Establishing a method to transport and deliver adipose derived stem cells to chronic wounds

Sam Beckett BEng

# Abstract:

Chronic wounds present a challenge to heal due to the surrounding complications they often present with. These wounds are both debilitating and distressing to patients and can lead to amputation of the afflicted limb. Stem cell therapy is currently being investigated as a method of healing these wounds, however delivery and transport methods of stem cells are not universally agreed on.

The aim of this thesis was to present a method of delivering adipose derived stem cells to a human skin model, keeping cells viable whilst out of conventional culture conditions in a ‘ready to use’ product requiring no further processing at point of use.

Specific objectives were to assess which biomaterials readily supported cells over a period of 3 days. In this investigation human derived keratinocytes, fibroblasts, adipose derived stem cells and bone marrow derived stem cells were seeded onto synthetic poly(lactic-co-glycolic acid) or encapsulated in fibrin gels and cell viability assessed using an MTT assay.

These cell and scaffold combinations were further assessed when in transport conditions, left in ambient conditions (~22°C) with or without supplementing airtight containers with 5% CO2 to aid the carbonate buffering of the cell media, compared to controls at 37°C in a conventional incubator with 5% CO2 supplementation. It was found that maintaining pH by use of 5% CO2 supplementation was a requirement for keeping these cell types viable over 3 days.

Finally, characteristics of adipose derived stem cells were investigated when encapsulated in fibrin gels after being in transport conditions for 48 hours by their ability to differentiate, ability to migrate to a skin wound mode and retention of common stem cell markers.

In summary, human cells can be supported and maintained when outside of conventional culture conditions providing that pH of cell media is maintained by supplementing air with 5% CO2. Furthermore, adipose derived stem cells encapsulated in fibrin gels can be transported in these conditions whilst retaining common stem cell markers.

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**”You've got to reach for the stars, not for the ceiling.”**

Dr Sam Beckett, Quantum Leap (1989 - 1993)

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# Abbreviations:

|  |  |
| --- | --- |
| ADSC | Adipose Derived Stem Cell |
| Alpha MEM | Minimum Essential Medium Eagle - Alpha Modification |
| AP2 | Apidocyte protein 2 |
| BMSC | Bone Marrow Derived Stem Cell |
| CD | Clusters of differentiation |
| CEPBα | CCAAT/enhancer-binding protein alpha |
| DCM | Dichloromethane |
| DED | De-epidermised Dermis |
| DLX5 | Distal-Less Homeobox 5 |
| DMEM | Dulbecco's Modified Eagle's Medium |
| DMSO | Dimethyl Sulphoxide |
| EDTA | Ethylenediaminetetraacetic acid |
| FACS | Fluorescence-activated cell sorting |
| FBS | Foetal Bovine Serum |
| hDF | Human Dermal Fibroblast |
| hDK | Human Dermal Keratinocyte |
| i3T3 | Irradiated 3T3 cell |
| LPL | Lipoprotein Lipase |
| MSC | Mesenchymal stem cell |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| NaCl | Sodium Chloride |
| OSX | Osterix |
| PBS | Phosphate Buffered Saline |
| PGA | Poly(glycolic acid) |
| PLA | Poly(lactic acid) |
| PLGA | Poly(lactic-co-glycolic acid) |
| Pparγ | Peroxisome proliferator-activated receptor gamma |
| RFP | Red fluorescent protein |
| RPM | Rotations per minute |
| RUNX2 | Runt-related transcription factor 2 |
| SEM | Standard error of the mean |
| SF | Significant Figure |
| TCP | Tissue culture plastic |

# Aim:

The aim of this project is to develop and evaluate a cell carrier to deliver adipose derived stem cells expanded in a laboratory to skin wounds in an out- patient clinic. To achieve this it is important to keep cells alive in transport conditions for at least 48 hours whilst maintaining ‘stemness’ as a priority to ensure cells will retain their capacity for wound healing when used as a treatment.

# Objectives:

1. Assess stem cell viability on two common biomaterials; fibrin gels and electrospun PLGA scaffolds.
2. Investigate a solution to keeping cells viable for 48 hours or more in transportation conditions in the absence of a conventional cell incubator which would normally maintain the pH and temperature of the cells.
3. Evaluate if cells seeded in scaffolds post transport conditions for 48 hours retain their differentiation potential into adipose, osteoblast and chondrocyte lineages and have the ability to then migrate from transport biomaterials to tissue engineered skin.

# Chapter 1:

# Introduction

## Skin:

The largest organ of the body is the skin, accounting for approximately 16% of the human body weight [1]. The skin is responsible for a variety of functions, including the regulation of body temperature through sweating or vasoconstriction of small blood vessels. Touch, pressure and vibration sensation is possible due to mechanoreceptors within the skin. The skin is able to protect cells from damaging ultraviolet B light owing to the production of melanin. The skin also synthesises vitamin D, a necessary vitamin for bone health.

The mechanical protection of skin is provided by the high concentration of keratin in the cell membranes of the keratinocytes, cells that form the epidermis. Lipids are secreted between the cells of the epidermis, forming a permeability barrier. Acidic sebum is secreted to inhibit growth of pathogens [2][3], and skin shedding limits the build up of bacteria. This causes epidermal acidity, which is necessary for the maintenance of the water barrier [4]. Despite the defences of the skin in preventing microorganism growth, bacteria can be found in the outermost regions of the epidermis [5]. However, the natural skin flora can act as a defence to further infections by pathogens. For instance, Staphyloccus epidermidis acts as a defence by inhibiting virulent strains of Staphyloccus aureus from binding to keratinocytes [6].

Langerhans cells are present in the basal layers of the skin, and present antigens to T cells in order to initiate an immune response [7]. This allows constant surveillance of the epidermis from pathogen invasion.

## Skin structure:

The heterogeneous structure of skin can broadly be separated into 3 layers, the hypodermis, dermis and epidermis. The hypodermis is the innermost layer of skin, lying beneath the dermis. In between the dermis and the epidermis is the basement membrane, a collagen based matrix which anchors the protective epidermis to the dermis underneath.

#### Hypodermis:

Also known as subcutaneous fat, the hypodermis is mainly comprised of lobules of fat and loose connective tissue. The hypodermis functions as an energy store, a shock absorber and an insulator from heat loss.

#### Dermis:

Located below the epidermis, the dermis contains both fibrous and elastic tissues, with fibroblast cells embedded within the matrix. Mechanically, the skin needs to be both elastic and resilient in order to withstand the stresses and torsion of movement. Elasticity of skin is provided from elastin, whereas collagens I and III provide resistance against strain forces. The most abundant protein in skin is collagen type I, 85%, whereas type III collagen makes up between 10-15% [8]. Fibroblasts synthesise and secrete procollagen, which is cleaved by proteases and eventually matured into collagen fibres.

The dermis contains blood vessels to supply the surrounding tissues and avascular epidermis with nutrients and oxygen. Hair follicles are situated in the dermis and produce hair, providing some insulation from heat loss.

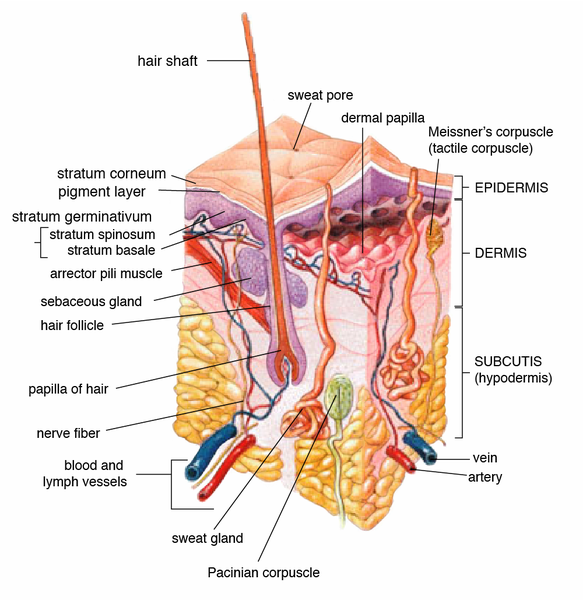


Figure 1: Structure of skin and structures. Figure from National Cancer Institute, SEER [9].

#### Epidermis:

As described by R. Wickett and M. Visscher, the epidermis is comprised of 5 integrated layers, the stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum and the stratum basale [1].

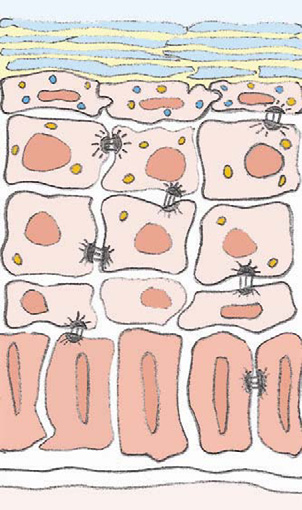
Stratum corneum

Stratum granulosum

Desmosomes

Lipid bilayer

Dead keratinocytes



Basement membrane

Stratum basale

Stratum spinosum

Figure 2: Structure of epidermis. (Adapted from A. Baroni et al, 2012) [10]

##### Stratum basale:

Basal cells joined to the basement membrane by hemidesmosomes, cell adhesion proteins, to form the stratum basale. This layer contains proliferative keratinocytes and a small number of melanocytes. Melanocytes are attached to the basement membrane and produce melanin, a pigment that protects cells from damaging ultraviolet B light. Melanosomes, melanin containing vesicles, are exported by the melanocyte dendrites to nearby keratinocytes, where they are uptaken [9].

Merkel cells can be found in the basal layer of the epidermis, their function is to act as a mechanoreceptor, and they may perform endocrine or paracrine processes [10].

##### Stratum spinosum:

As the basal cells proliferate, the cells move towards the surface and mature, forming the stratum spinosum. The keratinocytes are bridged by desmosomes, cell-to-cell adhesion proteins, giving them a ‘spiky’ appearance.

##### Stratum granulosum:

Keratinocytes mature into corneocytes in the stratum granulosum, whereby the nucleus is digested and lipids are secreted into the intracellular space. Corneocytes have a resistant cellular envelope of cross linked proteins, and contain keratin microfibrils to provide mechanical stiffness to the epidermis.

##### Stratum corneum:

The most external section of the epidermis consists of nonviable, stratified corneocytes known as the stratum corneum. The cells are arranged into layers, the number of which can vary a large amount depending on the anatomical location, from 10 layers on the neck to around 80 on the heel [11]. It takes approximately 28 days for cells to migrate from the stratum basale to the stratum corneum whereby the cell layers are shed to the environment. The outer layers of epidermis often have a high level of contamination from bacteria, which become a source of environmental contamination when they shed [1]. As the keratinocytes migrate towards the surface, they transform into corneocytes. Corneocytes gain chemical resistance from a cornified envelope consisting of proteins and a covalently bound lipid envelope [12]. Secretory organelles, or lamellar granules, are produced by corneocytes and contain enzymes which process lipids into hydrophobic products, and antimicrobial peptides [13].

Microfibrils of keratin form and lie parallel to the cellular surface. These act to reinforce the corneocytes from loading and limit swelling of the skin in the plane of the skin surface [1].

## Wound healing:

If the skin is injured deep enough to the extent of blood vessel damage, a vascular response is initiated to prevent further blood loss. Inactive von Willebrand factor (vWF) is continuously secreted by endothelial cells to blood plasma, and when vWF comes into contact with exposed collagen, binding and a subsequent conformational change activates vWF [14] [15]. The active form of vWF aids the adhesion of platelet receptors to collagen. Bound platelets become active and aggregate to prevent both blood loss and microorganism infiltration. When active, an intracellular increase in calcium leads to the release of histamine, clotting and growth factor containing granules.

Mast cells release histamine as part of the inflammatory process, increasing vasodilation and vessel permeability to allow white cells more rapid access to the wound, and simultaneously increasing the nutrients and oxygen available at the wound site [16]. Other proinflammatory cytokines are simultaneously released, which attracts neutrophils and macrophages to the wound [17].

Inflammatory cells migrate to the wound site by chemotaxis due to previously secreted factors. Neutrophils clear cellular debris and invading microorganisms whilst releasing reactive oxygen species (ROS) in the initial stages of inflammation to destroy bacteria and fungae, preventing invasion. Without neutrophil presence, severe infections and delayed wound healing occurs [18]. After the migration of neutrophils, monocytes migrate to the wound and differentiate into macrophages, continuing phagocytosis at the wound site and releasing additional ROS to the wound site.

The proliferative phase of wound healing is dominated by interactions between fibroblasts and keratinocytes, initially stimulated by macrophage secreted pro-fibrotic cytokines [19]. Angiogenesis is initiated and extracellular matrix is synthesised by fibroblasts to form granulation tissue, whilst keratinocytes proliferate and the wound edges begin to close. Some fibroblasts begin to exhibit contractile properties when under mechanical tension and differentiate to myofibroblasts, and act to contract the wound. Over the next few months and years the tensile strength of the wound increases as the collagen is remodelled from a random arrangement to a more regular and aligned structure, and excess capillaries regress such that the skin has a normal vascular density.

## Chronic wound formation:

Any impairment during the wound healing sequence can cause the wound healing to fail to progress. Many factors can influence negatively on wound healing, such as age, diabetes, poor nutrition and hypoxia. Most often, chronic wounds occur when the healing sequence is impaired in the inflammatory phase [20]. The depth, cause of the wound and surface area dictate the clinical prognosis of the wound and the management necessary to promote healing. Although often secondary to a disease, chronic wounds are often more difficult to treat than the primary disease [21].

Chronic wounds affect patients socially, mentally and physically. Current treatments do not effectively improve wound healing and as chronic wounds can last many years this is an area of high unmet clinical need.

### Age:

Age is a risk factor for chronic wound formation. The depth of the skin decreases with age, and the skin weakens as the rate of collagen and elastin breakdown exceeds the rate of production. With age comes a constant decrease in the number of fibroblasts in the tissue, which are chronologically aged, reducing collagen production and therefore inhibiting the wound healing response [22]. The thinning and increasingly disorganised connective tissue bundles in the skin reduce the mechanical strength of the tissue, increasing the risk of damage.

### Inflammation and skin ageing:

Since dermal collagen has a long half life, approximately 15 years [23], any additional accumulated damage is likely to exist for an extended period of time unless collagen remodelling occurs.

Matrix metalloproteinases (MMPs) degrade extracellular matrix proteins. MMP-1 degrades a wide range of collagens, including collagens I and III [24]. Comparing aged and young skin, aged skin contains a 4-fold increase in insoluble and partially degraded collagen. MMP-1 is expressed more in fibroblasts in aged patients and when there are elevated levels of fragmented collagen lattices [25]. This leads to positive feedback, since MMP-1 degrades collagen into fragments and reduces the fibre organisation leading to a detrimental impact on the mechanical properties of the skin. IL-1 expression can be activated by MMP-1, leading to increased inflammation and further decreasing collagen production [26].

Topical treatment of skin with Vitamin A (retinol) increased the numbers of fibroblasts in elderly patient skin and reduced the levels of MMP-1, but failed to increase the organisation of connective tissue [27]. Common limitations with using retinoids are the skin irritations it often causes after multiple treatments, countering the positive benefits. A multivariate analysis showed that reduced dietary intake of retinol increased the severity of chronic wounds, showing a correlation between retinol and wound healing [28]. A synthetic retinoid has been shown to reduce MMP-1 in skin, increase levels of tissue inhibitor of metalloproteinase and increase procollagen in skin models [29].

A slower inflammatory response with age leads to an inadequate healing process on an already unstable mechanical structure. It has been shown that elderly subjects given standardized biopsy punch wounds had delayed healing in all stages of wound healing [30].

There is a decrease in the number of dermal capillary loops in skin with age [31], leading to more oxygen and nutrient deprived tissues. Decrease in structural support of blood vessels due to ageing may lead to increased bruising [32]. Furthermore, with age comes a decrease in pro-angiogenic mast cells, this delays the regeneration of blood vessels when damage occurs.

### Photoageing:

In addition to the chronological ageing of tissues over time, skin is additionally subjected to a lifetime exposure to UV light, causing a detrimental effect on tissue mechanical function and appearance known as photoageing [33]. Reactive oxygen species (ROS), which are known to activate receptors for IL-1 and TNF-a, can be produced in skin from exposure to UV light [34]. ROS also damages the membrane lipids, attracting inflammatory cells to the area.

Within hours of low concentration exposure to ultraviolet-B irradiation, transcription factors AP-1 and NF-kB were upregulated, which are stimulatory for MMP genes and collagenase levels become elevated [35]. Since AP-1 inhibits procollagen gene expression, production of collagen is reduced [25]. Elastic fibre associated proteins, such as fibrillins, help to transmit mechanical forces and support cell attachment and are rich in UV chromophores [36]. These components are degraded by physiologically relevant UV light and can accumulate environmental damage.

### Vascular insufficiencies:

There is a clear correlation between vascular insufficiencies and the formation of chronic wounds; often leading to hypertension in the peripheral circulation, often the legs. Hypertension may damage to blood vessels, leading to the formation of fibrin cuffs around blood vessels, comprised of fibrin and extracellular matrix. This build up of extracellular matrix may prevent the transport of nutrients and oxygen to tissues, causing cell death and inflammation [37].

One alternative hypothesis is that venous insufficiency causes white cells to become trapped when blood circulation slows. These trapped cells then release proteolytic enzymes and superoxide radicals, causing damage to the endothelial cells [38]. Damage to endothelial cells can lead to an inflammatory response due to the cytokines released by the damaged cells. Evidence supporting this hypothesis is that patients treated with compression stockings showed increased blood flow and a release of trapped white cells. However, many platelets remained trapped in the capillaries [39].

The necessity for cellular metabolism at the wound site often causes mild and short lasting hypoxia as the wound heals. Furthermore the necessary oxygen consumption coupled with vascular insufficiencies can lead to chronic hypoxia. Severe hypoxia limits the bacterial killing capacity of neutrophils [40], and prevents collagen production by fibroblasts [41]. This causes an issue since the main early defence of wounds from bacterial invasion are neutrophils and the production of ROS.

