

Welsh School of Pharmacy

Cardiff University



**FISH OIL AS A TOPICALLY
APPLIED ANTI-PSORIATIC**

By

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of

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ABBREVIATIONS

AA	Arachidonic acid
Ab	Asebia
ALA	α -linolenic acid
APS	Ammonium persulphate
ASA	Acetylsalicylic acid
BD	Betamethasone dipropionate
BMI	Body mass index
BMP	Bone morphogenic protein
BP	Benzoyl peroxide
cAMP	Adenosine 3c, 5c, cyclic mono phosphate
cGMP	Cyclic guanosine 3c,5c-mono phosphate
COX	Cyclooxygenase
CPD	Chronic proliferative dermatitis
DfL	Defolliculated
DAPI	4',6-diamidino-2-phenylindole
DGLA	Dihomo- γ -linolenic acid
DHA	Docosahexaenoic acid
DMEM	Dulbecco's minimum essential medium
DMSO	Dimethyl sulphoxide
DPBS	Dulbecco's phosphate buffered saline
DPX	Di-n-butylphthalate in xylene
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
EFA	Essential fatty acids
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid
FA	Fatty acid
FBS	Foetal Bovine Serum
FO	Fish oil
Fsn	Flaky skin
GC	Gas Chromatography
GLA	γ -linolenic acid
H&E	Haematoxylin & eosin
HBSBS	Hank's balanced salt buffer solution
HEPE	Hydroxyeicosapentaenoic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HETE	Hydroxyeicosatetraenoic acid
HLA	Human leucocyte antigen
HPLC	High Performance Liquid Chromatography

HRP	Horse radish peroxidase
ICC	Immunocytochemistry
IHC	Immunohistochemistry
IL	Interleukin
INF	Interferon
Jss	Pseudo-steady state flux
KGF	Keratinocyte growth factor
LA	Linoleic acid
LOD	Limit of detection
LOX	Lipoxygenase
LT	Leukotriene
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
MTX	Methotrexate
NMR	Nuclear magnetic resonance
NSAIDs	Non-steroidal anti inflammatory drugs
OCT	Optical coherence tomography
OCTC	Optimum cutting temperature compound
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PG	Prostaglandin
PPAR	<i>Peroxisome proliferator-activated receptors</i>
PUFA	Polyunsaturated fatty acid
RA	Retinoic acid
RIPA	Radio immune precipitation assay
SA	Salicylic acid
SC	Stratum corneum
SD	Standard deviation
SDS	Sodium dodecyl sulphate
TBS	Tris-buffered saline
TEMED	N,N,N',N'-Tetramethylethylenediamine
TGF	Transforming growth factor
TNF	Tumour necrosis factor
US	United States
UV	Ultraviolet
VDR	Vitamin D receptor
VEGF	Vascular endothelial growth factor

PUBLICATIONS FROM THIS THESIS

Full papers

Zulfakar, MH; Abdelouahab N; and Heard, CM. 2009. Enhanced topical delivery and ex vivo anti-inflammatory activity from a betamethasone dipropionate formulation containing fish oil. *Inflammation Research*. Published online 31st July 2009

Zulfakar, MH; Edwards M; and Heard, CM. 2007. Is there a role for topically delivered eicosapentaenoic acid in the treatment of psoriasis? *European Journal of Dermatology* 17(4), pp. 284-291.

Conference Presentations

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SUMMARY

In this study, the topical delivery and anti-psoriatic properties of the major anti-inflammatory constituent of fish (eicosapentaenoic acid, EPA) were studied, in combination with other anti-psoriatic agents with a view to the development of a new therapeutic regimen. In *in vitro* models of skin permeation EPA was delivered successfully and presence of fish oil also enhanced the delivery of betamethasone dipropionate (BD) to the lower basal layers of the epidermis. The same enhancement was not seen with salicylic acid and aspirin. Investigations on the anti-inflammatory action properties of fish oil were also successfully carried out. Fish oil alone inhibited the expression of key inflammatory enzymes in the skin, cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) along with a major eicosanoid, prostaglandin E₂, comparable to that of BD. In HaCaT keratinocyte cell culture, the anti-psoriatic properties of fish oil were further demonstrated by both growth inhibitory effects and the induction of pro-apoptotic markers. The final part of the study investigated *in vivo* a potential new model of psoriasis: the defolliculated mouse. The model responded well to formulations containing BD, denoted by a reduction in the epidermal thickness associated with the mutation. This suggests a role in screening of new therapeutic compound. Treatment with fish oil, however, caused a thickening of the epidermis, contradictory to the initial hypothesis. This was further confirmed when expression of growth markers Ki67 and K17 were found to be increased. Concurrently, Optical Coherence Tomography was utilized successfully in the *in vivo* studies, providing a rapid, non-invasive technique of live measurement of skin modulation without the need of sacrificing the animals for individual observations. In summary, despite unexpected outcome with the *in vivo* studies, the study provided ample evidence to support the incorporation of fish oil in the treatment of inflammatory skin diseases such as psoriasis.

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Chapter 1:

General introduction

1. Overview

Millions of people in the UK, and around the world, are affected by skin diseases, such as eczema and psoriasis. Along with the discovery of newer and better pharmacological interventions, a substantial number of patients also turn to naturally-derived products to improve their symptoms and as supplement to existing therapies. Currently, one of the most popular natural products is fish oil, which is rich in polyunsaturated fatty acids. With a wealth of evidence supporting its anti-psoriatic and anti-inflammatory activity (Calder 2007; Dewsbury et al. 1989; Wolters 2005), this study investigated the feasibility of topically applied fish oil and probed on the mechanisms involved in its reported anti-psoriatic properties.

1.1 Psoriasis

Psoriasis is a chronic inflammatory disease affecting several sites of the body. Though commonly associated with the skin, psoriasis can also affect the nails, mucous membranes and the joints. It is clinically defined by the formation of sharply demarcated erythematous, scaly and sometimes pustular lesions, varying in extent and with a symmetrical distribution on the skin. Often the disease manifests itself in periodic flares which may necessitate additional therapeutic control (Bos and De Rie 1999)

It has a worldwide prevalence of approximately 2% (Mayser et al. 2002; Wolters 2005), although a higher number of cases are found in Caucasians compared to other racial types. The prevalence varies throughout the world, ranging from 4.6% in the United States, 1.5% of central Europe to 0.4% in Chinese population (Wolters 2005).

Historically, psoriasis was considered to be a variant of leprosy until Robert Willan, a British dermatologist, distinguished it as a separate clinical entity in the early 19th century (Griffiths and Barker 2007); the term 'psoriasis' is derived from the Greek word *psora* or 'to itch' (Glickman 1986). Its primary cause has been attributed to *de novo* hyperproliferation of the keratinocytes and abnormal epidermal differentiation; although the exact reason for this deviation from the normal skin physiology was not determined until the discovery of gene involvement in predisposing an individual to psoriasis. Several theories have been proposed to shed light on the increased proliferation of cells:

A. Cyclic nucleotides - Cells are blocked in the G₁ phase by high levels of cAMP (adenosine 3',5'-cyclic monophosphate). Any increase in cAMP or decrease in cGMP (cyclic guanosine 3',5'-monophosphate) would increase the number of proliferating cells (Voorhees et al. 1976)

B. Arachidonic acid metabolism - Diversion of the arachidonic acid (AA) metabolism pathway to the lipoxygenase pathway and release of inflammatory mediators, such as 12-hydroxyeicosatetraenoic acid (12-HETE), leukotriene B₄, and prostaglandins, stimulate cell multiplication (Kragballe and Voorhees 1983)

C. Polyamines - Putrescine, spermidine, and spermine are increased. These polyamines are important in cellular proliferation. Ornithine decarboxylase is the rate-limiting enzyme which increases in the early stages of hyperplasia (McDonald 1983)

D. Protease-antiprotease system - Plasminogen activator (α1 antitrypsin) and cathepsin 1 have a role in epidermal proliferation and differentiation. Protease activity increases in psoriasis. Cell surface proteases can be activated at the local level (Galadari et al. 2005)

It has also been suggested that the hyperproliferation is partly due to the reduction of cell-cycle time in psoriatic epidermis. In normal skin, cell cycle turnover time is estimated to take 457 hours, compared to 37 hours in psoriatic skin. Moreover, the transit time between the basal and the granular layer is reduced to 2 days instead of the normal 13. There is a mixture of resting and cycling cells in the basal layer of the epidermis, and the abnormality observed in psoriasis has been determined to be due to increased recruitment of cycling cells from the resting fraction.

It is now accepted that the immune system, mediated by primed T-lymphocytes and chemical mediators of inflammation or cytokines, e.g. interleukin (IL) 1, IL-2, IL-6, IL-8, TNF- α , IFN- γ , epidermal growth factor-like, fibroblast growth factor (FGF)-like, and platelet-derived growth factors, stimulates the proliferation of keratinocytes in focal skin regions. In other words, it is an inflammatory process. In psoriasis, there is a prolonged stimulation of keratinocytes by T-cells, probably triggered by antigens such as bacteria, viruses, or even autoreactivity to proteins of the keratinocyte themselves.

The hypothesis that psoriasis is mediated by T-cells is based upon several observations. Normal skin from a psoriasis-susceptible patient began to express a psoriasis-like condition after being grafted on an immunodeficient mouse (Nickoloff 2000). It had also been observed that graft or transplant patients who were also suffering from psoriasis showed an improvement after being treated with cyclosporine, which has an inhibitory effects towards T-cells (Galadari et al. 2005). The same improvements were also observed in patients treated with psoralen and UVA, which also acts on T-cells. Furthermore, by using Denileukin diftitox, a toxin towards T-cells, reduction of psoriatic plaques was apparent (Bagel et al. 1998). Reciprocally, a marked aggravation of the disease was observed in patients treated with pro-inflammatory agents, such as interferons.

The disease causes significant morbidity and ranges in severity from mild to severe. Apart from its obvious physiological effects (intense itching, pain), patients have also been known to suffer psychologically from the effects of psoriasis (Stein 2005), with significant deterioration in the quality of life of patients, as the severity of the disease increases (Krueger et al. 2001).

1.1.2 *Histological features of psoriasis*

Histological examination of the psoriatic skin reveals significant changes to the overall structure and organization of tissues within the skin. Figure 1.1 is a representative haematoxylin and eosin (H&E) stained tissue obtained from involved psoriatic skin.

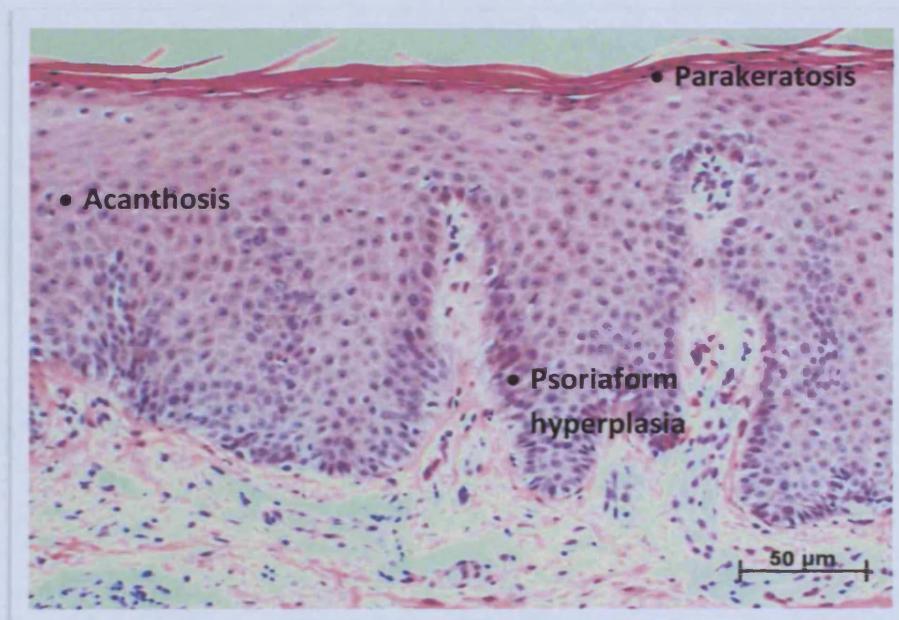


Figure 1.1 : H&E staining of involved psoriatic skin

Parakeratosis is the presence of nuclei within the stratum corneum (SC) (indicated by purple dots in the above figure). In normal skin, as the keratinocytes (primary skin cells) migrate upwards from the basal layer, differentiation of cells occurs and the cell loses its nucleus as keratinisation

progresses. The presence of nuclei in the SC indicates an incomplete ('para') differentiation and keratinisation process. There is a notable increase in the length of epidermal projections or psoriaform hyperplasia and the epidermal is generally thickened due to hyperproliferation of keratinocytes (acanthosis). Immunostaining reveals infiltration of polymorphonuclear leucocytes, and CD4+ and CD8+ lymphocytes in the epidermis and dermis, respectively.

1.1.3 *Classification of psoriasis*

Psoriasis can be classified by 2 methods. Association with human leucocyte antigen (HLA), age of onset and the course of the disease divides psoriasis into 2 types:

Type I normally occurs in young adults, with 80% of the patients expressing HLA-Cw6. There is a strong familial inheritance and often the manifestations of this type of psoriasis are more severe in nature. The course of the disease also tends to be more irregular. Incidence of the milder, more localized Type II psoriasis peaks between the ages of 50-60 years. HLA-Cw6 expression occurs in only 20% of the patients, and a positive family history is rare (Swanbeck et al. 1995). The second method of classification is based on the clinical presentation. Using this approach, several variants of the disease have been identified.



Figure 1.2 : Psoriasis vulgaris / plaque psoriasis (Hossain 2008). It is identified by the formation of plaques covered in silvery, white scales.

An estimated 80-90% of all psoriatic patients exhibit psoriasis vulgaris or plaque psoriasis (Figure 1.2), named as such due to the formation of clearly defined papulosquamous plaques which are red in colour and covered in silvery, white scales. The plaques occur most commonly on the scalp, elbows and knees, the lumbosacral region and the umbilicus with a symmetrical distribution. This variant is further characterized by the Koebner phenomenon, where developments of new lesions occur at sites of trauma (Crissey et al. 2002; Weiss et al. 2002).



Figure 1.3 : Inverse psoriasis of the buttocks, appearing as smooth patches with the absence of scales (Janjua 2004)

Inverse or flexural psoriasis (Figure 1.3) appears as smooth patches on the skin of the intertriginous areas, commonly on the genitals, armpits and under the breasts. The 'inverse' in this condition refers to the absence of scales as opposed to the most common feature found in psoriasis vulgaris (Lebwohl 2003).



Figure 1.4 : Guttate psoriasis, which manifests itself as numerous teardrop shaped spots often preceded by an infection (Cohen 2007)

Numerous teardrop-shaped spots over a large area of the body appear in what is known as guttate psoriasis (from *gutta*, latin for droplet) (Figure 1.4). These less than 1cm² papules are often preceded by infection with β -haemolytic streptococcus or a virus (Mallon et al. 2000). Resolution occurs within 3-4 months of onset and appears to be self-limiting.



Figure 1.5 : Pustular psoriasis (Khazal 2009)

Pustular psoriasis (Figure 1.5) manifests itself by formation of small pustules, which can either be localized to the hands and feet (palmoplantar pustulosis) or taking a more generalized and widespread form (von Zumbusch psoriasis). Patients with the generalized form often develop the condition after sudden withdrawal of systemic or ultra potent topical corticosteroids, and also intercurrent infections. Palmoplantar pustulosis is unique in the sense that it affects mostly women, current or previous smokers, and occurs late in life, often in the 4th or 5th decades of life. Research also suggests that it has different cause to psoriasis vulgaris, and thus being described as a comorbidity instead of a variant of psoriasis (Lebwohl 2003).

As the name suggests, psoriatic nail disease or nail psoriasis affects the nails, which undergo changes such as discolouration of the nail plate, pitting, onycholysis or nail plate separation, formation of 'oil-drop sign', thickening of

the skin under the nails and crumbling of the nails itself. The condition occurs in about 50% of psoriatic patients (Menter et al. 2004).



Figure 1.6 : Erythrodermic psoriasis (Cohen 2002)

Erythrodermic psoriasis (Figure 1.6) involves widespread inflammation across the area of the body and subsequent exfoliation of the areas affected. This is often a result of exacerbation of uncontrolled plaque psoriasis, which could occur with sudden withdrawal of systemic therapies. The reduced ability of the skin to provide barrier functions and thermoregulation leads to hypothermia, hypoalbuminaemia, cardiac failure and, on a number of occasions, death (Griffiths and Barker 2007).

Opinions are still divided whether psoriatic arthritis is a form of psoriasis or a co-morbidity affecting psoriatic patients. Approximately 25% of all psoriatic patients have been reported to present with a certain degree of joint involvement, most commonly in the fingers and toes. Gladman (1988) reported a prevalence of between 5-40%. Rheumatoid factors are typically absent, enabling the clinician to rule out other forms of rheumatism, such as rheumatoid arthritis and osteoarthritis.

1.1.4 Co-morbidities and risk factors in psoriasis

Aside from the joint involvement, long-term sufferers of severe psoriasis are linked with several other morbidities, both cutaneous and non-cutaneous (Henseler and Christophers 1995). A higher incidence of cardiovascular problems and mortality have been reported by several studies (Kaye et al. 2008; Kremers et al. 2007; Ludwig et al. 2007), with some suggesting that the cardiovascular problems, such as atherosclerosis, are in fact a systemic manifestation of psoriasis (Alexandroff et al. 2009).

Psoriasis is also associated with several conditions of metabolic origin; collectively, they are identified as the metabolic syndrome. According to the Adult Treatment Panel III, metabolic syndrome is defined as the presence of at least three of the following conditions: abdominal obesity, elevated serum triglycerides, low HDL cholesterol, elevated blood pressure and an elevated fasting glucose (Azfar and Gelfand 2008).

There is a high prevalence of psoriasis in obese individuals and a direct correlation with the body mass index (BMI) (Henseler and Christophers 1995; Naldi et al. 2005; Wolkenstein et al. 2009), with one study reporting a higher incidence of severe psoriasis (>20% body area affected) in their cohort of obese patients (Herron et al. 2005).

The estimated risk of developing psoriasis is also significantly higher with current and ex-smokers (Menter et al. 2004; Nancy and Yehuda 2009; Wolkenstein et al. 2009), stress (Arnetz et al. 1985), and alcohol consumption (Dediol et al. 2009; Kirby et al. 2008). With a concurrent association with smoking and metabolic syndrome, the prevalence of chronic obstructive pulmonary disease has been proven in a case-controlled study to be increased in patients with underlying psoriasis (Dreiherr et al. 2008).

Dermatological conditions which concurrently affect psoriasis patients include Candida infections, dermatophyte infections, acne, condylomata acuminata, erysipelas, zoster, allergic contact dermatitis, impetigo contagiosa, urticaria, eczema herpeticatum, atopic dermatitis. In the study carried out by Henseler and Christophers (1995) all these conditions were significantly associated ($p < 0.05$) with psoriasis.

Psoriasis can also be exacerbated by patients on concurrent medications, such as anti-malarials, beta-blockers, lithium, interferons and systemic glucocorticoids (Menter et al. 2004). Other anecdotal evidence for exacerbation of psoriasis has been reported for ACE-inhibitors, gold salts and NSAIDs.

1.1.5 Genetic and immunologic aspects of psoriasis

Though the precise causative factor of psoriasis has yet to be determined, a genetic link has been determined through population-wide studies. It has been shown to be an inheritable disease, particularly marked if inherited on the paternal side (Burden et al. 1998). First and second degree relatives of psoriatic patients have a higher incidence rate of presenting the disease themselves, with studies suggesting that upward of 30% psoriatic patients have the involvement of their first degree relatives. Concurrently, monozygotic twins have a 50-60% risk of getting psoriasis compared to dizygotic twins.

An array of genes, coupled with external and environmental factors, such as infection, stress, smoking, high alcohol intake and certain drugs, are accepted to be the general causes which lead to the development and the subsequent manifestation of psoriasis. Current genome studies have indicated at least 9

chromosomal loci (PSORS1-9). Much of the studies have been focused on the 300-kb PSORS1 locus near HLA-C on chromosome 6p21.3, which accounts for 30-50% of the genetic contribution to psoriasis (Trembath et al. 1997). Psoriasis has also been linked to 17q24-q25 (PSORS2), 4qter (PSORS3), 1q21 (PSORS4), and 3q21 (PSORS5) in different populations, although these linkages are not as reproducible as the one with PSORS1 (Galadari et al. 2005).

A separate study conducted by Luszczek et al (2004) on a Polish population also revealed a link to the KIR2DS1 gene, which activates a natural killer (NK) cells receptor; a finding confirmed independently by studies conducted on Japanese (Suzuki et al 2004) and American populations (Holm et al 2005). In the study done by Lesueur et al. (2007), they identified a susceptibility locus at chromosome 20p13 named ADAM33 which has a previous association with asthma, suggesting a common link between different diseases which are inflammatory in nature.

Initially, psoriasis was viewed as a primary disorder of keratinocytes. Over time, evidence began to suggest the immunologic nature of the disease. It is observed in developing lesions that infiltration by lymphocytes preceded epidermal and other changes. Transplant patients with concurrent psoriasis saw an improvement in their lesions as a result of taking cyclosporine, a potent inhibitor of T-cells, to prevent rejection of transplanted tissues. Further proofs were found in xenograft animal models, whereby uninvolved skin from psoriatic patients grafted on to an immune-deficient mouse began to show psoriaform lesions upon injection of T-cells (Galadari et al. 2005).

Interestingly, recent studies have resurrected the classic argument of keratinocytes as the primary instigators of the disease. Zenz et al. (2005) reported the successful creation of an animal model showing both psoriasiform lesions and joint manifestations by knocking out the genes encoding for the activator protein-1 (AP-1) components Junb and Jun within

basal layer keratinocytes. Apart from the lesions, genes encoding for the calcium binding proteins S100A8 and S100A9 were also found to be upregulated. In a remarkable resemblance to psoriasis, both these proteins are overexpressed, meanwhile the Junb gene in human is localized within the psoriasis susceptibility locus PSORS6 (Nickoloff 2006). Furthermore, following Jun deletion, the mouse epidermal keratinocytes began to produce proinflammatory cytokines, supporting previous proponents of keratinocytes as the instigators of psoriasis. It is however noted that certain criteria of human psoriasis were not reported in the mouse model, the composition of the infiltrated T-cells, for example. As yet, opinions are still divided over whether psoriasis is the sole result of an impaired barrier function and abnormalities of the keratinocytes or due to dysregulation of immune cells

1.1.6 *Biomarkers of psoriasis*

Currently, diagnosis of psoriasis is reliant solely on clinical presentations. As mentioned earlier, there is a prolonged and sustained immune reaction by T cells on keratinocytes; however, the exact antigen that triggers the process remains elusive. Experimental and clinical data revealed notable expression patterns for certain proteins and markers of oxidative stress (Table 1.1), which may provide the basis for biochemical diagnosis of psoriasis and potential new targets for treatment (Rashmi et al. 2009).

Apart from the markers listed below, it was also reported that the A₃ adenosine receptor is over-expressed in psoriasis. Similarly, it is overexpressed in rheumatoid arthritis and Crohn's disease. This receptor has now been identified as a novel target for anti-inflammatory agents (Ochaion et al. 2009).

Proposed marker	Expression in psoriasis	Source
Oxidative stress		
➤ Thiobarbituric acid	↑	Blood
➤ Malondialdehyde	↑	Tissue
➤ Superoxide dismutase	↓	Blood
➤ Glutathione peroxidase	↓	Blood
➤ Catalase	↑	Blood
➤ Neutrophil function	↑	Blood
➤ Oxidised LDL	↑	Tissue
➤ Anti-oxidised LDL antibody	↑	Blood
➤ Cytokeratins	+	Tissue
Keratinocyte hyperproliferation		
➤ Heat-shock proteins (27, 60)	+	Tissue
➤ Connexins (26, 30)	↑	Tissue
➤ Bcl-X, Bax, p53, Bak	↑	Tissue
➤ Epidermal growth factor	↑	Tissue
➤ TGF- α	↑	Tissue
➤ Ornithine decarboxylase and MAPK activities	↑	Tissue
➤ AP1, BMP-6	*	Tissue
➤ Keratin (6, 16)	↑	Tissue
Abnormal keratinocyte differentiation		
➤ TGase K	↑	Tissue
➤ Keratin 1/10	↓	Tissue
➤ Involucrin	↑	Tissue
➤ SKALP	+	Tissue
➤ MRP-8	+	Tissue
➤ Filaggrin	-	Tissue
Inflammation		
➤ Anti-calpastatin antibody	↑	Blood
➤ C-reactive protein	↑	Blood
➤ Fibrinogen	↑	Blood
➤ Haptoglobin	↑	Blood
➤ TNF- α , IFN- α , IFN- γ , interleukins (2, 6, 8, 12, 15, 17, 22, 23), LIF1	↑	Blood
➤ Interleukins (1, 4, 5, 10)	↓	Blood
➤ TGF- β , interleukin-18	↑	Blood
Neuropeptides		
➤ SP, VIP, PACAP-38, CGRP, NGF	↑	Tissue
➤ Somatostatin	↓	Tissue

Table 1.1 : Proposed biomarkers for psoriasis. Adapted from Rashmi et al (2009).
 +, Presence in psoriatic condition only; - absence in normal skin; ↑ increased expression; ↓ decreased expression; * changed expression; † combined measurement

1.2 Treatment options

Although incurable, there is a wide variety of treatments currently available to treat or control psoriasis. The mode of treatment is determined primarily by the extent and severity of the disease. Mild and localized forms are often treated topically; although more severe and widespread forms may necessitate systemic therapies. In these cases, topical agents would often still be included in the therapeutic regimens to control flares. Other factors for the clinician to consider before prescribing any therapeutic agents include safety concerns for systemic agents, cosmetic issues (several of the commonly used agents are highly staining), cost and economic issues, accessibility to treatment centres (for phototherapy), and patient acceptance and attitude toward the disease. Any single treatment may be employed alone, or in combination (Galadari et al. 2005; Gottlieb 2005a).

1.2.1 Topical agents

1.2.1.1 Corticosteroids

Topical corticosteroids have been the mainstay of topical anti-psoriasis therapy (Stein 2005). Therapy with this class of drug is generally effective and well tolerated, relatively rapid, and, compared to most other form of therapies, economically feasible. They are one of the most commonly prescribed topical agents in the US (Feldman et al. 2000) and their range of actions include immunosuppression, anti-inflammation, anti-proliferation and vasoconstriction (the degree of which determines the potency of a given corticosteroid formulation), which are largely mediated through binding with intracellular corticosteroid receptors and regulation of gene transcription, including those which code for cytokines (Hughes and Rustin 1997).

Steroids are broadly classified according to their potencies, ranging from Class 1 (superpotent) to Class 7 (least potent), and are used daily to provide initial control before switching to a twice-weekly regimen employing a lower potency agent or another substitute. Examples of corticosteroids include betamethasone dipropionate (Figure 1.7), betamethasone valerate, clobetasol propionate, fluticasone propionate and hydrocortisone, among others. Though commonly prescribed, corticosteroids are not used in patients with more than 20% of their body surface affected to limit systemic absorption which can lead to pituitary-adrenal axis suppression and Cushing's syndrome (Menter et al. 2004).

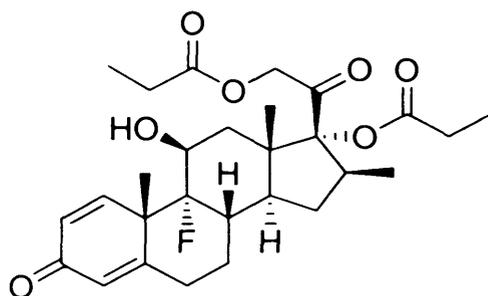


Figure 1.7 : Betamethasone dipropionate

Side effects commonly occurring with steroid use among others include atrophy or thickening of the skin, striae, telangiectases, acneform eruption, rosacea, contact dermatitis and the aforementioned secondary effects resulting from systemic absorption.

1.2.1.2 Vitamin D₃ analogues

The biologically active form of vitamin D₃, 1- α , 25-dihydroxyvitamin D₃ or calcitriol was first reported in the 1980s to have an effect on induction of cell differentiation and growth, in addition to its major role in calcium and bone metabolism. Further findings acknowledged its immunomodulatory activities (Mathieu and Adorini 2002). This had a profound effect in generating further

research on this compound and its analogues for a range of other conditions, including psoriasis, secondary hyperthyroidism and osteoporosis (Shimizu et al. 2006).

The analogues most commonly used in psoriasis are calcipotriene, tacalcitol and maxacalcitol (Yamaguchi et al. 2008), which act via several mechanisms, the first of such involves inhibition of keratinocytes proliferation and stimulation of keratinocyte differentiation (Takahashi et al. 2003). These are achieved by interactions of the analogues with nuclear vitamin D receptors (VDR) within the keratinocytes (Gottlieb 2005a).

The analogue-VDR interaction also leads to alterations in vitamin D-responsive gene transcription, which includes genes involved in keratinocyte differentiation and proliferation, as well as those controlling cell adhesion and movement. This alteration could possibly also be achieved by antagonizing effects of transcription regulators such as activator protein 1 (Nagpal et al. 2001). Lu et al.(1996), working with treated psoriatic plaques, found that topical application of calcitriol decreases T-cell infiltration and keratinocyte intracellular adhesion molecule-1 (ICAM-1).

The effectiveness of these agents, in particular calcipotriene, is significantly superior to anthralin; a fact also observed in combination therapy with potent corticosteroids (versus calcipotriene monotherapy). Clinical effectiveness varies between 6-8 weeks; however, therapeutic response could also be observed as early as two weeks after initiation of therapy (Witman 2001). Side effects include skin irritation, and, with high doses (upwards if 360 g/week), a decrease in serum parathyroid levels and increase in serum and urine calcium levels have been reported (Bourke et al. 1997).

1.2.1.3 *Retinoids*

Retinoids are a class of compounds which are chemically related to vitamin A. They include first generation agents, such as retinol, tretinoin, isotretinoin, alitretinoin, and newer generations, such as etritinate, tazarotene and bexarotene. Currently, only tazarotene is approved for topical treatment of plaque psoriasis (Koo et al. 2003), although oral retinoids have been used, albeit limitedly due to its toxicity.

The mechanism of action is believed to involve normalizing epidermal differentiation, reducing hyperproliferation and reducing the influx of inflammatory cells into the skin (Duvic et al. 1997). These actions are achieved by binding to retinoic acid receptors and modulating transcription of genes involved in psoriasis. These genes have been identified as TIG 1,2 and 3 (Gottlieb 2005b). Additionally, tazarotene has been shown to reduce cell proliferation and inflammation markers and to normalize markers of keratinocyte differentiation (Duvic et al. 1998).

1.2.1.4 *Anthralin*

Anthralin, more commonly known as dithranol (Figure 1.8), is another drug used widely as a topical agent to treat psoriasis. It is an anthracene derivative used topically with strengths ranging from 0.1-2%. Its anti-psoriatic action lies in its ability to produce free radicals in the cellular mitochondria, interfering with the cell's energy supply and, thus, retarding DNA replication and cell multiplication (Orojan et al. 2006). It has a slower onset of action compared to corticosteroids, although it has the advantage of not causing any rebound or withdrawal effects, which are typical with the former.

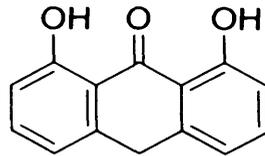


Figure 1.8 : Anthralin (Dithranol)

The poor efficacy and safety profile of anthralin compared to other topical therapies led to its reduction of use (Callis and Krueger 2003). Apart from that, it has undesirable property of leaving a stain and burning sensation (Agarwal et al. 2001). In the US, it is still used to treat moderate to severe plaque psoriasis in patients not adequately responding to other agents. In the UK, it has been in use for more than 80 years, with Dithrocream® and Micanol® as examples of proprietary preparations developed for home use (Menter et al. 2004)

1.2.1.5 Coal tar preparations

Coal tar is an effective treatment for chronic stable plaque psoriasis, scalp psoriasis, atopic dermatitis, and seborrheic dermatitis. It is perhaps one of the oldest preparation used in dermatological diseases, dating back to ancient time (Paghdal and Schwartz 2009). Though not properly understood, it is believed to exert its action via reduction of cellular proliferation by suppressing DNA synthesis (Smith et al. 2000). Its photosensitizing properties were utilized in combination therapy with ultraviolet B (UVB) radiation in what was called the Goeckerman regimen (Lebwohl 2003). Several concerns arising from its use and the development of newer topical agents led to a decline in its use. Apart from the staining, it is rather messy to use and has an undesirable odour. It can cause folliculitis, photosensitivity, and is a carcinogen in animal models, although its association with human cancers has not been shown (Gottlieb 2005a).

1.2.1.6 Other topical treatments

1.2.1.6.1 Salicylic acid

Salicylic acid (Figure 1.9) is a well-known agent employed widely in the treatment of dermatological diseases, such as psoriasis, acne, verruca, warts and other hyperkeratotic states (Lodén 2000; Tsai et al. 1999). This beta-hydroxy acid is originally derived from the bark of the willow tree, and often used in conjunction with other topical anti-psoriatics, such as dithranol, corticosteroids and coal tar.

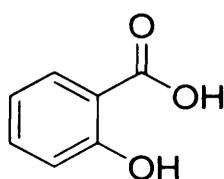


Figure 1.9 : Salicylic acid

The reason for this combination is that in psoriasis, the presence of highly keratinized plaques/scales hinders the delivery of topical agents which necessitates removal of the scales. Salicylic acid is often described as a 'keratolytic' and it has the property of promoting the softening and lifting of the scales by a mechanism not fully understood; although it is believed to be related to its ability to affect stratum corneum structure by "dissolving intracellular cement and reducing cellular cohesion between keratinocytes" (Bashir et al. 2005) and increasing the hydration and softening of the scales by reducing the pH of the applied area (Davies and Marks 1976).

It is available in preparations ranging from lotions, shampoos, and is also formulated together with other drugs, for example betamethasone dipropionate (Diprosalic©, Schering). A study by Krochmal et al (1989) showed that delivery of several corticosteroids were increased by 2-fold or more when formulated together with salicylic acid and showed no effects on

the physicochemical stability for up to 2 months. The increased delivery resulted in increased efficacy, as proven by Koo et al. (1998), whereby more than 75% improvement in skin lesions were seen in 53% of patients compared to 36% not treated with salicylic acid. This finding was further supported by a separate study by Katz et al (1998). Overall, the increased delivery supported the action of SA on the SC.

1.2.1.6.2 *Emollients & moisturizers*

Diseases such as psoriasis are commonly associated with dry and scaly skin conditions resulting from the impaired barrier (Bikowski 2001). Emollients and moisturizers are designed to soften the SC and reduce formation of scales by increasing hydration in the skin. An adjuvant in the treatment of psoriasis, these agents work by producing an occlusive film that limits water evaporation. This leads to increased skin hydration. Apart from treating the symptoms of dry skin, the use of emollients help to maintain the integrity of the skin barrier by supplying the compromised stratum corneum with vital lipids and accelerate barrier recovery (Lebwohl and Herrmann 2005). The concurrent use of emollients has also been found to increase the delivery of corticosteroids, leading to a decrease in steroid dosage and minimizing the associated side effects (Fluhr et al. 2008).

1.2.2 *Phototherapy*

It has been well observed that natural sunlight has the effect of improving the condition of psoriatic patients. Since then, these effects have been successfully replicated with the use of artificial lamps generating ultraviolet light. For decades, the Goeckerman regimen, combining UV lamp and

application of coal tar under occlusion, has been a mainstay in therapy (Zanolli 2003). Although the introduction of newer agents and home UV treatment led to its decline, it remained an important treatment modality. There have even been cases where successful remission of psoriasis was achieved using the regimen in recalcitrant patients who are not responsive to the latest biological therapies (Serrao and Davis 2009).

1.2.2.1 UVA and UVB

Natural UV radiation can be divided into 3 distinct types according to the wavelength: UVA, UVB and UVC. UVA (320-400nm) penetrates the deeper layers of the dermis. It is used in combination with systemic therapy with psoralen (Figure 1.10), a photosensitizer, as the radiation is largely ineffective on its own. This combination is commonly known as PUVA (Psoralen-UVA) and has been classed as 'photochemotherapy'. It is indicated for patients presenting with moderate to severe psoriasis not responsive to topical therapies. It can also benefit cases of localized disease, in combination with retinoids (Gottlieb 2005b).

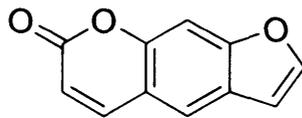


Figure 1.10 : Psoralen

As opposed to UVA, UVB works best at the epidermis where absorption is at its highest. Two sources of UVB are used in therapy. The broadband UVB (270-350nm) is more commonly available than the narrowband UVB (311-313). The latter, however, is generally regarded to be safer and more effective. UVB is indicated in extensive chronic plaque psoriasis or seborrheic psoriasis unresponsive to topical therapy and persistent guttate psoriasis (Menter et al. 2004).

Phototherapy is often used in combination with other forms of treatment. After initial remission or clearance is achieved, it is maintained using either topical or systemic therapy. Another reason for the combination is also to minimize exposure to UV light and reciprocally reduce the dose of maintenance drugs. The use of phototherapy requires proper observation from trained practitioners to reduce the associated side effects such as burns, and the long-term risk of skin malignancies.

1.2.2.2 *Excimer laser therapy*

Excimer laser (308nm) is produced from a mixture of a noble gas (argon, krypton or xenon) and a halogen (typically fluorine or chlorine) induced with a high energy current to form an unstable excited dimer or 'excimer' for short. As the excited dimers return to the ground state, the emitted energy, in the form of laser light, is then delivered to the target by means of an fibre optic cable (Baker 2008; Passeron and Ortonne 2005).

The use of excimer lasers for psoriasis began in the late 1990s. It is used to selectively target blood vessels within psoriatic lesions and leads to ablation of the targeted vessels. The principle behind this form of phototherapy came from the findings that showed changes in dermal papillary blood vessels during the initial development of lesions. By blocking these vessels, it was found that the developmental process can be halted and led to a clearing of lesions. The advantages of excimer lasers over conventional phototherapy are the overall effectiveness, clearing lesions quicker than UVB and providing a longer remission. The high precision also means that uninvolved tissues are spared from UV effects, thus reducing long-term risk associated with phototherapy (Baker 2008).

1.2.3 Systemic treatments

Systemic treatments are usually reserved for extensive cases of psoriasis, where topical treatments are impractical and the patients are not responsive to phototherapy (Menter et al. 2004). Typically, patients with more than 10-15% of body area affected by psoriasis are considered as 'severe', although the decision to initiate systemic therapy may be influenced by other factors, such as debilitation or serious reduction in quality of life, regardless of the severity in terms of body area (Feldman et al. 2005).

1.2.3.1 Methotrexate (MTX)

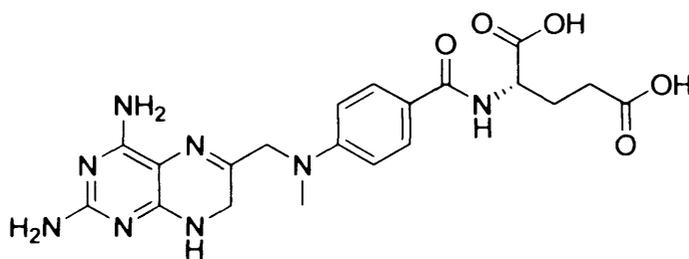


Figure 1.11 : Methotrexate

MTX (Figure 1.11) is widely accepted as the 'gold standard' in systemic therapy for psoriasis and has been in use for more than 40 years (Menter et al. 2004). It is a structural analogue of folic acid (vitamin B₃), an important trace element required for DNA synthesis and cellular division. MTX acts by competitively inhibiting the enzyme responsible for folic acid metabolism, dihydrofolate reductase, thus interfering with DNA synthesis and the subsequent cellular division (Robert et al. 1982). It is notoriously linked with several side effects resulting from folate deficiency (reversible with concurrent folic acid supplementation), such as nausea, diarrhoea, stomatitis and abdominal distress, and in severe cases can also lead to anaemia and

neutropenia as a result of bone marrow suppression. Long-term use can be toxic and has been associated with increased risk of malignancies and hepatotoxicity (Patel et al. 2009; Suzuki et al. 2007).

1.2.3.2 Cyclosporine/ciclosporin

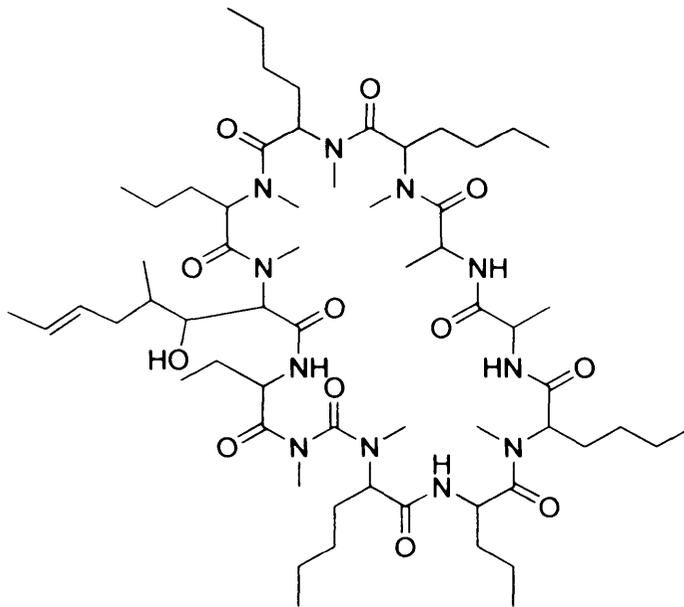


Figure 1.12 : Cyclosporine

Cyclosporine or ciclosporin (Figure 1.12), belongs to a group of drugs called the calcineurin inhibitors. It is derived from a fungus found in the soil, *Tolypocladium inflatum* Gams (Warren and Griffiths 2008). The effectiveness of cyclosporine (Figure 1.12) on the control of psoriasis symptoms was discovered when transplant patients with psoriasis prescribed with cyclosporine to prevent organ rejection coincidentally began showing signs of improvement on their psoriatic lesions (Galadari et al. 2005). It is a potent immunosuppressant, acting by binding on cyclophilin, a protein found in the cytoplasm of immunocompetent T-cells, leading to its inhibition. It was through cyclosporine use that the association of psoriasis with T-cell activity was established.

As with MTX, the use of cyclosporine is hindered by its dose dependent toxicity and associated risk of malignancy with long-term use (Ho 2004)

1.2.3.3 Systemic retinoids

The topical use of retinoids and the metabolite acitretin has been discussed in Section 1.2.1.3. It is given systemically, often in combination with UV treatment of other systemic agents, such as cyclosporine, to reduce dosage of both agents (Menter et al. 2004).

1.2.3.4 Hydroxycarbamide

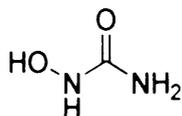


Figure 1.13 : Hydroxycarbamide

Hydroxycarbamide (Figure 1.13), also known as hydroxyurea, is an anti-metabolite typically used to treat malignancies of the blood. It inhibits the production of DNA by interfering with the enzyme ribonucleoside diphosphate reductase (Sharma et al. 2004). It is generally regarded as a second line therapeutic agent for psoriasis (Bhushan et al. 2001; Sharma et al. 2004) and brings about improvement much slower compared to MTX (Menter et al. 2004). Furthermore, its successful use in patients was also associated with bone marrow toxicity, with leukopenia or thrombocytopenia in 50% of patients (Lebwohl and Ali 2001).

1.2.3.5 Other systemic agents

Other immune-modifying agents that have been used in the treatments of severe psoriasis include azathioprine, 6-mercaptopurine, the cyclosporine-like tacrolimus and pimecrolimus, and the fungal derived mycophenolate mofetil (Kaaroud et al. 2007; Menter et al. 2004). These agents are normally used in cases non-responsive to typical systemic agents.

1.2.4 Immunobiologics

Immunobiologics are molecules or proteins, derived from living organisms or recombinant DNA technology, used to treat diseases. It is the latest class of therapeutic agents to be introduced as part of the arsenal against psoriasis. These agents act on specific immune responses involved with development or maintenance of disease (Gottlieb 2005b). The main types of immunobiologics are recombinant cytokines or growth factors, monoclonal antibodies, and fusion proteins. These agents are administered intravenously, and as yet there are no oral or topically applied immunobiologics available. The actions of immunobiologics can be classified in 4 different ways; elimination of pathogenic T-cells (fusion proteins, e.g alefacept), blockade of T-cell activation, stimulation and migration (monoclonal antibodies e.g efalizumab), alteration of T-cell proliferative signals/immune deviation (monoclonal antibody) and blocking of pro-inflammatory effector cytokines (fusion protein, recombinant cytokines and monoclonal antibody) (Menter et al. 2004).

The high target specificity of these biological responses provides a modality of treatment which allows minimal exposure to uninvolved targets, leading to

a reduction in toxic or side effects associated with systemic agents (Baker 2008).

1.3 Role of fatty acids in the treatment of psoriasis

There has been considerable interest in the use of naturally occurring products in improving the symptoms of psoriasis, apart from the pharmacological interventions listed previously. Anecdotal evidence report the successful use of a wide range of natural products, from aloe vera (Syed et al. 1996), honey (Al-Waili 2003) and traditional Chinese herbs (Koo and Arain 1998). Perhaps one of the most documented and popular natural treatment is the use of bioactive fatty acids and lipids from oils derived from sources such as fish.

1.3.1 Fatty acids – an overview

To better understand the use of fatty acids in treatment of psoriasis, it is important to understand some facts about fatty acids. Fatty acids are straight chain hydrocarbons possessing a carboxyl (COOH) group at one end (α) and a methyl group at the other (ω) end. As pictured in Figure 1.14, they can be divided into several groups based on their chemical structure. The saturated fatty acids, e.g palmitic acid (16:0) and lauric acid (12:0), have no double bonds or functional groups along the hydrocarbon chain. They are found mostly in animal tissues, as a form of energy store.

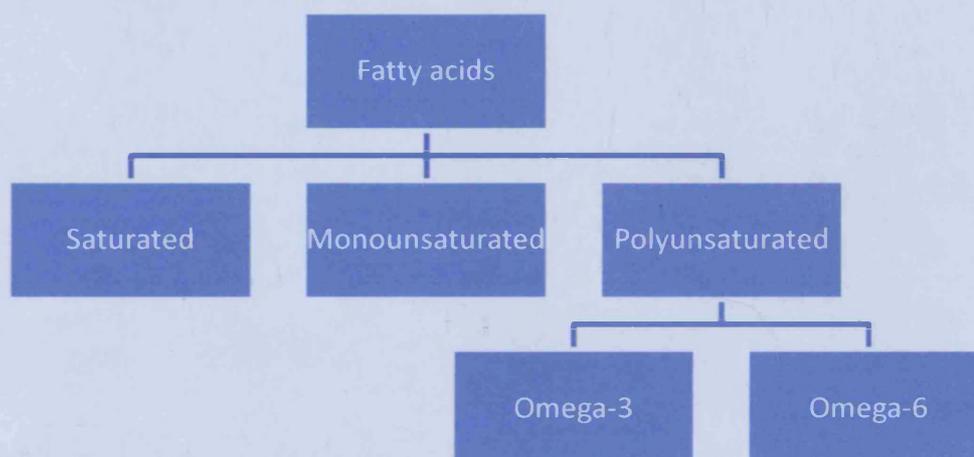


Figure 1.14 : General classification of fatty acids

The monounsaturated and polyunsaturated fatty acids, on the other hand, possess either one or several double bonds or functional groups, respectively.

1.3.2 Polyunsaturated fatty acids (PUFA)

PUFAs are fatty acids possessing 2 or more double bonds in their chemical structure. The position of the first double bond from the terminal methyl group is used to define and classify the classes of PUFAs. The carbon in the terminal methyl group is known by the Greek letter Omega (ω). Other designation may use the letter 'n' instead. Hence, PUFAs can be classified into ω -3, or n-3 (the double bond 3 carbons away from ω carbon) and ω -6 or n-6 (6 carbons away from ω) (Ziboh 1996).

1.3.3 Essential fatty acids (EFA)

EFA is the term often used to encompass the closely related n-3 and n-6 fatty acids. This term was derived after the discovery that these fatty acids are important for a lot of biochemical processes in the body and not just a fuel source. The use of the term is further strengthened by the fact that they cannot be synthesized *in vivo* and can only be obtained from dietary sources. A third group of PUFAs, the omega-9 FAs, the primary example being oleic acid (18:1), is not considered an EFA, as the human body possesses all the necessary enzymes required for its production (James et al. 2000).

The main fatty acids for each class of EFAs are linoleic acid, LA (18:2, n-6) and alpha-linolenic acid, ALA (18:3, n-3). From these 2 precursors, longer fatty acids with a higher degree of unsaturation can be synthesized, therefore, strictly speaking, only these 2 fatty acids are 'essential'. However, considering the role of the resulting long chain FAs, a lot of researchers use the term EFAs for all of the physiologically important PUFAs belonging to the 2 omega groups. The longer chain fatty acids are as follows:

n-3 fatty acids

- ❖ Stearidonic acid, SDA (18:4)
- ❖ All-*Cis* 8,11,14,17-Eicosatetraenoic acid, ETA (20:4) – an intermediary between SDA and EPA
- ❖ Eicosapentaenoic acid, EPA (20:5) (Figure 1.15a)
- ❖ Docosahexanoic acid, DHA (22:6) (Figure 1.15b)

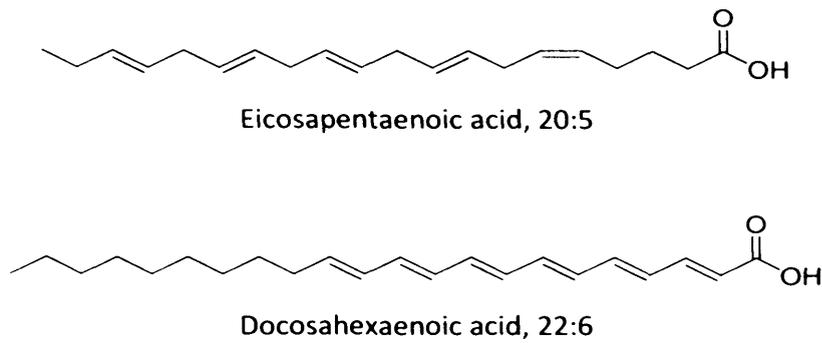


Figure 1.15 : a) Eicosapentaenoic acid (top) and b) docosahexaenoic acid (bottom)

n-6 fatty acids

- ❖ Gamma-linolenic acid, GLA (18:3)
- ❖ Di-homo Gamma-Linolenic acid, DGLA (20:3)
- ❖ All-Cis 5,8,11,14-Eicosatetraenoic acid (Arachidonic acid), AA (20:4)

1.3.4 Sources of EFAs

The EFAs are obtained through the diet, either as the precursor forms (LA and ALA) or the longer chain derivatives. Major sources of LA are egg yolk, lard, meat, and the oils of corn, peanut, safflower, soy, sunflower and walnut (Connor 1999; James et al. 2000), while flaxseed, canola, and green leafy vegetables are particularly rich sources for the n-3 counterpart, ALA. γ -linolenic acid (GLA), the elongation product of LA and the precursor for AA and dihomo- γ -linolenic acid (DGLA) can be found in substantial amounts in evening primrose oil, a popular dietary supplement taken for relieving symptoms of pre-menstrual syndrome. Other sources include borage, blackcurrant seed and hemp seed oil.

1.3.5 Fish oil as a rich source of n-3 FAs

Unlike its precursor, ALA, the 2 major n-3 fatty acids EPA and DHA can only be obtained from animal sources, apart from perhaps certain species of microalgae. Fish oil, particularly fatty or oily fishes, which are typically found in colder waters, are the primary source for the 2 FAs. There are at least 50 different fatty acids, ranging from C₁₄ to C₂₄ in chain lengths, fatty acids with differing degrees of saturation branching, different isomers, and other characteristics, with EPA and DHA accounting for 18-36% of total FAs in fish oil (Haraldsson and Hjaltason 2001). The fatty acids exist in the form of triglycerides or are incorporated in the membrane as phospholipids. In fact, the phospholipids in fish are even more enriched in EPA and DHA compared to the triacylglycerols, ranging from 40-55%. However, the phospholipids are not considered as a viable source of EPA and DHA because of their low amount (1-1.5% per body weight) and the difficult extraction processes required (Haraldsson and Hjaltason 2001)

The types of fish include salmon, menhaden, herring, mackerel, sardine, trout, anchovy, and sprats, among others. The long chain n-3 FAs are accumulated in the flesh of these fish as they pass up the food chain resulting from consumption of phytoplanktons.

1.3.6 Physiological functions of EFAs

In the body, the fatty acids are important towards a lot of physiological processes and functions. They are part of the phospholipids which form the cellular membranes. One of the most important roles of the PUFAs is the formation of inflammatory mediators or eicosanoids from this membrane

reservoir in response to noxious stimuli, and will be the main thrust of this study and will be elaborated further in the coming section.

The EFAs are also an important component in signalling pathways within the body. Formation of endogenous cannabinoids e.g. anandamide from arachidonic acid, is still an area which is poorly understood. This class of lipid messenger is found in most tissues and is at present believed to be involved in pain sensation (Pertwee 2001), mood regulation (Martin et al. 2002), appetite (Di Marzo et al. 2001; Kirkham and Tucci 2006), memory and cognitive learning (Marsicano et al. 2002; Martin et al. 2002)

The EFAs play a significant role in the development and maintenance of the nervous system, with DHA perhaps the most important FA of them all. The human brain is composed mainly of fat, and 40% of the constituent PUFAs are DHA (Singh 2005). 60% of the PUFAs in the human retina are also made up of DHA. There is a direct correlation between the levels of DHA in infants supplied through breast milk and the level of neurodevelopment and visual acuity, as reported by Jensen (2001).

