TISSUE ENGINEERED HUMAN SKIN MODELS TO STUDY THE EFFECT OF INFLAMMATION ON MELANOMA INVASION

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DEDICATION

I entirely dedicate this work to my husband Jefferson and to my daughter Emily for always being with me. Thank you very much for everything. I love you both.

Also dedicate it to my mum Dirce and my dad Benedicto (*in memoriam*) for their unconditional love and support.

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Increasing evidence suggests inflammation is associated with progression of a range of cancers. The phenomenon of local recurrence after surgical excision of melanoma also merits investigation – post-excision there is both wounding and inflammation. Following on from this the overall aim of this work was to investigate whether anti-inflammatories could help reduce melanoma cell migration, invasion and survival *in vitro*.

The first objective of this work was to investigate the effects of NSAIDS (ibuprofen released from a hydrogel or capsaicin) in reducing melanoma cell migration. The second objective was to investigate the role of NSAIDS in reducing melanoma invasion in a 3D wounded and inflamed tissue engineered skin model. This was designed to imitate the physiological conditions of an inflamed wound bed post-excision of primary melanoma. The third objective was to investigate the effects of a combined therapy of capsaicin and HA14-1 in inducing apoptosis in melanoma cells and normal skin cells as an alternative treatment for malignant melanoma.

Three melanoma cell lines of increasing metastatic potential were used - HBL, A375SM and C8161. Both ibuprofen and capsaicin reduced melanoma cell migration and opposed the inflammatory effects of the major pro-inflammatory cytokine TNF-α in stimulating cell migration.

A 3D skin model which was wounded and subjected to an inflammatory environment was established. Investigation of invasion of A375SM cells in this model showed that the addition of ibuprofen released from a hydrogel reduced melanoma cell invasion. Fibroblasts were also found to retard invasion in 2 out of 3 melanoma cells in this model.

Finally, a combined therapy of capsaicin and HA14-1, an apoptotic inducer, was shown to be a promising alternative in inducing apoptosis in all three tumours. Effects of the two agents were additive for HBL and A375SM cells and synergistic for the C8161 cells.

In summary this data suggests both NSAIDS and NSAIDS combined with a pro-apoptotic inducer could be useful approaches for reducing melanoma metastases and inducing apoptosis for patients suffering from primary and metastatic melanoma.

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PUBLICATIONS

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ABSTRACTS

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ABBREVIATIONS

AIF	apoptosis-inducing factor
ALI	air-liquid interface
ATP	adenosine triphosphate
BAK	BCL2L4
BAX	BCL2-associated X protein
BM	basement membrane
CAD	caspase-activated DNase
Campth	camptothecin
C5a	Complement 5a
COX1	cyclooxygenase enzymes - 1
COX2	cyclooxygenase enzymes - 2
DNA	deoxyribonucleic acid
DED	de-epidermised dermis
DMEM	Dulbecco's minimum essential medium
EMEM	Eagle`s modified essential medium
FCS	fetal calf serum
FCM	fibroblasts culture medium
HIF-α	hypoxia inducible factor-alpha
HRE	hypoxia response element
HA14-1	2-Amino-6-bromo-α-cyano-3-(ethoxycarbonyl)-4*H*-1-benzopyran-4-acetic acid ethyl ester
ICAD	inhibitor of caspase activated DNases
ICAM-1	intercellular adhesion molecule-1
ICE	inlerleukin converting enzyme
ILs	interleukins
KCM	keratinocyte culture medium
LPS	lipopolysaccharide
MTT	[3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide]
NADH	nicotinamide adenine dinucleotide

Nalbu	sodium ibuprofen
NBCS	newborn calf serum
ns	not significant
NSAIDs	non-steroidal anti-inflammatory drugs
NF-*k*B	nuclear factor kappa beta
PBS	phosphate-buffered saline
PS	phosphatidylserine
ROS	reactive oxygen species
SAIDs	steroidal anti-inflammatory drugs
Smac/DIABLO	second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI
STSGs	split-thickness skin grafts
TNF	tumour necrosis factor
UV	ultra violet
UVA	ultra-violet - longer wavelength
UVB	ultra-violet - medium wavelength
VEGF	vascular endothelial cell growth

CONTENTS

FIGURE CONTENTS

TABLE CONTENTS

CHAPTER 1

INTRODUCTION

1.1. CANCER – ORIGIN AND HISTORY

Continuous advances in science and technology, and developments in clinical and public health are leading to improvements in the quality of life and longevity. Many diseases have been found to be curable or controlled very well by treatments. However, there are still some diseases which are not treatable or are age-related degenerative diseases such as heart disease and cancer. Cancer is a disease of complex aetiology and there is still much to be studied and understood.

In this study the impact of inflammation on melanoma migration, invasion and survival was studied in a simple 2D model and in an engineered human skin model. Therefore, to start with, some background to the origin and history of cancer is described.

Cancer can be found in historical Roman documents by Aulus Cornelius Celsus (25BC-50) who translated the word *carcinos* into the Latin word *cancer*, meaning crab (Broxmeyer, 2004), as he described a solid mass which could resemble a crab-like form having the main body and the roots of cancer invading into other organs and tissues. Nowadays the word cancer can be understood as a degenerative disease in which a normal cell suffers many consecutive alterations in its DNA (Deoxyribonucleic Acid) and the cell itself loses its capability of being a "normal" and become a variant form. This can result in a cell which is highly proliferative, invasive and has an endless cell division cycle.

Cancer cells can be capable of invading blood and/or lymphatic vessels and implanting in other tissues/organs distant from their original location; this process is known as metastasis. There are many types of cancer and it can affect people at any age however, it tends to be more common with increasing age as the probability of DNA alteration and damage is higher.

Cancer can also be associated with genetic (inherited) factors (Norman and Chin, 2006), family history or immune surveillance failure as well as environmental factors such as long-term exposure to carcinogens such as radiation, alcohol, tobacco and viruses.

Neoplasia is the accurate term for defining abnormal cell growth or tumour formation. Basically there are two forms of tumours: 1) Benign tumours which demonstrate uncontrolled cell growth but maintain a similar phenotype to the original source of cells. Generally benign cancers are encapsulated and surrounded by a normal fibrous tissue which is induced by compression as the tumour expands. This capsule may prevent tumour cells leaking or spreading. The capsule also demarcates the tumour from normal tissue, making removal by surgical procedures feasable. A benign cancer may however transform into a malignant form of cancer. 2) Tumours are also characterised by an uncontrolled cell proliferation and growth, however, unlike benign tumours, these cells are able to invade and produce a metastasis. This infiltrating property of tumour cells can cause normal tissue destruction. As metastases occur, tumour removal only proceeds at sites where surgical resection is possible to remove the neoplastic cells with a wide margin of normal tissue. The presence of metastases distinguishes malignant tumours from benign tumours.

Tumours may be discovered as a swelling or lump associated with neoplasia or inflammation. However, not all forms of cancer appear as a solid tumour as there can be cases of circulating immune cells which give a range of forms of leukemia.

Cancer was not really treated until the improvement of microscopy and the realisation that the human body is made of living cells, making it possible to better understand the disease. At that time only rudimentary forms of treatments were made with poor outcomes from surgical procedures. Fundamental advances in the treatment of cancer only appeared at the end of the 19^{th} century when Marie Curie and Pierre

Curie implemented radiation as the first non-surgical treatment for cancer (Goodman *et al.*, 1984). Nowadays, radiotherapy and chemotherapy are commonly used in cancer patients to improve survival rates. Nevertheless much more investigation and research work is still needed to improve the pharmacological agents used to hopefully target the malignant cells without compromising healthy living tissue. The use of many anti-cancer drugs and treatments is still limited and associated with many unwanted side effects.

A significant contribution to understanding the causes of cancer occurred in 1953 when Francis Crick and James Watson unravelled the structure of the DNA. Since then many studies have been able to investigate the causes of cancer at a cellular and molecular level and hence better understand the fundamentals of the disease. In addition to this, the knowledge of the biochemical structure of the cell plasma membrane and cell physiology made it possible to get a closer look at the development of the disease and devise better treatments.

This study focuses on melanoma skin cancer and therefore a review on human skin physiology is required before discussing melanoma as a cancer.

1.2. HUMAN SKIN STRUCTURE

Skin is the largest human organ. It covers an area of approximately 1.5 to 2 m^2, and represents about one sixth of total body weight. It is estimated that each square inch of skin has 4.6 m of blood vessels, 3.6 m of nerves, 350 sweat glands, 100 sebaceous glands and, 1500 nervous receptors (Marieb and Hoehn, 2007a).

Skin has many physiological functions, such as serving as a barrier to the environment; protection from water loss and light mechanical shock; production of specialized pigment cells to form a shield against ultraviolet rays of the sun; production

of vitamin D in the epidermal layer; regulation of body temperature through sweat glands and provision of anti-infective properties by sebaceous gland which also helps it to regulate body metabolism.

A diagram of human skin structure can be seen in Figure 1.1.

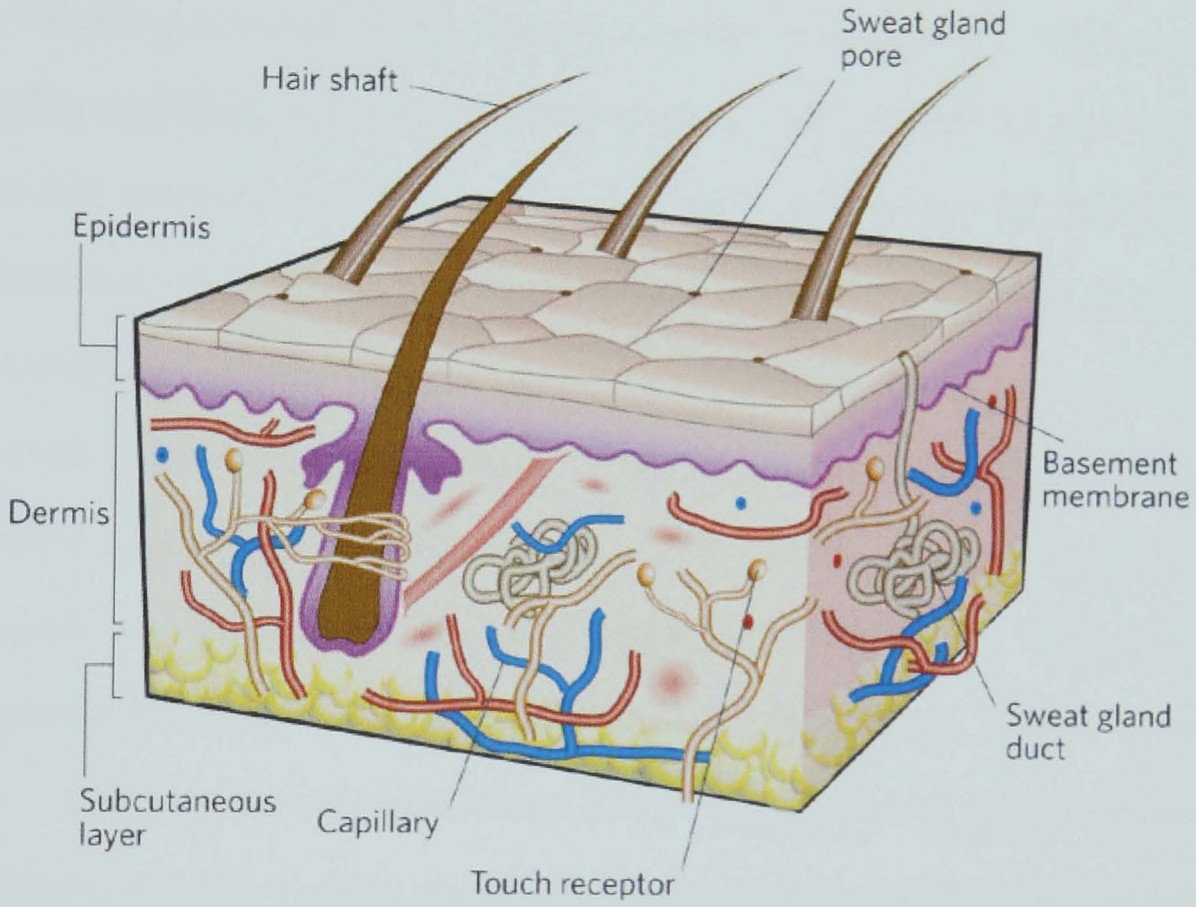


Figure 1.1. Structure of Human Skin (Shier *et al.*, 1999).

Skin is a heterogeneous structure consisting of various types of tissue and cells. The skin is organised in three overlapping layers: the epidermis, dermis and hypodermis (or subcutaneous fat tissue), (see Figure 1.1). Between the epidermis and the dermis there is a thin layer known as the basement membrane (BM). The BM is a structure of 2 layers of basal lamina, which consists of type IV collagen, glycoproteins and proteoglycans (Junqueira and Carneiro, 2005). The structural and molecular composition of the BM varies between tissues.

1.2.1. Epidermis

The epidermis survives through a self-renewal process. Clonogenic basal keratinocytes forming the innermost epidermal basal layer regularly undergo mitosis, differentiation and upward migration to replace terminally differentiated cornified cells that are continuously shed into the environment. As a result, human epidermis is completely renewed approximately every month. To accomplish this process and to face the emergency of wound healing, the epidermis relies on the presence of stem cells and transient amplifying cells which are located in the basal layer of the epidermis. The basic and essential characteristics of a stem cell is its capacity for extensive self-maintenance with proliferative self-renewal potential extending for at least one life-span of the organism irrespective of whether the cell is multipotent (as for the haematopoietic stem cell) or unipotent (as for epidermal stem cells) in nature. Conversely, the transient amplifying cell population, which arises from stem cells and will eventually generate terminally differentiated cells, has a high proliferative rate for only a limited period of time before becoming committed to terminal differentiation (Harris, 1996).

There are some factors associated with the conversion from stem cell to transient amplifying cell. Some studies suggest that the transcription factor p63 (a homologue of p53) is known to be expressed in epithelial stem cells of skin keratinocytes in vitro (Pellegrini *et al.*, 2001) and the proto-oncogene c-myc, a transcriptional regulator of proliferation in a large variety of cell types, including skin keratinocytes (Wong *et al.*, 2008).

The epidermis is composed of five layers, from the BM to the surface: 1- germinative layer (*stratum basale*); 2- spinous or prickle-cell layer (*stratum spinosum*); 3- granular layer (*stratum granulosum*); 4- clear layer (*stratum lucidum*); and 5- horny layer (*stratum corneum*) (Marieb and Hoehn, 2007a; Junqueira and Carneiro, 2005). Figure 1.2 illustrates the stratification of the epidermis.

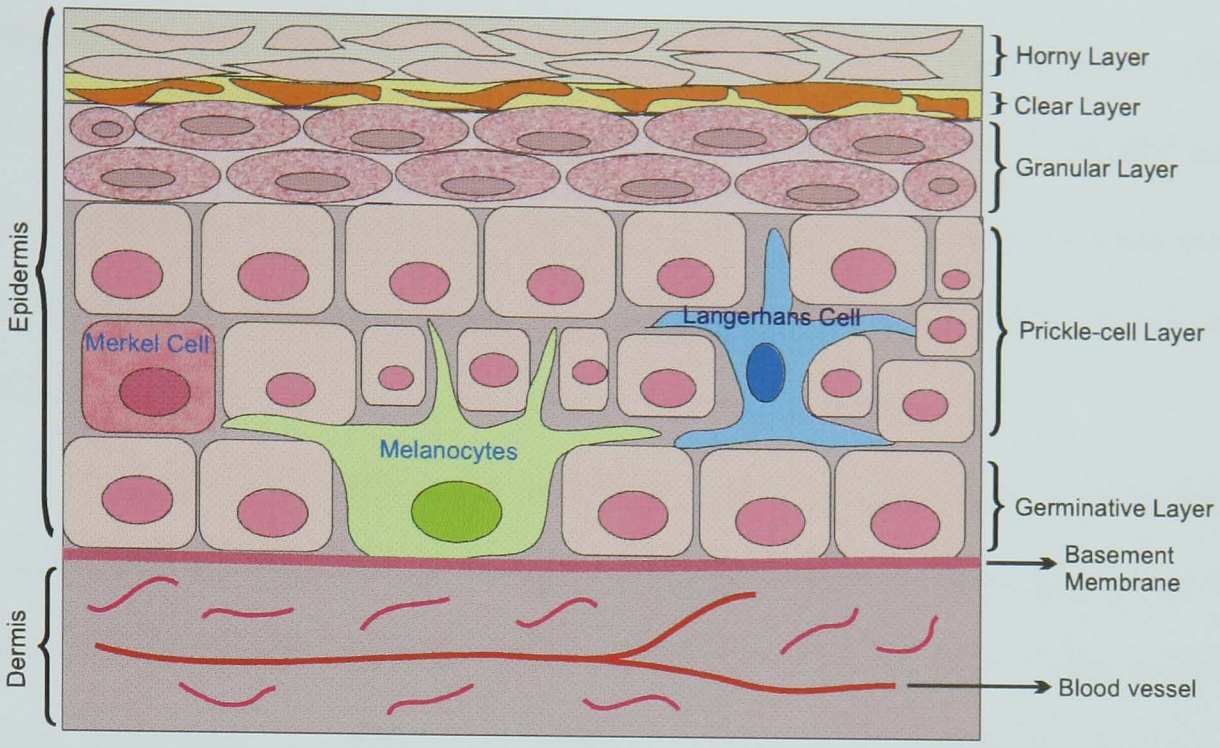


Figure 1.2. Cells of the epidermis.

The **germinative layer** is the lowest layer of the epidermis and lies on the BM. The BM separates the epidermis from the dermis. The keratinocytes are the predominant cell type.

When a basal epidermal cell undergoes a commitment to terminally differentiate, it ceases to divide and begins to migrate outwards towards the surface of the skin. Dramatic changes in its cytoskeletal architecture take place, accompanied by numerous changes in the expression of keratin and filaggrin (Smith and Dale, 1986). Keratins are a group of water-insoluble proteins (molecular weight range 40-70kD) that form 10-nm tonofilaments in a wide variety of epithelial cells. Monoclonal antibodies (AE1, AE2 and AE3) recognize all known keratin species. These keratins can be divided into two families according to their reactivity to AE1 and AE3 antibodies. Keratins of AE1 family are in general more acidic than those of the AE3 family. Keratin can be further divided, according to their molecular size into keratin classes: 50kD and 58kD keratins are present in all cell layers (which is a characteristic of all stratified squamous epithelia), whereas the 56.5kD and 65-67kD keratins are associated only with the more

differentiated cells above the basal layer (related to the keratinized epidermis) (Sun *et al.*, 1983; Woodcock-Mitchell *et al.*, 1982). Thus, the expression of specific keratin classes appeared to correlate with different types of epithelial differentiation (simple versus stratified; keratinized versus nonkeratinized) (Sun *et al.*, 1983; Tseng *et al.*, 1982).

As basal cells enter the spinous layer, they strengthen their cytoskeletal and intercellular connections. Once this task is completed the cells enter the granular layer, where they produce the epidermal barrier becoming corneocytes. The major constituents of the corneocytes are keratin intermediate filaments, which fill the inside of the corneocytes, and contribute to the mechanical stiffness of the stratum corneum architecture.

This makes up the epidermal barrier (localized primarily in the stratum corneum) which consists of protein-enriched cells (corneocytes with cornified envelope and cytoskeletal elements, as well as corneodesmosomes) and lipid-enriched intercellular domains. The nucleated cells in the epidermis have gap and adherens junctions, additional desmosomes and cytoskeletal elements which also contribute to the barrier. As keratinocytes differentiate, lipids are synthesized are extruded into the extracellular domains, where they form lipid-enriched extracellular layers. The cornified cell envelope, a robust protein/lipid polymer structure, is located below the plasma membrane of terminally differentiated keratinocytes (Hohl, 1990). Filaggrin, as mentioned before, is a cross-linked protein to the cornified envelope and aggregates keratin filaments into macrofibrils. Cytokines, cAMP and calcium influence the formation and maintenance of barrier function (Jensen and Proksch, 2009).

Involucrin plays a role in forming the cornified layer. It is a 66kD protein with a rod-like shape, which was the first cornified envelope precursor protein to be identified and it is localised in the outmost region of the cornified envelope (Vanhoutteghem *et al.*,

2008). It has extensive tandem repeated of specific amino acid sequence in which the glutamine/glutamine residues are used for extensive cross-links (Vanhoutteghem *et al.* 2008).

Filaggrin and trichohyalin are multifunctional proteins known to play an important role during cornification. Mutations in the genes encoding for filaggrin have been identified in patients with ichthyosis vulgaris and atopic dermatitis (such as eczema). Profilaggrin, which is processes to functional filaggrin monomers, and keratohyalin granules in the epidermis and hair follicle respectively (Markova *et al.*, 1993).

Filaggrin and trichohyalin also combine sequence repeats, similar to cornified envelope precursors, with two calcium binding EF-hand domains (a calcium-binding motif named after the E- and F-helices of parvalbumin), which are typical features of the S100 proteins. S100 proteins function as regulatory protein that are involved in different steps of the calcium signalling, following cell morphology, cell cycle and differentiation (Sun *et al.*, 2000). Calcium concentration increases as keratinocytes migrate to the stratum corneum and differentiate.

The human epidermis is renewed every 15-30 days, depending on many factors such as age and body location.

Around 10-25% of the cells in the germinative layer are melanocytes. Melanocytes are derived from neural crest cells. They appear as rounded-like cells with long irregular dendritic extensions into the epidermis reaching the cells of the germinative layer and prickle-cell layer. Melanocytes synthesise melanin granules (pigment) and transfer them into the cytoplasm of keratinocytes. Stimulation of melanin production by melanocytes is caused by increased exposure to UV (ultra-violet) radiation. This is an attempt to form a shield of protection in the supranuclear region of the cells against the deleterious effects of UV radiation, such as DNA damage. Longer

UV radiation exposure produces darkening or tanning of the skin caused by further synthesis of melanin transferred to keratinocytes.

Other cells found in the germinative layer are the Merkel's cells which are the neuroendocrine cells of the skin. Their highest density is within the epithelial mucosa, lips, palate, palms and fingertips. It is suggested that these cells are specialised in slow-adapting tactile mechanoreceptors.

In the **prickle-cell layer**, keratinocytes appear irregular (spiny) in shape, therefore they are called prickle cells. Keratinocytes are attached to one another by numerous intermediate intercellular filaments which span from their cytosol to attach to the desmosomes. This prevents the intercellular spaces from becoming markedly dilated and therefore helps to keep cells firmly attached one to another. During inflammation, these intercellular junctions may be damaged causing them to rupture and allowing the intercellular spaces to enlarge. Inflammatory cells and mediator factors often occupy these enlarged intercellular spaces. Other cells found in the prickle-cell layer are Langerhans cells which derive from precursors in the bone marrow. These cells have a star-like shape and are localised in the supra-basal epidermal layer among keratinocytes. Their function is to take up the antigens that reach the epidermis, process them and migrate to regional lymph node and present antigens to immunocompetent T cells. Therefore accordingly they appear to play a key role in cutaneous immunosurveillance.

The **granular layer** is a thin layer of three to five layers of keratinocytes with changes in appearance as they flatten and their organelles and nuclei disintegrate. These cells produce and accumulate two forms of granules: the keratohyaline granules which form keratin in the upper layers of epidermis and lamellated granules containing a waterproof glycolipid substance which is transferred to the extracellular space slowing down the water loss in the epidermis. Above the granular layer, cell nutrition is reduced

as these cells are far from the dermal capillaries. Accordingly the cells die producing clear horny layers.

The **clear layer** is so called as it is highly refractive. This layer is made of a few rows of clear flat dead keratinocytes. The cytoplasm is mostly made of keratin filaments. This layer of the epidermis is only seen in thick skin.

The **horny layer** is the uppermost layer of the epidermis. It is made of 20-30 flattened epithelial cells layers. This accounts for up to ¾ of the epithelial thickness. These layers are called keratinized layers because of the build-up of the protein keratin in these cells. This serves as a barrier against hostile exogenous factors and also prevents water loss.

Other specialised cell junctions to keep the epithelium impermeable are the *tight junction*s which form a seal between adjacent cells and also define the apical and basal sides of an epithelial cell. Just below the tight junctions are the *adhesion junctions* that hold cells in a fixed position and help to keep cells tightly united together in response to skin shear stress. Among adjacent cells are the *communications junctions* (or gap junctions) providing cell attachment as well as cell-to-cell communication of small molecules and signals. Therefore, keratinocytes work together to provide a barrier, keeping foreign and harmful substances out of the body, preventing water and other physiological substances from escaping the body as well as forming a network of communication between cells (Marieb and Hoehn, 2007a; Junqueira and Carneiro, 2005).

Changes in lipid composition and epidermal differentiation lead to a disturbed skin barrier, which allows the entry of environmental allergens, immunological reaction and inflammation in atopic dermatitis. A disturbed skin barrier is an important component in the pathogenesis of contact dermatitis, ichthyosis, psoriasis, and atopic dermatitis (Jensen and Proksch 2009).

Although the epidermis has a rich innervated network, its stratified layers of cells are non-vascular. Hence, epithelium metabolic exchange occurs by diffusion from the capillaries in the dermal papillae layer of connective tissue. The thickness of the epidermis varies between 0.04 to 1.6 mm.

1.2.2. Dermis

The dermis is an important tissue as it provides mechanical protection to the underlying body parts and serves as an interface and filling tissue to bind together all the superficial structures. The dermis is a specialised connective tissue made of cells, fibres and extracellular matrix. The cell types found in the dermis are typical of those found in any connective tissue proper such as fibroblasts, macrophages and occasional mast cells and white blood cells. This strong and flexible connective tissue layer is composed of a gel-like matrix heavily embedded with water collagen, elastin and reticular fibres. Collagen fibres and glycosaminoglycans (GAGs), can bind large amounts of water and collagen to determine the high intrinsic tension of skin. As proteins are synthesized in the rough endoplasmic reticulum, glycosaminoglycans bind to proteins in the Golgi Apparaus to form proteoglycans. Proteoglycans are exported in secretory vesicles to the extracellular matrix. Proteoglycans, collagen and water form a gel-like mass that can absorb and expel water like a sponge. This gel-like mass may also act as a physical barrier to foreign particles (Marieb and Hoehn, 2007a).

Differently, hyaluroran (a non-sulfated glycosaminoglycan), is synthesized without a protein core and is released at cell surface directly into the extracellular matrix. Hyaluronan contributes significantly to cell proliferation and migration, and may also be involved in the progression of some malignant tumours (Edward *et al.*, 2005).

Embedded in this layer are systems and structures common to other organs and tissues such as lymphatic and blood vessels, nerve fibres, and muscle cells. The main cell types are fibroblasts which are responsible for the synthesis of collagen and elastin. There are about 20 different types of collagen, but adult collagen consists mainly of types I and III, whereas type IV is a major constituent of the basal lamina. Other cell types include mast cells which are capable of producing potent inflammatory mediators; macrophages which are derived from blood monocytes and have phagocytic properties; and plasma cells which express immunoglobulin receptors on their surface and once activated, are able to synthesise immunoglobulins. There are also some types of leukocytes that can move from the circulation into the dermis through extravasation. These include: neutrophils, a phagocytic cell, eosinophils which recognise parasites and, lymphocytes that are cells capable of triggering an immune reaction. The dermis also contains a multitude of nerve endings that act as tactile and thermo receptors.

The dermis has two major layers, the papillary layer (or *stratum papillare*) and the reticular layer (or *stratum reticulare*).

The thin superficial papillary layer is made up of loose connective tissue clearly demarcated from the epidermis by the BM border. Its superior surface is thrown into projections called dermal papillae that indent into the epidermis above and contains small blood vessels that nourish the epidermis. Within the papillary layer are thin fibres of collagen which provide strength and flexibility and, contains receptors which communicate with the Central Nervous System - these include touch, pressure, hot, cold and pain receptors.

The thick reticular layer lying below the papillary layer is much denser. It contains bundles of interlocking collagen fibres that run in various planes parallel to the skin surface, elastin and reticular fibres which give the skin its stretch and recoil properties. It also houses hair follicles, nerves and certain glands. The lower part of the

reticular dermis shows a continuous transition to its lower adjacent layer, the hypodermis.

The hypodermis or subcutaneous fat tissue consists of a spongy connective tissue interspersed with energy-storing adipocytes (fat cells). The hypodermis is heavily interlaced with blood vessels, ensuring a quick delivery of stored energy and nutrients as needed. Other functions of the subcutaneous fatty tissue include the insulation of the body from cold and shock absorption like on the palms of the hands and soles of the feet (Marieb and Hoehn, 2007a). Skin appendices are hair follicles, sebaceous and sweat glands.

1.3. EPIDEMIOLOGY OF CANCER

It is estimated that one in three people in the United Kingdom will develop a malignancy by the time they reach the age of 70, with the incidence increasing with age. Unfortunately, the UK incidence of cancer is expected to increase by 2025 (Gabriel, 2004). This may however be applicable to any developed or developing region of the world as cancer is an age and degenerative disease, common where longevity is increased.

The incidence of cancer is a worldwide phenomenon. For example in Brazil, breast and prostate cancer in 2006 were estimated as the highest in the southern and northern regions respectively (Menezes *et al.*, 2007).

In the USA (United States of America), overall incidence of cancer and cancer deaths dropped, according to the trends based on data from 1975 through 2005 (Jemal *et al.*, 2009). This was due to largely decreases in the most common cancers: breasts, prostate, lung and colorectal cancers. However, there are more new cases of skin cancer (more than one million) diagnosed annually (America Cancer Society. Cancer Facts &

Figures, 2009). In addition to this, the incidence of melanoma continues to rise significantly fast. The US Skin Cancer Facts (2009) reported some data that should be carefully considered: 1) melanoma accounts for about 3 percent of skin cancer, but it causes more than 75 percent of skin cancer deaths; 2) although survival with melanoma increased from 49 percent between 1950 and 1954 to 92 percent between 1996 and 2003, one person dies of melanoma almost every hour (every 62 minutes): and, 3) about 65 percent of melanoma cases can be attributed to ultraviolet (UV) radiation from the sun.

In the United Kingdom (Westlake, 2008) breast cancer is the most common cancer for women (42,000 new cases a year). The most common type of cancer in men is prostate cancer with over 31,900 new cases a year. Lung cancer is the second most common form and men are more likely to be affected (37,200 new cases a year). Colorectal cancer is the third most common cancer in men (about 18,700 new cases a year) and the second most common cancer in women (nearly 16,200 cases a year). However an analysis of 1.6 million cases of cancer in people aged up to 79 in England, in an eight year period, showed that cancers of the bone and specific types of ovarian and testicular cancers disproportionately affected young people aged between 13 and 24 (O'Dowd, 2006). As for childhood cancer, while statistics show that over 160,000 children worldwide are newly diagnosed with cancer each year, the exact number of new cases is not known as cancer registers do not exist in many countries. Leukemia is the most common childhood cancer in Europe, America and East Asia. Other tumour types are more frequent in developing countries, such as lymphomas, Kaposi's sarcoma and retinoblastoma (International Union against Cancer, 2006).

Melanoma, the most serious form of skin cancer accounts for almost three per cent of all newly diagnosed cancers each year. There are about 3,500 new cases of melanoma in men and over 4,500 new cases in women each year in the UK (Westlake, 2008).

As melanoma is the skin cancer investigated in this study, it is discussed in more detail as follows.

1.4. SKIN CANCER - MELANOMA

There are two main types of skin cancer, melanoma and non-melanoma skin cancer. Melanoma is the most serious form of skin cancer as it grows and spreads rapidly in the body.

1.4.1. History of Melanoma

As described in "Texbook of Melanoma" (Thompson *et al.*, 2004), the first case of a patient with melanoma was published by John Hunter in 1787. His patient had a recurrent mass behind the angle of the lower jaw. Hunter's original specimen is still preserved in the Hunterian Museum of the Royal College of Surgeons of England. In 1968, Bodenham reported that microscopic examination of the specimen confirmed that it was a melanoma. However, in 1806 Rene Laennec was the first to describe melanoma as a disease entity and in 1812, used the term 'melanosis'. The first case in England was reported by William Norris in 1820. He referred to it as a case of fungoid disease but later described a patient who died from disseminated melanoma. Many other descriptions have been made, including "superficial spreading" melanoma by David Williams and, the first excision of metastasis in the lymph nodes in 1851. In the early 20^{th} century Frederick Eve remarked that 'it is generally stated that melanomas are the most malignant form of tumours'. He also recommended the removal of a primary tumour and the nearest chain of lymphatic glands. At this time, melanoma was usually very advanced when diagnosed and the prognosis was very poor. From 1970 to the present day, the management of melanoma went through a series of changes. Many

countries adopted rapid treatment for patients with melanomas presenting a tumour thickness of >1mm in an attempt to increase the survival rate. However, melanoma still causes many deaths worldwide as it produces metastasis very rapidly making treatment difficult.

1.4.2. Epidemiology of melanoma

The earliest descriptions of skin cancer were of slow inexorable growths destroying their entire path. The Latin term – *noli me tangere* (touch me not) was given because they were thought to be incurable and therefore not to be treated (Colver, 2002). In fact it is now known that melanoma if diagnosed at an early stage may be curable.

The incidence of invasive cutaneous malignant melanoma is increasing rapidly and mortality trends are increasing steadily upwards. Some authors state that there is an urgent need for better therapy of cutaneous malignant melanoma (MacKie, 1996).

Although melanoma is not a common source of cancer, in the United Kingdom, the incidence of melanoma has increased over three fold in the last 25 years to age-standardized rates of 11.1 per 1000,000 in men and 12.6 per 100,000 in women in 2003. As incidence rates have increased faster than any other major cancer melanoma is a public health concern as it is the eighth most common malignancy in the UK (Newton-Bishop, 2008).

Later stage melanoma incidences are particularly high in lower socioeconomic groups than in higher socioeconomic groups. This may be explained as individuals in low socioeconomic areas have less access to educational campaigns and screening examinations and effective treatments when compared to individuals from higher socioeconomic areas (Ortiz *et al.*, 2005).

Considering the increasing incidence of melanoma in both USA and UK, it can be assumed that melanoma incidence may be increasing world wide.

1.4.3. Skin types and skin cancer

Skin types can be referred to the different skin colours. Three pigments contribute to skin colour: melanin, carotene and haemoglobin. Melanin, a polymer made up of tyrosine amino acids, ranges in colour from yellow to orange to brown. As described before, melanin is transferred from melanocytes to keratinocytes. The melanocytes of black- and brown-skinned people produce much darker melanin than those of fair-skinned individuals. Melanocytes are stimulated to greater activity when the skin is exposed to sunlight. Prolonged sun exposure causes a substantial melanin build up, which helps to protect viable skin cells from ultraviolet radiation and in fair-skinned people causes visible darkening of the skin. Despite of melanin's protective effects, excessive sun exposure eventually damages the skin. It depresses the immune system and can alter the DNA of skin cells and may lead to skin cancer.

Carotene is a yellow to orange pigment found primary in the stratum corneum and in adipose tissue. Carotene is particularly abundant in the skin of Oriental people. Together with melanin, it accounts for the yellowish tinge of their skin.

Haemoglobin – the pinkish hue of Caucasian skin reflects the crimson colour of oxygenated haemoglobin in the red blood cells circulating through the dermal capillaries. Since Caucassian skin contains only small amounts of melanin, the epidermis is quite transparent and allows haemoglobin's red colour to show through. (Marieb, 2000).

Skin cancer is uncommon in skin colour people compared to Caucasians. However, skin cancer prevention and screening practices historically have been lower among Hispanics, Blacks and Asians (Bradford, 2009). On the other hand, white-skinned people living in sunny areas seem to be more aware of the need to protect skin against UV radiation.

1.5. IDENTIFICATION OF MELANOMA

Melanoma can be caused by ultraviolet (UV) radiation, genetic factors and ageing. Melanoma, also known as malignant melanoma, is a cancer that develops from melanocytes. As mentioned before, these cells produce melanin, a pigment that is passed into adjacent keratinocytes where it accumulates over the cell nucleus, forming a shield to protect the skin from the harmful effects of the UV radiation. Melanoma may develop in moles as well as elsewhere on the skin. It can also occur in the eyes, under the finger nails or on other body parts not usually exposed to the sun such as the palms of hands or the soles of feet. Approximately one third of melanomas develop from normal moles. The earlier melanoma is detected the better the chance of being curable. Despite the majority of melanomas arising in the skin, it may also arise on mucosal surfaces or at other sites to which neural crest cells migrate. Melanoma occurs more commonly in adults and more than 50 % of the cases arise in apparently normal exposed areas of the skin. Early signs in a nevus that suggest malignant change include a dark patch that is getting larger, a mole with a ragged edge, a mole with a mixture of different shades of brown and black colours, development of satellites, inflamed moles, bleeding or crusting of a mole and an itching mole. In suspected skin lesions an excision biopsy is processed by conventional histology and examined.

1.5.1. Cell's own mechanisms of protection

Both UVA (ultra-violet - longer wavelength) and UVB (ultra-violet - medium wavelength) rays cause damage, including genetic injury, wrinkles, lower immunity against infection, aging skin disorders, and cancer. The mechanisms inducing such changes are not yet fully understood. In order to counteract to these deleterious effects, the body takes some defensive mechanisms against DNA damage, as follows:

- Production of anti-oxidants to fight against oxidants (also called free radicals). These are unstable molecules produced by normal chemicals during metabolic process. High levels of oxidants may alter the genetic component, contributing to ageing and cancer. The large surface area of the skin makes this organ a prime target for oxidants.
- Repair of defective DNA. Cells can produce protective enzymes (such as DNA polymerases (Kunkel and Bebenek, 2000)) which help to repair damaged DNA. Melanoma and other skin cancers may be caused by a breakdown in this mechanism.
- Apoptosis. It is a natural process of cell self-destruction, which occurs when cells are severely damaged. Some genetic mutations or other factors inhibit apoptosis. Once this occurs, cells continue to proliferate in an uncontrolled manner and may result in clones of modified cells.
- Immune response against a non-recognizable or altered cell within the system. This is a very complex process which may include innate immune and/or specific responses, activating cells and soluble factors.

The initiation of cancer may occur therefore if one or more mechanisms of protection fail to destroy or eliminate an altered cell.

1.5.1.1. Cancer development

The multiplication of cells is carefully regulated and responsive to specific needs of the body. Very occasionally the mechanism of protection fails and the exquisite controls that regulate cell multiplication break down. A cell in which this occurs begins to grow and divide in an unregulated fashion, without regard to the body's need for further cells of its type. This results in a clone of cells able to expand indefinitely and tumour formation takes place. Some tumours do not have serious consequences but

tumours composed of cells that spread throughout the body usually cause the disease. Cancer therefore is caused by accumulation of mutations of the somatic cells. Approximately half of all cases of human cancer may be attributed to a defective p53 protein, caused by mutation s in the p53 gene, changing one amino acid in the protein to another. This may alter the binding of the protein to DNA or the transcription factors, corrupting the signal from p53 to the cellular machinery or it may unnaturally increase the stability of the protein, disrupting the delicate balance of synthesis and degradation that controls the level of p53 in the cell. Either way, p53 is unable to open the gates to cell cycle arrest or apoptosis, and cancerous cells are allowed to proliferate without control (Goodsell, 1999). The p53 tumour suppressor has been termed "The guardian of the cell". It is not essential for life but it is essential in its role protecting an organism.

Tumour progression is initially dependent on other cells, and the rate of tumour growth is determined by comparing the excess of cell production with cell loss. For tumour expansion to occur, vascularization (or angiogenesis) develop to "feed" tumour cells. With further progression, tumour become more independent (and aggressive) (Anderson *et al.*, 2009) and may generate blood vessels as they develop the ability to synthesize growth factors that stimulate formation of vessels. Further steps are the detachment to cells from solid tumours and metastasis formation.

1.5.2. Histopathological Detection of Melanoma

Malignant melanoma originates from altered melanocytes of the basal and spinous layers of the epidermis. Histopathological examination demonstrated that abnormal melanocytes proliferate (by mitosis) and spread into both the epidermis and the dermis. Superficial spreading of melanoma is represented by abnormal melanocytes growing radially within the epidermis and is known as Pagetoid spread (Figure 1.3 A). If only this horizontal spread occurs no metastasis are formed. Therefore this is a pre-

invasive form of melanoma. This type of melanoma can be surgically removed with a high success rate.

On the other hand, melanoma cells may also proliferate and invade in a vertical way, reaching deeper in the dermal layer (Figure 1.3 B). This type of vertical invasion is highly likely to develop into a metastatic form of melanoma with a poor clinical prognosis.

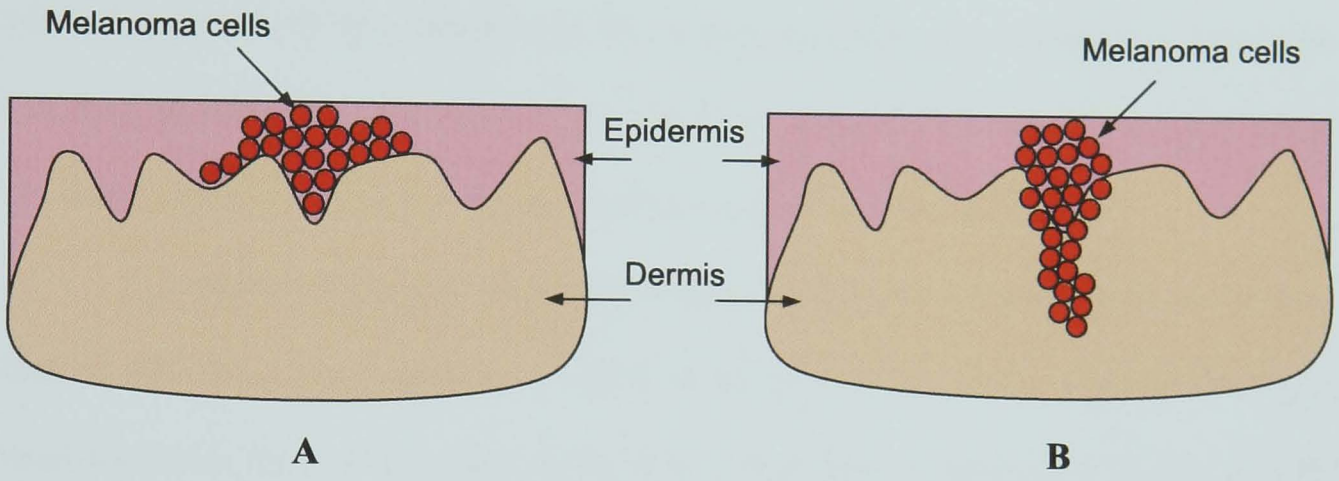


Figure 1.3. Melanoma cell invasion occurring in two different ways. Horizontal cell proliferation (A); vertical cell proliferation and invasion (B).

1.5.3. Clinical classification of melanoma

Clinical classification of melanoma is based on tumour spread from its place of origin to regional lymph nodes or distant sites. For tumours at the primary site, the greater the thickness and depth of local invasion of the melanoma, the higher the chance of lymph node or systemic metastasis and, therefore the worse the prognosis. Melanoma may spread into local lymphatic/blood vessels and to distant places via lymph/blood circulation. At this point, the lesion can be classed as an invasive malignant melanoma capable of producing distant metastases. Melanoma also varies in terms of pigmentation and size.

According to some authors (Buchan and Roberts, 2000), four main types of cutaneous melanoma are classified for clinical identification: 1) superficial spreading

melanoma; 2) nodular melanoma; 3) lentigo maligna melanoma; 4) acral lentiginous melanoma.

1. Superficial spreading melanoma. These include over half of the melanomas presenting on white skinned people. They are more common in women than in men. Lesions are pigmented with irregular borders and are slightly raised. Normal skin margins are lost and there may be evidence of inflammation. Superficial and spreading growth may last months. A change in thickness or the growth of nodules indicates a vertical invasion of the tumour and its growth indicates a worsening in prognosis. Crusting and bleeding can occur if the lesion is ignored. This type of melanoma can occur in normal skin, however it frequently arises in a pre-existing mole.

2. Nodular Melanoma. Malignant nodular melanomas are more common in men, later in life. This type comprises 20-25% of all melanomas. They occur on the trunk, head and neck. There is no horizontal growth. The tumour invades vertically and rapidly from the skin surface and, has a poor prognosis. The colour varies between reddish to brown or black. This type of melanoma may present as an elevated dome-shaped or, even tag-like lesion. It is common to find it as a red raised central lesion with a peripheral brown ring or crescent of melanin. This type of melanoma bleeds at an early stage and therefore is easily confused with a vascular lesion. The absence of horizontal invasion of cells around a nodular melanoma distinguishes it from other forms of melanoma.

3. Lentigo maligna melanoma. This type of melanoma is common in people over 60 years of age and those who have had long periods of sun exposure. Therefore it is often found in normally exposed body areas such as face, hands and legs. It may have a long pre-invasive phase of several years. During this phase, the lesion has a slow growth and flat brown even appearance. However, with time, the pigmentation becomes irregular with areas of dark, brown, blue or black. Red or purple dark areas are less

frequent. The development of an elevated nodule indicates a vertical invasive growth phase. If ignored, bleeding, oozing and ulceration may occur.

4. Acral lentiginous melanoma. These comprise 10 % of melanomas in the white skinned population and their common sites are palms, soles and around the nail bed. These lesions appear as a large flat pigmented area around a focus of a raised melanoma and may bleed or ooze. Lesions around the nail bed are often mistaken for old blood.

1.5.4. The Clark level of invasion and Breslow thickness

Both techniques are a measurement of the depth of invasion as an important prognostic factor in the pathology. Clark (Clark *et al.*, 1975) recognised that there was a relationship between the level of invasion of the tumour and the clinical prognosis. The higher the level of Clark's classification the deeper the invasion. Five levels of classification are used (See Table 1.1).

Table 1.1. Clark's classification level (Clark *et al.*, 1975).

Level	Description
1	Lesions involving only the epidermis (*in situ* melanoma)
2	Invasion of the papillary dermis by single cells
3	Invasion fills and expands the papillary dermis
4	Invasion into the reticular dermis
5	Invasion through the reticular dermis into the subcutaneous tissue

Breslow (1970) refined the above classification and demonstrated that the direct measurement of the tumour thickness (in millimetres), from granular layer to the

deepest abnormal tumour cell along with a safe margin of excision provides a more precise and reproducible prognosis for patient survival and, the likelihood of metastatic spread (Figure 1.4). Therefore, according to the Breslow thickness, the thicker the tumour the lower the rate of survival.

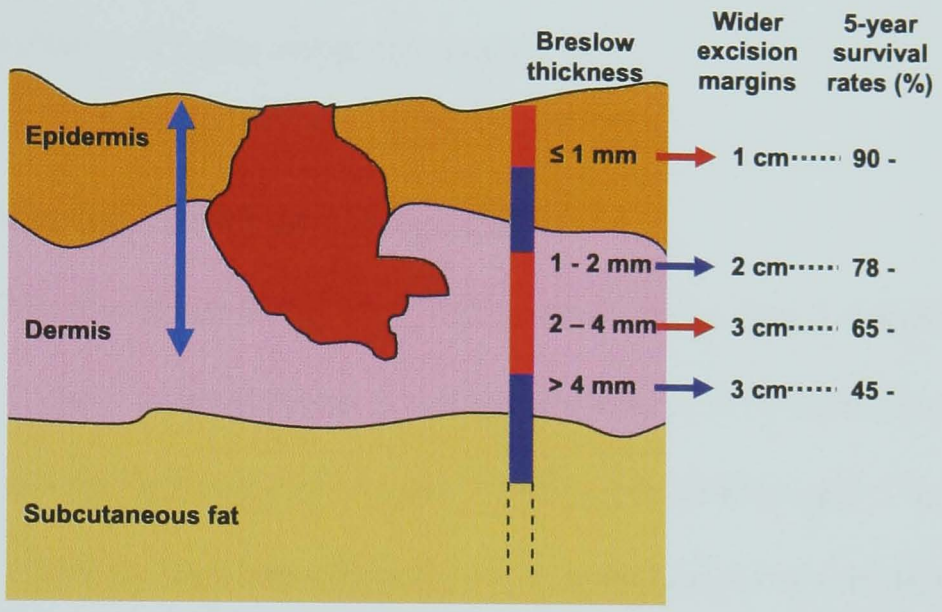


Figure 1.4. Breslow thickness and determination of the size of margins required for excision and prediction of 5 year survival rates. Adapted from Rajpar and Marsden (2008a).

1.5.5. Treatments for melanoma

As survival from primary melanoma is determined by Breslow thickness, treatments for melanoma can be described from early stages from surgical removal of superficial melanoma to the more intensive treatments and palliative care (Rajpar and Marsden, 2008a).

1.5.5.1. Treatment for early stage melanoma

Melanoma *in situ* is the very early stage in which abnormal melanocytes/melanoma cells are found in the basal layer and to a lesser extent in the

spinous layer of the epidermis. Treatment is by surgical removal of melanoma cells and a margin of healthy tissue around it. It can be totally curable if it is removed at an early stage.

Early melanoma, in its pre-invasive form is classified as a tumour of less than 1 mm in thickness. Treatment is by surgical excision and no further treatment is necessary as it has a high chance of being completely cured.

1.5.5.2. Treatment of stage 2 and 3 melanoma

Stage 2 is considered to be when a melanoma is more than 2 mm thick or more than 1mm thick and ulcerated. **Stage 3** is when melanoma cells have already invaded into the body and are found in one or more lymph nodes. Both stages 2 and 3 involve further tests to check for metastasis formation and, further treatment may be necessary.

Post-surgery treatments need to be more effective and stronger in an attempt to reduce recurrence in the lymph nodes and to prevent metastatic invasion of the melanoma. This makes metastasis treatment completely different when compared to the pre-invasive stage of the disease. Successful treatment is more likely if the melanoma is fully surgically removed, including a small area of normal surrounding skin or, if it remains localised around the original scar or when only regional lymph nodes are involved (Cancer Research UK, 2009; Rajpar and Marsden, 2008b).

Chemotherapy is used only in cases of advanced melanoma, including a combination of drugs and sometimes immunotherapy (where the patient's own immune system is activated). Interferon and interleukin 2 are the commonest approaches used for immunotherapy.

Treatments are therefore: surgery, isolated limb perfusion, laser treatments, chemotherapy, radiotherapy, interferon, vaccines and palliative care.

Some of the latest treatments and co-adjuvant drug therapies include some basic anti-inflammatory agents. This reflects the recent knowledge that inflammation seems to play a role in cancer development. Accordingly, a summary of inflammation is described next.

1.6. INFLAMMATION

Inflammation is a process in which immune cells and a cascade of cytokines and chemokines are stimulated in response to tissue trauma such as cuts or other mechanical trauma, *e.g.* burns, infection, autoimmune responses and toxic agents.

With acute inflammation (Playfair and Chain, 2005a; Playfair and Chain, 2005b; Kang *et al.*, 2006; Marieb and Hoehn, 2007b), mast cells and macrophages release chemical mediators such as histamine and heparin into the surrounding area. As a result, phospholipids, one of the main substances released, are converted to arachidonic acid by the enzyme phospholipase A2. In the next stage, the arachidonic acid may enter two different inflammatory pathways. One is the cyclic peroxides in which cyclooxygenase enzymes (COX1 and COX2) convert the arachidonic acid into prostaglandins. Prostaglandins mediate vasodilation and inhibit platelet aggregation. This leads to increased vascular permeability which allows serum and protein to accumulate in the tissue around the wound. The result of this is redness, swelling and heat apparent in and around the wound site.

If Arachidonic acid enters the lipoxygenase pathway, it is converted into leukotrienes. Leukotrienes are responsible for recruiting leukocytes to the inflamed area. This involves a complex specific immune response and may damage the surrounding tissue if inflammation is uncontrolled.

Thromboxanes are also produced by the release of phospholipids and mediate platelet aggregation and vasoconstriction which opposes the effects of inflammation. Therefore a balance between the production of prostaglandins and thromboxanes may control inflammation. See Figure 1.5.

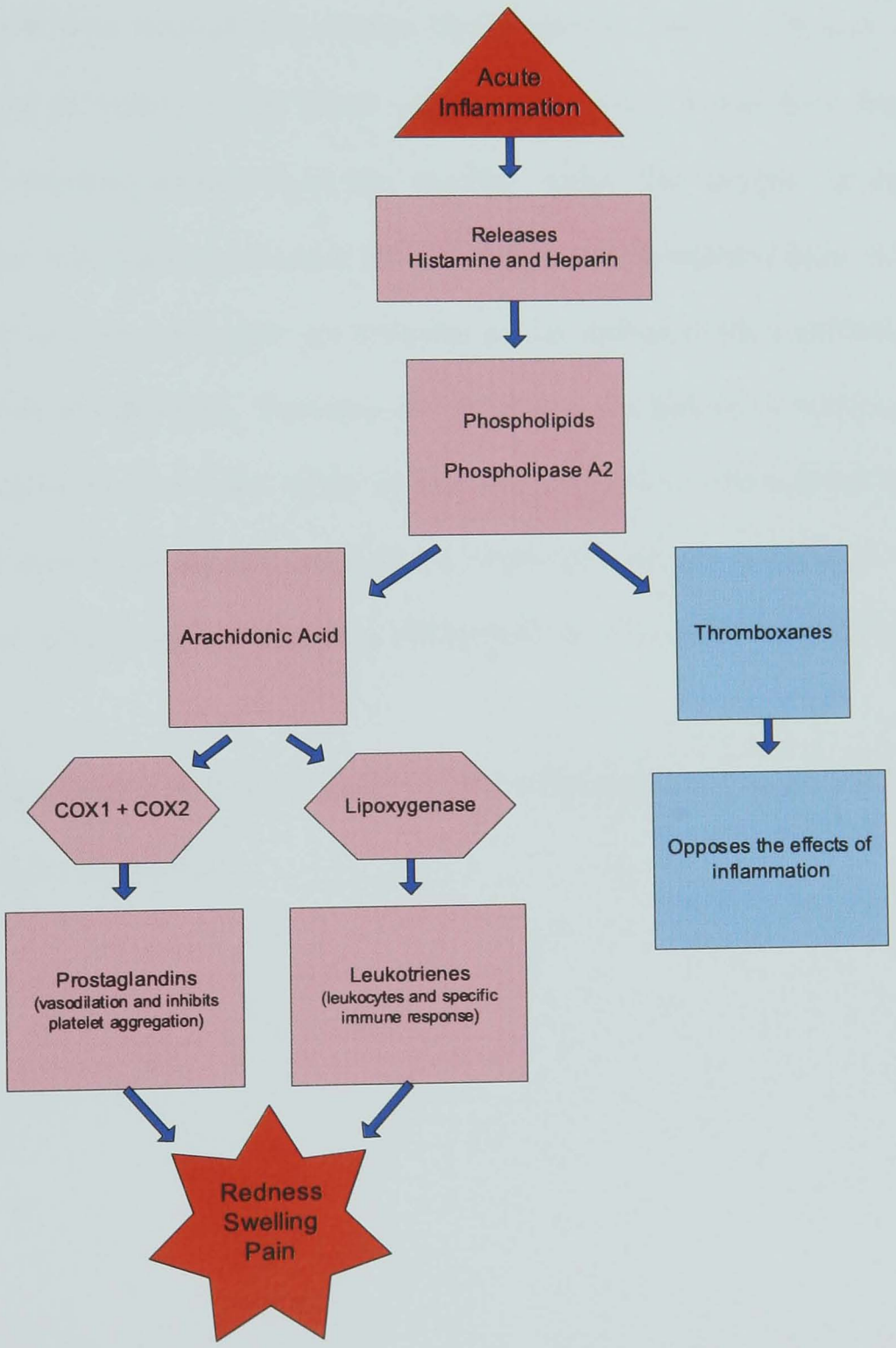


Figure 1.5. Flow chart of acute inflammation.

During inflammation, monocytes, macrophages and other cell types produce cytokines such as Tumour Necrosis Factor (TNF) and Interleukins (ILs), mainly IL-1 and IL-6. These cytokines initiate many of the changes in the vascular endothelium promoting leukocyte entry to the inflammatory site, as well as inducing tissue repair.

The beginning of inflammation involves some hemodynamic changes to slow down the blood flow through the smaller blood vessels. This is achieved by the vasoconstriction of nearby larger blood vessels. The slower blood flow facilitates adherence of activated leukocytes to the capillary walls. The increase in capillary permeability (or vasodilation) increases the spaces between endothelial cells. As blood flow is slower, adhesion molecules are activated on the surface of the endothelial cells on the inner wall of capillaries. Activation of integrins on the leukocyte enables surface attachment to adhesion molecules on the endothelial cell surface. This makes it easy for leukocytes to flatten and squeeze through the space between the endothelial cells, a process called diapedesis, extravasation (Ransohoff *et al.*, 2003) or transmigration (Figure 1.6).

Although there are many leukocytes in the circulating blood, neutrophils are the first responder to inflammation.

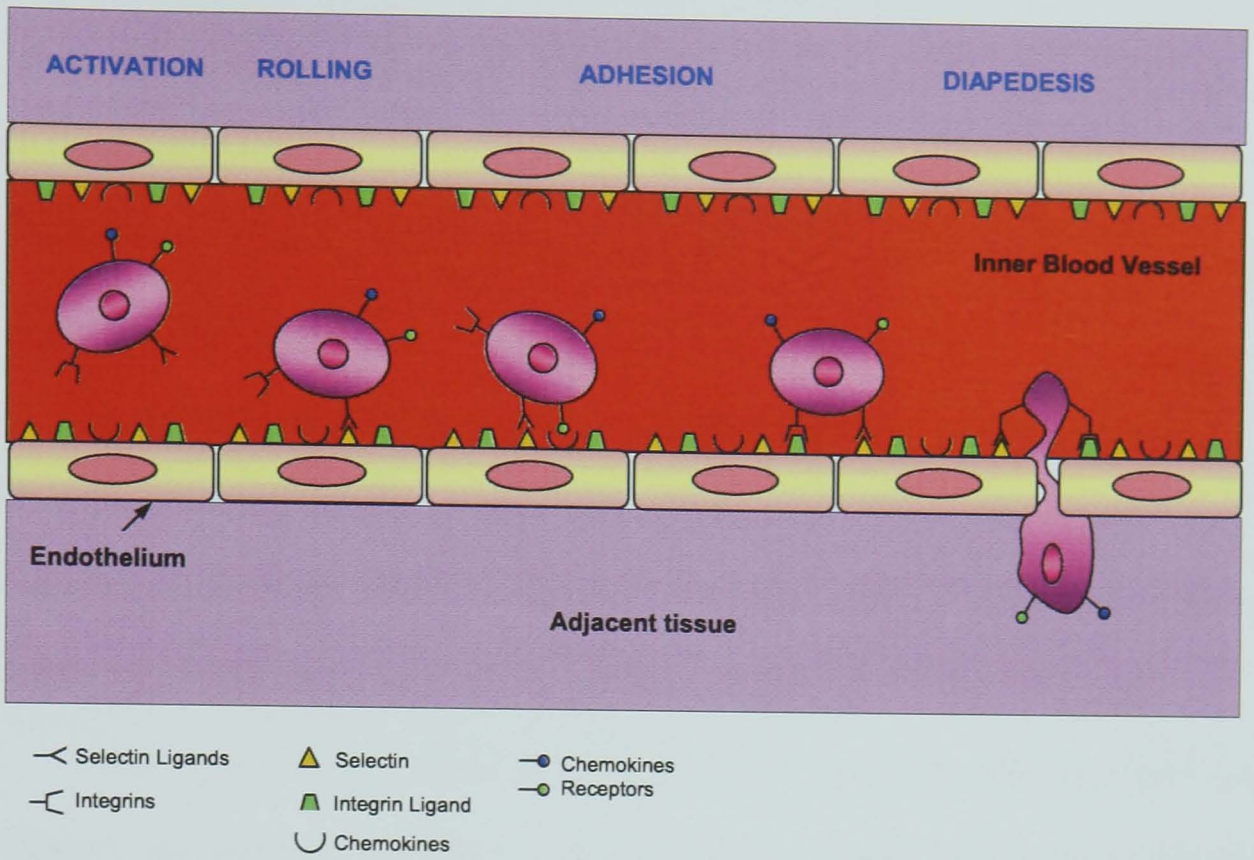


Figure 1.6. Inflammation and diapedesis. Stages of leukocytes attraction to the site of inflammation : Cell activation, rolling, adhesion and diapedesis. (Adapted from Ransohoff *et al.*, 2003).

This process is explained further in Figure 1.6 which shows the actions of neutrophils in an acute inflammatory response. This is divided into 5 subsequent phases: margination (the process in which free-flowing leukocytes exit the central blood stream, and adhere onto the internal surface of the vessel walls), rolling, adhesion, diapedesis and chemotaxis. These series of events are mediated by many biochemical changes such as:

1. The internal surface of endothelial cells express selectins (E-Selectin), integrins ligands, such as ICAM-1 (Intercellular Adhesion Molecule-1) and chemokines. Leukocytes express chemokines receptors, selectins ligands (L-Selectins) and integrins.
2. Leukocytes selectins ligands, and endothelium selectins (E-selectin) act as receptors to provide binding for rolling.

3. Engagement of the chemokine receptor on the leukocyte surface to the immobilised chemokines on the endothelial cell surface provides further rolling, adhesion and integrin activation.
4. Leukocytes integrin association with integrin ligand on endothelial cell surface promote diapedesis.

In acute inflammation chemotaxis is aided by C5a component from complement activation, along with the lipoxygenase pathway to attract phagocytes (such as macrophages which can engulf and destroy substantial numbers of bacteria, other cells or microorganisms) and induce specific immune responses.

These events are stimulated and enhanced by a variety of chemical inflammatory mediators, produced by leukocytes and macrophages. These include proinflammatory cytokines such as TNF-alpha, IL-1 and IL-6. As a result of these proinflammatory cytokines, IL-8, a type of chemokine is released. Chemokines play a key role in chemotaxis and regulation of leukocyte mobilization. The entire process results in redness, swelling, heat and pain. As inflammation finishes, the healing process begins to restore and repair the damaged tissue. Inflammation and wound healing process begin to restore and repair the damaged tissue. This is helped by the production of a clotting system in a form of a matrix into which fibroblasts migrate and initiate healing. This occurs by the production of a clotting system forming a matrix into which fibroblasts migrate and initiate healing.

In this study, reconstructed human skin models have been used to investigate melanoma invasion in an inflamed wound bed. Therefore some background on skin wound healing has been included.

1.7. SKIN WOUND HEALING

Wound healing is a complex and essential process in all damaged human tissues. Skin wound healing involves a collaborative effort of many different tissues and cells lineages during the phases of proliferation, migration, matrix synthesis and contraction. Growth factors and matrix signal also present at wound site also play a role in wound healing.

As skin is the barrier from the external environment any breakage must be rapidly and perfectly healed. Adult skin wound healing results in a scar that is neither aesthetically nor functionally similar to the same previous intact tissue. The same principles underlie the healing of physical damage such as cuts as well as other mechanical or chemical damages, such as ulcers and burns.

When the skin is wounded, the remaining healthy living tissue activates the healing process, which overlaps with the inflammatory response. The body immediately releases chemical mediators of inflammation which play an important role in cleaning the damaged tissue area and, growth factors which help a wound to heal. Healing involves the release of fibrinogen and thrombin to react and form a gel-form of fibrin which clots to fill in the empty wound space. This is important as sealing the open wound helps avoid possible contamination or infection. It also serves as a provisional scaffold for cells that proliferate and migrate in from the wound edges towards the centre of the wound. Healing itself occurs as fibroblasts proliferate and migrate in the fibrin clot produced within the wound. As fibroblasts proliferate and migrate, a new extracellular matrix is produced, including collagen and elastin. The proliferation of keratinocytes from the wound edges closes the epidermal gap over the new dermal tissue and, within a few days the tissue is healed and gradually returns to normal function.

There are important factors for the fast and healthy healing of wounds, such as adequate supply of nutrients and oxygen (importantly vitamin C an essential cofactor for collagen synthesis by dermal fibroblasts, and zinc for helping in wound healing (Lansdown *et al.*, 2007)); and an aseptic wound bed (Lippincott Williams & Wilkins, 2003). On the other hand, congenital disorders may contribute to a delay in wound healing, such as disorders of plasma fibrinogen synthesis (Flute, 1977) and diabetes (Lioupis, 2005).

A sequence of overlapping events about the skin wound healing process has been described by some authors (Marks, 2003; Lippincott Williams & Wilkins, 2003; Martin, 1997). Hence the steps of skin wound healing can be enumerated as follows:

1. Within seconds of tissue damage, release of serotonin, histamine and prostaglandins from the blood circulation enter the injured site.

2. In the initial few minutes, the small blood vessels constrict and platelets plug the endothelial gaps. However, as the wounded skin previously caused leakage of blood from damaged blood vessels, the formation of a clot serves as a temporary shield of the unprotected wound tissues and provides a provisional matrix for cell migration during the healing process. This clot is formed by platelets embedded in a mesh of cross-linked fibrin fibres provided by thrombin cleavage of fibrinogen. The clot acts as a reservoir of cytokines and growth factors that are released as activated platelets degranulate. This start of wound closure provides chemotaxis for recruitment of circulating inflammatory cells to wound site, initiates the tissue movements of reepithelialization, connective tissue contraction and wound angiogenic response. The wound edges swell. Leukocytes from surrounding vessels move in and digest cellular debris and digest bacteria, gradually demolishing the clot. Redness, warmth, swelling, and pain may occur.

3. Adjacent healthy tissue supplies blood, fibroblasts, and other building materials such as proteins. These form a highly vascular tissue known as granulation tissue, which begins to bridge the wounded area. At this stage, in a non-infected site, inflammation may decrease.

4. Fibroblasts in the granulation tissue secrete collagen which criss-crosses the area forming scar tissue.

5. Meanwhile (after 18-24 hours), epithelial cell at the wound edge multiply and migrate toward the wound centre forming a new layer of surface cells. For this to happen the leading-edge keratinocytes have to dissolve the fibrin clot barrier and move through or along the interface between clot and healthy dermis. The major fibrinolytic enzyme is plasmin derived from plasminogen within the clot itself and can be activated either by tissue-type plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA). Both of these PA's activators and receptors are upregulated in the migrating keratinocytes. In addition to this, various members of the matrix metalloproteinases (MMPs) family, can cleave an specific subset of matrix proteins and these are also upregulated by wound-edge keratinocytes. This may inclue MMP1, MMP2 and MMP10 which were detected by the high levels of proteolytic activity in chronic wound fluid. Keratinocytes that are normally basally restricted appear suprabasally at the wound margin. This is important for cell and tissue strength during reepithelialization of the wound. After 2-4 days, new capillaries develop in the wound cavity.

6. The lymphatic and blood vessels regenerate and the dermis is healed by tissue repair. Wound contraction occurs as a normal process of wound closure resulting in minimum scar. During granulation tissue formation migrating fibroblasts differentiate into myofibroblasts. These myofibroblasts are responsible for force generation and

produce tissue contraction. The scar diminishes in size with the return of normal tissue function.

7. Between day 4 and 10 the wound cavity has become covered with new epidermis and the granulation tissue is replaced by a new dermis. In the later stages, remodelling leads the orientation of the dermal collagenous bundles to the original lines of stress. The epidermis ultimately develops a normal profile and the dermis' vasculature is also restored to normal contractility. Scar formation occurs when there has been significant damage to the dermis. See Figure 1.7 for an illustration of skin wound healing at a glace.

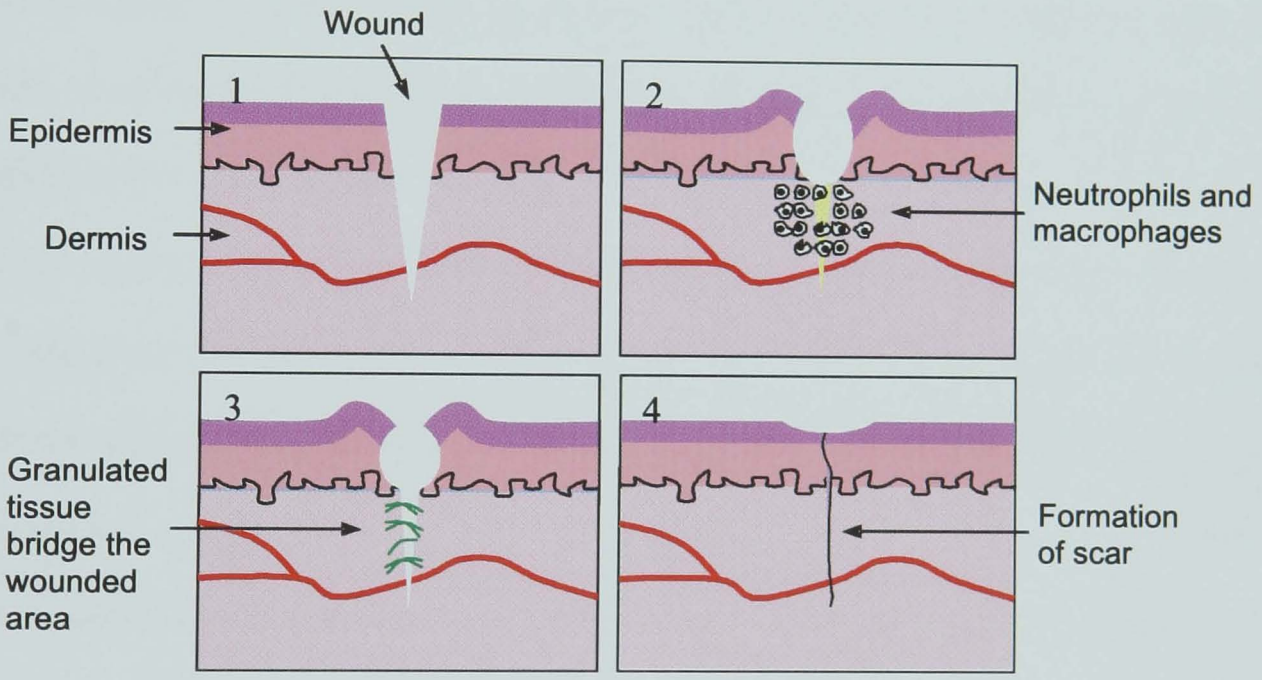


Figure 1.7. Skin wound healing at a glance. (1) Immediately serotonin, histamine and prostaglandins are released to initiate an inflammatory reaction. (2) Fibrin clotting binds the wound edges. Extravasation of leukocytes and plasma from dilated blood vessels. This is followed by oedema, redness, warmth and pain. Neutrophils and monocytes are attracted to wound site by inflammatory mediators, such as growth factors and peptides cleaved from bacterial proteins and the by-products of proteolysis of fibrin and other matrix components. Neutrophils are the first type of leukocytes to be attracted into the wound. The activity of neutrophils essentially cleanses the wound bed of bacteria and cellular debris and are also a source of pro-inflammatory cytokines (*e.g.* TNF-α, IL-1α and –β). The activated macrophages produce a variety of cytokines that regulate tissue repair (*e.g.* platelet-derived growth factor (PDGF) and fibroblasts growth factor (FGF)). (3) Adjacent healthy tissue supplies blood, fibroblasts and proteins forming the granulation tissue. This begins to bridge the wounded area. (4) Epithelial cells at the wound edge multiply and migrate toward the wound centre over the new reconstructed dermis and a scar tissue is formed.

1.8. ANTI-INFLAMMATORIES

Anti-inflammatories are substances which have properties to reduce or eliminate inflammation and pain as these medications may also contain analgesics. There are two forms of anti-inflammatories: the Steroidal Anti-Inflammatory Drugs (SAIDs) and the Non-Steroidal Anti-inflammatory Drugs (NSAIDs). Both anti-inflammatory drugs act against inflammation, however their mechanisms of action and effects are different.

The Steroidal Anti-inflammatory Drugs are specifically glucocorticoids, and can reduce inflammation by binding to cortisol receptors. These drugs are often referred to as corticosteroids. Glucocorticoids influence all types of inflammatory events by inducing lipocortin-1 synthesis, which then binds to cell membranes preventing phospholipase A2 from coming into contact with its substrate arachidonic acid. This leads to diminished eicosanoid production. Therefore, the two main products in inflammation (prostaglandins and leukotrienes) are inhibited and therefore cyclooxygenase expression (both COX-1 and COX-2) is also suppressed. Lipocortins escaping into the extracellular space bind to leukocyte membrane receptors and inhibit various inflammatory events such as epithelial adhesion, emigration chemotaxis and phagocytosis, including the inhibition of the release of various inflammatory mediators like lysosomal enzymes, cytokines and chemokines from neutrophils, macrophages and mastocytes. Hence, anti-inflammatory steroids suppress all elements of the immune response.

NSAIDs include among others, aspirin and ibuprofen. Aspirin or acetylsalicylic acid (acetosal) is a drug in the family of salicylates, often used as an analgesic, antipyretic and anti-inflammatory. It is also recognised for its anticoagulant effects and has been used in long-term low doses to prevent heart- attack and stroke (Miner and Hoffhines, 2007).

Once NSAIDs inhibit cyclooxygenase, prostaglandin production is limited and biosynthesis of COX-1 and COX-2 is reduced. COX-1 is constitutively produced at basal levels in most tissues and plays a central role in platelet aggregation and gastric cytoprotection. COX-2 is expressed constitutively in the human kidney and brain and its expression is induced in many tissues during inflammation, wound healing and neoplasia (Thun *et al.*, 2002; de Souza Pereira, 2009).

Ibuprofen is a NSAID widely known under various trademarks including Act-3, Advil, Brufen, Motrin, Nuprin and Nurofen. It is used for relief of symptoms of arthritis, primary dysmenorrhoea, fever, and as an analgesic, especially where there is an inflammatory component. Its mechanism of action is also through inhibition of COX-1 and COX-2 expression, inhibiting prostaglandin synthesis. Its analgesic, antipyretic and anti-inflammatory activities are achieved principally through COX-2 inhibition whereas COX-1 inhibition is responsible for its acting in platelet anti-aggregation and inducing gastro-intestinal mucosa ulcers (Andersohn *et al.*, 2006; Layton *et al.*, 2008).

The first generation of NSAIDs such as aspirin, can cause life-threatening stomach ulcers. More selective NSAIDs for example COX-2 inhibitors, which are designed to avoid side effects such as stomach ulcers, have unfortunately been associated with an elevated risk of AMI (Acute Myocardial Infarction) (Andersohn *et al.*, 2006; Brophy *et al.*, 2007; Greenberg *et al.*, 2009). However, there is also evidence to suggest that a regular intake of NSAIDs that non-selectively block COX-2 protects against the development of many types of cancer (Thun *et al.*, 2002; Greenhough *et al.*, 2009).

In order to discuss melanoma and the skin's essential defence mechanisms it is important to discuss inflammation and its role in cancer.

1.9. INFLAMMATION AND CANCER

The inflammation-cancer connection is especially intriguing as in some circumstances the immune system, which sustains inflammation, should inhibit tumour development. However, many studies (Balkwill and Mantovani, 2001; Coussens and Werb, 2002; Balkwill, 2004; Balkwill *et al.*, 2005; Kulbe *et al.*, 2004; Marx, 2004; Karin, 2005; Mantovani, 2005; Rollins, 2006, Hussain and Harris, 2007; Wang and Du Bois, 2008; Porta *et al.*, 2009) have shown that inflammation can also play a key role in exacerbating cancer development.

While many researchers have paid attention to observing the role of various genetic mutations in cell DNA in promoting abnormal cell growth that may lead to malignant cell clones, recently research workers are exploring the concept that both mutation and inflammation are major processes that if unchecked and untreated may lead to potentially malignant tumours with a poor prognosis.

Melanoma may be caused (among others) by excess of UV radiation exposure which suppresses the immune response in skin and it is believed that it might also be caused in part by uncontrolled inflammation of the skin. There is increasing evidence that strongly supports the link between inflammation, infection, and many types of cancer. Chronic inflammation has been associated with cancer of the lung, colon, pancreatic and bladder tumours (Shacter and Weitzman, 2002) whereas infections such as hepatitis B and C are associated with an increased risk for primary liver cancer (Perz *et al.*, 2006). Also, chronic infection with the ulcer–causing microorganism Helicobater pylori is the world's leading cause of stomach cancer (Ernst and Gold, 2000).

Hence, some types of cancer arise from tissues that have been irritated, infected, inflamed and wounded. During inflammation, cancer cells can produce chemokines to attract nutrients and other support structures to facilitate their survival. Some of these chemokines recruit neutrophils to a tumour. These in turn may facilitate cancer cells

travelling through blood vessels. It is therefore believed that cancer cells may take advantage of the expression of the adhesion molecules that help neutrophils to adhere to the blood vessel walls during extravasation and also use the same transport system from the vessels to the tissue. Infiltration of cancer cells may also occur during wound healing. Normal skin cells such as fibroblasts produce tissue remodelling substances and, neutrophils, macrophages, monocytes and lymphocytes produce growth factors which stimulate blood vessel growth. This allows normal tissue development that can also be utilized by tumour cells facilitating their survival and proliferation, promoting tumour development.