There is, however, a limit to the advantages of ROS in the wound. Hypoxia coupled with the oxygen depletion of the normal wound healing process can lead to chronic wound formation if the levels of ROS exceeds the ability of the naturally produced antioxidants to suppress the oxidative stress [18], and fibroblasts can become environmentally aged by prolonged exposure to ROS [42].

### Diabetes:

Type I and type II diabetes both lead to high blood glucose, despite the mechanisms behind both diseases differing. In type I diabetes, an autoimmune reaction destroys pancreatic β cells. Insulin is produced by these cells which has the effect of regulating glucose in the blood by promoting cellular uptake and storage. Without insulin, patients have chronic hyperglycaemia and this can lead to many negative health effects. Type 2 diabetes is much more common; in this disease cells become resistant to insulin due to consistently high insulin levels in the blood, caused by a high dietary intake of sugars.

In the UK, over 1.2 million people have been diagnosed or are living undiagnosed with diabetes. A diabetic patient has a 15-25% risk developing a foot ulcer over their lifetime [43][44], however if the patient has been diagnosed with peripheral neuropathy and vascular disease, there is an annual risk of between 5-7.5% [43]. Diabetes is associated with neuropathies; at least 20% of diabetic patients exhibit some form of a neuropathy [45]. Neuropathic foot ulcers are the most preventable through patient education regarding sensory loss and how to recognise potential foot problems. Despite the best clinical practice, diabetic foot ulcers have a 50% risk of recurrence after 3 years [46]. Over a two year period, the total cost to treat a foot ulcer is between £9533-15246 [47].

These ulcers present an issue due to the frequency of their incidence, the impact on the function of the patient and the high risk of amputation. Studies suggest in 48.9% of lower limb amputations, diabetes was noted as a co-morbidity. Patient data analysis shows that diabetes has an amputation risk of 23.3 times greater per year than a person without diabetes [48].

Diabetic patients have compromised wound healing for several reasons, including high blood glucose. High glucose levels inhibit chemotaxis of macrophages and lymphocytes, delaying the inflammatory response of white blood cells and increasing the time taken for wounds to heal [49]. Patients suffering from diabetes have a less effective immune system, preventing the inflammatory phase from being adequately completed.

The complement cascade is a vital process for the elimination of microorganism infiltration in the body and consists of 3 different pathways: classical, alternative and lectin. Microorganisms exhibit surface carbohydrate structures; mannan-binding lectin (MBL) binds specifically to these and activates the complement pathway. Raised concentrations of glucose inhibits the binding of MBL, and therefore inhibits the lectin pathway, but does not affect the classical or alternative pathways [50]. Perhaps to offset inhibited binding, there is an increase of serum MBL in type 1 diabetic patients [51], which can lead to chronic inflammation around the body. Both low [52] and high [53] levels of MBL indicate a risk of mortality for patients.

### Bacterial infections:

Chronic wounds are associated with a diverse bacterial burden, up to 60% of chronic wounds are infected with bacteria when compared to only 6% of acute wounds [54]. However, one study found the presence of Staphylococcus aureus in 93.5% of ulcers, with Pseudomonas aeruginosa in particular contributing to delayed healing [55].

Chronic wound bacteria are often densely aggregated and surrounded in an alginate biofilm, inhibiting the effectiveness of topical antibiotics and providing protection against host produced ROS [56].

### Serum iron:

An increased iron serum level is correlated with both healing impairment and risk of infection. Serum iron and MMP-9 vary in concentration depending on the body site, especially the arms and legs of patients with leg ulcers. Iron accumulates in tissues when venous valve insufficiencies and high blood pressure cause extravasation of red blood cells. These cells then degrade and are engulfed by macrophages, leading to iron release. An increased level of iron in serum causes a highly activated macrophage phenotype, leading to the secretion of ROS, pro-inflammatory cytokines including TNF-α and IL-1 and a range of MMPs, causing significant oxidative and degradative damage to the surrounding tissues and a chronic inflammatory state. Subsequent macrophage uptake of iron leads to the formation of intracellular iron-storage complexes, known as hemoseriden clusters [57]. Over activation of macrophages can cause adjacent fibroblasts in the tissue to age, causing senescence and inhibiting the ability for the cells to repair damaged tissues. Acute wounds differ from chronic wounds due to the absence of a raised iron concentration. Treating mice with iron-dextran prevented wound healing, with an observable increase in TNF-α producing macrophages compared to controls. Subsequent treatment with an iron chelator allowed the wounds to heal, showing a correlation between iron and impaired wound healing [58].

The homeostasis of iron is closely linked to inflammatory responses [59]. Antioxidants are raised in high serum iron patients to counteract the additional oxidative stress caused by high iron levels [60].

There is a link between iron deficiency and obesity which may be due to insufficient dietary intake or a greater iron requirement due to a higher blood volume [61]. This shows a multivariable cause for chronic wound formation, since obese patients are more at risk than non-obese patients despite iron deficiencies.

## Current treatments for diabetic ulcers:

Assistive ambulatory devices such as crutches or wheelchairs aimed at relieving mechanical loads are not often effective due to poor patient compliance. However, total contact casts are successful when changed regularly since patients are unable to remove them easily [47]. Other methods to relieve loading include modified half shoes and dressing materials underneath the foot, but the benefits of these again are dependant on the compliance of the patient.

Whilst surgery can help to heal foot ulcers, few randomised trials have been conducted to compare against non-surgical treatments and surgical interventions can lead to infections or ulcers at other sites. Some procedures leave patients with an altered gait, modifying the stress-strain cycle of the foot when walking and potentially leading to ulcer formation at a different site of the foot.

Hyberbaric oxygen therapy has been used successfully to treat non-healing diabetic foot ulcers [62] although the treatment is time intensive for the patient (often between 1-2 hours per session) and there are issues with potential patient claustrophobia. Additional side effects of this treatment are potential inner ear lesions or pain associated with pressure changes, or oedema of the lungs [63].

Dressings are almost always used on their own or in conjunction to treatments for chronic wounds in order to absorb wound exudate. There are many varieties of dressings, which vary in expense. However, a recent study comparing the cost effectiveness of commonly used dressing types found no major differences between a simple gauze with or without an antimicrobial agent or a hydrocolloidal preparation [64]. The more expensive dressings required less changes, however the cost was offset by the cost for professional time. The only noticeable difference between the dressings was a decrease in reported pain by use of non-adherent dressings.

## Future treatments for chronic ulcers:

In chronic non-healing ulcers, there is an increased level of MMPs and a decreased level of TIMPs (tissue inhibitor of matrix metalloproteinases), leading to excessive degradation and inflammation. It is possible to predict non-healing ulcers from the ratios of MMP-1 to TIMP-1 in wound fluid, with an optimal ratio of 0.39 predicting full healing at 12 weeks [65]. This allows for selectively treating ulcers predicted to not heal with more expensive therapies which would be unnecessarily expensive for broadly treating all non-healing ulcers. Alternatively, inhibitors of MMP and inhibitors of promoters of TIMPs could be developed.

### Bacteriophage therapies:

A bacteriophage is a virus which specifically infects bacteria, and come in two main forms, temperate and lytic bacteriophages. Temperate bacteriophages are dormant and reside within bacterial DNA either as a free plasmid or integrated within the bacterial chromosome. When the cells become damaged or begin apoptosis the phages are released. A lytic bacteriophage causes lysis of the infected bacterium as the replication of the bacteriophage is performed. Bacteriophages have been suggested as a treatment for antibiotic resistant bacteria due to their specificity and effectiveness in targeting bacteria [66].

A topically applied bacteriophage cocktail to treat a bacterially infected chronic wound model was used to treat both mice and pigs, consisting of punch biopsy wounds and inoculation with 3 separate strains of bacteria. After some time, these wounds were applied with a bacteriophage cocktail, leading to improvements in wound healing, particularly in Staphylococcus aureus and Pseudomonas aeruginosa infections, but less effective against Acinetobacter baumannii [67].

A major concern with bacteriophage treatments is the lack of clinical data in their usage, and since chronic wounds exhibit wide ranges of bacteria, therapies must ideally be broadly acting since there is no single bacterial strain present.

## Mesenchymal stem cell therapy:

Stem cell therapies may provide treatments against many of the aetiological components of chronic wounds, namely the lack of angiogenesis, chronic inflammatory response, and lack of extracellular matrix production. Conveniently, mesenchymal stem cells can be relatively simple to isolate and expand in vitro, whereby an autocrine response stimulates high proliferation allowing for rapid expansion of the cells whilst maintaining the cells potency for differentiation [68][69].

M Dominici et al provide guidance on cell surface marker expression for determination of mesenchymal stem cells, however this definition is varied between authors [70].

Table 1: Antigen markers for mesenchymal stem cells [70].

|  |  |
| --- | --- |
| Positive markers | Negative markers |
| CD105 | CD35 |
| CD73 | CD43 |
| CD90 | CD14 or CD11b |
|  | CD79α or CD19 |
|  | HLA-DR |

There are a variety of potential sources for mesenchymal stem cells (MSCs), such as bone marrow, adipose tissue and amniotic fluid. These cells can be applied at the wound site using a spray, scaffold or injection to the surrounding tissue, potentially providing a cost effective treatment to heal chronic wounds. However to date there is no consensus on the most effective method for cell delivery to the wound site, the numbers of cells needed or the latest passage number which can be used to achieve wound healing [21]. Both bone marrow derived mesenchymal stem cells (BMSCs) and adipose derived mesenchymal stem cells (ADSCs) have been shown in studies to elicit improved wound healing response in comparison to controls and provide a promising alternative to conventional therapies. Given the relatively convenient isolation of ADSCs in comparison to BMDSCs, and the comparatively similar repair properties, ADSCs may be the preferred future of stem cell based therapy.

### Beneficial effects of mesenchymal stem cells on surrounding cells and tissues:

ADSCs release factors which affect human dermal fibroblasts. During in vitro co-culture of fibroblasts with ADSCs, fibroblasts had increased proliferation compared to controls. Furthermore, a separate investigation of fibroblast cell culture in ADSC conditioned media increased fibroblast migration and proliferation [71]. ADSC conditioned media, prepared by culturing ADSCs in standard media for several days, aspirating and filtering, had a dose dependant effect of collagen production in fibroblasts, and down regulated expression of MMP-1 [72]. Conditioned media may provide beneficial promotion to fibroblasts due to the secretion of multiple growth factors, including VEGF, PIGF, PDGF, HGF, KGF, basic FGF and TGF-β 1 and 2. Fibroblasts treated with conditioned media by ADSCs are afforded protection from free radicals when compared to control media. This study showed that conditioned media was as anti-oxidative as dosing cells with 100 micromolar ascorbic acid [73]. This is of particular interest in the treatment of chronic wounds, since the issue of excess ROS needs to be addressed for wound healing to commence.

Macrophages stimulate MSC migration to sites of injury by use of cytokines, in particular IL-8, and can influence their molecular phenotype. Macrophage stimulated MSCs secrete IL-6, a cytokine which inhibits monocyte differentiation to a dendritic antigen presenting phenotype, at a level 56-fold higher than when unstimulated, inhibiting the inflammatory responses of macrophages [74][75]. BMDSCs have been used to reduce mortality in animal models of sepsis, by treating before or after perforating the cecum [76]. Results suggest that prostaglandin release by BMDSCs are responsible for eliciting an anti-inflammatory effect on macrophages, and IL-10 release by macrophages inhibits the migration of neutrophils which would otherwise cause oxidative organ damage leading to death [77]. Elimination of IL-10, monocytes or macrophages in the model prevented the beneficial effect of BMDSC treatment. This investigation, although unrelated to skin wound healing, provides a clear indication of the anti-inflammatory effect that MSCs can possess.

MSCs treatment has successfully treated severe graft versus host disease of the gut and liver, which was resistant to all attempted therapies after a bone marrow transplant. The MSC treatment was haploidentical and did not cause an immune reaction. Full recovery of the gut took many months, and MSC engraftment to the tissue may have occurred [78].

### Mesenchymal stem cells increase wound healing:

A wound healing investigation in which a 7 mm biopsy punch was used to create wounds in mice showed that treatments of human ADSCs encapsulated in a collagen gel solution increased wound healing by 34% compared to controls. No significant inflammatory response was noted, however the mice were immunocompromised [71].

Fibrin glue was used to adhere multiple cell sheets of ADSCs to form a multilayer composite of cells. Compared to controls, these composites substantially aided healing of full thickness dermal wounds in mice after a 12mm biopsy punch was applied. Composites of ADSC sheets were shown to increase blood vessel density and collagen production in these dermal wounds [79].

A rabbit wound model was treated topically with rabbit ADSCs and BMDSCs and a semi-occlusive dressing was placed on top. Subsequent analysis of the wound sites showed that the transplanted ADSCs were evenly distributed in the granulation tissue and wound bed. Many but not all of the ADSCs exhibited a myofibroblast cell type, with expression of myofibroblast markers present from both endogenous cells and transplanted cells. The wounds treated with ADSCs exhibited increased CD31, markers of endothelial cells, but there was no detection of blood vessel structures. Increased macrophage recruitment to wound was attributed to have increased wound healing. The study appeared to show that excessive numbers of applied cells (3x105 cells per 7 mm diameter biopsy punch) inhibited the re-epithelialisation of the wound [80]. The underlying causes were not discussed further in the study but may have been due to the low survival rate of stem cells in experiments, leading to subsequent inflammation at the wound site. In some investigations approximately 99% of transplanted cells die after treatment to the ischemic heart due to hypoxia and nutrient deficiencies. However, cells can be pre conditioned in hypoxic environments to aid survival rates [81].

## Current models for wound healing:

Wound healing models are often used to test new treatments in order to evaluate their safety, efficacy and efficiency before regulatory approval. Rodents are often used due to the relatively inexpensive cost, small size and the common use in laboratory research [82]. There are several differences between rodent and human skin, such as the contractile nature of rat skin reducing wound size rather than healing via epithelisation, giving the rodent model limitations.

For wounding models, pigs are preferred to rats as the wounds do not heal predominantly by contraction and the skin structure is comparable to human skin. The main disadvantages of pig models are the cost of housing the animals and the lack of transgenic pig models available for modelling different disease states [83].

There are difficulties in replicating the underlying conditions of a chronic wound such as diabetes and age, bacterial infiltration and the highly inflammatory and degradative state. Wounds tend to be generated from use of biopsy punches to create a full thickness wound, however this basic wound lacks the underlying processes of a chronic wound.

### Chronic wound models:

Despite the difficulties, many authors have attempted to achieve greater comparison to human chronic wounds by increasing co-morbidities into animal models.

### Ischemia:

Surgically limiting blood flow can in a rabbit ear creates an ischemic model. The lack of oxygen and nutrients hinders wound healing, in particular there is limited re-epithelialisation and an increased infection rate, however the inflammatory and degradative state is dissimilar to chronic wounds [84]. Additionally, the tissues involved will not have any aged or diabetic components of inhibited wound healing.

### Diabetes:

Mice with induced diabetes have markedly decreased wound healing compared to control mice and as such, diabetic mice are often used in wound healing studies. Diabetic mice have decreased growth factors and an increased number of macrophages and neutrophils at wound sites, contributing to delayed healing. The wound sites of diabetic mice are filled with abundant fat tissue, which causes the formation of lipogranulomas and inflammation of adipose tissues [85], however, these formations are not relevant to human chronic wounds.

### Age:

Unfortunately, use of aged animals increases the costs associated with animal housing, which may be a great disadvantage. An aged mouse wound model has been investigated, comparing young (6 months old), middle aged (18 months old) and old (30 months old) mice. The animals were cut incisionally and after sacrificing the animals, histology samples and growth factor analysis were performed. There was a decrease in the wound PDGF and PDGF receptors in the older animals, which may have led to decreased fibroblast and inflammatory cell migration [86]. It is debatable how translatable the wound healing process of aged mice is to humans.

### Bacterial infection:

Chronic wounds commonly have bacterial infiltration present. For this reason, investigations of wound healing models with bacterial elements have been investigated. A wound healing model consisting of rats injured with biopsy punches and subsequent application of either Staphylococcus epidermis or Staphylococcus aureus biofilms had significantly decreased re-epithelialisation compared to controls. The biofilms caused both chronic inflammation and a physical barrier for wound healing [87].

The previous model was improved on by the use of rats which were modified to show symptoms of diabetes mellitus. Wounding with biopsy punches and inoculation with Staphylococcus aureus or Pseudomonas aeruginosa caused wounds to increase in size until debridement was used. After debridement, wounds reduced in size due to the decrease in bacterial load [88].

In one recent study, pigs were chemically induced with diabetes before creating a full thickness wound and inoculating with several strains of clinically relevant bacteria [67]. This created a more physiologically relevant model, although the investigation only ran for 9 days.

## Skin substitutes for wound healing models:

It is difficult to justify the use of animals in research when the current models are not completely comparable to human chronic wounds. Most commonly, rodents are used in studies despite differences in their wound healing responses to humans, and often the wound models do not fulfil all requirements of a chronic wound.

Skin substitutes have been used in research as an animal model alternative due to their simplicity and reproducibility. Many models use acellular de-epidermised dermis or collagen matrices with cells added to create a skin substitute. The use of de-epidermised dermis retains the natural structure of skin whilst enabling control over the sources of cells or their density in the model. It is necessary to use both fibroblasts and keratinocytes for in vitro skin models since keratinocytes with no fibroblasts present will form an epidermal layer with no basement membrane [89]. This model would also be limited in terms of comparative physiology to skin since it lacks a dermal component. Inclusion of fibroblasts to models both increases the stability of keratinocyte attachment and the organisation of the epidermis [90][91].

Attempts have been made to introduce chronic wound elements into skin substitutes. Inoculation with bacteria after burning skin substitutes increases colonisation of the skin, and increased colonisation increases the loss of epidermal keratinocytes and loss of the basement membrane [92]. This model is still limited however by the lack of vascularisation and immune system response.