1.3.7 EFAs and inflammation

As mentioned earlier, the EFAs play a major role in the inflammation process within the body. They are the precursors of the mediators of the inflammation/AA cascade (Figure 1.16) termed the eicosanoids. The n-6 FA, arachidonic acid (AA, 20:4) is the primary FA in the phospholipid membrane of the cell, and upon hormonal release or physical stimulation, e.g. injury, trauma, is liberated from the membrane by the action of phospholipases. Depending on the subsequent enzymatic pathways (cyclooxygenases or lipoxygenases), it is converted to prostaglandins, leukotrienes and thromboxanes (Wada et al. 2007). Despite the beneficial role of protecting

the body, inflammation, when it becomes out of control, has been implicated in the pathogenesis of diseases such as cardiovascular problems, asthma, arthritis, diabetes, inflammatory bowel diseases, and skin diseases, including psoriasis.

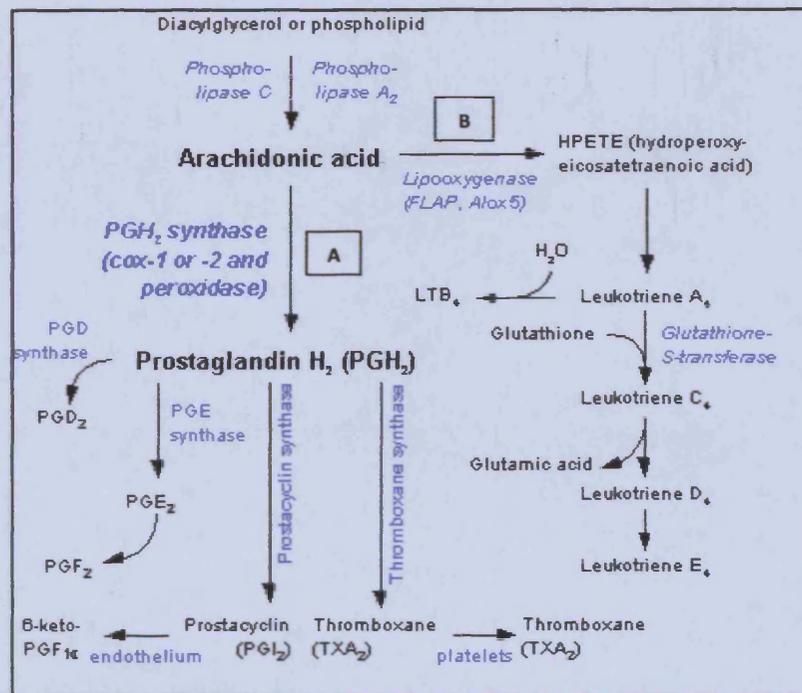


Figure 1.16 : Inflammation/AA cascade (Davis et al 2002). Arachidonic acid liberated from diacylglycerol of phospholipids are converted to different classes of eicosanoids depending on the enzymatic pathway. The cyclooxygenase pathway (A) results in production of prostaglandins (PGs) and thromboxane (TXAs), while the lipoxygenases pathway (B) lead to production of leukotrienes (LTs).

It is generally regarded that eicosanoids derived from AA are pro-inflammatory and have been reported to be over-expressed in inflammatory diseases. High levels of leukotriene B₄ (LTB₄) is characteristic of arthritis, Crohn's disease, ulcerative colitis and lupus erythematosus (Simopoulos 2002). Along with the oxidation product of AA, 12-hydroxyeicosatetraenoic acid (12-HETE), LTB₄ has been found in high amounts in psoriatic lesions and implicated in the pathogenesis of the disease (Fogh et al. 1989; Iversen et al. 1997). It is a powerful chemotactic and chemokinetic agent for leucocytes

and neutrophils. It has also been shown to induce DNA synthesis and promote hyperproliferation in keratinocytes (Fogh and Kragballe 2000).

Prostaglandin E₂ (PGE₂), another major product of AA metabolism by COX-2, plays a major role in tissue oedema, hyperalgesia, and IL-6 production at sites of inflammation (Ruzicka et al. 1986). It also has been implicated in psoriasis, although its exact role in the pathogenesis was not properly elucidated (Ikai 1999). In the lesions, up to 5 times increase were seen compared to normal skin (Reilly et al. 2000), while another study suggested that the increase is as high as 40% compared to non-involved skin (Ikai 1999).

The modern human diet tends to have an imbalance between the n-6 and n-3 fatty acids. This is related to the reduction of fish consumption caught from its natural habitat, and an increase in consumption of meat and domestically raised fish which are fed with high contents of n-6. NHANES III (United States Department of Health and Human Services 2004), a database compiling the nutrient consumption of Americans, reported a median intake of 0 g for EPA and DHA, and less than 1 g of ALA.

1.3.8 *n-3 Fatty acids and inflammatory diseases*

In his seminal work published in 1987, (Horrobin 1987) reported a low incidence of inflammatory diseases such as congestive heart diseases, rheumatoid arthritis, asthma and psoriasis among the indigenous Inuits of Greenland. This was attributed to their high EPA intake from fatty fishes, which is found in abundance in the cold waters surrounding the island. Since his findings, much effort has been taken to study the apparently beneficial effects of n-3 fatty acids in reduction and treatment of diseases with inflammatory components. Most of the studies over the years had focused on cardiovascular diseases, the leading killer of man of recent times, and the

role of n-3 as a cardioprotective agent (Davignus et al. 1997; Harper and Jacobson 2001; Lopez-Garcia et al. 2004; Weber and Leaf 1991). Nevertheless, Calder (2001) concluded that the use of high levels of EPA and DHA are supported in patients with other forms of chronic inflammatory and allergic diseases. A list of inflammatory diseases reported to be responsive to supplementation with n-3 is shown in Table 1.2.

The way n-3 FAs affect inflammatory process lie in its interaction with n-6 FAs. It has been well documented that supplementation of n-3 FAs lead to their incorporation into cellular membrane replacing AA as the major fatty acid in the phospholipids (DeFilippis and Sperling 2006). As a consequence, less n-6 will be available for liberation. Meanwhile, metabolism of n-3 FAs yield less potent inflammatory mediators, namely the 5-series LTs and 3-series PGs. LTB_5 , for example is 10 times less potent than LTB_4 (Mayser et al. 2002). Another aspect of the relation between n-3 and n-6 apart from the altered lipid profile of the cell membrane is the relative affinity of the FAs towards enzyme within the inflammation cascade. The n-3 FAs have a higher affinity towards the binding site of both COX and LOX, leading to competitive inhibition of n-6 metabolism.

Acute respiratory disease syndrome
Allergic diseases
Asthma
Atherosclerosis-related cardiovascular diseases
Inflammatory bowel diseases
Osteoarthritis
Psoriasis
Rheumatoid arthritis
Traumas of multiple aetiology
Viral and bacterial pneumonia

Table 1.2 : List of diseases responsive to n-3 supplementation (Gil 2002)

Apart from the n-3s competition with n-6 fatty acids, they are believed to assist alleviation of inflammation by the formation of newly identified lipid mediators or 'lipoxins' such as resolvins and protectins (Schwab and Serhan 2006). These bioactive compounds play a role in resolving inflammation –

sort of a braking mechanism which is switched on during the 'resolution phase' of inflammation. These lipoxins display potent anti-inflammatory, pro-resolving, immunoregulatory and neuroprotective properties (Serhan and Savill 2005).

1.3.9 *n-3 and psoriasis*

As mentioned earlier, inflammation plays a central role in the pathogenesis of psoriasis. Therefore, psoriatic patients could theoretically benefit from n-3 supplementation. Previous works by (Mayser et al. 2002) and Kojima et al (1991), working with intravenous EPA and highly purified EPA capsule supplements respectively, found a marked improvement in patients. This concurs with an earlier clinical trial done by (Bittiner et al. 1988) proving the benefits of fish oil in alleviating symptoms of psoriasis.

The effects of n-3 on inflammatory enzymes such as COX-2 and 5-LOX had also been studied (Thomas et al. 2007a). These two enzymes are the main metabolizing enzymes in the skin and the enzymes or the metabolic product have been implicated in inflammatory skin conditions such as psoriasis. The study employed immunohistochemical methods on freshly excised porcine skin treated with fish oil on its own and in combination with other agents such as ketoprofen. It was found that a mixture of the two provided the highest degree of enzyme inhibition, signified by the reduced intensity of the staining. This supported the hypothesis of a combined action of both agents in COX-2 inhibition; ketoprofen, a known COX-2 inhibitor and EPA which actively competes with arachidonic acid to produce less potent inflammatory mediators.

In the skin, EPA is metabolized by epidermal 15-lipoxygenase (15-LOX) to monohydroxylated metabolites, 15(S)-hydroxyeicosapentaenoic acid (15-

HEPE) and 15(S)-hydroxyeicosatrienoic acid (15-HETrE). These metabolites share the ability of EPA in competing with arachidonic acid within the inflammation cascade to produce less potent inflammatory mediators, with Vang & Ziboh (2005) suggesting that the metabolites are even more potent than EPA with regards to this process. Depth profile analysis by Thomas and Heard (2007) revealed that the greatest conversion of topically applied EPA to the more potent 15-HEPE occurred at the metabolically active basal layer of the skin. It is postulated that this is of particular benefit in treatment of psoriasis, as this layer is in a state of hyperproliferation (thus, higher metabolic activity) in psoriatic skin.

1.4 Models of psoriasis

The use of animals in scientific procedures and experimentations hark back to the time of the Greeks, where references of animal experimentation were recorded as early as the 3rd century BC (Cohen and Loew 1984). The contribution of animal studies is immense, especially with recent advances in development of humanized and transgenic animal models exhibiting genes implicated in the pathogenesis of human diseases (Laboratory Primate Advocacy Group 2005).

In the study of psoriasis, there have been numerous models, particularly murine, utilized in research over the years. Due to the complex nature of the disease, these models understandably have certain limitations in the reproduction of psoriasis and psoriatic arthritis characteristics, exhibiting different parts of the numerous pathogenic mechanism involved. Nevertheless, collectively these models have improved the understanding of how psoriasis develops and crucially, providing the next step in finding a potential cure. In recent times, there have also been claims of the discovery

of a 'true' animal model of psoriasis which exhibits switchable triggers for both psoriasis and psoriatic arthritis (Zenz et al. 2005).

In general, animal models for psoriasis can be divided into several categories, the first one being mutant models which occurred spontaneously. The next category involves animals that are genetically engineered, either by the insertion of a foreign gene or knocking out specific genetic target. This category also involves manipulation of the epidermis, endothelium or component of the immune system e.g. leucocytes, and those that are induced (either by immune transfer or by xenotransplantation of skin from psoriatic patients).

1.4.1 *Spontaneous animal model*

1.4.1.1 *Asebia (Ab) mouse*

The asebia mutation was first reported about 30 years ago in a colony of BALB/c Crg/Ga mice. The Ab mutant is characterized by hypoplastic sebaceous and modified sebaceous glands (meibomian, preputial, clitoral, and ceruminous glands), the mutation mapped to the gene Stearoyl-Coenzyme A Desaturase 1 (SCD-1) (Sundberg et al. 2000). They have short sparse coat of hair, scaly skin, predisposed to corneal opacities development, and the livers are deficient in triglycerides and cholesterol esters (Miyazaki et al. 2001). Other similar mutation exists, such as the asebia-J and the asebia-2J (Sundberg et al. 2000).

1.4.1.2 *Flaky skin (Ttc7^{fsn} / Ttc7^{fsn})*

Flaky skin or fsn mice exhibit epidermal hyperproliferation, with mixed inflammatory infiltrate with neutrophils accumulating in epidermal microabscesses, and increased dermal vascularity with dilated blood vessels (Schön 2008). It is perhaps the best described animal model for psoriasis (Danilenko 2008). Unlike wild type mice which have thickened epidermis that thins after 2 weeks of age, this spontaneous mutant progressively undergoes epidermal thickening until reaching a point of stabilization. Dysregulation in T- cell activation in fsn mice is a trait commonly shared with the pathogenesis of psoriasis in human, thus the skin disorder developed in this mutant exhibits many biochemical and histopathological features found in some forms of psoriasis (Abernethy et al. 2000). Interestingly, the mutation on chromosome 17 which gives rise to this phenotype is homologous to the 2p21-22 region of chromosome 1 in humans. This region has been identified to be a potential susceptibility region in human psoriasis (Bhalerao and Bowcock 1998)

1.4.1.3 *Chronic proliferative dermatitis (Sharpin^{cpdm} / Sharpin^{cpdm})*

This mutation, mapped to chromosome 15, results in development of scaly lesions that begin as patches which subsequently will cover the entire length of the body. This is accompanied by erythema and thinning of hair. Histological examination of the epidermis shows hyperplasia that is patchy in young mice but diffuse in mice 5 weeks of age and older. The prominent features are acanthosis with orthokeratotic hyperkeratosis and patchy parakeratotic hyperkeratosis (Schön 1999). The epidermis and dermis both exhibit increased immune cell infiltration, particularly eosinophils. Severe pruritus, exacerbated by scratching leads to ulceration which may necessitate culling of older mice. Apart from the skin, several other organs are also

affected, and the mechanism appears to be independent to T-cells (Schön 2008). Along with the *fsn*, this mutation does not appear to respond to certain anti-psoriatic medication (Gijbels et al. 2000; Sundberg et al. 1994) and driven by cytokines of Th2, instead of the Th1 and Th7 driven processes of psoriasis. As such it is not widely considered representative of the disease (Danilenko 2008).

1.4.2 Genetically engineered animal models

Alternatively, another approach in studying the pathophysiology of psoriasis in animal models involves modification of the genes central to the development of psoriasis. Transgenic and knockout animals constitute the largest form of animal models for psoriasis (Danilenko 2008). The genes involved include those which code for adhesion molecules, cytokines, transcription factors and other cellular mediators in which modulation in these proteins expression gives rise to psoriaform features. Examples of the molecules include transforming growth factor- α (TGF- α), interleukin-6 (IL-6), keratinocyte growth factor (KGF), IL-1 α , vascular endothelial growth factor (VEGF), bone morphogenic protein-6 (BMP-6), amphiregulin, p40, IL-20, collagenase, MEK1, leucocyte b2 integrins (CD18), IL-1ra, and IRF-2 (Schön 2008).

1.4.2.1 Xenotransplant model

Transplantation of human psoriatic skin (both involved and non-involved) onto animals have been attempted successfully and had contributed largely to the understanding of psoriasis. In relation to the other models discussed earlier, this model is perhaps the best representative to psoriasis in human. On the other hand, it is the most difficult to maintain and achieving consistency can be a hindrance (Danilenko 2008). The discovery of the

possible role of T-cells in psoriasis was a result of grafting skin from a psoriasis-susceptible patient to an athymic immunodeficient mouse (Nickoloff 2000). This model has been used extensively in the study of chimeric monoclonal antibody and fusion protein, in which the psoriatic lesions on the mice responded positively to agents such as infliximab, efalizumab and etanercept (Boyman et al. 2004; Xia et al. 2003). These agents were previously shown to work in humans, and the response of the animal models proves the close similarity to psoriasis.

1.5 *Defolliculated, GsdmA3^{Dfl}/+ (DfL) mouse – a new model for psoriasis*

This mouse model (Figure 1.17) belongs to the spontaneous mutation category. The first incidence of DfL mouse was reported in Bonn, Germany (Porter et al. 2002) as a spontaneous mutation occurring in a colony of BALB/c mice. The mutation has been mapped to the gasdermin gene at chromosome 11. The association was discovered when the finnegan (fng) mouse, induced by chemical mutagenesis, showed a similar phenotype with DfL (Lunny et al. 2005; Nolan et al. 2000).



Figure 1.17 : Defolliculated, $GsdmA3^{Dfl/+}$ at 20 weeks of age

The exact function of gasdermin is yet unclear, however immunohistochemistry analysis revealed that it may have a role in the differentiation of the epidermis and associated appendages (Lunny et al. 2005). Specifically, the gene was found to be localized in cells at the later stage of differentiation of the upper epidermis, hair shaft and the inner root sheath, and sebocytes of the sebaceous, preputial, meibomium, ceruminous and anal glands. Both mutants exhibited a defective hair cycle leading to a predictable, non reversible destruction of hair follicles beginning at the age of 9 weeks (Figure 1.18) (Lunny et al. 2005; Porter et al. 2002).

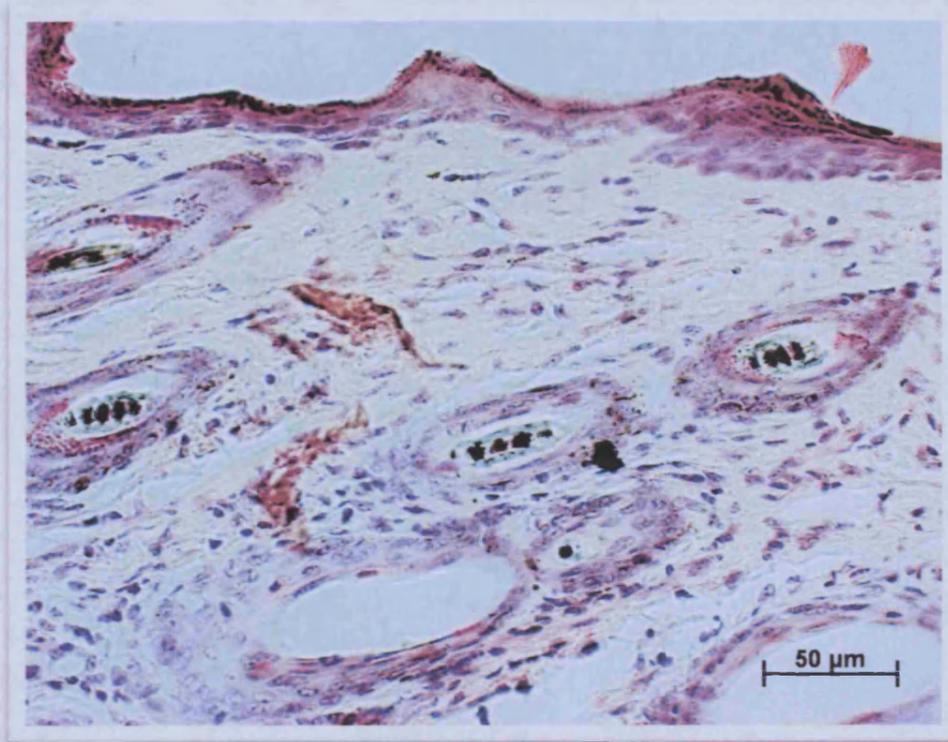


Figure 1.18 : H&E stain of a DfL skin section of the back showing the early phases of hair follicle destruction

Due to the localization of the gene in sebocytes, sebum production is defective in DfL, leading to dryness of skin, formation of scales, shortened hair shaft (prior to total destruction of the follicles), and formation of corneal opacities in older mice (Figure 1.19) (Porter et al. 2002). In regards to the defects in sebum production, this model bears a resemblance towards the asebia mutant described previously, although in asebia the mutation occurs in an enzyme involved in sebum production (Sundberg et al. 2000). Immediately from birth, DfL mice in the litter can be recognized through their thick and folded skin. As they age, the gradual hair loss begins, followed by acanthosis and hyperkeratosis of the epidermis. Concurrently, the expression of proliferative markers such as Ki67, K6, K16, and K17 is increased (Porter et al. 2002).



Figure 1.19 : Formation of corneal opacities and severe scaling in a 20 weeks old DfL

The dermis is highly populated with cells of the immune system. These include CD4+ T-cells, macrophages, fibroblast and mast cells, while the epidermis shows increased presence of CD3+ T cells. The continuous presence of immune cells during the period of hair loss led to the initial assumption that the destruction of hair follicles was immune-driven (Porter et al. 2002). A later study, however, suggested that both the phenotypic changes and the increased infiltration of the dermis and epidermis were in fact a result of the defect in sebum production (Lunny et al. 2005).

The hyperproliferative state of the epidermis and the increased infiltration of immune cells however are of interest in the study of psoriasis. As mentioned earlier, DfL mice show a resemblance toward another established psoriasis model, the *ab* mutation. Previously forwarded as a model for scarring alopecia in humans (Porter et al. 2002), it is now proposed that the mutation be studied as a model for psoriasis as an alternative to other existing animal models.

1.6 Topical drug delivery

The vast majority of therapeutic agents are delivered into the body via the oral route. It is the easiest route, and provides a convenient way for patients to self administer their medication. However, certain issues, such as hepatic first-pass metabolism and lower bioavailability at the intended target in skin diseases, meant that direct application of the drug on the skin is much more preferable. Apart from targeting the disease directly at the site, higher bioavailability at the treatment site cuts down on the dose of the drug that needs to be given, in effect reducing the potential side effects caused by non-specific modes of drug delivery. Often a major cause in decreased patient compliance, the reduction in toxicity also has the added benefit of increasing patient's acceptability and overall compliance (Berti and Lipsky 1995). Modes of drug delivery across skin can be categorized into *topical*, *transdermal* and *transcutaneous*. Topical delivery is intended to provide a local effect, with minimal absorption into systemic circulation. On the other hand, transdermal drug delivery involves delivery of the drug through the skin to provide a systemic effect. In transcutaneous delivery, the drugs are intended to be delivered into the tissues underlying the skin, for example muscles or joint tissues.

1.6.1 *The skin as a physiological barrier*

The skin (Figure 1.20), which represents 17% of total body mass, is the largest organ of the human body. The primary function of the skin is to serve as a barrier between the human body and the environment, providing protection against physical, chemical and radiation injuries, microbial invasion, and water loss. It is through the latter that the skin also acts a regulator of fluid and electrolyte balance (Berti and Lipsky 1995; Williams 2003).

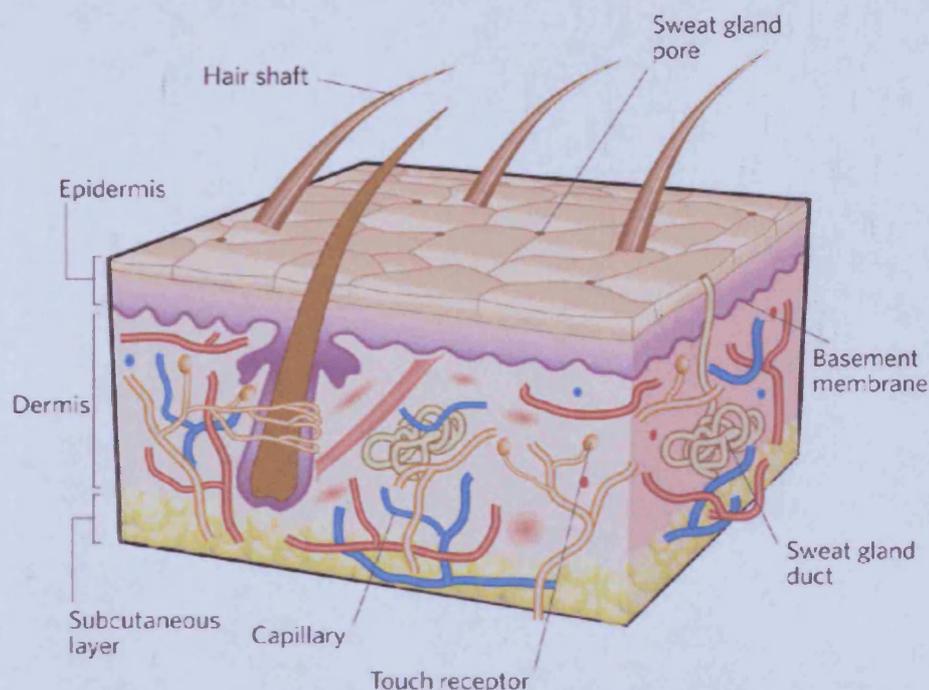


Figure 1.20 : A graphical representation of the skin structure (MacNeil 2007)

In relation to drug delivery into and across the skin, the most important layer is the epidermis. Separated from the dermis by a basement membrane, the epidermis can be further divided into 4 distinct layers (Figure 1.21). From outside to inside, they are the stratum corneum, stratum granulosum, stratum spinosum, and stratum germinatum or the basal layer. The top layer, stratum corneum, is made up of 10-15 layers of keratinized/cornified cells embedded in an intercellular lipid matrix. Underneath this layer is what has been termed the viable epidermis to distinguish it from the dead, anucleate cells of the SC (Berti and Lipsky 1995). The cornified cells or corneocytes are surrounded by a proteinaceous cell envelope, ~80% consisting of α and β -keratin. This is then bound to a lipid envelope (consisting of multiple lipid bilayers) through glutamate moieties of the protein envelope (Williams 2003). The cohesion between individual corneocytes is further strengthened by structures called desmosomes.

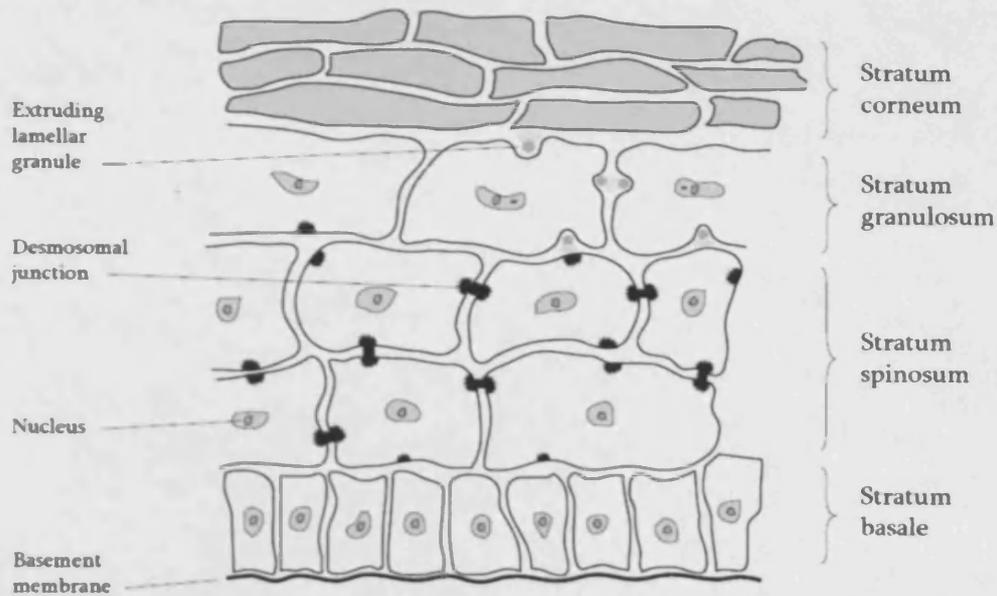


Figure 1.21 : The layers of the epidermis (Williams 2003)

The intercellular lipid is constituted by 40% ceramide, while the remainder consists of cholesterol, cholesteryl esters, cholesteryl sulfate, free fatty acids (mainly long chain members, such as 22:0 and 24:0). Phospholipids and glycolipids are present in a very minor amount (Motta et al. 1993). In healthy skin, the unique lipid composition of the matrix is an important attribute to enable the SC to be an effective barrier for water loss and entry of external matter. The major lipid of the SC, the ceramides, can be divided into 9 types according to their structure. There are ceramides which contain sphingosine and phytosphingosine with amide-linked non-OH fatty acids, α -OH fatty acids or ω -OH saturated fatty acids of varying chain length (Bouwstra and Ponec 2006). Also present are ester linked ceramides, and ceramides covalently-bound to the protein envelope (Motta et al. 1993). In the topical delivery of drugs, the continuous lipid matrix represents the primary pathway and the major limiting barrier by which drug molecules traverse across the skin (Williams 2003).

The integrity of the SC is also highly dependent on the water content. A well hydrated SC is important due to the presence of hydrolytic enzymes involved with SC desquamation and enzymes involved in the production of natural moisturizing factor, both regulated and mediated by water (Williams 2003).

1.6.2 *Factors affecting drug delivery to the skin*

The delivery of drug into skin is governed by several factors that can broadly be categorized into physiological and physicochemical factors.

1.6.2.1 *Physiological factors*

As with any pharmacological intervention, there exists a variation between individuals in terms of percutaneous absorption of drugs. With regards to in vitro studies with animal models of skin permeation, it is a pertinent point to acknowledge the variation that exists between different species as well. Age plays an important role in determining percutaneous absorption, in view of the structural changes associated with aging. In neonates, the dermis is approximately 60% the thickness of adults, while the SC is rudimentary and only a few cells thick. The majority of the evidence available suggests only minimal or negligible effects of aging over normal skin. Nevertheless, changes such as water content and hydration status (which decreases with age), effects of accumulated exposure to UV, chemicals and environment, and changes in blood flow, can theoretically affect drug absorption and clearance in the skin. Variation in SC thickness according to body sites is a huge contributing factor, as the SC is the principal barrier for drug delivery across the skin. This factor also influences the choice of treatment sites, particularly with transdermal formulations with intended systemic effects. In general, the genitals are considered to be the most permeable, followed by the head and

neck, trunk, arm, and finally the leg. Significant differences exist in the skin between races, such as the SC water content and levels of pigment/melanocytes. As with aging, available data suggests minimal or negligible contribution of race to drug permeation into the skin. The most relevant factor to this study is the effect of pathological conditions or disease of the skin. In most diseases, the barrier function of the skin is compromised, owing to structural defects and changes in hydration and lipid profile of the skin, among others. As treatment progresses, the barrier properties change as the condition improves (Washington et al. 2002; Williams 2003).

1.6.2.2 *Physicochemical factors*

The physicochemical properties of the permeant form a major determinant for skin permeation and absorption. In order to traverse the multiple layers of the SC, a permeant must first partition into the skin. The octanol-water partition coefficient ($\text{LogP}_{\text{octanol/water}}$) is an indicator of the lipophilicity of the permeants, with a value <1 indicative of a hydrophilic molecule, and >3 of a highly lipophilic molecule. The keratinized cells of the SC are hydrated, therefore a hydrophilic permeant is more likely to partition into the cell (intracellular route), while lipophilic permeants would partition into the lipoidal matrix surrounding the cells (intercellular route) (Williams 2003). The route chosen is not exclusive for any given permeants, as polar regions exist even within the lipoidal domain, e.g. desmosomes. Furthermore, even if a lipophilic molecule traverses mainly in the lipoidal domain, in order to exit the SC, it needs to partition into the highly hydrated viable epidermis. An ideal permeant would then need to be sufficiently soluble in both oil and water phases. Typically these molecules will then have a LogP value between 1 to 3 (Williams 2003).

The second factor is the molecular weight of the molecule. Most conventional agents selected for topical and transdermal delivery lie within the range of 100-500 Dalton. However, barrier defects in diseased states means that this rule may not be restrictive, as the delivery of larger molecules have been confirmed across diseased skin (Gribetz et al. 2004; Steele et al. 2005).

1.6.3 Barrier impairment in diseased state - psoriasis

In many skin conditions, the diseased skin is characterized by an impaired barrier function (Bouwstra and Ponc 2006). In psoriasis, the impairment is a result of several factors. Aberrant keratinization of the cells is a major contributor, with altered lipid content of the matrix also determined. There is an increase in free cholesterol, with a reciprocal reduction in cholesteryl esters. Examination of psoriatic scales also revealed an alteration in the levels of phytosphingosine-containing ceramides, with 25% in psoriatic scale compared to 44% in normal SC. This is accompanied by a concurrent increase in sphingosine containing ceramides. The conversion of sphingosine to phytosphingosine has been determined to involve water; therefore, the reduction in phytosphingosine is associated with the increased water loss observed in the dry, scaly skin of psoriasis (Motta et al. 1993).

Another variation is the reduction of the ω -OH ceramides, the bound fatty acid chain consisting mainly of linoleic acid. This is surprising considering the reported evidence of increased free linoleic acid and fatty acid esters in psoriatic scale. The products of AA metabolism, such as LTB_4 and PGE_2 , the precursor of which is linoleic acid, have also been found to be increased greatly in psoriasis (Logan 2005). The association with the reduction in linoleic acid bound ceramides is then thought to be due to impairment in the enzymatic process involved with the production of the ω -OH ceramides.

These defects in the barrier property in psoriatic skin undoubtedly alter the permeation of drug molecules across skin. Colombo et al. (2003) reported an increase in the cumulative amount of 8-methoxypsoralen recovered from psoriatic skin compared to normal skin. Wang et al.(1987) observed a variation in the permeation of various anthralin preparations across psoriatic skin in relation to the stage of the disease. In all cases, the permeation was not limited by the SC, as observed in normal skin; rather, it depended on the rate of release from the vehicle. This suggested the defective barrier of psoriatic skin provides minimal resistance to travelling drug molecules.

The permeation of two experimental therapeutic agents for psoriasis were also investigated. A C-5 propyne-modified antisense oligonucleotide, which was found not to permeate normal SC, was successfully delivered intact to the basal layer of psoriatic skin (White et al. 2002). Meanwhile, Gould et al. (2003) reported a ten-fold increase in the permeability of involved psoriatic skin compared to uninvolved skin using a radio-labelled protein, plasminogen activator inhibitor 2, as a permeant.

These are only few examples which show the altered barrier state of the psoriatic skin, and indeed this irregularity has been exploited successfully in the delivery of anti-psoriatic medication across the skin. The cyclosporine-like tacrolimus and pimecrolimus, which in normal conditions would be notoriously difficult to deliver across skin owing to their high molecular weights (803 and 810 MW, respectively), have been used successfully in the treatment of psoriasis (Gribetz et al. 2004; Hebert et al. 2001; Steele et al. 2005).

1.6.4 Topical delivery of fish oil and EPA

Previous work involving EPA/fish oil involved mostly delivery via oral or intravenous routes. It is hypothesized that delivering n-3 fatty acids directly to the skin via topical application would result in a better outcome. In fact, trials with topically applied fish oil and n-3 mixtures have been attempted to varying effects. It has been proven that topical application of n-3 fatty acids led to its their incorporation in the cell membrane of animal models (Mani et al. 1999), a process previously observed with dietary supplementation (Chapkin et al. 1987).

Several clinical trials have reported the use of topically applied fish oil and pure n-3 fatty acids on psoriatic patients, with varying outcomes. Dewsbury et al. (1989) applied a commercial fish oil preparation (MaxEPA 10%) compounded in an ointment base in a small trial of eleven patients, with eight patients showing clinical improvements.

Escobar et al (1992) determined the efficacy of topically applied fish oil in reducing psoriasis symptoms compared to liquid paraffin. Both treatments were applied daily under occlusive dressing for 6 hours and the duration of treatment was for 4 weeks. The parameters investigated were erythema, scaling, plaque thickness (induration) and itching, on a weekly basis. It was found that both treatments improved erythema and scaling compared to base values, while there was a significant difference between the two treatments in reduction of plaque thickness and scaling.

In contrast, a further study by Henneicke-von Zepelin et al. (1993) which used a higher purity EPA, DCHA mixture (80% EPA-ethyl ester and 20% DCHA-ethyl ester) did not show any statistically significant improvement compared to control. The difference in response was attributed to a pharmacokinetic problem or poor formulation arising from the fact that pure forms of n-3 do

not penetrate the skin as well as the mixed fish oil (Grimminger and Mayser 1995).

1.6.5 Fish oil as a drug delivery enhancer

As mentioned previously in Section 1.6.1, the SC poses a substantial barrier for delivery of drug across skin. There are several strategies in which the delivery of drugs can be enhanced, e.g. iontophoresis, increasing hydration of the skin through occlusion and the use of chemical enhancers. One of the chemical enhancers used are fatty acids. The use of fatty acids has been demonstrated to enhance the delivery of estradiol, progesterone, acyclovir, 5-fluorouracil, retinoic acid and salicylic acid, as examples (Williams 2003)

The mechanism of enhancement by fatty acids is related to the intercellular lipid domain, which, as mentioned previously, is the primary route of transport for drugs. The lipophilic nature of fatty acids allows it to traverse easily across the lipid domains of the stratum corneum. This fact can be utilized by polar drugs which are hydrophilic in nature.

In the study done by Heard et al. (2003), the delivery of NSAIDs were enhanced by EPA and DHA from a fish oil vehicle. It was found that the permeation rates of the NSAIDs were increased correspondingly with the increase in permeation rates of the vehicle and vice-versa, suggesting that a solute permeates skin complete with its vehicular solvation cage. This phenomenon was later termed the 'pull' or 'drag' effect (Heard et al. 2006). The effects of fish oil and delivery of NSAIDs were explored further (Puglia et al. 2005; Thomas and Heard 2004, 2007; Thomas et al. 2007b) and NMR studies suggested that the enhancement was due to a formation of complex between EPA and ketoprofen. Thus, the mechanism by which the delivery of ketoprofen was enhanced by EPA and vice versa can be explained by their

different properties across the skin layers. Once the complex is formed, the triglyceride/free fatty acid could aid permeation of ketoprofen into the lipophilic stratum corneum via the pull effect. Once permeated, the process is taken over by the more hydrophilic ketoprofen leading the permeation of the triglyceride/free fatty acid through the epidermis.

1.7 Aims and objectives

The objective of this study was to investigate the effects of combining fish oil with commonly used anti-psoriatic medication both on the delivery and its activity.

The associated aims were as follows:

- Determination of fish oil permeation and penetration profile across the skin
- Determination of fish oil enhancing properties for delivery of common anti-psoriatic medications
- Investigation of the anti-inflammatory properties of fish oil by its action on inflammatory markers and enzymes
- Determination of in vivo response of a proposed animal model for psoriasis to anti-psoriatic medications and fish oil

Chapter 2:

*In vitro transcutaneous delivery
of bioactive oils across excised
porcine ear skin*

2.1 Introduction

Bioactive oils are naturally occurring or synthetically produced lipophilic liquids which have an effect on or cause a reaction in living tissues. The action of these oils is dependent on the constituent fatty acids.

Fish oil, is one of the most commonly found bioactive oils in the market. Available in forms ranging from capsule to liquid suspension, the demand for fish oil is attributed to its many health benefits which were outlined in the Section 1.3.8 and 1.3.9. The principal fatty acids in fish oil are the omega-3 fatty acids, namely EPA and DHA. Many manufacturers often fortify their fish oil preparations with additional EPA and DHA. Far from being just a nutritional supplement, fish oil has also been licensed as an adjuvant treatment for post-myocardial infarction patients and control of hypertriglyceridaemia when dietary controls are insufficient (Bays 2006).

Although previous work has demonstrated the skin delivery of EPA and DHA from fish oil (Thomas and Heard 2004) and GLA from borage oil (Ho et al. 2004), the phenomenon is considered unlikely according to regular skin permeation theory owing to the high lipophilicity of the fatty acids. In the current chapter it was considered important to confirm such findings and to determine if similar observations could be obtained with further bioactive oils; thus, the skin delivery of dihomo- γ -linolenic acid (DGLA) and 15-Hydroxyeicosatetraenoic acid (15-HETE) were probed.

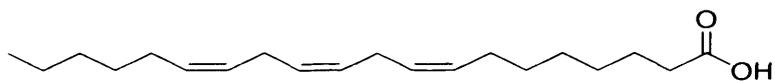


Figure 2.1 : Chemical structure of dihomo- γ -linolenic acid, 20:3,n-6

DGLA (Figure 2.1) is a 20 carbon long chain omega-6 fatty acid obtained from animal sources such as meat, egg, fish and breast milk (Kawashima et al. 2009). It is also available in precursor form (γ -linolenic acid) obtained from

sources such as evening primrose oil. It is derived from the desaturation and subsequent elongation of linoleic acid (C18:2, n-6). Unlike other n-6 fatty acids which are regarded as pro-inflammatory, e.g. arachidonic acid, DGLA possesses anti-inflammatory actions via production of 1-series prostanoids, thromboxanes and a 15-hydroxyl derivative which blocks the transformation of arachidonic acid (AA) to pro-inflammatory leukotrienes (Belch and Hill 2000; Fan and Chapkin 1998). Similarly to the n-3 fatty acids, this is achieved by active competition with AA for binding sites on cyclooxygenases.

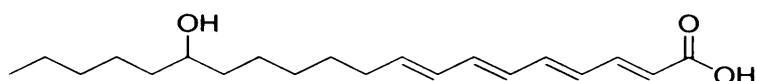


Figure 2.2 : Chemical structure of 15-(±) HETE

15-HETE (Figure 2.2) is derived from the metabolism of AA by 15-LOX (Vonakis and Vanderhoek 1992). Unlike products of 5- and 12-LOX metabolism, this monohydroxylated metabolite possesses anti-inflammatory activity by inhibiting the 2 enzymes; the products of which are pro-inflammatory in the skin (Fogh and Kragballe 2000; Fogh et al. 1988).

In the current study, the fatty acid content and delivery of fish oil, and refined oils containing 100% DGLA and 100% 15-HETE, across full-thickness porcine ear skin were investigated *in vitro*.

2.2 Materials and methods

2.2.1 Materials

EQT-101 (DGLA, lot PK-E13), EQT-104 (15-HETE, lot PK-E26) were obtained from Equatec Ltd (Isle of Lewis, UK). Fish oil capsules (n-3 Fish Oil 1000 mg high strength, lot 118202) were obtained from a local Boots outlet. Butylated hydroxyanisole (BHA), and cetrimide BP were obtained from Sigma-Aldrich Company Ltd., Poole, UK. Fatty acid standards were obtained from Nu-Chek Prep.Inc., Elysian, USA). Analytical grade hexane, methanol, potassium bicarbonate, sodium chloride, sodium sulphate, and sulphuric acid were all obtained from Fisher Scientific (Loughborough, UK).

2.2.2 Methods

2.2.2.1 Determination of fatty acid content

To determine the fatty acid content of the oils used in the study, aliquots of the oil were diluted in hexane at 1 in 100 dilutions and analysed using gas chromatography (GC)

2.2.2.2 Gas chromatography

Preparation of fatty acid methyl esters (FAME) and GC analysis of fatty acids were performed according to methods adapted from Christie (2003).

2.2.2.2.1 Preparation of fatty acid methyl esters (FAME)

FAMEs were prepared by transmethylation of the diluted aliquots with 2.5% H_2SO_4 in the solution of dry methanol : toluene (2:1 v/v) in Teflon lined methylation tubes, which were then placed in a heating block for 2 hours at 70°C. A known amount of internal standard (C15:0, 1 mg mL⁻¹) was added to each sample before methylation to allow quantification analysis. 3 mL of 5% sodium chloride was added to each sample followed by 3 mL hexane. Samples were centrifuged for 5 minutes, and the upper lipid layer was removed to a clean 10 mL centrifuge tube and dried under a stream of nitrogen. Another 3 mL hexane was added and samples re-centrifuged. The resulting lipid layer was added to the first lipid layer, and the whole process repeated.

For additional purification, 3 mL of 2% potassium bicarbonate was added to each sample to neutralize any remaining acid. The upper lipid layer was removed, taking care not to disturb the interface. A spatula of anhydrous sodium sulphate was then added to remove any water and each sample was then re-dissolved in hexane and transferred to GC injection vials.

2.2.2.2.2 Gas chromatography analysis

GC analysis was done on a Clarus 500 gas chromatograph (Perkin Elmer, Norwalk, US) fitted with a flame ionizing detector (Perkin-Elmer 8500, Norwalk, US) and 30 m x 0.25mm i.d Elite 225 phenylmethylpolysiloxane (50% Cyanopropylphenyl) capillary column (Perkin Elmer). The temperature was programmed as follows: 170°C for 3 minutes, then heated to 220°C at 4°C min⁻¹, and heated at 220°C for 15 minutes. Injection temperature was 200°C, and detection temperature was set at 220°C. The carrier was nitrogen at a flow rate of 20 mL min⁻¹. Sample (5µl) was injected, and a split ratio of

20:1 was employed. FAMES were identified by comparing retention times with those obtained from fatty acid standards (Nu-Chek Prep). Limits of detection for EPA and DGLA were $0.4 \mu\text{g mL}^{-1}$ ($1.32 \times 10^{-3} \mu\text{mol mL}^{-1}$) and $0.3 \mu\text{g mL}^{-1}$ ($9.78 \times 10^{-4} \mu\text{mol mL}^{-1}$), respectively.

2.2.2.3 *In vitro* skin permeation analysis

2.2.2.3.1 *Preparation of porcine ear skin membrane*

In the current study (and the subsequent following chapters) the membrane of choice for in vitro skin permeation studies was from porcine ear skin, unless stated otherwise. The skin from this area of a pig has been validated to be a comparable substitute for human skin based on close similarities in the anatomy, physiology and biochemistry between pig and human (Simon and Maibach 2000b; Ute et al. 2007). Pig ears obtained from the abattoir were cleaned under running water and the skin of the outer side of the ear removed by blunt dissection using a scalpel. The hairs were trimmed close to the skin using a pair of clippers, and the skin cut into sections of 2 x 2 cm, with care taken to choose the areas of the ear which were free from scarring or other noticeable defects which can compromise barrier integrity or provide resistance to permeating compounds.

2.2.2.3.2 *Receptor phase solution*

Previous research by Thomas and Heard (2004) reported the use of a 30 mg mL^{-1} cetrimide solution as an optimal receptor phase for permeation studies of lipophilic compounds. It provides a good sink condition, and is anti-microbial. It was also reported by Karim (2008) that the solution did not

compromise membrane integrity. 12 g of cetrimide was weighed, along with 0.05% w/w of BHA as antioxidant, and dissolved in 400 mL deionized water to give a final cetrimide concentration of 30 mg mL^{-1} . The resulting solution was stirred and then degassed by vacuum filtering through a 0.45 micron cellulose membrane.

2.2.2.3.3 *In vitro skin permeation*

The cut porcine ear skin sections were placed on pre-greased receptor compartment of glass Franz-type diffusion cells with the stratum corneum side facing upward (Figure 2.3).

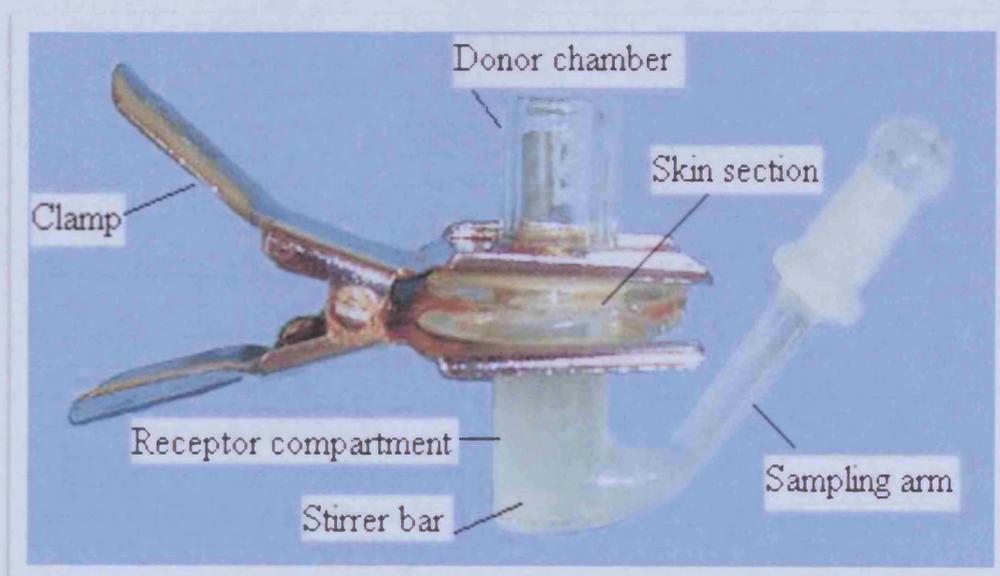


Figure 2.3 : Glass Franz-type diffusion cell mounted with a porcine ear skin membrane (Lau 2008)

Micro magnetic stirring bars were added before hand to ensure complete distribution of permeating solutes in the receptor solution. The donor cells were then pinch clamped on top, and the receptor compartment filled with temperature equilibrated receptor solution using a syringe. The completed

cells were placed on a magnetic stirring plate (Variomag, Daytona Beach, USA) in a water bath maintained at 37°C, providing a skin surface temperature of 32°C. After 10 minutes, the cells were dosed with 1 mL of the oils from a pipette. Both the sampling arm and donor cells were occluded. Six replicates were prepared for each oil, with the control cell dosed with deionized water. Entire receptor phases were removed using Pasteur pipettes at predetermined time points (3, 6, 12, 24, 36 & 48 hours) and replaced with temperature equilibrated receptor solution. The collected receptor phases were frozen in -20°C prior to GC analysis, as outlined in Section 2.2.2.2. For the skin sections dosed with fish oil, only the permeation of EPA was investigated, as the major fatty acid in the commercial fish oil used in the study. As mentioned in Section 1.6.4, previous studies reported pharmacokinetic issues arising from the use of pure fatty acid (Grimminger and Mayser 1995). Hence, all the studies contained within this thesis were based on the delivery and action of EPA from an oil mixture instead of the pure form.

2.2.2.4 Data analysis

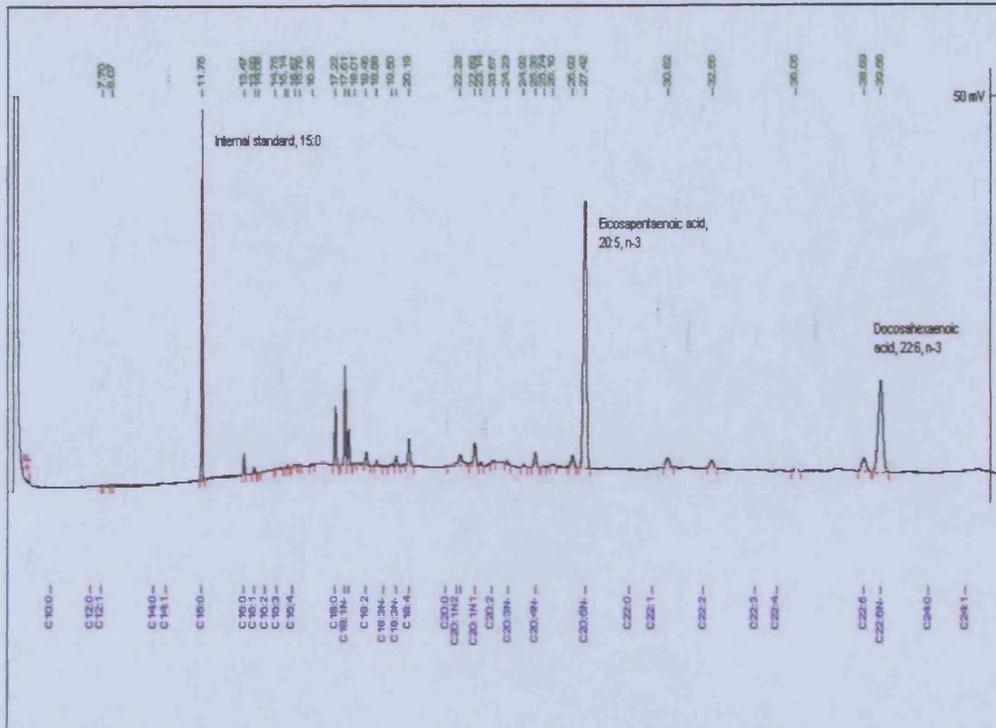
The data obtained were collected and analyzed using Microsoft Excel 2007, and expressed as mean \pm SD. Statistical tests were performed with Instat3 for Windows (GraphPad Inc. Elysian, US). Confidence interval was 95%, with p value < 0.05 considered significant.

2.3 Results and discussion

2.3.1 *Determination of fatty acid content*

2.3.1.1 *Fish oil (Boots Super Strength 1000 mg)*

The gas chromatogram and list of fatty acids contained within the fish oil are shown in Figure 2.4 and Table 2.1 respectively. As claimed by the manufacturer, the principal fatty acids contained within the capsule were EPA and DHA, with 33.46% and 20.9% respectively (listed contents – 33% EPA and 22% DHA). Other major fatty acids include oleic acid (6.88%), stearic acid (3.9%), C22:5 (2.57%), C20:1, n-12 (2.45%), C18:1, n-7 (2.43%) and arachidonic acid (2.23%). This particular brand of fish oil was fortified with additional EPA and DHA; both n-3 fatty acids that are marketed as a nutritional supplement of high value, the virtues of which have been discussed in the general introduction and explored further in the coming chapters.



Fatty acid	Average content per 1000 mg (mg)	Percentage (%)
Saturated		
C10:0	0.45 ± 0.01	0.045
C14:0 (myristic acid)	0.28 ± 0.03	0.028
C16:0 (palmitic acid)	10.72 ± 0.11	1.072
C18:0 (stearic acid)	39.01 ± 0.24	3.901
C22:0 (docosanoic acid)	0.55 ± 0.05	0.055
Monounsaturated		
C12:1	0.35 ± 0.03	0.035
C14:1	0.17 ± 0.02	0.017
C16:1	3.33 ± 0.01	0.333
C18:1, n-9 (oleic acid)	68.79 ± 0.41	6.879
C18:1, n-7	24.32 ± 0.12	2.432
C20:1, n-9	1.88 ± 0.21	0.188
C20:1, n-12	24.47 ± 0.21	2.447
C20:1, n-15	9.73 ± 0.22	0.973
C22:1 (erucic acid)	14.43 ± 0.1	1.443
C24:1	1.62 ± 0.11	0.162

Polyunsaturated		
C16:2	1.82 ± 0.25	0.182
C16:3	0.94 ± 0.05	0.094
C16:4	1.00 ± 0.06	0.1
C18:2, n-6 (LA)	9.64 ± 0.13	0.964
C18:3, n-3 (ALA)	5.40 ± 0.16	0.54
C18:3, n-6 (GLA)	6.10 ± 0.21	0.61
C20:2, n-6	3.35 ± 0.22	0.335
C20:3, n-3	1.88 ± 0.22	0.188
C20:3, n-6 (DGLA)	2.92 ± 0.012	0.292
C20:4, n-3	14.64 ± 0.13	1.464
C20:4, n-6 (AA)	22.99 ± 1.47	2.299
C20:5, n-3 (EPA)	334.6 ± 1.35	33.46
C22:2	11.15 ± 0.05	1.115
C22:3	0.44 ± 0.05	0.044
C22:4	3.78 ± 0.05	0.378
C22:5	25.74 ± 0.11	2.574
C22:6, n-3 (DHA)	209.38 ± 0.86	20.938
TOTAL	847.45	84.745

Table 2.1 : List of fatty acids in Boots Super Strength 1000 mg fish oil capsule

From Table 2.1, it can be seen that EPA represents the highest percentage of fatty acids contained within the oil. Therefore, the effects observed throughout all the studies involving the same fish oil was assumed to be attributed to EPA. The possibility of involvement by other fatty acids, however, cannot be discounted.

hydroxylated fatty acid remaining as fatty acid. There could have been polymerisation or intramolecular esterification under the reaction conditions, the temperature could have been too hot and would degrade 15-HETE upon injection. In hindsight, other analytical method such as gas chromatography-mass spectrophotometry (GC-MS) coupled with derivatization methods such as acetylation or isopropylidene derivatives (Christie 2003) might prove to be more suitable for the analysis of 15-HETE.

