# A Comparison of Equine Adipose Tissue, Bone Marrow and Peripheral Blood as Sources of Mesenchymal Stem Cells

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**Amy Redmond Hubbard**


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# ABSTRACT

Lameness is a significant cause of wastage in equine athletes, with reportedly 66% days lost from training being contributed to musculoskeletal injuries. The use of regenerative cell therapies has been suggested as an alternative to current treatments which have low tissue restorative success. Although stem cell treatments are currently utilised, the optimal tissue source of equine mesenchymal stem cells (MSC) for treatment has not been determined.

The aim of the project was to compare the recovery of MSCs from adipose tissue (AT), bone marrow (BM) and peripheral blood (pB). Stem cell characteristics including adherence, proliferation, multipotency, as well as gene expression for stemness and differentiation markers were investigated.

Adipose tissue, bone marrow and peripheral blood samples were isolated from six mares and grown to confluence to investigate MSC yield and proliferation rate. The samples were cryopreserved in liquid nitrogen and then placed into a trilineage differentiation assay to assess tissue-dependent plasticity. Messenger RNA was isolated before and during differentiation to assess changes in gene expression.

All three tissue sources yielded fibroblast MSC-like cells however, AT and BM were found to be superior source of MSCs compared to pB. Trilineage differentiation capacity was demonstrated in AT and BM derived MSCs while peripheral blood derived stem cells were not investigated for multipotency due to a low number of samples. The selected pluripotent ‘stemness’ markers were not widely expressed in any of the three cell types and may not be suitable for use with multipotent stem cells of equine origin. Bone marrow derived stem cells displayed superior expression of differentiation markers compared to adipose derived stem cells.

Adipose tissue was found to be a rich reliable source of MSCs however, this cell source displayed lower efficiency for chondrogenic differentiation. Overall, bone marrow derived stem cells appeared to be a superior source of equine MSCs. An

in vivo investigation of treating musculoskeletal injuries with BM and AT derived MSCs is required to determine which source of MSCs offers the best therapeutic benefit for injured horses.

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| | |
|---|---|
| ADSC | Adipose derived stem cell |
| ANOVA | Analysis of variance |
| ASC | Adult stem cell |
| AT | Adipose tissue |
| B2M | Beta-2 microglobulin |
| BCP | 1-Brom-3-chloropan |
| BM | Bone marrow |
| BMP-6 | Bone morphogenetic protein six |
| BMSC | Bone marrow derived stem cell |
| BODIPY | Bron-dipyrromethene |
| C/EBPδ | CCAAT-enhancer-binding-protein delta |
| Cλ | Carrageenan lambda |
| CD | Cluster of differentiation |
| cDNA | Complementary deoxyribonucleic acid |
| $CO^2$ | Carbon dioxide |
| DAPI | 4',6-diamidino-2-phenylindole |
| DMEM | Dulbecco's modified eagle medium |
| DMEM F12 | Dulbecco's modified eagle medium nutrient mixture F12 |
| DMEM HG | Dulbecco's modified eagle medium high glucose |
| DMEM LG | Dulbecco's modified eagle medium low glucose |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| EDTA | Ethylenediaminetetraacetic acid, |
| ELISA | Enzyme-linked immunosorbent assay |
| ESC | Embryonic stem cell |
| FBS | Fetal bovine serum |
| FCS | Fetal calf serum |
| feES | Fetal derived embryonic stem cell |
| G | Gentamicin |
| GAG | Glycosaminoglycan |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |

| | |
|---|---|
| HEPES | Hydroxyethyl piperazineethanesulfonic acid |
| HES | Hydroxyethylstarch |
| HGF | Hepatocyte growth factor |
| HS | Horse serum |
| IBMX | 3-isobutyl-1-methylxanthine |
| ITS+premix | Universal culture supplement: Insulin, Human Transferrin, And Selenous Acid |
| MgCl2 | Magnesium chloride |
| MHC | Myosin heavy chain |
| MHC I | Major histocompatibility complex 1 |
| MHC II | Major histocompatibility complex 2 |
| MNC | Mononucleated cell |
| MQ | MilliQ water |
| mRNA | Messenger ribonucleic acid |
| MSC | Mesenchymal stem cell |
| NaAc | Sodium acetate |
| NK | Natural killer cell |
| NSS | Normal sheep serum |
| Oct4 | octamer-binding transcription factor 4 |
| P- | Passage minus |
| P | Penicillin |
| P0 | Passage zero |
| P1 | Passage one |
| P2 | Passage two |
| pB | Peripheral blood |
| PBS | Phosphate buffered saline |
| pBSC | Peripheral blood derived stem cell |
| PCR | Polymerase chain reaction |
| PPARy2 | Peroxisome proliferator-activated receptor gamma 2 |
| RBC | Red blood cell |
| RNA | Ribonucleic acid |
| RS | Rabbit serum (normal) |
| RT-PCR | Real time polymerase chain reaction |

| | |
|---|---|
| RUNX2 | Runt-related transcription factor |
| SCF | Stem cell factor |
| SDFT | Superficial flexor tendon |
| SOX9 | Sex determining region Y |
| SPP1 | Secreted phosphoprotein 1 |
| TGF-β | Transforming growth factor beta |
| TGF-1 | Transforming growth factor-1 |
| TBS-T | Tris buffered saline with tween |
| TrisMn | tris(hydroxymethyl)aminomethane manganese |
| UCB | Umbilical cord blood |
| UTSC | Umbilical cord tissue stem cell |

# CHAPTER 1: LITERATURE REVIEW AND AIMS

## 1.1 INTRODUCTION

Mesenchymal stem cells (MSCs) have received a great deal of attention in recent years for their ability to self-renew and differentiate (Marfe et al., 2012). These adult-derived stem cells can be harvested from connective tissue found throughout the body, making it highly accessible for therapeutic use. Mesenchymal stem cells have been attributed with regenerative (Uccelli et al., 2008) and immunomodulatory (Chen et al., 2008; Uccelli et al., 2008) properties and have been widely suggested as a candidate for regenerative therapy (Bunnell et al., 2010, Carrade et al., 2011; Chen et al., 2008). Musculoskeletal injuries are the leading cause of lameness in racehorses and are the single largest reason for horses to miss days from training (Dyson et al., 2008). A regenerative therapy, which restores tissue functionality, would contribute to a reduction of musculoskeletal associated wastage. Currently, there are a wide range of protocols used to isolate and culture equine MSCs from raw tissue. There are also considerable differences in the protocols used for the isolation of MSCs for therapeutic application in equines. Due to this variability in techniques and approaches, it becomes difficult to compare published studies on equine MSC culture. This project evaluated three sources of connective tissue, adipose tissue (AT), bone marrow (BM) and peripheral blood (pB), to determine which source is optimal for harvesting MSCs. Adipose derived stem cells (ADSC), bone marrow derived stem cells (BMSC) and peripheral blood derived stem cells (pBSC) were isolated from adult tissue to compare growth characteristics, cryopreservation ability, differentiation capacity and expression of stemness genes. Gene expression of selected markers of differentiation were also investigated before and after differentiation.

### 1.1.1 WHAT IS A STEM CELL?

A mesenchymal stem cell is considered to be an archetypal cell which gives rise to identical daughter cells capable of differentiating into multiple cell types (Gattegno-Ho et al., 2012). Additional characteristics of MSCs are the ability to proliferate as adherent cells and exhibit fibroblast-like morphology (Verfaillie, 2002, Uccelli et al., 2008, Lakshmipathy and Verfaillie, 2005).

Stem cells sourced from the inner cell mass of a blastocyst are termed embryonic stem cells (ESC) and are capable of maturing into cells from all three germ layers (mesoderm, ectoderm and endoderm) (Gattegno-Ho et al., 2012). These pluripotent cells are considered to have particular significance in regenerative medicine, as they are capable of differentiating into any cell type. Adult stem cells (ASC) on the other hand are derived from fully developed adult tissues and have limited differentiation ability.

The development potential of ASCs into specific cell lineages is thought to be restricted to the type of adult tissue from which they are derived. Stem cells derived from connective tissue are able to differentiate into adipocytes, chondrocytes and osteocytes, which are of mesodermal lineage. The term mesenchymal stem cell refers to the capacity of these cells to self-renew and differentiate into cells of the mesodermal lineage (Uccelli et al., 2008). Stem cells of mesodermal origin are also referred to as multipotent stromal cells or mesenchymal progenitor cells (Bourzac et al., 2010). In comparison with ESCs, ASCs have limited differentiation potential but they can be successfully harvested from multiple organs. In addition, the use of ESCs for cell therapy has been associated with the development of tumours referred to as teratomas (Li et al., 2010). These are encapsulated tumours containing tissues from more than one germ layer (Prokhorova et al., 2009). The use of ASCs not only avoids the risk of teratoma formation but also the ethical concerns connected with using embryonic tissue. Although it is generally thought that MSCs can only differentiate into cells of mesodermal lineage, there have also been reports of MSCs being able to

differentiate into cells of ectoderm and endoderm lineages such as neural and muscle cells (Uccelli et al., 2008) (**Figure 1.1**).

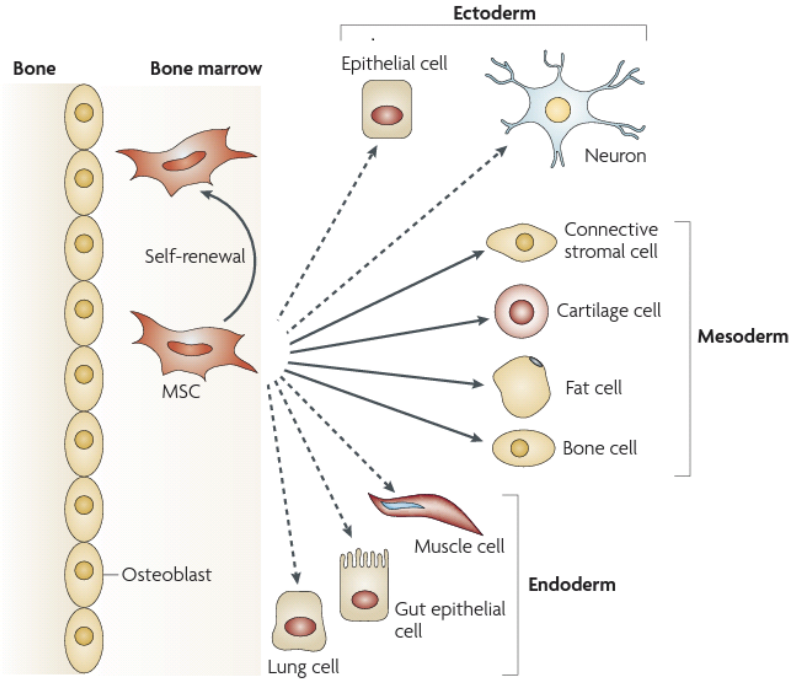



**Figure 1.1. Mesenchymal stem cell lineages. Diagram of bone marrow derived multipotent mesodermal differentiation capacity (straight solid arrows) and self-replication ability (curved, solid arrow). The potential ability of mesenchymal stem cells to transdifferentiate into endoderm and ectoderm lineages is indicated by dashed arrows. (Uccelli et al., 2008).**

Currently, this transdifferentiation ability is tentatively explained by referring to the developmental origin of MSCs during embryogenesis. During early development, mesenchymal tissues develop in the neuroepithelium and neural crest. This early neural origin suggests a potential capability of adult MSCs to differentiate into cells of non-mesodermal lineage. However, transdifferentiation has not been consistently demonstrated due to the low number of circulating MSCs of non-mesodermal embryonic lineages (Uccelli et al., 2008). Potentially, MSCs are able to differentiate into cells of almost any lineage although consistent *in-vitro* demonstration of this ability is still required.

### 1.1.2 *IN VIVO* ROLE AND MECHANISMS

Adult stem cells play a key role in homeostasis and regeneration within the body, however, the specific mechanisms of how MSCs carry out these functions are generally unknown, particularly in terms of *in vivo* differentiation (Chen et al., 2008; Kang et al., 2012; Shi et al., 2012). In the BM specifically, it is generally accepted that MSCs contribute to the formation of a hematopoietic stem cell niche. Within this niche they regulate cellular self-renewal, proliferation and differentiation of hematopoietic progenitor cells (Caplan 2009; Chen et al., 2008). Adipose tissue acts as a reservoir for MSCs which are situated around the mature adipocytes in subcutaneous white fat (Oñate et al., 2012) and visceral fat (Girolamo et al., 2013). After the occurrence of an acute injury, MSCs migrate to the site of tissue damage, either from the surrounding tissue or from the BM (Shi et al., 2012). The injured tissue environment provides the necessary signals for the MSCs to behave as precursors of bone, cartilage and fat (Uccelli et al., 2008). Blood vessels act as the primary route for MSC transportation around the body (Kang et al., 2012).

Although considerable emphasis has been placed on the differentiation capacity of MSCs, the ability of MSCs to ameliorate tissue damage may outweigh their role as cell precursors. Through the secretion of special regulators, including cytokines and growth factors, MSCs assist in tissue repair following injury or disease by altering the tissue microenvironment surrounding the site of injury (Chen et al., 2008). These molecules include hepatocyte growth factor (HGF), transforming growth factor-1 (TGF-1), various interleukin cytokines and stem cell factor (SCF) (Chen et al., 2008). Together, these soluble factors may improve the regeneration ability of injured tissues, stimulate proliferation and differentiation, as well as regulating inflammatory responses (Chen et al., 2008). Mesenchymal stem cells are also considered to elicit a poor immune response and specifically, may suppress the cellular functions of many T cells, B cells and natural killer cells (NK), as well as affecting dendritic cell activity (Chen et al., 2008; Uccelli et al., 2008).

### 1.1.3 CELL LIFE CYCLE

Mesenchymal cells are somatic cells which undergo mitosis to divide into two identical cells. Mitotic division comprises two main phases, the interphase and the mitotic phase (Colville and Bassert, 2002).

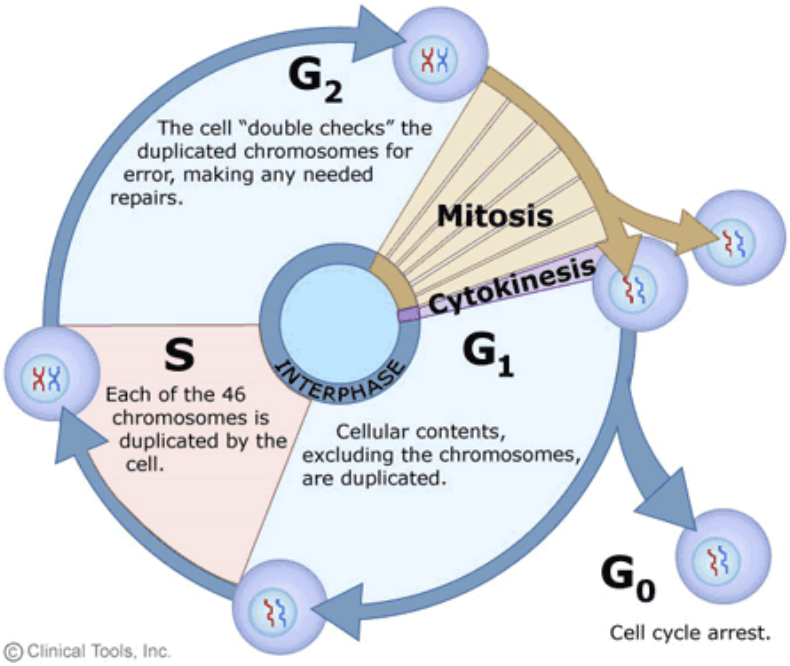



**Figure 1.2. Somatic cell cycle - stages of interphase in human cells. (University of Leicester, 2013).**

The interphase is the duration of time between cellular divisions where the cell carries out normal metabolic functions. The interphase has been subdivided into three consecutive stages (**Figure 1.2**). First, the growth one ($G_1$) phase is a period of intense growth and metabolic activity; second, the synthetic (S) phase is marked by deoxyribonucleic acid (DNA) synthesis and replication; and thirdly, the growth two ($G_2$) phase is a very short phase involving the synthesis of enzymes and proteins for cell division and growth (Freshney, 2010).

On completion of interphase the cell enters the mitotic phase. This is a time where the cell undergoes active division to produce two daughter cells with identical

copies of the original cell's DNA. Mitotic division comprises of five phases, prophase, prometaphase, metaphase, anaphase and telophase (**Figure 1.3**).

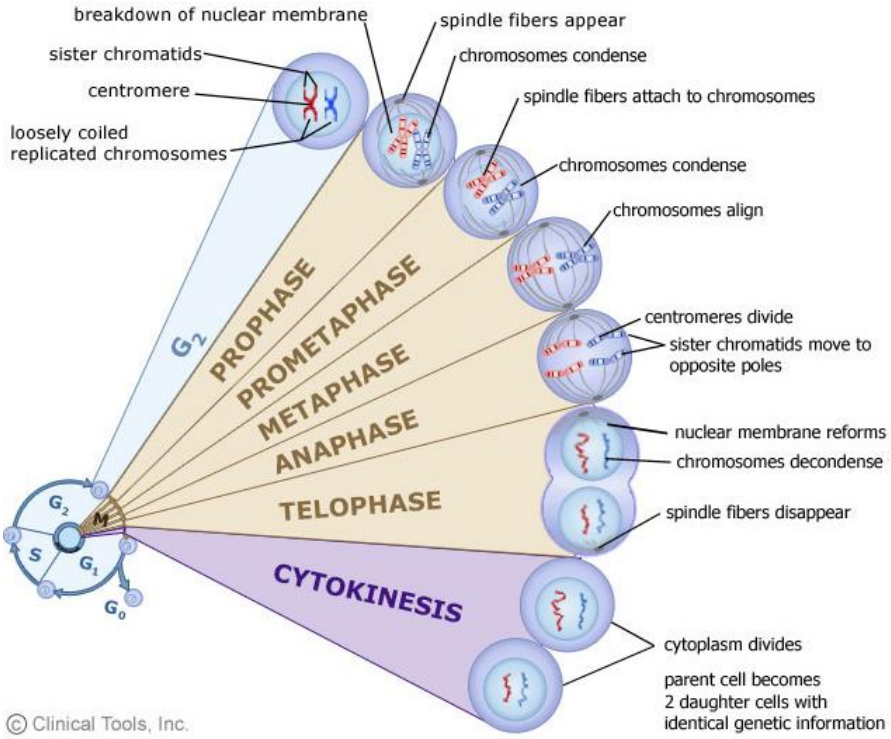



**Figure 1.3. Somatic cell cycle - stages of Mitotic Phase.**

**(University of Leicester, 2013).**

During prophase, the chromatin contracts and condenses and is associated with a cessation in metabolic function while the DNA strands are tightly bound. The nuclear envelope and the nucleoli disappear during prophase, and as the cell moves into prometaphase the centrioles divide and replicate prior to moving to either end of the cell for chromosome orientation in the metaphase stage. The metaphase stage is associated with the alignment of the chromosomes on the median plane of the cell located between the centrioles positioned at either end of the cell. Once the chromosomes are located correctly they begin to migrate away from each other to their respective centrioles at the far end of the cell. This movement characterises the anaphase stage of mitotic division. The telophase stage is marked with the reversion of the chromosomes back to being long-fibre chromatids. The nuclear envelope and nucleoli reappear and the separated

chromosomes are now located in two independent, identical daughter cells (Colville and Bassert, 2002).

The number of times a cell can self-replicate is a genetically controlled event known as senescence, which is regulated by several different genes (Freshney, 2010). Cells derived from adult tissue can only be expanded in culture for a limited number of times before the cells lose the ability to divide. With each cell division the DNA ends are not completely replicated due to the lack of telomerase expression in somatic cells (Mathon & Lloyd, 2001). Telomerase is a ribonucleoprotein required for the complete replication of DNA. Telomeres, repetitive DNA sequences located at the linear ends of the chromosomes, are therefore not fully replicated with each cell division and become progressively shorter (Mathon & Lloyd, 2001). Once the telomeres reach a critical length, the cell is signalled to undergo senescence and can no longer self-replicate (Freshney, 2010).

Cell senescence is also correlated to changes in differentiation capacity. With increasing passages, cultured MSC telomere integrity is reduced, with a corresponding decline in the efficiency of MSCs to develop into certain lineages. Significant decreases in differentiation capacity has been reported in murine BMSCs for adipogenic, chondrogenic and osteogenic lineages (Kretlow et al., 2008).

### 1.1.4 SOURCES OF STEM CELLS

Mesenchymal stem cells were first discovered in the bone marrow by Friedenstein and colleagues in 1976 (Mafi et al., 2011). Since then, multiple sources of MSC-like cells have been identified (Barry and Murphy, 2004; Payushina, et al., 2006). Additional sources of cells displaying MSC-like properties include adipose tissue (de Mattos Carvalho et al., 2011; Ranera et al., 2012), periosteum (Payushina et al., 2006), vascular wall (Abedin et al., 2004), synovial membrane, skeletal muscle, dermis, dental pulp (Chen et al., 2008), umbilical cord blood (UCB) (Reed and

Johnson, 2012) and peripheral blood (Ahern et al., 2011; Koerner et al. 2006; Marfe et al., 2012). However, the density and viability of MSC-like cells from different tissues can vary considerably (Payushina et al, 2006). Additionally, cell phenotype, proliferation rates and differentiation capacity is tissue dependant, with some cell sources being superior to others (Chen et al., 2008).

Adipose tissue isolation in the horse has received a significant amount of attention for its straightforward collection procedure, abundance of tissue and high yield of stem cells (de Mattos Carvalho et al., 2011, Ranera et al., 2012). Bone marrow aspiration has been investigated in a number of species (Payushina et al., 2006; Pittenger et al., 1999) including the horse (Vidal et al., 2008). Peripheral blood has received relatively recent attention, compared to AT and BM, as a source of MSCs. While highly accessible, with low collection costs, blood has a low concentration of MSCs, and there has been variable success in the isolation of adherent mononucleated cells from this source (Ahern et al., 2011; Koerner et al., 2006; Marfe et al., 2012). Adipose tissue, BM and pB all share the same characteristics of being retrievable from a live animal with relatively low levels of invasiveness. Indeed, the surgical manipulations performed on a horse to retrieve either AT or BM are considered to be minor manipulations which heal well with minimal complications. Due to ease of collection, and the ability to collect a large sample volume at any time from such a large animal, pB offers an exciting potential source of MSCs in equids.

Traditionally, BM has been considered as the richest and most reliable source of MSCs (Chen et al., 2008). However AT has recently been considered to be a high yielding source of MSCs in the horse, even more so than BM (Kang et al., 2012). Although pB is a low yielding MSC source, isolated cells can be cultured to amplify cell numbers.

### 1.1.5 ADIPOSE TISSUE

Adipose tissue is generally removed from the easily accessible dorsal gluteal region in the horse (Ahern et al., 2011; de Mattos Carvalho et al., 2011; Dhar et al., 2012; Ranera et al., 2012; Vidal et al., 2007). Adipose tissue appears to be a robust tissue source to harvest, as it is able to be kept at room temperature for up to 24 hours before significant tissue degeneration occurs (Bunnell et al., 2008). A single 10g collection of AT can be used to isolate stem cells for culture, expansion, differentiation and treatment (Ahern et al., 2011; Dhar et al., 2012). The minimum sample size reported in the literature was 5g from the dorsal gluteal region (de Mattos Carvalho et al., 2011), from which MSCs were able to be isolated and expanded in culture. The size of the surgical incision can be refined when harvesting a smaller volume of fat, resulting in reduced healing time and minimised scarring. The amount of fat deposited in the dorsal gluteal region will determine the size of the incision in order to collect a minimum of 5g of tissue. Athletic horses, such as Thoroughbreds, may have minimal deposits of fat available due to their lean build and, therefore, may require larger surgical incisions to collect the minimal amount of tissue required for processing.

A number of authors (Ahern et al., 2011; Bunnell et al., 2008 & Dhar et al., 2012) use a series of phosphate buffered saline (PBS) washes followed by incubating the sample in collagenase to dissociate the tissue. There are variable types and concentrations of collagenase reported in the literature (de Mattos Carvalho et al., 2011; Martinez- Lorenzo et al., 2009). Collagenase type 1 is predominately used for adipose tissue dissociation (Bunnell et al., 2008; Ranera et al., 2011; Vidal et al., 2008; Raabe et al., 2010; Raabe et al., 2011; Dhar et al., 2012) however, there are published protocols which utilise collagenase type 1A (Aguena et al., 2012) and collagenase type II (Braun et al., 2009). The collagen digestion unit (CDU) may vary from 125-250CDU/mg and significant lot to lot variation may require readjustment of collagenase concentrations to achieve tissue dissociation (Williams et al., 1995). The length of incubation time in collagenase also varies considerably ranging from a minimum of 30 minutes (Ranera et al., 2011) to 12 hours (de Mattos Carvalho et al., 2011).

### 1.1.6 BONE MARROW

The collection site of BM is largely influenced by the species from which it is being isolated. In small species, such as rodents, complete segments of the femur and tibia are removed from euthanized animals and the marrow is then extracted from within the bone cavity (Ferreira et al., 2012). In human studies, BM is extracted from the posterior iliac crest as well as the femur and tibia (Kemp et al., 2005). The three regions have all provided reliable and adequate yields of MSCs. Equine BMSCs are either collected from the sternum or iliac crest with or without the use of diagnostic ultrasonography (Bourzac et al., 2010; Kasashima et al., 2011; Koerner et al., 2006; Ranera et al., 2012; Smith et al., 2003; Sutter, 2007). As an alternative to sternal bone marrow collection, aspiration of the tuber coxae, which is a specific anatomical point on the ilium, can be performed (Sutter, 2007). This may be a preferable option to owners of valuable horses who do not want to risk the potential complication associated with sternal bone marrow collection. However, the yield of equine MSCs is comparatively lower from tuber coxae aspiration. Sternal aspiration provides a larger volume of bone marrow with a denser population of MSCs than currently obtainable from the tuber coxae in horses (Delling et al., 2012).

Typically, bone marrow aspirate is processed using a density gradient to isolate stem cells suspended in the aspirate (Bourzac et al., 2010; Spaas et al., 2013). Three different gradients are used in the literature, Ficoll (density 1.077g/ml) (Bourzac et al., 2012), Percoll (density 1.07g/ml) (Bourzac et al., 2012) and Lymphoprep (1.077g/ml) (Ranera et al., 2011) for equine BM aspirate processing. The entire cell fraction isolated from raw BM aspirate can also be plated without using a density gradient however, the use of a gradient concentrates the MNC fraction and reportedly results in higher density of cells at confluence (Bourzac et al., 2012).

### 1.1.7 PERIPHERAL BLOOD

The use of pB provides a minimally invasive collection technique when compared with BM or AT harvesting. However, the low concentration of stem cells in pB limits its use as a reliable source of MSCs. Blood can be collected from any accessible vein, however, veins in the lower leg or the jugular vein are predominately used in the horse (Dhar et al., 2012; Marfe et al., 2012; Spaas et al., 2013). The volume of blood collected from a horse at one time ranges from 10ml (Spaas et al., 2013) to 4000ml (Ahern et al., 2011).

The isolation of MNCs from raw pB utilises density gradient protocols similar to those used for isolating MNCs from BM. Percoll (Spaas et al., 2012), Ficoll (Giovannini et al., 2008; Koerner et al., 2006; Martinello et al., 2011) and Lymphoprep (Dhar et al., 2011) are all used to isolate equine blood MNCs. Isolation success of equine pBSCs ranges from 30.3% (Koerner et al., 2006, using Ficoll) to 100% (Spaas et al., 2013, using Percoll).

### 1.1.8 MESENCHYMAL STEM CELL CULTURE MEDIA AND SUPPLEMENTS

Culture media types and supplements used for the culture of equine MSCs from AT, BM and pB were reviewed (**Table 1.1**). Dulbeccos Modified Eagle Medium (DMEM) is consistently used for equine MSC culture. The supplement of F12 to DMEM provides additional amino acids, and can be used when culturing ADSCs, BMSCs and pBSCs (Ahern et al., 2011; Dhar et al., 2012; Giovannini et al., 2008).
Serum is added to media to promote cell proliferation and attachment. Protein is the major component of serum and acts as a carrier for minerals, fatty acids and hormones and as a source of growth factors. Protein also increases medium viscosity and may reduce damage to cell structure during pipetting and other physical manipulations (Freshney, 2010). Fetal bovine serum (FBS), also known as fetal calf serum (FCS), is the most widely used serum, although horse serum is preferred by some labs as it is considered to be more consistent batch to batch than FBS (Freshney, 2010).

Glutamine is required by all cells, and may be used as a source of energy and carbon. This amino acid was found to be used as a supplement by a number of researchers (Braun et al., 2009; Giovannini et al., 2008; Kemp et al., 2005; Martinello et al., 2010; Ranera et al., 2012; Spaas et al., 2013), however, it does not appear to be a standard additive.

Medium buffering is an important aspect of cell culture to ensure the pH levels remain stable throughout culture. Bicarbonate buffer or hydroxyethyl piperazineethanesulfonic acid (HEPES) are used as effective buffering systems. HEPES is a much stronger buffer than bicarbonate, however, bicarbonate is used more frequently due to its low toxicity and low cost (Freshney, 2010). Bicarbonate also reportedly provides a nutritional benefit to the cells whereas HEPES does not (Freshney, 2010). The exact nutritional benefit of bicarbonate is not stated in cell culture literature (Freshney, 2010). An investigation on bicarbonate supplementation in human patients with chronic kidney disease reported an improvement in protein uptake with increased bicarbonate ingestion (de Brito-Ashurst et al., 2009). This may or may not relate to cells in culture but potentially, bicarbonate may provide a nutritional benefit to culture cells by improving protein metabolism.

Primary tissue culture is fraught with potential contamination from a number of sources. Contaminated equipment, media, work spaces, tissue samples and poor aseptic techniques can all lead to culture contamination resulting in the loss of samples. Bacteria, fungi, yeast and mycoplasma are all types of contamination which can potentially occur with cell culture (Freshney, 2010). The addition of antibiotics and antifungals can reduce the rate of contamination, although the low concentrations used in culture, to minimize detrimental effect on cell viability and proliferation, can allow for contaminations to occur. While bacteria, fungi and yeast infections are visible, mycoplasma contaminations require more powerful methods for detection (Freshney, 2010).

Mycoplasma contaminations are important to detect as they can alter the behaviour and metabolism of cells, and can significantly affect the findings of a research project without the researchers knowing the cause. Detection of mycoplasma infections requires fluorescent staining, PCR, enzyme-linked immunosorbent assay (ELISA), immunostaining, autoradiography or microbiological assay. The most commonly used technique for mycoplasma detection is fluorescent staining of DNA (Freshney, 2010).

Antibiotics are added to reduce the chance of bacterial contamination, particularly when working with primary tissue cultures. It appears that equine stem cell culture relies on the addition of penicillin/streptomycin (P/S) to prevent bacterial contamination, although this combination of antibacterials target an ever narrowing range of bacteria, with resistant strains continuing to emerge. Gentamicin (G) is used for human stem cell culture with minimal detrimental effect on the viability and proliferation rates in culture being reported when used at low concentrations (Chang et al., 2006), however, it is not widely used for equine stem cell culture. Out of the range of literature reviewed only Spaas et al. (2013) used G (50µg/ml) for culturing equine pBSCs.

Fungal contaminations are particularly common during the warmer months when the air is filled with fungal spores. Amphotericin B is a commonly used antibiotic due to its activity against fungi and yeast (Freshney, 2010).

**Table 1.1. Medium, additives and antibiotics used for equine adipose, bone marrow and peripheral blood derived stem cells.**

| Adipose derived stem cells | | | |
|---|---|---|---|
| **Author** | **Culture medium** | **Additives** | **Antibiotics** |
| **De Mattos Carvalho et al., 2011** | DMEM | 10% FBS | Not reported |
| **Ranera et al 2012** | DMEM LG | 10% FBS, 1% glutamine | 1% P/S |
| **Ahern et al. 2011** | DMEM, | 10% FBS | 1% P/S |
| **Dhar et al, 2012** | DMEM-F12, | 10% FBS | 1% P/S |
| **Braun et al., 2009** | DMEM LG | 10% FBS, 1% glutamine, 25mM HEPES | 1% P/S |
| **Bone marrow derived stem cells** | | | |
| **Author** | **Culture medium** | **Additives** | **Antibiotics** |
| **Ranera et al., 2012** | DMEM LG | 10% FBS, 1% glutamine | 1% P/S |
| **Kasashima et al., 2011** | DMEM LG | 10% FBS | 2% P/S |
| **Giovannini et al., 2008** | DMEM-F12 | 10% FBS | 1% P/S |
| **Bourzac et al, 2010** | DMEM LG | 10% FBS, pyruvate | 1% P/S amphotericin B, |
| **Peripheral blood derived stem cells** | | | |
| **Author** | **Culture medium** | **Additives** | **Antibiotics** |
| **Spaas et al., 2013** | DMEM LG | 30% FBS, 1% ultraglutamine, $10^{-11}$ dexamethasone | 50µg/ml gentamicin |
| **Koerner et al., 2006** | DMEM | 20% FCS, | 1% P/S |
| **Ahern et al., 2011** | DMEM-F12 | 20% FBS | 1% P/S |
| **Martinello et al., 2010** | DMEM | 20% FBS glutamine. | P/S (percentage not stated) |
| **Giovannini et al., 2008** | DMEM, | 20% FBS l-glutamine & 15mM HEPES, , | 1% P/S |

**DMEM – Dulbecco's modified eagle medium, FBS – fetal bovine serum, FCS – fetal calf serum, HEPES - hydroxyethyl piperazineethanesulfonic acid, LG – low glucose, P/S - Penicillin/streptomycin**

### 1.1.9 CELL CULTURE

Cell culture can be divided into two stages, primary culture and secondary culture. Primary culture is the stage of culture after cell isolation from raw tissue but before the first subculture (Freshney, 2010). Following the first subculture the cells are referred to as secondary culture. Passaging is used to increase the number of cells in culture and maintain the population's self-replication ability. The first passage occurs once the primary culture has covered the available surface of a culture vessel. Cells are then replated into new culture vessels at low densities to allow for cell expansion during secondary culture (Freshney, 2010).

As with any non-immortalized somatic cell, MSCs have a limited lifespan in culture. For this reason, cells are cryopreserved during early passages to preserve the expansion ability for future culturing. There are variable reports for the number of passages a particular cell line can undergo before senescence occurs. Adult derived MSCs can reportedly be expanded for 20-50 passages before self-replication is lost (Lakshimpathy & Verfaillie, 2005). Adipose tissue derived stem cells have been found to reach senescence at later passages than BMSCs. Samples of ADSC could be passaged nine times while BMSC samples could only be passaged six times (Braun et al., 2010) although conflicting reports have been found in the literature.

