Assessment of bioprocess shear stress as a tool to enhance osteogenic induction of mesenchymal cells

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I, Zi Nie confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Shear stress is an unavoidable bioprocess force encountered during routine cell culture and large scale cell manufacture. It is generally considered as harmful for cells in bio-manufacturing, as it affects cell viability and function. Thus, reducing cell loss, maintaining cell integrity and function during processing are important for cell therapy. Also, based on the fact that mechanical cues can increase bone formation *in vivo*, we hypothesized that the capillary shear stress could enhance osteogenic differentiation and maturation of cells. This study assessed the effect of capillary shear stress on survival and osteogenic differentiation using rat bone marrow derived mesenchymal stromal cells (MSCs), human MG63 cells and human MSCs. Cells were exposed to defined shear stress by passing through a capillary device. Three key parameters were tested: capillary internal diameters (e.g. 0.254 mm, 0.203 mm), flow rates (e.g. 13, 20, 28 ml/min) and number of passes (e.g. 10, 20, 40 passes). Cell recovery and viability were measured immediately after exposure to shear stress and after 24 and 72 hours cell culture. A small decline in immediate recovery and viability at 24 hrs was evident for MSCs passed through capillaries compared to controls. High flow rates and hence higher shear stress (e.g. 258 Pa) resulted in greater cell loss and reduced cell viability after culture for 24 hrs ($p < 0.05$). Using the capillary diameters and flow rates we reported here, cell growth is permissible in spite of an initial reduction in viable cell growth, but cells recover from this rapidly in culture. Although longer exposure durations to shear stress lead to more osteogenic differentiation, the increasing trend of mineralization was not linear to the increase of shear stress exposure time. Alizarin red S staining of both MSCs and MG63 cells revealed that appropriate capillary wall shear stress has potential to enhance osteogenic differentiation. In conclusion, sub-lethal fluid shear stress that cells experience during bioprocessing can be used as a mechanical cue for osteogenesis.

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Heartfelt thanks to my parents who constant support and believe in me, I always get comfort from them during this challenging period. I would not have made it this far without their unconditional love and understanding.

Finally I would like to thank all people not mentioned by name, who provided help during my research.

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Abbreviations

AA	ascorbic acid
ALP	alkaline phosphatase
bFGF	basic fibroblast growth factor
BGP	β-glycerophosphate
BMSC	bone marrow stromal cell
cDNA	complementary DNA
CO_2	carbon dioxide
COL1A1	type I collagen alpha 1
DEX	dexamethasone
DM	differentiation medium
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate buffered saline
DW	distilled water
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GM	growth medium
hMSCs	human mesenchymal stromal cells
IFF	interstitial fluid flow
PBS	phosphate buffered saline
PFA	paraformaldehyde
PCR	polymerase chain reaction
qRT-PCR	quantitative reverse transcription PCR
rat-BMSCs	rat bone marrow derived mesenchymal stromal cells
Re	Reynolds number

Runx2	runt-related transcription factor 2
UK	United Kingdom
USA	United States of America

℃	centigrade
cm	centimeters
g	grams
G	gauge
hrs	hours
ID	internal diameter
min	minute
ml	milliliters
mm	millimeters
mM	millimoles
ng	nanograms
µl	microlitres
µm	micrometers
Pa	pascal
rpm	revolutions per minute
rt	room temperature
sec	seconds

Pressure unit conversion: 1 Pa = 1 N/m^2 = 10 $dyne/cm^2$

Chapter 1 Introduction

1.1. Stem cells

Stem cells are defined functionally as cells that have the capacity to self-renew as well as the ability to generate differentiated cells. They can renew themselves by cell division for a long period, and they always maintain the undifferentiated status. In certain circumstances, stem cells can be induced to give rise to specific types of cells that have special function, and this is triggered by internal and external signals.

1.1.1. Adult mesenchymal stem cells

Adult stem cells (ASCs) are undifferentiated cells with limited self-renewal capacity that originate from most adult tissues and organs. They can renew themselves and differentiate into a few particular cell types that are related to their origin. For instance, hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) are two kinds of adult stem cells from bone marrow. The former can form blood cells and the latter can yield, as a minimum, osteoblasts, chondrocytes and adipocytes. The main role of adult stem cells in the body is the replacement and replenishment of blood and tissues and repair of damaged tissue, meaning they can maintain a dormant state for a long time before being activated by disease or injury (Castillo and Jacobs, 2010). ASCs present several advantages for cell therapy. First, small samples of a patient's own autologous cells can be used for implantation without tissue rejection problems. Moreover, their application avoids the ethical issues that are associated with embryonic stem cell research.

Mesenchymal stem cells can be isolated from lots of tissues, such as bone marrow, adipose tissue and cord blood. Their phenotype can vary and show differences due to the effect of tissue source from which they are harvested.

The relative quantities of MSCs obtained from a tissue vary dependent on their origins. For instance, adipose-derived MSCs have high harvest frequency of about 557 per 10^6 stromal vascular cells, bone marrow-derived MSCs are isolated with a frequency of approximately 81 per 10^6 mononuclear cells, yet umbilical cord blood only yields about 0.002 MSCs per 10^6 mononuclear cells (Krawiec and Vorp, 2012).

MSCs were first discovered with an adherent fibroblast like shape in the bone marrow by Friedenstein (Friedenstein *et al*., 1968). MSCs derived from bone marrow are multipotent and possess high capacity to give rise to cells of connective tissue lineages, such as cartilage, bone and fat (Figure 1.1) (Kelly, 2007; Yourek *et al.*, 2010; Krawiec and Vorp, 2012). Three criteria were established by the International Society of Cellular Therapy to define mesenchymal stem cells: they must adhere to plastic under standard tissue culture condition; certain cell surface markers must be expressed by them, for instance, CD73, CD90 and CD105; they must be capable of differentiating into osteoblasts, chondroblasts and adipocytes during culture *in vitro*. Although MSCs only account for about 0.001-0.01% of total nucleated cells in bone marrow (Barry and Murphy, 2004), MSCs still have large therapeutic potential and value for tissue engineering and regenerative medicine due to the ease of culturing them *ex vivo* to expand and differentiate into multiple cell types under certain culture conditions.

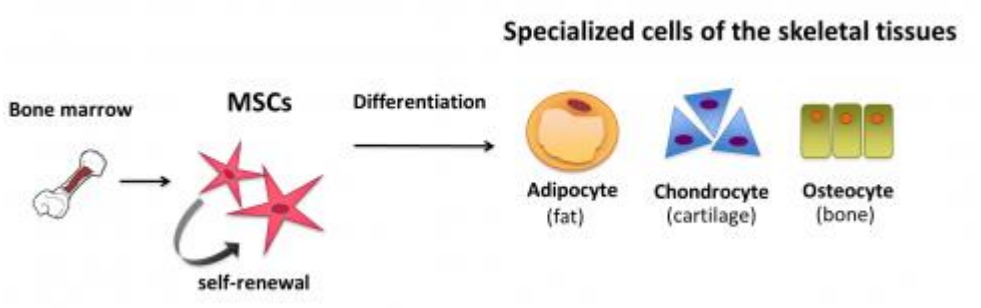


Figure 1.1: Differentiation capacity of bone marrow derived MSCs (EuroStemCell, 2012).

1.1.2. Bone tissue engineering

Tissue engineering is an interdisciplinary study that brings together principles of engineering, life sciences and medicine to develop biological tissues in the laboratory. These are applied to patients to restore, maintain and improve the function of damaged tissues that might result from congenital defects, disease, trauma or age-related degeneration. Tissue engineering generates new functional tissues *ex vivo* based on understanding of tissue formation and regeneration (Jennifer *et al*, 2011).

The skeletal system plays a prominent role in supporting motion-related muscular contraction, withstanding mechanical load and protecting internal organs. Injury and disease of bone (e.g. bone fracture, osteosarcoma and osteoporosis) can result in dramatically change quality of life (Salgado *et al*., 2004; Seong *et al*., 2010; Arvidson *et al.*, 2011). Millions of people suffer from various bone diseases and defects in the world, this becomes a major issue especially given that the aging population is increasing continuously at present and many bone defects occur more readily in aged patients. Compared to traditional transplantation and replacement therapies which use autologous bone graft or allograft, bone tissue engineering utilizes various stem cells to derive bone forming cells. Stem cell sources can include embryonic stem cells (ESCs), bone marrow derived MSCs, adipose tissue, muscle and dental pulp stem cells (Seong *et al.*, 2010). All these cells have been shown to have capacity to differentiate into osteoblasts *in vitro*. Bone marrow derived MSCs are the most used cell source for bone tissue engineering (Figure 1.2) (Seong *et al.*, 2010). Osteoblasts can also be used but are limited by the difficulty in their isolation and expansion to clinical numbers. MSCs overcome this problem as they can be isolated from more accessible donor sites. In addition to cells, two other critical elements are required for forming new bone tissue: scaffolds and growth factors. Typical procedures involved in bone tissue

engineering are shown in figure 1.3. The basic steps include: harvest of bone marrow from patients, isolation of MSCs via adherence to tissue culture plastic, expansion and differentiation of MSCs, an appropriate scaffold which allows MSCs to produce extracellular matrix (ECM) in regulated culture condition followed by implantation into patients (Arvidson *et al.*, 2011).

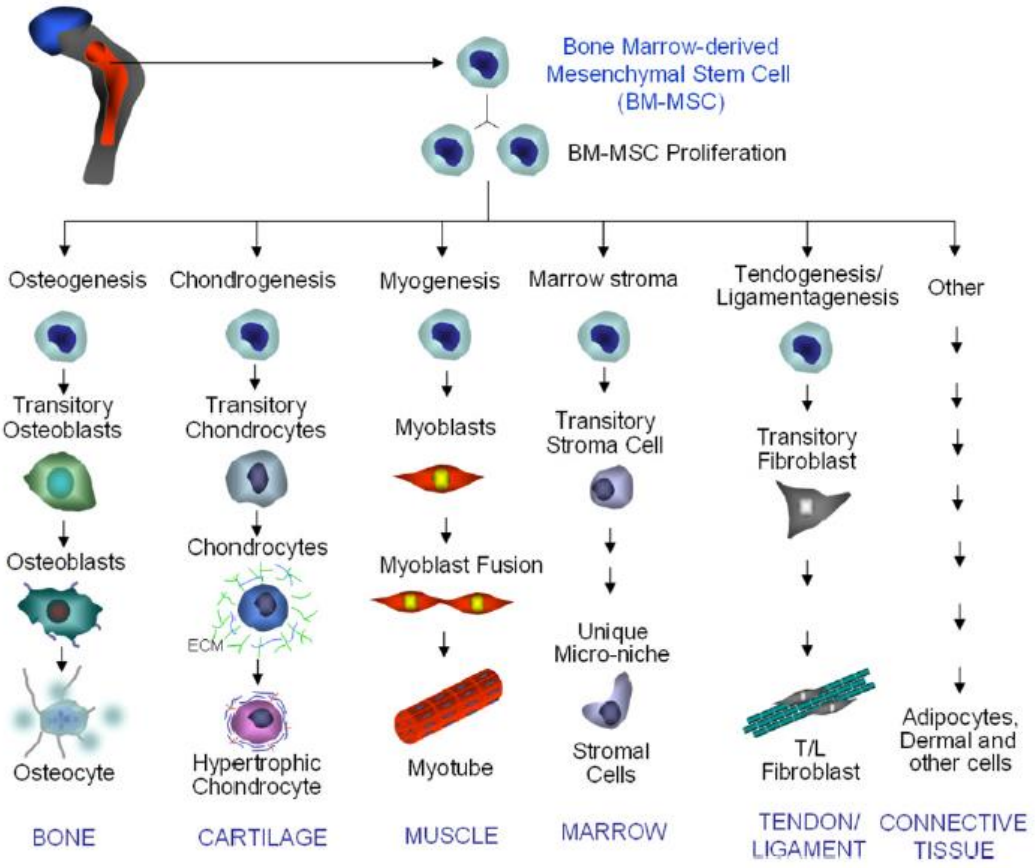


Figure 1.2: Differentiation of bone marrow derived mesenchymal stem cells for bone tissue engineering (Seong *et al.*, 2010).

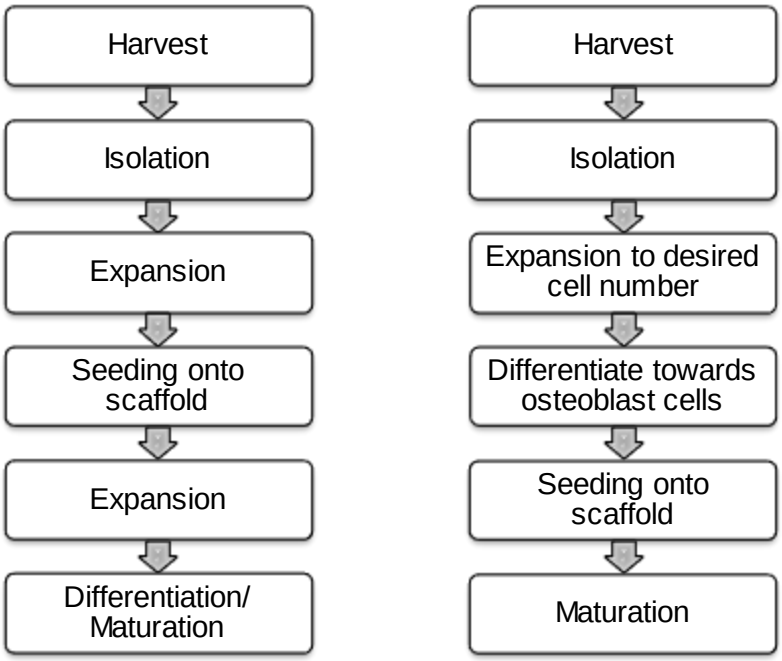


Figure 1.3: Two classical procedures of bone tissue engineering.

1.2. Bone formation

Bone is a rigid, porous and mineralized connective tissue. It possesses multiple functions in the human body, for instance, providing attachment surfaces for muscle, supporting and protecting internal organs and providing a calcium store for the body. There are two architecturally distinct forms of bone tissue. One is trabecular, also known as spongy bone with a high porosity (50-90%) and a honeycomb like arrangement. Approximately, 20% of the total skeleton is spongy bone. The remaining 80% of the total skeleton is compact bone, which is solid with a low porosity (10%) and comprises several subgroups: long bones, such as tibia and femur; flat bones, such as skull vault; and short bones, such as those in the ankle and wrist (Salgado *et al.*, 2004).

Bone cells and bone matrix form osseous tissue. About 90% of bone matrix is composed of type I collagen and the remaining 10% is non-collagenous proteins, such as osteocalcin and osteonectin, which play a role in

mineralization and regulating functional activity of bone cells (Arvidson *et al.*, 2011). Bone cells consist of osteoprogenitors, osteoblasts, osteocytes, osteoclasts and bone lining cells.

Osteoprogenitors are also known as preosteoblasts and possess the capacity to develop into osteoblasts and mediate bone growth. Osteoblasts can synthesize extracellular matrix (ECM) and osteocytes are mature differentiated osteoblasts that support bone structure. Both osteoblasts and osteocytes are responsible for the formation and repair of bones. On the contrary, osteoclasts participate in bone resorption. Homeostasis in osseous tissue can be maintained through bone remodeling processes that involve repetitive cycles of bone resorption by osteoclasts and bone formation by osteoblasts (Caetano-Lopes *et al.*, 2007; Arvidson *et al*., 2011). Bone-lining cells are resting osteoblasts which become elongated and no longer generate matrix, instead they can protect bone from the extracellular fluid space (Hall, 1990).

1.2.1. Osteoblasts and osteocytes

Osteoblasts are mononuclear bone forming cells that originate in the bone marrow and are located closely opposed to the bone surface. They are responsible for synthesizing bone matrix and also mineralizing this matrix during both bone formation and remodeling (Caetano-Lopes *et al.*, 2007). When they are inactive, they become elongated and do not form matrix, then numerous gaps and spaces appear between cells. However, when the osteoblasts become active, they display large Golgi apparatus and rough endoplasmic reticulum, as gene expression is increased and protein production ensues (Hall, 1990). Transcription factors, such as Runx2 and Osx, and the bone morphogenic protein (BMP) family of signaling proteins are critical for controlling osteoblasts formation (Caetano-Lopes *et al.*, 2007).

Osteocytes are the most abundant bone cells and have a stellate morphology.

They are a matured form of osteoblast at a later stage of bone development. Osteoblasts undergo a transition to osteocytes when they become trapped in their own matrix. They sit within spaces in the mineralized tissue and the body of the osteocyte sits in a space known as the lacunae and the cell processes extend to form connections with adjacent cells via channels called cannaliculi. The exchange of mineral ions between bone and extracellular fluid can be regulated through that canalicular system. They contribute to bone maintenance, and act as mechanosensors to instruct osteoclasts to resorb bone and osteoblasts to form bone at the required time and location.

1.2.2. Osteogenic differentiation

Bone formation occurs via a common process *in vivo*, throughout embryonic development, normal skeletal growth, remodeling, and fracture healing. At first, MSCs aggregate and proliferate to sufficient numbers of cells, then these cells synthesize and secrete matrix and differentiate into osteoblasts. Changes of matrix result in a suitable environment for calcification of bone matrix. During the mineralization process, cells increase the concentration and deposition of calcium and phosphate to produce mineralized matrix. Cells become trapped in that matrix and mature to osteocytes (Liu *et al.*, 2010).

Osteogenic differentiation can occur under specific growth conditions *in vitro*. Many researchers have reported that addition of β-glycerophosphate (BGP) to culture media can induce the mineralization of cells. It provides organic phosphate to the cells which leads to elevation of alkaline phosphate activity, resulting in greater calcification. Also, some studies have successfully demonstrated that adipose tissue and bone marrow derived MSCs can be induced to differentiate into bone cells *in vitro* when supplemented with dexamethasone (DEX), BGP and ascorbic acid (AA) (Kang *et al*., 2012; Li *et al*., 2015). In addition to the phosphate supply by BGP, dexamethasone is a hormone that can regulate bone morphogenetic proteins (BMPs) and ascorbic

acid is necessary for collagen production (Yourek *et al.*, 2010).

1.3. Shear stress

Cell behavior is affected by the environment that surrounds them, including chemical and mechanical factors. For instance, the contact and communication between cells, the chemicals secreted from cells, and the fluid shear stress in spaces around cells. All of these elements have impact on cell morphology, viability and metabolic activity.

Some mechanical cues play a pivotal role in function of tissues, such as muscle and bone. One critical factor is shear stress which is caused by fluid flow. Shear stress results from the force parallel to the material cross section which leads to the deformation of material. Laminar and turbulent flow are the two main types of fluid flow which are classified based on Reynolds number (Figure 1.4), the former is also known as streamline flow which means the fluid flows in parallel without disruption between adjacent flow layers. In other words, there is no cross currents perpendicular to the flow direction. The movement of the latter changes chaotically and stochastically, which has rapid variation of velocity in space and time. In brief, the direction of motion of each flow is random.

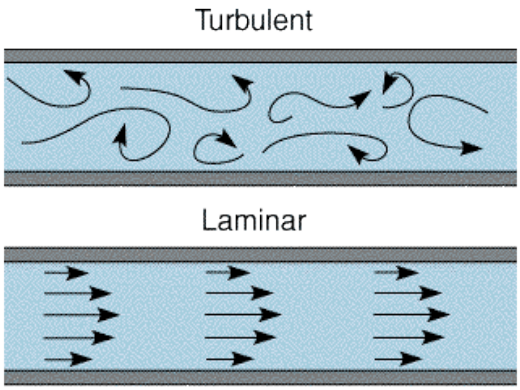


Figure 1.4: Turbulent flow and laminar flow ("Hydrodynamic-voltammetry", University of Cambridge teaching notes).

Reynolds number (*Re*), which is a dimensionless number that combines major variables (e.g. viscosity and velocity of flow), to analyze flow properties, is used to identify laminar and turbulent flows. For flows in a pipe, Laminar flow occurs at low velocity with a Reynolds number less than 2100 (Szewczyk, 2008). On the contrary, turbulent flow forms when the velocity is high and the Reynolds number is larger than 4000. And the flow is referred to as transitional flow when *Re* is between 2100 and 4000. Compared to laminar flow, turbulent flow causes more damage of cells due to the chaotic movement of flows. Cells encounter more intense shear forces and conflict, and they may lose membrane integrity and deform or even break up into pieces.

1.3.1. Shear stress in bioprocess

Regenerative therapies based on live cells are of considerable interest in modern medicine. However, large quantities of cells are required for many treatments, thus, production of cells on a large scale is a key challenge for the cell therapy industry. The whole process for manufacturing cells involves many steps, and the specific environment of each step can impact on the cell product (Figure 1.5). Therefore, reducing cell loss, maintaining their integrity and required function throughout the whole bioprocess is important for cell manufacture. Delicate cells are inevitably exposed to hydrodynamic forces

with different intensities during culture and other sub-processes. For instance, capillary transfer results in low levels of shear stress which might lead to alteration of the cell's phenotype. Also shear forces caused by agitation in stirred tank bioreactors, or hydrodynamic forces resulting from shaking and tapping the flasks to assist the detachment of adherent stem cells, or shear stress due to pipetting or aspiration when re-suspending cells can all impose a physical influence on cell phenotype (Brindley *et al.*, 2011).

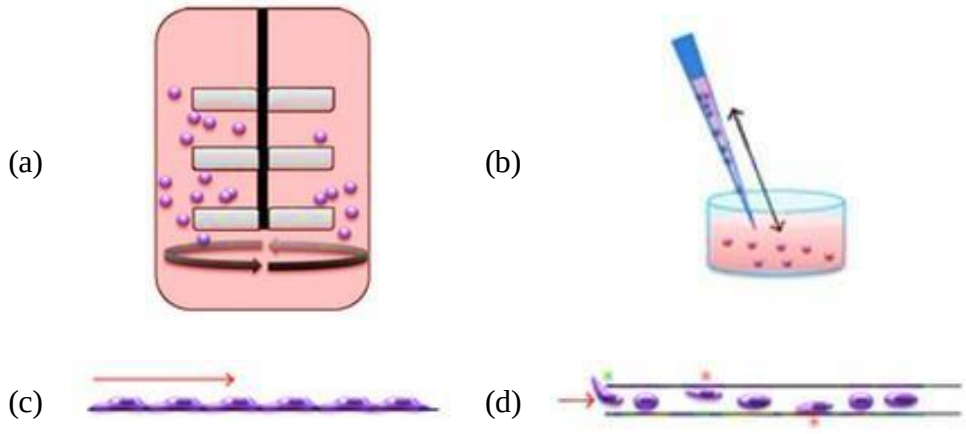


Figure 1.5: Shear stress in bioprocesses (a) non-adherent cells in suspension encounter shear stress caused by agitation when cultured in stirred tank bioreactor; (b) adherent cells in suspension face shear stress results from manual processes (e.g. pipetting and capillary transfer) during routine passage; (c) shear force in 2D culture condition; (d) shear force during aspiration capillary transfer (Brindley *et al.*, 2011).

1.3.2. Impact of shear stress on cells in bioprocessing

The biopharmaceutical industry commonly uses mammalian cells for manufacturing monoclonal antibodies and recombinant proteins. The regenerative medicine industry aims to deliver cells themselves as the product. Undesired cell loss during cell culture due to hydrodynamic shear stress is detrimental for obtaining high yield of therapeutic product. For this reason, understanding impact of shear stress on cells becomes necessary.

Animal cells are delicate. They are susceptible to shear forces because they lack a protective cell wall and different cells have different capacities to withstand shear stress. Much research has been established to analyse the sensitivity of mammalian cells to shear stress. It is widely believed that shear force is harmful for suspension-adapted mammalian cells when it exceeds a critical level. Shear stress will lead to the deformation of the cell membrane, physical and metabolic alteration and potentially cell death (Born *et al*., 1992; Zoro *et al.*, 2008; Acosta Martinez *et al.*, 2010). Compared to laminar shear stress, turbulent shear stress results in more damage to cells in stirred tanks (Brindley *et al.*, 2011). Mardikar and Niranjan demonstrated that for several animal cell lines, cell loss at low (1 Nm^{-2}) and high (100 Nm^{-2}) shear stress is greater than that at intermediate shear stress (10 Nm^{-2}) (Mardikar and Niranjan, 2000). At high levels of shear stress, deformation of the cell membrane and cell lysis were caused by applied shear force exceeding cell bursting force. On the contrary, at low levels of shear stress, damage of cells occured when turgor pressure pushed the cytoplasm out of cell and resulted in a papillate state of the cell (Brindley *et al.*, 2011). Also, low level shear stress affects enzyme release and cell metabolism.

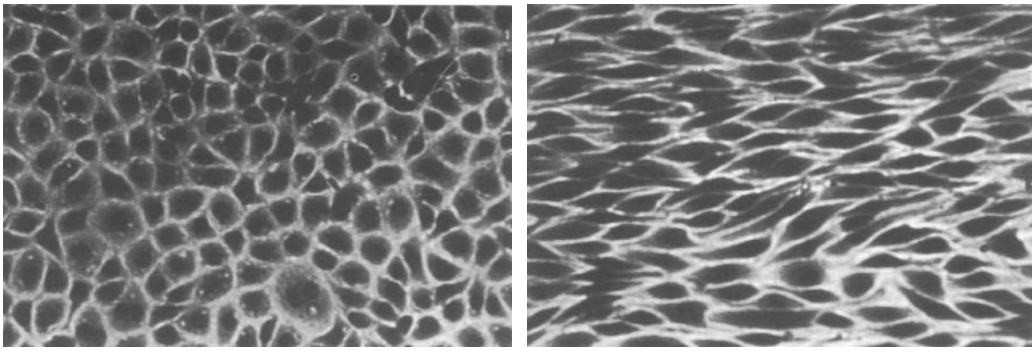
Ludwig and Kretzmer investigated the effect of shear force on cells through exposing recombinant baby hamster kidney (BHK) cells to shear stress (range: 0.1-1.55 Nm^{-2}) for 3 days. Results showed that number of viable anchorage dependent recombinant BHK cells between shear stress levels of 0.3 and 0.7 NM^{-2} was almost 25% higher than those at other lower and higher levels. However, continually increasing the level of shear stress led to dramatic decline in cell number. They found that more cell leakage resulted from long duration exposure to shear stress by evaluating the activity of the intracellular enzymes, such as β-D-galactosidase (β-Gal) and lactate dehydrogenase (LDH) (Ludwig and Kretzmer, 1992).

Another study conducted by Shiragami *et al*. indicated that activity of LDH in

Chinese hamster ovary (CHO-K1) cells increased about 4 fold when exposed to a shear stress of 0.5 Pa for 12 hours compared to cells without the influence of shear stress. However, activity of LDH dropped a lot when exposure time increased to 16 hours. This differential response is related to cell damage versus repair processes. Damage of cells caused by shear stress induces the activation of intracellular enzymes for repairing cells, thus, LDH activity increases. However, enzyme activity decreases when exposed to shear stress for a long period (Shiragami and Unno, 1994).