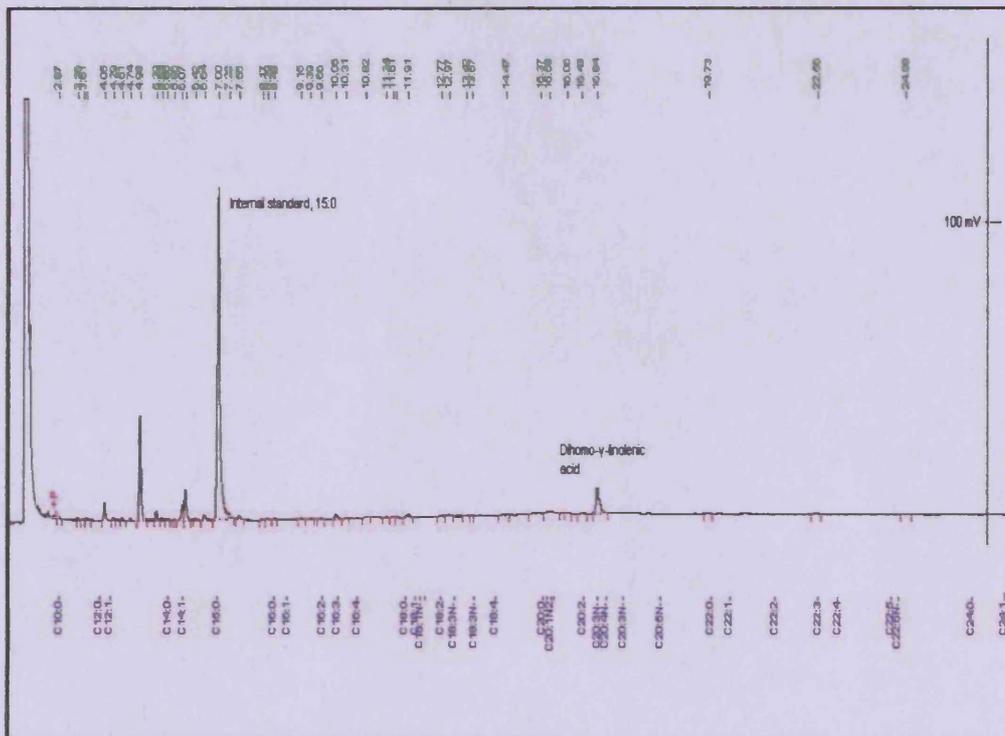


Figure 2.6 : Gas chromatogram of Equatec EQT-104 (15-HETE). Only DGLA was detected, suggestive of 15-HETE breakdown or unsuitable derivatization method and/or GC conditions

2.3.2 *In vitro skin permeation*

2.3.2.1 *EPA*

From the permeation profile (Figure 2.7), the cumulative amount of EPA permeating across porcine ear skin was $0.038 \pm 0.003 \mu\text{mol cm}^{-2}$, representing only 0.0034% percent of total EPA (contained within the fish oil) dosed on the skin. The remaining balance was not accounted for in this study. It was assumed that the majority remained within the donor phase, although further confirmation is needed to support this assumption. As comparison, a previous study done by Thomas (2006) reported an absence of EPA permeating across porcine ear skin using the same experimental setup. The low EPA amount can be attributed, at least in part, to the high lipophilicity ($\text{LogP} = 6.66$) of EPA, rendering it less effective to permeate across the more polar viable epidermis and dermis. The maximum flux (J_{max}) value was calculated to be $0.005 \pm 0.083 \mu\text{mol cm}^{-2} \text{h}^{-1}$. Based on the permeation profile, the typical plot for an infinite dosing setup was not obtained, hence the inability to determine a pseudo-steady state for calculation of J_{ss} (pseudo-steady state flux). The reason for this deviation might be caused by an incomplete esterification of fatty acids or loss during derivatization, as the amount dosed was hugely in excess, effectively ruling out depletion of permeant, which would have resulted in the same profile.

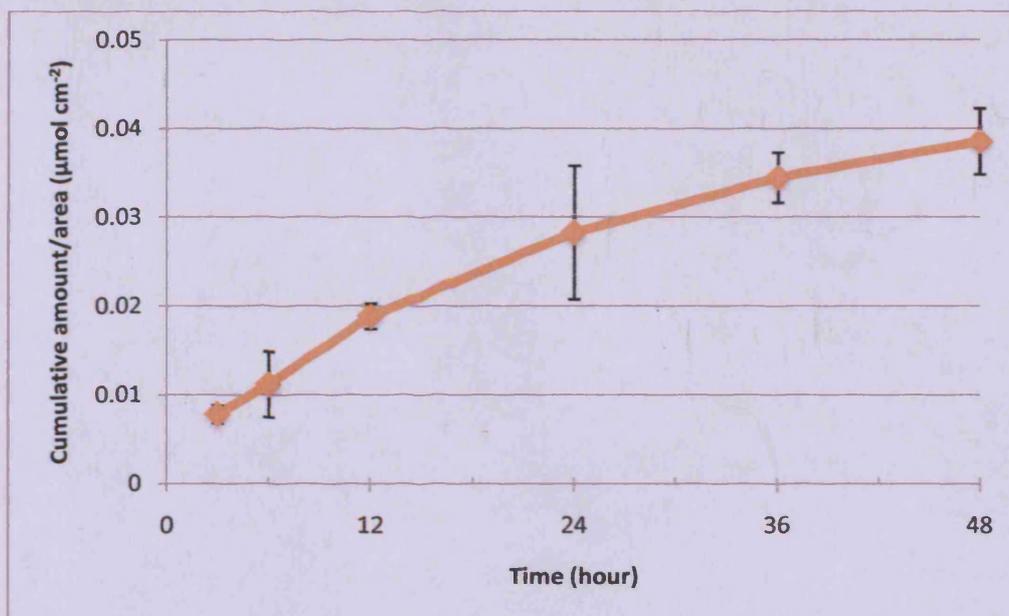


Figure 2.7 : Permeation profile of EPA across porcine skin over 48 hours (n=6, \pm SD). The maximum flux (J_{max}) value was calculated to be $0.005 \pm 0.083 \mu\text{mol cm}^{-2} \text{h}^{-1}$

2.3.2.2 EQT-101 (DGLA)

After 48 hours, the cumulative amount of DGLA permeated from EQT-101 was $0.045 \pm 0.003 \mu\text{mol cm}^{-2}$ and the J_{max} value calculated to be $5 \times 10^{-4} \pm 5.7 \times 10^{-5} \mu\text{mol cm}^{-2} \text{h}^{-1}$. The plot obtained (Figure 2.8) again did not reflect a typical permeation profile of an infinite dosing protocol, with the possible reasons discussed earlier in Section 2.3.2.1.

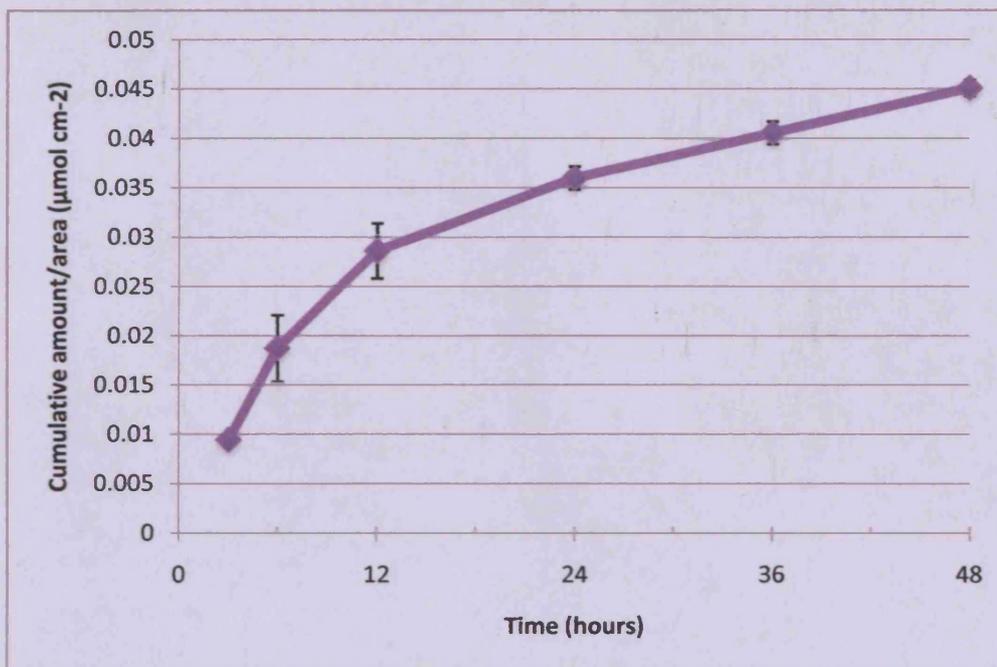


Figure 2.8 : Permeation profile of DGLA from EQT-101 over 48 hours ($n=6, \pm SD$). The maximum flux (J_{max}) value was calculated to be $5 \times 10^{-4} \pm 5.7 \times 10^{-5} \mu\text{mol cm}^{-2} \text{h}^{-1}$.

2.3.2.3 EQT-104

Analysis of the receptor phase collected from the skin section dosed with EQT-104, reflective of the analysis of the oil, only showed permeation of DGLA. Therefore the permeation profile shown in Figure 2.9 was based on DGLA values. The cumulative amount after 48 hours was $0.035 \pm 0.0007 \mu\text{mol cm}^{-2}$ and the J_{max} was $4 \times 10^{-4} \pm 6 \times 10^{-5} \mu\text{mol cm}^{-2} \text{h}^{-1}$, which was not different statistically ($p > 0.05$) from the J_{max} value for EQT-101.

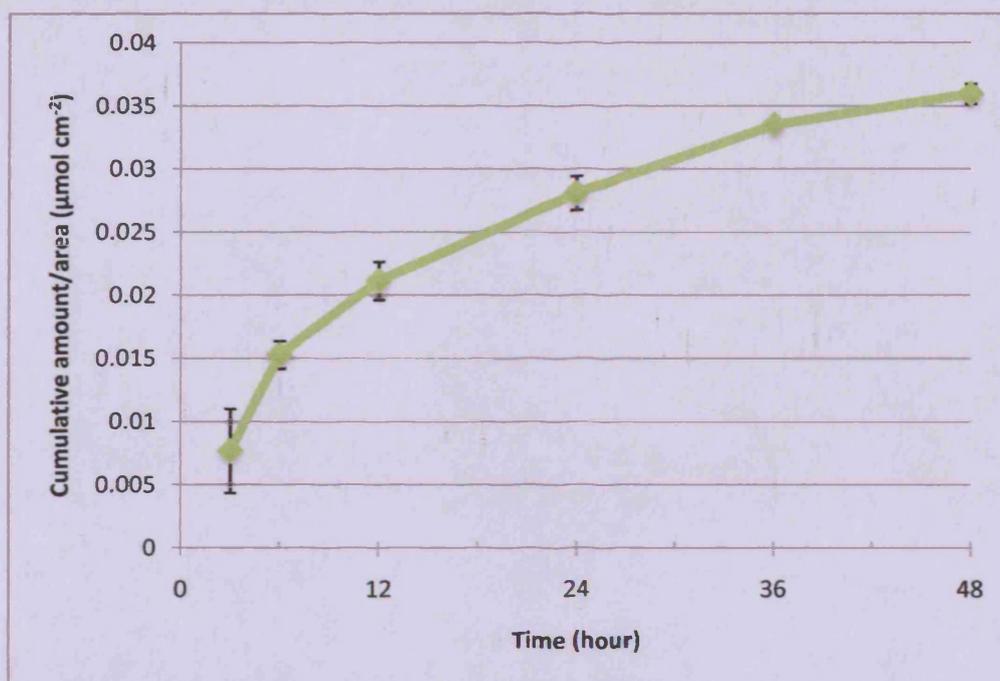


Figure 2.9 : Permeation profile of DGLA detected from EQT-104 ($n=6, \pm SD$). The maximum flux (J_{max}) value was calculated to be $4 \times 10^{-4} \pm 6 \times 10^{-5} \mu\text{mol cm}^{-2} \text{h}^{-1}$

It is acknowledged that one major limitation of the study was the lack of validation on the efficiency of the derivatization process, which may have not provided a 100% yield of FAMES. Therefore, this leaves room for improvement for future analysis of fatty acids, especially from bioactive oils such as the ones analyzed in this chapter.

2.4 Conclusion

EPA, the major fatty acid in a commercial fish oil preparation, and DGLA from custom manufactured oil was delivered successfully across porcine ear skin. However, the collected amounts were very low, considering the amount applied. This however does not necessarily affect their biological effect, as efficacy may be achieved with a small amount of the bioactive agent. The

delivery of 15-HETE was not successfully determined, owing to several factors, which may include unsuitable derivatization and/or analytical method. Refinements in these aspects could be beneficial in providing a more precise set of data, hence a better picture of the transcutaneous delivery of these bioactive oils.

Chapter 3:

*In vitro transcutaneous delivery
of EPA and acetylsalicylic acid /
salicylic acid from a fish oil
vehicle*

3.1 Introduction

Eicosapentaenoic acid is a long chain n-3 polyunsaturated fatty acid. These are associated with anti-inflammatory properties, resulting from formation of less potent inflammatory eicosanoids compared to those derived from n-6 fatty acids (Ziboh et al 2000). Trials using fish oil and EPA had shown that it can be beneficial in alleviating symptoms related to psoriasis (Mayser et al. 2002; Riku et al. 1993). Furthermore, it has been proven that EPA enhanced the delivery of other compounds when co-formulated (Thomas and Heard 2004; Thomas et al. 2007b).

Acetylsalicylic acid (ASA), more commonly known as aspirin, is the acetylated form of salicylic acid (SA) (Figure 3.1)

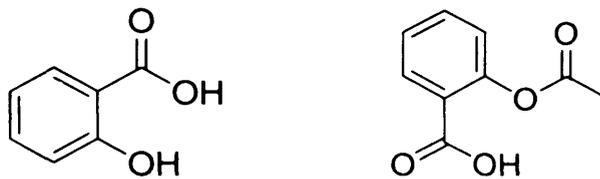


Figure 3.1 : Salicylic acid (left) and acetylsalicylic acid (right)

ASA was the first discovered non-steroidal anti-inflammatory drug (NSAID). Its non-specific inhibition of cyclooxygenases led to its decline in use, superseded by more specific and less side effects inducing COX inhibitors. However, it remained an important drug in cardiovascular treatments, owing to its anti-platelet effect. Long-term use of aspirin in high risk patient lowers the incidence of myocardial infarction, stroke and blood clotting. In some parts of the world, it also remained a popular over-the-counter pain relief and anti-fever medication. The anti-inflammatory action of ASA is attributed to its ability to inhibit the pro-inflammatory enzymes, COX-1 and COX-2, and inhibiting production of prostaglandin E₂ (Vane and Botting 2003).

The parent compound for ASA, salicylic acid, has long been used in topical preparations for conditions, including psoriasis, acne, and warts, in different concentrations as a 'peeling' or desmosolytic agent (Lodén 2000). It is believed to exert its action on the stratum corneum by affecting intercorneocyte cohesion and desquamation (Bashir et al. 2005), although the precise mechanism for this has never been elucidated.

The aim of this study was to investigate the plausibility of combining the two agents, namely acetylsalicylic acid in a fish oil vehicle by probing the permeation and penetration profile of both compounds. In particular, it was hypothesized that the EPA from fish oil could enhance delivery of ASA which would then exert an anti-inflammatory effect, while the breakdown of ASA to SA will promote desmosomolysis within the SC.

3.2 Materials and methods

3.2.1 Materials

Fish oil capsules 1000mg (Boots Super Strength, lot 5647, Composition 33% EPA) were obtained from a local store. Acetylsalicylic acid (lot 045KO101), butylated hydroxyanisole (BHA), cetrimide BP, and EPA were obtained from Sigma-Aldrich Company Ltd., Poole, UK. Methanol, acetonitrile, ethanol, hexane (all HPLC grade), sulphuric acid and phosphoric acid were obtained from Fisher Scientific, Loughborough, UK. Adhesive tape (3M, Bracknell, UK) was obtained from a local stationer. All other reagents were of analytical grade or equivalent. Freshly excised pig ears were obtained from a local abattoir prior to steam cleaning.

3.2.2 Methods

3.2.2.1 Preparation of test solutions

Saturated solutions of acetylsalicylic acid in fish oil were prepared by adding an excess amount of ASA into 10 mL fish oil obtained from squeezing out the content of fish oil capsules. In fish oil, the solubility of ASA was calculated to be 0.471 mg mL^{-1} (2.61 mol mL^{-1}). This was determined by adding an excess of ASA to 1 mL of fish oil followed by vortex mixing. The tube was then placed on a blood tube rotator inside an incubator set at 32°C for 24 hours. This was followed by centrifugation of the solution, and the concentration of the saturated FO-ASA solution determined by HPLC (Section 3.2.23).

3.2.2.2 In vitro skin permeation and depth profiling

3.2.2.2.1 In vitro skin permeation

In vitro skin permeation was carried out according to the method outlined in Section 2.2.2.3.3. Porcine skin sections mounted on glass Franz-type diffusion cells were dosed with 1 mL of the ASA-FO mixture using a pipette and rubbed gently using a glass rod. The receptor phase was a solution of 30 mg mL^{-1} cetrimide with 0.05% w/v BHA as an anti-oxidant. In this solution, the solubility of ASA and SA were determined using the method elaborated in Section 3.2.2.1, with the value calculated to be $4.34 \pm 0.38 \text{ mg mL}^{-1}$ ($24.1 \pm 2.1 \text{ } \mu\text{mol mL}^{-1}$) and $8.7 \pm 0.48 \text{ mg mL}^{-1}$ ($62.9 \pm 3.48 \text{ } \mu\text{mol mL}^{-1}$), respectively. The solubility of FO and EPA were determined by sequentially adding aliquots of FO into the cetrimide solution until it turned cloudy. The solubility of FO was determined to be $5.0 \pm 0.91 \text{ } \mu\text{g mL}^{-1}$. With EPA representing 33% of the

oil, this equated to a $1.65 \pm 0.3 \mu\text{g mL}^{-1}$ ($5.45 \times 10^{-3} \pm 0.99 \times 10^{-3} \mu\text{mol mL}^{-1}$) for EPA in cetrimide. Six replicates were prepared with one cell dosed with deionized water as a control. Whole receptor phases were removed at 3, 6, 12, 24, 36, and 48 hours and replaced with fresh temperature-equilibrated cetrimide solution. Aliquots of the receptor phase was immediately analysed after sampling for ASA and SA and the remainder stored at -20°C prior to GC analysis for EPA.

3.2.2.2.2 *Depth profiling via adhesive tape stripping*

Tape stripping (Löffler et al. 2004) was carried by placing adhesive tape on the permeated area of the skin sections after completing the permeation experiment for 24 hours. Excess test solutions were first wiped off and the first 2 strips were discarded. 20 strips were taken from the skin by applying the tape with a constant pressure and removing it with a constant velocity using stainless steel forceps. The 20 strips were then grouped 2 per vials (1-10) containing 5 mL methanol as an extracting solution and placed on a rocker overnight to desorb the penetrants from the strips. The remaining skin was also placed in methanol and left overnight. The solvent was then evaporated using a vacuum oven and 1 mL methanol was added to each vial and then centrifuged at 10000rpm for 10 minutes. The supernatants were then transferred to autosampler vials for HPLC analysis. It must be noted that the constituents of the adhesive used on the tape were not evaluated, and theoretically, might interfere with the detection of the penetrants. This possibility was minimized by analyzing samples from the control cell and comparing it with the chromatogram obtained from the cells dosed with the ASA-FO mixture, and comparing the retention time for ASA, EPA and SA as obtained with standards.

3.2.2.3 High Performance Liquid Chromatography (HPLC) analysis

ASA, SA and EPA from tape strip samples were analysed using an Agilent series 1100 HPLC system using a Gemini Cu18 150 x 4.6mm column (Phenomenex, Macclesfield, UK), fitted with a Phenomenex Securityguard guard column. The wavelength was set at $\lambda = 210$ nm. The mobile phase was methanol (95%) and deionized water (5%), pH adjusted with phosphoric acid to pH 2.5. The flow rate was 1 mL min^{-1} , the sample injection was $20 \mu\text{L}$ and the runtime was 15 minutes. SA (Figure 3.2), ASA (Figure 3.3) and EPA (Figure 3.4) were found to elute at 3.6 minutes, 4.4 minutes and 7.8 minutes, respectively. The HPLC method for EPA analysis was an adaptation of the method reported by Thomas (2006).

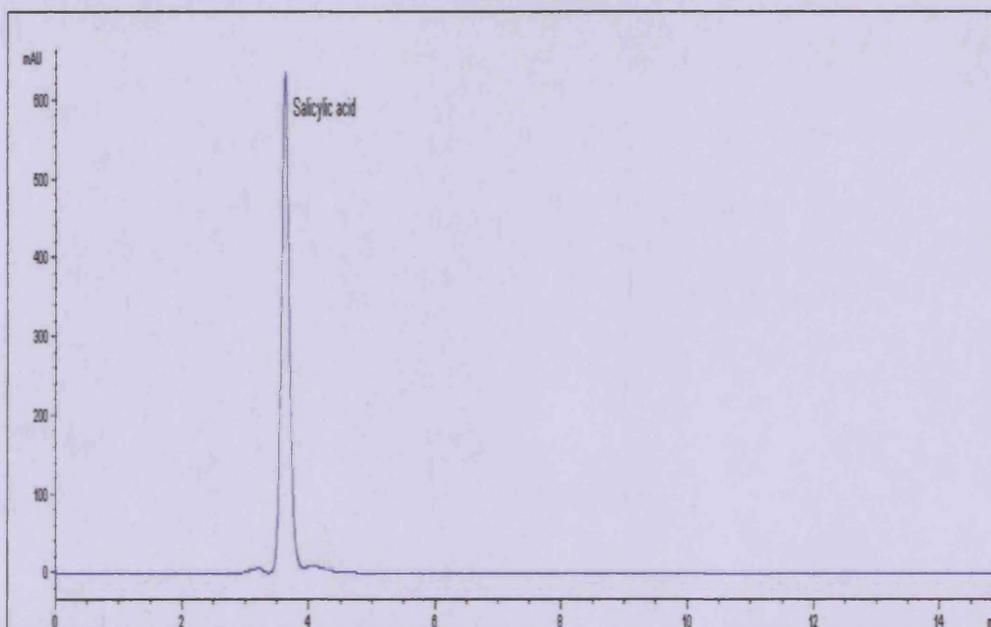


Figure 3.2 : HPLC chromatogram for salicylic acid, $\lambda=210$ nm. The retention time was 3.6 minutes with 95:5 methanol:deionized water, pH adjusted to 2.2 with phosphoric acid at a flow rate of 1 mL min^{-1}

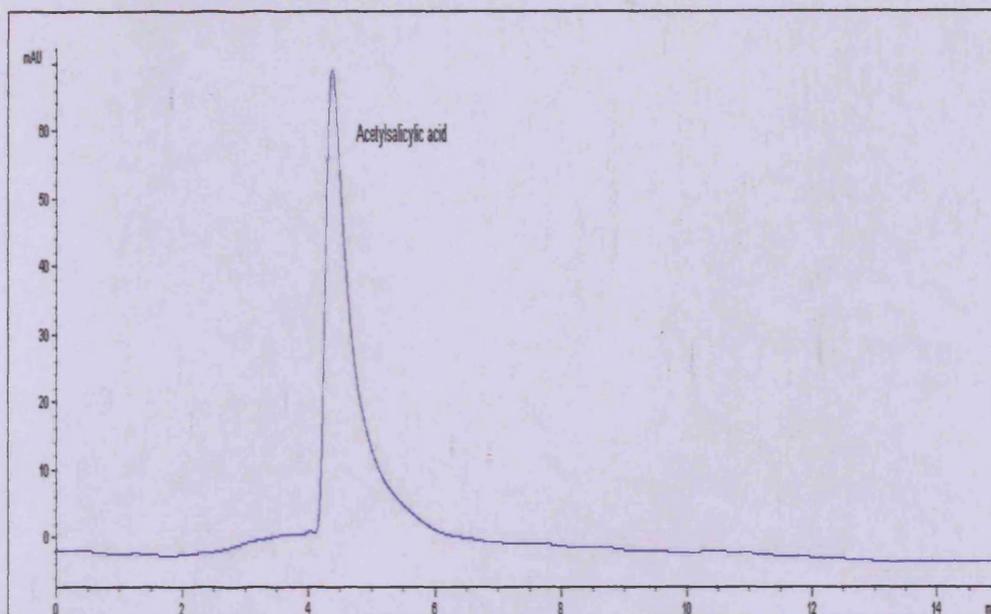


Figure 3.3 : HPLC chromatogram for acetylsalicylic acid, $\lambda=210$ nm. The retention time was 4.4 minutes with 95:5 methanol:deionized water, pH adjusted to 2.2 with phosphoric acid at a flow rate of 1 mL min^{-1}

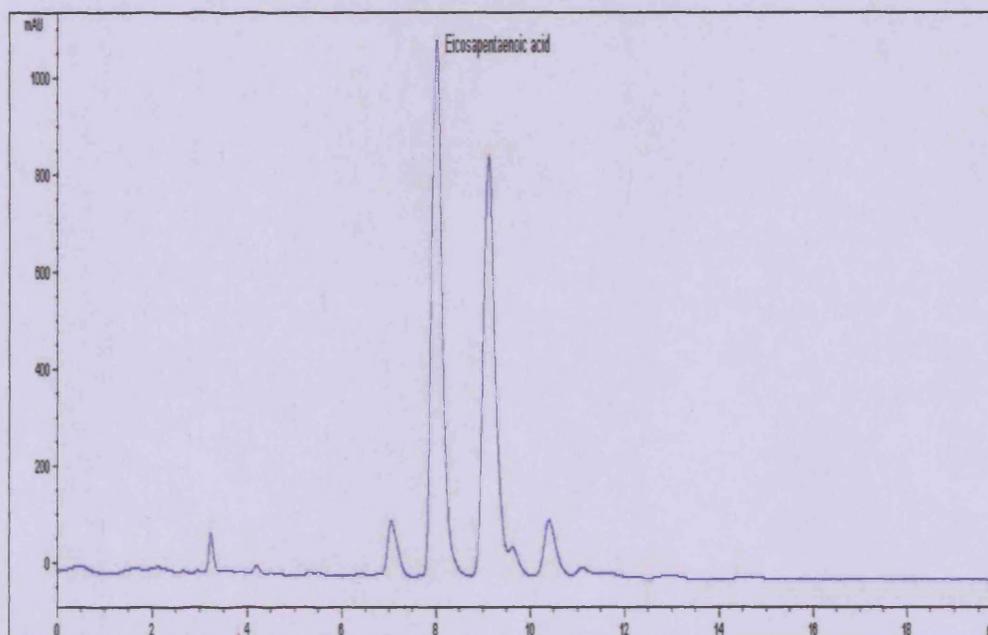


Figure 3.4 : HPLC chromatogram of EPA from fish oil, $\lambda=210$ nm. The retention time was 7.8 minutes with 95:5 methanol:deionized water, pH adjusted to 2.2 with phosphoric acid at a flow rate of 1 mL min^{-1}

Standard calibration curves were constructed over the range of 1-20 $\mu\text{mol mL}^{-1}$ for ASA and SA, both with an R^2 value of >0.99 . Standard curve for EPA was constructed over the range of 0.002-0.05 $\mu\text{mol mL}^{-1}$. Concentrations of the permeants were then calculated from the area under the curve. The limit of detections for SA and ASA were $0.12 \mu\text{g mL}^{-1}$ ($8.69 \times 10^{-4} \mu\text{mol mL}^{-1}$) and $0.09 \mu\text{g mL}^{-1}$ ($4.99 \times 10^{-4} \mu\text{mol mL}^{-1}$), respectively, while the LOD for EPA was $0.6 \mu\text{g mL}^{-1}$ ($1.98 \times 10^{-3} \mu\text{mol mL}^{-1}$).

3.2.2.4 GC analysis

EPA from permeation samples was analysed using GC with the method described previously in Section 2.2.2.2

3.2.2.5 Data analysis

The data obtained were collected and analyzed using Microsoft Excel 2007, and expressed as mean \pm SD. Statistical tests were performed with InStat3 for Windows (GraphPad Inc. Elysian, US). Confidence interval was set at 95%, with p value < 0.05 considered significant.

3.3 Results and discussion

3.3.1 *In vitro* transcutaneous delivery

3.3.1.1 ASA and SA

Both ASA and SA were found within the receptor phases at all sampling points (Figure 3.5). The pseudo-steady state fluxes (J_{ss}) were 0.17 ± 0.07 and $0.18 \pm 0.03 \mu\text{mol cm}^{-2} \text{h}^{-1}$ for ASA and SA, respectively (p 0.8012). The

presence of SA may be contributed by several possible sources. Hydrolysis of ASA prior to usage (attributed to less than optimal storage conditions i.e. exposure to water/water vapours), hydrolysis of ASA in the donor phase (ASA-FO mixture), enzymatic breakdown within the skin sections, or hydrolysis of permeated ASA within the receptor solution. Up to 12 hours, the cumulative amount of ASA and SA were significantly different, at 1.41 ± 0.42 and $0.80 \pm 0.20 \mu\text{mol cm}^{-2}$, respectively (p 0.0099). Over the 48 hours duration of the study, the amount of SA detected continued to increase, until the difference from ASA amount was not statistically significant (p 0.8548), with 7.79 ± 2.74 and $7.56 \pm 1.54 \mu\text{mol cm}^{-2}$ ASA and SA, respectively.

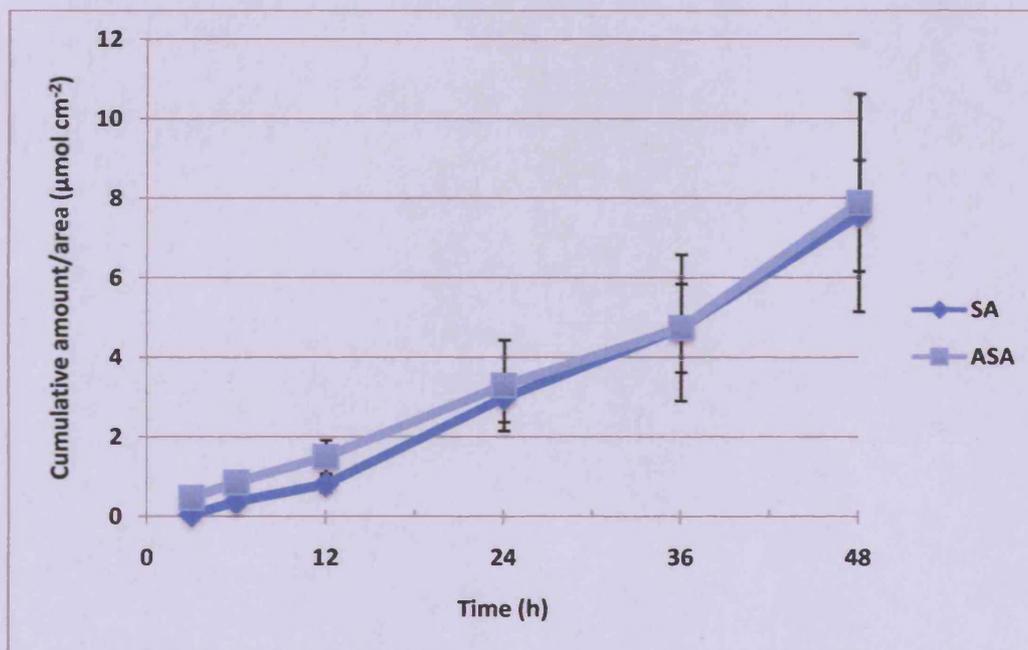


Figure 3.5 : Permeation profile of ASA and SA from a saturated solution of aspirin in fish oil (n=6, ±SD)

This was further illustrated with the molar ratio of SA to ASA, as shown in Table 3.1. In the first 12 hours, the ratios were significantly different over the time period, with p 0.026. As the amount of SA present in the receptor phase increased over the next 36 hours, the ratios of SA to ASA became statistically insignificant (p 0.8257).

Timepoint (h)	Molar ratio \pm SD
3	0.1052 \pm 0.09
6	0.4246 \pm 0.26
12	0.5693 \pm 0.19
24	0.9291 \pm 0.21
36	1.0170 \pm 0.27
48	0.9698 \pm 0.25

Table 3.1 : Molar ratio of SA to ASA at the sampling timepoints (n=6, \pm SD)

The rise in SA amount over time was presumably a total sum of permeated SA originating from the donor phase, skin metabolism of SA, and conversion of ASA in the receptor phase, as mentioned previously. Hypothetically, as time progress, it is a possible scenario that the amount of SA will continue to rise, until eventually all ASA are hydrolysed and only SA will be detected.

3.3.1.2 EPA

The permeation profile for EPA is shown in Figure 3.6. After 48 hours, the cumulative amount of EPA permeated was $0.0148 \pm 0.001 \mu\text{mol cm}^{-2}$ and the J_{ss} was calculated to be $0.0003 \mu\text{mol cm}^{-2} \text{h}^{-1}$. The amount permeated represented 0.0014% of total applied EPA contained within the oil, approximately half from the amount of EPA permeated from fish oil alone (Section 2.3.2.1). This suggested that the presence of ASA appeared to retard the delivery of EPA.

The molar ratio of EPA to ASA and SA is shown in Table 3.2. Analysis of variance (ANOVA) for the molar ratio across the time points revealed a

significant difference for EPA:ASA (p 0.0011), while it was the opposite for EPA:SA, with p 0.818.

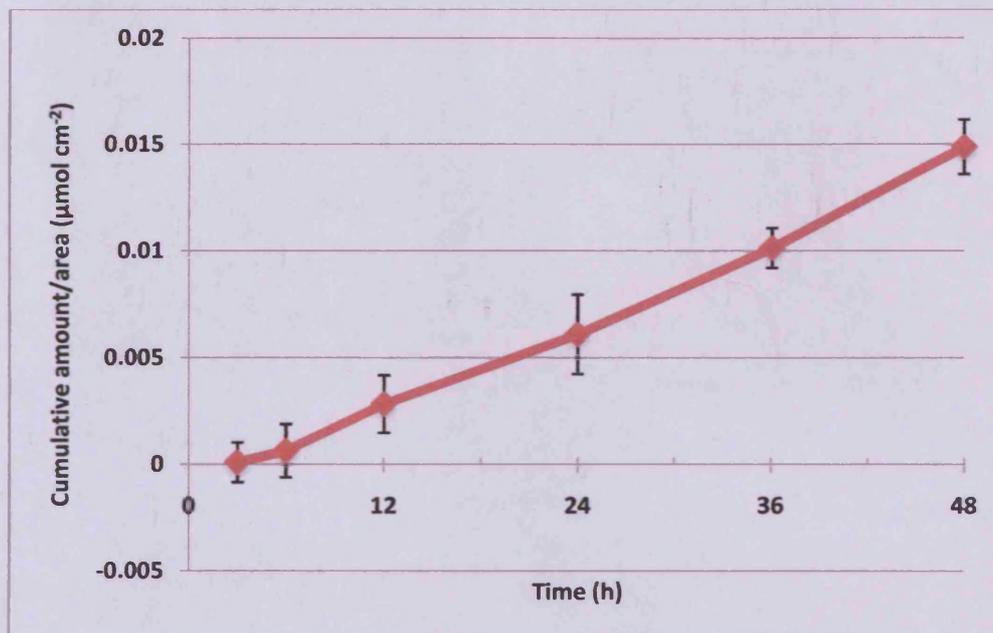


Figure 3.6 : Permeation profile for EPA from a fish oil-ASA mixture ($n=6, \pm\text{SD}$). After 48 hours, the cumulative amount of EPA permeated was $0.0148 \pm 0.001 \mu\text{mol cm}^{-2}$ and the J_{ss} was calculated to be $0.0003 \mu\text{mol cm}^{-2} \text{h}^{-1}$

Time (h)	Molar ratio EPA/ASA \pm SD	Molar ratio EPA/SA \pm SD
3	0.00024 ± 0.0001	0.0025 ± 0.002
6	0.00072 ± 0.0002	0.0018 ± 0.001
12	0.0021 ± 0.0005	0.0036 ± 0.0001
24	0.00234 ± 0.0021	0.0022 ± 0.001
36	0.0026 ± 0.0018	0.0023 ± 0.001
48	0.0022 ± 0.0013	0.0021 ± 0.005

Table 3.2 : Molar ratio of EPA to ASA and SA ($n=6, \pm\text{SD}$)

3.3.2 Depth profiling

Permeation data is of limited value for a topical anti-psoriasis formulation. Of greater importance is the level and location of drug localized within the skin tissue.

3.3.2.1 ASA and SA

Analysis of the permeant recovered from the adhesive tapes revealed only a small amount of ASA present across the layers and in the remaining skin (Figure 3.7). The highest amount was found in the first 2 strips and the remaining skin, at 0.013 ± 0.004 and 0.014 ± 0.002 μmol respectively. The total amount of ASA recovered from the tape strips, excluding the remaining skin, was 0.052 ± 0.01 μmol . The depth profile obtained for SA is shown in Figure 3.8.

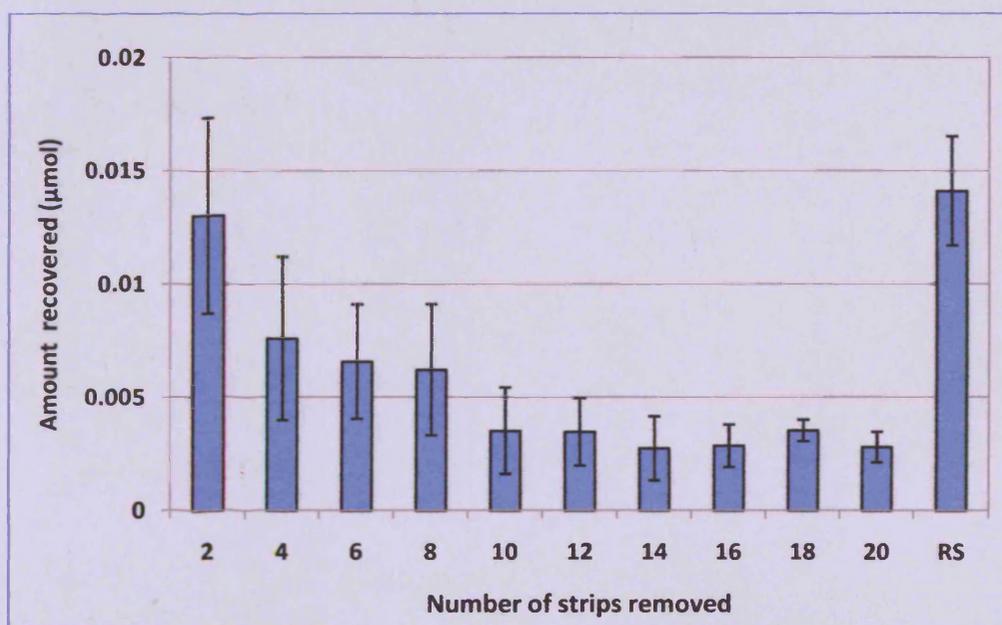


Figure 3.7 : Depth profile for ASA (n=6, \pm SD). RS= remaining skin

The highest amount was again recovered from the first 2 strips and the remaining skin, at 0.15 ± 0.03 and 1.91 ± 0.47 μmol . The ratio of SA to ASA is shown in Table 3.3. The total amount of SA recovered from the tape strips was 0.687 ± 0.23 μmol .

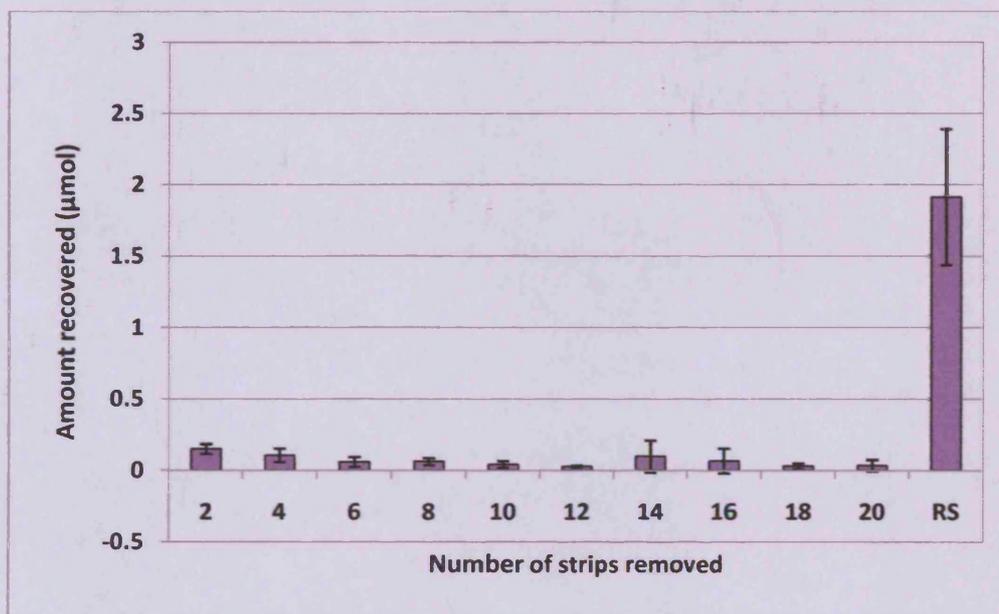


Figure 3.8 : Depth profile for SA (n=6, \pm SD). RS= remaining skin

Number of strips removed	Molar ratio \pm SD
2	14.6 \pm 7.95
4	10.8 \pm 4.75
6	5.97 \pm 2.38
8	10.87 \pm 2.61
10	5.87 \pm 0.87
12	8.12 \pm 3.84
14	12.7 \pm 5.72
16	9.85 \pm 6.21
18	8.35 \pm 6.90
20	5.77 \pm 1.82
Remaining skin	128.18 \pm 10.4

Table 3.3 : Molar ratio of SA to ASA across the tape strips and remaining skin. (n=6 \pm SD)

The unusually high ratio of SA to ASA (compared to the molar ratio of permeated SA and ASA) was suggestive of continuous skin esterases activity, hydrolyzing ASA molecules which had penetrated across the skin layers. There was also the contribution of liberated SA within the donor phase, as discussed in Section 3.3.1.1, these donor phase SA were readily available to traverse the skin, as opposed to being liberated from ASA *within* the skin. LogP of the 2 compounds may also be an important factor. At 2.26, SA has a higher calculated LogP value compared to ASA (1.19) (Virtual Computational Chemistry Laboratory 2005), hence, according to skin permeation theory, would partition more easily across skin.

Several clinical trials of topically applied ASA have been reported previously (De Benedittis and Lorenzetti 1996; Schmelz and Kress 1996; Steen et al. 1995, 1996). Although all of these publications reported successful outcomes in relieving pain, none of them discussed permeation data of ASA and SA. Additionally, there are now commercially available topical aspirin preparations marketed to relieve pain and inflammation arising from shaving, e.g. Paula's Choice Skin Relief Treatment (Paula's Choice Europe B.V., Amstelveen, Holland). The manufacturer's website listed "stabilized acetylsalicylic acid" in the ingredients of the lotion (Paula's Choice 2009), although no further details were available regarding the stabilization process and in vitro permeation and deposition of ASA/SA with the preparation.

3.3.2.2 EPA

The depth profile for EPA is shown in Figure 3.9. In this instance, the highest amount recovered was from the first 2 strips, with $0.203 \pm 0.05 \mu\text{mol}$, while the amount recovered from the remaining skin was $0.0865 \pm 0.013 \mu\text{mol}$. The total EPA recovered from the 20 strips was $0.654 \pm 0.25 \mu\text{mol}$. The retention

of EPA in the skin layers (as opposed to traversing the skin) is of particular importance in the treatment of psoriasis, with regards to the intended target, which is the basal layer of epidermis. This layer contains keratinocytes, which, in psoriatic skin, are in a state of hyperproliferation. The high metabolic activity of this layer increases metabolism of EPA to its hydroxylated metabolite, 15-hydroxyeicosapentaenoic acid (15-HEPE); which has been reported to have even more potent anti-inflammatory activity compared to EPA (Thomas and Heard 2007).

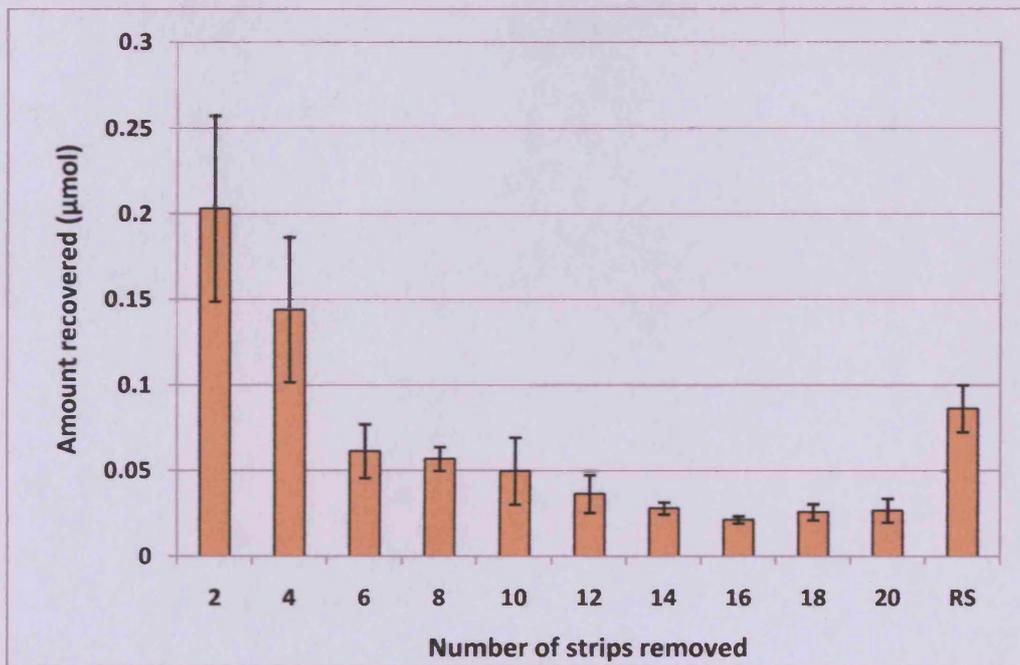


Figure 3.9 : Depth profile for EPA (n=6, ±SD). RS =remaining skin

3.4 Conclusion

The successful delivery of all three principal components of the mixture exhibited a potential in combining ASA with fish oil, either as a vehicle or a possible co-formulation. ASA, SA and EPA all possess anti-inflammatory activity, while SA has the added use as a desmosomolytic agent to soften and remove scales, making it useful in diseases such as psoriasis, where inhibition of inflammation is key, and the formation of plaques represents a challenge in topical drug delivery.

Chapter 4:

Quantification of desmosomolytic properties of salicylic acid, benzoyl peroxide and all-trans retinoic acid in a corticosteroid formulation via adhesive tape stripping and protein assay

4.1 Introduction

In the treatment of dermatological diseases, keratolytic agents, as they are generally known, are often included as part of the therapeutic regimen. Treatment of diseases such as acne, wart and psoriasis often require the sloughing or removal of thickened skin resulting from the hyperkeratinization commonly associated with those conditions. The term 'keratolysis' however, does not properly reflect the mechanism involved in removal of the stratum corneum. Proteolysis of desmosomes, which form a junction between keratinocytes, thus reducing cellular cohesion, are believed to be the main mechanism involved with most common keratolytic agents (Waller et al. 2006). Therefore, in this experiment, the term 'desmosomolysis' and 'desmosomolytic' will be used.

Retinoic acid, or in general retinoids, exert several effects when applied topically. Among others, they include comedolysis, alteration of keratinocyte proliferation, and thinning of SC (Bershad 2001; Boswell 2006). Benzoyl peroxide is most commonly used in the treatment of acne as a strong anti-microbial agent. It is also a mild anti-inflammatory and comedogenic agent, available in preparations ranging from 1-10% (Gollnick and Schramm 1998)

This main purpose of this study was to compare the desmosomolytic activity of two dermatological drugs, namely all-trans retinoic acid (RA) and benzoyl peroxide (BP), with a widely used desmosomolytic agent, salicylic acid (SA), in a formulation containing betamethasone dipropionate (BD) and fish oil (FO). To achieve this, adhesive tape stripping combined with protein assay, as previously described by Dreher et al. (2005), was employed.

4.2 Material and Methods

4.2.1 Materials

Fish oil capsules 1000mg (Boots Super Strength, lot 0336000100A, Composition 33% EPA) were obtained from a local store. BD (lot 066K1212), and cetrimide BP were obtained from Sigma-Aldrich Company Ltd., Poole, UK. Salicylic acid (lot A016882401), benzoyl peroxide (lot A0239820) and all-trans retinoic acid (lot A01533801) were obtained from Acros Organics, New Jersey UK. Sodium hydroxide and phosphoric acid were obtained from Fisher Chemicals, Loughborough, USA. Emulsifying ointment B.P (lot 72FG) was obtained from Thornton and Ross, Huddersfield, UK. All other reagents were of analytical grade or equivalent. Freshly excised pig ears were obtained from a local abattoir prior to steam cleaning.

4.2.2 Methods

4.2.2.1 *In vitro skin permeation and adhesive tape stripping*

4.2.2.1.1 *Preparation of test formulations*

Five test formulations were prepared for the purpose of the experiment, as shown in Table 4.1. Emulsifying ointment (5 g) was melted at 40°C, and the required amount of BD, BHA, SA, BP and RA were weighed and added to the melted base and stirred to ensure uniform distribution of the drugs. For formulations incorporating fish oil, the melted base was allowed to cool before squeezing the content of the fish oil capsule into the mixture and

stirring. All the formulations were kept at 4°C and excluded from light before use.

Formulation	Contents
BSA	20% fish oil, 0.1 % betamethasone dipropionate, 0.05 % butylated hydroxyanisole, 2% salicylic acid in emulsifying ointment B.P
BBP	20% fish oil, 0.1 % betamethasone dipropionate, 0.05 % butylated hydroxyanisole, 2% benzoyl peroxide in emulsifying ointment B.P
BRA	20% fish oil, 0.1 % betamethasone dipropionate, 0.05 % butylated hydroxyanisole, 0.05% all-trans retinoic acid in emulsifying ointment B.P
BFO	20% fish oil, 0.1 % betamethasone dipropionate, 0.05 % butylated hydroxyanisole in emulsifying ointment B.P
Base	0.05% butylated hydroxyanisole in emulsifying ointment BP

Table 4.1 : Test formulations containing different desmosomolytic agents

4.2.2.1.2 Receptor solution

A 30 mg mL⁻¹ cetrimide solution was chosen as receptor phase, as it provides a good sink condition apart from possessing intrinsic anti-microbial properties. The required amount of cetrimide was weighed, along with 0.05% w/w of BHA as antioxidant, and dissolved in distilled water. The resulting solution was stirred and then degassed by vacuum filtering through a 0.45 micron cellulose membrane.

4.2.2.1.3 *In vitro skin permeation*

In vitro skin permeation was performed using full thickness porcine skin according to the method outlined in Section 2.2.2.3.3. The skin sections were dosed with approximately 0.5 g of each formulation (n=3), the receptor phase was cetrimide 30mg mL⁻¹ with 0.05% w/v BHA.

4.2.2.2 *Adhesive tape stripping and protein assay*

The tape stripping and protein assay procedures were adapted from the method outlined by Waller et al. (2006). Adhesive tapes were placed on the skin sections after completing a regular permeation for 24 hours. 20 strips were taken from the skin by applying the tape with a constant pressure and removing it with constant velocity with a pair of forceps. The first 2 strips were discarded. The subsequent strips from every cell were grouped into glass vials containing 5 strips each with 10mL 1M NaOH as an extraction solvent. The vials were then placed on a mechanical rocker overnight to ensure complete extraction of the stripped SC from the adhesive tape.

Subsequently, 400 µL of the NaOH solution from each vial was transferred into 1.5 mL disposable polystyrene cuvettes and neutralized with an equal amount of 1M HCl. To the neutralized solutions, 200 µL of Coomassie (Bradford) Protein Assay Reagent (Pierce, Rockford, Illinois, USA) was added and left to stand for 15 minutes. The optical density of each sample was measured at 595nm using a Hitachi U-3000 spectrophotometer (Hitachi Ltd., Japan) with deionized water as blank. The readings were then converted into µg mL⁻¹ 1M NaCl using a calibration curve constructed with albumin, with a concentration range of 0.015625 to 2mg (R² >0.99).

