Enhancement of a bone substitute by chemical pre-treatment to mobilize growth factor stores

Thesis submitted to fulfil requirements for the degree of Master of Philosophy by:

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For Lisa and my parents.

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Abstract

Growth factors are extracellular molecules that have several regulatory roles during inflammation and bone regeneration. Bio-Oss® granules and Bio-Oss® Collagen are xenografts used in the management of periodontal and peri-implant defects, in addition to a variety of other clinical indications. The aims of this thesis were to investigate if Bio-Oss® contains residual growth factors that can be released by chemical pre-treatment. It also aimed to investigate if commercial Bio-Oss® granules or its extracted components alter cellular behaviour.

Bio-Oss® collagen and Bio-Oss® granules underwent extraction with either ethylenediaminetetraacetic acid, calcium hydroxide or hydrochloric acid,followed by guanidinium chloride and tris(hydroxymethyl)aminomethane. Extracted proteins were separated using sodium dodecyl sulphate - polyacrylamide gel electrophoresis and stained with silver stain. Stained protein bands were consistently demonstrated in Bio-Oss® extracts. Immunoreactivity was demonstrated using antibodies to TGF-β_1 and the Western blot technique. In order to investigate cellular behaviour, bone marrow stromal cells were harvested form Wistar rats prior to being cultured using mineralising media in the presence of Bio-Oss® granules and Bio-Oss® Collagen. Commercial Bio-Oss® in both forms was unable to support cell growth when seeded directly on their surfaces. In contrast, when cells were cultured with media supplemented with the extracted components of Bio-Oss® granules, cell behaviour was positively affected. Cell expansion was increased following exposure to DBBM extracts as measured with the MTS assay. Importantly, polymerase chain reactions revealed enhanced temporal expression of bone sialoprotein in cells cultured in the presence of DBBM extracted components.

The detection of residual protein within Bio-Oss® granules in particular may be of clinical significance. The protein may be in the form of a complex of TGF-β_1, a key growth factor, which may be osteoinductive. Alteration of gene expression profiles may suggest that faster bone regeneration is possible clinically if Bio-Oss® is chemically pre-treated to solubilise growth factors.

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Diolch.

Abbreviations

°C	Degrees celcius
µg	Microgram
µl	Microlitre
mm	Millimetre
mM	Millimolar
nm	Nanometre
BCA	Bicinchoninic acid
BMD	Bone mineral density
BMP	Bone morphogenetic protein
BMSC	Bone marrow stromal cells
BSM	Bone substitute material
CAL	Clinical attachment level
$Ca(OH)_2$	Calcium hydroxide
Cbfa-1	Core binding factor α 1
CSF-1	Colony stimulating factor 1
DBBM	Deproteinized Bovine Bone Mineral
EDTA	Ethylenediaminetetraacetic acid
EMD	Enamel matrix derivative
ECL	Enhanced chemiluminescence
FCS	Foetal calf serum
FGF	Fibroblast growth factor
GBR	Guided bone regeneration
GTR	Guided tissue regeneration
GuCl	Guanidinium chloride
HCl	Hydrochloric acid
HRP	Horseradish peroxidase
IGF	Insulin like growth factor
IgG	Immunoglobulin G

IL-1	Interleukin-1
LAP	Latency associated peptide
αMEM	α minimum essential medium
MCSF	Macrophage colony stimulating factor
Min	Minute
MTA	Mineralised trioxide aggregate
mRNA	Messenger ribonucleic acid
OC	Osteocalcin
OFD	Open flap debridement
ON	Osteonectin
OP	Osteopontin
OPG	Osteoprotegerin
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
RANKL	Receptor activator of nuclear factor kappa B ligand
RT-PCR	Reverse transcription polymerase chain reaction
Runx-2	Runt-related transcription factor 2
SDS	Sodium dodecyl sulphate
TBE	Tris / borate / EDTA
Tris	Tris(hydroxymethyl)aminomethane
TGF-β	Transforming growth factor β
TNFα	Tumour necrosis factor α
VEGF	Vascular endothelial growth factor

Chapter 1

Literature review

1.0 Introduction to study

Growth factors play a pivotal role in bone healing and regeneration within periodontal defects and implant-related bone augmentation. Importantly, growth factors are known to be sequestered in bone matrix. A commercial bone substitute material known as Bio-Oss® is derived from bovine sources. There is conflicting evidence regarding its residual protein content that requires further investigation. Furthermore, there is potential that residual protein within Bio-Oss® is in the form of growth factors. Consequently ,the release of these growth factors could have positive effects on bone regeneration.

This thesis will initially characterise the presence of residual protein within Bio-Oss®. In addition, it will focus on *in vitro* assessment of the impact that this bone substitute has on cellular activity. It will identify whether the effect of growth factors locked within bone matrix can be extracted by chemical pre-treatment of the bone substitute. This data will form the initial phase of a strategy to improve clinical techniques available for bone regeneration, particularly in conditions that result in bone resorption or negatively affect bone healing.

1.1 Introduction to bone biology

1.1.1 Macrostructure

In order to understand the requirement for bone substitutes in a given clinical situation, it is important to have an understanding of bone biology. Bone is known to be a dynamic, specialized connective tissue composed of a mineralised organic matrix. This matrix consists of collagen fibres and non-collagenous proteins surrounding mineral hydroxyapatite (Nanci 1999; McCauley and Nohutcu 2002). By necessity, bone has been described previously in terms of maturity, bone marrow content and location (Davies 2003; Bosshardt 2009). In terms of its maturity, immature woven bone, which is produced rapidly by the mineralisation of osteoid, has a disorganised fibrillar structure that is not capable of withstanding the same load as mature lamellar bone. In contrast, lamellar bone is mature, organized bone formed after a period of remodelling of woven bone and is considerably more adapted to functional requirements (Nanci 1999). The second characteristic often used to describe bone is its bone marrow content. Cancellous bone (spongy or trabecular bone), which is found in the centre of long bones and alveolar processes, contains islands of lamellar bone which have been termed trabeculae that are separated by bone marrow. Of vital importance to regenerative strategies, this bone marrow is known to be a potent source of multipotent mesenchymal stem cells that have the ability to self renew and divide into a variety of cell types (Davies 2003; Huang 2009). Cortical bone (or compact bone) on the other hand is located at the periphery of long bones, the mandible and maxilla but in different proportions depending upon location. This cortical bone is composed of dense lamellar bone without intervening bone marrow. Within these lamellae the basic structural units are cylindrical

structures termed haversian systems that contain vascular canals within their centre important for the vitality of bone.

It is worth noting that cortical bone contributes to high primary stability during implant placement and therefore its presence is of clinical importance. Cancellous bone is contained within this cortical bone and is composed of varying densities of trabeculae (Davies 2003). In Implantology, trabecular bone has been described by some as biologically superior bone due to its faster remodelling potential even though, as explained below, many clinicians reported that this type of bone is of “poor quality” (Davies 2003). It is well known that the bone in the mandible and maxilla has been previously classified clinically by Lekholm and Zarb based on the proportion of cortical and cancellous bone (Lekholm and Zarb 1985). Within this classification, class 1 bone is mainly cortical and located in the anterior mandible. Conversely class 4 has very little cortical bone and is located primarily in the posterior maxillae. This is a useful clinical classification, of particular relevance during the placement of endosseous titanium dental implants that has been shown to correlate with the success rate of early machined implants (Jaffin and Berman 1991). The poor clinical success of initial designs of dental implants reported by Jaffin *et al* (1991) led to type 4 bone being termed “poor quality bone”. In contrast, more recent clinical studies of modern implant surfaces using modified surgical techniques tentatively suggests that the success rate in type 4 bone is approaching that of other bone types, with one retrospective study reporting less than 3% failure in type 4 bone (Friberg et al. 2002).

The bone within the jaw is classified as either alveolar where the alveolar bone supports the teeth in the dentate subject or basal bone below this. The socket wall surrounding teeth has also been described as bundle bone due to the bundles of inserting collagen fibres, termed Sharpey’s fibres, between the tooth cementum and the bone (Araújo and Lindhe 2005). This bone has been shown in animal models to be entirely dependent upon these inserting periodontal

ligament fibres. In a histological study, following tooth extraction these fibres were torn and the alveolar bone was shown to be lost within 4 – 8 weeks (Araújo and Lindhe 2005). Clinically, this bone loss can make subsequent implant placement impossible without bone augmentation and therefore regenerative strategies have been developed to preserve the socket that will be described in detail in section 1.7.6.

1.1.2 Microstructure

Microscopically, bone is composed of mineral, cells, collagens, non-collagenous proteins and proteoglycans. It is often described that bone matrix is composed of approximately 70% mineral, 30% organic components and water, although slight variations appear in the literature (McCauley and Nohutcu 2002). Bone is similar to other connective tissues in that it relies on specific cell types for its production and remodelling. Specifically, three key cells are associated with bone, namely osteoblasts, osteocytes and osteoclasts (Katagiri and Takahashi 2002; Bosshardt 2009). Osteoblasts are fully-differentiated cells, derived from mesenchymal cells that are unable to migrate and proliferate and are uniquely involved in bone formation and remodelling (Mackie 2003; Hughes et al. 2006). Osteocytes are thought to coordinate bone homeostasis and are derived from osteoblasts that become trapped within bone. Lastly, osteoclasts are known to initiate and maintain bone resorption (Davies 2003). Osteoclasts are found within lacunae at the resorption front of bone, whereas osteoblasts are found on newly-formed organic matrix at the bone deposition front (Giannobile 2008; Bosshardt 2009). Further details on cellular behaviour will be discussed in other sections of this review.

As already mentioned, cancellous bone contains trabeculae of lamellar bone separated by bone marrow. Bone marrow is known to contain a rich vasculature and a potent source of multipotent mesenchymal stem cells that can

differentiate into multiple cell types (Bruder et al. 1994; Davies 2003). Stem cells, importantly, are not terminally differentiated, have unlimited division capability and are described as either multipotent or pluripotent (Kuhn and Tuan 2010). Pluripotent (embryonic) stem cells can differentiate into any cell type but, multipotent mesenchymal stem cells primarily differentiate down the mesenchymal lineages including the osteogenic lineage (Kuhn and Tuan 2010). Mesenchymal stem cells, which also originate from granulation tissue and periosteal surfaces, are self-renewing undifferentiated cells that are capable of extensive replication (Dimitriou et al. 2005). These cells must not be confused with osteoproginator cells which are undifferentiated cells of mesenchymal origin. They migrate to sites of new bone formation and can differentiate into osteoblasts when stimulated by appropriate signalling molecules termed, growth factors (Bruder et al. 1994). Osteoblast-like cells are demonstrated biochemically by biosynthesis of osteocalcin and activity of the enzyme alkaline phosphatase (McCauley and Nohutcu 2002). Alkaline phosphatase is known to promote hydrolysis of phosphate containing substrates, increase deposition of calcium phosphate, and enhance mineralisation of collagen matrices (Açil et al. 2000). As will be discussed later, the local production or therapeutic application of growth factors will have an osteogenic effect driving cells down an osteoblastic lineage.

1.1.3 Osteogenesis

Osteogenesis is the process of bone formation by osteoblasts (Nefussi 2007). In terms of bone grafting or healing around implants, it requires the migration and proliferation of mesenchymal stem cells from the marrow space to the site of healing adjacent to the graft or implant material. The process has been described elsewhere in terms of healing around implants, with the terms contact and distance osteogenesis being used, depending on whether bone formation occurs on the implant or bone surface respectively (Davies 2003). Once at the

site of bone formation, the cells will proliferate, commit to an osteogenic lineage and pass down the osteoblast proliferation pathway guided by an array of signalling molecules (Bruder et al. 1994; Katagiri and Takahashi 2002). Once fully differentiated, proliferation ceases and osteoblasts will secrete a mixture of proteins, including collagen type 1, proteoglycans and glycoproteins (Mackie 2003). However, the initial stages of bone formation involve the secretion of collagen free organic matrix, termed osteoid, containing these non-collagenous proteins that facilitate calcium phosphate mineralization (Davies 2003). As the osteoblast cell lays down new bone, they form an ability to migrate over or within particulate bone graft materials which is critical to the success of theses augmentation procedures and the biocompatibility of the graft material itself.

1.1.4 Cellular behaviour, bone healing and osseointegration

Bone healing following surgical trauma must involve bleeding, clot formation, recruitment of inflammatory cells and undifferentiated mesenchymal cells, the release of growth factors and cytokines and finally osteogenesis (Davies 2003). In alveolar healing, unlike in long bones, no intervening callous formation stage occurs (Nefussi 2007). Haemostasis initially involves the formation of the platelet plug following their adhesion and aggregation to wound surfaces. Subsequently the platelets degranulate and release the contents of their secretory vesicles and a coagulation cascade begins that will ultimately produce a stabilized fibrin clot (Vander et al. 2011). This fibrin clot undergoes replacement by granulation tissue and the inflammatory process results in the release of bone-derived growth factors. These will promote the migration of mesenchymal stem cells from the periosteum and medullary spaces and osteogenesis (Bruder et al. 1994).

Much later, bone remodelling includes an important series of events that occurs following trauma or infection, but is also a continuous process throughout life. Bone remodelling involves two key processes that must be considered as occurring in unison, but at different rates depending on the requirements of the remodelling site (Figure 1.1). These two processes are bone resorption and bone formation (Giannobile 2008). It has been suggested the resorptive phase occurs over 3 to 4 weeks and the bone formation process occurs over 3 to 4 months (Rodan 1997; McCauley and Nohutcu 2002). The remodelling cycle has been summarised in the review by McCauley and Nohutcu (McCauley and Nohutcu 2002). Briefly, pre-osteoclasts are recruited and differentiate into osteoclasts that resorb bone. Interestingly, through a process of coupling, pre-osteoblasts are recruited and differentiate into active secreting osteoblasts with some osteoblasts becoming entrapped as osteocytes. The ability of undifferentiated mesenchymal stem cells to migrate and proliferate is under the control of growth factors, cytokines and local nutrients before ultimately they become osteoblasts (Bruder et al. 1994). These signalling molecules are produced by inflammatory cells, platelet degranulation, exposed bone surfaces and, potentially, bone mineral substitutes (Schwartz et al. 2000; Davies 2003). Transcription factors such as Cbfa-1 (Runx-2) are also required for expression of osteoblast specific genes, such as osteocalcin and bone sialoprotein (Mackie 2003).

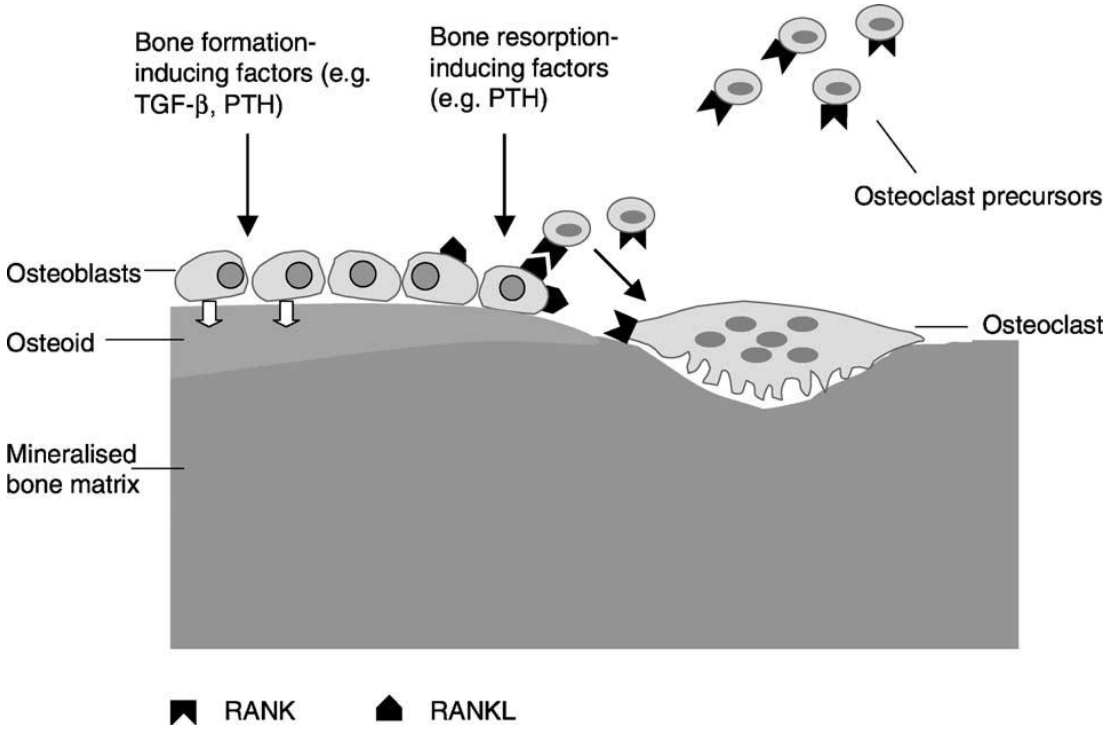


Figure 1.1 Diagrammatic overview of the cells involved in bone remodelling (Adapted from Mackie 2003)

As already stated, osteoblasts are derived from mesenchymal osteoprogenitor cells found in the bone marrow and periosteum through a process of differentiation that, although well described, is not fully understood (McCauley and Nohutcu 2002; Mackie 2003; Hughes et al. 2006). Briefly, mesenchymal cells differentiate through stages of being osteoprogenitor cells followed by preosteoblasts before ultimately becoming osteoblasts that can be identified by expression of specific markers. From an *in vitro* perspective, during matrix production the osteoblast expresses a variety of factors that can be detected to aid its identification, such as osteocalcin and bone sialoprotein (Franceschi 1999). Through this pathway, the cells retain the capability of proliferation until they become fully differentiated osteoblasts (Hughes et al. 2006). Signalling molecules termed growth factors such as bone morphogenetic proteins (BMP's), transforming growth factors (TGF's), platelet derived growth factors (PDGF), insulin-like growth factor (IGF) and fibroblast growth factor (FGF) are known to

be capable of initiating osteoblast differentiation from progenitor cells and importantly, osteoblasts themselves secrete these growth factors (Yamaguchi et al. 2000; McCauley and Nohutcu 2002).

The other key cell in bone homeostasis is the osteoclast. This is identified as a multinucleated cell and is derived from the haematopoietic lineage within the bone marrow (Katagiri and Takahashi 2002; Boyle et al. 2003). Importantly, in *in vitro* experiments these cells can be detected by their expression of the lytic enzyme, tartrate-resistant acid phosphatase (TRAP) (McCauley and Nohutcu 2002). Bone resorption by osteoclasts is known to be achieved by the degradation of the organic component by matrix metalloproteinases followed by liberation of bone mineral through secretion of hydrochloric acid (Teitelbaum 2000). Bone resorption was once thought to be purely mediated by osteoclasts. However, it is becoming established in the literature that osteoblasts play a key role in the differentiation of osteoclasts, in particular via receptor activator of nuclear factor kappa B ligand (RANKL)(Katagiri and Takahashi 2002; Boyle et al. 2003; Hughes et al. 2006). RANKL is a receptor located on the surface of osteoblasts and its activation has been shown to be a key stimulator of bone resorption by osteoclasts via interaction with the RANK receptor (Boyle et al. 2003). The effects of RANKL can be blocked by osteoprotegerin (OPG), a decoy factor produced by osteoblasts, which helps to regulate the bone remodelling process, but excessive expression leads to increased bone density (Horowitz et al. 2001). Furthermore, colony stimulating factor 1 (CSF-1), also known as macrophage colony stimulating factor (MCSF) that is secreted by osteoblasts has also been shown to play a role in the differentiation of osteoclast progenitors (Boyle et al. 2003). CSF-1 and RANKL are therefore required to induce expression of genes that characterise the osteoclast lineage. The combined evidence therefore clearly demonstrates that osteoblasts maintain bone not only through matrix deposition but also osteoclast regulation.

Other key mediators of bone resorption reported in the literature include, but not exclusively, interleukin 1, interleukin 6, prostaglandin E_2, tumour necrosis factor, parathyroid hormone, vitamin D, oestrogens and calcitonin (McCauley and Nohutcu 2002). It is beyond the scope of this project to discuss each factor in detail but certain factors will be discussed where appropriate.

As mentioned, the third fully-differentiated cell type within bone is the osteocyte and it has been described as the most differentiated cell of the osteoblast linage (Mackie 2003; Hughes et al. 2006). Briefly, this cell is believed to co-ordinate bone homeostasis via communications with osteoblasts and osteoclasts controlling both formation and resorption respectively (Rodan 1997; Mackie 2003).

From a clinical perspective, the ideal outcome of the remodelling process following surgical or non surgical trauma is the regeneration of mature, functional new bone. If this is not achieved, the osseous tissue may be repaired rather than regenerated forming fibrous scar tissue. This tissue is not adapted to functional demands like bone. During remodelling woven immature bone becomes replaced by lamellar bone which is adapted to resist functional load. Knowledge of the cell types involved in bone remodelling enables their detection via identification of signalling molecules or gene expression specific to them.

1.1.4.1 Osseointegration

Osseointegration can be defined as the direct functional and structural connection between living bone and the surface of a load carrying implant (Branemark 1983). The objective is to achieve a predictable tissue response to the placement of a dental implant which originally required bone growth from the bone towards the implant surface (Davies 2003). The response should be to produce a highly differentiated tissue that becomes organised depending on functional demands (Branemark 1983). Following implant placement,

processes of healing occur with bone remodelling starting after 7 days. This is the first stage of osseointegration. Using transmission electron microscopy, it is possible to see cell processes from bone and marrow cells adhering to the titanium oxide surface of implants (Berglundh et al. 2003). Osseointegration can only be achieved when the following factors are met: use of a pure titanium alloy, minimal surgical trauma, good quality bone and a sufficient healing period prior to loading (Adell et al. 1981). Conversely, failure of osseointegration occurs where a thick layer of connective tissue surrounds the implant prior to bone contact. Furthermore, failure is increased by surgical trauma, adverse loading, local bone quality and patient systemic factors. During implant placement, it is important that the implant is firm immediately following insertion. This concept is known as primary stability and is a determinant of long-term success. Where primary stability is unlikely to be achieved methods of bone augmentation may be required. Even if primary stability will be possible, bone augmentation is often required in the anterior aesthetic zone to prevent loss of buccal hard and soft tissue (Buser et al. 2004). This helps to ensure an integrated implant and an aesthetic prosthesis.

1.1.5 Systemic factors affecting bone quality and remodelling

Innate regulatory molecules are important in the systemic control of bone remodelling. This includes, but not exhaustively, parathyroid hormone, growth hormone and steroids, but the detail of their action is outside the scope of this review (Bosshardt 2009). Importantly, systemic conditions will affect bone density, volume and remodelling that clinically can alter periodontal disease progression and prejudice dental implant replacement (Karoussis et al. 2007). Knowledge of these conditions is therefore essential since it drives the search for improved bone regeneration techniques that may be used in the management of such conditions.

Osteoporosis is one such condition, defined as a skeletal disease that results in low bone mineral density (BMD) that predisposes long bones to fracture (Glaser and Kaplan 1997). Specifically, bone loss has been shown in the alveolar bone in patients with osteoporosis via bitewing radiograph densitometric analysis (Payne et al. 1997). Although the pathology of osteoporosis is not fully understood, it has been described as a metabolic disorder resulting from imbalances in the expression of bone signalling molecules that leads to altered bone homeostasis and inadequate osteoblastic bone formation in relation to osteoclastic bone resorption (Rodan 1997; Mackie 2003; Bosshardt 2009). Of relevance to dentistry, osteoporosis is a risk indicator for periodontal disease based on the majority of studies demonstrating an association between the two conditions (Stabholz et al. 2010). In the absence of a plaque induced inflammatory lesion, attachment loss will not occur in a patient with osteoporosis. However, where a lesion is present, it has been shown in several studies that there will be proportionally more bone loss as a result of periodontal disease compared to an individual without osteoporosis (Geurs 2007). Osteoporosis is also considered by some to be a risk indicator for implant placement (Alsaadi et al. 2007). It is important to be aware that increasingly over the last 15 years patients with osteoporosis have been treated with bisphosphonate medication. These agents inhibit osteoclast initiated bone resorption by promoting cell apoptosis and should therefore contribute to increased BMD (Rodan 1997). Unfortunately, the accumulation of bisphosphonates in surrounding bone has led to sporadic cases of bisphosphonate-associated osteonecrosis of the jaws (McCauley and Nohutcu 2002; Ruggiero et al. 2004). Although this destructive condition can occur spontaneously, it may be triggered by surgical trauma and therefore guidelines have been produced for the management of these patients when planning implant treatment (Madrid and Sanz 2009).

The second particularly important condition in terms of bone biology and healing is diabetes mellitus. This common endocrine disorder is increasing in incidence

in the western world (Giannobile 2008). It has most recently been classified as type 1, where insufficient insulin is produced and type 2, where there is relative insulin resistance (Giannobile 2008). Bone formation and remodelling is decreased in diabetes mellitus as a result of decreased osteoblast differentiation and a reduction of growth factors (Giannobile 2008).

Animal experiments using a diabetic mouse model have suggested that diabetes may affect bone repair through its effects on growth factors and osteoblast differentiation, demonstrated by decreased expression of osteocalcin (Lu et al. 2003). In terms of dental disease, individuals with diabetes mellitus have been shown in a number of studies to be at increased risk of periodontitis, progressive alveolar bone loss and ultimate tooth loss (Loos et al. 2005; Kinane et al. 2006). In addition, tooth replacement is also complicated by diabetes mellitus. In a review of both animal and human studies Kotsovilis and co-workers demonstrated impaired bony healing in diabetics (Kotsovilis et al. 2006). This has been confirmed in a recent study using a diabetic rat model following the insertion of implants into incisor sockets (Colombo et al. 2011). In this study, histological examination revealed delayed osteoblastic differentiation in diabetic rats. In humans, where diabetic control is good there will be little effect on implant prognosis, however, overall diabetic patients have higher implant failure rates (Moy et al. 2005; Kotsovilis et al. 2006). The combined evidence clearly shows that among systemic disorders osteoporosis and diabetes mellitus impact on bone healing, periodontal disease and implant placement. Predictable bone regeneration is therefore required in these patient groups.

1.2 Extracellular structure

1.2.1 Collagen

The extracellular structure of bone contains several types of collagen with type 1 being the most abundant representing 90% of the organic matrix (Mackie 2003; Hughes et al. 2006). This contributes to its physical properties. Type 1 collagen is synthesised by osteoblasts during bone formation arranged in fibrils and when in an ordered arrangement, as in lamellar bone, contributes to the strength of living bone (Nanci 1999). Indeed, type 1 collagen forms a scaffold, in combination with non-collagenous proteins around which calcium and phosphate ions can accumulate to form mineral crystals during the mineralization process (Nanci 1999; Bosshardt 2009). It appears that there may be a correlation between the amount and formation of collagen and non-collagenous protein content of bone (Nanci 1999). The relative distribution of these will impact on mineralisation and the physical properties of bone at that anatomic location. Importantly, in tissue culture experiments, the expression of type 1 collagen can be a useful indicator of osteoblastic differentiation (Lu et al. 2003).

1.2.2 Proteoglycans

Proteoglycans are glycosylated proteins that are composed of a core protein and one or more glycosaminoglycan side chains. Biglycan and decorin are proteoglycans demonstrated in bone tissue that have been identified as critical in bone remodelling with high affinity for calcium binding (Hughes et al. 2006; Smith et al. 2011). The mechanism through which they exert their affect *in vivo* is not fully known, but *in vitro* studies have demonstrated correlation between

expression of biglycan and decorin, collagen fibrillogenesis, cell proliferation and matrix deposition (Mackie 2003; Waddington et al. 2003). Biglycan and decorin are known to sequester growth factors and importantly may protect transforming growth factor β_1 (TGF-β_1) within the matrix or be involved in its presentation to the TGF-β_1 receptor (Takeuchi et al. 1994; Baker et al. 2009). As will be described later, TGF-β_1 is critical to bone remodelling. Biglycan and decorin may regulate cellular signalling promoting osteoblast differentiation and therefore are key extracellular matrix components critical in driving osteogenesis.

1.2.3 Glycoproteins

Several non-collagenous proteins are markers of bone remodelling that are detected in various concentrations in different regions of bone (Nanci 1999). These include osteocalcin, osteonectin, osteopontin and bone sialoprotein (Hughes et al. 2006; Bosshardt 2009). In histological studies using immunocytochemistry, bone sialoprotein and osteopontin have been detected in cement lines within bone (Nanci 1999). These so called glycoproteins are also proteins that contain oligosaccharide side chains that, like proteoglycans are found with the extracellular matrix.

Osteocalcin is widely accepted as the most abundant non collagenous protein in the mineralised bone matrix and is associated with bone formation (Franceschi 1999; Taba et al. 2005). Osteocalcin is a late marker of osteoblastic differentiation and bone formation produced only by osteoblasts and can therefore be used to detect their presence (Ducy et al. 1996). Studies using osteocalcin knockout mice have shown an increase in bone mass as a consequence of this deletion signifying its importance in bone remodelling (Ducy

et al. 1996; Hughes et al. 2006). This suggests osteocalcin may function to limit bone formation to produce brittle hypomineralised bone.

Osteopontin is an extracellular matrix protein produced by osteoblasts and osteoclasts. Preosteoblasts also demonstrate osteopontin expression and its levels have been shown to be increased at sites of active bone metabolism (Hughes et al. 2006). Osteopontin is therefore an early marker of osteoblastic differentiation. The detection of osteocalcin and osteopontin via the experimental technique, polymerase chain reaction, identifies that mature osteoblasts are present.

