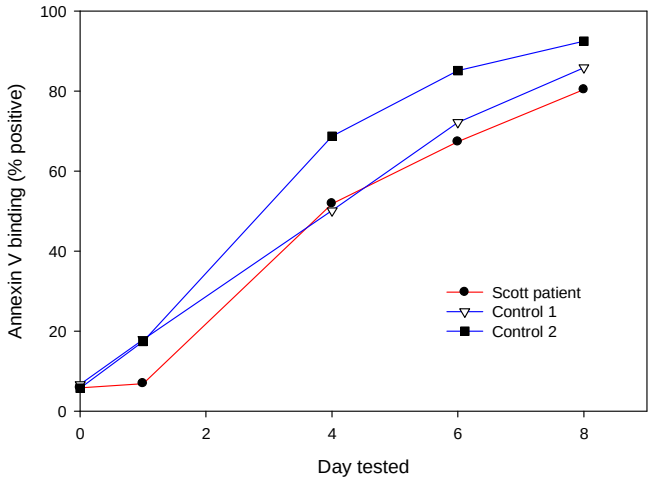


Figure 7.1:
Annexin V Binding in Scott patient study
(percent positive expression)

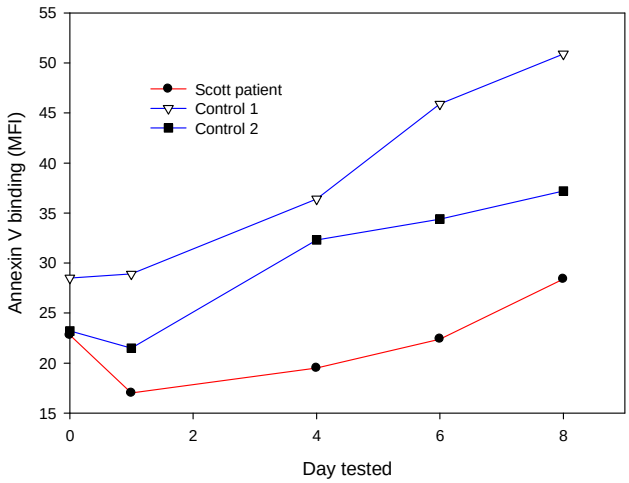


Figure 7.2:
Annexin V Binding in Scott patient study
(mean fluorescence intensity)

Table 7.2: Annexin V Binding in Scott patient study

DAY TESTED	Annexin V Binding (% Positive)			Mean Fluorescence Intensity		
	Scott patient	CONTROL 1	CONTROL 2	Scott patient	CONTROL 1	CONTROL 2
0	5.86	6.67	5.72	22.8	28.5	23.2
1	6.91	17.94	17.43	17.0	28.9	21.5
4	51.87	50.12	68.74	19.5	36.4	32.3
6	67.34	72.19	85.15	22.4	45.9	34.4
8	80.38	85.85	92.48	28.4	50.9	37.2

Thrombin Generation in Stored Scott Platelets

The pattern of annexin V binding in stored Scott platelets described above was duplicated by colleagues at UHW (figure 7.3) though the values were lower throughout storage in the Scott platelets.

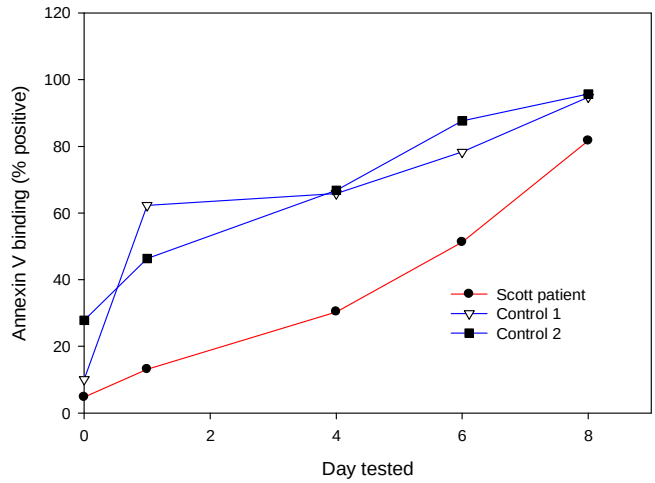


Figure 7.3: Annexin V Binding in Scott patient study (UHW study) (percent positive expression) (courtesy of B. Kerr)

Thrombin generation results for the Scott patient and the two controls over the eight-day storage period are presented individually in figure 7.4. Rates of thrombin generation on the day of collection were comparatively low with the Scott platelets but progressively increased with storage. By day 8, levels of thrombin were comparable to those observed in the controls, a finding more clearly illustrated in figure 7.5.

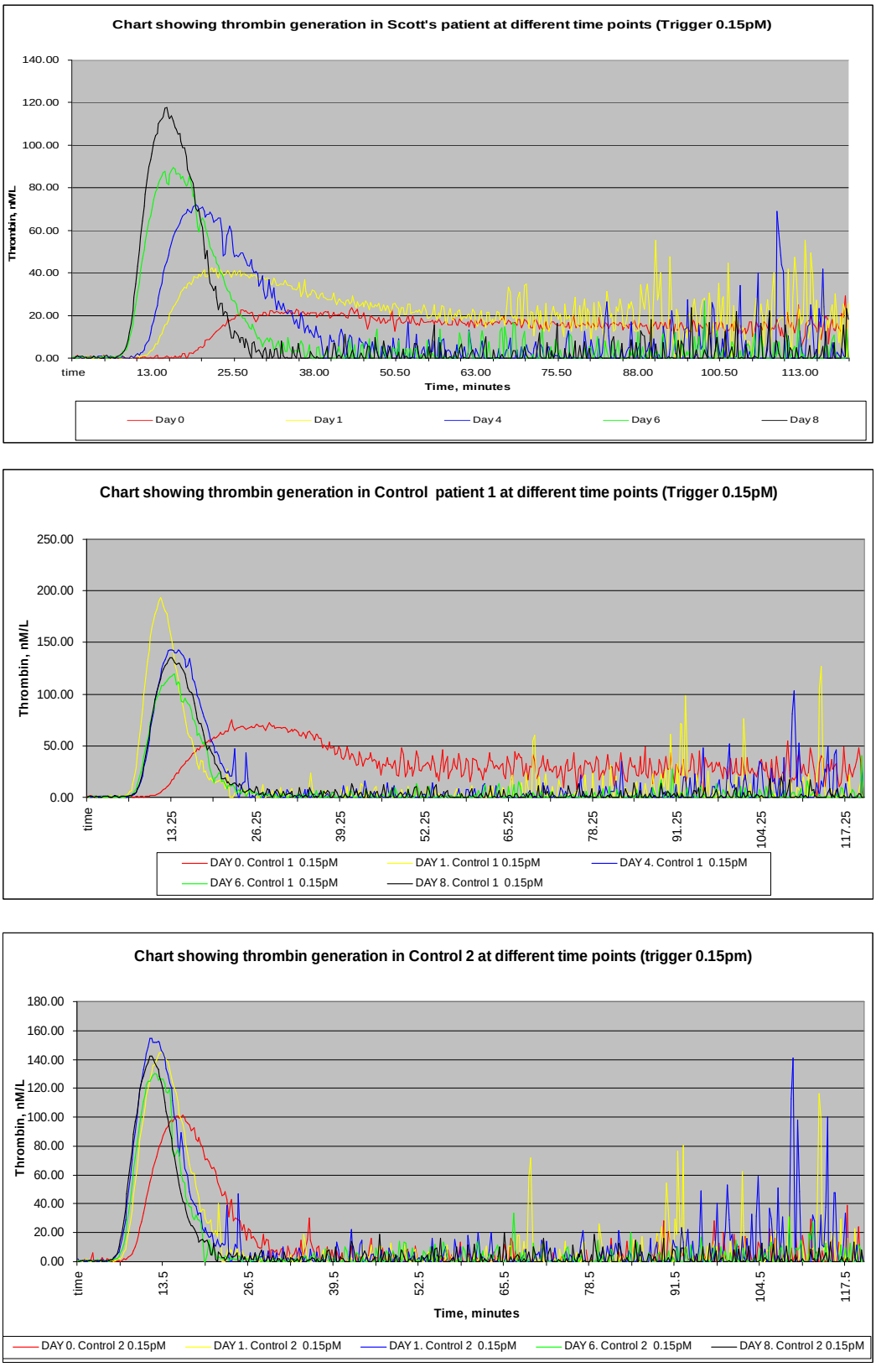
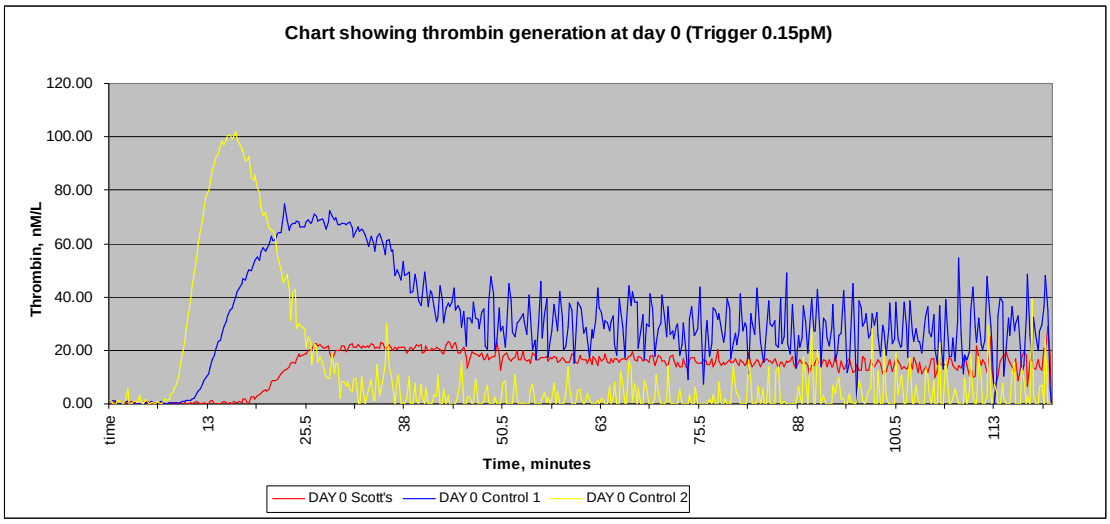


Figure 7.4: Thrombin generation in Scott and control platelets measured over the course of an 8-day storage period (all graphs courtesy of Dr C Jones, UHW).



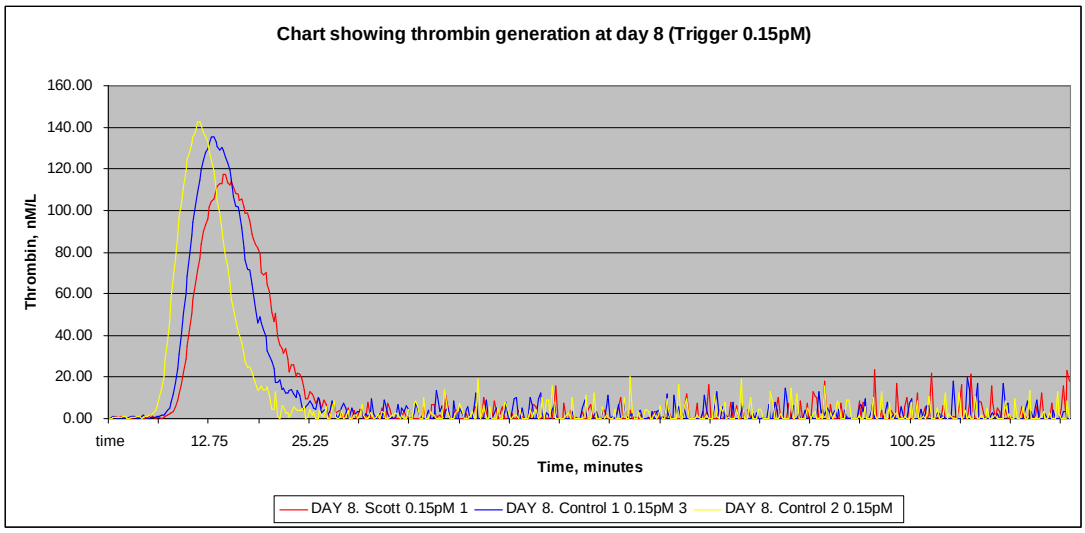


Figure 7.5: Thrombin generation on day 0 (day of unit preparation) and day 8 in Scott and normal control platelets (all graphs courtesy of Dr C Jones, UHW)

Response of Scott Platelets to the BH3 Mimetic ABT-737

At time 0, annexin V binding was virtually absent in both control and Scott platelets (figure 7.6). After a 4-hour incubation, the unmanipulated samples showed an approximately two to three fold increase in percent positive expression, with a marginal increase noted in the Scott platelets (table 7.3). Addition of CRP prompted a marked increase in annexin V binding in the controls, with a more modest increase to a value of 9.74% positive binding observed in Scott platelets. This was similar to the value of 10.85% positive binding obtained in these platelets after a 4-hour incubation with 20

µM ABT-737. Experiments by Prof. Bevers's team at Maastricht confirmed that the Scott platelets were annexin V positive after ABT-737 stimulation (data submitted).

Table 7.3: Annexin V binding on Platelets: Response to Incubation with ABT-737

TEST CONDITION	ANNEXIN V BINDING (% POSITIVE)		
	CONTROL 1	CONTROL 2	SCOTT PATIENT
TIME 0	1.65	2.11	1.03
CRP	61.05	80.24	9.74
NO ABT-737	3.37	5.66	1.25
ABT-737 POS	14.48	24.74	10.85
ABT-737 NEG	4.26	5.93	1.61

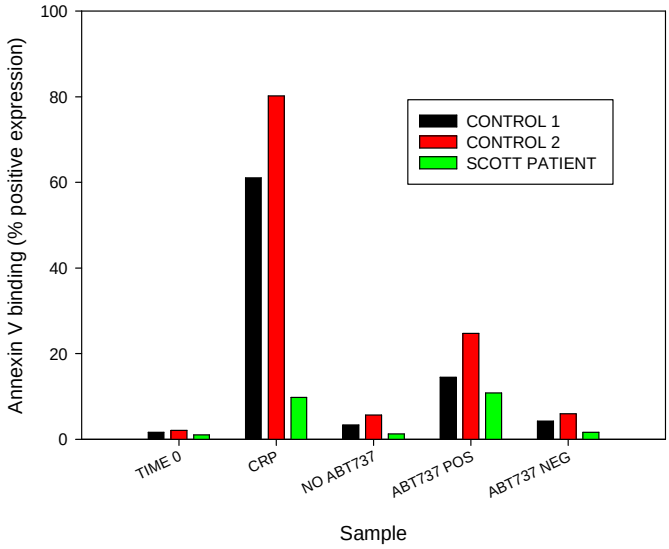


Figure 7.6: Annexin V binding in normal control and Scott platelets in response to a 4-hour incubation with 20µM ABT-737

DISCUSSION

The progressive increase in annexin V binding observed in Scott platelets was similar to that seen in control platelets and demonstrates that the expression of negatively charged aminophospholipids associated with in vitro aging was not due to TMEM-16F and Ca^{2+} dependent scramblase activity. The cellular mechanisms required for expression of negatively charged aminophospholipids through apoptotic pathways, however, appeared to be intact as demonstrated by the response to ABT-737. This demonstrates that the in vitro aging changes observed in the platelet storage lesion could be mediated, at least in part, by apoptotic pathways. These findings have been independently confirmed by a second laboratory.

The data presented here also suggest that the increased expression of negatively charged aminophospholipids (as demonstrated by annexin V binding) associated with *in vitro* ageing can have functional consequences. The ability of aged platelets to support coagulation reactions and the generation of thrombin shows that the annexin V positivity equates to expression of functionally active phospholipids. It is also possible that platelets expressing negatively charged aminophospholipids may be cleared from the circulation more rapidly (Hirt and Leist, 2003, Fadok et al., 2001).

In normal platelets, increased expression of PS on platelets provides a procoagulant surface for the enhanced activity of the prothrombinase complex and the resulting generation of thrombin (Monroe et al., 2002). The observed increase in thrombin generation with storage in the Scott platelets suggested that the PS expressed on the surface of these platelets retained one of its primary functions, though whether this functional activity is retained *in vivo* remains to be ascertained. A further study employing the apoptosis-inducing BH3 mimetic ABT-737 resulted in increased annexin V binding in Scott platelets. Although annexin V binding was lower compared to the normal controls, the results were consistent with the suggestion that at least part of the changes observed during the ageing study may be caused by a process of cell death akin to apoptosis. It was not possible to repeat the experiments on the Scott platelets because of limitations on the amount of blood that can be taken; however, these data were replicated by an external laboratory (Professor Bevers' laboratory, Maastricht).