4.2.2.4 Data analysis

Statistical analysis was performed using InStat3 (GraphPad Inc.). Assuming the data was not normally distributed, Kruskal-Wallis analysis with Dunn's Multiple Comparisons test was conducted. Graphical representation of the data was constructed with Microsoft Excel 2007.

4.3 Results and discussion

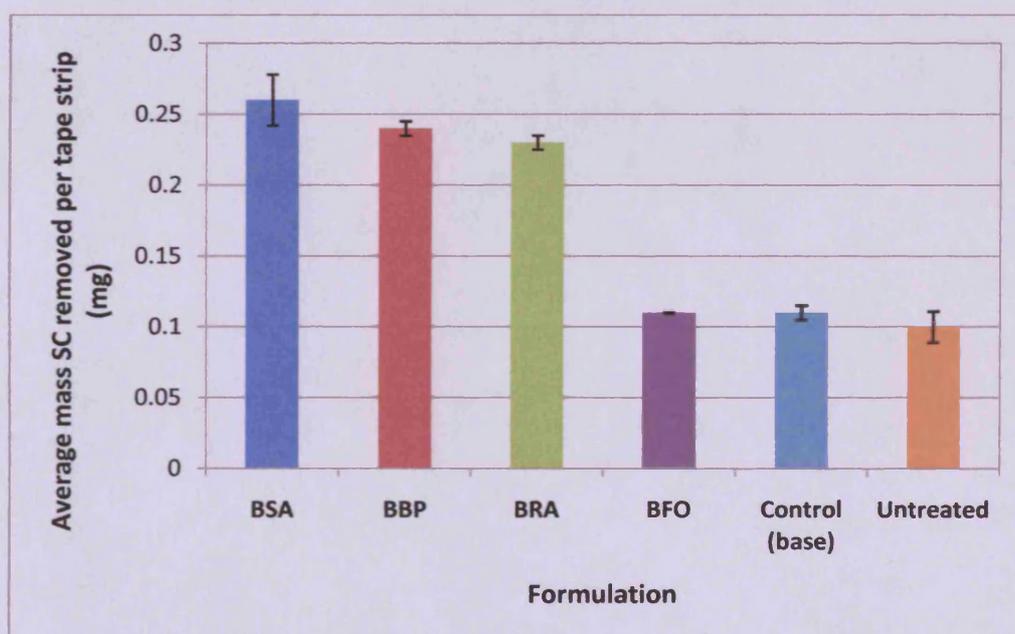


Figure 4.1 : Average SC mass removed per tape strip after 24 hours ($n=3, \pm SD$)

Protein analysis of the samples revealed that the average SC mass removed after 24 hours was significantly higher ($p < 0.05$) from skin treated with formulations incorporating the 3 test compounds (BSA, BRA, BBP) than untreated skin (Figure 4.1). It was also the case when comparing the 3 formulations with BFO and vehicle control (ointment base), with the

exception of BRA which showed no significant difference from both BFO and vehicle control.

The cumulative amount of SC removed (Figure 4.2), however, was not statistically significant ($p > 0.05$) among the 3 desmosomolytic agents, namely BSA (5.24 ± 0.37 mg), BBP (4.87 ± 0.11 mg) and BRA (4.62 ± 0.11 mg). The same was observed with the 3 remaining treatments, BFO (2.21 ± 0.01 μ g), Base (2.27 ± 0.21 mg, and untreated skin (2.02 ± 0.46 mg), where no statistical difference was observed in regard to the amount of SC removed.

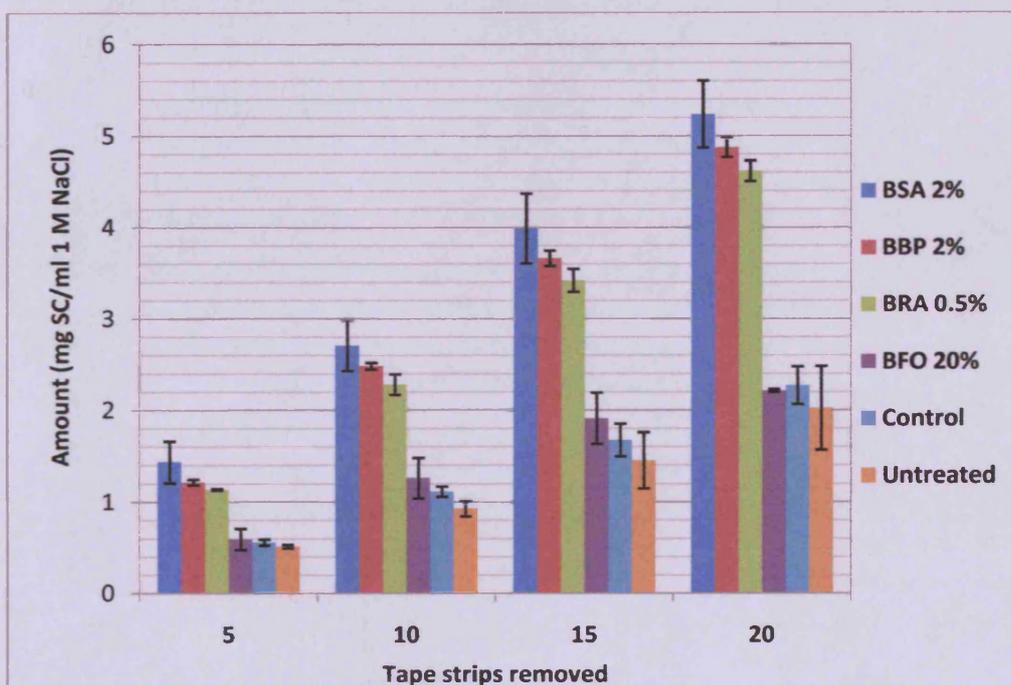


Figure 4.2 : Cumulative SC amount removed after 24 hours ($n=3$, \pm SD)

The data suggest that since there were a significant differences between the treatments and untreated skin, and the fact that there was no difference between untreated skin, FO, (the other major component of the formulation), and base (vehicle control), the increased SC removal was due to the presence of the desmosomolytic agents (SA, BP, RA).

Analyzed collectively in groups of 5 strips (Figure 4.3), SA was found to remove the highest amount of SC followed by BP and RA in the first 5 strips;

otherwise it was comparable across the remaining strips. As comparison, Waller et al (2006) also reported that SA removed the highest amount of SC in the first 10 strips, followed by BP and RA. However, for layers 11-25, BP was found to remove the most followed by SA and RA. It must be noted that the duration of the experiment differed (24 hours versus 6 hours).

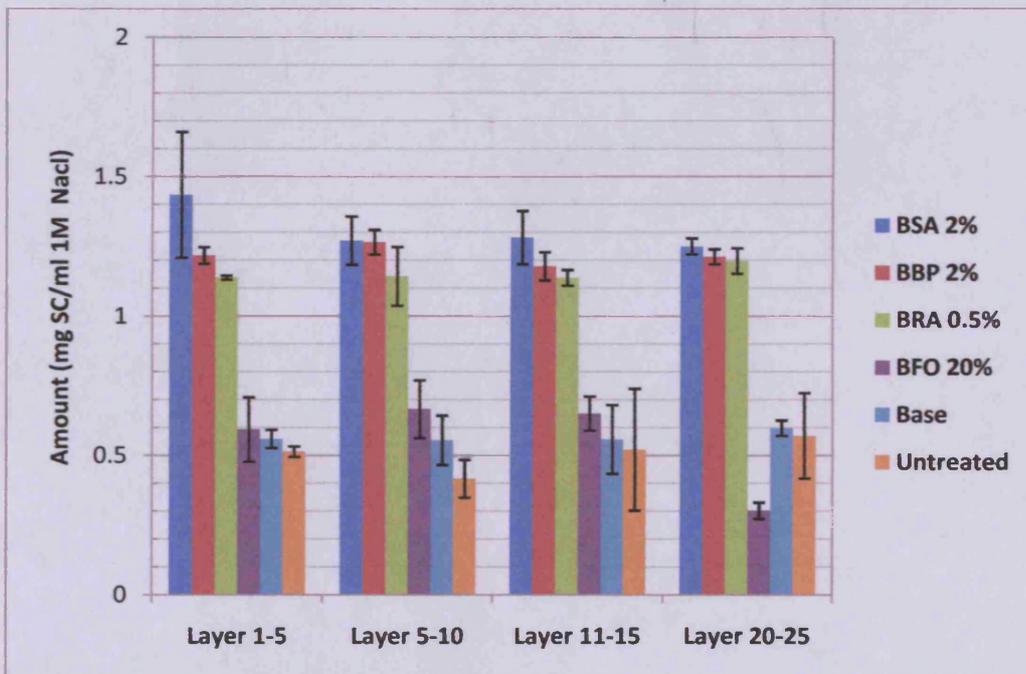


Figure 4.3 : Amount of SC removed – layer by layer comparison (n=3,±SD)

4.4 Conclusion

Employed widely in treatment of hyperkeratotic diseases (Lodén 2000), it was hypothesized that SA would remove the highest amount of SC. The mechanism by which this is achieved was mentioned before, with a proposed additional effect of the beta-hydroxy acid increasing the hydration and softening of scales by pH reduction of the applied area (Davies and Marks 1976). Interestingly, BP which is prescribed mainly for its anti-microbial activity possesses comparable desmosomolytic activity to established agents such as SA and RA, a finding confirmed by an earlier study (Waller et al. 2006). It is noted that one of the major side effects in topical acne therapy is peeling and scaling of the skin (Gollnick and Schramm 1998; Krowchuk 2000). This 'inconvenience' could in fact prove to be beneficial in other skin conditions which would require removal of skin layers with the added benefit of an anti-microbial. Another point of note is the relative potency of the 3 drugs used in this study. RA at a concentration of 0.05% exhibited a similar effect to 2% SA and BP. According to Bergfeld (1996) RA is a very potent comedolytic which acts by normalizing skin desquamation of epithelium, with the major drawback being the numerous associated side effects reported with higher concentrations.

As reflected from the data obtained, it can be suggested that since all 3 drugs have similar desmosomolytic properties, the selection of which to use in hyperkeratosis of the skin depends on the added benefit that the individual drug possess. Furthermore, the fact that BP, which previously was prescribed mostly for its activity against microbes, is also a desmosomolytic further widens the therapeutic options available for clinicians and encourages further assessment of other dermatological drugs for the same property.

Chapter 5:

*Comparative depth profiling of
betamethasone dipropionate,
salicylic acid and
eicosapentaenoic acid from an
ointment base*

5.1 Introduction

Corticosteroids, such as betamethasone and clobetasol, have long been a mainstay in the topical therapy of mild to moderate psoriasis. This class of drug exerts its anti-psoriatic effect via several mechanisms, including immunosuppression, anti-proliferation, and anti-inflammation via interaction with steroid receptors and modulation of gene transcriptions involved in the aetiology of the disease. In general, corticosteroids penetrate poorly into the skin. In order for these steroids to penetrate the skin, esters of these drugs have been synthesized, resulting in increased lipophilicity. These esters are then taken up by the skin and cleaved into the parent corticosteroid by skin esterases. Preparations containing esters such as betamethasone dipropionate and betamethasone valerate, to name a few, are available in different forms (lotions, creams, ointments) and classed according to their potency ranging from superpotent (Class 1) to the least potent (Class 7) (Kruger and Ellis 2003).

SA, on the other hand, does not exert an anti-psoriatic effect per se; instead its keratolytic action is employed to increase delivery of other topical agents by softening and removing the hyperkeratinized scales which pose a substantial barrier for drug delivery across the skin. Formulations containing 2-10% salicylic acid are the most commonly prescribed, although stronger concentrations (up to 20%) are also available to remove very thick plaques (Lebwohl 2005).

Often given separately, these 2 drugs are now also available as single, combined formulations. Examples include Diprosalic® (Schering-Plough) ointment, containing 0.05% betamethasone dipropionate and 3% salicylic acid. It has been reported that the delivery of non-steroidal anti-inflammatory drugs (NSAIDs), such as ketoprofen, was enhanced when co-formulated with EPA/fish oil and vice-versa (Thomas and Heard 2004).

The aims of the current study were to investigate the effects of fish oil addition to the delivery of BD & SA in a combination formulation replicating a commercially available preparation via adhesive tape stripping. In this study, also, the effects of freezing on skin permeability and the activity of skin esterase were probed.

5.2 Materials and methods

5.2.1 Materials

Fish oil capsules 1000mg (Boots Super Strength, lot 0336000100A, composition 33% EPA) were obtained from a local store. Betamethasone dipropionate (lot 066K1212), cetrimide BP, EPA and Hanks balanced salts were obtained from Sigma-Aldrich Company Ltd., Poole, UK. Salicylic acid (batch number A016882401) was obtained from Acros Organics, New Jersey US. Methanol and phosphoric acid were obtained from Fisher Chemicals, Loughborough, UK. Emulsifying ointment B.P (lot 72FG) was obtained from Thornton and Ross, Huddersfield, UK. All other reagents were of analytical grade or equivalent. Freshly excised pig ears were obtained from a local abattoir prior to steam cleaning.

5.2.2 Methods

5.2.2.1 Preparation of test formulations

Three test formulations were prepared. The first formulation (labelled 0% FO), intended to contained 3% salicylic acid, 0.05% betamethasone dipropionate (BD) in emulsifying ointment base with an addition of 0.05%

BHA as an antioxidant. The second formulation (20% FO), contained the same components as above in the same percentages, plus 20% fish oil obtained by squeezing the contents of fish oil capsules into the formulation. The content of EPA in this formulation was worked out to be approximately 6.6%. Emulsifying ointment was melted at 40°C, and the required amount of BD, SA and BHA were weighed and added to the melted base and stirred for 5 minutes to ensure uniform distribution of the drugs. The third formulation consisted of only the emulsifying ointment and BHA, to be employed as a control. All the formulations were kept at 4°C under light exclusion prior to use.

5.2.2.2 *In vitro skin permeation*

In vitro skin permeation was carried out using glass Franz-type diffusion cells, using the method outlined in 3.2.2.2.1. The receptor phase was again cetrimide 30 mg mL⁻¹ added with 0.05% w/v BHA. Solubility of BD and SA in this solution was 2.67 ± 0.14 (5.29 ± 0.27) and 8.7 ± 0.48 (62.98 ± 3.47) μmol mL⁻¹, respectively. Fish oil solubility was 5.0 ± 0.91 μL mL⁻¹. 500 mg of the test formulations were dosed on to the skin. To compare the effects of freezing on skin permeation, the skin sections used in the current study consisted of porcine ear skin sections which had been stored at -20°C (n=3) and freshly excised sections (n=3).

5.2.2.3 *Tape stripping*

Adhesive tape stripping of the permeated areas was carried out according to the method outlined in Section 3.2.2.2.2.

5.2.2.4 Hydrolysis of BD in homogenized porcine skin

To study the activity of skin esterases on BD in viable skin, freshly excised porcine ear skin was homogenized to release the enzymes from the skin. 1 g of the homogenate was then placed in vials containing a known concentration of BD dissolved in Hank's balanced salt buffer solution (HBSBS) incubated in 37°C. At t= 0, 6, 12 and 24 hours, aliquots of the BD solution in each vial were analysed using HPLC to determine changes in concentration.

5.2.2.5 HPLC analysis

BD and SA were analysed using an Agilent series 1100 HPLC system using a Gemini Cu18 150 x 4.6mm column (Phenomenex, Macclesfield, UK), attached to a Phenomenex Securiguard guard column. The wavelength was set at $\lambda=254\text{nm}$. The mobile phase was methanol (85%) and deionized water (15%), pH adjusted to 2.2 with phosphoric acid. The flow rate was 1 mL min^{-1} , the sample injection was $20\mu\text{l}$ and the runtime was 10 minutes. BD was found to elute at 6 minutes (Figure 5.1). Standard calibration curve for BD was constructed over the range of $1\text{-}20\ \mu\text{mol mL}^{-1}$, with an R^2 value of >0.99 . Concentrations of the permeants were then calculated from the area under the curve. SA and EPA were analysed according to the method previously outlined in Section 3.2.2.2.3. The LOD for BD, was $100\ \text{ng mL}^{-1}$ ($1.98 \times 10^{-4}\ \mu\text{mol mL}^{-1}$), while the LODs for SA and EPA were reported previously in the same section.

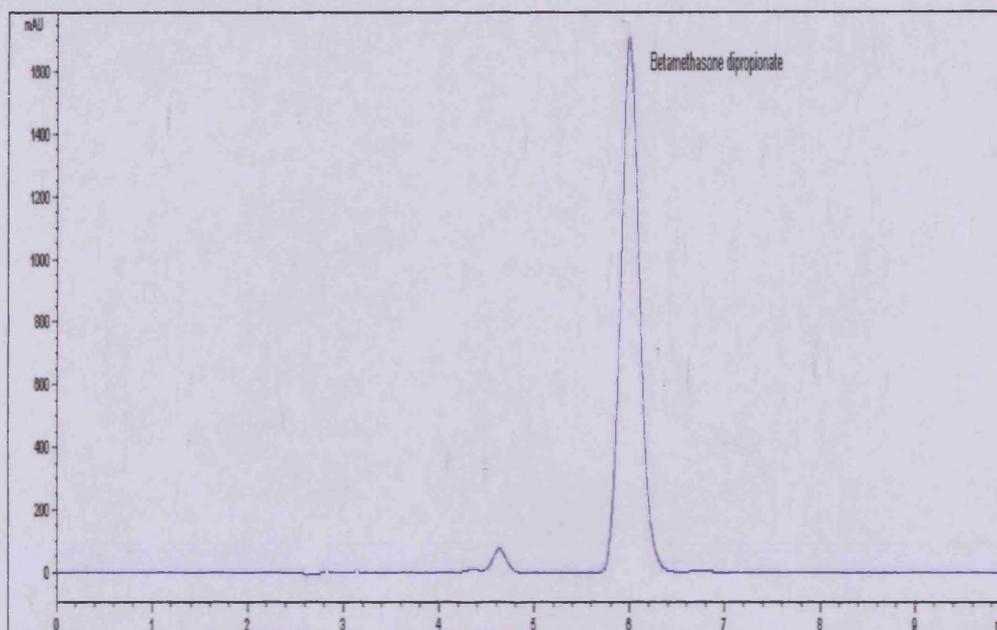


Figure 5.1 : HPLC chromatogram of BD, $\lambda=254$ nm. BD was found to elute at 6 minutes with 85:15 methanol:deionized water, pH adjusted to 2.2, Flow rate was set at 1 mL min^{-1} .

5.2.2.6 *Data analysis and statistical test*

Data from the HPLC analysis were then analysed using Microsoft Excel 2003 and statistical tests done with SPSS version 11.0

5.3 **Results and discussion**

5.3.1 *Depth profile – frozen skin sections*

5.3.1.1 *BD*

The depth profile for BD obtained from the frozen skin sections are shown in Figure 5.2. Comparison of the amount of betamethasone found within the

layers of the skin between the two test formulations shows that more BD was recovered from the first 20 layers treated with formulation containing 0% w/w fish oil (0% FO), which was significantly different from the skin treated with the formulation containing 20% w/w fish oil (20% FO), with a p value of 0.007. However, a higher amount of BD was recovered in the remaining skin treated with 20% FO. Again, a statistical difference was observed with a p value of 0.032 using Mann-Whitney test for two independent samples.

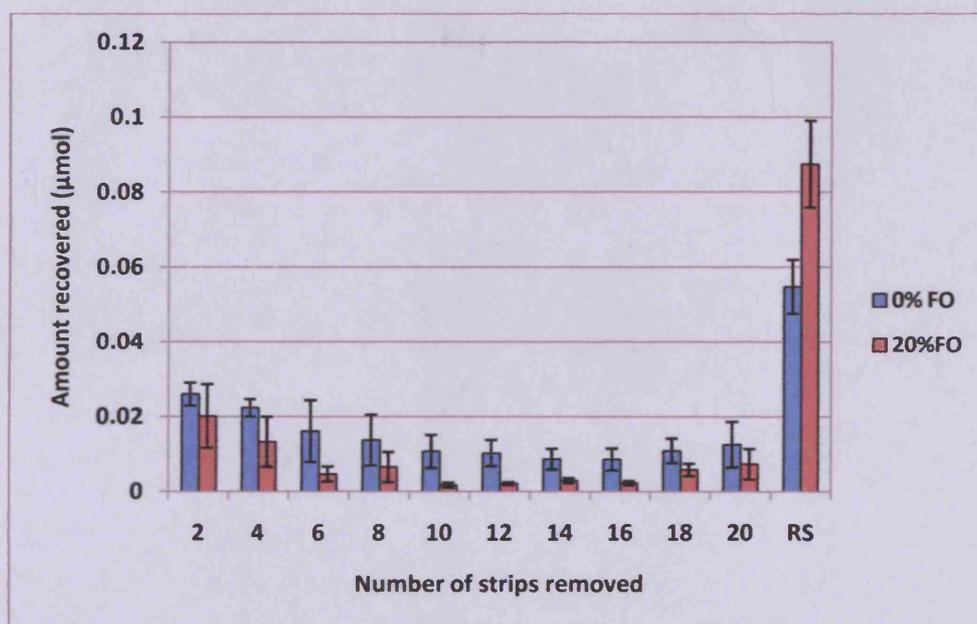


Figure 5.2 : Comparative depth profile of BD from frozen skin sections ($n=3$, \pm SD). RS= remaining skin

The presence of fish oil in the second formulation (20% FO) appeared to alter the deposition of BD within the skin layers, as seen in the plot above. It was suggestive of a reduced retention of BD, leading to enhanced delivery to the remaining layers of the skin. The exact localisation of the penetrants in the remaining skin (RS) in all the results was undetermined, as the remaining skin could possibly retain several layers of unstripped epidermis, in addition to the dermis. As such, the values for RS were a total sum of all remaining layers from the skin sections.

5.3.1.2 SA

A similar observation was made for the depth profile of salicylic acid between the 2 formulations (Figure 5.3), however the difference was not statistically significant for both the first 30 layers of skin (p 0.218) and the remaining epidermis and dermis (p 1.00). It appeared that the enhancement seen with BD was less apparent with SA.

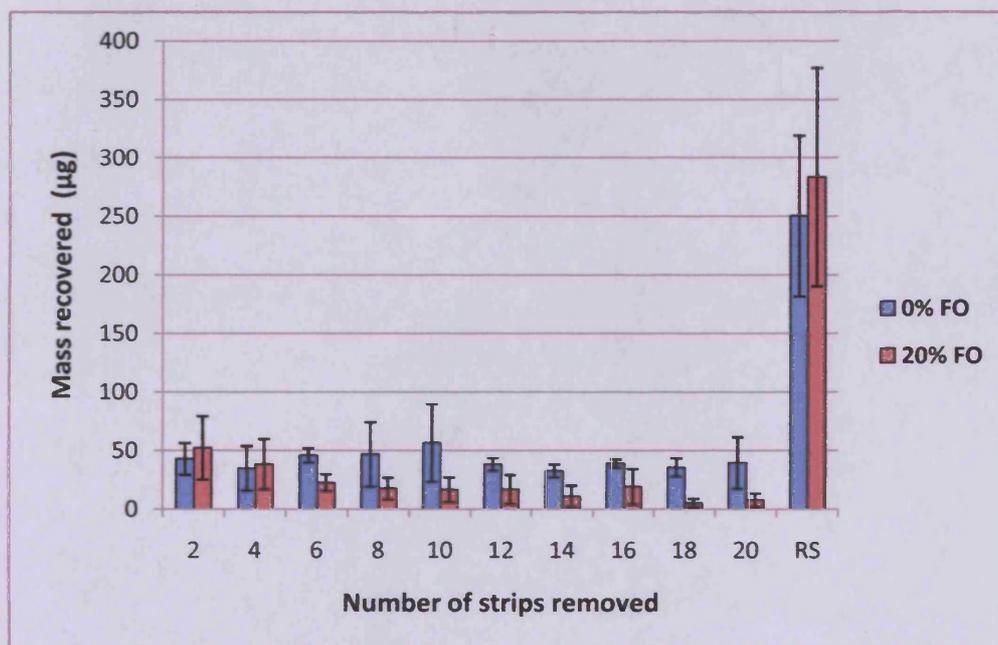


Figure 5.3 : Comparative depth profile of SA from frozen skin sections ($n=3$, \pm SD). RS= remaining skin

5.3.1.3 EPA

The depth profile for EPA is shown in Figure 5.4. The highest mass of EPA was recovered from the first 2 strips, with 0.31 ± 0.08 μ mol. The total amount recovered from the tape strips was 0.53 ± 0.09 μ mol while the amount recovered from the remaining skin was 0.014 ± 0.0069 μ mol.

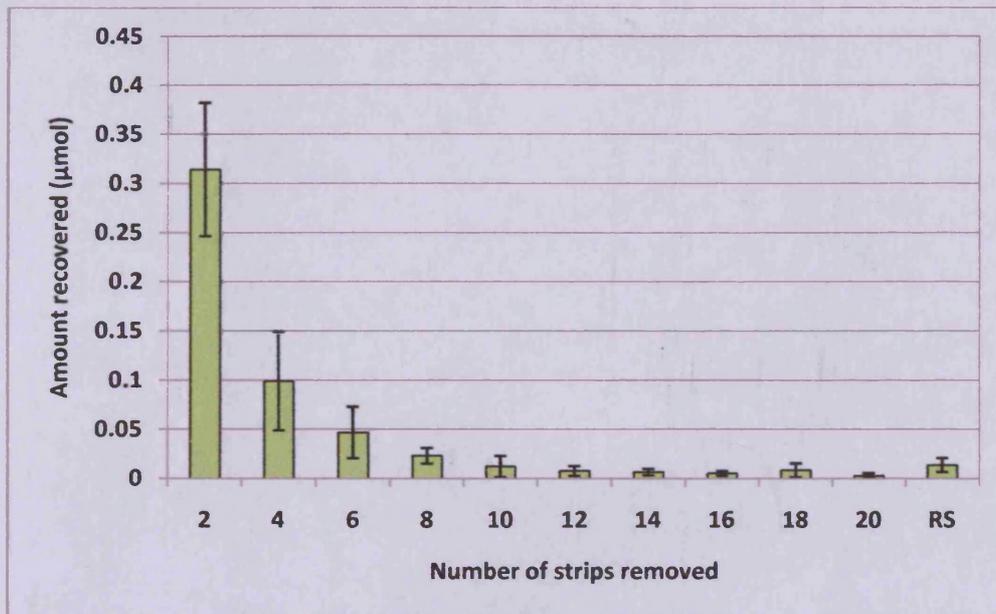


Figure 5.4 : Depth profile of EPA from frozen skin sections ($n=3$, \pm SD). RS= remaining skin

5.3.2 Depth profile – fresh skin sections

5.3.2.1 BD

Analysis of BD depth profiles obtained from the fresh skin sections (Figure 5.5) revealed the same observations with the profile obtained with frozen skin (Section 5.3.1.1). The BD amount recovered from the first 20 strips were not statistically significant between the 2 formulations, with p 0.7394, while there was a marked increase in the amount of BD recovered in the remaining skin treated with the combination formulation, with p 0.001. However, it was observed that the overall amount of BD recovered with the fresh skin section was greatly reduced. The highest amount recovered was from the remaining fresh skin sections in both 0% FO ($9.24 \times 10^{-3} \mu\text{mol}$) and 20% FO ($1.83 \times 10^{-2} \mu\text{mol}$). This, however represents only 17% and 21%, respectively, of the amount recovered from the frozen skin sections. It was also reflected in the

total amount recovered from the 1st 20 strips with 9% (0% FO) and 19% (20% FO), respectively.

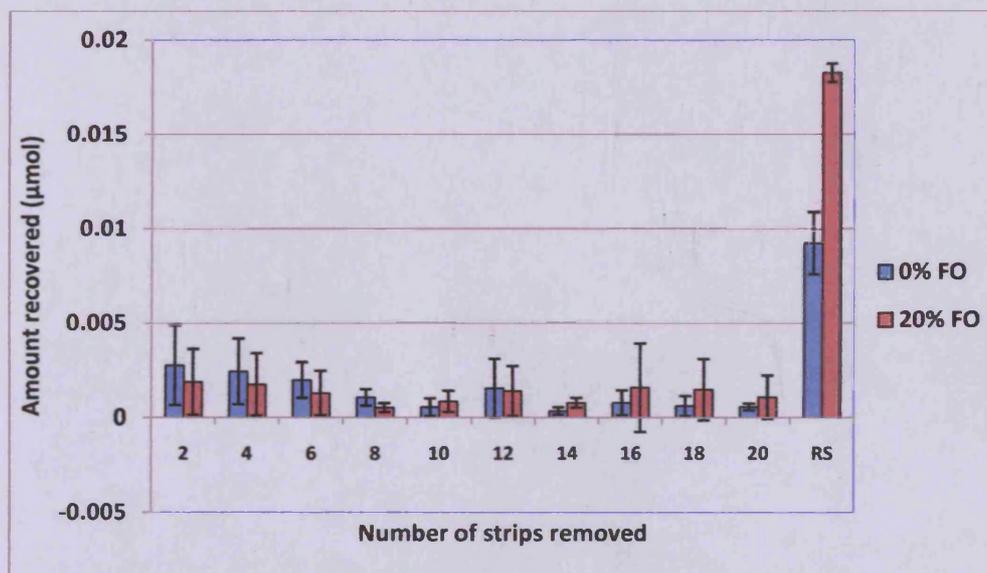


Figure 5.5 : Depth profile of BD from fresh skin sections (n=3, \pm SD). RS= remaining skin

The presence of fish oil in the second formulation (20% FO) appeared to alter the deposition of BD within the skin layers, as seen in both frozen and fresh skin sections. The data suggested decreased localisation of BD within the skin tissue. It was assumed that the presence of the fish oil made binding of BD to skin tissue less favourable. Therefore, there was more BD free to diffuse through the tissue, as evidenced by the 'remaining skin' data.

One possible explanation is that the excess fish oil effectively saturated the tissue, creating a lipoidal environment which would facilitate the movement of the lipophilic BD molecules ($\text{LogP} = 4.07$), at least partially solvated by the PUFA (Thomas et al. 2007b). This is generally referred to as the 'pull effect' (Heard et al. 2006; Kadir et al. 1987).

The interaction of long chain fatty acids, such as oleic acid (and other enhancers, including Azone) with the lipids of the stratum corneum has been reported previously. The classic explanation is that perturbation of the SC

lipids increases the fluidity of these lipids, and hence, leads to enhanced permeation. Lipid 'domains' within the barrier lipids of the stratum corneum in the presence of the fatty acid have also been suggested (Williams 2003). It is felt that such considerations are too simplistic to explain the current results, due to the fact that the fatty acid in question (EPA) was found to penetrate skin layers, and as will be seen in the following chapter (Chapter 6), found to permeate across skin along with the other two permeants (BD and SA).

5.3.2.2 SA

From the depth profile for SA shown in Figure 5.6, it can be gathered that the SA amount recovered from both the first 20 strips and remaining skin were not significantly different between the 2 formulations, with p 0.4183 and 0.9 respectively. In this instance, the amount recovered from the first 20 strips was higher (though not statistically significant) in the presence of FO, while the opposite was seen in the remaining skin. Comparing the frozen and fresh skin sections, the first 20 strips of the fresh skin sections recorded only 13.5% and 29.8% of the frozen skin. For the remaining skin, however, the amounts recovered were close between fresh and frozen, with 74.5% and 56.6% for 0% FO and 20% FO, respectively.

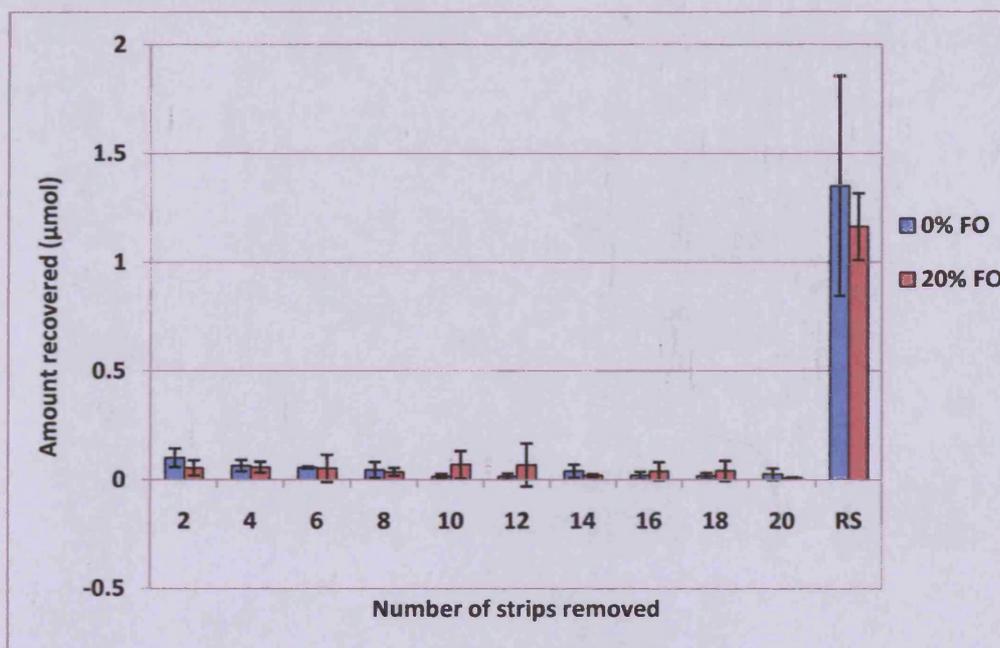


Figure 5.6 : Depth profile of SA from fresh skin sections ($n=3, \pm SD$). RS= remaining skin

5.3.2.3 EPA

In contrast with the depth profile obtained with frozen skin, the highest EPA amount in the fresh sections was recovered from the remaining skin (Figure 5.7). The EPA recovered from the 1st 20 strips (0.289 μmol) which represented 54.8% of the amount recovered in frozen sections. Comparing the effect of fresh and frozen sections, the EPA recovered from the remaining skin, however, was approximately 30 times the EPA recovered from the latter.

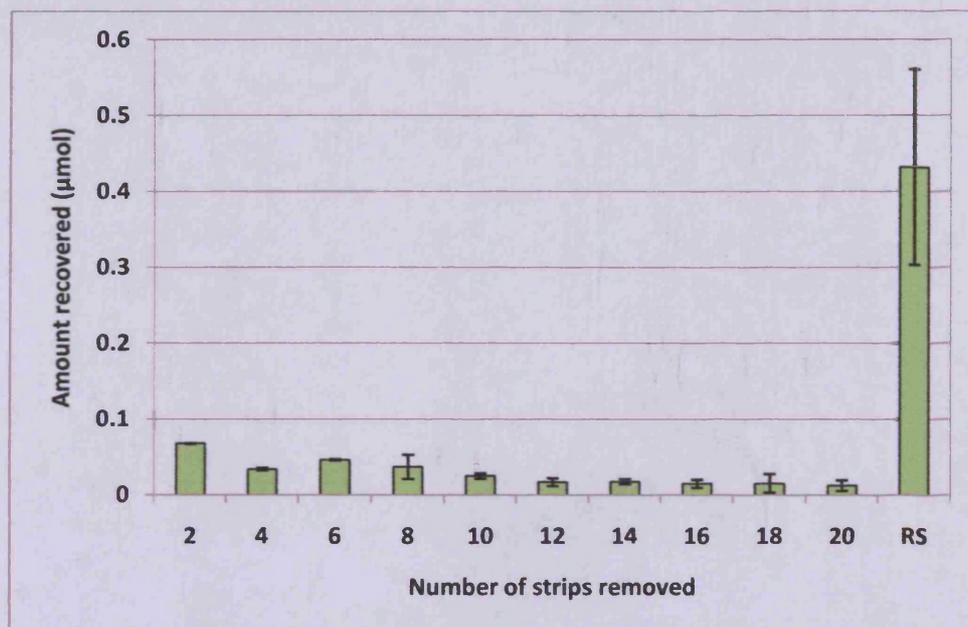


Figure 5.7 : Depth profile of EPA from fresh skin sections (n=3, \pm SD). RS= remaining skin

One possible explanation of this reversed effect is the freezing process may have disrupted the intercellular lipid matrix, which theoretically is the route favoured by the highly lipophilic EPA. The intact lipid in fresh skin then resulted in higher deposition of EPA to the lower layers.

In both cases (frozen vs. viable skin), the receptor phase was not analyzed for permeated BD, SA and EPA. While the data indeed showed an alteration in the delivery, particularly for BD, it is acknowledged that for a more complete picture on the effects of fish oil and in general, the fatty acids, on transcutaneous delivery of drugs the amount permeated across the membrane and amount remaining in the donor phase need to be accounted for. The efficiency of extraction from both the adhesive tape and skin sections, which were not validated, could also impact on the total recovered amount of penetrants in the study. These considerations must be taken into account for future studies of this nature.

5.3.3 Hydrolysis of BD in homogenized porcine skin

BD is one of the several esters of betamethasone developed specifically to increase their lipophilicity, hence increase their permeation across skin. In the skin, the ester bond is cleaved by esterases, liberating the parent compound.

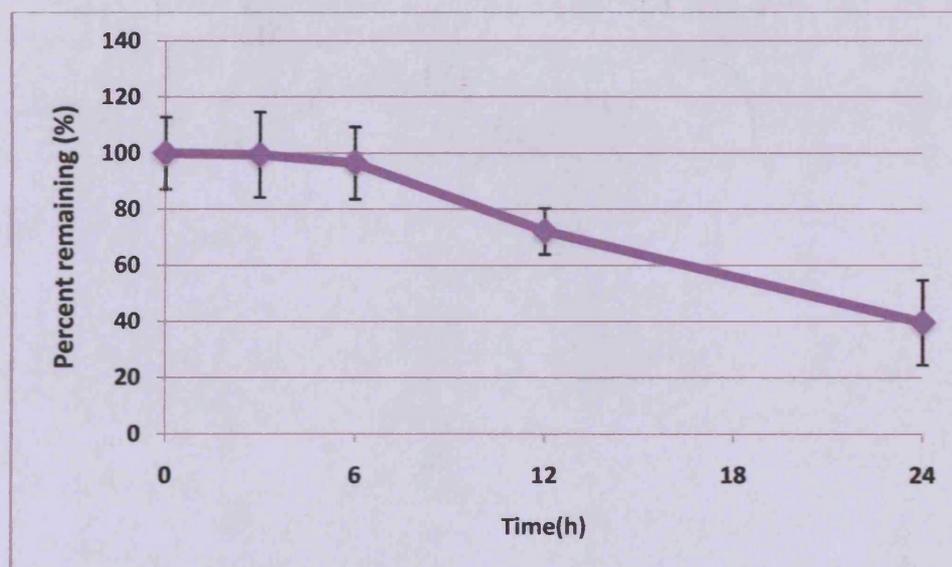


Figure 5.8 : % remaining BD incubated with homogenized porcine skin over time. (n=3, \pm SD)

Analysis of the concentration of BD, expressed as a percentage over the starting concentration, revealed that the concentration of BD declined progressively over 24 hours (Figure 5.7). The decline was most apparent after 12 hours incubation, the concentration at $72.22 \pm 8.3\%$ of the original concentration. After 24 hours, the remaining concentration was only $39.61 \pm 15\%$. This indicated continuous esterase activity in viable skin homogenate for up to 24 hours. The result also corresponded to the earlier depth profile, where the amount of BD recovered from fresh skin sections were approximately 20% of that from frozen skin.

The addition of fish oil in the test formulation replicating a commercially available corticosteroid appeared to enhance the delivery of BD, particularly to the lower layers of the skin. The lower basal layer of the epidermis is of particular importance, as this layer is metabolically active (Thomas and Heard 2007), and, in conditions such as psoriasis, are continuously dividing, presenting an important target for therapeutic agents such as BD. Enhancing delivery of these agents would, in theory, increase the efficacy and would allow dose reduction, particularly in view of the side effects commonly associated with prolonged use of corticosteroids, as discussed in Section 1.2.1.1. The addition of EPA, apart from the observed enhancement of corticosteroid delivery across the skin, possesses the extra benefit of reducing inflammation via production of less potent inflammatory mediators, inhibition of skin inflammatory enzymes, and promotion of the resolution phase of inflammation, as reported by several studies (DeFilippis and Sperling 2006; Riku et al. 1993; Schwab and Serhan 2006; Thomas et al. 2007a)

The effect of freezing on transdermal drug delivery and skin permeability has indeed been studied previously (Ahlstrom et al. 2007; Babu et al. 2003; Bosman et al. 1998; Shaikh et al. 1996). The majority of the studies were in agreement that the fluxes of topically applied compound across frozen skin were greatly increased, although permeation characteristics of the test compounds remained the same. The duration of freezing also were found to be crucial, with the suggestion that the longer the skin were frozen the greater the increase in flux (Ahlstrom et al. 2007; Babu et al. 2003; Bosman et al. 1998). The difference has been attributed to a variety of factors, from rearrangement of molecules within the skin (Weber 1993), structural damage and protein denaturation, and the release of protease and free radicals as a result of the freezing and thawing process (Kearney 1998).

The difference in metabolic capacity between fresh and frozen skin should also be considered. In a study looking at the activity of esterases, it was found

that one week- frozen porcine skin sections maintained enzymatic activity, although it was markedly reduced compared to fresh skin (Lau 2008). Contrastingly, Shaikh et al. (1996), using 8-methoxypsoralen as a test permeant, found that the metabolic capacity remained constant between frozen and fresh skin, despite increased permeability with the former. Perhaps the difference depended on the particular enzyme involved, the nature of the permeating compound, and factors such as the temperature and the length of storage in the different studies.

5.4 Conclusion

Based on the comparative depth profiles obtained, it is of a particular benefit to incorporate EPA or fish oil in topical formulations to employ both its anti-inflammatory activity and the enhancement properties of this n-3 fatty acid. The difference observed in the use of frozen/non-viable skin in the results highlights the importance of storage conditions and factors that may contribute to the outcome of permeation studies. Although there may be contrasting reports on the effects of freezing, the use of viable skin is logically more preferable to provide an in vitro condition as close as possible to in vivo conditions.

Chapter 6:

*In vitro transcutaneous delivery
of topical anti-psoriatic
medication and fish oil across
excised defolliculated mouse skin*

6.1 Introduction

In chapter 5, the delivery of BD, SA and EPA from FO across excised porcine ear skin was investigated. It was found that the addition of FO enhanced the delivery of BD and SA to the lower layers of the epidermis. As the first part of the study involving DfL mouse as a model for psoriasis, the transcutaneous delivery of the same agents was investigated. The aim of this study was to determine whether the same enhancing effect observed in porcine ear skin can be replicated in DfL mouse skin, *in vitro*. This was achieved through permeation studies using Franz-type diffusion cells and depth profiling via adhesive tape stripping.

6.2 Materials and methods

6.2.1 *Materials*

The materials used in this study were the same as listed previously in Section 5.2.1. D-squame© adhesive discs (CuDerm Corporation) were obtained from Fisher Scientific (Loughborough, UK). Excised DfL mouse back skin was obtained from Dr. Rebecca Porter of the Department of Dermatology, Cardiff University and frozen in -80°C prior to use. All skin sections were from mice of 15 weeks and above when the destruction of hair follicles is complete.

6.2.2 *Methods*

6.2.2.1 *In vitro skin permeation of EPA from FO*

To look at the delivery properties of EPA from FO across DfL skin, the contents of fish oil capsules were squeezed into a clean glass vial and 0.05% w/w BHA added as a preservative. 1mL of the mixture was then dosed onto

excised DfL skin (n=3) mounted on Franz cells with cetrimide 30mg mL⁻¹ added with BHA as a receptor solution. Permeation experiment were then carried out according to the method outlined in Section 2.2.2.3.3 with whole receptor phases removed at 3, 6, 12, 24 and 48 hours.

6.2.2.2 *In vitro skin permeation and depth profiling of topical formulation containing BD, SA and EPA from FO*

6.2.2.2.1 *In vitro skin permeation*

Permeation experiments were carried out according to the method previously described in Section 2.2.2.3.3. The skin sections were dosed with 100 mg of the formulations listed in Table 6.1 (n=3). The formulations were prepared according to the method described in Section 5.2.2.1. The required amount of each formulation was then weighed and massaged gently on to the skin sections. Whole receptor phases were collected at 3, 6, 12 and 24 hours.

Formulation	Contents
BDO	0.05% BD, 3% SA, 0.05% BHA in emulsifying ointment
BF	20% FO, 3% SA, 0.05% BHA in emulsifying ointment
BDF	0.05% BD, 20% FO, 3% SA, 0.05% BHA in emulsifying ointment
Control	0.05% BHA in emulsifying ointment

Table 6.1 : The test formulations used in permeation and depth profiling studies across excised DfL skin

6.2.2.2.2 *Depth profiling*

Skin sections from the above permeation experiments were sequentially stripped with D-squame® adhesive discs (CuDerm Corporation, Dallas, US) after 24 hours of permeation studies according to the method outlined in Section 3.2.2.2.2. Excess formulations were wiped off before stripping, with the first 2 strips discarded. The following 20 strips were then collected in batches of 2 and placed in methanol as an extracting solvent.

6.2.2.3 *Analysis of EPA*

Analysis of EPA from permeation experiment (Section 6.2.2.1) was conducted using GC with the method previously described in 2.2.2.2. The LOD of EPA for the GC analysis was reported previously in the same section. For depth profile studies, EPA was analysed using HPLC according to the method first described in Section 3.2.2.3. Both methods were adaptations of previously reported validated analysis (Christie 2003; Thomas 2006). However, in this study, the two methods were not validated against each other and was determined as a possible limitation.

6.2.2.4 *Analysis of BD and SA*

Quantification of BD and SA by HPLC used the same method described previously in Section 5.2.2.5. The LOD for BD and SA were also reported previously in the same section.

6.2.2.5 Data collection and statistical analysis

Data from both GC and HPLC were collated using Microsoft Excel 2007 and statistical analysis done with InStat 3 for Microsoft Windows (GraphPad Inc., Elysian, US).

6.3 Results and discussion

6.3.1 In vitro skin permeation of EPA from FO across excised DfL skin

The plot for cumulative amount of EPA over area across 48 hours is shown in Figure 6.1. After 48 hours, the amount of EPA permeated across DfL skin was $0.356 \pm 0.07 \mu\text{mol cm}^{-2}$. As a comparison, the amount of EPA permeated across porcine ear skin over the same time period was $0.046 \pm 0.003 \mu\text{mol cm}^{-2}$. Thus DfL skin was more permeable than porcine ear skin by a factor of 10. Mann-Whitney test for the fluxes (Table 6.2) of the two different skins also revealed a very significant difference, with p 0.0017, with EPA flux across porcine skin calculated to be $1.23 \times 10^{-3} \pm 8.46 \times 10^{-4} \mu\text{mol cm}^{-2} \text{h}^{-1}$, compared to $1.07 \times 10^{-2} \pm 1.25 \times 10^{-3} \mu\text{mol cm}^{-2} \text{h}^{-1}$ across DfL skin.

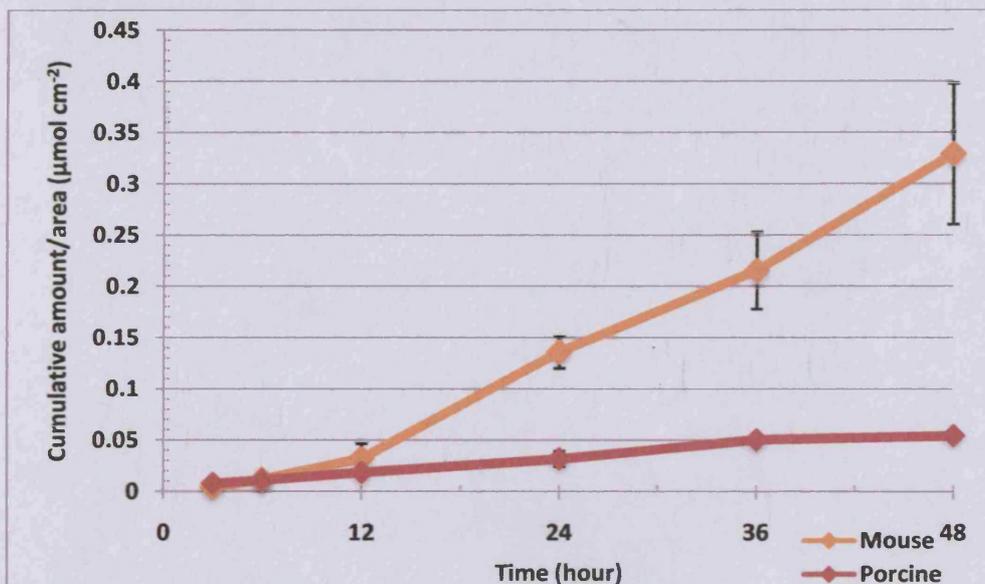


Figure 6.1 : Permeation profile of EPA across excised DfL mouse skin (n=3 ± SD).

Skin	Flux, $J_{ss} \pm SD$ ($\mu\text{mol cm}^{-2} \text{h}^{-1}$)
Porcine	$1.23 \times 10^{-3} \pm 8.46 \times 10^{-4}$
DfL	$1.07 \times 10^{-2} \pm 1.25 \times 10^{-3}$

Table 6.2 : Average pseudo-steady state flux of EPA across porcine and DfL skin (n=3,±SD)

The pig epidermis has an average thickness of 60-85 μm (Ute et al. 2007). The skin of the back of a DfL mouse of 15 weeks and above, contrastingly, has an epidermis which is between 13-40 μm . At this age, the DfL have already lost their hair follicles and there is a certain degree of epidermal hyperproliferation. Taking this into account, the DfL epidermis is still considerably thinner than the porcine ear. It was interesting to note that only 0.033% of the total EPA was detected across DfL skin, considering the thinness of the epidermis. With the porcine skin, only 0.0042% of total EPA was detected. This was comparable to the amount reported in Section 2.3.2.1 (0.0034% of total EPA)

It must be noted that, although the findings in this study were essential to determine the delivery properties of these agents prior to an *in vivo* study with live DfL mice, porcine ear skin is an established model for human skin in transdermal and transcutaneous studies (Simon and Maibach 2000a; Ute et al. 2007). Thus, the results obtained from DfL mice may not reflect conditions in humans; the same however, must be said even with the more established porcine model.

6.3.2 *In vitro* skin permeation of topical formulations containing BD, SA, and EPA from FO across DfL skin

6.3.2.1 *BD*

As clearly evident from the permeation plot of BD for both BDO and BDF, the addition of fish oil altered the delivery of BD. The cumulative amount of BD retrieved over 24 hours (Figure 6.2) from BDF was higher, at $9.12 \mu\text{mol cm}^{-2}$, compared to BDO ($4.99 \times 10^{-3} \mu\text{mol cm}^{-2}$). The Mann-Whitney test for 2 independent samples showed a significant difference between the cumulative amounts permeated from the two formulations, with p 0.0208. This was suggestive of an enhancing effect on behalf of fish oil on the delivery of BD.

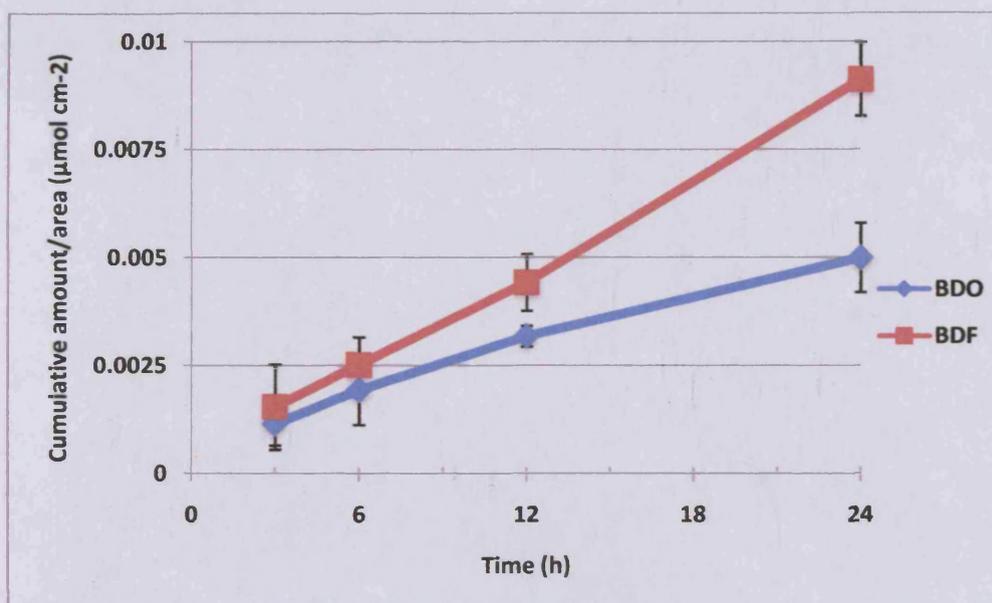


Figure 6.2 : Permeation profile of BD from BDO and BDF across excised DfL skin ($n=3$, \pm SD). Addition of fish oil increased the flux of BD (p 0.032), hence, increased cumulative amount of BD recovered (p 0.0208) from skin sections dosed with BDF.

The enhancing effect of fish oil on BD delivery was further strengthened by the J_{ss} value determined from the 2 formulations, shown in Table 6.3.

Formulation	Flux, J_{ss} ($\mu\text{mol cm}^{-2} \text{h}^{-1}$)
BDO	$1.43 \times 10^{-4} \pm 0.98 \times 10^{-4}$
BDF	$4.11 \times 10^{-4} \pm 0.08 \times 10^{-4}$

Table 6.3 : Average pseudo-steady state flux of BD from BDO and BDF across DfL skin. Presence of fish oil appear to increase the flux of BD by approximately 3 times.

