Tissue Engineering Group
School of Pharmacy, University of Nottingham

The analysis of cell fate post-ejection through parenteral devices, and, The development of systems that aid the transportation of cell therapy products

Kapil Agashi MRPharmS

Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy, September 2009

The ship wherein Theseus and the youth of Athens returned [from Crete] had thirty oars, and was preserved by the Athenians down even to the time of Demetrius Phalereus, for they took away the old planks as they decayed, putting in new and stronger timber in their place, insomuch that this ship became a standing example among the philosophers, for the logical question of things that grow; one side holding that the ship remained the same, and the other contending that it was not the same.

- Plutarch, Theseus

Abstract

Herein, two translational issues related to the development of cell therapy discoveries into therapeutic products, are addressed. Firstly, analysis of whether the manipulations required to transfer cells from cell culture conditions to a target tissue affect cellular characteristics was performed. It was shown that processing primary murine mesenchymal stem cells (MSCs) into a concentrated cell suspension, drawing them up into a syringe and immediately ejecting them, caused a significant viability decrease. Leaving the cells within the syringe chamber at room temperature for prolonged time periods caused to a further decrease in viability. However, cells that were viable post-ejection were found to be functional with regard to their ability to attach and proliferate. Reducing the ejection rate or using the antioxidant *n*-acetyl cysteine did not significantly improve viability, although using a wider bore 22g needle did improve viability.

Secondly, the feasibility of a cell transportation device that could store viable cells under room conditions was assessed. The development of such a transportation device would remove the need to cyropreserve cells during transit, which has many flaws. Human MSCs were found to enter a reversible proliferative arrest phase whilst under room conditions, allowing them to be stored for up to 11 days, before rapidly decreasing in viability. The accumulation of ammonia was identified within the cultures, and the introduction of a zeolite material was found to partially remove this ammonia and improve viability over 7 days. A means of developing a concentrated cell culture media, using a freeze-drying technique, was found not to comprise cell viability, whilst allowing the nutrient volume to be reduced, thus potentially making the transportation device more compact.

However, the introduction of a modified release nutrient gel, consisting of alginate, did not provide any significant effect on viability.

Publications, presentations and patents

The work presented in this thesis has given rise to the following publications, presentations and patents.

Publications in peer-reviewed journals

AGASHI K., CHAU D.Y.S. & SHAKESHEFF K.M. (2009) The effect of delivery via narrow-bore needles on mesenchymal cell viability, apoptosis and morphology. *Regen. Med.*, **4**, pp 49-64

CHAU D.Y.S., **AGASHI K.** & SHAKESHEFF K.M. (2008) Microparticles as tissue engineering scaffolds: manufacture, modification and manipulation. *Mater. Sci. Tech.*, **24**, pp 1031-1044

SOKOLSKY-PAPKOV M., **AGASHI K.,** OLAYE A., SHAKESHEFF K.M. & DOMB A.J. (2007) Polymer carriers for drug delivery in tissue engineering. *Adv. Drug Del. Rev.*, **59**, pp 187 -206

Oral Presentations

AGASHI K., CHAU D.Y.S. & SHAKESHEFF K. Life support systems for cell therapies. Presented at the British Pharmaceutical Conference, Manchester (UK), September, 2009

AGASHI K., CHAU D.Y.S. & SHAKESHEFF K. The influence of delivery via narrow-bore needles on mesenchymal stem cell viability, apoptosis and morphology. Presented at the UK-Taiwan International Networking for Young Scientists: Therapeutic and Research Applications of Human Stem Cells Meeting, Taipei (Taiwan), May, 2009

AGASHI K., CHAU D.Y.S. & SHAKESHEFF K. The influence of delivery via narrow-bore needles on mesenchymal stem cell viability, apoptosis and morphology. Presented at the Tissue & Cell Engineering Society Conference, Nottingham (UK), July, 2008

Poster Presentations

AGASHI K., CHAU D.Y.S. & SHAKESHEFF K. Delivery of MSCs Through Narrow-Bore Needles and its Effect on Cellular Characteristics. Presented at the 3rd UK Mesenchymal Stem Cell Meeting, January, 2009

AGASHI K., CHAU D.Y.S., BIBLE E., MODO M., PRICE J. & SHAKESHEFF K.M. The *In Vitro* Development of a Novel Microparticle-based Scaffold for Regenerative Medicine. Presented at the Tissue Engineering & Regenerative Medicine International Society North American Chapter, San Diego (USA), December, 2008

CHAU D.Y.S., BIBLE E., **AGASHI K.**, MODO M., PRICE J. & SHAKESHEFF K.M. Improving brain repair with neural stem cells and engineered scaffolds. Presented at the Tissue & Cell Engineering Society Conference, Nottingham (UK), July, 2008

LEE S.Y., **AGASHI K.**, CHAU D.Y.S. & SHAKESHEFF K.M. A novel self-sintering microparticle-based system for regenerative medicine. Presented at the Tissue & Cell Engineering Society Conference, Nottingham (UK), July, 2008

Patent

AGASHI K., SHAKESHEFF K.M. & QUIRCK R. Cell Storage System. Patent Number: 0912413.2, Filed 17/07/2009

Acknowledgements

There are many to whom I owe my gratitude, for, without their support, this degree would not have progressed as smoothly as it did, or may not even have been completed.

I would like to take this opportunity to thank my supervisors Dr. Richard France (during my first year) and Professor Kevin Shakesheff for their support throughout the 3 years. Many a time the focus of my research lost track, and they were able to bring my attention back to my main aims. Professor Shakesheff also provided me with many amazing opportunities, which I am also grateful for.

My thanks also goes to everyone within the Tissue Engineering group for their support and for making the working atmosphere relaxed and enjoyable. Specifically, I would like to thank Dr. David Chau for all his guidance with regard to article-writing and general laboratory skills, Lloyd Hamilton for my initial cell culture training, and Dr. Andrew Olaye for my training in microparticle fabrication. I would also like to acknowledge Dr. Virginie Sottile for her help in isolating primary murine mesenchymal stem cells, and Dr. Adrian Robins for his guidance with regards to obtaining and analysing the flow cytometry data.

I would like to thank the Biotechnology and Biological Sciences Research Council and Regentec® for funding. I would also like to thank Regentec® for the provision of the primary human mesenchymal stem cell line.

I would also like to thank all the friends I have made during my PhD for making working at Nottingham a joy, especially Dave, Hywel and Catherine.

Finally, a special thanks goes to my family for their support. And, last but not least, I would like to thank Kathy for all her enthusiastic encouragement and for always being there for me.

Abbreviations

AB	Alamar Blue
ACI/ACT	Autologous Chondrocyte Implantation or Transplantation
AD	Alzheimers Disease
ACGM	Advisory Committee on Genetic Modification
ATCC	American Type Culture Collection
BOOST	Bone marrow to enhance ST-elevation infarct regeneration
CBER	Center for Biologics Evaluation & Research
CHF	Congestive Heart Failure
CVA	Cerebrovascular Accident
DCM	Dichloromethane
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulphoxide
DTT	Dithiothreitol
ECM	Extracellular Matrix
EDTA	Ethylene diamine tetraacetic acid
emm	Emission
ex	Excitation
ESC(s)	Embryonic Stem Cell(s)
FCS	Foetal Calf Serum
FDA	Food and Drug Administration
g	Centrifugal G Force (Tilting Strain)
GO	Glucose Oxidase
GMP	Good Manufacturing Practice
GTP	Good Tissue Practice

GVHD	Graft Versus Host Disease
HBSS	Hanks Balanced Salt Solution
HDF	Human Dermal Fibroblast
HEPA	High Efficiency Particulate Air
hMSC(s)	Human Mesenchymal Stem Cell(s)
HPLC	High Performance Liquid Cromotrophy
IDDM	Insulin-Dependent Diabetes Mellitus
IND	Investigational New Drug
kÅ	Kilo Ångström
kDa	Kilo Dalton
LVEF	Left Ventricular Ejection Fraction
MHC	Major Histocompatibility Complex
MHz	Mega hertz
MI	Myocardial Infarction
mMSC(s)	Murine Mesenchymal Stem Cell(s)
MP	Microparticle
MSCM	Mesenchymal Stem Cell Media
MSC(s)	Mesenchymal Stem Cell(s)
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2H-tetrazolium
π	Pi
Pa	Pascal
PBS	Phosphate-buffered saline
PDMS	Polydimethylsiloxane
pH	$\log(^{1}/_{[H^{+}]})$
PLGA	Poly(lactic-*co*-glycolic acid)
ppAAm	Plasma polymerised allylamine
PVA	Poly Vinyl Acetate
RCT	Randomised Control Trial
REPAIR-AMI	Reinfusion of Enriched Progenitor Cells And Infarct Remodelling in Acute Myocardial Infarction

RNA	Ribonucleic Acid
rpm	Revolutions Per Minute
τ	Tau (Shear Stress
TB	Trypan Blue
TNFα	Tumour Necrosis Factor α
VEGF	Vascular Endothelial Growth Factor
$^{v}/_{v}$	Volume per Volume
$^{w}/_{v}$	Weight per Volume

Table of contents

List of figures

Chapter 4: Flow cytometric analysis of mesenchymal stem cell viability post-ejection

Chapter 5: The identification of a toxin adsorbent that could be utilised to transport cell therapy products

List of tables

1

General introduction

Here, the potential of regenerative medicine, and, more specifically cell therapy, as therapeutic applications, will be reviewed. Mesenchymal stem cells (MSCs) will be discussed as a cell source, as well as the clinical areas where MSC cell therapy techniques may be beneficial. The translational obstacles related to moving cell therapy from the laboratory to the bedside will be summarised, finishing with the aims of this thesis.

1.1 – The development of regenerative medicine

Regenerative medicine is an overarching term referring to the development of technologies that can restore the function of diseased or aged organs. The research strategies working towards this regenerative aim include tissue engineering (TR), cell therapy, organ and tissue transplantation, as well as therapies that induce endogenous regeneration. Currently, regenerative medicine is a research topic of great importance, mainly because of the potential that this form of therapy could provide in the clinic and because of the significant drawbacks of current tissue-restoration techniques.

There is no question that organ or tissue loss has a massive impact on healthcare, both on a global and an individual level; by 2010, the worldwide market for regenerative medicine has been estimated to be as high as $ 500 billion[1].

1.2 – Cell therapy

Cell therapy can be considered as the delivery of an active cell suspension without an extracellular matrix (ECM) component. Cell therapy is a technique applied to disease states where the cells require little structural or biochemical support, and

has the obvious advantage in that the systems tend to be less complicated than scaffold-based TE systems.

The earliest successful example of cell therapy was the blood transfusion, which is still used in modern medicine to replenish lost blood from trauma or surgery, or to treat severe anaemia and thrombocytopenia. Developments within this field aided our understanding of compatibility and sterility[2], which are useful in the development of current allogeneic cell therapy developments. Cell therapy methods are also applied in the form of allogeneic haemopoetic stem cell transplantation, performed to replenish the number functioning lymphocytes in patients with severe multiple myeloma or leukemia. If the patient's immune system can tolerate the transplant, this form of therapy can be effective.

Another disease state that has been shown to have benefited from cell therapy developments is insulin-dependent diabetes mellitus (IDDM). In IDDM, a specific discrete pancreatic β cell population is rejected by the immune system, which not only releases insulin, but, more importantly, constantly monitors blood glucose levels and releases the correct amount of insulin accordingly, providing a level of glucose control that no current technology can. The fact that the disease is solely caused by the lack of a certain cell type makes it ideal for cell therapy[3]. The transplantation of these cells using a well-established Edmonton Protocol can provide insulin independence in IDDM patient for up to two years[4]. Unfortunately, akin to kidney transplantation, there are a severe lack of donors, and this is made worse by the loss of functional cells during the operation, thus the operation requiring up to 3 donors per recipient. However strategies are being developed to provide a limitless supply of insulin releasing cells[3,5].

There are two main forms of cell therapy, depending on whether the cells are sourced from the patient intended to be treated (autologous) or elsewhere (allogeneic).

1.2.1 – Autologous cell therapy

Patients of all ages carry adult stem cells that are able to, to a certain extent, replicate and replace cells that become damaged or age (although the number of stem cells does decrease as a human ages)[6]. The aim of autologous cell therapy is to utilise these adult stem cells by extracting them from the body with minimal inconvenience to the patient, expanding them *in vitro* if necessary, and then delivering them to the area of required regeneration, where the cells hopefully engraft, differentiate into functional tissue, and ultimately treat the disease.

The main advantage of autologous cell therapy is that, because the cells delivered are the patient's own, there is no risk of immune rejection. However, autologous cell therapy would be complicated to perform in a hospital setting, as essentially two operations would be required (one to remove the stem cells, and one to deliver the cells), with a time period of up to a month between operations to allow the cells to replicate to an adequate population for delivery.

Another issue with autologous therapy is the high potential variability in effectiveness and adverse effects this treatment may have. No one procedure used to obtain stem cells from a patient could yield a pure stem cell population, but instead a mix of numerous cell types, so, even after isolating and expanding, it is unlikely that the delivered suspension will not have a variable number of other cells. And, even if the delivered cell suspension could be processed to be pure and of the same cell number for every patient, the level of bioactive molecules that the cells would release would be highly variable and ultimately affect how effective the treatment is from patient to patient[7].

Finally, in order for this form of therapy to be available in the future, cell culture facilities would be required either in or near to the clinic where the procedure would take place. Because cell therapy products cannot be sterilised after processing, like some medical devices, every facility would require stringent good manufacturing practice (GMP) guidelines[7,8] to ensure sterility is maintained

throughout the processing. This would ultimately be very costly to operate. This would mean that, if these therapies were found to be effective in the future, only the wealthy countries would have access to them.

Research has been carried out to assess whether autologous cell therapy is still effective if the cell population is not expanded, but delivered in the same operation as when they are removed. If this were possible, this would lead to a reduction in hospital time per patient and remove the need for any cell culture facilities. One report suggests that expanding human bone marrow stromal cells reduces their differentiation potential, and that using a non-expanded cell population is more effective in diseases involving osteogenic, chondrogenic or adipogenic differentiation[9]. Early cell therapy trials, focusing on improving cardiac tissue post myocardial infarction, delivered unselected bone marrow aspirate to the heart but showed little benefit, supposedly because bone aspirate has a very low percentage of functional progenitor cells (< 0.1 %)[10]. Hernigou and co-workers showed that concentrating the bone marrow aspirate by centrifugation led to an improvement in bone engraftment when using the marrow to treat tibial nonunion[11].

An exciting avenue of cell therapy may develop from recent insights into induced pluripotency. In 2006, it was shown that terminally differentiated fibroblasts could be reprogrammed into a stem cell-like state by introducing four transcription factors. These cells had many of attributes of ESCs; they were able to proliferate, were able to differentiate into all 3 germ layers and were able to form teratomas in immunodeficient mice[12]. The multiple viral vectors could, however, potentially cause genetic alterations and therefore could not be delivered to a patient. However, Kaji *et al* recently showed that it is possible to induce stemness in human skin cells using a transgene that could be removed once reprogramming had occurred[13]. This technology could potentially simplify how cells are harvested from the patient, but the limitations with directing specific cell differentiation could lead to the development of teratomas in patients treated with induced pluripotent stem cells; studies have suggested that the tumorigenic potential of

these cells is at least equal to hESCs, if not or more[14]. Moreover, the issues in terms of cell expansion facilities and patient-to-patient variability mentioned previously would still be present.

1.2.2 – Allogeneic cell therapy

All the flaws of autologous cell therapy previously mentioned could be at least partially rectified by using cells from allogeneic sources; because the cells would not need to be the patient's own, the cells could be processed at a specialised facility, and shipped to the clinic. Processing techniques could be optimized to ensure that batch-to-batch variability is kept to a minimum, and essentially only one procedure would be required in order to provide the treatment, thus reducing hospital costs and inconvenience to the patient.

Allogeneic cell therapy also provides the option of using embryonic stem cells (ESCs) to treat disease; these are, in theory, a more effective cell source than adult stem cells because their pluripotency allow them to potentially develop into all three germ layers, providing any cell required, provided that the correct biological cues are used. However, the ethical considerations involved with sourcing ESCs means that their availability is scarce, and this in turn encourages the research in induced pluripotency mentioned previously[13].

1.2.2.1 - Immune rejection

The main problem of allogeneic cell therapy is immune rejection; if the cells are not the patient's own, there is always a chance that the patient's immune system will recognize it as non-self and clear them. How significant this problem is has been under much debate; some studies have shown little or no immune response when allogeneic cells have been delivered *in vivo*, whilst some have shown a clear activation.

ESCs are thought to be "immune privileged", mainly because, during healthy pregnancy, the maternal immune system is not seen to reject the embryonic tissue

containing nonmaternal antigens inherited paternally[15]. However, even in their undifferentiated state, ESCs express low levels of major histocompatibility complex (MHC) class I molecules, and, as these cells differentiate, these levels are seen to increase, accelerating the immune response[16].

Allogeneic studies using mesenchymal stem cells (MSCs) have been shown to produce little immune response; Arinzeh *et al* showed that allogeneic MSCs were able to aid the repair of a critical size bone defect with no adverse immune response[17]. This is thought to be due to MSCs lacking MHC class II molecules on their surface[18], providing immunoprotection that CD28-mediated co-stimulation nor *y*-interferon pretreatment could activate[19].

However, in separate reports, MSCs have been less successful; De Bari and co-workers found that MSCs used for osteogenic engraftment were only effective in immunodeficient or immunosuppressed mice[20], and similar findings have been reported when MSCs were used for myogenic regeneration[21]. This contrast in results may be due to level of miss-match between donor and recipient[7], or may be due to an increased immune response as the MSCs differentiate, as with ESCs[16].

Methods are currently being developed to prevent the immune rejection of stem cells. One such method involves microencapsulating the stem cells in order to hide their immunogenic surface proteins. This method was seen to be effective in preventing rejection during xenograft murine ilset transplantation, though when scaled up to dog, primate or human models, no protection was seen[22].

1.3 – Mesenchymal stem cells

Mesenchymal stem cells (MSCs) have become a popular cell source for cell therapy research because they are easily expandable *in vitro*, and may have the ability to differentiate into useful cell lineages *in vivo*, such as bone, cartilage, fat, cardiac muscle and potentially neurons. They are also straightforward to isolate from patients through bone marrow aspiration or adipose tissue biopsy[6], though

the former can be a painful procedure for the patient. Because of their popularity within cell therapy research, all studies detailed in this thesis were carried out using primary MSCs.

Mesenchymal stem cells are broadly classed as the non-haemapoetic stem cells present the bone marrow, and are called mesenchymal because they can differentiate into multiple mesodermal cell lineages. MSCs represent a very small fraction of the bone marrow, but can proliferate to a graftable number within a month *in vitro*.

One key issue with MSCs is their broad characterization. The surface markers used by different groups to extract MSCs from bone marrow include CD13, CD29, CD31, CD44, CD54, CD63, CD73, CD105, CD106, CD140b, CD166 and Strol, among others[6], but no robust marker definition has been deduced for MSCs. What makes this definition more complicated is that culture expanded MSCs may lose some markers, but still remain active as a stem cell[23]. Until a definition of an MSC is determined, MSC-based therapeutics are likely to be highly variable from batch to batch.

The differentiating potential of MSCs into certain cell types has been heavily scrutinised. It is established that MSCs have the ability to form osteocytes, chondrocytes, adipocytes and myocytes. However reports on the neural and hepatic differentiation are mixed, as reports stating these forms of differentiation have been questioned on the grounds of the possibility of cell fusion[6,24].

It has become clear that the ability of an MSC to differentiate into a certain cell type *in vitro* does not necessarily correlate with its ability to form that cell type *in vivo*. For example, the reports showing myogenic differentiation *in vivo* may actually be *de novo* myogenesis or cell fusion with resident myoblasts, as described by Gonçlaves and co-workers[25].

Another issue regarding the use of MSCs for cell therapy relates to studies that show that they enter senescence and lose their stemness soon after isolation and *in vitro* culture[26]. In the majority of cases, even when tissues usually abundant in patients, such as adipose tissue, are used to source MSCs, expansion is required before delivery in order to yield an adequate cell number (especially concerning allogeneic therapy), but this expansion may reduce the quality of the cells. Studies have shown that, even under ideal culture conditions, passaging human mesenchymal stem cells (hMSCs) over 25 times can lead to a significant increase in cell doubling time and a decrease in differentiation potential[27]. However, recent research has shown that the stemness of the cells *in vitro* can be improved using methods such as lentiviral gene transfer[28].

The application of MSC-based cell therapy to treat specific disease states will now be discussed. Table 1 summarises the main clinical areas where MSC-based cell therapy has been attempted *in vivo*.

1.3.1 – Cardiac regeneration

The vast majority of cardiac tissue is composed of terminally differentiated myocytes that are unable to replicate nor repair damaged areas after a myocardial infarction (MI). This means that, even if a patient survives after an MI, the heart is weakened and the patient is then more prone to congestive heart failure (CHF). Cell therapy may be able to encourage the regeneration of damaged myocardium and reduce the risk of the patient suffering from CHF.

To date, numerous clinical trials have been completed where bone marrow stem cells have been delivered to the myocardium to promote healing, involving over a thousand patients[29,30,31,32,33]. Many of these studies show a significant increase in left ventricular ejection fraction (LVEF) after the intervention, but this increase tends to be small; in the REPAIR-AMI trial only a modest increase of 2.5 %

Condition	Human?	Results	Comment
Cardiac Regeneration[33]	Yes	Small improvement in LVEF, though not long-term	Tested on over 1000 patients
GVHD[43]	Yes	Significant improvements in the majority of patients	Phase II trial
Bone Regeneration[11]	Yes	Improvements in non-union fractures	Supportive scaffold required
Cartilage Regeneration[48]	Yes	ACI effective in regrowing articular and patellar cartilage	Chondrocytes much more effective than MSCs
Diabetes Mellitus[52]	Yes	Differentiated insulin-producing MSCs decreased insulin requirements by up to 50 %	Difficulty in producing cells that monitor blood glucose, like β cells
Skin Graft[53]	No	MSCs were able to prolong skin graft survival	MSCs possibly prevent immune rejection
Lung Inflammation[60]	No	MSCs reduce bleomycin-induced inflammation in mice	
Crohns Disease[54]	Yes	Adipose-derived MSCs healed 75 % of treated fistulas	Phase I trial
Corneal Burns[57]	No	MSCs prevented unfavourable corneal remodelling post-trauma in rats	
Multiple Scherosis[55]	No	MSCs effective in preventing demyelination in an autoimmune encephalomyelitis model	A more accurate model may be required
Cerebrovasular Accident[34]	No	IV administration of MSCs reduced infarct volume, though no evidence that they migrated to brain	

Table 1.1. **Overview of the mesenchymal stem cell-based cell therapy studies that have been carried out, both on human and animal subjects.** LVEF: left ventricular ejection fraction; GVHD: graft versus host disease; MSC: mesenchymal stem cell; ACI: autologous chondrocyte implantation; IV: Intravenous

compared to control was seen[35]. When considering that a patient surviving after their MI may still experience a decrease in LVEF by as much as 20 %, this increase is small indeed (though cell therapy has been seen to have a greater effect when the MI has caused a greater level of damage to the heart than average)[36]. Furthermore, Martin-Rendon and co-workers suggest that patient-centered outcomes should be measured as well as clinical outcomes, such as exercise capacity and quality of life[33]. This suggestion is supported by Chang *et al*, who found that maximal exercise capacity did not correlate accurately with LVEF six months after treatment[29], though this may be due to a discrepancy in the level of exercise the patients did and the level of exercise they reported after the procedure.

A somewhat irrational design approach has been used in choosing the correct cell type to provide maximum benefit to the patient; few studies exist comparing the regenerative capacity of different cell types, so the method of delivering an autologous unfractionated bone marrow cell suspension has become popular[36]. However, this approach is pragmatic in that, by not selecting a specific cell subset, more cells are can be harvested from a relatively lower volume of bone marrow aspirate (as the cells are not expanded *in vitro* to make the operation quicker), reducing the possible discomfort caused to the patient.

Little is known as to how the cells provide any benefit *in vivo*. Cell-tracking studies show that less than 5 % of the cells remain within the myocardium after 3 months[37], suggesting that the beneficial effects seen from the therapy are provided through a mechanism other than cell engraftment, forming functional tissue. It has been predicted that the cells may release cytokines that stimulate favourable outcomes, such as angiogenesis, the prevention of cardiomyocyte apoptosis and increased tissue perfusion[38]. This paracrine hypothesis is supported by the fact that this form of therapy did not show any benefit one year after treatment in the BOOST trial[39], suggesting that, after a year, the paracrine benefits on angiogenesis and perfusion had dwindled, and the level of surviving engrafted cells had decreased to a level that no longer provided significant benefit.

Assuming that this paracrine effect does occur, when the cells are delivered may relate to further benefit to the patient; it is known that levels of the cytokine vascular endothelial growth factor (VEGF) increase acutely (24-48 hours after infarction) and sub-acutely (7days after)[33]. The REPAIR-AMI trial showed that significant additional benefit was seen when the stem cells were delivered more than 6 days after reperfusion[35], suggesting that the cells may amplify the release of VEGF, providing additional benefit. Martin-Rendon *et al* suggested that, due to the acute increase in VEGF post-MI, cell therapy may be even more effective if delivered within 24 hours of the MI[33], although logistically this would be difficult to perform.

Recently, Korf-Klingebiel *et al* assessed whether bone marrow cells provide a paracrine effect by analysing the benefit provided by bone marrow supernatant. Here they showed the supernatant was able to protect rat cardiomyocytes from cell death induced by simulated ischaemia *in vitro*[40]. *In vivo* assessment is required, but this study suggests that the beneficial effects of the bone marrow cells could be mimicked by a cytokine delivery system, which would remove all the complications regarding cell therapy.

The cells are delivered to the myocardium either transvascularly (via a blood vessel) or via a direct injection. Transvascular strategies are suited for a recently reperfused heart, as that is when chemoattractants and cell adhesions molecules are more prevalent. The most effective delivery method in terms of delivering a high concentration of cells homogeneously is by intracoronary artery infusion using an over-the-wire balloon catheter. This catheter inflates to maximise the contact time between the cells and the intracoronary wall. For the experienced physician, this process is straightforward and can be completed within an hour[36].

Direct injections into the ventricular wall are the preferred route for patients with chronic myocardial ischaemia and when scar tissue has formed. However, this method creates islands of cells with limited blood supply that are prone to hypoxia.

Injecting into the border zone of the infarct can also be technically complicated, and it is unclear how well the perforation caused by the needle recovers[36].

1.3.2 – Graft-versus-host disease (GVHD)

One step further from the reports stating that allogeneic MSCs have little or no effect on the host's immune response are the reports that suggest MSCs have an immunomodulatory role[41]. Little is known about how MSCs modulate the immune response, but it has been hypothesized that MSCs may:-

- engage cell-to-cell contact with T cells (which is required before T-cell suppression takes place[42]),
- suppress effector T cells through the release of various growth factors, such as transforming growth factor β, hepatocyte growth factor, indoleamine 2,3-dioxygenase, prostaglandin E_2 and nitric oxide[43],
- down-regulate immunoglobulin production[43] and/or
- up-regulate MHC class II markers, reducing the natural killer cell cytotoxicity[43].

Studies also showed that MSCs, once delivered, were able to accumulate in either sites of organ damage, inflammation, or cancer[42,43], which means that MSCs may not have to be delivered to the specific required site of action to provide benefit.

In order to explore the immunomodulatory of MSCs, researchers have clinically assessed the effectiveness of MSC cell therapy for treating graft-versus-host disease (GVHD). GVHD is a common complication of allogeneic haematopoietic stem cell transplantation, where the host rejects the transplant. In a case report, transplanted allogeneic MSCs, delivered intravenously, were found to treat a patient with severe GVHD[44]. This led to a clinical trial, where 60 million MSCs were delivered, and this showed an impressive response rate of 70 %[45]. In a phase II study containing 55 patients with steroid resistant GVHD, no patients had any side effects from the therapy, 30 patients had a complete response and a further 9 showed improvement[46]. However, it was suggested by Miura and co-workers that this therapy may make the patients more prone to infections, due to its

immunosuppressive action. This may have led to 3 deaths in the study (2 from bacterial septicaemia, 1 from adenovirus infection)[47], though GVHD in itself does weaken the immune system also.

1.3.3 – Bone regeneration

During certain types of fracture, the MSCs within the skeletal tissue contribute towards remodeling the bone so that it is able to bear weight again, providing a pool of osteoblasts necessary for the formation of mineralized matrix. However, in certain situations, such as non-union fracture, or in certain diseases such as osteoporosis, arthritis and cancer, normal remodeling processes are impaired. MSCs have been shown to be an effective cell source to aid the repair in non-union fractures[11,23], but only with a scaffold present that can provide a three-dimensional environment for the MSCs to proliferate in, and provide some element of weight-bearing capacity at the injury site before the cells have had time to mineralize[48]. For these reasons, many developments in bone regeneration have been TE-based, rather than cell therapy-based.

1.3.4 – Cartilage regeneration

Autologous chondrocyte implantation (ACI) (or autologous chondrocyte transplantation) is mainly used to replace articular or patella cartilage (within the knee joint). Until recently, the most effective method of treating articular cartilage damage was to replace the joint with an artificial construct. However, because these artificial joints are stiffer than the surrounding bone, damage to the healthy bone often occurs[49].

ACI is a treatment aimed at patients suffering from grade III or grade IV cartilaginous lesions, which can form as a result of local trauma or a disease such as osteoarthritis. Within these lesions, the hyaline cartilage is avascular, aneural and, especially with age, with limited reparative capacity. Also, due to the limited migratory ability of chondrocytes, healthy cartilage surrounding a defect is often unable to fill the defect[49].

ACI is a very effective treatment for repairing injured cartilaginous areas, but only when performed with chondrocytes isolated from the joint (which may include MSCs also), rather than MSCs alone. MSCs are found to produce fibrocartilage that lack the mechanical properties required to support the underlying bone, especially for high-demand patients[50]. Also, isolating and expanding the chondrocytes is no more complicated a procedure than the equivalent MSC isolation.

Autologous chondrocyte implantation has also been assessed for regenerating the intervertebral disc to help alleviate lower back pain[51], and a recent EuroDISC clinical trial did suggest effectiveness[52], but only with chondrocytes isolated from the vertebrae.

1.3.5 – Diabetes mellitus

Trivedi and co-workers presented work where they were able to differentiate adipose-derived MSCs into insulin-secreting cells. These cells, when delivered to diabetic patients, were found to decrease insulin requirements by up to 50 %, with a mean follow-up of 2.9 months[53]. The authors carried out the study using allogeneic cells (obtained from family donors), but there appears to be no reason why the cells could not be extracted from the autologous source to reduce the level of immune rejection. Optimisation of the delivery process and the number of cells delivered may make this a curable treatment, though there was no evidence to show that these transformed cells, like β cells, are able to release insulin in response to increasing blood glucose levels.

1.3.6 – Other diseases

The breadth of clinical manifestations where the use of MSC therapy has been investigated is staggering. Due to the immunomodulatory effects of MSCs, they have been shown to prolong skin graft survival *in vivo*[54], and may be beneficial in treating Crohns disease[42,43,55] (an autoimmune disease affecting the gastrointestinal tract) and multiple sclerosis[56] (an autoimmune disease affecting the central

nervous system). Also, due to the cell's ability to accumulate in cancerous tissue, the cells may be used as a delivery device, enabling the targeting of therapeutics directly to the cancer[57]. The beneficial paracrine effects that MSCs provide in cardiac tissue have also been reported intraocularly, and show promise in treating corneal burns[58]. The cell's ability to target inflamed areas, with excessive ECM deposition, may make MSCs effective as a vector used to help treat diseases of the lung[59,60], and studies have shown that MSCs have the ability to reduce bleomycin-induced inflammation and fibrosis within the lungs of mice[61]. Finally, recent work suggesting that MSCs could differentiate into hepatocyte-like cells *in vivo* could lead to the development of MSC-based hepatic-cell therapy, though only if it can be proved that cell differentiation is not actually cell fusion[62].

1.4 - Understanding the cell's tolerance to cell therapy-based manipulations

With regard to many of the animal and clinical studies described above, methodological parameters, such as how many cells to deliver, which cells to deliver, how many doses would be required etc., were based on little if any *in vitro* evidence. Furthermore, assessment of the effectiveness of a cell therapy trial was often clinically performed[63], with little regard of how and why a specific cell therapy was effective.

When considering the survival of the cells during the cell therapy process, the procedure can be split into two; (i) the effects of the processing of cells from either a tissue-culture flask, or, if not expanded, from the tissue biopsy, to the target organ/tissue via a delivery device, such as syringe or catheter, and (ii) the effects on the cells *in vivo* once delivered (Figure 1.1).

1.4.1 – Tracking cell migration and activity once within the target organ

It is very difficult to track the migration of cells once administered, and the task becomes more complicated when requiring information regarding cell activity.
The following imaging methods have been used in order to follow the progress of the cell:-

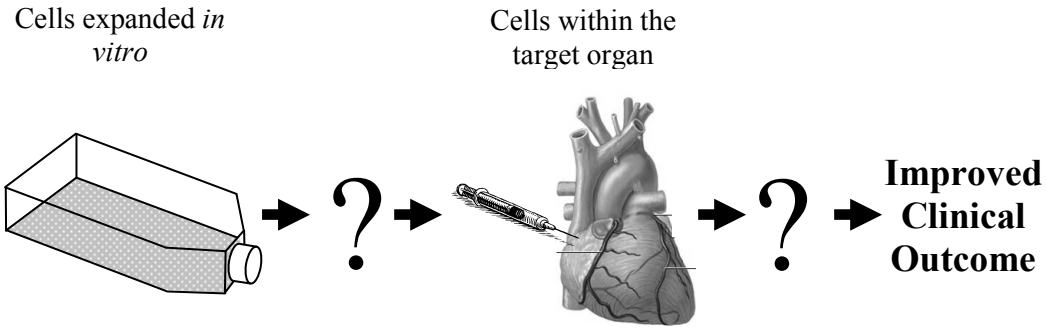


Figure 1.1 Simple schematic to show the areas of uncertainty during the cell therapy process. The first area of uncertainty assesses how cells are affected by the delivery process. The second area of interest questions how cells produce the required improved clinical outcome, and how the cells engraft *in vivo*

- Optical imaging. Bioluminescence imaging is a highly sensitive method, and has been used to assess the distribution of MSCs within an organ over time. Fluorescence reporter probes are used less often due to low signal-to-background ratios[57]. There is no radiation with this method, but this method is not, so far, clinically applicable[37].
- Two-photon microscopy, where a signal is produced when 2 photons of a low energy excite a fluorophore in a quantum event, have the advantage over optical imaging techniques in that the level of light scattering (which causes image blurriness) is much reduced. This means that imaging can be performed at much greater depths, from 100 μm with optical imaging, to upto 1 mm. This allows more opportunities for imaging to be performed without any damage to the tissue[64].
- Magnetic Resonance Imaging. Cells are directly labeled with magnetic resonance contrast agents, providing good resolution. Issues do also arise with regard to the sensitivity of detection, and whether the label affects the activity of the cells[37,57,65].
- Radionucleotide Imaging. These methods can have a good penetration depth, and provide quantifiable data, such as cell number *in vivo*[57,66]. However, the radiation may have an effect on the therapeutic cells.
- Reporter Gene Imaging. Cells are transfected with a viral reporter gene. Transfected cells can then be detected following the intravenous administration of a radio-labeled or optical reporter probe which binds to the specific translated protein. This is the most effective method of tracking active cells, as the expression of the protein requires the cell to be viable[57]. This method can also be used to track differentiation. However, the signal produced is weak, and there is a risk of genetic modification within the host[37].
- Quantum dot imaging. When delivered to murine ESCs, quantum dots were found to cause no significant effects on viability, proliferation or differentiation. These dots were found to be easily imaging fluorescently, even within deeper tissues, where MRI may not effective[67]. However, this technique still provides little information with regards to cell viability or function, and

there is the possibility of the quantum dots separating from the delivered cells *in vivo*.

None of these imaging methods are ideal for tracking the delivered cells, especially if the safety of the host is of concern, as is the case with clinical trials.

1.4.2 – Analysing the effect that the cell delivery process has on cell fate

Few studies have analysed the effect of processing the cells for cell therapy applications on cell viability and activity. The factors during this procedure that could have an effect on viability include:-

- the stresses experienced during the recovery of cells from culture i.e. trypsinisation from tissue culture plastic, centrifugation of cells, removal of cells from a tissue-culture incubator, resulting in an increase in pH and a decrease in atmospheric temperature,
- cell stresses resulting from a localised high cell concentration within the delivery system (produced when there are restrictions in the volume of solvent that the cells can be suspended in), such as alterations in pH, aeration levels, nutrient availability, toxic metabolite accumulation, and cell-cell signaling,
- physical cell insult due to the passage through the delivery system (such as the shear stresses induced by drawing up or expelling the suspension from a syringe needle/catheter, bubble entrainment/bursting by inversion of delivery device) leading to necrosis or induction of apoptosis,
- storage conditions, sterility and preparation of delivery device (e.g. “shelf life” of system, temperature, lag time before delivery).