### Summary:

There is no currently available model, animal or in-vitro based, which fully exhibits all aspects of human chronic wounds. Attempts have been made to include inhibitors of wound healing such as bacterial infection, diabetes and age to models, but so far no single model includes all aspects. Tissue engineered skin substitutes may provide an alternative to animal models due to their simplicity and reproducibility, however these models are currently lacking in their relevance to chronic wounds.

Current research indicates that stem cell therapies may be a promising treatment for chronic wounds due to their effects on angiogenesis, anti-inflammatory nature and ability to be rapidly expanded in-vitro.

## Delivery of cells to wounds:

While the literature widely supports the use of cell-based therapies to treat chronic wounds, there is not currently a universally accepted method of cell delivery.

Common approaches involve injection of cells, often in a medium to avoid cell run off from wound site [93][94][95][96][97][98], cells contained within a scaffold [99][100] [101] [102] [103], spraying of cells to the wound site [104][105][106][107], or cell sheets applied to the wound site [79].

There is much debate between using natural or synthetic materials as a cell delivery device. The general consensus is that the material of choice should provide support to the therapeutic cells and host tissue to induce regeneration of the tissue. Considering the scaffold fabrication, biocompatibility and degradation is vital [108] and should ideally replicate the natural extracellular matrix of the native tissue.

## Fabrication methods:

Possible fabrication methods vary by material choice, which imposes a limit on the full range of scaffold options. Common tissue engineering scaffold fabrication methods include 3D printing [109], solvent casting with particulate leaching [110] and electrospinning [111], resulting in unique a different scaffold morphology.

It is necessary to match the biomaterial properties for the intended purpose, since cell-extracellular matrix interaction affects cell migration, inflammatory responses and cell signalling. Growth factors and proteins can attach to any material introduced into the body and exert an influence.

### Electrospinning:

Electrospinning is a fabrication method in which a fibrous mat is formed by applying a strong electrical field onto a melted or solution of polymer. The usual components of this technique include a high-voltage power supply attached to the polymer supply (in the form of a melted polymer or solution) by an emitting electrode to a conductive nozzle. The grounding electrode of the power supply connects to a conductive collector and completes the electric circuit. As the electric field increases, the Coulomb repulsive force in the polymer at the end of the nozzle due to the same polarity causes the polymer to form a Taylor cone, or conical droplet. Increasing the electric field further overcomes surface tension in the polymer to form a charged stream which collects onto the collector as a fibrous scaffold.

Scaffold morphology can be altered by modifying the electrospinning process; for instance the ambient humidity, temperature, distance between application of current and the collector and if solution electrospinning; concentration of polymer and solvent used.

Ambient humidity can create surface pores in fibres, a higher humidity causes an increased number of pores in addition to the size [112].

A higher surface tension causes unstable jets and increases polymer spray whereas a low surface tension allows electrospinning with a lower electric field [113]. Increasing the ambient temperature whilst electrospinning creates a thinner fibre diameter, since the increase in temperature decreases the viscosity and surface tension of the polymer due to expansion of the polymer molecules [111].

Furthermore, the composition of the solvent used affects the electrospinning outcomes since conductivity, viscosity and surface tension are affected.

In addition to electrospinning synthetic polymers, some biological molecules have been electrospun including elastomimentic peptide sequences [114], lyophilised collagen [115], fibrinogen [116] [117], elastin and gelatin [118]. As with conventional electrospinning, the properties of these scaffolds can be altered by varying electrospinning parameters including concentration of solute to achieve a range of fibre thicknesses.

## Natural materials:

### Fibrin:

Fibrin is a necessary part of haemostasis and is integral to the formation of blood clots. Polymerised rapidly by the cleaving of the fibrin precursor fibrinogen by the protease thrombin, spontaneous assembly of fibrinogen proteins forms a fibrin network. Fibrin gels have been used as biomaterials for many years and have been used for a multitude of tissue regeneration and regenerative medicine purposes. This gel degrades over a matter of days, making the use of fibrin as a surgical glue ideal.

Fibrin has a vast range of literature describing its structure. The structure of fibrin and fibrin gel formation has been described in depth by (Wolberg, 2007) [119], therefore this will only be described in brief.

#### Thrombin:

A selective serine protease, thrombin is limited to acting on a small range of macromolecular substrates, one of which is fibrinogen. The precursor, prothrombin, has 2 reactive sites, Arg271 and Arg320, which must be cleaved to form thrombin protease.

Since prothrombin has 2 cleavage sites, they can be cleaved in an alternate order by separate pathways. Factor Xa cleaves prothrombin inefficiently [120], predominantly at Arg271 followed by cleavage of Arg320. This interaction activates the coagulation cascade. Efficient conversion of prothrombin to thrombin is achieved when factor Xa and cofactor protein Va assemble to form a prothrombinase complex [121]. The prothrombinase complex predominantly cleaves prothrombin at Arg320 before cleaving at Arg271 [120].

#### Fibrinogen:

The soluble glycoprotein fibrinogen is present at high (2-4 mg/ml) concentrations in blood plasma. 29 disulphide bridges link 3 different polypeptide chains Aα, Bβ and γ, 2 sets of these chains form fibrinogen protein. [122]. Thrombin acts to cleave 4 of the 6 fibrinogen chains, both pairs of the 2 Aαand Bβchains. Aαis proteolytically cleaved predominantly over the Bβchain, producing a new N-terminal group, this residue acts as an activation peptide.

Activated platelets are aggregated during haemostasis by fibrinogen, bound by surface integrin receptors on the platelet membrane. Low plasma fibrinogen is therefore associated with a risk of bleeding due to impaired haemostasis [123].

#### Fibrin clot degradation:

Breakdown of a fibrin clot, fibrolysis, often includes proteases such as plasmin to cleave fibrin polymers into degradation products.

In an *in vivo* investigation of rabbits after partial liver resection, human fibrin was used as a haemostat – (half life of 25 days, 9 week full granulation tissue). Antibodies peaked at 14 days to human components of the Beriplast P (Aventis Behring, Marburg, Germany) fibrin sealant used (fibrinogen, albumin, Factor XIII and thrombin) [124]. Whilst the degradation time in humans is unlikely to be similar since Beriplast is not antigenic, it is shown fibrin clots are organised by phagocytes then angiofibroblasts to form granulation tissue [125].

## Common fibrin uses:

Fibrin is readily manipulated by altering the fabrication methods, making it ideal for a range of uses for both surgical and tissue engineering uses.

The structure of the fibrin clot is readily altered by the surrounding pH, temperature and ionic strength [126]. Altering the ratios of thrombin and fibrinogen can modify the material properties of fibrin gels. A lower concentration of thrombin will form thicker yet loosely woven strands of fibrin, tightly woven thinner strands can be formed with a higher concentration of thrombin [119].

### Surgical glue:

Fibrin is the only FDA approved material for use as a haemostat, sealant and adhesive [127]. Surgical glues are beneficial over sutures due to the comparative speed of delivery by the surgeon.

An adhesive acts to glue articles together, whereas a sealant’s function is to block the passage of liquid or gas. The function of a haemostat is to cause blood to clot. Both sealants and adhesives may act to prevent blood loss however as a blood clot is not necessarily formed. Since fibrin can be used in all three major groupings of haemostatic agents, it is well researched. Other commercial products which act as haemostatic agents are reviewed by Galanakis et al [128] and by Sundaram and Keenan [129]. A large range of the surgical applications for fibrin glue including cardiovascular, urologic, thoracic, hepatic, reconstructive and neurosurgeries are reviewed in detail by D. Albala and J. Lawson [130].

Fibrin glue may be prepared from autologous or homologous fibrinogen and thrombin, sourced from human plasma. Sourcing from autologous plasma avoids disease transmission, however it is time consuming and expensive. Homologous fibrin is sourced from pooled human donor blood. Whilst disease transmission is of great concern, this can be minimised by use of viral screening and reduction methods through a variety of methods such as heat, solvent or detergent treatments, precipitation and filtration.

Fibrin kits can be purchased in the forms of lyophilised powders, which require reconstituting and mixing, or as frozen preloaded dual syringes that mix the liquid components of fibrinogen and thrombin when secreted from the syringe. Mixing of the liquid components begins the cross-linking process and the soluble liquids form insoluble fibrin polymers. Commercial fibrin kits often have additives to aid fibrin gelation. Plasma proteins such as Factor XIII are often added to the fibrinogen component to aid clotting, and ionic calcium is an additive in the thrombin component to activate Factor XIII [131][130]. Factor XIII is a necessary component since without it fibrin clots show severe lysis. The ideal concentration of Factor XIII is between 40-80U/ml.

### Fibrin in bioengineering:

Fibrin is not just a passive scaffold but readily binds the protein fibronectin, a cell adhesion molecule, vWF, involved in haemostasis, IL-1, an inflammatory cytokine, Vascular Endothelial Growth Factor (VEGF) and Fibroblast Growth Factor 2 (FBGF2) [122].

Cellular response to fibrin is in part determined by fibre thickness. Thicker fibres aid endothelial synthesis of vWF, and promote macrovascular structure formation in addition to having greater cell orientation along fibre axis when compared to thinner fibres [132]. Fibrin networks with thicker fibre bundles appeared to exhibit larger areas of fibrinolysis compared to thin fibres, demonstrating that fibrin degradation can be modified depending on the structure.

Skin:

Fibrin was used as a glue to hold together tri-layer ADSC sheets for aiding full thickness skin wound healing in mice [79].

Autologous keratinocytes were applied to full thickness skin wounds in pigs using a fibrin spray. The fibrin spray allowed more even coating of the wound surface compared to cells applied in culture medium [104]. Human keratinocytes delivered to human chronic wounds in a fibrin spray mostly or fully re-epithelised the wounds [105].

Cells from human adipose stromal vascular fraction grew readily in vitro after being sprayed in fibrin to well plates or Petri dishes [106]. Application of bone marrow derived stem cells in fibrin spray to chronic wounds in patients was attempted in 2007 by Falanga et al [107]. The number of cells delivered was correlated to decreasing wound size. The fibrin spray prevented run off of cell solution from the wound, but allowed migration of viable cells to the wound bed.

Cardiovascular:

Myocardial infarction causes irreversible damage to tissues of the heart and currently a transplant is the only treatment to replace the damaged tissue. Tissue engineered cardiac tissue has been created using fibrin as a scaffold for myofibroblasts derived from human cardiac tissue [99]. Up to 3 mm of tissue engineered cardiac tissue can be cultured without nutritional issue however the material stiffness is insufficient for direct implantation [100].

A more simple approach to treating a myocardial infarction is to inject cells to regenerate the tissue rather than tissue engineer the solution. Simple injection of cells contained within fibrin for myocardial infarction treatment has been investigated. Cardiac myoblast cells injected with fibrin glue have greater survival rate in than cells injected in bovine serum albumin and may reduce the area of infarction increasing in rats [93]. Injecting fibrin alone preserves cardiac function and retains the infarct wall thickness in a rat model [94].

Osteogenesis:

Bone tissue engineering to rectify bone disorders and conditions often use porous biomaterials that match the biomechanical strength of natural bone which fibrin lacks. Combining fibrin gels with bone cement to form a composite can better match the mechanical properties needed for bone regeneration. When fibrin-bone cement composites containing osteoblasts were implanted to nude mice, matrix calcification and cellular engraftment was noted, however normal bone formation was not apparent at 24 weeks post implantation. [101]

hMSCs were cultured in the commercial fibrin product Tisseel (Baxter, UK) and analysed for osteogenic lineage morphology and proliferation [133]. A lower concentration of fibrinogen was associated with higher proliferation, however higher concentrations of fibrinogen led to some osteogenic gene expression implying some partial differentiation down a osteoblastic lineage.

Cartilage:

Since fibrin lacks the mechanical properties necessary for bone tissue engineering, fibrin gels have been investigated for cartilage regeneration. *In vitro* fibrin gels containing chondrocyte structures are stable for at least 3 weeks [134].

Hyaluronic acid, a principle component of cartilage responsible for resilience against compression, was mixed with fibrin containing chondrocytes. These composites formed cartilage like tissue and extracellular matrix proteins associated with cartilage tissue when implanted to nude mice [102].

Full thickness cartilage defects in rabbits healed with hyaline-like cartilage after treatment with autologous adipose derived stem cells in fibrin scaffolds. 83% of the rejuvenated cartilage annealed to the natural cartilage and all rabbits showed healing to the subchondral bone after treatment [103].

### Fibrin as a cell delivery vehicle:

Fibrin glue has many beneficial wound healing and surgical applications, it has also been used as a medium for cell injection. This serves as a method for ‘gluing’ the cells at the area of the area, serving as both a degradable scaffold and cell delivery vehicle.

Fibrin has been used as a medium to inject endothelial cells [95], bone marrow derived cardiac stem cells [96], fibroblasts [97], mesenchymal progenitor cells [98] and skeletal myoblasts [93][94].

## Synthetic materials:

There is a huge range of synthetic biomaterials including esters, anhydrides, carbonates, urethanes, each with unique material properties. For the purposes of cell delivery, the material needs to meet criteria including biocompatibility (non allogenic), ideal degradation times, material properties which will withstand the local stress and strains whilst being easy to handle by the healthcare technician. Ideally the material should be approved by regulatory agencies such as the US FDA.

### PLGA:

One of the most common synthetic biomaterials, Poly (l-lactic – co – glycolic) acid (PLGA) is a random copolymer of the monomers lactic acid and glycolic acid and is a highly tuneable synthetic biomaterial. The polymer is readily dissolvable in a range of common solvents including cholorinated solvents, acetone and tetrahydofuran which allows the polymer to be processed by a wide range of techniques [135]. Furthermore, PLGA has been used to deliver a range of molecules including peptides, proteins and vaccines [136].

### PGA:

Poly(glycolic acid) (PGA) was one of the first degradable polymers investigated for biomedical use in the 1970s, most well known for degradable sutures [137]. With a rapid degradation rate and being insoluble in many common solvents, PGA is rarely used on its own.

### PLA:

Poly(lactic acid) has a much slower degradation rate than PGA, with degradation being as long as 5 years in vivo when at a high molecular weight [138]. For purposes of drug delivery, PLA is often blended with a more rapidly degradable polymer. PLA is a chiral molecule, but only poly(L-lactic acid) (PLLA), poly(D,L-lactic acid) (PDLLA) are studied for biomedical use.

Material properties of PLGA can be changed with altering ratios of lactic acid and glycolic acid and is often produced by ring opening copolymerisation of lactic acid and glycolic acid.

#### PLGA degradation:

PLGA exhibits degradation by hydrolysis of ester linkages. Degradation by hydrolysis affects properties including the glass transition temperature and molecular weight leading to differences in drug release over time when used as drug delivery vehicle [139]. Poly (lactic acid) features methyl side groups and is therefore more hydrophobic than PGA. A higher ratio of PLA to PGA leads to a slower degradation rate.

Degradation products of PLGA are lactic acid and glycolic acid, natural and harmless by-products of metabolic pathways in the body. These degradation products end up in the liver and kidneys following release of degradation products to blood stream [140]. With increased degradation the pH of the scaffold decreases due to the acidic degradation products, this can lead to autocatalysis in low porosity scaffolds due to the inability of these byproducts to diffuse away from the implant [141].

## Cell response to PLGA scaffolds:

Blood vessels:

Cells will readily align themselves with the direction of PLGA scaffolds. Microtubes formed by phase separated PLGA allowed a vascular smooth muscle cell line to orientate along the direction [142]. When electrospun with gelatin and elastin, PLGA scaffolds supported endothelial cells and smooth muscle cells to form a functional monolayer which was not thrombogenic, suggesting good functionality [118].

Nerves:

PLGA scaffolds allow neural stem cells to differentiate towards neurons and establish a neural network with functional synaptic vesicle response [143].

Bone:

Varying the hydroxyapatite ratios can optimise composites of PLGA and hydroxyapatite to maximise osteoblast attachment and viability [144]. These acellular scaffolds were able to bridge a bone defect after 24 weeks.

## Loading PLGA scaffolds:

Growth factors:

CO2 plasma treatment of PLGA scaffolds increases the binding efficiency of basic fibroblast growth factor (bFBGF), a growth factor involved in angiogenesis and fibroblast proliferation. Continuously released for up to 7 days, bound bFBGF also increased cell adhesion to scaffolds [145].

Stem cells:

Biodegradable microparticles of PLGA and Polyethylenimine have been reported to retain the stemness of MSCs and increase engraftment rates when injected to rats compared to controls [146].

PLGA sponges loaded with bone marrow derived stem cells (BMSCs) formed fibrous scar tissue when implanted to cartilage defects in rabbits. However, addition of fibrin gels to form a composite with PLGA repaired defects with cartilage-like tissue [147].

A spinal cord injury model using rats showed vast improvements when neural stem cells were implanted to the defect in a PLGA based scaffold, modelled to replicate the natural spinal cord tissue [148]. Comparing implanted cells with or without scaffold, only 17% of rats without a scaffold scored positively in a walking score assessment, compared to 69% of rats implanted with a scaffold.

# Transportation of cells:

Whilst there is much discussion over cell type and scaffold for therapeutic treatment of particular tissues, there is a lack of research into the actual delivery of cells from lab to clinic use. If autologous cells are isolated and used at the same clinic then this is not an issue, for instance, BMDSCs were delivered to a patient after an overnight isolation protocol at the same facility [149].

For further debate is how to conveniently maintain high numbers of viable cells from lab to clinic over several days until use. Current methods for shipping cells are either cryofrozen cells, or live cell cultures in sealed culture flasks.

### Transporting frozen cells:

In common lab practice; cells are frozen for long term storage at around −150°C in liquid nitrogen. Whilst it is possible to transport frozen cells in liquid nitrogen, in most cases this is not feasible due to the costly weight of the specialised container needed and use of a specialised courier company [150]. A more cost effective method involves cryofrozen cells which are packed in dry ice (around -78.5°C) for short shipping times (less than 24 hours).

### Transporting cells at ambient temperatures:

Cells can be readily maintained at lower temperatures than 37°C, M. Vergara et al report that whilst cell growth is inhibited at 33°C, metabolic activity is not affected [151].

