

***In vitro* Investigations of
Bioactive Glass Scaffolds
for Dental Tissue Engineering**

by

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for the Doctor of Philosophy degree (PhD) and the Diploma of Imperial College*

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Abstract

The regeneration of large bone and dental defects is hampered by the lack of remaining available tissue and the limited degree of vascularisation to support the healing process. Current treatment strategies using autologous tissue are inadequate for regenerative medicine. By contrast, the development of smart biomaterials that promote angiogenesis and bone deposition is gaining interest. This thesis describes the *in vitro* investigations of two bioactive glasses, namely strontium-containing bioactive glass (SrBG) and cobalt-containing bioactive glass (CoBG), for their potential use in the repair and regeneration of dental related structures. Strontium was incorporated into the bioactive glass (BG) structure by virtue of its dual role in promoting new bone formation and preventing osteoclast-mediated bone resorption. Cobalt is a known hypoxia-mimicking agent, and the incorporation of cobalt into the BG structure may also help create the hypoxic environment that benefits vascularisation during the regeneration of hard and soft tissue. Dental stem cells from permanent (dental pulp stem cells, DPSC) and deciduous teeth (stem cells from exfoliated deciduous tooth, SHED) were used in the current project since both appeared suitable for the regeneration of oral and dental related structures.

Strontium-containing BG (SrBG) with 0%, 10% and 100% substitution of strontium for calcium were studied. Their dissolution profiles in culture medium (DMEM and RPMI) were assessed using inductively coupled plasma-optical emission spectroscopy (ICP-OES), and the dissolution products were tested on human osteosarcoma cells (Saos-2) and mouse monocyte cells (RAW264.7). At the selected SrBG powder to liquid ratio (SrBG in DMEM was 6.0 mg/ml, and SrBG in RPMI was 1.5 mg/ml), the bioactive glasses were non-cytotoxic. In addition, when dental pulp stem cells from the permanent and deciduous teeth (DPSC and SHED, respectively) were seeded on the 10% SrBG discs, both cell types had a higher proliferation rate when compared to those seeded on tissue culture plastic (Thermanox) after 21 days of culture.

The use of DPSC and SHED as a source of stem cells for dental tissue engineering was investigated on cobalt-containing BG (CoBG). These glasses act as hypoxia-mimicking materials, a desirable property since hypoxia is known to enhance stem cell proliferation, stimulate VEGF secretion through the HIF-1 pathway, which help to improve vessel formation. First, the dissolution products from CoBG (0%, 1%, 2% and 4% cobalt for calcium substitution in mole percent) were incubated with the dental stem cells, and *in vitro* assays showed that the cobalt-containing BG-conditioned media had a dose-dependent effect on metabolic activity and proliferation. In addition, the CoBG succeeded in activating the hypoxia pathway, leading to VEGF secretion. The DPSC and SHED also secreted a substantial amount of collagen. Next, CoBG powder was incorporated into a biodegradable polymer, polycaprolactone (PCL), and three-dimensional cobalt-containing BG with PCL (CoBG/PCL) scaffolds were produced. The CoBG/PCL scaffolds also promoted the DPSC and human mesenchymal stem cells (hMSC) metabolic activity, and the cobalt released had no negative effect on cell survival. The CoBG/PCL scaffolds were also able to stimulate the hypoxia pathway and caused both DPSC and hMSC to secrete VEGF. Following the osteogenic induction of cells seeded on the CoBG/PCL scaffolds, there was lower ALP activity suggesting the cells remained undifferentiated when exposed to the hypoxia-mimicking CoBG.

Although the DPSC and SHED were seeded only on the 10SrBG discs (10% of strontium substitution with calcium in BG), the results presented in this thesis demonstrate the potential of SrBG and CoBG scaffolds for dental tissue repair and regeneration. The CoBG as dissolution products from the CoBG powder and the incorporation of CoBG powder into scaffolds demonstrated activation of HIF pathway, which has promising potential for dental tissue repair and regeneration.

Declaration

This thesis is a presentation of the research work carried out by me in the Department of Materials, and Institute of Biomedical Engineering at Imperial College London, United Kingdom and also Advanced Medical and Dental Institute, Universiti Sains Malaysia, Malaysia. The research work described in the thesis is my own. Every effort is clearly made to indicate any contributions from whose person who are involved in the project, and acknowledgement of collaborative research and discussions.

Conference contributions

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Dedication

This thesis is dedicated to my children:
Malihah Maisarah, Zuhrah Maisarah and Ibrahim

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List of abbreviations

ALP	Alkaline phosphatase
ARNT	Aryl hydrocarbon receptor nuclear translocator
ATP	Adenosine triphosphate
BG	Bioactive glass
BO	Bridging oxygen
bHLH	Basic helix-loop-helix
BrdU	Bromodeoxyuridine
CB	Charge balanced
CN	Coordination-number
COX	Cyclooxygenase
DAPI	4',6-diamidino-2-phenylindole, dihydrochloride
DFO	Desferrioxamine
DFSC	Dental follicular stem cell
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DPSC	Dental pulp stem cell
EC	Endothelial cell
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPC	Endothelial progenitor cell
ERK	Extracellular regulated kinase
FBS	Foetal bovine serum
FIH	Factor inhibiting HIF
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HA	Hydroxyapatite
HCA	Hydroxycarbonated apatite
HIF	Hypoxia inducible factor
HMSC	Human mesenchymal stromal cell
HRE	Hypoxia responsive element
HUVEC	Human umbilical vein endothelial cell

ICP-OES	Inductively coupled plasma-optical emissionspectroscopy
LDH	Lactate dehydrogenase
MAPK	Mitogen-activated protein kinases
mole %	Mole percent
MSC	Mesenchymal stromal cell
NBO	Non-bridging oxygen
NC	Network connectivity
NonCB	Non-charge balanced
ODDD	Oxygen-dependent degradation domain
PAS	Per-ARNT-Sim domain
PDLSC	Periodontal ligament stem cell
PGK	Phosphoglycerate kinase
PHD	Prolylhydroxylase
PI3K	Phosphoinositol 3-kinase
PSA	Particle size analysis
RACK	Receptor activator protein kinase
RANKL	Receptor activator of nuclear factor $\kappa\beta$ ligand
ROS	Reactive oxygen species
SBF	Simulated body fluid
SCAP	Stem cell from apical papilla
SEM	Scanning electron microscopy
SHED	Stem cell from human exfoliated deciduous teeth
TAD	Transactivation domain
T_c	Crystallization temperature
TE	Tissue engineering
T_g	Glass transition temperature
T_o	Onset crystallization temperature
VEGF	Vascular endothelial growth factor
VHL	Von Hippel Lindau tumour-suppressor protein
wt %	Weight percent
XRD	X-ray diffraction

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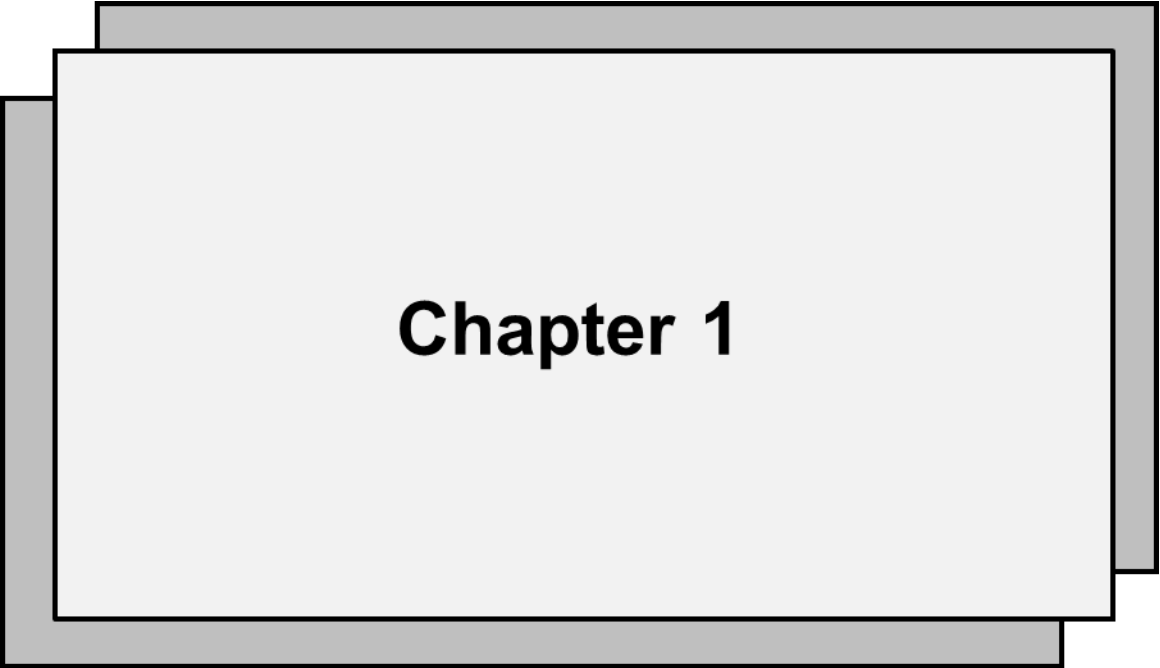
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Chapter 1

Chapter 1

Introduction

1.1 Scope of the Thesis

Humans have two sets of teeth: the permanent dentition (adult teeth) and the deciduous dentition (milk teeth). Tooth loss may occur for several reasons, including periodontal problems (where the teeth become mobile), motor vehicle accidents, trauma, or exfoliation, which normally occurs with deciduous teeth. There are many ways to replace missing teeth, such as partial dentures, resin-bonded bridges, fixed bridges, and the more permanent option of an implant. Implants are often the treatment of choice since they create the natural feeling of having a tooth in the mouth, and advances in biomaterial development have endowed implants with osseointegration properties. However, all these options are only temporary; dentures, crowns, and bridges may fracture, become loose and need replacing, and implants may fail due to patient factors such as heavy smoking habits, poor oral hygiene, and local factors associated with the environment of the implant inside the bone.

Dental stem cells can be harvested from teeth, which themselves are easily available and accessible. Indeed, teeth are often regarded as medical waste (1). Stem cells for regenerative medicine purposes are usually derived from the bone marrow (bone marrow stromal stem cells; BMSC) (reviewed in (2)), but the procedures to obtain them are laborious and can be associated with complications for the donor. Fortunately, there are alternative sources of stem cell in the human body that are more accessible. However, questions remain regarding their regenerative potential and whether these cells possess characteristics related their source.

Stem cells from the dental pulp of adult teeth (DPSC) were first isolated in 2000 (3, 4), and other types of stem cells from other dental tissues were subsequently harvested and characterised (5-7). Since then, many researchers have studied stem cells from dental tissues in the quest to understand how they behave immunologically and with other cell types (8, 9). To establish their use in tissue

engineering, it is of substantial interest to further investigate how dental stem cells respond to biomaterials involved in dental implantology. Furthermore, there is scarce information regarding the effect of the dental stem cells to BGs, especially in relation to the CoBG. Therefore, the main goal of the present work is to identify a role for dental stem cells as a cell source in dental tissue engineering and dental implantology. To achieve the goal, dental stem cells will be exposed to bioactive glasses (BG) in the form of BG discs, dissolution products from BGs, and also incorporation of BG into scaffolds, with their responses assessed through *in vitro* assays. Two types of BG will be used: strontium-containing (SrBG) and cobalt-containing bioactive glasses (CoBG). The knowledge gained from the current project will help to improve the understanding of dental stem cells' response to the SrBG and CoBG, and the potential future use of the BGs for dental tissue regeneration and implantation.

Autografts are limited in their potential use due to a lack of adequate tissue and vascular supply. These limitations enabled the development of allograft materials as promising alternatives, which include bioactive glasses and ceramic materials (10). In a recent facial injury survey in the West Ireland, it was shown that 40% of the facial injury cases come from patients aged 25-34 years, and approximately 51% sustained major facial injury requiring treatment and follow-up. Approximately 66% had suspected facial injury and 15% had damaged teeth (11, 12). The high incidence of facial injury may predispose the patients to acquire metal implants for facial reconstruction. Patients with damaged teeth may need suitable materials for dental pulp regeneration or prostheses such as dentures or implants to replace the missing teeth. Approximately 16.4% of patients with mandibular fractures have complications with metal implants within 4 years of placement, which predisposes patients to secondary surgeries that create the risk of additional bone loss and infection (13). Implants supporting teeth showed approximately 23.9% mild inflammation within one year after placement (14). Early implant-supported tooth failure may occur due to disruption of the healing process and lack of osseointegration (15). The number of implants replaced within one year was not reported but the authors stated that failures of implant-supported teeth from the record of 6 patients was mainly due to a failure of bonding between alveolar bone and implant, and also infection surrounding the implant site (15).

Bioactive glasses (BGs) are a biocompatible class of materials with various uses in regenerative medicine. The importance of using BGs in dentistry is clear. For more than ten years, bioactive glass (BG) particulates have been used to treat bone defects in maxillofacial surgery and dentistry (10, 16) because of their stimulation of new bone formation and ability to improve alveolar bone height (10, 16). BGs were also used to as treatment for dentine hypersensitivity (17), periodontal problems (16), sinus lift, and alveolar bone augmentation (18). Their composition can be tailored to contain elements that can influence cell behaviour. For example, strontium promotes osteoprogenitors to differentiate into pre-osteoblastic cells (19). In the form of strontium ranelate, strontium has been used to treat patients with osteoporosis, where it has been shown to reduce the risk of bone fracture (20). Although bone mineral density (BMD) increased among the subjects, there was a non-significance of cumulative incidences of new vertebral and non-vertebral fractures within 6 to 8 years after SrR consumption when compared to the cumulative incidence at the first 3 years (20). This may highlight the difficulty in assessing the SrR concentration within the body upon consumption; BGs might be better as an agent for the treatment in these patients. The recommended oral dose for SrR is approximately 2 g/day. It was estimated that when a patient consumed 2 g/day of SrR, this would be equivalent to 2.69 mole % of strontium in BG granules (21). If the BG granules are implanted in a bone defect, the strontium released from the granules in 12 months would be approximately 200 ppm. This estimated dose is many times less compared to the SrR oral dose, but the effect seen following implantation or local delivery far exceeds those taken systemically (21). The ionic dissolution from BGs can be tailored where BGs as particulates or scaffold are implanted at the fracture sites and then assessed for new bone formation. Previously studies revealed that strontium-containing BG-conditioned medium enhanced Saos-2 cell viability (21, 22). Beyond strontium, cobalt is a promising additive to BG composition. It is commonly present in green vegetables and fresh cereals, and is involved in vitamin B₁₂ synthesis (23). Cobalt is a known hypoxia mimic, and since hypoxia has been shown to promote wound healing, it is promising for the field of implantology. BMSC cultured on cobalt-containing mesoporous BG (Co-MBG) scaffolds showed proliferation that was dependent on the dose of cobalt (24). Therefore, the response of dental stem cells to

bioactive glasses was studied with the ultimate aim of demonstrating their use to promote repair in dental and oral tissues (Fig. 1.1).

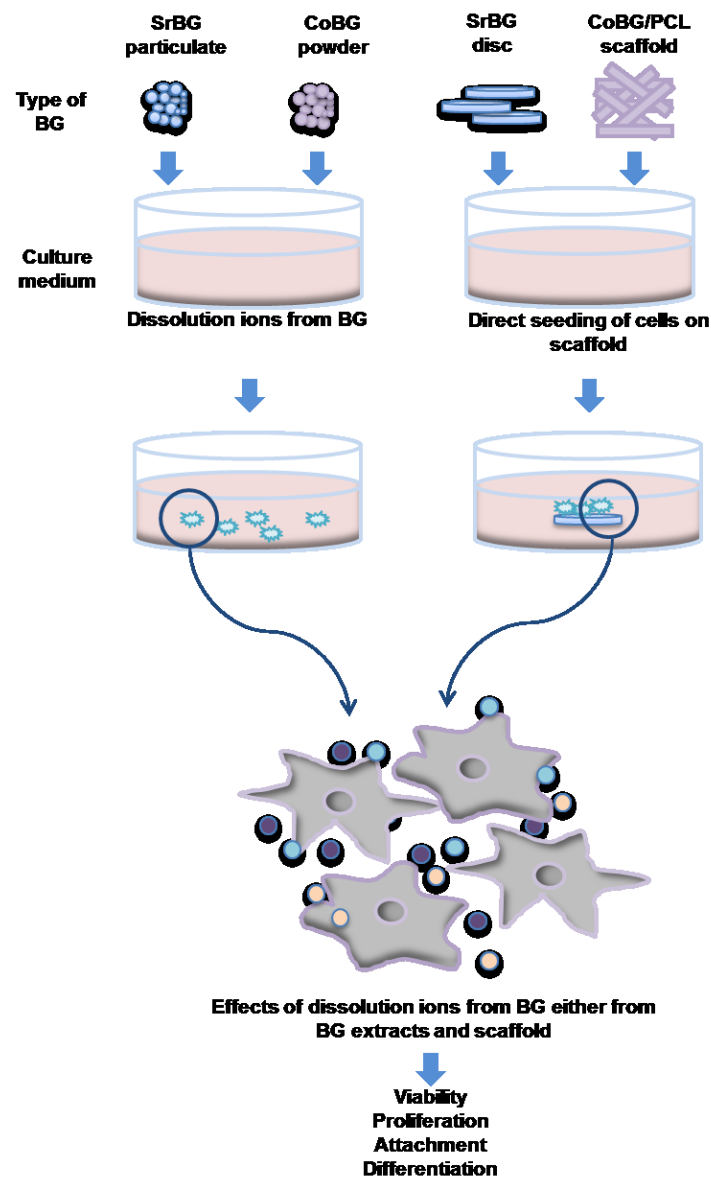


Fig. 1.1: The use of BGs in regenerative dentistry is due to the availability of various BG preparations such as powders or scaffolds. Strontium containing bioactive glasses (SrBG) and cobalt-containing BG (CoBG) allow for the controlled release of ions upon exposure to body fluids, which will lead to subsequent cellular responses such as proliferation and differentiation.

The specific objectives of the present work are to:

1. Isolate and characterise the stem cells from the dental pulp of human permanent and deciduous teeth.
2. Evaluate the response of the dental stem cells when exposed to BGs containing strontium or cobalt, two biologically active ions.

We hypothesise there will be a higher cell metabolic activity for the DPSC and stem cells from deciduous teeth (SHED) when these cells are exposed to the BGs.

Chapter 2 provides a review of dental stem cells from the tooth and the use of biomaterials in repair and regeneration. It focuses on the field of dental tissue engineering, regenerative medicine and tissue engineering (TE), and contains a summary of the current knowledge on BGs. Chapters 3-6 describe the main experimental results, including the related literature reviews, experimental methodologies and, when appropriate, suggestions for future work. Chapter 7 provides the final conclusions and overview of the present project.

The thesis is divided into three parts. The first part describes the preliminary assessment of BG on bone modifying cells: osteosarcoma cells with properties similar to osteoblasts (Saos-2) and monocytes (RAW264.7) (Chapter 3). The second part describes the isolation of dental stem cells and an assessment of the biological activity of BG. The dental stem cells were seeded on strontium-containing BG discs (Chapter 4), and were exposed to the dissolution products of cobalt-containing BG-conditioned medium (Chapter 5). The third part consists of the assessment of dental stem cells and human mesenchymal stem cells (hMSC) when seeded on a three-dimensional (3D) BG scaffold (Chapter 6).

1.2 Preliminary testing of BG

In the first part of the project, the effects of strontium-containing bioactive glass (SrBG) were studied on two bone modifying cell types. Strontium BG-conditioned media were incubated with cells involved in bone formation and remodelling, Saos-2 and RAW264.7 cells (Chapter 3). Saos-2 is an osteosarcoma cell line with properties similar to osteoblasts (bone forming cells) and RAW264.7 is a monocyte cell line, which under suitable conditions, can differentiate into osteoclasts (bone resorbing

cells). Alkaline phosphatase (ALP) activity, which acts as a marker of osteoblast differentiation and mineralisation, was assessed following the incubation of Saos-2 with the strontium BG-conditioned medium. For RAW264.7, the tartrate resistant acid phosphatase (TRAP) activity, which is one of the markers that can be found during osteoclastic differentiation, was also assessed following the incubation with the strontium BG-conditioned medium. The findings from this preliminary testing provided an initial characterisation of the SrBG that were then used in the following chapters in combination with dental stem cells.

1.3 Isolation and characterisation of dental stem cells and assessment of BG biological activity on dental stem cells

Dental stem cells were isolated from human permanent and deciduous teeth. Following ethical approval from the Internal Review Board (Universiti Sains Malaysia), teeth were collected from adult patients and children attending an oral health clinic. The tooth was subjected to cleaning procedures prior to isolation of dental stem cells from the tooth pulp tissue. The cells were characterised with immunohistochemistry followed by assessment of their proliferative activity. Bioactive glass discs of SrBG with a 10% substitution (10% strontium was substituted for calcium) were prepared, and dental stem cells were seeded on them. At designated time points, the viability of dental stem cells was assessed, and the SrBG discs were imaged by scanning electron microscopy (Chapter 4). The promising results obtained when using strontium-containing BG provided the motivation for the use of cobalt-containing BG (CoBG) in the following chapter.

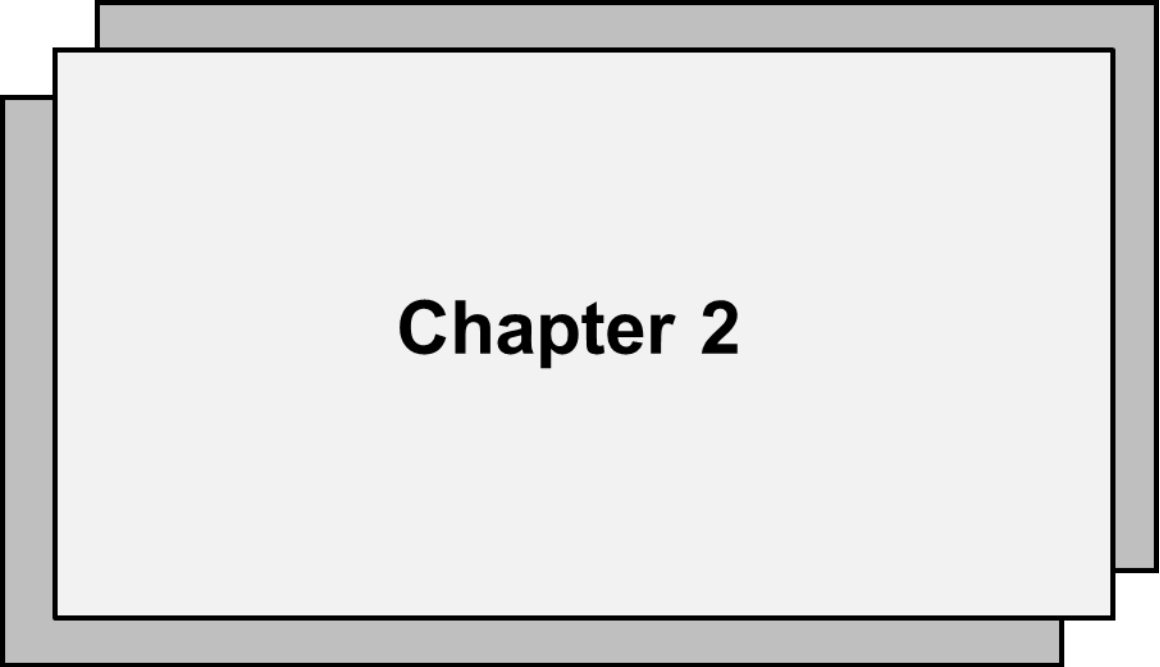
CoBG is a hypoxia-mimicking BG, which could improve repair and regeneration in dental tissue. The biological response of dental stem cells following incubation with cobalt-containing BG-conditioned medium was assessed (Chapter 5) using several assays including viability and proliferation. The compositions of the CoBG used were 0% Co, 1% Co, 2% Co and 4% Co (mole %). The activation of the hypoxia-sensing pathway by CoBG was determined by assessing the HIF-1 α activity. HIF-1 α is a transcription factor that is responsible in regulating the HIF-1 pathway. The nuclear amount of HIF-1 α protein and *HIF-1A* mRNA expression were also assessed in these cells upon exposure to cobalt-containing BG-conditioned medium. The subsequent cellular response was analysed by quantifying the expression of

vascular endothelial growth factor (VEGF), a regulator of blood vessel formation that is expressed in hypoxic conditions *via* the HIF-1 α pathway. Dental stem cells from the permanent teeth (DPSC) showed favourable results when exposed to the 0% Co, 1% Co, 2% Co and 4% Co (mole %) BG-conditioned medium compared to stem cells from the deciduous teeth (SHED). Thus, it was decided to further explore the potential of DPSC in the third part of the project.

1.4 Assessment of CoBG for dental repair in regenerative dentistry

Dental stem cells exposed to conditioned media by some compositions of CoBG showed activation of the HIF-1 pathway, which subsequently triggered the secretion of VEGF. The encouraging findings from the cobalt-containing BG-conditioned medium established the need for a scaffold that could release cobalt (from CoBG) over time to improve cell behaviour. Thus, in the final part of the thesis, 30 weight per cent (wt %) of the CoBG glass powder was mixed with a degradable polymer, polycaprolactone (PCL), for generation of 3D CoBG/PCL scaffold using the fused deposition method. The 3D scaffold had tailorable cobalt content, physical properties, and mechanical properties to address the specific needs for the application. Glass powders with particle size < 20 μm were prepared and sent to our collaborators at The Queensland University of Technology, Australia. The scaffolds were treated with 5M NaOH (detailed methodology in Chapter 6), after which the ion release profiles were characterised and the cell seeding method was optimized. Human mesenchymal stem cells (hMSC) and DPSC were seeded on the CoBG/PCL scaffold and were assessed for their proliferative capacity and their response to the hypoxia-mimicking effect of the CoBG/PCL scaffold. The angiogenic potential of the CoBG/PCL scaffold was determined by measuring the VEGF release from these cells when seeded on the scaffolds and a paracrine effect was measured with a chemotactic migration assay using human umbilical vein endothelial cells (HUVEC).

The cobalt released from the CoBG/PCL scaffolds is also tailorable, and did not negatively impair the cells viability. The cobalt within the CoBG/PCL scaffolds had triggered the hypoxia-mimicking effects on the cells *via* activation of the HIF-1 pathway, which results in the release of VEGF, suggesting the potential use of the CoBG/PCL scaffolds for dental tissue engineering.



Chapter 2

Chapter 2

Literature review

2.1 Tissue engineering

In many scenarios, the human body has the ability to heal upon injury, providing that its own defence and regeneration mechanisms are working properly. It was with this concept in mind that the tissue engineering approach was adopted (reviewed in (25)). Tissue engineering (TE) involves the interaction between cells, tissue-inducing factors and scaffolds for the purpose of organ restoration or reconstruction (reviewed in (26)), and under the appropriate stimuli, it promotes tissue regeneration. TE is defined as “an interdisciplinary field that applies the principles of engineering and the life sciences towards the development of biological substitutes that restore, maintain, or improve tissue function” (26). Three strategies have been adopted for the creation of new bone in TE such as the presence of isolated cells or cell substitutes, and the tissue-inducing substances and cells placed on or within matrices (Figure 2.1, (26)).

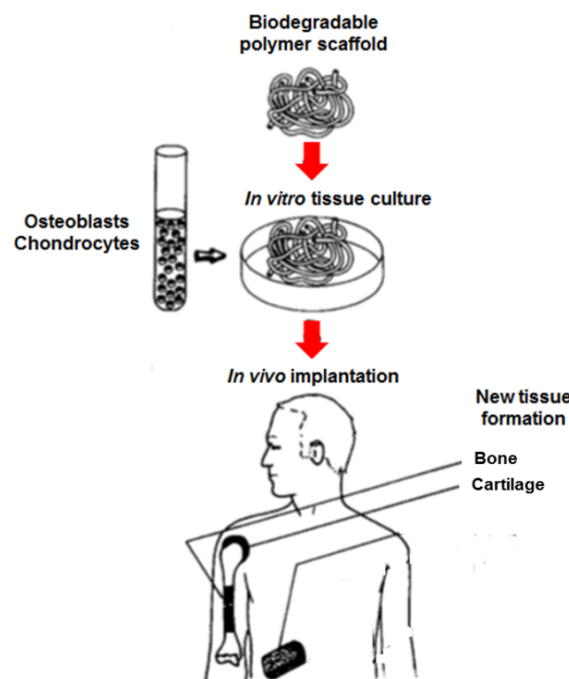


Fig. 2.1: An illustration of a typical tissue engineering approach. A biodegradable polymer scaffold is first infused with cells *in vitro*, with the resulting cell-polymer matrix implanted *in vivo*. Cell organisation occurs leading to new tissue formation. The scaffold eventually degrades. Adapted from (26).

The TE strategy involves the implantation of a scaffold, which is typically a biomaterial used to deliver cells that may be enriched with growth factors. In an ideal situation, the scaffold should be biodegradable and treated with factors or combined with cells that can expand upon implantation, and it should either be prevascularised or become vascularised upon implantation to ensure the survival of the implant (27). The unconventional TE approach to organ transplantation and tissue reconstruction has improved remarkably in the last two decades (28).

Advances in developmental and cellular biology, material science, and medicine have seen the adoption of TE principles in the generation of a large number of tissues, including bone (29, 30), skin (31), and cartilage (32). However, there are still challenges to address. Critically, there is a need to provide a blood supply to the new tissue, since cells can only survive within 100-200 μm of blood vessels (26, 33). The creation of a natural material to support cells and signalling factors is crucial, however, it may be influenced by batch-to-batch variation, especially in large-scale production (26). The individual variation among donors, and restrictions related to underlying diseases may also pose challenges to effective regeneration. Finally, the shortage of donors also reduces the possibility of transplantation procedures (26). Thus, the development of biomaterials that can overcome these problems is very much sought after.

Tissue engineering aimed at regenerating hard tissue is commonly known as bone tissue engineering, while in dentistry, the regeneration of dental pulp tissue is known as dental pulp tissue engineering, dental regeneration or regenerative dentistry. The application of TE in bone repair has received much attention as a promising approach to tackle the shortcomings of conventional clinical treatments of bone fracture due to disease or trauma. Various conventional treatment modalities exist for the replacement of hard tissue, which involve the removal of the defective tissue, replacement with autologous bone, or implantation of an allograft or natural or artificial prostheses (34). Rapid bone formation can also be achieved using distraction osteogenesis by applying a mechanical strain to the local tissue (35). However, autologous bone transplantation faces a shortage of tissue supply, and infection may arise following the implantation of an artificial prosthesis. Allograft prostheses may be used, but they expose patients to graft-versus-host disease,

which can be life threatening (26). The size of the bone defect may also affect the success of the surgery (34). The body may not be able to spontaneously close the gap left by large defects (35), especially in major facial bone reconstruction and severe non-union fractures (36).

The increasing number of maxillofacial trauma patients (11, 12) requiring metal implants, and the longevity of these prostheses also creates the need for suitable materials that can withstand high loads (reviewed in (37)), but metal implants predispose patients to secondary surgeries that may create the risk of additional bone loss and infection (13). Thus, TE of hard tissue in regenerative medicine is applied as an alternative approach for bone regeneration and repair (35). TE can further cater to the patient's individual needs with minimal surgical intervention, which could benefit patients by reducing the cost of surgery (26).

There are many challenges associated with bone tissue engineering. An adequate number of cells are needed prior to implantation, suitable scaffolds must be designed, and growth factors should enhance tissue formation. Various cell types have been harvested for use in TE, including embryonic stem cells (38) and adult mesenchymal stem cells (39). As mentioned earlier, cells can be harvested from allogeneic or autologous tissues, but there are risks of rejection or surgical trauma, respectively (26). The scaffolds fabricated from biomaterials can be enriched with cells or growth factors *in vitro* and then implanted *in vivo* to improve healing and regeneration. The scaffolds must meet certain criteria. They must be biocompatible and non-toxic, act as a template for bone growth, and possess porosity and surface topography to allow cell attachment and migration (27). The optimal chemical and physical interaction of scaffolds and cells must also be kept in mind (27). Small- and large-scale scaffold development may suffer batch-to-batch variation or scale-up differences (26). The availability of bioreactors allows for the improvement of tissue culture techniques, and scaffold infusion with cells prior to *in vivo* implantation can enhance bone repair (40).

The creation of biomaterials such as bioactive glass (BG) scaffolds with various compositions, and the combination of BG with other materials are promising as a route for the delivery of signalling molecules, and to act as signals for cell migration,

proliferation, and angiogenesis (35). In developing scaffolds for bone TE, the central aim is to produce a scaffold that can mimic the properties of bone, while providing additional benefits such as the release of growth factors or signalling molecules. Hence, BGs have potential as replacement materials for bone TE due to their inherent properties, which are discussed in Section 2.4.

2.2 Dental pulp tissue engineering

In regenerative medicine, more attention is paid to neural or cardiac diseases than dental diseases. This is due to the fact that teeth are non-essential for life, but stem cells from teeth are suitable for testing new cell-based therapies (41) and there is an enduring demand for repairing damaged or fractured teeth and replacing missing teeth (42). Missing teeth are currently replaced with dental prostheses that are either fixed (dental implants) or removable (dentures) (43). In addition, fractured teeth that are still vital are restored either using the fractured fragment or tooth coloured restorative materials (44). A tooth that has become non-vital would need root canal therapy and subsequently a post and crown if there is severe structure loss (45). Thus far, damaged teeth or oral tissues are replaced or repaired using biocompatible synthetic materials that do not have characteristics comparable to the native tissue (46). This is part of the reason why patients endure additional treatments (46), and why they suffer from tissue loss and the high cost of hospital bills. In the United States, dental treatment ranks second-highest in the number of procedures performed (10 million procedures) behind blood transfusions (18 million procedures) (26).

Dental problems are faced by diverse age groups. In babies and toddlers, rampant caries are one of the problems faced due to nursing bottles and breastfeeding on demand, which gave rise to problems such as dentoalveolar abscesses, early tooth loss, and a reduced quality of life (47-49). In addition, children born with birth defects such as cleft lip and palate have hypodontia (lower number of teeth) and insufficient bone at the defect for teeth to erupt inside the oral cavity, which also exposes them to long term corrective surgeries and prosthetic implants (50, 51). A significant number of young adolescents are involved in motor vehicle accidents, trauma and assaults (52, 53), which also increase the need for dental treatments to replace missing teeth, or maxillofacial surgery for facial reconstructions. Tooth loss is

replaced using dentures, implants or crowns, and fractured bone normally requires internal fixation and stabilisation using metal plates (54, 55). These procedures predispose patients to problems related to the implants or lack of support. The improvement in health care has also seen an increase in the number of adult patients, especially in developed countries where the life-span exceeds 75 years (56), and these adults may have more surgical complications (57). The number of complex dental cases such as correction of worn dentition, temporomandibular joint (TMJ) problems, and maxillofacial trauma cases to treat edentulous ridges is rising (58), and physicians are faced with many challenges. Hence, the concept of TE has been expanded to dentistry. Dental stem cells and cells from other related oral tissues are a suitable source for this purpose, including dental pulp stem cells (DPSC) (3, 4), stem cells from human exfoliated deciduous tooth (SHED) (59), periodontal ligament stem cells (PDSLC) (7), dental follicular stem cells (DFSC) (60, 61), and stem cells from apical papilla (SCAP) (5).

DPSC and SHED are a suitable source for the regeneration of oral and dental tissues compared to other type of adult stem cells (eg. bone marrow stromal stem cells [BMSC]) because DPSC and SHED are more related to the structures of the facial skeleton during developmental stages. Further, the availability of various types of dental stem cells generates opportunities for their use to regenerate oral and maxillofacial tissue. *In vitro* studies showed that DPSC exhibited higher proliferation rates compared with BMSC (3, 4). For example, the third molar tooth (where DPSC are commonly harvested from) was the last tooth to develop into the oral cavity, which contributed to the earlier state of development compared to adult bone marrow. For tooth healing and repair following pulpal injury, DPSC expressed the odontoblast-specific gene known as dentin sialoprotein and dentin phosphoprotein, which is encoded by a single gene known as dentin sialo phosphoprotein (DSPP) (3, 4). The expression of DSPP is important for pulpal healing and formation of reparative dentin. The deciduous tooth developed intra-uterine and may possess the more immature type of stem cells, which may be similar in one way or another to umbilical cord-containing stem cells (59). The availability of SHED offers a unique population of stem cells which have the potential for clinical applications in the field of neurological regenerative disease (62).

Stem cells have the capability of self-renewal, and are able develop into many different cell types (25). They can be categorised as embryonic, foetal or adult stem cells (38, 63). Embryonic stem (ES) cells are harvested from the inner cell mass of blastocysts during the early stages of embryonic development, and can be cultured *in vitro* with a nearly unlimited capability to differentiate. However, their use is limited by legal and ethical issues (38). In order to grow, ES cells need feeder cells (eg. mouse embryonic fibroblast feeder-cell layer or primitive mesenchymal cells), which may result in cross-reactivity between the ES and feeder cells. The addition of leukemia inhibitory factor (LIF) to mouse ES cells is sufficient to maintain the ES cells in an undifferentiated state (64). Furthermore, if ES cells are to be designed for human applications, they would need to be grown on a large-scale basis and checked for signs of malformation. Any disruption or deviation in ES cells differentiation may result in impairment of organ development upon implantation, which poses the need for a higher number of test compounds or antibodies (65). Adult stem cells are used as an alternative to overcome these issues, but they have more limited differentiation potential than ES cells (66). Nevertheless, their availability at every phase of life makes them a suitable source in regenerative therapies (63).

The process of obtaining stem cells from the human body for therapeutic use can be tedious, especially when it comes to identifying suitable sites for obtaining an adequate number of cells. The procedures may also pose risks such as infection and trauma for the donor (reviewed in (67)). When one compares the procedure of obtaining bone marrow stem cells with the procedure of obtaining dental pulp stem cells from a tooth, the latter is clearly much easier. The tooth can be collected after a routine dental extraction procedure, surgical removal, or following multiple tooth extraction under general anaesthesia. The availability of dental stem cells from teeth makes them suitable candidates for biomaterial testing in regenerative dentistry (67). Furthermore, the *ex vivo* expanded dental stem cells have various potential applications in the field of tissue engineering and regenerative medicine, such as the treatment of severe alveolar ridges, regeneration of ankylosed temporomandibular joints, implant placement, endodontics and bone fractures, which are summarised in Fig. 2.2 (68). Although the use of dental stem cells in tissue engineering is still in its infancy, they are an alternative type of stem cells, and are thought to be more

suitable for the regeneration of oral and maxillofacial tissues since they are more closely related thanks to their development from the neural crest during embryonic development (69). Thus, two types of dental stem cells will be used in this thesis for *in vitro* biocompatibility testing of bioactive glasses: dental pulp stem cells (DPSC) from the permanent tooth, and stem cells from exfoliated deciduous tooth (SHED). The BGs role in promoting dental tissue regeneration is highlighted in Fig. 2.3.

The availability of dental tooth structures and stem cells from dental tissues and teeth opens opportunities for these structures and their derivatives to be used in tissue engineering for the regeneration of dental related structures (Fig. 2.4). Furthermore, the tooth structure closely resembles bone based of its hydroxyapatite mineral content and the non-collagenous proteins (NCP), consisting of osteopontin (OP), matrix extracellular phosphoglycoprotein (MEPE), bone sialoprotein (BSP), dentin matrix protein 1 (Dmp1), and dentin sialoprotein phosphoprotein (DSPP), which opens possibilities to understand the role of these proteins in biomineralisation. For instance, a study showed that dentin phosphoprotein (DPP) from rat tooth formed distinct mineralised nodules similar to those observed in developing bone compared to the BSP and OPN (both from rat tibia), suggesting that DPP may play an important role in biomineralisation for construct development in tissue engineering (70).

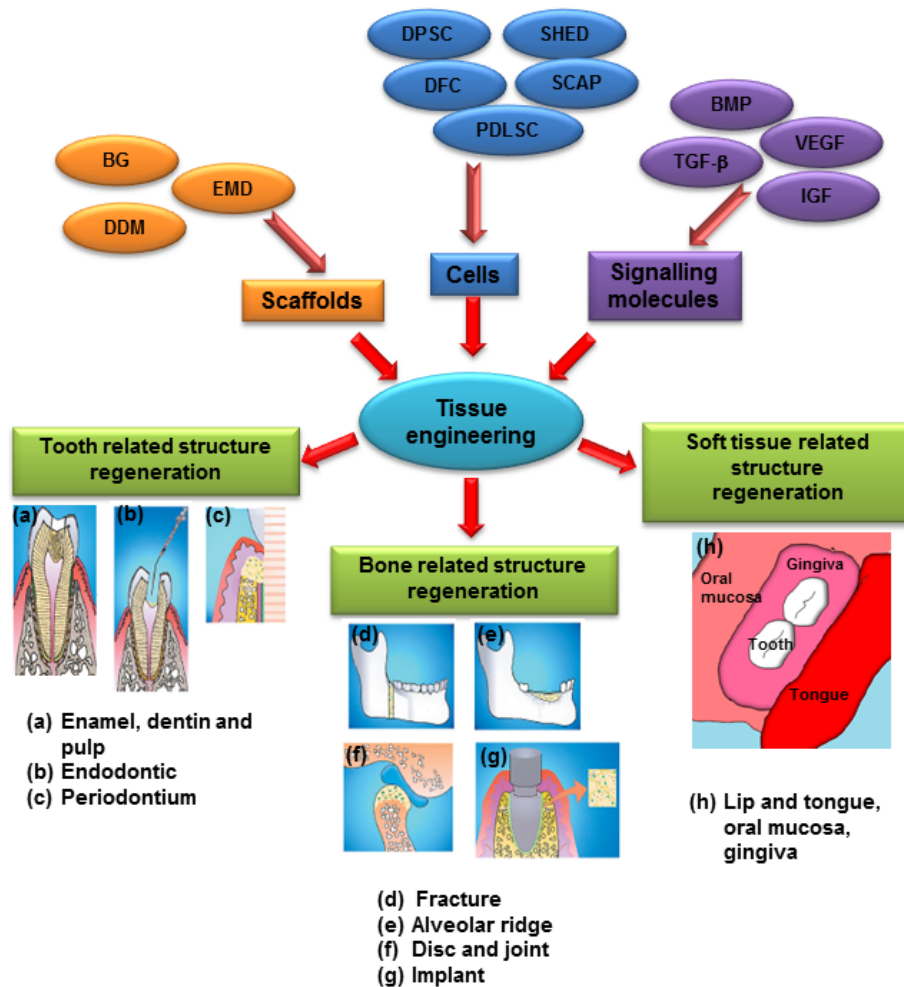


Fig. 2.2: The tissue engineering concept is applied in the regeneration of oral related structures using stem cells from the tooth, scaffolds and signalling molecules. Scaffolds from bioactive glass (BG), enamel matrix derivatives (EMD), and demineralised dentin matrix (DDM) are used either alone or in combination with cells, such as dental pulp stem cells (DPSC), stem cells from human exfoliated deciduous teeth (SHED), periodontal ligament stem cells (PDLSC), stem cells from apical papilla (SCAP), and dental follicular stem cells (DFC). They are also enriched with growth factors or signalling molecules such as bone morphogenetic protein (BMP), vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF- β) and insulin-like growth factor (IGF). Adapted from (68).

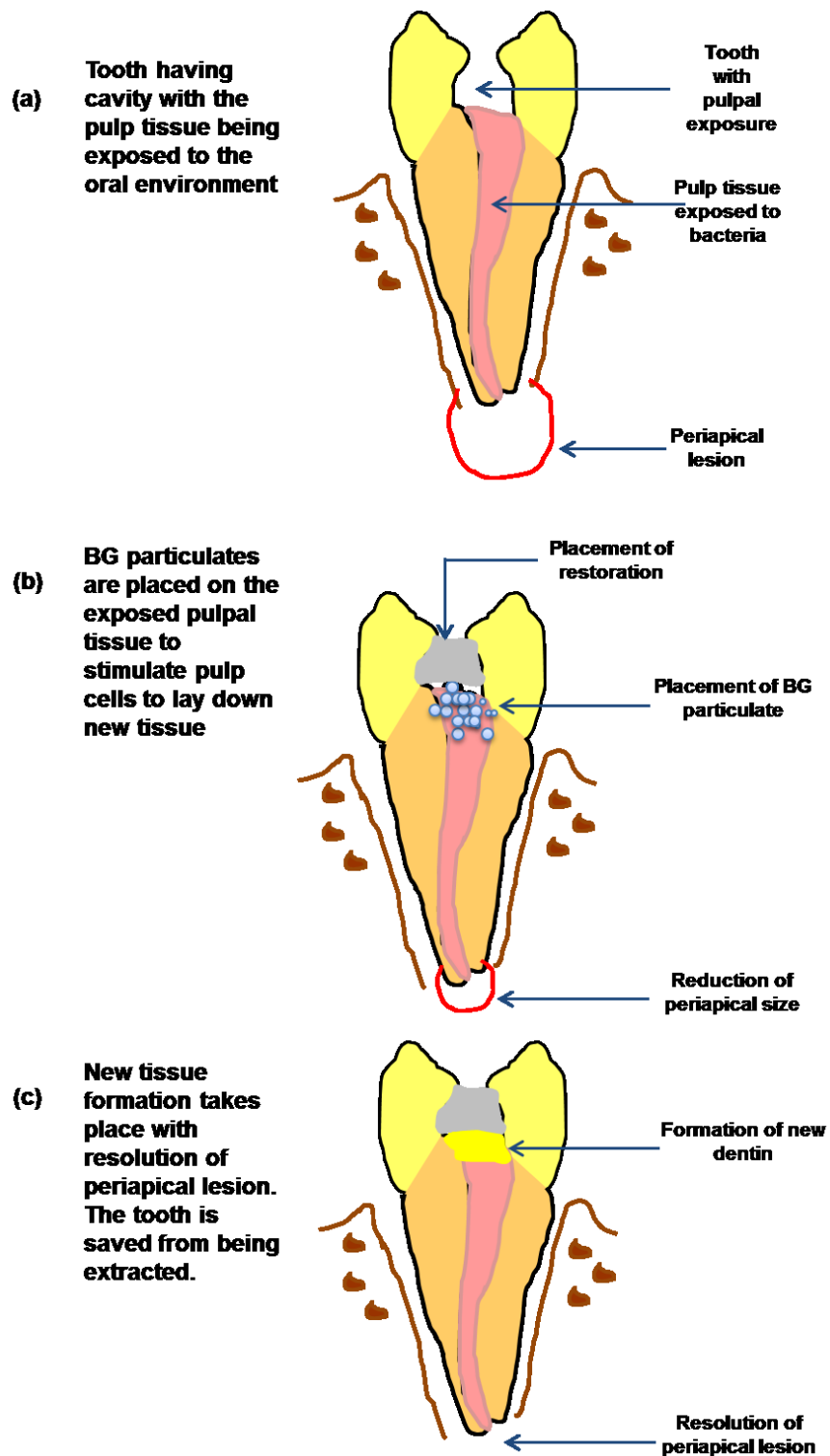


Fig. 2.3: The potential role of BGs in promoting dental tissue regeneration. BGs are used as a pulpal protection agent, which stimulates the underlying pulp cells to differentiate into odontoblast and lay down new dentin tissue, and prevents the tooth from being extracted.

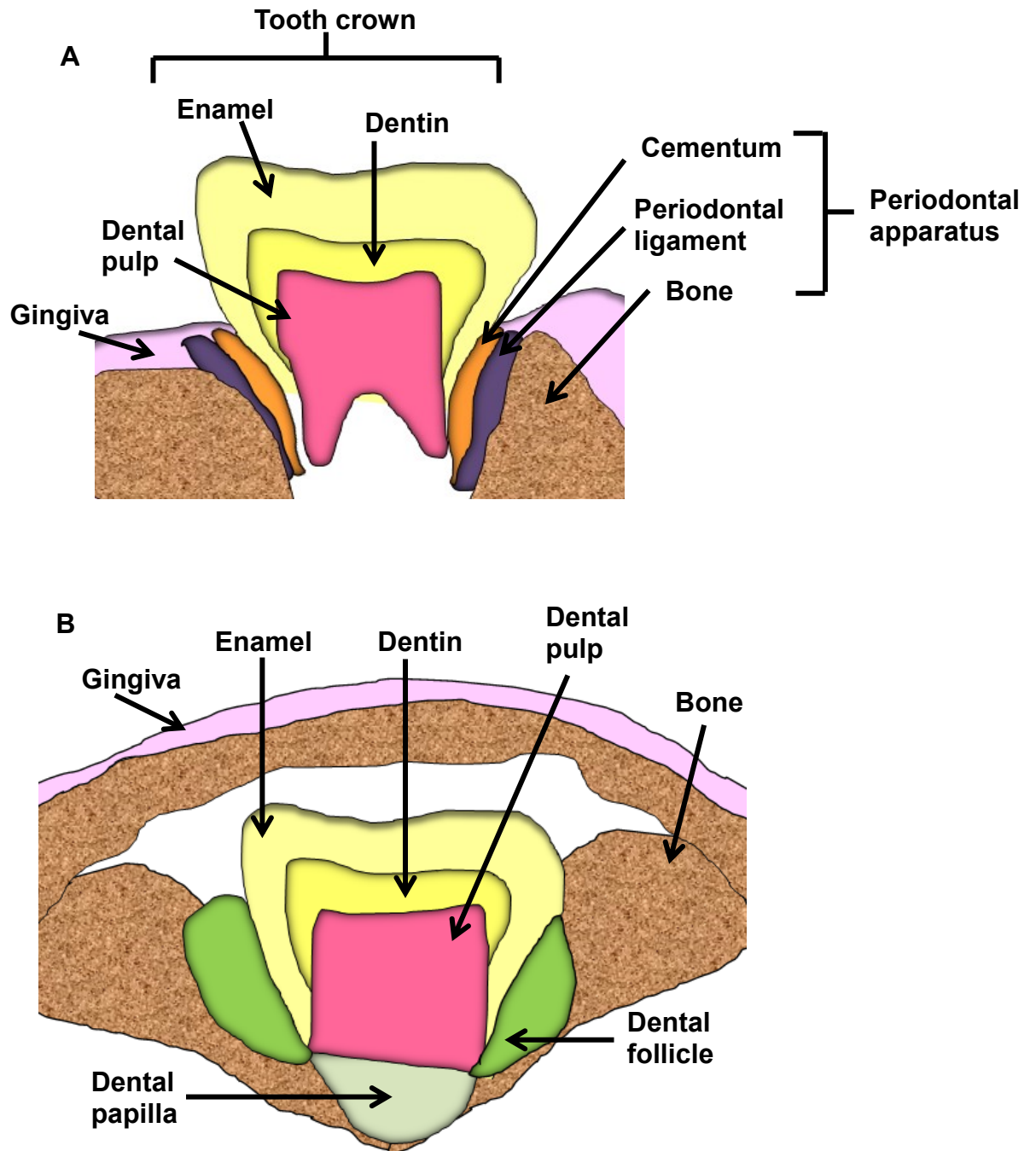


Fig. 2.4: Schematic diagram of dental tissues in an erupted (A) and unerupted tooth (B). Adapted from (71).

In the orofacial region, the process of repair and regeneration involves many tissues, including facial bone, soft tissues, and dental related structures. The facial bones consist of maxilla and mandible, which hold the tooth. There are soft tissue components from extra-oral to intra-oral structures, including facial skin, facial muscle, oral mucosa, tongue, and tooth. Tooth is made up of enamel, dentin and pulp and its supporting structures within the alveolar bone such as the periodontal ligament, cementum and root. Undoubtedly, the repair and regeneration of these tissues is very much interrelated and complex, and involves both hard and soft tissue regeneration.

The hard tissue repair and regeneration of dental related structures includes healing of the tooth socket following tooth extraction, tooth fracture within the alveolar bone, fractures of the jaw bone and also tooth movement in its bony socket either due to chewing or during orthodontic treatment, and also distraction osteogenesis treatment. Each bone repair process is unique to the underlying cause. For example in the fracture of the jaw, the surgeons will approximate the bone segments and apply metal and screw fixation without leaving any gap in between the bone. In this case, the repair process is known as primary bone repair (reviewed in (72)). On the other hand, the repair process following distraction osteogenesis in patients with skeletal malformations involves direct intramembranous ossification, where soft osteoid resembling woven bone is gradually replaced by lamellar bone (72). The similarities between tooth and bone in relation to its organic matrix also make it necessary to understand bone biology and its formation, which will be discussed in the next section (2.3).