Veraitch *et al* assessed the impact that manual handling had on ESC viability and directed differentiation capacity, promoting the need for automated cultivation systems. Exposing the ESCs to prolonged periods (up to 3 hours) under ambient temperatures saw a decrease in proliferation, as did the passage of the cells 45 times through a 500 μm diameter capillary (to simulate the pipetting of cells[68]). One could speculate from these results that, a prolonged period at room temperature for a cell therapy suspension would have a more drastic effect on the

cells (as the cells would be at a higher concentration). Similarly, the passage of mammalian cells through a narrow bore would have a more pronounced effect as the bore would be narrower than 500 μm. Moreover, in order to have the cells within the delivery device, in theory, all of the manipulations described in the paper would need to be carried out, adding a negative predisposition to the cells before they endure the passage through the delivery device. However, the extent at which these negative effects would occur is greatly unclear.

One of the only published studies assessing the viability of the cells post-ejection from a delivery device is described in Kondziolka *et al*. The paper assessed the viability of neuronal cells within a concentrated (3.3×10^7 cells/ml) suspension, using a trypan blue exclusion method, after being drawn up through a 25 gauge bore (260 μm internal diameter) and expelled through a catheter of similar diameter. Surprisingly, they found a decrease in viability by almost half after this manipulation as acceptable for their studies[69].

One could argue that, provided that the majority of cells survive the delivery process, information regarding the fate of the cells post-administration makes little difference to the number of cells delivered, as little is known with regard to the number of cells required to provide a clinical benefit. Furthermore, in some studies, a large variance in the number of cell delivered is seen as the researchers aim to deliver as many cells as possible in each individual case (Le Blanc *et al* is an example of this)[46]. However, a small level of cell death within a concentrated cell population could have a significant effect on the cellular characteristics of the remaining viable portion and the host environment through the release of cytotoxic agents such as tumour necrosis factor α[70]. Moreover, studies analysing the effect of the cell delivery process could also explore alterations to the process that may optimize cell viability, such as refrigerating samples, increasing the needle bore diameter, reducing the ejection rate, among others.

1.5 - The complications surrounding the provision of allogeneic cell therapy products

1.5.1 – Regulatory complications

With so little known with regard to the mechanisms involved in a cell's effectiveness to treat a clinical state, regulatory bodies, such as the Food and Drug Administration (FDA), want to make sure that these therapies are consistent, safe, pure and efficacious[71]. The FDA separates cell products into those that require "minimal manipulation", such as traditional blood products, which must be processed following Good Tissue Practice (GTP), and those that require more than minimal manipulations. The latter must be processed under Good Manufacturing Practice (GMP) as well as GTP, and are considered as investigational new drugs (INDs) with regards to the pre-market clinical trials required[8]. GMP, and to a lesser extent GTP, present the need for costly GMP facilities that ensure sterility within the cell therapy product.

Regulatory bodies also require cells that are free from cell debris and pyrogens, potent and of a specified cell number at the point of delivery[8]. A cell viability of 80 % or more is also expected, as well as a method of displaying repeatable potency[72].

1.5.2 – The storage and transportation of cell therapy products

Researchers accept that, for cell therapy to be clinically available in the future, research is required in effective storage and transportation methods for the final product[73]. The logistical limitations of transporting autologous cell therapy products was a significant issue that the company CellTran® faced with their product MySkin®. MySkin® is a product based on autologous keratinocytes delivered on transfer disc, developed to treat a range of dermatological conditions, such as burns and diabetic ulcers. They found that regular repeated applications of this product initiated wound healing in 18 out of 21 patients with diabetic ulcers in a randomized controlled trial[74], but found that the application of the products was

complicated by the logistical challenges associated with sampling, processing, storing and delivering the autologous cells.

One method that may provide a storage solution, and improve the ease of transportation, is cryopreservation. Bone marrow cryopreservation for the use in treating GVHD has been found in most reports to be at least as effective in treating the disease as freshly isolated bone marrow, although the number of patients treated in the studies was low. One report showed an improvement in treatment when using the frozen cells compared to fresh cells (though the improvement may have been due to adjuvant medicines administered, which were not kept constant throughout the studies). However, a significantly greater number of cryopreserved transfusions were found to be contaminated with bacteria, compared to fresh transfusions, and this is thought to be caused by the greater level of manipulation required for the preserved cells[75].

Another issue with cryopreservation is the use of cryopreservant. One cryopreservant used often is dimethyl sulphoxide (DMSO), which, at a 10 % ($^{v}/_{v}$) concentration (optimal for cryopreservation), was found to be toxic to the patient, causing a significant increase in nausea, vomiting and fever[75]. However, research in increasing the rate of freezing may reduce the concentration of cryoprotectant to non-toxic levels[76]. Strategies are also being developed in using alternative non-toxic cyropreservants, utilising the mechanisms used by freeze-hardy plants; by encouraging the accumulation of disaccharides, such as sucrose and trehalose, into the cells, the sugars can act as a cryoprotectant[77]. Olarewaju *et al* applied the disaccharide trehalose, together with propylene glycol, to embryonic stem cells and showed efficient viability retention following rapid freezing and thawing[78].

Flasza and co-workers describe storing and transporting human dermal fibroblasts (HDFs) under refrigerated (2-8 °C) conditions during the phase I trials of the Intercytex® product, ICX-SKN. The HDFs were stored on fibrin constructs in

sterile pouches containing CO_2-independent transport medium. This method was able to keep the HDFs alive and proliferative for up to 5 days[79].

Flasza *et al* state that the ability to transport cells without cryopreservation removes the need for specialised storage or complicated recovery methods[79]. For these reasons, companies, such as Genzyme® are developing cell-specific refrigeration technologies to possibly meet the demand of cell products in the future that would require distribution[80].

A study by Lane *et al* showed that the viability of human bone marrow stromal cells completely diminished when stored at room temperature at the concentration of 2×10^6 cells/ml[81]. This study suggests the need for complex systems in order to prolong cell viability under room conditions (or, arguably, that is it not possible to retain cell viability under room conditions). One group have developed a method of preserving limbal epithelial cells at room temperature for 7 days in organ culture medium, allowing them to be transported with ease. Limbal epithelial cells are used to treat patients with a ocular limbal stem cell deficiency and the preservation of these cells are important because human donors are in demand, and the cells can take several weeks to culture into an epithelial sheet, making the scheduling of the operation complicated. Utheim *et al*, found that, by culturing the cells onto an amniotic membrane, suturing the membrane onto a polyester membrane, and transferring this to a bath of organ culture media, 84 % of viable cells could be retained after a week[82]. Although an impressive finding, this study was carried out in media containing xenogeneic material, which may cause prion-based complications when delivered to the host. Moreover, the cells were kept at temperatures (23 °C) higher than the temperatures the cells may be exposed to if transported outside of a cell culture facility or clinic.

1.6 – Aims

There are two distinct aims of the research presented here. The first aim is to assess the effect that parenteral delivery has on the MSC's ability to thrive. Section 1.3 clearly describes the potential of MSCs to treat numerous diseases, but little is known as to whether cells are affected by the delivery process, and this may have fundamental implications for the development of future cell therapy products.

The objectives in order to achieve this aim are to:-

- assess how the time left within the syringe chamber at room temperature affects mMSC viability, apoptotic activation, morphology and proliferative ability (Chapter 3),
- assess whether seeding the cells onto microparticles before being left within the syringe chamber improves mMSC viability (by allowing the anchorage-dependent cells a surface to adhere to) (Chapter 3),
- assess how parameters such as ejection rate, needle gauge, anti-oxidant concentration, affect the viability of the mMSCs using flow cytometry as an analytical method (Chapter 4),
- compare the viability of mMSCs left within the syringe chamber against those left within an eppendorf (Chapter 3 and 4).

The second aim is to develop a means of transporting cell therapy products, keeping cells viable at room temperature and under atmospheric conditions for up to 7 days. The ability to keep cells alive under these conditions removes the need for special delivery, and keeping the cell alive over such a time period means that international distribution may be feasible. It is hypothesized that, if the required nutrients are delivered to the cells at a controlled rate, and the metabolites which are toxic are removed from the system, and gaseous exchange can occur through a permeable membrane, primary human MSCs should survive under these conditions.

The objectives in order to achieve this aim are to:-

- develop a proof-of-concept, where the effectiveness of a toxin adsorbent (Chapter 5) and a nutrient supply (Chapter 6) within a simple well-plate configureation is shown,
- assess which key metabolites need to be adsorbed (Chapter 5), and which key factors are needed to be delivered via the delivery system (Chapter 6),
- develop an adsorbent that is able to adsorb the key toxins (Chapter 5),
- develop a delivery system that can provide the required nutrients at a steady rate (Chapter 6).

2

Materials and methods

2.1 - Materials

2.1.1 – Cell cultures

Adult CD1 mice used to obtain the mMSCs were sourced from the Biomedical Science Unit, University of Nottingham, UK. Both the Biomedical Science Unit and the personnel have up-to-date Home Office licenses, allowing them to sacrifice the mice prior to isolation using asphyxiation.

Primary hMSCs were obtained from TCS CellWorks, Buckingham, UK. On receipt the cells were at passage 1.

2.1.2 – General chemicals

Sterile preparation of stock solutions and chemicals were performed either by filtration through a 0.22 μm Whatman sterile filter and/or autoclaving at 121 °C at 1 bar for 1 hour. Most general chemicals were purchased from Sigma-Aldrich (Poole, UK) unless otherwise as stated as below:

Alamar blue was bought from Invitrogen®, Paisley, UK. CellTiter AQ One Solution Cell Proliferation® assay kit was bought from Promega, Southampton, UK. High molecular weight 85:15 PLGA was purchased from Lakeshore Biomaterials, Birmingham, AL, USA. The zeolite used in this research was a kind gift from Bear River Zeolite, Preston, Idaho, USA. Other reagents can be found in the appendix.

2.1.3 – Apparatus and consumables

Primaria 6-well culture dishes and hypodermic needles were bought from Falcon, Becton and Dickinson Ltd., Oxford, UK. Tissue culture flasks (75 cm^2, 175 cm^2 and triple 175 cm^2) were bought from Nunclon, Denmark. Costar 96-well flat-bottom, non-treated, polystyrene assay plates and Transwell® polyester membrane inserts, 24 mm diameter, were purchased from Corning, NY, USA. Ten µl glass syringes with a luer lock tip (model 1701LT) were purchased from Hamilton®, Bonaduz, Switzerland. The Havard® syringe pump (model PHD 2000) was bought from Havard Apparatus®, Holliston, Massachusetts, USA. Magna nylon 0.45µm membrane filters, 500 µl eppendorfs, Sterilin® petri dishes, 15 and 50 ml sterile centrifuge tubes, 10 ml sterile pipettes, automatic pipette fillers, 1 ml 200 µl and 3 µl pipette tips, 0.22 µm filters were bought from Fisher Scientific, Loughborough, UK. Whatmann® filter paper was purchased from Sigma-Aldrich, Poole, UK. Scintillation vials were supplied by Canberra-Packard, Pangborne, UK. The Mettler-Toledo SevenEasy® pH meter, the improved Neubauer haemocytometer and parafilm were purchased from Scientific Laboratory supplies, Nottingham, UK. The Bio-Tek KC4 microplate reader was purchased from Fisher Scientific, Loughborough, UK. The MFX plate reader was bought from Dynex, Chantilly, VA, USA. The class II double HEPA-filtered flow safety cabinets were purchased from Walker®, Derbyshire, UK. The tissue culture Incusafe® incubators were bought from Sanyo, Loughborough, UK. The Leica DM-IRB/E inverted microscope was purchased from Leica Microsystems *Ltd*., Milton Keynes, UK, and the Volocity® image software was bought from Improvision, Coventry, UK.

2.2 - Methods

2.2.1 - Murine mesenchymal stem cell isolation

Primary murine mesenchymal stem cells (mMSCs) were isolated in-house following the standard protocol as previously described by Da Silva Meirelles and Nardi, which has recently been updated[83,84]. Unbroken bone was isolated from the hind legs of CD1 mice and the bone marrow was exposed using a simple end fracture. Bone marrow/cell homogenate was flushed out with complete mMSC

media using a hypodermic needle. Erythrocytes were removed with the use of 1 ml of red blood cell lysis buffer, incubating at 37 °C for 1 minute, centrifuging (250 g, 4 minutes), and removing the supernatant fraction. The resultant cell mass was transferred to a 25 cm^2 T-flask containing complete mMSC medium, and incubated for 72 hours before the non-adherent cells were removed.

2.2.2 – Culture conditions

Cells were cultured and maintained, *in vitro*, as monolayers in tissue culture flasks using either complete mMSC or hMSC medium (appendix 1.1 and 1.2 respectively). Cells were routinely cultured in a humidified-atmosphere tissue-culture incubator at 37 ˚C and with 5 % CO_2 and never allowed to reach greater than 80 % confluency at any one time. For routine detachment and passaging, a standard trypsinisation protocol was performed. The cell monolayer was rinsed once with phosphate-buffered saline (PBS) prior treatment with trypsin/EDTA solution (appendix 1.7), at 37 ˚C for approximately 5 minutes (based on visual detachment). Inactivation of the trypsin was achieved by the addition of an equal volume of complete medium. This suspension was then transferred to a centrifuge tube before being spun down at 250 g for 4 minutes. The cell pellet was then re-suspended in the appropriate volume of fresh complete medium or re-seeded at the desired cell density in the corresponding culture vessel. This was then returned to a tissue culture incubator or used for experimentation. Back-up cultures were maintained using a separate and independent supply of medium and solutions.

Both mMSCs and hMSCs were passaged up to 25 times (as suggested in Pal *et al*, 2008), or when noticeable differences in morphology or in cell doubling times were observed, before being used within the studies[27].

All aseptic techniques used were performed in accordance with the ATCC guidelines. All manipulative tasks were confined to class II safety cabinets under ACGM Containment level 2 regulations. The disposal of cells and other biological

wastes were either autoclaved and/or disinfected in accordance with ACGM regulations.

2.2.2.1 – Specific culturing into 6 well plates or transwells

A standard method was used to transfer cells to the well or transwell of a 6 well plate. After detachment using trypsin (2.2.2), cell counts and viability determination, to create the correct viable cell concentration, were performed using the trypan blue exclusion technique (appendix 4.3). Cells were then spun down at 1200 rpm for 4 minutes, the supernatant was removed, and cells re-suspended in a volume of media to provide a final cell concentration of 10^5 cells/ml. This final concentration (1 ml) was plated onto either the well of a tissue-culture 6-well plate or a Transwell® insert, and provided 24 hours to attach within a tissue culture incubator.

2.2.3 – CellTiter AQ one solution cell proliferation assay

The CellTiter AQ One Solution Cell Proliferation® assay kit detects viable cells through its conversion into a formazan product in the presence of active mitochondrial succinic dehydrogenase, resulting in a colorimetric change. This assay has been used previously for this purpose[85]. For assessing viability, 20 µl of CellTiter AQ reagent was added to 100 µl of cell suspension, under reduced lighting. The samples were then incubated in the tissue culture incubator for 1 hour before colorimetric analysis (492 nm) using a Bio-Tek KC4 plate reader. Blank values (or, samples containing MPs alone) were subtracted from sample readings, and, using a standard curve (Appendix 5.1), cell concentration and, therefore, percentage viability could be determined.

2.2.4 – Alamar blue assay

The alamar blue assay was also used to assess viability. This reagent is a REDOX indicator that fluoresces in response to the chemical reduction that occurs within the growth medium as a cell population metabolises. The main advantage of this assay is that it does not infiltrate the cells, and therefore is minimally toxic to

living cells, so the assay can be ran on the same batch of cells over various timepoints. However, contamination and infection can both lead to a chemical reduction, which in turn affects the results[86]. As the vast majority of cellular metabolism occurs via mitochondria, the results from MTT-based assays (such as the CellTiter AQ assay) have been found to closely correlate those from Alamar blue[87,88].

Within a 6-well plate or transwell configuration, StemPro® media was removed and replaced with 1 ml StemPro® media containing 100 µl of a 10 × concentration alamar blue solution. Cells were left to stand at room temperature for 2 hours, before the alamar blue solution was removed and replaced with the media they were cultured in previously. The alamar blue solution (100 µl) was transferred to a 96 well plate before fluorometric analysis (ex 560 nm/ emm 590 nm) was performed using a MFX plate reader. Blank values were subtracted from sample readings, and, using a standard curve (appendix 5.4), cell concentration and, therefore, percentage viability could be determined.

2.2.5 – Staining of viable cells using mitotracker® deep red

Mitotracker® reagent fluoresces upon binding to active mitochondria. The advantage of this stain over the live/dead® solution is the ability for the stain to be retained within the cells once they are fixed, allowing one to read all the samples together once all the time periods have passed.

To 500 µl of cell suspension, 250 nl of mitotracker® deep red was added, and the cells were incubated for 30 minutes in a tissue culture incubator. They were then fixed by a 10 minute incubation with 3.7 % ($^{w}/_{v}$) paraformaldehyde in an incubator.

2.2.6 – Flow cytometry analysis

Cell suspensions were analysed using a Beckman Coulter Cytomics FC500 flow cytometer (High Wycombe, UK). Fluorescence emission was collected with

interference bandpass filter sets for the spectral regions of 530 ± 30 nm (calcein) and 630 ± 22 nm (ethidium homodimer and Mitotracker Deep Red). Isoton II® diluent (Beckman Coulter, High Wycombe, UK) was used as sheath fluid, and typical flow rates were 60 µl/min, producing between 200 and 1000 events/sec. Forward scatter and side scatter data were also collected and cross-referenced with the fluorescence data. Data were analysed using Windows Multiple Document Interface for flow cytometry (WinMDI) software (Scripps Institute, CA).

Percentage viability was determined by dividing the number of viable events (either the events fluorescing at 530 nm when the Live/Dead stain was used, or the events within the gated region when the Mitotracker® stain was used) by the total number of events that occurred within the control. Using this methods allows one to take into account the number of cells that may have lysed and therefore not produced an event through the flow cytometer. However, this method does assume that no cells lysed within the control group.

2.2.7 – Preparation of zeolite

The zeolite granules were separated from residual zeolite powder through decantation in distilled water, retaining the zeolite granules (sediment). The decantation process is repeated at least 5 times in order to ensure all powder is removed. The particles were then dried overnight, using a freeze-drier, to remove residual moisture. The granules were then weighed into separate 2 or 4 g batches and autoclaved at 121°C at 1 bar for 1 hour.

2.2.8 – Glucose concentration determination

Glucose concentration was determined using a glucose oxidase (GO) assay. This assay works by specifically catalyzing the production of hydrogen peroxide from glucose, using glucose oxidase. This hydrogen peroxide then oxidises o-dianisidine, causing the colorimetric change. Recently prepared GO reagent (appendix 2.4) (200 µl) was added to 100 µl of sample solution, and incubated at 37 ºC for 30 minutes, before colorimetric analysis (540 nm) using a Bio-Tek KC4

plate reader. Blank values were subtracted from sample readings, and, using a standard curve (appendix 5.6), the concentration of glucose could be determined.

2.2.9 – Total protein concentration determination

Total protein concentration was determined using the Bradford assay (Chapter 3) and the Coomassie Plus assay. Both assays work on a similar principle; the reagents are able to form a blue complex upon binding to the hydrophobic pockets of the protein's tertiary structure. Few substances interfere with the production of the signal[89]. The Bradford assay was performed simply by the addition of 250 µl of Bradford reagent, incubating, at room temperature for 5 minutes, before colorimetric analysis (595 nm) using a Bio-Tek KC4 plate reader. Blank values were subtracted from sample readings, and, using a standard curve (Appendix 5.3), the concentration of total protein could be determined.

The Coomassie assay works by the same principle as the Bradford assay, but has the added advantages that the standard curve produced is more linear, and the variability of signal after protein interaction is half that of the standard Bradford assay (due to the assay's ability to bind to amino acids other than arginine with a greater affinity). Coomassie Plus reagent (300 µl) was added to 10 µl of PBS sample, shaken on a plate-shaker for 30 seconds and left to stand at room temperature for 10 minutes, before colorimetric analysis (595 nm) using a Bio-Tek KC4 plate reader. Blank values were subtracted from sample readings, and, using a standard curve (appendix 5.5), the concentration of total protein could be determined.

2.2.10 - Statistical analysis

Data sets were tested for normality and subsequent tests of comparisons were chosen appropriately. For simple paired comparisons, the student *t*-test, was utilised and expressed as mean ± standard error of the mean (SEM). For normally distributed multiple data comparisons, a one-way ANOVA test was utilised and expressed as mean ± SEM. Whenever the statistical significance between control

and treated samples were evaluated at a 95 % confidence level ($p < 0.05$) or, at a 99 % confidence level ($p < 0.01$), the data set would be considered to be statistically significant and represented with a (*) or a (**) respectively.

3

The effect of delivery via narrow-bore needles on mesenchymal stem cell viability, apoptosis and morphology

3.1 - Introduction

As discussed in 1.4.2, little is known as to whether the viability and characteristics of the delivered cell are affected by the delivery process itself[68,69]. In this chapter, the effect on mMSC viability and apoptosis are described after they were drawn up into a syringe, left within the syringe for periods of up to two days, and then ejected. Cell viability whilst left within the syringe chamber were compared to the viability when the cells were instead left within an eppendorf, either under ambient temperature, or 4 °C, in order to provide information regarding how cells should be stored prior to an operation. Characteristics important to the functionality of mMSCs, such as their ability to spread onto a favourable surface and their proliferative ability, are also discussed here.

The parameters regarding how the cells are manipulated, such as ejection rate, needle gauge and cell concentration, were based on how the neuronal cells were handled by Bible and co-workers;[90] this is a paper showing an improvement in cerebral recovery after neuronal cells were directly injected into an ischaemic brain region of a rat stroke model.

As discussed in depth within the general introduction, mesenchymal stem cell therapies have the potential to treat a vast array of disease states. For this reason, primary murine mesenchymal stem cells were chosen for this study.

The effectiveness of the antioxidant *n*-acetyl cysteine in this study was also based on the use of the antioxidant in the *in vivo* studies described by Bible *et al*[90]. *N*-acetyl cysteine is thiol-containing compound that has been shown to have an anti-apoptotic effect through a direct mechanism involving the reduction of reactive oxygen intermediates, and by stimulating the synthesis of glutathione, which, in turn, mediates apoptosis[91] (figure 3.1).

The mMSCs used in this experiment are anchorage-dependent. Studies have shown that following a prolonged period of detachment after trypsinisation has a negative effect on cell viability and increases the level of apoptotic activation via surface integrin signaling pathways[92]. To test this hypothesis, cells were seeded onto microparticles (MPs) composed of poly(lactic-*co*-glycolic acid) (PLGA), and the viability of the seeded cells were compared to a single cell suspension post-ejection. Previous work has shown that seeding cells onto pre-treated PLGA surfaces does not affect the viability of the cells compared to tissue-culture plastic[90]. Moreover, PLGA has an established history of showing biocompatible characteristics[93], allowing one to deliver the particles with the cells to the host. If the hypothesis was correct, one could envision the use of PLGA MPs not only to deliver important factors to the cells to aid host engraftment[93], prevent the clearance of the cells and provide a favourable environment to proliferate in[94], but MPs may also be used to improve the viability of the cells during delivery.

3.2 – Materials and methods

3.2.1 – Microparticle fabrication

PLGA MPs were fabricated using a single oil-in-water (O/W) emulsion method, and optimised for the production of particles with a diameter of between 50 and 100 μm. PLGA was dissolved in dichloromethane (DCM) at room temperature, overnight. Freshly prepared 0.3 % ($^{w}/_{v}$) polyvinyl alcohol (PVA) solution was slowly added to the vial before the solution was emulsified using a vortex mixer. This emulsion was then immediately poured into an 0.3 % PVA hardening bath and stirred constantly for at least 24 hours, within a fume hood, to allow the

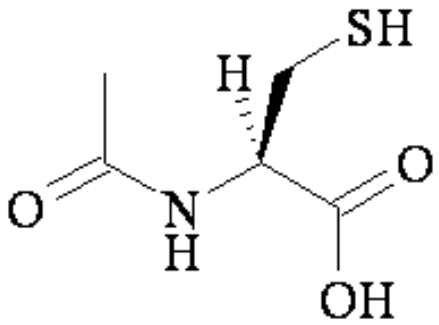


Figure 3.1. Chemical structure of *n*-acetyl cysteine

solvent to evaporate. The solidified MPs were then washed with distilled water and harvested via vacuum filtration. After drying the MPs, they were sieved and separated using a Retsch AS200 sieve shaker. MPs were stored in individual vacuum sealed packets at 4 °C.

3.2.2 – Surface treatment via plasma polymerisation

MP surface chemistry was modified using a plasma polymerised allylamine (ppAAm) deposition technique, using an in-house plasma reactor, as previously described[95]. The plasma reactor consists of a T-shaped borosilicate chamber, enclosed within stainless steel endplates, and sealed with Viton O-rings. Plasma formation was initiated by two external inductivity coupled copper band electrodes connected to a 13.56 MHz power source (Coaxial Power Systems Ltd.) and matched manually such that the reflected power was < 1 W. Petri-dishes containing MPs were placed into an evacuated chamber and etched with 300 mTorr of oxygen. The MPs were agitated before this was repeated so that a total of 5 random etching cycles were performed on the MPs. Following this, the chamber was then restabilised at 300 mTorr with allylamine vapour. Deposition of allylamine onto the MPs was maintained until a layer 0.5 kÅ thick had formed on the quartz crystal microbalance positioned opposite the substrates. A total of 5 ppAAm coatings were performed on each sample, in between agitating the particles, to ensure a complete and uniform surface coating.

3.2.3 – Surface treatment via adsorption of ECM proteins

MPs were sterilized using 70 % ($^{v}/_{v}$) ethanol on a shaking platform, at room temperature. After rinsing in PBS, an additional wash in 1 % ($^{v}/_{v}$) penicillin-streptomycin-amphotericin B solution, was performed, followed by another PBS wash.

MPs were pre-wetted with serum-free medium for 24 hours in a tissue-culture incubator before being coated with fibronectin, using a standard adsorption protocol. A 10 µg/ml fibronectin solution are applied to eppendorfs containing

MPs, for three hours in a tissue culture incubator. The MPs were agitated at times 15, 30, 45, 60 and 90 minutes of the incubation period. Unattached fibronectin was then removed, and the MPs were washed twice in PBS.

3.2.4 – Seeding mMSCs onto PLGA microparticles and syringe manipulation

Murine MSCs were isolated and cultured as described in 2.2.1 and 2.2.2 respectively. After detachment using trypsin (2.2.2), cell counts and viability determination to create the correct viable cell concentration were performed using the trypan blue (TB) exclusion technique (appendix 4.3). The cells were then spun down at 250 g for 4 minutes, supernatant was removed, and cells were re-suspended in a volume of Hanks Balanced Saline Solution (HBSS) containing 0.5 % ($^{w}/_{v}$) carboxymethyl cellulose (a biocompatible viscosity modifier that prevents the settling of the particles so that they effectively be drawn up into the syringe) to provide a final cell concentration of 5×10^{7} cells/ml. The cell suspension was either added to pre-coated PLGA MPs and incubated in a tissue culture incubator for 1 hour, gently manually agitating every 15 minutes (seeded cells), or drawn up directly into a syringe with a 22 gauge needle (internal diameter 394 μm, appendix 6) attached at a rate of ~30 μl/min and left to stand for pre-defined time periods at room temperature (unseeded cells) (a wider needle gauge was used here, compared to the other experiments within this study because the MP suspension could not be effectively drawn up into a narrower bore). Ten μl samples of both seeded cells and unseeded cells were processed to provide a control. Seeded cells were drawn up into a similar syringe and left to stand for pre-defined time periods at room temperature. The cell suspensions were ejected at the controlled rate of 5 μl/min into full media, as shown in figure 3.2. Samples were then analysed for viability using the CellTiter AQ One Solution assay as described in 2.2.3.

A preliminary experiment was carried out to determine the percentage of cells that attached to the microparticles and to identify the optimum particle to cell ratio. A ratio of 15 cells per microparticle was found to have to best seeding efficiency; 90 % of the cells attached to the microparticles, as determined using the CellTiter AQ

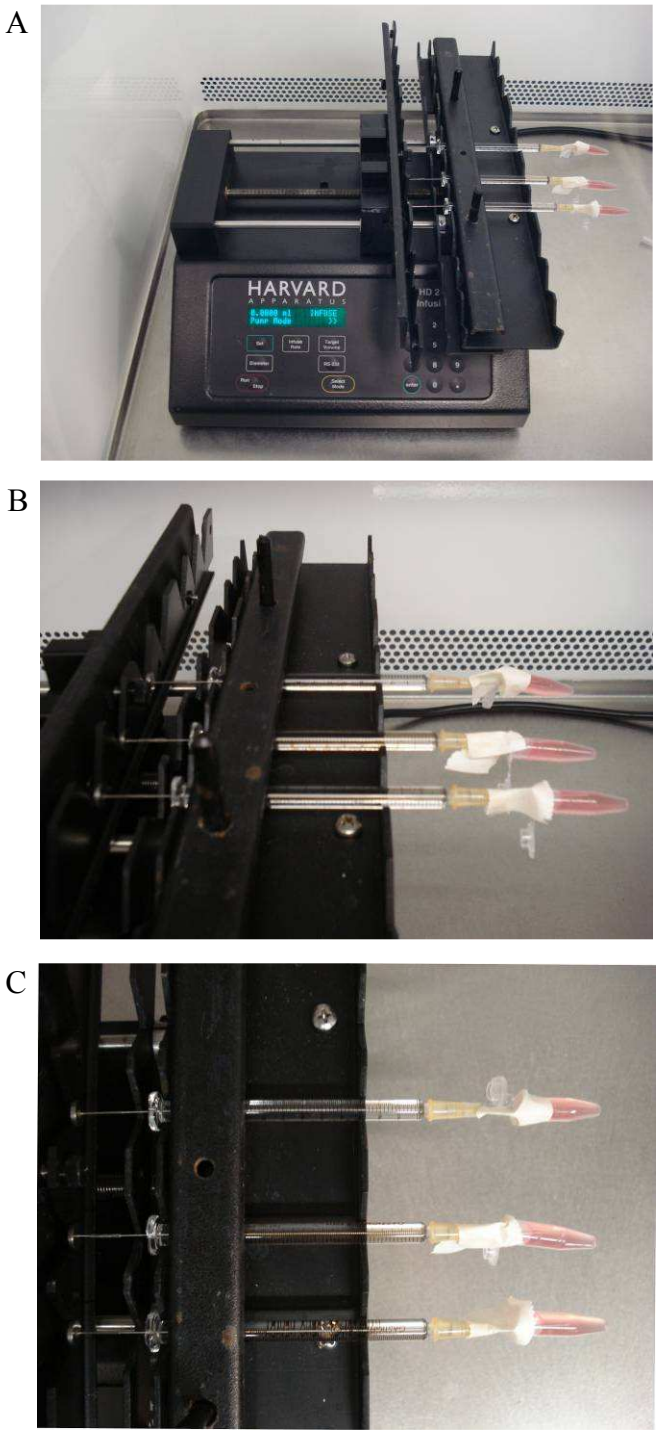


Figure 3.2. Experimental setup of syringes on pump. A general view of the apparatus (A), as well as focused side (B) and top (C) views of the syringes are provided. The Harvard® PHD 2000 syringe pump was sterilised using 70 % industrial methylated spirit and placed within a class II safety cabinet. Luer-lock needles of various gauges were attached to Hamilton 1701LT syringes. Ten µl of cell suspension was then drawn up into the needle and syringe before placing the syringes on the syringe pump. Eppendorfs containing 500 µl were then taped to the end of the needle to ensure no cell suspension was lost during ejection. Programming of syringe diameter allowed ejection to occur at the required rate determined by µl/min, rather than mm/min.

One Solution assay as described in 2.2.3(data not shown). Taking this percentage attachment allowed one to ensure that the same number of cells were being analysed when comparing the cells seeded to the MPs to the unseeded cells.

3.2.5 - Syringe manipulation of the cell suspension

After the mMSCs were detached using trypsin (2.2.2), cell counts and viability determination to create the correct cell concentration were performed using the TB exclusion technique (appendix 4.3). The cells were then spun down at 250 g for 4 minutes, supernatant was removed, and cells were re-suspended in a volume of HBSS or HBSS with 60 mM *n*-acetyl cysteine (nAC), to provide a final cell concentration of 5×10^7 cells/ml. Ten µl samples of this final concentration were processed to provide a control. Samples suspensions were either drawn up into the syringe, with a 26s gauge needle attached (internal diameter 114 µm, appendix 6) at a rate of ~30 µl/min and left to stand for pre-defined time periods at room temperature, or transferred into sterile 500 µl eppendorfs and left to stand at either room temperature, or 4 °C. They were then either ejected at the controlled rate of either 5 µl/min or 1 µl/min, into 500 µl of full media (figure 3.2), or added to 500 µl of full media directly (for eppendorf samples).

3.2.6 - Cell viability and proliferation

For assessing viability, after the cells were manipulated as described in 3.2.4 and 3.2.5, the CellTiter AQ One Solution Cell Proliferation assay was used as described in 2.2.3.

For the cell proliferation study, mMSCs were ejected into the well of a 6-well-plate containing 2 ml of complete mMSC medium. After 24 and 48 hours, the cells are detached from the well plate surface as described in 2.4, centrifuged and re-suspended in 500 µl complete medium. 100 µl of this suspension was then analysed using the CellTiter AQ assay as described in 2.2.3.

3.2.7 - Caspase-3 activity assay

Programmed cell death was assessed using the commercially available CaspACE® Assay system (Promega, Southampton, UK). The apoptotic index of the cell line is defined by the ability of caspase-3 to cleave an Ac-(aspartate-glutamate-valine-asparate)-p-nitroaniline substrate and release the detectable chromophore, p-nitroaniline (pNA). Caspase-3 is a key protease up-regulated in the vast majority of apoptotic pathways, though cannot account for all apoptotic pathways[96].

The cells experienced the appropriate culture conditions (3.2.5) before being transferred to a fresh eppendorf containing 10 µl HBSS and being treated to 3 freeze-thaw cycles to induce cell lysis. Cell debris was removed by centrifugation at 15,000 g for 20 minutes, at 4 °C. Clarified supernatant samples were incubated with the appropriate assay mixture, in a tissue culture incubator, for 4 hours before colorimetric analysis (405 nm) using a Bio-Tek KC4 plate reader. Apoptosis in the positive controls was induced using 1 µM of the protein kinase inhibitor staurosporine, whereas the irreversible and cell-permeable pan-caspace inhibitor, Z-VAD-FMK, was added (50 µM final concentration) to the negative control together with the staurosporine. The negative control values were subtracted from sample readings, and, using a standard curve (Appendix 5.2), the concentration of *p*-nitroaniline could be determined.

3.2.8 - Protein concentration

Protein concentration determination was performed in order to normalise the concentration of caspase-3 with respect to the level of total protein. Total protein was determined using the Bradford assay, as described in 2.2.9.

3.2.9 - Cell attachment and spreading assay

Cell attachment and spreading was assessed using the dual May-Grunwald and Giemsa staining technique for the cytoplasm and nucleus, respectively. After manipulating the mMSCs as described in 3.2.5, they were seeded on tissue-culture plastic at a density of 630 cells/mm^2. After allowing 2 hours for cells to attach and

spread within a tissue culture incubator, they were fixed with 3.7 % ($^{w}/_{v}$) paraformaldehyde, permeabilised by the addition of 0.1 % ($^{v}/_{v}$) Triton X-100, before staining with May-Grunwald (0.25 % ($^{w}/_{v}$) in methanol) and Giemsa stains (0.4 % ($^{w}/_{v}$) in methanol). Cells were then viewed at 200 × magnification using a Leica DM-IRB/E inverted microscope. Ten separate fixed-size, non-overlapping, random fields per sample were photographed with the in-built digital camera and the images subsequently analysed using Volocity® image software. Spread cells were distinguished and characterised based upon the presence of a clear halo of cytoplasm surrounding their nucleus, following the rearrangement within the actin skeleton, as previously described[97].

3.3 – Results

3.3.1 - Cell viability

Figure 3.3 documents the cell viability data following sample manipulation and assessment using the MTS assay. Regardless of the ejection rate, cell viability is significantly reduced from the 100 % control values, immediately, following ejection from the syringe needle (i.e. at the 0 hour time-point) ($p < 0.05$). No noteworthy differences in viability were observed when the ejection rate was altered or when nAC was present.

