BONE TISSUE ENGINEERING FOR CORRECTING OF CLEFT PALATE DURING ORAL AND MAXILLOFACIAL SURGERY

A Thesis Submitted in Partial Fulfilment of the Requirements for the degree of Doctor of Philosophy

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LIST OF ABBREVIATIONS

%	percentage
A	alpha
β	beta
γ	gamma
2D	two dimensional
3D	three dimensional
AA	Ascorbic acid-2-phosphate
AB	Alamar blue
ADP	Adenosine diphosphate
ALP	Alkaline phosphatase
ALT/GPT	Alanine Aminotransferase/glutamine-pyruvate transaminase
ANOVA	Analysis of variance
AST	Asparate Aminotransferase
AT	Adipose tissue
βGP	β-glycerophosphate
BSA	Bovine serum albumin
BCM	Basal culture medium
BCM+Dex	Basal media supplemented with either 10nM or 100nM of Dex.
BM	Bone marrow
BMP-2	Bone morphogenetic protein-2
hBMSC	Bone marrow stem cell
Ca^{2+}	Calcium
$CaCl_2$	Calcium chloride
CD	Cluster of differentiation

c-fms	colony-stimulating factor-1 receptor
CLSM	Confocal Laser Scanning Microscopy
Cl^-	Chloride ion
CO_2	Carbon dioxide
COL1	Collagen type I
COX-2	Cyclooxygenase 2
Cu^{2+}	Copper
DAPI	4‟,6 diamidino-2-phenylindole
DCM	Dichloromethane
Dex	Dexamethasone
diH_2O	Deionised water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dsDNA	Double stranded Deoxyribonucleic acid
ECGF	Endothelial cell growth factor
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
FBS	Foetal bovine serum
FDA	Food and drug administration
Fe^{2+}	Iron ion
FGF	Fibroblast growth factor
FNB6	Immortalised human normal oral keratinocytes
GM-CFU	Granulocyte-Macrophage-Colony forming cells
hBMSCs	human mesenchymal stem cells derived from bone marrow

HCO_3^-	Bicarbonate ion
hESMPs	human embryonic stem cell mesenchymal progenitor cells
HLA-DR	Human Leukocyte Antigen - antigen D Related
HJPs	Human jaw periosteal cells
hPL	human platelet lysate
hPRP	human platelet-rich plasma
IGF	Insulin-like growth factors
IL	Interleukin
ITS	Insulin-transferrin-selenium
K^+	Potassium ion
M	Molar
MAPK	Mitogen-activated protein kinase
MEM	Minimum essential medium
N	Individual biological repeat
n	Technical experimental repeat
M-CSF	Macrophage Colony-Stimulating Factor
Mg^{2+}	Magnesium ion
Mn^{2+}	Manganese
Mo^{6+}	Molybdenum
mRNA	Messenger Ribonucleic acid
MSCs	Mesenchymal stem cells
MSC-NutriStem	MSC NutriStem® XF medium
Na^+	Sodium ion
NGF	Nerve Growth Factor
Ni^{2+}	Nickel
NOCM	Non-osteogenic culture medium

NOFs	Human normal oral fibroblasts
OC	Osteocalcin
OCM	Osteogenic culture medium
ON	Osteonectin
OPG	Osteoprotegerin
OPN	Osteopontin
PBMNCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PC	Platelets concentration
PCL	Poly (ε-caprolactone)
PDAF	Platelet-derived angiogenesis factor
PDGF	Platelet-derived growth factor
PDEGF	Platelet-derived epidermal growth factor
PF	Platelet factor
PG	PicoGreen
PMP	Platelet-derived membrane microparticles
pNPP	Para-Nitrophenylphosphate
PO_4^{3-}	Phosphate anion
PSG	Penicillin/streptomycin/glutamine
PS	Penicillin/streptomycin
RANKL	Receptor activator of NF-kappa B ligand
PRP	Platelet-rich-plasma
rpm	Revolutions per minute
RUNX2	Runt-related transcription factor 2
Se^{8+}	Selenium
SD	Standard deviation

Si^{4+}	Silicon
SO_4^{2-}	Sulphate ion
SR1	PeproGrow-1 serum-free cell culture supplement
TGF-β	Transforming growth factor-beta
TNF	Tumor necrosis factor
v%	Percentage by volume
VEGF	Vascular endothelial growth factor
Vitamin A	Retinol/retinoic acid
Vitamin C	Ascorbic Acid
Vitamin E	α-Tocopherol
Vitamin D	1,25-dihydroxyvitamin D
wt%	Percentage by weight
XF2	Human Mesenchymal-XF Expansion Medium (2% (v/v) human serum)
XF1	Stem X Vivo™ xeno-free human MSC expansion medium
XO	Xylenol orange
Zn^{2+}	Zinc

Summary

One of the most common craniofacial congenital deficiencies is cleft palate with an incidence of 1.7:1000 live births. The clinical management of cleft palate involves multiple surgical interventions and gives rise to post-operative complications. Recently, tissue-engineering approaches have been developed to treat cleft palate using osteoprogenitor (bone forming) cells seeded on biodegradable electrospun mat scaffolds to repair the defects.

Animal sera are frequently employed as a regular supplement *in vitro* for osteoprogenitor cell proliferation and differentiation in culture conditions. However, serious drawbacks have been recorded, such as contamination, poorly defined components, and disease transmission. To overcome these obstacles, xeno-free media have recently been developed. This project aims to test a set of commercially available xeno-free media or supplements in which human-derived cells (hBMSCs and HJPs) can be grown to create a bone-like matrix suitable for craniofacial tissue engineering.

The investigation showed that a commercially available medium containing 2% (v/v) human serum is best able to support human Bone Marrow Stem Cells (hBMSC) growth and differentiation among the media tested. However, there was noticeable donor variability in cell growth rates and osteogenic potential in the different media studied. Although 10% (v/v) foetal bovine serum is frequently employed for the proliferation of cells supporting *in vitro* growth, it was shown to poorly to support osteogenic differentiation compared to human-derived culture media.

Polycaprolactone scaffolds were fabricated by electrospinning and these were shown support osteogenesis and bone-like matrix formation in selected xeno-free media with differential effects on secretion of angiogenic growth factor (VEGF). A tri-layer PCL mat scaffold could support and separate three different cell types (Fibroblasts, Keratinocytes, and hBMSCs) to allow osteogenic differentiation on one side and create an oral mucosa on the other side indicating this would be a promising scaffold to incorporate the cell types needed for cleft palate repair.

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CHAPTER ONE: LITERATURE REVIEW

1.1 Introduction

Cleft palate and cleft lip pose a highly significant birth problem or defect with a global incidence of approximately 1.7 per 1000 live births (Mossey et al., 2009).

The causes of the defects include both genetic and environmental factors, the latter including maternal ailments, alcohol abuse or side effects from prescription medication involving anti-convulsants or retinoic acid based medications.

All these factors can influence initial embryonic development during the first trimester especially between the fourth to tenth weeks of pregnancy (Stanier and Moore, 2004). Cleft palate is a morphological abnormality that commonly affects the maxillofacial region (i.e. jaw and teeth) with effects on speaking, feeding, hearing, and social relationships. Recently, multidisciplinary teams, including, oral and maxillofacial surgeons, plastic surgeons, paediatric surgeons and dentists have managed the treatments of cleft palate. These teams can play a crucial role in supporting and providing some surgical operations to achieve the final result (from childhood to adulthood) (Moreau et al., 2007).

Conventional surgical operations for bone regeneration often use bone grafts that are an autograft, isograft, allograft, xenograft, prosthetic material and/or combination grafts. The autologous graft is the gold standard to achieve bone regeneration, but it has a limited use. Autologous graft causes morbidity; threatening the donor site, creating bone resorption, disturbance of gait, and prolonged numbness and/or paraesthesia of the leg, especially when taken from the lateral aspect of the femoral bone (Elsalanty and Genecov, 2009). (Bergland et al., (1986) and Samee et al., (2008) review and present the limitations of harvesting autologous bone for grafting. Allograft and xenograft have the limitations of processing steps which affect the bone's mechanical properties and risk of disease transmission. A substitute bone source for bone regeneration should be further investigated to enhance cleft palate repair.

In the past decade, tissue engineering systems have been trialled to correct malformations during facial growth (Smahel et al., 2009). The approach showed promise for reconstruction of the alveolar ridge which reinforces teeth during the management of

cleft palate. However, the presence of a connective and/or scar tissue between the grafted tissue engineered bone and the cleft palate resulted in impaired facial growth and development warranting further investigations (Ophof et al., 2008; Smahel et al., 2009).

This project aims to develop a bone tissue engineering methodology which is appropriate for eventual clinical by using xeno-free (free of non-animal derived ingredients) media for reconstruction of bony defects in the oral cavity, especially in the treatment of cleft palate. The long-term purpose is to develop approaches to culture stem cells in xeno-free conditions as a pre-culture for clinical applications in cleft palate repair by investigating: 1) primary human mesenchymal stem cells, 2) electrospun 3D scaffolds, 3) appropriate media and supplements for extracellular matrix formation by the bone-like cells. The following literature review will focus on the fundamental principles of bone biology, cleft palate development, advance treatment of cleft palate, and current clinical strategies for bone tissue engineering.

1.2 Bone structure

1.2.1 Function and anatomy bone

Bones are a rigid part of our body and have blood vessels which preserve living cells and promote them to grow. They provide several functions such as movement, support of body structures, internal organ protection, and mineral homoeostasis. They store crucial elements which are essential for bone development such as growth factors, and cytokines; the red bone marrow houses blood-creating cells. A newborn human has around 300 bones while the human adult typically has 206 bones. Bone structure is comprised of two fundamental parts: cortical (compact) bone and trabecular (cancellous) bone. Cortical bone is a dense part of the skeleton with less than 5% porosity (Clarke, 2008), and is comprises 80% of healthy adult bone. In contrast, the trabecular bone is mainly porous with around 50-400 μm of strut thickness (Seeley et al., 2008) and corresponds to around 20% of healthy adult bone (Roger, 2011). Trabecular bone is the internal tissue of the bone filled with bone marrow.

1.2.2 Macroscopic anatomy

Bones are classified according to their function into four different shapes- long, short, flat, and irregular. The first includes the femur of the leg and humerus of the arm while the

second contains bones of the ankles and wrists. Flat bones include the craniofacial bones and ribs (Figure 1.2.2). The vertebra, coccyx and sacrum bones are irregular bones. The long bones are organised into three main parts: the diaphysis is a hollow part of the central region and is composed of cortical bone, the metaphysis is flared with a cone shape, and the epiphysis is an expanded portion which is located at each end of the long bones, and coated with articular cartilage. Flat bones have a significantly large surface to improve protection and muscular attachment specifically, craniofacial bones.

The outer surface of bones is firmly coated with a periosteal membrane which is essential for bone apposition on the exterior surface of the bone. The endosteal layer contacts the bone marrow with covers the interior surface of compact and cancellous bones. Furthermore, the endosteal sheet is essential for bone formation and resorption on the interior surface (Figure 1.2.2).

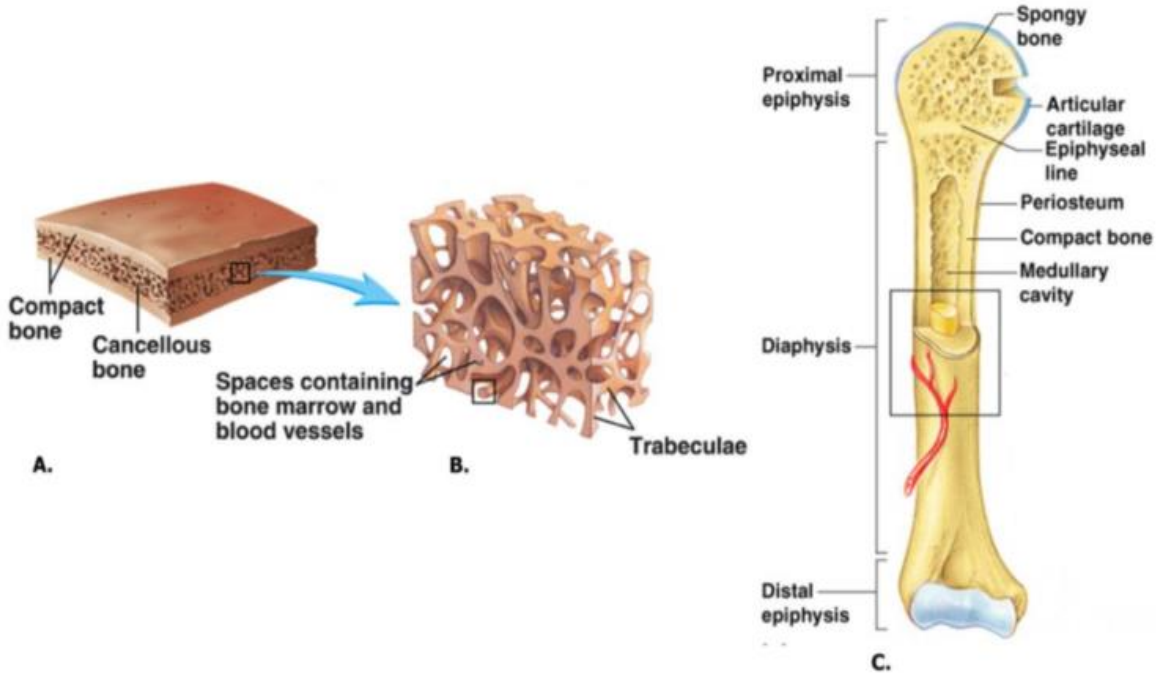


Figure 1.2.2: *(A) The diagram reveals a flat bone which comprises of compact and cancellous bone layer, (B) layer of spongy bone between the compact bone layers, (C) a major structure of a long bone consists of diaphysis and epiphysis. Reprinted from Seeley et al., (2008) with kind permission of McGRAW-HILL*

1.2.3 Microscopic anatomy

Bone is categorised into two types depending on the microscopic level, lamellar and woven bone (fibrous bone). Collagen type I is the main protein present in the bone matrix. Woven bone is distinguished by a random collagen fibre organisation which is mechanically weak, it is an immature bone and forms quickly. It is formed when osteoblasts construct osteoid rapidly and is considered to be the initial bone produced after fracture, more flexible than lamellar bone (Clarke, 2008). Lamellar bone is secondary bone formed after woven bone remodelling which has a regular dense well-aligned collagen and is mechanically robust. After one month of birth, human lamellar bone starts remodelling and a majority of the bone structure converts into lamellar bone by 4 years of age. The concise arrangement of individual lamella leads to the production of osteons surrounding a central canal which houses blood, lymph vessels, and nerves at the core to form a secondary osteon or Haversian system. A Volkmann's canal is a small canal located in the bone which transmits the blood vessels to the bone and communicates with each Haversian canal. Lacunae are the spaces which house the mature bone cells, termed osteocytes, located between lamellae. Osteocytes communicate with other osteocytes through long, thin channels known as canaliculi, through which nutrients can also pass. Haversian system and osteons represent a major structural component of the bone and line up parallel up to the long axis of the bone to enhance the mechanical strength. Cortical bone is produced from several osteons, which unite with circumferential (beneath periosteum and endosteum) and interstitial lamellae (between osteon) (Doll, 2005). Osteons are the essential structure of compact and cancellous bones (Clarke, 2008). There are no blood vessels at the centre of trabecular bone's osteon (Figure. 1.2.3) (Seeley et al., 2008).

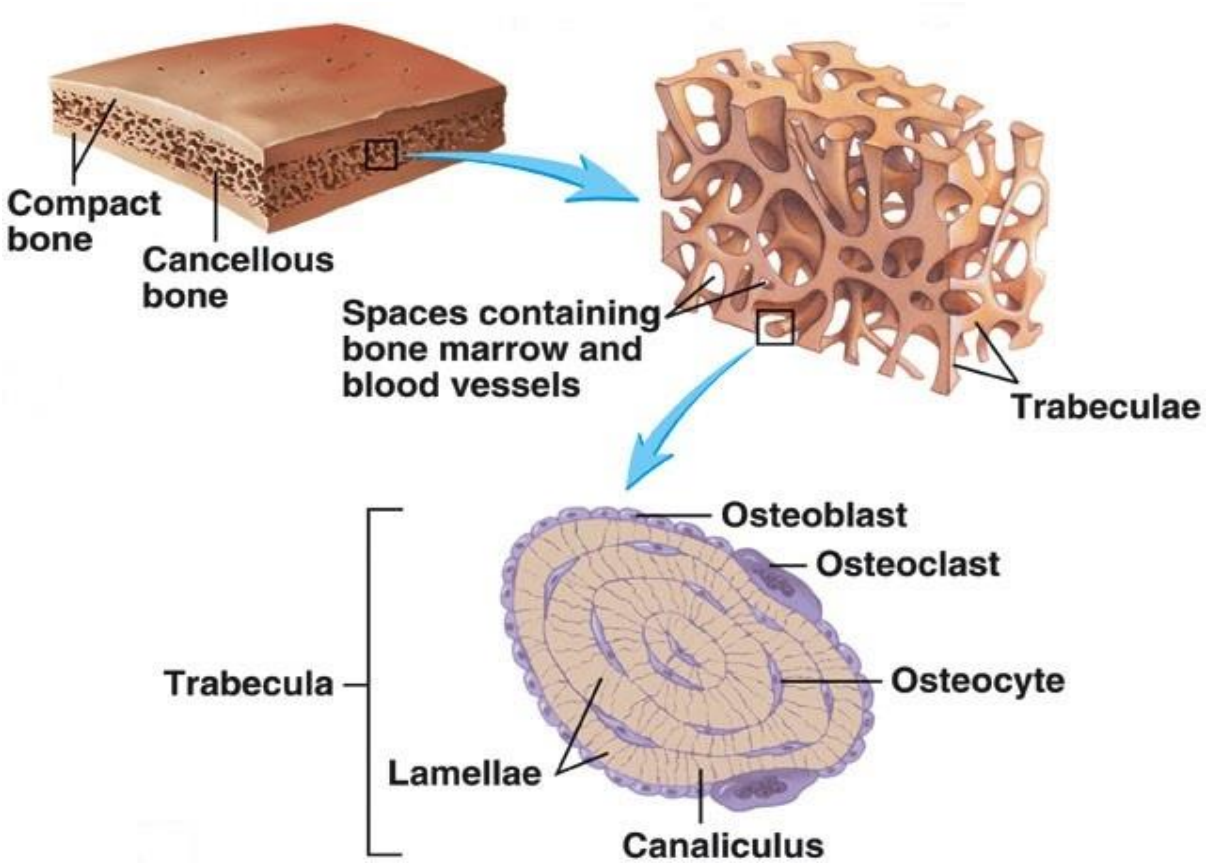


Figure 1.2.3: *A transverse region of the trabecular bone illustrating an osteon. Reprinted from Seeley et al., (2008) with kind permission of McGRAW-HILL*

1.2.4. The bone extracellular matrix composition

The substantial components of the extracellular matrix (ECM) of bone are the inorganic constituents (about 60%) and the organic matrix (up to 40%) together with trace quantities of lipids and water. The inorganic component is predominantly hydroxyapatite with a trace amount of carbon and magnesium which contributes to the mechanical compressive strength of bone. The organic matrix consists of non-collagenous proteins and collagen. Bone proteins are divided into about 90% collagenous which are mainly collagen type I and a minimal amount of Type III and V, collagen type I contributes to the tensile strength of bone. The remaining bone proteins are non-collagenous proteins which represent 10% of the total organic matrix and are composed of cytokines, matrix proteins, proteoglycans, and growth factors. Proteoglycans are ubiquitous molecules that consist of a core protein, covalently linked to either one or several glycosaminoglycan (repeating sugar unit) chains. The main functions of bone matrix are to provide structural support and to regulate the activity of bone cells, bone mineral deposition and bone

resorption. Furthermore, growth factors and cytokines are presented in a bone matrix such as bone morphogenetic proteins (BMP), transforming growth factor (TGF) and vascular endothelial growth factor (VEGF). These growth factors regulate bone cell proliferation, differentiation, angiogenesis and bone remodelling (Clarke, 2008; Lamoureux et al., 2007; Salbach et al., 2012).

1.3 Bone cell biology

Bone is a dynamic organ that contains many active different cells for processing of formation and resorption to maintain the stability throughout life such as osteoprogenitor cells, osteoblast cells (which form bone matrix), osteocyte cells (which support bone matrix), and osteoclast cells (which resorb bone matrix) (Martin et al., 1988).

1.3.1 Osteoprogenitor cells

Osteoprogenitor cells are a population of undifferentiated, mesenchymal stem cells, characterised by a fibroblast-like fusiform shape *in vitro* (Figure 1.3.1). They are derived from primitive mesenchymal stem cells which give rise to the more specialised bone-producing cells to form a new bone matrix. Bone marrow stroma contains mesenchymal stem cells that can differentiate into a variety of lineages such as bone, cartilage, or fibrous connective tissue depending on the supplementation (James and Anderson 2015). Mesenchymal stem cells are useful for bone tissue engineering because of their ability to osteogenic differentiation.

1.3.2 Osteoblasts

Osteoblast cells are large cuboidal cells which are located along bony surfaces covering 4-6% of the whole bone and are derived from mesenchymal stem cells. They communicate with bone cells, and bone matrix through specific receptors for activation of signalling pathways such as growth factors, hormones, and cytokines to encourage the cell function (Florencio-Silva et al., 2015). *In vivo*; osteoblasts can produce 0.5-1.5 µm of osteoid per day which is a non-mineralised organic portion of the bone matrix (Sommerfeldt and Rubin 2001). An early sign that a cell is becoming a preosteoblast is activity of the enzyme alkaline phosphatase. Eventually, the osteoblasts become trapped in the calcified matrix and transform their phenotypes to form osteocytes (Sommerfeldt

and Rubin, 2001; Florencio-Silva et al., 2015).

1.3.3 Osteocytes

Osteocytes are the most abundant of bone cells which comprise about 90-95% of total bone cells and represent the osteoblasts differentiation terminally. They are smaller than osteoblasts and have abundant cytoplasmic extensions number. Osteocytes are located within lacunae in the calcified bone matrix. Once the maturation of osteocytes occurs, several osteoblast markers are downregulated. The osteocyte's functions are to enhance intercellular communication and to regulate the mineral exchange within lacunae. Osteocytes can interconnect with adjacent cells by their cytoplasmic extensions and can survive in the lacunae for up to 25 years. In aged bone, an empty space in lacunae can be observed due to the cell apoptosis or cellular interaction disturbance (Franz-Odendaal et al., 2006; Verborgt et al., 2000).

1.3.4 Osteoclasts

Osteoclast cells originate from mononuclear hematopoietic stem cells (derived from blood cells) which are found within either periosteal or endosteal surfaces. Osteoclast cells are large, multinucleate (containing approximately 6-12 nuclei) and have ruffled borders. The main function of osteoclasts is remodelling of the bone through bone resorption. Osteoclasts secret acid to dissolve the inorganic component of the mineralised bone matrix, and its lysosome enzyme digests the organic component. An activated osteoclast can resorb a bone volume of 200,000μm^3/day (Jeon et al., 2012; Sommerfeldt and Rubin, 2001).

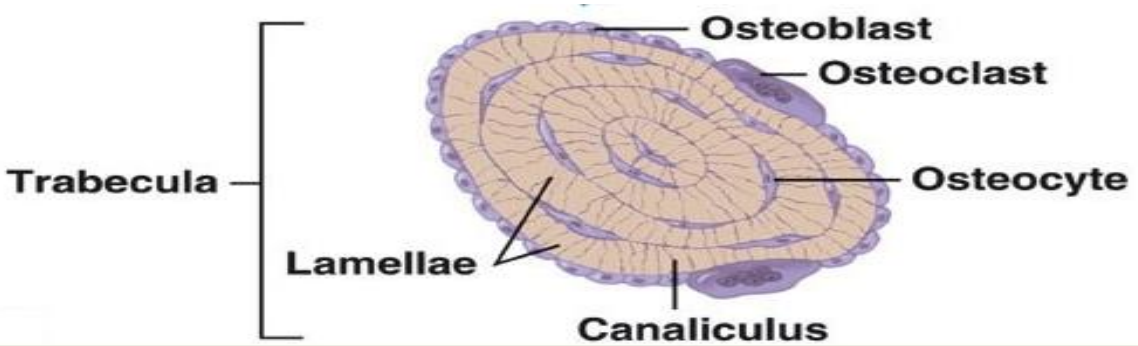


Figure 1.3.2: *transverse section of trabecular bone displaying the three typical bone cells osteoblasts, osteocytes and osteoclasts. Reprinted from Seeley et al., (2008) with kind permission of McGRAW-HILL.*

1.4 Morphogenesis of bone

In the first few weeks of prenatal development, the bony structure is formed and continues to grow into adulthood. There are two methods for ossification of bone; intramembranous and endochondral.

1.4.1 Intramembranous ossification

This type of ossification begins during embryonic development and occurs in flat bones. In intramembranous ossification, mesenchymal stem cells directly differentiate into osteoblasts. These osteoblasts can form and mineralise a bone matrix. The remaining osteoblasts are trapped in bone matrix to become osteocytes. Consequently, small trabecula is generated (Figure 1.4.1 A), which integrates to develop a network of trabecular bone. At the surrounding surface of trabeculae, some osteoblasts increase the thickness of trabeculae (Figure 1.4.1 B). Eventually, the remodelling process replaces the woven bone to mature lamellar bone (blue stain in Figure 1.4.1 C) that is more organised than woven bone. The cells inside the trabecular bone spaces form red bone marrow, while the osteoblasts in the periosteum develop a bone matrix and create compact bone (Seely et al., 2008)

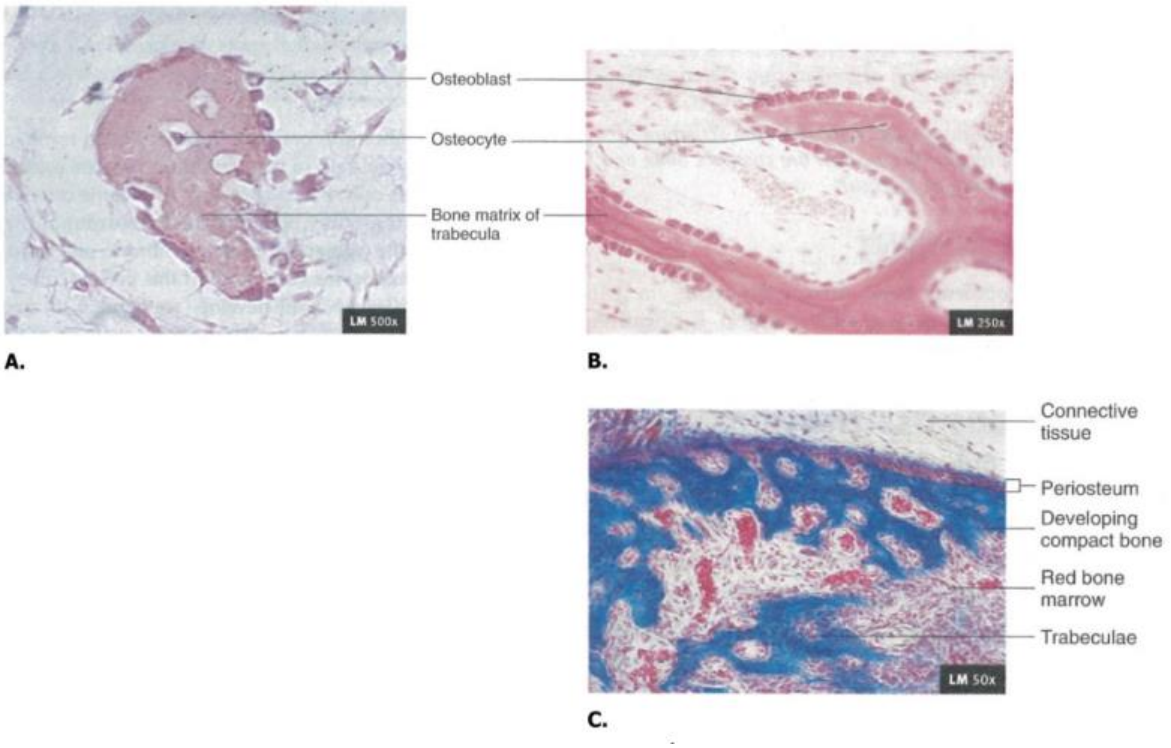


Figure 1.4.1: *Intramembranous ossification in a 12-week old foetus, (A) Osteoblasts at the outer surface create bone matrix and differentiate into osteocytes when they embed in the bone matrix. (B) Interconnection of many trabeculae forms cancellous bone. (C) The trabecular bone (blue colour) is in the centre and the spaces contain red bone marrow. Under the periosteum is developing compact bone. Reprint from Seeley et al., (2008) with kind permission of McGRAW-HILL.*

1.4.2 Endochondral ossification

Endochondral ossification begins with the differentiation of mesenchymal stem cells into chondroblasts (cartilage cells). Overtime the chondrocytes undergo apoptosis due to lack of nutrition, then vasculature develops at the centre of the cartilaginous template. The perichondrium is a membrane cover the external surface of the cartilage which is replaced by periosteum and progenitor cells that eventually differentiate to the osteoblasts. The bone collar is a layer of osteoblasts that produce bone which covers the outer part of diaphysis (Figure 1.412 A). Blood vessels are attracted into the medullary cavity and osteoblasts are recruited form these blood vessels creating the primary ossification centre. Osteoblasts start to establish the bone matrix at the centre of primary ossification (Figure 1.412 B). At the epiphysis regions, the secondary ossification centres form. The cartilaginous structure on both sides of the ossification centres become woven

bone, and through the remodelling process are replaced by lamellar bone (Figure 1.4.2 C). As a result of endochondral ossification, the centre of the long bone forms via two directions of growth either lengthening or widening, by adulthood only the articular cartilage is left at the joint ends (Figure 1.4.2 D). This ossification is a critical process in long bone formation and also occurs during natural healing of fractured bone (Yaszemski et al., 1996).

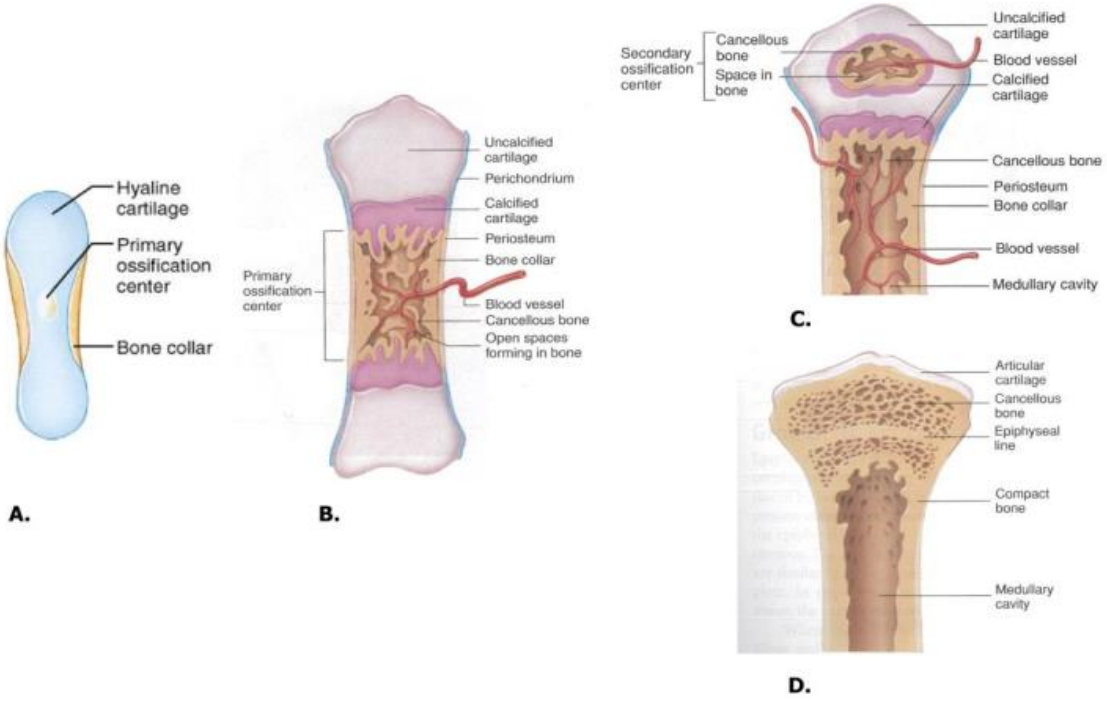


Figure 1.4.2: *Process of the endochondral ossification. (A) The cartilage was produced by chondroblasts. It has bone a collar at the diaphysis area. (B) The penetration of blood vessels become a primary ossification centre and bring osteoblasts to this area. (C) Secondary ossification centre occurs at the proximal of long bone. (D) The bone is fully developed, the cancellous and compact bones are mature, articular cartilage is the only cartilage present. Reprint from Seeley et al., (2008) with kind permission of McGRAW-HILL.*

1.5 Bone healing and remodelling

Bone healing is a proliferative physiological approach to repair fractured bone. There are three essential stages for bone healing the inflammatory stage, repairing stage, and finally the remodelling process. The inflammatory stage begins in the first few hours of injury and lasts for several days during which a heamatoma is created from white blood cells and fibroblasts, producing granulation tissue, initiating vascular ingrowth and promoting mesenchymal stem cell migration.

In the repair stage, the cells in the heamatoma create a callus which contains different cell and tissue types such as fibrous tissue, cartilage and immature bone. At the initiation of this stage, the fibrocartilage formation predominates, which is followed by mineralisation.

In the remodelling stage of bone repair, the chondrocytes differentiate and eventually become apoptotic. Cartilaginous calcification occurs, new blood vessels penetrate at the junction between the new bone formation and chondrocyte regions. New bone is generated by the co-ordinated activity of the osteoblasts and osteoclasts.

The bone remodelling cycle consists of three consecutive phases: resorption, reversal, and formation (Kini and Nandeesh, 2012).

The bone resorption starts with the activation and recruitments precursors of the mononuclear monocytes-macrophage osteoclasts to produce preosteoclasts (Roodman, 1999). Integrin receptors interact with RGD (arginine, glycine and asparagine) to facilitate the preosteoclast attachment to the bone matrix. Osteoclast creates extensively specialised folds called the “ruffled border”, which is surrounded by annular zones called “sealing zone”. Osteoclast-mediated bone resorption is regulated by secretion of the lysosome enzyme and breakdown of the bone matrix proteins. After the process, the apoptosis of osteoclasts takes place. The remodelling process has distinct sequential aspects that changeover from bone formation to resorption and vice versa. In the creation of bone, osteoblasts produce a new proteinaceous matrix that contained predominately collagen type I and minerals, controlling the mineralisation of bone matrix (Anderson, 2003; Clarke, 2008). They store a range of growth factors including platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), transforming growth factor-beta

(TGF-β), basic fibroblast growth factor (bFGF), and bone morphogenetic proteins (BMP). Osteoblasts regulate these growth factors by two mechanisms, autocrine and paracrine process. After new bone formation, nearly 50 to 70% of osteoblasts become apoptotic (Clarke, 2008; Jilka et al., 1998).

Bone remodelling is regulated by the Receptor activator of nuclear kappa-B ligand (RANKL) /RANK and osteoprotegerin (OPG). Remodelling can be activated by change in fundamental circumstances in the bone, such as fracture, bone diseases, calcium deficiency, and poverty. RANKL is a protein expressed by osteoblast that can be membrane bound or soluble, when it binds to the receptor of RANK locates on the surface of osteoclast precursors and mature osteoclast this activates an intercellular signal pathway inside the osteoclasts, driving differentiation and osteoclastogenesis. OPG protein is secreted by osteoblast cells and is a mimic of RANK that can bind to RANKL thereby blocking its ability to activate RANK and inhibiting osteoclastogenesis. The ratio between RANKL: OPG regulates the amount of bone resorption a given bone remodelling until will undergo (Figure 1.5) (Clarke, 2008; Lamoureux et al., 2007).

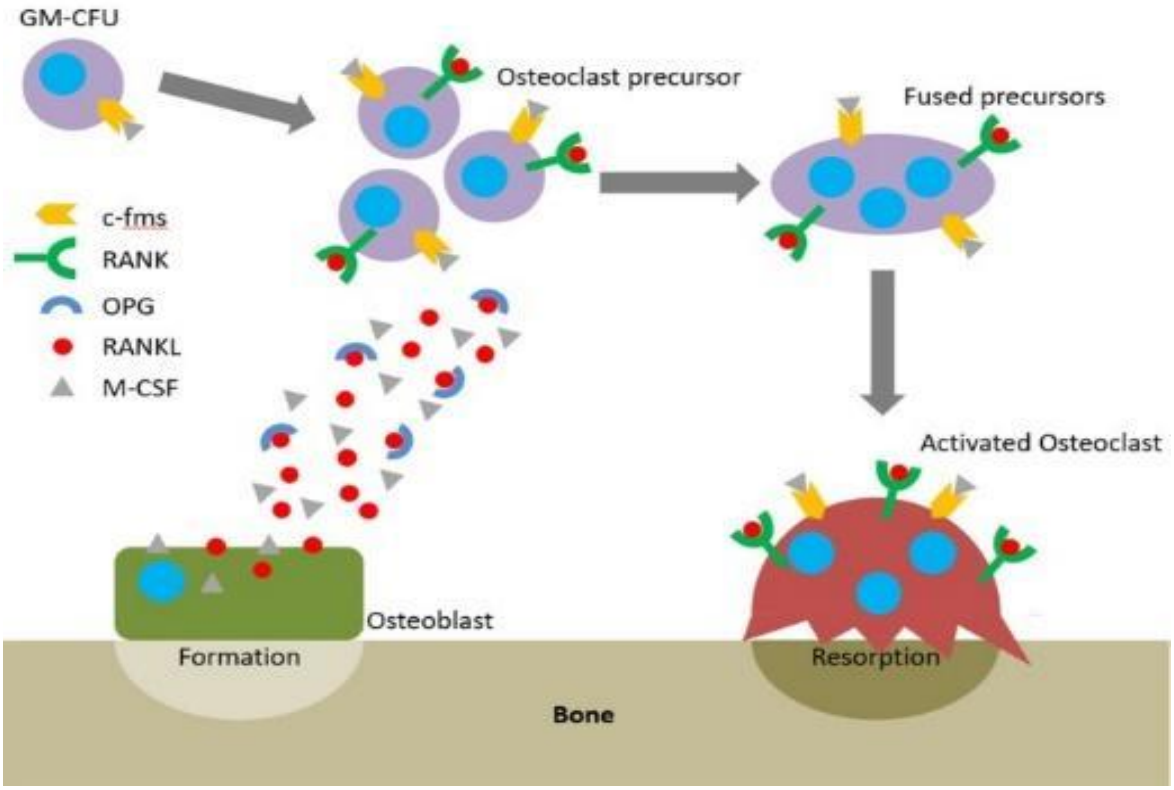


Figure 1.5: *The RANKL, RANK and OPG axis during bone remodelling (Owen and Reilly, 2018, creative commons liscence).*

1.6 Introduction to cleft palate

1.6.1 Development of the palate

The human face begins to develop four weeks after conception and this is followed by palatal shelf development (Stanier and Moore, 2004). Embryologically, palatal development begins at around the sixth-week post conception with the formation of three main structures; the lateral palatal shelves, median palatal processes, and the nasal septum. The median palatal processes represent the main segment of the intermaxillary region which is known as the anterior or primary palate and also forms the central segment of the lip, while the posterior or secondary palate forms from migration fusion of two lateral palatal shelves which arise from maxillary processes (Figure 1.6.1 A and B) (Yoon et al., 2000; Zuk, 2008).

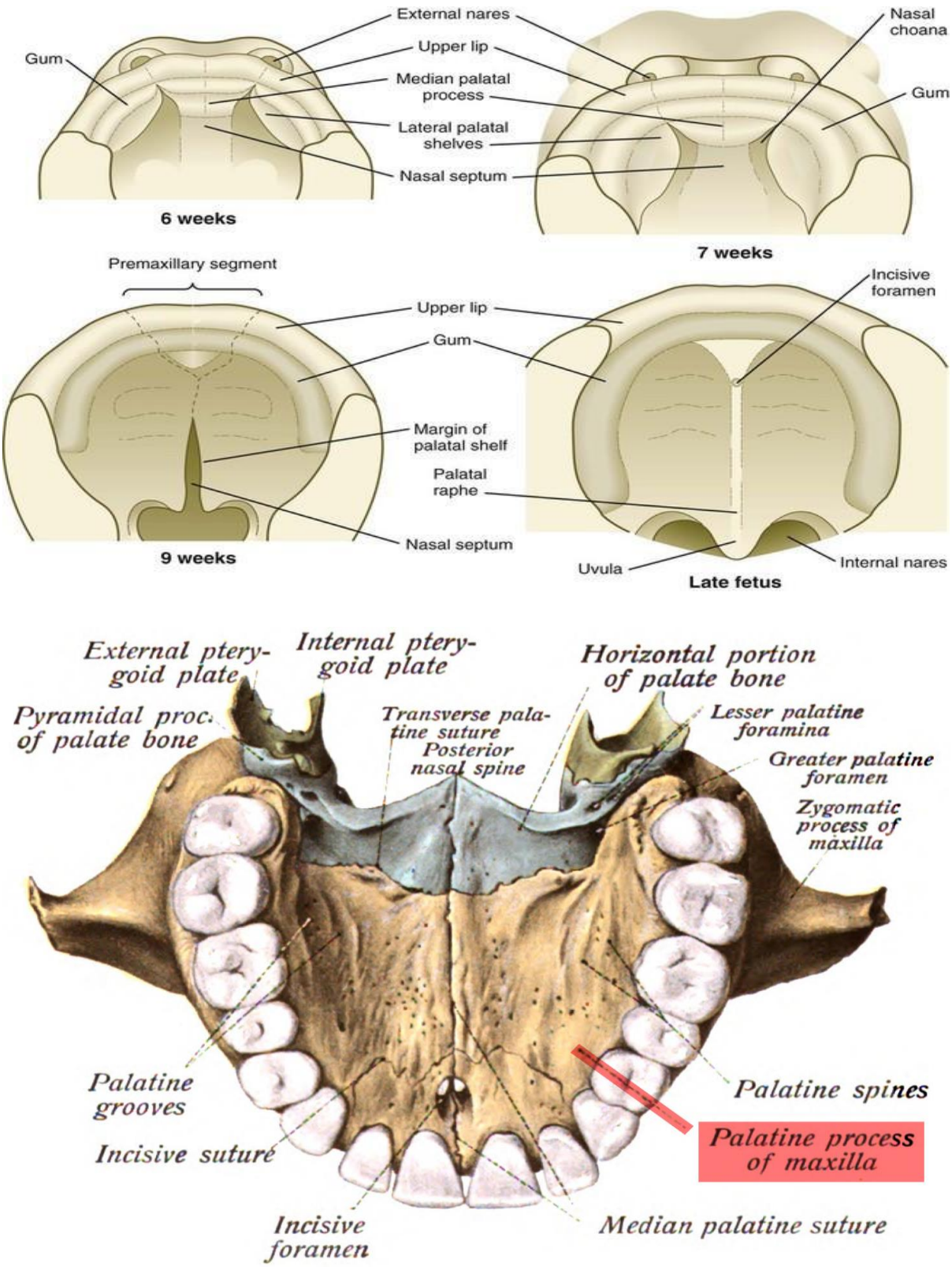


Figure 1.6.1: Embryonic formation and anatomical development of human palate. *(A) Development of human palate [(1)]. (B) Human anatomy of the maxilla (anterior and medial view) [(2)].*

1.6.2 Incidence and aetiology of cleft palate

Cleft palate is the most common craniofacial congenital abnormalities in new-borns. A permanent opening occurs in the roof of the oral cavity because of a failure of the palatal shelves to fuse correctly during the early stage of embryonic development. This leads to communication between the nasal and the oral cavity. The frequency of cleft lip with or without cleft palate is between 1 in 300 and 1 in 2500 per new-born, while the cleft palate defect only has an incidence of 1 in 1500 per new-born. Several factors play a crucial role in increasing the prevalence of these defects such as geographical area, ethnicity and socio-economic situation (Lidral and Murray, 2004). The causes of cleft anomalies have been elucidated and include (Sabbagh et al., 2015): 1) environmental factors, for example, maternal ailments, exposure to chemicals/smoking especially through the first trimester, alcohol abuse, drug use including anti-convulsant and retinoic acid based drugs, 2) genetic factors which are passed down from either one or both parents, 3) infection such as rubella "German measles" and 4) consanguinity. Although the pathogenesis of cleft palate is unknown so far, some suggested causes are teratogenic exposure and/or genetic effects via the combination effect of environmental causes with allele genes during embryonic face development. these can give rise to variant form of phenotypes (Wu et al., 2014). For example, in an animal study, retinoic acid medication of the treated group induced morphological changes such as excessive growth inhibition, orofacial deformities, failure of palatal shelves fusion, and Meckel's cartilage hypoplasia (Padmanabhan and Ahmed, 1997).

All these factors can influence initial embryonic development during the first trimester especially between the fourth and tenth weeks of pregnancy (Stanier and Moore, 2004). It is not life threatening but may commonly affect speaking, feeding, hearing and social relationships. The orofacial clefts are classified into syndromic and nonsyndromic types. In a syndromic orofacial cleft, the infant has mental and physical impairments which occur in about 30 percent of cleft defect patients. However, the remaining 70 percent of infants have nonsyndromic orofacial anomalies and do not have any mental and physical disturbances, as reviewed in the thesis of a colleague at University of Sheffield (Puwanun, 2014).

1.6.3 Treatments of cleft palate

The UK gold standard for treatment of cleft palate and/or lip (National Health Service, 2013) begins with case management, which includes examination and diagnosis of cleft lip and palate, hearing tests, and feeding control. A cleft lip is closed approximately in the third month of a child's life while the closure of cleft palate is usually achieved between 6-12 months of child's life using a soft tissue to close the defect. The objectives of this management are to enable speech acquisition and allow for the encouragement of maxillary bone growth (R J Rohrich et al., 1996) (Table 1.6.3).

Complications may occur after the operation including problems at the suture joint, which is the fibrous tissue joint between skull bones (Cohen, 1993) with morphological changes during growth and orthodontic treatment (Hahn et al., 2009) and ossification of the maxilla. The remaining bony defect in the cleft site leads to maxilla narrowing and distortion, shallow palate with shorting vault and may cause speech problems (Figure 1.6.3). The soft tissue may fill the cleft in the hard palate and develop scar formation which affects facial development (Smahel et al., 2009). If defects include the alveolar process, surgical intervention is performed to support teeth during eruption so that the bone graft frequently takes place between the ages of 8-11 years (Wolford and Stevao, 2002)

Table 1.6.3: Timeline of care of a patient with cleft palate/lip defects, indicating the key times during a child's growth (months/years) during which interventions would take place. Treatment is performed by multidisciplinary teams, including oral and maxillofacial surgeons, plastic surgeons, paediatric surgeons and dentists [3].

Standard Treatment of Cleft Palate	Birth	3 months	6 months	9 months	12 months	18 months	30 months	36 months	4 years	5 years	6 years	8 years	12 years	15 years	18 years
Lip Closure															
Team Care															
Periodic Speech Care															
Palate Closure															
Audiologic Care															
Routine Care by Family															
Orthodontic Intervention															
Prosthodontic Intervention															
Alveolar Cleft Treatment															

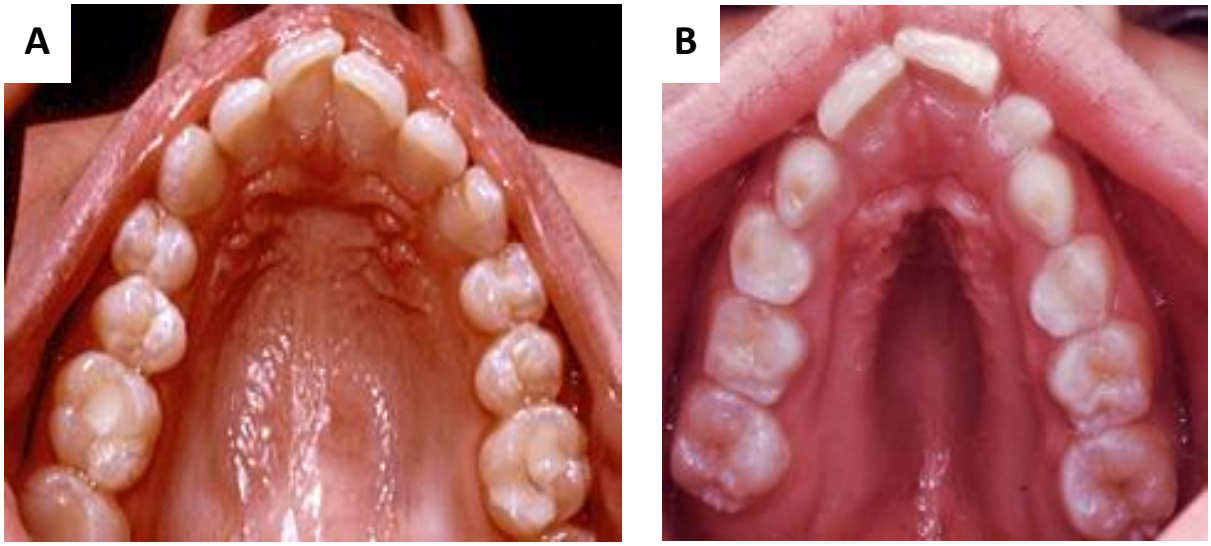


Figure 1.6.3: Post-operative complications after cleft palate correction surgery. (A) Shallow Palate [4]. (B) Short Palate with narrow maxilla [5

Conventional surgical operations for bone regeneration utilise bone grafts which can be an autograft, isograft, allograft, xenograft, prosthetic materials and/or combination grafts. The autologous graft is the gold standard to achieve bone regeneration. However, bone graft has limited use because of high morbidity and complications at the donor site such as bone resorption, disturbance of gait, impermanent numbness and/or paraesthesia of the leg, especially when taken from the lateral aspect of femoral bone (Elsalanty et al., 2009). Ross (1987) mentioned that 73 per cent of patients suffering from unilateral cleft lip and palate needed orthodontic treatment for the arrangement of teeth while the remaining per cent (27) required orthognathic surgery (surgical correction of the jaw). The management of cleft palate usually takes an extended period.

1.6.4 Arguments about the timing of cleft palate repair

Cleft palate management protocols still create controversy, in which the perfect time of surgical intervention for the closing of the cleft palate is not fully established. The surgical intervention depends on a number of factors including the kinds of cleft anomaly (cleft lip, cleft palate, cleft lip and cleft palate, unilateral and/or bilateral: Figure 1.6.4) (Shkoukani et al., 2014), patient-specific factors and the medical team's opinion. Cleft palate in children usually causes a delay in speech onset because of the opening between the oral and nasal cavity (Peterson-Falzone et al., 2001).

Berkowitz et al., (2005) reported that an appropriate age for repairing a cleft lip is around the age of 3 months, following by cleft palate correction between 6-18 months. Although this time is essential to improve the phonetics, maxillary growth may be affected. The study of Ysunza et al., (2010) claimed that an early repair of the cleft palate at around 4-6 months of infant`s age could improve speech development, and can be followed by orthodontic treatment to enhance the normal maxillary expansion and growth. Nevertheless, specific studies found scar formation in the anteroposterior direction which leads to retrusion of the midface (Puwanun, 2014; Ross, 1987b).

Many procedures can be undertaken for the reconstruction of cleft palate (Moreau et al., 2007; Shkoukani et al., 2014). The autologous graft is the gold standard to achieve bone regeneration at the alveolar ridge because it contains factors that improve the osteogenesis. The reconstruction of alveolar cleft depended on the fresh autologous bone graft that can be harvested from different sources such as iliac, tibia, rib, cranial bone, and

mandibular symphysis. These provide a corticocancellous bone block that can be reshaped depending on the defect shape and then transplanted to the alveolar cleft space (Rawashdeh and Telfah, 2008; Scalzone et al., 2019).

In bilateral cleft palate and lip, which is a more complicated case and more challenging to treat, maxillary arch development was shallower and narrower than in the unilateral cleft case. Furthermore, there was a slight enhancement of the maxillary arch provided by orthodontic treatment (Smahel et al., 2009). The reviews of Schweckendiek and Doz, (1978) and Prydso et al., (1974) claimed that the soft palate defect could be corrected in the first year of a child's age which led to improved speech development and diminished speech difficulty with age. The hard palate defect is repaired at around 12-14 years of patient's age due to the completed maxillary growth, which leads to minimising the maxillary disturbance. On the other hand, Rohrich et al., (1996) observed that there was no significant difference in maxillary growth disturbance in patients whose hard palate was closed early (10.8 months of age) compared with delayed (40.8 months of age).

However, greater speech difficulties are noticed in the delayed treatment group that can cause hearing loss, and there was a higher incidence of palatal fistula compared to the early closure group. They proposed a protocol in which intact cleft palate correction should occur before two years of the child's age to facilitate the healthy development of phonetics and also eliminate the risk of deafness (Rohrich and Gosman, 2004).

Recently, tissue engineering has been considered a promising technique for reconstruction of the alveolar ridge area which reinforces tooth eruption and also can integrate with a tissue in the palate region (Martin-de-Campo et al., 2019).

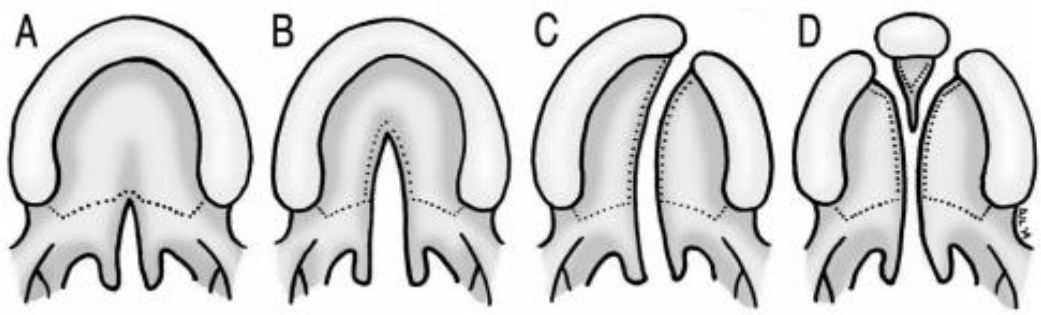


Figure 1.6.4: *Veau cleft lip and palate classification. (A) Class I_ cleft in soft palate. (B) class II_ Cleft in both hard and soft palate. (C) Class III_ complete unilateral cleft. (D) Class IV_ Complete bilateral cleft. Printed from Shkoukani et al., (2014) with kind permission of Birth defects research.*

1.7 Use of tissue engineering to repair cleft palate

A new technique for the regeneration of missing bone tissue is known as tissue engineering. This technique would reduce problems noted during conventional treatments such as immune rejection, donor site morbidity, bone resorption, disturbance of gait, impermanent numbness and/or paraesthesia of the leg, and pathogen cross-contamination. The ideal standard for bone tissue engineering is to collect cells from a patient's tissue and isolate the relevant cell population, usually adult stem cells, then culture the cells to allow proliferation and seed them into a scaffold for tissue development before re-implantation into a patient (Figure 1.7)

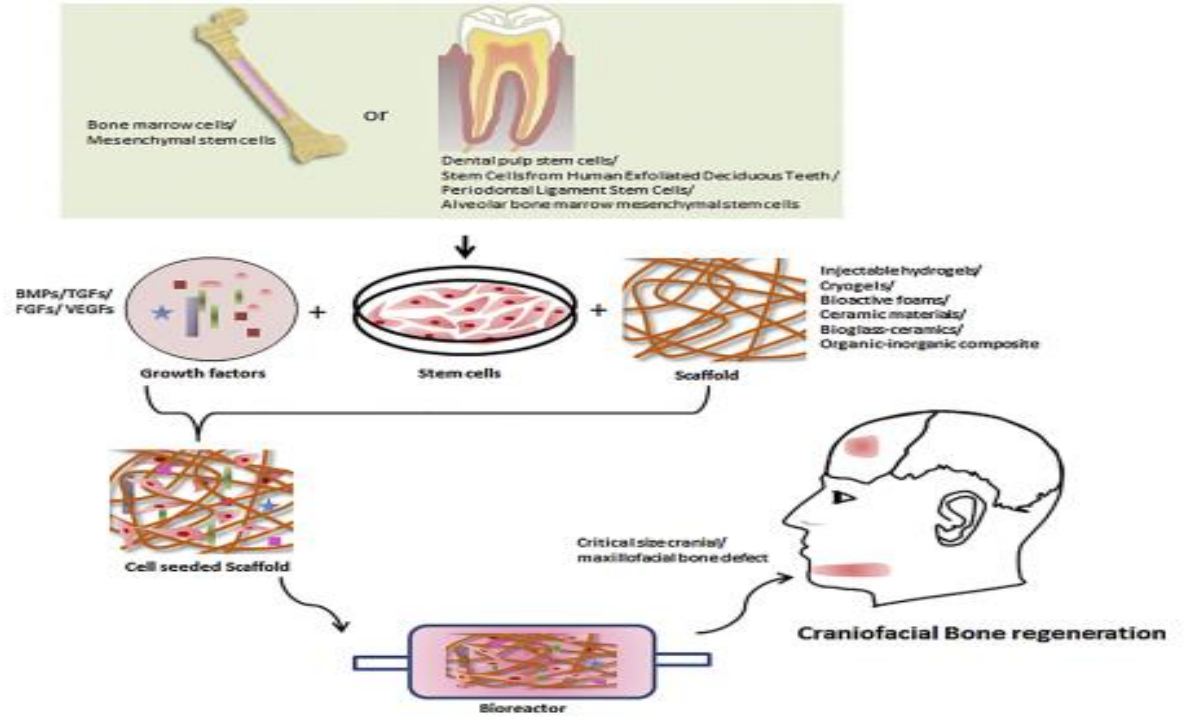


Figure 1.7: *Strategies of craniofacial bone tissue engineering,* Kumar and Kandalam (2017) *with kind permission of British Journal of Oral & Maxillofacial Surgery*

In a clinical study, Moy et al., (1993) reported that the quantity of bone formation in the craniofacial after implantation of a combination of autologous corticocancellous bone chips and hydroxyapatite granules was significantly more than when hydroxyapatite was implanted alone. Using cells in tissue engineering may be a reliable alternative to standard autologous grafting approaches to achieve clinically successful bone regeneration for craniofacial reconstruction. The cells used for bone tissue engineering should be able to incorporate into the adjacent tissue and be capable of osteogenic differentiation.

Scaffolds are either mechanically fabricated or natural biomaterials which can act as a carrier for specific biological elements such as cells, hormones, growth factors, and drugs. The scaffold requirements for bone tissue engineering are biocompatibility, adequate mechanical strength and stability, bioresorbability, ease of manipulation, and interconnected porosity (Tevlin et al., 2014). Hibi et al., (2006) described the use of a combination of autologous mesenchymal stem cells harvested from the bone marrow of patient's iliac crest, platelet-rich plasma (PRP), and a gel matrix to fill the alveolar cleft

defect in a 9-year-old patient. Consequently, the bone volume augmented about 79% when assessed nine months postoperative follow up. Moreover, the maxillary lateral incisor and canine teeth erupted after two-years post-operation at the location surrounding the defect. These results indicate that tissue engineering can support cleft palate repair. However, there are many crucial factors that affect the result of tissue engineering repair include donors' variation such as gender, age, habit, physiological status and anatomy.

The methods and sources of stem cell collection and isolation affect the outcome of any tissue engineering strategy as these will affect the quality of the cell and its proliferation, and differentiation *in vitro*. Finally, the bone defect in the maxillofacial region will have a variety of vascularisation and loading functions. Current research in tissue engineering is working to control these variables and minimise the subsequent healing time before implantation (Phinney et al., 1999).

1.8 Stem cells

Stem cells have the capability to self-renew and to differentiate into two or more cell types. Totipotent cells can differentiate into any cell type of an embryo including the placenta whereas pluripotent cells can differentiate into any cell of the body.

There are two significant sources of stem cell which have been used in medical sciences; embryonic sources called "embryonic stem cells", which are found in the inner cell mass of the early stage of the embryonic blastocyst and adult stem cells, which are already present in the adult tissue. Embryonic stem cells are pluripotent which means they are capable of being differentiated into all types of body cell; they also have an unlimited capacity of self-renewal. On the other hand, adult stem cells are considered multipotent as they have a more limited number of cell types that they can differentiate into. During the growth process, embryonic stem cells undergo significant proliferation which may be considered advantageous, as they provide an inexhaustible source of cells for tissue regeneration. Nevertheless, although embryonic stem cells can regenerate missing tissue, they can also form teratoma (Ben-David and Benvenisty, 2011). In addition, there is ethical resistance to using embryonic stem cells in the clinic. From another view, adult stem cells do not display the same ethical issues, although questions could be raised during therapeutic uses involving possible risk of cancer (Chandaet al., 2010). In general,

they are assumed to be safe as they are derived from adult tissue (Obokata and Vacanti, 2014).

A proportion of stromal cells found in bone marrow, are considered to be a source of mesenchymal stem cells. The bone marrow stromal cells were previously considered to be purely a supporting structure for hematopoiesis. Many studies have demonstrated that these stromal cells can be differentiated into many mesenchymal cell lineages, such as bone, muscle, fat, cartilage (Chamberlain et al., 2007).

Friedenstein and colleagues (1966) first isolated MSCs from bone marrow (Mafi et al, (2011). MSCs exhibit adherence to tissue culture plastic and are spindle-shaped. The expansion of mesenchymal stem cells has been undertaken from cells of many sources, for example; muscles (Dodson et al., 2010), fat tissue, dental pulp, umbilical cord, amniotic fluid, embryonic stem cells (Lian et al., 2007; Phinney and Prockop, 2007), periosteum (Choi et al., 2008), synovial membrane (Bari et al., 2001), skin (Belicchi et al., 2004), peripheral blood (Shi et al., 2009), primary teeth (Miura et al., 2003) and periodontal ligament (Seo et al., 2004). There are, however, difficulties in cell isolation which include the small numbers of isolated cells that can be obtained from a tissue biopsy or blood/marrow aspirate, and there also issues associated with segregating cell populations within tissues. For these reasons, stem cells derived from adults need be able to be expanded *in vitro* and undergo advanced manipulation before use in preclinical or clinical trials, and this leads to safety and efficiency issues (Obokata and Vacanti, 2014).

There has recently been a significant focus on the application of mesenchymal stem cells for several varieties of ailments (Wappler et al., 2013). Researchers have investigated the beneficial effects of mesenchymal stem cells for paracrine support and modulation of immunity (Horwitz and Rph, 2009; Meirelles et al., 2009; Patel et al., 2008).

Human mesenchymal stem cells are characterised by secretion of a range of growth factors (including basic fibroblast growth factor, epidermal growth factor, hepatocyte growth factor, platelet-derived growth factor, stromal-derived growth factor 1 α, transforming growth factor β, vascular endothelial growth factor) and cytokines (interleukin 1 β, interleukin-6, interleukin-8, tumor necrosis factor α) (A I Caplan, 2009; Führmann et al., 2010). They represent a small amount (1-2%) of whole bone marrow

cells, and hence, expansion to high cell numbers is important (Fibbe and Noort, 2003; Pittenger et al., 1999).

1.8.1 Mesenchymal stem cells

Mesenchymal stem cells can undergo differentiation and proliferation under the control of some critical factors which involve: growth factors, cytokines, hormones, and transcription agents (Alexander et al., 2014). Although bone marrow is an essential source of mesenchymal stem cells and is easy to access, there are still drawbacks to its use, which are: the morbidity and pain at the donor site and low cell numbers compared to the defect to be filled (Baksh et al., 2004; Campagnoli et al., 2015; Gregoryet al, 2005; Tuan et al., 2003).

The rate of proliferation, multipotency, tissue origin, donor gender and age, ailment stage, potential capability for differentiation, and ease of access are considered crucial factors which determine the usefulness of mesenchymal stem cells (Alexander et al., 2014). Biochemical signals are used to modify stem cell behaviour for example; the cytokines and growth factors discussed above (Delaine-Smith and Reilly, 2011). Environmental agents such as; surface chemical components and topography, also seem to influence stem cell fate (Dalby et al., 2007; Reilly and Engler, 2010), as do mechanical forces for instance; tension, fluid shear, and hydrostatic pressure (Delaine-Smith and Reilly, 2011).

Mesenchymal stem cells are commonly grown *in vitro* after selection by plastic adherence (Nombela-Arrieta et al., 2011). They are considered to be a heterogeneous population, and to date, no specific cell-surface marker identification for mesenchymal stem cells has been presented (Schauwer et al., 2012). Therefore, specific common guidelines are used for the identification of mesenchymal stem cells. As well as adherence to plastic tissue culture they should show positive expression of surface antigen markers including CD73, CD 90, and CD 105; and negative to CD11b, CD 19 or CD 34, CD 45, CD 79 α, and HLA-DR (Dominici et al., 2006; Alexander et al., 2014).

Mesenchymal stem cells are typically harvested from bone marrow aspirates collected from either the anterior or posterior iliac crest. MSCs hold great promise therapeutically and offer benefits either by direct tissue repair through differentiation into specific tissue

damage or indirect wound regeneration by releasing specific bioactive proteins such as growth factors, chemokines, and anti-apoptotic agents (Rehman et al., 2004). These proteins play crucial significant roles in angiogenesis and cell recruitment which is also very important for regeneration of damaged tissues (Meirelles et al., 2009). Mesenchymal stem cells also can create immunosuppression by acting together with T and B lymphocyte, monocytes, and Natural Killer Cells (Sioud, Mobergslien, Boudabous, and Fløisand, 2011) therefore could become a competent resource for allogeneic cell sources (Koç et al., 2002).

1.8.2 Mesenchymal stem cells differentiation *in vitro*

The differentiation of MSCs can be controlled *in vitro* through the preparation of specific culture conditions and tested using some phenotypic markers related to that tissue type. Growing cells are typically observed in an optimised medium which is prepared by adding specific supplements or growth factors. To determine if the cells have differentiated, expression of markers specific to differentiation pathways and the extracellular matrix (ECM) structure are observed (Delaine-Smith and Reilly, 2011). These conditions and markers are described in the following sections

1.8.3 Human periosteum cells as promising cells for osteogenicity

The periosteum has been considered to be a promising origin of immature osteogenic progenitor cells and also has a tri-layer membrane which envelops the whole outer surface of bone except the joint (Kawase et al., 2014; Olbrich et al., 2012). Its purpose is to enable growth, remodelling, and bone healing. The internal layer of the periosteal membrane contains mesenchymal stem cells which can differentiate into many different cells such as osteoblasts and chondroblasts; these are similar to mesenchymal stem cells that are harvested from bone marrow (Puwanun, 2014). Periosteal cells from older donors are still able to exhibit osteogenic properties; in contrast, this property is reduced in bone marrow mesenchymal stem cells (Stolzing et al., 2008).

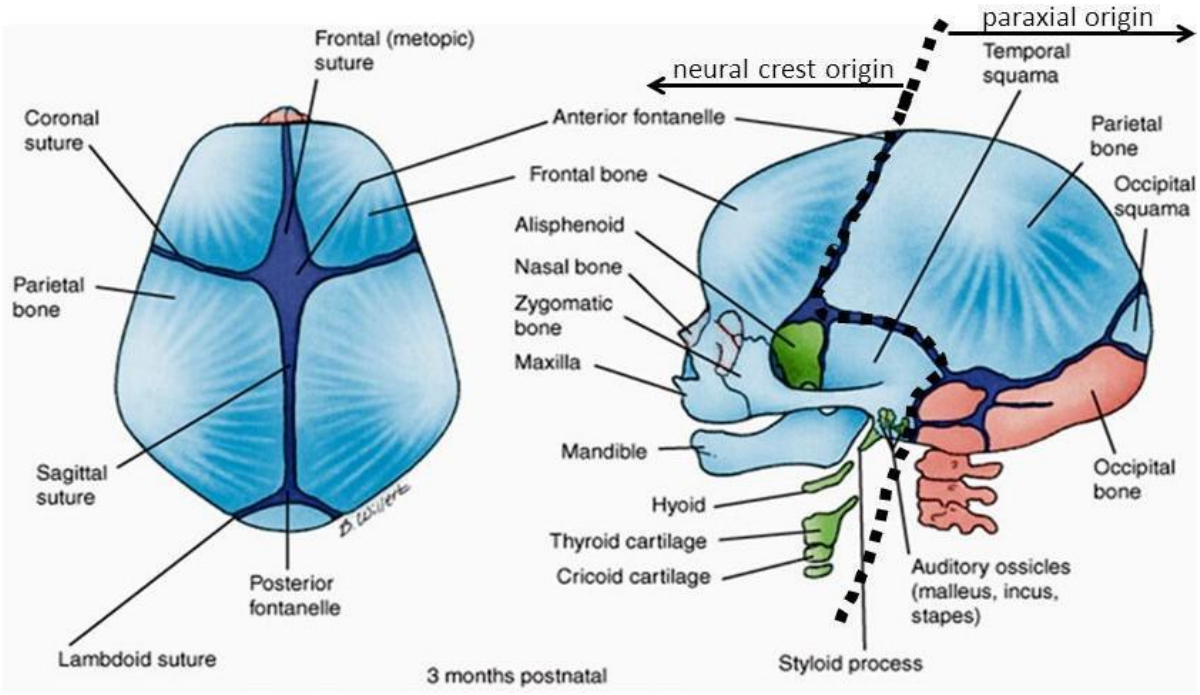


Figure 1.8.3: *Facial development by intramembranous ossification and endochondral ossification. pink colour and green colour "chondrocranium" - endochondral ossification forms the base of skull. Light blue "membranous" - intramembranous ossification forms superior-anterior of skull and face* (.)

There are arguments about what is the best harvesting area of mesenchymal stem cells for the regeneration of facial bone deformities. During development, the facial skeleton emerges from the cranial neural crest. Periosteal tissue from two different regions could differ in bone formation and healing (Puwanun, 2014). Fuji et al., (2006) and Choi et al., (2002) used animals to compare bone formation from periosteum harvested from the tibia and facial regions with implanted into suprahyoid muscle. The results showed that intramembranous ossification (Figure 1.8.3) was induced by the harvested periosteum from the facial bone. While the periosteum from the tibia initiated endochondral ossification (Figure 1.8.3). In alveolar bone augmentation, D'Addona and Nowzari (2001) reported that the graft bone harvested intraorally induced bone augmentation without cartilage formation. These previous studies indicated that human periosteum cells might be considered as an alternative source for the engineering of bone tissue and that Jaw periosteum might be more relevant to induce intramembranous ossification.