In both the extrinsic and intrinsic pathways of apoptosis, caspases are the cysteine proteases associated with the execution of cell death (Kumar, 2007). The addition of a pan caspase inhibitor to the suspending medium of a platelet concentrate unit would thus be expected to confirm or refute the role of apoptosis in platelet death during storage. This would be an area worthy of further investigation.

EXPLORATORY INVESTIGATION INTO THE ROLE OF APOPTOSIS IN PLATELETS STORED AS CONCENTRATES

INTRODUCTION

The Bcl-2 family of proteins are key regulators of cell death by apoptosis (Chipuk et al., 2010, Brunelle and Letai, 2009). If the machinery of apoptosis is viable in platelets stored as concentrates in additive solution, the addition of the BH3-only mimetic ABT-737 should result in enhanced expression of aminophospholipids on the platelet surface. Of particular interest in this respect is the role of glucose, in view of the association between glucose depletion and increased annexin V binding described in chapter 5. A comparative study was undertaken in which samples from identical platelet concentrates in SAS, with or without added glucose, were obtained at periodic intervals over a 14-day storage period and either incubated with ABT-737 or vehicle only. Cell death was determined by the expression of aminophospholipids on the platelet surface, as measured by annexin V binding.

METHOD

Unit and sample preparation

A buffy coat-derived platelet concentrate was centrifuged at 2400 *g* for 10 minutes at 22°C to pellet the platelets. As much of the plasma as possible was drained into a waste pack, and the pellets re-suspended by gentle manual mixing in 300 mL of SAS. Sodium bicarbonate and acetate were added aseptically to final concentrations of 20 mmol/L and 28 mmol/L, respectively. The unit was subsequently split into two neonatal packs with unit volumes of 50 mL. Glucose was aseptically added to one of the packs to a final concentration of 30 mmol/L, with an equal volume of diluent added to the remaining unit.

Samples were removed from the units on days 2, 5, 8, 10 and 14. The samples were split into pairs and diluted in autologous plasma to a platelet concentration of approximately 200×10^9/L. To one of the samples was added ABT-737 to a final concentration of 10 µmol/L. An equal volume of DMSO vehicle was added to the second sample. Units containing ABT-737 were incubated at ambient temperature for 3 hours before analysis.

Assays

Glucose levels were measured from the units before sample preparation on the days of testing. Due to the association observed in the glucose study (chapter 5) between annexin V binding, intracellular free calcium and ATP levels, the last two parameters were measured in addition to annexin V binding. All assays were performed as previously described.

RESULTS

The parent PC was confirmed to be leucoreduced and all four neonatal packs were negative for bacterial contamination at end of storage. Glucose levels in the two units with exogenous glucose were depleted between days 10 to 14. There was progressive disruption of platelets with increasing storage in units with no added glucose, determined by a decrease in platelet concentration (table 7.4 and figure 7.7).

Table 7.4: Impact of the BH3-only mimetic ABT-737 on platelet concentration in the presence and absence of glucose in an artificial storage medium

	PLATELET CONCENTRATION ($\times 10^9$/L)			
	No Glucose		30 mmol/L Glucose	
	ABT-737 POS	ABT-737 NEG	ABT-737 POS	ABT-737 NEG
Day 2	202	202	205	215
Day 5	172	188	204	179
Day 8	150	144	215	206
Day 10	108	128	198	204
Day 14	70	59	165	178

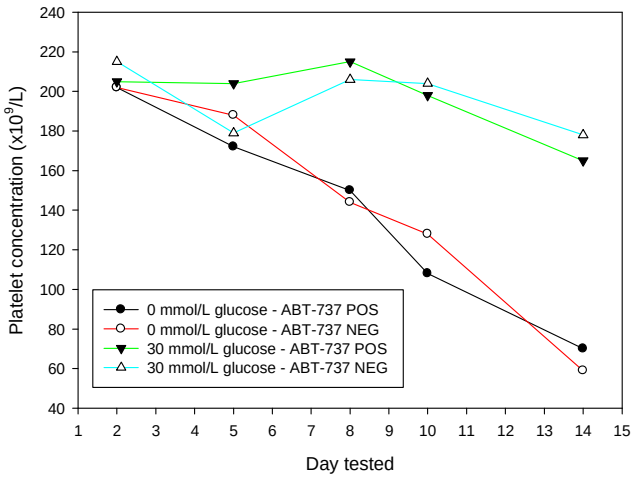


Figure 7.7: Impact of the BH3-only mimetic on platelet concentration in PC stored with or without glucose in the suspending medium (results from single experiment).

Annexin V Binding

On day 2, the addition of ABT-737 resulted in increased annexin V binding in samples with and without added glucose compared to controls (figure 7.8), indicating that the machinery that induces apoptosis was intact at this time. With the exhaustion of glucose in the unit, there was a rapid increase in annexin V binding between days 10 to 14. In units without glucose, there was a relatively rapid increase in annexin V binding with progressive storage, with no clear indication that the addition of ABT-737 caused an increase in percent positive expression beyond day 2 (table 7.5). This suggests that the machinery that induces apoptosis was not functional at these timepoints.

In the units with added glucose, the addition of ABT-737 resulted in increased annexin V binding on days 2 and 5 and possibly day 8, suggesting the apoptosis-inducing machinery was functional at these timepoints but was not functional after day 8.

Table 7.5: Impact of the BH3-only mimetic ABT-737 on annexin V binding in PC stored in SAS in the presence and absence of glucose

	ANNEXIN V BINDING (% POSITIVE)			
	No Glucose		30 mmol/L Glucose	
	ABT-737 POS	ABT-737 NEG	ABT-737 POS	ABT-737 NEG
Day 2	22.6	7.37	24.26	8.25
Day 5	44.38	37.66	25.42	14.12
Day 8	67.43	65.31	29.63	22.03
Day 10	83.14	81.19	24.07	25.66
Day 14	97.26	97.34	82.09	79.15

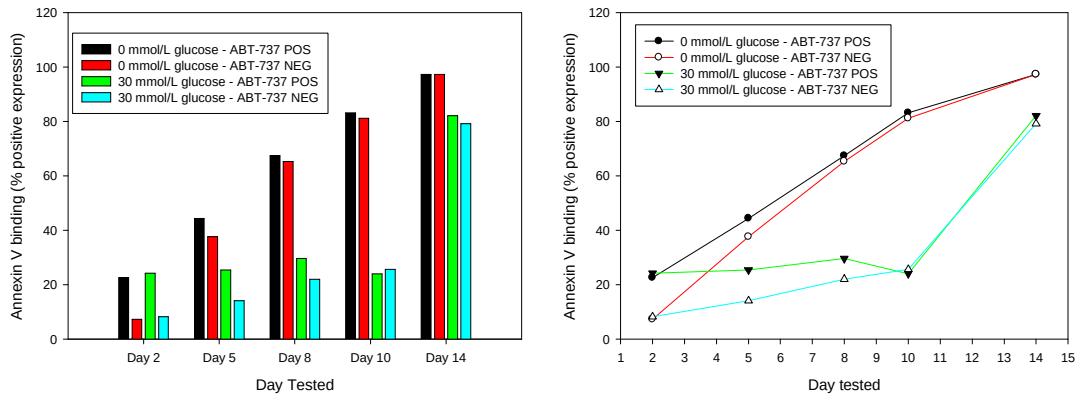


Figure 7.8: Impact of the BH3-only mimetic on annexin V binding as percent positive expression in PC stored with or without glucose in the suspending medium (results from single experiment).

Intracellular free calcium

The absence of glucose resulted in a progressive increase in intracellular free calcium throughout the storage. Glucose depletion coincided with a marked increase in intracellular free calcium after day 10 in units with added glucose (table 7.6 and figure 7.9). The addition of ABT-737, by contrast, did not result in a clear difference in intracellular free calcium compared to the control sample.

Table 7.6: Impact of the BH3-only mimetic ABT-737 on levels of intracellular free calcium in PC stored in SAS in the presence and absence of glucose

	INTRACELLULAR FREE CALCIUM (Fluo-4/FuraRed MFI ratio)			
	No Glucose		30 mmol/L Glucose	
	ABT-737 POS	ABT-737 NEG	ABT-737 POS	ABT-737 NEG
Day 2	0.31	0.23	0.35	0.22
Day 5	1.09	0.83	0.43	0.30
Day 8	2.12	1.77	0.52	0.42
Day 10	2.75	3.25	0.43	0.89
Day 14	4.63	3.80	2.87	2.80

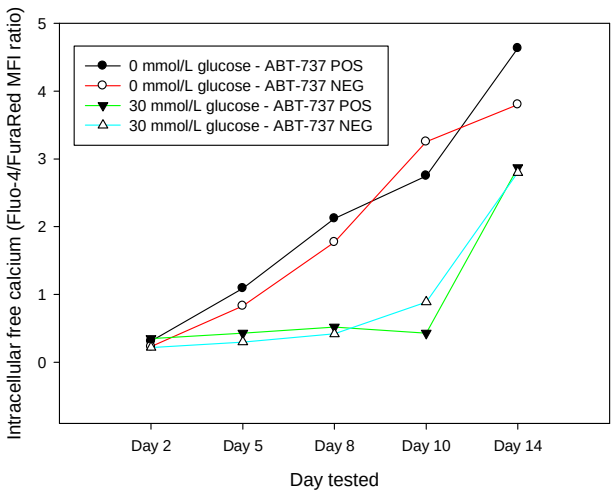


Figure 7.9: Impact of the BH3-only mimetic on intracellular free calcium levels in PC stored with or without glucose in the suspending medium (results from single experiment).

ATP Levels

As expected from the glucose study, an absence of glucose in the storage medium resulted in a more rapid decline in ATP levels with storage (table 7.7 and figure 7.10). As with intracellular free calcium, the addition of ABT-737 did not result in a clear difference in ATP levels compared to the control sample.

Table 7.7: Impact of the BH3-only mimetic ABT-737 on ATP levels in PC stored in SAS in the presence and absence of glucose

	ATP (µmol/10^{11} plts)			
	No Glucose		30 mmol/L Glucose	
	ABT-737 POS	ABT-737 NEG	ABT-737 POS	ABT-737 NEG
Day 2	5.112	5.488	4.419	4.965
Day 5	2.88	2.734	3.955	3.697
Day 8	1.68	1.729	3.416	3.398
Day 10	0.656	0.571	2.840	3.125
Day 14	N/A	0.026	0.485	0.408

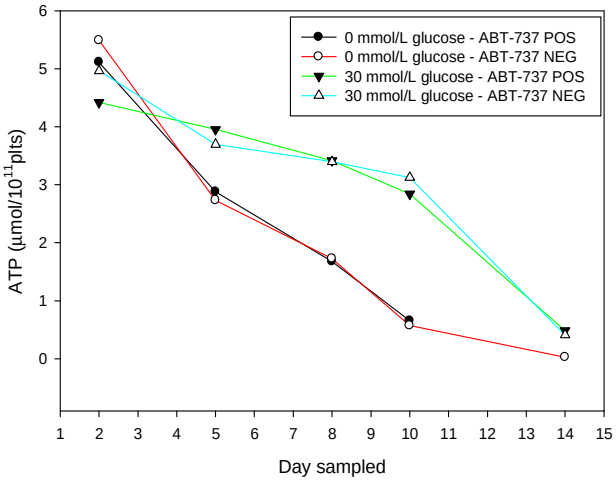


Figure 7.10: Impact of the BH3-only mimetic on ATP levels in PC stored with or without glucose in the suspending medium (results from single experiment).

DISCUSSION

On day 2, platelets responded to the addition of the BH3 mimetic ABT-737 with increased translocation of aminophospholipids to the platelet surface, a response that was still observed (though muted) in the unit with added glucose on day 8. This

suggested that mechanisms leading to apoptotic driven expression of aminophospholipids were intact and could be contributing to the annexin V positivity observed with *in vitro* ageing. After that time ABT-737 did not increase annexin V positivity, implying that mechanisms other than apoptosis would have been required to externalise aminophospholipids in old platelets.

In the unit with no added glucose, mechanisms for apoptotic cell death were intact at day 2 but not after that time, and other mechanisms would have been required to externalise aminophospholipids. ATP levels on day 5 had already declined to approximately 50% of day 2 values in the platelets with no added glucose. This may have prompted a mechanism of cell death that was more akin to necrosis (Leist et al., 1997, Skulachev, 2006). This was reflected in the increasing levels of intracellular free calcium which would also be compatible with necrosis (Rizzuto et al., 2003, Rasola and Bernardi, 2011), resulting in increased mitochondrial dysfunction and ultimately platelet disruption, as observed by the decrease in platelet concentration.

Thus, platelets stored as concentrates in additive solution initially have functional machinery for a Bcl-2 protein-mediated mechanism of cell death. This may be superseded with progressive storage by a mechanism more akin to necrosis as energy stores are depleted. This is exacerbated and occurs earlier during storage in media lacking glucose, where the maintenance of ATP levels by glycolysis may be aided by the maintenance of mitochondrial function via antioxidant mechanisms mediated via the pentose phosphate pathway (Brookes et al., 2004).

Following confirmation of the results reported above, the next logical step would be to undertake a similar storage study in the presence or absence of a caspase inhibitor. Addition of a broad spectrum caspase inhibitor such as Q-VD-OPh (Caserta et al., 2003) to the unit would be expected to negate an increase in annexin V binding caused by ABT-737 if caspase-dependent apoptosis is indeed a viable mechanism of cell death for platelets in storage.

8. DISCUSSION

A progressive deterioration of *in vitro* characteristics associated with platelet function, morphology and metabolism are characteristic of the storage of platelets as concentrates and collectively referred to as the platelet storage lesion (PSL). Platelet activation, the release of potentially harmful bioactive substances from cellular elements, changes in morphology and either the inability to maintain or suppress platelet metabolism, have all been associated with the phenomenon (Picker et al., 2009, Smyth et al., 2009). The suggestion that a programmed mechanism of cell death may be involved in the development of the PSL has been more recently considered following studies investigating the possibility that platelets are capable of displaying responses typically associated with apoptosis in other cell types (Leytin and Freedman, 2003, Rinder and Smith, 2003, Kile, 2009). However, it is difficult to assign a causative element to these observations, and no comprehensive model of the PSL is currently available. This thesis aimed to contribute to the current debate on the origin and mechanism of the PSL, proceeding under the hypothesis that a process akin to apoptosis serves as a central mechanism to explain the variety of changes associated with the phenomenon. A concurrent applied aim for the investigations was to provide insight into the manufacturing and storage of platelet concentrates that may enhance the viability and efficacy of platelets post-transfusion.

The progressive changes described in the literature in association with the PSL were confirmed in the fourteen-day storage study using PC suspended in plasma or a 70% SSP+™ additive solution, with the work adding to the currently limted body of data available for characteristics of the PSL beyond the storage periods recommended by official guidelines. The subsequent investigations suggested that a Bcl-2 protein-mediated mechanism of cell death was viable in platelets in the presence of adequate stores of energy in the form of glucose. Depletion or absence of glucose resulted in platelet death by a mechanism associated with decreased ATP levels, accelerated aminophospholipid translocation and platelet disruption which was more reminiscent of necrosis.

The study investigated the PSL in buffy coat-derived platelet concentrates suspended in a platelet additive solution containing minimal concentrations of plasma and leucocytes. The ability to more comprehensively control the composition of a relatively simple artificial medium allowed for comparative studies to be undertaken on the role of individual constituents of platelet additive solutions. White cells are known to affect the *in vitro* characteristics of platelets through the release of cytokines and other bioactive substances (Mueller et al., 2008), whilst the complex nature of plasma may complicate the interpretation of results. By substantially reducing the influence of these two factors it was possible to more directly interpret results as the response of platelets to the storage conditions. It has been suggested that the various changes associated with the PSL may be assigned to categories describing platelet metabolism, activation, morphology and senescence (Rinder and Smith, 2003, Li et al., 2005a). A range of assays was adopted to determine the response of platelets across these broadly-defined categories. In particular, repeated measures over the storage period may offer an insight into the timeline of events, which may in turn suggest avenues for further research into the relationship between the different aspects of the PSL.

An initial study was undertaken on buffy coat-derived PC suspended in either 100% autologous plasma or a combination of SSP+™ additive solution and plasma in an approximate 70:30 ratio over an extended storage period of 14 days. Current UK guidelines restrict the shelf-life of PC to a maximum of seven days (James, 2005). As a result, studies investigating the ability of novel suspension media to maintain platelet function and viability in storage have often understandably limited themselves to obtaining measurements up to the accepted shelf-life or shortly thereafter. To determine whether it is feasible to consider extending the shelf-life of PC beyond the current limits, it would be useful to obtain information on the *in vitro* characteristics of platelets over an extended period of storage using present manufacturing processes and storage conditions. Such comparative studies extending to storage periods beyond seven days are relatively uncommon in the literature (Cardigan et al., 2005), though more recent publications have begun to address the issue (Sandgren et al., 2010). In addition, results from the subsequent studies in additive solutions using the same assays would be more directly comparable than comparisons against published data, since significant variability in the values of assays can be found in the published literature for some of

the parameters of interest (Girotti et al., 1989, Curvers et al., 2008). A paired study design that pooled the requisite number of buffy coats to provide two identical starting PCs would have negated any donor variability and increased the power of the comparative analysis. However, such an approach would deviate from standard manufacturing processes and it was deemed more appropriate for this study to remain within the confines of established practice.