2.3 Bone structure and formation

Bone is a mineralised tissue composed mainly of calcium and phosphate. Mineral deposition, which requires collagen fibril deposition (73), occurs by hydroxyapatite precipitation with the formula of $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$. Modification of this formula occurs due to substitution with ions such as carbonate (CO_3^{2-}) (74). Bone matrix consists of an organic and inorganic component. The organic matrix consists of approximately 90% collagen type I and 10% non-collagenous proteins and water phases (wet weight) (75). The inorganic matrix is largely composed of hydroxyapatite, which contributes approximately 65% of the bone wet weight. In calcified bone, the organic

matrix accounts for 25% including cells (2-5%), 5% water, and 70% inorganic mineral (hydroxylapatite), while new bone matrix consists of 94% collagen, where the non-collagenous component of bone consists mainly of proteoglycans (76). Macroscopically, bone tissue is non-homogenous, with porosity ranging from 5 to 95%, which allows bone to be characterised as trabecular (cancellous) or cortical (compact bones) (reviewed in (77)). Trabecular bone, which has 50 to 95% porosity, can be found in flat bones, cuboidal bones and at the end of long bones, while cortical bone, which has 5 to 10% porosity, can be found at the shaft of long bones (77).

Bone is formed *via* two mechanisms: intramembranous ossification or endochondral bone formation (76). In intramembranous ossification, the differentiation of osteoprogenitor cells into bone forming cells results in bone deposition. Endochondral bone formation involves the replacement of a rudimentary cartilage template with bone tissue (78). Many of the facial bones are flat bones formed through intramembranous ossification, while the temporomandibular joints are formed through endochondral bone formation (78). Four types of bone cells exist: osteoblasts, osteoclasts, osteocytes and bone lining cells (77). Osteoblasts differentiated from mesenchymal stem cells and are involved in producing bone. Osteoclasts are multinucleated cells originating from the bone marrow and are involved in bone resorption. Osteocytes are mature osteoblasts located in lacunae surrounded by bone matrix and maintain communication with other cells through canaliculi. Finally, the bone lining cells are inactive osteoblasts that remain on the bone surface when bone formation stops, and become reactivated following chemical or mechanical stimuli (77).

2.3.1 Bone remodelling

Bone undergoes a remodelling process almost constantly. This process is complex, involving resorption of the bone surface and new bone formation (79). Remodelling therefore involves both osteoblast and osteoclast activity. Bone resorption and bone formation are normally balanced, occurring as bone responds to changes in mechanical and physical forces (79). Certain diseases result in an imbalance of bone remodelling, including osteoporosis (where bones have a tendency to fracture and undergo resorption) and osteopetrosis (where the rate of bone formation exceeds

that of bone resorption) (80, 81). Bone remodelling involves a few cyclic phases: resorption, reversal, formation and the resting phase (79). In the resorption phase, activated osteoclasts resorb an area of mineralised bone matrix. The reversal phase occurs when osteoblast precursor cells become activated and differentiate into osteoblasts, migrate to the resorption cavity, and enclose the osteoclastic activity. The formative phase begins when osteoblasts deposit new bone, which becomes mineralised and fills the resorption cavity, and the osteoblasts lying on the surface of the newly formed bone become trapped in the cavity, differentiate into osteocytes, and remain quiescent until activated. Bone remodelling is regulated by many growth factors and hormones, including parathyroid hormone, calcitonin, insulin and growth hormones. Examples of growth factors that play an important role in bone remodelling include the insulin-like growth factors (IGF) I and II, fibroblast growth factors (FGF) and platelet-derived growth factors (PDGF) (reviewed in (79)).

2.3.2 Bone metabolism

Bone metabolism is regulated by many factors, including the biochemical environment, extracellular matrix components and signalling molecules (reviewed in (82)). The signalling molecules involved in bone metabolism include hormones, cytokines, and growth factors. Vitamins A and D, and minerals such as phosphate, calcium, and pyrophosphate have essential roles in bone metabolism, matrix production and ossification. Collagen, mineral ions, and removal of both pyrophosphate and inhibitors are important for ossification (82). The role of these mineral ions, especially the inorganic metals, in bone metabolism is important for the development of scaffolds for bone tissue engineering and regenerative medicine (83). These inorganic metals ions are involved in various bone metabolic pathways and interact with enzymes (84). Collectively, these metal ions known as metallic ions as therapeutic agents (MITA), include strontium, cobalt, copper, and manganese (83). The effect of strontium and cobalt will be discussed further since these two ions are the topic of interest for this thesis.

2.3.2.1 Calcium and phosphorus ions

Calcium and phosphorus are the main ions of mineralised bone in the form of hydroxyapatite, and both are known to be involved in bone metabolism. Extracellular calcium ions maintain bone homeostasis by their direct action on parathyroid

hormone (PTH) secretion, absorption of calcium into the kidney, and activation of the calcium sensing receptor (CaSR) in osteoblasts, which triggers bone remodelling (85). Osteoblasts had higher proliferation when exposed to a low calcium concentration (2-4 mM), whereas higher calcium concentration (6-8 mM) in the culture medium enhanced osteoblast differentiation resulting in higher matrix formation (86). Further to that, osteoblast viability remained higher when exposed to 4.98-7.49 mM calcium in the culture medium compared to 12.49 mM (87). Bone stores approximately 99% calcium, and 85% phosphate. Bone resorption leads to an increase in local inorganic phosphate (Pi) concentration (88). It was shown that inorganic phosphate regulates the expression of matrix gla protein (MGP) and osteopontin (OP) in MC3T3-E1 cells in a process mediated by calcium (88). High inorganic phosphate (7-10 mM) reduced odontoblast viability and led to cell apoptosis, and it was suggested that high inorganic phosphate leads to the formation of calcium phosphate crystals that may play a role during caries formation on the tooth (89).

2.3.2.2 Strontium ions

Strontium ions are divalent cations that can be found as trace elements in biological fluids (90). The ionic radius of strontium (1.13 Å) is larger than that of calcium (0.99 Å) (91) resulting in a higher tendency for calcium to be substituted with strontium in the hydroxyapatite crystal lattice of bone and teeth (92, 93). This occurs either by surface exchange or ionic substitution, which is rapid in new bone and slower in old bone (91). Osteoblast, osteoclast and bone marrow stem cell activity are all affected by strontium. It helps to promote differentiation of osteoprogenitor cells to pre-osteoblastic cells, to prevent osteoclast formation, and to retard cell-induced adipogenic differentiation (19). Strontium inhibits osteoclastogenesis and prevents osteoclast formation through cell apoptosis (94).

Therapeutically, strontium as strontium ranelate (SrR) in low doses is used in osteoporotic women to reduce the risk of bone fracture (95, 96). Male patient with osteoporosis taking SrR also showed improvement in bone density (97). However, administration of high doses of strontium can result in ricket formation among children (98), and osteomalacia in rats with chronic renal failure (99). Strontium was shown to increase bone mass and stimulate bone formation and to reduce bone

resorption (100). Strontium has potential wide uses in diseases other than osteoporosis, including as bone substitutes following fractures due to trauma and old age, and also as a caries preventive agent. For example, in osteoporotic patients requiring dental rehabilitation, the current treatment regimens include the use of bisphosphonates, which predisposes patients to osteonecrosis of the jaw (101, 102), establishing the use of strontium as an alternative treatment.

Strontium-substituted bioactive glasses (SrBG) were developed in our group, and *in vitro* testing showed good biocompatibility for osteoblasts and the prevention of erosion by osteoclasts (22). Furthermore, murine osteoblasts seeded on calcium phosphate (CaP) films exposed to Sr-supplemented culture medium in varying concentrations showed that cells exposed to 1000 μM strontium had higher proliferation and differentiation compared to control and 1, 10, and 100 μM Sr-supplemented culture media, suggesting that strontium promotes osteoblast growth and differentiation to produce mineralised nodules (103). An *in vivo* study showed that the addition of strontium to apatite (HA) resulted in a greater formation of osteoid tissue compared to poly(methyl methacrylate) (PMMA), suggesting the biocompatibility of the Sr-HA for treating bone fractures (104). Scaffolds fabricated with various strontium concentrations were developed and tested *in vitro* (105, 106) and *in vivo* (107, 108), where they showed great potential as bone substitutes in the regeneration and repair of hard tissue.

2.3.2.3 Cobalt ions

Cobalt is one of the trace elements found in human muscle, bone and hair, and is normally available from green vegetables and fresh cereals. Cobalt is involved in the formation of Vitamin B₁₂ (hydroxycobalamin) (23). In healthy subjects, cobalt can be found as trace elements at a concentration of 0.07-0.17 $\mu\text{g/l}$ (109). Cobalt as cobalt chloride (CoCl_2) was used to mimic hypoxia in cells (110, 111), which was proposed either by affecting the *HIF1A* expression through iron reduction in the HIF-1 α prolyl hydroxylases (110), by directly inhibiting HIF-1 α to reduce its binding with von Hippel-Lindau (VHL) (112), or through phosphoinositol 3-kinase (PI3K) signalling by ROS stimulation (111). Cobalt uptake into cells is initially rapid, with a maximum level after 8-12 hours that remains unchanged after 24 hours, suggesting that cobalt

transport into cells may be mediated by active endocytosis through membrane bound ion pumps (113).

Cobalt may play role in bone formation due to its effect in mimicking hypoxia in endochondral bone formation at long bones and epiphysis, where the cartilage template created by chondrocytes forms the shape that guides the osteoblasts to lay down matrix (72). The programmed death of chondrocytes enables blood vessel invasion together with osteoblasts (114), and the interaction of cartilage resorption and osteogenesis is important for bone formation (115). Cobalt may also play role in the repair of bone. In normal bone healing, the release of platelets and inflammatory mediators triggers the release of many cytokines such as VEGF, BMPs and TGFs, which play roles in angiogenesis and osteogenesis (72). Cobalt as a hypoxia-mimicking agent may also trigger the release of these factors, especially VEGF mediated through hypoxia-inducible factor 1 (HIF-1), which may also promote angiogenesis and the repair process (116). Cobalt stimulates *HIF1A*, and induces VEGF secretion suggesting a role as an angiogenesis stimulator through HIF pathway activation (117). Murine bone marrow osteoclasts seeded on CaP-coated surfaces with cobalt addition showed higher osteolytic activity compared to surfaces without cobalt, suggesting that cobalt stimulates osteoclastic activity and that this phenomenon might be a way to enhance vascularisation following implant placement (118).

Although chronic exposure to cobalt may result in hard metal lung hypersensitivity reaction, low dose cobalt exposure mainly occurs due to occupational hazards, and cobalt is excreted in the urine (23). Volunteers taking cobalt (II) chloride as a dietary supplement for 90 days showed that approximately 96% cobalt ends up bound to proteins such as albumin, and within the physiological range, it did not trigger major side effects (119). Furthermore, cobalt-chromium (CoCr) based alloys have been widely used in dentistry as dentures (120). Cobalt was incorporated in BG as a hypoxia-mimicking agent (24, 121, 122), and *in vitro* results showed that the presence of cobalt within the BG scaffold did not affect bone marrow stem cell (BMSC) proliferation (24).

2.3.2.4 Silicon

Silicon (Si) is important for bone matrix formation and mineralisation (84, 123). It normally exists bound with oxygen due to their strong affinity (123). It was shown that silicon is important during the early stages of bone matrix formation, and results in higher collagen production within the bone matrix (124, 125), suggesting that silicon is important for the newly formed bone matrix. Silicon is also involved in CaP formation to enhance bone remodelling (126). The dietary intake of silicon has been shown to increase bone mineral density in men and premenopausal women, suggesting silicon can reduce bone resorption (127). Silicon may exist in a variety of natural and synthetic forms such as: (a) silicic acid and silane in small molecular species; (b) silicates in dicalcium silica; and (c) silica in quartz and amorphous silicon dioxide (128).

The role of silicon in bone homeostasis remains unclear and a proper silicon receptor *in vitro* is still under investigation (129). Human primary osteoblasts-like cells exposed to silicon in the form of orthosilicic acid (Si(OH)_4) showed higher collagen type I expression in the range of 10-50 μM compared to control, suggesting that silicon may play role in bone matrix synthesis (130). The 45S5 BG-conditioned medium contains calcium, phosphorus, and silicon ions that were shown to stimulate gene transcription in osteoblasts within 48 hours. These genes are related to osteoblast function such as RCL (growth-related gene) and Decorin (production and maintenance of extracellular matrix). Osteoblasts showed increased RCL expression up to 5-fold and Decorin expression up to 2.2-fold. This effect may be due to the high level of silicon present in the culture medium which increased up to 88-fold compared to control medium, whereas the calcium and phosphorus concentration were slightly higher (10% increase) compared to the control medium (129). In an earlier study, the authors also showed the 45S5 BG conditioned-medium resulted in higher osteoblast numbers (more than 100%) compared to the control medium, which suggests the 45S5 BG conditioned-medium promoted osteoblast proliferation (131).

2.4 Regeneration of oral and dental tissue

The repair and regeneration of dental related structures is explained through the simple procedure of a tooth extraction in order to demonstrate how bioactive glasses

(BG) have the potential to be applied as bone substitutes (either particulate or scaffold based), oral patches or films, or surgical sutures (to approximate the wound) to promote healing and repair. Following tooth extraction, the tooth socket undergoes changes to allow new bone formation in the empty socket left behind by the tooth. The soft tissue (gingiva and oral mucosa) will also undergo healing to close the gap.

Prior to tooth extraction, local anaesthesia is given to the area near the tooth to be extracted. Once the area is numb, the tooth is elevated and extracted using an elevator or tooth forceps. Then, the tooth is removed from the socket and blood will ooze from the socket. The patient is asked to bite gauze to apply pressure, which helps to stop the bleeding and initiate clot formation. Then, the socket will heal following the deposition of new bone and the soft tissue will approximate and close the wound, which typically occurs without suturing. In the case of a particularly large wound or incision, the gap will be sutured. In non-ideal conditions, for example when there is an abscessed tooth, there is a tendency for infection of the socket known as alveolar osteitis (AO) (132). The mechanism involved in the repair and regeneration of the alveolar socket bone (hard tissue) and oral mucosa (soft tissue) will be discussed in the next section.

2.4.1 Healing and repair of tooth socket bone

The healing of the socket occurs through intramembranous ossification (133), which is based on embryonic development, in which the flat bones of the facial skeleton including maxilla and mandible developed through intramembranous ossification (72). During the early stages of fracture healing, induction and inflammation occur. Bleeding occurs due to severed blood vessels and a haemorrhage fills the gap, resulting in haematoma formation (77). Bone necrosis and cell death occur, and intracellular by-products are released during induction, where low pH in the environment results in the release of chemotactic factors, such as prostaglandins (PG) and bone morphogenetic proteins (BMP) to attract local inflammatory cells (134). Inflammation is induced by neutrophils, macrophages, and mast cells (134). The macrophages remove the dead tissue and initiate initial granulation tissue formation upon which the undifferentiated mesenchymal stem cells (MSC) can reside (77). These undifferentiated MSC differentiate, and osteoblasts and fibroblasts

migrate to the area to form osteoid matrix and initiate capillary ingrowth to heal the fracture (134).

During the healing process of the socket after tooth extraction, a controlled and regulated repair and regeneration process takes place. New tissue will be generated in a complex process involving chemoattractants to the wound, migration of undifferentiated mesenchymal progenitor cells that will later differentiate into bone forming cells to lay down new bone, and extracellular matrix (ECM) formation together with the nutrient supply provided by the local neurovascular bundle (135). During normal healing of the socket, which relies on blood clot formation, the socket will be filled by the clot, followed by the coordinated events of inflammation, epithelisation, fibroplasia and remodelling (reviewed in (136)). A fibrin clot will fill the gap in the socket, then fibroblasts and capillaries from the tissue around the alveolar wall will enter the defect and interlace with the clot to form granulation tissue. Capillaries will penetrate the granulation tissue and new bone formation (woven bone) is observable after 31 days (136) (Fig. 2.5). The woven bone will be formed in an accelerated fashion to allow the bone deposition, and it was shown that sockets are filled by new bone one month after tooth extraction in a dog model (137).

Histological sections during socket healing showed a typical tissue repair process occurring in the socket. The presence of a blood clot and inflammatory infiltrate is observable within three days, and the clot will undergo maturation and infiltration by fibroblasts to form granulation tissue and osteoid matrix formation (138), which is further mineralised by calcium deposition as hydroxyapatite crystals. The alveolar bone neoformation begins from the apical and lateral wall of the socket, which moves centrally, and in the end, the socket is filled with trabecular bone (139). The progression of bone formation in the tooth sockets depends on many factors, including the gap size, blood supply, hormones, growth factors, and also drugs taken by the patient such as bisphosphonates or menopause therapy (140, 141).

The healing of a tooth socket results in a reduction in alveolar bone height at both its buccal and lingual plates, and also a buccal wall reduction horizontally. Thus, strategies to repair and heal the tooth socket must take into account the bone loss

and the importance of nutrient supply from the neurovascular bundles to ensure proper tissue regeneration.

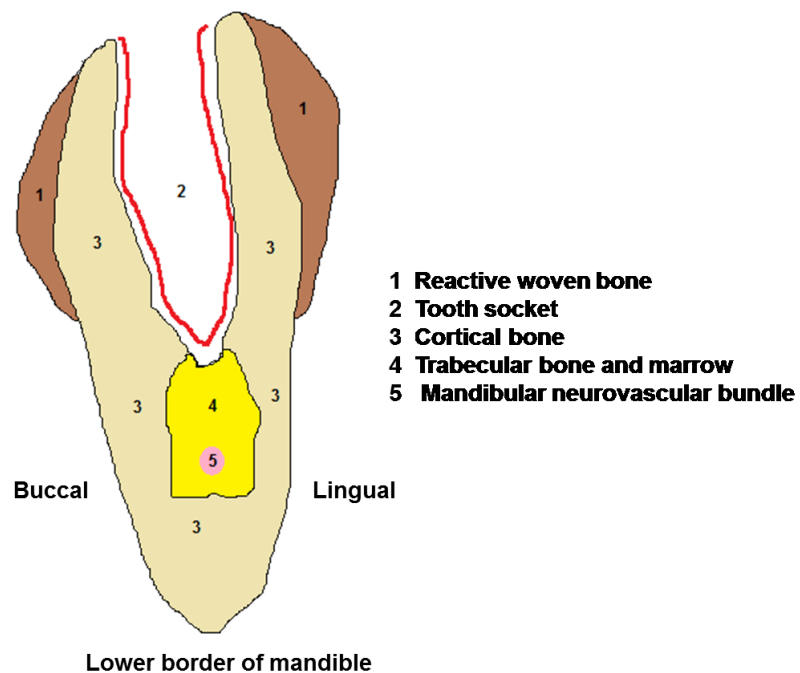


Fig. 2.5: The schematic representative of tooth socket following tooth extraction and the surrounding bone in the lower jaw. The woven bone is reactive owing to the regional acceleratory phenomenon, which is observable within a month (136). Adapted from (142).

Many strategies have been undertaken to reduce the bone resorption effect following tooth extraction. A flap is raised to provide additional support for a local haemostatic agent placed in the socket (143). The regeneration of the periodontal support is enhanced by the modification of gingival tissue prior to tooth extraction (144), and placement of enamel matrix derivatives (EMD) (145), demineralised dentin matrix (DDM) (146), or demineralised autologous bone (147) within the socket was also used to enhance and promote bone formation. Nevertheless, these strategies cannot prevent alveolar bone resorption, which tends to occur following tooth extraction (144).

Growth factors or signalling molecules were also used to enhance the repair process. The signalling molecules involved in the healing process include transforming growth factor beta (TGF- β), vascular endothelial growth factor (VEGF),

bone morphogenetic protein (BMP) and insulin-like growth factor (IGF) (135). It was shown that rabbits with untreated diabetes showed delayed healing after tooth extraction after 30 days due to a lower level of TGF- β and BMP-4, suggesting that these growth factors are important for bone formation in the socket after tooth extraction (135). Platelet derived growth factor (PDGF) can stimulate osteoblasts, fibroblasts and endothelial cells, and is also important for angiogenesis. It was shown that PDGF and its isoforms carried by liposomes and injected into the tooth socket of rats was able to promote bone formation compared to the control socket (only blood clot) (138). However, the use of growth factors or signalling molecules may need further exploration since a high cost is involved and the use in humans may also pose difficulties due to the targets of these factors or molecules within the human body (26). Thus, the use of BG as a scaffold-based strategy either infused with cells or without cells, is a suitable alternative. Indeed, BG has shown many benefits in *in vitro* (22, 148) and *in vivo* (149, 150) studies and the BG compositions can be tailored for specific needs. Although the use of BG particulates in cat tooth extraction sockets did not show significant changes when compared to the control and autogenous bone grafts within four weeks, the BG used did not affect bone formation, suggesting the biocompatibility of these materials for use as bone filler materials (133). Furthermore, when particulate materials were used and placed in the extraction socket, these materials provided additional benefits by preventing alveolar bone collapse, and maintained the shape of the socket prior to implant placement (151).

2.5 Oxygenation for healing and repair of dental structures

Early life organisms used solar for energy. Photosynthesis uses CO₂, water and light to generate energy, which is derived from glucose production that results in oxygen (O₂) formation (152). Mitochondria are capable of producing ATP from glucose and O₂, and to generate CO₂ and water as side products (152). During oxidative phosphorylation, a proton gradient is generated when an electron is transferred within the inner mitochondrial membrane, where O₂ acts as the electron acceptor, and water is produced. This process is catalysed by cytochrome c oxidase (COX), which is rather inefficient, resulting in superoxide radical production also known as reactive oxygen species (ROS). Superoxide dismutase enzymes convert ROS to

hydrogen peroxide (H_2O_2), and ROS can oxidize cellular proteins, lipids or nucleic acids leading to cell death (152). In mammalian cells, mitochondrial ROS is used as a signal to activate HIF-1 in hypoxia.

Cells require O_2 for their metabolism and for generating energy from ATP. The O_2 supply is also important for the repair and regeneration of tissues (153). O_2 availability helps to prevent infection, promote proliferation and enhance collagen production (154, 155). During the early stages of injury, the surrounding tissue may become hypoxic due to damaged vascular supply and the increased O_2 consumption by the metabolically active cells (153). Chronic wounds are hypoxic and have O_2 tension around 5 to 20 mm Hg compared to normal tissue, which is around 30 to 50 mm Hg (156). Chronic hypoxia impairs wound healing (154, 155). Hypoxia stimulates macrophages, keratinocytes and fibroblasts to release cytokines such as PDGF, TGF- β , VEGF, and TNF- α . These cytokines are important in the wound healing cascade (155). In normal wound healing, ROS such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-) act as cellular messengers. Hypoxia stimulates wound healing by triggering cells to release factors involved in angiogenesis, and O_2 is important to sustain the repair process (154). The role of HIF-1 α through the HIF-1 pathway in the regeneration of dental tissue is shown in Fig. 2.6.

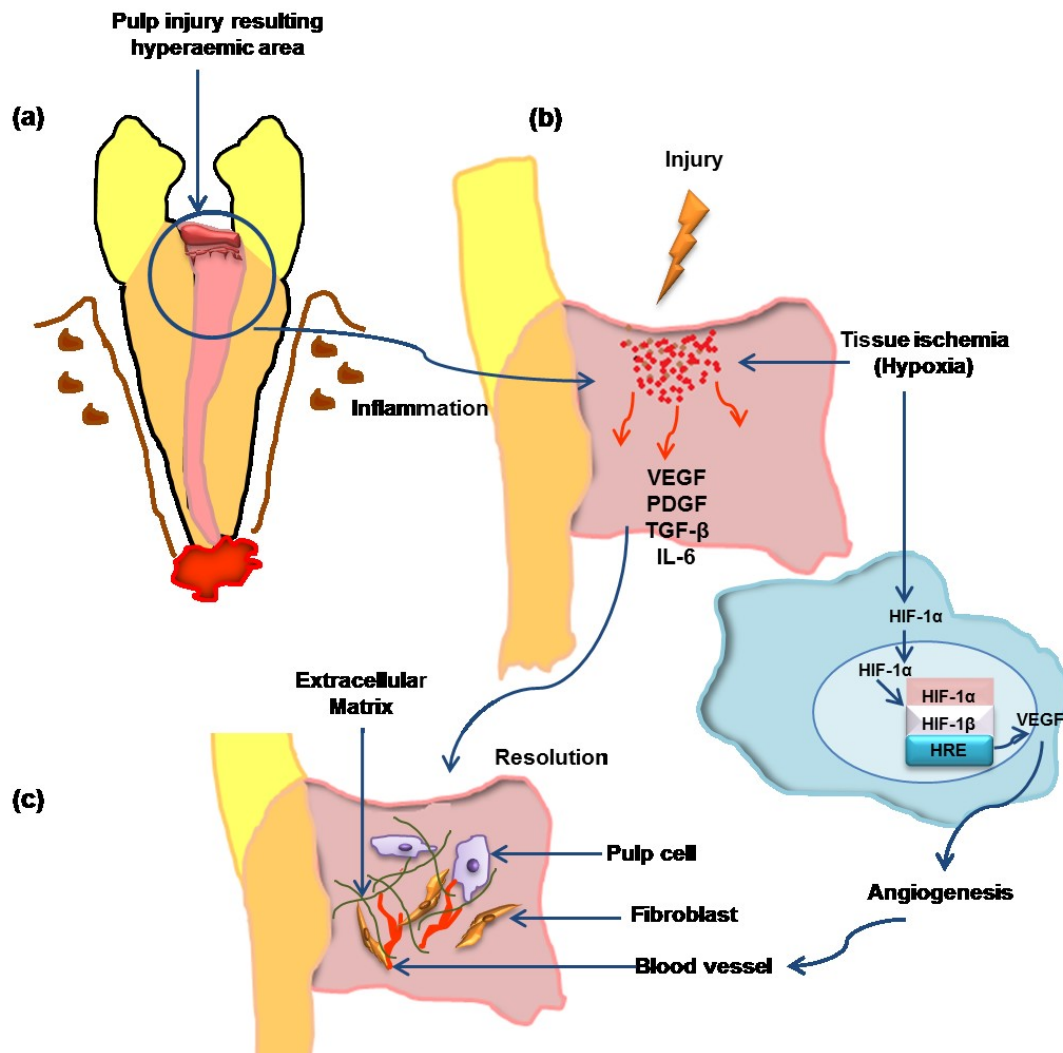


Fig. 2.6: The role of hypoxia through the HIF-1 pathway for the regeneration of dental tissue. Pulpal injury results from the exposed cavity (a). The injured pulp cells release signalling molecules which initiate an inflammatory cascade, and tissue ischemia (hypoxic) then triggers the HIF-1 pathway to promote angiogenesis via VEGF release (b). Once the cavity is restored and angiogenesis takes place, dental pulp tissue undergoes healing (c).

2.5.1 Hypoxia

Oxygen (O₂) plays a significant role in controlling the expression of the specific genetic programs involved in normal developmental processes and pathological conditions (114). Physiological hypoxia occurs during the early stages of organ development (embryogenesis) to enable vasculogenesis until the organs are fully formed and can control their own supply of oxygenation (114, 157). Hypoxia through HIF activated genes is involved in proliferation and differentiation during morphogenesis. Failure of the HIF pathway in modulating the transcriptional gene activation may lead to embryo lethality (158). In cells, oxygen concentrations are carefully maintained within a narrow physiological range to ensure the cells can function properly. Cells require ATP to function, while a reduction or lack of oxygen may lead to altered cells and tissue damage (159).

Hypoxia refers to below-normal levels of O₂ in air, blood, or tissue (normal O₂ levels in most mammalian cell types falls in the range of 2 to 9%) (157), which may lead to cellular dysfunction and death due to decreased blood, altered O₂ release from haemoglobin, and impaired blood delivery (160). When hypoxia occurs (114), the body responds by adapting to low levels of O₂ or by increasing O₂ delivery to the body, displaying a range of responses such as changes in carbohydrate metabolism or local blood vessel vasculature (157). The response can occur systemically, locally, or at the cellular level such as through remodelling of the local vascular network, increased production of erythropoietin (EPO), induction of tyrosine hydroxylase, and the stimulation of new blood vessels by upregulation of VEGF (160). Hypoxia also activates certain molecular pathways, including the hypoxia-inducible factor pathways, the environmental sensing mammalian target of rapamycin (mTOR), the endoplasmic reticulum stress response, the cyclic guanosine monophosphate (cGMP), and nuclear factor kappa-B (NF-κB) (reviewed in (157), Fig. 2.7). In this thesis, only the HIF pathway will be further discussed.

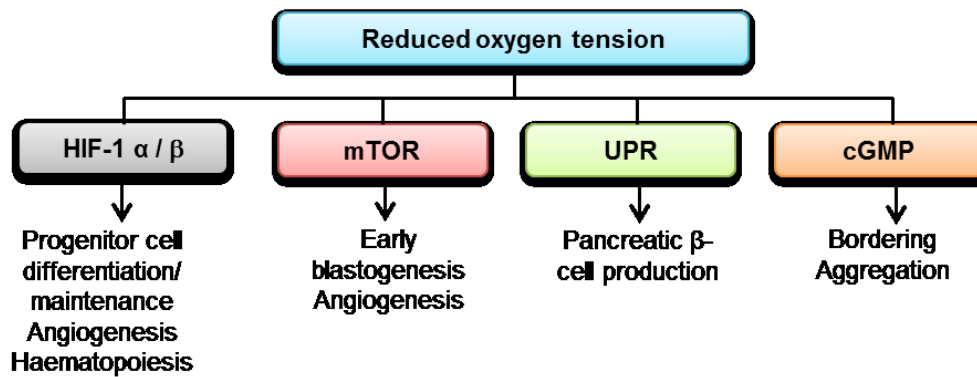


Fig. 2.7: Examples of pathways that are activated upon changes in oxygen availability and the effects following those changes. These include the hypoxia-inducible factors (HIF), mammalian target of rapamycin (mTOR), unfolded protein response (UPR), cyclic guanosine monophosphate (cGMP). Adapted from (157).

Some of the most studied transcriptional factors involved in hypoxia are the family of hypoxia-inducible factors (HIFs) (161, 162). HIFs are part of an environmental sensor known as bHLH–PAS (basic helix-loop-helix–Per-Arnt-Sim) transcription factors in a rapidly growing family of proteins. They are composed of O₂-labile α subunits (HIF-1α, HIF-2α and HIF 3α), and a stable β-subunit (HIF-1β) (157, 163). Hypoxia inducible factor-1, a heterodimer of two bHLH-PAS proteins (164), has two subunits: the hypoxia inducible factor-1α (HIF-1α) and the constitutively expressed hypoxia inducible factor-1β (HIF-1β) (114), which is also known as the aryl hydrocarbon receptor nuclear translocator (ARNT) that is principally insensible to the changes in O₂ tension (157) as shown in Fig. 2.8. The bHLH-PAS motifs and the transactivation domain (TAD) are involved in heterodimer formation with p300/CBP that results in transcriptional stimulation (165). HIF-2α is encoded by the endothelial PAS domain protein 1 (EPAS1) gene and its expression is strictly related to certain cell types (166), such as lung endothelial and epithelial cells (167) and hepatocytes, and it is involved in vascular endothelial functions in angiogenesis and erythropoiesis (163). HIF-1α and HIF-2α may induce similar hypoxia-target genes. HIF-3α is the third subunit of HIF-α, which contains inhibitory Per-Arnt-Sim (IPAS) as one of its loci. It lacks the C terminal transactivation domain and may be involved as a negative inhibitor of HIF pathway activation (168). Variants of HIF-3α were induced by hypoxia mediated by HIF-1α but not HIF-2α (169).

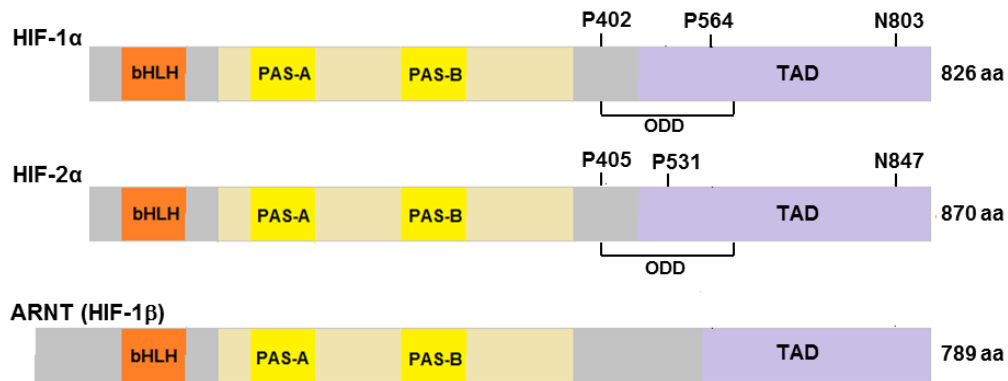


Fig. 2.8: Hypoxia-inducible factors (HIFs) have different subunits. HIF-1 α , HIF-2 α and HIF-1 β are part of the basic helix-loop-helix (bHLH) superfamily containing two PAS domains acids, known as PAS-A and PAS-B. The bHLH, PAS-A, and PAS-B of both HIF-1 α and HIF-1 β take part in HIF dimerization to activate the transcriptional program that mediates the hypoxic response. Proline residues on the oxygen-dependant degradation domain (ODDD) of HIF-1 α (P402, P564) and HIF-2 α (P405) are involved in HIF-1 α proteosomal degradation. HIF-1 β (ARNT) with no ODD is insensitive to O₂ changes. Adapted from (157).

In HeLa S3 cells, it was shown that upon exposure to anoxia and hypoxia (0, 0.02, 0.1, 0.5, 5 and 20% O₂), the HIF-1 α protein is detectable within 2 minutes, and nuclear accumulation occurs within 20 to 30 minutes. However, HIF-1 α degradation takes a longer time, suggesting that HIF-1 α supply is important to ensure cell survival (170). HIFs are also important during embryonic development. Homozygous-deficient mouse embryos lacking *HIF1A* (*HIF1A*^{-/-}) showed signs of altered developmental stages, defective neural tube formation, and cardiovascular defects, and died in 11 days. The histologic analysis showed that *HIF1A*^{-/-} mice lacked viable mesenchymal stem cells and had defective vascular endothelial cells, suggesting that *HIF1A* is required for embryonic development (171, 172). In addition, it was shown that defective vascularisation in *HIF1A*^{-/-} mice was due to a reduced number of mesenchymal stem cells supporting the vascular endothelium (173). In mouse embryos with normal *HIF1A*, many areas were found to be hypoxic, especially at the neural tube and neuronal mesenchymal areas including the yolk sac, suggesting that hypoxia may exist during embryo formation to assist in vascularisation (174). Embryonic stem cells (ESC) lacking *HIF1A* also failed to express the O₂-dependent *HIF1A* subunit, and showed reduced expression of genes

encoding glucose transporters and glucolytic enzymes compared to normal Hep3B cells, suggesting a role of *HIF1A* and hypoxia for cell maintenance (171).

Reduced O₂ tension and the hypoxic responses mediated by HIF-1 occur *via* the HIF-1 pathway as shown in Fig 2.9 below. In normal O₂ tension, the proline residues (prolines 402 and 564) in the O₂-dependant degradation domain (ODDD) of HIF-1 α are hydroxylated by prolyl hydroxylase (PHD) causing the degradation of HIF-1 α mediated by the product of von Hippel-Lindau (VHL) tumour suppressor protein through the ubiquitin-proteosomal degradation pathway within 5 minutes or less (114, 157, 164). VHL recruits HIF-1 α to elongin C in the elongin C binding domain (CBD) of VHL (175), and interacts with the E3 ubiquitin ligase complex to ubiquitinate HIF-1 α (152). Degradation of HIF-1 α in the ubiquitin complex involves covalent attachment of ubiquitin to the target protein and degradation occurs through the 26S proteasome (176). When O₂ levels fall below 5%, HIF-1 α accumulates after subsequent stabilisation by heat shock protein 90 (HSP90) (165), binds with HIF-1 β (ARNT) and translocates into the nucleus, where the HIF-1 complex binds to the hypoxia-responsive elements (HRE) in the promoter regions of hypoxia responsive genes such as VEGF, EPO and GLUT1 (165) to initiate transcription (114) as shown in Fig. 2.9a. The activated HRE consists of a pair of contiguous transcription binding sites for HIF-1 α and the HIF-1 β dimer, and the sequence is represented as 59-RCGTG-39, which is found in the mouse LDHA promoter (177) as shown in Fig. 2.9b.

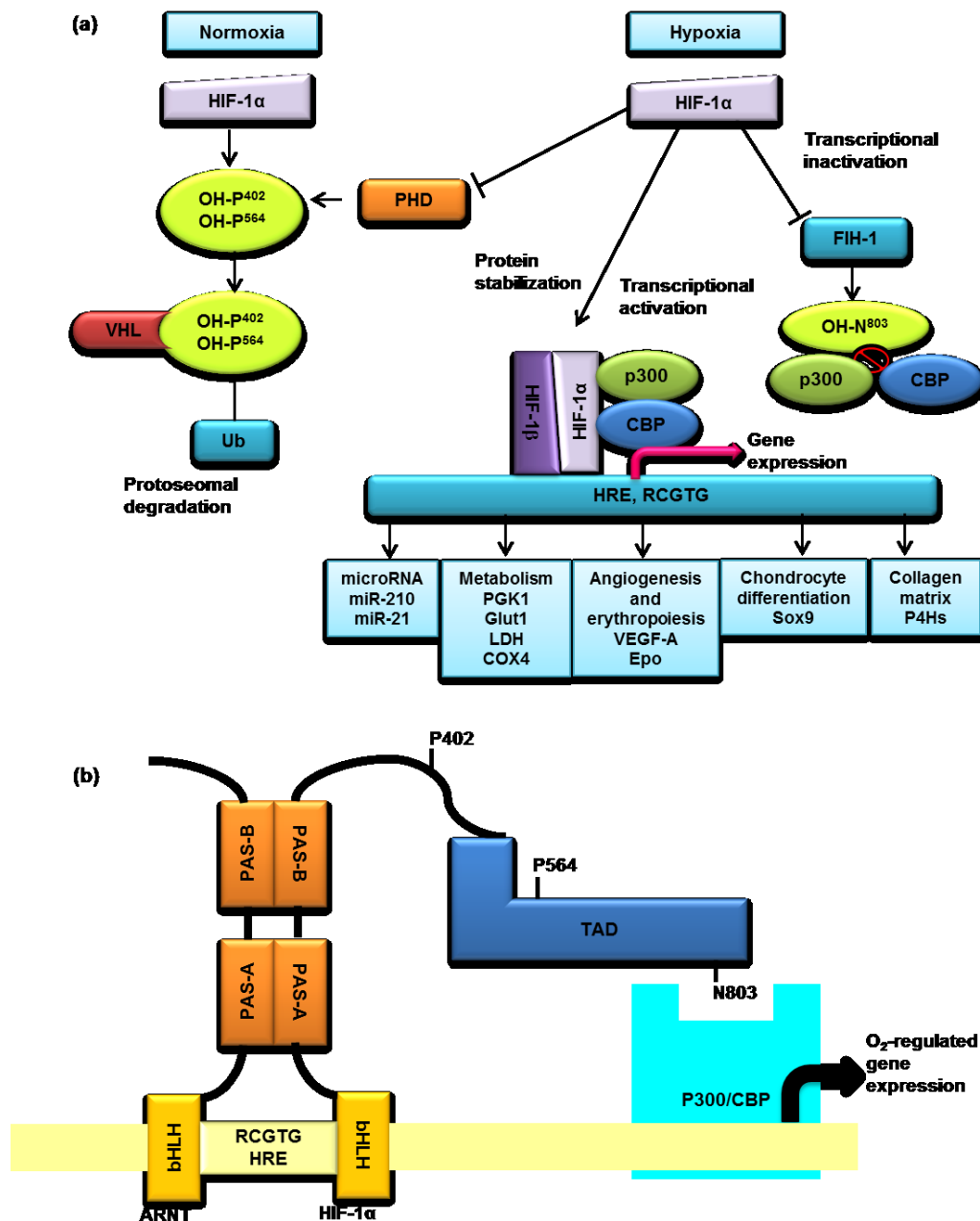


Fig. 2.9: The HIF pathway. During normoxia, HIF-1 α undergoes proteosomal degradation mediated by von-Hippel-Lindau ubiquitin ligase, and factor inhibiting HIF (FIH) hydroxylate HIF-1 α on Asp803 in its carboxy-terminal transactivation domain (C-TAD) resulting in HIF-1 α degradation. In hypoxia, HIF-1 α escapes hydroxylation by prolyl hydroxylase (PHD) and translocates to the nucleus, where it dimerises with HIF-1 β to bind to hypoxia-responsive element (HRE) sites on hypoxia-related genes (a). The HIF-1 dimer is formed between HIF-1 α and HIF-1 β through Per-Arnt-Sim (PAS) domains, which bind to the hypoxia-responsive element (HRE) of hypoxia regulated genes (b). Adapted from (114) and (157).

Currently, the mechanism by which the HIF-1 complex translocates into the nucleus is not fully understood. The nuclear envelope separates DNA and its transcriptional apparatus from other elements in the cytoplasm (178), and communication between these biomolecules occurs through tightly regulated nuclear-pore complexes (165). A specific amino acid sequence known as the nuclear localization signal (NLS) within the cargo protein to be transported mediates the import of nuclear protein into the cell nucleus. However, the NLS requires specific transporters known as karyopherins (179). These karyopherins mediate nuclear import (importins) and nuclear export (exportins) and interact directly with the cargo protein (179). It was stated that all importin α have the potential to mediate the nuclear import of NLS within the cargo protein, but have divergent functions (178). The translocation of the HIF-1 complex (cargo protein) into the nucleus first requires the binding of importin β -1 with importin α . The NLS of the cargo protein will then bind to the importin α and importin β -1 complex. Importin β mediates the passage of the complex through the nuclear pore complex (NPC) (179, 180). This mechanism of HIF-1 complex translocation was proposed for the activation of the VEGF gene during hypoxic stress (165).

Hypoxia mediated by HIF-1 activity is regulated in an O_2 -dependent and O_2 -independent manner (114). As mentioned earlier, HIF-1 α degradation in the O_2 -dependent regulation of hypoxia is mediated by prolyl hydroxylase 2 (PHD2). It is involved in the hydroxylation of proline residues (Pro 402 and Pro 564) residing in the ODD domain of HIF-1 α , which requires O_2 as substrate, 2-oxoglutarate (2-OG), iron (Fe^{2+}) as cofactor, and ascorbate (181, 182). During PHD hydroxylation, there is an O_2 atom split, where one oxygen atom is transferred to the proline residue, and the other oxygen atom interacts with 2-oxoglutarate to generate succinate and CO_2 (183-185). PHD hydroxylation occurs in the presence of reactive ferryl species, and is dependent on ascorbate in order to augment intracellular iron. The iron must be replenished to overcome damage by ROS generation (186). PHD activity also requires one iron atom, which is loosely bound to two histidine residues and one aspartic acid. Ascorbate helps to maintain iron in the ferrous state to ensure continued activity of PHD (187). In the same ODD domain, lysine residue (Lys532) upon acetylation by arrest-defective-protein-1 (ARD1) cooperates with the OH group, recruits pVHL and leads to HIF-1 α degradation (188). It was also shown that HIF-1 α suppression does not require ARD1. Instead, ARD1 may be involved in regulating

cell proliferation (189). Furthermore, hydroxylation of an asparagine residue (N803) in the C-TAD domain also inhibits HIF-1 α binding to CBP/p300, which then inhibits HIF-1 α transcriptional activity (190). In mammalian cells, other PHDs have been identified, known as PHD1, PHD2, and PHD3, which regulate the key proline residues in HIF- α (187). The iron can be displaced using iron chelating agents such as desferrioxamine (DFO) and transition metals such as cobalt, manganese and nickel (186).

Another mechanism is involved in controlling the HIF-1 activity in an O₂-dependent manner that may be modulated by the cellular microenvironment to affect the HIF pathway to precisely fine-tune the physiological responses to hypoxia (183, 191). Factor inhibiting HIF (FIH-1) is an asparagine hydroxylase that hydroxylates the N803 residue in the C-terminal transactivation domain of HIF-1 α allowing HIF-1 α interaction with the CBP/p300 co-activator complex. The residues range from 757-826 of carboxy-TAD on HIF-1 α (192). FIH also requires iron and ascorbate as cofactors (190). During hypoxia, FIH-1 does not hydroxylate N803, ensuring that HIF-1 α can interact with its transcriptional co-activators, p300 and CBP (CREB [cAMP-response-element-binding protein] binding protein), which enhances the hypoxic response (114).

The O₂-independent manner of regulating HIF-1 activity occurs *via* the activation of growth factors, environmental parameters, vascular hormones and cytokines involved in various signalling pathways such as PI3K, mTOR, AKT, NF- κ B, HSP90, RACK1 and cMyc (193, 194). These pathways allow cell-type-specific (195, 196), and organ specific (197) hypoxia responses. During hypoxia, cellular, local and systemic responses occur through the activation of genes and proteins to ensure sufficient O₂ supply to the site for cell survival (152, 191). Various HIF-1 target genes are listed in Table 2.1.

Table 2.1: HIF-1 target genes. Based on (198).

Gene Product
Adenylate kinase 3
Adrenomedullin
Aldolase A
Aldolase C
Endothelin-1 (ET-1)
Enolase 1
Erythropoietin (EPO)
Glucose transporter 1
Glucose transporter 3
Glyceraldehyde phosphate dehydrogenase
Heme-oxygenase-1
Hexokinase 1
Hexokinase 2
Insulin-like growth factor II (IGF-II)
IGF binding protein 1
IGF factor binding protein 3
Lactate dehydrogenase A
Nitric oxide synthase 2 (NOS2)
Phosphoglycerate kinase I
Transferrin
Vascular endothelial growth factor (VEGF)
VEGF receptor FLT-1

During hypoxia, synchronised responses occur at the local and systemic level to compensate. At the cellular level, cells increase their gene expression related to glycolysis. The reduced ATP level in mitochondria is compensated by anaerobic glycolysis (152, 193). Cell injury during hypoxia results in HIF-1 release, which also activates insulin growth factor 2 (IGF-2), epithelial growth factor (EGF) and fibroblast

growth factor 2 (FGF-2) to enhance proliferation and repair mechanisms mediated *via* the MAPK and PI3K pathways (184, 193). Cellular injury due to hypoxia may also result in apoptosis mediated by the upregulation of p53. Hypoxia also triggers nitric oxide (NO), which is the major regulator of vascular perfusion (199), and inhibits 2-oxoglutarate-dependent oxygenases, which competes with O₂ for binding to the iron active site *in vitro* (200).

2.5.2 Hypoxia and hard tissue regeneration

Bone is a special tissue that can undergo remodelling and repair, and is formed *via* two mechanisms: the endochondral and intramembranous bone formation (114, 201). Bone formation requires the interplay of osteogenesis and angiogenesis, and the genetic mechanisms are largely affected by hypoxia (114, 158). The vascular invasion into the area is crucial for the coupling between cartilage resorption and osteogenesis (115).

As shown in Fig. 2.10, in endochondral ossification, chondrocytes in the foetal growth plate undergo differentiation, synthesise collagen type II, and form a columnar layer as they piled up against each other. The most distal cells stop proliferating, exit the cycle and become hypertrophic. The hypertrophic chondrocytes lay down collagen type X, secrete VEGF (116), and undergo mineralisation. Programmed death of the hypertrophic chondrocytes enables blood vessels to invade the area and replace the cartilage with bone, the so-called *primary spongiosa* (114). The vascular invasion into the area is crucial for the coupling between cartilage resorption and osteogenesis (115).

Intramembranous bone formation gives rise to the flat bones of skull. During formation, the osteoprogenitors directly differentiate into bone forming cells, which lay down bone matrix and eventually lead to mineralisation and new bone formation (202). As endochondral bone formation coincides with capillary ingrowth and angiogenesis, the HIF-1 pathway may also play a major role in the intramembranous bone formation acting on bone progenitor cells (202). During bone fracture, disruption of blood occurs, which may lead to hypoxia of adjacent tissue. Based on these observations, osteoblasts and osteoclasts are able to respond to oxygen fluctuations (eg. reduced oxygen tension [hypoxia]) through the HIF-1 pathway by

increasing levels of HIF-1 α , which subsequently activates VEGF release and promotes angiogenesis and osteogenesis (202).

As mentioned in Section 2.3, there are four types of cells involved in bone formation: osteoblasts, osteoclasts, osteocytes, and bone lining cells. At the cellular level, osteoblasts mediate bone matrix formation and mineralisation, while osteoclasts mediated bone resorption. Reduced oxygen tension or hypoxia has been shown to regulate the activity of these cells. Various responses has been reported upon exposure of osteoblasts (159, 203) and osteoclasts (204, 205) to hypoxia, which will be discussed in the following section.

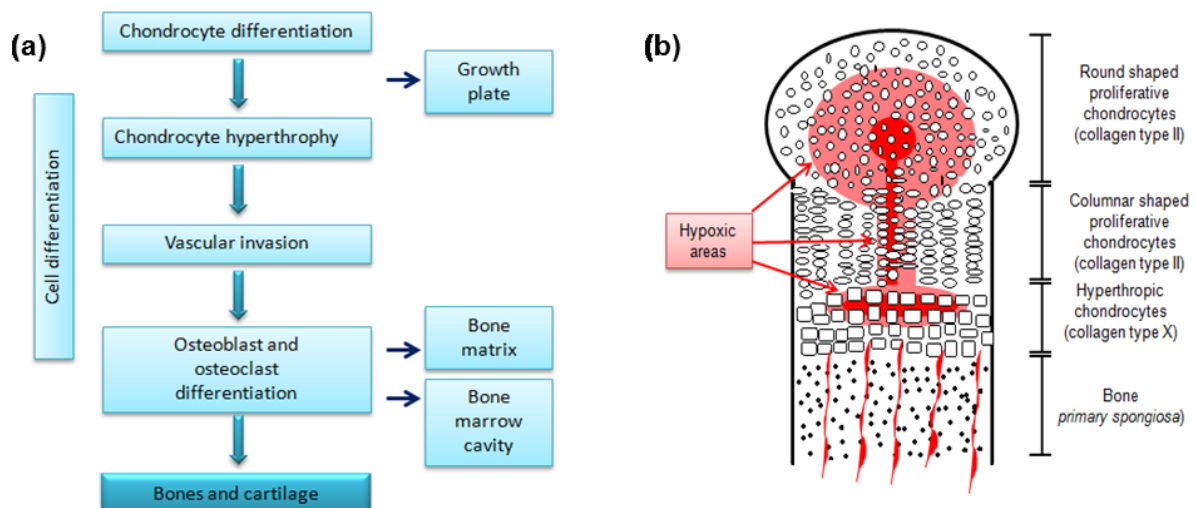


Fig. 2.10: Cells involved in bone formation are spatially distributed (a). Chondrocytes are found in growth plates and joints. Blood vessels invade the area and bring progenitor cells from the bone collar to the centre of future bone, resulting in bone marrow formation, which is represented in the schematic of the foetal growth plate (b). Mesenchymal cells differentiate into round chondrocytes, which pile up to form a layer of columnar proliferative chondrocytes. The most distal chondrocytes are hypertrophic and subsequently undergo apoptosis allowing the space to be invaded by blood vessels and osteoblasts to lay down primordial trabecular bone. Adapted from (201) (a). Adapted from (114) (b).

2.5.2.1 Hypoxia and osteoblast

Osteoblasts are the bone-forming cells that originate from the mesenchymal stem cells (MSCs) that have committed towards osteogenesis (77). During bone formation, angiogenesis allows the delivery of nutrients, oxygen, and growth factors. However, vascular disruption may lead to the formation of a hypoxic zone of injury, which may retard osteogenesis (206). Hypoxia up-regulates VEGF mRNA expression in osteoblasts, which peaks at 6 hours and gradually decreases at 24 and 48 hours (206). Hypoxia stimulates angiogenesis through VEGF release in the growth plate of developing bone (115).

Reduced O₂ supply may lead to the production of less ATP, which is essential for cellular function (203). Hypoxia causes reduced osteogenesis due to reduced osteoblast growth and differentiation, which in turn leads to reduced cell proliferation, collagen production, and alkaline phosphatase activity (207). Osteoblasts cultured on Melanex discs and subjected to 0.2-20% O₂ concentration for 18 days showed progressive inhibition of bone formation after alizarin red S staining, which coincided with a reduction in alkaline phosphatase (ALP) activity (203). Cells exposed to hypoxia (2% O₂) resulted in fewer collagen fibrils formation, which suggested that hypoxia (2% O₂) strongly mediated osteoblastic function and affected bone formation and the supporting matrix. Hypoxia caused a reduction in osteoblast precursor cells proliferation leading to a reduction in the number of differentiated cells important for bone formation. Further, osteoblasts subjected to cyclic exposure of hypoxia and normoxia were able to recover from the insults. This also suggested that hypoxia induced a state of quiescence in osteoblasts, which could indicate maintenance of 'stemness' in the cells (203). Long-term exposure to hypoxia and HIF-1 stabilisation on osteoblasts may result in defective collagen formation such as low fibril density and disorganised characteristics. A reduction in pH or acidosis can affect organic matrix mineralisation during bone formation by osteoblasts. A pH reduction from 7.4 to 6.8 on osteoblast culture led to a reduction of mineralisation, which became completely abolished at pH 6.9 (208). This was supported by the reduction in osteoblast size and number and ALP activity, which suggested that acidosis affected osteoblast activity to produce mineralised matrix but not osteoblast proliferation *per se*.