1.8.4 Osteogenesis

Two types of markers are commonly used to test the osteogenic differentiation of mesenchymal stem cells. The initial or early markers include: mesenchymal stem cells morphology switching from fibroblast, fusiform to a more cuboidal shape, and expression of mRNA for Runt-related transcription factor-2 (RUNX-2), alkaline phosphatase enzyme (ALP), bone morphogenetic protein 2 (BMP-2), osteonectin matrix protein (ON), transforming growth factor-β1 (TGF-β1), and the abundant matrix protein collagen type I (COLI). The late markers of osteogenic differentiation include the proteins osteocalcin (OC), osteopontin (OPN), continuing expression and deposition of COL I and finally bone mineral formation. The mineral present in the bone is a type of calcium phosphate in the form of carbonated hydroxyapatite. Studies have identified the presence of a calcium group via alizarin red staining or a phosphate group via Von Kossa staining (Delaine-Smith and Reilly, 2011).

VEGF comprises the secreted glycoproteins that play essential roles in the regulation of vasculogenesis (controlling of the sprouting of new blood vessels). Later VEGF studies discovered a family of VEGF, which are designed of seven secreted types; family comprises seven secreted glycoproteins that are designated VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, placental growth factor (PlGF) and VEGF-F (Ferrara et al., 2003). VEGF-A (is also alluded to VEGF) mostly exerts its biological influence through secretion of vasculogenetic protein and initiates the proliferation of endothelial cells, bone sprouting and new blood vessels. The human gene of VEGF comprises of four different mature isoforms (VEGF121, VEGF165, VEGF189 and VEGF206) (Tischer et al., 1991). VEGF189 and VEGF206 are wholly sequestered in the ECM that is indispensable for vascularisation of epiphyseal region in endochondral bone construction. While the significant isoform of VEGF165 is slightly diffused but is mainly attached to ECM that takes the essential effects of angiogenesis in the ossification of primary centre. VEGF directly interacts with three central receptors (VEGFR1, 2 and co-receptor neuropilin-1). VEGF and its receptors are indicated to human osteoblast cells and expressed the significant level in the late stage of osteogenesis, which contributed to osteoblast differentiation and bone regeneration via directly and indirectly effects of vascularisation promotion. Clinical investigation indicated that the highest expression of VEGF could lead to figuring out a variety of diseases such as rheumatoid arthritis, diabetic retinopathy and cancer (Risau, 1997). The

secretion of VEGF regulates by among factors such as hypoxic environments, prostaglandin, vitamin D, BMPs, transforming growth factor-beta (TGF-β), insulin-like growth factor 1 (IGF1), platelet-derived growth factor (PDGF) and fibroblast growth factor 2 (FGF-2). Whereas the glucocorticoid is diminished the secretion of VEGF (Maes and Carmeliet, 2008).

Ascorbic acid (AA), beta-glycerophosphate (βGP), and dexamethasone (Dex) are typically used during the preparation of osteogenic medium. These components are usually applied from one to several weeks. Ascorbic acid is essential for the stabilisation of collagen production, and it is essential for the formation of bone matrix proteins. It also plays a significant co-factor role for proline residue hydroxylation in collagen fibres (Kielty et al., 1993). βGP supplies inorganic phosphate for the production of bone-like mineral via the cells (Chang et al., 2000). Dexamethasone is considered as a steroidal anti-inflammatory agent, glucocorticoid, and is required for multi-differentiation media such as chondrogenic, adipogenic, and osteogenic media. Osteogenesis is inhibited if there is a lack of one or more of osteogenic inducers especially in 2D static culture (Porter et al, 2003). Although it stimulates early differentiation Dex has the general ability to inhibit collagen synthesis and high levels (≥100 nM) has been observed to facilitate adipogenesis (Beresford et al., 1992). BMP, transforming growth factor-β1, growth factors, and also vitamins D3 (VD3) all play crucial roles in the enhancement of osteogenesis when added to the supplements mentioned above (Seong et al., 2010; Vater et al., 2011).

BMPs are considered to be one of the superfamilies of transforming growth factors. They can be used for the stimulation of osteogenic differentiation in both in vivo and in vitro, but *in vitro*, the effect may be dependent on species (Diefenderfer et al., 2003). For example, to activate human mesenchymal stem cells during osteogenesis *in vitro*, specifically defined media, such as serum-free, are required when using BMP-2 (Osyczka and Leboy, 2005).

1.9 Soft tissue-engineering

1.9.1 Human normal oral fibroblasts

The oral epithelium contains basal cells that use the hemidesmosome contacts to adherence to the connective tissue of the basement membrane. They split and apically transfer into the suprabasal spinous layer, with express constant modifications in gene

morphology and expression, which consequently differentiate into cells that produce the specific layer called “granular layer”. Finally, this layer represents the primary permeability of the epithelium barrier. The cells of oral mucosa undergo further differentiation into either a highly keratinised layer that locates in the hard palate or non-keratinized layer that underlies the buccal mucosa and lingual regions. The basal cells express keratin; whereas, the suprabasal cells express keratin in the skin (Jennings et al., 2016). The paracrine interactions between the NOFs and NOKs are indispensable for the differentiation and growth of the keratinocytes to create a multilayer, stratified squamous epithelium (Costea et al., 2003).

1.9.2 Tissue-engineered oral mucosal models

The first time using of primary keratinocytes cultivation was by Rheinwald and Green in 1975. They employed mouse mature fibroblast cells cultured in Green’s media to create a feeder layer for keratinocytes and attained an appropriate epidermal cells proliferation (Kriegebaum et al., 2012). In the early 1980s, tissue-engineered oral mucosa (TEOM) has been introduced *in vitro* and *in vivo* (Tayebi and Moharamzadeh, 2017). The researchers cultured the epithelial sheets that took from different parts of the body to treat some injuries such as burns and oral mucosa defect (Madden et al., 1986; Lauer et al., 1991). Recently, investigators have focussed on the development of 3D multilayers of human oral mucosal. TEOM is increasingly being used to model drug delivery, toxicity and oral diseases (Jennings et al., 2016). Currently, TEOM mainly consists of co-culture of primary normal oral keratinocytes (NOKs) on top of an oral fibroblasts-containing matrix (Dongari-Bagtzoglou and Kashleva, 2006; Moharamzadeh et al., 2012) which, when cultured at an air-to-liquid interface, form a stratified epithelium and display histological features similar to their *in vivo* counterparts. Therefore, they are an excellent alternative for native tissue (Lee et al., 2004). However, a limited commercial supply of NOK challenges widespread application of these mucosal models. Furthermore, NOKs are restricted due to poor longevity and also donor-to-donor variability. NOKs have been immortalised by overexpression of hTERT using stable transfection (Dongari-Bagtzoglou and Kashleva, 2006). Consequently, this prevents telomere shortening, thereby incrementing the life-span with associated improvement of cell proliferation capacity while preserving their phenotype (Lee et al., 2004). Therefore, the creation of TEOM

based on commercial immortalised oral keratinocytes (FNB6) may provide wider access to NOK-based TEOM (Jennings et al., 2016).

1.10 Introduction of serum and serum-free media

The most common culture cell media used *in vitro* cell expansion is a base media, typically Dulbecco's Modified Eagle's Medium (DMEM) or alpha Minimum Essential Medium (αMEM) supplemented with foetal bovine serum (FBS).

The basal supplementation of culture media with different sera of animal origin is essential for cell growth metabolic activity, metabolism and for stimulating proliferation (Brunner et al., 2010). However, although various cell lines are cultured in medium supplemented with serum, in many cases cultures could be cultured and proliferated in serum-free media (Freshney, 2010).

FBS is a collection of numerous factors which are very important in cell attachment, growth and proliferation, and for these reasons are usually used as a growth supplements for many types of human and animal cell (Brunner et al., 2010). FBS is used in the most common media formulations utilized for isolation and expansion of human mesenchymal stem cells (hMSCs). These interact with the other basic conditions of temperature, pH, osmolarity and oxygen supply, (Davis, 1994) which are essential to control successful growth, cell survival, maintenance and differentiation of human and animal cells *in vitro* (Figure 1.10).

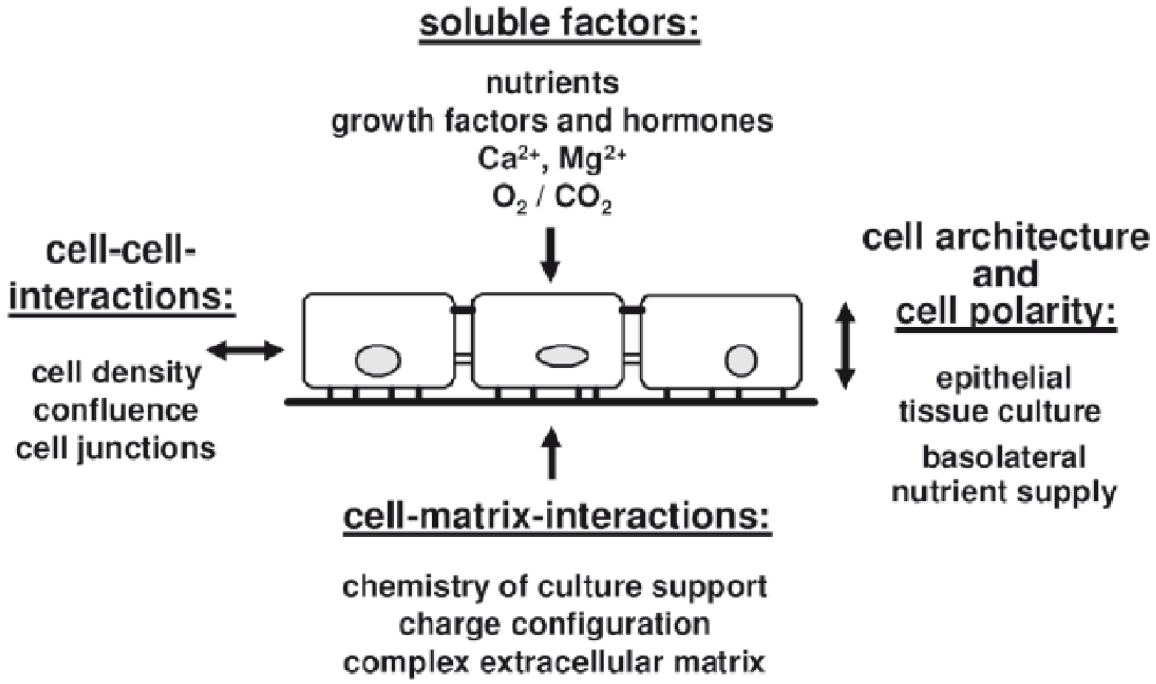


Figure 1.10: *Parameters controlling growth, proliferation and expression of differentiated functions of cultured cell in vitro. Printed from Brunner et al., (2010) with kind permission of Altex.*

Serum is a complex mixture of different molecular weight biomolecules that creates a balance between growth-promoting and growth-inhibiting actions (Werner and Grose, 2003; Freshney, 2010). The critical ingredients of serum and their major functions are shown in (Table 1.10 A).

Table 1.10: The critical ingredients of serum (Brunner et al., 2010).

Attachment Factors	Fibronectin Laminin
Hormones	Insulin Glucagon Corticosteroids Vasopressin Growth hormone
Growth factors	Epidermal growth factor Fibroblast growth factor Endothelial growth factor Insulin-like growth factor Platelet-derived growth factor Transforming growth factor
Cytokines	Interleukins Interferons
Carbohydrates	Glucose Galactose Fructose Mannose
Fatty acid and lipids	Free and Protein-bound fatty acids Triglycerides Phospholipids

The roles of serum in cell culture media include provision of many different types of growth factors, hormones and proteins for binding and transport. As well as, enzymes such as protease inhibitors and amino acids, vitamins and trace elements. Moreover, the serum provides detoxification and controls osmotic pressure (Brunner et al., 2010).

The advantages of serum in media are as follows (Brunner et al., 2010):

i. The natural coagulated serum is highly productive of cell proliferation by secretion of specific polypeptides from activated platelets during the clotting cascade (Balk et al., 1981; Gospodarowicz and Ill, 1980).

ii. FBS has been considered as a standard supplement for cell culturing because of its content high growth factors and low ϒ-globulin.

iii. FBS has a mixture of essential factors which are very important during cell proliferation and maintenance.

iv. Reduction the time and effort involved in preparing a media with all the factors needed for cell growth.

However, the use of animal serum in cell culture has many disadvantages and potential risks which include (Mannello and Tonti, 2007; Berger et al., 2006):

i. The risk that contamination could be transmitted to cell culture such as viruses, mycoplasma, bacteria and/or identification of zoonotic agent.

ii. In a clinical context, xenogeneic proteins could induce an immunological reaction in patients, especially during cell culture therapeutics.

iii. Batch-to-batch variability can lead to inconsistencies between experiments.

iv. The content of growth inhibitors, cytotoxic elements and serum could also contain some ingredients that can inhibit the growth of specific cell types. As well as this, serum is toxic for most cell type if it uses in too high concentration (Barnes and Sato, 1980).

v. A set of voracious quality requirement controls are needed to reduce contamination risk (Barnes and Sato, 1980).

vi. The serum can interfere with unidentified factors which affect hormones, growth factors and other additives (Barnes and Sato, 1980).

vii. Availability limitations (Barnes and Sato, 1980).

viii. Ethical concerns and animal welfare issues (Jochems et al, 2002.; Tekkatte et al., 2011).

1.10.1 Foetal bovine serum alternatives

Recently, due to the disadvantages and problems raised for serum used in cell propagation and maintenance, the reduction of FBS in culture media with alternatives of animal serum substitutions have been recommended (Culture & Gstraunthaler, 2003). The substitutions are extracted from other animal species such as horse, pig, goat, pituitary, chicken embryo or ocular fluid (Filipic et al., 2002), bovine milk fractions or bovine colostrum (Belford et al., 1995; Pakkanen and Neutra, 1994) or plants are used for extraction of protein fractions called vegetal serum (Pazos et al., 2004). Alternatively,

human platelet lysates could be extracted from donor red blood cells (Rauch et al., 2008), autologous serum has been described to induce faster proliferation of bone marrow stem cells (BMSCs) and reduce differentiation (Dahl et al., 2008; Shahdadfar et al., 2005) however, it still has problems of limited availability (Lindroos et al., 2009).

There are many methods for reduction of FBS including:

i. Existing culture media optimisation (Brunner et al., 2010; Jung et al., 2012).
ii. Serum media reduction (Brunner et al., 2010).
iii. Defined serum-free media (Gstraunthaler, 2003; Zimmerman et al., 2000) or serum deprived media (Solmesky et al., 2010).
iv. Allogeneic human serum (HS), xeno-free (XF) media formulations (Kocaoemer et al., 2007; Mannello and Tonti, 2007).

1.10.2 Serum-free media

Recently, the availability of commercial serum-free media has increased significantly. Most types of cells; both primary cultures and cell lines can be grown in growth media or "application media". Growth media could be considered as a primary media which can maintain the cells and enable proliferation; they are termed in the literature replacement media or basal media. Application media describes a media designed for specific functions of metabolism, for example, a simple washing solution, the medium of transport (Brunner et al., 2010).

There are plenty of factors which can be altered that affect cell maintenance, growth and characteristics. Furthermore, the attachment of anchorage-dependent cells such as hMSCs will be affected by the specific components of any serum-free media. Additionally, since hMSCs are obtained from adult human donors, the responses to the components of the media may be donor-specific. For these reasons, the processing of a defined serum-free medium especially for specific cell type is a complex task (Jung et al., 2012).

A number of methods for developing of serum-free medium either for specific cell line or initial culture have been described (Freshney, 2010; Jung et al., 2012).

The first approach is called Ham's approach which is partly reducing or eliminating serum amount in a medium. The essential concept of Ham's approach is minimising serum to a level that confines growth and then adding serum ingredients and observing

any changes in the medium or culture conditions that will create growth improvement (Jung et al., 2012). Using this approach, Ham and colleagues assorted the growth-promoting functions of serum into two basic classes, “replaceable” and “non-replaceable” (Jung et al., 2012; Ham and Mckeehan, 1978).

The second method is called Sato’s approach; it is different from Ham’s approach as it used an artificial method for serum replacement in culture media. It based on what functions serum plays for cell growth and maintenance in the culture media and then supplementation of the existing basal medium formulation with essential hormones and growth factors, stimulating the growth stimulation function of whole serum (Barnes and Sato, 1980). Jung and colleagues mentioned that Sato’s group distinguished different essential chief supplements, hormones, binding proteins, lipids and trace elements for medium basal addition. In particular, insulin, transferrin, and selenium were required for essential growth of most cells but some cell types additionally required hydrocortisone and epidermal growth factor (Jung et al., 2012).

The final methods are called a top-down and bottom-up approach, which could be effectively applied to cell growth by new serum-free medium formulation development (Jung et al., 2012).

The **top-down** approach uses existing medium formulations for a similar cell type, and the stimulatory ingredients are identified in serum present during target cell growth. This process is based mainly on the gradual reduction of serum concentration but when this approach is used the user should be careful as any cytotoxic or growth inhibitory elements in the medium may have a stronger effect with reduced serum (Jung et al., 2012).

The **bottom-up** approach uses an appropriate basal medium (cell type growth is used) and observation of different selected exogenous factors for their growth-stimulatory effect. Just the active elements necessary for growth cell are added to the medium (Jung et al., 2012; 2010).

Nutrients are chemical materials that are transferred to the cells and used as substrates in energy metabolism or biosynthesis, catalysts or structural elements of cellular organelles. In general, they are classified as organic nutrients, inorganic salts, and trace components. The organic nutrients are subclassified into amino acids, carbohydrates,

vitamins, lipids and other (Jung et al., 2012). Typically, the nutrients are a significant part or "defined" portion of culture media as they play essential roles for functional regulation (Ham and Mckeehan, 1978).

Among the organic nutrients are amino acids which are essential critical compounds of media as building blocks for the synthesis protein. Some amino acids, especially in serum-free conditions, have crucial roles in the multiplication of cells in culture, for example, glutamine shows an essential purpose in many metabolic pathways (Parker et al., 2007).

Carbohydrates are important for cell growth in culture and a vital source of energy for cells in culture, such as glucose. Although glucose can be harmful if used at high concentrations. Nevertheless, specific recent research appears to show that a high glucose concentration (~25mM) could lead to faster animal and human MSC growth (Li et al., 2007; Nauman and Deorosan, 2011; Weil et al., 2009).

Specific lipids that stimulate mammalian cell growth such as cholesterol and free linoleic acid are not involved in the defining part of cell culture media. Therefore, external lipids are supplemented in specific serum-free culture (Jung et al., 2010; Parker et al., 2007).

There are 12 vitamins which are divided into four fat-soluble vitamins (A, D, E and K) and eight members of the B complex, all these vitamins are required for mammals. Vitamins B act as enzyme cofactors and are required for the diploid cell (cell division). For this reason, all B vitamins are included in many cell culture media (Jung et al., 2012). Vitamin C (ascorbic acid) is essential for individual cell growth and its functions include playing a role as an oxygen acceptor in several mixed-function oxidase systems, and stabilising collagen fibrils factor (Rowe et al., 1977). Vitamin C was shown to be an essential element for increasing cell proliferation and stimulated the biosynthesis of extracellular matrix constructions in the primary or initial culture of human bone marrow cells under serum-free conditions (Gronthos and Simmons, 1995; Harada et al., 1991). Vitamin C has also been demonstrated to support specifically hMSC growth (Jung et al., 2010; Kuznetsov et al., 1997) and osteogenesis (Jung et al., 2010). Vitamin C is generally being utilised as a supplement in specific MSC differentiation media, and has been shown to maximise osteoblastic marker expression in hMSCs grown under serum-free condition (Mimura et al., 2011).

There are many salts founded in the media which include Calcium ions (Ca^{2+}), Chloride ions (Cl^{-}), Sulphate ions (SO_4^{2-}), Phosphate anions (PO_4^{3-}), Sodium ions (Na^{+}), Potassium ions (K^{+}), Magnesium ions (Mg^{2+}) and Bicarbonate ions (HCO_3^{-}), and these have many functions which are: firstly, maintaining the osmotic balance of the cells. Secondly, acting as divalent cation, especially Ca^{2+} for specific molecule cell adhesion. Thirdly, acting as an intermediate for signal transduction and regulation of membrane potential. Fourthly, the matrix and nutritional precursors of macromolecules need SO_4^{2-}, PO_4^{3-} and HCO_3. Finally, HCO_3^{-} is considered as a buffer in media and the concentration of Carbon dioxide (CO_2) in the gas phase determines the concentration of HCO_3^{-} (Jung et al., 2012).

As well as the inorganic salts, other inorganic compounds found in serum play a role as enzyme cofactors and are required for survival and growth of cells, such as Copper (Cu^{2+}), Manganese (Mn^{2+}), Molybdenum (Mo^{6+}), Zinc (Zn^{2+}), Selenium (Se^{8+}), Silicon (Si^{4+}), Iron (Fe^{2+}) and Nickel (Ni^{2+}) (Jung et al., 2012).

Mammalian cell growth requires additional factors as well as nutrient ingredients. These include growth factors and attachment proteins and hormones. These factors are not present in serum-free basal media and, therefore, supplementation is required. These growth-stimulating regulatory components have commonly been the priority in serum-free media optimisation (Chase et al., 2010; Jung et al., 2010; Ng et al., 2008). It is crucial to note that TGF-β 1 alone appears to have a growth-inhibitory effect on hMSCs while showing as considerable synergistic effect with bFGF (Jung et al., 2010). The influence of PDGF on hMSCs is debatable. Some research suggested that PDGF increases MSC proliferation (Lennon et al., 1995; Ng et al., 2008). Furthermore, plasma containing a high level of PDGF or platelet lysate has been demonstrated to enhance anchorage and hMSCs expansion; but their effect is often masked in the presence of other potent factors (Jung et al., 2010).

Binding proteins such as albumin and transferrin are commonly supplemented with serum-free media. The absence of albumin in serum-free formulations has reduced hMSC growth (Parker et al., 2007). Conversely, many other reports also show the growth-promoting effects of these proteins in many cell types (Freshney, 2010). The hormone hydrocortisone is considered to be a key ingredient in serum-free media for hBMSCs growth, and furthermore displays a significant positive effect on cell metabolism when

combined with fetuin. Fetuin is an essential plasma glycoprotein which is synthesised in the liver by hepatocytes and has the ability to regulate bone metabolism clinically and has also been shown to be an essential requirement for serum-free primary cultures of hMSCs and other cell types such as mouse fibroblast and epithelial cells (Jung et al., 2010; Wang and Haslam, S, 1994).

Heparin, a glycosaminoglycan anticoagulant factor has been shown to have both proliferative and anti-proliferation effects (Jung et al., 2012). It was shown to reduce the growth of hMSCs from adipose tissue and bone marrow (Jung et al., 2010; Kocaoemer et al., 2007). In contrast, Mimura et al. reported the growth-enhancing effect of heparin on a genetically modified hMSCs line (Mimura et al., 2011).

1.11 Human platelet-rich blood products

The life cycle of platelets begins with hematopoietic differentiation of stem cells in the surface endosteal layer of bone, megakaryocyte progenitors then migrate to the blood vessels in the bone marrow (Malhotra et al., 2013). Platelets have an essential role in wound healing where they attach to exposed surface promoting blood clotting and the inflammatory molecules and cytokines are attracted to the region (Figure 1.11 A).

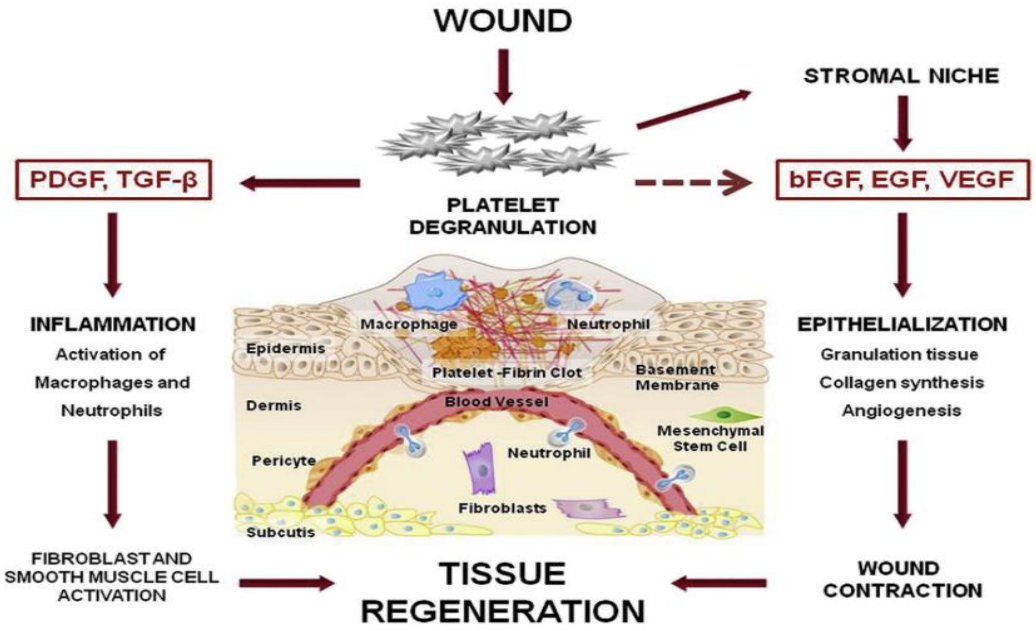


Figure 1.11 A: *The consequence of platelet activation during wound healing. Printed from Burnouf et al., (2016) with kind permission of Biomaterials.*

Platelets are small (2 μm in diameter), anuclear cells with a 7 to 10-day lifespan. They have many pseudopodia extensions, and the morphology is often described to be similar to the natural sea sponge (Marx and Garg, 2005).

Platelets contain three essential kinds of granules: dense, lysosomes and alpha granules (Blair and Flaumenhaft, 2009; Marx and Garg, 2005). The essential function of the lysosome is to serve as the storehouses for digestive enzymes (Malhotra et al., 2013; Marx and Garg, 2005). While the dense granules play a crucial role in storing and releasing adenosine diphosphate (ADP) which is responsible for the activation of other platelets. They also secrete calcium ions (Whiteheart, 2011). Therefore, the α-granule storehouse is considered to be a key source of growth factors. After degranulation, a considerable number of proteins are delivered either into the extracellular matrix or fused with the cell membrane. These proteins are collected from a cluster of chemotactic and mitogenic growth agents or factors, haemostatic agents, adhesion molecules and different cytokines (Blair and Flaumenhaft, 2009). Finally, the secretion of specific and nonspecific growth factors can initially control the platelets for tissue repair (Malhotra et al., 2013). The growth factors contained in platelets are platelet-derived growth factors (PDGFaa, PDGFbb, and PDGFab), transferring growth factors beta (TGFβ1, TGF β2), vascular endothelial growth factor (VEGF) and epithelial growth factor (EGF) (Marx and Garg, 2005) (Figure 1.11 B). As a consequence of platelet activation, the contents of granules are released and distributed to enhance wound healing (Burnouf et al., 2016).

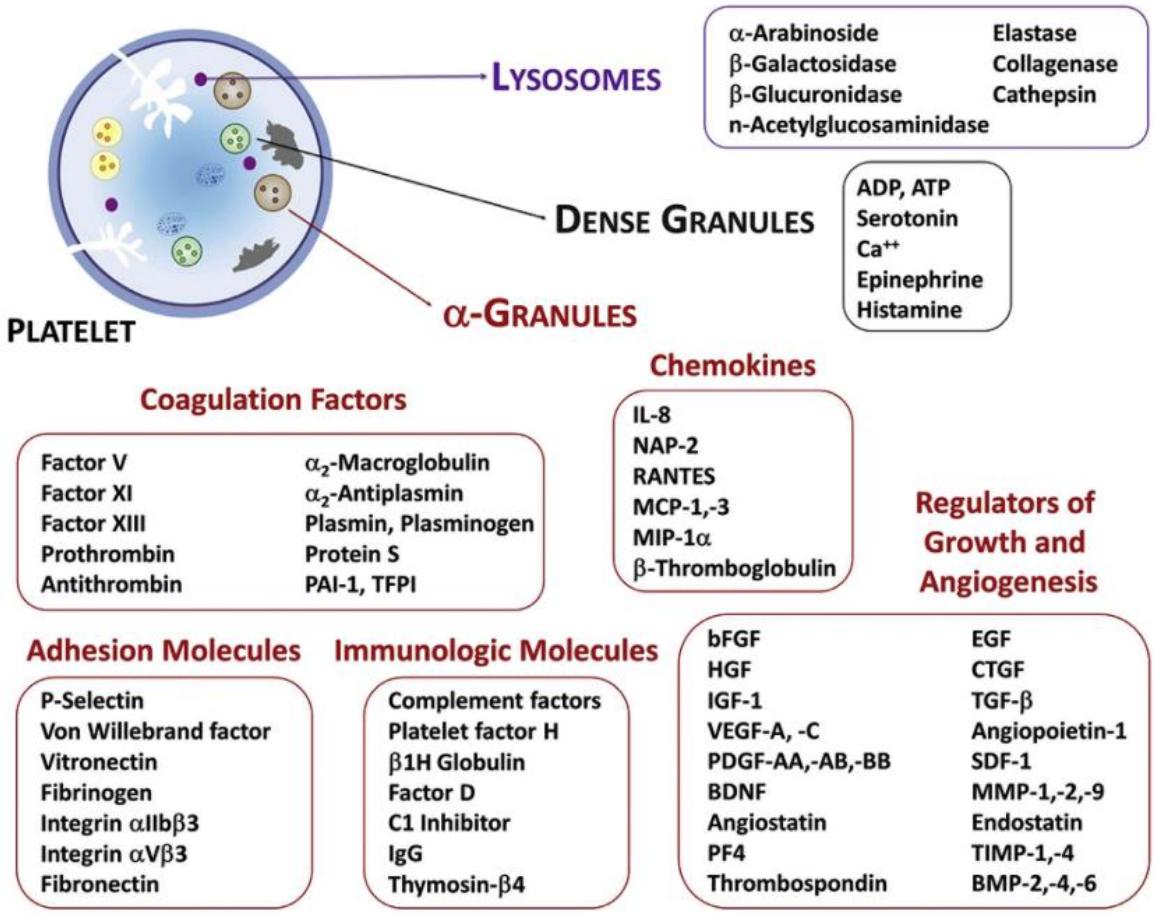


Figure 1.11 B: *The cargo of platelet granules. Three essential granules store an abundance of substances, there are lysosomes, dense granules and α-granules. Printed from Burnouf et al., (2016) with kind permission of Biomaterials.*

1.11.1 Human blood serum

Human blood serum is a complex body fluid that derives from human plasma in which blood cells are suspended and circulated around the body. With the removal of clotting factors plasma; serum contains various proteins ranging in concentration from 60 to 80 mg of protein per mL in addition to different small molecules including salts, lipids, amino acids, and sugars. The primary protein ingredients of serum include albumin, immunoglobulins, transferrin, haptoglobin, and lipoproteins (Burtis et al., 2014). Furthermore, these significant components, serum has several additional other proteins that comes through the lysis method of the cells and tissues throughout the body. It is predicted that up to 10,000 proteins may be commonly found in serum (Adkins et al., 2002). It is obviously that sera extracted from human and animal are routinely filtered at 0.22 µm. however, in particular clinical applications, human serum is taken place either filtered at 0.2 mm (Behnia et al., 2009) or without filter.

Human blood products are often applied in the clinical regenerative dentistry due to their easy preparation. For example, platelet-rich fibrin (PRF), can be separated by blood centrifugation without anticoagulation agents and applied clinically. It has been stated that the levels of growth factors are higher with high platelet concentrations (Huang et al., 2017; Kitamura et al., 2018), Figure 1.11.1. Although PRF is frequently identified as an advanced second-generation of PRP, it is differentiated from other PRP derivatives in terms of the removal of anticoagulant substances and coagulation elements (Dohan et al., 2006).

Previously, it was thought that PRF does not have a high quantity of growth factors (Gassling et al., 2009) however, this was contradicted recently (Masuki et al., 2016). Sera extracted from human and animals and used in the laboratory are routinely filtered at 0.22 µm. however, in particular clinical applications, human serum is either filtered at 0.2 mm (Behnia et al., 2009) or used without a filter for autologous uses.

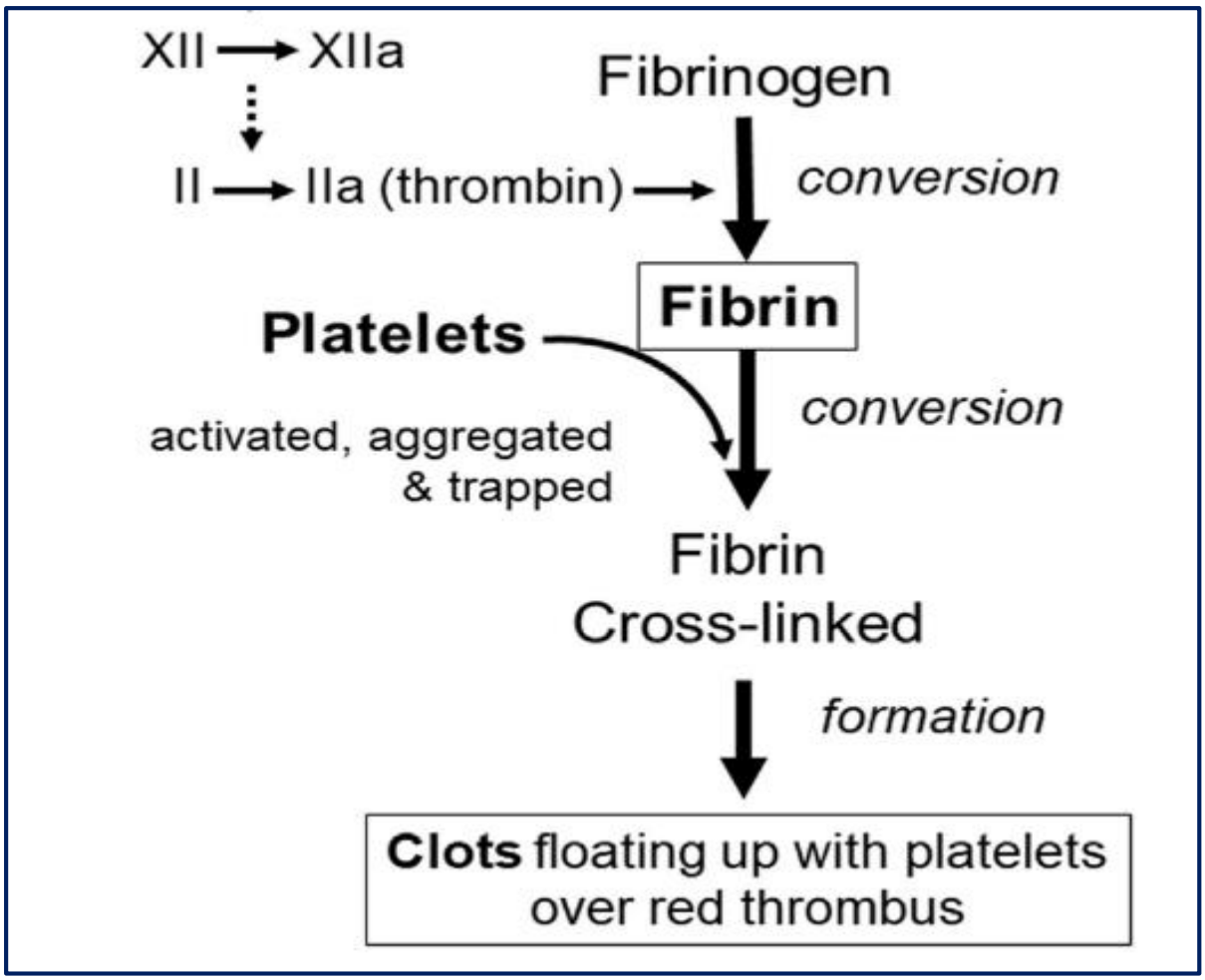


Figure 1.11.1: *mechanism of platelet-rich fibrin formation* (Kitamura et al., 2018)

1.11.2 Human plasma

Plasma is prepared by centrifugation after mixing of an anticoagulant agent with blood. It has numerous growth factors and nutrients which are used for cell proliferation, expansion, and can support differentiation into different cell lineages (Mannello and Tonti, 2007). Although platelet-poor plasma can be used for the initial growth of colonies-derived from bone marrow, the addition of platelet lysate is essential to enhance cell proliferation (Horn et al., 2010).

Both plasma and serum are the essential ingredient of blood and have different functions. Because they are frequently applied in the medical field, it is important to know what the differences between them are (Table 1.11.2) [7].

Table 1.11.2: the important differences between the human serum and plasma [7]

Aspects	Serum	Plasma
Definition and Extraction	Liquid obtained from blood after allowing the blood to clot and separated from the clot	Intravascular liquid obtained by centrifuging blood and separating the liquid from the blood cells
Contains coagulation proteins?	No	Yes
Anti-coagulant agents are needed to separate it?	No	Yes
Has undergone coagulation process?	Yes	No
Contains fibrinogen?	No	Yes
Medical applications	• Serological test • Investigation of blood groups Test for cholesterol and proteins	Diagnosis for certain blood diseases such as haemophilia and clotting disorder
Medical applications	• Serological test • Investigation of blood groups • Test for cholesterol and proteins	Diagnostic for certain blood diseases such as haemophilia and clotting disorder

1.11.3 Human platelet concentrate

Fractionated blood components have been used in the medical field for several decades. Extracted fibrin glue has also been used in intraoperative surgery as a topical haemostatic agent (Brennan, 1991). Platelets can be concentrated by three procedures using the anticoagulating agent in whole blood for clinical applications which include: 1) the buffy coat procedure (commonly used in Europe), 2) the PRP procedure (commonly used in Asia and USA), and 3) plateletpheresis methodology to create human platelet lysate (HPL) (globally used) (Figure 1.11.3) (Burnouf et al., 2016).

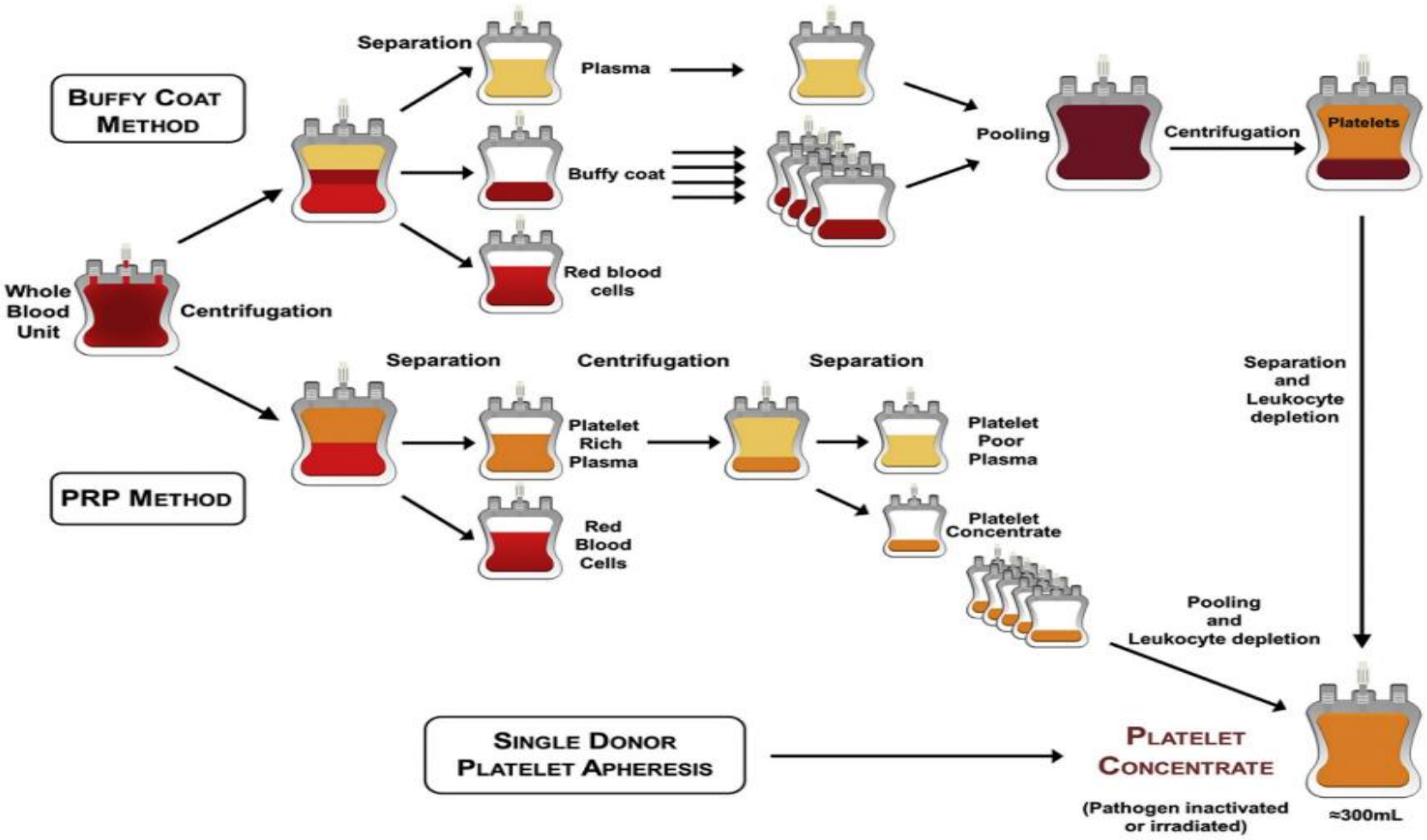


Figure 1.11.3: *the essential methodology for platelet concentrate preparation. Printed from Burnouf et al., (2016) with kind permission of Biomaterials.*

Platelets concentration (PC) is a standard of blood production; it has ten times the number platelets than PRP and is approximately independent from donors' gender and age. PC contains the growth factors in the cell-free supernatant which are secreted from the platelets after a freeze-thawing step (Krüger et al., 2012). It is clinically used for the management of different regenerative pathologies such as wounds and ulcerations (Dellera et al., 2014). In *in vitro* studies, PC was shown to enhance the wound healing processes (Backly et al., 2011). A further advantage of PC is that it can be stored frozen and can be analysed for growth factor content and other characteristics (Burnouf et al., 2016). Additionally, an allogenic PC can be based on clinical blood collections as these have already established safety and absence of known pathogens (Strunk et al., 2018).

Platelet-rich plasma (PRP) is a normal blood fluid consisting of a highly significant number of platelets and clotting factors. The concentration of platelets has been reported to be approximately ten million per micro-litre of plasma in 5 ml, and PRP contains a 3-5-fold higher concentration of growth factor which is associated with enhancement of healing (Foster et al., 2009). It is free of any transmissible diseases and any hypersensitivity reaction due to it being derived from the patient's own blood (Marx and Garg, 2005). It is prepared from whole venous blood combined with anticoagulants to avoid clotting (Middleton et al., 2012). Two methods of PRP production have been described, either a two-step centrifuge method which is the most common application or a one-step method (Malhotra et al., 2013). Marx et al., (1998) used PRP in the regeneration of mandibular bony defects and consequently reported positive results. This report led to more interest and research funding for the application of PRP in oral and maxillofacial surgery (Kassolis et al., 2000; Landesberg et al., 2000).

Some proteins can be used to activate PRP to initiate the clotting mechanism and secretion of growth factors. These proteins are bovine thrombin (Fréchette et al., 2005), calcium chloride (Nagata et al., 2010), mixed bovine thrombin and calcium chloride (Fufa et al., 2008; Marx and Grag, 2005), type I collagen (Fufa et al., 2008), thrombin receptor agonist peptide (TRAP)(Landesberg et al., 2005), autologous thrombin (Everts et al., 2006), freeze-thaw (Baik et al., 2014) and batroxobin activator (Middleton et al., 2012). While the methods used for activating growth factors and other bioactive substances from HPL can be categorised into four procedures either repeating freeze-thaw procedure (Baik et al., 2014; Schallmoser et al., 2007), sonication (Umeno et al., 1989),

solvent-detergent treatment (Shih and Burnouf, 2015), and those of proteins used for initiating PRP.

The principal factor important for tissue engineering of bony defects is the combination of signalling molecules, cell and matrices (Langer and Vacanti, 2015). PC can provide the molecules and cell-supporting matrix signalling through the provision of growth factors, and fibrin matrix respectively (Malhotra et al., 2013).

The clinical applications of PC have increased significantly and it is used in many areas, especially in dental surgical procedures such as sinus lifting augmentation (Schaaf et al., 2008); ridge augmentation or atrophy (Schuckert et al., 2011); third molar socket (Kaul et al., 2012); periodontal defect treatment (Kitoh et al., 2007); preservation of alveolar ridge (Marx and Garg, 2005) and in dental implants (Polimeni et al., 2010). It has also been used in craniofacial surgery for example, in the: reconstruction of the alveolar ridge (Luaces-Rey et al., 2010); mandibular reconstruction, split-mouth; double-mask (Hanna et al., 2004); maxillary and midface reconstruction (Kuvat et al., 2009).

1.11.3.1 Mechanisms of PC during regeneration of bony defects

Bone loss or bone defects can arise as a result of surgery, different ailments or serious injuries. Bone can rebuild itself with complex processing (Ingham and Fisher, 2005; Stea et al., 2000).

The principles of PC mechanisms through three phases of bone regeneration include the inflammatory cytokines phase with growth factors which are released during degranulation of α –granules, and angiogenesis (Zhang et al., 2013; Zimmerman et al., 2002).

1. **PC in the function of inflammatory cytokines enhances bone regeneration**

   Inflammatory mediators play an essential role in the early phase of both intrinsic and extrinsic clot formation. There are aggregation and secretion of cytokines and growth factors as well as haemostatic factors during bone deficiency healing (Glass et al., 2011; J. Zhang et al., 2012). Both histamine and serotonin are secreted from dense granules, and the primary function of them raises the capillary permeability which leads to accumulation of inflammatory cells on the wound site and stimulation of macrophages (McManus and Pinckard, 2000).

2. PC in the function of growth factors enhances bone regeneration

The increase of growth factor concentration and the released bioactive proteins can lead to tissue regeneration where there is low potential for healing and regeneration (Zhang et al., 2013).

Growth factors acts on the regulation of genes and control this through the feedback of normal wound healing. The relationship between growth factors and stages of endochondral healing and how the growth factors interact with main the cell types during bone healing are illustrated in Figures 1.11.3.2 (Malhotra et al., 2013).

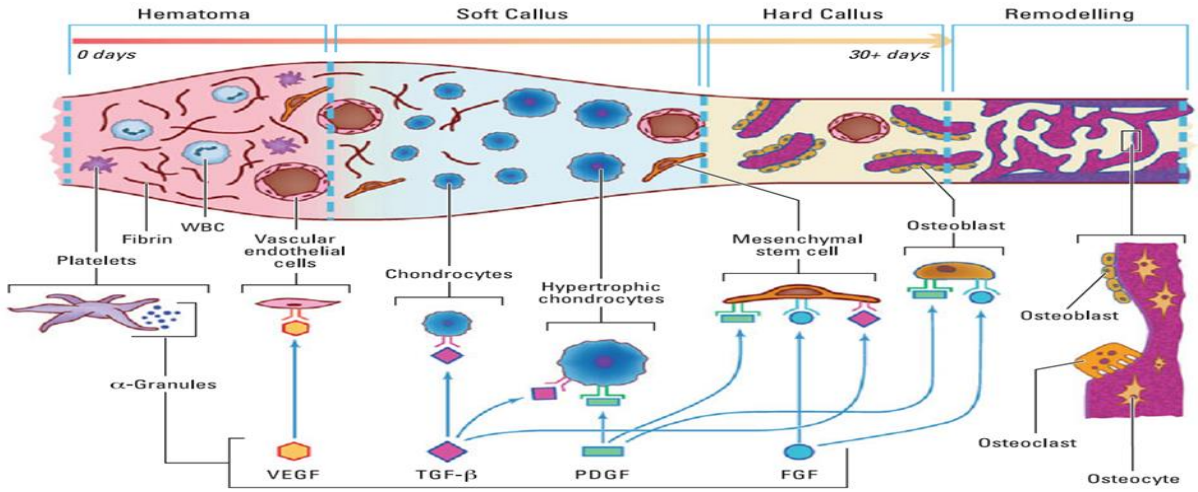


Figure 1.11.3.2: *the relationship between growth factors and stages of endochondral healing how the growth factors interact with main cell types during bone healing. Printed from Malhotra et al., (2013) with kind permission of Archives of Orthopaedic and Trauma Surgery.*

3. The Influence of PC in angiogenesis factors enhances bone repairing

A sufficient blood supply is needed for osteogenesis, in the last stage of endochondral ossification. The specific matrix metalloproteinase would break the cartilage and bone to lead to the new growth of blood vessels. There are two independent approaches to angiogenesis: the first one depends on VEGF, and the second depends on angiogenin. The renewal of vessel growth and a specific mitogen of endothelial cells are mainly affected by VEGF, while large vessels and formation of collateral circulation are mainly affected by angiogenin (Zhang et al., 2013).

1.12 Scaffolds for craniofacial bone tissue engineering

A scaffold is structure which acts as a vehicle for growth factors; cells and drugs and also may partially imitate the native structure of extracellular matrix. The biomaterial scaffold plays a crucial role in reconstructive surgery by allowing an adequate microenvironment for regeneration of the required cells (Florencio-Silva et al., 2015; Tevlin et al., 2014).

Tevlin et al., (2014) and Salgado et al., (2004) suggest that the properties of scaffolds for craniofacial bone tissue engineering must consider the:

i. Biocompatibility: the scaffold should be able to integrate with the neighbouring tissue without unfavourable immunological reaction.

ii. Bioresorbability or Biodegradability: the scaffold should be able to degrade with time and in a controlled manner.

iii. Porosity: the scaffold should be interconnective to facilitate essential nutrient diffusion, cell attachment and growth, oxygen diffusion, and removal of metabolic waste to ensure cell survival.

iv. Mechanical strength: the scaffold should have an adequate mechanical strength to overcome the implantation method and the mechanical forces which are generally applied to the scaffold-tissue construct after implantation, and also to preserve space for cell growth.

v. Pore size: the diameter of the pores plays a crucial role in cell penetration, production of bone matrix, and angiogenesis (blood vessel formation).

vi. Surface properties: cell attachment and proliferation are influenced by the properties of the scaffold surface such as hydrophobicity and ability to bind to proteins.

vii. Osteoinductivity: the scaffold should be able to encourage osteogenesis and bone matrix deposition.

1.12.1 Types of scaffolds

Biomaterials using in craniofacial bone tissue engineering scaffolds can be classified into several basic material types (Tevlin et al., 2014):

i. Polymers are commonly used for creating scaffolds because of controllable degradation, easy manipulation, and biocompatibility. The degradation of polymer scaffolds is usually achieved by hydrolysis, permitting the supporting tissue to recover functionality slowly. Biodegradable polymers can further be classified into two groups: firstly, natural materials such as polysaccharides (e.g., chitosan) and proteins (e.g., collagen) secondly, synthetic polymers such as poly (lactic acid) and polycaprolactone. Biodegradable polymers have some advantages especially in paediatric surgery, as they should be replaced by the patient's bone to allow growth and perform the therapy; they may also have clinical advantages during radiography as computed tomography scans can see the resorbed bone formation, Table 1.12.1, shows specific widely used scaffold materials approved by the US Food and Drug Administration (FDA) for medical use, for example in craniofacial regeneration.

Table 1.12.1: Characteristic features of synthetic and natural polymers utilised in bone tissue engineering

Materials	Origin	Characteristics
Collagen	Natural	Minimum immune activation. Cell adhesion is facilitated via cell adhesion molecules similarly to in the physiological environment (because collagen is the major ECM protein of human tissue). Assist with cell morphology, migration, modification, and differentiation. Poor mechanical properties (Kleinman et al., 1981).
Chitosan	Natural	Support to haemostatic processes. Increase osteoconductivity and wound healing (Pogorielov et al., 2017).
Poly(hydroxybutyrate)	Natural	A natural resource.

		Restricted usefulness because of brittleness (Chen and Wang, 2002).
Poly(Ɛ-caprolactone)	Synthetic	The aliphatic family of polyester. Approved by U.S. FDA. Degradation by hydrolysis (Hutmacher et al., 2001). Biocompatibility (Langer and Tirrell, 2004). Controllable degradation. No toxicity. Low cost (Yoshimoto et al., 2003). Adequate mechanical attributes (Delaine-smith et al., 2014).
Polylactic acid (PLA)	Synthetic	Inadequate mechanical attributes. Controllable degradation. Degradation by hydrolysis method. Inflammation might be created in vivo (Cheung et al., 2007).
Polyurethane (PU)	Synthetic	Biocompatibility. Degradable and nondegradable substances. A broad range of adjustable mechanical, biological, and degradable attributes. An essential problem is potential toxicity (Gunatillake et al., 2003).
Hydrogel	Synthetic	Absorption of a considerable amount of water. Act as matrices for tissue engineering. Mimic of extracellular matrix topography. Transferring of bioactive factors (Lee and Shin, 2007).

ii. Metals are solid and rigid and robust; commonly used metals for clinical applications include gold, cobalt-chromium, stainless steel, and titanium. Titanium is especially important for craniofacial applications (Yusop et al., 2012).

iii. Bioactive glass and ceramic (Hoppe et al., 2011).

iv. Composite scaffolds which are composed of two or more materials such as a scaffold constructed from both polymer and ceramic (Rezwan et al., 2006).

There are drawbacks of these materials for example, metals are non-degradable, ceramics are very brittle and difficult to shape for thin defects, and composites are complicated to produce in a reproducible way.

1.12.1.1 Poly (ε-caprolactone)

A poly (Ɛ-caprolactone) (PCL) is produced from aliphatic polyester (Figure 1.12.1.1), and is considered to be a biocompatible and biodegradable material. PCL is broken down by hydrolysis into biocompatible products, many studies have employed PCL clinically and it is officially approved by the U.S. Federal Drug Administration (FDA) for human applications such as Monocryl® sutures (Hutmacher et al., 2001; Reed et al., 2009). PCL is suitable for bone regeneration as a tissue engineering scaffold because it has a slow degradation rate. Therefore, it has been implicated as an adequate material for replacing bone in load-bearing areas to support bone while it undergoes mature development and forms a functional tissue. Hutmacher et al., (2001) indicated that a PCL scaffold could preserve its mechanical attributes for 5-6 months and be completely metabolised in more than two years. Furthermore, several researchers have employed PCL as a material for *in vitro* research, for example, to support human osteoblast and fibroblast-like cells for attachment, proliferation, extracellular matrix formation, and tissue development (Delaine-Smith et al., 2014; Tuan and Hutmacher, 2005; Williams et al., 2005). Moreover, PCL is inexpensive and easy to produce large quantities.

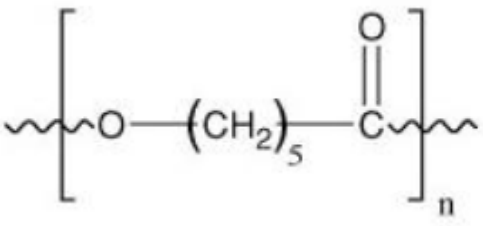


Figure 1.12.1.1: *Chemical formula of polycaprolactone.*

1.13 Electrospinning

1.13.1 Introduction

Electrospinning is a versatile system for scaffold preparation in tissue engineering. It usually utilises an electric force to create polymer fibres of different diameters from micrometres down to nanometres; many types of polymer can be used in this technique. The advantages of electrospinning over other fabrication techniques are that it is controllable, specific orientations can be achieved, a high surface area and high porosity is created and it is an inexpensive approach. However, this technique has limitations such as slow rate of production, and difficulties of scale-up (Delaine-Smith et al., 2014; Rim et al., 2013).

1.13.2 Spinning mechanism and theory

The first discovery of the electrospinning was by Rayleigh in 1897, while the first description of the technique in details was by Zeleny in 1914 (Zeleny, 1914). The main idea of his work is created an electrostatic repulsion via applied a considerable voltage on a polymer solution. The repulsion effect is to frustrate the value of surface tension of the polymer solution. Consequently, it causes a small drop to flare up and becomes stretch to create a Taylor cone. Polymer solution jet is released when the solution charge resists the tension of the surface (Taylor, 1964). Polymer fluid emerged and polymerised on the collector. A continuous flow of liquid has two stages; firstly, it is a stable stage (alignment form), polymer steam releases from the tip of the needle as an extending uniformly in one direction line to form a woven mat of fibre. Secondly, it is unstable stage (non-alignment form), in which the polymer steam presents a non-aligned whipping orientation to fabricate a non-woven fibre mat of thin fibres onto a collector (Reneker et al., 2000).

1.13.3 Electrospinning in tissue engineering

The electrospun scaffold is employed for tissue engineering as an extracellular matrix to support cell growth and differentiation. Both synthetic and natural polymers have been investigated for use in tissue engineering applications (Burger et al., 2006). Electrospinning can be adjusted to produce different fibre sizes which are appropriate for a particular tissue and has been the basis for studies on many different tissues such as bone (Ma et al., 2005), skin (Blackwood et al., 2008), tendon (Yin et al., 2010), heart (Zong et al., 2005), and nerve (Daud et al., 2012). Electrospun scaffolds can also be used

as delivery vehicles for bioactive components such as proteins, drugs, and DNA if using degradable polymers (Luu et al., 2003). The amount of agent released depends on the technique of consolidation between the bioactive component and the scaffold (Liu et al., 2010).

1.13.4 Electrospinning rig set up

Four main components comprise electrospinning equipment (Katta et al., 2004):

1) An electricity source with a controllable high voltage which charges the polymer solution.

2) A syringe pump which determines the amount and rate of polymer solution dispensing.

3) A syringe to hold the polymer solution and needle to charge the polymer solution.

4) An earthed collector for fibre collection.

Different kinds of collectors are designed to fit the objective such as a rotating drum or flat plate. An unevenly distributed mat may be created when using a flat plate. Therefore, a rotating drum collector was applied in our experiments (Table 1.13.4) (Figure. 1.13.4).

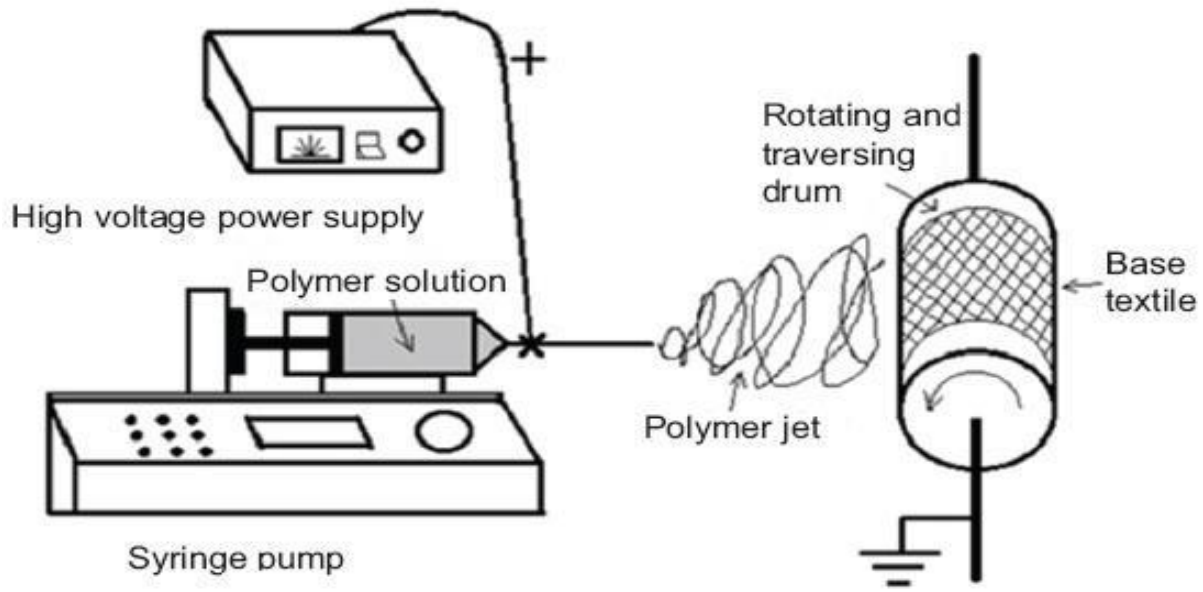


Figure. 1.13.4*: Schematic of electrospinning machine [8] illustrating the components described in section 1.13.4.*

Table 1.13.4: shows the effect of parameters on the fibre structure of an electrospun scaffold.

Parameters	Fibre morphology
Polymer concentration: ➢ low ➢ high	Breaking in the jet and bead formation No jet creation because of the dryness of droplets at the capillary tip
Working distance: ➢ close ➢ far	Wet beaded structure and fibres Bead morphology
The flow rate of polymer ➢ Low flow rate ➢ High flow rate	Reduced fibre diameter High fibre diameter and bead formation
Voltage supply ➢ Low voltage ➢ High voltage	High fibre diameter Low fibre diameter and beading morphology (Zuo et al., 2005).
Humidity ➢ High humidity (>60%)	Wet fibres and pore formation on the fibre surface called "breath figure". The breath figure is produced from the air condensing on the fibre surface because of the jet cooling and evaporation (Casper et al., 2004).
Temperature ➢ High temperature	Reduced fibre diameter

1.14 Aims and objectives of the research project

Hypothesis

The primary hypothesis of this project is that xeno-free media will have the ability to support osteogenic differentiation of human mesenchymal stem cells and to support creation of a bone-like matrix suitable for craniofacial tissue engineering. The long term goal is to create a co-culture construct of a human bone-like tissue layer and a human soft/fibrous-tissue layer. After clinical development such a strategy could be applied in the future to repair defects in the roof of the mouth for patients afflicted with cleft palate.

Objectives

The objectives of this PhD project were to characterise and evolve a novel methodology, which would be convenient for eventual clinical application for repair of bone defects in the oral cavity. Furthermore, to understand how commercial animal-free media may be used to engineer bone tissue for developing cleft palate repair in 2D and 3D. A tri-layer electrospun PCL scaffold was used to separate three different cell types; human normal oral fibroblast cells (NOFs), immortalised human normal oral keratinocytes (FNB6), and primary human mesenchymal stem cells derived from bone marrow (hBMSCs). This type of multi-layered tissue construct could permit normal development of facial bone and reduced the operational steps required to reconstruct the cleft palate.

The tasks to be undertaken to carry out the projects aims are to:

1. Evaluate animal-free component culture media on monolayer cultured primary human bone marrow mesenchymal stem cell and human jaw periosteal cells (HJPs).

1.1 Assess whether there are differences in the ability of xeno-free media to support osteogenesis in relation to the typically used osteogenic supplements: ascorbic acid-2-phosphate (AA) and β-glycerophosphate (βGP) using the indicators of osteogenic differentiation- Alkaline Phosphatase activity and calcium and collagen deposition.

1.2 Asses the effects of xeno-free media on proliferation of hBMSCs using flow cytometry.

2. Elucidate the ability of xeno-free media to support osteogenic differentiation of human cells on 3D poly (ε-caprolactone) electrospun scaffolds.

2.1 Evaluate cell metabolic activity on selected electrospun scaffolds (PCL) for hBMSCs and HJPs in a variety of xeno-free media.

2.2 To compare osteogenic differentiation of hBMSCs and HJPs cultured in different xeno-free basal culture media on PCL scaffolds.

2.3 Assess whether there is variability between different donor's cells when grown in xeno-free media.

2.4 Elucidate using VEGF secretion, as a marker of angiogenic potential whether the different culture conditions under investigation would influence the ability of cells to initiate angiogenesis.

3. Further, develop a tri-layer electrospun PCL scaffold to separate cell types on either side of a nano-fibre membrane.

3.1 Optimise growth conditions using xeno-free media for human normal oral fibroblast cells (hNOFs) and immortalised human normal oral keratinocytes (FNB6).

3.2 Visualise the osteogenic differentiation and oral mucosa development in the 3D tri-layered PCL scaffolds.

CHAPTER TWO: MATERIALS AND METHODS

2.1 Materials

1. 4‟-6-Diamidino-2-phenylindole(DAPI) (Sigma-Aldrich, Dorset, UK).
2. Alizarin red (stain which binds to calcium-containing) (Sigma-Aldrich, Dorset, UK).
3. Alkaline Phosphatase Yellow (pNPP) Liquid Substrate System ELISA (Sigma-Aldrich, Dorset, UK).
4. CellCrown™ (made from polyethene terephthalate: PET) (Scaffdex®, Tampere, Finland).
5. Click-iT™ EdU Alexa Fluor™ 488 Flow Cytometry Assay Kit (Invitrogen; ThermoFisher, UK).
6. Data analysis software: GraphPad Prism 7.
7. Dichloromethane (Sigma-Aldrich, Dorset, UK) for solubility polymers for electrospinning.
8. Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich, Dorset, UK).
9. Electrospinning apparatus contains a syringe pump (Genie™ Plus, Kent Science, USA), a high voltage electric source (Genvolt, UK), a rotating motor (Heidolph Instrument, Germany).
10. Flow Cytometry, FACS Calibur, BD Biosciences.
11. Formaldehyde (37% (v/v), grade 1) (Sigma-Aldrich, Dorset, UK).
12. Hexamethyldisilazane (HMDS) (Sigma-Aldrich, Dorset, UK).
13. Human bone marrow mononuclear cells (Lonza®, Bruxelles, Belgium).
14. Human mesenchymal-XF expansion medium (XF2) (Millipore, UK).
15. Human normal oral fibroblasts (NOF) were isolated from connective tissue from waste mucosa collected during routine dental surgeries at the Charles Clifford Dental Hospital, Sheffield, with written, informed consent with ethical approval granted from Sheffield Research Ethical Committee.
16. Image processing software: Flowjo-win64-10.2
17. Image processing software: image analysis and processing in Java-based (ImageJ) (developed by National Institutes of Health, USA).
18. Immortalised human oral keratinocytes (FNB6), kindly donated by Professor Keith Hunter, University of Sheffield.
19. Infinite F200 microplate fluorescence reader (PRO, TECAN, Labtech, UK).
20. Insulin-transferrin-selenium (ITS -G) (ThermoFisher, UK).

21. Laboratory digital balance (Mettler Toledo, UK).
22. Medical grade steel ring (2cm diameter, 1.2cm inner hole diameter) (produced by NHS medical workshop).
23. Minimum Essential Medium Alpha Eagle (α-MEM) (Lonza®, UK).
24. MSC NutriStem® XF medium with supplement mix solution (biological Industries BI, USA).
25. PBS without calcium (Ca^{2+}) and magnesium (Mg^{2+}) (Sigma-Aldrich, Dorset UK).
26. PeproGrow-1 serum-free cell culture supplement kit (Peprotech, London, UK).
27. Phosphate buffered saline (PBS) (Dulbecco A) (OXOID Limited, Hamshire, England).
28. Picric acid solution (Sigma-Aldrich, Dorset UK).
29. Sirius red stain (Sigma-Aldrich, Dorset UK).
30. Poly(ε-caprolactone) (PCL), 80kDa (Sigma-Aldrich-Aldrich, Dorset, UK) to manufacture the electrospun scaffolds.
31. Porcine gelatine (Sigma-Aldrich, Dorset, UK).
32. Primary human jaw periosteal (HJPs) cells were derived from periosteum tissue which was collected during routine wisdom tooth extraction at the Charles Clifford Dental Hospital, Sheffield.
33. Quantikine® ELISA Human VEGF Immunoassay Kit (RandD, USA).
34. Quant-iT™ PicoGreen® dsDNA Reagent Assay Kits (Invitrogen™, Paisley, UK).
35. Resazurin reduction assay (Sigma-Aldrich, Dorset, UK).
36. Scanning electron microscope (SEM, Philips XL-20 SEM, Netherlands).
37. Stem X Vivo™ xeno-free human MSC expansion medium (RandD, USA).
38. Stemulate™ pooled human platelet lysate (HPL) (COOK, USA).
39. Trypan blue (Sigma-Aldrich, Dorset, UK).
40. Trypsin-EDTA (Sigma-Aldrich, Dorset, UK).
41. Xylenol Orange tetrasodium salt (Sigma-Aldrich, Dorset, UK).

2.2 Methods

2.2.1 Media preparation

A range of media and supplements were tested during this thesis they will be referred to using the short-name or acronym as described below each was used with and without a range of supplements typically used in osteogenic media to identify interaction between the media type and the supplements:

BCM: Basal media: This refers to the selected media under investigation supplemented with FBS where required (see table 2) and 1% (v/v) antibiotics (either Penicillin - 100 units, Streptomycin – 100 µg/mL, L-Glutamine – 2 mM, or Penicillin and Streptomycin).

10% (v/v) FBS: alpha MEM supplemented with 10% FBS.

XF1: Stem X Vivo™ xeno-free human MSC expansion medium.

XF2: Human mesenchymal-XF expansion medium has 2% (v/v) human serum (the formulation for this product is confidential and therefore, can't be shared with our customers).

5% (v/v) HPL: Stemulate™ pooled human platelet lysate: alpha MEM supplemented with 5% HPL.

2% (v/v) FBS+ITS: Insulin-transferrin-selenium: alpha MEM supplemented with 2% FBS and ITS.

MSC-NutriStem: MSC NutriStem® XF medium with supplement mix solution

SR1: PeproGrow-1 serum-free cell culture supplement: PeproGrow-1 serum-free cell culture supplement kit, it contains two essential components: 1. Serum replacement solution containing a non-animal derived and chemically-defined salt. 2. Lipid mixture solution contains non-animal derived fatty acid and lipid, designed to improve cell growth in serum-free media.

Green's Medium: consists of Dulbecco's modified Eagle's medium (DMEM) (Gibco, Paisley, UK) and Ham's F12 medium (Biosera, East Sussex, UK,) in a 3:1 (v/v) ratio supplemented with 10% (v/v) FCS (Biosera), 0.1 mM cholera toxin, 10

ng/ml EGF, 0.4 mg/ml hydrocortisone, 0.18mM adenine, 5mg/ml insulin, 5mg/ml transferrin, 2mM glutamine, 0.2 nM triiodothyronine, 0.625mg/ml amphotericin B, 100 IU/ml penicillin, and 100mg/ml streptomycin. (All remaining products were obtained from Sigma-Aldrich Aldrich, Dorset, UK unless otherwise stated).

NOCM: Non-osteogenic culture medium: basal media supplemented with 50μM AA-2-Ph and 10mM β-GP (Sigma-Aldrich, Dorset, UK) this contains supplements required for matrix mineralisation but without the osteogenic factor Dexamethasone (Dex), know to stimulate differentiation.

OCM: Osteogenic culture medium: Dex containing osteogenic media; basal media supplemented with 50μM AA-2-Ph and 10mM β-GP, and either 10nM or 100nM Dex (Sigma-Aldrich, Dorset, UK), respectively.