Osteonectin and bone sialoprotein are other non-collagenous proteins that may play a role in bone remodelling and bone cell adhesion (Bradshaw and Sage 2001; McCauley and Nohutcu 2002; Mackie 2003). Bone sialoprotein is a marker of bone resorption and its detection during tissue culture experiments suggests the cells are either pre-osteoblasts or osteoblasts (McCauley and Nohutcu 2002; Hughes et al. 2006). Further, it has been suggested that bone sialoprotein acts as a nucleator for bone mineral deposition (Mackie 2003; Amerio et al. 2010). Meanwhile, osteonectin is proposed to modulate the activity of the growth factor TGF-β_1 by increasing production of its mRNA, promotes osteoblast differentiation, affects collagen synthesis and binds calcium ions (Bradshaw and Sage 2001; Amerio et al. 2010).

Fetuin (also known as α2 HS glycoprotein) is another glycoprotein that may have an important role in bone metabolism and accumulates in the mineralized matrix of bone (Schinke et al. 1996). *In vitro* studies have demonstrated that fetuin has a high affinity for calcium binding in foetal bovine serum (Suzuki et al. 1994). Furthermore, fetuin has also been shown to stimulate bone cell proliferation *in vitro* but inhibit hydroxyapatite formation in cell cultures (Schinke et al. 1996). In cell culture studies fetuin has been shown to block the

osteogenic activity of bone morphogenetic proteins and antagonises the antiproliferative effect of TGF-β_1 (Binkert et al. 1999).

Taken together, the detection of these selected non-collagenous proteins from cells cultured in association with bone substitute materials would be highly suggestive of osteoblastic activity. The aforementioned studies clearly show the importance of glycoproteins and proteoglycans in bone homeostasis. Subsequently, their identification in biomaterials may be suggestive of osteoinductive potential.

1.3 Signalling molecules of bone

1.3.1 Introduction

It is necessary to understand how a variety of signalling molecules can regulate bone homeostasis to appreciate why their detection in *in vitro* experiments is valuable. The signalling molecules of bone have been categorized elsewhere as belonging to three groups (Dimitriou et al. 2005; Hallman and Thor 2008):

- TGF-β superfamily, including BMP's and other growth factors (e.g. PDGF).
- Pro-inflammatory cytokines (e.g. IL-1).
- Angiogenic factors (e.g. VEGF).

Due to the scope of the project, only certain molecules will be discussed in detail but it must be appreciated that a cocktail of growth factors act together in the *in vivo* situation. Growth factors have been described as natural biological mediators secreted by cells that regulate tissue repair by binding to specific cell surface receptors that in turn activate genes that can alter cellular activity (Giannobile 1996; Lieberman et al. 2002). They regulate critical healing events including cell proliferation, chemotaxis, differentiation and matrix synthesis (Giannobile 1996; Bessade et al. 2007). This is achieved through activation of intracellular transcription factor reactions leading to gene expression, transcription of mRNA and ultimately protein release (Lieberman et al. 2002; Hallman and Thor 2008). Following an injury such as surgery, platelets and adjacent cells release these growth factors which exert their affects (Giannobile 1996). Furthermore, as will be discussed in detail later, bone and dentine matrix are known to be reservoirs for these growth factors (Graham et al. 2006; Smith

et al. 2011). The following is a summary of key molecules that are involved in the bone remodelling process.

1.3.2 TGF-β

Transforming growth factor-β 1, 2 and 3 belong to the transforming growth factor β (TGF-β) superfamily of polypeptide factors that control development and homeostasis. They have previously been ascribed with several important functions that include the regulation of cell growth, development, tissue remodelling and controlling the inflammatory response (Grande 1997). Their effects are known to be mediated through a variety of ligand-receptor reactions that activate intracellular signals that affect gene expression (Dimitriou et al. 2005). TGF-β binds to type-I and type-II serine / threonine kinase receptors which activates the SMAD 2 and 3 pathway (Dimitriou et al. 2005). SMADs have been described as a class of intracellular proteins that are involved in TGF-β signalling (Lieberman et al. 2002). The SMAD protein family contains eight members with SMAD 6 and 7 having inhibitory effects. Furthermore, *in vitro* and *in vivo* studies have shown these growth factors stimulate chemotaxis and survival of osteoblasts and affect osteoblast cell growth, migration and differentiation, deposition of matrix and mineralization (Pfeilschifter et al. 1990; Giannobile 1996; Binkert et al. 1999; Hallman and Thor 2008). TGF-β_1 is known to be produced by fibroblasts, osteoblasts, platelets and inflammatory cells and is expressed throughout the process of bone healing (Robey et al. 1987; Dimitriou et al. 2005). Its release is the result of activation of transcription factors, such as SMAD proteins. Importantly, TGF-β_1 reportedly acts on the differentiation of osteoproginator cells to pre-osteoblasts but conversely may inhibit the later stages of differentiation into osteoblasts and mineralisation (Giannobile 1996; Maeda et al. 2004). Therefore, it appears from this evidence that the affect of TGF-β_1 on osteoproginators is variable. This contrasting evidence shows that the effect of TGF-β_1 may be dose or location dependent. Indeed, in mouse bone marrow cultures, TGF-β_1 levels rise in the first 5 days of

osteogenic differentiation, then decrease and rise again late in the mineralization phase (Binkert et al. 1999). TGF-β also stimulates the production of a large number of extracellular matrix components, including the synthesis of type 1 collagen, osteopontin and osteonectin, that are important in bone remodelling (Giannobile 1996; Cochran and Wozney 1999; Dimitriou et al. 2005). In particular, TGF-β_1 stimulates the expression of BMP's and inhibits the activity of matrix metalloproteinases (Overall et al. 1989).
Importantly from a clinical perspective, *in vitro* studies suggest that when osteoblast-like cells are cultured on rougher biocompatible materials, TGF-β_1 production increases which would have the effect to increase osteoblast differentiation locally (Schwartz et al. 1997). This would suggest that if surface geometry and porosity of an implant or bone substitute material could be optimised bone regeneration would improve.

It has been demonstrated that TGF-β_1 is excreted in an inactive form as a latent complex with a latency associated peptide (TGF-β_1 – LAP) which requires subsequent activation following the binding of accessory molecules such as biglycan (Sloan et al. 2002). It follows that TGF-β_1 – LAP found in the bone matrix may therefore be responsible for the large amounts of latent transforming growth factor sequestered in bone matrix. Further, in some cells this latent complex binds with a 125 – 160kDa protein, termed the latent TGF-β binding protein, to form a larger latent complex (Grande 1997). It is noteworthy that, osteoclasts have the ability to activate bone-derived latent TGF-β_1, but TGF-β_1 itself can inhibit osteoclast proliferation by blocking the formation of osteoclast precursors and has an inhibitory effect on mature osteoclasts (Robey et al. 1987). TGF-β_1 therefore has key roles in mineralisation, by controlling the differentiation of pluripotent cells into mesenchymal cells, the differentiation and proliferation of osteoblasts stimulating collagen type 1 production and production of fibronectin and osteopontin (Robey et al. 1987). Conversely, *in vitro* experiments have clearly shown that excess TGF-β_1 will inhibit mineralisation completely (Binkert et al. 1999).

Taken together, the combined evidence suggests that this family of growth factors are critical in controlling the behaviour of both osteoblasts and osteoclasts and their expression occurs during bone repair. It follows that the ability to detect TGF-β_1 within bone substitute materials would show that residual growth factors remain in such materials that may be extracted by chemical pre-treatment.

1.3.3 Bone Morphogenetic Proteins

Bone morphogenetic proteins (BMP's) form a subgroup of the TGF-β superfamily, mentioned previously (Chen et al. 2004; Dimitriou et al. 2005). They promote osteogenesis and are involved in bone remodelling. The differentiation of bone forming cells has been described as the hallmark of these molecules (Giannobile 1996). BMP's are known to promote recruitment of mesenchymal cells, chondroblast differentiation, cartilage formation and vascular invasion (Chen et al. 2004). Currently, at least 30 BMP's have been identified and in particular BMP 2 , 4, 6, 7, 8 and 9 show high osteogenic activity in both *in vitro* and *in vivo* studies directing pluripotent cells down the osteoblastic lineage (Cheng et al. 2003; Dimitriou et al. 2005; Hughes et al. 2006; Hallman and Thor 2008). Specifically, BMP's are known to stimulate progenitor cell migration and osteoblast differentiation from undifferentiated mesenchymal cells (Giannobile 1996; Dimitriou et al. 2005; Hughes et al. 2006). These effects are mediated by type 1 and 2 BMP receptors and downstream molecules, such as Smad 1, 5 and 8 (Chen et al. 2004).

Although BMP's have the ability to induce bone formation, TGF-β_1 does not. However, it is currently agreed that both molecules are critical to osteoblast formation with TGF-β_1 increasing cell numbers and BMP's acting on later differentiation (Hughes et al. 2006). Indeed, only the combination of growth factors is likely to achieve biological effects *in vivo.* Importantly, mesenchymal

stem cells, which form osteoblasts, have several BMP-receptors which corroborates BMP's role in directing mesenchymal cells down the osteoblast cell linage (Katagiri and Takahashi 2002; Nefussi 2007). Osteoblasts and importantly osteoprogenitor cells secrete BMP's and their antagonists e.g. noggin, during bone formation and remodelling and some of the growth factor will become sequestered within the bone matrix (Mackie 2003; Dimitriou et al. 2005). In contrast, over-expression of BMP antagonists can lead to osteoporosis in mice.

Based on the evidence for the role of BMP in bone remodelling, its therapeutic use has understandably been investigated. Both clinical and *in vitro* studies demonstrate that BMP-2 has therapeutic use where bone healing is required (Cheng et al. 2003; Chen et al. 2004). In its recombinant form BMP-2 has been used therapeutically in bone defects , non-healing fractures, periapical surgery defects and around peri-implant defects (Cochran and Wozney 1999). Taken together this evidence summarises the critical roles BMP's have to play in bone remodelling. Subsequently, when BMP's are detected experimentally from cultured cells they are indicative that cells are becoming osteoblasts.

1.3.4 Other growth factors

Numerous other growth factors are involved in bone remodelling processes, including PDGF, FGF and IGF. PDGF is released by degranulating platelets, inflammatory cells and osteoblasts. PDGF has been shown to have a strong positive effect on the proliferation of both animal and human osteoblasts and their differentiation (Lynch et al. 1991; Giannobile 1996; Hughes et al. 2006). Histologically, using a rat tissue culture model, PDGF has been shown to promote collagen synthesis (Giannobile 1996). It is also known to be released at the very early stages of fracture healing (Dimitriou et al. 2005).

FGF's and IGF's are other growth factor families known to be involved in the osteoblast differentiation pathway and in particular, osteoprogenitor cell proliferation (Dimitriou et al. 2005; Hughes et al. 2006). Hughes *et al* also suggested that *in vitro*, FGF's mainly act to stimulate proliferation in immature cells, resulting in expansion of the osteoblast progenitor pool (Hughes et al. 2006). It has been suggested that in particular, IGF-1 becomes more critical later in the differentiation process than fibroblast growth factor (Dimitriou et al. 2005; Hughes et al. 2006). IGF, which is also produced by osteoblasts induces their proliferation, differentiation, chemotaxis and collagen 1 synthesis (Lynch et al. 1991; Giannobile 1996). Of potential clinical relevance, animal studies have shown that the combination of recombinant human IGF and BMP-2 may produce an osteoinductive effect around implants (Lan et al. 2006). Further, the combination of IGF and PDGF improved periodontal regeneration within bone defects in beagle dogs (Lynch et al. 1991).

1.3.5 IL-1, 6 and prostaglandin E_2

Another important group of signalling molecules that control inflammation and regeneration are the cytokines. Cytokines are glycoproteins which act as local signalling molecules to co-ordinate cellular behaviour and function. They are known to bind to cell-surface receptors resulting in the modulation of gene expression in their target cells (Hughes et al. 2006). In particular, interleukin 1 (IL-1) is a potent bone resorbing cytokine that is secreted by macrophages, neutrophils, lymphocytes, fibroblasts and epithelial cells (Boch et al. 2001; Dimitriou et al. 2005). It is widely accepted that IL-1 is one of the major cytokines produced at inflamed sites and is involved in the initiation and progression of connective tissue destruction, including bone, partly through osteoclast recruitment and activation (Boch et al. 2001). This inflammatory response is perpetuated by a chemotactic effect on other inflammatory cells. Importantly, IL-1 has been shown to initially stimulate the bone repair process in

addition to interleukin 6 and tumour necrosis factor-α, by enhancing the proliferation and differentiation of osteoblast progenitor cells; and extracellular matrix synthesis (Dimitriou et al. 2005; Hughes et al. 2006). Prostaglandin E_2 also stimulates bone resorption, potentially through activation of RANK ligand located on the osteoblast surface (Horowitz et al. 2001). This is further evidence of the role osteoblasts play as a regulator binding both signalling molecules and osteoclasts. Of potential clinical importance is the identification of IL-1 and prostaglandin E_2 in gingival crevicular fluid and its relationship to active periodontal diseases (Boch et al. 2001; Taba et al. 2005).

1.3.6 Growth factors within extracellular matrix

Growth factors are considered by many as a subset of cytokines (Hughes et al. 2006). It is known that during both bone and dentine formation, cells lay down growth factors within the bone matrix (Graham et al. 2006; Hughes et al. 2006; Bosshardt 2009; Smith et al. 2011). Furthermore, during tissue repair, growth factors are produced by local cells, but importantly are released from the bone matrix itself (Bosshardt 2009). For example, the extracellular matrix is thought to contain the main source of BMP's produced by osteoprogenitors and osteoblasts (Dimitriou et al. 2005). It has been suggested that the challenge for tissue engineering is to maintain growth factor activity over time since ultimately their degradation will occur (Lieberman et al. 2002). It has also been stated that it may be combinations of growth factors in healing tissues that produce optimal effects (Pfeilschifter et al. 1990; Bessade et al. 2007). This has been confirmed by both *in vitro* and *in vivo* studies showing increased bone formation when a combination of growth factors have been used (Giannobile 1996). The previous section has outlined the role that growth factors play in bone remodelling. The ability to harness the effects of these molecules, if identified within the matrix of bone substitute materials, may provide faster bone regeneration in the challenging clinical situations mentioned later.

1.4 Calcium Hydroxide and EDTA

1.4.1 Introduction

In order to appreciate the potential for growth factor release from bone tissue, it is important to review the evidence supporting the use of two important chemical agents in particular. As already mentioned, mineralised tissues have been shown to be a store of growth factors (Graham et al. 2006; Smith et al. 2011). Calcium hydroxide and ethylenediaminetetraacetic acid (EDTA) are important, external factors that have the ability to extract and activate growth factors. Their mechanisms of action can only be understood after their chemistry is reviewed.

EDTA is known to be a potent chelating agent used routinely as an endodontic irrigant that has recently been shown to improve the clinical outcome of endodontic retreatment (Bystrom and Sundqvist 1985; Ng et al. 2011). It is proposed to solubilise growth factors by mineral demineralisation, the chelation of calcium ions and the solubilisation of non-collagenous proteins (Hulsmann et al. 2003). Hulsmann has summarised the mechanism of action of EDTA on mineralised tissue as follows, "An equilibrium is established between the EDTA solution and the precipitate because ions from the mineral precipitate constantly go into solution while ions from the solution are precipitated as solids" (Hulsmann et al. 2003). EDTA forms a stable complex with calcium such that when all ions are bound no further dissolution takes place (Hulsmann et al. 2003).

Calcium hydroxide has already seen many uses in dentistry including direct and indirect pulp capping, apexification, apexogenesis and as an inter-appointment root canal dressing (Sathorn et al. 2007; Huang 2009; Mohammadi and Dummer 2011). There is, however, a trend for contemporary materials such as

mineral trioxide aggregate (MTA), to render the use of calcium hydroxide unnecessary for many clinical situations (Torabinejad and Chivian 1999). Nonetheless, calcium hydroxide remains in clinical use. Most of its therapeutic properties are due to its high pH of approximately 12.5 and is therefore a strong base. The ionic dissociation of calcium and hydroxyl ions affect vital tissues, induce hard-tissue deposition and has antibacterial properties (Mohammadi and Dummer 2011). Calcium hydroxide is a known extractant capable of solubilising proteins from mineralised surfaces and therefore, has potential for liberating proteins from the matrix of bone substitutes (Smith et al. 2011).

1.4.2 Effect on dentine matrix proteins

The application of calcium hydroxide to the dentine surface and exposed pulps has been shown to stimulate reparative dentine formation (Horsted-Bindslev et al. 2003). The formation of reparative dentine is similar to bone repair, requiring recruitment to and stimulation of progenitor cells at the injury site. The release of growth factors from dentine would potentiate this cellular response. *In vitro* experiments have examined the ability of chemical treatment to extract growth factors from powdered dentine. For example, chemical treatments with EDTA, and calcium hydroxide, have been demonstrated to solubilise TGF-β_1 and other bioactive molecules from the mineralised matrix of dentine using a variety of *in vitro* techniques, including enzyme linked immunosorbant assays and 1D polyacrylamide gel electrophoresis (Graham et al. 2006; Tomson et al. 2007). The release of this and other growth factors as a result of the caries process or dental intervention may result in dentinogenesis through increased expression of osteopontin and BMP-2 (Graham et al. 2006).

1.4.3 Effect on bone matrix proteins

Bone matrix and bone healing forms many parallels with dentine and the ability of calcium hydroxide and EDTA to release growth factors from the bone matrix have been investigated previously, in terms of beneficial effects on bone repair. An *in vitro* study using a rat model was used to demonstrate release of TGF-β_1 from bone slabs, following application of EDTA and calcium hydroxide (Smith et al. 2011). Importantly, this study also demonstrated increased osteoblastic differentiation from bone marrow stromal cells when bone slabs were chemically treated, compared to a PBS control. Although this study was limited to the investigation of the growth factor TGF-β_1, the authors recognised that the interplay of other growth factors probably contributed to the differences in osteoblastic differentiation. Indeed, the presence of growth factors within the bone matrix provides a reservoir of bioactive molecules that may influence cellular behaviour during bone regeneration. It follows that a variety of preparations of natural bone particles, including those from animal sources, may also contain these molecules.

1.5 Clinical requirements for bone augmentation and regeneration

Two main fields of clinical dentistry utilize bone augmentation and regeneration techniques. These are periodontology and implantology. In periodontal therapy, the ideal treatment goal is to replace bone lost as a consequence of the disease process. Prior to, or during implant placement a certain bone volume is required that is consistent with the specific implant system parameters. Furthermore, systemic conditions compromise the available bone quality or adversely affect the bone remodelling process. It is therefore advantageous for current bone regeneration techniques to be refined or modified to improve the biological response that will allow clinicians to manage these clinical scenarios. Faster and more predictable bone regeneration would be significantly beneficial in these situations. It has been summarised by others that predictable regeneration requires the elimination of bacteria, wound stability, elimination of undesirable tissue types and appropriate surface characteristics (Schwartz et al. 1997). What follows is a summary of the clinical challenges that require bone augmentation and regeneration procedures.

1.5.1 Periodontal disease

Periodontal diseases are a group of inflammatory disorders that can lead to the ultimate destruction of the supporting structures around teeth (Socransky et al. 1984; Löe et al. 1986). A variety of classification systems have been used over the years but most recently destructive periodontal diseases have been diagnosed as either aggressive or chronic (Armitage 1999). Both forms of disease result in an inflammatory lesion that leads to the loss of attachment and

bone (Löe et al. 1986). Following the irreversible destruction of tissue caused by advanced periodontal disease, there is often a decrease in bone height surrounding the dentition. Progressive bone resorption leads to the formation of craters of bone loss or intrabony defects where the periodontal pocket extends below the level of the surrounding bone (Cortellini and Tonetti 2000).
It is accepted that the presence of bacteria in dental plaque adjacent to the gingival crevice is the trigger for gingivitis (Löe et al. 1965). In susceptible patients bacteria stimulate monocytes, neutrophils, macrophages and other cells to release inflammatory mediators, such as IL-1, tumour necrosis factor (TNFα), and prostaglandin E_2, that can result in tissue damage and osteoclast mediated bone loss (Taba et al. 2005; Kinane 2008).

The treatment of periodontal disease has been described in three phases (Salvi 2008). Firstly, “initial cause related therapy”, which aims to improve plaque control and manage risk factors, a second “corrective phase”, where persistent pathological pockets can be reduced or lost tissue regenerated; and an ongoing phase of “supportive therapy” to reduce disease recurrence. As mentioned, destructive periodontal disease can result in intrabony defects. These defects cannot always be treated successfully by nonsurgical therapy. To reduce the pocket depth further, these defects are often amenable to guided tissue regeneration than can result in the formation of new cementum, bone and periodontal ligament that results in reduction of the defect (Nyman et al. 1982; Reynolds et al. 2003). The theory supporting the clinical use of grafting procedures is that the complete regeneration of the attachment apparatus (including new bone formation and new connective tissue attachment) is enhanced by biomaterials. This is either due to their osteogenic potential (if the graft contained viable bone-forming cells), osteoinductive capacities (exerted by the release of bone inducing substances), or osteoconductive properties (i.e. the possibility to create a scaffold to support bone formation) (Trombelli 2005). The clinical use of the guided tissue regeneration technique is discussed in more detail in section 1.7.2.

1.5.2 Dental implants

Dental implants are defined as prosthetic devices made of alloplastic materials implanted into the oral tissues within the bone to provide retention and support for a fixed or removable dental prosthesis (The Academy of Prosthodontics 2005). It is clear that the clinical use of dental implants absolutely depends on bone quality and volume. The majority of systems are composed of pure titanium alloy. Branemark who pioneered implant dentistry, first treated edentulous patients in 1965 (Branemark 1983). Dental implant systems have evolved through the years to number over 480 systems. These systems differ in design, surface treatments and surface roughness. Examples of some of the most popular systems include sandblasted and acid etched surfaces (SLA Active® *Straumann*) and oxidised moderately rough surfaces (Ti-unite® *Nobel Biocare*) and it has been known for some time that osseointegration is better on rough surfaces *in vivo* (Schwartz et al. 1997).

The original implant placement protocol involved the placement of the fixture which was then covered immediately by soft tissue, followed by exposure at 6 months after which the implant could be loaded. This first implant placement protocols suggested no loading for 3 – 4 months in the mandible and 5 – 6 months in the maxilla (Adell et al. 1981). As the technique has become more predictable time to loading has reduced and some clinicians place and load implants immediately although this may be less predictable (Grutter and Belser 2009). The success rates of dental implants have been well-documented in the literature (Adell et al. 1981; Lindh et al. 1998; Jung et al. 2008a). An extensive review of the literature by Esposito concluded that in edentulous patients, maxillary implant failure is about 3 times higher than that of the mandible. (Esposito et al. 1998). It is clear from these results that there are differences in success rates between the jaw bones and often it is maxillary implant sites that require bone augmentation, particularly bone width anteriorly and bone height

posteriorly. More recent studies looking at implant outcomes have quite rightly used the term implant survival rather than implant success to describe the maintenance of implant integration over time (Albrektsson et al. 1986; Pjetursson et al. 2007). According to a variety of authors (Albrektsson et al. 1986; Buser et al. 1997; Karoussis et al. 2004; Ong et al. 2008), in order to be a success an implant should demonstrate:

- The absence of mobility.
- The absence of recurrent peri-implant infection with suppuration.
- The absence of a continuous radiolucency around the implant.
- No probing pocket depth $\geq$5mm or bleeding on probing.
- No more than 0.2mm annual vertical bone loss after the first year of service.

Studies have shown implant supporting single crown survival of 96.8% and implant supporting fixed bridge survival of 95.2% at 5 years (Pjetursson et al. 2007; Jung et al. 2008a).

Low bone volume can occur following the loss of the dentition as a result of resorption patterns and pneumatisation of the maxillary sinus. In the anterior maxilla, loss of the buccal plate of bone can result in significant horizontal defects that can lead to functional and aesthetic compromise. This means that local conditions of the edentulous ridge may be unfavourable for implant placement, particularly vertical deficiency which results in insufficient volume for implants of adequate length (Chiapasco et al. 2007). Where there is insufficient bone anteriorly options for management include either separate staged block grafts or simultaneous particulate grafts placed at the time of implant insertion (von Arx and Buser 2006). The use of bone mineral particulate grafts for sinus augmentation and lateral ridge augmentation will be discussed in detail later. A systematic review has been performed to compare the survival of implants in

regenerated bone, versus implants placed in non-regenerated sites after 12 months of loading. The authors used well defined outcome criteria, however a limited electronic search was carried out. Eleven studies were included at varying levels of evidence. The majority of studies demonstrated survival of over 90% in regenerated bone, which is comparable to that of non regenerated bone (Hammerle et al. 2002).

Not all implants survive and can be deemed successful with a proportion of failures attributed to inflammatory processes. Peri-implant mucositis describes an inflammatory lesion that resides in the mucosa, while peri-implantitis also affects the supporting bone around a dental implant (Lindhe and Meyle 2008). Peri-implantitis presents with the cardinal signs of inflammation, suppuration and always with bone loss. Recent evidence suggests that the incidence is considerably higher than previously thought and per-implantitis may occur in as much as 28 – 56% of patients (Esposito et al. 1998; Zitzmann and Berglundh 2008). Currently, it is unclear what the most favourable treatment modality is although some guidelines do exist (Lindhe and Meyle 2008). Briefly, treatment involves anti-infective therapy and possibly surgical intervention using bone grafts in appropriate cases.

1.5.3 Indications for bone regeneration

Bone grafting or periodontal regeneration is required in several aspects of dentistry, including Implantology, Periodontics and Endodontics. Although the clinical use of the materials may be similar, the techniques have been described using different terminology dependent upon the indication. This includes block grafting with intraoral autogenous bone for significant alveolar defects, guided tissue regeneration in periodontal defects or endodontic microsurgery; and simultaneous guided bone regeneration procedures for smaller peri-implant

defects. The main indications for bone grafting or periodontal regeneration, therefore, include:

- Guided bone regeneration for peri-implant dehiscences or fenestrations.
- Horizontal or vertical ridge augmentation, prior to implant placement.
- Augmentation of the maxillary sinus.
- Surgical treatment of peri-implantitis.
- Guided tissue regeneration of periodontal bony defects.
- Guided tissue regeneration of large bony crypts following endodontic microsurgery.

1.5.4 Use and limitations of autogenous bone

Autogenous bone is bone derived from the host. In implant dentistry, the most popular sites have been the symphiseal region or mandibular ramus for moderate volumes of bone, or the iliac crest for larger volumes of bone. Autogenous bone is often reported as the gold standard with osteogenic, osteoinductive and osteoconductive effects (Block and Kent 1997). This is mainly related to its osteogenic effects, where viable cells and growth factors can be transferred to the donor site and stimulate new bone formation directly. Also as it is harvested from the recipient, there is no antigenic effect. There are several disadvantages which have propagated the development of alternatives including non-human particulate grafts. The disadvantages of autogenous bone include:

- The need for two operative sites with associated morbidity of the second site.
- Increased operative time and cost
- Finite volume particularly at intraoral sites
- The need for sedation or general anaesthesia to allow graft harvest

- Resorption of autogenous blocks with time and subsequent loss of volume (von Arx and Buser 2006).

Graft surgery also carries with it the possibility of graft loss, infection, neurological deficit and increased postoperative discomfort. In view of these challenges a variety of bone substitute materials have been developed and are gaining popularity that will be discussed in the next section.

1.6 Bone grafting

1.6.1 Introduction and classification

The aim of bone grafting or bone augmentation is to restore critical defects or alveolar bone volume to normal form and function, where the defect exceeds the regeneration potential of the adjacent bone itself (Giannobile 1996). In order for bone regeneration to be successful, there needs to be bone-forming cells (ultimately derived from mesenchymal stem cells), differentiation signalling molecules and a scaffold upon which tissue can proliferate (Dimitriou et al. 2005). Bone graft materials are commonly described as belonging to one of the four following groups: autografts, allografts, alloplasts and xenografts (Antoun 2007).

The mechanisms important in bone grafting include osteogenesis, osteoconduction and osteoinduction. These three processes may all occur together, depending on the grafting technique used. Osteogenesis is definitively the formation of bone. In bone grafting, this process occurs when viable mature osteoblast cells and their precursors are transplanted with the graft material into the defect and result in bone formation (Giannobile 2008). An autogenous bone graft has osteogenic properties by virtue of the transfer of osteoblasts, their precursors and signalling molecules within the graft (Bosshardt 2009). These facilitate direct bone formation upon the graft. An osteoconductive material works as a scaffold, over which cells migrate and around which new bone can form (Albrektsson and Johansson 2001). The process relies on the recruitment and migration of osteogenic cells to the site of healing through the blood clot (Davies 2003). Autogenous cortical bone and several bone substitutes can form such scaffolds including synthetic calcium phosphates and xenografts.

Resorption of the graft material and replacement by bone is variable between graft materials, and some materials remain in the defect in the long-term. A literature review by Hallman and Thor stated that using solely an osteoconductive grafting material in implant cases may prolong the healing period by 2–6 months, which may be of clinical significance (Hallman and Thor 2008) Finally, osteoinduction is the process by which primitive undifferentiated pluripotential cells are stimulated down the osteoblast cell lineage (Albrektsson and Johansson 2001; Dimitriou et al. 2005). An osteoinductive material has the ability to bind or release proteins, such as TGF and BMP, which are capable of stimulating undifferentiated mesenchymal cells to transform into preosteoblasts and osteoblasts to form new bone (Antoun 2007). Osteoinduction describes a process of accelerated bone formation that results in a shorter healing period, following surgical intervention (Hallman and Thor 2008). If the term is used to describe a bone substitute, it means the material can stimulate new bone formation by virtue of the proteins it contains. Further, the factors that contribute to osteoinduction have been summarised as osteoinductive signals, appropriate cell types and a supportive scaffold to carry cells and signalling molecules (Bosshardt 2009). Confusingly, several materials are often described as either osteoinductive or osteoconductive, which is attributed to contradictory results in *in vitro* studies. In summary, certain bone substitute materials may result in slower and less complete bone formation, compared to autogenous grafts, based on the depleted biological components mentioned above.