Collagen production depends on O₂ availability for the hydroxylation of proline and lysine to ensure stable triple helix collagen formation (186). Collagen prolyl 4-hydroxylases (P4Hs) are involved in the formation of 4-hydroxyproline and collagen (209, 210), and the 4-hydroxyproline requires O₂ for its enzymatic activity. Osteoblasts exposed to hypoxia may produce less collagen via reduction of hydroxylated proline levels needed for collagen triple helix formation, which suggests that hypoxia exerts inhibitory effects on osteoblast collagen production at the post-translational level (203).

2.5.2.2 Hypoxia and osteoclast

Osteoclasts are multinucleated, bone-resorbing cells originating from hematopoietic precursors of the monocyte-macrophage lineage (77). Studies have shown that hypoxia plays an important role in osteoclast formation (204, 205, 207, 211). Active pathological bone destruction usually occurred at an area with low oxygen tension (204). Osteoclasts seeded on ivory discs and then subjected to reducing O₂ concentration (ranging from 20-0.2% O₂) for 7 days remained relatively small with less than 3 nuclei per cell when exposed to 20% O₂. However, larger and higher number of osteoclasts were observed in hypoxia conditions (5% or 2% O₂) based on tartrate-resistant acid phosphatase (TRAP) staining. Many resorption pits and increasing osteoclast number were observed on the discs exposed to hypoxia compared to normoxia, suggesting that hypoxia promotes osteoclast formation and bone resorption (204). Further, cultures of osteoclasts showed that in short-term cultures (26 hours), a reduced TRAP positive cells was observed, in concordance with the reduction in O₂ concentration. A longer incubation period for up to 13 days showed an increased in resorption pit areas and cell number especially at 2% O₂ concentration. Their results suggested that prolonged exposure to hypoxia especially at 2% O₂ concentration enhanced bone resorption or osteolysis (204). This highlights the problem that may arise in cases with prolonged HIF-1 stabilisation such as reduced bone mass density due to dissolution of ions (especially calcium) from the bone.

Since osteoclasts may also originate from circulating peripheral blood mononuclear cells (PBMCs), studies have assessed the effects of hypoxia on these cells. Osteoclasts harvested from human PBMC cultures seeded on ivory discs were

subjected to 0.2-20% O₂ concentration and stained for TRAP positive cells (205). Larger and higher osteoclast numbers were observed in hypoxic conditions (1-2% O₂) compared to cells in normoxic cultures, and mediated by the HIF proteins (HIF1 α and HIF2 α). These findings were supported by the higher resorption surface area on the ivory discs. The RANKL (1 ng/ml) addition may have resulted in a higher expression of VEGF mRNA in osteoclasts exposed to hypoxia (2% O₂), although the VEGF protein level was almost similar to the normoxic group. The released VEGF stimulated osteoclast formation, activity, and survival of mature osteoclasts in the presence of RANKL. Osteoclast formation from the human PBMCs upon exposure to hypoxia also suggests the osteoclastogenic response to hypoxia is an intrinsic property of the circulating mononuclear cells (207) through the extravasation of mononuclear precursors into a hypoxic area.

The healing and repair process in hard and soft tissue regeneration highlights the importance of vascular endothelial growth factor (VEGF), which increases vascular permeability, is a mitogen for endothelial cells (212), promotes collagen deposition, and is involved in wound healing cascades, angiogenesis, and epithelisation (213-215). VEGF is a homodimeric glycoprotein and exists in five isoforms known as VEGF-A (VEGF₁₆₅), VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF), and VEGF-E and VEGF-Fs from viral proteins and snake venom (216). VEGF is produced by various cells involve in the wound healing cascade, and binds with VEGFR-1 (Flt-1), and VEGFR-2 (KDR) receptors located on endothelial cells, and also VEGFR-3 (Flt-4) (213). VEGF may also play role in regulating mature osteoclast activity during the repair process to prevent excessive bone formation at the site of injury, which is mediated by the VEGFR-1 (Flt-1), and KDR/Flk-1 present on these cells (217). In VEGF-A, its eight exons can be spliced to generate seven different protein isoforms (206, 189, 183, 165, 148, 145 and 121 amino acids), all of which can interact with VEGFR-1 and VEGFR-2 *via* its N-terminal ends (216). Endothelial cells required VEGF-A for precursor cell differentiation (EPC), proliferation, and cell assembly during vasculogenesis, angiogenesis and vessel remodelling (215).

Stimulation of angiogenesis by VEGF during wound healing and repair occurs through various modes, including vasodilation of blood vessels, epithelial membrane degradation, endothelial cell migration, and proliferation in a spatial temporal pattern.

A higher amount of VEGF is observed during the early days of wound repair and decreases within 7-10 days when the wound stabilises (218). In hypoxia-mediated angiogenesis through the HIF-1 pathway, hypoxia stimulates adenosine accumulation and enhances *VEGF* expression (219). VEGF is also released from foetal small intestinal epithelial cells independent of the HIF-1 pathway when exposed to iron chelators, suggesting a role of VEGF for activating ERK/MAPK signalling to maintain epithelial integrity (220). Hypoxia down-regulates VEGFR-2 expression, and increases VEGFR-1, which leads to increased angiogenesis during wound healing (221). Vascular invasion is crucial for osteogenesis to occur. Sequestration of VEGF decreases the number of endothelial cells leading to diminished blood vessel formation, providing evidence that VEGF is crucial for growth plate angiogenesis, and acts as a main regulator of angiogenesis (115).

Acting through the HIF-1 pathway, hypoxia also stimulates erythropoiesis through EPO directly on the endothelium to enhance NO release *in vitro* (222). This is important for vasodilation to occur and supply enough O₂ to the injury site. Hypoxia is also important for progenitor and MSC differentiation towards the osteoblastic lineage *in vitro* (223, 224) and *in vivo* (225), an important repair mechanism in hard tissue. The hypoxia-mediated effect on osteogenesis occurs through the HIF-1 pathway *via* activation of VEGF (202, 226), and fibroblast growth factor (FGF) (227). Hypoxia also promotes proliferation, survival and differentiation of human haematopoietic stem cell progenitors and bone marrow derived MSC (BMSC) (228) which is important to ensure a surplus cell supply for regeneration. A reduced oxygen level (1% O₂) resulted in increased stromal cell-derived factor (SDF-1) expression, suggesting O₂-dependent hypoxia-specific transcription during injury (229). TMJ synovial fibroblasts exposed to hypoxia released significantly higher VEGF compared to normoxic cells and to periodontal ligament cells, which was mediated by HIF-1 α (230).

2.5.3 Hypoxia and dental pulp tissue regeneration

Some compositions of dental materials may cause damage to the underlying dentin pulp complex. Tooth coloured materials (dental composites) contain residual monomers from acid and base reactions that may be harmful to the dental pulp complex (231). It was shown that methacrylate-based dental composites induced the

production of ROS that causes oxidative stress in the dentin pulp complex and may lead to reduced cell survival. The dental pulp responds to injury by laying down reactionary dentin produced by the underlying odontoblast progenitor cells, which differentiate to odontoblasts (232).

Vital pulp therapy is important to ensure the dental pulp remains viable to generate tertiary dentin at the injured site and to prevent bacterial invasion through the dentinal tubules (233). *In vitro* studies have shown the capability of dental stem cells to express VEGF, PDGF, and FGF (234, 235). A few *in vivo* studies showed the potential of using dental stem pulp cells for tissue repair and regeneration (236, 237). Rat brain showed improvement in ischaemic areas following injection of radiolabelled DPSC (237), and a tooth slice model implanted in severe combined immunodeficient (SCID) mouse remained viable after 7 days of implantation (236). Gelatine hydrogel microspheres coated with FGF-2 and embedded in collagen sponges were applied to exposed dental pulp and restored using α -TCP on rat molars. They showed formation of reparative dentin with calcified nodules compared to the control group, suggesting that the controlled release of FGF-2 enhances dentin-pulp complex regeneration compared to uncontrolled FGF-2 release (238). Human dental pulp stem cells were shown to possess better immunoreactivity than bone marrow stem cells (BMSC) for repair and regeneration of oral and dental related tissues partly due to the origin of specific tissues where the adult stem cells originated (3, 234).

Dental pulp is a soft tissue located in the innermost part of a tooth that receives nutrients and oxygen from the neurovascular complex that enters through the apical foramen (239). Hence, pulp tissue is susceptible to injuries arising from decreased blood supply and increased pressure, which may lead to ischemia and cell death. When porcine dental pulp cells (pDPCs) were exposed to anoxia ($< 0.1\% \text{ O}_2$) and hypoxia ($5\% \text{ O}_2$) in combination with low and high glucose conditions, cell death was the highest in medium without glucose in the anoxia group, suggesting that the cell death may occur due to cell necrosis and lack of intracellular ATP (239). Indeed, a lack of intracellular ATP causes cells to switch to anaerobic metabolism and increase ROS generation resulting in cell necrosis (239, 240). Inflamed dental pulp cells have higher expression of EPO and EPO-R compared to healthy pulp. When these cells were exposed to hypoxia, the EPO and EPO-R expression were upregulated

compared to normoxic pulp, suggesting that injury in the pulp may lead to local tissue hypoxia, triggering the HIF-1 pathway (241) to enhance the repair process. Hypoxia enhances human dental pulp cells (hDPC) proliferation compared to normoxia, and suppresses osteo/odontogenic induction of hDPC. Nonetheless, expression of STRO-1 was detected in these cells, suggesting that hypoxia causes the cells to be in immature state (242). Hypoxia also resulted in higher migration in a wound healing assay, and higher cell yield and faster population doubling time of SHED compared to DPSC. Together, this suggested that hypoxia is suitable for dental stem cells, and SHED are superior to DPSC for use in treatment of ischaemic dental tissue (243).

Periodontal repair and regeneration require angiogenesis, and strategies incorporating PHD inhibitors along the HIF-1 pathway may be beneficial. Exposure to PHD inhibitors such as dimethyloxallylglycine (DMOG), desferrioxamine (DFO), L-mimosine (L-MIM) and CoCl_2 resulted in decreased human gingival fibroblast (hGF) and periodontal ligament fibroblast (PDLF) proliferation and protein synthesis, but increased nuclear HIF-1 α and VEGF production. Importantly, treatment with a PHD inhibitor did not trigger an inflammatory response in these cells (244). Hypoxic conditions and re-oxygenation of periodontal ligament fibroblasts (PDLF) in osteogenic medium caused the cells to upregulate bone sialoprotein (BSP) mRNA, suggesting that PDLF maintain their osteogenic differentiation ability in hypoxia (245).

Orthodontic tooth movement may result in bone resorption at areas exposed to orthodontic pressure. Hypoxia promotes the transformation of blood mononuclear cells into osteoclasts when the cells were cocultured with osteoblast cells and may mediate the bone resorption seen during orthodontic tooth movement (211).

There are many blood vessels in the dental pulp, and any procedures conducted on a cavity may cause an injury to the pulp tissue, blood vessels, or pulp fibroblasts. The injured cells release signalling molecules that initiate an inflammatory cascade and the healing process within the dental pulp. In one study, pulp injury was stimulated on the confluent cultures of dental pulp using scalpel blades. A higher expression of VEGF after the induced injury was observed. This finding suggested

that pulp fibroblasts play an important role in angiogenesis by secreting soluble factors (246). It was also shown that VEGF is found in both inflamed normal pulp, which suggests that VEGF is locally produced by the pulp tissue (247). The development of suitable biomaterials for the protection of the dentin pulp complex is crucial.

2.5.4 Modifying cell behaviour to response to hypoxia

Hypoxia has proven beneficial for dental stem cell maintenance (237, 242, 243), and targeting hypoxia may be beneficial for treatment in related oral and dental diseases (248, 249). The HIF-1 pathway can be modulated in both normoxia and hypoxia (240) (Fig. 2.10). Different strategies have been adopted to stabilise HIF-1 α in normoxia. HIF-1 α degradation by proteosomal ubiquitination can be prevented by using prolyl hydroxylase inhibitors (244, 250, 251), and proteasome inhibitors (252), and transfection to generate a stabilised HIF-1 α gene (253). Nonhypoxic stimuli are also known to stabilise HIF-1 α , including ions chelators, nitric oxide (254, 255), and growth factors (194). Hypoxic stimulation *via* the HIF-1 pathway using metal ions such cobalt (112, 256), nickel, iron, and copper (257) are common in cell culture studies. The HIF-1 α /HIF-1 β dimer that binds to the HRE on target genes will also lead to transcriptional suppression in certain cases. The dimer binds directly and transactivates a gene encoding for TF_R (transcription factor repressors) or the HIF-1 dimer binds to TF encoding for histone demethylase (HDM) activity, which suppresses the response to hypoxia (240). The effect of cobalt ions (Co²⁺) in stimulating hypoxia will be further discussed as it is related to the CoBG used in this thesis.

Cobalt in the form of CoCl₂ is commonly used to mimic hypoxia in normoxic cell culture. Earlier studies were conducted to understand how cells respond to hypoxic conditions by synthesising a higher amount of erythropoietin (EPO). This protein reacts with the O₂ molecule through a heme moiety, and haemoglobin is a typical model of a heme protein (258). VEGF is a mitogen specific for endothelial cells, and it was shown that both EPO and VEGF share the same common sensing mechanism. The use of CoCl₂ also induces the release of both EPO and VEGF (259). In their study, the authors used CoCl₂ with three different concentrations, and it was shown that when HepB cells were cultured with 100 μ M CoCl₂ from 0 to 24

hours, the expression of VEGF peaked around 6 hours and tended to decrease in 24 hours. The findings were also supported when C6 cells were cultured with 200 μM and 300 μM CoCl_2 for 24 hours (258, 259), and it was demonstrated that cobalt induced the EPO expression in Hep3B cells *via* the same O_2 -sensing mechanism when the cells were exposed to cobalt and hypoxia. The O_2 sensor is a heme protein that influences EPO production and secretion once binding is achieved (258). The ability of cells to uptake cobalt is cell specific (191, 260), and depends upon the relative amount of heme being synthesised to stimulate the O_2 -sensing mechanism that results from hypoxia.

PHD activity also requires iron (184, 187), the potential site for cobalt mimicking hypoxic action. Cobalt affects the O_2 sensor through a number of proposed mechanisms including (a) substitution with iron in the porphyrin ring creating a molecule with lower O_2 affinity (258), (b) inhibition of proteosomal HIF-1 α degradation (110), (c) enhancing HIF-1 α stability through ROS generation, leading to cell apoptosis (261), and (d) inhibition of extracellular ascorbate transport into the cytoplasm for PHD activity (262). It was shown that cobalt replaces the iron in the iron residue of prolylhydroxylase and prevents HIF-1 α proteosomal degradation (162, 258). The substitution of cobalt⁺ with iron is partially due to the similarities of the ionic radius of cobalt (0.72 Å) and iron (0.77 Å) (263). Cobalt binds directly to the ODD domain of HIF-1 α leading to pVHL-mediated degradation (112). Kanaya *et al.* showed cobalt mediated HIF-1 α ubiquitination independent of prolyl hydroxylase hydroxylation, and found cobalt binding sites in both the PAS-A and PAS-B domains of the HIF-1 α gene (196). The authors also showed that cobalt binds to the N-terminus of HIF-1 α , which can also be ubiquitinated but resists proteosomal degradation, suggesting that cells exposed to cobalt may have ubiquitinated HIF-1 α accumulation. In addition, cobalt may inhibit other proteins that require divalent metal ions to be functional (253).

The hypoxia-mimicking effect of cobalt is different in each cell type including liver cancerous cells (166), mouse macrophage (264), and human renal carcinoma cells (196). Osteogenic differentiated BMSC treated with 100 μM CoCl_2 showed enhanced skull defect repair with blood vessels compared to control BMSC and induced BMSC, and it was found that cobalt as a hypoxia-mimetic promotes angiogenesis

and enhances osteogenesis through the activation of VEGF (265). Cobalt treatment increased Glut1 and Glut3 mRNA expression in the rat brain, suggesting that glucose is actively transported across the cell membrane in response to hypoxia (266). However, cobalt was also reported to be less effective in mimicking hypoxia than iron chelators such as desferrioxamine when assessed in human cell lines (264). Cobalt (150 μ M) and hypoxia (1% O₂) rapidly induce HIF-1 α and HIF-2 α expression through PI3K and ERK1/2. EPO also induced HIF-2 α in a dependent way, but cobalt inhibited EPO expression and HIF-2 α , but not HIF-1 α , suggesting that cobalt⁺ activates the HIF-1 dependent phosphoglycerate kinase (PGK) promoter (166).

Cobalt uptake is shared with calcium within the transport pathway of human red blood cells, and thus modification of the calcium transport system may also modulate the effects of cobalt. However, cobalt irreversibly enters the cytosol (reviewed in (267)), hence cobalt toxicity is related to calcium channel modifications and cobalt properties in its ability to displace divalent cations in metal-activated enzymes (263, 267). The effect of hypoxia must be balanced; the body has its own adaptive mechanisms to ensure enough O₂ is delivered to target tissues, as described in Section 2.5.1 (240).

The use of cobalt as a hypoxia-mimicking agent in the current study shows the potential for use of this technology in dental tissue regeneration. However, the controlled release of cobalt is crucial for cell survival. Prolonged exposure to cobalt may lead to HIF-1 stabilisation and produce unwanted effects such inflammation and chronic hypoxia. *In vitro* studies showed that although cobalt mimicked hypoxia, the effect of hypoxia (using various O₂ concentrations) is different from the effect seen when using cobalt as CoCl₂. Long-term exposure to cobalt may lead to apoptosis, and impairment of angiogenesis *in vitro* (117). Cobalt may also induce HIF-1 α protein level and cause ROS production through the PI3K-dependent mechanism (111). Cobalt may prevent HIF-1 α degradation through sustained PHD activity. PHD activity requires O₂, iron, ascorbate and 2-oxoglutarate (181). Cobalt may be displaced or substituted with iron *in vitro*, which causes iron levels to be unaltered since the presence of ascorbate helps to maintain iron in a ferrous state (187). This may reduce HIF-1 α degradation by VHL. Cobalt may also directly displace iron

because cobalt can bind more tightly than iron to the membrane transporter of the divalent metal ion 1 (DMT-1), which suppresses iron delivery into cells (263). In 1950s, cobalt was used to treat anaemia using the brand name Roncovite. However, high renal toxicity led to withdrawal of the drug from market (263). Another proposed mechanism for HIF stabilisation may occur through the binding of cobalt to HIF-2 α binding sites. Cobalt may occupy the pVHL-binding domain and prevent interaction between pVHL and hydroxylated HIF-2 α (112). Chronic wounds in the diabetic foot are an example of pathological hypoxia due to impaired microvascular remodelling (268). Detection of HIF-1 α in cancerous tissue indicated increased mortality (269, 270), and HIF-1 pathway-related mutations also lead to tumour formation (271). Current strategies use the HIF-1 pathway target genes for management of blood disorders and tumours. For example, VEGF inhibitors and similar family members have reached the clinical setting (216, 272). Nevertheless, potential harmful effects from these treatments still warrant further investigation. Hence, the development of a biomaterial with specific features (273) capable of enhancing tissue repair and regeneration for the patient's benefit is crucial.

2.6 Bioactive glasses (BG)

The choice of ceramics and glasses as biomaterials depends on physical and mechanical properties, their degradation inside the body, and biocompatibility (274). In developing scaffolds for bone and dental pulp TE, the ultimate aim is to produce a scaffold with bone-like properties and at the same time to promote the local tissue to produce their own matrix for healing and repair. Mature bone is composed of organic matrix (25%) including cells (2-5%), 5% water, and 70% inorganic mineral (hydroxylapatite), while new bone matrix consists of 94% collagen, and the non-collagenous component of bone consists mainly of proteoglycans, as mentioned in Section 2.3 (76). Enamel also contains organic and inorganic matrix, which is quite similar to bone (275). Thus, the similarities between natural bone and dentin allow the development of materials that can be used for both tissue defects. Bioactive glass is chosen as a suitable material to be used in bone and dental pulp TE and will be explained further.

Ceramic and glass materials can be prepared in the form of powders, coatings, and bulk devices. Special compositions present within the ceramics and glasses allow

them to be used as replacements in diseased or damaged tissues such as bones, joints and teeth. In the late 1960s, glass developed with a specific composition was found to bind to bone, and this glass has become known as bioactive glass (BG) (276). The first BG, 45S5 Bioglass®, has 45% SiO₂ – 24.5% Na₂O – 24.5% CaO – 6% P₂O₅ (weight %), which differentiates it from other glasses (277). The term bioactive glass was adopted based on the definition of a bioactive material: “a bioactive material is one that elicits a specific biological response at the interface of the material which results in the formation of a bond between the tissue and the material” (278). BG and ceramics possess common characteristics. They form a biological hydroxyl carbonate apatite (HCA) layer when immersed in biological fluid, which facilitates their bonding with bone (279). Many studies were conducted to understand the underlying principles of the bone bonding mechanism and modifications were made to the original BG composition to tailor for specific needs. The BG structure and properties, the degradation mechanism and their bioactivity will be summarised in the following sections.

2.6.1 BG structure and properties

The knowledge regarding BG structures is important for tailoring the BG composition for specific needs. As an introduction to glass structure, glass has been defined in various terms such as liquid, solid, and glassy state (280). Glass is described as supercooled liquids from the point of view of physical chemistry, and as solids from the perspective of elasticity (281). In principal, glass can be defined as an amorphous material that is brittle in nature. The glassy state is obtained when glass as a liquid is cooled below its freezing point to a rigid state without crystallising and is explained using the volume-temperature diagram below (Fig. 2.11) (280).

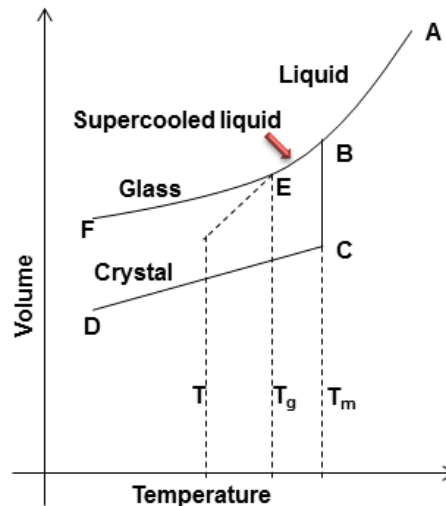


Fig. 2.11: Glass formation based on the volume and temperature changes, which defines glass into liquid, glass or crystal. Adapted from (280).

When liquid is cooled down from point A to B, a reduction in volume and temperature occurs. However, slow cooling from B to C will result in nuclei formation within the structure to become the site for crystallization to occur, and when the temperature falls below the melting temperature (T_m), a further reduction in temperature and volume will result in crystals being formed (280). Rapid cooling of the liquid from A to E will not produce crystals within the structure. In this case, supercooled liquid is formed at the B-E line, and with rapid cooling, the atomic structure is preserved and maintained. When the temperature is below the transition temperature (T_g), glass will be produced with a reduction in the volume. Glass formation is a kinetic phenomenon that involves transitions from liquid to glass structure. This is related to the molecular structure of the glass itself (280). The BG structure is based on a silicon tetrahedral (SiO_4), with silica at the centre bridged by four oxygen atoms (Fig. 2.12). This is a prerequisite for glass formation based on the Goldschmidt's criteria, where glass is formed by simple oxides (A_mO_m) dependent on the relative size of oxygen (R_O) and relative size of A atom (R_A) ratios, where R_A/R_O must be between 0.2 to 0.4 (280).

The concept of simple oxides is challenged by Zachariasen (281), who proposed that glass exists as a three dimensional (3D) network with oxides with three simple rules: the oxygen atom is linked to two or fewer A cations, the number of oxygen

atoms surrounding A must be small, and the oxygen polyhedra share corners with each other. For a 3D network, at least three corners of each polyhedron must be shared (280). Network forming cations include Si^{4+} , Ge^{4+} and As^{5+} , which form networks with bridging oxygens (BO) and non-bridging oxygens (NBO) (281) as shown in Table 2.2. Alkali ions, including Ca^{2+} , Na^{2+} and Ba^{2+} , modify the glass network situated in the free spaces between them, reduce the degree of network connectivity, and replace bridging oxygens (BO) with non-bridging oxygens (NBO) (282). They make the glass structure less rigid, decrease the glass transition temperature (T_g) and make the glass susceptible to degradation (281). Lower T_g produces glass with lower hardness, which may also increase its bioactivity (283).

Table 2.2: The role of oxides according to Zachariasen. Adapted from (284).

Network forming oxides	Network modifying oxides	Intermediates
B_2O_3	Na_2O	PbO
SiO_2	K_2O	Al_2O_3
GeO_2	CaO	TiO_2
P_2O_5	BaO	ZnO
As_2O_3	Li_2O	CdO
As_2O_5		
Sb_2O_3		
V_2O_5		

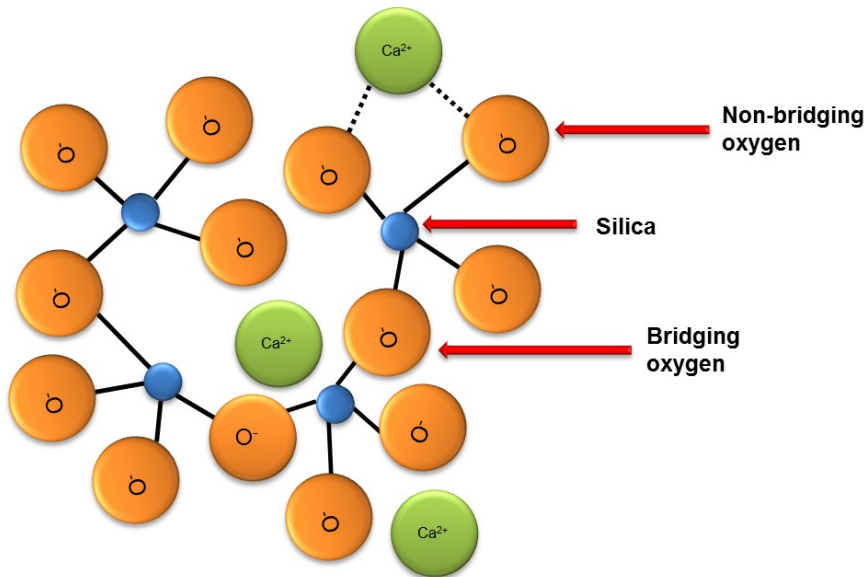


Fig. 2.12: Schematic illustration of a BG SiO₄ tetrahedra network with the presence of the network modifying oxide such as calcium oxide (CaO) that can affect the glass network. Adapted from (284).

Glass structures can be seen as inorganic polymers of oxygen crosslinked by silicon atoms crosslinked in places and charge-balanced by network modifiers (285, 286). Thus, glass behaviour such as surface reactivity and solubility can be predicted by measuring the glass network connectivity (NC). The NC assumes that the glass is homogenous and is an average value (285). It is determined by calculating the amount of BO and NBO per silicon tetrahedron (285-287), and can be expressed by the Q^n notation (288) shown in Equation 2.1. The notation Q^n gives the amount of NBO and BO per silicon tetrahedron (Q), where n is the number of BO (288). Q^4 structure corresponds to one molecule of SiO₂ with four BO and Q^2 corresponds to silicon surrounded by two BO and two NBO (287). The addition of network formers into the glass structure will reduce the NBO and increase the glass NC. For example, silicon as a network-forming oxide will hold the glass network together when bridged by oxygen. When a network-modifying oxide such as calcium oxide (CaO) is added to the network of SiO₂, the addition of oxygen ion will break the SiO₂ (Si-O-Si) bridge, and create a pair of non-bridging oxygen (NBO). The two negative charges on the oxygen will be neutralised by the positively charged calcium cations. Glass with NC less than 2 is bioactive, such as 45S5 with an NC of 1.9, while glass with NC more

than 2 are non-bioactive. For example, glasses containing more than 60% silica content will have a reduction in NBO and an increase in NC towards Q^3 (282, 285).

$$\text{Network connectivity (NC)} = \frac{\text{number of BO} - \text{number of NBO}}{\text{number of network former oxides}} \quad \text{Eq. 2.1}$$

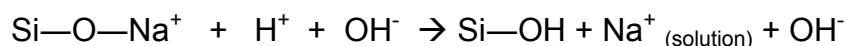
The NC, based on the inorganic polymer model, is suitable for soda-lime-silicate glass. However, the phosphate addition within the glass is not counted and is present as PO_4 tetrahedra (285). Studies have shown that P_2O_5 may enter the silicate network and form a separate phase into orthophosphate species involved in displacing Na and Ca from the glass network (282, 286).

In summary, the BGs used in this study are based on the soda-lime-silicate glass with phosphate addition ($Na_2O-CaO-Si_2O-P_2O_5$). Upon immersion in biological fluid, they will form silica-rich and calcium-phosphate-rich layers indicating the bioactivity of the glass (289). The silicate phase based on the Si-O network can be modified with the addition of alkali and alkali oxides, while the phosphate phase is mobile and can form a separate orthophosphate phase or network former entering the silicate network (283, 287). Hence, these unique properties allow the creation of BGs with specific compositions that can be tailored for specific needs.

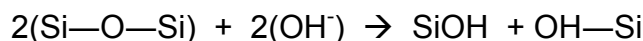
2.6.2 BG degradation mechanism and bioactivity

When BG is implanted into host tissue, a series of reactions occur at the material-bone interface resulting in chemical bonding between the material and host tissue. This phenomenon was later regarded as the BG degradation mechanism, where a hydroxyl carbonate (HCA) layer is formed on the BG surface (stage 1 to 6). Subsequent bonding of the materials to the host tissue involves protein adsorption to the BG surface, the action of cytokines and cells adhesion, and the generation of extracellular matrix by osteoblast cells to form bone. The mineralisation process is complete once bone is formed and mature osteocytes are enclosed within the composite structure (stage 6 to 11), as shown in Fig. 2.13 below (277, 290).

Stage 1. Rapid exchange of Na^+ from the glass with H^+ and H_3O^+ from the solution:



Stage 2. Soluble silica is lost to the solution when Si-O-Si bonds break resulting in formation of SiOH at the glass solution interface:



Stage 3. Condensation and repolymerisation of SiO_2 rich layer on the surface which is depleted in alkali and alkaline-earth cations:



Stage 4. Migration of Ca^{2+} and PO_4^{3-} groups to the surface through the SiO_2 rich layer forming a $\text{CaO—P}_2\text{O}_5$ rich film on top of the SiO_2 rich layer followed by growth of the amorphous $\text{CaO—P}_2\text{O}_5$ rich film by incorporation of calcium and phosphate from the solution.

Stage 5. Crystallisation of the amorphous $\text{CaO—P}_2\text{O}_5$ rich film by incorporation of OH^- and CO_3^{2-} anions from surrounding solution to form a HCA layer.

Stage 6. The HCA layer grows in size following agglomeration and chemical bonding of biological moieties within it, leading to the incorporation of collagen fibrils produced by osteoblasts or fibroblasts.

Stage 7. Action of macrophages.

Stage 8. Stem cell attachment.

Stage 9. Differentiation of stem cell to osteoblasts.

Stage 10. Generation of extracellular matrix by osteoblasts to form bone.

Stage 11. Mineralisation of matrix to enclose mature cells within the living construct.

Fig. 2.13: The schematic process of HCA layer formation as a reaction of BG degradation. Adapted from (290).

The process in stage 1 and 2 occurs rapidly. Rapid exchange of sodium from the glass into the solution with the ions from the solution (H^+ and H_3O^+) leads to the formation of silanol (Si-OH). An increase in hydroxyl concentration causes network dissolution. This also leads to a fast release of soluble silicon from the glass into the

solution in the form of orthosilicic acid (Si(OH)_4) at the glass solution interface, as a result of the breakage of Si-O-Si bonds. At stage 3, the condensation and repolymerisation of SiO_2 rich layer occurred on the surface of the glass. This SiO_2 rich gel-layer is depleted in alkali and alkaline-earth cations and allows the apatite formation to take place as stage 4: the formation of an amorphous calcium phosphate layer. The CaP layer on the SiO_2 rich gel-layer grew in thickness by incorporating calcium and phosphate from the solution. Later, this amorphous CaP layer crystallised by incorporating anions (OH^- and CO_3^{2-}) from the solution (277, 290). Stages 1 to 5 are the surface reaction stages that result in the formation of a HCA layer that creates a suitable environment for cellular reactions (290). Osteogenic differentiation will take place when osteoblasts from the bone colonize the HCA layer (stage 8), and undergo differentiation and proliferation (stage 9) leading to the formation of soft bone matrix (stage 10) that subsequently crystallises to generate bone growth (stage 11) (290). Stages 6 to 11 occur *in vivo*, requiring cell interaction with the host tissue. However, these events still need further investigation since *in vitro* and *in vivo* experiments may produce differences (291) and alternative theories for BG degradation were also proposed by different authors (285, 292). The HCA layer formation provides useful information about the interaction of BG with bone, however, BG-bone bonding upon implantation involves time dependent modifications of the implant surface. Thus, the controlled release of ionic dissolution products at specific compositions and the atomic glass structure are important to enhance bioactivity (277, 279). The BG bioactivity can be explained by the compositional diagram for bone bonding as shown in Fig. 2.14. The glass represented here has four components: SiO_2 , Na_2O , CaO and 6% P_2O_5 (in weight %). Glasses in region A are bioactive and bond to bone, and glasses in region E at the centre of the triangle are highly bioactive. In region B, the glasses are inert, having more than 60% SiO_2 content, while glasses in region S also bond to soft tissue (277). Other than tailoring the BG compositions with specific doses, the BG particle size, surface area and its topography also affect bioactivity (277, 291, 292). Hence, the next section will summarise the effects of modifying BG composition, and combinations of BGs with other materials on BG bioactivity especially in strontium-substituted and cobalt-containing BG.

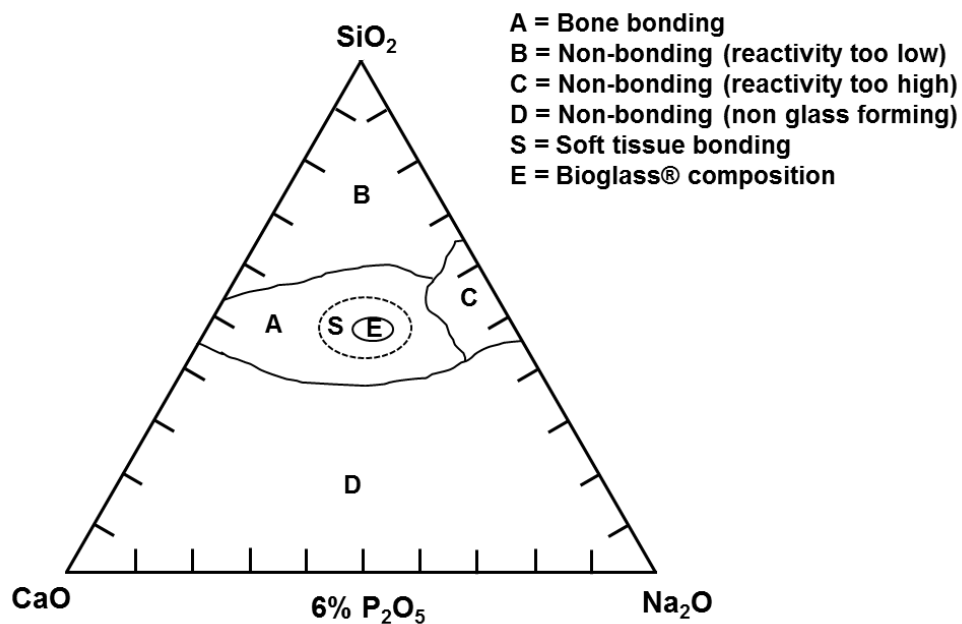


Fig. 2.14: The tissue bone-bonding compositional diagram showing glass bioactivity in relation to its composition (in weight percent). Adapted from (279).

2.6.3 Modifying BG composition

Glass dissolution rates and HCA formation on BG are dependent on the glass atomic structure (291). Modification of the glass composition with alkali and alkaline oxides affects network connectivity as previously discussed. Silicon acts as a glass network-forming atom, while sodium and calcium are network modifiers capable of creating NBO within the SiO_4 tetrahedra (291). The addition of network modifiers such as calcium results in glass network disruption, making the glass less dense due to the breakage of the Si-O-Si bridge and resulting in additional pairs of NBO in the structure (282). Examples of alkalis used to modify BG compositions include magnesium (293), silver (294), strontium (22), calcium, sodium, potassium (282), and cobalt (121), which all result in different BG properties.

Dissolution products from BG upregulate the expression of genes that control osteogenesis (295), including insulin-like growth factor II (IGF-2) (129) and collagen type I (296). When foetal osteoblast cells were cultured in BG-conditioned medium

without the addition of osteogenic supplements, the cells were able to form mineralised bone nodules, and showed an increased in ALP, osteonectin and bone sialoprotein gene expression (297). When polyglycolic acid (PGA) fibres coated with 45S5 particles were implanted subcutaneously *in vivo*, the VEGF level was higher compared to controls, suggesting that BG-conditioned medium plays a role in angiogenesis (298). When magnesium was substituted for calcium (in mole %) within the BG structure with $NC=2.13$, the resulting glass showed a decrease in bulk density. MgO addition displaces calcium and weakens the glass network, and the 100% magnesium substitution showed an absence of crystallinity suggesting that magnesium inhibits the glass crystallinity that is important in maintaining bioactivity (293). Zinc-containing BG was also developed, however, the apatite formation in Zn-doped BG is lower compared to undoped BG (299).

In the current investigations, strontium or cobalt substituted for calcium BGs were prepared using a melt-derived method to study the response of dental pulp stem cells. The compositions for strontium-containing BG (SrBG) and cobalt-containing BG (CoBG) are based on ICIE1, which contains 49.46 SiO_2 –26.38 Na_2O –23.08 CaO –1.07 P_2O_5 (in mole %), (282, 286). It was shown that substitution of strontium with calcium results in glass with increased crystal lattice size due to larger strontium cation compared to calcium, reducing the glass T_g and lowering the network connectivity to enhance the glass bioactivity (286, 287).

As mentioned earlier, strontium has shown potential benefits in the treatment of osteoporosis (95). The controlled strontium release from the SrBG is important to ensure the efficiency of strontium when used as therapeutics. Osteoclasts cultured in strontium preparations containing strontium chloride and strontium ranelate (concentration range from 0–30 mM) for 48 hours showed apoptosis in a dose-dependent manner (94). Upon calcium addition (concentration 1.8 to 20 mM) to the culture medium containing strontium, osteoclast apoptosis was still observed. The presence of strontium may potentiate the effect of calcium on osteoclast apoptosis especially at lower calcium concentration (1.8 mM) (94). This highlights the importance of controlling strontium ion release since targeting strontium to bone cells such as osteoblasts or osteoclasts may confer different responses (300).

The addition of strontium in the SrBG showed that the NC is not affected and has little influence on the glass structure, while the ^{31}P spectra indicates that phosphorus that is not part of the silicate network becomes separated and phased out and P_2O_5 is present as orthophosphate (287). Hill *et al.* (301) conducted a study using low, medium and high content strontium oxide (SrO), and substituted calcium of the same amount in the glass preparation. They also substituted strontium fluoride (SrF_2) with calcium fluoride (CaF_2) using the same method. Their results showed that there were minimal changes in the glass structure for both experiments, and concluded that this was because strontium had a similar charge size ratio to calcium. It was shown that strontium-substituted apatite can be prepared by a standard wet chemical route in which the strontium is easily interchanged with calcium (302). Strontium-containing borosilicate (Sr-BS) and calcium silicate (Sr-CS) were compared with Sr-substituted hydroxyl-apatite ($\text{Sr}_x\text{-HA}$) in terms of structure, composition, degradation behaviour, and biological responses. The results showed that there were no phase or structural changes in Sr-HA, Sr-BS and Sr-CS. The Sr10-HA (where 10% of the strontium was substituted for calcium) sample showed the highest MG-63 osteoblast proliferation throughout the seven day incubation period when compared with the HA samples (303). In another study, melt-derived BGs were prepared from a base 45S5 BG, where 6% SrO substitution for CaO was prepared (45S5.6SrBG) and transplanted in the intramedullary canal of rat tibiae. The results showed a good osteointegration between the bone and the materials with that there were no significant differences among the osteoconductive potentials of 45S5 and 45S5.6SrBG particles (304).

In CoBG, the cobalt acts as a network intermediate and enters the silicate network. Due to its charge to size ratio, cobalt as a divalent ion also acts as a higher coordination number (CN), and is present in silicate network in both octahedral and tetrahedral (305). Cobalt as a network modifier is able to replace BO with NBOs. It can also be substituted with calcium, suggesting that BG compositions can be tailored for specific needs. The CoBG used in this study is from the non-charged balance (NCB) group, where one CoO was substituted for CaO based on the assumption that cobalt acts as a network intermediate entering the silicate network, and also acts as a network modifying oxide. It was shown that the glass T_g decreased with the addition of Co in a concentration dependent manner for 0, 1, and 2% Co for calcium, but not for 4% Co (mole %), suggesting that lower T_g weakens

the glass network and renders the BG susceptible to degradation in physiological fluids to enhance the bioactivity (121). Further, the authors showed that incubation of CoBG powder in simulated body fluid (SBF) caused the phosphate ions to remain in solution for up to 15 days, which could be due to slower precipitation of orthophosphate ions forming a hydroxycarbonate apatite (HCA) layer or amorphous calcium phosphate on the CoBG. In longer incubation periods, the cobalt released from the CoBG may form stable complexes between the cobalt and phosphate compared to those from between calcium and phosphate. Cobalt has more valence electrons and is more electronegative than calcium, which may explain the higher stability of cobalt phosphate complexes (121). Mesoporous BG (MBG) containing cobalt (2CoMBG, and 5CoMBG) showed that cobalt incorporated into the scaffold did not affect bioactivity of the MBG, with continued slow release of cobalt from the MBG and promoted BMSC proliferation at day 7 (24).

BG composition modifications are also possible by combining them with other materials to develop BG composites. BG is combined with polymers such as polysulphone, and with reinforcing materials such as titanium and stainless steel fibres. BG composites were developed to overcome the brittle nature of BG and to improve fracture toughness, bending strength and stress shielding (277). The combination of BG with other materials (BG composites) enables the generation of nanocomposite nanoparticles, which are a powerful approach for the creation of 3rd generation bioactive materials (274). Bioglass® particles were combined with multi-walled carbon nanotubes (MWCNTs) and poly(3-hydroxybutyrate) (P(3HB)), and the results showed that the combined materials had increased electrical resistance when placed in stimulated body fluid, pointing to the fact that it is possible to create biomaterials with a sensing function (306). BG can be combined with other materials to enhance the properties of the composite material including biodegradable polymers such as poly(glycolic acid), poly(lactic acid), poly- ϵ -caprolactone (PCL) and their copolymers (307). In one study, different BG powders with or without PCL were developed: sol-gel derived BG powder (BGP), electrospun BG nanofibers (BGNF) and PCL/BG composite (307). Osteoblast responses were assessed after culturing the cells using these materials. Their results showed that there was increased ALP activity in the PCL/BG and PCL/BGNF group compared to the control PCL only group, with PCL/BGNF showing the highest activity, suggesting the benefits of BG

addition to PCL and the importance of the type of BG used either in powders or fibres. The use of BGNF supported the idea that there was higher surface area for cell interaction and the fibres can be uniformly distributed within the PCL matrix compared to powders. Additional information gathered was the use of 20 weight % combination of PCL/BGP and PCL/BGNF, which resulted in higher ALP activity compared to 10 or 30 weight % (307).

2.6.4 BG scaffolds

BGs are available in multiple types, including conventional silicates, phosphate-based glasses and borate-based glasses, and can be produced *via* two processing method: either melt-derived or sol-gel derived, and more recently by using additive manufacturing techniques (reviewed in (291)). In this thesis, the BGs are synthesised using the melt-derived method for the disc preparation and glass powder (22). The three dimensional (3D) scaffolds were subsequently fabricated using a bioextruder machine (308). The scaffold fabrication method is important to ensure the scaffold possesses optimal properties to enhance cell and tissue interaction (309).

2.6.4.1 The melt-derived BG scaffolds

The base components in most bioactive glasses and glass-ceramics are composed of SiO_2 , Na_2O , CaO , and P_2O_5 . The 45S5 is the first and most studied bioglass. It contains 45% SiO_2 , 24.5% Na_2O , 24.5% CaO and 6% P_2O_5 (weight %) (277) processed using a melt-derived method. Glass powders were placed in a platinum crucible and melted at high temperatures, normally between the glass transition and onset of crystallization temperature for amorphous glass, and are then quenched in water to obtain glass frit or graphite mould resulting in dense glass rods (291). This need further processing such as milling and sieving to obtain desired particle sizes. The milling process is usually done electronically using ball and mill machine. The sieving process is done using machines with different sieves sizes. In order to obtain melt-derived glass that is bioactive, the NC must be around 2 (NC~2), and to achieve this, glass substitution using calcium and sodium, for example, is done in mole % (291). In order to obtain glasses with large interconnecting pores and higher surface area, a few methods have been used which include sacrificial polymeric sponges to serve as template (24), surface functionalization to enhance surface roughness

(310), freeze casting using ice crystals (311), and foam or bubble creation within the glass structure (312, 313).

2.6.4.2 The BG scaffolds from sol-gel processing method

The sol-gel processing method is a chemically-based glass synthesis using solution and glass powders to assemble silica nanoparticles with inherent nanoporosity at room temperature, first creating gels that later need to undergo ageing, drying and stabilisation, which depends mainly on the surfactant concentration (313, 314). Commonly available compositions include ternary systems such as 58S (60 mole% SiO_2 , 36 mole % CaO , 4 mole % P_2O_5) and 77S (80 mole % SiO_2 , 16 mole % CaO , 4 mole % P_2O_5), and binary systems such as 70S30C (70 mole % SiO_2 , 30 mole % CaO) (reviewed in (291)). Glass produced using sol-gel processing methods possesses advantages over melt-derived such as nanoporosity, which improves cellular response and leads to a faster dissolution rate. Limitations for the sol-gel method include the difficulty in obtaining pores with similar size and shape or similar interconnecting strut length.

2.6.4.3 The BG scaffolds from additive manufacturing method

BG scaffolds can be manufactured using additive manufacturing methods, also known as rapid prototyping or solid freeform fabrication, where the glass is deposited in layers with a controlled design from a computer-aided design (CAD) file. These methods include fused deposition modelling, robocasting, and freeze extrusion (reviewed in (291)). In robocasting, flexible glass ink formulation was developed and printed on alumina substrate inside a non-wetting oil reservoir to obtain a 3D scaffold (315). In freeze extrusion fabrication, a suspension of glass particles mixed with camphene and isostearic acid is exposed to unidirectional freezing (316). Other methods to produce 3D scaffolds are membrane lamination, fibre bonding, particulate leaching, solvent casting, gas foaming, melt modelling, temperature-induced phase separation, and 3D printing (317).

In this study, 3D CoBG/PCL scaffolds were fabricated using a fused deposition modelling method (FDM). It is one of the rapid prototyping technologies that enables the creation of 3D scaffolds that are highly reproducible, accurate, and stable in their physical and mechanical properties with control over pore size and material

composition (317). Using the FDM method, a 3D scaffold is built by deposition of raw material arranged in layers according to designated sizes in terms of height, width and length (317), where the design can be predetermined using a computer aided design model (CAD) before the data are downloaded into the FDM machine (318). FDM also results in 3D scaffolds free of breakage of the struts because of the proficient mechanical force conduction during scaffold loading (319). It was shown that the mechanical properties of poly- ϵ -caprolactone (PCL) 3D scaffolds were not altered within 5 to 6 months, however they lost their physical properties and underwent degradation process after 2 years (317, 320). Recently, strontium-substituted BG composite using PCL (PCL/SrBG) was prepared using the FDM method, and had improved mechanical strength compared to a PCL only scaffold suggesting that PCL/SrBG scaffolds are suitable for bone TE (105). The use of synthetic polymers allows precise scaffold control based on its properties, and BG and polymer composite usage in TE is very much sought to enhance regeneration and repair (26), and the use of FDM is encouraged to produce scaffolds with high reproducibility (309, 317).

Tissue engineering depends on scaffolds with 3D architecture for support and early cell attachment (317), especially when used as temporary fillers in bone and cartilage defects where they provide extracellular matrix and maintain function until the regeneration process completes (319). The scaffolds can be implanted directly into the host site or embedded with suitable cells before being implanted (319). A scaffold for use in tissue engineering must meet certain criteria. They must be able to provide a 3D template for *in vitro* and *in vivo* bone growth with porous structures for cells to penetrate, exhibit biocompatibility, allow cell attachment, promote bone formation, resorb while new bone is forming without producing toxic by-products, unite with a stable interface to the host bone without creating scar tissue, possess similar mechanical properties as the host bone, accommodate any defects with various sizes or shapes, be produced in an environment following good manufacturing practice, and be commercialized for clinical use (27).

2.6.5 The role of BG in dental pulp tissue engineering

In the field of regenerative medicine, the use of bioactive glass has long being recognised since the first time Bioglass® 45S5 was used in humans as an inner ear

replacement material (321). Many other studies were conducted thereafter to test various 45S5 composition modifications that cater to the specific needs (reviewed in (291)). The current concept of dental treatment adopts aesthetic functions as the main goal, leaving the understanding of interaction between dental materials and the dentine-pulp complex as a major issue (reviewed in (322)). Dental materials for restorative treatments used to fill the gap inside the tooth include amalgam, glass ionomers, composites and compomers, or protective liners such as calcium hydroxide and mineral trioxide aggregate (MTA) to protect the pulp tissue from harmful irritants.

In dental pulp TE, various carrier materials as protective agents for the dentin-pulp complex were developed and tested for replacement therapy, especially in the case of pulpal exposure or near exposure. These include hydroxyapatite/tricalcium phosphate (HA/TCP) (3, 4), and combinations of glass ionomer cements and BG (323). Nevertheless, all are temporary measures. These materials may dislodge from the tooth cavity and may further damage the pulp tissue. The ease of use of these materials also depends on the presence of undifferentiated dental stem cells within the pulp itself (43) to give rise to tertiary dentin formation which seals the dentine tubule and protects the pulp from further damage by irritants. The use of bioactive molecules or chemical additives that trigger an internal response from the dentin-pulp complex for pulpal healing and repair is crucial, thus, the emergence of bioactive materials such as bioactive glass as an additive to the currently available dental materials is very much needed (reviewed in (322)).

In dentistry, a few types of BG have been formulated and used for craniomaxillary applications, sinus lift and alveolar ridge augmentation (10, 18), periodontal problems (16), treatment of dentin sensitivity (17), paste, and abrasion powders for tooth surface treatment, such as NovaMin®, Perioglass®, NovaBone®, Biogran®, and BonAlive® (reviewed in (291)) as shown in Table 2.3. Bioactive glass particles (BioGran®) were placed on a fresh socket of extracted tooth and after 6 months, the bony socket was biopsied during the implant placement procedure (10). The placement of bioactive glass into the empty socket has helped to maintain the bone height with the new formation of bone, which is important for long-term retention of the implant within the socket. Elderly patients with edentulous ridge will normally

encounter problems retaining dentures inside their mouth, especially during eating or talking. A study was conducted where these patients were subjected to maxillary sinus augmentation and a sinus lift to increase the bony height and make the upper bony ridges retentive for dentures (18). The authors used nine types of biomaterials, including demineralised freeze-dried bone allograft (DFDBA), Biocoral® (calcium carbonate based), Bioglass® (bioactive glass), PepGen P-15™ (peptide plus bovine bone mineral component), calcium sulphate powders, Bio-Oss® (deproteinised bovine bone), and Fingranule® (hydroxyapatite). Demineralised freeze-dried bone material resulted in the least amount of new bone formation with the some residual grafted leftover. On the other hand, the use of bioactive glass showed approximately the same amount of residual graft leftover as autologous bone grafting. There was no gaps or fibrous tissue formed around the glass-bone area, and this highlighted the benefit of long-term applications since the results were as close as possible to the natural process (18).