BCM+Dex: Supplemented with Dex medium; basal media supplemented with either 10nM or 100nM of Dex.

Table 2.2.1: Media compositions investigated

Media Compositions
Basal Culture Medium (BCM)
BCM supplemented with Dex 10 nM
BCM supplemented with Dex 100 nM
BCM supplemented with AA 50 µM and BGP 10 mM
BCM supplemented with Dex 10 nM, AA 50 µM and BGP 10 mM
BCM supplemented with Dex 100 nM, AA 50 µM and BGP 10 mM

General cell culture conditions

Cell maintain ace and passaging

Cells were routinely maintained in T-75 flasks in their respective culture media at 37°C in 5% CO_2 in a humidified atmosphere. The medium was changed every 2-3 days. Cells were passaged when they reached 80% confluence.

For passaging the medium was removed, and the cell monolayer washed twice with PBS. Three ml of trypsin-EDTA solution was added and incubated for five minutes to detach the cells enzymatically from the tissue culture plate. Cell detachment was monitored using a light microscope. Once all cells had detached, trypsin was neutralised by the

adding of fresh culture medium. The cell suspension was centrifuged at 112 g for 5 minutes. The supernatant was aspirated and the cell pellet resuspended in a known volume of fresh media. To determine the cell number and the percentage of viable cells a trypan blue exclusion assay was performed. Twenty µl of trypan blue cell solution was mixed with twenty µl of the cell suspension, ten µl was then transferred to a hemocytometer and the number of cells counted under a light microscope. 1 X 10^5 cells were transferred to new culture flask or seeded into tissue culture treated plates for the experiments described below.

Cryopreservation of cells

Cells were removed from the tissue culture flask, centrifuged for 5 min at 112 g and suspended in FBS or XF2 culture medium containing 10% (v/v) final concentration DMSO as a cryo-protectant. 1 X 10^6 cells were transferred into a cryovial and stored at -80ºC for 24 hours before moving to a liquid nitrogen dewar (-196ºC) for long-term storage.

2.2.2 Isolation of human jaw periosteal cells (HJPs)

The human jaw periosteum consists of two layers; the outer layer is called the "fibrous" layer which contains fibroblasts, collagen, nerves, and a microvascular network. This layer provides mechanical stability to the periosteum. The inner layer "cambium" comprises of high cellular content and presents with cells that encourage bone formation and repair (Allen et al., 2004; Lin et al., 2014). Periosteal tissues were collected by Mr. Robert Bolt during routine wisdom tooth extraction at the Charles Clifford Dental Hospital, Sheffield, with written, informed consent with ethical approval (number 09/H1308/66) granted from Sheffield Research Ethical Committee. The periosteum tissue was processed by Dr Helen Colley and supplied for the project after extraction from tissue, briefly; tissues were stored in DMEM basal culture medium containing 1% (v/v) of 100 mg/ml P/S at 4ºC for 4 hours before the cell isolation. Collagenase type II (0.25% (v/v) in serum-free medium) was added to the tissue and incubated at 37ºC for 3 hours (Samee et al., 2008) before resuspending in basal culture medium and centrifuging at 447 g for 5 minutes, the supernatant was removed and the cell pellet resuspended in basal culture medium and transferred to a T-25 culture flask. A 50% (v/v) medium change was performed after 72 hours and a complete medium change after seven days. These HJP cells were supplied for this project and used between passages 3 and 10.

2.2.3 Human mesenchymal stem cells derived from bone marrow (hBMSCs)

Primary bone marrow mononuclear cell samples obtained from six different donors were purchased commercially (Lonza®) (Table 2.2.3). The purchased cells contain both haematopoietic cells and hMSCs. The isolation of hMSCs is dependent upon their ability to adhere to tissue culture plastic (Chao et al., 2012; Dominici et al., 2006). The cell solutions were transferred to 5 ml of pre-warmed basal culture media (10% (v/v) FBS, XF1 and XF2) to observe cell proliferation. 2 ml of fresh basal culture media was supplemented with samples every 2-3 days for seven days. Floating cells and non-adherent cells were removed and fresh culture media was added. The cells were cultured until 80% confluence was reached and labelled passage 0. The cells were serially sub-cultured at a density of 5 X 10^5 per T-75 flasks until passage four for experiments (Lee et al., 2013). Cells from donors numbered 1 to 4 were cultured in 2D monolayer experiments. Cells from donors numbered 4, 5, and 7 were cultured in 3D PCL scaffolds.

Table 2.2.3: Information about human bone marrow mesenchymal stem cells acquired from six different donors. (Donor numbered 6 was used in the laboratory but not for any experiments reported in this thesis, so is not listed).

Assigned Donor no.	(Lot No.)	Supplier's Date	Age	Sex
1	081291B	05-Aug-2008	23	Male
2	081286A	05-Aug-2008	30	Male
3	081294A	06-Aug-2008	23	Male
4	080007B	02-Jan-2008	22	Female
5	0000497388	04-Aug-2015	32	Male
7	080030A	07-Jan-2008	18	Male

2.2.4 A measure of cell metabolism using the resazurin reduction assay

The resazurin reduction assay has been applied to investigate the cytotoxicity and metabolic activity of many different cells. The can be used to analyse continuous cell growth as it is a non-toxic stain. The blue non-fluorescent colour of the resazurin solution reduces to resorufin, a pink colour that is also fluorescent (Figure 2.2.4). The mechanism of reduction is currently unknown, but it is believed to be due to the intercellular activity of mitochondrial enzymes. The resazurin reduction product can be measured either by measuring absorbance or fluorescence in a spectrometer. However, the measurement of fluorescence is more sensitive than measuring the absorbance (O'Brien et al., 2000).

A 1 mM stock solution of resazurin reduction salt dissolved in distal water was prepared and diluted to a working solution of 0.1 mM in α-MEM culture medium (MW=251.17 g/mol). Media was removed from the cells (either in 2D monolayer or on 3D electrospun PCL scaffolds), and 2 ml working solution was added. Working solution was also added to an empty cell culture well to provide a blank, background reading. The samples were wrapped in aluminium foil and incubated for 4 hours at 37°C. 200 µl of production of pink resorufin was transferred to a 96-well plate (triplicate from each well) and assessed using a spectrofluorometer was setting 540 nm and 590 nm of an excitation wavelength, and an emission wavelength respectively.

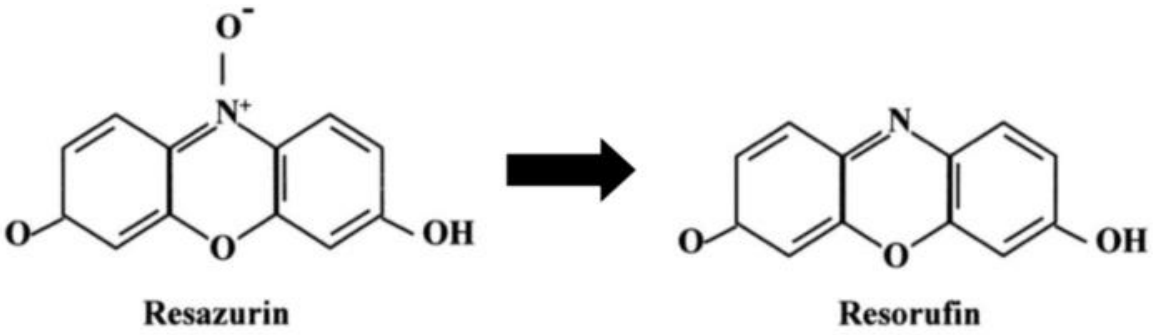


Figure 2.2.4: *A chemical structure of blue non-fluorescent resazurin and pink resorufin product. Printed from O'Brien et al., (2000) with kind permission of Wiley.*

2.2.5 DNA quantification as a measure of cell number using PicoGreen®

The fluorescent dye (PicoGreen®) was used to detect double-stranded DNA (dsDNA) for DNA quantification. The dye exclusively binds to dsDNA, and the fluorescence indicates the amount of dsDNA. The medium was removed from the cells and washed twice with PBS. The cell digestion buffer was added to the monolayer (200 µl) or to submerge the scaffold (500 µl) to extract cellular DNA. The cells were incubated at 37°C for 30 minutes before the lysates were transferred to microcentrifuge tubes. For 2D monolayer, the well was scraped using a pipette tip before transfer. For 3D culture, the whole scaffold was transferred with the lysate, vortexed for 15 seconds and refrigerated overnight, before storing at - 80°C. The lysate samples were thawed (-80C ten minutes, 37°C 15 minutes) thrice and centrifuged at 12298 g for 5 minutes immediately before analysis. The PicoGreen® working solution was made by mixing the PicoGreen® reagent (1x) with dilution assay buffer (200x) in a 10 ml tube then protected from the light. 90 µl of the PicoGreen® working solution and 10 µl of lysate were transferred into black 96-well plates and using the automated spectrofluorometer mixed for 10 seconds before being, incubated at room temperature for 10 minutes to allow the DNA to fully conjugate. The level of fluorescence in the samples was measured using a spectrofluorometer with an excitation of 480 nm; and an emission of 520 nm (Oliveira et al., 2006). The intensity of emission was measured from known DNA concentrations, which were used to create a standard curve (n=6) and fluorescence emission intensities extrapolated to the approximate cell number of DNA (ng/ml):

For 2D: DNA (ng/ml) = (Fluorescence emission – 279.8)/203.6

For 3D PCL scaffold DNA (ng/ml) = (Fluorescence emission + 215.1)/223.2

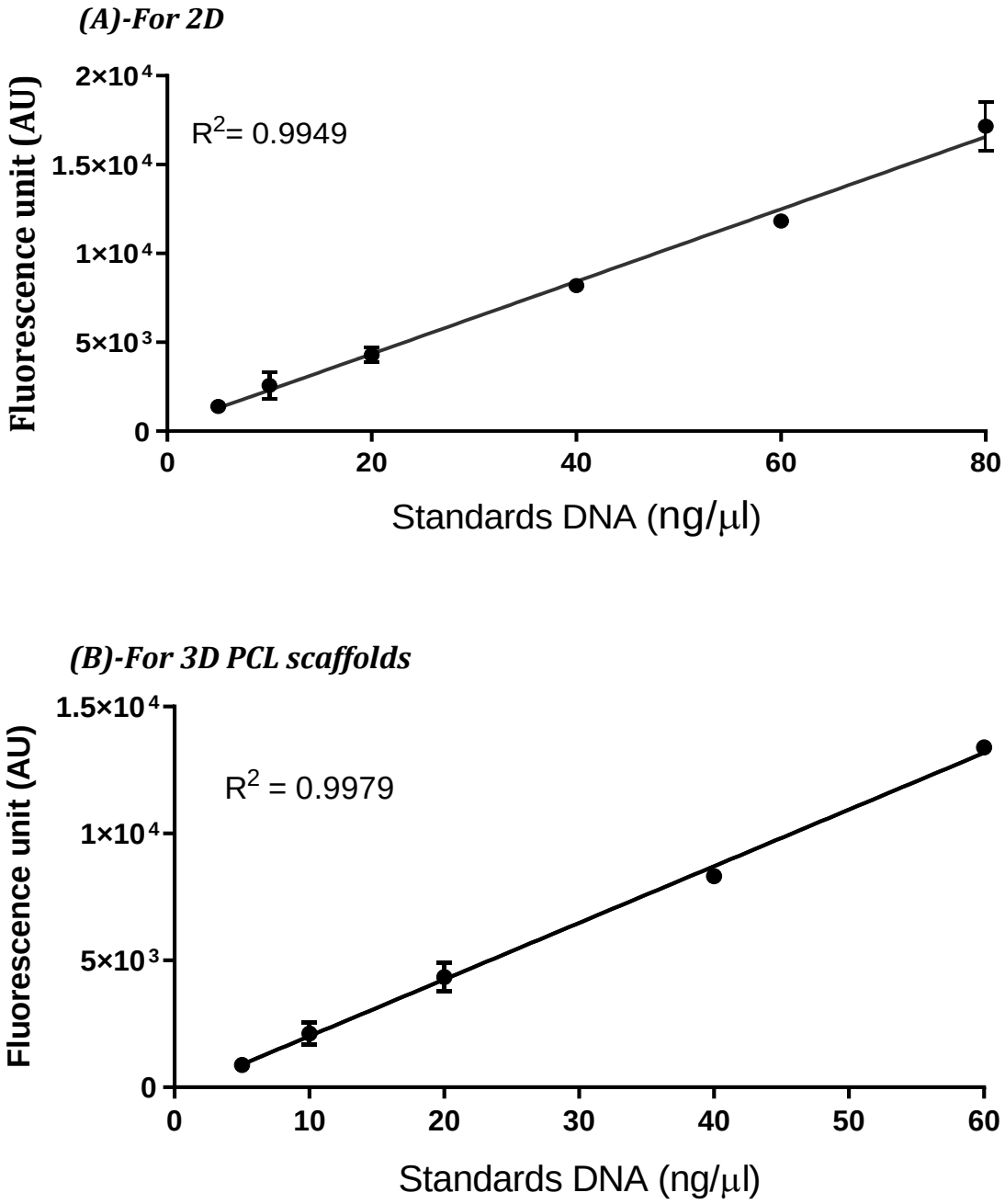


Figure. 2.2.5: *Linear quantitation of DNA using PicoGreen® reagent, from 0 to 80 ng of DNA/µL with linear regression analysis for (A) 2D monolayer (0 to 80 ng/µl DNA) and (B) 3D electrospun PCL scaffolds (0 to 60 ng/µl DNA).*

2.2.6 Assessment of osteogenesis

2.2.6.1 Alkaline phosphatase activity

Alkaline phosphatase (ALP) is an enzyme present during bone mineralisation; its activity is frequently employed as a marker for initial detection of osteogenic differentiation. ALP hydrolyses the inorganic pyrophosphate that causes the inhibition of mineralisation through inhibition of hydroxyapatite formation, and supplies inorganic phosphate to encourage mineralization. (Douglas et al., 2012; Orimo, 2010). The activity of ALP in hBMSCs was determined for monolayer cell culture and 3D electrospun PCL scaffolds between days 10 and 14.

Cells were washed three times with PBS. 500µl of cell digestion buffer was added; 10% (v/v) of cell assay buffer (1.5M Tris-HCl, 1mM $ZnCL_2$, 1mM $MgCL_2$ in deionised water, diH_2O) was diluted in diH_2O, and subsequently 1% (v/v) of Triton X-100 was mixed with assay buffer and left at room temperature for 30 minutes to produce the cell digestion buffer. The cells cultured on tissue culture plates were covered with a known volume of cell digestion buffer and incubated for 30 minutes, after this time they were scraped and the cell lysate transferred into a microcentrifuge tube, vortexed briefly and left overnight at 4°C. The cells were seeded onto electrospun PCL scaffolds were dipped into cell digestion buffer, transferred into a micro-centrifuge tube; vortexed briefly, and left overnight at 4°C. If not immediately used the samples were then stored at -80°C for longer term storage. For analysis all samples were exposed to repeated freeze-thaw cycle where lysates were thawed at 37°C for 15 minutes, then incubated at -80°C for 10 minutes and this was repeated three times before samples were vortexed for 15 seconds and centrifuged at 12298 g for 5 minutes. 180 µl of para-nitrophenylphosphate (pNPP) phosphatase substrate was prepared by diluting the buffer and dissolving the tablet in diH_2O (1 tablet: 1 ml buffer: 4 ml diH_2O) the assay buffer was mixed with 20 µL of lysate. It was transferred in triplicate to a transparent 96-well plate and incubated at room temperature until a colour change from colourless to yellow was observed or 30 minutes had passed. Absorbance was calculated spectrophotometrically with an emission wavelength of 405 nm every minute for 30 minutes. ALP activity was expressed as nmol of para-nitrophenol per minute (nmol pNP/min) where one absorbance value equals 19.75 nmol product. The ALP activity was normalised by dividing nmol pNP/min by the amount of DNA from the same well of cells.

2.2.6.2 Alizarin red staining for calcium deposition

Alizarin red staining is commonly used to evaluate calcium deposition *in vitro*. Alizarin red stain binds to calcium-containing mineral which can be visualised as an orange-red colour under a light microscope. The quantity of stain can be evaluated by eluting the red dye with 5% (v/v) perchloric acid and reading the optical density of the resulting solution spectrophotometrically, as previously described (Hild et al., 2011).

Both sample types, 2D monolayer and 3D electrospun PCL scaffolds were fixed in 10% (v/v) formalin for 20 minutes at room temperature and washed twice with PBS. 1 ml of 1% (w/v) alizarin red stain (see below) was added to each well and left for 30 minutes, after which time the alizarin red stain solution was removed, the sample washed with deionised water (diH_2O) under gentle orbital shaking, until the water was clear. 1 ml of 5% (v/v) perchloric acid was added on the fixed samples for 15 minutes to elute the dye with gentle orbital shaking. 150 µL of the eluate was read in triplicate in a transparent 96 well plate at an absorbance of 405 nm.

The absorbance of alizarin red was converted to a concentration of alizarin red stain (µg/ml) using a standard curve. A new standard curve was created for each experiment. A stock of ARS solution was prepared by dissolving in diH_2O at 1% (w/v). Different concentrations of ARS were produced from 1000 µg/mL to 0 µg/mL. Then, 0 µg/mL (blank) value was subtracted from all values of the standard curve. 150 µL of different concentrations of standard solution was transferred to a clear 96-well plate in triplicate. The level of absorbance in the samples was measured using a spectrofluorometer at 405 nm. A plot of absorbance at 405 nm against concentartion of alizarin red indicated a straight line relationship with R^2 value ≥ 0.975 (Figure 2.2.6.2).

Alizarin red concentration (µg/ml) = (Absorbance – 0.03046)/0.002892

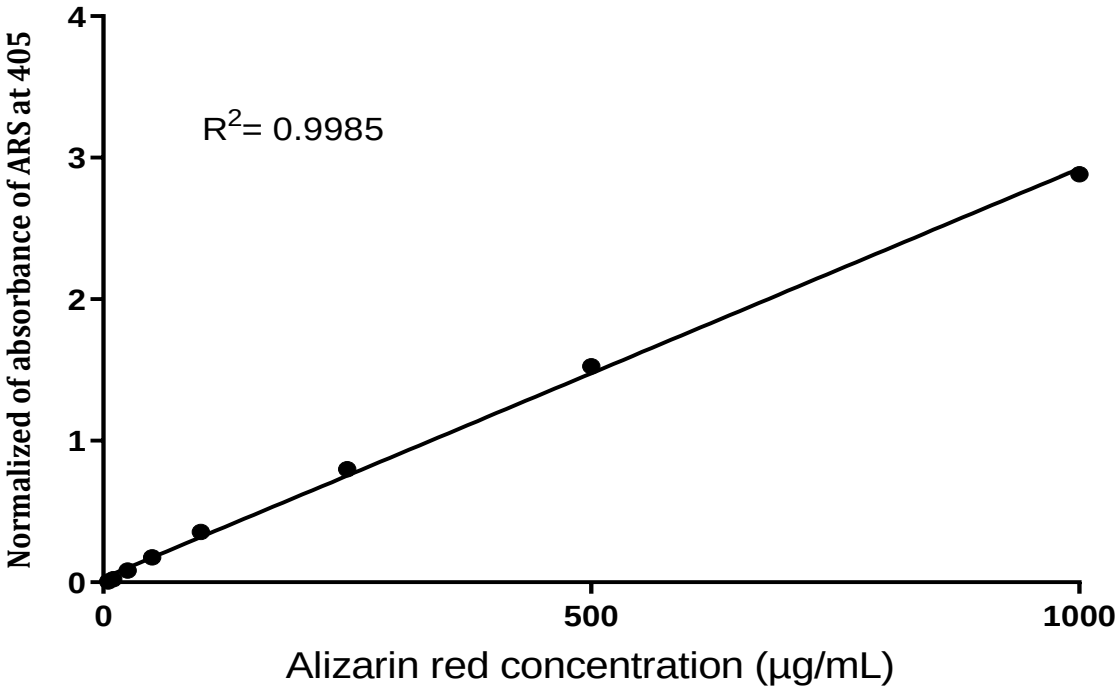


Figure 2.2.6.2: *The linear concentration of alizarin red staining, from 0 to 1000 µg of ARS/mL with linear regression analysis. n=3*

2.2.6.3 Analysis of cell-deposited collagen using sirius red staining

Sirius red staining is frequently employed to stain collagen and quantitatively measure the amount of collagen deposited *in vitro*. This stain is an anionic dye and can bind to a cation (the guanidine group of arginine) on the collagen molecules. This is a simple method for explicitly labelling of collagen type I and III (Tullberg-reinert and Jundt, 1999).

After the sample was assayed for calcium using alizarin red staining, they were washed thrice with diH_2O. The sirius red solution was prepared by dissolving 1 mg/ml of sirius red powder in saturated picric acid. 1 ml of the working solution was added to each well for 18 hours with orbital shaking at 100 rpm. After incubation, the solution was removed and washed with diH_2O until the water was clear. 1ml of 0.2 M sodium hydroxide (NaOH) and methanol in a ratio 1:1 NaOH: MeOH (v/v) was added for 20 minutes, with orbital shaking at 100 rpm, to elute the stain. 150 µL of the destain solution was read using a spectrometer in triplicate in a transparent 96 well plate at an absorbance of 405 nm.

The absorbance of sirius red was converted to a sirius red stain concentration (µg/ml) using a standard curve. As described for alizarin red. The intensity of absorption from known sirius red concentrations was used to create a standard curve of absorbance at 405 nm against concentration of SRS this gave a straight line relationship with an R^2 value ≥ 0.975 (Figure 2.2.6.3).

Sirius red concentration (µg/ml) = (Absorbance – 0.0546)/0.02596

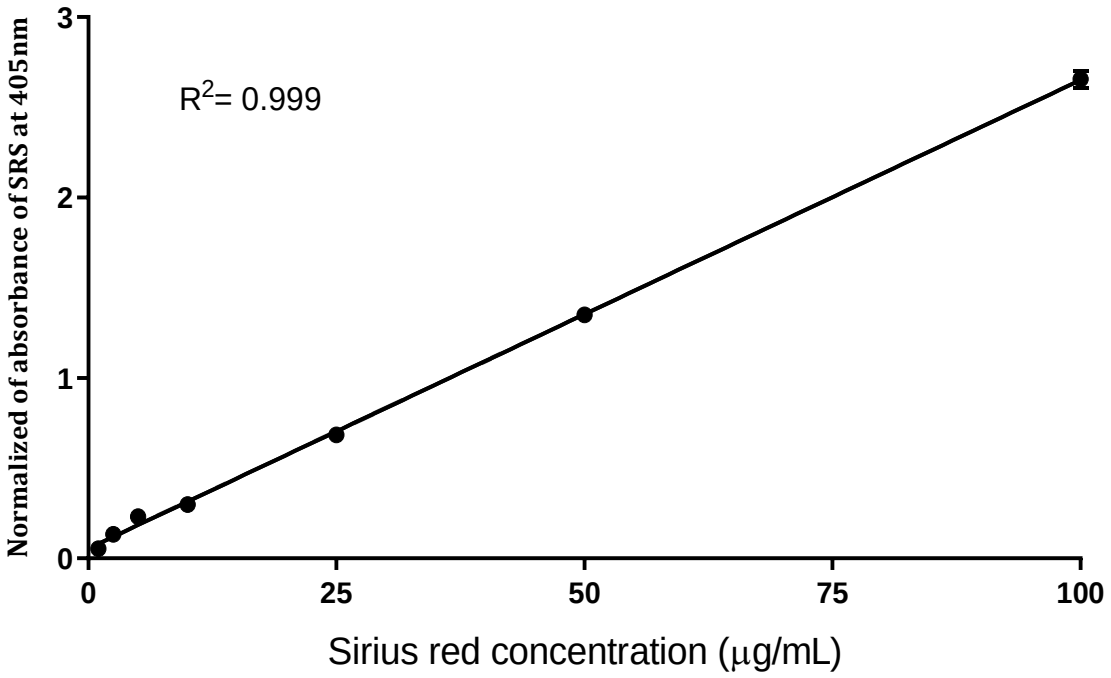


Figure 2.2.6.3: *The linear concentration of sirius red staining, from 0 to 100 μg of SRS/mL with linear regression analysis. n=3*

2.2.6.4 *Analysis of calcium deposition using xylenol orange staining*

Xylenol orange binds to calcium ions and is considered to be a non-toxic fluorochrome label (van Gaalen et al., 2010; Wang et al., 2006). The staining method is a widely accepted technique for labelling sequential bone formation *in vivo* by the intraperitoneal parenteral method at different time points (van Gaalen et al., 2010) and *in vitro* to identify mineralised osteoblastic cells matrix (Kuhn et al., 2010). Merits of this dye include; continuing observing of osteogenic formation without the need for sacrificing the specimens, it is an easy procedure and is high-throughput. 20 mM of stock xylenol orange solution was dissolved in diH_2O and saved at 4^oC. The stock solution of xylenol orange was diluted with the basal culture medium in ratio 1:1000 to produce a final working solution had the concentration of 20 µM with using within 12 hours before imaging (Kuhn et al., 2010). The xylenol orange solution was eliminated and washed with PBS two times and supplanted with new medium without β-GP to eliminate the fluorescent background from unbound stain. xylenol orange dyeing Images were visualised using a confocal microscope at an excitation wavelength of 543nM. Non-cell control scaffolds were dyed to determine whether there was any background xylenol orange staining.

2.2.7 Cell proliferation measured using EdU by flow cytometry

EdU is a nucleoside analogue to thymidine and incorporates into DNA during the active period of DNA synthesis. Therefore, it is an accurate approach for measuring cell proliferation.

EdU levels were measured using a Click-iTTM EdU Flow Cytometry Assay Kit as per the manufacturer's instructions. Briefly, 2 x 10^5 of hMSC were seeded in 6 well plates, after four days EdU (10 µM) was added to the culture media. After one hour, the cells were harvested using trypsin and transferred into a microcentrifuge tube, washed with PBS containing 1% (v/v) bovine serum albumin (BSA) and 100 µL of Click-iTTM fixative added for 15 minutes at room temperature in the dark. After this time, the cells with washed with PBS containing 1% (v/v) BSA, and resuspended in 100 µL of 1X Click-iTTM saponin-based permeability and incubated for 15 minutes at room temperature in the dark, followed by the addition of 0.5 mL of Click-iTTM with the reaction cocktail and incubated for a further 30 minutes at room temperature. After an additional three washes, EdU levels were measured by detection of Alexa FlourTM 488 picolyl azid using

a FACS Calibur flow cytometer using a 488 nm excitation wavelength and measuring emission at (530/30 nm).

2.2.8 Electrospinning rig set up and fabrication of PCL scaffolds

Electrospun Polycaprolactone (PCL) scaffolds were produced from a 10% (w/w) polymer concentration. 6g of polymer pellet, which is a semi-crystalline hydrophobic biodegradable polyester, were dissolved in with 54 g of DCM (Dichloromethane= 1.33g/cm3) to create a 10% (w/w) solution. For tri-layer scaffolds PCL was dissolved in a mixture of DCM and methanol ratio 9:1. The solutions were placed in a water bath at 37°C for 1 hour and then left on a magnetic stirrer plate overnight to ensure homogeneity. The electrospinning apparatus was set up as shown in Figure. 2.2.9. Scaffolds were constructed at 17 kV, a working distance of 17 cm, with a flow rate of 40µL/min, and a drum rotation speed of 300 rpm. The solutions were released by 12 needles (0.64 mm internal diameter) to the rotating drum, ground collector. Aluminium foil was used to cover the collector to support a fibrous sheet. The fibrous sheets collected were stored under vacuum at room temperature for 24 hours to evaporate any excess solvent before being stored in a self-sealed plastic bag at 4°C and used within six months (fabrication of tri-layer scaffolds will be described in chapter five).

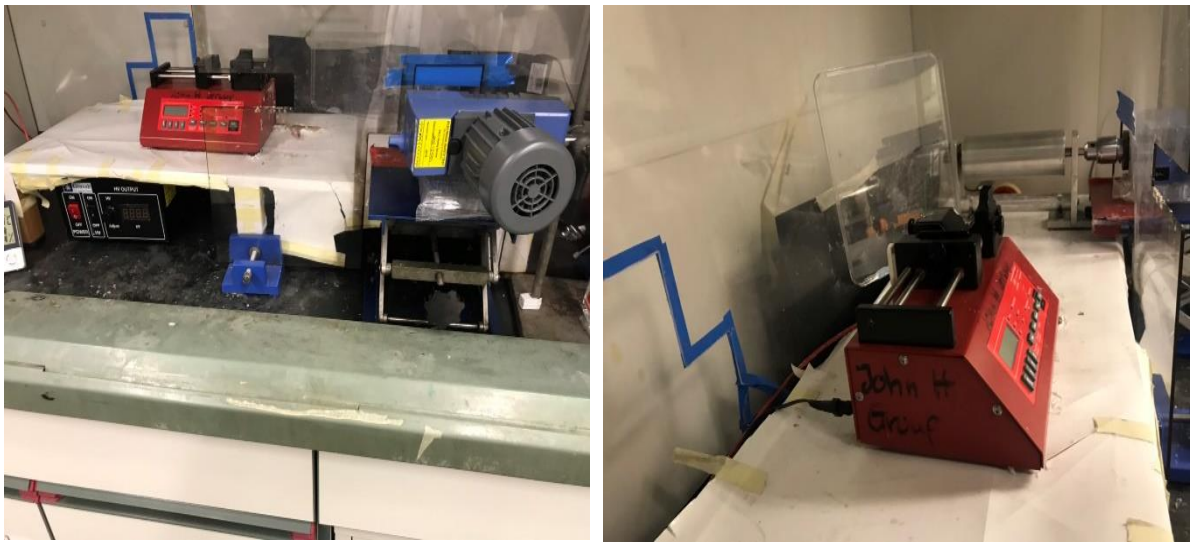


Figure. 2.2.8: *The electrospinning apparatus used to produce the 3D electrospinning PCL scaffolds as discussed in Chapter 1.*

2.2.9 Characterisation of electrospun PCL Scaffold using SEM

The morphology of electrospun PCL scaffolds was analysed by measuring fibre diameter by SEM. SEM can be used to visualise the surface topography and composition of material with a magnification capacity from 10 to 500,000 times. The scanning process uses electrons emitting from the electron gun to interact with the sample surface, while the emanated secondary electrons are detected. Electrospun scaffold samples were cut into 10 mm squares and mounted onto a specimen stub using electrically conductive double-sided adhesive tape. An ultrathin gold layer was sputter coated onto the scaffolds to enable the sample to be electrically conductive. The measurement set up will be described in chapter four.

2.2.10 Cell seeding onto polycaprolactone scaffolds

Different cell seeding techniques have been used to evaluate the effectiveness of cell attachment and differentiation within the scaffolds. Puwanun (2014) used different methods for cell seeding and mentioned that there were no meaningful differences among the methods however the large volume procedure presented less variation compared with other methods. Consequently, a significant volume medium procedure was carried out. The equilibrium was achieved through the media leaked to the outer, middle, and inner parts of PCL scaffolds to augment cell attachment. A day before seeding cells, the scaffolds were placed in a culture dish using a sterile stainless steel ring (Daud et al., 2012) (Figure 2.2.10 A) or within a CellCrown™ (Figure 2.2.9 B and C). Scaffolds were sterilised with 80% (v/v) ethanol for 2 hours, before washing three times in PBS. The samples were then immersed in the three different basal culture media (XF2, 10% (v/v) FBS, and 5% (v/v) HPL) overnight at 37ºC to enhance cell attachment. After overnight incubation, the media were removed from the well/plate, a small volume suspension of the hMSC (cell density 2 X 10^5 and no more than 100 µL) was directly seeded on the top of electrospun PCL scaffold and immediately incubated for 1 hour for cell attachment, after that 2 ml of each experimental media was added and left 24 hours in a cell incubator. The following day the PCL scaffolds were removed to the new well/plate which divided into different groups, and the scaffolds were then submerged in a fresh culture media along with the required supplements on day 1.

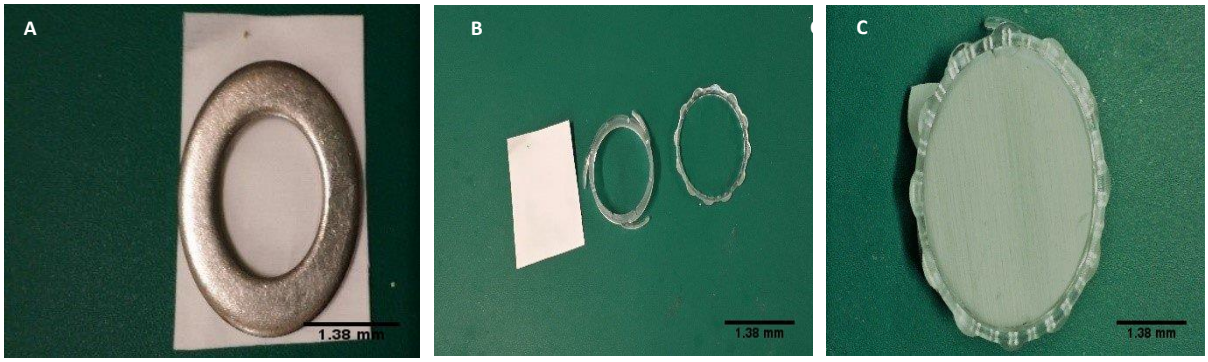


Figure 2.2.10: *(A) with sterile stainless steel ring was glutted with PCL scaffolds. (B) electrospun PCL scaffolds were cut into rectangular shape with CellCrown™. (C) The scaffolds were inserted into the CellCrown™. Scale bar = 1.38 mm*

2.2.11 Scanning electron microscopy (SEM) analysis of cells-seeded onto electrospun PCL scaffolds

After 21 days, culture media was removed, and scaffolds washed with PBS thrice and fixed with 3.7% (v/v) formaldehyde for 20 minutes (2D monolayer)/ 30 minutes (3D scaffolds) at room temperature to fix the cells. A dehydration step was undertaken to interchange water in the cells with ethanol. The samples were immersed in the following series of ethanols (v/v): 50%, 70%, 80%, 90% diluted in diH_2O, followed by 100% ethanol for 10 minutes. Following this, the samples were immersed in 100% (v/v) Hexamethyldisilazane (HMDS) for 3 minutes. The samples were left to air dry in a fume cupboard and subsequently sputter coated with gold and visualised using the SEM.

2.2.12 Assessment of vascular endothelial growth factor secretion by ELISA

Enzyme-linked immunosorbent assay (ELISA) is a quantification technique applied to ascertain the concentration of specific cytokines in cell conditioned media. Supernatants were collected to quantify VEGF secretion by cells 48 hours after the media was changed. The assessment protocol was pursued according to the manufacture's guidelines. Briefly, 50µL of diluent assay RD1 was added to the 96-well immunomicro plates, then 200 µL of standard, control and samples were added to each well. The well was sealed with an adhesive strip and incubated for 2 hours. All procedures were accomplished at room temperature. The well was then washed three times with 400 µL wash buffer solution. After being washed, 200 µL of VEGF conjugate was added to the well and sealed with a new adhesive strip and incubated for 2 hours. The well was washed as previously, and

then 200 µL of substrate solution was added to each well and incubated for 20 minutes. 50 µL of stop solution was added to each well and the plate gently tapped to ensure thorough mixing. The optical density was measured by using an absorbance microplate reader at a 540 nm wavelength. The intensity of fluorescence emission was converted to create a standard curve and the equation from the standard curve used to convert fluorescence emission intensities to approximate human VEGF concentration (picogram/ ml): (Figure 2.2.12).

human VEGF concentration (picogram/ ml) = (Fluorescence emission – 0.04475)/0.001532

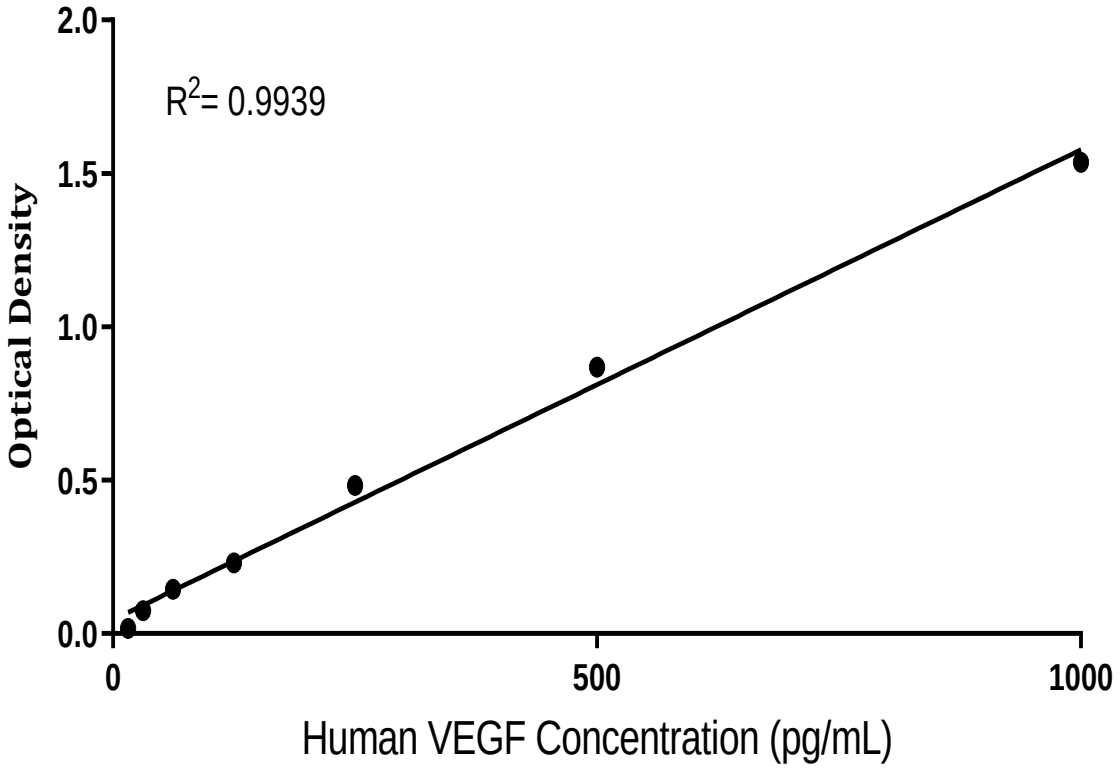


Figure. 2.2.12: *Linear quantitation of human VEGF concentration measured using an ELISA from 0 to 1000 pg of VEGF/ml with linear regression analysis*.

2.2.13 Staining of cell nuclei using DAPI

DAPI is a fluorescent dye which binds to A-T rich regions on dsDNA. DAPI can quickly permealise both live and fixed cells but it stains live cells more strongly (Zink et al., 2003).

Cells seeded onto scaffolds were fixed in 3.7% (v/v) formaldehyde for 30 minutes at room temperature, the fixative solution was removed and washed thrice with PBS. 1% (v/v) Triton X-100® in PBS was treated to the specimens and 10 minutes left to enhance cell membrane permeabilisation; then specimens were washed with PBS. The PCL mesh scaffolds were submerged until the scaffold was covered with 0.1% (v/v) DAPI in PBS; scaffolds were well wrapped in aluminium foil and left for 15 minutes. They were washed two times with PBS before imaging by ultraviolet absorbs at a maximum wavelength of 358 nm and emits at a maximum wavelength of 461 nm which is identified using a blue filter in the fluorescence microscopy (ImageXpressTM).

2.1.13 Statistical analysis

Statistical analysis was performed using Prism7 (GraphPad) to calculate the mean values and standard deviation (SD) of each group. Two-way ANOVA with Tukey's post-test and one-way ANOVA were employed to assess significant differences. A *p-value* of either less than or equal to 0.05 ($p \leq 0.05$) was considered to be statistically significant. Numbers of replicates are identified in each figure legend. "N" represents the number of donors and "n" represents a technical repeat within an experiment. Most experiments were repeated in triplicate.

Chapter Three: Evaluation of animal-free component culture media on monolayer cultured primary human osteogenic cells

3.1 Introduction

Paramount requirement of bone tissue engineering for cleft palate management is an appropriate culture medium and cells that have osteogenic differentiation capacity. The cells that have been used in clinical studies for the repair of bone defects intraorally such as alveolar cleft bone grafts for dental implants and maxillary sinus augmentation are human bone marrow derived mesenchymal stem cells hBMSCs (Behnia et al., 2009; Hibi et al., 2006) and human jaw periosteal HJPs (Trautvetter et al., 2011) as discussed in the previous chapter. Consequently, the selected cells to be used in this project are primary hBMSCs and HJPs. Animal sera are widely used during *in vitro* cell culture for hBMSC proliferation and differentiation. However, serious shortcomings have been noted such as potential contamination, poorly defined components, and potential for disease transmission (Freshney, 2010). Recently, xeno-free media have been developed to overcome the limitation of animal sera. However, little is known about their ability to support osteogenic differentiation and their applications in tissue engineering for cleft palate repair and similar craniofacial applications.

Recently, efforts have been focussed on the development of animal-free formulation culture media using human-derived growth supplements, such as human platelet lysates (HPL) (Hemeda et al., 2014), and autologous human serum (Dahl et al., 2008). Interestingly, the Federal Drug Administration (FDA) sanctioned a commercially produced pre-defined xeno-free culture medium particularly formulated for human MSC expansion (Oikonomopoulos et al., 2015)[9].

Osteogenic induction medium is supplemented with ascorbic acid-2-phosphate (AA), β-glycerophosphate (βGP), and dexamethasone (Dex) (required for osteogenic differentiation). (AA) is essential for producing stable collagen that is a substantial component of the extracellular matrix of bone (ECM). (βGP) is essential for providing an inorganic phosphate during mineralisation of ECM by differentiated cells. Dex is a synthetic glucocorticoid drug that can stimulate the differentiation of hBMSCs osteogenically (Coelho and Fernandes, 2000). Based on previous experience in the

laboratory, for all monolayer experiments cells were seeded at 10,000 cells/well unless otherwise stated.

In craniofacial surgery, autologous graft is the gold standard to an achieve facial bone regeneration because it has properties of native bone and integrates into the neighbouring tissue. Human jaw periosteum is a thin tri-layered tissue that covers the external surface of the jaw bone and contains osteogenic cells which have potential for creating of tissue engineered bone in oral and craniofacial surgery (Allen et al., 2004; Olbrich et al., 2012).

Researchers have focussed on hBMSCs for potential clinical regenerative medicine and tissue engineering because these cells can differentiate into osteoblasts and form bone. However, their osteogenic potential is adversely affected by various factors such as: donor-to-donor variability, donor age, *in vitro* expansion (Kretlow et al., 2008), physiological status of the patient, age and gender, the methods of cell collection, and source of isolation of hMSCs (Siddappa et al., 2007).

The aims of work in this chapter were to evaluate the effectiveness of different xeno-free media for expansion of hBMSCs and to obtain preliminary data about whether these media would also be suitable for culture of HJPs. All experiments were performed in standard 2D monolayer, culture with the long-term aim of establishing xeno-free culture protocols for future clinical applications of cleft palate repair. In order to do this, I set out the following objectives:

1. To investigate the effect of different commercial media containing animal-free components on the growth of hBMSCs.
2. To investigate the interaction between media formulations and the osteogenic supplements Dex, Ascorbate-2-phosphate and β-glycerophosphate, separately and in combination, on cell proliferation of hBMSCs.

Table 3.1: Experimental culture media with their abbreviations used in the project all basal media supplemented with antibiotics as described in section 2.2.1

Basal culture medium	Abbreviation
Foetal bovine serum (FBS) + α-MEM	**10% FBS (v/v)**
Stem X Vivo™ xeno-free human MSC expansion medium	**XF1**
Human mesenchymal-XF expansion medium	**XF2**
Human platelet lysate + α-MEM	**5% HPL (v/v)**
FBS + α-MEM + Insulin-transferrin-selenium	**2% FBS+ITS (v/v)**
PeproGrow-1 chemical-defined medium	**SR1**
MSC-NutriStem	**MSC-NutriStem**

3.2 Appraisal of various culture media formulations on the seeding efficiency and growth of hBMSCs in monolayer

The purpose of these experiments was to assess how varying the concentration of animal serum foetal bovine serum (FBS), and human platelet lysate (HPL) affected the metabolic activity of hBMSCs at different times-points.

Cell metabolic activity was investigated using the resazurin reduction assay after days 1, 4, and 7. On day 1 and 4, cell metabolic activity increased in all conditions except the non-serum formulation. While on day 7, the non-serum condition was significantly lower than the other conditions. Although there is no significant difference between 5% (v/v) and 10% (v/v) FBS, the 5% (v/v) FBS group underwent a decrease of cell metabolic activity over time ($P<0.05$). All the concentrations below 10% (v/v) FBS caused a decrease of cell metabolic activity over the time-points. Therefore, this project used a 10% (v/v) of animal-serum concentration (FBS) as gold standard control in all experiments (Figure 3.2 A).

A metabolic activity assay was also carried out to evaluate the most effective HPL concentration for hBMSCs. There was no significant difference between any media formulations on day one. However, significant differences were seen on days 4 and 7 between the conditions. Cell metabolic activity was significantly higher for both 5% (v/v) and 10% (v/v) HPL compared with other conditions and also increased over time for 7 days ($P<0.05$) (Figure 3.2 B).

Overall, the below results provided sufficient evidence that both 10% (v/v) FBS and 5% (v/v) HPL basal culture media formulations can support the growth of hBMSCs.

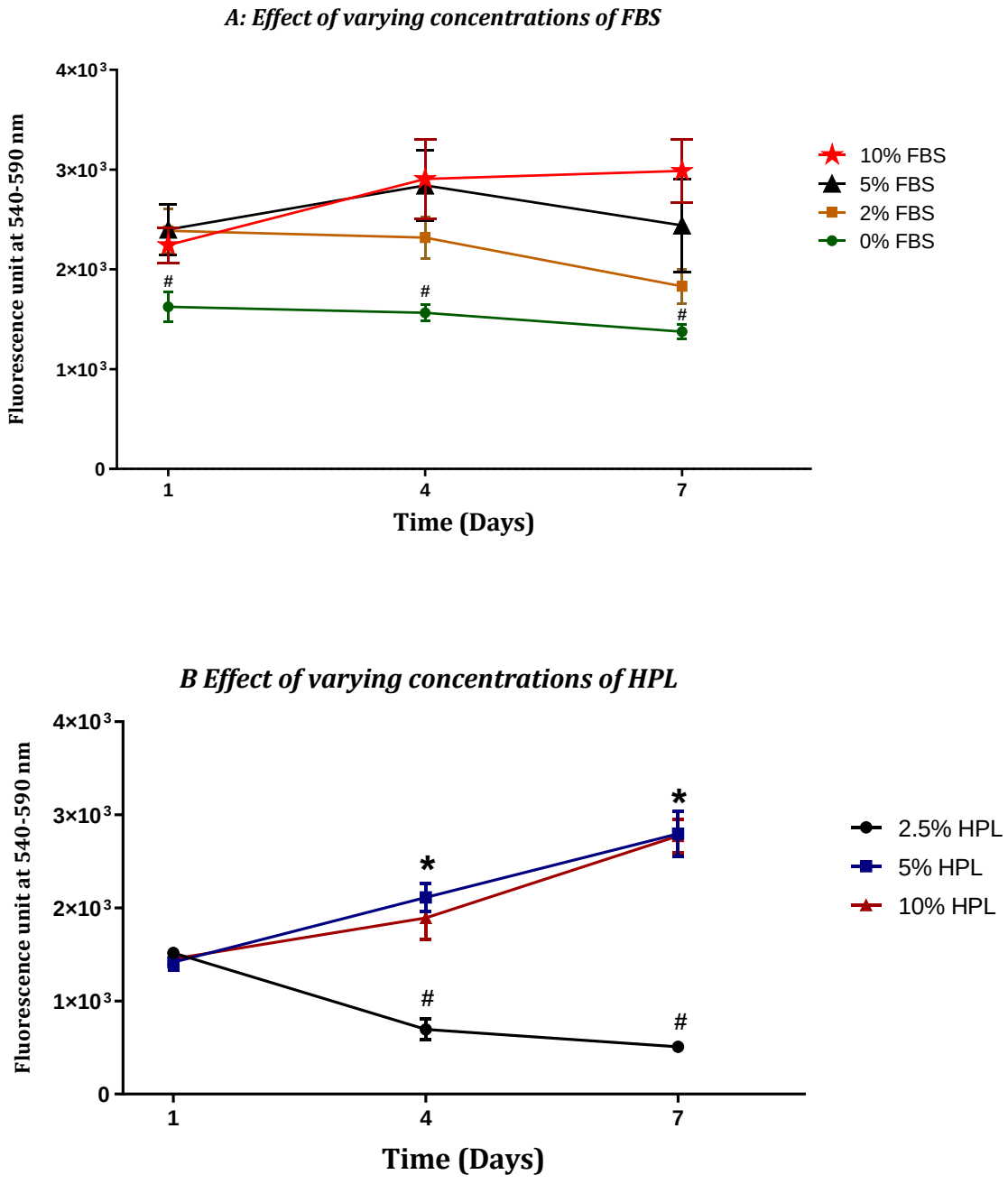


Figure 3.2: *Metabolic activity of hBMSCs donor 3 under different FBS concentrations and human platelet lysate (HPL) concentrations from day 1 to day 7. Symbol indicates mean and standard deviation for each condition Mean±SD. (A) Effect of varying concentrations of FBS, (B) Effect of varying concentrations of HPL (*) significantly higher than the other media at all-time points. (#) Significantly lower. (P <0.05). n=3. Two-way ANOVA, Tukey's multiple comparison test.*

3.3 Assessment of cell metabolic activity in a range of experimental media formulations

These experiments aimed at identifying some promising xeno-free media formulations to support hBMSCs, which could be taken forward for further work throughout this thesis. Donor 3 was used.

After 24 hours of cell seeding, the resazurin reduction assay was used to evaluate cell metabolic activity on day 1, which gave an indication of how many cells attached on the well plate. Statistically, most media enabled the same levels of cell attachment except the MSC-NutriStem that supported a higher cell attachment (Fig 3.3-Basal culture media). Moreover, the cells grown in SR1 (chemically defined medium) had significantly lower cell metabolic activity at 24 hours and then throughout the experiment. After 24 hours of culture, the supplements were added. Therefore, there is no day 1 data for supplemented media groups.

Metabolic activity on days 4 and 7 indicated how the different media formulations affected cell growth. The metabolic activity of cells cultured in XF2 (2% (v/v) human serum) increased sharply approximately 12 folds with significant differences compared to the other conditions over time for 7 days ($P<0.05$). Cell metabolic activity also increased with 10% (v/v) FBS throughout the 7 days; however, there was no significant difference between days 4 to 7. Although the XF1 (human plasma) initially exhibited suitable cell attachment at 24 hours, cell numbers declined steadily over the rest of the culture period (Figure 3.3).

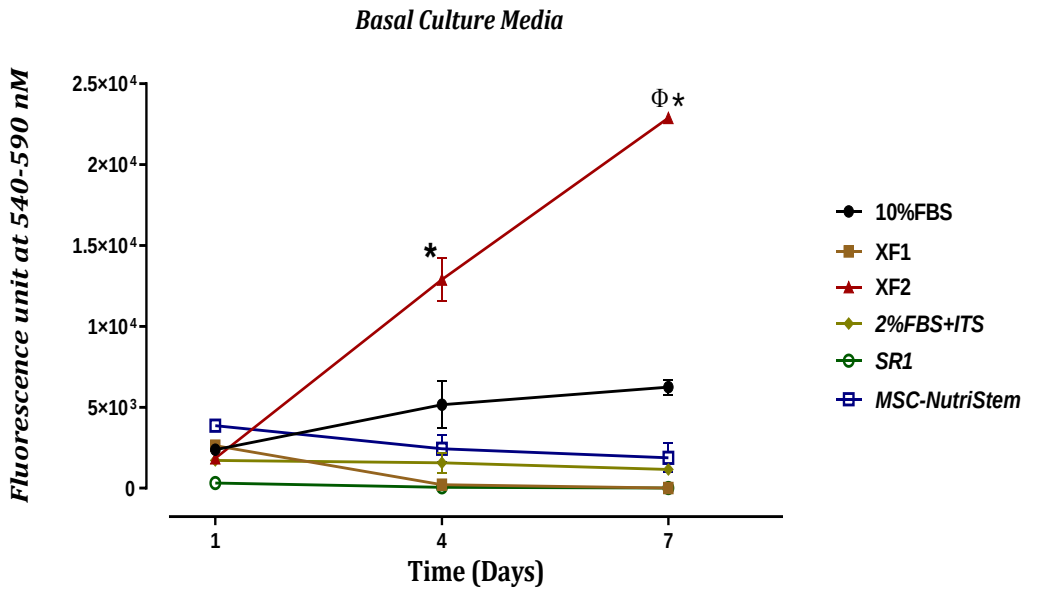
When supplemented with Dex 10 nM, on day 4 and 7, the metabolic activity of the metabolic cells cultured in XF2 increased over time at the highest rate indicating XF2 had a stronger effect on cell proliferation compared to the other groups the next fastest growth was seen in 10% (v/v) FBS followed by MSC-NutriStem. On the other hand, the XF1 and SR1 had a significantly lower cell growth ($P<0.05$) (Figure 3. 3).

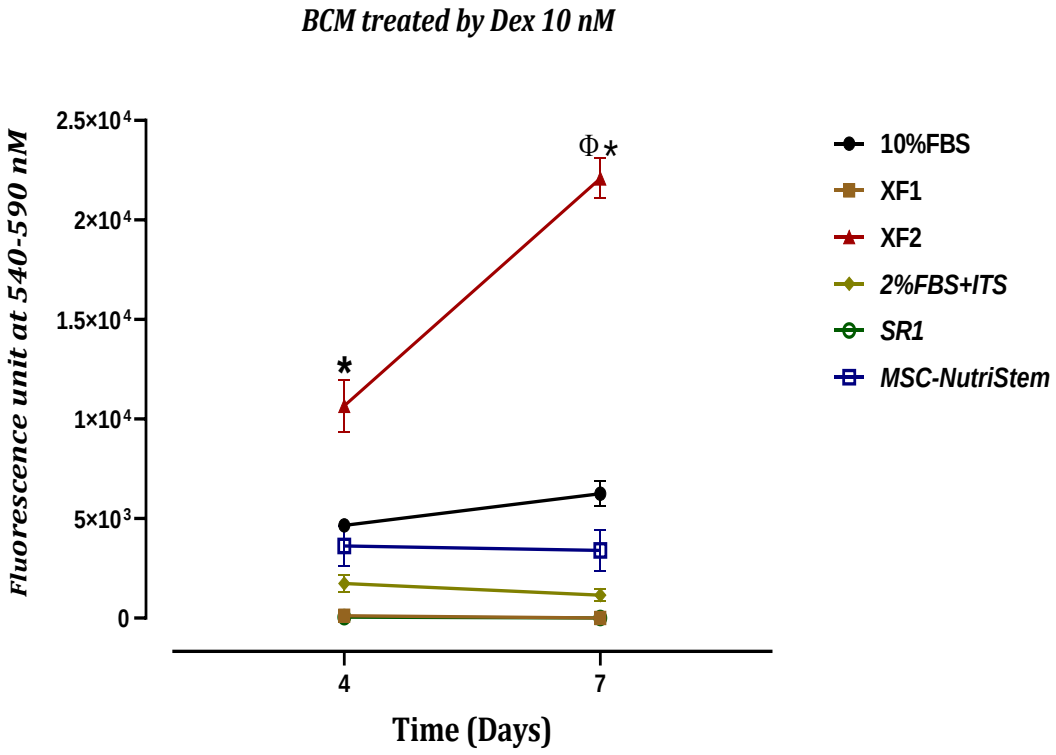
When supplemented with (AA) 50 µM and (βGP) 10 mM (without Dex), day 4 (represented with a grey outline bars in figure 3.4), the metabolic activity for cells cultured in XF2 suggested a high cell proliferation rate (increase in metabolic activity over time) compared to the other groups, followed by 10% (v/v) FBS. On the other hand, the other media supported significantly slower cell growth ($P<0.05$).

On day 7 (represented with coloured dot plots), XF2 media formulations improved the hBMSCs proliferation as indicated by increased cell metabolic activity. The growth of cells cultured in XF2 was augmented approximately 2.5-fold over 3 days after day 4. However, cells grown in 10% (v/v) of animal serum supplement reduced to 2-fold less over the same time ($P<0.05$). XF1 and chemical-defined medium (MSC-NutriStem and SR1) poorly encouraged the growth of hBMSCs (Figure 3.3).

When grown in full osteogenic media (Dex 10 nM, AA 50 μM, and βGP 10 mM), on day 4, the metabolic cell activity of cells cultured in XF2 suggested a higher cell proliferation compared to the other groups, followed by 10% (v/v) FBS and 2% (v/v) FBS+ITS. On the other hand, the XF1 and SR1 supported a significantly less cell proliferation ($P<0.05$)

Overall, the results informed that the substitution of animal serum formulation by the human serum could accelerate the hBMSCs growth in all conditions.


Basal Culture Media
Fluorescence unit at 540-590 nM
2.5×10⁴
2×10⁴
1.5×10⁴
1×10⁴
5×10³
0
1
4
7
Time (Days)
Φ*
*
10%FBS
XF1
XF2
2%FBS+ITS
SR1
MSC-NutriStem


BCM treated by Dex 10 nM
Fluorescence unit at 540-590 nM
2.5×10⁴
2×10⁴
1.5×10⁴
1×10⁴
5×10³
0
4
7
Time (Days)
Φ*
*
10%FBS
XF1
XF2
2%FBS+ITS
SR1
MSC-NutriStem

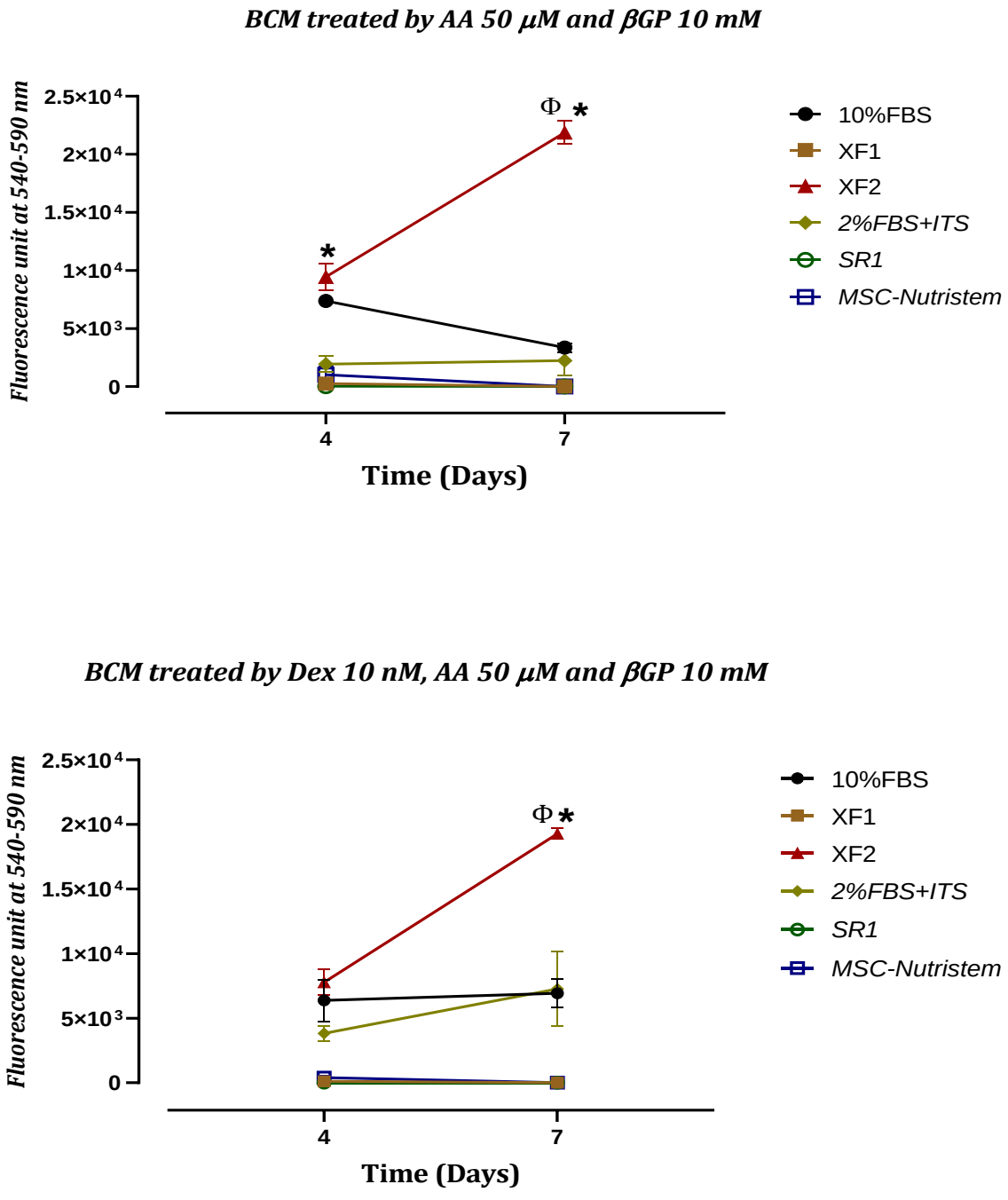


Figure 3.3: *Metabolic activity of hBMSCs donor 3 cultured in different culture media from day 1 to day 7. Each symbol represents a single well, and the line indicated the mean and standard deviation for each condition. Media supplemented with Dex 10 nM, ascorbic acid-2-phosphate (AA) 50 μM and β-glycerophosphate (βGP) 10 mM. (*) Significantly higher than the other media at the same time-point. (Φ) Significantly higher than all the other media formulations at all-time points. (ns) There is no significant difference. (P <0.05). n=3. ANOVA, Brown-Forsythe and Two-way ANOVA, Tukey's multiple comparison tests.*

3.4 The effects of different basal culture media on cell metabolic activity of cells from different donors and passages

This experiment aimed to investigate differences between donors and effects of passage on cell metabolic activity when cultured in two commercial xeno-free media compared with 10% (v/v) animal serum formulation. I used XF2 and XF1 for this next set of experiments as media that showed promising and less promising results in the previous preliminary experiments to investigate if these differences were consistent across a range of donors.

Resazurin metabolic activity assay was used to indicate metabolic activity of cells from different adult donors and passages of hBMSCs, with groups cultured in xeno-free basal culture medium and measured after 1,4 and 7 days.

The cell metabolic activity after 24 hours showed that the donor-1 and passage-1 cells cultured in all experimental culture media were significantly higher compared with cells from other donors and passages particularly in the XF2 basal culture medium. This suggests that a higher number of cells had attached. The next highest metabolic activity was seen in the donor-4 cells cultured in all experimental culture media. While passages 2, 3, and 4 exhibited significantly lower cell attachment with no significant differences (Figure 3.4 A and B).

On day 4, donors 1 and 4 cultured in XF2 exhibited an increase around 2-fold compared to their donors cultured in 10% (v/v) FBS with significantly higher cell metabolic activity compared to all other donors cultured in the same and different media ($P<0.05$). Both donors 1 and 4 cultured in 10% (v/v) FBS and donor-1 cultured in XF1 appeared to have higher cell metabolic activity compared to donor-4 cultured in XF1. However, these differences were not statistically significant. (Figure 3.4 C).

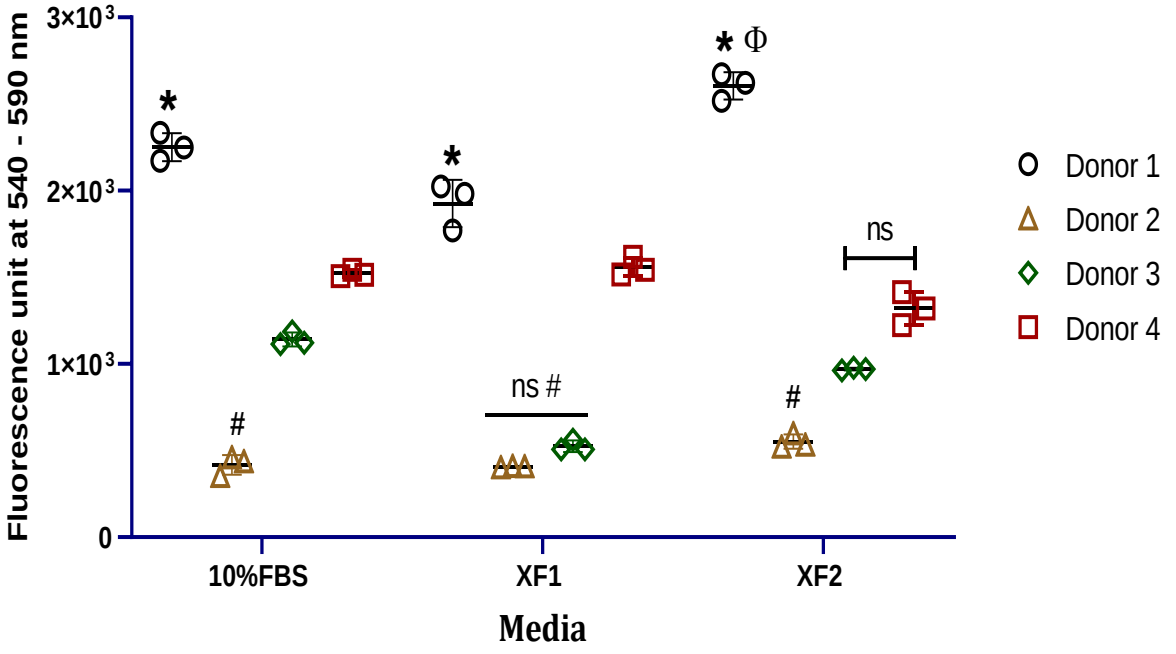
On day 7, the donors cultured in XF2 showed significantly higher metabolic activity compared with cells from the same donors cultured in other experimental media. While there were no significant differences between cells from donor-1 and -4. Donor-1 cells cultured in both 10% (v/v) serum medium and XF1 displayed significant difference compared with donor-4 cultured in the same experimental media. Whereas, the poorly cell metabolic activity indicated in donor-4 cultured in XF1 (Figure 3.4 D).

Overall, cell metabolic activity increased over the culture period (comparing figure 3.4 A with C and D) except for donor-4 cells culture in XF1.

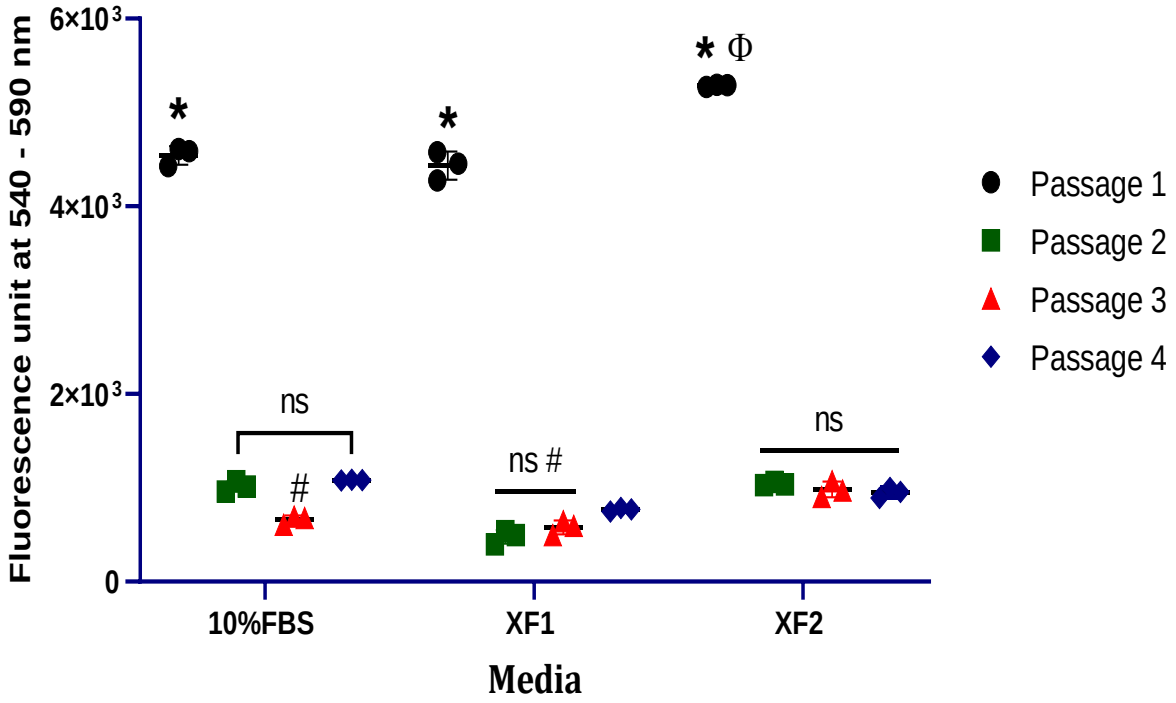
When examining cells from passages on day 4 and 7, the cell metabolic activity for both passages 3 and 4 had the lowest metabolic activity in xeno-free and 10% (v/v) FBS culture medium. Whereas the cell metabolic activity of both passages 1 and 2 cultured in XF2 were significantly different from the other passages cultured in the same and different culture media. Furthermore, these passages cultured in XF2 had a high metabolic rate, nearly 1.5-fold on day 7and 2.2–fold on day 4more with XF1 respectively ($P<0.05$) (Figure 3.3 E and F).

In summary, cells from passages 3 and 4 did not exhibit increases in metabolic activity under any conditions. This indicates that these cells are not appropriate to examine differences between media. Therefore, for further analysis passage 3 and 4 cells are excluded from my experiments and all donor cells reported for the following experiments were from passage 1 and 2 only. The results from passage 1 and 2 cells do indicate that the media containing human serum (XF2) supported a sustainable increase in cell metabolic activity compared with other experimental media.