The metastatic sequence involves the detachment of cells within a primary tumour, local migration and invasion through the local tissue, intravasation and transit in the blood and lymphatic vessels, extravasation from vessels, formation of small sites of metastasis, local migration, attachment and eventually proliferation (Chambers *et al.*, 2002).

It is believed that in human tissues an inflammation-induced protein called NF-*k*B (Nuclear Transcription Factor–kappaB) may play a key role in cancer development (Barnes *et al.*, 1997). It is an intermediary in promoting cellular changes leading to the uncontrolled growth of cancer cells and also, to later changes that help metastatic cells escape from the original tumour and spread to new sites in the body. Also, NF-kB activity leads to inhibition of the programmed cell death (apoptosis) that can eliminate defective cells, thus contributing both to cancer development and resistance to drug and radiation therapies (Bradley, 2008).

1.10. Anti-inflammatories and Cancer

A review study has indicated that NSAIDs particularly the highly selective COX inhibitors, show promise as anticancer drugs (Harris *et al.*, 2005; Kast, 2007). Chronic

intake of NSAIDs may reduce carcinogenesis by inhibiting production of prostaglandins, cytokines, and angiogenic factors. NSAIDs may act by reducing the production of selected inflammatory factors.

Another review study showed a significant decline in cancer risk with increasing intake of NSAIDs (primarily aspirin and ibuprofen) for at least four major types of cancer: breast, prostate, colon and lung (Harris *et al.*, 2007). Preclinical investigations provide consistent evidence that both selective and non-selective NSAIDs effectively inhibit chemically-induced carcinogenesis of epithelial tumours (Harris *et al.*, 2005).

A third component that has been recognised as a NSAID is capsaicin (Dasgupta *et al.*, 1998; Patel *et al.*, 2002a; Patel *et al.*, 2002b). Capsaicin (or 8-methyl N-vanillyl 6-nonamide), is a chemical component mostly found in chilli peppers and is recognised for its ability to produce the burning hot sensation of chilli-laced spicy foods. Capsaicin has been part of the human diet and used as a medication for hundreds of years. An old popular belief stated that high and continuous intake of capsaicin has a prejudicial effect in the stomach. In addition to this, a report states that dietary capsaicin is a suspected carcinogen (Verschoyle *et al.*, 2007). However a review study revealed that capsaicin is not the cause of ulcer formation as it does not stimulate, but actually inhibits acid secretion. In fact it stimulates alkali, mucus secretions and gastric mucosal blood flow which all help in the prevention and healing of ulcers (Satyanarayana, 2006).

Initial reports of using capsaicin as an anti-inflammatory/anti-cancer medication came from Dasgupta *et al.* (1998) as a treatment for human bladder cancer. In their study, 20 patients had repeated instillations of intravesical capsaicin (1-2 mmol/l). Cystoscopy and bladder biopsies were performed before and after capsaicin for surveillance. No further pre-malignant or malignant changes were found in the biopsies of the patients who had repeated capsaicin instillations for up to 5 years. However, as

the morphological effects of chemical carcinogens may not be apparent for 10 years, it was suggested that further surveillance was necessary and is being continued.

Morré *et al.* (1996) reported that NADH (nicotinamide adenine dinucleotide) oxidase activity was inhibited preferentially in the A375 melanoma cells but not in the primary melanocytes by capsaicin. These authors suggested that this inhibition of cell surface NADH oxidase activity may be correlated to the inhibition of growth and capsaicin-induced apoptosis.

Morré *et al.* (1997) reported that capsaicin-inhibited NADH oxidase activity was found in sera over a broad spectrum of cancer patients including patients with solid cancers (*e.g.* breast, prostate, lung, ovarium), leukemias and lymphomas.

Brar *et al.* (2001) reported that catalase, N-acetylcysteine, ebselen, dicumarol and capsaicin also inhibited growth of melanoma and other malignant cell lines. These results raise the possibility that ROS (Reactive Oxygen Species) produced endogenously by mechanisms involving the NAD(P)H:quinone oxidoreductase (NQO) can constitutively activate NF-kappaB in an autocrine way. Hence suggestions may come up for new antioxidant strategies for interruption of oxidant signalling of melanoma cell growth. See Figure 1.8 for further explanation about TNF-α induced ROS activation.

Patel *et al.* (2002a) reported that down regulation of constitutive and induced NF-*k*B activation leads to inhibition of IL-8 expression in malignant melanoma cells. In the same year, Patel *et al.* (2002b) suggested that inhibition of NF-kB activation impedes cellular growth and proliferative signals in human melanoma cells. Further results from these authors were that the NF-kB activation may trigger stress-signalling pathways causing enhanced HIF-α (Hypoxia Inducible Factor-alpha) expression and it's binding to HRE (Hypoxia Response Element). Hence this leads to an enhanced VEGF (Vascular Endothelial Cell Growth) production by the remaining melanoma cells.

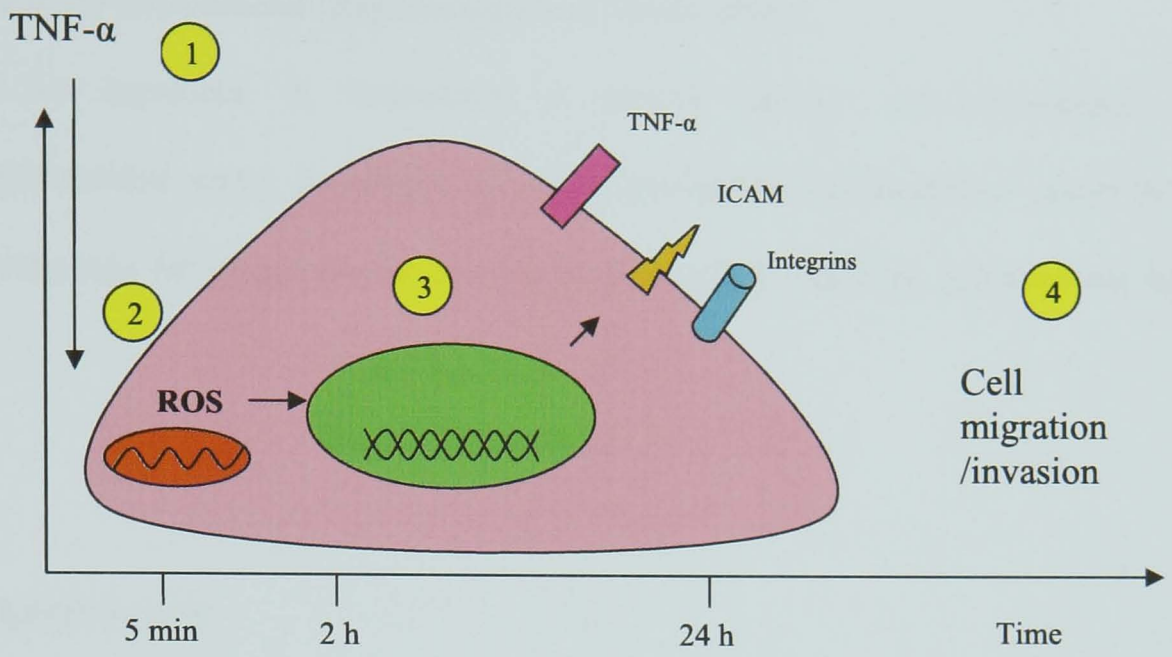


Figure 1.8. Inflammation at the molecular level: TNF-α induced ROS and NF-kB activation. (1) TNF-α entrance to the cell and inducing of ROS; (2) Increase of ROS in mitochondria; (3) Translocation of nuclear transcription factor NK-kB from cytoplasm to nucleus, activation of p65 gene. TNF-α protein synthesis and migration to cell periphery; (4) Extrusion of TNF-α receptors on cell surface, induces its own production by cell activation in an autocrine and paracrine fashion. This leads to enhanced ICAM-1 and integrin expression. As a result, increased cell surface adhesion and infiltration of cells is facilitated.

A China-Japan research group (Gong *et al.*, 2005) studied the mechanisms of capsaicin-induced apoptosis of human melanoma A375-S2 cells in a time- and dose-dependent manner. They found that capsaicin induces A375-S2 cell apoptosis and that down-regulation of ICAD (Inhibitor of Caspase Activated DNases) contributes to this process.

Mori *et al.* (1996) reported that capsaicin inhibited NF-kB activation by preventing its nuclear migration. They suggested that given the clinical tolerability of capsaicin a clinical trial of this agent seems appropriate in selected individuals with prostate cancer.

Capsaicin combined with genistein demonstrated to have an anti-inflammatory and anticarcinogenic properties (Hwang *et al.*, 2009), to act as non-steroidal anti-

inflammatory drug (Mózsik *et al.*, 2009) and to inhibit platelet aggregation and thromboxane biosynthesis (Raghavendra and Naidu, 2009).

As capsaicin is considered a natural dietary anti-inflammatory and chemopreventive agent, its effects on cancer prevention and treatment seems to be a promising area for people at risk and, for those needing treatment, and deserves further study.

1.11. APOPTOSIS

In this study, after observing the effects of NSAIDs on melanoma cell migration and viability, the effects of NSAIDs on melanoma cell viability was further investigated using apoptosis assays.

Apoptosis means "programmed cell death". It is an essential process that allows embryonic development (*e.g.* finger separation during foetal development) and physiological maintenance in multicellular organisms. Apoptosis enables the body to regulate homeostasis by eliminating dysfunctional and unwanted cells thus avoiding diseases or infection. Apoptosis can be induced by the tumour suppressor protein p53 which is a major senescence and cell death inducing transcription factor (Moll *et al.*, 2005; Speidel, 2009). p53 has also an additional extra cellular effect on cell-cycle arresting (Galluzi *et al.*, 2008).

Apoptosis differs from necrosis. Apoptosis affects individual cells within a multicellular tissue or organ which are phagocyted by macrophages and other phagocyte-like cells. It is stimulated by physiological inductions, such as lack or excess of signals (*e.g.* lack of growth factors or excess of ultraviolet light), and very importantly, it does not trigger an inflammatory response. Morphological changes in apoptotic cells are recognised by cell shrinkage and eventual whole cell fragmentation

without the release of cellular contents into the tissue vicinity. On the other hand, necrosis is caused by chemical and physical agents such as toxins, lack of oxygen, radiation and trauma. Morphological changes in necrotic cells are swelling and the appearance of holes in the plasma membrane. Once homeostasis is lost, plasma membrane and intracellular contents are released into the surrounding microenvironment. Necrosis affects partial or regional tissue or cell groups in which phagocytosis of macrophages occurs and an unavoidable inflammatory response takes place. Inflammation and necrosis may turn into an expanded process affecting the surrounding tissue area and resulting in a poor prognosis if not treated.

Basically, apoptosis involves activation of many cellular agents that lead to morphological and biochemical changes in the cell. The entire apoptosis process is not understood yet. Apoptosis is induced by stimulation of cell membrane surface receptors and/or activation of the mitochondrial apoptotic pathway, leading to changes in the cytoplasm and nuclear changes and ultimately, whole cell fragmentation.

1.11.1. Apoptosis pathways – The participation of the caspases cascade.

Apoptosis initiates as the signal molecules (ligands) released by other cells bind to specific transmembrane receptors. The accumulation of these receptors on the cell membrane recruits proteins in the cell cytoplasm which then activate the caspase cascade. Caspases are cysteine-dependent, aspartate-specific proteases, existing in the cell cytoplasm in an inactivated form of pro-caspases. Once caspases are activated the process reaches an irreversible apoptotic pathway.

The first mammalian or human caspase (caspase-1 or ICE – Interleukin Converting Enzyme) was identified as an important regulator of the inflammatory process (Yu and Finlay, 2008). Caspases can be divided into two different groups: the

initiator caspases, including caspase-2, -8, -9 and -10 and, the effector caspases which include caspase-3, -6, and -7 (Riedl and Shi, 2004).

Mediation of apoptosis can be divided into two pathways: (1) by stimulation of transmembrane receptors or via ***receptor-mediated or extrinsic pathways*** (such as Fas receptors by Fas ligand or TNF-α mediated) in the plasma membrane (Csipo *et al.*, 1998). The cell receptor-mediated pathway activates the initiator caspase-8; and, (2) by the release of mitochondrial factors or, via a ***mitochondrial or intrinsic pathway*** which occurs in the cell mitochondria (Riedl and Shi, 2004).

The apoptosis mitochondrial pathway is associated with the pro-apoptotic Bcl-2 family of proteins and this stimulates the release of cytochrome c and other proteins which activates the initiator caspase-9. The initiator caspase-9, cytochrome c and ATP (Adenosine Triphosphate) collaborate to form apoptosome, which in turn activates the effector caspase-3 and, cell degradation and DNA fragmentation begins. Cell fragments are engulfed by macrophages and a neat and organised clean up occurs within the surrounding tissue.

It is worth noting that the intrinsic and extrinsic pathways are activated through the caspase-8-mediated cleavage of BID, a BH3-only member of the Bcl-2 family of proteins, which eventually activate caspase-3 and initiates the release of mitochondrial proteins. This ends up in cell degradation and fragmentation, making this system very efficient.

In the cell cytoplasm, the outer membrane of the mitochondria becomes permeable and proteins such as cytochrome *c*, Smac/DIABLO (second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI), and Apoptosis-inducing Factor (AIF), located in the intermembrane space are released into the cytoplasm (Riedl and Shi, 2004). The release of cytochrome *c* is followed by the loss of the mitochondrial transmembrane potential which results in the release of

Ca^{2+} and the disruption of electron transport. The mitochondrial outer membrane permeabilization is regulated by proteins, specially the Bcl-2 family members of proteins Bax and Bak. It is believed that the so-called pro-apoptotic members of Bcl-2 proteins Bax and Bak support mitochondrial outer membrane permeabilization resulting in the activation of caspase 9, marking the end of this phase.

Alterations in the cell nucleus occur as DNA is cleaved into large fragments. This is followed by further cleavage into small nucleosomal units. The DNA fragmentation is a response of caspase-activated DNase (CAD). Normally in the cell, CAD is kept inactivate by a complex of inhibitor of CAD (ICAD). If an apoptotic signal is received, such as from caspase-3, ICAD is cleaved and CAD is released to fragment DNA.

1.12. APOPTOSIS AND CANCER

The study of apoptosis has gained much attention in health management as apoptosis has been shown to be closely related to several diseases such as autoimmune diseases, AIDS and cancer (Fischer and Schulze-Osthoff, 2008). In cancer, the build up of mutations within cells means that these cells lose their ability to follow normal apoptotic pathways and hence transform into immortal cells.

Alterations in the mitochondrial pathway are often a common cause in the first steps to cancer development. This is because mitochondrial stress is easily caused by DNA damage and heat shock. Mutation of the p53 protein is a common cause of tumour genesis as it regulates DNA repair, senescence and cell life-cycle (Guida *et al.*, 2008). In a recent study (Freedberg *et al.*, 2008) identification of $p14^{ARF}$ as the 9p21 gene was the most commonly inactivated in melanoma than p16. The role of p16 inactivation in melanoma is well known whereas p53 mutation is well established in the development

of many types of cancer (Rodolfo *et al.*, 2005). However the contribution of p53 in melanoma development needs to be elucidated.

Apoptosis is regulated by the Bcl-2 family of proteins, which modulate outer mitochondrial membrane permeability. This family of proteins is categorised as anti-apoptotic members (such as Bcl-2, Bcl-xL and, Mcl-1) as well as pro-apoptotic members, which consist of multidomain proteins (such as Bax, Bak, Bid, Bim, and Bik) (Tsujimoto, 2003).

As a prototypic member of this family, Bcl-2 can contribute to neoplastic cell expansion by preventing normal cell turnover caused by physiological cell death mechanisms. High levels of Bcl-2 gene expression are found in a wide variety of human cancers (Reed, 1999). In addition, Bcl-2 is implicated in chemoresistance as over-expression of Bcl-2 can inhibit the killing effect of many current anticancer drugs by blocking the apoptotic pathway (Bettaieb *et al.*, 2003; Fernandez-Luna, 2008; Marzo and Naval, 2008).

Therefore, the inhibition of over-expressed Bcl-2 protein in tumour cells could be used as a strategy for either restoring the normal apoptotic process or making tumour cells more susceptible for conventional chemotherapy.

It has been demonstrated that HA14-1 (2-Amino-6-bromo-α-cyano-3-(ethoxycarbonyl)-4*H*-1-benzopyran-4-acetic acid ethyl ester) can bind to Bcl-2 protein and induce apoptosis of tumour cells (Wang *et al.*, 2000). Thus this supports a role for Bcl-2 over-expression being linked to tumour development, inhibition of apoptosis and, being associated with chemotherapy resistance.

It has also been suggested by some authors that HA14-1 may represent a promising candidate for treatment of drug resistant cancer either as a monotherapy on in combination with current cancer therapies (Tian *et al.*, 2007).

In summary all of this research suggests that regulation of apoptosis may be a central part in tumour development.

1.13. BACKGROUND OF THIS WORK

The current research is based on a growing literature associating inflammation with cancer progression and in the case of melanoma, the phenomenon of "local recurrence".

With respect to inflammation and melanoma, recent studies from the MacNeil group have shown that TNF-alpha increases melanoma cell invasion and migration in vitro (Katerinaki *et al.*, 2003) and that anti-inflammatory agents such as α-MSH (alpha-Melanocyte Stimulating Hormone) can reduce melanoma cell invasion and protect cells against proinflammatory cytokine attack in cells with the wild-type receptor (HBL) (Eves *et al.*, 2003). Interestingly, α-MSH has been shown to act as a "protector" of melanoma cells from proinflammatory cytokine action, but also to have anti-invasive abilities which are consistent with α-MSH working in an anti-inflammatory capacity (Zhu *et al.*, 2002).

Another study (Cantón *et al.*, 2003), reported that TNF-α significantly increased the invasiveness of uveal melanoma cell lines in the aqueous humor , while α-MSH reduced the invasion of cells through human fibronectin by 45-50% and also protected uveal melanoma cells from the pro-invasive actions of TNF-α. These data are consistent with the hypothesis that inflammation is playing a major role in affecting the metastatic ability of uveal melanomas.

Recent results from this group have shown that sodium salicylate can effectively inhibit TNF-alpha-induced upregulation of NF-kappaB and ICAM-1 expression during *in vitro* migration and invasion of human melanoma cells. This indicates that non-

steroidal anti-inflammatory drugs may be a useful therapeutic approach to oppose inflammation-induced melanoma invasion and metastasis (Katerinaki *et al.*, 2006).

With respect to local recurrence of melanoma this is a phenomenon in which melanoma cells invade an inflamed wound created after surgical excision of a primary melanoma.

There are many interactions that may influence cancer cell progression within their environment and influence their survival and metastases. Therefore, to be able to study melanoma cell migration through a structure that resembles as much as possible an *in vivo* physiological microenvironment, a 3-D model of reconstructed human skin was used.

This model was also wounded and inflammation induced to represent some of the conditions which occurs following melanoma excision. In this model, normal skin cells are added to de-epidermised dermis (skin without the original epidermal layer), which retains a residual BM. Melanoma cell lines can then be added into the reconstructed human skin and melanoma cell migration and invasion occurs during the incubation period. The disadvantages of this methodology are the difficulty of tracking cell movements with time requiring the sacrifice of many samples of the reconstructed human skin for histology.

Tissue engineered skin was discovered by the classic experiments of Rheinwald and Green in 1977 (Rheinwald and Green, 1977; Price *et al.*, 2008). Reconstructed human skin as a 3-D model was established by Chakrabarty *et al.* (1999). This study demonstrated a successful method for sterilization of human dermis, achieving an acellular dermis and retention of collagen IV, allowing clinical use as dermal replacement material and also for production of dermal-epidermal composites in *in vitro* studies. A further study showed that the reconstructed skin model could be used to

investigate interactions between melanoma cell lines and normal fibroblasts and keratinocytes, as well as the components of the basement membrane (Eves *et al.*, 2000).

TNF-α is a primary mediator of the inflammatory response and has been ascribed a wide range of biologic activities. TNF-α regulates gene expression in epidermal keratinocytes and acts as a key component in organizing a response to skin injury. Hence it is involved in regulating a wide scope of biological processes ranging from immune responses to cell migration, epidermal differentiation and tissue repair (Banno *et al.*, 2004; Artuc *et al.*, 2006).

Experimental studies are consistent in terms of the tumour promoter effect of TNF- α (Pikarsky *et al.*, 2004). Some workers have demonstrated that TNF-α is a cytokine that has been associated with tumour progression (Szlosarek *et al.*, 2006). Others have suggested further exploration of TNF blockade as an adjunct to cancer therapies (Monk *et al.*, 2006).

1.13.1. Aims and Objectives

The aim of this work was to investigate the role of inflammation in melanoma invasion using a 3D model of tissue engineered skin and to study the role of NSAIDs in these models. The justification for this work is based on the serious nature of melanoma as a cancer which is particularly difficult to treat with a low survival rate upon late diagnosis. There are many studies re-enforcing the relationship between inflammation and cancer.

Our hypothesis is that NSAIDs may be an important approach to blocking or reducing the negative effects of inflammation on melanoma cell migration, invasion and survival.

The Specific objectives were:

1. To study the effects of inflammation on melanoma cell migration. This was achieved using a two-dimensional (scratch wound) model using three different melanoma cell lines. The effects of NSAIDs (ibuprofen released from a hydrogel and capsaicin) were studied on melanoma cell migration, opposing to the pro-inflammatory cytokine TNF-α.

2. To develop a 3D model of a fibrin clot to observe melanoma cell invasion.

3. To investigate melanoma cell invasion and the effects of NSAIDs in a 3D model of reconstructed human skin.

4. To develop and establish a skin wound model using a tissue engineered human skin model in which to study the effects of wounding and inflammation on melanoma invasion.

5. To examine the effect of TNF-α on melanoma cell NF-kB transcription factor activation.

6. To investigate the effect of two combined drugs (one NSAID, capsaicin and, a pro-apoptotic agent, HA14-1) on melanoma cell survival by assessing cell viability, and cell apoptosis assessed using Annexin V and DNA damage assays.

CHAPTER 2

MATERIALS AND METHODS

2.1. MATERIALS

Cell culture media, antibiotics and additives were purchased from Gibco BRL (Life Technologies, Paisley, UK) and Sigma Aldrich Ltd (Poole, UK). Foetal calf serum (FCS) was purchased from GlobePharm Limited (Esher, UK); newborn calf serum (NBCS) was obtained from Sigma-Aldrich Ltd (Poole, UK); trypsin/ethylenediamine-tetraacetic acid (EDTA) and phosphate-buffered saline (PBS) tablets were purchased from Oxoid Ltd (Basingstoke, UK). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide] for viability test; TNF-α, (Tumour Necrosis Factor-alpha), Sodium Ibuprofen (NaIbu) and Capsaiçin as NSAID were purchased from Sigma Aldrich Ltd (Poole, UK). Pluronic F-127 polymer was also purchased from Sigma Aldrich Ltd (Poole, UK). Fibrinogen and thrombin (from bovine plasma) were purchased from Sigma-Aldrich Ltd. For protein assay, BCA™ Protein Assay Reagent A and BCA™ Protein Assay Reagent B were purchased from Pierce US. HA14-1 was purchased from Tocris™. Twelve well culture plates were purchased from Costar Corning Incorporated; and inserts (ThinCert), pore diameter 8.0 μm, were purchased from Greiner Bio-One, UK. Six and twenty-four culture well plates were purchased from Costar Corning Incorporated.

For NF-kB activation in melanoma cells by immunolabelling for the intracellular position of the p65 gene, the following materials were used: TNF-α was purchased from Sigma Aldrich™; 10% (w/v) paraformaldehyde purchased from BDH/Merck, Poole, Dorset, UK in phosphate-buffered saline (PBS); unreacted binding sites were blocked with 5% (w/v) dried milk powder (in PBS). Primary anti-NF-kB/p65 (C-20) goat polyclonal IgG antibody sc-109 was purchased from Santa Cruz Biotechnology, California, and secondary biotinylated anti-goat IgG antibody BA-5000 from Vector Laboratories Inc., California. Fluorescein streptavidin SA-5001 was purchased from Vector and DAPI (4',6-diamino-2phenylindole) from Sigma-Aldrich™.

For DNA Damage Assay (fluorescent), the kit containing reagents were purchased from Active Motif Europe, Belgium.

For cell viability and apoptosis assay a Guava Nexin™ Kit was purchased from Guava Technologies Inc. USA. Camptothecin was purchased from Sigma Aldrich™.

Anticollagen IV antibody, rabbit-derived S-100 antibody: mouse-derived HMB45 antibody; and, mouse anti-cytokeratin AE1AE3 antibody were purchased from Dako (Carpintera, CA, U.S.A.). All other chemicals were of analytical grade.

2.2. CULTURE OF MELANOMA CELLS

2.2.1. Culture of C8161 melanoma cells

The human C8161 melanoma line was established from an abdominal wall metastasis from a menopausal woman with recurrent melanoma (kindly donated by Professor F. Meyskens, University of California, Irvine, USA, via Professor M. Edwards University of Glasgow, UK). Cells were cultured in Eagle's modified essential medium (EMEM) containing 4.5 g/L glucose, supplemented with 10% (v/v) FCS, 2mM L-Glutamine, 100 units/ml penicillin, and 100μg/ml streptomycin sulphate, 1.2 mg/ml amphotericin B, 1.5% (v/v) (of 100x stock solution) vitamin concentrate, 1mM sodium pyruvate, 1% (v/v) non essential amino acids (NEA) and 0.187% (w/v) sodium hydrogen carbonate (Sigma-Aldrich, Poole, UK). Cells were incubated at 37°C in a humidified 5% carbon dioxide/95% air environment under standard conditions. Cells were passaged when 80-90% confluent using 0.02% (w/v) EDTA for five to ten minutes, and pelleted at 300g for experiments and for expanding the cell stock. Cells were used between passages 10 – 60 for experimentation.

2.2.2. Culture of A375SM melanoma cells

The melanoma cell line A375-SM was a generous gift from Professor IJ Fidler (USA) via Professor MJ Humphries (University of Manchester, UK). The A375 melanoma cell line was established in culture from a lymph node metastasis. These cells are heterogeneous in nature and a highly metastatic variant (A375-SM) was established in culture from lung metastases produced by parental A375 cells growing subcutaneously in nude mice (Kozlowski *et al.*, 1984). C8161 and A375SM melanoma cells were cultured in Eagle`s modified essential medium (EMEM) containing 4.5 g/L glucose, supplemented with 10% FCS, 2mM L-Glutamine, 100 units/ml penicillin, and 100μg/ml streptomycin sulphate, 1.2 mg/ml amphotericin B, 1.5% (of 100x stock solution) vitamin concentrate, 1mM sodium pyruvate, 1% non-essential amino acids (NEA) and 0.187% sodium hydrogen carbonate, all purchased from Sigma-Aldrich Ltd (Poole, UK). Cells were incubated at 37 °C in a humidified 5% carbon dioxide/95% air environment under standard conditions. Cells were passaged when 80-90% confluent using 0.02% (w/v) EDTA for five to ten minutes, and pelleted at 300g for experiments and expanding stock. Cells were used between passages 30-60 for experimentation. When C8161 and A375SM melanoma cells were passaged prior to being added to the wells they were resuspended in EMEM.

2.2.3. Culture of HBL Melanoma Cells

The human cutaneous melanoma cell line HBL was originally established and described by Professor Ghanem Ghanem (Laboratory of Oncology and Experimental Surgery, Free University of Brussels, Belgium) from a lymph node metastasis of a nodular melanoma (Ghanem *et al.*, 1988). Cells were cultured in Ham's F10 medium (Gibco: Paisley, Scotland) containing 1 g/L glucose, supplemented with 5% (v/v) fetal calf serum (FCS), 5% (v/v) newborn calf serum (NBCS; Advanced Protein Products,

UK), 2mM L-Glutamine, 100 units/ml penicillin, 100μg/ml Streptomycin, 250μg/ml amphotericin. Cells were cultured in T75 vented flasks at 37°C in a humidified atmosphere of 5% CO_2/95% air. Cells were passaged when 80-90% confluent using 0.02% (w/v) EDTA for five to ten minutes, and pelleted at 300g for experiments and expanding stock. Cells were used between passages 17 and 50.

2.3. PREPARATION OF SOLUTIONS

2.3.1. TNF-α Solution Preparation

TNF-α solution was prepared from a 10μl stock containing 10.000 U/ml. TNF-α was dissolved in media to make concentrations as desired.

2.3.2. Pluronic Hydrogel Characteristics

Pluronic F-127 is a triblock copolymer consisting of polyethylene glycol (PEG) and polypropylene glycol (PPG) arranged as $[PEG]_n$-$[PPG]_m$-$[PEG]_n$ where n and m are the number of monomer units in the PEG and PPG blocks, respectively. Pluronic solutions undergo a reversible transition on warming from a liquid solution to a clear, transparent, soft gel on heating. This transition is reversible. Gel temperature decreases with increasing polymer content, and, for a 30% Pluronic gel, this occurs around 13°C. Below the transition temperature the gel reverts to a liquid which can be readily pipetted.

2.3.3. Ibuprofen Sodium Salt (NaIbu) Hydrogel Preparation

NaIbu (in powder) was weighed and dissolved in sterile PBS to give a desired concentration, then passed through a 0.20 μm pore filter (non-pyrogenic sterile – R). Hydrogels were prepared using 30% (w/v) Pluronic F-127 polymer dissolved in PBS.

Dissolution of the polymer was achieved by holding for approximately one week at approximately 5°C. On subsequent warming to room temperature, the liquid sol transformed to a soft gel. The gel was sterilised in an autoclave. The hydrogel was then cooled down to -20°C which enabled the incorporation of NaIbu solutions at a ratio of 87% volume hydrogel to 13% volume NaIbu to achieve final concentrations of $33x10^{-6}$, $66x10^{-6}$, $99x10^{-6}$, 131 $x10^{-6}$ M NaIbu hydrogel (which correspond to NaIbu concentrations of 0.05%, 0.10%, 0.15% and 0.20%). 13% volume of PBS only added to the hydrogel gave a concentration of 0% at unloaded gel.

2.3.4. Release of Ibuprofen Sodium Salt from F127 Hydrogel

The release profile of the drug from the gel loaded with $131x10^{-6}$M ibuprofen sodium salt was obtained by assessing the amount of drug released from gels held in PET cell co-culture baskets with porous PET membranes in 12 well plates. 300µl aliquots of gel cooled below the gel point were pipetted into the baskets. The baskets were suspended in 12 well plates in an incubator at 37°C, at which temperature gelation occurred. 1ml of distilled water was placed in each well on the outside of the basket. At times of 2, 4, 6, 8, and 24 hours baskets were removed. Three wells were used for each time point. The content of sodium ibuprofen which had been released from the gel into the distilled water was then determined from the characteristic ibuprofen UV absorbance at 264nm using a UV spectrometer.

2.3.5. Capsaicin (8-Methyl-N-vanillyl-*trans*-6-nonenamide) Solution Preparation

Capsaicin is an active ingredient in chilli peppers and it gives a pungent flavour to food. Capsaicin is used medically to deal with pain (Tesfaye, 2009). A stock solution of capsaicin (in powder) 0.328 M was made by dissolving 50 mg in 0.5 ml of ethanol.

Different concentrations of capsaicin were obtained by adding the stock solution into cell culture medium to give the desired concentrations in μM.

2.3.6. HA14-1 (2-Amino-6-bromo-α-cyano-3-(ethoxycarbonyl)-4*H*-1-benzopyran-4-acetic acid ethyl ester) solution preparation

HA14-1 is a small molecular compound which interacts with Bcl-2 protein and inhibits the anti-apoptotic effect of Bcl-2. A stock solution of HA14-1 (in powder) 0.024436 M was made by dissolving 10 mg in 1 ml of ethanol. Different concentrations of HA14-1 were obtained by adding the stock solution into cell culture medium to give the desired concentrations in μM.

2.4. ASSAYS

2.4.1. Migration Assay

Prior to seeding cells, the underside of the tissue culture wells (12 well plates) were marked with 3 horizontal lines with an indelible pen to provide reference points for relocating scratches post wounding. Cells were then plated in 12-well culture plates in supplemented EMEM + 10% FCS at a density of $1x10^5$ cells / ml per well and were incubated for 3 days under standard culture conditions. On the third day, a "scratch wound" for migration assay (Cha *et al.*, 1996) was made in each well using a plastic pipette tip, traversing the three horizontal lines. This created a cell free zone in each well. Scratches greater than 700μm or less than 300μm were omitted from the experiment at time point 0. For studies looking at drug effects on TNF-α stimulated migration, TNF-α was added on the second day, for a 24 hour period.

The culture medium was then removed and replaced with an equal volume of fresh medium. For experiments using Nalbu Hydrogels, inserts (ThinCerts™ Greiner

Bio One) containing 300µl Nalbu hydrogels were added to each well and submerged within the same medium that covered the melanoma cells to allow diffusion of drug from the hydrogels. For experiments using capsaicin, 1 ml of media was added to each well at the desired capsaicin concentration. Photographs were taken at predetermined intervals of 0, 4, 8 and 24 hours after the scratch was made on the cell monolayer, using an Apple MacIntosh video microscope (Open Lab 3.0.4 software). Measurements of the distance between the two edges of the scratch were recorded for each different time point. The reduction of distance between the scratch edges at different time points represented the migration (in µm) of melanoma cells. Triplicate measurements were obtained from each experiment guided by the three horizontal lines. The 12 well-culture plates with cells and inserts were incubated at 37 °C, 5% CO_2 during experiments.

2.4.2. Cell Viability Assay

At the end of migration and other viability assays, the same plates containing melanoma cells, fibroblasts, melanocytes and keratinocytes were used for an MTT assay. This was carried out to assess the toxicity of sodium ibuprofen, capsaicin or HA14-1 on melanoma and skin cells. MTT acts as an artificial hydrogen acceptor substrate which forms a coloured formazan product, which is then eluted from the cells using acidified isopropanol. This cyto-biochemical assay provides a direct indication of the viability of cells and can be used to provide an indirect reflection of cell number.

The inserts and media were removed and cells were washed in PBS. MTT solution (0.5mg/ml PBS/well) was added to each well and incubated at 37°C for 40 minutes. Following removal of MTT 300 µl acidified isopropanol was added to elute the stain. The result was read on a multi-well scanning spectrophotometer MRX –II Microplate Reader – Dynex Technologies using Revelation software set at a wavelength of 540 nm with a reference wavelength of 630 nm.

2.4.3. Melanoma Cell Degradative Capability in Fibrin Clot Scaffolds Assay

This method was used in order to evaluate the protein quantity from fibrin clots left in the wells by melanoma cells after a period of 24 hour incubation. Initially, a fibrin clot scaffold was added to the well and cells were added and incubated for a period of 24 hours. It was observed that after this incubation period, cells totally or partially degraded the fibrin clot, making it impossible to study the cell invasion. However, the degradative capacity of the melanoma cells could be measured using a simple protein assay in which the amount of fibrin clot protein could be stained and analyzed in a plate reader.

2.4.3.1. Fibrin Clot Scaffold Preparation

42 mg of plasminogen-free bovine fibrinogen was dissolved in 24 ml of sterile PBS, giving a 57% fibrinogen solution. After mixing thoroughly with bovine thrombin (10 U/ml), the fibrinogen solution was quickly transferred into a 24 well-culture plate at a volume of 0.5 ml/well. This was incubated overnight to in order allow thrombin to convert fibrinogen to fibrin which polymerizes forming an insoluble clot.

2.4.3.2. Seeding Melanoma Cells on a Fibrin Clot Scaffold

C8161 and A375SM melanoma cells were seeded at a density of $5x10^4$ cells/ml per well and HBL cells were seeded at a density of $4x10^4$ cells/ml per well. The cells were then incubated for 24 hours. The protein assay was then performed as described below (2.5.4).

2.4.3.3. Melanoma Cell Visualization on Fibrin Clot scaffold

Cell culture wells could provide melanoma cell visualization within the gel using light and immunofluorescence microscopy without sectioning the fibrin scaffold.

300 nM DAPI was used to stain all cell nuclei. After 24 hours, the wells were gently washed three times with PBS, fixed in 4% (w/v) paraformaldehyde for 30 minutes and permeabilised with 0.2% (w/v) Triton X-100 in PBS for 5 minutes. After 3 final washes with PBS, epifluorescent images were taken using an Apple MacIntosh video microscope (OpenLab™ 3.0.4 software) for DAPI/nuclei visualization.

2.4.3.4. Fibrin Clot Protein Assay

After the incubation period, the volume (ml) of medium left was measured in each well using a 1ml plastic pipette. A protein assay was performed to measure the amount of the fibrin protein not degraded by melanoma cells.

After removing the media, the wells were washed twice with PBS. 500µl of Digestion Buffer (0.04% SDS (sodium dodecyl sulphate) diluted in PBS plus 8M or 2 M urea solution) was added and incubated for 30 minutes. Using the Protein Assay Kit, a mixture of 200 µl of reagent B and 10 ml of reagent A was made. After the incubation period, 10 µl of degraded fibrin clot protein was transferred into a 96 well plate. 200 µl of the reagent mixture was added to each well and incubated for 30 minutes. The result was read on a multi-well scanning spectrophotometer MRX –II Microplate Reader – Dynex Technologies using Revelation software set at a wavelength of 540 nm with a reference wavelength of 630 nm.

2.4.4. DNA Damage Assay – Apoptosis Investigation

Double stranded DNA breaks were measured as a marker of apoptosis using a fluorescent DNA Damage Assay (Active Motif, Carlsbad, CA). Cells were seeded at a density of $1x10^5$ cell/250µl per well in a 96 well plate and were cultured in the presence of various concentrations of capsaicin for 24 hours. Control cell were incubated with medium alone (negative control) or 20 µM camptothecin (as positive control).

Camptothecin is originated from a deciduous tree found in southern China and was used as cancer treatment (Cao *et al.*, 2009). It is known to be a cell proliferation inhibitor and apoptotic agent (Ding *et al.*, 2009) which blocks the enzyme topoisomerase-I and stops DNA replication within the cell (Punchihewa *et al.*, 2009). Cell were fixed with ice-cold methanol for 10 minutes, washed, incubated with 5% BSA in PBS for 1 hour at RT and washed again. Cells were incubated at 4°C overnight with an anti-DNA break antibody (Anti-phospho-H2AX (ser 139)), washed and incubated with a fluorescently labelled secondary antibody (Chromeo™ 488 Goat anti-Rabbit IgG) for 1 hour at RT. Washed cells were incubated with propidium iodine (PI) for 30 minutes to stain all fixed cells. The plate was then scanned using a fluorescent plate reader and the ratio of DNA-damaged cells to total cells was calculated. A ratio of PI and Chromeo 488 was calculated and converted to percentage of viable cells.

2.4.5. Immunofluorescent Labelling of NF-kB/p65 in Melanoma Cells

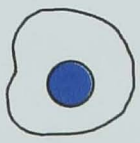
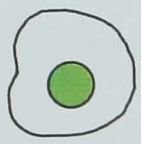
An immunofluorescent labelling assay was used to assess the NF-kB activation for the intracellular position of the p65 transcriptionally active subunit in cultured melanoma cells. Melanoma cells were cultured until 60% confluence and stimulated with TNF-α (U/ml) for 1-2 hours. Experiments were stopped by addition of 10% (wt/vol) paraformaldehyde in PBS. Cells were washed three times in PBS between treatments. Unreacted binding sites were blocked with 5% (w/v) BSA (Bovine Serum Albimin) or milk powder in PBS for 30 minutes. Cells were then incubated with the primary anti-NF-kB/p65 (C-20) goat polyclonal IgG antibody for 1 hour at room temperature (1:100 (v/v) in PBS). Cells were washed with PBS (x3) then incubated with biotinylated anti-goat IgG antibody (1:1000 vol/vol in PBS) for 1 hour at room temperature, washed with PBS (x 3), followed by incubation with fluorescein streptavidin (1:100 v/v in PBS) and 300 nM DAPI for 30 minutes.

NF-kB/p65 images were captured using Fluorescein isothiocyanate (FITC) fluorescence for NF-kB cellular position and DAPI for nucleus localisation (See Table 2.1). The boundary between the cell nucleus and cytoplasm was circumscribed by ImageXpress analysis software (ImageXpress Console 1.0)

Fluorescence micrographs of immunolabelled samples were taken by illumination at λ_{ex} = 495nm, λ_{em} = 515nm (for fluorescein isothiocyanate (FITC)/NF-kB visualisation) and λ_{ex} = 358nm, λ_{em} = 461nm (for DAPI/nuclei visualisation).

Cell nuclei were first located using DAPI (blue colour) signal alone giving the total number of cells in a focused area. NF-kB/p65 images were captured using Fluorescein isothiocyanate (FITC, green colour) for NF-kB cellular position in the cell nucleus. Therefore NF-kB activation was assessed by counting the total number of cells (100 to 200 cells located by DAPI, in a focused area at 20x magnification) and the number of cells showing NF-kB/p65 activation in the cell nucleus, giving a percentage of activated cells.

Table 2.1. TNF-α stimulated melanoma cells.
Modified from Moustafa *et al.*(2002) and Grubb(2007).

DAPI labelling for cell/nucleus location	FITC Immunolabelling of NF-kB for location of p65
	

2.4.6. Flow Cytometric Investigation of Apoptosis by Looking at Loss of Membrane Integrity and Annexin-V Binding

Cells were plated at a density of $5x10^4$ cells/ml per well in 24 well plates (Costar) in their respective culture media and incubated for 72 hours. After this incubation period cell media was removed and 1 ml of treatment solution (e.g. TNF-α, NaIbu, Capsaicin or HA14-1) at desired concentrations was added to the cells and incubated for 24 hours. Control samples were incubated with media only. Camptothecin (20μM) was used as a positive control.

Cells were prepared for flow cytometry by removing culture media, washing with PBS (x2) and detached using 0.02% (w/v) EDTA. Aspirated culture medium and PBS washes (potentially containing late apoptotic and dead cells) were combined together with detached cells and centrifuged at 300 g for 5 minutes. Cell pellets were resuspended in 150 μl cold PBS buffer and divided into three parallel samples. Samples were then used to measure cellular viability or investigate phosphatidylserine (PS) externalisation.

2.4.6.1. Determination of Externalised Phosphatidylserine by Annexin-V-PE Binding: Guava Nexin™

The Guava Nexin™ assay exploits the use of Annexin V which has a strong affinity and specificity in the presence of calcium for phosphatidylserine (PS). The externalisation of PS to the cell surface is one of the early apoptotic events. The kit includes Annexin V conjugated phycoerythrin (PE; λ_{ex} = 480nm; λ_{em} = 578nm) and 7-aminoactinomycin D (7-AAD; λ_{ex} = 555nm; λ_{em} = 655nm), a viability stain. 7-AAD is excluded from live cells and binds to GC rich regions of DNA within the cell allowing identification and distinguishing between apoptotic cells (Annexin V positive) in early (7-AAD negative) and late (7-AAD positive) stages. Annexin V is able to access

internal PS as a result of increased permeability so simultaneous staining with 7-AAD is essential. The use of the Nexin® kit to quantify external PS has recently been described (Moustafa *et al.*, 2002). Briefly, cells (~ $1x10^5$) suspended in 100µl cold PBS buffer were washed once with 1 ml ice cold 1 x Nexin® buffer (Guava Technologies) and centrifuged at 300 g for 5 minutes. Cells were resuspended in 40µl ice cold 1 x Nexin® buffer and incubated on ice in the dark with 5µl Annexin-V-PE and 5µl 7-AAD for 20 minutes. 450µl 1 x Nexin® buffer was added to each tube, gently agitated and measurement of 1000 single cell PE versus 7-AAD fluorescence events were made via flow cytometry (Guava PCA). Acquired data was analysed using Cytosoft (v 2.0) software, with differences in apoptosis determined by relative PE versus 7-AAD fluorescence.

2.5. SKIN COMPOSITES

2.5.1. History

Engineered human skin *in vitro* has been developed in the Kroto Laboratory with the objective of treating patients with burns, who need extensive full thickness skin replacement. Previously, a sterilization technique was developed for de-epidermised dermis (DED) using glycerol and ethylene oxide (EO). This was based on previous studies by Chakrabarty *et al.* (1999), comparing glycerol and EO treatment for dermis *in vitro* and *in vivo* in nude mice. However, in this work, samples of non-sterile DED were used for research as there was no requirement of this skin to be destroyed or utilized for clinical purpose.

2.5.2. Harvesting Split-Thickness Skin Grafts (STSGs)

DED was produced from STSGs obtained from routine plastic surgery of breast reduction and abdominoplasties. The STSGs were harvested and placed in sterile saline and stored at 4 °C until processing (within 24 hours).

2.5.3. De-Epidermisation of Split-Thickness Skin Grafts

STSGs were stored in PBS for a minimum of 48 hours and then immersed in sterile 1 mol/L sodium chloride for 18 hours, resulting in an acellular DED. The epidermis was separated from the underlying dermis using forceps and the remaining DED samples were washed several times in PBS (See Figure 3.1). The DED samples were stored in DMEM (Dulbecco's minimum essential medium) supplemented with 5% FCS, 2 x 10^{-3} mol/L glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin and 0.625 µg/mL amphotericin B. This medium is referred as to fibroblast culture medium (FCM).

As it was of interest to keep the basement membrane (BM) to observe the ability of melanoma and/or other human skin cells to migrate into the dermis, no attempt was made to remove the BM antigens.

2.5.4. Isolation and Culturing Human Skin Cells

2.5.4.1. Keratinocyte Culture

STSGs were obtained as previously described. Samples of this skin were cut into 0.5 cm^2 pieces using a scalpel blade (No.22) and were incubated overnight (12-24 hours) at 4°C in 0.1% w/v trypsin. FCS was added to neutralize the trypsin and the epidermal and dermal layers were carefully separated using a pair of forceps with fine points. A scalpel blade was used for gently scraping basal keratinocytes from the undersurface of the epidermis and the papillary surface of the dermis (See Figure 2.1).

The cells were collected into a mixture of FCS and PBS in sterile 25 ml universal containers. The cell suspension was then centrifuged at 300g for 5 minutes and the cells were resuspended in a known volume of keratinocyte culture medium (KCM). This medium consists of DMEM and Ham's F12 medium in a 3:1 ratio supplemented with 10% FCS, 10 ng/mL EGF; 0.4 μg/mL hydrocortisone; 1.8 x 10^{-4} mol/L adenine; 5 μg/mL insulin; 5 μg/mL transferrin; 2 x 10^{-3} mol/L glutamine; 2 x 10^{-7} mol/L tri-iodo thyronine; 0.625 μg/mL amphotericin B; 100 IU/mL penicillin and 100 μg/mL streptomycin.

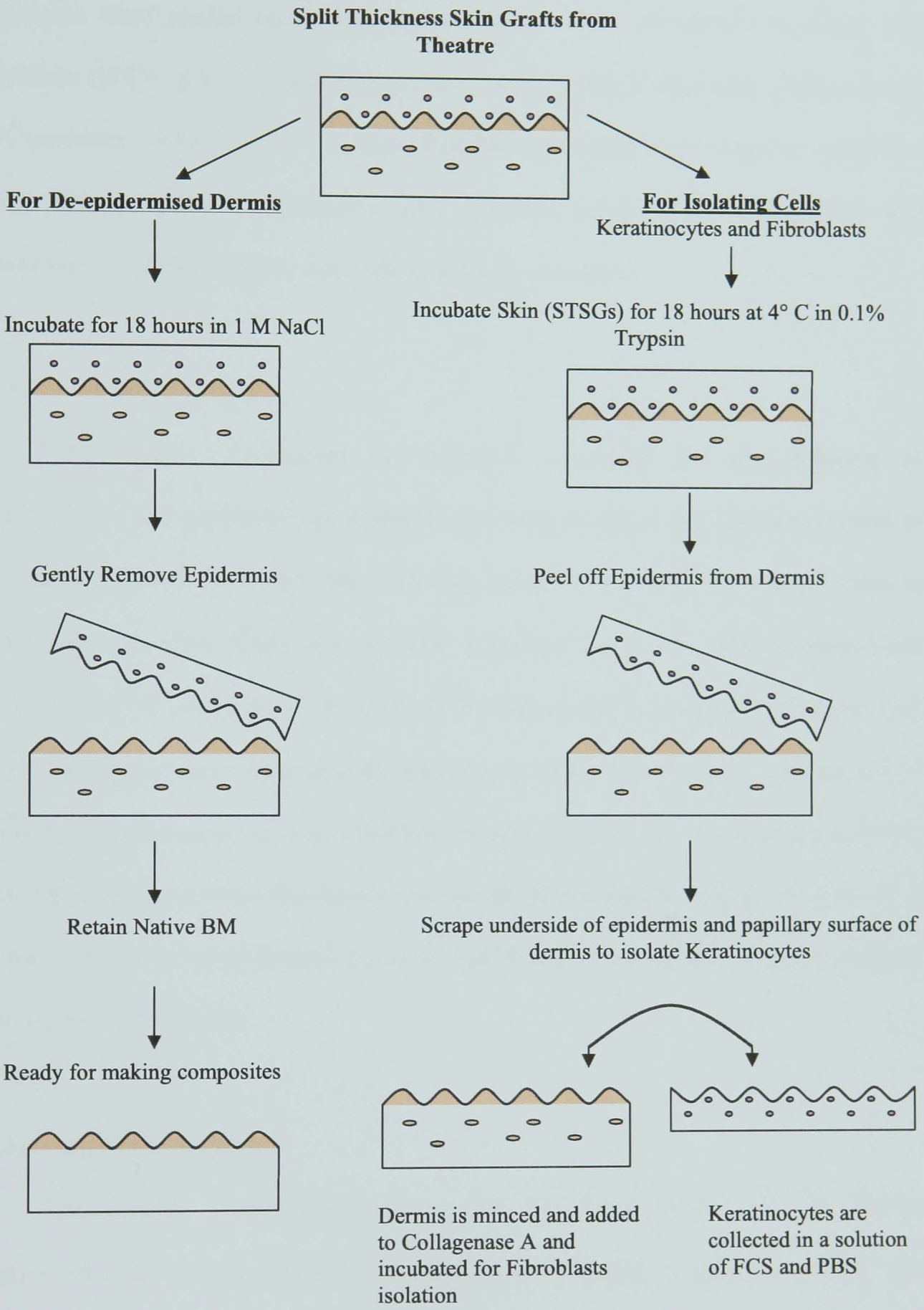


Figure 2.1. Step-by-step procedure for isolating skin cells and production of DED. (Modified from Eves, 2001).

For keratinocytes expanded in culture, freshly isolated keratinocytes in KCM suspension were seeded on to a pre-coated feeder layer of lethally irradiated 3T3 fibroblasts (i3T3) in KCM. Cell cultures were re-fed every 3 days until approximately 80% confluent, usually by 5 to 7 days. Residual i3T3 cells were removed with 2 ml 0.02% EDTA before keratinocytes were detached using 2 ml trypsin and used experimentally. Keratinocytes were not used above passage 3.

2.5.4.2. Fibroblast Culture

STSGs were trypsinized as described previously for the isolation of keratinocytes. The epidermal and dermal layers were separated and the dermal parts of the specimens were collected. These dermal samples were washed several times in sterile PBS and then finely minced with a scalpel blade. The dermal mince was incubated at 37°C overnight in 10 ml of a 0.5% collagenase A solution (See Figure 3.1). The following day, the collagenase digest was spun down in a centrifuge at 300g for 10 minutes, the supernatant was discarded and the pellet of cells was resuspended in FCM. Cells were passaged when fibroblasts reached 80-90% confluence using 2 ml of a 1:1 mixture of 0.1% w/v trypsin and 0.02 5 w/v EDTA per T25 flask. Fibroblasts were used between passages 4 and 9.

2.5.4.3. Melanocyte Culture

Melanocytes were isolated in exactly the same manner as for human keratinocytes (as described in 2.8.3. Isolation and Culturing Human Skin Cells; and 2.8.3.1. Keratinocyte Culture), using 0.1% trypsin. Once isolated, cell suspensions were seeded at 4 x 10^6 cells/T25 flask in a low calcium MCDB 153 basal media supplemented with 2 % chelated FCS, 25 µg/ml bovine pituitary extract (BPE), 0.6 ng/ml basic fibroblasts growth factor (bFGF), 10µg/ml insulin, 10µg/ml transferrin, 2.8

μg/ml hydrocortisone, 2 mM/l L-glutamine, 100 IU/ml; 100 μg/ml penicillin/streptomycin, 10 U/ml nystatin, 1 μg/ml α-tocopherol, 100 ng/ml cholera toxin and 10nM/l phorbol 12-myristate 13-acetate (PMA). Geneticin (100 μg/ml) was added to the media over the first few days to prevent fibroblast contamination. Melanocyte cultures were fed twice weekly with this Melanocyte Growth Medium. For co-culture, melanocytes were trypsinised using 2 ml of a 1:1 mixture of 0.1% w/v trypsin and 0.02% w/v EDTA. Once detached 1:2 trypsin inhibitor (TI) was used to block the effects of trypsin and melanocyte growth medium was added to the cells prior centrifuging. Cells were resuspended in this medium and seeded at the desired density. Melanocytes were not used for experiments after passage 4.

2.5.5. Engineered Human Skin Model

De-cellularised human skin was obtained as previously detailed which resulted in a DED, with retained basement membrane. The dermal pieces were then cut into approximately 2x2 cm pieces to fit into a six-well tissue culture plate. The DED was placed in each well with the reticular surface uppermost. Sterile $0.8cm^2$ stainless steel rings were placed in the middle of each piece of dermis and a seal was obtained between steel ring and dermis by application of gentle pressure. FCM was added to the inside of each ring to check for any leakage. The surrounding dermis was then flooded with FCM. Once satisfied that a sufficient seal had been obtained, FCM from inside each ring was removed and fibroblasts at density of $1x10^5$ cells per composite were added to each ring in a volume of 500 μl of FCM. After 24 hours incubation the medium within each ring was carefully removed and replaced with fresh FCM. Following a further overnight incubation at 37°C, to allow migration of fibroblast into the dermis reaching down the papillary layer, all the medium was carefully removed. Each ring was

removed and the pieces of DED were inverted so that the papillary surface was uppermost. Steel rings were then applied to the dermal surface as before and a check for leakage was again made using KCM. Once a tight seal had been demonstrated, 250 µl of melanoma cells at density of $5x10^4$ cells per composite ring were seeded. After a period of one to two hours, 250µl keratinocytes were seeded into each ring at density of $1x10^6$ cells. The following day, the medium in each ring was carefully removed and replaced with fresh KCM. After 24 hours of incubation the rings were removed and the composites were raised onto stainless steel grids in new six-well culture plates. Fresh KCM was added to the level of the base of the composite so that the KCM lapped over the edges of the composites (but not the seeded area) forming an air-liquid interface (ALI). Culture medium was changed every 2-3 days. When pharmacological agents (*e.g.* NaIbu, TNF-α) or fibrin clots were being used in composites experiments, KCM containing these freshly prepared agents was added and composites were then kept at 37°C. Following an examination of their morphology composites were ended after 10-19 days culture at an ALI. See Figure 2.3.

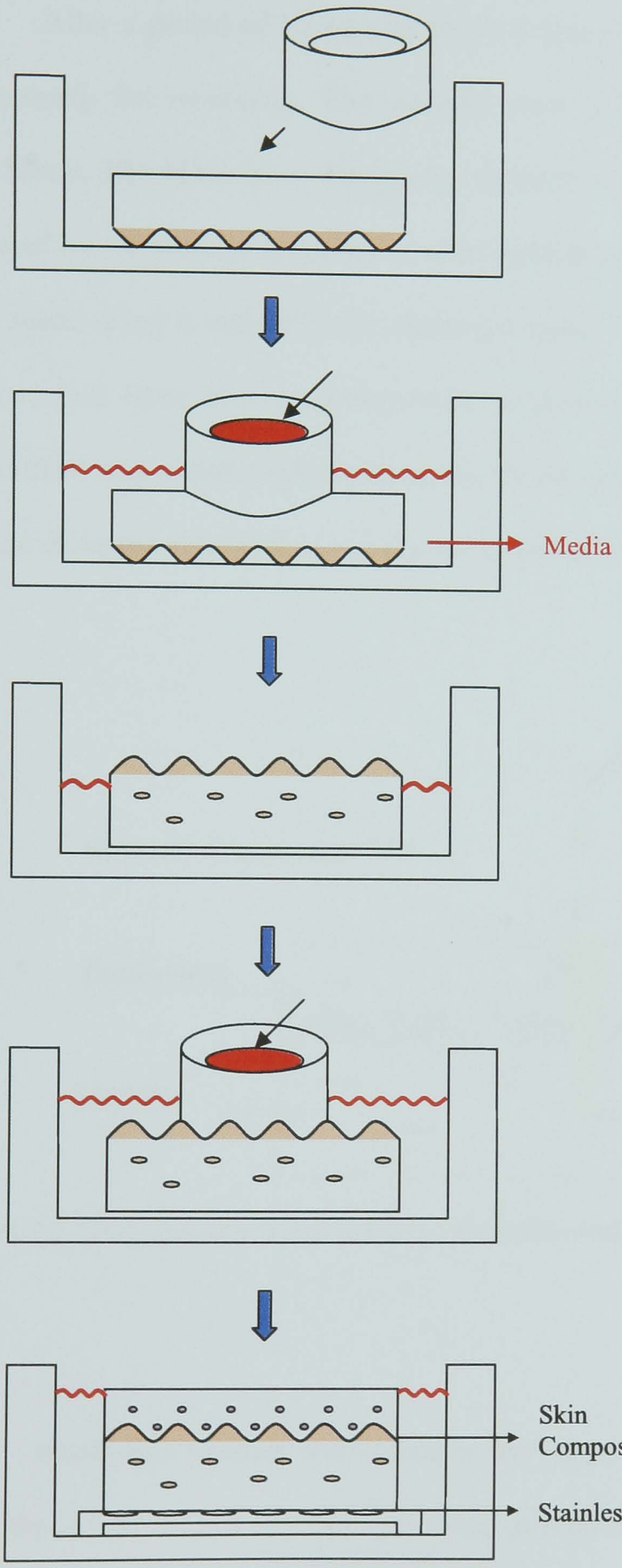


Figure 2.2. Making an Engineered Human Skin Model. (Modified from Eves, 2001).

2.5.6. Wounding Skin Composites

After a period of 10 days at an Air Liquid Interface (ALI), the skin composites were ready for wounding. The wounds were made firstly using a needle (19G 2" 1.1x50mm, BD Microlance Drogheda, Ireland). As these initial wounds could not be detected by subsequent histology of skin composites, larger full thickness wounds were then made using a scalpel blade, making a cross wound in the region where the cells were seeded. Once the wound was made, a fibrin clot solution with and without TNF-α (300 U/ml) was added on the composites. To the skin composites without fibrin clot, 2.5 to 3 ml of media with TNF-α at a concentration of 300 U/ml was added to each well.

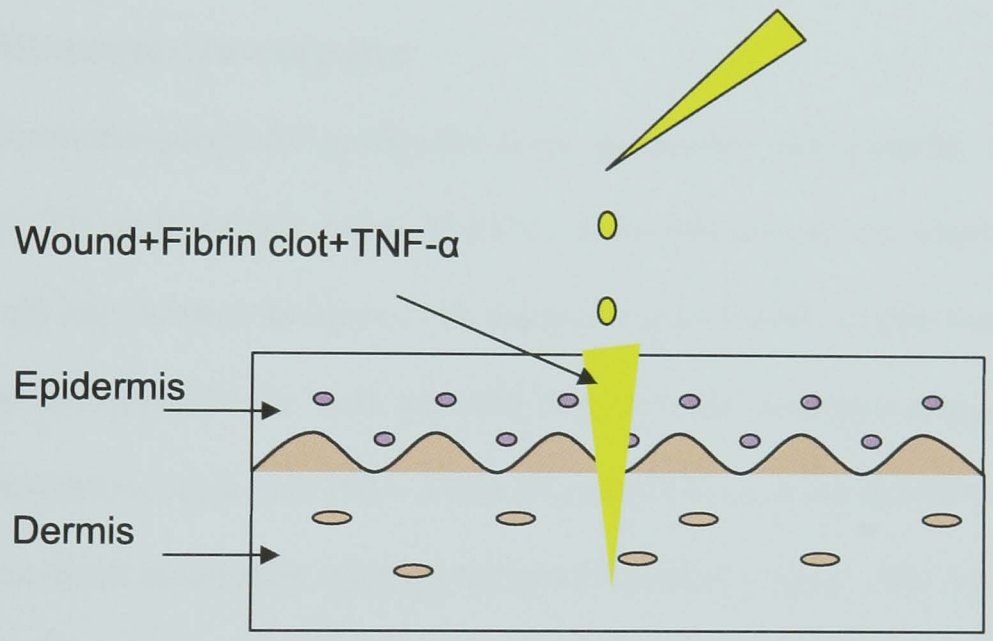


Figure 2.3. Diagram of a wounded skin composite and the addition of fibrin clot plus TNF-α.

Initial skin wounds were made in the shape of a cross but it proved difficult to keep the wound edges together. As these proved difficult to heal, wounds were then made with a single cut in to the seeded area. Skin composites were initially wounded after a period of 10 days at an ALI. However, this was later modified and wounds were made on the 3rd day after being lifted in ALI.

2.5.7. Assessment of Tissue Engineered Human Skin

ALI exposure was initially kept for 10-19 days. As this was considered a long time for incubation (increasing the risks for infection) this time was then reduced to 7 to 10 days. After an ALI period, tissue engineered human skin was processed for histological analysis. Samples were fixed in 10% phosphate buffered formaldehyde for at least 24 hours before routine histological procedure. All samples were assessed for overall morphology, the presence of BM, keratinocytes differentiation in the epidermis, fibroblast penetration into the dermis, melanoma invasion into the dermis and wound healing.

2.5.7.1. Histological Investigation

Formaldehyde-fixed specimens were processed into paraffin wax using an Automatic Tissue Processor (Leica TP1020 – Leica Microsystems), which over a period of 17 hours impregnates the tissue with paraplast (plasticized paraffin wax). The tissues were then embedded in the same paraffin wax, in tissue cassettes using an Automatic Tissue Processor (Leica EG1160 – Leica Microsystems). 4 μm sections were cut from each tissue block on a rotary microtome (Leica RM2145 – Leica Microsystems) and the sections were stained manually with Carazzi's Haematoxylin and Eosin (H&E). Sections were mounted using a glass cover slip.

2.5.7.2. Histological Immunostaining

Antibody staining for melanoma cells, keratinocytes and BM localization was done in the Department of Histopathology at the Northern General Hospital (Sheffield, UK). As the cells were invading into the dermis, an antibody staining technique was performed to be able to distinguish between the cell types in the skin composites and to detect the basement membrane. Standard H&E staining were done for overall contrast.

2.5.7.3. Preparation of Paraffin Wax Sections for Immunohistochemistry

Paraffin wax sections were cut, transferred to Poly-L-Lysine coated slides and then kept at 60°C overnight. These slides were dewaxed in xylene, rehydrated through descending grades of alcohol and washed in running tap water for at least 10 minutes. Any masking of antigenic sites by the processing procedure was reversed by the use of an antigen retrieval method – either enzyme digestion or ultra-heat treatment. The type of antigen retrieval method used was specific to the antibody used.

2.5.7.4. Enzyme Digestion

Sections were placed in distilled water at 37°C to equilibrate. To a water bath containing 500ml of distilled water at 37°C, 10 ml of 5% calcium chloride and 0.25g of trypsin were added. The trypsin solution was adjusted to pH 7.8 by the addition of sodium hydroxide. The slides were incubated in this solution for 14 minutes at 37°C and then transferred to running water for at least 10 minutes to prevent further enzymic digestion.

2.5.7.5. Ultra Heat Treatment

Sections were placed in a pressure cooker containing enough boiling citrate buffer (pH 6) to cover the tissue sections and then taken up to approximately 120°C for 2 minutes. The pressure was carefully released and the lid removed from the pressure cooker. The slides remained in the hot buffer solution for 20 minutes and were then transferred to a glass trough of running tap water.

2.5.7.6. Immunohistochemical Methodology

After antigen retrieval, all sections were placed in Tris-Buffered Saline (TBS) pH 7.8 with 5µl of 0.1% Tween for 10 minutes. Sections were then incubated for 10

minutes in blocking serum (1:25 horse serum in TBS/Tween for monoclonal antibodies and 1:25 swine serum in TBS/Tween for polyclonal antibodies) to decrease background staining. The blocking serum was tipped off the slides and the primary antibody applied for an incubation time of 60 minutes. After this primary antibody incubation, the slides were washed in TBS/Tween for 10 minutes. The sections were then incubated with the secondary antibody (biotinylated anti-mouse secondary antibodies for monoclonals and biotinylated anti-rabbit secondary antibodies for polyclonals) for 30 minutes, followed by a 10 minute wash in TBS/Tween.

The alkaline phosphatase Avidin Biotin Complex kit was prepared as per instructions and the slides incubated with the complex for 30 minutes. The slides were again washed in TBS/Tween for 10 minutes. The alkaline phosphatase substrate kit was prepared and applied to the slides for 10 minutes (checked microscopically) followed by a final wash in TBS/Tween. The sections were washed in running water, stained in Haematoxylin (20 seconds), differentiated in acid/alcohol (1 second), "blued" in running tap water, dehydrated, cleared and mounted for microscopic examination.

2.5.7.7. Antibodies Used

Anti-collagen IV antibody was used at a dilution of 1:40 to stain for the presence of the BM. Rabbit derived S100 antibody was used at a dilution of 1:2500 to stain for melanocytes and mouse derived HMB45 antibody (HMB45 recognizes premelanosomal vesicle in premature developing melanocytes and melanoma cells (Schaumburg-Lever *et al.*, 1991) and as such has been found to be very useful for distinguishing between melanocytes and melanoma cells (Gown *et al.*, 1986) was used at a dilution of 1:40 to stain for melanoma cells. A dilution of 1:50 of mouse anti-cytokeratin AE1AE3 antibody was used to stain keratinocytes.

2.5.8. Cell Invasion Assay

In order to quantify cell invasion into the composites, an invasion score was used as previously established by Eves *et al.* (2000) and Eves (2001). The score was based on the number of cells invaded into the dermis counted in each skin composite slide. A score above 1.5 was considered as evidence of clear invasion of cells.

Table 2.2. Cell invasion score.

Score	Number of cells
0	No cells present in the dermis
0.5	1-5 cells in the dermis
1.0	6-15 cells in the dermis
1.5	16-20 cells in the dermis
2.0	21-30 cells in the dermis
2.5	31-40 cells in the dermis
3.0	+50 cells in the dermis (florid invasion)

2.6. STATISTICS

For melanoma cell migration assay the statistical analysis compared control *versus* treated cells using a Multiple Comparison test and Bonferoni correction, presuming equivalent variances.

Melanoma cell viability and comparisons between invasion scores were analysed by comparing control versus treated cells using a repeated Student t-test for 2 samples presuming equivalent variances.

All statistical tests were considered as non-parametric. Differences between means were taken as significant at the $p<0.05$ (significant); $p<0.01$ (very significant); $p<0.001$ (extremely significant) level.

2.7. RESEARCH ETHICS APPROVAL

The Kroto Research Laboratory holds a Research Tissue Bank License (Human Tissue Authority (HTA) License Number 12179). All tissue cultures for this work came from patients who signed a Research Consent Form for tissue to be used for research purposes. See details in Appendix I.

CHAPTER 3

EFFECTS OF TNF-α, IBUPROFEN AND CAPSAICIN ON MELANOMA CELL MIGRATION AND VIABILITY

3.1. RELEASE OF IBUPROFEN FROM F127 PLURONICS HYDROGEL

Initially, NaIbu was incorporated into a hydrogel as described in section 2.3.3 in Methods (Chapter 2). The Pluronic hydrogel used (F127) was not cytotoxic to cells as will be shown later in this chapter. Figure 3.1 illustrates the release of NaIbu from a 30% F127 Pluronics hydrogel over a period of 24 hours. This was assessed by the content of NaIbu which had been released from the hydrogel into the distilled water and then determined from the characteristic ibuprofen UV absorbance at 264nm using a UV spectrometer.

In vitro suitable controlled drug delivery system based on Pluronic hydrogel has been previously demonstrated by Gong *et al.* (2009). The standard curve (see Figure 3.1) shows the expected release of ibuprofen from the hydrogel in cell culture medium as it was kept at the same Pluronic F127 concentration (30%) and incubated at the same temperature of 37 °C.

The release of NaIbu was rapid from 2 to 8 hours (from 0.4 to 1.48 μM approximately). After this period NaIbu released from the hydrogel was steady up to 24 hours (from 1.48 to 1.8μM).

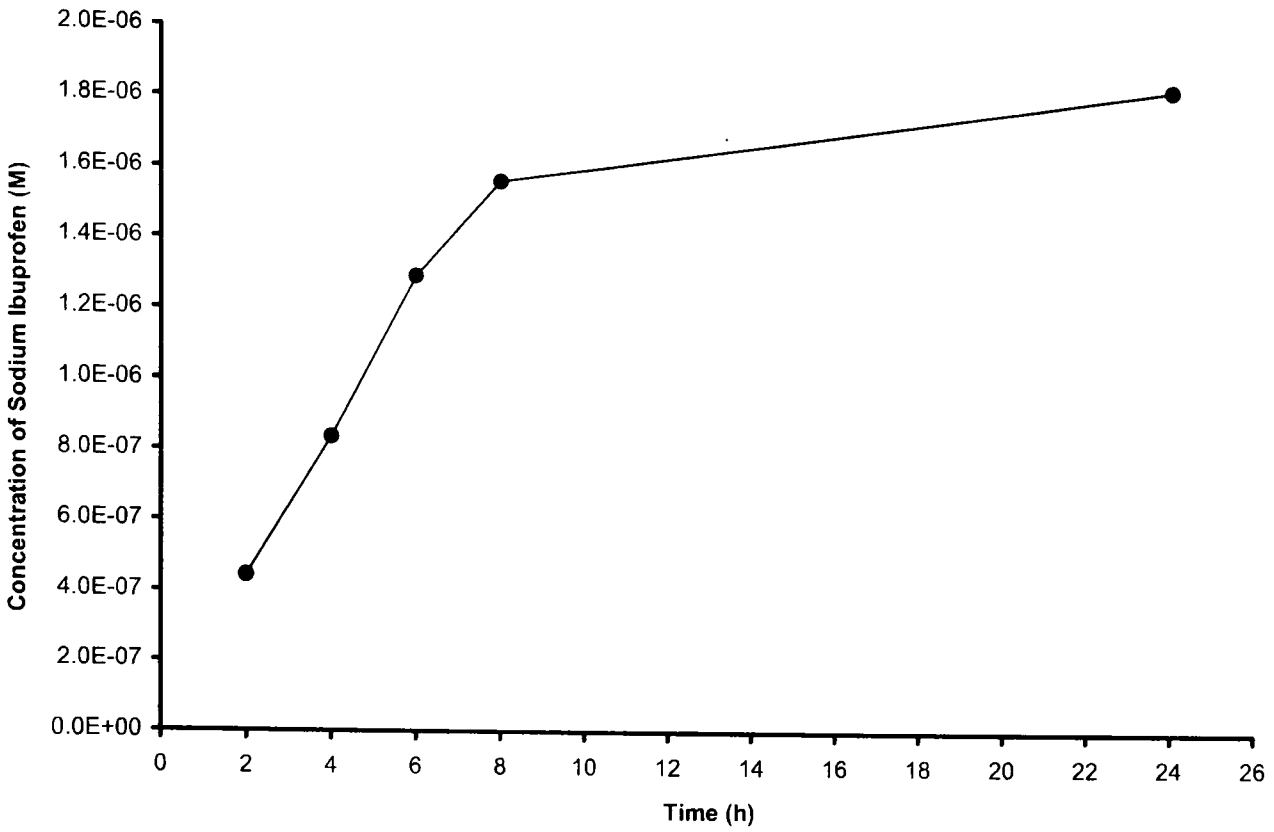


Figure 3.1. Release profile of NaIbu from 30% F-127 Pluronics hydrogel over 24 hours (h). The initial concentration of NaIbu in the hydrogel was $131x10^{-6}$ M and the test temperature was 37°C (Data obtained from Dr. Alan Waddon).

3.2. PATTERN OF MELANOMA CELL MIGRATION OVER TIME

Initially, the melanoma migration pattern was studied using three mean control values (treated with media only) obtained from three different experiments. These experiments were: 1) Effects of NaIbu hydrogel on melanoma cell migration; 2) Effects of NaIbu (in media) on melanoma cell migration; and 3) Effects of Capsaicin on melanoma cell migration. Figure 3.2 shows the pattern of migration of three different melanoma cell lines at different time points (2, 4, 8 and 24 hours).

A non-parametric Spearmans' Correlation test was used to examine how linear the rate of melanoma cell migration was over time. Overall the rate of migration was linear for all these cell lines at 24 hours with high correlation values (76% to 92%) as shown Figure 3.2. HBL cell migration showed an evident linear pattern over a period of 24 hours (correlation test resulted in $r=0.972$, $p<0.001$). Migration of A375SM was

slow until 4 hours however, overall over 24 hours migration was nearly linear (correlation test resulted in $r=0.756$, $p<0.05$). C8161 cell migration was faster at 8 hours and migration was linear over 24 hours (correlation test resulted in $r=0.972$, $p<0.001$).

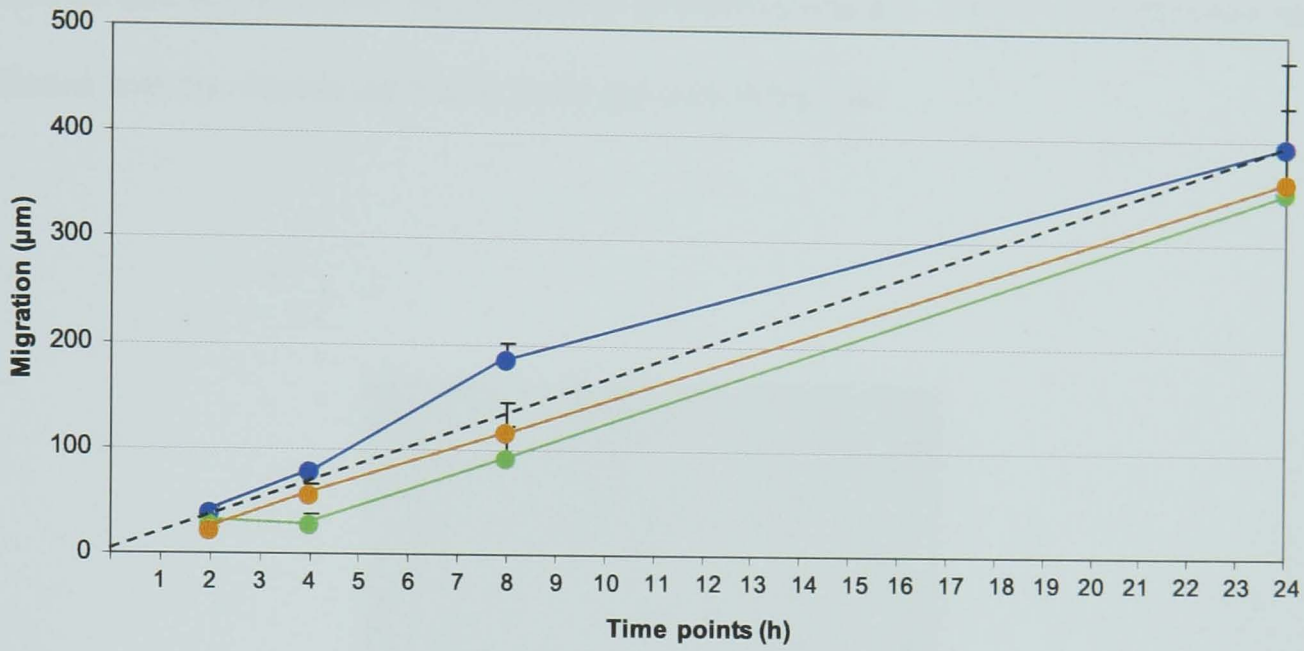


Figure 3.2. Pattern of melanoma cell migration over 24 hours (h). ● = HBL cells, ● = A375SM cells, ● = C8161 cells. Values are expressed as Mean+SEM. The dotted line shows a linear plot between 0 hour and 24 hours for the distance migrated by C8161 cells.

3.3. 2D MODEL FOR MELANOMA CELL MIGRATION

Melanoma migration was investigated using a 2D model in which a monolayer of cells was formed after seeding the cells in a 12 well plate. After 72 hours of incubation, a scratch wound was made using a sterile plastic Gilson's pipette tip. This created a zone free of cells. The media was removed and 1 ml/well of new fresh media was added to the wells.

Scratch photographs were taken from each well for later measurement of migration at time zero. This was made by measuring the distance between the two edges in micrometres (µm). The cells were then incubated until the next migration measurement time points of 2, 4, 8 and 24 hours. Migration was later calculated by

subtracting the distance between the edges at each time point from the initial scratch distance at zero time.

Figure 3.3 illustrates a scratch wound model in which C8161 cells were stained with DAPI at 24 hours (end of migration experiment). Cells were treated with unloaded hydrogel and the different concentrations of NaIbu hydrogel. C8161 cell migration was reduced with the increasing NaIbu hydrogel concentrations.