Perioglass® has been used to treat periodontitis (inflammation of soft tissue and hard tissue surrounding the tooth that leads to bone loss) with furcation involvement in molar teeth. Clinical results showed that there were statistically significant improvements in probing depth after 3 and 6 months. The authors stated that the addition of biomaterials to promote bone repair during surgical treatment is advantageous compared to surgical treatment alone (16). 45S5 BG powder (NovaMin®) was used in combination with CO₂ laser treatment *in vitro* on the exposed dentine surface to reduce dentin sensitivity. Each group was subjected to different treatments with or without 45S5 BG powder and laser exposure. The results showed that combination of both 45S5 BG powder and CO₂ laser on tooth surface resulted in the formation of a more compact layer compared to other groups (324). Another study used sol-gel based nano-structured bioactive glass (BAG) as an agent to reduce dentinal sensitivity in an *in vitro* study by measuring the fluid conductance inside the pulp chamber. Their results showed that the newly developed BAG helped to reduce fluid conductance and proposed the use of the BAG for treatment of dentinal hypersensitivity (17).

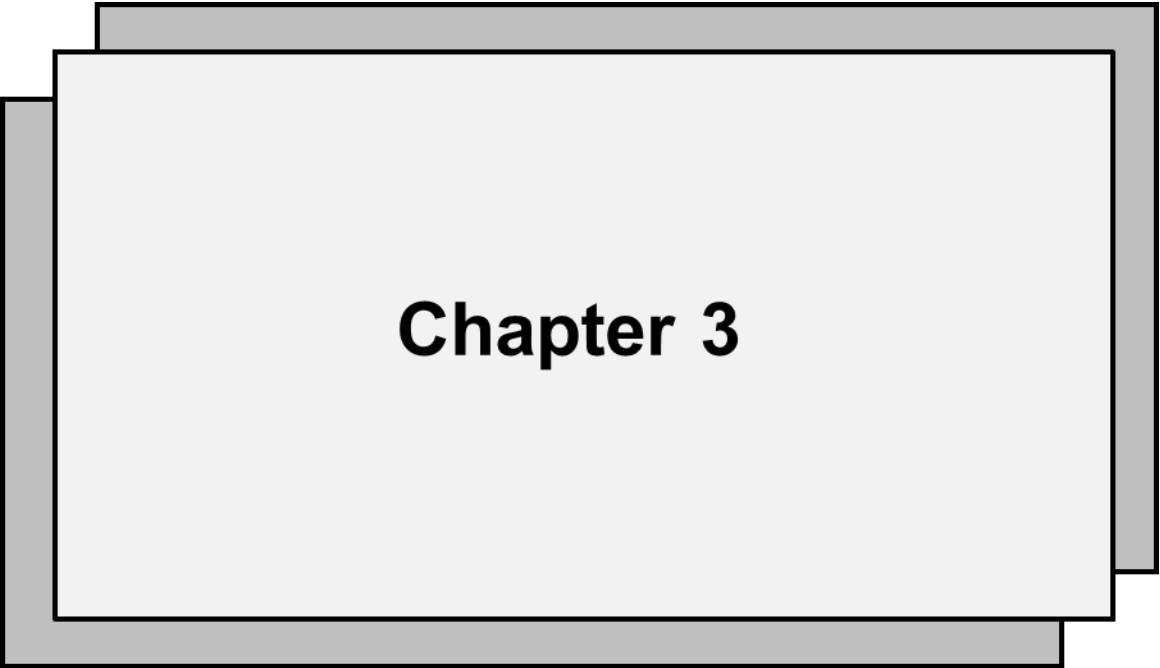
The combination of dental materials and bioactive glass to develop bioactive restorative materials is a novel way to save a tooth. Glass ionomer cements (GIC)

from conventional cure and light activated containing bioactive glass (S53P4) were successfully developed. Even though the initial results showed that S53P4 addition led to a decrease in the compressive strength and modulus elasticity (325), the authors proposed the use of this material for internal restorative usage such as root surface treatment, canal obliteration and cavity liners. Further to that, the authors conducted an *in vivo* study on dog teeth using the same material, S53P4, combining it with commercially available GIC, and exposing them to the oral environment. Their results showed that the light activated GIC with S53P4 was able to stimulate the formation of dentin mineralised matrix (323).

In the dentin-pulp complex, it is common to have a low blood supply of oxygen due the nature of the morphology of the tooth itself. Nutrients are supplied to the pulp chamber by blood vessels that pass through the apical foramen. In any event, there is a reduction of blood supply and the tooth may subsequently die, especially if the event is continuous. It was shown that a low concentration of 45S5 BG promotes VEGF release from fibroblasts *in vitro*, suggesting the angiogenic potential of BG (298). Collagen coated 45S5 particles promote bone formation in mouse calvarial defects, suggesting the angiogenic potential of low concentrations of BG (326). Hence, the use of SrBG and CoBG in dental pulp TE for regeneration and repair is very much sought.

Table 2.3: The product used in dentistry for dental tissue regeneration. Based on (18, 327).

Product name	Composition	Use	Form
Bioglass, US Biomaterials, Alachua, Florida	SiO ₂ (45%), CaO (24.5%), NaO ₂ (24.5%), and P ₂ O ₅ (6%) in weight %	Socket preservation, sinus lift	Granules, paste
Biogran, 3i Implant Innovations, Inc, Palm Beach Gardens, Florida	SiO ₂ (45%), CaO (24.5%), NaO ₂ (24.5%), and P ₂ O ₅ (6%) in weight % (size 300-355 µm)	Socket preservation, sinus lift	Granules
BonAlive S53P4, Bonalive Biomaterials Ltd, Finland	SiO ₂ (65 %), CaO (31%), and P ₂ O ₅ (4%) in mole %	Root surface treatment	Granules
Bio-Oss, Geistlich Pharma AG, Wohlhusen, Switzerland	Deproteinised sterilised bovine bone	Socket preservation, sinus lift	Granules (cortical-shape)
Biocoral, Inotek, St. Gonnelly, France	Calcium carbonate in the form of aragonite (97% to 98%), strontium, fluoride, magnesium, sodium, and potassium	Socket preservation	Granules
DFDBA, LifeNet, Virginia Beach, VA	Demineralised freeze-dried bone allograft	Socket preservation, sinus lift	Granules
Ethisorb, Ethicon, Norderstedt, Germany	Synthetic absorbable implant consisting of 97% poly lactic-co-glycolic acid and 3% polydioxanon	Socket preservation, sinus lift	Sponge, membrane
Fingranule, Fin-Ceramica, Faenza RA, Italy	Hydroxyapatite (HA)	Socket preservation, sinus lift	Granules (potato-shaped)
Fisiograft, Ghimas, Bologna, Italy	Synthetic resorbable sponge composed of a 50–50 lactide-glycolide polymer	Socket preservation, sinus lift	Sponge
Gelfoam, Upjohn, Kalamazoo, Massachusetts	Purified gelatin solution that is beaten, dried, sectioned, sealed, and dry-heat sterilised to form sponge	Socket preservation	Sponge
NovaMin, NovaMin Technology, Fluorida	SiO ₂ (45%), CaO (24.5%), NaO ₂ (24.5%), and P ₂ O ₅ (6%) in weight %	Dentin hypersensitivity	Granules
NovaBone Dental Putty, NovaBone Products, LLC	Calcium-phosphosilicate (bioactive glass with additives like polyethylene glycol and glycerine)	Dental bone defects	Putty
PerioGlas, US Biomaterials Alachua, Florida	SiO ₂ (45%), CaO (24.5%), NaO ₂ (24.5%), and P ₂ O ₅ (6%) in weight % (size 90-710 µm)	Alveolar bone graft in root bone resorption	Granules
Pura Matrix, 3DM	Synthetic 16-amino-acid peptide, forming nanofibres	Dental bone defects	Gel
PepGen P-15, Dentsply, Friadent, CeraMed, Lakewood, CO	Synthetic 15-amino-acid peptide plus bovine bone mineral component	Alveolar bone graft, sinus lift	Granules
Pro Osteon 500R, Biomet, Inc	Hydroxyapatite and calcium carbonate	Alveolar bone graft	Granules
ReproBone, Ceramisis, Sheffield, England	Contains 60% Hydroxyapatite and 40% β-Tricalcium phosphate (TCP)	Alveolar cleft osteoplasty	Granules
Surgiplaster sinus, ClassImplant, Rome, Italy	Calcium sulphate	Sinus lift, alveolar bone graft	Membrane



Chapter 3

Chapter 3

Preliminary assessment of strontium-containing bioactive glass dissolution products on bone modifying cells

The tissue engineering (TE) approach has been applied for the reconstruction of lost oral and maxillofacial structures. TE involves the interaction between scaffolds, cells and growth factors (26). The scaffolds alone can be used to act as templates for new bone formation, or cells can be incorporated *in vitro* into the scaffolds prior to implantation *in vivo*. In the field of oral and maxillofacial surgery, one of the major hurdles faced by clinicians is restoring bone and tooth structure loss due to disease (328), trauma (52). These will not create problems for the patient in cases with minimal tissue loss, but will require bone substitutes for large defects (329). Diseases such as radiation-induced or drug-induced bone necrosis (328, 330) are some of the conditions that could benefit from an improved bone substitute. Current treatment strategies include the use of drugs, hyperbaric oxygen (331), and radical neck dissection for mandibular osteoradionecrosis (ORN) (332). Autologous bone grafts (333), allografts (334), xenografts (335) and external devices, such as plates and screws (336), have been used to restore bone defects in oral and maxillofacial surgeries. However, the use of autologous bone grafts poses a threat to the patients because of pain and complications that occur during the bone harvesting at a secondary surgical site, and the pain arising from damage to the nearby tissue (reviewed in (337)). The use of allografts from human donors also poses challenges due to the immunological response that may arise following implantation (338). Bioactive glasses (BG) have the potential to overcome the problems associated with autografts and allografts. In the current chapter, strontium-containing BG (SrBG) is used to further understand the dual role of strontium in promoting new bone and preventing osteoclast-mediated resorption. The current project used the SrBG powder with particle size range from 0.1-100 μm . The wide range in particles size distributions may have an effect on the ion dissolutions profile from the glass.

3.1 Introduction

Bioactive glass (BG) has received wide acceptance in the field of tissue engineering and regenerative medicine, and has been used in many cases to treat oral and maxillofacial diseases (10, 321). Implantation of BG into living bone resulted in strong bonds between the BG and host tissue, which occurred *via* the formation of a hydroxyapatite layer (149, 339). In recent years, many studies have been conducted to understand the mechanism of the dissolution properties of BG and to demonstrate its potential as a bone replacement material as either particles or in a scaffold (21, 22, 297). Different BG compositions have been developed and are used widely for many applications, including dentistry, craniofacial reconstruction, and maxillofacial surgery. The different compositions are all based on the first BG (45S5), which was developed by Hench and colleagues (reviewed in (291)). Various ions such as magnesium (Mg) (293), silver (Ag) (294), and zinc (Zn) (299) have been substituted for calcium in order to enhance the BG properties and cater to the needs of specific applications.

Relatively small changes to BG compositions can have profound effects on biological responses. In one study, the 45S5 composition was modified, where the 45 weight percent (wt %) SiO_2 was changed to 46 wt % and the authors named the BG as 46S6 (299). The addition of a small amount of zinc (Zn) (0.1 wt %) to the BG 46S6 powder (46S6Zn) led to an increase in osteoblast viability when the particle size was between 500-600 μm , but not when the particle size was 40-60 μm , suggesting that cytotoxicity is related to the composition and reactivity of the compound (299). In our group, strontium (Sr) substituted for calcium in bioactive glass has been extensively studied (22, 302) and is the subject of ongoing research. Strontium is used because studies have shown that strontium taken orally as strontium ranelate helps to reduce the fracture risks in vertebral bones in postmenopausal women (95). The addition of strontium to other biomaterials, namely apatites (HA), calcium silicate (CS) and borosilicate (BS), with different strontium ratios (5, 6, 10, 12, 20, 40 and 100% substitution) showed that 10% strontium substitution in apatites and calcium silicate resulted in higher osteoblast viability and ALP activity, implying that an optimum dose may exist for strontium substitution in biomaterials (303). Furthermore, an *in vivo* study showed that strontium-containing hydroxyapatite (Sr-HA) injected in a rabbit iliac crest resulted in abundant osteoid tissue formation compared to a

poly(methyl methacrylate) (PMMA) bone cement, establishing a role for Sr-HA for treating osteoporotic fractures (104). It was further shown that BG containing strontium is able to promote osteoblast phenotype (340), affect osteoclast activity (22), and does not produce any cytotoxic effects on osteoblasts *in vitro* (21).

Considering the potential of SrBG as a bone replacement material, the effect of strontium-substituted BG-conditioned medium on the bone modifying cells, Saos-2 and RAW.264.7, will be investigated. These two cell lines can represent osteoblasts and osteoclasts, respectively. Osteoblasts play a role as the cells that lay down bone, while osteoclasts remodel bone (291). In biomaterial studies for tissue engineering, Saos-2 cells are commonly used as a model system for osteoblasts (22, 341) and also as a model to study osteoblasts transition to osteocytes (342). Saos-2 cells demonstrate mineralising capacity (343) and respond to parathyroid hormone (PTH) like primary osteoblasts (342). Osteoblast differentiation and maturation resulting in bone formation involves the increase of alkaline phosphatase (ALP) activity, which will be assessed in this chapter upon Saos-2 exposure to strontium-containing BG-conditioned medium. In bone remodelling, synergistic action occurs between osteoblasts and osteoclasts to promote the bone formation and resorption to occur simultaneously (reviewed in (344)).

The RAW264.7 cell is a monocyte cell, which, upon induction by RANKL, will differentiate into osteoclasts. RAW264.7 has been used as a model of osteoclast differentiation in *in vitro* cultures in the presence of RANKL with or without macrophage colony stimulating factor (MCSF) (345, 346), and has been widely accepted as a cellular model of osteoclast formation and function (347). The mouse monocyte/macrophage cell line RAW264.7 is a transfectable RANK expressing cell line able to retain the osteoclast-like cells in the presence of RANKL and secrete TRAP (348) and has been well characterised in terms of their functions (349). There are issues related to the RAW264.7 polymorphism and coexistence of macrophages with different phenotypic characteristics, particularly in relation to the capacity of RAW264.7 cells transforming into osteoclasts and causing bone resorption (350). However, upon treatment with RANKL, osteoclast precursors cloned from the RAW264.7 cell line caused mineralised matrix resorption, were capable of expressing TRAP, and provided an excellent *in vitro* model for studying osteoclast

differentiation and activation (345). The receptor activator of nuclear factor- κ B ligand (RANKL) belongs to the tumour necrosis factor (TNF) family, which is involved in osteoclast formation, activation and differentiation. Osteoblasts express and secrete RANKL, which is required for tartrate resistance acid phosphatase (TRAP) expression (reviewed in (344)). The receptor activator of nuclear factor- κ B (RANK) is the receptor for RANK ligand (RANKL). Activation by RANKL of osteoclast precursor cells will result in osteoclast formation. Osteoclasts belong to the monocyte/macrophage lineage, which is formed when the mononuclear precursors fuse together (351, 352). The activation, differentiation and formation towards mature osteoclasts, and the mature osteoclasts themselves can be blocked by bisphosphonates (BP), which consists of two major groups: the nitrogen-containing and non-nitrogen-containing BP. Examples of non-nitrogen-containing BP include Etidronate and Clodronate, while Pamidronate, Alendronate, Risendronate and Zolendronate belong to the nitrogen-containing group (reviewed in (352)).

Bone resorption occurs when osteoclasts release acidified matrix through their ruffled cellular membrane that creates a tightly sealed vacuole between the cells and the bone surface. In doing so, the osteoclasts activate TRAP and other enzymes resulting in hydroxyapatite (HA) dissolution (344). Bisphosphonates may also cause apoptosis *in vitro* (353) while *in vivo*, the inhibition of bone resorption by bisphosphonates may not require osteoclast apoptosis. However, long-term bisphosphonate treatment may result in more osteoclasts in the cancellous bone, which is dose-dependent and depends on the duration of treatment (354). Bisphosphonates have been used to treat bone related diseases mainly in the case of abnormal bone formation, such as Paget's disease and osteogenesis imperfecta (354, 355). Bisphosphonates can be administered orally or by injection and work by reducing bone resorption by osteoclasts. However, long-term treatment using bisphosphonates may not be suitable for the purposes of bone repair and remodelling. Bisphosphonates have been reported to induce osteonecrosis of the jaw (101, 102), and in rare cases, may complicate dialysis treatment (356). Thus, alternative strategies are needed to overcome this problem to find a suitable agent for bone remodelling and repair that will promote the formation of bone and mineralisation without affecting the bone structure.

Strontium in the form of strontium ranelate (SrR) was used to treat osteoporosis in post-menopausal women (20), and has recently been in male patients (97). In osteoporosis, bone density is reduced, which weakens the bone structure and makes it fragile, thereby predisposing patients to fractures (357). Strontium (Sr^{2+}) may also exert its positive effects on teeth and bone when consumed orally (357), but a high dose of strontium is detrimental. In fact, daily exposure to high strontium levels found in soil can lead to rickets in children (98), and osteomalacia in rats with chronic renal failure (99). Importantly, however, the high strontium was induced in these rats to stimulate long-term exposure and the pathology showed that the increase in strontium may be related to an effect on osteoblast activity itself rather than the mineralisation process. However, a low dose strontium is beneficial, and patients taking an oral dose SrR (2g/day) had increased bone mineral density (BMD) of their femoral neck, lumbar spine, and hip after 8 years. It was concluded that SrR treatment had stabilised the fracture incidence in these patients concurrent with the increased BMD (20). In a study where rats with chronic renal failure were exposed to increasing doses of strontium (Sr30, Sr75 and Sr150, which is equivalent to 0.03% (w/v), 0.075% (w/v), and 0.15% (w/v)), for nine months, it has been suggested that bone density was dependent on the strontium concentration, and a lower dose of strontium in a suitable therapeutic range may help to promote mineralisation (358).

Strontium effects on the cellular mechanisms of osteoblasts, osteoclasts, and bone marrow stromal cells were reviewed recently (359). It was shown *in vitro* that strontium helps to promote the formation of pre-osteoblastic cells, prevents osteoclast formation, and retards cell-induced differentiation towards adipogenesis. It is thought to exert its effects on bone modifying cells through some of the calcium (Ca^{2+}) sensing pathways including the calcium-sensing receptor (CaSR), the NFATc pathway, and other related pathways involve in bone modifying cells, such as Wnt signalling, the RANK/RANKL/OPG system and fibroblast growth factor signalling (reviewed in (359)) due to similar charge-size-ratio of strontium to calcium (287).

One of the best-described mechanisms of action for strontium is that it may exert its effects on osteoblasts through the CaSR pathway, as shown in a study using CaSR knockdown osteoblasts (19). Human osteoblasts without the CaSR were exposed to increasing concentrations of strontium in media (0.01 to 2 mM strontium). The

authors showed increased RANKL expression and decreased osteoprotegerin (OPG) mRNA compared to the wildtype, suggesting that reducing RANKL and increasing OPG expressions were mediated by the CaSR (19). In osteoclasts, strontium inhibits osteoclastogenesis by causing apoptosis of mature osteoclasts in a dose-dependent manner mediated by protein kinase C isoform β II (PKC β II) and translocation of NF- κ B (RANKL) (94). In the same study, the authors also observed that administration of calcium and strontium (20 mM each) to mature osteoclasts enhanced NF- κ B translocation compared to when calcium was administered alone, suggesting that strontium potentiates the calcium effect by activating the CaSR through the same NF- κ B pathway *via* activation of PKC (94).

Therapeutically, strontium has wide potential uses to treat conditions other than osteoporosis, including bone fractures, treatment for bone loss due to immobilisation and old age (reviewed in (359)), and also for treatment of caries and cavities in restorative dentistry. Thus, in this chapter, strontium-substituted BGs were studied as potential materials to promote bone formation and repair. Three different compositions of BG powder were used: 45S5 (0% strontium substitution with calcium; 0SrBG), 10SrBG (10% strontium substitution with calcium), and 100SrBG (100% strontium substitution with calcium) with particle sizes ranging from 0.1 to 100 μ m. We anticipated the wide range of BG particle size distribution may also have an effect on the ionic dissolution profile. The strontium substitution with calcium was done in mole % (which will be referred after this as mole %). The effects of strontium-containing BG-conditioned media were assessed on bone modifying cells with the aim of generating a better understanding of the potential of SrBG as bone replacement materials in the regeneration and repair of dental and oral related structures.

3.2 Materials and methods

3.2.1 Cell culture

In this chapter, two different cell lines were used: the human osteosarcoma cell line, Saos-2, and mouse monocyte cell line, RAW264.7. Saos-2 were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK) and initial culture was

performed in 75cm² flasks in a humidified atmosphere at 37°C with 5% CO₂ with RPMI 1640 (Gibco) supplemented with 10% (v/v) foetal bovine serum (FBS), 2 mM L-glutamine, and 1% (v/v) penicillin/streptomycin (Gibco). Once the cells reached 90% confluence, they were trypsinized with 0.25% (w/v) trypsin/EDTA (Gibco) and counted using a haemocytometer. The cells were plated at a density of 3x10⁴ cells/cm² and cultured for up to 21 days in a 24-well plate (Nunc, USA). The cells were supplemented with fresh medium every 2-3 days.

RAW264.7, a mouse monocyte cell line that can differentiate into osteoclasts when induced, was obtained from American Type Culture Collection (ATCC, Teddington, UK), and initial cells was performed in 75cm² flasks in a humidified atmosphere at 37°C with 5% CO₂ in DMEM (Gibco) supplemented with 10% (v/v) FBS, 4 mM L-glutamine and 1% (v/v) penicillin/streptomycin. Once the cells had reached 90% confluence, they were trypsinized with 0.25% (w/v) trypsin/EDTA and counted using a haemocytometer. The cells were plated at a density of 3x10⁴ cells/cm² and cultured for up to 6 days in a 96-well plate (Nunc, USA). The cells were supplemented with fresh medium every 2-3 days.

For experiments using RAW264.7 cells, the cells were subjected to eight different types of media including controls. The first control was DMEM alone without BG addition or any factors. The second control was culture medium with the addition of nitrogenous bisphosphonate pamidronate (PAM) (Sigma) at a concentration of 1 µM, which is known to prevent osteoclast-mediated bone resorption *in vivo* (354). The RAW264.7 monocytes will differentiate into osteoclasts upon the addition of 20 ng/ml soluble murine receptor activator of nuclear factor-κB ligand (RANKL) (PeproTech EC, London, UK). The third control was culture medium with the addition of RANKL. RANKL was also added to the culture medium containing BG dissolution ions. For comparison purposes, an additional control was prepared by adding 1 mM SrCl₂·6H₂O (Sigma) to cell culture medium containing RANKL. The type of media formulations prepared is listed in Table 3.1.

Table 3.1: The media formulations prepared for the assessment of osteoclast differentiation.

Name	Abbreviations	Content
Control	Control	Normal medium (DMEM)
Control+RANKL	RANKL	DMEM plus receptor activator of nuclear factor- κ B ligand (RANKL)
Control+RANKL+PAM	PAM	DMEM plus receptor activator of nuclear factor- κ B ligand (RANKL) plus pamidronate (PAM)
0SrBG+RANKL	0SrBG	Non strontium-containing BG-conditioned medium (0SrBG) plus receptor activator of nuclear factor- κ B ligand (RANKL)
10SrBG+RANKL	10SrBG	10% strontium-substituted for calcium (10SrBG) strontium-containing BG-conditioned medium plus receptor activator of nuclear factor- κ B ligand (RANKL)
10SrBG+RANKL	100SrBG	100% strontium-substituted for calcium (100SrBG) strontium-containing BG-conditioned medium plus receptor activator of nuclear factor- κ B ligand (RANKL)
HC+RANKL	HC	Reference material (HC) plus receptor activator of nuclear factor- κ B ligand (RANKL)
1 mM SrCl ₂ +RANKL	1 mM SrCl ₂	DMEM containing 1 mM strontium chloride solution plus receptor activator of nuclear factor- κ B ligand (RANKL)

3.2.2 Preparation of SrBG powder

Strontium-containing bioactive glasses (SrBG) used in the current project were a kind gift from Dr. Matthew O'Donnell, and prepared as previously described (21) with compositions listed in Table 3.2. Briefly, the precursor materials (SiO₂, Na₂CO₃, CaCO₃, SrCO₃ and Na₃PO₄) were prepared and the powder was melted at 1400°C

in a platinum crucible for 90 minutes followed by rapid quenching in deionised (DI) water. The glass frits were ground and sieved to yield glass powders with particle size 0.1-100 μm . The glasses were stored in a desiccator prior to experiments. A commercially available synthetic coralline based on hydroxyapatite and calcium carbonate (Pro Osteon, which will named as HC later in the chapter, Interpore International, USA) was used as a reference material.

Table 3.2: The composition of the glasses studied.

	Oxide	SiO₂	Na₂O	CaO	SrO	P₂O₅
Weight %	0SrBG	45.00	24.50	24.50	0.00	6.00
	10SrBG	44.08	24.00	21.60	4.43	5.88
	100SrBG	37.26	20.29	0.00	37.49	4.97
Mole %	0SrBG	46.13	24.35	26.91	0.00	2.60
	10SrBG	46.13	24.35	24.22	2.69	2.60
	100SrBG	46.13	24.35	0.00	26.91	2.60

3.2.3 Preparation of BG-conditioned media

Three different compositions of bioactive glass (BG) without calcium (0%) or with approximately 10 or 100% (in mole %) of the calcium was substituted with strontium were prepared using the melt quench route as previously described (21, 22). Initial assessments were performed to determine the BG powder to culture medium ratio in order to optimise the dissolution behaviour of ions from the BG powder. This was important to ensure the correct concentration of ions is released from the BG powder to the culture medium since we are using BG powder with different particle sizes. Upon exposure of BGs to body fluid, the apatite phase will be formed involving the reactions previously described in Section 2.6.2. Suitable silicon and calcium concentrations are needed to initiate the osteoblast response for mineralisation (range of 15-30 ppm for silicon and 88-100 ppm for calcium) (292, 360). The rate of the apatite layer formation is normally used as a measure of bioactivity, which can be related to factors such as the chemical composition of the material and its textural properties (292). Fine, medium and coarse 45S5 BG powders showed different silicon and calcium dissolution release profiles upon soaking in SBF and culture medium. This suggested the particle size of the BG powder affected the ionic

dissolution release profile, with smaller particle size showing a higher rate of dissolution (360). Initially, we prepared approximately 1.5 mg/ml of BG powder immersed in both DMEM and RPMI culture media. However, the 1.5 mg/ml BG powder in DMEM did not produce sufficient silicon and calcium released into the culture medium after a 4 hour incubation period (less than 20 ppm for silicon and less than 80 ppm for calcium, data not shown). Based on the initial results from the ICP-OES data, we decided to add 1.5 mg/ml of BG powder to RPMI 1640 (for Saos-2 cells) and 6.0 mg/ml of BG powder to DMEM (for RAW264.7 cells). These mixtures of BG glass powders and culture media were incubated with continuous rolling at 37°C for 4 hours, followed by filtration of the media through a 0.2 µm filter (Minisart, Sartorius, USA). The samples of dissolution ion media were collected after 10, 30, 60, 120 and 240 minutes for elemental analysis. The BG-conditioned media was supplemented with 10% (v/v) FBS, 2 mM L-glutamine (for Saos-2) or 4 mM L-glutamine (for RAW264.7), and 1% (v/v) penicillin/streptomycin. The BG-conditioned media was incubated in a vented flask in 37°C with 5% CO₂ for 4 hours in order to stabilise their pH prior to cell culture.

3.2.4 Elemental analysis

In order to assess the concentration of ions released from the BGs, the cell culture media collected were filtered (0.2 µm syringe filter) and samples containing dissolution ions from BGs were diluted by a factor of 10 in deionised (DI) water. The elemental concentrations of the following ions: calcium (Ca), silicon (Si), sodium (Na), phosphorus (P), and strontium (Sr) were analysed by inductively coupled plasma – optical emission spectrometer (ICP-OES) (iCAP 6000, Thermo Scientific, Waltham, MA, USA). A standard curve for each ion was prepared according to the manufacturer's instructions to ensure that the concentration of ions released was within the instrument's range. Each sample was measured in triplicate.

3.2.5 Alkaline phosphatase (ALP) activity

Substrate p-nitrophenyl phosphate was used to measure ALP activity, a marker of osteoblast differentiation and mineralisation. Cell culture medium was removed from each well, followed by washing steps and lysis using 0.9% (v/v) Triton X-100 with freeze-thaw cycles. The cell lysates were prepared and reacted with 1 mg/ml p-nitrophenyl phosphate in alkaline buffer (1.5 M) prepared according to

manufacturer's instructions. The colour development that occurred during the reaction was stopped with 1 M NaOH and the absorbance was measured using a microplate reader (SpectraMax M5, Molecular Devices) at a wavelength of 410 nm. The ALP concentrations were calculated using a standard curve prepared with paranitrophenol (Sigma) at concentrations between 0 and 200 μ M according to the manufacturer's instructions.

3.2.6 Tartrate resistant acid phosphatase (TRAP) activity

TRAP is a marker of osteoclast differentiation and resorption activity (361). In RAW264.7 cells, the TRAP activity can be quantified by measuring colour development during the conversion of p-nitrophenyl phosphate to p-nitrophenyl with sodium tartrate under acidic conditions. Cells were lysed in deionized (DI) water followed by freeze-thaw cycles. A 1 mg/ml p-nitrophenyl phosphate in 0.1 M glycine buffer with 0.1 mM ZnCl_2 , 0.1 mM MgCl_2 and 20 mM sodium tartrate (pH = 5.0) was added to the lysates. The colour development was stopped with 1 M NaOH and the absorbance was measured on a microplate reader (SpectraMax M5, Molecular Devices) at a wavelength of 405 nm. The TRAP concentrations were calculated using a standard curve prepared using TRAP stock solution with paranitrophenol (Sigma) at concentrations between 0 and 200 μ M according to the manufacturer's instructions.

3.2.7 Total DNA assay

The total DNA from the same cell lysates as used for ALP and TRAP quantification was determined using a Quant-iT PicoGreen Kit (Molecular Probes, UK) according to the manufacturer's instructions. Briefly, cell lysates (from Saos-2 and RAW264.7 cells) were prepared and Pico Green was added. The fluorescence was measured with an excitation wavelength of 485 nm and an emission wavelength of 535 nm on a microplate reader (SpectraMax M5, Molecular Devices). The amount of DNA was used to determine the number of total cells in the cell lysates and to normalise the ALP and TRAP activity. The DNA concentrations were calculated using a standard curve prepared with a DNA stock solution at concentrations between 0 and 1000 ng/ml according to the manufacturer's instructions.

3.2.8 Light microscopy for cells

Light microscopy was used to observe cell morphology upon exposure to BG-conditioned medium. Microscopy of cells inside the well-plate was performed using an Olympus BX-URA2 (Olympus UK Ltd, London) epifluorescence microscope, and the images were captured using a Zeiss Axiocam digital camera (21).

3.2.9 Statistical analyses

The data for the experiments are expressed as mean $\pm$ standard deviation (SD). Each experiments were performed at least twice, and prepared in $N=4$ for each composition at a designated time point. The data were analysed using SPSS version 20.0 (IBM, USA) using one-way analysis of variance (ANOVA) with subsequent post-hoc test (Bonferroni procedure), with p -values < 0.05 considered statistically significant.

3.3 Results and discussion

3.3.1 Ion studies release

The concentrations of calcium (Ca), silicon (Si), strontium (Sr) and phosphorus (P) of the BG-conditioned media are shown in Fig. 3.1 (RPMI 1640) and Fig. 3.2 (DMEM). For both RPMI 1640 and DMEM, the calcium content of the culture medium incubated with BG is inversely related to the strontium concentration present in the BG particulate, such that higher strontium concentration in the BG leads to a lower calcium concentration. This occurs as a result of strontium substitution for calcium, which is possible because the ionic radius of strontium is similar to calcium. It was shown by ^{29}Si NMR spectra that the glass network connectivity remained unaltered when strontium was substituted with calcium (287). The amount of calcium incorporated within the glass is however lower when strontium substitution increases, explaining the differences in calcium content observed between the conditioned media. A decrease in calcium content over time was obtained as a result of 100SrBG incubation into cell culture medium. This effect was observed for both the RPMI and DMEM culture media even though they differ in their ion formulations. RPMI 1640 has a calcium concentration of 100 mg/ml (0.424 mM) provided in the form of calcium nitrate, while DMEM has 200 mg/ml (1.802 mM) provided in the form of calcium chloride (Gibco).

Such a decrease in calcium content following incubation with BG has previously been reported. This was thought to indicate calcium phosphate (CaP) formation on the BG surfaces, which was supported by the decreasing level of phosphorus in the culture medium (22). However, in our BG dissolution profiles in DMEM, we could not observe a decrease in phosphorus concentration. The phosphorus level in the culture medium remained fairly constant and there was a minimal reduction in the calcium concentration, with approximately 50 ppm recorded after four hours (Fig. 3.2). This result may be explained by the size of the BG powder. We used particle sizes ranging from 0.1 to 100 μm and with a higher powder to liquid ratio (6 mg/ml). The strontium profile release, however, is similar between the two studies despite the different powder to liquid ratio and preparation of the formulation (powder versus particulates). The fact that a similar strontium release can be obtained from powder with different particle size further underlines the suitability of strontium-substituted BGs for dental tissue engineering applications. As mentioned above, a minimal change in phosphorus was measured for both RPMI 1640 and DMEM. The calcium and phosphorus dissolutions from HC particulates were almost unchanged during the four hour period. This was anticipated because HC is composed mainly of hydroxyapatite and calcium carbonate. HC is a calcium phosphate based ceramic that is normally used as a bone graft material, is resorbable and is commonly used to treat periodontal defects.

The silicon and strontium dissolutions into the culture medium increased over time, which was dependent on the type of culture media used and the BG composition, in agreement with a previous report (22). In RPMI 1640, the silicon dissolution into the culture medium depended on the concentration of strontium present in the BG powder, with a high strontium concentration leading to higher silicon dissolution into the culture medium. However, in DMEM, the same trend was not observed. In DMEM, 10SrBG had the highest silicon dissolution into the media over the four hour period, which was also observed in a previous study (22). This may occur as a result of the strontium concentration present in the BG powder. In BG with a higher strontium concentration (100SrBG), more strontium is involved in the glass silicate network, leading to a lower silicon release into the culture medium. Conversely, in BG with a lower strontium concentration (10SrBG), less strontium is present to

substitute for calcium, which leads to a higher silicon release into the culture medium. Furthermore, it was shown that higher strontium substitution resulted in the formation of a calcium/strontium carbonate phase, making the BG surface more reactive and thus allowing for more interactions with the other ions present in the BG (287).

Table 3.3: The strontium concentration at four hours determined by ICP-OES (in ppm and mM).

Strontium concentration	RPMI (1.5 mg/ml)		DMEM (6.0 mg/ml)	
	ppm	mM	ppm	mM
0SrBG	0.02	0.02	0.03	0.42
10SrBG	4.07	0.05	6.29	0.07
100SrBG	45.61	0.52	84.32	0.96

The strontium concentration after four hours of incubation was different in RPMI and DMEM (Table 3.3). This variation can be attributed to the differences in ionic composition of the two media as well as the change in the SrBG powder to liquid ratios that were used. This resulted in different strontium release from the BG particles into the culture medium, suggesting that the SrBG can be tailored for individual needs and has potential application for regeneration and repair in dental tissue engineering.

In the current project, we also wanted to see the effect of particle size on dissolution profiles upon immersion of the BG powder into culture medium. We could see that using such a wide particle size range (0.1-100 μm) had an effect on the dissolution profile of the BG. Our initial results for the 1.5mg/ml BG powder in DMEM did not show suitable silicon and calcium release profile from the BG, which differs from the findings of Gentleman *et al* (22). In that study, the authors used 1.5 mg/ml BG powder to culture medium and particle size < 38 μm . Sepulveda *et al* (360) also showed that particle size has a profound effect on ion dissolution release profile, with fine particles (~5-20 μm) showing a higher release profile compared to medium (~90-300 μm) and coarse (~90-710 μm) particles.

Our initial results also showed that when we used the 1.5 mg/ml BG powder in DMEM, a lower strontium dissolution profile was also observed, with less than 50 ppm strontium released into the DMEM. However, when we increased the BG powder to DMEM ratio to 6.0 mg/ml, we were able to show an increase in the strontium concentration to approximately 84.32 ppm as shown in Fig. 3.2. This showed that larger particle size in our SrBG powder released lower strontium upon immersion in DMEM culture medium. As shown previously (360), the use of dissolution media (eg. simulated body fluid [SBF] and culture medium) also affected the ionic dissolution profile. For example, the silicon release in SBF and cell culture medium from the 45S5 BG fine powder showed similar trends, although higher silicon released was observed in cell culture medium (which may occur due to slow release). The presence of serum in culture medium may have slowed down the ions dissolution from the BG powder. This was likely due to the saturation of the solution by the dissolved ions or by the formation of organic residues or protein complexes within the serum of the BG powder. This may delay the apatite layer formation (360).

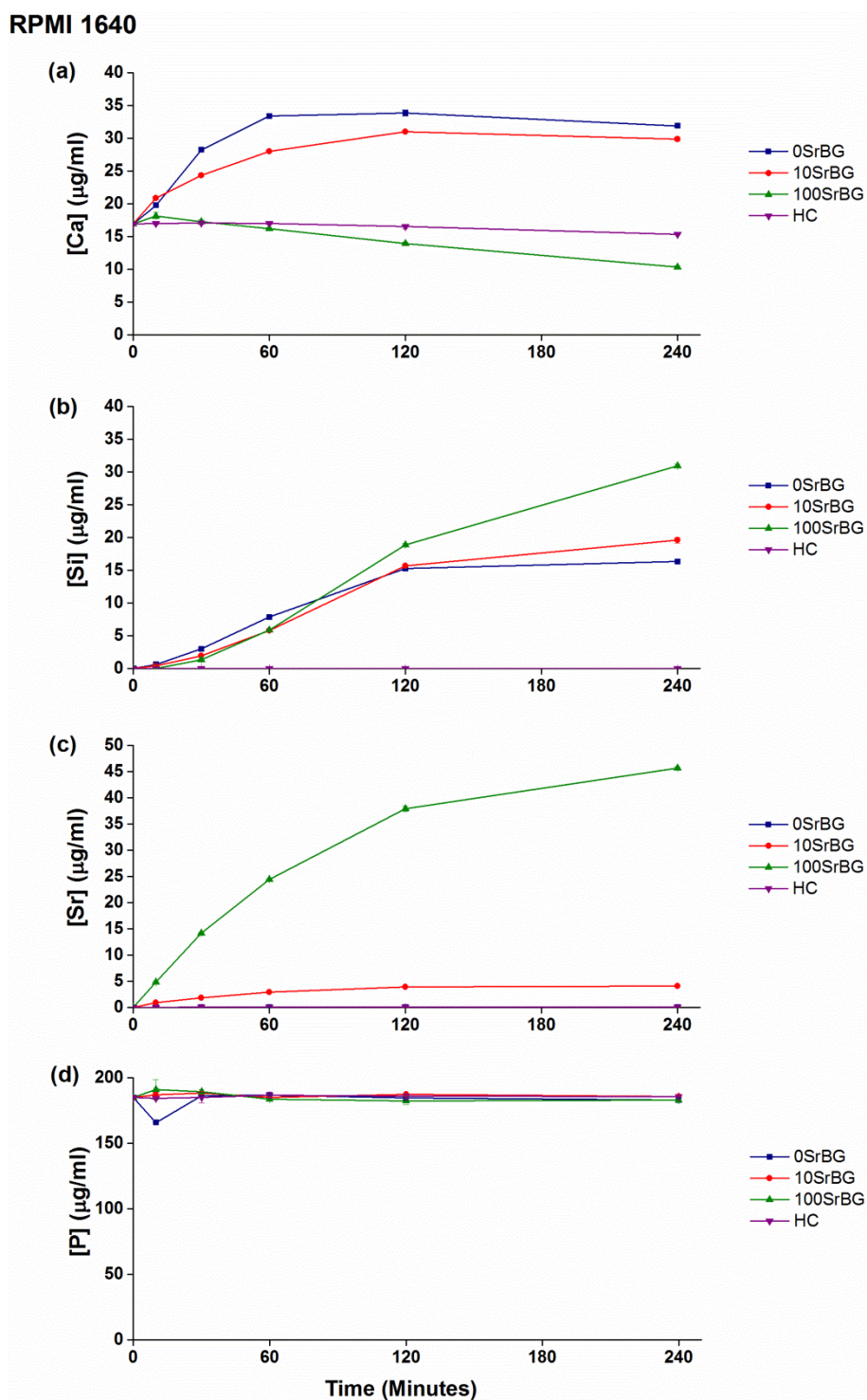


Fig. 3.1: The dissolution profile of BG powder incubated in RPMI 1640 culture medium over a four hour period was assessed using ICP-OES. The calcium (Ca) content (a) is inversely related to the strontium (Sr) concentration (c) present in the BG powder. The silicon (Si) dissolution (b) into the culture medium is dependent on the concentration of strontium present in the BG powder. Minimal changes were observed for the phosphorus (P) release (d) from the BG powder.

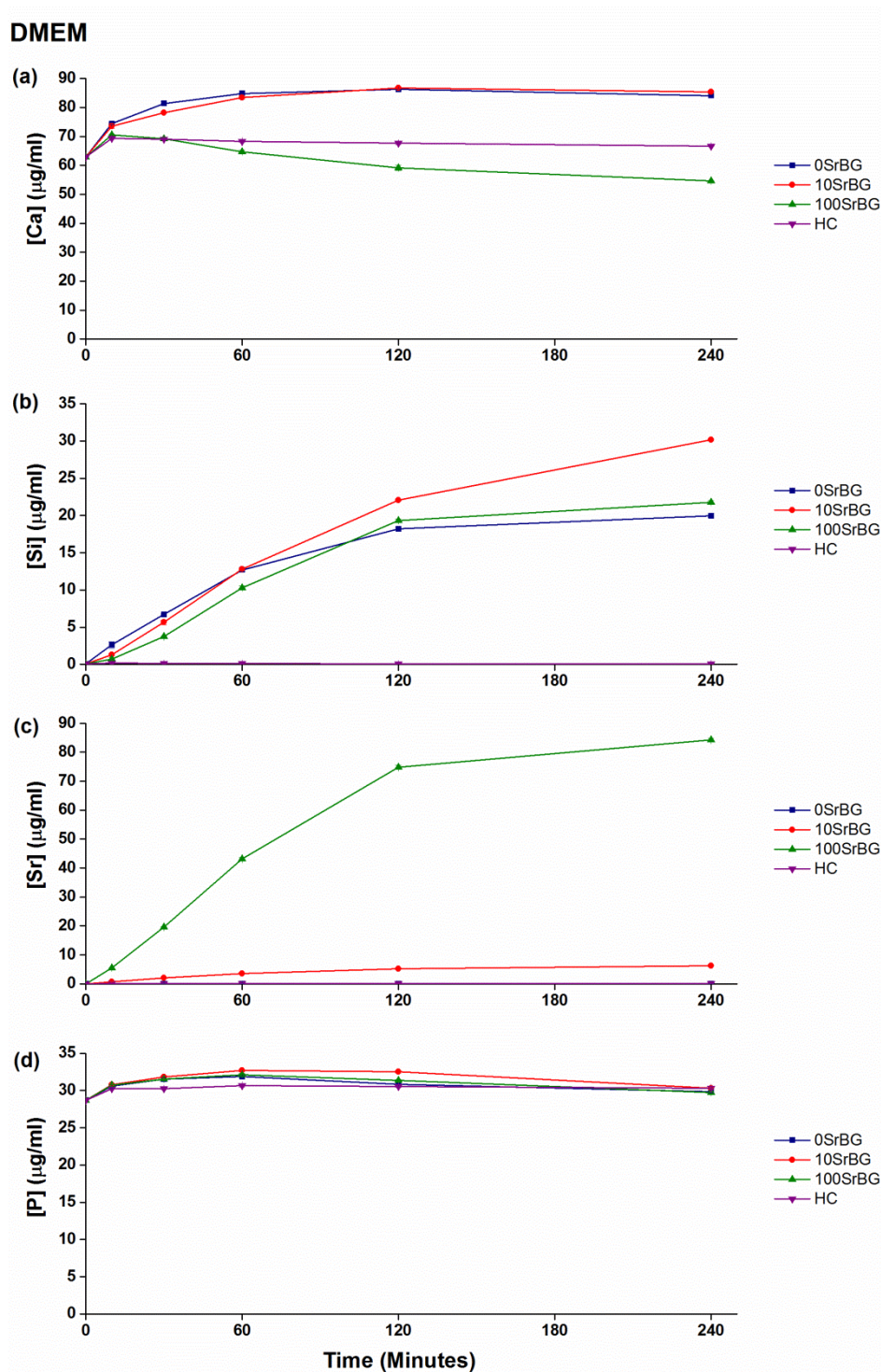


Fig. 3.2: The dissolution profile of BG powder incubated in DMEM culture medium over a four hour period was assessed using ICP-OES. The calcium (Ca) content (a) is inversely related to the strontium (Sr) concentration (c) present in the BG powder, which remained fairly constant. The 10SrBG showed a higher silicon (Si) dissolution (b) into the culture medium compared to 0SrBG and 100SrBG. Minimal changes were observed for the phosphorus (P) release (d) from the BG powder.

3.3.2 ALP activity and total DNA quantification

Hard tissue repair related to dental tissue engineering involves synergy between bone formation and bone resorption to allow proper remodelling, especially in fractures in the craniofacial region, tooth eruption, and orthodontic tooth movement (reviewed in (362)). For this reason, the effect of strontium-containing BG is assessed on two types of bone modifying cells: osteoblasts, which are responsible for bone formation, and osteoclasts, which are responsible for bone resorption. For bone formation, Saos-2 cells are used as a model cell type to determine osteoblastic differentiation upon exposure to strontium-containing BG-conditioned medium. The effect of BG dissolution products on osteoblast bone-forming activity was assessed by measuring alkaline phosphatase (ALP) activity in the cells. For this assay, 1 mM $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$ was used as additional control together with the non-conditioned control medium (control).

The quantity of DNA in the cell lysates was used to normalise the ALP activity per cell following exposure to strontium-containing BG-conditioned medium. The ALP activity normalised to DNA from Saos-2 cells cultured in the dissolution media is shown in Fig. 3.3a. In general, Saos-2 cells cultured with ion dissolution products from all BGs had increased total ALP activity over time from day 3 and up to day 21. There was minimal ALP activity at days 3 and 7. At day 3, cells cultured in 10SrBG and 100Sr BG-conditioned media showed significantly lower ALP activity compared to 0SrBG. The ALP activity from cells cultured in reference material (HC) and 1 mM SrCl_2 conditioned medium was too low to be detected at this time point. The same trend of low ALP activity was also observed at day 7 with cells cultured in 0SrBG, HC and 1 mM SrCl_2 conditioned medium, showing less ALP activity compared to other cells in other strontium-containing BG-conditioned medium. At day 14, the ALP activity in cells cultured in 10Sr BG-conditioned medium was slightly higher compared to cells cultured in the control and 0SrBG conditions. At this time point, ALP activity appeared higher in cells cultured in control, 0SrBG- and 10SrBG-conditioned media compared to cells in 100SrBG- and HC-conditioned media. Cells cultured in medium supplemented with 1 mM SrCl_2 showed the lowest ALP activity, which was significantly different when compared to 0SrBG. At day 21, the ALP activity from cells cultured with 0SrBG was significantly higher when compared to cells cultured in control media. Although the ALP activity from the SrBG appeared to

be inversely related to the strontium content in the BG, no statistical differences were found when compared to 0SrBG or the control condition. This may suggest that Saos-2 cultured in Sr BG-conditioned media is proliferating as shown by the higher cell number at day 21 (Fig. 3.3b). In general, the results showed that the strontium-containing BG-conditioned medium did not negatively affect Saos-2 cells ALP activity compared to controls and non-Sr BG-conditioned medium, suggesting the potential use of SrBG in dental tissue engineering.

The DNA assay (Fig. 3.3b) showed that at day 3, Saos-2 cells cultured in 1 mM SrCl_2 -containing medium had the highest DNA content, which was significant compared to control and 0Sr BG-conditioned medium. For strontium-substituted BG-conditioned medium (0Sr, 10Sr and 100SrBG), the amount of DNA present in the cells was dose-dependent. Higher strontium content led to a higher amount of DNA, which is statistically significant for 100SrBG compared to 0SrBG. This trend was observable at days 3 and 7. HC showed significantly higher DNA content compared to 0SrBG. At day 14, the amount of DNA was statistically higher in 10SrBG compared to 0Sr and 100SrBG, which was also observable at day 21. The amount of DNA from HC and 1 mM SrCl_2 conditioned medium was significantly lower compared to the control medium. The DNA content in 0SrBG was significantly lower compared to other groups at day 21, which was also observed at days 7 and 14. This finding is probably due to the lower number of Saos-2 cells present in the well plates, where mineralisation gradually occurred from day 7 to day 21. However, this finding requires further investigation. As shown in Fig. 3.4, the Saos-2 cells cultured in 0SrBG and in 10Sr BG-conditioned media showed some precipitation that could be contributed by the formation of hydroxy-carbonate apatite (HCA) within the well plates in Saos-2 cells cultured in 0SrBG and 10SrBG.

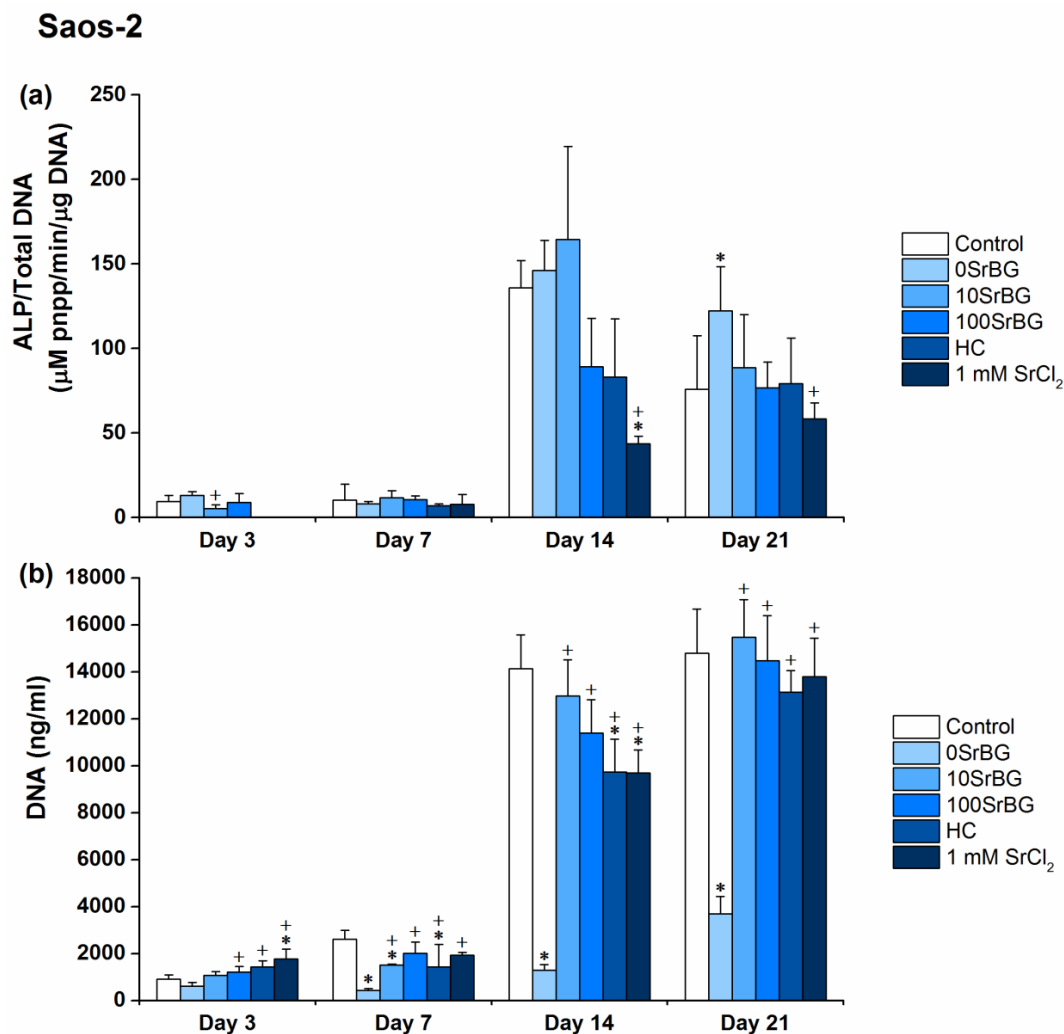


Fig. 3.3: The ALP activity from Saos-2 cultured in BG-conditioned medium normalised to total DNA content from the same cell lysates (a). The DNA assay represented the total number of cells from Saos-2 cultures in BG-conditioned medium (b). * denotes a significant difference of the marked bar compared to the control at the same time point. + denotes a significant difference of the marked bar compared to 0SrBG at the same time point.