One suggested method of shipping cell line cultures was to keep cells in suspension with buffered medium in cryovials at ambient temperatures [152]. However, since many cell types are adversely affected after culturing in suspension, more research may be needed to assess the practical uses of this technique. The aforementioned shipping method essentially had the effect of maintaining the pH of the cell solution by preventing cell medium exposure with air. Conventional pH buffering of cell media involves a carbonate additive, which maintains media pH when exposed to air supplemented with CO2 during incubation (usually 5%). As normal air has around 0.04% CO2 the equilibrium becomes unbalanced and the carbonate buffer is converted to CO2 and is released, rapidly transforming the media alkaline and cytoxic [153]. Filling the flasks to the brim with culture medium to remove as much air as possible and making the flasks air tight can allow shipping of adhered cells in conventional tissue culture flasks [154]. Unfortunately this technique of shipping can lead to the liquid exerting shear stress on the cells as the flasks are transported.

### Transporting cells at ambient temperatures on a carrier:

Care needs to be taken when shipping cells in the presence of medium alone to prevent shear stress as cell medium causes turbulence to the cells, and excess strain can cause apoptosis of cells [155]. Using a carrier material can prevent shear stress on cells since the lateral movement of cell media is restricted.

Agarose:

The polysaccharide agarose can be used to transport cells at ambient temperatures. 1% agarose solution was added to human cell lines in conventional well plates and after the agarose had set the plates were sealed to be airtight and kept in ambient room temperatures to be shipped [154]. To recover cells, the agarose layer is peeled away and cell medium is replaced. Cells can then be cultured as per standard practice. This method retains cell viability in transport conditions for up to 6 days, however peeling the agarose layer leads to loss of cells which adhered to the gel, and the relatively high temperature of the agarose solution needed (45°C) may be detrimental to the viability of cells.

Matrigel:

Matrigel, a gelatinous protein mixture, may be used as an alternative solution for using agarose as a transport method. Resuspending cells in a mixture of chilled cell media and Matrigel at 4°C before allowing the solution to set in the incubator at 37°C maintains over 90% of cells for up to 4 days in transit in ambient temperatures. Cells can be recovered by cooling the gel to liquefy the Matrigel and increasingly adding cell media and centrifuging [156]. However, Matrigel is derived from mouse sarcoma and from a regulatory perspective it is very doubtful that it would be approved to be used in the clinic due to the tumorigenic source of this material.

Gelatin:

When collagen is irreversibly hydrolysed, it forms the water-soluble protein gelatin. A fibroblastic cell line encapsulation in gelatin maintained viability for up to 7 days at 23°C out of incubation conditions and without supplementing the air with CO2. Gelation was rapid and controllable by altering the temperature of the gelatin solution [157].

Silicone:

Keratinocytes and melanocytes were seeded onto a plasma coated silicone carrier. These constructs were placed into an airtight container which was flushed with 5% CO2. The addition of CO2 maintained the pH of cell media and allowed the transport and delivery of the cells at room temperature (23°C) for over 48 hours. After these simulated transport conditions, cells were able to migrate to de-epidermised dermis from the carrier [158].

## Summary:

Not only are there many choices for the biomaterial to be used when delivering cells to the patient, the shipping conditions of the constructs must be taken into account. Focussing on how the scaffold will be used, considering ease of use by the surgeon (eg lack of processing before using the cells at point of delivery) would simplify the procedure needed at the healthcare clinic and reduce the need for a specialist on hand. The only method described above which would be most convenient for delivering cells was as described by P. Eves et al [158]. This shipping method allowed pre-seeded constructs ‘ready to use’ to be transported without much processing at the point of use, whereas the other mentioned techniques would require the cells to be recovered either from frozen conditions, the culture flask or transport gel before use.

# Chapter 2:

# Materials and Methods

## Preparation of culture medium:

All culture media was prepared under sterile conditions and stored in a fridge at 4°C for a maximum of 4 weeks. After each preparation of media, a 10ml aliquot was placed in an incubator at 37°C for 72 hours without antibiotic or fungicide to examine sterility. Before cell media use, the temperature was raised to 37°C in a water bath to prevent temperature shock.

Greens media (used for keratinocytes):

|  |  |
| --- | --- |
| 330ml | DMEM AQ Media |
| 108ml | Hams F12 |
| 50ml | Foetal Calf Serum (FCS) |
| 5ml | Penicillin and Streptomycin |
| 1.25ml | Amphotericin B |
| 2ml | Adenine |
| 2.5ml | Insulin |
| 0.5ml | 3,3,5-Tri-idothyronine/Apo-Transferrin |
| 0.025ml | Epidermal growth factor |
| 0.08ml | Hydrocortisone |
| 0.5ml | Cholera toxin |

Adipose derived mesenchymal stem cell media (AD-MSC media) (used for primary ADSCs):

|  |  |
| --- | --- |
| 500ml | Alpha MEM media - (ThemoFisher) |
| 25ml | Human platelet lysate |
| 500U | Heparin |
| 2.5ml | Ciproflaxin |

Dulbecco’s Modified Eagle’s Medium + 10% FCS (DMEM + 10% FCS) (used for hDFs, ADSCs passage 2 onwards and BMSCs):

|  |  |
| --- | --- |
| 446 ml | DMEM AQ Media |
| 50 ml | Foetal Calf Serum |
| 5 ml | Penicillin and Streptomycin |
| 1.25 ml | Amphotericin B |

## Cell isolation:

Split thickness skin grafts:

Fresh skin samples from epidermis to hypodermal fat layers were kindly donated by patients who underwent an abdominoplasty or breast reduction surgery. These grafts were kept in refrigeration at 4°C in sterile saline until cell isolation was performed (within 48 hours of harvest).

Lethal irradiation of murine 3T3 cells:

A feeder layer of cells allowed the rapid expansion of keratinocytes, in this project a murine fibroblast cell line was used. To prevent 3T3 proliferation, cells were irradiated with a dose of 60 Grays.

Primary keratinocyte isolation:

Skin samples were placed into a sterile petri dish containing a small amount of Greens media to prevent the grafts from drying out. Split thickness skin was harvested and a sterile scalpel used to cut samples into square sections approximately 0.5 cm2. These sections were incubated with 0.1% trypsin solution in phosphate buffered saline (PBS) and refrigerated at 4°C for at least 12 hours to enzymatically separate the epidermis.

Sections were moved to a sterile petri dish containing Greens media to inhibit trypsin, and sterilised forceps used to peel the epidermis from the dermis. A sterilised scalpel blade was used to gently scrape at the papillary side of the dermis and the underside of the epidermal surface to remove keratinocytes from the dermo-epidermal junction (containing the greatest numbers of primary keratinocytes). The resulting media then contained a rich source of keratinocytes in suspension, this was added to 20ml Greens media and centrifuged at 200g for 5 minutes. After discarding the supernatant, the pellet was then re-suspended in Greens media and seeded into T75 flasks, previously seeded with irradiated 3T3 fibroblasts (i3T3s) (2x106 per flask) at least an hour before keratinocyte seeding.

Cell media were changed every 2-3 days until approximately 80% confluent, at which point flasks were washed 3 times in PBS and 2ml of 0.02% EDTA used to detach i3T3 cells which were then discarded. 2ml of trypsin was used to detach keratinocytes, cells then further expanded or used experimentally.

Experimentally, human dermal keratinocytes (hDKs) were used at between passages 2-4 in Greens media.

Primary fibroblast isolation:

After keratinocyte isolation, the remaining dermis was used to isolate fibroblasts. Dermis samples were washed in PBS and finely minced using a scalpel blade. 10ml of 0.5% Collagenase A solution was added to minced dermis and incubated at 37°C for 24 hours to allow fibroblast release. The resulting cell suspension was centrifuged at 400g for 10 minutes, supernatant discarded and pellet was re-suspended in DMEM + 10% FCS, before seeding into a sterile T75 flask and incubating at 37°C. Unlike keratinocytes, fibroblasts do not need a feeder layer and were seeded directly onto tissue culture plastic.

Experimentally, human dermal fibroblasts (hDFs) were used at between passages 2-4 in DMEM + 10% FCS.

Adipose derived stem cell isolation:

Fat tissue from previously mentioned donations were finely minced using a scalpel in a petri dish containing 10ml of PBS (penicillin and streptomycin at 100µg/ml). These pieces were added to 15ml PBS and centrifuged at 335.4g for 5 minutes. After resuspending in collagenase A solution at a ratio of 1:2 and placed in an incubator for 30 minutes at 37°C, samples were periodically mixed to prevent aggregation. Digested tissues were centrifuged at 335.4 g for 5 minutes, and the surface oily layers of adipocytes discarded. The pellet, containing the stromal vascular fraction, were resuspended in DMEM + 10% FCS and seeded into a T25 flask and incubated at 37°C. Non adherent cells were discarded after 24 hours by gently washing with PBS.

Experimentally, human adipose derived stem cells (ADSCs) were used at between passages 4-7 in DMEM + 10% FCS in experimental chapters 1 and 2. Freshly isolated ADSCs were used in experimental chapter 3 and cultured in AD-MSC media.

Bone marrow derived stem cells:

Bone marrow derived stem cells (BMSCs) were kindly donated by Professor Suzanne Watt of the National Blood Transfusion Services Oxford and cultured in DMEM+10% FSC media. These cells were used experimentally between passages 6-8.

Cell freezing:

Cells were kept for long-term storage in liquid nitrogen. Briefly, culture flasks were washed 3 times with PBS before using trypsin to detach cells. The cell solution was centrifuged and the pellet resuspended in relevant cell media and cells counted using a haemocytometer. Cells were then centrifuged and resuspended in foetal calf serum (FCS) with 10% DMSO to yield known cell volumes in each cryovial. Vials were placed into an isopropanol-containing chamber which regulated the cooling rate when placed in a freezer at -80°C, cells could then be stored in liquid nitrogen until needed.

Cell thawing:

Cryovials were removed from liquid nitrogen storage and warmed by hand until partially melted. Cell solutions were gently pipetted to pre-warmed media and centrifuged to remove DMSO before resuspending in relevant cell media and seeding into culture flasks.

Electrospinning:

5 g of Poly(lactic-co-glycolic) acid (PLGA) was added to 20g of dichloromethane (DCM) and agitated until fully dissolved, forming a 20% w/w solution. Polymer solution was loaded into syringes fitted with blunt stainless steel tips with an internal diameter of 0.8 mm (I&J Fisnar Inc) before placing onto a programmable pump (Aladdin 1000). For electrospinning PLGA, four loaded syringes were used in tandem. A constant feed rate of 50μl per minute is used with an accelerating voltage of approximately 10kV provided by a power supply (Brandenburg, Alpha series III).

An earthed aluminium collector was covered in sterilised aluminium foil 20cm from syringe tips, with a rotating speed of 300 rpm.

## Preparation of de-epidermised dermis:

Human skin (Euroskin, Beverwijk, Netherlands) was kept in glycerol for long term storage and kept in a fridge at 4°C. To remove glycerol, skin was moved to a sterile container and repeatedly washed with PBS over a period 4 days in an incubator at 37°C.

After this washing stage, skin was incubated for 12 hours with 1M NaCl to salt-split the epidermis from the underlying dermis. This de-epidermised dermis (DED) was then be kept in DMEM 4°C for short-term storage. A sterile scalpel (blade size 10, Swann Morton) was used to cut the DED into pieces approximately 1.5cm by 1.5cm.

## MTT assay:

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide) assays measure cell metabolism by converting the water-soluble tetrazolium salt to an insoluble formazan. Dehydrogenases in the cells cleave the terazolium ring, which can be dissolved using common solvents such as isopropanol and measured spectrophotometrically for an absorbance reading, a function of the concentration of the formazan. Mitochondrial dehydrogenases of live cells convert the salt, providing an indication of cell viability. Cell metabolic activity correlates to the quantity of MTT reduction and therefore concentration of formazan formed, therefore a higher absorbance of light infers a greater number of viable cells. Formazan has an absorbance maximum nearing 570nm; which can be read in a conventional absorbance plate reader.

Samples were gently washed three times with PBS, all liquid was then pipetted and discarded. 1ml of MTT solution (M5655, Sigma Aldrich) at 5mg/ml in PBS was added to wells and samples incubated at 37°C for 45 minutes to allow cells to metabolise the MTT solution. After incubation, MTT solution was aspirated and 500μl of acidified isopropanol added to wells to dissolve the formazan. Each sample had 150μl of pigment solution extracted and placed into an absorbance plate reader in a 96 well plate. This was read with an absorbance reading at 570nm with a reference of 630nm, each sample could be read three times using this method and an average of the 3 readings used.

## Statistical Analysis:

Statistical analysis will be performed by using a One-Way ANOVA (Analysis of Variance) test on the StatPlus program (AnalystSoft). This examination determins whether the means of the test groups are statistically different, specifically the null hypothesis (H0) that the means are the same:

H0 = μ 1 = μ 2 = μ 3 = … = μ k

# A statistically different result determins that instead the alternative hypothesis is correct (HA) and at least two of the groups are statistically different to each other.Chapter 3:

# Effect of cell viability when seeded on natural or synthetic biomaterialsAim:

To examine the cell viability of keratinocytes, melanocytes, adipose derived stem cells and bone marrow derived stem cells on tissue culture plastic, fibrin and electrospun scaffolds.

To assess if these cell seeded scaffolds can be readily handled using tweezers or forceps as if used by a healthcare technician.

## Experimental protocol:

Electrospun scaffold preparation:

PLGA scaffolds were prepared by electrospinning PLGA 0.2g/ml DCM solution as previously described. Scaffolds were cut using a scalpel blade in a class 2 safety cabinet into squares approximately 1.5cm2 and briefly placed into 70% IMS solution before drying in a sterile petri dish. Once dried, scaffolds were thoroughly washed in PBS overnight to remove any remaining IMS and used immediately.

Fibrin gel preparation and cell encapsulation:

A reliable method for setting fibrin gels was investigated by mixing fibrinogen and thrombin solutions in PBS at varying concentrations. Concentrations were evaluated and assessed to determine whether gelation occurs in a rapid time frame (within 20 minutes) and if the mechanical strength was sufficient to allow movement of gels using forceps.

Once appropriate concentrations of Fibrinogen (F4883, Sigma Aldrich) and Thrombin (T6884, Sigma Aldrich) were found, PBS as a solvent for the powders was substituted with media to determine whether clots will still form. Finally cells were encapsulated within gels and their respective cell viabilities determined by an MTT assay.

Furthermore, a brief investigation into the effects of heparin on fibrin clot formation was analysed, since heparin is occasionally used as an additive in human cell culture media when platelet lysate is added as a source of growth factors.

Cell culture:

Keratinocytes, fibroblasts, adipose derived stem cells and bone marrow derived stem cells were cultured as previously described. These cells were seeded into 12 well plates at 1x105 as controls, onto electrospun PLGA or incorporated into fibrin gels. After 24, 48 or 96 hours, samples were assessed for cell metabolic activity using an MTT assay.

Ease of handling scaffolds after cell seeding:

Both fibrin and PLGA seeded scaffolds were briefly assessed for ease of handling after 24 hours incubation to determine whether they could be handled with forceps.

## Results:

Fibrinogen and Thrombin were combined at varying concentrations to find a solution which would set rapidly (in under 20 minutes) and be of sufficient mechanical strength to be picked up with forceps to allow being transported between well plates for experimental purposes.

Briefly, solutions of fibrinogen and thrombin were made by combining the lyophilised powder with PBS and filter sterilising with a 20μm syringe filter. In order to find an appropriate concentration of thrombin to fibrinogen, the concentrations shown in Table 1 were investigated and the gelation noted.

Table 1: Table describing consistency of fibrin gels with changing thrombin concentration after 1 hour incubation at 37°C:

|  |  |  |  |
| --- | --- | --- | --- |
| Fibrinogen (800μl) | Thrombin (350μl) | Notes: | Corresponding figure: |
| 20mg/ml | 0.5U/ml | The clot does not reach full gelation, instead a thin brittle gel forms over the remaining liquid (approximately 800μl).  It is difficult to pick up the gel with forceps due to considerable weakness and shearing of the gel. | Fig: A, A’ |
| 20mg/ml | 2.5U/ml | A greater volume of the clot solidifies, however a small quantity of liquid (<50μl) remains.  It is possible to pick up the gel with forceps however much of the gel remains in the well plate in a viscous liquid form. | Fig B, B’ |
| 20mg/ml | 5U/ml | This concentration of thrombin causes rapid solidification within 20 minutes whether in the incubator at 37°C or at room temperature. The gel is readily moved with forceps and can be replated with ease. | Fig C, C’ |
| 20mg/ml | 15U/ml | Despite a more rapid gelation than 5U/ml thrombin (approximately 5 minutes), the gel is brittle and unable to be picked up by forceps. Instead, the gel shears into multiple pieces. | Fig D, D’ |

Table 1 shows the different concentrations and the impact of replacing the fibrin gel in the well and picking with forceps. From these results the optimum mixture was 800μl fibrinogen (20mg/ml) and 350μl thrombin (5U/ml).

|  |  |
| --- | --- |
| **A** | **A’** |
| **B** | **B’** |
| **C** | **C’** |
| **D** | **D’** |
| Figure 1: Images of fibrin gels with different concentrations of thrombin to fibrinogen. A-D show fibrin gels after being replaced into the same well plate. A’-D’ show the gels being moved with forceps. Both A and B show the replaced gel floating in the ungelled fibrinogen solution. C and D show solid gels after being replaced into the well plate, however during transit gel D suffered shear and has split. | |

The effect of heparin on fibrin clot formation:

|  |
| --- |
| Macintosh HD:private:var:folders:3b:2h8mbl992k5556p3c7s6jjw00000gn:T:TemporaryItems:unspecified.jpg |
| Figure 2: The effects of heparin on fibrin clot formation. The ideal concentration of fibrinogen to thrombin was found to be 700μl fibrinogen solution (20mg/ml) and 350μl of thrombin solution (5U/ml). When heparin was added to fibrinogen solution at a concentration of 1U/ml clots would not form regardless of incubation time. Solutions with heparin are in the wells on the left hand side, labeled F+H: Fibrin + heparin. The second set of wells contain fibrin solutions without heparin additives, finally wells on the far right contain cell media (DMEM) only.  The 12 well plate is angled at 45° to allow solutions to accumulate at the base of the well, it can be shown that solutions with heparin added at 1U/ml are fully liquid and are of approximate viscosity to normal cell media, without heparin, fibrin gels have fully solidified. |

Incorporating cells into fibrin clots:

When modifying the protocol to dissolve fibrinogen in cell media (DMEM) rather than PBS, gelation and ease of handling the gels with forceps was found to be identical. The concentration of thrombin was found to be ideal when used at 5U/ml as this yielded a gel which could be handled with forceps and moved to a different well plate without causing damage to the clot. Therefore for one fibrin clot the ideal composition was that as previously found with PBS ie

800μl fibrinogen solution (20mg/ml)

350ul thrombin solution (5U/ml)

Cells could be incorporated into fibrin clots by resuspending a pellet of cells into the fibrinogen solution, 100µl cell suspension (1x105) + 700µl fibrinogen solution (20mg/ml in media) and mixing with thrombin solution 350µl of thrombin solution (5U per ml in PBS). After incubating for 20 minutes at 37°C the fibrin finished forming a clot.