Zoro and colleagues set a capillary system to investigate the effect of shear stress on suspended anchorage-dependent mammalian cells (Zoro *et al.*, 2008). When rat aortic smooth muscle cells were exposed to capillary shear stress (2-120 Nm^{-2}), both total cell number and number of intact cells decreased. The optimum shear stress for cell survival was found at 10-50 Nm^{-2}, as loss of cells in this region was less (Zoro *et al.*, 2008).

Others found that bovine aortic endothelial cells (BAEC) exposed to shear stress displayed alteration of their morphology and function (Nerem, 1991). A confluent monolayer of BAEC grown on Thermanox plastic surface presented a cobblestone-like appearance under static culture conditions. However, cells elongated and oriented their major axis to align with the direction of flow after being exposed to shear stress of 30 dynes/cm^2 over 24 hours. The shear stress level and the exposure time affected the degree of elongation (Figure 1.6). Ensley *et al.* also obtained a similar conclusion by exposing endothelial cells to laminar shear stress of 15 dynes/cm^2 for 24 hours (Figure 1.7) (Ensley *et al.* 2012).



(a) (b)

Figure 1.6: Bovine aortic endothelial cells under shear stress (30 dynes/cm^2) for 24 hours (b) compared to static control conditions (a), flow was from left to right (Nerem, 1991).

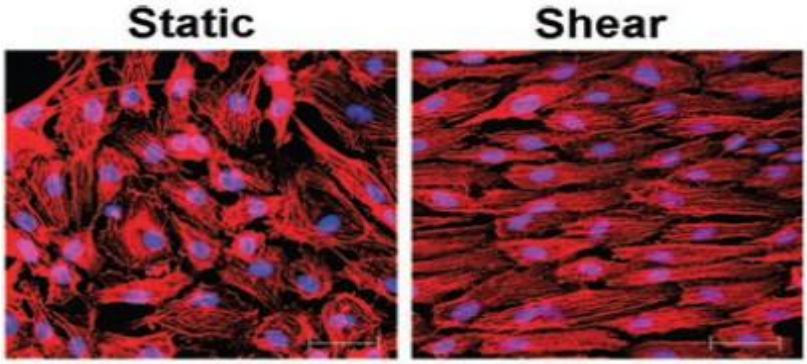


Figure 1.7: Rhodamine phalloidin staining of F-actin in endothelial cells (EC) expose to laminar shear stress (15 dynes/cm^2) for 24 hours, compared to static controls, flow was from left to right (Ensley *et al.* 2012).

In addition to negative effects such as cell deformation, loss of cell number and viability, shear stress can also exert positive influence on some cells. For instance, it can induce differentiation of stem cells where mechanotransduction is a cue. Yamamoto *et al.* exposed mouse Flk1$^+$ ESCs to shear stress in the range of 1.5-10 dynes/cm^2. Results showed that expression of endothelial cell specific markers, such as Flk-1, Flt-1, VE-cadherin and PECAM-1, were increased by exposure to shear stress. Moreover, Flk1$^+$ cells revealed increased ability to produce tube-like structures after exposure to shear stress (1.5 dyne/cm^2) for 24 hours. In contrast, shear stress had no impact on

expression of mural cell markers, epithelial cell markers or blood cell markers. In other words, shear stress only induced $Flk1^+$ ES cells to differentiate into the vascular endothelial cell lineage. Further studies based on this research showed that expression of the arterial endothelial marker EphrinB2 was induced when $Flk1^+$ cells were exposed to shear stress of 20 dynes/cm^2 (Yamamoto *et al.*, 2005; Adamo and Garcia Cardena, 2011).

Bai and colleagues isolated stromal cells from rat bone marrow, exposed them to shear stress in the range of 10-25 dynes/cm^2 for up to 48 hours, and tested whether shear stress can induce endothelial differentiation under conditions with or without vascular endothelial growth factor (VEGF). According to their results, greater expression of endothelial genes (e.g. Flk1, tPA) was induced by lower level shear stress (10 and 15 dynes/cm^2) at the 24 hour time point, and this effect was more prominent with addition of VEGF. However, expression levels of endothelial genes declined back to static levels after exposure to shear stress for 48 hours unless VEGF was added (Bai *et al.*, 2010).

In order to investigate whether shear stress can induce bone marrow mesenchymal stem cells to differentiate into cardiac valve-like tissue, Engelmayr *et al*. seeded BMSCs on polymeric scaffolds and introduced shear stress (1 dynes/cm^2) with and without cyclic stretch for 3 weeks. Shear stress alone enhanced cell proliferation, and when combined with cyclic stretch, expression of endothelial related markers (e.g. CD31, laminin) was promoted (Adamo and Garcia-Cardena, 2011).

1.3.3. Impact of shear stress on bone formation

Appropriate mechanical loading maintains homeostasis of bone in healthy individuals as the hard mineralized matrix protects cells against the effect of mechanical loads. Bone will remodel and become stronger with moderate

increased loading on bone. In 1892, the relationship between bone formation and mechanical loading was first presented (Wollf, 1892). Proposed mechanical strain and fluid flow-induced mechanisms explain how signals of mechanical loading transmit to bone cells at the micron scale. One hypothesis of fluid shear stress playing a role in bone remodeling caused by mechanical loading and injury was proposed by Hillsley and Frangos in 1990.

Two main fluidic stimuli in the human body are those induced by vascular or lymphatic flow and those induced by interstitial fluid flow (IFF) (Park *et al.*, 2010). Both of them contribute to bone maintenance and healing. Any change in them would change the remodeling and formation of bone. Interstitial fluid is a major component of the extracellular fluid which is found in tissue spaces that surround cells. Several factors cause the appearance of IFF in bones: (1) an outward radial flow results from the hydrostatic pressure drop across the cortex; (2) muscle contractions during exercise create pressure fluctuations in the marrow cavity which induce changes of IFF; (3) mechanical loading (e.g. during walking or running) induces stress on bone (Hillsley and Frangos, 1994; Gurkan and Akkus, 2008).

The primary stress generated by IFF is fluid shear stress (FSS). One *in vitro* experiment demonstrated that FSS is a critical activator of bone cells in response to mechanical loading through exposing bone cells to fluid with increasing viscosity. FSS acts as a mechanical stimulus to cells, then these stimuli initiate intracellular signal transduction that causes the biological effects on cells. They can affect the proliferation of osteocytes and the differentiation of MSCs and osteoblasts (Liu *et al.*, 2010).

The level of FSS induced by IFF in lacuna-canalicular spaces within bone tissue is about 8–30 dyne/cm^2 and it may change due to physical activities (Weinbaum *et al.*, 1994). One study investigated the dynamic interaction between mechanical forces, bone cells, and the continuously changing 3D

matrix architecture of bone, by culturing rat marrow stromal osteoblasts in 3D perfusion conditions with different flow rates to induce matrix generation and mineralization (Bancroft *et al.*, 2002). Results showed that more mineralized matrix was formed during flow perfusion culture for 16 days with all flow rates (0.3 ml/min, 1 ml/min and 3 ml/min) than under static culture conditions. Higher flow rate generated more mineralization and calcium content increased about 6 fold when flow rate was increased from 0.3 ml/min to 1 ml/min. Twice as much calcium was deposited when flow rate was further increased to 3 ml/min. However, this increasing trend in matrix mineralization throughout the range of flow rates was not linear which illustrated that there is a limitation of matrix production in this perfusion culture system (Bancroft *et al*., 2002).

Numerous studies have illustrated that appropriate FSS stimulation can enhance deposition of mineralized matrix and promote expression of genes that are related to osteogenesis by rat MSCs. Datta *et al.* pointed out that FSS had a positive effect on osteogenic differentiation of MSCs (Datta *et al.*, 2006). Results indicated that matrix mineralization of MSCs increased significantly when they were cultured under flow perfusion conditions in Ti/ECM constructs, where cells experienced larger shear stress. Furthermore, flow perfusion culture without dexamethasone resulted in more mineralization when compared to static culture with dexamethasone (Datta *et al.*, 2006).

In another study, human BMSC were cultured in a two dimensional parallel chamber on different substrates and cells were exposed to a uniform shear stress (1.2×10^{-3} N/m^2) for 10 days. Immunohistochemical staining for type I collagen revealed only weak stain under static culture conditions. However, stain was very intense after cells were exposed to FSS (Figure 1.8) (Scaglione *et al.*, 2008).

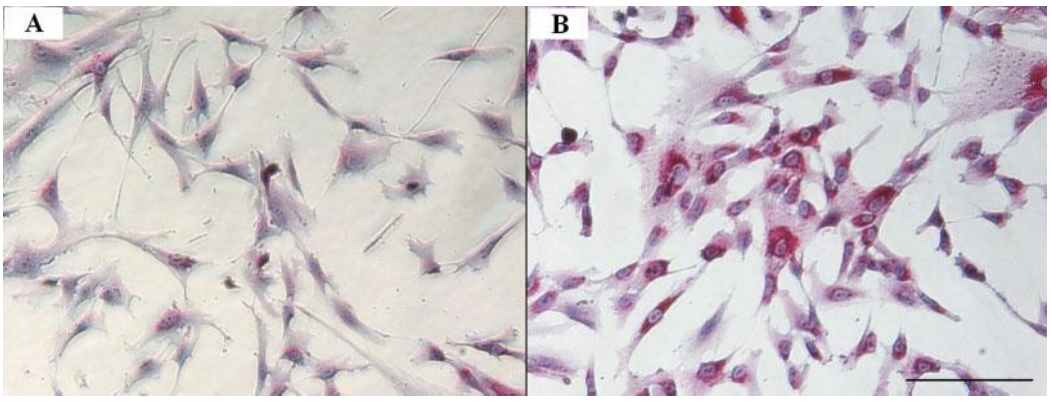


Figure 1.8: Immunohistochemical staining for type I collagen in human BMSCs cultured statically (A); exposed to fluid flow with flow rate of 3 ml/min (B). (Scale bar=100 μm) (Scaglione *et al.*, 2008).

Kreke and colleagues cultured rat derived BMSCs in a parallel-plate flow chamber with addition of osteogenic supplement, and then exposed the cell layers to repeated shear stress of 1.6 dynes/cm^2 for 5, 30 and 120 minutes on days 6, 8, 10 and 12. Results showed that expression of osteoblastic genes bone sialoprotein (BSP) and osteopontin (OPN) were increased at all shear conditions. The increase in BSP was significant when cells were exposed to shear for 30 and 120 min (Kreke *et al.*, 2005). Also, significant variation in osteogenic signaling pathways was observed when rat BMSCs were exposed to shear stress of 2.3 dynes/cm^2 in planar culture conditions. They also showed that upregulation of OPN, BSP, collagen 1α1 and osteocalcin (OCN) was observed after exposing BMSCs to shear stress for 24 hours followed by static culture for 13 days. In other words, an early transient stimulation on osteoblastic cells has influence on the later stages of osteogenic differentiation (Kreke *et al.*, 2008).

A parallel flow chamber that was developed by Grellier and his coworker was used to apply shear stress of 12 dynes/cm^2 for 30 and 90 minutes to research the impact of laminar fluid flow on osteoblastic differentiation of human BMSCs. Results revealed two fold increases in expression of the alkaline phosphatase

(ALP) gene after exposure to shear stress for 30 minutes and about 50% decrease of it after 90 minutes (Grellier *et al.*, 2009).

Oscillatory fluid flow is a significant regulator for the proliferation and differentiation of MSCs. Under the effect of OFF, intracellular Ca^{2+} mobilization increased. Also, proliferation of MSCs increased significantly which led to increased numbers of osteoprogenitors. Moreover, although the activity of ALP was low, gene expression of osteopontin (65% increase) and osteocalcin (44% increase) was up-regulated by OFF (Li *et al.*, 2004), suggesting a more mature osteoblast phenotype.

Patel *et al*. passed muscle derived precursor cells through a capillary for different durations (10, 20 and 30 passes), then cultured them with standard culture medium or osteogenic medium for 2 weeks. Results showed that osteogenic differentiation was promoted by shear stress for both early adherent pre-plate 1 (PP1) cells and late-adherent PP3 MDPCs. PP1 cells were capable of undergoing more osteogenic differentiation than PP3 cells. This is to be expected as PP1 cells attach to tissue culture plastic rapidly, as osteogenic cells do, yet the PP3 cells attached more slowly, characteristics of muscle-like cells. Comparing MDPCs that underwent 30 capillary passes with un-stimulated cells, those exposed to capillary transfer generated an additive effect to the osteogenic medium by inducing a significant increase in osteogenic differentiation (Patel *et al.*, 2011; Mulhall *et al*. 2011). It was correlated with cells being exposed to wall shear stress during capillary transfer.

1.3.4. Theory of mechanotransduction

Mechanotransduction of FSS consists of three processes. First, FSS activates cell surface receptors, and then an intracellular signal is transduced to the nucleus, where downstream transcription factors regulate the expression of

osteogenesis-related genes (Duncan and Turner, 1995). Mechanoreceptors on the cell surface assist hMSCs to recognize the physical stimulation of FSS. Ion channels and integrins play a vital role in the detection process. Ion channels will open and result in the influx of cations (e.g. Ca^{2+}, Na^{+} and K^{+}) into the cell when the cell membrane is deformed and proteins on the membrane are altered by the effect of FSS. Ca^{2+} channels are critical for responding to FSS. Intracellular Ca^{2+} concentration increased significantly when hMSCs were exposed to a shear stress of 20 dynes/cm^2 (Riddle *et al.*, 2006). In addition, Stiehler found more than tenfold increase of Ca^{2+} content in cells when hMSCs were stimulated by FSS under dynamic 3D culture conditions (Stiehler *et al*., 2009). Integrins are transmembrane receptors responsible for mediating cell adhesion, linking cells to extracellular matrix (ECM) by connecting ECM and cytoskeleton via focal adhesions. Due to the connection between integrins and ECM, they can detect extracellular mechanical stimulation and activate intracellular signaling pathways. One study in osteoblast-like MG63 cells indicated that β_1 integrins are critical for conduction of shear induced signalling and expression of genes that related to bone formation, e.g. type I collagen and fibronectin (Lee *et al.*, 2008).

Focal adhesions are assembled by integral membrane proteins and function as mechanoreceptors. They are critical for the production of cyclooxygenase-2 (COX-2) and prostaglandin E_2 (PGE_2) which are essential to mechanical induction of bone formation (Ponik and Pavalko, 2004; Young *et al.*, 2009). Focal adhesions kinase (FAK) plays a significant role in the mechanotransduction of fluid shear stress in hMSCs and regulating ECM-induced osteogenic differentiation of hMSCs (Salasznyk *et al.*, 2007).

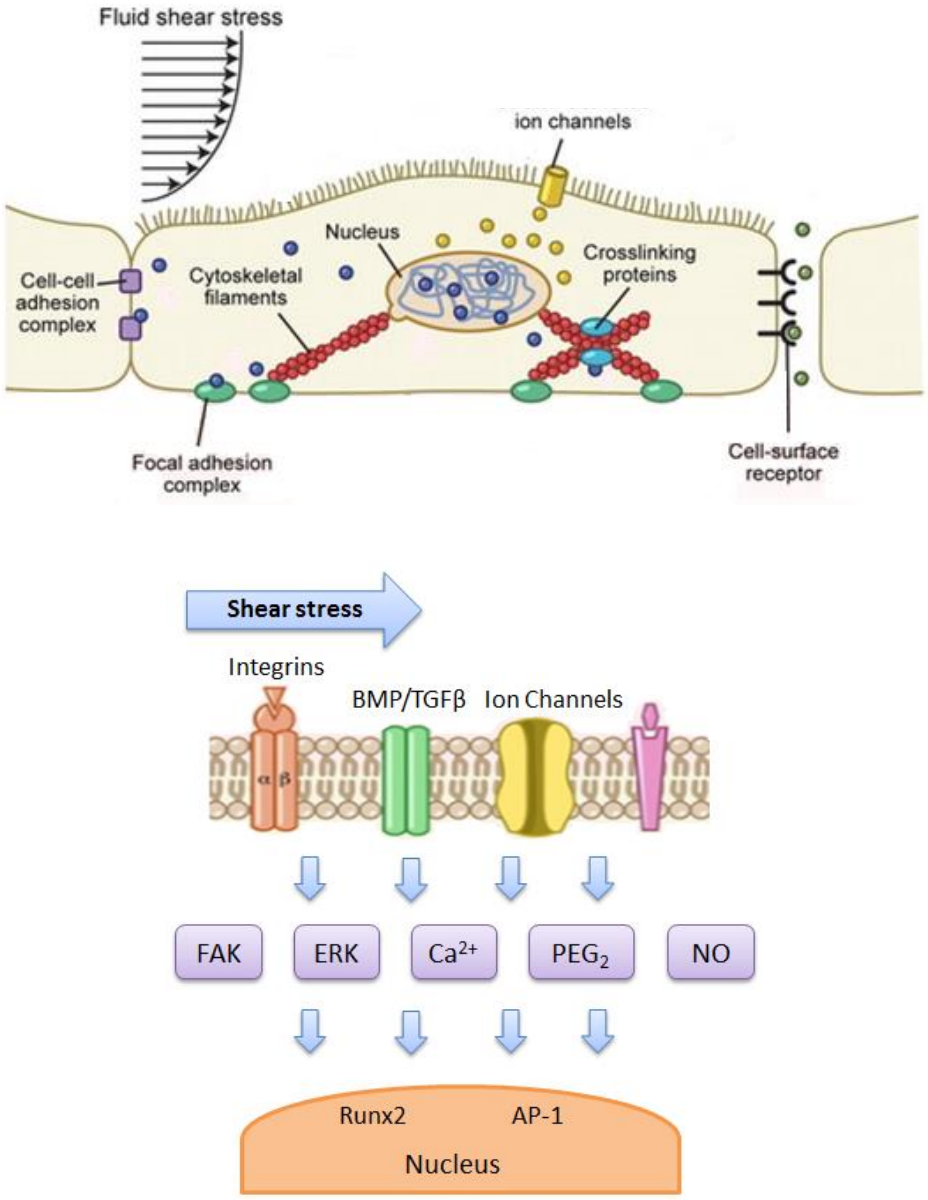


Figure 1.9: MSCs, osteoblasts and osteocytes response to mechanostimulation (fluid shear stress). Ion channels open and result in influx of cations into cells (e.g. Ca^{2+}); Focal adhesions detect mechanical stimulation and activate several signaling pathways (e.g. FAK); activation of transcription factors (e.g. Runx2) and promotion of transcription of osteoblasts related gene occur inside the nucleus (Nicolas and Serge, 2010).

Several different signaling pathways are responsible for FSS-induced osteogenic differentiation of hMSCs. These include nitric oxide/ protein kinase G (NO/PKG), prostaglandin E_2 (PGE_2)/PKA, Ca^{2+}/PKC and mitogen-activated

protein kinase (MAPK) signaling pathways (Iqbal and Zaidi, 2005; Haasper *et al.*, 2008; Stiehler *et al.*, 2009). The NO/PKG signaling pathway plays a critical role in osteoblastic differentiation. McAllister *et al*. found that FSS can increase the level of NO in MSCs (McAllister *et al.*, 2000). NO can rapidly diffuse through membranes due to its lipophilicity where it activates the cGMP dependent protein kinase through promoting synthesis of cGMP, then PKG can increase the activity of ERK by phosphorylating receptor serine/ threonine kinases. Finally, activity of runt related transcription factor 2 (Runx2) and activator protein 1 (AP-1) are increased which results in transcription of osteogenic genes (Rangaswami *et al.*, 2009). Ca^{2+} concentration is a critical intracellular signal that regulates cells proliferation and differentiation. Ca^{2+}/calmodulin dependent protein kinase adjust responses of cells to Ca^{2+} signals. For instance, calmodulin kinase II (CaMKII) will be activated when Ca^{2+} concentration increases, then ERK1/2 signaling is activated due to phosphorlation of ERK1/2 by CaMK II. ERK1/2 pathway then plays a significant role in osteogenesis (Shin *et al.*, 2008). MAPK pathways also play an important role in FSS induced mechanotransduction. Expression of genes in the MAPK signaling pathway, e.g. MAP kinase kinase kinase 8 (MAP3K8) and interleukin-1 beta (IL1B), can be increased by effect of fluid shear stress (Glossop and Cartmell, 2009).

Runx2 and AP-1 are two important transcription factors whose activation promotes osteogenic differentiation, they can trigger expressions of specific genes related to osteogenesis and then result in increased mineralization activity of cells. Runx2 is a crucial factor not only in differentiation of osteoblasts that control postnatal bone formation, but also in osteogenic differentiation of hMSCs. The DNA binding ability and expression of Runx2 can be increased through ERK1/2-MAKP pathway under the effect of FSS (Kim *et al.*, 2007). Runx2 modulates the expression of osteoblast related genes, such as ALP, osteocalcin and type I collagen. AP-1 proteins are dimers that consist

of Fos and Jun proteins that can be activated by FSS, they can regulate expression of osteogenic differentiation related genes through binding to the consensus sequence in their promoters (Haasper *et al.*, 2007; Rangaswami *et al.*, 2009).

1.4. Aims

It is clear from the literature that shear stress can in some contexts be harmful to cells, yet in others promote positive responses such as cell proliferation and differentiation. Therefore, the overall aim of my thesis was to determine whether fluid shear stress can be used as a cost-effective bioprocess tool to promote osteogenic differentiation of MSCs into osteoblasts.

The individual objectives were to:

1. Determine the response of mature osteoblastic cells to fluid shear stress and the critical level of shear stress that will cause cell death.
2. Determine whether immature MSCs are more sensitive to fluid shear stress and whether the critical level of shear stress will be less for these cells.
3. Determine whether there is a fluid shear stress regime that can enhance cell differentiation responses.

Chapter 2 Materials and methods

2.1. General cell culture and maintenance

2.1.1. Cell culture

Rat bone marrow derived MSCs (rat-BMSCS), human MG63 cells and human bone marrow derived MSCs (hBMSCS) were used in this project. All of them were cultured in standard growth media which composed of Dulbecco's Modified Eagle Medium (DMEM; Gibco, UK), 10% Fetal Bovine Serum (FBS; Gibco, UK) and 1% Antibiotic-Antimycotic (100×; Gibco, UK), and incubated at constant conditions of 37°C and 5% CO_2. Medium was changed every three days for providing sufficient nutrient for cell growth. The subculture was performed when cell monolayer reached 80% confluence. Spent medium was removed, cells were washed once with phosphate buffered saline (PBS; Gibco, UK) and trypsinized by incubating with an appropriate amount of 0.25% Trypsin/EDTA (Gibco, UK) for 3 min at 37°C/5% CO_2, and then the cell suspension was centrifuged at 1221 rpm for 3 minutes. The cells were resuspended in fresh culture medium and reseeded in new flasks at a density of $1x10^4$ cells/cm^2. Rat-BMSCs and hBMSCs were cultivated until passage 6 maximum, yet most of the experiments were carried out using passage 3-6 cells. Human MG63 cells were kindly provided by Dr. Vehid Salih (Eastman Dental Institute, UCL), they were cultured to maximum 19th passage.

Cell type	Advantages	Disadvantages
rat-BMSCs	1) easily available 2) possible to control the selection of rats	1) interspecies differences 2) genomic difference 3) limited expansion
human MG63 cells	1) easily available and unlimited number 2) no interspecies difference	1) limited osteogenic differentiation possibility
hBMSCs	1) no interspecies difference 2) relevant for clinical trial	1) long isolation procedure 2) limited expansion

Table 2.1: Advantages and disadvantages of rat-BMSCs, human MG63 cells and hBMSCs.

2.1.2. Cryopreservation

For cryopreservation, after cell trypsinization and counting, cells were centrifuged for 3 min at 1221 rpm, the supernatant was aspirated completely and the cell pellet was resuspended in a freezing medium which was composed of 90% FBS and 10% dimethylsulfoxide (DMSO), after this, the cell suspension was aliquoted in cryovials (1ml; Thermo Scientific, UK) which were then placed in a freezing container (Mr. Frosty^{TM}; Thermo Scientific, UK) and stored at -80°C freezer for 24 hrs. Subsequently, they were transferred to liquid nitrogen (-196°C) for long term storage. The upper limit of freezing cell number in each vial is about 1x10^6 cells in 1 ml freezing medium.

For retrieval, cryopreserved cell samples were placed in a dewar bottle that contained a small amount of liquid nitrogen, and then thawed in the water bath for 1 or 2 min at 37°C. Suspensions were immediately mixed with 5 ml standard growth medium and pelleted by centrifuging for 3 min at 1221 rpm. The supernatant was removed and the cell pellet was resuspended in the fresh medium and cultured in new flasks.

2.2. Shear system

2.2.1. Shear device

The shear system used in this project consists of a syringe pump, a capillary and syringes. A Harvard PHD 2000 infuse/withdraw syringe pump (Harvard Apparatus Ltd, UK) was used to set a range of flow rates (Figure 2.1A). Cells were loaded into a 20 ml Luer-Lock syringe barrel (BD, UK) connected to another 20 ml syringe by a small diameter stainless steel capillary (Cooper's Needleworks, Birmingham, UK; Figure 2.1B). The syringe with cells inside was clamped to the syringe pump, cells were passed through the capillary from one syringe to the other (Figure 2.1C).

Three major parameters, which determine the effect of shear stress, involved in this research are: (1) Internal diameter (ID) of capillary: capillary wall shear stress has little effect on cells when ID is too large, due to the fact that most cells might pass through the capillary without making contact with the wall, whereas wall shear stress has more effect on cells when pass them through a capillary with small ID, because the probability of cells randomly hit the capillary wall during transport increase. Three capillaries with different ID were tested, which are 25 gauge, 26 gauge and 27 gauge capillaries (Table 2.2).

(2) Length of capillary: 10 mm and 40 mm capillaries were utilized to detect whether the cell damage is caused by capillary wall shear stress or by shear stress and extensional forces from squared end capillary entrance. And longer capillary results in more exposure time to capillary shear stress when ID and flow rate were set to the same value.

(3) Flow rate: in addition to capillary diameter, flow rate is another critical factor that will affect the *Re* and determine type of fluid in tube. Based on the assumption that cell suspension viscosity is similar to that of water at 25℃

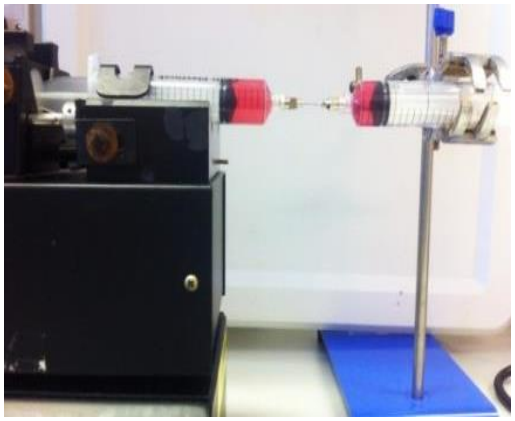
(8.9×10^{-4} Pa·s), the known diameter of capillary, the range of flow rate was set between 13 ml/min and 28 ml/min.

Gauge	ID (mm)	Wall (mm)
27	0.203	0.102
26	0.254	0.102
25	0.254	0.128

Table 2.2: Standard size stainless steel tubing dimension.