This observation is significantly different compared with the eppendorf system; a relatively smaller decrease in viability occurred over the 24 hour period ($p < 0.01$). After 4 hours, the cells stored at 4 °C decrease in viability at a significantly quicker rate compared to those left at room temperature, with less than half of the cells viable at 4 °C compared to 68 % at room temperature.

Figure 3.4 compares the viability of cells seeded onto PLGA MPs against single cell suspension post-ejection. Seeded cells onto MPs showed a significant decrease in viability compared to single cell suspension after 2 to 8 hours within the syringe chamber compared to the equivalent single cell suspension.

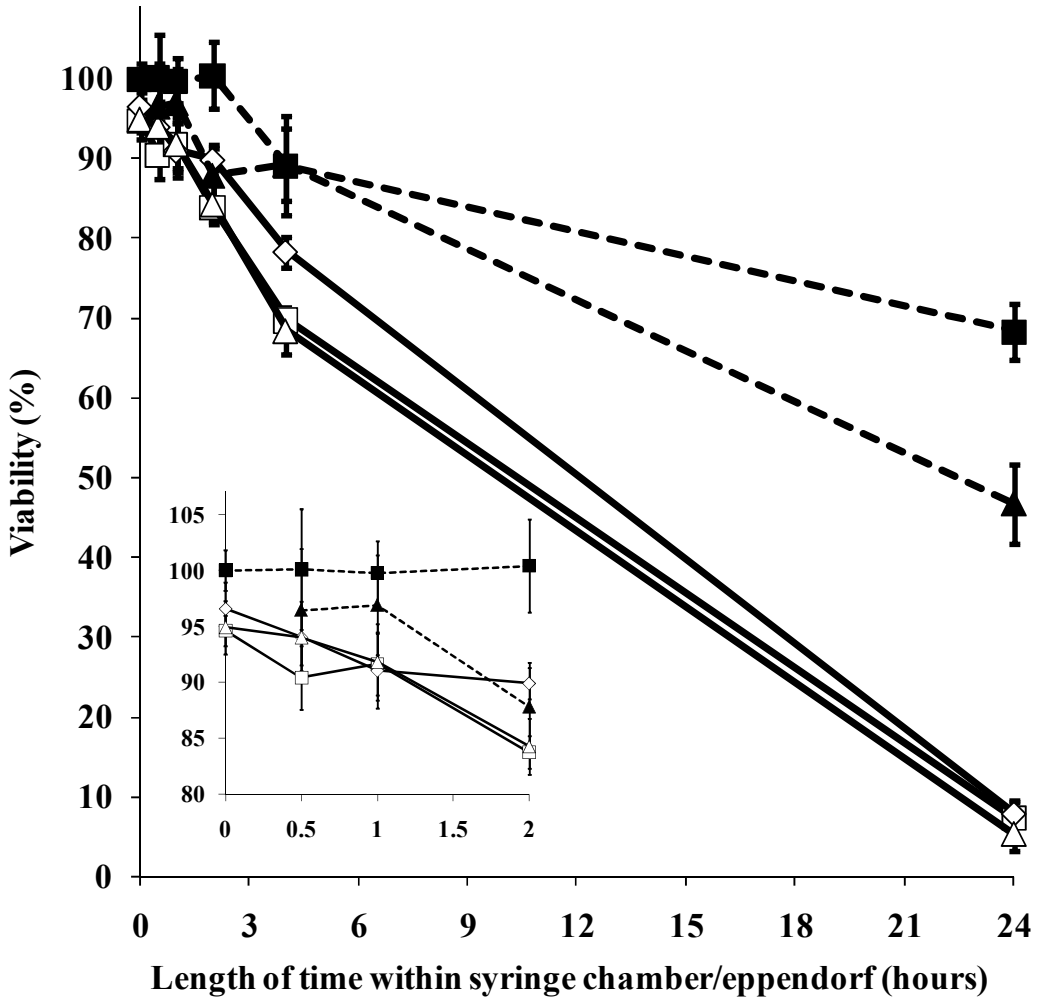


Figure 3.3. Viability of mMSCs following specific periods within either a syringe chamber (solid lines) or eppendorf (dashed lines). Cells within the syringe chamber were ejected at 5 µl/min (□), 1µl/min (◇) or 1 µl/min in the presence of *n*-acetyl cysteine (△). Cell within the eppendorf were kept at either room temperature (■) or at 4 °C (▲). Determined using the CellTiter AQ assay system (Promega). Results are the mean values ± SEM from 3 independent experiments, each having triplicate samples. Sample data were normalised against control values of 100 %. Superimposed graph focuses on the first 2 hours within either the syringe or eppendorf.

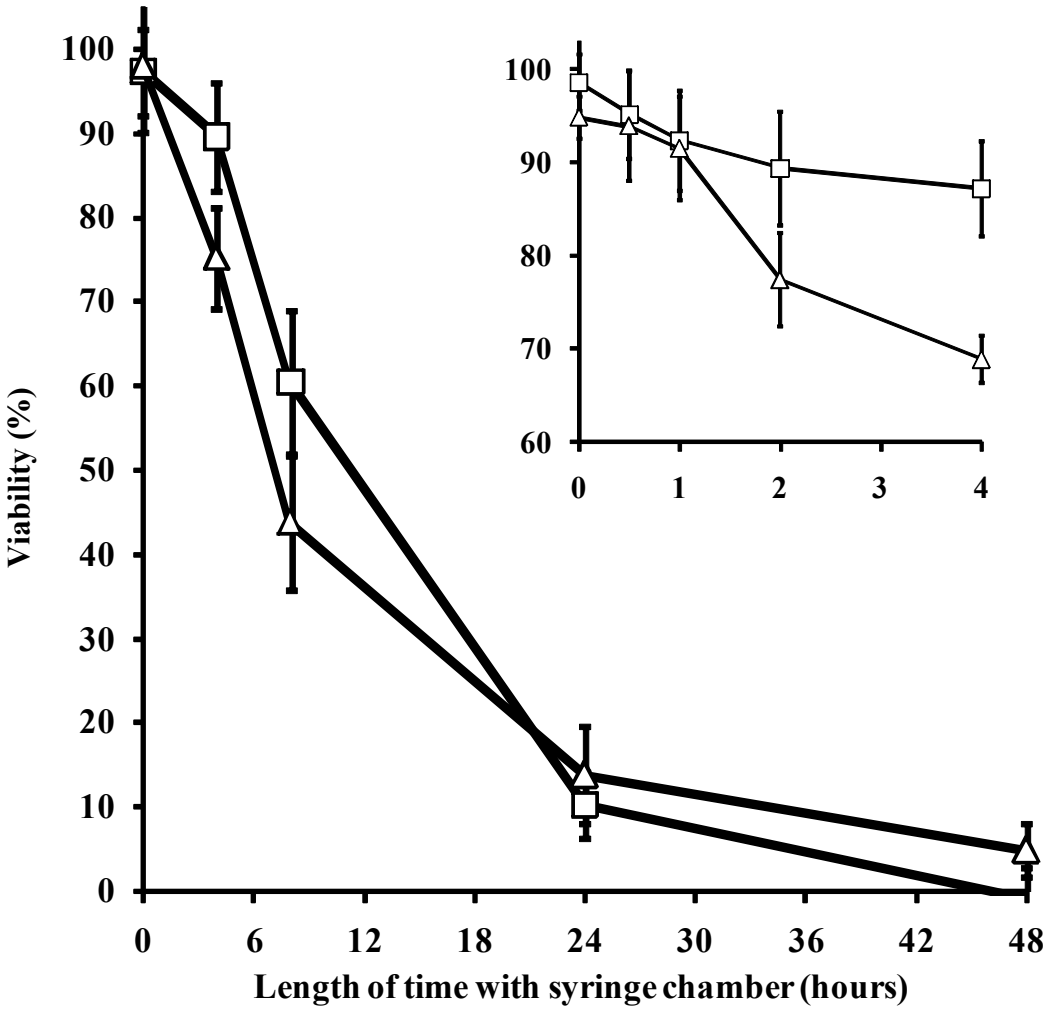


Figure 3.4. Viability of mMSCs following specific periods within a syringe chamber and ejection. Cells were drawn up into the syringe chamber either seeded onto PLGA microparticles (△) or as a single cell suspension (□). Determined using the CellTiter AQ assay system (Promega). Sample data were normalised against control values of 100 %. Superimposed graph represents a separate study focusing on the first 4 hours within the syringe.

Unlike what is observed in figure 3.3, there was no significant decrease in viability when either seeded cells or single mMSC suspension were ejected from syringes with a 22 gauge (394 μm diameter) needle attached.

3.3.2 - Apoptosis

Figure 3.5 shows an increase of caspace-3 activity with increased time as the cells were within the syringe chamber. On comparing the caspase-3 activity levels between the 5 μl/min and 1 μl/min ejection rates, the activity levels were significantly greater (at the 2 hour time-point onwards) for the higher ejection rate sample ($p < 0.05$), though this trend does not continue after 24 hours within the syringe. No significant differences were demonstrated between the 1 μl/min ejection rate samples supplemented with and without 60 mM nAC ($p < 0.05$) at all the time-points.

3.3.3 - Cell attachment and spreading

Representative images of attached and spread cells are presented in table 3.1 with figures 3.6 and 3.7 showing the capability of mMSCs to re-attach and spread on tissue culture surfaces, respectively, following stagnant periods within the syringe chamber. There was a significant reduction ($p < 0.05$) in the number of attaching mMSCs following the immediate ejection (0 hour time-point) and this decrease is maintained with increasing stagnant periods within the syringe chamber for both ejection rates ($p < 0.01$). The presence of 60 mM nAC caused a significant decrease in the number of cells attaching when the cells were subjected to 2 hours within the syringe ($p < 0.01$) as an almost complete loss of attachment was achieved. A similar end point was only reached following 4 hours within the syringe in samples ejected at 1 μl/min without nAC. In contrast, at the 5 μl/min ejection rate, over 30 % of the mMSCs were still able to attach after being within the syringe chamber for 4 hours.

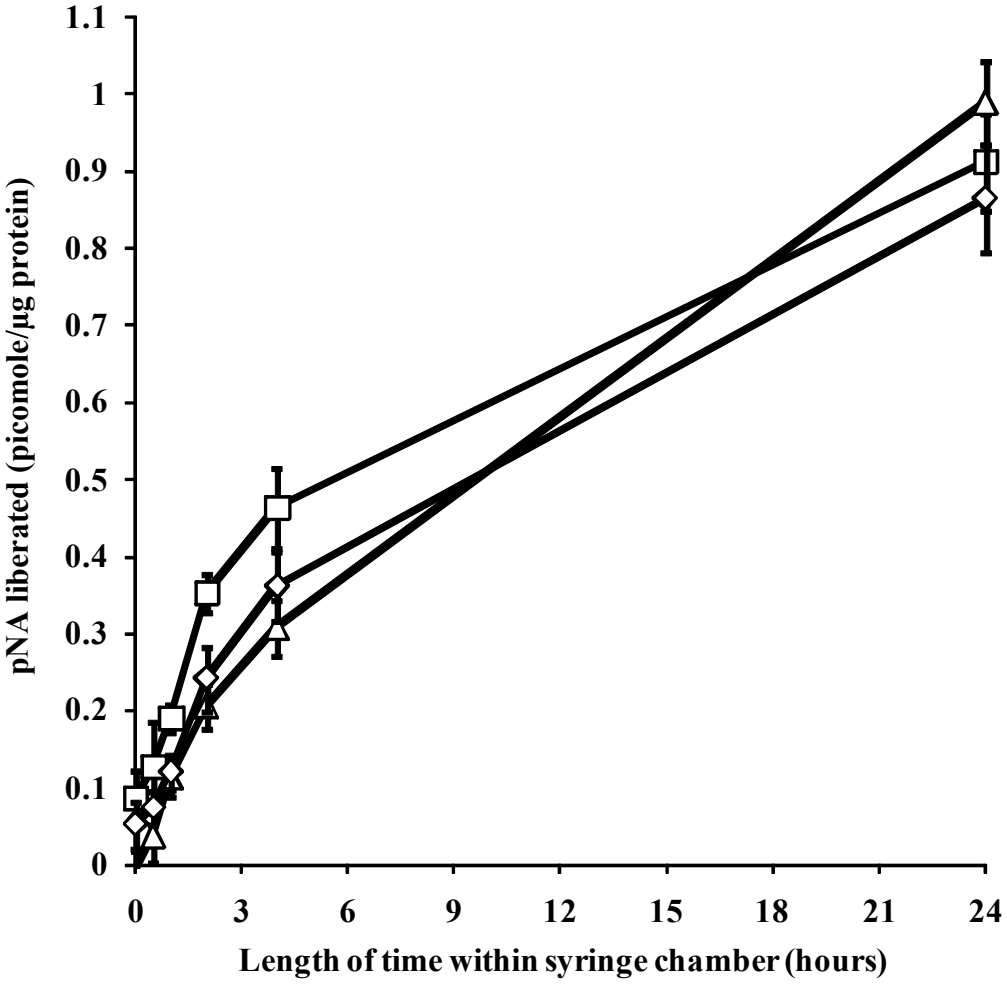


Figure 3.5. Caspase-3 activity levels of mMSCs following specific periods within the syringe chamber and ejection at 5 µl/min (□), 1 µl/min (◇) or 1µl/min in the presence of *n*-acetyl cysteine (△). Determined using the CaspACE assay system (Promega). Results are the mean values ± SEM from 3 independent experiments, each having triplicate samples. Sample data were normalised against control values.

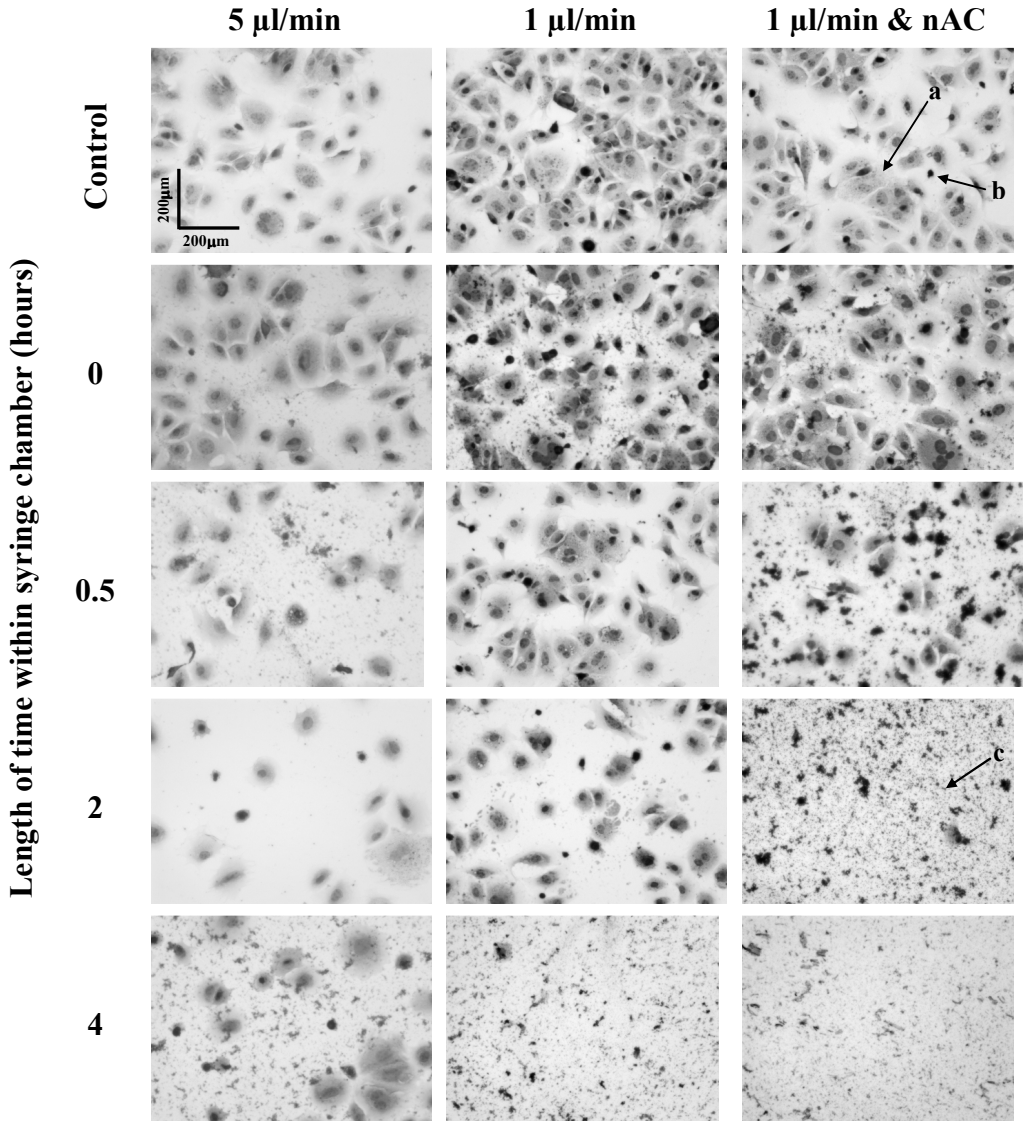


Table 3.1. Attachment and spreading of mMSCs following specific periods within the syringe chamber and ejection at 5 µl/min, 1 µl/min and 1 µl/min in the presence of *n*-acetyl cysteine. Following syringe manipulations, cells were re-cultured on tissue culture plastic, and placed within a tissue culture incubator for 2 hours to all the cells to attach. The cells were then fixed using 3.7 % ($^{w}/_{v}$) paraformaldehyde and stained with May-Grunwald and Giemsa before being viewed at × 20 magnification. 10 separate random fields per sample were imaged, showing examples of (**a**) spread cells (**b**) attached but unspread cells and (**c**) cell debris. nAC; *n*-acetyl cysteine.

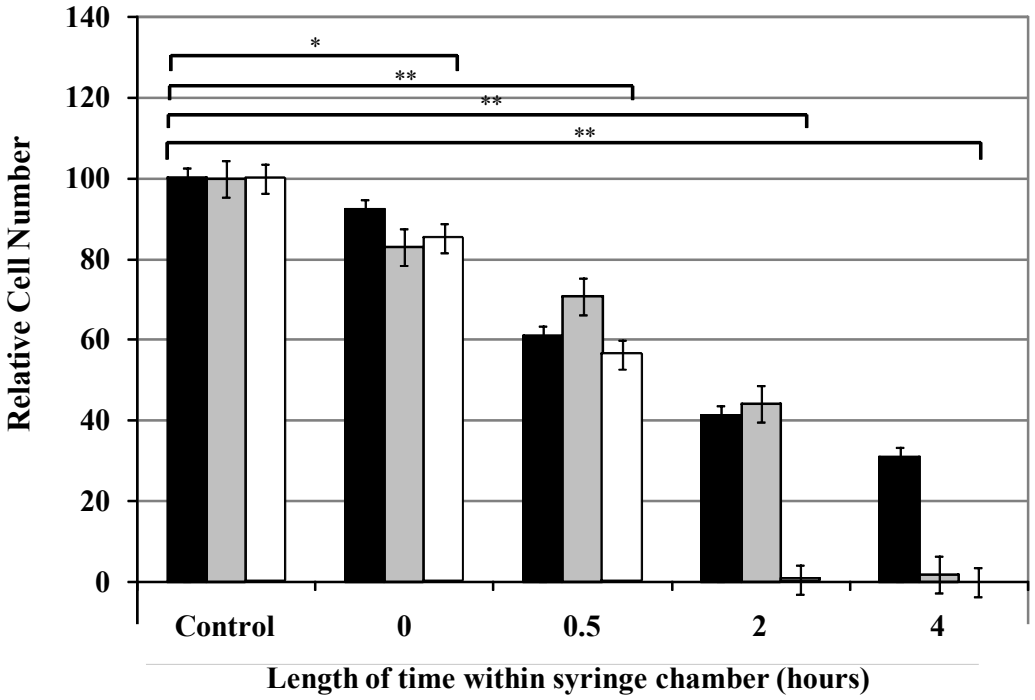


Figure 3.6. Attachment of mMSCs following specific periods within the syringe chamber and ejection at the rate of 5 µl/min (■), 1 µl/min (▒) and 1 µl/min with 60 mM *n*-acetyl cysteine (□). Ten images were taken from each of three samples. Sample data were normalised against control. Asterisks represent the level of significance compared to control sample (* where $p < 0.05$, ** where $p < 0.01$).

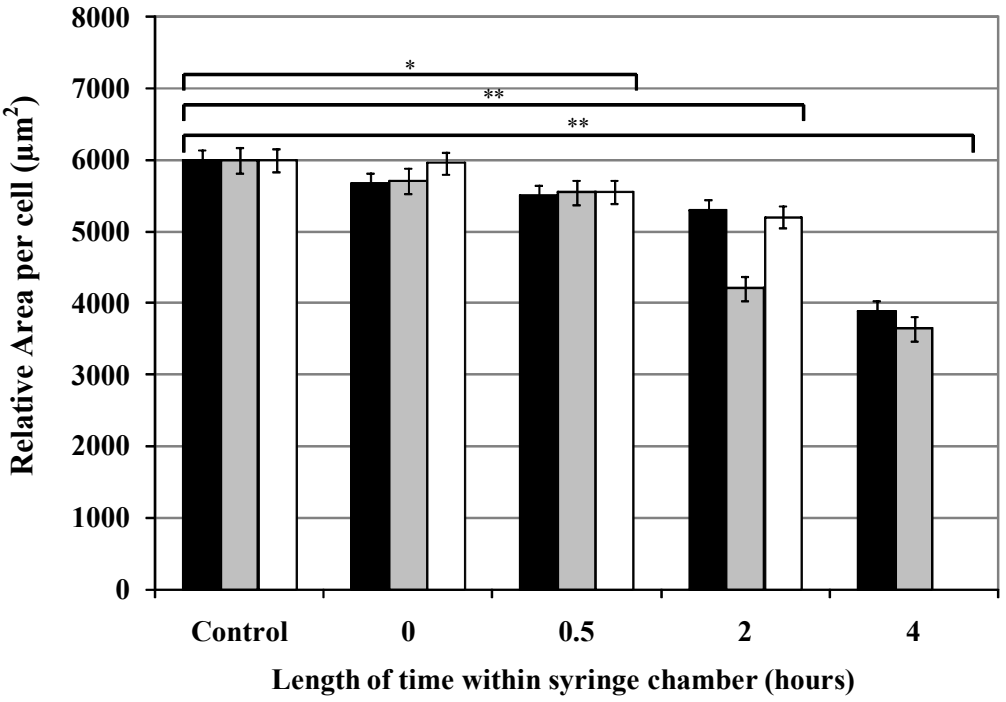


Figure 3.7. Extent of spreading of mMSCs following specific periods within the syringe chamber and ejection at a rate of 5 µl/min (■), 1 µl/min (▒) and 1 µl/min with 60 mM *n*-acetyl cysteine (□). Ten images were taken from each of three samples. Sample data were normalised against control. Asterisks represent the level of significance compared to control sample (* where $p < 0.05$, ** where $p < 0.01$).

Although the attached mMSCs number decreased as the time period within the syringe chamber increased, the *quality* of the cell spreading appeared to be unaffected based on the extent of area of spreading per cell (figure 3.7). A high total surface area per cell is characteristic of a positive degree of spreading. A significant decrease in spreading did not occur until after 30 minutes within the syringe chamber. After 4 hours, the bar representing the cell suspension containing nAC is not present, as, at this time-point, no cells were attached to assess the spreading of.

A characteristic with regard to cell spreading that is noticeable in table 3.1, but is not quantified in figure 3.6 or 3.7, is the apparent increase in cell debris when the cells are left within the syringe chamber for increasing periods of time. There was a visible increase in cell debris when the cells are drawn up into and immediately expelled, compared to control.

3.3.4 - Cell proliferation

The proliferative ability of the mMSCs that were still viable following time within the syringe chamber and ejection at 5 μl/min were not significantly affected ($p < 0.05$) until after 8 hours compared against control (figure 3.8) (the proliferation trend for cells left within the syringe chamber for 24 hours was not fully plotted, as the negative cell concentrations obtained could not be plotted on a logarithmic scale). On increasing the time period within the syringe chamber, the doubling time (t_d) of the mMSCs increased, but not significantly (table 3.2).

3.4 – Discussion

The overall aim of this study was to investigate the cellular response of mMSCs following their manipulation and varying time period within a syringe-based delivery system. “Clinical success” of current cell therapy applications is often based upon the analysis of post-operative response i.e. restoration of function, tissue integration and/or cell localization[63]. However, for the majority of these cases, cells are usually only tracked (using a variety of dyes or contrasting

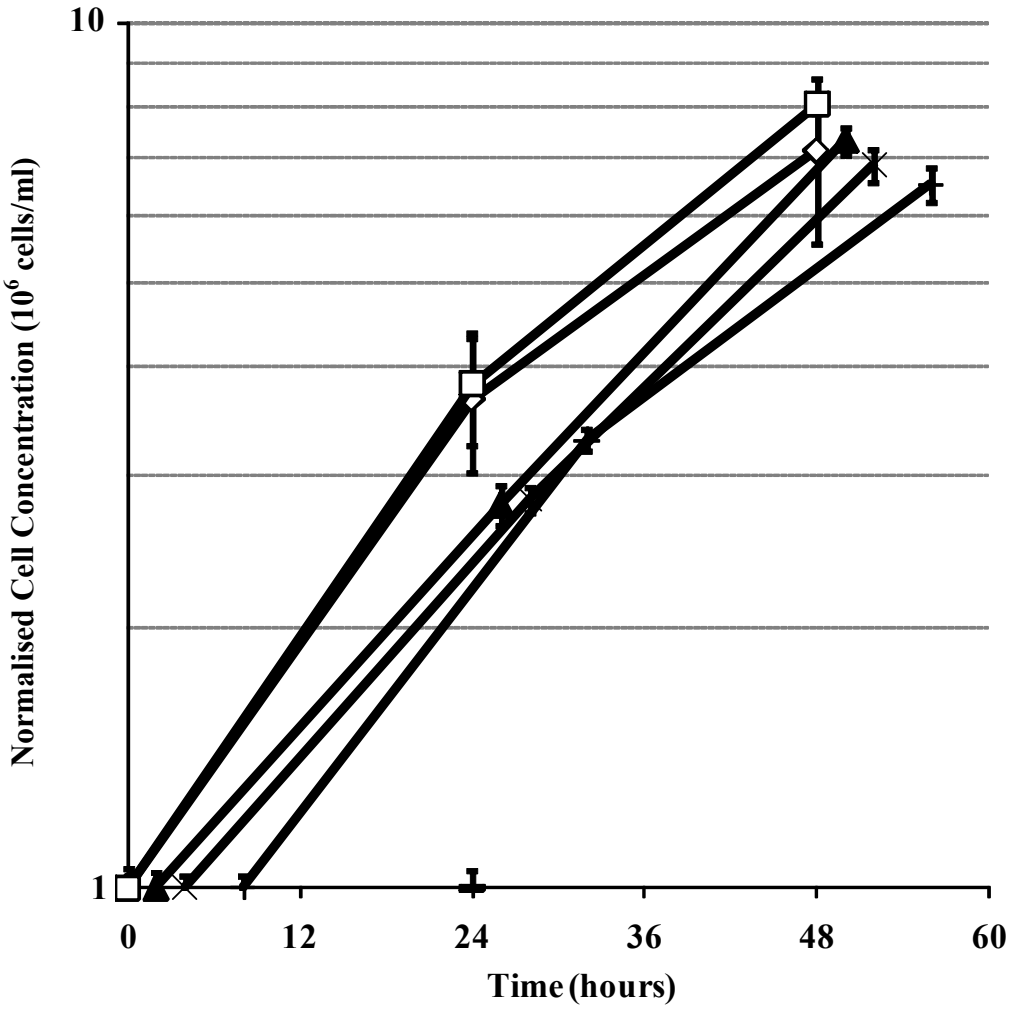


Figure 3.8. Proliferative count of mMSCs under favourable conditions following specific periods within the syringe chamber and ejection at 5 µl/min. Cells were left to stand within the delivery device for 0 (□), 2 (▲), 4 (×), 8 (+) and 24 hours (-) before being re-cultured on tissue culture plastic. Proliferation rates were determined using the CellTiter AQ assay system (Promega). Results are the mean values ± SEM from 2 independent experiments, each having triplicate samples. Sample data were normalised against control (◇) and plotted on an exponential axis.

Length of Incubation within syringe (h)	T_d (h)
Control	16.0
0	15.1
2	16.5
4	17.1
8	17.1
24	N/A

Table 3.2 Mean doubling rates (T_d) of mMSCs following specific periods of neglect and post-ejection at 5 µl/min.

agents)[65,66,67] for a brief period post-injection and there have been few recent studies investigating the actual effect of passing a cell suspension through a needle-based delivery device.

This study could be considered as a development of work published by Kondziolka and co-workers; they assessed the viability of neuronal cells passed through a 25 gauge needle and cannula using a simple trypan blue exclusion method[69]. Even though this work outlined a potential issue with delivering cells parenterally by showing a significant decrease in viability post-ejection, the method used to analyse viability has been reported to be inaccurate, due to the low number of cells analysed[98]. Furthermore, no assessment of whether the remaining viable cells post-ejection were functional. For these reasons, further investigation was required.

An informative finding from this study was that there was a significant (albeit small) decrease in viability when mMSCs were immediately ejected once drawn up into a syringe attached. This suggests that the shear stresses the cells experience as they are drawn through the narrow bore have a key detrimental effect on cell integrity. This is supported by the lack of significant viability decrease when the cells were drawn through a wider bore, or placed within an eppendorf. It could be argued that the decrease in viability could be attributed to a reduction of total cell number due to the volumetric loss of cell suspension in coating the internal surfaces of the needle and delivery device. However, this seems unlikely, as, if this were the case, one would expect to see the same or greater decrease in viability as the cells were passed through the wider bore needles (as the internal surface area of a wider bore would be greater). Moreover, if cells were lost in coating the syringe surface, this may be an issue that researchers would like to quantify when carrying out clinical studies.

Within the eppendorf study, cells were kept at either room temperature or 4 °C, in order to investigate whether temporary storage at reduced temperature would

prolong cell survival if a clinical procedure were delayed. A similar study to this has been carried out using similar marrow stromal cells (though human-derived), with the aim of analysing the effect of short-term cell transportation on viability[81]. This study was also similar to the one described here in that they also compared storage at room temperature to storage at 4 °C. Their results showed that storage of low passage (passage 3) hMSCs at 4 °C, at a concentration of 2×10^7 cells/ml (similar to the concentration of 5×10^7 cells/ml used in eppendorf study), had a similar effect on cell viability; after 24 hours of storage, they reported a loss of viability of 40 %, compared to the 50 % decrease shown in figure 3.3. This comparison suggests that the passage number and the species the cells are derived from have little influence on their tolerance to storage under such conditions.

As documented by our experimental results, incubation at a reduced temperature after 4 hours decreased cell viability compared to room temperature. Such an observation has been reported by Ma and co-workers[99] who analysed the effect of the preservation of human haemopoietic stem cells at 4 °C on viability. The study by Lane *et al* also showed no significant difference between storage at 4 °C compared to storage at room temperature[81].

When comparing a single mMSC suspension against cells seeded onto PLGA MPs (figure 3.4), the expected improvement in viability in the seeded cells did not occur, and, on the contrary, there was a significant decrease in viability within the seeded cells at some time-points. Compared to the detachment of mMSCs from tissue culture plastic, using trypsin that cleaves their surface-attachment proteins, the MPs were thought to allow the attachment proteins to re-grow and attach to a surface under favourable conditions before delivery. However, one issue that was not foreseen regarding MPs when delivering a highly concentrated cell suspension was the increase of solid mass that could increase the shear forces experienced by the cells whilst passing through the narrow bore. The seeding was found to be 15 cells per MP on average; if the amount of seeding was increased per MP, the

number of MPs required could be reduced and, in theory the shear stresses could be reduced. This may, in turn, improve cell viability.

With regard to the amount of time the mMSCs were within the syringe chamber, it was found that the longer this time period, the lower the viability of the cells following recovery in culture media. Such an effect may be attributed to a variety of culture conditions known to induce apoptosis, i.e. aeration levels, nutrient deficiency, toxic metabolite build-up and cell signaling[92,100,101,102]. However, there was little change in viability in the eppendorf system despite the cells being exposed to similar stresses. It is also plausible that the passage of cells through an extremely narrow channel and/or at a high rate may induce apoptosis- initiating the controlled cell death pathway and also contributing to the downstream decrease in viability state upon the point of ejection and analysis.

It has also been demonstrated that nAC provided no significant beneficial effects in cell viability compared to the non-supplemented control. Furthermore, no significant changes were detected in the corresponding caspase-3 activity ($p < 0.05$). Such an observation was found to be of contrast to the findings reported by Muscari and co-workers who specifically demonstrated an inhibition of apoptosis in rat MSCs[103]. Although other researchers have also demonstrated a similar effect of nAC supplementation (via the stimulation of glutathione synthesis and reduction of hydroxyl radicals), this attribute is very dependent on the cell-line involved[104,105,106,107,108]. It is also possible that, due to caspase activation being a later event within the apoptotic cascade, the beneficial effects of nAC were not seen because the control was within an early, and therefore undetectable state of apoptosis. This could be assessed by detecting phosphatidylserine exposure on the outer surface of the cell membrane (which is classed as an earlier apoptotic event), using annexin V[109].

Referring to figures 3.7 and 3.8, it is seen that the majority of cells that survive the syringe-based manipulations, after a short period of time within the syringe, tend

to function with regard to their ability to spread and proliferate. As cells would need to attach before proliferating[110,111,112], the fact that cells that were within the syringe chamber for up to 8 hours were able to proliferate at a rate similar to control suggests that a greater number of cells would have attached to the tissue culture substrate than observed in figure 3.6 if provided with a lengthier time period in which to attach. However, this attachment time needed to be low in order to ensure that the cells did not proliferate, which would have led to an increase in the recorded attached number.

As such, it may be suggested that the manipulation of mMSCs within a needle-based delivery device partially induces a delay on cell recovery. This delay may be required for the initiation of basic cellular repair mechanisms and, consequently, reduce the degree of cell attachment, spreading and proliferation on a culture substrate. Such behaviour is characteristic of apoptosis and, as such, these physical insults may cause the appropriate cellular mechanisms and energy diversion to initiate the controlled death programme. At greater shear stress levels (longer time periods within the syringe), these negative effects become more pronounced and, in situations of extended stress, cell death via membrane lysis occurs- explaining the presence of the cell debris, shown in table 3.1. Furthermore, in the presence of nAC, there was a compounded negative effect on the mMSCs recovery and downstream abilities in the majority of culture conditions.

3.5 – Conclusions

In summary, it has been shown that the manipulation of mMSCs within a syringe-based delivery device sensitises the cells by inducing significant effects on their cellular response namely, a reduction in cell viability, attachment and spreading characteristics and, to a lesser extent, proliferation rates. Moreover, the greater the cellular insult (i.e. a longer time period within the syringe chamber), the more pronounced and significant the effects on the recoverability of the cells. As such, evidence is presented suggesting that the manipulation process may not only induce cell death via necrosis but, also induce apoptosis and that this programmed

death cycle cannot be prevented by the addition of the anti-oxidising agent, nAC, nor the provision of a suitable attachment surface. Thus, for potential clinical cell therapy applications, it is important to consider the preparation and manipulation of the cell suspension and also to account for the viability and cellular response of these cells post-ejection.

4

Flow cytometric analysis of mesenchymal stem cell viability post-ejection

4.1 - Introduction

The results from Chapter 3 raise questions regarding how altering the parameters related to the syringe-based manipulations may affect cell viability. Three key questions raised are:-

- How does the needle bore diameter affect viability? Previous results suggest a key difference in viability when cells are drawn up and immediately ejected from a 22 gauge to a 26s gauge. Further analysis of these needle gauges was performed, incorporating studies using a 25 gauge needle also (corresponding internal diameters for these needle gauges can be found in appendix 6).
- How does varying the concentration of nAC affect viability? The concentration of nAC used in chapter 3 was based on the concentration used in Bible *et al*[90], but the rationale behind why this concentration was used was unclear. For this reason, various nAC concentrations were compared, with the aim of finding an optimum concentration.
- How does varying the ejection rate affect viability? There were no noteworthy differences between cells ejected at 5 µl/min compared to 1 µl/min, but both were relatively slow rates. The effects of the quicker ejection rate of 20 µl/min was assessed.