Table 2: Table summarising the impact of culturing cells in fibrin or PLGA on the viability of hDFs, hDKs, BMSCs and ADSCs compared to cells grown on TCP for 96 hrs. Viability was assessed using an MTT assay.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Cell type | Cell seeding condition | | | | | | |
|  | Control (TCP) | Encapsulated in Fibrin | | | PLGA scaffold | | |
|  | Absorbance at 570nm | Mean absorbance at 570nm | Percentage difference to control | Statistically different to control (p<0.05) | Mean absorbance at 570nm | Percentage difference to control | Statistically different to control (p<0.05) |
| hDF | 0.116 | 0.0617 | -46.7% | Yes | 0.0535 | -53.7% | Yes |
| hDK | 0.0492 | 0.0163 | -66.8% | Yes | 0.0158 | -68.0% | Yes |
| ADSC | 0.0897 | 0.0648 | -27.7% | Yes | 0.0502 | -44.1% | Yes |
| BMSC | 0.0798 | 0.0432 | -45.9% | Yes | 0.0395 | -50.5% | Yes |

Table 2: The impact of incorporating cells in fibrin or PLGA on cell viability after 96 hours compared to when seeded on tissue culture plastic is shown in Table 2 for hDF, hDK, BMSC and ADSC cells. Both fibrin encapsulation and PLGA scaffold reduced cell viability compared to culture of cells on TCP by 27.7 to 68.0%. The best viability was with tissue culture plastic controls, then fibrin gel encapsulation and the least with PLGA. These cell viabilities were statistically different to p<0.05 compared to tissue culture plastic controls.

Cells were seeded in triplicate, with 2 repeats of the experiment. Figures 2, 3, 4 and 5 illustrate the cell viabilities at 24, 48 and 96 hours for the different cell and substrate combinations. Figures 6 and 7 are photographs to illustrate scaffold appearance after 24 hours incubation at 37°C.

Handling cells seeded on PLGA scaffolds:

Figure 2: Graph to show hDF viability over 96 hours when seeded onto tissue culture plastic (Control), encapsulated in a fibrin gel or seeded onto an electrospun PLGA scaffold. Cells were seeded at 1x105 per condition.

Samples were assessed by an MTT assay at 24, 48 or 96 hours and the absorbance at 570nm with a reference of 630nm is plotted.

Cells were seeded in triplicate, with 2 repeats of the experiment.

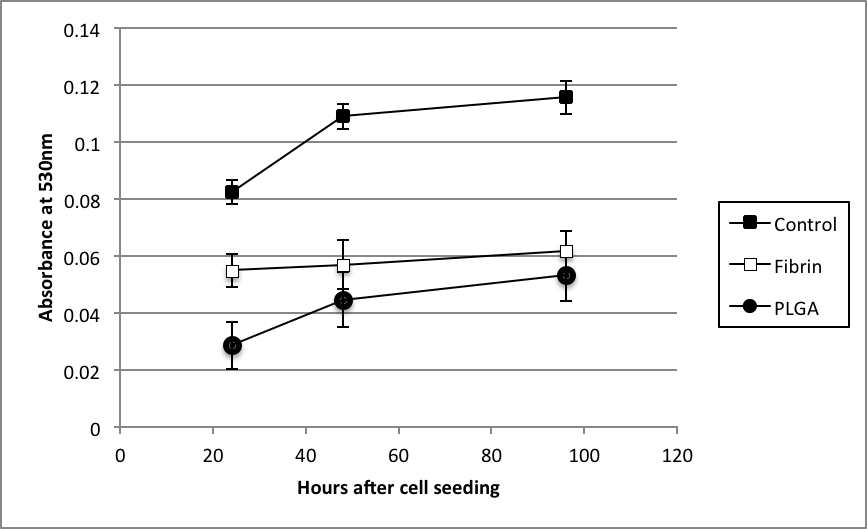


Figure 3: Graph to show hDK viability over 96 hours when seeded onto tissue culture plastic (Control), encapsulated in a fibrin gel or seeded onto an electrospun PLGA scaffold. Cells were seeded at 1x105 per condition.

Samples were assessed by an MTT assay at 24, 48 or 96 hours and the absorbance at 570nm with a reference of 630nm is plotted.

Cells were seeded in triplicate, with 2 repeats of the experiment.

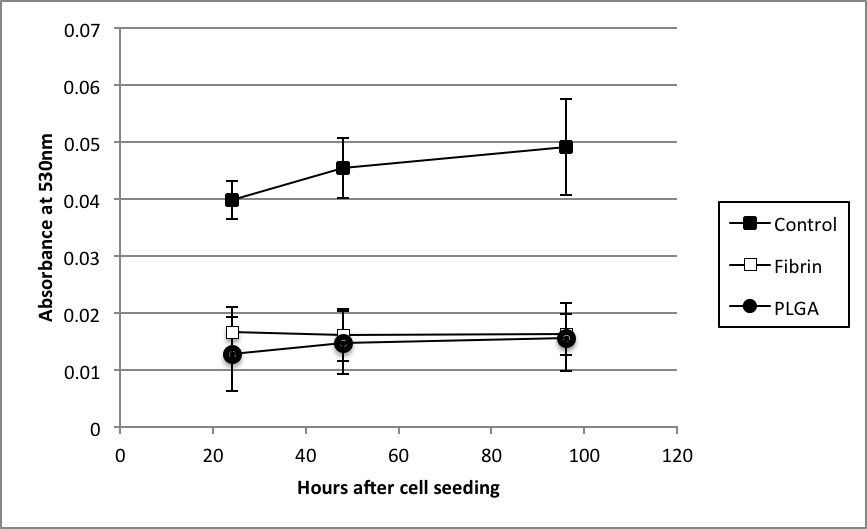


Figure 4: Graph to show BMSC viability over 96 hours when seeded onto tissue culture plastic (Control), encapsulated in a fibrin gel or seeded onto an electrospun PLGA scaffold. Cells were seeded at 1x105 per condition.

Samples were assessed by an MTT assay at 24, 48 or 96 hours and the absorbance at 570nm with a reference of 630nm is plotted.

Cells were seeded in triplicate, with 2 repeats of the experiment.

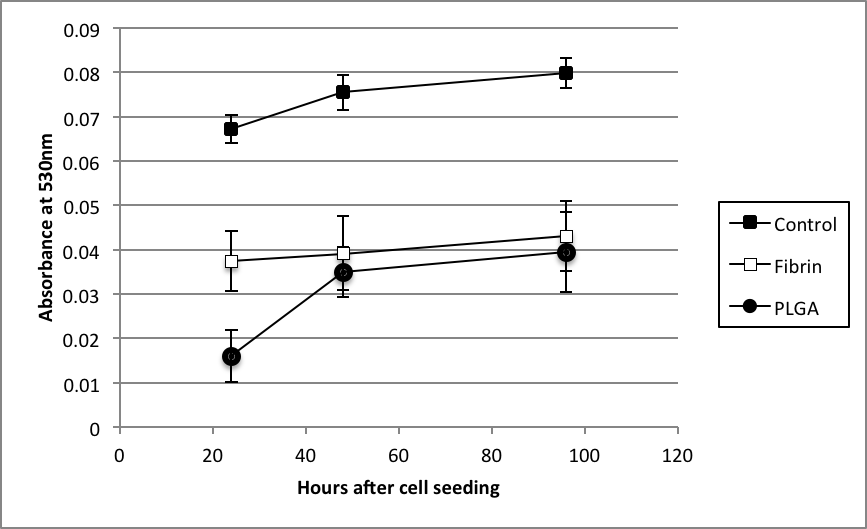
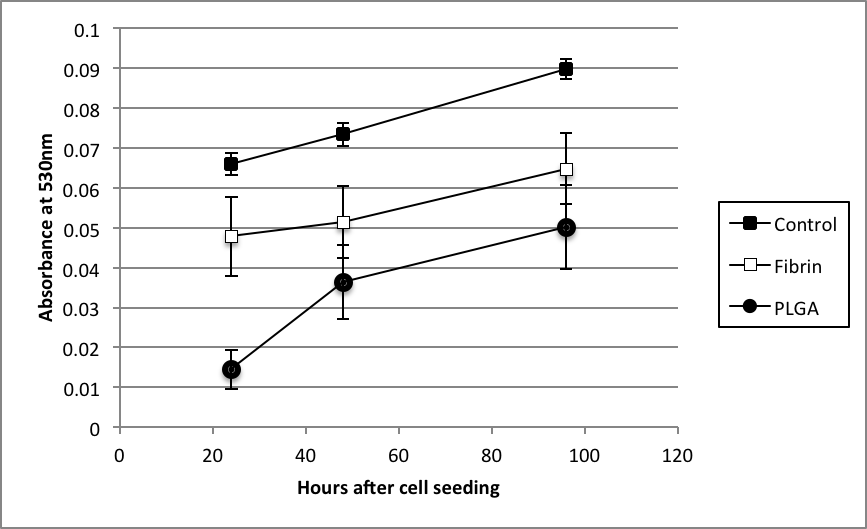


Figure 5: Graph to show ADSC viability over 96 hours when seeded onto tissue culture plastic (Control), encapsulated in a fibrin gel or seeded onto an electrospun PLGA scaffold. Cells were seeded at 1x105 per condition.

Samples were assessed by an MTT assay at 24, 48 or 96 hours and the absorbance at 570nm with a reference of 630nm is plotted.

Cells were seeded in triplicate, with 2 repeats of the experiment.



|  |  |
| --- | --- |
| **A** | |
| **B** | **B’** |
| Figure 6: Photographs showing electrospun scaffolds submerged in cell culture media after 24 hours at 37°C.  Image A shows PLGA scaffolds submerged in media with no cells, B and B’ are representative images of scaffolds 24 hours after fibroblast cell seeding. Regardless of cell type, cell seeded electrospun scaffolds exhibit contraction. It isn’t possible to uncurl scaffolds once contraction has started, however scaffolds are readily picked up and moved using forceps. | |

## Handling cells encapsulated in fibrin:

|  |  |
| --- | --- |
| C:\Users\mgadelorge\Desktop\Sans titre.png |  |
| Figure 7: Photographs demonstrating fibrin clots containing ADSCs can be transferred to a different well plate using tweezers, 1 hour after encapsulating the cells. | |

## Discussion:

This chapter discusses the ideal setting conditions to form a reproducible fibrin gel which sets in less than 20 minutes and is mechanically strong enough to be readily handled using forceps. Furthermore, it was possible to encapsulate cells within these gels by resuspending a cell pellet with stock 20mg/ml fibrinogen solution before combining with 5U/ml thrombin to initiate gelation. The gels formed were approximately 1ml in volume and of cylindrical shape, 18mm in diameter.

Platelet lysate is often an addition to human stem cell culture media to replace xenogenic serum. In order to prevent the clotting factors within platelet lysate causing an unwanted clot in the cell media, heparin is commonly added. Therefore the effect of heparin in a typical cell media concentration was investigated on the clotting potential of fibrin. As expected, the addition of heparin interfered with the fibrin clotting mechanism and clots would not form at a concentration of 1U/ml heparin.

Electrospun PLGA scaffolds are simple to produce, forming a sheet of reproducible scaffolds in a time efficient manner. Scaffolds are readily sterilized by a multitude of options, including using ethanol and gamma irradiation although the method of sterilization can affect mechanical stability of scaffolds. For this investigation however, the relatively short time of cell culture (96 hours) meant the long term mechanical properties of these scaffolds was not of interest [159].

Experimentally; keratinocytes, fibroblasts, ADSCs and BMSCs were cells seeded onto tissue culture plastic, fibrin or PLGA. Cell viability was assessed at 24, 48 and 96 hours using an MTT assay.

Broadly, cell viabilities hDKs, hDFs, ADSCs and BMSCs were highest when cultured on tissue culture plastic, the controls for this investigation. Cells encapsulated in fibrin had the next greatest cell viability, cells seeded onto PLGA scaffolds were the least viable. Cells seeded onto scaffolds were statistically different to controls at p<0.05 for all cases, irrespective of cell type or scaffold used.

Fibrin appeared to exert slightly less of a negative effect on cell viability, decreasing cell viabilities to 27.7-66.8% of control viability compared to 44.1-68.0% when seeded onto electrospun PLGA. ADSCs appeared to be less affected by scaffolds, having the closest cell viability to controls, 27.7% less when in fibrin and 44.1% less viability when seeded onto PLGA. Keratinocytes were most affected by cell seeding on scaffolds compared to controls, 66.8% less viable on fibrin and 68.0% less viable on electrospun PLGA.

The assay used to assess cell viability, MTT, is based on cell metabolism converting the MTT solution into the pigment formazan. The absorbance of this assay is used to indicate cell viability and is proportional to concentration and volume of absorbance according to Beers law. Since the MTT solution is likely to have been absorbed by the PLGA scaffolds and fibrin gels, this potentially explains the decreased readings across the range of test conditions when comparing scaffolds to controls. Additionally, any converted formazan is likely to have been absorbed or trapped within the PLGA or gels due to the large bulk of the scaffolds used, leading to falsely biased results against the use of scaffolds when comparing directly to tissue culture plastic alone.

Post cell seeding, it became clear that electrospun scaffolds readily contracted over a period of 24 hours. Whilst not detrimentally affecting cell viabilities over 96 hours or affecting the ease of handling scaffolds, scaffold surface area is affected which may in turn affect the ability of cells to migrate from the surface of the scaffold to provide the required therapeutic response.

## Conclusion

This chapter describes a simple method to form fibrin gels and encapsulate 4 different cell types; fibroblasts, keratinocytes, adipose derived and bone marrow derived stem cells. All cell types showed consistent cell metabolism over 96 hours. Additionally, PLGA electrospun scaffolds readily support these cell types over 96 hours, however scaffolds readily contract after being seeded with cells.

These biomaterials can readily support a variety of cell types over a period of 4 days.

# Chapter 4:

# Effect of transport conditions on cell viabilityAim:

To identify a method which enables ADSCs to be maintained out of incubator conditions for more than 48 hours.

To assess this, ADSCs were taken out of incubator conditions and supplemented with or without 5% CO2 at ambient temperatures in the lab (~22°C).

## Experimental protocol:

Transport container:

Firstly, an airtight plastic storage box was modified to allow 2 tubes to enter the box; an airtight seal was maintained around the tubing and container lid with remouldable synthetic rubber putty. These transport boxes were large enough to fit a conventional sized 12 well plate. Once well plates containing cell cultures were placed into the transport boxes, one of the tubes could be connected to a gas canister containing 95% air supplemented with 5% CO2 and the boxes gassed through. After gassing for 1 minute, both tubes could be sealed with a 3 point stopcock (Cole Parmer) to ensure the boxes remained airtight and retained the supplemented gas. Due to relative elasticity of the plastic container, the ‘end’ stopcock could be closed whilst the gas canister still permeated the chamber with gas, causing the container to swell but retaining the airtight seal. When the ‘front’ stopcock is closed to ensure a full seal and removal from the gas canister, the container remains airtight despite having marginally swollen in size.

Resealable container

Gas flow in

Gas flow out

Stopcock

Synthetic rubber adhesive

Figure 1: diagram representing the design of the transport containers. Petri dishes or culture well plates can be placed in the containers before being made airtight with a synthetic rubber adhesive around the container lid. Tubing can connect to a gas canister and gassing will flow through the container and out from the other tube. After gassing, external ambient air can be prevented from entering the container by closing the stopcocks.

Tubing

Gas flow

|  |
| --- |
| C:\Users\mgadelorge\AppData\Local\Microsoft\Windows\Temporary Internet Files\Content.IE5\YN56231O\IMG_20160317_113509.jpg |
| Figure 2: Photograph of the finished design for transport containers. This image shows a 12 well plate seeded with ADSCs inside the transport containers after gassing with 5% CO2. Stopcocks are closed immediately after gassing to prevent the gas inside the containers reaching equilibrium with ambient air. For simulated transport conditions with no CO2 supplement, no gassing was performed the stopcocks were left open for the entirety of the experiment. |

|  |
| --- |
| A  B  C  D |
| Figure 3: Photograph of DMEM in petri dishes, 24 hours after plating. From A-D; media kept at: A: 37°C with 5% CO2, B: 22°C with 5% CO2 gassing for 30 seconds, C: 22°C with 5% CO2 gassing for 1 minute, D: 22°C with no supplementary CO2.  The control media, A, is buffered to have a pH of 7.4 when at 37°C with 5% CO2 which is shown above. Compared to media sample A; B has a slightly more alkaline pH, C appears to be at a similar pH and D is clearly more alkaline than all other samples. |

From this brief visual investigation into media pH, it is shown that gassing with CO2 is necessary to prevent media pH alkalinity. The temperature of media does not appear to have an effect, however doubling the gassing time from 30 seconds to 1 minute leads to a pH almost identical to that of media kept in the incubator.Cell culture:

Human keratinocytes, fibroblasts adipose derived stem cells and bone marrow derived stem cells were cultured in an incubator at 37°C with 5% CO2. These cells were then resuspended and seeded into 12 well plates before being placed into the following culture conditions, either:

* In an incubator at 37°C with 5% CO2
* In transport boxes at ambient temperatures (~22°C) supplemented with 5% CO2
* In transport boxes at ambient temperatures (~22°C) with unsealed tubes exposed to air

The following cell/scaffold combinations were examined:

* Fibroblasts on tissue culture plastic
* Keratinocytes on tissue culture plastic
* BMSCs on tissue culture plastic
* ADSCs on tissue culture plastic
* ADSCs on electrospun PLGA scaffolds
* ADSCs encapsulated in fibrin gels

Whether on tissue culture plastic or scaffold, all cells were seeded at a concentration of 100,000 cells, and cell viability was assessed using an MTT viability assay 48 hours after being moved to the designated transport condition.