1.6.2 Bone substitute materials

Bone substitute materials (BSM's), including particulate bone grafts and pastes, have been developed as an alternative to human derived autogenous bone for the reasons already listed. Key considerations with these materials are biocompatibility, degrees of resorption, ease of use, patient acceptance, non-infectivity and preferably bioactivity (Antoun 2007). An ideal bone substitute

would also be osteoinductive and have a large surface area through porosity to allow revascularisation (Peetz 1997). Most commercially available materials are marketed as scaffolds with only osteoconductive properties, which biologically make them inferior to autogenous bone in this respect. The ability of these materials to demonstrate osteoinductive properties is thus appealing in clinical dentistry. BMP's and TGF-β_1 are osteogenic factors found in some osteoinductive bone graft materials that can differentiate mesenchymal cells into osteogenic cells (Tapety et al. 2004). The clinical availability of materials is expanding continuously so only selected materials will be discussed here briefly.

1.6.2.1 Alloplasts

Alloplasts are synthetic materials that are grouped as calcium phosphates, calcium sulphates, bioactive glasses and polymers. These materials have the advantages of varying degrees of resorbability, biocompatibility and the lack of antigenic effects. Alone they are unlikely to provide any osteoinductive effect as they are devoid of proteins. Biphasic calcium phosphate is commercially available as Straumann Bone Ceramic®, composed of synthetic 60% hydroxyapatite and 40% β tricalcium phosphate (Cordaro et al. 2008). B tricalcium phosphate has been shown to have favourable osteoconductivity and resorption rates over short follow up times (Antoun 2007). Vital®, produced by Fortoss, is a composite of β tricalcium phosphate in a calcium sulphate matrix that has demonstrated bone formation in animal models (Podaropoulos et al. 2009).

1.6.2.2 Allografts

Allografts are those bone products that come from other humans e.g. demineralised freeze-dried bone allograft from cadavers. It is biocompatible, resorbable and contains type 1 collagen. Bone allografts have been found to

contain BMP's that potentially could provide osteoinductive properties although less bone formation occurs compared to autogenous bone (Giannobile 1996; Mattout 2007). Human-derived alternatives are more popular in Northern America than the United Kingdom, with many clinicians concerned about potential transmission of infected material and the stimulation of an immune response.

1.6.2.3 Xenografts

Xenografts are materials derived from species other than human. Included in this group is bone derived from equine, porcine and bovine sources, but also corals. Xenografts are often described as purely osteoconductive space maintainers. Currently, the most researched bone substitute material is Bio-Oss®, produced by Geistlich. This is marketed as a natural bone substitute material obtained from the mineral portion of bovine bone. Bio-Oss® will be considered in detail in section 1.7

1.6.2.4 Advantages of bone substitute materials

The previously mentioned materials have limitless supply and do not require a second operative site which can make surgery more efficient. They are often supplied in blocks or specific carrier devices that facilitate easy placement in periodontal defects or the maxillary sinus. A recent review of bone substitutes listed several advantages of particulate bone grafting (Hallman and Thor 2008). These included the ability to place grafts in tiny grooves, the reduction in risk of soft tissue in growth between graft and bone; and possibly more rapid vascular in growth. It is unlikely that bone substitutes are an appropriate alternative to autogenous bone for onlay grafts, although case series have demonstrated some clinical success when combination grafts are used (von Arx and Buser 2006; Simion et al. 2007). The combined evidence demonstrates that bone

substitute materials have a major role in sinus grafting, peri-implant augmentation and ridge preservation.

1.6.3 Growth factors and bone grafting

Growth factors have also become commercially available and are now being added to a variety of bone graft materials or used alone with the intention of facilitating alveolar bone regeneration by chemo-attraction, differentiation and proliferation (Lieberman et al. 2002; Giannobile and Somerman 2003). As mentioned, growth factors are key signalling molecules released at different phases in bone healing. Previous evidence identified autogenous bone as having osteoinductive capacity, based on the transfer of growth factors to the recipient site (Block and Kent 1997). The many disadvantages of autogenous graft harvest, already discussed, have led investigators to combine growth factors with particulate bone grafts, thus using the graft as a scaffold or carrier for these growth factors. The use of exogenous growth factors may have therapeutic benefit to overcome the limitations of conventional regeneration techniques, but only if a suitable carrier device is used (Lieberman et al. 2002). Using a dog model, Giannobile (1996) demonstrated earlier bone formation, using guided tissue regeneration combined with growth factors to treat furcation defects (Giannobile 1996). Also, there is increasing evidence that growth factors alone or in combination with bone substitute materials can improve bone formation especially around dental implants (Giannobile 1996).

The use of enamel matrix proteins derived from embryonic tooth germs is another technique becoming ever more popular in the regeneration of periodontal defects (Giannobile and Somerman 2003). The main biological effects of enamel matrix derivatives (EMD's) have been attributed to their predominant protein, amelogenin (Hughes et al. 2006). Although clinically

successful, further discussion of the evidence regarding its use is outside the scope of this project.

Notwithstanding the above biological advantages of combining growth factors and bone substitute materials there is a growing body of opinion that this may not be a practical approach in view of short half lives of the growth factor and need for costly supraphysiological concentrations (Lieberman et al. 2002; Lee et al. 2011). The use of growth factors with Bio-Oss® in particular will be discussed in section 1.7.8.

1.7 Bio-Oss®

1.7.1 Characterization of material

Bio-Oss® is a grafting material that has been used as a bone substitute for several years. Its use in the literature has been documented since the early 1990's. Some confusion about the material is generated by the different terms that have been used to describe it. These have included cancellous bovine bone mineral (Berglundh and Lindhe 1997), deproteinized bovine bone mineral (Araujo et al. 2009), anorganic bovine bone mineral (von Arx and Buser 2006), natural bone mineral (Tonetti et al. 2004) and bovine porous bone mineral (Molly et al. 2008). The term Deproteinized Bovine Bone Mineral (DBBM) shall be used throughout this thesis.

DBBM is commercially available in two main forms: Bio-Oss® granules and Bio-Oss® collagen block. Bio-Oss® granules consist of cancellous bone of bovine origin and Bio-Oss® collagen consists of Bio-Oss® granules with the addition of 10% porcine collagen. The manufacturing process of DBBM has been described by Schwartz *et al* (2000) and Tapety *et al* (2004). The bone particles undergo treatment with a strong alkali and sintering at 300°C for 15 hours. It is then treated with an organic solvent and sterilized (Schwartz et al. 2000; Tapety et al. 2004). The manufacturers claim this eliminates any protein leaving only inorganic material.

One of the suggested favourable qualities of DBBM is that its structure closely resembles human bone (Peetz 1997). It contains wide interconnecting pores that acts as a scaffold and could promote migration and attachment of cells and vascularisation. The pore size is variable with macropores of 300-1500 microns, micropores corresponding to haversian canals and inter-crystalline

spaces of 3 – 13 nm. In comparison to commercially available synthetic particulate grafts, it has a large internal surface area 100 m^2 / g and porosity of 70 -75% (Tapety et al. 2004).

The clinical advantage of Bio-Oss ® collagen over Bio-Oss® granules lies in its handling characteristics, where it is possible to trim the material with scissors to fit the dimensions of periodontal or peri-implant defects. Bio-Oss® granules are available in grain sizes of 0.25 – 1mm diameter or 1 – 2 mm which are designed for different clinical indications. Figures 1.2 and 1.3 shows samples of Bio-Oss® granules and Bio-Oss® collagen. Bio-Oss® collagen is also supplied in a combination pack along with a resorbable collagen membrane called Bio-Gide®. This is composed of porcine collagen and is used to protect the granules or blocks in a variety of indications. Bio-Oss® is strongly hydrophilic and the particles stick to each other when combined with blood at the surgical site.

Figure 1.2 Bio-Oss® collagen block as supplied by manufacturer

Figure 1.3 Bio-Oss® granules as supplied by manufacturer

1.7.2 DBBM and guided tissue regeneration

Guided tissue regeneration (GTR) is a surgical procedure that specifically aims to regenerate the periodontal tissues, when the disease is advanced (Nyman et al. 1982). It is principally a form of tissue engineering attempting to control cell behaviour. The contemporary technique involves the use of a resorbable membrane to exclude epithelial down growth, while bone and connective tissue regeneration occurs. This can be supplemented by a variety of bone substitutes to fill the defect area. The concept promotes the cells derived from the periodontal ligament as the first cells to colonise the root surface, thereby permitting new attachment. A Cochrane systematic review has examined, amongst other things, the additional benefit of GTR with graft materials over open flap debridement (OFD) therapy and concluded there was a significant benefit of GTR with bone substitutes, though the magnitude itself remains unclear (Needleman et al. 2005). A contemporary application of the GTR technique involves the placement of DBBM usually in the form of Bio-Oss® collagen into the bony defect to support a collagen membrane (Bio-Gide®) to prevent epithelial down growth. Several studies have shown the clinical benefits of Bio-Oss® in the management of intra-bony and furcation defects with short and long term follow-up. Sculean *et al* performed a multi-centre randomised controlled trial showing results at 5 years. The difference in clinical attachment level (CAL) gain was shown to be significantly higher ($p<0.01$) in the group that received Bio-Oss® (Sculean et al. 2007). A clinical case treated by the author of the current thesis demonstrating the guided tissue regeneration technique, using Bio-Oss® and Bio-Gide®, is shown in Figures 1.4 to 1.6.

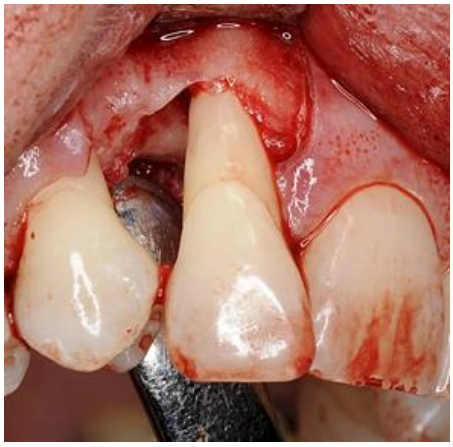


Figure 1.4 Three walled intrabony defect associated with the distal aspect of tooth 12 in a patient with localized severe chronic periodontitis

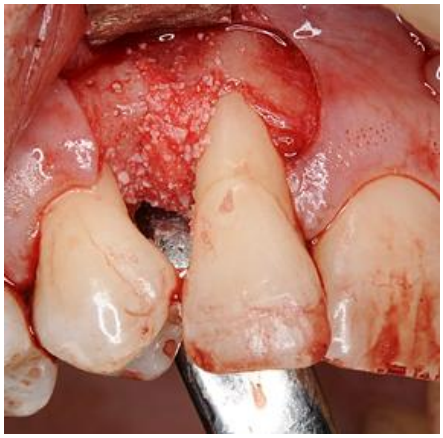


Figure 1.5 Placement of Bio-Oss® collagen to fill the intrabony defect.

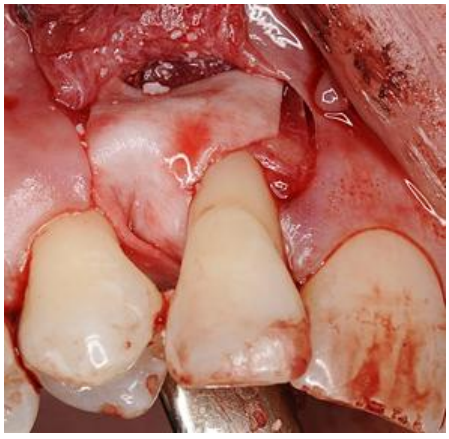


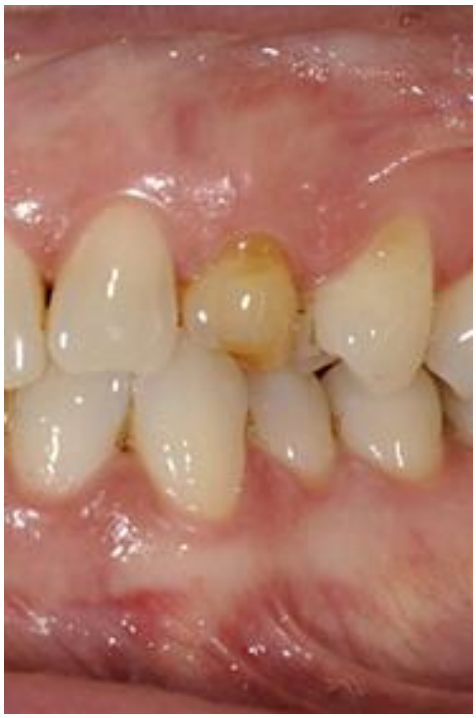
Figure 1.6 Coverage of graft with a Bio-Gide® collagen membrane. The surgical flap was closed with sutures.

1.7.3 DBBM and guided bone regeneration

It is no longer acceptable for an implant simply to osseointegrate, but it is critical that aesthetic hard and soft tissue profiles develop adjacent to the implant. Depending on the implant system used, the minimum crestal width of bone from buccal to palatal is between 5 and 7 mm, which allows at least 1mm of bone surrounding the implant. Where hard tissue is insufficient to achieve this goal, then bone augmentation becomes necessary. This may be performed during implant placement or where more severe deficiencies are present as a separate "staged" procedure. Bio-Oss® granules have been used as a sole graft or in combination with autogenous bone to augment potential implant sites as an alternative to the traditional autogenous block graft (von Arx and Buser 2006). In an alternative approach, Hammerle described 12 consecutive cases where only Bio-Oss® and a collagen membrane (Bio-Gide®) were used to horizontally augment bone prior to implant placement in sites with insufficient bone width (Hammerle et al. 2008). Implants were placed 9-10 months following augmentation and the authors commented that clinically, "integration" of Bio-Oss® particles into newly formed bone was observed. To promote passage of cells, particularly osteoprogenitors, crucial to the remodelling process, their methodology included perforating the host site cortical plates.

The principle of guided bone regeneration (GBR) at the time of implant placement is similar to GTR, where space is provided for bone-forming cells to expand bone volume. In this technique, only bone is laid down, in contrast to cementum and periodontal fibres in the GTR technique. Most commonly, a membrane is placed between the soft tissue and bone surface, either on its own or supported by autogenous or bone substitute materials. The membrane isolates the osseous site from the mucosal connective tissue, protects the clot, thus promoting osteoinduction (Mattout 2007). DBBM either alone or in combination with autogenous bone chips, is becoming popular during

simultaneous grafting procedures using the principles of GBR. In a comparative study in beagle dogs, 1.35mm circumferential defects with 5mm deep gaps surrounding implants were filled with either Bio-Oss® (0.25 – 1mm granules), autogenous bone or blood clot alone. A control with conventional implant placement was also used (Abushahba et al. 2008). Three months after placement, biopsies were taken and the distance from the top of the implant to first bone contact (FBIC) was significantly lower when either autogenous bone or Bio-Oss® was used, suggesting more bone deposition. There was a greater area of bone formation within the defects where a graft had been used; and their conclusion was that both autogenous bone graft and Bio-Oss® play an important role in hard tissue fill and osseointegration within marginal bone defects of 1.35mm. Clinical experiments confirm the findings of the aforementioned animal study for the use of DBBM in GBR (Zitzmann et al. 2001). A case treated by the author of the current thesis where an implant was placed combined with a GBR procedure, is shown in Figures 1.7 – 1.12**.**



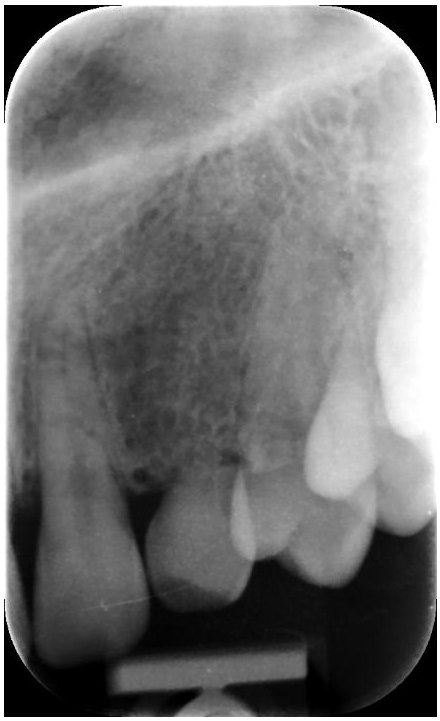
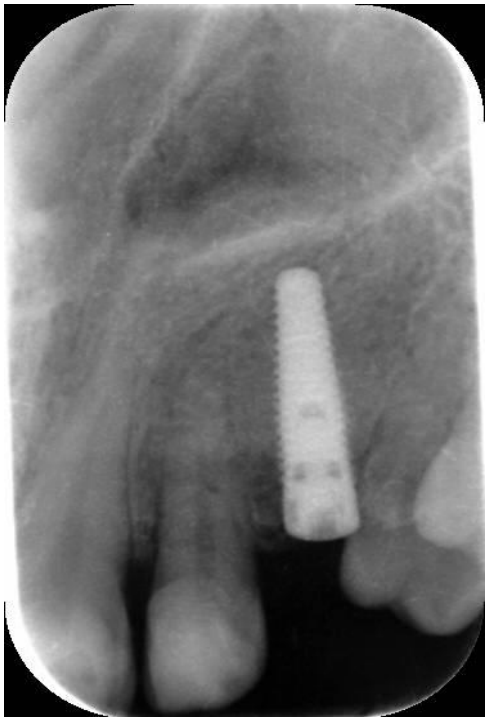


Figure 1.7 and 1.8 Developmentally absent permanent maxillary canines and associated reduction in bone width.



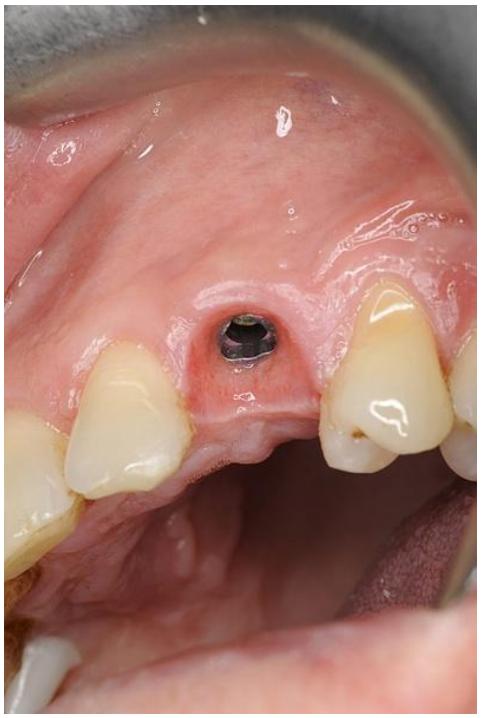
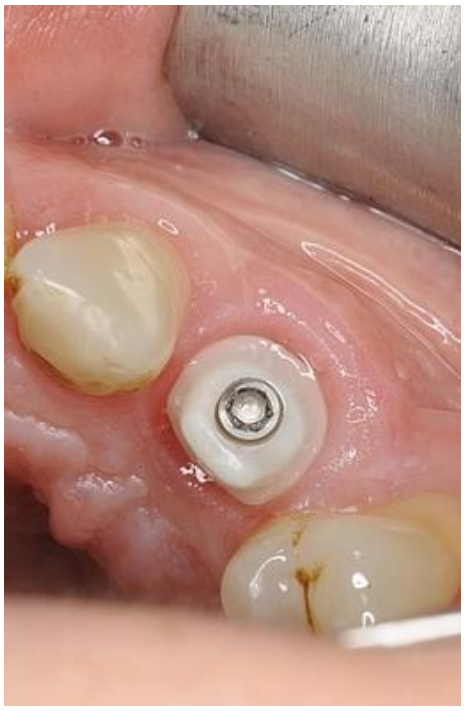


Figure 1.9 and 1.10 Implant placement resulted in a labial fenestration that was managed with Bio-Oss® granules and a Bio-Gide® membrane.



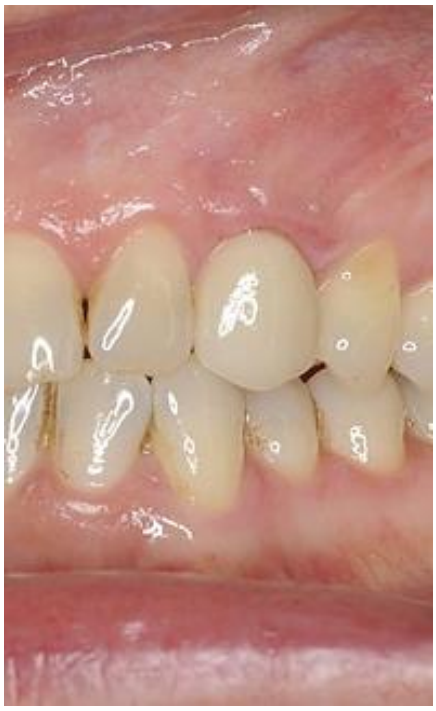


Figure 1.11 and 1.12 Following a period of integration the implant was restored with a cement retained crown. The figures show acceptable soft tissue architecture determined by the underlying bone volume.

1.7.4 DBBM and maxillary sinus augmentation

Over time, the maxillary sinus undergoes pneumatisation which reduces the available height for implant placement in the posterior maxilla. One solution to this problem is maxillary sinus augmentation to facilitate the placement of dental implants following a period of healing. This was first described by Boyne and James in 1980. Their technique used autogenous bone and a lateral window approach to access the maxillary sinus cavity (Boyne and James 1980).

A variety of bone substitute materials have been used for sinus augmentation, and DBBM can be used alone or mixed with autogenous bone in sinus grafting procedures. Hürzeler performed a 5 year clinical study comparing a variety of bone substitute materials. Although the subgroups were small, there were no differences noted between augmentation materials and the overall implant survival rate within augmented sinuses was 98.8%, which demonstrates the predictably of the procedure (Hurzeler et al. 1996). Indeed, the predictability of implant survival within augmented sinuses has been confirmed by others (Aghaloo and Moy 2007). The use of DBBM for this technique removes the necessity for harvesting bone from a second surgical site within the patient and therefore simplifies the procedure. There is some evidence showing that the use of DBBM alone can be successful and that the addition of autogenous bone is unnecessary (Valentini and Abensur 2003) and this is supported by a recent Cochrane systematic review (Esposito et al. 2010).

1.7.5 DBBM and peri-implantitis

Bio-Oss® has shown some promise in the management of bony defects as a result of peri-implantitis although this is not universally recommended (Lindhe and Meyle 2008). A prospective, randomized study by Schwarz *et al* (2008) showed that Bio-Oss® granules may have a role in the treatment of moderate intrabony peri-implantitis defects. Their sample included 22 patients, 11 of which received Bio-Oss® and Bio-Gide® treatment for defects with an intrabony component of 3 mm. The other 11 patients received an alternate graft material. The results demonstrated an approximate 2mm gain in clinical attachment level following treatment with DBBM at 24 months after surgery which was superior to the effect of the alternative graft material (Schwarz et al. 2008).

1.7.6 DBBM and extraction socket preservation

Following the extraction of teeth bone resorption will occur that can decrease residual bone volume horizontally and vertically. Two thirds of the resorption occurs in the first 3 months following tooth extraction (Schropp et al. 2003).
In an attempt to reduce the dimensional changes of the residual ridge following tooth extraction, techniques in socket and ridge preservation utilizing DBBM have developed. Techniques include the use of Bio-Oss® collagen or granules to support a gingival graft or collagen membrane as a space maintainer that should improve hard and soft tissue contour in the aesthetic zone. The effect of Bio-Oss® on ridge preservation has been studied in animal and clinical studies (Nevins et al. 2006; Heberer et al. 2008; Araujo et al. 2009). These studies generally show the width of bone at the extraction sites suffers less reduction in dimension of the marginal bone tissue in the grafted group compared to the control group. Although it is unlikely that DBBM increases bone formation within extraction sockets it does maintain site dimensions prior to implant placement.

Some clinicians are concerned that remaining graft material may interfere with bone deposition onto the implant surface, or it may promote overheating of the bone during the drilling procedure for the implant (Molly et al. 2008). A clinical case treated by the author of the current thesis where a Bio-Oss® collagen block was placed within an extraction socket to preserve volume is shown in Figures 1.13-1.15.

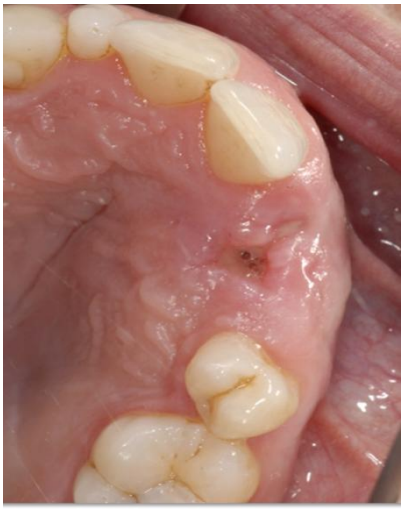
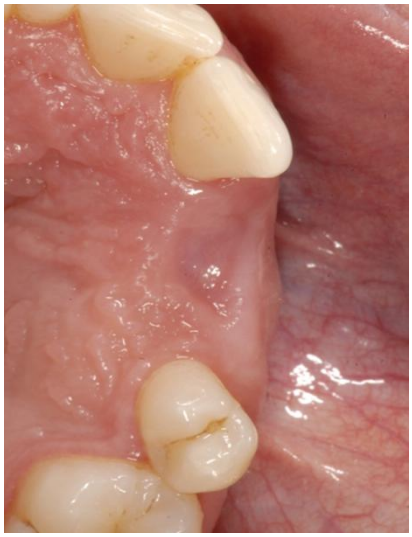


Figure 1.13 Following extraction of tooth 23 with apical periodontitis and microdont 22 Bio-Oss® collagen was immediately placed within the socket and the soft tissue sutured. This photograph shows the situation after 2 weeks



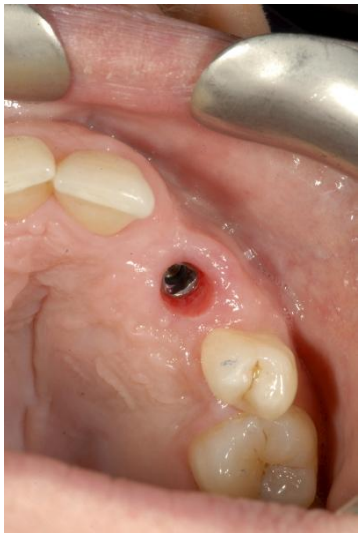
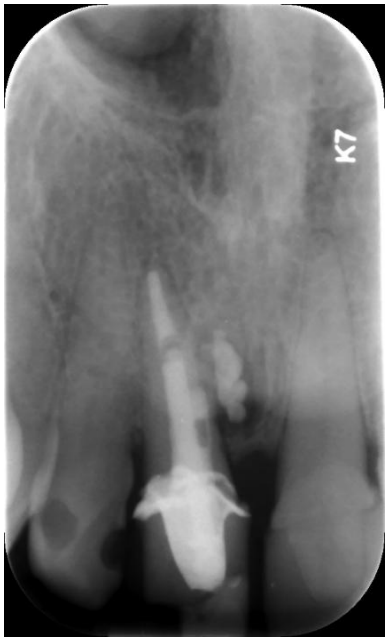


Figure 1.14 and 1.15 Following four months of healing good ridge width has been preserved facilitating implant placement.

1.7.7 DBBM and endodontic surgery

Apical periodontitis is an inflammatory lesion of the periapical tissues as a result of necrosis and infection of the root canal system. Although this is routinely treated with orthograde root canal treatment, a proportion of these treatments fail and surgical treatment may be necessary consistent with accepted guidelines (European Society of Endodontology 2006; Wu et al. 2006). Surgical treatment involves the curettage of inflammatory and potentially cystic tissue that often leaves a large residual bony defect. If the defect involves crestal bone loss, the prognosis of the procedure is known to be poor (Skoglund and Persson 1985). One method of managing these lesions is with the GTR procedure, although long term evidence to support this treatment concept is lacking (Lin et al. 2010; Tsesis et al. 2011). Indeed, a recent review on the topic concluded that GTR may improve the bony regeneration of certain defects during endodontic surgery, but that large scale clinical trials are needed to demonstrate additional benefits (Tsesis et al. 2011). A case treated by the author of the current thesis, is shown in Figures 1.16 – 1.19 and demonstrates the use of Bio-Oss® Collagen in the augmentation of a bone defect during endodontic surgery.



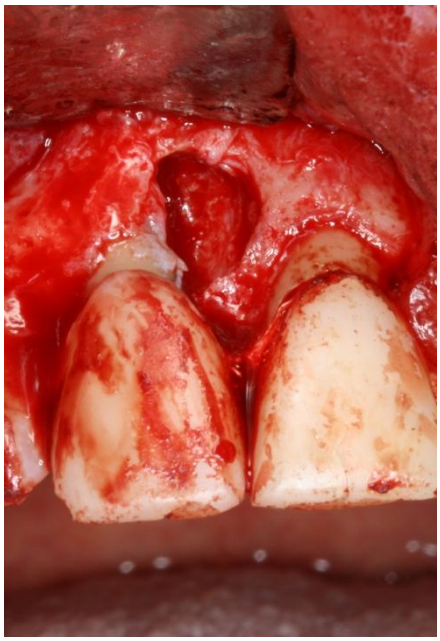
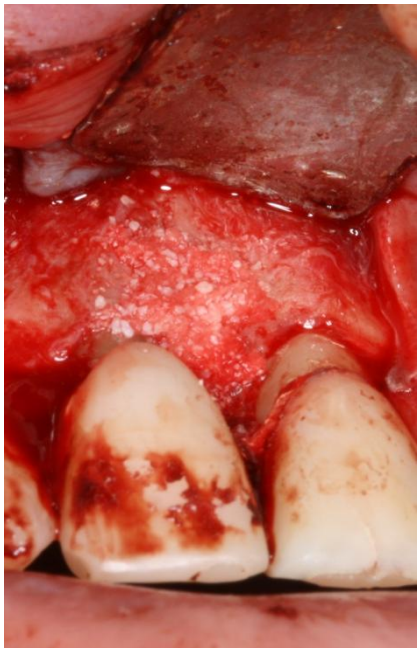


Figure 1.16 and 1.17 Tooth 11 demonstrates a lateral perforation, following the placement of a post crown, unsuccessfully repaired through an orthograde approach. After the elevation of a mucoperiosteal flap the inflammatory lesion was curetted.