Units were sampled on days 1, 2, 3, 6, 8 10 and 14. Regular sampling at the beginning of the storage period was important to characterise early changes in the *in vitro* characteristics of platelets stored in additive solutions in subsequent studies, as the storage conditions and processing methods were expected to be harsher than standard methods and thus possibly lead to accelerated expression of the PSL. In addition, it is possible that changes in parameters associated with the PSL early in the storage period may be evident in standard units and may offer insight into the subsequent development of the PSL. Published studies tend to omit measurements early in the storage period, as their generally stated aim is to determine the suitability of PCs for transfusion at the end of shelf-life. This study thus offered the opportunity to investigate this often neglected early period of storage. However, such an extensive series of measurements inevitably results in a marked volume loss. It is unclear whether such a change in the ratio of unit volume to pack surface area would artificially affect the *in vitro* characteristics towards the end of storage, or whether any impact would prove significant; thus limiting the study to a comparative investigation. Further work in which similarly processed units were only sampled on days 1 and 14 would help to address this question, but could not be undertaken at the time due to time and processing restrictions.

For clarity, the following discussion is separated into sections. The first section examines the results obtained in the study comparing the *in vitro* storage characteristics of platelets suspended in a 70:30 SSP+™ and plasma medium against platelets suspended in 100% autologous plasma. The second section discusses the results from the series of studies using variously-modified standard additive solution as a suspension medium. (However, the studies cannot be regarded in total isolation and the discussion will reflect this where appropriate.) The subsequent series of experiments undertaken in collaboration with the University Hospital of Wales to more directly explore the mechanisms for annexin V binding in platelet ageing by investigating the characteristics

of platelets from a Scott Syndrome patient, as well as the role of a Bcl-2 protein family-mediated initiation of cell death in platelets during storage are discussed next. These sections serve as the foundation for suggested models of platelet death in the storage conditions evaluated in the thesis and determine whether they support the hypothesis that apoptosis is a central player in the PSL. The limitations of the thesis are also evaluated as are suggestions for future potential studies that would build on the many questions generated from this work.

IN VITRO STORAGE CHARACTERISTICS OF PLATELET CONCENTRATES STORED IN 100% AUTOLOGOUS PLASMA VERSUS A MEDIUM COMPRISED OF 70% SSP^{+}™ AND 30% PLASMA

The expected decrease in extracellular pH with storage was evident after day 6 in PCs suspended in plasma. This is generally attributed to the consumption of bicarbonate which acts as the principal buffer in plasma by taking up H^+ ions produced by glycolysis and converting them to H_2O and CO_2 via carbonic acid. Carbon dioxide is able to escape through the gas-permeable plastic of the storage pack, thus maintaining pH levels (Wallvik and Akerblom, 1990, de Wildt-Eggen et al., 1998) (figure 8.1).

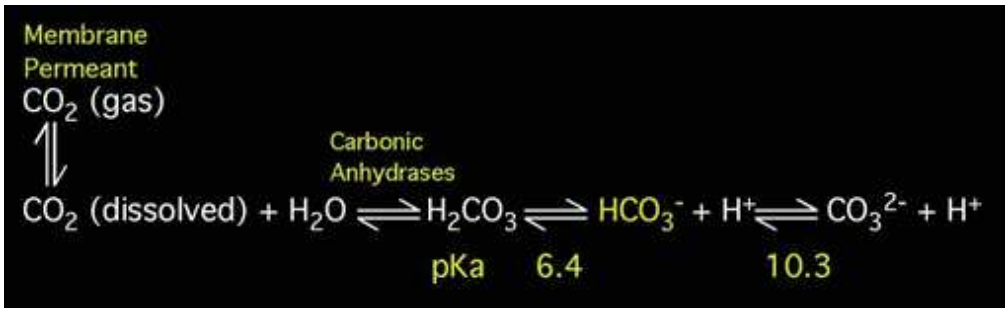


Figure 8.1: Reactions associated with the buffering capacity of bicarbonate in plasma (Cordat and Casey, 2009) (Reproduction permitted with accreditation, Portland Press, ©The Biochemical Society)

There is sufficient bicarbonate present in plasma to buffer the platelet concentrates until lactate levels reach approximately 20 mmol/L, after which pH levels have been shown to decrease at an accelerated rate (Murphy, 1999). It may be difficult to entirely attribute the decrease in pH after day 6 to exhaustion of bicarbonate in the plasma, since previous studies have shown bicarbonate levels in buffy coat-derived PCs remain relatively constant for up to seven days of storage (Sandgren et al., 2008, Gulliksson, 2001). The

increase in pH observed in plasma units during the first three days of storage has been previously reported, both in pooled units and in PCs collected by apheresis technologies (Krailadsiri et al., 2001, Goodrich et al., 2006). This may be related to the rapid decrease in pCO_2 levels noted in these units between days 1 and 3, suggesting accumulated CO_2 was being rapidly lost through the storage pack. The more alkaline conditions would also reduce the solubility of CO_2 – a further potential factor contributing to the marked decrease in pCO_2 in these units (Zhang et al., 2008b). In contrast to the plasma units, pH values in all SSP^{+}™ units remained above 7.0 throughout the storage period, with pH increasing markedly after day 10 due to the stalled lactate production following the depletion of glucose. In common with all commercially available additive solutions, SSP^{+}™ does not contain glucose. The only glucose available to these units would have originated from the reduced volume of autologous plasma, and this was effectively depleted by day 10.

The relatively reduced partial pressure of carbon dioxide in the SSP+™ units throughout the storage period reflects the reduced lactate production in this group. The difference in pCO_2 between plasma and SSP+™ units was most significant at the start of the storage period, suggesting the preparation of the units had an impact on this parameter. The only major difference in the preparation was the use of additive solution instead of plasma. The excess CO_2 in the plasma is most likely to have originated from glycolytic red cell activity in the whole blood unit and remained in the impermeable plasma pack ready to be transferred to the platelet concentrate. However, once in the gas-permeable PC storage packs, CO_2 was allowed to escape to the atmosphere resulting in a rapid decline of pCO_2 at the start of the storage period. It remains to be determined if the platelets are adversely affected by this transiently high level of CO_2. Levels of pO_2 and rates of oxygen consumption were similar in both groups of PCs. The steady increase in pO_2 in both groups over the storage period reflects the high gas permeability of the current generation of storage packs, since oxygen consumption was relatively stable throughout storage.

The stable rates of oxygen consumption coincided with the maintenance of ATP for the first ten days of storage in both plasma and SSP^{+}™ units, with day 10 levels remaining over 85% of starting values. The increase in ATP concentration over the first few days

of storage, particularly evident in plasma units, was somewhat surprising and was instrumental in maintaining ATP levels in both groups. However, a recent study in BC-PC has also reported increases in ATP levels that did not reduce below starting levels until after 7 days of storage (Sandgren et al., 2010). Absolute levels of ATP quoted in the literature vary considerably. A review of ATP levels in human platelets from whole blood samples found levels varied between 3.78 ± 0.68 to 7.5 ± 1.7 µmol/10^{11}plts. A major variable is the use of different assay methodologies, with methods ranging from high-pressure liquid chromatography (HPLC) to bioluminescence and spectrophotometric assays. However, even with methods employing the same general principles, considerable variation has been reported (Girotti et al., 1989). Similar variability is still evident in relatively recent storage studies, with levels in plasma units on day 1 varying between 4.7 ± 0.3 µmol/10^{11}plts (Gulliksson et al., 2000) to 8.28 ± 0.40 µmol/10^{11}plts (Sandgren et al., 2008).

On activation platelets release the contents of their storage granules, including the platelet agonists ADP and serotonin from the dense granules. ADP levels have not been commonly reported in the transfusion literature. An early study by Rao et al obtained mean levels of 3.5 ± 0.2 µmol/10^{11}plts in fresh PRP-PC; substantially lower than the mean levels obtained in the current study (Rao et al., 1981). Levels in the Rao study decreased by approximately 40% in 72 hours - a similar decrease was evident after day 8 of storage in the present study. A more recent study reported that levels in buffy coat-derived PC were maintained in the first four days of storage, but declined thereafter (Botchway et al., 2000). Both papers observe that the progressive decline in ADP with storage could be interpreted as an acquired storage pool defect, with Botchway et.al suggesting the effect may not be reversible even after transfusion, with implications for the efficacy of platelet transfusions. The δ-storage pool diseases are characterised by defective platelet dense granules, with mild to severe bleeding as one of the clinical manifestations (Simon et al., 2008, Sandrock and Zieger, 2010). An acquired defect of similar nature in stored platelets may be of clinical relevance and may account for some of the variation in the efficacy of PC transfusions to control bleeding (Apelseth et al., 2010). Particularly relevant scenarios include the transfusion of patients treated with anti-platelet agents such as clopidogrel which inhibit the ADP receptor $P2Y_{12}$ (Cattaneo, 2011) - such patients may benefit from the transfusion of fresh platelets to combat haemorrhage. The variable patient response to treatment with such anti-platelet

agents may be compounded by individual variability in the response to ADP by the transfusion recipient, possibly due to variation in the structure or density of the ADP receptors on the platelet surface (Michelson, 2004, Michelson et al., 2007, Fontana et al., 2003). ADP elicits its role in platelet aggregation by binding to neighbouring platelets via two G-protein coupled P2 receptors (Kunapuli et al., 2003). Binding to the $P2Y_{12}$ receptor releases the G_i protein subunits α_{Gi} and $\beta\gamma$. The former inhibits adenylyl cyclase (AC) which in turn decreases levels of cyclic adenosine monophosphate (cAMP) resulting in reduced phosphorylation of vasodilator-stimulated phosphoprotein (VASP); a process which would otherwise inhibit the activation of the GPIIb/IIIa receptor. The $\beta\gamma$ fragment activates phosphatidylinositol 3-kinase (PI3K), which promotes further granule secretion in a positive feedback loop (Woulfe et al., 2001, Johnston-Cox et al., 2011, Nguyen et al., 2005) (figure 8.2).

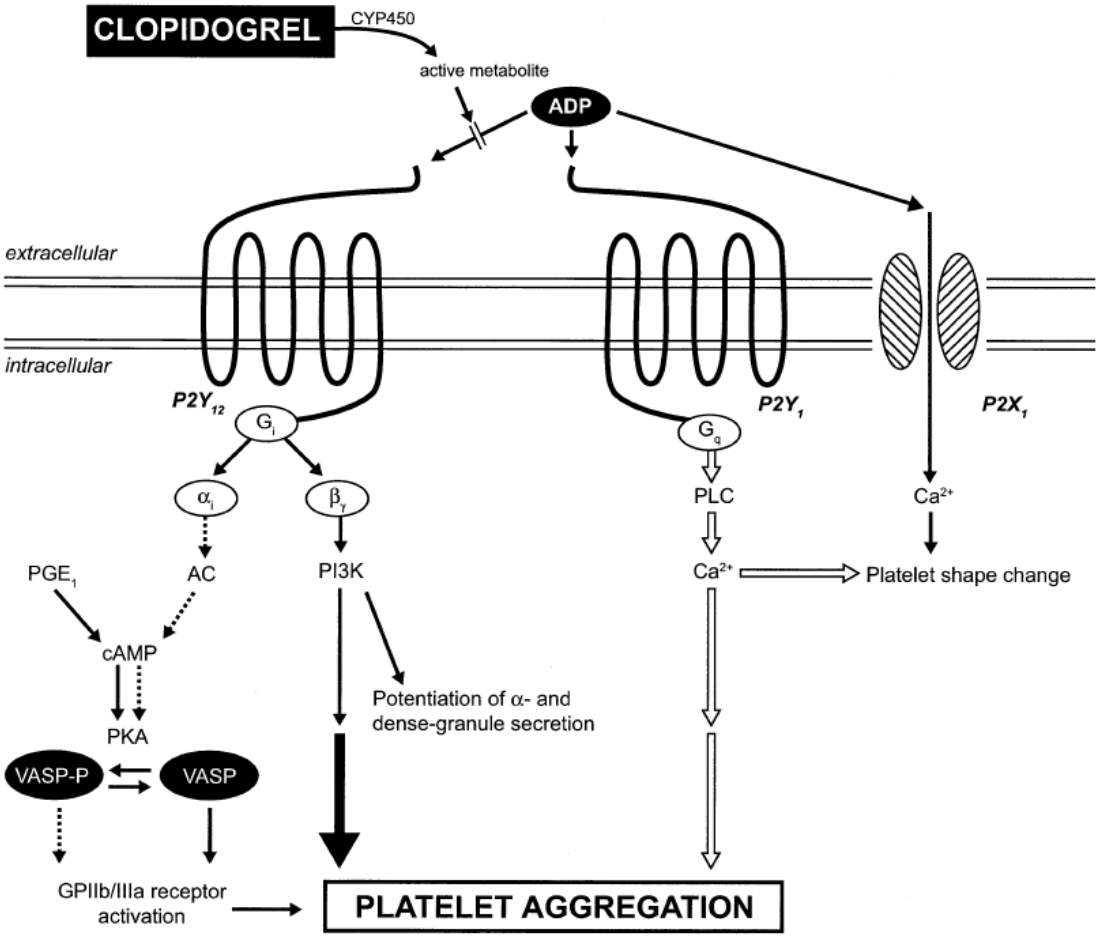


Figure 8.2: The relationship between ADP and platelet P2 receptors and their role in platelet aggregation, including the inhibitory effect of the anti-platelet drug clopidogrel which irreversibly binds to the $P2Y_{12}$ receptor (Nguyen et al., 2005) (Reproduced with permission; © American College of Cardiology Foundation)

A limitation of the study in this context was the lack of platelet aggregation assays which would have provided more direct information on platelet function. Nevertheless, the ADP results point to a different mechanism being associated with this aspect of the storage lesion that appears unrelated to the energy stores of the platelets.

Platelet activation as a consequence of processing and storage of PC has been commonly measured in recent years by the flow cytometric estimation of CD62P on the platelet surface. CD62P is constitutively expressed on the membrane of α-granules. As part of the secretory response following platelet activation, the membrane of the α-granules fuses with the plasma membrane, resulting in an increased expression of the CD62P glycoprotein on the platelet surface (Berman et al., 1986, Jurk and Kehrel, 2005). With increasing platelet activation expected during storage as the environment in which the platelets are suspended degrades, an increase in the percentage of platelets expressing CD62P would be expected. Interestingly, the percentage positive expression of CD62P decreased during the initial period of storage, only increasing after day 3 in plasma units and day 6 in SSP^{+TM} units; a similar, though more subtle decrease, was also evident with the median fluorescence intensity. A survey of the literature resulted in a recent study by Zhang et al that also reported a decrease between days 1 and 2 of storage in BC-PC suspended in various media (Zhang et al., 2008b). Such a decrease would suggest that CD62P is either being re-internalised or shed from the surface. Re-internalisation of CD62P would potentially allow the protein to be re-expressed on subsequent platelet activation. However, studies measuring the response of stored platelets to physiological agonists have concluded that an increased concentration of thrombin is required to elicit 50% positive expression of CD62P in platelets from 7 day-old PCs compared with platelets from PCs stored for 3 days (Cardigan and Williamson, 2003). This depressed response may be indicative of a reduced store of CD62P in α-granules that is not being replenished by the re-uptake of previously expressed CD62P (Jilma-Stohlawetz et al., 2008). Studies using a CD62P-deficient murine model described the presence of a soluble form of CD62P in the plasma of these animals within 15 minutes of the transfusion of activated wild-type platelets and identified it as a 100-kDa fragment by Western blotting (Berger et al., 1998). The same study affirmed the hypothesis that expressed CD62P was cleaved from the platelet surface by re-infusing thrombin-activated biotinylated platelets into mice. The re-infused platelets

were found to lose their surface CD62P expression and were not found to re-acquire it after exposure to thrombin or the calcium ionophore A23187.