Flow cytometric analysis could be argued to be a more accurate method to use to determine a level of viability. This is because metabolic assays, such as the one

used in Chapter 3, assesses viability through detecting mitochondrial activity, and assumes that each viable cell has a constant level of mitochondrial activity, therefore a decrease in signal from such an assay could be caused by not only a decrease in viable cell number, but also a decrease in metabolic activity. However, flow cytometry scans a sub-population of cells and provides a discrete number of viable cells, based on the characteristics of the dye used, so, assuming that the signal of a cell with low metabolic activity is detectable, the level of viability within a cell population determined using flow cytometry should not be affected by metabolic rate. Furthermore, flow cytometry provides information with regard to cell size and cell granularity, which can be related to whether a cell is undergoing apoptosis, and so provides additional information useful to this study. For the reasons above, it was decided that flow cytometry would be used as an additional analytical tool to assess the effect of syringe-based manipulations on cell viability.

4.2 – Materials and methods

4.2.1 - The effect of needle gauge and ejection rate

Murine MSCs were isolated and cultured as described in 2.2.1 and 2.2.2 respectively. After cell detachment using trypsin (2.2.2), cell counts and viability determination to create the correct cell concentration were performed using the trypan blue (TB) exclusion technique (appendix 4.3). The cells were then pelleted at 250 g for 4 minutes, supernatant removed, and re-suspended in HBSS to provide a final cell concentration of 5×10^7 cells/ml. Sample cell suspensions were then drawn up into syringes with either a 26s, 25 or 22 gauge needle (appendix 6) attached and ejected at a rate of either 20, 5 or 1 µl/min, into 500 µl of complete media, as shown in figure 3.2. Following the expulsion, samples were incubated for 30 minutes at room temperature in 1 ml Live/Dead® working solution, protected from light, before being analysed using flow cytometry as described in 2.2.5.

The Live/Dead® solution consists of calcein AM, which yields green fluorescence upon cleavage by active cell esterase, detecting viable cells, and a membrane impermanent ethidium homodimer, that fluoresces red after binding to nucleic acids.

4.2.2 - The effect of *n*-acetyl cysteine concentration

After mMSC detachment using trypsin (2.2.2), cell counts and viability determination to create the correct cell concentration were performed using the TB exclusion technique (appendix 4.3). The cells were then spun down at 250 g for 4 minutes before the supernatant was removed and the cells were re-suspended (5×10^7 cells/ml) in HBSS or HBSS supplemented with 20, 60 or 100 mM *n*-acetyl cysteine (nAC). These cell suspensions were then drawn up into syringes, left to stand for 2 hours (in order to induce partial cell death, so that the positive influences of nAC could be determined) before being ejected, via a 26s gauge needle, at 1 µl/min into 500 µl of complete media. Following the expulsion, samples were incubated for 30 minutes at room temperature in 1 ml Live/Dead® working solution, protected from light, before being analysed using flow cytometry as described in 2.2.5.

4.2.3 - The effect of sustained incubation

After mMSC detachment using trypsin (2.2.2), cell counts and viability determination to create the correct cell concentration were performed using the TB exclusion technique (appendix 4.3). The cells were then spun down at 250 g for 4 minutes, supernatant was removed, and cells were re-suspended in a volume of HBSS or HBSS with 60 mM *n*-acetyl cysteine (nAC), to provide a final cell concentration of 5×10^7 cells/ml. Samples suspensions were then drawn up into the syringe and left to stand for pre-defined time periods at room temperature before being ejected through a 26s gauge (internal diameter 114 µm, appendix 6) needle into 500 µl of mMSC media. These cells were then stained with mitotracker® deep red, as described in 2.2.5, before being analysed using flow cytometry as described in 2.2.6.

In a separate study, the concentrated cell suspension was separated into 10 µl aliquots, then left to stand for pre-defined time periods at either room temperature (18-20 °C) or 4 °C. Samples were then incubated with a mitotracker® solution (2.2.4), before again being analysed using flow cytometry (2.2.5).

4.3 – Results

4.3.1 - The effect of needle gauge and ejection rate

Table 4.1 and figure 4.1 summarise the flow cytometric analyses of cells once ejected at different ejection rates and through a variety of needle gauges. On comparing cell size and cell granularity, two distinct sub-populations are visible; large, clear cells (the events near to the x axis) and small, granular cells (the events near the y axis). These sub-populations correspond closely with the fluorescence data obtained; green fluorescent events correlated with the large, clear cells, and red fluorescent events were generally the small and granular cells. There was a visible increase of non-viable cells with the 26s gauge needle attached, as shown in table 4.1, but, as shown in figure 4.1, there were significant decreases in viability in both the 26s and the 25 needle gauges ($p < 0.05$) compared against control. Moreover, a noticeable level of cell debris (detected as events with a very low level of forward scatter, and therefore, of a small diameter) was seen with the 26s needle gauge, though this level of debris could not be quantified in terms of cell number (data not shown). No significant differences were observed between the various ejection rates.

4.3.2 - The effect of *n*-acetyl cysteine concentration

In table 4.2, a higher number of non-viable cell events were observed for samples left within the syringe chamber for 2 hours before ejection. At both the 20 mM and the 60 mM nAC concentrations, an insignificant increase in viability was observed compared to the control sample (figure 4.2). When a nAC concentration of 100 mM was used, all of the cells were found to be non-viable.

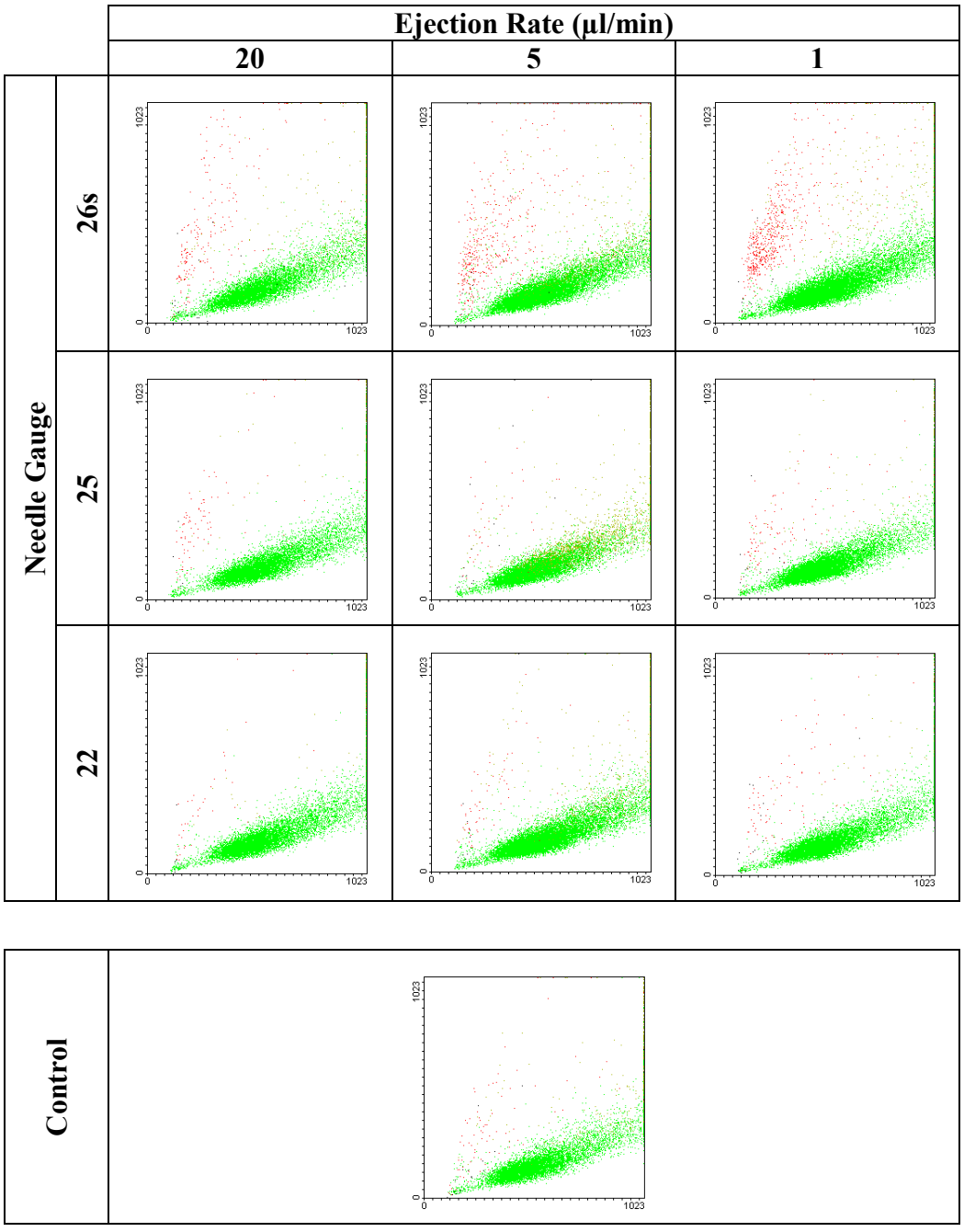


Table 4.1. Flow cytometry scatter plots comparing mMSC size (x-axis) against granularity (y-axis), post-ejection, at different rates through a variety of needle gauges. Cells were immediately expelled once drawn up into the syringe device. Following the expulsion, samples were incubated for 30 minutes with Live/Dead® solution (calcein AM/ethidium homodimer) according to the manufacturers' instructions before being analysed using a Beckman Coulter Cytomics FC500 flow cytometer (High Wycombe, UK) at 60 µl/min. Samples were analysed using WinMDI with viable events represented in green and non-viable events in red.

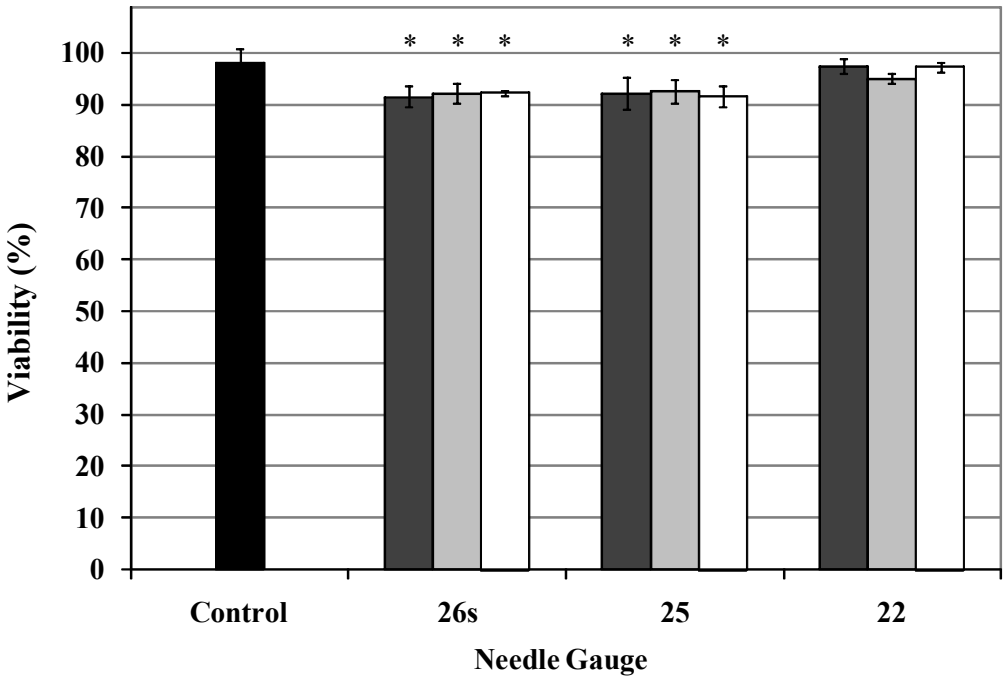


Figure 4.1. mMSC viability post-ejection, at different rates through a variety of needle gauges. Cells were immediately expelled once drawn up to into the syringe device at an ejection rate of either 20 (■) 5 (▒) or 1 (□) µl/min and compared against control (■). Following the expulsion, samples were incubated for 30 minutes with Live/Dead® solution (calcein AM/ethidium homodimer) according to the manufacturers' instructions before being analysed using a Beckman Coulter Cytomics FC500 flow cytometer (High Wycombe, UK) at 60 µl/min. Results represent mean percentage viability ± SEM. Asterisks represent the level of significance compared to control sample ($p < 0.05$).

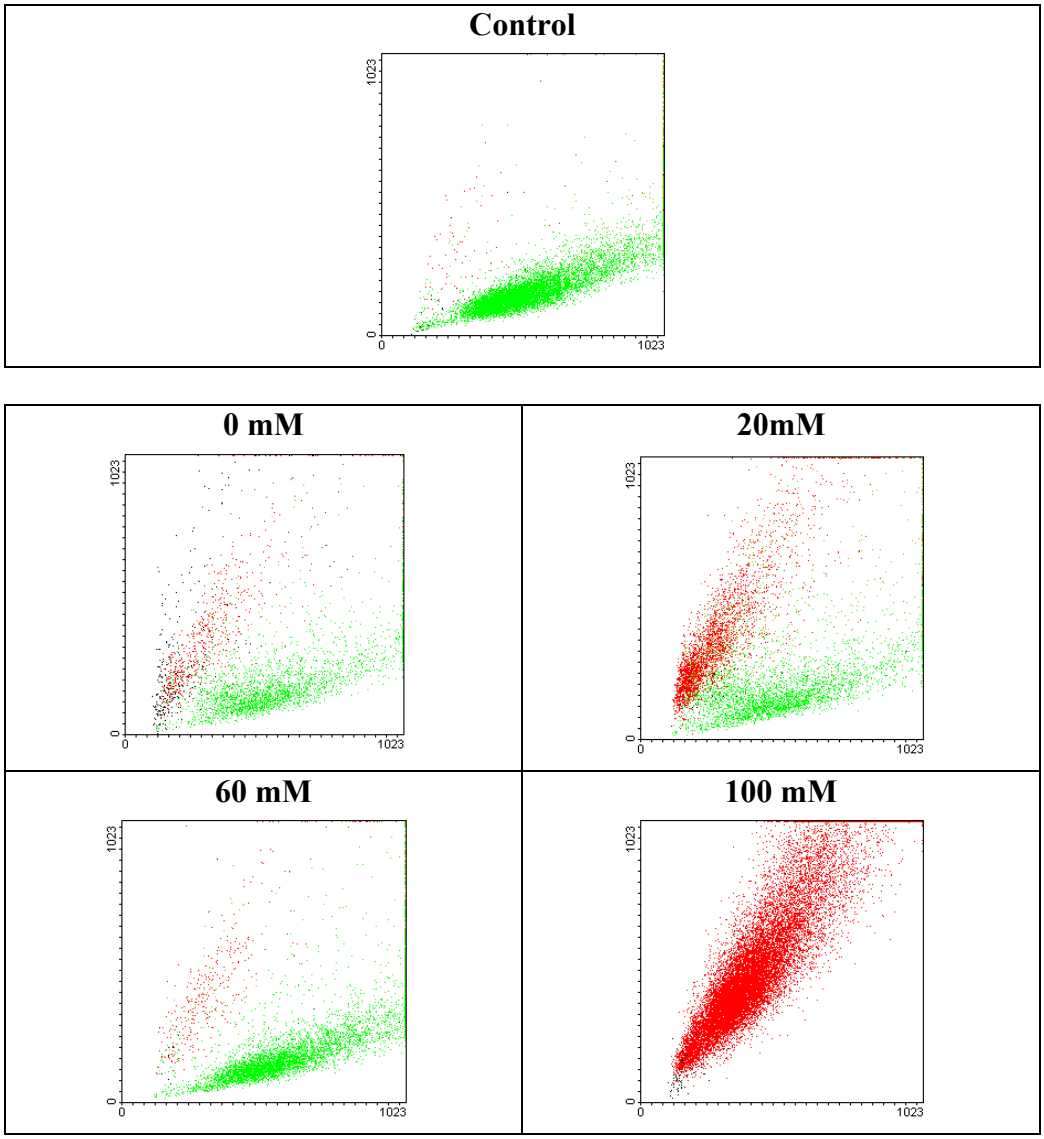


Table 4.2. Flow cytometry scatter plots comparing mMSC size (x-axis) against granularity (y-axis), post-ejection, within a suspension containing various concentrations of *n*-acetyl-cysteine. Sample cells were left to stand within the syringe chamber for 2 hours before being ejected at 1 µl/min via a 26s gauge needle, whilst the control sample was transferred for staining without incubation at room temperature or ejection. Samples were incubated for 30 minutes with Live/Dead® solution (calcein AM/ethidium homodimer) according to the manufacturers' instructions before being analysed using a Beckman Coulter Cytomics FC500 flow cytometer (High Wycombe, UK) at 60 µl/min. Samples were analysed using WinMDI with viable cells stained green and non-viable stained red.

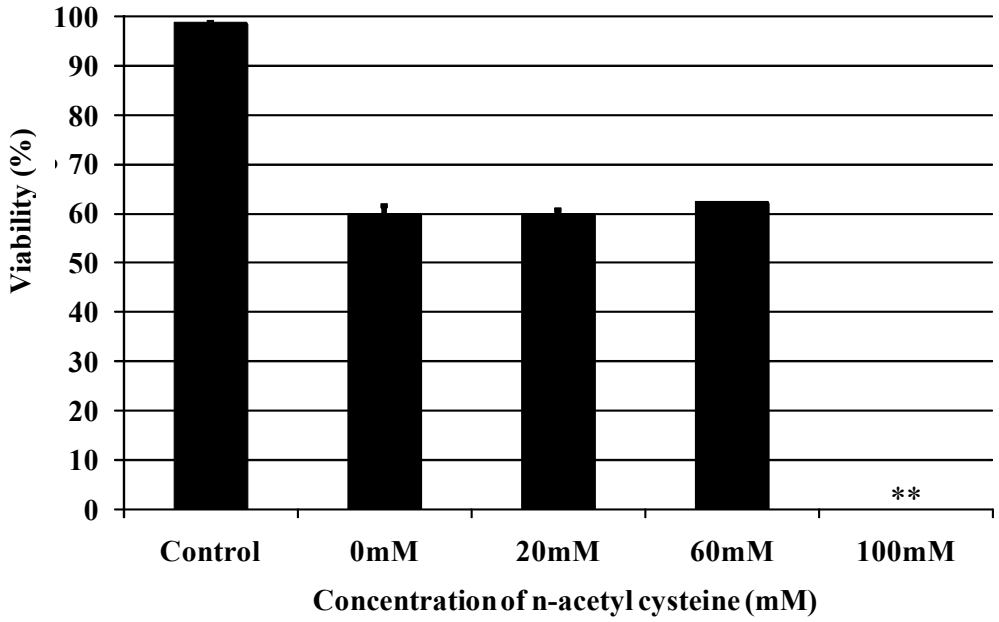


Figure 4.2. mMSC viability post-ejection, within a suspension containing various concentrations of *n*-acetyl-cysteine, compared to control. Following the expulsion, samples were incubated for 30 minutes with Live/Dead® solution (calcein AM/ethidium homodimer) according to the manufacturers' instructions before being analysed using a Beckman Coulter Cytomics FC500 flow cytometer (High Wycombe, UK) at 60 µl/min. Results represent mean percentage viability ± SEM. Asterisks represent the level of significance compared to control sample ($p < 0.01$).

4.3.3 - The effect of sustained incubation within the syringe chamber

Data summarised in table 4.3 and figure 4.3 compare cell diameter to the intensity of viable fluorescence for mMSCs following extended periods within a syringe chamber following expulsion at different ejection rates. The decrease in fluorescent intensity corresponding to the smaller cell-sized population may be explained by the fact that (i) smaller cells have less mitochondria and therefore have a lower maximum of dye that can be absorbed, or (ii) the cells have compartmentalised due to apoptosis and can subsequently absorb no dye. There was a rapid decrease in viability with increasing time periods within the syringe chamber and, at the 4 hour time-point, the majority of the MSCs were found to be non-viable. When comparing the differing ejection rates used, or the presence of nAC to without, no noteworthy changes were observed, and this agrees generally with the results discussed in Chapter 3.

4.3.4 – The effect of sustained incubation within the eppendorf

Table 4.4 shows very little change within the scatterplots for all time periods, and this is reflected in figure 4.3, which shows only a small decrease in viability over the 4 hour period. There was a significant decrease in viability only after 4 hours within the eppendorf at both room temperature and 4 °C, compared to control (time 0 in figure 4.3) ($p < 0.05$).

4.4 – Discussion

Within this investigation, the viability of mMSCs following their ejection through three different needle gauges was assessed; 26s, 25 and 22, which have the internal diameters of 114, 241 and 394 μm respectively (appendix 6). The selection of an appropriate needle gauge used during a cell therapy application is very much dependent on clinical parameters, namely the volume of fluid delivered, the size and fragility of the target location/organ, the number of cells required for a clinical effect etc. As such, various needle gauge sizes have been successfully demonstrated for medical applications, ranging from 22 gauge[113,114] to 25

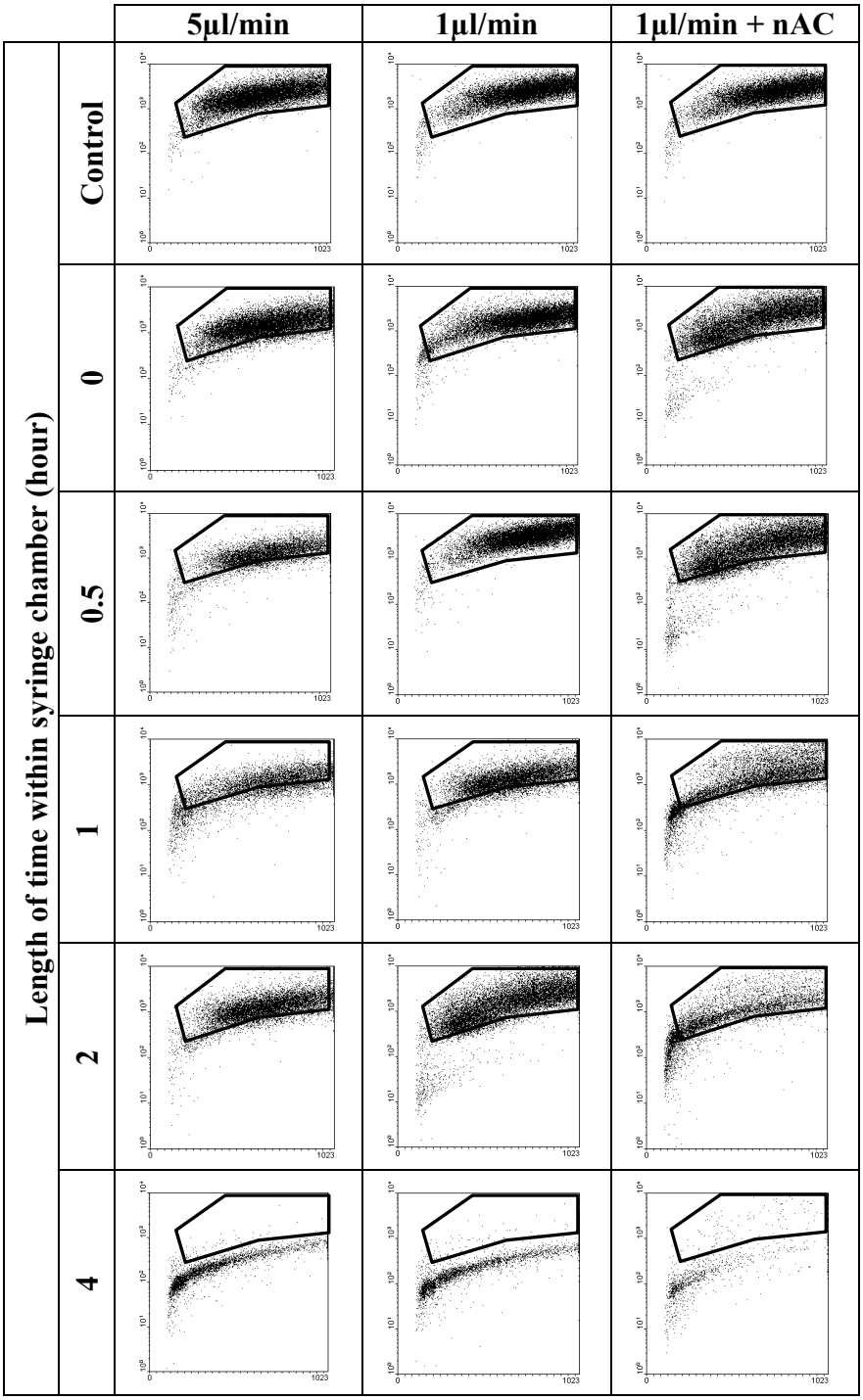


Table 4.3. Flow cytometry scatter plots comparing mMSC size (x-axis) against fluorescent intensity (y-axis) following specific periods within the syringe chamber at different ejection rates. At specific timepoints, samples were incubated mitotracker® deep red dye according to the manufacturers' instructions before being analysed using a Beckman Coulter Cytomics FC500 flow cytometer (High Wycombe, UK) at 60 µl/min. Samples were analysed using WinMDI with region of interest highlighted by the polygon. nAC; *n*-acetyl cysteine.

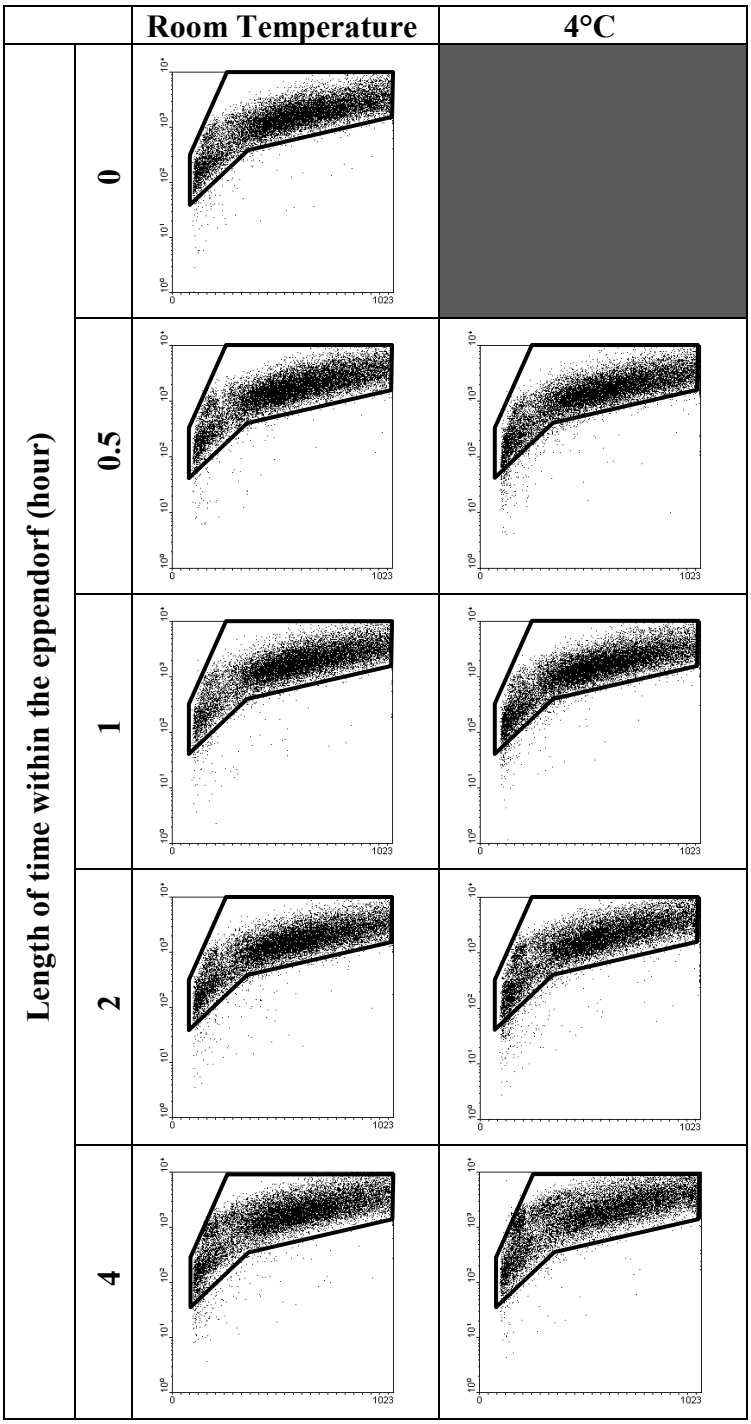


Table 4.4. Flow cytometry scatter plots comparing mMSC size (x-axis) against fluorescent intensity (y-axis) following specific periods within the eppendorf at either room temperature or 4 °C. At specific timepoints, samples were incubated mitotracker® deep red dye according to the manufacturers' instructions before being analysed using a Beckman Coulter Cytomics FC500 flow cytometer (High Wycombe, UK) at 60 µl/min. Samples were analysed using WinMDI with region of interest highlighted by the polygon.

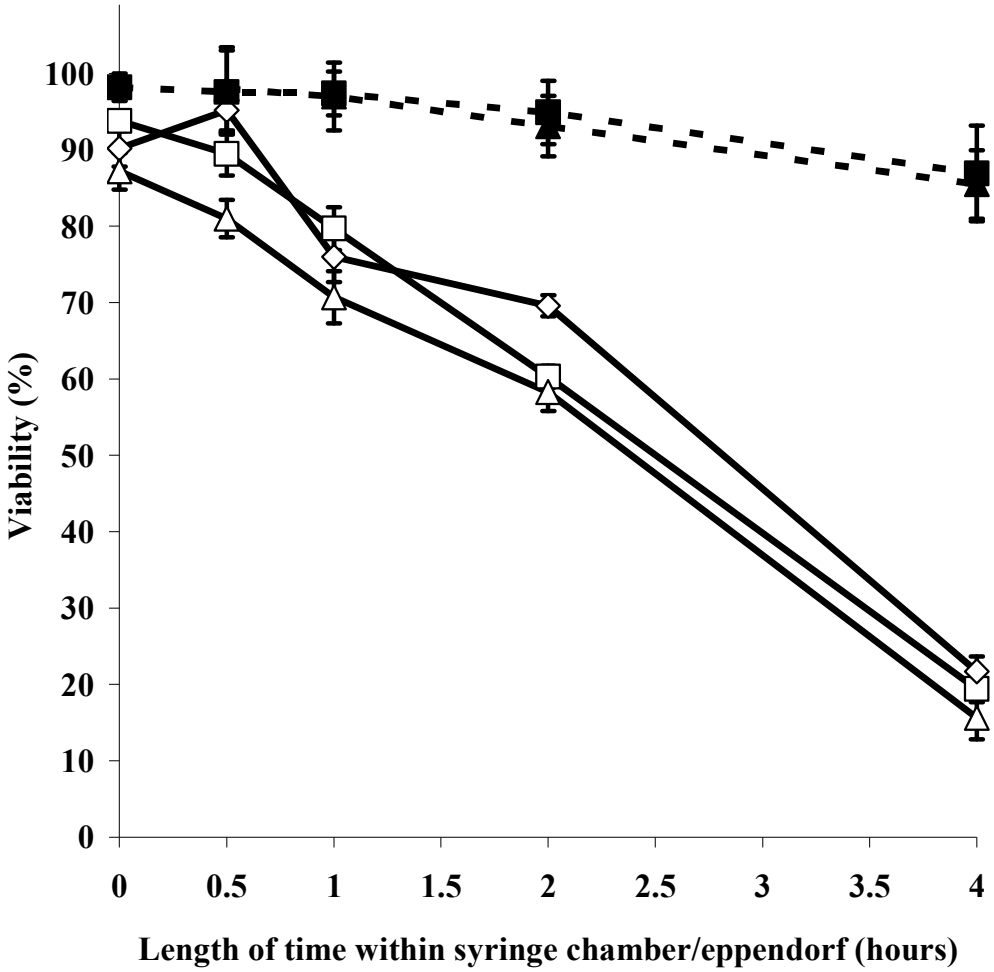


Figure 4.3. Viability of mMSCs following specific periods within either a syringe chamber (solid lines) or eppendorf (dashed lines). Cells within the syringe chamber were ejected at 5 µl/min (□), 1 µl/min (◇) or 1 µl/min in the presence of *n*-acetyl cysteine (△). Cell within the eppendorf were kept at either room temperature (■) or at 4 °C (▲). At specific timepoints, samples were incubated mitotracker® deep red dye according to the manufacturers' instructions before being analysed using a Beckman Coulter Cytomics FC500 flow cytometer (High Wycombe, UK) at 60 µl/min. Samples were analysed using WinMDI. Results represent mean percentage viability ± SEM.

gauge[69,115]. The narrow 26s gauge was used to minimise the quantity of residual suspension within the syringe following ejection.

Herein, we have demonstrated that mMSC viability significantly decreased upon immediate ejection from a 26s and a 25 gauge needle, but not following expulsion from a 22 gauge needle. This trend was identified for all the ejection rates studied.

As such, it may be speculated that the shear stress experienced during the expulsion process was a contributing factor for this reduction of cell viability and caused the reduction in cell number via direct cell insult and/or lysis. As shear stress is linearly inversely proportional to the area a substance is applied through[116], it could be deduced that the shear stress that a cell suspension experiences through a 26s needle is 12 and 4 times greater than that experienced through a 22 and 25 gauge needle respectively (appendix 6). However, when considering that, as a liquid moves from a wide bore (the syringe chamber) to a narrower bore (needle), the velocity at which the liquid moves increases, this would further increase the shear stresses the cells would experience within the narrower bores. Table 4.5 shows the shear stress values that a water solution, containing no cells, would experience when passing through the needles used in this study (26s, 25 and 22 gauge), at the ejection rates used in the study (mathematical derivation of the values can be found in appendix 7). When using the derived values from table 4.5, the cells would actually experience a shear stress that is 81 and 19 times greater than that experienced through a 22 and 25 needle respectively. The influential effect of the needle bore size on cell fate post-ejection was highlighted by Veraitch *et al* who found that 45 passages through a bore of 500 μm diameter were required before a significant decrease in viability was observed[68] (though the ESCs used in that study are smaller than the MSCs used here, and that would affect their results). Furthermore, Glossop and Cartmell reported the up-regulation of negative regulators of mitosis following induction under a controlled tensile strain and concluded that this increased strain may encourage an anti-proliferative transition[117].

		Ejection Rate (µl/min)		
		20	**5**	**1**
Needle Gauge	**26s**	1140.3	285.0	57.0
	25	60.8	15.2	3.0
	22	14.0	3.5	0.7

Table 4.5. Shear stress (mPa) experienced by water (without cells) as it is passed through needles of various gauges at a variety of ejection rates. Mathematical derivation of these shear stress is presented in appendix 7[118]

It is known that a higher ejection rate induces greater shear stresses to a cell suspension due to the increases in pressure and frictional forces within the needle. When comparing the shear stresses in table 5, it can be seen that there is a direct linear relationship between ejection rate and shear stress (i.e. the shear stress the cells would experience would be 20 and 5 times greater at ejection rates of 20 µl/min and 5 µl/min compared to 1 µl/min). However, no significant differences could be observed between the different ejection rates investigated here. A possible explanation could be based on how the samples were prepared; cells samples were loaded directly into the syringe via the needle at ~30 µl/min, which was at a higher rate than the highest ejection rate investigated. This loading rate was used to mimic the conditions during routine cell-therapy; often the ejection rate is controlled and slow in order to ensure minimal inflammation to the delivery site, but the loading rate is not controlled.

After finding no significant effect of nAC in improving cell viability or cell function in Chapter 3, it was important to explore whether the concentration of nAC was either too high or too low to elicit the beneficial effect that nAC has been reported to provide in the literature[103]. Table 4.2 and figure 4.2 show that concentrations below and above the 60 mM used in Chapter 3 provided no improvement in viability and, instead, a concentration of 100 mM was actually found to be toxic to the cells. However, when considering the mechanism by which nAC has been reported to provide its anti-apoptotic effect (not only through the direct interaction with oxygen-reactive species, but through the activation of glutathione)[91], one may speculate that nAC was not seen to be effective because an insufficient period of exposure to the cells occurred to significantly increase the levels of glutathione, and therefore, only the direct effects of nAC were being assessed. An effective way to assess this would be to culture the cells in media supplemented with nAC for 24 hours before the syringe studies were carried out.

When comparing the results shown in figure 4.3 and tables 4.3 and 4.4 against those shown in figure 3.3, it is intriguing to see such a level of agreement between

the different methods; they all show (i) a significant decrease in viability upon immediate ejection through the bore of a 25 gauge bore or narrower, (ii) a decrease in viability when cells are left within the syringe for increasing time periods compared to when left within an eppendorf, and (iii) minimal effects of nAC or ejection rate on cell viability. This suggests that cell death occurs via a pathway that initiates a whole host of alterations that can be detected. For example, there is a clear link between the changes in cell morphology and cell viability, as when cells undergo apoptosis, they shrink as they compartmentalise and become more granular as their cell membrane condenses[92]. However, there is a discrepancy in the rate of cell death within the syringe chamber, especially after one hour, as the Mitotracker detects a much quicker decrease in viability compared to the Celltiter colorimetric assay; reasons for this are unclear.