ADSC migration from fibrin to DED after 48 hours in transport conditions:

Furthermore, an investigation into whether cells would be able to migrate from scaffold to DED after being kept in transport conditions for 48 hours was undertaken. 1x106 ADSCs were encapsulated in fibrin gels and stored in the transport containers at ambient temperatures for 48 hours. After this time, gels were moved with forceps to DED and placed in the incubator for 24, 48, 96 or 168 hours. At each time point, a fibrin clot was moved to a separate well and DED incubated with 1ml MTT solution for 45 minutes. The results of this were photographed and compared.

## Hypothesis:

From previous data shown, it appears the pH of cell media can be kept similar out of the incubator by gassing a container with 5% CO2. The hypothesis tested will be that cell viability will not be affected by culture conditions outside of the incubator at ambient temperatures (~22°C) if flushed with 5% CO2 against controls in the incubator. This will be tested against a probability of p<0.05.

Secondly, the effect of CO2 gassing on cell cultures when in transport conditions at ambient temperatures (~22°C) will be analysed. The hypothesis tested will be that CO2 gassing is necessary for cells to remain viable. This will be tested against a probability of p<0.05.

## Results:

Comparisons were made of viabilities when cells were stored in an incubator at 37°C with 5% CO2 vs transport containers at ambient temperatures (~22°C) supplemented with 5% CO2 or without 5% CO2. These comparisons illustrate the impact of changing the storage temperature or altering the concentration of CO2 in the air of the container. Percentage changes in cell viability were calculated and statistical differences in cell viability calculated using an unpaired 2-tailed Students’ t test with the significance level set at p<0.05.

Table 1: Effect of cell viability when cell cultures are kept at 22°C in regular air, 22°C with 5% CO2 or 37°C with 5% CO2 (3SF):

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  |  | Culture condition, mean MTT viability data | | | Comparing viability of cells at 22°C vs 37°C + 5% CO2 | Comparing viability of cells at 22°C with CO2 vs without CO2 | Figure |
| Cell type | Substrate | 22°C | 22°C + CO2 | 37°C + CO2 | Percentage difference | Percentage difference |  |
| hDFs | **TCP** | 0.006 | 0.0974 | 0.0973 | -0.114% | 1520% | 4 |
| hDKs | **TCP** | 0.00178 | 0.0376 | 0.0432 | 13.1% | 2010% | 5 |
| BMSCs | **TCP** | 0.00211 | 0.0469 | 0.0664 | 29.4% | 2120% | 6 |
| ADSCs | **TCP** | 0.00178 | 0.0821 | 0.0801 | -2.50% | 4520% | 7 |
| ADSCs | **PLGA** | 0.00633 | 0.0274 | 0.0275 | 0.484% | 333% | 8 |
| ADSCs | **Fibrin** | 0.0236 | 0.0492 | 0.0517 | 4.73% | 109% | 9 |

Cells were seeded at a concentration of 1x106 cells/ml, 100μl, onto the designated substrate and cultured for 48 hours in different culture conditions, 22°C, 22°C with 5% CO2 or 37°C with 5% CO2 for 48 hours before assessing viability using an MTT assay at 570nm. hDFs – human dermal fibroblasts, hDK – human dermal fibroblasts, BMSCs – bone marrow derived stem cells, ADSCs – adipose derived stem cells. TCP – tissue culture plastic, PLGA, poly(lactic-co-glyocolic)acid. NS – not significant

The table shows that the cells hDFs, hDKs and ADSCs do not vary in viability from controls when cultured at 22°C with CO2 by more than 13.1%. BMSCs, however, are 29.4% less viable at the lower culture temperature. hDKs also appear to be sensitive to the change in culture temperature with a drop of 13.1% in viability when cultured at these lower temperatures compared to controls.

When comparing viability of cells at 22°C with CO2 or without CO2; TCP seeded cells vary in viability from 1520% to 4520%, favouring the supplement of CO2. When seeded onto scaffolds, ADSCs had greater cell viability, with a percentage difference of 109% (in fibrin) and 333% (on PLGA).

Figure 4: Effect of 5% CO2 gassing and temperature on hDF cell viability when seeded on tissue culture plastic in 12 well plates after 48 hours. Cell viability is calculated by measuring absorbance at 570nm after incubation with MTT solution. Mean ± SEM. Cells were seeded at 1x105, n=3 experiments, 3 replicates.

ANOVA (One-Way) read out from StatPlus:

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Source of Variation | SS | df | MS | F | p-value | F crit |
| Between Groups | 0.05011 | 2 | 0.02506 | 3,064.58 | 0.00E+00 | 3.40283 |
| Within Groups | 0.0002 | 24 | 8.18E-06 |  |  |  |
| Total | 0.05031 | 26 |  |  |  |  |

Since the p-value expressed is 0 with a multiple 0 exponential (too low for the software to express) it can be concluded that the null hypothesis (no difference between test conditions) is rejected and the test conditions are statistically different.

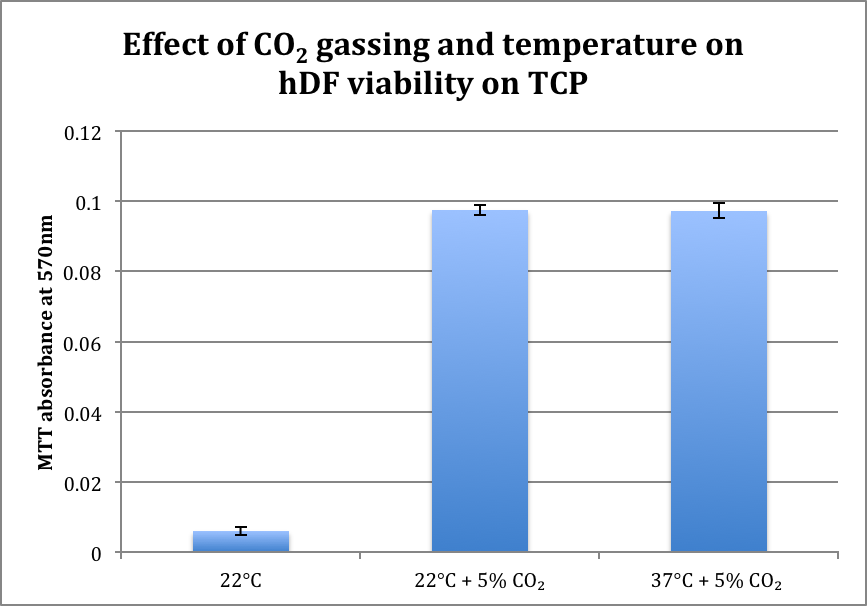


Figure 5: Effect of 5% CO2 gassing and temperature on hDK cell viability when seeded on tissue culture plastic in 12 well plates after 48 hours. Cell viability is calculated by measuring absorbance at 570nm after incubation with MTT solution. Mean ± SEM. Cells were seeded at 1x105, n=3 experiments, 3 replicates.

ANOVA (One-Way) read out from StatPlus:

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Source of Variation | SS | df | MS | F | p-value | F crit |
| Between Groups | 0.00909 | 2 | 0.00454 | 117.61994 | 0.00E+00 | 3.40283 |
| Within Groups | 0.00093 | 24 | 0.00004 |  |  |  |
| Total | 0.01002 | 26 |  |  |  |  |

Since the p-value expressed is 0 with a multiple 0 exponential (too low for the software to express) it can be concluded that the null hypothesis (no difference between test conditions) is rejected and the test conditions are statistically different.

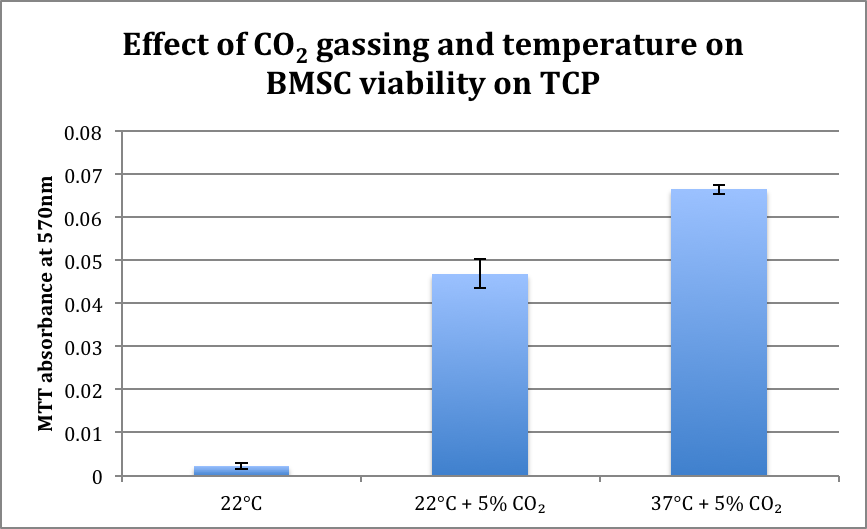
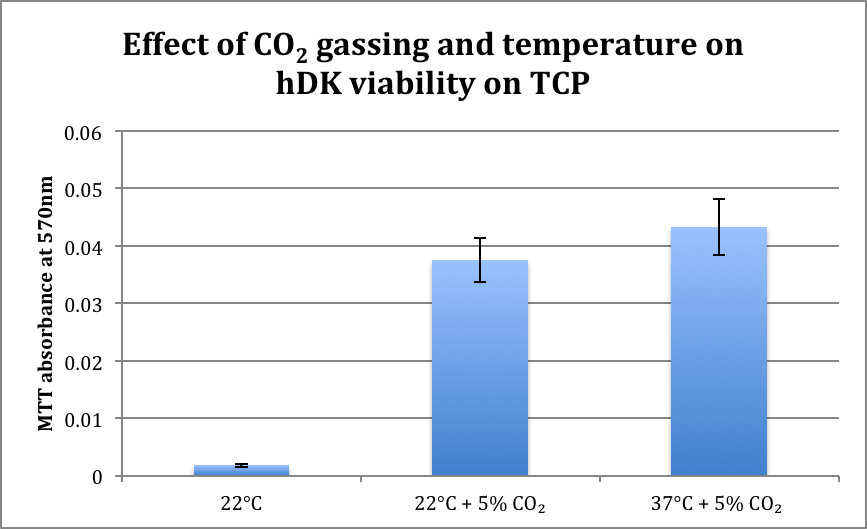


Figure 6: Effect of 5% CO2 gassing and temperature on BMSC viability when seeded on tissue culture plastic in 12 well plates after 48 hours. Cell viability is calculated by measuring absorbance at 570nm after incubation with MTT solution.

Mean ± SEM. Cells were seeded at 1x105, n=3 experiments, 3 replicates.

ANOVA (One-Way) read out from StatPlus:

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Source of Variation | SS | df | MS | F | p-value | F crit |
| Between Groups | 0.01958 | 2 | 0.00979 | 734.20278 | 0.00E+00 | 3.40283 |
| Within Groups | 0.00032 | 24 | 0.00001 |  |  |  |
| Total | 0.0199 | 26 |  |  |  |  |

Since the p-value expressed is 0 with a multiple 0 exponential (too low for the software to express) it can be concluded that the null hypothesis (no difference between test conditions) is rejected and the test conditions are statistically different.

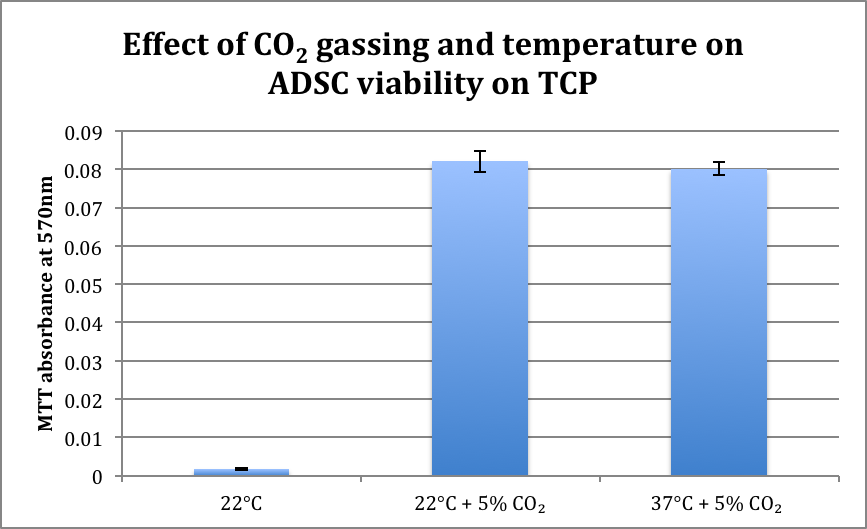


Figure 7: Effect of 5% CO2 gassing and temperature on ADSC viability when seeded on tissue culture plastic in 12 well plates after 48 hours. Cell viability is calculated by measuring absorbance at 570nm after incubation with MTT solution.

Mean ± SEM. Cells were seeded at 1x105, n=3 experiments, 3 replicates.

ANOVA (One-Way) read out from StatPlus:

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Source of Variation | SS | df | MS | F | p-value | F crit |
| Between Groups | 0.03778 | 2 | 0.01889 | 1,761.79 | 0.00E+00 | 3.40283 |
| Within Groups | 0.00026 | 24 | 0.00001 |  |  |  |
| Total | 0.03804 | 26 |  |  |  |  |

Since the p-value expressed is 0 with a multiple 0 exponential (too low for the software to express) it can be concluded that the null hypothesis (no difference between test conditions is rejected and the test conditions are statistically different.

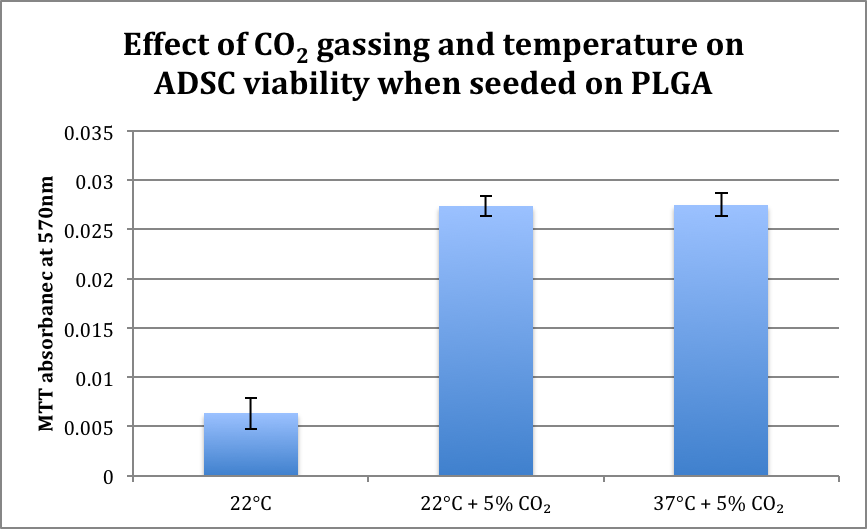


Figure 8: Effect of 5% CO2 gassing and temperature on ADSC viability when seeded on electrospun PLGA scaffolds after 48 hours. Cell viability is calculated by measuring absorbance at 570nm after incubation with MTT solution.

Mean ± SEM. Cells were seeded at 1x105, n=3 experiments, 3 replicates.

ANOVA (One-Way) read out from StatPlus:

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Source of Variation | SS | df | MS | F | p-value | F crit |
| Between Groups | 0.00268 | 2 | 0.00134 | 271.80934 | 3.26x10-17 | 3.40283 |
| Within Groups | 0.00012 | 24 | 4.92E-06 |  |  |  |
| Total | 0.0028 | 26 |  |  |  |  |

Since the p-value expressed is p<0.005 it can be concluded that the null hypothesis (no difference between test conditions) is rejected and the test conditions are statistically different.

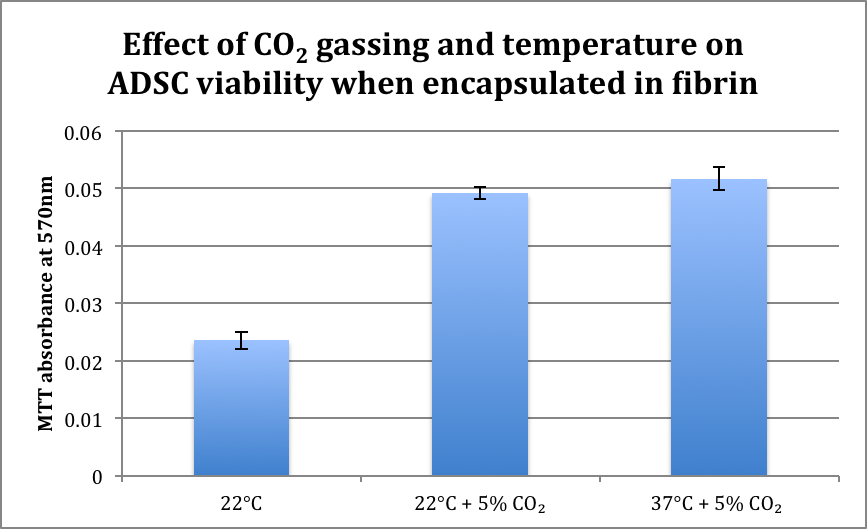


Figure 9: Effect of 5% CO2 gassing and temperature on ADSC viability when encapsulated in fibrin after 48 hours. Cell viability is calculated by measuring absorbance at 570nm after incubation with MTT solution.