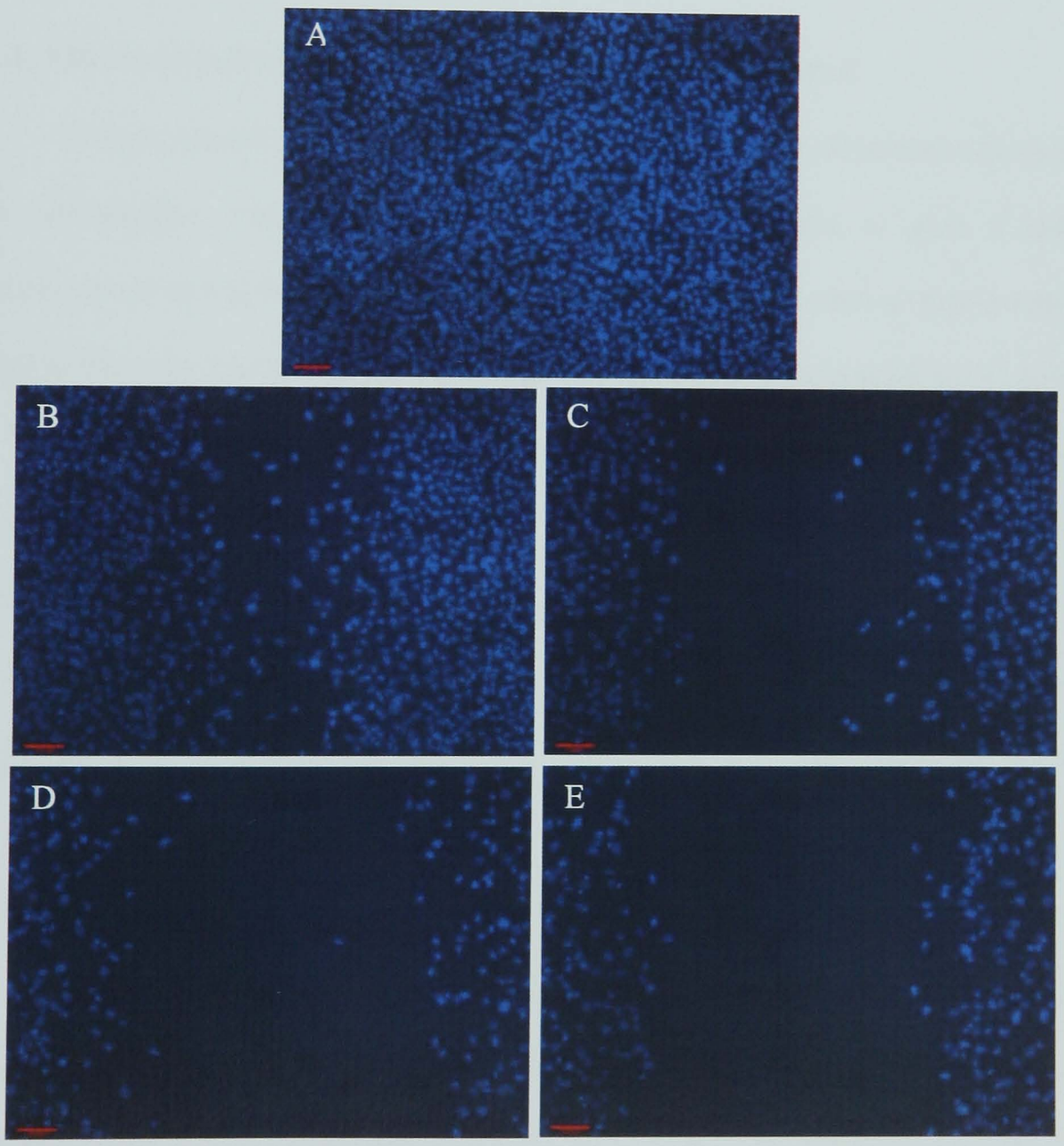


Figure 3.3. Scratch wound model. Migration of C8161 cells at (A) Unloaded hydrogel and (B) 33μM, (C) 66μM, (D) 99μM and (E) 131μM NaIbu hydrogel concentrations. Scale bar = 50μm.

3.4. EFFECTS OF TNF-α, IBUPROFEN AND CAPSAICIN ON MELANOMA CELL MIGRATION

In these experiments, the effects of the NSAIDs Ibuprofen and Capsaicin were investigated on three melanoma cell lines. The pro-inflammatory cytokine TNF-α was used to pre-stimulate melanoma cell migration and the addition of Ibuprofen or Capsaicin in reducing melanoma cell migration was investigated. In some experiments NaIbu was also incorporated into a Pluronics hydrogel for further investigation of the effects of NaIbu hydrogel on melanoma cell migration.

3.4.1. Effects of NaIbu (in media) on Melanoma Cell Migration

In these experiments the effects of NaIbu (in media) on melanoma cell migration was investigated. NaIbu (powder) was dissolved in media to give 2 different concentrations of 66μM and 131μM. 1 ml/well of NaIbu (in media) or media only were added to the cells. Melanoma cells treated with media only were considered as controls.

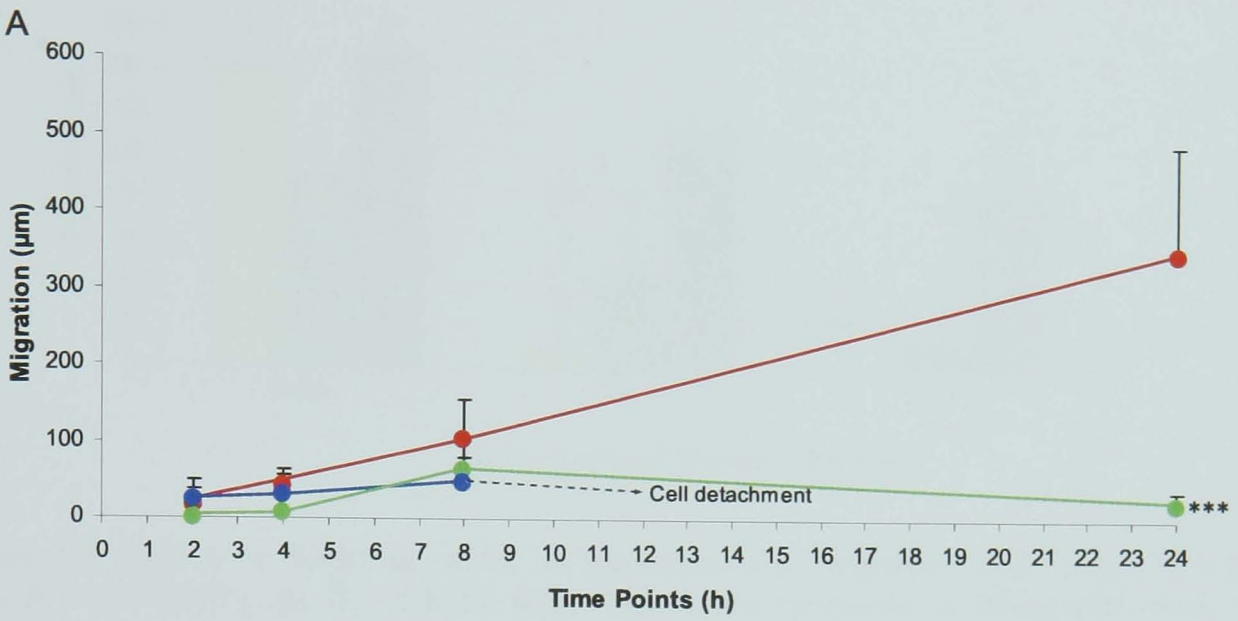
HBL cell migration was significantly reduced at 66μM NaIbu ($p<0.001$). However, at 131μM NaIbu concentration, HBL cells were detaching from the wells at 24 hours of incubation making migration measurements not possible. See Figure 3.4 A.

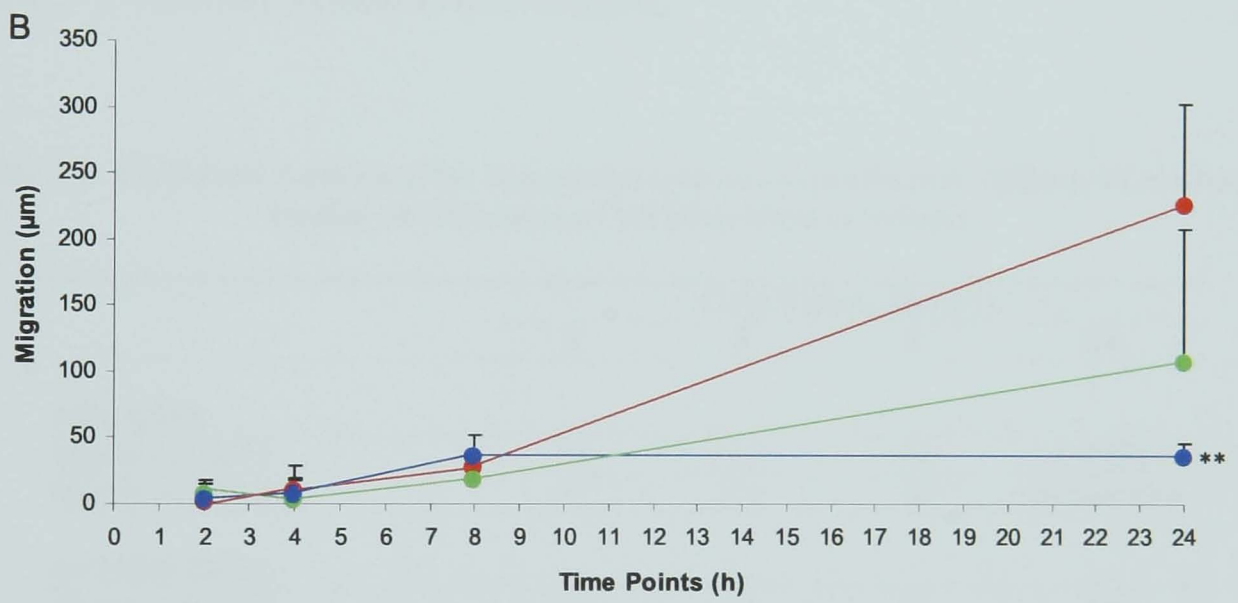
For A375SM cells a significant reduction occurred at a higher concentration of 131μM ($p<0.01$) of NaIbu at 24 hours. See Figure 3.4 B.

For C8161 cells, a significant reduction in migration was observed at 131μM NaIbu ($p<0.01$) at 24 hours. See Figure 3.4 C.

Although these are measures of melanoma cell migration only, it should be considered a combination of melanoma cell migration and cell proliferation, particularly for C8161 cells as they proliferate more rapidly than the other two melanoma cell lines (HBL and A375SM cells). Figure 3.5 illustrates the effects of NaIbu in media on the

three melanoma cell lines over 24 hours. Table 3.1 shows a statistical summary of these results.





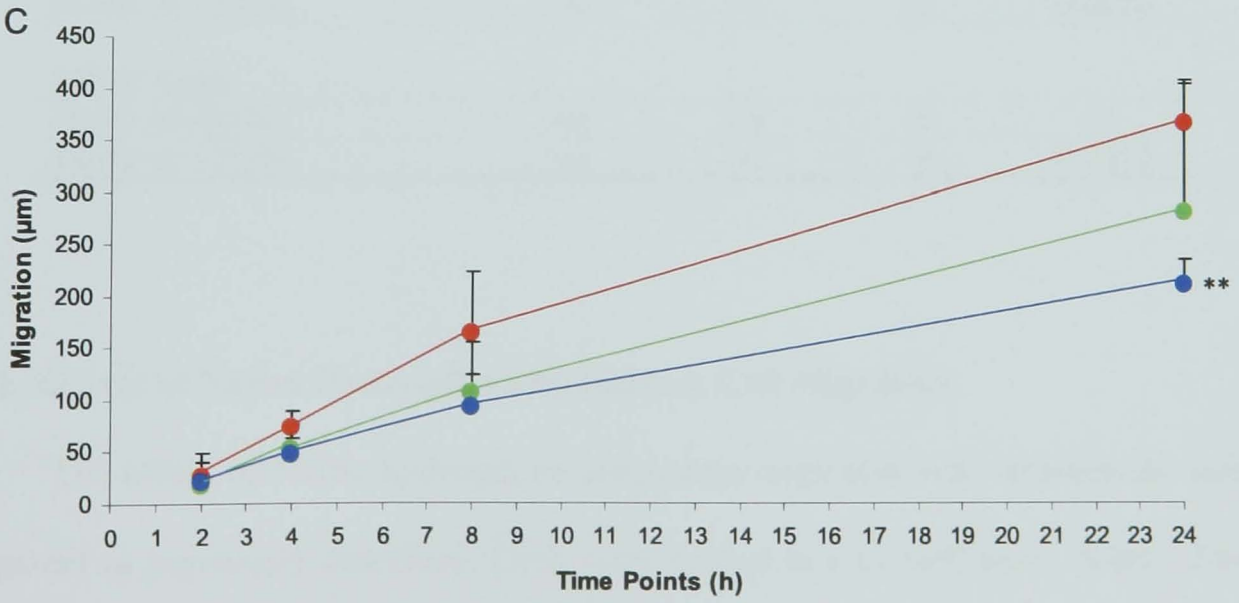


Figure 3.4. Effects of NaIbu in media on melanoma cell migration over 24 hours (h). (A) HBL cells, (B) A375SM cells, and (C) C8161 cells. ● = Media, ● = 66µM, ● = 131µM. Values are expressed as Mean+SD. N=4. ** = $p<0.01$; *** = $p<0.001$, relative to control media.

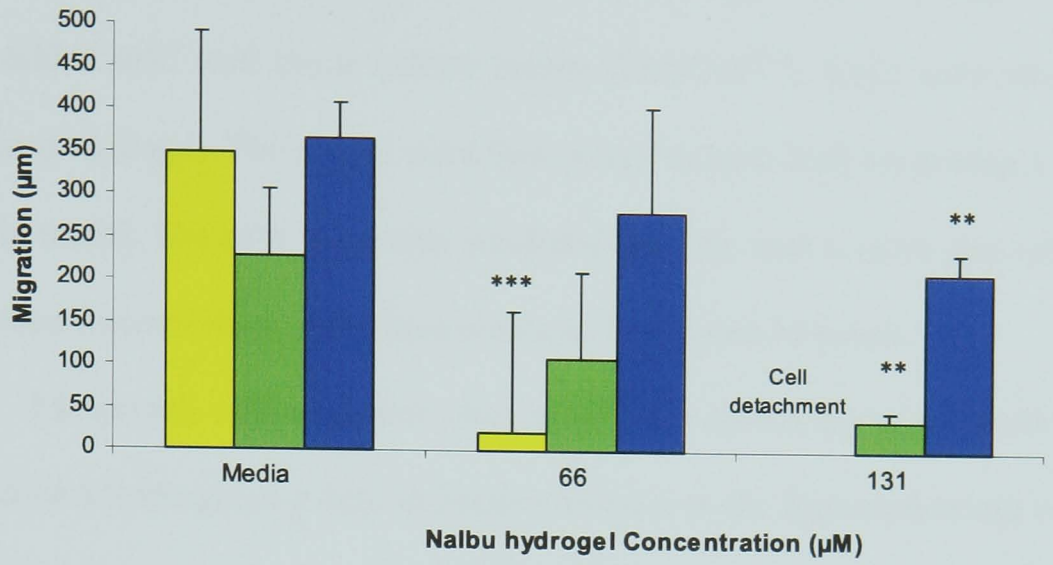


Figure 3.5. Effects of NaIbu in media on melanoma cell migration at 24 hours. ▯ = HBL cells, ▯ = A375SM cells, ▯ = C8161 cells. Values are expressed as Mean+SD. N=4. ** = p<0.01; *** = p<0.001, relative to control media.

Table 3.1. Statistical Summary for comparison between treatments - Effects of NaIbu (in media) on Melanoma Cell Migration (p values).

	Time Points (hours)			
	2	4	8	24
HBL Cells				
Media *vs* 66µM	*ns*	*ns*	*ns*	0.0001
Media *vs* 131µM	*ns*	*ns*	*ns*	Cell detachment
A375SM Cells				
Media *vs* 66µM	*ns*	*ns*	*ns*	*ns*
Media *vs* 131µM	*ns*	*ns*	*ns*	0.0050
C8161 Cells				
Media *vs* 66µM	*ns*	*ns*	*ns*	*ns*
Media *vs* 131µM	*ns*	*ns*	*ns*	0.0010

3.4.2. Effects of NaIbu Hydrogel on Melanoma Cell Migration

The effect of NaIbu hydrogel on melanoma migration was investigated using a 2D model as previously described. Cells were seeded in a 12 well plate. After 72 hours of incubation time a scratch wound was made and the media was removed and 1ml/well of fresh media was added to the wells.

Scratch photographs were taken from each well at time zero to measure cell migration. In these experiments 300μl of increasing NaIbu hydrogel concentrations were added to12 well tissue culture inserts (ThinCert^{TM}). Cells were also treated with unloaded hydrogel. The inserts were then placed in each well containing a monolayer of cells in media. The cells were then incubated until the next scratch photographs for later migration measurement at the time points of 2, 4, 8 and 24 hours.

Melanoma cell migration decreased with increasing concentrations of NaIbu loaded on a hydrogel in a dose-dependant manner in the three melanoma cell lines used. Statistical comparisons considered the unloaded hydrogel treated cells as control. Figure 3.6 illustrates the effects of NaIbu hydrogel on migration of three melanoma cell lines.

HBL cell migration was unchanged at different concentrations of NaIbu hydrogel at 2 hours ($p>0.05$) relative to the unloaded hydrogel. Migration was significantly reduced at 33μM and 131μM NaIbu hydrogel ($p<0.05$ and $p<0.01$ respectively) relative to the unloaded hydrogel, at 4 hours. HBL cell migration was also reduced at NaIbu hydrogel at 66μM and 131μM ($p<0.05$ respectively) relative to the unloaded hydrogel, at 8 hours. At 24 hours, HBL cell migration was markedly reduced with the increasing NaIbu hydrogel concentrations with a significant reduced migration at 131μM ($p<0.05$) compared to the unloaded hydrogel. See Figure 3.7 A for an illustration of these results.

For A375SM cells, migration was significantly reduced with the unloaded hydrogel ($p<0.05$) and to each NaIbu hydrogel concentration ($p<0.05$) relative to the cell treated with media only. A375SM cell migration at increasing NaIbu hydrogel concentration was not significantly reduced comparing compared to the unloaded hydrogel ($p>0.05$), at 24 hours. See Figure 3.6 B.

For C8161 cells, at 4 and 8 hours, migration was reduced at the unloaded hydrogel and at increasing NaIbu hydrogel concentrations compared to the cell treated with media only. C8161 cell migration was reduced by the increasing concentrations of NaIbu hydrogel, however this was not significant ($p>0.05$) relative to the unloaded hydrogel.

Overall, NaIbu hydrogel reduced migration of the three melanoma cell lines. This was more effective on HBL cells followed by A375SM and C8161 cells.

Reduced melanoma cell migration with increasing concentrations of NaIbu hydrogel at 24 hours is illustrated in Figure 3.7. A statistical summary of these results is in Table 3.2.

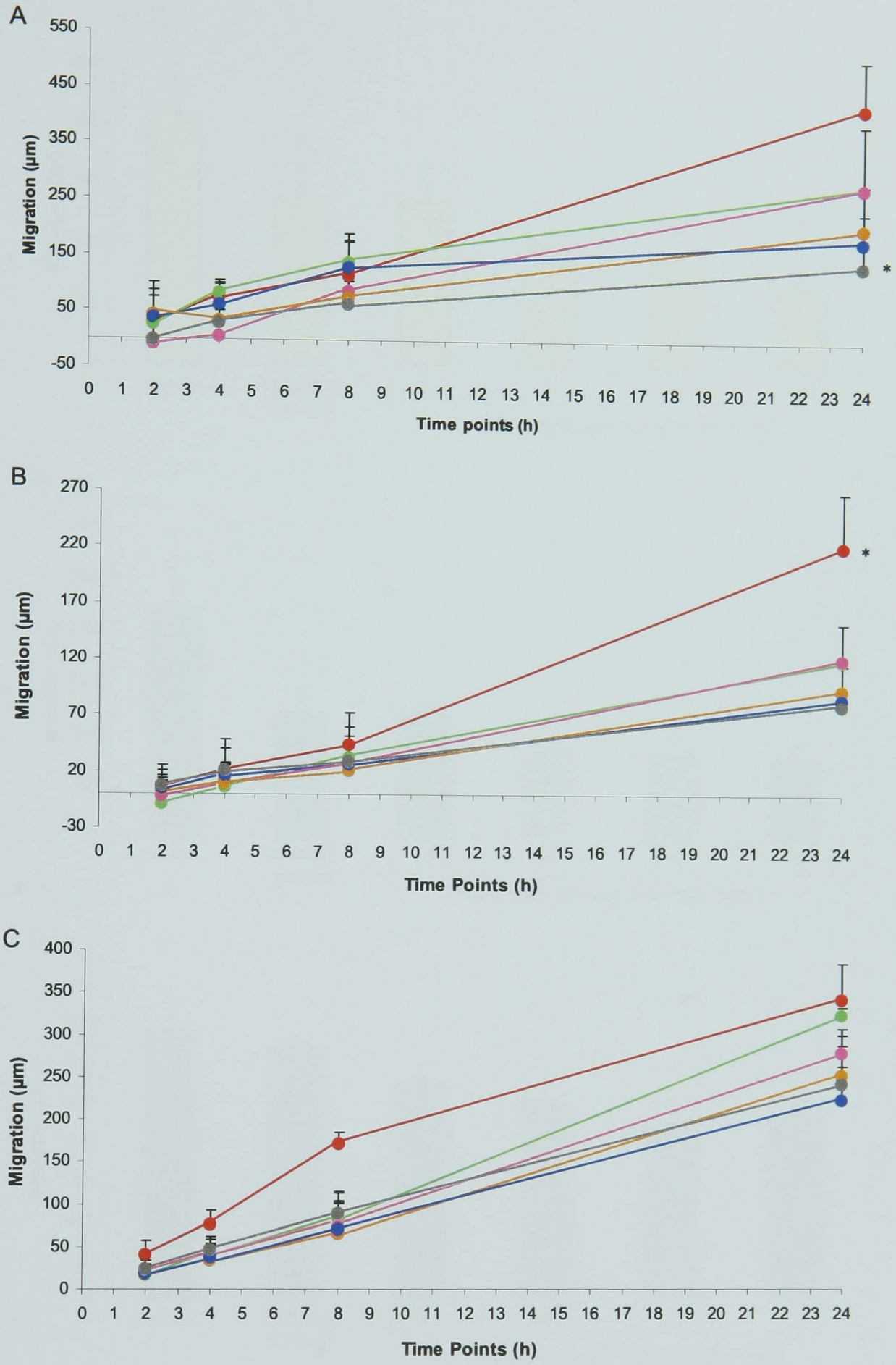
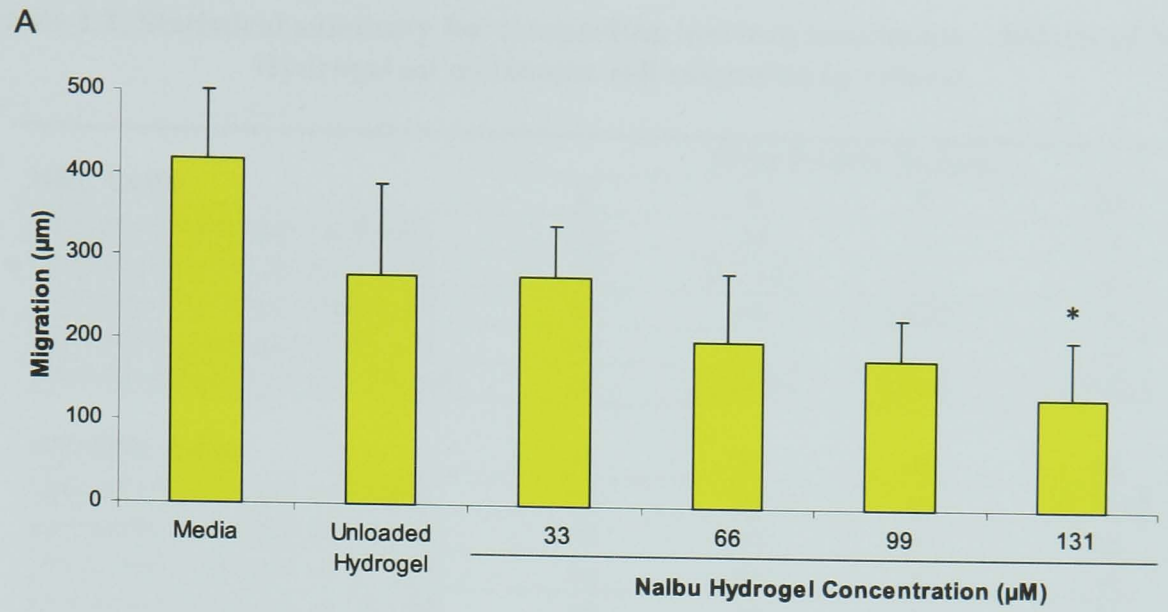
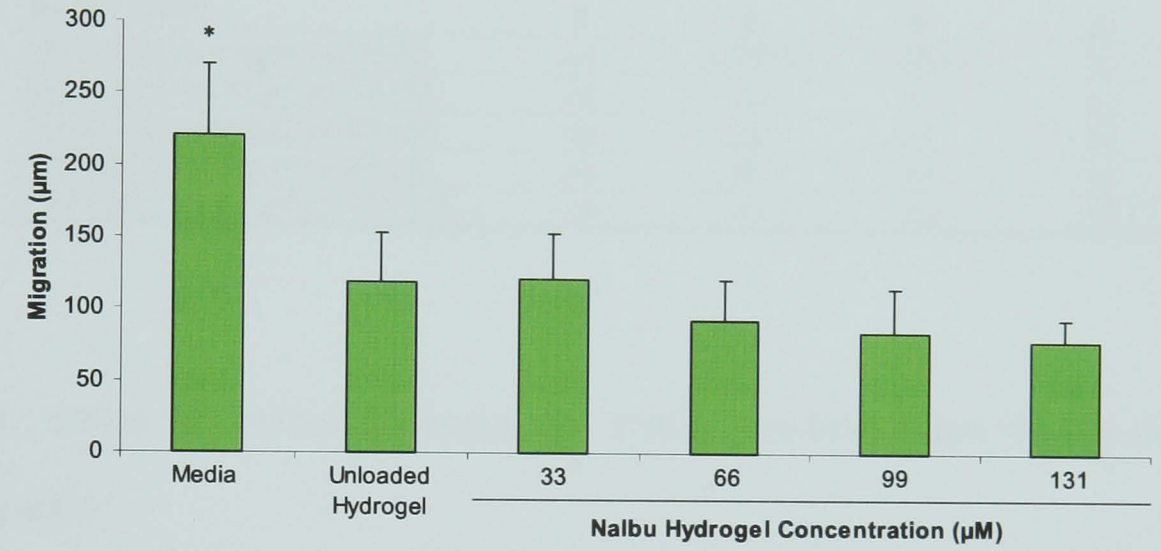


Figure 3.6. Effects of NaIbu hydrogel on melanoma cell migration over 24 hours (h). (A) HBL cells, (B) A375SM cells, and (C) C8161 cells. ● = Media, ● = Unloaded hydrogel, ● = 33µM, ● = 66µM, ● = 99µM, ● = 131µM. Values are expressed as Mean +SD. N=5. * = $p<0.05$, relative to the unloaded hydrogel.





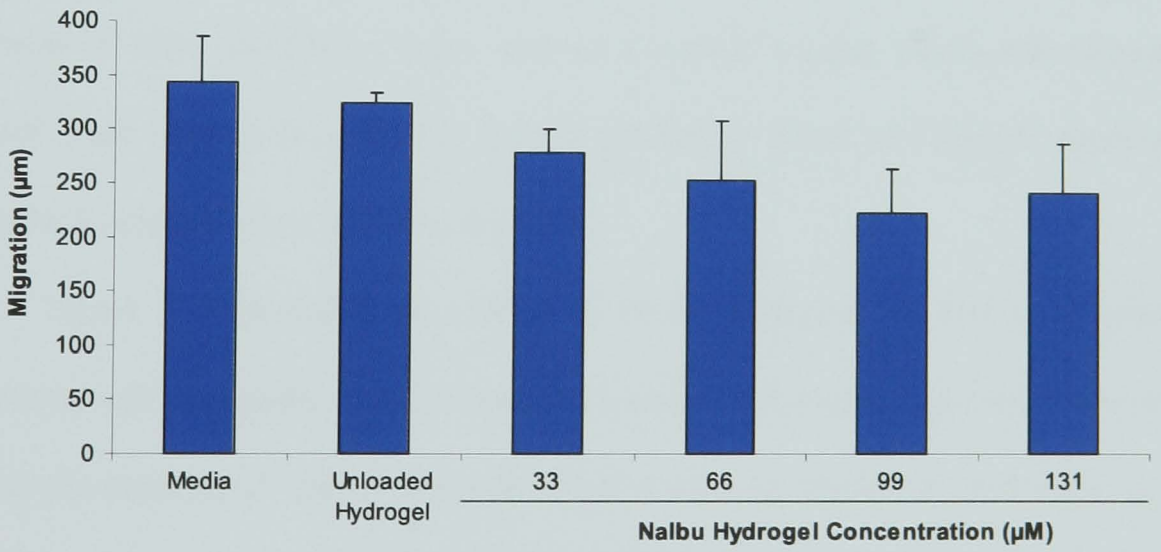


Figure 3.7. Effects of NaIbu hydrogel on melanoma cell migration at 24 hours. (A) HBL cells, (B) A375SM cells and (C) C8161 cells. Values are expressed as Mean+SD. * = $p<0.05$, relative to the unloaded hydrogel.

Table 3.2. Statistical summary for comparison between treatments – Effects of NaIbu Hydrogel on melanoma cell migration (p values).

	Time Points (hours)			
HBL Cells	**2**	**4**	**8**	**24**
Unloaded hydrogel *vs* Media	*ns*	*ns*	*ns*	*ns*
Unloaded hydrogel *vs* 33 µM	*ns*	0.0102	*ns*	*ns*
Unloaded hydrogel *vs* 66 µM	*ns*	*ns*	0.0207	*ns*
Unloaded hydrogel *vs* 99 µM	*ns*	*ns*	*ns*	*ns*
Unloaded hydrogel *vs* 131 µM	*ns*	0.0026	0.0107	0.0491
A375SM Cells	**2**	**4**	**8**	**24**
Unloaded hydrogel *vs* Media	*ns*	*ns*	*ns*	0.0348
Unloaded hydrogel *vs* 33 µM	*ns*	*ns*	*ns*	*ns*
Unloaded hydrogel *vs* 66 µM	*ns*	*ns*	*ns*	*ns*
Unloaded hydrogel *vs* 99 µM	*ns*	*ns*	*ns*	*ns*
Unloaded hydrogel *vs* 131 µM	*ns*	*ns*	*ns*	*ns*
C8161 Cells	**2**	**4**	**8**	**24**
Unloaded hydrogel *vs* Media	*ns*	0.0231	0.0003	*ns*
Unloaded hydrogel *vs* 33 µM	*ns*	*ns*	*ns*	*ns*
Unloaded hydrogel *vs* 66 µM	*ns*	*ns*	*ns*	*ns*
Unloaded hydrogel *vs* 99 µM	*ns*	*ns*	*ns*	*ns*
Unloaded hydrogel *vs* 131 µM	*ns*	*ns*	*ns*	*ns*

3.4.3. Effect of NaIbu Hydrogel on TNF-α Pre-Stimulated Melanoma Cell Migration

In these experiments melanoma cells were pre-stimulated for 24 hours with 1ml/well of 300U/ml TNF-α. After making a scratch wound, media was changed to 1 ml/well fresh new media and then inserts containing 300µl of different concentrations of NaIbu hydrogels were added to the cells.

Figure 3.8 illustrates the effects of NaIbu hydrogel on TNF-α pre-stimulated melanoma cell migration. There were cells treated with media only and these were not TNF-α pre-stimulated. There were also TNF-α only (in media) treated cells. Unloaded and NaIbu loaded hydrogel treated cells were therefore also pre-stimulated with TNF-α. The unloaded hydrogel treated cells were considered as control for comparisons.

HBL cell migration was not significantly reduced by NaIbu hydrogel in TNF-α stimulated cells at 2, 4 and 8 hours. Increasing NaIbu hydrogel reduced HBL cell migration in TNF-α stimulated cells at 24 hours, however this was not significant

($p>0.05$) relative to the unloaded hydrogel. Only 99μM NaIbu hydrogel significantly reduced HBL cell migration in TNF-α stimulated cells ($p<0.05$) relative to the unloaded hydrogel.

For A375SM cells, migration was significantly reduced with the unloaded hydrogel at 2, 4 and 8 hours compared to media (not stimulated with TNF-α) and TNF-α only (see Table 3.3 for statistical details). However, at 24 hours, increasing concentration of NaIbu hydrogel of 66μM, 99μM and 131μM significantly reduced A375SM cell migration in TNF-α stimulated cells compared to the unloaded hydrogel ($p<0.05$, $p<0.01$ and $p<0.001$, respectively).

For C8161 cells, migration was significantly increased by 300U/ml TNF-α compared to the unloaded hydrogel ($p<0.05$), at 2 hours. At 8 hours, migration was significantly increased by 300U/ml TNF-α and the unloaded hydrogel treated cells ($p<0.01$, respectively) relative to media treated cells. Also, higher NaIbu hydrogel concentrations of 99μM and 131μM reduced migration ($p<0.05$, respectively) compared to the unloaded hydrogel, in TNF-α stimulated cells. At 24 hours, C8161 cell migration was significantly reduced by 300U/ml TNF-α compared to the unloaded hydrogel ($p<0.001$). Either TNF-α treated cells and unloaded hydrogel significantly increased C8151 migration compared to media treated cells ($p<0.001$, respectively). Increasing NaIbu hydrogel concentrations reduced C8161 cell migrations at 33μM and 66μM ($p<0.01$ for both concentrations) and at 99μM and 131μM ($p<0.001$ for both concentrations) in TNF-α stimulated cells. The maximum NaIbu hydrogel concentration of 131μM reduced cell migration to a similar level of migration of cells treated with media only which demonstrated the stimulatory effect of TNF-α on melanoma cell migration and the suppressive effect of NaIbu hydrogel on melanoma cell migration. See Figures 3.8 C and 3.9 C and Table 3.3 for a summary of these results.

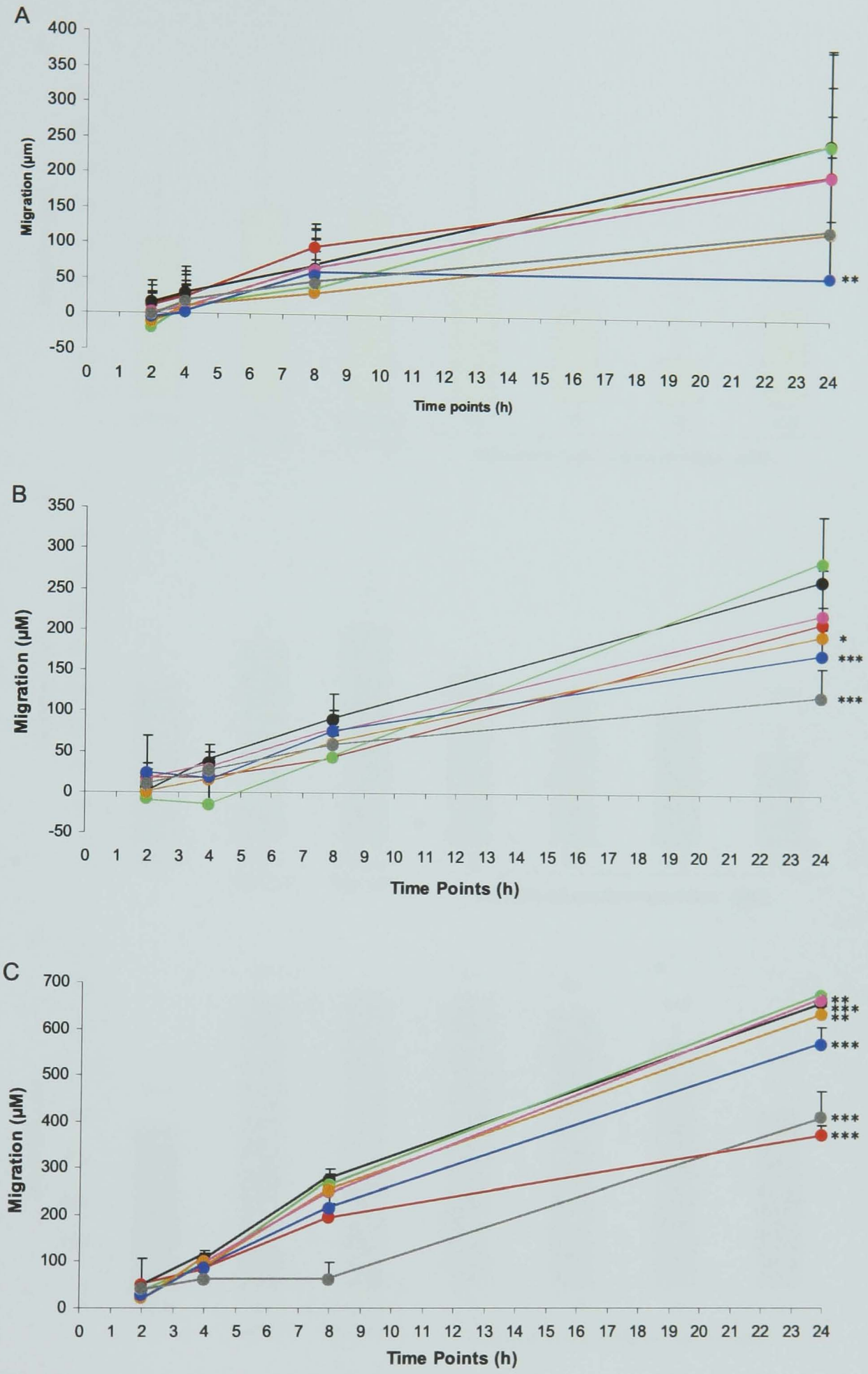
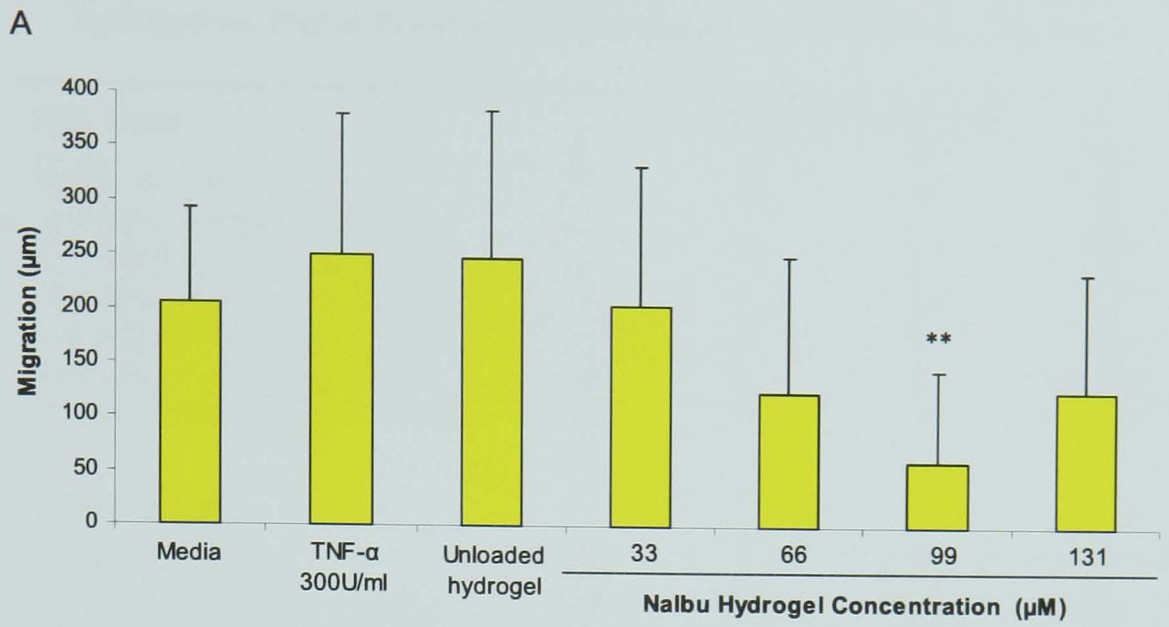
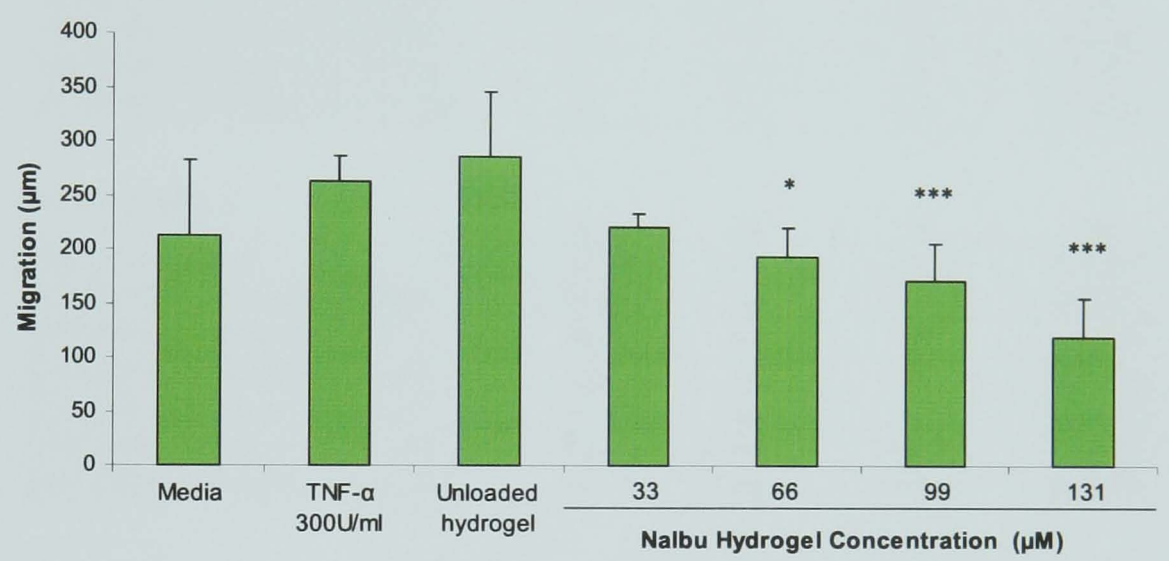


Figure 3.8. Effects of NaIbu hydrogel on TNF-α pre-stimulated melanoma cell migration over 24 hours (h). (A) HBL cells, N=6. (B) A375SM cells, N=6. (C) C8161 cells, N=5. ● = Media, ● = TNF-α, ● = Unloaded hydrogel, ● = 33µM, ● = 66µM, ● = 99µM, ● = 131µM. Values are expressed as Mean+SD. * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$, relative to the unloaded gel.





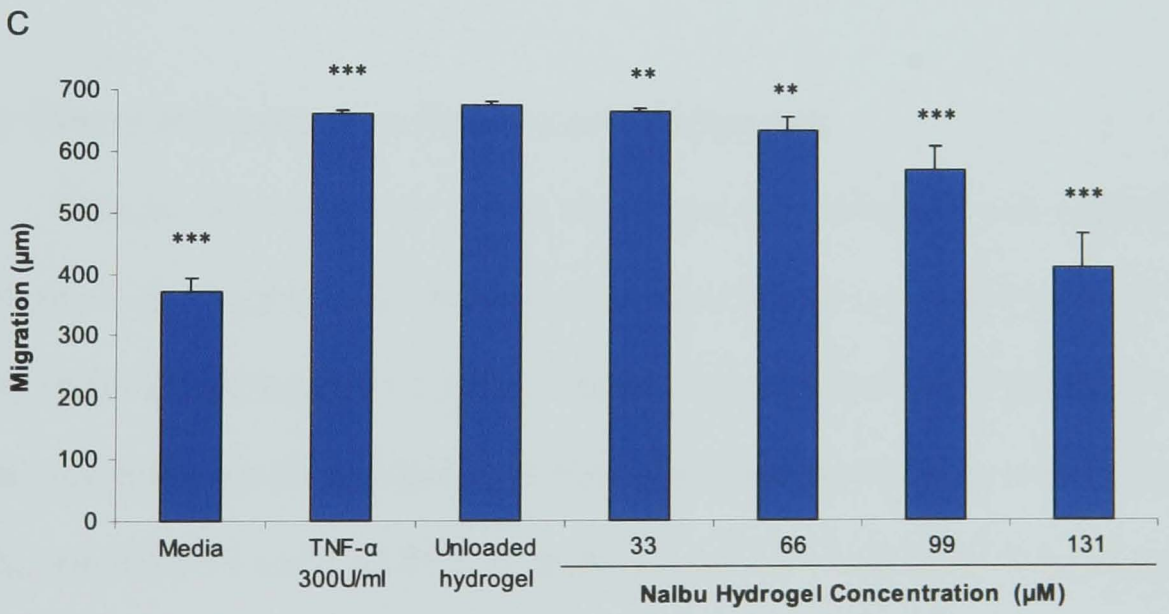


Figure 3.9. Effects of NaIbu hydrogel on TNF-α pre-stimulated melanoma cell migration at 24 hours. (A) HBL cells, (B) A375SM cells, and (C) C8161 cells. Values are expressed as Mean+SD. * = p<0.05; ** = p<0.01; *** = p<0.001, relative to the unloaded gel.

Table 3.3. Statistical Summary for comparison between treatments – Effects of NaIbu hydrogel on TNF-α Pre-stimulated Melanoma Cell Migration (p values).

HBL Cells	Time Points (hours)			
	2	4	8	24
Media *vs* TNF-α	*ns*	*ns*	*ns*	*ns*
Unloaded hydrogel *vs* Media	*ns*	*ns*	*ns*	*ns*
Unloaded hydrogel *vs* TNF-α	*ns*	*ns*	*ns*	*ns*
Unloaded hydrogel *vs* 33 μM	*ns*	*ns*	*ns*	*ns*
Unloaded hydrogel *vs* 66 μM	*ns*	*ns*	*ns*	*ns*
Unloaded hydrogel *vs* 99 μM	*ns*	*ns*	*ns*	0.0178
Unloaded hydrogel *vs* 131 μM	*ns*	*ns*	*ns*	*ns*

A375SM Cells	2	4	8	24
Media *vs* TNF-α	*ns*	*ns*	*0.002*	*ns*
Unloaded hydrogel *vs* Media	0.0217	*ns*	*ns*	*ns*
Unloaded hydrogel *vs* TNF-α	*ns*	0.0359	*0.0052*	*ns*
Unloaded hydrogel *vs* 33 μM	*ns*	*ns*	*ns*	*ns*
Unloaded hydrogel *vs* 66 μM	*ns*	*ns*	*ns*	0.0184
Unloaded hydrogel *vs* 99 μM	*ns*	*ns*	*ns*	0.007
Unloaded hydrogel *vs* 131 μM	*ns*	*ns*	*ns*	0.0007

C8161 Cells	2	4	8	24
Media *vs* TNF-α	*ns*	*ns*	0.002	0.0001
Unloaded hydrogel *vs* Media	0.0436	*ns*	0.004	0.0001
Unloaded hydrogel *vs* TNF-α	*ns*	*ns*	*ns*	0.0004
Unloaded hydrogel *vs* 33 μM	*ns*	*ns*	*ns*	0.0029
Unloaded hydrogel *vs* 66 μM	*ns*	*ns*	*ns*	0.0018
Unloaded hydrogel *vs* 99 μM	*ns*	*ns*	0.0292	0.0002
Unloaded hydrogel *vs* 131 μM	*ns*	*ns*	0.0141	0.0001

3.4.4. Effects of Capsaicin on Melanoma Cell Migration

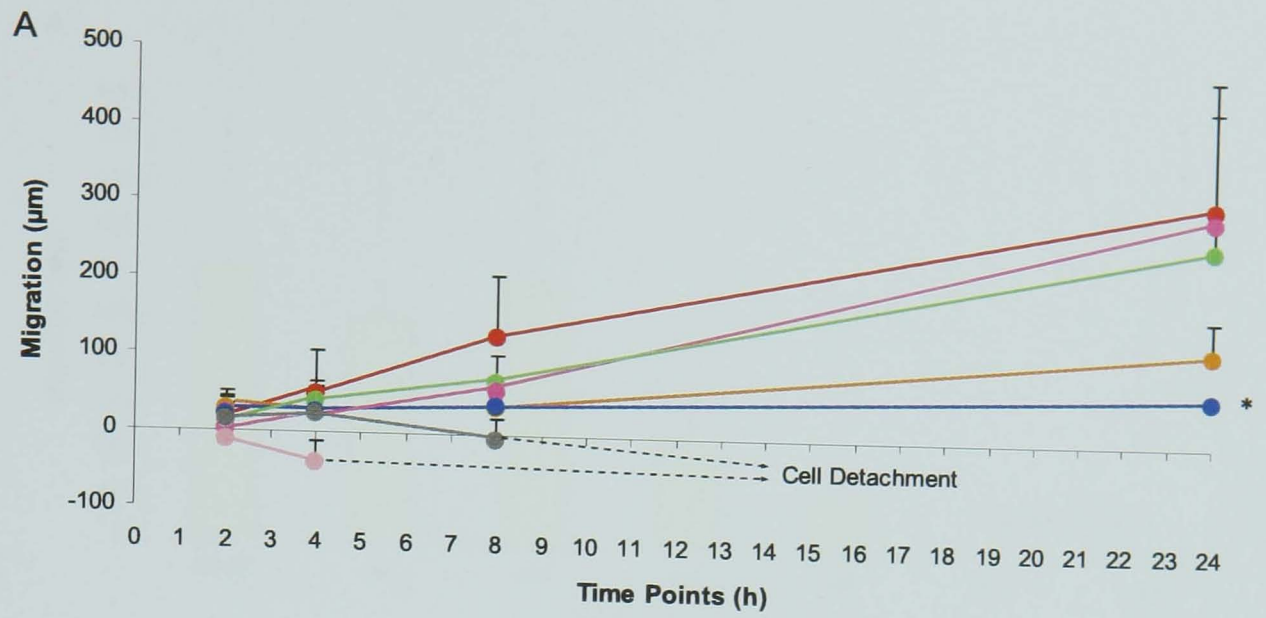
In these experiments the effects of capsaicin on melanoma cell migration was investigated. See Figure 3.10. Melanoma cell were seeded at a density of $1x10^5$ cells / ml per well and incubated for 3 days in media. After this incubation period, a scratch-wound was made and photographed for migration measurement as previously described. Media was removed and 1 ml of fresh media per well (as control) or different capsaicin concentrations were added to the cells.

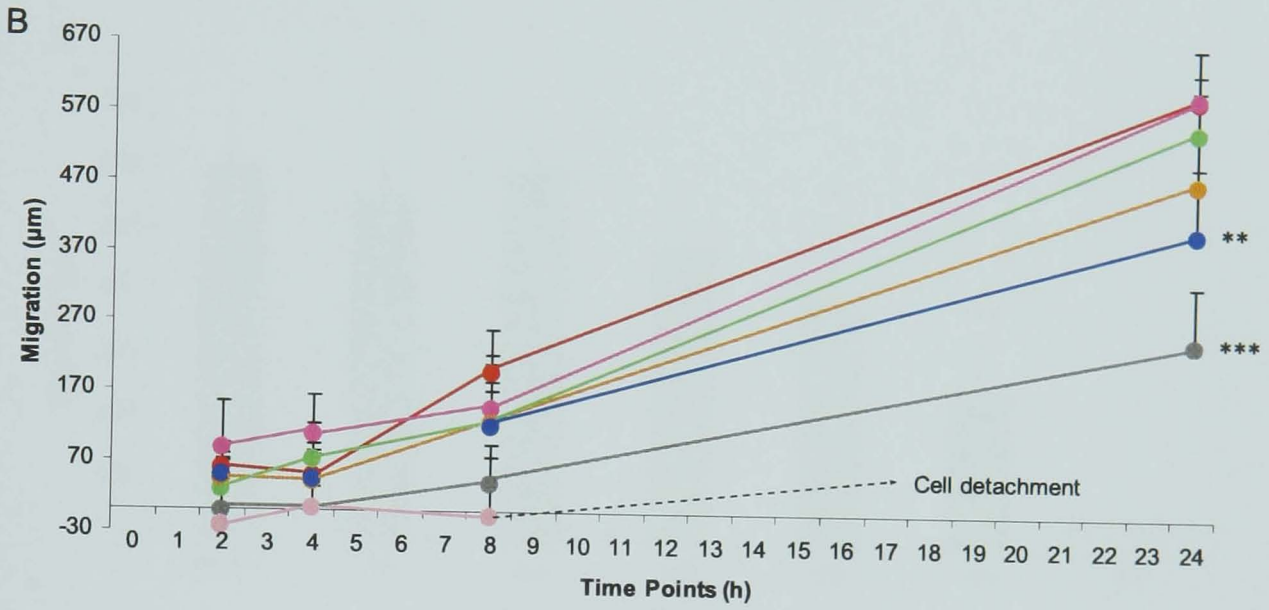
For HBL cells, overall, increasing capsaicin concentrations reduced cell migration compared to control (media). At 8 hours, 500μM capsaicin detached the cells from the wells. At 24 hours 500μM capsaicin also detached HBL cells from the wells

indicating toxic effect of capsaicin on HBL cells. At 8 hours, HBL cell migration was significantly reduced at a capsaicin concentration of 300μM and 400μM ($p<0.05$ respectively), compared to media. At 24 hours migration was further reduced at capsaicin concentration of 300μM ($p<0.05$). See Figures 3.10 A and 3.11 A for an illustration of these results and Table 3.4 for statistical summary of results.

Overall, at 8 hours, A375SM cell migration was reduced by the increasing capsaicin concentrations. A375SM cell migration was significantly reduced at 400μM and 500μM capsaicin ($p<0.05$ respectively) relative to media. At 24 hours A375SM cell migration was significantly reduced at capsaicin concentrations of 300μM and 400μM ($p<0.01$ and $p<0.001$ respectively) relative to media. At capsaicin concentration of 500μM however the cells detached from the wells indicating that this concentration was toxic to the cells. See Figures 3.10 B and 3.11 B for an illustration of these results and Table 3.4 for statistical summary of results.

Overall, up to 8 hours, C8161 cell migration was slightly increased at a capsaicin concentration of 400μM compared to media. At 24 hours, C8161 cell migration was slightly increased at capsaicin concentrations of 100μM and 200μM respectively. Migration was then reduced at capsaicin concentrations of 300μM, 400μM and 500μM which reduced C8161 cell migration below the media value. However, none of these were statistically significant ($p>0.05$). See Figures 3.10 C and 3.11 C for an illustration of these results and, Table 3.4 for statistical summary of results.





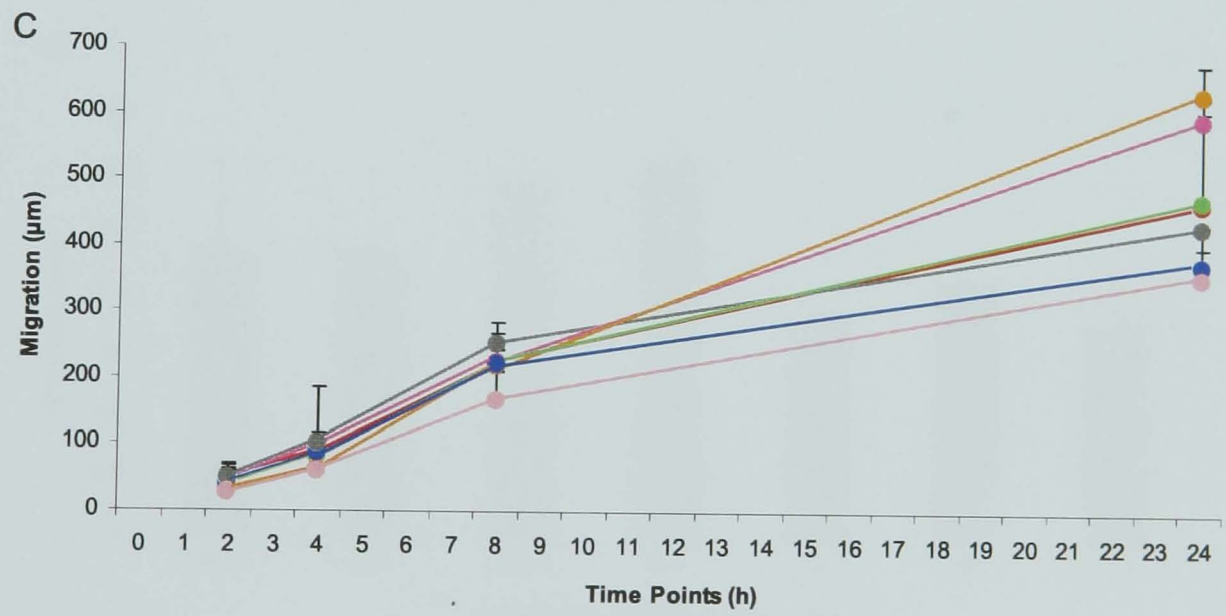
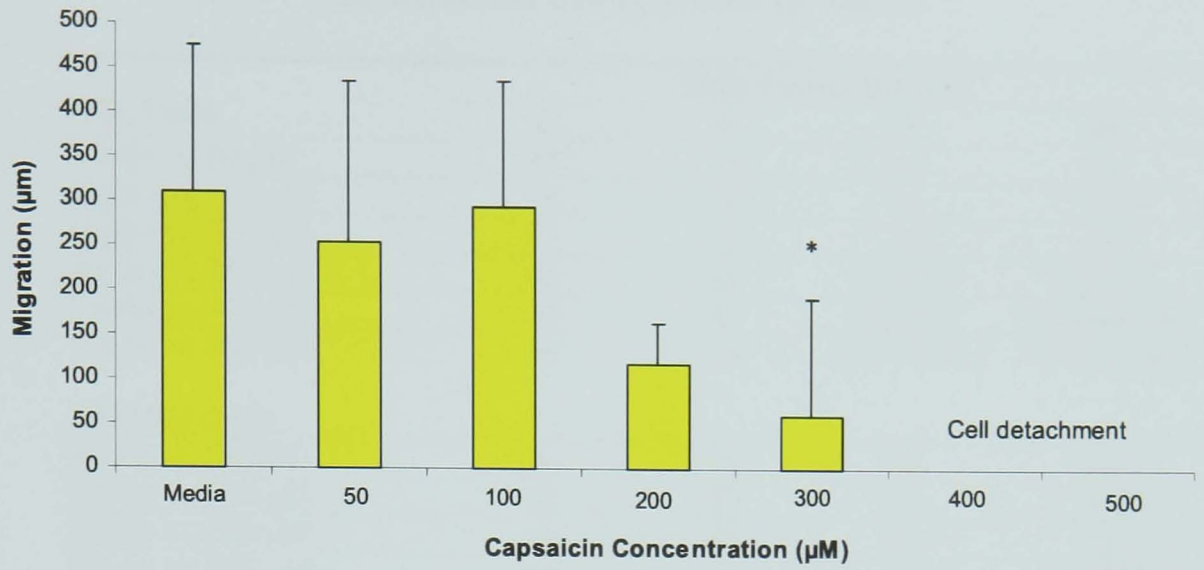


Figure 3.10. Effects of Capsaicin on melanoma cell migration over 24 hours (h). (A) HBL cells, N=6. (B) A375SM cells, N=5. (C) C8161 cells, N=6. ● = Media, ● = 50µM, ● = 100µM, ● = 200µM, ● = 300µM, ● = 400µM, ● =500µM. Values expressed as Mean+SD. * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$, relative to media.

A



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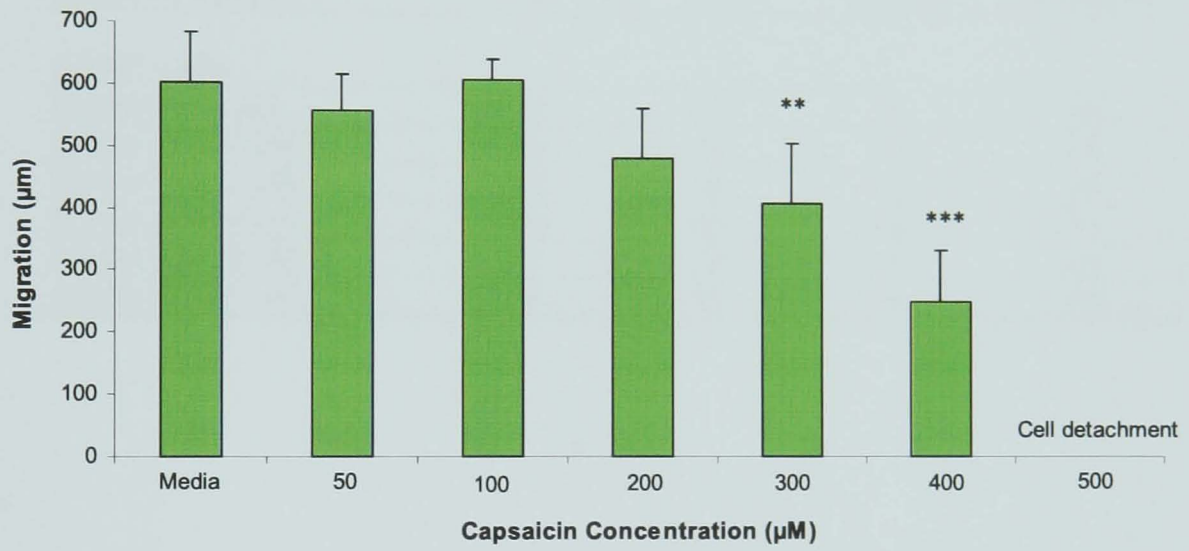


C

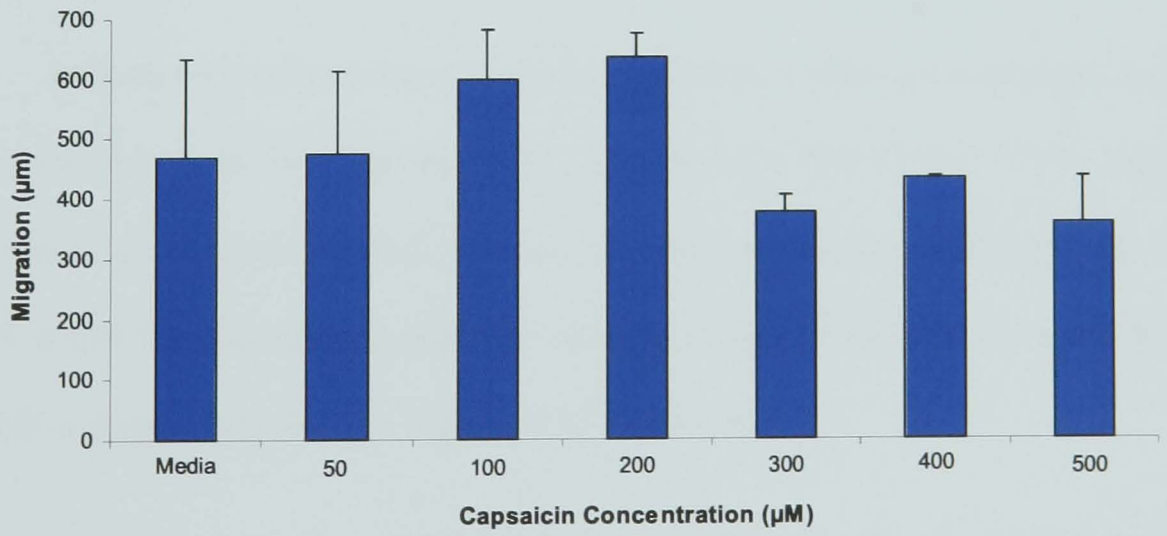


Figure 3.11. Effects of Capsaicin on melanoma cell migration at 24 hours. (A) HBL cells, (B) A375SM cells, and (C) C8161 cells. Values are expressed as Mean+SD. * = $p<0.05$; **= $p<0.01$; ***= $p<0.001$, relative to media.

Table 3.4. Statistical Summary for comparison between treatments - Effects of Capsaicin on Melanoma Cell Migration (p values).

	Time Points (hours)			
HBL Cells	**2**	**4**	**8**	**24**
Media *vs* 50 µM	*ns*	*ns*	*ns*	*ns*
Media *vs* 100 µM	*ns*	*ns*	*ns*	*ns*
Media *vs* 200 µM	*ns*	*ns*	*ns*	*ns*
Media *vs* 300 µM	*ns*	*ns*	0.0150	0.0150
Media *vs* 400 µM	*ns*	*ns*	0.0130	Cell detachment
Media *vs* 500 µM	*ns*	*ns*	Cell detachment	Cell detachment
A375SM Cells				
Media *vs* 50 µM	*ns*	*ns*	*ns*	*ns*
Media *vs* 100 µM	*ns*	*ns*	*ns*	*ns*
Media *vs* 200 µM	*ns*	*ns*	*ns*	*ns*
Media *vs* 300 µM	*ns*	*ns*	*ns*	0.0040
Media *vs* 400 µM	*ns*	*ns*	0.020	0.0001
Media *vs* 500 µM	*ns*	*ns*	0.010	Cell detachment
C8161 Cells				
Media *vs* 50 µM	*ns*	*ns*	*ns*	*ns*
Media *vs* 100 µM	*ns*	*ns*	*ns*	*ns*
Media *vs* 200 µM	*ns*	*ns*	*ns*	*ns*
Media *vs* 300 µM	*ns*	*ns*	*ns*	*ns*
Media *vs* 400 µM	*ns*	*ns*	*ns*	*ns*
Media *vs* 500 µM	*ns*	*ns*	*ns*	*ns*

3.4.5. Effects of Capsaicin on TNF-α Pre-stimulated Melanoma Cell Migration

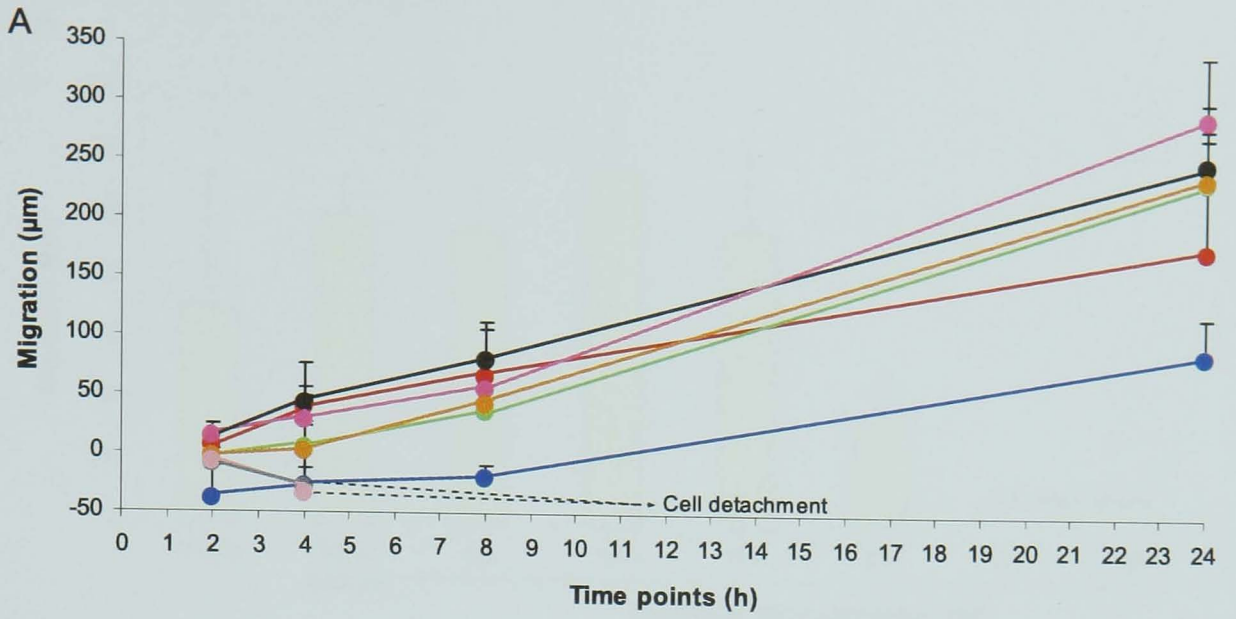
In these experiments the effects of capsaicin on TNF-α pre-stimulated melanoma cell migration was investigated. Cells treated with 300 U/ml TNF-α only were considered as controls. Overall, TNF-α increased melanoma cell migration over 24 hours whilst the increasing capsaicin concentrations reduced melanoma cell migration in TNF-α stimulated cells (see Figures 3.12 and 3.13).

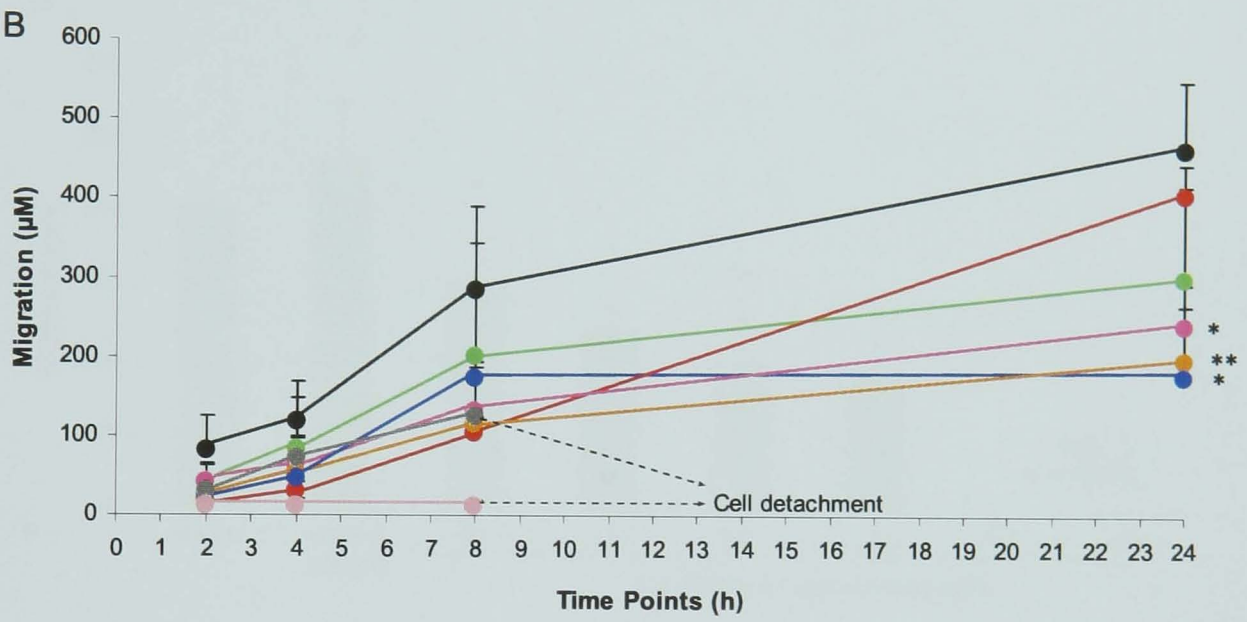
For HBL cells, increasing capsaicin concentrations reduced migration in TNF-α pre-stimulated cells, however this was not significant ($p>0.05$). However, at 8 hours capsacin concentrations of 400µM and 500µM detached the cells in TNF-α pre-stimulated cells, meaning that these were toxic to the cells. At 24 hours, HBL cell

migration was slightly reduced at 300μM capsaicin in TNF-α pre-stimulated cells ($p>0.05$) relative to TNF-α treated cells.

At 2, 4 , 8 and 24 hours, A375SM cell migration was significantly increased by 300U/ml TNF-α compared to media treated cells ($p<0.05$, $p<0.01$, $p<0.001$ and $p<0.001$ respectively). At 2 hours, A375SM cell migration was reduced at 200μM, 300μM and 500μM capsaicin in TNF-α pre-stimulated cells ($p<0.05$ in all cases) compared to TNF-α treated cells. At 4 hours, 300μM and 500μM capsaicin concentrations reduced cell migration in TNF-α pre-stimulated cells ($p<0.05$ and $p<0.01$, respectively) relative to TNF-α treated cell. At 8 hours, A375SM cell migration was significantly reduced at 100 and 200 capsaicin in TNF-α pre-stimulated cells ($p<0.05$ for both cases) and, at 400μM and 500μM ($p<0.05$ and $p<0.01$, respectively) relative to TNF-α treated cells. At 24 hours 100μM, 200μM and 300μM capsaicin significantly reduced A375SM cell migration in TNF-α pre-stimulated cells ($p<0.05$, $p<0.01$ and $p<0.05$, respectively) relative to TNF-α treated cells. However 400μM and 500μM capsaicin detached A375SM cells from the wells and these concentrations were toxic to cells. Figures 3.12 B and Figure 3.13 B illustrate these results.

Overall, 300U/ml TNF-α slightly increased C8161 cell migration compared to media treated cells and different capsaicin concentrations in TNF-α stimulated cells. This was significant at 2 and 4 hours, comparing TNF-α treated cells and media treated cells ($p<0.01$ and $p<0.05$, respectively). At 8 and 24 hours, C8161 cell migration was significantly reduced at capsaicin concentration of 500μM in TNF-α stimulated cells ($p<0.05$) compared to TNF-α treated cells. Figures 3.12 C and Figure 3.13 C illustrate these results and Table 3.5 for a statistical summary of these results.