Although MSCs are usually cultured in heterogeneous populations, there has been a more recent push to culture clonal populations to validate the stemness of isolated cells. Indeed, Bianco et al. (2013) is of the opinion that adherent cells derived from adult tissue cannot be termed MSCs unless they have been amplified from a single colony derived from a single stem cell and proven to have trilineage capability. Clonal growth is not widely used in MSC research; indeed, all of the papers reviewed for this project used heterogeneous cell populations which were referred to as MSCs.

### 1.1.10 CRYOPRESERVATION

The cryopreservation allows cells to be retained for future use, reducing the need for repeated tissue sampling. As MSCs undergo a limited number of passages before senescence, preservation of early passage cells is particularly important.
The freezing process causes significant stress to the cells and as such, precautions need to be made to protect against cell damage during freezing and thawing (Haack-Sorensen & Kastrup, 2011). During the cryopreservation process, intracellular and extracellular water transforms into ice crystals, and cellular metabolism functions cease. Cryoprotectants are used to prevent the formation of ice crystals within the cells during freezing, maintaining the integrity of the cellular membrane which is particularly vulnerable to rupture during freezing and thawing (Berz et al., 2007). Dimethyl sulfoxide (DMSO) (Seo et al., 2011), hydroxytheyl starch (HES), trehelose and polyvinal (Naaldijk et al., 2012) have all been used successfully as cryoprotectants.

Dimethyl sulfoxide (DMSO) is a hygroscopic polar compound commonly used in cryopreservation due to its ability to attract and hold on to water molecules from the surrounding environment (Seo et al., 2011). This ability makes it particularly applicable to stem cell cryopreservation as it prevents cells from drying out (Seo et al., 2011). Although this compound works effectively as a cryoprotectant it can be toxic to cells at room temperature, as it is highly permeable. As a precautionary measure, it is recommended to use DMSO at temperatures less than 4°C to prevent additional damage to cells (Hunt, 2011). However, it has been reported that, when using low concentrations of DMSO with high concentrations of serum, the adverse effects are greatly reduced even when working at ambient temperatures as long as slow freezing and rapid thawing protocols are used (Hunt, 2011).

Serum is added to the cryogenic medium to further protect the cell walls from damage due to ice crystal formation (Haack-Sorensen & Kastrup, 2011). Fetal bovine serum is commonly used as a source of protein in cell culture and in

cryopreservation. There is a range of FBS concentrations used for freezing, from 10% (M. Green, personal communication, November, 2012) to 90% (Hunt, 2011).

The rate at which cells or tissues are frozen can significantly impact on their ability to recover post thaw (Seo et al., 2011; Vajti & Kuwayama, 2006). The traditional slow rate freezing has been used in cell culture and reproductive technology for many years (Vajti & Kuwayama, 2006). A slow, controlled rate of freezing reduces the quantity of intracellular ice build-up and prevents the rupture of cellular membranes. A slow rate of freezing is achieved through the use of an alcohol based freezing container, commonly marketed as a Mr Frosty™ freezing container. This container holds a number of cryovials which can be slowly frozen at a controlled rate of -1°C per minute when placed in a -80°C freezer.

When cryopreserved cells are required for further research or treatment applications, a fast thawing process should be used to prevent morphological damage to the cells (Berz et al., 2007). Cells are generally thawed rapidly in a 37ºC water bath followed by dilution with culture medium (Berg et al., 2009). Following thawing, it is important to remove the DMSO quickly by immediately diluting the cell suspension in culture medium and washing the cells thoroughly to remove traces of DMSO (Bunnell et al., 2008; Naaldijk et al., 2012; Wu et al., 2012). This is of particular importance if the sample is to be administered directly or plated out for culture. Indeed, samples containing traces of DMSO at the time of patient treatment can result in adverse reactions (Berz et al., 2007; Hunt, 2011).

Cryopreservation can also be used to store intact tissue samples for later processing for MSC isolation. Human AT is able to be frozen intact, stored in liquid nitrogen, thawed and then processed for MSC isolation. The cells from frozen tissue samples were compared to cells from fresh tissue samples, with no significant difference found in proliferation capacity, morphology, differentiation capacity, and gene expression (Choudhery, 2013).

### 1.1.11 DIFFERENTIATION

Differentiation is a diagnostic method applied to MSCs to assess plasticity and confirm them as MSCs (Lakshmipathy et al., 2005). Plasticity is the capacity of a cell to give rise to one or more lineages. Unipotent cells give rise to only one lineage, multipotent cells give rise to more than two lineages, pluripotent cells may give rise to several lineages and totipotent cells are able to give rise to all known cell types (Freshney, 2010). Mesenchymal stem cells are considered multipotent due to their ability to differentiate into more than two lineages.

Adipogenic, chondrogenic and osteogenic lineages are the most commonly differentiated cell types for confirmation of MSC plasticity, although tenogenic (tendon) and myogenic (muscle) differentiation has been confirmed for equine MSCs (Ahern et al., 2011, Kasashima et al, 2011, Ranera et al., 2012) (**Table 1.2**). Equine ADSCs, BMSCs and pBSCs all have proven trilineage differentiation capacity although variability in differentiation efficiency has been reported between the three cell sources (**Table 1.2**).

**Table 1.2. Differentiation success of equine adipose, bone marrow and peripheral blood derived stem cells for adipogenic, chondrogenic, osteogenic, tenogenic and myogenic lineages.**

| Adipose derived stem cells | | | | | |
|---|---|---|---|---|---|
| **Author** | **Adipogenic** | **Chondrogenic** | **Osteogenic** | **Tenogenic** | **Myogenic** |
| **Burk et al., 2013** | Yes | Yes | Yes | N/D | N/D |
| **Colleoni et al., 2009** | N/D | Yes | Yes | N/D | N/D |
| **Vidal et al., 2007** | Yes | N/D | Yes | N/D | N/D |
| **Braun et al., 2010** | Yes | Yes | Yes | N/D | N/D |
| **Bone marrow derived stem cells** | | | | | |
| **Author** | **Adipogenic** | **Chondrogenic** | **Osteogenic** | **Tenogenic** | **Myogenic** |
| **Burk et al., 2013** | Yes | Yes | Yes | N/D | N/D |
| **Colleoni et al., 2009** | N/D | Yes | Yes | N/D | N/D |
| **Violini et al., 2009** | N/D | N/D | Yes | Yes | N/D |

| | | | | | |
|---|---|---|---|---|---|
| **Bourzac et al., 2010** | Yes | Yes | Yes | N/D | N/D |
| **Peripheral blood derived stem cells** | | | | | |
| **Author** | **Adipogenic** | **Chondrogenic** | **Osteogenic** | **Tenogenic** | **Myogenic** |
| **Dhar et al., 2012** | Yes | Yes | Yes | N/D | N/D |
| **Spaas et al., 2013** | Yes | Yes | Yes | N/D | N/D |
| **Martinello et al., 2010** | Yes | N/D | Yes | N/D | Yes |

The differentiation potential of MSCs is not guaranteed within a specific population of cells. Colonies of MSCs grown from a single precursor cell have reportedly formed a population of cells with varied multilineage differentiation potential (Baksh et al., 2004). Additionally, Muraglia et al. (2000) found that only 30% of cells derived from nonimmortalised cell clones expressed trilineage differentiation capacity, while the remaining cells expressed bi-lineage or uni-lineage potential.

#### *1.1.11.1 ADIPOGENIC DIFFERENTIATION*

Adipogenic differentiation requires a combination of specific medium, serum and additional reagents (**Table 1.3**). The basal medium DMEM, either containing low glucose (LG), or with F12 combined with FBS or rabbit serum (RS) or a combination of both, has been shown to induce adipogenic differentiation. Rabbit serum is widely used due to it containing specific fatty acids which are responsible for strong adipogenic differentiation (Diascro et al., 1998). The supplements used for adipogenesis and their associated actions are listed in **Table 1.6**.

**Table 1.3. Media, serum and additives used for adipogenic differentiation of equine mesenchymal stem cells.**

| Author | DMEM/ SERA | Dexamethasone | IBMX | Indomethacin | Insulin |
|---|---|---|---|---|---|
| Schwarz et al., 2012 | DMEM, 10% autologous serum | 1µM | 1mM | 0.2mM | 1.6µM |
| Ranera et al., 2012 | DMEM LG10% FBS, 15% RS | 1µM | 0.5mM | 0.2mM | - |
| Burk et al., 2013 | DMEM F12 15% RS | 1µM | 0.5mM | 0.1mM | 0.7µM |
| Vidal et al., 2007[1] | DMEM Hams F12 3% FBS, 5% RS | 1µM | 0.5mM | - | 0.1µM |
| Braun et al., 2010[2] | DMEM LG 10% FBS, 5% RS | 1µM | 0.5mM | 0.1mM | 0.1µM |
| Raabe et al., 2011 | DMEM, 10% FCS | 1µM | 0.5mM | 0.1mM | 10µM |
| Giovannini et al., 2007 | DMEM F12 5% RS | 1µM | 0.5mM | 0.1mM | 1.7µM |
| Spaas et al., 2013 | DMEM LG 15% RS | 1µM | 0.5mM | 0.2mM | 0.1µM |

[1] Biotin - 33µmol/L, Pantothenate - 17µmol/L, Rosiglitazol - 5µmol/L also added
[2] D-glucose - 4.5g/L also added

The detection of adipogenic differentiation is achieved through determination of morphological characteristics, the use of specific stains and evaluating gene expression in differentiated cells. For adipogenic fate, oil red O is typically used to stain lipid vesicles deposited within adipocyte cells. While the use of BODIPY was not reported in any of the equine adipogenic differentiation papers, this fluorescent stain has been used with human ADSCs and BMSCs to confirm intercellular accumulation of lipid droplets (Bunnell et al., 2008; Fink et al., 2004).

Gene expression of specific adipogenic markers have been used in a number of papers to complement histological findings (Poloni et al., 2013; Raabe et al., 2011; Ranera et al., 2012). Peroxisome proliferator-activated receptor gamma (PPARγ2) is one of the key positive transcriptional regulators of adipogenesis (Payushina et al., 2006; Poloni et al., 2013). The activation of PPARγ2 triggers the expression of

additional adipogenic genes required for lipid accumulation. Dexamethasone is considered to be a trigger of the transcription factor CCAAT-enhancer-binding-protein delta (C/EBPδ). This factor binds to the promoter of PPARγ2, leading to its activation (Payushima et al. 2006). Although PPARγ2 is considered to be the highest expression towards the end of differentiation (Ranera et al., 2012), upregulation of this gene has been reported after six and seven days in adipogenic medium (Braun et al., 2010, Bracegirdle et al, submitted).

#### *1.1.11.2 CHONDROGENIC DIFFERENTIATION*

Chondrogenic differentiation requires DMEM, generally with high glucose (HG), although LG has also been used (**Table 1.4**). Varying amounts of FBS are used, ranging from 0% to 10%. The supplements used for chondrogenesis and their associated actions are listed in **Table 1.6**.

In addition to the specific supplements required for differentiation, the cell culture system used has a significant impact on whether chondrogenic differentiation can occur. By plating the cells at high densities in 96 well plates or equivalent, a three dimensional cell aggregate is able to form (Berg et al., 2009; Pilz et al., 2011). A tertiary structure is vital to chondrocyte formation and care must be taken during medium changes to prevent disturbing the cell pellet (Berg et al., 2009; Payushina et al., 2006). If the pellet is disrupted, the aggregate collapses and cell to cell communication is lost. The close association of cells with one another mimics the cell-cell interactions which occur during embryonic cartilage formation and is important for chondrogenic differentiation to occur (Giovannini et al., 2008).

**Table 1.4. Media, serum and additives used for chondrogenic differentiation of equine mesenchymal stem cells.**

| Author | DMEM | TGF-β | *ITS+ premix | Proline | Ascorbate-2-phosphate | Dexamethasone |
|---|---|---|---|---|---|---|
| Ranera et al., 2012 | DMEM HG, 10% FBS | TGF-β3 10ng/ml | Yes | 40µg/ml | 0.05mM | 0.1µM |
| Colleoni et al., 2009 | Not stated | TGF-β1 5ng/ml | Yes | - | 0.25mM | 0.1µM |
| Burk et al., 2013 | DMEM HG | TGF-β1 10ng/ml | 1% | 400µM | 0.1mM | 0.1µM |
| Vidal et al., 2008[1] | DMEM HG | TGF-β3 10ng/ml | Yes | - | 0.05mM | 0.1µM |
| Braun et al., 2010[2] | DMEM LG, 10% FBS | TGF-β3 10ng/ml | - | 350µM | - | 0.1µM |
| Ahern et al., 2011 | DMEM, serum not stated | TGF-β3 5ng/ml | 1% | - | 0.1mM | 0.1µM |
| Giovannini et al., 2007 | DMEM HG | TGF-β3 5ng/ml | 1% | - | 0.1mM | 0.1µM |

1 BMP-6 - 10ng/ml also added
2 D-glucose – 4.5g/L also added
*ITS+premix: culture supplement containing insulin, transferrin, and selenium

The detection of chondrogenic differentiation is achieved through determination of morphological characteristics, such as the shape and density of a tertiary structure, the use of specific stains and evaluating gene expression in differentiated cell pellets. Chondrogenic differentiation is detected by a variety of stains including Alcian Blue (Ahern et al., 2011), toluidine blue (Bunnell et al., 2008) and Safranin O for glycosaminoglycan (GAG) deposits (Burk et al., 2013), hematoxylin and eosin (H&E) for cell structure (Vidal et al., 2008) and Massons Trichrome (Burk et al., 2013) for collagen fibre formation.

In conjunction with histological stains, gene expression is used to confirm that chondrogenic differentiation has occurred. Chondrogenic-specific lineage markers include collagen type 2, collagen type 1, Aggrecan, SOX9 and cartilage-derived retinoic acid-sensitive protein (CD-RAP) (Berg et al., 2009). The marker SOX9 is a cartilage-specific transcription factor used by a number of authors for analysis of chondrogenic differentiation in MSCs in a range of species including dogs (Csaki et

al. 2007), horses (Berg et al., 2009) and humans (Ullah et al., 2012). This gene has an essential role in the commitment of cells towards chondrogenic fate. Upregulation of SOX9 stimulates the reduction of intercellular spaces within cell aggregates (Tacchetti et al., 1992), expression remains high during differentiation and is then 'turned off' at the end of differentiation, as the cells begin to undergo prehypertrophy (Lefebvre et al., 2005).

#### *1.1.11.3 OSTEOGENIC DIFFERENTIATION*

Osteogenic differentiation medium appears to be relatively consistent across the literature (**Table 1.5**). Basal medium (DMEM), either with LG or with F12 is used with a combination of dexamethasone, β-glycerophosphate and ascorbate 2-phosphate. Only one reviewed article (Ahern et al., 2011) did not use or did not report the use of dexamethasone, however osteogenic differentiation was still achieved. The supplements used for osteogenesis and their associated actions are listed in **Table 1.6**.

**Table 1.5. Media, serum and additives used for osteogenic differentiation of equine mesenchymal stem cells.**

| Author | DMEM | Dexamethasone | β-glycerophosphate | Ascorbate 2-phosphate |
|---|---|---|---|---|
| Ranera et al., 2012 | DMEM 10% FBS | 0.1µM | 10mM | 0.05mM |
| Colleoni et al., 2009 | DMEM/TCM199 10% FBS | 0.1µM | 10mM | 0.25mM |
| Burk et al., 2013 | DMEM F12,10% FBS | 0.1µM | 10mM | 0.1mM |
| Vidal et al., 2007 | DMEM Hams F12, 10% FBS | 0.2µM | 10mM | 0.05mM |
| Braun et al., 2010 | DMEM LG, 10% FBS | 1µM | 10mM | 0.1mM |
| Ahern et al., 2011 | DMEM – serum not specified | - | 5mM | 0.1mM |
| Raabe et al., 2011 | DMEM 10% FCS | 0.1µM | 10mM | 0.05mM |
| Schwarz et al., 2012 | DMEM, 10% autologous serum | 0.1µM | 10mM | 0.05mM |
| Giovannini et al., 2007 | DMEM F12 10% FBS | 0.1µM | 10mM | 0.1mM |
| Spaas et al., 2013 | DMEM LG, 10% FCS | 0.1µM | 10mM | 0.2mM |

The detection of osteogenic differentiation is achieved through determination of morphological characteristics, the use of specific stains which target mineral deposits in the cells and evaluating gene expression in differentiated cells. Osteogenic differentiation can be detected with Alizarin Red stain which highlights calcium mineral content (Vidal et al., 2007).

A range of osteogenic differentiation markers have been investigated in equine MSCs. The expression of osteogenic lineage specific markers includes alkaline phosphatase (ALP), osteocalcin (BGLAP), collagen type I (COL1A1), runt-related transcription factor 2 (RUNX2) and secreted phosphoprotein 1 (SPP1) (Ranera et al., 2012). The early expression of RUNX2 and SPP1 markers leads to a cascade of gene expression resulting in osteogenic differentiation (Eyckman et al., 2012; Ranera et al., 2011). The expression of RUNX2 in particular is correlated to the expression of other osteogenic markers indicating closely linked upregulation. The markers RUNX2 and SPP1 are reportedly expressed at similar stages of differentiation. However, RUNX2 appears to precede SPP1 expression by three days in equine BMSCs undergoing osteogenic differentiation (Ranera et al., 2011).

**Table 1.6. Differentiation medium supplements and corresponding actions**

| Name of reagent | Action | Reference |
|---|---|---|
| Dexamethasone | Growth factor. A synthetic hydrocortisone commonly added to media to increase the attachment capacity of cells | Baker et al., 1978 |
| IBMX | Nonspecific inhibitor of phosphodiesterases which possess adenosine agonist activity | IBMX - Life technologies, 2013 |
| Indomethacin | Non-steroidal anti-inflammatory which increases PPARy2 protein levels | Styner et al., 2010; Dhar et al., 2012. |
| Insulin | Hormone that triggers induction transcription factors which assist in differentiating pre-adipocytes into mature adipocytes | Klemm et al., 2001 |
| TGF-β | Family of cytokines which regulate cell differentiation and proliferation | Clark and Coker, 1998 |
| ITS+premix | Culture supplement containing insulin, human transferrin, and selenous acid which stimulates cell proliferation | ITS+premix - BD Sciences, 2013 |
| Proline | An amino acid which induces differentiation. Important for maintenance of pluripotency in ESC | Washington et al., 2010 |
| Ascorbate 2-phosphate | Vitamin supplement capable of stimulating cell differentiation, specifically collagen synthesis and alkaline phosphatase activity | Takamizawa et al., 2004 |
| β-glycerophosphate | Serine-threonine phosphatase inhibitor. Functions as an organic phosphate donor for osteogenic differentiation | β-glycerophosphate –Sigma, 2013 |

### *1.1.11.4 DIFFERENTIATION EFFICIENCIES OF EQUINE MESENCHYMAL STEM CELLS*

There are varying reports for adipogenic differentiation efficiency between different sources of equine MSCs. Adipocyte potential is reportedly similar for equine ADSCs and BMSCs but poor adipogenic differentiation capacity has been observed in umbilical cord blood and tendon derived stem cells (Burk et al., 2013). Ahern et al. (2011) however, found that ADSCs stained strongly for adipogenic differentiation while no detectable difference in staining intensity was seen for BMSCs following exposure to adipogenic medium and control cells. There have also been varied results for pBSC differentiation into adipocytes. Koerner et al. (2006) reported weak staining for lipid vesicles following adipogenic differentiation, while Dhar et al. (2012) reported strong positive staining of lipids for pBSCs. Dhar et al. (2012) also compared two combinations of adipogenic media and found that ADSCs demonstrated lipid vesicle development in both combinations of media, while pBSCs were only able to achieve adipogenic differentiation in medium supplemented with indomethacin. Overall, it appears that ADSCs have superior adipogenic differentiation efficiency compared to other sources of equine MSCs.

Previous studies looking at chondrogenic differentiation in ADSCs and BMSCs have produced conflicting results. Burk et al. (2013) found that BMSCs demonstrated the weakest chondrogenic potential compared to cells isolated from UCB, UCT, AT and tendon tissue. However, in a study by Giovannini et al. (2008), BMSCs displayed intense chondrogenic differentiation, and Vidal et al. (2008) reported that BMSC chondrogenic potential was greater than ADSC chondrogenic potential. Furthermore, expression of collagen type II (COL1A2) was found to be higher for BMSC chondrocytes than ADSC chondrocytes (Vidal et al., 2008). Positive staining for GAG deposits could also be seen as early as day three after activation in BMSC chondrocytes, while GAG deposits in ADSC chondrocytes could only be seen from day 14 (Vidal et al., 2008).

Bone marrow derived stem cells reportedly have superior osteogenic potential compared to ADSCs (Vidal et al., 2008). Vidal et al. (2008) also reported that a

longer duration of exposure to osteogenic medium is required for ADSC differentiation to occur. Additionally, ADSCs expressed lower levels of alkaline phosphatase (ALP) than BMSCs. Alkaline phosphatase is an important component in the formation of hard tissue and is strongly expressed in mineralised tissue cells (Golub & Boesze-Bottaglia, 2007). The expression levels of ALP reported by Vidal et al. (2008) contradicts the results found by Ranera et al. (2012). When comparing ADSC expression with BMSC expression Ranera et al, (2012) found that ALP expression was significantly higher in ADSCs. Colleoni et al. (2009) reported that the levels of osteonectin, a stimulant of cell division and differentiation, in osteogenic differentiated ADSCs and BMSCs were similar when the cells were treated with basic fibroblast growth factor (bFGF). Osteonectin levels were increased to similar levels for both cell types following exposure to this treatment (Freshney, 2010).

### 1.1.12 IDENTIFICATION OF *IN VITRO* EQUINE MSCS

Although a great deal of research has been carried out on equine MSCs, a uniform approach to characterising these cells is yet to be established (De Schauwer et al., 2011).

In human medicine, only cells which express or lack specific surface markers can be labelled as MSCs (De Schauwer et al., 2011). This specific list in human medicine uses a panel of antigens that unequivocally identifies MSCs. Such a list or even a standard approach to identification is lacking in equine stem cell culture. Currently, researchers rely on cell attachment and differentiation into fat, cartilage and bone as proof of stemness. As a third approach to confirm stemness, it has been proposed that a panel of cell surface antigens be established for use with equine MSCs (De Schauwer et al., 2011).

Cell surface marker expression and messenger RNA (mRNA) gene expression have both been used for evaluating stemness in equine MSCs. Although gene expression at the mRNA level does not necessarily translate to protein level (Guest

et al., 2008), mRNA analysis is still a valuable characterisation tool when suitable antibodies are not available (De Schauwer et al., 2011). Cell surface marker analysis is reliant on the availability of species-specific or cross-reacting antibodies for proper immunophenotyping. Many equine studies use anti-human antibodies for the determination of equine cluster of differentiation (CD) markers, but difficulties arise due to the small reactivity of available human antibodies with equine epitopes (Ranera et al., 2012). Currently, this is the greatest limiting factor to the establishment of cell marker analysis in veterinary medicine (De Schauwer et al., 2011).

A range of different cell surface markers and gene markers have been evaluated in equine MSCs isolated from AT, BM and pB (**Table 1.7**). Recently, De Schauwer et al., (2012) published a list of suggested cell surface markers which may be used for positively identifying equine umbilical cord tissue stem cells (UTSC). However, this is yet to be incorporated into research to determine whether these markers are expressed in additional sources of equine MSCs. Although a range of markers have been investigated, there is still no proven selection of markers for routine stem cell identification.

The pluripotent markers octamer-binding transcription factor 4 (Oct4) and Nanog are reportedly positive in ADSCs (Raabe et al., 2010) and BMSCs (Violini et al., 2009) and, although expression at protein level is variable (Guest et al., 2008), they have been suggested as markers of MSCs (Raabe et al., 2010; Violini et al., 2009). Oct4, and Nanog are also reportedly expressed in MSCs derived from equine umbilical cord blood (Mohanty et al., 2014). Both of these markers are involved in maintaining self-replication and pluripotency in embryonic stem cells (Poloni et al., 2013). As the expression of Oct4 and Nanog in adult derived stem cells has been established in a number of studies (Poloni et al., 2013; Raabe et al., 2010; Raabe et al., 2011; Violini et al., 2009) it has been suggested that the distinction between adult and embryonic stem cell markers is not as clear cut as originally thought (Violini et al., 2009). Further research into the expression of additional embryonic markers has been recommended (Violini et al., 2009).

Bone marrow derived stem cells have been found to positively express CD105 (Ranera et al., 2012), CD29, CD44, CD90, CD11a/CD18 and CD45RB (Radcliffe et al., 2010) while CD45, CD79α and major histocompatibility complex II (MHC II) are not expressed (De Schauwer et al., 2012). Adipose derived stem cells are also reportedly negative for the expression of CD45 (Dhar et al., 2012) but positive for CD29 (Ranera et al., 2012), CD51, CD90 and CD105 (Raabe et al., 2011). Peripheral blood derived stem cells are positive for CD45, CD34, CD51, CD90 and CD105 (Dhar et al., 2012).

There appears to be minimal overlap in the expression of tested markers for equine MSCs. CD29 appears to be expressed in ADSCs and BMSCs (Radcliffe et al., 2010; Ranera et al., 2011) but not in pBSCs. Similarly, CD51 is expressed in ADSCs and pBSCs but not in BMSCs. The marker CD90 was found to be expressed in ADSCs (Raabe et al., 2011), BMSCs (Radcliffe et al., 2010) and pBSCs (Dhar et al., 2012). The only other marker that appears to be expressed in all three MSC types is CD105 however, a study by De Schauwer et al., (2012) reported variable expression of CD105 when umbilical cord tissue was used.

**Table 1.7. Overview of different cell surface markers and gene markers evaluated in equine mesenchymal stem cells from adipose tissue (AT), bone marrow (BM) and peripheral blood (pB).**

| Tissue type | Author | Cell surface marker | Gene marker |
|---|---|---|---|
| BM | Guest et al., 2008 | CD14, CD29, CD44,CD79α, CD90, MHC-I, MHC-II | ND |
| | Radcliffe et al., 2010 | CD11a/CD18, CD29, CD44, CD45RB, CD90 | CD11a, CD13, CD29, CD44, CD45, CD90 |
| | Ranera et al., 2012 | ND | CD31, CD34, CD45, CD73, CD90, CD105, CD166, ALP, BGLAP, COL1A1, RUNX2, SPP1, LPL, PPARγ, |
| | Ranera et al., 2011 | CD13, CD29, CD31, CD34, CD44, CD45, CD45d, CD73, CD90, CD105, CD106, CD146, CD166 | CD13, CD29, CD31, CD34, CD44, CD45, CD45d, CD73, CD90, CD105, CD106, CD146, CD166 |

| | | | |
|---|---|---|---|
| | Violini et al., 2009 | ND | CD34, Oct4, Nanog, Sox2 |
| | Berg et al., 2009 | ND | COL1A1, COL1A2, Aggrecan, SOX9, CD-RAP |
| AT | Ranera et al., 2012 | ND | CD31, CD34, CD45, CD73, CD90, CD105, CD166, ALP, BGLAP, COL1A1, RUNX2, SPP1, LPL, PPARγ, |
| | Ranera et al., 2011 | CD13, CD29, CD31, CD34, CD44, CD45, CD45d, CD73, CD90, CD105, CD106, CD146, CD166 | CD13, CD29, CD31, CD34, CD44, CD45, CD45d, CD73, CD90, CD105, CD106, CD146, CD166 |
| | Raabe et al., 2011 | ND | Oct4, Nanog, CD90, CD105, PPARγ2, AR, PP, Runx2, OC |
| | Dhar et al., 2012 | CD34, CD45, CD51, CD90, CD105 | ND |
| pB | Dhar et al., 2012 | CD34, CD45, CD51, CD90, CD105 | ND |
| | Giovanni et al, 2008 | ND | COL1A1, COL1A2, Aggrecan, Versican |
| | Martinello et al., 2009 | CD13, CD44, CD45, CD90, CD117, CD140a. | ND |

### 1.1.13 THERAPEUTIC APPLICATION OF MESENCHYMAL STEM CELLS IN HORSES

The equine industry is associated with a high percentage of wastage, a large proportion of which is attributed to musculoskeletal injury (Ramzan & Palmer, 2011). An Australian study of wastage in two-year-old Thoroughbreds, stated that 66% of lost training days are due to musculoskeletal injuries (Bailey et al., 1997). Additionally, Dyson et al. (2008) reported that in the UK, lameness remained the major cause of days lost from training. A further review of UK equine industry wastage determined that joint problems account for 15 – 25% of horses lost from training, and tendon and ligament injuries account for up to 46% of limb injuries (Paris and Stout, 2010). There is also a high incidence of re-injury once a rehabilitated horse has returned to training. A case controlled study of 400 horses in the UK revealed that the re-injury rate of tendon and ligament injuries was 53% (O'Meara et al. 2010).

Current treatments focus on restoring functionality, however, they do not appear to demonstrate a significant improvement in tendon structure (Schnabel et al., 2009). Furthermore orthopaedic injuries, such as subchondral bone cysts, bone fractures, cartilage defects and tendon damage, significantly contribute to wastage of otherwise healthy horses (Richardson et al., 2007).

The most common musculoskeletal injury in racehorses is superficial digital flexor tendonitis of the forelimb (O'Meara et al., 2010). A number of risk factors contribute to superficial digital flexor tendon (SDFT) injuries including age, sex, race distance, frequency of high speed work, heavier bodyweight, track surface and longer racing careers. During maximal exercise the SDFT is at its highest likelihood of over-strain injury, as the tendon is pushed to its maximal potential (O'Meara et al., 2010; Richardson et al., 2007). Tendon over-strain injuries present a situation where the tendon tissue heals naturally but the resultant scar tissue is considerably weaker than uninjured tendon tissue, resulting in reduced performance and increased risk of re-injury (Richardson et al., 2007).

Over the past few years, an intense interest in stem cell therapy has developed due to the potential benefits of these self-renewing, multi-lineage cells (Lopez and Daigle, 2013; Richardson et al., 2007; Undale et al., 2009). An increasing amount of research is being carried out on whether the perceived benefits of MSC treatment can be proven through equine *in vivo* studies. However, there are relatively few reports which demonstrate long term benefits from these treatments (Schnabel et al., 2013) (**Table 1.8**).

**Table 1.8. *In vivo* mesenchymal stem cell research in equine cell therapy.**

| Site of treatment | Results | Reference |
|---|---|---|
| **Adipose derived stem cells** | | |
| **Superficial digital flexor (chemical lesion)** | Treated limbs had increased organisation and uniformity of tendon tissue | de Mattos Carvalho et al., 2011 |
| **Middle carpal joint (bilateral arthroscopic surgery)** | No statistically measurable effect | Frisbie et al., 2009 |
| **Superficial digital flexor (chemical lesion)** | Histological improvement compared with control (PBS) | Nixon et al. 2008 |
| **Bone marrow derived stem cells** | | |
| **Superficial digital flexor (clinical cases)** | Reinjury rate 27.4% (lower than published controls circa 55%) | Godwin et al. 2012 |
| **Superficial digital flexor (surgical lesion)** | No difference to control (supernatant) | Canigala et al. 2011 |
| **Suspensory ligament and superficial flexor (clinical cases)** | 11 / 13 (85%) horses returned to competition | Torricelli et al. 2011 |
| **Superficial digital flexor** | Improved healing | Crovace et al. 2010 |
| **Stifle joint** | Improved healing of cartilage | Fortier et al. 2010 |
| **Middle carpal joint (bilateral arthroscopic surgery)** | Reduction in PGE2 concentration, minimal overall effect on injury | Frisbie et al., 2009 |
| **Superficial digital flexor (chemical lesion)** | Histological improvement compared to control (PBS) | Schnabel LV et al. 2009 |
| **Superficial digital flexor (surgical lesion) – autologous vs allogeneic transfer** | No cell mediated response to allogeneic transfer | Guest et al. 2008 |
| **Stifle joint** | Improved early healing | Wilke et al. 2007 |

| | | |
|---|---|---|
| **Superficial flexor tendon (clinical cases)** | Minimal change in lesion size. 5% reduction at 6 weeks post implantation. | Smith et al., 2003 |
| **Peripheral blood derived stem cells** | | |
| **Superficial digital flexor (clinical cases)** | Improved healing - regenerated tendon tissue | Marfe et al. 2012 |
| **Embryonic stem cell and mesenchymal stem cell comparison** | | |
| **Superficial digital flexor** | ESC survival was better than MSC in the host. Neither cell source initiated a host immune response or tumour formation | Guest et al. 2010 |
| **Foetal derived embryonic stem cells (feES)** | | |
| **Superficial digital flexor (chemical lesion)** | Improved histological markers of healing. | Watts et al. 2011 |

### 1.1.14 AIMS OF RESEARCH AND HYPOTHESIS

The aim of this research was to compare the recovery of stem cells from AT, BM and pB. The three tissues were collected from six adult horses to compare cell yield and viability at isolation, and cell proliferation differences during culture. All three tissue types were investigated for stemness markers in undifferentiated and differentiated cells. Adipose tissue and BM were also evaluated for multipotent differentiation capacity.

In addition to the main aims of the project, several questions were investigated through the course of the project. Due to contamination issues during AT isolation an antibiotic trial was carried out to compare two combinations of antibiotics for minimising bacterial contamination. Two combinations of cryopreservation medium were also compared for cellular viability after thawing.

Through the use of validated techniques, the aim was to determine which of the three selected tissue sources should be used for routine harvest of equine MSCs. Determining the optimal source of equine MSCs would be greatly beneficial for veterinary practitioners wishing to utilise stem cell therapies to treat lameness in horses.

# CHAPTER 2: MATERIALS AND METHODS

Ethics approval was gained from the University of Waikato School of Science and Engineering Animal ethics committee prior to the commencement of this study. Ethics approval was also received from the AgResearch Ltd Animal Ethics Committee prior to the study commencing.

## 2.1 PILOT TRIAL

A pilot trial, in association with AgResearch Ltd, was carried out using post-mortem samples to enable training in the necessary skills and techniques required to complete the project's laboratory work. The pilot trial was also used to ensure the protocols were successful before the live samples were collected.

### 2.1.1 ANIMALS

AC Petfoods Ltd very generously allowed the collection of AT, BM and pB from horses they had purchased for meat processing. Samples were collected from 13 horses of various breed and age. Mares (11) and geldings (2) were included. The individuals are identified in **Table 2.1.** Post-mortem (PM) samples were used for training and verifying isolation, cryopreservation and differentiation protocols.