Mean ± SEM. Cells were seeded at 1x105, n=3 experiments, 3 replicates.

ANOVA (One-Way) read out from StatPlus:

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Source of Variation | SS | df | MS | F | p-value | F crit |
| Between Groups | 0.00436 | 2 | 0.00218 | 291.35723 | 1.47x10-17 | 3.40283 |
| Within Groups | 0.00018 | 24 | 7.49E-06 |  |  |  |
| Total | 0.00454 | 26 |  |  |  |  |

Since the p-value expressed is p<0.005 it can be concluded that the null hypothesis (no difference between test conditions) is rejected and the test conditions are statistically different.

## Cell delivery to DED after 48 hours in container:

|  |  |  |  |
| --- | --- | --- | --- |
| A | B | C | D |
| 24 | 48 | 96 | 168 |

Figure 10: Photographs showing DED after incubation for 45 minutes with MTT solution. Fibrin clots were made containing 1x106 cells and kept at 22°C with 5% CO2 for 48 hours before being removed from culture and placed onto DED on the papillary side of the dermis. Images shown are of DED constructs 24, 48, 96 or 168 hours after initial fibrin placement onto DED.

At hours 24, 48, 96 and 168 after being moved onto the dermis, the fibrin clot was moved to a separate well and both wells were flooded with 1ml of MTT solution. From images A-D, cells have distinctively begun to migrate from the fibrin to dermis in increasing numbers due to the colouration of the dermis. Image A shows minimal staining due to low cell number migration, but by 48 hours onwards staining is clear. At 168 hours cells have migrated almost 1cm from the initial placement of the fibrin clot.Discussion:

Cell survival relies on maintenance of many different factors, from temperature, pH, osmotic potential, glucose concentration and other salts within tight constraints for optimum growth, proliferation and function. In conventional cell culture, many of these needs are addressed by the use of an incubator and appropriate cell growth media.

*In vitro* cell culture pH balance is often stabilised to *in vivo* levels by use of a carbonate buffer additive in cell culture media. If exposed to normal air, this carbonate buffer scavenges H+ ions in the media leading to an alkaline pH in the media; hence it is necessary for the air to be supplemented with raised CO2 (often at 5%).

In this chapter the design of an airtight box which can be flushed through with gas is described. The design is modelled on the transport container detailed by P. Eves et al (2011) [158], although this design differs by allowing the gas exit stopcock to be closed for a short time during gas perfusion of the container, preventing back flow of regular air and maintaining the internal air of the container at 5% CO2.

By a visual assessment at the colour of cell media in figure 3, it is possible to make an estimate at the pH of the media due to the colour change of phenol red in the media. Comparing the colour change, it is clear that media kept at 22°C will readily turn alkaline. If conditions are kept identical but the container is flushed with 5% CO2, it can be seen that media retains a similar pH to that of media kept in the incubator at 37°C supplemented with 5% CO2.

Fibroblasts on TCP, ADSCs on TCP, PLGA or fibrin retained cell viability within 5% of controls when comparing culture conditions with 5% CO2 at 22°C and 37°C. Keratinocytes and BMSCs on TCP were more viable at 37°C than 22°C + 5% CO2 with the percentage differences being 13.1% and 29.4% respectively. Therefore we can conclude that cell viability can be maintained in these conditions at ambient temperatures with near identical cell survival within 48 hours. However, it should be noted that cell metabolic activity, which is measured by the MTT assay used in these experiments, does not necessarily correlate to cell growth, which should be expected to decrease with lowered culture temperatures or longer term viability after 48 hours [151].

Experimentally, cell viabilities were investigated at identical temperatures, 22°C. Each cell type; fibroblasts, keratinocytes, adipose derived and bone marrow derived stem cells were treated identically except for one condition with supplemented 5% CO2.. For fibroblasts, keratinocytes, bone marrow derived stem cells and adipose derived stem cells on TCP the percentage improvement on viability ranged from 1520% to 4520% when supplemented with 5%CO2. Since there was almost no detectable absorbance during the MTT assay, it is strongly inferred there were no remaining live cells in these conditions. This is confirmed when analysing the one-way ANOVA test results, concluding the culture conditions are statistically different.

Adipose derived stem cells are being extensively researched due to the positive effects they exert on reducing inflammation, promoting angiogenesis, fibroblast migration and proliferation [71][72][73]. Therefore further research has been undertaken on whether cell viability during transport conditions can be improved when cells have been preseeded onto scaffolds. We had the intent in providing a technique to improve the ‘shelf life’ of a cellular therapeutic device which could be transported to a healthcare clinic in a ready to use fashion. We aimed to maintain the cells in transport conditions for a number of days in order for a a courier to deliver cells internationally or globally with no detrimental effect on cell viability, with the intent that these cells could either then migrate from scaffold to the wound in question or exert a wound healing response via paracrine signalling.

These cells were investigated in this chapter when encapsulated in fibrin or seeded onto an electrospun polymer, PLGA. In the cases where ADSCs had been seeded onto PLGA scaffolds or encapsulated in fibrin gels, samples kept at 22°C with no 5% CO2 remained more viable, compared to other cell types on tissue culture plastic alone. This finding confirms that seeding cells onto a scaffold increases viability when kept out of incubated conditions [156][154][157], although the impact on viability is not as great as supplementing with 5% CO2. Despite the increase in viability when cultured on scaffold, the one-way ANOVA analysis confirms the statistical difference between culture conditions.

The final investigation of this chapter involved determining whether ADSCs would migrate from the fibrin gel to a skin model after being maintained in transport conditions for 48 hours. This would infer cells can exhibit a wound healing response and can function as intended once transported to a healthcare clinic in a ‘ready to use’ product. The cells in this instance were exposed to MTT solution in order to dye the cultures with the visible purple crystal formazan, and clear photographs could be taken of the cell populations which had migrated to the epidermis over a period of 7 days. This will be further evaluated in the next chapter to determine whether ADSCs retain characteristics such as their differentiation potential, stem cell markers, and the depths to which the migration potential into de-epidermised dermis after 48 hours in transport conditions.

# **Conclusion:**

Airtight transport containers were constructed which can be flushed through with a gas comprised of 95% air with 5% CO2. These containers can fit a conventional well plate and support cell viability out of a normal cell culture incubator at ambient temperatures for up to 48 hours at ambient temperatures. However, without 5% CO2 gassing, cell viability is severely diminished for all cell types and substrates. The clear difference in cell viabilities for each culture condition was verified using a one-way ANOVA. Fibroblasts, keratinocytes, adipose derived stem cells and bone marrow derived stem cells were supported in these containers when seeded on tissue culture plastic, additionally adipose derived stem cells maintained their cell viability when seeded on PLGA electrospun scaffolds or encapsulated in fibrin.

After 48 hours in simulated transport conditions, ADSCs encapsulated in fibrin were placed onto de-epidermised dermis. Over several days these constructs were removed and dermis incubated with MTT solution. Clear images show metabolically active ADSCs which had migrated over the dermis.

# Chapter 5:

# Post transport effect on adipose derived stem cells

## Aim:

The aims of this chapter are to expand on the previous work of the other two results chapters and assess the post transport performance of ADSCs after 48 hours in fibrin with respect to their ability to differentiate, whether they retained their stem cell markers and their potential to migrate to a simulated wound bed. All work in this chapter was carried out in partnership with StromaLab, Inserm, Toulouse, France with the kind help of Mélanie Gadelorge.

## Experimental plan:

ADSCs were assessed both in fibrin clots and on tissue culture plastic at 1x106 in order to deliver a relevant therapeutic dose of cells. Therapeutic potential of ADSC delivery from fresh isolation was investigated by using cells of freshly isolated cells, kindly donated by Mélanie Gadelorge from StromaLab, Inserm, Toulouse, France.

Culture conditions of cells were as follows:

* 37°C with 5% CO2 on tissue culture plastic
* 37°C with 5% CO2 in fibrin gels
* transport conditions at ambient temperatures (~22°C) supplemented with 5% CO2 on tissue culture plastic
* transport conditions at ambient temperatures (~22°C) supplemented with 5% CO2 in fibrin gels

Cells were assessed for viability using an MTT assay over a period of 24, 48 and 72 hours in the above conditions.

For cell conditions without fibrin, adhered cells were recovered from the well plate using trypsin. Fibrin encapsulated cells were recovered by mechanically agitating the gels using a sterile pipette tip and before addition of trypsin to recover cells before reseeding into a culture flask for 24 hours to allow ADSCs to adhere to tissue culture plastic. The following investigations were then performed:

* Fluorescence-activated cell sorting (FACS) to assess the effect of culture conditions on cell surface antigens. The antigens which were assessed were: CD90, CD73, CD14, CD105, CD31, CD34, CD45, CD29, HLADR, CD13 and CD36; this was kindly performed by Mélanie Gadelorge.

* A differentiation study to assess whether ADSCs retain their ability to differentiate into different lineages, namely adipose and osteoblast cells, after encapsulation in fibrin and culture in transport conditions at 37°C or ambient temperatures (~22°C) supplemented with 5% CO2. Cells were assessed by further culture in adipogenic culture media or osteoblast induction media and cell surface markers assessed; this was investigated by Mélanie Gadelorge.
* To investigate the migration potential of these cells to a wound bed, fibrin clots stored in an incubator at 37°C with 5% CO2 or transport conditions at ambient temperatures (~22°C) supplemented with 5% CO2 for 48 hours were placed onto de-epidermised dermis. These samples were imaged using confocal microscopy for up to 14 days to determine whether cells would migrate from the fibrin clot to the dermis. ADSCs were labeled with fluorescent lentiviral particles for the purpose of imaging. Constructs were imaged with the help of Mélanie Gadelorge and Corinne Bareau, StromaLab, Inserm, Toulouse, France).

Fluorescent Lentiviral labelling of cells:

Cells were counted and lentiviral particles tagged with red fluorescent protein were thawed from -80°C. Lentiviral particles were diluted to yield 200,000 particles per cell in 500µl of serum free cell media.

The viral suspension was added to the cells with protamine sulphate at 20µg/ml to improve the rate of viral transduction and cells incubated for 10 hours at 37°C. After incubation, culture medium was removed and cells washed with serum free media to remove excess particles. Cell media with serum was then replaced and cultured as normal. ADSCs were kindly prepared using this method by Anne Prel, StromaLab, Inserm, Toulouse, France.

All cells were incorporated into fibrin clots under either transport or incubated conditions. Then 48 hours post seeding, gels were removed from the well plate and placed onto de-epidermised dermis, prepared as above.

## Results:

The aim of this chapter was to assess cells in the following conditions, however due to lack of cells recovered from experiments or samples lost to infection, not all experiments could be performed.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | 37°C with 5% CO2 | | 22°C with 5% CO2 | |
|  | Without fibrin | With fibrin | Without fibrin | With fibrin |
| Cell viability in fibrin clots | Not tested | ✔ | Not tested | ✔ |
| Differentiation potential | ✔ | ✔ | Lack of cells | Lack of cells |
| FACS | ✔ | ✔ | ✔ | ✔ |
| Cell migration to de-epidermised dermis after fibrin encapsulation | ✔ | ✔ | ✔ | ✔ |

|  |
| --- |
|  |
| Figure 1: MTT viability of ADSCs encapsulated in fibrin at 24, 48 and 72 hours post seeding.  Different cell donors were used in the investigation, results are n=3. Error bars are +/- SEM. Data shown is n=3.  Percentage difference between the mean cell viability after culturing at different temperature conditions are as follows; when culturing at 22°C, donor 1 had a 65.5% variance in cell viability and donor 2 had a 108.7% variance in cell viability, both favouring culture conditions at 37°C. To p<0.05 these variances were significantly different. |

|  |  |
| --- | --- |
| 37°C with 5% CO2 | 22°C with 5% CO2 |
|  |  |
| Figure 2: Fibrin gels containing 1x106 ADSCs were imaged in greyscale after incubating samples with MTT solution for 45 minutes. Images show fibrin gels at day 4 post gelation after being stored at 37°C or at 22°C with 5% CO2. Cell viability at 4 days post encapsulation in cells is indicated clearly by the dark staining of the samples. | |

ADSC ability to differentiate to osteoblasts:

|  |
| --- |
|  |
|  |
| Figure 3: Showing the osteogenic differentiation potential of ADSCs after encapsulation in fibrin after 48 hours.  ND – not differentiated, Diff – differentiated.  Cells were grown over 9 days using osteogenic culture media and the following gene expression markers; OSX - Osterix, RUNX2 – Runt-related transcription factor 2 and DLX5 – Distal-Less Homeobox 5, were assessed to determine levels of differentiation to osteogenic lineages. Results are inconclusive due to the small test size (n=1) however it appears the previous encapsulation of cells in fibrin inhibits ADSC ability to differentiate down the osteogenic lineage. |

ADSC ability to differentiate to adipocytes:

|  |
| --- |
|  |
|  |
| Figure 4: Showing the adipogenic differentiation potential of ADSCs after encapsulation in fibrin after 48 hours.  Cells were grown over 14 days using adipogenic culture media and the following gene expression markers; LPL – Lipoprotein lipase, PParγ – Peroxisome proliferator-activated receptor gamma and CEPBα – CCAAT/enhancer-binding protein alpha, were assessed to determine levels of differentiation to adipogenic lineages. Results are inconclusive due to the small test size (n=1) however it appears the prior encapsulation of cells in fibrin does not affect ADSC ability to differentiate down the adipogenic lineage. |

ADSC ability to differentiate to adipocytes (continued):

|  |
| --- |
|  |
|  |
| Figure 5: Showing the adipogenic differentiation potential of ADSCs after encapsulation in fibrin after 48 hours.  Cells were grown over 14 days using adipogenic culture media and the following gene expression markers; Leptin, AP2 – adipocyte protein 2 and AdipoQ were assessed to determine levels of differentiation to adipogenic lineages. Results are inconclusive due to the small test size (n=1) however it appears the previous encapsulation of cells in fibrin does not affect ADSC ability to differentiate down the adipogenic lineage. |
|  |

FACS analysis of cells encapsulated in fibrin clots or seeded on tissue culture plastic, in incubated conditions or transport conditions:

|  |
| --- |
|  |
| Figure 6: FACS analysis of 3 donors 48 hours after being kept in incubated or transport conditions, with or without fibrin. Supplementary data is shown in the appendix. There is no significant difference between the stem cell markers. CD90, CD73, CD14, CD105, CD31, CD34, CD45, CD29, HLADR, CD13, CD36 were assessed. Error bars are standard deviation of the mean. |

|  |  |
| --- | --- |
| Incubator conditions Transport conditions | |
| A  A’  B  B’  C  C’  D  D’ | 24 hours  48 hours  72 hours  96 hours |
| Figure 7: Light microscopy images of ADSCs encapsulated in fibrin. Images were taken after 30 minutes incubation with MTT solution. A, B, C, D samples were kept in the incubator, A’, B’, C', D’ samples were kept in transport conditions after 24, 48, 72 and 96 hours. Formazan staining indicates cell viability in these conditions. Cell morphology in incubated conditions is spindly and have spread out, cells in transport conditions at 22°C are spheroidal and aggregated. | |

|  |  |  |
| --- | --- | --- |
| Incubator conditions | Transport conditions | Day of imaging |
|  |  | 1 |
|  |  | 2 |
|  |  | 5 |
|  |  | 9 |
|  |  | 13 |
|  |  | 15 |
| Figure 8: Confocal microscopy images taken 1-15 days after fibrin clots kept in incubator conditions (37°C) or ambient temperatures (~22°C) for 48 hours were placed onto de-epidermised dermis (DED). Images were taken at 1, 2, 5, 9, 13 and 15 days post movement to dermis.  Fibrin clots contained 1x106 ADSCs.  In this experimental set up, images were taken of ADSCs labelled with RFP-lentivirals.  The images above are representative of samples when scanning through the xyz axis. In each image there are a greater number of visibly stained cells when fibrin clots had prior culture at 22°C compared to prior culture at 37°C. This was the case for all time points for these particular cells.  Images of cells kept at 37°C:  Cell morphology for cells in the incubated conditions appear to be mostly spherical, this is particularly of note for time points 1, 2, 9, and 13, however by day 5 some cells appear to be slightly more elongated. At day 15 elongated cells were visible.  Images of cells kept at 22°C:  A much greater number of cells are visible, most of which appear to be elongated and spindle-like. Between days 1-5, cells appear to have no particular order or orientation, however from day 9 and onwards ADSCs have a clear orientation. | | |

|  |  |  |
| --- | --- | --- |
| Incubator conditions | Transport conditions | Day of imaging |
|  |  | 1 |
|  |  | 2 |
|  |  | 5 |
|  |  | 9 |
|  |  | 13 |
|  |  | 15 |
| Figure 9: Confocal microscopy images taken 1-15 days after fibrin clots kept at incubator conditions (37°C) or ambient temperatures (~22°C) for 48 hours were placed onto de-epidermised dermis (DED). Images were taken at 1, 2, 5, 9, 13 and 15 days post movement to dermis.  Fibrin clots contained 1x106 ADSCs.  In this experimental set up, images were taken of ADSCs labelled with RFP-lentivirals.  Contrary to figure 8, a similar number of cells are visible from DED and fibrin gel constructs when fibrin gels were initially kept at 37°C than from clots kept at 22°C. This is particularly the case from days 5 onwards where cell confluency in the images appears similar, however at days 1 and 2, the number of visible cells appears slightly greater when fibrin clots were previously cultured at 22°C compared to 37°C.  Images of cells kept at 37°C:  Mostly spherical cells are noted at days 1 and 2, however at day 5 some cells appear to be slightly more elongated and spindly. Additionally, from day 5-15 the number of cells appears to markedly increase and the images taken show almost 90% confluency, although these cells are not orientated along a particular axis.  Images of cells kept at 22°C:  Similar to cells kept at 37°C, cells become less spherical over time, becoming clearly more dendritic with subsequent time points. At day 9, some orientation can be seen, this is markedly clear at day 13 and 15. | | |

## Discussion:

In this chapter, the effect of encapsulating primary cell cultures of ADSCs in fibrin gels at a concentration of 1x106 per gel was studied. These were further investigated compared to cultures seeded on tissue culture plastic when in transport conditions at ambient temperatures of ~22°C supplemented with 5% CO2 or 37°C in an incubator.