In the presence of fish oil, the flux for BD increased by approximately 3 times (p 0.032). The exact mechanism of this enhancement on BD was not determined, however, several possibilities were discussed earlier in Chapter 5, and more importantly, the delivery enhancing effects of fish oil have been reported previously (Thomas et al. 2007b).

6.3.2.2 SA

From Figure 6.3, the cumulative amount of SA permeated over 24 hours was the highest from BF, followed by BDO and finally, BDF. These values however were not statistically significant, with p 0.0734.

The SA fluxes from all three formulations (Table 6.4) were also not significantly different (p 0.4979), suggesting there is no enhancement of SA delivery either by BD, FO, or both of them when co-formulated with SA. This was in contrast with the enhancement seen earlier with BD. It did, however, concur with the findings in Chapter 5, whereby the delivery of SA was not enhanced significantly in both fresh and frozen porcine skin sections.

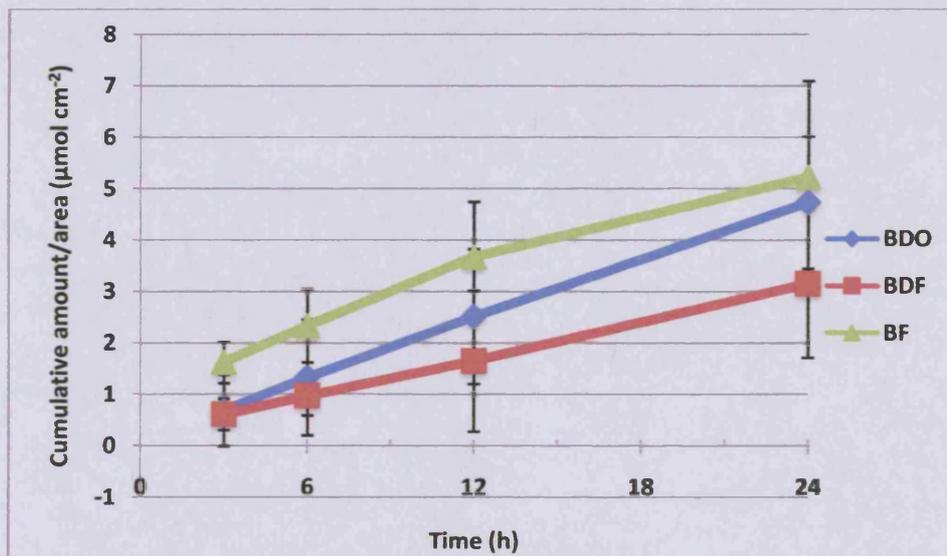


Figure 6.3 : Permeation profile of SA from BDO, BDF, and BF across DfL skin.

Formulation	Flux, J_{ss} ($\mu\text{mol cm}^{-2} \text{h}^{-1}$)
BDO	0.189 ± 0.014
BDF	0.164 ± 0.012
BF	0.201 ± 0.002

Table 6.4 : Average pseudo-steady state flux of SA from BDO, BDF and BF

($n=3, \pm\text{SD}$)

6.3.2.3 EPA

Analysis of samples that were treated with FO-containing formulations (BF and BDF) by GC revealed that the levels of EPA were under the limit of detection in both cases. Considering that only 0.033% of EPA permeated when delivered as the stock oil (containing 330 mg EPA), the non-detection of EPA, which in both formulations constituted only 6.6 mg per 100 mg, was not surprising. This did not reflect poorly on the overall delivery of EPA, as will be discussed further in the following depth profiling studies.

6.3.3 Comparative depth profiling of BD, SA, and EPA from topical formulations across excised DfL skin.

6.3.3.1 BD

Figure 6.4 shows the comparative depth profile of BD from two different BD formulations. After 20 strips, the amount of BD recovered from BDO and BDF were 8.03×10^{-3} and 7.35×10^{-3} μmol , respectively. The difference was not statistically significant, with p 0.5787. For the remaining skin, however, there was a huge difference (p 0.0067) with the amount of BD recovered from the

combined formulation ($2.99 \times 10^{-3} \mu\text{mol}$), at 3 times higher compared to BDO ($9.95 \times 10^{-4} \mu\text{mol}$). This again was suggestive of an enhancing effect of EPA on the delivery of BD, as discussed in Chapter 5, and concurred with the permeation results in Section 6.3.2.1

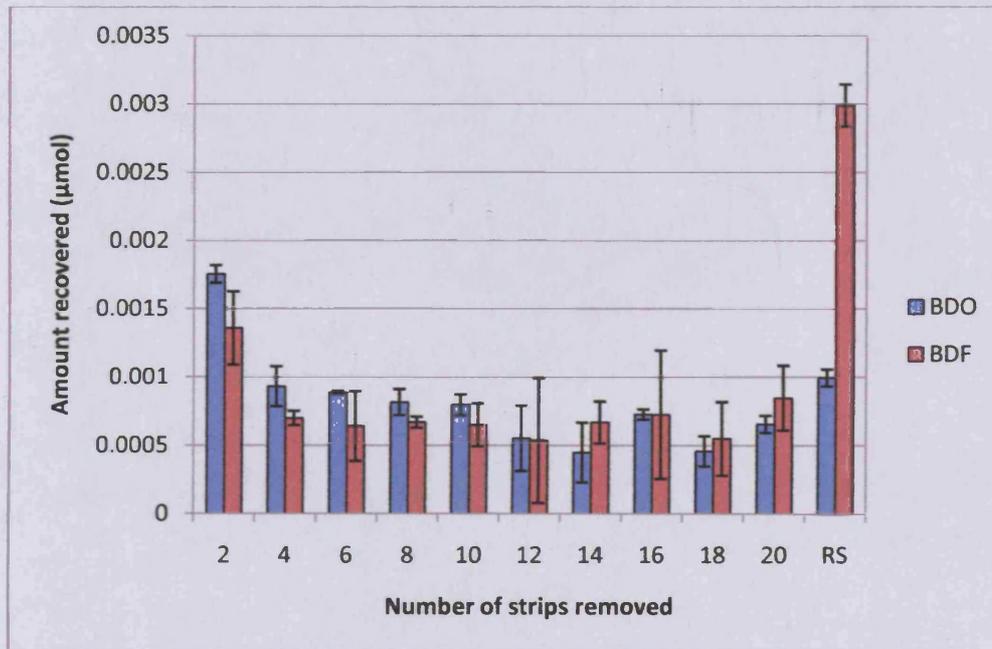


Figure 6.4 : Comparative depth profile of BD recovered from BDO and BDF treated DfL skin (n=3, \pm SEM). RS = remaining skin

It was stated earlier in Chapter 5, that this enhancement may, in part, be attributed to the 'pull' effect. It was also stressed that the basal layer of the epidermis contains metabolically active cells, and IHC staining (will be explained in Chapters 7 & 8) also revealed that major inflammatory enzymes within the epidermis were localized in this layer.

6.3.3.2 SA

Analysis of the depth profile for SA (Figure 6.5) found no significant difference (p 0.0539) in the amount of SA recovered from BF, BDO, and BDF within the first 20 strips. The amount of SA recovered from the remaining skin was highest in BDF, followed by BDO, and BF (p 0.0001). This was the opposite of the permeation profile in Section 6.3.2.2; the low amount of SA permeating from the combined BD, SA and FO formulation translated to a higher deposition in the remaining skin. It was noted that the deposition within the skin was influenced by the presence of BD instead of EPA.

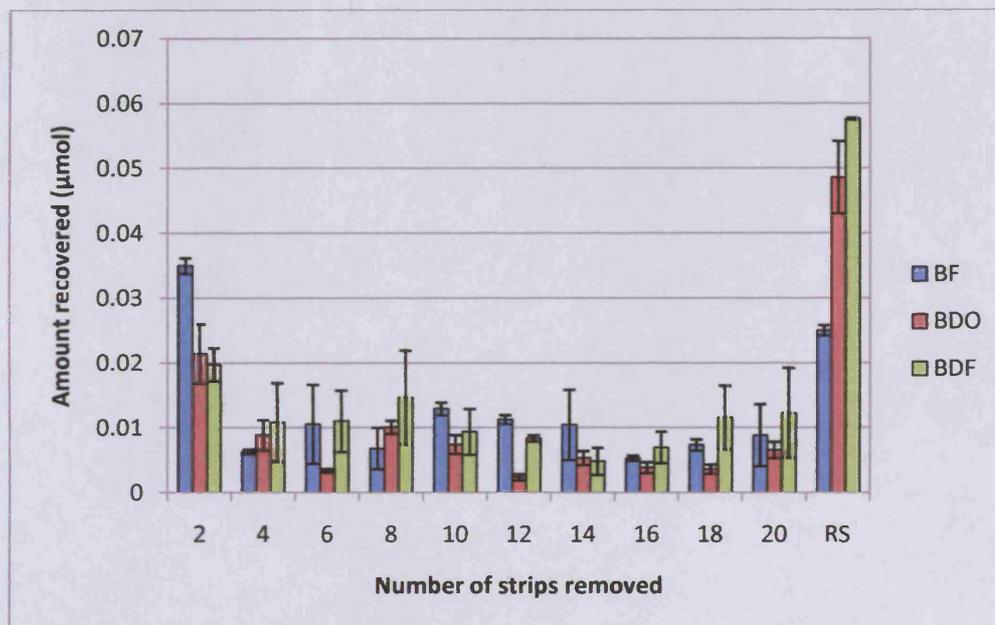


Figure 6.5. : Comparative depth profile of SA recovered from BF, BDO and BDF treated DfL skin ($n=3$, \pm SEM). RS= remaining skin.

6.3.3.3 EPA

Comparative depth profile of EPA (Figure 6.6.) obtained from the two FO-containing formulations (BF and BDF) revealed that less EPA was recovered from the first 20 strips in the combined BD+FO formulation. However, Mann-

Whitney test for two independent samples revealed no statistical difference, with the p value at 0.7394. Meanwhile, the amount recovered from the remaining skin was 0.086 μmol for BF and 0.113 μmol for BDF. This supported the observation of the 'pull' effect between EPA and BD reported in 6.3.3.1. However, this difference was not statistically significant, with p 0.1650.

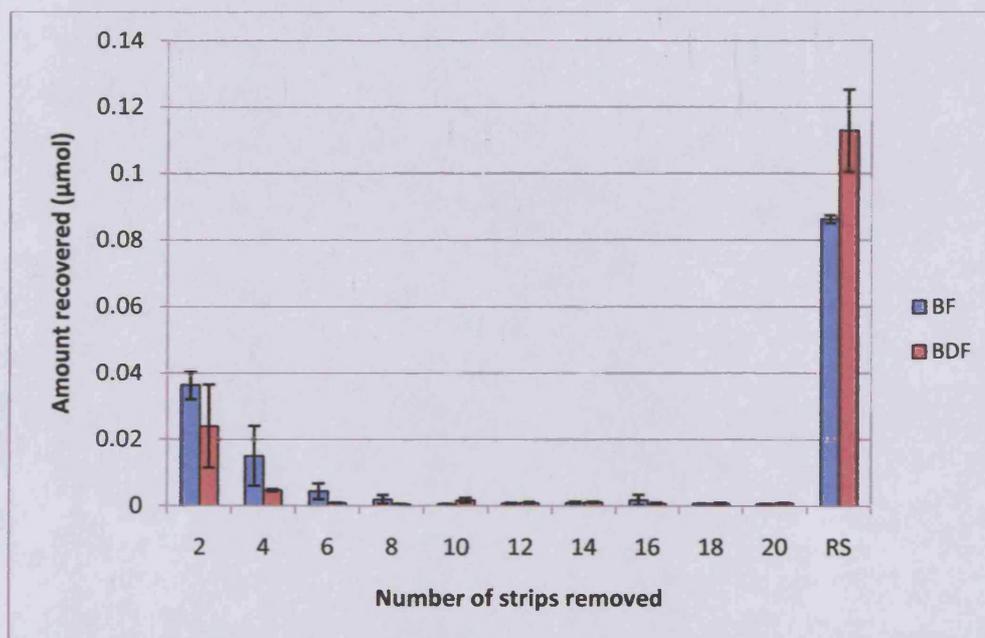


Figure 6.6 : Comparative depth profile of EPA recovered from BF and BDF treated DfL skin ($n=3$, \pm SEM). No significant difference ($p > 0.05$) was observed in the amount of EPA from the 2 formulations for both the first 20 strips and remaining skin (RS).

In Chapter 5, we have discussed the depth profile of these agents in porcine skin. To recap, the amount of BD in the remaining skin was significantly higher with the presence of FO. In the case of salicylic acid, the difference in the amount recovered was not significant, although, again, there was an increase in its delivery to the lower layers of the epidermis.

These findings were replicated with the depth profiles obtained using DfL skin. Note however, that the total amount being recovered was lower

compared to the study using porcine ear skin. The vast difference in thickness between the two types of skin and impaired barrier meant that DfL skin were more permeable to the compounds, hence the reduced deposition within the skin layers.

6.4 Conclusion

The study into the transcutaneous delivery of topical anti-psoriatic agents using DfL skin showed that BD, SA and EPA were delivered successfully. The delivery enhancement action of EPA seen with porcine skin was again proven in this murine model; however, there were differences in the extent and the amount of drug recovered. These findings represent the first step towards establishing this spontaneous mutant in an in vivo setting, which will be explored in Chapters 12 and 13.

Chapter 7:

*Enhanced anti-inflammatory
activity of a betamethasone
dipropionate formulation
containing fish oil*

7.1 Introduction

In Chapters 5 and 6, it was found that delivery of a corticosteroid (BD) was enhanced, especially to the lower layers of the skin, when co-formulated with SA and fish oil. Depth profiling of porcine and DFL skin treated with a formulation containing BD, SA and 20% FO showed a higher deposition of BD in the remaining skin retrieved post-adhesive tape stripping compared to a same formulation without FO. The purpose of this work was to investigate whether this enhancement translates to an increased biological activity *in vitro*, determined here by immunohistochemistry (IHC) staining and Western blot (WB) analysis.

Cyclooxygenase 2 (COX-2) and 5-lipoxygenase (5-LOX) are the main enzymes involved in the production of inflammatory mediators or eicosanoids within the arachidonic acid (AA) cascade, otherwise known as the inflammation cascade. The products of this cascade, such as leukotriene, prostaglandin, and thromboxane, help to protect the body from injury; released act in response to noxious stimuli (Yamazaki et al. 2006). Prolonged inflammatory processes have been implicated as the main cause of diseases, such as cardiovascular problems, asthma, and skin diseases such as psoriasis (Gil 2002). Modulation of cellular fatty acid components from the pro-inflammatory arachidonic acid to n-3 fatty acids, such as EPA, has been reported to improve symptoms and ameliorate inflammatory diseases.

Considering their important role in inflammation, these two enzymes were chosen in this study, and how fish oil and anti-psoriatic medication affect their expression was investigated.

7.2 Materials and methods

7.2.1 Materials

Fish oil capsules 1000 mg (Boots Super Strength, lot 0336000100A, 33% EPA) were obtained from a local store. Betamethasone dipropionate (lot 066K1212) was obtained from Sigma-Aldrich Company Ltd., Poole, UK. Salicylic acid (lot A016882401) and bromophenol blue (lot A0251435) were obtained from Acros Organics, New Jersey US. COX-2 (#4842) and 5-LOX (lot 123241-12342) antibody were obtained from Cell Signalling Technology and Cayman Chemical Company, respectively. Aprotinin, glycine, leupeptin, ammonium persulphate (APS, lot 16531JH), β -actin (A2228), bovine serum albumen (BSA), acrylamide/bis-acrylamide (30% solution v/v, 29:1 ratio), dimethyl sulphoxide (DMSO), di-n-butylphthalate in xylene (DPX) tissue mounting media, dithiothreitol (DTT) 1M in water, methyl green, N,N,N',N'-tetramethylene-diamine (TEMED, lot 0768871), phosphate buffered saline (PBS), Ponceau S, sodium chloride (NaCl), sodium dodecyl sulphate (SDS, lot 0806443), and Trizma (Tris base) were purchased from Sigma-Aldrich, Poole, UK. Bromophenol blue was sourced from BDH Chemicals Ltd, Poole, UK. Chemiluminescent Supersignal® West HRP substrate (Pico, Dura and Femto) came from Pierce and Warriner Ltd, Chester, UK. Rainbow™ protein molecular weight markers (RPN800E, 12-225 kDa) were from GE Healthcare Life Sciences (Buckinghamshire, UK). Emulsifying ointment B.P (lot 72FG) was obtained from Thornton and Ross, Huddersfield, UK. All other reagents were of analytical grade or equivalent. Freshly excised porcine ears were immersed in Hanks balanced salt buffer solution (HBSBS) and used within 1 hour of slaughter. The hair was removed with a clipper and full thickness skin from the dorsal side of the ear was then excised and cut into sections of approximately 2x2cm.

7.2.2 Methods

7.2.2.1 *Preparation of test formulations*

Two test formulations were prepared. The first formulation (0% FO), intended to replicate the dosage of commercially available formulation (Diprosalic® ointment, Schering-Plough) contained 3% salicylic acid and 0.1% betamethasone dipropionate in emulsifying ointment base with an addition of 0.05% BHA as an antioxidant. The second formulation (20% FO), contained the same components as above in the same percentages, plus 20% fish oil. The content of EPA in this formulation is approximately 6.6%. Emulsifying ointment was melted at 40°C, and the required amount of BD, SA and BHA were weighed and added to the melted base and stirred to ensure uniform distribution of the drugs. All the formulations were kept at room temperature under light exclusion prior to use.

7.2.2.2 *IHC staining*

IHC is a technique used to determine the localization of a protein within a tissue. It is based on the principle of specific binding between an antibody and an antigen, which can then be visualized in several ways. Common visualization strategies include antibodies bound to a fluorescent molecule, or an enzyme which, in the presence of a substrate, results in a coloured product (Ramos-Vara 2005).

7.2.2.2.1 In vitro permeation & tissue fixation

Freshly excised porcine skin sections mounted on Franz-type glass cell were treated with 100 mg of the test formulations gently rubbed onto the sections using a glass rod. The cells were maintained in a water bath set at 37°C and both the donor and receptor phase occluded. The diffused area was excised at 0, 3, and 6 hours post application, placed in disposable cassettes and fixed in 4% formaldehyde overnight.

7.2.2.2.2 Dehydration of tissues and embedment in wax

The skin sections were dehydrated with ethanol of increasing concentration and chloroform according to the protocol shown in Table 7.1.

Solution	Time
70% ethanol	2 x 30 mins
90% ethanol	2 x 30 mins
100% ethanol	2 x 30 mins
Chloroform	1 x 1 hour, followed by 2 x 30 mins

Table 7.1 : Solutions used for tissue dehydration

The cassettes were then immersed in 3 different wax baths containing molten embedding medium according to the protocol in Table 7.2.

Wax bath	Instructions
1	Vacuum turned off for 15 mins, on for 10 mins, and turned off again for 5 mins
2	Vacuum turned off for 10 mins, on for 15 mins, and turned off again for 5 mins
3	Vacuum turned off for 10 mins, on for 15 mins, and turned off again for 25 mins

Table 7.2 : Protocol for the wax baths

The sections were then taken out from the cassettes, placed in moulds, and arranged on their sides so the layers of the skin were perpendicular to the surface of the mould. In this position, molten medium were then poured on from a reservoir ensuring no bubbles were formed. The cassettes, with their lids removed, were then quickly placed on top of the molten medium and allowed to solidify overnight. The blocks (Figure 7.1) were then taken out of the mould for sectioning.



Figure 7.1 : Porcine skin section embedded in paraffin wax blocks. Sections of 5 μm thicknesses were then cut from these blocks

7.2.2.2.3 Sectioning

Sections of 5 µm thicknesses were microtomed from the wax blocks using a Shandon Finesse microtome and mounted on pre-cleaned Super Frost™ microscope slides (Surgipath). To ensure the slices were completely flat before mounting, the wax slices were floated on a beaker of water heated to 40°C which will then automatically remove any kinks or folding. The slices were then quickly picked up using the slides. They were then allowed to dry in an oven set at 40°C overnight.

7.2.2.2.4 Dewaxing and rehydration

Prior to staining, the slides need to be dewaxed by first placing the slides in an oven set at 60°C. This is to facilitate dewaxing by melting the embedding medium. Complete removal of the paraffin was ensured by soaking the slides in 2 x 7 minutes chloroform. Rehydration of the tissues was done using 2 x 3 minutes of ethanol in decreasing concentrations (100, 90 and 70%). The slides were then soaked in distilled water followed by PBS at 5 minutes each.

7.2.2.2.5 Blocking of endogenous peroxidases

Blocking of endogenous peroxidases is an important step in immunostaining with HRP linked antibodies. Using HRP conjugated antibody may result in high, non-specific background staining caused by the peroxides (Gao et al. 2008). 3% Hydrogen peroxide (H₂O₂) is the traditional blocking reagent, and was applied on the skin sections for 5 minutes. Afterwards, excess H₂O₂ was removed and the slides washed in 2 changes of PBS for 3 minutes.

7.2.2.2.6 Antigen retrieval

Antigen retrieval or recovery is conducted to retrieve the antigen of interest that might have been masked during the fixation process. This step also allows for a better staining (Shi et al. 2001). This entails a simple process of boiling the slides in a suitable buffer. For this study, the slides were microwaved in 1 litre citrate buffer, prepared by dissolving 2.94 g sodium citrate in deionized water and adjusting the pH to 6 with hydrochloric acid. The boiling time was 15 minutes, followed by *in situ* cooling for 30 minutes and under running tap water for 20 minutes. The slides were then rinsed with PBS and the skin sections outlined with a wax pen (Dako).

7.2.2.2.7 Blocking

Sections were blocked to reduce unspecific binding by incubating the slides in PBS-Tween for 20 minutes. This step and the subsequent ones were all done inside a humidity chamber to avoid desiccation of the slides.

7.2.2.2.8 Primary antibody

The principle behind an indirect IHC detection is the application of an unlabelled primary antibody directed toward the antigen or protein of interest. This is followed by a second labelled antibody, directed towards the immunoglobulin of the species in which the primary antibody was raised, e.g. anti-rabbit, anti-mouse. Excess blocking solution was removed and 75 μ L primary antibody for COX-2 at 1 in 50 dilution pipetted on the sections and left overnight at room temperature. The stock antibody was diluted with the

necessary volume of PBS. The following day, the slides were washed with PBS followed by PBS-Tween.

7.2.2.2.9 *Detection & staining*

Detection was conducted using Dako Envision+ System horseradish peroxidase labelled polymer anti-rabbit, which consisted of a polymer backbone conjugated with streptavidin-horseradish peroxidase on one end and secondary antibody on the other end. This reagent was applied to the section direct from the dispenser and left for 2 hours at room temperature. This was followed by rinsing in PBS and PBS-Tween. The next step of the detection involved a substrate which reacts with the HRP. The Dako Chromogen system contains 3, 3'-Diaminobenzidine (DAB) that change colour to brown in the presence of hydrogen peroxide. One drop of the stock solution was made up to 1 mL with the buffer and applied to the sections for 10 minutes, followed by rinsing in deionized water.

7.2.2.2.10 *Counterstaining*

The sections were counterstained using an aqueous solution of 0.5 % methyl green for 2 minutes, followed by rinsing in deionized water. The slides were then left to dry before mounting in DPX and covered with cover slips. The slides were examined and imaged using a microscope (Optihop, Nikon Corporation, Tokyo, Japan) equipped with image capture facilities (Axiovision LE, Carl Zeiss Ltd., Welwyn Garden City, UK).

7.2.2.3 Western blot analysis

The expression of both COX-2 and 5-LOX were determined using Western blot with a method adapted from Leong et al. (1996). The principle behind WB analysis is the detection of specific proteins from a tissue or cellular lysate/extract combining separation of proteins using gel electrophoresis with immunoprobings of the separated proteins blotted on to a membrane (Burnette 1981). Freshly excised porcine skin sections were mounted on Franz-type glass cells with HBSBS solution as a receptor phase and treated with both formulations and left for 6 hours in a water bath maintained at 37°C. After 6 hours, the diffused area was excised, homogenized and incubated with 1 mL of radioimmunoprecipitation assay (RIPA) buffer (Sigma Aldrich, lot 096K6068) added with 1µL aprotinin and 50 µL leupeptin. The samples were then centrifuged at 10000 rpm for 15 minutes twice and the resulting supernatant kept frozen at -20°C prior to use.

7.2.2.3.1 Protein estimation

Protein estimation was done using a method based on the Coomassie (Bradford) protein assay and the values obtained from a calibration curve constructed from bovine serum albumin (Sigma Aldrich) with a range of 5-25 µg mL⁻¹ diluted in deionized water. Sample protein lysates were diluted 1 in 200, also in deionized water. 800 µL of each standard concentration and protein samples (in duplicates) was prepared in 1.5 mL disposable cuvettes. Afterwards, 200 µL of protein assay reagent (Bio-rad, Munich, Germany, lot no 107862) was added to each cuvette and mixed by turning the cuvettes upside down several times and left to stand for at least 10 minutes. The intensity of the resulting blue colour was then read at 595 nm using an UV spectrophotometer (Cecil Instruments CE2041, Cambridge, UK. The

concentration of protein in each diluted sample can then be obtained using the standard curve, and the concentration in the total sample worked out by multiplying by the dilution factor.

7.2.2.3.2 Polyacrylamide gel electrophoresis (PAGE)

Aliquots of the sample equating to 30-40 μg protein were added with equal amounts of loading/sample buffer (10% SDS, glycerol, 1M Tris-HCl 20mM pH 6.8, bromophenol blue, 0.1M DTT in water) were denatured at 100°C for 5 minutes. The denatured samples were then stored at -20°C or used straight away for PAGE.

PAGE was done using Xcell SureLock™ 1-D electrophoresis system (Invitrogen, Paisley UK) pictured in Figure 7.2. Separating gel (3.3 mL 30% acrylamide, 4 mL deionized H₂O, 2.5 mL 1.5M tris HCL (pH 8.8), 0.1 mL 10% SDS, 0.1 mL 10% APS, 10 μL TEMED) was cast in disposable plastic cassettes, allowing 20-30 minutes for it to set. Then, stacking gel solution (1.7 mL 30% acrylamide, 5.8 mL deionized H₂O, 2.5 mL 1.5M tris HCL (pH 6.8), 0.1 mL 10% SDS, 0.1 mL 10% APS, 10 μL TEMED) was pipetted on top of the separating gel, and a well-casting comb applied. Gel setting time was approximately 15 minutes. Once set, the comb was removed and the wells washed with deionized water, before locking the cassette in the apparatus. 1x running buffer (10x stock solution - 15 g Tris, 72 g glycine, 5 g SDS, diluted with deionized water) was pipetted into the wells and filled up the tank followed by application of sample and 5 μL protein molecular weight markers. Electrophoresis was conducted at 125 V until separation of protein markers had reached the desired molecular weight. The cassette was then removed and broken apart to remove the gel for Western blotting.

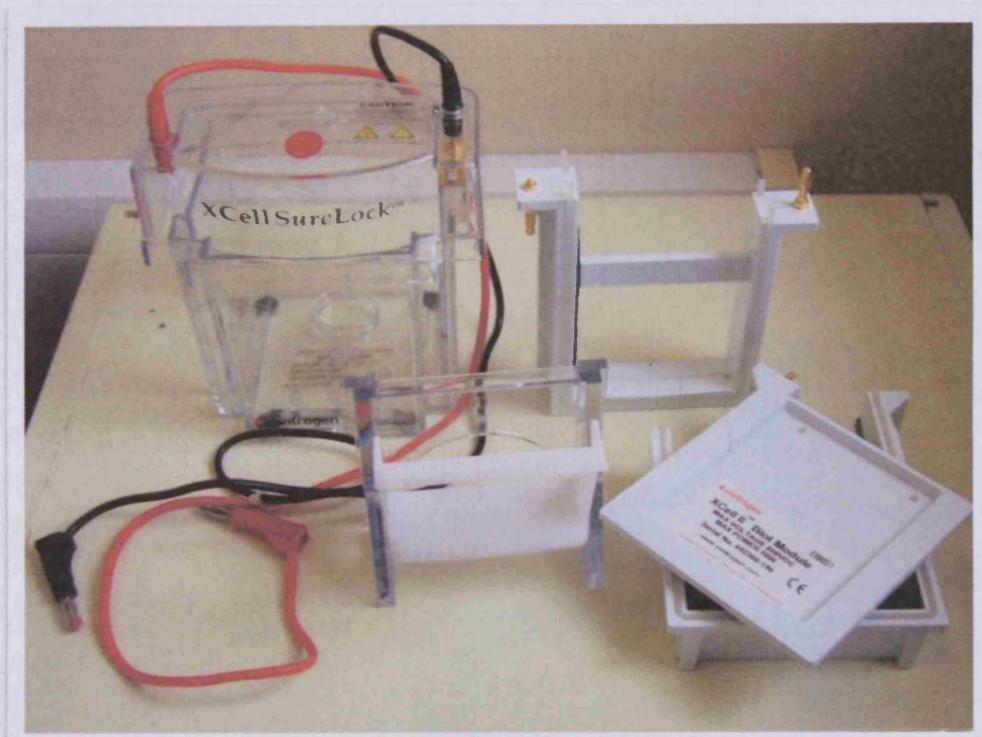


Figure 7.2 : Invitrogen Xcell SureLock PAGE and blotting module as used in the study

7.2.2.3.3 *Western blotting*

In preparation for blotting, filter paper and sponge pads were pre-soaked in western blot buffer (3.03 g Tris Base, 14.4 g glycine, 200 mL methanol in 800 mL deionized water) and transfer membrane wetted in methanol and then shaken in deionized water. The gel was then assembled on a flat surface in this order: filter paper, gel, transfer membrane, filter paper. The whole assembly was then placed on sponge pads and into the blotting module (XCell II™, Invitrogen). The module was filled with western blot buffer and placed in a tank filled with deionized water. Running time was 1-2 hours, with a voltage set at 25 V.

7.2.2.3.4 Immunohistochemistry

The resulting blot from the previous procedure was then subjected to immunohistochemistry procedure for the protein of interest. To ensure protein loading was equal and the transfer was complete, the blot was placed in a solution of Ponceau S (0.1% w/v Ponceau S in 5% acetic acid) for several minutes followed by deionized water. The intensity of the resulting red colour on each band was directly correlated to the relative amount of protein present. The blot was cleared using Tris Buffered Saline with 0.1 % v/v Tween-20 (TBS-Tween) made up with a stock 10x solution (24.2 g Trizma base, 80 g NaCl) diluted to 1x.

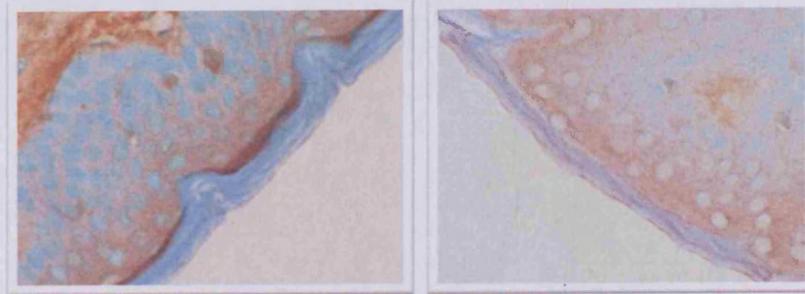
The blot was blocked using 5% milk (Marvel™ Original Dried Skimmed Milk, Chivers Ireland Ltd.) in TBS-Tween for 1 hour in room temperature with constant rocking. This was followed with COX-2 and 5-LOX antibody (1/1000 dilution) first for 1 hour in room temperature and then overnight at 4°C. After washing with 2 changes of TBS-Tween (5 minutes, then 25 minutes) the blot was incubated with secondary antibody (horseradish peroxidase linked anti-rabbit IgG) at 1/10000 dilution in TBS-Tween with 1% milk for 1 hour. Visualization of the blot was done using Chemiluminescent Supersignal® West HRP substrate (Pierce) and exposed to radiography film (Kodak) for 5 minutes before development.

7.3 Results and discussion

7.3.1 IHC staining

Figure 7.3 illustrates the change in COX-2 levels over 6 hours. The brownish-red stain observed denotes the presence of the enzyme which was found to be localized in the viable epidermis. For the first 3 hours, no discernable changes were observed for both treatments. However, at t=6 hours, a reduction in the intensity of the staining, i.e. anti-inflammatory effect, was observed with 20% FO especially exhibiting a marked effect.

0 hour



0% FO

20% FO

3 hours



0% FO

20% FO

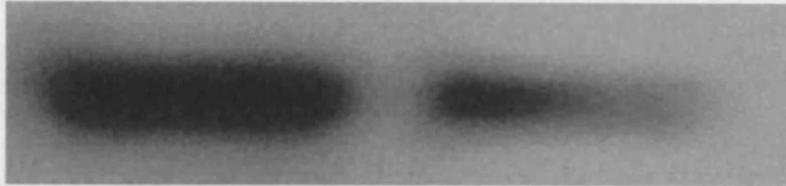
6 hours



0% FO

20% FO

Figure 7.3 : IHC staining for COX-2 at 40x magnification. After 6 hours, the relative levels of COX-2 was greatly reduced in the combined BD-FO treatment compared to BD alone.

7.3.2 Western blot analysis5-LOX

0% FO

20% FO

COX-2

0% FO

20% FO

Figure 7.4 : Western blot for 5-LOX and COX. The expression of 5-LOX and COX-2 was found to be decreased with addition of FO. This reduction is attributed to the inhibitory effects of n-3 fatty acids on both enzymes, by a mechanism yet to be determined.

Figure 7.4 shows the results obtained from the Western blot analysis. The blot on the left represents 0% FO, and on the right, 20% FO. As observed, there is an appreciable decrease in the size of the blot signifying the reduction of the enzyme level in the protein sample, noticeably for 5-LOX. Although the same is observed with COX, it was less apparent. Since the Western blot was done qualitatively, it was not known whether the difference in the enzyme levels was statistically significant. However, both results alluded to an enhanced anti-inflammatory effect in the sample treated with 20% FO.

Since both formulations contain the same percentage of both salicylic acid and betamethasone dipropionate, a known anti-inflammatory, the increased effect observed in both experiment can be attributed to the presence of fish oil in 20% FO.

Two possibilities are suggested to explain this apparent enhancement. First, fish oil, which is rich with essential fatty acids, has been reported to possess anti-inflammatory effect both in vitro and in a clinical setting. Indeed, several trials with fish oil and/or the fatty acids (in particular EPA) have proven the benefits of fish oil in improving inflammatory skin diseases, such as psoriasis (Dewsbury et al. 1989; Escobar et al. 1992). Incorporation of EPA into cellular membranes (through ingestion or application) and its subsequent release results in production of less potent eicosanoids through competition with arachidonic acid (AA), with the net effect of reducing the extent of inflammation. In relation to this experiment, Thomas et al (2007a) concluded that fish oil has a direct inhibitory effect on both COX-2 and 5-LOX, demonstrated via ICC staining on skin dosed with fish oil alone or co-formulated with ketoprofen. Again, an additive effect was observed when fish oil was present in the formulation along with a known anti-inflammatory. A similar observation was noted by Puglia et al. (2005) with transcutaneously delivered fish oil.

The second possibility can be attributed to the enhancement of BD delivery across skin, as discussed previously in Chapters 5 and 6. The increased amount of BD present would then translate to a greater inhibition of the enzymes by BD.

7.4 Conclusion

This study showed that the addition of 20% FO in a corticosteroid formulation resulted in an increased inhibition of COX-2 and 5-LOX, two main inflammatory enzymes found in the epidermis. This was reflected from the reduced staining observed with ICC (COX-2), and the reduction of the blot size, hence, the level of the protein/enzyme from WB analysis (COX-2 and 5-LOX). It is uncertain how FO enhanced the inhibition and arrested the inflammatory process, but it was postulated that it is achieved via a two pronged mechanism: Enhanced delivery of BD and possession of intrinsic anti-inflammatory effect.

Although the data represented here is qualitative, this would only encourage further research into the plausibility of incorporating FO into the topical regiment of dermatological drugs, with a view of targeting inflammatory skin diseases, such as dermatitis and psoriasis. It is hoped that quantification of the WB results, coupled with other methods, such as cell culture would further strengthen the available data and ultimately highlight the benefits of topically applied FO/EPA.

Chapter 8:

*IHC staining and WB analysis for
COX-2 and 5-LOX in porcine skin
sections treated with
betamethasone dipropionate,
salicylic acid and fish oil*

8.1 Introduction

An earlier study using a combined formulation of an anti-inflammatory agent (ketoprofen) and fish oil reported an additive inhibitory effect of FO towards COX-2 and LOX (Thomas et al. 2007a). This also was supported with the previous work in Chapter 7, which combined betamethasone dipropionate and salicylic acid, intended to replicate a commercially available anti-psoriatic medication, with the addition of fish oil. Therefore, the current study would look further into the effect of each individual component on epidermal COX-2 and LOX via immunohistochemistry (IHC) staining and quantitative WB analysis.

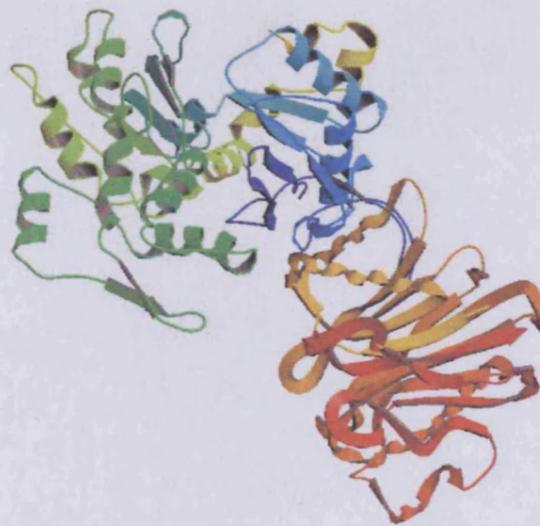


Figure 8.1 : β -actin (42 kDa) (Schutt et al. 1993)

In WB assays, although care is taken to ensure an equal amount of protein is loaded during electrophoresis, errors are invariably present. In order to minimize loading error, apart from replication, the result for the protein of interest is often normalized with other proteins that are found in abundance within tissues. One such protein is β -actin (Figure 8.1). The 42 kDa protein is

one of the cytoskeletal actins, with primary functions in cell motility, structure and integrity (Gunning et al. 1997).

8.2 Materials & method

8.2.1 Materials

Monoclonal antibody for β -actin (clone AC-74 produced in mouse, A2228) was obtained from Sigma Aldrich (Poole, UK). Western blot stripping buffer (Restore™ Western Blot Stripping Buffer) was from Thermo Scientific (Loughbororugh, UK). All other reagents and materials were the same as listed in Section 7.2.1

8.2.2 Methods

8.2.2.1 Preparation of test formulation

Five test formulations (Table 8.1) were prepared according to the method outlined in section 5.2.2.

Group	Treatment
BD	0.05% w/w BD, 0.05% w/w BHA in emulsifying ointment BP
BF	20% w/w FO, 0.05% w/w BHA in emulsifying ointment BP
SA	3% w/w SA, 0.05% w/w BHA in emulsifying ointment BP
BDF	0.05% w/w BD, 20% w/w FO, 0.05% w/w BHA in emulsifying ointment BP
Control	0.05% w/w BHA in emulsifying ointment BP

Table 8.1 : Test formulations used in the current study

8.2.2.2 *In vitro skin permeation*

Approximately 100 mg of each formulation was applied with gentle rubbing using a glass rod onto excised porcine ear skin sections mounted on Franz diffusion cells with HBSBS as a receptor solution (n=6)

8.2.2.3 *IHC staining*

At t=0 h, 3, 6 and 24 hours, the diffused areas were excised and fixed in formaldehyde overnight. The following procedures for tissue processing and the subsequent IHC staining were explained previously in Section 7.2.2.2

8.2.2.4 *PAGE and western blot analysis*

At t=0 h, the diffused areas were excised and lysed in 1 mL of RIPA buffer and stored at -20°C. The amount of protein lysate from each treatment equivalent to 30 mg total protein were denatured and subjected to PAGE and western blotting as outlined previously in Section 7.2.2.3. The resulting blots were then subjected to immunohistochemistry procedures using primary antibodies for COX-2 and 5-LOX (both 1/1000 dilution in 5% w/v Marvel milk in TBS), and developed. Afterwards, the blots were soaked in 10 mL of WB stripping buffer for 15 minutes with gentle shaking at room temperature to remove all traces of the chemiluminescent agent used in the previous development. The blots were then washed with TBS-Tween 3 times for 5 minutes. Subsequently, the blots were incubated with primary antibody for β -actin (diluted 1 in 50 000 in 5% w/v Marvel milk in TBS) for 1 hour in room temperature, followed by the secondary antibody (anti-mouse) at 1 in 10 000 dilution also for 1 hour.

After development, the film for the COX-2 and 5-LOX blot and the corresponding β -actin film were scanned and analysed using a densitometer (GS-690 Imaging Densitometer, Bio-Rad Laboratories Ltd, Herts, UK) equipped with Alpha DigiDoc software. The levels of COX-2 and 5-LOX in each lysate were normalized with the levels of β -actin of each corresponding blot, and presented as a percentage, with control = 100%.

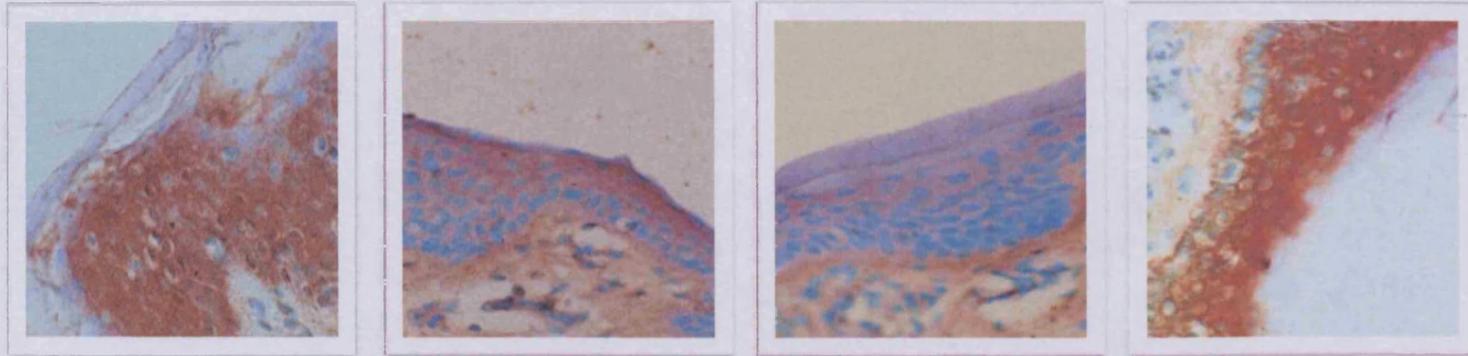
8.3 Results and discussion

8.3.1 IHC staining

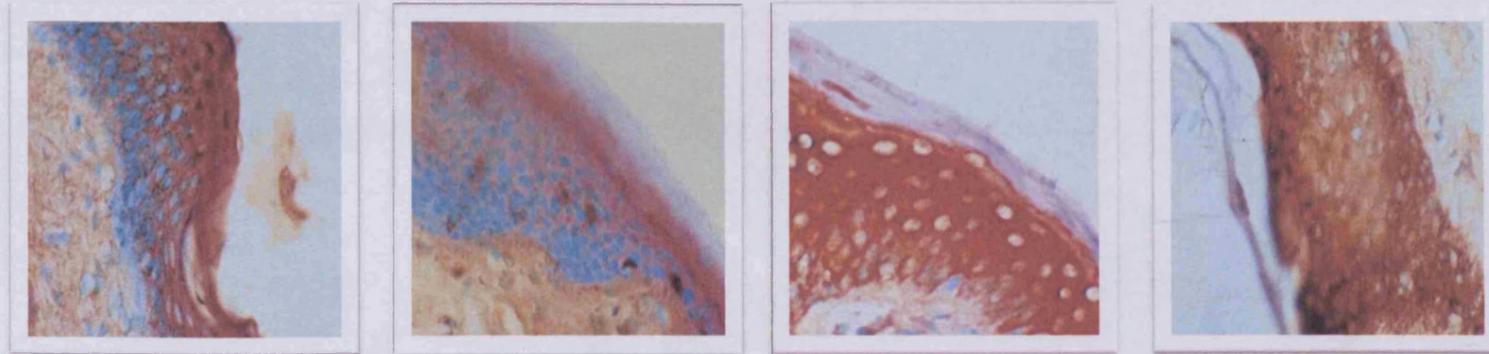
8.3.1.1 COX-2

All the skin sections exhibited an expression of COX-2 (Figure 8.2) at t=0, presumably due to the trauma inflicted during removal of the porcine ears at the abattoir. A noticeable reduction can be seen in the skin section treated with BD (Figure 8.2A) at 3 hours, and especially more pronounced at 6 hours post-treatment.

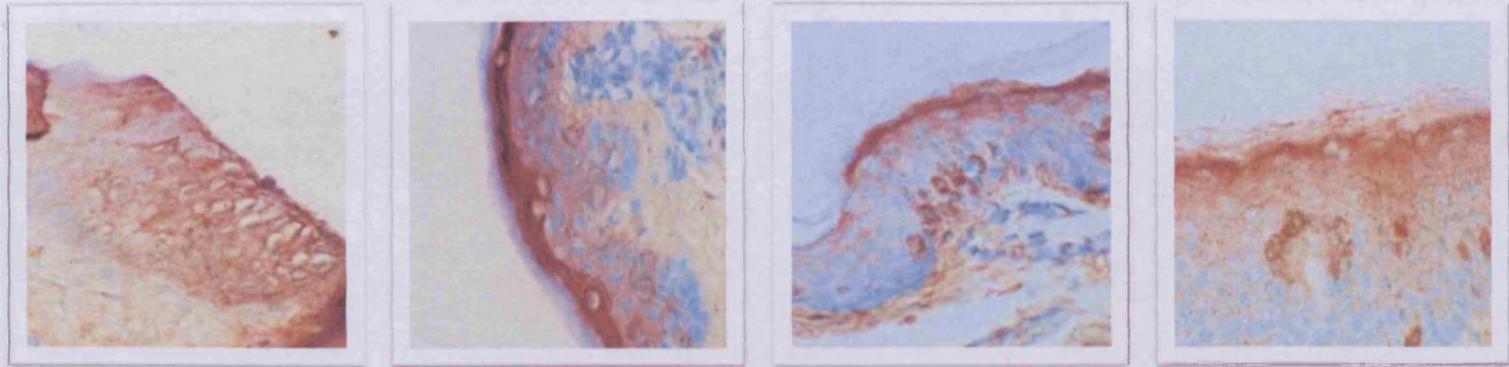
Treatment with SA (Figure 8.2B) showed a slight reduction in the staining after 3 hours; however, from 6 hours onward, there was a noticeable increase, signifying continuous expression of COX-2. The skin treated with FO (Figure 8.2C) however, showed no discernable changes over 6 hours, while the control displayed increasing levels of COX-2 over the same time period. For all skin sections, the staining pattern at t=24 hours revealed a general and widespread staining, suggesting a non-specific staining and/or the lost of viability in the skin sections (Thomas et al. 2007a).



A : Betamethasone dipropionate 0.05% (l-r) : 0h, 3h, 6h, 24h)



B : Salicylic acid 3% (l-r) : 0h, 3h, 6h, 24h)

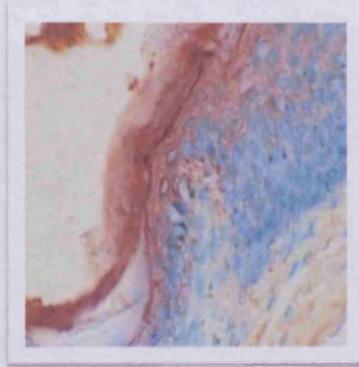
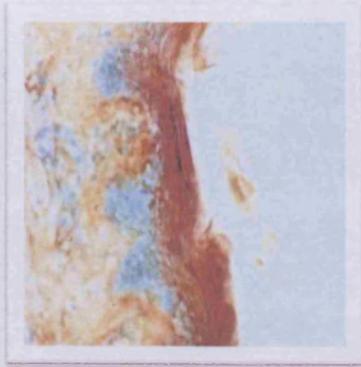


C : Fish Oil 20% (l-r) : 0h, 3h, 6h, 24h)

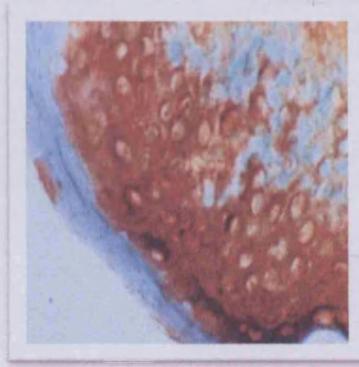
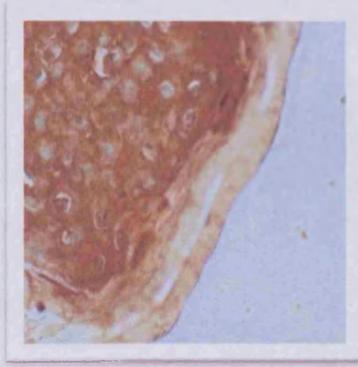


D : Control (l-r) : 0h, 3h, 6h, 24h

Figure 8.2 : COX-2 IHC staining at 0, 3, 6, and 24 hours (40x magnification)



A : Betamethasone dipropionate 0.05% (l-r) : 0h, 3h, 6h, 24h)



B : Salicylic acid 2% (l-r) : 0h, 3h, 6h, 24h)



C : Fish Oil 20% (l-r) : 0h, 3h, 6h, 24h)



D : Control (l-r) : 0h, 3h, 6h, 24h)

Figure 8.3 : 5-LOX IHC staining at 0, 3, 6, and 24 hours (40x magnification)

8.3.1.2 5-LOX

Figure 8.3 shows the expression of 5-LOX obtained qualitatively from IHC staining. Apart from a slight reduction at t=6 hours in the skin treated with BD (Figure 8.3A), no noticeable change was observed in all the treatments across the sampling period. While the control (Figure 8.3D) exhibited increased staining over 6 hours, SA (Figure 8.3B) and FO (Figure 8.3C) remained relatively unchanged. As with IHC staining for COX-2, all the skin sections showed a widespread staining at t=24 hours.

8.3.2 WB analysis

8.3.2.1 COX-2

The blot obtained for COX-2 and the corresponding β -actin in the tissue lysates collected from all the treatments after 6 hours is shown in Figure 8.4. Quantification of the protein levels was achieved by normalizing the values with that of β -actin. It was found that relative to control, BD exhibited the greatest reduction of COX-2 at 97.2 %, (p 0.0001) followed closely by FO (90.8 %, p 0.0001), SA at 71.33 % (p 0.0001), and the combined BD-FO (BDF) at 44.41 % (p 0.0002) (Figure 8.5). The reduction in COX-2 level by BD and FO corresponded with the previous IHC staining. The combined treatment also significantly inhibited the expression of COX-2, although in this instance it did not appear to enhance the inhibition. Interestingly, in contrast to the IHC staining, SA on its own also reduced the expression of COX-2 at t=6. It was noted however, that at t=3 hours the COX-2 expression was reduced in the IHC staining, before the noticeable increase at t=6 hours.

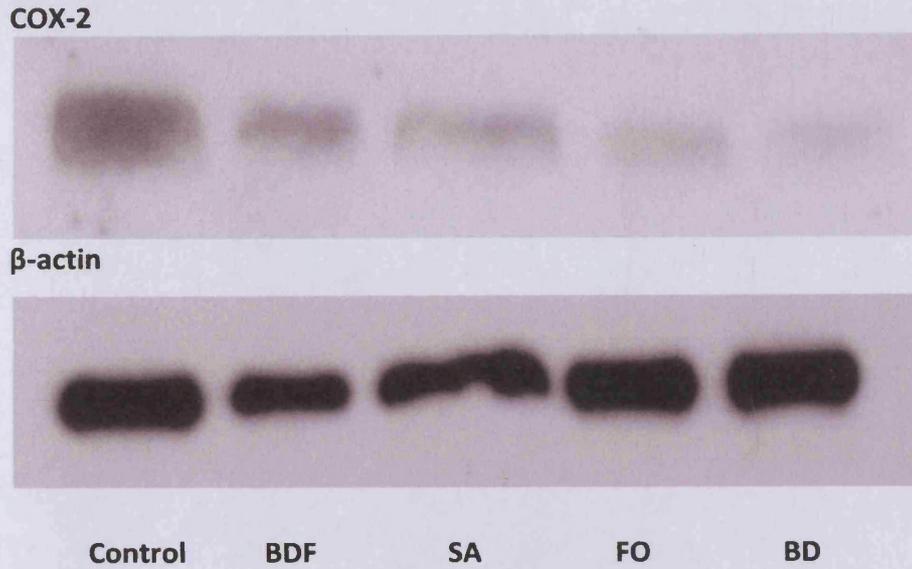


Figure 8.4 : Representative blots for COX-2 (top) and β -actin (bottom). The intensity of the bands is directly correlated to the amount of the protein of interest.