**Table 2.1. Post-mortem horse information, age, sex, breed and tissue type collected from each individual. Thoroughbred (TB), Warmblood (WB), Standardbred (SB). Gelded male (GM), Female (F).**

| Sample name | Tissue collected | Breed | Sex | Age |
|---|---|---|---|---|
| PM1 | AT | TB | GM | 6 |
| PM2 | AT, pB | TB | F | 7 |
| PM3 | BM | TB | F | 7 |
| PM4 | AT, BM | WB | F | 12 |
| PM5 | AT, pB | TB | F | 23 |
| PM6 | AT, pB | TB | GM | 10 |
| PM7 | AT, pB | TB | F | 7 |
| PM8 | AT | TB | F | 9 |
| PM9 | AT | TB | F | 7 |
| PM10 | AT | TB | F | 6 |
| PM11 | AT, pB | SB | F | 7 |
| PM12 | AT | TB | F | 6 |
| PM13 | AT | TB | F | 11 |

### 2.1.2 TISSUE COLLECTION

Adipose tissue and BM samples were collected within 30min after the horses were euthanized. Adipose tissue was collected after the horses had been skinned. Using a sterile scalpel holder (Appendix 2) and blade (Appendix 2) a 10g section of tissue was sliced away from the subcutaneous fat deposits and transferred to a sterile collection container (Appendix 2) using sterile forceps. The sample was covered with PBS 2% P/S (Appendix 1) and sealed with a lid. A total of 10g each was collected from 12 horses. Bone marrow was collected using the same technique described in **section 2.2.1.2** without the use of local anaesthetic and a total of 20ml was collected from two horses. Peripheral blood was collected from the jugular vein immediately after the horse was euthanized. A 20ml syringe (Appendix 2) and an 18g needle (Appendix 2) was used to collect blood. The blood was then injected into sterile EDTA blood tubes (Appendix 2) a mean of 27ml +/- 6.6ml of blood was collected from five horses.

### 2.1.3 TISSUE PROCESSING, CULTURE AND DIFFERENTIATION

Adipose tissue was processed using the protocol described in section 2.3.1, BM and pB were processed using two different protocols. The initial protocol used Percoll density gradient (1.08g/ml) but this did not appear to suit the composition of equine BM or pB. An alternative method using Lymphoprep (1.077g/ml) resulted in a well-defined interface containing mononucleated cells and minimal erythrocytes. This protocol was used for the live BM and pB samples and is detailed in **section 2.3.2** and **section 2.3.3** respectively.

Two different media were used for pB culture, DMEM F12, 20% FBS, 1% P/S, 0.1% fungizone and DMEM High Glucose, 20% FBS, 1% P/S, 0.1% fungizone.

To establish differentiation techniques, ADSCs and BMSCs from two individual horses were activated into adipocytes, osteocytes and chondrocytes using the protocols set out in **sections 2.7.1** (adipogenic), **2.7.2** (chondrogenic) and **0** (osteogenic). The adipogenic protocol was refined by altering plating densities and

comparing two different matrixes. Laminin was determined to be superior to Matrigel for adipogenic seeding as more cells adhered to Laminin than to Matrigel. Myogenic differentiation was carried out for one BMSC sample using the protocol set out in **section 2.1.4**. Peripheral blood was not placed in a differentiation assay due to the low number of pBSC samples (2) reaching confluence.

### 2.1.4 MYOGENIC DIFFERENTIATION

The materials and methods for differentiating equine MSCs into myogenic linages are based on the protocol used at AgResearch Ltd. A single BMSC sample was plated in three wells at a density of $1x10^4$ cells in a 24 well plate. Coverslips were placed on the surface of the three wells (Appendix 2). The cells were cultured in myogenic media (Appendix 3) for 21 days. The cells were then fixed using the protocol set out in **section 2.7.4.1**. Staining was carried out using an ICC protocol to identify myosin heavy chain proteins which may have formed during differentiation. The fixed coverslips were permeabilized with 0.1% triton X-100 in PBS for 10 min at room temperature. The coverslips were then washed with 1 x Tris buffered saline with 0.2% tween (TBS-T) wash. The cells were then blocked with the polysaccharide carrageenan lambda (Cλ) in PBS with 10% normal sheep serum (NSS) for 30-60min at room temperature. Mouse anti-MHC (myosin heavy chain) antibody (1:200) in 0.35% Cλ in PBS + 5% NSS was added to the wells containing the coverslips and left overnight at 4°C wrapped in parafilm. The following day the coverslips were washed three times in 1 x TBS-T for 5mins for each wash. Biotinylated Sheep anti-Mouse (1:300) in 0.35% Cλ + 5% NSS was aliquoted into each well and incubated for one hr at room temperature. Streptavidin Alexa Fluor 488 was then added at 1:400 in 0.35% Cλ and incubated for one hr at room temperature. Streptavidin is a biotin binding protein which covalently attaches to the fluorescent label Alexa Fluor. The coverslips were washed twice in TBS-T for 5min each. A DAPI counter stain (1:1000) in PBS was added and incubated for 5min followed by two PBS washes. The cells were then mounted using the protocol set out in **section 2.7.4.2**. The coverslips were then

viewed under the fluorescent microscope (Appendix 2) at 20x magnification using SPOT software.

## 2.2 *MAIN PROJECT*

Adipose tissue, BM and pB samples were collected from six adult female horses (mean age 10.5 +/- SEM 2.04) (**Table 2.2**). The horses were located at Equibreed NZ Ltd for the duration of the project. All surgical procedures were carried out at this location which has purpose built facilities for safely restraining horses. Following the surgical procedures the mares were monitored twice daily for seven days until the sutures were removed from the AT harvesting site.

**Table 2.2. Tissue collected from live horses, adipose tissue (AT), bone marrow (BM), and peripheral blood (pB). Sexually mature female (F) Thoroughbred (TB) and Standardbred (SB) horses were of various age (7-20 years old).**

| Sample name | Tissue collected | Breed | Sex | Age |
|---|---|---|---|---|
| Live horse 1 (LH1) | AT, BM, pB | SB | F | 11 |
| Live horse 2 (LH2) | AT, BM, pB | TB | F | 9 |
| Live horse 3 (LH3) | BM, pB | TB | F | 7 |
| Live horse 4 (LH4) | AT, BM, pB | TB | F | 20 |
| Live horse 5 (LH5) | AT, BM, pB | TB | F | 6 |
| Live horse 6 (LH6) | AT, BM, pB | SB | F | 10 |

### 2.2.1 TISSUE COLLECTION

The number of samples collected is displayed in **Table 2.3**. Multiple sampling was carried out for AT as a number of samples were lost due to bacterial contamination at isolation. Bone marrow was collected twice from three horses due to contaminated medium. Peripheral blood was collected three times from each

horse, six samples were lost due to fungal contamination and the remaining samples did not reach confluence.

**Table 2.3. Total number of adipose tissue (AT), bone marrow (BM) and peripheral blood (pB) samples collected from live horses.**

| Tissue type | AT | BM | pB |
|---|---|---|---|
| Number of samples collected | 9 | 9 | 18 |

#### *2.2.1.1 ADIPOSE TISSUE COLLECTION*

The horses were handled by experienced handlers and were adequately restrained in stocks for pre-analgesic administration. The sterile surgical site was clipped and prepared aseptically with chlorhexidine scrub (Appendix 2) and ethanol (Appendix 2). The horses were then sedated with Dormosedan and Torbugesic (1mg/kg, i.v.) (Appendix 2), followed by infiltration of the skin and subcutaneous tissues with Bomacaine (Appendix 2) using an inverted L-block. Ultrasound was used to identify a deposit of adipose tissue before an incision was made.

Adipose tissue collection procedure was based on previously published methods for equine AT harvesting (Ahern et al., 2011; Ranera et al., 2011). An incision of approximately 10cm in length was made parallel to and approximately 15cm below the spinal column, permitting visualization of a layer of AT between the skin and musculature. An average of 12g of tissue was collected from each horse into a sterile collection container. A sterile penrose drain (Appendix 2) was incorporated into the surgical site to aid the healing process. The surgical site was then sutured closed with Supramid Braun suture material (Appendix 2) using a single uninterrupted suture pattern (**Figure 2.1**).

**Figure 2.1. Adipose tissue collection site with incorporated penrose drain.**

#### *2.2.1.2 BONE MARROW COLLECTION*

Following sedation with Dormosedan and Torbugesic (1mg/kg, i.v.) the sternal region was clipped with electronic clippers then scanned with an ultrasound (Appendix 2) to identify an intersternebral space shown as an inverted V shape on the ultrasound screen. Two of these images were marked on the horse's skin with a marker pen (on light-coloured horses) or white correction pen (on dark-coloured horses). These V shapes indicated the $5^{th}$- $6^{th}$ intersternebral space and the caudal end of the $7^{th}$ intersternebral (**Figure 2.2**). Bone marrow aspiration methodology was based on the protocols published by Kasashima et al. (2011).

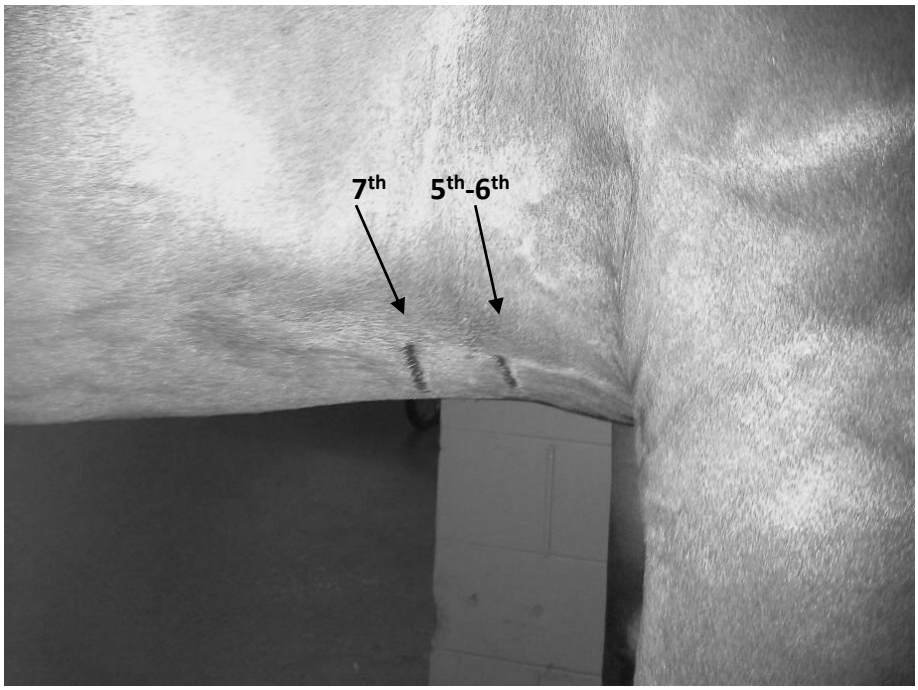



**Figure 2.2. Location of intersternebral spaces to determine insertion point of Jamshidi® needle.**

The sternal area was then prepared aseptically with chlorhexidine scrub and 70% ethanol. Six millilitres of Bomacaine was injected into the tissue surrounding the predicted insertion point to desensitise the skin. At this point two 20ml luer slip (Appendix 2) syringes were preloaded with 0.5ml of 5000 iu/ml of heparin (Appendix 2) to give a final concentration of 125 iu/ml when 20ml of BM was aspirated. The predicted insertion points were checked a second time via ultrasound to ensure the correct entry point was identified. The area was scrubbed a final time with the chlorhexidine scrub and ethanol. The horses were also thoroughly assessed at this point to ensure adequate sedation. If required, additional sedative was administered by the veterinarian. After the final aseptic preparation of the insertion site, a sterile 11 gauge scalpel blade was used to make a small stab incision through the skin. A sterile 11 gauge Jamshidi® needle (Appendix 2) was inserted through the skin opening and advanced slowly until the needle point made contact with the ventral surface of the sternebrae. The needle was gradually advanced through the ventral cortex into the medullary cavity using a rotating movement until the handle end of the Jamshidi® needle was no more than 5cm away from the skin surface. A 20ml syringe preloaded with heparin was

attached and the BM was slowly aspirated (**Figure 2.3**). This process was repeated with an additional syringe to collect a total of 40ml of BM aspirate from each horse. Upon collection, the samples were gently inverted to ensure even distribution of heparin to prevent clotting.

**Figure 2.3. Bone marrow aspiration using a Jamshidi® needle.**

#### *2.2.1.3 PERIPHERAL BLOOD COLLECTION*

Forty millilitres of pB was collected from the jugular vein in four 10ml EDTA vacutainers with an 18 gauge needle. During pB collection the horses were restrained in a stock and an experienced technician collected the blood. The vacutainers were inverted immediately after collection to ensure adequate mixing of EDTA and blood to prevent clotting.

## 2.3 TISSUE PROCESSING

Samples were processed to yield a mononucleated cell (MNC) fraction which contains MSCs, additional MNCs and RBCs.

### 2.3.1 ADIPOSE TISSUE

Mononucleated cells from AT were isolated using enzymatic digestion and washes to produce a cell pellet. Briefly, MNCs were isolated from the tissue samples using the following methods and materials set out in **Table 2.4**. The procedure was in accordance with an established preadipocyte isolation protocol for bovine ADSCs at AgResearch Ltd (M. Senna Salerno, personal communication, November, 1, 2012) and is similar to other published ADSC isolation protocols although specific details were not provided in the published papers (Ahern et al., 2011; Ranera et al., 2011).

**Table 2.4. Isolation protocol for adipose tissue.**

| Step | Process |
|---|---|
| 1 | Mechanical separation of tissue, wash with PBS |
| 2 | PBS wash and spin |
| 3 | Retrieve fat layer, resuspend and spin |
| 4 | Digest in 0.2% Type 1A collagenase |
| 5 | Neutralise, spin, remove supernatant |
| 6 | Resuspend in medium |
| 7 | Filter (70µm strainer) and spin |
| 8 | Resuspend, lysis |
| 9 | Wash in PBS and spin |
| 10 | Resuspend cells in DMEM 10% FBS, 1% P/S, 0.1% fungizone |
| 11 | Count, record viability and plate |

The cell pellet was resuspended in DMEM, 10% FBS, 1% P/S, 0.1% fungizone (Appendix 1) for counting and viability assessment. The mononucleated cells were seeded at a density of 2 x$10^5$ cells per $cm^2$ in 75$cm^2$ flasks (Appendix 2) and placed in the $CO^2$ incubator (Appendix 2).

### 2.3.2 BONE MARROW

Bone marrow aspirate was isolated using a lymphocyte gradient technique established at Equibreed NZ Ltd (**Table 2.5**) and was based on a lymphocyte density gradient protocol published by Ranera et al., (2011) with some minor modifications.

**Table 2.5. Isolation protocol for bone marrow.**

| Step | Process |
|---|---|
| 1 | Strain BM through a 70um cell strainer |
| 2 | Dilute 1:3 with PBS |
| 3 | Dispense 10ml of aspirate over 5ml of Lymphoprep |
| 4 | Centrifuge at 1400rpm for 15min |
| 5 | Collect interface and dilute with 30ml of PBS |
| 6 | Centrifuge at 2000rpm for 5min |
| 7 | Discard supernatant, add 5ml RBC lysis and leave for 10min |
| 8 | Add 30ml of PBS, mix and centrifuge at 2000rpm for 5min |
| 9 | Discard supernatant |
| 10 | Resuspend cells in DMEM/F12, 10% FBS, 1% P/S, 0.1% fungizone |
| 11 | Count, check viability and plate |

Briefly, the sample was diluted 1:3 in PBS (Appendix 1) and filtered through a 70µm cell strainer (Appendix 2). Ten millilitres of diluted aspirate was layered over 5ml of Lymphoprep (Appendix 2) in 15ml falcon tubes using sterile disposable pipettes (Appendix 2). The falcon tubes were centrifuged for 15min at 1400rpm without a brake. The samples separated into a red blood cell fraction, a Lymphoprep fraction and a PBS fraction. An interface containing MNCs was isolated between the Lymphoprep fraction and the PBS fraction (**Figure 2.4**).

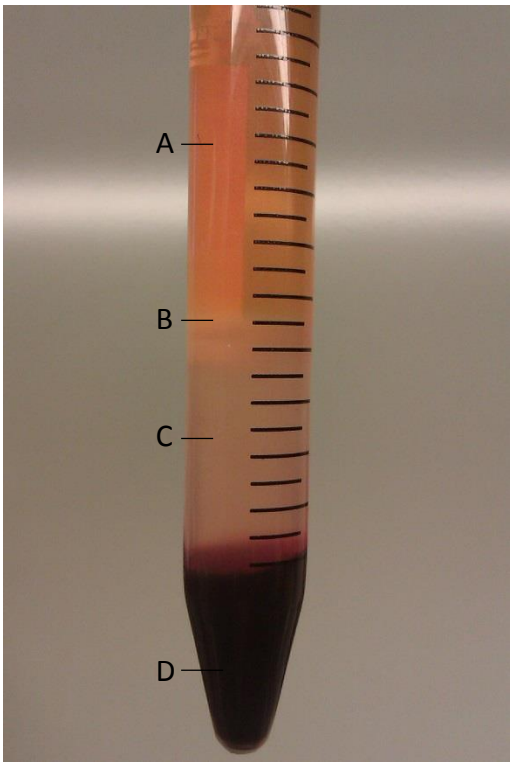



**Figure 2.4. Bone marrow aspirate separation using Lymphoprep. A - PBS fraction, B - interface, C - Lymphoprep fraction, D – red blood cell fraction.**

Using sterile 3ml disposable pipettes, the interface was isolated and transferred to a 50ml falcon tube. The interface from each density gradient was combined in a single 50ml falcon tube which was then diluted with 30ml of PBS and centrifuged for 5min at 2000rpm to remove traces of Lymphoprep. The MNCs formed a pellet at the base of the falcon tube and the supernatant was tipped off into a waste collection bottle. The pellet was loosened by flicking the base of the tube and resuspended in 5ml of RBC lysis for 10min to reduce red blood cell contamination. The resuspended sample was diluted a final time in 30ml of PBS to remove traces of RBC lysis. The pellet formed from this wash was resuspended in 20ml of DMEM/F12, 10% FBS, 1% P/S, 0.1% fungizone (Appendix 1) and underwent counting and viability assessment. The mononucleated cells were seeded at a density of 2 $x10^5$ cells per $cm^2$ in 75$cm^2$ flasks (Appendix 2) and placed in the $CO^2$ incubator.

### 2.3.3 PERIPHERAL BLOOD

Peripheral blood samples were processed using a combined protocol adapted from Spaas et al. (2013) and Dhar et al. (2011) with some minor modifications.

**Table 2.6. Isolation protocol for peripheral blood.**

| Step | Process |
|---|---|
| 1 | Centrifugation 2200rpm, 20min |
| 2 | Remove buffy coat, dilute 1:2 with PBS |
| 3 | Buffy coat layered on 3ml of Lymphoprep |
| 4 | Centrifuge at 1400rpm for 15min |
| 5 | Collect interface |
| 6 | Add 30ml of PBS |
| 7 | Centrifuge at 2000rpm for 15min |
| 8 | Discard interface resuspend pellet in 30ml of PBS |
| 9 | Centrifuge at 2000rpm for 15min |
| 10 | Discard interface and resuspend cells in DMEM F12, 20% FBS, 1% P/S, 0.1% fungizone |
| 11 | Count, check viability and plate |

Briefly, 40ml of peripheral blood was centrifuged (Appendix 2) for 20min at 2200rpm before being processed using a Lymphoprep density gradient protocol established at Equibreed NZ Ltd (**Table 2.6**). The initial centrifugation resulted in a buffy layer forming on the surface of a dense layer of red blood cells with a large fraction of plasma located above the buffy layer. The buffy layer and the plasma was then collected from each of the four EDTA tubes using sterile 3ml disposable pipettes and combined into a single sample in a 50ml falcon tube. The buffy layer was then diluted 1:3 in PBS and 5ml was layered on 3ml of Lymphoprep density gradient. The samples were then centrifuged for 15min at 1400rpm to form the four fractions of density separation (**Figure 2.5**).

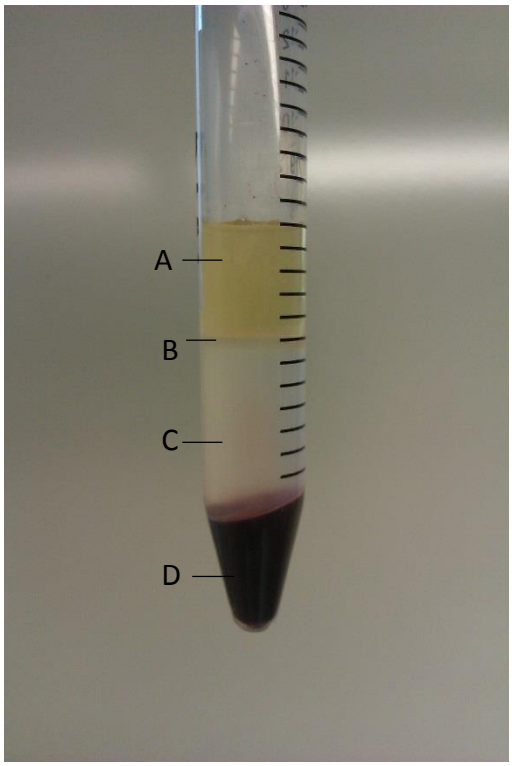



**Figure 2.5. Peripheral blood separation using Lymphoprep. A - PBS fraction, B - interface, C - Lymphoprep fraction, D – red blood cell fraction.**

The MNC interface from each tube was combined in a single 50ml falcon tube, diluted with 30ml of PBS and centrifuged for 5min at 2000rpm to remove traces of Lymphoprep. The MNCs formed a pellet at the base of the falcon tube and the supernatant was tipped off into a waste collection bottle. The pellet was loosened by flicking the base of the tube and resuspended in 30ml of PBS followed by another centrifugation for 5min at 2000rpm. The pellet formed from the final wash was resuspended in 10ml of DMEM/F12, 20% FBS, 1% P/S, 0.1% fungizone (Appendix 1) and underwent counting and viability assessment. The cells were seeded at a density of 2 x$10^5$ cells per $cm^2$ in 25$cm^2$ flasks (Appendix 2) and placed in the $CO^2$ incubator (**Table 2.6**).

### 2.3.4 COUNTING AND VIABILITY ASSESSMENT

A Neubauer counting chamber (Appendix 2) (**Figure 2.6)** was used to determine the concentration and viability of cells at isolation, replating and freezing. Methodology was based on techniques used at AgResearch Ltd and published cell

counting protocols by Freshney (2010). The specialised coverslip was adhered to the surface of the counting chamber by moistening the sides of the chamber with 70% ethanol and sliding the cover slip on top until Newton's rings were visible. This method ensured the coverslip would stay firmly attached during counting and prevented the cells from moving around the grid and being miscounted. A 20µl sample of cell suspension was aliquoted into an eppendorf tube and resuspended with 10µl of trypan blue exclusion dye (Appendix 1). A 20µl sample was evenly distributed under the cover slip, ensuring the counting chamber was covered with the cellular suspension.

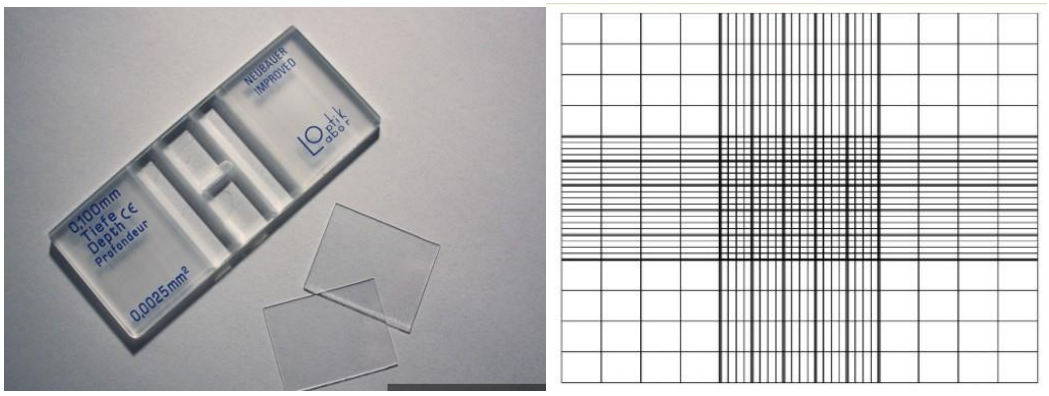



**Figure 2.6. Neubauer improved counting chamber with gridlines shown.**

The sample was immediately viewed under the microscope (Appendix 2) and methodically counted using either a five square count if there was a high density of cells or a 25 square count if there was a low density of cells. The gridlines in the centre of **Figure 2.6** show the central 25 square grid on which the cells are counted. Viability was determined by visually identifying dead cells stained with trypan blue dye. The formula used for determining cell count is shown below.

Cell count = (n x $10^4$) x (Dilution volume of trypan blue) x (dilution volume of total cell suspension)

## 2.4 CELL CULTURE

Mononucleated cell samples were plated and incubated until confluence was reached. During pBSC culture, the samples were incubated for 21 days before discarding the flasks if no cell colonies had appeared.

All of the cells isolated from each tissue type were plated as a single population of cells with no clonal growth protocol. The cell populations grown are heterologous with a mixture of cell types and a range of cell growth stages are represented in each flask of cells. As no live pB samples reached confluence, cell culture data was collected from live ADSCs and BMSCs only. Peripheral blood derived stem cell growth and culture behaviour data was recorded from the two post-mortem pB samples which reached confluence. For evaluation of isolation counts, data from tissue collected on the same day or within the same week were used for comparison to reduce day effect on cell yield.

### 2.4.1 PASSAGES AND CONFLUENCE

The primary stem cells collected directly from raw tissue were labelled as passage – (P-) as they had not yet been plated. Passage zero (P0) cells had been plated but had not yet been removed, divided and replated. Passage one (P1) cells had undergone one removal, division and replating and passage two (P2) cells had undergone two removals, divisions and replatings (**Figure 2.7**). Cells at P2 were frozen for future use from all samples that reached this passage stage.

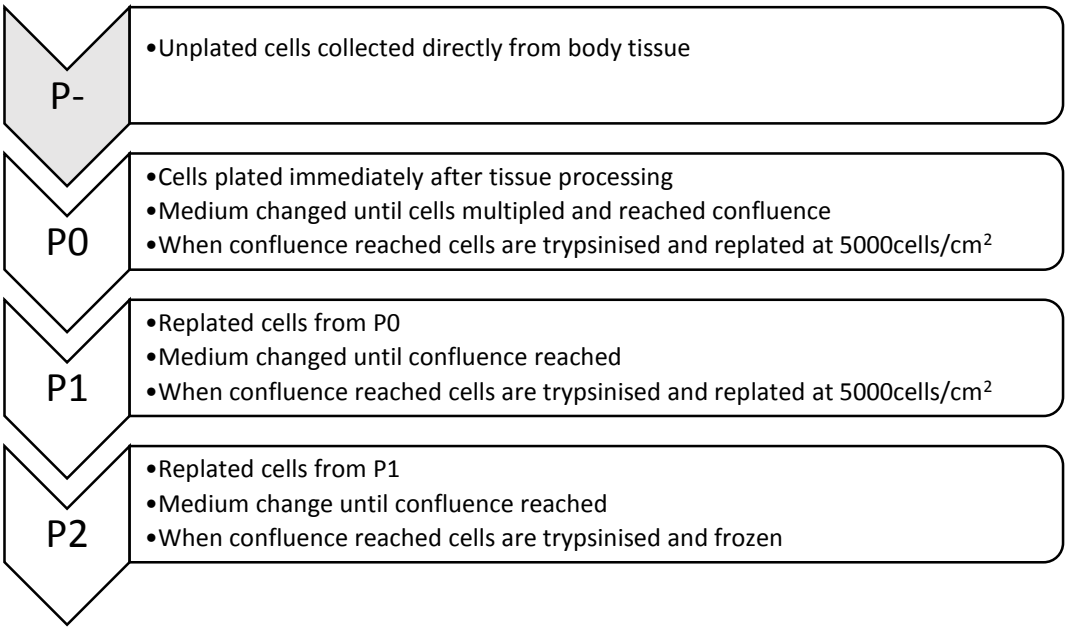



**Figure 2.7. Passage stages during culture, passage minus (P-), passage zero (P0), passage one (P1) and passage 2 (P2). Grey passages are primary cell from raw tissue. White passages are secondary cells which had been replated.**

The cells were removed from the flasks when they reached 80% confluence, determined by evaluating the entire surface of the flask to gauge overall cell density.

Adipose derived stem cells, BMSCs and pBSCs from P0 were plated at a density of 5000 cells/cm$^2$ in 75cm$^2$ nunclon flasks in medium prescribed for each tissue type. The interval of time from point of plating to day of confluence was recorded. This plating density was repeated for an additional passage until the cells reached P2. The length of culture time until confluence was recorded for each passage, as was the number of cells and cell viability. Adipose derived stem cells from LH4 and LH6 were passaged to P2 and then cryopreserved. Bone marrow derived stem cells from LH2, LH3, LH4, LH5 and LH6 were passaged to P2 then cryopreserved. For use in the differentiation assays, ADSCs and BMSCs were thawed and passaged until the required number of cells were available. The maximum passage reached was passage 6 (P6) and the cells used for differentiation range from P4 to P6 cells.

### 2.4.2 MEDIUM CHANGES

Every three days the flasks were removed from the $CO_2$ incubator, evaluated under a light microscope (Appendix 2) and placed in the laminar flow hood. Medium changes were carried out following the protocol published by Freshney (2010). Briefly, the medium stock bottle was prewarmed in a 37°C water bath to ensure the cells remained at a stable temperature. The lids of the flasks were removed and the medium was removed using a sterile 10ml pipette (Appendix 2). The medium was then discarded into a waste bottle. The replacement medium was added via a sterile 10ml pipette by releasing the liquid along the side of the flask to minimise disturbing the attached cells. The cells were then placed back into the $CO_2$ incubator. At each medium change the cells were assessed for morphology, attachment and proliferation. The medium was examined for changes in pH and clarity which can be an indication of cell growth and contamination. At day one in culture, the ADSCs and BMSCs were examined under the microscope to assess cell attachment. The flasks were then left to attach before changing the medium at day three. Medium changes were carried out every three days until confluence. The pBSC flasks were examined at day one in culture and the medium was replaced in each flask to remove the red blood cells which were contained in the isolated cell fraction. On day five, the pBSC medium was replaced and changed every three days until the cells were either confluent or the flasks were discarded due to lack of cell adherence. Flasks containing the mononucleated cell fraction from pB were maintained in culture for a maximum of 20 days to allow time for cell attachment. This generous allowance in time was seen in previously published papers on equine pBSC culture (Dhar et al., 2011; Giovannini et al., 2008).

### 2.4.3 SUBCULTURING

When the adherent cell layer approached late log phase, demonstrated by reaching 80% confluence, the cells were removed from the flask using a subculture protocol published by Freshney (2010). Briefly, the flasks were removed from the $CO_2$ incubator and washed with sterile PBS to remove traces of medium prior to

the cells being trypsinised. Two ml of Trypsin/EDTA (Appendix 2) was added to the washed flasks, swirled around the entire surface and the excess solution was tipped out of the flask into a waste bottle to leave a residual film on the flask surface. The flasks were incubated at 37°C for 2min until the cells become rounded and detached from the flask surface. The trypsin was neutralised by adding DMEM 10% FBS and the cells were collected in a 50ml falcon tube. A cell count was determined following the protocol set out in **section 2.4.4**. The cells were then replated at 5000cells/cm$^2$. Cell plating density was based on published papers using culture flasks for cell proliferation analysis (Ranera et al., 2011; Vidal et al., 2008). Cell counts, viability and duration to confluence was recorded at each passage.

The method used for estimating confluence involved assessing the concentration of cells throughout the plate and estimating a percentage of the flask surface that was covered with cells. Accurately assessing cell confluence requires experience with cell behaviour in culture which was gained during the course of the project.

### 2.4.4 CELL CALCULATIONS

The proportion of MSCs contained in raw tissue was calculated based on the number of MNCs per ml of raw tissue at isolation (P-) and the number of MSCs per ml that reached initial confluence (P0).

## 2.5 ANTIBIOTIC TRIAL

Five AT primary cell samples were plated at 5000cells/cm$^2$ and cultured in two separate combinations of antibiotics in 25cm$^2$ flasks. Standard culture medium for ADSC was used with the addition of either penicillin/streptomycin (P/S) (100U/100µg) or gentamicin/vancomycin (G/V) (40µg/ml). Once the cells reached confluence the number of cells per flask and viability percentage was measured and compared.

# 2.6 CRYOPRESERVATION

### 2.6.1 FREEZING

Cryopreservation of cell populations followed the freezing protocols used by AgResearch Ltd with some minor differences in cryopreservation medium composition. The minor changes were made based on the results of a summer project carried out at EquiBreed NZ Ltd. The project compared cell viability after freezing in 5% DMSO, 20% FBS and 75% DMEM to cell viability after freezing in 5% DMSO, 90% FBS and 5% DMEM. As no significant difference was found in cell viability after freezing 5% DMSO, 20% FBS and 75% DMEM was suggested as a potential cryopreservation medium for routine freezing and storage of equine MSCs.

The methodology for MSC cryopreservation is based on the protocols used by AgResearch Ltd and the protocols published by Freshney (2010). The flasks were trypsinised (**see section 2.4.3**), counted and washed to remove trypsin and pellet the cells. The cell pellet was resuspended in cryopreservation medium consisting of 5% DMSO, 20% FBS and 75% DMEM medium. The cells were diluted in enough cryopreservation medium to produce a final concentration of approximately 1 x $10^6$ cells per ml and 1ml of cell suspension was aliquoted into 2ml cryovials (Appendix 2). The vials were placed into a Mr Frosty™ freezing container (**Figure 2.8**) and stored in a -80°C freezer for 24 hours before being transferred into a liquid nitrogen tank.


**Figure 2.8. Mr Frosty™ freezing container.**

### 2.6.2 THAWING

The cryovials were retrieved from the liquid nitrogen tank and placed in a 37°C waterbath for 2min until the ice crystals had disappeared as described by Naaldjik et al. (2012). Cell samples were then diluted in the standard medium for each cell type (Appendix 1) and cultured in a $CO_2$ incubator at 37°C. A 20µl aliquot of thawed cells were counted and assessed for viability after cryopreservation.

### 2.6.3 CRYOPRESERVATION MEDIUM COMPARISON

Five post-mortem ADSC samples (PM4, PM5, PM7, PM8 and PM9) were used to compare the effect of two different concentrations of FBS (20% and 90%) on cell viability and number after cryopreservation. The cells were trypsinised and counted using the protocols set out in **sections 2.4.3** and **2.3.4.** The samples were then frozen in cryopreservation media comprising of either 20% FBS, 5% DMSO and 75% DMEM or 90% FBS, 5% DMSO and 5% DMEM. After approximately three weeks after freezing, the cells were thawed, counted and assessed for viability using trypan blue stain. The cell count and viability results, before and after freezing, was compared to obtain a percentage of cell survival post-thaw.

## 2.7 DIFFERENTIATION

Differentiation was carried out in triplicate design which was duplicated to provide one plate for determining differentiation morphology changes and another for determining changes in gene expression during differentiation. The differentiation assay for ADSC included the two live horse samples (LH4 and LH6) and two of the post-mortem samples to increase the number of samples in the assay (**Table 2.7**). The samples used for differentiation had been previously stored in liquid nitrogen before being thawed, plated and amplified.