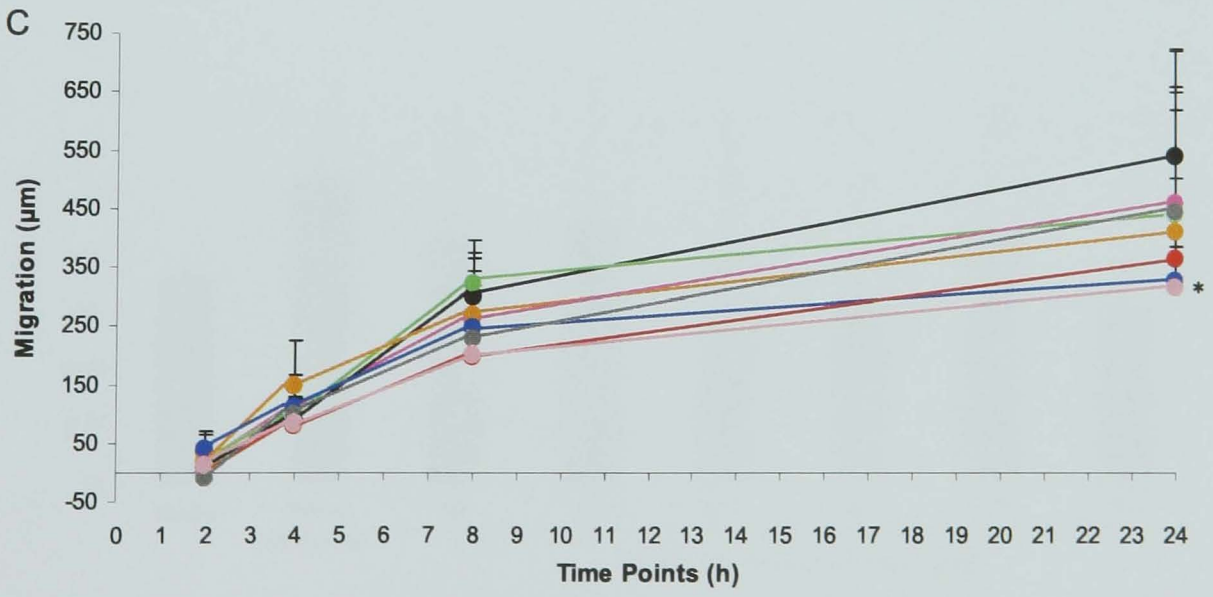
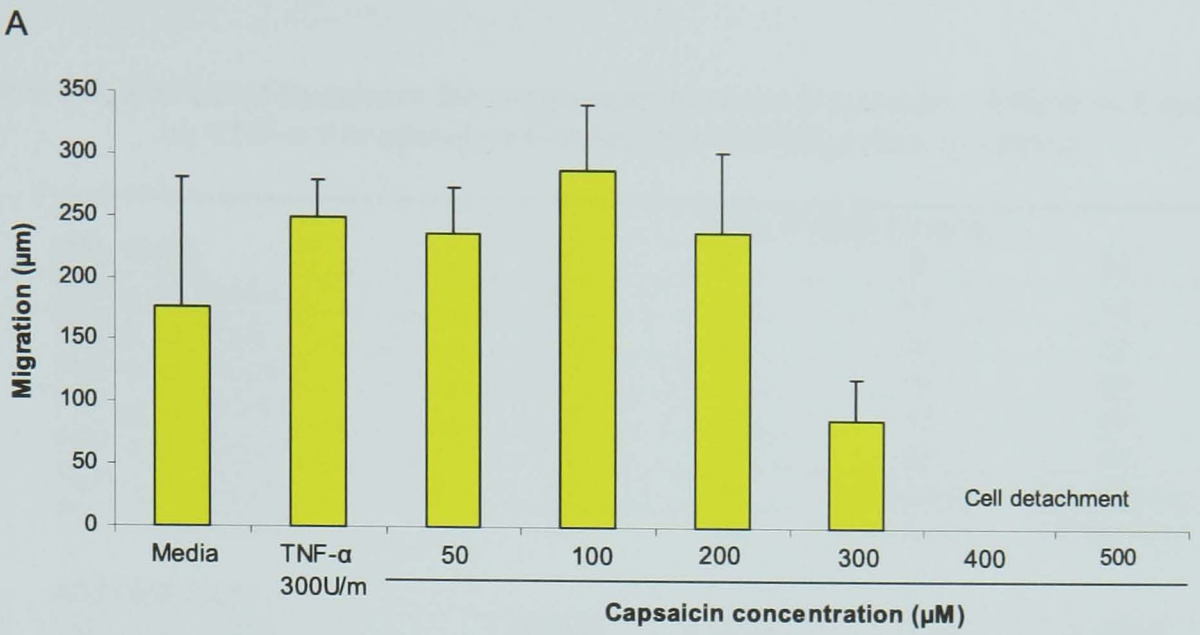
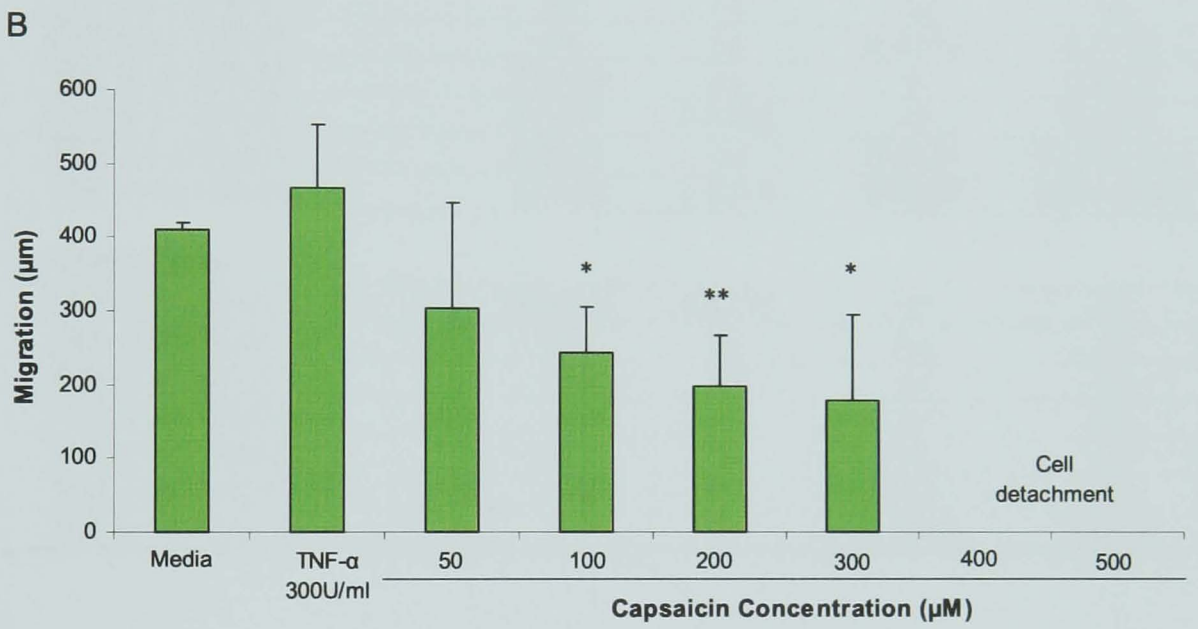


Figure 3.12. Effects of Capsaicin on TNF-α pre-stimulated melanoma cell migration over 24 hours (h). (A) HBL cells, N=6. (B) A375SM cells, N=5. (C) C8161 cells, N=6. ● = Media, ● = TNF-α, ● = 50µM, ● = 100µM, ● = 200µM, ● = 300µM, ● = 400µM, ● =500µM. Values are expressed as Mean+SD. * = $p<0.05$; ** = $p<0.01$, relative to TNF-α treated cells.





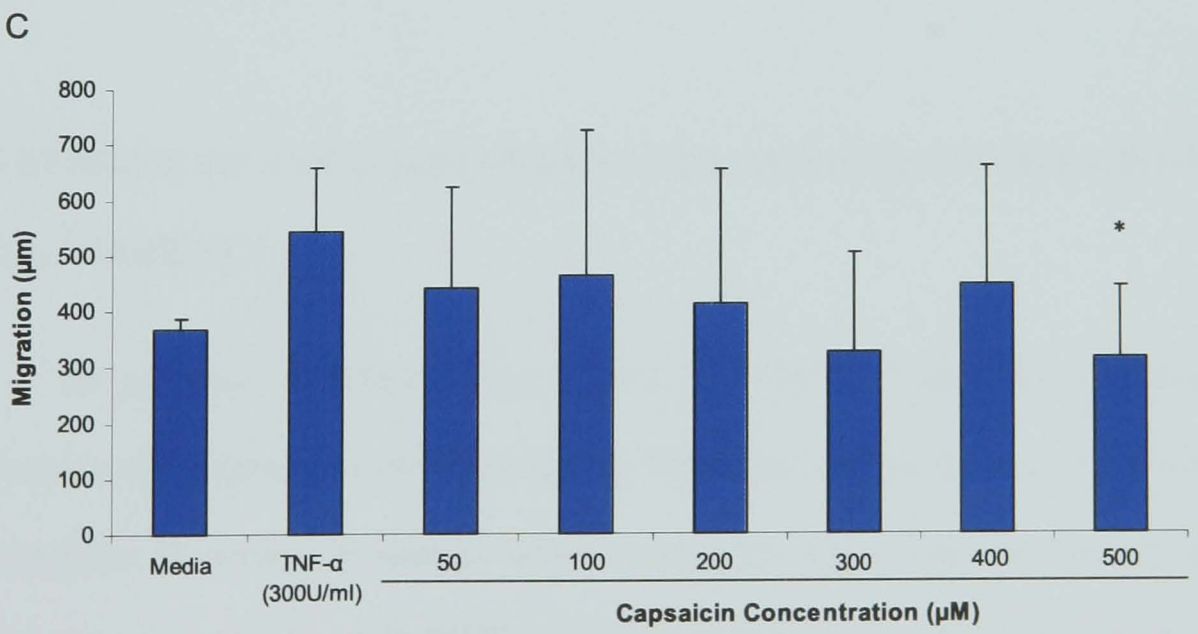


Figure 3.13. Effects of Capsaicin on TNF-α pre-stimulated melanoma cell migration at 24 hours. (A) HBL cells, (B) A375SM cells, (C) C8161 cells. Values are expressed as Mean+SD. * = $p<0.05$; ** = $p<0.01$, relative to TNF-α treated cells.

Table 3.5. Statistical Summary for comparison between treatments - Effects of Capsaicin on TNF-α Pre-stimulated Melanoma Cell Migration (p values).

	Time Points (hours)			
HBL Cells	**2**	**4**	**8**	**24**
TNF-α *vs* Media	*ns*	*ns*	*ns*	*ns*
TNF-α *vs* 50μM	*ns*	*ns*	*ns*	*ns*
TNF-α *vs* 100μM	*ns*	*ns*	*ns*	*ns*
TNF-α *vs* 200μM	*ns*	*ns*	*ns*	*ns*
TNF-α *vs* 300μM	*ns*	*ns*	*ns*	*ns*
TNF-α *vs* 400μM	*ns*	*ns*	Cell detachment	Cell detachment
TNF-α *vs* 500μM	*ns*	*ns*	Cell detachment	Cell detachment
A375SM Cells				
TNF-α *vs* Media	0.0316	0.0030	0.0004	0.0001
TNF-α *vs* 50μM	*ns*	*ns*	*ns*	*ns*
TNF-α *vs* 100μM	*ns*	*ns*	0.0243	0.0168
TNF-α *vs* 200μM	0.0159	*ns*	*ns*	0.0032
TNF-α *vs* 300μM	0.0331	0.0493	*ns*	0.0274
TNF-α *vs* 400μM	0.0521	*ns*	0.0234	Cell detachment
TNF-α *vs* 500μM	0.0105	0.0014	0.0051	Cell detachment
C8161 Cells				
TNF-α *vs* Media	0.0023	0.0211	*ns*	*ns*
TNF-α *vs* 50μM	*ns*	*ns*	*ns*	*ns*
TNF-α *vs* 100μM	*ns*	*ns*	*ns*	*ns*
TNF-α *vs* 200μM	*ns*	*ns*	*ns*	*ns*
TNF-α *vs* 300μM	*ns*	*ns*	*ns*	*ns*
TNF-α *vs* 400μM	*ns*	*ns*	*ns*	*ns*
TNF-α *vs* 500μM	*ns*	*ns*	0.0293	0.0186

3.5. EFFECTS OF TNF-α, IBUPROFEN AND CAPSAICIN ON MELANOMA CELL VIABILITY

In previous experiments the effects of TNF-α, Nalbu and capsaicin on melanoma cell migration were investigated. Melanoma cell migration was affected by these agents. In order to investigate whether migration was affected by the toxic effects of these agents a viability test (MTT assay) was used. These experiments used the same cells cultured in 12 well plates after finishing with the migration experiments (24 hours). The MTT assay provided a reading for cell viability which was converted to a percentage of viable cells.

3.5.1. Effects of NaIbu (in media) on Melanoma Cell Viability

In these experiments, melanoma cells were treated with NaIbu in media and melanoma cell viability was investigated (See Figure 3.14). Increasing concentrations of NaIbu in media reduced viability in three melanoma cell lines at two NaIbu concentrations used (66μM and 131μM) compared to control media.

HBL control cell viability was normalised to 100%. HBL cell viability was significantly reduced to 14.3% and to 2.7% at NaIbu concentrations of 66μM and 131μM ($p<0.05$ and $p<0.01$), respectively.

A375SM control cell viability was normalised to 100%. A375SM cell viability was significantly reduced to 25.6% and 2.5% at NaIbu concentrations of 66μM and 131μM ($p<0.01$), respectively.

C8161 control cell viability was normalised to 100%. C8161 cell viability was reduced to 61.4% at NaIbu concentration of 66μM. This was not significant ($p>0.05$). Viability was significantly reduced to 33% at 131μM NaIbu ($p<0.01$). Figure 3.14 and Table 3.6 illustrate a summary of these results.

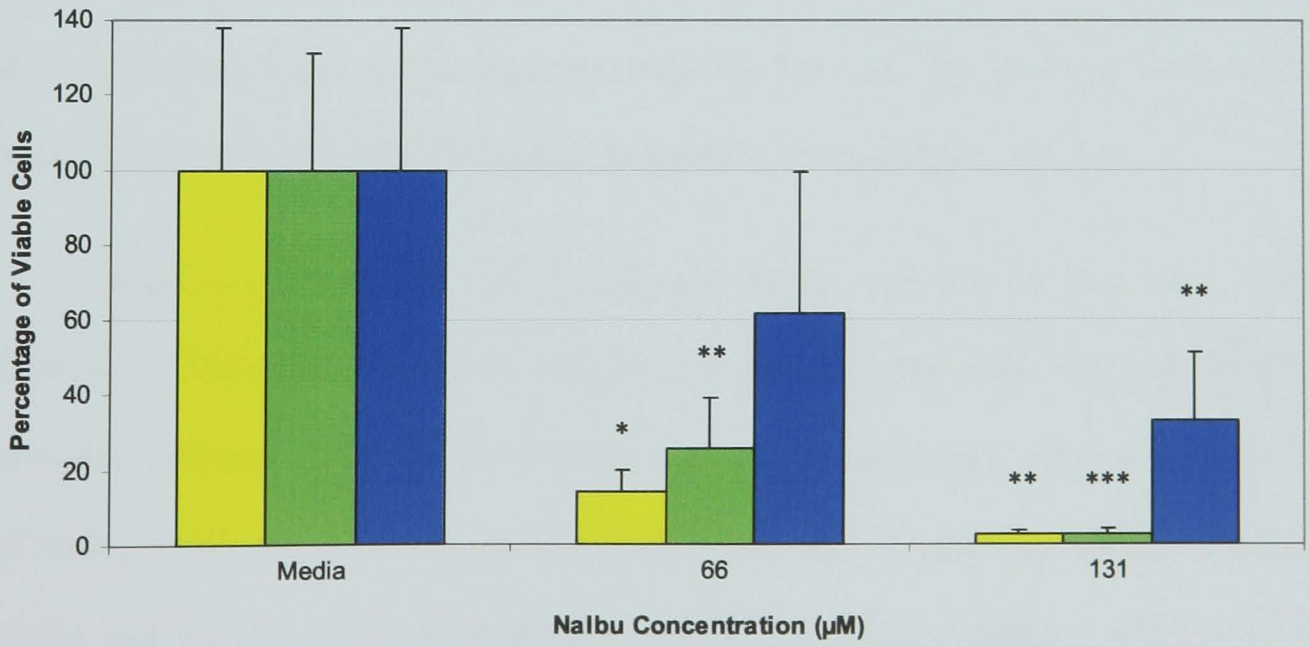


Figure 3.14. Effects of NaIbu in media on melanoma cell viability. ▯ = HBL cells, N=4. ▯ = A375SM cells, N=4. ▯ = C8161 cells, N=5. Values are expressed as Mean+SD. * = $p<0.05$; ** = $p<0.01$; *** = $p < 0.001$, relative to media.

Table 3.6. Statistical Summary for comparison between treatments - Effects of NaIbu in media on melanoma cell viability (p values)

HBL Cells	p Value
Media *vs* 66 μM	0.0125
Media *vs* 131 μM	0.0021
A375SM Cells	
Media *vs* 66 μM	0.0045
Media *vs* 131 μM	0.0007
C8161 Cells	
Media *vs* 66 μM	*ns*
Media *vs* 131 μM	0.0074

3.5.2. Effects of NaIbu hydrogel on Melanoma Cell Viability

In these experiments, melanoma cells were treated with different concentrations of NaIbu hydrogel and cell viability was investigated (See Figure 3.15).

Viability of HBL cell treated with media only was normalised to 100%. HBL cell viability was unchanged by the unloaded hydrogel (98.2% viable cells) compared to media treated cells. HBL cell viability increased to 118% at 33μM NaIbu hydrogel concentration and was reduced to 87% and 60% at NaIbu hydrogel concentrations of 66μM and 99μM, respectively. However, these were not significant ($p<0.05$) relative to the unloaded hydrogel. A significant reduction in HBL cell viability to 26% occurred at NaIbu concentration of 131μM ($p<0.01$) relative to the unloaded hydrogel.

Viability of A375SM cell treated with media only was normalised to 100%. Overall, A375SM melanoma cell viability was reduced with increasing concentrations of NaIbu hydrogel. A375SM cell viability was slightly reduced to 78%, 62% and 57% at NaIbu hydrogel concentrations of 66μM, 99μM and 131μM respectively. Although 99μM and 131μM NaIbu hydrogel reduced A375SM cell viability compared to the unloaded hydrogel, these effects were not significant ($p>0.05$).

Viability of C8161 cells treated with media only was normalised to 100%. C8161 cell viability was unchanged by the unloaded hydrogel (97.6% of viable cells) compared to media treated cells. Overall, C8161 melanoma cell viability was slightly reduced with increasing concentrations of NaIbu hydrogel compared to the unloaded hydrogel ($p>0.05$).

These results suggest that NaIbu hydrogel had no toxic effect on A375SM and C8161 cells. See Figure 3.15 for an illustration of these results.

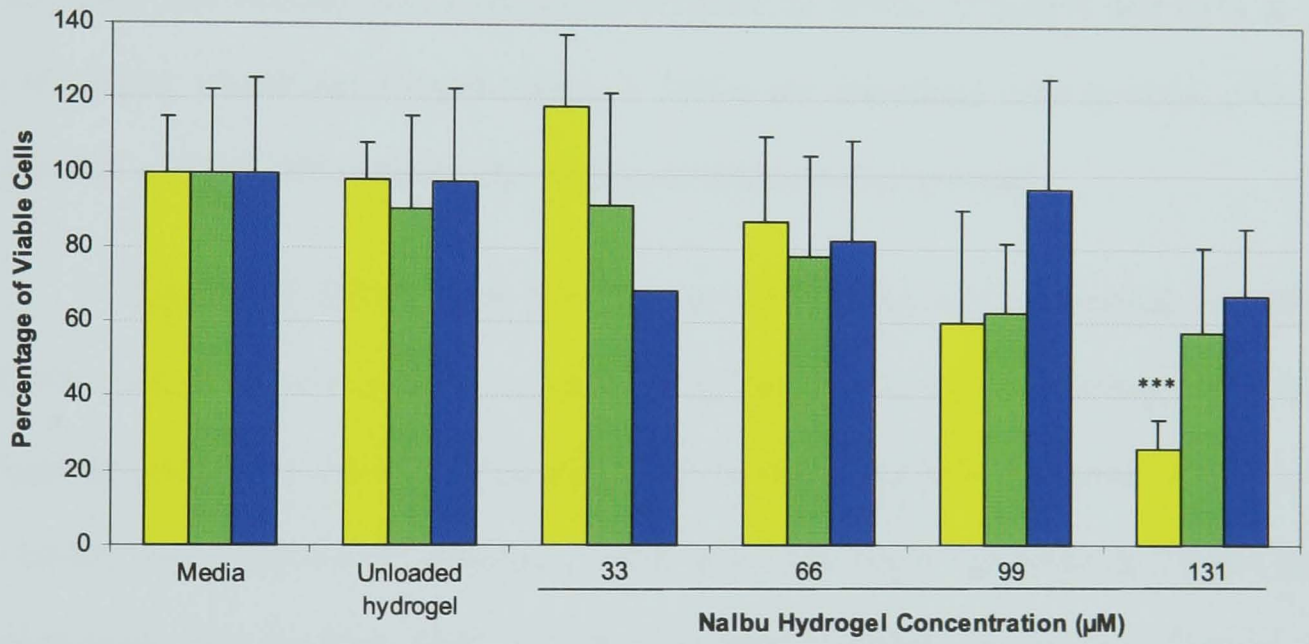


Figure 3.15. Effects of NaIbu hydrogel on melanoma cell viability. ▯ = HBL cells, ▯ = A375SM cells, ▯ = C8161 cells. Values are expressed as Mean+SD. N=4. * indicates $p<0.001$, relative to the unloaded hydrogel.**

3.5.3. Effects of NaIbu hydrogel on TNF-α Pre-stimulated Melanoma Cell Viability

In these experiments melanoma cells were pre-stimulated for 24 hours with 300U/ml of TNF-α. After this incubation period, media was removed and fresh media was added to the cells. Inserts containing 300μl of increasing NaIbu hydrogel concentrations were added to the wells. The cells exposed to unloaded hydrogels were also TNF-α pre-stimulated and were considered as controls. See Figure 3.16.

For the three melanoma cell lines, viability was unchanged at the unloaded hydrogel in TNF-α pre-stimulated cells compared to the cells treated with media or TNF-α only ($p>0.05$ respectively).

Viability of HBL cells treated with media only was normalised to 100%. HBL cell viability was significantly reduced to 62%, 21% and 4% at 66μM, 99μM and 131μM NaIbu hydrogel in TNF-α pre-stimulated cells ($p<0.05$, $p<0.001$ and $p<0.001$ respectively) relative to the unloaded hydrogel.

Viability of A375SM cells treated with media only was normalised to 100%. A375SM cell viability was significantly reduced to 79.6%, 73%,65% and 48% at 33 μM, 66μM, 99μM and 131μM NaIbu in TNF-α pre-stimulated cells ($p<0.05$, $p<0.01$, $p<0.001$ and $p<0.001$ respectively) relative to the unloaded hydrogel.

Viability of C8161 cells treated with media only was normalised to 100%. C8161 melanoma cell viability was reduced with the increasing concentrations of NaIbu hydrogel in TNF-α pre-stimulated cells relative to the unloaded hydrogel. C8161 cell viability was significantly reduced to 56% at 131μM NaIbu hydrogel in TNF-α pre-stimulated cells ($p<0.05$) relative to the unloaded hydrogel. See Figure 3.16 for an illustration of these results and Table 3.7 for a statistical summary.

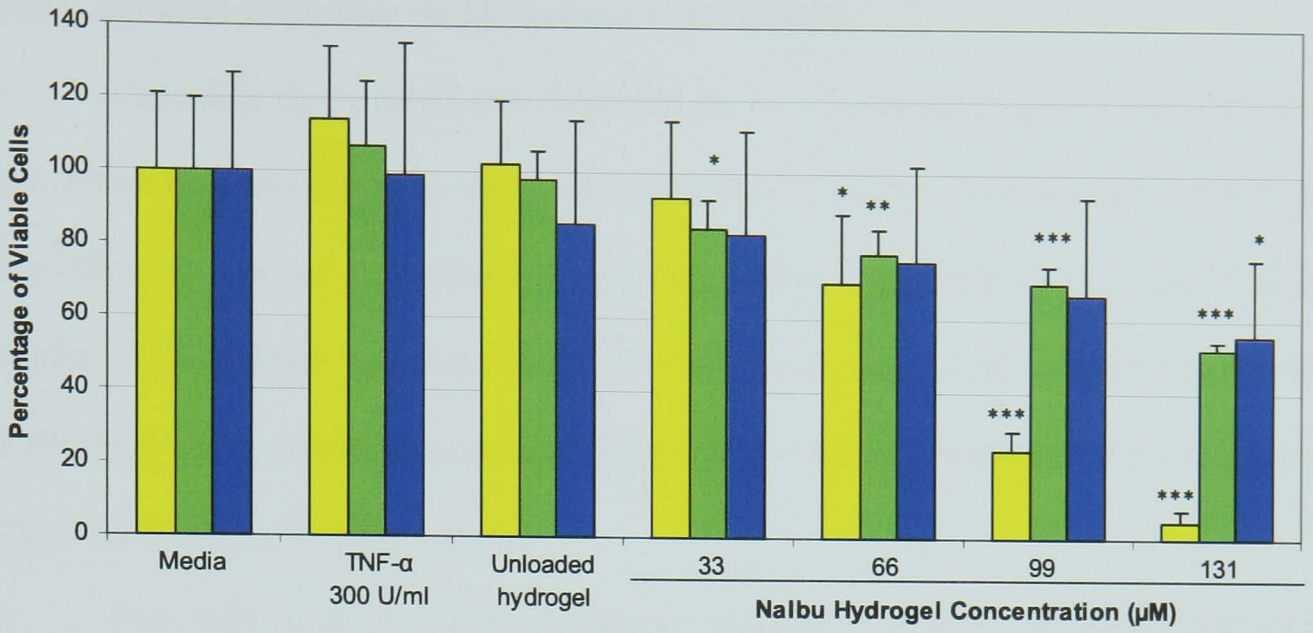


Figure 3.16. Effects of NaIbu hydrogel on TNF-α pre-stimulated melanoma cell viability. ▯ = HBL cells, ▯ = A375SM cells, ▯ = C8161 cells. Values are expressed as Mean+SD. N=3. * = p<0.05; ** = p <0.01; *** = p < 0.001, relative to the unloaded hydrogel.

Table 3.7. Statistical Summary for comparison between treatments - Effects of NaIbu hydrogel on TNF-α pre-stimulated melanoma cell viability (p values)

HBL Cells	**p values**
Media *vs* TNF-α	*ns*
Unloaded hydrogel *vs* Media	*ns*
Unloaded hydrogel *vs* TNF	*ns*
Unloaded hydrogel *vs* 33 μM	*ns*
Unloaded hydrogel *vs* 66 μM	0.0226
Unloaded hydrogel *vs* 99 μM	0.0001
Unloaded hydrogel *vs* 131 μM	0.0001

A375SM Cells	
Media *vs* TNF-α	*ns*
Unloaded hydrogel *vs* Media	*ns*
Unloaded hydrogel *vs* TNF-α	*ns*
Unloaded hydrogel *vs* 33 μM	0.0204
Unloaded hydrogel *vs* 66 μM	0.0014
Unloaded hydrogel *vs* 99 μM	0.0001
Unloaded hydrogel *vs* 131 μM	0.0001

C8161 Cells	**p Value**
Control *vs* TNF-α	*ns*
Unloaded hydrogel *vs* Media	*ns*
Unloaded hydrogel *vs* TNF-α	*ns*
Unloaded hydrogel *vs* 33 μM	*ns*
Unloaded hydrogel *vs* 66 μM	*ns*
Unloaded hydrogel *vs* 99 μM	*ns*
Unloaded hydrogel *vs* 131 μM	0.0455

3.5.4. Effect of Capsaicin on Melanoma Cell Viability

Capsaicin (in powder) was dissolved in 0.5 ml ethanol to give a stock solution for further dilutions in media. An initial experiment was done in triplicate to investigate the effects of ethanol on melanoma cells. A volume of pure ethanol was added to the media. This volume was equivalent to the volume used from the capsaicin stock solution to make a 600μM capsaicin concentration (18.24 μl of ethanol in 10 ml media), resulting in media 0.18% ethanol.

Increased capsaicin concentrations reduced viability of melanoma cells comparing cells treated with media only or media containing ethanol over 24 hours. As there was a single experiment of media containing ethanol, two controls were used for comparisons - media only or media containing ethanol compared to the increasing capsaicin concentrations. These results are included in Figure 3.17.

Viability of HBL cells treated with media only was normalised to 100%. HBL cell viability was reduced to 61% with media containing ethanol, compared to cells treated with media only. This was not significant ($p>0.05$). Capsaicin concentrations significantly reduced HBL cell viability to 16.8%, 2.4% and 2.5% at capsaicin concentrations of 300μM, 400μM and 500μM respectively ($p<0.001$, $p<0.01$, $p<0.05$ respectively) relative to media containing ethanol. HBL cell viability was significantly reduced to 60%, 50%, 16.8%, 2.4% and 2.5% at capsaicin concentrations of 100μM, 200μM, 300μM, 400μM and 500μM respectively ($p<0.05$, $p<0.05$, $p<0.001$, $p<0.001$ and $p<0.001$ respectively), compared to media only.

Viability of A375SM cells treated with media only was normalised to 100%. A375SM cell viability was reduced to 66% with media containing ethanol, compared to cells treated with media only. Capsaicin reduced A375SM cell viability to 51.5%, 16.1% and 1.56% at concentrations of 300μM, 400μM and 500μM ($p<0.01$, $p<0.05$, and $p< 0.01$, respectively) compared to media containing ethanol. A375SM cell

viability was significantly reduced at capsaicin concentrations of 100μM, 200μM, 300μM, 400μM and 500μM ($p<0.01$, $p<0.01$, $p<0.001$, $p<0.001$ and $p<0.001$ respectively), compared to media treated cells.

Viability of C8161 cells treated with media only was normalised to 100%. C8161 cell viability was reduced to 65% at media containing ethanol compared to cells treated with media only, however this was not significant ($p<0.05$). Capsaicin at 500μM reduced C8161 cell viability to 9.3% ($p<0.01$) relative to media containing ethanol. C8161 cell viability was significantly reduced to 71.6%, 47.6%, 74.5%, 40.6% and 9.3% at capsaicin concentrations of 100μM, 200μM, 300μM, 400μM and 500μM respectively ($p<0.05$, $p<0.01$, $p<0.05$, $p<0.01$ and $p<0.05$ respectively), compared to media treated cells. See Figure 3.17 for a summary of these results and Table 3.8 for a statistical summary of results.

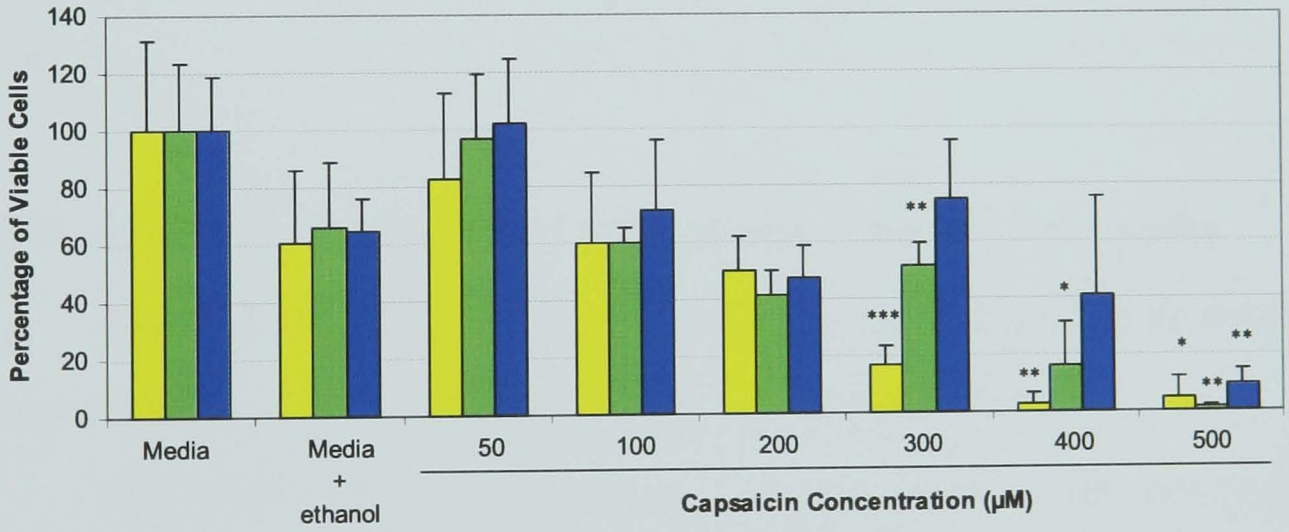


Figure 3.17. Effect of capsaicin on melanoma cell viability. ▯ = HBL cells, ▯ = A375SM cells, ▯ = C8161 cells. N=2. For ethanol, N=1 (triplicate). Values are expressed as Mean+SD. * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$, relative to media containing ethanol.

Table 3.8. Statistical Summary for comparison between treatments - Effect of capsaicin on melanoma cell viability (p values)

HBL Cells	p Value	HBL Cells	p Value
Media *vs* Media+ethanol	*ns*	Media *vs* Media+ethanol	*ns*
Media+ethanol *vs* 50 μM	*ns*	Media *vs* 50 μM	*ns*
Media+ethanol *vs* 100 μM	*ns*	Media *vs* 100 μM	0.0478
Media+ethanol *vs* 200 μM	*ns*	Media *vs* 200 μM	0.0255
Media+ethanol *vs* 300 μM	*ns*	Media *vs* 300 μM	0.0001
Media+ethanol *vs* 400 μM	*ns*	Media *vs* 400 μM	0.0001
Media+ethanol *vs* 500 μM	0.0111	Media *vs* 500 μM	0.0005
A375SM Cells		**A375SM Cells**	
Media *vs* Media+ethanol	*ns*	Media *vs* Media+ethanol	*ns*
Media+ethanol *vs* 50 μM	*ns*	Media *vs* 50 μM	*ns*
Media+ethanol *vs* 100 μM	*ns*	Media *vs* 100 μM	*ns*
Media+ethanol *vs* 200 μM	*ns*	Media *vs* 200 μM	0.0018
Media+ethanol *vs* 300 μM	0.0021	Media *vs* 300 μM	0.0001
Media+ethanol *vs* 400 μM	0.0402	Media *vs* 400 μM	0.0001
Media+ethanol *vs* 500 μM	0.0060	Media *vs* 500 μM	0.0001
C8161		**C8161**	
Media *vs* Media+ethanol	*ns*	Media *vs* Media+ethanol	*ns*
Media+ethanol *vs* 50 μM	*ns*	Media *vs* 50 μM	*ns*
Media+ethanol *vs* 100 μM	*ns*	Media *vs* 100 μM	0.0413
Media+ethanol *vs* 200 μM	*ns*	Media *vs* 200 μM	0.0011
Media+ethanol *vs* 300 μM	0.0003	Media *vs* 300 μM	0.0156
Media+ethanol *vs* 400 μM	0.0015	Media *vs* 400 μM	0.0018
Media+ethanol *vs* 500 μM	0.0285	Media *vs* 500 μM	0.0358

3.5.5. Effect of Capsaicin on TNF-α Pre-stimulated Melanoma Cell Viability

Increasing concentrations of capsaicin in TNF-α pre-stimulated melanoma cells reduced viability in the three melanoma cell lines (see Figure 3.18).

HBL media treated cell viability was normalised to 100%. HBL cell viability was unchanged by TNF-α only treated cells ($p>0.05$) relative to media. However increasing capsaicin concentrations significantly reduced HBL cell viability to 50%, 53% and below at concentrations of 50μM, 100μM, 200μM and 300μM ($p<0.001$ in all cases) in TNF-α pre-stimulated cells, relative to TNF-α treated cells. No viable cells were found at capsaicin concentrations of 400μM and 500μM showing a very toxic effects on these cells.

A375SM media treated cell viability was normalised 100%. An almost significant reduction to 80% in A375SM cell viability occurred with TNF-α treatment ($p=0.0559$). A375SM cell viability was then reduced to 57%, 4%, 10%, 3%, 24%, and to 1.3% at capsaicin concentrations of 50μM ($p<0.05$), 100μM ($p<0.001$), 200μM, 300μM, 400μM and 500μM ($p<0.001$) in TNF-α pre-stimulated cells relative to TNF-α treated cells.

C8161 media treated cell viability was normalised to 100%. A significant reduction to 68% in C8161 melanoma cell viability occurred in TNF-α only treated cells ($p<0.05$). This was not expected as C8161 are quite resistant cells. C8161 cell viability was then reduced to 49%, 34%, 36%, and to 10% at capsaicin concentrations of 200μM, 300μM, 400μM and 500μM ($p<0.01$, $p<0.001$, $p<0.001$ and $p<0.001$ respectively) in TNF-α pre-stimulated cells, compared to TNF-α treated cells. See Figure 3.18 for an illustration of these results and Table 3.9 for a statistical summary.

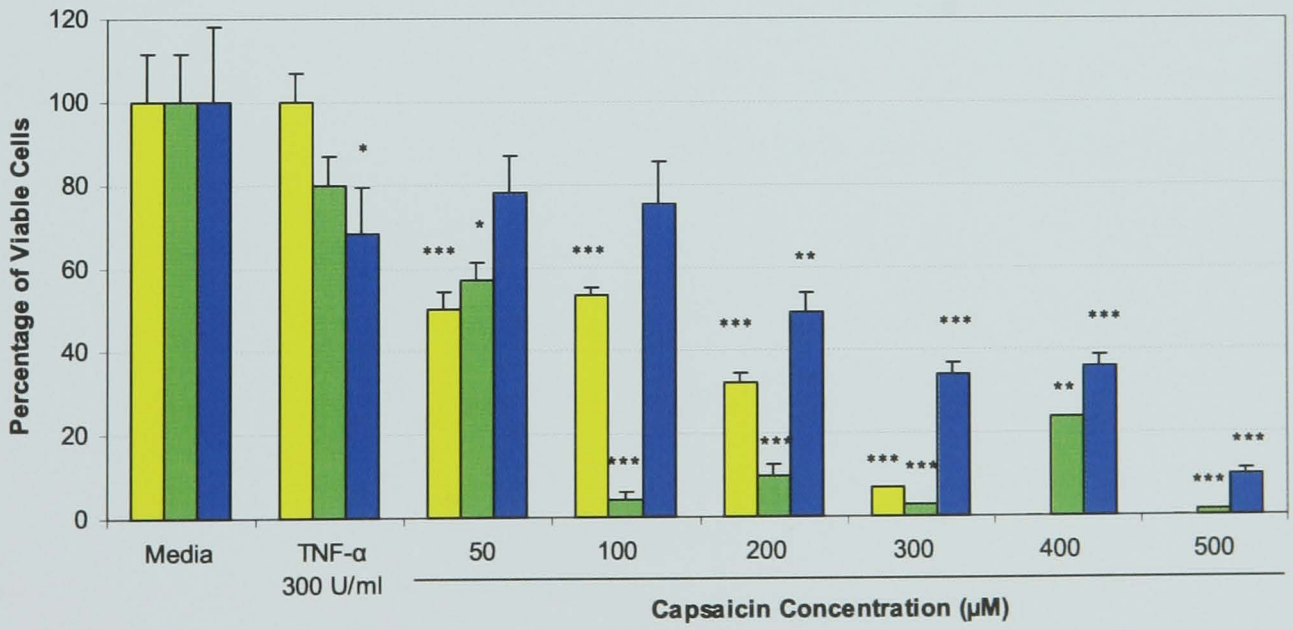


Figure 3.18. Effect of capsaicin on TNF-α stimulated melanoma cell viability. ▯ = HBL cells, N=6. ▯ = A375SM cells, N=5. ▯ = C8161 cells, N=6. Values are expressed as Mean+SD. * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$, relative to TNF-α treated cells.

Table 3.9. Statistical Summary for comparison between treatments - Effect of capsaicin on TNF-α pre-stimulated melanoma cell viability (p values)

HBL Cells	p Value
TNF-α *vs* Media	0.0001
TNF-α *vs* 50 μM	0.0001
TNF-α *vs v* 100 μM	0.0001
TNF-α *vs* 200 μM	0.0001
TNF-α *vs* 300 μM	0.0001
TNF-α *vs* 400 μM	--------
TNF-α *vs* 500 μM	--------
A375SM Cells	
TNF-α *vs* Media	0.0559
TNF-α *vs* 50 μM	0.0108
TNF-α *vs* 100 μM	0.0002
TNF-α *vs* 200 μM	0.0001
TNF-α *vs* 300 μM	0.0001
TNF-α *vs* 400 μM	0.0001
TNF-α *vs* 500 μM	0.0001
C8161 Cells	
TNF-α *vs* Media	0.0113
TNF-α *vs* 50 μM	*ns*
TNF-α *vs* 100 μM	*ns*
TNF-α *vs* 200 μM	0.0097
TNF-α *vs* 300 μM	0.0002
TNF-α *vs* 400 μM	0.0004
TNF-α *vs* 500 μM	0.0001

3.6. DISCUSSION

Part of the justification behind this study is the phenomenon of local recurrence of malignant melanoma following surgical excision. Cancer cells that leave the primary tumour can seed metastases in distant organs. However, circulating tumour cells (CTCs) can also colonize their tumours of origin, a process known as "tumour self-seeding" of many types of cancer, such as breast, colon and melanoma tumours. Tumour self-seeding explains this common phenomenon of local recurrence seeded by disseminated cells following complete excision (Kim *et al.*, 2009). Surgical excision creates an inflammatory wound bed which may be favourable to melanoma cell invasion and metastasis.

The link between inflammation and cancer development is getting stronger and the use of NSAIDs as therapy to reduce or block cancer development is being explored.

In this chapter the role of the NSAID ibuprofen and of capsaicin in opposing the migratory effect of the pro-inflammatory cytokine TNF-α on HBL, A375SM and C8161 human melanoma cell lines was investigated. Melanoma cell migration (in a 2D model) and cell survival were investigated after cells were exposed for 24 hours to TNF-α, ibuprofen or capsaicin.

Three types of melanoma cells used were of different levels of invasiveness and origin. HBL cells were obtained from a lymph node metastasis of a malignant melanoma, meaning that these cells may have originated from a previous stationary site of a cutaneous primary melanoma tumour. Culturing HBL cells demonstrated their lower proliferation rate compared to the other two melanoma cell lines used.

A375SM cells were obtained from an established A375 melanoma cell line from a lymph node metastasis. These cells then became highly metastatic as a A375-SM subline once established in culture from lung metastasis by parental A375 cells growing

subcutaneously in mice. Culturing A375SM demonstrated an intermediate proliferation rate compared to HBL cells and the highly proliferative C8161 cells.

C8161 cells were obtained from a metastatic tumour of the abdominal wall and this indicates a very aggressive (invasive and proliferative) melanoma, capable of further metastatic cascade and tumour formation.

Melanoma cells migrated at a linear rate over 24 hours. However this linearity changed by the effects of ibuprofen or capsaicin, both reducing melanoma cell migration.

In the first experiments it was investigated the effect of ibuprofen on melanoma cell migration. Ibuprofen [(±)-(R, S)-2-4(4-isobutylphenyl)-propionic acid] is known as a NSAID, used to relieve pain and inflammation (Davies, 1998). It works by inhibiting non-selectively cyclooxygenase COX-1 and COX-2 forms, with analgesic and anti-inflammatory effects.

Previous studies have looked at non-steroidal anti-inflammatory agents with and without COX-inhibition properties; α-MSH, indomethacin and aspirin respectively, as many investigators (Benamouzig *et al.*, 2005; Borthwick *et al.*, 2006) have suggested that anti-cancer properties of NSAID and selective COX-2 inhibitors may be independent of their COX-inhibition properties. COX-2 blockade is effective for both cancer prevention and therapy (Harris, 2007).

Initially, Ibuprofen dissolved in media significantly reduced migration in melanoma cells. However, it was also toxic to HBL and A375SM cells at the lower concentrations of 66μM Ibuprofen. This could be attenuated by incorporating ibuprofen in a hydrogel resulting in a slow release of ibuprofen.

Ibuprofen incorporated into a Pluronic F-127 hydrogel demonstrated a slow release in distilled water. The hydrogel may had the same pattern of ibuprofen delivery in cell culture media, as it was observed a progressive reduction of cell migration

associated with low cytotoxicity. Although the release of ibuprofen from the hydrogel was measured only in distilled water, the same conditions were kept during the melanoma migration experiments for a slow released of ibuprofen from the hydrogel in media *i.e.*, the same polymer concentration of 30% and temperature of 37°C during incubation. Pluronic F-127 hydrogel has been clinically used. It is a copolymer which forms thermo-sensitive hydrogels and may by advantageous as an injectable therapeutic formulation for an anti-cancer treatment (Hsu *et al.*, 2003) and, as a carrier for other routes of administration such as oral, nasal, topical and ocular (Escobar-Chávez *et al.*, 2006).

Ibuprofen hydrogel (NaIbu hydrogel) reduced migration of the three melanoma cell lines without inducing toxicity to the cells. As expected, migration of HBL cells was reduced more than that of A375SM and C8161 cells. The hydrogel itself had some slightly inhibitory effect on cell migration but this was not significant. This may have demonstrated that a slower release of Ibuprofen from a hydrogel was more effective in reducing melanoma cell migration and yet not toxic to the cells. The only significant toxic effect of NaIbu hydrogel was observed on HBL cells at a maximum NaIbu concentration of 131μM.

In this study an *in vitro* inflammatory-like environment was created to study an association between inflammation and cancer. Therefore, the first steps in this investigation were to find the optimal concentration of TNF-α for melanoma cell migration and the concentration at which sodium Ibuprofen becomes toxic. Previous work in the Kroto Institute (Redpath *et al.*, 2009) established that a concentration of around 300-400 U/ml and exposure for 24 hours produced maximal effects on cell migration and, this is consistent with previous studies on HBL melanoma (Katerinaki *et al.*, 2006). Therefore in this study 300U/ml TNF-α was used to stimulate an inflammatory condition to melanoma cells in culture.

Melanoma cells were 24 hours pre-stimulated with 300U/ml TNF-α and Ibuprofen was added to the cells. Accordingly, TNF-α (on its own) increased melanoma cell migration for the three melanoma cell lines. Addition of Ibuprofen incorporated into the hydrogel reduced migration in melanoma cells below the control level of migration for HBL and A375SM cells and, just above control migration level for C8161 cells. For hydrogels loaded with 99μM and 131μM of ibuprofen this effect was significantly greater than the effect of the gel on its own. HBL and A375SM melanoma cell survival was significantly reduced by 40% at 66μM and above concentrations of NaIbu hydrogel. A higher concentration of 131μM NaIbu hydrogel was necessary to significantly reduce C8161 cell survival. This is in fair agreement with another study by Janssen *et al.* (2008) who demonstrated that both induction of a cell cycle block and apoptosis after S- and R-ibuprofen treatment was in part dependent on p53 in human colon cancer cells.

NSAIDs have been used as an anti-cancer therapy, however, the mechanism of these COX inhibitors remain unclear. Melanoma progression is often associated with supra-normal levels of IL-1 and IL-6. During inflammation, TNF-α is a potent inducer of IL-6 gene transcription factor and synthesis. IL-6 is therefore thought to be another primary growth factor driving the progression of human melanoma. The likely mechanism of COX inhibition leading to IL-6 lowering is due to the tendency for IL-6 levels to be controlled by intracellular cyclic adenosine monophosphate (cAMP). COX inhibitors lower prostaglandin E levels. This lowers intracellular cAMP levels. Lower cAMP results in lower IL-6 synthesis (van Kemper *et al.*, 2006), and therefore reduces inflammation.

TNF-α has been described as a pro-inflammatory cytokine which promotes melanoma cell migration, invasion and survival and to upregulate ICAM-1, integrin and NF-κB expression, with increased melanoma cell migration and invasion (Zhu *et al.*,

2002). All of these effects can be inhibited by α-MSH (Cantón *et al.*, 2003) and sodium salicylate (Katerinaki *et al.*, 2006).

Melanoma cell migration was also investigated under the effects of another NSAID, capsaicin. Capsaicin is an extract from the red chilli peppers and it is used topically to relieve pain such as in peripheral neuropathy (Tesfaye, 2009). There are many suggestions that capsaicin utilizes a vanilloid receptor-1 (VR1) which functions as a calcium-permeable non-specific cation channel and it is utilized in painful thermal stimuli *in vivo* (Caterina *et al.*, 1997). Capsaicin has an immunological and neurological response against established tumours (Beltran *et al.*, 2007). It has also been recognised as an anti-inflammatory agent (Southall *et al.*, 2003; Zegarska *et al.*, 2006) and as a pro-apoptotic agent (Kim *et al.*, 2004).

Capsaicin significantly reduced HBL and A375SM cell migration at a higher concentration of 300μM but had no effect on C8161 cell migration. However, 100μM capsaicin significantly reduced melanoma cell viability by 30-40%. This suggests that the suppressive effect of capsaicin on melanoma cell migration may be due to the anti-inflammatory and pro-apoptotic effects of capsaicin. This is in accordance with another study (Patel *et al.*, 2002a) which demonstrated that capsaicin inhibited cellular proliferation and NF-kB transcription factor activity over 96 hours on A375P cells, reducing cell viability 50% at 100μM capsaicin.

Capsaicin concentration above 300μM significantly reduced migration in the three melanoma cell lines. This reduction in migration is associated with the cytotoxic effect of capsaicin as comparisons between media containing ethanol reduced cell viability at the same capsaicin concentration (300μM). Considering media only as control, capsaicin was toxic at a lower concentration of 100μM.

There was one viability experiment in which ethanol in media was used as control for comparisons. In this, melanoma cell viability was largely reduced, but not

statistically significant. In addition to this, ethanol concentration in media to be used as control was higher than the maximum ethanol concentration in media to make up the maximum capsaicin concentration of 500μM used in the experiments. For this reason, for the remaining experiments the investigation of the effects of capsaicin on melanoma cells and fibroblasts viability, it was decided to use media treated cells as control.

The effect of capsaicin on TNF-α stimulated melanoma cell migration was also investigated. When melanoma migration was pre-stimulated with 300U/ml TNF-α migration was increased in three melanoma cell lines however, increasing capsaicin concentration suppressed melanoma cell migration. HBL cell migration was stopped above 300μM capsaicin. At 400μM capsaicin, migration carried on with just a few A375SM cells (data now shown). This capsaicin concentration was toxic for most cells however, some resistant and residual cells within the cell culture continued migrating. This may represent what occurs *in vivo*, where a few melanoma cells escape from chemotherapy or radiotherapy and continue to migrate and invade. TNF-α stimulated C8161 cell migration however, only 500μM capsaicin significantly reduced migration of theses cells. This capsaicin concentration started to be toxic to these cells, showed by significant reduction in C8161 cell viability.

In terms of cell viability TNF-α pre-stimulation and further treatment with capsaicin showed toxic effect on three melanoma cell lines at 50μM. This is in fair agreement with Shin *et al.* (2008) who reported a suppressive effect of capsaicin on B16-F10 melanoma cell migration, targeting P13-K/Akt/Rac1-mediated cellular events.

The anti-inflammatory and anti-migratory effect of capsaicin on melanoma cells can also be confirmed by Philip and Kundu (2003) who used curcumin, a known anti-inflammatory agent which inhibited osteopontin-induced NF-kB transcriptional activity and MMP-2 activation, reducing murine (B16-F10) melanoma cell migration and tumour growth in nude mice.

Similar to the proposed mechanisms of ibuprofen, capsaicin is reported to modulate the expression of inflammatory iNOS (inducible Nitric Oxide synthase) and COX-2 (cyclooxygenase-2) genes in macrophages and may provide new insights into the potential benefits of capsaicin in inflammatory conditions (Chen *et al.*, 2003).

Reports on the effects of capsaicin as an apoptosis inducer are not yet completely understood, however capsaicin may involve plasma membrane or mitochondrial targets. It has been therefore suggested that this vanilloid agent may be useful for preventing or treating skin cancers or other hyperproliferative skin disorders (Hail and Lotan, 2002).

The fact that TNF-α on its own significantly reduced A375SM and C8161 cell viability was unexpected as TNF-α is known to promote melanoma cell survival. Overall, the accelerated migration in melanoma cells induced by TNF-α was attenuated by capsaicin, without the latter being very toxic to these cells. These findings are similar to those of other authors (Shin *et al.*, 2008).

The stimulatory effect of TNF-α on melanoma cell migration and invasion has been previously reported by Katerinaki *et al.* (2003). However, the inhibitory effect of NSAIDs on melanoma cell migration pre-stimulated with TNF-α has also been demonstrated with the utilization of alpha melanocyte stimulating hormone (Zhu *et al.*, 2002) and with sodium salicylate (Katerinaki *et al.*, 2006).

In conclusion, this data on three very different melanoma cell lines confirmed that capsaicin and a hydrogel releasing ibuprofen sodium salt were capable of inhibiting human melanoma cell migration. This adds more evidence to previous data on inflammation and melanoma migration in demonstrating an anti-migration effect of Ibuprofen and Capsaicin on TNF-α stimulated cells.

CHAPTER 4

EFFECTS OF CAPSAICIN ON MELANOMA CELLS DEGRADATION ABILITY IN FIBRIN SCAFFOLD

4.1. MELANOMA CELLS AND FIBRIN SCAFFOLD DEGRADATION

Initially, the aim of this experiment was to look at melanoma cell invasion in a simple fibrin 3D model. This was made by adding fibrin clots to a 24 cell culture well plate. The volume of fibrin clot added was 0.5ml which made a scaffold 2mm in thickness in each well of a 24 culture well plate. The plate contained a mixture of fibrinogen plus thrombin and was incubated for a period of 24 hours, to allow the fibrinogen and thrombin to clot. After this incubation period, melanoma cells were added to the fibrin clot at a concentration of $4x10^4$ cells in 1 ml of media per well and were again incubated for 24 hours. See Figure 4.1 for an illustration of this method.

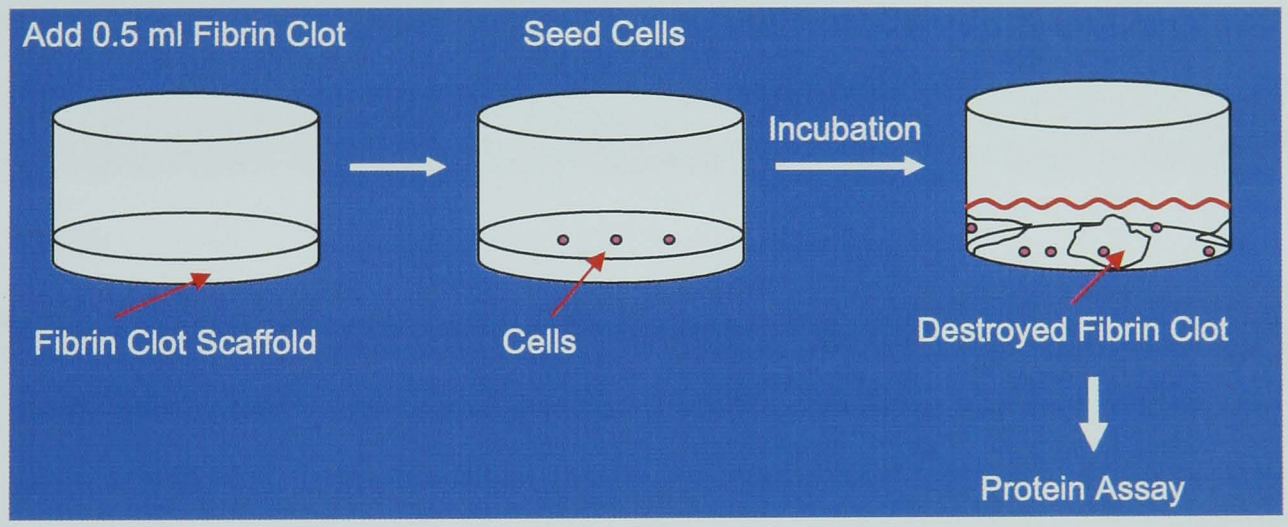


Figure 4.1. Fibrin degradation after 24 hours incubation with melanoma cells. This was followed by a protein assay.

After this incubation period, it was observed that the fibrin clot was broken into pieces by HBL and A375SM cells added to the scaffold. However, fibrin to which C8161 cells were added remained intact.

These results suggest that HBL and A375SM melanoma cells have a degradative capability and hence destroy the fibrin scaffold. Micrographs illustrate what have happened in Figure 4.2 in which HBL and A375SM cells are attached to broken pieces of fibrin (see light and fluorescent microscopy (DAPI) staining for cell nuclei).

Surprisingly, C8161 cells did not affect the structure of the fibrin clot scaffold. The C8161 cell nuclei were not distinguished by fluorescent staining as these cells seemed to be invaded into the scaffold. This was unexpected as C8161 cells are the most invasive and aggressive of the 3 cell lines as previously demonstrated.

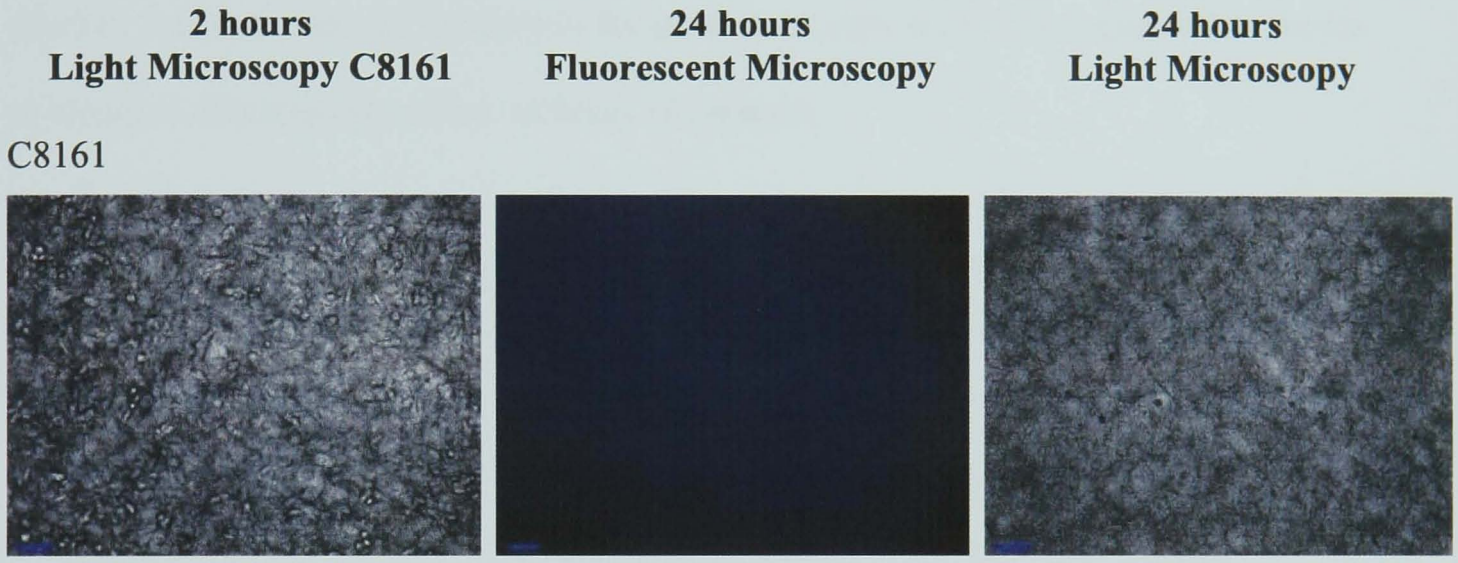


Figure 4.2. Micrographs of light and fluorescent microscopy at 2 and 24 hours showing melanoma cells on fibrin clot scaffold. 20x magnification, scale bar = 50μm.

An attempt was made to observe what happened to the melanoma cells after 6 hours of incubation. In this experiment, 6 hours after seeding melanoma cells on the fibrin clot scaffolds did not show any degradative response. In fact, melanoma cells seemed to be forming some clusters or isolated cells not attached to the surface of the fibrin clot, as the cells were rounded up and clustered together (see Figure 4.3). Although these are not good images for visualisation of what had happened, one can observe that at 6 hours of incubation the melanoma cells were still suspended in media or trying to attach to the surface of fibrin underneath.

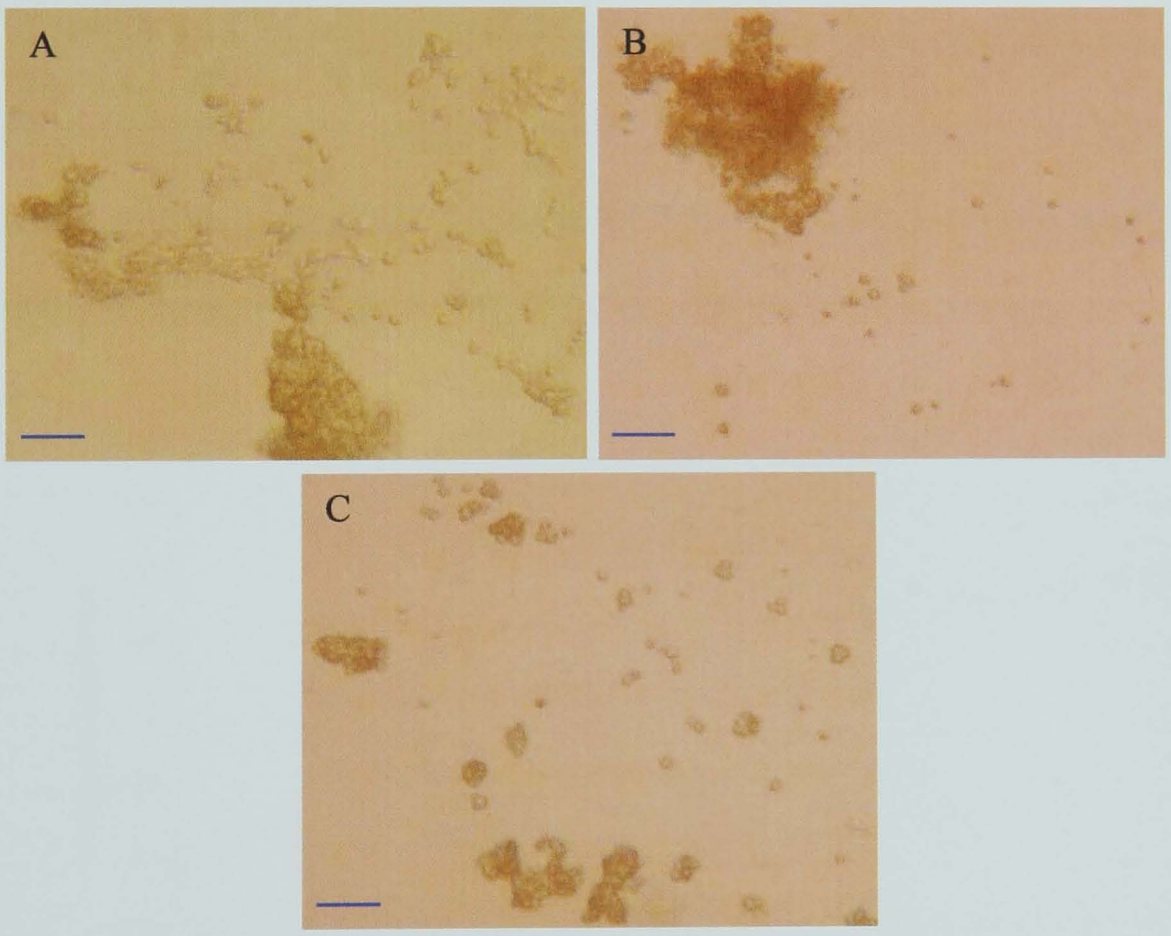


Figure 4.3. Melanoma cells seeded on a fibrin scaffold. A = C8161, B= A375SM, C = HBL cells. Melanoma cells were still not attached to the fibrin clot after 6 hours incubation period. Scale bars = 100μm.

The exact time which melanoma cells start their degradative enzymatic activity remains to be clarified. Therefore, the next experiments were still carried out incubating

melanoma cells for 24 hours to observe the degradative capability of these melanoma cells.

4.2. ASSESSMENT OF THE ABILITY OF MELANOMA CELLS TO DEGRADE THE FIBRIN SCAFFOLD

A protein assay was conducted using different volumes of the fibrinogen and thrombin mixture, digesting with the digestion buffer as referred in Materials and Methods (section 2.4.3.4).

Absorbance readings were taken at 540 mm with reference of 630 mm. As it can be seen in Figure 4.4 the absorbance reading for the protein assay increased with the increased volume of fibrin clot added to the wells. This was then used as an assay for estimating the volume of fibrin clot remaining in these experiments.

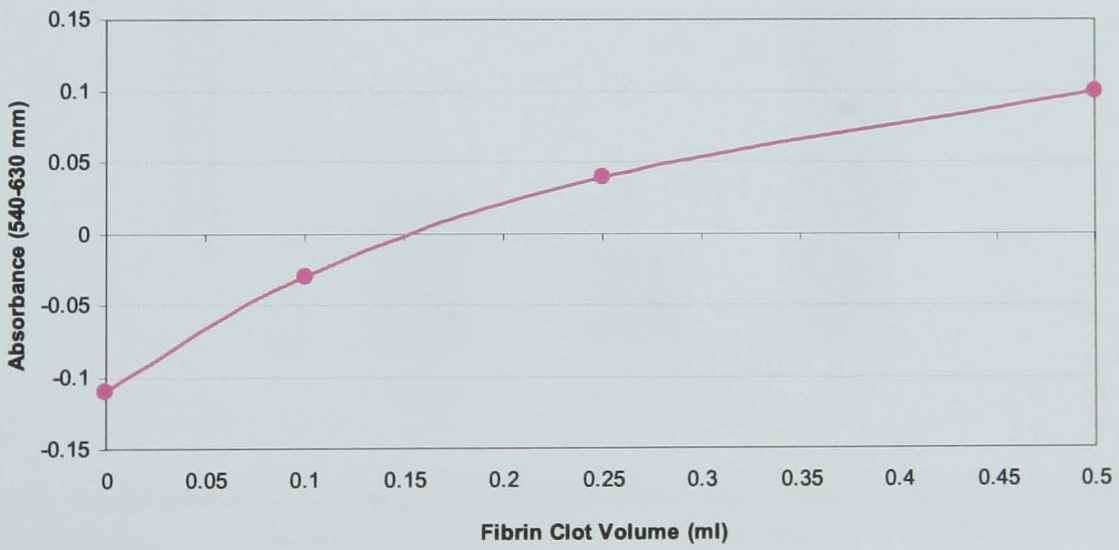


Figure 4.4. Assay of fibrin clot volume following protein digestion.

In the next experiments melanoma cells were incubated for 24 hours on a fibrin clot scaffold. HBL and A375SM melanoma cells (but not C8161 cells) were shown to have the ability to degrade and destroy a fibrin clot scaffold during this incubation

period. In order to be able to obtain an accurate measure of the amount of fibrin clot left by the melanoma cells a protein assay was then used to quantify the amount of fibrin clot remaining. At the end of the 24 hours incubation media was removed (this was the same volume for all cell lines – not shown) and the remaining fibrin clot quantified.

The protein assays demonstrated that the amount of fibrin clot protein left by melanoma cells was higher in HBL cells than for C8161 ($p=0.0475$) and A375SM cells ($p=0.0114$) (see Figure 4.5). However, this was not consistent when observing by eye the amount of fibrin clot left in the wells, after 24 hours incubation with melanoma cells. Looking at the wells it was possible to observe that the amount of fibrin clot left by C8161 was higher than the other two types of melanoma cells (A375SM and HBL), as is shown in Figure 4.2.

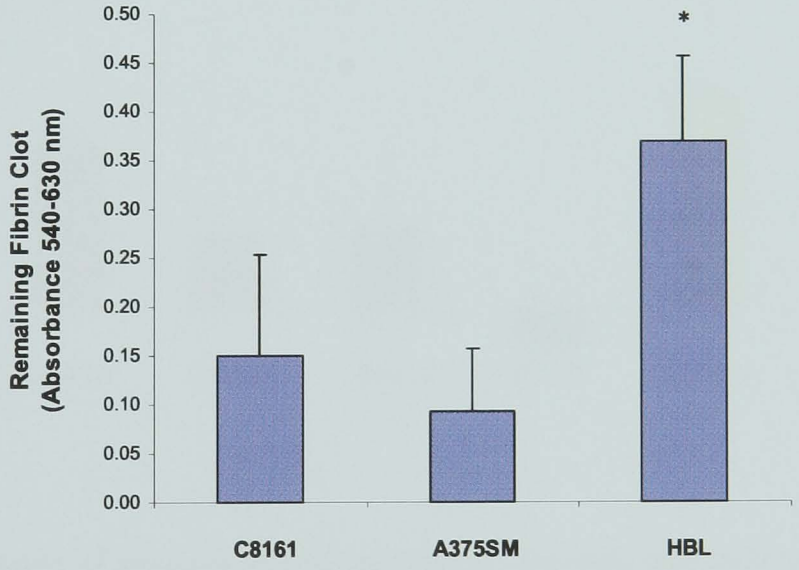


Figure 4.5. Assessment of amount of residual fibrin clot after melanoma cell digestion. Incubation period = 24 hours. Values are expressed as Mean + 1SD. N=3. * indicates $p<0.05$, relative to C8161 cells.

As this experiment was not consistent in terms of measuring the amount of fibrin clot, it was possible that the FCS present was preventing digestion of the fibrin clot by

the digestion mixture of SDS and urea, this was then investigated in the following experiments.

4.3. ASSESSMENT OF THE ABILITY OF MELANOMA CELLL TO DEGRADE THE FIBRIN SCAFFOLD IN THE PRESENCE AND ABSENCE OF FCS

In this experiment a comparison was made between the ability of melanoma cells to digest fibrin in a medium with and without FCS. This was made to investigate whether FCS was blocking the ability of melanoma cells to digest fibrin clot (see Figure 4.6).

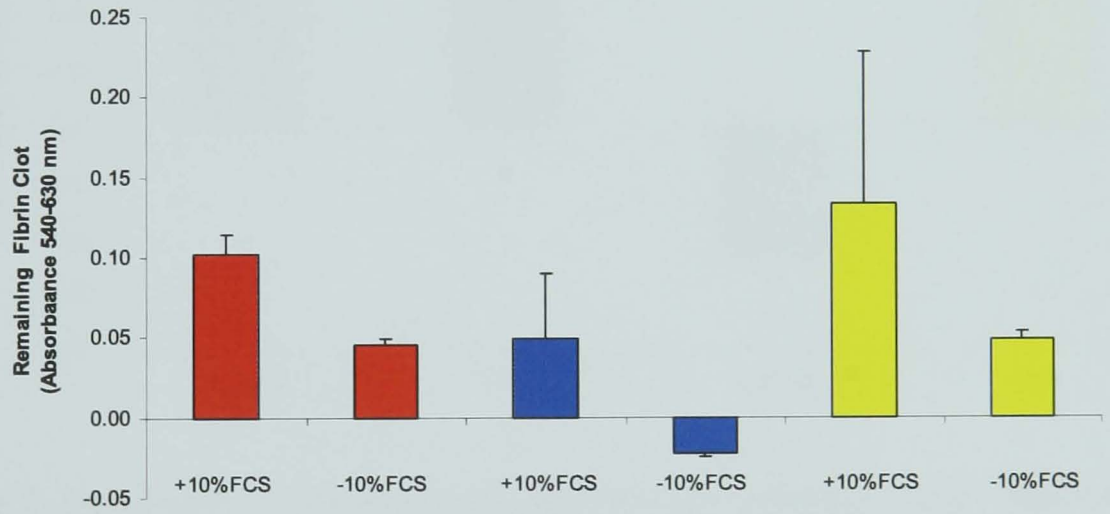


Figure 4.6. Assessment of amount of residual fibrin clot with and without FCS. ■ = C8161, ■ = A375SM and ■ = HBL melanoma cells. Incubation period = 24 hours. Values expressed as Mean +SD from calculations of triplicate wells. N=1.

The results showed that in the absence of FCS there was less residual content from the fibrin left by the three melanoma cells. This time, A375SM cells in FCS-free media showed degraded all of the fibrin clot scaffold.

In the next experiment, degradation of the fibrin clot in the absence of FCS was examined. Figure 4.7 showed that C8161 and HBL cells did not degrade the fibrin clot after 24 hours incubation period. On the other hand, A375SM cells destroyed most of the fibrin clot (see Table 4.1 for a statistical summary of these results). However, there was a considerable variance between the experiments as shown by the high SD values. A control value of fibrin without any added melanoma cells was used in these experiments as a reference.

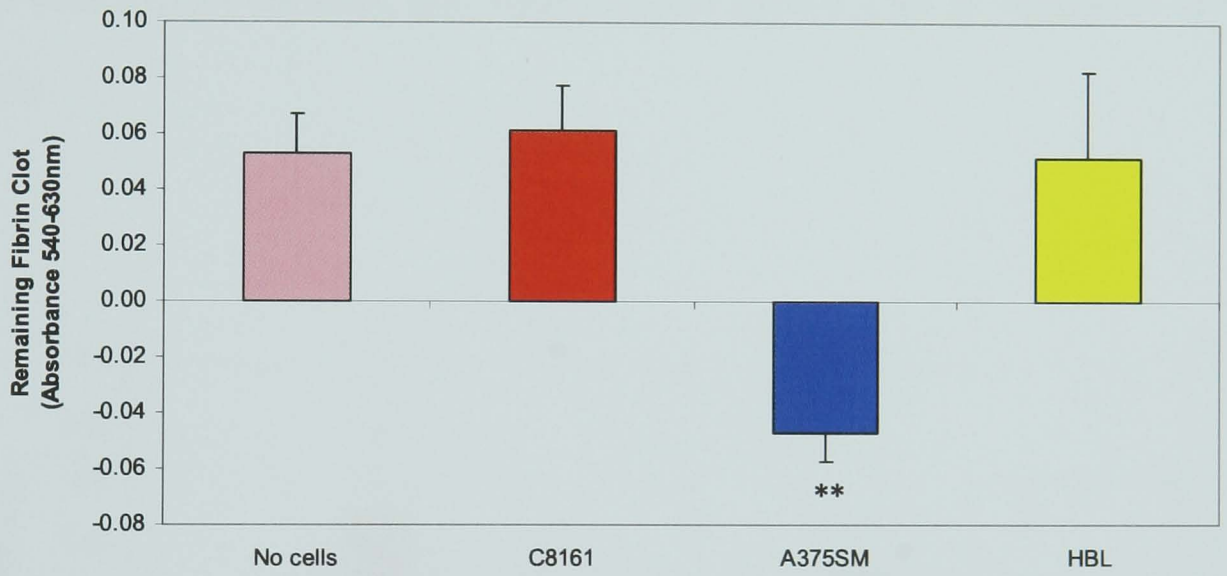


Figure 4.7. Assessment of amount of residual fibrin clot without FCS. ▯ = shows the fibrin clot without any added cells, ▯ = C8161, ▯ = A375SM and ▯ = HBL melanoma cells. Incubation period = 24 hours. Values expresses as mean +SD. Student t-test for 2 samples presuming equivalent variances. N=3. ** indicates p<0.01, relative to fibrin clot.

Table 4.1. Assessment of residual fibrin clot without FCS. Statistical summary.

Comparisons	p values
C8161 vs A375SM	p=0.006
A375SM vs HBL	P=0.072
C8161 vs HBL	P=0.073
C8161 vs PBS (control)	P=0.712
A375SM vs PBS (control)	P=0.006
HBL vs PBS (control)	P=0.893
PBS vs Fibrin Clot	P=0.779

4.4. EFFECT OF CAPSAICIN ON MELANOMA CELL DEGRADATIVE ABILITY

There are many studies suggesting that capsaicin can be used as a NSAID and also as an anti-cancer drug. This experiment examined the effects of capsaicin on the ability of melanoma cells to degrade fibrin clot.

The same procedure was followed in this experiment in which 0.5 ml fibrin clot was added to a 24 well plate. C8161 and A375SM melanoma cells were seeded at a density of $5x10^4$ cells/ml per well and, HBL cells were seeded at a density of $4x10^4$ cells/ml per well. Capsaicin was added to treated wells at 300 μM/ml per well. Cells were incubated for 24 hours. See Figure 4.8 and Table 4.2 for an illustration of these results.

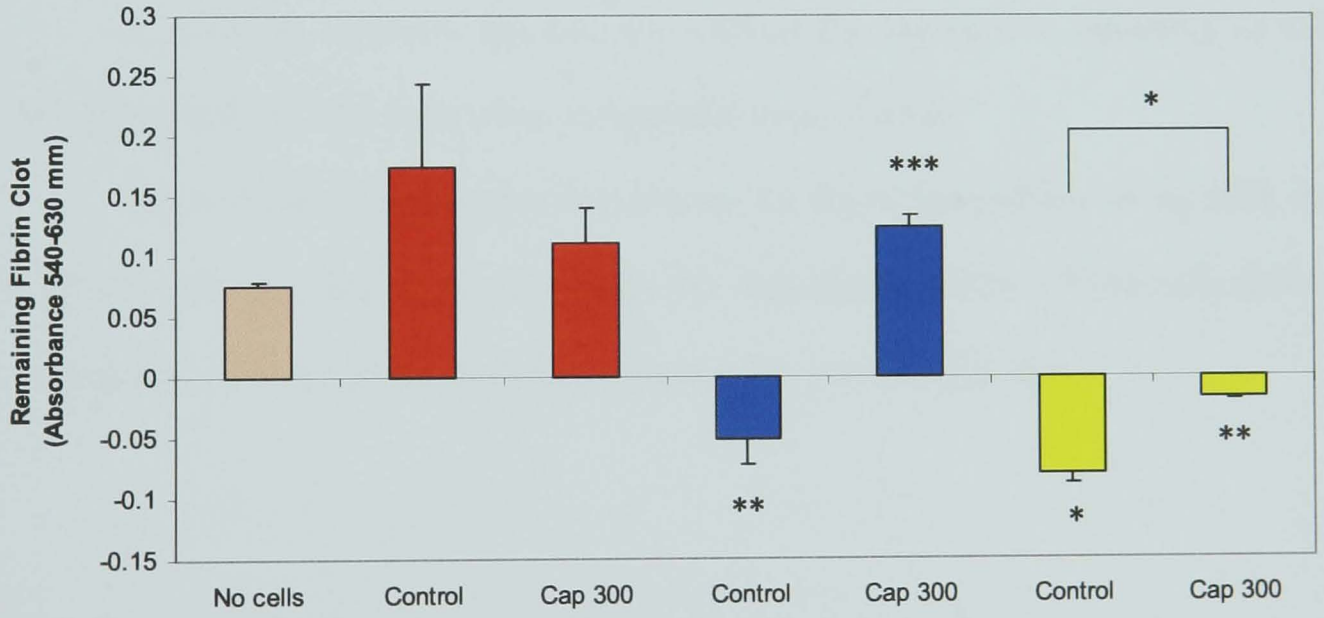


Figure 4.8. Effects of capsaicin on melanoma cell fibrin degradation capability. Assessment of amount of residual fibrin clot in FCS free media. □ = no added cells, ■ = C8161, ■ = A375SM and ■ = HBL melanoma cells. Cap 300 = 300μM Capsaicin. Incubation period = 24 hours. Student t-test for 2 samples presuming equivalent variances. (N=3). Mean +SD. * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$, relative to their respective control.

Table 4.2. Effects of capsaicin on melanoma cell degradative capability in fibrin clot. Statistical summary.

C8161	p values
Control vs 300	p=0.2285
PBS vs Control	p=0.0743
PBS vs 300	p=0.0970
A375SM	
Control vs 300	p=0.7765
PBS vs Control	p=0.0018
PBS vs 300	p=0.0003
HBL	
Control vs 300	p=0.0219
PBS vs Control	p=0.0383
PBS vs 300	p=0.0018

For C8161 cells (Figure 4.8) the amount of fibrin clot remaining was not significantly reduced by the addition of these cells with or without capsaicin ($p>0.05$).