4.5 - Conclusions

Flow cytometric analysis confirms the results shown in Chapter 3; a similar decrease in viability occurred when mMSCs were left within the syringe chamber for prolonged lengths of time, and this viability decrease was more rapid when compared to cells left within an eppendorf. Increasing the ejection rate to 20 µl/min did not significantly decrease cell viability, yet increasing the needle gauge diameter to a 22g did significantly improve viability, suggesting that the shear stresses the cells experience through the syringe have a key impact on cell fate. Analysis of varying nAC concentrations on cell viability suggests that the antioxidant is not able to provide any beneficial effects at any concentration.

5

The identification of a toxin adsorbent that could be utilised to transport cell therapy products

5.1 - Introduction

There will be a need for effective transportation methods for cell-based products if, in the future, allogeneic cell therapies are used routinely in the clinic to treat disease. As discussed previously, cryopreservation is currently the most common way to maintain cell viability during transit, but this is plagued with problems such as a significant loss of cell viability and function, toxic cyropreservants, special and expensive storage conditions, and an increased risk of infection due to an increased level of handling[75]. Intercytex® have developed a means to transport cells in a refrigerated state for short time periods[79], but the ideal would be to develop a way to transport cells under ambient room temperature.

It has been hypothesized that primary cells could survive at room temperature for prolonged periods within an enclosed vessel, if the following was provided:-

- a modified-release, serum-free nutrient delivery system, delivering nutrients to the cells at a constant rate,
- a toxin adsorbent, which would be able to adsorb any toxic metabolites released,
- a surface for anchorage-dependent cells to attach to,
- access to atmospheric gases.

A key assumption surrounding this hypothesis is that the transported cells would remain viable at room temperature.

In order to test the hypothesis made above, studies were configured where the cell viability was assessed after primary human mesenchymal stem cells (hMSCs) were left at room temperature within a well-plate format for up to 7 days. Seven days would allow cell therapy products to be transported internationally, and potentially allow parts of the world that lack cell culture facilities to access these treatments.

The aims of this experimental chapter were first to determine whether it was possible to culture hMSCs under room conditions if all other parameters were optimal, and secondly to focus on the identification of an absorbent or adsorbent material that would effectively remove metabolic toxins from the system without having any detrimental effects on the cells. Such a material would allow the liquid volume that the cells were transported in to be reduced (as, without such a material, a large volume would be required to dilute the toxins to a non-harmful level), allowing for the transportation vessel to be more compact.

Throughout this chapter, the cells were incubated at room temperature within a medium free from xenogeneic serum. This was to ensure that, if a transportation device were to be developed, no xenogeneic substances could be delivered to the patient, which may potentially be harmful.

5.2 – Materials and methods

5.2.1 – The effect of media volume on hMSC culture under room conditions

The cells were transferred to transwells as described in 2.2.2.1. The transwells were then moved to either wells or beakers (for when the media volume was greater than 10 ml) containing the following volumes of StemPro® media, pre-warmed to 37 °C, in ml: 2, 5, 10, 20 and 50. After a day elapsed, the cells were analysed for viability using the alamar blue assay (2.2.4).

After 7 days of culture at room temperature, one transwell from the remaining viable volume groups were placed back into a tissue culture incubator, whist the remaining 2 transwells per group remained at room temperature. Viability analysis of all transwells continued using the alamar blue assay (2.2.4).

This study was repeated using differing volumes of pre-warmed StemPro® media: 2, 5, 8 and 10 ml. After 1, 4 and 7 days, the cells were analysed for viability with the use of the Mitotracker® Deep Red stain (2.2.5) followed by flow cytometric analysis (2.2.6).

5.2.2 – The effect of metabolic toxins and prolonged detachment on cell viability

After the hMSCs were processed as described in 2.2.2.1, the transwells were transferred to wells containing 5 ml of pre-warmed StemPro® media. The following toxins were added to the wells: 500 nl of a 35 % ammonia solution (1.81 mM) or 9 mg lactic acid (20 mM). To assess prolonged cell detachment, the cells were plated onto non-tissue plastic well plates and incubated within a tissue culture incubator for 24 hours before culturing the cells at under room conditions. To determine the level of viability, the alamar blue assay was used (2.2.4), using centrifugation (250 g, 4 minutes) to ensure that solutions could be changed without losing viable detached cells.

5.2.3 – The ammonia adsorptive properties of zeolite and activated carbon

Ammonia solution (35 % $^{v}/_{v}$) was added in 5 µl increments to 10 ml of phosphate-buffered saline (PBS) (control) or 10 ml of PBS containing 4 g of either pre-prepared zeolite (2.2.7) or activated carbon. Due the mild acidic effects of the zeolite, this was compared to zeolite, in PBS, with the pH pre-adjusted to be the same as control (pH 7.4) using molar sodium hydroxide (this pre-adjustment was not necessary for the PBS containing activated carbon). After each ammonia addition, the pH was measured and recorded using a Mettler Toledo SevenMulti® pH meter.

5.2.4 - Nutrient adsorption from media containing either zeolite or activated carbon

Pre-prepared zeolite (2.2.7) or activated carbon (4 g) was placed into 10 ml of complete StemPro® media. After each day at room temperature, the media was replaced with fresh media, and the extracted samples were analysed for both glucose (2.2.8) and total protein content (2.2.9).

5.2.5 – The effect of zeolite on cell viability under room conditions

After culturing the hMSCs onto wells in a 6 well plate (2.2.2.1), the ammonia was managed either through the addition of 4 g of pre-prepared zeolite (2.2.7), within a transwell (figure 5.1) or through the manual adjustment of the pH to 7.4, every 12 hours, using 100 mM hydrochloric acid (HCl). All cell samples contained 5 ml of pre-warmed StemPro® and 5 ml PBS (as the volume needed to be more than 5 ml in order to submerge the zeolite within the solution). After 1, 2, 4 and 7 days of culture under room conditions, 3 transwells from each group were sacrificed, and the cells were analysed for viability using the CellTiter AQ One Solution Cell Proliferation Assay (2.2.3).

5.2.6 – Ammonia release from hMSCs cultured under room conditions

After the cells were cultured and analysed for viability as described in 5.2.5, the cell culture media was retained. This media (50 µl) was mixed with 50 µl of working Biovision® ammonia assay reagent (appendix 2.5) and incubated at 37 ºC for 60 minutes. Colorimetric analysis was carried out at 570 nm using a Bio-Tek KC4 plate reader. Blank values were subtracted from sample readings, and, using a standard curve (appendix 5.7), the concentration of ammonia was determined.

5.3 – Results

5.3.1 – The effect on media volume on hMSC culture under room conditions

Figure 5.2 documents the number of hMSCs present during 11 days of culturing with varying volumes of StemPro® media. Cells within all volumes showed the cell number almost doubled after 1 day of culture. Cells cultured within a media

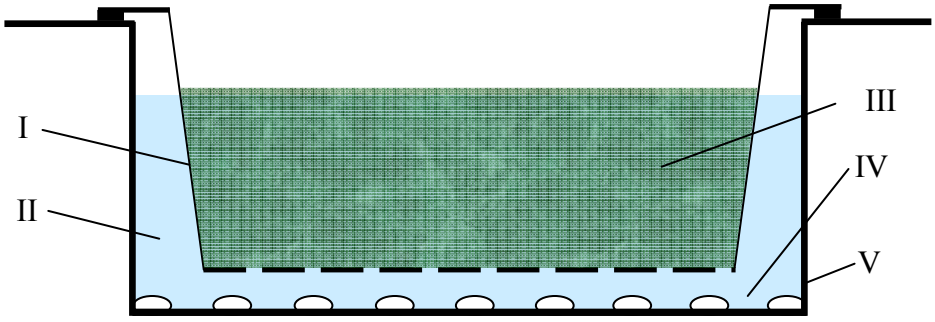


Figure 5.1. Schematic of well plate configuration to assess the effectiveness of the zeolite (III) in supporting cell viability at room conditions. The hMSCs (IV) were seeded onto a well of a 6-well plate (V), and the zeolite was suspended above the cells using the a Corning Transwell® (I). The cells were cultured in a solution (II) containing 5 ml StemPro® media, with 5 ml PBS to ensure that the entirety of the zeolite was in solution.

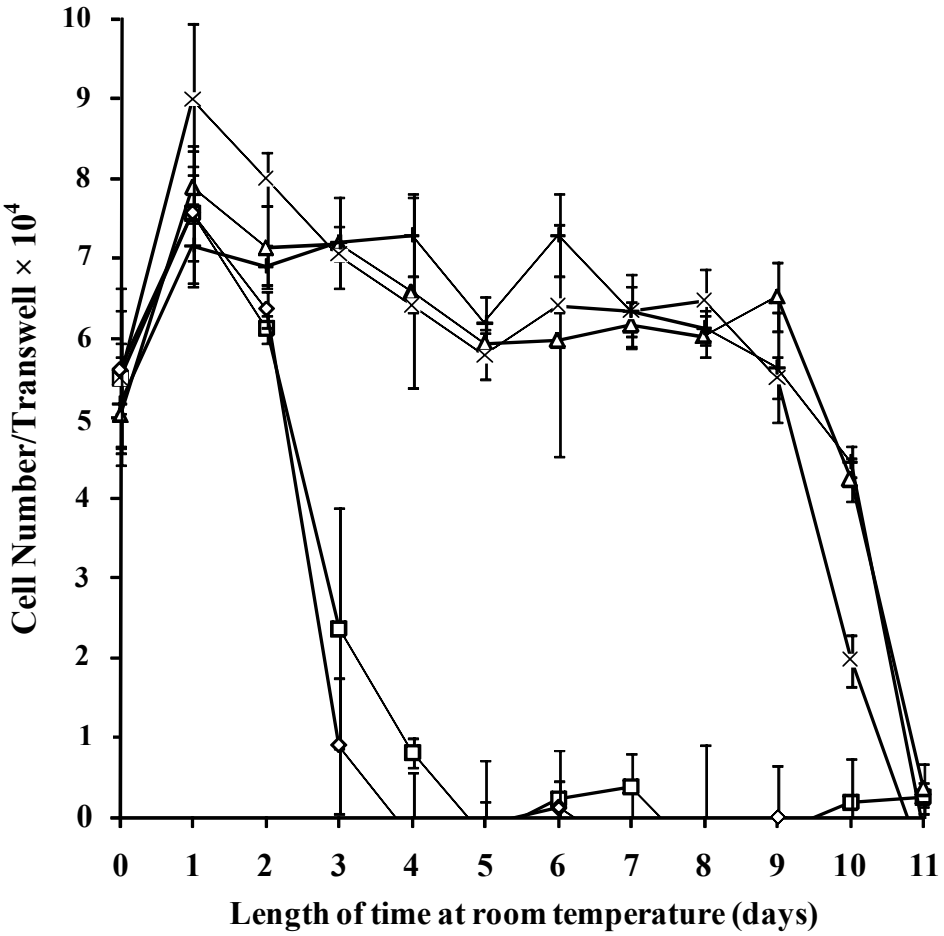


Figure 5.2. Viability of hMSCs following specific periods under ambient room conditions within the following volumes of StemPro® media (ml): 2 (□), 5 (◇), 10 (△), 20 (×) and 50 (+). Determined using the alamar blue assay. Results represent mean viability ± SEM. The study was carried out in triplicate up to day 7, after which point the study was performed in duplicate.

volume of 10 ml and above saw a gradual decrease in viable cells from days 1 to 9, followed by a dramatic decrease in viability, leading to a small number of cells being viable at day 11, regardless of the volume of media they were in of 10 ml and above. Cells cultured in volumes of 5 ml and below showed a very different viability profile; a dramatic fall in viability was seen after day 1 so that few cells were viable at day 4.

The subset of cells that were placed back within favourable conditions within a tissue culture incubator after 7 days under room conditions were found to proliferate, after a lag phase of 2 days, at a rate similar to that seen at the first day of culture within room conditions (figure 5.3). This proliferation was found to cease after 5 days within the tissue culture incubator; this is thought to be due to a restriction in expansion space.

When repeating this study using a mitotracker® stain, followed by flow cytometric analysis, the results were found to be vastly different to those seen in figure 5.2; no significant increase in cell number was seen after the first day of culture under room conditions in any of the volumes of media, and the majority of cells were found to be non-viable after 7 days of culture (figure 5.4). Only the volume of 10 ml was found to show no significant decrease in viability after 4 days of culture at room temperature ($p < 0.05$).

5.3.2 – The effect of metabolic toxins and prolonged detachment on cell viability

The number of viable hMSCs decreased significantly when cultured on to non-tissue culture plastic and when ammonia was introduced into the culture, compared to control ($p < 0.01$) (figure 5.5). However, the cells tolerated the introduction of lactic acid and this led to a significantly increased level of viability after 5 days compared to control ($p < 0.01$).

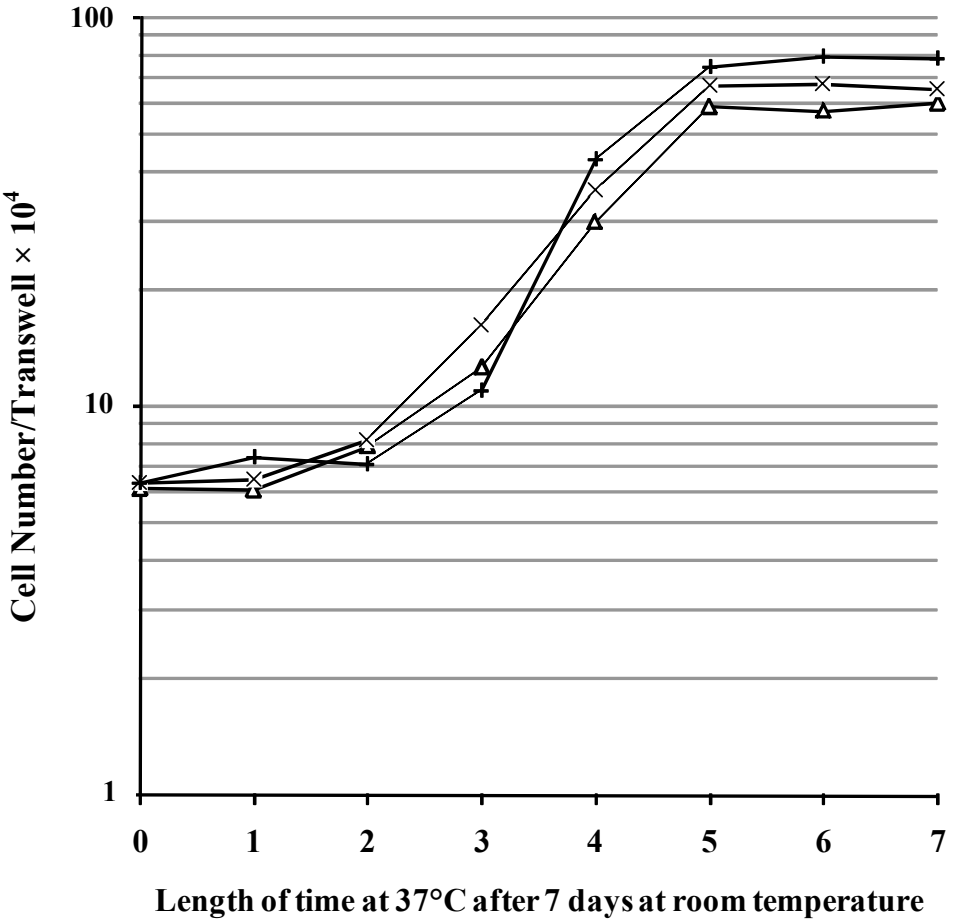


Figure 5.3. Proliferative count of hMSCs under favourable conditions following 7 days under room conditions within the following volumes of StemPro® media (ml): 10 (△), 20 (×) and 50 (+). Proliferation rates were determined using the alamar blue assay. The results did not have any replicates (hence no error bars) Sample data were plotted on an exponential axis.

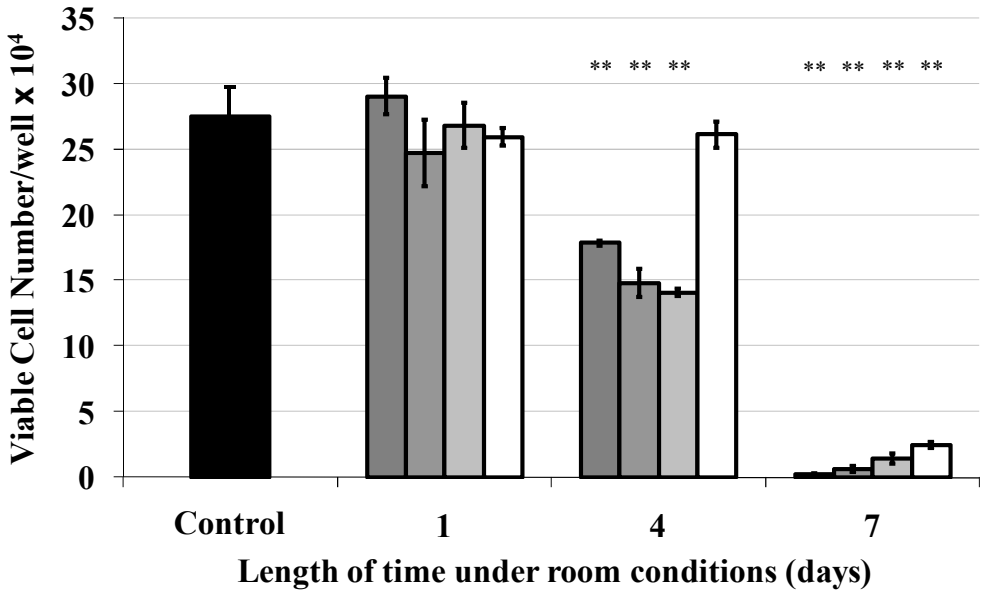


Figure 5.4. Viability of hMSCs following specific periods under ambient room conditions within the following volumes of StemPro® media (ml): 2 (■), 5 (■), 8 (■) and 10 (□). Samples were compared against control (■). At specific timepoints, samples were incubated mitotracker® deep red dye according to the manufacturers' instructions before being analysed using a Beckman Coulter Cytomics FC500 flow cytometer at 60 µl/min. Samples were analysed using WinMDI. Results represent mean viable cell number ± SEM. Asterisks represent the level of significance compared to control sample ($p < 0.01$).

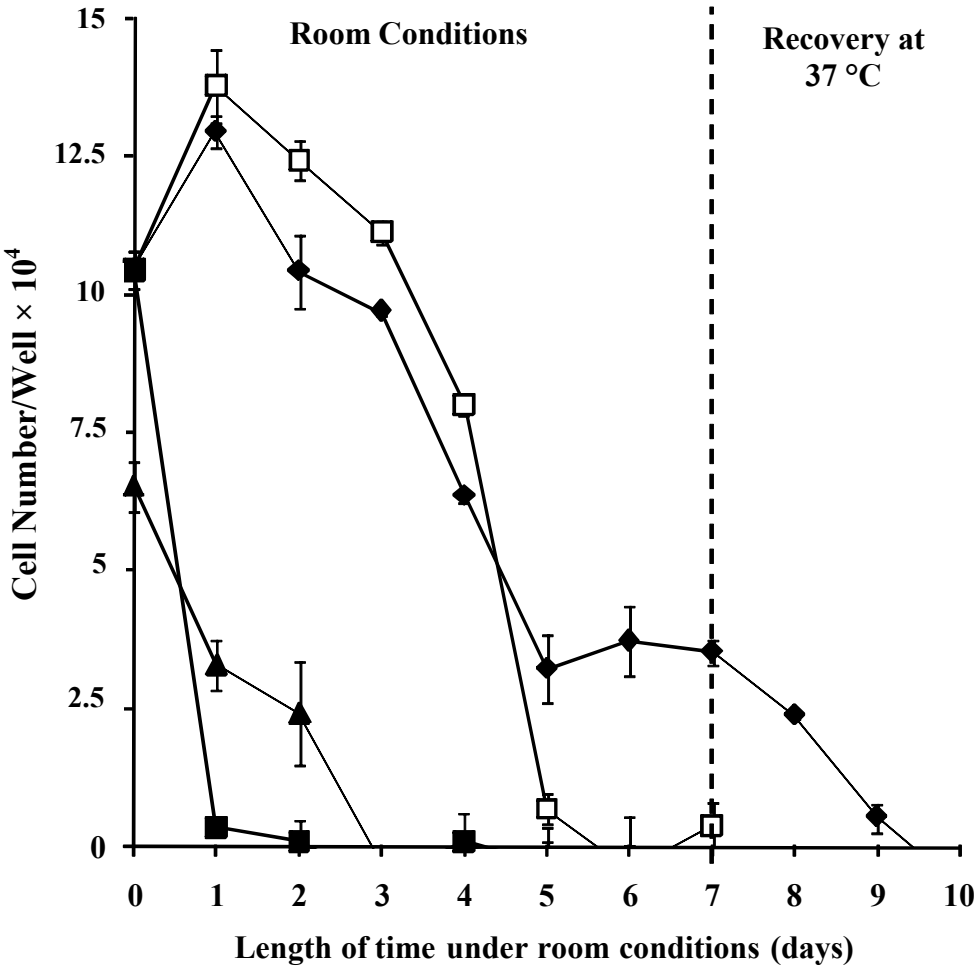


Figure 5.5. Viability of hMSCs following specific periods under ambient room conditions within 5 ml of StemPro® media introduced to a single dose of toxin, compared against no toxin introduction (□). The following toxins were added: 500 nl of a 35 % ammonia solution (1.81 mM) (■) and 9 mg lactic acid (20 mM) (♦). Cells were also seeded onto non-tissue culture plastic (▲). Viability was determined using the alamar blue assay. Results represent mean viability ± SEM.

5.3.3 – The ammonia adsorptive properties of zeolite and activated carbon

Figure 5.6 shows the increase of pH when ammonia was added to PBS containing zeolite or activated carbon. Both zeolite and activated carbon both significantly reduced the pH increase with each addition of ammonia ($p < 0.01$). The pH-reducing effect of the zeolite was not hindered by the addition of molar sodium hydroxide (NaOH), used to increase the slightly acidic solution to pH 7.4.

5.3.4 - Nutrient adsorption from media containing either zeolite or activated carbon

Activated carbon significantly reduced the level of total protein from complete StemPro® over 2 days, compared to complete media ($p < 0.05$), but did not have any significant affects on the glucose concentration. Zeolite had no significant effect on glucose or total protein levels (figure 5.7). It was also observed that, during the first 3 days, the yellow colour of the media disappeared from the activated carbon samples, and turned colourless (data not shown).

5.3.5 – The effect of zeolite on cell viability under room conditions

Figure 5.8 compares the viability of samples containing zeolite to samples that were manually pH controlled, compared to control. All samples were seen to increase in viability after 1 day of culture at room temperature. After days 4 and 7, both the pH controlled group and the zeolite-containing group showed a significantly higher level of viability compared to control. No significant differences were observed between the zeolite group and the pH-controlled group.

5.3.6 – Ammonia release from hMSCs cultured under room conditions

Figure 5.9 compares the level of ammonia present in the media when the hMSCs were cultured under room conditions for samples containing zeolite, or that were manually pH controlled, compared to control. Within all samples, ammonia was seen to increase drastically during the first day of culture, then decrease slowly over the next six days. A significantly lower concentration of ammonia was present in the zeolite-containing group compared to the other groups, though the

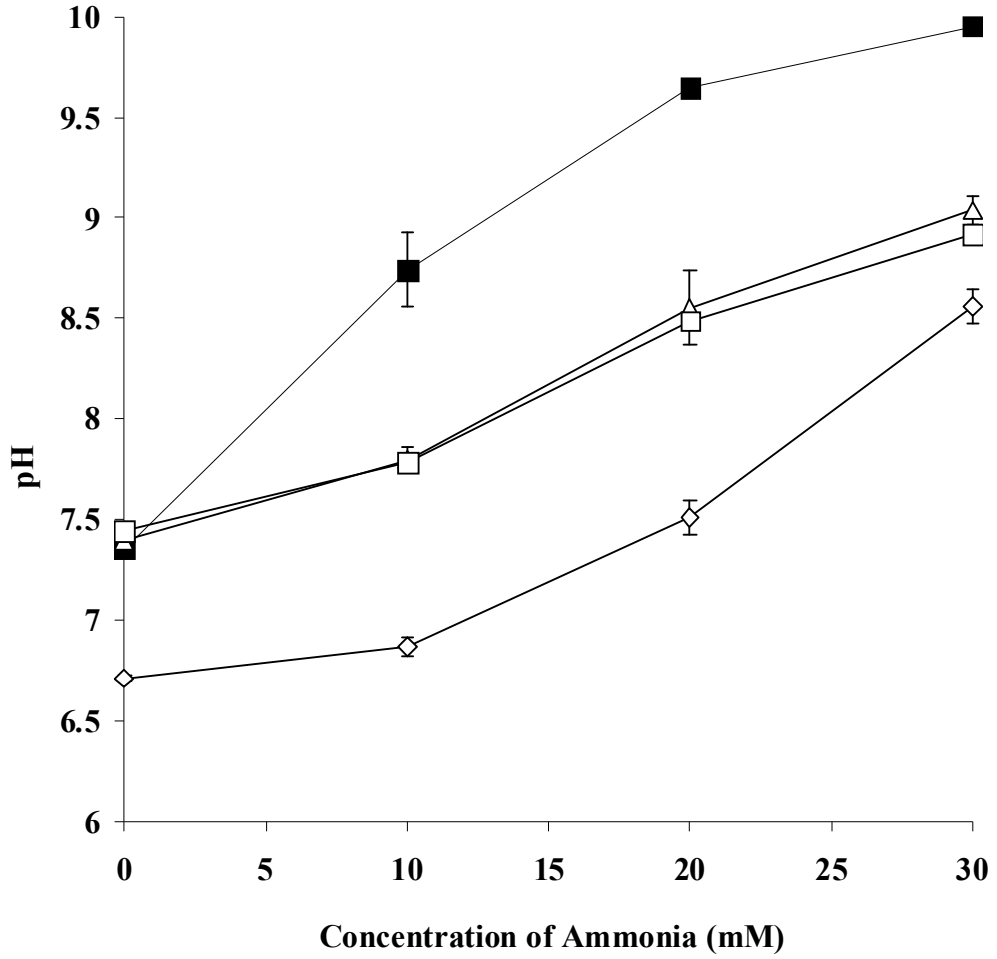


Figure 5.6. pH values of 10 ml ammonia solutions in PBS, containing either (i) 2 g pre-prepared zeolite granules (◊), or (ii) 2 g pre-prepared zeolite, pH pre-adjusted to 7.4 using 1 M NaOH (△), or (iii) 2 g activated carbon (□) compared to solution containing no adsorbant (■). pH was measured using a calibrated Mettler Toledo SevenMulti® pH meter. Results represent mean pH ± SEM

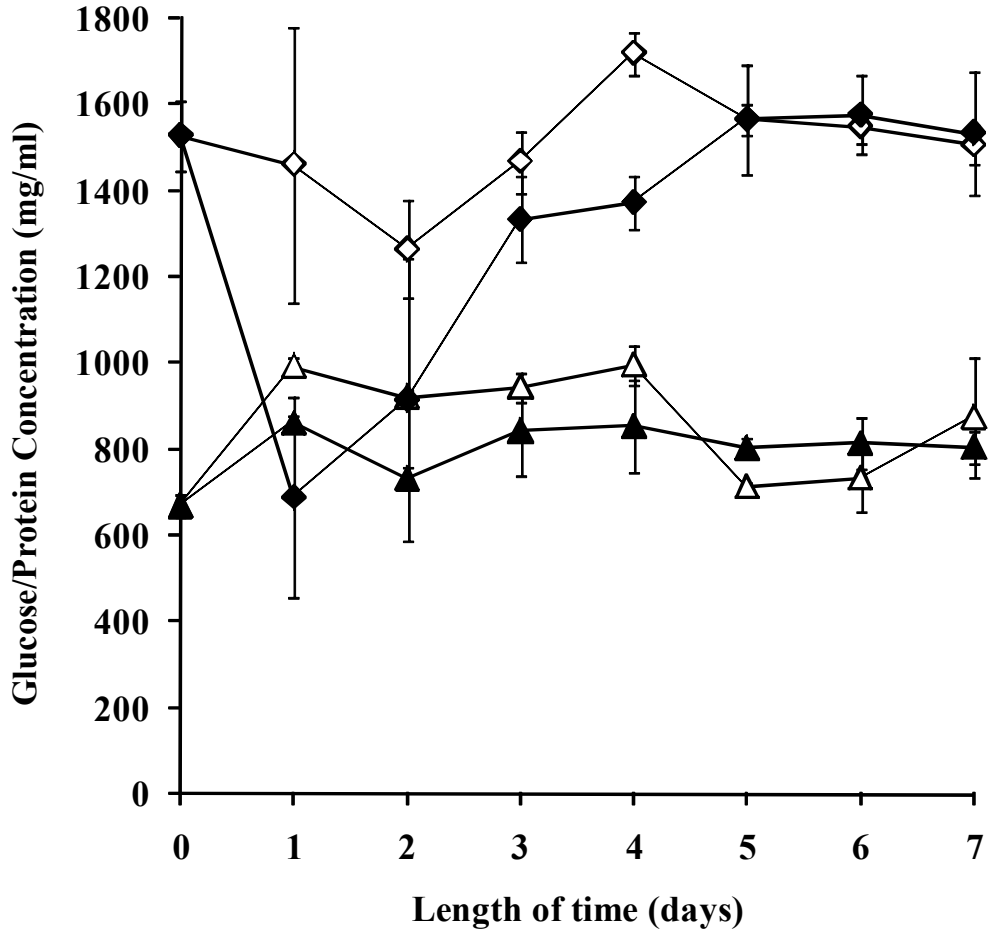


Figure 5.7. Glucose (△▲) and total protein (◇♦) concentration within 10 ml complete StemPro media following adsorption from 2 g of either zeolite granules (white) or activated carbon (black). After each day, media was extracted from the adsorbent, either through simple pipetting (zeolite) or through vacuum filtration (carbon). The extracted media was replaced with fresh media. The glucose concentration within the extracted samples was determined using the glucose oxidase assay. The total protein concentration was determined using the Coomassie Plus assay. Results represent mean concentration ± SEM

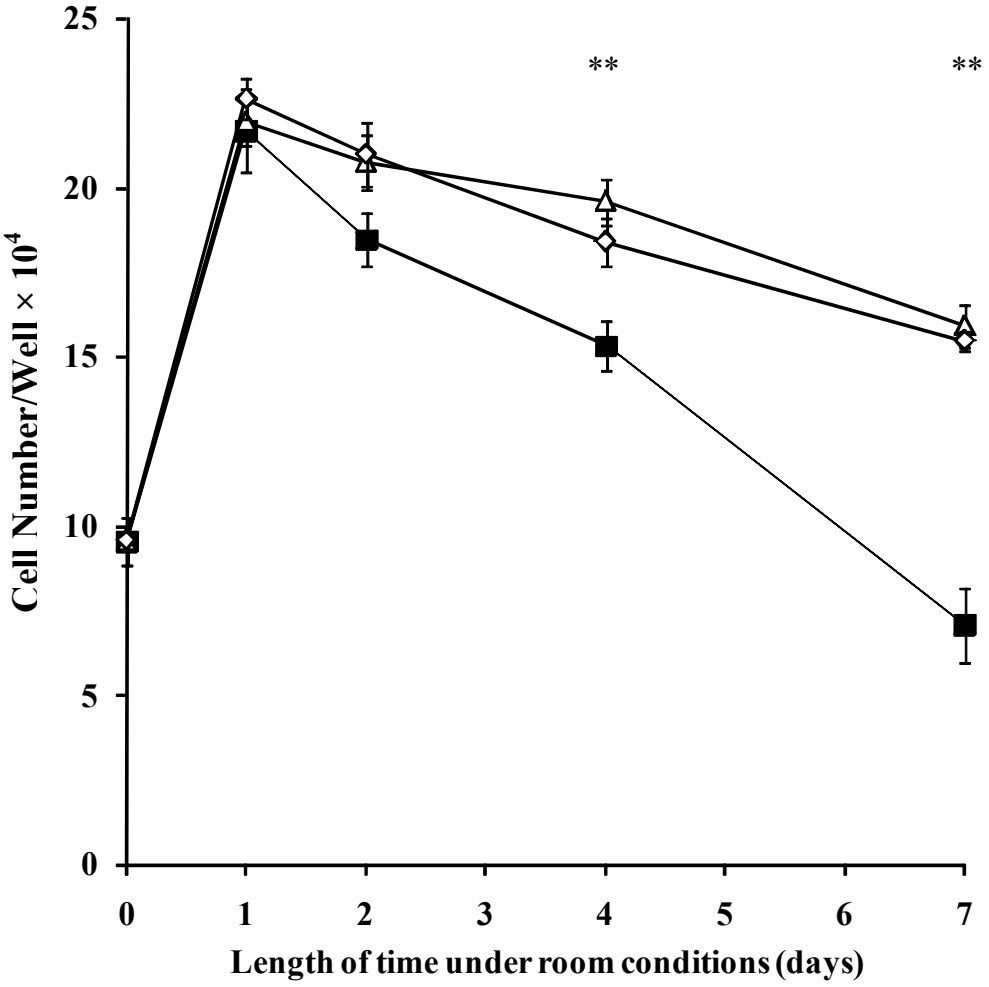


Figure 5.8. Viability of hMSCs following specific periods under ambient room conditions within 5 ml of StemPro® media (■). Ammonia accumulation was managed either through the introduction of 4 g zeolite (△) or by the adjustment of pH every 12 hours to 7.4 using 100 mM HCl (◇). Determined using the CellTiter AQ Proliferation assay. Results represent mean viability ± SEM. Asterisks represent the level of significance compared to control sample ($p < 0.01$).

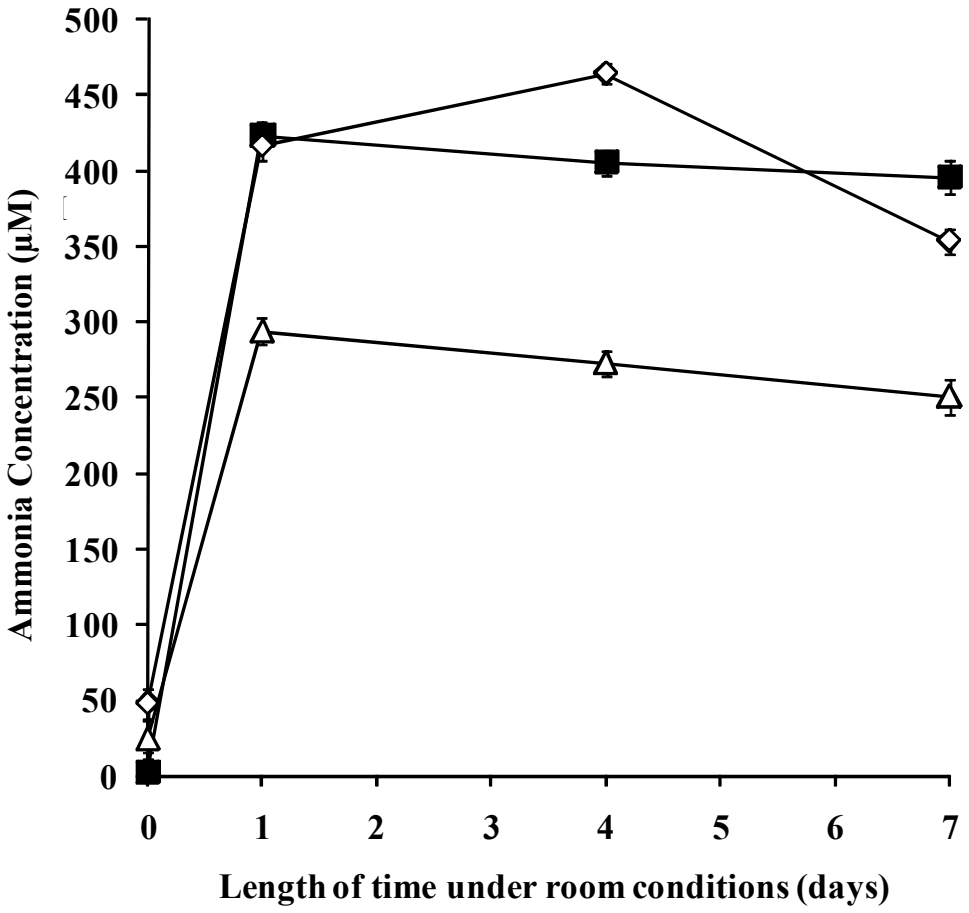


Figure 5.9. Ammonia concentration within 5 ml StemPro® media samples cultured with hMSCs under ambient room conditions, either in the presence of 4 g zeolite (△), or with the 12 hourly adjustment of pH to 7.4 using 100 mM HCl (◊), compared to control (■). Determined using a Biovision® ammonia detection assay. Results represent mean viability ± SEM.

level of the ammonia within the zeolite-containing group was still significantly higher than the ammonia level of fresh media ($p < 0.01$).