CD62P is a member of the selectin family of adhesion molecules. Initial interest in the introduction of an assay to measure surface-expressed CD62P on platelets was based on the observation that its ligand, PSGL-1, was present on leucocytes (Larsen et al., 1989). This led to the suggestion that binding of platelets expressing CD62P to leucocytes, particularly neutrophils and monocytes (Gutensohn et al., 2002), could lead to accelerated clearance of these platelets from the circulation and thus explain the decrease in recovery and survival of platelets after transfusion, especially following the transfusion of older PCs with increased expression of the protein. Two studies often discussed in relation with this hypothesis reported inverse correlations between the degree of platelet activation, as determined by the surface expression of CD62P, and platelet recovery up to one hour post-transfusion in either normal volunteers or thrombocytopenic patients (Rinder et al., 1991, Triulzi et al., 1992). Subsequent studies with animal models, however, have found that thrombin-activated, degranulated platelets are able to circulate and function after shedding the expressed CD62P (Berger et al., 1998, Michelson et al., 1996). It is possible that the two seemingly contradictory results reflect a time-course of events, with CD62P expression involved in an initial clearance of a proportion of the transfused platelet population. A time limit would be imposed by the shedding of CD62P from the surface. Indeed, studies have demonstrated that 50% of CD62P is lost from the platelet surface during the first 5.5 hours after transfusion (Leytin et al., 2004). Loss of CD62P from the platelet surface *in vivo* may render platelets less adhesive to leucocytes expressing the PSGL-1 receptor and thus prevent excessive leucocyte recruitment to the site of injury, which could otherwise lead to damaging levels of inflammation (Li et al., 2011).

If the decrease in the percentage positive expression was due to the proteolytic cleavage of surface-expressed CD62P, an increase in the soluble fraction of CD62P (sCD62P) would be expected. Increases in sCD62P are indeed evident throughout the storage period, but the rate of increase is relatively stable and does not reflect the variation in surface expression of the molecule. A tentative explanation would involve a proportion of the initially activated population of platelets shedding the majority of the surface-expressed CD62P into the medium, accounting for the initial increase in sCD62P and

apparent recovery from activation measured by flow cytometry (also see (Zhang et al., 2008b). Subsequent rates of CD62P externalisation and cleavage may occur in a more measured manner as conditions during storage change more gradually or simply as a result of progressive ageing of the platelet population, resulting in a more direct relationship between surface expression and shedding of CD62P. A clearer quantitative association between the surface expression of CD62P and the concentration of the soluble fraction would be necessary to explore this further. The increase in median fluorescence intensity (MFI), particularly evident in plasma units after day 10 of storage, suggests that externalisation of CD62P on individual platelets is not an all-or-nothing response. The possibility of a graded response is also evident from a study by Rinder et al which demonstrated a four-fold higher CD62P expression per platelet following activation with thrombin compared to activation by a combination of epinephrine and ADP (despite similar percent positive expression) (Rinder et al., 1991). If an increase in MFI is related to a stronger stimulus, the rise noted in this parameter after day 10 may suggest a deterioration of the storage environment. For plasma units, this may be related to the decrease in extracellular $pH_{22°C}$ to median levels of 6.6.

Two further observations related to the externalisation and shedding of CD62P require discussion. Firstly, the levels of sCD62P in SSP^{+}™ units at the start of the storage period and secondly, the relatively high levels of percent positive expression of CD62P in comparison to the published literature. Median levels of sCD62P in plasma units were 89.7 ng/mL at the start of the storage period. This reflects levels in normal human plasma ranging between 36 – 250 ng/mL (Ushiyama et al., 1993, Katayama et al., 1992, Dunlop et al., 1992). Considering that only about 30% of plasma was carried over into the SSP^{+}™ units, the expected median concentration of sCD62P in SSP^{+}™ units would be 26.9 ng/mL. Measured levels were approximately twice this value (median of 52.0 ng/mL). Similar proportional levels were reported in a recent study comparing various *in vitro* characteristics in PC suspended in plasma or two different platelet additive solutions in a 70:30 PAS to plasma ratio (Cardigan et al., 2008). A possible explanation would be an increase in platelet activation caused by the processing of the two groups of PCs. However, the only difference in the processing of the two groups was the replacement of 70% of plasma with SSP^{+}™. In addition, other *in vitro* parameters were similar in both study groups on day 1; in particular, both measures of the surface expression of CD62P. It is possible that the source of the plasma carried over into the

SSP^{+}™ units is the source of the high sCD62P levels. This plasma remnant is obtained from the residual plasma bathing the buffy coats, which are held overnight prior to processing. Berger et al reported that infusion of platelets incubated in whole blood led to a significant decrease in CD62P expression after re-infusion into mice compared with platelets incubated in either platelet-poor-plasma or a buffer solution, suggesting increased shedding of CD62P from platelets in the presence of other cellular elements (Berger et al., 1998). If the overnight storage of platelets in a buffy coat promoted the externalisation and shedding of CD62P into the residual plasma, these proportionately high levels of sCD62P would have been retained in the SSP^{+}™ units and reflected in the day 1 values. Measuring the concentrations of soluble CD62P in buffy coat-derived plasma as well as in the plasma units retained separately for the preparation of BC-PC in 100% plasma would help to determine if this explanation accounts for the measured levels of sCD62P.

A number of storage studies have reported percent positive values for surface CD62P ranging between 12.6% and 36.6% on day 1 (with units in either 100% plasma or a PAS-IIIM formulation) (Keuren et al., 2006, Dijkstra-Tiekstra et al., 2004, Cardigan et al., 2008, Wagner et al., 2008, Wagner et al., 2002, Hornsey et al., 2006). These published values compare with median levels of 53.5% in the plasma group and 55.7% in SSP^{+}™ units on day 1. As the units were processed by standard procedures, it seems unlikely that the relatively high values in this study reflect greater platelet activation compared to other studies. It is more likely that the relatively high values are due to assay methodology. In the studies referenced above in which details of the methodology were provided, the samples were fixed either before or after staining. The assay used in these studies omitted fixing the samples in preference of immediate analysis after staining. In addition, the assay combined the measurement of CD62P with annexin V binding. Binding of CD62P occurred initially with a 15-minute incubation, with the annexin-V-FITC probe requiring a second incubation in the same sample tube. Without fixation, it is possible that platelet activation could continue in the sample tube during the second incubation, resulting in higher surface expression of CD62P. However, the combined incubation of 30 minutes with anti-CD62P is not excessive compared to published methodologies. A further variable which may be of significance is the use of phycoerythrin (PE)-conjugated anti-CD62P instead of a FITC-conjugated antibody, as PE is an inherently brighter fluorescent stain than FITC (Shapiro, 2003) (though both

stains are referred to in the literature). Further variables posited as having an impact on CD62P surface expression include the instrument used, the choice of antibody clone and the definition of the gating for the isotypic control; that is, the percentage of negative events included in the gate used to define CD62P positivity (Curvers et al., 2008). The adopted method used a Beckman Coulter FC500 and Immunotech clones (Beckman Coulter, Milton Keynes, UK) for both the anti-CD62P stain and the isotypic control. The CD62P gate was set on one percent of negative events, following common practice (Ormerod, 2000). Additional investigations to determine whether particular aspects of the assay methodology are responsible for the relatively high percentage positive expression should initially centre on the role of fixation. The study by Curvers et al described a statistically significant reduction in CD62P staining when cells were fixed, though the gross differences did not appear sufficient to account entirely for the variation in results between the adopted assay and the referenced literature. The approach of combining the anti-CD62P and annexin V binding and its possible role may also be worth re-visiting, though the initial development of the assay did not suggest an impact on results.

The possible involvement of programmed cell death in the platelet storage lesion was investigated through the increased expression of aminophospholipids on the outer leaflet of the plasma membrane and the disruption of the mitochondrial membrane potential ($\Delta\Psi m$). The former was measured by the binding of fluorescently-conjugated annexin V to aminophospholipids on the platelet surface, whilst disruption of the membrane potential was measured by changes in the mitochondrial uptake of the cationic dye JC-1. As described in the introduction, the loss of phospholipid asymmetry that leads to a proportionate increase in aminophospholipids on the outer leaflet of the plasma membrane of activated platelets is important for normal coagulation. The increased expression of phosphatidylserine on the outer leaflet, as demonstrated by annexin V positivity, is also commonly seen in cells undergoing apoptosis and is generally regarded as an early indication of a cell entering a process of programmed cell death (Martin et al., 1995, Schlegel and Williamson, 2001, Leventis and Grinstein, 2010). (However, it should be stressed that increased phosphatidylserine expression is not confined to an apoptotic mechanism of cell death and has been also been reported in various examples of cell death by necrosis (Krysko et al., 2004, Galluzzi et al., 2009, Jackson and Schoenwaelder, 2010, Hirt and Leist, 2003, Poon et al., 2009)). The higher

levels of percent positive expression in units suspended in 100% plasma compared with SSP^{+}™ contrasted with relatively lower mean fluorescence intensities in the same units; the latter indicating a lower expression of aminophospholipids per platelet in units suspended in plasma. The kinetics of annexin V binding per platelet in the two groups throughout the storage period was similar. MFI levels generally decreased in both groups between days 1 and 10. One possible interpretation is that externalisation of aminophospholipids was transiently increased during processing, with the normal phospholipid asymmetric distribution gradually re-established in the more controlled conditions of standard storage. The relatively higher MFI levels in SSP^{+}™ suggests the initial phospholipid disruption was more marked in these units (and may have continued in the initial period of storage, as suggested by the increase in levels between days 1 and 2 in these units). The similar rate of decrease in MFI levels between the two study groups over time suggests a standard rate of activity for the ATP-dependent translocase associated with the inward movement of aminophospholipids.

The higher percent positive expression during most of the storage period in platelets suspended in plasma suggests that although more platelets were expressing detectable levels of aminophospholipids, the concentration of these phospholipids was lower per platelet than for platelets in SSP^{+}™ units. The processing-related increase in annexin V binding per platelet in both study groups is limited to a small subpopulation of platelets, as less than 5% of platelets exhibit annexin V binding at the start of storage. Similar starting values have been reported in the literature for PCs prepared by various processes and suspended in a variety of media (Perez-Pujol et al., 2004, Shapira et al., 2000, Sweeney et al., 2006). Levels on day 8 were similar to those published by Cardigan et al for plasma-suspended BC-PC stored in ELX packs at comparable time periods (days 7 - 9) (Cardigan et al., 2008). Despite the accelerated increase in positive percentage expression after day 8 in both groups, day 14 values of approximately 30% suggested a relatively large pool of platelets remained viable, although their *in vivo* survival and ability to support coagulation reactions remains to be conclusively determined. A loss of aminophospholipids on the surface of platelets could be related to increased formation and shedding of microparticles in plasma units (Heijnen et al., 1999, Perez-Pujol et al., 2005), although microparticles were not quantified in this study.

The progressive decline in the red to green (R/G) fluorescence ratio of the JC-1 cationic dye used to investigate the effect of storage on the mitochondrial membrane potential indicates a relative reduction of J-aggregates in the mitochondria with respect to green-fluorescing monomers restricted to the cytosol; a change attributed to the depolarisation of the inner mitochondrial membrane with PC storage. There is limited data on the impact that processing and subsequent storage of platelet concentrates has on mitochondrial membrane potential. Some studies have found little difference over the first seven days of storage (Perrotta et al., 2003); others have described an initial increase in the fluorescence ratio over the first few days of storage followed by a decrease (Verhoeven et al., 2005), whilst still others have shown steady decreases in the ratio with storage (Li et al., 2005b). A number of groups have reported results in terms of percentage of cells expressing red (polarised) and/or green (depolarised) fluorescence (Perrotta et al., 2003, Li et al., 2004). Interestingly, the limited number of studies that have reported results using both approaches have found disparate patterns between the two methods over the storage period. The study by Li et al reported limited decrease in the percentage of polarised cells over 7 days of storage, but an approximate 50% decrease in the red/green fluorescence ratio (Li et al., 2005b). Similarly, a recent study by Albanyan et al reported significant decreases in the red/green fluorescence ratio in both apheresis and buffy coat-derived PCs, but failed to detect a significant increase in the percentage of depolarised platelets in the BC-PC units (though marked increases were noted in the apheresis-derived PCs) (Albanyan et al., 2009). The last study suggested that the two approaches for reporting $\Delta\Psi m$ reflect different aspects of mitochondrial membrane depolarisation, with the R/G fluorescence ratio indicating a platelet population displaying partial as well as full depolarisation of mitochondrial membranes whilst percentage of depolarised platelets indicates solely complete depolarisation. The decrease in the R/G ratio in the first half of the storage period may thus reflect partial as well as complete depolarisation of the mitochondrial membrane. Depolarisation was evident from the beginning of the storage period, without the delay evident for the externalisation of aminophospholipids; thus agreeing with observations that suggest changes to the $\Delta\Psi m$ are an early indication of programmed cell death (Mignotte and Vayssiere, 1998, Castedo et al., 1996, Newmeyer and Ferguson-Miller, 2003). In addition, the platelets in both study groups were able to adequately compensate for any disruption to oxidative phosphorylation, since ATP production during the first half of the storage period was well-maintained.

In summary, platelets suspended as concentrates in a storage medium comprising a ratio of 70:30 SSP+™ and plasma performed at least as well as platelets suspended in 100% autologous plasma for up to 10 days of storage. Beyond this time-point, an accelerated deterioration was observed, possibly related to the depletion of glucose in these units, which was not reflected by the routine quality parameters of pH and swirling. The increase in the percentage positive expression of annexin V binding and decrease in $\Delta\Psi m$ suggest an apoptosis-like process may be involved in the platelet storage lesion, with mitochondrial changes preceding an increase in the percentage of platelets expressing annexin V. However, subsequent studies suggested that whilst this may have played a role in the first few days of storage, this was not the case at later timepoints. The subsequent studies targeting constituents of an artificial additive solution and the response to the BH3 mimetic ABT-737 were undertaken to further investigate this hypothesis. Although the assessment of platelet *in vitro* characteristics is a logical starting point for the investigation of platelet viability beyond 7 days of storage, the questions of haemostatic function as well as platelet recovery and survival following transfusion would need to be addressed prior to any consideration of extending the storage period of PCs beyond seven days.

INVESTIGATION INTO THE ROLE OF ALBUMIN, GLUCOSE AND ACETATE ON THE *IN VITRO* STORAGE CHARACTERISTICS OF PLATELETS STORED AS CONCENTRATES IN AN ARTIFICIAL ADDITIVE SOLUTION WITH MINIMAL PLASMA CARRY-OVER

Albumin Study

Human albumin is the most abundant protein in plasma, comprising 50 – 60% of the total serum protein, with a normal concentration of 40 – 50 g/L (Farrugia, 2010). It is a 65 kDa glycoprotein with a flexible molecular structure, a negative surface charge and a large number of disulphide bridges (He and Carter, 1992) - all attributes of the molecule which relate to its varied functions. Albumin is the most significant contributor to normal colloid osmotic pressure due to a combination of its molecular weight and high plasma concentration as well as its negative surface charge (Nicholson et al., 2000). The flexible nature of the molecule allows it to bind to a wide variety of molecules and serve

as a carrier for free fatty acids which may be incorporated in the metabolism of other lipids (Okuma et al., 1971). Other suggested roles for albumin include an ability to act as a buffer (McAuliffe et al., 1986) and as an antioxidant. The latter may be due to the large number of sulfhydryl groups which serve to remove oxygen free radicals and other oxidising agents (Nicholson et al., 2000). In addition, studies on cultured endothelial cells have identified albumin as an inhibitor of apoptosis (Zoellner et al., 1996). The suggested role of albumin in fatty acid transport, plasma buffering, the scavenging of oxidants and inhibition of apoptosis suggest a potential beneficial effect on *in vitro* platelet viability, and suggested albumin may be partly responsible for the requirement to retain a significant proportion of plasma in the suspending medium. The presence of albumin in the suspending medium was thus hypothesised to aid in maintaining platelet viability in storage.

The principal observation from the three replicate experiments which incorporated a 20% human albumin solution (Zenalb®20, BioProducts Laboratory, Elstree, UK) to the standard additive solution was the rapid deterioration of the platelets after day 3. This was evident across all the parameters measured. For a significant proportion of the platelet population disruption was total, as evidenced by the marked decrease in platelet concentration, suggestive of platelet lysis. The results suggested the disruption was directly related to the addition of the albumin solution, as a dosage-dependent response was clearly evident. The loss of swirling in albumin-containing units was also indicative of gross morphological changes. The possibility that differences in osmotic balance were responsible appeared remote since the concentrations of albumin in the media were not expected to result in a hyperosmotic environment. For confirmation, the osmolality of a sample from a unit re-suspended with SAS containing the highest concentration of albumin (120 ml of Zenalb®20) was measured with an Osmomat 030 cryoscopic osmometer (Gonotec GmBH; Berlin, Germany). The result of 292 mOsm/kg was comparable to normal levels in extracellular fluid of approximately 300 mOsm/kg (Bourque, 2008).

Glucose was consumed at an accelerated rate in the presence of albumin, leading directly to the cessation of lactate production by day 6. This was reflected by the pH levels which failed to decrease beyond day 6; reinforcing the observation made in the comparative study of plasma and SSP^{+}™ units that extracellular pH is highly dependent

on the presence of glucose in the suspending medium. It also highlights the limits of employing pH in isolation as an indicator of platelet viability in routine quality control for PC components with limited glucose stores, since pH values remained stable despite obvious deterioration of the components. As well as the collapse of glycolysis by day 6, the partial pressures of oxygen and carbon dioxide indicated oxidative metabolism was also compromised. The relatively high pO_2 levels on day 2 in the presence of albumin suggest oxygen diffusing into the pack was not being utilised, even at this early stage in the storage period, and may indicate early damage to the mitochondria. The loss of both mechanisms of ATP synthesis in the albumin-containing units was reflected in the absence of ATP by day 6. ATP levels on days 2 and 3 were similar to units suspended in SAS in the absence of albumin, suggesting that the increased rate of glycolysis in the albumin-containing PC early during the storage period was compensating for any disruption of oxidative metabolism.