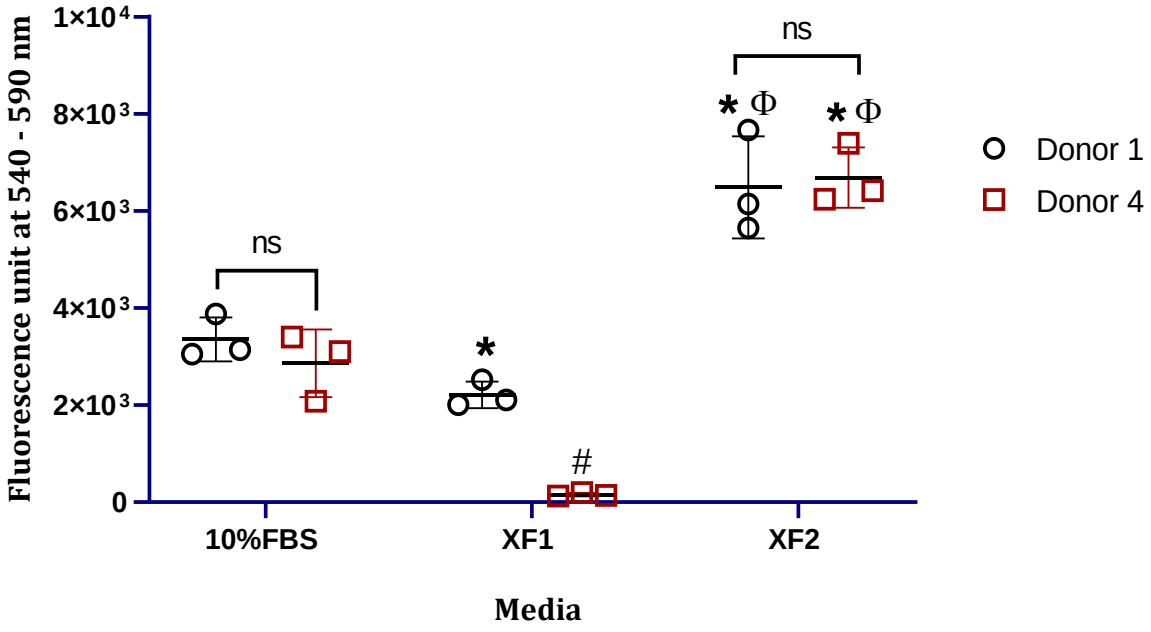
A: Cell attachment of different donors cultured in BCM at 24 hours



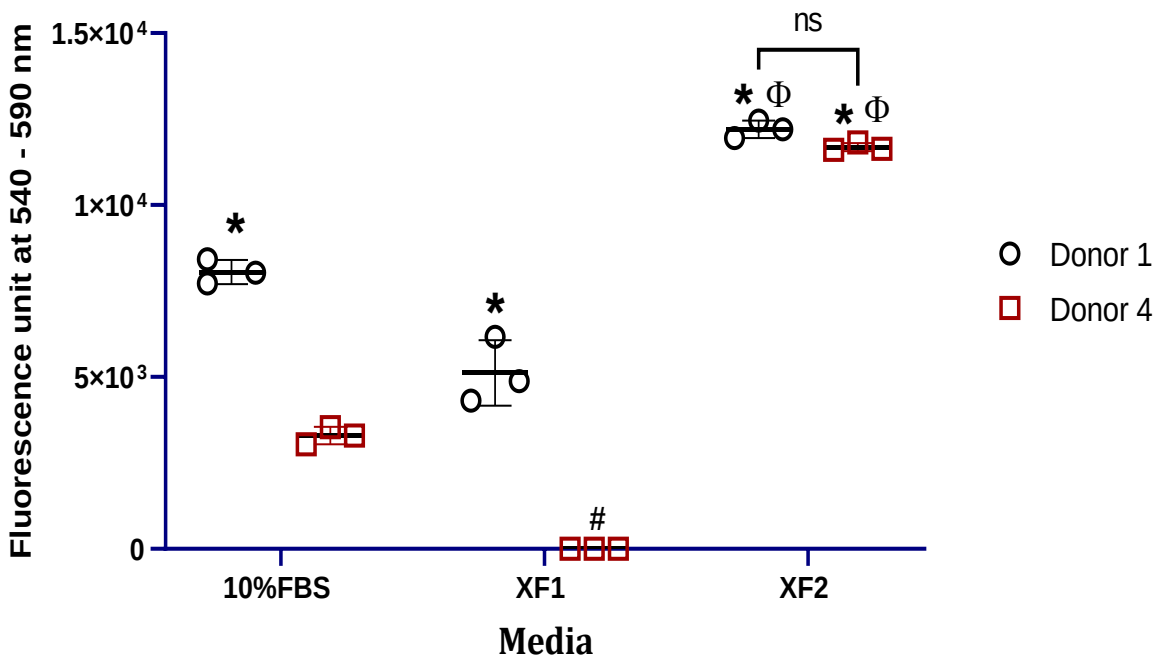
B: Cell attachment of different passages cultured in BCM at 24 hours



C: Different Donors cultured in BCM on day 4



D: Different Donors cultured in BCM on day 7



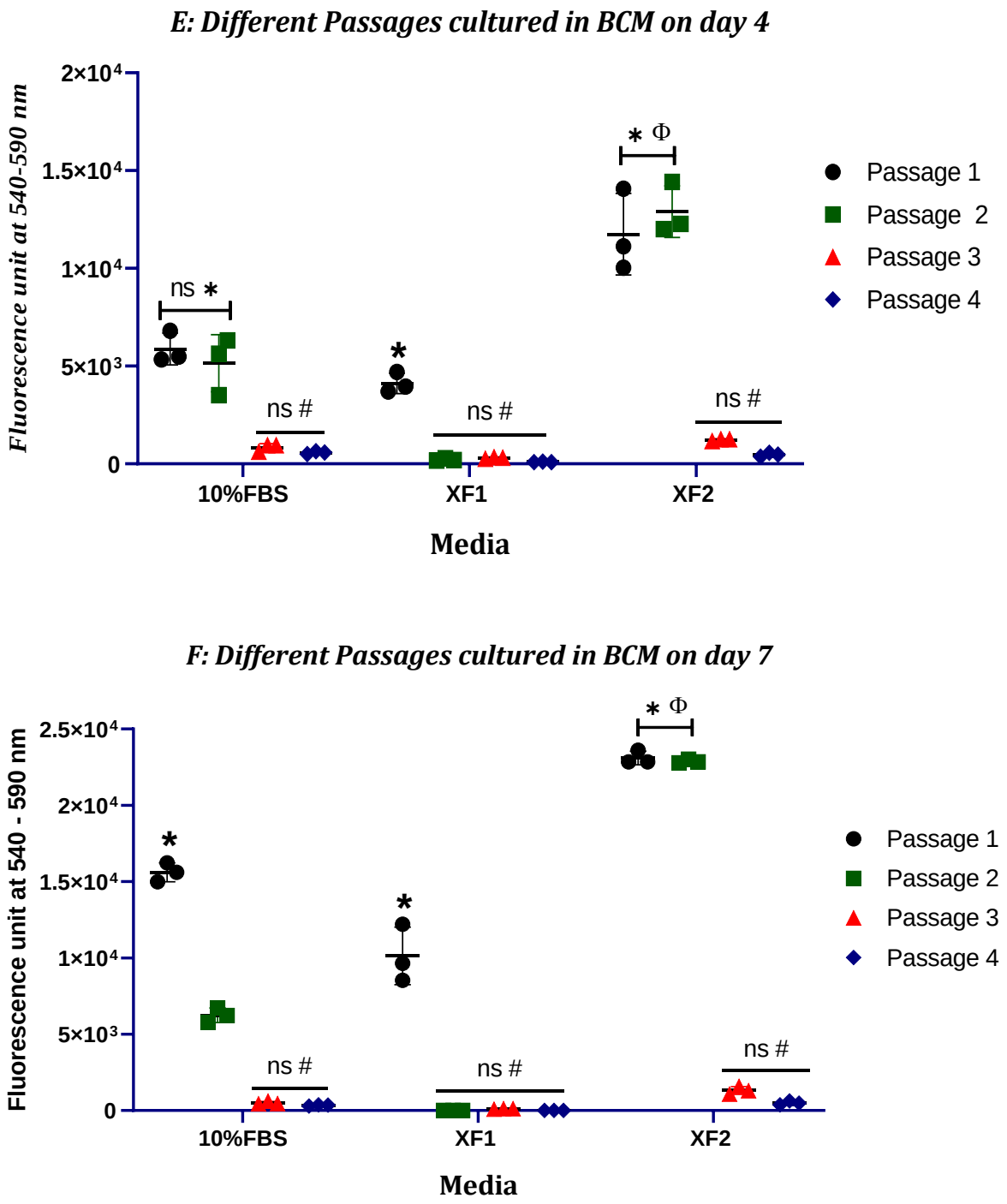


Figure 3.4: *Metabolic activity of different donors of hBMSCs using different media in monolayer culture from day 1 to day 7. Symbols indicate mean and standard deviation for each condition. (*) Higher than the other cells cultured in the same medium. (#) Significantly lower. (Φ) Significantly higher than the same cells in all the other culture media. (ns) There is no significant difference. (P <0.05). n= 3. Two-way ANOVA, Tukey's multiple comparison test.*

3.5 The effects of dexamethasone supplementation on culture of hBMSCs in monolayer in various media

Because I aim to induce osteogenesis in these cells and osteogenic supplements may also affect cell growth the interaction between osteogenic supplements separately and in combination are examined in the next set of experiments. Initially I examined dexamethasone alone at two different concentrations. Dexamethasone was added after 24 hours when cells were well attached on the well plate.

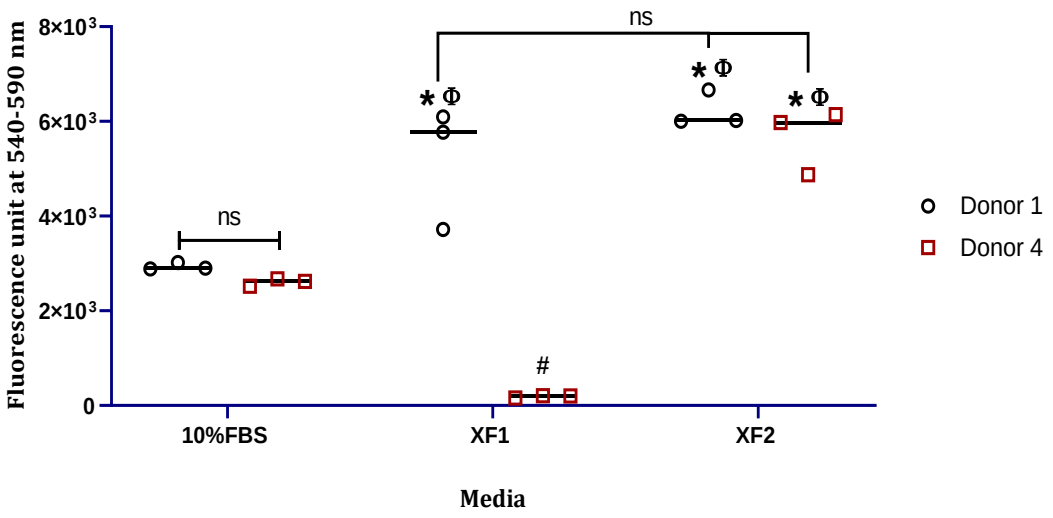
3.5.1 The effects of two different xeno-free basal culture medium supplemented with Dex 10 nM on cell metabolic activity of different donors

The cell metabolic activity of cells from donor-1 and -4 cultured in XF2 and donor-1 cultured in XF1 indicated significantly enhanced cell growth compared toc ells cultured in 10% (v/v) serum medium and the donor-4 cells cultured in XF1 ($P<0.05$) increasing about 2 folds over the same donors and passages cultured in 10% (v/v) FBS. Donors-1 and -4 cultured in 10% (v/v) FBS also showed higher metabolic activity compared with donor-4 cultured in XF1. However, the donor-4 cultured in XF1showedvery low metabolic activity indicating lower cell numbers (Figure 3.5.1 A).

On day7, the cell metabolic activity of both donors 1 and 4 cultured in XF2 supplemented with 10 nM were significantly higher than the same donors cultured in both 10% (v/v) FBS and XF1 culture media supplemented with 10 nM Dex. Moreover, the donors cultured in XF2 supplemented with 10 nM Dex had a high metabolic activity around 2-fold compared with 10% (v/v) FBS and XF1; while it was approximately 3.5 times higher than donor-4 when cultured in 10% (v/v) FBS (*$P<0.05$*) (Figure 3.5.1 B).

Overall, the results showed that when supplemented with Dex 10 nM the media containing human serum (XF2) supported the cells better than both the human plasma and animal serum medium formulations. Even though human plasma (XF1) enhanced cell proliferation in donor-1 initially, cell growth diminished overtime for 7 days.

A: Different Donors treated by Dex10 nM on day 4



B: Different Donors treated by Dex10 nM on day 7

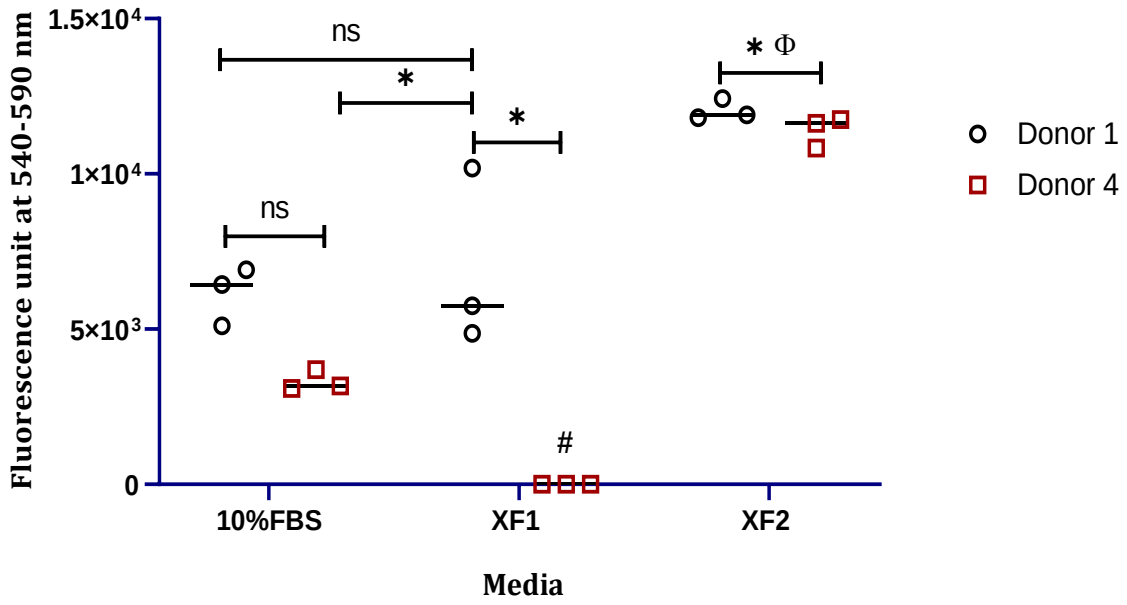


Figure 3.5.1: *Metabolic activity of different donors of hBMSCs in different xeno-free media supplemented with Dex 10 nM on monolayer culture from day 4 to day 7. The scatter dot plots indicated individual wells and horizontal lines mean and standard deviation for each condition. (*) Higher than the other groups in the same donor. (#) Significantly lower. (P <0.05). n=3. Two-way ANOVA, Tukey's multiple comparison tests.*

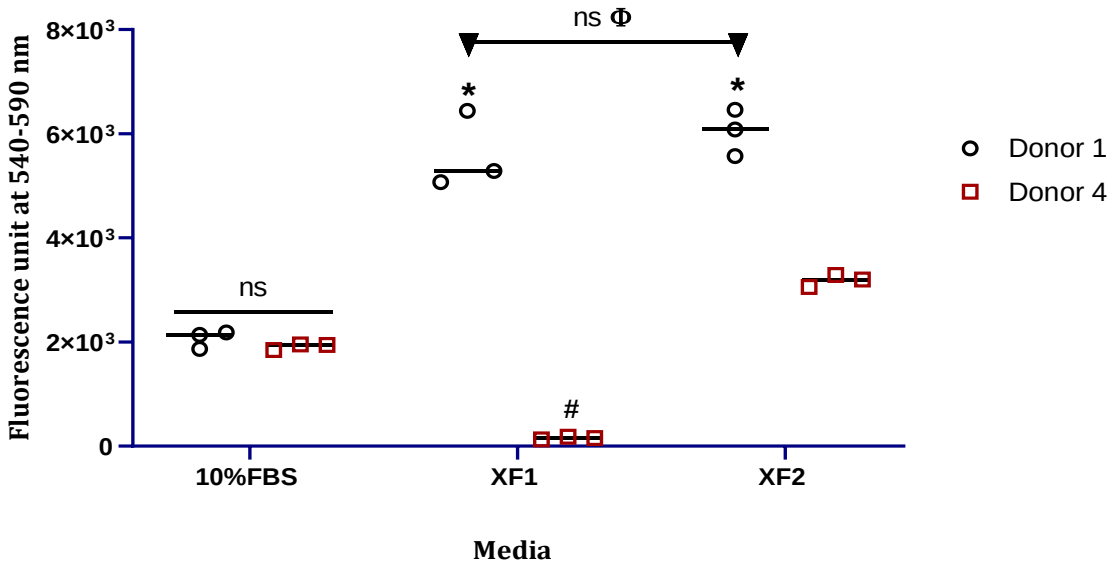
3.5.2 The effects of two different xeno-free basal culture medium supplemented with Dex 100 nM on cell metabolic activity of different donors

Cells from donors on day 4, donor-1 cultured in human-derived formulations exhibited a significantly higher cell proliferation compared to donor-4 cultured in all experimental media as well as donor-1 cultured in 10% (v/v) FBS. The metabolic activity of cells from donor-4 cultured in XF2 supplemented with Dex 100 nM indicated a steady upward trend of nearly 1.5-fold and 13 folds when cultured in 10% (v/v) FBS and XF1 respectively ($P<0.05$) (Figure 3.5.2A)

On day7, the cell metabolic activity of donor-1 cells cultured in XF2 media supplemented with 100 nM was significantly enhanced compared to all other groups cultured in experimental culture media. Donor-1 cultured in XF2 supplemented with 100 nM had a high cell growth of around 2.5-fold and 1.5-fold compared to when cultured in 10% (v/v) FBS and XF1 respectively. Moreover, when donor-1 cells were cultured in XF1 metabolic activity was 1.5 times higher compared to 10% (v/v) FBS (*$P<0.05$*). Cells of Donor-1 had significantly higher cell proliferation compared to donor-4 cells cultured in the same media (Figure 3.5.2 B).

Overall, the results demonstrated that the human serum supplemented with Dex 100 nM supported cell growth better than the human plasma and animal serum medium formulation cultured in the same circumstances.

A: Different Donors treated by Dex 100 nM on day 4



B: Different Donors treated by Dex 100 nM on day 7

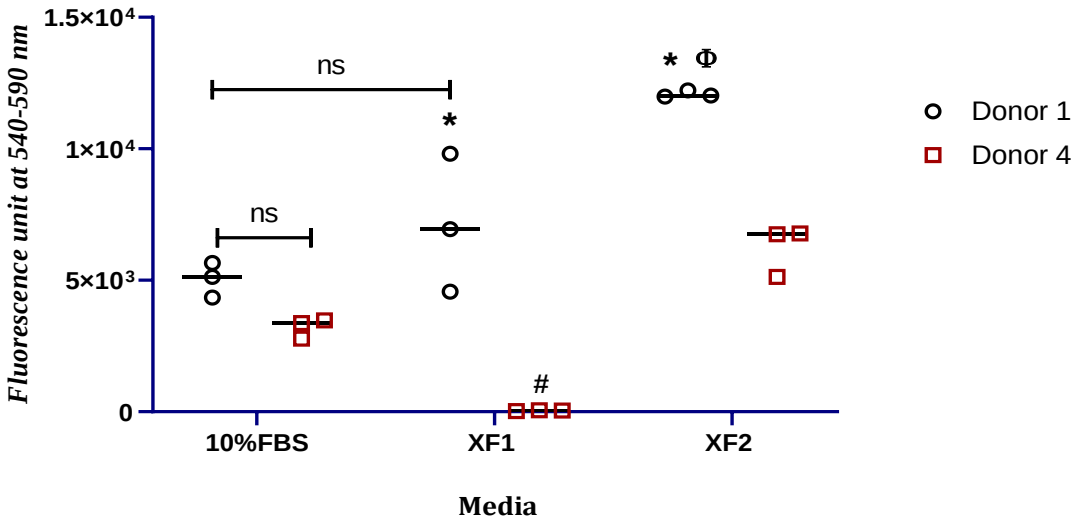


Figure 3.5.2: *Metabolic activity of different donors of hBMSCs in different xeno-free media supplemented with Dex 100 nM on monolayer culture from day 4 to day 7. The symbols indicate individual wells and horizontal lines the mean and standard deviation for each condition. (*) Significantly higher than the other groups in the same medium. (#) Significantly lower. (Φ) Significantly higher than all the other conditions. (ns) There is no significant difference. (P <0.05). n=3. Two-way ANOVA, Tukey's multiple comparison tests.*

3.6 The effects of two different xeno-free basal culture medium supplemented with ascorbic acid-2-phosphate (AA) and β-glycerophosphate (βGP) on cell metabolic activity of different donors

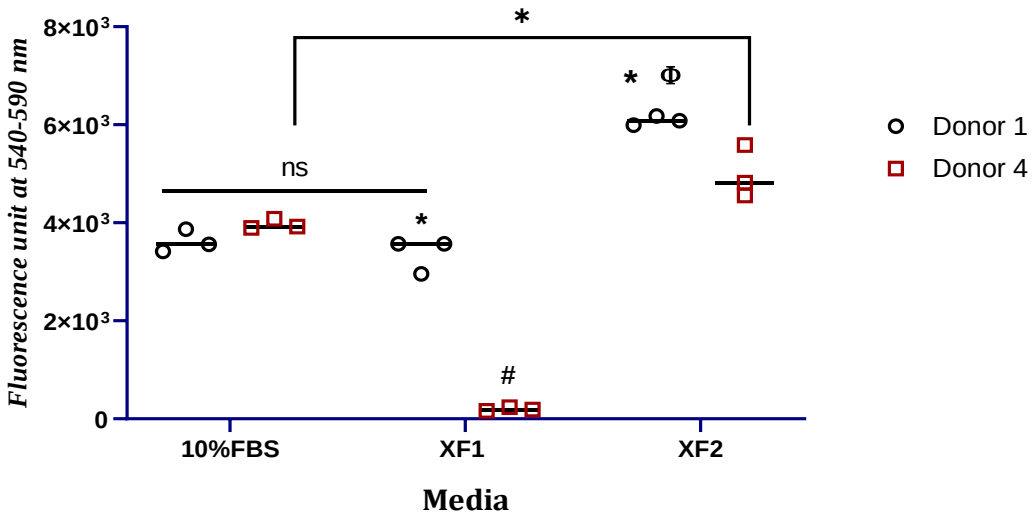
The ascorbic acid-2-phosphate 50 µM and β-glycerophosphate 10 mM were added after 24 hours when the cells were well attached to the well plate.

Donor-1 cells cultured in XF2 exhibited enhanced cell growth, about 2-fold, compared to when grown in animal-derived growth supplements and significantly higher than all the other conditions in the different media, followed by donor-4 (Figure 3.6 A).

On day7, the cell metabolic activity of cells cultured in XF2 exhibited substantially higher metabolic activity indicating high proliferation compared to the other groups. However, these were not significantly different compared with DONOR-1 treated with 10% (v/v) FBS. Whereas the cell metabolic activity of DONOR-4 cultured in XF1 was significantly impaired in all conditions ($P<0.05$) (Figure 3.6 B).

Overall, the results showed that human serum supplemented with (AA) 50 µM and (βGP) 10 mM performed better of metabolic activity than the human plasma and animal serum medium formulations cultured in the same conditions.

A: Different Donors treated by AA 50 µM and βGP 10 mM on day 4



B: Different Donors treated by AA 50 µM and βGP 10 mM on day 7

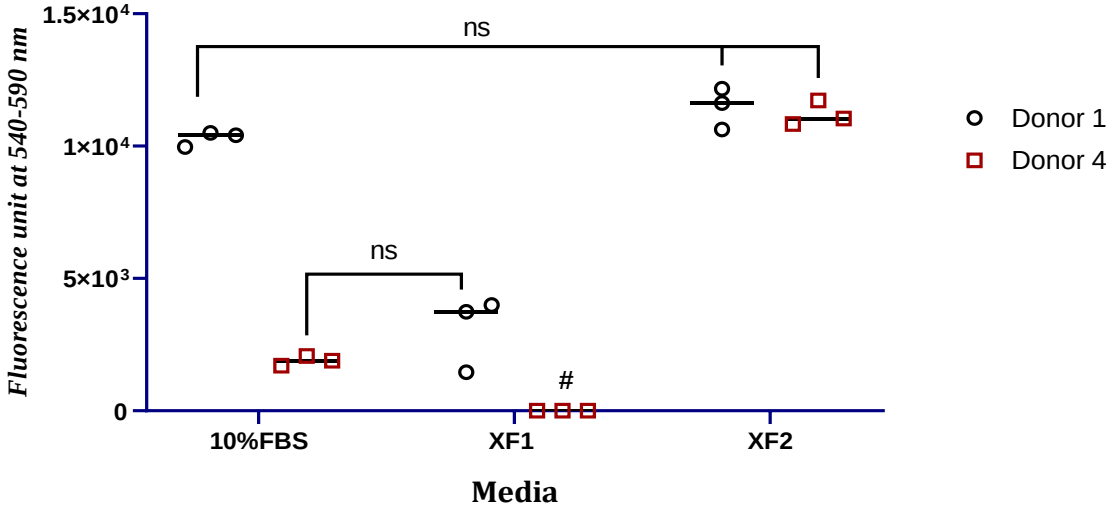


Figure 3.6: *Metabolic activity of different donors of hBMSCs in different xeno-free media supplemented with ascorbic acid-2-phosphate (AA) 50 µM and β-glycerophosphate (βGP) 10 mM on monolayer culture from day 4 to day 7. The symbols indicated individual wells and the horizontal lines the mean and standard deviation for each condition. (*) Significantly higher than the other groups in the same medium. (#) Significantly lower. (Φ) Significantly higher than all the other conditions in the different media. (ns) There is no significant difference (P <0.05). n=3. Two-way ANOVA, Tukey's multiple comparison tests.*

3.7 The effect of osteogenic-inductive supplements on cell metabolic activity of a monolayer cultured hBMSCs

The osteogenic-inductive substances Dex, AA, and βGP were added after 24 hours when the cells were well attached on the well plate.

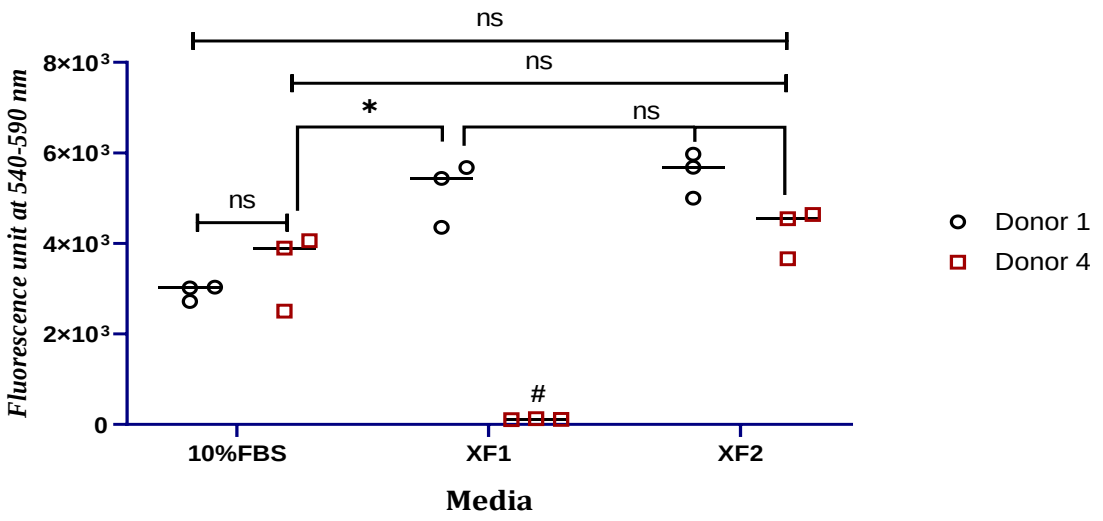
3.7.1 The effects of two different xeno-free basal culture medium supplemented with osteogenic-inductive elements Dex 10 nM, AA 50 µM, and βGP 10 mM on cell metabolic activity of different donors

The cell metabolic activity of donor-1 cells cultured in human-derived substitutes was higher, nearly 2-fold, compared to the same donors cultured in 10% (v/v) FBS. They had a significant difference compared to donors cultured in 10% (v/v) FBS and donor-4 cultured in XF1. Even though donor-4 cells cultured in XF2 appeared to have lower cell metabolic activity compared to donor-1 cultured in XF2 and XF1, there was no significant difference ($P<0.05$). Significant differences were seen between donor 4 cells cultured in XF2 with both donors cultured in 10% (v/v) FBS (Figure 3.7.1 A).

On day7, the cell metabolic activity of both donors cultured in XF2 indicated a substantial enhancement of potential cell proliferation; there were no significant differences between them but they had significantly higher metabolic activity compared to cells grown with different media. Consequently, they were about 2.5-fold higher compared to the same donors treated with XF1 and 10% (v/v) FBS. Donor-4 cells cultured in 10% (v/v) FBS had a rise in cell growth, but retained a low cell number ($P<0.05$) (Figure 3.7.1 B).

Overall, the results demonstrated that the human serum supplemented media with traditional osteogenic-inductive factors supported high cell proliferation compared to the human plasma and animal-serum supplementations cultured in the same conditions.

A: Different donors treated by Dex 10 nM, AA 50 µM, and βGP 10 mM on day 4



B: Different donors treated by Dex 10 nM, AA 50 µM, and βGP 10 mM on day 7

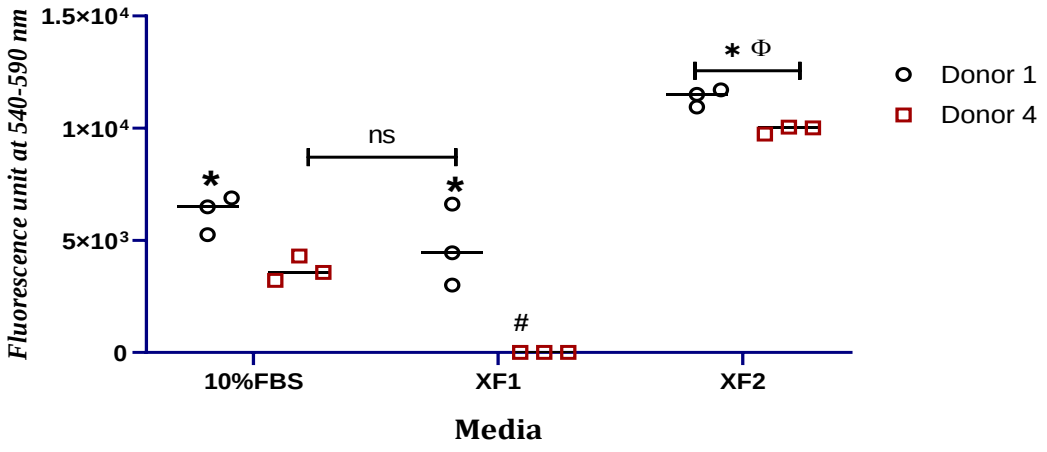


Figure 3.7.1: *Metabolic activity of different donors of hBMSCs on a different xeno-free medium supplemented with osteogenic-inductive ingredients {Dex 10 nM, ascorbic acid-2-phosphate (AA) 50 µM and β-glycerophosphate (βGP) 10 mM} on monolayer culture from day 4 to day 7. The symbols represent an individual well and the horizontal lines mean and standard deviation for each condition. (*) Higher than the other donors in the same medium. (#) Significantly lower. (Φ) Significantly higher than all the other conditions. (P <0.05). n=3. Two-way ANOVA, Tukey's multiple comparison tests.*

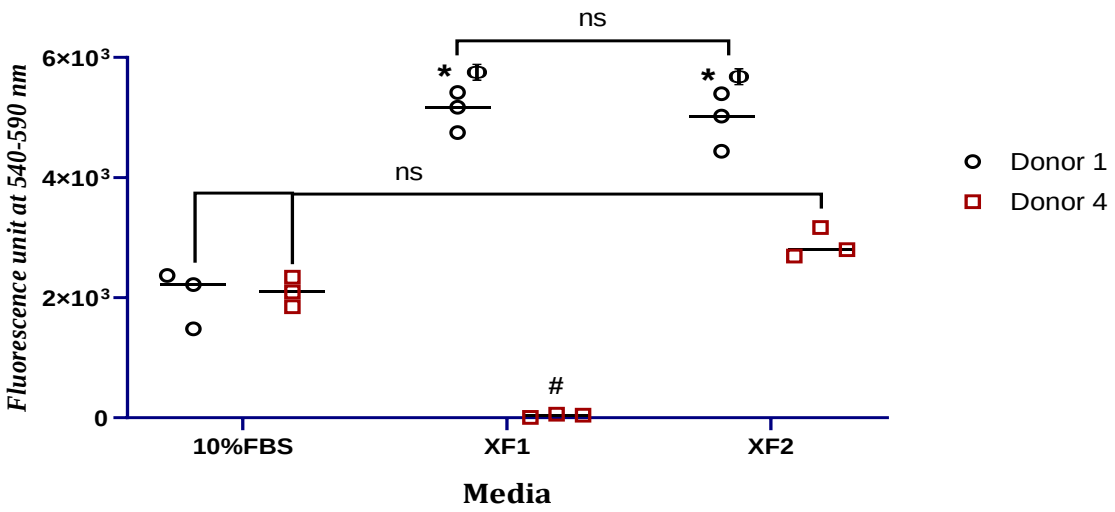
3.7.2 The effects of two different xeno-free basal culture medium supplemented with osteogenic-inductive elements Dex 100 nM, AA 50 µM, and βGP 10 mM on cell metabolic activity of different donors

Similarly, at the higher dose of Dex in the osteogenic medium the cell metabolic activity of donor-1 cultured in human-derived formulations was significantly higher compared to other donor cultured in the same media and 10% (v/v) serum medium. Donor-1 cells cultured in human-derived media were nearly 2.5-fold higher compared to culture in 10% (v/v) FBS and compared to donor 4 cells cultured in XF2 and 10% (v/v) FBS. There was no significant difference between donors cultured in 10% (v/v) FBS and donor-4 cultured in XF2 (*P<0.05)* (Figure 3.7.2 A).

On day7, the metabolic activity of donor-1 cultured in XF2 increased dramatically compared to day 4 indicating proliferation. Moreover, donor-1 exhibited higher cell numbers around 2.5-fold and 4.5-fold compared to when cultured in 10% (v/v) FBS and XF1 respectively (*P<0.05*) (Figure 3.7.2 B).

Overall, the results informed that the human serum supplemented with osteogenic-inductive media containing the higher dose of Dex 100 nM also supported higher cell proliferation compared to the animal serum medium formulation.

A: Different donors treated by (AA, βGP, Dex 100 nM) on day 4



B: Different donors treated by (AA, βGP, Dex 100 nM) on day 7

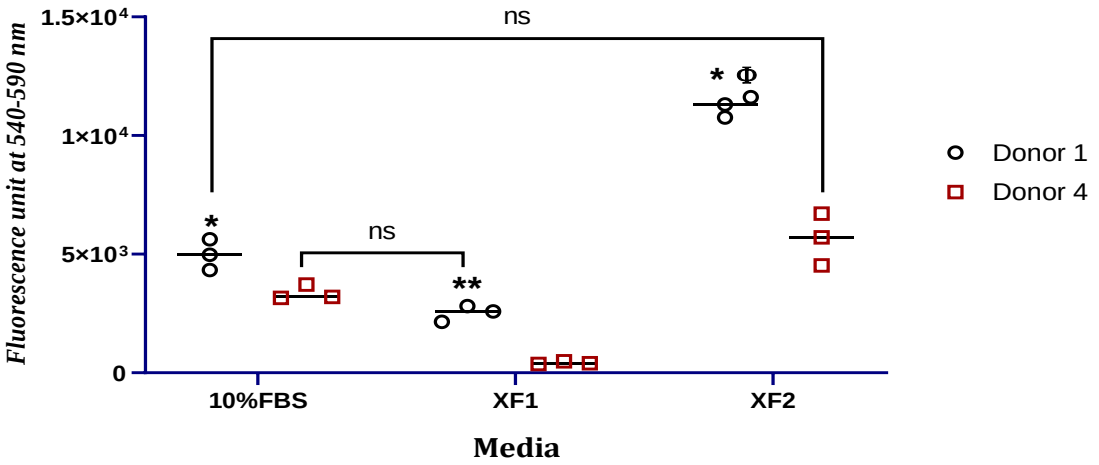


Figure 3.7.2: *Metabolic activity of different donors of hBMSCs on a different xeno-free medium supplemented with osteogenic-inductive ingredients {Dex 100 nM, ascorbic acid-2-phosphate (AA) 50 µM and β-glycerophosphate (βGP) 10 mM} on monolayer culture from day 4 to day 7. The symbols represent an individual well and the horizontal lines mean and standard deviation for each condition. (*) Higher than the other donors in the same medium. (#) Significantly lower. (Φ) Significantly higher than all the other conditions. (P <0.05). n=3. Two-way ANOVA, Tukey's multiple comparison tests.*

Summary of the effect of osteogenic medium on cell metabolic activity

In the previous reporting I deliberately kept the different supplemented groups separate to examine the effect of the different media within groups. However, it is also important to examine the overall effects of the osteogenic supplements on metabolic activity. For this the metabolic activity at day 7 is compared between the basal media groups and the OM-10 groups (Dex 10 nM, AA 50 μM, and βGP 10 mM), this shows that there is no consistent effect of Dex containing osteogenic media on metabolic activity

Table 3.7.2: Examination of the effect osteogenic supplements (Dex 10 nM, AA 50 μM, and βGP 10 mM) on the metabolic activity of cells. "Higher" indicates osteogenic-inductive medium caused significantly higher resazurin fluorescence when comparing data in figure 3.7.1 and figure 3.7.2 "Lower" indicates osteogenic-inductive medium caused significantly lower fluorescence and "NS" indicated no significant difference between basal media and OM within this group on day 7

Media	Donor 1	Donor 4
BCM-OM10	Higher	NS
XF1-OM10	Higher	Lower
XF2-OM10	NS	Higher

3.8 Determining the quantity of DNA

Because the resazurin assay depends on both cell number and metabolic rate of cells, a DNA assay was also performed for some of the conditions investigated. The aim of this experiment was to assess the DNA content through the binding of a fluorescence dye with double-stranded (dsDNA) and assess which xeno-free medium can support high numbers of hBMSCs to be presented by day 10.

These experiments were used to investigate the cell numbers independently of metabolic changes. As the metabolic activity results for XF1 were disappointing some additional conditions were used in the preliminary experiments including 2% (v/v) FBS+ITS and 5% (v/v) HPL.

3.8.1 Assessment of cellular DNA in animal-derived supplemented serum media and human-derived culture media

DNA was analysed quantitatively on day 10, the cells grown in XF2 basal medium had undoubtedly more cells present as the quantity of DNA was significantly higher ($P < 0.05$) compared with all other media at all-time points. Although the 5% (v/v) HPL culture medium also supported higher cell number reaching around 13-fold compared to the animal-derived formulations, it was about 5-fold lower compared with XF2. 10% (v/v) FBS, 2% (v/v) FBS+ITS and XF1 basal medium all supported notably lower cell number *(P <0.05)*.

On supplementation with dexamethasone at 10 nM, the higher growth rate of hBMSCs grown in XF2 culture media was evident and statically higher compared to the other media with the next highest amount of DNA being seen in 5% (v/v) HPL. Even though the 5% (v/v) HPL culture media supported higher DNA than the animal-derived formulations, it supported about 1.5 less compared with XF2. On the other hand, the remaining groups grown in XF1, 2% (v/v) FBS+ITS, and 10% (v/v) FBS media supported lower DNA content *(P <0.05)*.

When the basal culture media was supplemented with ascorbic acid-2-phosphate (AA) 50 µM and β-glycerophosphate (βGP) 10 mM, the cells grown in XF2 had increased cell proliferation as seen by 25-fold and 2-fold higher DNA content than the animal-derived

media and 5% (v/v) HPL respectively. Whereas the cell grown in 5% (v/v) HPL were about 13-fold higher compared to the animal-derived media *(P <0.05)*.

When supplemented with complete osteoinductive ingredients (AA, βGP and Dex) the cells grown in human-derived media, particularly XF2 and 5% (v/v) HPL had significantly higher DNA compared to these grown in the animal-derived osteogenic medium and XF1. In contrast, the quantity of DNA in animal-derived and XF1 osteoinductive culture medium indicated lower cell proliferation *(P <0.05)*.

As we have seen above, the findings provided evidence that the human-derived media formulations and particularly XF2 and 5% (v/v) HPL media could augment the proliferation potential of hBMSCs in both non-osteogenic and osteogenic conditions (Figure 3.8.1).

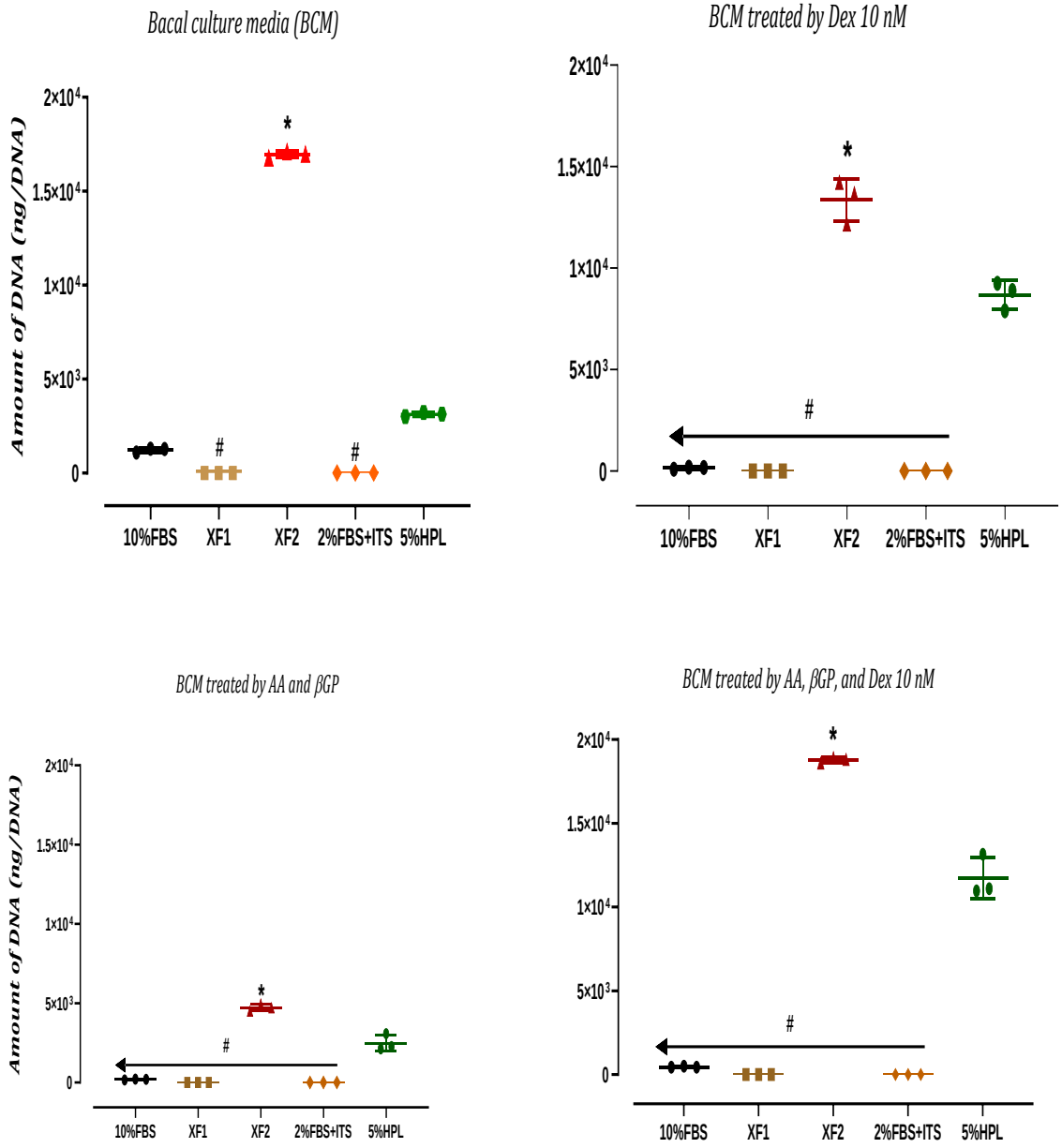


Figure 3.8.1: *DNA quantification of hBMSCs donor-3 passage-2 cultured with different animal-serum concentrations, and commercial human-derived basal culture medium measured by Quant-iT™ PicoGreen® on monolayer on day 10. The symbols indicate individual wells and the horizontal lines mean and standard deviation for each condition. (*) Significantly higher than the other media at all-time points. (#) Significantly lower. (P <0.05). n=3. ANOVA, Brown-Forsythe test.*

3.8.2 The effects of different basal culture medium on the DNA content of different donors

Further experiments were undertaken using selected culture media (10% (v/v) FBS as a standard, XF1 as a poorly performing media and XF2 and a good performing media) on a range of donor numbers to see if the effects of the culture media would be consistent across donors. DNA assay was undertaken at 10 days.

The donors' cells cultured in 10% (v/v) FBS and three out four donors cultured in XF1 generally had lower DNA content. It is noteworthy that all donors cultured in XF2 contained more DNA than all other groups, and each donor sample had significantly more DNA when cultured in XF2 compared to its respective cells cultured in other media. Furthermore, donor-1 and donor-2 cultured in XF2 were indicated significantly different, nearly 8-fold higher in DNA content, compared to cells cultured in 10% (v/v) FBS ($P<0.05$) (Figure 3.8.2).

Overall, it can be seen that the DNA content was the highest in human serum (XF2) and lowest in animal-derived and human plasma (XF1) basal culture media for all donors.

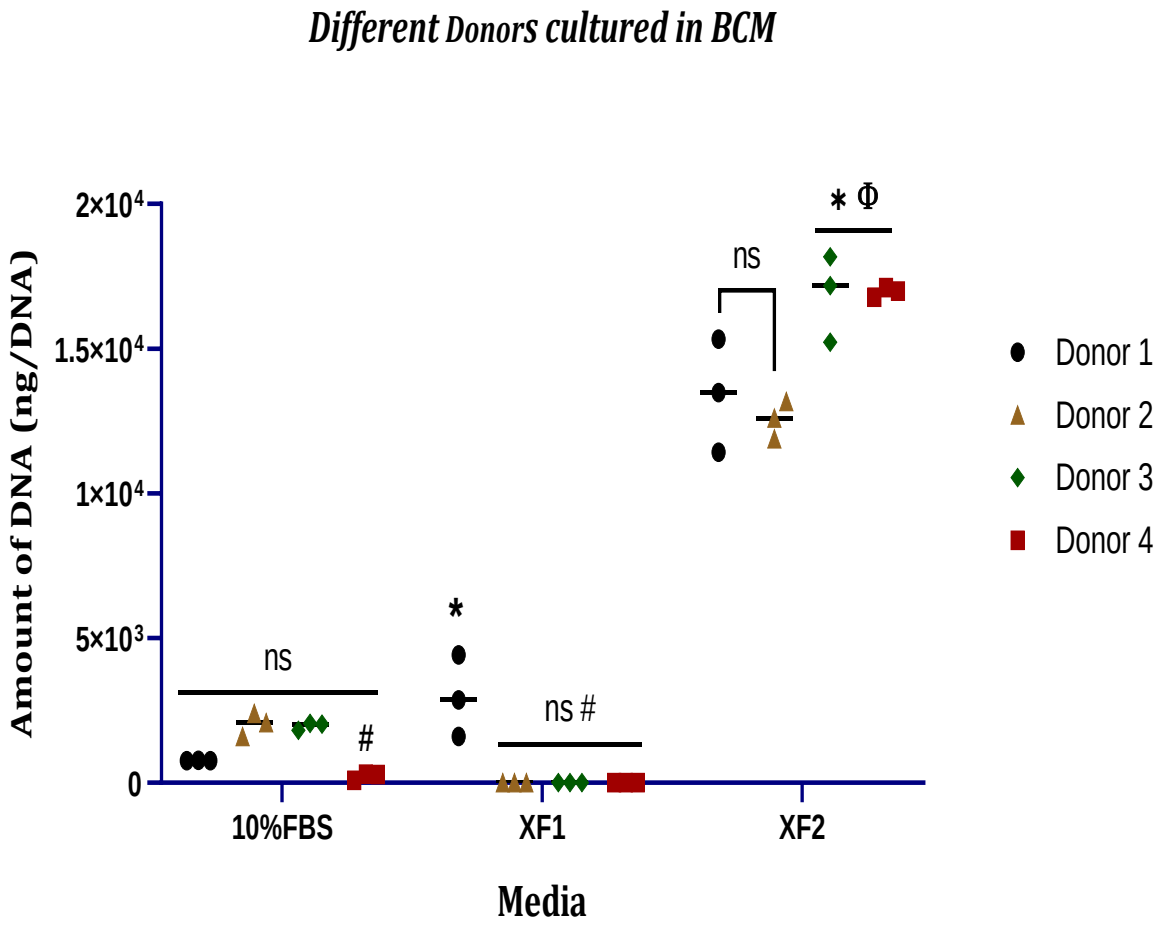


Figure 3.8.2: *DNA quantification of hBMSCs cultured in standard FBS, and commercial human-derived basal culture medium measured by Quant-iT™ PicoGreen® on monolayer on day 10. The symbols indicate individual wells and the horizontal lines mean and standard deviation for each condition. (*) Higher than the other donors in the same medium. (#) Significantly lower. (Φ) Significantly higher than all the other conditions. (P <0.05). n=3. Two-way ANOVA, Tukey's multiple comparison tests.*

3.8.3 The effect of varying concentrations of dexamethasone supplemented with basal culture medium on the amount of DNA of a monolayer cultured hBMSCs

Dexamethasone was added after 24 hours after the cells had attached on the well plate.

3.8.3.1 Determine total DNA content in two different xeno-free basal culture media supplemented with Dex 10 nM on different donors

Three donors out of four cultured in XF2 treated by Dex 10 nM contained statistically significantly higher cellular DNA content than cells from the same donors cultured in the different culture medium supplemented with Dex 10 nM ($P<0.05$) (Figure 3.8.3.1).

Overall, the results demonstrated that when treated with Dex 10 nM cells cultured in XF2 also contained higher numbers compared with other media as was seen in basal culture media alone.

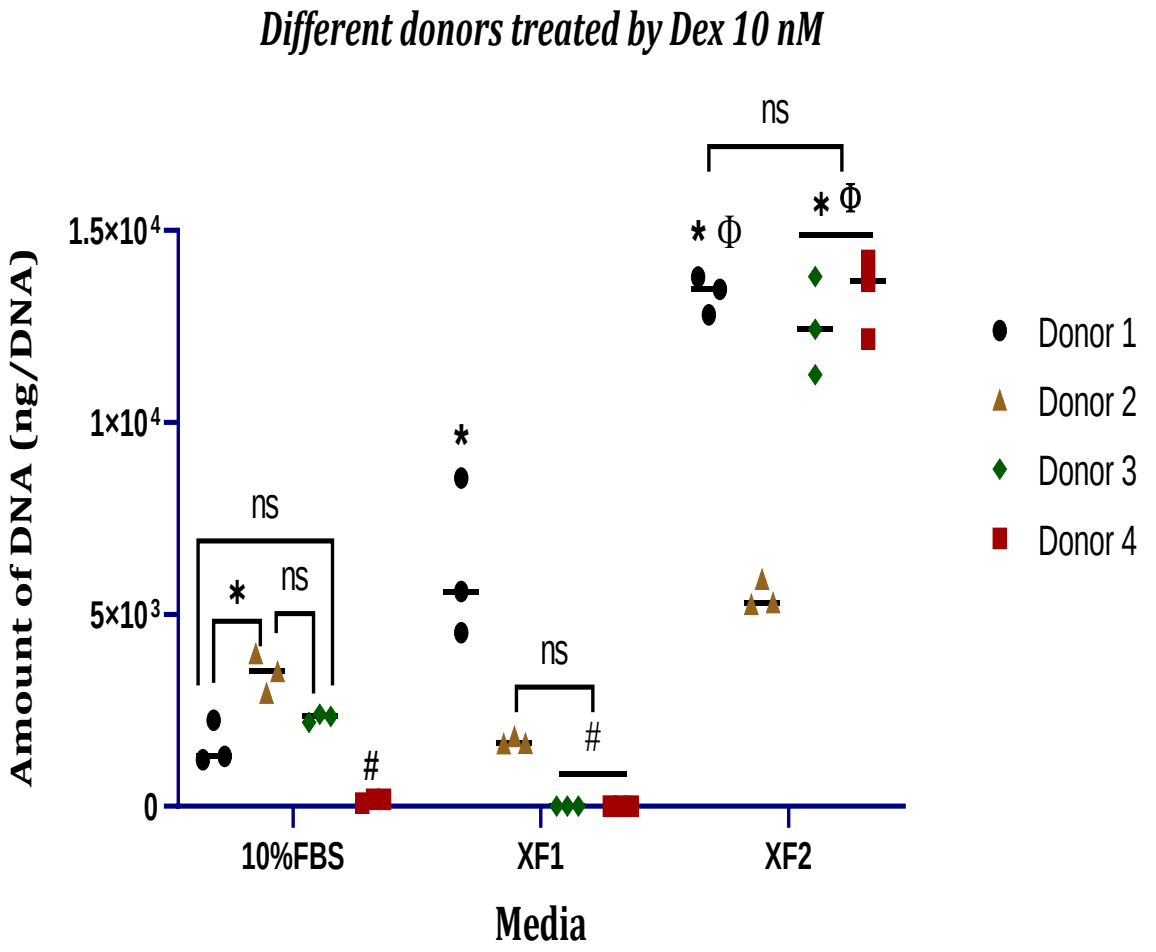


Figure 3.8.3.1: *DNA quantification of hBMSCs cultured on two commercial human-derived basal culture media compared with 10% (v/v) animal-serum concentration treated with Dex 10 nM and measured by Quant-iT™ PicoGreen® on monolayer on day 10. The symbols indicate individual wells and the horizontal lines mean and standard deviation for each condition. (*) Higher than the other donors in the same medium. (#) Significantly lower. (Φ) Significantly higher than all the other conditions. (P <0.05). n=3. Two-way ANOVA, Tukey's multiple comparison tests.*

3.8.3.2 Determine total DNA content in two different xeno-free basal culture media supplemented with Dex 100 nM on different donors

On supplementation of the basal culture media with Dex 100 nM, peak DNA content was lower than in 10 nM Dex indicating there may have been some inhibition of cell growth at the higher Dex concentration. However, the pattern of behaviour of different donor numbers were similar with effect of Dex 10 nM except in cultured XF2. Donor-1cells cultured in human-derived media and Donor-2 cells cultured in XF2 contained significantly higher DNA over the others (*P<0.05)* (Figure 3.8.3.2).

Overall, the results confirmed that also in Dex 100 nM cells of donor 1 and 2 cultured in XF2 usually had significantly more DNA content than the same cells cultured in other media, the exception being Donor-1 that had just as high DNA content in XF1.

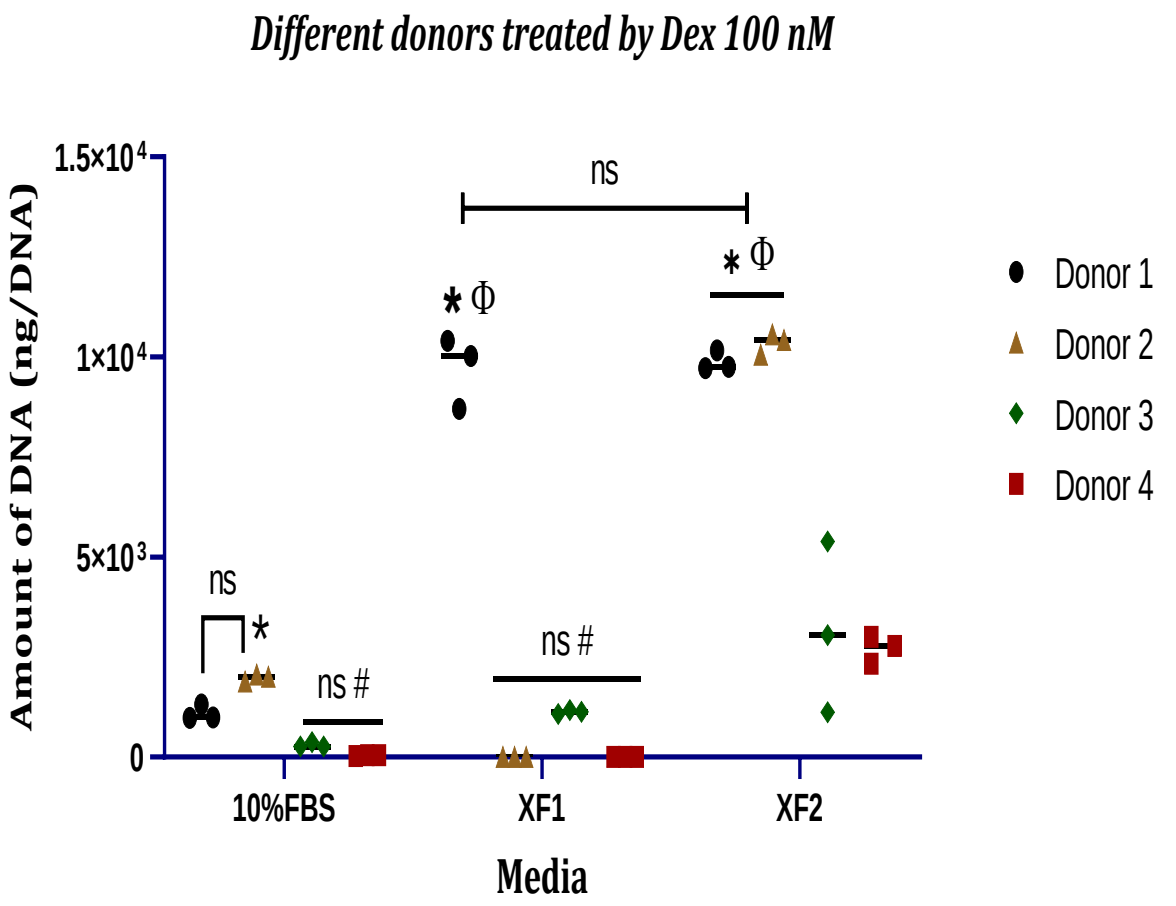


Figure 3.8.3.2: *DNA quantification of hBMSCs cultured on two commercial human-derived basal culture media compared with 10% (v/v) animal-serum concentration that treated by Dex 100 nM and measured by Quant-iT™ PicoGreen® on monolayer on day 10. The symbols indicate individual wells and the horizontal lines mean and standard deviation for each condition. (*) Higher than the other donors in the same medium. (#) Significantly lower. (Φ) Significantly higher than all the other conditions. (P <0.05). n=3. Two-way ANOVA, Tukey's multiple comparison tests.*

3.8.4 The effects of two different xeno-free basal culture medium supplemented with ascorbic acid-2-phosphate (AA) and β-glycerophosphate (βGP) on the amount of DNA in different donors

When the basal culture media was supplemented with (AA) 50 µM and (βGP) 10 mM, the amount of DNA in all donors cultured in XF1 and donor-4 cultured in 10% (v/v) serum medium was lower. Donor-2 cells cultured in XF2 exhibited distinctly higher DNA, approximately 20-fold compared to when cultured in 10% (v/v) FBS. While cells from DONOR-1 had high DNA indicating cell proliferation (about 2-fold and 3.5-fold compared to those cultured in 10% (v/v) FBS and XF1) respectively ($P<0.05$) (Figure 3.8.4).

Overall, the results confirmed that also when supplemented with (AA) 50 µM and (βGP) 10 mM, which are added to media to support matrix production, cells generally grew better in XF2 human derived serum than in human-derived plasma and animal-derived serum medium formulations, although there is donor to donor differences affecting whether the ells are responsive to XF2.

Different donors treated by AA and βGP

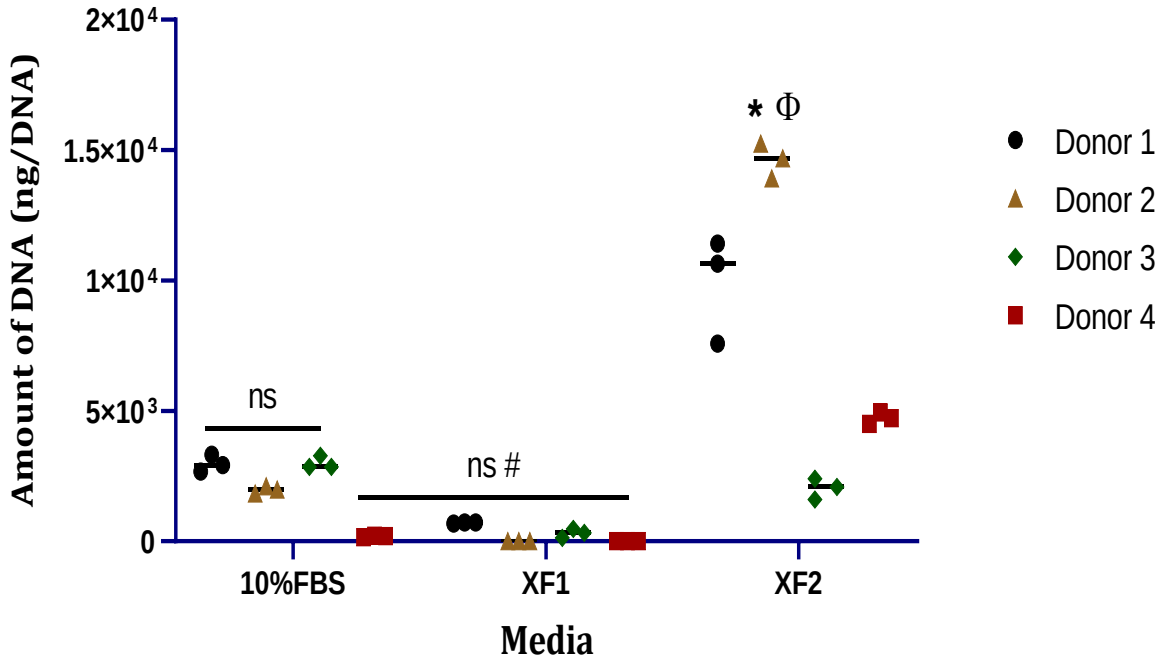


Figure 3.8.4: *DNA quantification of hBMSCs cultured on two commercial human-derived basal culture media compared with 10% (v/v) animal-serum concentration that treated by ascorbic acid-2-phosphate (AA) 50 µM and β-glycerophosphate (βGP) 10 mM and measured by Quant-iT™ PicoGreen® on monolayer on day 10. The symbols indicate individual wells and the horizontal lines mean and standard deviation for each condition. (*) Higher than the other donors in the same medium. (#) Significantly lower. (Φ) Significantly higher than all the other conditions. (P <0.05). n=3. Two-way ANOVA, Tukey's multiple comparison tests.*

3.8.5 The effect of osteogenic-inductive supplements on the quantity of DNA on a monolayer cultured hBMSCs

The osteogenic-inductive substances Dex, AA, and βGP were added after 24 hours when the cell was entirely attached on the well plate.

3.8.5.1 DNA content in culture media supplemented with osteogenic-inductive substances at a Dex concentration 10 nM.

When the basal culture media was supplemented with the full set of osteogenic-inductive substances including Dex 10 nM, the cell numbers of DONOR-1 cultured in XF2 were again very high and significantly from other donors. Whereas all donors cultured in 10% (v/v) serum media and three of four donors cultured in XF1 had very low DNA content. There was no significant difference among the donors when cultured in 10% (v/v) FBS (*P<0.05*) (Figure 3.8.5.1).

Overall, the results demonstrated that human serum supplemented with osteogenic-inductive media induced higher cell numbers compared to human plasma and animal-derived growth supplementation, cultured in the same conditions. It is interesting to note that the effect of XF2 media there is higher DNA content in with osteogenic supplements compared to in basal culture media alone (Table 3.8.5.1).

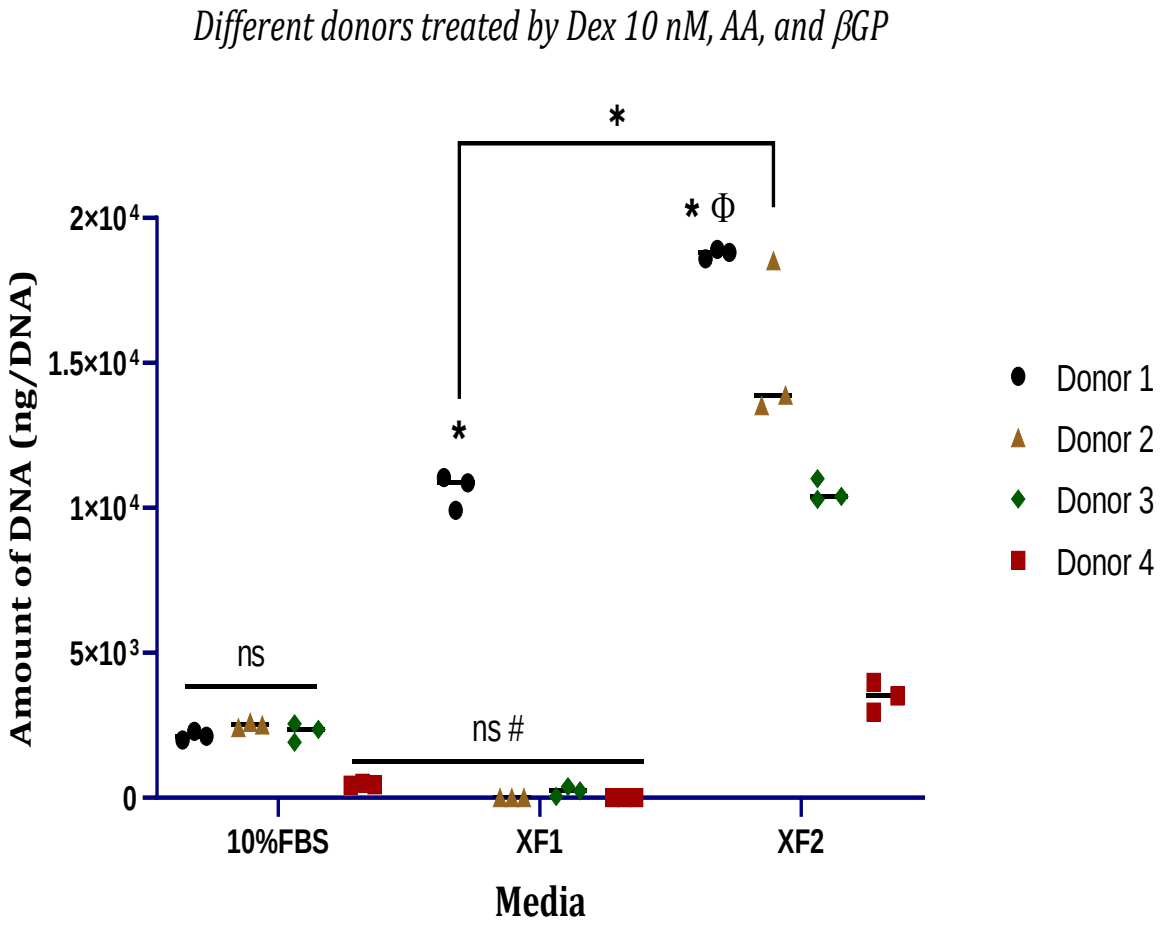


Figure 3.8.5.1: *DNA quantification of hBMSCs cultured on two commercial human-derived basal culture media compared with 10% (v/v) animal-serum concentration that treated by Dex 10 nM, ascorbic acid-2-phosphate (AA) 50 µM, and β-glycerophosphate (βGP) 10 mM and measured by Quant-iT™ PicoGreen® on monolayer on day 10. The symbols indicate individual wells and the horizontal lines mean and standard deviation for each condition. (*) Higher than the other donors in the same medium. (#) Significantly lower. (Φ) Significantly higher than all the other conditions. (P <0.05). n=3. Two-way ANOVA, Tukey's multiple comparison tests.*

Table 3.8.5.1.: The effect of osteogenic media (containing Dex 10 nM and 100 nM) on DNA content within the media groups examined. “Higher” indicates osteogenic-inductive medium caused significantly higher when comparing data in figure 3.8.5.1 and figure 3.8.5.2 “Lower” indicates osteogenic-inductive medium caused significantly lower DNA and “NS” indicated no significant difference between two OM within this group. There was only a consistent effect of osteogenic media for XF2.

Media	Donor 1	Donor 2	Donor 3	Donor 4
10% (v/v)FBS-OM10	Lower	Higher	Higher	Lower
XF1-OM10	Higher	NS	NS	NS
XF2-OM10	Higher	Higher	Higher	Higher

3.8.5.2 DNA content in culture media supplemented with osteogenic-inductive substances at a Dex concentration 100 nM

Because some researchers recommend 100 nM Dex for osteogenic culture medium but a high concentration of Dex may inhibit cell proliferation I also examined the effects of a higher Dex concentration in the osteogenic media. Overall, the results gave similar results to osteogenic medium with 10 nM Dex concentration although the donor cells that yielded the highest amounts of DNA in 10 nM Dex did have lower DNA in 100 nM Dex. Two out four donors' cells supplemented with human serum and osteogenic media containing 100 nM Dex had higher cell numbers than all other donors cultured in XF1 and 10% (v/v) serum medium ($P<0.05$) (Figure 3.8.5.2).