A375SM and HBL cells however significantly reduced the amount of fibrin remaining in the well. See Table 4.2 for significant values.

In contrast, capsaicin significantly reduced the degradative capability of both A375SM cells and HBL cells when compared to control wells.

In conclusion, these results demonstrate the degradative ability of the HBL and A375SM cells and that capsaicin reduced this degradative ability. C8161 cells showed no degradative enzymatic activity and capsaicin did not influence this.

4.5. DISCUSSION

Initially the aim of this study was to investigate melanoma cell invasion in a 3D fibrin clot scaffold as a model scaffold which occurs naturally in wound healing. However, 24 hours after seeding the melanoma cells, the fibrin clot scaffold was destroyed by two of the three melanoma cell lines.

The differing degradative ability of the melanoma cell lines was interesting and was investigated further. HBL and A375SM cells were very capable of degrading the fibrin clot scaffold whilst C8161 cells did not.

A recent review indicates that inflammatory leukocytes contribute to cancer development either via the release of vesicle-stored growth and survival factors and diverse proteolytic enzymes which result in altered pericellular matrix remodelling activity (Felding-Habermann *et al.*, 1992).

To remove a primary melanoma there will always be a wound followed by the healing process. Melanoma cells may remain either in the blood/lymphatic circulation or melanoma cells may still be present within the wound site. As part of the wound healing process inflammation will take place together with the production of inflammatory cytokines and chemokines. This is followed by removal of cell debris and chemotaxis of new and inflammatory cells, together with the production of fibrin clots for tissue regeneration. If melanoma cells are still present in a wound healing site, they may contribute to the reoccurrence of melanoma. Invasion may be facilitated by the pressure of the fibrin clot which occurs early in wound healing. In recent years this hypothesis of inflammation contributing to melanoma invasion have been investigated in the Kroto Institute laboratory (Eves *et al.*, 2003); Cantón *et al.*, 2003; Katerinaki *et al.*, 2006).

It is believed that melanoma cell invasion and migration involves the activation of proteolytic enzymes. However, Katerinaki *et al.* (2003) demonstrated that the pro-inflammatory cytokine TNF-α increased melanoma migration at 24 hours and invasion through fibronectin, but did not upregulate/activate the expression of latent MMP-2 and MMP-9 (Metalloproteinases 2 and 9). This study suggested that migratory and invasive characteristics of melanoma cells may be helped by other skin cells' enzyme activity (as MMP-2 and MMP-9 were found to be elevated in the skin cells of the tissue engineered

skin) which is capable of degrading the components of extracellular matrix. Some authors (Goldbrunner *et al.*, 1996) have examined integrin signalling and integrin-GTPase interplay in the context of cancer cell growth and behaviour. A study by Felding-Habermann *et al.* (1992) suggested that the expression of a single integrin αvβ3 is correlated with the most malignant phenotype of human melanoma *in situ* which can interact with fibrinogen and its natural breakdown products suggesting a significant cell adhesion mechanism that may contribute toward the metastatic phenotype of human melanoma cells.

Some studies support the theory of down-regulation of integrin expression as a potential suppressor of invasion by melanoma cells (Zhu *et al.*, 2002; Balzac *et al.*, 1994; Henriet *et al.*, 2000; Iida *et al.*, 2004).

The idea of using a fibrin clot scaffold was to be able to observe melanoma cells invasion in a simple 3-D model. However, after 6 hours of seeding time, cells had not attached to the fibrin clot and, after a 24 hours incubation period unfortunately, the fibrin clot scaffold was destroyed by some cells. The exact time over which melanoma cells start their degradative enzymatic activity remains to be clarified. They may start their enzymatic activity after 6 hours of incubation or perhaps develop a sudden activity after 20 hours of incubation which may be linked to the increased number of cells in the culture. However, this does not seem likely as HBL cells were the melanoma cell line which most degraded the fibrin scaffold yet have lowest proliferation compared to the other two melanoma cell lines.

It was much easier to see melanoma cell digestion of the fibrin clots in the absence of FCS as the presence of FCS seemed to block the ability of the digestion buffer to breakdown the fibrin clot. In the presence of FCS the results obtained did not result in what one saw by eye. In the absence of FCS the results did reflect the extent of visible breakdown of the fibrin clots.

Capsaicin reduced the degradative capability of HBL and A375SM melanoma cells but had no effect on C8161 cells. This may help in the prevention of invasion of these cells and metastasis formation.

Interestingly the degradative capability was reduced by capsaicin, as shown for A375SM and HBL cells. Surprisingly C8161 cells were not affected by capsaicin. These cells seemed to invade through the fibrin scaffold without showing any evident degradative capability. This may be explained by the low concentration of capsaicin used (300 μM) on C8161 cells.

The effects of capsaicin in reducing the degradative ability of 2 out of 3 melanoma cells could be attributed to the suppressive effect of capsaicin on the enzymatic activity of the melanoma cells or, on the integrin binding to fibrin or, a combination of these two effects.

In conclusion, this simple model of just melanoma cells and a fibrin clot shows how different the degradative ability of melanoma cells was *in vitro*. HBL and A375SM cells were capable of degrading the fibrin scaffold whilst C8161 cells were not. These experiments also demonstrated the effects of capsaicin in reducing the degradative capability of HBL and A375SM cells. In reality, *in vivo* melanoma cells need to invade through a more complex skin environment which contains skin cells which will themselves remodel the extracellular matrix following wounding and inflammation. Accordingly melanoma cell invasion may be influenced by adjacent skin cells and wounding.

CHAPTER 5

INVESTIGATION OF THE IMPACT OF WOUNDING AND INFLAMMATION ON MELANOMA INVASION IN 3D TISSUE ENGINEERED SKIN

5.1. COMPARISON BETWEEN SKIN COMPOSITES MADE OF STERILISED AND NON-STERILISED DED

In this study, tissue engineered human skin was used to investigate the impact of wounding and inflammation on melanoma invasion. The use of acellular dermis was previously reported by Chakrabarty *et al.* (1999) as a dermal scaffold for skin composites. Previous skin composites were produced using sterilised human dermis using the glycerol/ethylene oxide method described in Eves *et al.* (2000). This use of a sterilisation protocol was primarily because of developing this as tissue engineered skin to take to the clinic.

Firstly in this study the question of whether the model could be used with non-sterilised DED was investigated. Figure 5.1 comprises composites produced using sterilised (A, C and E) and non-sterilised (B, D and F) DED. As can be seen from this figure, the standard skin composites (A and B) had a layer of epidermis and, as expected a normal dermis. Skin composites produced using DED to which skin cells and C8161 melanoma cells were added are shown in Figure 5.1 (C and D). Figure 5.1 E and F) show C8161 cells on DED and there was an evident invasion into the dermis.

Figure 5.1 shows that there was no difference in morphology between the pairs of sterilised and non-sterilised skin composites. Therefore, future studies were carried out using non-sterilised (or fresh) DED for the production of human skin composites.

The advantage of using non-sterilised DED is that the human skin is only treated with 1Molar NaCl for 18 hours receiving no further treatments. This helps in the retention of natural collagen and basement membrane components yet achieves an acellular dermis. The disadvantages are the greater risks of infection using this non-sterilised DED. Table 5.1 and Table 5.2 show a summary of these skin composites results. Later in this chapter an analysis of the incidents of infection and integrity of the composites on long term storage is described in section 5.5.8.

Sterilised DED **Non-sterilised DED**

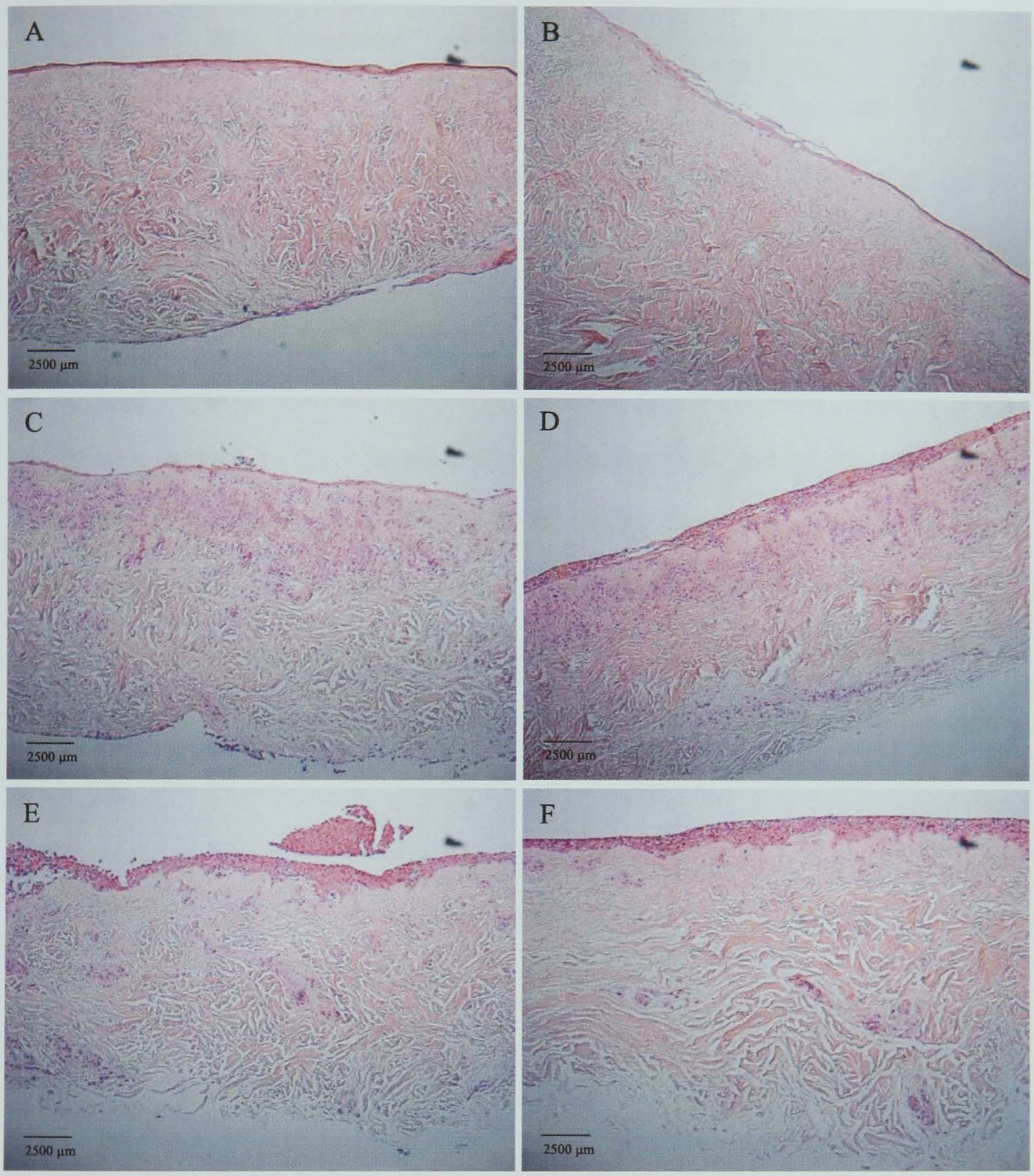


Figure 5.1. Morphological comparison between glycerol/ethylene oxide-sterilised DED (left) and non-sterilised DED (right). Standard skin composites to which fibroblasts and keratinocytes were added (A and B). Keratinocytes, fibroblasts and C8161 melanoma cells were added to DED (C and D); DED to which C8161 melanoma cells were added (E and F). 4x magnification.

Table 5.1. Sterilised and Non-sterilised Skin composites.

Skin Composite	Number of Experiments	
	Sterilised	Non-sterilised
DED+F+K	2	2
DED+F+K+C8161	2	2
DED+C8161	2	2

5.2. STANDARD SKIN COMPOSITES

More standard skin composites were produced for investigation of cell invasion and later comparison to the melanoma skin model. Figure 5.2 shows skin composites containing keratinocytes and fibroblasts. In all, twenty-two standard skin composites were produced (one lost due to infection) and an invasion score was used to quantify invasion of any cells into the dermis (see section 2.5.8 in Chapter 2 – Methods). All of these standard skin composites showed an invasion score of 0, meaning that in these composites there was no invasion of keratinocytes into the dermis (see Table 5.2).

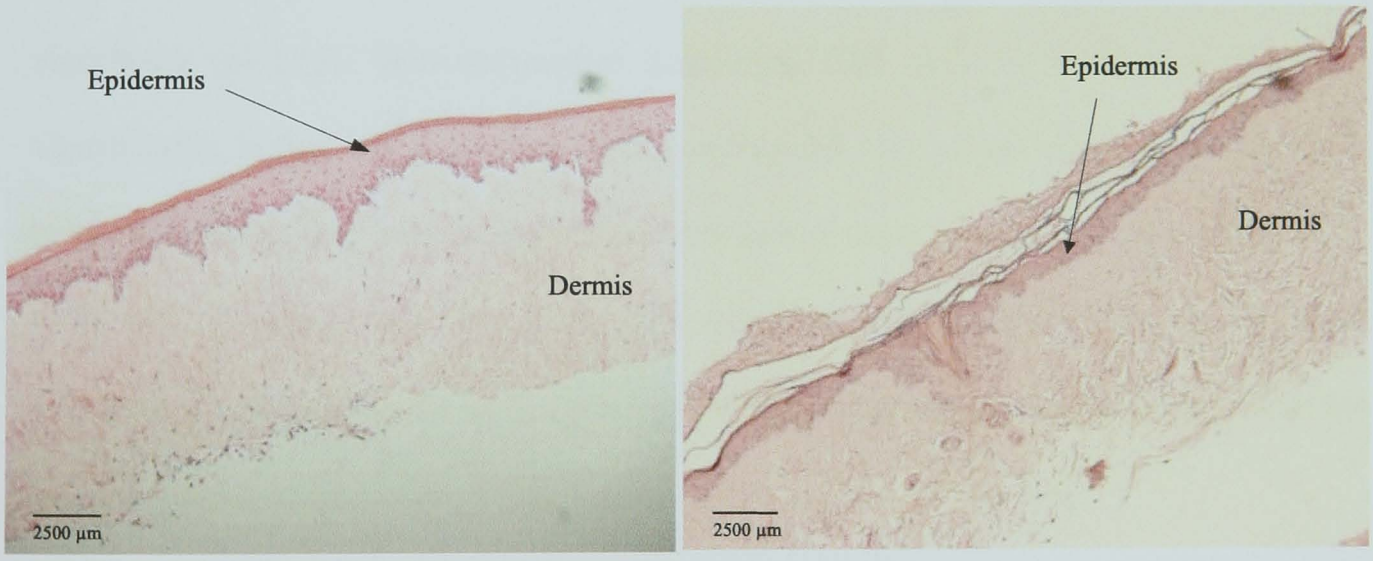


Figure 5.2. Standard Skin Composites to which fibroblasts and keratinocytes were added to the DED. 4x magnification.

5.3. MELANOMA INVASION IN HUMAN SKIN COMPOSITES

In these composites, melanoma cell invasion in reconstructed human skin was studied. Three melanoma cell lines were used which were known to have different invasion levels. Figure 5.3 shows examples of the invasion of all three cell types, Figure 5.6 shows the extent of melanoma cell invasion into the dermis and, Table 5.2 shows the invasion score for melanoma cell invasion into the dermis.

In four composites (four were lost due to infection) to which HBL cells were added on their own to the DED, a mean invasion score of 1 was reported (See Table 5.2 and Figure 5.6). In seven composites (one was lost due to infection) to which HBL cells were added with fibroblasts a lower mean invasion score of 0 was observed. In six composites to which fibroblasts and keratinocytes were added together with HBL cells, four had an invasion score of 0 and, one two had an invasion score of 1 (mean score = 0.33). Non-parametric Mann-Whitney t-test demonstrated that the invasion score of skin composites containing HBL cells, fibroblasts and keratinocytes compared to the invasion score of skin composites containing HBL cells and fibroblasts was not significant ($p>0.05$). Skin composites containing HBL cells on their own showed a significantly higher invasion score compared to the skin composites containing HBL cells, fibroblasts and keratinocytes ($p<0.05$) and to skin composites containing HBL cells and fibroblasts ($p<0.01$). Thus this demonstrated that there was clearly more invasion of melanoma cells in the presence of fibroblasts and keratinocytes and that the presence of fibroblasts only stopped HBL cell invasion in the dermis.

A375SM melanoma cells when added to the DED on their own were a little less invasive when compared to the invasion of HBL cells into the dermis (See Table 5.2 and Figure 5.5). In seven composites to which were added only A375SM cells, six had an invasion score of 0 and one had a score of 3 (mean score = 0.43). Skin composites containing A375SM melanoma cells added with fibroblasts showed no cell invasion. In

six composites (one was lost due to damaged DED), all had an invasion score of 0. A375SM melanoma cells when added together with fibroblasts and keratinocytes, in nineteen composites, seven had an invasion score of 0, two scored 0.5, one scored 1, two scored 1.5, two scored 2, two scored 2.5 and three scored 3 (mean score = 1.21), (three composites were lost due to damaged DED). Skin composites containing A375SM cells and skin cells had a significantly increased cell invasion score compared to skin composites containing A375SM cells and fibroblasts ($p<0.05$). There was no statistical significance between skin composites containing A375SM cells on their own compared to skin composites containing A375SM cells and fibroblasts ($p>0.05$). This may be explained by the occurrence of just one out of seven skin composites containing A375SM cells on their own with an invasion score of 3 resulting in variability of the sample. Cell invasion in skin composites containing A375SM cells, fibroblasts and keratinocytes compared to skin composites containing A375SM cells on their own was also not significant ($p>0.05$).

In order to distinguish between A375SM cells and keratinocyte invasion into the dermis, S100 antibody staining, specific for A375SM melanoma cells was used. The A375SM melanoma cells showed little invasion. Surprisingly, A375SM cells when added together with normal skin cells "encouraged" keratinocyte invasion into the dermis as can be seen in Figure 5.4 A and B. It was possible to observe that some groups of cells invaded into the dermis (Figure 5.4 A). The cells invading into the dermis were confirmed to be keratinocytes (in purple colour) in the presence of A375SM cell (in red colour) (Figure 5.4 B). It appeared that the cells of the epidermal layer had entered the dermis, possibly by degradative enzymes facilitating invasion of keratinocytes and melanoma cells.

C8161 melanoma cells were very invasive when added together with keratinocytes and fibroblasts. The results are summarized in Table 5.2 and Figure 5.6.

In fifteen of these composites (two were lost due to infection), two had an invasion score of 1, one had a score of 2, and twelve had a score of 3 (mean score = 2.67). For C8161 cells when added with fibroblasts to the DED, in five composites, two had an invasion score of 0.5 and three composites had a score of 3 (mean score = 2). In thirteen composites when C8161 cells were added on their own to the DED, one composite had an invasion score of 1 and twelve composites had a score of 3 (mean score 2.85). Although composites to which were added fibroblasts and C8161 melanoma cells resulted in a lower invasion score when compared to the other composites also containing C8161 cells, this was not significant ($p>0.05$). Therefore, C8161 cells showed the same level of invasion no matter what other cells were present. In order to observe keratinocyte invasion, an antibody staining (AE1AE2) for keratinocytes (in brown colour) was used. It is worth noting that although C8161 melanoma cells invaded the dermis, in these composites, there was also an infiltration of keratinocytes into the dermis, as illustrated in Figure 5.4 C and D.

These results indicate that C8161 melanoma cells are highly invasive, A375SM cells have an intermediate level of invasion in the presence of skin cells and HBL cells had the lowest invasion level in the presence of skin cells when compared to the other two melanoma cells. These results confirm those published by Eves *et al.* (2000).

It was interesting to note that for two of the weakly invasive melanoma cells (A375SM and HBL's) the presence of fibroblasts almost completely stopped cell invasion. However A375SM cells in these composites could make some "pocket-like" openings in the dermis, demonstrating degraded areas by these melanoma cells. A summary of these results can be found in Table 5.2 and Figure 5.5.

Table 5.2 also lists 11 skin composites experiments that were lost to infection or damaged DED.

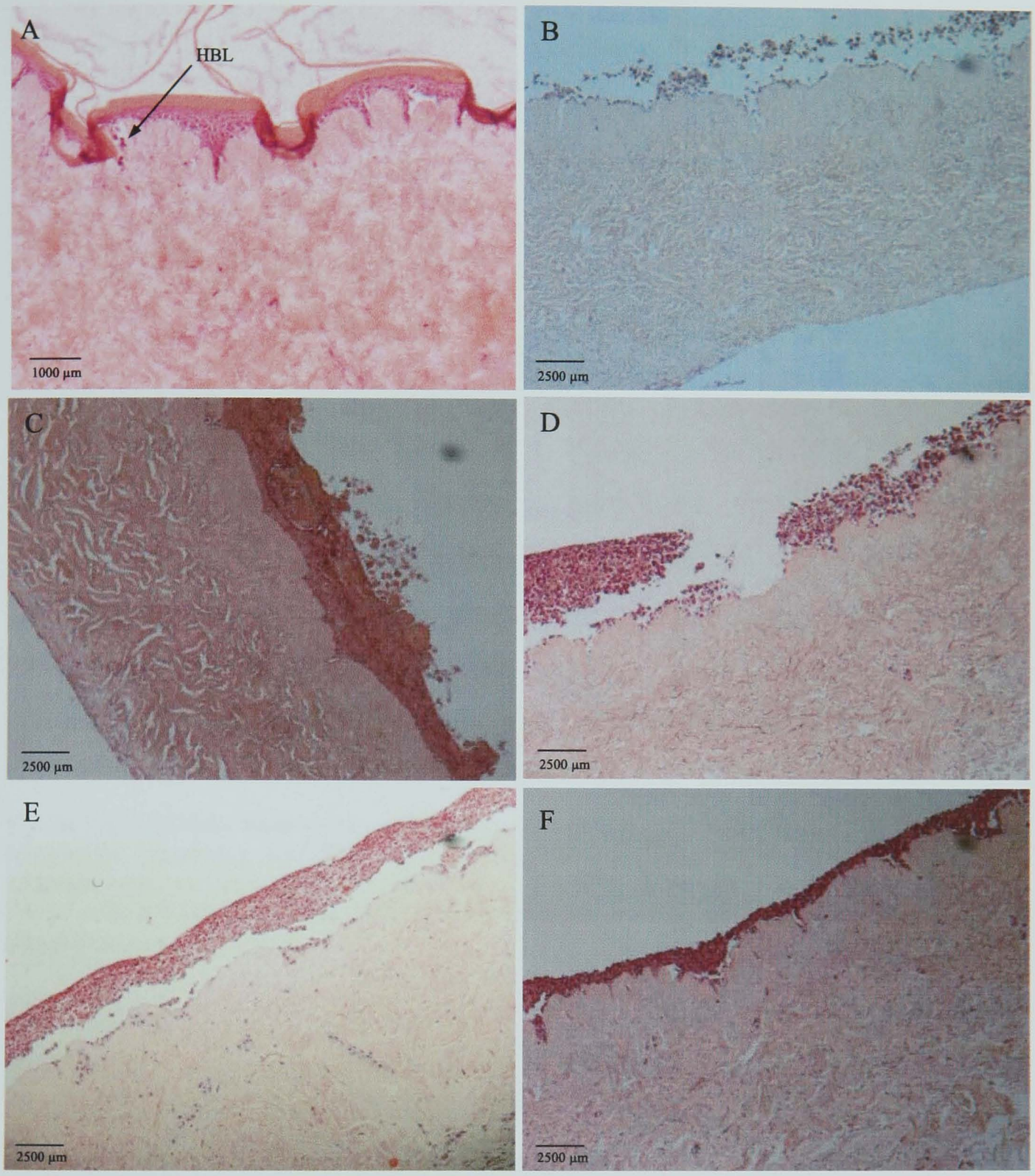


Figure 5.3. Skin composites to which were added melanoma cells. (A) Skin cells (fibroblasts and keratinocytes) and HBL melanoma cells in DED (10x magnification); (B) HBL melanoma cells only in DED; (C) Skin cells and A375SM melanoma cells in DED; (D) A375SM melanoma cells only in DED; (E) Skin cells and C8161 melanoma cells in DED; (F) C8161 melanoma cells only in DED (4x magnification).

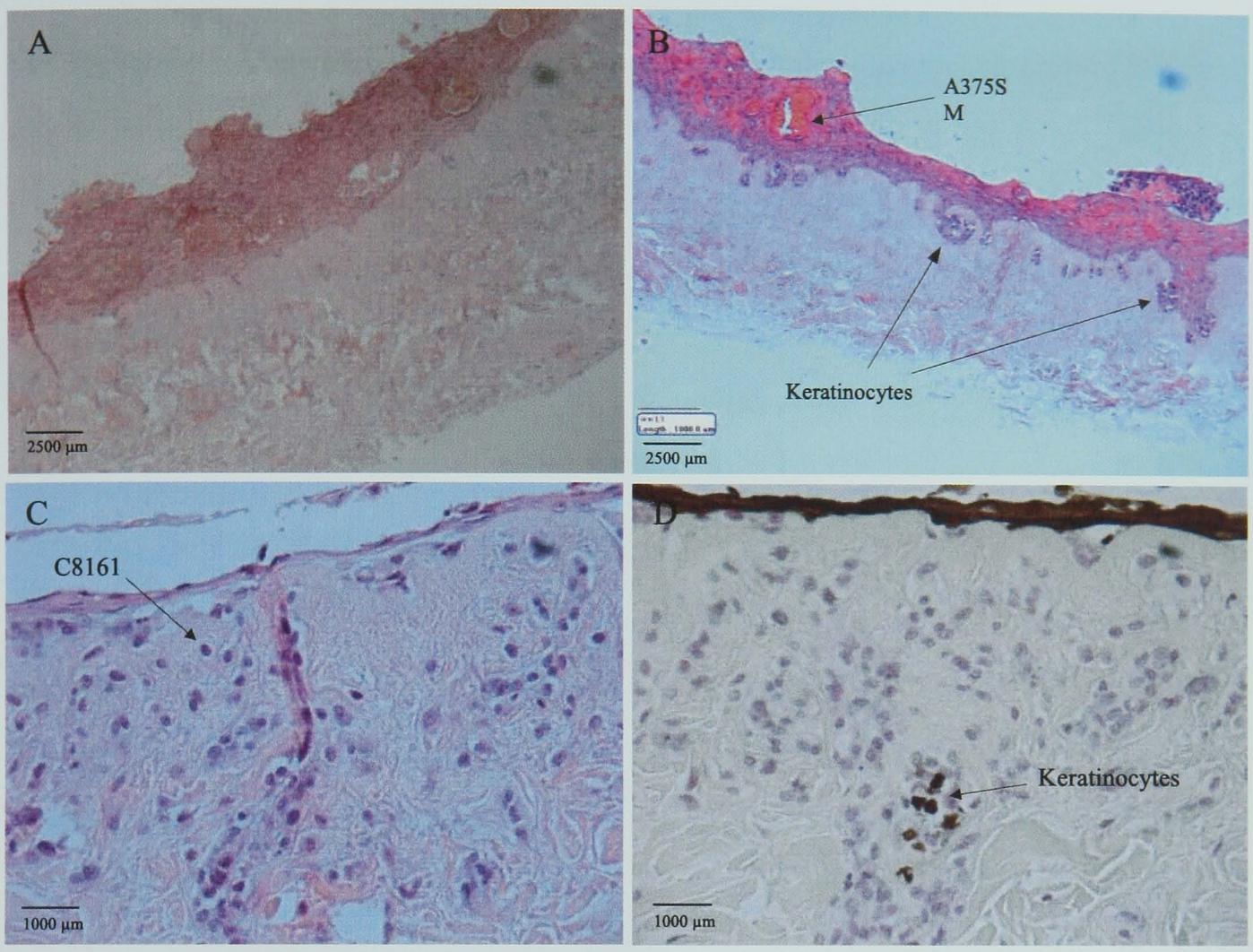


Figure 5.4. Antibody staining for melanoma cell invasion visualization. A and B show skin composites containing A375SM cells (A) H&E staining, (B) S100 antibody staining for A375SM cells, 4x magnification respectively. (C and D show skin composites containing C8161 cells (C) H&E staining, (D) AE1AE3 antibody staining for keratinocytes only, 10x magnification respectively.

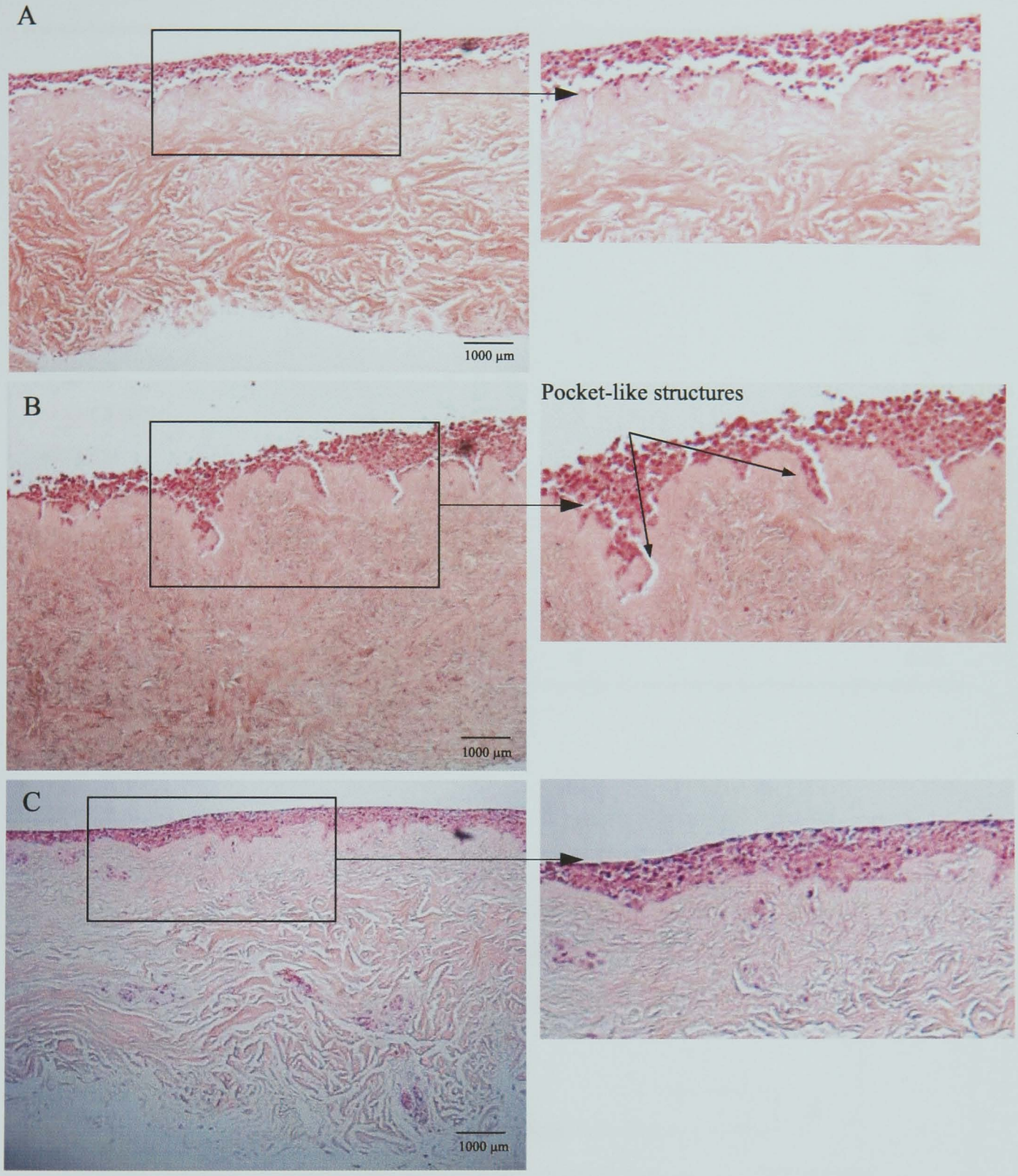


Figure 5.5. Skin composites to which were added fibroblasts and melanoma cells only. (A) HBL, (B) A375SM and (C) C8161 cells. 10x magnification and 50% zoom on the right hand side of each composite.

Table 5.2. Invasion Score for melanoma cell invasion into the dermis.

			Invasion Score							
Skin Composite	**No. Comp**	**Lost**	**0**	**0.5**	**1**	**1.5**	**2**	**2.5**	**3**	**Mean Score**
Sterile F+K	2		2							0
Sterile C8161	2								2	3
Sterile F+K+C8161	2								2	3
F+K	22	1	22							0
C8161	13				1				12	2.85
F+C8161	5			2					3	2
F+K+C8161	15	1			2		1		12	2.67
A375SM	7		6						1	0.43
F+A375SM	6	1	6							0
F+K+A375SM	19	3	7	2	1	2	2	2	3	1.21
HBL	4	4			4					1
F+HBL	7	1	7							0
F+K+HBL	6		4		2					0.33

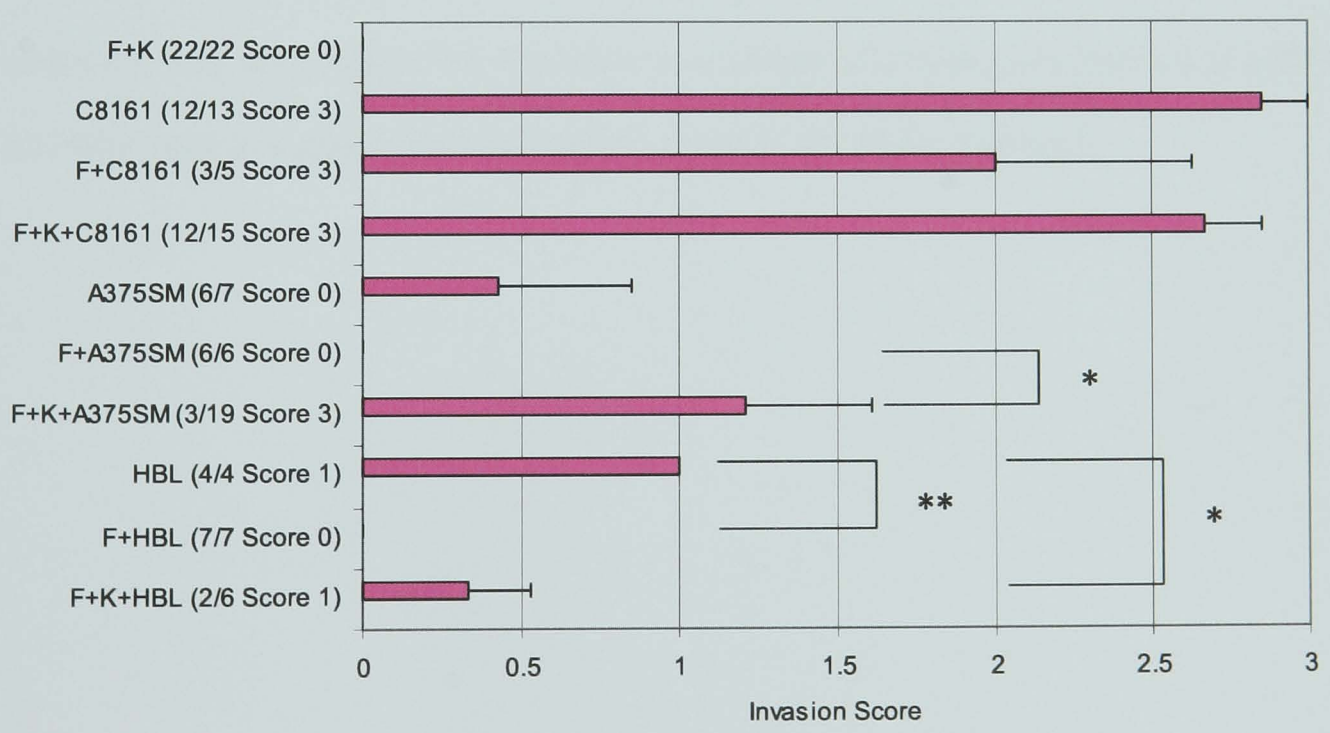


Figure 5.6. Differences in the extent of melanoma cell invasion into the dermis. The skin composite types are described followed by the number of skin composites in which the invasion was scored. The maximum invasion score is indicated in brackets. The histograms show the mean +1SEM of all scores. * indicates $p<0.05$; ** indicates <0.01.

5.4. EFFECTS OF NSAIDs ON MELANOMA SKIN COMPOSITES

In these experiments, two melanoma cell lines (C8161 and A375SM cells) were added to the skin composites to investigate the effects of NaIbu hydrogel (containing 131μM Ibuprofen) on melanoma cell invasion. Control skin composites without NaIbu hydrogel produced skin composites composed of an epidermal layer and a normal dermis with invaded melanoma cells, as expected. However, the addition of 100μl of NaIbu hydrogel at concentration of 131μM produced skin composites with no cells. Figure 5.7 shows examples of melanoma skin composites treated with media only (control) and the ones treated with 100μl NaIbu hydrogel.

Skin composites which were added fibroblasts and melanoma cells also produced the same pattern of result. In 131μM NaIbu hydrogel treated composites, no cells were present. However controls showed cells attached on composites (see Figure 5.8).

The same kind of results was obtained in composites which were added melanoma cell only. Figure 5.9 illustrates an example of a composite which was added A375SM cells as a control and a composite treated with NaIbu hydrogel.

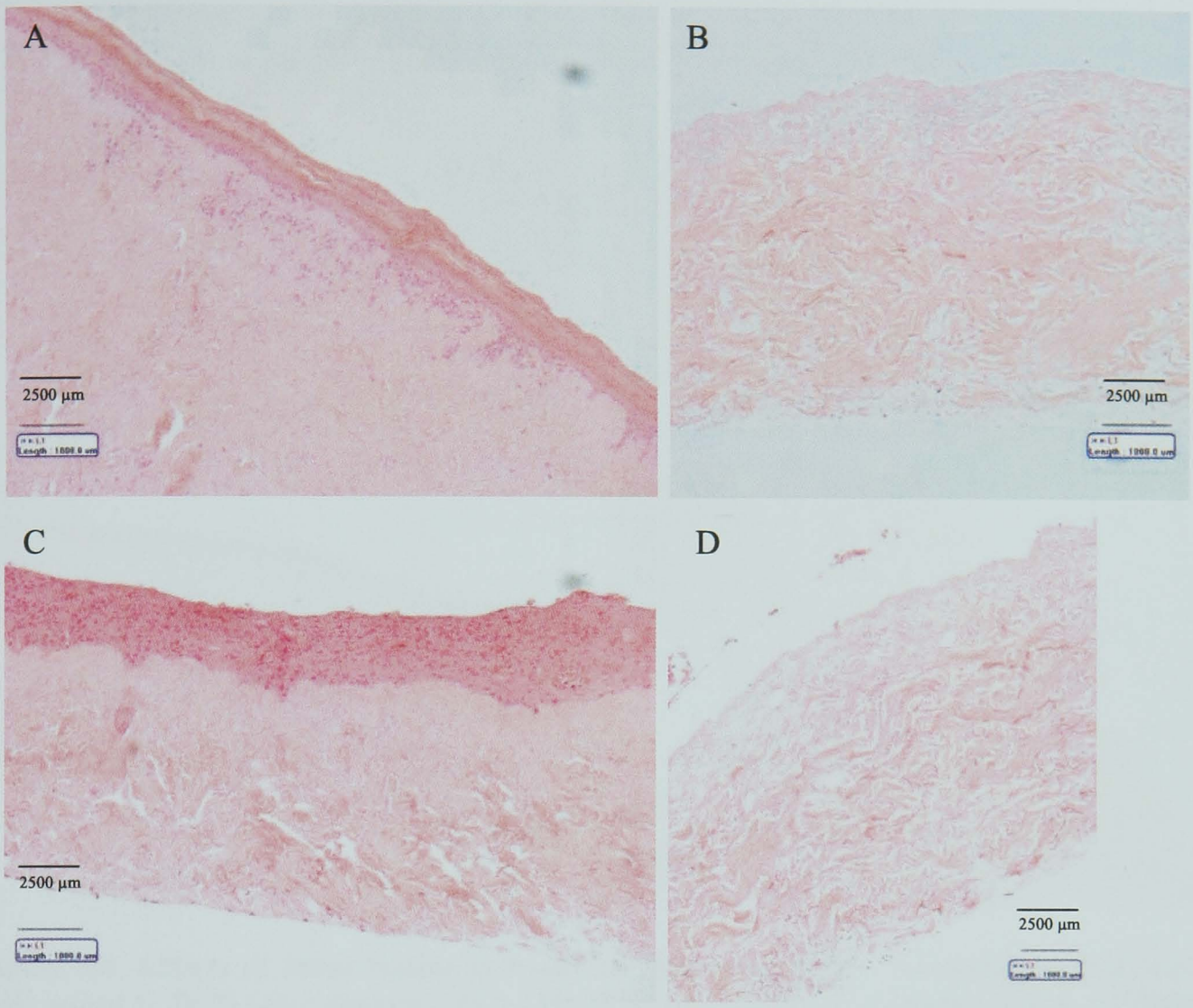


Figure 5.7. Effects of 100µl NaIbu hydrogel on skin composites. Skin composite containing C8161 cells (A) = control, (B) = 131µM NaIbu hydrogel. Skin composite containing A375SM cells (C) = control, (D) = 131µM NaIbu hydrogel. 4x magnification.

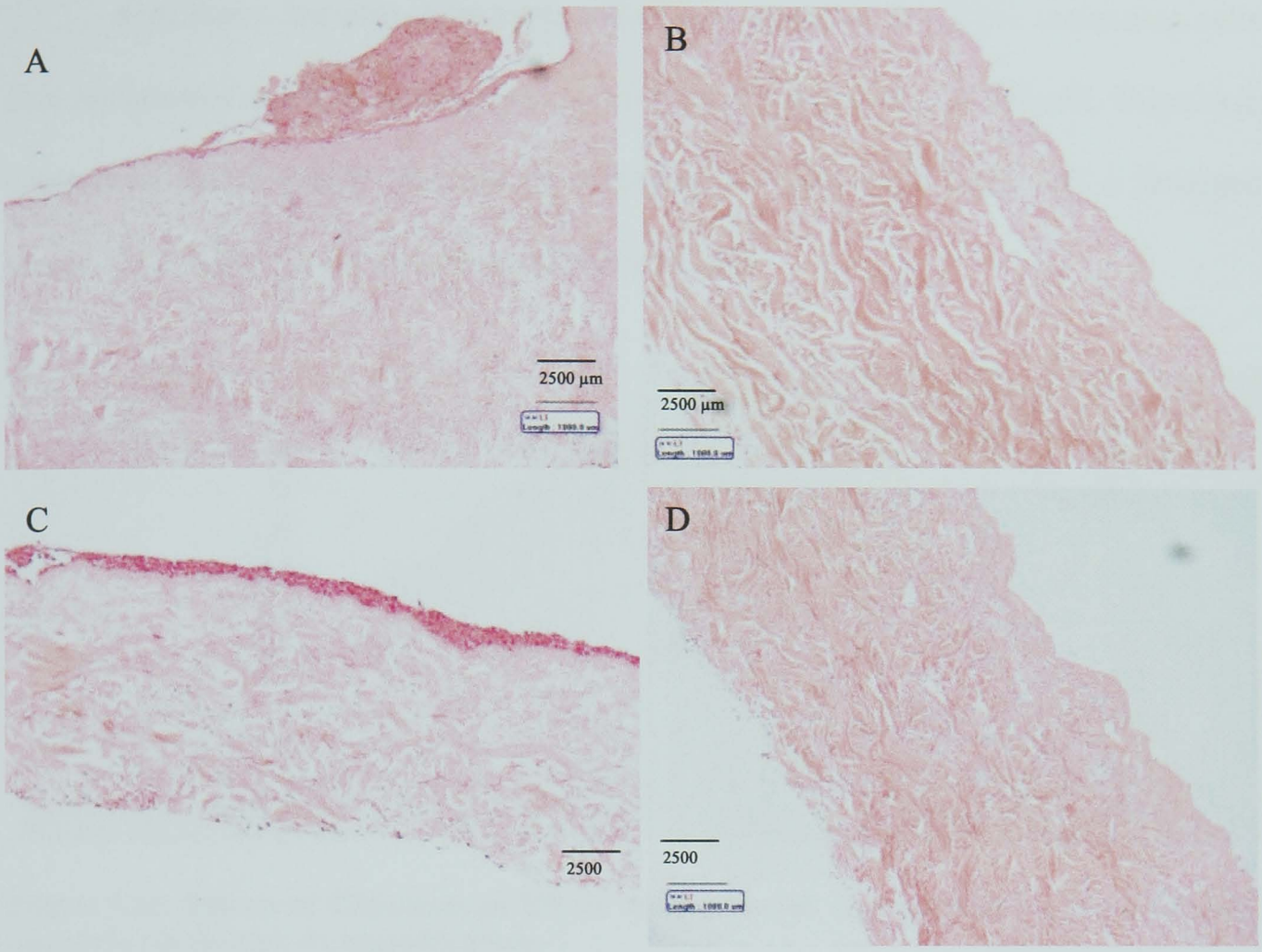


Figure 5.8. Effects of 100µl NaIbu hydrogel on skin composites. Fibroblasts and C8161 cells added to DED (A) = control, (B) = 131µM NaIbu hydrogel. Fibroblasts and A375SM cells added to DED (C) = control, (D) = 131µM NaIbu hydrogel. 4x magnification.

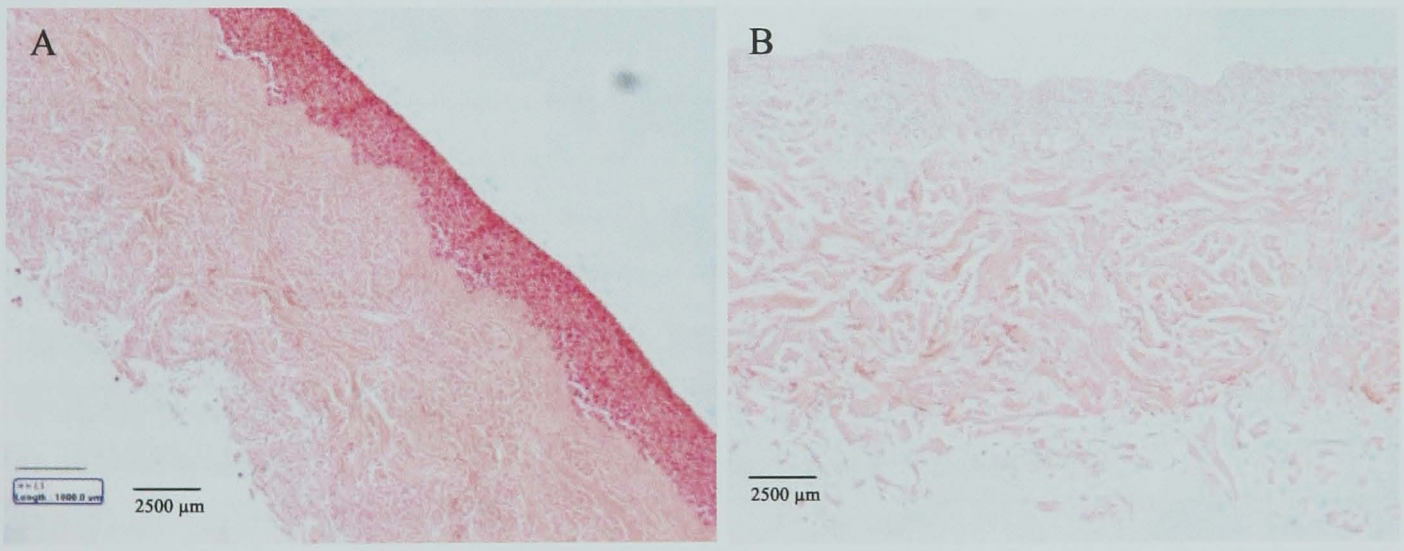


Figure 5.9. Effects of 100µl NaIbu hydrogel on A375SM cells only added to DED. (A) = control, (B) = 131µm NaIbu hydrogel. 4x magnification.

A different but also deleterious effect was observed in C8161 melanoma cells skin composites to which were added capsaicin at a concentration of 350μM. These skin composites had a poorly attached epidermis (see Figure 5.10 A+B) and a damaged dermis. See Table 5.3 for a summary of these results.

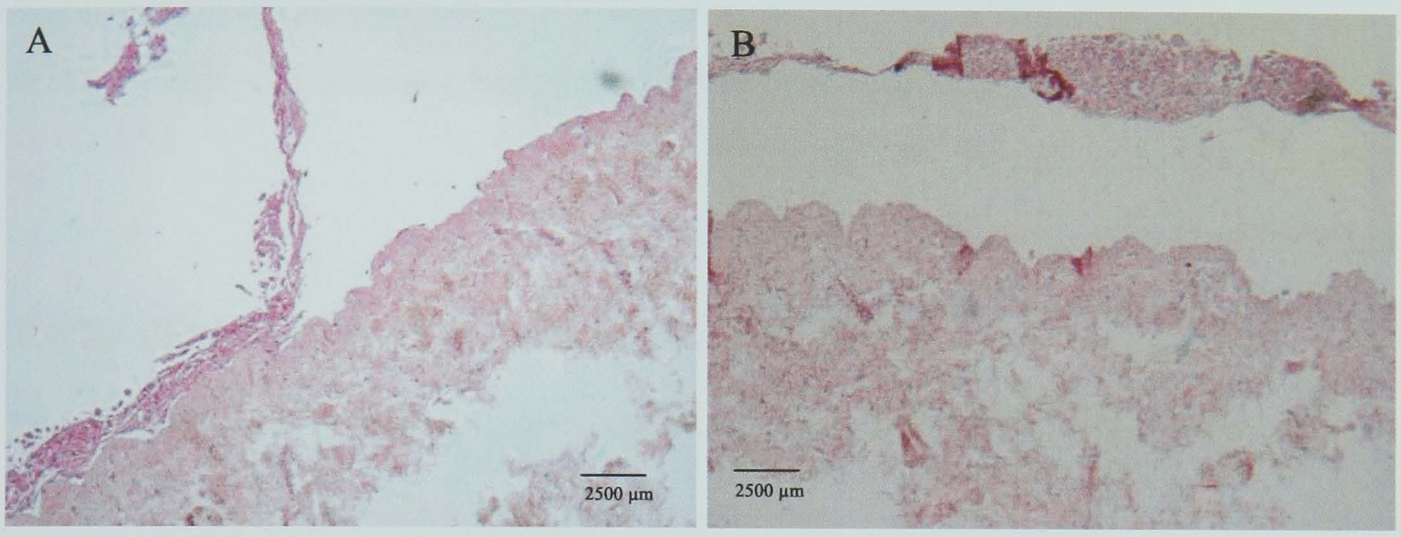


Figure 5.10. Effects of Capsaicin on C8161 melanoma cell skin composites. (A+B) 350μM capsaicin (in media). 4x magnification.

Table 5.3. Effects of NaIbu hydrogel (100μl volume) and Capsaicin (in media) on Skin Composites.

Skin Composite	Results	Number of Experiments
F+K+C8161 (Control)	Good composites. Invasion score = 3	1
F+K+C8161+131μM NaIbu hydrogel	No cells	1
F+K+A375SM (Control)	Good composites. Invasion score = 2	1
F+K+A375SM+131μM NaIbu hydrogel	No cells	1
F+C8161 (Control)	Good composites. Invasion score = 3	1
F+C8161+131μM NaIbu hydrogel	No cells	1
F+A375SM (Control)	Good composites. Invasion score = 0	1
F+A375SM+131μM NaIbu hydrogel	No cells	1
C8161 (Control)	Good composites. Invasion score = 3	1
C8161+131μM NaIbu hydrogel	No cells	1
A375SM (Control)	Good composites. Invasion score = 0	2
A375SM+NaIbu hydrogel 131μM	No cells	2
F+K+C8161+ 350μM Capsaicin	Dislodged epidermis and damaged dermis	2

In these initial composite experiments 100µl of hydrogel containing NaIbu was used but irrespective of the cell combination used there were no cells detected in these composites after 10 days incubation. Also, 350µM capsaicin was demonstrated to detach the epidermis from the dermis. The conclusion was that this local concentration of NaIbu was toxic to the cells. Accordingly future experiments used only 30µl of hydrogel. (Lower capsaicin concentrations should also be used in future experiments).

5.5. DEVELOPMENT OF 3D WOUNDED SKIN MODEL

In this part of the work, wounded tissue engineered human skin was developed and melanoma cell invasion was investigated. Fibrin clots and TNF-α were added to simulate some of the conditions of the normal inflamed wound bed which would have a high concentration of fibrin and TNF-α.

5.5.1. Skin Composites Wounded with a Syringe Needle

All of these composites contained normal skin cells and HBL or C8161 melanoma cells and were then wounded – initially using a syringe needle. Following wounding, fibrin clots and TNF-α were added to mimic inflammatory wound healing conditions.

Firstly, normal cells and melanoma cells were added to the DED to make a melanoma skin model. These composites were incubated in an Air-Liquid Interface (ALI) for 7 days and, after this period a wound using a syringe needle was made.

After wounding, a plastic ring made of a section of a PVC (polyvinyl chloride) tube (4mm height x 6mm diameter inside the ring) was placed on the top of the cell seeded area of the composite. 300µl of fibrin clot containing 300U/ml TNF-α was added in the ring (See Figure 5.11). These composites were then incubated at an ALI for a

further 12 days. The histology results showed no signs of wounds and also, the dermis showed no cells, suggesting that these composites were infected (See Figure 5.12 A, B, C and D). Table 5.4 shows a summary of 3 such experiments. Clearly wounding with a needle alone did not produce any visible wounds after culture for 12 days at an ALI.

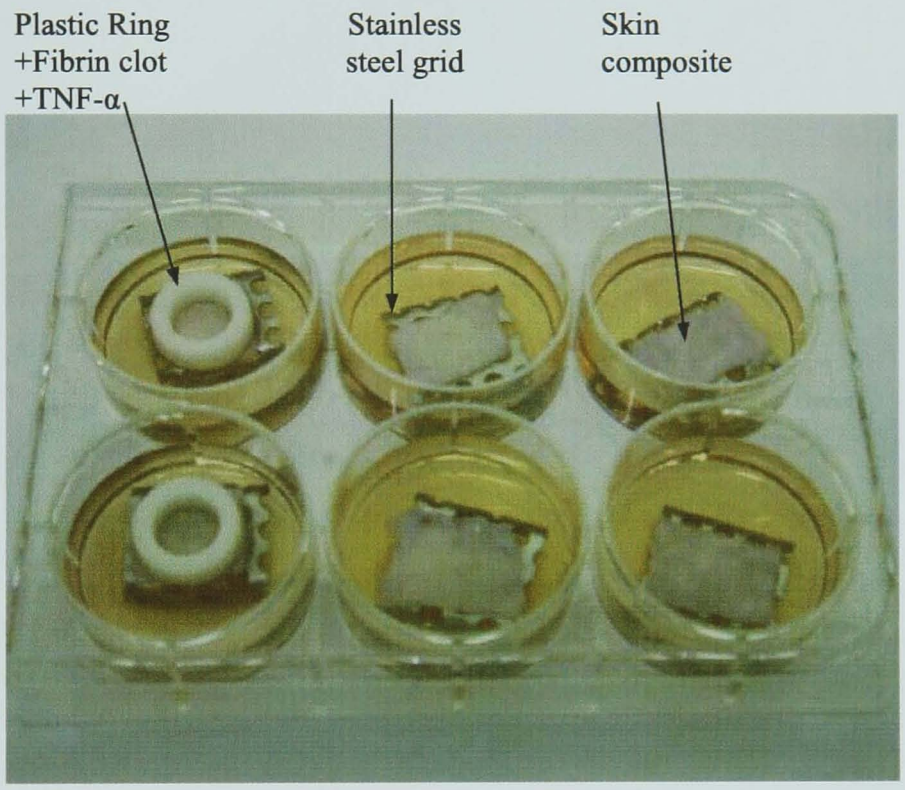


Figure 5.11. Skin composites at an ALI. A fibrin clot (300µl) and TNF-α (300U/ml) were added into the plastic ring.

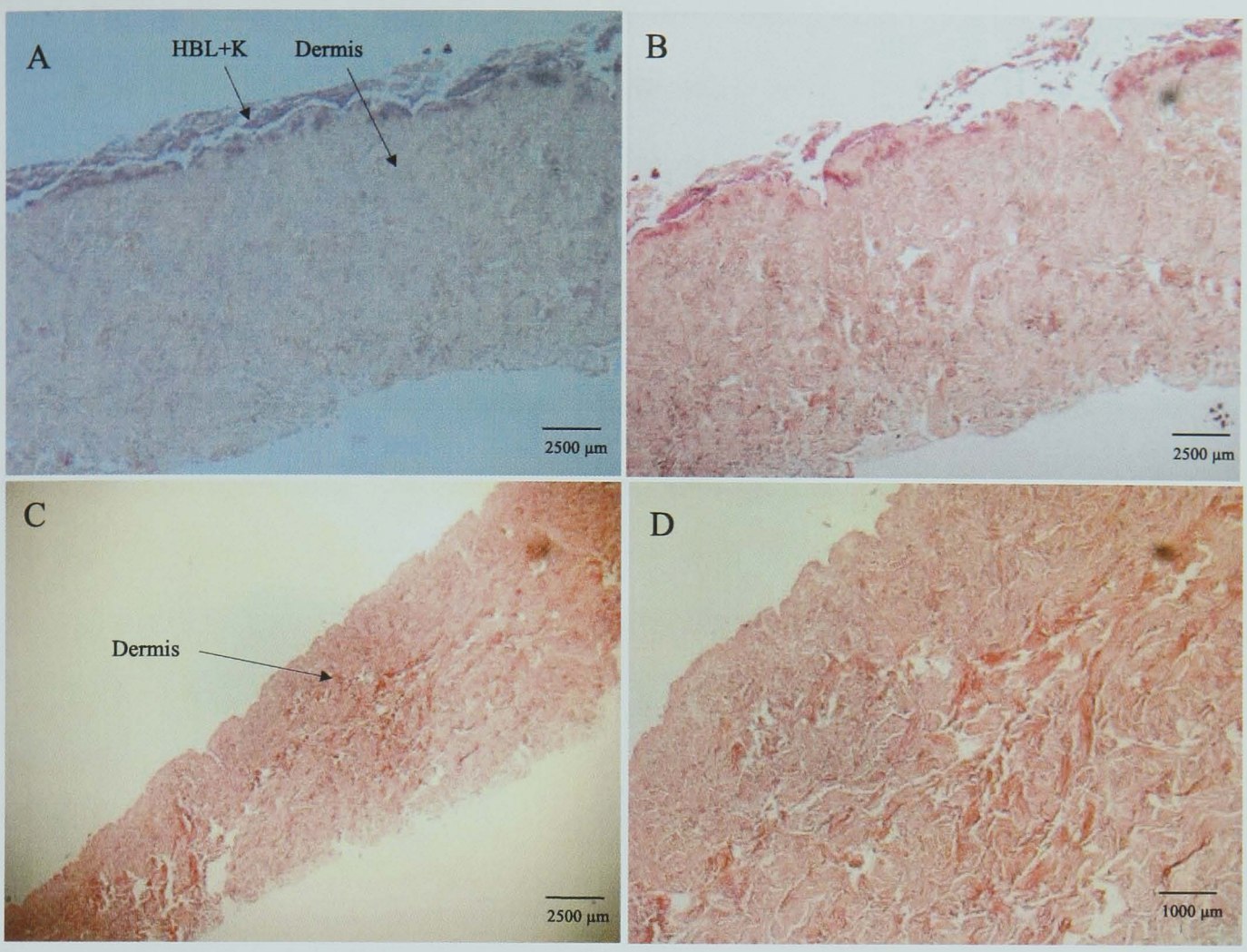


Figure 5.12. Syringe needle wounded skin composites. (A and B) Skin composite containing HBL cells to which were added fibrin clots and TNF-α; 4x magnification. (C and D) Skin composites containing C8161 cells to which were added fibrin clots and TNF-α. No wound was visible and no cells were present; 4x and 10x magnification respectively. All images are from the middle of each composite.

Table 5.4. Syringe needle wounded skin composites.

No. Experiments	Fibroblasts	Melanoma	Keratinocytes	Fibrin Clot	TNF-α	Visible wound	
						Yes	No
1	+	HBL	+	+	+		1
2	+	C8161	+	+	+		2

(+ = present)

5.5.2. Skin Composites Wounded with a Scalpel Blade

As the technique for wounded skin composites using a needle was not successful in producing visible wounds, full thickness wounds in a shape of a cross (See Figure 5.13) were then made using a scalpel blade in the following skin composites. After wounding, a plastic ring made from a section of a plastic Pasteur pipette (7mm height x 12mm diameter) was placed on the top of the cell seeded area of the composite. 300µl of a fibrin clot containing 300U/ml TNF-α was added into the ring (See Figure 5.14).

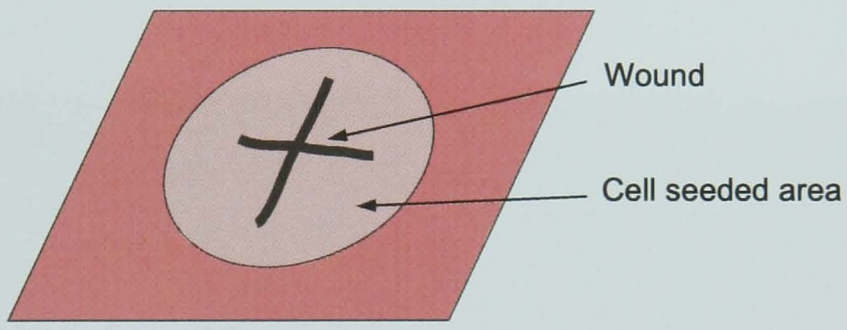


Figure 5.13. Diagram of a Skin Composite. Full thickness wounds in a shape of a cross.

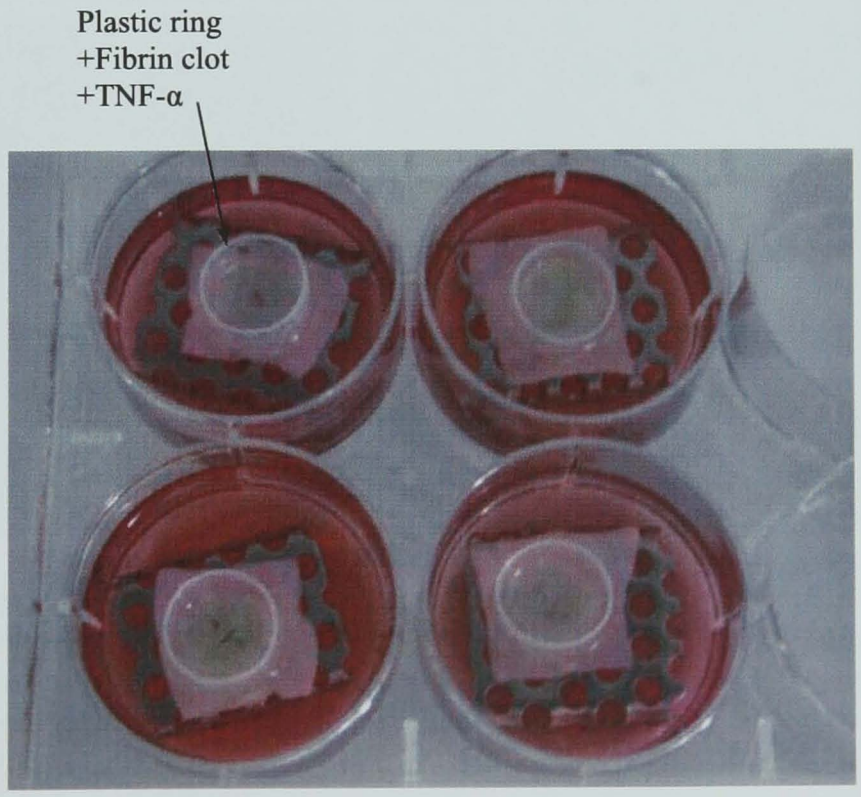


Figure 5.14. Scalpel blade full thickness wounded skin composites in ALI. A fibrin clot (300µl) and TNF-α (300U/ml) were added into the plastic ring.

In this experiment skin composites containing fibroblasts, keratinocytes and C8161 melanoma cells were incubated at an ALI for 10 days then a wound was made and composites incubated for a further 7 days. Histology of skin composites revealed a full thickness skin wound as shown in Figure 5.15 (A). However no cells were present in this composite suggesting an infected composite. In Figure 5.15 B, C and D it is possible to observe that more cells were present in the composite, however no wounds were visible. This experiment resulted in 2 full thickness wounds of 4 composites wounded with a scalpel blade. See Table 5.5 for results.

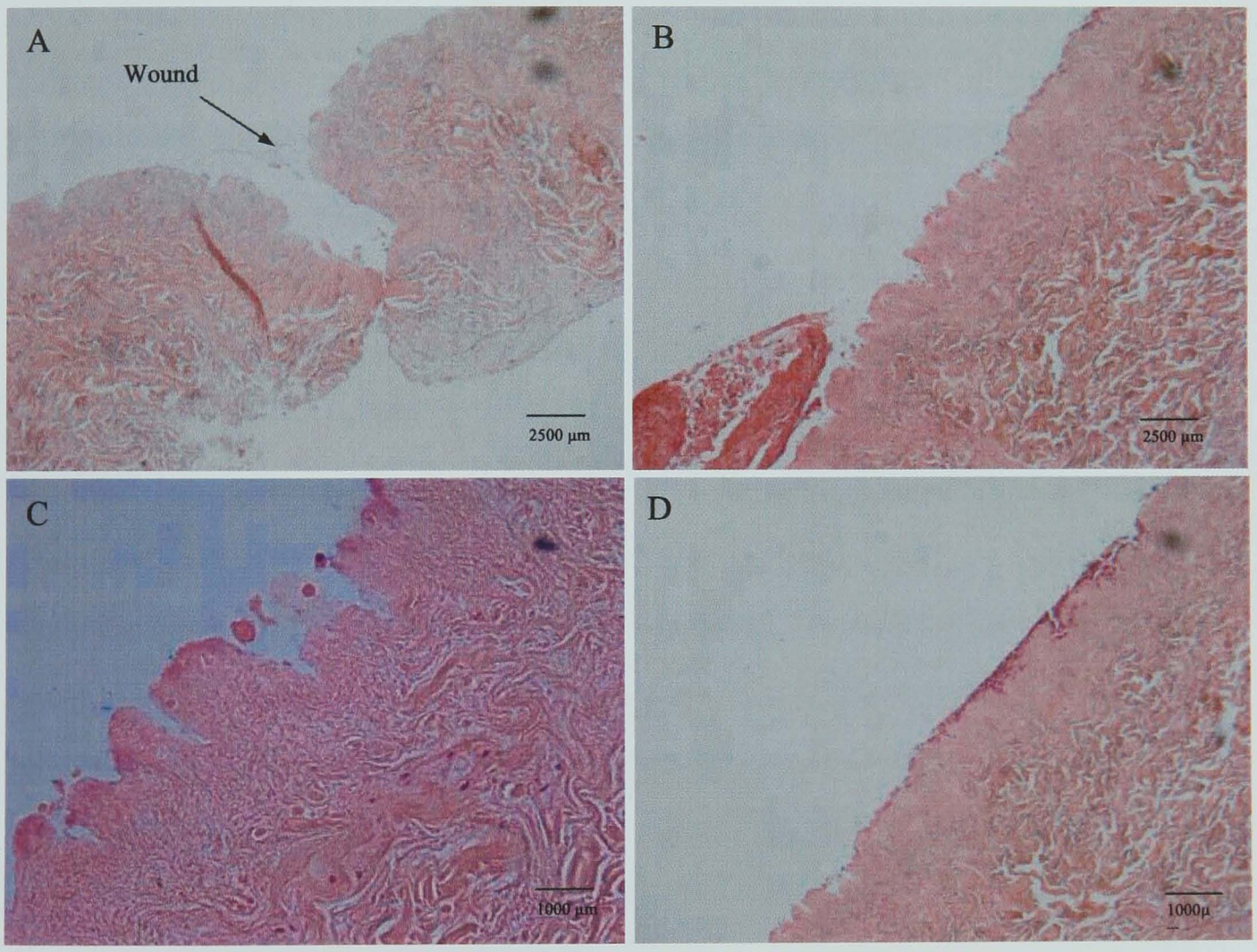


Figure 5.15. Effects wounding made by a scalpel blade on healing of C8161 cell skin composites to which was added 300µl of a fibrin clot plus 300U/ml TNF-α. (A) Visible wound (4x magnification). (B) far left (10x magnification), (C) middle (10x magnification) and (D) far right pictures of the same composite with no visible wounds (4x magnification). F=Fibroblasts; K=keratinocytes; C8161 melanoma cells.

Table 5.5. Scalpel blade full thickness wounded skin composites.

No. Experiments	Fibroblasts	Melanoma	Keratinocytes	Fibrin Clot	TNF-α	Visible wound	
						Yes	No
4	+	C8161	+	+	+	2	2

(+ = present)

As in these composites there were not many cells present, in the next experiments the amount of fibrin clot was reduced from 300µl to 30-50µl to see if this improved the composites. Also the fibrin clot was now added in a more localized fashion to the wound site rather than using a ring. This was done to increase the skin composite surface area not directly in contact with the fibrin clot. In these next composites, it is possible to observe then that there were more cells present and closer to the wounded site. See Figure 5.16.

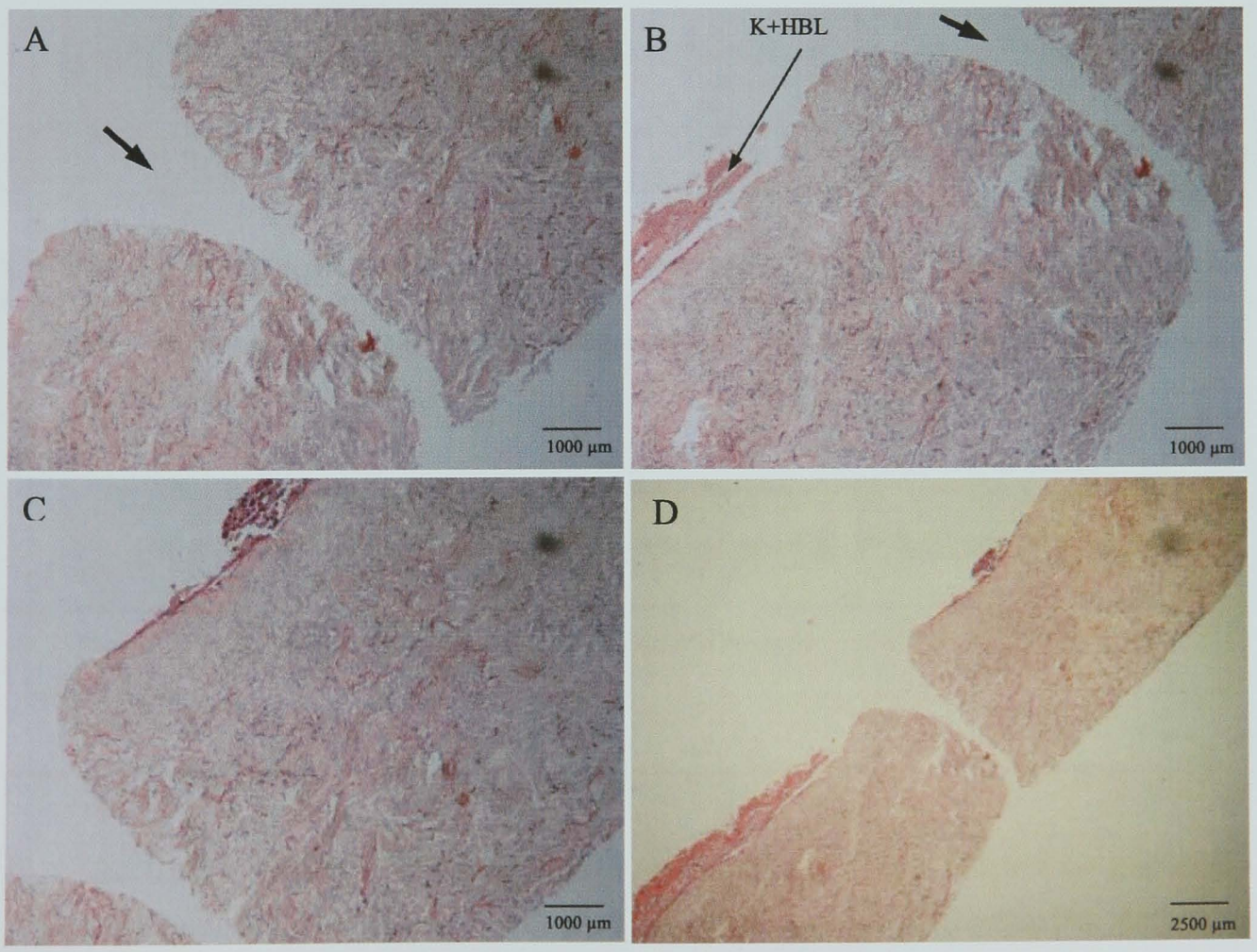


Figure 5.16. Scalpel blade wounded skin composite to which was added skin cells, HBL cells, fibrin clots and 300U/ml TNF-α. (A) middle, visible wound, (B) right, (C) left (10x magnification), (D) middle (4x magnification). Wound is indicated by an arrow.

In order to examine whether it was the fibrin clot or the TNF-α that was reducing the cells in the composites, in the next experiment, TNF-α was added in media without any fibrin clot. In 4 experiments treated with TNF-α added in the fibrin clot, 3 showed visible wounds and only one showed a closed wound. In 2 experiments treated with TNF-α in media, 2 of them showed a visible wound (see Figure 5.17). Table 5.6 shows a summary of these composites produced. Therefore, there was not much difference between skin composites with and without a fibrin clot, although one skin composite showed a layer of epidermis.

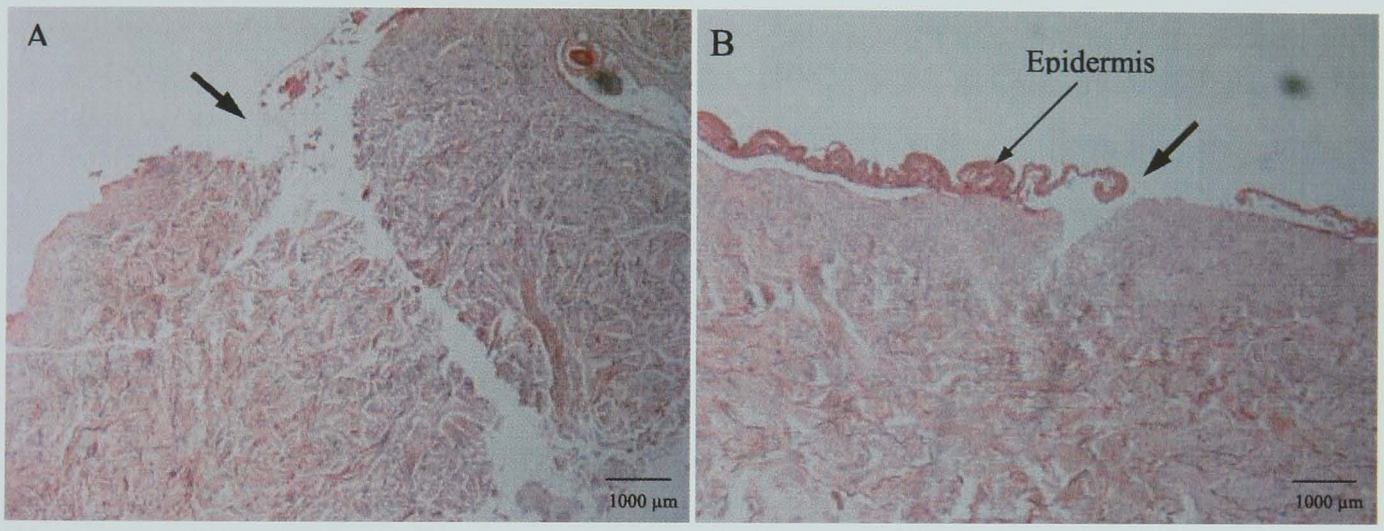


Figure 5.17. Scalpel blade wounded skin composites to which were added fibroblasts, keratinocytes and HBL cells and, TNF-α in media. (A) visible wound, (B) small wound and damaged epidermal layer (10x magnification). Wound is indicated by an arrow.

Table 5.6. Effect of fibrin clot on wound healing of skin composites.

No. Experiments	Fibroblasts	Melanoma	Keratinocytes	Fibrin Clot	TNF-α	Visible wound	
						Yes	No
4	+	HBL	+	+	+	3	1
2	+	HBL	+	-	+ in media	2	0

(+ = present)

5.5.3. Effect of Fibrin Clot on Wound Healing

In these early wounded skin composites, there were in retrospect many variables included in each experiment, making interpretation difficult. Therefore, simpler skin composites were next produced in order to observe the effects of a fibrin clot on wound healing. Some skin composites contained no cells, others contained only fibroblasts, others contained keratinocytes and fibroblasts. Wounds were made in these composites on the 11^{th} day of incubation at ALI and fibrin clots plus TNF-α were added at this time. For these fibrin clot studies the volume was kept at 30-50µl. These were then incubated for a further 7 days. Feeding was done every 2 days by removing old media and replacing with fresh media and a fresh fibrin clot plus TNF-α solution was made and added to the composites.