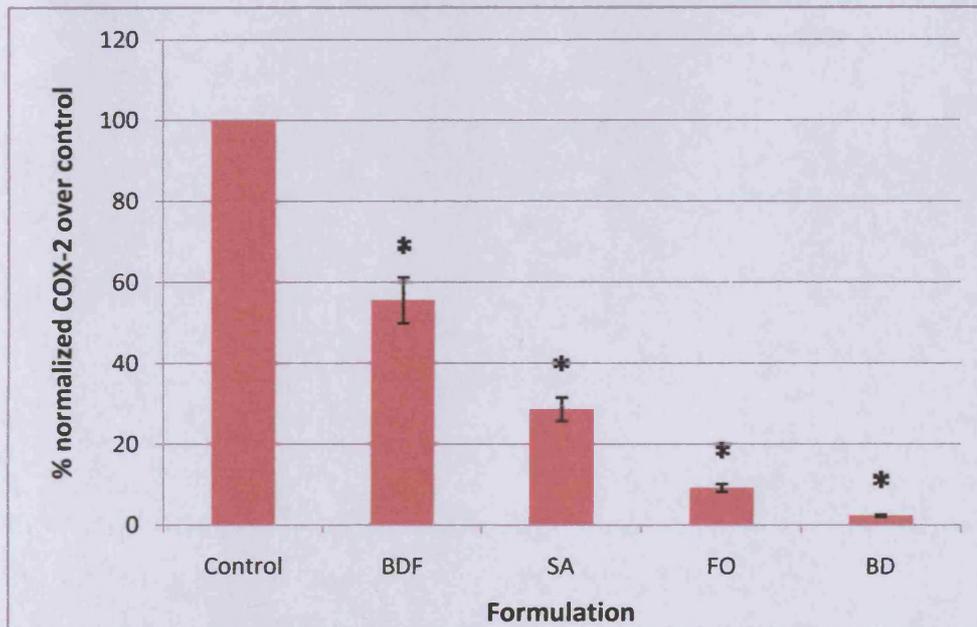
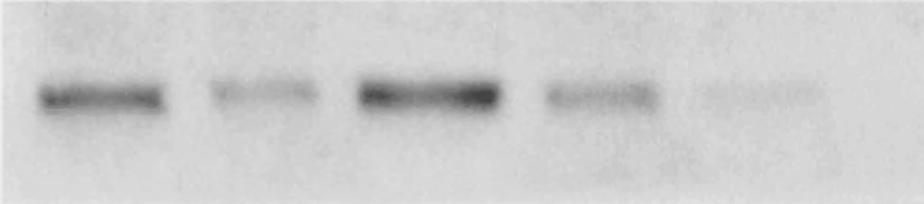


Figure 8.5 : The percentage of normalized COX-2 level relative to control (n=3, \pm SD), * = $p < 0.05$

8.3.2.2 5-LOX

The blots obtained for 5-LOX are shown in Figure 8.6, while the relative 5-LOX levels for each treatment are shown in Figure 8.7. The BD only formulation again proved superior to the other treatments in reducing the levels of 5-LOX, with 95.13% reduction (p 0.001). Similarly with COX, this was followed by the FO-only formulation (51.94%, p 0.0177). As observed with the IHC, SA increased 5-LOX expression by 2.4%, although compared to control it was not statistically significant (p 0.9222). The combined formulation, although resulted in a significant reduction (p 0.02) again did not translate to a greater inhibition, with only 41.96% reduction. It did not however differ significantly (p 0.5827) from the reduction caused by the FO-only formulation.

5-LOX **β -actin**

Control

BDF

SA

FO

BD

Figure 8.6 : Representative blots for 5-LOX (top) and β -actin (bottom)

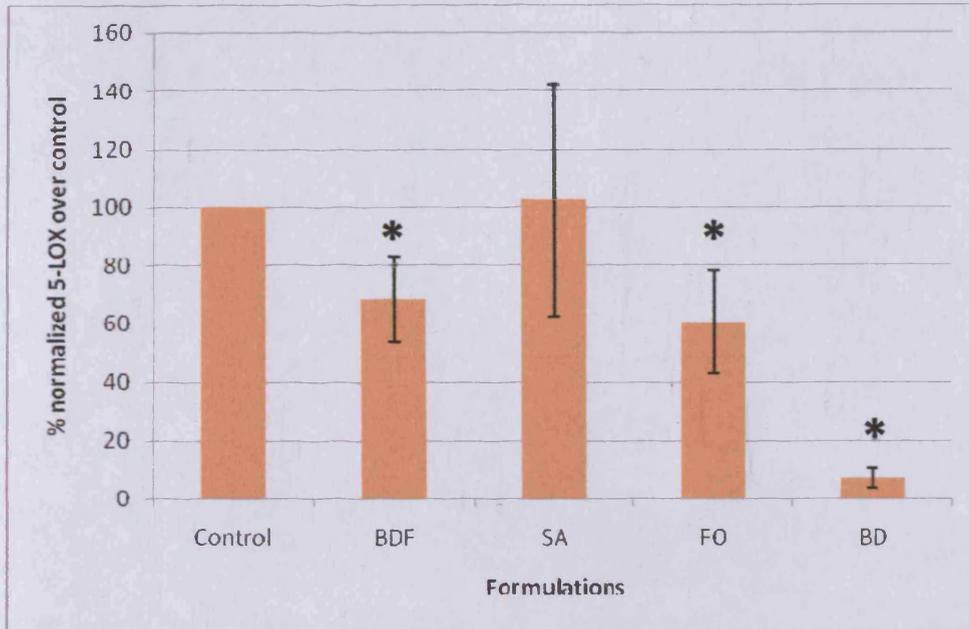


Figure 8.7 : The percentage of normalized 5-LOX level relative to control. (n=3, \pm SD). * = $p < 0.05$

From the results, one might have expected BD to have an inhibitory effect on both inflammatory enzymes. Being a mainstay of topical therapy particularly psoriasis (Stein 2005), BD and the corticosteroids in general are effective immunosuppressors and anti-inflammatory, exerting their effects through binding with intracellular receptors and the regulation of transcription for genes coding for cytokines (Hughes and Rustin 1997).

It has now been demonstrated that FO on its own possesses the ability to inhibit the expression of both inflammatory enzymes, particularly in the WB analysis. This supported the long standing belief in the anti-inflammatory properties of fish oil and its primary fatty acids (Mayser et al. 2002; Puglia et al. 2005; Thomas et al. 2007a; Wolters 2005).

Contrary to this, Chene et al. (2007) reported that n-3 and n-6 fatty acids, in fact induced the expression of COX-2 in human keratinocyte cell lines. This, however, was not readily apparent with the current study, as no observable increase in COX-2 level was seen. It was also suggested that this enzyme, which is pro-inflammatory, also plays an important role in the resolution of

inflammation in the later phase via production of PGD_2 , one of the products of AA metabolism by COX-2 (Rajakariar et al. 2006). This would be at odds with the belief that the anti-inflammatory effects of FO/fatty acids are due to direct antagonism of AA. It also highlights the need for a better understanding of the inflammation cascade.

Combining BD and FO in this instance however, did not result in a greater inhibition of both COX-2 and 5-LOX, as seen with the IHC and qualitative staining in Chapter 7. To put delivery issues in perspective, the delivery of BD was found to be increased in the lower layers of the skin (Chapter 5). Therefore, with the two mechanisms involved (increased delivery and intrinsic anti-inflammatory properties of both BD & FO) it was anticipated that an enhancing effect would be seen. One possible explanation is the saturation of binding sites on the enzyme by FO, thus rendering the effects of BD less apparent in the combined formulation.

SA, unlike its acetylated form aspirin, is more often used as a desmosomolytic agent in the treatment of dermatological diseases. Generally, salicylates, in their various forms (acetylated, methylated), have been used in anti-inflammatory preparations and pain relief medications by acting on COX and prostaglandin E2 production (Vane and Botting 2003). As aspirin is rapidly metabolized to SA, it is long believed that the anti-inflammatory action of aspirin is a result of its parent metabolite. However, opinions are still divided on the true extent of SA's contribution (Amann and Peskar 2002).

It was reported that both aspirin and salicylic acid are equipotent PGE_2 inhibitors, although there exists a difference in their activity against cyclooxygenase. Aspirin, by virtue of its acetyl group, exerts its effect by acetylating the enzyme; a process impossible to salicylic acid by the absence of the functional group. This paradox between anti-inflammatory activity and differences in COX inhibition by salicylic acid and aspirin was partly explained when it was found that SA inhibited PGE_2 production through a pathway

independent of COX-2 inhibition (Amann and Peskar 2002; Xu et al. 1999). It can be concluded then, although SA is anti-inflammatory, it does not affect the expression of COX. Therefore the reduction seen with the WB analysis, a direct contrast to the IHC staining at the same time point is attributed to the difference in COX-2 levels in the different tissue samples. With regards to SA activity on lipooxygenases (LOX), the LOX are generally not regarded as a target for salicylate action (Lapenna et al. 2009), which was clearly demonstrated in both IHC and WB analysis in the current study.

8.4 Conclusion

The current study provided strong evidence of the intrinsic anti-inflammatory nature of FO in the skin via its action of epidermal inflammatory enzyme. This explained the finding in Chapter 7, and shed light on the possible mechanism by which FO potentiates activity of BD. However, contrasting results on the effects of the combined formulation deserved further study, particularly with regards to the ideal composition of both components in the formulation.

Chapter 9:

*Investigating the effects of
topical formulations on skin
levels of prostaglandin E₂ (PGE₂)*

9.1 Introduction

In Section 1.3.7 the AA inflammation cascade was discussed, along with its potential modulation by n-3 fatty acids. One of the end-products of the cascade is a series of mediators or prostanoids termed prostaglandins.

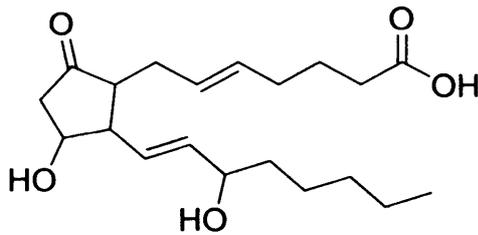


Figure 9.1 : Prostaglandin E₂ (PGE₂)

PGE₂ (Figure 9.1) is the most abundant prostaglandin in the human body (Serhan and Levy 2003), produced both constitutively and in response to stimuli or trauma from cellular AA via the action of cyclooxygenases and prostaglandin E₂ synthase (PGES), as shown in Figure 9.2. Like all prostaglandins, PGE₂ is synthesized de novo and there is no cellular reservoir. Delayed synthesis of PGE₂ (in response to stimuli and injury), mediated by COX-2 and microsomal PGES-1 (mPGES-1) has been implicated in the pathophysiology of many inflammatory diseases, fever, and cancer (Nakatani and Kudo 2002). PGE₂ plays a major role in tissue oedema, hyperalgesia, and IL-6 production at sites of inflammation (Ruzicka et al. 1986). This effect on IL-6 leads to induction of activator protein-2 mRNA and protein production by keratinocytes. Subsequently, this causes skin inflammation, and this has been shown to lead to hyperproliferation in the growth of keratinocytes (Oyama et al. 1999).

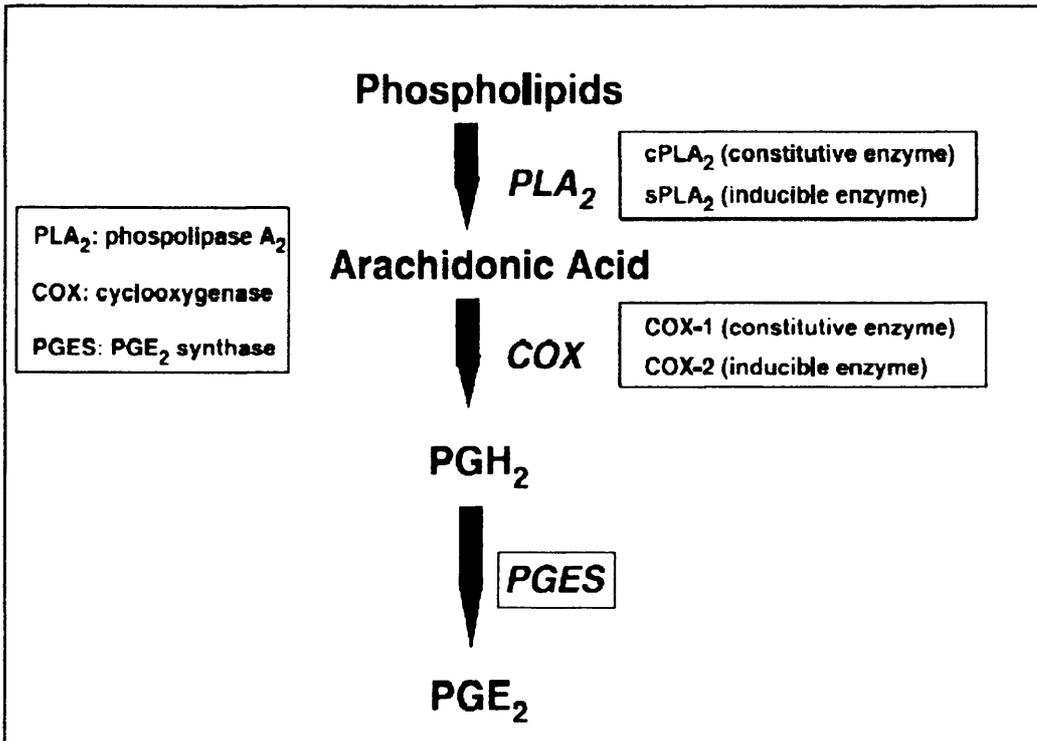


Figure 9.2 : Conversion of cellular phospholipids to PGE₂ (Nakatani and Kudo 2002)

Therefore, PGE₂ is an attractive marker to probe modulations within the inflammation process. The aim of this chapter was to investigate the effect of fish oil and other common anti-psoriatic medications on the levels of PGE₂ in an ex vivo porcine ear skin model of inflammation.

9.2 Materials and method

9.2.1 Materials

Fish oil capsules 1000 mg (Boots Super Strength, lot 0336000100A, 33% EPA) were obtained from a local store. Betamethasone dipropionate (lot 066K1212), BHA, RIPA buffer (lot 096K6068), and Hanks' balanced salts were obtained from Sigma-Aldrich Company Ltd., Poole, UK. Pure water was from Fisher Scientific (Loughborough, UK) Emulsifying ointment B.P (lot 72FG) was

obtained from Thornton and Ross, Huddersfield, UK. Enzyme immunoassay (EIA) kit for prostaglandin E₂ (PGE₂) (lot 0408969) was obtained from Cayman Europe, Estonia. All other reagents were of analytical grade or equivalent. Freshly excised porcine ears were immersed in Hanks balanced salt buffer solution (HBSBS) and used within 1 hour of slaughter. The hair was removed with electric clippers and full thickness skin from the dorsal side of the ear was then excised and cut into sections of approximately 2x2cm. During excision, the ear was continuously bathed with HBSBS and the cut sections soaked in the same buffer prior to mounting on the diffusion cells.

9.2.2 *Methods*

9.2.2.1 *Preparation of formulations*

The test formulations used in the current study were the same as the ones used in Chapter 6, namely BD, BF, BDF and control containing the blank ointment base.

9.2.2.2 *In vitro skin permeation*

Freshly excised porcine skin sections were mounted on Franz-type glass cells with HBSBS solution as a receptor phase and treated with the formulations and left for 6 hours in a water bath maintained at 37°C. After 6 hours, the diffused area was excised, homogenized and incubated with 1 mL of RIPA buffer added with endogenous protease inhibitors aprotinin (1 µL) and leupeptin (50 µL). The samples were then centrifuged at 10 000 rpm, 4°C for 10 minutes twice and the resulting supernatant kept frozen at -20°C prior to

enzyme immunoassay (EIA). EIA was conducted with a kit specific for PGE₂ according to the manufacturer's instructions.

9.2.2.3 *Enzyme immunoassay (EIA)*

The principle behind the EIA, as depicted in Figure 9.3, is the competition between a constant amount of tracer molecules (acetylcholinesterase linked to PGE₂) and PGE₂ in the sample for a limited amount of monoclonal antibody towards PGE₂. The amount of bound PGE₂ tracer is thus inversely proportional to the amount of PGE₂ available in the sample. Incubated together, the PGE₂- or tracer-antibody complex then binds with IgG pre-coated in the wells, directed towards the PGE₂ antibody. Subsequently, a reagent containing a substrate of acetylcholinesterase is added. The enzymatic reaction produces a coloured substance which absorbs strongly at 412 nm. Using a spectrophotometric plate reader (Sunrise™, Tecan UK Ltd., Reading, UK), the intensity of colour is proportional to the amount of bound tracer. In using this assay the relative modulation of PGE₂ could be used to compare an anti-inflammatory (or pro-inflammatory) response to the formulation dose onto the ex vivo skin.

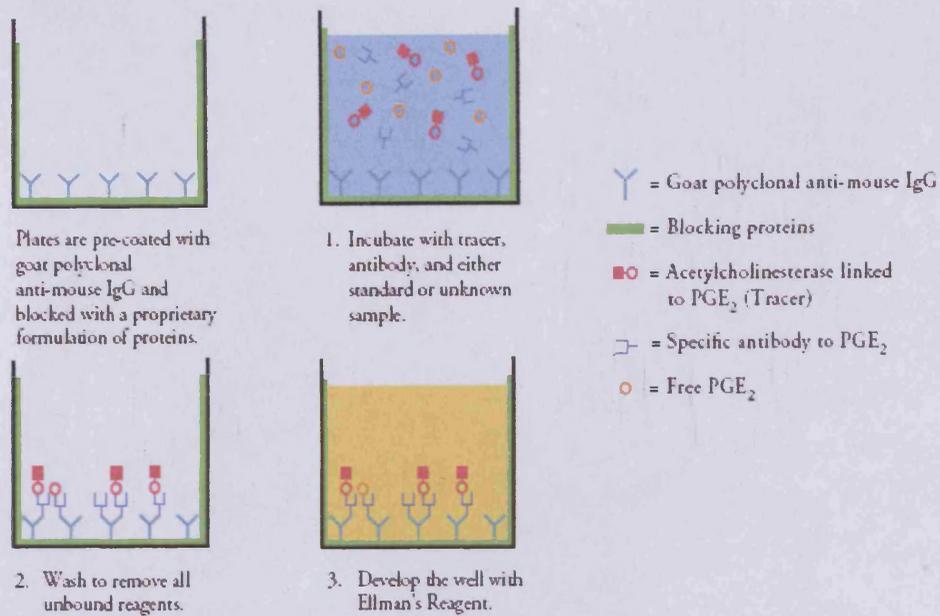


Figure 9.3 : Schematic of the EIA for PGE₂ (Cayman Europe, Estonia)

9.3 Results and discussion

The levels of PGE₂ in all 4 treatments after 6 hours are shown in Figure 9.4. The greatest reduction was seen with BD (398.12 pg mL⁻¹), representing a 54% reduction from control levels (p 0.0258). This was followed by BF, with a level of 473.23 pg mL⁻¹ (p 0.0435), and the combined formulation, BDF with 505.85 pg mL⁻¹ (p 0.0362). It was interesting to note that fish oil alone had the ability to reduce PGE₂ levels comparable to that of BD (p 0.1552), indicative of an intrinsic anti-inflammatory action. The combination of both BD and fish oil in a single formulation did not translate to a greater inhibition, although compared to BD and FO the levels were not statistically significant (p 0.2242 and 0.6745 respectively).

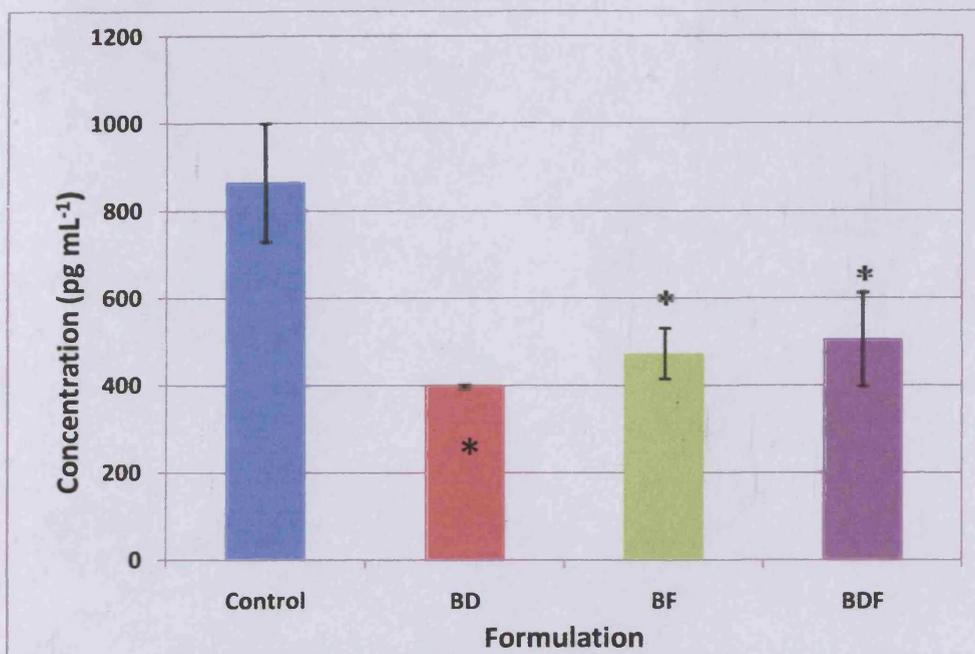


Figure 9.4 : Levels of PGE₂ after 6 hours post treatment (n=3,±SD) (*= $p < 0.05$).

This finding is significant in view of the role played by PGE₂ in inflammatory diseases. Suction blisters of psoriasis for example, were found to have up to 5 times higher PGE₂ levels compared to non-psoriatic skin (Reilly et al. 2000).

On the other hand, there is a conflicting view of the role played by PGE₂ in psoriasis. Namazi (2004, 2005) observed a low incidence of psoriasis in an African population. In his report, he attributed this fact to the high intake of corn and corn based products, a staple diet of Africans. Corn is rich with linoleic acid (LA), the precursor of PGE₂ (Calder and Gimble 2002). This in turn implied that the increased production of PGE₂ in the tissues is, in fact, protective against psoriasis. This is achieved via the ability of PGE₂ to suppress certain components of cellular immunity, i.e. interleukin-1, interferon-gamma and tumour necrosis factor alpha.

However, there is considerable evidence contrary to the above report. Logan (2005) questioned the validity of the assumption by directing the attention towards the effects of increased n-6 consumption. It is known that psoriatic

patients display elevated levels of the n-6 arachidonic acid (AA), which is derived from linoleic acid (Reilly et al. 2000). Furthermore, leukotriene B₄ (LTB₄), an important product within the inflammation cascade, is also elevated with increased LA intake. It is widely reported that the levels of AA and LTB₄ have been found to be elevated in the scales, epidermis, serum and suction blister fluid of psoriatic patients (Grimminger and Mayser 1995; Ikai 1999) and the presence of LTB₄ in early lesions implicates it in the pathogenesis of psoriasis (Fogh et al. 1989). It is apparent now that any increase in LA level would be detrimental in psoriasis.

As mentioned in previous sections, n-3 fatty acids provide an active competition with AA for binding sites on COX-2, the catalytic enzyme responsible for prostaglandin synthesis. The production of the less potent PGE₃ from EPA contributes to the overall natural anti-inflammatory processes in the body (Traub and Marshall 2007).

Another plausible explanation on the reduction of PGE₂ by fish oil can be related to its inhibitory action on COX-2, as mentioned in Chapter 7. In a similar finding, fish oil on its own appear to inhibit COX-2. Although the mechanism of this inhibition remains inconclusive, the reduced presence of COX-2 will only impair the production of PGE₂.

It was anticipated that the anti-inflammatory property demonstrated by FO would impart an additive or synergistic effect and provide a greater reduction of PGE₂. This, surprisingly, was not the case, as clearly evident from the assay.

9.4 Conclusion

One facet of the anti-inflammatory property of fish oil could be explained by its inhibitory action on pro-inflammatory prostanoids, either directly or indirectly through inhibition of associated enzyme. As demonstrated in Chapters 7 and 8, fish oil exerts an appreciable anti-inflammatory activity on its own, although in this experiment co-formulation with BD did not impart a potentiation effect.

Chapter 10:

Investigating the effects of fish oil and anti-psoriatic medication on cultured keratinocyte cell line

10.1 Introduction

Cell culture is a collection of technique and processes in which cells are grown in a controlled environment. The availability of immortalized cell lines by mutation or modification allows specific cells to be grown indefinitely, providing a continuous source for researchers (Arnetz et al. 1985). Cell culture has been used in various areas of biomedical research, from development of vaccines, and understanding the mechanism of cancer. It also served as the basis for tissue culture and tissue engineering.

The development of the spontaneously transforming HaCaT cell line was first reported by Boukamp et al. (1988), the designation given based on its origin (**H**uman **a**dult **k**eratinocytes) and growth conditions (low Ca^{2+} and elevated temperature). Prior to this discovery, the only available transformed keratinocyte cell lines were obtained via simian viral infection and DNA transfection. These cells had altered growth properties and substantial reduction of normal keratinization, as well as partial re-expression of fetal characteristics (Boukamp et al. 1988).

This immortal cell line possesses the capacity to differentiate normally with specific stable marker chromosomes even after multiple passages, providing a stable model to study keratinization. It is also non-tumourigenic and non-invasive upon in vivo transplantation; it has the capacity to reconstitute a well structured epidermis, and spatial distribution of epidermal differentiation products indicate a normal degree of morphologic differentiation. Expression of keratins was also found to be similar to transplanted normal keratinocytes.

The aims of the current study were to start and maintain HaCaT cell line cultures and investigate the growth characteristics of the culture via generation of growth curves. The inhibitory effect of BD and FO were also studied.

10.2 Materials and methods

10.2.1 Materials

Gibco® Dulbecco's modified Eagle medium 1x (DMEM, high glucose, with L-glutamine and phenol red, no HEPES, no sodium pyruvate), foetal bovine serum, trypsin 0.05% with EDTA·4Na 1x were obtained from Invitrogen (Paisley, UK). Amphotericin B ($250 \mu\text{g mL}^{-1}$) and penicillin (10,000 units)-streptomycin (10,000 μg) mixture were purchased from Lonza Group Ltd, (Basil, Switzerland). Corning cell culture flasks, 24- and 96-well plates, disposable pipettes, dimethylsulfoxide (DMSO) and ethanol were obtained from Fisher (Loughborough, UK). Betamethasone dipropionate, Dulbecco's phosphate buffered saline (DPBS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich (Poole, UK).

10.2.2 Methods

10.2.2.1 Routine cell culture

All cell culture procedures were carried out in aseptic conditions inside MDH Class II laminar-flow safety cabinet in a tissue culture suite. Before any procedures were carried out, all work surfaces, including any equipment, consumables and reagents brought into the cabinet, were disinfected with

70% ethanol. This was repeated after completion of work, followed by 1 hour of UV light. The HaCaT cells were grown in DMEM supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin mixture ($100 \mu\text{g mL}^{-1}$ and 100 U mL^{-1} respectively). 50 mL Aliquots of the medium were prepared from the stock. Inactivation of the FBS was achieved by placing the serum in 56°C for 30 minutes.

Temperature of the incubator was set at 37°C and flow of CO_2 at 5%. The medium was replenished every 2 days to keep the cells healthy, until the monolayer cultures reached 70-80% confluency. Once the suitable confluency is achieved, the cultures were passaged or split to maintain cells in exponential growth (Masters and Stacey 2007).

10.2.2.2 Cell splitting/passaging

Flasks of cells ready to be passaged were taken out of the incubator into the safety cabinet. The cells were then washed with 2 changes of DPBS, followed by incubation with trypsin-EDTA (the volume used determined by the size of the flask) at 37°C for 5 minutes or until all the cells had detached. Detached cells can be determined visually under the microscope; they appear as rounded, individual cells and fast flowing within the suspension. Detachment can be aided by gently tapping the flask to dislodge the cells or by the use of a cell scraper. The trypsin was then deactivated by pipetting a small amount of DMEM. The cell suspension was then transferred to a sterile centrifuge tube and centrifuged at 1000 rpm for 5 minutes. The resulting supernatant was removed carefully so that the cell pellet remained undisturbed. 8-10 mL Fresh medium was then added, followed by re-suspending the cells by gently pipetting the suspension up and down several times. The suspension was then ready to be used to seed cells in new flasks, frozen down for storage, used for experimental work and for cell counting.

10.2.2.3 Cell counting

The counting of cells is necessary to determine the number of cells required for seeding, and are particularly important when constructing the growth curve. This procedure is made possible by the use of cell counters or haemocytometers (Figure 10.1).

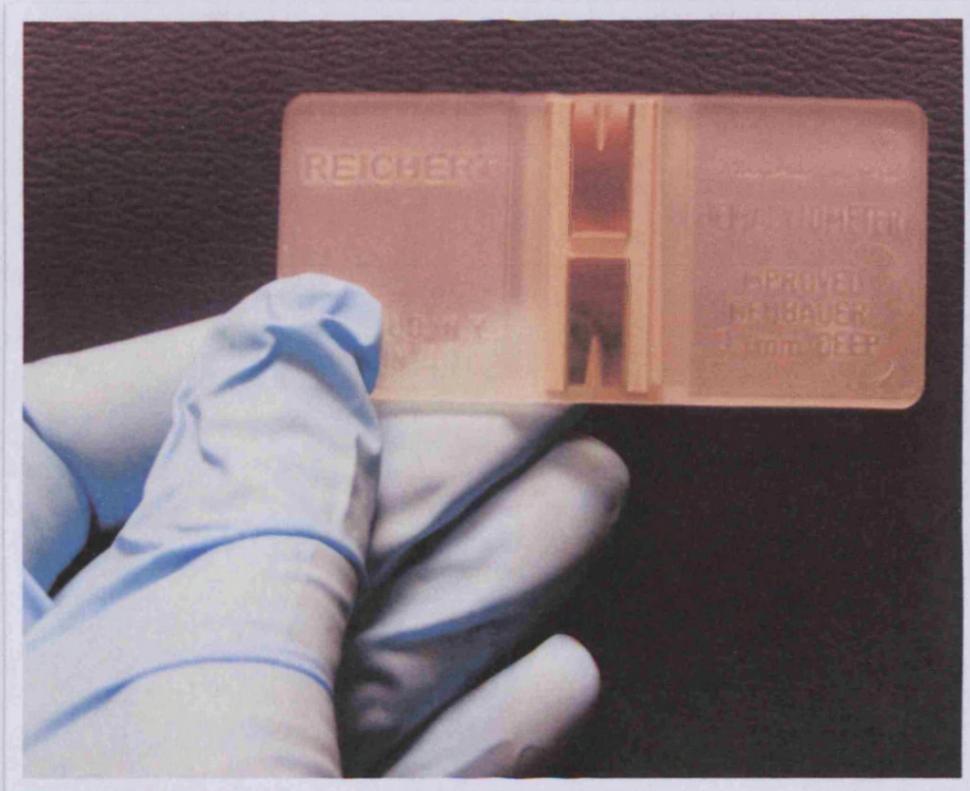


Figure 10.1 : A typical Improved Neubauer haemocytometer used for counting cells

Originally devised to count blood cells, they are also now used to count other cell types. The most commonly used haemocytometers are the improved Neubauer haemocytometers. The device consists of a heavy glass microscope slide with 2 indentations or counting chambers. These chambers are divided into $9 \times 1\text{mm}^2$ squares etched in a grid, both of them separated by a trough. The edge of the trough is raised by 0.1 mm off the grid. With the cover slip in place and cell suspension loaded into filling notches at either end of the

chambers, each square has a defined volume of liquid. Graphical description of the chambers and grids are shown in Figure 10.2.

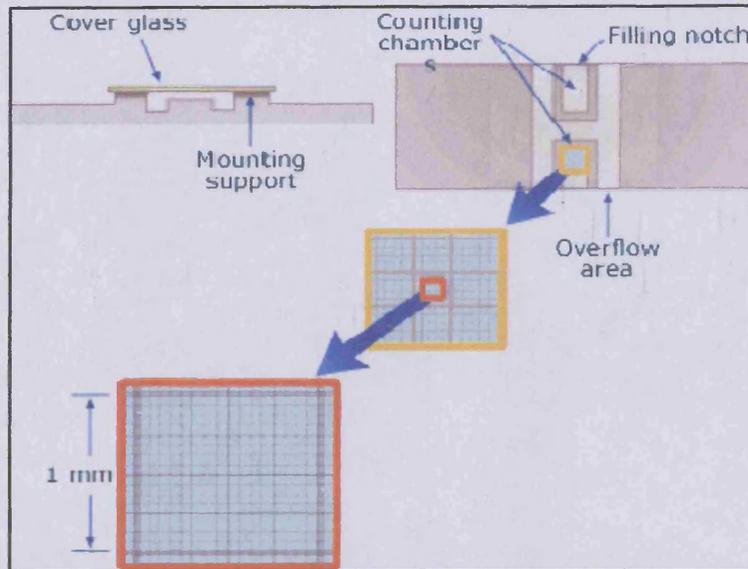


Figure 10.2 : Diagram showing the counting chamber and grid on a haemocytometer

As mentioned earlier, the chambers are divided into 9 squares of 1 mm^2 . The corner squares are further divided into 16 squares of 0.0625 mm^2 . The central square meanwhile is divided into 25 squares of 0.04 mm^2 ; each of them further divided into squares of 0.0025 mm^2 . The volumes of each different level of squares are shown in the Table 10.1.

Area (mm ²)	Volume (nL)
1	100
0.0625	6.25
0.05	5
0.04	4
0.025	0.25

Table 10.1 : Volumes of each level of squares on the counting grid of a haemocytometer

From the cell suspension prepared earlier, 10 μL was loaded in each counting chamber. Dispersion of the suspension was aided by capillary action. It is assumed that this volume is a random sample of the whole suspension. Thorough mixing is important to ensure that the cells do not clump, which would give a less accurate result. If the number of clumps is more than 10%, fresh sample should be taken. The haemocytometer is then placed under a microscope set at 100x magnification. In an improved Neubauer haemocytometer, the cells to be counted are those that lie within the area bordered by the 3 lines; the cells overlying those lines are not counted. It is important to be thoroughly consistent in choosing whether or not a cell should be counted. Five squares are counted from each chamber (typically the central 1 mm² square and the 4 corner squares). The total number of cells per mL can be obtained by multiplying the average number of cells from the 10 squares with 10 000.

10.2.2.4 *Freezing cells for storage*

Although caution is exercised during handling of the cells, often contamination might occur which would lead to cell death and depletion of supply. In order to counteract this possibility, the cells can be frozen to provide a cache of cells for long-term storage. The cell suspension, as obtained in Section 10.2.2.2 was placed in suitable cryo tubes. Next, dimethylsulfoxide (DMSO) was added slowly to the tubes dropwise to avoid osmotic breakdown of the cells. An equal volume of DMSO must be added to the volume of serum enriched medium, giving a total of 1 mL. The tubes were allowed to chill on ice for 30 minutes. The tubes were then placed in a pre-chilled freezing box, insulated by wrapping the tube with cotton wool. The box was placed in a -80 °C freezer overnight for slow cooling before transferring the tubes into liquid nitrogen for indefinite storage.

10.2.2.5 *Thawing frozen cells*

The key point to remember when thawing cells is to avoid osmotic shock to the cells. In order to achieve this, thawing must be carried out rapidly, followed by a slow dilution of the suspension. As soon as the suspension had thawed, the contents were transferred to a larger centrifuge tube and pre-warmed medium was added drop by drop to a suitable volume. Before use, the tube was spun down and the medium replaced to remove the DMSO. The re-suspended cells could now be seeded to flasks and allowed to grow for 2 passages before any experimental procedures were to be conducted.

10.2.2.6 HaCaT cell growth study

Before further experiments were conducted using the HaCaT cell lines, the growth characteristics of the cell line in the current laboratory setting were determined. The growth pattern can be determined via counting of cells over a certain period of time, and plotting a growth curve as a function of cell number over time. From a growth curve, the lag time, population doubling time, and saturation density can be determined (Mather and Roberts 1998). A typical cell growth curve can be divided into several distinct phases: inoculation/lag phase, exponential/log phase, stationary phase and death phase (Figure 10.3).

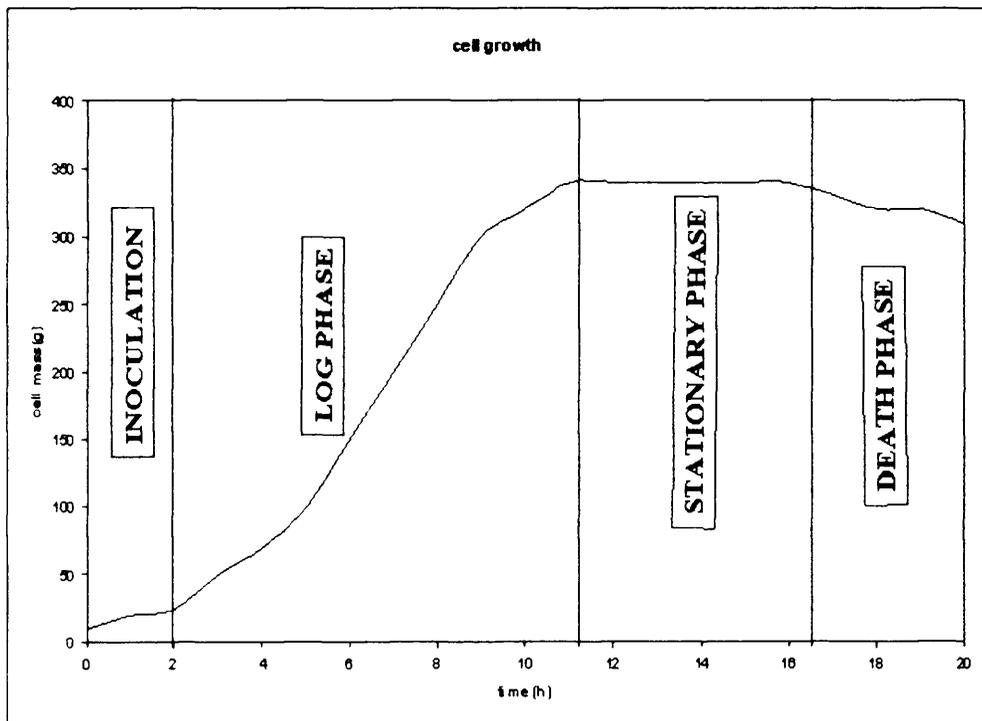


Figure 10.3 : A typical cell growth curve. Adapted from Tan (2001)

During the lag phase, the cells are acclimatizing to the growth surroundings/conditions, and cell division hardly occurs. As the cells enter the log/exponential phase, it is characterized by cell doubling at a constant rate, which can be determined by the slope of the resulting linear plot. During the

stationary phase, the growth rate is slowed down by the depletion of nutrient within the medium and the accumulation of cellular waste and toxic by-products. This phase can be prolonged as long as nutrients continue to be replenished. Otherwise, cellular growth will enter the death phase, whereby the remaining nutrient can no longer sustain growth of cells and leads to cell death.

Cells were seeded by preparing a cell suspension of 1×10^4 cells mL^{-1} in medium and placing 1 mL of the suspension in each well of a 24 well plate, which has a surface area of 2 cm^2 . This will give a cell density of 5×10^3 cells per cm^2 . Two plates were prepared for the study. The plates were then placed in the incubator at the setting mentioned earlier (37°C , 5% CO_2) and allowed to settle for 24 hours before the first counting was performed. For each sampling time, 2 wells were counted by first aspirating the medium, washing with DPBS and 1 mL of trypsin pipetted into the wells. The trypsin was allowed to dislodge the cells from the well surfaces for 10 minutes at 37°C , before being neutralized with an equal amount of medium. The resulting suspensions were then sucked out, placed in a micro-centrifuge tube and counted using the method described in Section 10.2.2.3. The cells were counted every 24 hours to a maximum of 8 days with the medium being replenished every 2 days. The number of cells versus the sampling time was plotted to construct the growth curve.

10.2.2.7 MTT colorimetric assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is one of the common assays used to determine cell viability. The principle behind this colorimetric assay is the ability of viable cells to reduce MTT into insoluble purple formazans via mitochondrial reductase. The insoluble dye will then be dissolved in a solvent, such as DMSO, and the absorption of the

resulting solution measured using a spectrophotometer at a wavelength between 500 to 600 nm. The absorption values are directly correlated with the number of viable cells present, as the reductase enzyme are only active in viable cells. The assay is also commonly used to assess toxicity of a compound, based on the compound's ability to induce cell toxicity, hence decreased viability. From this, a dose response curve of a compound can be plotted.

For the assay, HaCaT cells were seeded into 96-well plates at a density of 5×10^4 cells per mL. Each well had a surface area of 0.16 cm^2 and working volume of $200 \mu\text{L}$. After seeding, the wells were left overnight in the incubator. Stock solution of BD was also prepared by diluting in DMSO and mixing it with DMEM (containing 10% v/v FBS) to provide a series of concentrations ranging from $0.78125 \text{ mg mL}^{-1}$ to 2 mg mL^{-1} . The stock solution was prepared with care taken to limit the final DMSO concentration in the medium at 0.5% v/v. Mixing of the solution was done aseptically by filtering the stock BD solution with a syringe attached with a $0.2 \mu\text{m}$ filter before addition to DMEM. 4 Wells were filled for each concentration (at $200 \mu\text{L}$ per well), with a further 4 wells filled with DMEM containing 0.5% v/v DMSO (without BD) as vehicle control and 4 wells with only DMEM and 10% v/v FBS as control. The cells were allowed to grow within the treated medium for 48 hours in the incubator. After this period, the medium was removed from all the wells, and replaced with $20 \mu\text{L}$ of MTT in DPBS at a concentration of 5 mg mL^{-1} . The plates were incubated for a further 4 hours. Afterwards, all the wells were aspirated, leaving only the formazan precipitate at the bottom of the wells. $100 \mu\text{L}$ of DMSO was then pipetted into each well to dissolve the formazans, and the absorption of each well was read at 550 nm using a spectrophotometer (Sunrise™, Tecan Trading, Switzerland) after 20 minutes, preceded by a gentle shaking of the plates for 15 seconds to completely dissolve the formazans. The number of viable cells was calculated as a percentage of the absorbance of the test wells over the vehicle control. The

% inhibition was obtained by subtracting the number from 100. The results were then plotted as a dose response curve (% inhibition versus \log_{10} BD concentration). The GI_{50} value for BD, which will be used in all further cell experiments, was determined by extrapolating the concentration of BD which resulted in 50% growth inhibition in the dose response curve.

The assay was also repeated using a combination of BD at GI_{50} and fish oil. It was found that the maximum amount of fish oil that can possibly be dissolved in the medium was limited by the amount of ethanol allowed in cell medium. Typically, this is kept at 0.5% v/v or lower. Therefore, the fish oil was dissolved in ethanol at a 2:1 ratio, and the final concentration of the fish oil solution in the medium was 1% v/v (the ethanol concentration in the final medium solution kept at 0.5% v/v). 5 x 8 Wells were dosed with medium containing BD at GI_{50} , and an equal number of wells were dosed with medium containing BD at GI_{50} and 1% v/v FO:EtOH (equating to 0.66 mg mL⁻¹ EPA in each well of a 96-wells plate). As control, 2 x 8 wells were dosed with medium containing DMSO and EtOH, the vehicles used to dilute BD and FO at 0.5% v/v each.

10.3 Results and discussion

10.3.1 *HaCaT growth curve*

The growth curve obtained for HaCaT cells is shown in Figure 10.4. The typical growth curve pattern was achieved, with the different phases clearly shown. From the linear section of the plot, the cell doubling time was calculated to be 22.1 hours, while the lag time was 20.6 hours. Any further experiments with HaCaT cells were conducted at least 20.6 hours after seeding and ran for a minimum of 22.1 hours. For these reasons, the

subsequent growth assay and the ICC staining in Chapter 11 were carried out according to these times.

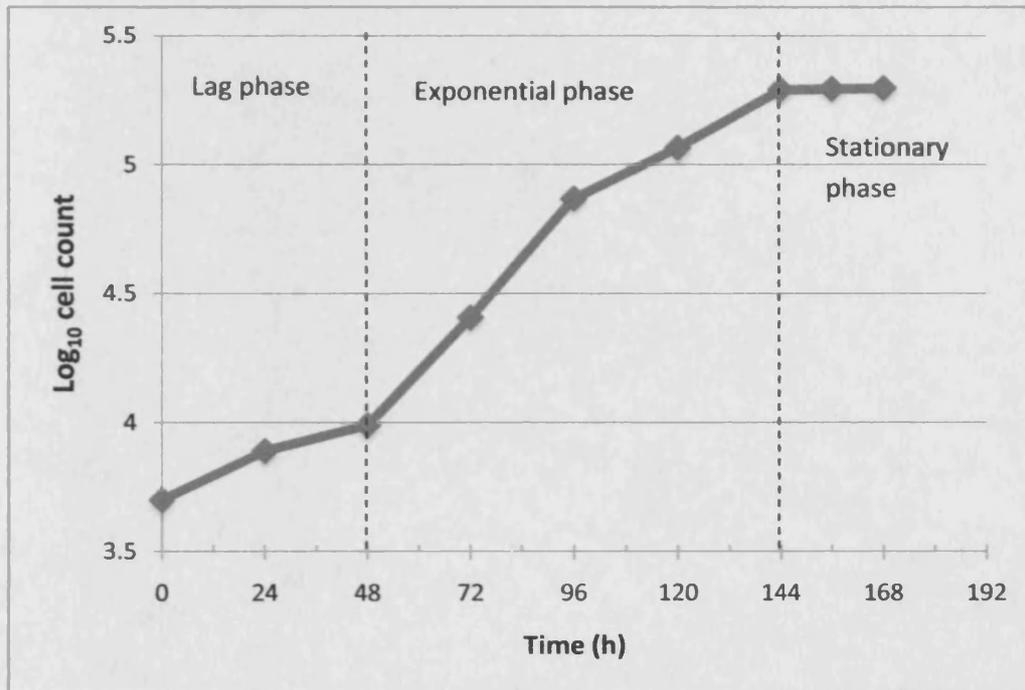


Figure 10.4 : Growth curve for HaCaT cells over 7 days

10.3.2 MTT assay – BD dose response curve and GI₅₀ determination

The dose response curve for BD is shown in Figure 10.5. Extrapolation of the plot revealed that the GI₅₀ value was 0.22 mg mL⁻¹. To facilitate preparation of test solutions, the concentration was rounded to 0.25 mg mL⁻¹, and all subsequent studies were conducted using this value.

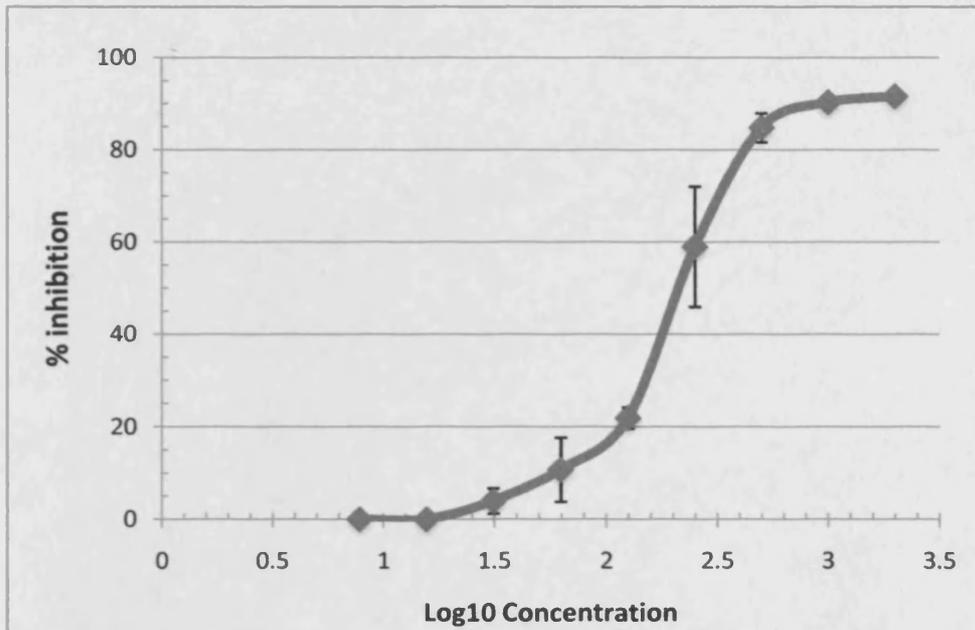


Figure 10.5 : BD dose response curve. The GI_{50} determined from this curve was 0.22 mg mL^{-1} . For convenience, this value was rounded up to 0.25 mg mL^{-1} and used in all subsequent assays.

In this study, the solubility issue surrounding fish oil, as mentioned in Section 10.2.2.7 made it impossible to obtain GI_{50} value for fish oil. Initial growth inhibition assay at the maximum fish oil concentration allowed only resulted in an average of $7.63 \pm 3.66 \%$ growth inhibition. All subsequent assays involving fish oil (either alone or in combination) were then performed at the maximum allowed concentration as reported in Section 10.2.2.7.

10.3.3 MTT assay – growth inhibition of HaCaT cells by BD and effects of FO addition

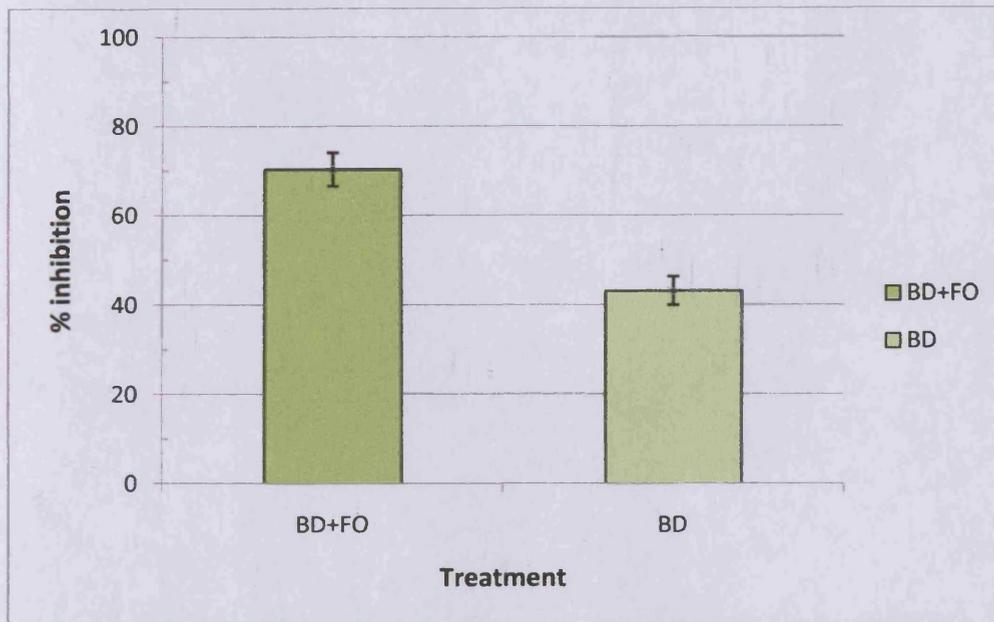


Figure 10.6 : % inhibition of BD and BD-FO mixture on HaCaT cells, $n = 4 \pm SD$. The addition of fish oil increased the inhibition of HaCaT cells by 27.2%, from 43.15 to 70.35 % compared to BD alone ($p 0.034$)

The mechanisms by which BD exert its activity in the treatment of psoriasis include anti-proliferative effects via disruption of DNA synthesis (Gottlieb 2005b). In this study, the treatment of HaCaT cells with BD at GI_{50} led to 43.15 ± 3.14 % ($p 0.0001$) growth inhibition. The addition of fish oil appeared to increase cellular growth inhibition, raising it up to 70.35 ± 3.74 % ($p 0.034$). This was suggestive of a synergism in inhibitory action between BD and fish oil. The exact mechanism of this synergism is unknown. Although at the maximum fish oil concentration used in this study growth inhibition was only less than 10%, the anti-proliferative effects of fish oil and its constituent fatty acids have been repeatedly demonstrated in many cell lines, particularly tumour cells (Chen and Auburn 1999; Kapoor 2009). The effect of EPA, one of the primary fatty acids found in fish oil, on the growth of cultured keratinocytes has also been reported (Riku et al. 1993). This inhibition is

believed to be attributed to the ability of EPA to modulate the various steps of signal transduction by growth factors (Terano et al. 1997).

10.4 Conclusion

The findings from the growth assay provide further support to combining fish oil with conventional anti-psoriatic medications. In previous chapters, we have seen the enhancing effects of fish oil on inflammatory enzyme inhibition by BD, and also the added benefit of modulating the delivery of BD across epidermal layers. It is now also proven, on a cellular level, another mechanism by which FO and the n-3 fatty acids exert their effects on keratinocytes, supporting the findings by which supplementation or treatment with FO can be advantageous in inflammatory skin diseases such as psoriasis.

Chapter 11:

*Immunocytochemical staining in
HaCaT cells for cleaved caspase-
3 (Asp175) and COX-2*

11.1 Introduction

In Chapter 10, the basic principles of culturing HaCaT cell lines were discussed. The dose response curve of BD was also determined. Investigations on the effects of both BD and FO to cellular growth revealed that addition of FO enhanced cellular growth inhibition by BD. In this chapter, the effects of FO and BD on apoptosis were investigated by looking at the relative levels of the apoptotic marker cleaved caspase 3 (Asp175) by immunocytochemistry, a variation of IHC methods used earlier in Chapters 7 and 8. Using ICC, the expression of COX-2 after exposure of HaCaT cells to the agents was also investigated.

Caspase-3 (Figure 11.1) is a member of the cysteine-aspartic acid protease (caspase) family, associated with cellular apoptosis (Janicke et al. 1998). Sequential activation of caspases is an important process in the execution-phase of apoptosis (Nicholson 1999). Physiologically, it is available as an inactive proenzyme, which will undergo proteolysis and dimerization to form the active enzyme. The larger fragment resulting from the enzyme activation (17/19 kDa), cleaved at the Asp175 site, is a novel epitope which is not present in normal cells, thus providing a sensitive and unique indicator of apoptosis. This has led to the development of cleaved caspase-3 detection as an improved assay for determination of apoptotic cells (Gown and Willingham 2002).