Different donors treated by Dex 100 nM, AA, and βGP

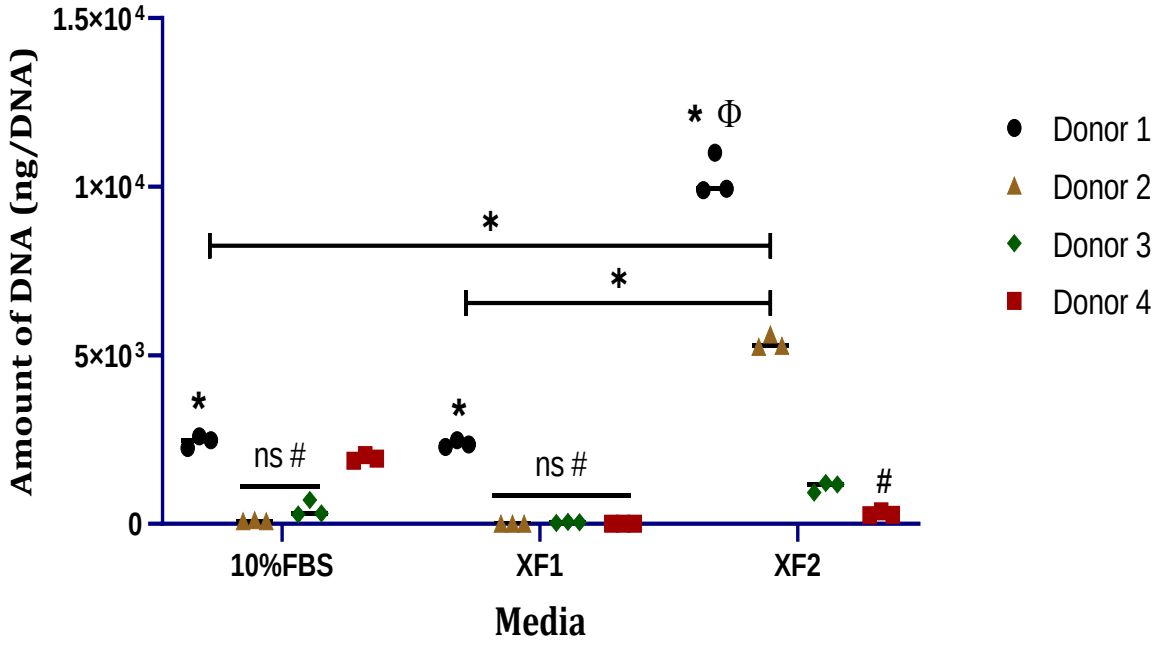


Figure 3.8.5.2: *DNA quantification of hBMSCs cultured on two commercial human-derived basal culture media compared with 10% (v/v) animal-serum concentration t treated by Dex 100 nM, AA 50 µM, and βGP 10 mM and measured by Quant-iT™ PicoGreen® on day 10. The symbols indicate individual wells and the horizontal lines mean and standard deviation for each condition. (*) Higher than the other donors in the same medium. (#) Significantly lower. (Φ) Significantly higher than all the other conditions. (P <0.05). n=3. Two-way ANOVA, Tukey's multiple comparison tests.*

3.9 Human jaw periosteum as an alternative source (preliminary data)

The target of this experiment was to assess the HJP behaviour when grown in xeno-free culture media and treated by different supplements as an alternative source of cells.

3.9.1 Appraisal of various culture media formulations on the seeding efficiency and growth of HJPs in monolayer

In the basal culture media after 24 hours of cell, seeding the resazurin reduction assay was used to evaluate cell attachment on the well plate. HJPs grown in all media attached, and there was no significant difference in cell number between media types. On day 4, the cell metabolic activity illustrated how different media formulation affect cell metabolic activity. The cell metabolic activity cultured in human-derived formulation media was higher with a significant effect compared to the other conditions.

The cell metabolic activity was 2.5-fold higher compared with 24 hours and 1.5-fold higher compared with 10% (v/v) FBS (*P<0.05)*. There was no significant difference between XF2 and XF1. There was no significant difference in cell metabolic activity between the human-derived growth media on day 4 and XF2 on day 7. For the remaining culture media, the cell metabolic activity dropped nearly 3-fold, indicating cell death. Although the XF1 had high the cell metabolic activity on day 4, this declined by day 7 (*P<0.05)* (Figure 3.9.1 A).

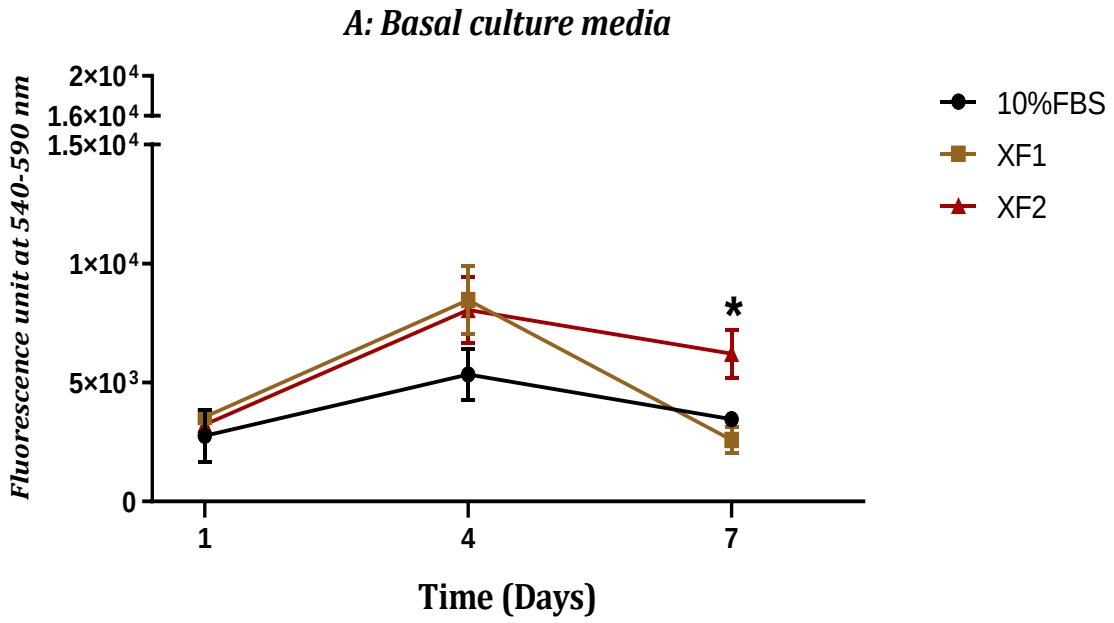
For basal culture media treated with Dex 10nM the cell metabolic activity of cells cultured in human-derived growth media was noticeably increased about 3-fold by day 7 compared with 10% (v/v) FBS at the same time (*P<0.05)* (Figure 3.9.1 B).

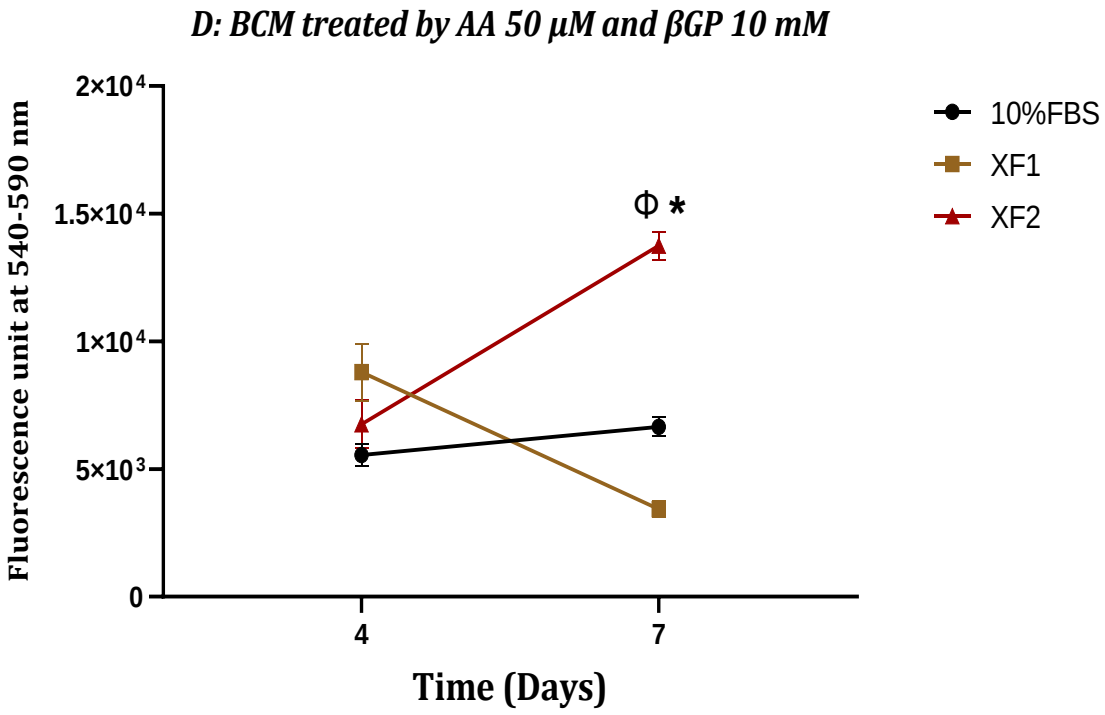
For basal culture media treated with Dex 100nM on day 4, the cell metabolic activity cultured in XF2 was significantly higher about 2-fold and 2.5-fold over the 10% (v/v) FBS and XF1 respectively (*P<0.05)* (Figure 3.9.1 C).

In the basal culture media treated by AA 50 µM and βGP 10 mM. On day 4, the cell metabolic activity in XF1 was about 1.5-fold significantly higher than in the other media. While the 10% (v/v) FBS culture medium supported nearly 2-fold cell metabolic activity over the XF1 culture medium, which was unable to support good cell metabolic activity. By day 7 cell metabolic activity in XF2 was two times higher compared with all conditions on day 4 (*P<0.05)* (Figure 3.9.1 D).

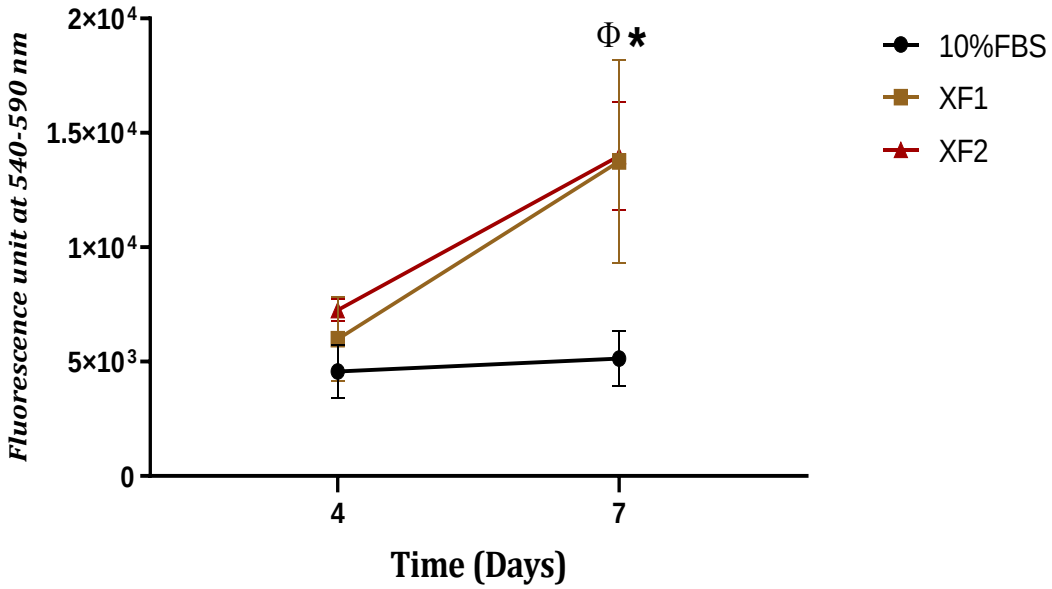
In culture media containing with osteogenic supplementations with Dex 10nM the human-derived growth media supported slightly higher cell numbers compared to animal-derived serum medium, but there was no significant difference. By day 7, the cell metabolic activity in XF2 was about 2.5-fold and 8-fold higher over the 10% (v/v) FBS and XF1 respectively. While the 10% (v/v) FBS culture medium supported nearly 3.5-fold metabolic activity compared to XF1 culture medium (*P<0.05)* (Figure 3.9.1 E).

In culture media containing with osteogenic supplementations with Dex 100nM all conditions supported lower of the cell metabolic activity and there was no significant difference among them at day 4 but by day 7, the cell metabolic activity in XF2 was 4.5-fold higher than in 10% (v/v) FBS (*P<0.05)* (Figure 3.9.1 F).

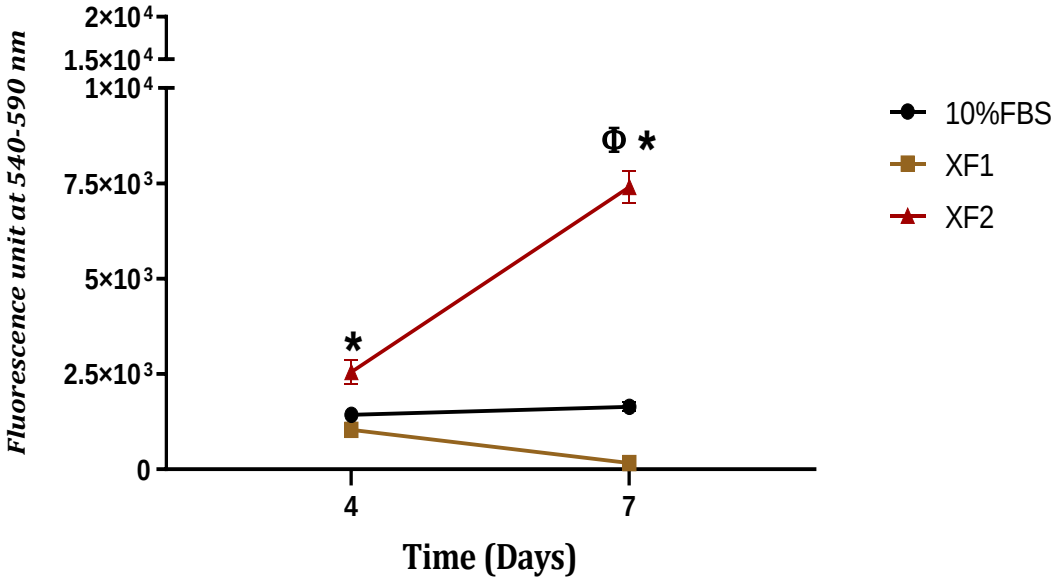

A: Basal culture media
Fluorescence unit at 540-590 nm
2×10⁴
1.6×10⁴
1.5×10⁴
1×10⁴
5×10³
0
1
4
7
Time (Days)
*
10%FBS
XF1
XF2


D: BCM treated by AA 50 µM and βGP 10 mM
Fluorescence unit at 540-590 nm
2×10⁴
1.5×10⁴
1×10⁴
5×10³
0
4
7
Time (Days)
Φ *
10%FBS
XF1
XF2

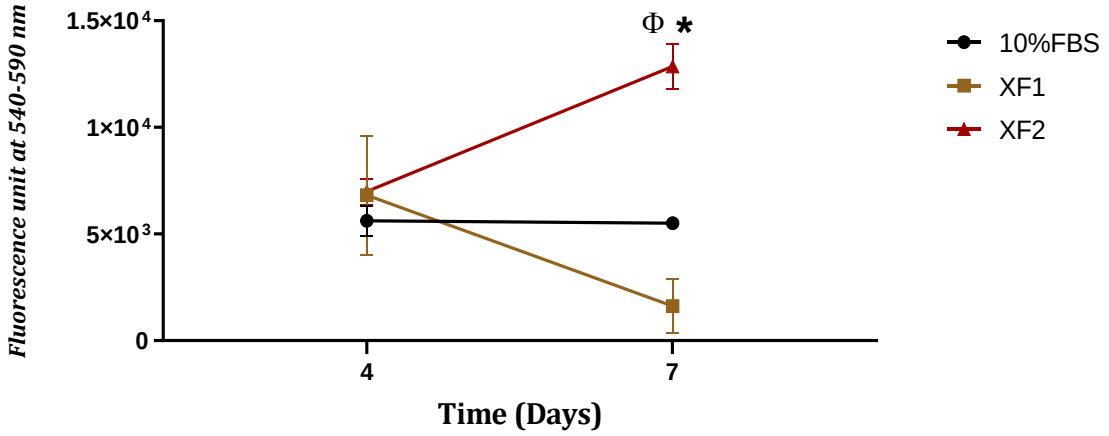
B: BCM treated by dex 10 nM



C: BCM treated by dex 100 nM



E: BCM treated by Dex 10 nM, AA 50 µm, and βGP 10 mM



F: BCM treated by Dex 100 nM, AA 50 µm, and βGP 10 mM

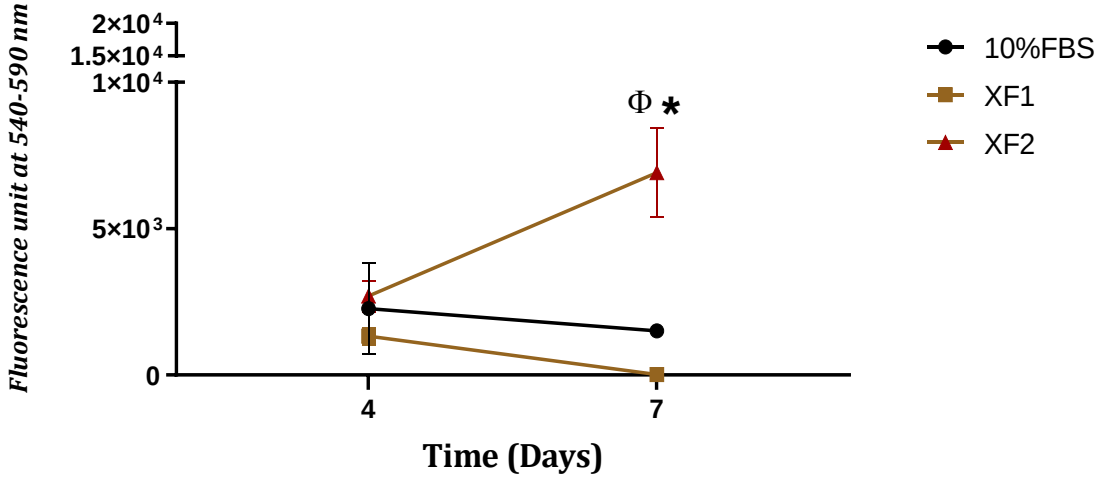


Figure 3.9.1: *Metabolic activity of HJPs cultured in commercial human-derived growth basal culture medium compared with 10% (v/v) animal-serum concentration measured by resazurin reduction assay on monolayer over time for 7 days. The scatter dot plot manifests mean and standard deviation for each condition. (*) Significantly higher than the other media at all-time points. (#) Significantly lower. Φ) Significantly higher than all the other conditions. (ns) There is no significant difference (P <0.05). n=3. Two-way ANOVA, Tukey's multiple comparison tests.*

3.9.2 Evaluation of the total amount of DNA of HJPs

In the basal culture media on day 10, the amount of DNA in XF2 was a noticeably different compared with other conditions. It was boosted about 1.5-fold and 3-fold compared with 10% (v/v) FBS and XF1 respectively, whereas the 10% (v/v) FBS had higher DNA, nearly 2-fold, over the XF1 and the XF1 groups had lower DNA quantity on day 10 (*P<0.05*) (Figure 3.9.2 A).

For the media supplemented with Dex 100 nM; XF2 was revealed to increase the DNA quantity significantly about 13-fold and more compared with 10% (v/v) FBS and XF1 respectively (P<0.05). For medium supplemented with Dex 10 nM; the human-derived formulations supported about 5-fold times the DNA (cell number) compared with animal-derived serum. In this group although the XF2 medium augmented the cell proliferation over the XF1, there was no significant difference. The human-derived growth media treated with 10 nM Dex in general supported higher DNA content compared to Dex at 100 nM (*P<0.05*) (Figure 3.9.2 B).

In media supplemented with AA 50 μM and βGP 10 mM, DNA in the XF2 group was a significantly different from the other conditions ,7-fold and 25-fold, compared with10% (v/v) FBS and XF1 respectively. Whereas the 10% (v/v) FBS increased the quantity of DNA approximately 3.5-fold compared to XF1, and XF1 was the poorest performing media in terms of supporting cell numbers (*P<0.05*) (Figure 3.9.2 C).

When full osteoinductive supplements were added at Dex 10 nM, the XF2 was revealed to increase the DNA quantity about 4-fold and 9-fold compared with 10% (v/v) FBS and XF1 respectively. 10% (v/v) FBS supported twice the DNA content, compared to XF1 (P<0.05). At the higher Dex concentration of100 nM, the XF2 supported higher DNA, almost 24-fold and 11-fold over the 10% (v/v) FBS and XF1 respectively. On comparing between the two groups with osteoinductive supplementation for the 10% (v/v) FBS group Dex at 10 nM supported more cells compared to Dex at 100 nM 10 (*P<0.05*) (Figure 3.9.2 D).

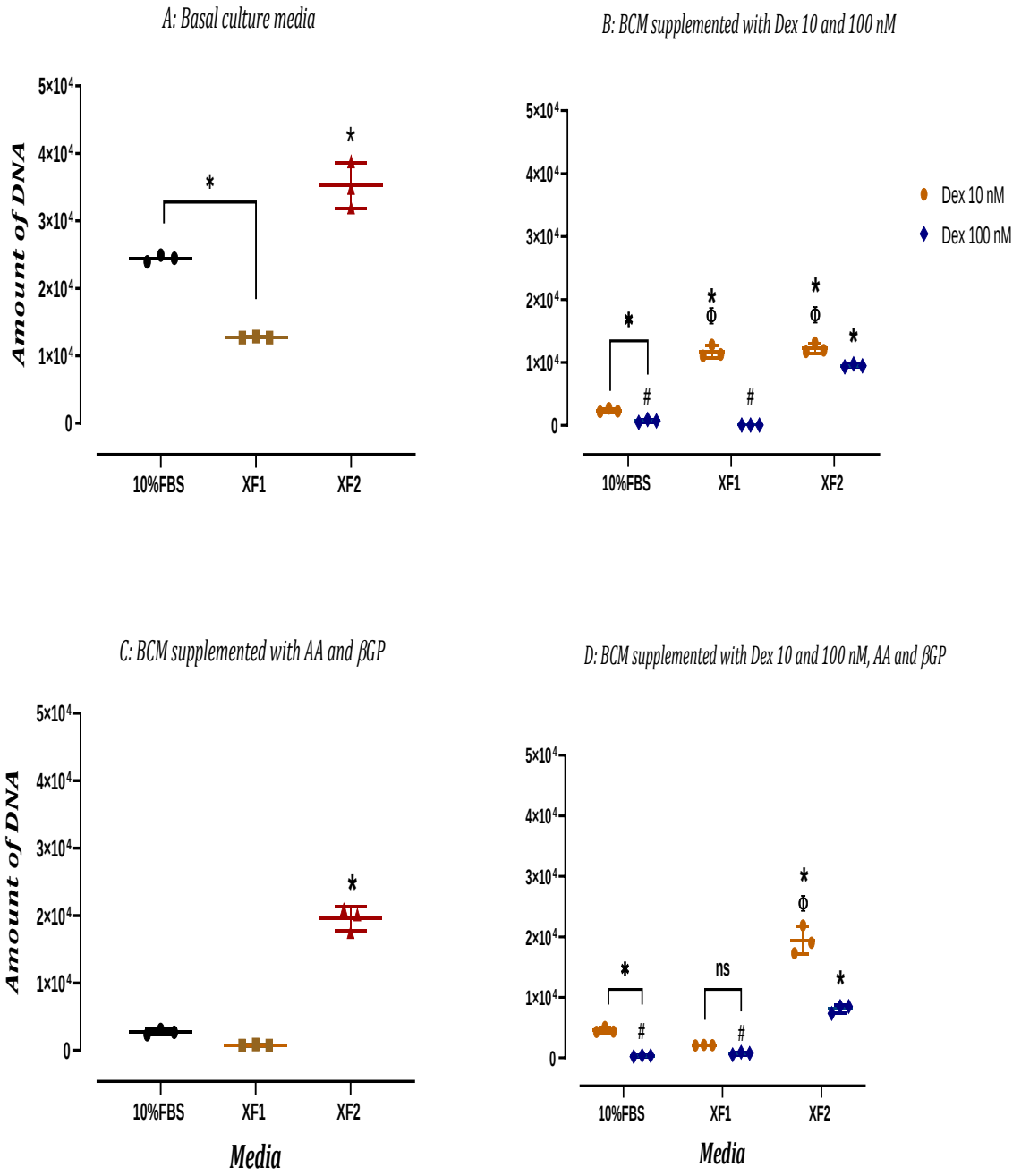


Figure 3.9.2: *DNA quantification of HJPs cultured on two commercial human-derived basal culture media compared with 10% (v/v) animal-serum concentration measured by Quant-iT™ PicoGreen® on monolayer on day 10. The scatter dot plot manifests mean and standard deviation for each condition. (*) Significantly higher than the other media at all-time points. (#) Significantly lower. Φ) Significantly higher than all the other conditions. (ns) There is no significant difference (P <0.05). n=3. ANOVA, Brown-Forsythe test. Moreover, two-way ANOVA, Tukey's multiple comparison tests.*

3.10 EdU proliferation assay for monolayer culture

The therapeutic application of hBMSCs in craniofacial bone regeneration requires rapid proliferation and expansion of cells in a medium that does not compromise the cells. The resazurin and DNA assays both indicated higher cells numbers in XF2 and HPL media, however neither of these assays directly measures cells proliferation. Thus, we investigated the effect of human-derived media on hBMSCs proliferation via EdU incorporation assay on day 4. EdU is a nucleoside analogue to thymidine and incorporates into DNA during the active period of DNA synthesis. We excluded the XF1 (human plasma containing) and chemically derived media from the experiments because they had shown low resazurin and DNA values, indicating poor cell growth.

EdU labelling experiments were performed for three adult donors. Although the animal-derived serum supplements are commonly used to support cell proliferation, lower amounts of newly synthesised DNA were seen in animal serum compared with human-derived supplements. Two out of three donors cultured in XF2 were shown to proliferate more rapidly compared to in other media particular cells from donor-1 that were statistically significantly different from the other donors. In donor-3, the amount of newly synthesised DNA was shown to be higher in 5% (v/v) HPL rather than other media. Although the amount of newly synthesised DNA in 5% (v/v) HPL was significantly different from 10% (v/v) FBS, it was not different from XF2 ($P<0.05$) (Figure 3.10).

Overall, the human-derived growth supplements enabled higher synthesis of DNA particularly XF2 compared to the animal-derived serum supplements. The donor effect and types of media played a crucial role in determining the rate of cell proliferation.

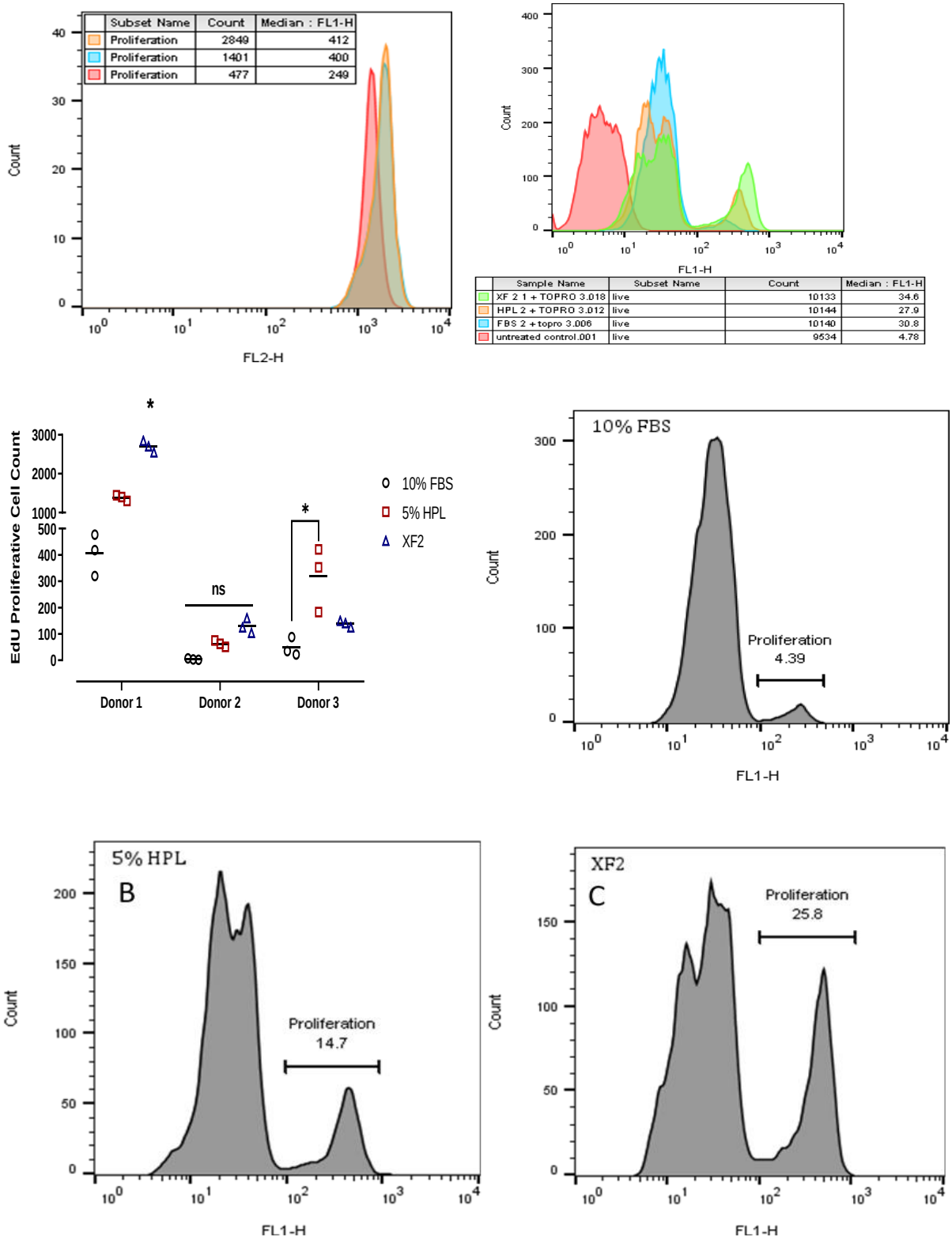


Figure 3.10: *Fluorescence Signal from Alexa Fluor® 488 Click-iT® EdU Flow Cytometry Assay Kits. The figures show proliferating cells that have incorporated EdU. (A) 10% (v/v)FBS showed few cells proliferation, (B) 5% (v/v)HPL showed upregulation of cells proliferation, (C) XF2 showed higher cells proliferation.*

3.11 Discussion

Bone tissue engineering presents a considerable opportunity for clinical cleft palate repair. The essential ingredients for this strategy to be effective are the osteoinductive cell source and the biochemicals for bone formation. Bone marrow derived cells have demonstrated therapeutic potential for the treatment of cleft palate and other disorders and diseases because they contain a subpopulation of cells called "precursor cells" which have the natural ability to differentiate toward bone forming cells (Coelho and Fernandes, 2000; Manolagas, 1995) and also present self-renewal capacity with multi-potency (Solmesky et al., 2010). It has been predicted that bone marrow contains only between 0.001 to 0.01% mesenchymal stem cells (MSCs) as a sub-population of whole bone marrow mononuclear cells (Pittenger et al., 1999; Schallmoser et al., 2007). While a sufficient amount of MSCs that are considered to be a clinical dose is at least 2 x 10^6 MSCs/kg body weight of the adult patient (Ringdén et al., 2006; Schallmoser et al., 2007). Therapeutically human bone marrow MSCs (hBMSCs) to be used in a patient need comprehensive *in vitro* cell expansion, usually using animal-serum components Foetal Bovine Serum (FBS) as the fundamental growth supplement. However, there are problems related to employing animal component medium. Thus, the purpose of this research is to identify satisfactory media to support hBMSCs and move from animal component media to a defined xeno-free product. In addition, the aim was to evaluate this in different donors, since there is well-demonstrated variability in the responses of hBMSCs to media supplements. Human serum and human platelet lysate could be clinically used and have been approved by the US Federal Drug Agency (FDA) and are considered as safe alternative media for hBMSCs.

The majority of studies have been focused on cell proliferation, and expansion and recent endeavours have been aimed at isolation and expansion of MSC in short culture times using xeno-free components (Battula et al., 2007). A minority of researchers have investigated the ability of xeno-free conditions to facilitate expansion and differentiation of hBMSCs over long term culture (Lindroos et al., 2009). Therapeutic implementation of hBMSCs in cleft palate requires rapid expansion and proliferation of cells in a medium that does not compromise the cell properties. Prosperous bone tissue engineering utilising hBMSCs primarily depends on cell quality, the capability of proliferation, differentiation *in vitro*, and bone formation *in vivo* (Siddappa *et al.,* 2007). This study

concerns xeno-free media that will support osteogenic cell differentiation with the long-term aim of establishing xeno-free cultures for clinical applications of craniofacial bone defects in particular cleft palate repair. The first step was to see how the interaction between the different media compositions and the different osteogenic supplements would affect cell numbers and cell proliferation in culture conditions. Resazurin reduction assay was performed on seeded hBMSCs in 2D monolayer to infer cell attachment and metabolic activity while PicoGreen was used to evaluate the DNA content.

I initially compared five commercial xeno-free media and a reduced concentration of animal serum supplemented with ITS (Insulin-Transferrin-Selenium) compared with 10% (v/v) FBS. These media include three media derived from human sources such as XF2 (2% (v/v) human serum), XF1 (human plasma), 5% (v/v) HPL, and two chemically defined media herein denoted as SR1 and MSC-NutriStem. It is noteworthy that hBMSCs cultured in two chemically defined media showed lower numbers and slower growth. The ITS culture supplement provides insulin, transferrin, and selenium as required in cultured cells, and has been shown to enable lower animal serum concentration to be used for example the FBS concentration to 2% (v/v). ITS has been routinely supplemented in most serum or a low concentration of bovine serum-free culture media (Jeong et al., 2008). However, in this study cells cultured in ITS with 2% (v/v) serum showed poor cell growth and therefore the use of this supplement was not continued.

Resazurin reduction assay was performed after 24 hours to understand the cell attachment to well plate base, and the results showed no significant difference among different media. Cells grown in xeno-free media particularly in 5% (v/v) HPL and XF2 adopted a spindle-shaped morphology with evidence of cell proliferation. These media allowed cells to grow at much higher densities over a short time compared to the cells grown in 10% (v/v) animal-serum supplemented media.

As would be expected the standard formulation basal culture medium had allowed good cell proliferation (Kyllönen et al., 2013) with the time. Surprisingly XF1 which also contained human-derived ingredients did not support cell growth as well as XF2, 5% (v/v) HPL or even 10% (v/v) FBS.

hBMSCs cultured in 10% (v/v) FBS and human-derived media (except XF1) supported upregulation of metabolic activity at a later time-points of the culture period. The reason

for this may be that these formulations containing human-derived media sustain the hBMSCs by delivery of growth factors required for hBMSCs proliferation and expansion. Chase et al., (2010) cultured primary hBMSCs in serum-free supplemented with growth factors, the authors concluded that there was a synergistic effect between them. Thus, our human-derived media have growth factors because they are extracted from human blood. However, the amount and types of growth factors are unknown. Because these media come from commercial sources and are protected by intellectual property rights, the company does not give the precise composition of the media. This makes it difficult to understand why XF1 supports cell growth much less than other formulations. Chemically defined media offers advantages in that the concentration of each growth factor and biochemical is consistent however in this study the chemically defined media could not support the primary hBMSC expansion, probably due to deficiency of essential bioactive factors.

Currently, there is limited consensus and conflicting information regarding the effects of donor variability and cell processing on adult mesenchymal stem cell function. Some previous researchers have revealed no age-related differences in differentiation using human BMSCs (Leskelä et al., 2003). However, several studies have seen changes in proliferation, attachment, senescence or self-renewal in hBMSCs although they have not seen effects on differentiation (Mareschi et al., 2006). Here there was a clear difference between donors and cell passage number in their ability to attach during 24 hours. For example, cells from donor one and passage one when cultured in XF2 media attached significantly better than others donors and passages cultured in the same and different media. Proliferation rates seemed to also differ between donors for example the highest cell number was achieved in XF2 basal culture medium and was the most different from other media at day 4. The cell metabolic activity was lower at higher cell passage numbers, whereas the human serum (XF2) showed a sustainable increase in cell metabolic activity compared with other media. However, our findings are disagreement with Kyllönen et al., (2013) who demonstrated that the proliferation of cell cultured in a basal medium of 10% (v/v) FBS was marginally higher than xeno-free media.

In my present study, I used the synthetic, glucocorticoid steroid hormone dexamethasone as the main biochemical to induce osteogenesis (Delaine-Smith and Reilly, 2011). it is clinically employed as anti-inflammatory pharmaceutical but long-term use of

dexamethasone or any types of steroid may give rise to many severe diseases such as hypertension, cushing's syndrome, osteoporosis, and diabetes mellitus type two (Yuasa et al., 2015). Dexamethasone is commonly used for MSC differentiation toward the osteogenic lineage (Oshina et al., 2007). Although dexamethasone stimulates osteogenesis, it can inhibit cell proliferation. The results revealed that a low concentration of dexamethasone supported hBMSCs proliferation better than a high concentration, which was particularly notable in XF2. Although dexamethasone has been used to promote osteogenesis, it was shown to have an adverse effect on cell metabolic activity when used at a high dose, hBMSCs cultivated in low dexamethasone concentration conserved a higher proliferation potential (Xiao *et al.,* 2010). In this study, the high concentration of dexamethasone of 100 nM inhibited growth of hBMSCs measured at day 4 and 7. In contrast, our data indicated that hBMSCs cultured without dexamethasone maintained their proliferation status better than cultivated with dexamethasone. It is noteworthy that Wang *et al.*, (2012) mentioned that apoptosis levels correlated with dexamethasone concentration. Their data demonstrated that a high concentration of dexamethasone (10^{-8}, 10^{-7} and 10^{-6} mol/L) in xeno-free media induced apoptosis of UC-MSC, while apoptosis was protected when a lower concentration of dexamethasone (10^{-10} and 10^{-9} mol/L) was used. Seong et al., (2010) observed that both concentrations have been used by researchers and reported no difference in the effects on cells whereas Alm and colleagues (2012) noted 100 nM dexamethasone treatment for 1 week reduces intra- and inter-donor variation during osteogenesis. Walsh et al., (2001) stated that the optimal concentration of dexamethasone to achieve proper mineralisation of bone nodules is similar to the physiological concentration of glucocorticoids (10 nM).

AA is an enzymatic cofactor for the hydroxylation of lysine and proline in pro-collagen (Vater et al., 2011). In the absence of ascorbic acid, proline hydroxylation processing does not occur which leads to improper helical structure of the collagen chain (Langenbach and Handschel, 2013). βGP is considered as a source of inorganic phosphate for bone-like hydroxyapatite mineral synthesis and facilitates osteogenic induction (Chang *et al.*, 2000; Langenbach and Handschel, 2013). In routine culture of MSCs for bone formation cells are subjected to the combination of Dex, AA and βGP and this termed 'osteogenic media'. Therefore, in this study I examined the effects of these supplements separately and combined. The metabolic activity in most of the experiments described

for cells cultured in XF2 was higher. AA and βGP support high proliferation of hBMSCs, more importantly, the concentration of both AA-2-Ph and βGP may promote proliferation without a reciprocal reduction of differentiation potential indicating that MSC proliferation or differentiation seems to depend on the concentration of ascorbic acid. Choi and co-workers (2008) reported that the proliferation and cell differentiation depends on the AA concentration, the absence of cell density reduction is consistent with metabolic activity, and the toxicity recognised with 5-250 μM concentrations of AA. The results here showed that the combination of human-derived ingredients and (AA 50 μM and βGP 10 mM) components indicated to accelerate the hBMSCs growth over the all groups. Where Dex, AA, and βGP are combined and added to the media this is termed 'osteogenic media' while AA and βGP are combined and added to the media, this is termed 'non-osteogenic media' throughout this thesis. The metabolic potency of cells in osteogenic media containing dexamethasone at 10 nM was approximately the same as results of non-osteogenic media on 4 and 7-day.

As well as assessing cell number by resazurin assay of metabolic activity, I used a DNA assay to assess cell number at a specific end-point. The advantages of the DNA assay are that the amount of DNA per cell for a given cell type should be constant and it would not be affected by factors that may affect metabolic rate within cell culture. However, the DNA assay does not reflect the health of the cells and would measure dead cells if they are still present in the culture. Therefore, it is helpful to include both assays to get an overall picture of the cell number in a culture situation. In these experiments, some cell culture wells did not contain sufficient measurable levels of DNA and were below the detection limit of my assay therefore these are not included in the data reported. All donors and passages cells cultured in XF2 basal culture medium with or without supplements contained more DNA and therefore more cells then when cells from the same donors and passages were cultured other media. Most significantly, for the passages 1, 3, 4 and 1, 2 cells supplemented with Dex at 10 and 100 nM respectively with all donors. The DNA results verified the metabolic activity data showing that XF2 media supported higher cell number. In osteogenic media, the cellular DNA quantity was also affected by Dex concentration. The results showed that decreased DNA content is consistent with the increase of Dex concentration. It is known that Dex inhibits hBMSCs proliferation and increases osteogenic differentiation. Overall, results may provide strong evidence that

xeno-free media and particularly XF2 can augment the quantity of DNA. Furthermore, XF1 did not support good cell growth in terms of total DNA in a donor-4.

The results of resazurin metabolic activity tests and DNA assay indicated that XF2 was having a strong effect on proliferation, however neither of these assays directly measure cell proliferation. Therefore, to investigate this hypothesis I used the EdU test on selected samples. EdU is a nucleoside thymidine analogue that incorporates into the DNA during functional DNA synthesis. Cell culture in human-derived media, particularly XF2 showed two out of three donors underwent faster cell proliferation. In donor 3 this effects were only observed using XF2 which induced higher cell proliferation than 10% (v/v) FBS. As described by Oikonomopoulos *et al.,* (2015), who used 10% (v/v) HPL, media can increase the cell proliferation of hBMSCs as measured by BrdU incorporation rate. Interestingly, my results are in agreement with this data although XF2 culture medium showed an even better ability to induce proliferation compared to 5% (v/v) HPL. Therefore, this study agrees with Oikonomopoulos *et al.,* (2015) that the xeno-free media is a promising substitute for animal-derived media, reducing the number hBMSCs needed and the manufacturing time.

3.12 Conclusions

My results indicate that media containing human serum can have a significant impact on hBMSCs behaviours, such as proliferation, expansion, and cell metabolic activity. Metabolic activity of hBMSCs was weakest in stem x vivoTM xeno-free human MSC expansion media (XF1). However, human mesenchymal-XF expansion medium (XF2) was able to enhance metabolic activity and support proliferation of hBMSCs strongly compared to the standard media conditions commonly used in the media of 10% (v/v) FBS. 5% (v/v) human platelet lysate also supported hBMSCs proliferation with increased the amount of DNA although not well as XF2.

Importantly, both 5% (v/v) HPL and XF2 are a therapeutically meaningful pre-clinic media and a safe substitute for FBS proliferation, expansion. I further identify that XF2 may be a trustworthy medium in which to grow human mesenchymal stem cells for clinical bone repair such as repairing of the bone defect in cleft palate.

3.13 Summary of results

A significant factor in the success of tissue engineering is the cell source and culture medium chosen, which should enhance the cell growth potential. This chapter aimed to evaluate suitable xeno-free culture conditions for both hBMSCs and HJPs for increasing the proliferation and encouraging a new DNA synthesis with maintenance of cells potency to undergo osteogenic differentiation, with the long-term aim of establishing xeno-free cultures for clinical applications of cleft palate repair. From these studies, I established that;

- A suitable commercially available xeno-free media for proliferation compared to 10% (v/v) FBS is human mesenchymal-XF expansion medium (XF2) which contains 2% (v/v) human serum then basal media containing 5% (v/v) human platelet lysate. Thus, these were used as an alternative media compared to 10% (v/v) FBS.
- XF2 augmented the metabolic activity and enhanced the DNA quantity of hBMSCs compared to any other culture condition tested here.
- XF2 may be a useful xeno-free pre-clinical medium for the expansion and proliferation of hBMSCs towards therapeutically bone differentiation for tissue engineering applications.
- It was confirmed that suitable concentration of Dex for hBMSCs is 10 nM that induces the highest cell maintenance, while the 100 nM of Dex concentration causes a reduction in cell number.
- A low concentration of animal serum supplemented with ITS did not well support the cell proliferation. Therefore, I discontinued this for further experiments.
- Donor variability, media composition and supplements all contributed a vital role in determining cell proliferation, preservation cell potency and DNA synthesis.
- There is lower metabolic activity with increased passage number.

CHAPTER FOUR: EVALUATION OF THE ABILITY OF ANIMAL-FREE COMPONENT CULTURE MEDIA TO SUPPORT DIFFERENTIATION OF OSTEOGENIC PRECURSOR CELLS

4.1 Introduction

Osteogenic differentiation medium is treated with ascorbic acid-2-phosphate (AA), β-glycerophosphate (βGP), and dexamethasone (Dex) (required for osteogenic differentiation). (AA) is essential for producing stable collagen that is a substantial component of the extracellular matrix (ECM) of bone. (βGP) is essential for providing an inorganic phosphate during mineralisation of ECM by differentiated cells. Dex is a synthetic glucocorticoid drug that can stimulate the osteogenic differentiation of hBMSCs (Coelho and Fernandes, 2000). Recently, cell therapy and tissue-engineering application have been performed in dental regenerative surgery to repair the soft tissue damage and skeletal defects to considerable attaint outcomes.

HJPs have been identified as auspicious provenance cells of osteogenesis (Kawase et al., 2009). The remarkable characteristics of HJPs are described: (1) deposition of ECM (Uematsu et al., 2013). (2) Support the osteogenesis through the significant achievement of osteogenic cell differentiation. (3) Promising origin of osteoinduction. (4) significant sources of growth factors that control the metabolism of bone (Kawase et al., 2009). (5) HJPs can be still functionally active in the ageing donor over hBMSCs (Kawase et al., 2014). The isolation of HJP is achieved from an aseptically dissection of a healthy buccal region of the different part of the oral cavity, particularly during the surgical removal of third molars and retromolar area of the mandible. Then the cells collections are grown in 10% (v/v) FBS.

The purposes of work in this chapter were to find out whether the various media examined in chapter 3 can support differentiation of hBMSCs and HJP cells and to obtain preliminary data about the induce of osteogenesis in HJPs. All experiments were performed using the experimental set up described in chapter 3, in standard 2D monolayer. The long-term aim is to establish xeno-free culture protocols capable of supporting bone matrix production and angiogenesis for future clinical applications in craniofacial repair. In order to do this, I set out the following objectives;

1. To determine whether xeno-free media that have been predominantly advertised as promoting cell expansion are suitable for the cell types, hBMSCs and HJPs, and also affect osteogenic differentiation with and without the commonly used osteogenic supplement combinations Dex, AA and βGP.
2. To elucidate whether animal component free media can support extracellular matrix production of osteogenic cells by measuring collagen and calcium production.
3. To determine whether HJPs may be an alternative cell source to bone marrow MScs by assessing their differentiation potential under the same conditions.

4.2 The effects of two different xeno-free basal culture medium on ALP assay of different donors

ALP activity was measured to indicate whether cells were progressing along the osteogenic differentiation pathway. This was undertaken using four adult donors (Chapter 2, Table 2.2.3) on day 10. These experiments were performed to compare the osteogenic potential of XF1 and XF2 compared to standard 10% (v/v) FBS supplemented media.

Although animal-derived serum supplements are commonly used for osteogenic differentiation in *in vitro*, lower ALP activity was seen in 10% (v/v) FBS compared with XF2, even when no osteogenic supplements were added. Three out of four donors cultured in XF2 had higher ALP activity in XF2 versus 10% (v/v) FBS and for donors 1 and 3 this was statistically significant. Although ALP activity in cells from donors 1 and 3 was higher than for donor 2, cells from donor 2 also exhibited significantly higher ALP activity in XF2 compared to other basal culture media. Cells cultured in 10% (v/v) FBS and XF1 had low ALP activity, and there was no significant difference between them ($P < 0.05$) (Figure 4.2).

Overall, the human-derived serum growth supplements (XF2) appeared to stimulate osteogenic differentiation over the animal-derived serum and the other human-derived plasma media formulation (XF2) on day 10.

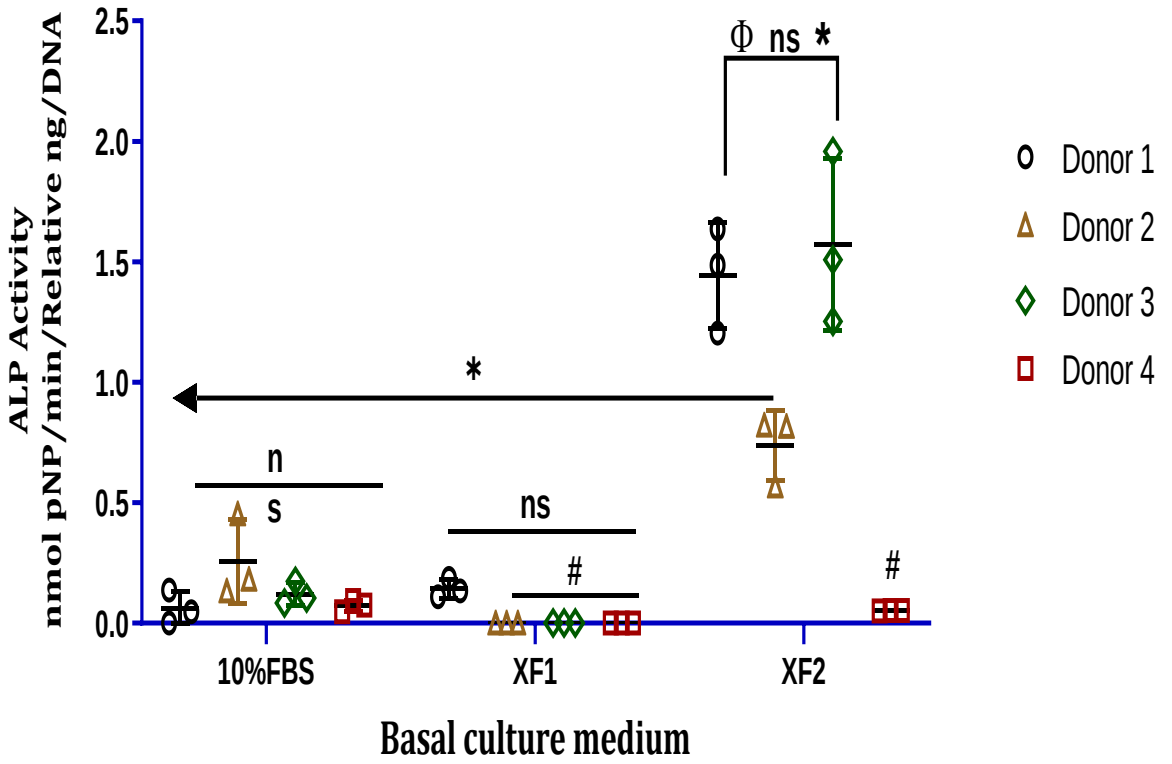


Figure 4.2: *Normalized ALP activity of hBMSCs from different adult donors cultured with two commercial human-derived basal culture media compared with 10% (v/v) animal-serum concentration on monolayer on day 10. Each point represents a single well and the horizontal bar indicates Mean±SD for each donor in that condition. (*) Higher than the other donors in the same medium. (#) Significantly lower. (Φ) Significantly higher than all the other conditions. (ns) There is no significant difference (P <0.05). n=3. Two-way ANOVA, Tukey's multiple comparison tests.*

4.3 The effects of dexamethasone alone on osteoinduction of hBMSCs

The culture media was treated with two dose dependent concentrations of Dex 10 and 100 nM to test whether there is an osteogenic effect of Dex alone on the ALP production in hBMSCs.

Within the groups treated with only Dex at 10 nM the differences in ALP due to the different media compositions was similar to that in Basal Media. In human-derived serum supplementation (XF2), three out of four donors had higher ALP activity. Although the donor-3 presented statistically higher ALP compared to the other donors cultured in XF2 and other media, it was not significantly different from donor-2 ($P < 0.05$) (Figure 4.3).

Because it was not known whether the different media compositions would lead to the need for a different concentration of Dex all ALP experiments were also performed with 100nM Dex. Within the groups treated with 100nM Dex three out of four donors cultured in 10% (v/v) FBS and XF2 had upgraded ALP activity. While two out of four cultured in XF1 showed elevated ALP activity. Cells from donor-2 had higher ALP activity compared to the other donors in 10% (v/v) FBS, and cells from donor-2 cultured in XF2 had 1.5-fold and 4-fold ALP compared with 10% (v/v) FBS and XF1 respectively. All donors cultured in 10% (v/v) FBS and XF1 exhibited no significant effects of supplement or differences between each other. The amount of ALP secretion in donor-1 cultured in XF2 showed 4.5-fold and 1.5-fold increase compared with 10% (v/v) FBS and XF1 respectively. Whereas donor-3 cells cultured in XF2 increased ALP activity around 4.5-fold and more compared with 10% (v/v) FBS and XF1 respectively (Figure 4.3).

Overall, although Dex had a small effect on the promotion of ALP activity in hBMSCs compared to basal media without supplements, there was no significant difference. Indicating that Dex alone is not sufficient to induce differentiation.

Table 4.3: The effect of dose dependent dexamethasone (containing Dex 10 nM and 100 nM) on normalised ALP activity within the media groups examined. “Higher” indicates Dex caused significantly higher ALP activity when comparing data in figure 4.2. “Lower” indicates osteogenic-inductive medium caused significantly lower ALP activity and “NS” indicated no significant difference between Dex compared with BCM within this group.

Media-Donors	**Donor 1**		**Donor 2**		**Donor 3**		**Donor 4**	
Dex (nM)	10	100	10	100	10	100	10	100
10% (v/v)FBS	NS	NS	NS	NS	NS	Higher	Higher	Lower
XF1	Higher	NS	NS	Higher	NS	NS	NS	NS
XF2	NS	Lower	NS	Higher	Higher	Higher	NS	Lower

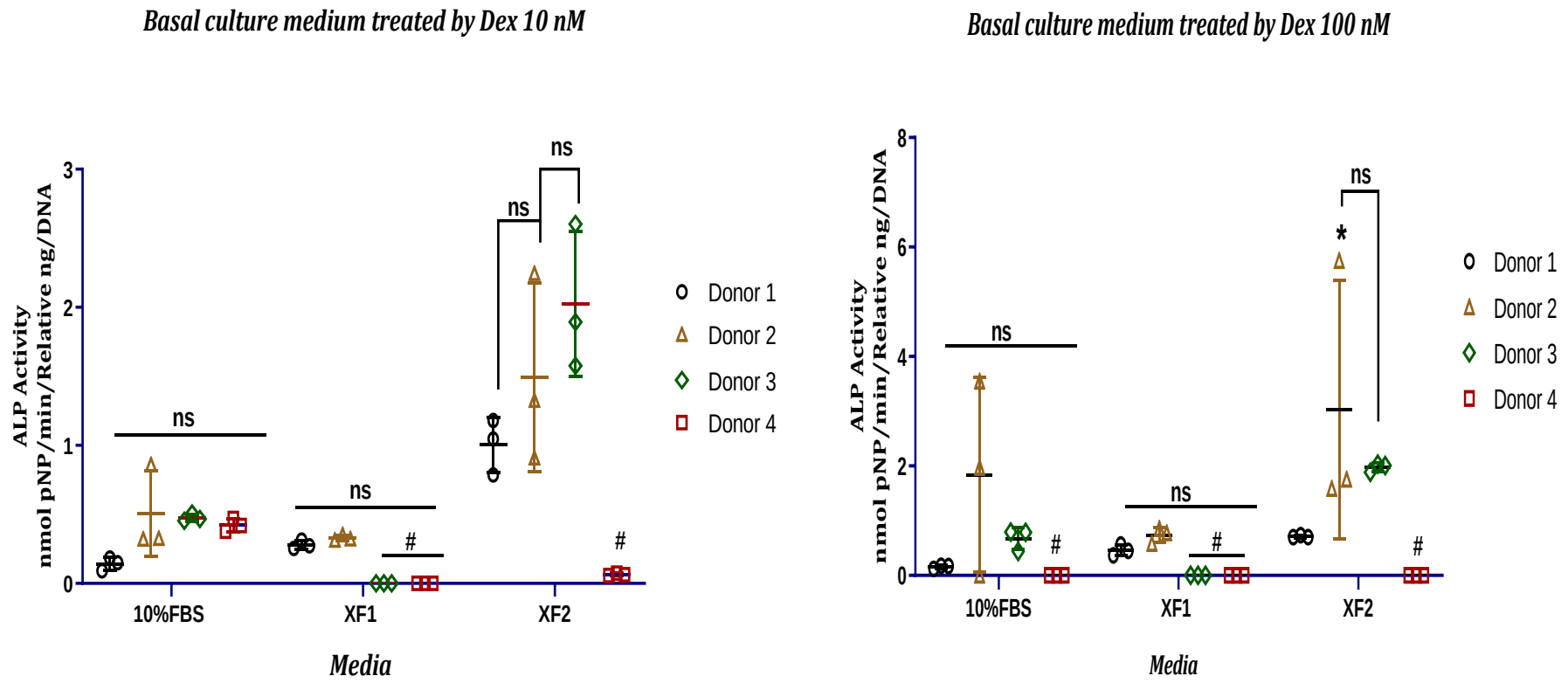


Figure 4.3: *Normalized ALP activity of hBMSCs from different adult donors cultured on two commercial human-derived basal culture media compared with 10% (v/v) animal-serum concentration were treated with Dex 10 and 100 nM on monolayer on day 10. The scatter dot plot manifests mean and standard deviation for each condition. (#) Significantly lower. (ns) There is no significant difference (P < 0.05). n=3. Two-way ANOVA, Tukey's multiple comparison tests*

4.4 The effects of two different xeno-free basal culture medium supplemented with AA 50 µM, and βGP 10 mM on osteoinduction of different donors

The culture media was treated with AA 50 µM and βGP 10 mM alone, this was to test whether dexamethasone supplementation was necessary to support osteogenic differentiation in the xeno free media. AA 50 µM and βGP 10 mM support bone matrix formation but they are not expected to stimulate ALP at day 10. As seen in the other conditions three out of four donors cultured in the human-derived serum supplements had higher ALP activity compared to other media with the same supplements. Donor-2 cultured in XF1 had significantly higher 7-fold ALP activity than cells from the other donors that was not seen in the basal conditions. All cells cultured in animal-derived serum supplementations had a little ALP activity, and there was no significant difference between them. The highest ALP activity was seen in cells from donor-2 cultured in XF2 that was significantly different from all other donors that were cultured in both the same and different culture media, it had about 18.5-fold increase of ALP activity over the 10% (v/v) FBS and approximately 3-fold compared with XF1. Both cells from donor1 and donor-3 cultured in XF2 also exhibited a high ALP activity, about 13-fold compared with these cells cultured in XF1 and 10% (v/v) FBS ($P<0.05$) (Figure 4.4).

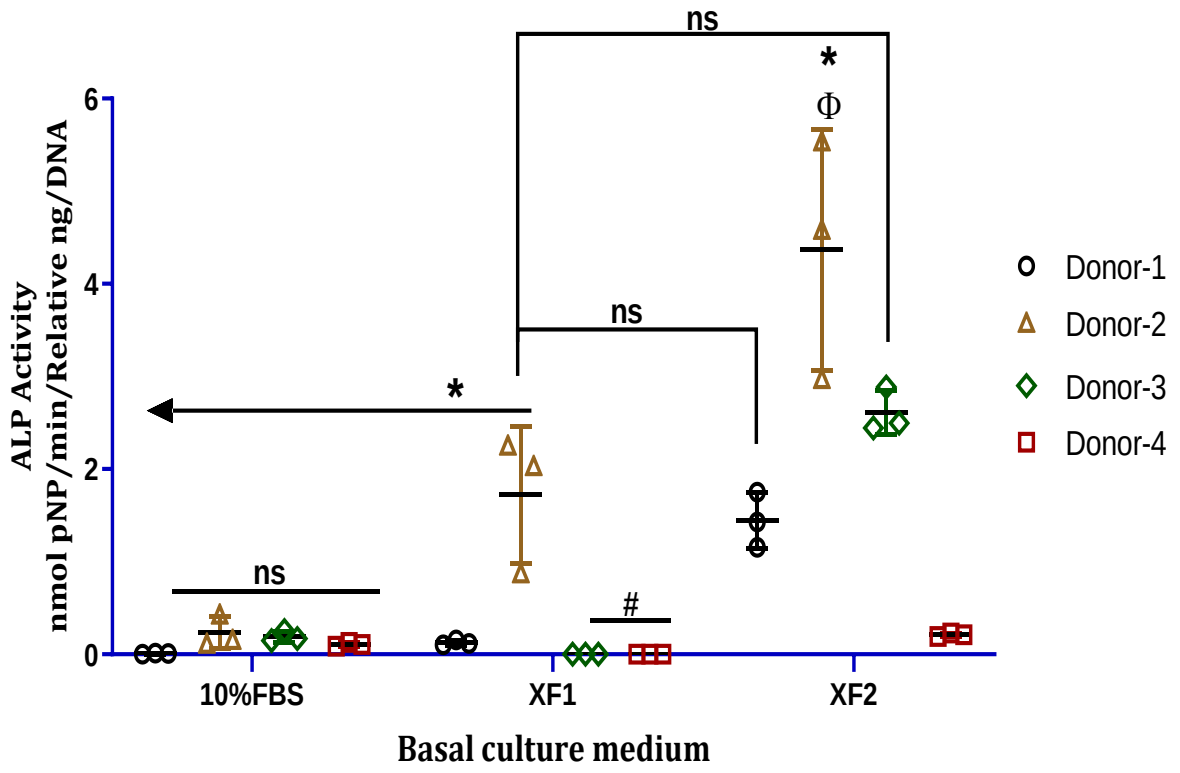


Figure 4.4: *Normalized ALP activity of hBMSCs from different adult donors cultured on two commercial human-derived basal culture media compared with 10% (v/v) animal-serum concentration were treated by AA 50 µM and βGP 10 mM on monolayer on day 10. The scatter dot plot manifests mean and standard deviation for each condition. (*) Higher than the other donors in the same medium. (#) Significantly lower. (Φ) Significantly higher than all the other conditions. (ns) There is no significant difference (P <0.05). n=3. Two-way ANOVA, Tukey's multiple comparison tests.*

4.5 Effect of full complement of osteogenic-inductive substances on ALP activity of hBMSCs on 2D monolayer

The most standard mixture of osteogenic supplements used for hBMSCs is Dex, AA, and βGP, therefore the three media were supplemented with this combination after 24 hours of culture when the cells were entirely attached on the well plate. ALP was measured at day 10.

Osteogenic-inductive substances with Dex 10 nM, as expected ALP activity increased overall in osteogenic media Three out of four donors cultured in the human-derived serum supplements (XF2) had higher ALP activity than in other media. Additionally, donor-2 cultured in XF1 had higher ALP activity compared to in 10% (v/v) FBS and significantly higher than the other donors which were cultured in the same medium. The ALP activity increased about 12-fold compared with the same donor cultured in animal-derived serum supplementations. Although there was a nearly 2.5-fold increase of ALP activity compared with the same donor and donor-3 cultured in XF2, there was no significant difference among them. Likewise, the ALP activity rose about 5-fold and experienced a statistically significant difference compared with donor-1 cultured in XF2. Whereas the all donors cultured in animal-derived serum supplementations had a little ALP activity, and there was no significant difference among them. Even though the three out of four donors cultured in XF2 had high ALP activity approximately 10-fold over their donors cultured in 10% (v/v) FBS, they had no significant difference among them ($P<0.05$) (Figure 4.5).

To check whether a higher dose of Dex was needed in the osteogenic differentiation media to stimulate ALP 100 nM Dex was also applied to the cells. Donor-2 cultured in all different medium exhibited an upward trend of ALP activity. Besides, cells cultured in XF2 presented a significant increase of ALP compared to cells that all donors were cultured in the same media and other media. Although ALP was nearly 1.25-fold higher than for cells cultured in XF1, there was no significant difference.

Additionally, ALP activity increased 10 times compared to culture in 10% (v/v) FBS and 9 times compared with donor-3 cultured in the XF2. Whereas the donor-3 cultured in XF2 showed the augmentation of ALP activity nearly 3 times and more compared with when was cultured in 10% (v/v) FBS and XF1 respectively. Donor-2 cultured in XF1 had about

8-fold increase of ALP activity and more compared with its donor and with other donors cultured in 10% (v/v) FBS respectively. While there was no significant difference throughout all the donors cell that were cultured in 10% (v/v) FBS ($P<0.05$) (Figure 4.5).

Overall, within the osteogenic supplemented groups the human-derived serum growth supplements (XF2) supported the hBMSCs to osteogenic differentiation better than the animal-derived serum supplements, although there is high door variability in this response.

Table 4.5: The effect of osteogenic media (containing Dex 10 nM and 100 nM) on normalised ALP activity within the media groups examined. "Higher" indicates osteogenic-inductive medium caused significantly higher when comparing data in figure 4.2. "Lower" indicates osteogenic-inductive medium caused significantly lower ALP activity and "NS" indicated no significant difference between two OM compared with BCM within this group.

Media	Donor 1		Donor 2		Donor 3		Donor 4	
	10 nM	100 nM	10 nM	100 nM	10 nM	100 nM	10 nM	100 nM
10% (v/v)FBS	NS	NS	NS	NS	NS	Higher	Higher	NS
XF1	Higher	NS	NS	Higher	NS	NS	NS	NS
XF2	NS	Lower	NS	Higher	Higher	Higher	NS	Lower

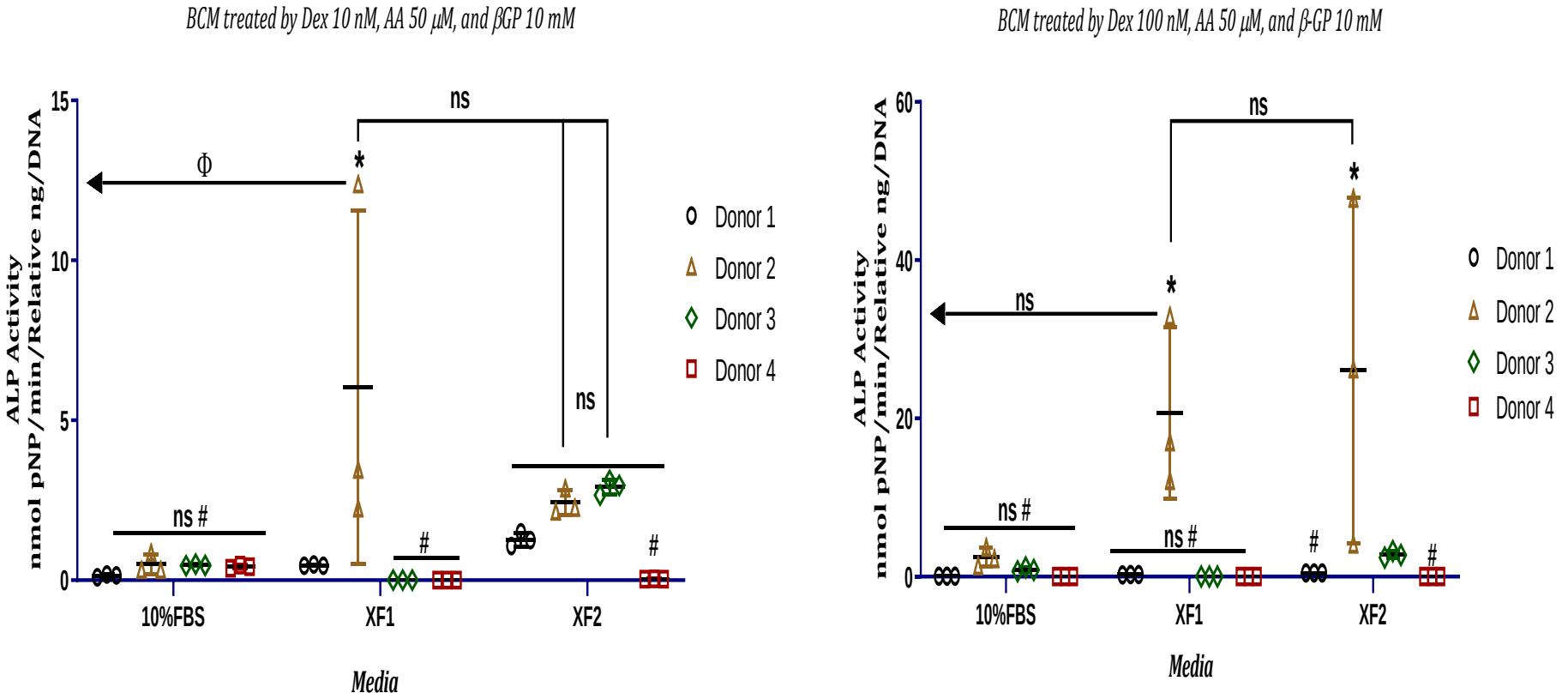


Figure 4.5: *Normalized ALP activity of hBMSCs from different adult donors cultured on two commercial human-derived basal culture media compared with 10% (v/v) animal-serum concentration were treated by Dex 10 and 100 nM, AA 50 µM, and βGP 10 mM on monolayer on day 10. The scatter dot plot manifests mean and standard deviation for each condition. (Φ) Significantly higher than all the other conditions. (*) Higher than the other donors in the same medium. (#) Significantly lower. (ns) There is no significant difference (P <0.05). n=3. Two-way ANOVA, Tukey's multiple comparison tests.*

4.6 Appraisal of extracellular matrix formation

One of the most important aims of the experiments in this chapter was to assess the late stage of osteogenic differentiation of hBMSCs to examine their bone-like matrix production.

I investigated both the calcium formation and collagen deposition for detection of the extracellular matrix formation by cells from the four adult donors with different passages on day 21. Because cells grown in XF1 showed disappointing results in both the proliferation and differentiation assays these experiments were not performed with XF1. However due to its promising effects on cell proliferation shown in chapter 3 selected cells were supplemented with 5% (v/v) HPL to compare this to the other media formulations. 100nM Dex was not used in this set of experiments as it had not shown any beneficial effect on ALP activity and inhibited proliferation for some donor types.

4.6.1 Effect of varying media components and supplements on collagen production of hBMSCs

Although animal-derived serum supplements are commonly used for osteogenic differentiation and collagen formation in *in vitro*, there was lower collagen production compared with cells grown in human-derived growth supplementation particularly in XF2 in basal culture media.

In basal media the amount of collagen production by both donor-1 and donor-3 cells cultured in XF2 and 5% (v/v) HPL was higher, about 3-fold, compared with the same donor cells cultured in 10% (v/v) FBS. Whereas cells from donor-4 deposited about 9-fold and 2.5-fold more collagen in XF2 media compared with in 10% (v/v) FBS and 5% (v/v) HPL respectively (Figure 4.6.1 A).