A

C



B

Figure 2.1: A. Harvard PHD 2000 infuse/withdraw syringe pump; B. Stainless steel capillary (with square end, Luer lock on both sides); C. Shear system.

2.2.2. Shear experiments

Rat BMSCs suspensions (2.5 ml of each group) of specific cell concentration (1.2×10^5 cells/ml) were loaded into 20 ml Luer-Lock syringe and passed through 10 mm capillary of known diameter (25G, 26G and 27G) from one syringe to the other, at a constant flow rate of 13ml/min for 10 passes, without rest or hold periods between passes.

Rat BMSCs suspensions (2.5 ml of each group) of specific cell concentration (1.2×10^5 cells/ml) were loaded into 20 ml Luer-Lock syringe and passed through 10 mm 25G capillary from one syringe to the other, at different flow rates (i.e. 13 ml/min, 16 ml/min, 19 ml/min and 22 ml/min) for 10 passes, without any passes or hold periods between passes.

Both human MG63 cell and human MSCs suspensions (4 ml of each group) of specific cell concentration (3×10^5 cells/ml) were loaded into 20 ml Luer-Lock syringe and passed through 10 mm 26G capillary from one syringe to the other, at different flow rates (i.e. 13 ml/min, 20 ml/min, 28 ml/min) for 10 passes, without any passes or hold periods between passes.

Both human MG63 cell and human MSCs suspensions (4 ml of each group) of specific cell concentration (3×10^5 cells/ml) were loaded into 20 ml Luer-Lock syringe and passed through 10 mm 26G capillary from one syringe to the other at flow rate of 20 ml/min for different numbers of pass (10, 20, 30 and 40 passes), without any passes or hold periods between passes.

All experiments were carried out at room temperature. A non-sheared sample kept at the same temperature for the duration of the experiment was used as control.

2.2.3. Reynolds number and shear stress

Two important parameters were considered: Reynolds number (*Re*) which was

used to determine the flow pattern; and the capillary wall shear stress (τ) that cells experienced in the capillary. Formulas for calculation of *Re* and τ (Mulhall *et al.*, 2011; Patel *et al*., 2011; Zoro *et al.*, 2008) in the pipe are:

$$Re = \frac{\rho v d}{\mu} \qquad \tau = \frac{32Q\mu}{\pi d^3}$$

Where, ρ is density (kg/m^3), v is velocity of flow (m/s), d is diameter of capillary (m), μ is viscosity of cell suspension (Pa·s) and Q is fluid flow rate (ml/min), Some assumptions were made for calculation: (1) Internal surface of capillary was assumed to be smooth; (2) ρ was same as water's density (1000 kg/m^3); (3) μ was same as viscosity of water at 25°C (0.89 mPa·s) (Mulhall *et al.*, 2011; Patel *et al*., 2011; Zoro *et al.*, 2008). The value of *Re* and τ are described in Table 2.3.

Capillary dimension	Capillary ID (mm)	Flow rate (ml/min)	*Re*	*Capillary Wall shear stress* τ (Pa)
25/26G	0.254	13	1220	120
		20	1877	184
		28	2628	258
27G	0.203	13	1527	235
		20	2349	361
		28	3289	506

Table 2.3: Reynolds number and shear stress of different capillaries (25, 26 and 27G) at different flow rates (13, 20 and 28 ml/min).

2.3. Cell recovery

The trypan blue exclusion method was used to assess cell recovery immediately after exposing cells to capillary wall shear stress. Live cells exclude trypan blue due to intact cell membranes. In contrast, membranes of nonviable cells are permeable, they will uptake of trypan blue dye and the

cytoplasm will become blue. 100 µl cell suspension was taken out and mixed with 100 µl trypan blue solution, and then 10 µl mixed sample was drawn up to fill Neubauer cell counting chamber and counted under the microscope. The cell concentration was calculated by the formula (where 2 is the dilution factor):

$$\text{cells/ml} = \frac{\text{total cells in 4 corner sections}}{4} \times 2 \times 10^4$$

The total cell number was calculated by the formula:

$$\text{total cells} = \text{cells/ml} \times \text{the original volume of the cell suspension}$$

The viability was calculated by the formula:

$$\text{viability } \% = \frac{\text{live cell number}}{\text{total cell number}} \times 100\%$$

2.4. Cell proliferation

Proliferation capacities of both non-sheared and sheared cells were determined using the cell counting kit-8 (CCK-8; NBS Biologicals, UK) assay after culture of 24 and 72 hrs. CCK-8 is a convenient and sensitive colorimetric assay. Tetrazolium salt WST-8 is reduced by dehydrogenases in viable cells to generate a yellow colored medium soluble formazan which has an absorbance at 450nm. The amount of the formazan is directly proportional to the number of living cells (NBS Biologicals technical manual).

The cell proliferation assay includes several steps. First, cells were placed in 96-well plates with seeding density of 2 or 5×10^3 cells per well in 100 µl growth medium. Non-sheared cells were used as a control. Two plates (24 and 72 hrs) were set up in a humidified incubator at 37°C in 5% CO_2. After 24 or 72 hrs, CCK-8 working solution was prepared by mixing standard growth medium with CCK-8 at ratio of 10:1. At this stage, old medium was removed and plates were washed once with Dulbecco's phosphate buffered saline (DPBS), then the

CCK-8 working solution was added (110 µl/well) according to manufacturer's instruction. After three hours incubation at 37°C in 5% CO_2, cell viability was determined colorimetrically by reading absorbance at 450 nm using a Tecan Safire^2 microplate reader (Tecan, Switzerland).

2.5. Quantitative reverse transcription PCR

2.5.1. RNA and complementary DNA (cDNA) preparation

After culture non-sheared and sheared cells in 6 well plates for 72 hrs, total RNA was extracted using Qiagen RNeasy Micro kit (Qiagen). Briefly, cell pellets were disrupted in RLT buffer and filtrated through QIAshredder spin column, cell lysates were mixed with 70% ethanol (ratio 1:1) and transferred to RNeasy MinElute spin column. 10 µl DNase was added and worked at room temperature for 15 min to digest contaminants from genomic DNA. Columns were then washed three times (RW1 buffer, RPE buffer and 80% ethanol) and dried by full speed centrifugation. Subsequently, total RNA was eluted in 14 µl RNase-free water. RNA concentration and purity were evaluated by analyzing spectrophotometrically using NanoDrop^{TM}1000 (Thermo Scientific, UK). Absorbance 260/280 and 260/230 ratios were used to assess the purity of RNA. Generally, A260/A280 value of 2.0 and A260/230 value in the range of 2.0-2.2 is accepted for pure RNA (Thermo Scientific, UK).

Around 1 µg RNA from each sample was reverse transcribed to cDNA using QuantiTect Reverse Transcription Kit (Qiagen). First, genomic DNA was eliminated from RNA by adding gDNA wipeout buffer and RNase-free water and incubating for 2 min at 42°C, and then the RNA was added to a mixture of 1 µl quantiscript reverse transcriptase, 4 µl quantiscript RT buffer and 1 µl RT primer mix, this mixture was incubated for 15 min at 42°C followed by 3 min at 95°C. The newly synthesized cDNA was proceeded directly with real-time PCR, otherwise, it was stored at -20°C for long term storage.

2.5.2. Quantitative PCR

The mRNA expression of target genes was quantified by real-time polymerase chain reaction (PCR) using the QuantiTect SYBR Green PCR Kit (Qiagen). A mixture of cDNA, primer, SYBR green and RNase-free water was loaded in 96-well PCR plate. The plate was sealed and placed on CFX connect^{TM} real-time PCR detection machine (BioRad, USA). The quantitative RT-PCR program ran 55 cycles of amplification as each cycle consisted of denaturation (94°C for 15 sec), annealing (55°C for 30 sec), and extension (72°C for 30 sec) steps. The amplified products were analyzed using BioRad CFX manager software (BioRad, USA). The gene expression of ALP, Runx2 and COL1A1 was quantified with GAPDH as internal reference.

2.6. Osteogenic differentiation

Non-sheared and capillary sheared cells were seeded into 24-well plates at a density of 2×10^4 cells/well in standard growth medium and incubated for 24 hrs at 37°C in 5% CO_2. The medium was then replaced with osteogenic differentiation medium consisting of standard growth medium, 10 nmol dexamethasone (DEX), 10 mmol β-glycerophosphate (bGP) and 100 µmol ascorbic acid (AA) (Sigma-Aldrich, UK), cells were cultured for 21 days and the medium was changed twice a week. Non-sheared cells cultured in standard growth medium were used as negative control, and those grown in osteogenic differentiation medium used as positive control.

Supplements	Solute	Solvent	Dilution
Dexamethasone	0.003 g	1.5 ml DMSO	2×
β-glycerophosphate	4.3205 g	20 ml DW	100×
Ascobic acid	0.05 g	1 ml DW	1000×

Table 2.4: Differentiation supplements stock solution (store at -20 ℃).

2.7. Alizarin Red S quantification of mineralization

Undifferentiated cells do not produce extracellular calcium deposits. On the contrary, differentiated osteoblasts generate extracellular calcium deposits. Therefore, calcium deposition is a critical indication of osteogenic differentiation. Alizarin Red S was used to detect matrix mineralization because it can react with calcium to form an alizarin red s-calcium complex which is visible as an orange-red color.

After 3 weeks cultivation, osteogenic cultures were processed for Alizarin Red S staining. Medium was removed from the 24-well plate, cells were rinsed once with DPBS and then fixed with 4% paraformaldehyde for 10 minutes at room temperature. After fixation, cells were rinsed twice with distilled water and stained with 2% w/v Alizarin Red S solution (pH 4.3, 0.5% ammonium hydroxide was used to adjust pH; Sigma-Aldrich, UK) at room temperature for 30 minutes. Lastly, cells were washed several times with distilled water to remove the excess non-bound stain. Images were captured using a Nikon Eclipse TE2000-U microscope (Nikon, Japan). Quantitation of mineralization was performed using Millipore osteogenic differentiation Kit (Milipore, UK), the Alizarin Red S stain was extracted and absorbance was measured spectrophotometrically at 405 nm.

Solution	Solute	Solvent
4% Paraformaldehyde	2 g	50 ml PBS
2% Alizarin Red S	0.5 g	25 ml DW
0.5% Ammonium hydroxide	2 ml	8 ml PBS

Table 2.5: Staining Solution preparation.

2.8. Statistical analysis

All experiments were carried out in triplicate. Data are presented as mean ± standard deviation. Differences were statistically analyzed by one-way ANOVA with post-hoc t-test. Statistical significance was assumed if $p < 0.05$.

Chapter 3 Exposure of rat bone marrow derived mesenchymal stem cell to fluid shear stress

3.1. Introduction

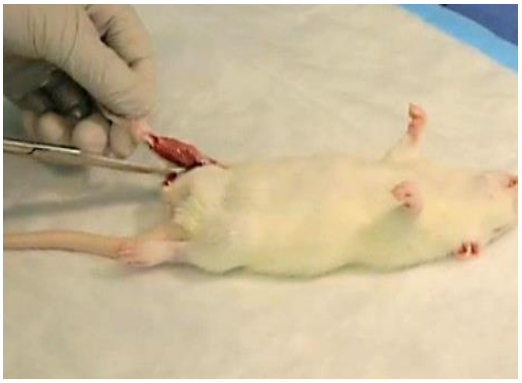
Initial experiments were carried out using rat bone marrow derived mesenchymal stem cells. MSCs were isolated from young male rats to ensure cell quality, they grow in adherent conditions, maintain multipotency and possess high capacity to give rise to cells of connective tissue lineages. Capillary diameter and flow rate were two main parameters that were researched to assess the effect of capillary wall shear stress on cells in this chapter. Other variables, such as capillary length and number of passes, were maintained the same for each experiment. The detailed description of different conditions that performed during experiments is in the sections below.

3.2. Rat dissection and cell isolation

MSCs were obtained from 8-week old male Sprague-Dawley rats. Briefly, rats were sacrificed by asphyxiation with CO_2 and cervical dislocation, ligaments and muscles of hind limbs were detached to expose the femurs and tibias. The head of the femur was separated from the acetabulum (Figure 3.1A), after sterilized using 70% ethanol, a 21 gauge needle was inserted into the shaft of the bone to flush out bone marrow using standard growth medium (Figure 3.1B) (Karaoz *et al.*,2009), which consisted of low glucose Dulbecco's Modified Eagle Medium (DMEM; Gibco, UK), supplemented with 10% Fetal Bovine Serum (FBS; Gibco, UK), 1% Antibiotic-Antimycotic (100×; Gibco, UK) and basic fibroblast growth factor (bFGF). Marrow suspension was then dispersed and centrifuged at 1221 rpm for 3 min, supernatant was discarded and the cell pellet was resuspended and seeded in 25 cm^2 or 75 cm^2 flasks, and then

incubated at 37°C in 5% CO_2 (Figure 3.1C).

A



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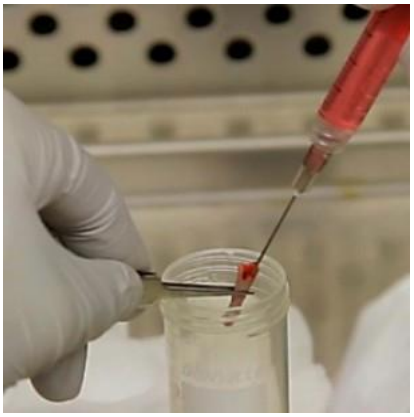


C

Figure 3.1: Rat bone marrow derived mesenchymal stem cells isolation processes. (A) Isolate femurs and tibias from rats. (B) Flush out of bone marrow. (C) Primary culture in T25 and T75 flask.

3.2. Primary culture and subculture

The cells isolated from rat bone marrow contain not only MSCs, but also other cell types such as blood cells and adipocytes, amongst others. MSCs were selected based on their adherence to TC plastic. The number of cells increased over time. Some of them started to adhere to the surface of the flask from day 0, and more cells adhered after three days. MSCs formed colonies and continued proliferating and they exhibited a fibroblast-like morphology, with characteristic spindle shape (Figure 3.2A, B). After this, fresh medium was replaced every 3 days, blood cells and other non-adherent cells were removed during the media removal leaving the adherent growing cells. By around day 6, cells in the flask reached 50% confluence (Figure 3.2C, D). The adherent cells were defined as passage zero (P0) cells when they grew to 80% confluence. Subculture was then performed, the P0 cells were washed with PBS and detached from flask by incubating with 0.25% Trypsin/EDTA (Gibco, UK) for 3 min at 37°C, standard growth medium was added and cell suspension was centrifuged at 1221 rpm for 3 min, re-suspended cells were plated in 75 cm^2 flasks (split ratio 1:3). All new passages were performed at confluence of 80-90%.

The viability and size of the rat bone marrow derived MSCs in cell suspension were determined using a Vi-Cell XR cell viability analyzer (Beckman coulter, USA). The Vi-Cell took pictures of cell samples to record cell size and calculate cell concentration, and used trypan blue to stain damaged cells to calculate cell viability. The average diameter of MSCs was around 18 microns. It was used as a reference for selecting capillary size. Cell viability was greater than 95%, indicating the healthy nature of MSCs.

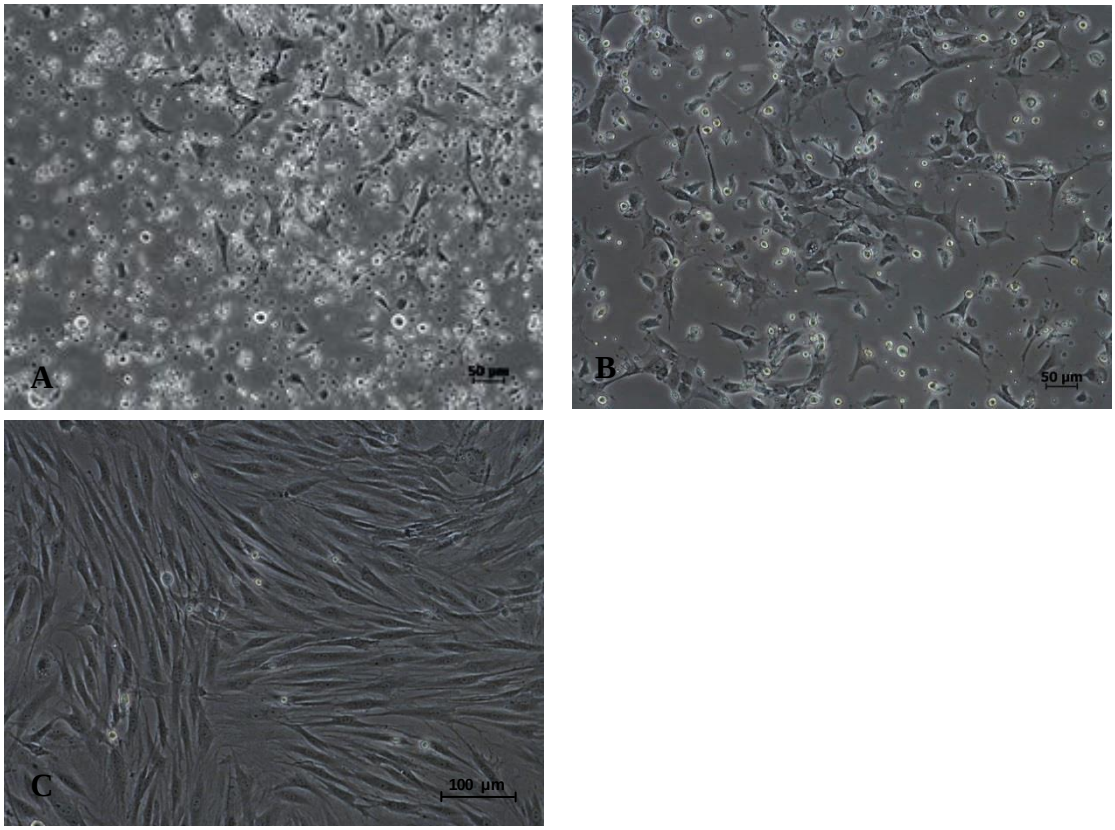


Figure 3.2: Culture of rat bone marrow derived MSCs in T25 flask. (A, B) P0 cells at day 4 and day 6 (Scale bar = 50µm); (C) Passage 1 cells (Scale bar = 100µm).

3.3. Effect of capillary diameter

Three different capillaries were assessed in the preliminary experiments. The 25G and 26G capillaries had the same internal diameter (0.254 mm), whereas the diameter of the 27G capillary was 0.203 mm. However, the wall of the 25G (0.128 mm) capillary was thicker than those of the 26G and 27G (0.102 mm). The deformation of the capillary with the thin wall was additional consideration for this study. The Reynolds number and capillary wall shear stress of different capillaries were shown in table 3.1. Flow rate was set at 13 ml/min to ensure experiments were conducted under laminar flow condition.

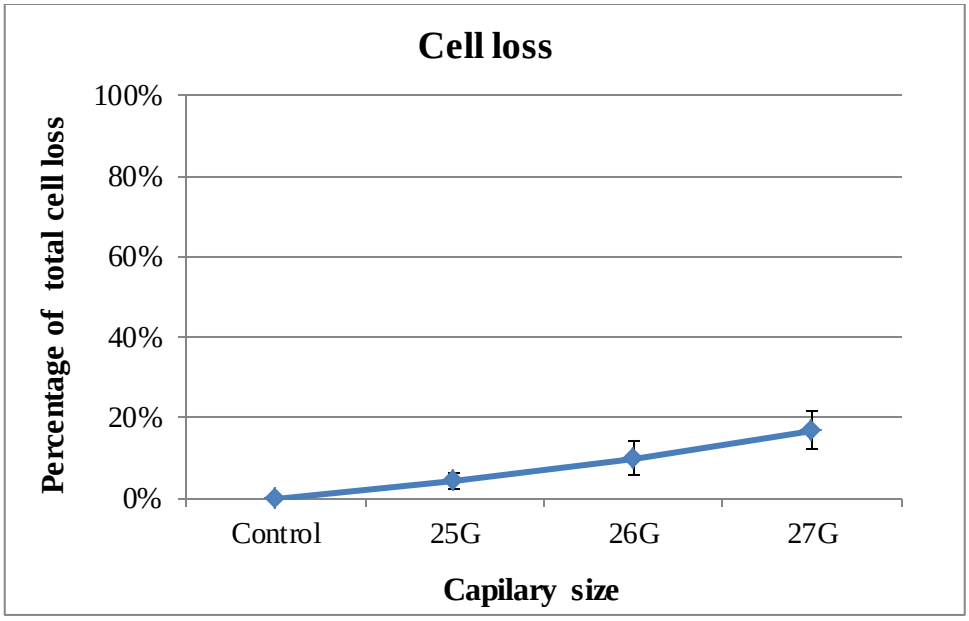
When assessing cell loss immediately after MSCs were passed through these different capillaries under constant conditions of pass number (10 passes) and flow rate (13 ml/min), results indicated that the 27G capillary caused more cell loss (17%) than the others, and the 25G capillary caused the least cell loss (4%)(Figure 3.3A). After cell culture for 24 hours, a CCK-8 assay revealed that cell viability of shear stress exposed cells was reduced compared to that of non-sheared cells. However, after 72 hours in culture, cell viabilities of both non-sheared cells and shear stress exposed cells were similar (Figure 3.3B), which indicates that a time period is required for cells to recover from the effect of shear stress. According to results of cell loss and cell viability, more cells experienced higher wall shear stress in 27G capillary with smaller diameter, although the remaining cells can recover from the negative effect of shear stress, as many cells still died, this would not be a wise choice when increasing scale of cell manufacture. In addition, we considered that the pressure in the syringe might cause deformation of the capillary with the thin wall, and therefore 25G capillary was chosen to perform subsequent studies.

Capillary	Capillary ID (mm)	Flow rate (ml/min)	*Re*		*Capillary Wall shear stress* (Pa)
25G	0.254	13	Laminar flow	1220	120
26G	0.254			1220	120
27G	0.203			1527	235

Table 3.1: Capillary wall shear stress of different capillaries (25, 26 and 27G) at flow rate of 13 ml/min.

	Rat BMSCs (passage)	Capillary length (mm)	Flow rate (ml/min)	Capillary	Capillary ID (mm)	*Re*	*Capillary Wall shear stress* (Pa)	Cell recovery	Cell viability	Osteogenic differentiation
Exp. 1	4	10	13	25G	0.254	1220	120	✓	✓	✓
				26G	0.254	1220	120	✓	✓	✓
				27G	0.203	1527	235	✓	✓	✓
Exp. 2	2 , 3	10	13	25G	0.254	1220	120	✓	✓	✓
			16			1502	148	✓	✓	✓
			19			1784	175	✓	✓	✓
			22			2065	203	✓	✓	✓
Exp. 3	6	10	13	25G	0.254	1220	120	✓	✓	✓
			18			1689	166	✓	✓	✓
			23			2159	212	✓	✓	✓
			28			2628	258	✓	✓	✓

Table 3.2: Experiments (Exp.) that were performed using rat bone marrow derived MSCs.



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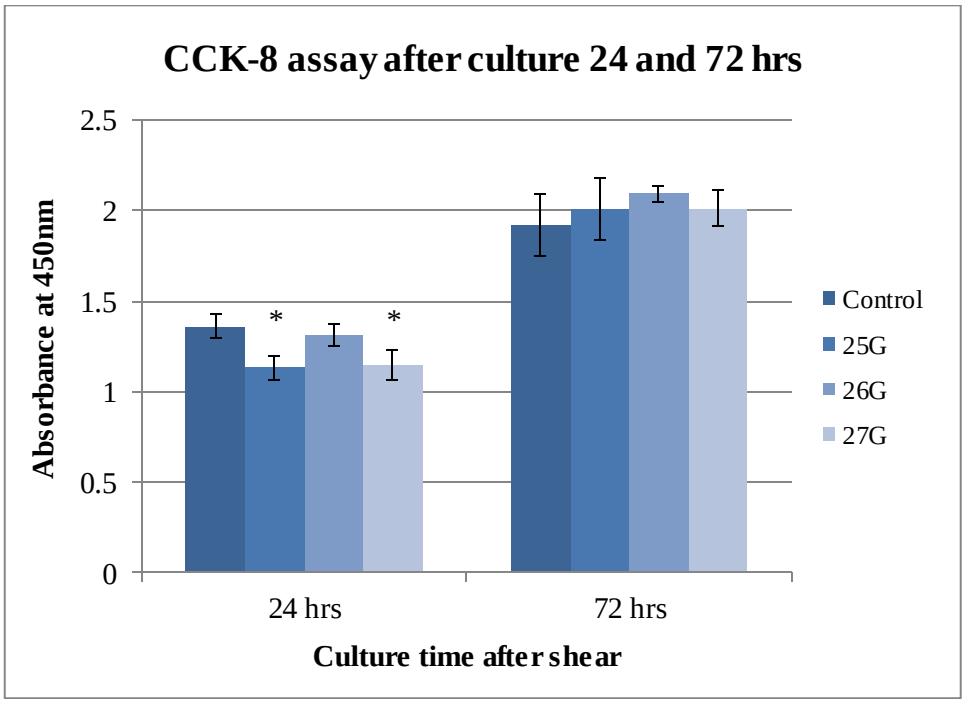


B

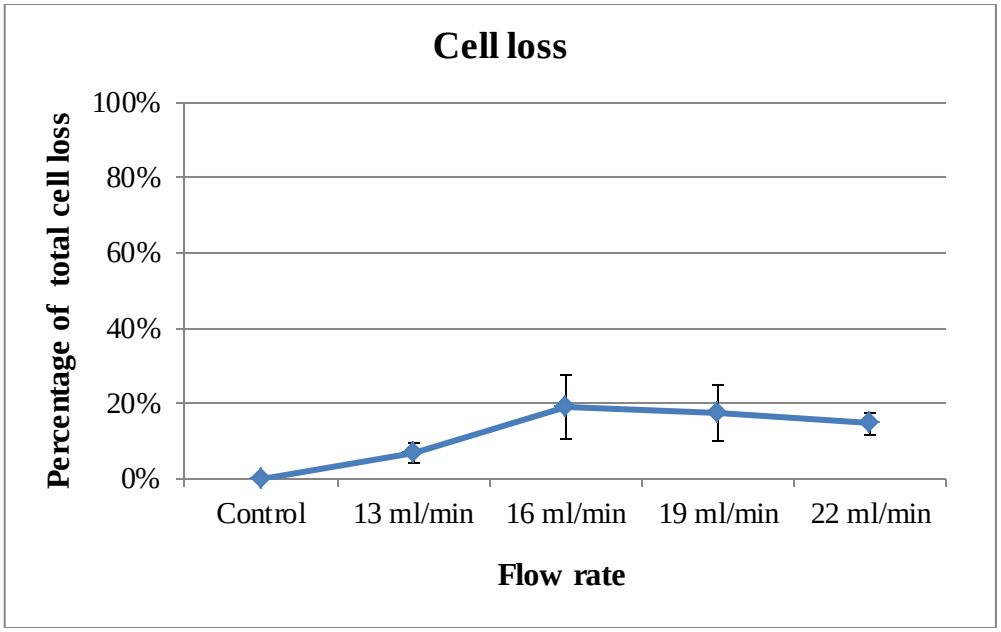
Figure 3.3: Effect of different capillary diameters (25, 26 and 27G) on cell loss and cell viability of P4 rat bone marrow derived MSCs (*P < 0.05). Length of capillary is 10 mm, at flow rate of 13 ml/min. (A) Cell loss; (B) Cell viability.