Osteoblasts lay down bone by secreting alkaline phosphatase, type I collagen, proteoglycans, bone sialoprotein and osteopontin (362). The higher ALP activity in Saos-2 cells exposed to 10Sr BG-conditioned medium observed at days 14 and 21 showed that lower strontium concentration or no strontium (for control BG, 0SrBG)

may be beneficial for bone formation. Human osteoblasts exposed to increasing strontium concentration (0-25 mM SrCl_2) showed that the percentage of cells undergoing cell division (>4 cell division) was significantly higher in cells exposed to 5 mM strontium compared to 1 and 10 mM strontium, suggesting that SrR promoted osteoblast proliferation (363). Furthermore, the authors showed that 10 mM strontium concentration induced the cells to deposit the most mineral, and Von Kossa staining showed that the mineralisation was dependent on the strontium concentration at day 28 and 42 (363).

When strontium is substituted for calcium in the BG structure, it may function differently due to being a larger cation than calcium, which in turn makes the BG crystal lattice bigger (287). The strontium-containing BG-conditioned medium promotes cell growth and increases cell number as shown by DNA measurement, which was significantly higher than 0SrBG and indicated the strontium-containing BG-conditioned medium was non-toxic and allowed for proliferation. When 45S5 BG-conditioned medium was prepared from BG powder (1% w/v) and human osteoblast cells were cultured in the conditioned medium for 4 hours, the results showed an increase in cell number during the four-day incubation, suggesting the dissolution products from the BG stimulated cell proliferation (148).

Bioactive glass powders prepared with different strontium substitution (10, 40 and 100 mole %) with calcium and hydroxyapatite (HA) added to the powder were previously tested on human osteoblast-like cells after 1, 3 and 7 days of incubation (303). The ALP activity was assessed and the results showed that the Sr10-HA promoted the highest activity compared to HA alone. This is accordance with our study, where 10SrBG resulted in the highest ALP activity per cell, suggesting that lower strontium promotes bone mineralisation. In the same study, the authors tested the osteoblasts exposed to strontium with calcium silicates with 5, 10 and 20 (mole %) strontium substitution with calcium and again their results showed that Sr10-CS led to the highest ALP activity compared to CS glass alone. This further suggests that strontium incorporation enhanced cell proliferation and differentiation and could enhance bone formation (303).

Strontium is thought to exert its effects on bone modifying cells through some of the calcium (Ca^{2+}) sensing pathways including the calcium-sensing receptor (CaSR) pathway (reviewed in (359)). The CaSR involvement was studied by using osteoblasts from CaSR knockout animals ($\text{CaSR}^{-/-}$) (364). Osteoblasts from $\text{CaSR}^{-/-}$ and wildtype ($\text{CaSR}^{+/+}$) mice were exposed to strontium ranelate (SrR) (0-10 mM strontium) in medium with or without serum, with the latter inducing apoptosis within 24 hours. In the presence of strontium, osteoblasts deprived of serum did not undergo apoptosis, which was determined to be *via* the CaSR (364). In our BG system however, both calcium and strontium can be released depending of the BG composition. Since, calcium and strontium often act on similar pathways, identifying the pathway activated as a result to strontium incorporation within BG would need further investigations.

The reference material, HC, used in this study also allowed osteoblast maturation as observed by the increase of ALP activity over time. However, in this condition, the ALP activity was lower than in the presence of strontium-containing BG-conditioned medium, indicating the benefit of ion released from the SrBG on Saos-2 anabolic activity. The HC is an HA and calcium carbonate based material, hence the presence of calcium in the composition may also enhance ALP production and promote cell growth if the cells were cultured in contact with the material. However, in our case, Saos-2 were cultured in presence of the HC conditioned medium, which showed an ionic concentration close to the control medium, as observed by ICP-OES.

Based on these results, a lower strontium or no strontium (for control BG, 0SrBG) concentration appeared to be able to promote the osteoblasts to undergo differentiation and mineralise without the addition of osteogenic supplements, establishing the potential of the SrBG to promote mineralisation in the field of bone tissue engineering.

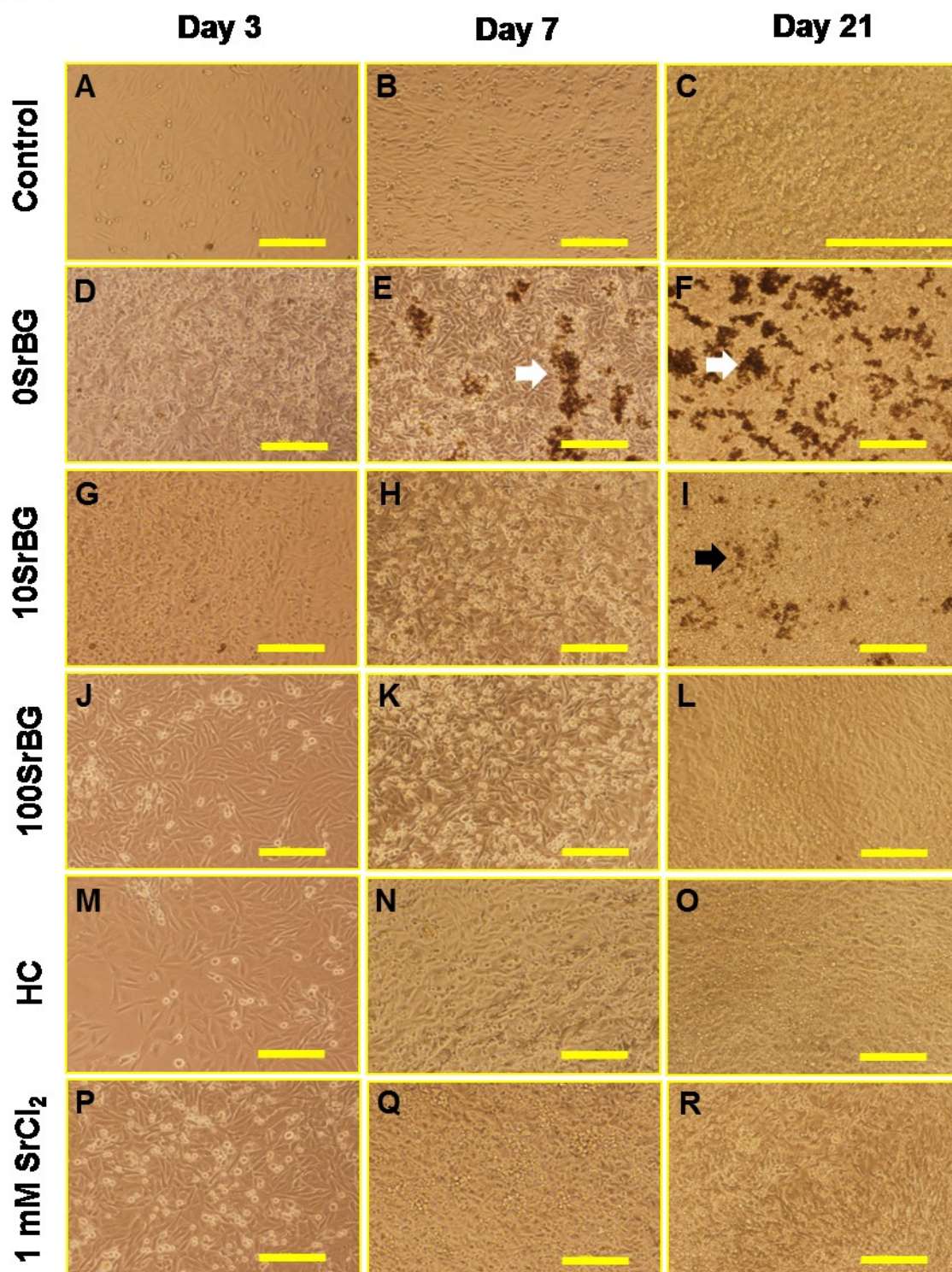
Saos-2

Fig. 3.4: Light microscopy images of Saos-2 cells in the ion dissolution media at day 3, 7, and 21. An increase in possible nodule formation (white arrow) is observed in Saos-2 cultured in 0SrBG (control BG) at days 7 (E) and 21 (F). Saos-2 cultured in 10SrBG-conditioned media also showed signs of possible nodule formation (black arrow) at day 21 (I). Scale bar represents 200 μm in all images.

3.3.3 Osteoclast differentiation, TRAP activity, and total DNA quantification

The murine monocytes RAW264.7 differentiate into osteoclasts when induced using RANKL (receptor activator of nuclear factor- κ B ligand) (351). In this study, 20 ng/ml RANKL was used to induce the differentiation of RAW264.7 to osteoclasts. To prevent osteoclast-mediated bone resorption, 1 mM pamidronate (PAM) was added during media preparation. There were eight different media formulations prepared for these tests, including one control medium (control) without BG and RANKL, as shown in Table 3.1. Control medium with the addition of RANKL is referred to as RANKL, and another control medium with the addition of RANKL and PAM is referred to as PAM. An additional control was prepared by adding 1 mM SrCl_2 to the control DMEM, referred to as 1 mM SrCl_2 . The RAW264.7 monocyte cell line was then cultured using the prepared media and also the strontium-containing BG-conditioned medium plus addition of RANKL to promote osteoclast differentiation.

The TRAP activity indicates whether cells are undergoing osteoclastic differentiation and causing bone resorption. The total cell quantification was performed on the same cell lysate by quantifying the amount of DNA present and using that to normalise the TRAP activity, which is shown in Fig. 3.5a. The TRAP activity of cells cultured in strontium BG-conditioned medium tended to decrease when the strontium concentration present in the BG increased as observed at day 6, however, no significant difference was detected. There was a slightly higher TRAP activity in cells cultured in RANKL, PAM, 100SrBG, and HC conditioned media compared to the control at day 2. At day 4, the TRAP activity was significantly higher in cells cultured in all strontium BG-conditioned media, including 0SrBG, when compared to control. TRAP activity was higher in cells cultured in control medium containing RANKL compared to control and medium containing RANKL and PAM. The TRAP activity was lower in HC-conditioned medium compared to all strontium BG-conditioned medium (0Sr, 10Sr and 100SrBG). At day 6 however, there was a reduction in the TRAP activity in cells in the entire conditioned medium group, with cells in 1 mM SrCl_2 showing the highest amount of TRAP activity, which was significant compared to control medium. The TRAP activity was higher in cells cultured in control medium containing RANKL compared to the control and medium containing RANKL and

PAM. The TRAP activity in cells cultured in strontium BG-conditioned medium appeared to be dose-dependent. A higher strontium concentration caused lower TRAP activity, and the reduction was nearly comparable to the control+RANKL group, which may suggest that incorporation of strontium within the BG can reduce TRAP activity. At this time point, indeed, from all SrBG, only 0Sr was significantly higher than the control group. As observed at day 4, the HC-conditioned medium led to a lower TRAP activity compared to cells cultured in the strontium BG-conditioned medium, a result also present at day 6.

RAW264.7

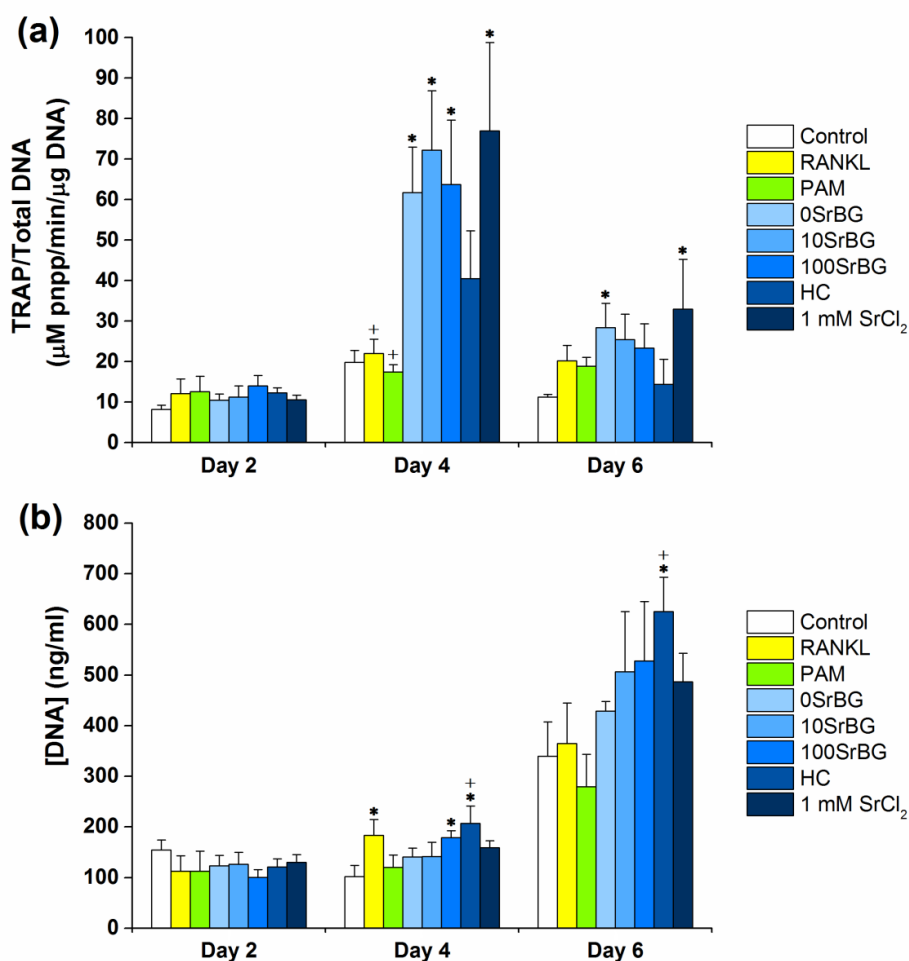


Fig. 3.5: The TRAP activity from RAW264.7 cultured in BG-conditioned medium normalised to total DNA content from the same cell lysates (a). The DNA assay as a representative of the total number of RAW264.7 cultured in BG-conditioned medium (b). * denotes a significant difference of the marked bar compared to the control at the same time point. + denotes a significant difference of the marked bar compared to 0SrBG at the same time point.

The total cell quantification based on DNA measurements is shown in Fig. 3.5b. At day 2, there were no significant differences. However, at days 4 and 6, HC recorded the highest amount of DNA content, which was significant when compared to control medium and 0Sr BG-conditioned medium. DNA amount tended to increase with strontium substitution within the BG, even though no statistical differences between the glasses were found. Only 100Sr showed an increased DNA quantity when compared to control at day 6. In general, there was an increase in the total DNA from day 2 to day 6 in the entire group of BG-conditioned media and their respective controls, RANKL, PAM, 1 mM SrCl₂, indicating cell proliferation over time.

RAW264.7 cultured in BG-conditioned media are shown in Fig. 3.6. The addition of RANKL into the BG-conditioned media caused the cells to change their morphology from small monocytes to large multinucleated osteoclasts. The addition of pamidronate (PAM) into the culture medium tends to prevent the RANKL-induced RAW264.7 from becoming osteoclasts. Bisphosphonates are effective in preventing the RANKL induction of RAW264.7, with fewer large multinucleated osteoclasts forming depending on the bisphosphonate concentration (351). The action of bisphosphonates is cell-specific (365), where 20 ng/ml RANKL was used to induce bone marrow cells from the mandible and long bones from the same mouse to become osteoclasts. PAM (1-100 µM) was administered to the cells at early (0-3 days) and later (3-6 days) time points, and the results showed that PAM administered either early or later produced the same inhibition of osteoclast formation (365).

Osteoclasts may possess up to eight nuclei before undergoing apoptosis (366), which can also be induced by exposure to supraphysiological extracellular calcium (20 mM) for 72 hours. This in return resulted in the reduction of osteoclast activity and prevented bone resorption (366). During apoptosis, when the calcium was removed, the osteoclast lifespan was extended. The subsequent formation of hypernucleated osteoclasts following the fusion of osteoclasts with their progenitor cells (354) may lead to bone resorption. Thus, the presence of calcium is crucial for maintaining bone homeostasis. The addition of strontium may help to retain more calcium in the bone, as shown in a study of ovariectomised (OVX) rats (367). The

rats were treated with bisphosphonates (RIS), strontium ranelate (SrR) and a combination of both, and after 16 weeks of treatment, the rats with SrR and RIS+SrR treatments showed appositional bone growth at the periosteal surface of cortical and trabecular bones enriched with strontium, although no significant difference was detected in bone strength compared to the OVX rats or sham (367). Nevertheless, the authors showed that strontium treatment helps to maintain bone microstructure, and the presence of strontium may encourage the dual incorporation of both calcium and strontium in the newly formed bone. Furthermore, when bisphosphonates were supplied in combination with SrR, they helped to produce stiffer bone compared to SrR treatment alone (367).

In this study, RANKL was added to all the medium and Sr BG-conditioned medium except the control (as shown in Fig. 3.6). In the presence of RANKL, osteoclasts undergo structural changes, releasing acidic enzymes and creating a vacuole beneath them. The activated TRAP degrades the bone and collagen matrix resulting in an increase in collagen fragments and calcium at the resorption front (344). Thus, the higher TRAP activity observed in our results may occur as a response to the ionic content of the SrBG-conditioned media. The Sr BG-conditioned medium has high calcium content resulting from both the culture medium itself and because of the calcium release from 0Sr and 10SrBG. Although we observed an increase of TRAP activity for all BG-conditioned medium conditions, incorporation of strontium within the BG tended to reduced TRAP activity, indicating a potential effect of the released strontium to osteoclast maturation. It is postulated that in the presence of the strontium-containing BG-conditioned medium, the RAW264.7 monocytes did not fully differentiate into osteoclasts, even with the addition of RANKL. Furthermore, although there might be some osteoclasts in the medium induced by RANKL, SrBG-conditioned medium did not completely abolish the osteoclast differentiation (22). Continuous exposure of osteoclasts to strontium may also reduce TRAP activity leading to a reduction of total surface area of resorption pits and osteoclast number (300).

The interplay between osteoclasts and osteoblasts is important during bone remodelling (362). In one study, the combination of calcium (0-20 mM) and strontium (0-24 mM) resulted in osteoclast apoptosis in a dose-dependent manner compared

to calcium treatment alone (94). In that study, there was an increase in osteoclast apoptosis with the increasing strontium concentration. Their finding was supported by an increase number of apoptotic TRAP positive cells, suggesting that strontium potentiates the calcium effect in reducing bone resorption by inhibition of osteoclastic activity (94). In our study, it would have similarly been interesting to determine the number of apoptotic TRAP positive cells to evaluate the effect of strontium content within the BG on osteoclast apoptosis.

It was surprising that RAW264.7 cells treated with RANKL cultured in 1 mM SrCl_2 -containing culture medium had the highest TRAP activity at day 4 and 6 compared to the control and Sr BG-conditioned medium. This may be attributed to the amount of strontium present in the culture medium, and also the particle size of the BG (0.1-100 μm). Larger particle size may lead to lower release of strontium compared to smaller particles. As shown in Table 3.3, the strontium concentration present in the DMEM at four hours in 10SrBG was 0.072 mM and 0.962 mM for 100SrBG, which is still lower than the control 1 mM SrCl_2 . This may have resulted in the higher TRAP activity observed in cells cultured in 1 mM SrCl_2 -containing medium. It also suggests that Sr BG-conditioned medium allows tailorable strontium concentration without affecting cell number and growth.

In this study, 1 mM PAM concentration was used. It was shown that when pharmacological doses of alendronate are used to treat osteoporosis and prevent bone resorption *in vivo*, the local concentration detected beneath the osteoclasts during the resorption process can reach a maximum of 1 mM. This is therefore considered an effective dose to be used *in vitro* (reviewed in (352)). Osteoclasts generate TRAP during differentiation, but it has been shown that the TRAP activity was less on tissue culture plastic compared to in osteoclasts seeded on bone slices (361). TRAP activity release is therefore affected by the surface substrate, and while it represents osteoclast activation, it does not indicate the maturation of osteoclasts *per se*. The rapid release of TRAP by osteoclasts on tissue culture plastic was not due to cell death, but may be due to a delay in replacing the TRAP or the generation of new osteoclasts in the culture (361). Strontium treatment may also result in the monocyte/macrophage cells lines differentiating into pre-osteocytes (363) or it may be due to the presence of undifferentiated stromal cells present in culture (365).

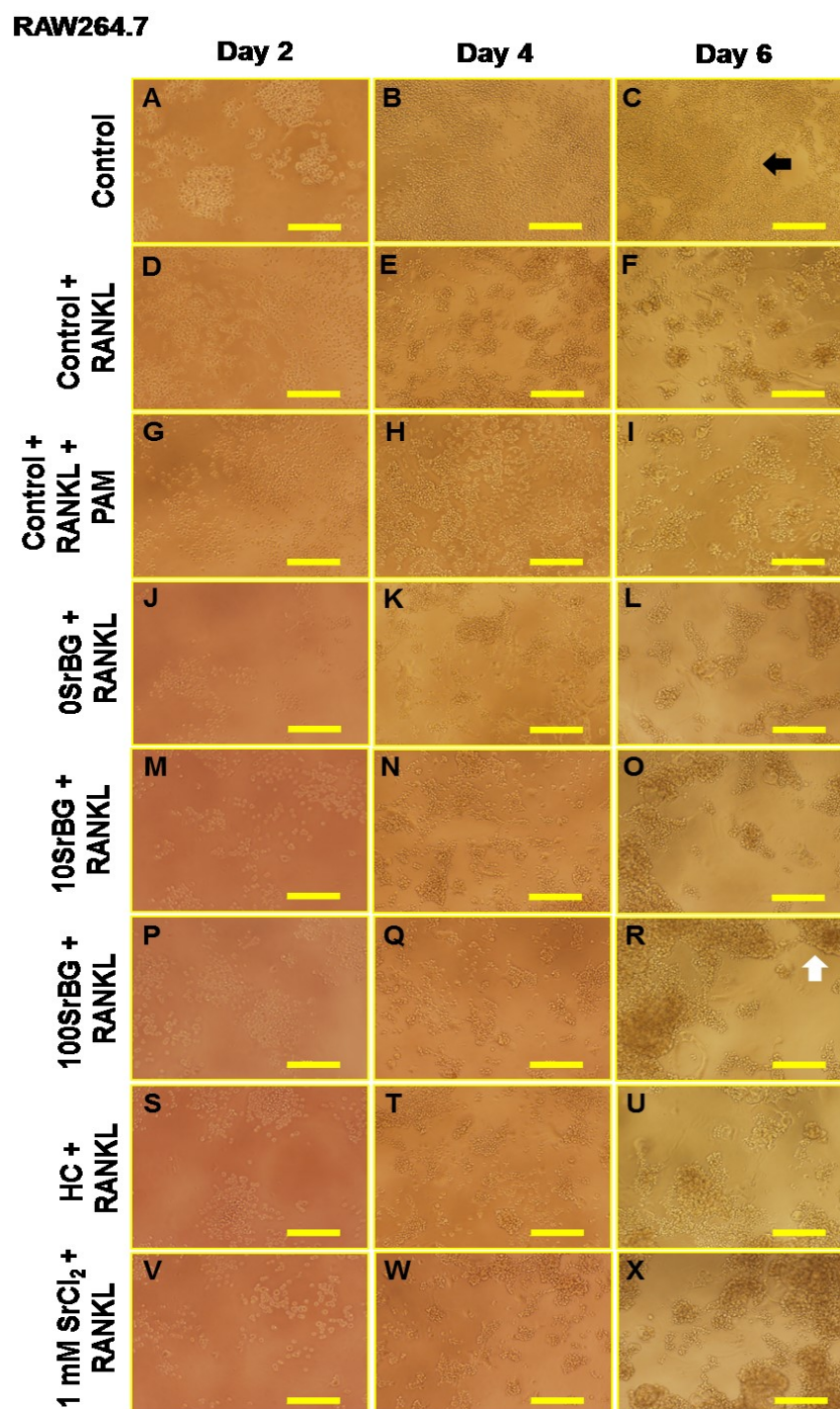


Fig. 3.6: Light microscopy images of RAW264.7 cells in the ion dissolution media at day 2, 4, and 6. RAW264.7 in control medium (C) were small monocytes cells (black arrow) at day 6 compared to RAW264.7 in other BG-conditioned medium containing RANKL (e.g. cells in 100SrBG+RANKL, R, white arrow). Scale bar represents 200 μ m in all images.

3.4 Conclusion

In this chapter, the effect of Sr BG-conditioned medium is assessed on Saos-2, an osteosarcoma cell line with osteoblastic properties, and RAW264.7 a monocyte/macrophage cell line. Both cells were chosen since they had previously been used to assess the effect of Sr BG-conditioned medium, and to observe the morphological changes that occurred when these cells were seeded on SrBG discs (22). Furthermore, Saos-2 and RAW264.7 have been widely used as cells representing osteoblasts and osteoclasts for biomaterial studies in the field of tissue engineering (341, 347).

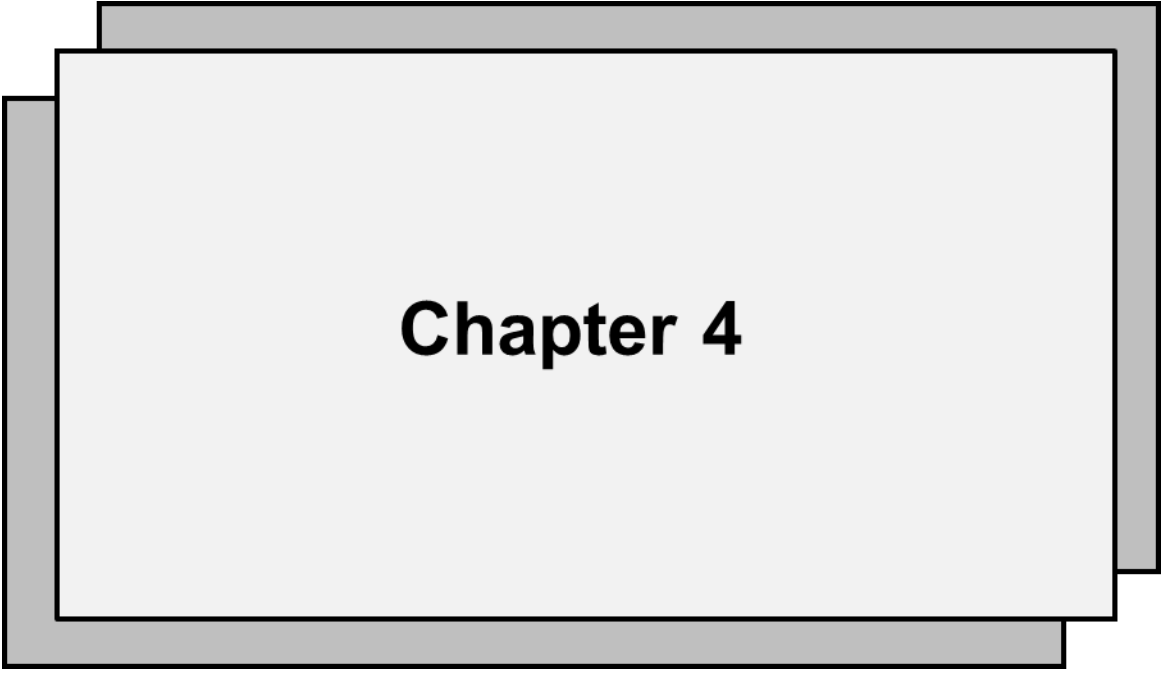
The Sr BG-conditioned medium was prepared in RPMI and DMEM and subjected to ICP-OES study to determine the BG dissolution profiles. The ICP-OES results showed the strontium released from the SrBG were dose-dependent on the strontium concentration within the SrBG and remained fairly constant, and could also be tailored to specific applications. The BG powders used in the current project have a wide range of particle size (0.1-100 μm) and affected the ionic dissolution profiles of the BGs powder, especially in DMEM culture medium. Larger particles tend to have slower dissolution rate due to lower surface area available for reaction in the culture medium. This also might cause lower strontium release from the BGs, particularly those in DMEM culture medium, which caused us to use higher concentrations of BG powder for DMEM (6.0 mg/ml). Our results showed that the strontium release after 4 hours in the culture medium was below 1 mM strontium, which is lower than the concentration normally used for patients receiving strontium ranelate to treat osteoporosis (352).

The selected SrBG concentration of 1.5 mg/ml for RPMI and 6.0 mg/ml for DMEM was chosen for Saos-2 and RAW264.4 cells. The cells were cultured in the Sr BG-conditioned medium and alkaline phosphatase activity in Saos-2 and TRAP activity and osteoclastic differentiation of RAW264.7 cells was assessed. Controls included control medium, culture medium containing 1 mM $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$, and a commercial product, HC. For the Saos-2 cells, the ALP activity showed an increasing trend in cells cultured in Sr BG-conditioned medium at days 3 to 14, with a decrease in ALP activity at day 21. The DNA assay as an indication of cell proliferation increased

steadily in cells cultured in Sr BG-conditioned medium but not those in 0Sr BG-conditioned medium. The lower proliferation in 0Sr BG-conditioned medium may be attributed to the mineralisation process which reduced cell numbers as mineral was deposited in the well plate. For RAW264.7 cells, the results showed the TRAP activity was higher at days 2 and 4 and then decreased at day 6. However, the total DNA that represents cell proliferation showed an increasing trend from days 2 to 6. This may suggest the presence of strontium in the BG reduces osteoclast activity at day 6 but does not prevent osteoclast proliferation.

Nodule formation was not examined in the current study. Future work should include the assessment of nodule formation in terms of qualitative and quantitative assays such as Alizarin Red or Von Kossa staining, and also XRD. The use of SBF may also be beneficial. For studies using RAW264.7, an additional control such as bone mineralising agent (eg. drugs or growth factors such as parathyroid hormones, Vitamin D, and bone morphogenetic proteins) is suggested. Strontium chloride without RANKL addition may also be added in future studies.

In general, our results showed that the strontium Sr BG-conditioned medium was non-toxic and did not negatively affect osteoblast and osteoclast activity and differentiation. Strontium containing BGs with lower strontium concentration such as 10SrBG (10% strontium substitution for calcium in the BG structure) may be beneficial for treatment and repair, which has the potential to be used in dental tissue engineering applications.



Chapter 4

Chapter 4

Investigation of strontium-containing bioactive glass on dental pulp stem cells

The anti-osteoporotic properties of strontium, demonstrated by treatment of post-menopausal women with strontium ranelate (SR) (95, 96), motivated the development of strontium-containing bioactive glasses (BGs) with potential use as bone replacement materials for tissue engineering and regenerative medicine (22). Strontium-containing BGs have great potential to advance regenerative dentistry. Strontium combined with existing restorative materials may help to retard the progression of caries beneath the restoration and prevent bacterial invasion, which would otherwise negatively affect pulp vitality. Strontium-containing BGs may also be used as filler materials in miniplates or screws for treatment of oral and maxillofacial fractures. Upon implantation, these BGs will degrade and resorb within a stipulated time frame, thus removing the need for a second surgery to remove the metal implants. BGs powders may be difficult to apply in a clinical setting, and BG fabrication into a three-dimensional (3D) shape may provide ease of use for the clinician. In this chapter, the effect of strontium-containing BGs in the form of BG discs will be assessed on dental pulp stem cells from both the permanent and deciduous teeth. The effect of surface topography and surface chemistry as well as ion release are therefore likely to influence cell behaviour.

4.1 Introduction

Mesenchymal stem cells (MSC) have been derived from embryonic stem cells (38) and isolated from various tissues including the bone marrow (39), cartilage (32), and skin (31). The isolation of these cells, for example from bone marrow, poses challenges. Special attention is needed to identify suitable donor sites for obtaining an adequate number of cells, and the surgical procedure may cause infection or trauma (67). The use of ES cells can expose the researchers to ethical issues while factors such as the disruption or deviation in ES cells differentiation may lead to impairment of organ development upon implantation and also teratoma formation (38, 368). This encouraged the search for other types of stem cells as the fields of

cellular and molecular biology advanced (63). The discovery of dental stem cells allows innovative therapeutic strategies to enhance oral and maxillofacial tissues regeneration (63). Human stem cells from the tooth were first isolated by Gronthos *et al.* (3, 4), which was followed by other types of stem cells from the tooth and its surroundings, such as stem cells from the deciduous tooth (SHED) (59), periodontal ligament stem cells (PDLSC) (7), dental follicular stem cells (DFSC) (60, 61), stem cells from the apical papilla (SCAP) (5), and also side populations of cells within the dental pulp itself (369). The availability of various types of dental stem cells generates opportunities for their use to regenerate oral and maxillofacial tissue (reviewed in (327)) (Figure 4.1).

The tooth developmental process has been reviewed at length (69, 370-372). In short, tooth formation is complex (69) and unique, involving the interaction of epithelial and mesenchymal stem cells (370, 373). These mesenchymal stem cells originate from the neural crest, which migrated at length to colonize the branchial arches, a process that has been studied mostly in mouse and chick embryos (371). There are six branchial arches that are involved in the formation of the structures in the head and neck region, such as the face, mouth and teeth, with the first branchial arch involved in tooth formation (371). Thickening of the dental lamina occurs at day 11.5 in the embryonic mouse, which later grows into the underlying mesenchyme of the first branchial arch to form epithelial tooth buds at day 13.5. These mesenchymal cells accumulate around the epithelial bud and undergo changes to become dental papilla, which later gives rise to the dental pulp and odontoblasts (372). At day 14.5, the epithelial compartment undergoes specific folding to form a cap, and changes its shape from cap-like to bell-shaped at day 15.5, which eventually gives rise to ameloblasts and the deposition of enamel matrix. This then triggers the underlying undifferentiated mesenchymal stem cells within the dental pulp to lay down dentin matrix (372).

Beginning as early as five months in an infant when the root formation is about two-thirds complete, the tooth will erupt inside the oral cavity. Root formation is essential for the tooth to function properly inside the oral cavity (41), and continues for up to three years within the jaw bone. The cells involved in the root formation are more embryonic-like than other sources of dental stem cells (41). The tooth is situated

within the maxillary and mandibular jaw bones, and is enclosed by the periodontal ligament and cementum, separating it from the jaw bones (374). The enamel layer that protects the tooth consists of apatite crystals of calcium and phosphate (reviewed in (370)), which become harder (41) as the tooth erupts. Dentin is situated beneath enamel, forming the main part of tooth, while soft dental pulp forms the most inner layer (370). The dental pulp protected by the dentin is further divided into four layers. The external layer is made up of odontoblasts that produce dentin. The second layer is known as the “cell-free zone”, which is devoid of cells but rich in extracellular matrix (67). The third layer is called the “cell-rich zone”, which has many progenitor cells, and the innermost layer is composed of the vascular area and the nervous plexus (67).

The enamel layer of the human tooth cannot renew once it has erupted into the oral cavity (375). Therefore, once it is lost or destroyed due to dental caries, the only available alternative to save the tooth is to place a suitable dental material into the tooth cavity. A tooth cannot function properly without the presence of a viable root (5). During the tooth eruption process, the crown (the part of a tooth that is visible in the oral cavity) will penetrate the gingival tissue and move up from within the jaw bone to enter the oral cavity (61). Once the tooth is present in the oral cavity, it is exposed to saliva containing ions, lipids, polysaccharides and proteins (376). This oral environment is suitable to study biomaterial interactions for use in dentistry (reviewed in (377)).

The role of strontium to prevent caries has received much attention. Strontium prevents intracellular polysaccharide accumulation in bacteria, which is involved in acid production (378). For example, strontium combined with chlorhexidine and fluoride gels prevented both the pH of the solution from dropping and the softening of enamel when compared to fluoride gels alone during bacterial fermentation on an enamel surface (379). Its addition to dental composites helps to increase radiopacity, which is beneficial for comparisons between tooth and dental materials during radiographic assessment (380).

Composites containing strontium and barium aged in artificial saliva for 4 and 8 months resulted in a higher release of ions from the composite surface compared to

leaching in air, suggesting the leaching process occurred at the surface of the composite samples (380). Surface leaching may provide additional benefits. Strontium ions leached from the composite create a charge imbalance, which leads other ions present in the saliva to counteract this effect and charge balance the environment. Strontium may also bind with existing calcium or fluoride ions to trigger surface crystallisation on the composite and prevent acidic degradation (377, 380). The combination of strontium and fluoride action on caries prevention depends on the correct concentration of each. A low level of fluoride and strontium were beneficial for caries prevention, where 10 ppm strontium worked better with 0.05 ppm fluoride compared to 20 ppm strontium with 0.1 or 1.0 ppm fluoride (381).

The main part of the tooth is composed of inorganic hydroxyapatite, which may dissolve following a severe acid attack and lead to the dissolution of minerals from the tooth to result in cavity formation. The substituted calcium hydroxyapatite ($\text{Ca}_5\text{OH}(\text{PO}_4)_3$) with different ions, including fluoride, sodium and magnesium, for hydroxyl affects the apatite behaviour by making the tooth tougher to acid dissolution (reviewed in (382)). Other chemical agents have also been investigated as caries preventive modalities, including aluminium, strontium or combinations thereof (383). This was originally based on the ionic elements found in human enamel and dentine, dental plaque and drinking water (384, 385), and the strontium level was used as a measure of caries susceptibility. The strontium concentration in water (5 to 6 mg/l) showed the lowest mean decayed, missing, and filled tooth score in children aged 12 to 14 years old suggesting the potential benefit of strontium and fluoride combined to prevent caries formation (384). Strontium may also exert its effect in reducing caries prevalence in areas with low fluoride (386). The strontium concentration was higher in healthy teeth compared to carious teeth, suggesting that naturally occurring strontium helps to reduce caries formation. There are probably several mechanisms of action (93). The similarities between the strontium and calcium ionic radius may enhance strontium ion substitution with calcium, which increases the hydroxyapatite crystal size and makes the tooth less susceptible to acid attack to prevent cavity formation on the surface (93).

The enamel and dentine inorganic component is calcium-deficient and has a carbonate apatite phase, making it vulnerable to acid attack following carbonate

substitution with hydroxyl ions in the crystal lattice when compared to hydroxyapatite (HA) samples (387). It is postulated that in the presence of fluoride, strontium eluted from the teeth is lower because fluoride prevents strontium dissolution (388). Solutions containing strontium, aluminium and fluoride with or without combinations of each ion were prepared and cultured with pre-softened bovine enamel. Results showed that the strontium and fluoride group had the highest acid resistance compared to fluoride either alone or with aluminium, suggesting their combination may be suitable to prevent acid attacks on teeth (383). Strontium combined with chlorhexidine and fluoride gels also helps to reduce acid attacks by preventing the pH from dropping on enamel, and further prevents enamel demineralisation *in vitro* depending on the strontium concentration (379). Overall, strontium has great potential to be used in regenerative dentistry. Thus, in this preliminary study, the effect of strontium-containing BG discs on dental stem cell metabolic activity was assessed.

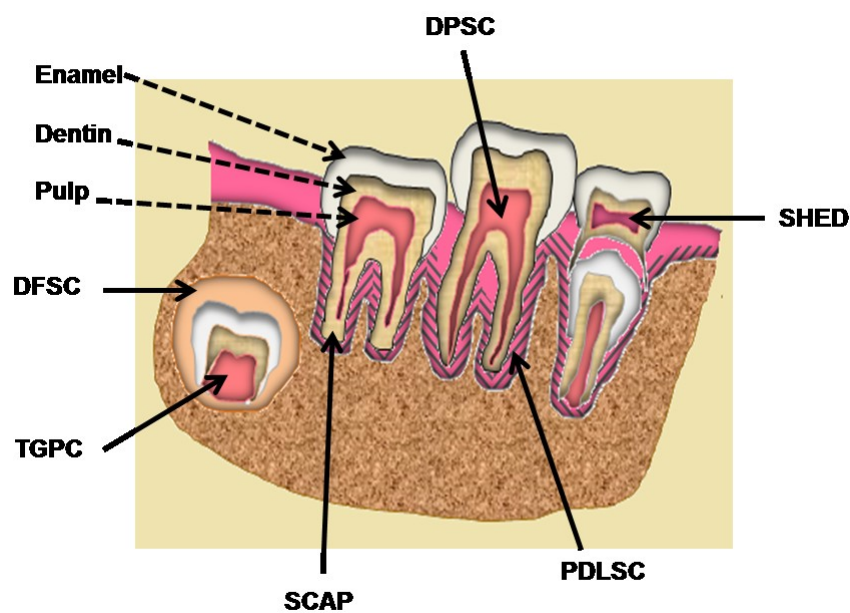


Fig. 4.1: The types of stem cells that can be obtained from a tooth. DPSCs: dental pulp stem cells; SHED: stem cells from human exfoliated deciduous teeth; PDLSCs: periodontal ligament stem cells; DFSCs: dental follicle stem cells; TGPCs: tooth germ progenitor cells; SCAP: stem cells from the apical papilla. Adapted from (389).

Dental stem cells are able to differentiate into osteogenic, chondrogenic, adipogenic, neurogenic, and myogenic lineages while maintaining their proliferative capacity (5, 9, 59). The gene expression profile of dental stem cells has been explored (390), but their propensity towards specific lineages remains poorly understood (391). Dental stem cells are known to possess therapeutic potential (63, 392), including their use in cell-based therapies for neurodegenerative and cardiac disease (393, 394). Studies have also shown the potential of dental stem cells to be differentiated into other tissues. Combined with their continuous availability, there is strong evidence that dental stem cells can be a source of stem cells for numerous applications (7, 9). The generation of induced pluripotent stem cells (iPS) from dental tissues created promise that dental stem cells can also be used for a wide range of regenerative and therapeutic purposes (62, 395).

Many studies of dental stem cells seeded on polymer scaffolds or natural tooth for cell-based therapies have shown promising results. The development of injectable materials for dental pulp tissue engineering and non-scaffold based therapy using dental stem cells for regeneration of oral and dental tissues is still in its infancy although it is gaining much interest (4, 63, 392).

Studies on the therapeutic potential of biomaterials in terms of dissolution ions from bioactive glass, especially on dental stem cells, are scarce. Once a tooth structure is lost, for example during cavity formation, pulp tissue is exposed to outside toxins that can penetrate through the dentinal tubules, which may kill the pulp. Thus, the search for a material that is capable of protecting the outer structures of the tooth and at the same time allows repair of pulp tissue is on-going. Hence, the use of bioactive glass (BG) that allows the underlying undifferentiated cells within the pulp tissue to differentiate into odontoblast cells and lay down tertiary dentine (396) for dentinal and pulpal repair is very much sought.

BGs have been used widely in the field of oral and maxillofacial surgery, including applications in middle ear prostheses (321), ridge augmentation (10), alveolar regeneration of periodontal pockets (16), and dentin hypersensitivity (17). Most of these studies use the original composition with slight modifications. However, the demand for biomaterials with potential to regenerate dental tissue creates

opportunities for researchers to develop BG with various compositions modifications to cater to specific needs. To date, scarce information can be found on the use of strontium-containing BG for dental treatment or regenerative dentistry.

Metal implants have also been widely used in prosthodontics and maxillofacial surgery (14, 397), but implant failures have been reported mainly due to failure of bonding between alveolar bone and implant, and also infection surrounding the implant site (15). Hydroxyapatite (HA) and BG coating (398), calcium phosphate (CaP) (14), and titanium web mesh (399) were used to provide additional adherence of the implants to bone. As proposed by Hench, the BG degradation leads to the formation of a hydroxyl-carbonate apatite (HCA) layer (278, 400), and thus using BGs as coatings for metal implants is a promising method to improve osseointegration. Implants coated with BG and HA resulted in a higher volume of bone formation compared to HA coated and non-coated implants (398), revealing another benefit of using BG as coatings for dental implants.

BGs have several advantages related to their dissolution products. They up-regulated the expression of genes related to osteogenesis and stimulated human osteoblast proliferation (129). Foetal osteoblast cells cultured in BG-conditioned medium without the addition of osteogenic supplements were able to form mineralised bone nodules, and had increased ALP, osteonectin and bone sialoprotein gene expression (297). Osteoblasts treated with strontium-containing BG-conditioned medium had increased metabolic activity (22). Recently, strontium-containing borosilicate (Sr-BS) and calcium silicate (Sr-CS) was compared with Sr-substituted hydroxyl-apatite (Sr α -HA) (0, 10, 40, and 100 mole % substituted HA) in terms of structure, composition, degradation behaviour, and biological responses. There were no phase or structural changes detected in all samples of Sr-HA, Sr-BS and Sr-CS, and the 10 mole % of strontium-substituted hydroxyl-apatite (Sr10-HA) sample showed the highest cell proliferation rate throughout the seven-day incubation period when compared with the HA samples (303). The 10SrBG that is used in this study was previously characterised (287) and has the potential to be used as a bone substitute (22). It was shown that the substitution of strontium with calcium in the bioactive glasses did not produce any cytotoxic effects on Saos-2

osteosarcoma cells, and the cells had normal attachment and morphology throughout the study period (21).

The promising results from chapter 3 provided the motivation to test the strontium-containing BG on dental stem cell viability. In this chapter, the isolation and characterisation of dental stem cells is described and the methodologies involved are discussed. Immunohistochemistry is used to confirm their presence within the dental pulp. The proliferation rate was measured, and initial assessment of the response of dental stem cells to strontium-containing BG disc was performed by an Alamar Blue (AB) assay.

4.2 Materials and methods

4.2.1 Isolation and culture of dental stem cells from pulp

The permanent tooth samples were selected from healthy adult patients undergoing dental surgery for third molar removal or tooth extraction for orthodontic and periodontal reasons. The deciduous tooth was collected from healthy patients with tooth mobility due to the exfoliation process. All teeth were collected from patients with ages ranging from six to 40 years who attended the oral health clinic at the Advanced Medical and Dental Institute following ethical approval from the Internal Review Board of the Universiti Sains Malaysia (USM). The teeth used in this study are normally discarded as clinical waste (63), and were obtained following informed consent.

4.2.2 Isolation and culture of dental stem cells from pulp

The tooth surface was cleaned and any remaining periodontal ligament attached to it was removed. The tooth was sectioned at the cemento-enamel junction (CEJ) using a standard high-speed dental-cutting unit with copious water irrigation to separate the crown from the root, and then was briefly immersed in 75% ethanol followed by soaking in phosphate-buffered saline (PBS) (Sigma Aldrich, UK) before the retrieval of pulp tissue using a sterile barbed roach (Dentsply, USA) (Fig. 4.2). The pulp tissue was digested in 3 mg/ml collagenase (Gibco) and 4 mg/ml dispase (Gibco) for 1 hour at 37°C and 5% CO₂ in an incubator. Single-cell suspensions were obtained by passing the cells through a 70 µm strainer (BD Falcon, USA), and the cells were

cultured in a 25 cm² flask (Nunc, USA) at 37°C and 5% CO₂ in Alpha Modification of Eagle's Medium (αMEM, Gibco) supplemented with 20% (v/v) foetal bovine serum (FBS), 100 μM L-ascorbic acid 2-phosphate, 2 mM L-glutamine and 1% (v/v) penicillin/streptomycin (4). After a 24-hour incubation period, the cells were washed twice in Dulbecco's phosphate-buffered saline (DPBS) (Gibco) to remove non-adherent cells. The medium was changed every 2 to 3 days. DPSC and SHED at passages three to seven were used in this study. The stem cell lineage of dental stem cells was confirmed by using immunohistochemistry with antibodies specific for stem cell markers. Human mesenchymal stem cells (hMSC) were purchased from Lonza (Cambrex, USA) and grown in mesenchymal stem cell basal medium kit (MSCBM) (Lonza Cambrex, USA) supplemented with growth factors and antibiotics according to the manufacturer's instructions. Human breast cancer cells (MCF7) were purchased from American Type Culture Collection (ATCC, USA) and grown in Eagle's minimum essential medium (EMEM) (ATCC, USA) supplemented with growth factors and antibiotics according to manufacturer's instructions. All images were captured using an inverted microscope (DigiAcquis 2.0 Aquisition Software, MatrixOptics, Japan).

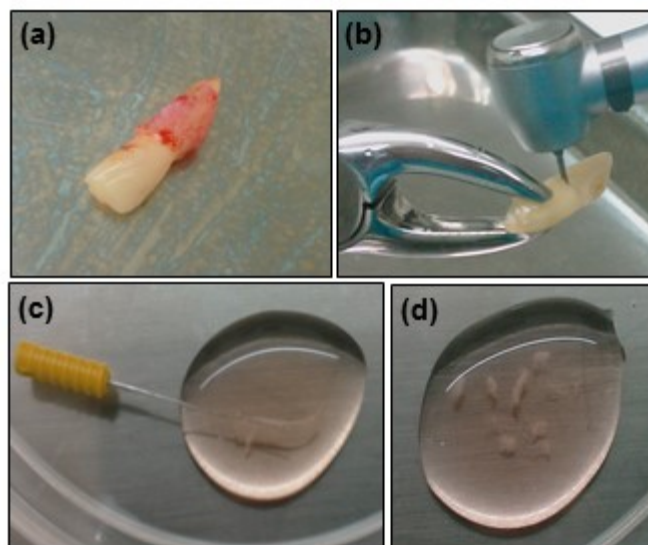


Fig. 4.2: Photographs showing the canine tooth from the lower right molar before (a) and during (b) sectioning. The pulp tissue is removed using a barbed roach (c) and is minced (d) prior to culture in digestive solution.

4.2.3 Characterisation of dental stem cells using immunohistochemistry

Immunostaining was performed using by biotin-horseradish peroxidase (HRP) complexed antibodies to detect the primary antibody. Briefly, DPSC, SHED, hMSC (serving as a positive control), and MCF7 (serving as a negative control), were subcultured into 4-chamber slides (Nunc, Denmark) at a density of 4×10^4 cells/well. After the cells were 80-90% confluent, they were fixed in 4% (v/v) paraformaldehyde (Merck, Germany) and incubated at 4°C for 20 minutes. Blocking reagent was then added for 10 minutes, and the cells were incubated with one of monoclonal mouse anti-human Endoglin (CD105: Thermo Scientific, USA) with dilution 1:100, monoclonal mouse anti human ALCAM (CD166: Epitomics, USA) with dilution 1:100, STRO-1 (R&D Systems, USA) with dilution of 1:100, or negative control isotype IgG (BD BioSciences, USA) overnight. Primary antibodies were then detected using a kit (UltraVision secondary detection system Anti-Polyvalent HRP/DAB, Thermo Scientific, USA) according to the manufacturer's protocol.

4.2.4 Colony-forming assay

Single-cell suspensions of day zero (passage zero) cells were seeded at 1×10^4 cells/well in 6-well plates (Nunc, USA) and grown in α MEM (Gibco) supplemented with 20% (v/v) fetal bovine serum (FBS), 100 μ M L-ascorbic acid 2-phosphate, 2 mM L-glutamine, 1% (v/v) penicillin/streptomycin (4), and incubated at 37°C in 5% CO₂ until day 14. The medium was changed every 2-3 days. At day 14, the cells were fixed with 4% (v/v) paraformaldehyde (Merck, Germany) and stained with 0.1% (w/v) toluidine blue (Fine, UK). Cell aggregates of ≥ 50 cells were scored as colonies.

4.2.5 Alamar Blue (AB) assay

4.2.5.1 AB assay for DPSC and SHED

Once DPSC and SHED were 80-90% confluent, approximately 2×10^4 cells/well were subcultured in 24-well plates in triplicate in growth medium at 37°C and 5% CO₂. After 24 hours, the medium was replenished. Approximately, 10% (v/v) of metabolic indicator dye AB (Biosource International, Inc, USA) was added to each well and to the culture medium without cells (serving as the negative control), and then further incubated for four hours. Approximately 100 μ l of the solution from each well was then transferred to a black Costar 96-well plate. The fluorescence was detected

using a microplate reader (FLUOstar Omega, BMG Labtech) at excitation wavelength of 544 nm and emission at 590 nm. The medium was subsequently changed and the process of AB staining and fluorescence readings was repeated for the time points of 1, 3, 7, 14 and 21 days.