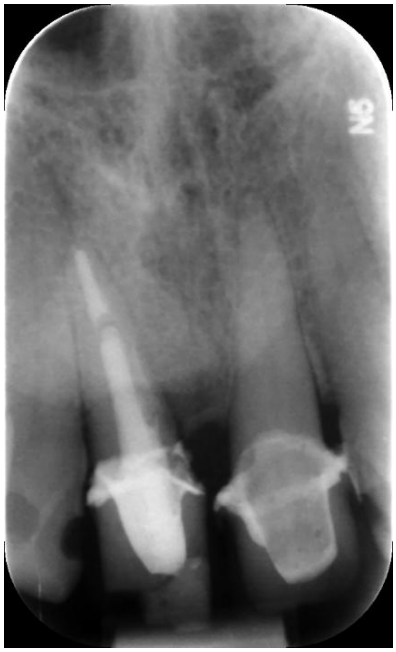


Figure 1.18 and 1.19 The perforation was repaired with BioDentine® (Septodont) and the lateral and crestal bony defect was repaired with Bio-Oss® collagen and a Bio-Gide® membrane.

1.7.8 Modifications of DBBM with growth factors

Modification of the commercially available forms of Bio-Oss® with growth factors has been carried out to improve the osteoinductive properties of the material. One case series reports on the use of Bio-Oss® to preserve extraction sockets where the Bio-Oss® is combined with recombinant human PDGF (Nevins et al. 2009). No membrane was used in this report to protect the DBBM, but primary closure was achieved with mucoperiosteal flap advancement and sutures. Implants were placed in half of the subjects at 4 months following extraction and the other half at 6 months following extraction. Although the authors concluded that the Bio-Oss® with PDGF up-regulated bone metabolism, since there was no control group it was not possible to quantify improvements in ridge preservation, as compared to no Bio-Oss® treatment. Further, the remaining particles of Bio-Oss® collagen demonstrated demineralization lines on the surface in intimate contact with multinucleate giant cells which is suggestive of active remodelling (Nevins et al. 2009). BMP-2 is also currently being investigated in combination with a deproteinized bovine bone mineral. Human histomorphometric studies have demonstrated increased bone to graft contact with the addition of BMP-2 over control (Jung et al. 2008b). It is the potential of growth factors to up-regulate osteogenic wound healing that merits further investigation.

1.8 Aims of research

1.8.1 Introduction

This research aims to investigate if there are methods for enhancing further the clinical performance of DBBM, by promoting faster healing. The results arising from this project are intended to form the early stages of a process to establish modified clinical protocols to enhance the properties of DBBM in implantology and periodontology.

1.8.2 Hypothesis

Endogenous growth factors bound in the bone matrix of DBBM can be released from the bone mineral by pre-treatment with calcium hydroxide or EDTA. The released growth factors will provide optimised signalling potential to stimulate recruitment of progenitor stem cells to the site of injury and provide the osteoinductive signals to promote bone formation.

1.8.3 Research questions

DBBM may contain the “ideal” cocktail of growth factors to promote bone repair within its bone matrix. Through a series of *in vitro* studies to investigate growth factor release and cellular behaviour, this research intends to investigate the application of calcium hydroxide and EDTA to mobilise growth factor stores from the matrix of DBBM. Specifically the following questions will be answered:

1. Does the chemical pre-treatment of DBBM increase growth factor release from the substitute?
2. How does DBBM or its extracted components influence cell behaviour in a tissue culture model?

Chapter 2

Characterisation of protein content of Demineralised Bovine Bone Mineral following extraction by chemical pre-treatment

2.0 Introduction

Bio-Oss®, or demineralised bovine bone mineral (DBBM), has been used extensively in the management of the consequences of periodontal disease and is used during the placement of dental implants. It is now clear there is conflicting research reporting on the protein content within DBBM. Moreover, investigation is required to identify if residual protein is in the form of growth factors, which may be released by chemical pre-treatment.

Bio-Oss® collagen blocks clearly contain protein of porcine origin, as described in chapter 1. However, there have been conflicting reports in the literature on whether Bio-Oss® granules themselves contain any residual protein. The majority of the literature states that all the protein is removed by the pre-treatment procedures explained in chapter 1 section 1.7.1 (Tapety et al. 2004). This pre-treatment performed by the manufacturers includes the treatment with a strong alkali, followed by sintering at 300°C for 15 hours. It is then treated with an organic solvent and sterilized (Tapety et al. 2004). The manufacturers claim this eliminates any protein leaving only inorganic material; however it is unlikely that an alkali alone will be sufficient to extract all residual proteins. Hallman and Thor suggest that the proteins in deproteinized bovine bone mineral have been extracted by the manufacturer to avoid immunologic rejection after implantation (Hallman and Thor 2008). They also state that the absence of protein results in DBBM losing any osteoinductive properties, this certainly suggests it behaves only as an osteoconductive scaffold (Hallman and Thor 2008).

Benke *et al* were amongst the first to carry out protein chemical analysis of DBBM in 2001 and concluded that it contained no protein to any measurable extent (Benke et al. 2001). Following chemical extractions, this group performed

SDS-PAGE, silver staining and also Western blotting, but failed to demonstrate proteins. In contrast, when this group used Coomassie blue to stain Bio-Oss® it resulted in an intense irreversible stain that the authors attributed to carbonate content rather than protein. The results of this study obviously also allows the conclusion that protein is actually present, but in amounts that are difficult to measure. Indeed, this group used a protein assay with detection limit of 0.25µg and so it is noteworthy that the ability to detect residual protein within DBBM is linked to the sensitivity of the technique used. Contradictory evidence is also available that demonstrates residual protein within Bio-Oss® granules. In 1999, Honig briefly reported on a single case of a failed Bio-Oss® spongiosa block graft. This graft had failed 10 months following placement. Following removal of the graft, it was analysed using polarisation microscopy. The graft stained positive with Comassie Blue stain which identified the presence of proteins within the failed graft. Obviously, the source of the protein could be due to protein deposition from the host and not protein within the graft itself. Nevertheless, the authors warned that residual protein could lead to transmission of Creutzfeldt-Jakob disease (CJD) or other infections (Honig et al. 1999).

A key study by Schwartz *et al* in 2000 further analysed the protein content of DBBM (Schwartz et al. 2000). They used laboratory based protein analysis techniques and suggested a small amount of residual protein may be present, but importantly, closely associated with the mineral phase. Using spectrophotometry, they demonstrated 11µg/g protein content and positive staining with silver stain. They were able to demonstrate via Western blot analysis the protein in the form of TGF-β and BMP-2 in DBBM particles (Schwartz et al. 2000). It is important to realise that this demonstration of protein does not mean that it is in an active form.

Another approach to the detection of protein is to use tissue culture techniques. Taylor *et al* concluded that residual protein may be present in DBBM following

the culturing of rabbit osteoclasts directly on a variety of bone substitutes, including DBBM. This group identified residual nitrogen content of between 0.17 – 0.47% within DBBM samples, although this was reduced compared with normal bovine bone controls (6.01 – 9.25%). They also identified collagen type 1 using antibody staining on the surface of DBBM after osteoclast activity, although this could have been laid down by the tissue culture cells (Taylor et al. 2002).

Taken together, there is evidence stating that residual proteins may be present in DBBM particles, in the form of Bio-Oss® granules, and therefore the material may be osteoinductive (Honig et al. 1999; Schwartz et al. 2000; Taylor et al. 2002), however there is also evidence reporting that DBBM is devoid of organic matter and therefore is only osteoconductive (Açil et al. 2000; Benke et al. 2001; Norton et al. 2003; Hallman and Thor 2008). Norton *et al* recognised that further studies are required, as it remains unclear whether proteins are present in DBBM (Norton et al. 2003). In contrast, there is a lack of existing evidence analysing specifically the nature of the protein content of DBBM in its other form, namely Bio-Oss® collagen. Indeed, the body of research has focussed on the original material in the form of Bio-Oss® granules. Although it is expected that Bio-Oss® collagen will contain protein, more detailed investigation is required to establish if it too contains bioactive molecules. This is likely, as it has been suggested by others that bioactive molecules can be sequestered along with proteoglycans closely associated with the collagen network within the extracellular matrix of mineralised tissues (Schonherr and Hausser 2000; Baker et al. 2009; Smith et al. 2011). It is clearly important to determine whether residual protein is present in DBBM, as this may help determine whether it behaves as an osteoconductive or osteoinductive material.

In light of the aforementioned conflicting evidence, the characterisation of residual protein potentially in the form of growth factors within DBBM, merits further investigation and will be a focus of this research project. It is evident that

the protein content within Bio-Oss® granules themselves may be low, but sufficient to be bioactive, therefore the aim of this chapter was to demonstrate and characterise protein in DBBM. In view of the challenges that some groups have faced in demonstrating residual protein, a carefully chosen extraction protocol was developed. In this chapter, experimental techniques were used to determine whether protein is trapped in the mineral phase and can therefore be demineralised with hydrochloric acid, or ethylenediaminetetraacetic acid or extracted with calcium hydroxide. Bioactive components were extracted from the substitute using either 0.02M $Ca(OH)_{2,}$ 10% EDTA or 0.5M HCl. Subsequent extracts were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) and Western Blot analysis for molecules known to be important in regulating bone regeneration, specifically TGF-β_1.

2.1 Materials and Methods

2.1.1 Extraction protocol

DBBM samples were prepared by weighing 5 grams of Bio-Oss® granules (Lot 080305, 080374, Geistlich Pharmaceuticals) and 1000mg of Bio-Oss® Collagen (Lot 070522, 080425, Geistlich Pharmaceuticals). Accurate samples were obtained in triplicate using an electronic balance. Double distilled water (DDW) was used throughout the experiments, with ultrapure water (UPW) being used where stated.

To each Bio-Oss® granules or Bio-Oss® Collagen sample was added either 150ml of 0.5M HCl (Fisher Scientific), 150ml of 0.02M $Ca(OH)_2$ (Fisher Scientific) pH 11.7 or 150mls of 10% EDTA (Fisher Scientific) pH 7.2. These extraction solutions contained protease inhibitors, 5mM

phenylmethanesulfonylfluoride (PMSF) (Sigma-Aldrich, UK) and 10 mM n-ethyl maleimide (NEM) (Sigma-Aldrich, UK). A final extraction solution was used on all 6 samples following treatment with either HCl, $Ca(OH)_2$ or EDTA. This final solution contained 4M guanidinium chloride (GuCl) (Fisher Scientific), 50mM tris(hydroxymethyl)methylamine (Tris) (Fisher Scientific) pH 7.4. All pH adjustments were carried out using a pH meter by adding either concentrated HCl or NaOH.

The Bio-Oss® granules and Bio-Oss® collagen samples were extracted in the HCl solution for 24 hours, and the EDTA and $Ca(OH)_2$ solutions for 48 hours at a constant temperature of 4°C, with continuous agitation with a magnetic stirrer. Following 24 or 48 hours, the soluble fractions were decanted after centrifugation for 15 minutes at 4000 rpm. Samples were nominated as either HCl, $Ca(OH)_2$ or EDTA "Extraction 1" for each sample and stored at -20°C for later volume reduction. The insoluble fractions were re-suspended in 4M GuCl, 50mM Tris, 5mM PMSF and 10 mM NEM, pH 7.4 for 18 hours at 4°C. The soluble fractions were again removed after centrifuging for 15 minutes at 4000rpm and stored at -20°C as HCl, $Ca(OH)_2$ or EDTA "Extraction 2" for each sample. The remaining undisolved samples were also stored. This extraction protocol is summarized in figure 2.1.

2.1.1.1 Buffer Exchange

The extraction process produced 12 extract solutions which were subsequently concentrated. The volume of each was reduced to approximately 10ml with the use of 15ml Amicon ultra centrifugal filter devices (Millipore, UK) and the solutions were buffer exchanged with DDW. This process of ultrafiltration reduces the volume overall. The solutions were continuously suspended in the spin columns within a centrifuge at 3500rpm for 30 minutes and the waste pipetted from the column. This resulted in a concentrated sample. The HCl, $Ca(OH)_2$ and EDTA DBBM extracts were stored at -20^{o}C, prior to use. These extracts were subsequently analysed by protein analysis, SDS-PAGE and Western Blot analysis.

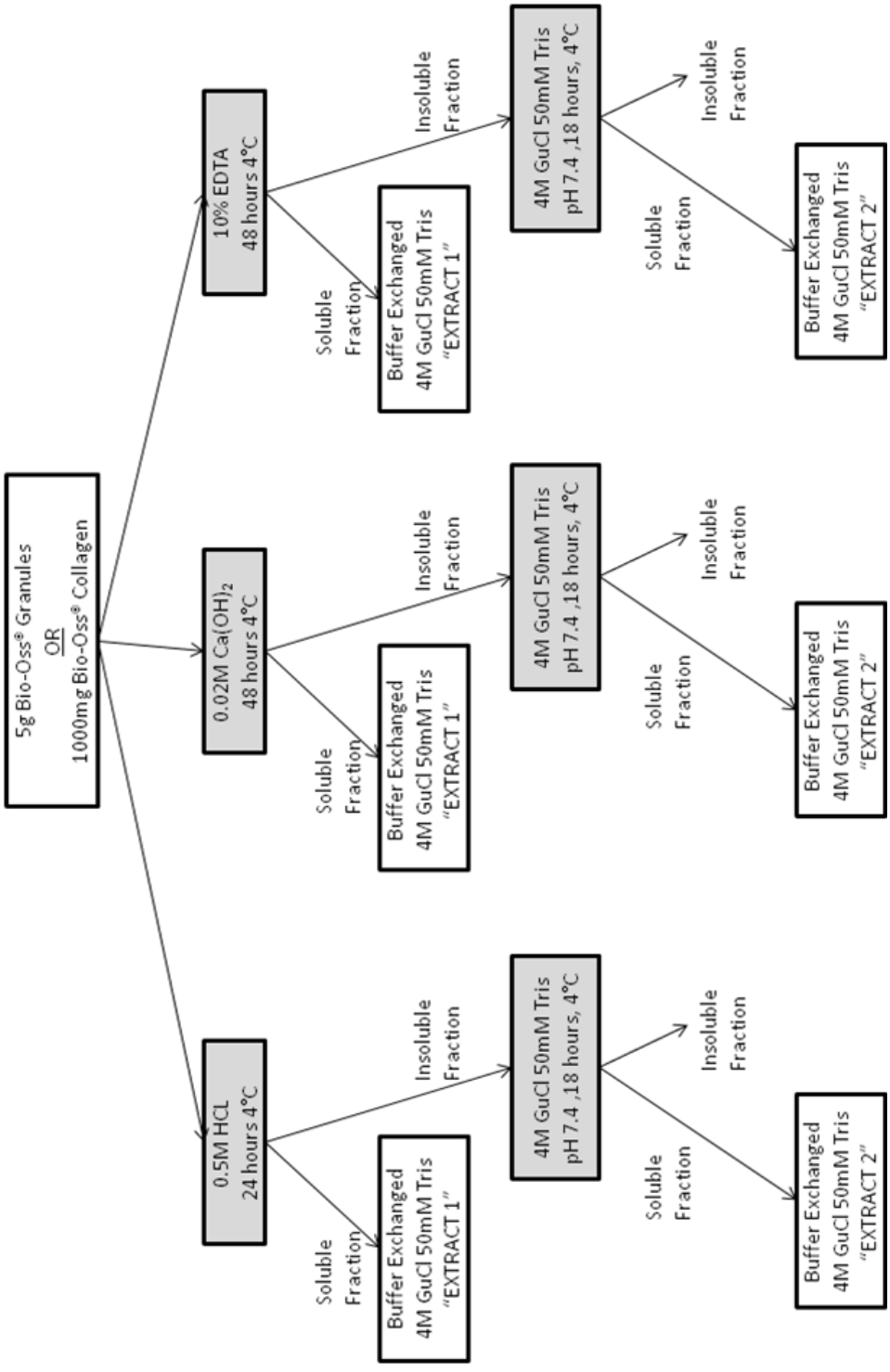


Figure 2.1 DBBM Extraction protoocol followed to release residual proteins

2.1.2 Protein Analysis

2.1.2.1 BCA assay

The bicinchoninic acid (BCA) assay (Pierce, UK) was used to determine the total concentration of protein within the extract solutions. A series of standards was prepared from bovine serum albumin (BSA) at a concentration of 0 – 2000 µg/ml, to produce a calibration curve against which the concentration of protein in the sample could be quantified. Using a 96 well plate, 10 µl of the test solution was added to 190 µl of BCA buffer and the degree of colour change was measured after 30 minutes following incubation at 37°C, using a Microplate reader (Bio-Tek Instruments Limited) by measuring the absorbance at 570nm. A control well contained water in place of extract sample.

2.1.2.2 Concentration of samples

Based on the results of the BCA assay (see section 2.2.2), the HCl extracted samples were further concentrated prior to analysis with SDS-PAGE. Concentration was carried out via a process of lypophilization. The extract solutions produced from Bio-Oss® granules (Extract 1 and 2) were concentrated by a factor of 10, by freeze drying 500 µl and reconstituting it in 50 µl of water. The extract solutions from Bio-Oss® collagen (Extract 1 and 2) were concentrated by a factor of 4 by freeze drying 200 µl and reconstituting in 50 µl of water. Different concentration factors were used, since the BCA assay had demonstrated a detectable protein concentration in the Bio-Oss® collagen extraction samples only and not in the Bio-Oss® granules. Therefore, it was not possible to equalize the protein concentrations.

2.1.3 SDS-PAGE

Proteins were separated from the extract samples by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS – PAGE). SDS – PAGE was performed using NuPage Novex 4 - 12% Bis Tris mini gels (Invitrogen, UK). Prior to placement in the electrophoresis tank (*XCell 2* blotting module, Invitrogen UK), the gels were washed thoroughly with DDW, the electrode exposed and the plastic comb removed from the gel cartridge. 200 ml of running buffer (NuPage MES SDS running buffer, Invitrogen, UK) containing 500 µl of antioxidant (NuPage, Invitrogen, UK) was prepared in the inner chamber of the electrophoresis tank and 300ml of running buffer alone prepared in the outer chamber. 12µl of each extract sample, 3µl of LDS sample buffer (NuPage, Invitrogen, UK) and 1.5µl of sample reducing agent (NuPage, Invitrogen, UK) were heated at 70 °C for 10 minutes and centrifuged for 1 minute at 3000 rpm before and after thermal treatment. The prepared samples were loaded into the gel along with 10µl of a molecular weight marker (*See Blue Plus 2* Pre-stained Standard, Invitrogen, UK) in a separate well. The pre-stained marker contains 10 pre-stained bands and allows visualization of molecular weights. The gels were electrophoresed for 35 minutes at 200V, 120mA. Upon completion, the gels were either silver stained or processed for Western Blot analysis.

2.1.3.1 Silver Staining

Prior to silver staining with the Color Silver stain Kit (Pierce Biotechnology Inc), the gels were fixed with 5 ml 5% acetic acid and 50 ml 50% alcohol for 1 hour. Fixation was complete when each gel ceased to shrink. The gels were washed with 4 changes of ultrapure water for 40 minutes per change, until they returned

to their original dimensions. The gels were incubated in the silver working solution for 30 minutes, followed by a 20 second rinse. The gels were then incubated in the reducer working solution for 5 minutes (prepared by mixing the reducer aldehyde and reducer base working solutions immediately before use). Following a 5 second water rinse, each gel was incubated at room temperature in the stabilizer working solution for a further 60 minutes. After the application of each solution, the gels were agitated using a platform shaker. Upon completion of the staining procedure, the gels were visualized on a white light box and photographed.

2.1.4 Electroblotting

Electroblotting was performed using unstained gels loaded with each extract sample, as described in section 2.1.3. Individual proteins were detected following their transfer to a nitrocellulose membrane and incubation with appropriate primary and secondary antibodies conjugated to horseradish peroxidise, as detailed below.

Gels were electrophoresed initially for 35 minutes at 200V, 120mA using the electrophoresis tank. The gel was placed in a blotting box adjacent to a nitrocellulose membrane (Hybond ECL, GE Healthcare) and sandwiched between 4 foam sponges and 4 filter paper layers, all soaked in NuPage transfer buffer (20X) (Invitrogen, UK). The blotting box was placed inside the electrophoresis tank filled with NuPage transfer buffer (20X). A NuPage Western blot cycle was run for 1 hour at 25V and 160mA. Following completion of the cycle, the filter paper and the gel were discarded and the membrane retained and stored in TBS, prior to analysis.

2.1.4.1 Ponceau staining

Ponceau staining was performed to detect protein on the nitrocellulose membrane produced using the HCl extracted components. The membrane was soaked in Ponceau stain (0.1% Ponceau in 5% acetic acid) for 3 minutes with gentle agitation. The stain was poured off and the membrane rinsed with TBS, prior to inspection. Ponceau will adhere to proteins transferred to the membrane resulting in red banding. Following use, a destaining procedure was performed prior to Western blotting by submerging the membrane in DDW until all traces of stain were removed.

2.1.4.2 Immunodetection

Nitrocellulose membranes were stained for the presence of protein following transfer. All experiments used primary antibodies and secondary antibodies, conjugated with horseradish peroxidase (HRP) enzyme and were detected via enhanced chemiluminescence (ECL). Antibodies were prepared to the appropriate concentration (Table 2.1) using 5% TBS nonfat dried milk. Prior to staining with antibodies, the membranes were blocked overnight with 5% TBS milk. Milk contains casein which can bind to nonspecific proteins to reduce the likelihood of non specific binding. The following day, the milk was poured off and the membrane incubated with primary antibody and agitated for 1 hour on a platform shaker. The membrane was washed 3 times for 5 minutes with 5% Tween / TBS to remove unbound primary antibody. The secondary antibody was added and agitated for 1 hour followed by 3 further washes with 5% Tween / TBS.

Immunoreactivity was detected using ECL Plus Western Blot Detection Reagents (Amersham Biosciences, Amersham, UK). Following staining with the appropriate antibodies, the membrane was treated using the ECL Plus Western

Blot Detection Reagent for 3 minutes with agitation. Following this stage, the membrane was drip dried, wrapped in saron wrap and placed face up inside a radiographic cassette and secured with insulation tape. In the dark room, radiographic film was placed in the radiographic cassette for various time intervals, ranging from 30 seconds to 10 minutes, depending on the intensity of the staining observed.

As a negative control, the primary antibody was replaced with an IgG isotype control that was diluted to the working concentration of the primary antibody. Membranes were also stained after the primary antibody was pre-incubated overnight with a 10 fold excess of the TGF-β_1 blocking peptide. The antibody bound to the blocking peptide should no longer be available for binding to the protein on the membrane. This procedure was used to identify nonspecific binding.

	Antibody name	**Antibody Source**	**Dilution**
TGF-β_1 primary	Anti-TGF-β_1 (v) Rabbit polyclonal IgG	Santa Cruz Sc 146	1:200 – 1:2000
TGF-β_1 secondary	Polyclonal swine anti-rabbit IgG HRP	Dako	1:2000 - 1:10000
TGF-β_1 Blocking peptide	TGF-β_1 (v) P Blocking peptide	Santa Cruz Sc 146p	10 X primary antibody concentration
Non-immune IgG (Isotype control)	Rabbit IgG	Santa Cruz	Same concentration as primary
Fetuin A primary	Anti Fetuin-A goat polyclonal IgG	Santa Cruz Sc 9663	1:200
Fetuin A secondary	Donkey anti-goat IgG HRP	Santa Cruz Sc 2020	1:2000

Table 2.1 Details of antibodies used during Western blotting

2.2 Results

2.2.1 Extraction solutions

At the end of the extraction process, the following 12 solutions were produced as described in figure 2.1:

- Bio-Oss® Granules Extract 1 HCl treatment only
  Extract 2 HCl and GuCl, Tris treatment

- Bio-Oss® Collagen Extract 1 HCl treatment only
  Extract 2 HCl and GuCl, Tris treatment

- Bio-Oss® Granules Extract 1 $Ca(OH)_2$ treatment only
  Extract 2 $Ca(OH)_2$ and GuCl, Tris treatment

- Bio-Oss® Collagen Extract 1 $Ca(OH)_2$ treatment only
  Extract 2 $Ca(OH)_2$ and GuCl, Tris treatment

- Bio-Oss® Granules Extract 1 EDTA treatment only
  Extract 2 EDTA and GuCl, Tris treatment

- Bio-Oss® Collagen Extract 1 EDTA treatment only
  Extract 2 EDTA and GuCl, Tris treatment

2.2.2 BCA assay

BCA assay was performed on the extract samples to determine protein content. The protein concentration within each sample was determined from the standard curve shown in Figure 2.1, by reading the absorbance value for each solution following subtraction of control. The protein content of the HCl extracted components are shown in Table 2.2. Analysis of these results demonstrated undetectable protein in the Bio-Oss® granules sample (Extract 1 and 2). The Bio-Oss® Collagen samples treated with HCl only (Extract 1) demonstrated a protein concentration of 132 µg /ml. Following further extraction with GuCl / Tris solution (Extract 2), the protein concentration increased to 955 µg/ml (Table 2.2). On the basis of these results, all HCl extract solutions were concentrated as described in section 2.1.2.2, prior to SDS-PAGE. Protein was undetectable in other extract solutions.

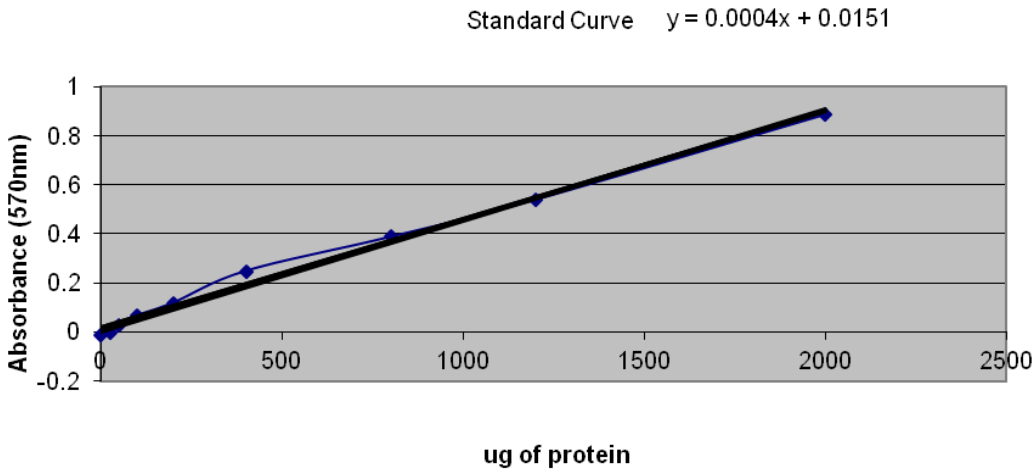


Figure 2.1 Standard curve demonstrating relationship of protein standard and BCA absorbance values.

		Control	Bio-Oss Granules Extract 1	Bio-Oss Granules Extract 2	Bio-Oss Collagen Extract 1	Bio-Oss Collagen Extract 2
Absorbance values	First experiment	0.086	0.087	0.085	0.153	0.482
	Repeat experiment	0.084	0.088	0.087	0.152	0.491
	Mean	0.085	0.088	0.086	0.153	0.487
Protein Concentration (µg/ml)			0	0	132	955

Table 2.2 Protein content of Bio-Oss® granules and Bio-Oss® Collagen following extraction with HCl (Extract 1) followed by GuCl and Tris (Extract 2). Table demonstrates BCA absorbance values and protein concentration as calculated using graph 2.1.

2.2.3 SDS-PAGE with silver stain

To determine the protein profile present in the extraction solutions, SDS-PAGE was performed. Figures 2.2 to 2.4 illustrate the banding pattern revealed by silver staining of the extraction solutions. Silver banding is present in both the concentrated and neat solutions of the Bio-Oss® granules Extract 1 and 2 groups, following treatment with HCl. Strong banding is present at approximately 62 kDa and fainter banding at 98 kDa. In the Bio-Oss® collagen samples, there were no distinct bands only non distinct streak-like staining (Figure 2.2). It is clear from the figure that a similar level of staining was detected in both neat and concentrated extraction components. In view of this finding, SDS-PAGE was only subsequently performed on neat extracted components from EDTA and $Ca(OH)_2$ solutions without an additional concentration procedure.

Figure 2.3 shows the silver staining profile following extraction with $Ca(OH)_2$. SDS-PAGE revealed silver stained banding in Bio-Oss® granules Extract 1 and 2 and Bio-Oss® collagen Extract 1, after treatment with $Ca(OH)_2$. Strong banding was detected at 62 kDa. Streak like staining was detected in the Bio-Oss® collagen extract 2 after calcium hydroxide and GuCl / Tris treatment (Figure 2.3). This pattern was similar in Bio-Oss® samples treated with EDTA with the most intense banding in the Bio-Oss® granules Extract 2 after EDTA and GuCl / Tris treatment (Figure 2.4).