3.4. Effect of flow rate

Flow rate plays a key role in determining capillary wall shear stress. High flow rate leads to high shear stress when the ID of the capillary is constant. However, lots of cell damage and loss in cell number might be caused when setting flow rate beyond a certain range. Therefore, the Reynolds number was used to ensure flow rate was kept in laminar flow regime to minimize cell death in preliminary studies. Experiments were performed using 25G capillary with different flow rates that were no more than 23 ml/min. The Reynolds number of each flow rate is less than 2100, indicating that laminar flow is maintained in the capillary (Table 3.3).

Flow rate (ml/min)	*Re*		*Capillary Wall shear stress* (Pa)
13	Laminar flow	1220	120
16		1502	148
19		1784	175
22		2065	203

Table 3.3: Reynolds number and capillary wall shear stress of 25G capillary at different flow rates (13, 16, 19 and 22 ml/min).



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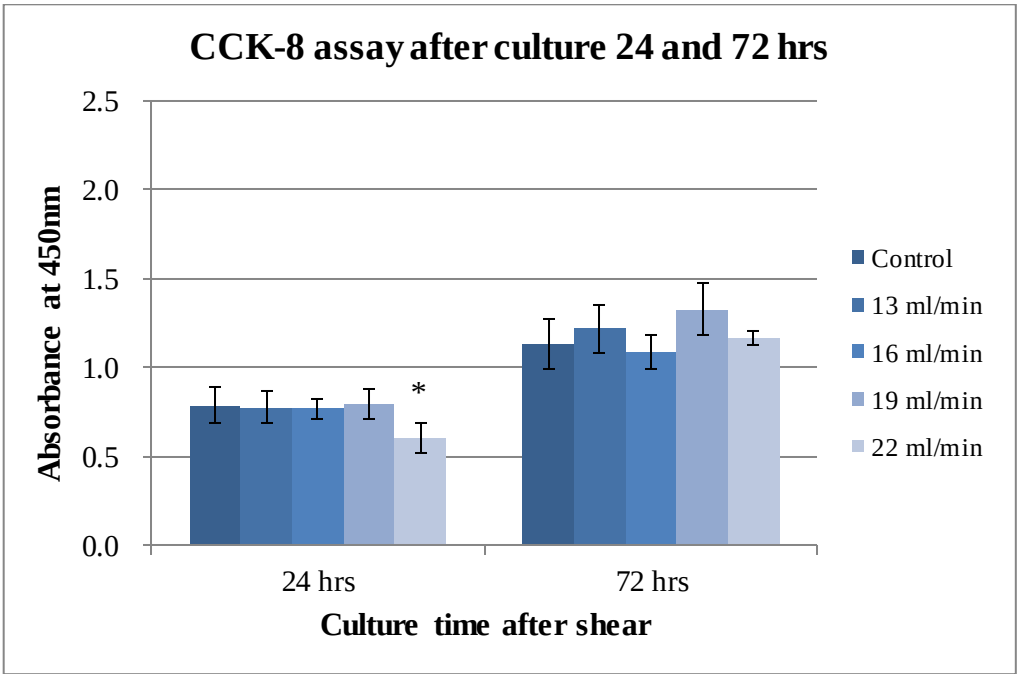


B

Figure 3.4: Effect of capillary wall shear stress at different flow rates (13, 16, 19 and 22 ml/min) on cell loss and cell viability of P2 rat BMSCs. 25G capillary with length of 10 mm, 10 passes. (A) Cell loss; (B) Cell viability (*P < 0.05).

Passage 2 rat BMSCs were harvested when they reached 90% confluence，then cell suspensions were passed through a 10 mm long, 25G capillary at 13, 16, 19 or 22 ml/min for 10 passes. The non-sheared control sample was hold on the bench for the duration of the experiment at room temperature to ensure that hold time was controlled in untreated and treated cells. Cell count data showed that shear stress under flow rates of 16, 19 and 22 ml/min resulted in more cell damage and cells loss (15%-19%). In contrast, only about 7% cell loss due to the flow rate of 13 ml/min in capillary (Figure 3.4A). The improper operation was a potential reason why most cell loss occurred at lower flow rate of 16 ml/min and fewer cells died under the higher flow rate of 22 ml/min. For the CCK-8 assay, it was clear that shear stress exposed cells under the high flow rate of 22 ml/min had the least cell viability after 24 hrs ($P<0.05$). After 72 hrs, the cell population recovered from the negative influence of shear stress and continually proliferated. Furthermore, sheared cells under flow rate of 22 ml/min got most increase of cell growth compared to other sheared cells (Figure 3.4B).

Meanwhile, both shear stress exposed cells and non-sheared cells were seeded into 24-well plates and exposed to osteogenic differentiation media for three weeks. After that, Alizarin Red S staining was performed. The control cells cultured in standard growth medium did not stain positive, which indicates they did not produce calcified matrix, typical of bone forming cells. All wells that were cultured with osteogenic differentiation media exhibited large areas of red stain. The red area in photos (Figure 3.5) indicates the mineralization of matrix of cells, which suggests differentiation from MSCs to osteoblasts, as they generate lots of calcium deposits under induction of osteogenic differentiation medium. Upon passing cells through a capillary, they were capable of producing more calcium compared to non-sheared cells. Based on the staining photos and also staining coverage data from analysis of Image J, cells that were sheared at high flow rate of 22 ml/min showed that more than 60% area was stained (Figure 3.5F; Figure 3.6). This illustrates that shear stress can enhance osteogenesis to some extent, in support of previous observations in other mesenchymal cell types (Mulhall *et al.*, 2010; Patel *et al.*, 2010).

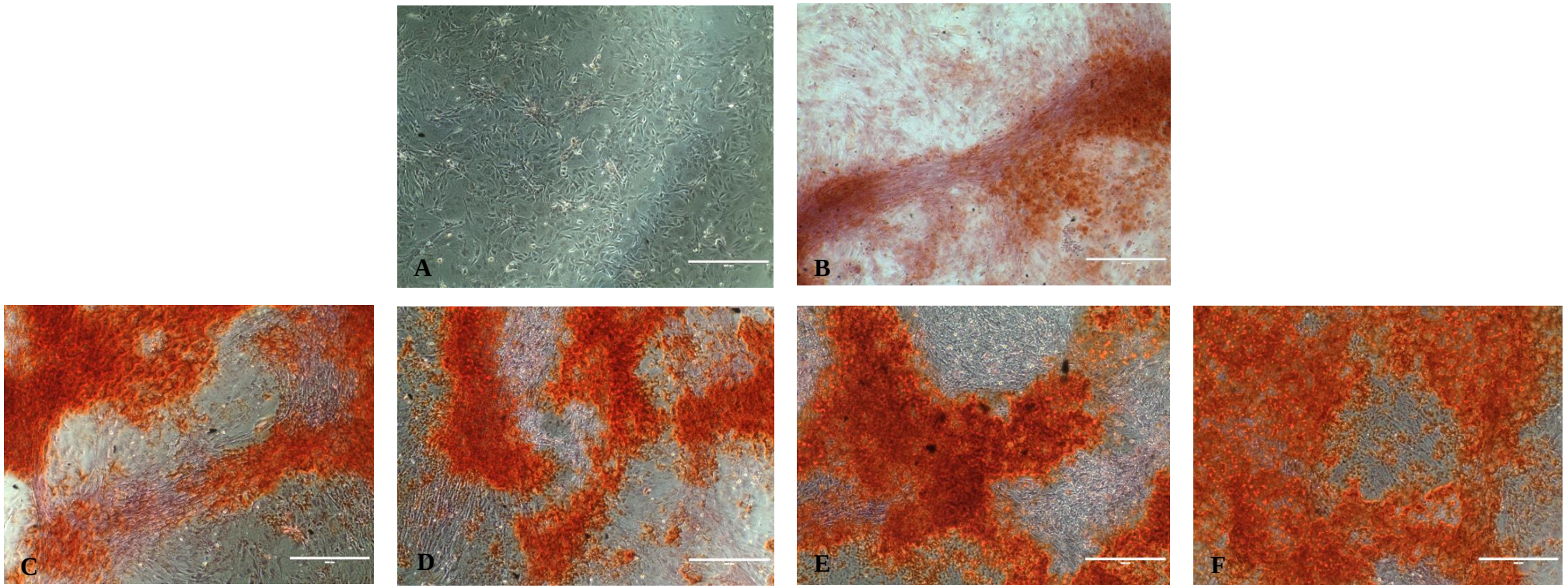


Figure 3.5: Both non-sheared and shear stress exposed rat bone marrow derived MSCs were cultured in standard growth medium and osteogenic differentiation medium for 21 days, and then were stained with Alizarin Red S solution. Non-sheared cells cultured in standard growth medium (A) or osteogenic differentiation medium (B) as the negative and positive controls respectively; Shear stress exposed cells that were sheared at 13 ml/min (120 Pa) (C), at 16 ml/min (148 Pa) (D), at 19 ml/min (175 Pa) (E) and at 22 ml/min (203 Pa) (F) cultured in osteogenic differentiation medium (Scale bar = 400µm).

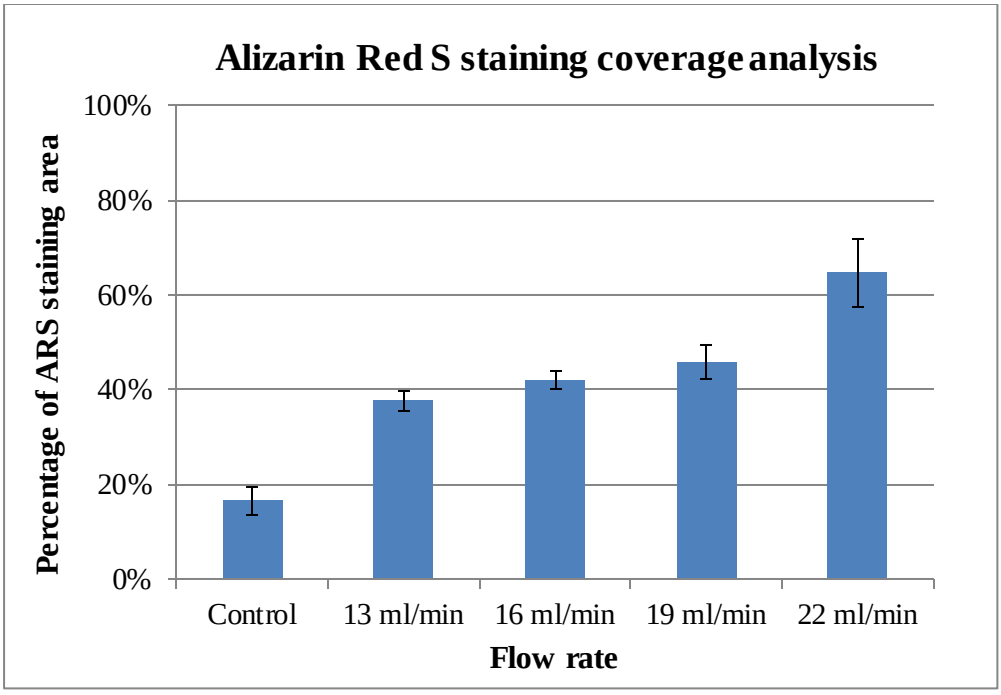
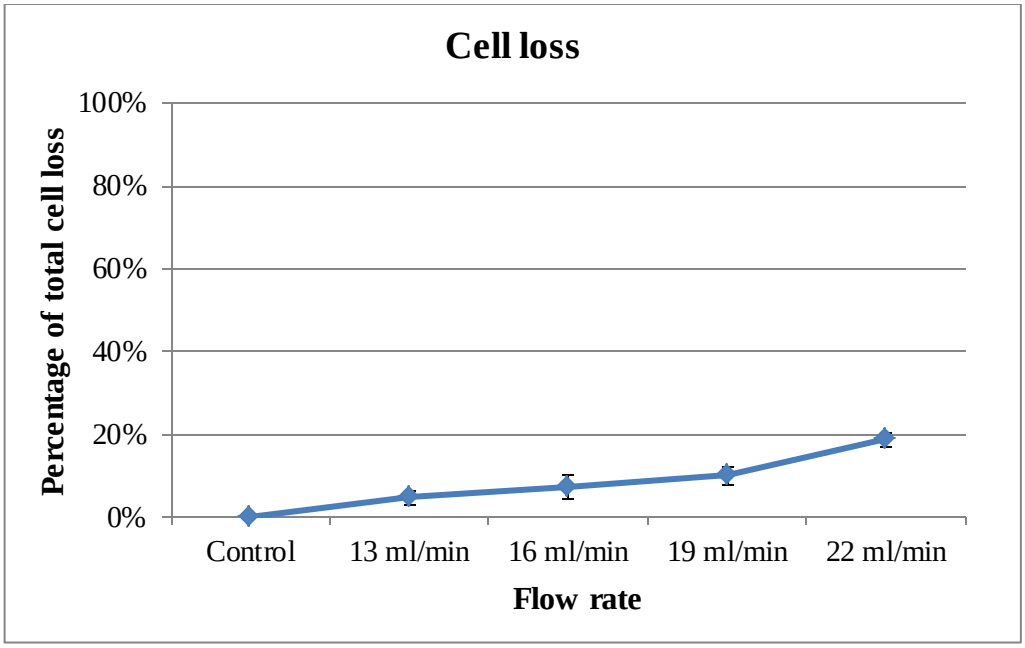


Figure 3.6: Analysis of Alizarin Red S staining area for both non-sheared and shear stress exposed P2 rat BMSCs that cultured with osteogenic differentiation media by Image J.

The same experiment was conducted using passage 3 rat bone marrow derived MSCs. The amount of cell loss increased with increases in flow rate. The least cell loss (5%) was seen at a flow rate of 13 ml/min, and the highest flow rate of 22 ml/min induced the most cell damage (19%)(Figure 3.7A). However, unlike the previous experiment, there was no loss of viability at 24 hrs following exposure to high shear stress. By 72 hrs, the absorbance values of sheared cells at low flow rate (13, 16 and 19 ml/min) only increased a small amount, which indicates that the cells continued to grow but that the growth rate decreased relative to untreated cells (Figure 3.7B), so the negative influence of shear stress on these cells possibly remained, and they needed more time to recover.

Similar differentiation results were obtained to experiment 2. Unlike cells cultured in standard growth medium (Figure 3.8A), cells cultured in osteogenic differentiation medium generated lots of calcium deposits (Figure 3.8B-F), illustrated by highly positive red staining. Higher flow rates (Figure 3.8E, F) with accompanying higher shear stress (175 Pa and 203 Pa) resulted in generation of more calcified matrix compared with lower shear stress (120 Pa and 148 Pa) (Figure 3.8C, D; Figure 3.9). Then, the quantification of staining using Millipore osteogenic differentiation Kit demonstrated that significantly more MSCs differentiated into bone forming cells under the effect of the highest shear stress (203 Pa; Table 3.3). The formation of calcified matrix at a flow rate of 22 ml/min was nearly three times of that at flow rate of 13 ml/min, and also it was almost four times that of non-sheared cells (Figure 3.10). This indicates that capillary wall shear stress has potential to induce osteogenic differentiation and the magnitude of shear stress is also important to induction of bone regeneration.



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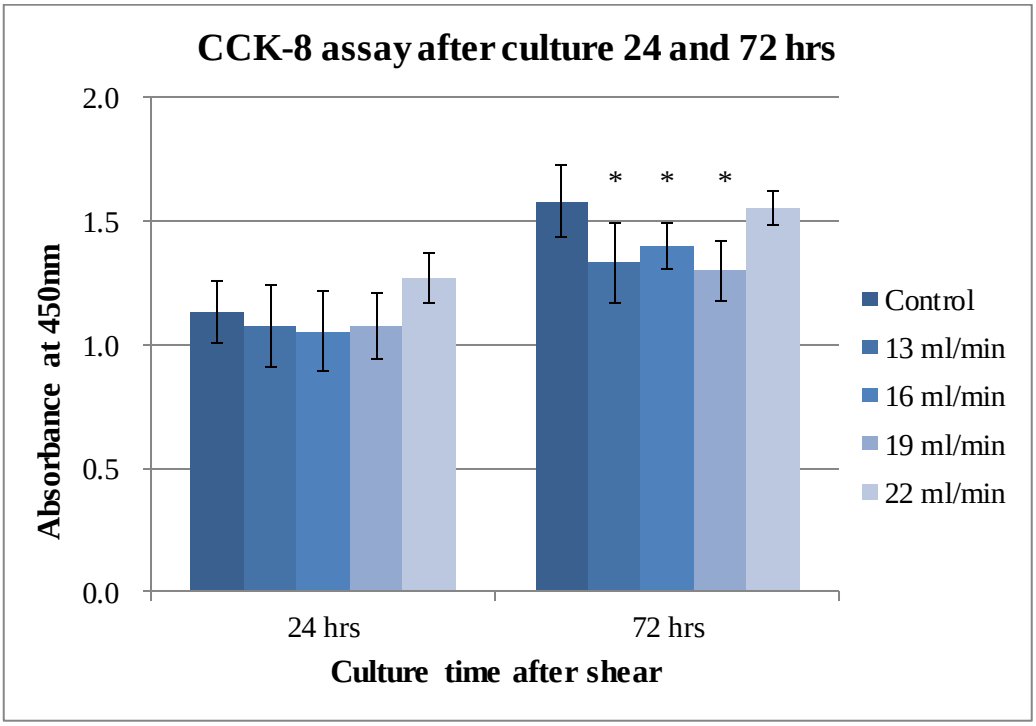


B

Figure 3.7: Effect of different flow rates (13, 16, 19 and 22 ml/min) on cell loss and cell viability of P3 rat BMSCs. 25G capillary with length of 10 mm, 10 passes. (A) Cell loss; (B) Cell viability (*$P < 0.05$).

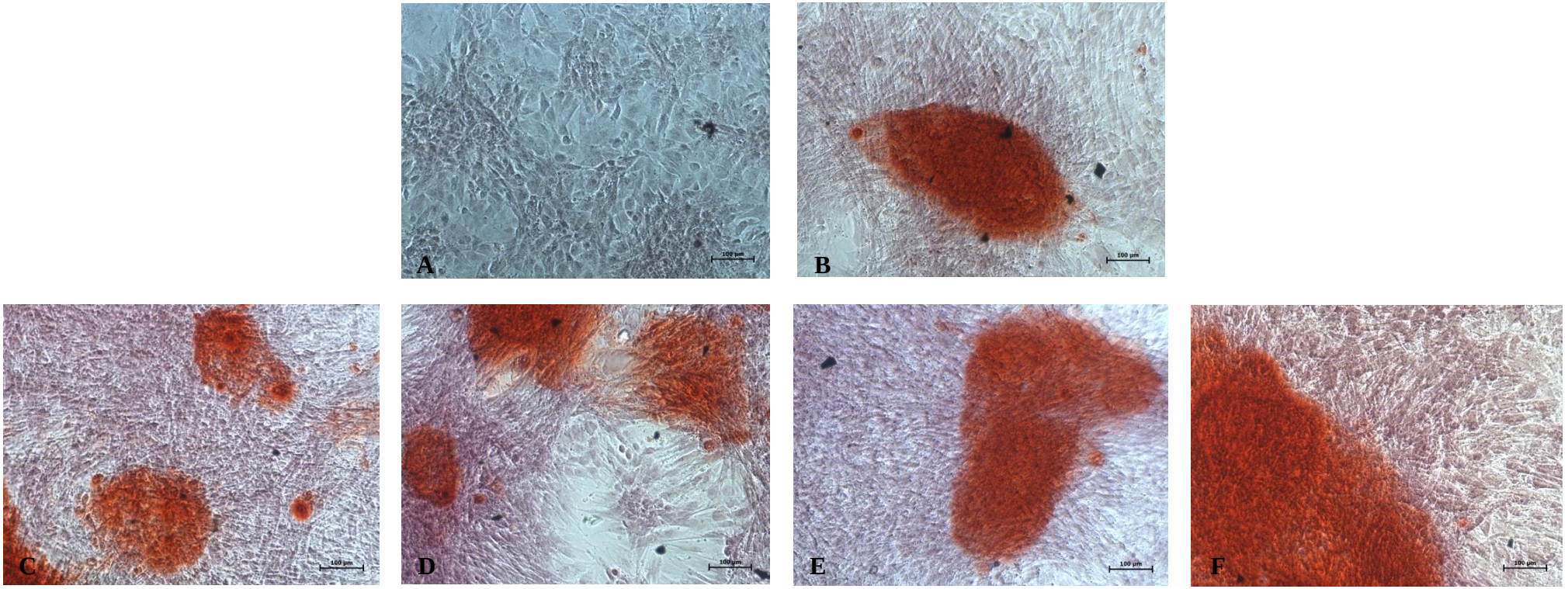


Figure 3.8: Both non-sheared and shear stress exposed P3 rat bone marrow derived MSCs were cultured in standard growth medium and osteogenic differentiation medium for 21 days, and then were stained with Alizarin Red S solution. Non-sheared cells cultured in standard growth medium (A) or osteogenic differentiation medium (B) as the negative and positive controls respectively; Shear stress exposed cells that were sheared at 13 ml/min (120 Pa) (C), at 16 ml/min (148 Pa) (D), at 19 ml/min (175 Pa) (E) and at 22 ml/min (203 Pa) (F) cultured in osteogenic differentiation medium (Scale bar = 100μm).

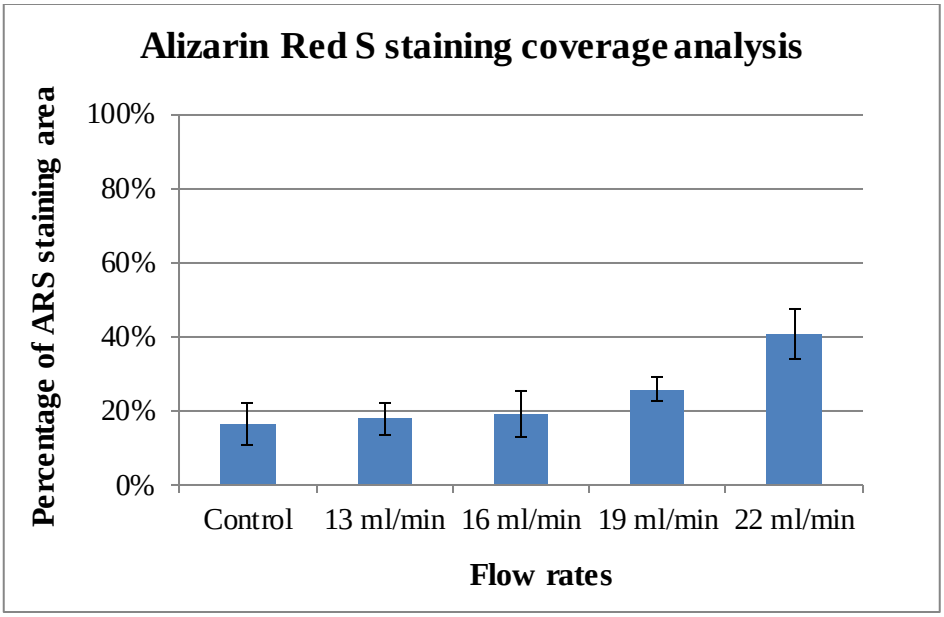


Figure 3.9: Analysis of Alizarin Red S staining area for both non-sheared and shear stress exposed P3 rat MSCs that cultured with osteogenic differentiation media by Image J.

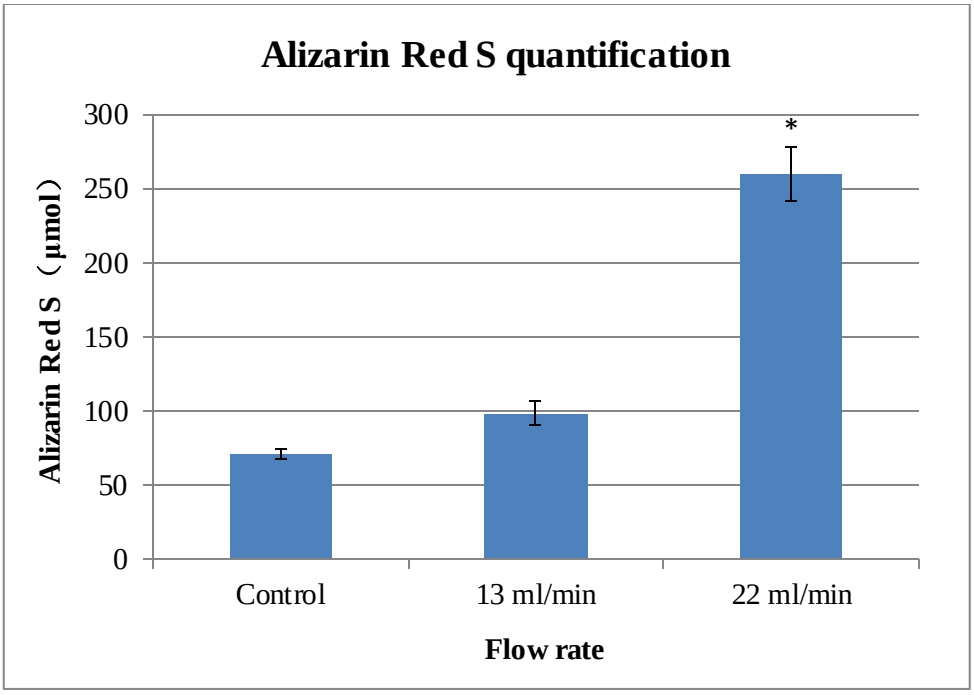
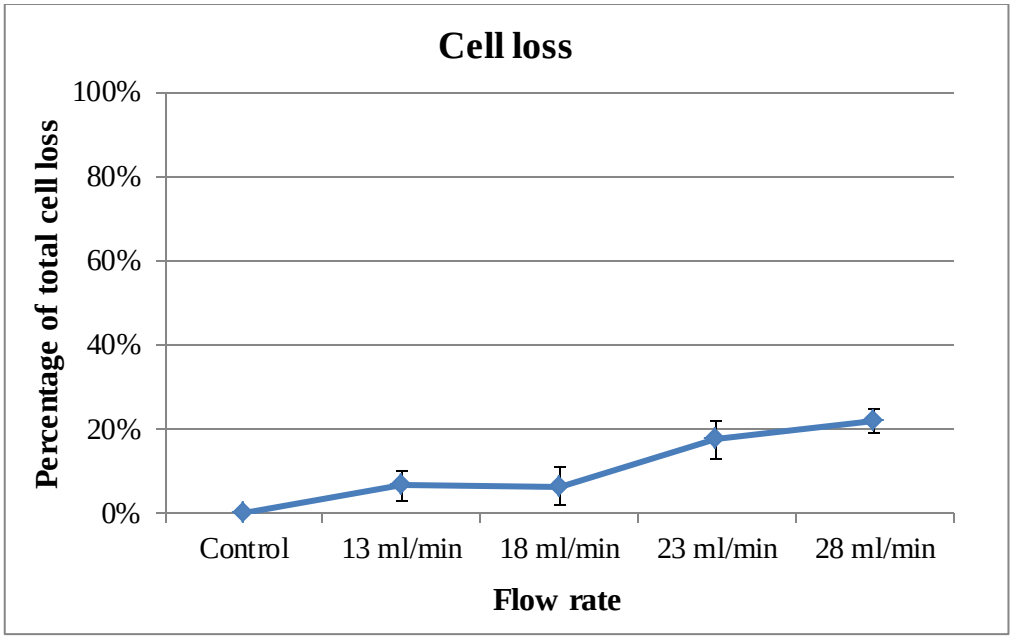


Figure 3.10: The quantification of Alizarin Red S staining of both non-sheared and shear stress exposed (at 13 ml/min and 22 ml/min) rat BMSCs that were cultured in osteogenic differentiation medium (*P<0.05).