Figures 5.18 (A and B) show that there was no morphological difference between the cell-free DED and the DED added fibrin clot. However, the wounded DED resulted in a wide open wound while a narrow wound was demonstrated when the fibrin clot was added to the wounded DED (Figure 5.18 C and D respectively).

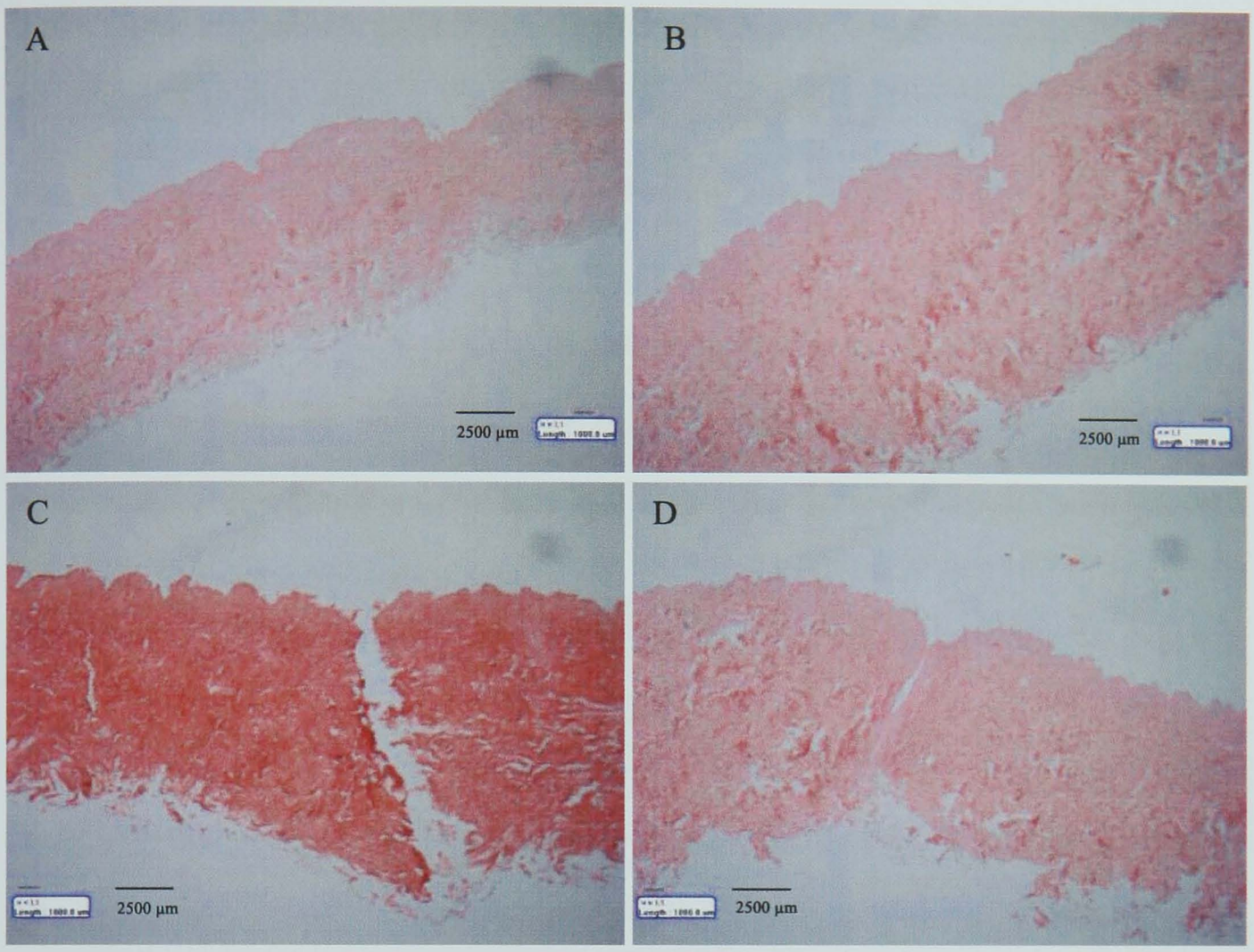


Figure 5.18. Addition of wounds and fibrin clot to cell-free DED to which were added Fibrin clot and with/without wound. (A) DED; (B) DED+Fibrin; (C) DED+Wound; (D) DED+Wound+Fibrin (4x magnification).

In the experiments illustrated in Figure 5.19, normal dermis was found (A) when fibroblasts only were added to DED. The addition of fibrin clots to DED did not show any difference when compared to the added fibroblasts only composites (B). As expected in DED containing fibroblasts, the wounded site showed a healed DED with very little remaining of the wound (C). However, the presence of the fibrin clot the skin composite showed an open wound (D). This may indicate the importance of participation of fibroblasts in the wound healing process. Table 5.7 shows a summary of these skin composites produced.

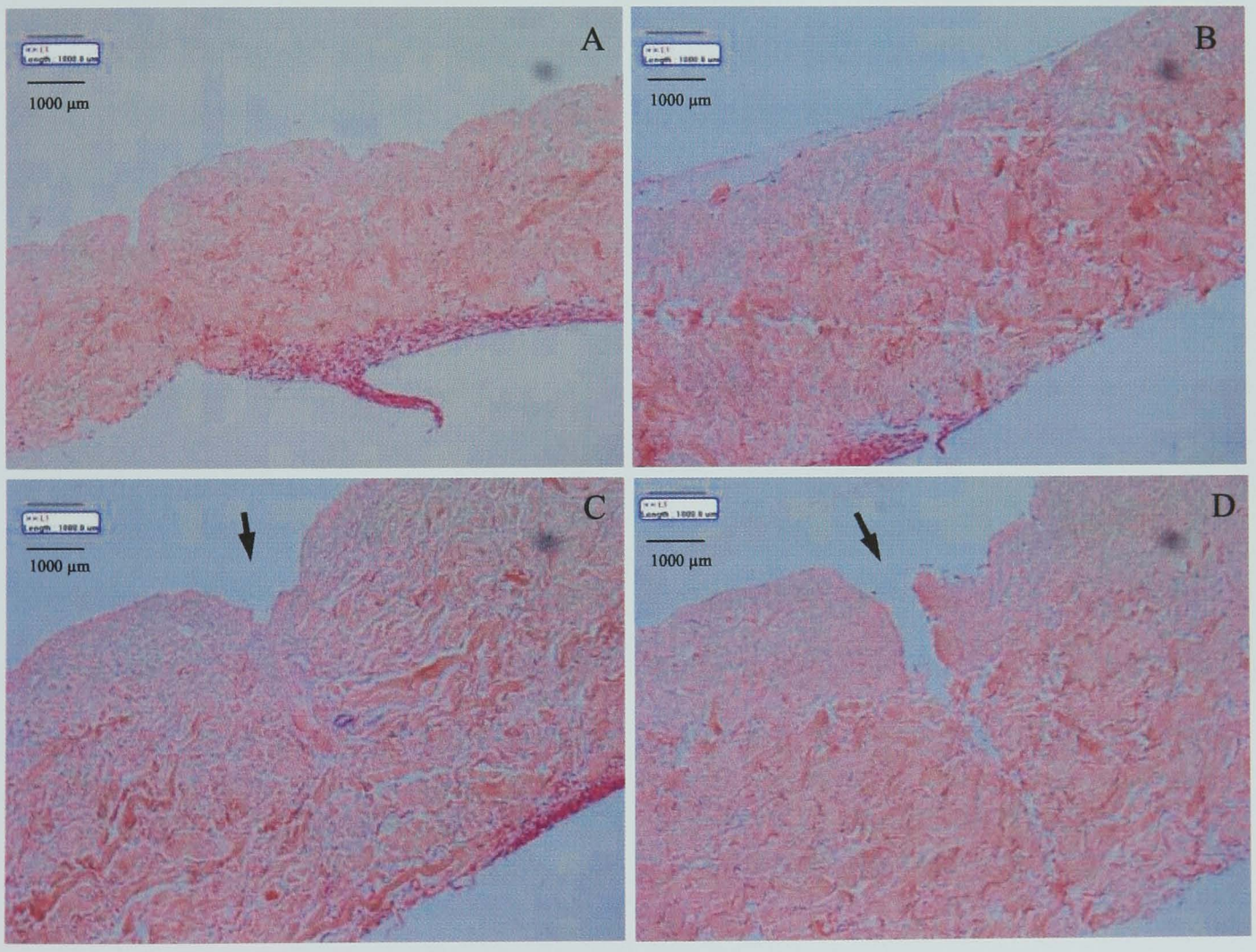


Figure 5.19. Addition of wound and fibrin clots to DED plus fibroblasts. The site of the wound is indicated by an arrow. (A) DED+Fibroblasts; (B) DED+Fibroblasts+Fibrin; (C) DED+Fibroblasts+Wound; (D) DED+Fibroblasts+Wound+Fibrin. 10x magnification.

In the next experiment a wounded standard skin composite was produced to compare the wound and healing process with and without the addition of fibrin clots and TNF-α. See Figure 5.20.

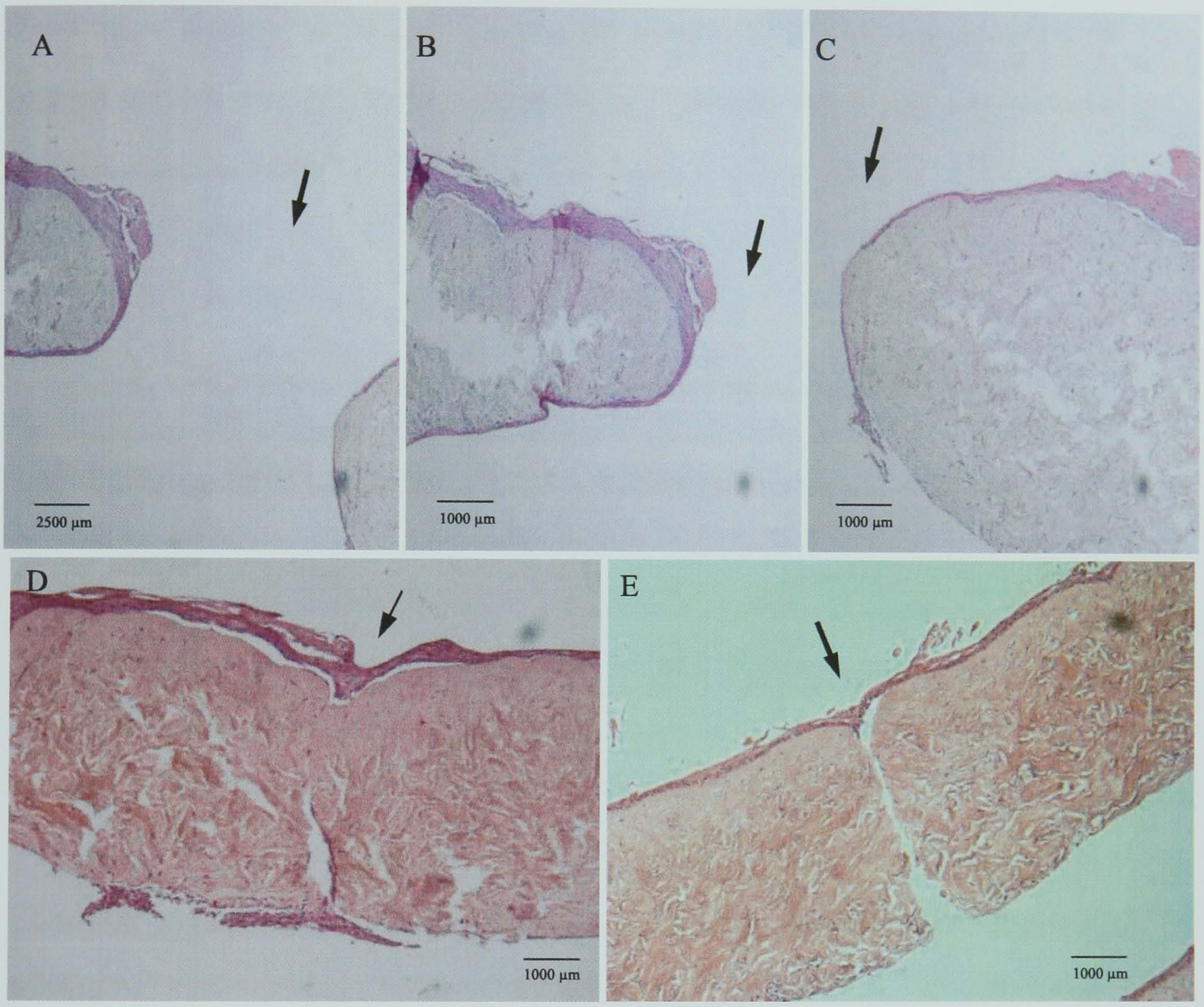


Figure 5.20. Wounded standard skin composites (A, B and C) 4x, 10x and 10x magnifications respectively. Standard skin composites with the addition of fibrin clots and TNF-α (D and F) 10x magnification. The site of the wound is indicated by an arrow.

With one exception of an experiment that was lost due to damaged DED, in two experiments, in which keratinocytes were added, the epidermis reformed over the still openly wounded dermis. Figures 5.20 (A, B and C) show this skin composite. A low magnification is used in A (4x) to illustrate the width of the wound at the end of the 10

day experiment. B and C are 10 x magnifications to show continuous epidermal, layer around the wound edges.

In another experiment when fibrin clots and TNF-α were added to the composites smaller wounds and a closed epidermal layer formed at 10 days as illustrated in Figures 5.20 D and E. It was not possible from these 2 experiments to interpret the influence of inflammation given by TNF-α so far. A summary of these results is shown in Table 5.7.

Table 5.7. Effect of fibrin clot and normal skin cells on wound healing.

Fibroblasts	Wound	Fibrin	TNF-α	Keratinocytes	Result
-	-	-	-	-	Normal DED
-	-	+	-	-	Normal DED
-	+	-	-	-	Open wide wound
-	+	+	-	-	Narrowed wound
+	-	-	-	-	Normal Dermis + F
+	-	+	-	-	Normal Dermis + F
+	+	-	-	-	Healed
+	+	+	-	-	Narrowed wound
+	+	-	-	+	Open wide wound
+	+	+	+	+	Small wound
+	+	+	+	+	Epidermis healed, narrowed wound

(+ = present; - = absent)

5.5.4. Wound Healing in Composites Containing Melanoma Cells

At this stage only HBL melanoma cells were added together with the normal skin cells to the skin composites and the effect of wounding was examined.

In two experiments produced containing HBL cells, the wounds were open however, an epidermal layer formed around the edges of the wound, showing invasion of cells in the wound edges site (Figure 5.21 A and B). A summary of this result is in Table 5.8.

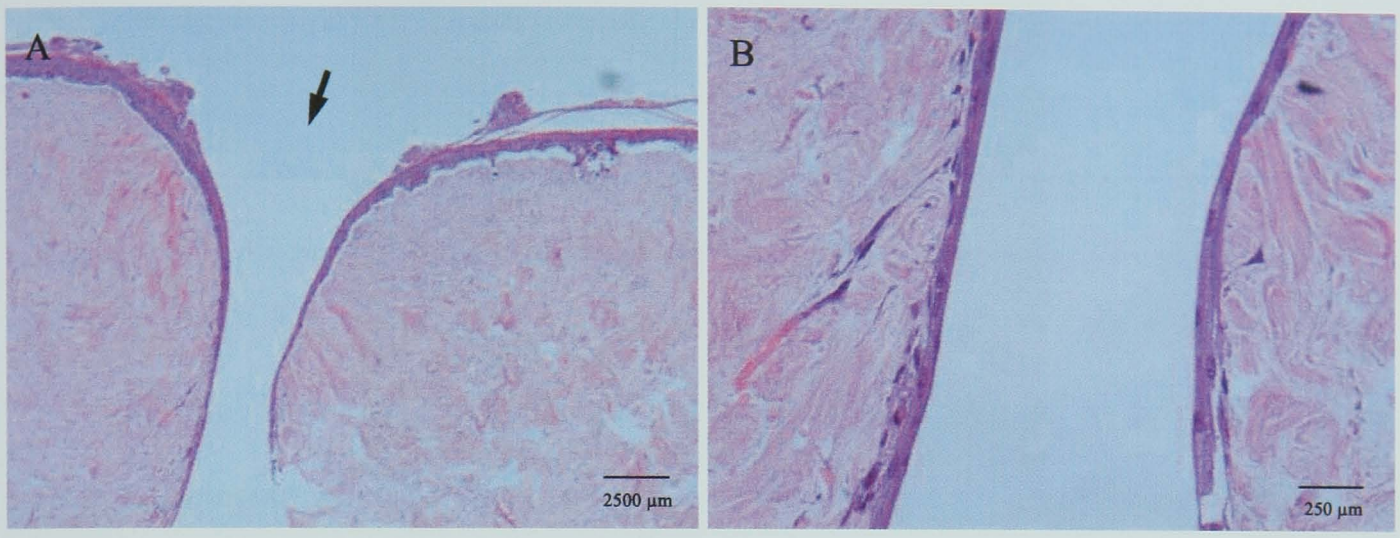


Figure 5.21. Wounded melanoma skin composites without the addition of fibrin clots and TNF-α. (A and B) Skin composites containing HBL cells (10x and 40x magnification respectively). The site of the wound is indicated by an arrow.

Table 5.8. Wounding and healing in melanoma skin composites.

Number of Experiments	Fibroblasts	Keratinocytes	HBL Cells	Results
2	+	+	+	Open wide wound

(+ = present)

In the next skin composites, to the DED were added fibroblasts, keratinocytes and A375SM melanoma cells, fibrin clots and TNF-α. These composites were incubated at an ALI for 10 days then a wound was made and 30-50µl fibrin clot was added and the composite was incubated for a further 7 days. As shown in Figure 5.22 (A), one skin composite without TNF-α was healed or demonstrated a very narrow wound. However in those composites to which fibrin clots and TNF-α were added a partially healed or open wound was demonstrated. These composites also showed cells in the wound (See Figure 5.22 B, C, D and E). This can be better seen in Figure 5.22 E, where cells invade in a badly made wound. (This particular wound was more clumsily made than others but the epidermis was continued to completion to observe what happened). A summary of these results is show in Table 5.9.

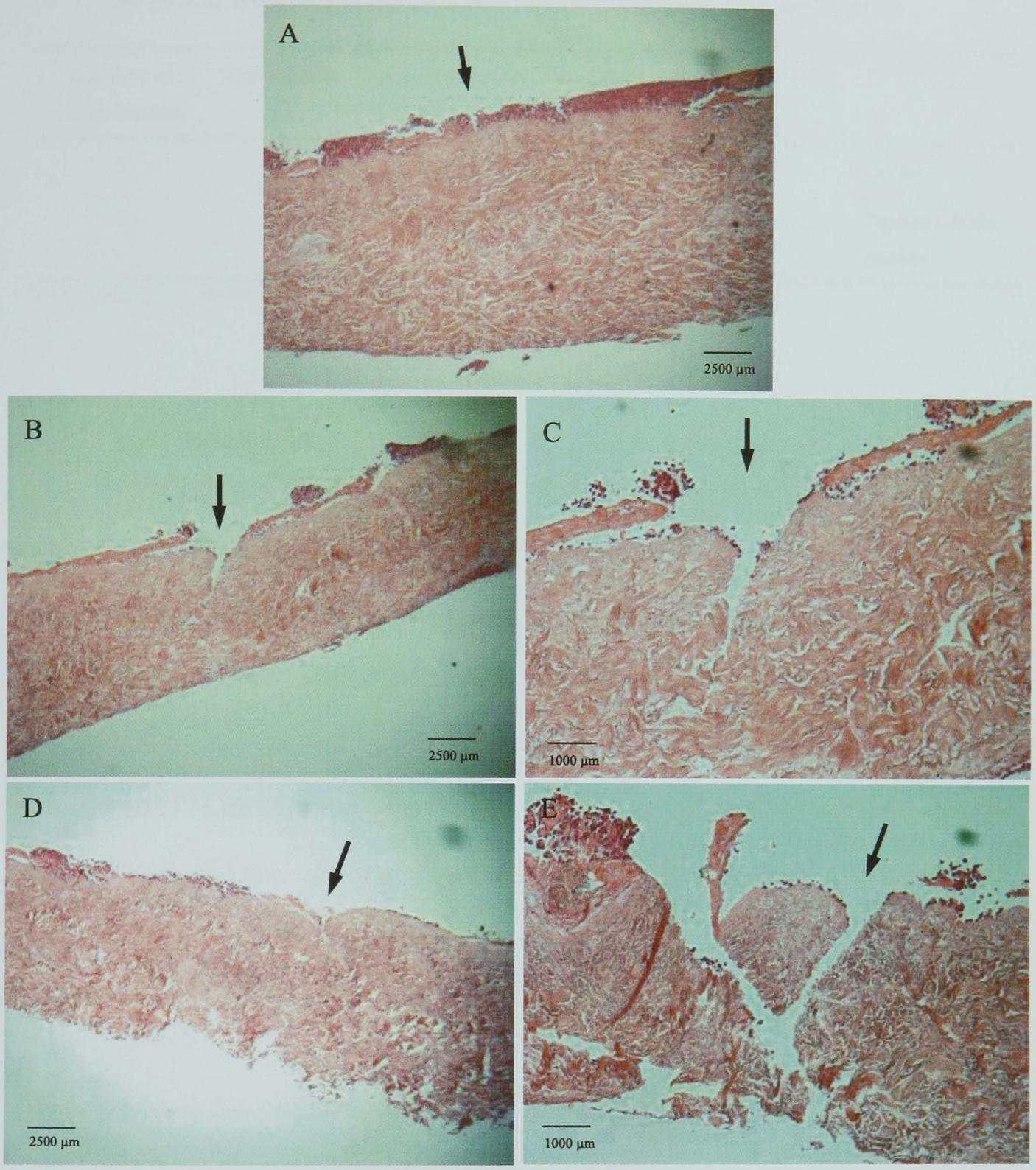


Figure 5.22. Effect of TNF-α on melanoma cell invasion in wounded skin composite model. All figures show composites containing DED+F+K+A375SM that have been wounded and a fibrin clot placed in the wound. In addition Figures B, C D and E have also received TNF- α (300U/ml). The site of the wound is indicated by an arrow. A, B and D, 4x magnification. C and E, 10x magnification.

Table 5.9. Wounded melanoma skin composites, fibrin clots and TNF-α.

Number of Experiments	Fibroblasts	Keratinocytes	A375SM Cells	Fibrin Clot	TNF-α	Result
1	+	+	+	-	-	Healed
3	+	+	+	+	+	Open/partially healed

(+ = present; - = absent)

Following these results it was decided to simplify the wound by making a single scalpel cut rather than a cross with two cuts for future experiments.

For these next skin composites, fibroblasts, melanoma cells and keratinocytes were added to the DED. However, wounds were made on the 3rd day after lifting the skin composites into an ALI. A full thickness wound was made by just a single cut using a scalpel blade (See Figure 5.23). After wounding, fibrin clots and TNF-α were added to the wound site. The skin composites were then incubated for a further period of 10 days.

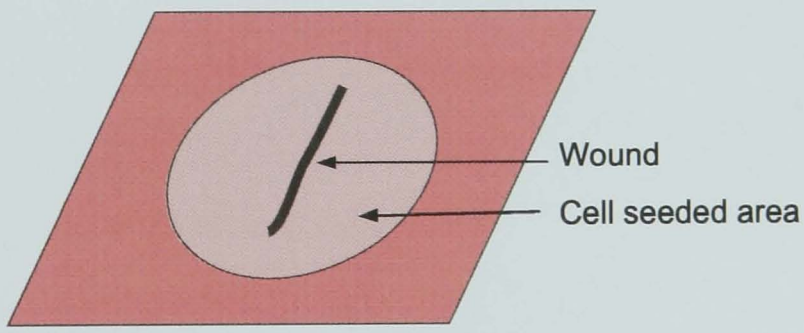


Figure 5.23. Diagram of a Skin Composite. Full thickness wound made by a single cut.

As an example of this method control wounded skin composites (without TNF-α), are shown in Figure 5.24 (A). This figure shows 3 skin composites to which were added fibroblasts, keratinocytes, A375SM and the open wound was made using a single cut using a scalpel blade on the 5th day after wounding. To these composites were also

added fibrin clots in the wounded area. After 10 days of incubation, the wounds were visibly healed. See Figure 5.24 (B). These skin composites were then sacrificed for histology (Figure 5.25).

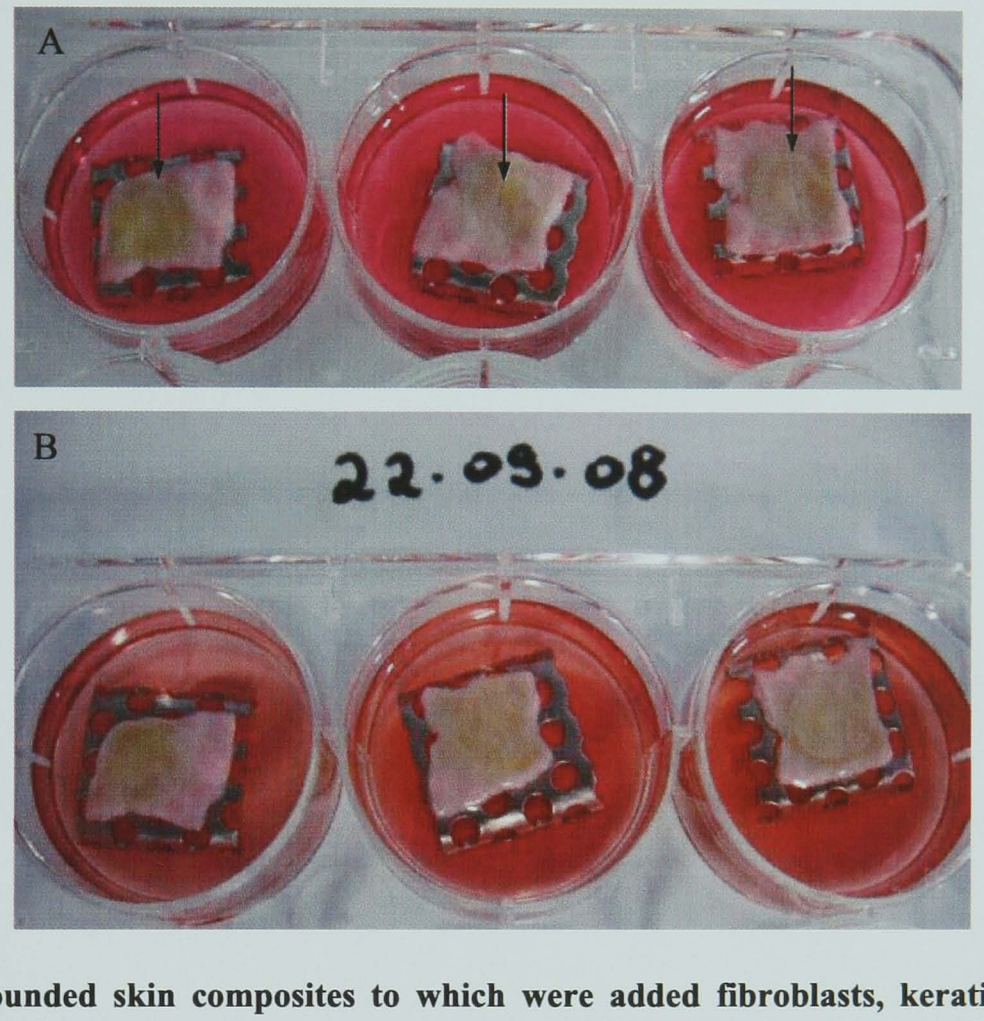


Figure 5.24. Wounded skin composites to which were added fibroblasts, keratinocytes, A375SM cells and fibrin clots. (A) open wounds 5 days after wounding. (B) Healed wounds in skin composites after 10 days incubation into ALI.

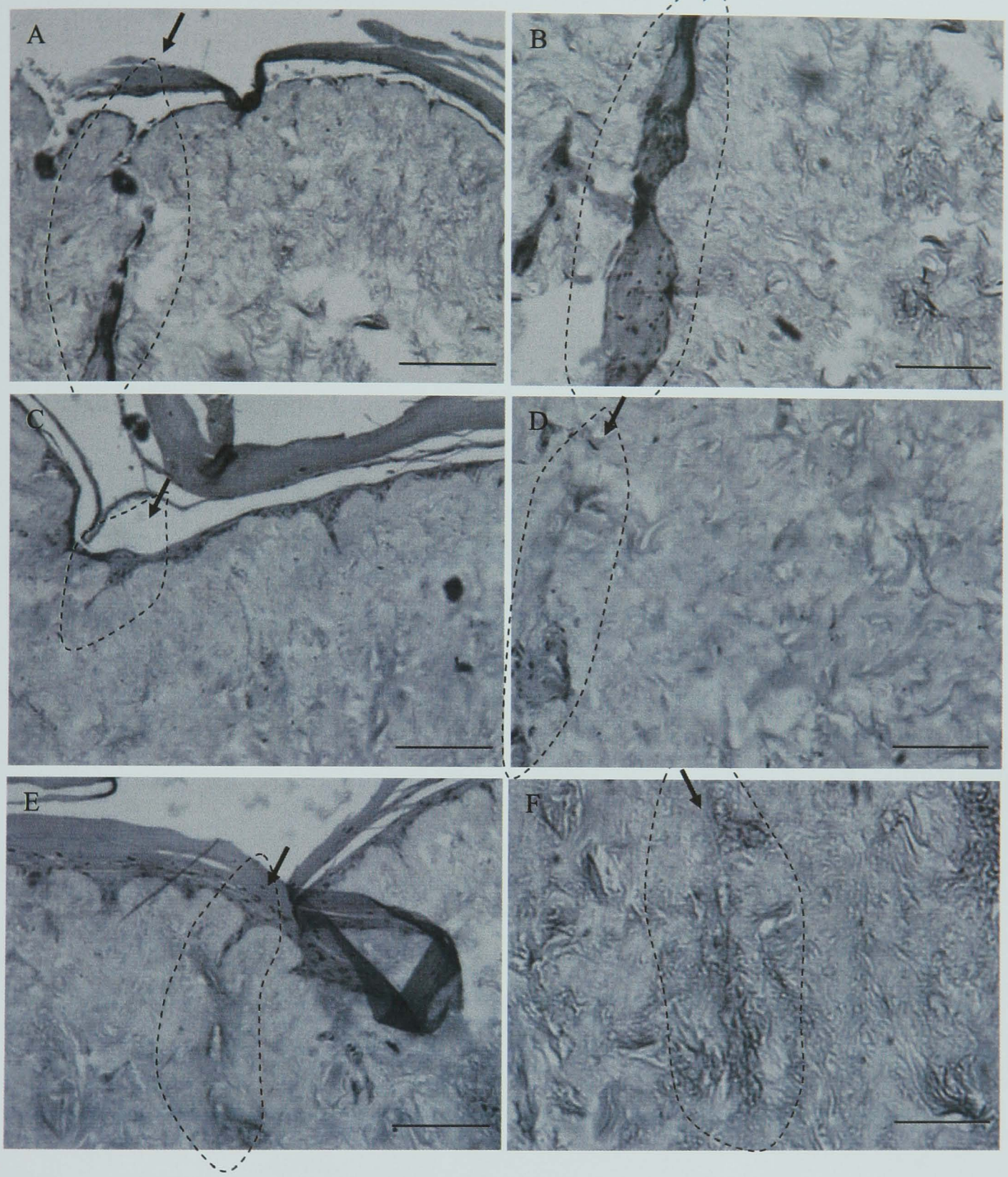


Figure 5.25. Wounded skin composites to which were added fibroblasts, keratinocytes, A375SM cells and fibrin clots. A, C and E show the top of each composite. B, D and F show the top-middle with the wound sites indicated by an arrow and a dotted line. Scale bars = 100 μm.

The histological results show that these three skin composites containing A375SM cells had achieved good healing and the site of the wounds can be seen as demonstrated in Figure 5.25. See Table 5.10 for a summary of these results.

Melanoma skin composites containing A375SM cells to which were added fibrin clots and TNF-α, demonstrated partially healed wounds. Histological observations of these wounds in these composites showed they were more evident than the ones without TNF-α. This may indicate that TNF-α delayed the wound healing process (Figure 5.26 A, B and C).

Wounded melanoma skin composites containing HBL cells to which were added fibrin clots and TNF-α, showed an inconsistent pattern of wound healing. These composites showed either a totally open wound in which the epidermal layer formed around the open wound edge (Figure 5.26 D) and a wound which had almost completely healed as shown in Figure 5.26 (E). A summary of these results are in Table 5.10

Table 5.10. Effects of fibrin clot and TNF-α in full thickness wounded skin composites containing melanoma cells.

Number of Experiments	F	Melanoma	K	Fibrin Clot	TNF-α	Open wound	Partially healed wound	Extensively healed wound
3	+	A375SM	+	+	-	0	0	3
3	+	A375SM	+	+	+	0	3	0
4	+	HBL	+	+	+	2	1	1

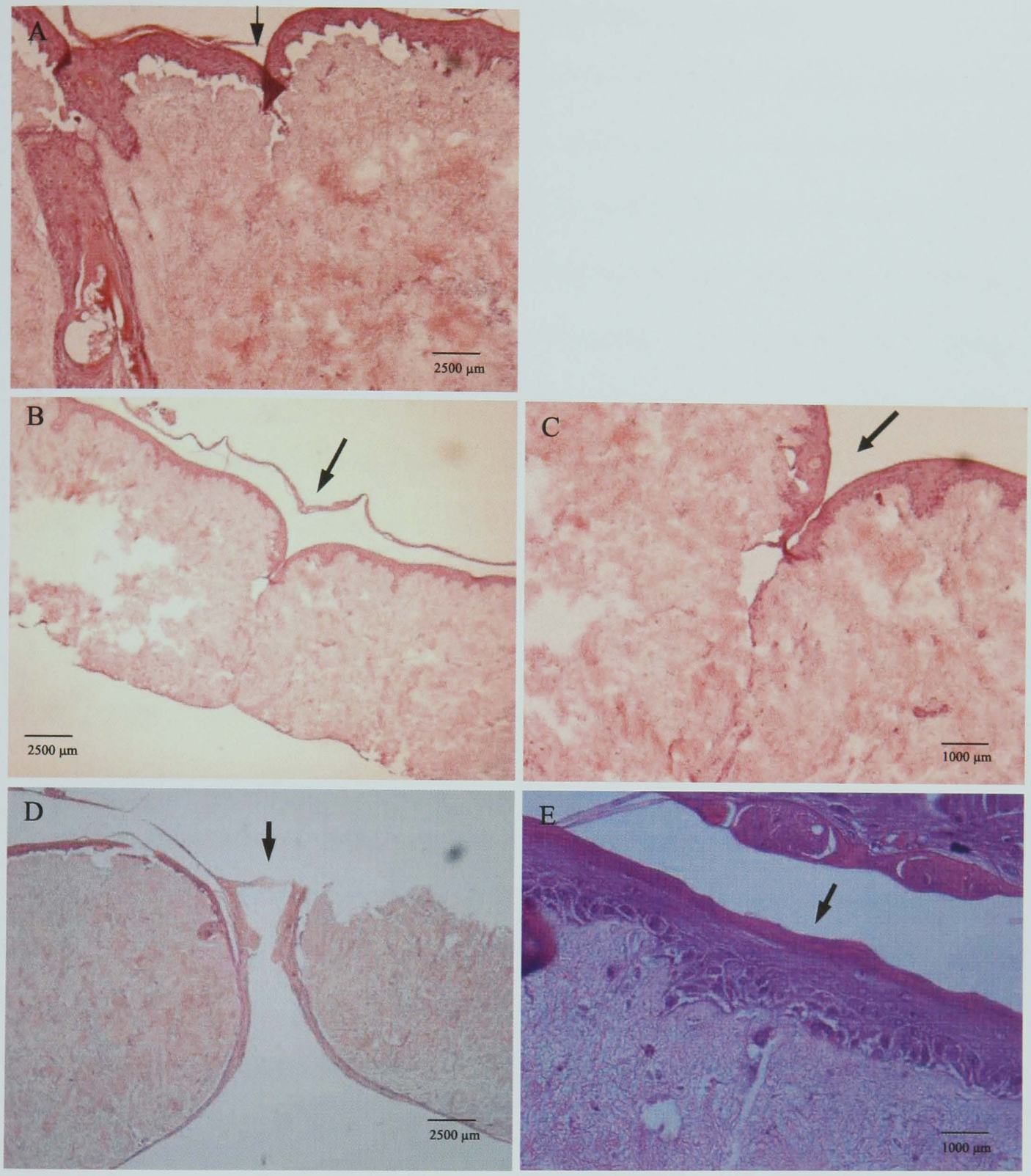


Figure 5.26. Wounded skin composites containing DED+F+K+ Melanoma cells to which were added fibrin clots and TNF-α. (A, B and C) Skin composites to which were added A375SM cells, fibrin clots and TNF-α showing partially healed wounds (10x, 4x and 10x magnification respectively). (D and E) Skin composites to which were added HBL cells, fibrin clots and TNF-α showing one open wound (D) and a wound which had almost completely healed (E) (4x and 10x magnification respectively). The site of the wound is indicated by an arrow.

5.5.5. Effect of NSAIDs on Wounded Melanoma Skin Composites

In these experiments, skin composites were produced by adding normal skin cells and C8161 melanoma cells. The wounds were made after 7 days of incubation at an ALI. A volume of 30µl hydrogel releasing ibuprofen sodium salt (NaIbu hydrogel) was added on the top of the wound site. Unloaded hydrogels and NaIbu hydrogel at concentration of 131µM were used. These composites were incubated for a further period of 7 days and were fed every 2-3 days with fresh media followed by a fresh topical application of NaIbu hydrogel. One skin composite to which was added skin cells and C8161 cells without any treatment demonstrated an almost healed wound (this served as a control sample). Both unloaded hydrogel and 131µM NaIbu hydrogel treatments on skin composites also showed healed wounds in these C8161 melanoma skin models. Figure 5.27 shows the histology of these skin composites and Table 5.11 shows a summary of the results for this experiment.

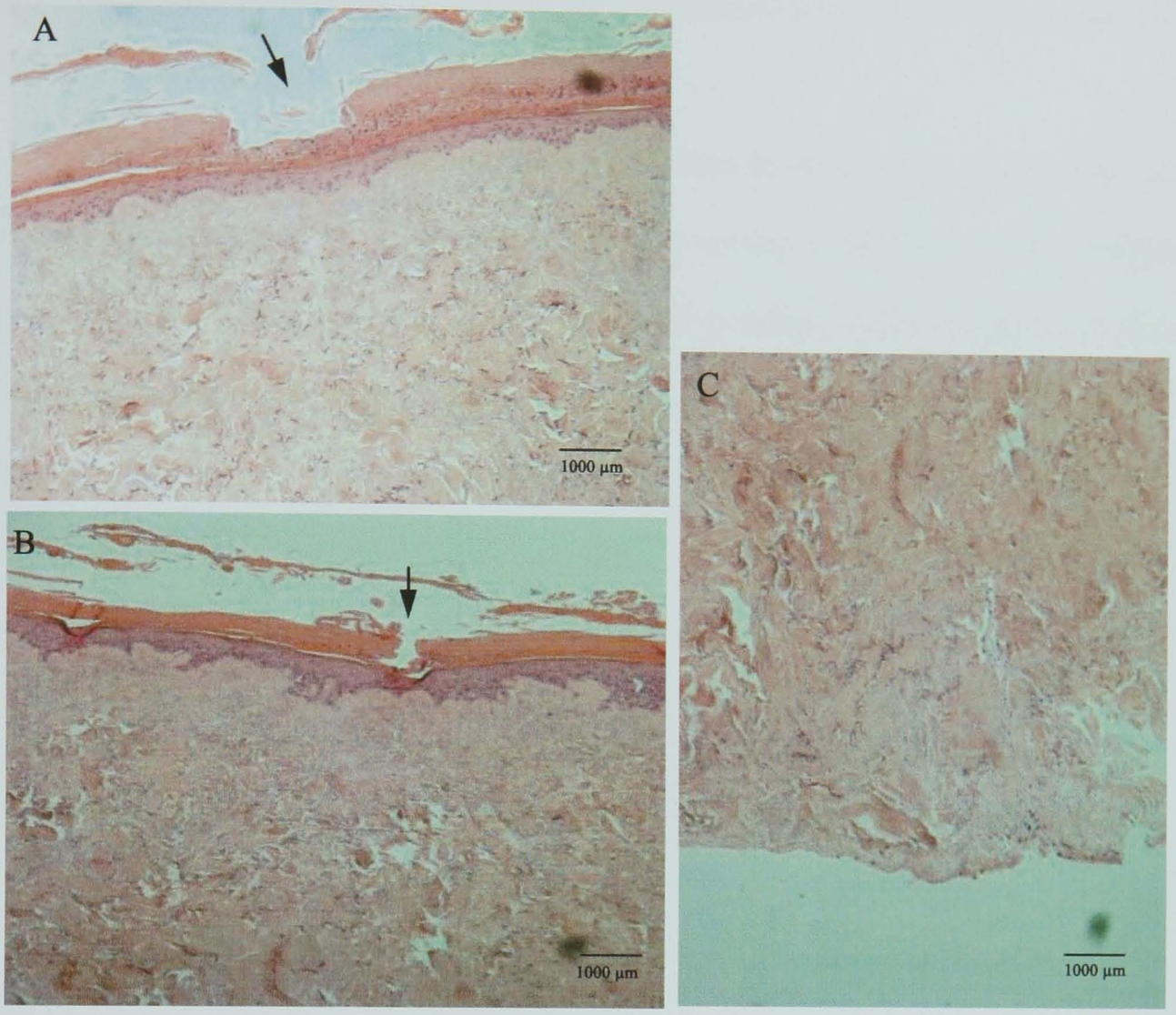


Figure 5.27. Effects of NaIbu hydrogel on wound healing of wounded melanoma skin composites. DED+F+K+C8161 (A). DED+F+K+C8161+unloaded hydrogel – top (B). DED+F+K+C8161+unloaded hydrogel – bottom (C). F+K+C8161+NaIbu hydrogel 131µM (D). 10x magnification. The site of the wound is indicated by an arrow

In the next experiments HBL melanoma skin composites were treated with capsaicin or NaIbu sodium salt, both added to media.

Figure 5.28 shows wounded skin composites to which were added HBL cells and different treatments with NSAIDs. Open wide wounds were found in these samples. Figure 5.28 (A and B) shown an open wide wound in media treated composites (control samples). The 40x magnification micrograph (B) showed some cells invading into the wound edges. Open wide wounds also occurred when 100μM capsaicin (in media) was added to the wounded composites (C and D), however there may be less cells invading into the wound edges. Wounded skin composites treated with 100μM NaIbu in media also demonstrated wide open wounds and some infiltrating cells on the wound edge (E and F). A summary of these results is shown in Table 5.11.

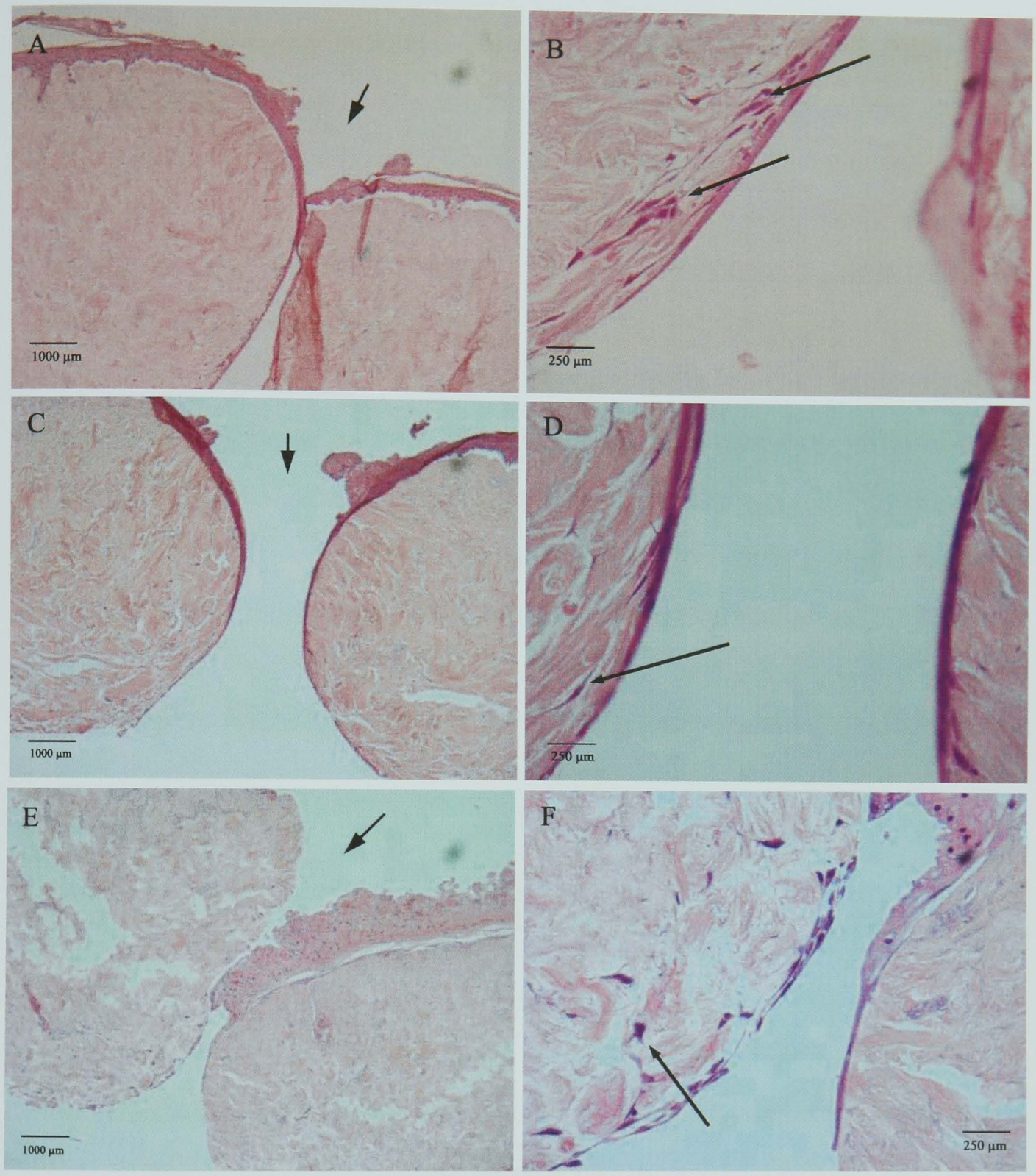


Figure 5.28. Wounded skin composites. DED+F+K+HBL cells (A and B). DED+F+K+HBL cells+ 100µM Capsaicin (C and D). F+K+HBL cells+100µM NaIbu in media (E and F). 10x and 40x magnifications respectively. The site of the wound is indicated by short arrow. Invading cells are indicated by long arrows.

Table 5.11. Effects of NSAIDs on wounded skin composites.

No. of Composites	Fibroblasts	Melanoma	Keratinocytes	Treatment	Wound
1	+	C8161	+	Control	Healed
2	+	C8161	+	Unloaded hydrogel	Healed
2	+	C8161	+	Nalbu hydrogel 131μM	Healed
2	+	HBL	+	Control	Open
2	+	HBL	+	Capsaicin 100μM	Open
2	+	HBL	+	Nalbu 100μM (in media)	Open

In the next set of experiments, skin composites containing A375SM cells were wounded on the 3rd day after being lifted to an ALI. These composites were fed with fresh media every 2-3 days, however some of these composites also received different treatments as follows:

1. Control – wounded A375SM skin composites treated with media only;
2. Treatment with fibrin clots plus TNF-α – to these composites, fibrin clots plus TNF-α (300U/ml) were added in the wound site at every feeding;
3. Treatment with fibrin clots plus TNF-α and a further treatment with Nalbu hydrogel – these composites were treated as previously described in item 2, and also received a further treatment with unloaded hydrogel or 100μM Nalbu hydrogel (See Figure 5.29).

All of these skin composites were ended on the 9th or 10th day after wounding.

Figure 5.29 (A) shows the A375SM skin composites after 2 days of wounding. It shows a full thickness wound made in all skin composites. Figure 5.29 (B) shows each

skin composite after treatment with fibrin clot plus TNF-α (300U/ml). The lower line of wells containing skin composites were the ones which also were treated with NaIbu hydrogel (100μM). Figure 5.29 (C) shows the skin composites at the end of the experiment (9^{th} day after wounding). One out of the three skin composites which was not treated with NaIbu hydrogel showed a visible open wound. However all three skin composites which were treated with 100μM NaIbu hydrogel showed apparent healed wounds.

Figure 5.30 shows the histological results of control wounded skin composites containing skin cells and A375SM cells. One of these composites had an open epidermal layer and cell invasion (See Figure 5.30 A, B and C). Another composite showed a disrupted epidermal layer, cell invasion and a small breakage of the lower part of the dermis (see Figure 5.30 D, E and F). These composites showed a poor quality dermal layer.

A375SM skin composites to which fibrin clots plus TNF-α were added to the wounds are shown in Figure 5.31. The histology results from this experiments confirmed one open wound and two healed wound composites which were not treated with NaIbu hydrogel. One of these skin composites showed a thicker epidermal layer when compared to the other two skin composites. In these three skin composites, the dermal layer was very poor quality.

A375SM skin composites to which were added fibrin clots plus TNF-α and a further treatment with the unloaded hydrogel showed healed composites but with extensive destruction of the lower part of the dermis. Figure 5.32 shows an example of these composites which had a very disrupted dermis and an apparently healed epidermis. However, it is worth observing that in this composite, cells invaded into the dermis and formed a "rounded mass" of cells at the bottom of the dermis (See Figure 5.32 C and D).

Figure 5.33 shows A375SM melanoma skin composites to which were added fibrin clots plus TNF-α followed by 100μM NaIbu hydrogel treatment. In these composites the wounds were healed and there was a thin but evident epidermal layer present. The dermis was also intact and there were no signs of broken regions. A summary of these results is shown in Table 5.12.

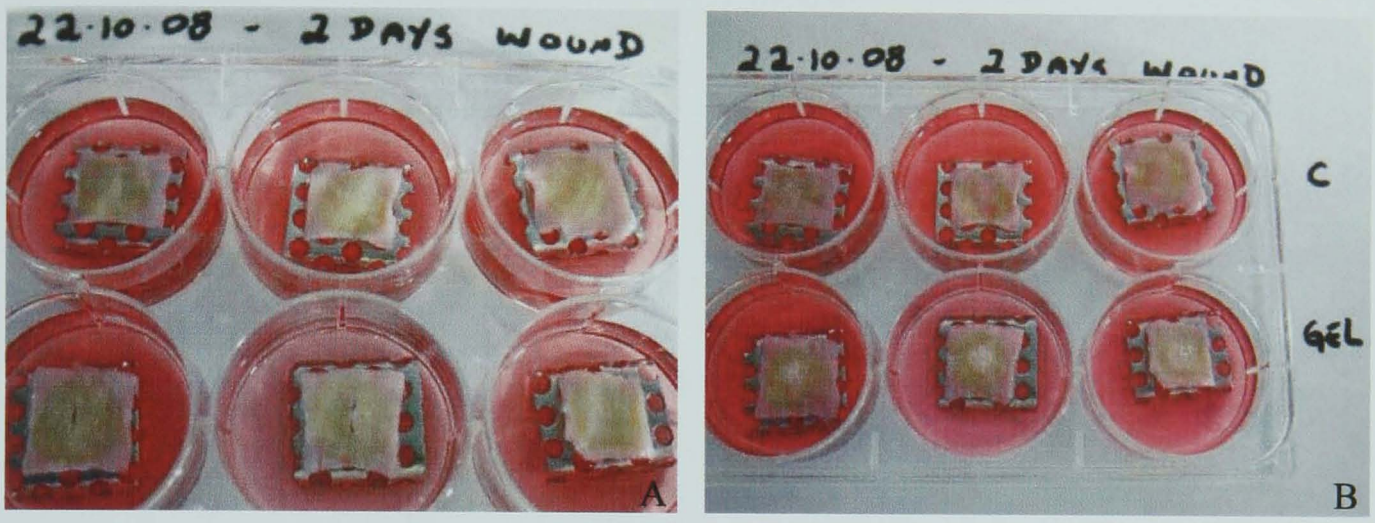


Figure 5.29. A375SM Melanoma skin composites to which were added with fibrin clots plus TNF-α (300U/ml) and a further treatment with 100μM NaIbu hydrogel. (A) NaIbu hydrogel treated skin composites after 2 days of wounding. (B) Each skin composite after treatment with fibrin clot plus TNF-α (300U/ml). Only the lower line of wells containing skin composites were the ones which also were treated with NaIbu hydrogel (100μM). (C) Shows the skin composites at the end of the experiment (9th day after wounding).

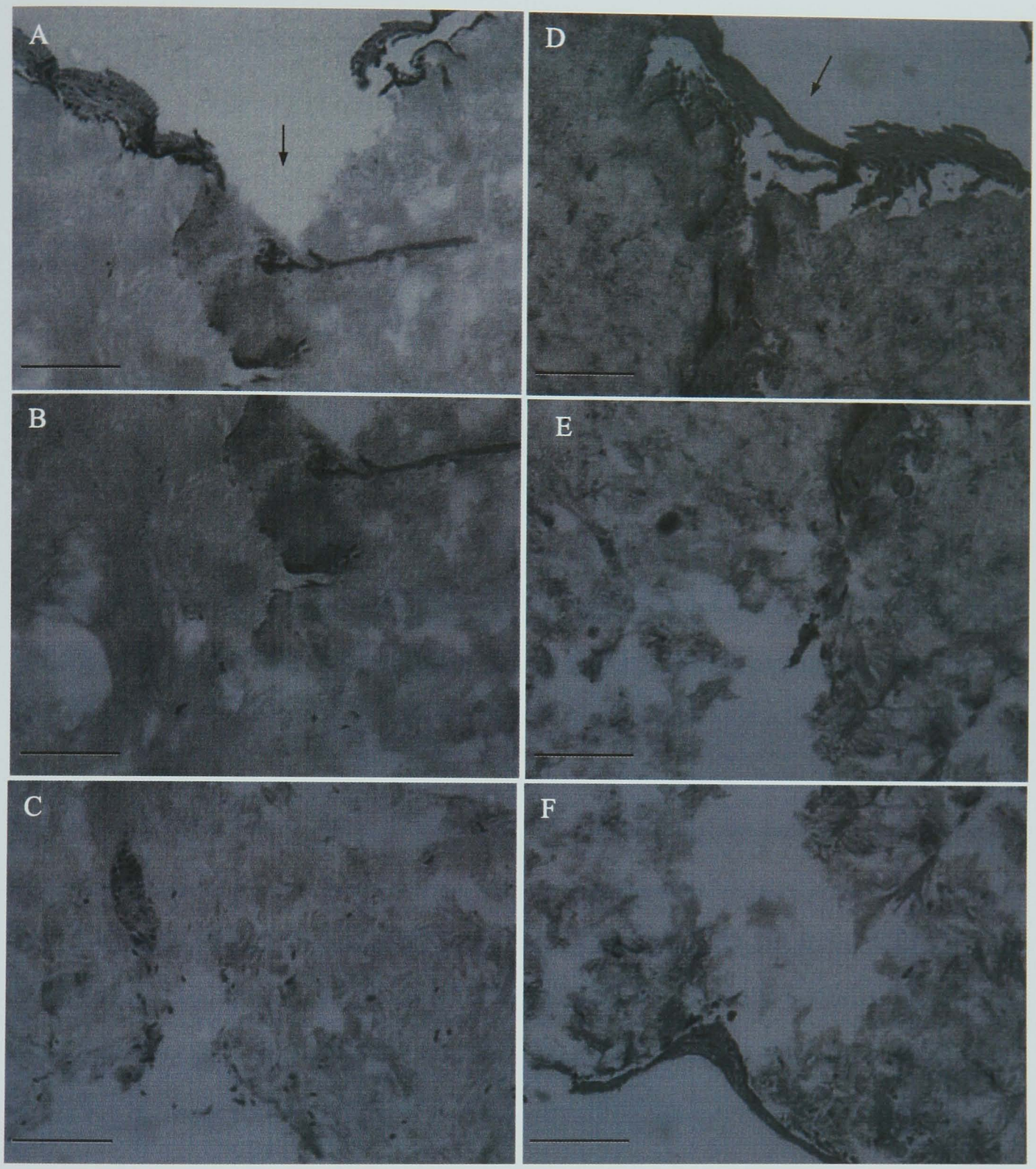


Figure 5.30. Wounded A375SM skin composites - controls. (A, B and C) = broken epidermis with healed dermis and cell invasion. (D and E) = disrupted epidermis with invasion of epidermis into the dermis. (F) = broken DED and invasion of cells. Wounds are indicated by an arrow. Scale bars = 100μm.

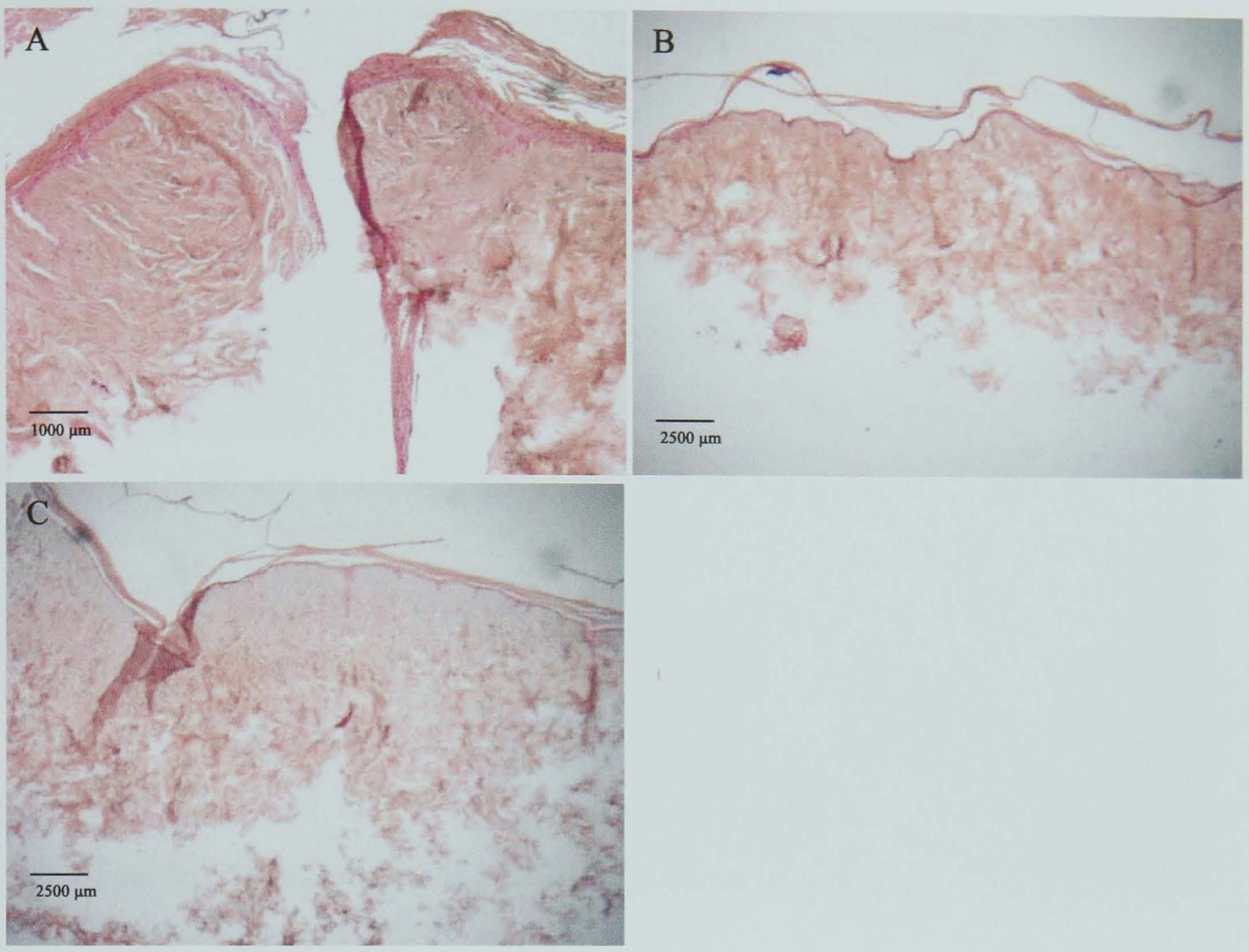


Figure 5.31. Wounded skin composites containing A375SM melanoma cells to which were added fibrin clots plus TNF-α (300U/ml). (A) Open wound (10x magnification), (B and C) healed wounds, damaged DED (4x magnification).

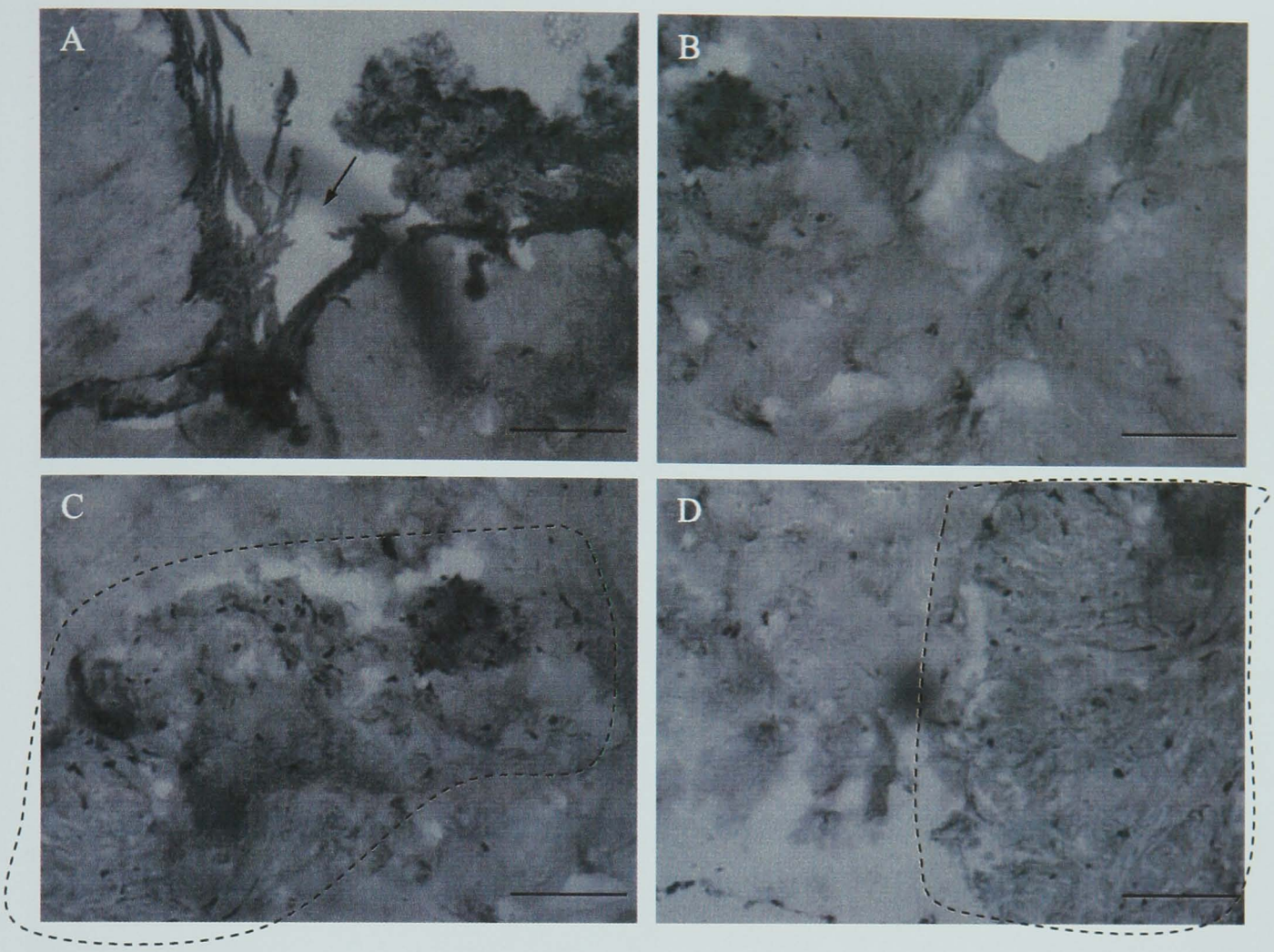


Figure 5.32. A375SM Melanoma skin composites to which were added with fibrin clots plus TNF-α (300U/ml) and a further treatment with unloaded NaIbu hydrogel (30µl volume). (A, top) = disrupted epidermis; (B, half of middle) = healed wound with invasion of cells; (C, lower middle) = formation of a mass of invasive cells, indicated by a dotted line. (D, bottom) = cell invasion (also indicated by a dotted line) and slight breakage of dermis. 5x magnification, scale bars= 100µm.

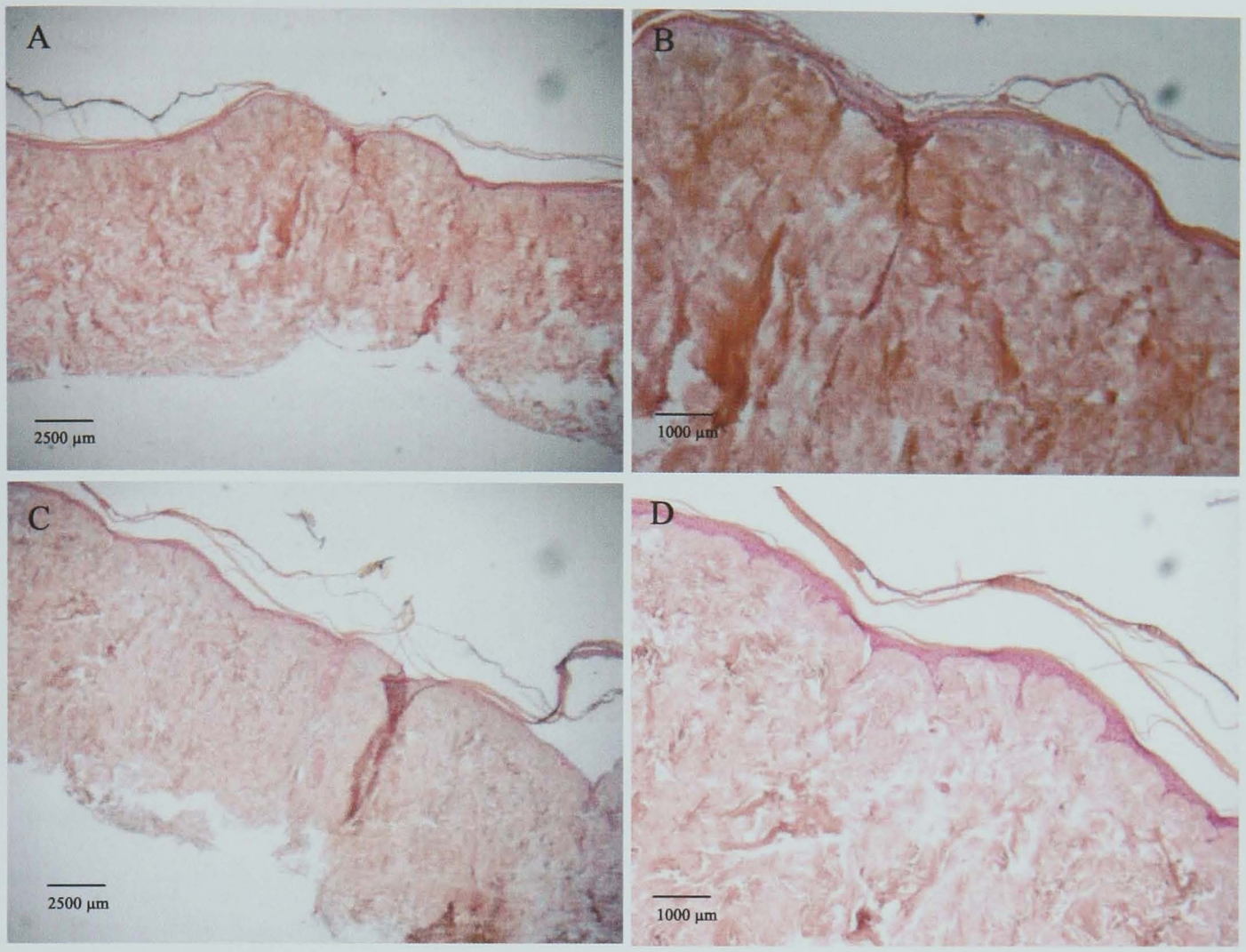


Figure 5.33. A375SM Melanoma skin composites to which were added with fibrin clots plus TNF-α (300U/ml) and a further treatment with 100µM NaIbu hydrogel (30µl volume). (A and C) 4x magnification; (B and D) 10x magnification.

Table 5.12. Effects of Nalbu hydrogel on A375SM cell wounded skin composites.

Treatment Description For A375SM wounded composites	Results	Total No. Composites
No treatment	1 partially healed wound and 1 healed wound but with destruction of the lower DED	2
Fibrin Clot+TNF-α	1 open wound and 4 healed but with destruction of the lower DED in all cases	5
Fibrin Clot+TNF-α Nalbu Unloaded Hydrogel Treated	2 healed but with some destruction of the lower DED in all cases	2
Fibrin Clot+TNF-α 100μM Nalbu Hydrogel Treated	5 healed good skin composites Good quality DED	5

A summary of these skin composites can be seen in Figure 5.34.

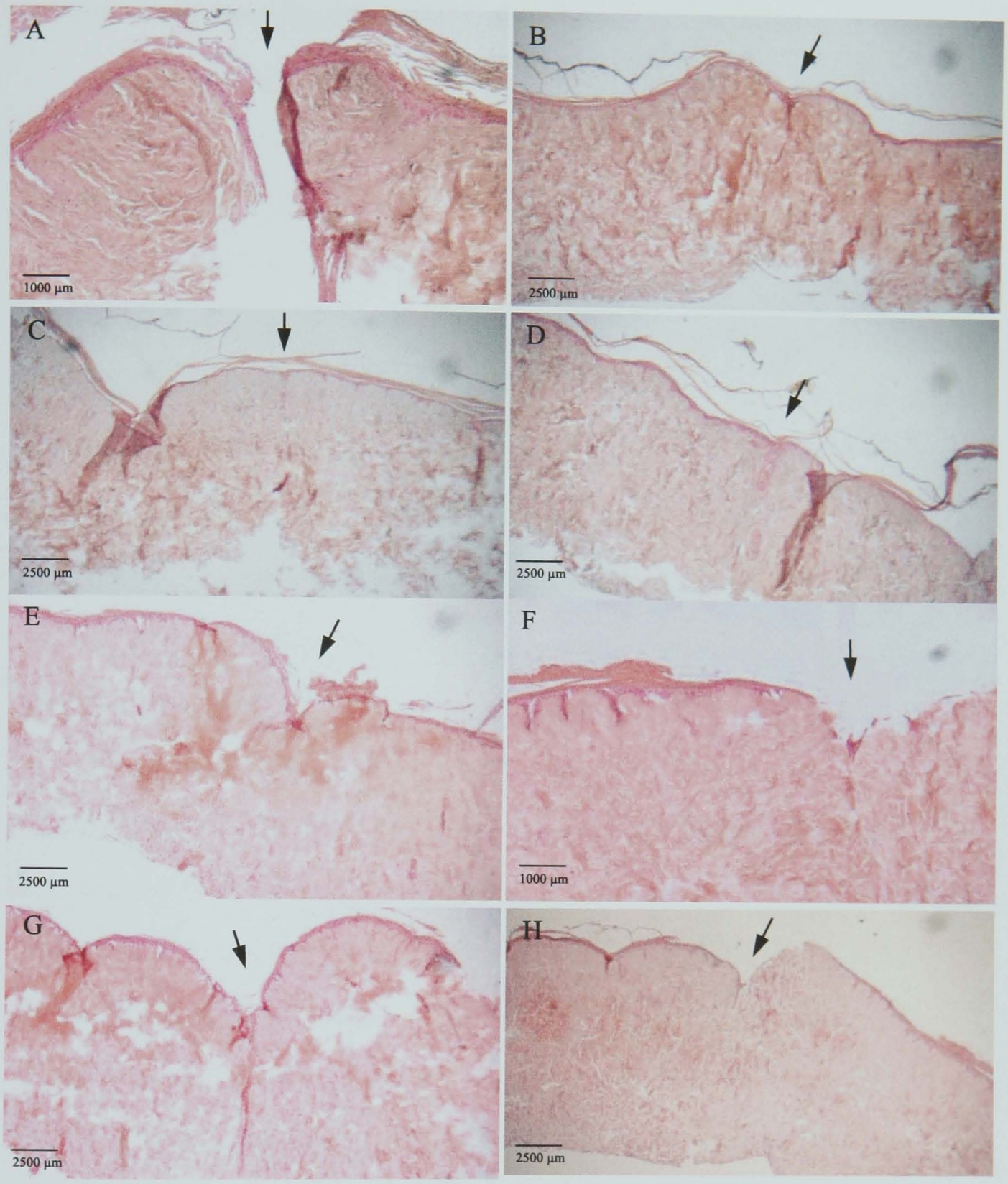


Figure 5.34. Effects of Ibuprofen on wounded melanoma skin composite model. All composites contained fibroblasts, keratinocytes and A375SM cells and fibrin clots plus 300 U/ml TNF-α (as in A and C). To E and G was added unloaded hydrogel. To B, D, F and H were added 100µM NaIbu hydrogel. B, C, D, E, G and H = 4x magnification; A and F = 10x magnification.

5.5.6. Effects of Wounding and of NSAIDs on Melanoma Cell Invasion

The effects of wounding and of NSAIDs on melanoma cell invasion are illustrated in Table 5.13. Melanoma invasion was scored in non-wounded and wounded skin composites to which were added Ibuprofen or Capsaicin.

Firstly, only one wounded standard skin composite was analysed for the invasion score of normal skin cells. There was no apparent invasion in a wounded skin composite. This invasion score was the same for the twenty-two non-wounded standard skin composites (mean invasion score = 0), previously demonstrated in this study. In three wounded standard skin composites to which were added fibrin clots and TNF-α (300U/ml), two scored 0 and one scored 2.5 (mean invasion score = 0.83). This high invasion score in one of these composites may have been due to infiltration of keratinocytes induced by TNF-α.

In six non-wounded skin composites containing HBL cells the mean score was 0.3 (see Table 5.13). In six wounded skin composites, to which were added skin cells and HBL cells, two scored 1.5, one scored 2, one scored 2.5 and two scored 3 (mean invasion score = 2.25) which was significantly higher than the non-wounded skin composites ($p<0.001$). This suggests that the wounds may have helped cell invasion into these composites. In two wounded skin composites containing HBL cells and TNF-α added in the media, one scored 0 and one scored 1 (mean invasion score 0.5). This was significantly lower compared to wounded HBL cell skin composites ($p<0.05$). The low invasion score shown by TNF-α in these composites was unexpected and TNF-α had previously been demonstrated to increased melanoma cell migration in the 2D model. A similar result was observed in wounded skin composites to which were added skin cells, HBL cells, fibrin clots and TNF-α. In six of these composites, three scored 0 and three scored 1 (mean invasion score = 0.5). This was also significantly lower compared to wounded HBL cell skin composites ($p<0.001$). The low invasion score shown by TNF-α

in these composites was unexpected as TNF-α had previously been demonstrated to increase melanoma cell migration in the 2D model.

Addition of 100μM Nalbu (in media) inhibited HBL cell invasion (mean invasion score = 0.75) ($p<0.05$) and, capsaicin (100μM) slightly (non-significantly) reduced cell invasion (mean invasion score = 1.83), relative to wounded HBL skin composites.

In nineteen non-wounded skin composites containing A375SM cells, the mean invasion score was 1.21 (see Table 5.13). In two wounded A375SM cells skin composites the mean invasion score was 2.5 (one scored 2, one scored 3). This showed that the mean invading score doubled in wounded skin composites, however this was not significant ($p>0.05$) relative to wounded A375SM cell skin composites. In three wounded skin composites to which were added A375SM cells and fibrin clots the invasion score was 0, suggesting that fibrin clots may have contributed to a faster healing and therefore the cells were not invading into the dermis ($p<0.001$) compared to wounded A375SM cell skin composites. On the other hand, in seven wounded skin composites to which were added skin cells, A375SM, fibrin clots and 300U/ml TNF-α, three scored 0, three scored 1 and one scored 3 (mean invasion score = 0.86). This demonstrates that when TNF-α was added to the fibrin clots in these composites, the invasion score was slightly increased compared to the ones treated with fibrin clot only. Cell invasion was reduced in wounded A375SM skin composites containing fibrin clot and TNF-α, however this was not significant ($p>0.05$) related to wounded A375SM cell skin composites.