2.2.4 Ponceau Staining

Figure 2.5 demonstrates that Ponceau staining was unable to detect any distinct banding in any of the extracts following treatment with HCl. Only streak like staining was detected on the membrane throughout the molecular weight range in Bio-Oss® collagen Extract 2 following GuCl / Tris treatment (Figure 2.5).

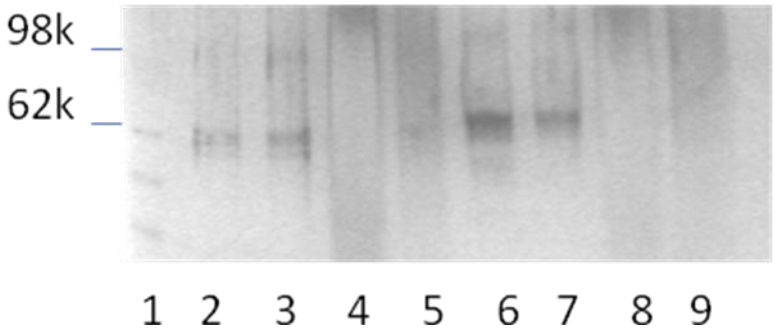


Figure 2.2 SDS PAGE and silver stain of HCl extracted components (Typical gel). Lane 1 molecular weight marker, lane 2 Bio-Oss® granules Extract 1, Lane 3 Bio-Oss® granules Extract 2, Lane 4 Bio-Oss® collagen Extract 1, Lane 5 Bio-Oss® collagen Extract 2. Lanes 6 – 9 were loaded with concentrated solutions in the same order as lane 2 to 5.

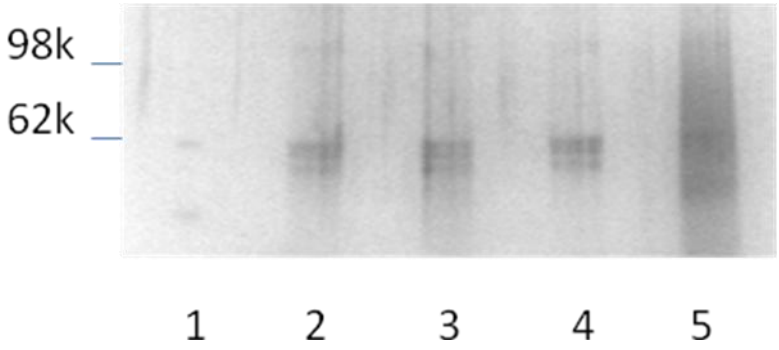


Figure 2.3 SDS PAGE and silver stain of $Ca(OH)_2$ extracted components (Typical gel). Lane 1 molecular weight marker, Lane 2 Bio-Oss® granules Extract 1, Lane 3 Bio-Oss® granules Extract 2, Lane 4 Bio-Oss® collagen Extract 1, Lane 5 Bio-Oss® collagen Extract 2.

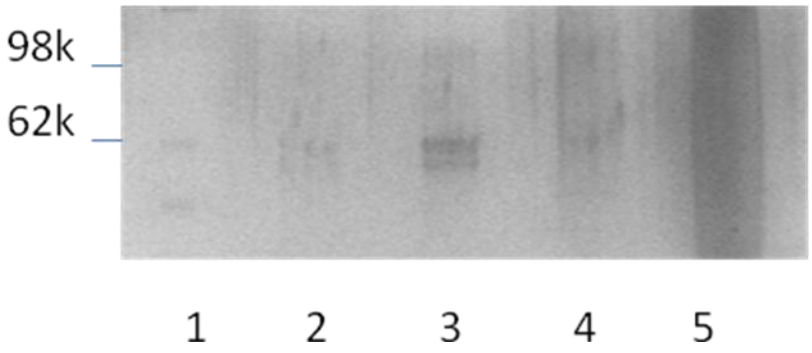


Figure 2.4 SDS PAGE and silver stain of EDTA extraction components (Typical Gel). Lane 1 molecular weight marker, lane 2 Bio-Oss® granules Extract 1, Lane 3 Bio-Oss® granules Extract 2, Lane 4 Bio-Oss® collagen Extract 1, Lane 5 Bio-Oss® collagen Extract 2.

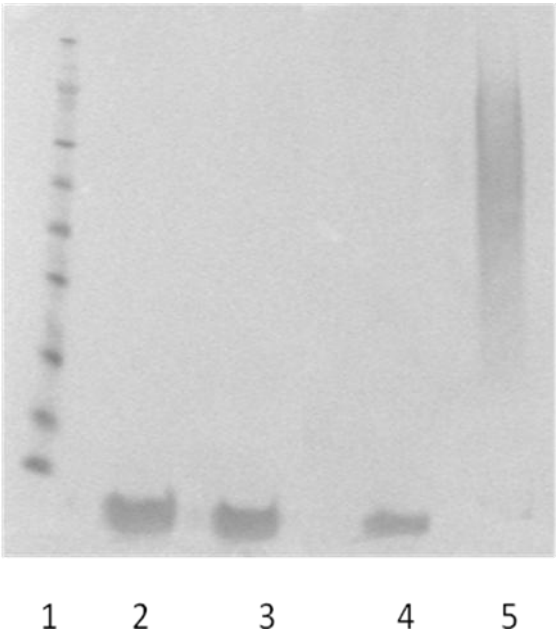


Figure 2.5 Staining patterns following Ponceau staining of nitrocellulose membrane after transfer of HCl extraction components. Lane 1 molecular weight marker, Lane 2 Bio-Oss® granules Extract 1, Lane 3 Bio-Oss® granules Extract 2, Lane 4 Bio-Oss® collagen Extract 1, Lane 5 Bio-Oss® collagen Extract 2.

2.2.5 Western blot analysis

Western blot analysis was used to demonstrate immunoreactive protein on nitrocellulose membranes. Figure 2.6 demonstrates immunoreactivity using antibodies for TGF-β_1 in all extracts following treatment with HCl, apart from Bio-Oss® collagen Extract 2. There was an intense band present at approximately 62kDa and a less intense band at 98kDa. The intensity of the band was strongest in extracts recovered from Bio-Oss® granules Extract 1 and Bio-Oss® collagen Extract 1, after only HCl treatment and not with guanidinium chloride treatment (Figure 2.6). Western blot analysis following overnight pre-incubation with TGF-β_1 blocking peptide continued to demonstrate immunoreactive protein at 62kDa following treatment with HCl, but importantly with less intensity (Figure 2.7)

Figure 2.8 demonstrates Western blot analysis of the $Ca(OH)_2$ extracts revealing immunoreactivity for TGF-β_1 in all extract groups. Banding at 62kDa was particularly intense in the Bio-Oss® collagen Extract 1 prior to further suspension in GuCl / Tris. Following pre-incubation with the blocking peptide, the intensity of the banding decreased in all $Ca(OH)_2$ extract groups and disappeared in the Bio-Oss® collagen Extract 1 (Figure 2.9).

Western Blot analysis of the EDTA extracts revealed immunoreactivity for TGF-β_1 in all extract groups, except Bio-Oss® collagen Extract 2 (Figure 2.10). Intense staining was present at 62kDa with slightly less intense staining in 98kDa. Following pre-incubation with the blocking peptide banding was only reduced significantly in the Bio-Oss® granules Extract 2 group (Figure 2.11). Interestingly, with the blocking peptide a band at 62kDa appeared in Bio-Oss® collagen Extract 2 which had not appeared when the blocking peptide had not been used.

Figure 2.12 demonstrates the negative control when non-immune IgG replaced the primary antibody. Western blotting failed to demonstrate immunoreactive proteins in the HCl, $Ca(OH)_2$ or EDTA derived samples using non-immune IgG (Figure 2.12).

Western blot analysis was unable to demonstrate immunoreactivity using antibodies for Fetuin A in extracts produced following treatment with HCl (Figure 2.13).

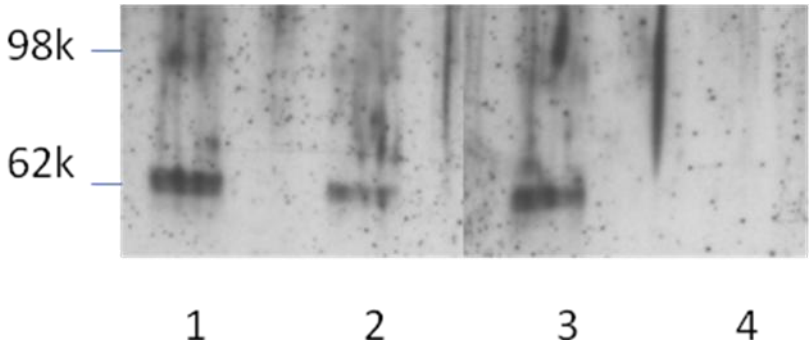


Figure 2.6 Western blot analysis for immunodetection of TGFβ1 after HCl extraction. Lane 1 Bio-Oss® granules Extract 1, Lane 2 Bio-Oss® granules Extract 2, Lane 3 Bio-Oss® collagen Extract 1, Lane 4 Bio-Oss® collagen Extract 2.

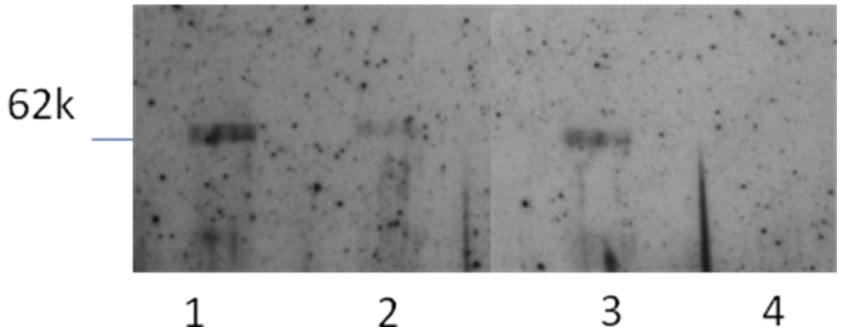


Figure 2.7 Western blot analysis following overnight incubation with TGFβ1 blocking peptide after HCl extraction. Lane 1 Bio-Oss® granules Extract 1, Lane 2 Bio-Oss® granules Extract 2, Lane 3 Bio-Oss® collagen Extract 1, Lane 4 Bio-Oss® collagen Extract 2.

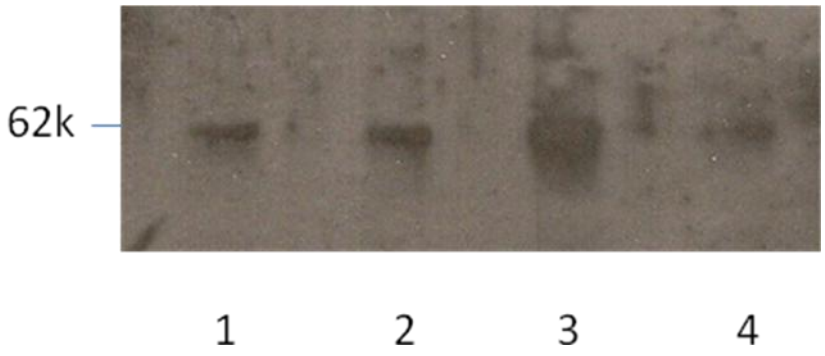


Figure 2.8 Western blot analysis for immunodetection of TGFβ1 after $Ca(OH)_2$ extraction. Lane 1 Bio-Oss® granules Extract 1, Lane 2 Bio-Oss® granules Extract 2, Lane 3 Bio-Oss® collagen Extract 1, Lane 4 Bio-Oss® collagen Extract 2.

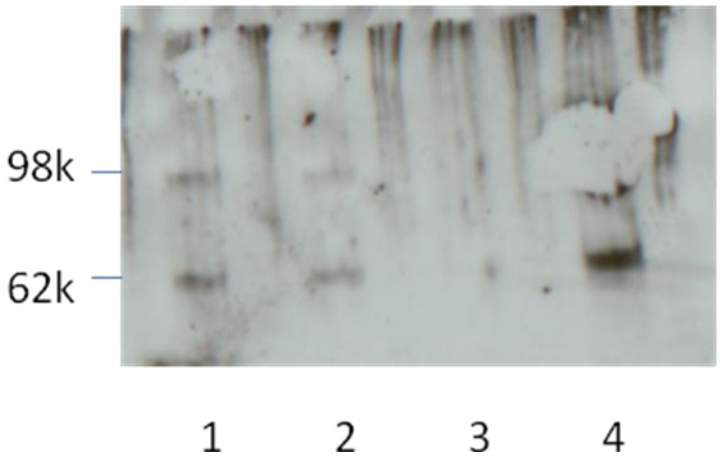


Figure 2.9 Western blot analysis after overnight incubation with TGFβ1 blocking peptide after $Ca(OH)_2$. Lane 1 Bio-Oss® granules Extract 1, Lane 2 Bio-Oss® granules Extract 2, Lane 3 Bio-Oss® collagen Extract 1, Lane 4 Bio-Oss® collagen Extract 2.

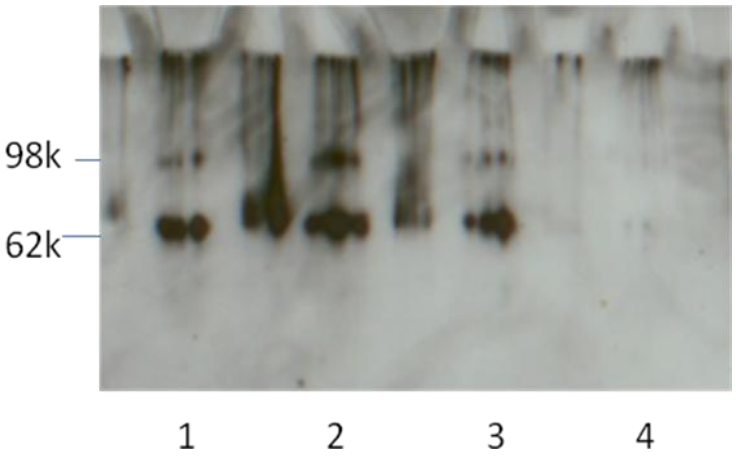


Figure 2.10 Western blot analysis for immunodetection of TGFβ1 after EDTA extraction. Lane 1 Bio-Oss® granules Extract 1, Lane 2 Bio-Oss® granules Extract 2, Lane 3 Bio-Oss® collagen Extract 1, Lane 4 Bio-Oss® collagen Extract 2.

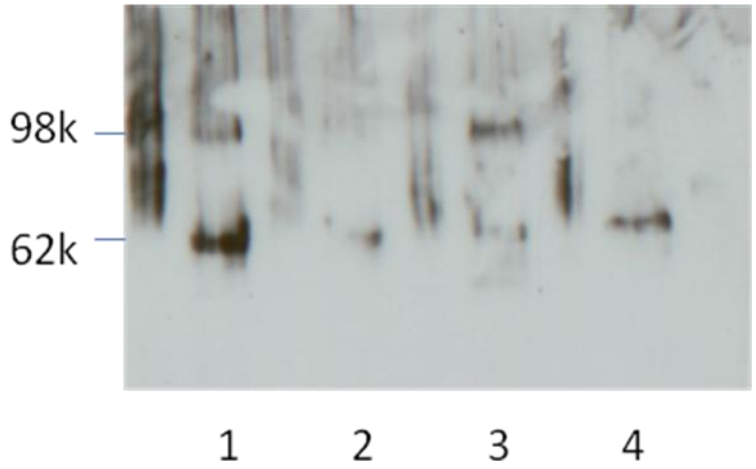


Figure 2.11 Western blot analysis after overnight incubation with TGFβ1 blocking peptide after EDTA. Lane 1 Bio-Oss® granules Extract 1, Lane 2 Bio-Oss® granules Extract 2, Lane 3 Bio-Oss® collagen Extract 1, Lane 4 Bio-Oss® collagen Extract 2.

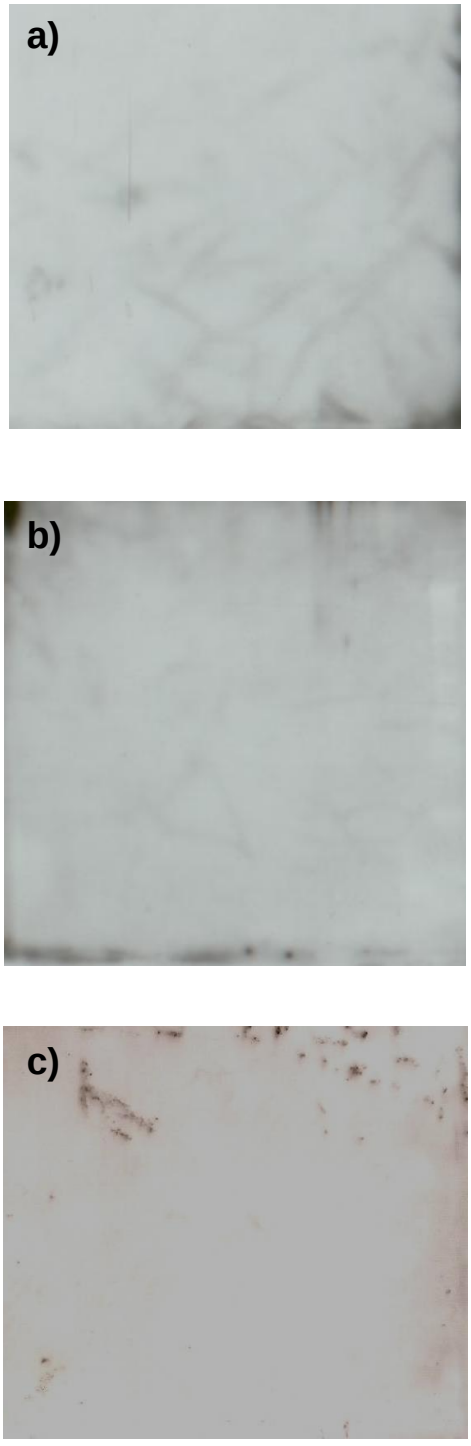


Figure 2.12 Western blot analysis following substitution of primary antibody with non-immune IgG. Membranes were produced using a) HCl extracted components, b) $Ca(OH)_2$ extracted components and c) EDTA extracted components of DBBM.

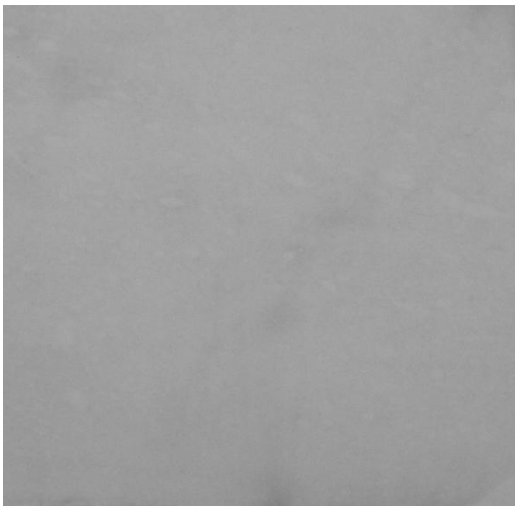


Figure 2.13 Western blot analysis for immunodetection of Fetuin A using HCl extracted components of DBBM

2.3 Discussion

This chapter provides evidence that there may be small amounts of residual protein in Bio-Oss® granules, that although not detectable by BCA assay, can be demonstrated using SDS-PAGE and Western blotting. This chapter has for the first time examined the nature of residual proteins within Bio-Oss® collagen block, in addition to Bio-Oss® granules.

The bicinchoninic acid assay (BCA) was used to determine the total concentration of protein within the extraction solutions. The results in this chapter show that only the Bio-Oss® collagen samples demonstrated detectable protein levels using the BCA assay. This is unsurprising considering that during the manufacture process 10% porcine collagen is added to the DBBM particles. When performed on Bio-Oss® granules, the BCA assay failed to detect protein. These results contrast with that of Schwartz *et al* 2000 who were able to detect protein at 11µg/g in Bio-Oss® granules based on absorbance of 280nm (Schwartz et al. 2000). Indeed, difficulty in detecting protein at a measurable level has also been reported by other research groups (Benke et al. 2001). It would appear from the present project that it is difficult to measure protein concentration within DBBM using certain assay techniques.

SDS – PAGE is a method for analyzing proteins that separates them based on their size. Silver staining is 5 – 200 fold more sensitive than another stain, Coomassie Blue, and is able to detect bands containing less than 1-2ng of protein, according to the manufacturers of the staining kit. Conventionally, 10 µg of protein are used for experimental techniques involving gels and blotting. In this chapter, it was not possible to equalize the proteins in each sample to a known concentration prior to loading gels, since the BCA assay had failed to record a protein level in the Bio-Oss® granules groups. The results for silver

staining in this chapter demonstrated clear bands at 62kDa in both neat and concentrated solutions from HCl extracted components of Bio-Oss® granules. Fainter banding was also detected at 98kDa. In the extracts produced by both $Ca(OH)_2$ and EDTA, similar banding was detected at 62kDa. Staining at this molecular weight was only detected in the Bio-Oss® collagen Extract 1 after $Ca(OH)_2$ treatment alone. The remaining Bio-Oss® collagen extracts failed to demonstrate a distinct band which is possibly a reflection of the denaturing of the collagen network following the extraction process. The ability of extracts to stain positively with silver staining mirrors the results of Schwartz *et al* 2000 who demonstrated particularly intense staining in Bio-Oss® granules, following extraction with EDTA and guanidinium chloride (Schwartz et al. 2000). However, this group also point out that silver stain bands may be produced by binding to phospholipids which means that silver staining alone is only suggestive of protein. Ponceau staining was also used as it is a relatively fast method of identifying proteins once transferred to a nitrocellulose membrane during the electroblotting process. The benefits of the stain are that it is rapid, reversible and can be washed off prior to Western blotting; however the technique is non-specific. In this chapter, Ponceau staining failed to detect protein in the HCl extraction group and was not used further. This appears to confirm the dependence of technique sensitivity on the detection of protein within DBBM.

Western blotting was used to demonstrate specific proteins. It is a technique for protein analysis whereby fractionated proteins are transferred via a current to a nitrocellulose membrane and detected using monoclonal and polyclonal antibodies. Primary antibodies bind to the protein under investigation and secondary antibodies are active against the primary antibodies. This binding can be detected using enhanced chemiluminescence. The results in this chapter show that following incubation with TGF-β_1 antibody, immunoreactivity occurred at approximately 62 kDa in all the Bio-Oss® granules extracts following treatment with HCl, EDTA and $Ca(OH)_2$ which may suggest the presence of this

growth factor within DBBM. This protein complex may have been released by chemical pre-treatment In addition, immunoreactivity was also detected at 98kDa, with particular intensity in the EDTA extraction group. However, the results must be interpreted with caution, since the presence of bands at these two molecular weights is not equivalent to that of TGF-β_1 which is 12.5kDa or 25kDa when it exists in dimer form. These uncharacteristic results mirror that of Schwartz *et al* 2000, who using anti-TGF-β antibody suggested that the immunoreactive band at 60 – 65kDa in their study was due to a high molecular weight complex of TGF-β (Schwartz et al. 2000). This high molecular weight complex could be in the form of latent TGF-β binding proteins which are complex carrier molecules involved in the regulation of TGF-β. Secondly, another possibility is that the antibodies are bound to TGF-β receptors type I and type II, which have molecular weights that may correspond to 62kDa and 98kDa, respectively (Grande 1997). In addition since TGF-β is known to bind to decorin and biglycan this would significantly increase their molecular weight during immunodetection. It is noteworthy that TGF-β is secreted associated to its latency associated peptide with a molecular weight of 45kDa (Grande 1997, Sloan et al. 2002)

In the present study, it was not possible to detect differences in the ability of HCl, EDTA or $Ca(OH)_2$ to liberate protein from the DBBM matrix. This conflicts with research from other groups on dentine matrix where EDTA was shown to liberate more TGF-β_1, although this group were able to measure the absolute concentration of growth factor, which was not possible in the present study (Tomson et al. 2007). In the present study the blocking peptide was relatively ineffective. This can be for several reasons which include an insufficient concentration, excess TGF-β_1 in the sample, non specific binding or a faulty blocking peptide.

Western blot analysis was unable to demonstrate immunoreactivity for Fetuin A. Even when the concentration of antibodies was increased substantially,

immunoreactivity was not demonstrated suggesting that it is highly unlikely that residual protein within DBBM is in this form. Antibodies for Fetuin A were used since the initial silver staining of gels had detected protein at 62 kDa which is the approximate molecular weight for Fetuin A (more precisely 59kDa).

Taken together, the observations in this chapter suggest that residual protein is present within Bio-Oss® granules, in addition to Bio-Oss® collagen. Potentially, the pre-treatment of DBBM with HCl, EDTA and $Ca(OH)_2$ may liberate the protein complex from the matrix of this bone graft material. It would appear, therefore, that these proteins are able to evade the manufacturers own pre-treatment procedures. This may be due to growth factor binding associated with the mineral phase of bone, as suggested by other groups, which requires extensive extraction and demineralisation for their release (Schwartz et al. 2000; Smith et al. 2011). Therefore, demineralisation of the DBBM with HCl and the chelating effect of EDTA in this chapter, may have liberated this protein. Equally important is the suggestion for the first time that a TGF-β_1 antibody binding site may be present in the protein complex within Bio-Oss® Collagen Block. The intense protein banding in Bio-Oss® collagen following suspension in calcium hydroxide may be accounted for by its behaviour as a protein extractant. The existing descriptions in the literature of DBBM (primarily in Bio-Oss® granule form) being devoid of organic matter may be a result of the sensitivity of the assay technique used by the manufacturer. It has been reported that the Lowry method is one of three methods used to demonstrate the absence of protein, with a detection limit of 135ppm (Peetz 1997). This may be insensitive to smaller protein concentrations protected within the mineral phase.

The potential effects of TGF-β_1 if locked within the mineral phase of Bio-Oss® granules or closely associated with collagen in Bio-Oss® Collagen block, are far reaching. Critically, it is the presence of stem cells, an appropriate scaffold and signalling molecules, such as TGF-β_1, that form the triad of factors required for

bone regeneration (Malhotra et al. 2009). In terms of tissue engineering, this growth factor will affect stem cell growth, differentiation, promote chemotaxis of osteoblasts, their migration and differentiation and ultimately the deposition of matrix and its mineralization (Giannobile 1996; Grande 1997; Binkert et al. 1999; Hallman and Thor 2008). It will also impact on tissue remodelling and controlling the inflammatory response. It must be recognised that TGF-β_1 will not act alone and an appropriate cocktail of growth factors is likely to be more effective. It is therefore possible that Bio-Oss® contains other growth factors not investigated in this study, such as BMP-2, 6, 7 and IGF. The potential presence of growth factors within this graft material, in addition to that released from the tissue bed as a result of surgical trauma, is of clear benefit. As previously mentioned, mineralised matrix in other forms is known to contain TGF-β_1 that has the ability to exert the aforementioned bioactive effects. These bioactive molecules can be sequestered along with proteoglycans bound to the collagen network in mineralised tissues (Schonherr and Hausser 2000; Baker et al. 2009; Smith et al. 2011). Further, it has been suggested that these molecules control the bioavailability of growth factors and their presentation to cell receptors.

The potential presence of growth factors in DBBM, even at low concentration, may help explain the effects on cells both *in vitro and in vivo.* This is important since it is recognised that only low concentrations of growth factor are needed to exert biological effects, probably at the nanogram level. Indeed, it has been suggested that the administration of supra-physiological concentrations of growth factors may lead to side effects due to the initial high concentration. This may lead to their rapid degradation and not allow sufficient concentration of the growth factors to reach target tissue for enough time to exert their effects (Lee et al. 2011). The next chapter will investigate the effect that the extracted components produced in this chapter; and the effect that commercial DBBM, have on cell behaviour in an *in vitro* cell culture model.

Chapter 3

The effect of Demineralised Bovine Bone Mineral and its extracted components on cell behaviour

3.0 Introduction

The previous chapter has demonstrated that residual proteins are highly likely to be present within commercial Bio-Oss® granules, in addition to Bio-Oss® Collagen block. Tentatively, this maybe in the form of growth factors. The focus of this chapter was to investigate the effect that DBBM and its extracted components have on the behaviour of animal cells *in vitro*.

Several previous *in vivo* animal studies have demonstrated the biocompatibility of DBBM by investigating cellular behaviour over the graft surface. In 1997, Berglundh examined the healing around implants placed in bone defects in Beagle dogs, where the defects had been filled with DBBM three months previously. This group reported that DBBM was biocompatible and its volume was observed to reduce over a 7 month period, which they presumed to be due to a process of resorption. Importantly, the graft particles were in direct contact with new bone and the level of osseointegration of the titanium implants was observed to be similar to non-grafted sites (Berglundh and Lindhe 1997). Using cell culture techniques, Açil *et al* (2000) studied the growth and extracellular matrix synthesis of human osteoblast-like cells, derived from iliac crest grafts, on DBBM. Following culturing for 6 weeks, this group observed osteoblasts forming a three dimensional structure on the surface of the Bio-Oss® block and the accumulation of mature collagen fibrils was visible on transmission electron microscopy and scanning electron microscopy. This is evidence of the ability of DBBM to support cell growth and matrix synthesis. Importantly, their biochemical analysis demonstrated increasing concentrations of hydroxylysylpyridinoline, lysylpyridinoline and hydroxyproline (the constituents of collagen) when cells were cultured on DBBM demonstrating, the ability of osteoblast-like cells to deposit collagen on the DBBM scaffold (Açil et al. 2000). These studies together confirm DBBM is a biocompatible scaffold.