Considering that the high flow rate with high shear stress could induce greater osteogenic differentiation (measured by Alizarin Red S staining), in the next experiment, flow rates of 23 ml/min and 28 ml/min were introduced for further study. This was done to determine whether even higher flow rates that move beyond the bounds of lamina flow could support osteogenic induction even more. Fluid flow at these flow rates is defined as transitional flow based on *Re*, it is not quite turbulent but beyond the limit of laminar. Although the flow in transition state was more unstable, which might cause serious damage of cells, it might also has more possibility to enhance differentiation. As shown in table 3.4, it generated the highest level of shear stress (258 Pa), twice as much as that at 13 ml/min (120 Pa) when the flow rate reached 28 ml/min.

Flow rate (ml/min)	*Re*		*Capillary Wall shear stress* (Pa)
13	Laminar flow	1220	120
18		1689	166
23	Transitional flow	2159	212
28		2628	258

Table 3.4: Reynolds number and capillary wall shear stress of 25G capillary at different flow rates (13, 18, 23 and 28 ml/min).



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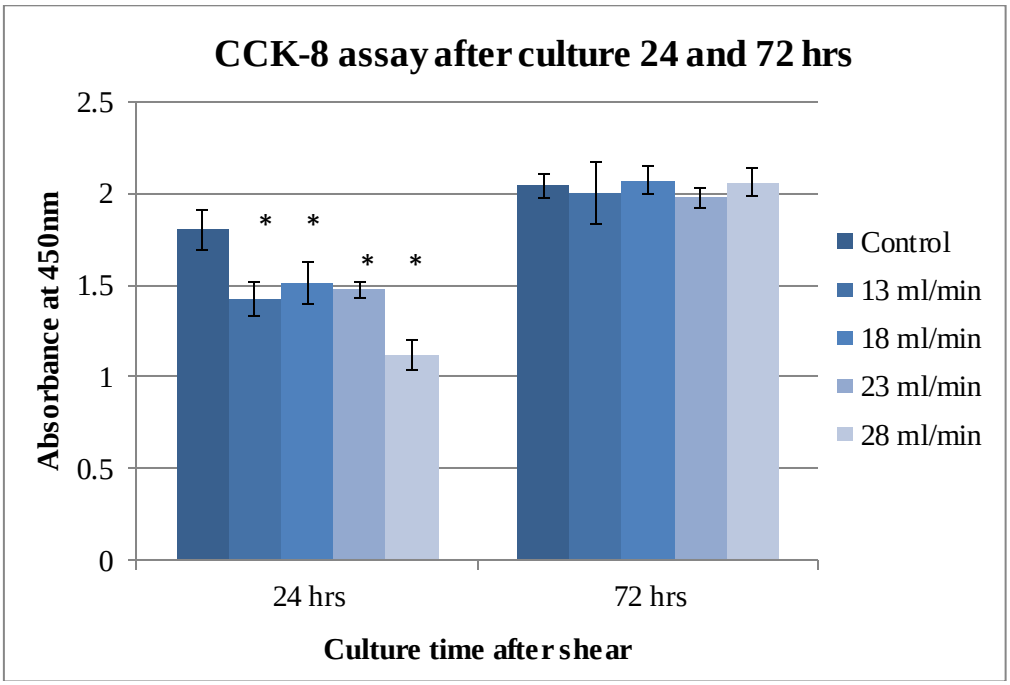


B

Figure 3.11: Effect of different flow rates (13, 18, 23 and 28 ml/min) on cell loss and cell viability of P6 rat BMSCs. 25G capillary with length of 10 mm, 10 Passes. (A) Cell loss; (B) Cell viability (*P<0.05).

Passage 6 rat BMSCs were passed through a 10 mm 25G capillary at four different flow rates (13 ml/min, 18 ml/min, 23 ml/min and 28 ml/min) for 10 passes. Laminar flow was achieved in capillaries when flow rate was between 13 and 18 ml/min. On the contrary, it became transitional flow which has more chaotic fluid movement at flow rate of 23 and 28 ml/min. The results illustrate that higher flow rate with higher capillary wall shear stress caused a greater degree of cell damage (18-22%) compared to lower flow rates with lower corresponding shear stress (6-7%)(Figure 3.11A). At higher flow rates, more cell debris and blue cells were observed when counted using trypan blue stain under the microscope. Shear stress exposed cells underwent a compromise in viability, which decreased compared to non-sheared cells during the subsequent 24 hours culture period. This was especially the case for shear stress induced by 28 ml/min flow, where the greatest reduction in cell viability was seen compared to the others. However, after culturing for 72 hours, both non-sheared and shear stress exposed cells had a similar level of cell viability (Figure 3.11B). This indicates that shear stress exposed cells survived and recovered from the negative influence of shear stress and continually proliferated, even under the transitional flow condition. Therefore, moving on to use 28 ml/min to shear cells will be acceptable, providing favourable osteogenic differentiation (e.g. determined via Alizarin Red S staining) positively adds to the findings.

3.5. Chapter discussion

The preliminary experiments with the rat bone marrow derived MSCs showed early success of research. Using the capillary diameters and flow rates that were reported above, cell growth is permissible in spite of an early reduction in viable cell growth, and appropriate capillary wall shear stress has potential to induce osteogenic differentiation. However, our initial hypothesis was that sub-lethal levels of shear stress would favour osteogenic differentiation, based on the fact that the literature typical reports that shear stress is exclusively detrimental (Born *et al.*, 1992; Zoro *et al.*, 2008; Acosta Martinez *et al.*, 2010; Brindley *et al.*, 2011) or favourable (Bancroft *et al.*, 2002; Datta *et al.*, 2006; Scaglione *et al.*, 2008; Patel *et al.*, 2011; Mulhall *et al.*, 2011), according to the

context and shear stress levels assessed. Whereas, here we found that the same levels of shear stress that caused short-term damage were also positive overall, in the context of longer term mineralized matrix formation. However, many lessons were learned from this preliminary study. First, the Harvard PHD 2000 syringe pump needs to be calibrated after working for a certain time to ensure the right setting of flow rates. The 25G capillary was not ideal for future experiments due to the irreversible deformation after using several times, by contrast, 26G with same internal diameter but thinner wall was more flexible for experimental operation. The higher cell concentration of cell suspensions in capillaries was needed for shear stress to work on it and need to be increased in the future research.

Chapter 4 Exposure of human MG63 cell line cells to fluid shear stress

4.1. Introduction

MG63 cells are derived from human osteosarcoma. They present obvious advantages to be widely used as an *in vitro* research model: they are a robust cell population with rapid growth rate and immature osteoblast phenotype. Shear experiments that compare different flow rates, different numbers of capillary passes and different capillary lengths were carried out using MG63 cells. This was done to define the operating parameters that were permissive for OB cells, prior to working with the immature human MSCs. To assess effect of fluid flow shear stress on cells, analysis was improved and more assays were added, for instance, the viability of cells that were collected immediately after exposing to shear stress was recorded, cells that remained in capillary were washed out using PBS and counted, and the expression level of osteogenesis related genes was analysed by qRT-PCR.

4.2. Culture of human MG63 cells

MG63 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, UK), supplement with 10% Fetal Bovine Serum (FBS; Gibco, UK) and 1% Antibiotic-Antimycotic (100×; Gibco, UK) and incubated at 37°C in 5% CO_2. Normally, they need to be passaged every three days and split at a high ratio (e.g. 1:4 ratios). Their growth rate is maintained at high levels. They are flat, well-separated from each other and exhibit mixed morphology with some polygonal and some spindle-shape similar to fibroblasts when cultured in monolayer (Figure 4.1). MG63 cell size was measured in suspension using the Vi-Cell instrument. Their average diameter is around 19 microns, cells maintain a healthy growth condition even at 19 passage.

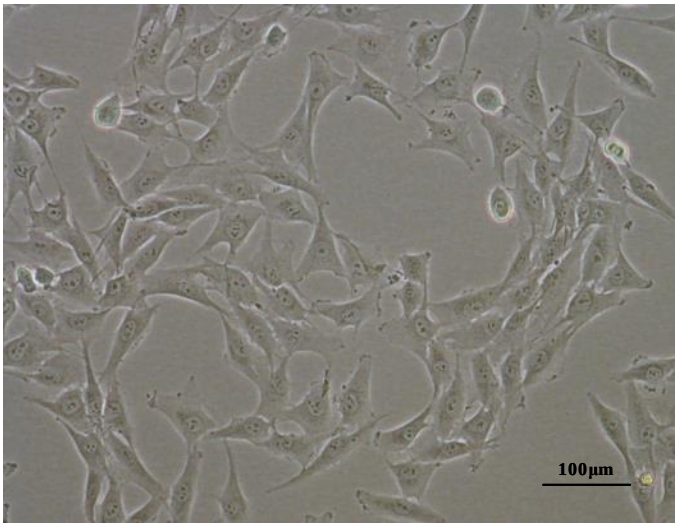


Figure 4.1: Passage 19 MG63 cells cultured in a T75 flask (Scale bar = 100 µm).

	Rat MSCs (passage)	Capillary length (mm)	Pass NO.	Flow rate (ml/min)	Capillary	Capillary ID (mm)	*Re*	*Capillary Wall shear stress* (Pa)	Cell recovery	Cell viability	qRT-PCR	Osteogenic differentiation
Exp. 1	19	10	10	13	26G	0.254	1220	120	✓	✓	×	✓
				20			1877	184	✓	✓	×	✓
				28			2628	258	✓	✓	×	✓
Exp. 2	12	10	10	13	26G	0.254	1220	120	✓	✓	✓	✓
				20			1877	184	✓	✓	✓	✓
				28			2628	258	✓	✓	✓	✓
Exp. 3	19	40	10	20	26G	0.254	1877	184	✓	✓	✓	✓
			40						✓	✓	✓	✓

Table 4.1: Experiments (Exp.) that performed using human MG63 cells.

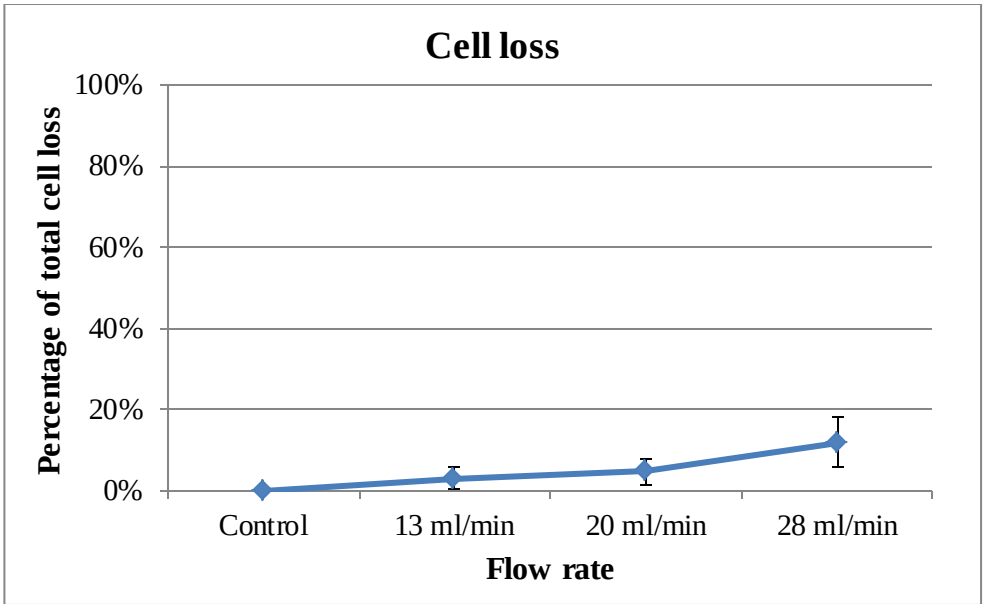
4.3. Effect of flow rate

A flow rate comparison experiment was performed by exposing passage 19 MG63 cells at flow rates of 13 ml/min, 20 ml/min and 28 ml/min for 10 passes using 10 mm 26G capillary. The shear stress and *Re* for each flow rate are shown in table 4.2. Results show that reduction in total cell number is variable, with least loss (3%) observed at a flow rate of 13 ml/min and most loss (12%) at a flow rate of 28 ml/min (Figure 4.2A). The viability of recovered cells that were collected immediately after exposure to shear stress at all flow rates are indistinctive and similar (96%-97%)(Figure 4.2B). Results of the cell viability assay illustrate that the highest shear stress (258 Pa) at 28 ml/min had a mild negative effect on cell viability after culture for 24 hrs, cells that exposure to all shear stress (120, 184 and 258 Pa) at all flow rates continually proliferated and had similar level of growth as non-sheared cells (Figure 4.3). This data indicates that MG63 cells were more robust than MSCs, as they exhibited more resistance to negative impact from shear stress and maintained their viability and growth.

The next experiment assessed the effect of capillary wall shear stress on osteogenic differentiation capacity. By culturing these non-sheared and shear stress exposed MG63 cells in osteogenic differentiation medium for 3 weeks, a similar trend in staining results were found. The stained calcium deposit was detected in all wells that cultured with osteogenic differentiation medium. Non-sheared MG63 cells only displayed a small amount of stain compared with shear stress exposed MG63 cells. High shear stress (184 Pa and 258 Pa) experienced at 20 ml/min and 28 ml/min (Figure 4.4D, E) induced more mineralization compared to low shear stress (120 Pa) at 13 ml/min (Figure 4.4C). These data demonstrate that the capillary wall shear stress has positive influence on osteogenic differentiation in the premise of not affecting cell viability.

Flow rate (ml/min)	*Re*		*Capillary Wall shear stress* (Pa)
13	Laminar flow	1220	120
20		1877	184
28	Transitional flow	2628	258

Table 4.2: Reynolds number and capillary wall shear stress of 26G capillary at different flow rates (13, 20, 28 ml/min).



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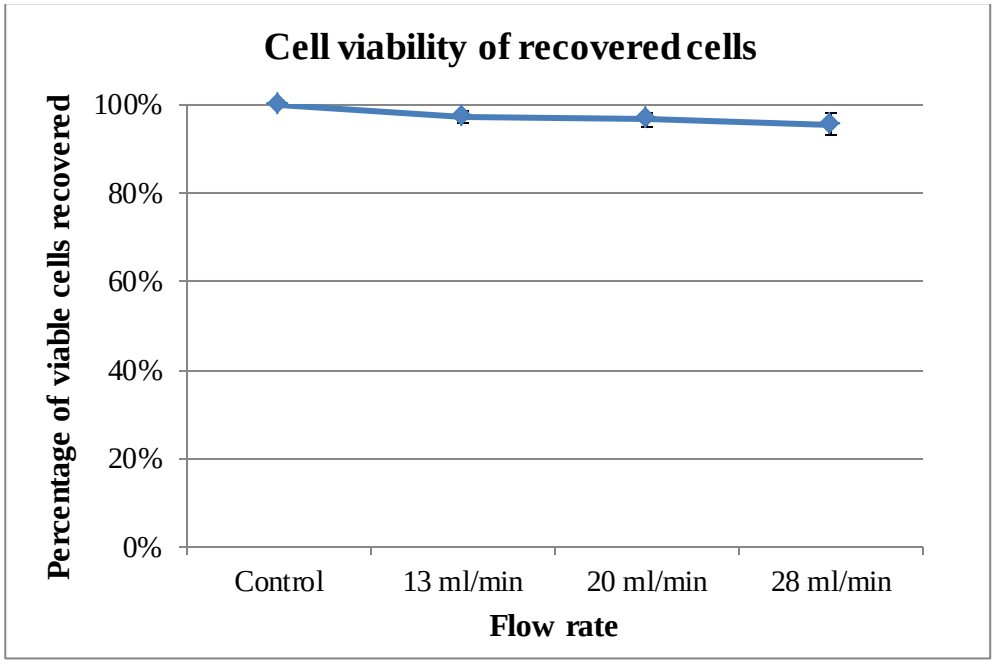


B

Figure 4.2: Effect of different flow rates (13, 20 and 28 ml/min) on cell loss and cell viability of P19 MG63 cells. 26G capillary with length of 10 mm, 10 passes (A) Cell loss; (B) Cell viability tested immediately after shear.

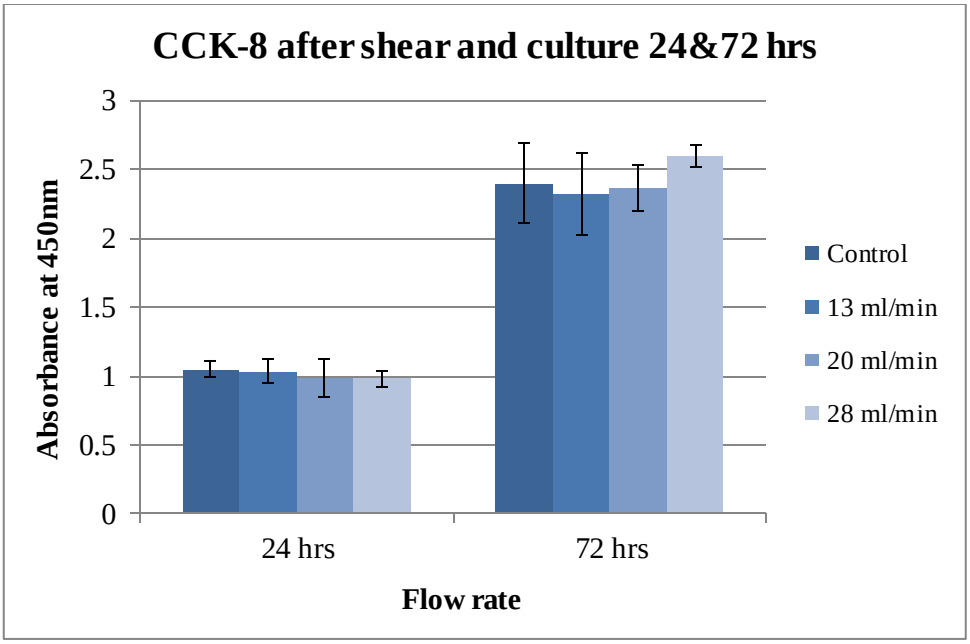


Figure 4.3: Effect of different flow rates (13, 20 and 28 ml/min) on cell viability of P19 MG63 cells after culture for 24 and 72 hours. 26G capillary with length of 10 mm, 10 passes.

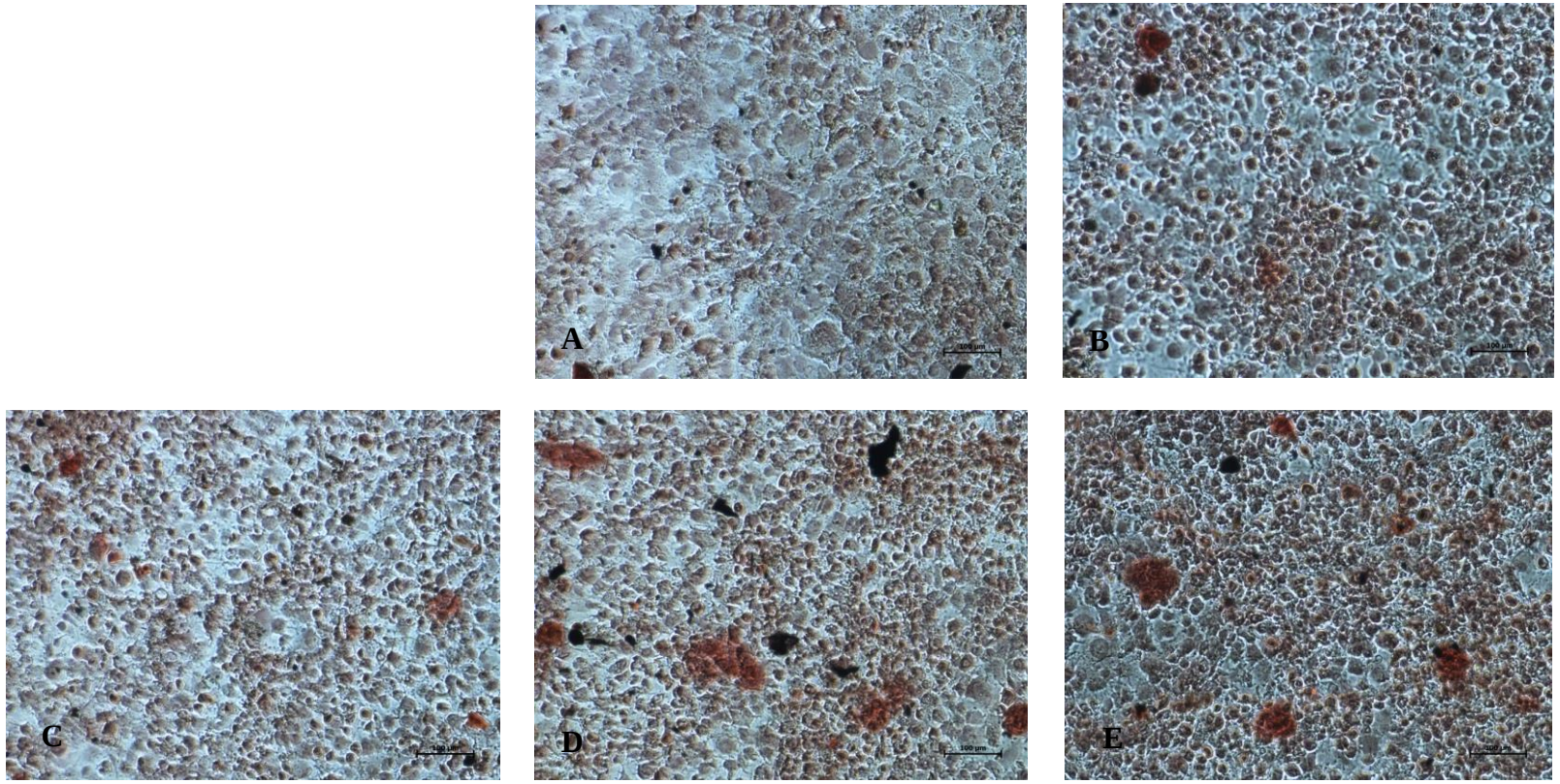
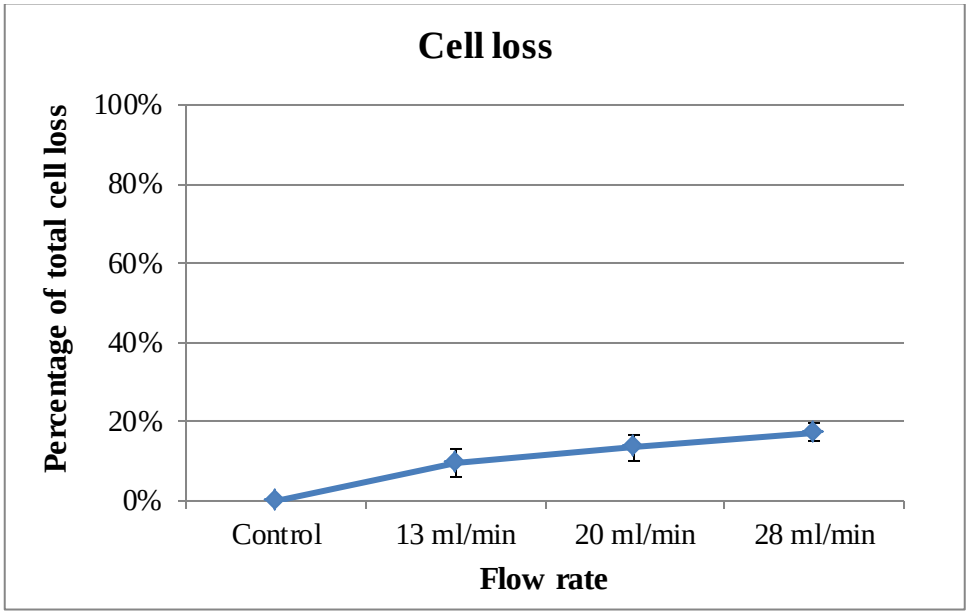


Figure 4.4: Sheared and non-sheared P19 MG63 cells were cultured in standard growth medium and osteogenic differentiation medium for 21 days, staining with Alizarin Red S dye: (A) Non-sheared cells cultured in standard growth medium; (B) Non-sheared cells cultured in differentiation medium, as the negative and positive controls respectively; (C) Sheared cells (flow rate of 13 ml/min) cultured in differentiation medium; (D) Sheared cells (flow rate of 20 ml/min) cultured in differentiation medium; (E) Sheared cells (flow rate of 28 ml/min) cultured in differentiation medium (Scale bar = 100 μm).

The same experiment was also carried out using passage 12 MG63 cells. Cell loss that may result from retention in syringe was evaluated this time, results show that a part of cells remained in the capillary and syringes, and might be reduced by careful manual operation. Some cells were destroyed and became dead cells or cell debris due to the negative influence of shear stress (Figure 4.5B), viability of the remaining cells was not affected by shear stress as it remained in the regime of 97-99% (Figure 4.6A). A reduction of total cell number was seen with increasing the flow rate (Figure 4.5A). Although the viability of control treated cells and those from the 28 ml/min condition have large deviations at 72 hrs, mostly they still exhibit similar values across different conditions at 24 hrs and 72 hrs (Figure 4.6B).

Shear stress cues are recognized to induce osteogenic differentiation and maturation due to activation of mechanotransduction pathways (Duncan and Turner, 1995; Iqbal and Zaidi, 2005; Riddle *et al.*, 2006; Kim *et al.*, 2007; Salasznyk *et al.*, 2007; Lee *et al.*, 2008; Shin *et al.*, 2008; Glossop and Cartmell, 2009; Stiehler *et al.*, 2009). Expressions of several candidate gene markers that indicate osteogenic differentiation were next assessed. To do this, quantitative reverse transcription PCR was used to study the effect of shear stress on osteogenic differentiation at gene expression level. Three key genes that are related to different stages of differentiation were assessed: type I collagen alpha 1 (COL1A1), alkaline phosphatase (ALP) and Runx2. COL1A1 and ALP activity are considered to be early markers of osteogenesis. Runx2 on the other hand is widely regarded as the master regulator of osteoblast formation. PCR data revealed that at the transcription level, shear stress was clearly a positive inducer of osteogenic gene expression. A relationship between duration of exposure and gene expression level could not be established but all genes underwent up-regulation (Figure 4.7). Much more red stain was observed at flow rate of 28 ml/min, which indicates high shear stress around 258 Pa has most effect on differentiation of MG63 cells (Figure 4.8). It illustrates that the shear stress exposed cells have more tendency to differentiate compared with non-sheared cells. It also reinforces the positive impact of shear stress on osteogenesis that has been reported in the literature (Bancroft *et al.*, 2002; Kreke *et al.*, 2005; Datta *et al.*, 2006; Scaglione *et al.*,

2008; Patel *et al.*, 2011; Mulhall *et al.*, 2011).