**Table 2.7. Adipose derived stem cell (ADSC) and bone marrow derived stem cell (BMSC) samples used for trilineage differentiation assay.**

| Cell type | Adipogenic | Chondrogenic | Osteogenic |
|---|---|---|---|
| ADSC (4) | LH4, LH6, PM1, PM13 | LH4, LH6, PM1, PM13 | LH4, LH6, PM1, PM13 |
| BMSC (5) | LH2, LH3, LH4, LH5, LH6 | LH2, LH3, LH4, LH5, LH6 | LH2, LH3, LH4, LH5, LH6 |

### 2.7.1 ADIPOGENIC DIFFERENTIATION

The methodology for adipogenic differentiation was a combination of two published protocols (Raabe et al., 2011; Spaas et al., 2013) with some minor adjustments for insulin concentration during adipogenic maintenance. The use of a matrix or coverslips was not published in the literature but were added to the protocol to facilitate cell surface adherence and ease of staining.

Adipogenic differentiation was carried out in 24 well plates (Appendix 2) with the addition of 13mm cover slips (Appendix 2) and a laminin matrix (Appendix 2) (1:20 dilution). The coverslips were placed in the well plates and coated with 200µl of laminin and incubated for two hours before use. The laminin was removed and the wells were washed three times with PBS. Undifferentiated ADSC and BMSC samples were trypsinised, counted and resuspended in DMEM, F12, 10% FBS, 1% P/S, 0.1% fungizone at a density of 1.5 x $10^5$ cells per well. The cells were cultured until confluence which took approximately 24-48 hours from seeding. Once

confluence was reached, the medium was replaced with adipogenic induction medium (Appendix 3). The cells were incubated for three days then cultured in adipogenic maintenance medium (Appendix 3).

The adipogenic assay was incubated for a total of 14 days from the day of exposure with adipogenic induction medium. Medium changes were carried out every three days and the cells were assessed regularly for cellular development and morphology. A semi-quantitative scoring system was developed for assessing the percentage of differentiated cells and the size and arrangement of lipid droplets within the cells (**Table 2.8**).

**Table 2.8. Semi-quantitative scoring system used to evaluate adipogenic differentiation of mesenchymal stem cells under fluorescent microscopy after staining with BODIPY and DAPI.**

| % of differentiated cells | | Lipid vesicle size | | Number and orientation of lipid vesicles | |
|---|---|---|---|---|---|
| 0 | >0-25 | 0 | No vesicles | 0 | No vesicles |
| 1 | >25-50 | 1 | Small, < 1/3 nucleus | 1 | Few individual vesicles throughout cell |
| 2 | >50-75 | 2 | Medium sized 1/3 of nucleus | 2 | Few individual vesicles surrounding nucleus |
| 3 | >75-100 | 3 | Large > 1/3 of nucleus | 3 | Many vesicles surrounding nucleus |

### 2.7.2 CHONDROGENIC DIFFERENTIATION

The protocol used for chondrogenic differentiation was based on a protocol published by Pilz et al. (2011). Chondrogenic differentiation was carried out in 96 well round-bottomed plates (Appendix 2). The wells were seeded with 4 x $10^5$ cells in 20µl of incomplete chondrogenic medium (Appendix 3). After an incubation period of two hours 180µl of complete chondrogenic medium (Appendix 3) was pipetted into the treated pellet wells. For each horse, ADSCs and BMSCs were seeded in two control wells and three treatment wells. The control pellets were exposed to incomplete chondrogenic medium throughout the length of

incubation. Medium changes were carried out every three days. The chondrogenic assay was incubated for 21 days from seeding. A semi-quantitative scoring system was developed to assess structural changes to the pelleted cells and the intensity of Alcian Blue staining (**Table 2.9**).

**Table 2.9. Semi-quantitative scoring system used to evaluate chondrogenic differentiation of mesenchymal stem cells after staining with Alcian Blue.**

| Pellet shape | | Pellet structure | | Lacunae formation | | Staining intensity of pellet | |
|---|---|---|---|---|---|---|---|
| 0 | No distinct shape | 0 | No tertiary structure | 0 | No gaps | 0 | No colour change |
| 1 | Variable rounded shape | 1 | Soft | 1 | < 10% gaps throughout section | 1 | Light blue |
| 2 | Irregular spherical shape | 2 | Medium hardness | 2 | 10-50% gaps throughout section | 2 | Medium blue |
| 3 | Symmetrical sphere | 3 | Hard pellet | 3 | >50 % gaps throughout section | 3 | Intense blue |

### 2.7.3 OSTEOGENIC DIFFERENTIATION

Osteogenic differentiation was carried out in 24 well plates (Appendix 2) in a triplicate design. The cells were directly seeded onto the surface of the wells without the addition of cover slips or a matrix. Following trypsinisation and counting, the cells were resuspended in medium consisting of DMEM, F12, 10% FBS, P/S and fungizone at a density of 3 x $10^5$ per well with a total of 9 x $10^5$ cells used for osteogenic differentiation per horse. The first medium change was carried out when the cells reached confluence, the medium was removed and changed to osteogenic induction medium (Appendix 3).

The osteogenic assay was incubated for 10 days from the point of exposure to osteogenic medium. Medium changes were carried out every three days. Differentiated cells were assessed using a semi-quantitative scoring system to

measure the percentage of differentiated cells and the formation and staining intensity of cell clusters (**Table 2.10**).

**Table 2.10. Semi-quantitative scoring system used to evaluate osteogenic differentiation of mesenchymal stem cells after staining with Alizarin Red.**

| Size of cell clusters | | Region of staining | | Staining intensity of cells | |
|---|---|---|---|---|---|
| 0 | No clusters | 0 | Small areas 0-25 | 0 | No stained cells |
| 1 | Small | 1 | Small/medium areas 25-50 | 1 | Light red |
| 2 | Medium | 2 | Medium 50-75 | 2 | Red |
| 3 | Large | 3 | Large 75-100 | 3 | Bright red |

### 2.7.4 DIFFERENTIATION ANALYSIS

#### *2.7.4.1 FIXING CELLS*

The differentiation plates were removed from the $CO^2$ incubator and washed three times in 1ml of prewarmed PBS to remove medium. Care was taken with the adipogenic well to ensure the cover slip remained in the same position. Prewarmed fixative (Appendix 3) was pipetted into each well and incubated for 20sec at room temperature. Fixative was discarded into a designated formalin collection container. The wells were then washed three times with PBS. The osteogenic and chondrogenic plates were stained immediately after fixing, the adipogenic plate had 1ml of PBS added to each well, was wrapped in parafilm (Appendix 2) then stored at 4°C.

#### *2.7.4.2 ADIPOGENIC STAINING*

The fluorescent stains BODIPY and DAPI were used to determine adipogenic differentiation. This procedure took place in a darkened room due to the fluorescent nature of the stains. The PBS was aspirated out of the wells. The BODIPY/DAPI stain (Appendix 1) was added, 1ml per well, and incubated in a dark place at room temperature for 20min. The stain was removed using a 1ml pipette

(Appendix 1) and washed three times with PBS. The first and second wash was left for 3min, the third wash was removed straight away. The cover slips were then mounted on a Polysine slide (Appendix 2) using Dako mounting medium (Appendix 2) and were covered with a 22 x 60mm cover slip (Appendix 3). Each Polysine slide was placed in a separate slide holder, wrapped in tin foil and stored at 4°C overnight. The cover slips were viewed using a fluorescent microscope (Appendix 2) at 20x magnification using SPOT software.

#### *2.7.4.3 CHONDROGENIC STAINING*

Alcian Blue stain, 1% pH 1.0 (Appendix 1) was used to visualise glycosaminoglycan (GAG) deposits within the chondrogenic pellets. The pellets were washed three times with MQ water, 100µl of Alcian Blue (Appendix 1) was added to each well and incubated for 10min at room temperature. This method did not appear to result in efficient staining so an alternative incubation method, 24hrs at 37°C in 100µl of Alcian Blue was used.

Chondrocyte pellets were prepared for embedding in tissue freezing medium by washing three times in 30% sucrose solution (Appendix 1). The pellets were incubated overnight at 4°C on a rocking platform. The pellets were then incubated in 100µl of 30% sucrose mixed with two drops of tissue freezing medium (Appendix 2) over night at 4°C on a rocking platform. One cryomold per pellet was filled with tissue freezing medium and the pellet was placed in the centre of the mold and covered with more tissue freezing medium. The molds were snap frozen with freezing spray (Appendix 2) and sectioned on a cryostat (Appendix 2) in 6µm sections. The sections were placed on Apex slides (Appendix 2) and air dried overnight.

The sections of pellets were placed in a 3% Acetic acid wash (Appendix 1) for 3min. The slides were then placed in 1% Alcian Blue stain for 30min and rinsed in distilled water for 1min. After being dipped briefly in alcohol to dehydrate the sections the slides were allowed to air dry. Using mounting medium (Appendix 2) and

coverslips (Appendix 2), the slides were covered and viewed under phase contrast microscopy (Appendix 2). Staining of GAGs and the formation of lacunae were then assessed under phase contrast microscopy.

#### *2.7.4.4 OSTEOGENIC STAINING*

Alizarin Red stain pH 4.1 (Appendix 1) was used to stain the calcium deposits which develop during osteogenic differentiation. The fixed cells were washed three times in MQ water to remove PBS. One ml of Alizarin Red was added to each well and incubated for 3min at room temperature. The stain was removed and the wells were washed three times with MQ water. One ml of MQ water was left in each well to facilitate visualization under the Leica microscope (Appendix 2).

## 2.8 RT-PCR ANALYSIS

Cell RNA was extracted from the ADSCs and BMSCs before differentiation and during differentiation for all three tissue lineages. Due to the high number of cells detaching during the assay, RNA was extracted on day six for adipogenic cells and day seven for osteogenic cells. Extraction of RNA for chondrogenic cells occurred at day eight. An RNA sample was extracted from one post-mortem undifferentiated pBSC sample. Undifferentiated cells were collected from the samples of cells used for the differentiation assays to enable comparison of the same population of cells before and during differentiation. A selection of primers were made and purchased from Sigma Aldrich (**Table 2.11**). The samples used for mRNA extraction are displayed in **Table 2.12**.

**Table 2.11. RT-PCR Primers.**

| Gene | NCBI sequence # | Forward Primer 5' – 3'<br>Reverse Primer 5' – 3' | Product size (bp) | Annealing Temp (°C) |
|---|---|---|---|---|
| **GAPDH (HK)** | NM_001163856 | GGCAAGTTCCATGGCACAGT<br>CACAACATATTCAGCACCAGCAT | 128 | 66 |
| **B2M (HK)** | NM_001082502 | TCGTCCTGCTCGGGCTACT<br>ATTCTCTGCTGGGTGACGTGA | 81 | 67 |
| **NANOG (P, DP)** | XM_001498808.1 | TACCTCAGCCTCCAGCAGAT<br>CAGTTGTTTTTCTGCCACCT | 118 | 62 |
| **OCT4 (P, DP)** | XM_001490108.3 | CGAGAAGGACGTGGTACGAG<br>GTGGTGACAGACACAGAGGG | 248 | 64 |
| **CD34 (HM)** | XM_001491596 | CACTAAACCCTCTACATCATTTTCTCC TA<br>GGCAGATACCTTGAGTCAATTTCA | 100 | 65 |
| **PPARγ2 (AD)** | XM_001492411 | TGCAAGGGTTTCTTCCGGA<br>GCAAGGCATTTCTGAAACCG | 124 | 67 |
| **RUNX2 (OD)** | XM_001502519 | GAACCCAGAAGGCACAGACA<br>GGCTCAGGTAGGAGGGGTAA | 249 | 64 |
| **SPP1 (OD)** | XM_001496152 | CTCACATCACCTGTGGAAAGCA<br>CACGGCTGTCCCAATCAGA | 96 | 67 |
| **SOX9 (CD)** | XM_001498424.3 | ATGAAGATGACCGACGAGCA<br>GTTCAGCAGTCTCCAGAGCTT | 419 | 64 |

HK – Housekeeping, P – Proliferation, DP – Differentiation potential, HM – Hematopoietic marker, AD – Adipogenic differentiation, OD – Osteogenic differentiation, CD – Chondrogenic differentiation.

**Table 2.12. Adipose derived stem cell (ADSC), bone marrow derived stem cell (BMSC) and peripheral blood derived stem cell (pBSC) samples used for mRNA extraction. Samples derived from undifferentiated or differentiated states.**

| Cell type | Undifferentiated | Adipogenic | Chondrogenic | Osteogenic |
|---|---|---|---|---|
| ADSC | LH4, LH6, PM1, PM13 | LH4, LH6, PM1, PM13 | LH4, LH6, PM1, PM13 | LH4, LH6, PM1, PM13 |
| BMSC | LH2, LH3, LH4, LH5, LH6 | LH2, LH3, LH4, LH5, LH6 | LH2, LH3, LH4, LH5, LH6 | LH2, LH3, LH4, LH5, LH6 |
| pBSC | PM13 | - | - | - |

### 2.8.1 RNA EXTRACTION

Undifferentiated cells were pelleted in 2ml eppendorf tubes by centrifuging for 5min at 2000rpm. The supernatant was tipped off into a waste container and 300ul of TRIzol® reagent (Appendix 3) was added via a pipette. The contents were resuspended by pipetting six times and frozen in a -80°C freezer until required.

The RNA from differentiated cells was collected from each well by firstly removing the medium and adding 100µl of TRIzol® in each well. The TRIzol® was pipetted over the surface of the wells then removed and collected in a single 2ml eppendorf tube to form a total of approximately 300µl. Samples were pooled from the triplicate differentiation assay due to the high number of cells lifting from the well surfaces and the low number of cells available for RNA extraction. The chondrogenic pellets were collected intact as they proved too solid to break up via pipetting. A total of three pellets were collected in approximately 300µl of TRIzol®. The RNA from the differentiated samples was then placed in a -80°c freezer until required.

### 2.8.2 RNA PROCESSING, PCR AMPLIFICATION AND GEL ELECTROPHORESIS

Undifferentiated cells, adipogenic cells and osteogenic cells were thawed and resuspended with additional TRIzol® so that all tubes contained 500µl of TRIzol®/RNA mix. Chondrogenic pellets were processed with a BeadBeater

(Appendix 3) to break up the three pellets in each eppendorf tube. The pellets were broken up by adding glass and silica beads (Appendix 3) to the RNA and mixing twice at 4800rpm for 30sec on the BeadBeater. All samples were then processed using the protocol set out in **Table 2.13**.

**Table 2.13. Steps for RNA processing from TriZol® extraction.**

| Step | Process |
|---|---|
| 1 | Add 50µl of 1-Brom-3-chloropan (BCP) |
| 2 | Mix thoroughly by hand for 15s |
| 3 | Place on emulsifier table 10min at room temp. |
| 4 | Centrifuge for 10min, 13 rcf, 4°C |
| 5 | Remove supernatant and place in eppendorf |
| 6 | Add NaAc (2M, pH 4.0), acid phenol, BCP, emulsify for 15 sec |
| 7 | Centrifuge for 10min, 13 rcf, 4°C |
| 8 | Collect supernatant, add equal volume of isopropanol |
| 9 | Centrifuge for 10min, 13 rcf, 4°C |
| 10 | Add 1ml 70% ethanol |
| 11 | Centrifuge, 10sec at 6 rcf |
| 12 | Remove supernatant and add 20µl of TrisMn |

Processed RNA was then measured using a Nanodrop spectrophotometer (Appendix 2). One ml of RNA sample was loaded on to the Nanodrop pedestal and the sample absorbance at 260 and 280 nm was determined. The RNA samples were then treated with DNase (Appendix 2) by adding 1µl of DNase per 10µl of samples. The mixture was vortexed and centrifuged briefly before being incubated for 30min at 37°C. Two microlitres of DNase stop was added and the samples were incubated for 10min at 65°C then placed on ice.

RNA was then converted to cDNA with BioRad Kit iScript™ (Appendix 2) following the manufacturer's instructions (Appendix 4). Complementary DNA was evaluated using conventional PCR, performed with GAPDH and B2M primers as house-keeping genes. These two endogenous controls are widely used in equine MSC research (Raabe et al., 2011; Ranera et al., 2011). A master mix (2M $MgCl_2$) was mixed with Taq (Appendix 2) and forward and reverse primers. For 125µl of master mix 0.65µl of Taq and 2.5µl of primer mix was added. The mixture was vortexed and aliquoted to make 24µl per tube. One microlitre of cDNA was then added to

each tube and the samples were amplified in a thermal cycler (Appendix 2) using the stages set out in **Table 2.14**.

**Table 2.14. Stages of thermal cycling for PCR amplification.**

| Stage number | Temp | Duration | # of reps |
|---|---|---|---|
| 1 | 95c | 15 | Single |
| 2 | 95c | 20 | |
| | 65c | 20 | X 10 |
| | -1c | 1 cycle | |
| | 72C | 30 | |
| 3 | 95c | 20 | X 35 |
| | 55c | 20 | |
| | 72c | 30 | |
| Finish | 68c | 5min | single |

The amplified PCR products were run on a 1% gel (Appendix 2) containing 3µl of ethidium bromide (Appendix 2). Ten microlitres of PCR product was mixed with 2µl of loading dye buffer and dispensed into the gel wells covered with 1% TAE (Appendix 2). The gels were run for 30min at 90V using an electrophoresis unit (Appendix 2). Gels were viewed on a UV illuminator (Appendix 2) using Scion Image software.

### 2.8.3 RT-PCR AMPLIFICATION AND ANALYSIS

Real-Time PCR amplifications were carried out for nine genes including two housekeeping genes for each of the 36 samples using SYTO®82 Orange fluorescent Nucleic Acid Stain (Appendix 2). Each RT-PCR run comprised of 27 cDNA samples and nine negative control samples (**Table 2.15**).

**Table 2.15. RT-PCR run sample set up example.**

| Replicate | GAPDH | B2M | CD34 | NANOG | OCT4 | PPARγ2 | RUNX2 | SPP1 | SOX9 |
|---|---|---|---|---|---|---|---|---|---|
| 1 | cDNA | cDNA | cDNA | cDNA | cDNA | cDNA | cDNA | cDNA | cDNA |
| 2 | cDNA | cDNA | cDNA | cDNA | cDNA | cDNA | cDNA | cDNA | cDNA |
| 3 | cDNA | cDNA | cDNA | cDNA | cDNA | cDNA | cDNA | cDNA | cDNA |
| -ve control | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve |

Each RT-PCR run used a reaction mix containing of 750µl of 4M MgCl2, 0.3µl Syto 82 and 5µl of Taq. An aliquot of 450µl of reaction mix was divided into nine tubes and combined with 1µl of forward and reverse primers for each gene. The remaining 300µl of reaction mix was divided into three 100µl aliquots and combined with 9µl of cDNA. A 10µl aliquot of diluted cDNA was added to each of the nine primer tubes. Each cDNA sample was run in a cycle profile optimised for SYTO®82 using a RT-PCR machine (Appendix 2) (**Table 2.16**).

**Table 2.16. Steps for RT-PCR amplification using SYTO®82 optimised cycling.**

| Cycle stage (40 repeats) | Temperature | Time |
|---|---|---|
| Hold | 95°C | 15 min |
| Step 1 | 95°C | Hold 20sec |
| Step 2 | 55°C | Hold 20sec |
| Step 3 | 68°C | Hold 30sec |
| Step 4 | 80°C | Hold 10sec |

Relative quantification was determined by using the comparative CT method. Expression of target genes NANOG, OCT4, CD34, PPARγ2, RUNX2, SPP1 and SOX9 relative to the reference genes GAPDH and B2M were calculated using $2^{\Delta\Delta CT}$. The efficiencies of the target gene amplification and the efficiencies of the reference gene amplification were calculated to ensure they were equal. Comparative quantification analysis was performed using Corbett software (Appendix 2). Gene expression as normalised to the housekeeping gene B2M and calculated using the following equation by Pfaffl et al. (2002).

$$\text{Ratio} = (E_{target})^{\Delta CP}\text{target(control - sample)}/(E_{ref})^{\Delta CP}\text{ref(control - sample)}$$

### 2.8.4 PRIMER SEQUENCING

Primers used for RT PCR were sequenced at the Waikato University sequencing unit. Twenty-five microlitres of amplified primer product was combined with 1.0µl of Exo Nucl 1, 3.0µl SAP and 7.6µl of water (Appendix 2). The samples were

amplified in a thermal cycler (Appendix 2) for 30min at 37°C and for 5min at 95°C before being sent for sequencing.

## 2.9 STATISTICAL ANALYSIS

Statistical analysis was limited due to the low number of samples available across the different tissue types. A post-hoc Tukey's multiple comparison test was used following ANOVA analysis of P- cell counts and viability for AT, BM and pB MNCs. An ANOVA was also used for analysis of ADSC and BMSC proliferation in culture. Differentiation analysis was carried out using students two sample T-Test, assuming unequal variance. A two sample paired T-Test was also used to analyse paired observations for the antibiotic trial. Cryopreservation medium effect was analysed using a two sample paired T-Test. Data was calculated using the mean and standard error of the mean (SEM). Comparison of differentiation gene expression between ADSCs and BMSCs was carried out using a two sample paired T-Test.

# CHAPTER 3: RESULTS

## 3.1 PILOT TRIAL

The pilot trial was carried out using post-mortem sampling to refine cell isolation, culture and differentiation protocols. Isolated cells from all three raw tissue types, adipose tissue (AT), bone marrow, (BM) and peripheral blood (pB) were plated and cultured until confluence was reached. Cells directly isolated from raw tissue for primary culture were referred to as P- mononucleated cells (MNC) before plating, and as P0 mesenchymal stem cells (MSC) once they had been plated. The P- cells were a heterologous mixture of MNCs while the P0 cell population contained a mixture of fibroblast-like cells. Once in culture, adherent cells were then referred to as MSCs from their tissues of origin, namely ADSCs, BMSCs and pBSCs from adipose tissue, bone marrow and peripheral blood respectively. Although this appears to be a complicated nomenclature, it is important to distinguish the two different populations of cells as they contain different proportions of cell types.

### 3.1.1 POST-MORTEM STEM CELL ISOLATION AND CULTURE

Of the 12 post-mortem AT samples collected, nine samples reached confluence and three were discarded due to bacterial contamination. One sample was isolated from behind the point of the elbow as no adipose tissue deposits were available in the typical dorsal gluteal region. The sample isolated from this alternative region was not included in the data due to the unusual morphology of the tissue sample and the low number of MNCs isolated. Therefore, a total of eight samples were available for analysis. Two post-mortem BM samples were obtained and both reached confluence. Five post-mortem pB samples were collected, two of which reached confluence (**Table 3.1**). Post-mortem pBSCs grown in DMEM F12 (**see section 2.1.3**) reached confluence at day 20 while the pBSCs cultured in high glucose (HG) DMEM (**see section 2.1.3**) reached confluence at day 33.

**Table 3.1. Number of post-mortem samples from adipose tissue (AT), bone marrow (BM) and peripheral blood (pB) collected and the number of samples which reached confluence.**

| Tissue type | Number of samples collected | Number of samples which reached confluence |
|---|---|---|
| AT | 12 | 9* |
| BM | 2 | 2 |
| pB | 5 | 2 |

*Three samples were discarded due to bacterial contamination.

Minimal differences in MNC yield at isolation were seen for AT and pB however, BM yielded approximately half the number of cells per 10ml of raw tissue (**Table 3.2**). Mononucleated cell viability was found to be significantly higher for pB and BM cell samples ($P<0.05$) before plating (99% and 98% respectively) compared to AT MNC viability which was 91% at isolation.

**Table 3.2. Post-mortem viability and yield of mononucleated cells per 10 ml of raw adipose tissue (AT), (11 samples), bone marrow (BM), (2 samples) and peripheral blood (pB), (5 samples). Data shown as mean (SEM). One AT sample was not included as the sample was collected from a different anatomical location.**

| Tissue type | Viability | Cells/10ml of raw tissue |
|---|---|---|
| AT (11) | 91 (1.7) | $94.45 \times 10^5$ ($19.22 \times 10^5$) |
| BM (2) | 98 (1.5) | $52.61 \times 10^5$ ($3.88 \times 10^5$) |
| pB (5) | 99 (0) | $92.71 \times 10^5$ ($29.82 \times 10^5$) |

The eight post-mortem samples of MNCs isolated from raw AT reached confluence in an average of 4.88 days (+/-0.52) after plating. Mononucleated cells isolated from the two raw BM aspirate samples reached confluence in an average of 27 days (+/-1) after plating. Mononucleated cells from two of the five raw pB samples reached confluence in an average of 26.5 (+/-6.5) days after plating (**Table 3.3**). However, these two samples had been cultured in two different culture media. The cells that reached confluence were then referred to as mesenchymal stem cells (MSC) as they demonstrated the ability to adhere to the surface of the culture flask and proliferate as well as display fibroblast-like morphology. No statistical

comparison was made between the three tissue types due to the low number of BM and pB samples.

**Table 3.3. Post-mortem culture of primary cells from adipose tissue (AT) (8), bone marrow (BM) (2) and peripheral blood (pB) (2) which reached confluence. Days of confluence, viability % and number of cells at confluence x $10^5$ shown as mean (SEM). One AT sample was not included as the fat sample was collected from a different anatomical location.**

| Tissue type | Days to confluence | Viability at confluence % | Number of cells at confluence X $10^5$ |
|---|---|---|---|
| AT (8) | 4.88 (0.52) | 97.13 (0.77) | 36.56 (7.59) |
| BM (2) | 27 (1) | 92.5 (2.5) | 49.50 (7.5) |
| pB (2) | 26.5 (6.5) | 89 (9) | 111.25 (64.75) |

The initial cell population isolated from raw tissue contains a range of MNCs. After plating, the varied nature of the cell population becomes refined to cells capable of plastic adherence, which is a characteristic associated with MSCs. By comparing the number of cells displaying the ability to adhere, to the number of MNCs obtained from the original cell fraction, an indication of the proportion of MSC contained in a MNC fraction can be inferred (**Table 3.4**). Eight AT samples from post-mortem horses (PM2, PM4, PM5, PM6, PM7, PM8, PM9 and P13) were used for this calculation (**see materials and methods section 2.4.4**). From an average of 6.91 x $10^5$ (+/-1.52 x $10^5$) MNCs at P- and an average of 3.65 x $10^5$ (+/-.75 x $10^5$) MSCs at P0, MSCs made up 52% of MNCs in 1ml of raw AT. This implies that equine derived AT may contain a dense population of MSCs. As the protocols used for isolating cells from BM and pB were modified with progressive sampling, the cell counts were not compared.

**Table 3.4. Percentage of mesenchymal stem cells (MSCs) in 1ml of raw AT from eight post-mortem horses. One AT sample was not included as the sample was collected from a different anatomical location.**

| Cell type | Cells/ml of raw tissue x $10^5$ | Cells/ml x $10^5$ at confluence | Percentage of MSCs per ml of raw tissue |
|---|---|---|---|
| AT (8) | 6.91 (1.52) | 3.65 (.75) | 52% |

Once the cells from P- reached confluence (P0) they were then termed ADSCs, BMSCs or pBSCs as the populations contained adherent MSCs capable of proliferation. In addition to analysing MSC proportions in each sample, two post-mortem samples, one BMSC sample and one ADSC sample were used for trialling four differentiation protocols, adipogenic, chondrogenic, osteogenic and myogenic lineages (**see materials and methods sections 2.7.1-0**). Differentiation was confirmed for adipogenic and osteogenic lineages using the appropriate stains, while the chondrogenic lineage was not confirmed with staining due to time and resource constraints. Myogenic differentiation was evaluated in the BMSC sample only but positive differentiation was not observed using immunocytochemistry with anti-myosin heavy chain antibodies (**see materials and methods section 2.1.4**).

## 3.2 MAIN PROJECT

Following the completion of the pilot trial, AT, BM and pB samples were collected from six live horses. The same nomenclature previously used in **section 3.1** for post-mortem tissue culture was used for live tissue culture.

The samples collected from each horse and the identification of each horse is outlined in **Table 2.2.** Due to bacterial contamination of some of the samples, repeated sampling was carried out from some of the horses. Nine AT samples were collected from LH1, LH2, LH4, LH5 and LH6, of which, seven samples were lost due to bacterial contamination and the two remaining samples reached confluence. One horse (LH3) did not have available AT in the dorsal gluteal region and no sample was collected from this animal. Of the nine BM samples, three samples were discarded due to media contamination and six samples were cultured to confluence. One BM sample (LH1) reached initial confluence (P0) but when replated, the cells did not attach and proliferate. Of the 18 pB samples collected from live horses, no samples reached confluence (**Table 3.5**).

**Table 3.5. Number of live adipose tissue (AT), bone marrow (BM) and peripheral blood (pB) samples collected and the number of samples which reached confluence.**

| Tissue type | Number of samples collected | Number of samples reaching confluence |
|---|---|---|
| AT | 9 | 2 |
| BM | 9 | 6 |
| pB | 18 | 0 |

### 3.2.1 COMPARISON OF ISOLATION COUNTS AND VIABILITY

The MNC fractions isolated from the live raw tissue samples were counted to determine cell number per ml of raw tissue. The three sources of stem cells did not demonstrate significant differences in MNC yield per ml of raw tissue (**Table 3.6**). Five horses were used for isolation comparison as no AT could be collected from one horse (LH3). Although multiple samples were collected from some of the individuals, only the samples collected on the same day or in close proximity of each other were used, in an attempt to reduce variability due to date of collection.

**Table 3.6. Live horse sample viability % and yield of mononucleated cells x $10^5$ per 10 ml of raw adipose tissue (AT), bone marrow (BM) and peripheral blood (pB) from five horses* collected on the same day or within the same week. Data shown as mean (SEM). Data from horses with tissue samples sourced from all three tissue types.**

| Tissue type | Viability % | Cells/10 ml of raw tissue x $10^5$ |
|---|---|---|
| AT (5) | 93 (1.81) | 134.00 (19.90) |
| BM (5) | 99 (0.21) | 169.17 (31.20) |
| pB (5) | 100 (0.24) | 111.13 (39.36) |

*Data from one horse (LH3) was excluded as adipose tissue could not be collected and compared to bone marrow or peripheral blood.

The count carried out on freshly isolated cells demonstrated some minor tissue-specific differences. Bone marrow had the highest number of MNCs per 10ml of raw tissue (mean - 169.17 x $10^5$ (SEM - 31.20)) with AT and pB being the second lowest and lowest respectively (mean AT - 134.00 x $10^5$ (SEM 19.90), mean pB - 111.13 x $10^5$ (SEM 39.36)). However, no statistically significant differences were found between the three tissue sources ($P < 0.05$). Viability was highest for MNCs

isolated from live pB and BM, at 100% and 99% respectively. Mononucleated cells from live AT had an average of 93% viability at isolation and were found to be significantly lower than the viability of MNCs isolated from BM and pB ($P < 0.05$). No statistically significant difference was found between the viability of BM and pB ($P > 0.05$). A post-hoc Tukey's multiple comparison test was used for statistical analysis of MNC counts and viability. In summary, the data showed no significant difference in the number of MNCs isolated from the same volume of raw AT, BM and pB tissues, while cell viability was significantly higher for MNCs from BM and pB ($P<0.05$). Mononucleated cells from AT demonstrated significantly lower viability, perhaps due to the more extensive processing required to isolate the cells (**see section 2.3.1**).

The cell samples were cultured until the first confluence was reached (P0). Two AT samples (LH4 and LH6) reached confluence and six BM samples reached confluence (LH1, LH2, LH3, LH4, LH5 and LH6). The two matched pairs of samples were from LH4 and LH6 both of which had one AT sample and one BM sample reaching confluence. These paired samples were used for initial confluence analysis.

The duration of time required for initial confluence to be reached for LH4 and LH6 ADSC samples was 5.5 days (SEM-0.5) while BMSCs from LH4 and LH6 reached confluence after 15 days (SEM-0) (Table 3.7). The mean day of P0 confluence for all six of the BMSC samples was 15.83 (SEM-1.27) (Data not shown). From the AT and BM samples isolated from LH4 and LH6, it appears that ADSCs reach confluence faster than BMSCs when harvested directly from raw tissue, confirming the results obtained from post-mortem derived stem cells.

**Table 3.7. Day of confluence for adipose derived stem cells (ADSCs) and bone marrow derived stem cells (BMSCs) isolated from LH4 and LH6. Data shown as mean (SEM).**

| Cell type | Day of confluence |
|---|---|
| ADSCs (2) | 5.5 (.5) |
| BMSCs (2) | 15 (0) |

The proportion of MSCs contained in the freshly isolated MNC fraction was calculated using the methods set out in **section 2.4.4**. One ml of raw AT contained a higher percentage of MSCs than 1ml of raw BM (53% vs 11%). (**Table 3.8**). The higher proportion of MSCs contained in the MNC fraction from AT may account for the reduced number of days required for MNCs from AT to reach confluence.

**Table 3.8. Percentage of mesenchymal stem cells (MSCs) per ml of raw adipose tissue (AT) and bone marrow (BM) from two horses (LH4 and LH6). Data shown as mean (SEM).**

| Tissue type | Cells/ml of raw tissue x $10^5$ | Cells/ml x $10^5$ at confluence | Percentage of MSCs per ml of raw tissue |
|---|---|---|---|
| AT (2) | 9.8 (0.3) | 5.15 (0.85) | 53% |
| BM (2) | 16.00 (9.5) | 1.77 (0.13) | 11% |

In summary, AT appears to be a better source of MSCs compared to BM aspirate as the higher proportion of MSCs per ml of raw AT resulted in reduced time to achieve confluence.

### 3.2.2 COMPARISON OF CELL PROLIFERATION RATES AND CELL YIELD IN CULTURE

Secondary culture counts and proliferation rate assessment was carried out using live ADSCs and BMSCs only. No pBSCs were included as no live samples reached confluence. The two ADSC samples from LH4 and LH6 and the five BMSC samples from LH2, LH3, LH4, LH5 and LH6 were used for assessment. The ADSC and BMSC samples from LH4 and LH6 were used for statistical analysis. The ADSCs and BMSCs that reached initial confluence (P0) were plated at 5000 cells per $cm^2$ and passaged twice to provide confluence counts. Passage one count is referred to as P1 and the passage two count is referred to as P2. The comparison of growth rates from both cell sources, AT and BM, provides an indication of any difference in proliferation rate due to cell tissue type origin. A subjective assessment of confluence was used to determine whether the cell population had covered 80% of the culture flask surface. Both of the ADSC samples were cultured to P2 however, only five of the

BMSC samples could be cultured to P2 as the BMSC sample from LH1 failed to attach and proliferate after passaging (**Table 3.9**).