The capacity of DBBM to display osteoconductive and osteoinductive behaviour has also been investigated. Tapety *et al* examined the response of bone cells to DBBM to assess the osteoconductive capacity. This group performed histochemical analysis following placement of the graft into rat femur defects, compared with controls. Importantly, at day 3, alkaline phosphatase immunoreactivity was only detected in defects filled with DBBM, which is characteristic of the presence of osteoblasts within the defects treated with the graft. Furthermore, bone formation occurred by day 5 in both groups and the DBBM that was adjacent to new bone demonstrated osteocalcin immunoreactivity. Using transmission electron microscopy, the authors were able to demonstrate osteoblasts depositing bone matrix on the surface of DBBM. These results clearly demonstrate that DBBM acts as a scaffold for bone formation with an affinity for osteogenic cells (Tapety et al. 2004). The authors concluded that there may be growth factors bound within DBBM but as the graft does not induce ectopic bone in other tissues, it may not contain sufficient volumes of growth factors to be effective. It is possible that local host growth factors accumulate on the graft particles following surgical trauma that promote cell migration, attachment and differentiation. Hämmerle and co-workers investigated the ability of DBBM to treat peri-implant dehiscence defects using a guided bone regeneration technique. Importantly, in this study, DBBM particles were shown to integrate into newly-formed bone within the defects when examined histologically and therefore, the group concluded that DBBM is osteoconductive (Hammerle et al. 1998). The osteoconductivity of DBBM has been further studied in humans (Schwartz et al. 2000; Norton et al. 2003). Schwartz *et al* describe a case where DBBM was used as part of a sinus augmentation procedure. Histologically bone cores demonstrated new bone and multinucleated giant cells on the surfaces of DBBM particles (Schwartz et al. 2000). This appearance has been replicated in a larger clinical study on 15 consecutive patients, where 22 bone cores taken from sites previously grafted with DBBM were analysed (Norton et al. 2003). In this study, trephines were

used to collect bone cores at least 4 months following grafting, prior to implant placement and were found to contain 26.9% new bone, 25.6% residual graft and 47.4% connective tissue. The results demonstrated that in 3 of the 22 specimens, less than 5% of the core was composed of bone, suggesting a failure of some of the grafts.

Another controversial subject is the ability of Bio-Oss® to resorb over time and be replaced by human bone which relates to its ability to promote the attachment and migration of cells upon its surface. Opinions in the literature have changed on the resorbability of DBBM over the last 15 years. In Berglundh et al's early study of peri-implant defects in Beagle dogs, a reduction in the amount of DBBM from 17% to 11% in biopsies obtained during a 3 to 7 month time period was observed (Berglundh and Lindhe 1997). Although these authors also commented that the remodelling of DBBM was similar to pristine bone, their conclusions were only based on subjective assessment of bone biopsies. This early research suggested that DBBM is resorbed with time, however, more recent studies suggest that this assumption may be incorrect. In order to understand why opinions have changed, it is important to appreciate how graft resorption occurs. As previously mentioned, the cells responsible for bone resorption are osteoclasts and the process of resorption requires adhesion molecules for the attachment of the osteoclastic cells (Benke et al. 2001). These include fibronectin, fibrinogen, vitronectin, type I collagen, osteopontin and bone sialoprotein. Evidence already reviewed from Benke *et al* and other groups suggested that DBBM is free of proteins which would theoretically mean that resorption could not occur as osteoclastic adhesion would not be possible (Benke et al. 2001). However, the results in chapter 1 and the evidence summarised earlier from both clinical and animal models suggests that residual protein is indeed present in DBBM and that partial resorption occurs. Further, Taylor *et al* used a rabbit osteoclast model to evaluate resorptive capacity of DBBM and a variety of other grafting materials. Following 4 days of culture the DBBM samples were examined using scanning electron microscopy, tartrate

resistant acid phosphatase (TRAP) staining, immunohistochemical staining and surface chemistry analysis. DBBM demonstrated less TRAP staining compared to controls; and with atypical resorptive pits under SEM unlike the other bone-derived materials. This suggests slow resorption activity within DBBM (Taylor et al. 2002). This group agreed with the findings of Benke in that a reduction in non-collagenous protein content may reduce cell attachment and therefore, resorptive capacity. Tapety has also reviewed the literature on the resorbability of Bio-Oss® noting that some studies demonstrate delayed resorbability and others a complete lack of resorbability (Tapety et al. 2004).

Clinical evidence demonstrating slow resorption of DBBM also exists. Using bone cores taken from grafted maxillae, Tadjoedin demonstrated TRAP stain positive osteoclasts, signifying resorption, in close approximation to DBBM treated sinus' (Tadjoedin et al. 2003). Piattelli retrieved Bio-Oss® samples up to 4 years, after sinus augmentation procedures in humans. Their samples showed that osteoclastic resorption was occurring at grafted sites as much as 4 years after grafting, confirming a slow resorption process (Piattelli et al. 1999). Over a longer follow-up time, another study has examined the histology of DBBM within a sinus over 10 years; and although is limited to a single case, provides useful information (Sartori et al. 2003). In this study, at 8 months following augmentation, a bone sample showed 29.8% of native bone tissue within the augmented sinus. When samples were repeated at 10 years, the bone content increased to 86.7% with a corresponding decrease in DBBM particles, suggesting its resorption (Sartori et al. 2003). Although this study demonstrates new bone formation and partial DBBM resorption over time, the reproducibility of the sampling techniques must be questioned. Another study looked at the resorptive capacity of DBBM in extraction sockets rather than the sinus; and failed to demonstrate osteoclastic activity on the particle surfaces (Molly et al. 2008). This study also demonstrated 20% by volume material remaining in bone biopsies taken 4 months after placement of DBBM into extraction sockets (Molly et al. 2008). Furthermore, in a dog study where DBBM

(in the Bio-Oss® Collagen form) was placed into extraction sockets, no resorption was observed over 6 months (Araujo et al. 2009). Recently, a study of Bio-Oss® Collagen modified with PDGF was placed in human extraction sockets. This study demonstrated that the modified DBBM showed signs of resorption at 4 and 6 months (Nevins et al. 2009). This may be due to the presence of proteins acting as chemoattractants for osteoclasts. Hallman *et al* reviewed much of the evidence for and against resorption by concluding that DBBM acts as a non-resorbable grafting material in humans (Hallman and Thor 2008).

The aforementioned evidence from animal and human studies demonstrates the biocompatibility of DBBM, but it is unclear on the absolute behaviour of DBBM within human bone. There is, however, convincing clinical and *in vitro* evidence that bone is laid down on DBBM particles which confirms its ability to behave, at least, as an osteoconductive scaffold. In this chapter, an *in vitro* rat bone marrow stromal cell model was used to investigate whether DBBM or its extracted components influence cell behaviour, as assessed by cell expansion and gene expression. Cell culture techniques allowed the assessment of the behaviour of osteoblast like cells on Bio-Oss®.

3.1 Materials and methods

3.1.1 Harvesting rat bone marrow stromal cells

Bone marrow stromal cells (BMSC's) were isolated from the femurs and ulnars of 28 day old male Wistar rats, following sacrifice via CO_2 asphyxiation. Rats were dipped in 70% ethanol and bones were dissected and placed in a 10 X antibiotic media consisting of α-MEM (Invitrogen, UK), and 10% penicillin / streptomycin (Sigma-Aldrich, UK). The marrow of the femurs and ulnars were flushed with 15ml culture media into falcon tubes containing α-MEM (Invitrogen, UK), 10% heat inactivated foetal calf serum (Sigma-Aldrich, UK), 1% penicillin / streptomycin (Sigma-Aldrich, UK), 250 ng/mL amphotericin B (Invitrogen, UK). Cells were centrifuged at 1500 rpm for 5 minutes and the pellet was dispersed into 500 µl of collagenase / dispase (Sigma-Aldrich, UK) and incubated for 15 minutes at 37°C. Following incubation, 5 ml of culture media was added to the tube and it was centrifuged at 1500 rpm for 5 minutes; and the supernatant decanted. The pellet was re-suspended in 10 ml α-MEM and passed through a 40 µm microfilter (Falcon) to generate a single cell suspension. Cells were seeded onto 10 µg/ml fibronectin-coated T75 flasks at 2 bones per flask and incubated for 20 minutes at 37°C. After 20 minutes non-adherent cells were flushed off using fresh media. Cells were subsequently cultured in an incubator at 37ºC, 5% CO_2 in air with medium changes every 2 days using 20 ml α-MEM, 10% FCS and 1% antibiotic mixture. After approximately 2 weeks, near confluent cells were trypsinised with 1ml trypsin (Invitrogen, UK). The cell suspension was centrifuged at 1500 rpm for 5 minutes before re-suspension in 1 ml of media. The cells were seeded at $1x10^4$ cells/cm^2 onto either 96-well polystyrene plates for an MTS assay or 6-well polystyrene plates for mRNA extraction.

3.1.2 Culture of mesenchymal cells in the presence of DBBM

All experimental protocols used either Bio-Oss® granules (Lot numbers 080305) or Bio-Oss® collagen block (Lot numbers 070522, 080156).

3.1.2.1 Analysis of cell expansion

In order to assess cell expansion, BMSC were seeded directly into wells containing Bio-Oss® granules or Bio-Oss® collagen, for analysis with an MTS assay. The MTS assay is a colorimetric method for determining the number of viable cells and cell proliferation during tissue culture experiments. The MTS compound is reduced by cells into a formazan product that is soluble in tissue culture medium. The quantity of formazan product is measured by the amount of absorbance at 490nm. This correlates with the number of living cells in the wells. Bio-Oss® granules were added to the 96-well plates until the base of the well was completely covered (10 mg approximately). A 250 mg Bio-Oss® collagen block was divided into 20 approximately equal slices and a single slice was placed into each well. Control wells did not contain Bio-Oss® and wells were prepared in triplicate. Cells were seeded at 10^4 cells per cm^2 and separate plates were prepared for 5 days. A no cell control consisted of plain media.

The cells were cultured in osteoblast differentiation media (containing α-MEM (Invitrogen, UK), 10% heat inactivated foetal calf serum (Sigma-Aldrich, UK), 1% 10mM β-glycerophosphate (Sigma-Aldrich, UK),), 1% 10mM dexamethasone (Sigma-Aldrich, UK) 1% 50µg/ml ascorbic acid (Sigma-Aldrich, UK), 1% penicillin / streptomycin (Sigma-Aldrich, UK) for 5 days, with media changes every 2 days. On each day of the experiment, a plate was removed from the incubator. Subsequently, 100µl of media was removed from each well

and 20µl of the MTS assay (CellTiter 96® Aqueous Solution Reagent) solution added to the well to make the total volume remaining within each well to120µl, as per the manufacturer's instructions. This was performed under low lighting conditions due to the photosensitive nature of the MTS assay. The plate was returned to the incubator at 37°C and 5% CO_2 for 2.5 hours, after which absorbance was measured using a FLUOstar™ Optima plate reader at 490nm. Data was captured using the Optima software programme.

Cells were also cultured in 6-well plates containing either 300 mg Bio-Oss® granules, Bio-Oss® collagen block or control. A 250mg Bio-Oss® collagen block was divided into 5 approximately equal slices with a scalpel and 2 slices placed into each well of a 6-well plate. An empty well acted as control. BMSC were seeded at 10^4 cells per cm^2 directly onto the Bio-Oss® or control wells in duplicate. Cells were fed osteoblast differentiation media as described previously, with media changes every 2 days. Cell growth was examined using an inverted light microscope (Eclipse TS100, Nikon) and images were captured with a digital camera (Canon Powershot S5 IS).

3.1.3 Culture of mesenchymal cells in the presence of DBBM extracts

Cells were harvested from rats, as described in section 3.1.1.

3.1.3.1 Preparation of media supplemented with DBBM extracts

Freeze-dried samples from DBBM extracted components produced during the protein content experiments, outlined in chapter 2, were used to produce supplemental media. Specifically, these samples were the extraction components of the Bio-Oss® granules alone prior to extraction with GuCl. To make the media, 5mg of each powdered sample (EDTA extract, $Ca(OH)_2$ extract and HCl extract) were added to 3 separate falcon tubes and mixed with double distilled water (DDW) to a concentration of 10µg/ml. This solution was then passed through a 0.2 micron sterilizing syringe filter. Each extract solution was diluted to a final concentration of 100 ng/ml in standard osteoblast differentiation media (containing α-MEM, 10% heat inactivated foetal calf serum, 1% 10mM β-glycerophosphate, 1% 10mM dexamethasone, 1% 50µg/ml ascorbic acid and 1% penicillin / streptomycin).

3.1.3.2 Analysis of cell expansion

In order to investigate the effect of DBBM extracted components on cell expansion, an MTS assay was performed as described in section 3.1.2.1. BMSC were seeded at 10^4 cells per cm^2 onto 96-well plates and were cultured with standard media on day 1. From day 2 onwards cells were cultured with media supplemented with $Ca(OH)_2$, EDTA or HCl extraction components and

control media, as prepared above for 5 days. The MTS assay was performed each day. Differences in mean absorbance (as correlated to cell number) between cells cultured in the presence of media supplemented with DBBM extracts and control media, were analysed statistically using one way analysis of variance using the Instat® programme (Graphpad software). *P* value <0.05 were considered significant.

3.1.3.3 Analysis of bone matrix markers

In order to investigate the effect of DBBM extracted components on gene expression of bone matrix markers, mRNA extraction followed by reverse transcription polymerase chain reaction (RT-PCR) was performed. BMSC were seeded onto 6-well plates and were cultured on first day with standard osteoblast differentiating media. From day 2 onwards, cells were cultured in media supplemented with one of three extraction components from Bio-Oss® granules, or a control media, as prepared above. These supplemented media contained EDTA extraction components, calcium hydroxide extracted components, and HCl extracted components, described in chapter 2 and a plain control. Control media consisted of α-MEM (Invitrogen, UK), 10% heat inactivated foetal calf serum (Sigma-Aldrich, UK), 1% 10mM β-glycerophosphate (Sigma-Aldrich, UK), 1% 10mM dexamethasone (Sigma-Aldrich, UK) 1% 50µg/ml ascorbic acid (Sigma-Aldrich, UK). A 6-well plate was prepared for day 5, 9, 12 in duplicate.

In order to extract mRNA at day 5, 9, 12, the following protocol was followed. All working areas were cleaned with 70% alcohol. Excess media was removed from each well and the well washed with PBS. 350µl of a solution containing 10µl of β-mercaptoethanol and 1ml of RLT lysis buffer (containing guanidine thiocyanate) was added to each well and agitated to disrupt cell walls. Total mRNA was isolated after homogenization, using the RNeasy™ mini kit (Qiagen,

UK). The lysate was pipetted into a QIAshredder™ and centrifuged at 13,600 g for 2 minutes; to homogenise. 350µl of 70% ethanol was added to the cell solution. The sample was transferred to a spin column to bind total RNA and centrifuged for 15 seconds at 8000 g (10,000 rpm). 700µl of buffer RW1 was added and centrifuged, as above, and the flow through discarded. 500 µl of buffer RPE was added and column centrifuged for 15 seconds and the flow through was discarded. This stage was repeated and centrifugation performed for 2 minutes, followed by placement of the RNeasy column in a fresh tube, and centrifugation for 1 minute. 30 µl of RNase-free water was added and centrifuged for 1minute at 8000 g. Total RNA was subsequently measured at an absorbance of 260nm using Nanovue™ Spectrometry (GE Healthcare), to enable equalisation of the RNA concentration during RT-PCR. The solution was pipetted into 1.5ml sterile eppendorff's and stored at -80°C until needed later for RT-PCR.

3.1.4 RT-PCR

Where measurable mRNA was detected, RT-PCR was used to characterise for changes in differentiation potential of cells by expression of osteopontin, bone sialoprotein, osteonectin and osteocalcin. β-actin was used as a housekeeping gene.

The first stage of the RT-PCR process is the production of copy DNA from the RNA extracted. All work areas were cleaned with 70% ethanol and RNA stability was maintained by performing procedures on ice. 1 µg of RNA was added to a sterile PCR tube along with 1 µl of random primer and DEPC (RNAase free) water, to a final volume of 15 µl. This was incubated at 70°C for 5 mins then stored on ice. A master mix solution using Promega® reagents of

5 µl MMLV reaction buffer, 1.25 µl dNTPS, 0.6 µl RNasin, 1 µl MMLV Reverse Transcriptase (Promega, UK) and 2.15 µl DEPC water was made to sufficient volume for the number of PCR reactions. 10 µl of the master mix was added to each of the RNA / random primer mix solution and incubated at 37°C for 1 hour. A negative control (labelled RT negative) was performed in the absence of RNA, using water as a replacement. All reverse transcription reactions were run on G-Storm™ GS1 Thermal Cycler (Genetic Research Instrumentation Ltd). The product of the above reaction is copy DNA which was stored at -20°C until required for PCR reactions.

The PCR reactions were performed using Promega reagents. A 10 mM stock of dNTP's was made from 50 µl each of ATP, CTP, GTP, TTP (adenosine, cytosine, guanine and thyamine triphosphates, respectively) and 300 µl PCR grade water. A master mix was prepared containing, 5 µl Buffer, 0.5 µl dNTP's PCR Nucleotide Mix (Promega, UK), 1.25 µl forward primer 1, 1.25 µl reverse primer 2, 1 µl magnesium chloride, 14.75 µl PCR grade water, 0.25 µl GoTaq flexi DNA polymerase (Promega, UK). The PCR reaction solution consisted of 24 µl of the master mix and 1 µl of cDNA. Negative controls consited of the PCR reaction, performed with only the mastermix (labelled PCR negative) and the PCR reaction with mastermix and cDNA reaction in the absence of mRNA (labelled RT negative). The primers used were rat β-actin, osteopontin, osteonectin, octeocalcin, bone sialoprotein and their primer sequences are shown in table 3.1. The reactions were run on a G-Storm™ GS1 Thermal Cycler and the PCR cycle is shown in table 3.2.

The PCR product was visualised using 2% agarose gel electrophoresis. 10x TBE buffer was used to make the agarose gels and used as a running buffer. TBE buffer contained 1M tris-base (Sigma-Aldrich, UK), 1M boric acid (Sigma-Aldrich) and 0.06M EDTA at a pH of 8.0. The buffer was diluted to 0.5x prior to use. Agarose powder was disolved in 0.5x TBE buffer and this was agitated in a microwave for 20 second cycles until the solution became clear. Following

cooling, 5 µl of 10 mg/ml ethidium bromide (Promega, UK) was added to the solution, prior to being poured into a gel cast with combs *in situ*. Once set, the gel was placed into a electrophoresis tank containing TBE buffer and the combs removed. 10 µl of each PCR reaction was added to each well. A seperate well contained a 100 base pair DNA ladder (Promega, UK). The gel was run at 80V for approximately 45 minutes. The gel was visualised using Gel Doc™ scanner (BioRad, UK) and images captured with Quantity One image analysis software (BioRad, UK).

Primer	Product size	Sequence
β-actin	180 bp	F: TGAAGATCAAGATCATTGCTCCTCC R: CTAGAAGCATTTGCGGTGGACGATG
BSP	211bp	F:CTGCTTTAATCTTGCTCTG R: CCATCTCCATTTTCTTCC
Osteopontin	189bp	F: GGAGTCCGATGAGGCTATCAA R: TCCGACTGCTCAGTGCTCTC
Osteocalcin	293bp	F: ATGAGGACCCTCTCTCTGCTC R: GTGGTGCCATAGATGCGCTTG
Osteonectin	395bp	F: CTGCAGAAGAGATGGTGGCGG R: CAGGCAGGGGGCAATGTATTTG

Table 3.1 Details of rat pimer sequences for bone matrix markers used in PCR reactions. BSP, Bone sialoprotein; β-actin as housekeeping gene.

Temperature	Time	Number of cycles
95°C (Denaturing)	4 MINS	1
95°C	1 MIN	35
56°C (Annealing)	1 MIN	
72°C (Extension)	1 MIN	
72°C	10 MINS	1

Table 3.2 Details of PCR cycle including temperature, time and number of cycles

3.2 Results

3.2.1 Influence of DBBM on cell expansion

The MTS assay demonstrated no cell proliferation after 3 days when cells were cultured in the presence of Bio-Oss® granules or Bio-Oss® collagen. The ability for cells to survive in association with DBBM particles and the morphological features of cells in control wells are demonstrated in figure 3.1. Both Bio-Oss® granules and Bio-Oss® collagen appeared to inhibit cell growth in close proximity to the particles by Day 5. However, cells in the control well were viable at Day 5 (Figure 3.1). Subsequently total mRNA collection could not be performed.

3.2.2 Influence of DBBM extracts on cell expansion

The MTS assay results produced from cells cultured in the presence of DBBM extracts, produced by HCl, EDTA or $Ca(OH)_2$ extraction, are shown in Figure 3.2. The graphs demonstrate the variation in absorbance over time detected using the MTS assay, which is directly proportional to cell number. The cell numbers for all test media was higher than control media by Day 4 and this was maintained at Day 5. There was a 2.6 fold increase in mean cell number in the $Ca(OH)_2$ group compared to control at Day 4. The EDTA and HCl groups demonstrated 2.4 fold and an almost 2 fold increase in mean cell number over control respectively at Day 4. EDTA and HCl extracts continued to support increased cell expansion up to Day 5. The cell numbers decreased slightly between Day 4 and Day 5 in media supplemented with $Ca(OH)_2$ extracts. Importantly, between Day 2 and Day 4 there was approximately a 2 fold increase in mean cell number in the calcium hydroxide extract group and a 2.5

fold increase in mean cell number in the EDTA extract group. Although cell expansion was slower in HCl extract group, between Day 4 and Day 5 mean cell number increased by 2.9 fold. Overall, cell numbers in control media decreased steadily over the first 3 days, before increasing at Day 4 and 5. The differences in cell expansion were not statistically significant between cells cultured with EDTA, $Ca(OH)_2$ or HCl extracts, compared to control. This may be a reflection of low *n* number, however there were trends towards differences in cell expansion between groups.

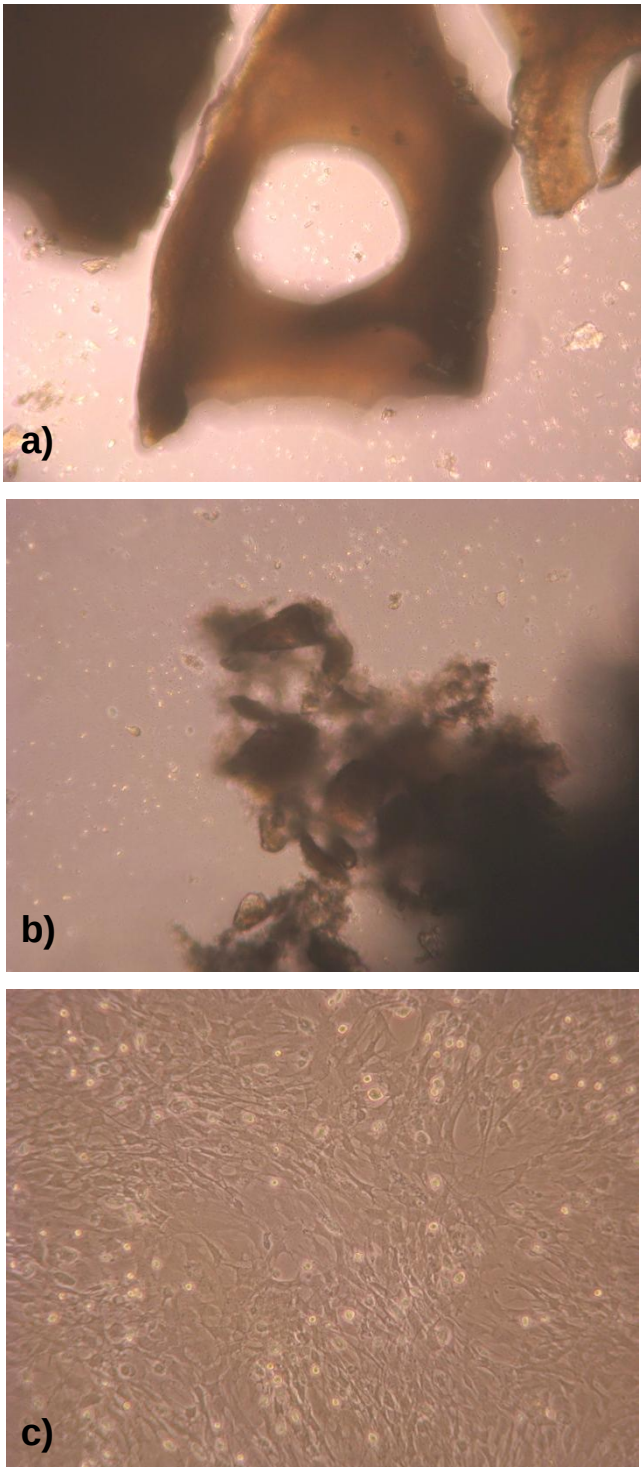
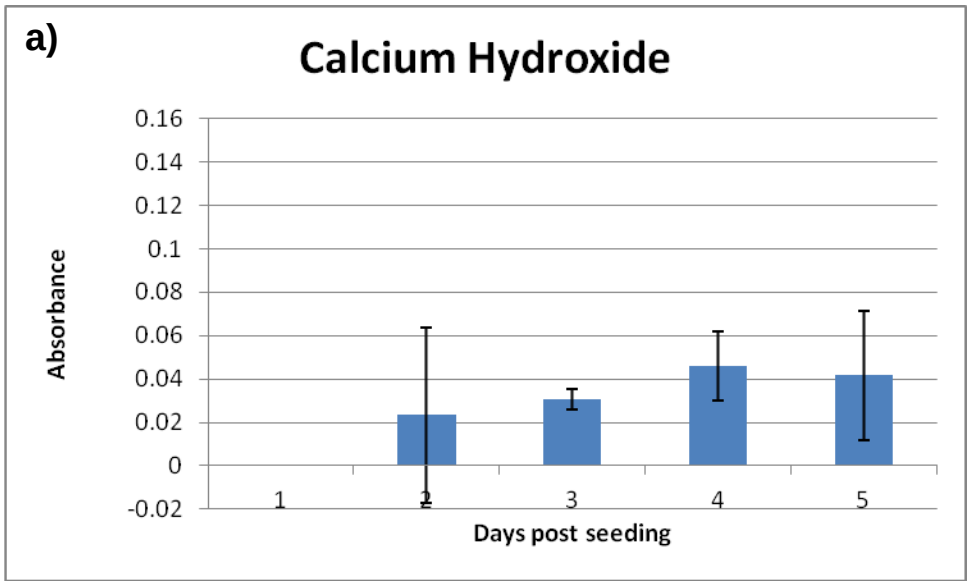


Figure 3.1 Cell growth in wells containg Bio-Oss® granules, Bio-Oss® collagen and control wells at Day 5 post seeding (Magnification x 10). a) Lack of cell growth around io-Oss® granules. b) Lack of cell growth around Bio-Oss® collagen. c) Morphological features of viable cells in control well



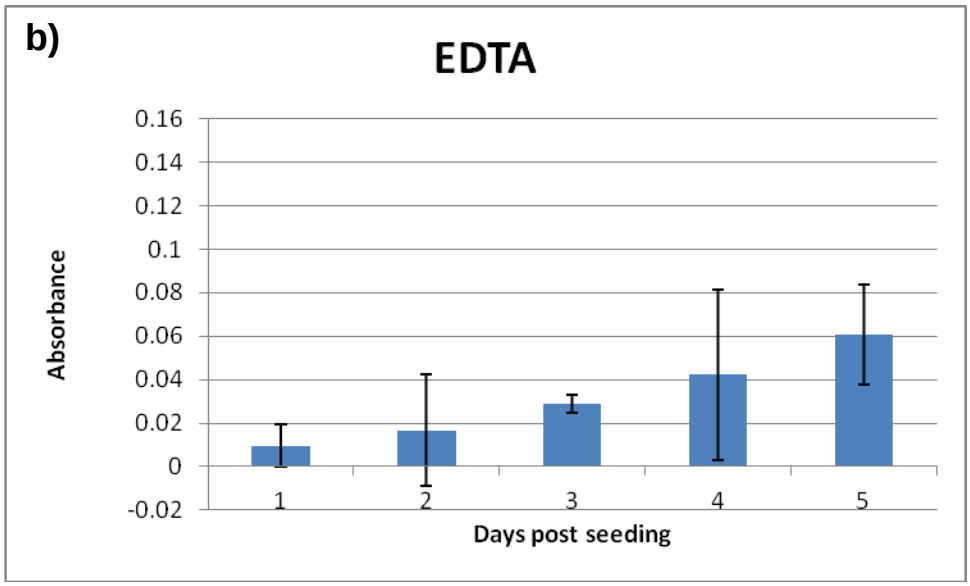
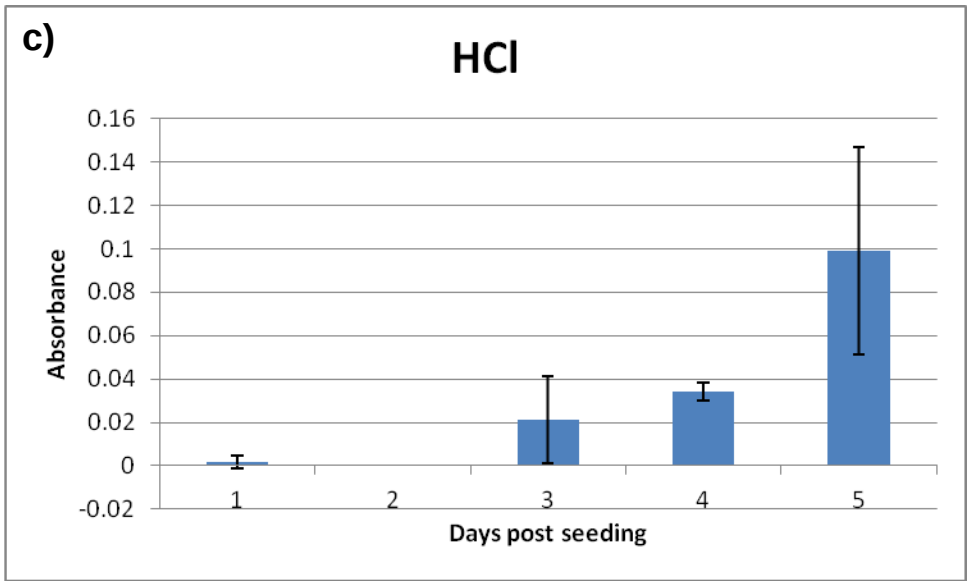


Figure 3.2a, b Effects of DBBM extracts on cell expansion. Mean MTS assay absorbance values following subtraction of absorbance reading from no cell control. Graphs demonstrate cell expansion over 5 days with media supplemented with DBBM extracts following treatment with a) $Ca(OH)_2$, b) EDTA. Error bars represent 1 standard deviation.