ADSCs from 2 separate donors were assessed for viability in fibrin when kept at over a period of 24, 48 and 72 hours using an MTT assay when kept at 22°C or 37°C with 5% CO2. Each donors cells retained cell viabilities over these time points and cell viability increased when cultured at 37°C with 5% CO2. ADSCs cultured at ambient temperatures (~22°C) supplemented with 5% CO2 decreased in cell viability over time, with a final cell viability between 65.5-108.7% difference in cell viability, favouring culture conditions at 37°C. To p<0.05 these variances were significantly different, showing that in this instance cell viability was not maintained at 22°C. This is potentially due to use of freshly isolated cells since these had not previously been maintained in culture, and may have been more sensitive to fluctuations in culture conditions. A higher passage of cells may have selected for cells which were more robust and could survive variations in culture conditions, however this experiment aimed to assess freshly isolated ADSCs as these had not been assessed for viability in fibrin or at 22°C transport conditions.

FACS

Flow cytometry was performed and ADSCs sorted by fluorescent labels. Cells from 3 separate donors were investigated under four different conditions; cells cultured with or without fibrin at 22°C or 37°C with 5% CO2. The results from this (figure 6) strongly indicated that these culture conditions did not impact on cell phenotypes and cells retained their ‘stemness’ after culturing in fibrin or at non-physiological temperatures in transport conditions.

Differentiation:

The effect of fibrin on cell differentiation of ADSCs was assessed by their differentiation into osteoblasts or adipose cells. Cells were readily able to differentiate into both of these lineages, however due to low numbers of recovered cells it was not possible to examine the effect of transportation conditions at 22°C in this examination.

The results for ADSCs cultured at 37°C however could be assessed with or without encapsulation in fibrin. The results, whilst n=1, strongly indicate that ADSCs are inhibited from differentiation down the osteogenic lineage after fibrin encapsulation compared to controls on tissue culture plastic, as noted by the lack of relevant gene expression, especiallyDLX5 and OSX (figure 3). Whilst fibrin encapsulation of ADSCs prevents osteogenic differentiation, any differentiation down this lineage would not be desirable for the treatment of skin wounds.

When assessed for their potential for differentiation down adipocyte lineages, ADSCs were able to do so after fibrin encapsulation, with strong gene expression for LPL, although gene expression for PParγ, CEPBα, Leptin, AP2 and AdipoQ were found to be less than controls on tissue culture plastic (figure 4,5). This investigation indicated that ADSCs retain some capacity for differentiation down the adiopogenic lineage, but not osteogenic lineages after fibrin encapsulation.

Initial experimental plans were to assess these differentiation potentials after 22°C when supplemented with 5% CO2 in addition to controls at 37°C, however insufficient cells were recovered after culturing for 48 hours in these conditions. This was due to the decreased cell viability after culturing at 22°C.

Migration of ADSCs from fibrin to de-epidermised dermis:

48 hours after encapsulation in fibrin, ADSCs kept at 22°C or 37°C with 5% CO2 supplementation were placed onto de-epidermised dermis and these skin composites were placed back in the incubator. Constructs were taken to the confocal suite and imaged over 15 days post fibrin cell delivery, imaged on days 1,2,5,9,13 and 15 (with help of Mélanie Gadelorge and Corinne Bareau, StromaLab, Inserm, Toulouse, France).

The images in figures 8 and 9 appear to show that fibrin gels initially cultured at 22°C for 48 hours deliver a greater number of ADSCs to dermis compared to fibrin gels cultured at 37°C. Over the subsequent imaging time points, cell confluency for both conditions appear to increase although the earlier time points of fibrin gels stored at 22°C showed a much greater initial cell density.

Cells encapsulated in fibrin gels at 37°C appear to show a much more spherical cell morphology than those kept at 22°C, although both conditions led to the appearance of dendritic cells by day 9. Additionally, cells initially stored at 22°C clearly showed orientation along an axis, implying cellular organisation, whereas cells from fibrin clots initially at 37°C do not appear to acheive any particular orientation

Unfortunately it was found during the confocal imaging that it was not possible to visualise the dermis in conjunction with the labelled cells due to lack of appropriate confocal laser. The RFP used during the lentiviral labelling of cells had an excitation wavelength of 488 and emission wavelength of 588, collagen has an autofluorescence when excited at 270-370nm, detectable at 305-450nm. The intention was to use the autofluorescence of collagen to visualise the dermis in conjunction with the RFP labelled ADSCs to determine whether the cells had migrated to the dermis from the fibrin clots, however the confocal microscope had been set up to focus on a narrow range of specific set of fluorophores and due to time constraints it was not possible to reconfigure the set up to test for collagen autofluorescence. Therefore it is not possible to determine the exact location of the imaged cells and conclude whether images were taken at some location within the fibrin clot or on the dermal sample. Morphological characteristics of ADSCs appear to be similar between the 2 sets of donor cells between the assessed time points, which infers that cells in the fibrin clots are initially spheroidal but as cells migrate from the gels, they are become dendritic and fibroblastic-like healthy cells.

## Conclusion

ADSCs can be placed within a fibrin clot at 1 million cells per ml, which are mechanically stable enough to be moved with forceps.

These fibrin clots were kept at 37°C in an incubator or in transport conditions at ambient temperatures ~22°C supplemented with 5% CO2. These conditions maintained cell viability for 72 hours, as assessed using an MTT assay.

Cells were incubated with red fluorescent protein and again encapsulated into fibrin gels. After 48 hours these gels were moved to a skin model and imaged using confocal microscopy. Unfortunately it was not possible to image the dermis on which the gels had been placed, therefore the exact location of cells in the dermis is not known.

Cell surface markers of ADSCs when encapsulated in fibrin or on tissue culture plastic, at 37°C in an incubator or at transport conditions at ambient temperatures ~22°C supplemented with 5% CO2 were assessed using fluorescence-assisted cell sorting. ADSCs in these four conditions retained their stem cell markers regardless of the culture conditions.

A differentiation study was performed on ADSCs when cultured at 37°C in fibrin gels, unfortunately due to a lack of recovered cells it was not possible to assess ADSCs when cultured at 22°C supplemented with 5% CO2 in fibrin gels or tissue culture plastic. ADSCs retained their ability to differentiate into adipocytes, however osteoblastic differentiation was inhibited when cells had previously been encapsulated in fibrin gels.

# Chapter 6:

# Summary

With an ageing population and the prevalence of diabetes, the rate of chronic wounds is increasing at a vast rate. These sores are not only painful, embarrassing and life threatening to the patient, these wounds are also a time consuming and a financial burden to the healthcare system, with an approximate cost between £9533-15246 for management of each ulcer over 2 years [47].

The current gold standard for treating these wounds is for painful debridement sessions coupled with bandaging and gauze, however patients often present with a reduced capacity for wound healing and repair and these treatments are ineffective.

An improved method to heal chronic wounds would decrease the burden for healthcare systems; free up nursing time and improve the quality of life for affected patients. Several studies report stem cells to aid chronic wound healing and stem cells can be sourced from a variety of autologous or allogeneic sources.

A source of debate is how to deliver these cells to the patient whilst minimising the need for a skilled technician at a healthcare clinic – as this would otherwise be utilising valuable resources. This study demonstrates a method to encapsulate adipose derived stem cells within fibrin, and the effect of this on their cell viabilities.

Several studies describe methods of retaining cell viability whilst in transport conditions out of the incubator at ambient temperatures [154][156][157]. In an incubator, media is prevented from becoming alkaline due to addition of carbon dioxide to the air preventing carbonate ions in the cell media being converted to carbon dioxide as this reaction is in equilibrium. These studies focussed on preventing loss of carbon dioxide to the flask air by filling cell media to the brim of the container, inhibiting carbon dioxide formation. Whilst maintaining their cells of choice for up 2 to 6 days, these methods are unsuitable for ‘off the shelf’ therapeutic cell products for a number of reasons; eg the need to remove materials which are not approved by regulatory bodies (matrigel), cells adhered to culture flasks need to be detached before therapeutic use, vast cell loses due to cell sheet shear, and unsuitable initial processing temperatures. Eves et al [158] proposed a technique to instead supplement the container air with 5% CO2, preventing carbon dioxide loss to the air (which would cause alkalinity) and retaining cell viability. Eves et al investigated keratinocytes and melanocytes seeded onto a non-biodegradable scaffold. The work in this document builds on their protocol by investigating multiple cell types (keratinocytes, fibroblasts, adipose derived stem cells and bone marrow derived stem cells) and evaluating the effect on cell viability when seeded or without scaffold (PLGA or fibrin). This protocol also differs from their protocol by omitting the use of agar gel as a means of providing nutrients and for simplicity cell media was used instead. Cells are readily maintained and viabilities retained at ambient temperatures for 72 hours using this method, which is enough time for delivering cells internationally.

Stem cells are readily acknowledged for their beneficial wound healing properties[71], inflammation suppression [72][73][74][75][77] and promotion of angiogenesis [79]. This work demonstrates both adipose and bone marrow derived stem cells can be maintained out of the incubator in transport conditions, however work focussed on adipose derived stem cells due to their ready availability and simple method of extraction.

This body of work shows maintaining of media pH is more important than temperature conditions of the cells. Although effects of supplementing containers with CO2 on media pH is inferred by visually assessing the colour change of phenol red DMEM, further investigation should be performed into actual change in pH over time using a digital meter to fully evaluate the detrimental effects of pH on cell viability.

This study uses the widely used surgical glue, fibrin, and the synthetic polymer PLGA, which are both approved by regulatory authorities and used worldwide in the clinic. These were chosen for these reasons and cell viability was assessed when in transport conditions without the need for complex material processing before transport or at point of use.

PLGA was electrospun to form a fibrous mat within which sterilisable and biodegradable fibres replicate natural extracellular matrix. Synthetic materials avoid the biological variability and potential disease risk which can occur in natural products.

Fibrin gels were investigated since they are used on a day-to-day basis as surgical glue. Whilst some natural materials may confer a disease or immune response or vary in consistency, the commonplace use of fibrin and the familiarity of healthcare staff with its use (along with widespread regulatory approval) made it particularly attractive for clinical adoption.

Results show the ease of handling both of these scaffolds, although PLGA scaffolds were found to contract and negatively impact cell viability after culture for 72 hours. Therefore fibrin encapsulation was further investigated and effects of maintaining cells at ambient temperatures with supplementation with 5% CO2.

After ADSCs were encapsulated in fibrin, surface antigen expression was assessed using fluorescence assisted cell sorting, with cell markers remaining similar to controls cultured on tissue culture plastic and comparing transport conditions at 22°C or incubated at 37°C. These results inferred that these different culture conditions did not cause cell differentiation (since these cell markers did not vary) and cells retained their stemness. However, when comparing differentiation the potential of ADSCs to osteoblastic or adipocyte lineages after fibrin encapsulation, osteoblastic differentiation was inhibited, as inferred by gene expression, whereas adipocyte differentiation was less affected.

Further research should be performed into the differentiation potential of ADSCs after culture in fibrin, since there appears to be an decreased effect of osteoblastic differentiation potential. It may be of clinical interest to determine whether other lineages are affected, for instance chrondrocyte differentiation lineages, where ADSCs are being investigated as a treatment for articular cartilage damage when delivered using fibrin clots.

ADSCs were moved to 3D *in vitro* skin wounds after encapsulation in fibrin gels and storage in transport conditions or an incubator to investigate the migration potential of cells. Unfortunately the experiment failed to discover the exact location of ADSCs within the dermis, and therefore the depth of penetration of cells, if at all. Whilst we have previously shown (Walker et al) that ADSCs promote dermal wound healing, further research should be performed in order to investigate the wound healing potential of ADSCs post encapsulation in fibrin and effect of transport conditions on rate of wound closure.

Transport conditions in this body of work were simulated by keeping cells on the laboratory bench in a polystyrene box at ambient temperatures to minimise temperature fluctuations, however in reality temperatures will fluctuate and vibrations occur from movement which was not evaluated. It would be of interest to further examine conditions when cells are transported from lab to lab and ensure cell viabilities can be maintained during real life transport conditions.

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# Appendix:

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  |  | **Donor 1** | **Donor 2** | **Donor 3** | **Mean** | **Expected specifications** |
|  |  | **% positive cells** | **% positive cells** | **% positive cells** | **% positive cells** | **% positive cells** |
| **Incubator without fibrin** | **CD90** | 99.18 | 99.68 | 99.99 | 99.62 | ≥ 90% |
| **CD73** | 99.05 | 99.76 | 99.92 | 99.58 | ≥ 90% |
| **CD14** | 0.00 | 0.00 | 0.19 | 0.06 | < 5% |
| **CD105** | 85.74 | 99.12 | 99.51 | 94.79 | ≥ 90% |
| **CD31** | 0.00 | 0.00 | 0.00 | 0.00 | No specification |
| **CD34** | 0.00 | 0.5 | 0.03 | 0.18 | ≤ 10% |
| **CD45** | 0.00 | 0.00 | 0.00 | 0.00 | < 5% |
| **CD29** | 99.03 | 99.93 | 99.94 | 99.63 | No specification |
| **HLADR** | 0.00 | 0.00 | 0.00 | 0.00 | No specification |
| **CD13** | 98.95 | 99.77 | 99.93 | 99.55 | No specification |
| **CD36** | 10.55 | 13.29 | 5.37 | 9.74 | No specification |

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  |  | **Donor 1** | **Donor 2** | **Donor 3** | **Mean** | **Expected specifications** |
|  |  | **% positive cells** | **% positive cells** | **% positive cells** | **% positive cells** | **% positive cells** |
| **Incubator with fibrin** | **CD90** | 99.15 | 99.81 | 99.61 | 99.52 | ≥ 90% |
| **CD73** | 98.90 | 99.62 | 99.23 | 99.25 | ≥ 90% |
| **CD14** | 2.82 | 0.36 | 0.51 | 1.23 | < 5% |
| **CD105** | 98.41 | 95.72 | 91.19 | 95.11 | ≥ 90% |
| **CD31** | 0.00 | 0.56 | 0.27 | 0.28 | No specification |
| **CD34** | 0.00 | 1.64 | 0.61 | 0.75 | ≤ 10% |
| **CD45** | 0.00 | 0.00 | 0.00 | 0.00 | < 5% |
| **CD29** | 98.87 | 99.54 | 99.06 | 99.16 | No specification |
| **HLADR** | 0.00 | 0.00 | 0.00 | 0.00 | No specification |
| **CD13** | 99.04 | 99.54 | 99.08 | 99.22 | No specification |
| **CD36** | 8.85 | 32.39 | 17.86 | 19.70 | No specification |

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  |  | **Donor 1** | **Donor 2** | **Donor 3** | **Mean** | **Expected specifications** |
|  |  | **% positive cells** | **% positive cells** | **% positive cells** | **% positive cells** | **% positive cells** |
| **Transport box without fibrin** | **CD90** | 99.81 | 99.92 | 99.92 | 99.88 | ≥ 90% |
| **CD73** | 99.85 | 99.63 | 99.57 | 99.68 | ≥ 90% |
| **CD14** | 0.31 | 0.23 | 0.85 | 0.46 | < 5% |
| **CD105** | 98.75 | 98.45 | 96.55 | 97.92 | ≥ 90% |
| **CD31** | 0.19 | 0.03 | 0 | 0.07 | No specification |
| **CD34** | 0.00 | 1.32 | 0.00 | 0.44 | ≤ 10% |
| **CD45** | 0.00 | 0.00 | 0.00 | 0.00 | < 5% |
| **CD29** | 99.79 | 99.71 | 99.57 | 99.69 | No specification |
| **HLADR** | 0.00 | 0.00 | 0.00 | 0.00 | No specification |
| **CD13** | 99.70 | 99.89 | 99.7 | 99.76 | No specification |
| **CD36** | 5.87 | 17.07 | 10 | 10.98 | No specification |

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  |  | **Donor 1** | **Donor 2** | **Donor 3** | **Mean** | **Expected specifications** |
|  |  | **% positive cells** | **% positive cells** | **% positive cells** | **% positive cells** | **% positive cells** |
| **Transport box with fibrin** | **CD90** | 99.54 | 99.87 | 99.81 | 99.74 | ≥ 90% |
| **CD73** | 99.02 | 99.25 | 99.29 | 99.19 | ≥ 90% |
| **CD14** | 1.10 | 0.22 | 0.26 | 0.53 | < 5% |
| **CD105** | 96.49 | 96.91 | 91.09 | 94.83 | ≥ 90% |
| **CD31** | 0.08 | 0.06 | 0.00 | 0.05 | No specification |
| **CD34** | 0.00 | 0.43 | 0.00 | 0.14 | ≤ 10% |
| **CD45** | 0.00 | 0.00 | 0.00 | 0.00 | < 5% |
| **CD29** | 98.93 | 99.22 |  | 99.08 | No specification |
| **HLADR** | 0.00 | 0.00 | 0.00 | 0.00 | No specification |
| **CD13** | 98.84 | 99.71 | 99.55 | 99.37 | No specification |
| **CD36** | 6.21 | 17.68 | 12.57 | 12.15 | No specification |

1. Chaka Kahn, ‘Ain’t Nobody’, 1984, *Stompin’ at the Savoy* [↑](#footnote-ref-1)