**Table 3.9. The number of adipose derived stem cell (ADSC) and bone marrow derived stem cell (BMSC) samples reaching confluence at passage one (P1) and passage two (P2)**

| Cell type (number of samples) | P1 | P2 |
|---|---|---|
| ADSC (2) | 2 | 2 |
| BMSC (6) | 5 | 5 |

The passage data generated from the ADSC and BMSC samples from LH4 and LH6 were analysed to determine if there were any differences in cell yield and time to confluence. An ANOVA test found that ADSCs yielded a significantly higher number of cells than BMSCs (P = 0.014) from the same seeding concentration for both passages. Adipose derived stem cells yielded 4.5 x $10^5$ cells/ml while BMSCs yielded 2.04 x $10^5$ cells/ml in the same number of days to confluence. Interestingly, there was no significant difference in the day cells reached confluence for ADSCs and BMSCs once the samples were established in culture.

Cell morphology and behaviour was evaluated during culture (**Figure 3.1**). All three tissue types contained cells which attached to the flask surface and displayed fibroblast-like morphology. Adipose derived stem cells grew in a monolayer and developed distinctive swirl patterns throughout the culture flask when reaching near confluence. Bone marrow derived stem cells also demonstrated a monolayer in culture with distinctive swirl patterns although there were also areas of densely clustered cells. The two pBSC samples available for analysis from post-mortem origin grew in clusters which eventually spread out to a monolayer although the areas of densely packed cells remained. These clusters were visible even without the use of a microscope. Adipose derived stem cells and BMSCs from live horses and pBSCs from post-mortem horses were able to be passaged to P2 and cryopreserved.

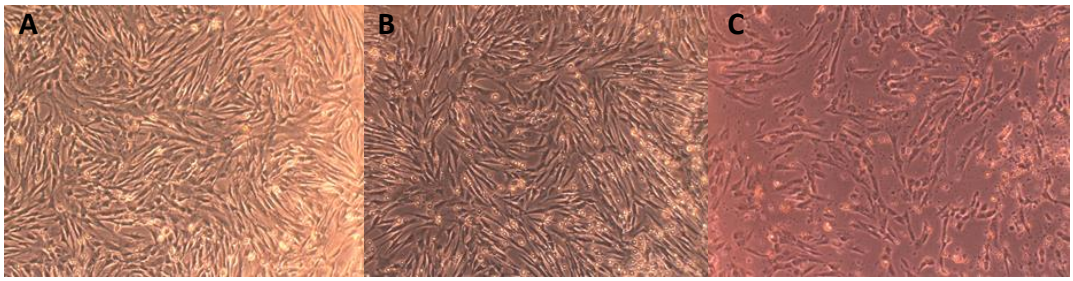



**Figure 3.1. Comparison of cell morphology in culture at P2, A – adipose derived stem cells, B – bone marrow derived stem cells, C – peripheral blood derived stem cells (from post-mortem sample). Phase contrast microscopy. (10 x magnification).**

***Cell culture summary***

All three tissue sources yielded fibroblast-like cells capable of adherence and proliferation. However, AT and BM appeared to be considerably more reliable sources of MSCs than pB. Based on the percentage of MSCs per ml of raw tissue, AT appeared to be a better source of equine MSCs compared to BM and pB. Adipose tissue reached initial confluence faster than BM or pB derived cells. Interestingly, ADSCs and BMSCs reach confluence at similar rates once the populations have been established in culture, however, the density of the cell population at confluence was significantly higher for ADSCs ($P=0.014$). Overall, the cell culture phase of the project suggests that ADSCs outperform BMSCs and pBSCs during initial cell culture. However, once in culture, rate of ADSC and BMSC confluence is not significantly different although the number of cells at confluence was significantly higher for ADSC samples.

## 3.3 CRYOPRESERVATION

The cryopreservation phase of the project investigated the capacity of all three tissue types to withstand freezing in liquid nitrogen. The two ADSC samples from LH4 and LH6 and the five bone marrow derived stem cell samples from LH2, LH3, LH4, LH5 and LH6 were cryopreserved. As no live pBSCs were obtained, the two post-mortem pBSC samples were cryopreserved. Cultured cells from all three sources were placed in a cryopreservation medium to preserve cell integrity and were slowly cooled to -80°C. The samples were then stored in liquid nitrogen for

a minimum of four weeks before being fast-thawed and plated to observe any changes in cell behaviour.

All of the samples that were thawed and plated reached confluence, demonstrating the retention of growth characteristics after controlled exposure to the extremely cold temperatures required for long-term storage.

In addition to investigating the effect of cryopreservation on ADSCs, BMSCs and pBSCs, two different combinations of cryopreservation media were compared using post-mortem ADSC samples (PM4, PM5, PM7, PM8 and PM9) (**Methodology: 2.6.1**). A two-sample paired T-Test analysis found no significant differences between the number and viability of ADSCs before and after cryopreservation (P value = 0.218) (**Figure 3.2**). As there was no statistically significant difference between the two different combinations of media, the use of a low-serum medium, containing 20% FBS, may be a more cost-effective approach for cryopreserving equine MSCs.

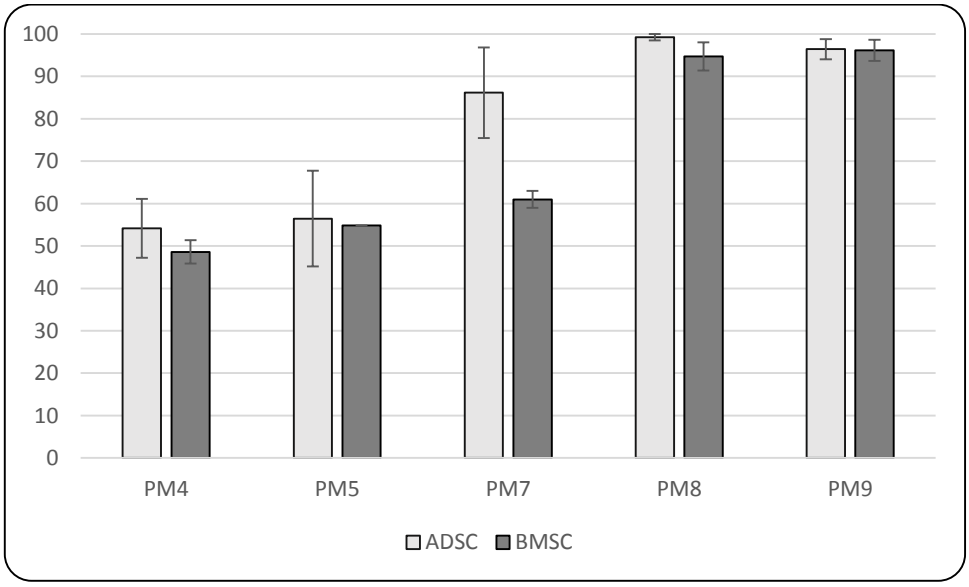



**Figure 3.2. Survival percentages of post-mortem adipose derived mesenchymal stem cell samples following cryopreservation in DMSO with two different fetal bovine serum concentrations.**

Overall, it appears that equine MSCs can be stored using a low-serum cryopreservation medium in liquid nitrogen, without detrimental effect on cell attachment and proliferation ability.

## 3.4 ANTIBIOTIC TRIAL

Due to the loss of samples early in the project, an antibiotic trial was carried out to determine if a combination of wide spectrum antibiotics would provide greater protection from bacterial contaminations. Two combinations of antibiotics were trialled using post-mortem ADSC samples. The cells from the same horse were plated at the same densities in P/S medium and G/S to compare cell attachment and proliferation. One sample placed in G/V culture medium did not reach confluence while all of the samples placed in P/S culture medium reached confluence. However, no significant difference in proliferation rate between the two antibiotic combinations could be seen ($P>0.05$). It appears that as all of the five samples cultured in P/S reached confluence, this combination of antibiotics has less detrimental effect on cell attachment and growth than G/V. However, as four of the five samples cultured in G/V reached confluence at a similar rate to P/S cultured cells this combination of antibiotics is a viable option for equine ADSC culture, particularly in conditions where contaminations are more likely to occur.

## 3.5 POST-MORTEM AND LIVE HORSE SAMPLE COMPARISON

During the pilot trial, post-mortem samples were collected for training purposes. After observing the live surgical procedures, I began to question the use of live samples for culture research purposes. If post-mortem samples could be used for isolating MSCs from various tissue sources, there would be no need to put research horses through surgery to collect samples.

Live and post-mortem samples at isolation were compared to see if there were differences in cell yield and viability. Due to the low number of samples, statistical

analysis could not be performed however, the means and standard error of the means were compared. There appeared to be a slightly higher yield of MNCs from live tissue for all three tissue sources compared to post-mortem tissue. Cellular viability did not appear to be affected by live or post-mortem collection. The number of MNCs isolated from live horse BM was higher than the number of MNCs isolated from post-mortem BM, however, this may be due to the different isolation techniques used and the smaller volume of post-mortem BM collected. Mononucleated cell yield from pB was also higher for live samples (**Table 3.10**).

**Table 3.10. Live and post-mortem comparison of adipose tissue (AT), bone marrow (BM) and peripheral blood (pB), viability and cells/ml of tissue. Data shown as mean (SEM).**

| Tissue type | Viability % | Cells/ml of tissue x $10^5$ |
|---|---|---|
| Live AT (5) | 92 (1.81) | 13.4 (1.99) |
| PM AT (11) | 91 (1.7) | 9.57 (1.90) |
| Live BM (5) | 99 (0.21) | 16.9 (3.12) |
| PM BM (2) | 98 (1.5) | 5.26 (0.38) |
| Live pB (5) | 99 (0.16) | 11.11 (3.93) |
| PM pB (5) | 99 (0) | 9.21 (1.75) |

Although cell counts from live horse samples appear to be higher than for post-mortem samples, this comparison shows that MSCs can be isolated from both live and post-mortem tissues without any effect on cell viability.

## 3.6 HISTOLOGICAL ANALYSIS OF TRILINEAGE DIFFERENTIATION

Multipotency of ADSCs and BMSCs was assessed through trilineage differentiation into adipogenic, chondrogenic and osteogenic lineages. The samples used for differentiation are displayed in **Table 2.7**. Adipose derived stem cell samples included two live horse samples (LH4, LH6) and two post-mortem samples (PM1, PM13). Bone marrow derived stem cell samples were from five live horse samples (LH2, LH3, LH4, LH5 and LH6). Adipogenic, chondrogenic and osteogenic differentiation assays were carried out over 14, 21 and 10 days respectively. At the

end of each assay, histological stains were used to assess the morphological degree of differentiation in each sample.

### 3.6.1 ADIPOGENIC DIFFERENTIATION

Adipogenic differentiation was carried out over 14 days using ADSC and BMSC samples. Adipose derived stem cell samples were from two live samples (LH4 and LH6) and two post-mortem samples (PM1 and PM13). Bone marrow derived stem cell samples were from five live samples (LH2, LH3, LH4, LH5, and LH6). The cells were incubated in adipogenic induction medium for three days and then cultured in adipogenic maintenance medium for the remaining duration of the assay. Changes in cell morphology and behaviour were assessed during medium changes which occurred every three days. On the final day of differentiation, the samples were fixed and stained with BODIPY and DAPI to observe morphological changes within the cells (**Methodology: 2.7.4.2**).

#### *3.6.1.1 EFFICIENCY OF ADIPOSE DERIVED STEM CELLS*

Changes in cell morphology were evident from day three after exposure to adipogenic induction medium (**Figure 3.3**). Some of the cells began to lift from the well surface while the attached cells began to convert from a fibroblast-like to a spherical shape. By day six, the attached cells displayed spherical morphology and cell detachment continued to occur. By day nine, small droplets had formed within the cells. At this stage there were cells with rounded morphology, mostly due to resettlement of floating, detached cells, and some which had retained fibroblast-like morphology. The adipogenic differentiation staining was performed at day 14.

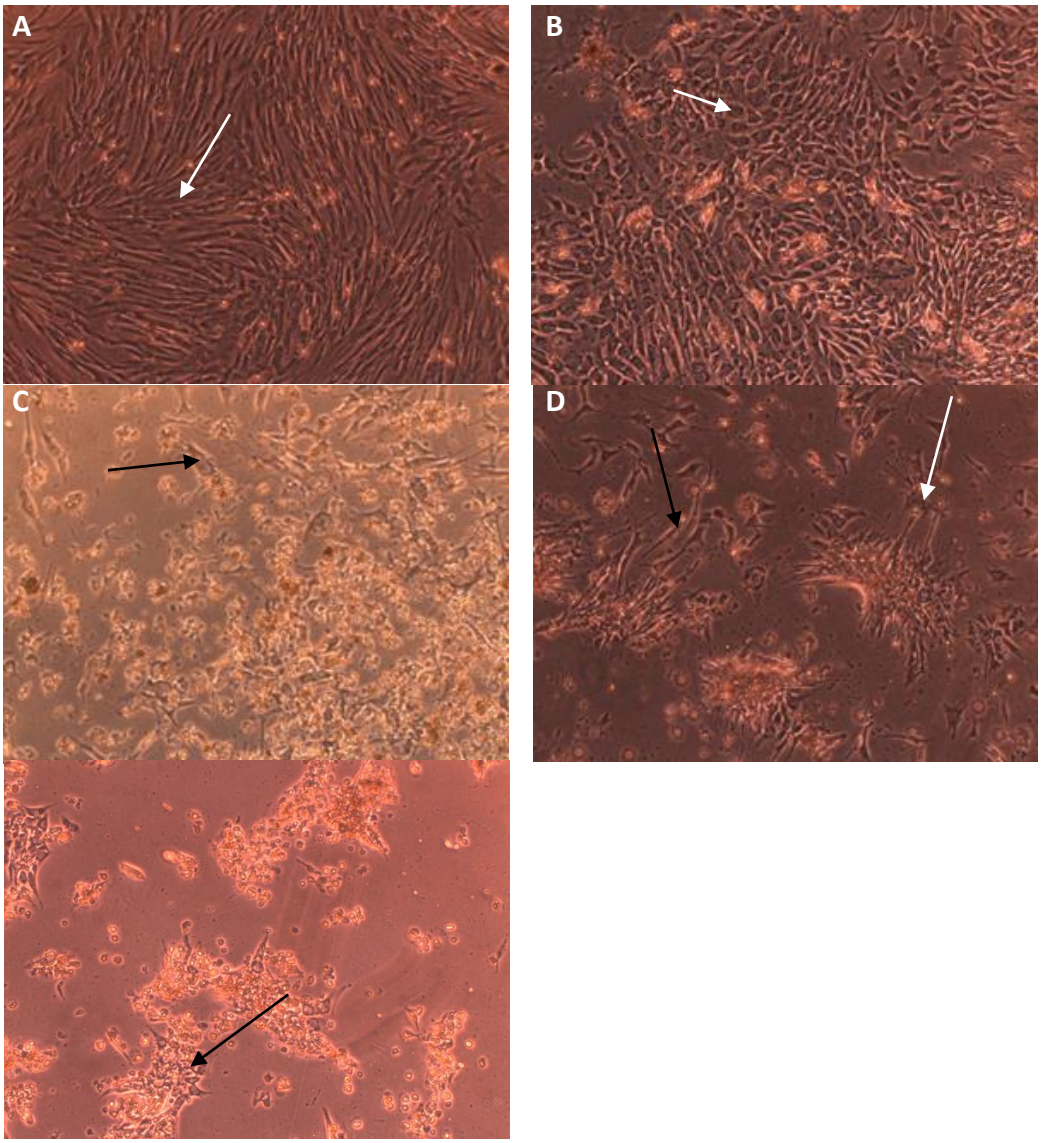



**Figure 3.3. Stages of adipogenic differentiation in adipose derived stem cell samples. A - Day 0, pre-exposure, arrow indicates near-confluent fibroblast-like cells, B - Day 3 after exposure, arrow indicates development of spherical morphology, C - Day 6 after exposure, arrow indicates increase in cell width, D - Day 9 after exposure, black arrow indicates increase in cell width, white arrow indicates attached fibroblast-like cell, E - Day 14 after exposure, black arrow indicates areas of attached cells. Phase contrast microscopy. (10 x magnification).**

Adipose derived stem cells were evaluated using BODIPY fluorescent stain to determine the degree of lipid accumulation within the cells as well as the size of the lipid containing vesicles. The lipid vesicles were stained green with BODIPY giving an indication of lipid vesicle size and density. The nuclei of the cells were stained blue with DAPI, providing an estimate of how many cells were attached

and how the lipid droplets were orientated around the nuclei of each cell (**Figure 3.4**).

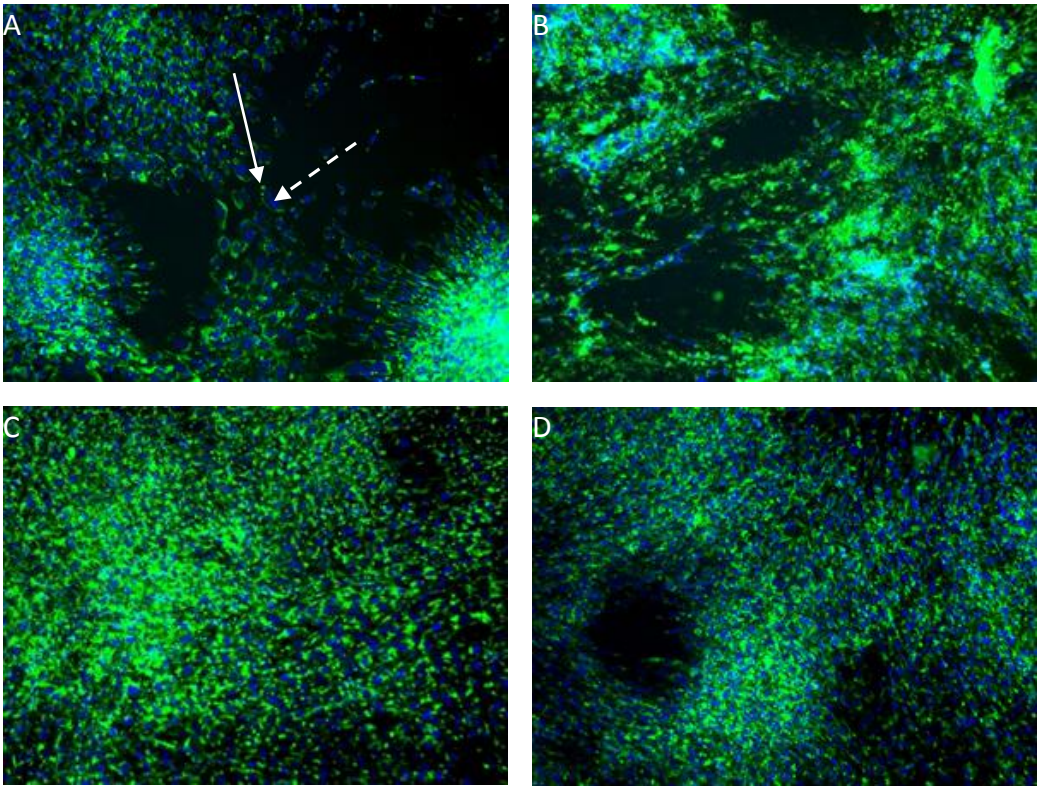



**Figure 3.4. Adipose derived stem cells from four different horses following adipogenic differentiation, stained with BODIPY (green) and DAPI (blue), A – LH4, B – LH6, C – PM1, D – PM13. Fluorescent microscopy. (20 x magnification). Dashed white arrow indicates a cell nucleus stained with DAPI, white arrow indicates lipid vesicles stained green with BODIPY surrounding the nucleus.**

Adipogenic differentiation was obtained in the four ADSC samples, all of which displayed strong staining for differentiation. There was a range of lipid droplet sizes across the samples, LH6 displayed strong BODIPY staining due to the high number of large lipid droplets present (**Table 3.11**). The sample from PM1 appeared to have the most intense level of staining however, the size of the lipid droplets were smaller than for LH6. Live horse four (LH4) and PM13 displayed strong staining with BODIPY, although the lipid vesicle size was smaller than seen for LH6 and PM1. (**For scoring system see methodology section: Table 2.8**).

**Table 3.11. Differentiation and lipid grades for adipogenic differentiated adipose derived stem cells from live horse four (LH4), live horse six (LH6), post-mortem horse one (PM1) and post-mortem horse 13 (PM13).**

| Horse | Differentiation grade | Lipid size | Lipid number and orientation |
|---|---|---|---|
| LH4 | 3 | 2 | 3 |
| LH6 | 3 | 3 | 3 |
| PM1 | 3 | 2 | 3 |
| PM13 | 3 | 2 | 3 |

#### *3.6.1.2 EFFICIENCY OF BONE MARROW DERIVED STEM CELLS*

The activated BMSCs developed a more spherical morphology by day three and only a small number of floating detached cells were visible (**Figure 3.5**). Changes in morphology continued to be seen at day six with the centre of the cells increasing in width. Detached cells were also evident at this stage. Cells at day nine retained the same morphology seen at day six and there appeared to be a range of cell morphology throughout the plate. Some cells retained the distinctive fibroblast-like shape whereas other attached cells had a spherical shape. Attached cells at day 14 in culture also showed a range of morphologies and the cells were stained at this point with BODIPY and DAPI (**see staining methodology: 2.7.4.2**).

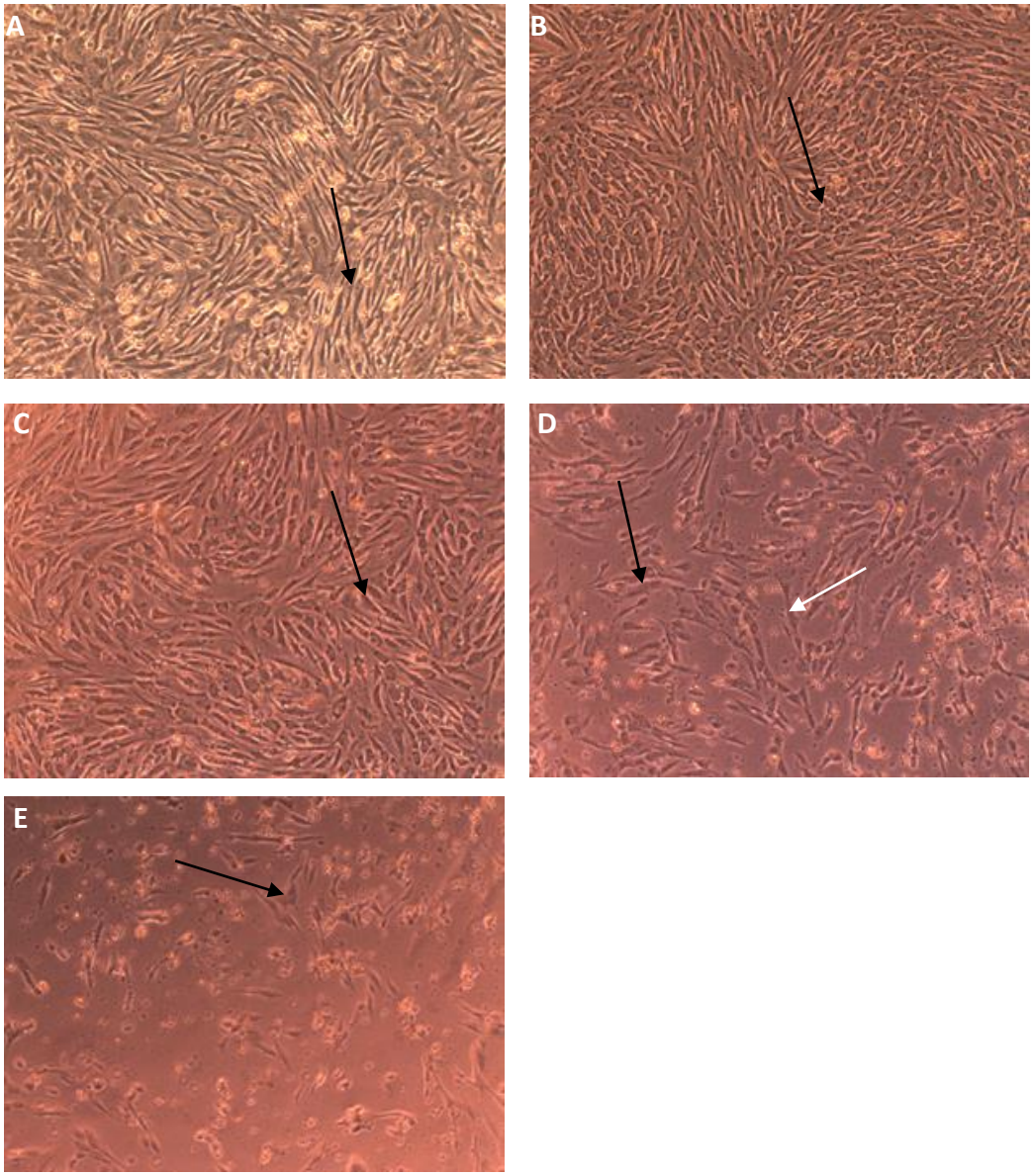



**Figure 3.5. Stages of adipogenic differentiation in bone marrow derived stem cells. A - Day 0, pre-exposure, arrow indicates near-confluent fibroblast-like cells, B - Day 3 after exposure, arrow indicates development of spherical morphology, C - Day 6 after activation, black arrow indicates cell increasing in width, D - Day 9 after exposure, black arrow indicates cell increasing in width, white arrow indicates attached fibroblast-like cell, E - Day 14 after exposure, black arrow indicates cell with enlarged centre. Phase contrast microscopy. (10 x magnification).**

Adipogenic differentiation was obtained in all of the five BMSC samples used in the assay (**Figure 3.6**) although the differentiation grade and lipid vesicle size varied between samples (**Table 3.12**). Three of the samples differentiated well with lipid deposits surrounding the nuclei of the cells. Two of the samples differentiated weakly and demonstrated a low number of attached cells which were surrounded by few, small lipid deposits, particularly in LH6.

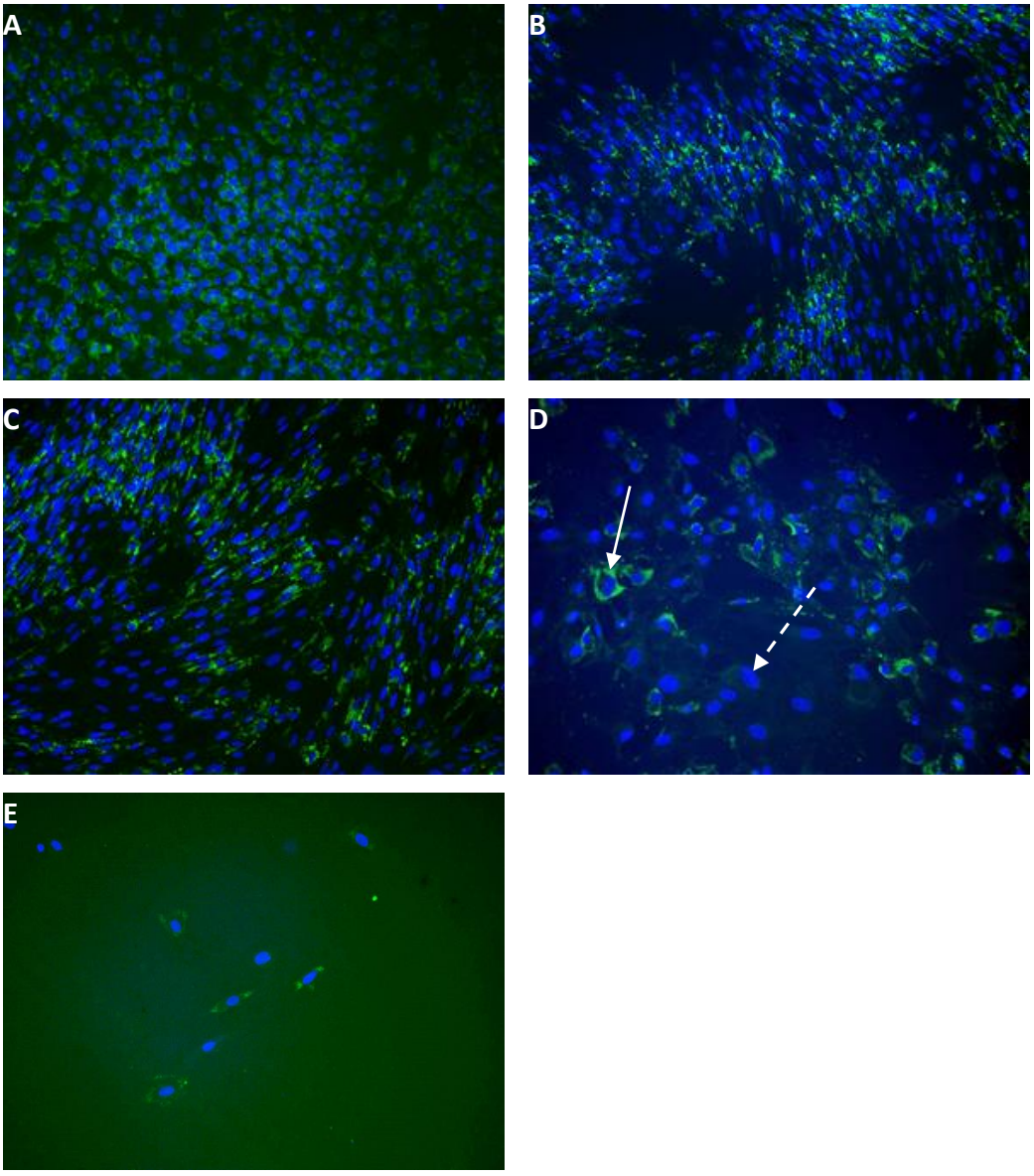



**Figure 3.6. Bone marrow derived stem cells from five different horses following adipogenic differentiation stained with BODIPY (green) and DAPI (blue), A – LH2, B – LH3, C – LH4, D – LH5, E – LH6. Fluorescent microscopy. (20 x magnification). Dashed white arrow indicate a cell nucleus stained with DAPI, white arrow indicates lipid vesicles stained green with BODIPY surrounding the nucleus.**

Bone marrow derived stem cells displayed weak staining with BODIPY due to the low density of lipid vesicles within the cells (**Table 3.12**). The sample from LH2 displayed the highest grade of differentiated cells with many small lipid vesicles. Live horse three (LH3) and LH4 had a lower grade of differentiation with many small lipid vesicles. Live horse five (LH5) showed an even lower grade of differentiation with very few small lipid vesicles surrounding the cells. Live horse six (LH6) displayed poor differentiation with a low number of attached cells surrounded by few, small lipid vesicles.

**Table 3.12. Differentiation and lipid grades for adipogenic differentiated bone marrow derived stem cells from five horses.**

| Horse | Differentiation grade | Lipid size | Lipid number and orientation |
|---|---|---|---|
| LH2 | 2 | 1 | 3 |
| LH3 | 2 | 1 | 3 |
| LH4 | 2 | 1 | 3 |
| LH5 | 1 | 1 | 2 |
| LH6 | 0 | 1 | 1 |

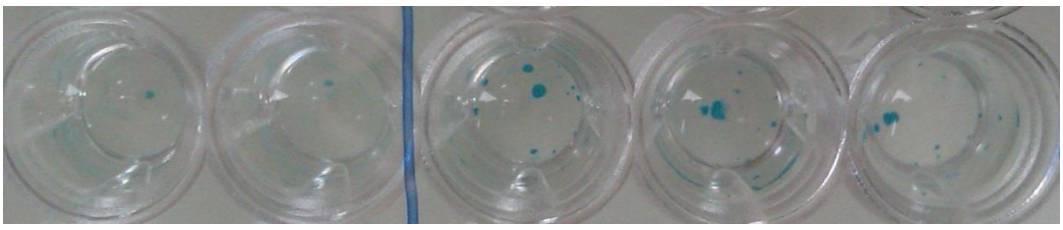
In summary, adipogenic differentiation efficiency appeared to be greater for ADSCs. All of the four ADSC samples displayed similar levels of differentiation efficiency while BMSC samples displayed a range of efficiencies and overall, exhibited lower staining scores for lipid vesicle development. Although ADSCs appear to be superior to BMSCs for adipogenic differentiation, both sources of cells were able to be pushed into the adipogenic lineage.

### 3.6.2 CHONDROGENIC DIFFERENTIATION

Chondrogenic differentiation was carried out over 21 days. Adipose derived stem cell pellets were from two live samples (LH4 and LH6) and two post-mortem samples (PM1 and PM13). Bone marrow derived stem cell pellets were from five live samples (LH2, LH3, LH4, LH5, and LH6). Control pellets were exposed to incomplete chondrogenic medium for the duration of the assay. Control and treated pellets from LH4 and LH6 were sectioned to compare chondrogenic differentiation efficiency in ADSCs and BMSCs. The sectioned pellets were stained with Alcian Blue after being fixed to a slide and dehydrated. The Alcian Blue stain is an indicator of chondrogenic differentiation as it highlights deposits of glycosaminoglycans (GAG) strongly associated with the formation of chondrocyte cells. Pellet shape and structure was also assessed as an indicator of chondrogenic differentiation.