4.2.5.2 AB assay for cell seeding on bioactive glass disc

The BG discs were sterilized using ultra violet (UV) exposure for 2 hours per side. The discs were passivated with culture medium inside a 24-well plate in triplicates for 12 hours in an incubator (Binder, Thermo Forma, USA) at 37°C with 5% CO₂. The soaking process or passivation of the discs into the culture medium helped to wet the disc surface for cell attachment. DPSC and SHED cell suspensions of approximately 2×10^4 cells suspension in 100 µl were seeded at the centre of the discs, and the discs were further incubated for 1 hour to allow cell attachment (306). 1 ml of growth media was added to each well of the plate, which was incubated for another 18 hours followed by the addition of 10% (v/v) metabolic indicator dye Alamar Blue (AB, Biosource International, Inc, USA) to each well containing the discs, and a further four hours incubation. Approximately, 100 µl of the culture medium from each well was transferred to a black Costar 96-well plate. The fluorescence was detected using microplate reader (FLUOstar Omega, BMG Labtech) with an excitation wavelength of 544 nm and emission at 590 nm. The percentage of AB reduction was calculated using the manufacturer's protocol. Subsequently, the discs were washed twice in PBS, the medium was changed, and AB staining and fluorescence readings were repeated for the time points of 1, 4, 7, 14 and 21 days. Control glass slides (Thermanox, Nunc, USA) were used as a control.

4.2.6 Preparation of bioactive glass disc

Bioactive glass with 10 mole % of the calcium was substituted with strontium (10SrBG) was prepared (Table 4.1) and all of the contents were placed in a 300 ml platinum crucible. The powders were melted for 1 hour at 1450°C in an electric furnace. The glass melt was poured into a graphite mould that was pre-heated to just above the glass transition temperature (Table 4.2). Glass rods of 10 mm in diameter were obtained and discs of 1.0 mm thickness were cut from the rod using a low speed diamond cutter (Isomet 1000, Buehler, Germany) with isopropanol as a coolant.

Table 4.1: The composition of the glasses studied.

	Oxide	SiO₂	Na₂O	CaO	SrO	P₂O₅
Weight %	45S5	45.00	16.64	24.50	0.00	13.86
	10SrBG	44.09	16.30	21.60	4.43	13.58
Mole %	45S5	48.67	17.45	28.39	0.00	5.49
	10SrBG	48.67	17.45	25.55	2.84	5.49

Table 4.2: The glass melting temperature. Adapted from (21).

Glass	Glass transition temperature (°C) T_g	Onset of crystallisation temperature (°C) T_x
45S5	538	677
10SrBG	525	674

4.2.7 Scanning electron microscopy (SEM) of dental stem cells

Scanning electron microscopy (SEM) was used to observe cell morphology and attachment on the BG discs. The BG discs with cells attached were rinsed twice with PBS and fixed in 2.5% (v/v) glutaraldehyde (Merck, Germany) in 0.1 M phosphate buffer solution (PBS, Sigma Aldrich, UK) for 4 hours at 4°C. The BG discs were washed three times in PBS, and then dehydrated through a series of increasing ethanol concentrations starting from 30%, 50%, 70%, 90%, 95% and twice in 99.9% (v/v) for 10 minutes each. The specimens were dried at ambient temperature and sputter-coated with gold-palladium at a ratio of 80:20 and examined by SEM at a voltage of 3kV and working distance of 6 mm (VPSEM SUPRA 35VP).

4.2.8 Statistical analysis

The data for the experiments are expressed as mean $\pm$ standard deviation (SD). Each experiments were performed at least twice, and prepared in $N=3$ for each composition at a designated intervals. The data were analysed using SPSS version 20.0 (IBM, USA) using one-way analysis of variance (ANOVA) with a post-hoc test (Bonferroni procedure) with p -values < 0.05 considered statistically significant.

4.3 Results and discussion

4.3.1 Isolation and characterisation of dental stem cells

The dental pulp from a tooth, a primary tissue source, has to undergo a digestion process in order to break up the tissue prior to the retrieval of dental stem cells. Both DPSC and SHED were isolated using the enzyme digestion method with collagenase and dispase. A study conducted comparing the biological behaviour of deciduous teeth stem cells (DTSCs) isolated either by enzymatic dissociation (ED) or by a direct outgrowth method from pulp tissue explants (OG) showed that DTSC-ED cultures display significant heterogeneity comprising different cell sizes and morphologies even within the same colonies, whereas the DTSC-OG cultures have more uniform migration of fibroblast-like cells (8). No difference could be detected in the growth rates of these two types of cultures. In our study, the enzyme digestion method was chosen to isolate the dental stem cells because the ED method was previously shown to yield a higher number of stem/progenitor cells (8). Single cell suspensions from the dental pulp were obtained after digestion and after passing through a strainer. It was shown that different sizes of strainer or sieves used to obtain the single cell suspension may result in the isolation of different cell populations (401). Smaller sized strainers allows a higher yield of small-sized stem cell populations, and cell strainers with sizes of 3, 20, and 70 μm are commonly used to isolate dental stem cells. Our study used a 70 μm strainer in order to yield the single cell suspensions as previously reported (4). The DPSC and SHED primary cultures (passage 0, Fig. 4.3) were 80-90% confluent within 7 to 12 days of incubation and they were passaged at a ratio of 1:3. After 24 hours of incubation, both DPSC and SHED attached to the flask surface (day 1). At day 7, the cells started to form colonies, and at day 11, the colonies grew bigger, and became interconnected with one another. SHED exhibited faster colony formation than DPSC as the number of passages increased from two to five.

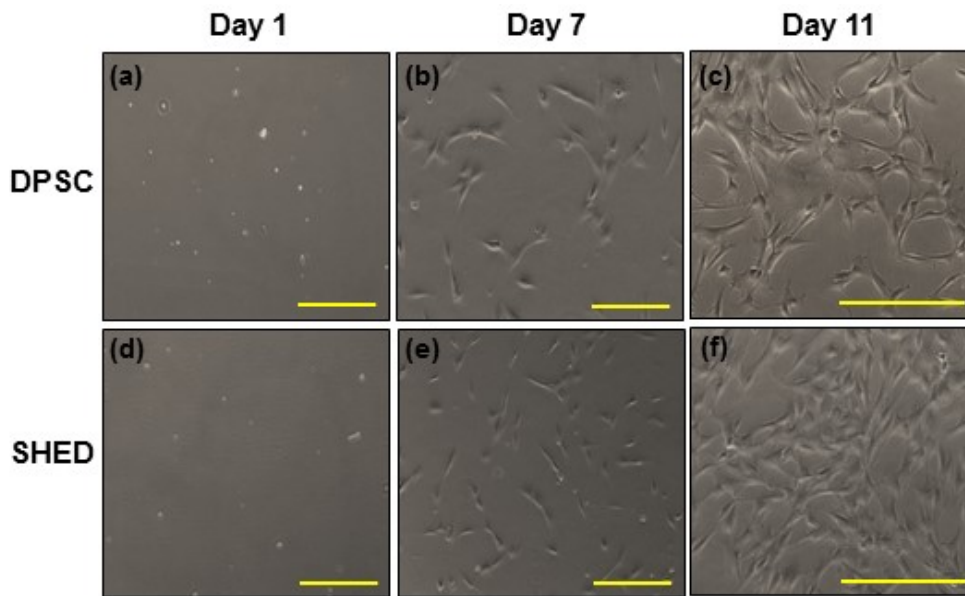


Fig. 4.3: Bright field microscopy images showing DPSC at day 1 passage 0 (a), day 7 passage 0 (b), and day 11 passage 0 (c). SHED are shown at day 1 passage 0 (d), day 7 passage 0 (e), and at day 11 passage 0 (f). Scale bar is 200 μm .

The characterisation of mesenchymal stem cells can be done using various techniques, including immunohistochemistry, immunocytochemistry, Western blotting, flow cytometry and polymerase chain reaction. Cell identification and characterisation is commonly done using cluster of differentiation (CD) markers. CD markers are surface proteins such as integrins, glycoproteins, and other molecules that can be detected using antibodies (402). The identification of mesenchymal stem cells (MSC) must meet certain criteria as proposed by the International Society for Cellular Therapy (ISCT). The isolated MSC must show adherence to plastic surfaces and specific antigen expression. The MSC must express more than 95% CD105, CD73, and CD90 measured by flow cytometry, and show multi-potential differentiation towards osteoblasts, adipocytes, and chondrogenic lineages in standard *in vitro* culture media (403).

The presence of stem cells in the dental pulp cultures from the permanent and deciduous teeth was confirmed with immunohistochemistry for mesenchymal stem cell using antibodies against human endoglin CD105 (Thermo Scientific, USA), ALCAM CD166 (Epitomics, USA) and STRO-1 (R&D Systems). These antibodies were chosen since they are commonly used to identify the MSC population. It

remains a challenge to identify specific markers for dental stem cells from the tooth, so markers normally used to identify MSC were chosen for the identification of dental stem cells. Many studies have shown that DPSC and BMSC have similar expression of cell surface markers commonly used in MSC characterisation (404). The monoclonal antibody (mAb) against CD105 (endoglin), a type I transmembrane protein, is characterised under the term SN6 from a hybridoma primary culture (405). This mAb was previously tested on both healthy and leukaemic human peripheral blood cells and it was shown that it did not react with the healthy cells (405), thus suggesting that cells expressing CD105 may be related to stem cells with a mesenchymal origin. CD166, is also known as activated leukocyte adhesion molecule (ALCAM), and the SB-10 that is a mAb clone for CD166 was shown to react with an antigen present on the surface of undifferentiated human MSC *in vitro* (406). CD166/ALCAM is a type I membrane glycoprotein and is a member of the immunoglobulin superfamily (IgSF) of cell adhesion molecules (reviewed in (407)). CD166/ALCAM expression can be detected in various tissues. Cells expressing CD166/ALCAM are involved in the development and maintenance of tissue architecture, and possess dynamic growth, proliferation and/or migration capabilities (reviewed in (407)). It was detected on human MSC, and also in other species such as canine, rabbit and rat MSC (408). STRO-1 identifies a small subset of mesenchymal cells. In bone marrow stromal cells (BMSC), the BMSC expressing STRO-1 developed colonies, while BMSC that did not express STRO-1 failed to form colonies after two weeks in culture leading to the conclusion that STRO-1 is expressed by BMSC (409). STRO-1 was previously used to sort the population of stem cells within the rat dental pulp, and the results showed that the rat STRO-1 positive dental pulp stem cells are capable of differentiating into multiple lineages (410).

In this study, immunohistochemistry analysis revealed DPSC and SHED were positive for CD105, CD166 and STRO-1 (Fig. 4.4), suggesting that the dental pulp derived from both the permanent and deciduous teeth contains stem cells (3, 4, 59). These cells can be isolated in high numbers and expanded *ex vivo*, which provides a unique and accessible population of stem cells (59). The immunohistochemistry provided qualitative information for CD105, CD166 and STRO-1 expression. The identification of STRO-1 in BMSC using fixed and paraffin-embedded tissue may be

limited, since STRO-1 expression can also be found in the microvasculature of many tissues (411), and it is also expressed by all dental stem or precursor cells (reviewed in (375)).

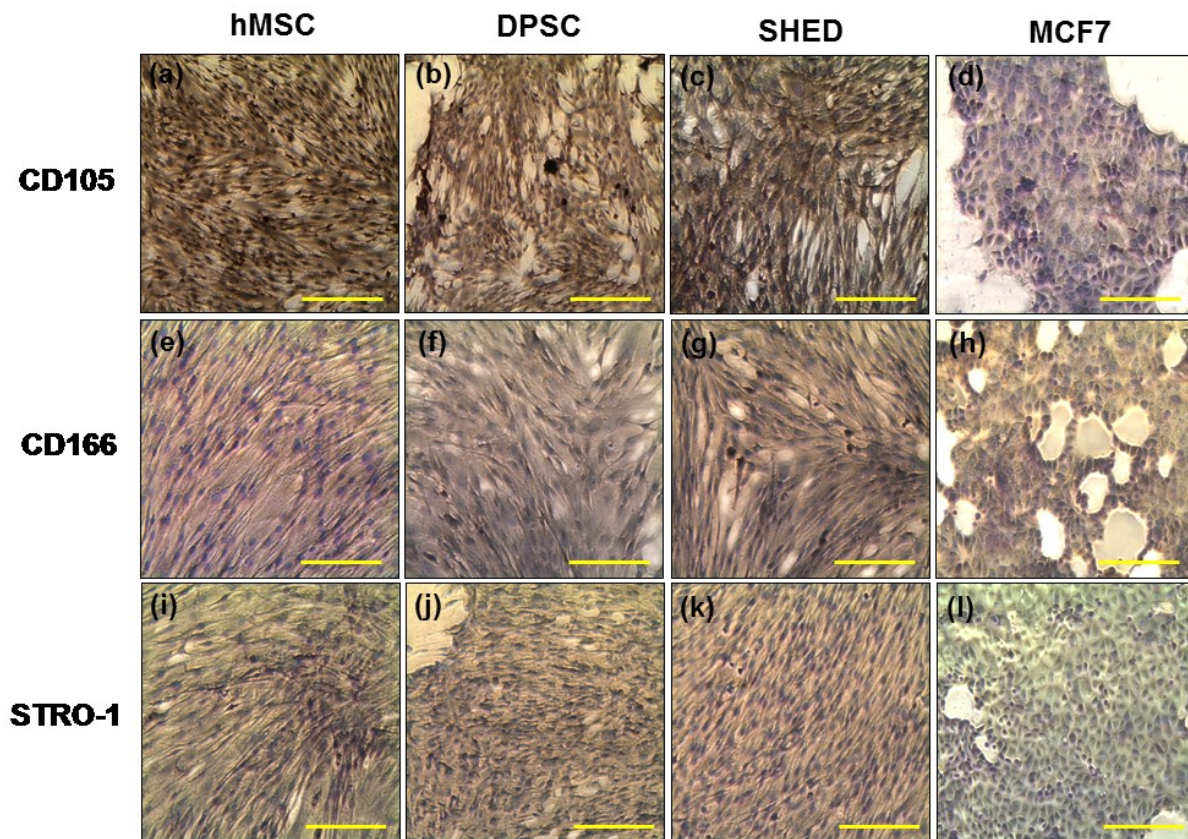


Fig. 4.4: Bright field microscopy images of plates showing immunohistochemistry for CD markers at day 5, with positive staining indicated by a brownish colour. hMSC (a, positive control), DPSC (b) and SHED (c,) expressed CD105, while no CD105 was detected in MCF7 (d), which served as the negative control. CD166 expression was detected on hMSC (e), DPSC (f) and SHED (g). No CD166 was detected on MCF7 (h). STRO-1 expression was detected on hMSC (i), DPSC (j) and SHED (k). No expression of STRO-1 was detected on MCF7 (l). Scale bar is 200 μm .

4.3.2 Dental stem cells colony-forming efficiency and cell metabolic activity

The colony-forming efficiencies of DPSC and SHED were assessed from primary cultures at passage zero after 14 days. Cell aggregates with ≥ 50 cells were scored as colonies (4). The assay was conducted to investigate the ability of dental stem cells to generate colony-forming units (CFU), which is normally observed in MSCs (2). The cells showed a typical fibroblastic-like morphology with both high and low density colonies as shown in Fig. 4.5. SHED cells possessed a higher frequency of colony-forming cells (17 colonies/ 10^4 cells plated, Fig. 4.6b) than DPSC (4 colonies/ 10^4 cells plated, Fig. 4.6a) when these cells were seeded at 1×10^4 cells/well. The higher CFU observed in SHED compared to DPSC is in accordance with a previously reported study (391). The higher number and size of the colonies observed in SHED compared to DPSC also suggested that SHED possessed higher proliferation capacity than DPSC (391).

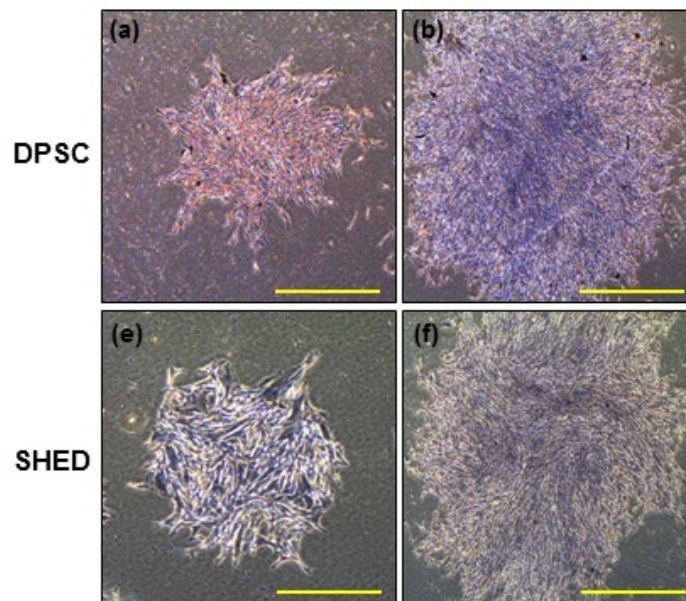


Fig. 4.5: The colony-forming efficiency of DPSC and SHED *in vitro*. Bright field microscopy images of high (b, DPSC and d, SHED) and also low (a, DPSC and c, SHED) density colonies after 14 days. Scale bar is 500 μm .

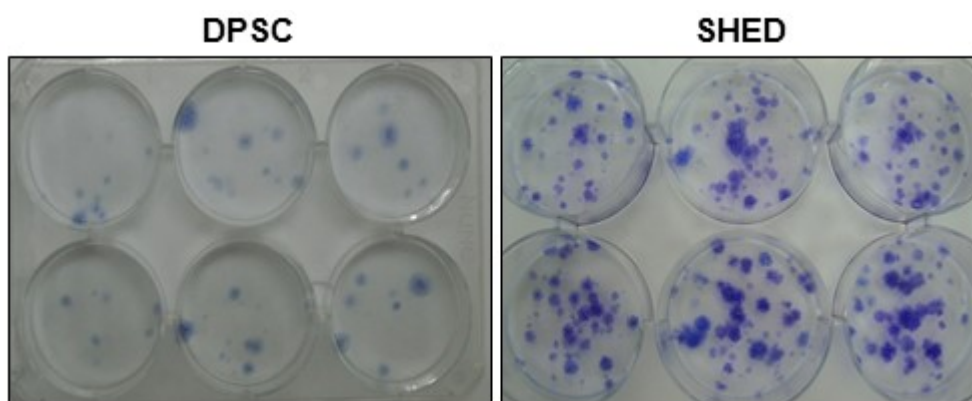


Fig. 4.6: Representative image showing the colony-forming ability of DPSC (a) and SHED (b) at passage 0 after incubation for 14 days. SHED exhibited higher colony-forming ability than DPSC.

The Alamar Blue (AB) cell viability assay (Biosource International, USA) was used to assess the metabolic activity of DPSC and SHED. At day 1, no significance difference was detected. However, at day 3 to day 14, SHED showed significantly higher metabolic activity compared to DPSC (Fig. 4.7). At day 21, there was a decrease in the DPSC and SHED metabolic activity.

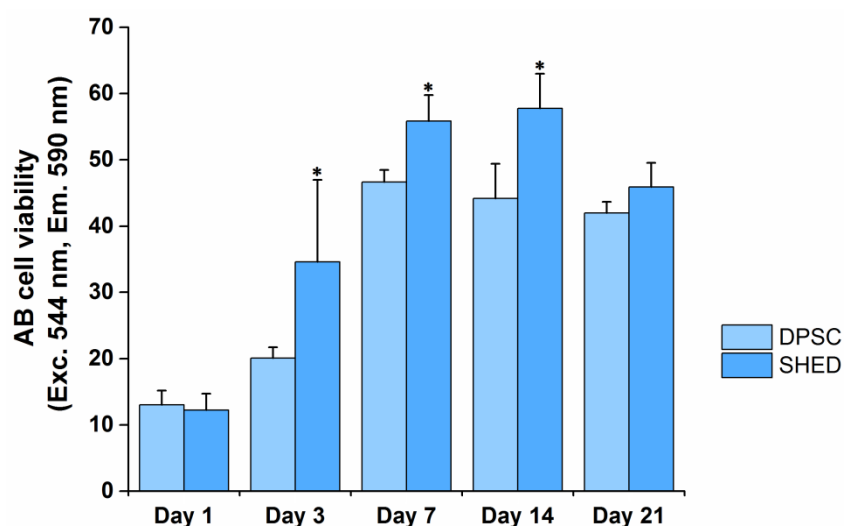


Fig. 4.7: The Alamar Blue assay. SHED had a higher metabolic cell activity than DPSC from day 3 to day 21. * denotes a significant difference of the marked bar compared to DPSC at the same time point.

During the study period, the number of DPSC and SHED seeded into the 24-well plate was kept constant at 2×10^4 cells/well, and cells at passage three were used for this assay to ensure that the metabolic activity measured was comparable with one another. Throughout the 21-day incubation period, except at day 1, SHED displayed a higher metabolic activity than DPSC, suggesting that SHED have higher proliferation capacity than DPSC as previously reported (391). This observation supports the notion that the stem cells present within the dental pulp of deciduous teeth may be 'immature' and possess multi-lineage potential (59). At day 21, the metabolic activity of SHED was significantly lower than day 14. This may be due to the higher number of cells present within the 24-well plate, which resulted in cell death due to the lack of nutrients. The lower SHED metabolic activity may also have occurred when they became too confluent, where they might have experienced contact inhibition and became senescent. This cellular senescence is known to occur in MSC (412). However, this finding needs to be investigated further. For longer incubation periods, the use of a larger plate or flask would be desirable. Furthermore, DPSC are from the permanent tooth (especially the third molar), which was the last tooth to develop post-natal, while SHED were harvested from the deciduous tooth that developed intra-uterine. Thus, SHED from the dental pulp of deciduous tooth may be immature in nature compared to DPSC, which could explain their high proliferative capacity (4, 59).

4.3.3 The strontium-containing BG effects on dental stem cells metabolic activity

The metabolic activity of DPSC and SHED was assessed following seeding on 10SrBG (bioactive glass with 10 mole % of the calcium was substituted with strontium) discs (DPSC-10SrBG and SHED-10SrBG). Both cell types were also seeded in the well plates directly (DPSC and SHED) and on control glass slides (Thermanox) to be used as a controls (DPSC-Thx and SHED-Thx).

For DPSC at day 4 and 7, cells cultured on 10SrBG discs (DPSC-10SrBG) showed significantly lower cell metabolic activity compared to DPSC cultured on Thermanox (DPSC-Thx). At day 14, the cell metabolic activity was similar in both groups. At day

21, DPSC cultured on 10SrBG discs showed slightly higher cell metabolic activity compared to DPSC seeded on Thermanox (Fig. 4.8a).

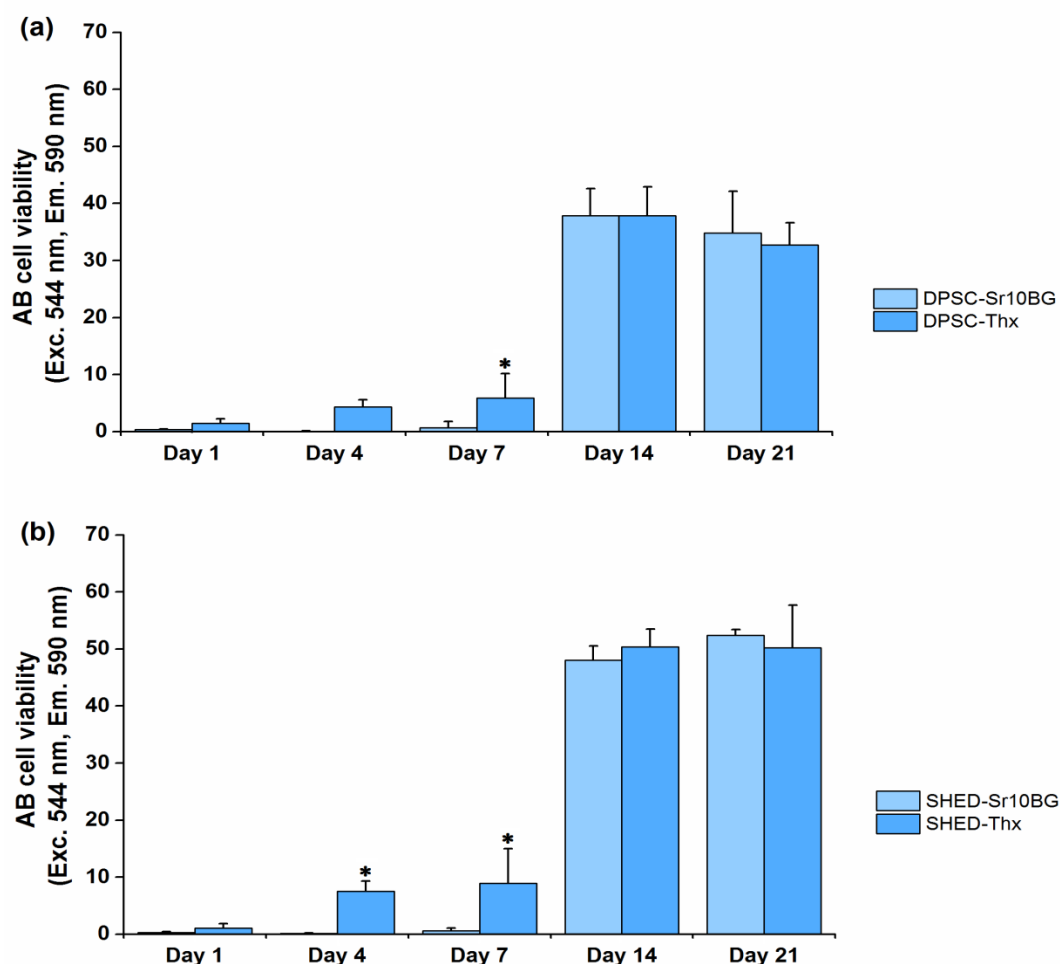


Fig. 4.8: The Alamar Blue assay. DPSC (a) and SHED (b). DPSC and SHED seeded on 10SrBG discs (DPSC-10SrBG, SHED-10SrBG) showed almost similar cell metabolic activity with cells seeded on control glass slide Thermanox (DPSC-Thx, SHED-Thx). * denotes a significant difference of the marked bar compared to 10 SrBG at the same time point.

For SHED, a similar trend to DPSC was observed. At day 4 and 7, metabolic activity was significantly higher on Thermanox (SHED-Thx) compared to SHED on 10SrBG (SHED-10SrBG). At day 14, the metabolic activity was similar in both conditions. At day 21, SHED cultured on 10SrBG discs (SHED-10SrBG) showed slightly higher metabolic activity compared to SHED on Thermanox (SHED-Thx) (Fig. 4.8b). The

findings of this preliminary assessment of DPSC and SHED seeded on the 10SrBG discs showed that strontium-containing BG may promote dental pulp stem cell growth on its surface and did not impair metabolic activity. At earlier time points (day 4 and 7), the metabolic activity on 10SrBG was lower than SHED on Thermanox. This observation may be due to the acclimatisation process for DPSC and SHED when cultured on the disc, or may occur due to fewer cells being adhered onto the disc surface or cell death during the first day. By day 21, cells cultured on the 10SrBG discs had the highest metabolic activity. However, this finding needs to be interpreted carefully. The metabolic activity may have already reached a plateau by day 14, so at day 21, it was lower because confluence was achieved. This is especially true for DPSC and SHED seeded on Thermanox. Nevertheless, the higher metabolic activity observed in DPSC and SHED at day 21 may indicate that the 10SrBG discs are biocompatible and support cell adhesion. This also suggests that strontium-containing BG with 10% strontium substitution for calcium may be a suitable material for the field of maxillofacial surgery and dentistry, and is also promising for the use in repair in oral and dental related tissues in cases that require longer healing and repair periods.

In this study, the BG discs were not preconditioned in culture medium as previously reported (22). The DPSC and SHED were exposed directly to the bursting ionic release from the BG discs, which resulted in lower proliferation during early days of culture. However, after 14 days of culture, DPSC and SHED seeded on the 10SrBG discs showed increasing cell metabolic activity. It was previously shown that the 45S5 Bioglass® conditioned medium without the addition of osteogenic supplements promoted osteoblast proliferation and nodule formation at day 14 and 21, suggesting that BG on its own has great potential as a bone substitute (297). Human dental pulp stromal cells seeded on a 45S5 Bioglass® 3D scaffold produced by a polymeric sponge method were viable and proliferating, and underwent osteogenic differentiation which was marked by higher Runx2 and osteocalcin production (413).

An ideal scaffold for bone tissue engineering may act as a template for *in vitro* and *in vivo* bone growth, must be biocompatible, and able to bond to the host tissue (27). The scaffold used in this study was developed for use in the field of regenerative medicine with 10% strontium substitution within the glass network (10 mole % of the

calcium was substituted with strontium). It was shown that strontium-substituted bioactive glass is able to stimulate cell proliferation, especially at 10% strontium substitution, which suggests that mixed strontium and calcium (Sr/Ca) glasses may be effective for increasing cell proliferation (21). The addition of strontium into the glass structure was shown to accelerate the speed of ion dissolution within the culture media (22). Strontium substituted bioactive glass (BG) discs (0, 10, 50, and 100%) were previously used for Saos-2 seeding, and after 14 days of culture, the number of cells per disc was the highest on the 50 and 100% strontium substituted bioactive glass discs (22). Many studies have been conducted to determine the genetic basis of BG ionic dissolution products, and the commonly used cell types include primary human osteoblasts, foetal human osteoblasts, murine embryonic stem cells, human embryonic stem cells, and murine foetal long bone cells (reviewed in (290)). Dental stem cells can be induced into osteoblastic, chondrogenic, adipogenic, and neurogenic lineages (3, 4, 59). Thus, the use of dental stem cells to study the response of the strontium-containing BG is crucial for the development and application of the strontium-containing BG in the field of oral and maxillofacial dentistry and tooth tissue engineering.

The DPSC and SHED were cultured on 10SrBG discs for the specified time frame (day 1, 4, 7, 14 and 21) and Thermanox (Nunc, USA) was used as a control. Scanning electron microscopy revealed a well spread morphology of DPSC and SHED on the 10SrBG discs (Fig. 4.9 and 4.10), and there was an increase in the number of cells attached on the discs as the incubation period continued. DPSC and SHED seeded on Thermanox appeared elongated and flat.

In one study, hMSC seeded on 45S5 BG discs showed a rounded nodule formation with patchy distribution compared to hMSC seeded on tissue culture plastic controls, which appeared elongated and flattened (414). Surface morphology on the BG disc was shown to affect the cell attachment. Discs with rough surfaces promoted faster nodule formation, and an HA layer formed faster on rough surfaces compared to discs with smooth surfaces (415). The HA formation on the BG discs can be created by incubating the 45S5 discs in simulated body fluids (414). However, in our study, the HA formation on the 10SrBG discs surface was not done prior to the experiment. The discs prepared in our study were not subjected to polishing, and this may have

resulted in the rough surface promoting cell attachment. In our study, the properties of the ionic dissolution products from 10SrBG discs were not analysed. We have shown in Chapter 3 that 10% strontium substitution for calcium did not hamper Saos-2 cell metabolic activity (22). The calcium ions released from the disc may promote calcium phosphate (CaP) layer formation on the disc surface, which becomes an attraction site for cell attachment. It would be interesting to understand the behaviour of DPSC and SHED cultured on 10SrBG discs, and investigate the changes in the gene expression profile of these cells in the future. The comparison with other strontium-substituted bioactive glass may be conducted in the future for better understanding of the effects of different concentrations of strontium on stem cells from the dental pulp.

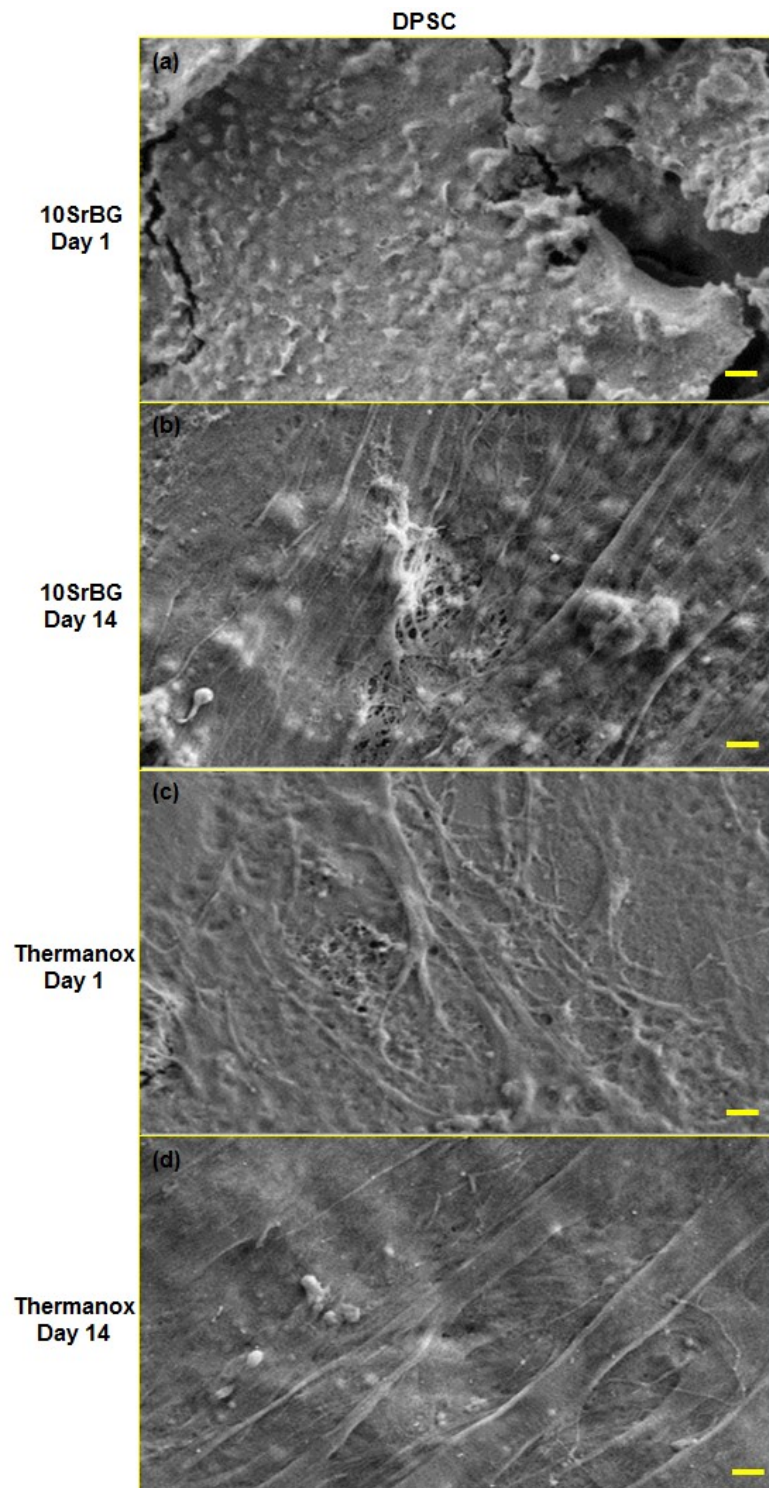


Fig. 4.9: Representative SEM micrographs showing DPSC attachment when cultured on the 10SrBG discs at days 1 and 14. At day 1 (a), there were minimal cells attached to the disc, and clumps of cells are observable at day 14 (b) with a few cells showing cytoplasmic extensions. DPSC cultured on Thermanox (c, d) were flatter compared to cells on 10SrBG disc. Scale bar is 10 μm .

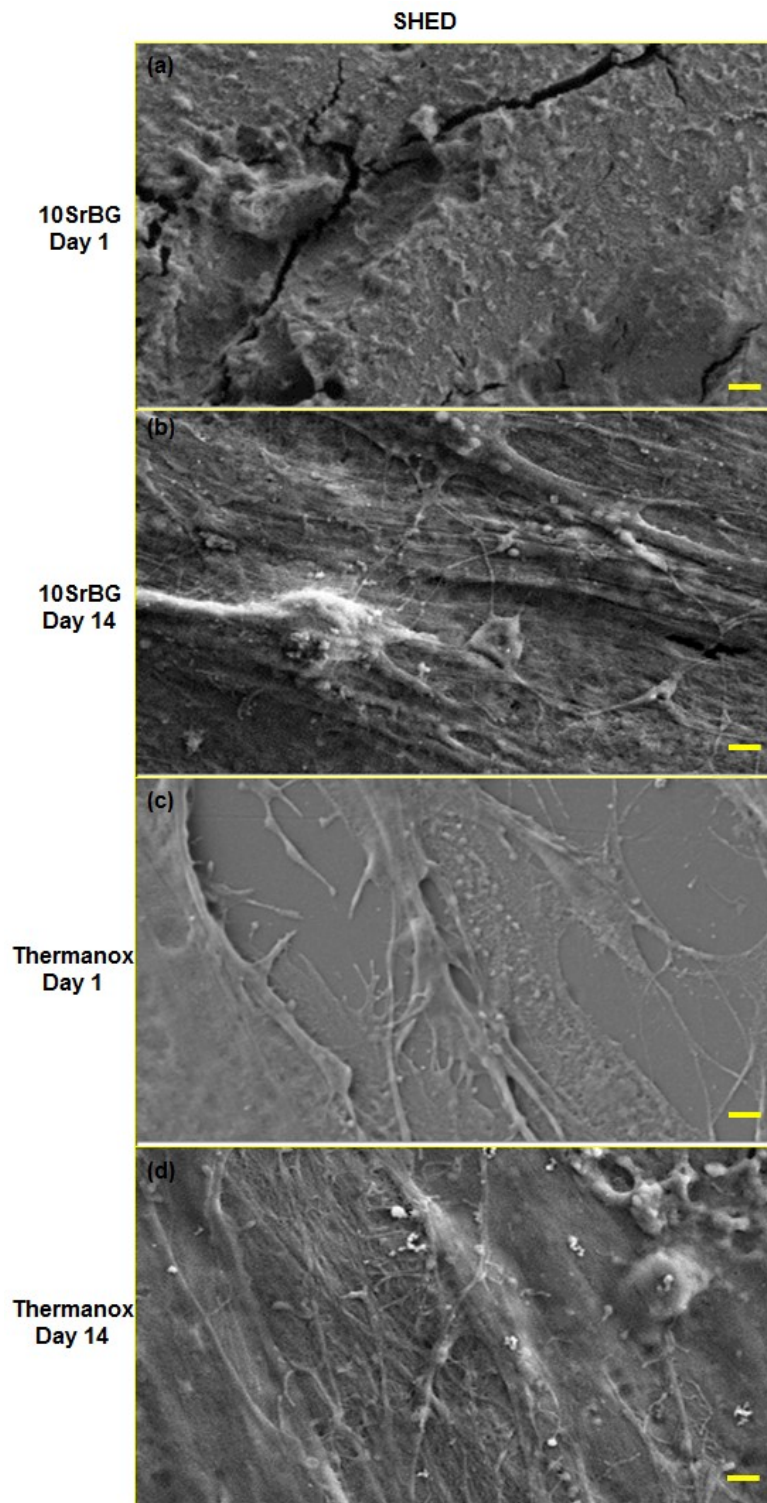
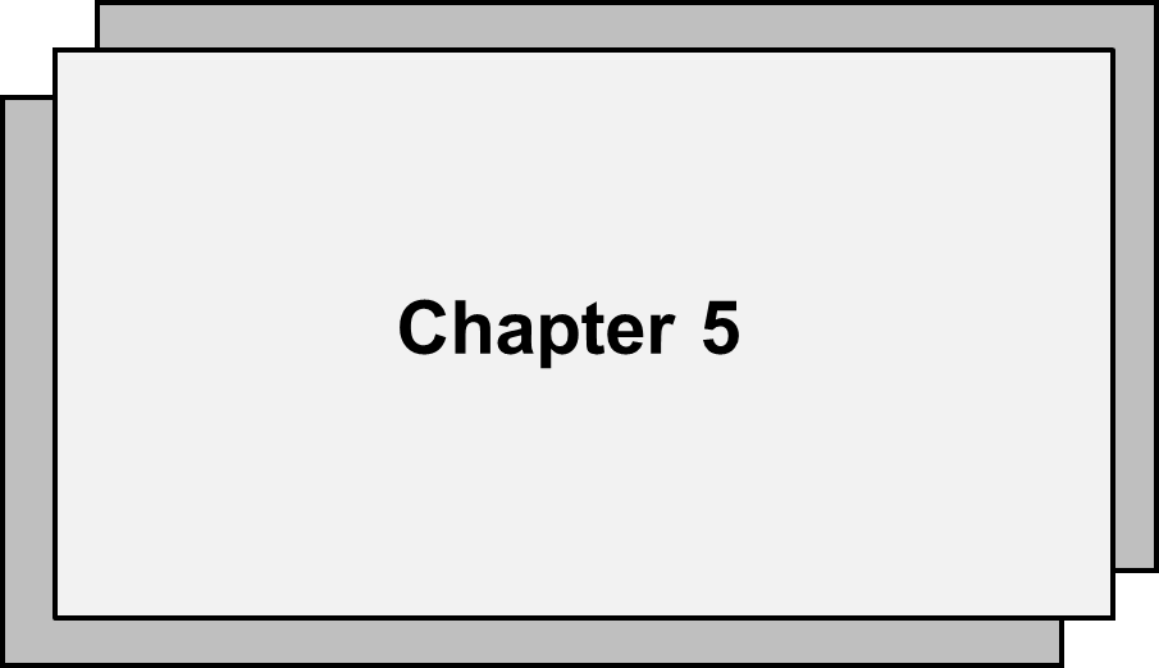


Fig. 4.10: Representative SEM micrographs showing SHED attachment when cultured on the 10SrBG discs at days 1 and 14. At day 1 (a), there were minimal cells attached to the disc, and cell clumps are observable at day 14 (b) with a few cells showing cytoplasmic extensions. SHED cultured on Thermanox (c, d) were flatter compared to cells on 10SrBG disc. Scale bar is 1 μm .

4.4 Conclusion

In the current project, tooth stem cells DPSC and SHED were isolated using an enzymatic digestion method with collagenase and dispase. The cells were subjected to immunohistochemistry analysis to confirm the presence of stem cells in the dental pulp. The positive expression of CD105, CD166 and STRO-1 confirmed the presence of stem cells within the dental pulp tissue (3, 4, 59). Based on the Alamar Blue assay, SHED had a higher proliferation rate than DPSC, and exhibited a higher colony-forming unit ability, which may be attributed to their immature nature in the dental pulp of deciduous teeth (59). The effect of strontium-containing BG was assessed on the dental pulp stem cells by seeding the cells on the 10SrBG discs (10 mole% of the calcium was substituted with strontium). DPSC and SHED showed higher proliferation rates when cultured on the 10SrBG disc compared to DPSC and SHED on Thermanox after 21 days of incubation. This suggests that strontium-substituted bioactive glasses have great potential for use in the field of maxillofacial surgery and dentistry for various applications, including as a caries-preventing agent (379, 381, 383) or through improving bone height in alveolar ridge augmentation (10).

Further investigations are needed to assess the effect of these dental stem cells on strontium-containing BGs. Comparison to other SrBG compositions are needed, and the effect of ionic dissolution from the discs must also be examined. The different types of media used to assess the dissolution profiles, such as simulated body fluid and culture medium, are also crucial to understand responses to the SrBGs. Future work should look into the effect of ionic dissolution profiles from the BG discs on the cells metabolic activity, with the use of positive controls such as hMSC. Flow cytometry should be used to isolate and characterise stem cells based on the expression of markers such as CD133 (multipotent progenitor cell), CD73 (common precursor cells), CD34 (hematopoietic stem progenitor), and CD45 (common leukocyte antigen).



Chapter 5

Chapter 5

Investigation of hypoxia-mimicking bioactive glass conditioned medium on dental pulp stem cells

Vascularised scaffolds play an important role in tooth tissue engineering and the regeneration of dental pulp. Pioneering research and new approaches are currently underway to overcome problems arising from dentistry based treatment and oral surgery. Tooth loss due to caries or dental trauma requires treatment to ensure the survival of the restored tooth. This is also the case for facial and bone reconstruction following major trauma, where patients are also affected by the high cost for treatment. Thus, strategies that uphold the concept of tissue regeneration in the repair and replacement of the lost tissue may help to decrease the financial burden (374) and provide new hope to patients.

5.1 Introduction

In regenerative dentistry, the applications of microscale technologies (reviewed in (374)) may help to ease some problems faced in treatment. From removable appliances, such as dentures, to fixed appliances, such as implants, these treatment modalities have their own problems that warrant an innovative approach for replacing missing teeth for the benefit of patients (42). Enamel and dentin provide protection to the dental pulp that contains nerves and blood vessels that enter the tooth through the apical foramen at the apex of the tooth (374). During cavity preparation on a tooth, a substantial number of reactions are taking place within the dental pulp itself, causing the undifferentiated progenitor cells to migrate from the centre of the pulp to the area of cavity preparation and differentiate into odontoblasts to form tertiary dentin and helped to protect the pulpal tissue from further damage (396). When the pulp is exposed to the oral cavity, as is the case for untreated caries or exposure of the pulp chamber, bacteria may enter through dentinal tubules, which may result in pulp death and lead to tooth abscess formation (reviewed in (416)). In some cases, this may be fatal if measures are not taken to overcome the problem (417, 418). Tooth structure does not regenerate (375), and if there is a cavity present on tooth, the remaining infected tooth structure will need to be removed before the

placement of dental restorative materials. Dental disease such as caries affects young children, especially those with prolonged bottle feeding, and negatively impacts their quality of life (48), and may extend into adulthood in the case of neglected oral hygiene (419). Elderly people with tooth loss may have a lower quality of life compared to those with teeth (420). Strategies that help to promote healing and repair in the dental pulp are crucial to ensure tooth vitality. Thus far, the quest is still on going for a material that would be able to replace complex tooth structures, involve the interplay between cell types such as ameloblasts or odontoblasts, and support the possibility of seeding these cells onto the scaffolds. The material should also allow healing and repair without compromising the blood and nutrient supply to the dental pulp (42).

Current strategies for regeneration and dental repair involve tissue engineering (TE) techniques using concept from developmental biology and wound repair (374). For example, the dental pulp nutritional supply relies solely on blood vessels that pass through the apical foramen and it is known that oxygen supply from blood vessels for cell survival is limited to 150-200 μm (33). Any imbalance between oxygen supply and demand may create a hypoxic condition, which signals the generation of angiogenic cytokines such as vascular endothelial growth factor (VEGF), which can activate signal transduction pathways resulting in the increased sprouting of blood vessels (214).

As mentioned in Chapter 2, in hypoxia there is a reduction of oxygen compared to normal. This may lead to cellular dysfunction, and death (157), and result in activation of the cellular hypoxia response such as higher erythropoietin (EPO) release, induction of tyrosine hydroxylase, and the stimulation of new blood vessels by upregulation of *VEGF* (160). In hypoxia, HIF-1 α accumulates (117) after its stabilisation by heat shock protein 90 (HSP90) (165) and dimerises with HIF-1 β (214) to translocate to the nucleus. Once inside the nucleus, the HIF-1 complex binds to the hypoxia-responsive elements (HRE) in the promoter regions of hypoxia-responsive genes such as *VEGF*, *EPO* and glutathione-1 (*GLUT1*) (165), which results in the transcriptional reaction to hypoxia (114).

In normoxia, proline residues 402 and 564 in the oxygen-dependant degradation domain (ODDD) of HIF-1 α are hydroxylated by prolyl-hydroxylase (PHD), causing the degradation of HIF-1 α through the ubiquitin-proteasome pathway (114, 157, 164) mediated by the von Hippel-Lindau (VHL) protein that acts together with protein elongin C and leads to the formation of an E3 ubiquitin-ligase complex (214).

HIF-1 activity can be assessed by the amount of steady-state HIF-1 α protein levels although other measures can be used, such as the mRNA expression, nuclear localisation and transcription (198). Hypoxia can be imitated by the use of cobalt (Co²⁺), which induces the hypoxia signalling pathway by a pVHL-dependent (181), and pVHL-independent system (196). It was demonstrated that cobalt induced the expression of both *VEGF* (259) and *EPO* in Hep3B cells *via* the same oxygen-sensing mechanism (258). Cobalt also directly influences cells to produce EPO, and at the same time it brings on a synchronized upregulation of HIF-responsive genes such as *VEGF* and certain glycolytic enzymes (267).

In this study, hypoxia-mimicking bioactive glasses were developed as a new means to promote dental repair. At present, the response of dental pulp stem cells to hypoxia and the underlying mechanisms are still very much sought after (260). Cobalt-containing bioactive glass (CoBGs) that mimicked hypoxia with different cobalt concentrations (121) were used to assess the response of dental pulp stem cells. The incorporation of cobalt into bioactive glass may help to promote vascularisation in a defect, which could thus improve bone-material bonding to be used as dental materials in the field of regenerative dentistry, especially for pulpal repair and healing. In addition, BG is biodegradable and can partially dissolve in simulated body fluids. The incorporation of cobalt within the BG structures allows the release of cobalt ions into the surrounding environment (22, 121). Thus, the aim of this study was to assess the response of dental stem cells from the permanent and deciduous teeth following exposure to the dissolution ions from the hypoxia-mimicking bioactive glass.

5.2 Materials and methods

5.2.1 Cell culture

The dental stem cells were isolated as previously described in Chapter 4. For their assessment in CoBG-conditioned medium, single-cell suspensions of DPSC and SHED were cultured and expanded in low glucose Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% (v/v) foetal bovine serum (FBS, Gibco), and 1% (v/v) penicillin/streptomycin (Gibco). Initially, the cells were seeded and grown in a 75 cm² flask in a humidified atmosphere of 95% air with 5% CO₂ at 37°C. The growth medium was changed every 2-3 days. Once the cells reached 90% confluence, they were detached using 0.25% trypsin-EDTA (Gibco). The cell were counted and resuspended in complete medium in the appropriate concentration for the designated assay in this chapter. Cells at passage 3 to 6 were used. The cells were seeded for different time periods in cobalt -containing BG-conditioned DMEM in tissue culture treated well plates, where the media were refreshed every 2-3 days (Fig. 5.1). CoCl₂-containing medium was used as a positive control for the presence of cobalt.

5.2.2 Preparation of BG-conditioned media

BG-conditioned medium was prepared in DMEM with cobalt-containing BGs from the non-charge-balance series (NCB) at a concentration of 1.5 mg/ml. The previously prepared CoBG powders (121) were kept in a desiccator until their use (Table 5.1). Briefly, the culture medium was incubated with cobalt-containing BG powder (< 38 µm) on a roller for 4 hours at 37°C and passed through 0.2 µm filters. The BG-conditioned media were supplemented as mentioned in Chapter 5.2.1 and were incubated in a vented flask in 37°C with 5% CO₂ for 4 hours in order to stabilise the pH before being used on the cells.

Table 5.1: The designed compositions of the CoBG used in this study in mole percentages. Adapted from (121).

Series	BGs	SiO ₂	P ₂ O ₅	CaO	Na ₂ O	CoO
	0Co	49.46	1.07	23.08	26.38	0.0
NCB	1C0	49.46	1.07	22.08	26.38	1.0
	2Co	49.46	1.07	21.08	26.38	2.0
	4Co	49.46	1.07	19.08	26.38	4.0

5.2.3 Elemental analysis

The cell culture medium samples containing dissolution ions from BG were diluted by a factor of 10 in deionised (DI) water. The elemental concentrations of calcium (Ca), silicon (Si), cobalt (Co) and phosphorus (P) ions were analysed by inductively coupled plasma – optical emission spectrometer (ICP-OES) (iCAP 6000, Thermo Scientific, Waltham, MA, USA). The standard curve of each ion was prepared according to the manufacturer's instructions to ensure that the amount of ions released was within the normal range, and each sample was read in triplicate.

5.2.4 Alamar Blue assay

One of the assays available to assess cell viability is the Alamar Blue (AB) (Invitrogen, Gibco) assay. Living cells maintain a reducing environment within their cytosol, and are able to convert resazurin (blue, non-fluorescent), the active ingredient of the AB reagent, to resofurin (red fluorescence) once the dye enters the cells. Viable cells continuously convert resazurin to resofurin through a mitochondrial enzymatic reaction that causes an increase in the overall fluorescence and colour of the media surrounding the cells. The amount of fluorescence produced is a quantitative indicator of cell viability since it is proportional to the number of living cells. The AB assay was performed according to the manufacturer's instructions. Once DPSC and SHED were 90% confluent, they were trypsinized, counted, and seeded in 96-well plates (5×10^3 cells/cm²). The cells were treated with cobalt - containing BG-conditioned media and incubated in a CO₂ incubator (Binder, Thermo Forma, USA) at 37°C with 5% CO₂. At designated time points (1, 2, 4 and 7 days), the cell culture media were aspirated from the well plates followed by washing steps

using 100 μ l of PBS. Approximately, 150 μ l of 10% (v/v) AB in DMEM with no phenol red (Gibco) was added per well (including one with no cells to be used as blank) and the well plates were further incubated for 2 hours at 37°C. Following the 2 hours incubation period, 100 μ l of the reaction product was transferred to a black Costar 96-well plate. The fluorescence of AB was read at an excitation wavelength of 544 nm and emission of 590 nm using a microplate reader (FLUOstar Omega, BMG Labtech).