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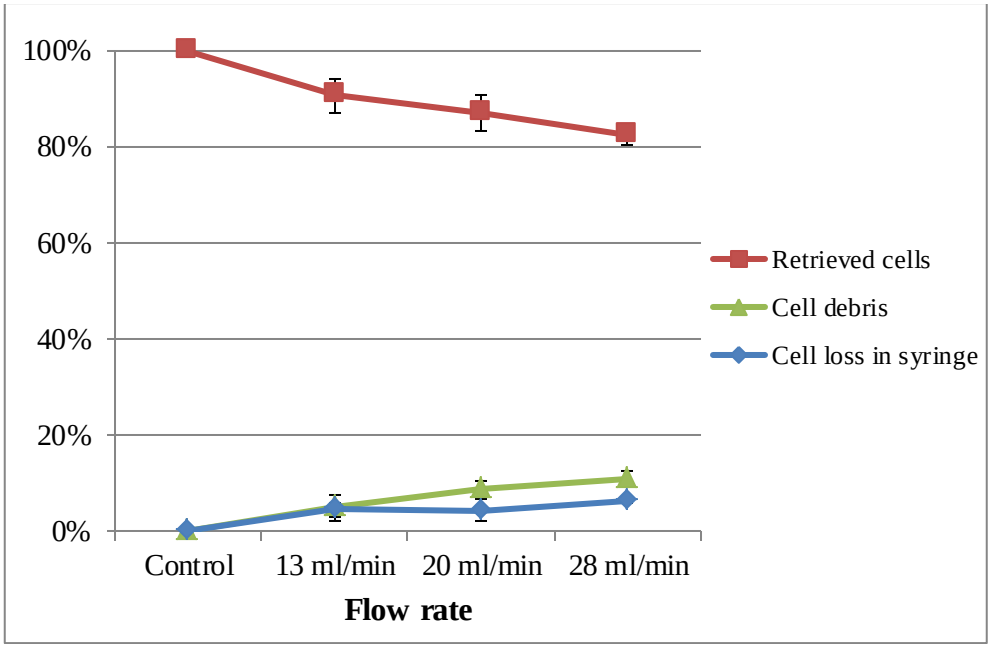
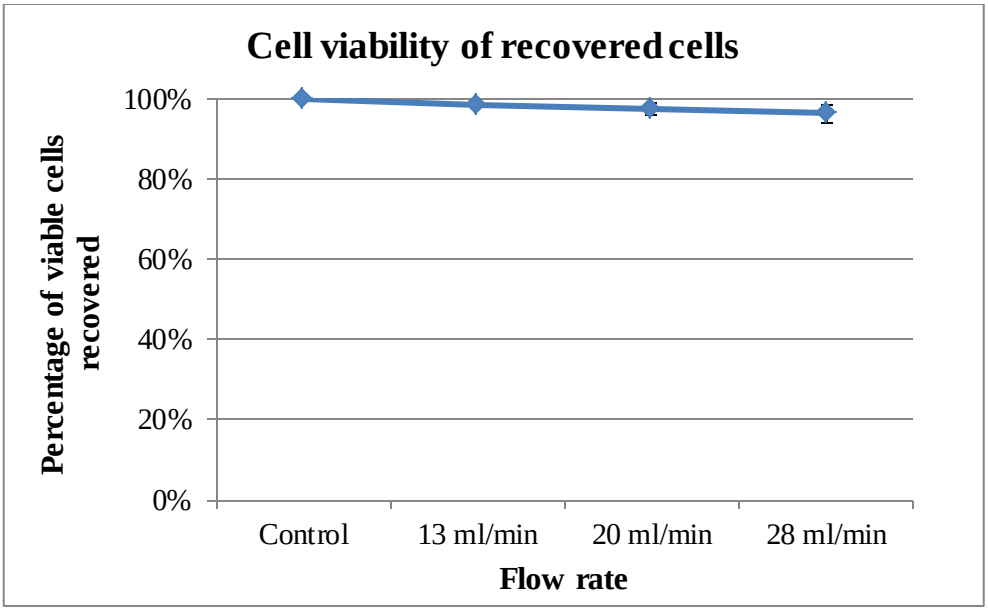


B

Figure 4.5: Effect of different flow rates (13, 20 and 28 ml/min) on cell loss of P12 MG63 cells. 26G capillary with length of 10 mm, 10 passes (A) Cell loss; (B) Different parts of total cells after exposure to capillary wall shear stress.



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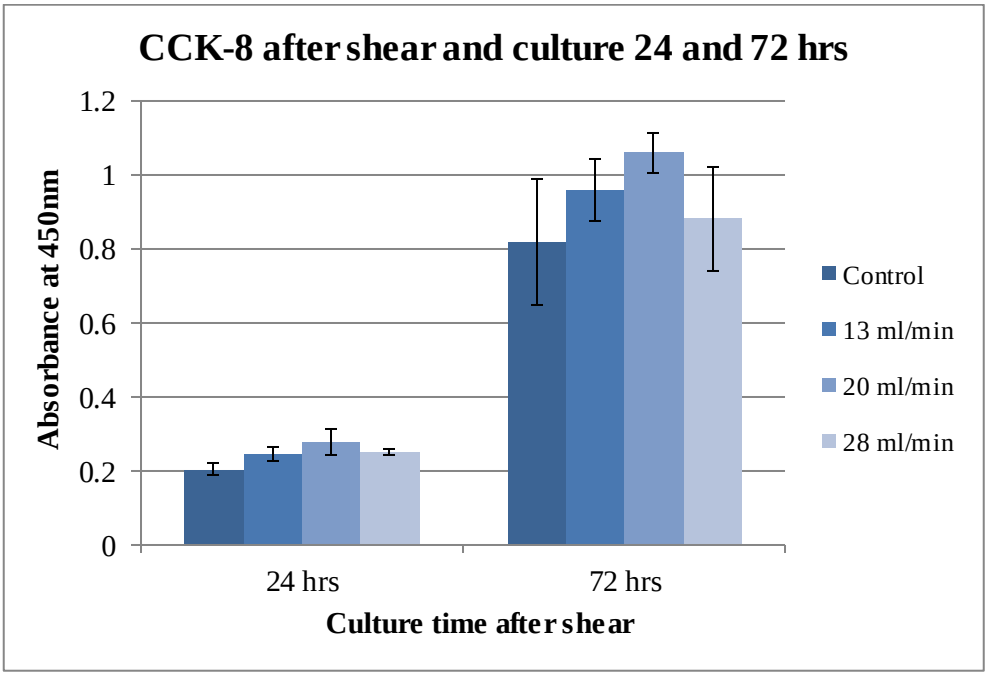


B

Figure 4.6: Effect of different flow rates (13, 20 and 28 ml/min) on cell loss and cell viability of P12 MG63 cells. 26G capillary with length of 10 mm, 10 passes. (A) Cell viability tested immediately after shear; (B) Cell viability after culture for 24 and 72 hours.

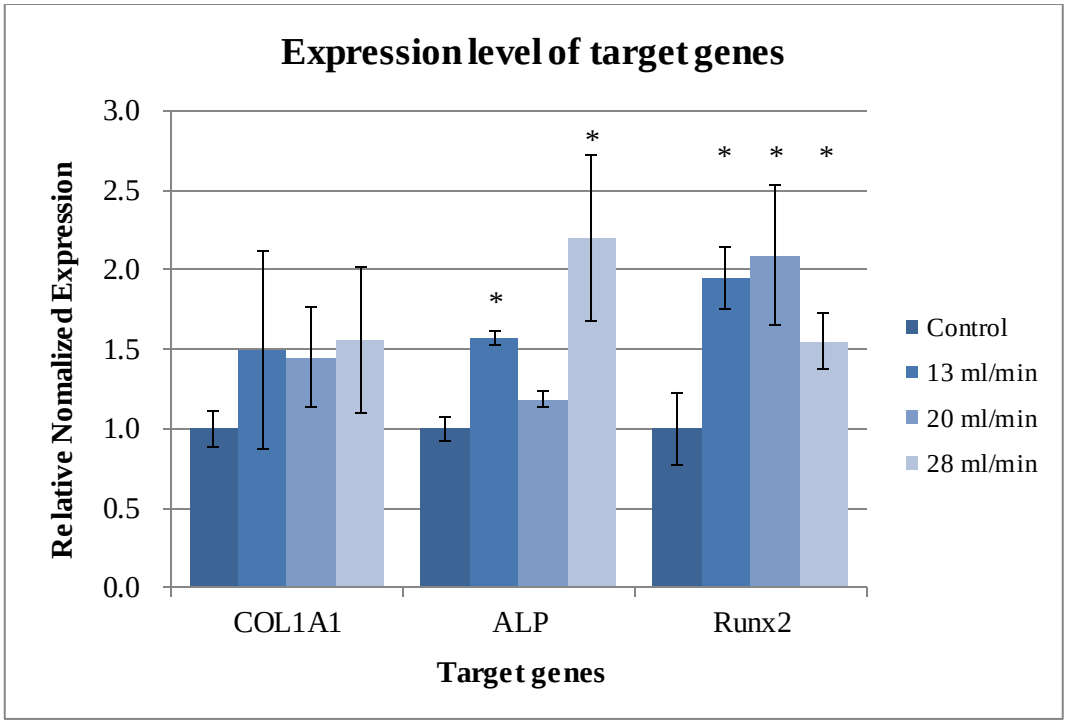


Figure 4.7: mRNA levels of COL1A1, ALP and Runx2 were measured after culture the sheared and non-sheared P12 MG63 cells for 72 hours (*P<0.05).

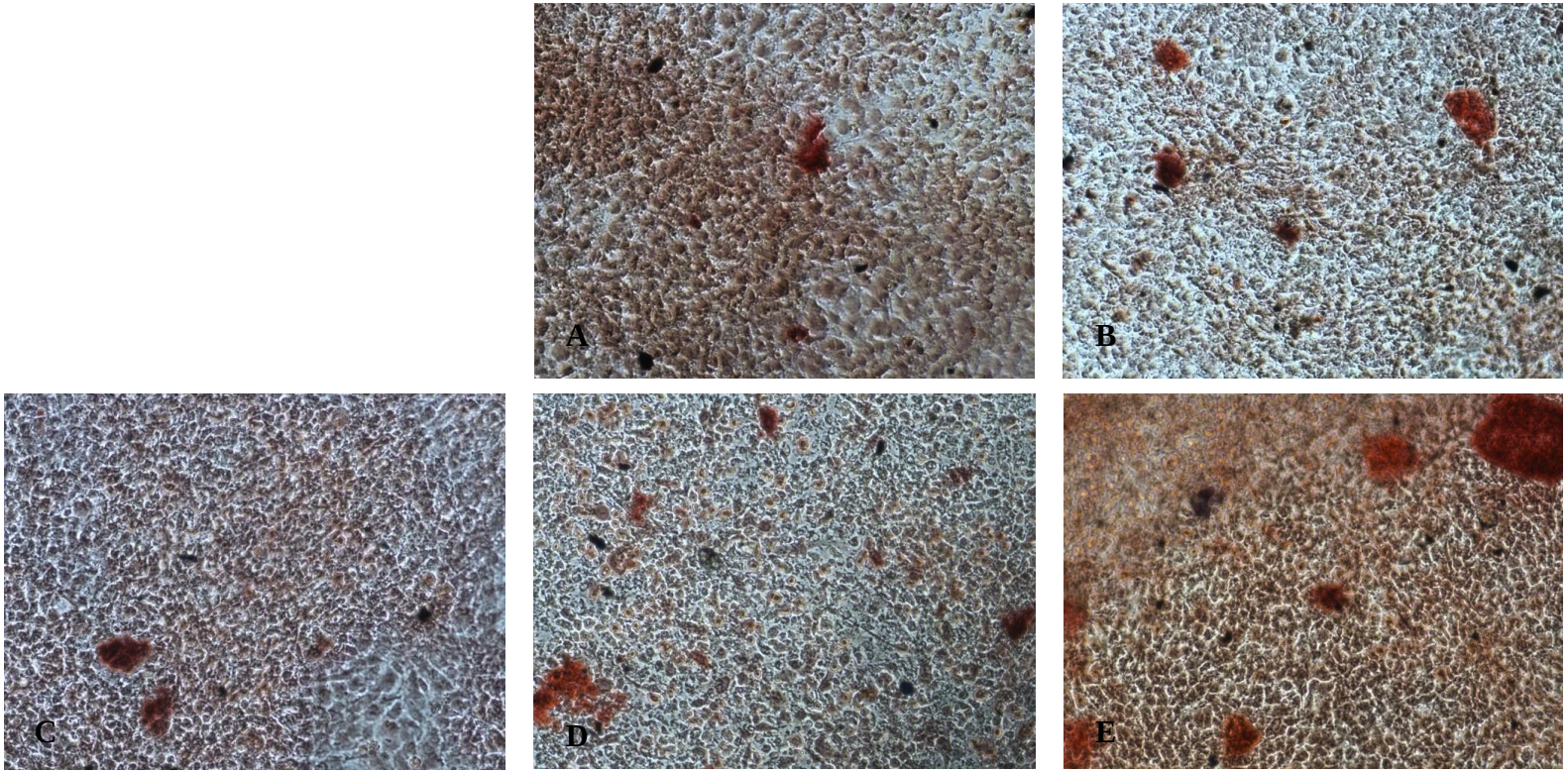


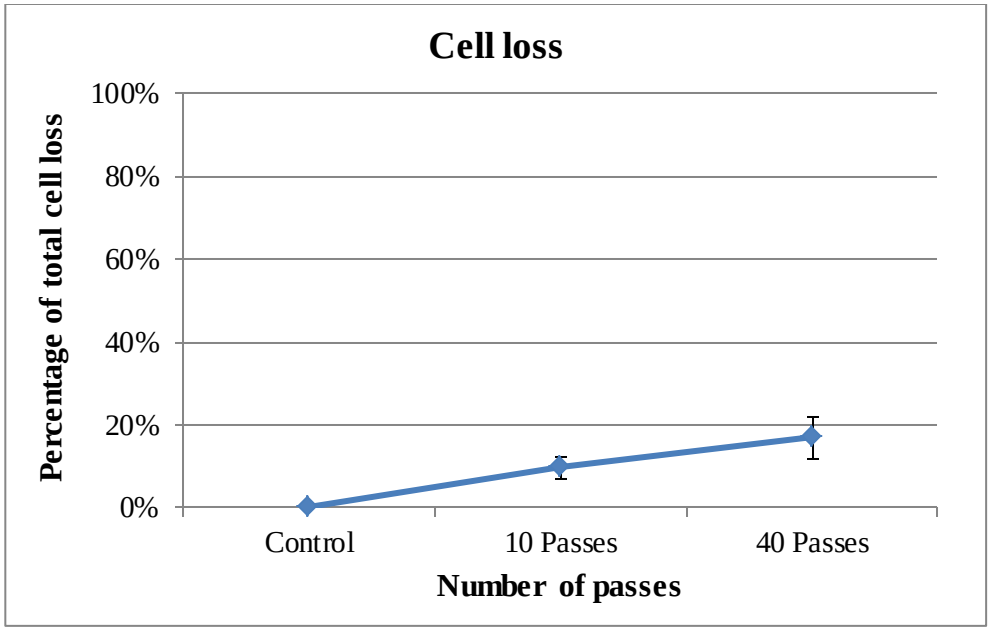
Figure 4.8: Sheared and non-sheared P12 MG63 cells were cultured in standard growth medium and osteogenic differentiation medium for 21 days, staining with Alizarin Red S dye. (A) Non-sheared cells cultured in standard growth medium; (B) Non-sheared cells cultured in differentiation medium; (C) Sheared cells (flow rate of 13 ml/min) cultured in differentiation medium; (D) Sheared cells (flow rate of 20 ml/min) cultured in differentiation medium; (E) Sheared cells (flow rate of 28 ml/min) cultured in differentiation medium (Scale bar = 100μm).

4.4. Effect of pass number

The effect of pass number on cell loss, cell viability and differentiation capacity was researched by passing passage 19 MG63 cells through 40 mm 26G capillary at 20 ml/min for 10 and 40 times. High pass number (e.g. 40 passes) implies a longer exposure time of cells to shear stress. Based on the data in Figure 4.9A, 40 passes (17%) caused more cell loss than 10 passes (10%), which suggests that more cells died and became cell debris during experiments. Results of cell viability and cell proliferation show that the negative effect of shear stress on cells is negligible. MG63 cells are stronger to maintain their integrity under shear stress compared with MSCs.

The quantitative reverse transcription PCR (qRT-PCR) was conducted after non-sheared and shear stress exposed cells were cultured for 72 hrs. Runx2 transcription was consistently in response to shear stress and was up-regulated significantly at 10 Passes, also a greater expression of ALP was found after 10 capillary passes compared to 40 passes. These data indicate that shear stress has a potential to induce osteogenic differentiation by enhancing the expression of bone related genes, the same conclusion has been reported in many other previous research (Haasper *et al.*, 2007; Kim *et al.*, 2007; Rangaswami *et al.*, 2009). However, type I collagen expressed early in the osteoblastic maturation process, was down regulated especially for 40 Passes, indicating that overall shear stress promoted a later stage of phenotype that bypassed COL1A1 production (Figure 4.11). Alizarin Red S staining assay was performed to examine calcified matrix after 21 days. The calcified matrix deposition capacity of MG63 cells was not as much as for MSCs, but positive staining results were still observed. Shear stress exposed cells generated more extracellular calcium deposits which were shown as red (Figure 4.12). Then, calcified deposits in extracellular matrix were quantified by calculating Alizarin Red concentration based on absorbance at 405 nm (Figure 4.13). Shear stress exposed MG63 cells that were cultured in osteogenic differentiation medium produced more calcified matrix compared to non-sheared cells at both pass numbers. MG63 cells sheared for 10 passes

formed more calcified matrix than those sheared for 40 passes, not only in osteogenic medium but also in standard growth medium, which was about 1.5 times of that produced by non-sheared cells. The increasing trend of mineralization was not linear to the increase of shear duration. This result indicates that appropriate shear stress can enhance osteogenic differentiation, but the long test time might bring long-time adverse effects on cell differentiation and there seems to be an optional level of shear stress that will promote differentiation. Same findings were shown in other studies, for example, in Bancroft's 3D perfusion research system and Grellier's parallel flow chamber research system (Bancroft *et al.*, 2002; Grellier *et al.*, 2009). Therefore, the pass number needs to be controlled in a reasonable range to maximize the cell differentiation and avoid unnecessary loss of cell function, especially when moving on to work with human MSCs which is more vulnerable to shear stress compared with MG63 cells.



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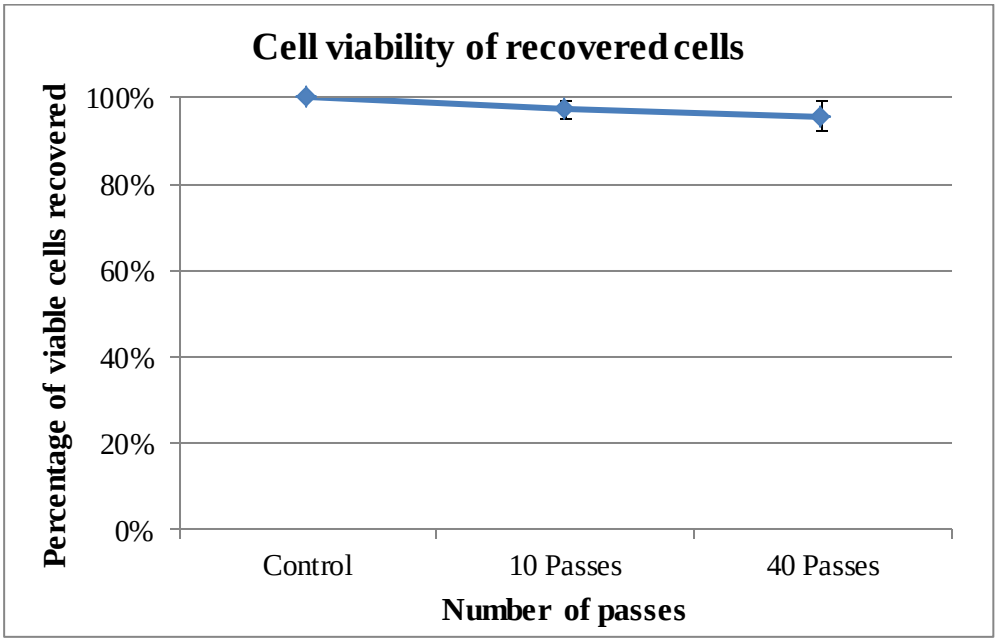


B

Figure 4.9: Effect of different numbers of passes (10 and 40 passes) on cell loss and cell viability of P19 MG63 cells. 26G capillary with length of 40 mm and flow rate of 20 ml/min. (a) Cell loss; (b) Cell viability tested immediately after shear.

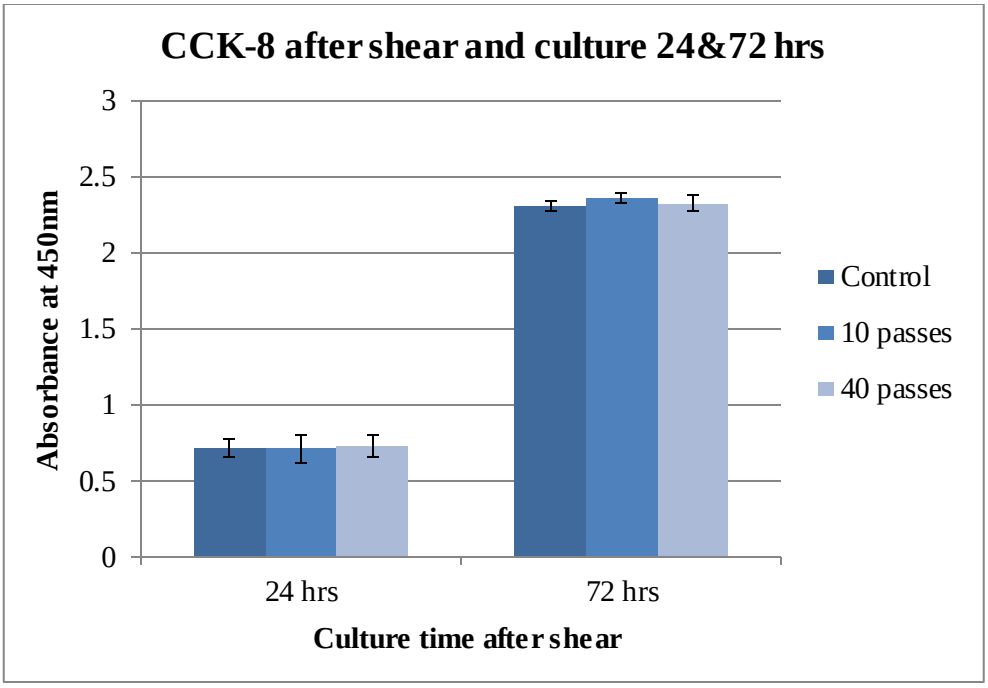


Figure 4.10: Effect of different numbers of passes (10 and 40 passes) on cell viability of P19 MG63 cells after culture for 24 and 72 hours. 26G capillary with length of 40 mm and flow rate of 20 ml/min.

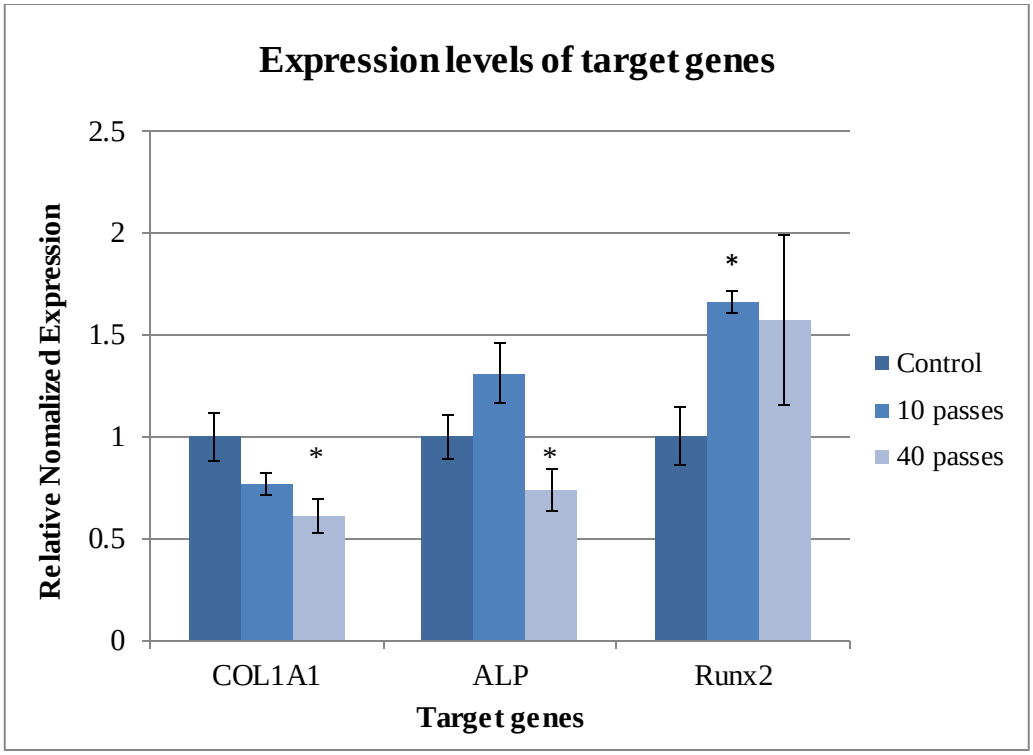


Figure 4.11: mRNA levels of COL1A1, ALP and Runx2 were measured after culturing the sheared and non-sheared P19 MG63 cells for 72 hrs (*P<0.05).