In culture media treated with Dex 10 nM alone all donors cultured in the human-derived growth supplementations had higher collagen production compared with animal-derived serum supplements. However, they were no significant difference between them. Although donor-4 cells produced less collagen than all others donor cells cultured in human-derived growth media, they still produced statistically more collagen compared with any donor cells cultured in 10% (v/v) FBS. Whereas the donors cultured in 10%

(v/v) FBS had lower collagen formation, and there was no significant difference among them ($P<0.05$) (Figure 4.6.1 B).

When the culture media was treated with AA 50 µM and βGP 10 mM donor-4 cells again exhibited lower collagen formation, however when these were cultured in XF2 the collagen formation was improved about 3-fold compared to the same donor cultured in either 5% (v/v) HPL or 10% (v/v) FBS. Moreover, cells from both donor-1 and donor-3 cultured in all different media produced high amount of collagen and this was significantly higher than for donor-4. Furthermore, cells cultured in 5% (v/v) HPL showed significantly higher collagen production compared with all other donors cultured in different media ($P<0.05$) (Figure 4.6.1 C).

The culture media was treated with the osteogenic supplementations that are Dex 10 nM, AA 50 µM, and βGP 10 mM. Both donor-1 and donor-3 cultured in the human-derived growth supplements upregulated collagen formation, significantly higher than donor-4 cultured in the same medium, and all donors cultured in animal-derived serum supplementations. Collagen production increased about 4-fold compared with the donors cultured in animal-derived serum supplementations. For cells cultured in 5% (v/v) HPL, collagen formation was about 3-fold higher compared to donor-4 cultured in the same medium. While for cells cultured in XF2 collagen deposition was about 2-fold higher compared to donor-4 cultured in the same medium ($P<0.05$) (Figure 4.6.1 D).

Overall, the human-derived growth media treated by various supplements supported the hBMSCs to produce collagen and enhance the extracellular matrix over the animal-derived serum supplementation when assayed on day 21. Notably, the effect of donor and varieties of media played a vital role in enhancing the late osteogenic cell differentiation via promoting the cells to form the collagen.

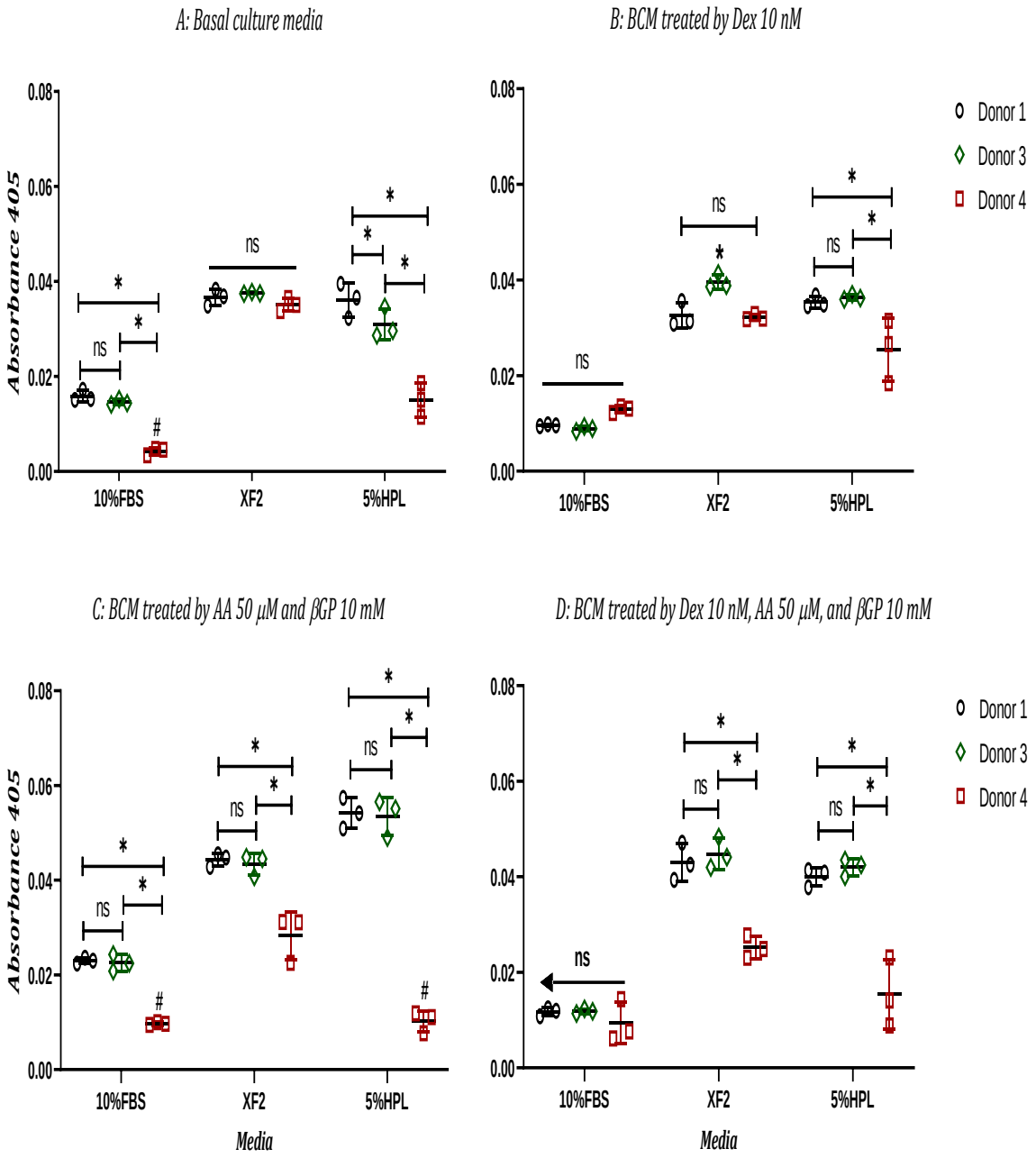


Figure 4.6.1: *Collagen formation by hBMSCs as measured with Sirius Red in day 21, cultured in three different media. The scatter dot plot manifests mean and standard deviation for each condition. (*) Significantly Higher than the other donors in the same medium. (#) Significantly lower. (ns) There is no significant difference (P <0.05). n=3. Two-way ANOVA, Tukey's multiple comparison tests.*

4.6.2 Effect of varying media components and supplements on calcium deposition of hBMSCs

In the basal culture media. Although the animal-derived serum supplements are often used for osteogenic differentiation and calcium deposition in *in vitro*, there had lower calcium deposition compared with cell in human-derived growth supplementation. Thus, two out of three donors cultured in XF2 and 5% (v/v) HPL were statistically higher throughout all donors cultured in 10% (v/v) FBS and donor-4 cultured in both XF2 and 5% (v/v) HPL.

The amount of calcium deposition in donor-1 cultured in XF2 showed an increase of about 2.5-fold and 16-fold compared with two out of three donors and donor-4 cultured in 10% (v/v) FBS. Whereas donor-4 cultured in XF2 increased calcium deposition about 5-fold compared with the same donor cultured in 10% (v/v) FBS and 5% (v/v) HPL. Donor-1 and donor-3 cells cultured in 5% (v/v) HPL both improved the calcium deposition nearly 1.5-fold compared with the same donors cultured in 10% (v/v) FBS. The donors cultured in 10% (v/v) FBS and donor-4 cultured in 5% (v/v) HPL exhibited low calcium deposition, and there was no significant difference between them (*P<0.05*) (Figure 4.6.2 A).

The culture media was treated with Dex 10 nM. Two out of three donors cultured in the human-derived growth supplementations increased the calcium deposition compared with other donors cultured in the same media and animal-derived serum supplements. In addition, they showed an increase of calcium deposition around 3-fold and 6-fold over the same donors and donor-4 cultured in 10% (v/v) FBS respectively. Whereas all the donors cultured in 10% (v/v) FBS had low the calcium deposition, donor-1 displayed a significant difference compared to donor-4, while it was shown no significant difference with donor-3 (P<0.05) (Figure 4.6.2 B).

The culture media was treated with AA 50 μM and βGP 10 mM. Although the donor-4 cultured in different conditions had low calcium deposition, cells cultured in XF2 calcium deposition was about 2.5-fold higher over the same donor cultured in both 5% (v/v) HPL and 10% (v/v) FBS. Moreover, both donor-1 and donor-3 cultured in all different media had high calcium deposition and significantly higher than donor-4. The donor-1 cultured in animal-derived serum supplementations significantly upgraded calcium deposition

nearly 1.5-fold and 11-fold over the donor-3 and donor-4 cultured in the same medium respectively ($P<0.05$) (Figure 4.6.2 C).

The culture media was treated with the osteogenic supplements are Dex 10 nM, AA 50 μM, and βGP 10 mM. Both donor-1 and donor-3 cultured in the human-derived growth supplements had higher calcium deposition and were significantly different compared to the donor-4 cultured in the same medium and all donors cultured in animal-derived serum supplementations. However, there was no significant difference between them. Furthermore, calcium deposition increased about 3-fold compared with the donors cultured in animal-derived serum supplementations. Cells cultured in 5% (v/v) HPL increased the calcium deposition about 7-fold over donor-4 cultured in the same medium ($P<0.05$) (Figure 4.6.2 D).

Overall, the human-derived growth media treated by various supplements supported the hBMSCs to produce the calcium deposition and extracellular matrix compared to the animal-derived serum supplementations particularly when supplemented with AA 50 μM, and βGP 10 mM on day 21.

Table 4.6.2: The effect of osteogenic media on collagen formation and calcium deposition within the media groups examined. "Higher" indicates osteogenic-inductive medium caused significantly higher. "Lower" indicates osteogenic-inductive medium caused significantly lower, and "NS" indicated no significant difference of OM-10 compared with BCM within this group.

Media-Donors	**Donor 1**		**Donor 3**		**Donor 4**	
	Collagen	Calcium	Collagen	Calcium	Collagen	Calcium
10% (v/v) FBS	NS	NS	NS	NS	NS	Higher
XF2	Higher	NS	NS	NS	Lower	Lower
5% (v/v) HPL	NS	NS	NS	Higher	NS	Higher

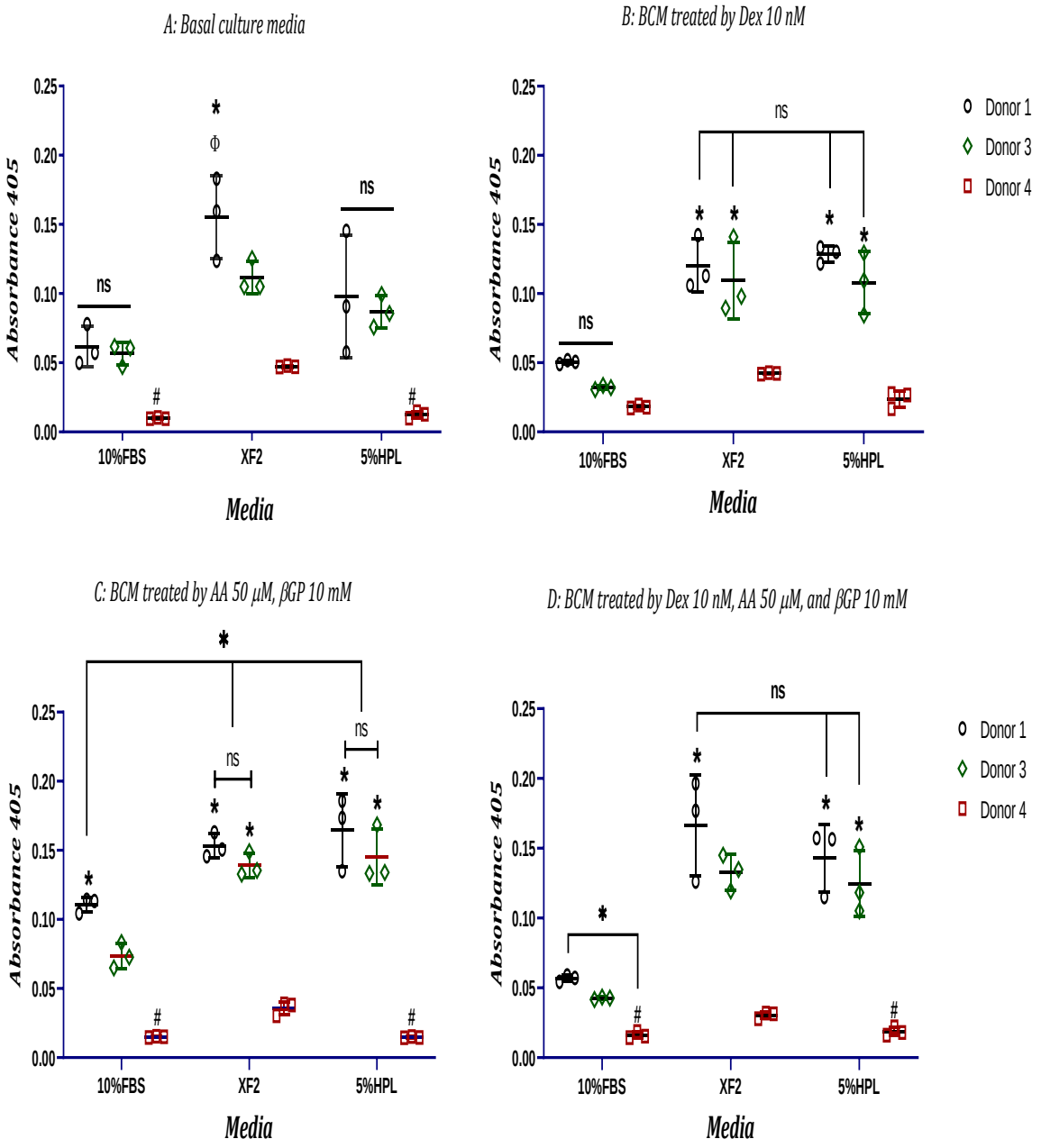


Figure 4.6.2: *The calcium deposition of hBMSCs cultured on an animal-derived serum supplementation, and commercial human-derived basal culture medium measured by alizarin red staining on monolayer on day 21. The scatter dot plot manifests mean and standard deviation for each condition. (Φ) Significantly higher than all the other conditions. (*) Higher than the other donors in the same medium. (#) Significantly lower. (ns) There is no significant difference (P <0.05). n=3. Two-way ANOVA, Tukey's multiple comparison tests.*

4.7 Evaluation of varying supplementation on ALP activity of a monolayer cultured HJPs

For human jaw periosteal cells (HJPs), the amount of ALP activity in BCM-XF1 was a noticeably significantly higher than in other BCM, 29-fold and 5-fold compared with 10% (v/v) FBS and XF2 respectively. Whereas the XF2 increased the ALP activity nearly 5.5-fold over the 10% (v/v) FBS ($P<0.05$) (Figure 4.7), what basal culture media was treated by varying dexamethasone concentrations the XF1 medium again supported the highest ALP activity but only in Dex 10 nM, 11-fold and 4-fold compared with 10% (v/v) FBS and XF2 respectively ($P<0.05$) (Figure 4.7).

In basal culture media treated by AA 50 µM and βGP 10 mM. ALP activity in XF1 also appeared to be higher than for other conditions however, there was no significant difference between them. While the amount of ALP activity in BCM-XF1 appeared significantly higher than XF1 treated by AA and βGP only.

In osteoinductive, media with different dexamethasone supplements ALP was surprisingly lower with no clear effect of media although Dex 100nM supported higher ALP than Dex 10nM. Whereas, all groups supplemented with osteogenic reagents had significantly lower of ALP activity compared with BCM-XF1.

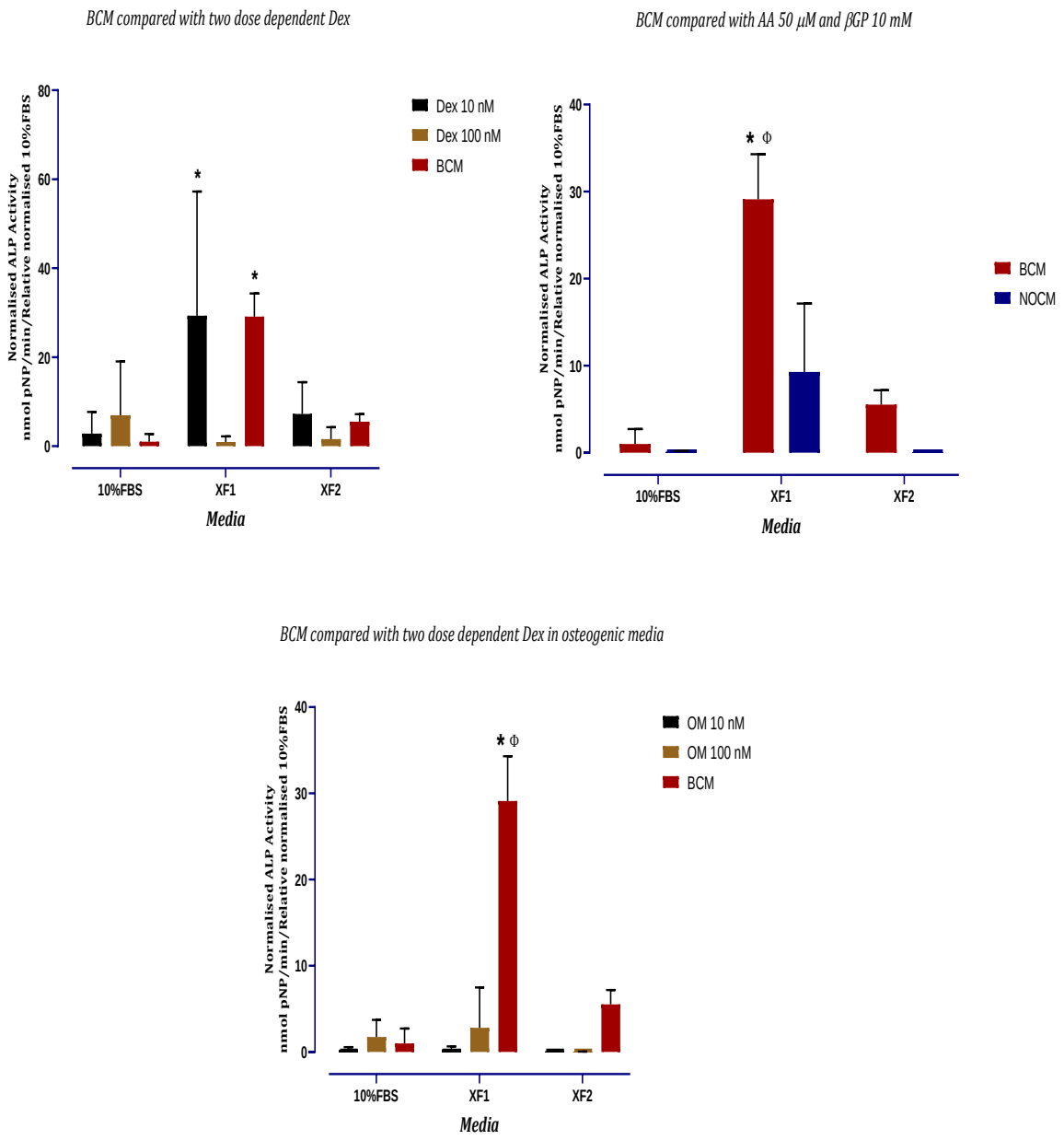


Figure 4.7: *Normalized of ALP activity of HJPs cultured on two commercial human-derived basal culture media compared with 10% (v/v) animal-serum concentration on monolayer on day 10. The bar manifests mean and standard deviation for each condition. (*) Significantly higher than the other media at all-time points. (Φ) Significantly higher than all the other conditions. (ns) There is no significant difference (P <0.05). n=3. ANOVA, Brown-Forsythe test. Moreover, two-way ANOVA, Tukey's multiple comparison tests.*

4.8 Discussion

Bone tissue engineering presents a considerable opportunity for clinical cleft palate repair. The essential ingredients for this strategy to be effective are the osteoinductive cell source and the biochemicals for bone formation. Bone marrow derived cells have demonstrated therapeutic potential for the treatment of cleft palate and other disorders and diseases because they contain a subpopulation of cells called "precursor cells" which have the natural ability to differentiate toward bone formatting cells (Coelho and Fernandes, 2000; Manolagas, 1995) and also present self-renewal capacity with multi-potency (Solmesky et al., 2010).

The aims of this chapter were to identify whether the various xeno-free culture condition can support osteogenic differentiation of cells including hBMSCs and HJPs. ALP activity and mineralisation tests analysed the capacity of osteogenic induction. In early stages of hBMSCs osteogenic differentiation ALP activity increases, while later stages are marked by the formation of collagen type 1 and calcium phosphate deposition. The peak in ALP activity was clearly seen in three out four donors when cultured in the XF2 basal culture media. Donor-3 cultured in XF2 demonstrated the highest ALP activity over all donors cultured in different basal culture media. Contrary to expectations, the amount of osteogenic differentiation was the lowest in 10% (v/v) FBS basal media as measured by ALP activity. Where supplements were applied separately, dexamethasone had effects on osteogenic induction by the elevated ALP activity either used alone (Fig 4.3) or combined with AA and βGP (Fig. 4.5). The inductive effect of non-osteogenic media was most evident in XF2 cultures and resulted in increased ALP activity, even without osteogenic supplements.

Prior researchers have established the importance of AA-2PH and βGP in the osteogenic differentiation of hBMSCs and osteoblast formation (Choi et al., 2008; Takamizawa et al., 2004; Wang et al., 2012). βGP plays a crucial role in providing phosphate for mineralisation by differentiation of hBMSCs to the osteogenic lineage. However, βGP supplementation can lead to false-positives by enabling chemical (non-cell-controlled) mineralisation via precipitation of calcium phosphate salts, that it means the investigation result improperly indicates the presence of osteogenic differentiation (Langenbach and Handschel, 2013). The concentration of dexamethasone plays a crucial role to induce the initial osteogenesis. However, over the long term Dex in high

concentrations has shown to suppress the osteogenic lineage (Wang et al., 2012) whereas low concentrations of Dex seem to be necessary for the efficient osteoinduction of MSCs (Atmani et al., 2002; Wang et al., 2012) this may be related to its suppression of collagen formation in many studies. In this study, variation between individuals was notably observed in ALP activity. Jaiswal and his colleagues (1997) detected that the level and peak time of ALP varied remarkably between different donors regardless of donor age. Similar results have been observed with human adipose stem cells (Bieback et al., 2012; Zuk et al., 2011). The microenvironment of culture medium compositions may significantly affect the results, the heterogeneity of the sample, the variation in the composition during aspiration and committed progenitor's population (Siddappa et al., 2007). The other possibility of interpretation is that differential procedure, and various physicians collecting the bone many may result in different heterogeneity (Phinney et al., 1999a).

In the present study, in my initial tests it became clear that the constituents of XF1 were insufficient to differentiate the hBMSCs towards the osteogenic lineage. Therefore, I used 5% (v/v) HPL as an alternative to XF1 basal culture medium. Higher calcium deposition and collagen formation were detected in human-derived media alone with and without supplementation compared to animal serum components. Notably, the results showed that the cells from two out of three individual donors cultured in human-derived media exhibited an elevation in *in vitro* mineralisation and more ECM compared with 10% (v/v) FBS. Donor 4 cells cultured in 5% (v/v) HPL had weak ECM formation, while they produced even less ECM when cultured in XF2. Human-derived growth media treated by various supplements enabled the hBMSCs to form collagen and deposit calcium and enhance extracellular matrix formation compared to animal-derived serum supplementation, which was measurable by day 21. However, in the same donor, there was no significant effect of the supplementations for augmenting the calcium deposition and collagen formation. The variability between donors show that there may be differences due to the variety of physiological behaviours and clinical background of the patients (Siddappa et al., 2007).

The components of the XF2 media have not been disclosed by the company but it contains 2% (v/v) human serum in which there is likely to be extracellular matrix elements including vitronectin, hyaluronic acid, fibronectin, and other components, which permit

cell attachment, prevent apoptosis (Uhm et al., 1999) and support mineralisation. 5% (v/v) HPL is also a successful medium for mineralisation and this may be because it contains the alpha granules which are a fundamental source of growth factors.

Periosteal-derived jaw cells have osteogenic potential and can also be considered as a suitable cell source for tissue engineering. In a clinical setting, the principal advantages of using the periosteum for bone tissue engineering are that it is comparatively accessible and easy to collect (Park et al., 2008; Young-Mo Ryu et al., 2011). There are limited previous studies on the use of xeno-free culture formulations for osteogenic differentiation of HJPs. Therefore, I cultured HJPs in two types of xeno-free culture media. The limitation of this study was that accomplished only one experiment in triplicate and only assessed the early-stage marker of osteogenic differentiation, ALP. However, some interesting information was obtained. HJP cell morphology in three different basal culture media was similar with cells exhibiting a small, spindle-shaped and characteristic fibroblast-like morphology, they were flattened. The HJPs attachment was the same in all conditions at 24 hours. As shown for hBMSCs the media XF1 was not able to support the cells over long-term culture. Although a significant difference was observed in XF1 compared to 10% (v/v) FBS basal medium on day 4, there were much lower numbers of cells when cultured in XF1 at day 7 (chapter 3). The cell number of HJPs was higher with Dex 10 nM supplementation compared to the same medium supplemented with 100 nM in which the cells distinctly dropped in numbers by the end of the period. This result indicated that the higher Dex concentrations were toxic to the cells. HJP cells have recently utilised in several researchers as the alternative sources for bone regeneration because they have the osteogenic progenitor cells (Samee et al., 2008). Marolt and his colleagues (2010) and Cicconetti and co-workers (2007) informed that HJPs extracted from the patients' jaws expressed nearly the same surface markers of MSC. While Akintoye and his groups (2006, 2003) mentioned that in their assessment of periosteal cells site-specific markers expression varied, added facts that the development of craniofacial bones differ from the other bones formation in the body (Simonds et al., 2002). The existence of orofacial periosteal surface markers variations is based on the basic anatomy of extraction sites and donors' variableness and is affected by issues such as donor tissue-samples age, health and habit. My studies indicated that HJPs produced higher levels of ALP activity than the hBMSCs indicating that the HJPs may be more

responsive to osteogenic induction than hBMSCs. It will be essential to understand whether HJPs extracted from the mature donor could have a subpopulation of cells that undergo osteogenic differentiation *in vitro* (Arnold I. Caplan, 2005). hMSCs derived from bone marrow have been demonstrated to contain a heterogeneous populations of cells in terms of their osteoinduction potential (Akintoye et al., 2006; Anagnostou et al., 2005). MSCs cell surface marker expression can be different based on the harvested sites; for example the observation that MSCs from craniofacial bone marrow display better osteogenesis and regenerative capacity compared to iliac crest hBMSCs (Akintoye et al., 2006; Osyczka et al., 2009). Orofacial bone marrow comprises a smaller amount of hematopoietic marrow precursors and tissue than iliac bone marrow. Park et al., (2012) performed comparisons of four different cell sources from rabbits; mandible bone marrow and periosteum, tibia bone marrow and periosteum. They found that these cells all had potential to be stem cells *in vitro*. Their study indicated that periosteum from the mandible exhibited the highest osteoblastic lineage differentiation.

Chen and co-workers (2011) analysed the osteogenesis induction between human MSCs extracted from both periosteal and bone marrow harvested from at the same donors and site, they saw that the periosteal MSCs had expressed more osteogenic genes such as BMP-2, OPN and OCN with high mineralization potential compared to hBMSCs. HJPs might be implemented as an alternative of autologous cells in cleft palate healing because they have capacity for promoting osteogenically induction *in vitro* which is an important step for pre-clinical application. Accordingly, it would be interesting in future studies to investigate the osteogenesis of HJPs from adult and child donors to compare their ability to act as a cell source for tissue engineering.

4.9 Conclusions

My results indicate that human serum has a significant impact on hBMSCs osteogenic potency. Osteogenic differentiation of hBMSCs was weakest in stem x vivo™ xeno-free human MSC expansion media (XF1). However, human mesenchymal-XF expansion medium (XF2) was able to enhance and support osteogenic differentiation of hBMSCs more strongly compared to the standard media conditions commonly used in the media of 10% (v/v) FBS. Although in standard FBS conditions osteogenic components are essential for differentiation along the osteogenic lineage, the human mesenchymal-XF expansion medium has measurable effect on osteogenesis even without osteogenic supplements compared to the 10% (v/v) serum medium. 5% (v/v) human platelet lysate also supported hBMSCs differentiation to the osteoblast cells.

Importantly, both 5% (v/v) HPL and XF2 are a therapeutically meaningful pre-clinic medium and a safe substitute for FBS proliferation, expansion, and differentiation of hBMSCs. I further identify that XF2 may be a trustworthy medium in which to grow human mesenchymal stem cells for clinical bone repair such as repairing of the bone defect in cleft palate.

4.10 Summary of results

A significant factor in the success of bone tissue engineering is the cell source and culture medium chosen that should be had osteogenic differentiation. This chapter aimed to assess suitable xeno-free culture conditions for hMSCs and HJPs for osteogenic induction with the long-term aim of establishing xeno-free cultures for clinical applications of cleft palate repair. From these studies, we established that;

- A suitable commercially available xeno-free media for osteogenic differentiation is human mesenchymal-XF expansion medium (XF2) and basal media containing 5% (v/v) human platelet lysate. Thus, these were used as an alternative media compared to 10% (v/v) FBS in osteogenic differentiation.
- XF2 augmented increased the ALP and mineralised ECM construction.
- XF2 may be a useful xeno-free pre-clinical medium for osteoblast-like cells differentiation of hBMSCs towards therapeutically bone differentiation for tissue engineering applications.

- Although osteogenic inductive XF2 medium treated by Dex 10 nM induced osteogenic differentiation of hBMSCs by increasing ALP activity compared to 10% (v/v) serum medium, collagen formation and calcium deposition showed no difference compared to 10% (v/v) serum medium.
- The human-derived growth media (XF2 and 5% (v/v) HPL) supported hBMSCs to synthesis ECM at higher levels than the animal-derived serum supplementations.
- Cell source (donor), media composition and supplements all played a vital role in determining osteogenic cell differentiation and ECM deposition.
- HJPs were also shown to have strong osteogenic potential in human derived culture conditions and in these cells cultured in XF1 could support proliferation and differentiation showing different outcomes to the hBMSCs.

CHAPTER FIVE: GROWTH AND OSTEOGENIC DIFFERENTIATION OF PRIMARY HUMAN BONE MARROW MESENCHYMAL STEM CELLS AND HUMAN JAW PERIOSTEAL STEM CELLS IN 3D POLY (ε -CARPOLACTONE) ELECTROSPUN SCAFFOLDS IN XENO-FREE MEDIA

5.1 Introduction

Tissue engineering is a novel method which may achieve improvements in bone reconstruction for repair of the cleft palate. The three basic strategies of tissue engineering include firstly; selection of a relevant cell source, then; the design of an engineered scaffold and lastly; identification and use of suitable biologic signalling molecules. The selection of an appropriate cell source and use of suitable biologic signalling molecules were discussed in the previous chapters. This chapter will use the conditions identified in the previous chapter to create thin 3D bone-matrix constructs. The aim is to synthesise xeno-free bone-like tissue in electrospun poly (ε-caprolactone) (PCL) scaffolds.

Electrospinning is a simple uncomplicated technique to fabricate fibre's mats in different diameters from nanometres to many micrometres (Zargarian and Haddadi-Asl Haddadi-Asl, 2010). The impact of the physical characteristics of such scaffolds has recently been of considerable interest to the biomaterials community and there has been an evolution of innovative scaffold materials with distinctive features determined by the chemistry of polymer. Meanwhile, the target is engineered bone tissue, ingredients should have the necessary strength, be easy to manufacture, flexible, biodegradable, biocompatible, easy to sterilise, and be able to be handled in the operating room. Such scaffolds should support the attachment and proliferation of osteoprogenitor cells.

In some clinical scenarios the scaffold would be implanted alone with the aim to prompt host cell migration to the injury site and initiate tissue regeneration. Other scaffolds, may be considered as a carrier for cells to achieve an appropriate cell replacement therapy. In recent studies, poly(ε-caprolactone) (PCL) has been assessed as biocompatible and biodegradable for tissue engineering materials. PCL is also approved by the U.S. FDA for medical use. PCL polymer was chosen for this project because it has higher mechanical

strength and a and has a minimise rate of biodegradable compared with natural materials like collagen type I (Hutmacher et al., 2001).

Bone is a natural composite material containing cells and the extracellular matrix that is formed from protein (mostly collagen) and calcium-phosphate based mineral plates (mostly hydroxyapatite). As mentioned in the introduction of chapter one, bone is a dynamic tissue that requires adequate nutrients to maintain its activity. It has mentioned in a few kinds of research that the osteogenic differentiation of human osteoprogenitor cells is improved by xeno-free formulation media (Lindroos et al., 2009). The cells cultured on electrospun PCL scaffolds are bounded by fibres and attach to others in different directions, which could better mimic the in vivo environment. Thence, by culturing cells on human-derived growth supplements in 3D culture, the results may be more comparable to in vivo than monolayer culture, and this may be contemplated as a good strategy for creating a layer of bone.

VEGF is a vasculogenesis growth factor construct that is secreted by numerous cells such as osteoblasts. Vasculogenesis is an essential process for the bone tissue engineering to survive in vivo and to encourage the blood vessels construction to bring oxygen and nutrients to cells within the implanted scaffold.

In chapters 3 and 4 it was demonstrated that human osteoprogenitor cells including; hBMSCs and HJPs cultured in a human-derived xeno-free medium with and without the supplement Dex, AA and βGP, (osteogenic media) exhibited enhanced osteogenesis and mineralisation compared to when grown in 10% (v/v) bovine serum medium. There was an effect of donors and human-derived growth supplements on ALP activity (an early marker of osteogenic differentiation) and mineralisation (calcium deposition and collagen type I formation). Therefore, the studies presented here will further assessed the potential of human-derived growth supplements to stimulate hBMSCs in 3D PCL scaffolds culture to stimulate osteogenic differentiation.

The objective of this chapter were to:

1) Investigate hBMSCs distribution and growth when seeded on the electrospun micro fibre scaffolds of PCL for bone tissue engineering.

2) Enhance osteogenic differentiation of mesenchymal progenitors using human-derived supplements incorporated into the cell culture media.
3) Identify whether there are donor-specific effects on the differentiation potential of cells incorporated into electrospun PCL scaffolds.

5.2 Methods

5.2.1 Fabrication of poly (ε-caprolactone) electrospun scaffold

Six g of polymer pellet of PCL was added into 54 g of DCM (density of DCM = 1.33g/cm^3). The mixture was left in the water bath for less than 1 hour then a mixture of 10% (w/w) of polymer solution was achieved using a magnetic stirrer at room temperature. The solution was then spun employing the technique of electrospinning (see chapter 2). The scaffold morphology and fibre diameter was evaluated from SEM images. Fibre diameter was measured utilising ImageJ-image analysis software. To ensure fibres were selected randomly a grid was designed comprising of intersections spaced at 35 X 35 micron evenly spaced points. Each image was overlaid with this grid (Figure 5.2.1). Then fibres diameter was measured only at the point where the intersection was present over the fibre.

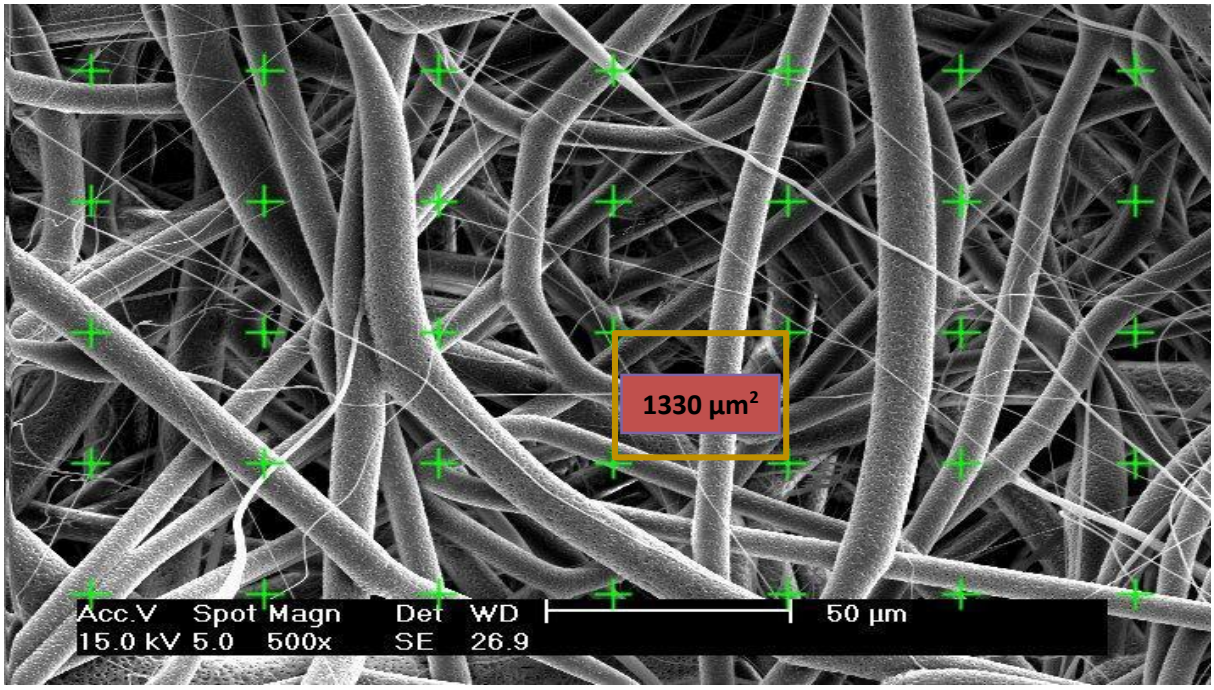


Figure 5.2.1: *Schematic of fibre diameter assessments from SEM images. The grid was overlaid on the images utilising an automated image J function. The elected 35 points under the intersections of the lines had the fibre diameter evaluated. Scale bar = 50 µm*

5.2.2 Optimising cell seeding on 3D PCL scaffolds

The PCL electrospun scaffolds were cut to a diameter of 1.5 cm and a sterile stainless steel ring was adhered using PCL solution (Figure 2.2.9 A). The PCL scaffolds were sterilised with 80% (v/v) ethanol in PBS for 2 hours inside the cell culture hood and washed thrice with PBS to remove the ethanol. Then, the scaffolds were immersed in the respective culture media overnight in the incubator at 37°C, 5% CO_2, in a humidified atmosphere. Two different cell densities (100,000 and 200,000) of hBMSCs were seeded on top of PCL scaffolds using a small volume technique of no more than 100 µL of cell suspension. Scaffolds were then left for 1 hour in an incubator, subsequently 2 mL of the appropriate media was added into the well plate to cover the scaffolds and the plates were left for 24 hours for cell attachment in the cell culture incubator. The cell-cultured scaffolds were transferred to new well plates after 1 day; the resazurin reduction assay for metabolic activity was used to assess the cell seeding efficiency. The quantity of cells, as estimated by metabolic activity, that attached on the original tissue culture wells was compared with the number of viable cells attached to the PCL scaffolds. The quantity of viable cells in the PCL scaffolds was measured by resazurin reduction assay on day 4 and 7, which indicated the efficiency of cell proliferation.

5.2.3 Evaluation of osteogenesis of various donors of hBMSCs cultured in different xeno-free culture media on PCL scaffolds

Circular PCL scaffolds with a diameter of 1.5 cm were cut and cultured on 2 X 10^5 hBMSCs; passage number is 1. Samples were divided into main two groups the basal culture medium (BM) which contained basal α-MEM for all media except XF2, and the BM medium treated by osteogenic supplementations which were 10 nM of Dex, 50 µM of AA, and 10 mM of β-GP (OM_10). The osteogenic differentiation of hBMSCs was measured using PicoGreen fluorescence (dsDNA) and alkaline phosphatase activity as the initial osteogenic differentiation indicator test after day 14, and alizarin red and picro-sirius red staining after 21 days. The scaffold sterilisation and cell seeding proceeded as experiment 5.2.2.

5.3 Results

5.3.1 Fabrication of poly (ε-caprolactone) electrospun scaffold

A smooth fibre morphology of PCL was successfully produced with very little bead formation (Figure 5.3.1 B). The result of fibre diameter measurements of ten scaffolds indicated that 76% of fibres had a diameter less 15 µm, 20% of fibres had diameters between 15 to 30 µm, and only 4% of fibres had a diameter over 30 µm (Figure 5.3.1 A). The average fibre diameter of PCL was 9.76 µm.

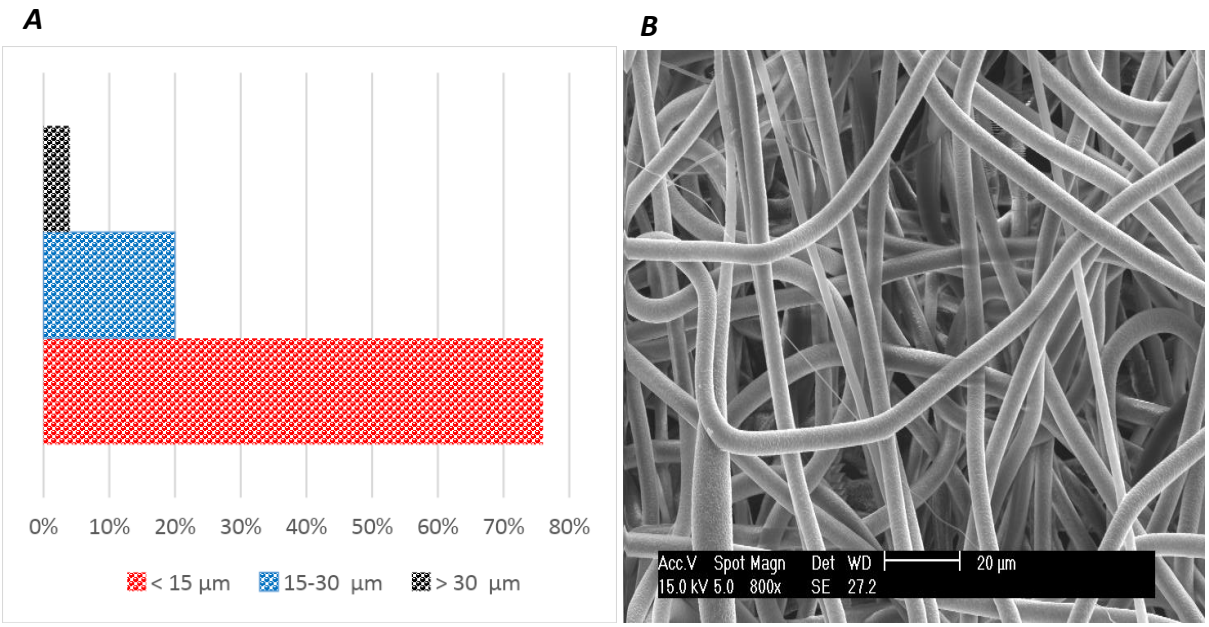


Figure 5.3.1: *10wt% PCL electrospun scaffold characterisation; A: the percentage of fibres at different diameter ranges B: SEM image of electrospun fibre morphology and diameter. The polymer pellets were dissolved in DCM and spun in the 40µL/min flow rate, 17kV, 17cm working distance, 300 rpm (rotating collector). Scale bar = 20µm.*

5.3.2 Optimising cell seeding on 3D PCL scaffolds

Two different cell densities (100,000 and 200,000) were tested for seeding the electrospun PCL scaffolds. Resazurin colour change for the background control (scaffold without cells cultured in αMEM) was measured then removed from the individual values for each scaffold during the time of data collection. After 24 hours, resazurin assay indicated that cell attachment to the scaffold was high for both seeding densities compared with quantity of cells that had come off the scaffold and attached to the underlying well plates. As expected, higher cell numbers were seen for cells seeded at 2 X 10^5. XF2 and 5% (v/v) HPL media supported statistically significantly more viable cells on the scaffolds than 10% (v/v) FBS at the high cell density. While 10% (v/v) FBS supported slightly more cells on the scaffolds than the other conditions when seeded at 1 X 10^5 cells per scaffold (*P<0.05*) (Figure 5.3.2 B).

The amount of cells that did not attach to the PCL scaffolds (attached to the plastic well plates rather than the scaffolds) was higher in XF2 followed by 10% (v/v) FBS at a cell density 200,000. Very few non-attached cells were observed in 5% (v/v) HPL at the same density. At the lower cell density very few viable cells were found not attached to the scaffold in any condition with no significant difference among them (*P<0.05*) (Figure 5.3.2 A).

On day 4, XF2 medium for both cell densities was observed to better support cell growth as seen by approximately 4-fold higher metabolic activity compared to the other groups. Cell metabolic activity was also slightly but significantly higher than for the 100,000 cell density. Although the cell metabolic activity in 5% (v/v) HPL was about 2-fold higher than 10% (v/v) FBS at cell density 100,000, there was no significant difference between them (Fig. 5.3.2 C).

By day 7, cells that had grown in XF2 media exhibited much higher cell metabolic activity, about 8 fold, compared to the other groups and there was no difference between 5% (v/v) HPL and 10% (v/v) FBS (*P<0.05*) (Figure 5.3.2 D).

The cell metabolic activity in XF2 indicated cell proliferation between days 4 and 7 but the cell metabolic activity in both 10% (v/v) FBS and 5% (v/v) HPL showed a gradual decrease this implies that these cell numbers could be not supported in these media.

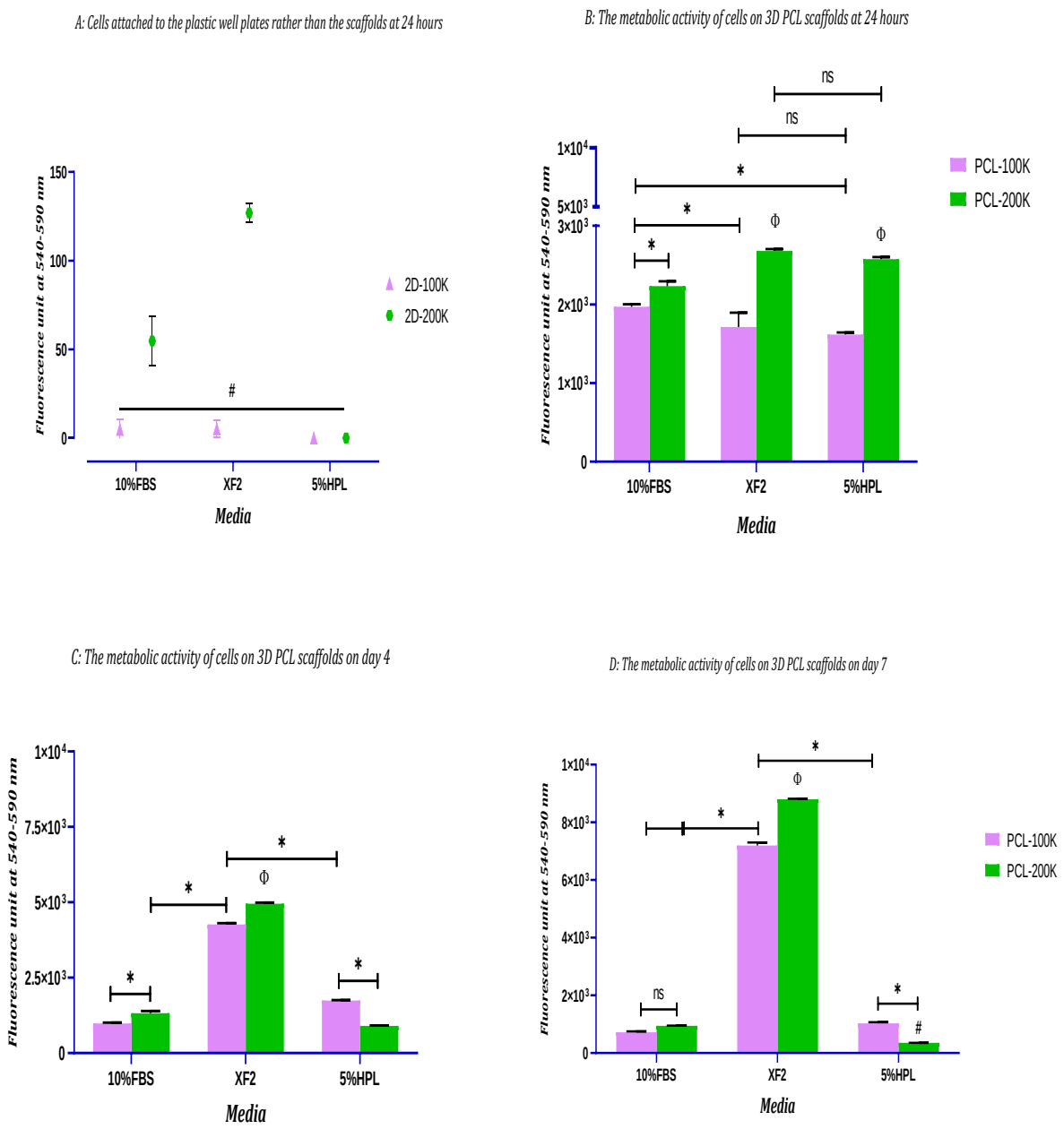


Figure 5.3.2: *The ability of 3D PCL electrospun scaffolds to support cell metabolic activity over 7 days. The metabolic activity of cell was assayed by resazurin reduction test after 1, 4 and 7 days. Bar indicates Mean ± S.D for each condition. (K=1000). (*) Significantly higher than the other media at the same cell density and time point. (Φ) Significant higher than the other*

conditions at that time point. (ns) There is no significant difference (P <0.05). n=3. Two-way ANOVA, Tukey's multiple comparison tests.

5.3.3 Determining the cellular DNA in a different cell density

DNA was analysed quantitatively on day 14, picoGreen fluorescence for the of background control (scaffold without cells cultured in αMEM) was measured, then removed from the individual values. The cells seeded at density of 100,000 in XF2 contained 9-fold and 4-fold the number of cells compared to the 10% (v/v) FBS and 5% (v/v) HPL groups respectively. Furthermore, at the seeding cell density of 200,000 in cultures grown in XF2 there was a statistically higher amount of DNA (indicating cell number) about 13-fold and 21-fold over the 10% (v/v) FBS and 5% (v/v) HPL respectively. There was no higher DNA seen at the higher cell seeding densities in 10% (v/v) FBS and 5% (v/v) HPL (*P <0.05*) (Figure 5.3.3).

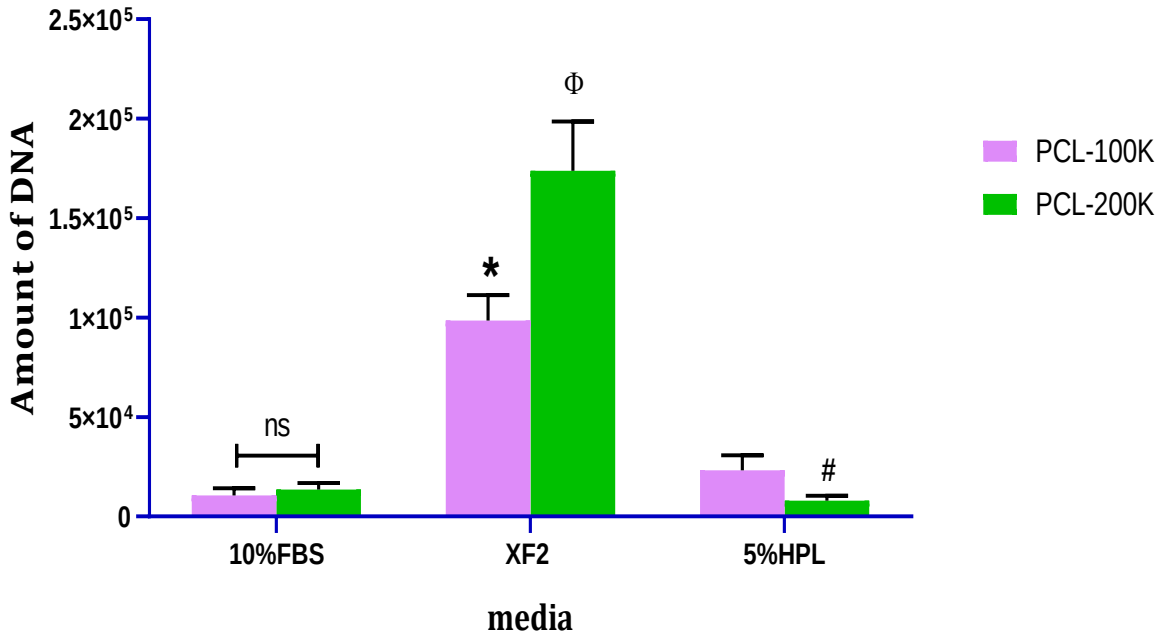


Figure 5.3.3: *DNA quantification of hBMSCs seeded on 3D PCL scaffolds cultured on a different commercial human-derived basal culture medium measured by Quant-iT™ PicoGreen® on monolayer on day 14. Horizontal line indicates Mean ± S.D for each condition, each point is a single scaffold. (*) Significantly higher than the other media at the same cell density. (#) Significantly lower. (Φ) Significantly higher than all the other cultured in different cell density overtime for 14 days. (P <0.05). n=3. Two-way ANOVA, Tukey's multiple comparison tests.*

5.3.4 Evaluation of alkaline phosphatase activity at different cell densities

ALP activity was analysed on day 14 in the basal culture media; the results indicated that despite the absence of dexamethasone the ALP activity in both groups of cell densities was higher in XF2 over the other conditions and statistically higher in the 200,000 cell seeding density compared to 100,000 cell density. ALP is normalised to DNA so the higher ALP activity in the 200K group indicates ALP activity per cell is higher and this is not just a reflection of the higher cell numbers (*P<0.05*) (Figure 5.3.4).

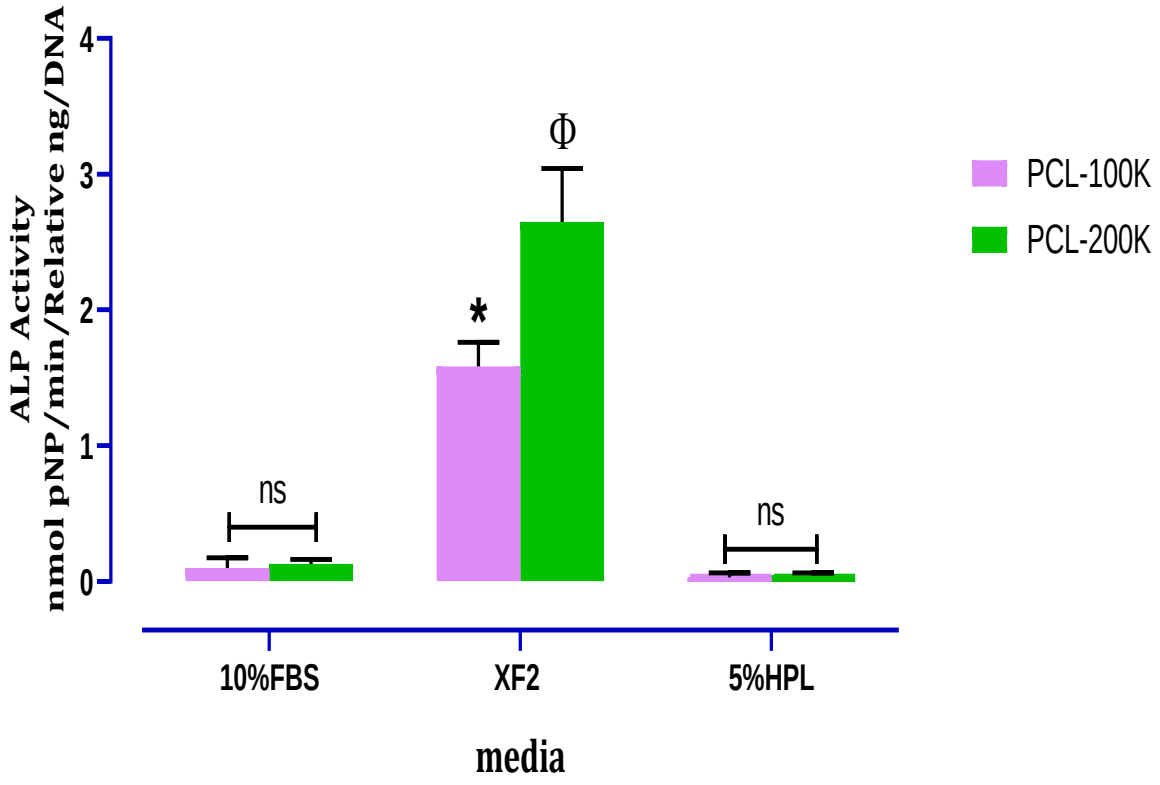


Figure 5.3.4: *The ALP activity of hBMSCs cultured on two commercial human-derived basal culture media compared with 10% (v/v) animal-serum concentration on 3D PCL scaffolds on day 14. Bar indicates mean ± S.D for each condition. (*) Significantly higher than the other media at the same cell density. (Φ) Significantly higher than all the other cultured in different cell density overtime for 14 days. (P <0.05). n=3. Two-way ANOVA, Tukey's multiple comparison tests.*

5.4 Evaluation of primary hBMSCs metabolic activity of cells from different donors cultured in basal media on electrospun PCL scaffolds

To identify whether there are donor-specific effects of the media under investigation in this study cells from different donors at the cell density of 200,000 per scaffold were cultured in the two commercial media containing human-derived components and with 10% (v/v) animal serum on 3D PCL scaffolds. The resazurin reduction test was used to assess the cell attachments on day 1 and the metabolic activity of cells on day 4 and 7.

hBMSCs from both donors 4 and 5 exhibited higher cell attachment on PCL scaffolds particularly in the XF2 culture medium followed by 10% (v/v) FBS culture media. However, the 5% (v/v) HPL supported a lower cell attachment.

Furthermore, rapid increase of cell metabolic activity was seen for cells cultured in XF2 in both donors, particularly between days 1 and 4. While the metabolic activity in 10% (v/v) FBS was slightly reduced over time for donor-4 and marginally increased for donor-5. In the meantime, the cell metabolic activity in 5% (v/v) HPL was increased over the first 4 days, and then appeared to slightly decrease at the end of the period ($P<0.05$) (Figure 5.4).

In donor-7, a sharp increase of cell metabolic activity in both of the media containing human-derived components was observed over the first 4 days of culture, which then levelled. Although cell metabolic activity increased in 10% (v/v) FBS culture media over the first 4 days, it deteriorated between day 4 and day 7 ($P<0.05$) (Figure 5.4).

Overall, metabolic activity of cells from donor-7 cultured on 3D PCL scaffolds was higher than the other donors cultured in the same conditions which implies that the hBMSCs of donor-7 cultured on PCL scaffolds underwent higher cell proliferation.

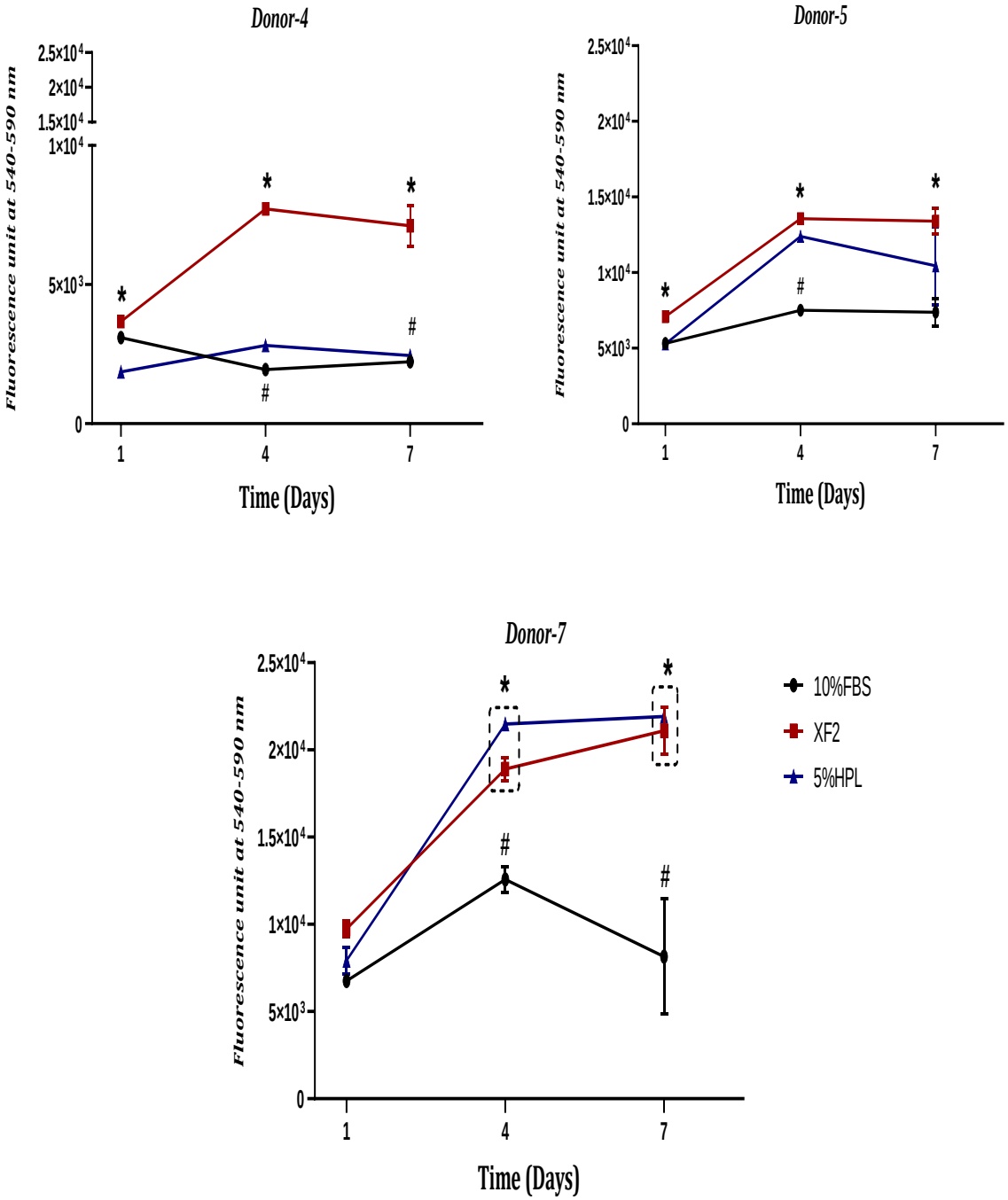


Figure 5.4: *Metabolic activity of three different donors of hBMSCs seeded on 3D PCL scaffolds cultured in two different xeno-free basal media from day 1 to day 7. The superimposed symbols with connecting line indicate mean±SD for each condition. (*) Higher than the other media with cells from the same donor. (#) Significantly lower. (P <0.05). n=3. Two-way ANOVA, Tukey's multiple comparison tests.*

5.5 Effects of osteogenic supplements on cell metabolic activity for different donors

To test whether osteogenic supplements affected cell metabolic activity, media were supplemented with Dex 10 nM, AA 50 µM, and βGP 10 mM. After 24 hours, the cells were expected to be entirely attached on the 3D PCL scaffolds. The resazurin reduction test was used to assess the metabolic activity of cells on day 4 and 7.

hBMSCs of donor 4 cultured in XF2 media exhibited statistically higher cell metabolic activity on PCL scaffolds over time for 4 days, the metabolic activity then slightly reduced at day 7 but was significantly higher compared to the other conditions. Cells cultured in 5% (v/v) HPL also exhibited higher cell metabolic activity than 10% (v/v) FBS but there was a gradual decline to day 7. The cell metabolic activity in 10% (v/v) FBS indicated a marginal increase on day 7. Metabolic activity in OM-10 was lower compared with the BCM and there was a significant difference. However, XF2-OM-10 had a significantly higher metabolic activity of cells compared with its BCM.

Donor-5, cells cultured in 5% (v/v) HPL showed statistically higher cell metabolic activity over time for 4 days and then the metabolic activity remained steady to the end of the period. Cells cultured in both human-derived formulations also exhibited higher cell metabolic activity than 10% (v/v) FBS but they remained stable to day 7 ($P<0.05$) (Figure 5.5).

In donor-7, the results were slightly different from the other two donors, overall this donor exhibited higher cell metabolic activity at all time-points and the cell metabolic activity in both human-derived media was higher compared to 10% (v/v) FBS at day 4. Although a significant difference was observed between 5% (v/v) HPL and 10% (v/v) FBS, there was no significant difference between XF2 and 10% (v/v) FBS. In all conditions the cell metabolic activity increased between days 4 and 7 in both human-derived media supporting significantly higher cell metabolic activity than 10% (v/v) FBS at day 7 ($P<0.05$) (Figure 5.5). When comparing OM-10 and its BCM OM-10 of 10% (v/v) FBS decreased metabolic activity of cells and there was a significant difference. On the other hand, OM-10 of human-derived medium revealed no significant difference compared with their BCM on day 7.

Overall, there was no consistent effect of osteogenic media on cell metabolic activity as previously seen in monolayer in chapter 3 (Table 5.5)

Table 5.5: The effect of osteogenic supplements on the metabolic activity of cells in PCL scaffolds. "Higher" indicates osteogenic-inductive medium caused significantly higher resazurin fluorescence when comparing data in figure 5.4 and figure 5.5. "Lower" indicates osteogenic-inductive medium caused significantly lower fluorescence and "NS" indicated no significant difference between basal media and OM within this group.

Media-Donors	**Donor 4**		**Donor 5**		**Donor 7**	
	Day 4	Day 7	Day 4	Day 7	Day 4	Day 7
10% (v/v) FBS	Higher	Higher	NS	Higher	NS	Higher
XF2	Lower	Lower	NS	NS	NS	NS
5% (v/v) HPL	Higher	Higher	NS	Higher	NS	NS

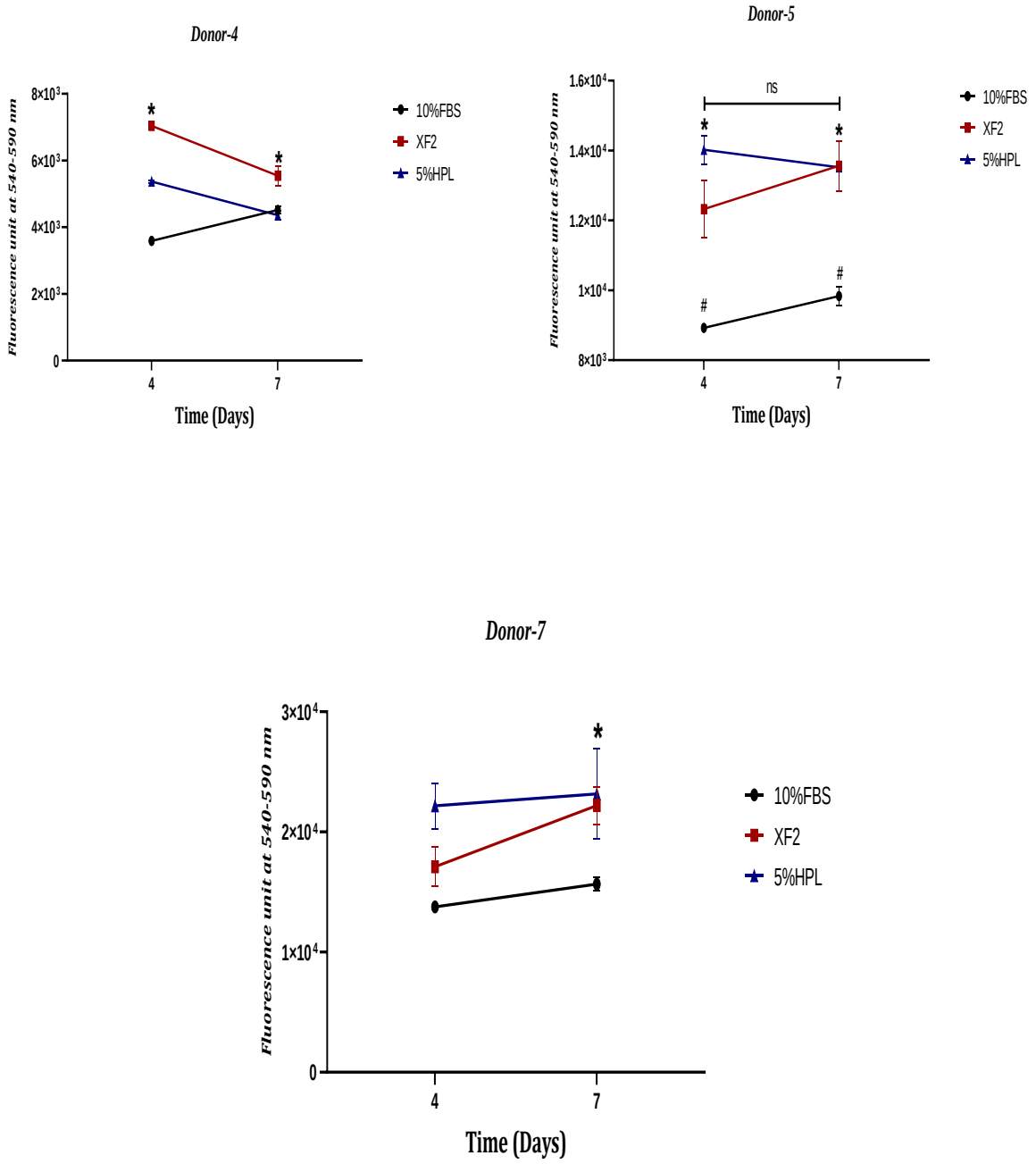


Figure 5.5: *Metabolic activity of cells from three different donors of hBMSCs seeded on 3D PCL scaffolds cultured in two different xeno-free basal medium treated with osteogenic media (OM-10) containing Dex 10 nM, AA 50 µM, and βGP 10 mM from day 1 to day 7. Horizontal line indicates Mean ± S.D for each condition, each point is a single scaffold. (*) Significant higher than the other condition. (#) Significantly lower. (P <0.05). n=3. Two-way ANOVA, Tukey's multiple comparison tests.*

5.6 Comparison of osteogenesis of various donors of hBMSCs cultured in different xeno-free culture media on PCL scaffolds

5.6.1 Alkaline phosphatase activity

Alkaline phosphatase (ALP) is an enzyme found in bone mineralisation, and its activity is frequently employed as a marker for initial detection of osteogenic differentiation. ALP activity was divided by the amount of DNA after converting to approximate cell number ng/mL= {(Fluorescence emission + 215.1)/223.2} (Figure 5.6.1 A) as described in chapter two.

In all donors, total DNA content was very high in XF2 basal culture medium and higher in 5% (v/v) HPL compared to 10% (v/v) FBS basal culture medium. The human-derived basal culture media had a significant difference compared to the 10% (v/v) FBS basal culture media.

In donor-4, even though there was no significant difference between both types of human-derived osteogenic media, they were 2-fold higher compared to 10% (v/v) FBS osteogenic media ($P<0.05$).