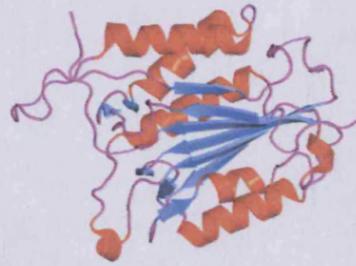


Figure 11.1 : Caspase-3 (Chao-Zhou et al. 2003)

Apoptosis, as a physiological process, is a series of biochemical events culminating in cell death (Fadeel and Orrenius 2005). This programmed cell death is initiated by the cell itself or from surrounding and inflammatory cells when a cell is damaged beyond repair, as a result of infection, DNA damage from ionizing radiation and toxic chemicals or stress. It also has an important role in embryonic development of organs, for example the formation of limbs and digits (Guha et al. 2002); and maintaining the balance in the number of cells within a living organism (Thompson 1995).

In psoriasis, the induction of apoptosis is found to be impaired as a result of overexpression of Human β -defensins (HBD), cathelicidin, psoriasin and other anti-microbial peptides in psoriatic plaques (Peric et al. 2009). With reduced cell death, the population of keratinocyte increases at a great rate, creating an imbalance in cell numbers and development of epidermal hyperproliferation and thickening which is characteristic of the disease.

As mentioned in section 7.1, the cyclooxygenases are important components in inflammation; catalyzing the production of mediators central to the whole process, e.g. prostaglandins. The COX-2, in particular, is almost undetectable or present at low levels in normal, non-neoplastic tissue. Distribution of this inducible enzyme is limited to certain tissues, including the epidermis

(keratinocytes). In the epidermis, it is highly inducible by UV rays and has been implicated in the pathogenesis of skin tumours (Flockhart et al. 2008).

In this chapter, the effects of fish oil and BD on cellular apoptosis, using caspase-3 as a marker, and expression of COX-2 were investigated using ICC on a HaCaT cell line treated with the two compounds and the effects of combining the two. In Chapter 7, BD and FO individually inhibited the expression of COX-2 in a full thickness porcine ear skin model of inflammation, and in combination resulted in a greater inhibition. It is of particular interest to see whether the same can be replicated on a cellular level.

11.2 Materials and methods

11.2.1 Materials

Gibco® Dulbecco's modified Eagle medium 1x (DMEM, high glucose, with L-glutamine and phenol red, no HEPES, no sodium pyruvate), foetal bovine serum, trypsin 0.05% with EDTA·4Na 1x were obtained from Invitrogen (Paisley, UK). 3-Aminopropyltriethoxysilane, dimethylsulfoxide (DMSO), ethanol, formaldehyde, methyl green, PBS sachets, and sodium chloride were from Fisher (Loughborough, UK). Dulbecco's phosphate buffered saline (DPBS), and Tween 20 were obtained from Sigma-Aldrich (Poole, UK). EnVision+ System HRP Labelled Polymer Anti-Rabbit, liquid DAB+ substrate Chromagen System were from Dako (Ely, UK). Antibodies for cleaved caspase 3 Asp175 (#9664) and COX-2 (#4842) were both from Cell Signalling. Fish oil capsules (Boots n-3 Fish Oil 1000mg high strength) were obtained from a local store. 3-Aminopropyltriethoxysilane (TESPA) covered microscope slides were coated in-house

11.2.2 Methods

11.2.2.1 Cell seeding and treatment

HaCaT cells were seeded at a density of 1×10^5 per cm^2 onto a TESPA-coated cover slip inside tissue culture treated dishes with a culture area of 8cm^2 . Post-seeding, the cultures were left overnight to allow the cells to attach to the surface of the cover slip. The following day, the medium in each dish was replaced with medium containing the test compounds. The incubation time was 24 hours at 37°C and 5% CO_2 . The treatments used were BD (in DMSO) at 0.25 mg mL^{-1} , a 2:1 mixture of FO: EtOH at 1% v/v, a combination of 0.25 mg mL^{-1} BD and the FO: EtOH mixture (also at 1% v/v) and control containing the vehicles (0.5% v/v DMSO, 0.5% v/v EtOH) and 10% v/v FBS in medium.

11.2.2.2 Fixation and ICC staining

Fixation of cells was carried out at the end of the incubation period using formalin saline (4.5 g NaCl, 50 mL formaldehyde in 450 mL water) by first removing the medium and replacing it with 1 mL of formalin saline and leaving it for 10 minutes. This was followed by 1 mL of 100% EtOH for 5 minutes, and 2 changes of PBS at 5 minutes each. At each rinse, the cover slips were lifted up with forceps to ensure complete washing and to release trapped medium beneath the cover slips.

The dishes were then transferred to a humidity chamber to avoid desiccation of the cover slips. The slips were given another rinse of PBS, this time with the addition of a few drops of PBS-Tween for 15 seconds as a blocking reagent. Prior to incubation with primary antibody of the desired proteins, the blocking solution was completely removed. The primary antibodies were prepared in PBS at 1 in 100 dilutions for cleaved caspase-3 (Asp175) and 1 in

50 for COX-2. 75-80 μL of the antibody were applied on top of each cover slip and incubated at room temperature and incubation time of 1 hour for Asp175 and overnight for COX-2.

Post-incubation, the cover slips were rinsed with 3 counts of PBS at 5 minutes each, followed by PBS Tween. The cover slips were then incubated with Dako Envision+ secondary antibody at 25 °C for 90 minutes. The cover slips were then washed with PBS for 3 min followed by PBS Tween (2 \times 5 minutes). Visualization of the protein was achieved by incubating the cover slips with 75 μL of DAB chromogenic HRP substrate (Dako) for 10 minutes at room temperature. The cover slips were then rinsed with deionized water (3 \times 2 minutes) and counter-stained using an aqueous solution of 0.5% methyl green solution for 35 minutes. The cover slips were rinsed several times with deionized water and then left in an oven set at 40°C to dry overnight. They were then mounted onto pre-cleaned microscope slides using DPX and dried at 60°C before inspection and imaged under a microscope (Nikon Optihop, Tokyo, Japan) equipped with image capture facilities (Axiovison LE, Carl Zeiss Ltd., Welwyn Garden City, UK)

11.3 Results and discussion

11.3.1 *Cleaved caspase-3 (Asp175)*

Figure 11.2 shows the ICC staining for Asp175. The staining of nuclei to brown indicates the positive presence of the protein, and the number of positive nuclei was a direct indication of the extent of cellular apoptosis in each slides. The BD treated cells showed approximately 75% of nuclei positive for Asp175, compared to the almost complete absence in the control slide. In cells treated with FO, approximately 25% were positive, while it was increased to

approximately 50% when combined with BD at the same concentration as the first treatment. The number of positive nuclei however was less than with BD alone.

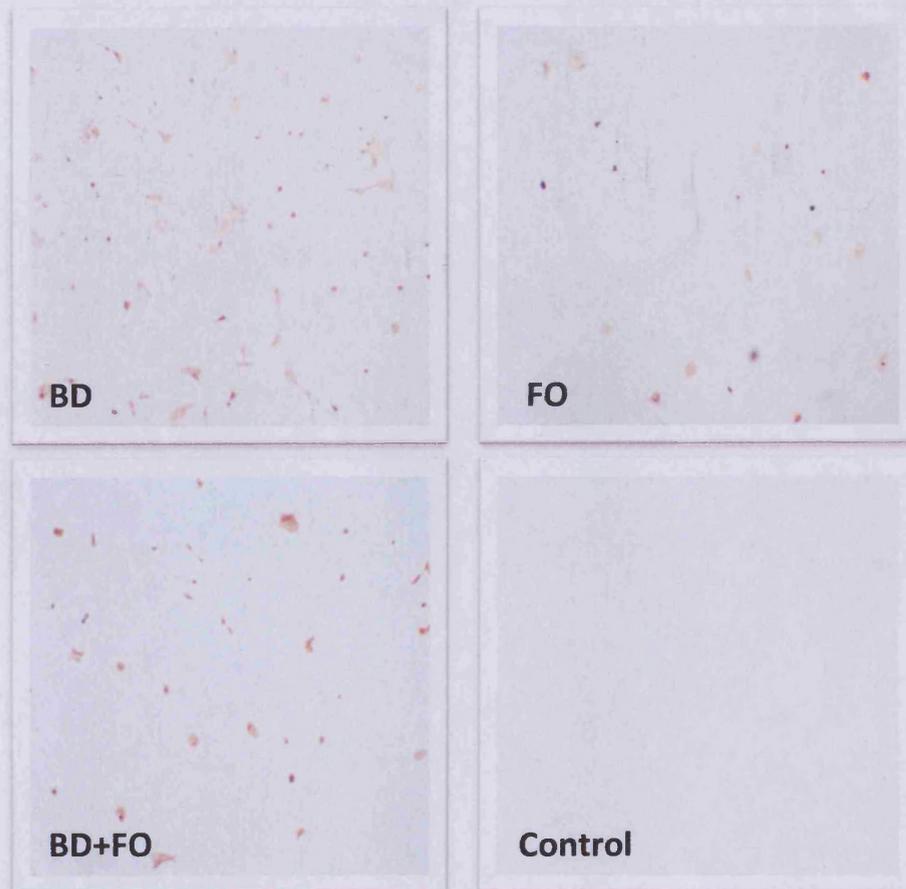


Figure 11.2 : ICC staining for Cleaved Caspase-3 (Asp175). BD = BD 0.25 mg mL⁻¹, FO = 1% FO-EtOH, BD+FO = BD 0.25mg mL⁻¹ + 1% FO-EtOH.

The efficacy of corticosteroids, e.g. betamethasone and other anti-psoriatics, in the treatment of psoriasis depends, among others, on their ability to exert anti-proliferative effects on keratinocytes. Although the mechanism of action varies between the agents, most of the treatments possess the ability to induce apoptosis in keratinocytes (Ceović et al. 2007).

The ability of fish oil/n-3 fatty acids to induce apoptosis in tumour and other cell lines has been well-documented (Hong et al. 2003; Sanders et al. 2004;

Siddiqui et al. 2001). In a study done by Pupe et al. (2002), it was reported that markers of cellular apoptosis were increased in UVB-irradiated keratinocytes following pre-treatment with EPA compared to the monounsaturated FA oleic acid. The mechanism involved has been identified to be a synergistic over-expression of TNF- α between EPA and UVB irradiation itself. This supported the protective ability of fish oil in reducing the incidence of skin cancer as a result of UV exposure by inducing death of damaged cells that can potentially induce tumour formation.

In the current study, the ability of FO to induce apoptosis was again demonstrated in HaCaT cells, an immortalized keratinocyte cell line, again supporting the role of FO in the treatment of psoriasis. It was also interesting to note the apparent potentiation of action observed in the combined corticosteroid-FO treatment. The slightly reduced number of positive cells may not be due to reduced effectiveness. It can also be influenced by the possible variation in the number of cells seeded initially, or even reflective of the greater number of dead cells that might have been detached from the cover slip as a function of increased efficacy of the combined treatment.

11.3.2 COX-2

Figure 11.3 shows representative images for COX-2 staining. Again, the presence of brown staining indicates the positive presence of COX-2 in the cytoplasm. The control slides indicated that COX-2 was expressed, though at a very low level. Treatment with BD, FO and combined BD+FO all resulted in a reduction of COX-2 staining, indicative of an inhibitory effect on COX-2 expression. These findings correspond to the study carried out on an excised porcine skin model of inflammation, outlined in Chapter 7 & 8. As a brief recap, the expression of COX-2 was found to be inhibited by BD, and by FO

alone. The greatest inhibition was also observed when the two agents were combined in a single formulation, suggesting a potentiation effect.

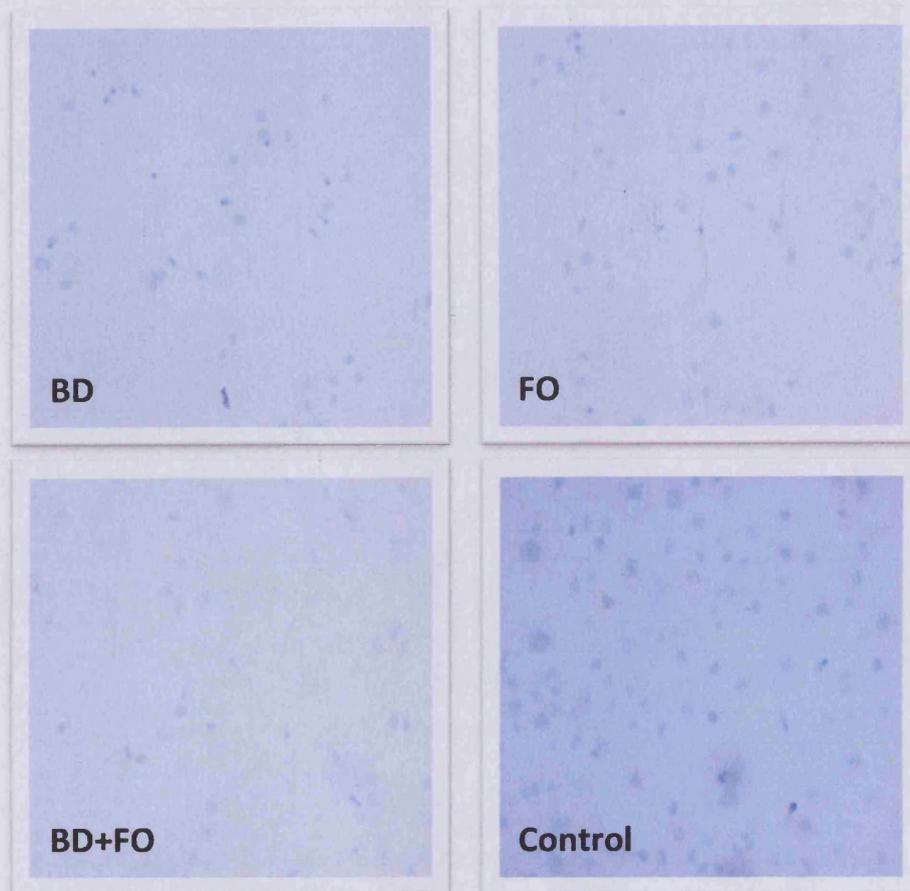


Figure 11.3 : ICC staining for COX-2 : BD = BD 0.25 mg mL⁻¹, FO = 1% FO-EtOH, BD+FO = BD 0.25mg mL⁻¹ + 1% FO-EtOH.

In the current study, the effect on COX-2 was more pronounced compared to experiments using excised skin, as the primary cells of interest were directly exposed to the treatments, as opposed to the substantial barrier posed by the layers of keratinized dead cells before reaching the viable epidermis.

Interestingly, the study done by Chêne et al. (2007) found that the expression of COX-2 in a HaCaT cell line was enhanced by both n-3 (EPA) and n-6 (γ -linoleic acid) mediated by peroxisomal proliferator-activated receptor (PPAR)- γ . It was also suggested that the protective/anti-inflammatory effect

of n-3 PUFAs was in fact dependent on COX-2. This sheds light on the 'duality' of the inducible enzyme. It is traditionally viewed as a pro-inflammatory enzyme, and the deleterious effects of excess inflammatory mediators produced by COX-2 metabolism in diseases, such as colorectal cancer, is well established (Dommels et al. 2003). However, it was also discovered that, in the resolution phase, products of COX-2 metabolism such as PGD₂ and PGF_{2α} possess pro-resolution/anti-inflammatory activity (Bertolini et al. 2001; Gilroy et al. 1999). Considering the activity of COX-2, which encompasses both ends of the spectrum (strongly pro-inflammatory through to strongly anti-inflammatory), it is reasonable to assume that induction of COX-2 might prove to be beneficial, or deleterious, depending on the phase of inflammation.

Contrastingly, several studies showed that induction of PPAR-γ by its ligands had an inverse relation with COX-2 expression (Kulkarni et al. 2008; Yang and Frucht 2001). Both EPA and DHA have been found to activate PPAR-γ (Allred et al. 2008; Li et al. 2005; Yang and Frucht 2001), and this activation is central to the anti-tumour effects of fish oil. Several publications extolled on the virtues of PPAR-γ ligands as a potential new class of therapeutic agent for psoriasis and psoriatic arthritis, given its action on cell proliferation and inhibition of pro-inflammatory mediators (Bongartz et al. 2005; Ellis et al. 2000; Friedmann et al. 2005). It is in this regard that the induction of COX-2 as reported by Chêne et al. (2007) requires further investigation, particularly in the knowledge that the opposite was observed in the current study involving HaCaT cells and also in Chapters 7 and 8.

11.4 Conclusion

In this chapter, it was shown that FO was able to induce apoptosis in an immortalized keratinocyte cell line. This supported numerous other studies reporting the effects of fish oil in inducing programmed cell death. The value of this in psoriasis therapy has also been elaborated, further supporting the use of FO. Mirroring the inhibition seen with a model of skin inflammation, the expression of COX-2 was inhibited on a cellular level. There seem to be contrasting reports on the action of FO/n-3 PUFAs on COX-2 in different cell lines, although the majority reported an inhibitory effect and accepted this as the basis of the anti-inflammatory action of FO/n-3 PUFAs. The importance of COX-2 in the resolution phase of inflammation is noted, bringing the issue of addressing the balance between inhibition and induction of COX-2 into focus.

Chapter 12:

In vitro response of defolliculated mice to topically applied fish oil and anti psoriatic agents I : effects on epidermal thickness as determined by Optical Coherence Tomography

12.1 Introduction

Among the prominent characteristics of DfL mice are the epidermal thickening and formation of scales. One of the targets in the treatment of psoriasis is a reduction of epidermal thickness, as many of the agents currently being used exert an anti-proliferative action on keratinocytes, for example corticosteroids, retinoids, and anthralin (Section 1.2)

Optical coherence tomography (OCT) is a technique of obtaining slice images of three-dimensional objects (Fercher et al. 2003). In simple terms, OCT can be described as the optical equivalent of ultrasound imaging, which is based on soundwaves (Drexler and Fujimoto 2008a). One of the main applications of this rapidly developing technique is to provide a real time, rapid, non-invasive imaging method for sub-surface tissue structures with micrometer resolutions. The principles behind the operation of OCT involves measurement of echo time delay and the magnitude of a backscattered light generated from a broadband light source employing super-luminescent diodes or extremely short pulse lasers (femtosecond lasers) (Drexler and Fujimoto 2008a; Schuman et al. 2004)

OCT works best in acquiring images from clear or transparent objects, of which the prime example is ocular media. The structural images of the retina obtained with OCT were of the quality unparalleled with other non-invasive diagnostic techniques, hence ophthalmic imaging and diagnostics are the most clinically developed OCT applications at the present time (Drexler and Fujimoto 2008b; Schuman et al. 2004).

The technology is currently finding more biomedical applications outside the field of ophthalmology. It has been successfully utilized to image the invasion of cancer cells in the liver (Kuo et al. 2009), bladder lesions (Karl et al. 2009),

determination of gastric cancer in murine models (Xiong et al. 2009), imaging of dentin and the pulp chamber of human teeth (Fonseca et al. 2009), and cardiology (Brezinski 2006; Regar et al. 2003; Tanaka et al. 2009). Of the highest relevance in the current study is the imaging of the skin and diagnosis of skin cancers (Kirillin et al. 2009; Mette et al. 2009; Yang et al. 2009).

The aim of this study was to investigate the effects of commonly used topical anti-psoriatics and fish oil on DfL mouse via changes in epidermal thickness. This study was also designed to compare the relatively new method of measuring epidermal thickness using OCT, compared to the more conventional method of H&E staining coupled with microscopy. One of the advantages in using the OCT is that it allows a reduction in the number of animal used in studies, as the same animal can be monitored progressively over time, and stress to the animals is reduced compared to methods such as punch biopsy.

12.2 Materials and methods

12.2.1 Materials

BD, BHA, and SA were obtained from Sigma Aldrich (Poole, UK). Fish oil capsules (Boots n-3 Fish Oil 1000mg high strength) were obtained from a local store. Emulsifying ointment and isoflurane were from Thornton and Ross and Merial Animal Health Ltd. (Harlow, Essex, UK) respectively. Tissue-Tek® O.C.T™ Compound (OCTC) was from Sakura Finetek Europe B.V (Zoeterwoude, Holland). All other reagents were of analytical grade or equivalent.

12.2.2 Methods

12.2.2.1 Breeding and maintenance of DfL colony

DfL mice were bred by mating the mice with C57BL/6, often referred to as "C57 black 6" or just "black 6", which is a common inbred strain of lab mouse. It is probably the most widely used "genetic background" for genetically modified mice for use as models of human disease. They are the most commonly used lab mouse strain due to the availability of congenic strains, easy breeding, robustness, and their relationship to GM models, making them ideal controls. The C57BL/6 mice were obtained from Harlan UK Ltd. (Loughborough, UK). Typically, half of the litter will be DfL. In order to prevent contamination and infections, the mice were housed in scantainers (Figure 12.1) operated under negative pressure up to the commencement of the study. The mice were fed with regular diet of pellets and water and put under a 12 hour light cycle. All procedures within this study were conducted under Home Office license and with prior review by the local Ethical Review committee.

All the mice used in the study were between 20-22 weeks of age, a stage where hair follicle destruction is complete. They were further divided into 4 treatment groups according to Table 12.1. These formulations were the same as used in Chapter 6. The formulations were prepared according to the method outlined in section 5.2.2

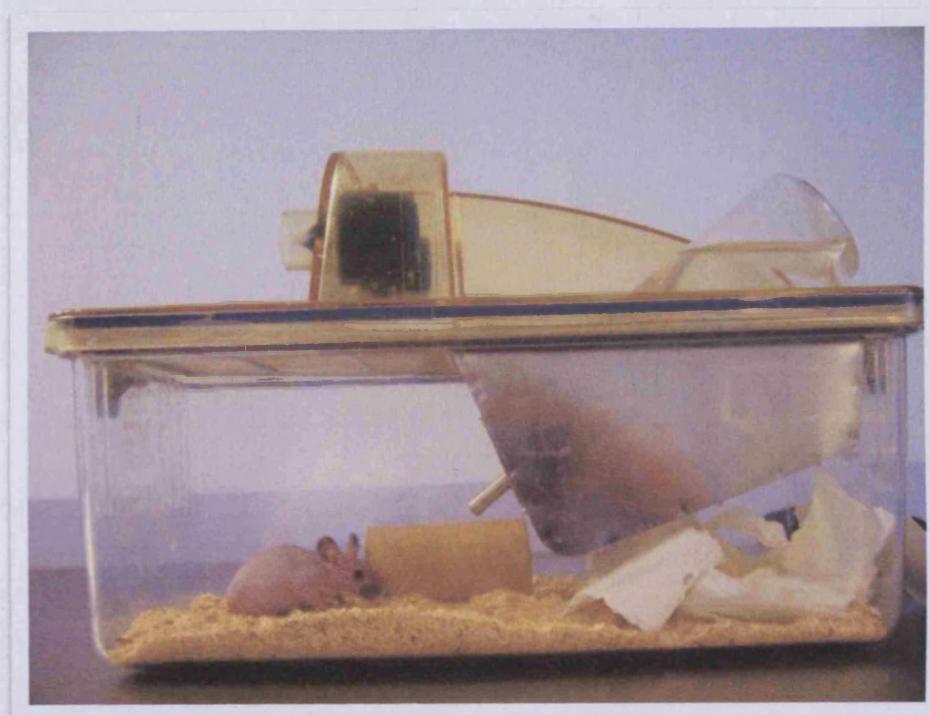


Figure 12.1 : A representative scintainer cage containing a single DfL mouse.

Group	Treatment
BDO	0.05% w/w BD, 3% w/w SA, 0.05% w/w BHA in emulsifying ointment BP
BF	20% w/w FO, 3% w/w SA, 0.05% w/w BHA in emulsifying ointment BP
BDF	0.05% w/w BD, 3% w/w SA, 20% w/w FO, 0.05% w/w BHA in emulsifying ointment BP
Control	0.05% w/w BHA in emulsifying ointment BP

Table 12.1 : The treatment groups of the study and its corresponding formulation. Each formulation was applied on a daily basis for 10 days

12.2.2.2 *Epidermal thickness measurement*

Figure 12.2 shows the area where the formulations were applied and scanned for thickness measured. The area was approximately 2x2 cm. This area was chosen as it was considered the area least accessible for the mice to groom off the formulation. To further avoid this, the formulations were applied as a thin layer with gentle rubbing using a glass rod to ensure uniform distribution on the applied area. At the beginning of the study, an initial measurement was made to provide baseline values of epidermal thickness, and the area marked with indelible, non toxic pen to ensure the same site was used consistently throughout the whole study, thereby the data obtained were directly compatible. Each treatment group consisted of 5 mice of mixed gender to offset any possible variation in response between male and female mice.

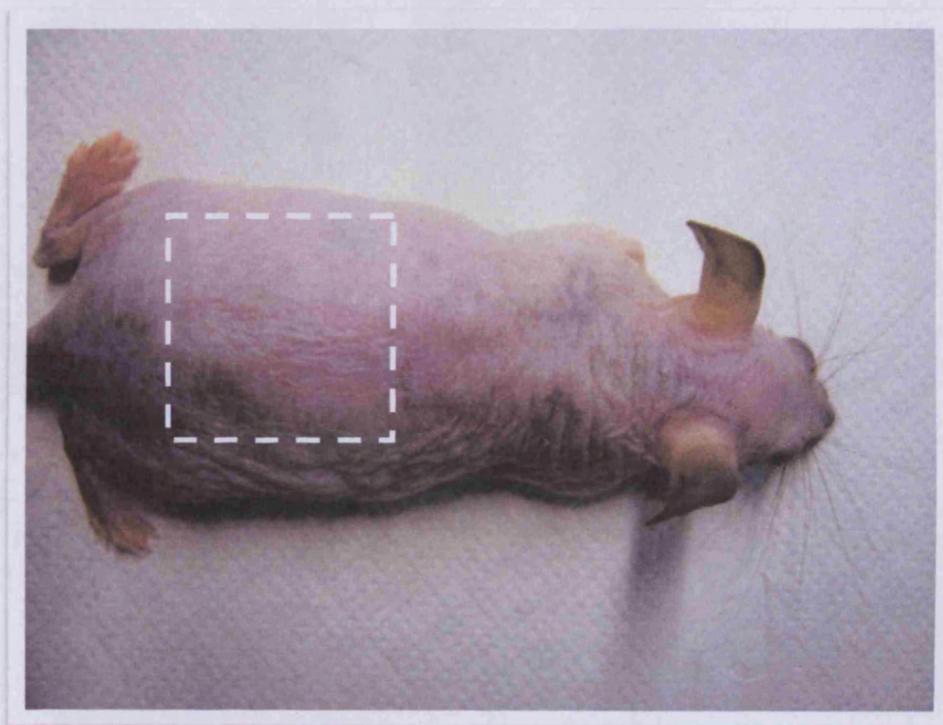


Figure 12.2 : The outlined area showing the site of formulation application and epidermal thickness measurement

Approximately 100 mg of the formulations were then applied on a daily basis and the mice scanned under anaesthesia. This was done in order to keep the animal as still as possible without the need for any physical restraint. Induction of anaesthesia was done with the aid of an anaesthetic chamber (Figure 12.3), using Isoflurane at a rate of 5 L min^{-1} with oxygen as a carrier.

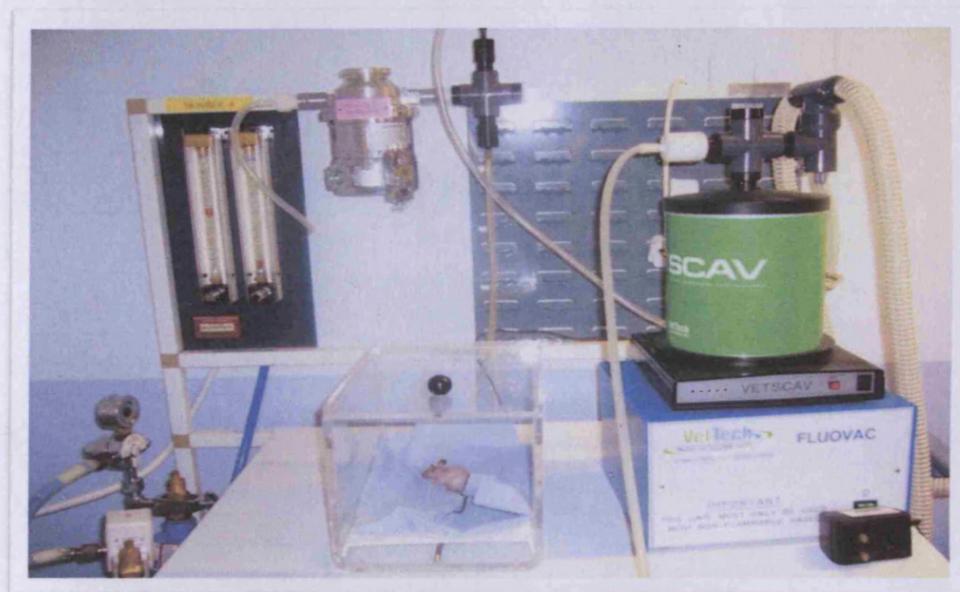


Figure 12.3 : Typical setup of an anaesthetic chamber connected to a scavenger machine

Following this, maintenance of anaesthesia was achieved with the use of an anaesthetic mask, with the flow rate reduced to $1\text{-}2 \text{ L min}^{-1}$. Prior to being connected with the mask, the eyes were lubricated with eye drops since these mice have a tendency to develop dry eyes, which can be further exacerbated by the gas flowing through the mask. For this reason also, the scan was only done on alternate days to minimize distress to the mice as a result of the anaesthesia. The setup for scanning is shown in Figure 12.4. The OCT system used was THORLABS swept source OCT imaging system (Thorlabs Ltd., Ely, UK). The wavelength for the laser used for the OCT was set at 800 nm. Spectral width was set at 100nm and axial-scan rate was 16kHz. Scanning dimensions were set at 5, 5, and 3 mm at the x, y and z axes. Post-scanning,

the mice were allowed to regain consciousness in warmed cages and full recovery was ensured before returning to the holding area. Any animal which showed signs of illness or deterioration of health was excluded from scanning and observed until deemed fit to undergo the procedure. These animals, however, continued to receive topical application of the test formulations.

OCT probe

Laser source

Computer

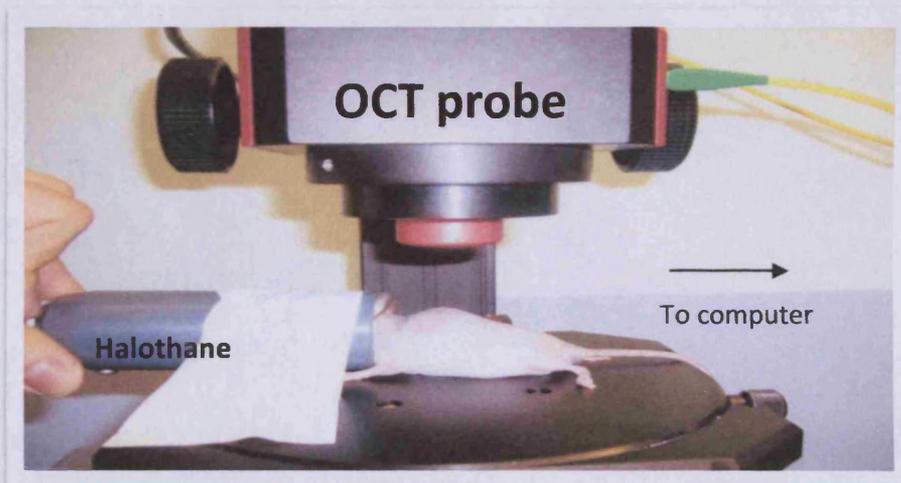
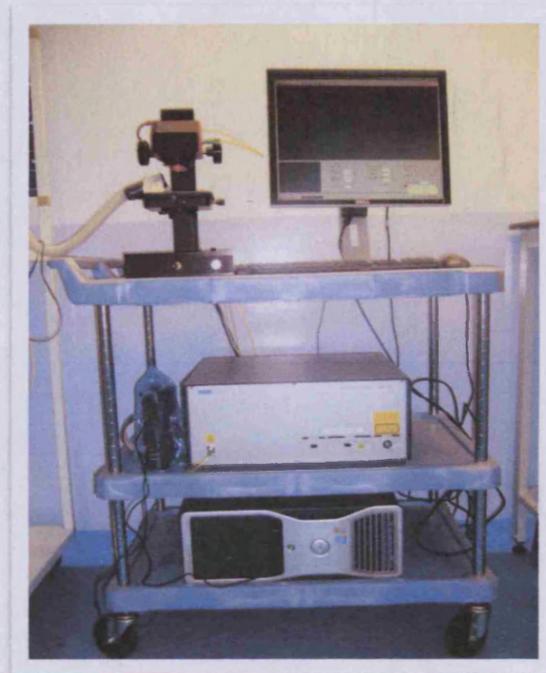


Figure 12.4 : Top : The OCT system connected to a laser source and computer processor. Bottom : Close up of the setup for measuring epidermal thickness of the mice

The OCT scans were then processed using ImageJ software (<http://rsbweb.nih.gov/ij/>); 5 measurements were taken from each scan and the values tabulated with Microsoft Excel 2007.

12.2.2.3 Tissue harvesting

At the conclusion of the experiment, all the mice were killed using Schedule 1 methods and the treated areas harvested for post-processing and analysis. The skin from this area was cut into 2 thin strips of approximately 3 cm x 0.5 cm, the first strip embedded in OCTC tissue embedding medium and allowed to solidify on a bed of dry ice. The remaining strip was soaked overnight in PBS-buffered formalin prior to embedding in paraffin wax.

12.2.2.4 H&E staining

Sections allocated for H&E staining were embedded in Paraplast embedding medium (Sigma) and then cut into sections of 5 μm on pre-cleaned X-tra™ Adhesive microscope slides (Surgipath). Prior to staining, the sections were dewaxed by first placing the slides in an oven set at 60°C for 30 minutes and then soaked in 2 changes of xylene, followed by descending concentrations of ethanol (100%, 90%, 70%) in duplicates. Subsequently, the sections were washed with running tap water for 3 minutes and rinsed with distilled water.

The sections were then stained in Mayer's haematoxylin for 10 minutes, and washed thoroughly in running tap water, followed by rinsing in distilled water. Blueing of the section was done in Scott's Tap Water Substitute. At this stage, the sections were examined under a light microscope to determine the extent of staining. Excess staining can be removed with 1%

acid alcohol, followed by rinsing in running tap water and then, distilled water.

Counter-staining was done with 1% eosin for 5 minutes, followed by a brief wash in tap water. The sections were then dehydrated in ascending concentration of alcohol, starting with 90% and 3 changes of 100% alcohol for 5 minutes each. This was then followed by 3 changes of xylene at 5 minutes each. Finally, the sections were mounted with DPX and left to dry overnight in an oven set at 45°C.

Slides were then observed under a microscope equipped with image-capture facility (Nikon). Five measurements were made on each section using the image capture software (Axiovision LE, Carl Zeiss, Welwyn Garden City, UK)

12.2.2.5 *Statistical analysis*

Statistical tests for non-parametric data were conducted using InStat 3 for Microsoft Windows (GraphPad Software, Inc.)

12.3 Results and discussion

12.3.1 *Epidermal thickness – OCT*

Figure 12.5 to Figure 12.10 shows the representative scans obtained from the 4 treatment group from Day 0 to Day 10. Initial measurement of all the mice yielded an average epidermal thickness of $26.92 \pm 1.17 \mu\text{m}$, with a *p* value of 0.5286. A change in epidermal thickness was already apparent by the next scan, revealing a pattern which was maintained throughout the whole duration of the study, as shown in Figure 12.11. The fish oil treated group

(BF) exhibited a sustained increase in thickness, with an average increase of 105% from baseline values. Conversely, the BD treated group showed a significant reduction in thickness (average 38.8%). The combined BD-FO treatment appeared to also reduce epidermal thickness although not to the same extent as BD alone, while the control group did not show appreciable changes over 10 days.

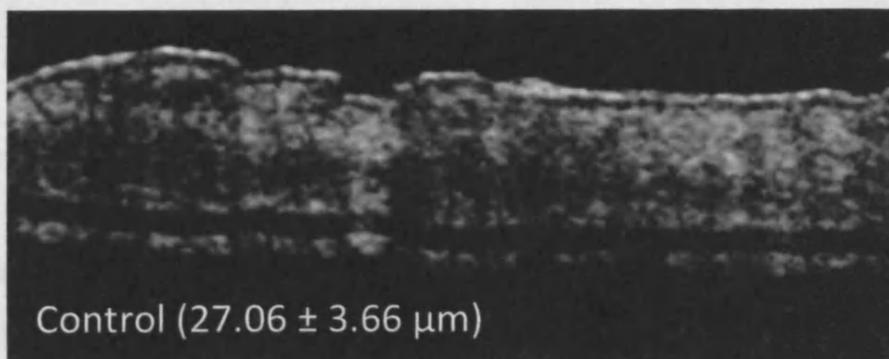
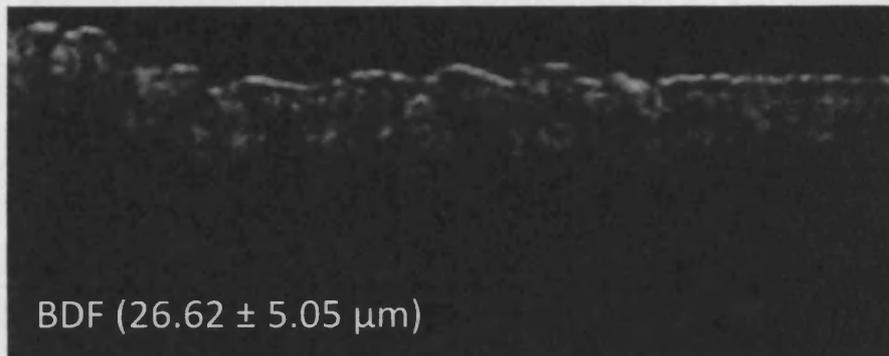
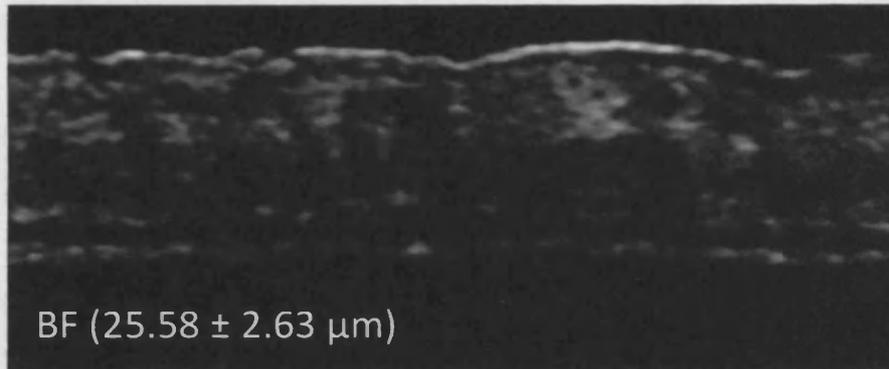
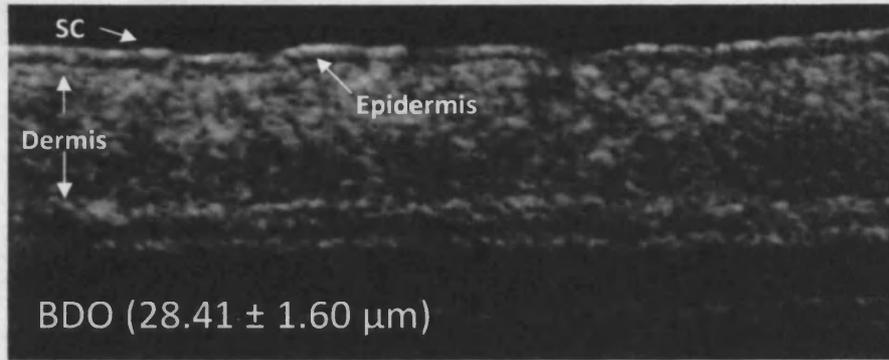


Figure 12.5 : Representative OCT scan, day 0. Epidermal thickness in each treatment group was not statistically significant (p 0.5286) at this point

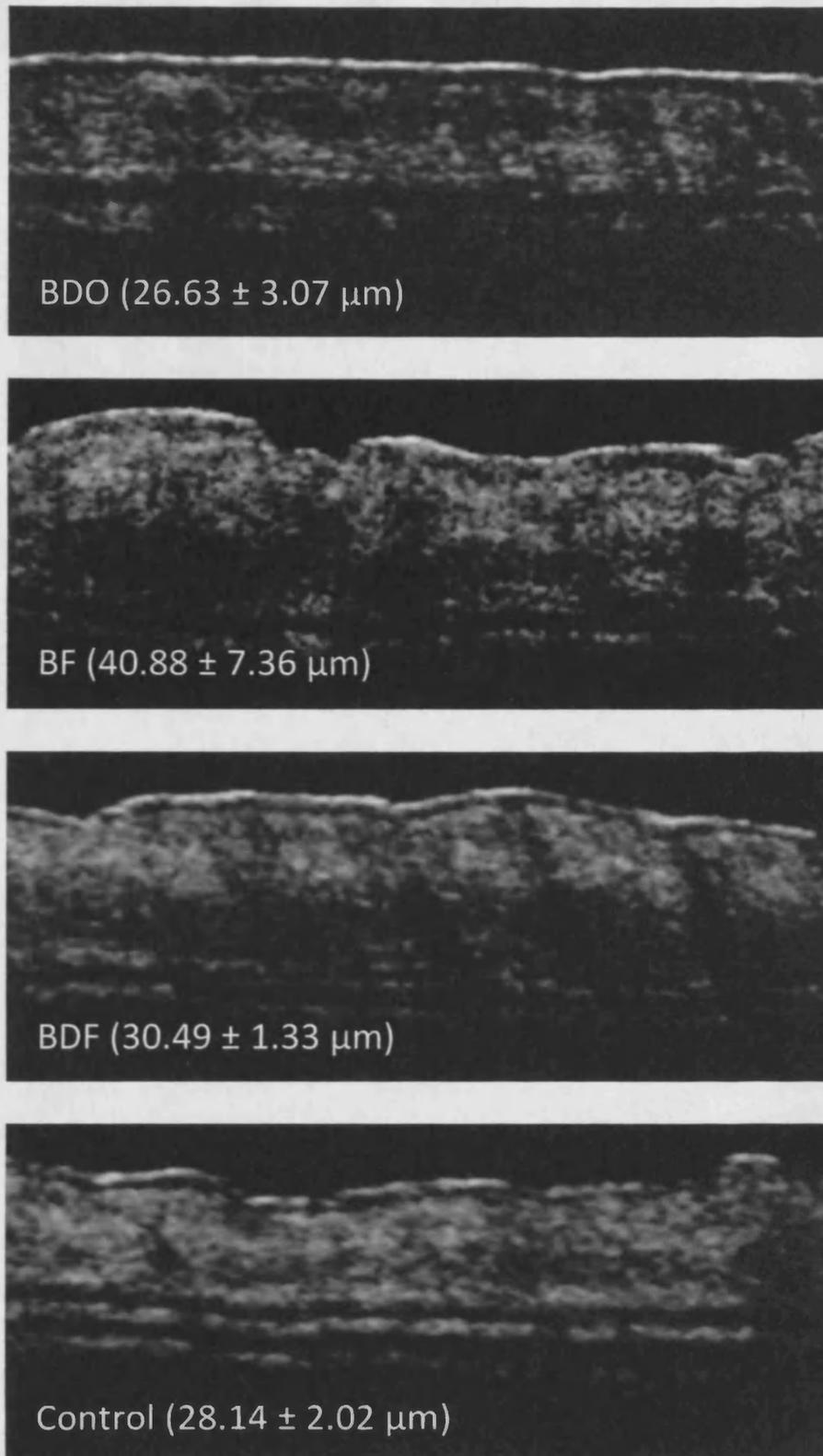


Figure 12.6 : Representative OCT scan, day 2. The group treated with BF had begun to show an increase in epidermal thickness, and the opposite with BDO.