#### *3.6.2.1 EFFICIENCY OF ADIPOSE DERIVED STEM CELLS*

All four ADSC samples formed spherical aggregation of cells which developed into solid, hard pellets after 21 days in chondrogenic culture medium. Two of the ADSC chondrogenic pellets, LH4 and PM1, showed staining around the peripheral edge of the pellet and the remaining two pellets, LH6 and PM13 did not appear to be visually different following staining. When compared to the differentiated pellets, the control pellets appeared smaller, less dense and did not have tidy, spheroid formations. Alcian Blue staining was also more intense with the treated pellets compared to the control pellets (**Figure 3.7**).



| Control pellets | Treated pellets |
|---|---|

**Figure 3.7. Control and treated chondrocyte pellets from adipose derived stem cells stained with Alcian Blue after 21 days in culture.**

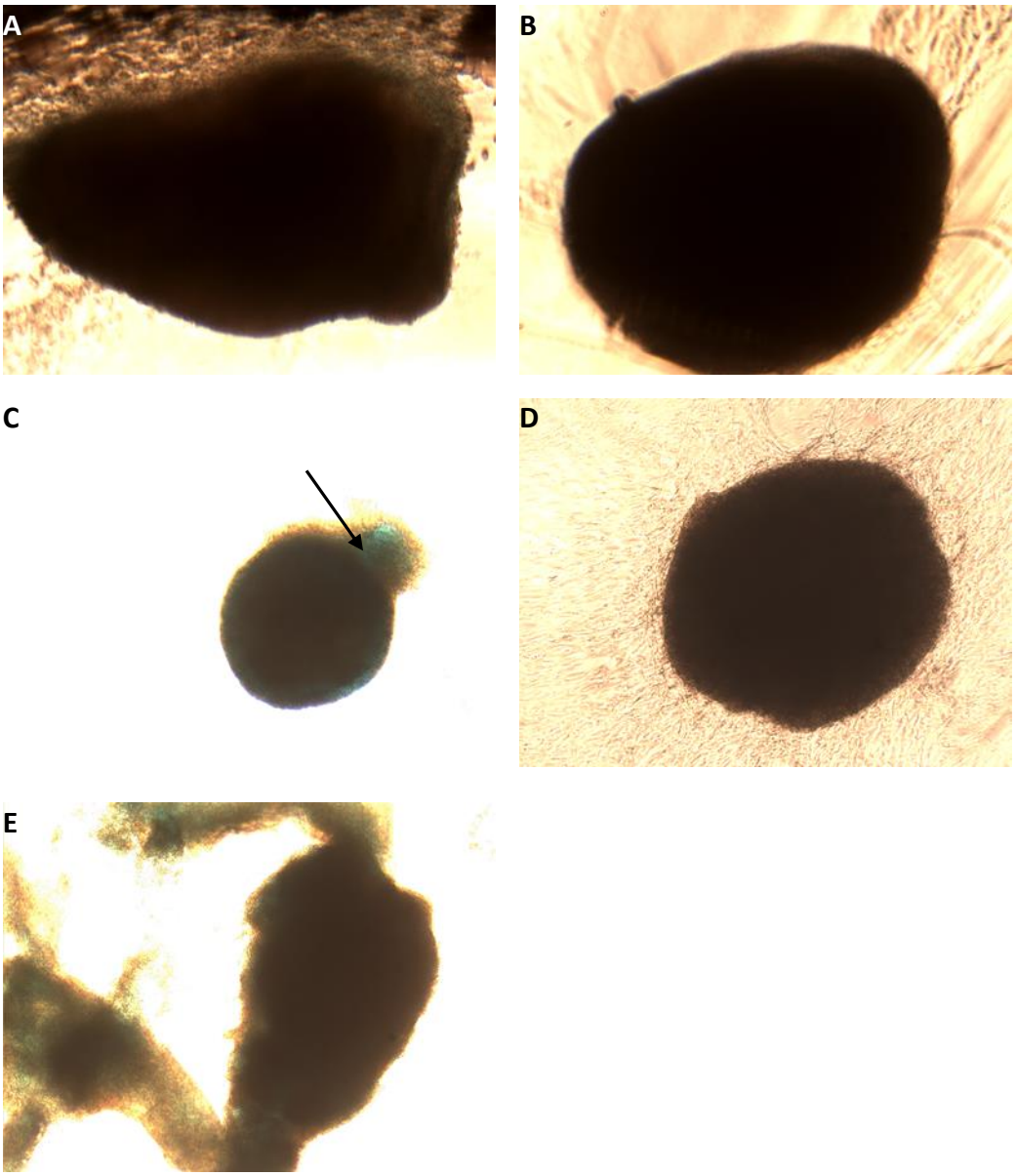



**Figure 3.8. Adipose derived stem cells from four different horses following chondrogenic differentiation and stained with Alcian Blue. A – LH4, B – LH6, C – PM1, D – PM13, E – control pellet. Phase contrast microscopy. (10 x magnification). Black arrow indicates Alcian Blue staining.**

Sectioned ADSC pellets from LH4 and LH6 stained with Alcian Blue displayed light staining indicating low level GAG deposits in the extracellular matrix (**Figure 3.9**). Chondrocyte-specific lacunae formation could be seen throughout the pellet for LH6, however LH4 did not appear to form lacunae.

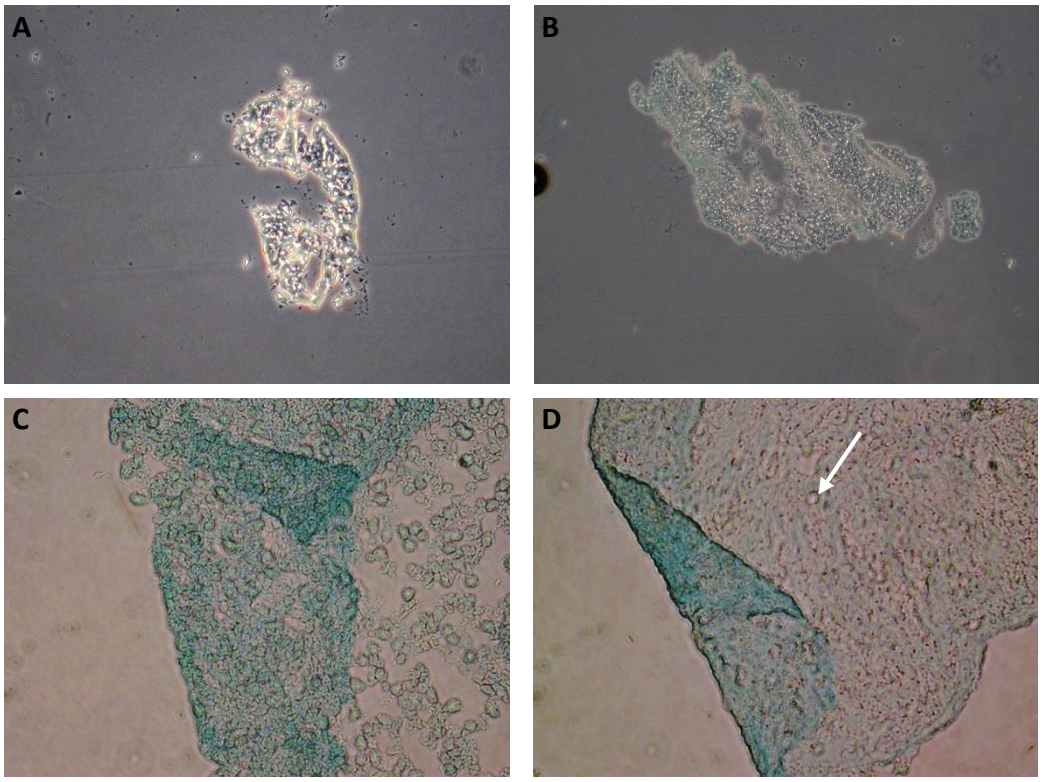



**Figure 3.9. Sectioned adipose derived stem cell chondrocyte pellets from LH4 and LH6 stained with Alcian Blue for GAG deposits. A- LH4 control pellet, (10 x magnification), B – LH6 control pellet (10 x magnification), C- LH4 treated pellet (20 x magnification), D – LH6 treated pellet (20 x magnification). Phase contrast microscopy. White arrow indicates lacunae.**

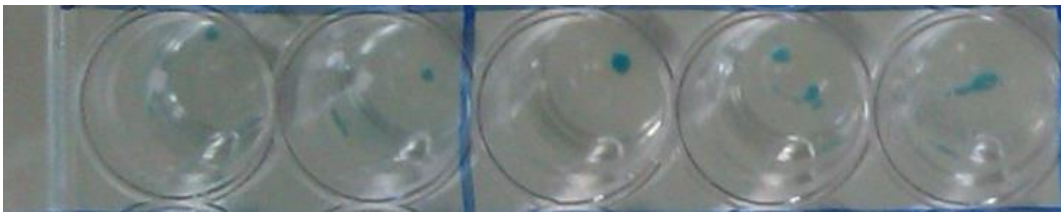
Pellet shape, structure, GAG staining intensity and lacunae formation was assessed in LH4 and LH6 sectioned ADSC pellets (**Table 3.13**) (**see scoring methodology Table 2.9**). Pellet shape, structure and staining intensity was similar for both samples however lacunae formation could be seen in only one sample. The sectioned pellet from LH4 appeared to be poorly structured, however, chondrogenic differentiation was confirmed by the development of GAGs which stained medium blue.

**Table 3.13. Pellet shape, structure, glycosaminoglycan staining intensity and lacunae formation grade for chondrogenic differentiation in sectioned adipose derived stem cell pellets from LH4 and LH6. Pellet shape and structure grade is from intact pellets before sectioning. Lacunae and staining intensity is from sectioned pellets.**

| Horse | Pellet shape | Structure | Lacunae formation | Staining intensity |
|---|---|---|---|---|
| LH4 | 2 | 3 | 0 | 2 |
| LH6 | 2 | 3 | 1 | 2 |

### *3.6.2.2 EFFICIENCY OF BONE MARROW DERIVED STEM CELLS*

All of the five BMSC samples formed defined spherical pellets and four (LH2, 3, 4, 5) were visibly blue after being stained although the extent of the stain could not be visualised in the intact pellets (**Figure 3.11**). Without the use of a microscope, all of the differentiated pellets appeared blue however, the treated pellets appeared to be more intense blue that the control pellets (**Figure 3.10**). All of the pellets were densely formed and although the control pellets also had distinctly spherical shapes, the cell density were visibly lower than that of the TGF-β1 treated pellets.



| Control pellets | Treated pellets |
|---|---|

**Figure 3.10. Control and treated chondrocyte pellets from bone marrow derived stem cells stained with Alcian Blue after 21 days in culture.**

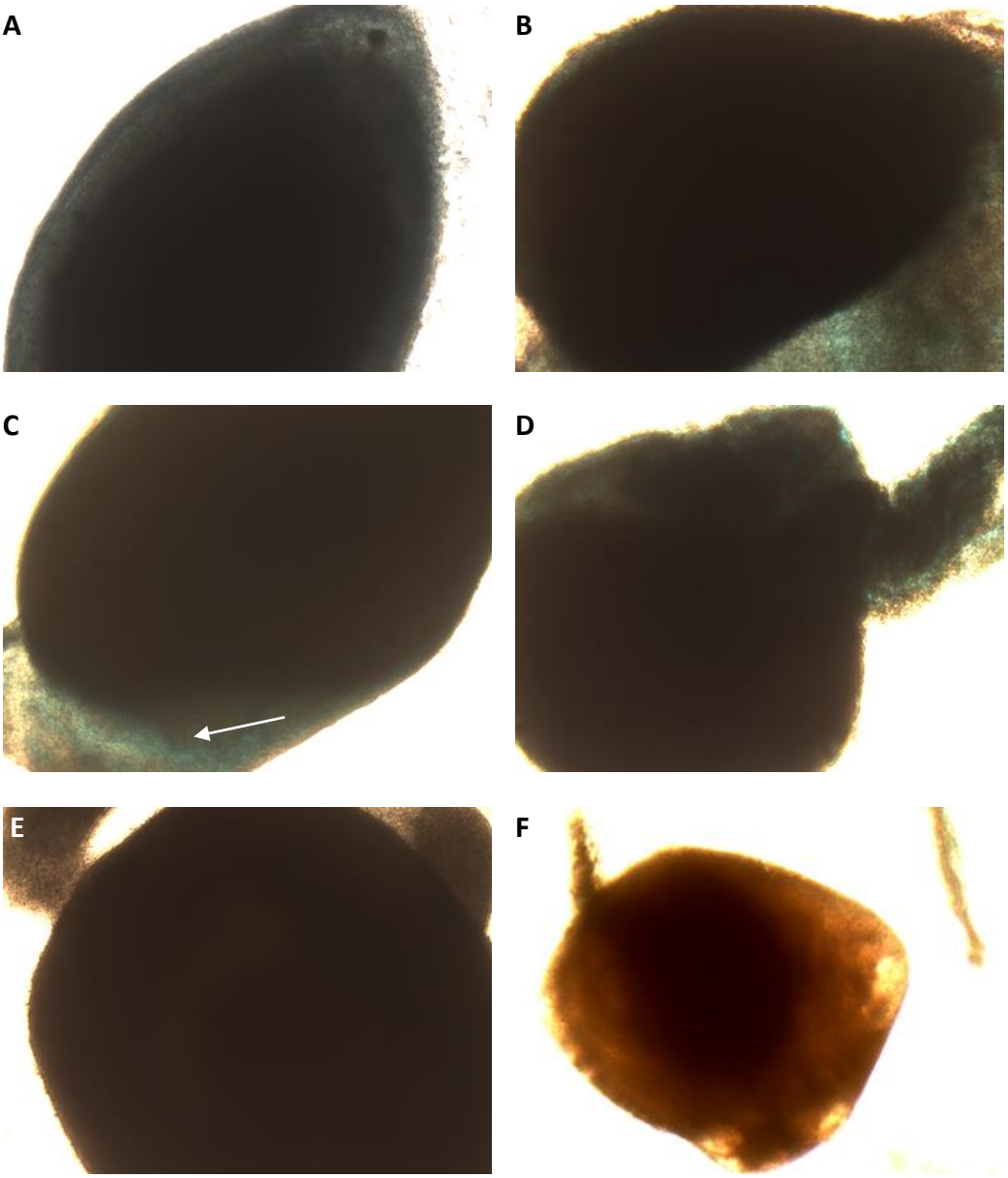



**Figure 3.11. Bone marrow stem cells from five different horses following chondrogenic differentiation and stained with Alcian Blue, A – LH2, B – LH3, C – LH4, D – LH5, E – LH6, F – control pellet. Phase contrast microscopy. 10 x microscopy). White arrow indicates Alcian Blue stain.**

Sectioned BMSC pellets from LH3, LH4 and LH6 were compared for lacunae formation and staining intensity (**see scoring methodology: Table 2.9**). The control pellets from all three horses displayed light blue staining, indicating GAG deposits. Treated pellets showed more intense staining than the control pellets indicating higher levels of GAG deposits. Lacunae formation was evident in the treated pellets from all three horses (**Figure 3.12**).

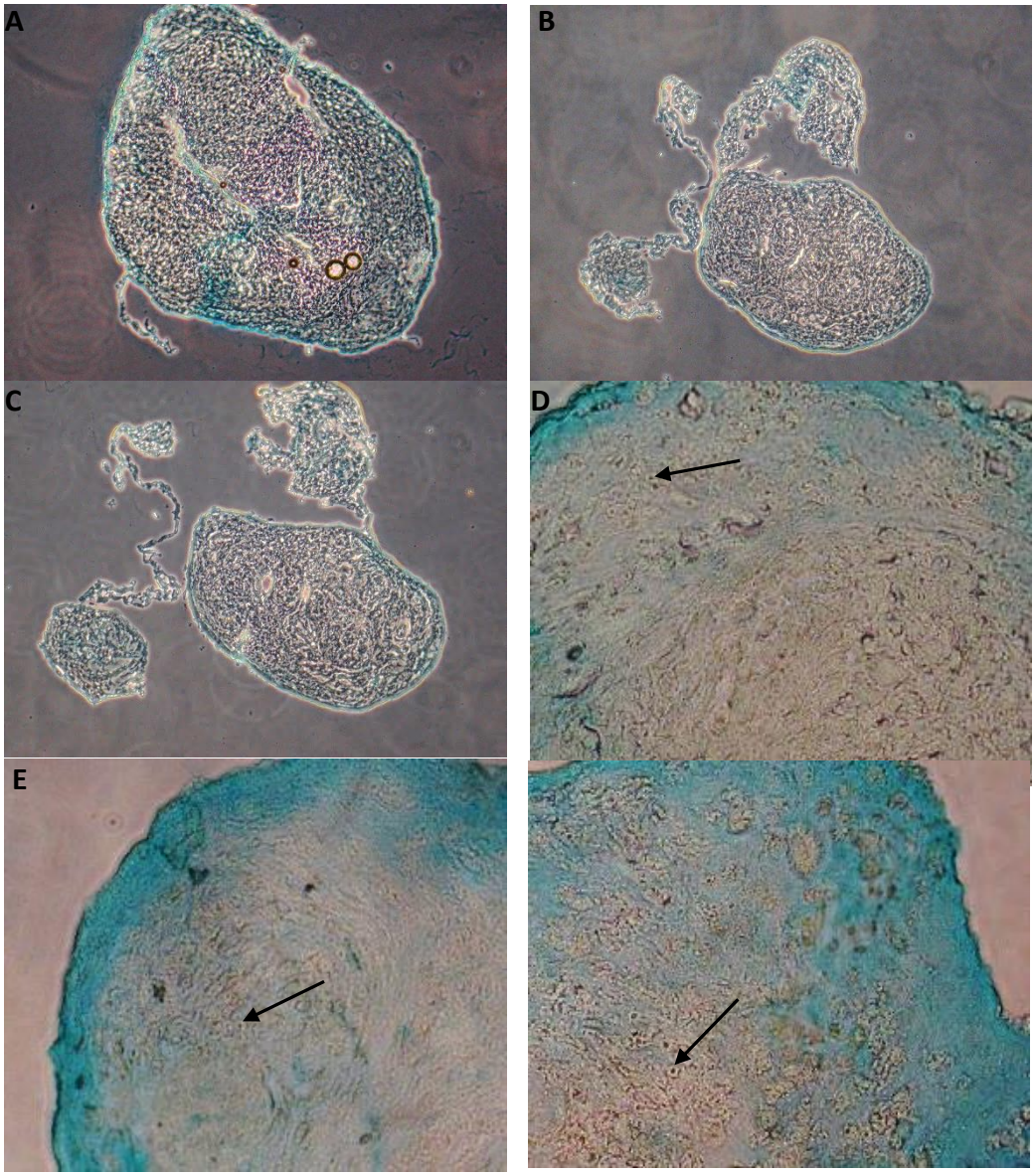



**Figure 3.12. Sectioned bone marrow stem cell chondrocyte pellets stained with Alcian Blue for GAG deposits. A- control pellet – LH3, B – control pellet – LH4, C – control pellet – LH6, D – treated pellet – LH3, E – treated pellet – LH4, F – treated pellet, LH6. Phase contrast microscopy (20 x magnification). Black arrows indicate lacunae.**

Pellet shape, structure, GAG staining intensity and lacunae formation was assessed in LH3, LH4 and LH6 sectioned BMSC pellets (**Table 3.14**) (**see scoring methodology Table 2.9**). The treated pellets from all three horses displayed irregular spherical shapes with hard pellet structures. When the pellet from LH3 was sectioned, lacunae formation was evident throughout the pellet and Alcian Blue staining could be seen around the edges and in some places within the pellet. Sectioning of the LH4 pellet displayed lacunae formation across 10-50% of the pellet and intense blue staining was seen around the edge and in places within the pellet. The treated pellet from LH6 displayed the highest score for chondrogenic differentiation. Lacunae formation was evident throughout the pellet and deposits of GAGs, as determined by the uptake of Alcian Blue stain, were seen around and throughout the pellet section.

**Table 3.14. Pellet shape and staining intensity score for bone marrow derived stem cell chondrogenic differentiation**

| Horse | Pellet shape | Pellet structure | Lacunae formation | Staining intensity |
|---|---|---|---|---|
| **LH3** | 2 | 3 | 3 | 2 |
| **LH4** | 2 | 3 | 2 | 3 |
| **LH6** | 2 | 3 | 3 | 3 |

In summary, BMSCs appear to be more efficient in differentiating into chondrocytes than their ADSC counterparts. The control pellets for BMSCs displayed light staining with Alcian Blue, suggesting that chondrogenic differentiation may occur in BMSCs, even without the necessary differentiation supplements, namely, TGF-β1.

### 3.6.3 OSTEOGENIC DIFFERENTIATION

Osteogenic differentiation was evaluated at day 10 by staining with Alizarin Red for the presence of calcium deposits. Adipose derived stem cell samples were from two live sample (LH4 and LH6) and two post-mortem samples (PM1 and PM13) and bone marrow derived stem cell samples were from five live samples (LH2, LH3,

LH4, LH5, and LH6). Morphological changes in the samples were documented every three days prior to the medium change.

### *3.6.3.1 EFFICIENCY OF ADIPOSE DERIVED STEM CELLS*

Morphological changes in ADSCs could be seen from day three following the start of differentiation (**Figure 3.13)**. The cells began to contract and individual cells began to group together, developing dense clusters of cells in various regions throughout the plate. By day six the clusters became quite distinct and individual cells were no longer visible within the clusters. At day nine the cells had developed into defined clusters of densely layered cells.

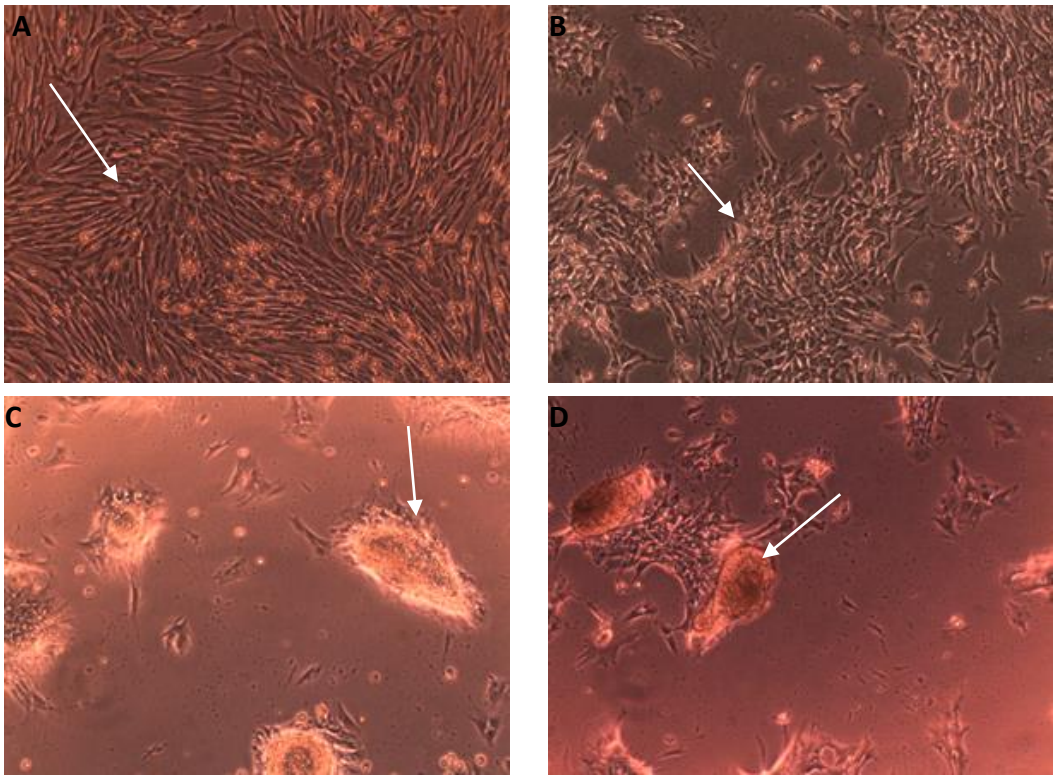



**Figure 3.13. Stages of osteogenic differentiation in adipose derived stem cell samples. A - Day 0, pre-exposure, white arrow indicates fibroblast-like morphology of confluent cells. B - Day 3 after exposure, white arrow indicates cell contraction. C - Day 6 after exposure, white arrow indicates formation of cell cluster. D - Day 9 after exposure, white arrow indicates formation of dense cell cluster. Phase contrast microscopy. (10 x magnification).**

All of the four ADSC samples demonstrated the formation of cell clusters which stained positively for calcium deposits, indicative of osteogenic differentiation (**Figure 3.14**). The clusters were large and distinct with dark red centres indicating dense deposits of calcium. Surrounding cells which had not undergone differentiation could be clearly seen with visually distinct fibroblast-like morphology and no uptake of Alizarin Red (**Figure 3.14**).

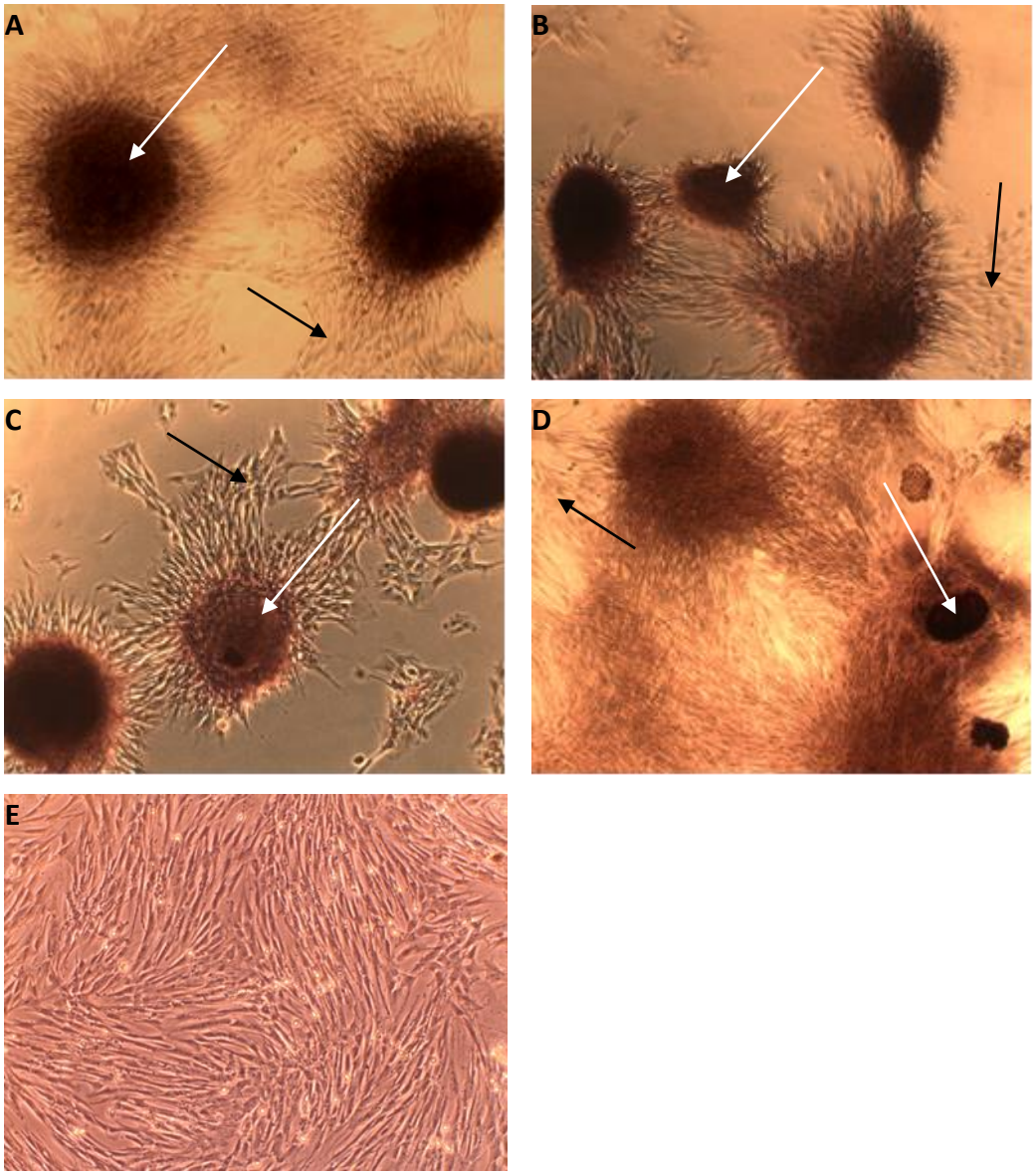



**Figure 3.14. Adipose derived stem cells from four horses following osteogenic differentiation stained with Alizarin Red, A – LH4, B – LH6, C – PM1, D – PM13, E – control, Phase contrast microscopy. (10 x magnification). White arrows indicate Alizarin Red staining, black arrows indicate non-stained fibroblast-like cells.**

All four ADSC samples appeared to have similar efficiencies for osteogenic differentiation as similar cell cluster formation, region of staining and staining intensity were observed (**Table 3.15**) (**see scoring methodology Table 2.10**).

**Table 3.15. Stain and cluster grade for osteogenic differentiated adipose derived stem cells from two live horses (LH4 and LH6) and two post-mortem horses (PM1 and PM13).**

| Horse | Cluster size | Region of staining | Staining intensity |
|---|---|---|---|
| LH4 | 3 | 2 | 2 |
| LH6 | 3 | 2 | 2 |
| PM1 | 3 | 2 | 2 |
| PM13 | 3 | 2 | 1 |

### *3.6.3.2 EFFICIENCY OF BONE MARROW DERIVED STEM CELLS*

Morphological changes in five BMSC samples were seen as early as day three after exposure to osteogenic differentiation medium. The BMSCs had the tendency to pull away from a monolayer distribution into clusters of cells (**Figure 3.15**). By day six a high proportion of cells had detached from the well surface and clusters of fibroblast-like cells could be seen throughout the well. Dense clusters of cells had formed by day nine and areas of single fibroblast-like cells could also be seen throughout the well.

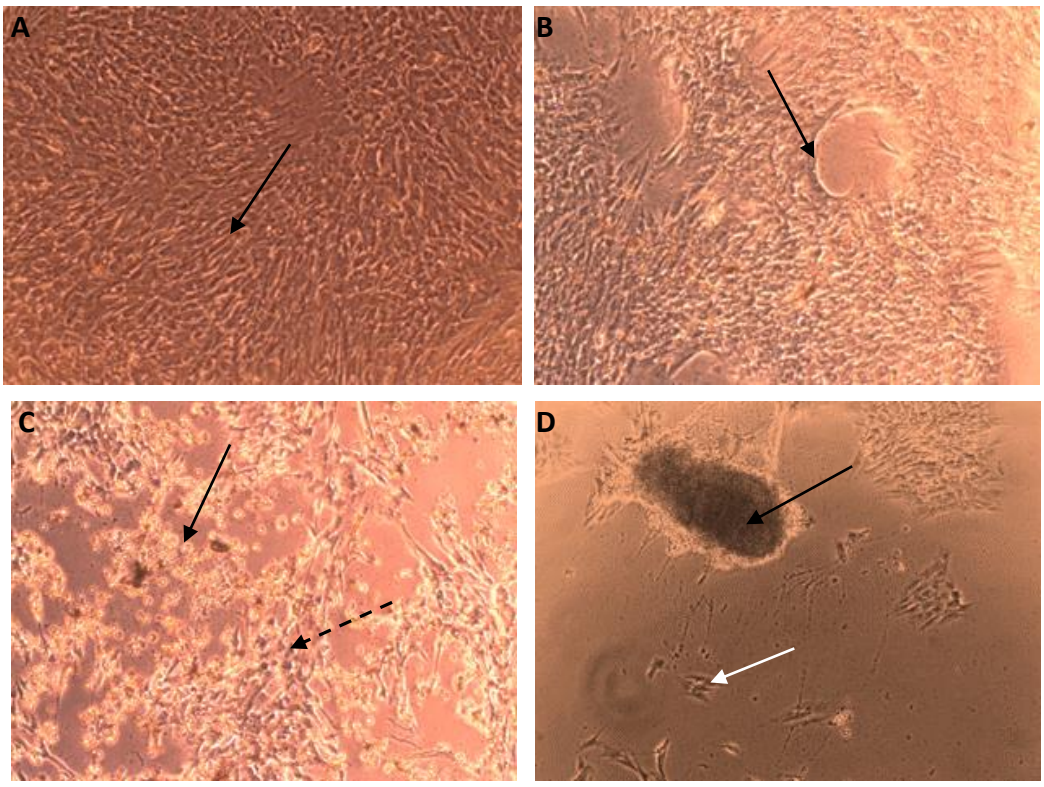



**Figure 3.15. Stages of osteogenic differentiation (BMSC) A - Day 0, pre-activation, black arrow indicates near-confluent fibroblast-like cells, B - Day 3 after activation, black arrow indicates cells pulling away from a monolayer, C - Day 6 after activation, black arrow indicates detached cells resettled on well surface, dashed black arrow indicates formation of cell clusters, D - Day 9 after activation, black arrow indicates dense cluster of cells, white arrow indicates single fibroblast-like cells. Phase contrast microscopy. (10 x magnification).**

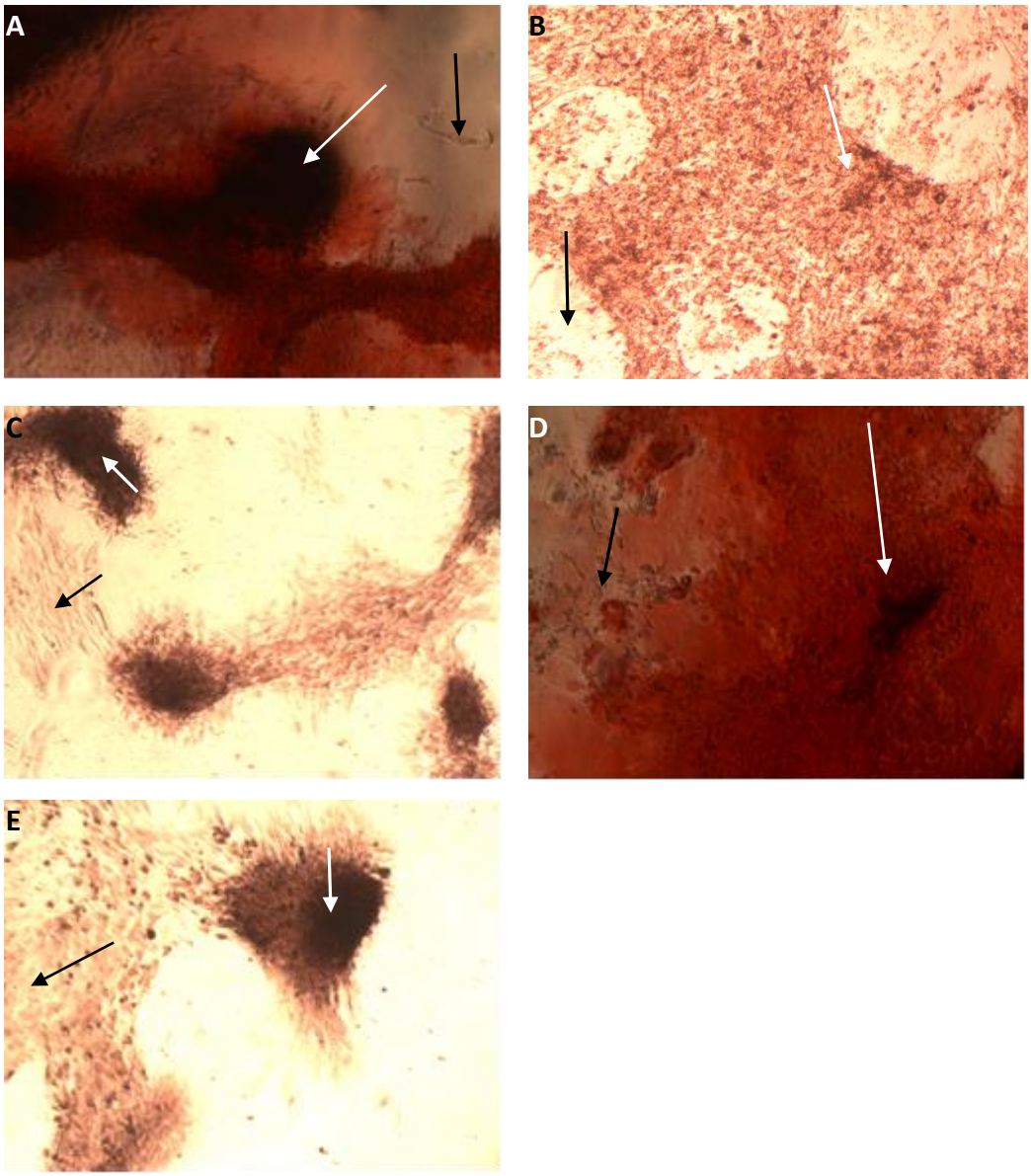



**Figure 3.16. Bone marrow derived stem cells from five horses following osteogenic differentiation stained with Alizarin Red. A – LH2, B – LH3, C – LH4, D – LH5, E – LH6. Phase contrast microscopy. (10 x magnification). White arrows indicate Alizarin Red staining, black arrows indicate non-stained fibroblast-like cells.**

Bone marrow derived stem cells demonstrated variable degrees of osteogenic differentiation (**Figure 3.16**). Although all five samples stained positively for calcium deposits, the intensity of the staining was variable and the formation of distinct osteogenic clusters was also variable (**Table 3.16**). Live horse two (LH2), LH4 and LH5 received higher grades compared to the two remaining samples (LH6 and LH3) due to the formation of medium sized but distinct clusters which stained positively for calcium deposits (**Figure 3.16**). Live horse six (LH6) demonstrated

small cell clusters which stained positively, while LH3 had no distinct clusters. Interestingly, the LH3 sample stained a pale pink, indicating deposits of calcium in the monolayer of cells.