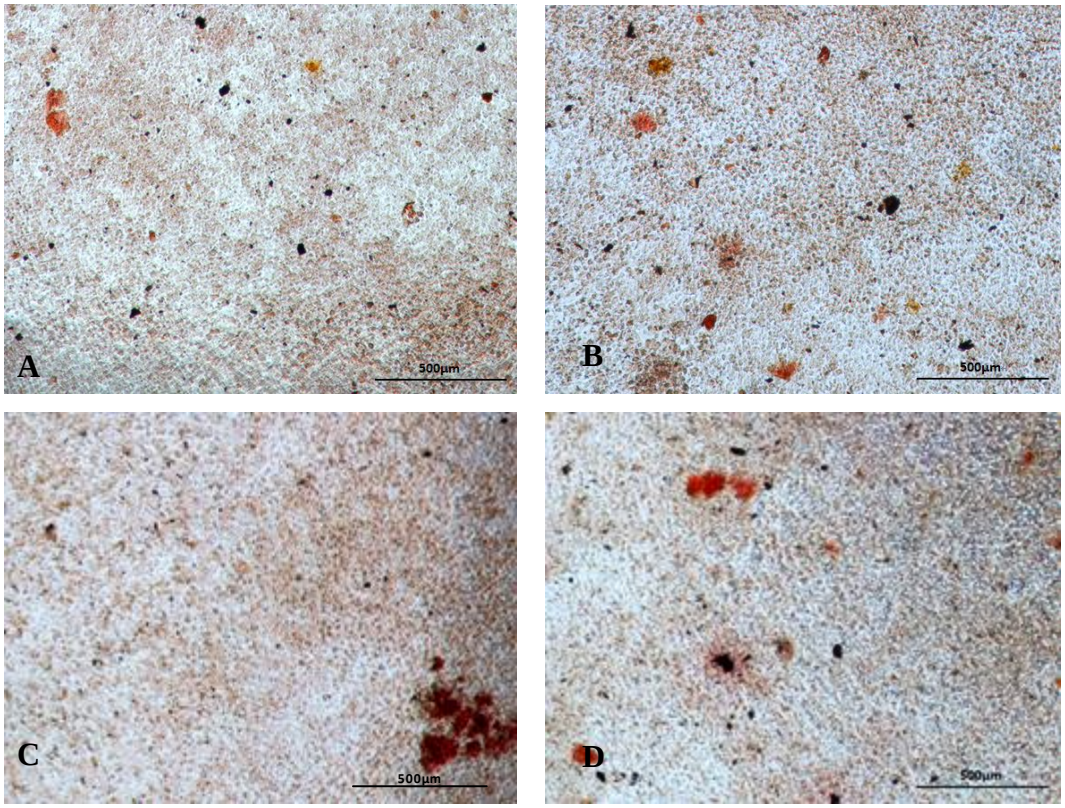


Figure 4.12: Sheared and non-sheared P19 MG63 cells were cultured in standard growth medium and osteogenic differentiation medium for 21 days, and stained with Alizarin Red S dye: (A) Non-sheared cultured in standard growth medium; (B) Non-sheared cells cultured in differentiation medium; (C) Sheared cells (10 Passes) cultured in differentiation medium; (D) Sheared cells (40 Passes) cultured in differentiation medium (Scale bar = 500 µm).

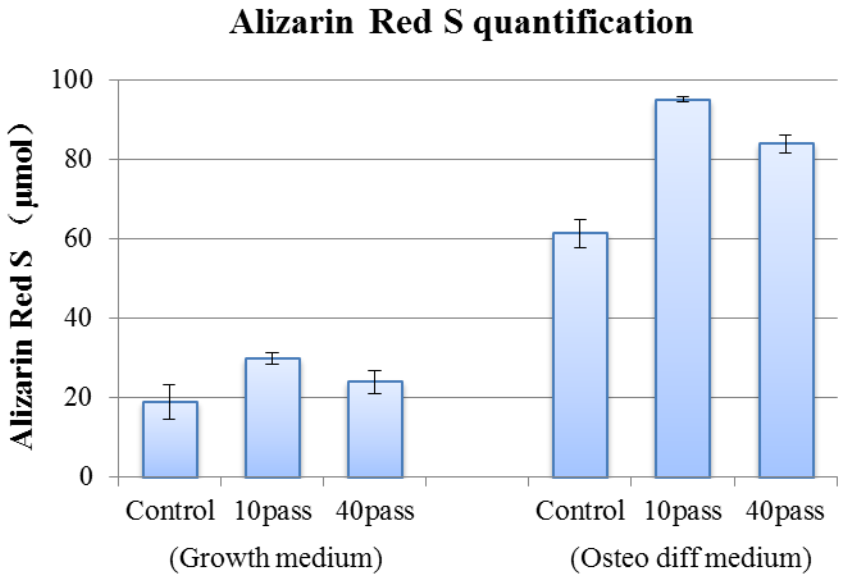


Figure 4.13: Alizarin Red S quantification of osteogenesis of both sheared and non-sheared P19 MG63 cells that were cultured in standard growth medium and osteogenic differentiation medium for 21 days.

4.5. Chapter discussion

MG63 cells were found to be more resilient than MSCs with respect to cell viability after exposure to shear stress. This is to be expected because mature osteoblasts experience FSS signals *in vivo* and respond favourably to them. However, in terms of defining a set of bioprocess parameters that might positively enhance bone cell responses, we identified duration of exposure to shear stress and flow rate as two key parameters that can influence osteogenic responses at the gene level and at the function level. By counting the number of viable cells and cells that remained in syringes, the effect of shear stress on cell loss has been further understanding. The total cell loss can be divided into three parts: the first part is cell lysis that due to the shear stress exceed bursting membrane tension; the second part is dead cells that stained by trypan blue dye because of the membrane leakage which is caused by shear stress; the third part is cells remaining in the capillary and syringes during experiment operation. This illustrates that the loss of total cell number can be reduced to some extent when operating with care, especially when increasing experiment scale. Although the highest shear stress (258 Pa) resulted in the most cell loss, remaining cells still maintained high viability. And after cultured these cells for 24 and 72 hrs, they still showed similar growth as non-sheared cells. The fatigue and apoptosis did not observe in survived MG63 cells, the negative effect of capillary wall shear stress on the proliferation of MG63 cells was negligible. In addition, there was no decrease in the differentiation capacity of cells that experienced shear stress. On the contrary, the osteogenic differentiation of cells was up-regulated by the positive effect of capillary wall shear stress based on the PCR and Alizarin Red S assay results. However, the up-regulation of mineralization was not linear to the increase of exposure duration of shear stress, which indicates that there is a limitation of the positive effect of shear stress on osteogenic differentiation. In general, results further verified the hypothesis that capillary wall shear stress can be used as a positive regulator for enhancing osteogenic differentiation without resulting in loss of a large number of cells.

Chapter 5 Exposure of human bone marrow derived mesenchymal stem cells to fluid shear stress

5.1. Introduction

All shear experiments were repeated by using human mesenchymal stem cells (hMSCs). These cells were isolated from patients' bone marrow, after expansion, passage 2 human MSCs were stored in the liquid nitrogen for long term use. Bone marrow derived MSCs have high capacity to differentiate into bone forming cells which makes them as a wildly used cell source for bone tissue engineering. Also, MSCs are advantageous because they can expand to increase numbers for autologous therapy.

5.2. Culture of human MSCs

Human MSCs were cultured *in vitro* based on their capacity to adhere to TC plastic. They possess a spindle-shaped morphology and possess high capacity to differentiate into bone, cartilage and fat cell. MSCs isolated from two different patients were expanded and labeled as GX09 and GX11. They were cultured in low glucose (1 g/L) Dulbecco's Modified Eagle Medium (DMEM; Gibco, UK), supplement with 10% Fetal Bovine Serum (FBS; Gibco, UK) and 1% Antibiotic-Antimycotic (100×; Gibco, UK), incubated at 37°C in 5% CO_2. Medium were changed every 3 days, and the subculture was performed when they reached 80% confluence. GX09 cells stopped growing at passage 5, and they became flat, enlarged and blebby, whereas GX11 cells grew well until passage 6. Therefore, GX11 cells were used to implement experiments. The Vi-cell data showed the average diameter of passage 5 human MSCs was around 19 microns and the considerably high viability (99.7%) exhibited their healthy growth.

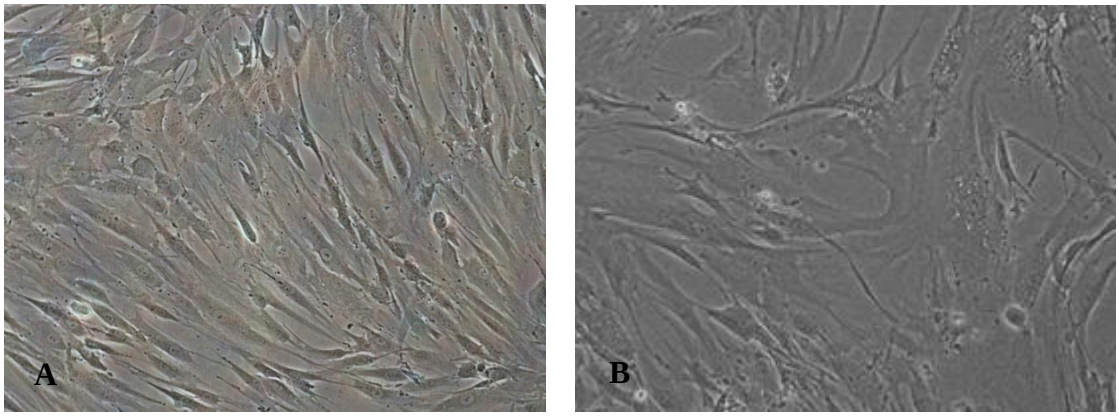


Figure 5.1: Passage 5 human MSCs from different patients cultured in T25 flask (A) label as GX11; (B) label as GX09 (10×).

5.3. Effect of flow rate

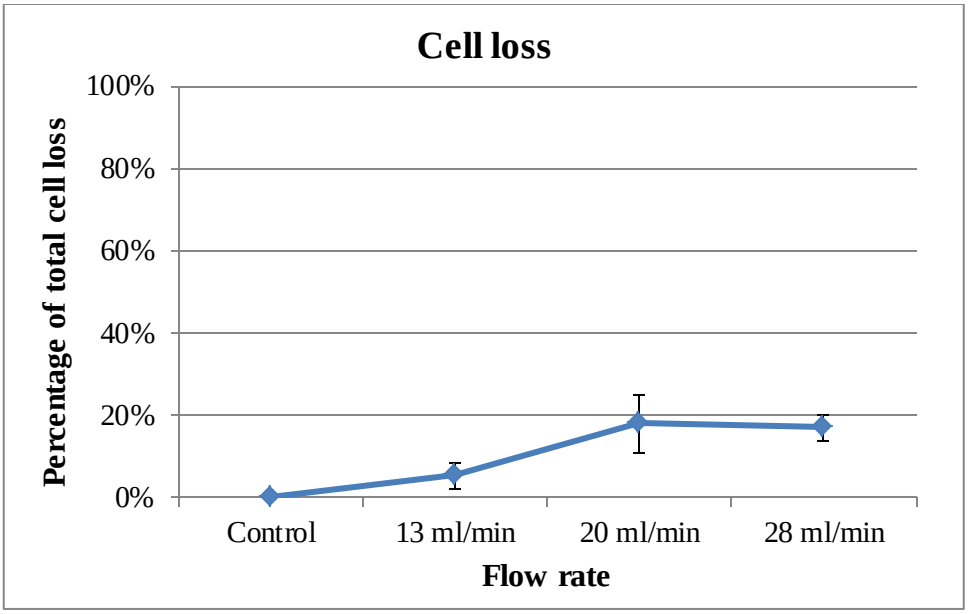
Passage 5 GX11 human MSCs were exposed to capillary wall shear stress by passing through a 10 mm 26G capillary at different flow rates (13, 20 and 28 ml/min) for 10 passes. The exposure time of each shear condition was recorded (Table 5.1).

The greatest cell losses were found at flow rates of 20 ml/min and 28 ml/min whereas low flow rate (13 ml/min) with low shear stress caused least cell loss. Almost all remaining cells were alive at the point of collection after shear (Figure 5.3A). However, cell viability data show significant reduction at 28 ml/min compared with control group after culture for 24 hrs, indicating that although cells appear viable immediately after recovery, the adverse effect of shear stress on cell proliferation reflects after a period of time, shear stress lead to cell fatigue that showed by slow growth of cells, it might be due to the change of cell metabolism that caused by shear stress induced cell membrane deformation and change of intracellular environment. Despite all this, both non-sheared and shear stress exposed cells show a similar level of cell growth after culture for 72 hrs, no apoptosis was observed, this illustrates that fatigue cells can recover from negative effect of shear stress and continue to grow back to their normal level (Figure 5.3B), the error bar shows the differences between repeated experiments, which might be because of different growth rates of different batches of human MSCs and their different withstand capacities to shear stress.

PCR results revealed that shear stress had an overall positive impact on expression of osteogenesis related genes but to varying degrees. Shear stress exposed cells exhibited elevated expression of alkaline phosphatase and type I collagen transcripts compared with non-sheared cells. Transcriptional levels of Runx2 increased a lot under the influence of higher shear stress (184 and 258 Pa) at higher flow rates (20 and 28 ml/min), and almost twice as high as those of non-sheared cells. This indicates that higher shear stress (184 and 258 Pa) has more positive effect on osteogenesis (Figure 5.4).

Flow rate (ml/min)	*Re*		*Capillary Wall Shear stress* (Pa)	Exposure duration
13	Laminar flow	1220	112	4 min 30 sec ± 30 sec
20		1877	184	3 min 30 sec ± 20 sec
28	Transitional flow	2628	258	3 min 10 sec ± 15 sec

Table 5.1: Shear stress and exposure duration of 10 mm 26G capillary at different flow rates (13, 20 and 28 ml/min) for 10 passes.



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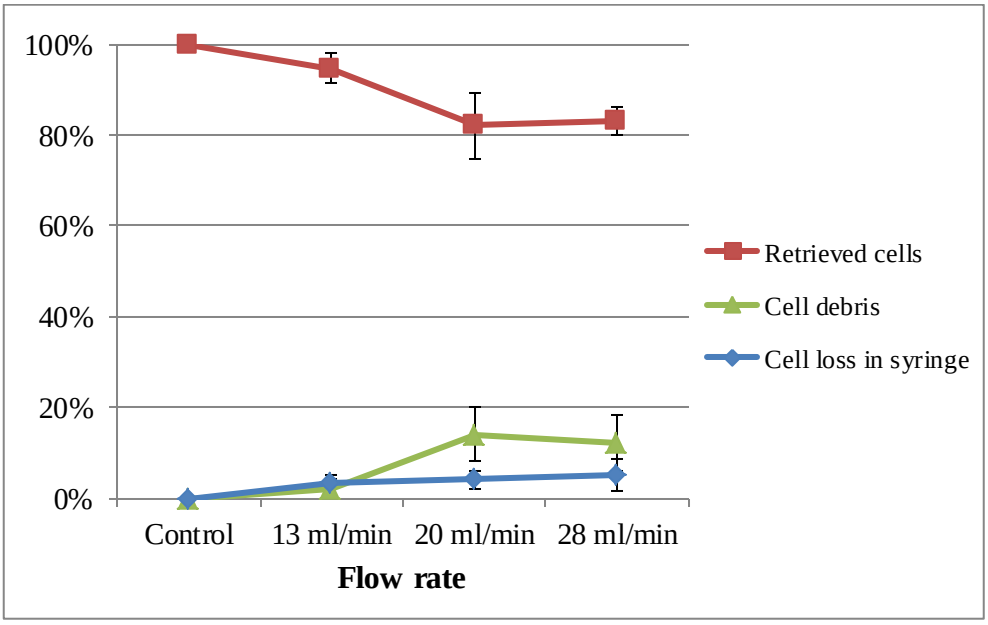
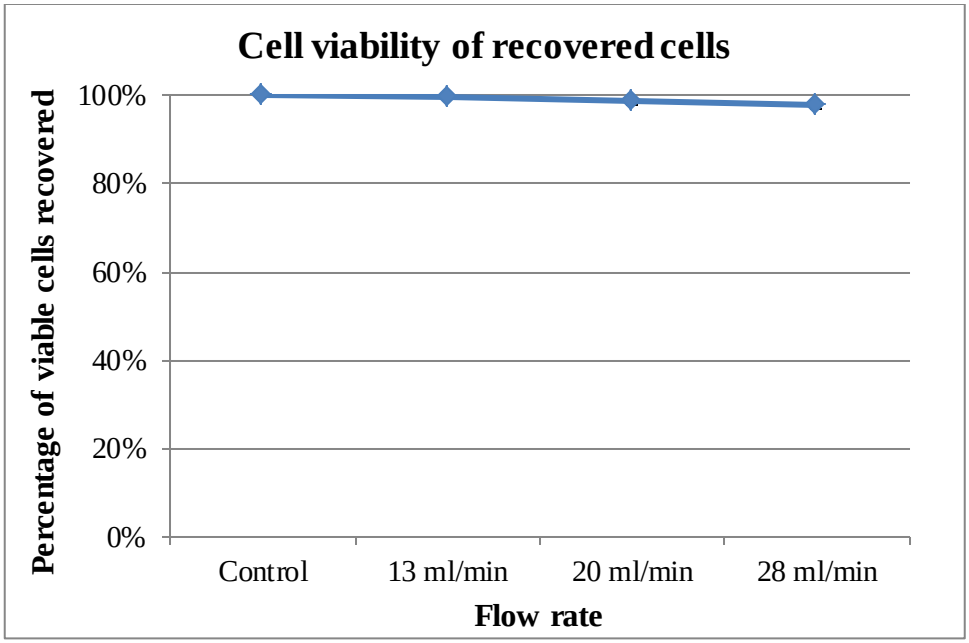


B

Figure 5.2: Effect of different flow rates (13, 20 and 28 ml/min) on cell loss of P5 GX11 human MSCs. 26G capillary with length of 10 mm, 10 passes. (A) Cell loss; (B) Different parts of cells after exposure to capillary wall shear stress.



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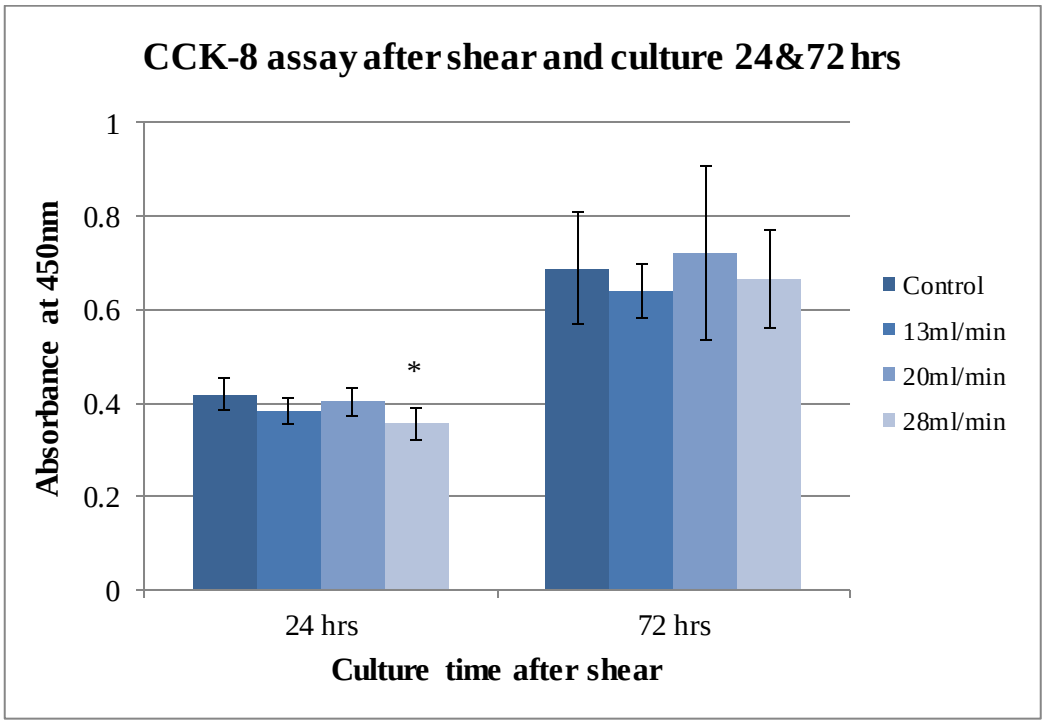


B

Figure 5.3: Effect of different flow rates (13, 20 and 28 ml/min) on cell viability of P5 GX11 human MSCs. 26G capillary with length of 10 mm, 10 passes. (A) Cell viability tested immediately after shear; (B) Cell viability after culture for 24 and 72 hours (*P<0.05).

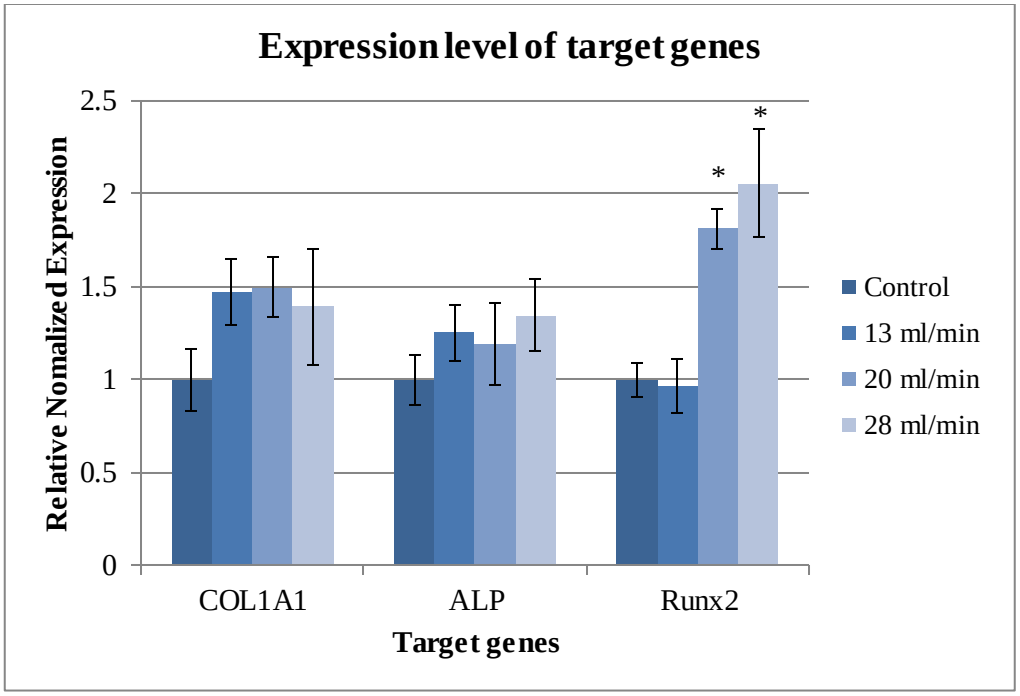


Figure 5.4: mRNA levels of COL1A1, ALP and Runx2 were measured after culture the sheared and non-sheared P5 GX11 human MSCs for 72 hrs.

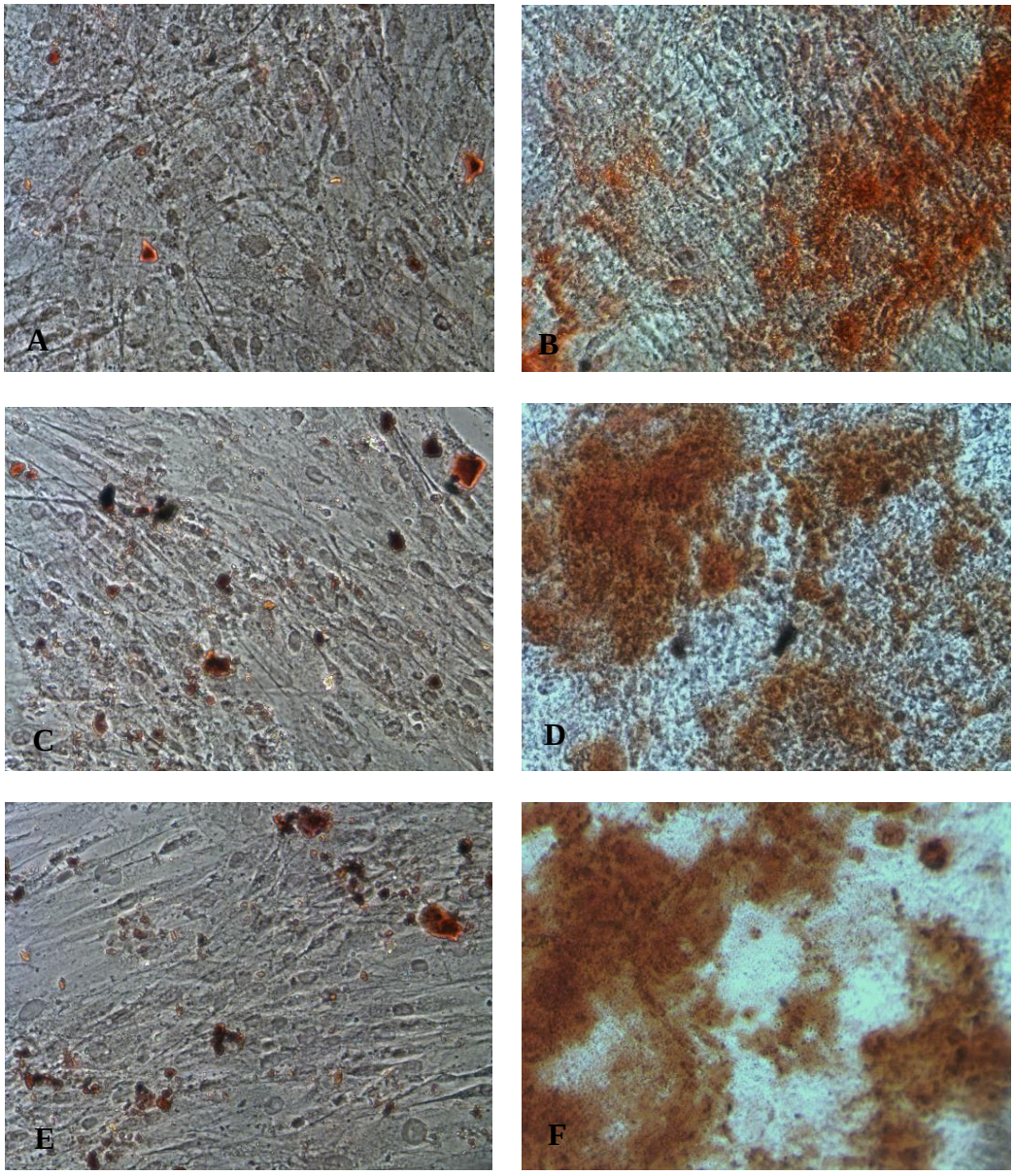


Figure 5.5: Both non-sheared and sheared P5 GX11 human MSCs were cultured in standard growth medium and osteogenic differentiation medium for 21 days, and then were stained with Alizarin Red S dye. (A) Non-sheared cells cultured in standard growth medium; (B) Non-sheared cells cultured in differentiation medium; (C) Sheared cells (at 20 ml/min) cultured in standard growth medium; (D) Sheared cells (at 20 ml/min) cultured in differentiation medium; (E) Sheared cells (at 28 ml/min) cultured in standard growth medium; (F) Sheared cells (at 28 ml/min) cultured in differentiation medium. (Note: no photo for 13 ml/min due to cell detachment and contamination).