Starting levels of surface-expressed CD62P with all four groups in this study were high compared to BC-PC processed by standard methods; a finding which was not unexpected considering the stress imposed on the platelets during processing by the hard spin required to pellet the platelets and remove the majority of the plasma. Interestingly, though, similarly-processed PC re-suspended in 100% plasma showed markedly lower percentage positive expression of CD62P at the start of storage, suggesting that a component in plasma not characterised by the albumin in solution reduced platelet activation to levels more comparable to standard BC-PCs. The progressive decrease in percent positive expression with storage suggested that shedding of the CD62P occurred preferentially with higher concentrations of albumin. This finding appeared to be corroborated by the higher concentrations of soluble CD62P in albumin-containing units compared with the group lacking albumin as the storage period increased. However, the clear dosage-dependent effect of albumin noted with percent positive expression was not matched by higher concentrations of soluble CD62P in units with higher concentrations of albumin. It is possible that lysis of the platelets resulted in CD62P-bearing fragments that were not quantified by the flow cytometer protocol employed, since this was aimed towards the counting of intact platelets. The increased platelet lysis associated with higher albumin concentrations may thus account for the more pronounced decrease in the percentage of platelets expressing CD62P, as well as the decrease in CD62P expression per platelet. As the

CD62P on these fragments would remain membrane-associated, it may also be "hidden" from the ELISA assay adopted to measure concentrations of the soluble 100 kDa fragment of the molecule.

The parameters selected to investigate apoptosis provided a comprehensive pattern that confirmed the conclusion derived from the metabolic and morphological markers that the platelets suspended in albumin-containing SAS had experienced a catastrophic disruption of function and viability. Mitochondrial membrane potential decreased rapidly in these units from the beginning of the storage period, which may be related to the impaired oxidative phosphorylation implied by the increase in pO_2 from day 2. By comparison, the comparative delay in the increase in annexin V binding and intracellular free calcium, with levels only increasing after day 3, suggest that aminophospholipid externalisation and release of calcium into the cytosol occur subsequent to mitochondrial disruption during platelet storage. The loss of phospholipid asymmetry is expected to result in an increase of both PE and PS on the outer leaflet of the platelet membrane. The nature of the aminophospholipids expressed on the platelet surface was further characterised by mass spectrometry on a representative sample. As expected, both PE and PS increased with storage in albumin-containing units. However, expression of all four isoforms of PE in non-albumin containing units remained stable throughout storage, suggesting that the relatively minor increase in the percentage of annexinV-binding platelets during storage was principally due to the increase in PS expression observed by mass spectrometry. Mass spectrometric measurements were performed on a representative sample, and further replicates would be required to confirm whether this observation is reproducible.

The addition of human albumin to the additive solution resulted in a clear degradation of platelet function and viability in excess of changes associated with the platelet storage lesion in more conventional media. This comprised an early increase in both aerobic and anaerobic metabolism, with total consumption of exogenous glucose by day 6 coinciding with the depletion of ATP stores. This in turn was associated with results from the cell death and platelet morphology parameters indicative of irreversible platelet degradation. The human albumin solution used was a 20% solution of commercially available human albumin (Zenalb® 20, Bio Products Laboratory, Elstree, UK). Different batches of Zenalb®20 were used for the three replicate experiments; a

possible artefact caused by a manufacturing anomaly of a single batch was thus excluded as a possible explanation.

Further experiments were undertaken with a different commercially available albumin solution (Baxter Healthcare, Norfolk, UK) as well as with fatty acid-free lyophilised albumin from human serum (Sigma-Aldrich, Dorset, UK) to determine whether the formulation of the albumin solution selected was responsible for the observed results. Both experiments strongly suggested that the rapid deterioration in platelet characteristics noted with Zenalb®20 was caused by the formulation of the albumin solution, though Zenalb® solutions are well-established in the clinical setting, with no reported effects on platelets. Therefore, the observed changes appear to be limited to *in vitro* phenomena. Details of the composition of the human albumin solutions (as determined from the publicly available package inserts) suggest the two products are similar, with both manufacturers adopting sodium octanoate as a stabilizer against the relatively high temperatures of the pasteurisation steps during manufacture and the Baxter formulation additionally employing sodium n-acetyltryptophanate to reduce oxidation (Yu and Finlayson, 1984, Anraku et al., 2004) (table 8.1).

Table 8.1: Composition of commercial human albumin solutions as provided by the manufacturers in their publicly available literature. (Sodium n-octanoate and sodium caprylate are synonyms)

Zenalb®20	Baxter 20% human albumin
Sodium 50-120 mmol/L	Sodium chloride 3.0 g/L
Potassium	Sodium caprylate 2.7 g/L
Chloride	Sodium n-acetyltryptophanate
Citrate	
Sodium n-octanoate	
<200 µg/l of aluminium	

Differences in the methods employed for the manufacture of the two commercial albumin solutions may provide an alternative explanation for the varying platelet response to the two solutions. BPL has introduced diafiltration to remove excess water and ethanol after the initial fractionation steps and a chromatographic step to further

remove contaminating proteins (Matejtschuk et al., 2000). Baxter has retained the Cohn cold fractionation method for the manufacture of plasma products, although it is difficult to determine from the published literature whether significant alterations to the original method have been adopted.

Questions over the safety of albumin were raised by the publication of a meta-analysis of 30 randomised controlled trials which concluded there was a 6% increase in the risk of death in patients treated with albumin (Berger, 1998). Subsequent studies have failed to confirm these conclusions (Wilkes and Navickis, 2001, Vincent et al., 2004, Dubois et al., 2006). In light of the markedly different impact on platelet characteristics resulting from the inclusion of albumin solution from two different manufacturers to the storage media, the question arose as to whether the use of albumin solutions from different manufacturers could explain the heterogeneity of results regarding the safety and efficacy of albumin in the clinical literature. A review of the publications used in the meta-analyses referenced above failed to yield any further insight due to the failure in a large proportion of the studies to identify the albumin manufacturer as well as the wide variety of manufacturers used in the various studies where the albumin source was quoted. However, different albumin preparations have been reported to demonstrate variable upregulation of endothelial cell adhesion molecules, even between batches from the same manufacturer (Nohe et al., 1999). Variable responses in blood pressure have also been reported on infusion of different batches of 5% human serum albumin (Heringlake et al., 2000). Thus, although it is not possible at this stage to definitively identify the causative factor behind the disparate effect on platelets in storage of the BPL and Baxter albumin solutions, it is clear that despite advances in the manufacture of human albumin solutions, there remains significant variability in the *in vitro* characteristics of different albumin solutions.

The inclusion of human albumin in the storage medium was hypothesized to aid in the maintenance of platelet viability *in vitro*. In this regard, the experiments with the Baxter formulation and powdered human serum albumin failed to demonstrate improved platelet storage characteristics, though care is required in deriving a conclusion from a single data point.

Glucose Study

The studies on platelet metabolism by Kilkson et al led to the conclusion that glucose was not an important substrate for aerobic metabolism, and called into question the relevance of glucose as a component of the storage medium (Kilkson et al., 1984). The possibility that it may even be harmful was suggested due to the promotion of glycolysis by glucose with a resultant decrease in pH. The consensus from more recent work on PC storage has increasingly converged on the notion that glucose is required throughout the storage period (Gulliksson, 2000, Gulliksson, 2003). However, the mechanism of action remains elusive and a consensus on its role remains to be reached (Gyongyossy-Issa, 2011).

Glucose was utilised by the platelets from the beginning of the storage period. The rate of decline was independent of the initial concentration of glucose and correlated closely with the rate of lactate production, suggesting glucose was being consumed via glycolysis from the beginning of the storage period. This was despite unrestricted access to oxygen through the gas-permeable packs and the presence of oxidisable fuels, as noted in early studies on platelet metabolism during storage (Holme et al., 1987). A precedent for the continued generation of lactate from glucose via the glycolytic pathway in environments with abundant oxygen was first described by Warburg in relation to the metabolism of proliferating cancer cells (Warburg, 1956) and has since been recognised as a feature in healthy dividing cells (Lunt and Vander Heiden, 2011). A posited explanation is that aerobic glycolysis, though inefficient in terms of the overall number of ATP molecules generated per glucose molecule compared to the oxidative phosphorylation pathway, is able to produce ATP at a faster rate (Pfeiffer et al., 2001). In rapidly proliferating cells which may be competing for limited resources, a faster rate of ATP may thus be more desirable (Vander Heiden et al., 2009, Lunt and Vander Heiden, 2011). It is unclear at this time, however, how this could translate to the physiology of platelets stored as concentrates. The rate of glucose consumption increased over the storage period. It is possible that the platelets are preferentially using the exogenous acetate as the principal fuel to maintain energy levels and progressively converting to glucose as a metabolite as the acetate is consumed (Murphy, 2002, Vetlesen et al., 2007, Ringwald et al., 2006).

Results from other metabolic parameters suggest that the role of glucose in platelet metabolism is not restricted to glycolysis. The absence of glucose in the medium resulted in a decrease in pCO_2 – in isolation, this may be indicative of reduced CO_2 generation as a result of impaired oxidative phosphorylation. However, as already discussed, various studies investigating the contribution of glucose to oxidative pathways of metabolism have concluded that it plays a minor role (Guppy et al., 1990, Murphy, 1995). A possible alternative explanation could relate the reduction in CO_2 generation to reduced activity of the bicarbonate buffering system as lactate levels cease to rise with the exhaustion of glucose. Loss of glucose was also associated with a reduced oxygen consumption rate and a decrease in ATP concentration; indeed, ATP levels were markedly lower from the start of the storage period in units lacking exogenous glucose. Mean ATP levels on day 2 in the units lacking glucose were 78% of mean levels in the group containing the highest concentration of glucose. It is possible that this difference simply reflects the proportion of ATP that would otherwise be supplied by glycolysis, estimated to be approximately 15-25% of total ATP production (Guppy et al., 1997, Bertolini et al., 1992). ATP levels in units lacking glucose declined steadily with storage, suggesting the exogenous acetate and endogenous fuels were unable to compensate for glucose in the production of ATP, unlike the results observed with units that retained reserves of glucose to the end of storage.

The glucose study showed that early changes in mitochondrial membrane polarisation were followed by increases in cytosolic Ca^{2+} and surface expression of aminophospholipids which were linked to a decrease in ATP levels coincident with the exhaustion of glucose in the media and ultimately resulted in platelet degradation indicated by decreased platelet concentration. The depletion of glucose may have provided a trigger for these events. Though some of the observations seemed to concur with the proposal that a process akin to apoptosis was a central component of the platelet storage lesion, the loss of ATP and cellular disruption coincident with the exhaustion of glucose stores are not consistent with the classical description of apoptosis since this is an energy dependent process (Kung et al., 2011). Regardless of the mechanism of cell death, the implication remains that glucose is required for the maintenance of platelets stored as concentrates and would be a beneficial component of artificial additive solutions, particularly if the aim is to reduce patient exposure to donor plasma by minimising plasma load in PC units.

Acetate Study

The addition of acetate to the SAS resulted in reduced glucose consumption (with the concomitant decrease in lactate production), a more reduced drop in pH and an increased oxygen consumption rate. Similar results have been reported in the literature (Gulliksson, 2000, Murphy, 2005) and are likely related to the preferred use of acetate as an oxidisable fuel by platelets as well as to its buffering capacity. However, although the decrease in pH and glucose during the first half of the storage period was not as marked, levels were not found to be stabilised by the inclusion of acetate. In addition, also in accordance with the published literature, the addition of acetate failed to maintain ATP levels and resulted in a concentration-dependent depression of the response in the HSR and ESC assays (Holme, 1992). The seemingly paradoxical decrease in ATP levels may be related to the metabolism of acetate into acetyl coA, a reaction that requires the consumption of an ATP molecule and results in the formation of AMP and a pyrophosphate (Liang and Lowenstein, 1978, Knowles et al., 1974). Studies associated with the use of acetate in haemodialysis have noted that a rapid ATP-consuming process occurred at high concentrations of acetate in cardiac myocytes, actually reducing the concentration of ATP and increasing AMP levels. Mitochondria are incapable of rephosphorylating AMP, instead forming two ADP molecules with the additional use of ATP by the adenylate kinase reaction (Vinay et al., 1987). Animal studies have also noted a decrease in ATP/ADP ratios and an increase in AMP levels following the infusion of acetate into heart or skeletal muscle (Liang and Lowenstein, 1978, Spydevold et al., 1976).

The reduced responses in HSR and ESC assays evident at the start of the storage period were accompanied with enhanced platelet activation and platelet lysis, as inferred from lower platelet concentrations. Subsequent measurements of these parameters during the storage period tended to occur at similar rates, suggesting the initial exposure to acetate was the principal stressor rather than progressive changes imposed over the storage period. Also evident was a direct relationship between the concentration of exogenous acetate and the level of the adverse response. Although it was not possible to infer a mechanism to explain these findings from this study, the possibility that acetate may have adverse effects has been suggested by clinical observations of the use of acetate and whole-body studies on animals. Acetate has been employed as a buffer in various

clinical solutions but been found to be associated with vasodilation effects, impaired contraction of cardiac myocytes and in some cases hypotension (Olinger et al., 1979, Jacob et al., 1997, Vinay et al., 1987). The impact of added acetate on the markers of apoptosis was less clear, with only a suggestion that the highest concentrations of acetate may be enhancing cell death. The marked increase in anionic phospholipid externalisation observed after day 8 may be related to the exhaustion of glucose stores rather than a storage-related effect of acetate in the medium.

The inclusion of acetate in artificial storage media has been principally based on its buffering action and associated ability to maintain pH at relatively higher levels. However, its addition to SAS in this study resulted in lower levels of ATP as well as decreased performance in assays related to platelet function (HSR/ESC). Further studies on the impact of exogenous acetate on platelet function, morphology, activation and apoptosis would help to clarify its role in the progression of the PSL. If the adverse effects suggested by this study were confirmed, the possibility remains that with adequate levels of sodium bicarbonate in the storage medium, acetate may not be required for the maintenance of platelets as concentrates.

INVESTIGATION INTO THE MECHANISM OF AMINOPHOSPHOLIPID TRANSLOCATION AND THE ROLE OF BCL-2 FAMILY INITIATION OF PLATELET DEATH IN RELATION TO THE PSL

Efforts to determine the optimal conditions for the storage of platelets as concentrates initially centred on the metabolic characteristics of platelets *in vitro*. Application of this work led to the adoption of gas-permeable plastics and storage under constant agitation at 22°C, with clear practical benefits evident in the maintenance of pH levels and platelet survival in the circulation post-transfusion (Murphy et al., 1982, Holme et al., 1978, van der Meer and Korte, 2011). However, current guidelines for PC storage continue to result in changes that adversely affect the survival and function of platelets in storage. Interest in the possibility that a controlled process of cell death may play a significant role in this phenomenon has been increasing, with the applied aim that a

better understanding of the phenomenon may allow the manipulation of the storage conditions to reduce the adverse effects of the PSL. The opportunity arose to investigate the mechanism of platelet death in storage more directly by examining the translocation of aminophospholipids in platelets from a Scott syndrome patient stored for up to ten days alongside platelets from normal volunteers. Specifically, this would determine whether the increase in aminophospholipid expression on the platelet surface with storage was due to platelet ageing or activation.

Scott syndrome is a rare platelet coagulation disorder characterised by an inability to express PS on the platelet surface following activation (Rosing et al., 1985). A recent study showed that transmembrane protein 16F (TMEM16F) is a required component of Ca^{2+}-dependent phospholipid scrambling, with a point mutation on the *TMEM16F* gene of a Scott syndrome patient resulting in premature termination of the protein at the third transmembrane region (Suzuki et al., 2010). Two further mutations have since been identified in this gene in peripheral blood leucocytes of a second Scott patient (the patient investigated in this study), confirming the role of this protein on Ca^{2+}-dependent PS expression (Castoldi et al., 2011). First recognised in lymphocytes, surface expression of PS is also associated with cells entering apoptosis and is believed to act as a recognition signal for cell clearance by phagocytes (Fadok et al., 1992, Bevers and Williamson, 2010). A study on B lymphocytes from a Scott patient detected normal PS exposure in cells stimulated to undergo apoptosis but failed to detect a similar change in cells exposed to a calcium ionophore, suggesting that two distinct pathways of PS exposure may be operating in haemopoietic cells (Williamson et al., 2001, Zwaal et al., 2004). A recent study investigated this concept in platelets using mice with variable expression of the pro-apoptotic Bcl-2 family members Bak and Bax. *Bak*$^{-/-}$*Bax*$^{-/-}$ platelets exposed to the apoptosis-inducing BH3 mimetic ABT-737 failed to express PS on the surface, whereas exposure to either a calcium ionophore or CRP/thrombin resulted in normal levels of PS exposure. These results confirmed the hypothesis that expression of PS on the platelet surface follows at least two distinct pathways depending on whether the platelets are subjected to a physiologic agonist, an apoptotic process (Schoenwaelder et al., 2009) or another mechanism. They also indicate that the scramblase remains unaffected by the Scott gene mutation and serves as a juncture for the convergence of the two pathways (Bevers and Williamson, 2010) (figure 8.3). Recent work on platelets from the Scott patient investigated in this study further

suggests that PS translocation occurs by at least two pathways. One pathway observed in both Scott and normal platelets is independent of functional TMEM16F and exhibits a relatively slow response to incubation with ABT-737. The second pathway is TMEM16F-dependent, absent from Scott platelets, and associated with high intracellular Ca^{2+} and rapid scrambling (van Kruchten; revised manuscript submitted).