5.4 – Discussion

Here, the feasibility of a transportation system that maintains viable cells under ambient room conditions was assessed. The development of such a system would significantly reduce the transportation costs compared to cryopreserved delivery. This system would also minimise the level of processing on the cell suspension that would be required before delivery to the patient, thus leading to a lower risk of contamination and a reduction of the processing facilities required at the clinic where the patient would be treated.

The development of such a transportation system relied upon the assumption that primary cells would remain viable at room temperature if all other variables were optimal. Developing a study where the cells were incubated within a high volume of media was used not only to provide a plentiful supply of nutrients, but also to thoroughly dilute any metabolic toxins released from the cells. Fortunately, with regard to hMSCs, our initial findings showed that cell viability did not decrease significantly over a 9 day period ($p < 0.05$). Similar results were reported in Lane *et al*; they showed no decrease in viability when the cells were stored at room temperature, though they only assessed viability over a shorter 24 hour period[81]. However, after 1 day, the cells did not proliferate either, but entered a state of proliferative arrest. This arrest phase appeared to be reversible, as when the cells were placed at 37 °C, they were able to proliferate at rate similar to cells not exposed to a temperature change, after a lag period of 2 days. There are other reports of a reversible proliferative arrest state; Macip and co-workers reported such a reversible state in cancer cells when exposed to a certain concentration of reactive oxygen species[119], and Stolzing reported a similar observation with MSCs[120].

A note-worthy observation is that, regardless of the level of nutrients provided, all of the cells became non-viable soon after 9 days at room temperature. Nine days may be the window of reversible arrrest with this cell type, after which cell death through apoptosis ensues.

If this reversible arrest state does not damage the cells, this state would be beneficial for the transportation device; if the cells are not proliferating, the nutrient demands, and the level of the metabolic toxin release would be lower. The cell number within the vessel after a 7 day period could also be easier to accurately predict. However, the lag phase when the cells are returned to 37 °C (when delivered to the patient) of 2 days before proliferation occurs may be too long, as the cells may need to engraft within the delivered site quickly to ensure they are not cleared. This may be easily dealt with by recommending a clinic to incubate the transportation vessel at 37 ºC for 2 days before administering the suspension, though this would require the clinic to have facilities to do so.

Between chapters 3 and 4, a close viability correlation was seen between cells analysed using mitochondrial staining and flow cytometry compared to cells analysed using a colorimetric mitochondrial assay. However, when comparing the viable cell number in figure 5.2 (determined using alamar blue) against figure 5.4 (determined using flow cytometry), differing results are observed. This difference increases the importance for further analysis of this arrest state, as it may be possible that, between day 4 and 7, where the large difference in viability is seen, there is early damage to the mitochondria that has not led to a change in chemical reduction within the media (detected by the alamar blue).

With regard to the study incorporating metabolic toxins within the culture, the main aim was to assess whether the cells were more sensitive to the toxins when at room temperature rather than at 37 ºC. The toxins that would be analysed, and their concentration, were determined from a review by Schneider *et al*, who stated that lactic acid and ammonia concentrations of 20 and 2 mM respectively could

inhibit cell growth[121]. The hMSCs were indeed more sensitive to ammonia; this concentration caused cell death within 2 days, although it is worth noting that the ammonia added to the culture would be in addition to any ammonia the cells released, this further increasing the concentration. A similar observation has been seen previously by Macip and co-workers, who found that cancer cells within the reversible arrest state are more sensitive to chemotherapeutic agents[119]. The lactic acid caused an increase in viability, and this may be due to the acid partially neutralising the ammonia released, keeping the pH closer to 7.4. This may also be due to the lactic acid causing an activation of metabolism as the cells attempt to repair themselves or activate an apoptotic cascade, as detailed in Boyle and Hickman[122].

The hMSCs, not surprisingly, reacted unfavorably when they were not attached to a surface. A decrease in viability and an increase in apoptosis within anchorage-dependent cells followed prolonged periods of detachment have been reported previously[92]. This finding suggests that a transportation system incorporating the poly(lactic-*co*-glycolic) acid microparticles developed in Chapter 3, as a scaffold that the cells can attach to, may be of benefit.

As ammonia is deemed a more toxic metabolite under aerobic conditions compared to lactic acid[121], and the results above showed that the cells were sensitive to ammonia, it was decided that an ammonia adsorbent would be identified and utilised. The two toxin adsorbents assessed were activated carbon and zeolite. Activated carbon, derived from charcoal, is an effective and cheap adsorbent, due to its extremely porous structure (figure 5.10). Activated carbon is found to be non-toxic and is currently used to adsorb poisons ingested by overdose patients[123].

Zeolites are natural, abundant, crystalline, hydrated aluminosilicates, that consist of pores containing alkali and alkaline earth cations, such as as Na^+, K^+, Ca^{2+} and Mg^{2+}. These cations are often loosely held, allowing the easy exchange of other

cations, such as ammonium (NH_4^+), which is contained with a higher affinity. Zeolites are currently used to filter ammonia from industrial and agricultural waste. Zeolites are classed as biocompatible and are used to filter blood as part of kidney dialysis equipment[124].

In a simple pH experiment, activated carbon and zeolite were found to adsorb ammonia equally as effectively. For this reason, the toxin adsorbent that would be used was chosen based on its ability adsorb ammonia *specifically* and not adsorb useful nutrient components. Even though glucose and total protein, which were both analysed in this study, do not represent all that a cell requires from cell culture media, the concentration of these two nutrients were analysed for two reasons; (i) both nutrients are vitally important to the cells and excessive adsorption of either component would have detrimental effects on the cells, and (ii) both components are very different from one another, mainly in terms of molecular weight and size. Therefore, analysing the adsorption of both nutrients could provide information with regards to whether preferential adsorption occurs for one molecule over another.

The activated carbon was found to adsorb significant amounts of protein from complete StemPro® media compared to the same weight of zeolite. These results link with work published by Petit *et al*, who found that activated carbon is not as effective at adsorbing ammonia than other substances (the opposite to the adsorption profile of the zeolite) and were developing means to increase its affinity towards ammonia[125]. These experimental results, together with the current literature, do suggest that zeolite would be more specific to ammonia than activated carbon if not totally specific, and for these reasons, the zeolite was chosen as the toxin adsorbent to use in the cellular studies.

Zeolite was seen to increase the viability of the cells to a similar extent as the manual pH adjustment. It was expected that zeolite would provide a higher level of viability over the pH-adjusted group because the zeolite would prevent the

ammonia from having negative molecular effects on the cells, as well as maintain the pH at 7.4. At a molecular level, ammonia is thought to be able to increase additional ammonia release through the activation of glutaminase (as well as reduce glutamine levels) and cause futile energy dissipation through the interference of a glutamate dehydrogenase pathway. However, the removal of these negative molecular effects may be countered by the effect of potential nutrient adsorption.

The zeolite was also found to significantly reduce the level of ammonia present in the culture compared to the pH-controlled group or the control ($p < 0.05$), though was not able to totally remove the ammonia. The plateau in ammonia, seen in all groups, was probably due to the low level of metabolism occurring within the cell samples during their arrest phase. However, this could also be due to the ammonia evaporating off from the samples as it was being produced by the cells. Interestingly, the results shown in figure 5.8 and 5.9 suggest that an increase in ammonia of as little as 120 μM, can cause a significant decrease in viability after 4 days, displaying the high level of sensitivity the cells have towards ammonia at room temperature.

5.5 – Conclusion

In this chapter, the overall aim was to develop a means of transporting viable primary cells under room conditions. Displayed here is initial evidence that hMSCs are able to survive under these room conditions for between 4-9 days. Even though the cells appear to enter a proliferative arrest phase under these conditions, the arrest was found to be reversible when the cells were placed back into favourable conditions.

Under room conditions, hMSCs were found to be sensitive towards the presence of low levels of ammonia, so it was decided that an adsorbent that could remove ammonia from the system would be identified. Even though activated carbon and zeolite showed similar ability to adsorb ammonia, the literature, together with the

findings here, suggested that carbon would adsorb a substantial amount of nutrients compared to zeolite, so zeolite was chosen for further analysis.

The initial results suggest that the presence of zeolite leads to a substantial improvement in hMSC viability, and that this improvement in viability is due to the zeolite removing ammonia from the system.

5.6 – Future Work

The methods used act as simple surrogate markers, providing an insight into the plausibility of cells remaining functional after transportation under room conditions and whether a toxin adsorbent could improve the likelihood of the cells remaining viable. Further studies are required in order to fully determine whether these initial results are correct.

Further analysis of the reversible proliferative arrest phase seen whilst the cells are under room conditions is required, such arrest-associated β-galactosidase staining and cell cycle analysis, to determine the mitotic checkpoint the cells arrest at, in order to ensure that cells are not damaged irreversibly after incubation under room conditions. In addition, a more robust assessment of cell morphology and function is required in order to fully determine whether the incubation under room conditions has had a detrimental impact on the cells. Analysis of cell attachment and spreading, and the level of apoptosis that occurs, using methods described in Chapter 3, would be beneficial to this study.

Ideally, flow cytometric analysis would have been used throughout this chapter, not only for the reasons stated in 4.1, but also because of the large discrepancy in results obtained from flow cytometric and colorimetric analyses, as described above. However, the particulate zeolite matter could not be effectively removed from the cell suspensions, which could have potentially caused false positives when analysed through flow cytometry. For this reason, when the zeolite was present, the CellTiter AQ One Solution assay was used instead.

Assessing the ability of these adsorbents to adsorb protein and glucose alone is by no means a fair representation of all cellular nutrients, and the zeolite may adsorb other important nutrients that have not been tested; reports have found that clinoptilolite, a form of zeolite, inhibited tumour cell proliferation, and it was predicted that this inhibition was through an adsorption of calcium from the media[126]. Ideally, a high performance liquid chromatography (HPLC) method would have been applied to the adsorbed samples in order to fully identify what nutrients had been removed. The use of HPLC would also be able to assess whether any substances are leaching out from the zeolite that may be harmful to the patient.

As the zeolite used was a natural mixture of many different aluminosilicates, using a specific zeolite with a high affinity for ammonia, such as clinoptilolite, may have reduced the ammonia levels further, as suggested by the literature[126,127,128]. This specific zeolite, however, was found to be very difficult to source.

6

The development of a nutrient release system to complement the cell transportation device

6.1 - Introduction

Chapter 5 identified zeolite as an effective adsorbent for the metabolic toxin ammonia that was able to significantly improve human mesenchymal stem cell (hMSC) viability after 7 days under room conditions. Although these findings are encouraging, the volume of cell culture media used was high; to culture 100,000 cells for 7 days, 5 ml of serum free media was used. Typically, for cell therapy studies, the number of cells delivered could be as high as 10^{10} (Martin-Rendon *et al*, [33] requiring respectively up to 500 litres of media. Even if a cell transportation vessel could be designed to ensure this high volume of liquid was not delivered to the patient when the vessel arrived at the clinic, this volume would make the vessel very cumbersome to transport and would significantly increase the costs of the media and the materials required.

Here, a method of forming a concentrated nutrient supply for hMSCs under ambient conditions was developed, which then led to the development of a controlled nutrient release system. The controlled nutrient release system was developed for two hypothetical reasons:-

- Concentrating a nutrient supply could have effects on osmolarity, as well as direct cellular effects, that could be detrimental rather than beneficial to the cells. A controlled release system would theoretically ensure that the nutrients were released at a rate similar to consumption and therefore, the concentration

of nutrients within the liquid phase would remain at non-toxic levels throughout transportation.

- Despite the initial results suggesting that the adsorbent material does not adsorb glucose or proteins from cell culture media (Chapter 5), the zeolite may adsorb other nutrients, as well as ammonia; reports have shown that calcium level could be depleted by the presence of zeolite[126]. Within a system containing concentrated nutrients, it is possible for the zeolite to adsorb important nutrients soon after its introduction, causing both an immediate reduction of nutrients for the cells and a reduced adsorptive capacity of the zeolite. Within a system containing a nutrient release gel, a diffusion gradient could be achieved, where nutrients pass the cells before being potentially adsorbed by the zeolite.

An alginate gel was developed to form the controlled release system. Due to alginate's ability to polymerise at room temperature through the use of multivalent cations (such as Ca^{2+} and Ba^{2+}) interacting with carboxylic acid groups on the polysaccharide backbone, forming gels in this fashion could ensure that any required protein nutrients would not denature. Alginate also has the advantages of being biocompatible and cheap to source[129].

6.2 – Materials and Methods

6.2.1 – The effect of specific nutrients on cell viability

After the hMSCs were processed as described in 2.2.2.1 into wells of a 6 well plate, the following nutrients were added on a daily basis: 2 mg glucose (2.2 mM), 2 mg fructose (2.2 mM), 50 µl glutamine (200 mM), 50 µl glutamax® (200 mM), 100 µl StemPro® supplement or 10 mM HEPES buffer. To determine the level of viability, the alamar blue assay was used (2.2.4).

6.2.2 – Comparison of concentrated StemPro® against standard media

Concentrated StemPro® media was created as described in appendix 1.4. The concentrated media was reconstituted with tissue culture water in order to make

the media standard strength once more. After the hMSCs were transferred to a 6 well plate at the concentration of 10^4 cells/well, as described in 2.2.2.1, the cells were cultured in a tissue culture incubator for up to 7 days. The number of viable cells was determined daily using the alamar blue assay (2.2.4).

6.2.3 – Alginate Gel Fabrication

A stock solution of 5 % ($^{w}/_{v}$) alginate was prepared by dissolving pre-autoclaved (121 °C at 1 bar for 1 hour), high viscosity alginate in Dulbecco's modified Eagle medium (DMEM) or concentrated StemPro®. After initial mixing, the solution was left on a heated magnetic stirrer for up to three hours at 37 °C to completely dissolve the alginate.

In order to polymerise the gel, a hole was made on the underside of a 34 mm diameter petri dish using a heated scalpel blade and inverted onto nylon filter paper. The alignate gel was weighed onto the filter paper and smoothed to the edges. The alginate, together with filter paper and petri dish, were placed into a 100 mm diameter petri dish containing 1 % ($^{w}/_{v}$) barium chloride polymerisation buffer. This was moved to a cell culture incubator and incubated for 16 hours.

The polymerising method was optimised after it was found that the gel failed to become stiff enough. The following parameters were altered: (i) the concentration of barium within the polymeristation buffer increased to 2 %, (ii) a layer of polymerisation buffer was slowly pipetted on top of the gel within the smaller petri dish, (iii) 2 hours after incubation, the gel was slowly peeled away from the filter paper, and placed upside-down into a petri dish containing fresh polyerisation buffer. The gel was adequately stiffened after a further 2 hours (figure 6.1).

The lowest weight of alginate that could be practically weighed and spread evenly into the petri dish was 2 g. Therefore, when gels of 500 mg and 1 g were required, the gel was cut into quarters or halves after it was stiffened. The sections were then

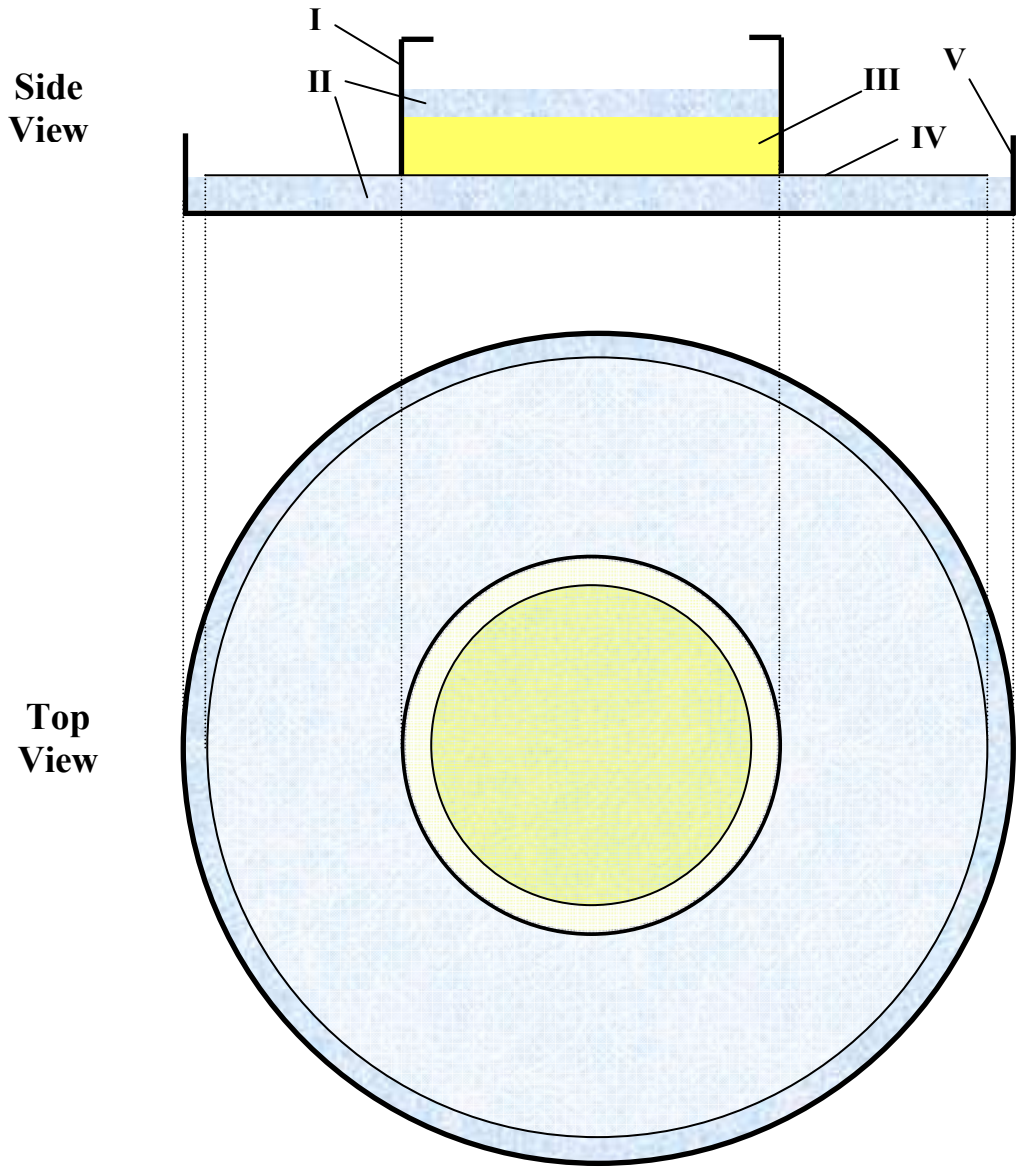


Figure 6.1 – Schematic showing the optimized polymerisation of alginate gels. A hole was made on the underside of a 34 mm diameter petri dish using a heated scalpel blade (I) and inverted onto nylon filter paper (IV). Alginate gel (5 % $^{w}/_{v}$) (III) was weighed onto the filter paper and smoothed to the edges. The alginate, together with the filter paper and petri dish, were placed onto a 100 mm diameter petri dish (V) containing 2 % ($^{w}/_{v}$) barium chloride polymerisation buffer (II). Additional polymerisation buffer was slowly added on top of the alginate gel. This was moved to a cell culture incubator. After 2 hours, the gel was slowly peeled away from the filter paper, and placed upside-down into a new petri dish containing fresh polymerisation buffer. The gel was adequately stiffened after a further 2 hours. If necessary, the gels were cut into halves or quarters and placed back into fresh polymerisation buffer for a further hour in order to stiffen the sections exposed by the sectioning.

placed back into fresh polymerisation buffer for 1 hour in order to stiffen the areas exposed by the sectioning.

6.2.4 – Release kinetics from alginate gels

The gels were placed into 5 ml of phosphate buffered saline (PBS). After each day at room temperature the PBS was replaced with fresh PBS and the extracted samples were analysed for both glucose (2.2.8) and total protein content (2.2.9).

The reasons for analysing glucose and total protein release are similar to those discussed in 5.2.4; both glucose and protein are important nutrients for cell viability, and the molecules differ so greatly in size and molecular weight that analysis of both release profiles could provide information regarding whether preferential release for one over the other occurs.

6.2.5 – The effect of the developed nutrient release system on cell viability

After the hMSCs were transferred to the transwells as described in 2.2.2.1, the cells were arranged in a disposable beaker as shown in figure 6.2. The control consisted of the configuration without the gel or zeolite, but instead with 500 µl of 5 × concentrated StemPro® media in 5 ml of PBS within the transwell. All the other samples contained 4 g of pre-prepared zeolite (2.2.7). The transwells were placed on top of the zeolite, which was submerged in 5 ml of PBS. A pre-fed group was arranged, which was similar to the control except that it did contain zeolite. A regularly fed group was configured in a similar manner to the pre-fed group except with the addition of 125 µl concentrated media to the transwell at day 0, 2, 4 and 6, instead of 500 µl at day 0. Finally, two cell groups containing a 500 mg nutrient gel were configured, one group with the gel placed with the zeolite, and the other where the gel was placed floating above the cells within the transwell.

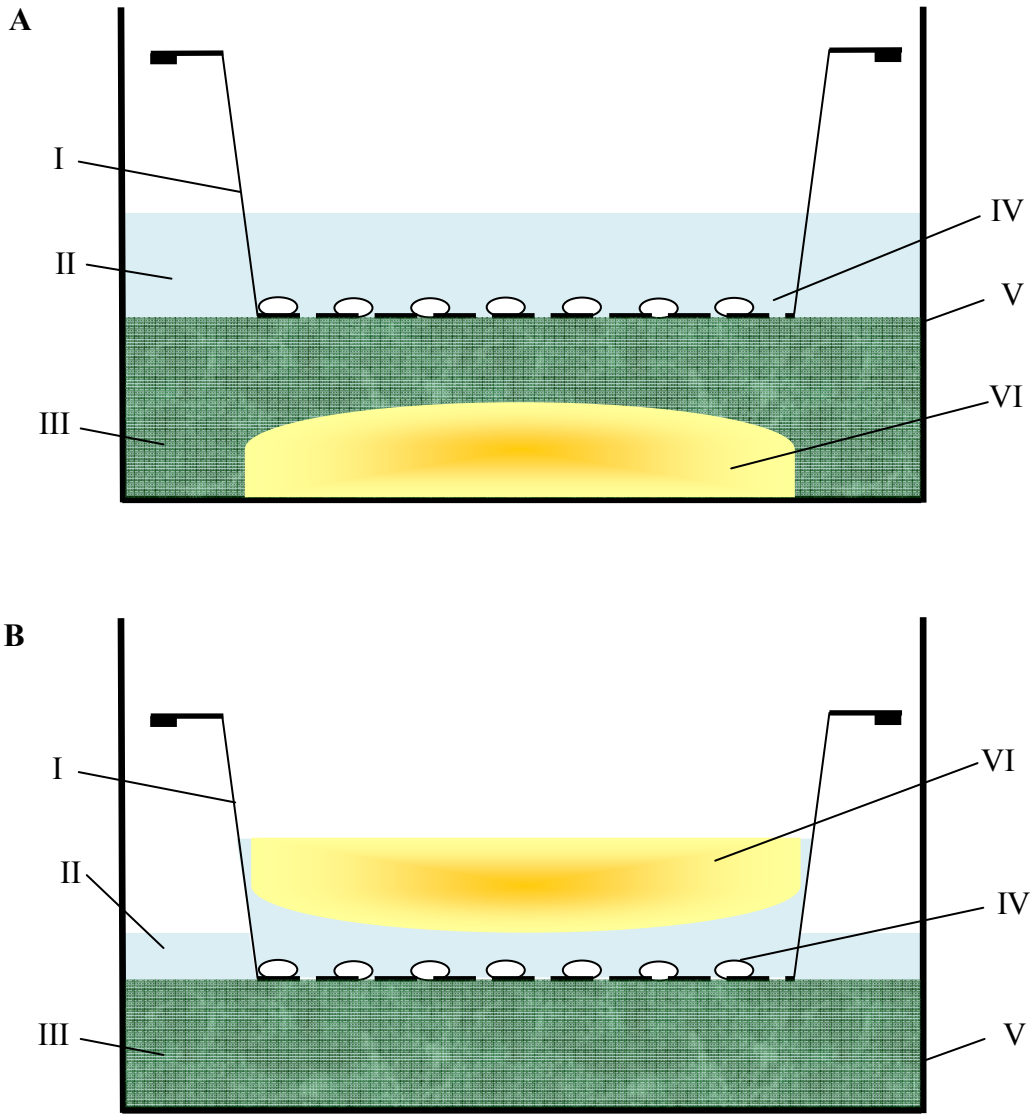


Figure 6.2. Configurations used to assess the effectiveness of the zeolite (III) and the alginate nutrient gel (VI) in supporting cell viability at room conditions. The study needed to be set up within a disposable 100 ml beaker (V), rather than a well plate, in order to accommodate the 4 g zeolite and the 500 mg gel. After the hMSCs (IV) were seeded onto the transwells (I) two set ups were used, one where the gel was placed with the zeolite within the beaker (**A**), and the other where the gel floated above the cells within the transwell (**B**). PBS (5 ml) was placed in the beaker, with an additional 5 ml within the transwell (II) so that the zeolite and the cells were in solution.

After 1, 2, 4 and 7 days of culture under room conditions, three transwells from each group were sacrificed, and the cells were analysed for viability using the CellTiter AQ One Solution Cell Proliferation Assay (2.2.3).

6.3 – Results

6.3.1 – The effect of specific nutrients on cell viability

In general, no one specific supplement provided a clear improvement in viability compared to control throughout the study (although daily supplementation with glutamax® did provide a significant improvement after days 3 to 5 of culture under room conditions) ($p < 0.05$) (figure 6.3). HEPES buffer caused a significant decrease in viability compared to control after one day of culture ($p < 0.01$). The majority of cells were found to be non-viable after 5 days of culture at room temperature.

6.3.2 – Comparison of concentrated StemPro® against standard media

After extensive shaking, the freeze-dried basal media had not fully dissolved within the liquid and, therefore, some supplements were lost when the media was filter-sterilised (data not shown). However, despite this loss of supplements, the concentrated media, following reconstitution, was found to be as effective as standard media in aiding the hMSC proliferation under optimal conditions within a tissue culture incubator (figure 6.4).

6.3.3 – Release kinetics from alginate gels

Using the method developed initially to polymerise the alginate gel, all of the glucose was found to be released into the PBS within 3 days, together with the majority of the total protein (figure 6.5). The optimized method provided a more linear release profile for both the glucose and the protein over a 7 day period, though a clear burst phase was still observed with relation to the glucose.

When the optimised polymerisation technique was applied to the concentrated StemPro® media, a reasonably constant release of protein was seen from day 4 to 7

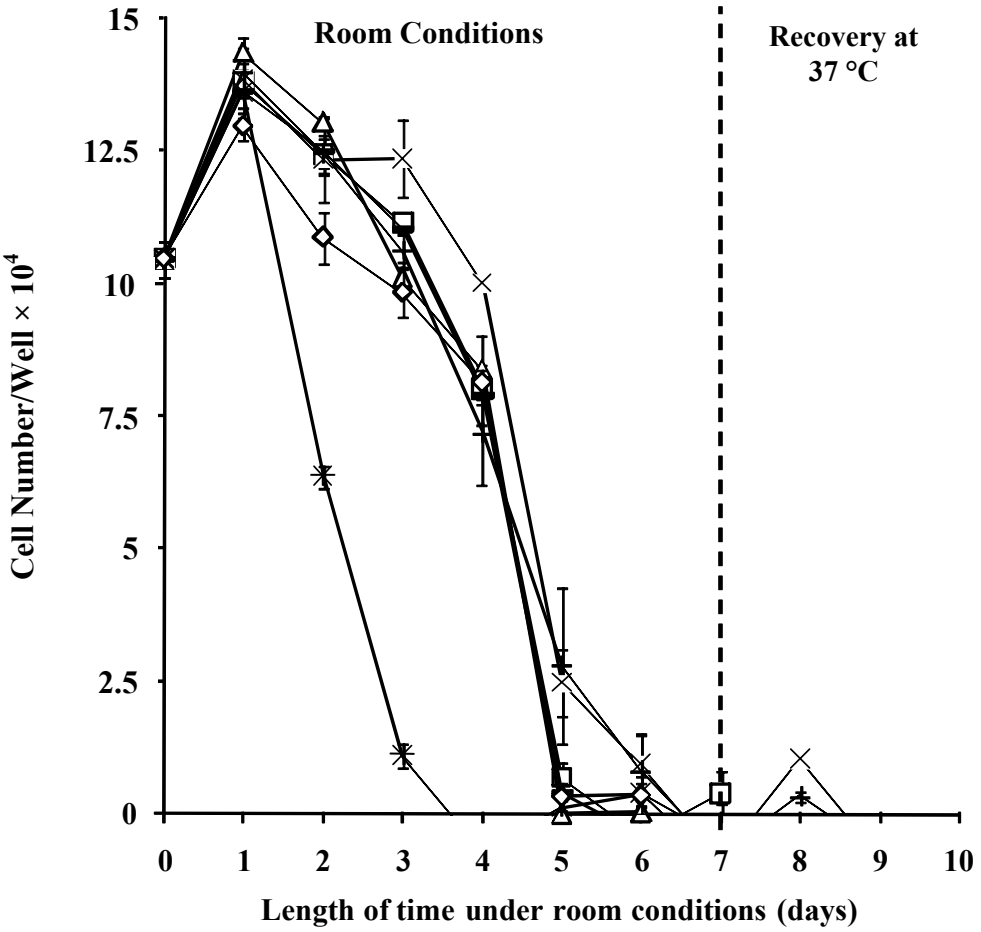


Figure 6.3. Viability of hMSCs following specific periods under ambient room conditions within 5 ml of StemPro® media supplemented daily with various nutrients, compared against no supplementation (□). The following nutrients were added to the media on a daily basis: 2 mg glucose (2.2 mM) (◇), 2 mg fructose (2.2 mM) (△), 50 µl glutamine (200mM) (+), 50 µl glutamax® (200mM) (×), 100 µl StemPro® supplement (-) and 10mM HEPES buffer (✳). Viability was determined using the alamar blue assay. Results represent mean viability ± SEM.

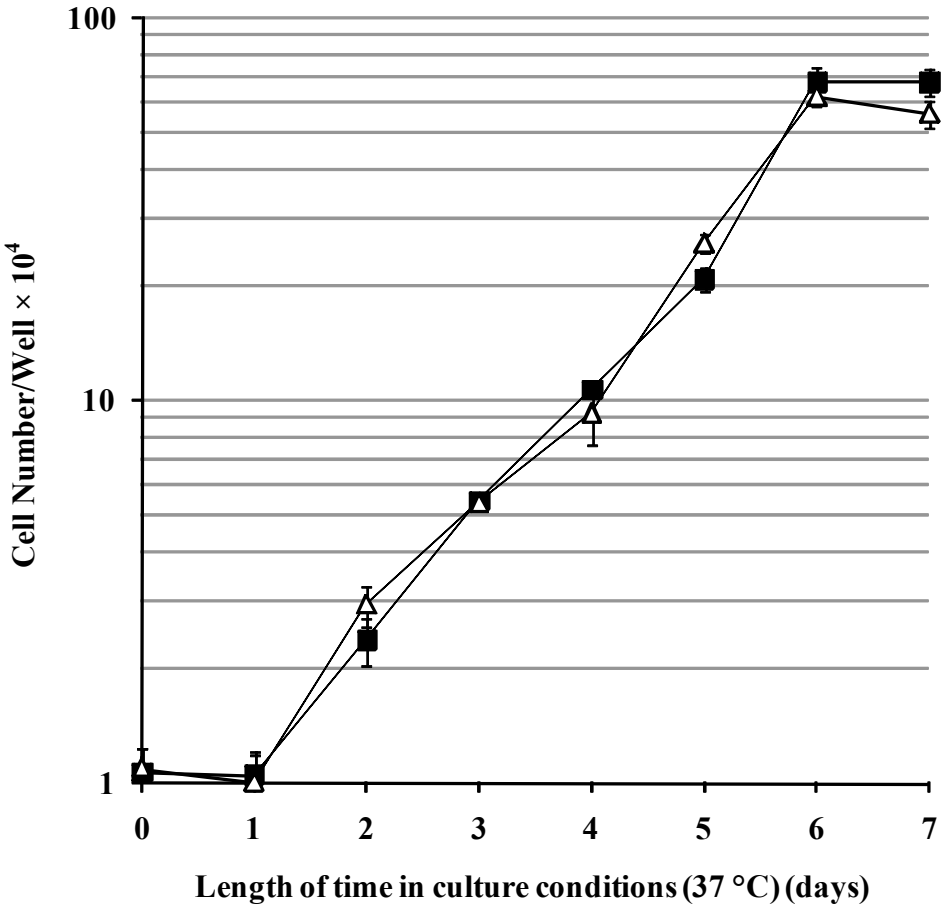


Figure 6.4. Proliferative count of hMSCs under favourable conditions, comparing complete StemPro® media (■) with 5 × concentrated media reconstituted to normal strength using tissue culture water (△). Proliferation rates were determined using the alamar blue assay. Results represent mean viability ± SEM. Sample data were plotted on an exponential axis.

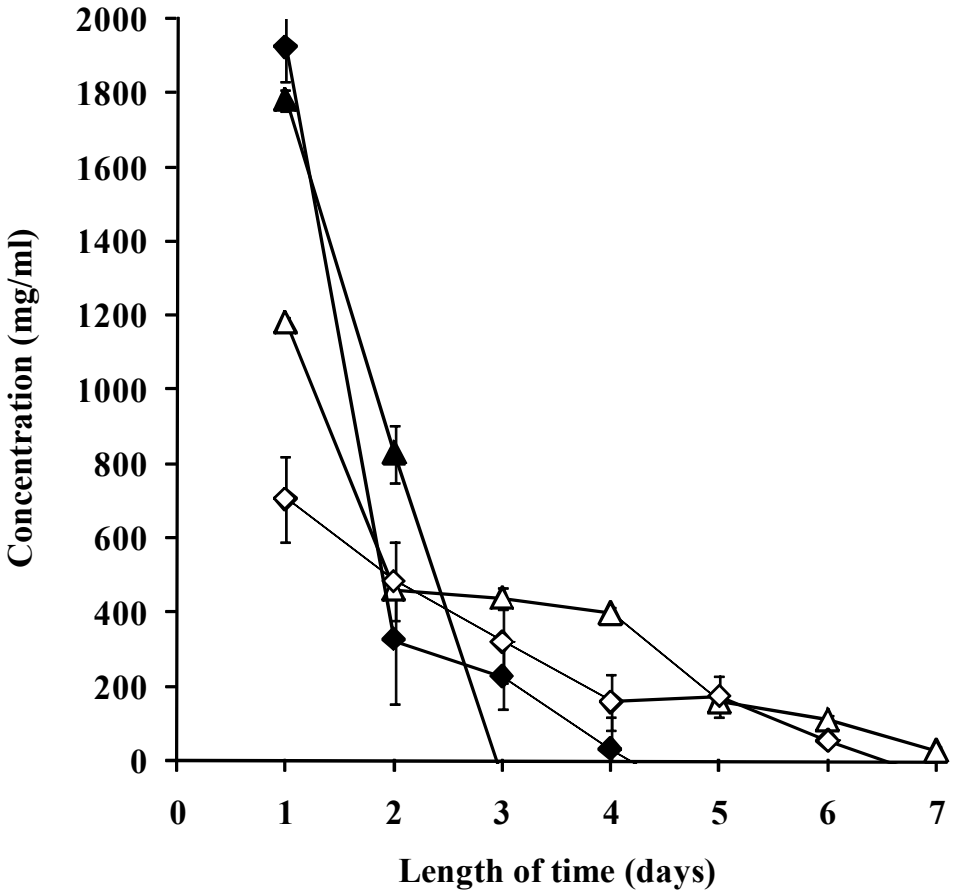


Figure 6.5. Glucose (△▲) and total protein (◇♦) concentration within 5 ml PBS following release from alginate gels (5 g) containing complete Dulbecco's modified Eagle's medium, fabricated using an initial method (black) or through an optimised method (white). After each day, the PBS was removed and replaced with fresh phosphate-buffered saline. The glucose concentration within the extracted samples was determined using the glucose oxidase assay. The total protein concentration was determined using the coomassie plus assay. Results represent mean concentration ± SEM

(figure 6.6). However, the concentration of glucose was found to be lower within the gel, leading to very low levels of glucose released at day 6 and 7.