After three weeks of culture, all cells that were cultured in the presence of osteogenic differentiation medium yielded positive staining with ARS, but only little red stain was observed in control wells that cultured in standard growth medium. Shear stress exposed cells produced more calcium depositions compared with non-sheared cells, and the highest shear stress (258 Pa) at flow rate of 28 ml/min resulted in greatest mineralization compared with the others. This illustrates that appropriate shear stress can induce MSCs to differentiate into osteoblasts, and the higher the shear stress the more differentiation (Figure 5.5).

The Alizarin Red S staining assay of another batch of P5 GX11 hMSCs showed a similar increasing trend of osteogenic differentiation that under the influence of shear stress. Cells that were cultured in differentiation medium showed positive staining results. And different batches of cells possess different levels of differentiation capacity. A morphology change of this batch of cells was observed, as cell sheets contracted and gathered together, especially those exposed to higher shear stress (184 Pa and 258 Pa). This structure could be a primary structure of bone formation. The highest shear stress (258 Pa) at flow rate of 28 ml/min induced the greatest mineralization (Figure 5.6E). This result further proved that appropriate shear stress has potential to induce osteogenic differentiation, and the magnitude of shear stress is important in the induction of bone regeneration.

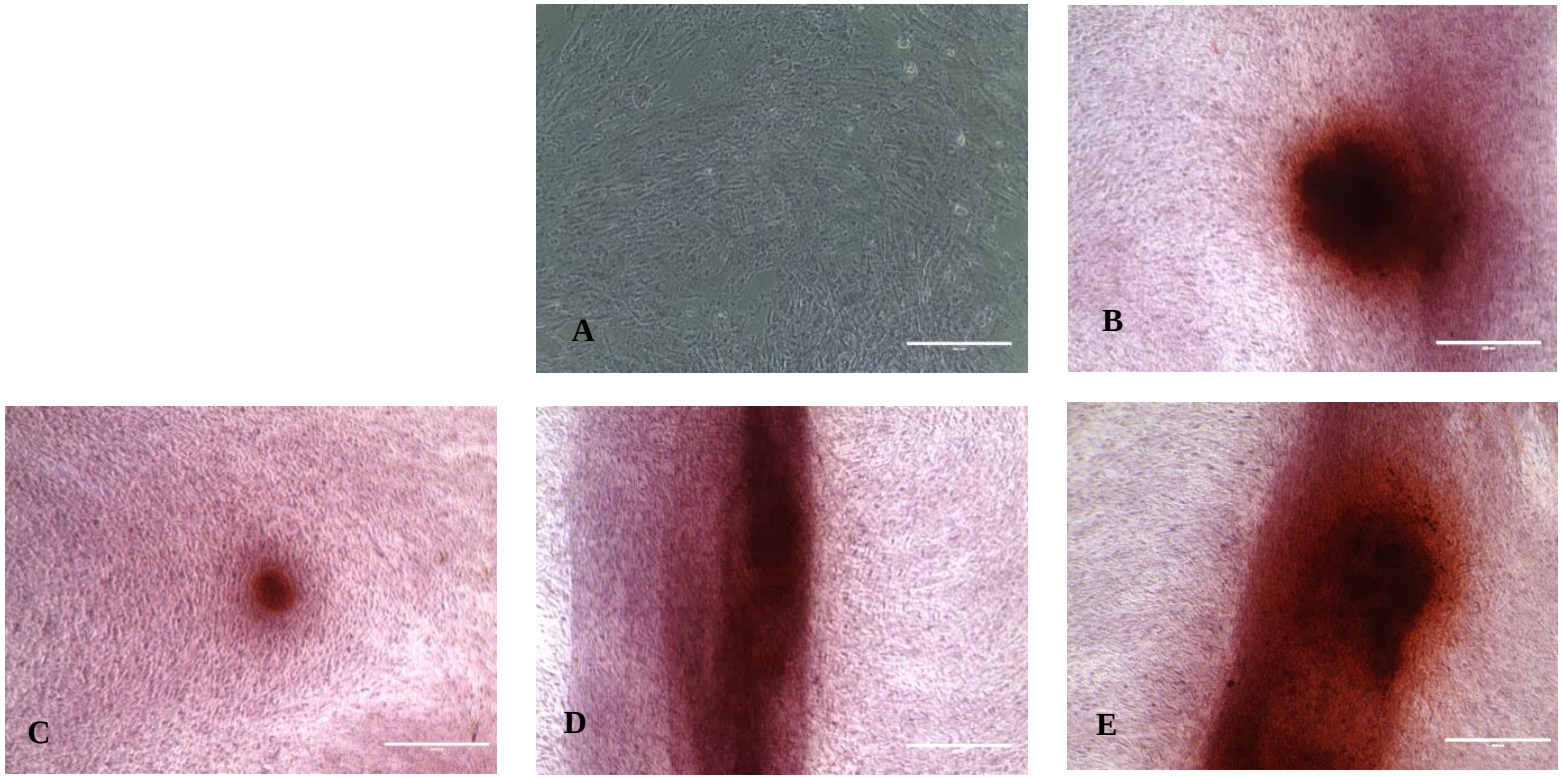


Figure 5.6: Both non-sheared and sheared P5 GX11 human MSCs were cultured in standard growth medium and osteogenic differentiation medium for 21 days, and then were stained by Alizarin Red S solution. (A) non-sheared cells cultured in standard growth medium; (B) non-sheared cells cultured in differentiation medium; (C) sheared cells (at 13 ml/min) cultured in differentiation medium; (D) sheared cells (at 20 ml/min) cultured in differentiation medium; (E) sheared cells (at 28 ml/min) cultured in differentiation medium (Scale bar = 100 μm).

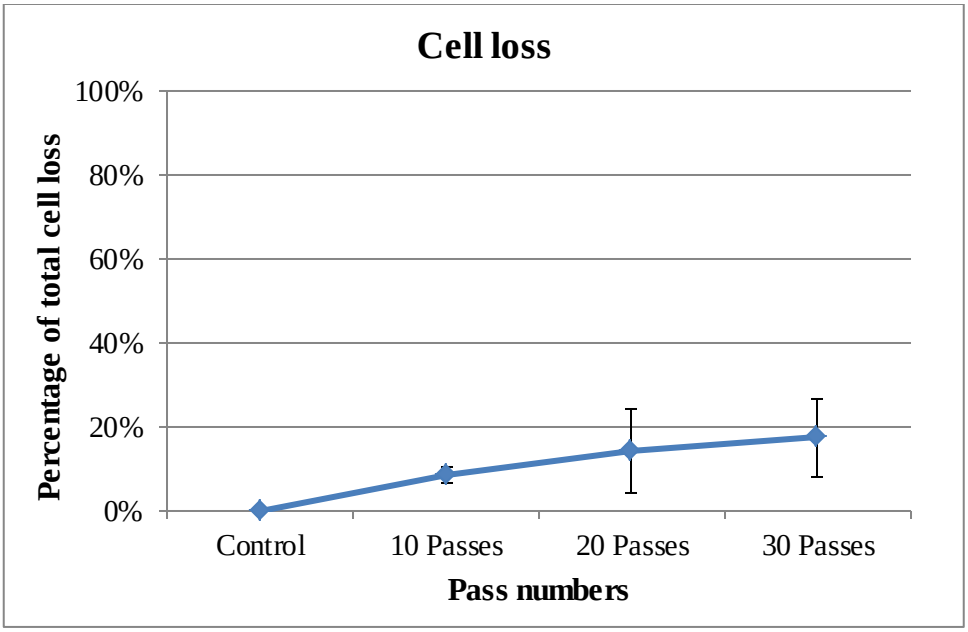
5.4. Effect of pass number

Different pass numbers were researched by passing passage 4 human bone marrow derived MSCs through a 10mm 26G capillary at flow rate of 20 ml/min for 10, 20 and 30 passes. More pass numbers lead to more shear stress exposure time (Table 5.2), the exposure duration for 30 passes is about three times of that of 10 passes, which means all cells experienced more influence of shear stress at higher pass numbers when the other parameters were constant. As revealed in Figure 5.2, 20 and 30 passes caused more total cell loss (14% and 18%), the large standard deviation might because of different capacities for withstanding shear stress of different batches of cells or instable manual operation. It shows that large proportion of cells was broken and became unrecoverable cell debris due to the harmful effect of shear stress (Figure 5.7B). The remaining cells that were collected immediately after exposing to shear stress had a very high viability that was more than 98% (Figure 5.8A). No apoptosis was detected after culturing both non-sheared and shear stress exposed cells for up to 72 hrs. Both shear stress exposed cells and non-sheared cells shows similar proliferation at both 24 and 72 hrs.

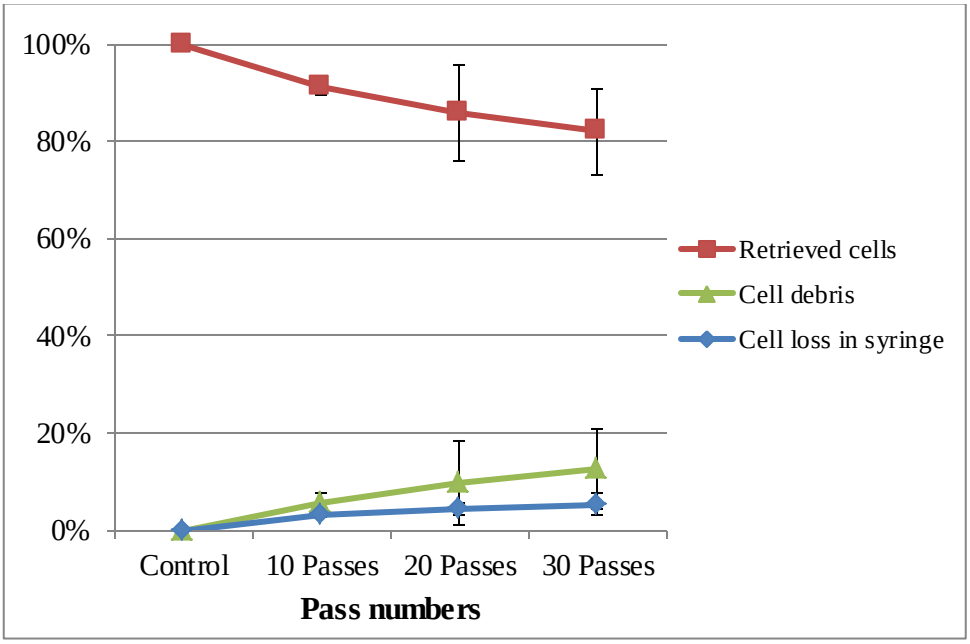
Osteogenic differentiation assay was conducted for 21 days, shear stress exposed cells exhibited considerably high capability of osteogenesis. Some parts of their cell monolayer contracted together and formed specific structure, which can be presumed as the basic architecture of bone formation. Cells that affected by capillary wall shear stress (184 Pa) for longer time at 20 and 30 passes showed more change of structure. One the contrary, non-sheared cells maintained a monolayer status even cultured with osteogenic differentiation supplements, also, cells that sheared for 10 passes displayed less structure alteration (Figure 5.9). This indicates that exposing human bone marrow derived MSCs to appropriate capillary wall shear stress for a proper length of time, their bone formation can be enhanced.

Flow rate (ml/min)	*Re*		*Capillary Wall Shear stress* (Pa)	Pass numbers	Exposure duration
20	Laminar flow	1877	184	10	3 min 30 sec ± 40 sec
				20	6 min 40 sec ± 35 sec
				30	9 min 30 sec ± 30 sec

Table 5.2: Shear stress and exposure duration of 10 mm 26G capillary at flow rate of 20 ml/min for different pass numbers (10, 20 and 30 Passes).

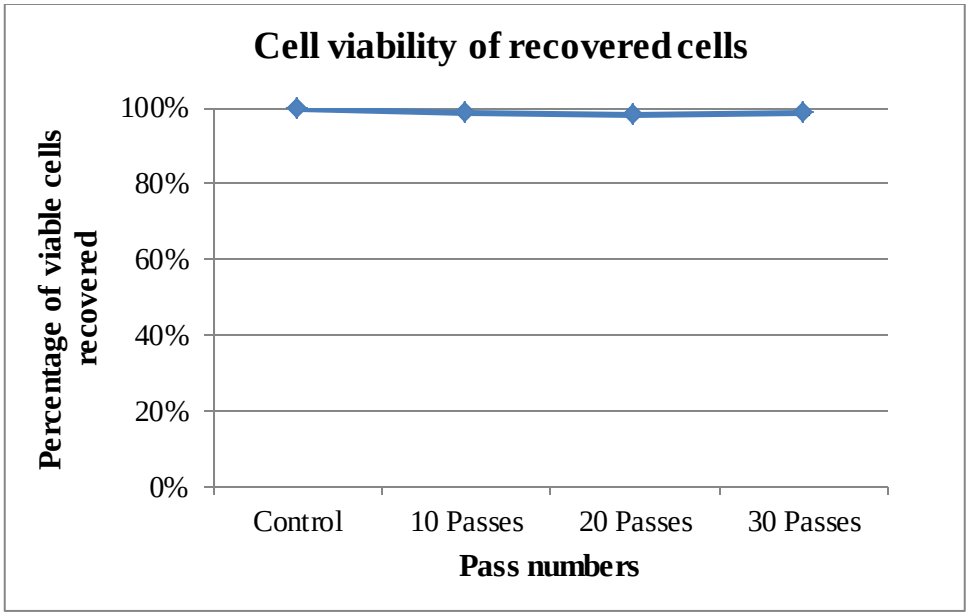


A

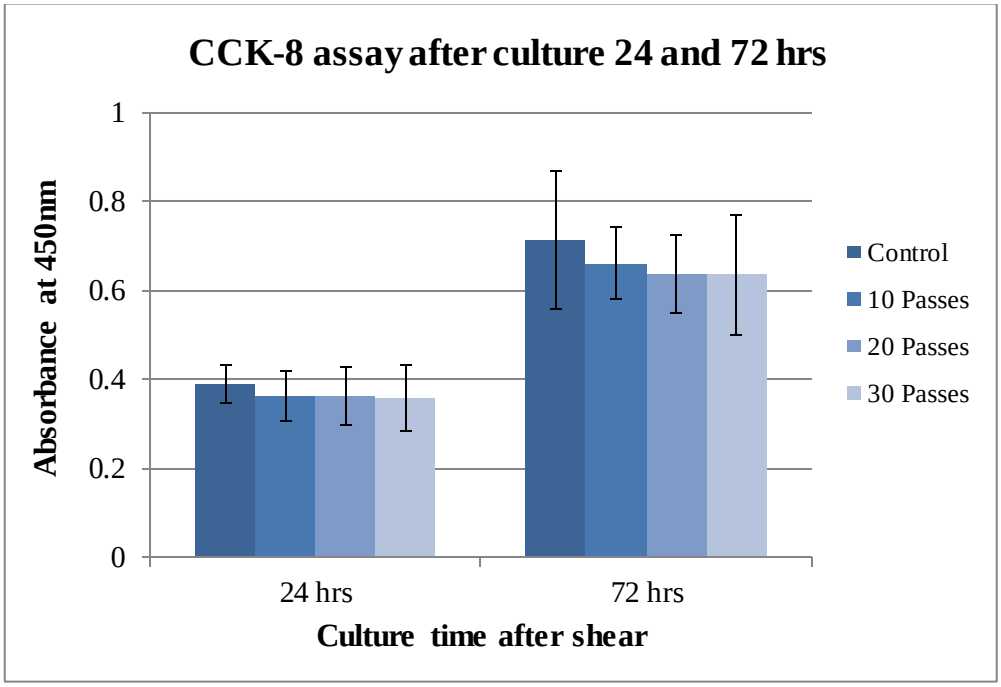


B

Figure 5.7: Effect of different numbers of passes (10, 20 and 30 passes) on cell loss of P4 GX11 hMSCs. 26G capillary with length of 10 mm, at flow rate of 20 ml/min. (A) Cell loss; (B) Different parts of cells after exposure to capillary wall shear stress.



A



B

Figure 5.8: Effect of different numbers of passes (10, 20 and 30 passes) on cell viability of P4 GX11 hMSCs after culture for 24 and 72 hours. 26G capillary with length of 10 mm, at flow rate of 20 ml/min. (A) Cell viability tested immediately after shear; (B) Cell viability after culture for 24 and 72 hours.

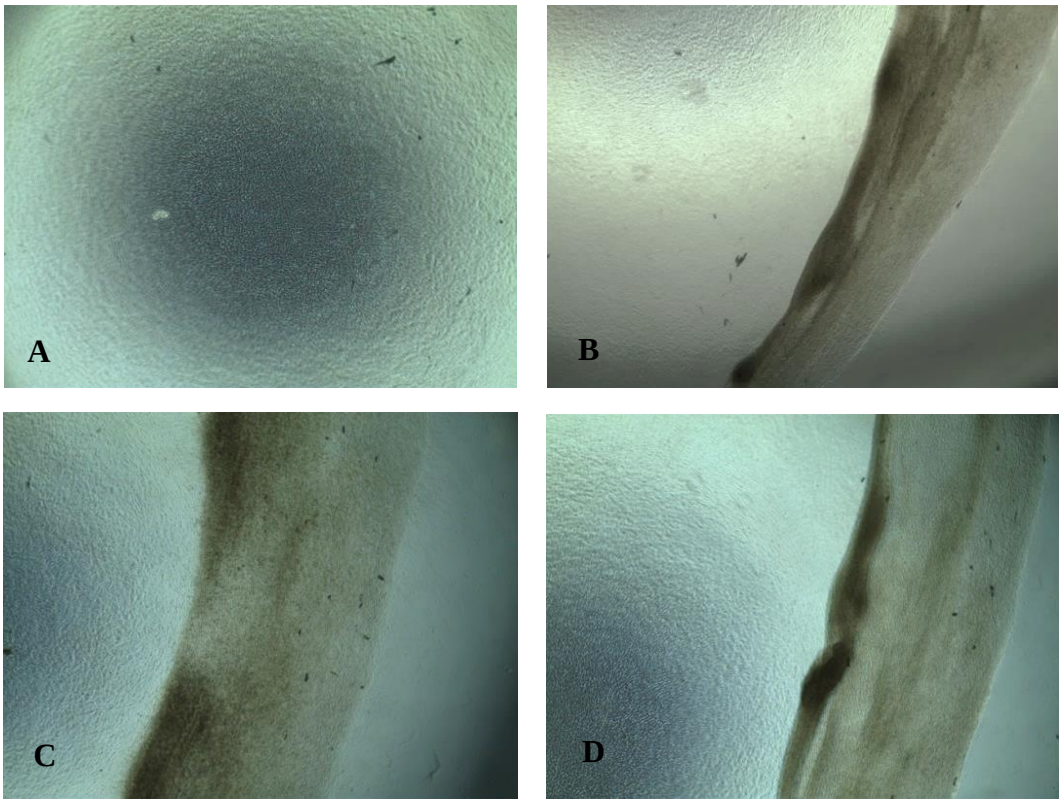


Figure 5.9: Both non-sheared and sheared P4 GX11 human MSCs were cultured in osteogenic differentiation medium for 21 days. (A) non-sheared cells; (B) sheared cells (10 passes); (C) sheared cells (20 passes); (D) sheared cells (30 passes).

Discussion

Three types of cells were examined in this thesis, which included rat bone marrow derived mesenchymal stem cells, human MG63 cell line and human bone marrow derived mesenchymal stem cells. Rat and human MSCs were found to be more delicate and sensitive to fluid shear stress signals compared to MG63 cells. MSCs from different rats/human bone marrow or from different isolation batches have different growth rates, which means the time interval for cells to achieve confluence is different. Also, growth of MCSs typically slow down with increase of passage number, so for example, passage 6 cells grow slower than passage 1 cells. Young passage MSCs expand rapidly, it takes less time to achieve confluence compared to older passage MSCs. Cells are spindle shape, and display elongated fibroblast-like morphology at low passage, whereas, high passage number cells become a flat and enlarged shape. Cell viability was assessed in this project by culturing cells in 96-well plates for 24 hrs and 72 hrs. There should in theory be a demonstrable increase in cell growth after 72 hrs compared to 24 hrs. However, sometimes the results of repeated experiments did not show the increase, this might be because of poor growth of cells, especially when this occurred in young MSCs. In addition, consider they need sufficient nutrition, growth factor and physical contact for proliferation, thus seeding density, medium change and instantaneous passage are important for healthy growth of cells. On the contrary, MG63 cells are more robust compared to MSCs, they grow well even at really high passage number, such as passage 20. One further reason for occasionally not seeing clear growth from 24 hrs to 72 hrs could be that the cells had already reached confluence and so could be contact inhibited and could not divide further due to space constraints in the dish. It was necessary to reduce the seeding density from five thousand cells to two thousand cells per 96 well for CCK-8 assay, in order to obtain real results for detecting how the capillary wall shear stress affects the cell proliferation.

The shear stress-inducing system that was used in this project supplied a relatively simple operation. The cell suspension (e.g. 4 ml, 3×10^5 cell/ml) was

loaded into the syringe and passed through the capillary under sterile conditions. Several parameters were studied and compared to assess the influence of capillary shear stress on cells. These included internal diameter of the capillary, flow rates, number of passes and capillary length. Any change of them will result in the alteration of intensity of shear stress that works on cells. Capillary size is a critical factor that should be studied first. Cells will not experience any effect if the size of capillary is too large, also it might cause more cell damage due to the crowded environment if the capillary is too narrow. The IDs of capillaries in this research were chosen as around 10 times of the average diameter of MSCs. When rat bone marrow derived MSCs were exposed to shear stress using capillaries of different sizes (25G, 26G, 27G) and other parameters remained the same, the 27G capillary, with a smaller ID compared to those of the 25G and 26G capillaries, resulted in more cell damage and cell loss when cell suspensions were passed though it for several times. This negative effect will become a huge issue when scales up the production. In addition, the pressure generated by passing cell suspension through 27G capillary could result in deformation of the capillary, especially at high flow rate. Therefore, the 25G and 26G capillaries were considered to be used to build the shear system to control the cell damage within a certain range.

Flow rate is another crucial parameter of shear experiments. For preliminary design, in order to reduce cell loss, all flow rates were set for laminar flow. Results showed that higher flow rates caused more cell loss than lower flow rates. A small decline in viability at 24 hrs was evident for MSCs passed through capillaries compared to controls. This is likely due to the onset of programmed cell death, which is not detected immediately after capillary passing. This is a critical observation because typically cells are delivered to a patient via a syringe and it is assumed that because cells have high viability at the point of delivery that the cells are therefore healthy. However, success or failure of cell therapy may relate to cell death that occurs in the hours after delivery due to shear stress triggering apoptosis. However, both non-sheared cells and sheared cells had similar level of cell viability after cultured of 72 hours, which indicates that cells that survived were able to overcome or

recover from the negative effect of shear stress, and they continually proliferate. This flow rate comparison experiment was repeated many times using different young passages MSCs from different rats. The Alizarin Red S staining assay, which was performed after culturing both sheared and non-sheared MSCs under osteogenic differentiation condition for 3 weeks, showed that High flow rate with high shear stress (e.g. 203 Pa, 258 Pa) resulted in more matrix mineralization compared to low shear stress (e.g. 120 Pa, 148 Pa). By contrast, human MG63 cells were stronger than MSCs, they exhibited more capability to adapt themselves to shear stress. Less cell loss and fewer dead cells were observed and even the cell proliferation capacity of both sheared and non-sheared cells after culturing for a period of time were still similar to each other. The PCR assay is a great method to reveal the effect of shear stress on cells at the transcription genetic level. The expression of bone formation related genes (e.g. ALP, Runx2, and CoL1a1) indicates that shear stress makes positive contribution to osteogenesis at varying degrees. Similar staining results as MSCs were found with high shear stress (258 Pa) at a flow rate of 28 ml/min induced more calcified matrix compared to low shear stress (184Pa) at flow rate of 13 ml/min. Although the differentiation potential of MG63 cells is limited, the staining results still show the increasing trend calcified matrix production as flow rate increases. By repeating experiments using human MSCs, the hypothesis of shear stress having a positive effect on bone formation was further confirmed, especially when applying a high shear stress at high flow rate. Human bone marrow exhibited more osteogenic differentiation capacity compared with other cell types. However, more cell loss was caused by high shear stress, the reason being that compromises in quantity of cells produced cannot be simply traded off against improvements in late differentiation efficiency.

Lastly, but also most important, is shear duration. Two parameters play a vital role in determining duration of shear, which are capillary length and number of passes through the capillary. The shear duration was varied between 3 minutes to 18 minutes based on the different choices of capillary length and number of passes. In spite of increasing number of passes causing greater cell loss, prolonged exposure to shear might result in more osteogenic

differentiation. However, the relationship was not linearly proportional. When duration of exposure reached 18 minutes (due to passing cells through 40 mm capillary for 40 times), it left cells in an unfavorable state, thus less calcium was deposited, which means less positive influence of shear stress on osteogenic differentiation. Therefore, to maximize the positive effect of shear stress on osteogenic differentiation, the combination of capillary length and pass number should be carefully chosen and optimized.

In general, data of whole experiments match the expectation, and the design of the system is reasonable and practicable. Using the capillary diameters, flow rates and pass numbers that have been reported here, cell growth is permissible in spite of an early reduction in viable cell growth. Shear stress shows potential to enhance osteogenic differentiation. Different from other research, which focuses on increasing bone formation by introducing low shear stress (e.g. 1.2×10^{-3} N/m^2; 1.6 dynes/cm^2)to MSCs for a long time (e.g. 10 days; 120 min) in 2D culture chambers and 3D perfusion systems (Scaglione *et al.*, 2008; Kreke *et al.*, 2005), this project developed a capillary shear system to create high shear stress (e.g. 258 Pa) to work on cells in a short time (e.g. 4 min), and the increase in osteogenic differentiation was achieved as expected. Also, for the future work, scale up of capillary size is possible, the capillary length is easily be increased, and thus this system can be used as an automated step in manufacturing processes to up regulate osteogenic differentiation. Briefly, after optimization of all parameters that related to determine shear stress, this will become a useful method with wide prospect in application to improve differentiation efficiency before MSCs are transplanted to patients.

Future work

Experiment results have proved our hypothesis that appropriate capillary wall shear stress has potential to induce osteogenic differentiation of MSCs. High magnitude of shear stress can result in more differentiation although it might cause more cell loss. Cells that experienced negative effect of shear stress,

which showed an early reduction in viability, can recover and continue to grow. To improve the design of the shear stress-inducing system and find a reasonable setting of all parameters (capillary IDs, capillary length, flow rates, pass number) that involved in determining the shear stress to minimize the cell loss and maximize the osteogenic differentiation are key objectives for the future research of this project.

A better understanding of cells response to shear stress can be obtained by introducing more specific biological and chemical analysis. For instance, recording images of trypan blue stained dead cells and cell debris, looking at apoptosis markers in cells after 24 hours, detecting alkaline phosphatase activity of both sheared and non-sheared cells, immunohistochemistry staining of osteocalcin and assessing the osteogenic differentiation by testing expressions of osteoblastic genes (e.g. osteocalcin, osteopontin and bone sialoprotein). A DoE approach can be used to analyse more variables and build a model to optimize parameters in the future. Also, looking at how cells interact with biomaterial scaffolds for bone repair after shear stress will be another aspect to be considered.

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