In donor-5, the quantity of DNA increased rapidly in all osteogenic media. There was no significant difference between the 10% (v/v) FBS osteogenic medium and 5% (v/v) HPL osteogenic medium or the XF2 basal culture medium. XF2 osteogenic medium had high cell proliferation and was significantly different compared to all conditions while the basal culture medium of 10% (v/v) FBS supported a lower number of cells.

In donor-7, the osteogenic human-derived media supported high numbers and was significantly different compared to 10% (v/v) FBS osteogenic medium. There was no significant difference between XF2 basal culture medium and 5% (v/v) HPL osteogenic medium. The growth of hBMSCs was augmented in XF2 osteogenic culture medium over the other conditions throughout all donors. Although 10% (v/v) FBS osteogenic media supported higher cell numbers over 10% (v/v) FBS basal culture medium, they revealed lower potential cell growth (*$P<0.05$*) (Figure 5.6.1 B).

In all donors, the ALP activity was normalised to ng/DNA and standardised to 10% (v/v) FBS basal culture medium for each donor. The normalised ALP activity was expressed

highly in both groups of human-derived culture medium over 10% (v/v) FBS basal and osteogenic culture medium. However, there was no significant difference between basal culture media and their osteogenic media in all donors. Meanwhile, hBMSCs of donor-4 exhibited high ALP activity over other donors and followed donor-5. Similarly, hBMSCs cultured in 10% (v/v) serum osteogenic media revealed the lowest ALP activity for all donors and was significantly different compared to XF2 osteogenic medium and human-derived osteogenic media in donors 4 and 5 respectively. While there was no significant difference between all osteogenic media in donor 7 ($P<0.05$) (Figure 5.6.1 C).

These data confirmed the results in monolayer culture which showed osteogenic medium containing Dex 10 nM to increase the osteogenic cells differentiation (Table 5.6.1). Although ALP in 10% (v/v) FBS media is relatively low a significant effect on osteogenic medium can be seen. Whereas in the media containing human-derived ingredients where the ALP is much higher even when there are no supplements present the effect of supplements is smaller and not significant for all donor.

Overall, the normalised ALP activity and DNA quantity of hBMSCs on 3D PCL scaffolds was highest in both human-derived growth media and even higher than all donors from any groups (basal and osteogenic) with 10% (v/v) animal serum supplementation. This implied that the primary hBMSCs seeded on 3D PCL scaffolds and cultured in human-derived basal and osteogenic media had undergone osteoblast differentiation.

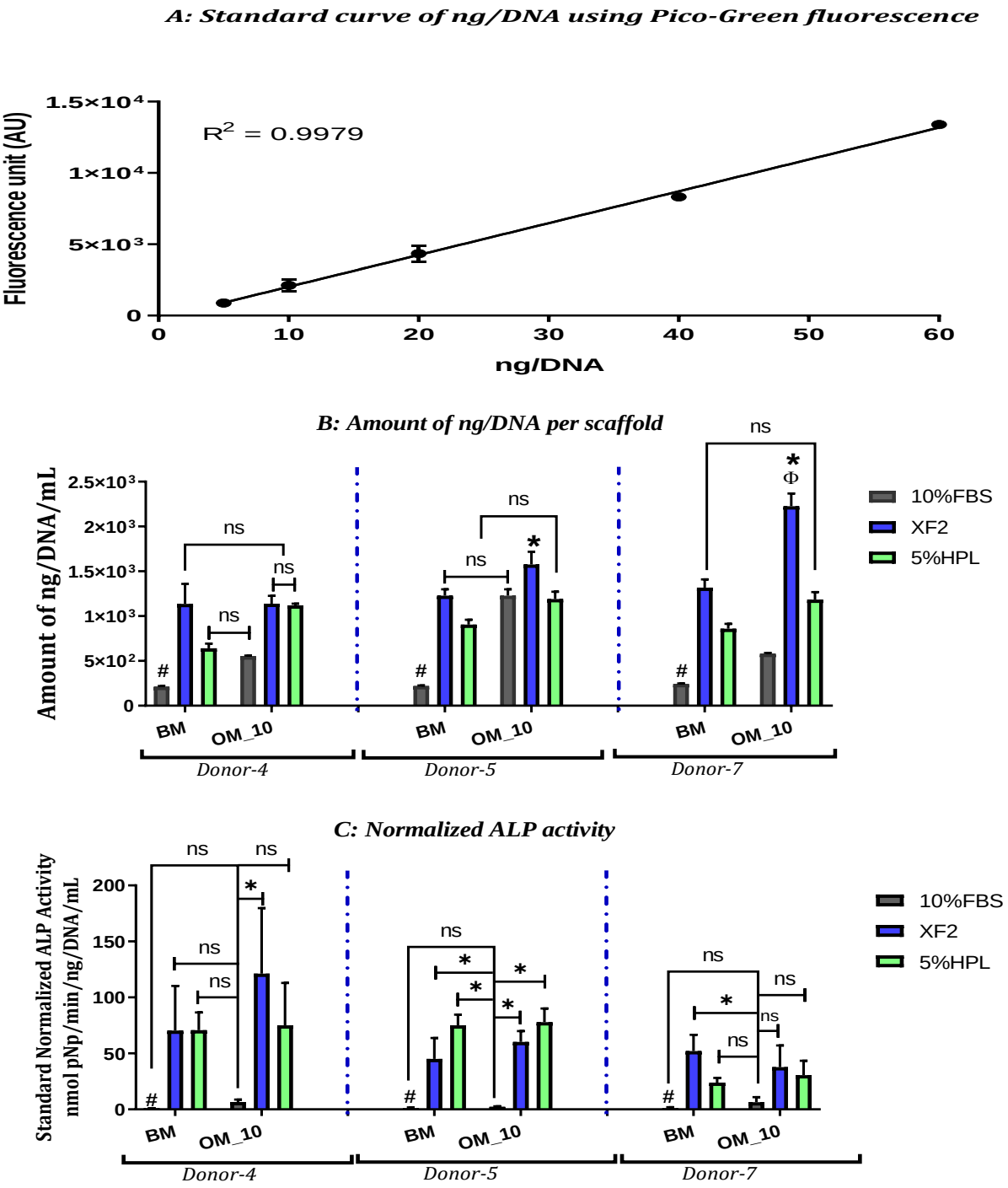


Figure 5.6.1: *hBMSCs from different adult donors seeded on 3D PCL scaffolds cultured on two commercial human-derived basal and osteogenic culture media compared with 10% (v/v) animal-serum concentration on day 14. The bar indicates mean±SD for each condition (n=3). (*) Significantly higher than the other groups in the same donors. (#) Significantly lower. (Φ) Significantly higher than the other groups in the different donors. (ns) There is no significant difference (P <0.05). n=3. Two-way ANOVA, Tukey's multiple comparison tests.*

Table 5.6.1: The effect of osteogenic supplements on the amount of DNA and ALP activity of cells in PCL scaffolds respectively. "Higher" indicates osteogenic-inductive medium caused significantly higher. "Lower" indicates osteogenic-inductive medium caused significantly lower and "NS" indicated no significant difference between basal media and OM within this group

Media	Donor 4	Donor 5	Donor 7
BCM	Higher	Higher	Higher
XF2	NS	Higher	Higher
5% (v/v) HPL	Higher	Higher	Higher

ALP activity

Media	Donor 4	Donor 5	Donor 7
BCM	Higher	NS	Higher
XF2	Higher	NS	Lower
5% (v/v) HPL	NS	NS	NS

5.6.2 Assessment of extracellular matrix formation

The aim of this study was to assess the late stage of osteogenic differentiation of primary hBMSCs on 3D PCL scaffolds, in terms of production of extracellular matrix (ECM) and then identify media containing only xeno-free ingredients as an alternative formulation to FBS supplements to repair bone defects in the cleft palate.

5.6.2.1 Collagen production

Collagen was measured using Sirius Red as described in chapter 2. It was interesting to observe that the media containing supplements supported higher deposition of collagen on PCL scaffolds over basal culture media. As was expected osteogenic media, which contains ascorbic acid increased collagen production in 10% (v/v) serum medium (evident in donors 4 and 7). The xeno-free media XF2, even without osteogenic supplements also supported significantly higher collagen production on PCL scaffolds compared to other conditions. When XF2 media was supplemented with typical osteogenic supplements collagen deposition was even higher ($P<0.05$) (Figure 5.6.2.1).

There were some differences in collagen production between donors. In donors 4 and 5, the total collagen production of cells cultured in XF2 osteogenic media on 3D PCL scaffolds was significantly higher than all the other groups and but for donor-7 cells cultured in osteogenic and FBS supplemented media collagen production was as high as in osteogenic 5% (v/v) HPL media. In donor-4, the collagen production in osteogenic media in both 5% (v/v) HPL and 10% (v/v) FBS was about 3-fold higher compared to basal media. (*P<0.05*) (Figure 5.6.2.1).

In donor-5, 5% (v/v) HPL osteogenic medium supported higher collagen production, approximately 1.5-fold, compared to basal culture medium while collagen production from both 10% (v/v) FBS basal and osteogenic media on PCL scaffolds remained steady (*P<0.05*) (Figure 5.6.2.1). Cells from donor-7 were the only cells in which osteogenic supplements in XF2 media did not increase collagen production over basal XF2.
These results imply that collagen production by primary hBMSCs seeded on 3D PCL scaffolds can be supported to produce collagen ECM in human-derived osteogenic media.

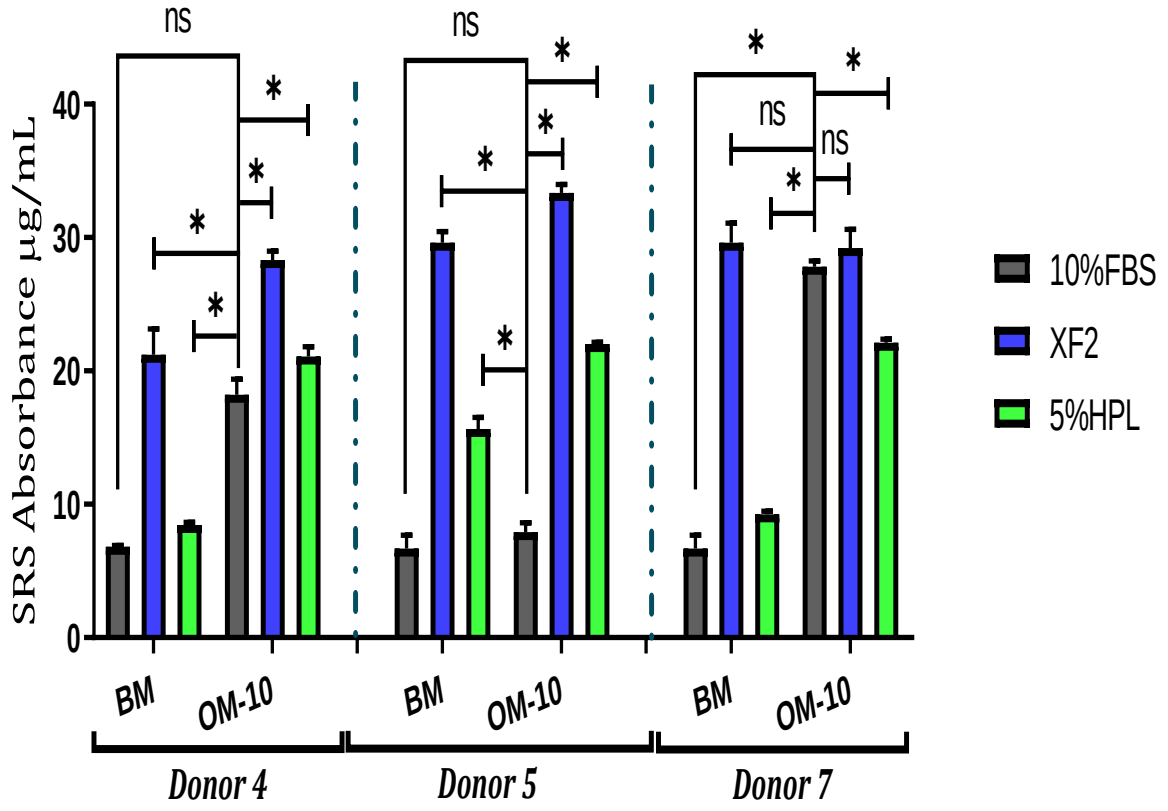


Figure 5.6.2.1: *The ability of primary hBMSCs from different adult donors to produce collagen when seeded on 3D PCL scaffolds cultured on two commercial human-derived basal and osteogenic culture media compared with 10% (v/v) animal-serum on day 21. The bar indicates mean±SD (n=3) for each condition. (*) Significantly higher than the other groups in the same donors. (ns) There is no significant difference (P <0.05). n=3. Two-way ANOVA, Tukey's multiple comparison tests.*

5.6.2.2 Calcium deposition

Calcium deposition was assessed using Alizarin red staining (ARS) as described in chapter 2.

There was considerable variety in mineral deposition between different donors which has also been shown in other studies (Sittichokechaiwut et al., 2010). In donors 4 and 7, the total calcium deposition by hBMSCs cultured in XF2 osteogenic media on 3D PCL scaffolds was highest followed by XF2 basal culture medium. As expected, osteogenic supplements supported higher calcium deposition on PCL scaffolds ($P<0.05$) (Figure 5.6.2.2).

Cells from donor-5 exhibited significantly lower mineralisation than the other donors in all media and differences were small. For this donor the amount of calcium measured by ARS was highest in osteogenic media with FBS which was significantly higher than the other media conditions ($P<0.05$) (Figure 5.6.2.2).

Table 5.6.2.2: The effect of osteogenic-inductive media on collagen formation and calcium deposition within the media groups examined. "Higher" indicates osteogenic-inductive caused significantly higher when comparing data in figure 5.6.2.1. "Lower" indicates osteogenic-inductive medium caused significantly lower and "NS" indicated no significant difference between osteogenic-inductive compared with BCM within this group.

Media-Donors	**Donor 4**		**Donor 5**		**Donor 7**	
	Collagen	Calcium	Collagen	Calcium	Collagen	Calcium
10% (v/v) FBS	Higher	Higher	NS	Higher	Higher	Higher
XF2	Higher	Higher	Higher	NS	NS	Higher
5% (v/v) HPL	Higher	Higher	Higher	NS	Higher	Higher

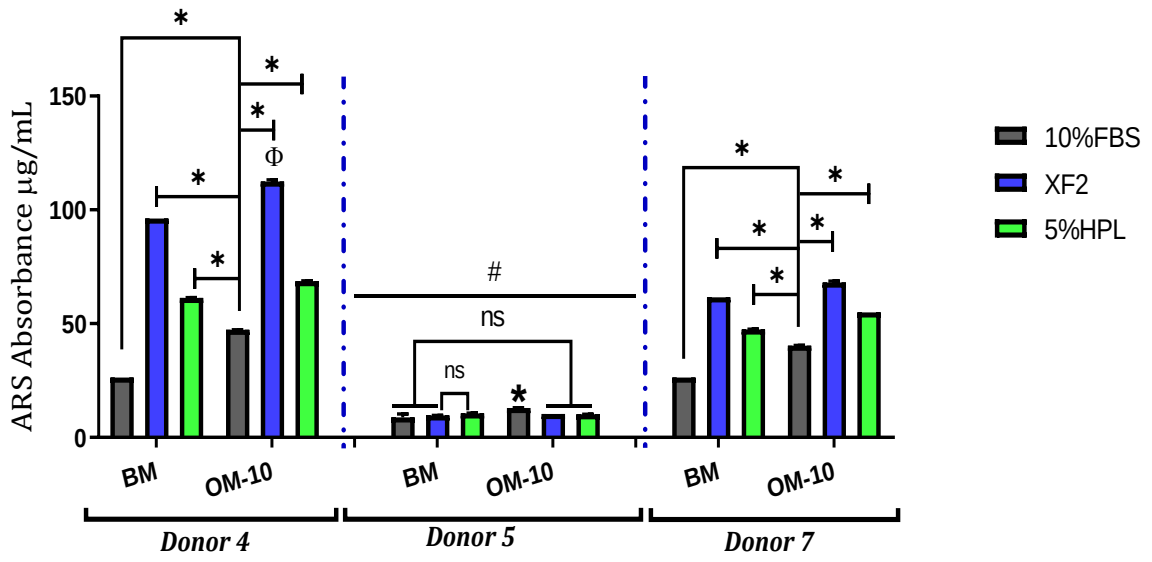


Figure 5.6.2.2: *The ability of primary hBMSCs from different adult donors seeded on 3D PCL scaffolds to deposit calcium, when cultured in two commercial human-derived basal and osteogenic culture media compared with 10% (v/v) animal-serum n on day 21. The bar indicates mean ± SD for each condition. (*) Significantly higher than the other groups in the same donors. (Φ) Significantly higher than all the other donors. (#) Significantly lower. (ns) There is no significant difference (P <0.05). n=3. Two-way ANOVA, Tukey's multiple comparison tests.*

5.7 Nuclei staining

The PCL scaffolds were stained with a fluorescent cell nucleus dye after 21 days. In all groups, the results showed some DAPI fluorescence signals with different brightness that depended on the depth and spread of cell in the PCL scaffolds. A significant build-up of DAPI fluorescent signals was observed on the PCL scaffolds treated by XF2 osteogenic media rather than the other conditions this supports the resazurin and DNA data (Figure 5.7).

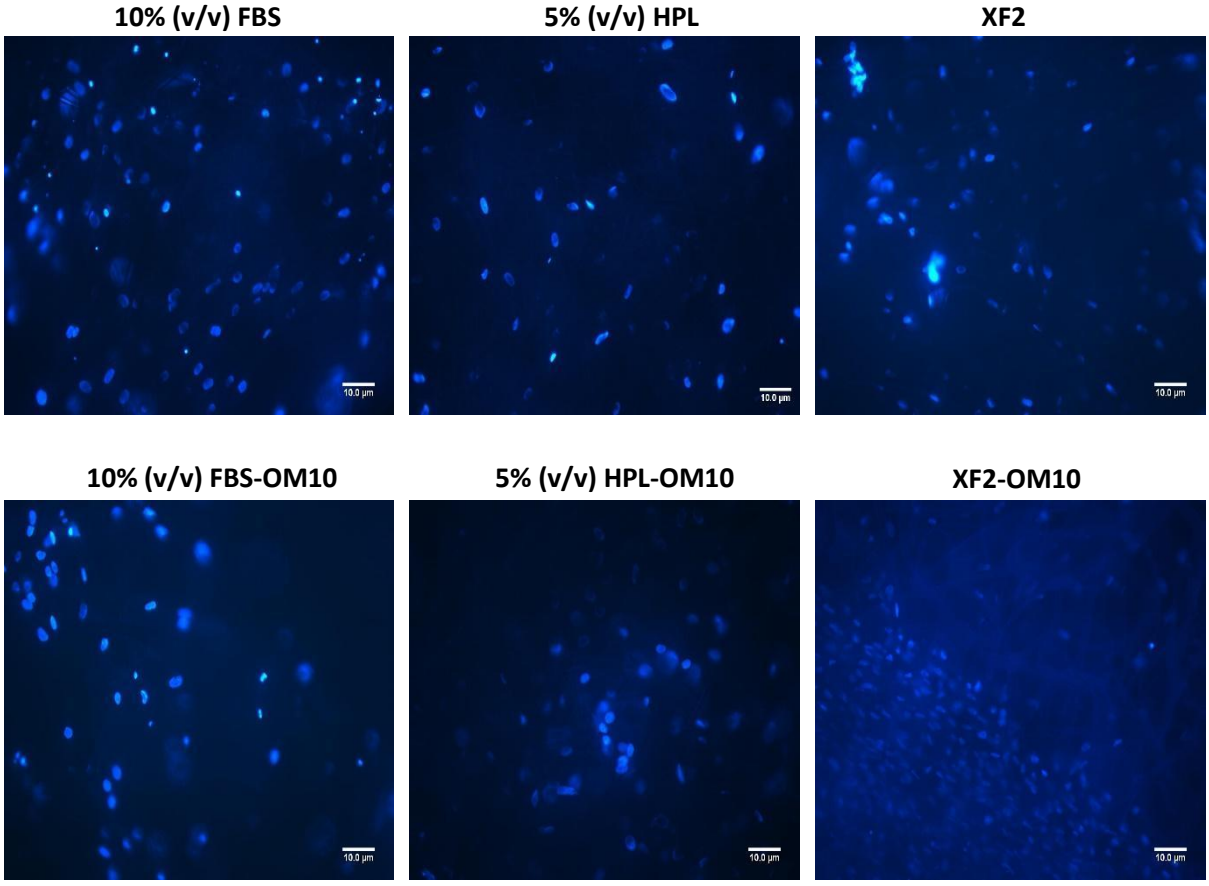


Figure 5.7: *The fluorescent microscopy images of 3D PCL scaffolds stained with DAPI in two commercial human-derived basal and osteogenic culture media compared with an animal-serum concentration on day 21. Bar = 10 µm.*

5.8 Scanning Electron Microscope (SEM) evaluation of extracellular matrix on PCL scaffolds

The SEM images of hBMSCs cultured on the 3D PCL scaffolds after 21 days indicated that the cells were well-attached, extended on the large surface area and underside of the scaffolds. For hBMSCs grown in XF2, in both osteogenic and basal culture media there was bone-like matrix formation over the surface of PCL scaffolds, which covered all the scaffold fibres. Although cells grown in 5% (v/v) HPL also produced a bone-like layer which covered most of the surface area of PCL scaffolds, it did not cover the whole surface. Although cells grown in 10% (v/v) FBS formed a thin layer of bone on the surface of PCL scaffolds, it did not cover all the fibres of PCL scaffolds and round structures which appeared to include ECM were presented underneath the scaffold surface. However, in 10% (v/v) FBS osteogenic media bone-like layer was thicker than in basal culture media. These results indicate that the presence of human-derived media either with or without osteogenic supplementation could lead to enhanced ECM deposition and create a new layer of pre-bone matrix compared to 10% (v/v) FBS. Moreover, the PCL scaffolds and human-derived supplementation could encourage bone repair for craniofacial bone defects, particularly in cleft palate (Figure 5.8).

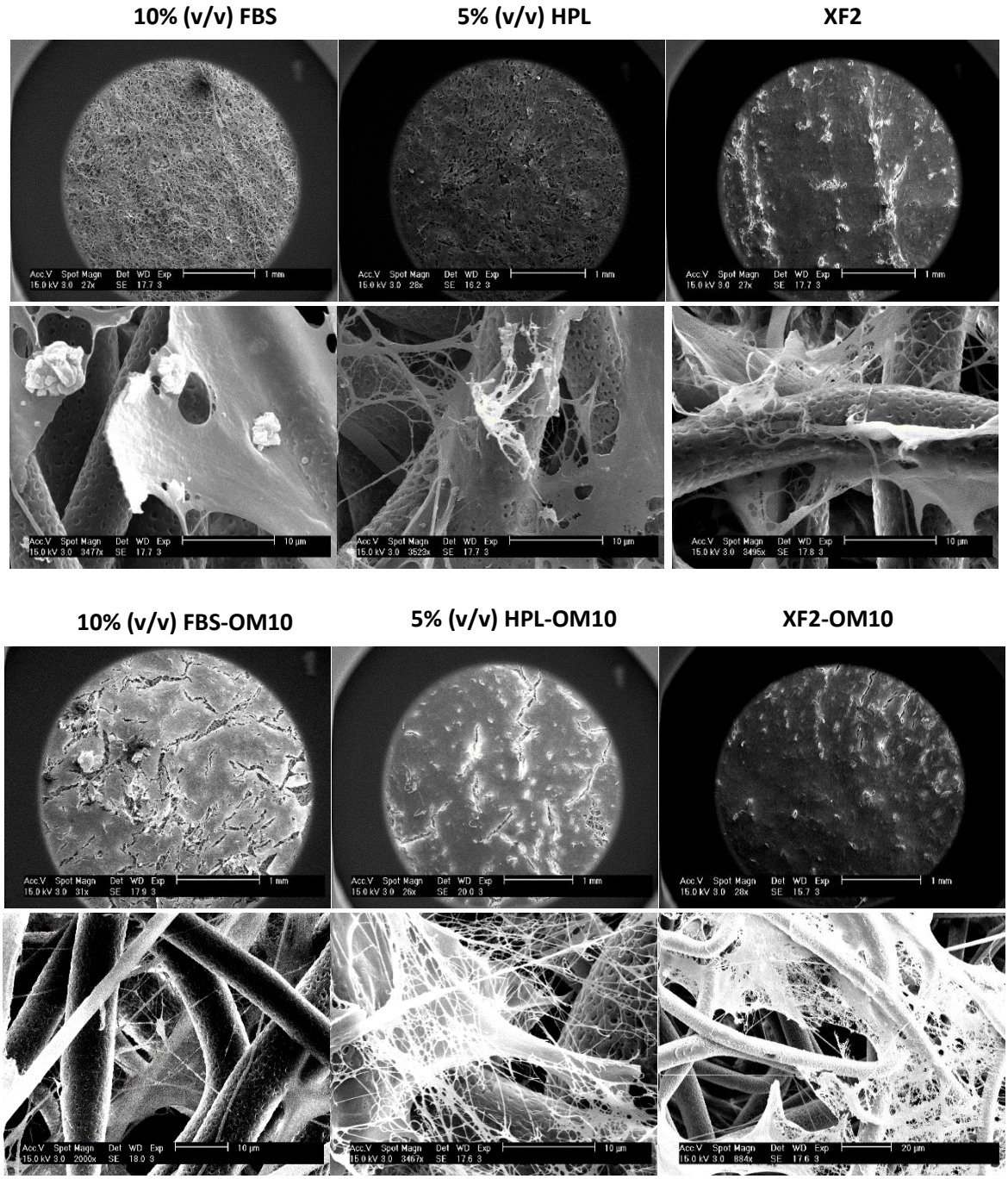


Figure 5.8: *The SEM images of the hBMSCs donor 4 seeded on 3D PCL scaffolds cultured on two commercial human-derived basal and osteogenic culture media compared with 10% (v/v) animal-serum concentration on day 21. The bone layer formation with mineralised ECM. Scale bar = 10µm to 1mm*

5.9 Xylenol orange fluorescence staining

Xylenol orange ($C_{31}H_{28}N_2O_{13}SNa_4$) is a non-toxic fluorochrome which binds to calcium ions. This staining has been using to detect the mineralised matrix by live osteoblastic cells *in vitro.*

CLSM fluorescence images of cultured hBMSCs confirmed that mineral deposition occurred on the PCL scaffold. The PCL only control did not give off a signal indicating that there was no non-specific binding of Xylenol orange on the PCL. Xylenol orange could be seen in all groups but was considerably higher in those that had been subjected to osteogenic supplementation. Moreover, the xylenol orange staining confirmed that XF2 osteogenic media supported substantial mineral deposition compared to other conditions. Human-derived basal media also enhanced mineral deposition on the PCL scaffolds compared to 10% (v/v) FBS. Xylenol orange staining confirmed the alizarin red results indicating that XF2 basal media supports mineralisation even without osteogenic supplements (Figure 5.9).

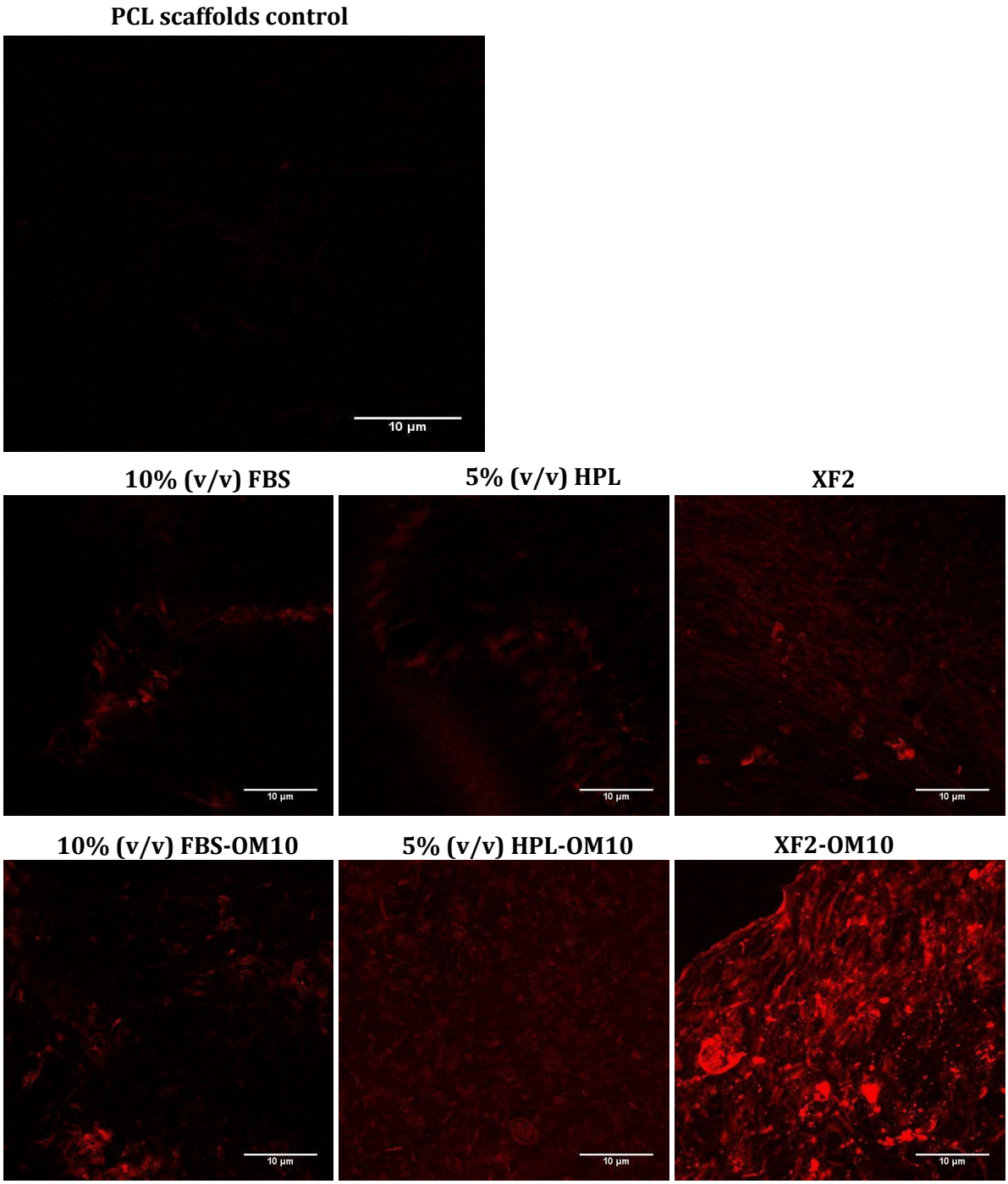


Figure 5.9: *CLSM fluorescence images of cultured hBMSCs on 3D PCL scaffolds. The matrix mineralisation of hBMSCs was measured by labelling using xylenol orange (red colour) staining after 21 days of culture. The PCL scaffold with no staining was imagined to show that it was not auto-fluorescent (no cells or mineralisation). Scale bar = 10 µm, n=2.*

5.10 Assessment of vascular endothelial growth factor (VEGF) secretion from commercial human-derived media using enzyme-linked immunosorbent assay (ELISA)

The aim of this study was to assess the ability of the human-derived media to enhance vasculogenesis through the secretion of VEGF. The importance of the blood supply for bone regeneration is well-known during oral and maxillofacial surgery. Impairment of the vascular supply considers diminished growth and repair, bone loss, and eventually, necrosis.

hBMSCs from both 10% (v/v) FBS and 5% (v/v) HPL basal culture media had significantly higher VEGF levels compared to XF2 basal culture medium. However, there was no significant difference between them. Whereas, XF2 basal medium seemed to support particularly low VEGF secretion($P<0.05$) (Figure 5.10 A). After subtraction of the amount of VEGF of basal culture media from each experimental media, the results indicated that the amount of VEGF per cell cultured in 10% (v/v) FBS and 5% (v/v) HPL basal medium, and 5% (v/v) HPL osteogenic medium was significantly higher compared to the other groups, and there was no significant difference among them. XF2 basal culture medium did not supported cell VEGF secretion, while the XF2 osteogenic medium augmented VEGF secretion approximately 5-fold compared to basal culture medium. A considerable reduction of the VEGF secretion in 10% (v/v) FBS osteogenic medium was observed over its basal medium ($P<0.05$) (Figure 5.10 B).

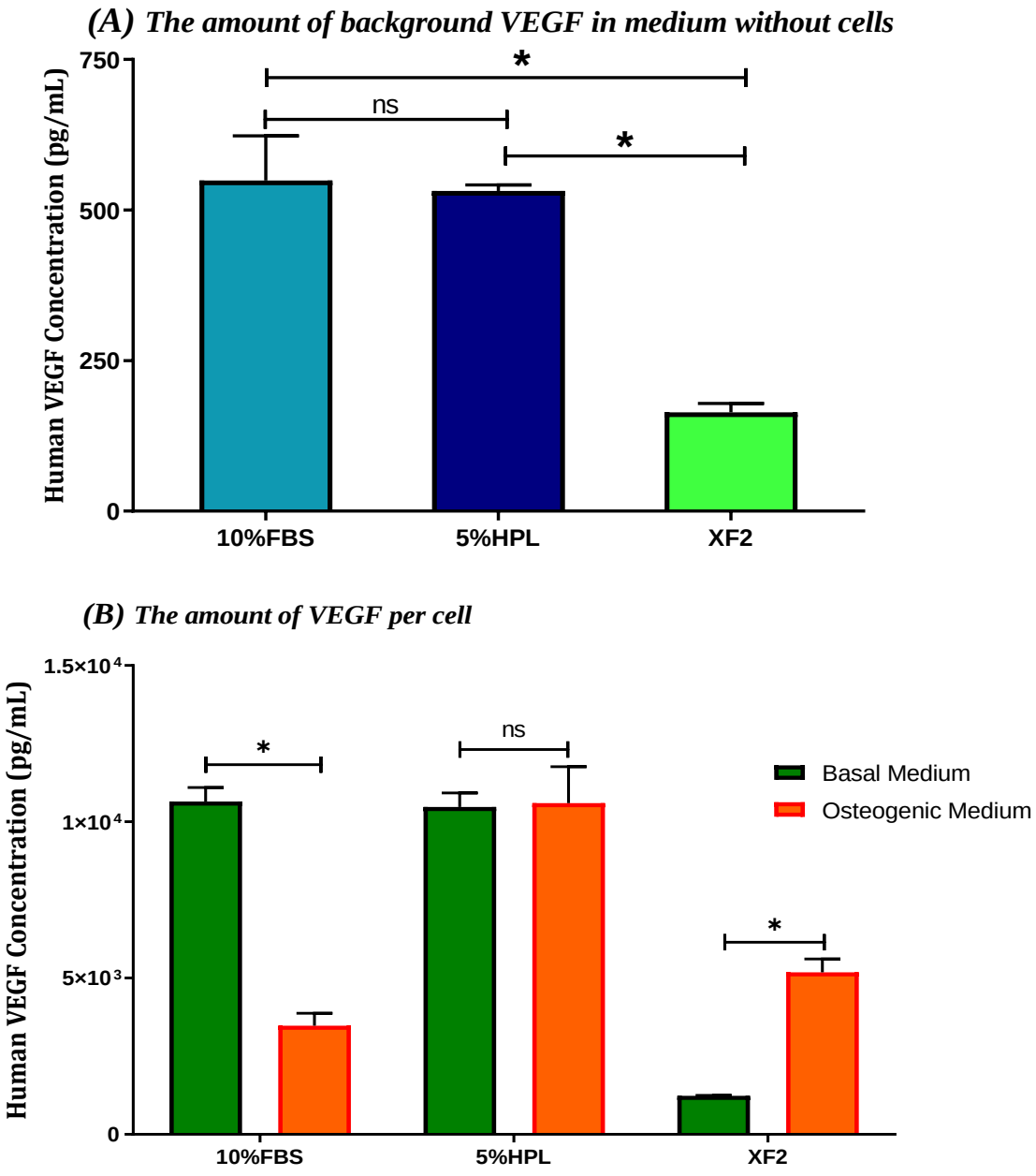


Figure 5.10: *Effect of two commercial human-derived basal and osteogenic culture media on VEGF secretion of hBMSCs seeded on 3D PCL scaffolds on day 21 (during 48 hours of collection period). The bar manifests mean±SD for each condition. (A) The amount of background VEGF in medium without cells, (B) the amount of VEGF per cell (not different scale bars). (*) Higher than the other conditions. (ns) There is no significant difference (P <0.05). n=3. One-way ANOVA, Brown-Forsythe test, and Two-way ANOVA, Tukey's multiple comparison tests.*

5.11 The ability of Human jaw periosteum cells to grow on 3D PCL scaffolds (preliminary alternative cell sources)

As the 2D culture results indicated that HJPs may be a promising osteogenic cell these were also cultured on PCL scaffolds to determine their ability to support bone tissue engineering. Experiments were performed as described for hBMSCs in this chapter. In terms of cell attachment, cells in XF2 had the highest amount of cell detachment as detected by resazurin reduction assay on 2D well plate, there was no cells detected on the 2D well plate from in either 5% (v/v) HPL or 10% (v/v) FBS ($P<0.05$) (Figure 5.11 A).

On days 1 and 4, the cells from both human-derived growth basal culture media were attached to the PCL scaffold fibres and numbers increased significantly. However, there was no significant differences between them. There was a sharp increase of cell metabolic activity for cells cultured in both human-derived growth basal media on PCL scaffolds compared to 10% (v/v) FBS basal medium. On day 7, both human-derived basal media supported higher cell metabolic activity compared to 10% (v/v) FBS basal medium. In XF2 basal medium cell number was significantly higher, about 1.5-fold and 3-fold compared to both 5% (v/v) HPL basal and 10% (v/v) FBS basal media respectively (Figure 5.11 B).

In osteogenic media, on days 4 and 7, the cells from both human-derived growth basal culture media on PCL scaffolds had robust growth and cell metabolic activity was significantly higher 3-fold and 2-fold compared to 10% (v/v) FBS on days 4 and 7 respectively. Then, cell metabolic activity in 5% (v/v) HPL appeared to reach a plateau, while the cells in XF2 continued to increase in number towards the end of the period. Although the metabolic activity of the cells in 10% (v/v) FBS increased gradually, they were significantly lower compared to other media throughout the period ($P<0.05$) (Figure 5.11 C).

Overall, the results indicated that both human-derived basal and osteogenic culture media supported cell growth better than 10% (v/v) FBS basal and osteogenic media.

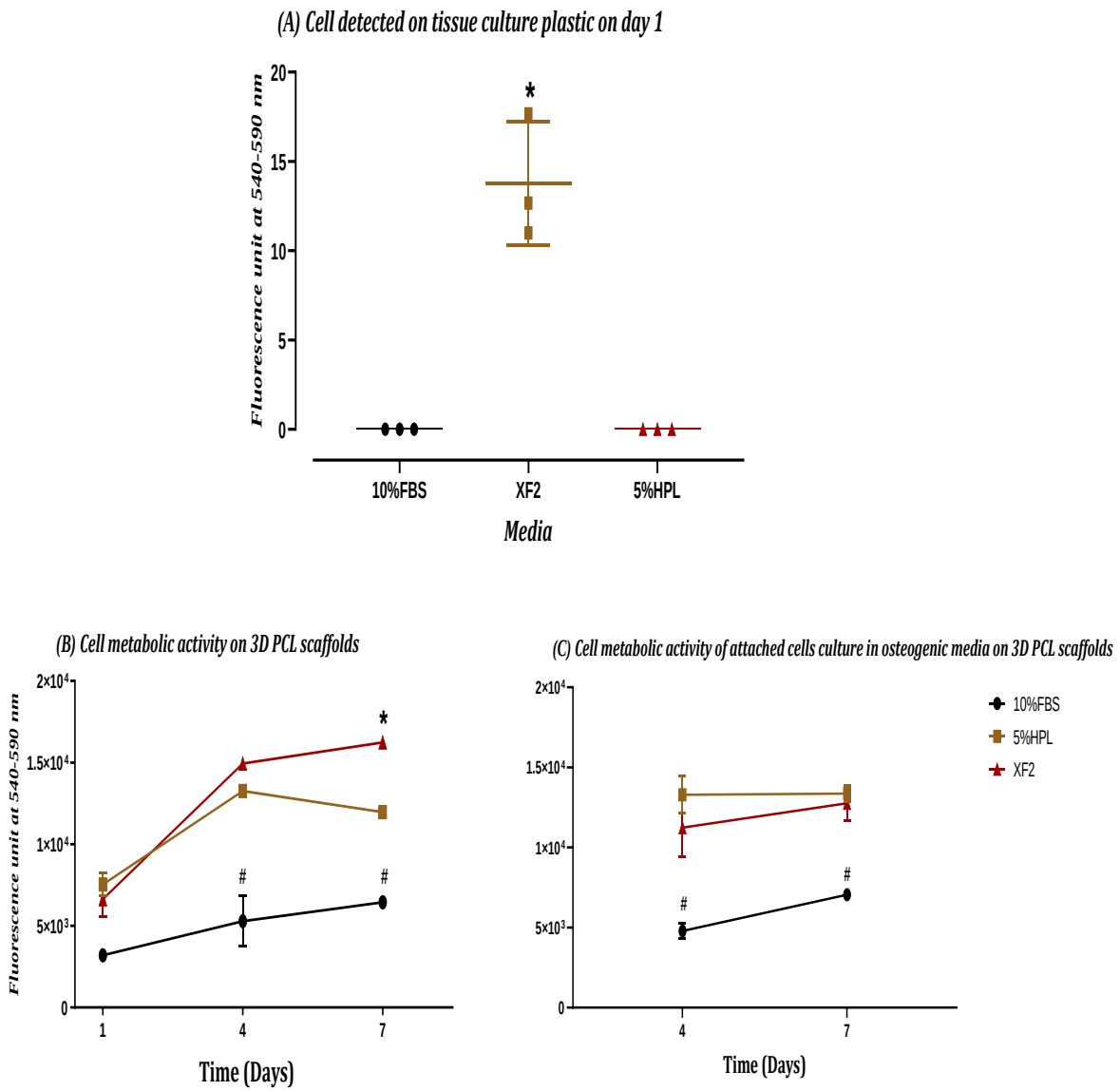


Figure 5.11: *The ability of 3D PCL electrospun scaffolds to support HJPs cell metabolic activity cultured in two commercial human-derived basal and osteogenic culture media over 7 days. The cell metabolic activity was assayed by resazurin reduction test after 1, 4 and 7 days. (A) cell detected on tissue culture plastic on day 1, (B) cell metabolic activity of attached cells in basal culture media on 3D PCL scaffolds on days 1, 4, and 7 (C) cell metabolic activity of attached cells culture in osteogenic media on 3D PCL scaffolds on days 4 and 7 respectively. (*) Significantly higher than the other media. (#) Significantly lower than other media. (P <0.05). n=3. One-way ANOVA, Brown-Forsythe test, Two-way ANOVA, Tukey's multiple comparison tests.*

5.11.1 Alkaline phosphatase activity of HJP cells

In both groups the basal culture media (BCM) and osteogenic media at a concentration of 10 nM (OM10), the results showed that both human-derived media had a high ALP activity and this was significantly higher than for cells in10% (v/v) FBS. Although the level of ALP activity in XF2 appeared to be higher than 5% (v/v) HPL, it was not significantly different. The ALP activity increased in 5% (v/v) HPL-OM10 compared to XF2-OM10, however, there was no significant difference. No ALP activity was seen in 10% (v/v) FBS-BCM while only a low amount of ALP activity was observed in 10% (v/v) FBS-OM10. As expected the osteogenic supplementation increased ALP activity in both 10% (v/v) FBS and 5% (v/v) HPL, while in XF2 ALP activity was no higher in osteogenic supplements than in basal media. (Figure 5.11.1).

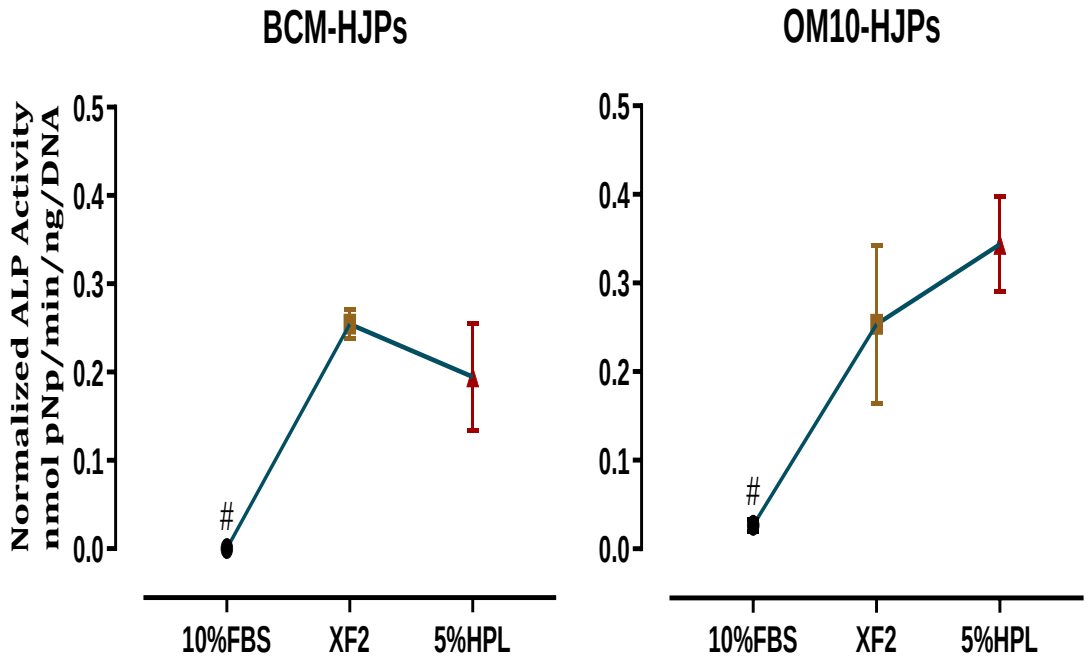


Figure 5.11.1: *The Alkaline phosphatase activity of HJPs seeded on 3D PCL scaffolds cultured on two commercial human-derived basal and osteogenic media compared with 10% (v/v) animal-serum on day 14. Mean±SD for each condition. (#) Significantly lower than other conditions. (P <0.05). n=3. One-way ANOVA, Brown-Forsythe test.*

5.12 Discussion

The purpose of bone tissue engineering is to make available a biocompatible material as an alternative to the well-regarded of surgical procedures that uses the autologous bone grafts for stimulating bone regeneration and implant fixation. The requirements for a polymer scaffold are to be biodegradable and to have sufficient mechanical strength, biological and medical properties to stimulate the cell proliferation and adhesion to form a temporary mineralised ECM until the regeneration of bone has occurred. The relationship between the mineralised ECM and the surrounding conditions affect the cell behaviours by direct and indirect signal transmission. The foremost goals of this chapter were to construct electrospun PCL scaffolds to regenerate cleft palate osseous defect that would be stimulated normal development of orofacial bones. To perform this aim, firstly, the selection of biodegradable polymer electrospun scaffold was undertaken utilising FDA confirmed PCL polymer and the efficiency of hBMSCs to grow and mineralise within this scaffold was investigated. Secondly, commercial xeno-free culture media derived from human blood were evaluated as a media, for the pre-clinical culture of tissue-engineered bone in cleft palate and other craniofacial applications, such as human serum media (XF2) and human platelet lysate. Finally, the variability of donor populations in growth characteristics and osteogenic potential was assessed.

In this chapter, the fabrication of PCL micro-fibre non-woven mat scaffolds was described. These had a smooth fibre morphology without bead formation and were non-aligned. Spasova et al., (2006) mentioned that the bead formation is usually dependent on the polymer solution surface tension and viscosity during and formed a significant defect in electrospun PCL scaffolds. Furthermore, Chen and co-workers (2007) mentioned that the beaded structure on the scaffolds had reduced cell proliferation and adhesion on the PCL scaffolds uniformly because of the decrease of the volume-to-surface area ratio of the PCL scaffold. Consequently, a pure fibre mat of electrospun PCL scaffold is essential for optimising tissue engineering.

To simplify the transition of human MSCs from conventional studies to clinical applications, several advances in the reagents used to expand these therapeutically relevant cells is needed. One of the essential steps in this study was the transition from animal serum supplementations to a better-defined xeno-free media which included

human-derived formulation media. Recently, many efforts have demonstrated that human bone marrow-derived MSCs can be isolated and expanded over the short term in serum-free medium (Battula et al., 2007). However, no published work has shown the ability of electrospun PCL scaffolds to support human MSCs under human-derived formulation media for long-term culture. To achieve such a feat, the selection of xeno-free media was performed as discussed in chapters 3 and 4. It is assumed that a reason these xeno-free formulations are able to support MSC expansion is because they contain required growth factors and ECM elements. The most significant growth factors and ECM reagents that play a crucial role in both hBMSCs proliferation and differentiation are PDGF-BB, bFGF, TGF-β1, vitronectin, hyaluronic acid, and fibronectin (Ng et al., 2008; Uhm et al., 1999). Interestingly, the combination of any two growth factors seemed to provide minimal or no significant improvement of cell proliferation over the single factors, however, the combination of all factors ameliorated a noticeable synergistic effect (Chase et al., 2010) .

The variability in hBMSCs ability to sustain their phenotype in long-term has been established for a long time (Collins and Dorshkind, 1987; Kuznetsov et al., 1997). Phinney and his colleagues (1999) investigated the growth properties, and osteogenic differentiation from over 50 donors and the results revealed a striking variation. Donor to donor variability is likely to be the consequence of heterogeneity in the population at the time of harvest. Consequently, accurate analysis of hMSC donor populations is essential before administering cells to patients in need of cell therapy regimens (Phinney et al., 1999). Therefore, our study investigated the growth properties and osteogenic differentiation of three donor samples cultured in human-derived supplements and reported each donor separately to give an overview of the consistency of any effects seen.

In these projects, I utilised hBMSCs as a cell model to identify cell attachment and metabolic activity. Results of the hBMSC cells cultured in PCL scaffolds and cultured in human serum basal media (XF2) showed a promoting resazurin assay that indicated the cell attachment at 24 hours (see Fig 5.3.2). Furthermore, they exhibited a non-toxic effect and supported adherence and proliferation after 7 days. Our results agreed with Xue et al., (2017) who found that PCL scaffolds can sustain human MSC metabolic activity and proliferation potency. The osteogenic supplementations to the experimental media showed increased cell metabolic activity and proliferation potential in human-derived

culture media compared to 10% (v/v) FBS media on day 4. It is known that Dex supresses the proliferation of mesenchymal stem cells and upregulates differentiation (Taira et al., 2003) and is required for stimulation of hBMSCs differentiation to osteoblasts in 3D culture. Subsequently, the reducing the amount of Dex in the PCL scaffolds culture media may be given the opportunity for enhanced cell proliferation. The cell metabolic activity in this experiment was assessed while metal, stainless steel rings were placed on the PCL scaffolds to prevent scaffolds floating in the media. However, these might minimise the reaction between the resazurin salt solution and cells underneath the metal rings. Therefore, the metal rings were removed for the cell metabolic activity assessment for subsequent experiments.

To assess longer term growth (14 days) DNA per scaffold was used as a measure of cell numbers. This is because this value can be measured in the same lysate as ALP and therefore used to normalise ALP to cell number and because resazurin can be difficult to measure once extensive ECM has been made and cells are surrounded by ECM. The results supported the early metabolic activity measurement using resazurin and showed that there were distinct differences in cell populations between scaffolds cultured in human-derived media compared to 10% (v/v) FBS. This augmentation in PCL scaffold cellularity could be partially interpreted by the large number of cells seeded. However, Thibault et al., (2010) mentioned that the augmented cells number could not be interpreted by the greater seeding density. The presence of dexamethasone is well known to reduce the proliferation of MSC and allows to initiate differentiation of MSCs toward the osteogenic lineage (Fromigué et al., 2008; Castano-Izquierdo et al., 2007; Holtorf et al., 2005; Thibault et al., 2010). Another interpretation of the observed high DNA could be the cellular DNA after cell lysis was kept within a fabricated PCL fibre mesh mats and was not likely released in its entirety into the cell lysate solution. Thibault et al., (2010) seeded MSC on PCL scaffolds and observed that the amount of cellular DNA increased during the first week culture and decreased in the second week of seeding which is similar to the results of studies from Datta et al., (2006 and 2005) using titanium as a scaffold. Although our studies calculated the cellular DNA after two weeks, the result demonstrated the osteogenic media supported the cellular activity of DNA in the presence of Dex. A paper by Nishimura et al., (2015) mentioned that there was an increase in cell numbers and higher cell distribution density in the scaffolds for 14 days

compared to that for 7 days under osteogenic differentiation media. The possible explanation for such differences under human-derived media could be either a more efficient release of nutrients, and the deterioration of metabolic waste or the eventuality of human-derived formulations media effect on the cells might be superior to the dexamethasone's effect.

ALP activity is an initial-stage marker of the osteogenic differentiation of MSCs (Douglas et al., 2012), PCL has been suggested to be a promising material for bone tissue engineering. However, the shortcomings of PCL are that it can be hydrophobic and does not have any demonstrated osteoinductive properties. This study hypothesises that hBMSCs seeded into electrospun PCL scaffolds cultured in xeno-free media particular human-derived formulations could undergo accelerated osteogenesis compared to in 10% (v/v) animal-serum supplements. A comparison of ALP activity was investigated under both human-derived basal and osteogenic cultivation after 14 days. ALP activity is upregulated, and the speed of bone differentiation is promoted in both human-derived cultivation compared to that in 10% (v/v) animal-serum cultivation. Although the ingredients of osteogenic media increased ALP within 10% (v/v) FBS groups above a very low baseline, they appeared to have limited additive effect in the non-FBS media. However, in10% (v/v) FBS osteogenic media, ALP activity was significantly lower than in human-derived media. This indicates that using human-derived media for culture of hBMSCs when in electrospun PCL fibre mesh scaffolds can facilitated osteogenic differentiation. The human derived supplement appear to contain substantial components which support to differentiate the hBMSCs onto osteogenic lineage. Phosphate and calcium ions are an essential factor for supporting osteogenic differentiation activity by stimulating osteogenic marker genes; such as ALP, collagen type I (Klammert et al., 2009). The xeno-free media used in this study were derived from pooled human blood that contains the serum, platelets, growth factors and cytokines. Therefore, the possible pathway of human-derived formulations media to induce the bone-forming potential of the cells and stimulate genes of the c-Jun and c-fos that upregulate of gene expression of ALP at an initial stage of differentiation osteogenically (Meyer et al., 1993). Although Lin and colleagues (2009) and Maeno with co-workers (2005) informed that an extreme amount of calcium-phosphate deposition in the culture medium (> 10mM of calcium) might lead to increase cell toxicity, my results did not

illustrate drop of metabolic activity of cell even though there was reduced ALP in 10% (v/v) FBS osteogenic media cultured PCL scaffolds. Stein and Lian, 1995; Stein with his groups (2004) followed ALP activity over time and observed that the maturation of the matrix could after maximal ALP levels which then drop during the time of mineralisation. Consequently, the low ALP levels do not necessarily indicate slower cell differentiation along the osteogenic lineage but could be due to the time points of the sample collection.

For successful bone tissue engineering, a scaffold is required to be biodegradable and osteoconductive, which also can support the attachment and proliferation of bone cells as well as guide bone formation. Recently, investigators have looked to use hydroxyapatites to enhance the osteoconductive potential of PCL scaffolds. Although HA is a constituent of the natural bone, it limits in some applications as a load-bearing implant scaffold material (Sun et al., 2011). This study hypothesises that culture of electrospun PCL scaffold in xeno-free media derived from human origin could enable accelerated osteogenesis compared to the animal serum supplementations. The mineralised matrix formation is marked for a later stage of osteogenic differentiation. A later-stage of osteogenic differentiation of MSCs are marked by collagen formation and the deposition of calcium phosphate. Mineralised matrix formation is commonly identified using measurements of calcium content. The assessment of ECM in electrospun PCL mesh scaffolds under different commercial human-derived basal and osteogenic culture regimes was investigated using SRS and ARS for explicitly labelling of collagen and calcium deposition under these conditions. The collagenous matrix formation in electrospun PCL mesh scaffolds by hBMSCs cultured in human-derived supplemented with osteogenic reagents was much higher than in basal culture media. XF2 basal culture medium containing human serum supported significant increased collagen production on PCL scaffolds over other conditions except medium treated by osteogenic supplements. Calcified matrix formation was also significantly higher in XF2 osteogenic media compared to other groups. Mineralisation is an indispensable stage of bone formation, taking place after collagenous matrix maturation. Collagen type I is considered to be the structural framework for connective tissues and plays a fundamental role in the cascade of events and leads to the construction of new bone from stem cells (Ryu et al., 2011), and it is enforced by calcium deposition on the network of collagen. The high

amount of calcium in the scaffold reveals that the matrix has mineralised and the cells are trapped within the mineralised matrix.

Although the dexamethasone maintains the osteogenic differentiation of MSCs, it has a complication for prospective *in vivo* studies because it does not occur naturally in the body, includes delays wound healing, includes osteoporosis, increases risk of infection and diabetes mellitus (Schäcke et al., 2002; Wagner et al., 2007). Many growth factors are associated with bone regeneration and enhance wound healing within short times. They are present in plentiful in the platelets and can be used with MSCs for bone regeneration. Therefore, the explanation of the mineralised matrix formation in electrospun PCL fibre mesh scaffolds may be due to the fibres absorption and retention of growth factors from the media for a long time, and continued secretion during the construction of ECM.

The cellular differentiation typically causes discrepancies in growth rates, which may reflect disparities in the number of active cells within each donor hBMSCs population. To investigate this possibility, I evaluated levels of ALP activity in each donor population following growth for 14 days. The levels of this assay in cultures should reflect the number of osteogenic cells differentiation within the population. In our culture system, the normalised of ALP activity was expressed highly in both groups of human-derived culture medium over 10% (v/v) FBS basal and osteogenic culture medium. However, there was no significant difference between the human-derived media in all donors and conditions. This implied that the primary hBMSCs seeded on 3D PCL scaffolds and cultured in human-derived basal and osteogenic media had considerably undergone to osteoblast cells differentiation. My results were supported by Ciapetti and co-workers (2006) who reported an increase of ALP on PCL scaffolds under osteogenic stimulation. The concentration of Dex 10 nM did not substantially increase ALP activity. It is noteworthy that Wang et al., (2012) mentioned that the apoptosis levels correlated with high Dex concentration, and Dex dosage 100 nM might deteriorate MSC potential (Malladi et al., 2006). Contrary to expectations, our findings showed that human-derived basal media supported the initiation differentiation of hBMSCs to the osteogenic lineage even in the absence of dexamethasone, this may be because the formulation of human-derived culture media could contain intrinsic signalling factors that stimulate osteogenesis. The Wnt/β-catenin and Smad3 signalling pathways stimulated in the osteogenesis of human MSCs on PCL mesh mats. The Wnt signal can modulate various developmental process

like cell adhesion, gene expression, and tissue homeostasis (Clevers and Nusse, 2012; Blaschuk et al., 2002). Additionally, the Wnt/β-catenin signalling pathway represents an essential role in MSC growth and differentiation (Kim et al., 2013). Mbalaviele co-workers (2005) revealed using human and mouse genetics analysis that Wnt/β-catenin signalling supports the modulation of bone formation. Therefore, it would be interesting to observe the effects of the media examined in this thesis on the Wnt pathway.

The total collagen production of hBMSCs from different adult donors on 3D PCL scaffolds was higher in all osteogenic media in most samples. XF2 basal culture medium supported a significant deposition in collagen production on PCL scaffolds. This implies that primary hBMSCs seeded on 3D PCL scaffolds and cultured in human-derived osteogenic media had deposited an extracellular matrix. The total calcium deposition by hBMSCs cultured in XF2 osteogenic media on 3D PCL scaffolds in both donors 4 and 7was significantly higher than the other groups and followed by XF2 basal culture medium. The osteogenic supplementation ingredients were continually augmented the calcium deposition on PCL scaffolds. There were no significant signs of biodegradation of PCL were indicated after 21 days *in vitro* which confirms with the observation from Ciapetti co-workers (2006) who testified there were no significant signs of biodegradation of PCL were after 28 days *in vitro.* Interestingly, cells of donor-5 had low calcium deposition on PCL scaffolds over other donors ($P<0.05$). As a result, I suggest that the ECM construction was more efficient of collagen matrix formation than mineralisation of the ECM, this concluded lead to soft and not hard tissue formation. The results of this study showed that the presence of a human-derived growth formulation and Dex are significant factors for ameliorating of the osteogenic differentiation of hBMSCs cultured *in vitro* in 3-D culture. Indeed, the ingredients presented in human-derived basal culture media could have a sufficient level of biological activity to nurture the osteogenic induction of hBMSCs over 10% (v/v) animal-serum supplementations in the absence of exogenous osteogenic molecules like dexamethasone. Likewise, it might be considered as a novel strategy for delivering the bioactive features that support the construction of an ECM by cells in the engineered bone circumstances. The characterisation of the proteins and minerals component present within the human-derived media is paramount for understanding the compositions essential to sustain the osteogenic induction of MSCs and will be the subject of forthcoming researches.

In clinical tissue engineering, the essential question has been continuously how to transfer hMSCs to the bone defect site, and one option is to custom biodegradable scaffolds. hBMSCs from both XF2 osteogenic and basal culture media showed the bone layer formed on the whole surface of PCL scaffolds and covered all the scaffolds fibres, and produced an abundant ECM which well attached with PCL scaffold fibres and extended across of the pore architecture. Although 5% (v/v) HPL also produced the bone layer and covered the most surface area of PCL scaffolds, it did not cover the whole surface of the scaffolds whereas the 5% (v/v) HPL osteogenic media demonstrated more bone formation on surface of PCL scaffolds than its basal culture media. These results implied that the presence of human-derived formulation either with or without osteogenic supplementations could lead to enhance mineralised deposition and create a new bone layer better than 10% (v/v) FBS. Based on the SEM image, it is noticeable that the human-derived formulations could lead to enhance mineralised deposition. Thibault et al., (2010) suggested that the seeded of MSCs on PCL scaffolds cultured in 10% (v/v) FBS osteogenic media can create ECM constructs and sustain the osteogenic differentiation of cells. Based on my findings, we agreed with this data, and likewise, the human-derived basal media constructed ECM even in the absence of Dex. Human serum found in XF2 has ECM components such as vitronectin, hyaluronic acid, and fibronectin which permit attached cells and prevent apoptosis (Uhm et al., 1999). Therefore, I suggest that human-derived media particular XF2 might act as "glue" to increase the adhesiveness between the cells and fibres, and secret these crucial elements to sustain the cell potential activity, spreading and growth and support gap junction communication between cells (Wang et al., 2010; Xue et al., 2013). Schiller and co-workers (2001) and mentioned that the gap junctions induced OPN which led to mineralised ECM constructs.

XO was employed to demonstrate mineralisation of osteogenic cells. Although it has not been regularly applied, in tissue engineering it is a robust methodology to visualise tissue mineralisation in 3D culture scaffolds. In an animal experiment, the XO stain is applied for constant observing of a new bone production through injected stain into the bloodstream in the same time where it expected a newly mineralised tissue creation (Kuhn et al., 2010; Y. Wang et al., 2006). CLSM fluorescence Xylenol orange stained scaffolds confirmed mineral deposition on the PCL scaffolds. The results contain that although the mineralisation was formed in all groups, it was considerably increased with

osteogenic supplementation. Moreover, the XF2 osteogenic media was confirmed to support substantial mineral deposition compared to other conditions.

Supporting vasculogenesis is a requirement for successful bone repair and is needed to replenish nutrients and expel waste. The creation and growth of new blood vessels in PCL mesh scaffolds are considered essential for the success of implant (Kanczler and Oreffo, 2008). VEGF release was analysed to appraise the effect of experimental media on cells in electrospun PCL scaffolds. My results showed that hBMSCs from both 10% (v/v) FBS and 5% (v/v) HPL basal culture media had significantly higher VEGF level compared toXF2 basal culture medium. In contrastXF2 osteogenic medium had augmented VEGF secretion, whereas there was a considerable reduction of VEGF secretion in 10% (v/v) FBS osteogenic medium compared to its basal medium. Further work could ascertain a suitable concentration of Dex for supporting bone mineralisation and secretion of VEGF. My findings indicated that a fundamental biological component present in the human-derived media induced angiogenesis. The adding of stem cell-based therapies to the PCL mesh scaffold will ultimately yield vascular and osteogenic precursors that will intensify the development of tissue-engineered structure.

The periosteal layer is considered to be an optimal source of stem cells for augmenting of bone healing (Agata et al., 2007; Zhang et al., 2006; Zhu et al., 2006) and clinically, the bone does not heal without a periosteal layer (Zhang et al., 2008). The primary essential components of periosteum have been grown on hyaluronic acid nanofibers (Ji et al., 2006). Polymers that permit adherence to bone (or used to manufacture scaffolds for bone tissue engineering) will possibly be the more applicable for growing periosteum (Reed et al., 2009) though no specific work has examined periosteal growth on PCL scaffolds cultured in xeno-free media. HJPs are a suitable stem cell source for future studies; the limitation in this study was that there was a limited cell supply for further experiments. Schimming and Schmelzeisen, (2004) and Schmelzeisen with co-workers (2003) demonstrated that HJP cells seeded in 3D composite scaffolds could form a new bone that supports the dental implant and maxillary augmentation. Ryu and colleagues (2011) also exhibited that HJP cells enhance the mineralisation of ECM in 3D collagen scaffolds *in vitro*. The results collected from these studies revealed consistently with my outcomes that MSC derived from bone marrow and jaw periosteum needs 10 nM of Dex to enhance osteogenic differentiation. Reed with colleagues (2009) showed that

microfibrous PCL scaffolds require mimicking ECM structural design to enhance the cells proliferation, adhesion, migration, and differentiation osteogenically. Furthermore, this research indicated that HJP cells could proliferate and differentiate to the osteoblasts in 3D PCL scaffolds.

5.13 Conclusion

The primary objective of this chapter was to assess suitable human-derived medium on hMSCs cultured on electrospun PCL scaffolds to sustain growth and osteogenically stimulate repair for the bone part of cleft palate. This objective was accomplished that PCL scaffolds could be an appropriate material as it exhibited biocompatible and permitted progenitor cells differentiation osteogenically. We further supported that the long-term goal of using human-derived media to create a bone layer on PCL scaffolds for repairing of cleft palate and eventually minimize the quantity of surgical operations required for cleft palate reconstruction and drop the postoperative complications. The next objective was to investigate the ability of human-derived culture medium for increasing osteogenic differentiation of osteoprogenitor cells on electrospun PCL mesh scaffolds. This objective was supported that xeno-free media encouraged the human serum (XF2) and human platelet lysate could support both hMSC and HJP cell proliferation with increased mineralization on electrospun PCL scaffolds even in the absence of dexamethasone. The other objective was to evaluate the different donors' differences in cell proliferation, expansion, and osteogenic stimulation to generate the ECM construct. Although our results indicated that total mineral deposition of hMSCs within the PCL scaffolds in all donors was sustained and increased, there was differential results between cells from different donors for example cell of one donor increased in collagen formation but reduced in calcium deposition. CLSM fluorescence images of culture hBMSCs in human-derived osteogenic media confirmed mineral deposition was presented on the cells seeded PCL scaffolds. The VEGF secretion of hBMSCs by osteogenic supplementations was inhibited in 10% (v/v) FBS and XF2, while no effect could be seen in 5% (v/v) HPL. The human bioactive molecules contained in human-derived formulations media particular in XF2 were a therapeutically meaningful pre-clinic medium and a safe substitution for propagation, expansion, and osteogenic stimulation of hMSCs with and without a requirement of exogenous drugs.

5.14 Summary

- The PCL electrospinning mats are compatible with the engineering of bone tissue.
- Both hBMSCs and HJPs are suitable for cell sources to create a bone matrix in the electrospun PCL scaffolds; therefore, they are a beneficial model cell for further research.
- hBMSCs and HJPs on PCL scaffolds cultured in human-derived media were induced to significantly increase mineralization.
- A completed thin layer of bone covered the top surface of the electrospun PCL scaffolds with deeply distributed hBMSCs detected on the SEM images, and the greatest amount of ECM was seen in both human-derived media supplemented with osteogenic molecules and basal culture of human-derived formulations.
- The different donors were affected differently by stimulates of *vitro*-generated matrix mineralisation.
- The application of hBMSCs cultivated on the electrospun PCL scaffolds using human-derived media can have formed a structural of the bone matrix creation that can implant to the bone defect of the cleft palate.
- CLSM fluorescence images of culture hBMSCs confirmed mineral deposition was presented on the PCL scaffold.

5.15 Future work

- Future-upcoming studies could promote appraise osteogenically differentiation of cells seeded on electrospun PCL fibres scaffolds cultured in human-derived medium by determining osteogenic gene expression.
- Altered concentrations of PCL scaffolds could be formed to evaluate the suitable PCL electrospun scaffold for improving osteogenic differentiation such as 2.5 or 7.5 wt% PCL.
- Evaluate the electrospun nanofibers PCL scaffolds as a membrane for improving osteogenic differentiation.
- Different concentrations of human serum and HPL could be applied and the osteoblasts-like cell differentiation on 3D culture assessed.
- To determine the increasing mineralization of cell numbers on the top level of scaffolds and VEGF secretion on osteoprogenitor cells in the absence of exogenous osteogenic drug delivery such as Dex.
- The cell distribution should be determined throughout scaffolds culture in human-derived formulations.
- The comparison of the effectiveness of PCL scaffolds on the mineralization of cells with other scaffolds that use in clinical applications.
- Future work could be necessary to realise completely the effect of human-derived medium on hMSCs biology *in vivo*.
- More investigation of HJP cells behave in human-derived media to increase propagation and enhance mineralisation.

CHAPTER SIX: GENERATION OF TRI-LAYER POLY (ε-CARPOLACTONE) SCAFFOLDS TO CREATE ORAL MUCOSA AND BONE CO-CULTURES FOR CLEFT PALATE REPAIR

6.1 Introduction

The common obstacle facing oral and maxillofacial surgeons during palatal closure is often the deficiency of oral mucosa. After palatal closure the most important issues are midface growth inhibition because of soft tissue intrusion and scar tissue formation at the palatal and alveolar regions (Moharamzadeh et al., 2012; Payne et al., 2014). To overcome this unmet clinical need evaluation of an alternative cell source for bone augmentation to enhance the cleft palate repair is warranted (Bueno et al., 2009; Lohberger et al., 2013). Some tissue engineering approaches have focussed on utilising the patient's own MSCs with the results demonstrating much potential for the cells in cleft defect repair in the alveolar ridge region. Hibi et al. (2006) successfully undertook a cleft repair for a 9-year old female patient, bone formation with the support of the canine eruption at the anterior segment of alveolar bone occurred in postoperative two years. A successful of the grafted region for repairing of cleft palate need to close the communication between the oral and nasal cavities. The repaired region should enable healthy orofacial development with bone growth and to avoid soft tissue migration into the bony cleft walls inside the cavity.