6.3.4 – The effect of the developed nutrient release system on cell viability

Figure 6.7 documents the number of viable hMSCs after being incorporated within various nutrient release configurations. The viability profile was found to be similar to what was observed in figure 5.8; an increase in viability was seen at day 1, followed by a gradual decrease in viability. After 7 days under room conditions, the presence of the zeolite was again shown to significantly improve viability compared to no zeolite being present ($p < 0.01$). Providing the nutrients gradually every other day provided no significant improvement in viability compared to providing the nutrient at day 0 ($p < 0.05$). Neither of the gel configurations provided a significant improvement over pre-fed or regular feeding groups, instead, the gel incorporated with the zeolite was found to significantly decrease the level of viability compared to all other groups after 4 days.

6.4 – Discussion

Providing the cells with a concentrated form of all the nutrients they require would allow for the development of a more compact transportation vessel for the cells, which would, in turn, reduce the costs both of materials and transporting bulky items.

Figure 6.3 clearly shows that the cells requirements are complex and various; one nutrient alone does not provide any substantial benefit, instead, the cells require numerous nutrients at the correct levels. The cell's requirement for complex culture media has been documented previously[92]. However, this study did suggest the use of some supplements over others within the media; cells supplements with glutamax® supplement showed a significant improvement in viability at days 3 to 5 compared to both control and cells supplemented with glutamine. Glutamax® contains a stable dipeptide form of glutamine, L-alanyl-L-glutamine, that is less prone to degradation (producing ammonia) than single glutamine amino acids[130].

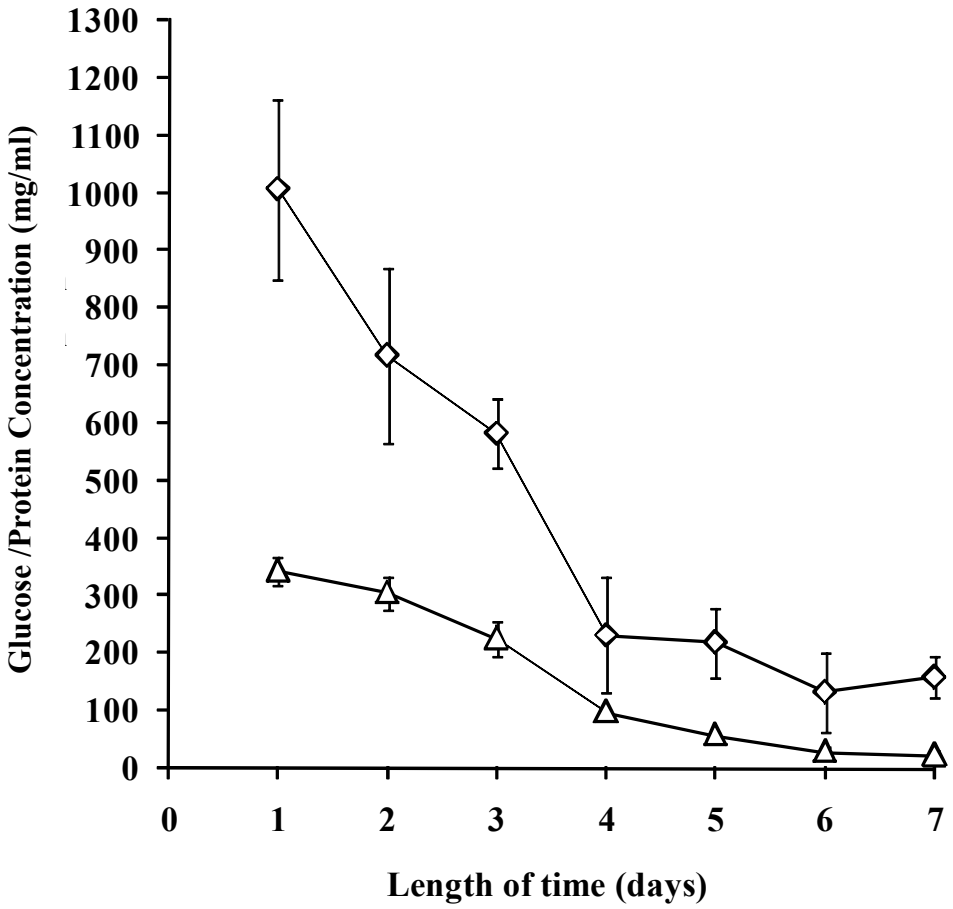


Figure 6.6. Glucose (△) and total protein (◊) concentration within 5 ml PBS following release from alginate gels (1 g) containing 5 × concentrated StemPro® media, fabricated using the optimised method. After each day, the phosphate-buffered saline was removed and replaced with fresh PBS. The glucose concentration within the extracted samples was determined using the glucose oxidase assay. The total protein concentration was determined using the Coomassie Plus assay. Results represent mean concentration ± SEM

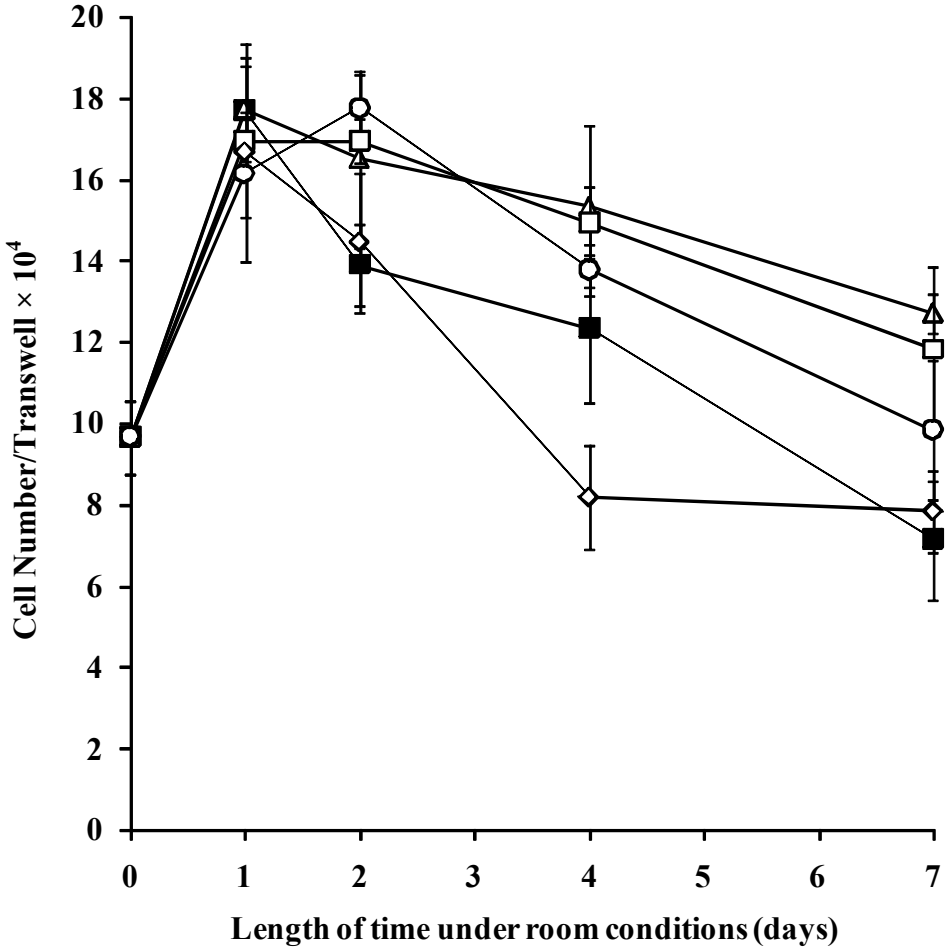


Figure 6.7. Viability of hMSCs following specific periods under ambient room conditions within PBS containing zeolite. Nutrients were provided to the cells either through pre-feeding (□), feeding at regular intervals (△), or through the use of a controlled nutrient release system (◊○) and compared to pre-fed controlled samples containing no zeolite (■). The pre-fed groups contained 500 µl of 5 × concentrated StemPro® media (equivalent to 2.5 ml standard strength media) within 5ml of phosphate-buffered saline (PBS) within the transwell, and the transwells were placed in an additional 5 ml PBS, either containing or not containing 4 g zeolite, within a 100 ml disposable beaker. The regularly fed group received 125 µl of concentrated media at days 0, 2, 4 and 6. Alginate nutrient gels (500 mg) were fabricated as described in 6.2.3, and added to the transwell set up as depicted in figure 6.2; the gel was placed either with the zeolte with the beaker (◊) or placed floating above the cells within the transwell (○). Viability was determined using the CellTiter AQ Proliferation assay. Results represent mean viability ± SEM.

Using the glutamax® supplement is thought to reduce the level of ammonia in the culture, thus improving viability, and, for this reason, glutamax supplement was used in further experiments.

It was not surprising that the level of viability would require the correct concentration of various nutrients, as, even under favourable conditions within a tissue culture incubator, the primary hMSCs were found to only proliferate in 2 culture medias specific to hMSCs, one of which being the StemPro® media. Both medias also have a secret recipe that the manufacturers do not divulge, so a concentrated form could not be created from its individual components. Because the cells prefer StemPro® media to DMEM media, means of concentrating the effective media was developed, rather than reconstituting basal DMEM media powder, which is commercially available, with a smaller volume of water than recommended. Figure 6.4 showed that the freeze-drying method was an effective means of concentrating the media, with no obvious damage or loss of vital nutrients.

The glucose and protein release profiles were not ideal; instead of providing a constant level of protein and glucose, a gradual decrease in the release of both was seen over a 7 day period. However, a burst release phase of nutrients may actually be beneficial in this circumstance; we have seen previously that cell proliferation only occurs during the first day under room conditions, suggesting that the majority of the cellular nutrient consumption would occur on that day.

A lower level of glucose was released from the gels formed with concentrated media compared with gels formed with standard media throughout the 7 days release period. This may be because there is a lower concentration of glucose in the StemPro® media than standard DMEM. This may also be due to more glucose leaching out of the gel during the prolonged period within the polymerisation buffer (as the gel was placed back into polymerisation buffer after being cut into quarters in order to stiffen the exposed edges).

When applying the concentrated media to the cellular configuration, there was no significant benefit of providing the nutrients every other day to providing the nutrients at day 0. When taking into account the cellular trend of proliferation only on the first day, one would have expected the cells that were fed every other day to have a lower level of viability compared to the pre-fed group, as the regularly fed group would have only had a quarter of nutrients required to proliferate on day 1. This raises questions regarding the level of nutrients the cells actually require.

Within the cellular experiment, half the level of nutrients (equivalent to 2.5 ml standard strength media) was applied to the system compared to the experiments carried out with the zeolite alone in Chapter 5. This was done deliberately to assess whether the level of nutrients that were required decreased with the presence of a toxin adsorbent. What is interesting is the similarity in the viability profile between that seen in figure 6.7 and figure 5.8; at day 7, a similar level of viable cell number is seen, and the rate of viable cell decrease is slightly greater in figure 5.8 than in figure 6.7. This similar level of viability may be because the nutrients were loaded into the transwell in figure 6.7, so the only way means of the zeolite accessing the nutrients was through the narrow pores (400 nm in diameter) of the transwell, which may have been preventing their passage.

Also, the cellular configurations within this chapter contained 10 ml of PBS, rather than the 5 ml of PBS used in the previous chapter, allowing for a greater volume of liquid to buffer any changes in pH and to dilute any toxins released. Unfortunately, it is difficult to incorporate both the nutrient gel and the toxin adsorbent in solution and keep the volume of liquid to a minimum.

The use of the nutrient gel did not significant improve viability compared to the pre-fed group and, instead, with regard to the gel placed with the zeolite, a significant decrease in viability occurred. This observation may have occurred for one of the following reasons; (i) the zeolite was adsorbing a nutrient vital to the cells from the gel before the it could reach the cells, (ii) the gel floating above the

cells led to a diffusion gradient through the cells, producing a steady flow of nutrients across the cells, or (iii) the transwell surface was preventing the passage of the nutrients to the cells. An effective way to test the third suggestion would be to form incisions on the transwell to ensure nutrients could flow through.

The cells cultured with the nutrient gel placed in the transwell were found to be slightly less viable than the pre-fed or the regularly fed cells. This may have been due to the lower level of glucose in the gel, or because, when the beakers were moved, the gel would move and sometimes touch the cells. This viability profile may have also been because the breakdown products of the alginate released were harmful to the cells; this reason could have been tested by producing a group that contained an alginate gel containing only PBS.

6.5 – Conclusions

No one particular nutrient was found to significantly improve hMSC viability under room conditions apart from glutamax® supplement, which was added instead of glutamine to the media used in all subsequent experiments.

It was not possible to produce a gel that produced a steady release of glucose and total protein, though it was envisioned that a burst release of nutrients may have been of benefit to the cells whilst they were proliferating during their first day in culture at room temperature.

A method of creating a 5 × concentrated StemPro® media was developed, and this media was found to be as effective as standard strength media under favourable conditions in a tissue culture incubator.

Neither regular feeding of nutrients, nor the use of a nutrient release gel, led to a significant improvement in viability compared to pre-fed cells, though the concentrated media was effective in retaining viability within the cell culture at room temperature.

7

General discussion, conclusions and future development

This thesis aimed to address, at least in part, two separate, fundamental issues with regards to translating cell therapy discoveries made within the laboratory to therapeutic products that can be routinely used to treat disease. The first fundamental issue was to establish whether delivery through narrow, parenteral devices, had a detrimental effect on cell viability, apoptosis and function (specifically cell attachment, spreading and proliferation) (covered in Chapters 3 and 4). The second fundamental issue was to determine a means of transporting viable cells at room temperature to a clinic without the need for processing at the end destination (Chapters 5 and 6).

7.1 – The effect of delivery via narrow-bore needles on cell viability, apoptosis and morphology

7.1.1 – General Discussion and Conclusions

Recent guidance issued by the regulatory body CBER (Center for Biologics Evaluation & Research) suggests that cell therapy products should have a viability of 80 % or more and show a repeatably high level of potency[72]. However, this guidance does not suggest at what stage, from culturing the cells within favourable *in vitro* conditions to delivering the cells to the patient, this level of viability is expected. The research shown in Chapters 3 and 4 assessed whether the delivery process, specifically through parenteral devices, affected viability and function of

primary murine mesenchymal stem cells (mMSCs), in order to determine whether cell viability and potency is affected by the manipulations related to cell delivery.

A significant decrease in viability was seen when mMSCs were processed from culture into a concentrated cell suspension, drawn up into a syringe with a 26s needle attached (appendix 6) and immediately ejected. This decrease in viability was not seen when the cells were processed into a cell suspension but not passed through a needle, suggesting that the physical shear stresses experienced as the cells move through the narrow bore had a detrimental effect to their survival. The noticeable increase in cell debris when comparing cells passed through a needle to control suggest that the decrease in viability is due to necrosis, although the significant increase in caspase 3-dependent apoptotic activation is also seen.

Leaving the cells within the syringe chamber at room temperature for prolonged time periods caused to a further decrease in viability and an increase in apoptosis. However, cells that were viable post-ejection were largely found to be functional with regard to their ability to spread onto tissue culture plastic (TCP) and their ability to proliferate.

When assessing whether changes in processing parameters reduced the decrease in cell viability, it was found that reducing the ejection rate or using the antioxidant *n*-acetyl cysteine did not significantly improve viability. However, using a wider bore needle (22g, 394μm) did improve viability.

Key recommendations for researchers to consider in order to reduce cellular damage during administration are to (i) use as wide a needle gauge as possible; (ii) restrict the time the cells are within a detached, concentrated cell suspension and (iii) if a delay in a surgical procedure does occur, store the cells separate from the syringe at room temperature.

7.1.2 – Future Development

The results suggested that the cells that were viable after the delivery process were able to perform simple functions, such as attaching to TCP and proliferation. However, some other functions that would essential if delivered to a patient, were overlooked. An example of this is cell differentiation; it is plausible that this form of delivery may impair the cells ability to differentiate into the required cell type, or even cause premature differentiation into a cell type different to the required cell type. Further studies are required to determine whether these effects occur.

In the study, murine mesenchymal stem cells (mMSCs) specifically were used, mainly because of the interest in MSCs as a cell source for cell therapies, as discussed in chapter 1. However, it could be argued that MSCs of a murine origin may not be representative of human-derived MSCs. There are reports showing significant differences in the cellular characteristics between different species[131], though studies similar to those reported in chapters 3 where the cells were kept within eppendorfs, performed using human marrow stromal cells, were found to be comparable to the results shown in figure 3.3[81]. Repeated studies using hMSCs are required to fully determine whether this is the case. It is hypothesised that, with regard to syringe-based manipulations, because the main insult on the cells seemed to be caused by the physical shear stresses that the cells experienced as they are passed through the needle, the physical parameters of cell size and morphology would be related to how they reacted to said stresses. These physical parameters differ only slightly when comparing MSCs sourced from humans and mice. In addition, it would be expected that cells differing in type or genetic predisposition (with regard to autologous cell therapy), with a similar size and morphology, would react in similar manner. An acceptable approach that could be used to test this hypothesis would be to repeat the study with clinically relevant primary cells that clearly differ in size, such as human embryonic stem cells (hESCs) (with hESCs being half the diameter of mesechymal stem cells[132]).

Studies have shown that smaller cellular size fractions show more favourable proliferation, cell cycle and cell differentiation characteristics compared to their larger counterparts[133]. A reason for this, suggested in some studies, relate to isolating a purer stem cell fraction (which are usually charactistically smaller) by isolating the smaller cells. However, this difference in size and function may also be related to the smaller cells being able to endure the shear stresses related to passaging and processing for the studies (described in[68]) to a greater extent than the larger cells. An interesting study would be to assess the passage of cell populations differing in size through narrow-bore needles, and assess their viability, apoptosis and morphology.

With regard to MP studies, it may be the case that cell viability had decreased with the cells seeded to the MPs because of the increased solid mass within the syringe chamber, increasing shear stress. This shear stress may be reduced by improving the number of cells that were seeded per MP, and therefore reducing the number of MPs required. This may show an improvement in viability with the seeded cells compared to single cell suspension, as was expected.

Finally, as mentioned in section 4.4, these studies have only assessed the short-term, direct effects of nAC on the cell cultures, as nAC was only exposed to the cells for limited periods of time. By culturing the cells within media containing nAC a day before the syringe-based studies were carried out, one may determine whether the indirect effects of nAC (namely the activation of glutathione) allows the cells to be more tolerant towards the stresses related to the delivery-based manipulations.

7.2 – Development of the cell transportation system

7.2.1 – General Discussion and Conclusions

The current means of transporting allogeneic cell therapy products are in a cyropreserved form, but this form of transportation has a number of drawbacks with regard to transport costs, the toxic effects of the cryopreservents and the

required processing of the cell suspension at the clinic, compromising sterility. Developing a means of transporting viable cells at room temperature would not only reduce transportation costs, but the cells could then be carried within a vessel that allows the delivery of the cells directly to the patient without any prior processing.

In order for such a transportation strategy to be feasible, evidence that clinically relevant cells would remain viable under room conditions, was required. The results shown in Chapter 5 suggest that, when placed under room conditions, and when nutrient supply and toxin dilution is ample through the provision of large volumes of culture media, primary human mesenchymal stem cells (hMSCs) enter a proliferative arrest phase that is reversible when returned to favourable temperatures. Research after this point focused on developing means of reducing the volume of culture media in order to make the end transportation vessel more compact, reducing production and shipping costs.

It was identified that ammonia was a key toxic metabolite within the culture. Zeolite and activated carbon were found to effectively adsorb ammonia from solution, but further development was carried out using zeolite alone, as our findings, together with the literature[125], suggested that zeolite would be able to adsorb ammonia more specifically and not adsorb nutrients from the solution to the same extent as activated carbon. Zeolite was found to significantly improve viability through the partial removal of ammonia. This is one of the first reports of using the adsorptive properties of such a material for an application in regenerative medicine.

It was realised that the nutrient needs of the cells were complex and that the best form of nutrient supply would be a modified form of the xeno-free StemPro® media that the cells were cultured in. The freeze-drying method used to concentrate the cell culture media appeared to be an effective means of making the end transportation device more compact without significantly comprising cell

viability. However, it was difficult to dissolve all of the nutrients into the solution at a 5 × concentration, so it is unlikely that this method could be used to make a media of a higher concentration.

A nutrient release system, using alginate, was developed to assess whether, through the prevention of the nutrients reaching toxic concentrations within the solution and through the prevention of nutrients being adsorbed by the zeolite before being consumed by the cells, the viability of the cells could be further improved. However, the nutrient release system did not provide any additional benefit compared to providing all the required nutrients at day 0.

In conclusion, the results showed herein suggest that hMSCs remained viable in a reversible proliferative arrest phase for up to 4 days under room conditions, suggesting that it is feasible to develop a vessel that is able to transport cells under such conditions. Zeolite is a toxin adsorbent that showed an ability to adsorb ammonia from solution, and its introduction improved the viability of cells cultured under room conditions. The use of a controlled nutrient release system provided no improvement in cell viability compared to the provision of all nutrients at day 0, though a means of concentrating the cell culture media did allow a smaller volume of media to be used without compromising cell viability.

7.2.2 – Future Development

The reversible arrest phase observed is not only a beneficial finding with regard to the development of the transportation device, but it is also an interesting observation with regards to the cellular mechanisms involved, as there are very few reports of reversible arrest in stem cells. Moreover, this is a useful finding for transporting cell lines for research purposes; if a cell line needs transporting over a short period of time, rather than incurring the costs linked with cyropreserved transportation, transportation can instead occur within a sealed container under room conditions with little loss in viability. However, as this arrest phase has only

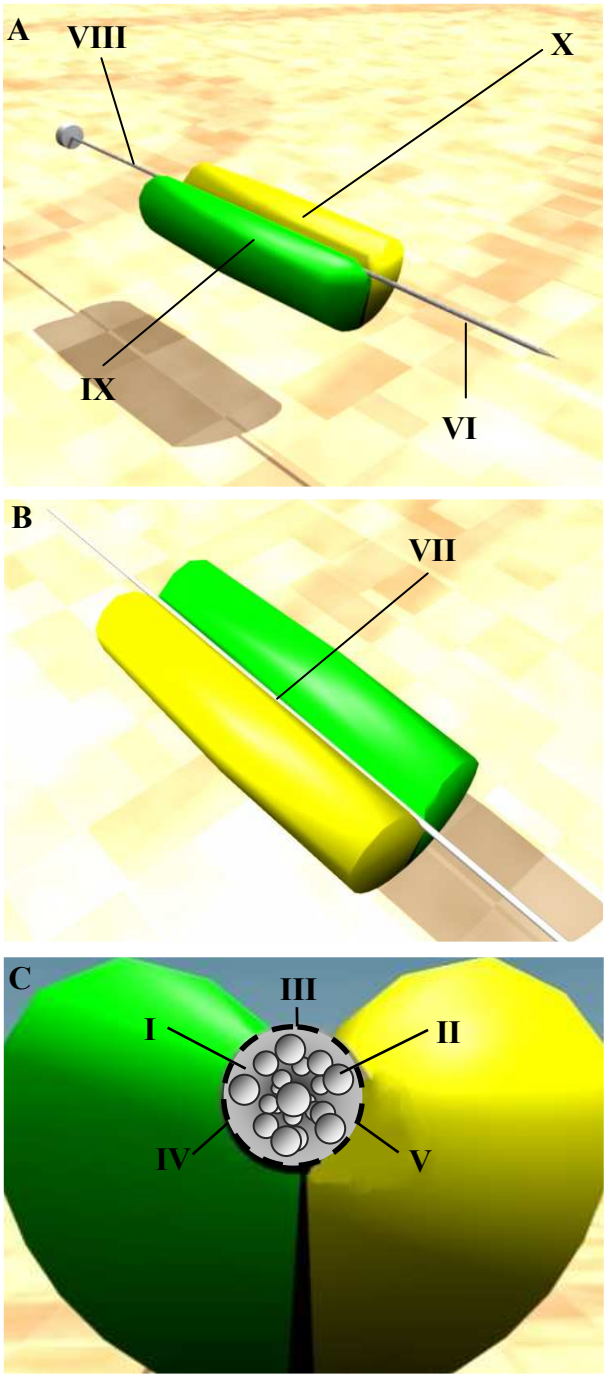


Figure 7.1. Schematic of final cell transportation device, showing a side (A), top (B), and a focused frontal view of the syringe chamber (C). The syringe chamber (I), containing cells seeded onto microparticles (II), would consist of a gas-permeable membrane (III), a toxin permeable membrane (IV) and a nutrient permeable membrane (V). The cell transportation device would be designed as a ready-to-use syringe, with needle (VI), syringe chamber (VII) and plunger (VIII) to allow ease of use in the clinic. The system would mainly consist of a toxin adsorbent (IX) and a nutrient supply (X).

been observed in hMSCs, these studies would need to be applied to a wide variety of cells in order to determine whether a similar cell state is seen.

Research is also required in order to assess how cells react to a variety of temperatures other than what has been referred to as "room" temperatures. All previous experiments had been carried out within cell culture facilities that were maintained at a temperature of about 20 °C, but it would be envisioned that, during transportation throughout the winter months across continental countries, the temperatures the cells would experience would be much lower than 20 °C. For this reason, it would be important to determine whether cell viability was significantly affected by temperatures closer to freezing.

With regards to the development of the transportation device, figure 7.1 depicts how the vessel may appear. Key components of the transportation device include the following:-

- The device would be in the form of a syringe, with a needle (or a luer lock adaptor to allow the attachment of a needle) and plunger, so no manipulations of the cell suspension are required other than the delivery into the patient. This would ensure that the cell suspension remains sterile.
- The device would consist largely of 3 sections, the syringe chamber (containing cells), the nutrient release and the toxin adsorbent. Through the results shown in figure 6.7, the nutrient supply and toxin adsorbent should be kept separate to create an osmotic gradient of nutrients across the cells and to ensure that no nutrients are adsorbed from the nutrient supply by the adsrobent before reaching the cells.
- The syringe chamber would be a relatively small part of the syringe device, to ensure that the volume of liquid delivered to the patient is kept to a minimum. If the cells being transported are attachment-dependent, the cells would be pre-seeded onto a microparticle (MP) scaffold, such as the poly(glycolic-*co*-lactic acid) (PLGA) MPs described in chapter 3. Because polymers such as

PLGA are approved by regulatory bodies to be inserted into the body, the MPs could be delivered to the patient together with the cells.

- The toxin adsorbent would consist of a form of zeolite material for ammonia removal. This adsorbent would need to be in solution for effective adsorption to occur. Analysis of zeolite modification is required to determine whether the adsorptive capacity can be improved, which would allow the volume of zeolite required to be reduced. Modifications include those suggested by Sprynskyy *et al*; they showed that zeolite that had been cation-modified had improved ammonia-adsorptive properties[128].
- The nutrient supply would not be in the form of a gel, but would be concentrated media liquid. The nutrient compartment may not be the same size as the toxin adsorbent section, in contrary to what is shown in figure 7.1, as the volume of zeolite used in chapter 6 was greater than the volume of concentrated media.
- Separating the nutrient supply, syringe chamber and the toxin absorbent would be a membrane that is permeable to nutrients and toxins, but not to cells. The design of the membrane would be simplified if the cells were seeded onto MPs, as the pore size of the membrane could be increased to one that only retains the MPs. The membrane would need to be stiff, so that the plunger can flow smoothly through the syringe chamber without any flex, to ensure all of the cell suspension is delivered and not left within the syringe. The volume of liquid used to fill the syringe would also need to be very accurate to ensure that no air bubbles were within the syringe, so that, when the plunger is pushed, only liquid cell suspension would be ejected.
- An opening at the top of the transportation device would allow the passage of gases to and from the syringe chamber containing the cells via a gas permeable membrane. This part of the syringe would be complicated to design, as the membrane would still need to be stiff for the reasons discussed above, but allow gases through without liquid. Polytetrafluoroethylene (Gore-tex®) material, (currently used in waterproof, breathable clothing), has been shown to be biocompatible and used as breathable surgical barrier fabric[134].

Polydimethylsiloxane (PDMS) is another polymer known for its gas permeable properties, and has been shown to be effective as a cellular substrate within a bioreactor[135]. These polymers may have the properties required for this device.

- The transportation device would be disposable, being made of relatively cheap materials similar to those utilised in syringes used currently in the clinic. A disposable device would ensure that the cell suspension delivered was sterile. The toxin adsorbent, although difficult to source, is inexpensive, so the most expensive part of the device would be the cell culture media.

The demand for such a device relies heavily on the successful identification of a specific allogeneic cell source being effective in treating a disease state. Furthermore, the global demand to treat the identified disease state would need to be great, both in terms of the number of patients with the disease and in terms of the current cost for treating the disease. This is because the cost of developing and producing such a cell therapy product would arguably be far greater than the cost of developing the equivalent pharmaceutical product. However, the number of reports suggesting that effectiveness of ESCs and induced pluripotent stem cells in a wide variety of disease states is increasing. Moreover, some of these allogeneic therapies are now moving from the developmental and animal trial stages to clinical trials; the FDA has recently approved its first clinical trial using ESC-derived products in the form of a glial oligodendrocyte injection for complete spinal cord injury repair, developed by the Geron Corporation[136]. These oligodendrocytes are not seeded onto any form of scaffold or conduit; instead this product is a form of cell therapy that is cyropreserved for transportation purposes[137]. The development of such a product is very encouraging for the work described in chapters 5 and 6, as the first trial approval gives confidence to the other companies developing cell therapy products[138], all of which may benefit from the cell transportation device described here, thus establishing a potential stable market for this product in the future.

References

1 Salter, B. & Harvey, O. Stem Cell Innovation in the USA: the benefits of the minimal state. *Regenerative Medicine* **3**, 597-610 (2008).

2 Overfield, J., Dawson, M. & Hamer, D. *Transfusion Science*. 2nd edn, (Scion Publishing Ltd., 2007).

3 Kobayashi, N. Artificial Cell for the Development of Cell Therapy. *Cell Transplantation* **17**, 3-9 (2008).

4 Shapiro, A. M. J. *et al.* International Trial of the Edmonton Protocol for Islet Transplantation. *New England Journal of Medicine* **355**, 1318-1330 (2006).

5 Jiang, J. J. *et al.* Generation of insulin-producing islet-like clusters from human embryonic stem cells. *Stem Cells* **25**, 1940-1953 (2007).

6 Jackson, L., Jones, D. R., Scotting, P. & Sottile, V. Adult mesenchymal stem cells: Differentiation potential and therapeutic applications. *Journal of Postgraduate Medicine* **53**, 121-127 (2007).

7 De Bari, C. & Dell'Accio, F. Cell Therapy: A challenge in modern medicine. *Bio-Medical Materials and Engineering* **18**, S11-S17 (2008).

8 Dietz, A., Padley, D. & Gastineau, D. Infrastructure development for human cell therapy translation. *Clinical Pharmacology & Therapeutics* **82**, 320-324 (2007).

9 Banfi, A. *et al.* Proliferation kinetics and differentiation potential of ex vivo expanded human bone marrow stromal cells: Implications for their use in cell therapy. *Experimental Hematology* **28**, 707-715 (2000).

10 Engelmann, M. & Franz, W. Stem Cell Therapy after myocardial infarction: Ready for clinical application? *Current Opinion in Molecular Therapeutics* **8**, 396-414 (2006).

11 Hernigou, P., Poignard, A., Baeujean, F. & Rouard, H. Percutaneous Autologous Bone-Marrow Grafting for Nonunions. *Journal of Bone & Joint Surgery America* **87**, 1430-1437 (2005).

12 Zhao, R. & Daley, G. From fibroblasts to iPS cells: induced pluripotency by defined factors. *Journal of Cellular Biochemistry* **105**, 949-955 (2008).

13 Kaji, K. *et al.* Virus-free induction of pluripotency and subsequent excision of reprogramming factors. *Nature* **458**, 771-775 (2009).

14 Knoepfler, P. Deconstructing stem cell tumorigenicity: a roadmap to safe regenerative medicine. *Stem Cells* **27**, 1050-1056 (2009).

15 Fändrich, F., Dresske, B., Bader, M. & Schulze, M. Embryonic stem cells shar immune-privileged features relevant for tolerance induction. *Journal of Molecular Medicine* **80**, 343-350 (2002).

16 Swijnenburg, R. *et al.* Embryonic Stem Cell Immunogenicity Increases Upon Differentiation After Transplantation Into Ischemic Myocardium. *Circulation* **112**, I-166-I-172 (2005).

17 Arinzeh, T. *et al.* Allogeneic Mesenchymal Stem Cells Regenerate Bone in a Critical-Sized Canine Segmental Defect. *Journal of Bone & Joint Surgery America* **85**, 1927-1935 (2003).

18 Niemeyer, P. *et al.* Mesenchymal Stem Cell-Based HLA-Independent Cell Therapy for Tissue Engineering of Bone and Cartilage. *Current Stem Cell Research & Therapy* **1**, 21-27 (2006).

19 Tse, W., Pendleton, J., Beyer, W., Egalka, M. & Guinan, E. Suppression of Allogeneic T-Cell Proliferation by Human Marrow Stromal Cells: Implications in Transplantation. *Transplantation* **75**, 389-397 (2003).

20 De Bari, C. *et al.* Skeletal muscle repair by adult human mesenchymal stem cells from synovial membrane. *Journal of Cell Biology* **160**, 909-918 (2003).

21 Grinnemo, K. *et al.* Xenoreactivity and engraftment of human mesenchymal stem cells transplanted into infarcted rat myocardium. *Journal of Thoracic and Cardiovascular Surgery* **127**, 1293-1300 (2004).

22 Orive, G., Hernandez, R. & Gascon, A. Cell encapsulation: promise and progress. *Nature Medicine* **9**, 104-107 (2003).

23 Jones, E. & McGonagle, D. Human bone marrow mesenchymal stem cells in vivo. *Rheumatology* **47**, 126-131 (2008).

24 Wang, X., Willenbring, H. & Akkari, Y. Cell fusion is the principal source fo bone marrow-derived hepatocytes. *Nature* **422**, 897-901 (2003).

25 Goncalves, M. *et al.* Human mesenchymal stem cells ectopically expressing full-length dystrophin can complement Duchenne muscular dystrophy myotubes by cell fusion. *Human Molecular Genetics* **15**, 213-221 (2006).

26 Bonab, M. *et al.* Aging of mesenchymal stem cell in vitro. *BMC Cell Biology* **7**, 1-7 (2006).

27 Pal, R., Hanwate, M., Jan, M. & Totey, S. Phenotypic and functional comparison of optimum culture conditions for upscaling of bone marrow-derived mesenchymal stem cells. *Journal of Tissue Engineering and Regenerative Medicine* **3**, 163-174 (2008).

28 Böcker, W. *et al.* Introducing a single-cell-derived human mesenchymal stem cell line expressing hTERT after lentiviral gene transfer. *J. Cell. Mol. Med.* **12**, 1347-1359 (2008).

29 Chang, S. *et al.* Restoration of left ventricular synchronous contraction after acute myocardial infarction by stem cell therapy: new insights into the therapeutic implication of stem cell therapy for acute myocardial infarction. *Heart* **94**, 995-1001 (2008).

30 Gyöngyösi, M. *et al.* Combined delivery approach of bone marrow mononuclear stem cells early and late after myocardial infarction: the MYSTAR prospective, randomized study. *Nature Clinical Practice: Cardiovascular Medicine* **6**, 70-81 (2009).

31 Huikuri, H. *et al.* Effects of intracoronary injection of mononuclear bone marrow cells on left ventricular function, arrhythmia risk profile, and

restenosis after thrombolytic therapy of acute myocardial infarction. *European Heart Journal* **29**, 2723-2732 (2008).

32 Hirsch, A. *et al.* Intracoronary infusion of autologous mononuclear bone marrow cells in patients with acute myocardial infarction treated with primary PCI: Pilot study of the multicenter HEBE trial. *Catheterization and cardiovascular interventions* **71**, 273-281 (2008).

33 Martin-Rendon, E. *et al.* Autologous bone marrow stem cells to treat acute myocardial infarction: A systematic review. *European Heart Journal* **29**, 1807-1818 (2008).

34 Wakabayashi, K. *et al.* Transplantation of human mesenchymal stem cells promotes functional improvement and increased expression of neurotrophic factors in a rat focal cerebral ischemia model. *Journal of Neuroscience Research*, doi:10.1002/jnr.22279 (2009).

35 Schachinger, V. *et al.* Intracoronary Bone Marrow-Derived Progenitor Cells in Acute Myocardial Infarction. *New England Journal of Medicine* **355**, 1210-1221 (2006).