**Table 3.16. Stain and cluster grade for osteogenic differentiated bone marrow derived stem cells. Live horse two (LH2), live horse three (LH3), live horse four (LH4), live horse five (LH5) and live horse six (LH6).**

| Horse | Cluster size | Region of staining | Staining intensity |
|---|---|---|---|
| LH2 | 2 | 2 | 3 |
| LH3 | 1 | 2 | 1 |
| LH4 | 2 | 2 | 1 |
| LH5 | 1 | 2 | 3 |
| LH6 | 2 | 2 | 1 |

Osteogenic efficiency appeared to be similar for ADSC and BMSC samples. The uptake of Alizarin Red stain displayed similar distribution within the wells, and although the formation of cell clusters was more distinct in the four ADSC samples, two of the BMSC samples (LH2 and LH5) displayed intense staining with Alizarin Red for calcium deposits.

### 3.6.4 DIFFERENTIATION EFFICIENCY SUMMARY

The differentiation assays were carried out to compare the capacity of ADSCs and BMSCs to differentiation into three cell lineages. The results described in sections 3.6.1, 3.6.2 and 3.6.3, obtained through the use of semi-quantitative scoring systems, were analysed using a two sampled unpaired T-Test with unequal variance. It appears that ADSCs differentiate more efficiently than BMSCs into adipogenic lineage (P=0.026) and BMSCs differentiate more efficiently into chondrocyte lineage than ADSCs (P=0.042). No significant difference was found between ADSC and BMSC osteogenic differentiation (**Table 3.17**).

**Table 3.17. Comparison of adipose derived stem cells (ADSC) and bone marrow derived stem cells (BMSC) total differentiation grade. Data: mean (SEM). Adipogenic and osteogenic data is from all ADSC samples and three BMSC samples (LH2, LH3 and LH5). Chondrogenic data from LH3, LH4 and LH6.**

| Cell type | Adipogenic grade average | Chondrogenic grade average | Osteogenic grade average |
|---|---|---|---|
| ADSC | 8.25 (0.25) | 7.50 (.5) | 6.75 (0.25) |
| BMSC | 5.33 (0.52) | 10.33 (.3) | 5.66 (2.33) |
| P value | = 0.026 | =0.042 | = 0.358 |

In summary, there were significant tissue dependent differences in differentiation efficiencies for adipogenic and chondrogenic lineages between the two sources of MSCs. No significant difference in osteogenic differentiation efficiency was found. The ability of BMSCs to differentiate efficiently into osteocytes and chondrocytes, suggests that this source of MSCs may have potentially higher therapeutic benefit for treating lameness in horses than ADSCs.

## 3.7 GENE EXPRESSION

Gene expression was carried out using mRNA extracted from samples that were undifferentiated and from samples undergoing differentiation into adipogenic, chondrogenic and osteogenic lineages as displayed in **Table 2.12**. Undifferentiated samples served as controls for measuring upregulation of differentiation markers as well as to investigate the levels of expression of stemness markers. The housekeeping gene B2M was used as an endogenous control. Due to the high number of cells detaching during adipogenic and osteogenic differentiation, mRNA was extracted on day six for adipogenic cells and day seven for osteogenic cells. Extraction of mRNA from chondrogenic cells occurred at day eight. Stemness in equine MSCs was investigated using Oct4 and Nanog as positive markers of stemness while CD34 was used as a negative marker of stemness. The expression of four tissue specific differentiation markers, PPARγ2 for adipogenesis, SOX9 for chondrogenesis and RUNX2 and SPP1 for osteogenesis, was investigated to determine whether these genes were upregulated during differentiation.

The fold increase in gene expression during differentiation for ADSCs is displayed in **Table 3.18** and in **Table 3.19** for BMSCs. Gene expression is relative to the house keeping gene B2M for each sample.

**Table 3.18. Fold increase in gene expression for trilineage differentiation in adipose derived stem cells from four different horses relative to the housekeeper B2M.**

| Gene | Fold increase in gene expression relative to B2M for each sample | | | |
|---|---|---|---|---|
| | **LH4** | **LH6** | **PM1** | **PM13** |
| PPARγ2 | 0.000 | 2.431* | 0.094 | 0.000 |
| RUNX2 | 0.404 | 0.492 | 0.261 | 1.701 |
| SPP1 | 0.140 | 1.155 | 0.633 | 3.732* |
| SOX9 | 0 | 0 | 0.072 | 0.118 |

PPARγ: adipogenic differentiation, RUNX2, SPP1: osteogenic markers, SOX9: chondrogenic marker.
*Fold increase over 2 demonstrated upregulation of a gene

**Table 3.19. Fold increase in gene expression for trilineage differentiation in BMSCs relative to the housekeeper B2M**

| Gene | Fold increase in gene expression relative to B2M for each sample | | | | |
|---|---|---|---|---|---|
| | **LH2** | **LH3** | **LH4** | **LH5** | **LH6** |
| PPARγ2 | 0 | 0 | 0 | 0 | 0 |
| RUNX2 | 4.523* | 11.417* | 3.498* | 10.229* | 4.731* |
| SPP1 | 0.323 | 28.341* | 3.220* | 1.273 | 6.987* |
| SOX9 | 0 | 28.828* | 3.272* | 0 | 0 |

PPARγ: adipogenic differentiation, RUNX2, SPP1: osteogenic markers, SOX9: chondrogenic marker.
*Fold increase over 2 demonstrated upregulation of a gene

### 3.7.1 OCT4 EXPRESSION

Oct4 is a pluripotent marker required for self-replication and pluripotent differentiation. The positive expression of Oct4 has been found in some studies of equine MSCs, however, there have been conflicting results as to whether this marker is a reliable indicator of stemness in multipotent cells. In the present study, no relative expression of Oct4 could be detected using RT-PCR in any of the ADSC, BMSC or pBSC samples. The results found in the present study imply that this

pluripotent marker may not be suitable for identifying stemness in equine multipotent stem cells from AT, BM, or pB.

### 3.7.2 NANOG EXPRESSION

Nanog is a pluripotent marker required for self-replication and multilineage differentiation. Similarly to Oct4, Nanog expression has been found in equine MSCs although variable expression of this marker has been reported. In the present study, Nanog was found to be expressed by an undifferentiated ADSC sample (LH4). This expression disappeared when the cells underwent differentiation in the trilineage assay. As with Oct4, the results of the present study imply that this pluripotent marker may not be suitable for identifying stemness in equine multipotent stem cells from AT, BM, or pB.

### 3.7.3 CD34 EXPRESSION

The hematopoietic marker CD34 is positively expressed in hematopoietic stem cells but is thought to be negatively expressed in equine MSCs. Due the negative expression of this marker, it is widely used as a negative marker of stemness. In the present study, all of the BMSCs were found to be negative for CD34 expression, this was an encouraging finding as the BM contains a well characterised population of CD34+ hematopoietic cells. The negative expression of CD34 in the BMSCs in the present study indicates that the samples used were free of hematopoietic precursor cells. However, one undifferentiated ADSC sample (PM13) had positive expression of CD34.

### 3.7.4 PPARγ2 EXPRESSION

Peroxisome proliferator-activated receptor gamma 2 is an adipogenic differentiation marker upregulated as early as day six after adipogenic induction although expression is highest towards the end of differentiation. This marker is a key positive transcriptional regulator of adipogenesis and triggers the expression of additional adipogenic genes required for lipid accumulation. Although mRNA

from adipogenic samples was collected on day six after adipogenic induction, PPARγ2 was not found to be widely expressed. Indeed, only one ADSC sample (LH6) expressed for PPARγ2 (**Table 3.18**) and no expression of PPARγ2 was found in any of the BMSC samples (**Table 3.19**). The day of mRNA collection may have preceded the upregulation of PPARγ2, particularly in the BMSC samples which did not appear to differentiate efficiently into adipocytes and may be associated with delayed upregulation of adipogenic transcription factors. The extensive lipid accumulation displayed in the ADSC sample from LH6 (Figure 3.4) may therefore be due to the earlier upregulation of PPARγ2 found in this sample.

### 3.7.5 SOX9 EXPRESSION

The primary transcription factor involved with chondrogenic differentiation is thought to be SOX9. This marker is responsible for early activation of cells into chondrocytes and maintaining the commitment of cells towards the chondrogenic lineage. In spite of the essential role SOX9 plays throughout chondrogenic differentiation, expression of this marker was only seen in two BMSC samples (LH3, LH4) following chondrogenic differentiation (**Table 3.19**) while no expression was seen in any of the ADSC samples (**Table 3.18**).

### 3.7.6 RUNX2 EXPRESSION

Runt-related transcription factor two is expressed during early osteogenic differentiation and precedes the expression of additional transcription factors, necessary for osteogenic differentiation to occur. The present study found that while RUNX2 was expressed in all of the BMSC samples on day seven after osteogenic induction (**Table 3.19**), no expression of RUNX2 in ADSC samples was detected (**Table 3.18**). The expression of this early osteogenic marker in all of the BMSC samples indicates that BMSCs differentiate into osteocytes at a lower rate than ADSCs. Indeed, the day of mRNA collection may have been after the peak of RUNX2 had occurred in the ADSC samples.

### 3.7.7 SPP1 EXPRESSION

Secreted phosphoprotein 1 (SPP1) is an additional early osteogenic marker widely used for analysing osteogenic differentiation. The expression of this marker was found in three of the BMSC samples (**Table 3.19**) and in one of the ADSC samples (**Table 3.18**). This marker is preceded by RUNX2 and the expression of SPP1 in the single ADSC sample, compared to the expression seen in the three BMSC samples, indicates that osteogenic differentiation may occur at different rates for the two cell sources.

### 3.7.8 COMPARISON OF MATCHED ADIPOSE DERIVED AND BONE MARROW DERIVED STEM CELL SAMPLES

The ADSC and BMSC samples from the same horse were compared to see if any differences could be observed (**LH4: Figure 3.17, LH6: Figure 3.18**). It appears that BMSCs have a higher relative expression of markers for osteogenic differentiation (RUNX2 and SPP1) than ADSCs. Relative expression of SOX9 was increased in LH4 BMSC chondrocytes but not in LH4 ADSCs. There was no expression of SOX9 in LH6 ADSC or LH6 BMSC chondrocytes. An increase in PPARγ2 relative expression was seen for LH6 ADSC (2.431), however, this was the only MSC sample to show a fold increase in PPARγ2 expression following adipogenic differentiation.

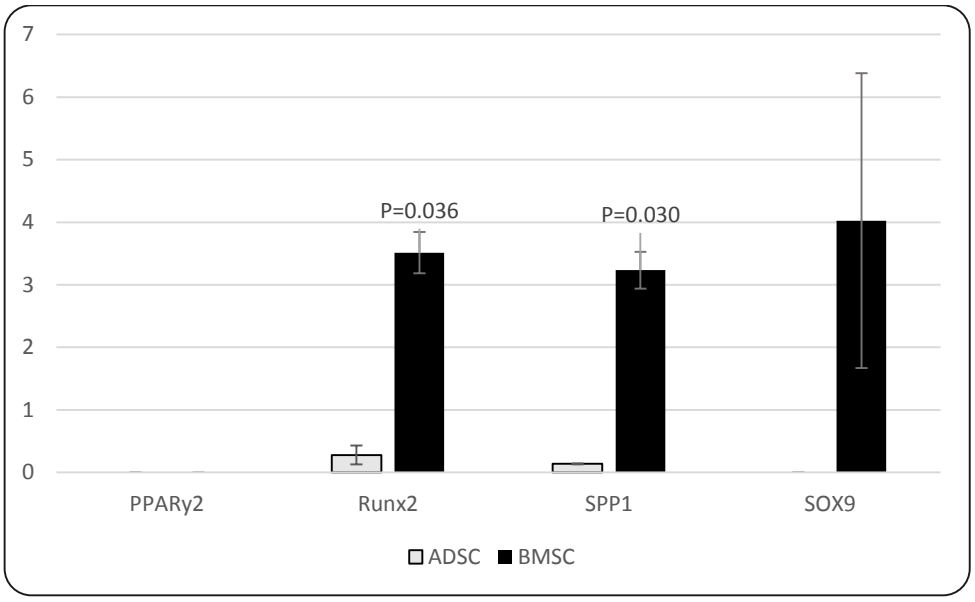



**Figure 3.17. Gene expression. Comparison of adipose derived stem cells (ADSC) and bone marrow derive stem cells (BMSC) from LH4 during differentiation into adipocytes, chondrocytes and osteocytes.**

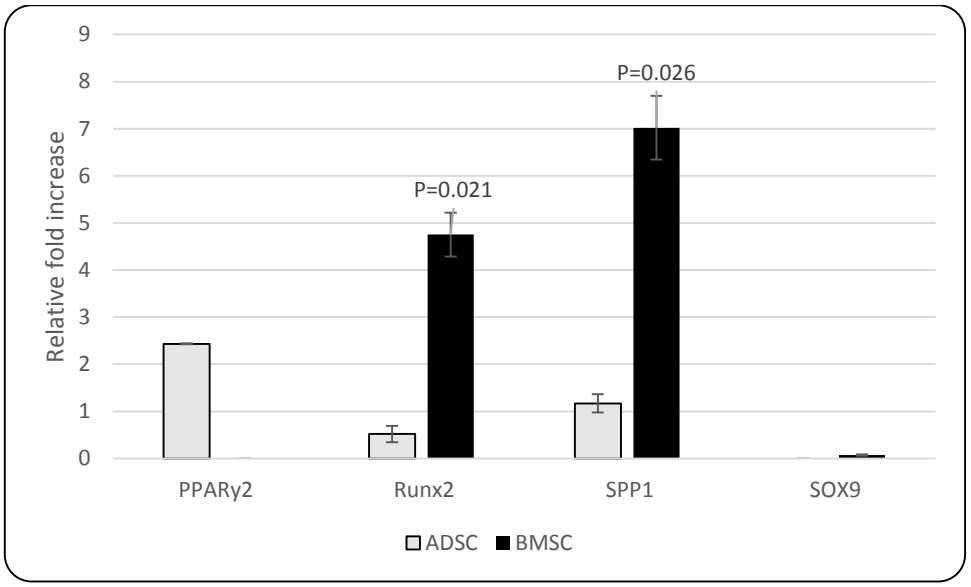



**Figure 3.18. Gene expression. Comparison of adipose derived stem cells (ADSC) and bone marrow derived stem cells (BMSC) from LH6 during differentiation into adipocytes, chondrocytes and osteocytes.**

In summary, the pluripotent markers Oct4 and Nanog were not consistently expressed in ADSC and BMSC samples. Oct4 was not expressed in any samples while Nanog was expressed in only one undifferentiated ADSC sample. This implies that pluripotent markers are not widely expressed by equine MSCs. The

hematopoietic marker CD34 was expressed in one ADSC sample but was not expressed in any of the BMSC samples. Despite displaying better morphological differentiation when compared to BMSCs, only one ADSC sample expressed PPARγ2 during adipogenic differentiation, with no expression in any of the BMSC samples. On the other hand, BMSCs exhibit higher upregulation of osteogenic and chondrogenic markers than ADSCs during differentiation.

Expression of these markers at protein level was not investigated in the present study and as such, the results indicating expression at mRNA level need to be interpreted with caution. Although it has been reported that gene expression can carry through to protein level this is highly gene dependent and mixed results have been reported for the markers used in this study.

### 3.7.9 GENE EXPRESSION AND DIFFERENTIATION STAINING

The correlation between morphological differentiation and relevant gene expression was investigated. No correlation between gene expression and histological grade of differentiation could be found. However, this may be due to the day on which the mRNA was isolated for analysis.

# CHAPTER 4: DISCUSSION

Mesenchymal stem cells have been used in recent years for the treatment of various diseases and injuries in humans (Wilson et al., 2011), dogs (Guercio et al., 2012) and horses (Richardson et al., 2007) due to their ability to self-renew and differentiate (Marfe et al., 2012). Multiple sources have been identified for the isolation of equine MSCs (Barry and Murphy, 2004). Adipose tissue and BM are two sources which have been used extensively for routine MSC harvest and therapeutic application (Schnabel et al., 2013; Vidal et al, 2008). Peripheral blood derived stem cells have been used to a lesser extent and has had mixed success reported in the literature in terms of culturing success (Giovannini et al., 2008; Koerner et al., 2006). What appears to be lacking in the literature is a standardised approach for identifying stem cells isolated from various sources. Although the ability to adhere to a culture vessel, differentiate into multiple lineages and express for markers of 'stemness' are the current requirements for MSC identification, some therapeutic approaches do not use a validated approach to determine whether the cells isolated from raw tissue are indeed MSCs (Schnabel et al., 2013). Due to the lack of verification, it is difficult for veterinary practitioners to confidently recommend the harvesting of a specific tissue to isolate MSCs for treatment. A comparison of MSCs isolated from AT, BM and pB, and their capacity into different cell fates in vitro, would therefore confirm whether the cells routinely harvested for stem cell therapy do indeed display the criteria to be characterised as MSCs.

## 4.1 MAIN PROJECT

### 4.1.1 ISOLATION OF MESENCHYMAL STEM CELLS FROM DIFFERENT TISSUES

The isolation of AT, BM and pB required three specialised approaches for tissue harvesting. There were tissue specific advantages and disadvantages in terms of animal welfare and collection practicality.

Adipose tissue collection was an invasive procedure and had the highest perceived levels of surgical morbidity. Even so, the horses recovered well from the procedure and only one horse developed complications, a small abscess that resolved quickly with treatment. The scarring from the surgery was minimal and less obvious than expected. The use of an ultrasound to identify pockets of adipose tissue was particularly advantageous. The surgeons were able to make a small incision directly over a dense layer of fat, resulting in faster and more efficient tissue recovery. Of the six horses in the study, AT was collected from five individuals. The horse which did not have available adipose tissue was a thoroughbred of light build. This implies that adipose tissue collection may not be a viable option for owners of light or lean horses. Specialised breeds of horse, such as thoroughbreds and standardbreds, may have minimal fat deposits while in training due to rigorous exercise regimes. For horses with minimal fat deposits available, bone marrow collection may be a viable alternative for obtaining tissue for stem cell isolation.

Three of the horses used for live tissue collection were euthanized later, for reasons unrelated to the project. The opportunity was taken to observe how the adipose tissue harvest site had healed. The tissue surrounding the incision was deeply mottled and purplish in colour indicating tissue necrosis. On consideration of the surgical procedure, the region of discoloured tissue reflected the inverted L-block pattern used by the veterinarians to inject local anaesthetic. Local anaesthetic is required to prevent the animal suffering during the procedure but is associated with tissue damage (B. Fraser, personal communication, March 18, 2013). Therefore, AT harvesting is associated with unavoidable surgical morbidity.

Human ADSCs are routinely isolated from lipoaspirate sampling (Dubois et al., 2008). The use of liposuction may potentially be used for the collection of equine AT in an attempt to reduce surgical morbidity. It has been suggested by Dubois et al. (2008) that only minor modifications of human liposuction protocols are required to enable liposuction in other species. An investigation into the use of a

modified liposuction technique for equine AT harvest would determine if this approach is a viable alternative to open surgery.

Bone marrow aspiration from the sternum has associated risks due to the close proximity of the collection site to the heart. A biopsy needle inserted too deeply into the sternum could potentially have fatal consequences. No complications occurred during BM collection due to the experience of the veterinarian team. Indeed, this became the preferred method of tissue collection for the veterinarians involved with this project.

Of the three tissue types collected, pB was the least invasive and caused little impact on animal welfare. Repeated samples can be taken from the same animal without unnecessary distress and, due to the large body mass of the horse, large volumes of blood can be isolated at one time without impacting negatively on the health of the animal.

The number of MNCs isolated from raw tissue samples was compared. Bone marrow aspirate yielded the highest number of MNCs from 10ml of raw aspirate however, the MNC yield difference between the three tissue types was not significantly different. The number of MNCs isolated from AT, BM and pB is comparable to the isolation counts reported by Burk et al. (2013). Although the number of AT MNCs isolated in the present study was slightly lower than reported by Burk et al. (2013), the cells from both studies reached confluence at similar rates. This indicates that the proportion of MSCs per ml of raw tissue may indeed have been higher in the present study.

Interestingly, the variability seen between donors and within donors for AT MNCs collected in the present study was similar. Bone marrow MNC and pB MNC counts showed high variability within and between horses, while AT MNC counts remained comparable. Mononucleated cells yielded from AT appeared to have a wider variability between horses in the literature (Burk et al., 2013) than seen in the present study. Cell count variability seen between repeated samples of BM

from the same horse may be due to uneven distribution of MNCs within the marrow. The variability reported in this project and in the literature suggests that each horse has wide variability of MNC concentrations within the sternum. Kasashima et al. (2011) reported an insertion site dependent yield of MSCs from BM aspirate. Additionally, Kasashima et al. (2011) also reported that the first 5ml of aspirate contained the densest concentration of MSCs. Variability of isolation counts may be due to where the biopsy needle is inserted and where the cell clusters are located in each horse. Bone marrow isolated in the present study was aspirated from either the $5^{th}$ and $6^{th}$ sternebrae. Isolation from either sternebrae appears to be suitable for equine BMSC harvesting. Although MSCs appear to be unevenly distributed within the marrow, this does not appear to impact on total MSC isolation success, as all of the BM samples collected from the six horses in this project reached P0 confluence.

The jugular vein is an easily accessible source of pB for MNC isolation. The average yield of pB MNCs has been reported as approximately 12-24 x $10^5$ cells/ml of pB (Dhar et al., 2012), however, a lower variability in MNCs per 10ml of pB was seen in the present study. Dhar et al. (2012) used a Ficoll method of cell isolation while the current study used a Lymphoprep method. Although the density of both gradients are the same, additional methodology was not reported by Dhar et al. (2012) and a more thorough comparison of culture techniques could not be carried out. The collection of pB for the isolation of MSCs is less well established. Generally, pB is processed in a similar manner to BM using Ficoll (Koerner et al., 2006; Giovanini et al., 2008; Martinello et al., 2011) and Percoll gradients (Spaas et al, 2012) or lymphocyte separation media (Dhar et al, 2011). The protocol used in this project consistently obtained a MNC fraction from 40ml of pB, however, the MSC component of the cell fraction was not able to attach and proliferate in any of the live samples.

Overall, adipose tissue appears to be a stable source of MNCs, while BM and pB MNC yields can vary considerably between and within individuals.

Body condition can significantly impact on the volume of AT available for harvest. As previously mentioned, one of the horses used in this study was of light build and no AT could be found at the typical site of collection. Health status may affect the number of MSCs available in BM and pB, and potentially AT if weight loss has occurred due to illness. A previous study looking at MNC yield from BM aspirate in human leukaemia patients compared to healthy individuals showed a decreased yield of MNCs from compromised individuals (Jing et al., 2011). Interestingly, although the initial yield of MNCs was decreased, the proliferation rate of leukaemia patient MSCs was comparable to healthy patient MSC proliferation rates, indicating that donor health may not affect *in-vitro* behaviour of BMSCs. As all of the horses used for the present study were of good health, no comparison of health status on cell recovery was made.

While performing the cell count at isolation, a viability assessment using trypan blue dye was carried out to determine the percentage of live cells within the freshly isolated cell population (**Methodology: 2.3.4**). The viability of freshly isolated cells was found to be significantly higher for BM MNCs and pB MNCs than for AT MNCs ($P<0.05$). This may be due to the more extensive processing that AT undergoes compared to BM and pB. Adipose tissue processing requires a number of washes before and after collagenase digestion which can be damaging to cells. The enzymatic digestion used for processing raw AT can also cause damage to the cells. Although the initial viability of AT MNCs was significantly lower than the viability of BM and pB MNCs, this did not appear to affect ADSC culture success. Indeed, the cells isolated from AT appeared to be considerably robust once in culture. Although the viability in AT MNCs was significantly lower than BM and pB MNCs, it still had a 93% viability, which indicates a high percentage of live cells.

It appears that the count at isolation does not provide a definitive guide to how fast a sample may reach confluence. Indeed, the isolation count can only provide an indication of MNC concentrations and it is only at the first confluence count that we can begin to evaluate what proportion of those MNCs are indeed MSCs. Primary cell culture demonstrated that AT contains a significantly higher

proportion of MSCs than BM from the same size volume of raw tissue. Across the literature, it appears that AT yields a higher quantity of adherent fibroblast-like cells (Ahern et al., 2011; Burk et al., 2013; Kern et al., 2006; Ranera et al., 2012) than BM. This observation complements the findings of the present study as ADSCs reached confluence on average 9.5 days faster than BMSCs and yielded a higher population of cells at confluence. This rapid proliferation rate of ADSCs is thought to be due to the higher density of MSCs found in raw AT (Ahern et al., 2011). Indeed, the MNC fraction isolated from one ml of raw AT contained an estimated 53% of MSCs capable of adherence and expansion. The MNC fraction isolated from BM aspirate contained only 11% of MSCs per ml of raw BM.

The pBSCs from two post-mortem blood samples were cultured and counted at confluence, however, it was observed that the cells had a tendency to clump during trypsinisation making it difficult to obtain an accurate count. This clumping may be linked to the unusual cluster-like growth pattern seen in both pBSC samples during culture. These cells adhered and displayed fibroblast-like morphology, although the mRNA from the samples did not express any markers of stemness. Therefore, the stem cells isolated from pB in this project may not necessarily be termed as mesenchymal without further validation.

Interestingly, two different types of media were used to culture pBSCs, DMEM F12 with 20% FBS and DMEM-high glucose with 20% FBS. Peripheral blood stem cells were able to be grown in both types of media however, differences were seen in the proliferation rate of the two cell samples. The sample cultured in DMEM F12 reached confluence considerably faster than the sample cultured in DMEM HG. However, more work is required to determine whether DMEM F12 or DMEM HG is the superior medium for pBSC culture.

Peripheral blood stem cell culture demonstrated initial cell attachment followed by progressive cell detachment. The use of laminin as a matrix did not assist with cell attachment and the two samples which reached confluence were grown in Nunclon™ Delta treated flasks. Delta is a proprietary cell culture surface treatment

used to improve the adhesion ability of a range of cell types. Successful culturing of pBSCs may require a comparative study of various combinations of matrixes and reagents to determine the optimal culture conditions for pBSCs to efficiently reach confluence. A number of authors have demonstrated that pBSCs are capable of trilineage differentiation (Dhar et al., 2012; Giovannini et al., 2008) and express markers of pluripotent stem cells (Marfe et al., 2012). These findings suggest that pB can be used as a source of MSCs, and highlights the need for future research in pB cell culture to determine the optimal culture conditions for this easily accessible source of MSCs. However, whether this source of MSCs will ever be comparable to AT and BM is currently unknown.

In comparison to ADSCs and BMSCS, the two post-mortem pBSC samples took an average of 26.5 days to reach confluence while ADSCs and BMSCs from live samples took an average of 5.5 and 15 days to confluence respectively. With this in mind, MSCs isolated from AT and BM may still prove to be a superior source of MSCs than pB due to the extensive time required for the blood derived samples to reach confluence.

Of the two sources of live stem cells that reached confluence, raw AT contained a significantly higher percentage of MSCs than raw BM. In terms of using MSCs for therapeutic application, the tissue type with the highest proportion of MSCs at isolation would likely be the optimal cell source for immediate transfer. However, further research is required to verify the therapeutic benefit of injecting freshly isolated cells from raw tissue.

The current stem cell treatment kits available on the market, Regeneus Adicell and Stem Vet NZ Ltd, use freshly isolated stem cells from adipose tissue for immediate transfer into animal patients. This suggests that ADSCs are of greater potential benefit than BMSCs or pBSCs when using primary cells for treatment. The higher concentration of MSCs in the AT MNC fraction is correlated to reduced time in culture as the isolated ADSCs reached confluence significantly faster than BMSCs. Therefore, not only are ADSCs potentially a more suitable candidate for immediate

transfer, they can also be amplified within five days of raw tissue extraction and used for cell therapy.

Bone marrow derived stem cells are found in lower concentrations and require additional time to reach confluence, however, these cells can be amplified and used for cell therapy in approximately 15 days after raw tissue isolation. Currently, it is unknown at what point in time after an injury has occurred that cell therapy should be applied (Smith et al., 2003). It has been suggested that stem cell therapy should be carried out soon after an injury has occurred but a specific interval of time has not been established (Godwin et al., 2011 cited in Burk et al., 2013). Smith et al. (2003) injected cultured BMSCs into a five week old superficial digital flexor tendon injury and suggested that a delay between injury and cell therapy may be advantageous as angiogenesis and granulation tissue formation may assist MSCs by forming a 'graft bed'. If further research indicates that there is a wide window of time where stem cell treatment is effective, then the differences seen between ADSC and BMSC culture would become less significant.

The superiority of ADSCs in culture continued through to secondary culturing. Adipose derived stem cells yielded a significantly higher number of cells at confluence than BMSCs from the same seeding density. This finding is supported by the literature in which ADSCs are found to be superior to BMSCs for both the total number of cells and cell doubling time (Burk et al., 2013; Colleoni et al., 2009).

Of the six BMSC samples cultured, only five reached passage one. One sample could be cultured to confluence but when replated, the cells failed to attach to the flask surface. Interestingly, the ADSC and BMSC samples reached confluence at similar rates, however, the number of cells counted at confluence was significantly different. This may be due to the subjective nature of assessing confluence, which is influenced by experience and personal judgement. Due to an increase in experience as the project progressed, my assessment of confluence became more accurate. Additionally, the size of the individual cells in culture may vary with tissue source. As there was a consistently lower count of BMSCs, these cells may

indeed have been larger than ADSCs and reached confluence at a similar day but with a lower number of larger cells.

Mesenchymal stem cells can only be maintained in culture for a limited number of passages before the ability to self-replicate is lost and the population undergoes senescence. Culturing of MSCs therefore aims to expand the number of cells in culture through dividing the population, replating and expanding the cells to confluence through a minimal number of passages. When culturing for therapeutic application, cells can be passaged two or three times to retain a portion of the population for storage while being able to supply cells for treatment. The ADSC and BMSC samples used for differentiation in the present project were able to be passaged six times. For veterinary purposes, the amplification of cells to passage six would provide ample amounts of cells for treatment and storage. The duration of time in culture before senescence occurs is reportedly greater in ADSCs compared to BMSCs (Vidal et al., 2012). Burk et al. (2013) compared five sources of equine MSCs, including AT and BM. In this experiment, AT and BM cells were passaged eight times, with all of the ADSC samples reaching passage eight, while only three of the ten BMSC samples reached passage eight. On the other hand, Braun et al. (2010) reported that only two of six ADSC samples could be cultured to passage nine which conflicts with the results reported by Burk et al. (2013). Although there are conflicting results reported in the literature, it would appear that BMSCs have a more limited number of passages in culture than ADSCs, however, the present study found that both ADSCs and BMSCs were able to be passaged six times without any detrimental effect on cell plasticity.

***Cryopreservation***

All of the ADSC, BMSC and pBSC samples that reached confluence were cryopreserved in 20% FBS and 5% DMSO. A slow freeze and rapid thaw protocol resulted in viable ADSCs, BMSCs and pBSCs. All samples reached confluence following thawing, demonstrating that cryopreservation can be used to store MSCs from AT, BM and pB for future use. Although cryopreservation impacts on

cell viability at thaw, the ADSCs and BMSCs recovered from liquid nitrogen storage demonstrated cell proliferation, fibroblast-like morphology and trilineage differentiation. Thawed pBSCs were able to be amplified after long term storage in liquid nitrogen and retained fibroblast-like morphology when placed back into culture. The effect of cryopreservation on pBSC differentiation capacity was not determined due to the small number of samples available.

The ability to collect a single sample of raw tissue, either AT or BM, amplify the MSC population and store the cells for future use is a particularly advantageous aspect of MSC culture. Storage of cells for successive treatments negates the need to re-harvest tissue, thereby avoiding putting horses through repeated surgery and risk, while reducing the cost for the owner.

The ability to cryopreserve MSCs for long term storage is supported by the literature. Preservation of MSC characteristics have been reported for ADSCs (Mambelli et al. 2009; Ranera et al., 2011; Vidal et al., 2008) BMSCs (Berg et al., 2009; Ranera et al., 2011) and pBSCs (Martinello et al., 2010) after cryopreservation of approximately four weeks. As there are reports of DMSO causing adverse reactions in patients receiving thawed cells, a thorough washing protocol, or culturing the cells before transfer is recommended (Hunt, 2011).

Adipose derived stem cells from post-mortem collections were used to investigate two different concentrations of cryopreservation medium (Appendix 1) on cell viability at thawing. A combination of 90% FBS with 5% DMSO and 20% FBS with 5% DMSO was compared. No significant difference was found between the two media, however, a cryopreservation medium containing 20% FBS would be the most cost-effective storage approach.

Overall, the cryopreservation phase of the project supports the possibility that equine MSCs from AT and BM can be frozen for future therapeutic use.

***Differentiation***

The differentiation phase assessed the trilineage differentiation capacity of ADSCs and BMSCs. Peripheral blood derived stem cells were not assessed as there were not enough samples to carry out a statistically valid assay. The samples used for differentiation are displayed in **Table 2.7**.

One of the main features of MSCs is their trilineage differentiation potential. Adipose derived stem cells and BMSCs were differentiated into adipocytes, chondrocytes and osteocytes to assess multipotency. Adipogenesis was confirmed by using the fluorescent stains BODIPY and by measuring the expression of the adipogenic marker, PPARγ2. Chondrogenic differentiation was confirmed by sectioning the pellets and staining with Alcian Blue to highlight deposits of GAGs. The mRNA isolated from pellets during differentiation was measured for expression of the chondrogenic marker SOX9. Osteogenic differentiation was confirmed by staining with Alizarin Red and measuring the expression of two osteogenic markers, RUNX2 and SPP1.

*Adipogenic differentiation*

In the present study, a superior adipogenic differentiation efficiency was seen in ADSCs when compared to BMSCs. Adipogenic differentiation occurred in all of the ADSC and BMSC samples although not all BMSC samples differentiated equally. The four ADSC samples showed a high proportion of adipogenic differentiation based on the number of adhered cells containing lipid vesicles and the size and distribution of lipid vesicles within the cells. The nuclei of the individual cells was visible and surrounded by lipid deposits. The five BMSC samples showed a lower efficiency for adipogenic differentiation than ADSCs with two samples showing poor lipid deposits. The triplicate design of the assay showed high variability in the distribution of cells across the surface of the wells for ADSCs and BMSCs. At the beginning of the assay, prior to differentiation, the cells had reached 80% confluence and were evenly distributed in the wells. However, by the end of the assay, many of the cells had lifted and only small patches of cells remained attached.