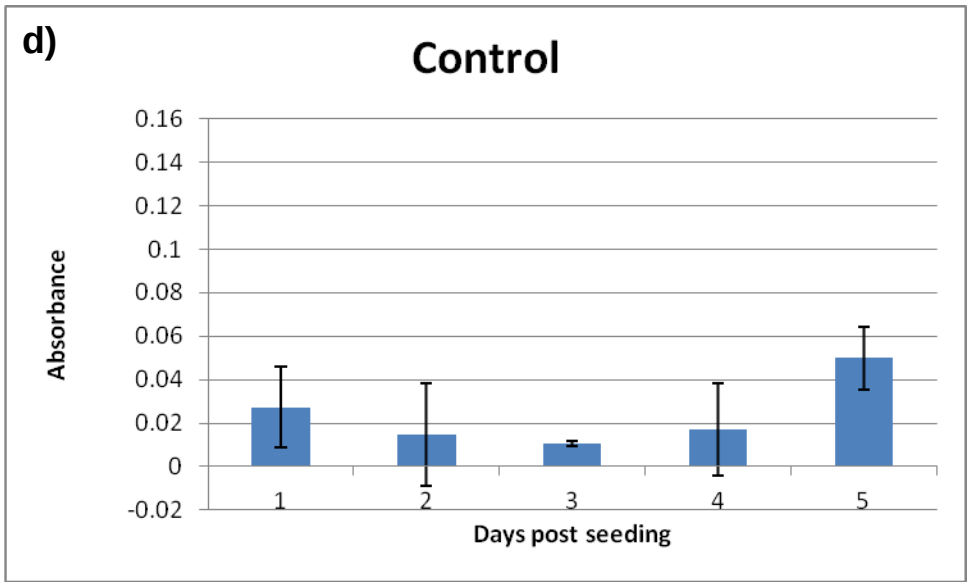


Figure 3.2c, d Effects of DBBM extracts on cell expansion. Mean MTS assay absorbance values following subtraction of absorbance reading from no cell control. Graphs demonstrate cell expansion over 5 days with media supplemented with DBBM extracts following treatment with c) HCl and d) control media. Error bars represent 1 standard deviation.

3.2.3 Influence of DBBM extracts on expression of bone matrix markers

Cells that were cultured in the presence of DBBM extraction components survived the experimental period and mRNA was successfully extracted. The temporal expression of bone matrix markers following culture of bone marrow stromal cells in the presence of DBBM extracted components, are shown in figure 3.3. All osteoblast markers were found to be expressed by BMSC cultured with all three supplemental media types and control media. Osteopontin and osteonectin were expressed intensly and consistently at all time points and in all four media groups during the culture period with little differences between groups. Within all supplemental media groups, BSP was expressed more intensly at Day 5, compared to control. At Day 9 and Day 12, BSP expression in the control media group had risen to that demonstrated in the three medias containing DBBM extracts. In contrast at Day 5, the band for osteocalcin was most intense in the BMSC's cultured with HCl supplimented media. In addition, all groups demonstrated osteocalcin expression at Day 9 and 12, although the banding was less intense as compared to other markers. Of note, at Day 12, the expression of osteocalcin in the supplimental media groups was less than control. Neither RT negative controls nor PCR negative controls demonstrated product formation.

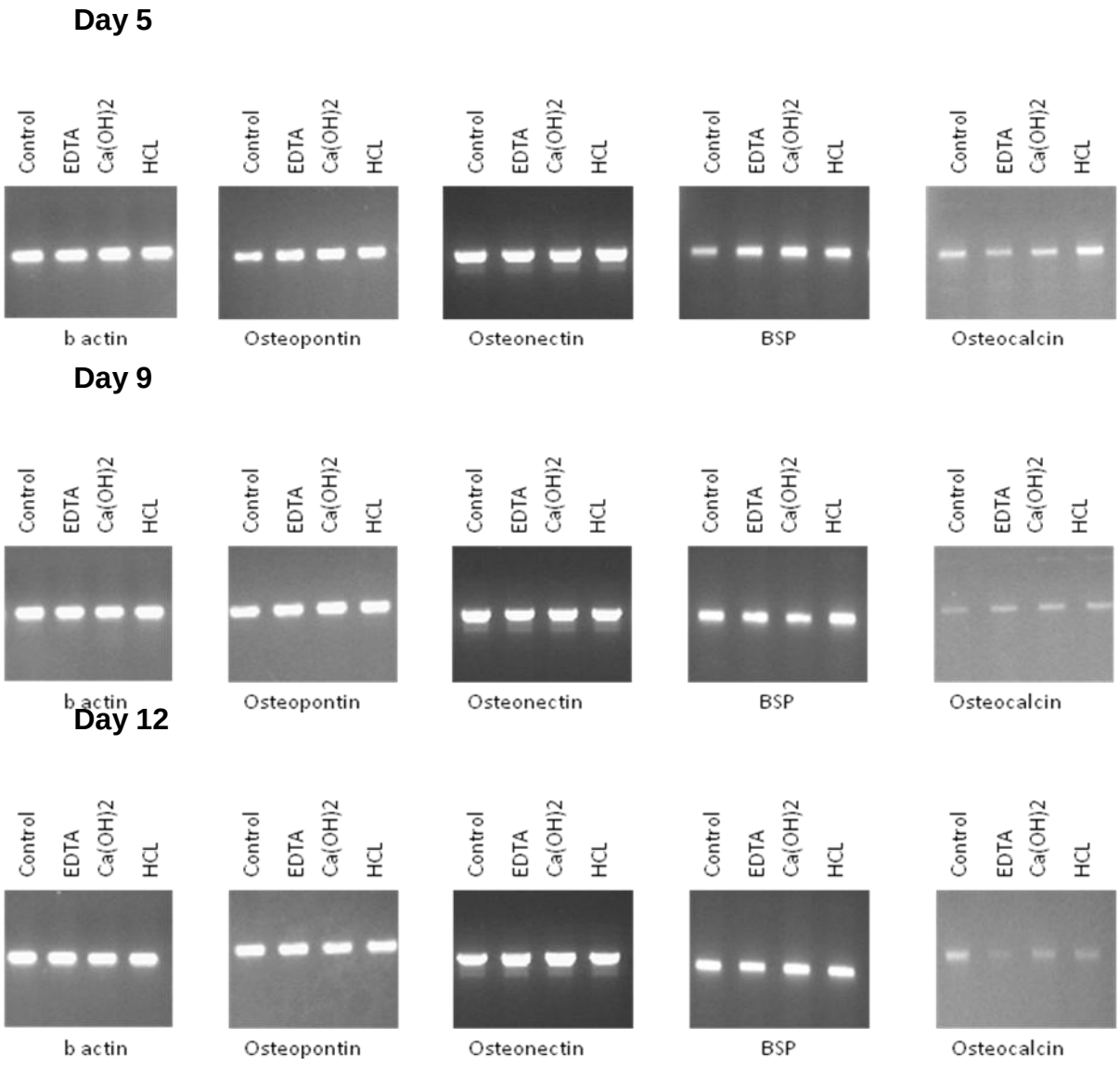


Figure 3.3 Effects of DBBM extracts on temporal gene expression. Typical RT-PCR result showing mRNA expression profiles for osteogenic markers of BMSC's cultured in the presence of DBBM extracts. β-actin was used as a housekeeping gene.

3.3 Discussion

The results within this chapter demonstrate trends towards increased proliferation of bone marrow stromal cells cultured with media supplimented with DBBM extracted components. Also, the results demonstrate this media may induce mRNA production and altered bone matrix gene expression profiles. In addition and importantly, the results suggest that BMSC are not capable of growing in close proximity to Bio-Oss® granules or the Bio-Oss® collagen block in this cell culture model.

The results presented suggest it was not possible to grow bone marrow stromal cells in close proximity to commercially available Bio-Oss® granules and Bio-Oss® collagen in this *in vitro* model. It has been accepted recently that surface characteristics of DBBM may have an impact on cell attachment and the behaviour of cells of the osteoblastic lineage (Ayobian-Markazi et al. 2012). In contrast to the present study, other researchers have successfully cultured human-derived osteoblasts directly on Bio-Oss® granules in a petri dish, although these cells were cultured longer for 6 – 28 days (Turhani et al. 2005; Amerio et al. 2010). Interestingly, in Turhani's study, cells from human mandibular blocks that were grown on Bio-Oss® granules were only able to form a monolayer by day 2,1 compared to the multilayers observed on a graft that they supplemented with a cell binding peptide (Pep Gen P-15™), suggesting less favourable cell growth. Other groups have also been able to demonstrate attachment of human osteoblasts and ultimately mature collagen production on DBBM in cell culture and the potential for tissue engineered growth of human bone (Açil et al. 2000). Interestingly, a very recent *in vitro* study that cultured primary human osteoblasts (SaOS-2 cells) on Bio-Oss® revealed good cell differentiation, as shown by ALP staining, but lower rates of cell proliferation and less favourable morphological features of the cell layer (Ayobian-Markazi et al. 2012). Early investigations performed with rat

osteoblasts, demonstrated attachment and proliferation of cells on DBBM, although observations only occurred for less than 48 hours (Stephan et al. 1999).

The difficulty in supporting cell growth in proximity to DBBM in the present study may be related to several factors that include calcium concentration. It is known that high calcium and phosphate concentrations suppress cell growth (Knabe et al. 2000; Maeno et al. 2005; Saldana et al. 2009). An *in vitro* study demonstrated that osteoblasts were unable to survive in a calcium hydroxide content of 2.5 mg ml^{-1} (Narita et al. 2010). It is possible that cells are unable to survive in close association with the high calcium concentration of the surface of DBBM in this *in vitro* situation. In addition, surface roughness of biomaterials has a dramatic effect on cellular attachment and migration. Importantly the ability to culture cells on any biomaterial is critically related to cell type, particularly if they are immortalised, and culture conditions (Ayobian-Markazi et al. 2012). Other studies have utilized mature cell lines such as mature osteoblasts which possess a high ability to adhere to surfaces and proliferate (Stephan et al. 1999; Petrovic et al. 2006; Amerio et al. 2010). In contrast, the cells used in the present study, were relatively immature and may therefore lack specific cell surface receptors required for effective attachment to the Bio-Oss® surface. Petrovic *et al* suggest that difficulty in culturing cells on biomaterials may be a function of the seeding density in tissue culture (Petrovic et al. 2006). In keeping with their study the seeding density in the present project is significanly lower than other groups demonstrating more favourable cell growth (Açil et al. 2000).

The results of this project support the findings of other groups that have assessed the potential osteoinductive effect of commercially available DBBM, when implanted into mouse calf muscle (Schwartz et al. 2000). This group failed to demonstrate new bone formation or inflammation associated with these particles suggesting inhibition of local cells in close proximity to the DBBM

substance. These findings are significant and support the observations that Bio-Oss® does not fully resorb, particlulalry if cells are unable to attach directly to DBBM. However, when Bio-Oss® is used at a more ususal site, such as the alveolar bone, it has been widely reported that new bone formation occurs upon the surface of DBBM particles (Norton et al. 2003; Tadjoedin et al. 2003). Following on from these observations, it is likely that in the *in vivo* situation, the fibrin clot rich in inflammatory molecules that surround Bio-Oss® granules during clinical use, acts as a matrix to support and sustain cell viability allowing the secretion of bone matrix proteins and mineralisation. Indeed, Schwartz group used demineralized freeze-dried bone allograft (DFDBA) as a carrier or matrix for extracted components of DBBM, produced by suspension in HCl, GuCl and EDTA. New bone formation was observed histologically adjacent to the DFDBA particles supplemented with extracts produced from DBBM. This data suggests that extracted components from DBBM released by acidic chemical treatment may have osteoinductive effects.

In this chapter, cell expansion was not observed in association with DBBM directly. This conflicts with evidence from Petrovic *et al* 2006 using mature human osteoblast like cells, (NHOst cells) which showed measureable but reduced proliferation and differentiation of cells grown on Bio-Oss® granules and Bio-Oss® collagen, compared to control. When the results of this study are examined closely, the proliferation of cells on the biomaterial, as measured by absorbance values, was actually negligible by the fifth day (Petrovic et al. 2006). In this chaper, assessment of cell expansion in the presence of DBBM extracted components, however, showed a trend towards faster proliferation than those cells cultured with control. This trend of increasing cell expansion over time occurred with calcium hydroxide, EDTA and HCl extracted components of Bio-Oss® granules. In particular, by Day 4, there was a 2.4 fold increase in mean cell number in wells cultured in the presence of EDTA extracted components over control, although this was not statistically significant. The reason for this increased proliferation could be the ability of chemical pre-

treatment to release growth factors locked within the mineral phase of the DBBM particles. Growth factors (e.g. TGF-β_1), in conjunction with proteoglycans (e.g. biglycan), are known to increase the proliferation of mesenchymal stem cells and cells of the osteoblast linage (Robey et al. 1987; Waddington et al. 2003). Indeed, the chemical pre-treatment of bone slabs has been able to extract growth factors from within the matrix (Smith et al. 2011). It is likely that *in vivo,* only small concentrations (at the nanogram level) are required, due to several growth factors acting together and the protection afforded by other proteins (Lee et al. 2011).

To investigate the gene expression of osteogenic markers in the presence of DBBM extracted components, RT-PCR was used. Reverse transcription polymerase chain reaction utilises the reverse transcriptase enzyme which can transcribe RNA into DNA. In this chapter, the gene expression profiles demonstrated through PCR were largely similar between the different supplimental media, suggesting that cellular activity was similar. All media types were, therefore, capable of supporting cells of an osteoblastic linage through the maintenance of osteonectin and osteopontin expression. It is worth noting that BSP had more expression in the DBBM supplemental media groups at Day 5, suggesting the formation of pre-ostoblasts and osteoblasts may occur more rapidly in the presence of DBBM extracted components. Since BSP is known to aid attachment of osteoblasts and act as a nucleator for bone mineral deposition, these effects could lead to faster mineralisation if translated to the *in vivo* situation (Mackie 2003; Hughes et al. 2006). It is noteworthy that in Amerio *et al* 2010 study with cells cultured directly on DBBM granules, BSP expression was shown to be reduced (Amerio et al. 2010). The altered gene expression in the experimental media groups, compared with control, could reflect the fact that proteins extracted from Bio-Oss® granules are osteoinductive and increase cellular activity. It was not possible to extract mRNA from cells seeded directly on DBBM and as mentioned these cells failed to survive. These results contrast with those of Amerio *et al* 2010, who demonstrated expression of mRNA for a

variety of osteogenic and inflammtory markers when human osteoblasts (NHOst cells) were cultured on Bio-Oss® granules (Amerio et al. 2010).

Taken together, the observations in this chapter suggest that the extracted components of Bio-Oss® granules support an osteoblastic phenotype and could influence bone marrow stromal cell behaviour, as demonstrated by an increased overall production of mRNA. In addition, earlier differentiation of cells down the osteoblastic linage is suggested by increased expression of bone sialoprotien. If the liberation of growth factors occurs *in vivo* this could result in osteoinduction and, for example, account for improved first bone to implant contact in the management of circumferental perimplant defects (Abushahba et al. 2008).

The inability of DBBM to directly support cell growth in this *in vitro* model may go some way to explain the clinical observations outlined previously. Bio-Oss® is able to maintain ridge width when used in extraction sockets, possibly a reflection of minimal resorption over short time periods due to a lack of direct cellular attachment (Nevins et al. 2006; Molly et al. 2008). If resorption of DBBM occurs at all, it appears to be a slow process taking many years particularly when used in sinus augmentation (Sartori et al. 2003). If DBBM in the form it is manufactured does not resorb, then what remains in grafted sites would be non-vital, non-active, mineralised bone. Certainly, from a clinical point of view, Bio-Oss® augmented sites demonstrate a different texture and density on surgical re-entry compared with native bone. However, it would seem from long-term studies and clinical experience, this has little if any clinical significance.

Chapter 4

General discussion

The management of periodontal diseases and the placement of dental implants require a biological approach to the resolution of bone defects associated with the periodontal or alveolar tissues. Bone substitute materials and specifically DBBM, in the form of Bio-Oss® granules and Bio-Oss® collagen, have been used extensively in periodontal and implant-related treatment modalities (von Arx and Buser 2006; Sculean et al. 2008). In order to be effective a bone substitute material, such as Bio-Oss®, must be at the very least osteoconductive and ideally, osteoinductive. To demonstrate osteoinductive behaviour, it is necessary to demonstrate bioactive proteins within DBBM particles, or the ability of these particles to bind growth factors released from the tissue bed during surgical trauma. Harnessing key molecules locked with the mineralised matrix of DBBM could facilitate bone regeneration. The objective of this research was to investigate the ability of chemical pre-treatment to release residual protein in the form of growth factors within DBBM and investigate if this protein is bioactive such that it alters the behaviour of cells in an *in vitro* tissue culture model.

4.1 Growth factor release

Conflicting existing evidence has been presented with regard to the protein content of Bio-Oss® granules, based on a variety of experimental techniques. The results in chapter 2 suggest that Bio-Oss® granules are highly likely to contain residual protein within its matrix demonstrated by silver staining of gels produced by SDS PAGE. Potentially this protein is in the form of growth factors and the Western blot results suggest the potential for TGF-β_1 to be part of the protein complex. It is clear that the concentration of this protein is small and can therefore not be detected via BCA assay or other relatively insensitive techniques, such as Ponceau stain. The results of electroblotting also suggest TGF-β_1 may be present associated with the porcine collagen that is added to DBBM to form the Bio-Oss® Collagen block. The ability of EDTA, $Ca(OH)_2$ and

HCl to release these proteins from close association with the mineral phase has been demonstrated in chapter 2. These investigations provide further evidence that residual protein remains in DBBM, despite the manufacturing process and can be liberated by the application of $Ca(OH)_2$, EDTA and HCl. This detection of protein corroborates the findings of other groups (Schwartz et al. 2000). Specifically, antibody staining using western blot was positive using antibodies for TGF-β_1, although this did not occur at the expected molecular weight. The inability to identify protein at high concentration with BCA assay is consistent with the work of other groups investigating the protein content of DBBM. Schwartz *et al* 2000 suggested a small amount of protein was present associated with the mineral phase; however, Benke *et al* 2001 stated that protein was not present at a detectable level and questioned the methodology of Schwartz (Benke et al. 2001). The ability of acidic chelating agents and $Ca(OH)_2$ to augment the release of TGF-β_1 from mineralized matrices has been confirmed recently in an *in vitro* model (Smith et al. 2011). The present study provides pilot data which are consistent with the findings of Smith *et al* 2011, in terms of the mineralised matrix of a xenographic bone graft, namely DBBM. In the case of Bio-Oss® collagen, it is likely that growth factors are sequestered with proteoglycans, such as biglycan and decorin, associated with the collagen fibrils. Biglycan and decorin may protect TGF-β_1 within the matrix or be involved in its presentation to the TGF-β_1 receptor (Takeuchi et al. 1994; Baker et al. 2009).

The present study may have clinical implications given the wide range of functions that growth factors, such as TGF-β_1, possess. The TGF-β superfamily possess several important functions in bone healing and remodelling that include; the regulation of cell growth , chemotaxis, osteoblast cell growth, migration and differentiation, deposition of matrix and mineralization (Pfeilschifter et al. 1990; Giannobile 1996; Grande 1997; Binkert et al. 1999; Hallman and Thor 2008). It also stimulates the production of type 1 collagen, osteopontin and osteonectin, which are important in bone healing (Giannobile

1996; Cochran and Wozney 1999; Dimitriou et al. 2005). Although this project focussed on the identification of TGF-β_1, it is unlikely that this growth factor acts alone on the osteoblast cell lineage. As outlined in the first chapter, the interplay of the ideal cocktail of growth factors, including bone morphogenic proteins (BMP's), platelet derived growth factor (PDGF), Insulin-like growth factor (IGF) and fibroblast growth factor FGF is necessary to positively impact on bone formation. The potential of DBBM to contain TGF-β_1 and other growth factors, would help explain the bone regeneration seen with its use clinically.

4.2 Cell behaviour

Following the confirmation of residual protein in DBBM, a series of cell culture experiments were performed to enable investigation of differences in cell behaviour in response to Bio-Oss® granules, Bio-Oss® collagen or DBBM extracted components. It is noteworthy that in order to regenerate any tissue type, three key elements are required. These are a supply of stem cells, a biocompatible scaffold, and the presence of appropriate growth factors as signalling molecules to control the regenerative process (Petrovic et al. 2006). Although a number of osteoconductive scaffolds are in clinical use in dentistry as an alternative to autogenous bone, it is desirable for graft materials to be osteoinductive. This would enable the release of intrinsic growth factors or the ability to bind locally released growth factors upon its surface that would drive bone regeneration by committing undifferentiated mesenchymal cells down an osteoblastic lineage.

The results shown in chapter 3 demonstrated increased cell expansion and increased gene expression of BSP by rat derived BMSC when cultured under osteogenic differentiation conditions. Specifically, cells were cultured with media supplemented by the extraction components released from DBBM following chemical pre-treatment. The MTS assay suggested increased cell numbers in association with all DBBM extracted media over control. This

suggests that once solubilised by chemical pre-treatment with EDTA, $Ca(OH)_2$ or HCl, residual proteins within DBBM are able to support cell proliferation and growth. Later, the increased concentration of mRNA transcribed would be used for protein translation and this data suggests that the components of DBBM have a positive osteoinductive effect on bone regeneration. The osteoblastic phenotype was confirmed via RT-PCR with the continued expression of osteopontin, osteonectin and osteocalcin throughout the culture period. The specific bone matrix marker BSP demonstrated earlier temporal expression when cells were cultured with media supplimented with DBBM extracts suggesting more rapid osteoblastic differentiation.

In this study it was observed that cells were unable to survive in media in close proximity to DBBM particles, even though the direct growth of cells upon DBBM itself this has been demonstrated by other authors (Açil et al. 2000; Amerio et al. 2010). The osteoinductive effect of Bio-Oss® was suggested elsewhere (Schwartz et al. 2000). This group concluded Bio-Oss® may support osteoblast attachment and proliferation, such that growth factors on or near the xenograft influence osteogenesis. Further, the release of osteoinductive factors from the matrix of DBBM may be tissue specific. These contrasting results to the present study clearly show the sensitivity of cell culture techniques. Monolayer cell cultures, as used in the present study, consistently exhibit osteogenic potential when stem cells are cultured in the presence of dexamethasone, ascorbic acid and β-glycerophosphate (Karner et al. 2007). Cell culture studies on other materials demonstrate that the cell response to a biomaterial may depend on many factors including cell types used, duration of study, freshness of material, frequency of media changes, direct contact of cells on materials or extraction components of materials and the concentration of material used (Bonson et al. 2004; Hakki et al. 2009; Torabinejad and Parirokh 2010). Nevertheless, in *in vivo* studies, it is repeatedly shown that cells have the ability to grow and form new bone on the surface of DBBM granules histologically (Norton et al. 2003). This may reveal the importance that bleeding, the inflammatory response and

extracellular components have to provide a matrix for osteoblasts to migrate through to reach the bone graft surface prior to bone formation.

4.3 Future research

There are several potential avenues with which to progress this research further to identify specific growth factors and confirm bioactivity. Future studies are envisaged which include electroblotting to identify other growth factors in addition to TGF-β_1, since the combination of growth factors are likely to be important. Although this study demonstrated mRNA synthesis and expression of osteogenic markers via PCR, their expression by cells could be confirmed in future at protein level, via Western blot or immunocytochemistry.

Mass spectrometry could be utilized to identify the composition of the stained bands identified with silver stained gels. Although some preliminary work has been carried out by the author, it was not possible to complete this within the time constraints of the research programme.

Further, animal models could be utilized to compare chemically pre-treated Bio-Oss® and commercial Bio-Oss® in bone defects. Although supplementation of DBBM with recombinant growth factors has been performed by other groups, to the authors knowledge, this has not yet been performed with chemically pre-treated DBBM. It is noteworthy that the application of growth factors, such as recombinant BMP and scaffolds (e.g collagen), can only be effective if the carrier and delivery sytem is appropriate, the growth factor is delivered at an appropriate dose and the tissue dynamics of the recipients site are favourable (Lieberman et al. 2002). If animal studies showed promise, the clinical pre-treatment of Bio-Oss® particles with either calcium hydroxide or EDTA, prior to placement in extraction sockets or at the time of GTR procedures, may release proteins locked within the DBBM matrix. This may result in faster bone regeneration in these clinical scenarios. Future studies are envisaged that will

investigate whether, as an alternative approach, chemical pre-treatment of the host bone surface itself will enhance growth factor release that may improve regeneration in concert with Bio-Oss®, that acts as a scaffold.

The aformentioned future strategies aim to provide the biological justification for optimisation of current treatment protocols associated with implant placement or the management of periodontal defects. In terms of implant dentistry, more predictable bone augmentation procedures using this xenograft will reduce the need for autogenous bone. This is a valid goal since autogenous bone harvesting is often more complex, associated with more morbidity and is usually more demanding of healthcare resources, which are already under significant pressure. Alternative approaches being investigated by others in this research field include the abandonment of grafts altogether, by altering the behaviour of host cells directly. For example, gene therapy using viral vectors to tranfer genetic material directly into cells can upregulate specific protein production by host cells (Lieberman et al. 2002).

4.4 Conclusions

This project has demonstrated that $Ca(OH)_2$, EDTA and HCl can extract residual protein from Bio-Oss®. The results of the present study indicate that DBBM in the form of both Bio-Oss® granules and Bio-Oss® collagen, contain protein that may be in the form of TGF-β_1. The results from cell culture demonstrate that chemical pre-treatment of Bio-Oss® alters mRNA production and gene expression of osteoprogenitor cells, specifically BSP.

In conclusion, these data suggest that the biocompatibility and bone regeneration associated with Bio-Oss® clinically may be related to residual protein, in the form of growth factors within the particulate bone material.

References

Abushahba, F. *et al*. 2008. Effect of grafting materials on osseointegration of dental implants surrounded by circumferential bone defects. An experimental study in the dog. *Clinical Oral Implants Research* 19(4), pp. 329-334.

Açil, Y. *et al.* 2000. Three-dimensional cultivation of human osteoblast-like cells on highly porous natural bone mineral. *Journal of Biomedical Materials Research* 51(4), pp. 703-710.

Adell, R. *et al.* 1981. A 15-year study of osseointegrated implants in the treatment of the edentulous jaw. *International Journal of Oral Surgery* 10(6), pp. 387-416.

Aghaloo, T. L. and Moy, P. K. 2007. Which hard tissue augmentation techniques are the most successful in furnishing bony support for implant placement? *The International Journal of Oral & Maxillofacial Implants* 22 Suppl, pp. 49-70.

Albrektsson, T. and Johansson, C. 2001. Osteoinduction, osteoconduction and osseointegration. *European spine journal : official publication of the European Spine Society, the European Spinal Deformity Society, and the European Section of the Cervical Spine Research Society* 10 Suppl 2, pp. S96-101.

Albrektsson, T. *et al.* 1986. The long-term efficacy of currently used dental implants: a review and proposed criteria of success. *The International Journal of Oral & Maxillofacial Implants* 1(1), pp. 11-25.

Alsaadi, G. *et al.* 2007. Impact of local and systemic factors on the incidence of oral implant failures, up to abutment connection. *Journal of Clinical Periodontology* 34(7), pp. 610-617.

Amerio, P. *et al.* 2010. The effect of deproteinized bovine bone on osteoblast growth factors and proinflammatory cytokine production. *Clinical Oral Implants Research* 21(6), pp. 650-655.

Antoun, H. 2007. Bone substitutes. In: Khoury, F. ed. *Bone augmentation in oral implantology*. London: Quintessence, pp. 341 - 372.

Araujo, M. *et al.* 2009. Effect of a xenograft on early bone formation in extraction sockets: an experimental study in dog. *Clinical Oral Implants Research* 20(1), pp. 1-6.

Araújo, M. G. and Lindhe, J. 2005. Dimensional ridge alterations following tooth extraction. An experimental study in the dog. *Journal of Clinical Periodontology* 32(2), pp. 212-218.

Armitage, G. C. 1999. Development of a classification system for periodontal diseases and conditions. *Annals of periodontology / the American Academy of Periodontology* 4(1), pp. 1-6.

Ayobian-Markazi, N. *et al.* 2012. Comparison of cell viability and morphology of a human osteoblast-like cell line (SaOS-2) seeded on various bone substitute materials: An *in vitro* study. *Dental Research Journal* 9(1), pp. 86-92.

Baker, S. M. *et al.* 2009. TGF-β/extracellular matrix interactions in dentin matrix: a role in regulating sequestration and protection of bioactivity. *Calcified Tissue International* 85(1), pp. 66-74.