36 Wollert, K. Cell therapy for acute myocardial infarction. *Current Opinion in Pharmacology* **8**, 202-210 (2008).

37 Beeres, S. *et al.* Role of imaging in cardiac stem cell therapy. *Journal of the American College of Cardiology* **49**, 1137-1148 (2007).

38 Ward, M., Stewart, D. & Kutryk, M. Endothelial progenitor cell therapy for the treatment of coronary disease, acute MI, and pulmonary arterial hypertension: current perspectives. *Catheterization and cardiovascular interventions* **70**, 983-998 (2007).

39 Meyer, G. P. *et al.* Intracoronary bone marrow cell transfer after myocardial infarction - Eighteen months' follow-up data from the randomized, controlled BOOST (BOne marrOw transfer to enhance ST-elevation infarct regeneration) trial. *Circulation* **113**, 1287-1294 (2006).

40 Korf-Klingebiel, M. *et al.* Bone marrow cells are a rich source of growth factors and cytokines: implications for cell therapy trials after myocardial infarction. *European Heart Journal* **29**, 2851-2858 (2008).

41 Le Blanc, K., Tammik, L., Sundberg, B., Haynesworth, S. & Ringdén, O. Mesenchymal Stem Cells Inhibit and Stimulate Mixed Lymphocyte Cultures and Mitogenic Responses Independently of the Major Histocompatibility Complex. *Scandinavian Journal of Immunology* **57**, 11-20 (2003).

42 Ozawa, K. *et al.* Cell and gene therapy using mesenchymal stem cells (MSCs). *Journal of Autoimmunity* **30**, 121-127 (2008).

43 Abdi, R., Fiorina, P., Adra, C., Atkinson, M. & MH, S. Immunomodulation by mesenchymal stem cells: a potential therapeutic strategy for type 1 diabetes. *Diabetes* **57**, 1759-1767 (2008).

44 Le Blanc, K. *et al.* Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. *The Lancet* **363**, 1439-1441 (2004).

45 Ringdén, O. *et al.* Mesenchymal stem cells for treatment of therapy-resistant graft-versus-host disease. *Transplantation* **81**, 1390-1397 (2006).

[46] Le Blanc, K. *et al.* Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. *Lancet* **371**, 1579-1586 (2008).

[47] Miura, Y., Kami, M., Tsubokura, M., Takei, N. & Komatsu, T. Mesenchymal stem cells for acute graft-versus-host disease. *Lancet* **372**, 715-716 (2008).

[48] Arthur, A., Zannettino, A. & Gronthos, S. The therapeutic applications of multipotential mesenchymal/stromal stem cells in skeletal tissue repair. *J Cell Physiol* **218**, 237-245 (2009).

[49] Jones, D. & Peterson, L. Autologous chondrocyte implantation. *Journal of Bone & Joint Surgery* **88**, 2501-2520 (2006).

[50] Robinson, D. & Nevo, Z. Articular cartilage chondrocytes are more advantageous for generating hyaline-like cartilage than mesenchymal cells isolated from microfracture repairs. *Cell Tissue Bank* **2**, 23-30 (2001).

[51] Richardson, R. T. *et al.* The effect of polypyrrole with incorporated neurotrophin-3 on the promotion of neurite outgrowth from auditory neurons. *Biomaterials* **28**, 513-523 (2007).

[52] Meisel, H. *et al.* Clinical Experience in cell-based therapeutics: disc chondrocyte transplantation A treatment for degenerated or damaged intervertebral disc *Biomolecular Engineering* **24**, 5-21 (2007).

[53] Trivedi, H. L. *et al.* Human Adipose Tissue-Derived Mesenchymal Stem Cells Combined With Hematopoietic Stem Cell Transplantation Synthesize Insulin. *Transplantation Proceedings* **40**, 1135-1139 (2008).

[54] Bartholomew, A. *et al.* Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. *Experimental Hematology* **30**, 42-48 (2002).

[55] García-Olmo, D. *et al.* A phase I clinical trial of the treatment of Crohn's fistula by adipose mesenchymal stem cell transplantation. *Diseases of the colon and rectum* **48**, 1416-1423 (2005).

[56] Kassis, I. *et al.* Neuroprotection and immunomodulation with mesenchymal stem cells in chronic experimental autoimmune encephalomyelitis. *Archives of Neurology* **65**, 753-761 (2008).

[57] Wang, H. & Chen, X. Imaging mesenchymal stem cell migration and the implications for stem cell-based cancer therapies. *Future Oncology* **4**, 623-628 (2008).

[58] Oh, J. *et al.* The Anti-Inflammatory and Anti-Angiogenic Role of Mesenchymal Stem Cells in Corneal Wound Healing Following Chemical Injury. *Stem Cells* **26**, 1047-1055 (2008).

[59] Loebinger, M., Sage, E. & Janes, S. Mesenchymal stem cells as vectors for lung disease. *Proceedings of the American Thoracic Soceity* **5**, 711-716 (2008).

[60] Siniscalco, D., Sullo, N., Maione, S., Rossi, F. & D'Agostino, B. Stem cell therapy: the great promise in lung disease. *Therapeutic Advances in Respiratory Disease* **2**, 173-177 (2008).

[61] Ortiz, L. *et al.* Interleukin 1 receptor antagonist mediates the antiinflammatory and antifibrotic effect of mesenchymal stem cells during

lung injury. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 11002-11007 (2007).

62 Xu, Y. & Liu, Z. Therapeutic potential of adult bone marrow stem cells in liver disease and delivery approaches. *Stem Cell Reviews* **4**, 101-112 (2008).

63 Burt, R. *et al.* Clinical applications of blood-derived and marrow-derived stem cells for nonmalignant diseases. *Journal of the American Medical Association* **299**, 925-936 (2008).

64 Helmchen, F. & Denk, W. Deep tissue two-photon microscopy. *Nature Methods* **2**, 932-940 (2005).

65 Kraitchman, D., Gilson, W. & Lorenz, C. Stem cell therapy: MRI guidance and monitoring. *Journal Of Magnetic Resonance Imaging* **27**, 299-310 (2008).

66 Doyle, B. *et al.* Dynamic Tracking During Intracoronary Injection of ^{18}F-FDG-Labeled Progenitor Cell Therapy for Acute Myocardial Infarction. *Journal of Nuclear Medicine* **48**, 1708-1714 (2007).

67 Lin, S. *et al.* Quantum dot imaging for embryonic stem cells. *BMC Biotechnology* **7** (2007).

68 Veraitch, F., Scott, R., Wong, J.-W., Lye, G. & Mason, C. The Impact of Manual Processing on the Expansion and Directed Differentiation of Embryonic Stem Cells. *Biotechnology and Bioengineering* **99**, 1216 (2008).

69 Kondziolka, D., Steinberg, G., Cullen, S. & McGrogan, M. Evaluation of Surgical Techiques for Neuronal Cell Transplantation Used in Patients With Stroke. *Cell Transplantation* **13**, 749-754 (2004).

70 McCarthy, N. & Bennett, M. in *Apoptosis: The molecular biology of programmed cell death* eds MD Jacobson & NJ McCarthy) 200-234 (Oxford University Press, 2002).

71 Gastineau, D. Will regulation be the death of cell therapy in the United States? *Bone Marrow Transplantation* **33**, 777-780 (2004).

72 Center for Biologics Evaluation and Research, C. B. E. R. *Guidance for Industry. Potency tests for cellular and gene therapy products.*, <http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/CellularandGeneTherapy/ucm072571.htm> (2008).

73 Weber, D., McFarland, R. & Irony, I. Selected Food and Drug Administration review issues for regulation of allogeneic islets of langerhans as somatic cell therapy. *Transplantation* **74**, 1816-1820 (2002).

74 Moustafa, M. *et al.* Randomized, controlled, single-blind study on use of autologous keratinocytes on a transfer dressing to treat nonhealing diabetic ulcers. *Regenerative Medicine* **2**, 887-902 (2007).

75 Frey, N., Lazarus, H. & Goldstein, S. Has allogeneic stem cell cryopreservation been given the 'cold shoulder'> An analysis of the pros and cons of using frozen versus fresh stem cell products in allogeneic stem cell transplantation. *Bone Marrow Transplantation* **38**, 399-405 (2006).

[76] Fahy, G., Wowk, B. & Wu, J. Cryopreservation of Complex Systems: The Missing Link in the Regenerative Medicine Supply Chain. *Rejuvenation Research* **9**, 279-291 (2006).

[77] Brockbank, K. Stabilization of tissue-engineered products for transportation and extended shelf-life. *Annals of the New York Academy of Sciences* **961**, 265-267 (2002).

[78] Olarewaju, O., Pegg, D., Genever, P. & Hunt, C. Designing an optimal cryopreservation protocol for human embryonic stem cells: A methodological approach. *Cryoletters* **30**, 83 (2009).

[79] Flasza, M. *et al.* Development and manufacture of an investigational human living dermal equivalent (ICX-SKN). *Regenerative Medicine* **2**, 903-918 (2007).

[80] Heroux, A. & Seymour, B. Thermally insulated transport container system for cultured cells, comprises closable container having a thermally insulated portion with a blown polyurethane or styrofoam, and sealable canister and refrigerant within the container. WO2007033051-A2; US2008276643-A1 (2008).

[81] Lane, T., Garls, D., Mackintosh, E., Kohli, S. & Cramer, S. Liquid Storage of Marrow Stromal Cells. *Transfusion* **49**, 1471-1481 (2009).

[82] Utheim, T. *et al.* A novel method for preserving cultured limbal epithelial cells. *British Journal of Ophthalmology* **91**, 797-800 (2007).

[83] Da Silva Meirelles, L. & Nardi, N. B. Murine marrow-derived mesenchymal stem cell: Isolation, in vitro expansion, and characterization. *British Journal of Haematology* **123**, 702-711 (2003).

[84] Soleimani, M. & Nadri, S. A protocol for isolation and culture of mesenchymal stem cells from mouse bone marrow. *Nature Protocols* **4**, 102-106 (2009).

[85] Shamovsky, I., Ivannikov, M., Kandel, E., Gershon, D. & Nudler, E. RNA-Mediated Response to Heat Shock in Mammalian Cells. *Nature* **440**, 556-560 (2006).

[86] Ahmed, S. A., Gogal, R. M. & Walsh, J. E. A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [3H]thymidine incorporation assay. *Journal of Immunological Methods* **170**, 211-224 (1994).

[87] De Fries, R. & Mitsuhashi, M. Quantification of Mitogen Induced Human Lymphocyte Proliferation: Comparison of AlamarBlue Assay to ^{3}H-Thymidine Incorporation Assay. *Journal Of Clinical Laboratory Analysis* **9**, 89-95 (1995).

[88] Hamid, R., Rotshteyn, Y., Rabadi, L., Parikh, R. & Bullock, P. Comparison of alamar blue and MTT assays for high through-put screening. *Toxicology in Vitro* **18**, 703-710 (2004).

[89] Bradford, M. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. *Analytical Biochemistry* **72**, 248-254 (1976).

[90] Bible, E. *et al.* The support of neural stem cells transplanted into stroke-induced brain cavities by PLGA particles. *Biomaterials* **30**, 2985-2994 (2009).

91 Cotgrave, I. A. N-acetylcysteine: pharmacological considerations and experiemental and clinical applications. *Advances in Pharmacology* **38**, 205-227 (1996).

92 Singh, R. & Al-Rubeai, M. Apoptosis and bioprocess technology. *Advances in Biochemical Engineering/Biotechnology* **62**, 167-184 (1998).

93 Sokolsky-Papkov, M., Agashi, K., Olaye, A., Shakesheff, K. M. & Domb, A. J. Polymer Carriers for drug delivery in tissue engineering. *Adv Drug Deliv Rev* **59**, 187-206 (2007).

94 Chau, D., Agashi, K. & Shakesheff, K. Microparticles as tissue engineering scaffolds: manufacture, modification and manipulation. *Materials Science and Technology* **24**, 1031-1044 (2008).

95 Barry, J. J. A., M.M.C.G., S., Shakesheff, K. M., Howdle, S. M. & Alexander, M. R. Using Plasma Deposits to Promote Cell Population of the Porous Interior of Three-Dimensional Poly(D,L-Lactic Acid) Tissue-Engineering Scaffolds. *Advanced Functional Materials* **15**, 1134-1140 (2005).

96 Hughes, D. & Mehmet, H. *Cell Proliferation and Apoptosis*. (BIOS Scientific Publishers Limited, 2003).

97 Jones, R. A., Nicholas, B., Mian, S., Davies, P. J. & Griffin, M. Reduced expression of tissue transglutaminase in a human endothelial cell line leads to changes in cell spreading, cell adhesion and reduced polymerisation of fibronectin. *Journal of Cell Science* **110**, 2461-2472 (1997).

98 Humpe, A., Beck, C., Schoch, R., Kneba, M. & Horst, H.-A. Establishment and optimization of a flow cytometric method for evaluation of viability of CD34+ cells after cryopreservation and comparison with trypan blue exclusion staining. *Transplantation and Cellular Engineering* **45**, 1208-1213 (2005).

99 Ma, E. *et al.* The Effect of Superoxide Dismutase on the Recovery of Human Bone Marrow Hemopoietic Stem Cells Stored at 4°C. *Cryobiology* **37**, 372-375 (1998).

100 Cotter, T. & Al-Rubeai, M. Cell death (apoptosis) in cell culture systems. *Trends in Biotechnology* **13**, 150-155 (1995).

101 Thomas, C., Al-Rubeai, M. & Zhang, Z. Prediction of mechanical damage to animal cells in turbulence. *Cytotechnology* **15**, 329-335 (1994).

102 Al-Rubeai, M., Emery, A., Chalder, S. & Goldman, M. A flow cytometric study of hydrodynamic damage to mammalian cells. *Journal of Biotechnology* **31**, 161-177 (1993).

103 Muscari, C. *et al.* Long-term treatment with N-acetylcysteine, but not caloric restriction, protects mesenchymal stem cells of aged rats against tumor necrosis factor-induced death. *Experimental Gerontology* **41**, 800-804 (2006).

104 Arakawa, M. & Ito, Y. N-acetylcysteine and neurodegenerative diseases: Basic and clinical pharmacology. *Cerebellum* **6**, 308-314 (2007).

105 Ying, M. *et al.* MSFTZ, a flavanone derivative, induces human hepatoma cells apoptosis via a reactive oxygen species and caspase-dependent mitochondrial pathway. *Journal of Pharmacology and Experimental Therapeutics* **325**, 758-765 (2008).

106 Tsai, J.-C. *et al.* Induction of Apoptosis by Pyrrolidinedithiocarbamate and *N*-Acetylcysteine in Vascular Smooth Muscle Cells. *Journal of Biological Chemistry* **271**, 3667-3670 (1996).

107 Qanungo, S., Wang, M. & Nieminen, A.-L. N-Acetyl-L-cysteine Enhances Apoptosis through Inhibition of Nuclear Factor-□B in Hypoxic Murine Embryonic Fibroblasts. *Journal of Biological Chemistry* **279**, 50455-50464 (2004).

108 Kelly, G. Clinical Applications of N-acetylcysteine. *Alternative Medical Review* **3**, 114-127 (1998).

109 Tait, J. Imaging of apoptosis. *Journal of Nuclear Medicine* **49**, 1573-1576 (2008).

110 Lock, J., Wehrle-Haller, B. & Stromblad, S. Cell-matrix adhesion complexes: master control machinery of cell migration. *Seminars in Cancer Biology* **18**, 65-76 (2007).

111 Martin, K., Slack, J., Boerner, S., Martin, C. & Parsons, J. Integrin connections map: to infinity and beyond. *Science* **296**, 1652-1653 (2002).

112 Schwarz, U. S. & Bischofs, I. B. Physical determinants of cell organization in soft media. *Medical Engineering & Physics* **27**, 763-772 (2005).

113 Arnalich-Montiel, F. *et al.* Adipose-Derived Stem Cells Are a Source for Cell Therapy of the Corneal Stroma *Stem Cells* **26**, 570-579 (2008).

114 Pompilio, G. *et al.* Autologous Peripheral Blood Stem Cell Transplantation for Myocardial Regeneration: A Novel Strategy for Cell Collection and Surgical Injection. *The Annals of Thoracic Surgery* **78**, 1808-1813 (2004).

115 Hung, S.-C. *et al.* Mesenchymal Stem Cell Targeting of Microscopic Tumors and Tumor Stroma Development Monitored by Noninvasive In vivo Positron Emission Tomography Imaging. *Clinical Cancer Research* **11**, 7749-7756 (2005).

116 Allahham, A., Stewart, P., Marriott, J. & Mainwaring, D. Flow and injection characteristics of pharmaceutical parenteral formulations using a micro-capillary rheometer. *International Journal of Pharmaceutics* **270**, 139-148 (2004).

117 Glossop, J. & Cartmell, S. Mesenchymal stem cells and tensile strain. *Tissue Eng* **13**, 1724-1724 (2007).

118 Langstrom, P. *Laminar Flow Theory*. (Princeton University Press, 1996).

119 Macip, S., Kosoy, A., Lee, S., O'Connell, M. & Aaronson, S. Oxidative stress induces a prolonged but reversible arrest in p53-null cancer cells, involving a Chk1-dependent G2 checkpoint. *Oncogene* **25**, 6037-6047 (2006).

120 Stolzing, A., Sethe, S. & Scutt, A. Stressed Stem Cells: Temperature response in aged mesenchymal stem cells. *Stem Cells and Development* **15**, 478-487 (2006).

121 Schneider, M., Marison, I. & von Stockar, U. The importance of ammonia in mammalian cell culture. *Journal of Biotechnology* **46**, 161-185 (1996).

122 Boyle, C. & Hickman, J. Toxin-induced increase in survival factor receptors: Modulation of the threshold for apoptosis. *Cancer Research* **57**, 2404-2409 (1997).

123 Eddleston, M. *et al.* Multiple-dose activated charcoal in acute self-poisoning: a randomised controlled trial. *Lancet* **371**, 579-587 (2008).

124 Mumpton, F. La roca magica: Uses of natural zeolites in agriculture and industry. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 3463-3470 (1999).

125 Petit, C. & Bandosz, T. J. Complexity of ammonia interactions on activated carbons modified with V2O5. *Journal of Colloid and Interface Science* **325**, 301-308 (2008).

126 Katic, M., Bosnjak, B., Gall-Troselj, K., Dikic, I. & Pavelic, K. A clinoptilolite effect on cell media and the consequent effects on tumor cells in vitro *Frontiers in Bioscience* **11**, 1722-1732 (2006).

127 Capiaumont, J. *et al.* Methods for reducing the ammonia in hybridoma cell cultures. *Journal of Biotechnology* **39**, 49-58 (1995).

128 Sprynskyy, M. *et al.* Ammonium sorption from aqueous solutions by the natural zeolite Transcarpathian clinoptilolite studied under dynamic conditions. *Journal of Colloid and Interface Science* **284**, 408-415 (2005).

129 Augst, A. D., Kong, H. J. & Mooney, D. J. Alginate hydrogels as biomaterials. *Macromolecular Bioscience* **6**, 623-633 (2006).

130 Christie, A. & Butler, M. Glutamine-based dipeptides are utilized in mammalian cell culture by extracellular hydrolysis catalyzed by a specific peptidase. *Journal of Biotechnology* **37**, 277-290 (1994).

131 Godin, S. J. *et al.* Species differences in the in vitro metabolism of deltamethrin and esfenvalerate: Differential oxidative and hydrolytic metabolism by humans and rats. *Drug Metabolism and Disposition* **34**, 1764-1771 (2006).

132 Amit, M. & Itskovitz-Eldor, J. in *Handbook of Stem Cells* Vol. 1 eds R Lanza *et al.*) 419-436 (2004).

133 Li, J. *et al.* Enrichment of putative human epidermal stem cells based on cell size and collagen type IV adhesiveness. *Cell Research* **18**, 360-371 (2008).

134 Gore. *Gore(r) Surgical Barrier Fabrics*, <http://www.gore.com/en_xx/products/medical/fabrics/index.html> (2009).

135 Leclerc, E., Sakai, Y. & Fujii, T. Microfluidic PDMS (Polydimethylsiloxane) Bioreactor for Large-Scale Culture of Hepatocytes. *Biotechnology Progress* **20**, 750-755 (2004).

136 Trounson, A. New perspectives in human stem cell therapeutic research. *BMC Medicine* **7** (2009).

137 Geron, C. *Manufacturing: Production and Qualification of GRNOPC1*, <http://www.geron.com/grnopc1clearance/grnopc1-sec5.html> (2009).

138 Couzin, J. Celebration and Concern Over U.S. Trial of Embryonic Stem Cells. *Science* **323**, 568 (2009).

Appendix

1 – Media and Supplements

1.1 – mMSC Complete Medium

Dulbecco's modified Eagle's Medium (DMEM) (GibcoBRL, UK)	500 ml
L-Glutamine (GibcoBRL, UK) (200 mM)	5 ml
Anibiotic/antimycotic Solution (containing penicillin (10000 units), streptomycin (10000 μg) and amphotericin B (125 μg) in 0.85 % $^{w}/_{v}$ saline (GibcoBRL, UK))	5 ml
Foetal calf serum (FCS) (heat-inactivated (56ºC for 1h)) (Sigma-Aldrich, Poole, UK)	50 ml
Non-essential amino acids (Sigma-Aldrich, Poole, UK)	5 ml

All supplements were passed through a 0.2 μm filter into the medium. Stored at 4 °C

1.2 – Serum-supplemented hMSC Medium

Mesenchymal stem cell medium (MSCM) (TCS Cell Works, UK)	500 ml
FCS (TCS Cell Works, UK)	25 ml
Mesenchymal stem cell growth medium (TCS Cell Works, UK)	5 ml
Penicillin/streptomycin solution (TCS Cell Works, UK)	5 ml
L-Glutamine (GibcoBRL, UK) (200 mM)	5 ml
Non-essential amino acids (Sigma-Aldrich, Poole, UK)	5 ml

All supplements were passed through a 0.2 μm filter into the MSCM. Stored at 4 °C

1.3 – Serum-free hMSC Medium

StemPro® MSC serum free basal medium (Invitrogen, UK)	500 ml
StemPro® MSC serum free medium supplement (Invitrogen, UK)	75 ml
Anibiotic/antimycotic Solution (containing penicillin (10000 units), streptomycin (10000 µg) and amphotericin B (125 µg) in 0.85 % $^{w}/_{v}$ saline (GibcoBRL, UK))	5 ml
Glutamax® Supplement (Sigma Aldrich, Poole UK)	5 ml
Non-essential amino acids (Sigma-Aldrich, Poole, UK)	5 ml

All supplements were passed through a 0.2 µm filter into the basal media. Stored at 4 °C

1.4 – Concentrated StemPro® Medium

StemPro® MSC serum free basal medium (Invitrogen, UK)	500 ml
Freeze-dry solution, so that all liquid is removed	
Tissue Culture Water	28 ml
StemPro® MSC serum free medium supplement (Invitrogen, UK)	75 ml
Anibiotic/antimycotic Solution (containing penicillin (10000 units), streptomycin (10000 µg) and amphotericin B (125 µg) in 0.85 % $^{w}/_{v}$ saline (GibcoBRL, UK))	5 ml
Glutamax® Supplement (Sigma Aldrich, Poole UK)	5 ml
Non-essential amino acids (Sigma-Aldrich, Poole, UK)	5 ml

Placed on shaker at 37 ºC for 2 hours. pH-adjusted using 100 mM hydrochloric acid. Passed through a 0.2 µm filter. Stored at 4 °C

1.5 – Phosphate buffered saline (PBS) (0.01 M pH 7.4)

PBS (Dulbecco's A) tablets (Oxoid, UK)	1 tablet

Made up to 100 ml in distilled water and heat sterilized.

1.6 – Ethylenediaminetetraacetic acid (EDTA) stock solution (2 %)

EDTA (Sigma, UK)	0.5 g

Made up to 25 ml with PBS

1.7 – Trypsin/EDTA solution

Trypsin (Sigma, UK) 2.5 %	10 ml
EDTA (2 %)	1 ml

Made up to 100 ml with PBS, filter sterilized and stored at - 20 °C

1.8 – Freezing Medium (Sigma-Aldrich, Poole, UK)

DMSO	2 ml
Foetal calf serum (FCS) (heat-inactivated (56ºC for 1h))	18 ml

Passed through a 0.2 µm filter and used immediately

2 - Stains and assay reagents

2.1 – CaspACE® Assay (Promega, UK)

Caspase Assay Buffer	160 µl
DMSO (Sigma-Aldrich, Poole, UK)	10 µl
DTT (Sigma-Aldrich, Poole, UK)	50 µl
Cell Extract	10µl
Distilled H_2O	to 490 µl

Gently vortexed, incubated at 37 °C, 5 % CO_2, for 30 minutes

Caspase-3 substrate (10 mM)	2.5 µl
DMSO	7.5µl

2.2 – Live/Dead® Viability/Cytotoxicity Kit L-3224 (Invitrogen, UK)

Ethidium homodimer-1 (EthD-1) (2 mM in DMSO/H_2O 1:4 $^{v}/_{v}$)	20 µl
PBS	10 ml

Vortex solution

Calcein AM (4 mM in DMSO)	5 µl

Used immediately

2.3 – Mitotracker® Deep Red M-22426 (Invitrogen, UK)

Mitotracker® Deep Red	50 µg
DMSO	92 µl

Once reconstituted, the solution was added immediately to 200 ml of serum-supplemented hMSC medium, resulting in a 500 nM solution of Mitotracker® Deep Red

2.4 – Glucose Oxidase assay (Sigma-Aldrich, Poole, UK)

Glucose oxidase/peroxidase reagent	4.9 ml
o-dianisidine reagent	100 µl

2.5 – BioVision® Ammonia Assay (Cambridge Bioscience, UK)

Ammonia Assay Buffer	44 µl
OxiRed Probe	2 µl
Enzyme Mix	2 µl
Converting Enzyme	2 µl

3 – Other Solutions

3.1 – Paraformaldehyde solution (3.7 % $^{w}/_{v}$)

Paraformaldehyde (Sigma-Aldrich, Poole, UK)	3.7 g
Sodium Hydroxide (1 M)	8 ml
0.2 M phosphate buffer	50 ml
Distilled H_2O	100 ml

Stored at -20 °C for upto 1 year

3.2 – Triton-X 100 solution (0.1 % $^{v}/_{v}$)

Triton-X 100 (Sigma Aldrich, Poole, UK)	20 µl
PBS	to 20 ml

3.3 – Poly vinyl acetate (PVA) solution (0.3 %)

PVA (Sigma-Aldrich, Poole, UK)	3 g
Distilled water	1 L

Stirred at room temperature overnight and filtered before use

3.4 – Alginate Polymerisation Buffer

20 % Barium Chloride solution (Sigma-Aldrich, Poole, UK) (1 or 2 %)	5 or 10 ml
Sodium Chloride (Sigma-Aldrich, Poole, UK) (145 mM)	877 mg
100 mM HEPES solution (Sigma-Aldrich, Poole, UK) (10 mM)	10 ml
Distilled H_2O	to 100 ml

4- General Cell Maintenance

4.1 – Cryopreservation

Prior to storage, cell cultures were maintained in an actively growing state to ensure optimum health and good recovery. A minimum concentration of 5 x 10^6 cells/ml were detached from the culture vessel before being re-suspended in a freezing medium (appendix 1.8). This cell homogenate was then aliquotted into cryogenic storage vials and placed into an isopropanol-containing cryo-container (Mr Frosty, Sigma, Poole, UK), before being 'stepped-frozen'- by placement into a -80 °C freezer for 24 hours, after which the vials were transferred to liquid nitrogen for further long-term storage.

4.2 – Cell Revival from Storage

Cells were removed from storage and placed directly into a tissue culture incubator until thawed. Immediately after thawing, the cell suspension was slowly introduced into a T 75 cm^2 flask containing complete, pre-warmed media. The cell were allowed to attach within a tissue culture incubator for 24 hours before the medium was changed to remove the DMSO from the culture.

4.3 – Trypan Blue Exclusion Assay

Cell counts and viability estimations were performed using the trypan blue exclusion technique by means of a 0.22 µm sterile filtered 0.5 % ($^{w}/_{v}$) trypan blue solution and a haemocytometer. An equal volume of the cell suspension and of the stain were mixed and transferred onto a haemocytometer. The cells within at least 5 separate squares (and at least 100 cells) were counted under a light-inverted microscope. The average cell number per square was determined, and a multiplication of 10^4 gave the cellular concentration (cells/ml).

Non-viable cells stained blue due to the loss of their membrane integrity and, hence, allowed the passage of dye into the cell. Viable cells remained colourless.

5 – Standard Curve Derivation

5.1 - CellTiter AQ One Solution Cell Proliferation® assay

Cell suspensions were created into 100 µl aliquots, using mMSC media, containing the following cellular concentrations in 10^4 cells/ml: 100, 80, 60, 40, 30, 20, 10, 5, 2.5 0. These solutions were added to 20 µl CellTiter AQ Reagent (Promega, Southampton, UK), incubated in a tissue culture incubator for 1 hour, and analysed colorimetrically (492 nm) using a Bio-Tek KC4 plate reader (Fisher Scientific, Loughborough, UK).

5.2 – CaspACE® assay

p-nitroaniline (100 mM solution) (Promega, Southampton, UK) 1 µl

Made up to 1 ml using PBS, resulting in a 100 µM solution. From this solution, 100, 50, 10, 5, 2, 1 and 0 µl were diluted with PBS to create 100 µl solutions of the following concentrations in µM: 100, 50, 10, 5, 2, 1 and 0. The solutions were transferred to a 96-well plate and analysed colorimetrically (405 nm) using a Bio-Tek KC4 plate reader (Fisher Scientific, Loughborough, UK).

5.3 – Bradford assay

Bovine γ Globulin (Sigma-Aldrich, Poole, UK) 2 mg

Made up to 1 ml with serum-free mMSC media. From this solution, serial dilutions were performed to provide 5 µl of solution at the following concentrations in µg/ml: 2000, 1000, 500, 250, 125 and 0. These solutions were added to 250 µl Bradford reagent (Sigma-Aldrich, Poole, UK), agitated for 5 minutes, and analysed colorimetrically (595 nm) using a Bio-Tek KC4 plate reader (Fisher Scientific, Loughborough, UK).

5.4 – Alamar Blue assay

Alamar Blue Reagent (Invitrogen, Paisley, UK) 15 ml

StemPro® media (Invitrogen, Paisley, UK) 150 ml

Cell suspensions were created into 10 ml aliquots, using the Alamar Blue solution, containing the following cellular concentrations in 10^3 cells/ml: 10, 5, 2.5, 1.25, 0. These solutions incubated in a tissue culture incubator for 2 hours, and analysed fluorometrically (ex 560 nm/ emm 590 nm) nm using a Dynex MFX plate reader (Chantilly, VA, USA).

5.5 – Coomassie Plus assay

Bovine γ Globulin (Sigma-Aldrich, Poole, UK) 2 mg

Made up to 1 ml with PBS. From this solution, serial dilutions were performed to provide 30 µl of solution at the following concentrations in µg/ml: 2000, 1000, 500, 250, 125 and 0. These solutions were added to 1.5 ml Coomassie Plus reagent (Thermo Fisher Scientific, Loughborough, UK), agitated for 1 minute, and analysed colorimetrically (595 nm) using a Bio-Tek KC4 plate reader (Fisher Scientific, Loughborough, UK).

5.6 – Glucose oxidase assay

Glucose Standard (100 mg/ml) (Sigma-Aldrich, Poole, UK) 500 µl

From this solution, 100, 80, 60, 40, 20 and 0 µl were diluted with PBS to create 100 µl solutions of the following concentrations in mg/ml: 100, 80, 60, 40, 20 and 0. The solutions were added to 200 µl of glucose assay reagent (appendix 2.4), incubated within a tissue culture incubator for 30 minutes, and analysed colorimetrically (540 nm) using a Bio-Tek KC4 plate reader (Fisher Scientific, Loughborough, UK).

5.7 - Biovision® Ammonia Assay

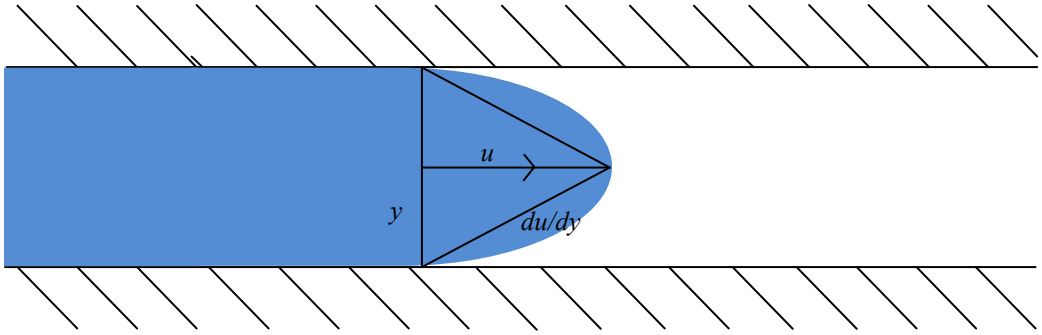
Ammonium Chloride Standard Solution (100 mM) (Cambridge Bioscience, UK) 10 µl

This stock solution was diluted with 990 µl tissue culture water to provide a 1 mM concentration. From this solution, 10, 8, 6, 4, 2 and 0 µl were diluted with ammonia assay buffer (Cambridge Bioscience, UK) to create 50 µl solutions of the following concentrations in µM: 200, 160, 120, 80, 40 and 0. The solutions were added to 50 µl of ammonia assay reaction mix (Appendix 2.5), incubated within a tissue culture incubator for 60 minutes, and analysed colorimetrically (570 nm) using a Bio-Tek KC4 plate reader (Fisher Scientific, Loughborough, UK).

6 – Corresponding needle diameters and needle areas for the needle gauges used

Needle Gauge	Internal Needle Diameter (µm)	Circular Needle Area (μm^2)
26s	114	10207
25	241	45617
22	394	121922

7 – Mathematical derivation of the shear stresses experienced by water through narrow bores (Table 4.5)



The figure above shows how a liquid would flow in a laminar manner through a bore under non-slip conditions, i.e. the velocity at the boundary interface = 0. Using the figure above, shear stress (τ) can be defined as $\mu \times (du/dy)$, where μ is the co-efficient of viscosity (for water, this value is 1.003×10^{-3} Pa.s) and *du/dy* is the gradient of the velocity compared to distance from the boundary, as shown in the figure[118].

The velocity at which the liquid travels through the bore is dependent on the diameter of the bore. In order to convert the velocity from µl/min to mm/s, the equation $\pi r^2 l$ was used to determine the volume of the bore (where r is the bore radius and l is the length). Substituting the rate in µl/min (which is equivalent to mm^3/min) for the volume and the length for the rate in mm/s gives this equation; Rate (mm/s) = rate (mm^3/min) / ($\pi r^2 \times 60$) (divide by 60 to convert minutes to seconds).

The derived velocity in mm/s is used in the 1st equation;
τ (Pa) = (μ (Pa.s) × rate (mm/s) × 2) / r (the equation needs to multiplied by 2, as, from the side on view of the bore in the figure, the liquid is affected by the shear stress both at the bottom and the top of the bore, therefore the shear stress is doubled.

As a working example, if one were to determine the shear stress experienced on water as it moved through a 26s needle at a rate of 1 µl/min, one would first need to convert the velocity from µl/min to mm/s:

Rate (mm/s) = rate (mm^3/min) / (πr^2 × 60 s/min)

= 1 mm^3/min / (π × (0.057mm)^2 × 60 s/min) = 1.62 mm/s

This velocity is then applied to the shear stress equation:

τ (Pa) = (μ (Pa.s) × rate (mm/s) × 2) / r

(1.003 × 10^{-3} Pa.s × 1.62 mm/s × 2) / 0.057 mm = **57.0 mPa**

Some assumptions were made in order to derive these shear stress values. The assumptions made were:-

- The temperature was at 20 ºC (as the viscosity of water changes with temperature)[118]
- The flow through the needle was thought to be laminar. This might not have been the cases through the narrower needles at the faster ejection rates, where turbulent flow may have occurred[118]
- The boundary layer (the layer near the boundary where the liquid is not flowing at its maximal velocity) was assumed to be equal to the radius, as shown in the figure (i.e. the liquid was only flowing at maximal velocity in the centre of the bore). The thickness of the boundary layer is dependent on the level of friction caused by the inner surface of the needle, and this could not be determined[118]
- The average gradient of velocity with respect to distance from boundary (*du/dy*) was assumed to be equal to the maximal velocity with respect to bore radius. As shown in the figure, this average gradient would probably be greater than what is calculated, but this is difficult to determine mathematically and also depends on the level of friction that occurs[118]