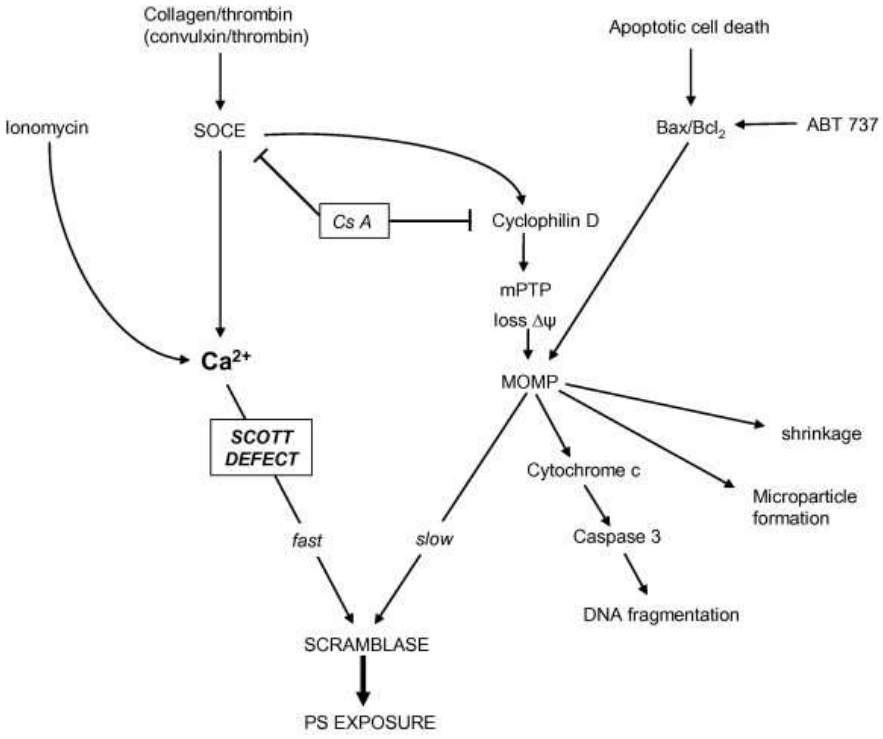


Figure 8.3: Two distinct pathways leading to PS exposure in platelets. Exposure of PS following Ca^{2+} ionophore or agonist stimulation is blocked in Scott syndrome patients. The alternative pathway, initiated by the Bcl-2 family of proteins, is unaffected by the Scott defect and is able to lead to PS translocation via regulation of mitochondrial changes (SOCE – store-operated calcium entry) (Bevers and Williamson, 2010) (Reproduced with permission, © Elsevier B.V.)

If scramblase independent mechanisms were associated with the expression of aminophospholipids during platelet storage, the platelets from a Scott patient would be expected to increasingly express PS on their surface over the course of the storage period, similar to platelets from normal individuals. Results from the current study confirmed this, with similar levels of annexin V binding on platelets from a Scott patient and two normal volunteers over the course of 9 days of storage. Subsequent experiments at the University Hospital of Wales (UHW) and by Professor Bevers' team at Maastricht confirmed these findings. The team at UHW subsequently investigated the relationship between this increased expression of aminophospholipids in Scott platelets

and their ability to support thrombin generation. An increased expression of PS in activated platelets from normal individuals serves to spatially concentrate the activities of the tenase and prothrombinase complexes and thus promote thrombin generation (Heemskerk et al., 2002). If the PS progressively expressed on the surface of stored Scott platelets retains this function, an increase in thrombin generation would be observed with increased storage. The results were consistent with this, with levels of thrombin generation in Scott platelets similar to control platelets by day 8 of storage. The question remains as to whether aged Scott platelets retain this functional capacity *in vivo*. The studies thus confirmed that the increase in annexin V binding observed with storage was not due to activation mediated by scramblase. A further experiment was undertaken to determine whether an increase in aminophospholipid translocation could be observed in platelets stored in additive solution on addition of a promoter of a controlled mechanism of cell death.

The central role played in the apoptosis of nucleated cells by the Bcl-2 family of proteins has been confirmed in numerous studies (for recent reviews see (Danial, 2007, Chipuk et al., 2010, Brunelle and Letai, 2009, Dewson, 2010)). Following exposure to an apoptotic stressor, the conformational change and oligomerisation of pro-apoptotic members Bax and/or Bak on the outer mitochondrial membrane is thought to form pores allowing for the release into the cytosol of intermembrane space proteins such as cytochrome c and Smac/DIABLO which proceed to initiate or enhance caspase activation leading to apoptosis (Youle and Strasser, 2008, Westphal et al., 2011). Two major models have been postulated to explain the regulation of apoptosis by Bcl-2 family proteins; both involving the interaction of BH3-only proteins as facilitators of apoptosis. The indirect activation model suggests the role of the anti-apoptotic Bcl-2 and Bcl-X_L proteins is to inhibit pro-apoptotic Bax and Bak. BH3-only proteins in this model sequester the anti-apoptotic Bcl-2 proteins is response to apoptosis-inducing signals, thus releasing Bax and Bak and allowing them to oligomerize (Willis et al., 2005, Fletcher and Huang, 2008). The direct activation model further classifies the BH3-only proteins into sensitizers and activators. Activators such as Bim and tBid directly bind and promote the oligomerization of Bax and Bak. The role of anti-apoptotic Bcl-2 proteins in this model is to bind and inhibit the activators. BH3-only sensitizers such as Bad are able to promote apoptosis by binding to anti-apoptotic Bcl-2

and/or Bcl-X_L and thus release the activators (Kim et al., 2006). A further model referred to as "embedding together", which incorporates aspects of both the direct and indirect activation models, has also been proposed that emphasises the vital role played by the mitochondrial membrane in the initiation of apoptosis by the Bcl-2 protein family (Leber et al., 2007, Bogner et al., 2010). A common theme of these models is that the interaction between pro- and anti-apoptotic members of the Bcl-2 family acts to regulate the process of cell death (Leytin and Freedman, 2003, Brown et al., 2000) (figure 8.4).

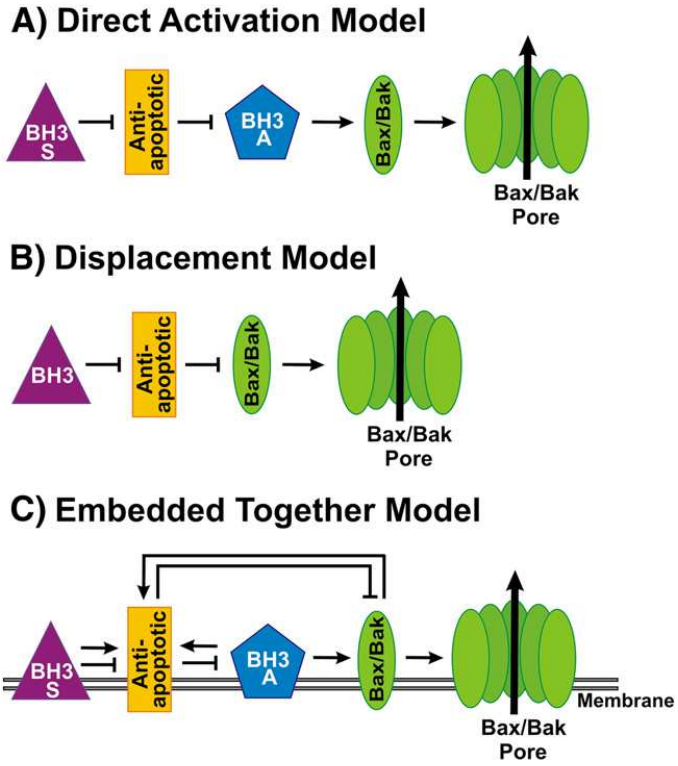


Figure 8.4: Direct and indirect activation models of Bax and Bak activation. A). In the direct model, proapoptotic Bax and Bak require activator BH3-only proteins to function. These are preferentially bound to antiapoptotic Bcl-2s in viable cells. Bax and Bak are able to oligomerise and form pores in the mitochondrial outer membrane function when the concentration of activator proteins exceeds the capacity of the antiapoptotic proteins to sequester them. Sensitizer BH3-only proteins (e.g. Bad, Noxa) facilitate the process by binding to the antiapoptotic proteins and promoting the release of activators. B). In the indirect or diaplacement model, antiapoptotic Bcl-2 members bind to Bax and Bak, inhibiting their ability to oligomerise. BH3-only proteins bind to antiapoptotic Bcl-2s, thus releasing the proapototic proteins. C). Anti-apoptotic Bcl-2 proteins mobilised to the mitochondrial outer membrane bind to and inhibit both activator BH3-only proteins such as tBid and pro-apoptotic Bax and Bak. Sensitiser BH3-only proteins (e.g. Bad) may displace the activators, freeing them to bind to pro-apoptotic proteins and initiate mitochondrial outer membrane permeabilisation (Shamas-Din et al., 2011) (Reproduced with permission, © Elsevier B.V.).

ABT-737 binds avidly to the hydrophobic groove of Bcl-X_L, mimicking the action of the Bad BH3-only protein (figure 8.5) (Dewson, 2010). An increase in annexin V

binding in response to the addition of ABT-737 would thus indicate that aminophospholipid translocation in platelets stored in additive solution was able to proceed through a process of cell death regulated by the Bcl-2 family of proteins.

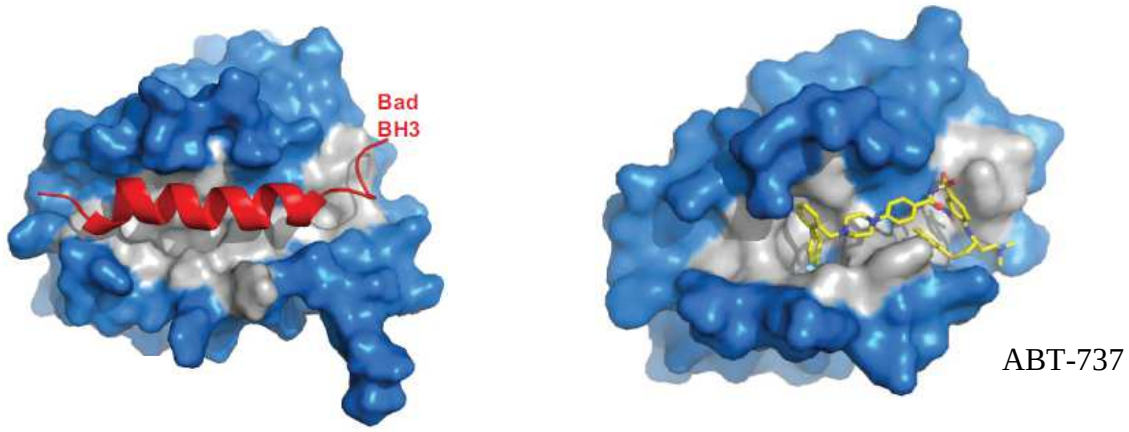


Figure 8.5: Interaction of the BH3-only protein Bad with the hydrophobic groove of the antiapoptotic Bcl-X_L and the corresponding activity of the BH3 mimetic ABT-737 (Dewson, 2010) (Open access, ©Dove Medical Press Ltd)

In the unit with added glucose in the storage medium, the addition of ABT-737 increased annexin V binding early in the storage period compared to control samples, with the effect gradually decreasing over time. The reduced response to ABT-737 was temporally related to falling levels of glucose and ATP. The depletion of glucose was also temporally related to a marked increase in annexin V binding, but with no additional increase observed in samples with ABT-737 compared with the control. This suggests that after glucose and ATP levels fall, the platelets are no longer able to express aminophospholipids through an apoptotic mechanism and another mechanism needs to be postulated.

In the unit with no added glucose, ABT-737 resulted in an increase in annexin V binding compared to controls only on day 2. Beyond this time-point, annexin V binding increased rapidly with storage but with no additional expression caused by ABT-737. The results indicated that early during the storage period, a mechanism regulated by Bcl-2 proteins was present that could increase aminophospholipid translocation in platelets. In addition, the depletion or absence of glucose in the storage medium markedly increased aminophospholipid translocation without the involvement of this

Bcl-2 protein-regulated mechanism, by a process coincident with a marked decrease in ATP levels, an increase in intracellular free calcium and progressive platelet disruption.

SUGGESTED MODEL FOR THE MECHANISM OF PLATELET DEATH IN PLATELETS STORED AS CONCENTRATES

The increase in annexin V binding following the addition of ABT-737 suggested a pathway of cell death mediated by the Bcl-2 family of proteins could be initiated in platelets at the start of storage and prevailed for up to eight days in the presence of energy stores able to maintain ATP levels. This is consistent with apoptosis being an energy-dependent process (Nicotera and Melino, 2004). Recent studies have confirmed the release of cytochrome c and activation of caspase 3 in stored platelets in response to the BH3 mimetic ABT-737 (Dasgupta et al., 2010), showing that apoptosis via a Bcl-2 family-mediated mitochondrial pathway is a credible mechanism for platelet death in storage. A Bcl-2 protein-mediated cell death mechanism may have been present in PC stored for 14 days in autologous plasma, where an increase in annexin V binding was observed after day 8, despite glucose still being available in the medium and ATP levels retained close to day 1 values. These results are consistent with the concept of an internal clock limiting platelet lifespan, as suggested by Mason et al in their study with Bak knockout mice in which they proposed that levels of anti-apoptotic Bcl-X_L inherited from the parent megakaryocyte degrade more rapidly than those of pro-apoptotic Bak. Once free from the inhibitory activity of Bcl-X_L, the remaining Bak is able to initiate platelet death via apoptosis (Mason et al., 2007, Qi and Hardwick, 2007). Various lines of evidence have suggested a role for the Bcl-2 protein family in platelet death. In mature platelets, levels of pro-apoptotic Bax and Bak have been found to increase with platelet storage (Brown et al., 2000). Anti-apoptotic members of the family are predominantly represented by Bcl-X_L, with minimal levels of Bcl-2 having been detected by Western blot (Sanz et al., 2001, Zhang et al., 2007). Bcl-X_L levels have been reported to decline after 7 days in platelets stored as concentrates in standard storage conditions, with the rate of decline accelerated markedly with an increase in storage temperature to 37°C (Bertino et al., 2003). An additional factor is the progressive mitochondrial dysfunction suggested by the gradual loss of mitochondrial

membrane potential observed with storage, which may lead to increased strain on the antioxidant defences of the platelets and facilitate Bcl-2 protein mediated cell death (Voehringer, 1999, Howard et al., 2009). Thus, a TMEM16F-independent mechanism of controlled cell death - mediated by the Bcl-2 family of proteins and dependent on the retention of adequate levels of ATP - is suggested to be a plausible mechanism of cell death for platelets stored as concentrates. The viability of this mechanism may be limited by the "shelf-life" of internal stores of anti-apoptotic Bcl-X_L and may be exacerbated by a gradual loss of mitochondrial function.

In the absence of glucose in the storage medium, the translocation of aminophospholipids was markedly accelerated in the absence of a Bcl-2 family-mediated mechanism, as evidenced by the lack of any additional annexin V binding on addition of ABT-737. An alternative mechanism is thus required to explain platelet death in a glucose-depleted environment.

A consistent observation in this study has been that the absence or depletion of glucose in the storage medium resulted in an increased expression of the markers associated with cell death that coincided with a decline in the levels of ATP. It seems likely that the ability of glucose to generate ATP via glycolysis at least partly compensated for a decrease in ATP generation via oxidative phosphorylation and helped to maintain ATP levels during storage. In addition to its ability to regenerate ATP via glycolysis, the question arose as to whether the absence of glucose may be having an adverse effect on platelet biology with indirect consequences on the ability of platelets to maintain or regenerate ATP. A clue may lie in the markedly greater disruption in the mitochondrial membrane potential evident from the start of storage in units lacking glucose.