In wounded A375SM skin composites to which were added fibrin clots plus TNF-α and a further treatment with the unloaded hydrogel the mean cell invasion score was 2.75 (one scored 2.5 and one scored 3). This was a similar level of invasion as demonstrated in wounded A375SM cell skin composites.

Interestingly, in wounded A373SM melanoma skin composites to which were added fibrin clots, 300U/ml TNF-α and a further treatment with 100μM NaIbu hydrogel, melanoma invasion was significantly lower as in five composites four scored 0 and one scored 1 (mean invasion = 0.2), than the control wounded A375SM cell skin composites ($p<0.001$) compared to wounded A375SM skin composites. In addition, 100μM NaIbu hydrogel treatment significantly reduced cell invasion compared to the unloaded hydrogel treatment ($p<0.001$). See Table 5.13, Figure 5.35 for a summary of these results.

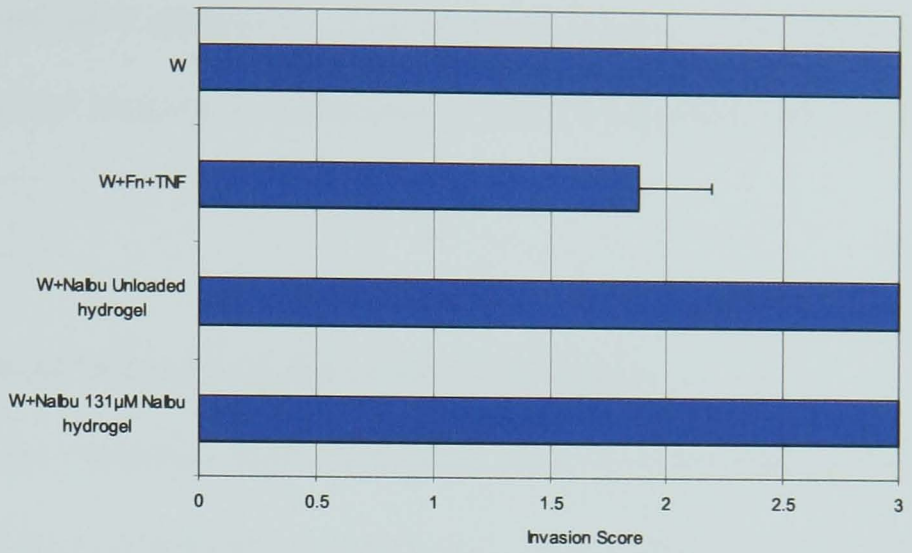
In 15 non-wounded skin composites containing C8161 cells the invasion score was 2.67. A wounded skin composite containing C8161 cells also had a high invasion score of 3. This invasion score of 3 was as expected as invasion in the non-wounded skin composites was already so high. In four wounded skin composites to which were added skin cells, C8161 cells, fibrin clots and TNF-α (300U/ml) one scored 1, two scored 2, and one scored 2.5 (mean invasion score = 1.88) as shown in Figure 5.33 and Table 5.13. In wounded skin composites to which were added C8161 cells, 30-50μl volume of unloaded hydrogel or 131μM NaIbu hydrogel were added to the wounded area. Both treatments had no effect on C8161 melanoma cell invasion in four composites, as a florid invasion occurred in all cases.

An overview of these skin composites invasion score is shown in Table 5.13 and Figure 5.35.

Table 5.13. Effects of NSAIDs on melanoma invasion in wounded skin composites.

Skin Composite	Total No.	Invasion Score							
		0	0.5	1	1.5	2	2.5	3	Mean Invasion Score
F+K	22	22							0
F+K+W	1	1							0
F+K+W+Fn+TNF-α [#]	3	2					1		0.83
F+K+HBL	6	4		2					0.3
F+K+HBL+W	6				2	1	1	2	2.25 ***
F+K+HBL+W+TNF-α (in media)	2	1		1					0.5 *
F+K+HBL+W+Fn+TNF-α	6	3		3					0.5 **
F+K+HBL+W+ Nalbu 100μM	2		1	1					0.75 *
F+K+HBL+W+Capsaicin 100μM	3				1	2			1.83
F+K+A375SM	19	7	2	1	2	2	2	3	1.21
F+K+A375SM+W	2					1		1	2.5
F+K+A375SM+W+Fn	3	3							0 **
F+K+A375SM+W+Fn+TNF-α	7	3		3				1	0.86
F+K+A375SM+W+Fn+TNF-α+Unloaded hydrogel	2						1	1	2.75
F+K+A375SM+W+Fn+TNF-α+Nalbu 100μM hydrogel	5	4		1					0.2 **
F+K+C8161	15			2		1		12	2.67
F+K+C8161+W	1							1	3
F+K+C8161+W+Fn+TNF-α	4			1	2		1		1.88
F+K+C8161+W+Unloaded hydrogel	2							2	3
F+K+C8161+W+Nalbu 131 μM hydrogel	2							2	3

[#] =Invasion of Keratinocytes; * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$.



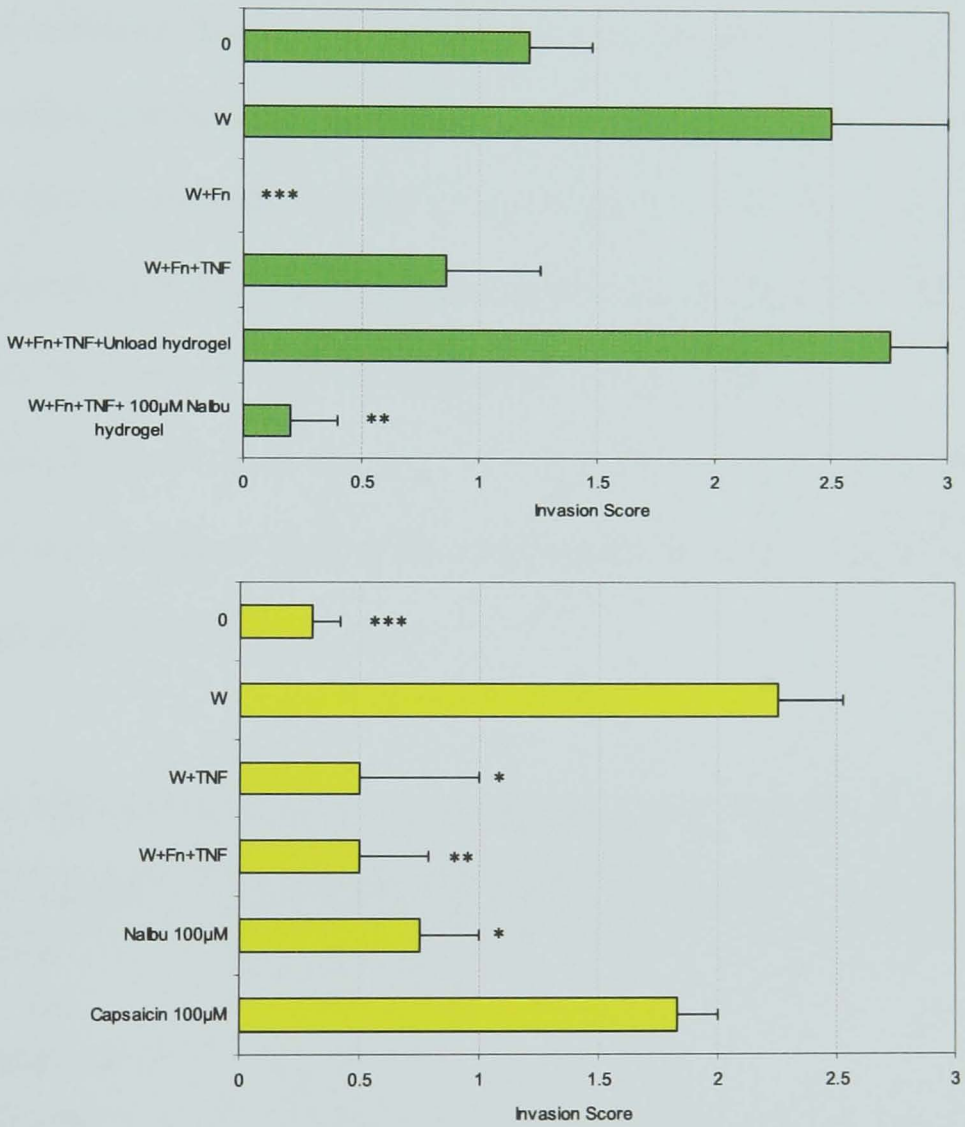


Figure 5.35. Differences in the extent of melanoma cell invasion in wounded skin composites and the different treatments used. All composites contained keratinocytes and fibroblasts but in addition (A) contained C8161 cells; (B) contained A375SM cells and (C) contained HBL cells. To the composites were added W=Wound, Fn=Fibrin clots, TNF=300U/ml TNF-α, NaIbu=Ibuprofen hydrogel as indicated. Values (bars) are expressed as Mean+SEM of invasion score. * indicates $p<0.05$; ** indicates $p<0.01$; * indicates $p<0.001$, relative to wounded skin composites.**

5.5.7. Effects of Melanoma Cells on Wound Healing and Dermal Quality

Wounded skin composites were analysed looking at the impact of wounding healing and dermal integrity in composites to which were added different melanoma cell lines.

5.5.7.1. Effects of Melanoma Cells on Wound Healing

For these, wounded skin composites were analysed looking at the impact of wounding healing in composites to which were added different melanoma cell lines and received no treatment. Initially this was observed in standard (control) skin composites. In two wounded standard skin composites, one healed and another did not. HBL skin composites did not to heal in 4 out of 4 composites while A375SM showed some healing characteristics as 2 out of 3 healed and the one C8161 skin composite showed good healing. See Table 5.14 for an illustration of these results.

Clearly however these numbers of composites are too low for real assessment. Therefore it was decided to look at the composites which had wounds (irrespective of whether they had fibrin, TNF-α or not.

Table 5.14. Effects of Melanoma Cells on Wound Healing (number of skin composites).

Skin Composite	No Healing	Intermediate Healing	Good Healing
Standard	1	0	1
HBL Cells	4	0	0
A375SM Cells	2	0	1
C8161 Cells	0	0	1

The impact of the melanoma cells on wound healing was then examined irrespective of the treatments used. These results are summarised in Table 5.15.

HBL skin composites proved to be the most difficult ones to heal as 15 out of 20 (75%) did not heal. A few of these skin composites (3 out of 20, or 15%) showed some intermediate healing and only 2 out of 20 (10%) showed good healing.

A375SM cells were slightly better at healing as 9 out of 20 (45%) healed, with an intermediate healing in 6 out of 20 (30%) whilst only 5 out of 20 (25%) did not heal.

For C8161 cells 7 out of 7 wounded composites healed completely (100%).

Table 5.15. Effects of Melanoma Cells on Wound Healing (number of skin composites).

	Skin Composites		
	HBL Cells	**A375SM Cells**	**C8161 Cells**
No Healing	15	5	0
Intermediate Healing	3	6	0
Good Healing	2	9	7

5.5.7.2. Effects of Melanoma Cells on Dermal Quality

The effects of melanoma cells on dermal quality was analysed by looking at damage of the dermis as a result of cell invasion. Table 5.16 illustrates these results.

Normal skin cells did not significantly affect the quality of the dermis in standard (control) skin composites.

The effects of melanoma cells on dermal integrity varied according to the melanoma cell type. In six non-wounded skin composites containing HBL cells, 2 showed a reduction in the quality of the dermis. Similarly 2 out of 6 composites post wounded had a dermal quality that was suboptimal. However, 6 out of 6 HBL skin composites to which were added fibrin clots and TNF-α showed a good dermis quality.

Wounding and the addition of Ibuprofen resulted in good dermal integrity in 2 out of 2 composites.

In nineteen non-wounded skin composites containing A375SM cells, 15 showed good a dermis while 4 had a less good dermis. Wounding was associated with a poor dermal integrity in 2 out of 2 composites. Wounding and the addition of fibrin clots plus TNF-α significantly reduced dermal quality in 5 out of 7 composites. Two A375SM skin composites treated with unloaded gel showed lower quality of dermis. Wounding and the addition of fibrin clots plus TNF- α and a further treatment with NaIbu hydrogel showed good quality of dermis in 5 out of 5 composites.

Interestingly, all 22 skin composites containing C8161 cells showed good dermal quality irrespective of wounding or TNF-α or Ibuprofen. See Table 5.16 for a summary of these results.

Table 5.16. Effects of Melanoma cells on dermal quality (number of skin composites).

		Control	Wounding	Wounding +Fibrin+TNF-α	Wounding +Ibuprofen	Total Number Composites
Standard Skin Comp	Good	20	1	2	Not done	26
	Reasonable	2	0	1	Not done	
HBL Cells	Good	4	4	6	2	20
	Reasonable	2	2	0	0	
A375SM Cells	Good	15	0	2	5	33
	Reasonable	4	2	5	0	
C8161 Cells	Good	15	1	4	2	22
	Reasonable	0	0	0	0	

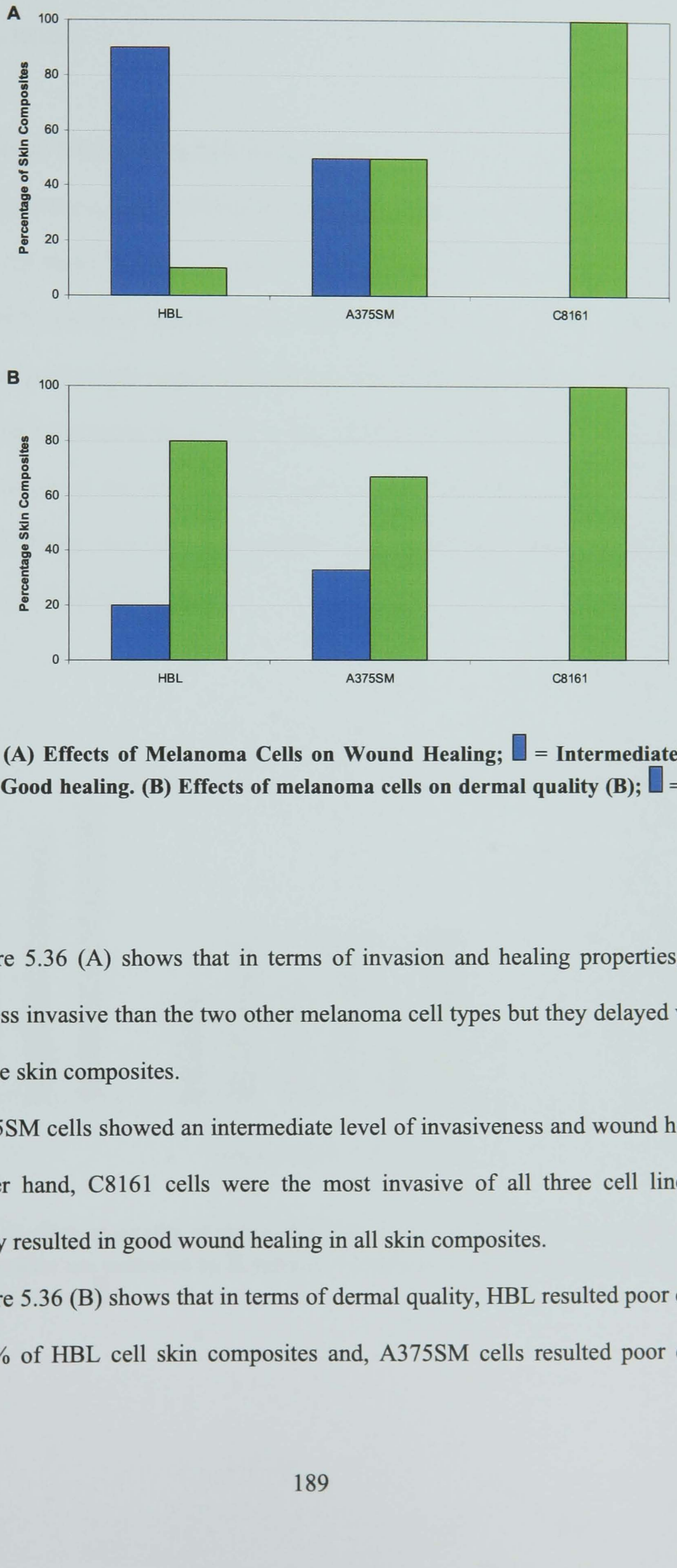


Figure 5.36. (A) Effects of Melanoma Cells on Wound Healing; ▮ = Intermediate or no healing, ▮ = Good healing. (B) Effects of melanoma cells on dermal quality (B); ▮ = Poor, ▮ = Good.

Figure 5.36 (A) shows that in terms of invasion and healing properties, HBL cells were less invasive than the two other melanoma cell types but they delayed wound healing in the skin composites.

A375SM cells showed an intermediate level of invasiveness and wound healing. On the other hand, C8161 cells were the most invasive of all three cell lines and unexpectedly resulted in good wound healing in all skin composites.

Figure 5.36 (B) shows that in terms of dermal quality, HBL resulted poor quality DED in 20% of HBL cell skin composites and, A375SM cells resulted poor quality

DED in 33% of A375SM cell skin composites whilst C8161 cells always demonstrated good quality DED.

5.5.8. Quality of DED used in Skin Composites

In this study a total of 208 skin composites were made based on DED from 22 individuals. Of these 192 skin composites were of good quality. 11 were lost due to infection and 5 were poor quality due to damage to the dermis. As it was possible that some of these poor results might have been due to the length of storage of the DED this was examined by plotting the quality of the DED (good, infected or clearly damaged) against the length of the time the DED was stored in the fridge (at 4°C) as shown in Figure 5.37. There was no clear pattern that would have been consistent with deterioration of DED with time.

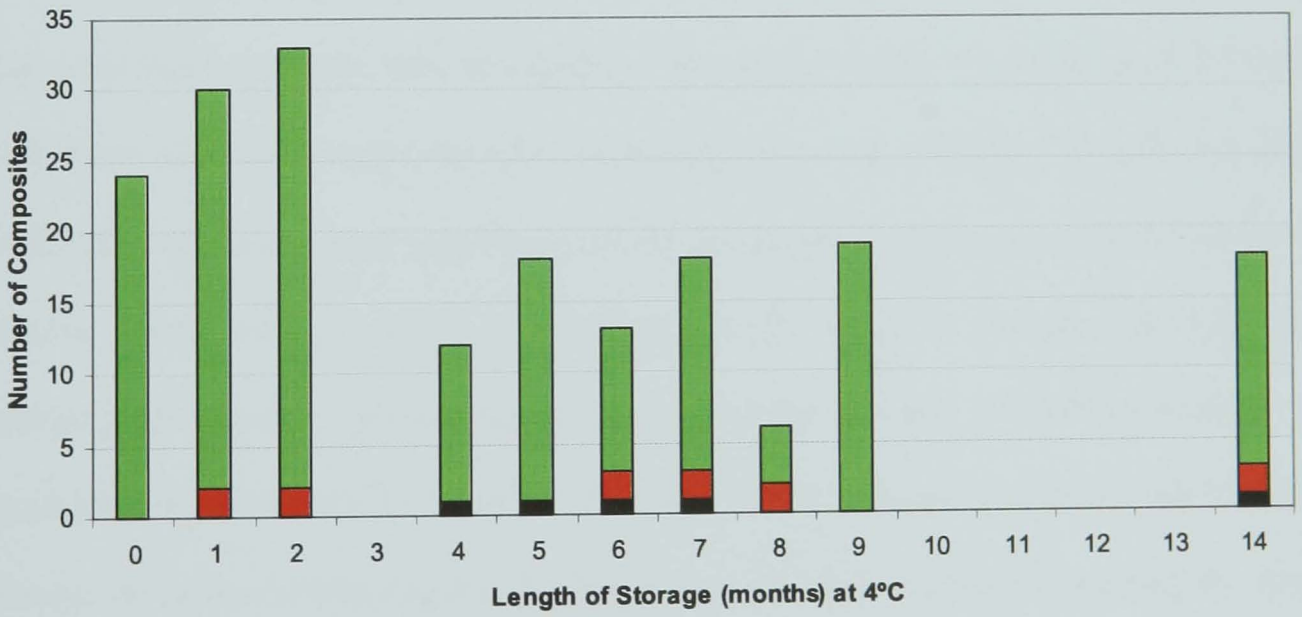


Figure 5.37. Analysis of quality of skin composites versus length of storage of DED. Good quality composites are indicated by ▯, infected composites by ▯ and composites with clear damage to the dermis ▮.

5.6. DISCUSSION

In this study, tissue engineered human skin was used to investigate the impact of wounding and inflammation on melanoma cell invasion. An initial comparison between skin composites made of sterilised and non-sterilised DED confirmed that non-sterilised DED produced skin composites identical to the sterilised DED. These were suitable to be used for the reconstruction of human skin in this work.

Tissue engineered human skin resulted in a normal skin structure which has a defined epidermal layer securely attached to the underlying dermis with a reasonable number of keratinocytes organising an epidermal layer ranging from basal cells with large nuclei to flat-shaped keratinocytes lacking nuclei. The dermis contained a relatively low density of cells identified as fibroblasts. This reconstructed skin has been previously demonstrated by Chakrabarty *et al.* (1999), Eves *et al.* (2000), Eves (2001) and Lee *et al.* (2003). However, the addition of melanoma cells resulted in a disrupted morphology of these reconstructed human skin.

The addition of HBL cells had slightly affected the organisation of the epidermal layer but there was very little disruption to the dermal layer. The addition of A375SM cell shows melanoma cells (identified by antibody staining for S100 cell marker) mainly within the epidermal layer which was poorly organised. There were also cell within the dermis which were identified as keratinocytes (by antibody staining AE1AE2 cell marker) not normally present in the dermis in the absence of melanoma cells. In contrast, the addition of C8161 cells led to an extensive invasion of these cells into the dermis. Very few of these were identified as keratinocytes by specific staining for these cells into the dermis. The residual epidermis was poorly organised.

Influence of skin cells on melanoma cell invasion

Tissue engineered composites were prepared so that they contained melanoma cells alone, melanoma cells and fibroblasts or melanoma cells and fibroblasts and keratinocytes. The extent of cellular invasion into the dermis was then quantified. In standard tissue engineered skin composites there was no evidence of epidermal cell invasion into the dermis, as expected.

The three melanoma cell lines used were previously known to have different invasion levels. This is in agreement with previous work by Eves *et al.* (2000). For HBL cells, these were moderately invasive into the dermis on their own but the presence of fibroblasts completely blocked their invasion and the presence of keratinocytes and fibroblasts slightly reduced their invasion.

A375SM cells were not very invasive on their own in the dermis and as observed with HBL cells, invasion was completely blocked by the addition of fibroblasts (unfortunately because of the large variability in the invasion of the cells on their own this was not significant). However, these cells were most invasive in the presence of keratinocytes and fibroblasts. Comparing the invasion seen in the presence of both skin cells to that seen in the presence of fibroblasts alone it is clear that the fibroblasts tended to reduce the less invasive cells (HBL and A375SM) invasion whereas keratinocytes tended to exacerbate it.

In contrast, C8161 cells were extremely invasive. The addition of fibroblasts had no significant effect on this neither did the addition of fibroblasts and keratinocytes. This melanoma cell line remained aggressively invasive in all experiments.

This model indicates that invasion through dermis is more difficult for certain melanoma cell lines in the presence of fibroblasts. Fibroblasts are the cells with the major responsibility for tissue repair. Thus fibroblasts repair of the dermis by synthesising new collagen or by also down-regulating MMPs (Matrix

Metalloproteinases) activation may make this a more difficult matrix for melanoma cells to invade through.

It is known that normal cells need contact with the ECM (Extra-Cellular Matrix) which regulates the cell cycle and mediates cell anchorage. The loss of these requirements is a hallmark of malignant cells. This idea is supported by the fact that integrin mediated contact between malignant cells and the ECM influences their behaviour (Goldbrunner *et al.*, 1996). For example, human M24met melanoma cells remain responsive to growth regulatory signals that result from contact with type I collagen and that effect on proliferation depends on the physical structure of the collagen. On polymerised fibrillar collagen, M24met cells are growth arrested at the G1/S checkpoint and maintain high level of $p27^{KIP1}$ mRNA and protein. In contrast, on non-fibrillar (denatured) collagen, the cells enter the cell cycle and $p27^{KIP1}$ is down regulated (Henriet et *al.*, 2000). Also, Iida *et al.* (2004) reported that MT1-MMP (Membrane Type 1 Matrix Metalloproteinase) in Bowes melanoma cells promoted selective invasion into matrigel but not matrices consisting of type-I collagen. Furthermore, MT1-MMP expressing melanoma cells exhibit increased migration in response to laminin 1 but not to type-I or type-IV collagen.

The addition of keratinocytes helped cell invasion into the dermis. One hypothesis for this is based on the fact that keratinocytes release soluble factors which inhibit fibroblast matrix production (Harrison *et al.*, 2005) and melanoma cells ruled the interaction between normal skin cells. An inflamed environment (with the addition of TNF-α) stimulates melanoma cells and keratinocytes to invade the dermis. Keratinocytes respond to the pro-inflammatory cytokine TNF-α, expressing integrins for cell adhesion and invasion (Franchi *et al.*, 2008).

Prior work from this group has shown that for some melanoma cells to invade in 3D reconstructed skin model the presence of fibroblasts and keratinocytes are required

(Eves *et al.*, 2000). Almost certainly the wound bed these cells create in modifying the dermal matrix with upregulation of matrix metalloproteinases to break down mature collagen is conducive to melanoma cell invasion. However it does not follow that the melanoma cells themselves upregulate degradative enzyme – another study from this group have shown that MMP-2 and MMP-9 are not upregulated in invading melanoma cells but in the surrounding keratinocytes and fibroblasts (Katerinaki *et al.*, 2003).

Therefore, from these results it is suggested that while fibroblasts synthesized components (collagen and matrix) for a stronger dermis, keratinocytes may have contributed to melanoma cell invasion.

Effect of mechanical wounding and addition of fibrin and TNF-α on wound healing in tissue engineered skin

This tissue engineered skin study is based on the clinical fact that when cutaneous malignant melanoma is excised surgically an inflammatory environment is created, as occurs at any injured tissue site. It has been suggested that local melanoma recurrence following excision of a primary melanoma may be initiated by the inflammatory environment at the excision site - establishing an environment that is favourable to neoplasia/metastasis. As discussed in Katerinaki *et al.* (2003), this has been demonstrated in animal models (Bogden *et al.*, 1997; Hofer *et al.*, 1998; Hofer *et al.*, 1999), where inflammatory mediators promote the growth, invasion and metastatic potential of human melanoma and other malignant tumour xenografts.

Initial attempts at wounding skin composites were done using a syringe needle. However, these composites showed to be impossible to observe signs of the wound sites in histology. Also, in many events, there were some infections in the tissue culture resulting in poor skin composites. In addition to this, the volume of fibrin clot and

hydrogel initially used covered much of the tissue surface area, contributing to unsuccessful skin composites.

However, full thickness wounds made with a single scalpel blade cut were more successful resulting in either open wound or less healed wounds, depending on the skin composite type and the cells added to it.

Simple experiments of wounded acellular DED showed that fibrin clots help wound healing and DED plus fibroblasts are essential for wound healing. From this it appears that a wounded model containing fibroblasts or fibroblasts plus fibrin has a good chance of healing. This is explained by the provision of temporary fibrin clot scaffold for cell migration and fibroblasts to synthesise new dermal extracellular matrix components, as demonstrated by Rizzi *et al.* (2010).

Standard skin composites showed an open wound with continuation of the epidermis around the wounded edge. Standard skin composites to which were added fibrin clots plus TNF-α surprisingly showed largely healed wounds with a continuous epidermis or evident dermal wound with continuous epidermal layer. In wounded skin composites, fibrin clots plus TNF-α were added in the wounds to imitate an inflamed healing process. Surprisingly fibrin clots plus TNF-α did not interfere with wound healing.

The impact of the melanoma cells on wound healing depended on the melanoma cell line. HBL cells added to the engineered skin proved to be the most difficult wounds to heal. A375SM cells added to engineered skin were slightly better with an intermediate level of healing. Surprisingly, C8161 cells added to engineered skin showed complete normal healing. In terms of invasion and healing properties, HBL cells were less invasive than the other melanoma cell lines but they clearly delayed wound healing in the skin composites. A375SM cells showed an intermediate level of invasiveness and wound healing. On the other hand, C8161 cells were the most invasive

of all three cell lines and unexpectedly resulted in good wound healing in all composites.

The slow healing process in wounded composites containing two of the three melanoma cells can be related to the unhealed wounds and cancer or to the ulceration present at wound sites. This is in fair agreement with a clinical report which showed a cutaneous malignant melanoma 4.6mm deep with signs of ulceration (Schöttler *et al.*, 2009). Also, according to the final version of melanoma staging and classification (Balch *et al.*, 2009), it was reported that patients with regional metastasis also had ulceration of the primary melanoma. Therefore, melanoma cells may have a role to play in deteriorating DED and maintaining a wound that does not heal *in vitro*, by the expression or activation of the MMPs. One possible explanation for C8161 cell invasion without influencing wound healing may be related to the expression of CD44 and hyaluronan on C8161 cells, as reported by Edward *et al.* (2005). However, the differences in melanoma cell invasion and healing process remains to be clarified.

Effects of Sodium Ibuprofen on Melanoma Cell Invasion

Initially experiments using ibuprofen and capsaicin added to non-wounded skin composites used high concentrations and therefore no cell attachment to the dermis was found. Lower concentrations gave better results on skin composites.

The interaction between melanoma cells invading into wounded skin composites and the impact of adding ibuprofen was complex. HBL cells appeared to be in the dermis following wounding however, following the addition of ibuprofen gel there was less invasion and growth of cells in an unhealed wound.

With respect to A375SM cells these were very moderately invasive without wounding and more invasive following wounding. The addition of ibuprofen gel in wounded A375SM skin composites showed reasonable healing and less invasion. Also

very strikingly, although A375SM cells invasion caused damage in the dermis, this was very effectively blocked by the addition of ibuprofen.

With respect to C8161 cells, in non-wounded and wounded engineered skin there was still extensive invasion despite good wound healing and ibuprofen had no input in this.

Topical treatment with ibuprofen has been successfully developed as a therapeutic approach to the reduction of wound pain in patients with chronic, exudating leg ulcers (Sibbald *et al.*, 2007; Price *et al.*, 2007). However, there are no reports on the effects of ibuprofen as a topical treatment in pre- and post-surgery primary melanoma removal. While further studies on the effects of topical ibuprofen still remains to be elucidated, there have been some approaches on the use of systemic COX inhibitors in clinical trials, such as Pioglitazone and Rofecoxib combined with Trofosfamide in the treatment of far-advanced melanoma and soft tissue sarcoma (Reichle *et al.*, 2004) and, Treosulfan chemotherapy in combination with Rofecoxib (Spieth *et al.*, 2003). This result is in fair accordance with Lejeune *et al*, (2006), who demonstrated a case study on a patient with a metastatic melanoma of the leg who experienced a complete and sustained regression of skin metastases upon continuous single treatment with the cyclooxygenase-2 inhibitor rofecoxib.

Quantitation of the effects of wounding and inflammation on melanoma cell invasion in tissue engineered skin

This analysis is based on a large number of experiments looking at the impact of wounding and the pro-inflammatory cytokine TNF-α and ibuprofen on melanoma invasion into tissue engineered skin. HBL cells were weakly invasive but much more cells are present in the dermis following mechanical wounding. Paradoxically this was reduced by the addition of TNF-α or fibrin plus TNF-α but the addition of sodium

ibuprofen clearly reduced, by approximately 50%, the extent of invasion seen in response to wounding.

A375SM cells are moderately invasive cells. Invasion was slightly increased following wounding but was reduced by the addition of fibrin. Addition of TNF-α slightly reduced cell invasion. The addition of a hydrogel carrier did not reduce invasion which was very high in these wounded composites but when the hydrogel contained sodium ibuprofen this invasion was massively decreased.

Finally, C8161 cells showed extensive invasion pre-wounding and post wounding. This was not changed by the addition of fibrin plus TNF-α or the addition of ibuprofen. Essentially these cells remained aggressively invasive under all conditions.

Effects of melanoma cell on dermal integrity

The effects of melanoma cells on dermal integrity was analysed by looking at damage of the dermis as a result of cell invasion. Normal skin cells did not significantly affect the quality of the dermis in tissue engineered skin. The quality of the dermis did not seem to be much affected by wounding and the addition of fibrin clots and TNF-α.

The effects of melanoma cells on dermal integrity were then analysed. All experiments were used for this analysis except for those with ibuprofen. The effects of melanoma on dermal integrity varied according to the melanoma cell type. HBL cells reduced the quality of the dermis in 4 out of 20 skin composites. A375SM cells reduced the quality of the dermis in 11 out of 33 skin composites whilst composites containing C8161 cells resulted in good dermal quality at all cases.

Similarly in the presence of wounding and the addition of fibrin and TNF-α HBL cells or A375SM cells reduced the quality of the dermis.

In all cases the addition of ibuprofen resulted in good quality dermis. Surprisingly C8161 cells showed good quality of dermis under all circumstances.

Although all the DED samples used in these experiments were not sterilised, there was no indication of deterioration of DED with storage over 14 months. This excluded the possibility that poor dermal integrity was due to any storage problem with the DED.

These results confirm and extend previous findings from the Kroto Institute. They illustrate the complexity of melanoma cell interactions with skin cells and the differences in invasion, wound healing and dermal integrity achieved with different melanoma cell lines. They provide further evidence for a wound healing environment being conducive to melanoma invasion but encouragingly they also provide evidence that the local addition of a major anti-inflammatory cytokine such as sodium ibuprofen can attenuate this invasion. These are in fair agreement with many other studies (Balkwill and Mantovani, 2001; Coussens and Werb, 2002; Thun *et al.*, 2002; Balkwill, 2004; Harris *et al.*, 2005; Balkwill and Coussens, 2004, Harris, 2007; Hussain and Harris, 2007; de Souza Pereira, 2009).

Conclusions

These results extend our previous studies and confirm that melanoma cells interact with skin cells in the extent to which they invade into the skin but this relationship appears to be dictated by the melanoma cells. Although there was some variability in the experiments, some melanoma cells become more aggressively invasive in the presence of skin cells, for others, the skin cells reduce the invasion of the melanoma cells.

These results also show for the first time that fibroblasts on their own tend to inhibit the invasion of some melanoma cell lines. This was found for the two least invasive melanoma cell lines. It is possible that fibroblasts tend to repair any damage to the dermis. Mechanical wounding increased invasion of the two least aggressive

melanoma cell lines. The addition of TNF-α and fibrin also paradoxically tended to reduce the invasion of these melanoma cell lines. Possibly this also tended to reduce damage to the dermis.

Finally ibuprofen significantly reduced invasion of melanoma cells in this complex wound model. This was seen for HBL cells where the extent of the invasion following wounding was reduced by at least 50% by the addition of 100μM sodium ibuprofen. It was also seen for A375SM cells. Invasion of these cells was associated with considerable degradation of the underlying dermis. This was very much reduced by the use of a hydrogel releasing ibuprofen with invasion reduced from around 2.75 to 0.25. Unfortunately ibuprofen was without effect on the invasion of the very aggressive C8161 cells in this model.

CHAPTER 6

INFLAMMATORY CYTOKINE TNF-α ACTIVATION OF THE NUCLEAR TRANSCRIPTION FACTOR NF-kB IN MELANOMA CELLS

6.1. EFFECTS OF TNF-α ACTIVATING THE NUCLEAR TRANSCRIPTION FACTOR NF-KB IN MELANOMA CELLS

The next experiments set out to investigate what concentrations of TNF-α lead to activation of the pro-inflammatory NF-kB transcription factor in melanoma cells.

The major pro-inflammatory cytokine TNF-α was used to activate the transcription nuclear factor NF-kB. In a 2D model of migration, melanoma cells were exposed to 300U/ml TNF-α for 24 hours. The same concentration was used in media used in 3D tissue engineered skin for the same purpose.

Figure 6.1 shows photographs of melanoma cells exposed to the inflammatory effects of TNF-α. As it can be observed, melanoma cells were responding to the 500U/ml TNF-α stimulus and activated the nuclear transcriptor factor NF-kB which was translocated from the cytosol to the cell nucleus, indicated by the green fluorescent dye FITC.

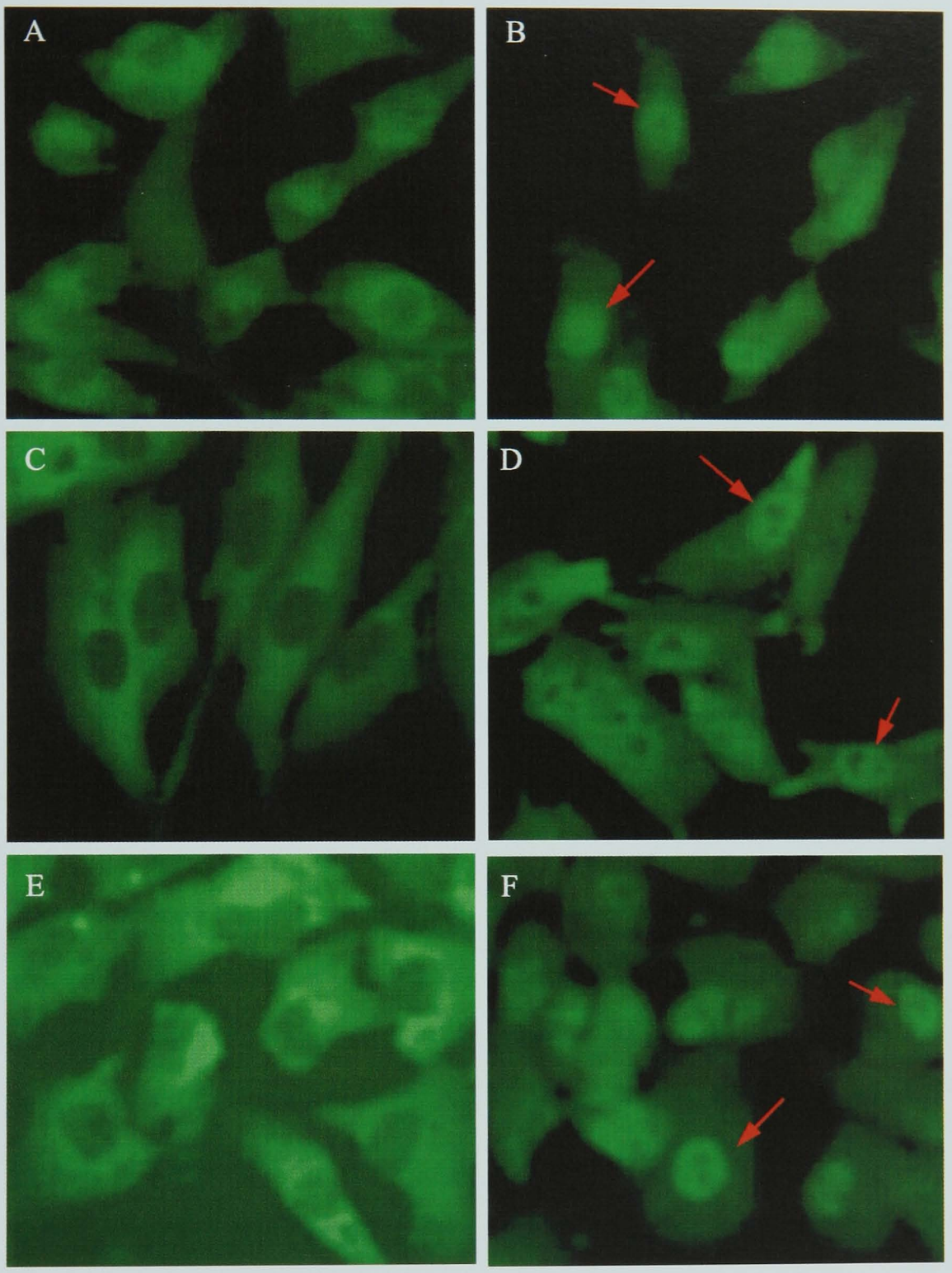


Figure 6.1. Micrographs of melanoma cells exposed to 500U/ml TNF-α for 1.5 hours and the activated nuclear transcription factor NF-kB. HBL cells (A) control, (B) TNF-α stimulated; A375SM cells (C) control, (D) TNF-α stimulated; C8161 cell (E) control and (F) TNF-α stimulated. Examples of NF-kB (FITC staining) activated cells are indicated by a red arrow.

Figure 6.2 illustrates the effects of TNF-α in stimulating the nuclear transcription factor NF-kB in the three melanoma cells lines.

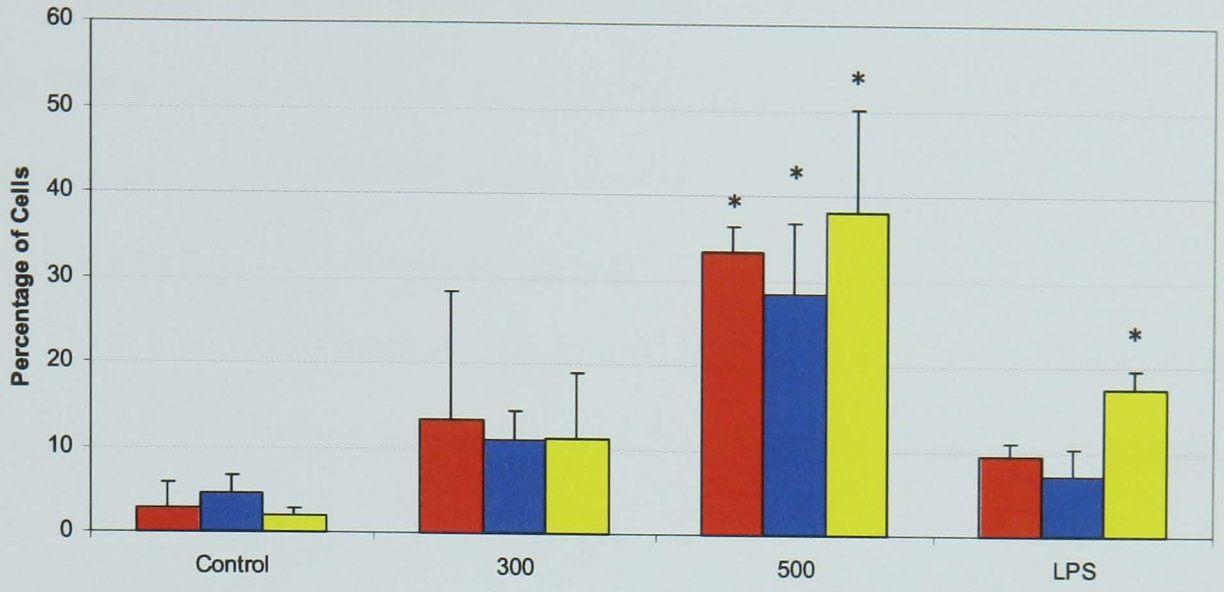


Figure 6.2. NF-kB p65 gene stimulated by TNF-α (300 and 500 U/ml) for 1-2 hours in melanoma cells. ▮ = C8161, ▮ = A375SM, ▯ = HBL cells. 100 U/ml LPS was used as a positive control. Values are expressed as Mean +SEM. N=3.

In these experiments (Figure 6.2) it was demonstrated that 300U/ml TNF-α slightly increased the activation of NF-kB transcription factor. However, 500U/ml TNF-α significantly increased NF-kB activation in C8161, A375SM and HBL cells ($p<0.05$). These melanoma cells were exposed to TNF-α for a period of 1-2 hours. Although 300U/ml TNF-α only slightly increased NF-kB activation, this was probably enough to mimic an inflammatory microenvironment either in the 2D melanoma migration and 3D tissue engineering skin models used in this study.

100 U/ml LPS was used as a positive control, and also slightly increased NF-kB activation in C8161 and A375SM cells whilst HBL cells were significantly affected ($p<0.05$).

6.2. DISCUSSION

In this study, two different TNF-α concentrations (300 and 500U/ml) were demonstrated to activate the nuclear transcription factor NF-kB in melanoma cells.

Although 300U/ml TNF-α only slightly increased the NF-kB activation, the same TNF-α concentration was enough to demonstrate an increase in melanoma cell migration and viability in a 2D model of migration and to increase viability in HBL cells, the least aggressive melanoma cell line.

It is known that TNF-α induced activation of NF-kB activity is linked to the upregulation of integrins and ICAM-1 expression which facilitates melanoma cell attachment. invasion (Zhu *et al.*, 2002) and proliferation (Kim *et al.*, 2006).

Although 300U/ml TNF-α only slightly increased activation of NF-kB, it was related to an increase of dermal degradation in melanoma wounded tissue engineered skin. The effect of TNF-α in attenuating melanoma cell invasion in wounded tissue engineered skin may be due to the simultaneous addition of TNF-α and fibrin clots which were demonstrated to help to heal the wounds. However it seems that this low TNF-α concentration was associated with some degree of melanoma cell invasion, which was attenuated with the administration of ibuprofen in wounded tissue engineered skin. Therefore, a slightly inflamed wound bed was created for the investigation of melanoma cell invasion which was demonstrated in Chapter 5.

These results confirm that the pro-inflammatory cytokine TNF-α activated the nuclear transcription factor NF-kB in melanoma cells. This created an inflamed microenvironment that is known to upregulate integrins expression (Zhu *et al.*, 2002). This is essential for intercellular membrane adhesion for lymphocyte interaction such as that which occurs during inflammation. Therefore, NF-kB activation is the central indicator of cellular responses to inflammation. Similarly, this scenario involves facilitating melanoma cell migration/invasion and survival.

NF-kB is a genetic intracellular transcription factor which regulates the pre-synthesis of genes involved in the inflammatory and immune response. If NF-kB is inactivated it is located in the cell cytoplasm, however it can be rapidly activated if

exposed to inflammatory cytokines such as TNF-α, lipopolysacharide (LPS), ultraviolet light or viral infection. The activity of NF-kB is tightly regulated by its interaction with inhibitory IkB proteins. This interaction blocks the ability of NF-kB to bind to DNA and results in the NF-kB complex being primarily localized in the cytoplasm due to strong nuclear export signal in IkBα. Therefore following exposure to the inflammatory cytokines, the NF-kB signaling cascade is activated, leading to the complete degradation of the IkB. This allows the translocation of unmasked NF-kB to the nucleus where it binds to the enhancer genes and regulates their transcription.

In this study, a major pro-inflammatory cytokine TNF-α was used to activate NF-kB in melanoma cells either in a 2D (Zhu *et al.*, 2002; Katerinaki *et al.*, 2006) wound model of migration and in a 3D tissue engineered skin model. Measurement of NF-kB activity was possible in tissue engineered skin as an extremely sensitive indicator of inflammatory stress (Tao *et al.*, 2004). As mentioned before in the Introduction Chapter, TNF-α is a major mediator of inflammation that orchestrates a series of immune and stromal cell responses necessary for the destruction of damaged cells but also for the repair of tissues during wound healing. Its production has been demonstrated in a number of human epithelial and haematological malignancies and it appears that both the protein itself and its receptor can be expressed in both the malignant and/or the stromal cells, suggesting potential autocrine and paracrine actions.

In this study, melanoma cells were exposed to TNF-α for 1 to 2 hours and this also showed NF-kB activation by the inflammatory stress. The transcription factor NF-kB is known to be activated within minutes after stimulation with TNF-α cytokine. The ability of NF-kB to be activated by inflammatory cytokines and to regulate genes involved in inflammatory function links the idea of inflammation to disease progression.

Evidence from previous studies suggests that TNF-α may be acting via up-regulation of NF-κB and integrin expression (Zhu *et al.*, 2002; Cantón *et al.*, 2003;

Katerinaki *et al.*, 2006; Banno *et al.*, 2004; Pikarsky *et al.*, 2004). Both normal and tumour cells express integrins, which are cell surface molecules that are involved in attachment to the extracellular matrix, motility and degradative enzyme production in malignant cells (van Kemper *et al.*, 2006).

NSAIDs or glucocorticoids block NF-kB activation and they can be used for long term administration. Thus, the long-term use of NF-kB inhibitors is a valid strategy for a variety of human disease (Baldwin, 2001).

Although the inflammatory cytokine TNF-α was used for the expression of the transcription factor NF-kB as the basis of the experiments on the effects of melanoma cell migration, invasion and viability, this final part of the discussion is focused on the link between the cytokine TNF-α in facilitating melanoma cell survival.

Studies point to a correlation between the activation of cellular gene expression by Rel/NF-kB factors and their participation in the malignant process (Rayet and Gélinas, 1999). Successful efforts in the development of small molecule inhibitors of NF-kB (Conner *et al.*, 1997) provide a logical basis for an adjuvant approach in cancer therapy to inhibit NF-kB and its associated anti-apoptotic function (Wang *et al.*, 1999; Sarkar *et al.*, 2008; Sánchez-Duffhues *et al.*, 2009).

Some studies reported that TNF-α can mediate either apoptotic or anti-apoptotic effects (Aggarwal, 2003; Aggarwal *et al,* 2004; Pikarski and Ben-Neriah, 2006; Lin and Karin, 2007). However, a clinical study already suggests TNF-α blockade as an intervention to improve chemotherapy in cancer patients (Monk *et al.*, 2006) and for eradication of the primary causes of cancer and NF-kB inhibition to halt tumour progression (Naugler and Karin, 2008 Yun *et al.*, 2009).

A review study reported that NF-kB activation is known to suppress cancer cell apoptosis (Balkwill and Coussens, 2004). Continuous nuclear NF-kB activity protects cancer cells from apoptosis and enhances their growth activity.

In Chapter 3 it was demonstrated that TNF-α enhanced melanoma cell migration and that ibuprofen reduced melanoma cell migration and viability in TNF-α stimulated HBL cells. This is in accordance with other studies (Zhu *et al.*, 2002; Katerinaki *et al.*, 2003; Eves *et al.*, 2003; Katerinaki *et al.*, 2006) which demonstrated that NSAIDs attenuated the migration and invasion of melanoma cells.

Therefore, this study supports the hypothesis that NSAIDs may be blocking the effect of TNF-α induced activation of NF-kB transcription factor and hence, make melanoma cells more susceptible to apoptosis.

In conclusion, this study supports the hypothesis that a pro-inflammatory cytokine TNF-α activates the transcription nuclear factor NF-kB creating an inflammatory condition which facilitated melanoma cell migration, invasion and survival and the fact that NF-kB activation is linked to apoptosis inhibition in melanoma cells.

CHAPTER 7

COMBINED CAPSACIN AND HA14-1 IN INDUCING APOPTOSIS IN MELANOMA CELLS

7.1. EFFECTS OF IBUPROFEN, CAPSAICIN AND HA14-1 IN INDUCING APOPTOSIS IN MELANOMA CELLS

In previous results (Chapter 3) it was demonstrated that ibuprofen and capsaicin reduced melanoma cell migration and viability in TNF-α stimulated cells. In the next experiments the mechanism of the effects of TNF-α and the NSAID ibuprofen on melanoma cell survival and apoptosis were assessed. This also included the investigation of capsaicin or HA14-1 and a combination of capsaicin and HA14-1 to observe any additive or synergistic effect in inducing melanoma cell apoptosis. In these experiments cell viability was determined by phosphatidylserine (PS) externalisation on cell membranes (Annexin-V) and cell membrane integrity, using a Guava ViaCount assay.

7.2. EFFECTS OF TNF-α AND IBUPROFEN IN INDUCING APOPTOSIS IN MELANOMA CELLS

In order to observe the effects of ibuprofen sodium salt affecting melanoma cell migration and survival, cells were 24 hours pre-incubated with 300U/ml TNF-α. After this incubation period, media was removed and media containing 10^{-3}M or 10^{-4}M NaIbu was added to the cells and incubated for a further 24 hours. Viability was determined using a Guava ViaCount assay (see Figure 7.1).

HBL late apoptotic cells were significantly increased when exposed to 300U/ml TNF-α on its own ($p<0.01$) and 10^{-3} M NaIbu (<0.001). Live HBL cells were significantly reduced by TNF-α on its own ($p<0.05$). 10^{-4}M NaIbu ($p<0.05$) and 10^{-3}M NaIbu ($p<0.001$) relative to media, see Figure 7.1 A.

On the other hand, TNF-α on its own and NaIbu did not induce apoptosis in C8161 melanoma cells. All aspects of apoptosis were unchanged and live viable cells

were slightly increased at 300U/ml TNF-α ($p>0.05$). Slight reductions in live C8161 cells occurred at 10^{-4}M and 10^{-3} M NaIbu ($p>0.05$ respectively). 20μM camptothecin showed a significant increase in nuclear debris and late apoptosis whilst a significant reduction in cell viability was observed ($p<0.05$), relative to control media, see Figure 7.1 B.

In summary, TNF-α on its own and 10^{-3}M NaIbu had a marked effect on HBL cells in inducing cell apoptosis and reducing live cells. However, TNF-α on its own and 10^{-4}M and 10^{-3}M NaIbu did not affect C8161 cell viability and neither induced apoptosis. See Figure 7.1 and Table 7.1 for an illustration of these results.

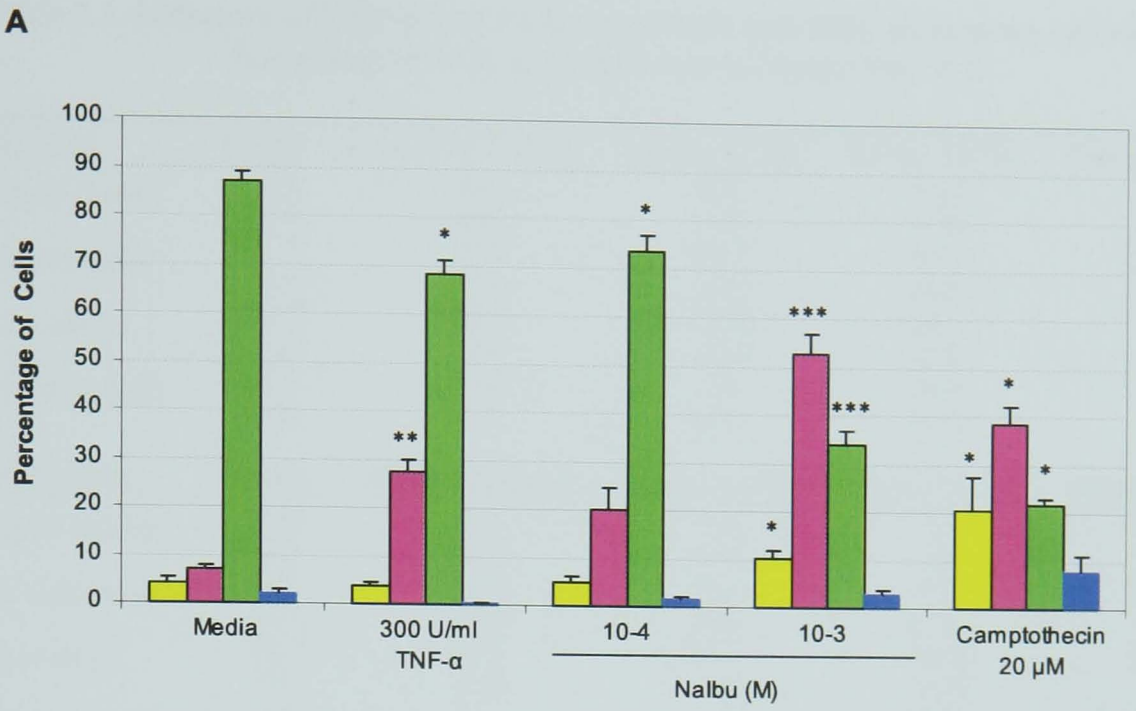


Figure 7.1. Influence of TNF-α and NaIbu on melanoma cell viability. (A) = HBL cells, (B) = C8161 cells. ▯ = Nuclear debris, ▯ = Late apoptotic, ▯ = Live viable, ▯ = Early apoptotic. Values expressed as Mean+SEM. N=2 in duplicates. * = $p<0.05$; ** = $p < 0.01$; *** = $p < 0.001$, relative to media.

Table 7.1. Influence of TNF-α and Nalbu on C8161 and HBL melanoma cell viability. Percentage of cells are expressed as Mean+SD. N=2.

HBL Cells	Media	300U/mlTNF-α	Nalbu 10^{-4}M	Nalbu 10^{-3}M	Camptothecin
Nuclear debris	3.9 (1.5)	3.8 (0.6)	4.7 (1.4)	10.4 (1.5)	38.5 (6.8)
Late apoptotic	7.1 (0.7)	27.5 (2.1)	20.2 (4.4)	52.7 (4.2)	20.6 (3.4)
Live viable	86.8 (2.4)	68.3 (2.5)	73.6 (3.2)	34.1 (2.5)	21.8 (1)
Early apoptotic	2.2 (0.8)	0.40 (0.1)	1.5 (0.4)	3.0 (0.7)	7.9 (3.2)
C8161 Cells	**Media**	**300U/mlTNF-α**	**Nalbu 10^{-4}M**	**Nalbu 10^{-3}M**	**Camptothecin**
Nuclear debris	3.9 (0.8)	2.9 (0.4)	7.6 (4.5)	3.4 (1.2)	45.1 (3.5)
Late apoptotic	6.6 (2.4)	3.73 (0.3)	7.1 (2.8)	5.3 (1.8)	26.5 (7)
Live viable	88.1 (3.9)	92.1 (0.2)	83.7 (8.5)	89.8 (4.2)	26.9 (6)
Early apoptotic	1.4 (0.8)	1.2 (0.5)	1.6 (1.2)	1.7 (1.2)	8.2 (3)

7.3. EFFECTS OF CAPSAICIN AND HA14-1 ON MELANOMA CELLS, MELANOCYTES AND FIBROBLASTS VIABILITY

Initially, a dose-response curve for each drug and the combination of both was tested using MTT assays to assess cell viability and to determine appropriate concentrations for investigation of the effects of both drugs in inducing apoptosis in these cells.

Figures 7.2 A, B and C show the effects of capsaicin and Figures 7.2 D, E and F show the effects of HA14-1 on melanoma cells, melanocytes and fibroblast viability respectively. Overall, capsaicin reduced melanoma cell viability. For HBLs the IC50 value was 100μM. (Capsaicin significantly reduced HBL cell viability to 16.5% at 300μM ($p<0.001$), and to 5% at 400μM ($p<0.001$). No viable cells were found at 500μM capsaicin).

For A375SM cell the IC50 value was 133μM capsaicin. (Capsaicin significantly reduced cell viability at concentrations of 200μM up to 400μM and no viable cells were found by 500μM).

For C8161 cells the IC50 value was 166μM capsaicin. (Cell viability was significantly reduced to 35% at 200μM ($p<0.05$) and to 6.5% at 500μM capsaicin ($p<0.01$))

For melanocytes the IC50 value was 150μM capsaicin ($p<0.001$) and concentrations of 50-200μM significantly reduced cell viability. For fibroblasts the IC50 value was 350μM capsaicin and cell viability was significantly reduced by 400μM and 500μM ($p<0.001$).

In summary, the three melanoma cell lines and melanocytes showed a similar sensitivity to capsaicin with capsaicin concentrations above 200μM becoming cytotoxic. However, fibroblasts tolerated capsaicin up to 300μM without any effect with an IC50 of around 350μM, showing these cells were more resistant to the metabolic inhibitory effects of capsaicin.

HA14-1 also reduced melanoma cell viability. The IC50 value for HA14-1 in HBL cells was around 60μM HA14-1 ($p<0.05$). For A375SM cells the IC50 value was around 100μM HA14-1. For C8161 cells the IC50 value was greater than 100μM HA14-1. For melanocytes the IC50 value was around 100μM HA14-1 (see Figure 7.2 E). Fibroblasts were relatively resistant to HA14-1 up to concentrations of 150μM but 200μM reduced viability to only 1.3% (see Figure 7.2 F).

In summary, the HA14-1 IC50 values for melanoma cells, fibroblasts and melanocytes were: HBL (55μM) followed by melanocytes (80μM), A375SM (95μM), C8161 (higher than 100μM) and fibroblasts (180μM).

In conclusion, these results suggest that melanoma cells and melanocytes had similar sensitivities to HA14-1 whilst fibroblasts were more resistant to HA14-1 (by a factor of two-fold).

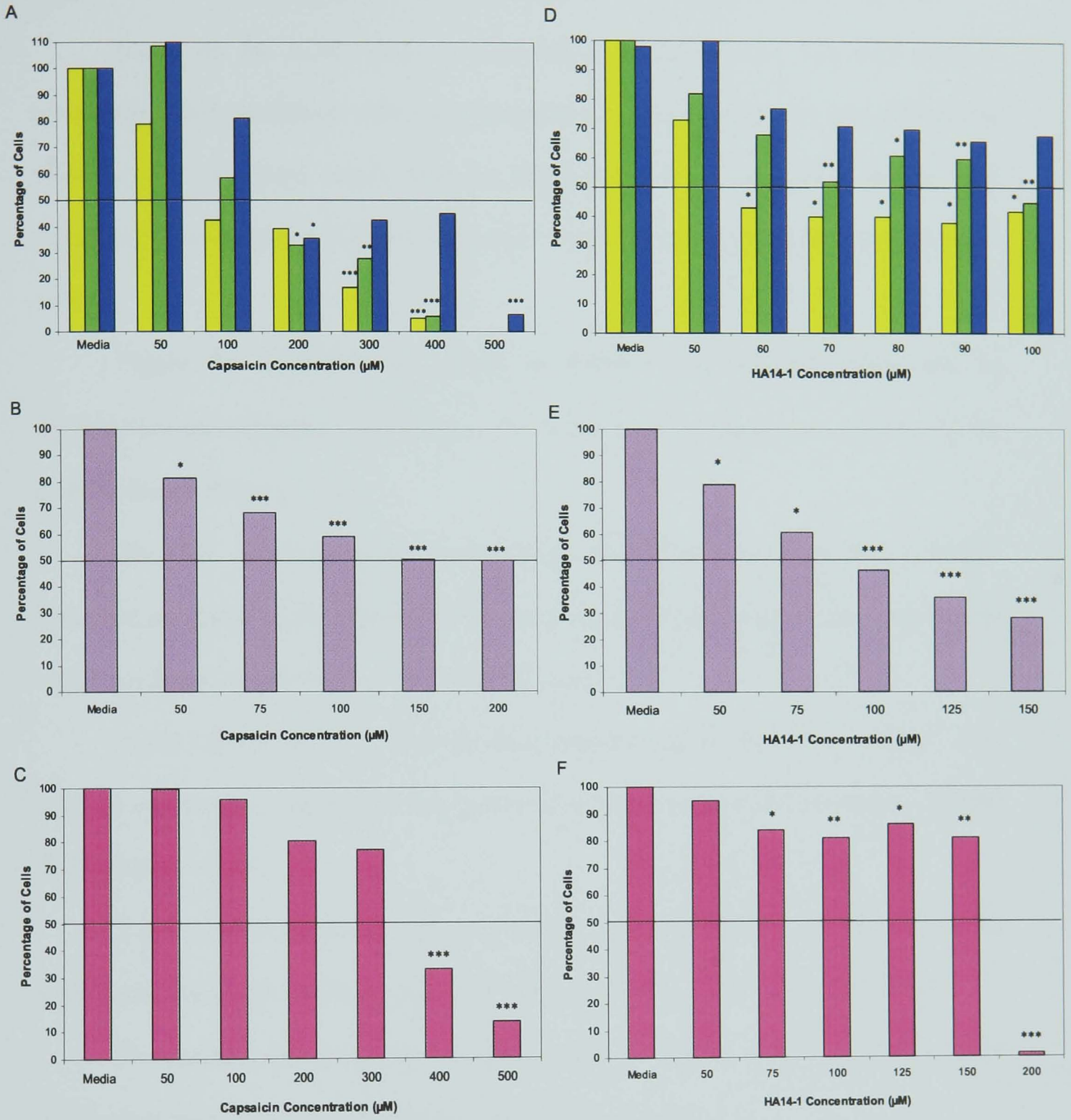


Figure 7.2. Effects of Capsaicin or HA14-1 on melanoma cells, melanocytes and fibroblasts viability (MTT assays). (A) = Capsaicin on melanoma cells, N= 2. (B) = Capsaicin on melanocytes, N= 2. (C) = Capsaicin on fibroblasts, N=2. (D) = HA14-1 on melanoma cells, N= 2. (E) = HA14-1 on melanocytes, N= 4. (F) = HA14-1 on fibroblasts. ☐ = HBL, ☐ = A375SM, ☐ = C8161, ☐ = Melanocytes, ☐ = Fibroblasts Values expressed as Mean+SD. N= 3 in duplicates * = p<0.05; ** = p<0.01; *** = p<0.001, related to media.

7.3.1. Effects of Combined HA14-1 and Capsaicin on Melanoma Cell Viability

Based on the IC50 values a combination of both drugs was then used to investigate their combined effect on melanoma cells, melanocytes and fibroblasts viability. The predicted values from an additive effect of using both agents were calculated. Capsaicin and HA14-1 treated cell were compared to control media treated cells.

Figure 7.3 illustrates the effects of HA14-1 and capsaicin alone and in combination on melanoma cells (Figure 7.3 A, B and C), melanocytes (Figure 7.3 D) and fibroblasts (Figure 7.3 E).

For HBL cells both agents significantly reduced cell viability to 50% ($p<0.05$). This was not significantly different to the predicted additive effects of these two agents which predicted a reduction in HBL viability to 30%.

For A375SM cell the combined drugs reduced viability to 57% ($p<0.001$). This was not significantly different to the predicted additive value of reduction in A375SM cell viability to 47%.

For C8161 cells the two drugs combined reduced viability to 56% ($p<0.001$) which was exactly the predicted value for an additive effect (57%) of these agents.

For melanocytes the combined agents reduced viability to 19% ($p<0.05$) which was exactly as predicted for an additive effect of these agents.

For fibroblasts the combination of the two agents reduced viability to only 0.4% ($p<0.001$) which showed a strong synergistic effect as the predicted effect was 35% ($p<0.001$). However, it should be noted that higher concentrations of HA14-1 and capsaicin were deliberately used for these cells as they had been shown to be more resistant to both of these agents compared to the other melanoma cells and melanocytes.

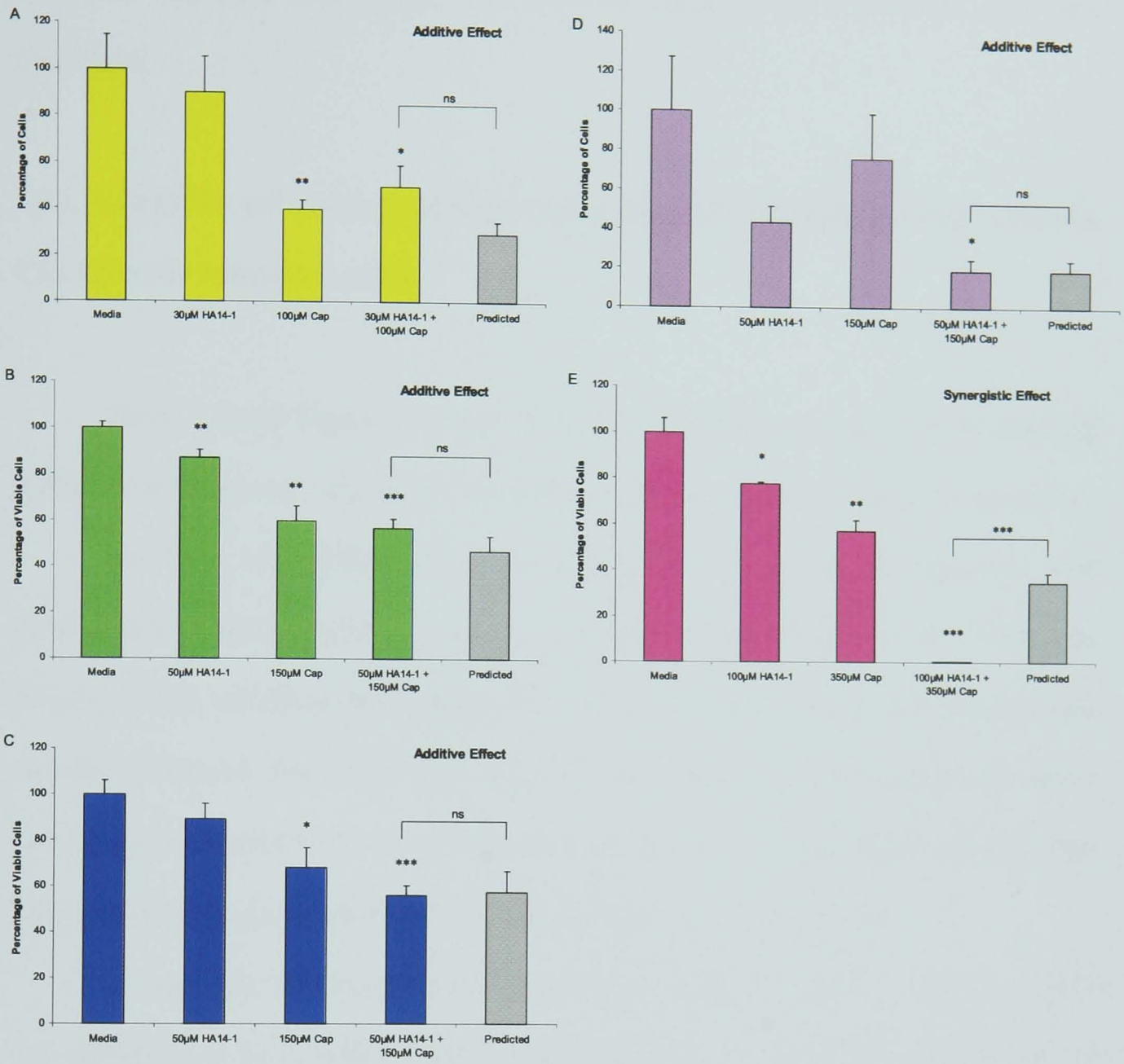


Figure 7.3. Additive and synergistic effects of combined HA14-1 and Capsaicin on (A) HBL, (B) A375SM, (C) C8161 melanoma cells, (D) melanocytes and (E) fibroblasts viability. HA14-1 (μM), Cap=Capsaicin (μM). Combined agents were maintained at the same concentrations as used isolated. Predicted values are shown by the grey coloured columns. Values are expressed as percentage of viable cells. N=2 in triplicates. * = $p>0.05$; ** = $p<0.01$; *** = $p<0.001$, related to media.

7.4. EFFECTS OF CAPSAICIN AND HA14-1 ALONE AND IN COMBINATION IN INDUCING APOPTOSIS IN MELANOMA CELLS AND FIBROBLASTS

In the following experiments, the effects of capsaicin or HA14-1 in inducing apoptosis in melanoma cells and fibroblasts were investigated. IC50 values of the

effects of capsaicin or HA14-1 were observed for a further investigation of the effects of combined capsaicin and HA14-1 in inducing apoptosis in melanoma cells and fibroblasts.

7.4.1. EFFECTS OF CAPSAICIN IN INDUCING APOPTOSIS IN MELANOMA CELLS AND FIBROBLASTS

Table 7.2 and Figure 7.4 show the effects of increasing capsaicin in inducing apoptosis in melanoma cells and in fibroblasts using the Annexin-V assay for apoptosis.

For HBL cells (Figure 7.4 A), a significant increase in late apoptosis with reduced cell viability started at capsaicin concentration of 300μM. For A375SM cells (Figure 7.4 B) capsaicin significantly increased late apoptotic cells and reduced cell viability at 400μM. For C8161 cells (Figure 7.4 C) capsaicin increased nuclear debris and reduced cell viability at around 400μM capsaicin. Overall, fibroblasts tolerated with capsaicin at the highest concentration tested, 500μM, see Figure 7.4 D.

In summary, the concentration of capsaicin which reduced cell viability to 50% was calculated to be 220μM for HBL cells, 350μM for A375SM cells and 320μM for C8161 cells. Capsaicin had no effect on fibroblasts at concentrations up to 500μM.

20μM camptothecin was used as a positive control and significantly induced apoptosis in 2 of 3 melanoma cell lines and fibroblasts. This was not significant for HBL cells due to sample variation.

Table 7.2. Effects of capsaicin in inducing apoptosis in melanoma cells and fibroblasts.

Values are expressed as Mean+SD

HBL	Nuclear Debris	Late Apoptosis	Live viable	Early Apoptosis
Media	17.6 ± 13.7	20.8 ± 11.9	58.3 ± 22.4	3.4 ± 1.7
50	9.3 ± 4.8	28.2 ± 2.4	59.2 ± 7.1	3.4 ± 1.6
100	13.5 ± 6.8	41.8± 8.1	36.3 ± 12.3	8.5 ± 2.7
200	13.3 ± 9.8	18.6 ± 3.5	61.2 ± 7	7.1 ± 4.5
300	26.9 ± 17.6	62.5 ± 13.2	9.0 ± 3.5	1.6 ± 1.4
400	10.5 ± 6.5	84.9 ± 5.9	2.8 ± 1.1	1.9 ± 1.1
500	3.3 ± 3.1	93.4 ± 3.1	1.2 ± 0.5	2.1 ± 0.7
Camptothecin	20.6 ± 12.7	38.5 ± 10.9	33.1 ± 13.7	7.9 ± 6
A375SM	**Nuclear Debris**	**Late Apoptotic**	**Live viable**	**Early Apoptotic**
Media	0.3 ± 0.3	7.5 ± 13.4	88.9 ± 10	3.2 ± 6.8
50	0.5 ± 0.3	2.7 ± 0.9	96.2 ± 1.1	0.7 ± 0.4
100	0.6 ± 0.3	2.8 ± 0.3	95.4 ± 0.7	1.2 ± 0.4
200	0.7 ± 0.5	6.0 ± 2.9	90.3 ± 4.5	2.9 ± 2.1
300	1.1 ± 1.1	15.1 ± 2.1	82.6 ± 2.3	1.3 ± 0.8
400	0.4 ± 0.1	76.4 ± 5.4	21.9 ± 5.9	1.3 ± 0.5
500	0.4 ± 0.2	68.8 ± 9.9	27.8 ± 10.4	2.9 ± 0.5
Camptothecin	2.6 ± 2.6	36.2 ± 16.5	53.9 ± 19.3	7.3 ± 8.2
C8161	**Nuclear Debris**	**Late Apoptotic**	**Live viable**	**Early Apoptotic**
Media	15.9 ± 2.6	9.4 ± 5.2	68.4 ± 9.1	6.6 ± 6.6
50	14.2 ± 4.9	12.3 ± 2.4	66.7 ± 1.9	7.0 ± 4.4
100	12.2 ± 3.8	11.4 ± 3.7	67.6 ± 7.2	7.7 ± 5.4
200	11.7 ± 2.3	7.9 ± 4.0	75.7 ± 7	4.9± 5.3
300	25.2 ± 12.7	11.8 ± 1.3	60.4 ± 8	2.8 ± 3.5
400	49.7 ± 20.4	23.4 ± 16.4	24.8 ± 1.1	2.3 ± 3.1
500	53.3 ± 12.9	16.5± 9.4	29.9 ± 2.9	0.5 ± 0.6
Camptothecin	43.6 ± 12.2	26.7 ± 4.7	26.9 ± 8.1	1.6 ± 1.9
Fibroblasts	**Nuclear Debris**	**Late Apoptotic**	**Live viable**	**Early Apoptotic**
Media	5.0 ± 1.4	5.6 ± 3.7	87.1 ± 5.5	2.4 ± 1
50	4.7 ± 2.9	11.8 ± 8.5	78.5 ± 13.2	5.1 ± 2.4
100	12.4 ± 3	23.1 ± 1.4	59.9 ± 4	4.6 ± 2.1
200	6.2 ± 2.7	15.9 ± 14.2	74.8 ± 18.6	3.1 ± 1.8
300	5.3 ± 4.5	15.5 ± 15.9	77.1 ± 22	2.1 ± 1.7
400	8.4 ± 4.1	19.6 ± 18.3	69.8 ± 22.5	2.2 ± 1.2
500	6.3 ± 5.7	15.7 ± 15.9	74.2 ± 22.2	3.9 ± 1.5
Campothecin	8.8 ± 3	40.0 ± 4.2	44.3 ± 5.7	6.9 ± 1

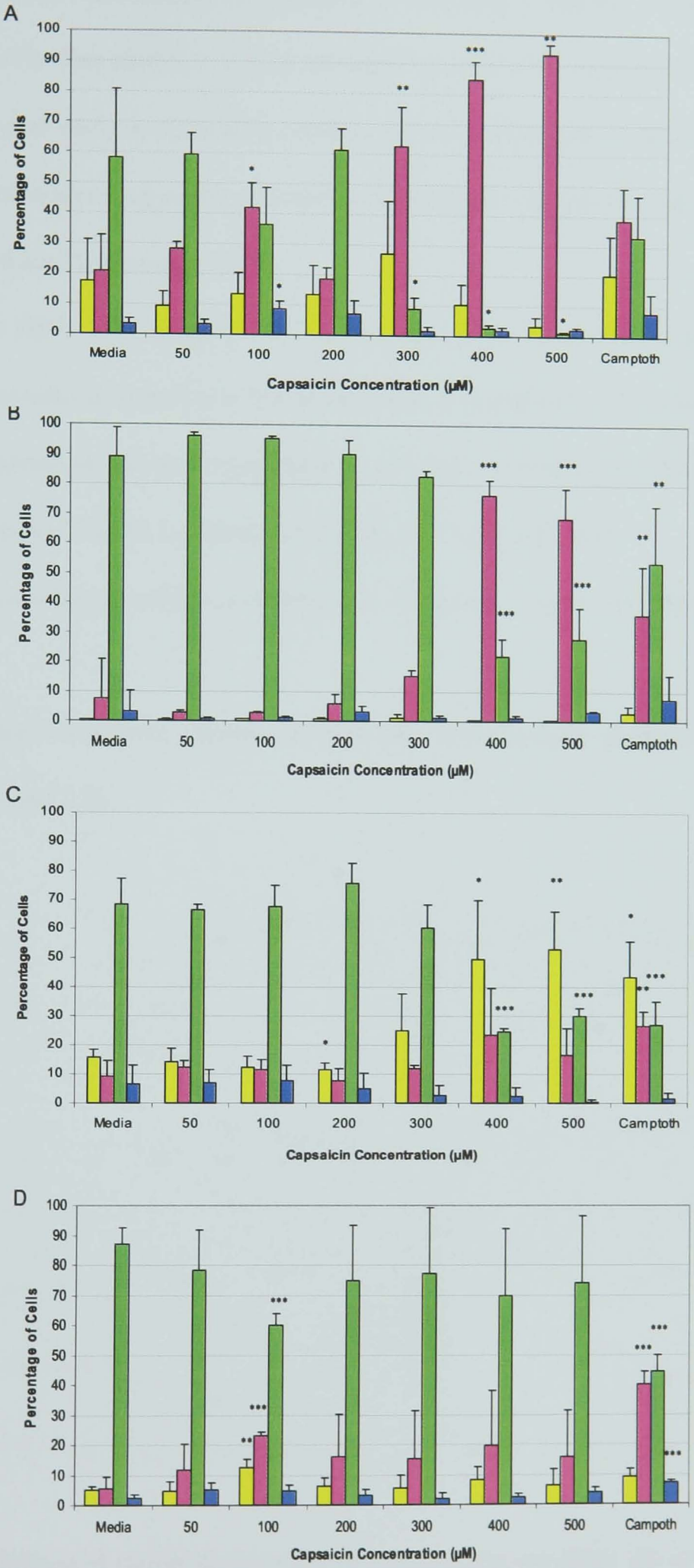


Figure 7.4. Effects of capsaicin in inducing apoptosis in (A) HBL, (B) A375SM, (C) C8161 melanoma cells and (D) fibroblasts. ■ = Nuclear debris, ■ = Late apoptotic, ■ = Live viable, ■ = Early apoptotic. Values are expressed as Mean+SD. N=2 in duplicates. * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$, relative to media. Camptoth = 20μM camptothecin.