5.2.5 MTT assay

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue) is a water soluble yellowish dye that is readily taken up by viable cells. The viable cells convert this dye to insoluble purple formazan crystals through cleavage of the tetrazolium ring by mitochondrial dehydrogenase enzymes. The water insoluble formazan was solubilized using DMSO and the dissolved material was measured spectrophotometrically yielding absorbance as a function of concentration of converted dye that is proportionate to the activity of living cells. Briefly, DPSC and SHED were seeded in 96-well plates (5×10^3 cells/cm²). The cells were treated with cobalt -containing BG-conditioned media and incubated in a CO₂ incubator (Binder, Thermo Forma, USA) at 37°C with 5% CO₂. At designated time points (1, 2, 4 and 7 days), 5 mg/ml MTT salt solution (thiazolyl blue tetrazolium bromide, Sigma) was added to cell culture media followed by four hours of incubation to allow cellular tetrazolium conversion. The medium was removed from the wells and 200 μ l of DMSO was added to solubilise the formazan crystals. The absorbance was measured using a microplate reader (FLUOstar Omega, BMG Labtech) at a wavelength of 570 nm with a reference wavelength of 620 nm.

5.2.6 BrdU assay

BrdU (5-bromo-2'-deoxyuridine) is permanently integrated into DNA of living cells during proliferation. In this study, an ELISA BrdU kit (Roche, Japan) was used for the evaluation of BrdU incorporation in place of thymidine into the DNA of proliferating cells. The assay was performed following the instructions from the manufacturer. Briefly, DPSC and SHED were seeded in 96-well plates (5×10^3 cells/cm²) using DMEM (Gibco) supplemented with 10% (v/v) FBS (Gibco), and 1% (v/v) penicillin/streptomycin (Gibco) as mentioned in Section 5.2.1 and incubated in a CO₂

incubator (Binder, Thermo Forma, USA) at 37°C with 5% CO₂. After the cells were attached, they were starved in culture media supplemented with only 1% (v/v) FBS for 24 hours in order to synchronise their cell cycle. The medium was removed and the cells were then cultured in cobalt-containing BG-conditioned media. At designated time points (1, 2, 3 and 4 days), approximately 20 µl of BrdU-labelling solution was added to each well and the cells were further incubated for 24 hours to allow BrdU incorporation into the DNA of proliferating cells. Once the 24 hour period was completed, the cell culture medium was removed from each well followed by fixation steps. The cells were then incubated with the anti-BrdU antibody prior to washing steps for removal of unbound antibodies. The formation of immune complexes was detected by the subsequent substrate reaction. The absorbance was measured using a microplate reader (FLUOstar Omega, BMG Labtech) at a wavelength of 450 nm with a reference wavelength of 690 nm.

5.2.7 Total DNA assay

The total DNA assay was performed using DNA quantification kit that uses the fluorescent dye, bisBenzimide H 33258 (Hoechst dye, Sigma) to measure the DNA concentration. This dye binds to DNA and, when excited at 360 nm, the fluorescence emission at 460 nm increases in the presence of DNA. Briefly, DPSC and SHED were seeded in 96-well plates (5×10^4 cells/cm²) supplemented with cobalt - containing BG-conditioned media. At designated time points (2, 4 and 7 days) cell culture medium was removed from each well, followed by washing steps in PBS. 100 µl of distilled water was added into each well and the cells were subjected to freeze and thaw cycles. Then 50 µl of the lysates was collected and transferred to a black Costar 96-well plate and 50 µl of Hoechst dye diluted to 20 µg/ml was added to each well followed by 5 minutes incubation in the dark. Fluorescence was measured with an excitation wavelength of 360 nm and an emission wavelength of 460 nm using a microplate reader (FLUOstar Omega, BMG Labtech). The DNA concentrations were calculated using a standard curve prepared with calf thymus DNA (Sigma) at concentrations between 0-1000 µg/ml. The total DNA assay results were used to normalise the VEGF assay results.

5.2.8 HIF-1 α activity assay

Hypoxia inducible factor-1 (HIF-1) is a transcription factor that is activated when there is a low oxygen level, for example in hypoxic or ischemic conditions. The HIF-1 α activity in dental stem cells exposed to the BG-conditioned medium was assessed using the TransAM assay kit (Active Motif). The DPSC and SHED were seeded in 25cm² flasks (5x10⁴ cells/cm²) using DMEM (Gibco) supplemented with 10% (v/v) FBS (Gibco) and 1% (v/v) penicillin/streptomycin (Gibco) as mentioned in Section 5.2.1 until approximately 90% confluent. The cell culture medium was removed and the dental stem cells were treated with cobalt -containing BG-conditioned media for another 8 hours, followed by the assay according to the manufacturer's instructions. Nuclear extracts were harvested from the dental stem cells using the Nuclear Extract Kit (Active Motif) following the manufacturer's instructions, and quantified using ProStain (Active Motif). Approximately 4 μ g of nuclear extract were prepared and added to a 96-well plate which was pre-coated with a specific oligonucleotide containing the a hypoxia response element (HRE) from the *EPO* gene. Next, a primary antibody that recognizes the epitope on HIF-1 α was added to the well plate, which was later followed by the addition of a secondary antibody conjugated to horseradish peroxidase. Developing solution was prepared and added to each well and the reaction was stopped by the addition of stop solution. The absorbance was measured using a microplate reader (FLUOstar Omega, BMG Labtech) at a wavelength of 450 nm with a reference wavelength of 655 nm.

5.2.9 Analysis of VEGF release

The VEGF in the dental stem cells culture supernatant was quantified with a VEGF A₁₆₅ ELISA kit (Quantikine Human VEGF Immunoassay, R&D Systems, USA) that uses a quantitative sandwich enzyme immunoassay technique, and performed according to the manufacturer's instructions. Briefly, the DPSC and SHED were seeded in 96-well plates (5x10⁴ cells/cm²) and treated with cobalt -containing BG-conditioned media for 2, 4 and 7 days before the cell culture supernatants were collected and added to the provided 96-well plate (pre-coated with a monoclonal antibody specific for VEGF A₁₆₅) together with the standards and blanks. Any VEGF present inside the well was bound by the immobilized antibody during the 1 hour incubation period. Later, each well was washed to remove any unbound substances,

which was followed by the addition of an enzyme-linked polyclonal antibody specific for VEGF. Following a washing step to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and the colour developed in proportion to the amount of VEGF A₁₆₅ bound from the supernatant. The colour development was stopped and the intensity was measured using a microplate reader (FLUOstar Omega, BMG Labtech) at a wavelength of 450 nm with a reference wavelength of 540 nm.

5.2.10 Assessment of collagen production

The collagen production by dental stem cells following treatment with BG-conditioned media was assessed with a Sircol Collagen assay (Biocolor, UK) which uses a dye-binding method designed for the analysis of acid and pepsin-soluble collagens. The Sircol Dye reagent binds to the [Gly-X-Y]_n helical structure found in collagen and the intensity of colour of the reaction product is proportional to the amount of collagen in the sample. Briefly, the DPSC and SHED were seeded in 12-well plates (5×10^4 cells/cm²) and treated with cobalt -containing BG-conditioned media for 5 and 10 days. Cell culture supernatants were collected from test samples, and blanks and collagen was isolated and concentrated according to the manufacturer's instructions. Sircol collagen dye was added to each tube and incubated for 30 minutes on a gentle shaker to allow the formation of collagen-dye complex and precipitation of the unbound dye. The precipitate was then washed, and an alkaline reagent was added to allow the release of Sircol dye from the collagen-dye complex. A collagen reference standard with the concentration range from 0-50 µg was prepared and total collagen production from dental stem cells was calculated using a standard curve. The absorbance was measured using a microplate reader (FLUOstar Omega, BMG Labtech) at a wavelength of 555 nm.

5.2.11 Reverse transcription and real time polymerase chain reaction

The hypoxic response of DPSC and SHED was further assessed by real-time quantitative RT-PCR to measure the *HIF1A* mRNA expression. DPSC and SHED (5×10^4 cells/cm²) were seeded with cobalt-containing BG-conditioned medium in 6-well plates. At 0-24 hours, total RNA from cell cultures were extracted using an RNeasy Mini Kit (Qiagen, Germany). Approximately 1000 ng of total RNA was used to synthesise cDNA (Quantitect Reverse Transcription Kit, Qiagen, Germany).

Master mix was prepared containing SYBR Green (Quantitect SYBR Green RT-PCT kit, Qiagen, Germany), and the RT-PCR reaction was performed on a RotorGene (Applied Biosystems) with a 15 min holding step (95°C), followed by 40 cycles of denaturation at 94°C (15 sec), annealing at 60°C (30 sec), and extension at 72°C (30 sec). All samples and controls were run in quadruplicate. The *HIF1A* mRNA levels were calculated following normalization to a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (Table 5.2), and expressed as relative expression when compared to the T0 control samples using the $2^{-\Delta\Delta C_t}$ method (421). The T0 samples are cells incubated with normal cell culture medium, which was collected at the same time of the start of the incubation time for the other samples.

Table 5.2: The human primer sequences used for real-time PCR

Gene	Primers	References
<i>GAPDH</i>	Forward: GGCATGGACTGTGGTCATGAG Reverse: TGCACCACTGCTTAGC	(422)
<i>HIF1A</i>	Forward: CAAGCCCTGAAAGCGCAAGTCCTC Reverse: GTGGCAGTGGTAGTGGTGGCATTAGC	(117)

5.2.12 Statistical analysis

The data for the experiments are expressed as mean $\pm$ standard deviations (SD). Each experiment was performed at least twice, and prepared in $N=4$ for each composition at a designated time point. The data were analysed using SPSS version 20.0 (IBM, USA) using one-way analysis of variance (ANOVA) with a post-hoc test (Bonferroni procedure) with p values < 0.05 considered statistically significant.

5.3 Results and discussion

5.3.1 Ion studies release

The incubation of the cobalt-containing bioactive glass (CoBG) powders in cell culture medium for four hours resulted in an increase of calcium and silicon content and a decrease of phosphorus concentration, which is accordance with a previous study (24). These modifications were similar for all glass compositions and were independent of the cobalt content of the glass (Table 5.3).

Table 5.3: The concentration of different ions (Si, Ca, P, Co) in cobalt-containing BG-conditioned DMEM ($\leq 38 \mu\text{m}$) (in ppm) was determined by ICP-OES following four hours incubation.

	Si	Ca	P	Co
Control	0.1 ± 0.5	73.6 ± 0.9	28.8 ± 0.05	0 ± 0.01
0CoBG	71.4 ± 0.5	94.7 ± 0.5	6.17 ± 0.01	0 ± 0.01
1CoBG	66.4 ± 0.6	88.1 ± 0.1	6.55 ± 0.07	4.62 ± 0.02
2CoBG	61.4 ± 0.3	80 ± 0.8	6.06 ± 0.06	9.04 ± 0.01
4CoBG	74.6 ± 0.4	94 ± 0.9	8.21 ± 0.06	20.91 ± 0.04
100 μM CoCl_2	0.5 ± 0.3	67.1 ± 0.9	26.34 ± 0.11	5.2 ± 0.01

As expected, the control medium and 100 μM CoCl_2 -containing cell culture medium did not contain any silicon ions, and the levels of calcium (73.6 ppm, 1.82 mM) and phosphate (28.8 ppm, 0.92 mM) were found to be consistent with the DMEM composition, confirming the validity of the results by ICP-OES. The high calcium and silicon present in the cobalt -containing BG-conditioned medium may result from the continuous release of calcium and silicon, where a network modifier (calcium) is being replaced by a cation that takes part in the silicate network (22, 121). The lower phosphorus concentration may result from the slow phosphorus release from the glass in the culture medium associated with a rapid precipitation of a CaP layer on the surface of the glasses, which involves the phosphorus (22, 121).

In this study, cobalt-containing BG from the non-charge balanced (NonCB) group was used where the CoO was substituted for CaO to give cobalt contents of 0, 1, 2 and 4 mole % (121). The other BG components were kept constant. As the cobalt was added to the glass composition, the BG structure became weaker and easily disrupted, lowering the T_g from 0 to 2 mole %, as shown previously (121). This

suggested that cobalt is entering the silicate network forming the Co-O-Si bonds and replacing the stronger Si-O-Si network, leading to lower silicon being released into the culture medium as observed in the 1Co and 2Co BG after 4 hours incubation. But in the 4 mole % CoBG, the T_g increasing suggested that as more cobalt is available, the effect of replacing a network modifier (calcium) with cobalt predominates. At higher cobalt concentrations, as the cobalt replaces calcium, the degree of polymerisation of the silicate network increases as more cobalt enters the silicate network, causing an increased T_g , and more silicon released into the culture medium. Further, based on magic-angle-spinning nuclear magnetic resonance (MAS-NMR) spectroscopy, the ^{29}Si MAS-NMR spectra remain constant as a function of cobalt concentration in the BG throughout the NonCB series. This suggests that cobalt may play a role as network modifier in the CoBG, and that the effect seen may be due to the compositional design over the role of the cobalt cation (121).

The cobalt release from CoBGs can be tailored. In this study, the cobalt released from the BGs was directly proportional to the amount added to the glass. The concentration of cobalt was $78.4 \pm 0.2 \mu\text{M}$, $153.5 \pm 0.3 \mu\text{M}$ and $355.0 \pm 0.2 \mu\text{M}$ for 1CoBG, 2CoBG and 4CoBG, respectively. The highest cobalt concentration was within the range of 20 ppm (equivalent to 355 μM), which is in accordance with the result of a recent study where mesoporous-bioactive glass with 5% cobalt released approximately 20 ppm cobalt when incubated with DMEM for up to 7 days (24).

5.3.2 The hypoxia-mimicking BG effects on dental stem cells proliferation and viability

AB and MTT assays were used to assess the DPSC and SHED viability and proliferation following exposure to the hypoxia-mimicking BG-conditioned media. From the AB assay, DPSC and SHED (Fig. 5.1a and b) cultured in cobalt-containing BG-conditioned medium showed increasing cell number from day 1 to day 14, especially in culture media with the lower cobalt concentration. At day 4, DPSC cell number cultured in 1Co BG-conditioned medium was significantly higher than control and 0Co BG-conditioned medium. At day 7 and 14, DPSC cell number was significantly lower in 4Co BG-conditioned medium compared to control and 0Co BG-conditioned medium. Although DPSC AB activity in 2Co BG-conditioned medium was significantly lower than control and 0Co BG-conditioned medium, the cell

number increased over time, indicating that the 2Co BG-conditioned medium did not entirely affect DPSC cell number as observed in 4Co BG-conditioned medium. For SHED, from day 4 to day 14, the cell number in the cobalt-containing BG-conditioned medium, especially 2Co BG-conditioned and 4Co BG-conditioned medium, was significantly lower compared to the control and 0Co BG-conditioned medium. Higher cobalt concentration in the BGs, especially in 4Co BG-conditioned medium, led to significantly lower cell number, suggesting a toxic effect of the CoBG.

The MTT activity assay supported the findings of AB assay. DPSC and SHED metabolic activity was affected by exposure to cobalt-containing BG-conditioned media in a dose-dependent manner, where higher cobalt concentration in CoBG led to a lower metabolic activity. For DPSC (Fig. 5.2a) as early as day 2, the effect of high cobalt on cell viability is readily observable, where 4Co BG-conditioned medium caused significantly lower viability compared to other CoBG compositions. These effects persisted up to day 14. DPSC cell viability in control, 0Co BG-conditioned and 1Co BG-conditioned medium, and 100 μ M CoCl_2 -containing medium remained higher throughout the incubation period, which is also observable in SHED cultured in these media (Fig. 5.2b). From day 1 to 14, the effect of high cobalt (4CoBG) led to a decrease in SHED viability. In general, incubation of DPSC and SHED with cobalt-containing BG-conditioned media affects their metabolic activity and proliferation in a cobalt dose-dependent manner. However, while no increase was observed for cells incubated in the presence of 4Co BG-conditioned medium over time, the cell metabolic activity increased from day 1 to day 14 for all other conditions. Minimal information is available in the literature regarding the response of dental stem cells to hypoxia-mimicking BG. A recent study had been conducted assessing the effect of hypoxia-mimicking BG, but the authors used bone marrow stromal stem cells (BMSC) in their study (24) and assessed proliferation with an MTT assay. Their results showed that the mesoporous BG with 5% cobalt (5Co-MBG) resulted in lower cell proliferation compared to mesoporous BG with 2% cobalt (2Co-MBG), in accordance with our study which showed that higher cobalt concentration led to reduced cell proliferation, suggesting the toxic effects of high cobalt concentration.

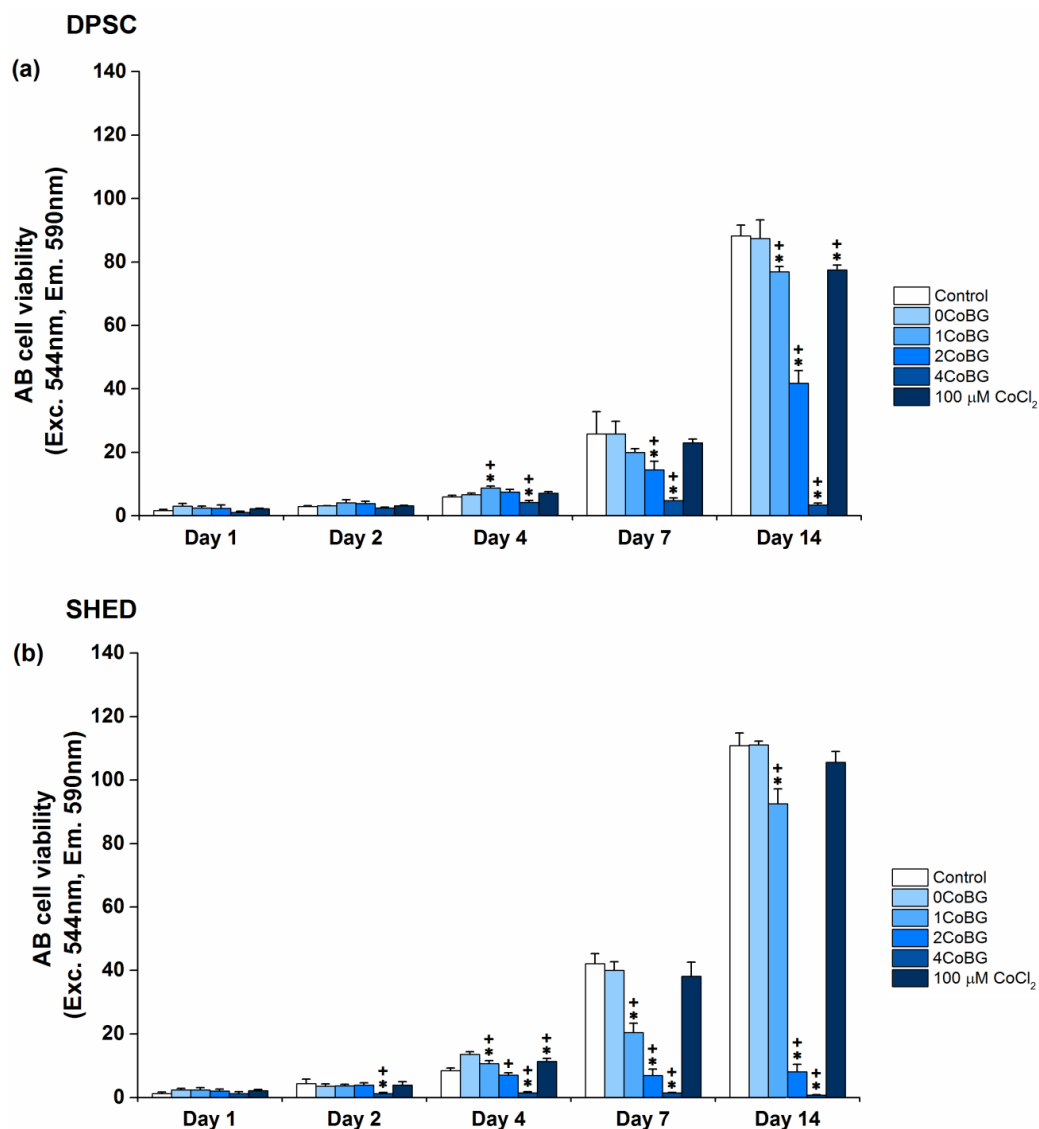


Fig. 5.1: The Alamar Blue assay. DPSC (a) and SHED (b) supplemented with cobalt-containing BG-conditioned medium at different time periods. * denotes a significant difference of the marked bar compared to the control at the same time point. + denotes a significant difference of the marked bar compared to 0Co at the same time point.

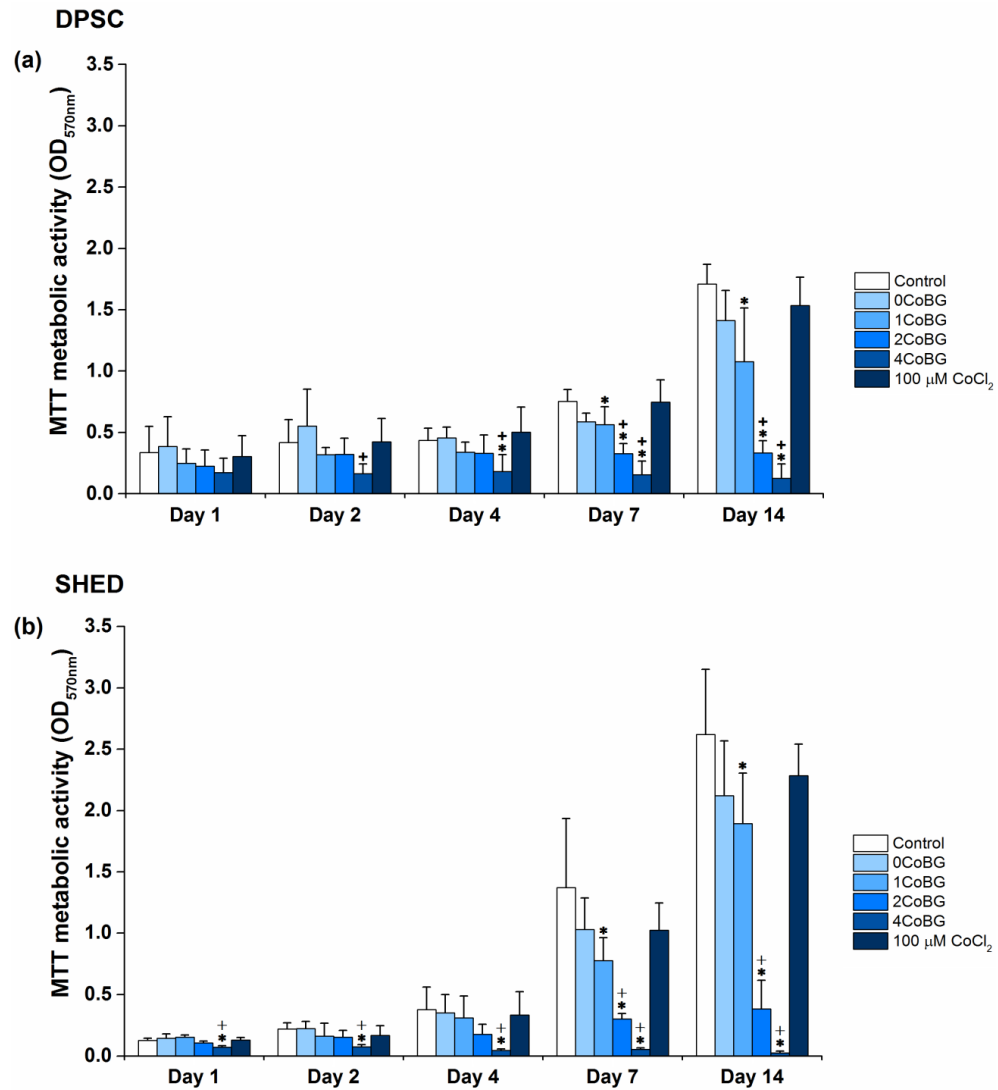


Fig. 5.2: The MTT metabolic activity assay. DPSC (a) and SHED (b) supplemented with cobalt - containing BG-conditioned medium at different time periods. * denotes a significant difference of the marked bar compared to the control at the same time point. + denotes a significant difference of the marked bar compared to 0Co at the same time point.

BrdU incorporation results supported the findings of the AB and MTT assays. The incubation of the dental stem cells with 2Co BG-conditioned medium resulted in a decrease of metabolic activity when compared to the control and 0Co BG-conditioned media samples at various time points. These effects appeared to result from the diminished proliferation of the cells, as shown by a lower BrdU incorporation in the presence of the released cobalt. Although the 4Co BG-conditioned medium strongly inhibited cell growth, incubation with 2Co BG-conditioned medium still allowed the metabolic activity to increase slightly over time. Proliferation rates were

not statistically different in DPSC (Fig. 5.3a) and SHED (Fig. 5.3b) cultured in control, 0Co, 1Co, 2Co BG-conditioned media, and 100 μM CoCl_2 -containing medium at days 1 to 4. For SHED, the cell proliferation was statistically lower in 4Co BG-conditioned medium compared to the control for each time point. Treatment of SHED with 100 μM CoCl_2 -containing medium slightly affected BrdU incorporation, while not modifying MTT activity. At day 2, SHED proliferation was significantly lower in 100 μM CoCl_2 -containing medium compared to control and 0Co BG-conditioned medium. At day 3 and 4, however, it was similar to 0Co and 1Co BG-conditioned medium. Generally, SHED had higher proliferation compared to DPSC over time. These findings also supported the results obtained from the AB and MTT assays, where a high cobalt concentration in the culture medium lowered cell viability and proliferation, which suggested there was cell toxicity associated with high cobalt.

Based on the AB, MTT and BrdU assays, the incubation of the conditioned media with DPSC and SHED cells showed a dose-dependent effect on cell metabolic activity and proliferation. With higher cobalt concentrations, there was a time-dependant decrease of proliferation (117). High cobalt present in the cobalt-containing BG-conditioned medium may have led to apoptosis (Fig. 5.4 and 5.5), since low oxygen availability is known to result in increased mitochondrial reactive oxygen species (ROS) (240). It is also possible that the high cobalt present in the medium is directly cytotoxic to the cells, and that, it mimicked hypoxia too strongly leading to lower proliferation. Nevertheless, apoptosis was not assessed in our study. A recent study (423) looked into the effect of hypoxia on human periodontal ligament cells (PDLCs). The authors isolated fresh PDLCs from adult teeth and subjected the cells to various concentrations of CoCl_2 . Some of the cells were pre-treated with *N*-acetylcysteine (an anti-oxidant) for 2 hours prior to CoCl_2 exposure for 48 hours, and based on the cell viability assay, 200 μM and 400 μM CoCl_2 concentrations led to reduced cell viability compared to controls, and 400 μM CoCl_2 concentration resulted in the highest number of apoptotic cells (423). The authors also showed that the half maximal inhibitory concentration (EC_{50}) of CoCl_2 to PDLCs was 169 μM . Our result is in accordance with their study, where high CoCl_2 concentration led to decreased cell viability in a dose-dependent manner. In our study, 100 μM CoCl_2 -containing medium was used as the positive control, and the 2CoBG released approximately 153.5 μM of cobalt into the medium, which can still

be considered a safe concentration (258). However, whilst the cell metabolic activity decreased in correlation to the amount of cobalt added to the glass, an additive effect of the medium ion content modification resulting from incubation with the glass can be observed. For example, the metabolic activity of cells treated with 100 μM CoCl_2 -containing medium was higher than 1Co BG-conditioned medium that contained only 78.4 μM . Taking these factors in consideration, we suggest that the suitable amount of cobalt to be incorporated within BG for use with stem cells from oral and dental tissues origins should be equal or lower than 2 mole %.

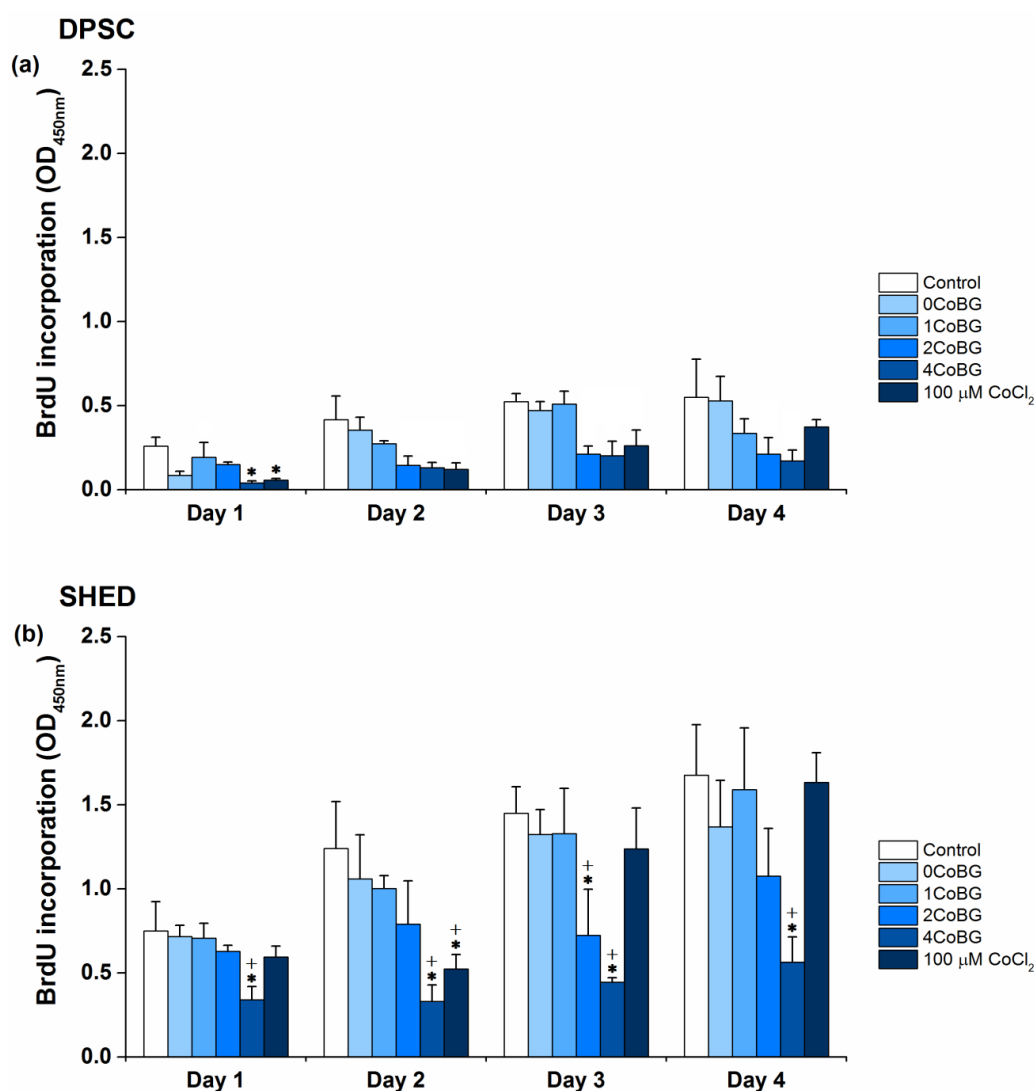


Fig. 5.3: The BrdU proliferation assay. DPSC (a) and SHED (b) supplemented with cobalt - containing BG-conditioned medium at different time periods. * denotes a significant difference of the marked bar compared to the control at the same time point. + denotes a significant difference of the marked bar compared to 0Co at the same time point.

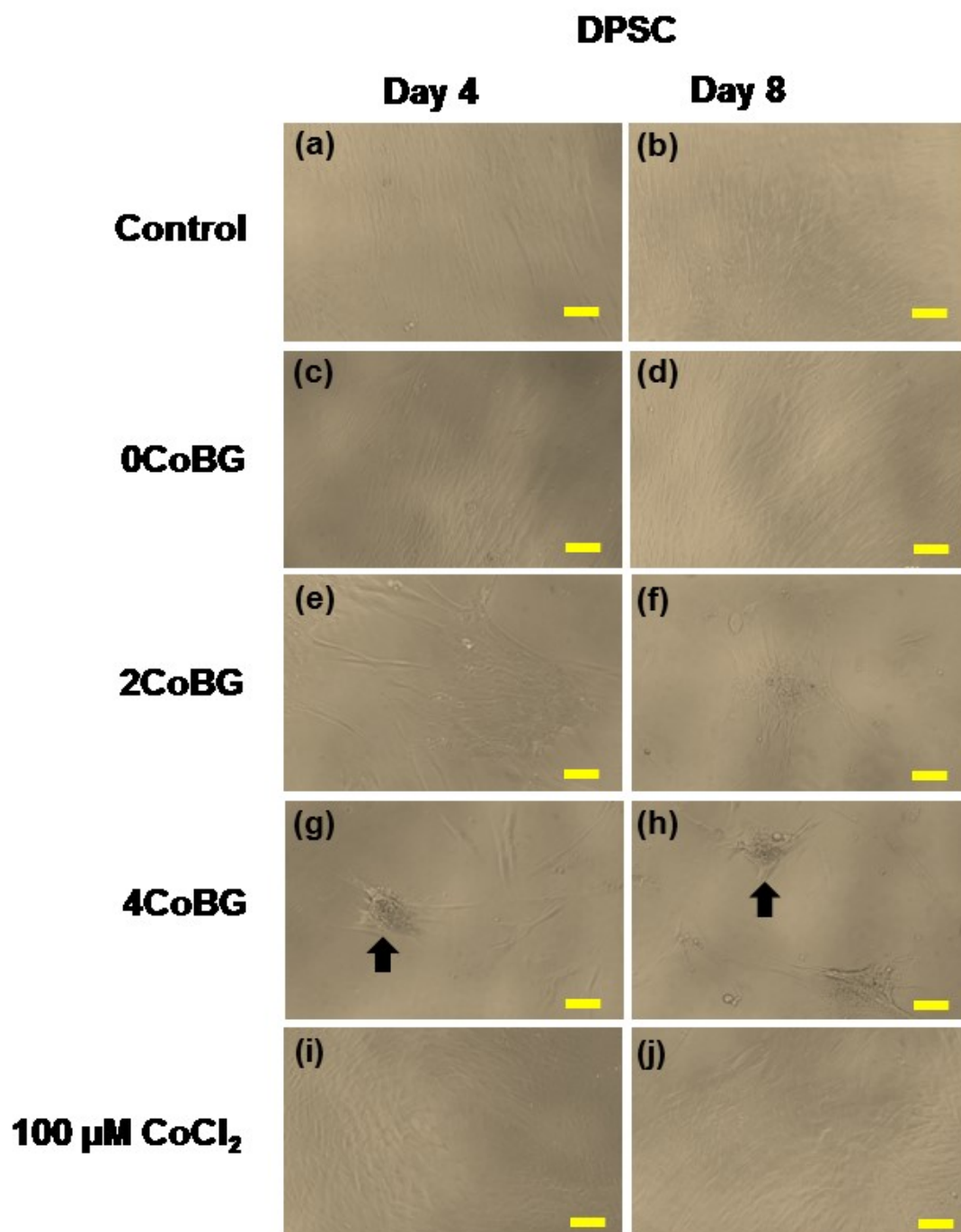


Fig. 5.4: Light microscopy images of DPSC (a - j) supplemented with cobalt-containing BG-conditioned medium at days 4 and 8. Cells in 4Co BG-conditioned medium are probably showing cell membrane asymmetry and nuclear fragmentation (black arrow) (g, h, q). Scale bar is 200 μm .

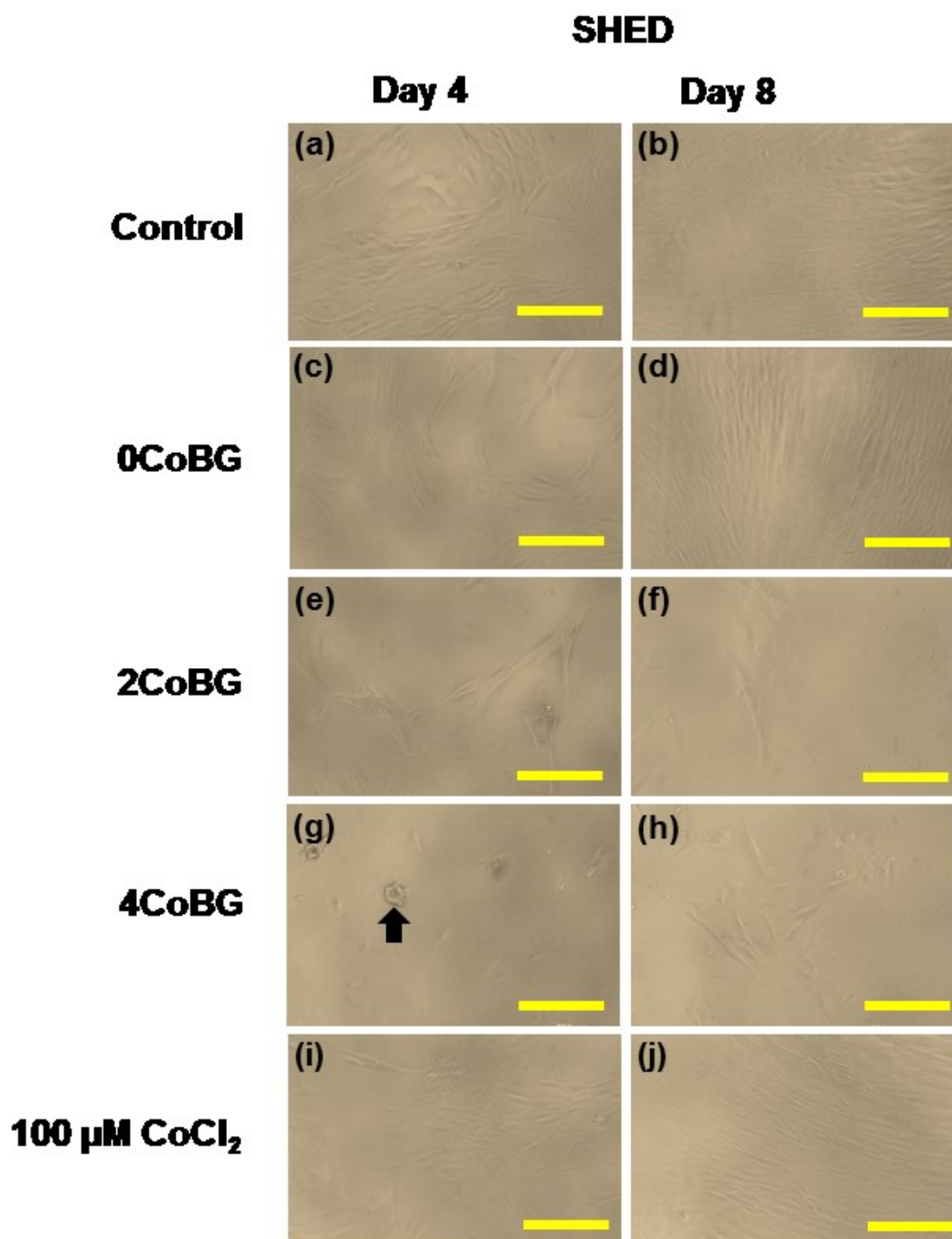


Fig. 5.5: Light microscopy images of SHED (a - j) supplemented with Co^{2+} -containing BG-conditioned medium at days 4 and 8. Cells in 4Co BG-conditioned medium are probably showing cell membrane asymmetry and nuclear fragmentation (black arrow) (g). Scale bar is 500 μm .

In our study, the 0Co BG-conditioned medium did not significantly affect the metabolic activity of DPSC nor SHED. In another study, human DPCs exposed to control BG-conditioned medium (45S5) showed the highest proliferation rate compared to cells cultured in conditioned medium from 58S nano-sized BG (nano-58S) and 58S (58S BG), suggesting that control BG-conditioned medium (45S5) was able to promote DPC proliferation (424).

In our study, when the cells were exposed to hypoxia-mimicking BGs, SHED had higher cell proliferation and viability compared to DPSC, which is in accordance with the study by Kanafi *et al.* (243). In their study, SHED were capable of a higher cell yield and a shorter population doubling time when exposed to hypoxia (the authors used 2.3% O₂ concentration to mimic hypoxia) compared to DPSC, and hypoxia promoted greater SHED migration compared to DPSC (243), which may be attributed by the developmental differences of DPSC and SHED (4, 59), as mentioned earlier in Chapter 4. Hypoxia (3% O₂) had also been shown to promote human DPC proliferation two-fold higher than cells exposed to normoxia (20% O₂), and higher STRO-1, a marker for mesenchymal progenitor cells, suggesting that hypoxia is able to maintain cells in an undifferentiated state (425). Although different dental stem cell origins were used, hypoxia (8% O₂) promotes human periapical follicular stem cells (PAFSC) and human PDLSC proliferation when compared to their respective controls (426). Hypoxia (3% O₂) enhanced human DPC proliferation when plated at a lower cell density compared to 21% O₂, suggesting that hypoxia may be suitable for promoting dental pulp stem cell growth since they originated from a low oxygen environment (242). Although such effects were not observed in our study where hypoxia was mimicked by cobalt incorporation in BG, CoBG with a cobalt percentage lower or equal to 2% appeared adapted to be used in the field of regenerative dentistry.

5.3.3 The hypoxia-mimicking BG effects on activation of hypoxia pathway and subsequent hypoxia response

The present study was conducted with the aim of saving a tooth from becoming non-vital by offering regenerative strategies using CoBGs to mimic hypoxia and promote angiogenesis and healing within the pulp. Cobalt was chosen as the agent to mimic hypoxia due to its activity on the HIF-1 pathway (117, 258) and its suitability to be incorporated within bioactive glass structures (121). The activation of the hypoxia pathway was assessed by the quantification of *HIF1A* mRNA expression and HIF-1 α nuclear amount that could then trigger the secretion of VEGF. HIF-1 activity in cells can be assessed by the expression and activity of steady-state HIF-1 α protein levels, mRNA expression, nuclear localization and transcriptional machinery in the nucleus, since hypoxia upregulates many genes (reviewed in (198)). Both DPSC and SHED were exposed to cobalt-containing BG-conditioned medium for up to 8 hours prior to the assessment of *HIF1A* expression. A previous report highlighted that an eight hour exposure of cells to 0.7 mM cobalt ions may cause a sudden decrease of *HIF1A* expression up to 24 hours, and then an increase to normal level by 48 hours (117).

During dental repair, diminished blood supply to the pulp may lead to hypoxia, so to assess these responses, we treated the dental stem cells from the dental pulp of the permanent and deciduous teeth to cobalt-containing BG-conditioned medium, which creates hypoxia-mimicking conditions, and 100 μ M CoCl₂ as a positive control along with cells grown in normal growth medium as a control. HIF-1 α pathway activation was determined by quantifying the amount of HIF-1 α translocated to the nucleus after exposure to the cobalt-containing BG-conditioned media. While no differences in nuclear extract content of HIF-1 α was found after treatment of DPSC and SHED with control, 100 μ M CoCl₂, 0Co and 1Co BG-conditioned medium (Fig. 5.6a and b), exposure of DPSC to 2Co BG-conditioned medium enhanced the amount of HIF-1 α in the nuclear extracts compared to the other conditions. Cells under normoxia may have lower HIF-1 α level in the nucleus, since HIF-1 α is degraded by proteasome ubiquitination (196). Although statistical significance was not reached, a similar trend was observed for SHED treated with 2Co BG-conditioned medium.

Exposure of DPSC (Fig. 5.7a) and SHED (Fig. 5.7b) to 2Co BG-conditioned medium for 8 and 24 hours led to a decrease of *HIF1A* expression compared to controls and other groups of cobalt-containing BG-conditioned medium. Such a decrease in *HIF1A* mRNA level has been reported as a result of HIF-1 pathway activation (423).

These results indicated that dental stem cells in control medium experience normoxia, thus have low levels of nuclear HIF-1 α , while dental stem cells exposed to 2Co BG-conditioned medium are experiencing a hypoxia-mimicking environment due to the action of the cobalt released from the glass.

Our results are in accordance with a recent study (24) where the authors developed a hypoxia-mimicking mesoporous BG scaffold by incorporating Co (molar: 2 and 5%) into the BG and assessed bone marrow stromal stem cells (BMSC) for activation of the hypoxia pathway. They showed that incorporation of 2% cobalt into their BG enhanced HIF-1 α content and VEGF secretion after seven days of BMSC incubation with the mesoporous BG scaffold compared to the 5% of cobalt into the BG (24). However, the authors did not assess the production of VEGF at early time points, which may give a better picture of the secretion in response to the mesoporous BG.

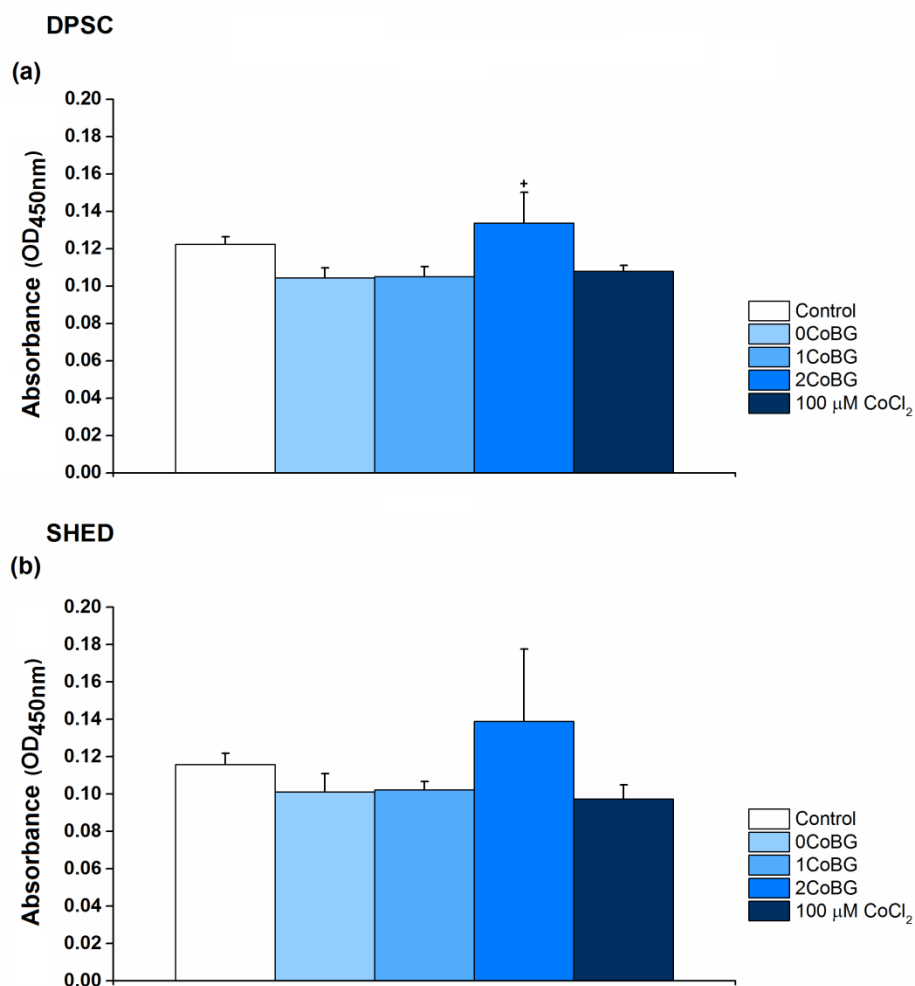


Fig. 5.6: The assessment of hypoxia-mimicking BGs on the activation of the hypoxia pathway of dental stem cells using a HIF-1 α activity assay. DPSC (a) and SHED (b) supplemented with cobalt -containing BG-conditioned medium for 8 hours. * denotes a significant difference of the marked bar compared to the control at the same time point. + denotes a significant difference of the marked bar compared to 0Co at the same time point.

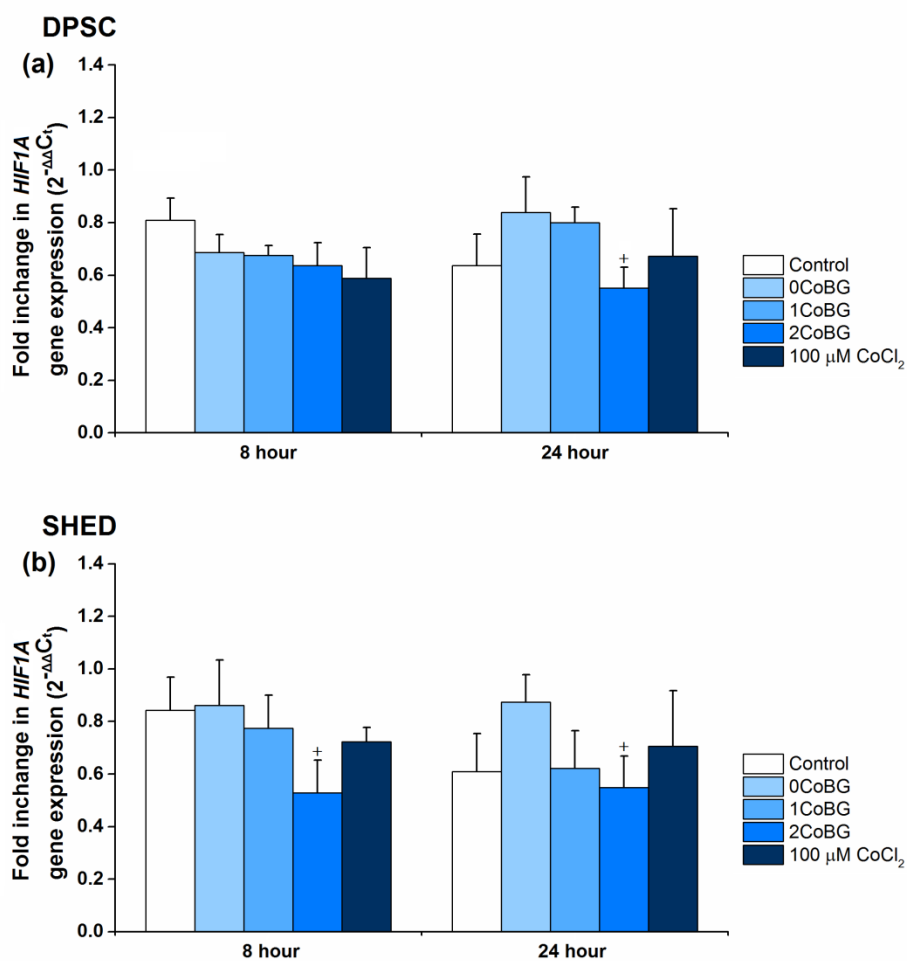


Fig. 5.7: The assessment of hypoxia-mimicking BGs on the activation of the hypoxia pathway of dental stem cells using RT-PCR for *HIF1A* transcript expression. DPSC (a) and SHED (b) supplemented with cobalt-containing BG-conditioned medium at different time periods. * denotes a significant difference of the marked bar compared to the control at the same time point. + denotes a significant difference of the marked bar compared to 0Co at the same time point.

The activation of the hypoxia response was further assessed by determining the amount of VEGF release from the cells in response to cobalt-containing BG-conditioned medium. Exposure of DPSC to 2Co BG-conditioned medium resulted in significantly higher VEGF secretion compared to 0Co BG-conditioned medium at day 2. At day 4, significantly higher VEGF was observed in DPSC exposed to 1Co and 2Co BG-conditioned media when compared to control and 0Co BG-conditioned media (Fig. 5.8a). The VEGF release at day 4 reached a concentration of 1008.4 ± 83.7 and 1148.2 ± 379.9 pg/ μ g DNA for 1Co and 2Co BG-conditioned media, respectively, compared to 382.1 ± 38.2 pg/ μ g DNA for 0Co BG-conditioned medium. SHED, however, did not show an increase in VEGF release when exposed to cobalt-containing BG-conditioned media at days 2 to 7, and the total quantity of VEGF secreted from SHED remained very low (Fig. 5.8b).

Mimicking hypoxia using cobalt may result in different responses from different cell types because the response to hypoxia is cell type-specific (260). For example, it was shown that exposure of MG63 cells to 0.1 mM cobalt in culture medium resulted in 1.0 ng/ml of VEGF release, and upon exposure to hypoxia (3% O₂) and anoxia (0% O₂), the release was 0.57 and 1.3 ng/ml, respectively (117). For fibroblasts, the amount of VEGF released was undetectable in 0.1 mM cobalt and in hypoxia. However, the VEGF release during anoxia was 0.15 ng/ml (117). Thus, variation may exist in the amount of VEGF release from different cell types. Based on these observations, our study confirmed an activation of the HIF-1 pathway by 2Co BG-conditioned medium.