The gene expression for adipogenic differentiation was evaluated on day six following activation with adipogenic induction medium. Although PPARγ2 is expressed highest at the end of adipogenesis (Ranera et al., 2012), an increase in expression can reportedly be seen after six and seven days in adipogenic medium (Braun et al., 2010, Bracegirdle et al, submitted). Although mRNA collection was originally planned for day eight, due to the large amount of cells detaching from the well surfaces, mRNA collection was moved forward two days to ensure there would be an adequate amount of mRNA for analysis. The earlier mRNA extraction date may account for the lack of PPARγ2 expression in eight of the differentiated samples. Only one ADSC sample demonstrated an upregulation in PPARγ2 even though all of the ADSC samples differentiated efficiently. This particular sample also received the highest grade for adipogenic differentiation due to the high density of large lipids deposited within the cells. The early upregulation of PPARγ2 in this sample may account for more extensive differentiation seen at the end of the adipogenic assay.

The cell fraction isolated from raw adipose tissue contains a mixture of cells including fibroblasts and preadipocyte cells. Preadipocytes are capable of self-replication and are thought to be in a committed state of adipogenic differentiation (Cawthorn et al., 2012). As higher numbers of these pre-committed cells are likely to be found in AT MNCs than in BM MNCs, the increased adipogenic differentiation capacity of ADSCs may be due to the presence of preadipocytes in the cell sample.

*Chondrogenic differentiation*

All of the ADSCs and BMSCs samples aggregated into hard pellets during the chondrogenic differentiation assay, indicative of chondrocyte development. A tertiary structure is required for chondrogenic differentiation to occur and cell clusters are created prior to differentiation stimulation by micromassing undifferentiated cells. The structure of chondrogenic pellets is used as a measure of differentiation (Vidal et al., 2008) and as such, this parameter is an important indicator of chondrogenic efficiency. The pellet structure of control and treated

pellets were compared. Treated pellets appeared to develop into defined aggregates of cells while control pellets displayed less uniform aggregates. It appears that the presence of TGF-β1 is an important supplement for tertiary structure development in chondrocytes. Ten pellets, including control and treated pellets, were sectioned to compare the extent of GAG deposits and lacunae formation in ADSCs and BMSCs. The pellets used for sectioning were from live horse three (BMSCs), live horse four (ADSCs and BMSCs) and live horse six (ADSCs and BMSCs). The sample from live horse three was sectioned due to the high level fold increase in SOX9 expression. The sectioned BMSC pellets showed greater chondrogenic differentiation efficiency than ADSC pellets. Alcian Blue staining was more intense in BMSC pellets and there was a greater proportion of lacunae. Adipose derived stem cell pellets stained positively for GAG deposits however, the stain was pale and the formation of lacunae was less obvious. Additionally, the chondrogenic marker SOX9 was expressed in two BMSC samples but no expression was detected in any of the ADSC samples. One BMSC sample (LH3) had a 28.83 fold increase change in SOX9 expression during differentiation. This pellet was sectioned and compared to the other sectioned BMSC pellets, one of which expressed SOX9 but at a lower level (3.27). Interestingly, the high fold change in SOX9 expression did not correlate to higher chondrogenic differentiation efficiency. Indeed, the only BMSC sectioned sample that did not have any expression of SOX9 demonstrated more defined lacunae formation and more intense staining of GAG deposits than the two samples with SOX9 upregulation. It is important to remember that mRNA expression does not necessarily translate to protein level. This may account for the discrepancy between histological findings and mRNA gene expression.

Previous studies looking at chondrogenic differentiation in ADSCs and BMSCs have produced conflicting results. Burk et al. (2013) found that BMSCs demonstrated the weakest chondrogenic potential compared to cells isolated from umbilical cord blood, umbilical cord tissue, AT and tendon tissue. However, in a study by Giovannini et al. (2008), BMSCs displayed intense chondrogenic differentiation and Vidal et al. (2008) reported that BMSC chondrogenic potential was greater

than ADSC chondrogenic potential. Expression of collagen type II (COL1A2) was found to be higher for BMSC chondrocytes than ADSC chondrocytes (Vidal et al., 2008). Furthermore, positive staining for GAG deposits could be seen as early as day three after activation in BMSC chondrocytes while GAG deposits in ADSC chondrocytes could only be seen from day 14.

An interesting observation was the positive staining of the BMSC control pellets for GAG deposits. Staining in the ADSC control pellets was not seen, however a small amount of blue stain could be seen in all three BMSC control pellets. The only difference between the control and treated pellets is the exposure to TGF-β1. This growth factor is from a family of cytokines which regulate cell proliferation and differentiation and is considered to be essential for the induction and maintenance of chondrogenic differentiation (Goessler et al., 2005). As the staining in the control pellets was restricted to BMSC samples only, it may be that BMSCs have greater potential for chondrogenic differentiation and may begin to deposit GAGs even when exposed to incomplete induction medium. Nevertheless, chondrogenic differentiation was greater in BMSC samples exposed to TGF-β1.

*Osteogenic differentiation*

Osteogenic differentiation was achieved in all ADSC and BMSC samples, however there were clear differences between some of the BMSC samples. Adipose derived stem cells displayed similar osteogenic differentiation capacities, as all four samples formed distinctive cell clusters which stained positively for calcium. The BMSCs displayed localised staining of cell clusters in four of the five osteogenic differentiated samples. In contrast to the samples forming cell clusters, one sample demonstrated generalised staining of individual cells. Variability between individual differentiation capacities was not found to be reported in the literature. The differentiation grades of ADSCs and BMSCs were not found to be significantly different ($P= 0.358$), indicating that the osteogenic differentiation efficiencies of ADSCs and BMSCs are comparable.

The expression of two osteogenic markers, Runt-related transcription factor two (RUNX2) and secreted phosphoprotein 1 (SPP1), were analysed on day seven after exposure to osteogenic medium. While RUNX2 expression in BMSC samples ranged from 3.49-11.41, no increase in expression was seen in any of the ADSC samples. The osteogenic marker SPP1 was expressed in three BMSC samples and in one ADSC sample. The BMSC sample (LH3) with the highest RUNX2 expression increase did not form any clusters, however the individual cells along the well surface stained positively for calcium and covered a large surface area. This sample also demonstrated the highest fold change of SPP1 (28.34). As all of the ADSC samples stained positively for calcium, osteogenic differentiation can be confirmed for these cells, although gene expression of specific osteogenic markers did not complement the histological findings. Ranera et al. (2012) showed that not only is RUNX2 expressed in ADSC osteocytes, it is upregulated by day three in activation medium. These findings are in contrast to what was observed in the present study. The mRNA analysis was a snapshot approach as the mRNA was collected from day six only. The expression of RUNX2 and SPP1 on this day may differ between tissue types due to tissue specific upregulation or the amount of cells available for mRNA collection. The expression of either osteogenic marker did not appear to correlate with the histological findings, however, as previously mentioned, mRNA does not necessarily translate to protein level and the selected genes may indeed have been upregulated. Furthermore, ADSCs and BMSCs may differentiate at different rates and as such, gene expression evaluated on the same day would not necessary show the same pattern for both cell sources.

There appears to be a range of osteogenic differentiation observations in the literature and it remains unclear whether ADSCs or BMSCs have superior osteogenic differentiation capacity. However, what can be reported from the current project is that both ADSCs and BMSCs are capable of osteogenic differentiation. Adipose derived stem cells demonstrated higher morphological differentiation than BMSCs, although this was not significantly different. As BMSCs demonstrated superior gene expression of markers for osteogenesis, this suggests

that the optimal day for collecting mRNA for assessing the two osteogenic markers may have already passed for ADSCs.

Trilineage differentiation of ADSCs, BMSCs and pBSCs has been demonstrated by a number of authors (Bourzac et al., 2010; Braun et al., 2010; Burk et al., 2013; Dhar et al., 2012; Giovannini et al., 2008; Koerner et al., 2006; Ranera et al., 2012; Ranera et al., 2011; Spaas et al., 2013; Vidal et al., 2007), with adipogenic, osteogenic and chondrogenic lineages the most commonly used lineages to demonstrate multipotency in equine MSCs. Additionally, tenogenic differentiation capacity has been established in BMSCs (Violini et al., 2009) and myogenic differentiation capacity has been established in pBSCs (Martinello et al., 2010).

The wide range of protocols used for trilineage differentiation may account for the variable results reported for MSC differentiation capacity. For example, the protocol used by Schwarz et al. (2012) for adipogenic differentiation of equine ADSC, differed to the protocol used in the present project as rabbit serum was used instead of autologous serum. Additionally, the passage at which the cells are differentiated has been found to influence differentiation success (Braun et al., 2010). The ADSCs and BMSCs used for differentiation in this study were passaged four to six times before being utilized in the trilineage assay. At these higher passages, both cell types retained their capacity for trilineage differentiation. Adipose tissue derived stem cells from passage two and passage four were compared by Braun et al. (2010) to determine if two additional passages can effect differentiation capacity. It was found that chondrogenic and osteogenic lineages differentiated better at passage two than differentiating at passage four, although differentiation did occur in both groups of cells. Additionally, gene expression of collagen type I, II, and X (COL1AI, COL1A2, COL1A10) for chondrogenic differentiation and osteopontin, COL1A1 and β-actin for osteogenic differentiation was increased in cells differentiated at passage two, but not four. Adipogenic differentiation demonstrated the opposite effect where passage four cells developed higher concentrations of lipid deposits within the cells and had increased expression of PPARγ2. As MSCs used in equine cell therapy are likely to

be treating joint or tendon injuries the use of early passage cells may be beneficial for treatment. However, the present study demonstrated that ADSCs and BMSCs retain osteogenic and chondrogenic differentiation when passaged up to six times.

Cellular detachment of ADSCs and BMSCs during adipogenic and osteogenic differentiation was concerning. The protocols used for adipogenic and osteogenic differentiation were optimised for ovine stem cell differentiation and may account for cell loss during induction. Although cellular detachment occurred across all of the samples used for adipogenic differentiation there were limited reports of this phenomenon in the literature. Koerner et al. (2006) reported high cell detachment rates when pBSCs were exposed to adipogenic induction medium. The cells in the adipogenic assay in the present study were thoroughly assessed for bacterial contamination during culture and no bacterial growth was observed in any of the wells. Although cell loss during adipogenic differentiation has not been reported in the literature as a standard phenomenon, photographic evidence of differentiation in a number of papers clearly show distinct clusters of cells attached to the culture plate surface (Braun et al., 2010; Burk et al., 2013). This would suggest that cellular detachment is commonly seen with adipogenic differentiation with no detrimental effect on differentiation capacity. In the present project, one ADSC sample demonstrated particularly high rates of cell detachment. However, even with the loss of many cells, this sample differentiated well and scored highly for lipid development and percentage of differentiated cells. It appears that the number of cells that detach during differentiation is not an indication of the differentiation capacity of the individual cells in the sample. Due to the heterologous nature of the cell population used for differentiation, the loss of cells may be related to the portion of cells that do not possess multipotency.

The type of matrix used during differentiation may influence the rate of cell detachment during adipogenic differentiation. In the post-mortem pilot trial two matrixes, Matrigel and Laminin, were compared for BMSC adipogenic

differentiation. Laminin appeared to be a superior matrix with less cellular detachment occurring than with Matrigel.

Cell detachment also occurred during osteogenic differentiation in the present project. A human MSC osteogenic differentiation kit by R&D Systems states that cell detachment during differentiation can occur (R&D Systems, 2011). The osteogenic differentiation potential of equine MSCs and equine adult fibroblasts were compared by Colleoni et al. (2009). It was found that the fibroblast samples demonstrated extensive cell loss during differentiation and the few cells that remained attached did not stain positively for alkaline phosphatase or calcium. These findings suggest that the ADSCs and BMSCs seeded for osteogenic differentiation in the present study were a mixture of fibroblast and mesenchymal cells. As the population of cells used for differentiation are of heterologous nature this potentially explains the extensive cell loss seen during osteogenic and perhaps adipogenic differentiation of ADSCs and BMSCs.

***Stemness***

Stemness of MSCs isolated from AT, BM and pB was assessed through the expression of Oct4, Nanog and CD34. The markers Oct4 and Nanog are key regulators in the formation and maintenance of pluripotent embryonic stem cells (Mohanty et al., 2014). Positive expression of Oct4 and Nanog and the negative expression of CD34 in equine MSCs has been reported by Raabe et al. (2011) (in ADSCs) and Violini et al. (2009) (in BMSCs). The expression of these pluripotent markers in multipotent MSCs is debated in the literature. Indeed, Guest et al. (2008) has reported that multipotent cells, such as MSCs, do not express Oct4 or Nanog, and that these two markers are only expressed in ESCs. Irrespective of differing opinions, positive expression of Oct4 and Nanog has been found in equine ADSC, (Raabe et al., 2011) BMSC, (Violini et al., 2009) and UBC (Mohanty et al., 2014).

Although Oct4 expression was tested in all undifferentiated and differentiated samples, no expression was detected. Nanog was expressed in one

undifferentiated ADSC sample however, the CT values for the sample was above 35, indicating a low copy number for the mRNA. Interestingly, this ADSC sample demonstrated negative expression of Nanog once the cells underwent differentiation. The expression of Nanog in the ADSC samples prior to differentiation and subsequent loss of Nanog expression suggests that the 'stemness' of the MSCs was lost once the cells committed to a specific lineage fate. The results of the present study indicate that Oct4 cannot be used as a reliable marker of stemness in equine MSCs. Similarly, Nanog was only weakly expressed in one sample and may not be suitable for routine confirmation of stemness in equine MSCs. These findings are supported by Guest et al. (2008) but conflict with Raabe et al. (2011) and Violini et al. (2009).

There were two undifferentiated samples (ADSC PM13 and BMSC LH3) which expressed CD34. The CD34 gene is from a family of glycoproteins that can potentially influence cell adhesion, proliferation, migration, and differentiation. The expression of this gene has been seen on hematopoietic progenitor cells and is closely associated with this cell type (Cawthorn et al., 2012). No expression of CD34 has been reported in BMSCs but there are reports of ADSCs being positive for CD34, while maintaining trilineage differentiation capability (Dhar et al., 2012; Ranera et al., 2011). The expression of this marker is thought to decrease with time in culture although it is reportedly still present in ADSCs at passage three (Ranera et al., 2011). The ADSCs and BMSCs samples which expressed CD34 in this study had been passaged six times demonstrating that CD34 expression can continue for a number of passages. The ADSC and BMSCs samples which expressed for CD34 demonstrated trilineage differentiation, and the BMSCs from LH3 had the highest fold increase for chondrogenic and osteogenic differentiation markers. Therefore, the expression of CD34 does not appear to affect differentiation potential and may not be suitable as a negative marker of stemness for equine MSCs. As previously mentioned with the markers of differentiation and stemness, mRNA does not necessarily translate to protein level. As gene expression at protein levels were not tested, it cannot be determined whether there was a corresponding expression of CD34 in the post-translational protein level.

***Antibacterial trial***

Primary tissue culture is particularly vulnerable to bacterial and fungal contamination. In this project, cell culture presented a significant challenge due to environmental contamination apparently connected with the 2013 drought. Seven AT samples became contaminated with various strains of bacteria from an unknown source. All potential sources of contaminations were considered and several steps were taken to rule out common sources of bacterial contamination. Alternative autoclaves were used, samples were transported to AgResearch Ltd for processing and sterile surgical gowns, including face masks and hairnets were used. These extra measures did not reduce the occurrence of bacterial contamination. The samples were evaluated by Gribbles Veterinary Pathology and were found to consist of several different strains of bacteria. Due to this finding it was suggested that the primary source of contamination was from dust circulating in the environment when the tissue samples were collected. These contaminations coincided with the drought conditions during the summer of 2013, specifically during the months of January and March. It was decided to delay further collections until the drought conditions ended and the veterinarians and I then proceeded to collect AT from two horses.

Prior to the re-commencement of live surgeries, two post-mortem AT samples were collected and processed to assess for contamination risk. These samples were collected after the drought had finished to determine whether the change in environmental conditions had an effect on contamination rate. Gribbles Veterinary Pathology ran a minimum inhibition concentration test of the various strains of bacteria found in the contaminated samples. It was found that the bacteria, namely Coryneform bacteria and Enterrococcus, were resistant to penicillin (P) but susceptible to vancomycin (V). This prompted an antibiotic trial to compare penicillin/streptomycin (P/S) with gentamycin/ vancomycin (G/V) containing medium on cell proliferation and viability in culture and to determine if G/V could be used for routine equine ADSC culture.

A combination of P/S is routinely used in cell culture as it prevents contamination from many strains of bacteria and has minimal effect on cellular viability (Parker et al., 2012). However, many forms of bacteria are resistant to penicillin and streptomycin resulting in bacterial growth if only P/S is used. The antibacterial combination of G/V is less commonly used than P/S in equine stem cell culture and is thought to be more detrimental to cell health, although low concentrations of gentamicin have been found to have minimal effect on cell viability (Parker et al., 2012). Antibacterial action against a wide range of bacteria is provided by G/V, and this combination would be ideal in culture if there was no detrimental effect on cell proliferation or gene expression. It has been reported that if the concentrations of gentamicin are increased in culture, there is a corresponding decline in total RNA and expression of certain genes, although low concentrations are thought to be suitable for cell culture (Parker et al., 2012). Conversely, concentrations of penicillin as high as 500ug/ml do not result in any change to cell viability or RNA levels (Parker et al., 2012).

The antibiotic trial in the present project compared the rate of growth and viability of cells grown in medium containing G/V at 40ug/ml to controls cultured in P/S at 100U/100µg. There were no significant differences between growth rates and cell viability of any of the ADSC samples in the trial. There were no bacterial contaminations in any of the samples grown in either combination of antibiotics. However, as the antibiotic trial took place after the drought had ended, an improvement in environmental conditions appeared to have reduced the risk of bacterial contaminations.

Bone marrow and pB appeared to be more sterile tissue types to harvest cells from compared to AT. There was a lower incidence of bacterial or fungal contaminations occurring on the BM and pB and the bacterial contamination that did occur was due to contaminated media.

***Post-mortem vs Live***

An important point for discussion is the comparison of post-mortem (PM) and live samples for research purposes. All of the non-contaminated AT and BM samples collected PM yielded fibroblast-like cells which grew to confluence, and the samples selected for the differentiation trials demonstrated multipotency. Two of the PM pB samples yielded fibroblast-like cells, although these cannot be confirmed as MSCs as their trilineage capacity was not investigated. What can be inferred from the result obtained in the present study is that PM tissue, harvested soon after euthanasia, is a viable source for ADSCs, BMSCs and potentially pBSCs for research purposes.

Live BM and post-mortem AT from the same horse has been used as a scientifically valid approach to compare multiple tissue types for MSC research (Burk et al., 2013). To compare growth and differentiation characteristics of equine MSCs from different sources, Burk et al. (2013) collected tissue samples from umbilical cord blood and umbilical cord tissue of 12 foals and BM from 12 adult horses. Seven of the adult horses were euthanized to enable harvesting of post-mortem AT and tendon tissue. The post-mortem AT samples yielded the fastest growing MSCs with the highest yield of cells at confluence. Furthermore, all tissue types demonstrated multipotency (Burk et al., 2013). While compiling articles for the literature review, it was noticed that the vast majority of researchers used live tissue samples rather than harvesting from healthy horses being euthanized for reasons other than research. The present study showed that MSCs obtained from freshly euthanized animals displayed similar differentiation capacity to cells harvested from live animals. From an ethical point of view this is a valuable finding and should be considered for future equine stem cell research and indeed for many other species as well.

## 4.2 CONCLUDING REMARKS AND FUTURE DIRECTIONS

The aim of the project was to compare the recovery and stemness of MSCs harvested from adipose tissue (AT), bone marrow (BM) and peripheral blood (pB). Tissue recovery practicality was considered and compared. The number and

viability of cells at isolation, and cell proliferation differences were evaluated and compared. Bone marrow derived stem cells and ADSCs were also evaluated for trilineage differentiation efficiency. Confirmation of stemness was investigated through gene expression using specific stem cell markers.

Adipose tissue and bone marrow are two reliable sources of equine MSCs. Peripheral blood does not appear to be a reliable source and is not recommended for routine MSC isolation. In matched samples, AT appeared to be a superior source of MSCs than BM, particularly during primary culture. However, both tissue sources contained adherent cells capable of expansion in culture.

Trilineage differentiation was achieved in ADSC and BMSC samples. Adipogenic differentiation efficiency was superior for ADSCs while BMSCs displayed higher efficiency for chondrogenic differentiation. Osteogenic differentiation efficiency appeared to be similar for both sources of cells. These findings imply that BMSCs are a more suitable candidate for treating lameness in horses due to their superior plasticity into relevant cell lineages. Whether these superior efficiencies translate into *in vivo* application requires investigation.

Stemness of ADSCs, BMSCs and pBSCs could not be confirmed using pluripotent markers or the negative hematopoietic marker. Further research is required to establish which markers of stemness should be used for routine identification of stem cells.

Although a single source of stem cells was not consistently superior throughout the project, when taking into account tissue collection practicality, differentiation efficiency and gene expression, BM appears to be a superior source of equine MSCs. This tissue source can be collected safely provided the veterinarian is experienced with sternal BM aspiration. Although BMSCs take longer initially to reach confluence than ADSCs, once in culture, confluence rate is comparable. The superior chondrogenic differentiation efficiency seen in BMSCs is of clinical relevance as joint injury is a leading cause of wastage in horses.

Currently, there is no available kit in New Zealand capable of processing BM aspirate for equine MSC isolation. The development of a BMSC kit for reinjection into equine patients would fulfil a vacant niche in the market. Although the findings of this project suggests that BMSCs are superior to ADSCs, the next step is to investigate how these two sources of cells assist in regenerative therapy using a controlled *in vivo* trial. Findings of an *in vivo* trial would be of particular value in recommending a specific tissue source for treating equine musculoskeletal injuries.

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# APPENDIX 1: MEDIA AND STAINS

Media

**Media for ADSCs – (DMEM x 2, 10% FBS, 1% P/S, 0.1% fungizone)**

| Ingredients | Volume | Source |
|---|---|---|
| DMEM X 2 | 100ml | Gibco (powder) |
| $HCO_3$ | 10.6ml | AgResearch |
| FBS | 20ml | Gibco |
| P/S | 2ml | Sigma Aldrich |
| Fungizone | 200ul | Sigma Aldrich |
| Sterile MQ water | To make total volume of 200ml | AgResearch |

Filter sterilised stock ingredients

**Media for BMSCs (DMEM F12, 10% FBS, 1% P/S, 0.1% fungizone)**

| Ingredients | Volume |
|---|---|
| DMEM X 2 | 50ml |
| F12 X 2 | 50ml |
| $HCO_3$ | 10.6ml |
| FBS | 20ml |
| P/S | 2ml |
| Fungizone | 200ul |
| Sterile MQ water | To make total volume of 200ml |

Filter sterilised stock ingredients
F12: Gibco

**Media for pBSC (DMEM F12, 20% FBS, 1% P/S, 0.1% fungizone)**

| Ingredients | Volume |
|---|---|
| DMEM X 2 | 50ml |
| F12 X 2 | 50ml |
| $HCO_3$ | 10.6ml |
| FBS | 40ml |
| P/S | 2ml |
| Fungizone | 200ul |
| Sterile MQ water | To make total volume of 200ml |

Filter sterilised stock ingredients
F12: Gibco

**PBS (% P/S, 0.1% fungizone) for adipose tissue processing**

| Ingredients | Volume | Source |
|---|---|---|
| PBS | 100ml | Thermofisher |
| P/S | 1ml | Thermofisher |
| Fungizone | 100ul | Thermofisher |

Autoclaved before adding fungizone

**Media for tissue processing**

| Media | Quantity | Source and contents |
|---|---|---|
| DMEM x 2, F12, 10% FBS | 200ml | Thermofisher |
| Vigro™ complete flush medium | 200ml | Containing surfactant and antibiotic; Bioniche, Animal Health, USA -1000 ml |

Sterile stock

**Cryopreservation medium**

| Ingredients | Volume (20ml stock) | Source |
|---|---|---|
| 75% DMEM | 15ml | Thermofisher |
| 20% FBS | 4ml | Life Technologies/Gibco |
| 5% DMSO | 1ml | Thermofisher |

Sterile stock

**Red blood cell lysis**

| Ingredients | Volume (10ml stock) | Source |
|---|---|---|
| NH4Cl (0.83% w/v in water) | 9 ml | Sigma Aldrich |
| Tris (2.06% w/v in water) | 1 ml | Sigma Aldrich |

Filter sterilised

**Collagenase**

| Ingredients | Quantity/volume (10ml) | Source |
|---|---|---|
| Collagenase, Type 1A, 125 CDU/mg solid | 0.02g | Sigma Aldrich |
| PBS | 10ml | Thermofisher |

Filter sterilised, used immediately.

**30% Sucrose solution**

| Ingredients | Quantity/volume 500ml | Source |
|---|---|---|
| Sucrose | 150g | Sigma Aldrich |
| PBS 10x | 50ml | Thermofisher |
| Sterile dH20 | To 500ml | Waikato University |

Filter sterilised and stored at 4°C

## Stains

**Trypan blue**

| Ingredients | Source |
|---|---|
| Trypan blue solution 0.4% in PBS | Thermofisher 100ml |

**Alcian Blue** – Provided by AgResearch, pH 1, 1% Alcian Blue in acetic acid.

**Alizarin Red** – Provided by AgResearch, pH 4.1. (Sigma)

**BODIPY/DAPI stain**

| Ingredients | Source |
|---|---|
| 10ul of 1mg/ml BODIPY | Life Technologies |
| 10ul of 1mg/ml DAPI | Life Technologies |
| 10ml 150mM NaCl | |

# APPENDIX 2: INSTRUMENTS, MATERIALS, CHEMICALS, DRUGS

| Equipment/ materials | Supplier | Specifications |
|---|---|---|
| Cell flasks | Thermofisher | Nunclon T75 |
| Cell flasks | Thermofisher | Nunclon T50 |
| Cell strainer | Ray Lab | 100um |
| Cell strainer | Ray Lab | 70um |
| Centrifuge (AgResearch) 50/15ml (no brake) | Sorvall | H-2000B |
| Centrifuge (AgResearch) 50/15ml tubes (with brake) | Eppendorf | 5702 |
| Centrifuge (Equibreed) 15ml tubes | MSE | VWR |
| Centrifuge (Equibreed) 50ml tubes | Hettich | Rotofix 32 |
| Centrifuge tubes | Interlab | 50ml |
| Centrifuge tubes | Interlab | 15ml |
| Collection containers | Thermofisher | 50ml |
| Coverslips (rectangular) | Sigma Aldrich | 22x60 |
| Coverslips (round) | Thermofisher | Nunc 13mm |
| Cryostat | Leica | CM1850UV-Cryostat |
| Cryovial inserts | Ray Lab | Greiner |
| Cryovials | Ray Lab | Greiner 2ml |
| EDTA tubes | SVS | Vacutainer 10ml |
| Fluorescent microscope | Olympus | BX50 U-ND25-2 |
| Fluorescent microscope | Olympus | BX50 U-ND25-2 |
| Freezing medium | Jung Tissue Freezing Medium | |
| Freezing spray | Surgipath® Frostbite® Spray | |
| Inverted microscope | Zeiss | Axiovert 40 CFL |
| Jamshidi™ needles | Shoof | 11g |
| Microscope (EquiBreed NZ) | Olympus | CK2 ULWCD 0.30 |
| Needles 18G | SVS | 18G, 21G |
| Parafilm | Interlab | 2 inch |
| Pasteur pipettes | Interlab | 3ml |
| Penrose drains | Shoof | 13mm, 46cm |
| Pipette bulb | Thermofisher | |
| Pipette tips | Interlab | 10ul, 200ul, 1000ul |
| Rocking platform | Waikato university | |
| Shaking incubator (AgResearch) | Ratek | Orbital mixer incubator |

| | | |
|---|---|---|
| Shaking incubator (Equibreed) | Interlab | S1-45 environmental shaker |
| Slides | Polysine | 25x75x1mm |
| Slides | Surgipath Apex superior slides | 25x75x1mm |
| Stripette | Interlab | 5ml |
| Stripette | Interlab | 10ml |
| Stripette | Interlab | 25ml |
| Supramid Braun suture | SVS | 4met, 75m |
| Syringe filter | Interlab | 0.22µm |
| Syringe filter (AgResearch) | Millipore | 0.27µm Millex-GP |
| Tissue tek cryomold | FisherScientific | 10x10x5mm |
| **Chemicals** | **Supplier** | **Specifications** |
| Ammonium chloride | Thermo Fisher | 500g |
| Amphotericin B | Thermo Fisher | 20ml 250ul g/ml |
| Antibiotics -P/S | Thermo Fisher | 50ml, |
| $CO^2$ | BOC | Carbon dioxide food fresh size G |
| Collagenase | Sigma Aldrich | Type 1A 100g |
| DMSO | Thermo Fisher | 100ml |
| Lymphoprep | Medica Ltd | 1.077g/ml |
| Mounting medium | Dako | |
| Percoll | Sigma Aldrich (AgResearch) | 1.08g/ml |
| Trypsin/EDTA | Thermo Fisher | 0.25% (1x) 100ml |
| **Drugs** | **Supplier** | **Specifications** |
| Depocillin | SVS | 100ml |
| Dormosedan | SVS | 5ml 10mg/ml |
| Phenylbute paste | Caledonian Holdings | 200mg/ml |
| Torbugesic | SVS | 50ml 10mg/ml |

**PCR equipment and enzymes**

| Equipment/enzymes | Supplier | Specifications |
|---|---|---|
| Ethidium bromide | | 10mg/ml |
| 100bp Ladder | GenScript | M102R 500ul |
| Loading dye | Solis BioDyne | 6 x DNA Loading Dye Buffer |
| Hot Start Thermostable DNA Polymerase | Hot Firepol®(TAQ) | Waikato University |
| Nucleic acid stain | Syto82 Orange Fluorescent | Waikato University |
| $MgCl_2$ | 2M and 4M | |
| Electrophoresis unit | Owl tank and power packs | |

| | | |
|---|---|---|
| Thermal cycler | BioRad | T100 |
| Real Time PCR machine | Corbett Research | Rotor-Gene 6000 |
| UV illuminator | Gibco BRL | UV-TFX-35M |
| Spectrophotometer | Nanodrop 2000 | |
| PCR eppendorfs | | 200ul, 500ul |
| Refrigerated centrifuge | Eppendorf 5415R | |
| Vortex | TALBOYS | |
| Thermo mixer | Compact Eppendorf | 1.5ml tube tray |

**1% Agarose gel**

| Ingredients | Quantity | Source |
|---|---|---|
| Agarose powder | .33g | |
| 1xTAE buffer | 33ml | Waikato University |

**50x TAE stock**

| Ingredients | Quantity | Source |
|---|---|---|
| Tris | 242g | Sigma Aldrich |
| glacial acetic acid | 57.1ml | Sigma Aldrich |
| 0.5M EDTA | 100ml | Sigma Aldrich |
| dH20 | To 1L | Waikato University |

1 x TAE (20ml 50x TAE into 980 ml dH20)

**Myogenic staining reagents supplied by AgResearch Ltd**

| Ingredients | Quantity | Source |
|---|---|---|
| Triton | 0.1% w/v in PBS | Sigma Aldrich |
| Tris buffered saline | 1 x | Sigma Aldrich |
| Cλ | | Sigma Aldrich |
| Mouse anti-MHC | 1:200 | Sigma Aldrich |
| Normal sheep serum | 5% | Sigma Aldrich |
| Biotinylated sheep anti-mouse | 1:300 | Sigma Aldrich |
| Streptavidin Alexa Fluor 488 | 1:400 | Life Technologies |

# APPENDIX 3: DIFFERENTIATION MEDIA

## Adipogenic differentiation

**Induction medium**

| Medium/reagents | Concentration used (stock concentration) | Source |
|---|---|---|
| DMEM 10% NRS, 1% P/S, 0.1% fungizone, buffer | 1ml per well | NRS - Gibco |
| IBMX | 100x stock (500µM) | Sigma Aldrich |
| Dexamethasone | 50x stock (50µM) | Sigma Aldrich |
| Indomethacin | 200x stock (100µM) | Sigma Aldrich |
| Insulin – bovine | 50 x stock (10mg/ml, 25mM) | Sigma Aldrich |

**Maintenance medium**

| Medium/reagents | Concentration used (stock concentration) | Source |
|---|---|---|
| DMEM 10% NRS, 1% P/S, 0.1% fungizone, buffer | 1ml per well | |
| Dexamethasone | 50x stock (50µM) | Sigma Aldrich |
| Insulin – bovine | 500x stock (10mg/ml) | Sigma Aldrich |

## Chondrogenic differentiation

**Incomplete medium** (control medium)

| Medium/reagents | Concentration used (stock concentration) | Source |
|---|---|---|
| DMEM, 1% FBS, 1% P/S, 0.1% fungizone, buffer | 20µl/well for treated induction, 200ul/well for control pellets | |
| Ascorbic acid 2-phosphate | 100x stock (100µM) | Gibco |
| Dexamethasone | 500x stock (100µM) | Sigma |
| ITS+1 premix | 1% | BD Science |

**Complete medium**

| Medium/reagents | Concentration used (stock concentration) | Source |
|---|---|---|
| DMEM, 1% FBS, 1% P/S, 0.1% fungizone, buffer | 20µl/well for treated induction, 200ul/well for control pellets | |
| Dexamethasone | 500x stock (100µM) | Sigma Aldrich |
| ITS+1 | 1% | BD Science |
| TGF-β1 | 10ng/ml | R & D Systems |

TGF-β1- R&D systems

**Osteogenic differentiation**

**Induction/maintenance medium**

| Medium/reagents | Concentration used (stock concentration) | Source |
|---|---|---|
| DMEM, 10% FBS, 1% P/S, 0.1% fungizone | 1ml/well | |
| Ascorbate 2-phosphate | 100x stock (100µM) | Sigma Aldrich |
| β-Glycerophosphate | 100x stock (100µM) | Sigma Aldrich |
| Vitamin D | 1000x stock | Sigma Aldrich |
| Dexamethasone | 500x stock (100µM) | Sigma Aldrich |

**Myogenic differentiation**

**Induction/maintenance medium**

| Medium/reagents | Concentration used (stock concentration) | Source |
|---|---|---|
| DMEM, 5% FBS, 2% horse serum (HS), 1% P/S, 0.1% fungizone | 1ml/well | HS - Gibco |
| Hydrocortisone | 1000x stock (3.5µm per 3.5ml medium) | Sigma Aldrich |