Loss of mitochondrial membrane potential was evident before marked changes in other markers of cell death, suggesting an early and progressive mitochondrial dysfunction with storage. Synthesis of ATP via the electron transport chain is a critical role of mitochondria and is mediated by four complexes associated with the inner membrane of the mitochondria. The transfer of electrons between complexes I to IV is coupled to the extrusion of H^+ ions from the mitochondrial matrix to the intermembrane space via complexes I, III and IV, resulting in an electrochemical gradient across the inner mitochondrial membrane of approximately 180 mV (negative on the matrix side of the

membrane). The flow of protons back into the mitochondrial matrix through the fifth complex (ATP synthase) subsequently drives the synthesis of ATP from ADP (Nicholls, 2002, Bayir and Kagan, 2008) (figure 8.6). Disruption of the proton-motive force would result in depolarisation of the membrane potential and reduction in the rate of ATP synthesis.

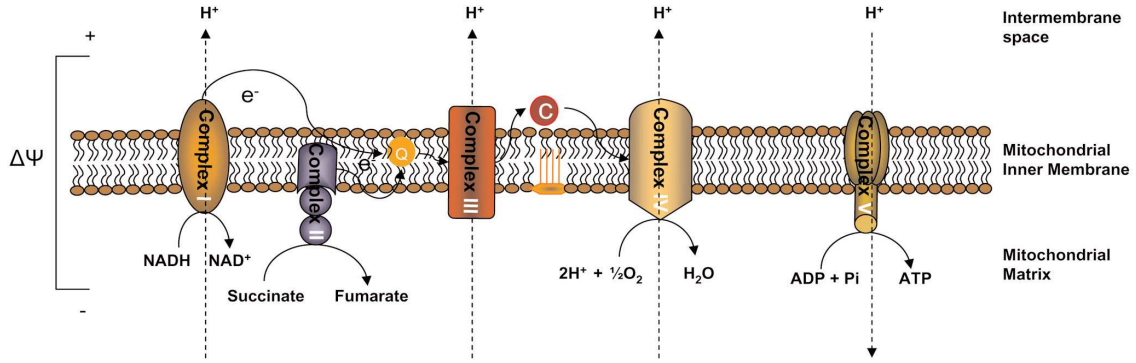


Figure 8.6: Synthesis of ATP via the mitochondrial electron transport chain (Bayir and Kagan, 2008) (Open Access, © BioMed Central Ltd)

Deviation of electrons from the orderly progression through the mitochondrial transport chain can lead to their sequestration by oxygen and result in the generation of superoxide ($O_2^{\bullet-}$) (Adam-Vizi and Chinopoulos, 2006, Murphy, 2009). Platelets were first reported to generate $O_2^{\bullet-}$ by Marcus et.al (Marcus et al., 1977). Reactive oxygen species have been reported to promote platelet activation, platelet aggregation and platelet sequestration and recruitment to developing thrombi (Iuliano et al., 1997, Krotz et al., 2004). Various lines of evidence further support the concept that reactive oxygen species can participate in cell death (Kroemer et al., 1995, Zhao et al., 2003), with the possibility that the levels of ROS in the cell may help to decide its ultimate fate (Mignotte and Vayssiere, 1998). A possible link between platelet death in storage and the lack of glucose may involve a reduction in the ability of the platelets to combat oxidative stress due to reduced activity of the pentose phosphate pathway. Glycolysis and the pentose phosphate pathway both require glucose as a starting substrate, linked via glucose-6-phosphate (figure 8.7).

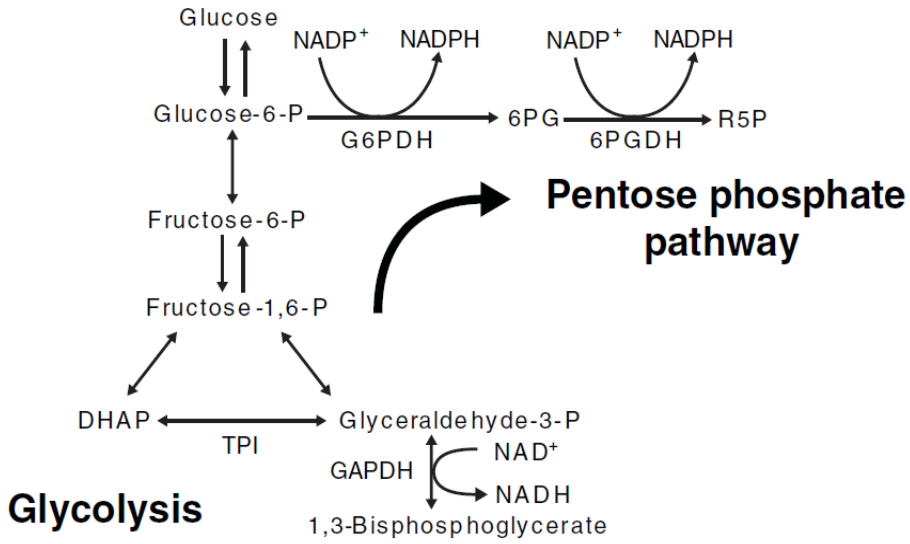


Figure 8.7: The role of glucose as a substrate in both glycolysis and the pentose phosphate pathway (Grant, 2008) (Open Access, © BioMed Central Ltd)

The pentose phosphate pathway generates NADPH which is able to reduce oxidised glutathione. Reduced glutathione acts as the substrate for a peroxidase-catalysed conversion of peroxides such as H_2O_2 to water, helping to limit damage from these highly reactive molecules (Grant, 2008). High levels of intracellular glutathione levels have been previously reported to prevent apoptotic methods triggered by ROS such as H_2O_2 (Pierce et al., 1991, Simon et al., 2000). Although ROS and glutathione metabolism were not investigated in the thesis, and discussion of their role in the observed results must remain speculative, it is possible that a similar antioxidant mechanism was compromised from the start of storage by the total absence of glucose, leading to disruption of the respiratory chain and enhanced depolarisation of the inner mitochondrial membrane.

Also associated with ATP metabolism is the concentration of mitochondrial and intracellular calcium. Three of the dehydrogenases involved in the TCA cycle are known to be activated by increases in Ca^{2+} concentrations (Duchen, 1999, Denton, 2009), with a primary function of Ca^{2+} in the mitochondria being the stimulation of oxidative phosphorylation (Balaban, 2002, Hansford and Zorov, 1998). Oscillations in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) can cause similar spiking in the mitochondrial Ca^{2+} concentrations ($[Ca^{2+}]_m$) which result in a relatively prolonged increase in NADH in the stimulated cell which can enhance the proton-motive force and increase ATP

levels (Rizzuto et al., 2000, Pozzan and Rizzuto, 2000). Indeed, a direct relationship between $[Ca^{2+}]_m$ and ATP levels has been established in cultured cells (Jouaville et al., 1999). It has been suggested that this mechanism offers an attractive method of targeting ATP supply to demand, since increases in cytosolic calcium are associated with a large number of cellular functions (Duchen, 2000, Pozzan and Rizzuto, 2000). It may also help explain the increase in ATP levels observed during the first few days of storage in PC suspended in plasma or SSP+™, though some authors remain cautious and cite that evidence in intact systems is limited (Szabadkai and Duchen, 2008).

One of the principal calcium stores in platelets is located in the dense tubular system, comprised of residual smooth endoplasmic reticulum (Jurk and Kehrel, 2005). A close physical association between ER membranes and mitochondria has been identified which allows for the rapid and highly localised uptake of Ca^{2+} by mitochondria (Rizzuto and Pozzan, 2006). This has suggested a role for mitochondria as a buffer against the general propagation of calcium into the cytosol, with the potential for recycling of Ca^{2+} back into the DTS via ATP-dependent SERCA pumps (Wang and El-Deiry, 2004). Cytochrome c may also act as a signalling molecule and induce a moderate release of calcium ions from the intracellular stores of the dense tubular system. Cytochrome c is able to interact with the $InsP_3$ receptors on the membrane of the DTS, promoting calcium release from the intracellular stores (Boehning et al., 2003). This may be reflected in the results, which showed minimal increases in cytosolic calcium concentrations whilst glucose was still available, despite a moderate reduction in mitochondrial membrane potential. This essentially physiological role for Ca^{2+} may be subsequently overwhelmed by the pathological changes comprising the PSL (Rasola and Bernardi, 2011).

Progressive depletion of the DTS calcium pool may amplify the process of mitochondrial dysfunction, as suggested by the two-hit hypothesis in which calcium's nominally benign physiological role is altered on exposure to a pathological stimulus such as ROS. Calcium itself may exacerbate this process, as Ca^{2+}-induced permeabilisation of the outer mitochondrial membrane has been observed to lead to loss of mitochondrial GSH and reduced antioxidant capacity (Brookes et al., 2004). Thus, the changes in mitochondrial membrane potential that preceded the increase in cytosolic calcium in the additive solution studies may reflect an early change in mitochondrial

function at least partly driven by an increase in Ca^{2+} levels within the mitochondrial matrix. A measured increase in cytosolic Ca^{2+} was only evident following the exhaustion of glucose and is suggested to be a consequence of the increased mitochondrial dysfunction possibly caused by the accumulation of ROS; a sequence of events previously suggested in other cell systems (Tan et al., 1998, Macho et al., 1997) and emphasising the close relationship between mitochondrial function and the dynamics of intracellular calcium. Extracellular calcium was minimally available to the platelets in the additive solution studies, since the SAS did not contain any Ca^{2+}-containing compounds. Thus, the principal source for the increase in cytosolic Ca^{2+} would be the intracellular stores, which appeared adequate to maintain calcium's physiological functions until mitochondrial dysfunction triggered adverse changes to the mechanisms for Ca^{2+} homeostasis. The associated loss of ATP, phospholipid asymmetry and continued mitochondrial membrane depolarisation were also able to proceed in the absence of extracellular calcium (see also (Lopez et al., 2007)).

The results and above discussion suggest a complex interaction between mitochondrial function, ATP generation, intracellular Ca^{2+} and the redox state of the platelet was possibly initiated and likely exacerbated by the depletion of glucose in the storage medium. The absence of glucose in the medium would have resulted in a loss of glycolytic ATP generation, compounding any decline in ATP regeneration by oxidative phosphorylation due to depolarisation of the mitochondrial membrane potential. Mitochondrial calcium levels may have increased in an attempt to promote ATP generation. With continually decreasing energy stores, however, Ca^{2+} re-uptake by the DTS via the ATP-dependent SERCA pump may have been compromised, leading to excessive levels of Ca^{2+} in the mitochondrial matrix. The lack of glucose may also have led to a reduced antioxidant capacity in the platelets due to decreased GSH levels. The combination of increased ROS, $[Ca^{2+}]_m$ overload and ATP depletion resulted in permanent opening of the mitochondrial permeability transition pore (Zong and Thompson, 2006), leading to further ROS production and mitochondrial disruption (Baines, 2010). Persistent opening of the pore allows for the unselected entry of small molecules into the mitochondrial matrix. Due to the high protein concentration in the matrix this will include an influx of water, leading to mitochondrial swelling which may terminate in rupture of the organelle due to the higher surface area of the highly convoluted inner membrane relative to the outer mitochondrial membrane (Desagher

and Martinou, 2000). Organelle and plasma membranes may be disrupted by the increased cytosolic Ca^{2+} concentrations, exacerbated by the lipid peroxidation activity of ROS. Cellular lysis would be the inevitable outcome. Disruption of the normal phospholipid asymmetry would also be expected as a consequence of the increased levels of cytosolic Ca^{2+} and reduced ATP. The general scenario is of a necrotic pathway to cell death (Golstein and Kroemer, 2007) (figure 8.8).

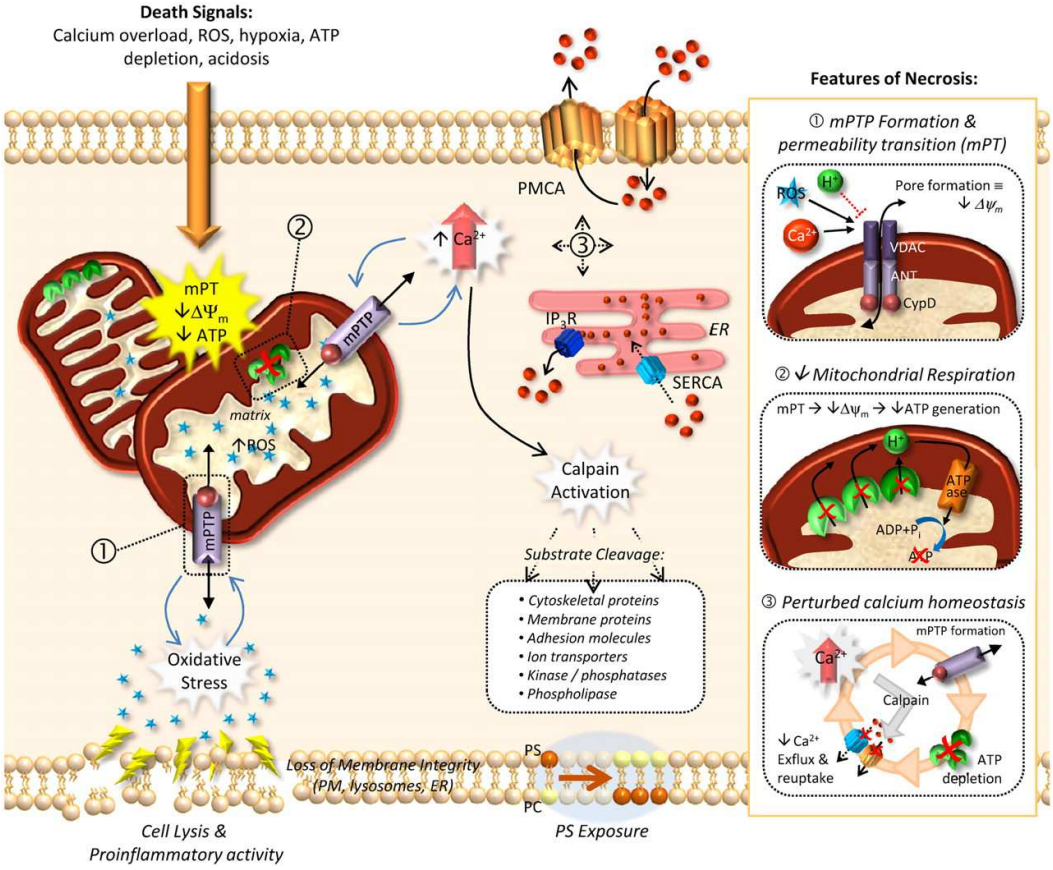


Figure 8.8: Mechanisms associated with necrosis, including a reduction in mitochondrial membrane potential (ΔΨm), decreased ATP production, increased ROS generation and enhanced opening of the mitochondrial permeability transition pore (mPTP) (Jackson and Schoenwaelder, 2010) (Reproduced with permission, © The American Society of Hematology).

Taken as a whole, the results suggest a Bcl-2 mediated mechanism of cell death was present in platelets stored with adequate energy stores which may be initiated under normal storage conditions by the interplay between pre-existing concentrations of the Bcl-2 family proteins. Further studies involving the manipulation of caspases would help to confirm whether this is in fact an apoptotic process. A further consideration is

the suggestion that apoptosis and necrosis may not be processes to be viewed strictly in isolation, but may form part of a graduated response to cellular insult. In this context, the cell's response to ATP depletion may involve a critical threshold beyond which the energy-dependent process of apoptosis is superseded by necrosis (Chen, 2009, Kim et al., 2003). Of particular relevance to the artificial environment of platelet storage is the phenomenon of secondary necrosis, whereby apoptosis culminates in a necrotic outcome following the failure of apoptotic cells to be removed by scavenging phagocytes (Lauber et al., 2004, Zong and Thompson, 2006). By contrast, the lack of a critical component such as glucose in an artificial additive solution led to a primary necrotic mechanism being imposed on platelets due to a critical depletion of energy stores. Further insight into the PSL may be gained by extending investigations into the role of both necrosis and apoptosis on platelet death, with particular emphasis on the central part played by mitochondria in the control of both processes.

SUGGESTIONS FOR FUTURE INVESTIGATIONS

- A more comprehensive investigation of the $\Delta\Psi m$ during prolonged platelet storage would be beneficial in describing the role of mitochondria in the PSL. In addition to further work with JC-1, other fluoroprobes sensitive to changes to membrane potential such as tetramethylrhodamine methyl ester (TMRM) could be used in concert for confirmation of the results (Castedo et al., 2002, Galluzzi et al., 2007).
- To differentiate between inner and outer mitochondrial membrane permeabilisation, it may be possible to adopt the calcein quenching method. Calcein in the form of an acetoxymethyl ester is able to diffuse into mitochondria whereas cobalt ions, which are able to quench the fluorescence signal from calcein, are excluded unless the IM has been compromised (figure 8.9) (Petronilli et al., 1999, Poncet et al., 2003).