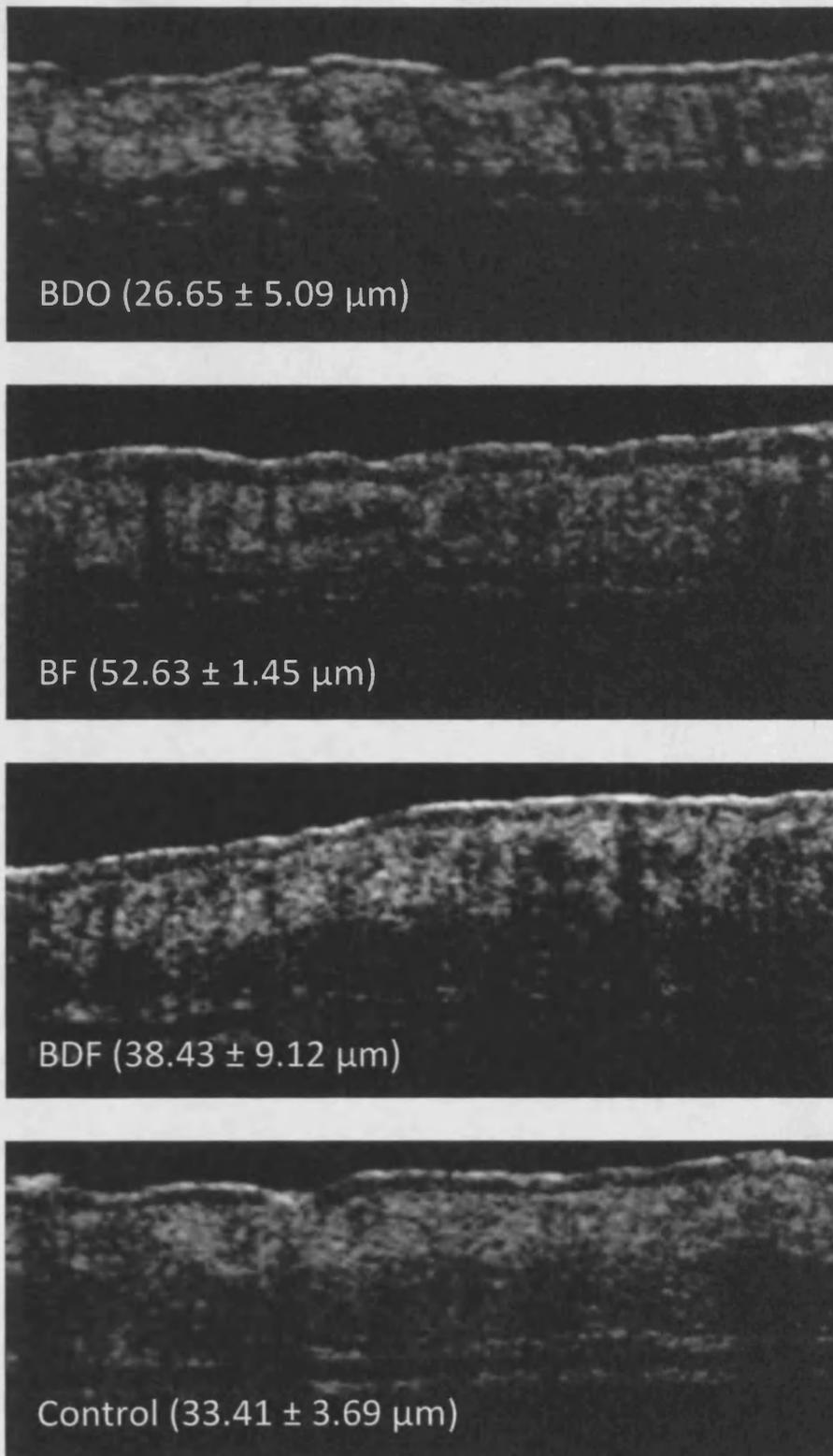


Figure 12.7 : Representative OCT scan, day 4

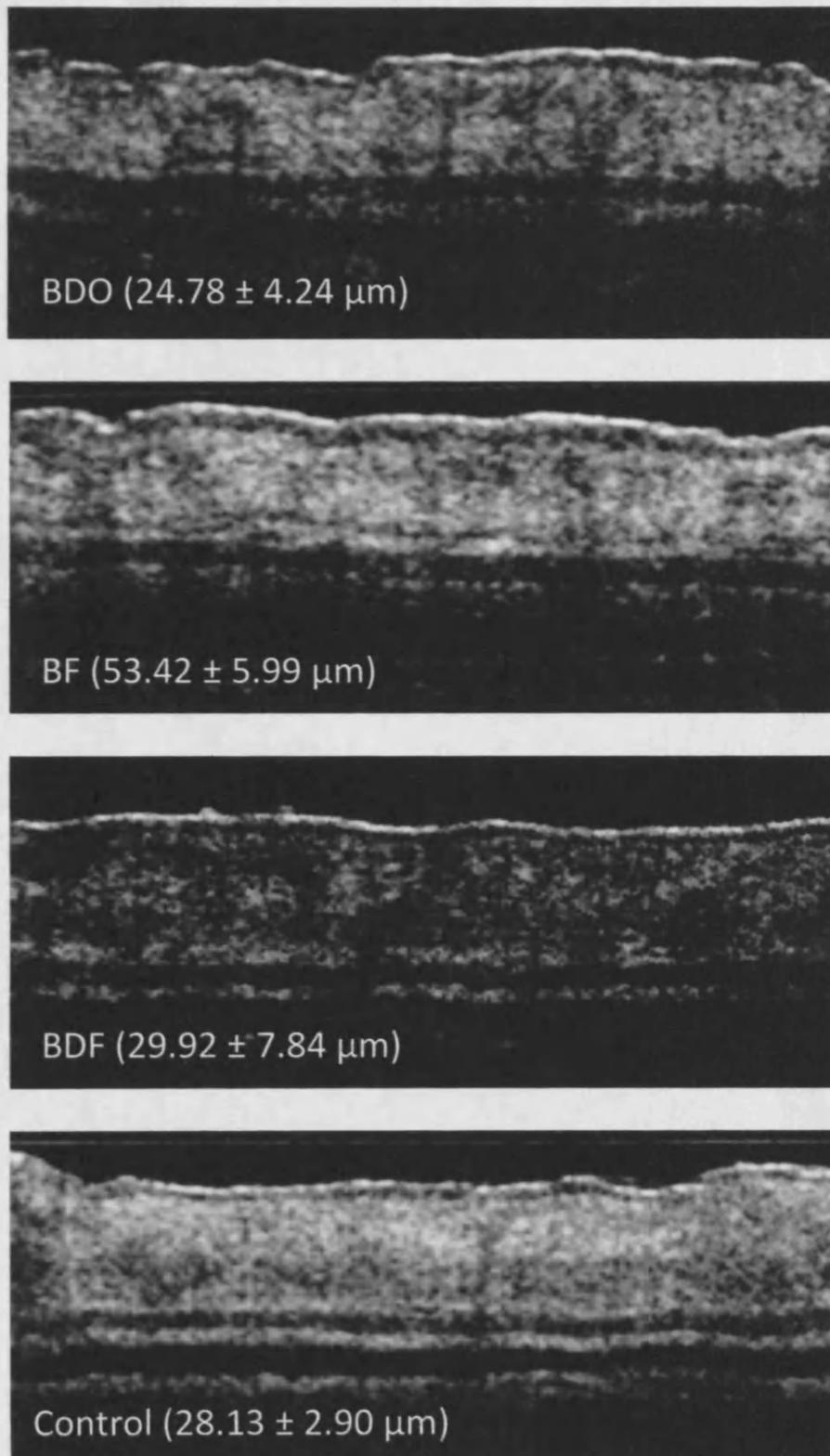


Figure 12.8 : Representative OCT scan, day 6

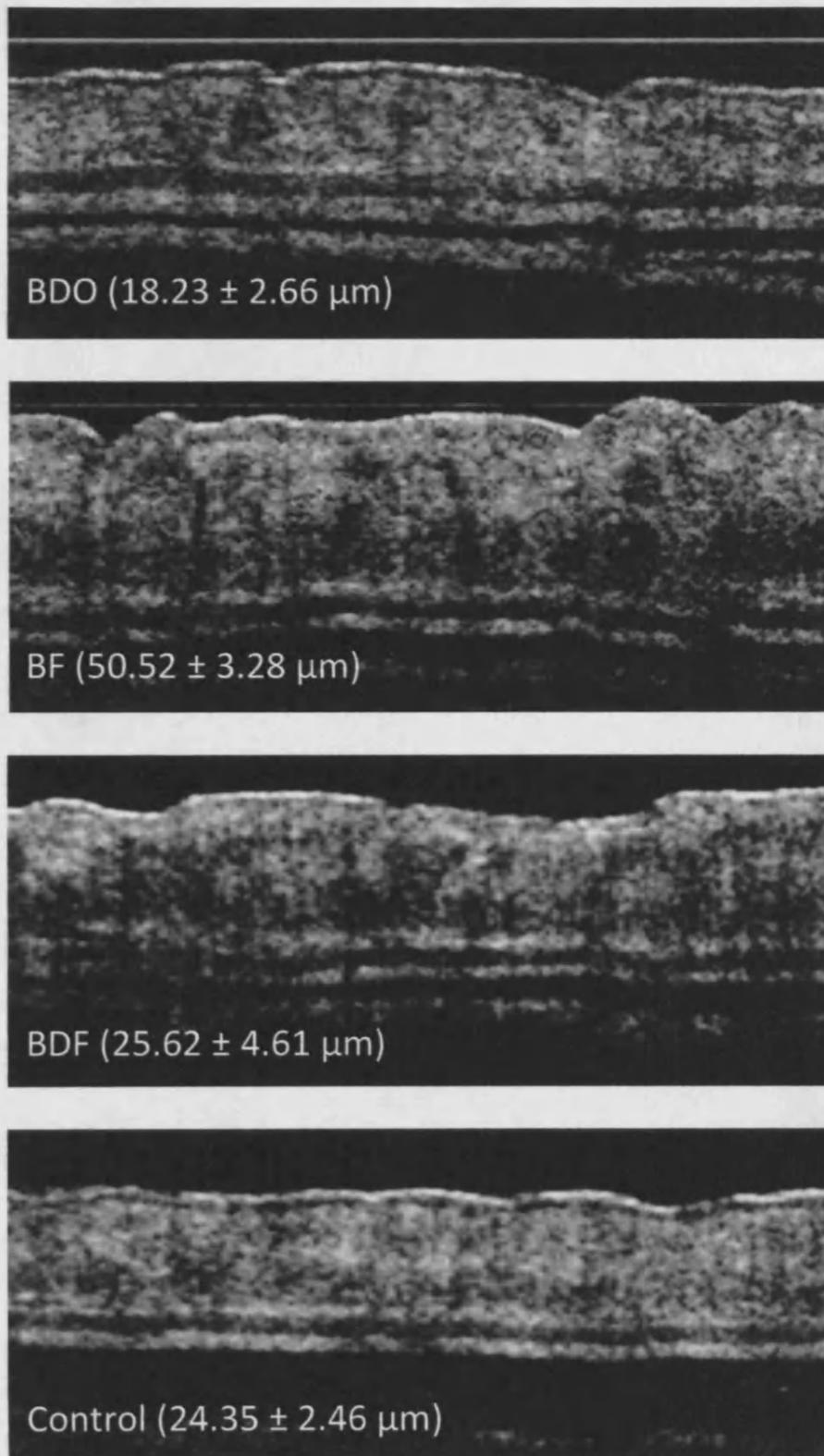


Figure 12.9 : Representative OCT scan, day 8

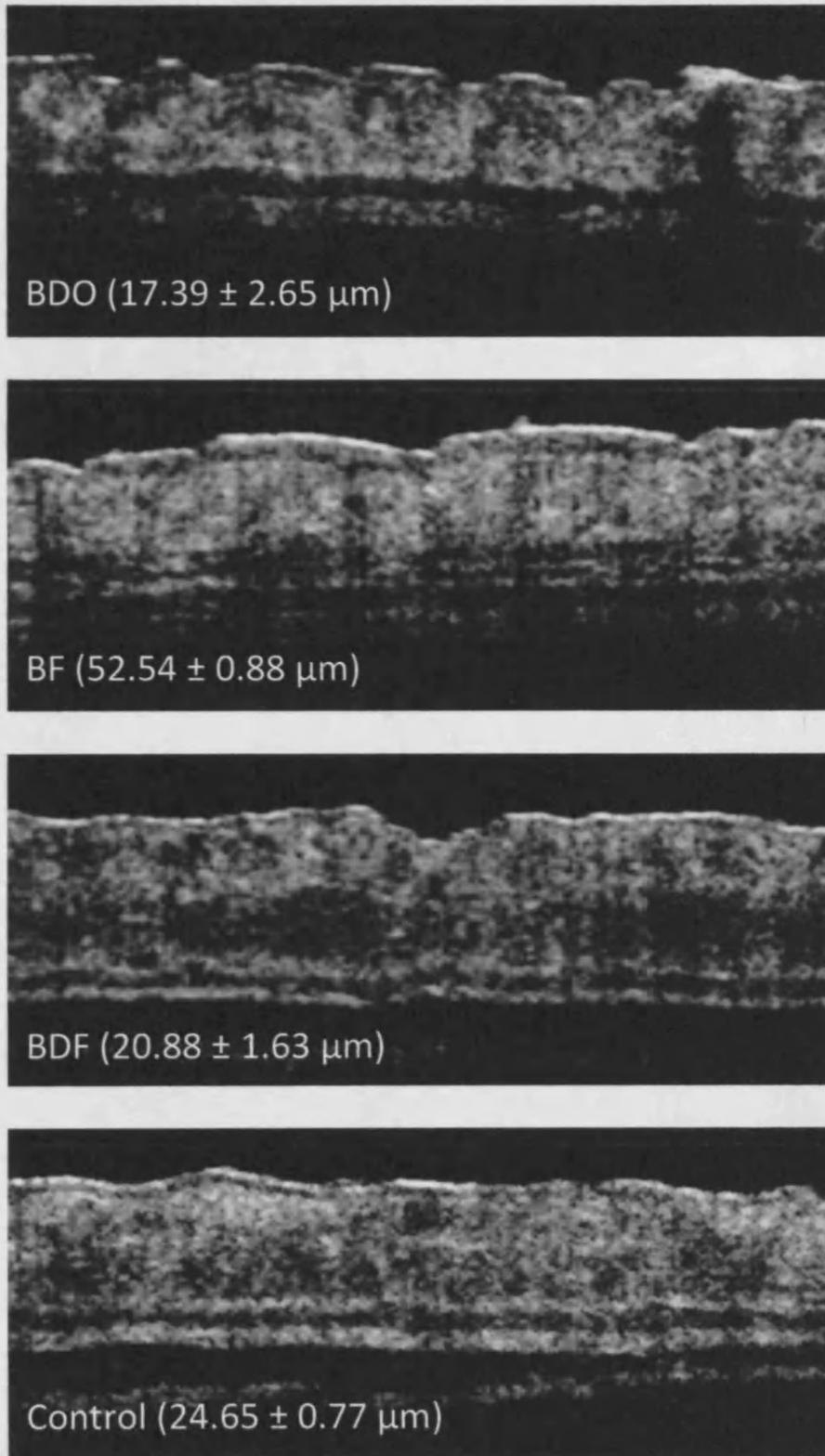


Figure 12.10 : Representative OCT scan, day 10

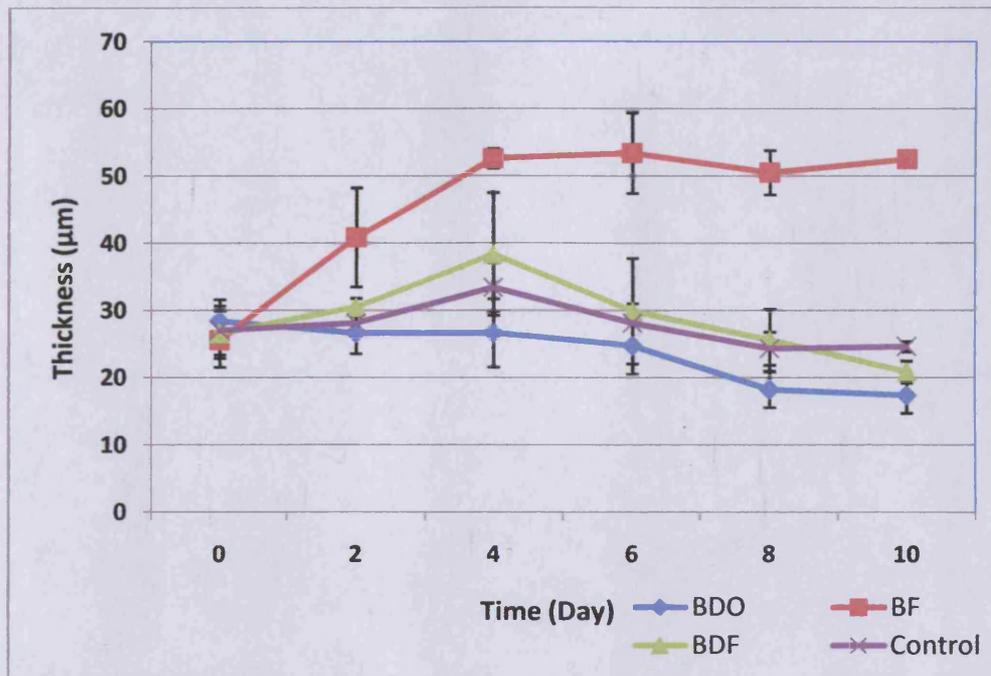


Figure 12.11 : Epidermal thickness over 10 days obtained with OCT ($n=5$, \pm SD). Treatment with BF increased epidermal thickness as early as the 2nd day of treatment and sustained throughout the whole duration of the study. The opposite was seen with BDO treatment. Treatment with BDF did not cause any notable changes compared to control

Figure 12.12 depicts the average pre- and post-study measurements for the 4 groups. BDO, BF and BDF treated group showed statistically significant values at the conclusion of the experiment (p 0.0001, 0.0001, and 0.0136 respectively). As mentioned earlier, changes in the control group were not statistically significant (p 0.3755).

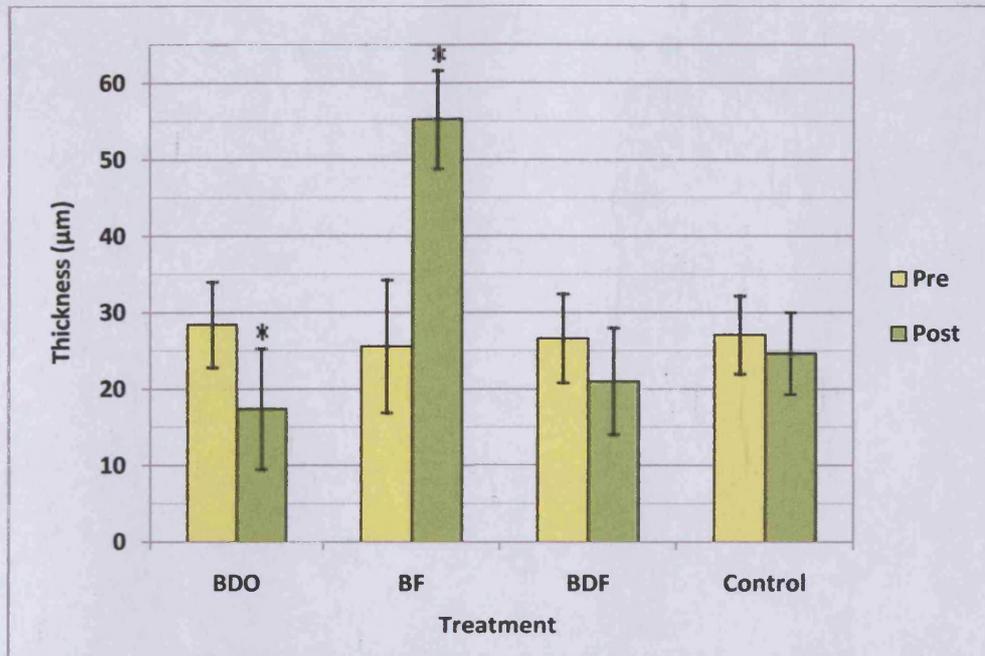


Figure 12.12 : Average pre- and post- treatment epidermal thickness (n=5, \pm SD). * = $p < 0.05$

12.3.2 Epidermal thickness - H & E

Figure 12.13 are representative skin sections of the 4 groups stained with H&E. Visual observation confirmed the OCT finding reported earlier. The BDO treated sections were noticeably thinner and, inversely, treatment with BF caused a gross thickening of the epidermis. Again, the combined treatment did not differ from control. The cells of the basal layer were found to be enlarged and columnar in nature in the BF treated group.

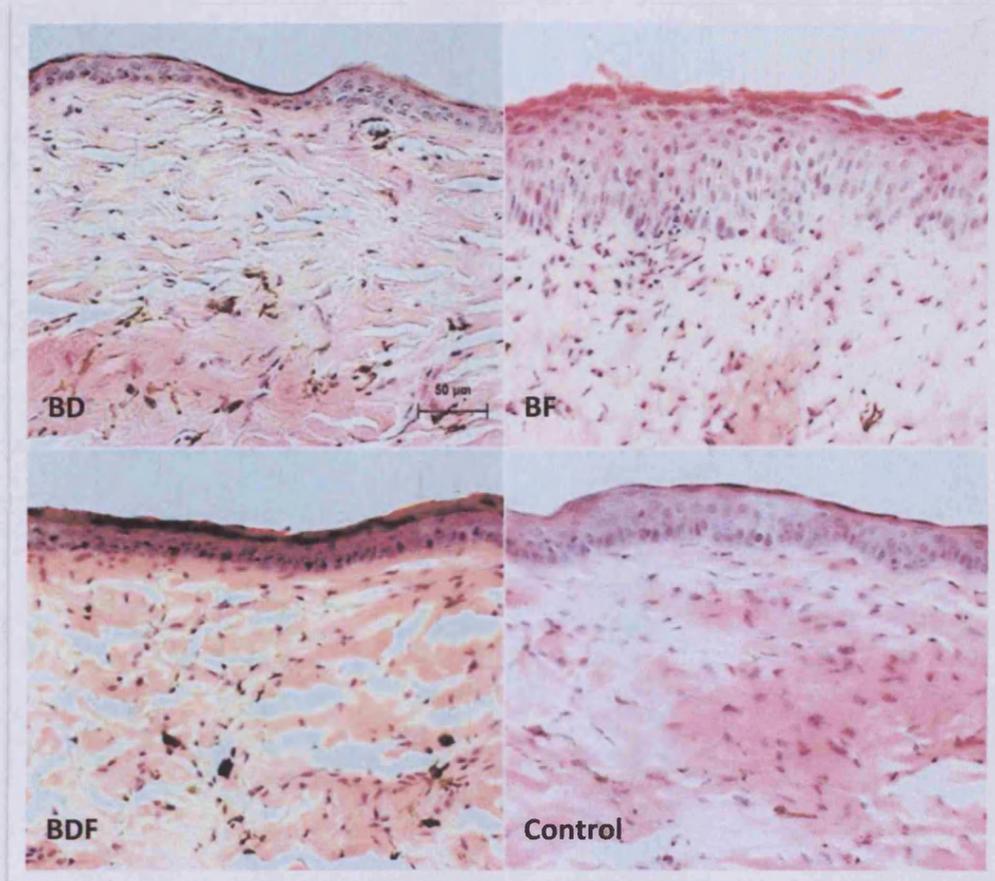


Figure 12.13 : Representative images of the H&E stained sections obtained from the 4 treatment groups (n=5). Note the apparent change in thickness for BDO and BF, while BDF remained unchanged.

12.3.3 *Epidermal thickness measurement - comparison between OCT and H&E*

The average epidermal thicknesses measured using the 2 techniques are shown in Figure 12.14. Mann-Whitney test revealed that the values obtained were comparable between the two. It must be noted that in order to achieve the measurement with H&E, sacrifice of the animals was required, compared to the non-invasive OCT which permits live measurement.

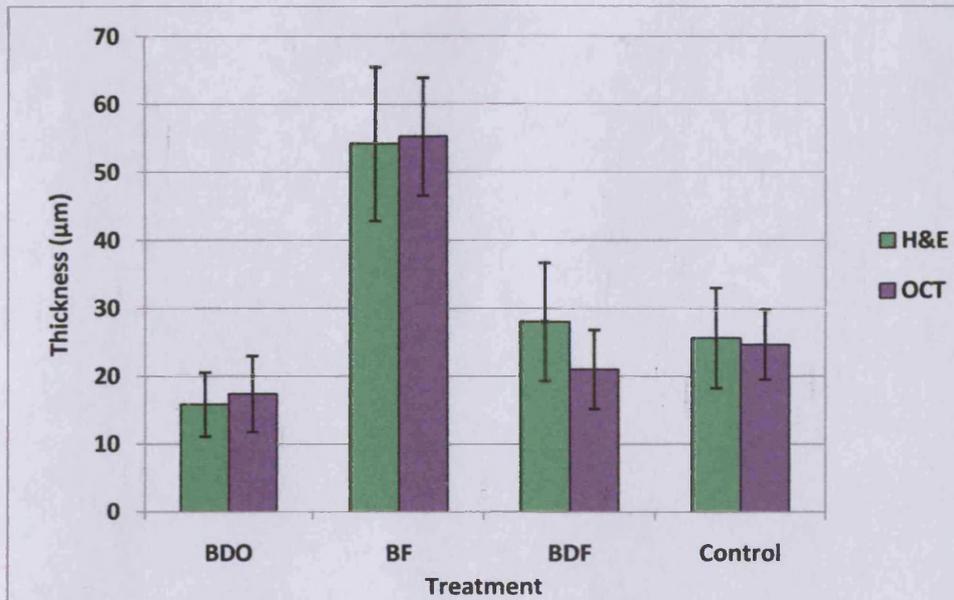


Figure 12.14 : Comparison of average epidermal thickness as measured by H&E vs OCT after 10 days ($n=5$, \pm SD). No significant differences were observed between the 2 techniques ($p > 0.05$).

The reduction in epidermal thickness seen with BDO is a direct effect of the corticosteroid. This class of drug exerts an anti-mitotic and anti-inflammatory effect through its action on DNA synthesis (Valencia and Kerdel 2003). As described previously, the phenotypic changes observed in the skin of DfL compared to wild-type mouse can possibly be attributed to increased infiltration of immune cells. Therefore the reduction in thickness can either be due to direct anti-proliferative effects of BD towards keratinocyte growth or the inhibition of immune cells which contributed to the increased growth in the first place.

The inverse effect seen with FO treatment is more difficult to explain. It was initially hypothesised that the anti-inflammatory effect possessed by FO would result in a similar outcome to that of the corticosteroid. This would imply that the hyperproliferation in DfL is not immune driven, or due to an unknown effect of fish oil or its constituents on the epidermis. In an earlier

study using a guinea pig model of epidermal hyperproliferation, a similar finding was reported in which both topical application and dietary supplementation with fish oil (or components of) resulted in acanthosis of the epidermis (Chapkin et al. 1987; Mani et al. 1999). This was attributed to the effects of DHA in reducing the epidermal level of 13-hydroxyoctadecadienoic acid (13-HODE) (Mani et al. 1999) derived from linoleic acid, an n-6 fatty acid found in abundance in normal skin (Miller and Ziboh 1990). In normal skin, the level of 13-HODE is high, and the reduction seen in the animal models, corresponding with the increased epidermal thickness, suggested an important role for 13-HODE in maintaining a normal epidermis. The exact mechanism, however, is still poorly understood. It is known that n-3 fatty acids, when present in sufficient amount, will displace n-6 FAs from cellular membranes, and perhaps the changes observed with DfL reflected the same condition seen with the guinea pig model.

From one of the studies, Chapkin et al.(1987) also came to the conclusion that although FO-derived fatty acids possess an inhibitive effect on the formation of pro-inflammatory cytokines, they are unable to reverse epidermal hyperproliferation in the animal model, as the FAs lack EFA functional properties in skin.

The relatively unchanged epidermal thickness seen in the combined BD+FO treatment group suggested an opposing effect of the 2 principal constituents. It was initially assumed that FO would enhance the activity and delivery of BD, thus would result in a greater reduction in epidermal thickness. The delivery enhancing property of fish oil towards other compounds has been documented previously (Thomas et al. 2007b), attributed to what has been termed the 'pull effect' (Heard et al. 2006). This only serves to strengthen the notion that in vitro results may not necessarily be translated well in vivo.

12.4 Conclusion

This study marks the first time that the *in vivo* effects of commonly used topical formulations and fish oil have been studied on DfL mouse. Although the findings were not as predicted, and the fact that a lot remains unknown with this particular animal model, it only warrants further investigation into the mechanism by which the phenotypic changes occur. It will also be interesting to see the effects of other classes of topical anti-psoriatics on DfL to further establish its potential as an animal model for psoriasis. Observations in this study also brings into focus the implication of topical fish oil application in psoriasis and/or other dermatoses, as it appears to contradict the reported benefits in patients receiving oral supplementation of the oil and the associated fatty acids.

On another aspect of the study, which is to evaluate the use of OCT in dermatological studies, it is encouraging to observe that it was successful as a means of obtaining continuous measurement of epidermal thickness, without the need of sacrificing test animals, compared to methods such as conventional H&E staining coupled with microscopy. In the long run, it is hoped that this will reduce the number of animals used in laboratories in line with the 3Rs principle of biomedical research (reduction, replacement, refinement) and possibly establish the use of OCT in clinical settings, particularly for dermatological studies.

Chapter 13:

In vitro response of defolliculated mice to topically applied fish oil and anti psoriatic agents II : Effects on cellular markers and components of the immune system

13.1 Introduction

In the previous chapter, the effects of fish oil and anti psoriatic agents on epidermal thickness were investigated. It was found that topical treatment with the agents revealed significant changes in the thickness of the epidermis over the whole duration of the study. In this chapter the investigations focused on finding a link between the earlier findings with markers of cellular proliferation, and also the protein and cells of the immune system.

Ki67 is an antigen produced by the gene MKI67 which is found to be expressed in the active phases of the cell cycle, namely the G₁, G₂ and S phase in all cells. It is totally absent during the resting (G₀) phase (Jörn et al. 2006). This strict association makes Ki67 a useful marker for cell growth and division (Mirko et al. 2002), and has been extremely useful in the detection of tumours and other forms of malignancies (Scholzen and Gerdes 2000). In normal epidermis, it is found to be weakly expressed in the basal and suprabasal layers, while in psoriatic epidermis distribution of Ki67 were spread throughout all the layers of the epidermis (Tao et al. 2008).

K17 is one the numerous epithelial-associated keratins, which are further classified as Type I and Type II keratins. K17, along with other Type I keratins are acidic in nature, while Type II keratins such as K1-K5 are basic to neutral (Moll et al. 2008). In normal conditions, K17 is only expressed in the nail bed, hair follicles, sebaceous glands, internal epithelia and developing interfollicular epidermis (Bonnekoh et al. 1995). However, in psoriatic lesions, there is an increase of suprabasal K17 expression concurrent with overexpression of K16 (Leigh et al. 1995). The increase in K17 is attributed to the rise seen in the levels of IFN- γ in psoriatic skin, both of which are believed to be contributing factors to the development of psoriasis (Bonnekoh et al. 1995).

Macrophage-1 antigen (MAC-1, also known as CR3; integrin alphaMbeta2, ITGAM) is a complement receptor consisting of CD11b and CD18. It has an affinity towards complements C3b and C4b. MAC-1 is expressed on the cells of the innate immune system, such as macrophages, monocytes, neutrophils and natural killer (NK) cells (Ross and Vetvicka 1993). Identification of C3b and C4b on foreign cells facilitates phagocytosis and the subsequent destruction of the foreign cells by the MAC-1 positive cells. As widely agreed, there is an accumulation of inflammatory/immune cells in both the dermal and epidermal layers of the psoriatic skin. These include lymphocytes and also cells of the innate immune system, such as neutrophils (Menter et al. 2004).

With regards to the DfL mouse, it was found that acanthosis and hyperkeratosis of the epidermis can be detected as early as 1 week of age, and the associated increase in Ki67 expression. Other hyperproliferation markers, such as keratins 6, 16, and 17, which are normally expressed only in the hair follicle, were found to be induced in the epidermis. This indicates an altered state of differentiation typical with thickened epidermis (Porter et al. 2002). Throughout all stages, from morphogenesis to the different stages of hair follicles destruction, an increase in macrophage infiltration has been confirmed in DfL mouse (Ruge and Porter Unpublished data).

13.2 Materials and methods

13.2.1 Materials

Tissue-Tek® O.C.T™ Compound (OCTC) was obtained from Sakura Finetek Europe B.V. Antibody towards Ki67 clone MM1 (lot 111849) was obtained from Novocastra Laboratories Ltd. (Newcastle, UK). Alexa Flour 488 goat anti-mouse IgG (lot 412441) and 4',6-diamidino-2-phenylindole (DAPI) were from

Invitrogen (Paisley, UK).Hydromount aqueous non-fluorescing mounting medium (lot 030805) was from National Diagnostics (Atlanta, US). Vectastain™ avidin-biotin complex (ABC) reagent (lot V0310) was from Vector Labs, Inc., California. Donkey (D9663) and goat serum (G9023) were from Sigma Aldrich. COX-2 and MAC-1 CD11b Rat anti-mouse antibody (lot 98414) were from Cell Signalling and BD Bioscience Pharmingen, respectively. Biotinylated anti-rat (lot 345191) and anti-rabbit (lot 372217) antibody were both from GE Healthcare, Buckinghamshire and K17 antibody was produced in-house. All other reagents were of analytical grade or equivalent.

13.2.2 *Methods*

13.2.2.1 *Immunofluorescence staining – Ki67*

Frozen OCTC embedded tissue sections (from Section 12.2.4) were cut to a thickness of 7 µm using a Shandon Cryotome and mounted on pre-cleaned microscope slides and left for 1 hour before being stored at -80°C until required. The slides, after being taken out of storage, were fixed with freshly thawed 4% paraformaldehyde for 15 minutes, followed by rinsing in PBS for 5 minutes. The skin sections were then circled with wax pen and blocked with 5% w/v BSA in PBS for 30 minutes. The slides were then placed in a humidified chamber and incubated with primary antibody for Ki67 (diluted 1:50 in PBS with 5% w/v BSA) at 4 °C overnight.

The following day, the slides were washed 3x with PBS/0.1% v/v Triton X100. Subsequently, the slides were incubated with anti-mouse Alexa 488 IgG diluted 1:500 in PBS for 30 minutes. After washing with PBS/0.1% Triton X100, DAPI at a concentration of 1 in 5000 (in PBS) was applied to the sections, and immediately tapped off before mounting in Hydromount™ and covered with glass cover slips. The slides were then left to dry for at least 1

hour under a silver foil covered lid to prevent fading. Imaging was done under a microscope (Nikon) set at 20x magnification aided by a UV lamp and image capture facilities (Carl Zeiss). The presence of Ki67 was identified by green nuclei, while negative cells were stained blue. The ratio of dividing cells was obtained by counting the number of positive nuclei over a distance of 700 basal cells. A higher ratio indicates an increase in Ki67, i.e. an increase in cellular proliferation, and vice versa.

13.2.2.2 IHC staining – COX-2, K17, and MAC-1

Frozen slides were allowed to equilibrate to room temperature after removal from -80°C freezer and then fixed in acetone for 15 minutes. The slides were then left to air-dry for 15 minutes, followed by washing with 3 changes of PBS for 5 minutes. Tissue sections were outlined with a wax ring using Dako wax pen and incubated for 20 minutes with 5% normal serum according to the species the secondary antibodies were made of. For COX-2 and K17, it was donkey serum and goat serum for MAC-1. From this point onwards, the slides were placed in a humidity chamber to avoid desiccation.

Excess serum was then tapped off and primary antibody for COX-2 (1 in 50 dilution, K17 (1 in 500 dilution) and MAC-1 (1 in 100 dilution) applied onto the section. After 1 hour incubation at room temperature (overnight for COX-2), the slides were washed with 3 x 5 minutes PBS, followed by incubation with biotinylated 2° antibody (anti-rabbit for COX-2 and K17, anti-rat for MAC-1) at 1 :200 dilution (in PBS) for 30 minutes. Next, the slides were incubated with Vectastain™ avidin-biotin complex (ABC) reagent, again for 30 minutes. After washing with PBS, development of slides with DAB was done for 10 minutes, and the slides dehydrated and finally mounted with DPX and left to dry overnight.

The slides were then observed under a microscope equipped with image capture facilities. The presence of COX-2, K17 and MAC-1 were determined by a reddish-brown stain.

13.3 Results and discussion

13.3.1 COX-2

IHC for COX-2 (Figure 13.1) revealed no appreciable difference in COX-2 staining, taking into account the change in thickness observed with the different treatment. It is suggested that COX-2 does not play a significant role in the development of the phenotypic changes seen in DfL. Unpublished data indicates that levels of COX-2 and COX-2-mediated AA derivatives were insignificant (Thomas and Porter Unpublished data).

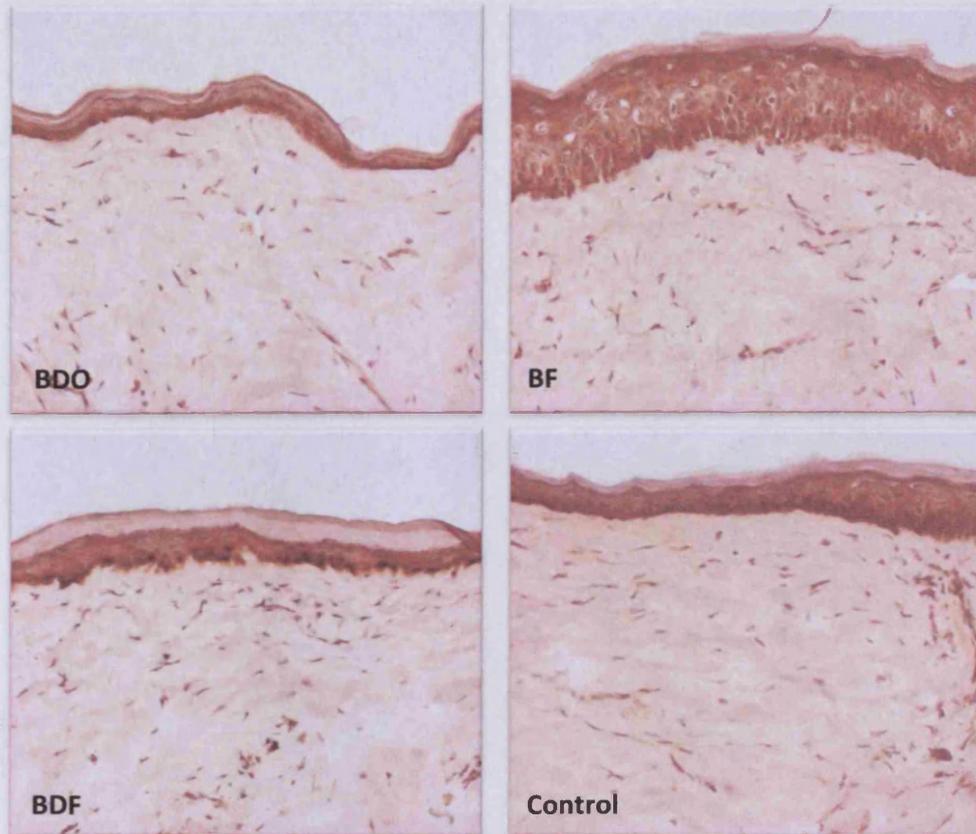


Figure 13.1 : IHC staining for COX-2 at 20x magnification. Clockwise from top left : BDO (BD only), BF (fish oil only), Control (blank ointment base), and BDF (combined BD and FO)

13.3.2 K17

The expression of K17, as indicated in Figure 13.2, mirrored the changes seen in the epidermal thickness with the different treatments. The staining was very intense in BF, corresponding to the gross increase in the thickness of the epidermis. On the other hand, the reduction in K17 staining with BDO (compared to control) supported the thinning seen earlier. As before, the relative levels of K17 for both BDF and control did not differ significantly.

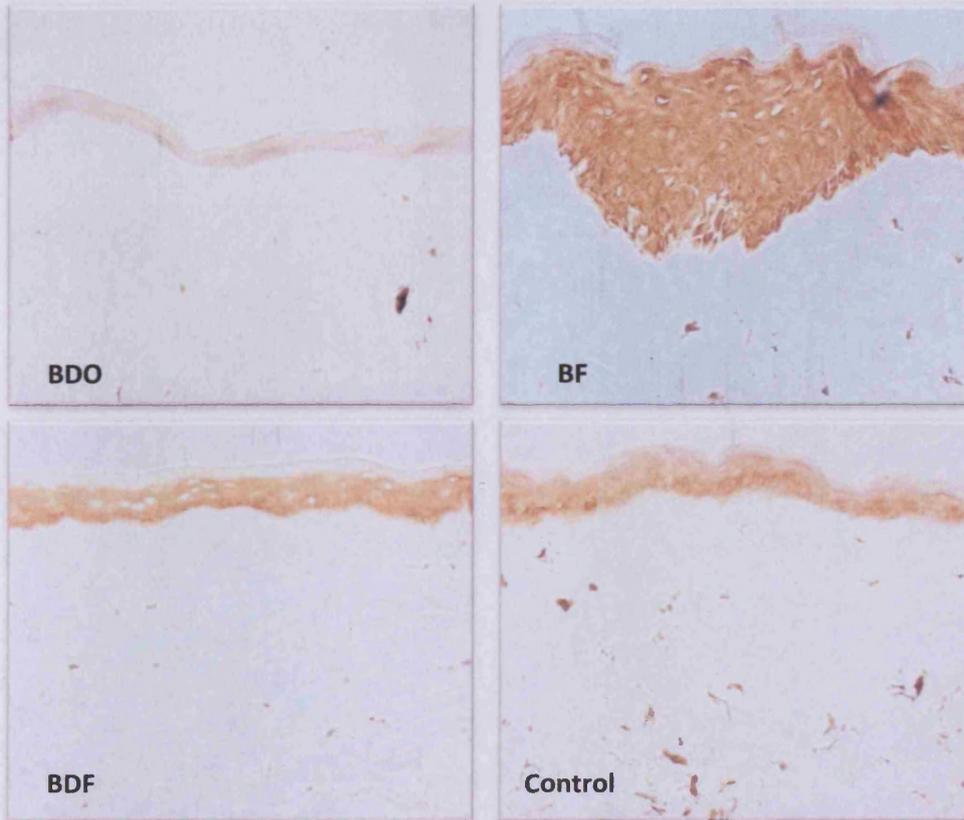


Figure 13.2 : IHC staining for K17 at 20x magnification. Clockwise from top left : BDO (BD only), BF (fish oil only), Control (blank ointment base), and BDF (combined BD and FO)

13.3.3 *Ki67*

Visual determination of positive nuclei from the images shown in Figure 13.3 showed an increased presence of Ki67 in the sections treated with FO, again reflecting the increased thickness of the epidermis. The antigen was weakly expressed in the basal layers of the BDO treated sections, and in BDF the expression was comparable to that of the control. Quantitatively, the ratios of Ki67 positive cells over total basal cells were 0.156, 0.703, 0.2063 and 0.226 for BDO, BFO, BDF and control (Figure 13.4). Both BDO and BFO ratios showed a statistically significant difference compared to control, with p

0.0415 and 0.0001, respectively. The ratio for BDF was not significant compared to control, with p 0.5732.

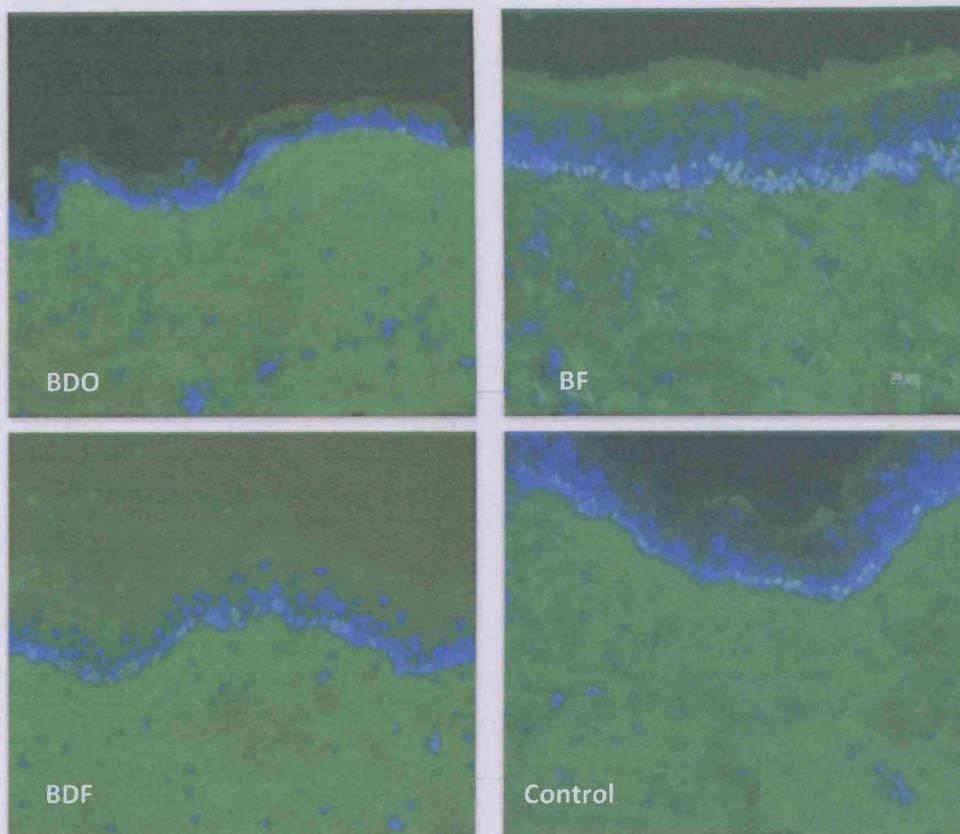


Figure 13.3 : Immunofluorescent staining for Ki67 at 20x magnification. Clockwise from top left : BDO (BD only), BF (fish oil only), Control (blank ointment base), and BDF (combined BD and FO). Nuclei positive for Ki67 are shown in bright green, while negative cells are in blue. In all cases Ki67 was only found to be expressed at the basal layer of the epidermis.

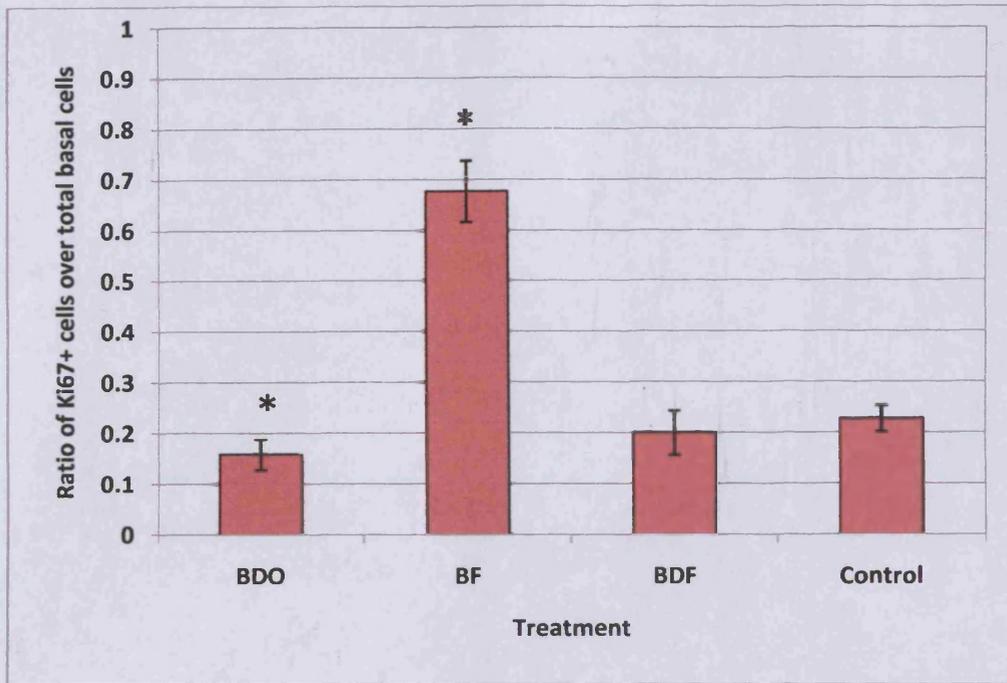


Figure 13.4 : Ratio of Ki67 positive cells per 100 epidermal basal cells. $* = p < 0.05$. The increase in Ki67 ratio with BF treatment corresponded with increase in epidermal thicknesses of the mice within the group. The opposite was seen with BDO treatment.

13.3.4 MAC-1

The IHC staining for MAC-1 is shown in Figure 13.5. In the control sections, the MAC-1 positive immune cells were found to be localized throughout the dermis, though particularly concentrated close to the epidermis and at the bottom of the dermis. This support the findings by Porter et al. (2002) which reported increased infiltration of DfL dermis with immune cells. Treatment with BD, a potent corticosteroid resulted in decreased presence of MAC-1 positive cells, especially near to the epidermis. A small amount of MAC-1 remained, though confined to the lower parts of the dermis.

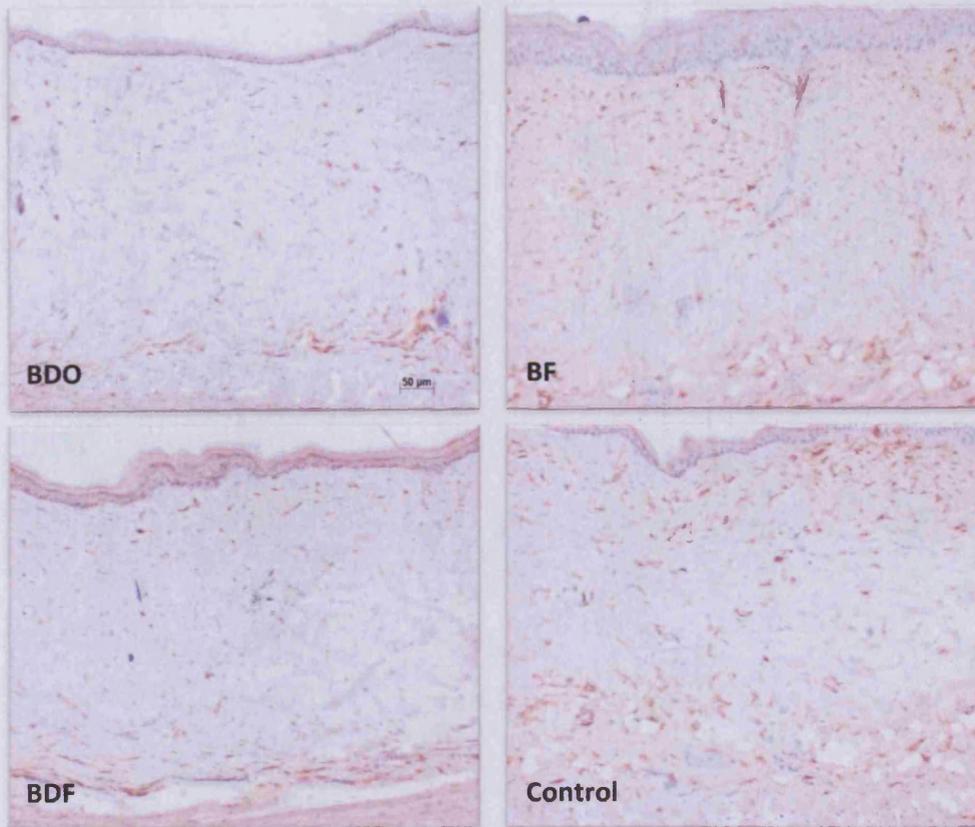


Figure 13.5 : IHC staining for MAC-1 antigen at 10x magnification. Clockwise from top left : BDO (BD only), BF (fish oil only), Control (blank ointment base), and BDF (combined BD and FO). The presence of MAC-1 was confirmed with the brown staining seen in the sections. Note the intense staining seen with BF and Control, and the opposite with the BD-containing treatment group (BDO and BDF)

The same was also observed with BDF, the other formulation which contained BD. The treatment with FO, however, did not reduce MAC-1 as expected. In fact, it was arguably increased compared to control. This was in contrast to the anti-inflammatory activity seen in previous chapters, although it might explain the increased epidermal growth seen with FO application. The anti-inflammatory activity of the principal components of the fish oil, the n-3 fatty acids, has been long supported and extensively documented. Thus, the result from this study was puzzling, to say the least. One possible explanation is the immune cells were reacting to other components of the oil,

or perhaps an allergic response to the fish oil. Sensitivity towards topical application of fish oil has been reported in clinical trials with psoriatic patients (Escobar et al. 1992), therefore this possibility cannot be discounted.

13.4 Conclusion

Evidence gathered from immunostaining of the different proteins and markers drew several conclusions. First, the relatively unchanged levels of COX-2 expression in all treatment groups suggest a non-involvement of COX-2 in the aetiology of the DfL phenotype. Though COX-2 is a major component in the inflammatory cascade, and there is evidence to suggest that the destruction of hair follicles and the epidermal changes are immune-driven, other mediators may play a larger role in this particular mutation. This will also explain why, in light of the inhibitory effect on COX-2 and its product by fish oil, it did not appear to reverse the epidermal thickening; in fact, the opposite was seen.

This leads to the second conclusion. Increased expression of Ki67 and K17, both markers associated with hyperproliferation, corresponded with the epidermal thickening seen with fish oil treatment. Although the exact cause is unknown, the increased presence of MAC-1 positive cells could explain, in part, why the epidermal changes were in great contrast to initial assumptions.

Furthermore, Porter et al. (1998), in their work with a mouse model for bullous congenital ichthyosiform erythroderma (BICE), which also exhibited epidermal thickening, acanthosis and focal keratosis much like DfL, concluded that hyperproliferation of keratinocytes was only partly responsible. Other mechanisms are likely to be involved, such as decreased desquamation.

Chapter 14:

General discussions

14.1 General discussion

There is an increasing body of evidence supporting the numerous therapeutic benefits of fish oil, encompassing a wide range of conditions, from skin diseases and cardiovascular diseases, to several types of malignancies. Although these benefits are well documented, little published work has involved the topical delivery of fish oil. Apart from several documented clinical trials and investigative work, the majority of the body of work has focused on orally and systemically delivered fish oil. Previous work has also established the skin penetration enhancing properties of fish oil and its major constituent fatty acids, especially EPA on the delivery of topically applied therapeutic agents. The objective of this thesis was therefore to investigate both the activity and the topical delivery of fish oil, with a view towards incorporation in therapeutic regimens for psoriasis, or co-formulated with other active agents.

In the beginning of the study, the delivery of EPA from commercially available fish oil was investigated, along with several bioactive oils containing bioactive long chain fatty acids and metabolite products. It was found that the delivery of fatty acids were indeed possible from fish oil across in vitro models of skin permeation. While this generally supported earlier work involving fish oil, it was also contradictory to established topical drug delivery theory. The following chapters were then focused on the delivery of several commonly used anti-psoriatic medications, with a view to incorporating fish oil together with these agents in a single formulation, providing a multiple action formulation. In the first instance, the delivery of ASA from a fish oil vehicle was attempted. Owing to the anti-inflammatory action of ASA, the desmosomolytic capability of its metabolite SA and the benefits of fish oil, both in its intrinsic anti-inflammatory and delivery enhancing properties, it

was hypothesised that this would lead to a multi-action preparation beneficial in the treatment of psoriasis.

The delivery of ASA, SA and EPA were indeed successful; however, contrary to the initial hypothesis, the delivery of EPA was retarded in the presence of ASA/SA, and the amount permeated was only half of that delivered from fish oil alone.

Nevertheless, subsequent work in Chapters 5 and 6, involving a combined BD and SA formulation (intended to replicate a commercially available preparation), with the addition of FO, was more successful. Increased deposition of BD was observed in the lower layers of the epidermis, a very important site of action for inflammatory skin diseases, in the presence of fish oil. This enhancement was observed in both non-viable and viable porcine skin membranes, and the skin of a potential new animal model for psoriasis, the DfL mouse. Reflective of the work in Chapter 2, the delivery of SA was not enhanced in all cases, suggesting that the enhancement of EPA depended on the chemical structure of the intended co-drug.

In Chapter 5, the impact of skin viability on drug permeation was also investigated. The findings indicated that it is an important factor to be considered in the design of experimental work in topical and transdermal studies, particularly with compounds that are metabolized in the skin. The amount of BD and SA recovered from viable skin sections using adhesive tape stripping were reduced by approximately 80% due to a more intact barrier and active metabolizing enzymes.

As the first part in the studies aimed at establishing the DfL mouse as a model for psoriasis, the delivery properties of the same agents were investigated with excised DfL skin sections in Chapter 6. The overall reduced thickness and the barrier defects of the DfL skin led to a higher permeability compared to porcine skin, with a 10-fold increase in the amount of EPA permeating across

the skin, while the permeation of BD was also increased, although to a lesser extent. The permeation of SA, yet again, was not affected by the presence of fish oil, with the fluxes obtained not statistically different between SA+FO, SA+BD+FO and SA alone. These two factors (thickness and impaired barrier) may have been a contributing factor in the outcome of the adhesive tape stripping work, with all three agents (BD, SA and EPA) not showing any statistically significant increase ($p > 0.05$) in the deposition to the lower epidermis.

To summarise, the earlier chapters (2 to 6) reported on the delivery properties of the anti-psoriatic medication and the effects of fish oil on enhancing drug delivery. The following chapters (7-9) then focused on investigating the anti-inflammatory properties of fish oil. Utilizing IHC methods and WB assays with experimental model of skin inflammation, the effects of fish oil on the inflammatory enzymes COX-2 and 5-LOX were investigated. Both methods provided conclusive proof of the intrinsic anti-inflammatory activity of fish oil, as shown by the inhibition of the expression of said enzymes even by fish oil alone. The combination of fish oil with the anti-psoriatic agents, however, did not translate to the anticipated increased activity, possibly attributable to saturation of inhibition pathways. In Chapter 9, the inhibitory action of fish oil on the major product of COX-2 metabolism, PGE₂, was demonstrated. Reflecting the IHC and WB results, the EIA analysis of PGE₂ showed greater activity of BD and FO on their own, while the combined formulation failed to support the initial hypothesis of increased inhibition. The implication of these chapters is crucial, in view of the role played by products of the enzyme in the pathogenesis and progression of inflammatory skin diseases, such as psoriasis. The findings showed that topical delivery of fish oil was indeed feasible, and exhibited the anti-inflammatory activity reported with other forms of administration.

The response seen in whole skin may possibly be affected by the different types of cell that constitute the tissue. Therefore, in Chapters 9 and 10 the effects of FO and anti-psoriatic medications were studied in the primary cells of the skin, keratinocytes, in the form of the immortalised cell HaCaT cell line grown in culture. The addition of fish oil led to an approximately 30% increase in the anti-proliferative effect of BD. As there was no associated delivery issue (other than across the cell membrane), this enhancement was attributed directly to the properties of fish oil in arresting cellular growth. This fact has indeed been demonstrated by previous researchers, particularly in tumour cell lines and also keratinocytes. Perhaps as a contributing mechanism for growth arrest, the expression of the apoptotic marker cleaved caspase-3 was induced by fish oil in the culture, and it was more pronounced in combination with BD. The effect of fish oil on COX-2 expression was again demonstrated, this time at the cellular level. Inhibition of COX-2 expression was achieved by fish oil alone, comparable to the inhibition by BD.

In summary, the anti-inflammatory activity of fish oil was proven at both tissue and cellular level, with the activity encompassing several components of the inflammatory process. This was indicated by the inhibition of both inflammatory enzymes in the epidermis, COX-2 and 5-LOX, and one of the major products and an important pro-inflammatory eicosanoids, PGE₂. The anti-psoriatic spectrum of fish oil activity also has been expanded to include its growth inhibition and induction of pro-apoptotic pathway in HaCaT cell line.

In the final part of the study, the main thrust was to attempt to replicate the above promising results in an *in vivo* situation. At the same time, the potential of the DfL mouse mutant presenting with skin defects, much akin to psoriasis, was investigated with a view to establishing a new murine model for the disease. Furthermore, the potential value of OCT technology in 'real time' skin modulation was assessed. These studies were successful on the

basis that the animal model responded to treatment using a common anti-psoriatic agent. At this stage, only two anti-psoriatic agents (BD and SA) were tested; therefore, the findings will hopefully support a larger role for the DfL mouse in the screening of new therapeutic compounds, and no less important, to better understand the pathogenesis and the mechanisms involved in the development of inflammatory skin diseases.

On the other hand, a major surprise was the observation that treatment with fish oil resulted in the opposite of the initial hypothesis of reversing the epidermal thickness seen in DfL owing to the anti-inflammatory properties showed by FO in the previous chapters. It was, however, no less interesting, as the findings only served to generate more interest in the role of fatty acids, particularly within the skin. It also served as proof that *in vitro* findings may not always be successfully replicated *in vivo*. Furthermore, there is still much to be understood about the animal model itself. As mentioned in the chapter, this represented the first time a study of anti-psoriatic medication was attempted on DfL; therefore, it is hoped that the findings will provide a basis for further work. Furthermore, OCT technology was successfully utilized in the study, providing a method to measure epidermal thickness which was less stressful compared to strenuous methods such as punch biopsy and more precise than the use of conventional measuring instruments, e.g. callipers. This would allow a reduction in the number of animals for further studies and allows refinement of previously used techniques, in line with the 3Rs (reduction, replacement, refinement) principle of biomedical research (Goldberg et al. 1996)

14.2 Future work

The potential value of the topical delivery of fish oil has been demonstrated in this study and, in tandem with anti-psoriatic medications, has been proven to potentiate their effects by possessing delivery enhancement and intrinsic anti-inflammatory activity of its own. A refinement in the formulation aspect of combining these agents is thus an important aspect that warrants further investigations. Other aspects that can be considered, including optimizing the amount of fish oil to be incorporated, is whether the same effects can be replicated or bettered with the use of the individual fatty acids. The exact mechanism of inhibition of inflammatory enzymes and their products, and other anti-psoriatic/anti-inflammatory properties of fish oil, can be elucidated further and perhaps investigated on a molecular level. An expansion to the animal study is also recommended, both to better understand the mutation itself, and in establishing DfL as a screen for therapeutic agents and understanding of inflammatory skin diseases. Investigation into the lipid content of DfL skin, particularly the different ceramides in the SC might shed light on the similarity in the barrier defect seen in DfL with human skin diseases. The successful use of OCT is also hoped to generate interest and for incorporation of this technology for future dermatological studies, and perhaps other fields of research as well.

14.3 Concluding remarks

The hypothesis of this study, which was to establish the potential and benefits of incorporating fish oil in the therapeutic regimen of psoriasis and/or other inflammatory skin disorders, has been achieved. Through the associated aims of the study, the delivery enhancing properties and the anti-inflammatory/anti-psoriatic action of fish oil has been documented

successfully. This study provides strong support for an expansion in the role of fish oil and other naturally occurring products in the treatment of disease.

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