Figure 7.5 shows the effects of capsaicin in inducing apoptosis in melanoma cells and fibroblasts. For this analysis, it was calculated a sum of all aspects of apoptosis (nuclear debris, late and early apoptosis) in relation to live viable cells. In this simpler way it was estimated at which capsaicin concentration increased apoptotic cells and crosses-over with the reduced live viable cells.

For HBL cells (Figure 7.5 A) the cross-over of increased apoptotic cells and reduced live cells occurred at a lower capsaicin concentration of 220μM. Figures 7.5 (B and C) illustrate an increase in apoptotic cells and a simultaneous reduction in live cells that occurred at 350μM capsaicin for C8161 and A375SM cells.

Increasing capsaicin concentrations showed no effects on fibroblasts (Figure 7.5 D).

These cross-over values confirm the IC50 values demonstrated above (see Figures 7.4 and 7.5).

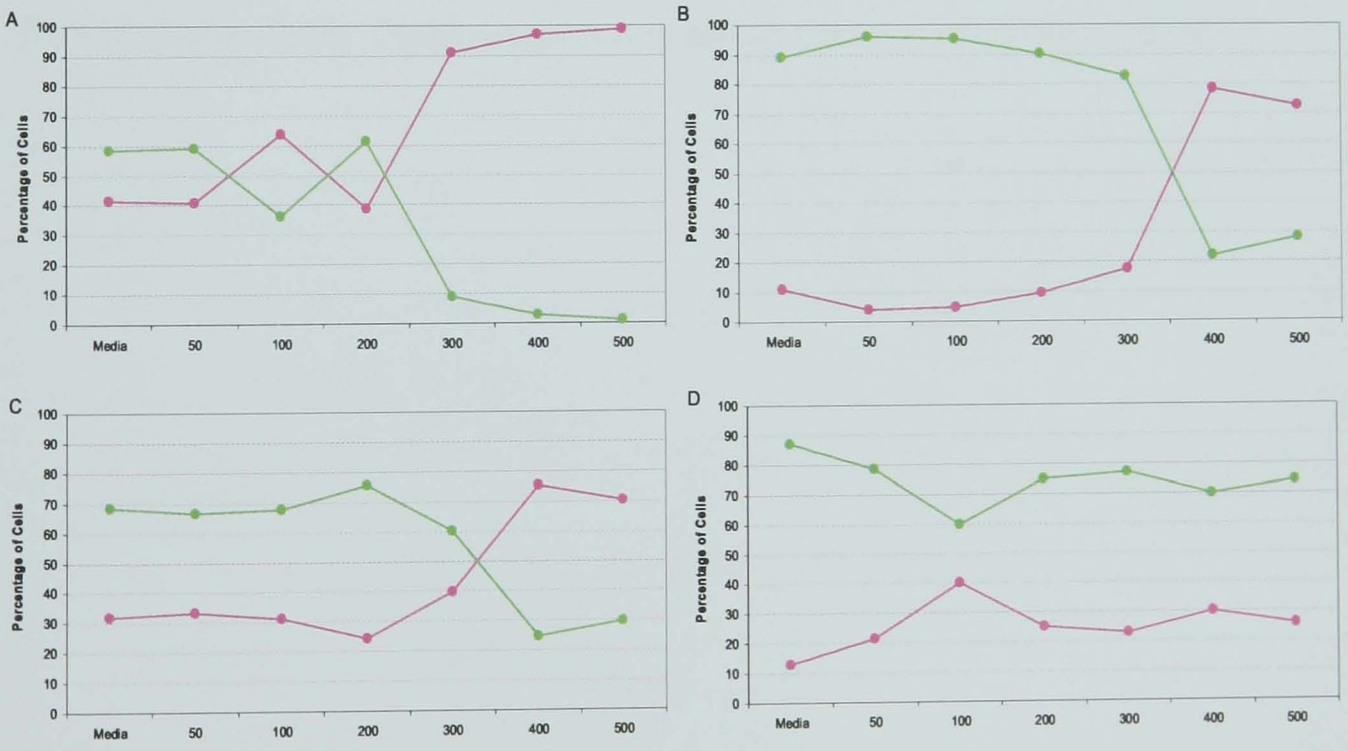


Figure 7.5. Effects of capsaicin in inducing apoptosis in (A) HBL, (B) A375SM, (C) C8161 melanoma cells and (D) fibroblasts. ● = Live viable, ● = Apoptotic. Values are expressed as Mean. N=2 in duplicates.

7.4.1.1 EFFECTS OF CAPSAICIN IN INDUCING APOPTOSIS IN MELANOMA CELLS ASSESSED BY DNA DAMAGE ASSAY

The effects of capsaicin in inducing apoptosis in melanoma cells were also investigated using a DNA Damage assay (see Figure 7.6). Capsaicin treated cells were compared to control media treated cells.

For HBL cells capsaicin concentrations of 300μM, 400μM, and 500μM significantly reduced HBL cells viability to 57.8%, 57.1% and 48% ($p<0.001$ in all cases). The IC50 value was around 480μM capsaïcin.

A375SM cell viability was significantly reduced to 68.7% at 300μM capsaicin concentrations ($p<0.01$) and the IC50 value was 440μM. The IC50 value for C8161 cells was 500μM capsaicin ($p<0.05$).

Fibroblasts were not affected by the effects of capsaicin up to 500μM ($p>0.05$).

20μM camptothecin, which was used as a positive control, significantly reduced viability of HBL and A375SM cells ($p<0.001$) whilst not significantly reducing C8161 cell viability ($p>0.05$). 20μM camptothecin had no effect on fibroblasts viability.

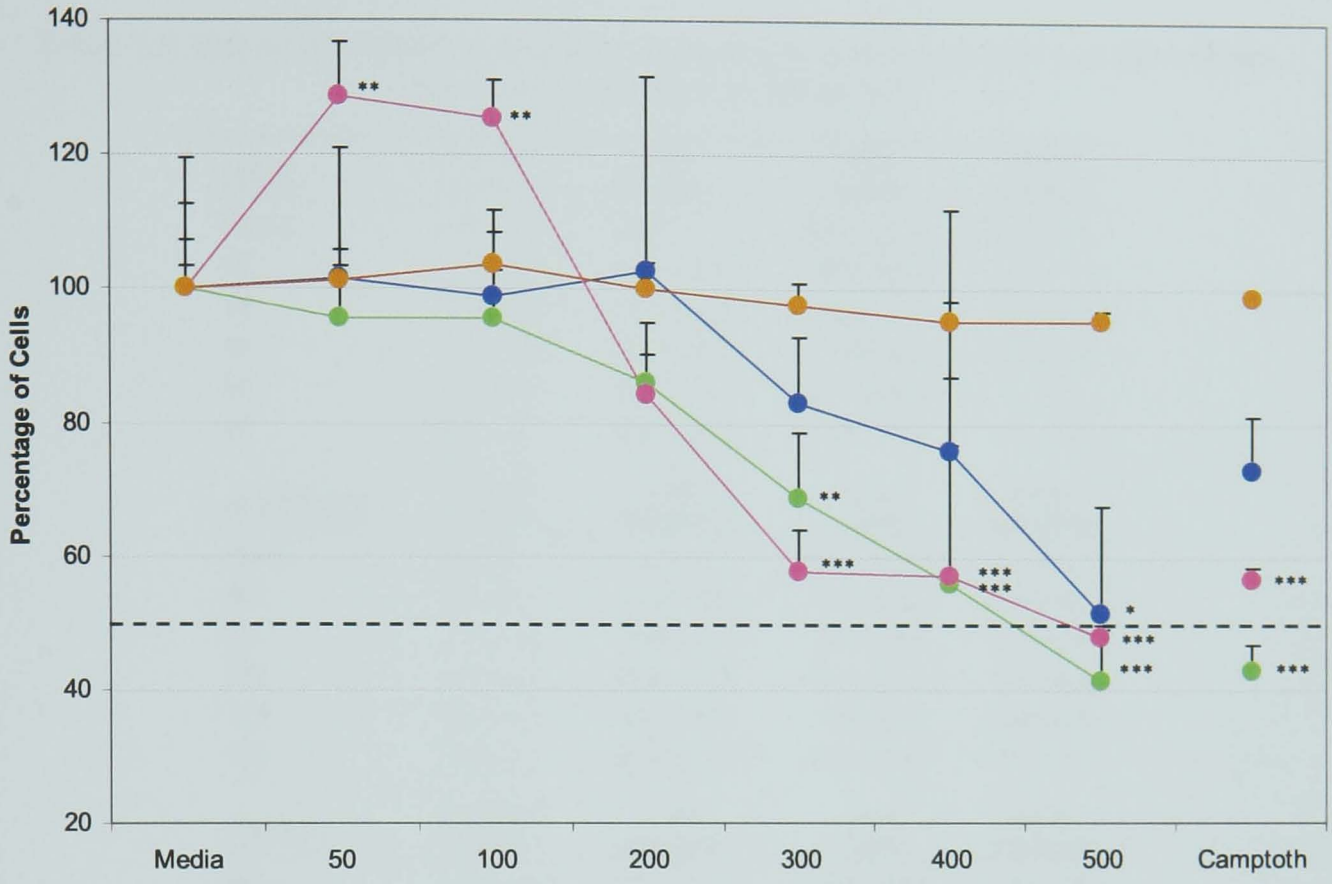


Figure 7.6. Effects of capsaicin (μM) in inducing apoptosis in melanoma cells and fibroblasts - DNA damage assay. ● = HBL cells, ● = A375SM cells, ● = C8161 cells, ● = Fibroblasts. Values express percentage of cells ± SD. N=2 in duplicates. IC50 values are indicated by a black dotted line. * = p<0.05; ** = p < 0.01; *** = p < 0.001, relative to media. Campth = 20μM camptothecin.

7.4.2. EFFECTS OF HA14-1 IN INDUCING APOPTOSIS IN MELANOMA CELLS AND FIBROBLASTS

Table 7.3 and Figure 7.7 show the effects of increasing HA14-1concentrations in inducing apoptosis in melanoma cells and fibroblasts, compared to control media. For HA14-1 the concentration which reduced cell viability to 50% was 102μM for fibroblasts. This is higher than the concentration required to demonstrate the same effects on HBL cells which was 48μM and 85μM for A375SM and C8161 cells respectively. Fibroblasts were more resistant to higher HA14-1 concentrations compared to melanoma cells. The IC50 value was 112μM HA14-1.

Table 7.3. Effects of HA14-1 in inducing apoptosis in melanoma cells and fibroblasts. Values are expressed as Mean+SD.

HBL	Nuclear Debris	Late Apoptotic	Live viable	Early Apoptotic
Media	0.85 ± 0.1	5.3 ± 1.8	90.0 ± 5.48	3.9 ± 3.5
20	1.3 ± 0	12.1 ± 8.5	84.5 ± 9.6	2.3 ± 1
30	1.8 ± 0.6	24.3 ± 9.9	70.9 ± 11.3	3.1 ± 0.9
40	2.9 ± 0.3	36.2 ± 8.8	58.8 ± 8.3	2.2 ± 0.9
50	3.6 ± 2.0	45.2 ± 46.9	48.1 ± 45.9	3.1 ± 3
60	4.4 ± 1.2	60.4 ± 29.2	33.2 ± 29.1	2.0 ± 1.6
A375SM	**Nuclear Debris**	**Late Apoptotic**	**Live viable**	**Early Apoptotic**
Media	1.8 ± 1.5	11.9 ± 11.5	81.7 ± 18.1	4.6 ± 5.2
50	1.7 ± 0.7	11.5 ± 0.8	81.1 ± 6.6	5.8 ± 6.5
75	1.7 ± 0.2	15.9 ± 0.1	76.7 ± 5.4	5.7 ± 5.6
100	1.2 ± 0.4	65.9 ± 11.9	25.8 ± 18.2	7.2 ± 6.8
125	0.9 ± 0.1	80.3 ± 6.6	13.0 ± 0.1	5.8 ± 6.9
150	1.8 ± 1.9	66.7 ± 27.6	22.7 ± 14.6	8.8 ± 11
C8161	**Nuclear Debris**	**Late Apoptotic**	**Live viable**	**Early Apoptotic**
Media	3.5 ± 1.9	15.2 ± 11.9	80.3 ± 14.5	1.0 ± 0.7
50	3.3 ± 1.1	18.5 ± 9.3	77.3 ± 10.7	0.9 ± 0.4
75	4.5 ± 1.3	22.8 ± 3.1	71.6 ± 1.4	1.2 ± 0.3
100	5.3 ± 2.9	64.9 ± 28.5	28.5 ± 32.4	1.3 ± 1
125	5.2 ± 2.9	84.1 ± 2.4	9.5 ± 6.8	1.1 ± 0.4
150	3.9 ± 1.1	56.1 ± 45.4	37.6 ± 41	2.4 ± 3.4
Fibroblasts	**Nuclear Debris**	**Late Apoptotic**	**Live viable**	**Late Apoptotic**
Media	3.8 ± 2.2	16.3 ± 0.4	76.5 ± 2.9	3.6 ± 0.4
50	2.5 ± 0.4	12.4 ± 2.1	82.9 ± 1.9	2.3 ± 0.6
75	3.9 ± 0.7	22.1 ± 4.3	70.7 ± 3.6	3.3 ± 1.1
100	1.4 ± 0.3	8.8 ± 2.3	85.9 ± 2.9	3.9 ± 0.5
125	5.3 ± 0.1	80.4 ± 2.7	12.8 ± 3.1	1.5 ± 0.6
150	2.9 ± 0.9	82.8 ± 0.4	12.1 ± 0.2	2.2 ± 1.2

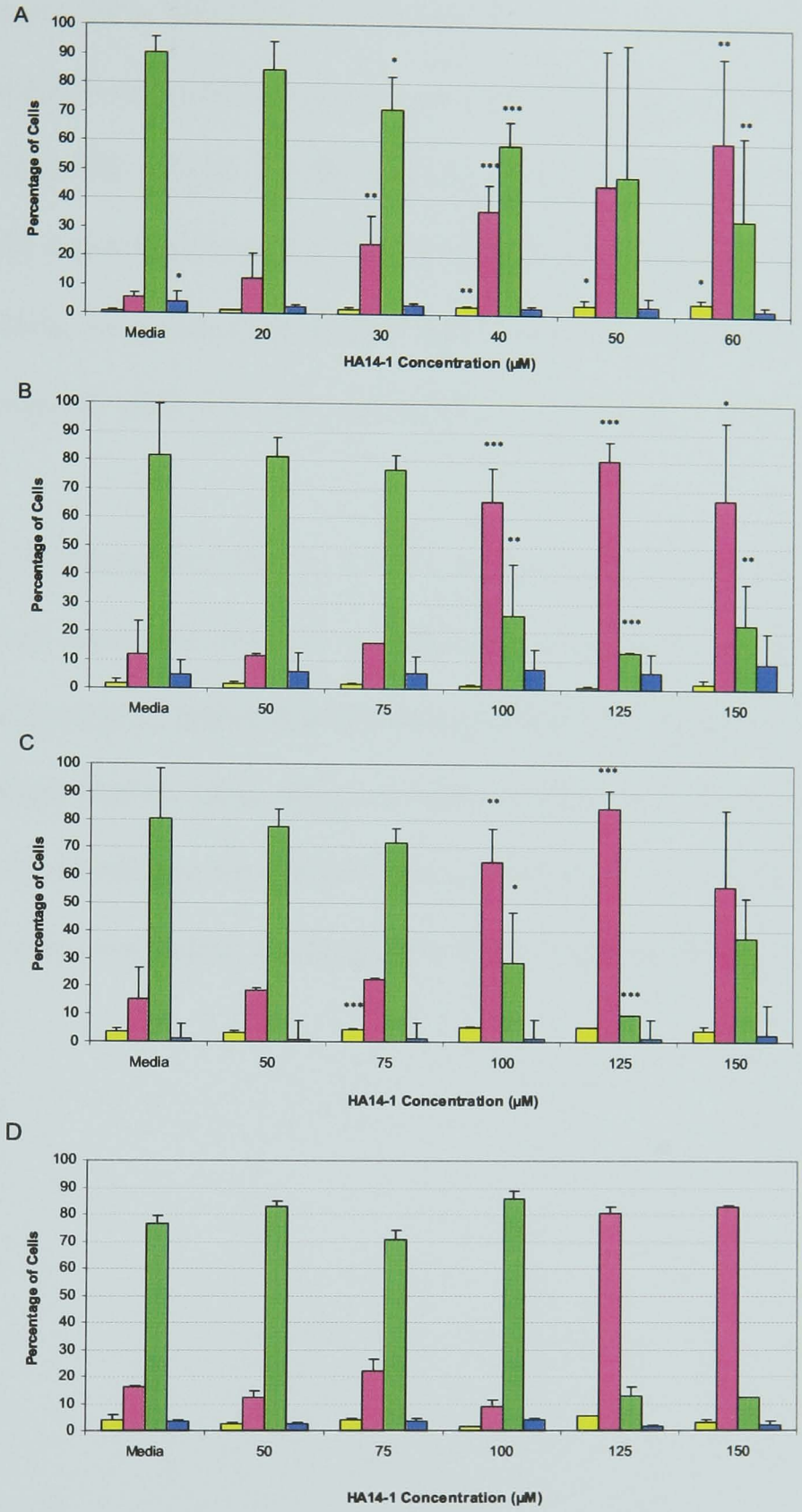


Figure 7.7. Effects of HA14-1 in inducing apoptosis in (A) HBL, (B) A375SM, (C) C8161 melanoma cells and (D) fibroblasts viability. Values are expressed as Mean+SD. ☐ = Nuclear debris, ☐ = Late apoptotic, ☐ = Live viable, ☐ = Early apoptotic. Values are expressed as Mean+SD. N=2 in duplicates. Fibroblasts N=1 in duplicates. * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$, relative to media.

Figure 7.8 illustrates the effects of HA14-1 on all aspects of apoptosis and live viable melanoma cells. For HBL cells (Figure 7.8 A), a cross-over of reduced live and increased apoptotic cells occurred at a HA14-1 concentration just below 50μM.

For A375SM (Figure 7.8 B), the cross-over of reduced live cell to increased apoptotic cells was estimated at HA14-1 concentration just below 90μM.

Similarly, for C8161 cells (Figure 7.8 C) the cross-over of reduced live cells and increased apoptotic cells was also estimated at an HA14-1 concentration just below 90μM.

For fibroblasts (see Figure 7.8 D), a cross-over for live and apoptotic cells occurred at an estimated HA14-1 concentration just above 110μM. This HA14-1 concentration is slightly higher than the estimated HA14-1 concentrations of 50μM and 90μM to demonstrate the same effect on HBL, A375SM and C8161 cells respectively. This shows that fibroblasts were slightly more resistant to HA14-1 than melanoma cells.

The cross-over values confirmed the IC50 values as shown above (see Figures 7.7 and 7.8).

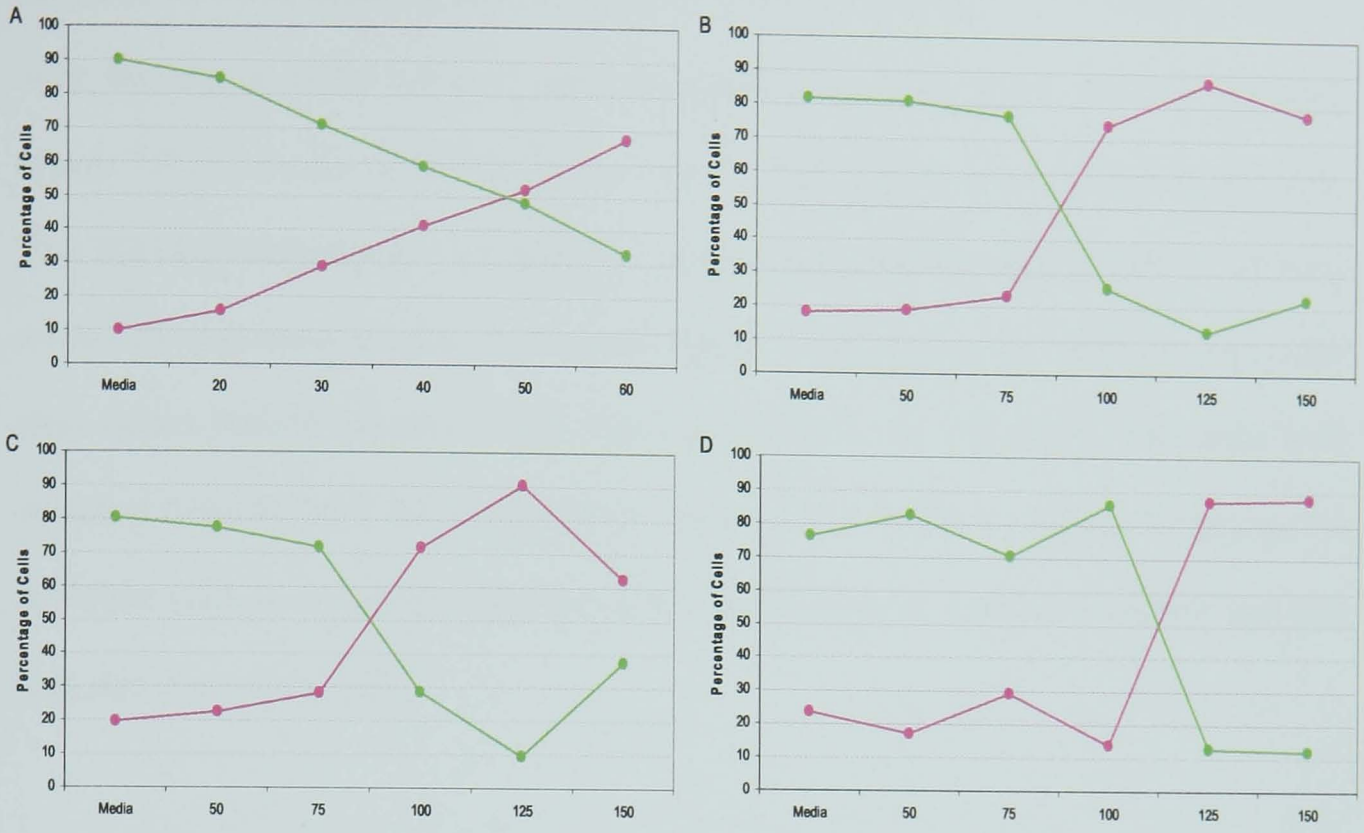


Figure 7.8. Effects of HA14-1 in inducing apoptosis in (A) HBL, (B) A375SM, (C) C8161 melanoma cells and (D) fibroblasts. ● = Live viable, ● = Apoptotic. Values are expressed as Mean. N=2 in duplicates. For fibroblasts N=1.

7.4.3. EFFECTS OF COMBINED CAPSAICIN AND HA14-1 IN INDUCING APOPTOSIS IN MELANOMA CELLS

In previous experiments, capsaicin and HA14-1 added individually were shown to reduce live cells and induce apoptosis in melanoma cells. In the next experiments capsaicin and HA14-1 were combined to investigate whether these agents would show a synergistic or additive effect in inducing apoptosis in melanoma cells. Figure 7.9 shows micrographs of A375SM melanoma cells to which were added capsaicin and HA14-1 individually and combined. Figure 7.9 (A) shows A375SM cells in media only as a control. The cells have a long or irregular shape and a fairly central nucleus. These cells are flattened to the culture well indicating that they are attached to the bottom of the well. There are a few dead cells shown by the bright round-shaped cells floating in the media. This is expected after incubating A375SM cells in media for 24h. Figure 7.9 (B) shows A375SM cells to which were added 300μM capsaicin. This capsaicin

concentration was demonstrated to be toxic to the A375SM cells. The majority of cells were floating in media however, there were also some cells still attached to the well. Figure 7.9 (C) shows A375SM cells to which 75μM HA14-1 was added to the cells. This HA14-1 concentration became toxic as there were floating cells in media with only a few cells remained attached to the well. Figure 7.9 (D) shows A375SM cells to which were added 300μM capsaicin combined with 75μM HA14-1 in media. The cells were detached from the well, indicating a toxic effect on A375SM cells. Figure 7.9 (E) shows A375SM cells to which was added 20μM camptothecin as a positive control and this was also toxic to the cells.

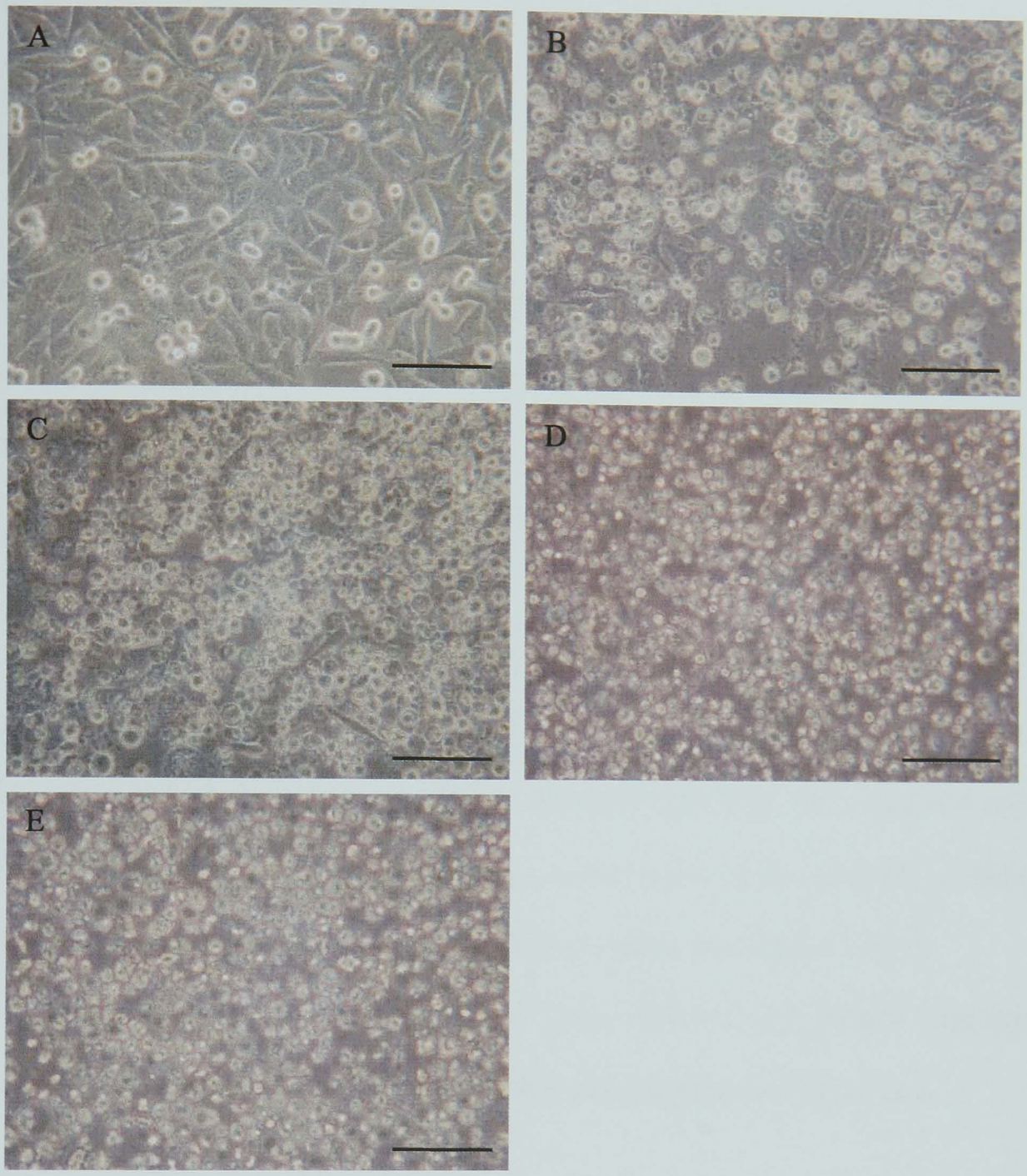


Figure 7.9. Micrographs of A375SM melanoma cells at 24 h incubation in different treatments (A) Control; (B) Capsaicin 300μM, (C) HA14-1 75μM; (D) Capsaicin 300μM+ HA14-1 75μM; (E) Camptothecin 20μM. Scale bars=100μm, 20 x magnification.

Figure 7.10 shows the apoptotic effects of HA14-1 and capsaicin alone and combined compared to the predicted values for the additive effects of these agents in inducing apoptosis.

Figure 7.10 (A) shows that the individual treatments with 30μM HA14-1 and 200μM capsaicin had little effect on live and apoptotic cells ($p>0.05$). Combined HA14-1 and capsaicin showed an additive effect on HBL cells which significantly reduced live

HBL cells from 76% of control to 40% ($p>0.05$) and increased late apoptotic cells from 18% in control cells to 52% ($p<0.05$). These results were as predicted for an additive effect.

For A375SM cells (Figure 7.10 B), 75μM HA14-1 on its own had little effect. 300μM capsaicin on its own reduced live cells to 44% and increased apoptotic cells to 55%, but this was not significant ($p>0.05$). However, the combined agents significantly reduced live A375SM cells to 18% ($p<0.001$) and significantly increased late apoptotic cells to 80% ($p<0.01$), showing an additive effect of these combined agents as predicted.

For C8161 cells, HA14-1 and capsaicin individually had no effect. However the combined agents significantly reduced live C8161 cells from 84% in control cultures to 50% ($p<0.05$) and significantly increased late apoptotic cells from 12% in control cells to 44% ($p<0.01$). This outcome clearly shows a synergistic effect ($p<0.05$) of these combined agents compared to the predicted additive values, (see Figure 7.10 C).

For fibroblasts (see Figure 7.10 D), 75μM HA14-1 and 200μM capsaicin individually had no effect on cell viability, neither did a combination of the two.

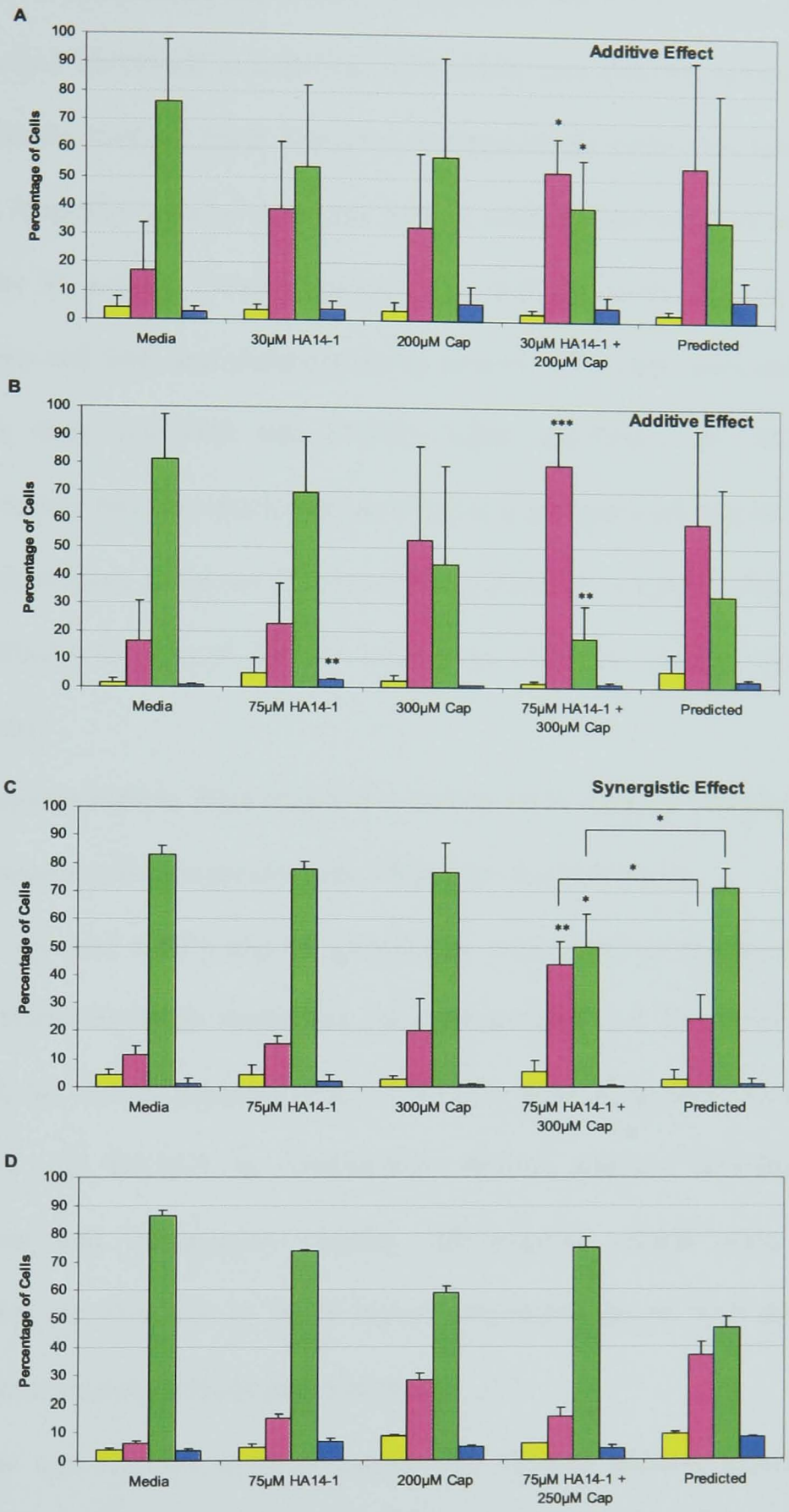


Figure 7.10. Effects of combined HA14-1 and Capsaicin on (A) HBL, (B) A375SM, (C) C8161 melanoma cells and (D) fibroblasts. ■ = Nuclear debris, ■ = Late apoptotic, ■ = Live viable, ■ = Early apoptotic. Values are expressed as Mean+SD. N=2 in duplicates. For fibroblasts N=1. * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$, relative to control media.

Table 7.4 summarises the effects of capsaicin and HA14-1 on melanoma cells, melanocytes and fibroblasts in reducing cell viability and inducing apoptosis comparing the three different methods used. The main findings of this study were that:

1. 10^{-3} μM ibuprofen induced apoptosis in HBL cells but had no effect on C8161 cells.
2. Capsaicin at around 150μM reduces the total metabolic activity of the three melanoma cell lines and melanocytes by around 50% (HBL cells are slightly more sensitive than A375SM and C8161) while fibroblasts are able to tolerate approximately twice as much capsaicin before any significant loss of viability.
3. Capsaicin induces apoptosis (assessed by the Annexin-V assay) with an IC50 range from 220μM to 320μM for the melanoma cells but greater than 500μM for fibroblasts.
4. Higher concentrations were required to induce DNA damage: 440μM to 500μM for the melanoma cells but greater than 500μM for the fibroblasts.
5. HA14-1 induced a 50% loss of viability at concentrations ranging from 60μM to greater than 100μM for melanoma cells and melanocytes. For fibroblasts the IC50 was 175μM. Similar concentrations of HA14-1 induced apoptosis in these cells.
6. Capsaicin and HA14-1 in combination showed additive inhibitory effects on melanoma and melanocyte viability. (Synergistic effects were observed on fibroblasts but this was at much higher concentrations of both drugs than were tested on melanoma cells or melanocytes).
7. Capsaicin and HA14-1 in combination also showed additive effects in inducing HBL and A375SM apoptosis and, surprisingly, a synergistic effect on C8161 cells. These combined agents (at similar concentrations) showed no effect in inducing fibroblast apoptosis.

Table 7.4 also shows that fibroblasts were sensitive to the combined effects of capsaicin and HA14-1 when used at concentrations that were sufficient to reduce viability by 50% (350μM) and induce 50% apoptosis (100μM), at which point a synergistic effect was seen.

Table 7.4. Summary of the effects of capsaicin and HA14-1 viability and apoptosis in melanoma cells and normal skin cells. (IC50 values).

	Capsaicin (μM)			HA14-1 (μM)		Combined	
	MTT	**Annexin V**	**DNA Damage**	**MTT**	**Annexin V**	**MTT**	**Annexin V**
HBL	100	220	480	60	48	100μM Capsaicin 30μM HA14-1 ADDITIVE	200μM Capsaicin 30μM HA14-1 ADDITIVE
A375SM	150	350	440	70	85	150μM Capsaicin 50μM HA14-1 ADDITIVE	300μM Capsaicin 75μM HA14-1 ADDITIVE
C8161	150	320	500	>100	85	150μM Capsaicin 50μM HA14-1 ADDITIVE	300μM Capsaicin 75μM HA14-1 SYNERGISTIC
Melanocytes	150	Not done	Not done	100	Not done	150μM Capsaicin 50μM HA14-1 ADDITIVE	Not done
Fibroblasts	350	>500	>500	175	112	350μM Capsaicin 100μM HA14-1 SYNERGISTIC	250μM Capsaicin 75μM HA14-1 NO EFFECT

7.5. DISCUSSION

The aim of this study was to examine 1) the mechanisms of ibuprofen in reducing melanoma cell migration by inducing apoptosis and, 2) whether a combination of capsaicin and HA14-1 would have a useful pro-apoptotic effect on melanoma cells. To do this, the effects of both drugs were examined on melanoma cells, melanocytes and fibroblasts looking at their effect on cell viability and apoptosis. Viability was assessed using a simple metabolic assay (MTT test) and two methods for examining apoptosis were used, Annexin V binding to PS on the cell membrane and DNA damage.

Initial experiments with ibuprofen showed that C8161 were not affected by TNF-α and ibuprofen at any concentration used. However 10^{-3}M ibuprofen had a marked effect in inducing apoptosis in HBL cells. The anti-inflammatory effects of ibuprofen on melanoma cells are linked to the inhibition of cyclooxygenase synthesis and the inhibition of prostaglandin production and hence an anti-inflammatory effect. However, ibuprofen has been reported only once as an apoptotic inducer in murine melanoma and in a human cell line of colon carcinoma (Lönnroth *et al.*, 2001). How ibuprofen induces apoptosis in the less aggressive melanoma cells remains to be clarified.

The main findings of this study were that capsaicin at around 150μM reduces the total metabolic activity of the three melanoma cell lines and melanocytes by around 50% (HBL cells are slightly more sensitive than A375SM and C8161) while fibroblasts are able to tolerate approximately twice as much capsaicin before any significant loss of viability. Capsaicin induces apoptosis (assessed by the Annexin-V assay) with an IC50 range from 220μM to 320μM for the melanoma cells but greater than 500μM for fibroblasts. Higher concentrations were required to induce DNA damage: 440μM to 500μM for the melanoma cells but greater than 500μM for the fibroblasts.

HA14-1 induced a 50% loss of viability at concentrations ranging from 60μM to greater than 100μM for melanoma cells and melanocytes. For fibroblasts the IC50 was 175μM. Similar concentrations of HA14-1 induced apoptosis in these cells.

Capsaicin and HA14-1 in combination showed additive inhibitory effects on melanoma and melanocyte viability. (Synergistic effects were observed on fibroblasts but this was at much higher concentrations of both drugs than were tested on melanoma cells or melanocytes).

Capsaicin and HA14-1 in combination also showed additive effects in inducing HBL and A375SM apoptosis and, surprisingly, a synergistic effect on C8161 cells. These combined agents (at similar concentrations) showed no effect in inducing fibroblast apoptosis. Fibroblasts were also sensitive to the combined effects of capsaicin and HA14-1 when used at concentrations that were sufficient to reduce viability by 50% (350μM) and induce 50% apoptosis (100μM), at which point a synergistic effect was seen.

Capsaicin is a well known inducer of apoptosis in cancer cells (Dasgupta *et al.*, 1998; Morré *et al.*, 1997; Mori *et al.*, 1996) and in melanoma cells (Patel *et al.*, 2002a Patel *et al.*, 2002b; Gong *et al.*, 2005). In this study, experiments using a DNA damage profile demonstrated that capsaicin induced apoptosis in HBL and A375SM cells at 300μM. C8161 cells were affected only at a higher capsaicin concentration of 500μM. At 500μM capsaicin all three melanoma cells had more than a 50% reduction in cell viability. Fibroblasts were not affected by capsaicin at up to 500μM. These results suggest that capsaicin may cause DNA breakage more readily in melanoma cells than in normal skin cells such as fibroblasts. The selective apoptosis inducing effect of capsaicin has been previously reported (Macho *et al.*, 1999; Kang *et al.*, 2003).

There are many reports suggesting mechanisms of action of capsaicin inducing apoptosis. For example, extra production of ROS by mitochondrial NADH oxidase

(Morré *et al.*, 1997; Surh, 2002), *ras* activation inducing apoptosis in transformed cells (Gong et al., 2005), Bcl-2 down-regulation and caspase 3 activation (Jun *et al.*, 2007; Jung *et al.*, 2001) and inhibition of IL-6-induced STAT3 activation (Bhutani *et al.*, 2007). In this study, the effects of capsaicin on inducing DNA damage are very clear suggesting that this is one mechanism for explaining apoptosis.

What are the molecular mechanisms by which capsaicin selectively induces apoptosis in transformed cells without affecting normal skin cells? One report from Bodó *et al.* (2004) demonstrated that functional VR1 (Vanilloid Receptor -1) which capsaicin interacts with, was present on human epidermal cells, specifically keratinocytes and Langerhans cells, but not on melanocytes; it was also present in dermal cells, mast cells, sweat gland epithelium, sebocytes, endothelial cells and smooth muscle cells but not, interestingly, in fibroblasts. The lack of response of fibroblasts to capsaicin may be based on their lack of receptors but on the other hand, Kim *et al.* (2004) reported the existence of VR1 receptors on fibroblasts. Clearly this is an area that requires further investigation.

As summarised in Table 7.4 capsaicin and HA14-1 each reduced cell viability and induced apoptosis in the melanoma cells, melanocytes and fibroblasts but at different concentrations. With respect to metabolic activity of the cells (which is what the MTT assay measures) this was reduced by capsaicin with the melanoma cells and melanocytes showing a similar sensitivity. Fibroblast viability was also reduced by capsaicin (arguing for the presence of VR1 receptors on these cells) but it required approximately twice the concentration of capsaicin compared to that which reduced viability in melanocytes and melanoma cells. For all cells higher concentrations of capsaicin (approximately twice as high) were required to show induction of apoptosis as evidenced by using the Annexin-V assay and still higher concentrations as measured by the assay for DNA damage.

The differences found using these different assays are much as expected. The MTT assay detects a reduction in cell viability and as such is quite sensitive. Not all cells with a reduced viability will necessarily go on to become apoptotic or die. Many cells may recover.

For the apoptotic assessment (Annexin-V and DNA damage assays), higher capsaicin concentrations were needed to demonstrate a similar result, showing the same sensitivity pattern as HBL were more sensitive followed by A375SM and C8161 cells and fibroblasts.

Annexin-V binds specifically to phospholipids at early stage of the apoptotic process (a disruption of membrane phospholipids asymmetry exposes PS on the outer cytoplasmatic membrane). In contrast the DNA damage assay is based on breaks in doubled-stranded DNA resulting in the phosphorylation of the histone variant H2AX at serine 139. This measures late apoptosis (by which time physical breaks in the DNA have occurred) so it is entirely as expected that the IC50 values for capsaicin assessed using this assay were higher than when using Annexin-V.

With respect to HA14-1, its mechanism of action was identified by Wang *et al.*, (2000). These authors verified that HA14-1 interacts with Bcl-2 *in vitro*. This protein induces apoptosis in a variety of tumour cell lines and cooperates with other drugs (Campàs *et al.*, 2006; Mena *et al.*, 2007).

Melanoma cells and melanocytes had a roughly similar sensitivity to HA14-1 as assessed by a loss of cell viability with IC50 values ranging from 60μM to over 100μM. For fibroblasts the IC50 was 175μM, again suggesting that these cells are more resistant than melanocytes and melanoma cells.

With respect to induction of apoptosis slightly lower concentrations of HA14-1 were required to produce apoptosis (compared to reducing cell viability) as assessed by Annexin-V (DNA damage was not undertaken in these experiments). The IC50 values

were 48-85μM for the three melanoma cells and 112μM for fibroblasts. This again is much as expected as this drug is known to interact directly with Bcl-2 to induce apoptosis directly.

The two agents were then tested together to see if agents thought to act by different mechanisms would show any useful additivity or synergy in their actions on melanoma cells. Combination therapies have been reported by others (Namkoong *et al.*, 2006; Mena *et al.*, 2007; Dal Lago *et al.*, 2008).

For both HBL and A375SM cells the effects of combining both were additive rather than synergistic, but interestingly for C8161 cells there was evidence of some synergy when Annexin-V was measured as an early indicator of apoptosis. This was encouraging as C8161 melanoma cells are particularly aggressive with respect to metastases. Concerning loss of viability the effects of combining the two were additive rather than synergistic.

For melanocytes combining the two gave a loss of viability that was additive (effects on apoptosis were not studied in these cells). For fibroblasts the results appear contradictory at first glance (Table 7.4) in that there is apparent synergy with respect to loss of viability but no effect when these two agents were combined on apoptosis. However this is explained by the concentrations used.

The concentrations of capsaicin and HA14-1 that were used in measuring early stage apoptosis in fibroblasts were similar to those used for the melanoma cells. However, in studying viability, as the fibroblasts had proven relatively resistant to capsaicin and HA14-1, higher concentrations of both were studied and then a combination of the two was found to be synergistic. This argues strongly for fibroblasts having a lower expression of the VR1 receptor rather than being entirely lacking in it.

Capsaicin and HA14-1 showed a synergistic effect on C8161 cells. This study suggests that these agents may have different mechanisms of action in inducing

apoptosis in melanoma cells. As previously reported capsaicin induces oxidative stress (Morré *et al.*, 1996; Brar *et al.*, 2001), regulates activation of NF-kB and IL-8 (Patel *et al.*, 2002a) and hypoxia inducing factor-1-alpha in human melanoma (Patel *et al.*, 2002b), but also inhibits Bcl-2 anti-apoptotic activity (Jung *et al.*, 2001; Jun *et al.*, 2007), whilst HA14-1 blocks the anti-apoptotic Bcl-2 protein (Tian *et al.*, 2007; Wang *et al.*, 2000) inducing melanoma cell apoptosis. Thus there is some overlap in their activity in that both are reported to inhibit Bcl-2 anti-apoptotic protein.

In conclusion this comparative study has shown that melanoma cells and melanocytes have a similar sensitivity to capsaicin while fibroblasts are more resistant to this agent. HA14-1 induces apoptosis at relatively low concentrations and a combination of the two agents produces a useful additive affect for 2 out of 3 of the melanoma cancer lines studied and encouragingly for the most metastatically aggressive cancer cell line (C8161), a combination of the two showed some evidence of synergy.

The results suggest that a natural agent (which is perhaps common in one's diet) such as capsaicin can be used in combination with another organic compound, HA14-1, as a pro-apoptotic agent. The advantages of a combined therapy include using the two drugs at lower concentrations which reduces toxicity and side effects for the patient while promoting improved cell apoptosis. This may be a promising alternative therapy for those patients with malignant melanoma for whom at present there are few chemotherapy approaches.

CHAPTER 8

GENERAL DISCUSSION

8.1. GENERAL DISCUSSION

Melanoma is the most dangerous type of skin cancer and affects millions of people worldwide. Its incidence is increasing every year and therefore prevention and treatment of melanoma has become a major health issue. Treatment of melanoma is difficult as it spreads quickly in the body if no treatment is undertaken or if treatment of a primary melanoma is not successful.

Treatments for malignant melanoma do exist and are successful in thin and superficial melanoma which can be surgically removed. However, melanoma thicker than 1mm indicates a poor prognosis as melanoma cells invade and spread quickly in the body. As these cells travel in the body, the next stage for melanoma is their installation in the lymph nodes. At this stage, the treatments available are the surgical removal of melanoma from the lymph nodes followed by chemotherapy. Unfortunately the drugs available so far are not as effective for melanoma as for other types of cancer, making it difficult to maintain a good quality of life and improved survival for patients.

This study was based on the growing realisation of the role played by inflammation in cancer and on the phenomenon of local recurrence after surgical excision of primary melanoma. In the latter there is always a wound environment and inflammation associated with wound healing.

Accordingly this study investigated how inflammation accelerates melanoma cell migration and invasion and to what extent anti-inflammatories may be useful in blocking migration and invasion and inducing apoptosis. More specifically, it investigated whether anti-inflammatories can block or reduce 1) melanoma cell migration, 2) melanoma cell invasion in a wounded and inflamed tissue engineered human skin model and 3) melanoma cell survival.

Initially in this study the role of the NSAIDs (ibuprofen and capsaicin) in opposing the effect of the pro-inflammatory cytokine TNF-α on human melanoma cell

migration were examined. Either ibuprofen or capsaicin reduced melanoma cell migration in unstimulated and TNF-α-stimulated cells at concentrations which were not toxic to the cells. The melanoma cell lines were previously described to have different levels of migration. Although this was a simple model of cell migration, the effects of ibuprofen and capsaicin were seen to be more effective in the 2 least aggressive melanoma cell lines. This study supports the hypothesis that a pro-inflammatory cytokine TNF-α stimulates melanoma cell migration as demonstrated by (Katerinaki *et al.*, 2003; Katerinaki *et al.*, 2006 Lejeune *et al.*, 2007; Bachmann *et al.*, 2008) and many type of tumour development (Lin *et al.*, 2010) and that NSAIDs such as ibuprofen and aspirin is effective for cancer prevention and therapy (Harris, 2007).

Following the studies on migration the work next progressed to studying invasion of melanoma cells in 3D models. Two models were studied - a simple model of cell migration through fibrin and a more complex physiologically relevant model of cell invasion through 3D tissue engineered skin.

In the 3D model of melanoma cell invasion in a fibrin scaffold some of the cell lines (HBL and A375SM cells) destroyed the scaffold after being incubated with it for 24 hours. Therefore it was not possible to study melanoma cell invasion in this model.

Observations by eye were that the melanoma cells showed different patterns of interaction with the fibrin clot. For this reason, it was decided to investigate the degradative capability of melanoma cells cultures in a fibrin scaffold using a protein assay.

Surprisingly, the two least aggressive melanoma cells were the ones which most degraded the fibrin scaffold. On the other hand, C8161, the most aggressive cell line did not. This suggests that these more aggressive cells quickly invaded the fibrin without degrading the fibrin scaffold.

Degradation of fibrin clot scaffold, probably occurred by the action of MMPs produced by melanoma cells. Accordingly, Hofmann *et al.*, (2000) reported that members of the MMP family with their natural tissue inhibitor (TIMP) are not only over-expressed in tumours cells but also by stromal fibroblasts. However, in this 3D fibrin model, only melanoma cells were present suggesting that MMPs were active in the least aggressive melanoma cell lines.

One possible way to reduce melanoma cell invasion and degradation of a fibrin clot scaffold is NSAIDs treatment, as reported by Tsai *et al.* (2009). An interesting finding was that capsaicin reduced the degradative capability of the two (the least aggressive) melanoma cell lines which most degraded the fibrin scaffold. The reasons for this may be an anti-inflammatory effect of capsaicin (Zegarska *et al.*, 2006; Southall *et al.*, 2003) in reducing integrin-binding to fibrin or down-regulating the membrane-bound enzymes (MMP-2 and MMP-9), attenuating the degradative capability of these cells. Another explanation for this is the apoptotic effects of capsaicin in reducing melanoma cell survival (Patel *et al.*, 2002a; Patel *et al.*, 2002b; Mori *et al.*, 1996; Morré *et al.*, 2003; Gong *et al.*, 2005; Jun *et al.*, 2007). However, the exact mechanisms by which the melanoma cells degraded the 3D fibrin scaffold remains to be clarified.

Degradation and remodelling of extracellular matrix by proteolytic enzymes are indispensable for melanoma cell invasion. This is well described by Hofmann *et al.* (2000) and Hoffmann *et al.* (2005). Other studies showed that NSAIDs suppressed the expression of MMP-2 (Pan *et al.*, 2001) and MMP-9 (Murono *et al.*, 2000) reducing cancer cell invasion.

In conclusion, the 3 melanoma cell lines studied were found to degrade the fibrin scaffold differently. The least aggressive ones (HBL and A375SM cells) were capable of degrading the fibrin scaffold whilst the most aggressive C8161 cells were not.

Capsaicin reduced the degradative capability of HBL and A375SM cells and had no effect on C8161 cells.

These results were very interesting, however, it was decided to develop a more challenging model of tissue engineered skin in which a wound was made and an inflammatory microenvironment was created to investigate melanoma cell invasion.

There are many types of scaffolds used in tissue engineered skin models (Price *et al.*, 2008). Tissue engineered skin models showed more consistent outcomes and imitate more closely the structural and physiological conditions of skin (Chakrabarty *et al.*, 1999; Eves *et al.*, 2000; Eves, 2001). Accordingly, this model proved to be a good model of tissue engineered human skin for studies of inflammation and melanoma cell invasion.

Following the experiments in tissue engineered skin with addition of melanoma cells, confirm that melanoma cell interact with keratinocytes and fibroblasts in the extent to which they invade into the skin composite but this relationship appears to be dictated by the melanoma cells. Different patterns of cell invasion were confirmed in which HBL and A375SM cells were less invasive and C8161 cells were the most invasive cells. This has been previously reported by Eves *et al.* (2000). Initial attempts to add ibuprofen or capsaicin to tissue engineered skin showed poor skin composites because the melanoma cells did not survive in higher concentrations of ibuprofen or capsaicin. Lower concentrations were used in future experiments.

Cutaneous melanoma cells are well known for their invasiveness and rapid metastatic capacity and this is regulated by adhesion molecules, matrix metalloproteinases, chemokines and growth factors (Zbytek *et al.*, 2008). The expression of adhesion molecules and proteases can be associated with inflammation (Eves *et al.*, 2003; Zhu *et al.*, 2002).

In terms of melanoma cell invasion in tissue engineered skin, one important result was that when fibroblasts were added with melanoma cells, cell invasion stopped (no keratinocytes were present in these tissue engineered skin). This is clearly seen when one compares melanoma cell invasion on their own and when melanoma cells are added together with the other skin cells (fibroblasts and keratinocytes). It may be that fibroblasts maintain a stronger dermis by producing more or better organised collagen making it difficult for the two least aggressive melanoma cells to invade, as reported by Quintanilla-Dieck *et al.* (2009) who suggested that a possible upregulation of skin conditions (such as scarring and inflammation) in which the prominent matrix degrading properties of cathepsin K in fibroblasts are thought to require tight regulation to maintain the homeostasis of the extracellular matrix.

Melanoma cells and fibroblasts activate MMPs to facilitate melanoma cell invasion as demonstrated by Hofmann *et al.* (2000). Conversely, in this study, a different result was found as fibroblasts blocked melanoma cell invasion. However, it was suggested by (Ntayi *et al.*, (2003) that fibroblasts may influence melanoma cell invasion after the beginning of tumour progression through the dermis. Therefore, it may be possible that fibroblasts did not facilitate melanoma cell invasion as there was no previous invasion by melanoma cells in the reconstructed skin.

The presence of keratinocytes influenced melanoma cells to invade in the dermis, as seen for A375SM cells. Therefore equilibrium between the beneficial work of fibroblasts in blocking or stopping melanoma cell invasion and the encouragement of cell invasion by keratinocytes should be investigated further.

Once the pattern of melanoma cell invasion in tissue engineered skin was established, studies progressed on to the development of wounded tissue engineered skin with the aim of using these to carry out studies of the impact of wounding and of inflammation on melanoma invasion.

Initial attempts of mechanical wounding with a syringe needle were unsuccessful as the wounds were not visible. However a single cut with a scalpel blade showed a clear visible full thickness wound and this was used for experiments.

For a better understanding of wound healing, simple experiments were conducted in which there was a gradual addition of fibrin clots and cells to the tissue engineered skin. The addition of fibrin clots and fibroblasts in acellular DED demonstrated that fibroblasts are essential for healing and keeping the dermis in good condition. This is explained by the synthesis of extracellular matrix by dermal fibroblasts.

Mechanical wounding increased invasion of the two least aggressive melanoma cell lines in tissue engineered skin.

Wounded tissue engineered human skin was found to "heal" after mechanical wounding, even without any innate immune cells or vasculature. One process that may have helped healing in this skin model was contraction, a common process in skin wound healing. During tissue repair, fibroblasts can be transformed into a proliferative and contractile type of cells called myofibroblasts. These cells show the same characteristic of smooth muscle cells and take part in skin wound contraction (Nedelec *et al.*, 2000; Darby and Hewitson, 2007). Basically, by contraction, myofibroblasts pull in the edges of the wound resulting in a scar.

Addition of fibrin clots and TNF-α helped to heal the wounds and reduced melanoma cell invasion in 2 out of 3 cell lines. This paradox may be explained by the quick healing helped by the fibrin clots plus TNF-α which are both normally present in a wounded and inflamed healing bed.

Evidence from previous studies suggests that TNF-α may be acting via up-regulation of NF-κB and integrin expression. Both normal and tumour cells express integrins, which are cell surface molecules that are involved in attachment to the

extracellular matrix, motility and degradative enzyme production in malignant cells. Malignancy of melanoma cells has been shown to be related to integrin expression, and therefore TNF-α stimulation of these molecules may play a pivotal role in malignant cell migration and invasion (Zhu *et al.*, 2002). MMPs are described as being crucial in the invasion of malignant cells, in many cancer cells types including melanoma (Katerinaki *et al.*, 2003). However, there was little to suggest that melanoma cells used their own degradative enzymes for invasion, rather the results were consistent with the melanoma cells invading through a dermal matrix in which keratinocytes and fibroblasts were expressing activated levels of MMP2 and 9. This leads one to suggest that melanoma cells in a wounded bed may easily invade in the dermis in which the matrix is already being modelled as part of the wound healing programme.

Another important result in this work was that mechanical wounding increased melanoma cell invasion and this was associated with significant breakage or damage in the dermis in 2 out of 3 melanoma cell lines added to the tissue engineered skin. If this is related to the phenomenon of local recurrence of a primary melanoma excision, this is a demonstration that residual cells or "circulating" cells would invade further into the dermis and, facilitate further local or proximal melanoma recurrence.

In terms of melanoma cell invasion, wound healing and dermal integrity, the results varied. HBL cells, the least aggressive melanoma cell lines did not facilitate healing the wounds and damaged the dermis. A375SM cells had an intermediate level of invasion and were demonstrated to heal the wounds, with less damage to the dermis. C8161 cells, the most aggressive of the three cell lines, invaded the dermis independently of the other cells added to the skin composites and treatment used. Surprisingly C8161 cells promoted complete wound healing with a good dermal integrity. The different patterns of melanoma cell invasion in this model may be explained by the MMPs activation. Increased expression and activation of MMPs has

been associated with melanoma progression (Hofmann *et al.*, 2005). MMP-2 was only observed in highly invasive cell lines and MMP-9 was expressed in cell lines derived from advanced primary melanomas (MacDougall *et al.*, 1995; Hoffmann *et al.*, 2000) whereas TIMP-1 and TIMP-2 reduced murine melanoma cell metastatic potential (Khokha, 1994). There are some studies suggesting that a proteolyitc balance between MMPs and TIMPs (Tissue Inhibitor of Metalloproteinases) determines melanoma progression (Henriet *et al.*, 1999, MacDougall *et al.*, 1999, Cruz-Munoz and Khokha, 2008). However, there is still some evidence that tumour growth and metastasis may be also directed by surrounding fibroblasts (Kurihara *et al.*, 2009). Another possible contributor for tumour cell invasion and growth is EMMPRIN (Extracellular Membrane Metalloproteinase Inducer) which is highly expressed in tumour cells (Nabeshima *et al.*, 2005) and stimulates MMPs production in surrounding fibroblasts (Gabiscon *et al.*, 2005) and may contribute for melanoma cell invasion.

The tissue engineered skin model which was mechanically wounded to study inflammation and melanoma cell invasion prove to be very complex. Therefore it was decided to choose one melanoma cell type for a complete study of the effects of inflammation and melanoma invasion in this model. For this, A375SM cells were chosen as they were known to have intermediate levels of invasion, wound healing and dermal integrity in tissue engineered skin models.

In tissue engineered skin, A375SM melanoma cells were showed to invade and damage the dermis. Wound healing in these composites occurred at an intermediate level. Wounds were healed with the addition of a hydrogel carrier but there was still considerable damage to the dermis. Ibuprofen however reduced these deleterious effects of melanoma cell invasion when delivered as a topical treatment from a hydrogel.

One explanation for this is the anti-inflammatory effects of ibuprofen being released at a slow rate on the inflamed and wounded site of a tissue engineered skin.

The exact mechanism of ibuprofen which caused this effect remains to be clarified. However, the rationale behind this is that ibuprofen blocks COX activation in the cells which inhibits the production of prostaglandins and therefore inflammation is stopped or reduced.

In tissue engineered skin, the addition of ibuprofen may also inhibit the inflammatory condition stimulated by the addition of TNF-α to the cells. Hence, it may down-regulate integrins and metalloproteinase enzyme activation which are responsible for melanoma cell attachment and degradation, facilitating melanoma cell invasion. This is in fair agreement with MacDougall *et al.* (1995) who reported that MMP-9 production in melanoma cells was induced by either IL-1ß or TNF-α. Therefore NSAIDs could reduce melanoma cell invasion by suppressing the effects of inflammatory cytokines.

This wounded and inflamed tissue engineered skin model for melanoma cell invasion was found to be a useful model which supports the hypothesis of the link between inflammation and melanoma invasion.

Macroscopic observations of inflammation around solid tumours have been reported for a long time ago (Balkwill and Mantovani, 2001; Thompson *et al.*, 2004). However nowadays, it is possible to observe the micro-inflammatory condition in cells and tissues which is made possible by advances in molecular biology. The microenvironment around the tumours contains proinflammatory cytokines such as TNF-α which is known to be responsible for the activation of NF-kB transcription factor (Zhu *et al.*, 2002; Cantón *et al.*, 2003; Katerinaki *et al.*, 2006; Banno *et al.*, 2004; Pikarsky *et al.*, 2004).

NF-kB activation induced by TNF-α is becoming another "hallmark" of inflammation and cancer development. It is known that the switch-off of NF-kB activity prevents inhibition of apoptosis. Apoptosis is a "fail-safe" mechanism to keep tumor

formation at its lowest by the natural process of self-destruction of transformed cells, avoiding their proliferation and metastasis.

Finally, the last part of the study examined apoptosis in these melanoma cells and to what extent inflammation (induced by TNF-α) or the use of anti-inflammatories affected this.

Experiments conducted to observe the effects of ibuprofen on TNF-α stimulated cells showed that HBL cells were very sensitive to TNF-α on its own and the maximum concentration of 10^{-3}M ibuprofen induced significant apoptosis in these cells and reduced HBL survival. On the other hand, C8161 cells did not respond to the effects of TNF-α alone or TNF-α stimulated with ibuprofen. This was expected as C8161 are the most aggressive cell line. These cells proved resistant to the induction of apoptosis by pro or anti-inflammatory agents.

The different sensitiveness of melanoma cells to the same drug was demonstrated in this study. One intriguing result is how TNF-α on its own induced HBL cell apoptosis as TNF-α is known as a pro-inflammatory cytokine which promotes melanoma cell survival. The important result in these experiments is that prior stimulation with TNF-α and ibuprofen induced HBL cell apoptosis.

For the next set of experiments, capsaicin or HA14-1 were administered individually or combined and cell viability assessed.

There are some suggestions that combined therapies are better approach for melanoma treatment (Namkoong *et al.*, 2006; Mena *et al.*, 2007; Dal Lago *et al.*, 2008; Valero *et al.*, 2009). A combined therapy containing capsaicin as a natural component and HA14-1 a small molecule organic compound showing pro-apoptotic properties was used.

These results showed that melanoma cells and melanocytes a had similar sensitivity to capsaicin or HA14-1 (used individually or combined) and that fibroblasts were shown resistant to these drugs.

For apoptosis, different methods were used to observe the effects of capsaicin and HA14-1 on melanoma cells and fibroblasts. The first method was Annexin V which showed early events of apoptosis and the second method was the DNA damage assay which shows later events of apoptosis. Comparison of these two methods showed that higher concentrations of capsaicin and HA14-1 were required to observe induced apoptosis in these.

Combined administration of capsaicin and HA14-1 in inducing apoptosis in melanoma cells showed an additive effect on the least aggressive melanoma cells and fibroblasts at similar concentrations. Surprisingly and importantly, a synergistic effect of combined capsaicin and HA14-1 in inducing apoptosis was shown in the most aggressive cell line C8161 cells.

Similar results on the apoptotic effects of capsaicin (Morré *et al.*, 1996; Gong *et al.*, 2005; Jun *et al.*, 2007); and synergistic effect of HA14-1 and a epidermal growth factor receptor ErbB tyrosine kinase inhibitor (lapatinib) in human breast cancer cells (Witters *et al.*, 2007) were previously reported. The synergistic effects of capsaicin and HA14-1 on the most aggressively melanoma cell line may be due to the different mechanisms of action of capsaicin and HA14-1 and the overlap effects of both drugs. However, this needs further investigation.

Isolated or combined therapies of capsaicin and HA14-1 demonstrated that fibroblasts tolerated higher concentrations of these agents. This seems to be an important result for melanoma target therapies, in which normal cells would remain less affected.

In conclusion, this comparative study of apoptosis showed 1) how melanoma cells and melanocytes have a similar sensitivity to capsaicin while fibroblasts cope with higher concentrations; 2) how HA14-1 induces apoptosis at relatively low concentrations; and 3) that a combination of the two in the main produces the expected results of an additive affect for 2 of 3 melanoma cancers but encouragingly for one particularly metastatic aggressive cancer, a combination of the two shows synergy.

8.2. FINAL CONCLUSIONS

In conclusion the NSAIDs ibuprofen reduced melanoma cell migration and invasion, and accelerated repair to the dermis and wound healing in tissue engineered human skin. This suggests that NSAIDs could be used as a topical treatment to reduce melanoma metastatic spread following initial excision of primary melanoma.

Also combined therapy of a natural NSAID (capsaicin) and a pro-apoptotic agent (HA14-1) reduced melanoma cell survival without extensive cytotoxicity suggesting a promising alternative chemotherapy for advanced melanoma.

8.3. LIMITATIONS OF THIS STUDY

Clearly, the interpretation of these results must be with caution as in the 2D model for melanoma cell migration there was no assessment of melanoma cell proliferation or density. Cell proliferation is an important aspect in a migration model, in particular, for melanoma cells, as they can proliferate faster than normal cells.

The 3D fibrin clot model proved impossible to observe melanoma cell invasion. Therefore an investigation of melanoma cell degradative capability in a fibrin clot scaffold was carried out. This was a simple 3D model of melanoma cell invasion and there was no interaction between melanoma cells, fibroblasts, keratinocytes or a complex extracellular matrix.

The 3D tissue engineered human skin model for melanoma cell invasion result must be also interpreted with caution. These models of tissue engineered human skin did not have either innervation or vascularity. In addition to this, there were no innate immune cells to interact and to result in a more complex immunological system of melanoma cell invasion and wound healing. In addition to this, although the inflamed and wound skin model "healed" in the presence of ibuprofen delivered from a hydrogel, the extension and depth of the wound were not considered for a controlled wound healing. However, the evidence presented from this 3D reconstructed skin suggests that mechanical wounding is associated with increased invasion of melanoma cells but that this can be attenuated by the addition of ibuprofen (either as free drug or released from a hydrogel). If one relates these findings to the phenomenon of local recurrence of melanoma then there is an argument to be made for reducing inflammation at the site of melanoma excision following excision of a primary melanoma. This could be achieved by the addition of ibuprofen either systemically or possibly, more effectively released from a hydrogel preparation and placed on the wound bed post surgery. Interestingly the results also indicated that there was less damage to the dermis following wounding when ibuprofen was administered in this model.

There was a comparative study on the effects of a natural component (capsaicin) or a pro-apoptotic agent (HA14-1) on melanoma cells, melanocytes and fibroblasts viability and, the induction of apoptosis in melanoma cells and fibroblasts after treatment with capsaicin or HA14-1 alone or the combination of these agents. Different methods were used to evaluate viability and apoptosis in melanoma and control cells (melanocytes and fibroblasts). However, it could be investigated the possible mechanisms of action of capsaicin and HA14-1 in inducing apoptosis in melanoma cells.

The results suggest that the advantages of a combined therapy include using the two drugs at lower concentrations which reduces toxicity and side effects for patients with malignant melanoma as an alternative therapy.

8.4. FUTURE WORK

After this work, it would be worthwhile to continue the investigation of melanoma cell invasion in an inflamed and wounded tissue engineered skin. Some of these aspects that could be look at are:

1 To investigate the mechanisms of NSAIDs on melanoma cell migration, invasion and survival, looking at MMPs expression/production and the role of EMMPRIN.

2. To carry out more work on the reconstructed skin model to carry out the effects of ibuprofen released from a hydrogel and other NSAIDs on migration of other melanoma cell lines in a wounded and inflamed bed.

3. To create a computational model of melanoma cell migration and invasion in 2D and wounded 3D skin model respectively and, consequently, simulate the effects of NSAIDs and pro-inflammatory cytokines in a wounded tissue engineered model.

It would also be very interesting:

1. To investigate closely the apoptotic mechanisms of capsaicin and HA14-1 looking at Bcl-2 levels in melanoma cells. Also it would be interesting to look at the effect of other natural agents with pro-apoptotic properties in melanoma cells.

2. To further investigate the effects of combined new drugs and identify targets for metastatic melanoma looking at pro-apoptotic agents.

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The University Of Sheffield.

PATIENT INFORMATION SHEET

Donating clinically surplus human skin for laboratory-based research

Sheffield Teaching Hospitals NHS
NHS Foundation Trust

Professor Sheila MacNeil
Tissue Engineering Group
The Kroto Research Institute.
North Campus.
University of Sheffield.
Broad Lane.
Sheffield. S3 7HQ
South Yorkshire. UK

Tel: (0)114 2225995

You are about to undergo surgery (abdominal reduction, breast reduction or body lift) that involves the removal of excess skin from your body. This skin would normally be discarded at the end of the operation. However, I would like to ask you whether you would be willing to donate this skin to our research laboratories at the Kroto Research Institute, Sheffield. You will not have to undergo any extra tests or procedures as a result of donating your skin and no extra skin will be removed as a result of your participation in this study.

Please take time to read the following information and discuss it with others if you wish. Ask, if there is anything that is not clear, or if you would like more information. Take time to decide whether or not you wish to donate the skin that will be removed in your operation.

All patients undergoing breast, abdominal reduction surgery or body lift will be approached regarding the donation of their skin to the research laboratories. However, it is up to you to decide whether you would like to donate your skin for this purpose. If you do decide to donate your skin, you will be given this information sheet to keep and will be asked to sign a consent form. If you decide not to donate your skin, your treatment will not be in any way affected by your decision.

Background Information

Over the last 15 years, my research group has been developing a skin-substitute for patients with burns or chronic wounds. Eventually we hope to be able to offer this skin substitute to patients with severe burns who do not have enough of their own skin to use for skin grafts. We also use this skin substitute to simulate the behaviour of normal skin under laboratory conditions.

The laboratory-based component of this research involves obtaining skin from patients such as yourself who are having skin removed, and isolating and growing skin cells from these samples. We also sterilise samples of skin to remove all the cells from them and use these sterilised samples to make new skin. If you agree to donate your skin, it will only be used in the laboratory to help us with our research. It will not be used on any patients or animals.

Skin donation

If you donate your skin, it will be treated as a freely donated gift from you to the Tissue Engineering Group. However, the knowledge gained from the gift of skin samples such as yours will hopefully be used to benefit many patients in the future.

Once you have donated your skin sample, it will be given an anonymous reference number. The only records kept by the research group are a record of your hospital number, the date of your operation and the anonymous number of your skin sample. No other personal details are kept on record. The skin will be used for experiments within the Tissue Engineering Group, University of Sheffield and will be stored in the Kroto Research Institute Laboratories, Sheffield. Once the experiments using your skin sample have been completed, it will be disposed of properly.

My research group will only use your skin to isolate cells and to make new skin in the laboratory. We will not do any tests on the skin that have any relevance to your current or future health.

My research group will aim to publish the results of our research in medical journals and present the results at national and international conferences related to plastic surgery and skin. If you would like to receive details of the results of our research, please write to me at the address on the front of this information sheet.

Thank you for taking the time to read this information sheet

Professor Sheila MacNeil

Sheffield Teaching Hospitals

Consent form for using samples of human skin for research

Thank you for reading the information about our research.
If you would like to take part, please read and sign this form,
a copy of which will be retained in your medical records.

Patient identification sticker

Centre:
Department of Plastic Surgery, Northern General Hospital, Sheffield

Title of project:
Development of tissue engineered wound models for wound healing research for patients with burns and chronic wounds.

Name of researcher: Professor Sheila MacNeil

Contact details for research team: Professor Sheila MacNeil, Tissue Engineering Group. The Kroto Research Institute. North Campus. University of Sheffield. Broad Lane. Sheffield. S3 7HQ. Tel (0114) 2225995

Please initial boxes

1. I have read the attached information sheet on this project, (Dated July 2006, version 3) and have been given a copy to keep. I have been able to ask questions about the project and I understand why the research is being done and any risks involved.

2. I agree to give a sample of skin for research in this project. I understand how the sample will be collected, that giving a sample for this research is voluntary and that I am free to withdraw my approval for the use of the sample at any time without giving a reason and without my medical treatment or legal rights being affected.

3. I understand that I will not benefit financially, or otherwise, if this research leads to the development of a new treatment or medical test.

4. I know how to contact the research team if I need to, and how to get information about the results of the research.

Please initial boxes

5. Consent for storage and use in possible future research projects

I agree that the sample I have given can be stored at the University of Sheffield for possible use in future projects to tackle some of problems which occur following burns injuries as described in the attached information sheet.

All donated skin samples will be stored anonymously and used only for laboratory research to benefit patients supervised by Professor MacNeil. All skin samples will be destroyed at the end of the experiments.

Name of patient (BLOCK CAPITALS)	Date	Signature
Name of person taking consent (if different from researcher)	Date	Signature
Professor Sheila Mac Neil Name of researcher	Date	Signature

Would you like to be sent information about the progress of this project? Please circle once.

YES / NO

Thank you for agreeing to participate in this research

Professor Sheila MacNeil
Professor of Cell and Tissue Engineering

Consent form version 3. June 16th, 2006