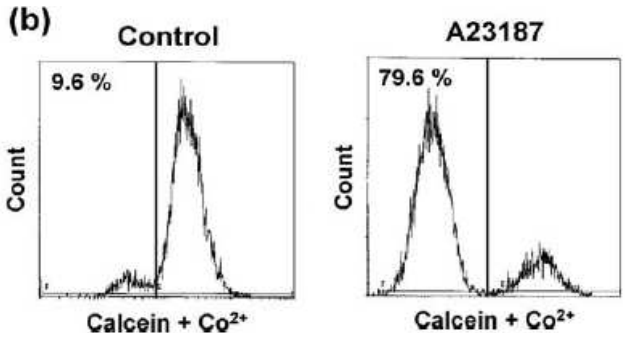


Figure 8.9: HeLa cells stained with calcein and its quencher Co^{2+}. Cells either left untreated or treated for 24 hours with the calcium ionophore A23187(Galluzzi et al., 2007) (Reproduced with permission, ©Springer Science)

- Disruption of the plasma membrane typical of necrotic cells has been previously studied with vital dyes such as trypan blue and the release of intracellular contents such as lactate dehydrogenase (LDH). However, some authors have suggested caution in the use of trypan blue with platelets (Brown et al., 2000), whilst measuring the loss of proteins such as LDH may not be sufficient to discriminate between cell death pathways (Galluzzi et al., 2009). Since morphological changes continue to provide some of the more definitive indicators differentiating between apoptosis and necrosis, direct observation of platelet morphology by electron microscopy may help to elucidate which mechanism is involved in platelet death during storage (Martinez et al., 2010) (figure 8.10). However, the approach is limited in that only a few cells can be imaged, making it difficult to interpret results in relation to the population dynamics whereby various sub-groups of platelets may be experiencing different stages of cell death at the same time point.

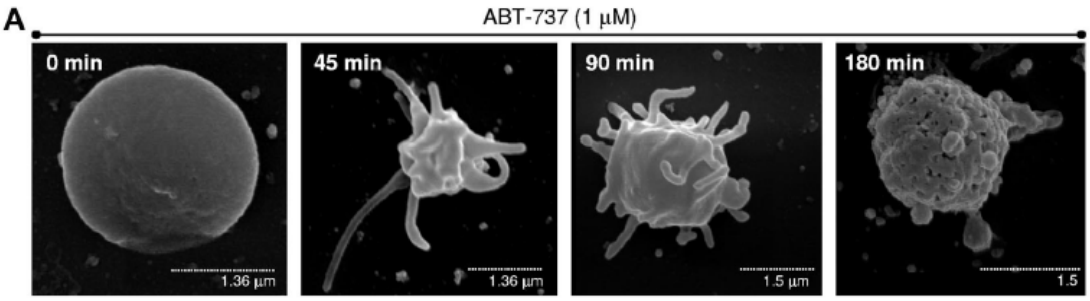


Figure 8.10: Scanning electron micrographs showing morphological changes in platelets incubated for the annotated periods of time with ABT-737 (Schoenwaelder et al., 2011) (Reproduced with permission, © The American Society of Hematology).

- Measurement of extracellular Ca^{2+} in addition to intracellular levels would improve the picture on Ca^{2+} movements (Sandgren 2010 found no increase in PCs with

storage) (Sandgren et al., 2010). Quantitation of intracellular calcium levels may be possible by the use of standard lines derived from commercially available buffers with known concentrations of Ca^{2+} (Dustin, 2000).

- The temporal correlation between the exhaustion of glucose and increasing activity in the markers suggestive of platelet death evident in this study led to the suggestion that ROS generation promoted by the loss of glutathione-mediated antioxidant capacity was an initiator of platelet death in storage. Further studies that directly measure ROS and glutathione levels, preferably in relation to mitochondrial function, would be necessary to test this hypothesis (Cossarizza et al., 2009). Possible methods include the use of fluorescent dyes such as 2′-7′-dichlorodihydrofluoroscein diacetate (DCFH-DA) for determining the redox state of the cell as well as dyes which may localise reactive species to specific organelles (Curtin et al., 2002). Various techniques for the measurement of H_2O_2 and $O_2^{\bullet-}$ have been described, though caution is required in their application to reduce the influence of confounding factors in the interpretation of results (Degli Esposti, 2002, Halliwell, 2007).

- Measurement of levels of Bcl-2 family members in platelets (Leytin et al., 2006) stored as concentrates, with emphasis on Bax, Bak and Bcl-X_L, may serve to complement published studies on knockout mouse models postulating that the decline of Bcl-X_L levels is a principal controller of platelet death (Mason et al., 2007).

- The pivotal role played by cytochrome c in the formation of the apoptosome and the subsequent activation of caspases during apoptosis makes the protein an attractive target to help determine the role of apoptosis in the PSL. Isolation of the mitochondria from the cytosol and progressive measurements of cytochrome c in the two fractions over the course of the storage period would help to show the time course of cytochrome c release. Its relation to other parameters such as ATP loss may help determine the nature of the cell death pathway in platelets during storage. The rate of caspase 3 activation would be an interesting parallel measure suggestive of the initiation of caspase-dependent apoptosis (Dasgupta et al., 2010, Martinez et al., 2010).

- Experiments employing inhibitors to initiators or regulators of cell death processes such as specific caspases or inhibitors of Bcl-2 family members may provide a systematic approach to aid in the identification of the cell death pathways involved in the PSL. Studies into the role of cyclophilin D, which plays a central role in promoting MPTP opening, have suggested a function as a regulator of necrotic death with little or no impact on events associated with apoptosis (Kung et al., 2011). Addition of the cyclophilin D inhibitor, cyclosporine A, to PC may provide further insight into platelet death in storage as well as the procoagulant activity of aged platelets (Jobe et al., 2008, Jackson and Schoenwaelder, 2010). A barrier with all these investigations in the context of a large-volume system such as platelet concentrate storage is the cost. Thus, the development of a small-volume model of PC in storage may be required before they can be practically considered.

- The partial pressure of oxygen in arterial blood ranges between 10.6 to 13.3 kPa and is lower still in capillaries (6.0 – 8.0 kPa) (Kumar and Clark, 2009, Halliwell and Gutteridge, 2007). Thus, platelets *in vivo* are seldom exposed to the levels of oxygen that were observed in the storage packs even at the start of the storage period. The hypothesis that ROS generation by platelets during storage is at least partly causative of the changes comprising the PSL would suggest that storage of platelet concentrates in a limited oxygen environment in gas-permeable packs that allow for the loss of CO_2 may help to limit some of these changes. A storage study using a paired design would help to answer this question.

- It would be interesting to investigate platelet death in storage against methods developed to measure platelet function. Such methods could include classical aggregation studies due to the long history of published work and continued relevance in the clinical setting. More recent technologies aim to mimic platelet function *in vivo* somewhat more faithfully. They include the measurement of platelet aggregation under conditions of high shear in response to agonists (PFA-100 analyer: Dade-Behring, Marburg, Germany) (Tanaka, 2006), and platelet adhesion and aggregation to a matrix expressing various glycoproteins associated with the platelet surface (Impact R: DiaMed, Switzerland) (Harrison, 2005). Both technologies were designed for use with whole blood but can be modified to study PCs by reconstituting the units with leucodepleted whole blood or leucodepleted red cells plus plasma (Morrison et al., 2007, Cardigan et al., 2005). A recent study has

reported that treatment of mice with the BH3-mimetics ABT-737 or ABT-263 led to the shedding of GP1bα vWF receptor and the collagen receptor GPVI, with defective adhesion to thrombogenic surfaces (Schoenwaelder et al., 2011). Combining platelet function studies with flow cytometric measurements of platelet surface glycoproteins as well as measurements of Bcl-2 family proteins may help to determine whether Bcl-2 proteins commonly associated with cell death can also help regulate the function of stored platelets post-transfusion.

- Finally, an increasingly detailed understanding of the mechanisms behind the PSL must be related to the practical clinical setting, with *in vivo* studies to determine platelet function post-transfusion as well as patient morbidity in addition to the more traditional measures of platelet survivability.

LIMITATIONS OF THE STUDY

- Experience with the methods adopted and developed as part of the study identified some limitations that, if addressed, could improve their robustness and applicability for the study of the platelet storage lesion. The relatively high levels of surface CD62P expression in comparison with some of the published literature have already been discussed in depth. Published results on mitochondrial membrane potential measured flow cytometrically with cationic dies such as JC-1 have resulted in differing interpretations regarding the role of changes in mitochondrial functional integrity in the progression of the storage lesion. It would be advantageous to use a second technique to investigate the role of $\Delta\Psi m$ in platelet death during storage and confirm the timeframe of mitochondrial changes in relation to the other markers indicative of platelet death. Additionally, although ATP levels were measured against a standard line, a reliable ATP control could not be sourced in a timely manner. Such a control would increase confidence in any direct comparison of the numerical results with the published literature.

- Relatively low levels for the HSR and ESC assays were evident in all PCs suspended solely in additive solution when compared with levels obtained for PC in plasma or a standard 70:30 ratio of additive solution and plasma. The harsher

processing method used to generate the former units could only account for a small proportion of this disparity, as units similarly manufactured but re-suspended in plasma showed a relatively small decrease in HSR and ESC responses. Existing recommendations based on PC suspended in plasma suggest that HSR values below 60% correspond to platelet recovery levels below 50% following transfusion (Diedrich et al., 2008). It would be difficult to apply such limits to PC in 100% additive solution if the results from this study are confirmed. It has also been suggested that platelet recoveries below 50% are observed when HSR values fall below 70-75% of levels in fresh platelets (Cardigan et al., 2005). Such a self-contained comparison appears more applicable to the results from the additive solution studies. Similarly, using limits based on percentage decrease of starting levels would be more applicable for ESC, with platelet recovery below 50% corresponding with ESC values below 60% of day 1 levels (Holme et al., 1998). An additional consideration is that the autologous plasma used as the sample diluent was stored at ambient temperature. A comparison of HSR and ESC results subsequent to the work for this thesis did not confirm that the storage temperature of the plasma was a significant factor. Buffering of the plasma was not undertaken until the day of testing – a further variable that would be worth investigating.

– Logistical considerations limited the number of replicates to five for the studies investigating the role of albumin, glucose and acetate. However, results from the preliminary study - which measured the storage characteristics of platelets stored in either autologous plasma or a medium of SSP+™ plus plasma over an extended storage period - provided confidence in the reproducibility of the assays adopted.

CONCLUDING REMARKS

The platelet storage lesion is a term used to encompass the varied changes observed in platelets stored as concentrates. The study employed a wide range of methodologies to provide a relatively comprehensive picture of the nature of the PSL in a variety of both standard and manipulated storage media. The aim was to provide a better understanding of the phenomenon in the context of the hypothesis that apoptosis plays a central role in platelet death in storage.

Results suggested that in storage media with adequate energy stores in the form of glucose, a Bcl-2 protein-mediated mechanism of cell death was viable, though possibly storage-time dependent and limited by the pre-existing levels of anti-apoptotic Bcl-2 proteins in the platelets. Further studies would be required to determine if this mechanism is akin to caspase-dependent apoptosis. In media lacking glucose, a mechanism more reminiscent of necrosis was observed, associated with decreased ATP levels, accelerated mitochondrial dysfunction, elevated intracellular free calcium and culminating in platelet disruption.

The thesis has certainly raised far more questions than it has answered, illustrating the complexity of the processes involved and the need for a more fundamental approach to the understanding of platelet senescence, with the ultimate aim of enhancing the efficacy of platelet transfusions and improving patient care.

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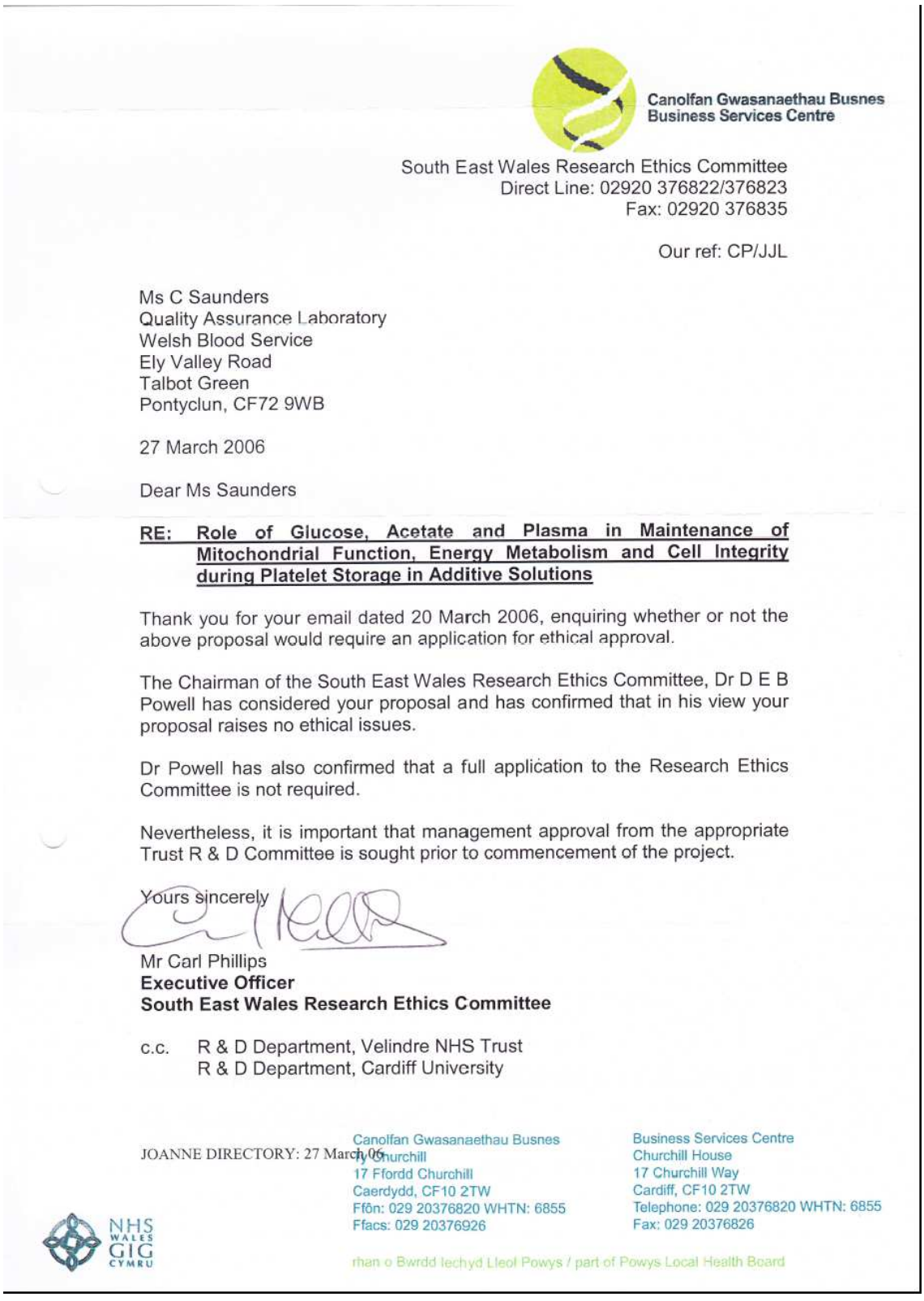
APPENDIX 1



Canolfan Gwasanaethau Busnes
Business Services Centre

South East Wales Research Ethics Committee
Direct Line: 02920 376822/376823
Fax: 02920 376835

Our ref: CP/JJL

Ms C Saunders
Quality Assurance Laboratory
Welsh Blood Service
Ely Valley Road
Talbot Green
Pontyclun, CF72 9WB

27 March 2006

Dear Ms Saunders

RE: Role of Glucose, Acetate and Plasma in Maintenance of Mitochondrial Function, Energy Metabolism and Cell Integrity during Platelet Storage in Additive Solutions

Thank you for your email dated 20 March 2006, enquiring whether or not the above proposal would require an application for ethical approval.

The Chairman of the South East Wales Research Ethics Committee, Dr D E B Powell has considered your proposal and has confirmed that in his view your proposal raises no ethical issues.

Dr Powell has also confirmed that a full application to the Research Ethics Committee is not required.

Nevertheless, it is important that management approval from the appropriate Trust R & D Committee is sought prior to commencement of the project.

Yours sincerely

Mr Carl Phillips
Executive Officer
South East Wales Research Ethics Committee

c.c. R & D Department, Velindre NHS Trust
R & D Department, Cardiff University

JOANNE DIRECTORY: 27 March 06

Canolfan Gwasanaethau Busnes
Ty Churchill
17 Ffordd Churchill
Caerdydd, CF10 2TW
Ffôn: 029 20376820 WHTN: 6855
Ffacs: 029 20376926

Business Services Centre
Churchill House
17 Churchill Way
Cardiff, CF10 2TW
Telephone: 029 20376820 WHTN: 6855
Fax: 029 20376826

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