Benke, D. *et al.* 2001. Protein-chemical analysis of Bio-Oss bone substitute and evidence on its carbonate content. *Biomaterials* 22(9), pp. 1005-1012.

Berglundh, T. *et al.* 2003. De novo alveolar bone formation adjacent to endosseous implants. *Clinical Oral Implants Research* 14(3), pp. 251-262.

Berglundh, T. and Lindhe, J. 1997. Healing around implants placed in bone defects treated with Bio-Oss. An experimental study in the dog. *Clinical Oral Implants Research* 8(2), pp. 117-124.

Bessade, J. *et al.* 2007. Growth factors and bone morphogenic proteins. In: Khoury, F. ed. *Bone augmentation in oral implantology*. London: Quintessence, pp. 373 - 389.

Binkert, C. *et al.* 1999. Regulation of osteogenesis by fetuin. *The Journal of Biological Chemistry* 274(40), pp. 28514-28520.

Block, M. S. and Kent, J. N. 1997. Sinus augmentation for dental implants: the use of autogenous bone. *Journal of Oral and Maxillofacial Surgery : official journal of the American Association of Oral and Maxillofacial Surgeons* 55(11), pp. 1281-1286.

Boch, J. A. *et al.* 2001. Interleukin 1 signal transduction--current concepts and relevance to periodontitis. *Journal of Dental Research* 80(2), pp. 400-407.

Bonson, S. *et al.* 2004. Root-end filling materials alter fibroblast differentiation. *Journal of Dental Research* 83(5), pp. 408-413.

Bosshardt. 2009. Biological basis of bone regeneration. In: Buser, D. ed. *20 years of Guided Bone Regeneration in Implant Dentistry*. 2nd ed. Hanover Park, IL: Quintessence Pub. Co., pp. 15 - 45.

Boyle, W. J. *et al*. 2003. Osteoclast differentiation and activation. *Nature* 423(6937), pp. 337-342.

Boyne, P. J. and James, R. A. 1980. Grafting of the maxillary sinus floor with autogenous marrow and bone. *Journal of Oral Surgery* 38(8), pp. 613-616.

Bradshaw, A. D. and Sage, E. H. 2001. SPARC, a matricellular protein that functions in cellular differentiation and tissue response to injury. *The Journal of Clinical Investigation* 107(9), pp. 1049-1054.

Branemark, P. I. 1983. Osseointegration and its experimental background. *The Journal of Prosthetic Dentistry* 50(3), pp. 399-410.

Bruder, S. P. *et al.* 1994. Mesenchymal stem cells in bone development, bone repair, and skeletal regenaration therapy. *Journal of Cellular Biochemistry* 56(3), pp. 283-294.

Buser, D. *et al.* 2004. Optimizing esthetics for implant restorations in the anterior maxilla: anatomic and surgical considerations. *International Journal of Oral Maxillofacial Implants* 19 Suppl, pp. 43-61.

Buser, D. *et al.* 1997. Long-term evaluation of non-submerged ITI implants. Part 1: 8-year life table analysis of a prospective multi-center study with 2359 implants. *Clinical Oral Implants Research* 8(3), pp. 161-172.

Bystrom, A. and Sundqvist, G. 1985. The antibacterial action of sodium hypochlorite and EDTA in 60 cases of endodontic therapy. *International Endodontic Journal* 18(1), pp. 35-40.

Chen, D. *et al.* 2004. Bone morphogenetic proteins. *Growth Factors* 22(4), pp. 233-241.

Cheng, H. *et al.* 2003. Osteogenic activity of the fourteen types of human bone morphogenetic proteins (BMPs). *The Journal of Bone and Joint Surgery. American volume* 85-A(8), pp. 1544-1552.

Chiapasco, M. *et al.* 2007. Autogenous onlay bone grafts vs. alveolar distraction osteogenesis for the correction of vertically deficient edentulous ridges: a 2-4-year prospective study on humans. *Clinical Oral Implants Research* 18(4), pp. 432-440.

Cochran, D. L. and Wozney, J. M. 1999. Biological mediators for periodontal regeneration. *Periodontology 2000* 19, pp. 40-58.

Colombo, J. S. *et al.* 2011. Delayed osteoblast differentiation and altered inflammatory response around implants placed in incisor sockets of type 2 diabetic rats. *Clinical Oral Implants Research* 22(6), pp. 578-586.

Cordaro, L. *et al.* 2008. Maxillary sinus grafting with Bio-Oss or Straumann Bone Ceramic: histomorphometric results from a randomized controlled multicenter clinical trial. *Clinical Oral Implants Research* 19(8), pp. 796-803.

Cortellini, P. and Tonetti, M. S. 2000. Focus on intrabony defects: guided tissue regeneration. *Periodontology 2000* 22, pp. 104-132.

Davies, J. E. 2003. Understanding peri-implant endosseous healing. *Journal of Dental Education* 67(8), pp. 932-949.

Dimitriou, R. *et al.* 2005. Current concepts of molecular aspects of bone healing. *Injury* 36(12), pp. 1392-1404.

Ducy, P. *et al.* 1996. Increased bone formation in osteocalcin-deficient mice. *Nature* 382(6590), pp. 448-452.

Esposito, M. *et al.* 2010. Interventions for replacing missing teeth: augmentation procedures of the maxillary sinus. *Cochrane Database of Systematic Reviews* (3), p. CD008397.

Esposito, M. *et al.* 1998. Biological factors contributing to failures of osseointegrated oral implants. (I). Success criteria and epidemiology. *European Journal of Oral Sciences* 106(1), pp. 527-551.

European Society of Endodontology. 2006. Quality guidelines for endodontic treatment: consensus report of the European Society of Endodontology. *International EndodonticJjournal* 39(12), pp. 921-930.

Franceschi, R. T. 1999. The Developmental control of osteoblast-specific gene expression: role of specific transcription factors and the extracellular matrix environment. *Critical Reviews in Oral Biology & Medicine* 10(1), pp. 40-57.

Friberg, B. *et al.* 2002. Clinical outcome of Branemark System implants of various diameters: a retrospective study. *Int J Oral Maxillofac Implants* 17(5), pp. 671-677.

Geurs, N. C. 2007. Osteoporosis and periodontal disease. *Periodontology 2000* 44(1), pp. 29-43.

Giannobile, W. V. 1996. Periodontal tissue engineering by growth factors. *Bone* 19(1 Suppl), pp. 23S-37S.

Giannobile, W. V. 2008. Bone as a tissue. In: Lindhe, J. ed. *Clinical Periodontology and Implant Dentistry*. 5th ed., Vol. 1. Oxford, UK ; Ames, Iowa: Blackwell Munksgaard, pp. 86 - 98.

Giannobile, W. V. and Somerman, M. J. 2003. Growth and amelogenin-like factors in periodontal wound healing. A systematic review. *Annals of Periodontology / the American Academy of Periodontology* 8(1), pp. 193-204.

Glaser, D. L. and Kaplan, F. S. 1997. Osteoporosis. Definition and clinical presentation. *Spine (Phila Pa 1976)* 22(24 Suppl), pp. 12S-16S.

Graham, L. *et al.* 2006. The effect of calcium hydroxide on solubilisation of bio-active dentine matrix components. *Biomaterials* 27(14), pp. 2865-2873.

Grande, J. P. 1997. Role of transforming growth factor-β in tissue injury and repair. *Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine* 214(1), pp. 27-40.

Grutter, L. and Belser, U. C. 2009. Implant loading protocols for the partially edentulous esthetic zone. *The International Journal of Oral & Maxillofacial Implants* 24 Suppl, pp. 169-179.

Hakki, S. S. *et al.* 2009. Effects of mineral trioxide aggregate on cell survival, gene expression associated with mineralized tissues, and biomineralization of cementoblasts. *Journal of Endodontics* 35(4), pp. 513-519.

Hallman, M. and Thor, A. 2008. Bone substitutes and growth factors as an alternative/complement to autogenous bone for grafting in implant dentistry. *Periodontology 2000* 47, pp. 172-192.

Hammerle, C. H. *et al.* 1998. The effect of a deproteinized bovine bone mineral on bone regeneration around titanium dental implants. *Clinical Oral Implants Research* 9(3), pp. 151-162.

Hammerle, C. H. *et al.* 2002. A systematic review of the survival of implants in bone sites augmented with barrier membranes (guided bone regeneration) in partially edentulous patients. *Journal of Clinical Periodontology* 29 Suppl 3, pp. 226-231; discussion 232-223.

Hammerle, C. H. *et al.* 2008. Ridge augmentation by applying bioresorbable membranes and deproteinized bovine bone mineral: a report of twelve consecutive cases. *Clinical Oral Implants Research* 19(1), pp. 19-25.

Heberer, S. *et al.* 2008. Histomorphometric analysis of extraction sockets augmented with Bio-Oss Collagen after a 6-week healing period: a prospective study. *Clinical Oral Implants Research* 19(12), pp. 1219-1225.

Honig, J. F. *et al.* 1999. Risk of transmission of agents associated with Creutzfeldt-Jakob disease and bovine spongiform encephalopathy. *Plastic and Reconstructive Surgery* 103(4), pp. 1324-1325.

Horowitz, M. C. *et al.* 2001. Control of osteoclastogenesis and bone resorption by members of the TNF family of receptors and ligands. *Cytokine and Growth Factor Reviews* 12(1), pp. 9-18.

Horsted-Bindslev, P. *et al.* 2003. Direct capping of human pulps with a dentin bonding system or with calcium hydroxide cement. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics* 96(5), pp. 591-600.

Huang, G. T. 2009. Apexification: the beginning of its end. *International Endodontic Journal* 42(10), pp. 855-866.

Hughes, F. J. *et al.* 2006. Effects of growth factors and cytokines on osteoblast differentiation. *Periodontology 2000* 41(1), pp. 48-72.

Hulsmann, M. *et al.* 2003. Chelating agents in root canal treatment: mode of action and indications for their use. *International Endodontic Journal* 36(12), pp. 810-830.

Hurzeler, M. B. *et al.* 1996. Reconstruction of the severely resorbed maxilla with dental implants in the augmented maxillary sinus: a 5-year clinical investigation. *The International Journal of Oral & Maxillofacial Implants* 11(4), pp. 466-475.

Jaffin, R. A. and Berman, C. L. 1991. The excessive loss of Branemark fixtures in type IV bone: a 5-year analysis. *J Periodontol* 62(1), pp. 2-4.

Jung, R. E. *et al.* 2008a. A systematic review of the 5-year survival and complication rates of implant-supported single crowns. *Clinical Oral Implants Research* 19(2), pp. 119-130.

Jung, R. E. *et al.* 2008b. Assessment of the potential of growth factors for localized alveolar ridge augmentation: a systematic review. *Journal of Clinical Periodontology* 35(8 Suppl), pp. 255-281.

Karner, E. *et al.* 2007. Bone matrix formation in osteogenic cultures derived from human embryonic stem cells *in vitro*. *Stem Cells and Development* 16(1), pp. 39-52.

Karoussis, I. K. *et al.* 2004. Effect of implant design on survival and success rates of titanium oral implants: a 10-year prospective cohort study of the ITI Dental Implant System. *Clinical Oral Implants Research* 15(1), pp. 8-17.

Karoussis, I. K. *et al.* 2007. A comprehensive and critical review of dental implant prognosis in periodontally compromised partially edentulous patients. *Clinical Oral Implants Research* 18(6), pp. 669-679.

Katagiri, T. and Takahashi, N. 2002. Regulatory mechanisms of osteoblast and osteoclast differentiation. *Oral Diseases* 8(3), pp. 147-159.

Kinane, D. F. 2008. Pathogenesis of periodontitis. In: Lindhe, J. ed. *Clinical periodontology and implant dentistry*. 5th ed. Oxford, UK ; Ames, Iowa: Blackwell Munksgaard, pp. 285-299.

Kinane, D. F. *et al.* 2006. Environmental and other modifying factors of the periodontal diseases. *Periodontology 2000* 40(1), pp. 107-119.

Knabe, C. *et al.* 2000. Evaluation of calcium phosphates and experimental calcium phosphate bone cements using osteogenic cultures. *Journal of Biomedical Materials Research* 52(3), pp. 498-508.

Kotsovilis, S. *et al.* 2006. A comprehensive and critical review of dental implant placement in diabetic animals and patients. *Clinical Oral Implants Research* 17(5), pp. 587-599.

Kuhn, N. Z. and Tuan, R. S. 2010. Regulation of stemness and stem cell niche of mesenchymal stem cells: Implications in tumorigenesis and metastasis. *Journal of Cellular Physiology* 222(2), pp. 268-277.

Lan, J. *et al.* 2006. The effect of combination of recombinant human bone morphogenetic protein-2 and basic fibroblast growth factor or insulin-like growth factor-I on dental implant osseointegration by confocal laser scanning microscopy. *Journal of Periodontology* 77(3), pp. 357-363.

Lee, K. *et al.* 2011. Growth factor delivery-based tissue engineering: general approaches and a review of recent developments. *Journal of the Royal Society, Interface / the Royal Society* 8(55), pp. 153-170.

Lekholm, U. and Zarb, A. 1985. Patient selection and preparation. In: Branemark, P.I. ed. *Tissue-integrated prostheses : osseointegration in clinical dentistry*. Chicago: Quintessence, pp. 199-210.

Lieberman, J. R. *et al.* 2002. The role of growth factors in the repair of bone. Biology and clinical applications. *The Journal of Bone and Joint Surgery. American volume* 84-A(6), pp. 1032-1044.

Lin, L. *et al.* 2010. Guided tissue regeneration in periapical surgery. *Journal of Endodontics* 36(4), pp. 618-625.

Lindh, T. *et al.* 1998. A meta-analysis of implants in partial edentulism. *Clinical Oral Implants Research* 9(2), pp. 80-90.

Lindhe, J. and Meyle, J. 2008. Peri-implant diseases: Consensus Report of the Sixth European Workshop on Periodontology. *Journal of Clinical Periodontology* 35(8 Suppl), pp. 282-285.

Löe, H. *et al.* 1986. Natural history of periodontal disease in man. Rapid, moderate and no loss of attachment in Sri Lankan laborers 14 to 46 years of age. *Journal of Clinical Periodontology* 13(5), pp. 431-445.

Löe, H. *et al.* 1965. Experimental gingivitis in man. *The Journal of Periodontology* 36, pp. 177-187.

Loos, B. G. *et al.* 2005. Identification of genetic risk factors for periodontitis and possible mechanisms of action. *Journal of Clinical Periodontology* 32, pp. 159-179.

Lu, H. *et al.* 2003. Diabetes interferes with the bone formation by affecting the expression of transcription factors that regulate osteoblast differentiation. *Endocrinology* 144(1), pp. 346-352.

Lynch, S. E. *et al.* 1991. The effects of short-term application of a combination of platelet-derived and insulin-like growth factors on periodontal wound healing. *Journal of Periodontology* 62(7), pp. 458-467.

Mackie, E. J. 2003. Osteoblasts: novel roles in orchestration of skeletal architecture. *The International Journal of Biochemistry and Cell Biology* 35(9), pp. 1301-1305.

Madrid, C. and Sanz, M. 2009. What impact do systemically administrated bisphosphonates have on oral implant therapy? A systematic review. *Clinical Oral Implants Research* 20, pp. 87-95.

Maeda, S. *et al.* 2004. Endogenous TGF-β signaling suppresses maturation of osteoblastic mesenchymal cells. *The EMBO journal* 23(3), pp. 552-563.

Maeno, S. *et al.* 2005. The effect of calcium ion concentration on osteoblast viability, proliferation and differentiation in monolayer and 3D culture. *Biomaterials* 26(23), pp. 4847-4855.

Malhotra, N. *et al*. 2009. Current strategies and applications of tissue engineering in dentistry--a review part 1. *Dental Update* 36(9), pp. 577-579, 581-572.

Mattout, P. 2007. Pre- and peri-implant guided bone regeneration. In: Khoury, F. ed. *Bone augmentation in oral implantology*. London: Quintessence, pp. 299 - 320.

McCauley, L. K. and Nohutcu, R. M. 2002. Mediators of periodontal osseous destruction and remodeling: principles and implications for diagnosis and therapy. *Journal of Periodontology* 73(11), pp. 1377-1391.

Mohammadi, Z. and Dummer, P. M. 2011. Properties and applications of calcium hydroxide in endodontics and dental traumatology. *International Endodontic Journal* 44(8), pp. 697-730.

Molly, L. *et al.* 2008. Bone formation following implantation of bone biomaterials into extraction sites. *Journal of Periodontology* 79(6), pp. 1108-1115.

Moy, P. K. *et al.* 2005. Dental implant failure rates and associated risk factors. *Int J Oral Maxillofac Implants* 20(4), pp. 569-577.

Nanci, A. 1999. Content and distribution of noncollagenous matrix proteins in bone and cementum: relationship to speed of formation and collagen packing density. *Journal of Structural Biology* 126(3), pp. 256-269.

Narita, H. *et al.* 2010. An explanation of the mineralization mechanism in osteoblasts induced by calcium hydroxide. *Acta Biomaterialia* 6(2), pp. 586-590.

Needleman, I. *et al*. 2005. Guided tissue regeneration for periodontal intrabony defects - a Cochrane Systematic Review. *Periodontology 2000* 37, pp. 106-123.

Nefussi, J. 2007. Biology and physiology of the implant bone site. In: Khoury, F. ed. *Bone augmentation in oral implantology*. London: Quintessence, pp. 1 - 27.

Nevins, M. *et al.* 2006. A study of the fate of the buccal wall of extraction sockets of teeth with prominent roots. *The International Journal of Periodontics & Restorative Dentistry* 26(1), pp. 19-29.

Nevins, M. L. *et al.* 2009. Human histologic evaluation of mineralized collagen bone substitute and recombinant platelet-derived growth factor-BB to create bone for implant placement in extraction socket defects at 4 and 6 months: a case series. *The International Journal of Periodontics & Restorative Dentistry* 29(2), pp. 129-139.

Ng, Y. L. *et al.* 2011. A prospective study of the factors affecting outcomes of nonsurgical root canal treatment: part 1: periapical health. *International Endodontic Journal* 44(7), pp. 583-609.

Norton, M. R. *et al.* 2003. Efficacy of bovine bone mineral for alveolar augmentation: a human histologic study. *Clinical Oral Implants Research* 14(6), pp. 775-783.

Nyman, S. *et al.* 1982. Bone regeneration in alveolar bone dehiscences produced by jiggling forces. *Journal of Periodontal Research* 17(3), pp. 316-322.

Ong, C. T. *et al.* 2008. Systematic review of implant outcomes in treated periodontitis subjects. *Journal of Clinical Periodontology* 35(5), pp. 438-462.

Overall, C. M. *et al.* 1989. Independent regulation of collagenase, 72-kDa progelatinase, and metalloendoproteinase inhibitor expression in human fibroblasts by transforming growth factor-β. *The Journal of Biological Chemistry* 264(3), pp. 1860-1869.

Payne, J. B. *et al.* 1997. The association between estrogen status and alveolar bone density changes in postmenopausal women with a history of periodontitis. *Journal of Periodontology* 68(1), pp. 24-31.

Peetz, M. 1997. Characterization of Xenogenic Bone Material. In: Boyne, P.J. ed. *Osseous reconstruction of the maxilla and the mandible : surgical*

techniques using titanium mesh and bone mineral. Chicago: Quintessence, pp. 87 - 100.

Petrovic, L. *et al.* 2006. Different substitute biomaterials as potential scaffolds in tissue engineering. *The International Journal of Oral & Maxillofacial Implants* 21(2), pp. 225-231.

Pfeilschifter, J. *et al.* 1990. Chemotactic response of osteoblastlike cells to transforming growth factor-β. *Journal of bone and mineral research : The Official Journal of the American Society for Bone and Mineral Research* 5(8), pp. 825-830.

Piattelli, M. *et al.* 1999. Bone reactions to anorganic bovine bone (Bio-Oss) used in sinus augmentation procedures: a histologic long-term report of 20 cases in humans. *The International Journal of Oral & Maxillofacial Implants* 14(6), pp. 835-840.

Pjetursson, B. E. *et al.* 2007. Comparison of survival and complication rates of tooth-supported fixed dental prostheses (FDPs) and implant-supported FDPs and single crowns (SCs). *Clinical Oral Implants Research* 18 Suppl 3, pp. 97-113.

Podaropoulos, L. *et al.* 2009. Bone regeneration using β-tricalcium phosphate in a calcium sulfate matrix. *The Journal of Oral Implantology* 35(1), pp. 28-36.

Reynolds, M. A. *et al.* 2003. The efficacy of bone replacement grafts in the treatment of periodontal osseous defects. A systematic review. *Annals of Periodontology / the American Academy of Periodontology* 8(1), pp. 227-265.

Robey, P. G. *et al.* 1987. Osteoblasts synthesize and respond to transforming growth factor-type beta (TGF-β) in vitro. *The Journal of Cell Biology* 105(1), pp. 457-463.

Rodan, G. A. 1997. Bone mass homeostasis and bisphosphonate action. *Bone* 20(1), pp. 1-4.

Ruggiero, S. L. *et al.* 2004. Osteonecrosis of the jaws associated with the use of bisphosphonates: a review of 63 cases. *Journal of Oral and Maxillofacial Surgery* 62(5), pp. 527-534.

Saldana, L. *et al.* 2009. Calcium phosphate-based particles influence osteogenic maturation of human mesenchymal stem cells. *Acta Biomaterialia* 5(4), pp. 1294-1305.

Salvi, G. E. 2008. Treatment planning of patients with periodontal diseases. In: Lindhe, J. ed. *Clinical periodontology and implant dentistry*. 5th ed., Vol. 2. Oxford, UK ; Ames, Iowa: Blackwell Munksgaard, pp. 656 - 674.

Sartori, S. *et al.* 2003. Ten-year follow-up in a maxillary sinus augmentation using anorganic bovine bone (Bio-Oss). A case report with histomorphometric evaluation. *Clinical Oral Implants Research* 14(3), pp. 369-372.

Sathorn, C. *et al.* 2007. Antibacterial efficacy of calcium hydroxide intracanal dressing: a systematic review and meta-analysis. *International Endodontic Journal* 40(1), pp. 2-10.

Schinke, T. *et al.* 1996. The serum protein α2-HS glycoprotein/fetuin inhibits apatite formation in vitro and in mineralizing calvaria cells. A possible role in mineralization and calcium homeostasis. *The Journal of Biological Chemistry* 271(34), pp. 20789-20796.

Schonherr, E. and Hausser, H. J. 2000. Extracellular matrix and cytokines: a functional unit. *Developmental Immunology* 7(2-4), pp. 89-101.

Schropp, L. *et al.* 2003. Bone healing and soft tissue contour changes following single-tooth extraction: a clinical and radiographic 12-month prospective study. *The International Journal of Periodontics & Restorative Dentistry* 23(4), pp. 313-323.

Schwartz, Z. *et al.* 1997. Underlying mechanisms at the bone-surface interface during regeneration. *Journal of Periodontal Research* 32(1) pp. 166-171.

Schwartz, Z. *et al.* 2000. Ability of deproteinized cancellous bovine bone to induce new bone formation. *Journal of Periodontology* 71(8), pp. 1258-1269.

Schwarz, F. *et al.* 2008. Two-year clinical results following treatment of peri-implantitis lesions using a nanocrystalline hydroxyapatite or a natural bone mineral in combination with a collagen membrane. *Journal of Clinical Periodontology* 35(1), pp. 80-87.

Sculean, A. *et al.* 2008. Ten-year results following treatment of intra-bony defects with enamel matrix proteins and guided tissue regeneration. *Journal of Clinical Periodontology* 35(9), pp. 817-824.

Sculean, A. *et al.* 2007. Five-year results of a prospective, randomized, controlled study evaluating treatment of intra-bony defects with a natural bone mineral and GTR. *Journal of Clinical Periodontology* 34(1), pp. 72-77.

Simion, M. *et al.* 2007. Vertical ridge augmentation by expanded-polytetrafluoroethylene membrane and a combination of intraoral autogenous

bone graft and deproteinized anorganic bovine bone (Bio Oss). *Clinical Oral Implants Research* 18(5), pp. 620-629.

Skoglund, A. and Persson, G. 1985. A follow-up study of apicoectomized teeth with total loss of the buccal bone plate. *Oral Surgery, Oral Medicine, and Oral Pathology* 59(1), pp. 78-81.

Sloan, A. J. *et al.* 2002. TGF-β latency-associated peptides (LAPs) in human dentin matrix and pulp. *Connective Tissue Research* 43(2-3), pp. 381-386.

Smith, E. L. *et al.* 2011. TGF-β1 exposure from bone surfaces by chemical treatment modalities. *European Cells & Materials* 21, pp. 193-201.

Socransky, S. S. *et al.* 1984. New concepts of destructive periodontal disease. *Journal of Clinical Periodontology* 11(1), pp. 21-32.

Stabholz, A. *et al.* 2010. Genetic and environmental risk factors for chronic periodontitis and aggressive periodontitis. *Periodontology 2000* 53(1), pp. 138-153.

Stephan, E. B. *et al.* 1999. Anorganic bovine bone supports osteoblastic cell attachment and proliferation. *Journal of Periodontology* 70(4), pp. 364-369.

Suzuki, M. *et al.* 1994. Calcium-binding properties of fetuin in fetal bovine serum. *The Journal of Experimental Zoology* 270(6), pp. 501-507.

Taba, M., Jr. *et al.* 2005. Diagnostic biomarkers for oral and periodontal diseases. *Dent Clin North Am* 49(3), pp. 551-571.

Tadjoedin, E. S. *et al.* 2003. Deproteinized cancellous bovine bone (Bio-Oss) as bone substitute for sinus floor elevation. A retrospective, histomorphometrical study of five cases. *Journal of Clinical Periodontology* 30(3), pp. 261-270.

Takeuchi, Y. *et al.* 1994. Bone matrix decorin binds transforming growth factor-β and enhances its bioactivity. *The Journal of Biological Chemistry* 269(51), pp. 32634-32638.

Tapety, F. I. *et al.* 2004. A histological evaluation of the involvement of Bio-Oss in osteoblastic differentiation and matrix synthesis. *Clinical Oral Implants Research* 15(3), pp. 315-324.

Taylor, J. C. *et al.* 2002. *In vitro* osteoclast resorption of bone substitute biomaterials used for implant site augmentation: a pilot study. *The International Journal of Oral & Maxillofacial Implants* 17(3), pp. 321-330.

Teitelbaum, S. L. 2000. Bone resorption by osteoclasts. *Science* 289(5484), pp. 1504-1508.

The Academy of Prosthodontics. 2005. The glossary of prosthodontic terms. *The Journal of Prosthetic Dentistry* 94(1), pp. 10-92.

Tomson, P. L. *et al*. 2007. Dissolution of bio-active dentine matrix components by mineral trioxide aggregate. *Journal of Dentistry* 35(8), pp. 636-642.

Tonetti, M. S. *et al.* 2004. Clinical outcomes following treatment of human intrabony defects with GTR/bone replacement material or access flap alone. A multicenter randomized controlled clinical trial. *Journal of Clinical Periodontology* 31(9), pp. 770-776.

Torabinejad, M. and Chivian, N. 1999. Clinical applications of mineral trioxide aggregate. *Journal of Endodontics* 25(3), pp. 197-205.

Torabinejad, M. and Parirokh, M. 2010. Mineral trioxide aggregate: a comprehensive literature review--part II: leakage and biocompatibility investigations. *Journal of Endodontics* 36(2), pp. 190-202.

Trombelli, L. 2005. Which reconstructive procedures are effective for treating the periodontal intraosseous defect? *Periodontology 2000* 37, pp. 88-105.

Tsesis, I. *et al.* 2011. Effect of guided tissue regeneration on the outcome of surgical endodontic treatment: a systematic review and meta-analysis. *Journal of Endodontics* 37(8), pp. 1039-1045.

Turhani, D. *et al.* 2005. *In vitro* study of adherent mandibular osteoblast-like cells on carrier materials. *International Journal of Oral and Maxillofacial Surgery* 34(5), pp. 543-550.

Valentini, P. and Abensur, D. J. 2003. Maxillary sinus grafting with anorganic bovine bone: a clinical report of long-term results. *The InternationalJournal of Oral & Maxillofacial Implants* 18(4), pp. 556-560.

Vander, A. J. *et al.* 2011. Blood and Haemostasis. In: Widmaier, E.P. ed. *Vander's human physiology : the mechanisms of body function*. 12th ed. New York: McGraw-Hill Higher Education, pp. 417- 427.

von Arx, T. and Buser, D. 2006. Horizontal ridge augmentation using autogenous block grafts and the guided bone regeneration technique with collagen membranes: a clinical study with 42 patients. *Clinical Oral Implants Research* 17(4), pp. 359-366.

Waddington, R. J. *et al.* 2003. Differential roles for small leucine-rich proteoglycans in bone formation. *European Cells & Materials* 6, pp. 12-21; discussion 21.

Wu, M. K. *et al.* 2006. Consequences of and strategies to deal with residual post-treatment root canal infection. *International Endodontic Journal* 39(5), pp. 343-356.

Yamaguchi, A. *et al.* 2000. Regulation of osteoblast differentiation mediated by Bone Morphogenetic Proteins, Hedgehogs, and Cbfa1. *Endocrine Reviews* 21(4), pp. 393-411.

Zitzmann, N. U. and Berglundh, T. 2008. Definition and prevalence of peri-implant diseases. *Journal of Clinical Periodontology* 35(8 Suppl), pp. 286-291.

Zitzmann, N. U. *et al.* 2001. Long-term results of implants treated with guided bone regeneration: a 5-year prospective study. *The International Journal of Oral & Maxillofacial Implants* 16(3), pp. 355-366.