PCL electrospun scaffolds should be biocompatible, biodegradable, interconnected pore network, and the appropriate mechanical strength to encourage the healthy growth of soft and bone tissue (Cipitria et al., 2011; Delaine-smith et al., 2014; Reed et al., 2009). Recently, a spinning tri-layer scaffolds technique has been developed using a nanofibre layer as a barrier between two microfibre layers (Bye et al., 2013; Puwanun, 2014). The tri-layer electrospun PCL scaffolds may be suitable for supporting two different cell types while maintaining their separation by the incorporation of the nanofibrous layer compared to a specific time of culture media.

Bone tissue engineering combined with oral mucosa has appreciably been achieved autonomously. The essential purpose of this chapter was to create and investigate a novel combination of the bone (top layer) and soft tissue (bottom layer) with barrier to

separate them to create a multi-tissue PCL scaffolds for reconstruction of cleft palate *in vitro*. The objective of this chapter was to investigate the simple, unique process to fabricate 3D trilayer electrospun PCL scaffolds then, to assess whether they can assist cell metabolic activity, attachment and adhesion, subsequent proliferation and cells differentiation. Thus, I tested whether the tri-layer PCL scaffolds can encourage the engineered bone matrix establishment and separate three different types of cells include hBMSCs and both immortalised oral keratinocytes (FNB6) with normal oral fibroblast (NOF).

6.2 Methods

6.2.1 Culture of immortalised oral keratinocytes cell line (FNB6)

FNB an immortalised oral keratinocyte cell line originally isolated from buccal mucosa, which was acquired from the School of Clinical Dentistry, University of Sheffield. Cells were chosen to avoid the shortage of gingival tissue resection clinically and the variation of inter-donors that construct a beneficial and consistent pattern for normal oral epithelial cells. The morphology and proliferation of cell were observed using a light microscope. The cells were cultured in Green's medium and changed every 2-3 days. Cells were passaged when they reached 80% confluence.

6.2.2 Culture of normal oral fibroblasts (NOF)

Normal oral fibroblasts cells were acquired from the School of Clinical Dentistry, University of Sheffield. These cells were selected as an alternative of dermal cell and to produce a valuable model of normal oral fibroblasts during tissue engineering of oral mucosa construction. The morphology and proliferation of cell were observed using a light microscope. The cells were typically cultured in DMEM supplemented with 10 % FCS (v/v) and changed every 2-3 days. Cells were passaged when they reached 80% confluence.

6.2.3 Tri-layer PCL scaffolds fabrication

A 10% (w/w) PCL micro-fibre solution was created by dissolving PCL Mn=80,000 in DCM. A 5% (w/w) PCL nano-fibre solution was performed by dissolving PCL in a mixture of methanol and DCM in the weight ratio of 1:9 (methanol/DCM). Both solutions were put

in a water bath for 1 hour and then left on a magnetic stirrer at room temperature to ensure homogeneity.

The fabricated tri-layer PCL scaffolds were fabricated in an electrospinning rig as previously described in chapter 2 (Bye et al., 2013; Delaine-Smith et al., 2014). Briefly; a tri-layer of PCL microfibres was fabricated at 17kV, a working distance of 17cm, a flow rate of 40µL/min, and a 300 rpm of drum rotation speed at room temperature. The solutions were released by 12 needles. The parameters of the nano-fibre electrospinning were the same as the microfibre scaffolds (above) except that the working distance was 10 cm. Scaffolds were developed using 40 mLs of 10% (w/w) PCL polymer solution to create the first layer of the micro-fibres. Then 20 mLs of 5% (w/w) PCL polymer solution was dispensed to create a middle layer (the nano-fibres), while 40 mLs of 10% (w/w) PCL polymer solution was continuously spun to produce the third layer of the micro-fibres (Figure 6.2.3).

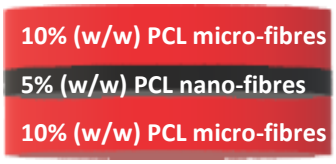


Figure 6.2.3: *Schematic of the final tri-layered scaffold created with three layers: microfibers (red), nanofibers (black), and microfibers. These layers are made from poly (caprolactone) with different concentrations.*

6.2.4 Cell seeding on tri-layer PCL scaffolds

Three different cell types were used in these experiments: 1) hBMSCs, 2) NOFs and 3) FNB6. The scaffolds were cut and mounted on a CellCrown™ and sterilised as previously described in chapter 2. Briefly, after sterilisation with 80 % (v/v) ethanol, the samples were immersed within a well plate in two different basal culture media (XF2 and Green's media) to enhance cell attachment and incubated at 37°C in 5% CO_2 humidified environment. Before cell seeding, the basal culture media was removed and hBMSC (2 x 10^5) in 100 µL directly seeded onto the top of the electrospun tri-layer PCL scaffold within the inner diameter of the CellCrown™ and incubated for 1 hour to enable the cells to attach. 2 mL of both of the two experimental media was added and the cultures incubated for 24 hours at 37°C. After this incubation period, the PCL scaffolds mounted on the CellCrowns™ were moved to fresh well plates and submerged in fresh culture media

supplemented with osteogenic supplementation (Dex 10 nM, AA 50 µm, and βGP 10 mM) on day 1 and cultured for a further 10 days with twice-weekly media changes.

At day 10, the media was removed, and NOF (5×10^5) in 100 µL seeded onto the other side of tri-layer PCL scaffolds and left for 1 hour for attachment. 5 mL of both of the two osteogenic and basal culture media was added and cultured for 24 hours. After 24 hours, the CellCrowns™ were transferred to fresh well plates and cultured for 5 days before the addition of FNB6 (5×10^5) in 100 µL to the upper layer. After 5 days, the cultures were raised to air to the liquid interface for 7 days with media changes twice weekly.

6.2.5 Fixing the tri-layer PCL scaffolds seeded cells

The scaffolds were fixed in 2 mL of 4% (v/v) paraformaldehyde (PFA) for 20 min then removed it and washed with PBS and finally stored in PBS at 4° C. To prepare 4% (v/v) PFA, 100 mL of diH_2O was heated to 60°C 4 g of PFA was added and stirred until dissolved after which 1 mL NaOH (1M) was added with continual stirring until the solution became clear.

In preparation for cryosection, the scaffolds were bisected. One section was fixed while the second section was prepared for cryosectioning. The scaffolds were embedded in optimal cutting temperature (OCT) and submerged in liquid nitrogen. 6–30µm thick sections were cut using a cryostat at -20°C. The cut sections were immediately transferred to Poly-L-lysine-coated microscope slide before staining with DAPI (Fischer et al., 2008).

6.3 Results

The purpose was to observe the morphology and evaluate qualitatively the behaviour of the cells cultured in different media under a microscope.

6.3.1 Evaluation of the morphology and growth of immortalised oral keratinocytes cell line (FNB6) in osteogenic media

To ensure that epithelial growth could be supported in the media used within this study, the cells were transferred from Green's media into the experimental media using stepwise reductions (v/v): (100%, 50%:50%) compared to a 4-day culture period. Cell morphology was assessed using an inverted light microscope. Cell proliferation was supported in all conditions, the characteristic features of the morphology of cells at high confluence were noted with cobblestone-like in shape and consistent with standard culture conditions. (Figure 6.3.1).

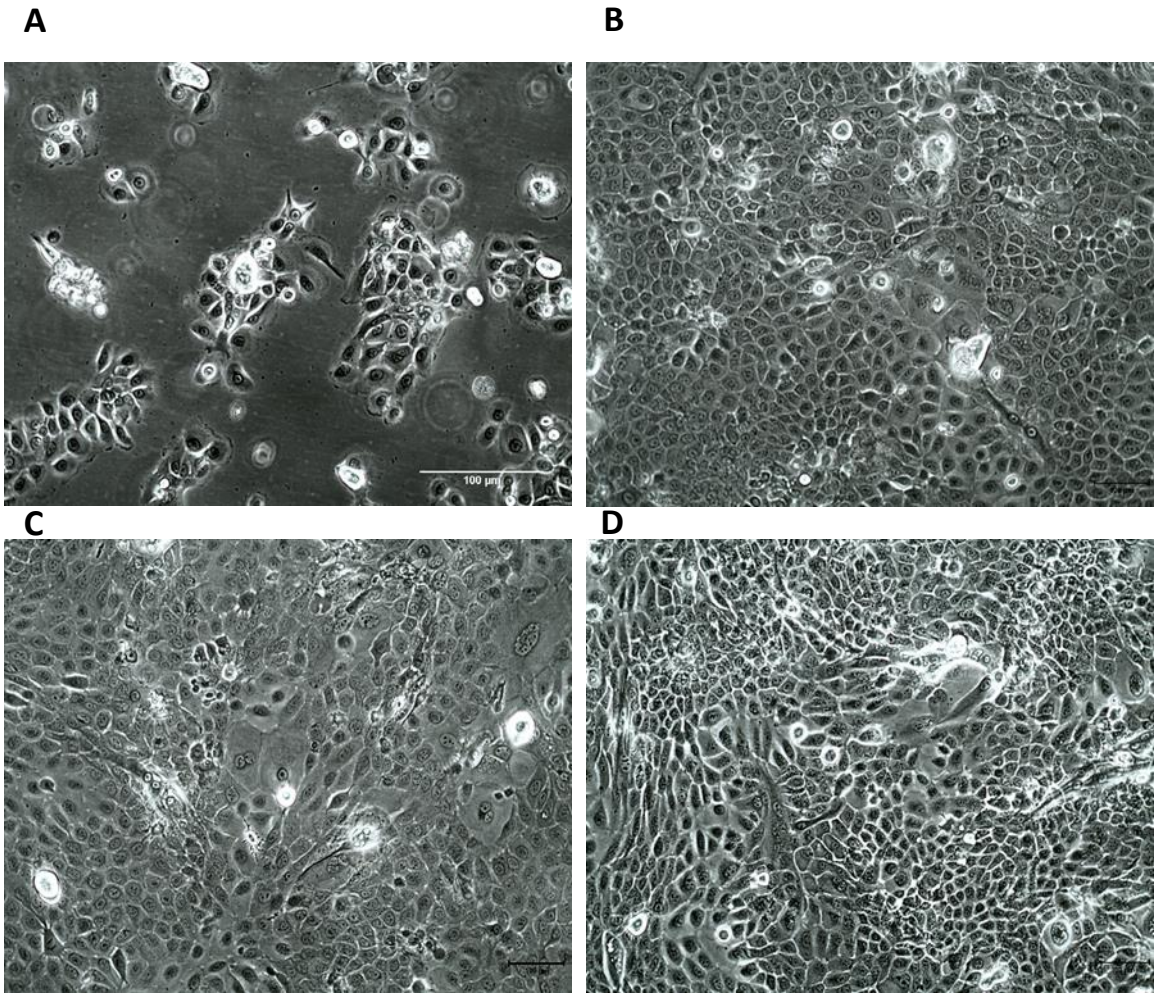


Figure 6.3.1: *Morphology of FNB6 cells in a commercial human-derived basal culture media (XF2) compared to Green's media from 1 to 4 days. (A) Control Green's media at 24 hours while (B) control Green's media on day 4, (C) 10% (v/v) FBS (α-MEM supplemented with 10% (v/v) of FBS), and (D) XF2. Scale bar = 100 µm, n=3*

6.3.2 Evaluation of the morphology and growth of normal oral fibroblast (NOF) in osteogenic culture media

Normal oral fibroblasts are typically cultured in DMEM supplemented with 10% (v/v) FCS. To ensure that the media in this study had no detrimental effect on NOF growth cells were transferred into the experimental media using stepwise reductions (v/v): (100%, 50%:50%) compared to a 4-day culture period. Cell morphology was assessed using an inverted light microscope at high confluence cells had an elongated, spindle, fusiform morphology, consistent with standard culture conditions. Cell proliferation was supported in all conditions with confluence reached quickest in XF2 compared to 10% (v/v) DMEM and alpha-MEM (Figure 6.3.2).

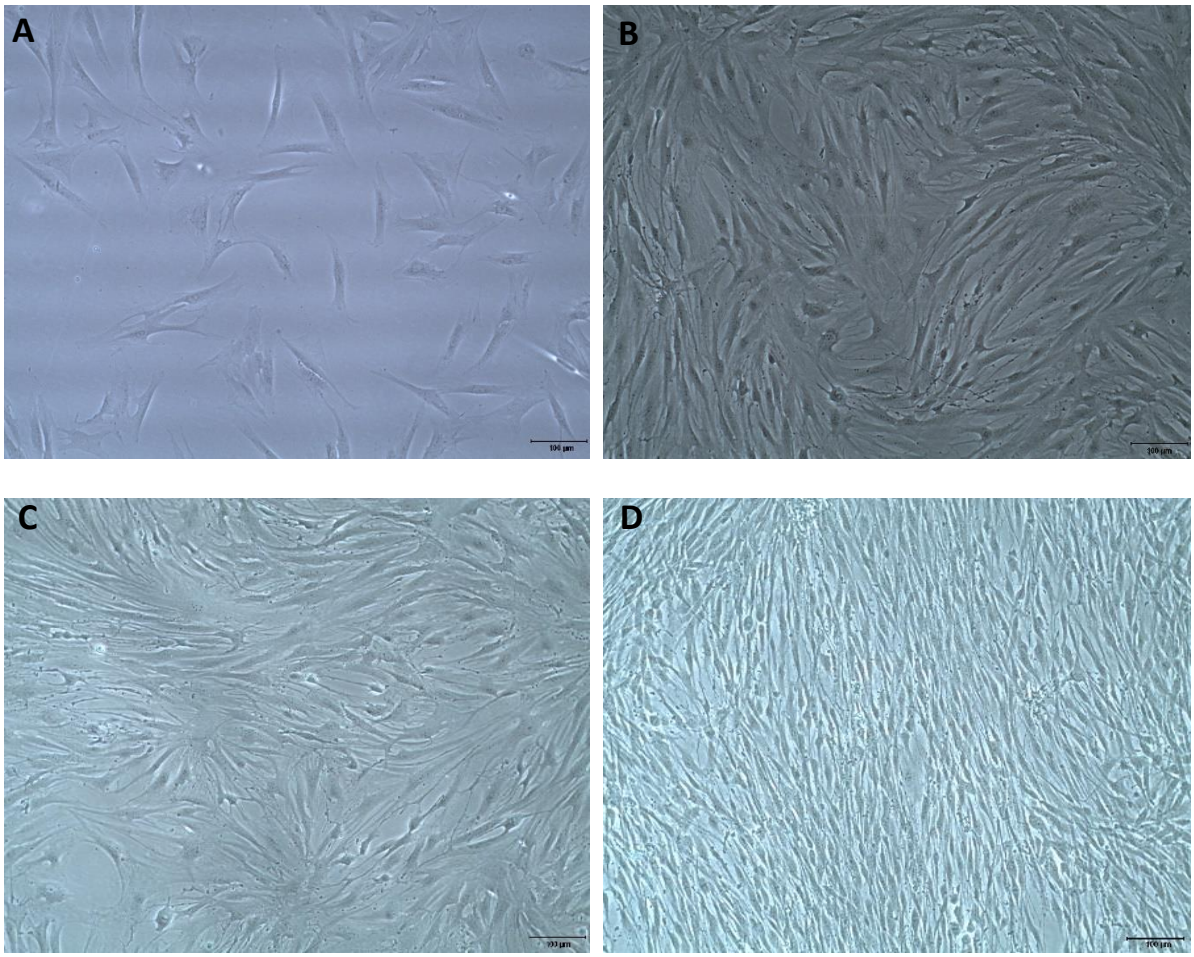


Figure 6.3.2: *Morphology of NOFs cultured in a commercial human-derived basal culture media compared with 10% (v/v) FBS-DMEM media (v/v) from 1 to 4 days. (A) control 10% (v/v) FBS-DMEM media at 24 hours while (B) control 10% (v/v) FBS-DMEM media on day 4,*

(C) 10% (v/v) FBS (α-MEM supplemented with 10% (v/v) of FBS), and (D) XF2. Scale bar = 100 µm, n=3

6.3.3 Evaluation of the morphology and growth of hBMSCs in Green's media and XF2

hBMSCs passage 4 were seeded in at a density of 1 x 10^4 cell/cm^2 in Green's media (as a described in chapter two), XF2, and 10% (v/v) FBS (as a control) media for 4 days. The morphology of the cells adhesion was similar to NOF cells. The results showed that the cells cultured in XF2 had higher cell proliferation compared to both the control media and Green's media. The cells cultured in Green's media had marginally increased in density compared to the control media at the end of the period (Figure 6.3.3).

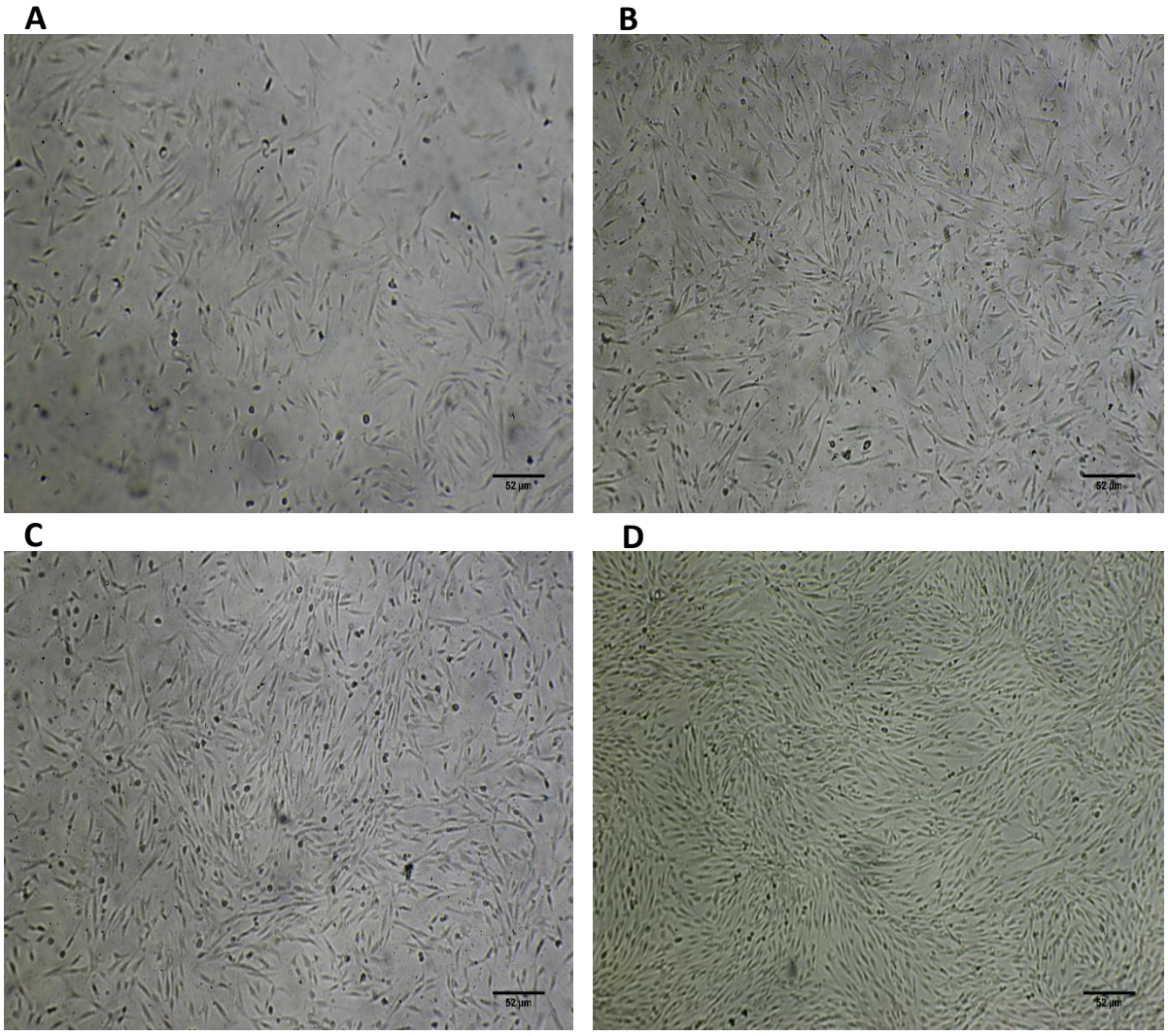


Figure 6.3.3: *Morphology of hBMSCs cultured in Green's media and XF2 media compared with 10% (v/v) FBS media from 1 to 4 days. (A) Control 10% (v/v) FBS media at 24 hours while (B) control 10% (v/v) FBS media on day 4, (C) Green's media, (D) XF2 media. Scale bar = 52 µm, n=3*

6.4 Examination of the cell seeded tri-layer PCL scaffolds

Examination of the tri-layer PCL scaffolds using light microscope after 24 days revealed a dense dark layer on the middle of scaffolds which represented the nano-fibrous separating membrane (Figure 6.4 A, red arrowheads). When, the tri-layer PCL scaffolds were visualised using a fluorescence microscope a dense layer could be seen which likely represents a barrier region (Figure 6.4 B, red arrowheads). A nuclear stain (DAPI) was used to detect cells. Cells were seen on both sides of tri-layer PCL scaffolds in XF2 osteogenic media (Figure 6.4 C, yellow arrowheads). This implies that XF2 osteogenic media with 10 nM Dex supported and encouraged both bone tissue and oral mucosa tissue. Although the Green's osteogenic media also showed a build–up of a layer of cells on one side, this was not seen on the other (Figure 6.4 D, yellow arrowheads). The limitation of this result was that it difficult to distinguish the layer of bone cells from the layer of oral mucosa tissues. Furthermore, I did not perform the characterisation, therefore, it seems that the layers differ only in concentration of PCL solution used in the dispensing liquid.

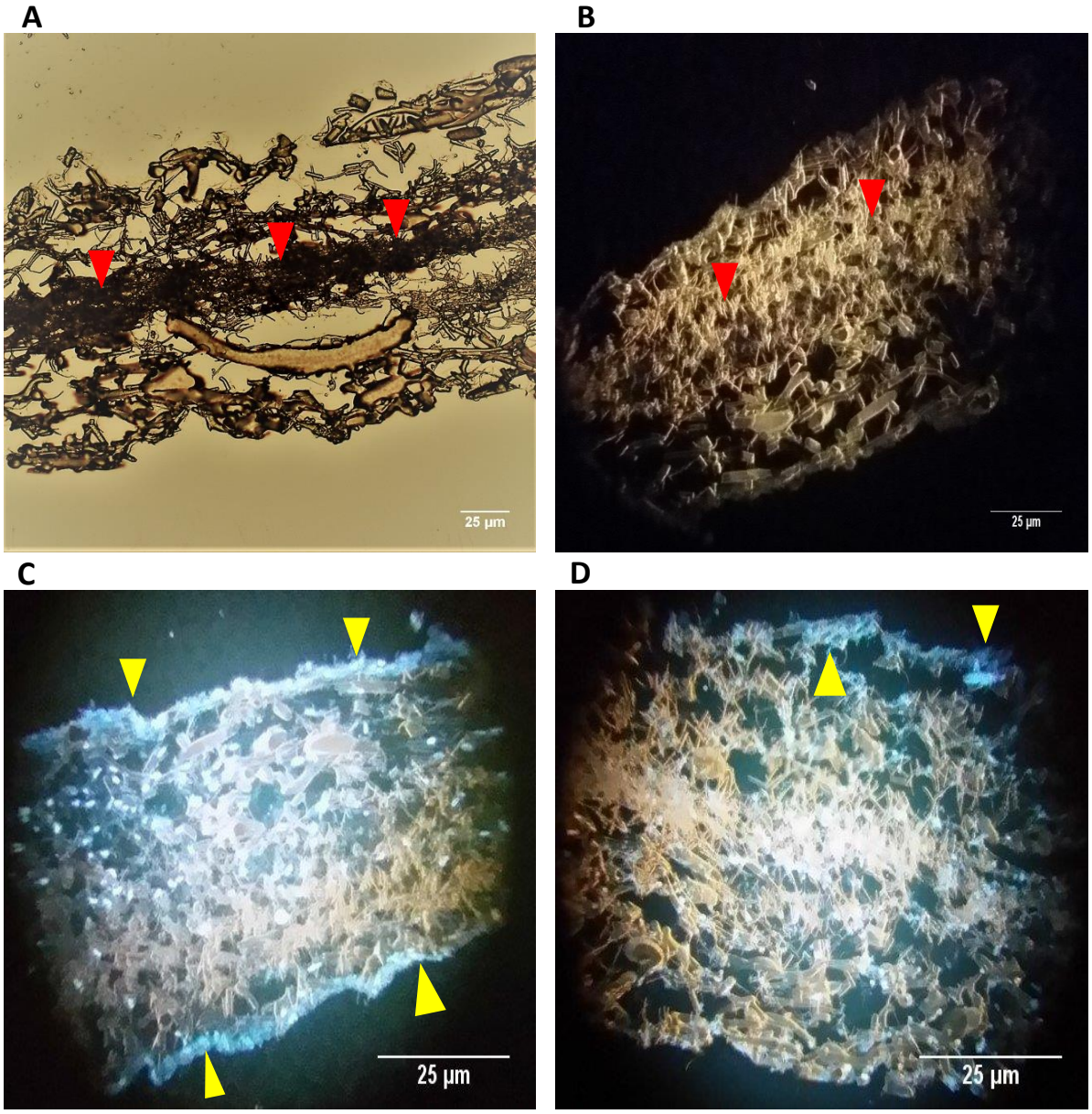


Figure 6.4: *Examination of the lateral aspect of the tri-layer PCL scaffolds co-cultured with hBMSCs and NOFs and FNB6 cells. Cell presence was assessed by labelling with DAPI (pale blue) after 24 days of culture. (A and B) control scaffolds under an inverted light microscope and fluorescent microscope respectively, the red arrowheads indicate the middle layer. (C and D) Tri-layer PCL scaffolds after 28 days with cells cultured in osteogenic media (Dex 10 nM) XF2 and Green's media respectively, the yellow arrowheads indicated the layer of cell growth. Scale bar = 25 µm, n=3*

6.5 Discussion

The purpose of cleft palate treatment is to repair both the bone and soft tissue. The previous chapter discussed how to manage the bone defect via support of the osteoblasts and creation of a bone layer in the 3D PCL scaffolds. Consequently, in this chapter, I have exhibited the implementation of a tissue engineering method for treating of cleft palate through creating a single trilayer electrospun PCL scaffold to encourage osteoblast and fibroblast growth with the perspective of filling the cleft defect.

The notions of segregation of tissue in regenerative treatments are not new. They have been recognised in dentistry where a guided tissue has been created to separate the bone forming tissue from the soft tissue in periodontal regeneration (Moreau et al., 2007; Retzepi and Donos, 2010) such as collagen membrane, guided tissue and guided bone regeneration (GTR and GBR), Teflon, and biodegradable polymers (Lundgren and Slotte, 1999; Sabir et al., 2009). Although the commercial collagen membrane has good biocompatibility and a rapid biodegradation rate, it can be problematic to remove if there are problems and can introduce a risk of prion disease transmission if extracted from animal tissue (bovine or porcine) (51). Consequently, the material sources used in clinical tissue engineering treatment must be mindful to avoid any risk of xenoantigens transmitted to the recipient (Oikonomopoulos et al., 2015) (51).

The technique of electrospinning was selected to fabricate the tissue engineered scaffolds because it has a versatility for creating the different diameters of polymer fibres from nano-fibres to micro-fibres, it can be functionalised for many purposes, has good mechanical properties, and can be used with different kinds of polymers such as poly-D-lactic acid (PLA) and poly-glycolic acid (PGA). However, the drawback of electrospinning is the low rate of production (Rim et al., 2013). Bye and colleagues (2013) successfully produced tri-layer scaffolds using polyhydroxybutyrate-polyhydroxyvalerate (PHBV) for the nano-fibrous membranes and PLA for the micro-fibres membranes. However, the polymers used were not optimal for producing bone tissue because of the rapid degradation rate of PLA. Many researchers used a composite scaffold for bearing a different cell type such as ceramic/collagen. Herein, the technique was adjusted to create a trilayer scaffolds from PCL following the methods of Bye and colleagues.

Observing of the cryosection of the tri-layer of PCL scaffolds under the microscope indicated that the middle layer was dense indicating successful deposition of the nano-fibrous layer. The aim is that this layer would act as the separation membrane and prevent cells to cross to the opposite side of the scaffold. Whereas, the top and bottom layers contained mostly micro-fibres and were occupied by hBMSCs on one side and both NOF and FNB6 cells on the opposite side, assuming that the cells remained segregated according to how they had been seeded. The shortcoming of this research is that it only used one experiment in triplicate. Furthermore, although the samples of PCL cryosection were immediately transferred to the Poly-L-lysine-coated microscope slide, they were physically unstable, fragile and easily removed from the coated slide.

In soft tissue regeneration, Puwanun (2014) and Hutmacher et al., (2001) previously chose human dermal fibroblasts (HDFs which are responsible for repairing connective tissue in skin injury) to produce the soft tissue layer on PCL scaffolds, and the result showed that the PCL scaffolds could encourage HDFs growth. However, in the clinical application of the soft tissue correction it would be better to use oral fibroblasts harvested from oral mucosa which can be collected from either gingival biopsies or the first surgical step for the closure of cleft lip (cheiloplasty).

Prior to this study it was not known how the NOF and FNB6 cells would grow in the xeno-free media used in this study. The cell proliferation of NOF and FNB6 cells indicated that the human-derived formulation media support them in a similar way to control media and supported superior growth of cells compared to 10% (v/v) FBS basal media. These results agreed with Tsugeno et al., (2014) who suggested that human gingival fibroblasts grow well when cultured in STK media (chemically-defined serum-free media) during 4 days.

DAPI fluorescent staining was utilised to detect and visualise the position of hBMSCs and oral mucosa cells. Although the images look strange and there is not the usual blue colour this could be because of the thickness of the scaffold, DAPI images exhibited that cells co-cultured on tri-layer PCL scaffolds can sustain separation on their specific regions; the dense dark layer in the middle region indicates that there was no cells migration in this region. However, some cell displacement into the trilayer PCL scaffolds was observed. This supports my hypothesis that the middle layer obstructs the crossover of cells in the

scaffolds because of its low porosity and is in agreement with Puwanun (2014) results on tri-layer membranes made from the same polymer and other polymers Bye et al. (2013).

6.6 Conclusion

This study provides preliminary evidence, that tri-layer PCL scaffolds could preserve and encourage the multi-culture of hBMSCs (on top) and both NOF with FNB6 cells (on bottom) and permits to the differentiation of osteogenic progenitor hBMSC cells. The scaffold does not permit the different cells to migrate to the opposite side during 24 days, probably because of the nanofibre structures of the middle layer of the tri-layer PCL scaffold, which has low porosity so is able to isolate different cells. The interest of this tri-layer PCL scaffold could be in its ability to assist and isolate of hard and soft tissue until a newly healed tissue becomes mature. I suggest that the healing of the soft tissue would be faster and prevent movement to the bone tissue area that indicates slower healing. This could decrease the amount of wound tension created from the scar at the surgical area after the surgery of cleft palate, reduce the inhibition of the palate growth that occurs when a bone graft is implemented too early. The tri-layer PCL membrane may have a beneficial in reconstructive oral and craniofacial surgery that comprises multi-types of tissue such as GTR "guided tissue regeneration" in periodontology and alveolar ridge augmentation for a dental implant.

6.7 Summary

- PCL polymer fabricated trilayer electrospun fibre mat was revealed to sustain cell metabolic activity, attachment, and permit subsequent proliferation and cell differentiation compared to 24 days, in the meantime, it assisted segregation between bone formation and oral mucosa cells.
- Trilayer PCL electrospun scaffold is considered as a suitable basement membrane for promoting the intrinsic repair of cleft palate.

CHAPTER SEVEN: CONCLUSIONS AND FUTURE DIRECTION

The cleft palate therapy is one of the considerable challenges to face the orofacial surgeon and has several shortcomings such as numerous surgical interventions advance, the long time needed to complete the treatment, and donor site morbidity from the bone harvesting procedure. The perspective of using strategy of bone tissue engineering remains to increase the attention of the oral and craniofacial surgeon, may conquer the impediments to successful surgery through combination of biomaterial engineering scaffolds and other biochemical reagents with cells transplantation to emerge as new techniques for reconstructive and repairing of craniofacial bone and cleft palate.

7.1 Primary human bone marrow mesenchymal stromal stem cells

Human BMSCs are commonly applied in the engineering of bone. Chemical reagents scaffold topography and characteristics of surface coatings can stimulate hBMSCs differentiation. My results emphasized on hBMSCs and HJPs are able to osteogenesis through the ability of cells to differentiate into osteoblasts and deposit collagen and calcium on their ECM cultured in different xeno-free media and supplementations. Interestingly, all these cell types dramatically increased their proliferation, differentiation, and ECM deposition when they were cultured in human-derived formulations compared to 10% (v/v) FBS culture in both 2D monolayer and 3D scaffolds, although the size of the difference depended on donor and supplements used. HJPs have recently been used in bone tissue engineering because they can be straightforwardly harvested from the oral periosteum with minimum oral surgery compared to harvesting of bone marrow mesenchymal stem cells.

Dexamethasone (Dex) is a glucocorticoid which clinically exerts an anti-inflammatory and immunosuppressant effect and is used for the treatment of different systemic diseases. Dex is widely used *in vitro* to induce early cell differentiation for a range of mesenchymal lineages. The mechanism of Dex is via its action on glucocorticoid receptors. Dex can have a paradoxical biphasic influence through increased proliferation after exposure to low-dose Dex and inhibition of growth after exposure to high-dose Dex on hBMSC and HJP cells. My results confirm that Dex influences the physiological function of hBMSC and HJP cells in vitro.

VEGF is a polypeptide substance derived from platelets and osteoblast cells to stimulate new blood vessels during wound healing. Insufficient vascularisation is a common feature of non-healing wounds and bone graft failure. Vascularisation is essential to the success of the engineered bone tissue at the site of an implant as it encourages the neighbouring endothelial cells to create new blood vessels to penetrate into the construct. My experiments on primary hBMSCs seeded in electrospun 3D PCL scaffolds on day 21 indicated that osteogenic media in 10% (v/v) FBS significantly inhibited the secretion of VEGF although it did not completely diminish. Whereas osteogenic human-derived media, specifically, 5% (v/v) HPL significantly promoted angiogenesis indicating they would support creation of blood vessels inside the 3D electrospun PCL scaffolds.

Mesenchymal stem cells are the best choice for bone tissue engineering because they can differentiate into different lineages. However, the best source of stem cells cannot easily recognise because the performance of extracted cells can differ based on several factors such as the site of the defect (either craniofacial or long bone), patient age, medical state, the cell collection approaches, and cell passages. The donor source from which cells were obtained affected cell proliferation, differentiation and in vitro-generated matrix mineralisation in 2D monolayer and 3D electrospun PCL scaffolds. In the cleft palate treatment, it requires further effort to elucidate what would be the best source of appropriate cells.

Future investigation is required:

- Evaluating the surface markers of osteogenic differentiation of cells such as cytokines, antigens, and genes expression of implicated in the early and late steps of cell differentiation.
- Evaluating the of the osteoprogenitor cells differentiation osteogenically from many various anatomic harvesting regions.
- Appraising the quality of mineralisation relative to a real bone by FT-IR, XRD, or EDX.

7.2 Xeno-free media

Xeno-free media defined in terms of cell culture is a media extracted from different sources, particularly human, which does not contain any animal-derived components. Xeno-free media has been shown to enhance cell proliferation, ECM, and calcium deposition in different cell types. My study used different commercial xeno-free media

for culture of two cells types, hBMSCs and HJPs, then compared this to 10% (v/v) foetal bovine serum (as a control) on both monolayer and 3D culture. My results showed that a type of media containing human serum commercially supplied as 'human mesenchymal-XF expansion medium' (XF2) with supplementation could significantly enhance both cells type's proliferation and osteogenic differentiation and support ECM construction by enriching calcium deposition and collagen formation compared to the animal-derived serum supplementation. XF2 augmented the metabolic activity, increased the ALP with mineralisation, and DNA quantity of hBMSCs to a greater extent compared to any other culture condition tested here. In addition, it was confirmed that a suitable Dex concentration for hBMSC is 10 nM, which induces the highest cell maintenance. While the 100 nM of Dex concentration causes a reduction in cell number, this may be related to the physiological concentration of glucocorticoids, which has also been reported to be around is 10 nM. Hence, XF2 may be a useful xeno-free pre-clinical medium for proliferation and differentiation of hBMSCs towards therapeutic bone differentiation for tissue engineering applications.

Osteogenic differentiation of hBMSCs was poor in 'stem x vivoTM xeno-free human MSC expansion media' (XF1), XF1 and these cells exhibited an insufficient proliferation and early osteogenic differentiation marker compared to the XF2 and 10% (v/v) FBS.

Many factors will influence the cellular response to a media composition such as cell source (donor), and the biological and chemical supplements used to stimulate osteogenesis. Providing the appropriate balance of these factors could enable scale-up of clinical applications for bone graft substitutes during cleft palate repair and engineered bone tissue construction. Improvement of the methods to promote osteogenesis and vasculogenesis by the cells, would substantially improve integration between the construct acting as a bone graft substitute and the surrounding implanted region.

Further investigation will be needed to:

- Characterise of the biological activity present when cells are grown in human-derived medium to establish if this is sufficient to induce osteogenic differentiation.
- Investigate fresh human-derived media collected from donors to support the cells and increase mineralisation

- HJPs indicated variability in growth rate and differentiation properties. Thus, it would be interesting to investigate the osteogenic potential of HJPs from more donors to compare the outcomes.

7.3 Three-dimensional model platform for understanding the bone tissue engineering

Several methodologies have been performed to fabricate the suitable scaffolds to restore the tissue damage by repairing the function. In the last decade, electrospinning has been used in the regeneration of different tissue because it is easy to fabricate a wide size of fibrous diameter and can be adjusted to modify a quantity of parameter. In my experiments, 3D electrospun PCL scaffolds were applied because their good processability and mechanical strength are preferred for bone reconstructions and they are well biocompatibility with neighbouring implanted structure. They can assist and maintain the hBMSC and HJP cells for propagation, expansion, and differentiation to the osteoblast-like cell with 10 nM Dex concentration.

On the other hand, the limitations of PCL scaffolds are that they have neither osteoconductive nor osteoinductive characteristics. Based on a previous study, I choose the 10wt% PCL electrospun scaffold in my experiments, which was revealed to be an appropriate membrane for supporting cell, propagation, and improving osteogenesis. I found that HJPs and hBMSCs could form the bone matrix in electrospun PCL scaffolds. Moreover, HJPs and hBMSCs seeded on PCL scaffolds cultured in human-derived media induced a significantly higher mineralised matrix deposition than 10% (v/v) FBS.

The SEM images showed a completed layer of bone covered the top surface of the electrospun PCL scaffolds with hBMSCs distributed throughout. Furthermore, the greatest matrix mineralisation was induced in the human-derived media supplemented with osteogenic substances and basal culture of human-derived formulations. Likewise, donor source affected in *vitro*-generated matrix mineralisation. The results indicated that total mineral deposition of hBMSCs within the PCL scaffolds in all donors was sustained and increased. VEGF secretion of hBMSCs by osteogenic supplementation was inhibited in 10% (v/v) FBS and enhanced in XF2 compared to basal culture media, while no effect could be seen in 5% (v/v) HPL. Therefore, the osteogenic media of human-derived media supported vasculogenesis in the scaffolds. Thus, this study could be open the gate to solve a significant obstacle of large-scale tissue-engineered grafts, which is a deficiency of new

vasculature inside the graft. Interestingly, the individual bioactive molecules contained in human-derived formulation media particular in XF2 were a therapeutically meaningful pre-clinic medium and a safe substitution for proliferation, expansion, and osteoblast-like cells differentiation with and without a requirement of exogenous drugs.

Further investigation will be needed to:

- Appraise the osteogenic differentiation of the cells seeded on PCL scaffolds culture in the human-derived medium by investigating osteogenic gene expression.
- Evaluate the electrospun microfibers PCL scaffolds as a membrane for improving osteogenic differentiation in vivo
- Assess different concentrations of human serum and HPL in 3D culture to verify whether I have indeed identified the best concentrations for osteogenesis.
- Determine whether the higher mineralisation and VEGF secretion observed on scaffolds can be routinely produced in the absence of exogenous osteogenic inducers such as dexamethasone.
- Compare the effect of PCL scaffolds on the mineralisation of cells with other scaffolds used in clinical applications.
- Future work could be necessary to understand the effect of human-derived medium on hBMSCs biology in vivo.

7.4 Trilayer PCL fibrous mat scaffolds

Several kinds of research have addressed the formulation of tissue engineering strategies for oral and maxillofacial reconstruction and characterise what architectures of scaffold and materials would have the best function. In this respect, the scaffold's design should give a physiological condition and mimicking the natural maxillofacial construction. My proposal is to use a trilayer PCL electrospun membrane, attempting to imitate the functional construction of the orofacial region by possessing oral mucosa in one side and bone tissue on the other segregated by a membrane. The soft tissue (NOF and FNB6) cells were seeded on one side within a micro-fibre layer and hBMSCs on the other side also within a micro-fibre layer and these were separated by the nano-fibrous layer located at the middle of the scaffold. My study indicates that the tri-layer scaffold can preserve and encourage the coculture of cells between hBMSCs and soft tissue cells and permit differentiation of the osteogenic progenitor hBMSC cells. In addition, the scaffold maintained separation of both hard and soft tissue for long time culture (24 days).

Further inquiry is included:

- Assess the ECM formation and confine the collagen production on the membranes for long time culture.
- Investigate the different cell populations that comport with trilayer PCL membrane in both short and how they support the engineered tissue over the long term *in vivo*.
- Compare the amount of VEGF secretion from the biomaterial scaffolds with the quantity of VEGF secretion in the implanted bone graft for inducing angiogenesis.

7.5 Clinical implementation of craniofacial reconstruction

Recently, the surgical treatment of cleft palate starts with management when the child is 6 months to one-year-old. However, the disturbance of orofacial development is occurred in several dimension because of the formed fibrosis tissue may intrude into the alveolar defects. Wolford and Stevao, (2002) mentioned that the bone cleft palate repair occurs between 8 to 9 years (before the permanent canine teeth erupt on the defect area) using autologous bone graft which is recommended in the treatment of cleft palate. This procedure can also lead to inhibition of maxillary growth. The ideal time for cleft palate management is still controversial. The autologous graft is the gold standard to achieve bone regeneration. However, it has several drawbacks such as the insufficient quantity and quality of grafts, morbidity, threatening the donor site, creating bone resorption, and prolonged numbness or paraesthesia of the leg. While allografts could cause immunogenic reactions and the risk of contamination from pathogen could transmit to the patient. The functional reconstruction of cleft palate using tissue-engineering technology that may mitigate the concerns of the multiple surgical repairs. The advantages of tissue-engineering are grafting of the healthy tissue takes from the body, cells can be isolated, proliferated and expanded under an appropriate cell culture conditions then seeded on biocompatible scaffolds, and re-implanted back at the defect area.

The suitable time of scaffolds for cleft palate engineering is so far controversial. It could be best before two years old to give the necessary time for developing speech with mitigate the concerns of hearing loss and decrease the multiple surgical repairs. On the other hand, it could be used at 8 to 9 years old to assist more bone growth before the adult cuspid teeth eruption. The tissue-engineering technique would be to minimise the

donor site morbidity, short operating time and stay in hospital compared with autologous iliac crest bone graft. In my viewpoint, the tissue-engineering of cleft palate is a talented strategy, whatever the selected time point is, better than the current treatment. My study demonstrated that both HJPs and hBMSCs cultured in human-derived media (XF2 and 5% (v/v) HPL) can differentiate to osteoblast-like cells and form ECM with deposition of calcium on monolayer and the biocompatible 3D electrospun PCL scaffolds.

Human-derived formulation media was widely impactful at stimulating mineralisation on the surface of PCL electrospun membrane. These media could be clinically developed to evaluate bone augmentation on 3D PCL scaffolds and biocompatible of scaffolds with surrounding area after re-implanted at the defect site using clinical cone-beam computed tomography (CBCT) scan (it is a new type of dental radiograph which creates 3D images of the jaw and teeth). The several hindrances need to be conquered before bone tissue engineering applies on routinely clinical protocols. Furthermore, short and long-time investigations of the implanted bone tissue engineering that demands to appraise the effectiveness results compared with the current treatment, where the essential consideration is whether the recent management progress enhances patient outcomes.

7.6 Final conclusion

- Primary osteoprogenitor cells (hBMSCs and HJPs) displayed the proliferation and osteogenic differentiation by deposition of calcium on 2D monolayers (chapter 3 and 4) and 3D PCL electrospun scaffolds fabrication structure (chapter 5) under the culture conditions studied.
- Human-derived media (XF2 and 5% (v/v) HPL) improved the deposition of calcium in all different types of cells on monolayers (chapter 4) and 3D structures (chapter 5) in osteogenic media supplemented with Dex 10 nM.
- Cell metabolic activity was reduced when cells were supplemented with 100 nM Dex.
- The human-derived media (XF2 and 5% (v/v) HPL) enabled the faster synthesis of DNA (cell proliferation) compared to 10% (v/v) FBS.
- XF1 (human plasma) and chemically-defined media poorly supported cell proliferation in monolayer.
- Donor variability was seen in the culture of monolayer (chapter 3 and 4) and 3D PCL electrospun scaffolds structures (chapter 5) under the culture conditions studied.

- There is lower metabolic activity with increased passage number.
- PCL is a suitable carrier in bone tissue engineering to construct a reanimation ECM for successful of pre-clinical (chapter 5).
- Amount of VEGF from hBMSCs was decreased by osteogenic supplemented with Dex 10 nM in 10% (v/v) FBS and increased in XF2 compared to basal culture media in 3D culture, while there was no effect of 5% (v/v) HPL (chapter 5).
- The tri-layer PCL mate membranes could assist and isolated three types of cell (NOFs, FNB6 and hBMSCs) to allow the bone and oral mucosa formation during 24 days (chapter 6).

Appendix

Oral presentation

- the 96th General Session and Exhibition of the International Association for Dental Research (IADR) and IADR/PAN European Regional Congress **"Appraisal of Xeno-Free Media for Craniofacial Bone Tissue Engineering"** 25-28 July 2018, London, England, UK.

Poster presentation

1. The 16th Annual Meeting of the Tissue and Cell Engineering Society: '**The effect of xeno-free media on human MSC metabolic activity and differentiation for bone tissue engineering**', 4-6 July 2016, University College London, London, UK.

2. The 16th MEIbioeng conference: '**Xeno-Free Media can Accelerate Human MSC Expansion and Differentiation for Efficient Bone Tissue Engineering**', 5-6 September 2016, Keble College, Oxford, UK.

3. The 3rd International Conference on Dental and Craniofacial Stem Cells: '**Comparison of Xeno-Free Media for Human MSC Expansion, and Differentiation in Tissue Engineering for Cleft Palate**', 25-28 October 2016, Columbia Global Centers Europe, Paris, France.

4. The 10th Annual Mesenchymal Stem Cell (MSC) Meeting: '**Assessment of Xeno-free Media on Human MSC Metabolic activity and Osteogenic Differentiation Capacity in Cleft Palate**', 5th December, University of York, York, UK.

5. The 95th General Session and Exhibition of the International Association for Dental Research (IADR), held in conjunction with the 46th Annual Meeting of the American Association for Dental Research and the 41st Annual Meeting of the Canadian Association for Dental Research: '**Identifying Xeno-Free Media for Productive Craniofacial Bone Tissue Engineering**', 22–25 March 2017, in San Francisco, Calif., USA.

6. European Chapter Meeting of the Tissue Engineering and Regenerative Medicine International Society (TERMIS-EU 2017): '**Evaluation of the Effectiveness of Xeno-free Media for Human MSC osteogenesis in 2D and Poly(ε-Caprolactone) Scaffolds**', 26-30 June 2017, Davos, Switzerland.

7. The 28th Annual Conference of the European Society for Biomaterials (ESB 2017): '**Assessment of Xeno-Free Efficacy for Human MSC Osteogenesis in 3D Poly(ε-Caprolactone) Scaffolds**', 4-8 September 2017, Megaron Athens International Conference Centre, Athens, Greece.

8. Biomaterials and Tissue Engineering Group 19th Annual White Rose Work in Progress Meeting: **'Effect of Different Xeno-Free Media on Osteogenic Differentiation of Human MSC in 2D Monolayer and Poly(ε-Caprolactone) Scaffolds'** 18th December 2017, University of Leeds, UK.
9. 8th World Congress of Biomechanics **'Osteoinductive Xeno-Free Culture of Human Bone-marrow MSCs on 3D Poly(ε-caprolactone) Scaffolds to Repair Cleft Palate'** 8-12 July 2018, Dublin, Ireland.
10. Biomaterials and Tissue Engineering Group 20th Annual White Rose Work in Progress Meeting: **"Feasibility of Human-derived Growth Supplements Formulations for Craniofacial Bone Tissue Engineering"** 17th December 2018, University of Sheffield, UK.

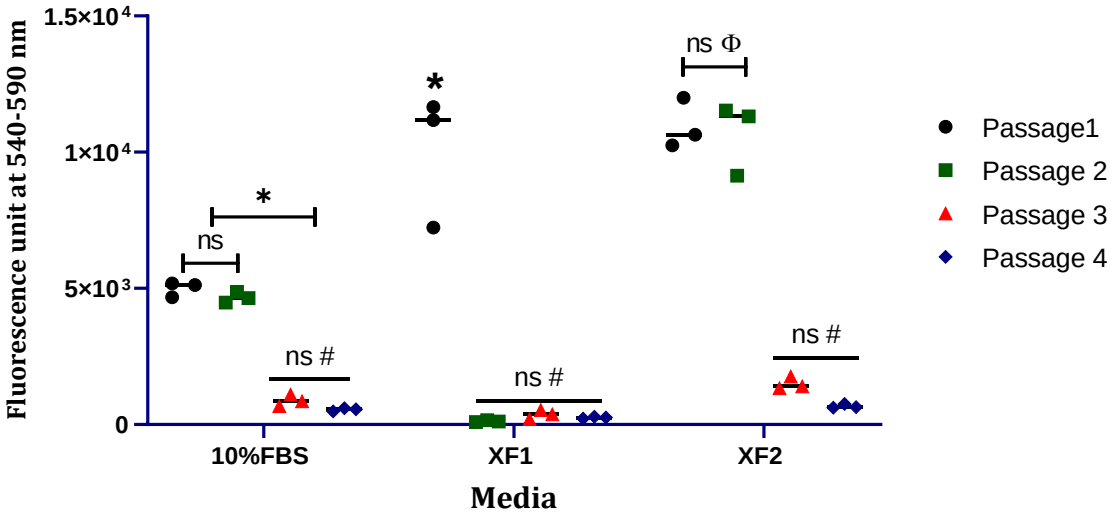
Publication

1. [Abstract] '**The effect of xeno-free media on human MSC metabolic activity and differentiation for bone tissue engineering' in Journal European Cells and Materials**, Vol.32, Suppl. 4, 2016 (page 66).

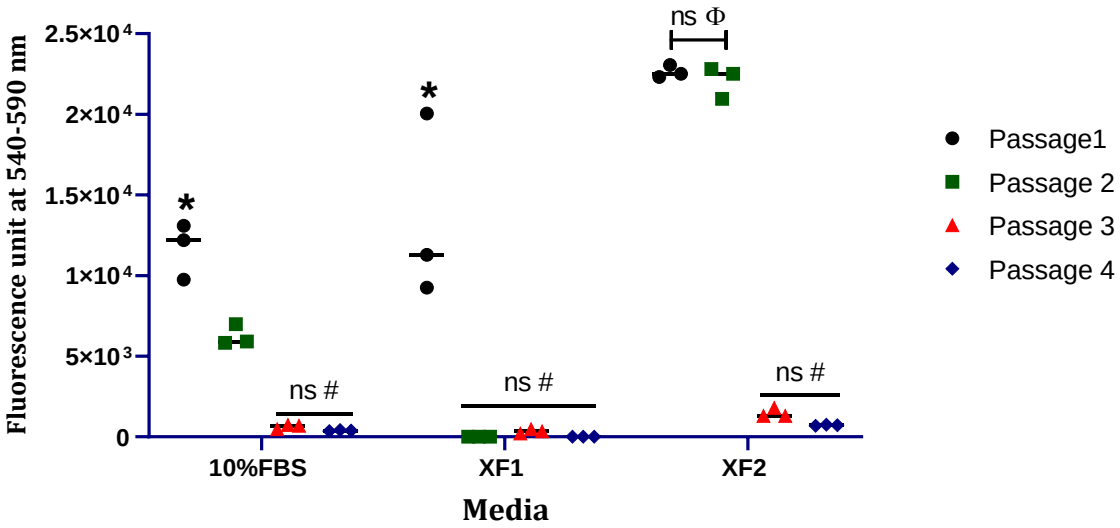
2. [Abstract] '**Evaluation of the effectiveness of xeno-free media for human MSC osteogenesis in 2D and poly(ε-Caprolactone) scaffolds**' **in Journal European Cells and Materials**, Vol.33, Suppl. 2, 2017 (page 181).

Figures

Different passages treated by Dex 10 nM on day 4

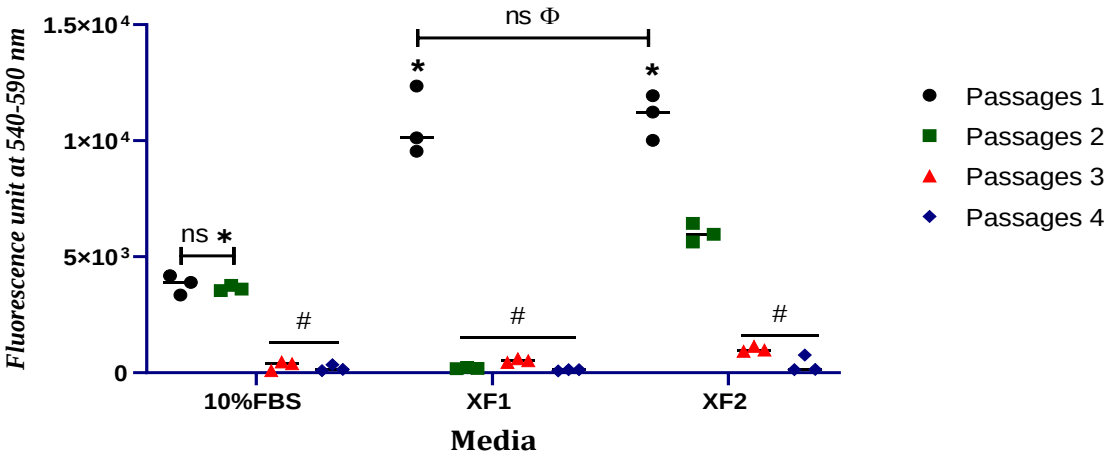


Different passages treated by Dex 10 nM on day 7

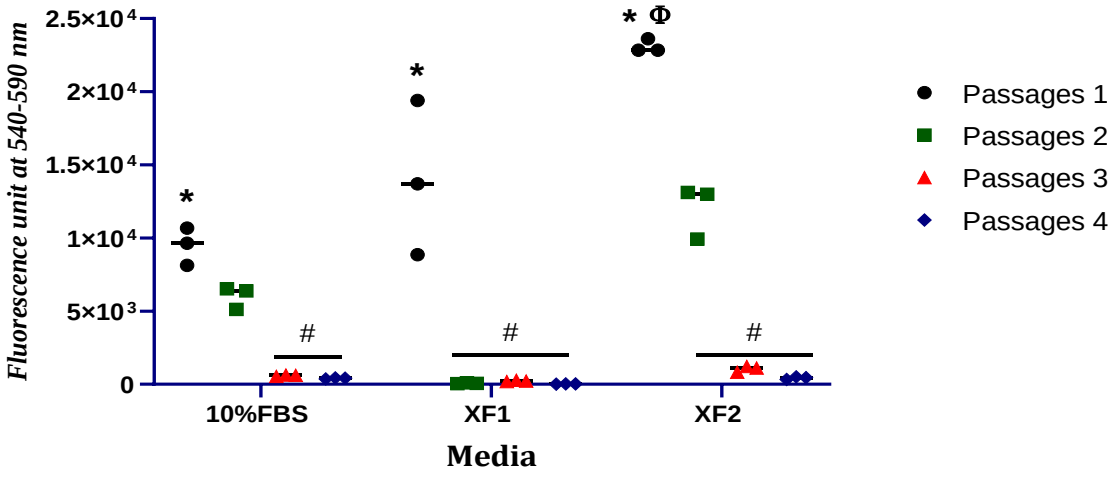


Metabolic activity of different donors of hBMSCs in different xeno-free media supplemented with Dex 10 nM on monolayer culture from day 4 to day 7. The scatter dot plots indicated individual wells and horizontal lines mean and standard deviation for each condition. (*) Higher than the other groups in the same donor. (#) Significantly lower. (P <0.05). n=3. Two-way ANOVA, Tukey's multiple comparison tests.

Different Passages treated by Dex 100 nM on day 4

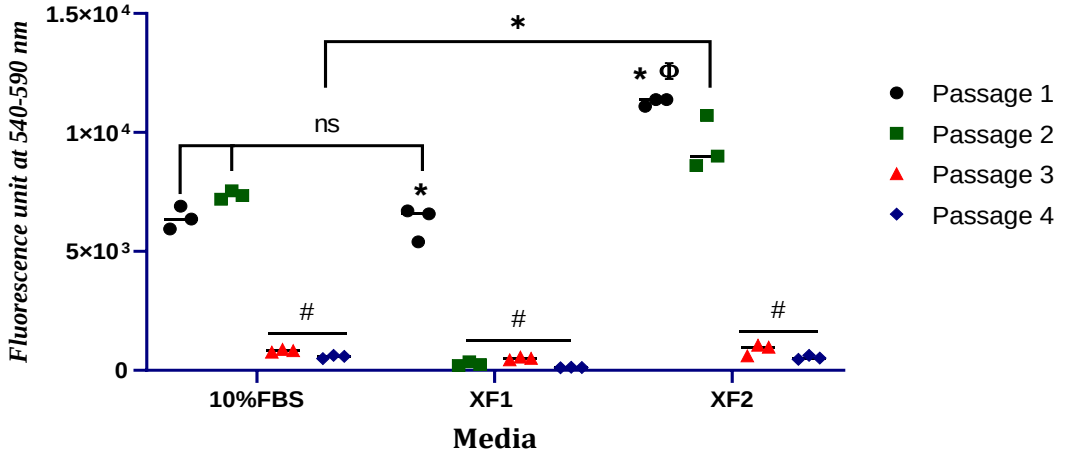


Different Passages treated by Dex 100 nM on day 7

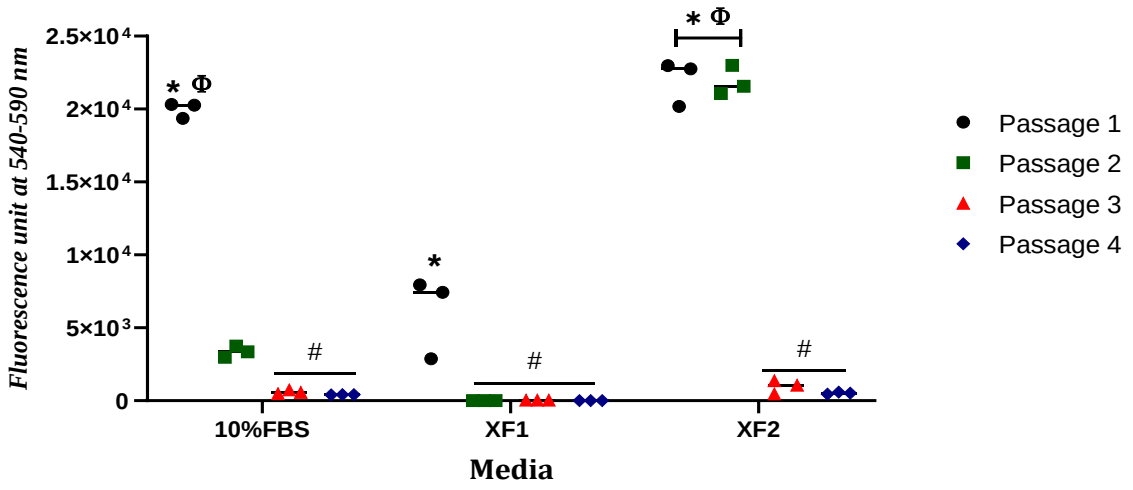


Metabolic activity of different passages of hBMSCs in different xeno-free media supplemented with Dex 100 nM on monolayer culture from day 4 to day 7. The symbols indicated individual wells and horizontal lines the mean and standard deviation for each condition. (*) Significantly higher than the other groups in the same medium. (#) Significantly lower. (Φ) Significantly higher than all the other conditions. (ns) There is no significant difference. (P <0.05). n=3. Two-way ANOVA, Tukey's multiple comparison tests

Different Passages treated by AA 50 µM and βGP 10 mM on day 4

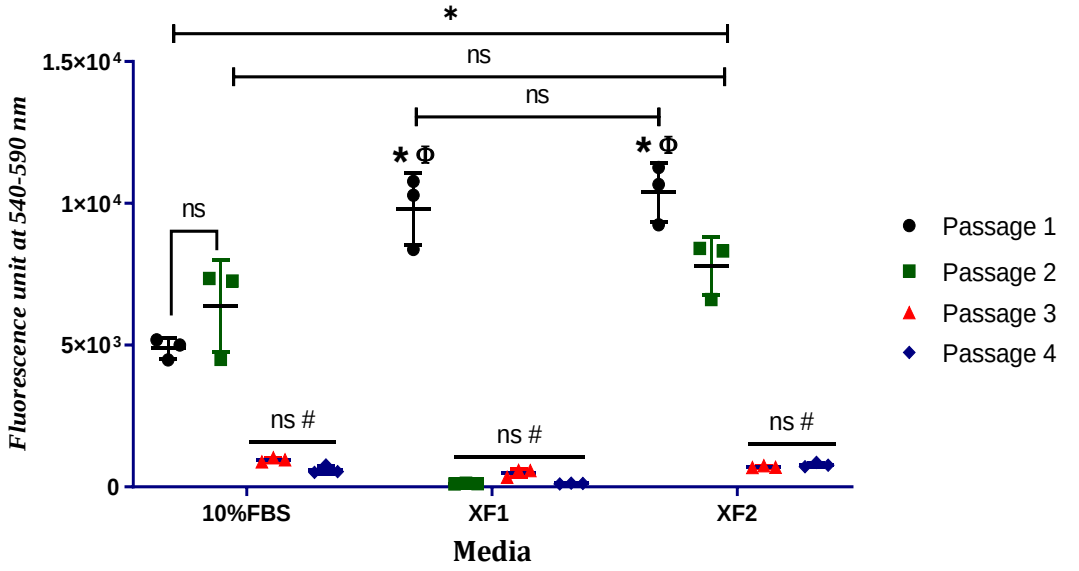


Different Passages treated by AA 50 µM and βGP 10 mM on day 7

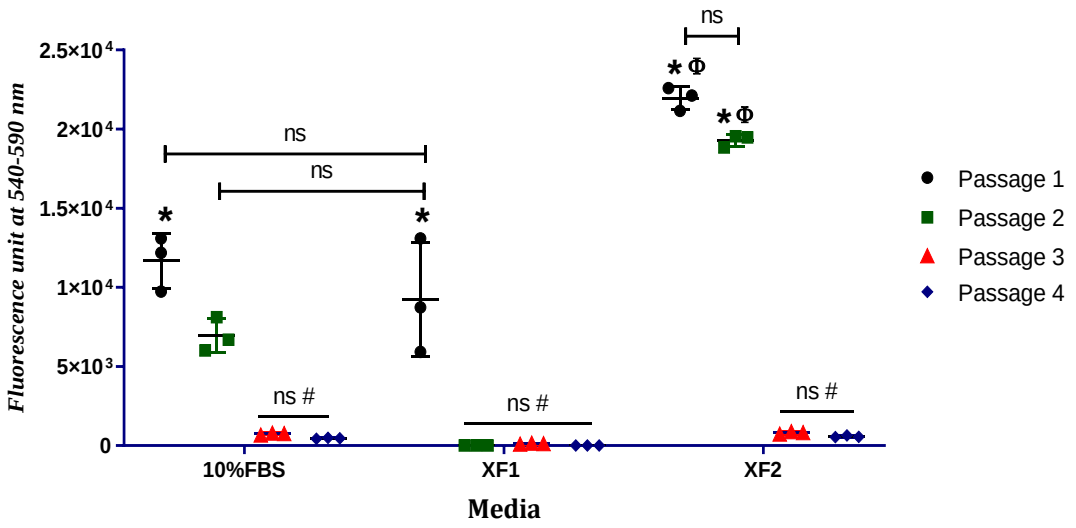


Metabolic activity of different passages of hBMSCs in different xeno-free media supplemented with ascorbic acid-2-phosphate (AA) 50 µM and β-glycerophosphate (βGP) 10 mM on monolayer culture from day 4 to day 7. The symbols indicated individual wells and the horizontal lines the mean and standard deviation for each condition. (*) Significantly higher than the other groups in the same medium. (#) Significantly lower. (Φ) Significantly higher than all the other conditions in the different media. (ns) There is no significant difference (P <0.05). n=3. Two-way ANOVA, Tukey's multiple comparison tests.

Different passages treated by Dex 10 nM, AA 50 µM, and βGP 10 mM on day 4

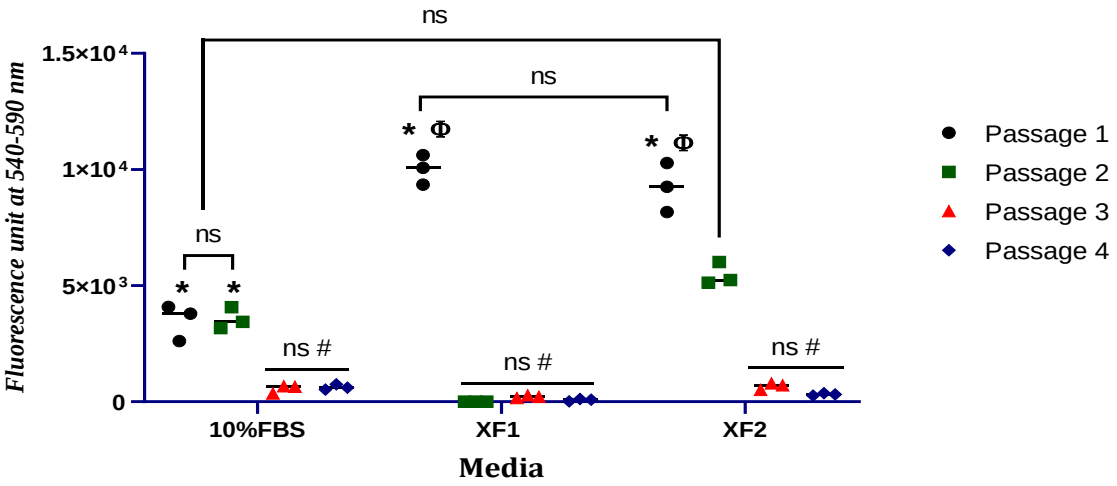


Different passages treated by Dex 10 nM, AA 50 µM, and βGP 10 mM on day 7

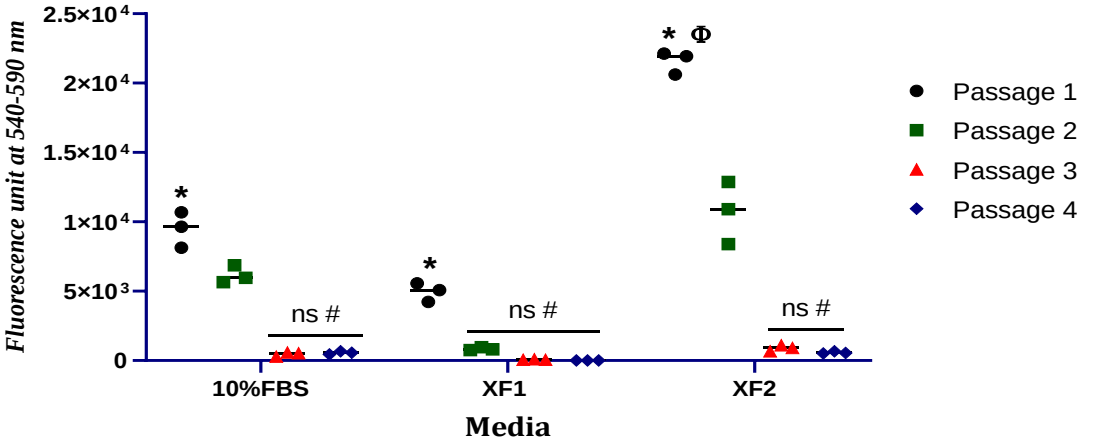


Metabolic activity of different passages of hBMSCs on a different xeno-free medium supplemented with osteogenic-inductive ingredients {Dex 10 nM, ascorbic acid-2-phosphate (AA) 50 µM and β-glycerophosphate (βGP) 10 mM} on monolayer culture from day 4 to day 7. The symbols represent an individual well and the horizontal lines mean and standard deviation for each condition. (*) Higher than the other donors in the same medium. (#) Significantly lower. (Φ) Significantly higher than all the other conditions. (P <0.05). n=3. Two-way ANOVA, Tukey's multiple comparison tests.

Different passages treated by (AA, βGP, Dex 100 nM) on day 4



Different passages treated by (AA, βGP, Dex 100 nM) on day 7



Metabolic activity of different passages of hBMSCs on a different xeno-free medium supplemented with osteogenic-inductive ingredients {Dex 100 nM, ascorbic acid-2-phosphate (AA) 50 µM and β-glycerophosphate (βGP) 10 mM} on monolayer culture from day 4 to day 7. The symbols represent an individual well and the horizontal lines mean and standard deviation for each condition. (*) Higher than the other donors in the same medium. (#) Significantly lower. (Φ) Significantly higher than all the other conditions. (P <0.05). n=3. Two-way ANOVA, Tukey's multiple comparison tests.

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