Based on the results of this study, the amount of cobalt concentration present within the BG appears to play a significant role in initiating the activation of the hypoxia pathway *via* HIF-1 α . We have demonstrated that 2Co BG-conditioned medium promotes activation of the HIF-1 α pathway through HIF-1 α nuclear translocation and the secretion of VEGF (Fig. 5.6 to 5.8). The effect of CoBG in mimicking hypoxia in normoxic environment may have lead to the HIF-1 α stabilisation (as shown by the slightly higher HIF-1 α nuclear extracts in Fig. 5.6). HIF-1 α would then have dimerised with HIF-1 β (as shown by the lower *HIF1A* expression by the RT-PCR results in Fig. 5.7). Upon dimerisation of the HIF-1 complex at the hypoxia-responsive element, this triggered a cellular response as assessed by the release of

VEGF from the cells (Fig 5.8). Based on this observation, we suggest the CoBG as a hypoxia-mimicking agent has successfully activated the HIF-1 pathway and show potential in the field of regenerative dentistry. Although, there are no known previously published data about the exposure of dental stem cells to hypoxic cobalt-containing BG-conditioned medium, it is important to highlight that hypoxic conditions or mimicry of hypoxia by cobalt exposure to human dermal microvascular endothelial cells (HDMEC) did lead to an increase of *HIF1A* within a few hours of exposure to 0.7 mM cobalt which later decreased from 8 to 24 hours (117). This decrease in *HIF1A* expression 8 and 24 hours after exposure to 2CoBG is consistent with such results. Recently, hMSC treated with the hypoxia-mimicking cobalt-containing BG-conditioned medium showed upregulation of several hypoxia-responsive genes including *HIF1A* and *VEGF* (427). This finding clearly suggests activation of hypoxia pathway mediated through HIF-1 α .

In order to engineer a vascularised scaffold, further research is required to understand cell response upon seeding on the scaffold, which can be done using *in vitro* co-culture systems (33). A study was conducted to assess the *in vitro* effect of cobalt concentrations (0.01-0.7mM) on endothelial cells, which showed that a hypoxia led to increased angiogenesis (117). During injury, hypoxia plays a critical role in activating the repair and healing cascade by promoting new blood vessel formation to re-establish blood supply and transfer oxygen and nutrients for the healing process to occur (165).

In studying the effect of hypoxia on dental stem cells, studies were conducted to assess proliferation by using rats dental follicle stem cells (428), human periodontal ligament cells (423), by modifying the culture conditions to subject the dental stem cells to various oxygen concentrations (260), and more recently, subjecting the cells to hypoxia and co-culture with endothelial cells (EC) (422). However these studies (242, 260, 422) only use the dental stem cells from the adult teeth and none from the deciduous teeth. This is the first study to report the response of dental stem cells from both the permanent and deciduous teeth following exposure to cobalt - containing BG-conditioned medium. In order to fully understand and develop materials for dental repair, subjecting the dental stem cells directly to the material either by using a scaffold or conditioned medium, and analysing their responses is a

step forward in the field of regenerative dentistry. In our study, DPSC and SHED had different responses to hypoxia-mimicking BG, especially regarding the secretion of VEGF. This might be due to the differences in the developmental timing between permanent and deciduous tooth. SHED showed higher proliferation, and may be considered an immature multipotent stem cell type compared to DPSC (59). Although the role of VEGF in the dental pulp was extensively reviewed (429), information regarding the relationship between VEGF and SHED is still scarce.

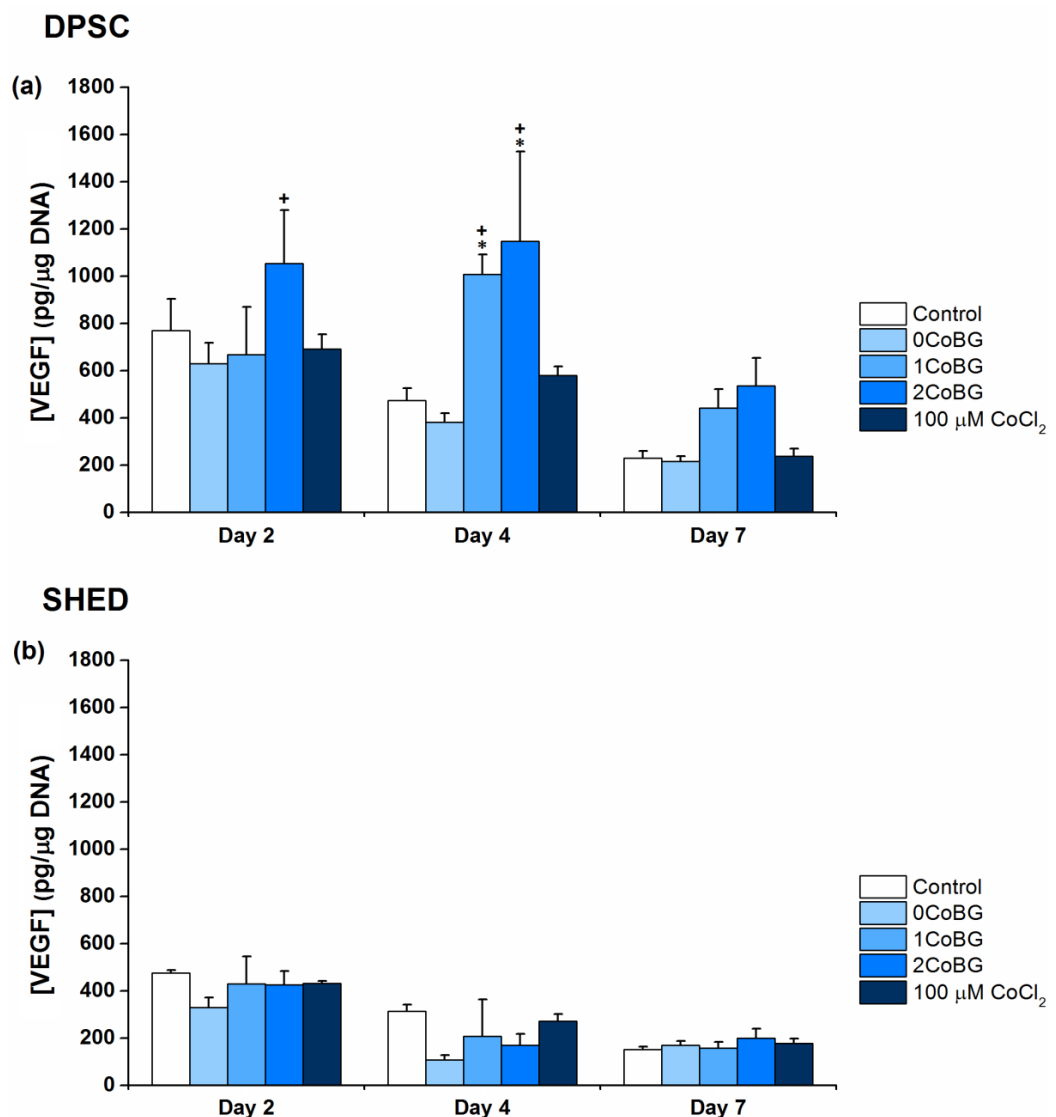


Fig. 5.8: The assessment of hypoxia-mimicking BGs on the hypoxia response dental stem cell using a VEGF secretion assay. DPSC (a) and SHED (b) supplemented with cobalt-containing BG-conditioned medium at different time periods. * denotes a significant difference of the marked bar compared to the control at the same time point. + denotes a significant difference of the marked bar compared to 0Co at the same time point.

5.3.4 The hypoxia-mimicking BG effects on dental stem cells collagen production

Extracellular matrix (ECM) formation is important in providing cues to interact with cell surface receptors and direct cellular organisation to activate signal transduction pathways and induce physiological responses (430). ECM formation is also important during tooth remodelling (431). Collagen prolyl 4-hydroxylase (P4H) is involved in the formation of 4-hydroxyproline and also plays a vital role in collagen biosynthesis (186). It was shown that hypoxia (1% O₂) through the activation of HIF-1 α induced expression of collagen prolyl hydroxylases (P4HA1 and P4HA2) resulting in increased collagen type I production in human newborn fibroblast cells (430). The formation of 4-hydroxyproline from proline residues is catalysed by collagen prolyl hydroxylases (P4Hs). The P4Hs are important for collagen synthesis due to their role in proper folding of collagen polypeptide chains to form a stable triple helix (430).

During hypoxia, the HIF-1 α hydroxylation by HIF P4H (also known as PHD in human) is blocked, resulting in binding of HIF-1 α with HIF-1 β , which then translocates to the nucleus and binds to the HRE on hypoxia-inducible genes. Thus, the level of P4H is expected to increase and catalyse proline residue hydroxylation in procollagen molecules to give rise to collagen formation when cells are exposed to hypoxia (432). In one study, foetal rat lung fibroblasts were exposed to prolonged hypoxia (0% O₂) for 9 days with or without supplemented ascorbic acid (432). In presence of ascorbic acid, denser procollagen and collagen was produced in the hypoxic group, suggesting that hypoxia accelerates the rate of collagen production resulting in higher amount of collagen deposition *via* posttranscriptional modulation (432). Inhibition of P4H or its domain, prolyl hydroxylase domain-containing protein (PHDs) by dimethyloxallylglycine (DMOG), desferrioxamine (DFO), L-mimosine (L-MIM) and CoCl₂ led to higher levels of HIF-1 α (167, 433). Hence, the use of CoBG could help to generate collagen production from DPSC and SHED following exposure of these cells to cobalt-containing BG-conditioned medium.

In response to the hypoxia-mimicking effect from the cobalt-containing BG-conditioned medium, no significant differences were observed for the collagen production from both the DPSC (Fig. 5.8a) and SHED (Fig. 5.8b). For DPSC, there

was a slight increasing trend for higher collagen production in cells exposed to increasing concentrations of cobalt in the cobalt-containing BG-conditioned medium at day 10 when compared to day 5, but this finding was not significant. However, for SHED, a decreasing trend in collagen production was observed from day 5 to day 10, except in SHED exposed to 2Co BG-conditioned medium.

In order to generate suitable materials as pulp protecting agents, human DPC have been previously cultured in extracts of pulp capping agents, calcium hydroxide (CaOH) supplemented with PHD inhibitors (DMOG, DFO, L-MIM, CoCl₂), or water for 24 hours (250). Cell proliferation and viability were lower compared to controls, while the VEGF levels and HIF-1 α expression were higher. However, when comparisons were made in the VEGF level and HIF-1 α expression in cells treated with combination of PHD inhibitors and CaOH extracts, no significant difference was detected. The authors suggested that the use of PHD inhibitors in combination with pulp capping materials for regeneration and repair of pulp tissues requires further exploration (250). Although statistical significance was not reached for both DPSC and SHED, upon exposure to the cobalt -containing BG-conditioned medium, collagen production was not altered. No previous studies are available regarding the collagen production from these cells when exposed to the cobalt -containing BG-conditioned medium. Nevertheless, further investigation is needed to assess the capability of DPSC and SHED in collagen production upon exposure to the cobalt -containing BG-conditioned medium. The type of collagen produced upon induction of these dental stem cells to cobalt-containing BG-conditioned medium was also assessed. The type of collagen produced may affect the regeneration of dental tissues. Dental pulp tissue consists of four rich zones, and within the cell-rich zone, odontoblasts are abundant and possess organelles that produce collagen and proteoglycan (434). The collagen fibres found in dental pulp are predominantly collagen type III and less collagen type I (435). Collagen type III is important for tissue elasticity and is mainly present in loose connective tissues. Collagen type I are present in dense connective tissue that is necessary for tissue architecture and stabilisation (435). Although higher amounts of collagen type III are present in the dental pulp, the collagen concentration in pulp tissues are determined by the type of tooth and degree of cross-linking between the collagen fibres (436). The solubility of

the collagen is also affected by the digestion process. The Sircol collagen assay did not discriminate between the collagen types and its binding to triple helix. This may also contribute to the non-significant differences in collagen production from these cells.

The effect of non-cobalt-containing BG (0CoBG) also requires discussion. In both cells, 0Co BG-conditioned medium showed substantial collagen production comparable to 1Co BG-conditioned medium. BG has a stimulatory effect on collagen type I production due to the presence of soluble silicon (296) and also enhances the laying down extracellular matrix for bone regeneration (313).

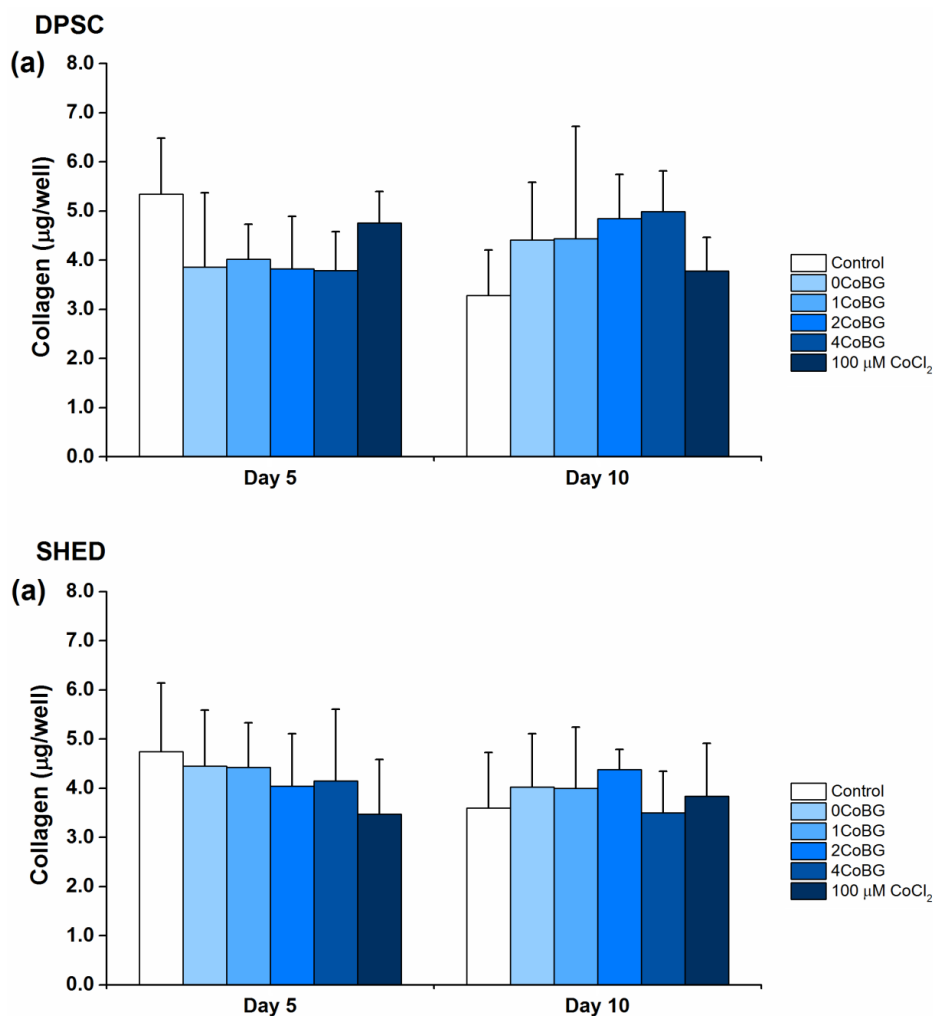


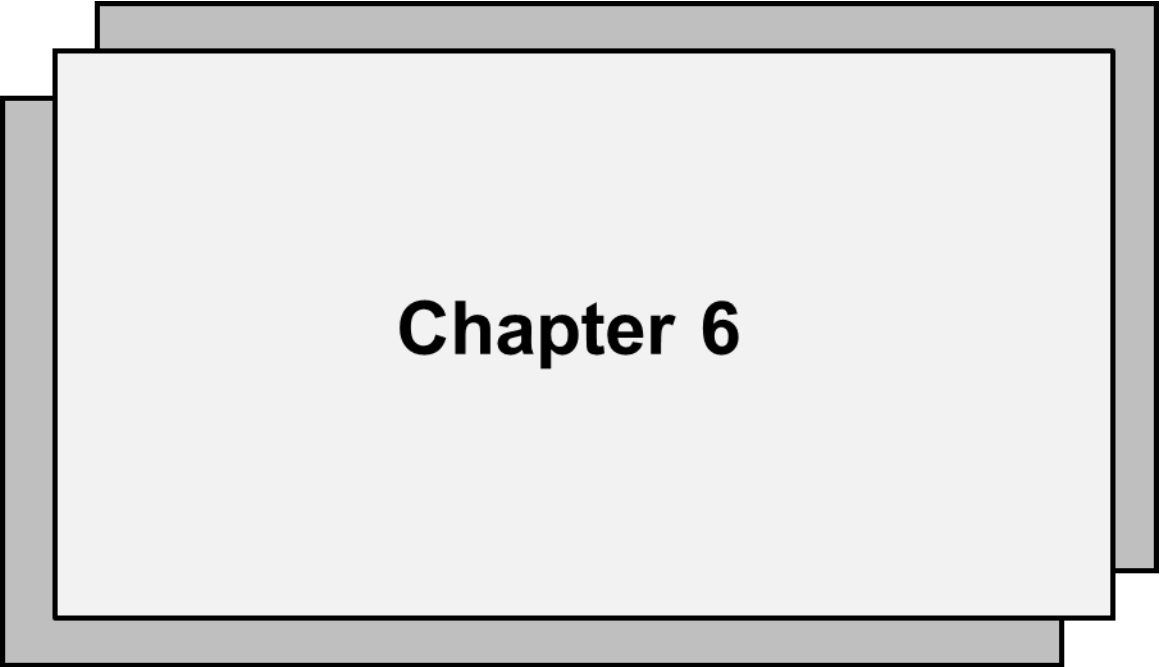
Fig. 5.8: The assessment of hypoxia-mimicking BGs on dental stem cell response on collagen production using a Sircol Collagen assay. DPSC (a) and SHED (b) supplemented with cobalt-containing BG-conditioned medium at different time periods. There were no statistically significant differences.

5.4 Conclusion

Human dental pulp stem cells have been shown to be promising candidates for stem cell based therapy and autologous tissue regeneration. Dental stem cells also possess therapeutic potential (392, 404) and provide an additional source to make iPS cells in the future (62). Dental pulp stem cells from permanent and deciduous teeth are suitable cells for testing the hypoxia-mimicking CoBG due to the nature of their growth, since they originated from a low oxygen environment (242), are easily accessible (7, 9), and are normally discarded as medical waste (63). Hypoxia is one of the factors that regulates stem cell proliferation and growth, and has been shown to enhance dental stem cells proliferation (243).

The effects of hypoxia-mimicking BG-conditioned medium exposure to dental stem cells were investigated in this chapter. The ICP-OES results showed that the cobalt released from the hypoxia-mimicking BGs could be tailored and are dose-dependent on the cobalt concentration present in the CoBG. The CoBG with a lower cobalt concentration provided benefits and did not strongly affect cellular viability or proliferation as assessed by AB, MTT and BrdU assays. Lower cobalt concentration did not affect DPSC and SHED viability and proliferation. However, high cobalt concentration (4CoBG) reduced viability and proliferation as shown by the AB, MTT and BrdU assays. These hypoxia mimicking CoBGs succeeded in activating the hypoxia pathway, resulting in hypoxic responses. These effects were observed through HIF-1 α stabilisation and translocation into the nucleus leading to dimerisation with HIF-1 β , which subsequently triggered a cellular response, ie. the release of VEGF. The cobalt released from the CoBG can be tailored for suitable applications in the field of regenerative dentistry. One of the CoBG (2Co BG-conditioned medium) successfully activated the hypoxia pathway and subsequently triggered the hypoxic responses in DPSC and SHED. The detection of nuclear HIF-1 α , the decrease of *HIF1A* expression, and the release of VEGF were used to identify the activation of HIF the pathway (240). Although further activation of the hypoxia pathway was assessed in terms of collagen production, more investigation is warranted to understand the hypoxia-mimicking effect of the CoBG on dental stem cells and other related oral tissues. The type of collagen produced upon exposure of these dental stem cells to the hypoxia-mimicking BG-conditioned medium may need

to be assessed. Further investigations are needed to examine the metabolic switch to anaerobic metabolism due to hypoxia. In conclusion, the results obtained in this chapter provide insight into the potential use of hypoxia-mimicking BG in the field of tissue engineering and regenerative dentistry.



Chapter 6

Chapter 6

Investigation of hypoxia-mimicking CoBG/PCL scaffold on dental pulp stem cells and human mesenchymal stem cells

In Chapter 5, it was found that the hypoxia-mimicking BGs with lower cobalt (Co^{2+}) concentrations of 0%, 1% and 2% CoBG have promising effects on dental stem cells from the permanent and deciduous tooth. The study conducted in Chapter 5 used the ion dissolution products from CoBG released into the environment. The 2Co BG-conditioned medium was able to stimulate the hypoxia pathway, which was signified by HIF1 α nuclear translocation and the release of VEGF. In large bony defects, the implantation of autologous bone alone may not meet the needs for repair. Thus, scaffolds may need to be used to provide structural support while delivering growth factors or cells to the host environment. Scaffolds fabricated for bone formation must possess the characteristics of normal bone. Porosity, pore size and surface roughness are some of the factors that need to be considered when designing such scaffolds (reviewed in (291)). In this chapter, we further investigate the effect of incorporating these glasses into 3D porous polymer-based scaffolds on DPSC and hMSC behaviour. The potential of the cobalt-containing BG/polycaprolactone (CoBG/PCL) scaffolds to activate the hypoxia HIF-1 pathway and secrete VEGF is assessed, as is their potential to induce endothelial cell migration *in vitro*.

6.1 Introduction

To promote vascular and bone regeneration in dentistry, materials must be developed carefully before they can be used as scaffolds. BGs as stand-alone products are difficult to shape in three dimensional (3D) porous structures, but they are known to be beneficial for the repair of bone and dental tissues. The produced 3D scaffolds are often brittle and may not suffice for use in load bearing areas. Blending BG powder, or other inorganic compounds, with various classes of polymers has already shown great potential for the production of mechanically strong and easily shaped 3D materials with high bioactivity and bone regenerating performance (105). In this chapter, the use of CoBG/PCL as a three dimensional (3D) scaffold is proposed. The CoBG powder is mixed with a semicrystalline

bioresorbable aliphatic polyester, polycaprolactone (PCL). Polymer-based scaffolds have been used in the tissue engineering field since 1980s. In 3D structures, the scaffolds must be biocompatible and provide mechanical and structural support for cells to proliferate and generate new bone, while at the same time undergoing degradation without compromising the newly formed tissue (reviewed in (291)).

In this study, a 3D CoBG/PCL scaffold was manufactured using fused deposition modelling (318) to be used as a scaffold to accommodate the space following bone loss or tooth loss in the maxillofacial region. PCL was chosen as the carrier material for the CoBG/PCL scaffold development due to its favourable properties, such as a low melting temperature of 60°C, a high decomposition temperature of 350°C, and also thermal stability (318, 437). PCL is biodegradable in its natural form and also *in vivo*, and it received approval by the United States Food and Drug Administration (317) as a base for drug delivery and in tissue engineering (438). The PCL degradation process is slow, taking up to 3-4 years, which makes it less popular compared to other polymers such as poly (L-lactic acid) (PLLA) and poly(lactic-co-glycolic acid) (PLGA). PCL degrades via hydrolytic degradation through surface and bulk degradation process with or without autocatalysis (439). In surface degradation, the polymer backbone undergoes hydrolytic cleavage causing erosion of the PCL surface, for example, when PCL is placed into water. Slow water intrusion rate into the PCL compared to the hydrolysis cleavage of the PCL polymer backbone causes thinning of the PCL surface as surface erosion takes place. When water enters into the whole bulk of the PCL structure at a faster rate, hydrolysis cleavage occurs in the entire PCL structure. This causes bulk degradation of the PCL and leads to a reduction of the molecular weight. The inherent properties of PCL allow it to be easily manipulated by a co-polymerisation process or by blending with other polymer materials or biomaterials such as starch, acrylates or silica. Many formulations have been prepared for use in drug delivery and tissue engineering (317, 438). The use of PCL based medical devices is well-known, and includes Osteoplug (Osteopore), Relison (Sigma), and PCL-based dental materials. They have gained wide acceptance for use in the dental field as implant rings, root canal filling materials, and dental impression trays (reviewed in (438)). The advancement in the field has seen many PCL/BGs composites being developed (440, 441). As mentioned earlier, BG powder as a stand-alone product is undesirable to use clinically because of the

difficulty in determining the powder-to-body fluids ratio (such as blood or serum) during surgery. Thus, the use of PCL/BG composite may help to overcome this problem. However, the use of these products, particularly BGs with incorporated cobalt, is still new for the regeneration and repair of oral and dental related tissues.

The CoBG/PCL scaffolds that were developed using the fused deposition modelling (FDM) method (308) for the current project may hold promise for future use. The FDM method allows the PCL to be blended with the BG powders to ensure that the component mixtures are uniform with a well-arranged pore size distribution prior to the 3D scaffold development. The FDM method also allows for control over the chemical, mechanical and physical properties of the scaffold while maintaining the reproducibility of the scaffold production (105, 442). For the FDM method, the glass powders must have a specific diameter size for it to be able to pass through the nozzle tip, and polymeric materials with a specific melting temperature need to be used (318). So far, studies using glass powders or ceramics mixed with PCL to fabricate 3D scaffolds had particle sizes ranging from 38 to 50 μm (105, 443). The FDM method is also interesting as it allows the production of highly porous scaffolds (classically 70 to 75% porosity (105, 442, 444, 445)) with variable pore sizes and with different lay-down patterns. By modifying such lay-down patterns and the blending conditions, it is possible to create scaffolds with tailored mechanical and *in vivo* performances.

When PCL and bioactive glass composites (PCL/BAG) (10 weight per cent, particle size $\leq 50 \mu\text{m}$) were fabricated with a lay-down pattern of 0-90° and 0-60-120°, and were subjected to compressive testing (443), the scaffold possessed the highest compressive strength in the x-axis compared to the PCL only scaffolds. The addition of BAG particles into the PCL structures improved the PCL/BAG performance compared to PCL alone (443). In the same study, it was also shown that the scaffold with a lay-down pattern of 0-60-120° and 0-90° designs with 400 μm vertical pores possessed the stiffest geometries in the z-axis direction during compression testing (443).

In a recent study, Berner *et al.* fabricated silanized polycaprolactone/tricalcium phosphate scaffolds (PCL/TCP(si)) (80:20) and carbonated hydroxyapatite-coated scaffolds (PCL/TCP(si)-CHA) (80:20) with lay-down patterns of 0-90° and 0-60-120°, where they had 67-71% porosity with pore sizes ranging from 420 to 500 µm (442). Both types of the coated scaffolds were implanted in a critical-size defect within the cranial bone of male Lewis rats (442). After 12 weeks of implantation, the scaffolds with a lay-down pattern of 0-90° had generated significantly more bone within the pores and possessed higher tissue stiffness compared to the scaffolds with a lay-down pattern of 0-60-120°. The scaffolds with a lay-down pattern of 0-60-120° had generated bone at the periphery near the host tissue. Although the findings of their study were not favourable towards the lay-down pattern of 0-60-120° *in vivo* (442), such a design remains appropriate for the initial characterisation of CoBG/PCL construct *in vitro*. In our study, we decided to use the CoBG/PCL scaffold with a lay-down pattern of 0-60-120° to assess the biological potential of these scaffolds with DPSC and hMSC. The knowledge gained will provide insight into the potential of using CoBG as bone replacement agents and dental materials for regenerative therapy. Our initial results showed that the CoBG/PCL scaffolds showed good biocompatibility for both DPSC and hMSC. They also triggered the hypoxic response in these cells by the cobalt released from the scaffold, which activated the HIF-1 pathway and subsequently resulted in the secretion of VEGF and are therefore likely to promote vessel formation.

6.2 Materials and methods

6.2.1 Cell culture

In this chapter, dental stem cells from the permanent tooth (DPSCs) and human mesenchymal stem cells (hMSC) were studied. Human umbilical vein endothelial cells (HUVEC) were also used in the migration assay.

6.2.1.1 Dental stem cells from permanent tooth (DPSC)

Dental pulp stem cells (DPSC) were cultured and expanded in low glucose Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% (v/v) foetal bovine serum (FBS, Gibco), and 1% (v/v) penicillin/streptomycin (Gibco). During initial culture, the cells were seeded and grown in a 75 cm² flask in a

humidified atmosphere of 95% air with 5% CO₂ at 37°C. The growth medium was changed every 2-3 days. Once the cells reached 90% confluence, they were detached using 0.25% (w/v) trypsin-EDTA (Gibco). The cell count was performed using a haemocytometer. The cells were resuspended in complete medium according to the appropriate concentration for the designated assay. Only cells at passage 4 to 8 were used in this chapter. The cells were seeded for different time periods on the CoBG/PCL scaffolds in growth medium in tissue culture treated well plates, where the cell-culture medium was changed every 2-3 days. 100 µM CoCl₂-containing media was used as a positive control.

6.2.1.2 Human mesenchymal stem cells (hMSC)

Human bone-marrow derived mesenchymal stem cells (hMSC) were purchased from PromoCell GmbH (Heidelberg). The cells were initially expanded in the proprietary PromoCell MSC growth medium (PromoCell GmbH Heidelberg) until passage 5. Prior to use, the cells were expanded in low glucose DMEM supplemented with 10% (v/v) foetal bovine serum, and 1% (v/v) penicillin/streptomycin. During initial culture, the cells were seeded and grown in a 75 cm² flask in a humidified atmosphere of 95% air with 5% CO₂ at 37°C. The growth medium was changed every 2-3 days.

6.2.1.3 Human umbilical vein endothelial cells (HUVEC)

The endothelial cell growth medium with bullet kit (EGM-2, Lonza, USA) were used to culture and expand the HUVEC cells purchased from Lonza (USA). They were expanded in tissue culture flasks and passaged when they reached 80% confluence. The medium was changed every 2-3 days. Only cells at passage 7 to 9 were used.

6.2.2 Preparation of CoBG/PCL scaffolds

6.2.2.1 Preparation of CoBG glass powder

Cobalt-containing bioactive glasses (CoBG) were prepared by a melt-quenched route as previously described (121), with compositions listed in Table 6.1. The glass powders were sieved to obtain particles ≤ 20 µm, and were characterised using a particle size analyser (Malvern Instruments, UK). The particle size distribution analysis showed that 50% of the glass powders had a particle size less than 11 µm and 90% of the glass powders have particle size distribution less than 26 µm (Table

6.2). The glass powders prepared were then sent to our collaborators for fabrication of the 3D scaffold.

Table 6.1: The designed compositions of the CoBG used in this study in mole percentages. Adapted from (121).

BGs	SiO₂	P₂O₅	CaO	Na₂O	CoO
0Co	49.46	1.07	23.08	26.38	0.0
1Co	49.46	1.07	22.08	26.38	1.0
2Co	49.46	1.07	21.08	26.38	2.0
4Co	49.46	1.07	19.08	26.38	4.0

Table 6.2: The particle size distribution for CoBG glass powder[†] (Mean $\pm$ SD).

Samples	D₁₀ (μm)	D₅₀ (μm)	D₉₀ (μm)
0CoBG	2.16 $\pm$ 0.02	10.86 $\pm$ 0.08	25.15 $\pm$ 0.09
1CoBG	2.22 $\pm$ 0.01	10.92 $\pm$ 0.00	25.55 $\pm$ 0.08
2CoBG	2.06 $\pm$ 0.01	10.03 $\pm$ 0.08	23.80 $\pm$ 0.26
4CoBG	2.11 $\pm$ 0.01	10.02 $\pm$ 0.01	23.68 $\pm$ 0.06

[†]D₁₀ (approximately 10% of the cumulative volume particle distribution for CoBG glass powder were < 3 μ m), D₅₀ (approximately 50% of the cumulative volume particle distribution for CoBG glass powder were < 11 μ m) and D₉₀ (approximately 90% of the cumulative volume particle distribution for CoBG glass powder were < 26 μ m)

6.2.2.2 Preparation of CoBG/PCL scaffold sheet

The CoBG/PCL scaffolds were prepared by Professor Dietmar Hutmacher's group from Queensland University of Technology (QUT) in Australia (Appendix 6.1). They were stored in a desiccator prior to the experiments. Bioactive glass powder (control/CoBG) with particles size of $\leq 20\mu$ m were incorporated into the PCL bulk by fast precipitation into excess ethanol. Briefly, 10% w/v PCL solution was prepared by

dissolving the PCL pellet (3.0 mm, CAPA 6500) in chloroform (100% concentration, MERCK) at room temperature. Then, 30 weight percent (wt %) of BG relative to the PCL mass was added into the PCL solution and stirred until a homogenous mixture was achieved. The PCL/BG solution mixture was then precipitated into 5-fold excess of 100% ethanol (MERCK). The solid PCL/BG composite was then isolated and air dried. All scaffolds were fabricated with melt extrusion-based additive manufacturing technology using an in-house screw-based extrusion system (317). Briefly, composite material (CoBG/PCL) was loaded into the material reservoir and melted at 95°C for PCL and 110°C for composite material. As pressure is applied, molten material moved towards the rotating screw chamber and extruded through the nozzle tip aided by the rotational movement of the screw. All scaffolds were designed with a lay-down pattern of 0-60-120°, filament gap of 2 mm, and slice thickness of 0.35 mm. Scaffolds of 50 × 50 × 2.5 mm were fabricated. All scaffolds were fabricated using 21G nozzle. Ambient temperature in the lab was maintained at 25 ± 2°C

6.2.2.3 Preparation of CoBG/PCL scaffold prior to cell seeding

The CoBG/PCL sheets that were obtained (approximately 50 x 50 x 2.5 mm) were cut into small individual scaffolds measuring 4 x 4 x 2.5 mm in size. Each of the cut scaffolds was weighed to ensure that all had a similar weight (13.4 ± 0.1 mg). The samples were then soaked in 5M NaOH for 1 hour to reduce the hydrophobicity of the surface (446) to promote cell attachment. Following that, the scaffolds were washed twice with PBS and sterilized with 70% (v/v) ethanol/water solution for 10 minutes. To remove the ethanol, the scaffolds were submerged into PBS (10 ml) then spun at 1000 rpm for 15 minutes, which was repeated three times. The CoBG/PCL scaffolds were then dried overnight under laminar flow and kept in a sterile environment until ready to be used. Prior to culturing with cells, the scaffolds were placed inside 48-well plates (1 scaffold/well) and exposed to UV light for 40 minutes. Then, 500 µl of supplemented cell culture medium (DMEM + 10% (v/v) FBS + 1% (v/v) penicillin/streptomycin) was added to the wells and incubated at 37°C for 24 hours for equilibration. These media were removed prior to cell seeding on the scaffold.

6.2.4 Elemental analysis

The concentrations of ions released from the CoBG/PCL scaffolds incubated with cell culture medium (DMEM) were assessed. Initially, the measurements were without cells (medium refresh daily), but the later assessments were done on culture medium from the scaffolds with cells seeded on them. The CoBG/PCL scaffolds were placed in 48-well plates and 500 μ l of DMEM cell culture medium was added in each well. At the indicated time points, the medium was collected and replaced by fresh culture medium. The collected cell culture media were filtered (0.2 μ m syringe filter) and stored at -20°C prior to the measurement. The samples were then diluted by a factor of 20 in deionized (DI) water, and the concentrations of calcium (Ca), silicon (Si), phosphorus (P), and cobalt (Co) ions were quantified by inductively coupled plasma – optical emission spectrometer (ICP-OES) (iCAP 6000, Thermo Scientific, Waltham, MA, USA). The standard curve of each ion was prepared according to the manufacturer's instructions, and each sample was read in triplicate.

6.2.5 Cell seeding method on the scaffold and adhesion assay

Due to the high porosity of the scaffolds, various methods of cell seeding onto the scaffolds were initially tested and compared.

- Seeding using a rotator

For the rotator method, the scaffold was placed in a microcentrifuge tube with 500 μ l of cell culture medium, and then 10 μ l of cell suspension (1×10^5 cells/scaffold) was added to the microcentrifuge tube, which was then placed on a rotator at 37°C. After 2 hours, the scaffolds and the remaining cell culture medium were collected and placed into a separate 48-well plate and incubated for 2 hours prior to assessment using an Alamar Blue (AB) (Invitrogen) assay. Occasionally, the rotator direction was changed from a clockwise to an anti-clockwise direction to promote uniform attachment.

- Seeding in static conditions

For the static seeding method, 10 μ l of cell suspension (1×10^5 cells /scaffold) was seeded on the scaffolds inside a 48-well plate, and 500 μ l of growth medium was added to each well. In an initial study, the culture medium was added directly to

each well resulting in fewer cells present on the scaffold (data not shown). Then, the plate was incubated for 2 hours at 37°C. After that, the scaffolds and the remaining cell culture medium were collected and placed into a new 48-well plate prior to assessment using AB. It was then decided to leave the cells on the CoBG/PCL scaffold for 1 hour prior to the addition of culture medium. A standard curve for each cell type was prepared following the incubation period of the test cells, and the number of cells attached to the scaffolds and the remaining cells inside each microcentrifuge tube and well plate were counted.

An adhesion assay was performed to evaluate the efficiency of the seeding method on the scaffold. Briefly, 10 µl of cell suspension (1×10^5 cells /scaffold) was seeded on the scaffolds in a 48-well plate and incubated for 1 hour before the addition of 500 µl of cell culture medium per well. Then, the plate was covered and incubated for 2 hours at 37°C. After that, the scaffolds and the remaining cell culture medium were collected and placed in a new 48-well plate prior to assessment of cell adhesion using AB. A standard curve for each cell type was prepared following the incubation period of the test cells.

The static method and adhesion assay protocol was adopted for each subsequent experiment performed in this chapter. The cells were left undisturbed for 1 hour at 37°C after cell seeding prior to the addition of 500 µl culture medium, and then incubated for 2 hours at 37°C followed by transferring the scaffolds with attached cells to a new 48-well plate. This is important to ensure that only cells attached on the scaffold were measured in the assays.

6.2.6 Alamar Blue assay

Alamar Blue (AB) (Invitrogen) was used to assess the viability of DPSC and hMSC seeded on the CoBG/PCL scaffold over time. The AB assay was performed following the manufacturer's instructions. Cells were seeded on the scaffold with different concentrations of CoBG and incubated in a CO₂ incubator (Panasonic, USA) at 37°C with 5% CO₂ as described in Section 6.2.4. At the designated time points (1, 2, 4, 7, and 14 days), the cell culture media were taken out from the well plates followed by washing steps with 250 µl of PBS. 500 µl of 10% (v/v) AB in DMEM with no phenol red (Gibco) was added per well (including one with no cells to be used as blank), and

the well plates were incubated for 2 hours at 37°C, after which 200 µl of the reaction product was transferred to a black Costar 96-well plate in duplicate. The fluorescence of AB was read with an excitation wavelength of 544 nm and emission at 590 nm using a microplate reader (SpectraMax M5, Molecular Devices).

6.2.7 Analysis of VEGF release

The VEGF release in the DPSC and hMSC culture supernatant was analysed using the VEGF A₁₆₅ ELISA kit (Quantikine Human VEGF Immunoassay, R&D Systems, USA), which uses the quantitative sandwich enzyme immunoassay technique. The test was performed according to the manufacturer's instructions. Briefly, DPSC and hMSC were seeded on the COBG/PCL scaffold in 48-well plates (1×10^5 cells/scaffold), as described in Section 6.2.4. The medium in the well plates was changed every 2 days, and at 2, 4 and 6 days the cell culture supernatants that have been in contact with cells for 48 hours were collected and added to the provided 96-well plate (pre-coated with a monoclonal antibody specific for VEGF A₁₆₅) together with the standards and blanks. Any VEGF present inside the well is bound by the immobilized antibody during the 2 hour incubation period. Later, each well was flooded with washing buffer to remove any unbound substances. It was then followed by the addition of an enzyme-linked polyclonal antibody specific for VEGF. Following the washing step to remove the unbound antibody-enzyme reagent, a substrate solution was added to the wells. This step resulted in colour development and the colour developed within the wells was in proportion to the amount of VEGF A₁₆₅ present. The colour development was stopped and the intensity of the colour measured using a microplate reader (SpectraMax M5, Molecular Devices) at a wavelength of 450 nm with a reference wavelength of 540 nm.

6.2.8 Alkaline phosphatase (ALP) activity

Substrate p-nitrophenyl phosphate was used to measure ALP activity, as a marker of osteoblast differentiation and mineralisation. Cells were seeded onto the scaffolds as described above and cultured in osteogenic medium for up to 7 days. The osteogenic medium was prepared fresh as previously described (4): DMEM containing 10% (v/v) foetal bovine serum, 1% (v/v) penicillin/streptomycin, 100 µM ascorbic acid (Sigma-Aldrich, UK), 100 nM dexamethasone (Sigma-Aldrich, UK), and 10 mM β-glycerophosphate (Sigma-Aldrich, UK). Cell culture medium was removed

from each well, followed by washing steps and lysis using 0.9% (v/v) Triton X-100 with freeze-thaw cycles. The cell lysates were prepared and reacted with 1 mg/ml p-nitrophenyl phosphate in alkaline buffer (1.5 M) prepared according to manufacturer's instructions. The colour development that occurred during the reaction was stopped with 1 M NaOH and the absorbance was measured using a microplate reader (SpectraMax M5, Molecular Devices) at a wavelength of 410 nm. The ALP concentrations were calculated using a standard curve prepared with paranitrophenol (Sigma) at concentrations between 0 and 200 μ M according to the manufacturer's instructions.

6.2.9 Total DNA assay

Total DNA from the same cell lysates was determined using a Quant-iT PicoGreen Kit (Molecular Probes, UK) according to the manufacturer's instructions. Briefly, cell lysates were prepared and Pico Green was added. The fluorescence was measured with an excitation wavelength of 485 nm and an emission wavelength of 535 nm on a microplate reader (SpectraMax M5, Molecular Devices). The amount of DNA was used to determine the number of total cells in the cell lysates and to normalise the VEGF and ALP activity. The DNA concentrations were calculated using a standard curve prepared with DNA stock solution at concentrations between 0 and 1000 ng/ml according to the manufacturer's instructions.

6.2.10 Analysis of *HIF1A* Expression using Reverse-Transcription and Real-Time Polymerase Chain Reaction

The hypoxia-mimicking effect on DPSC and hMSC was further assessed by real-time quantitative PCR to measure the *HIF1A* mRNA expression. DPSC and hMSC (5×10^4 cells/cm²) were seeded on the CoBG/PCL scaffold in 48-well plates. At 0-24 hours, total RNA from the cells that adhered on the scaffolds were extracted using an RNeasy Mini Kit (Qiagen, Germany). Approximately 100 ng of total RNA was used to synthesise cDNA (Quantitect Reverse Transcription Kit, Qiagen, Germany). Quantitative PCR (Quantitect SYBR Green RT-PCT kit, Qiagen, Germany) was performed on a RotorGene (Applied Biosystems) with a 15 min holding step (95°C), followed by 40 cycles of denaturation at 94°C (15 sec), annealing at 60°C (30 sec), and extension at 72°C (30 sec). All samples and controls were run in quadruplicate. The *HIF1A* mRNA expression was calculated following normalization to a

housekeeping gene, *GAPDH*, and expressed as relative expression compared to the T0 control samples (Table 6.3) using the $2^{-\Delta\Delta C_t}$ method (421). The T0 samples are cells seeded on the scaffold in growth medium collected at the same time of the start of the incubation time for the other samples, as mentioned in Section 6.2.4, where the T0 samples were collected during the change to a new 48-well plate.

Table 6.3: The human primer sequences used for real-time PCR

Gene	Primers	References
<i>GAPDH</i>	Forward: GGCATGGACTGTGGTCATGAG Reverse: TGCACCACCAACTGCTTAGC	(422)
<i>HIF1A</i>	Forward: CAAGCCCTGAAAGCGCAAGTCCTC Reverse: GTGGCAGTGGTAGTGGTGGCATTAGC	(117)

6.2.11 Migration assay

A migration assay was performed on HUVEC as a means to assess the hypoxia-mimicking effect of CoBG/PCL scaffolds on endothelial cells migration directed by the release of VEGF from DPSC and hMSC. Cells in hypoxia produce and release VEGF into the surrounding medium, which has a chemotactic effect on endothelial cells. The CoBG/PCL scaffolds were prepared as mentioned above. The scaffolds used were pre-treated with cell culture medium before being used on cells (DPSC and hMSC). Two sets of DMEM were prepared: For set 1, DMEM without any supplements was conditioned with CoBG/PCL scaffold exempt of any cells for 48h at 37°C. For set 2, DMEM, without supplements, was incubated for 48h at 37°C with scaffolds previously seeded with DPSC or hMSC. In this experiment, only four conditions were tested for each set of DMEM: control (no BG), 0% and 2% CoBG, and 100 μ M CoCl₂. The schematic for scaffold preparation is shown in the timeline below (Fig. 6.1).

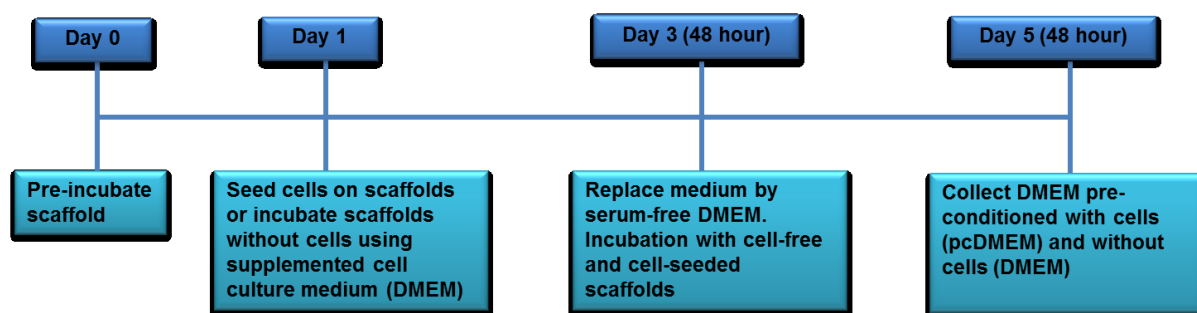


Fig. 6.1: The schematic for pre-conditioned DMEM preparation for migration assay.

In the migration assay, Transwell inserts (Corning, USA) with 8 μm pore size were used, which was based on the Boyden chamber concept. The upper part of the chambers was filled with cells, and the chemoattractant (in our case the DMEM conditioned with or without cells) is placed at the bottom of the well, as shown in Fig. 6.2. The cells will then migrate towards the chemoattractant and the migrated cells are quantified using techniques such as crystal violet dye or 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) staining.

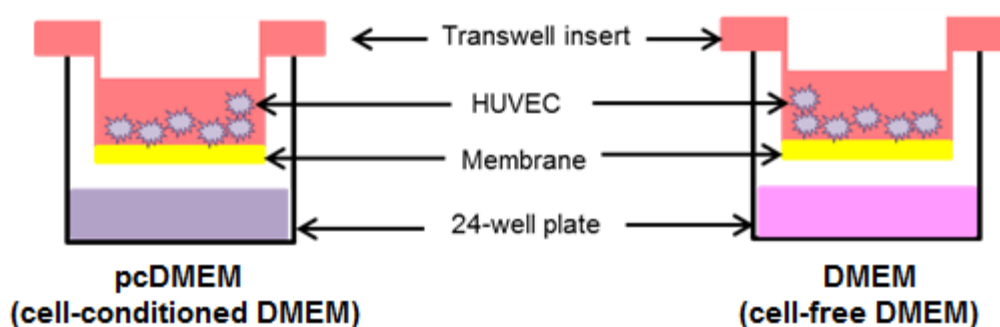


Fig. 6.2: The migration assay where HUVEC will migrate towards the chemoattractant based on the Boyden chamber concept.

For insert preparation, the inner surface of the insert was coated with Fibronectin (0.05mg/ml). The inner surface was coated for 20 minutes, and after that, the outer surface of the inserts (outside base) was also coated for another 20 minutes. The inserts were allowed to dry prior to being used for HUVEC seeding. For cell preparation, HUVEC were cultured using endothelial basal media (EBM-2) complete

growth medium (EGM-2, Lonza, USA) in a 175 cm² flask, and once they reached 80% confluence, they were starved by using EBM-2 containing 1% bovine serum albumin (BSA) for 24 hours. Following that, HUVEC were trypsinized using trypsin/EDTA and resuspended in serum-free DMEM containing 1% BSA for cell counting.

Prior to cell seeding, the prepared serum-free DMEM CoBG-conditioned media (Sets 1 and 2) were mixed with 1% BSA, and 500 μ l of the set 1, set 2, and positive control media were added to each 24-well plate. The migration chambers were then placed on the well plate, and 5×10^4 HUVEC were seeded on the upper part of the migration chamber inserts. The plate was covered and incubated for 6 hours at 37°C. Following that, the cells and remaining media in the upper surface of the migration chamber were carefully discarded using pipette tips and cotton swabs. The migration chambers were transferred to a new 24-well plate containing 500 μ l PBS for a washing step. Then, the cells at the bottom part of the migration chamber were fixed using 3.7% paraformaldehyde (PFA) for 15 min at room temperature (RT) followed by a washing step in 500 μ l PBS. The migration chambers with cells at the bottom were then stained with 200 nM DAPI for 5 min at RT, followed by two washing steps in 500 μ l PBS. For cell counting, the migration chambers were cut at the edges of the insert using a scalpel blade, and mounted between a slide and a cover slip using ProLong Gold Antifade (Invitrogen) mounting medium. The DAPI staining of the nuclei was then captured using a fluorescence microscope, where five different areas (1 mm² each) of squares of inserts were captured, and the nuclei were counted using ImageJ software (<http://rsb.info.nih.gov/ij/>) (447). The percentage of migration was determined using a standard formula based on the manufacturer's guidelines (Corning Lifesciences, USA).

6.2.12 Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) was used to monitor cell morphology and attachment on the CoBG/PCL scaffolds. The cell-seeded CoBG/PCL scaffolds were fixed in 4% (v/v) paraformaldehyde (PFA) in PBS for 30 min at 4°C. The cultured cells adhering to the scaffolds were washed three times using PBS, and then dehydrated through a series of increasing ethanol concentrations starting from 30%, 50%, 70%, 90%, 95% and twice in 99.7% for 10 minutes each. The specimens were

dried at ambient temperature and sputter-coated with gold at 20 mA for 10 min and then scanned using SEM (Jeol JSM6010 LA) operated at 22 kV.

6.2.13 Acti-stain 488 Phalloidin and DAPI staining

The cells seeded on the CoBG/PCL scaffold were rinsed twice with PBS, fixed in 4% (v/v) PFA in PBS for 30 min at 4°C, and washed twice in PBS. They were permeabilized using 0.5% Triton-X-100 for 5 min at RT, and then washed once using PBS followed by incubation with mixtures of 200 nM Acti-stain 488 Phalloidin and 200 nM DAPI for 30 min at RT. Then the scaffolds were washed in PBS and viewed on an inverted confocal fluorescence microscope (LSM-510, Carl Zeiss STM Lta).

6.2.14 Statistical analysis

The data for the experiments are expressed as mean $\pm$ standard deviations (SD). Each experiments were performed at least twice, and prepared in $N=4$ for each composition at a designated time point. The data were analysed using SPSS version 20.0 (IBM, USA) using one-way analysis of variance (ANOVA) with subsequent post-hoc test (Bonferroni procedure) with p -values < 0.05 considered statistically significant.

6.3 Results and discussion

6.3.1 CoBG/PCL scaffold structure and compositions

The CoBG/PCL scaffolds were fabricated in Professor Hutmacher's group using a Bioextruder machine with a lay-down pattern of 0-60-120°, aligned interconnected pores, and a uniform distribution of 70 to 76% porosity. The scaffolds had wide triangular pores and a filament gap (FG) of 2.0 mm (Fig. 6.3). The preparation of the CoBG/PCL composites involved the incorporation of 30 wt % of CoBG containing 0%, 1%, 2% and 4% cobalt (mole percentage) substitution with a particle size ≤ 20 μm (Appendix 6.1). Non-blended PCL scaffolds were produced to be used as control. Prior to cell seeding or ICP-OES analysis, CoBG/PCL scaffolds were immersed in 5M NaOH for 1 hour, which had no effect on pore dimensions or interconnecting pores as previously reported (446, 448). Even though the CoBG/PCL scaffolds were not characterised in regards to mechanical properties or degradation rate, the advantage of NaOH pre-treatment of inorganic compound/PCL composites

on *in vitro* and *in vivo* performance has already been demonstrated. Scaffold pre-treatment with NaOH was indeed shown to enhance PCL/TCP composite degradation rate, surface characteristics and wettability for enhanced matrix deposition and cell attachment *in vitro*, and NaOH treated samples promoted higher bone formation compared to untreated surfaces *in vivo* (444, 446).

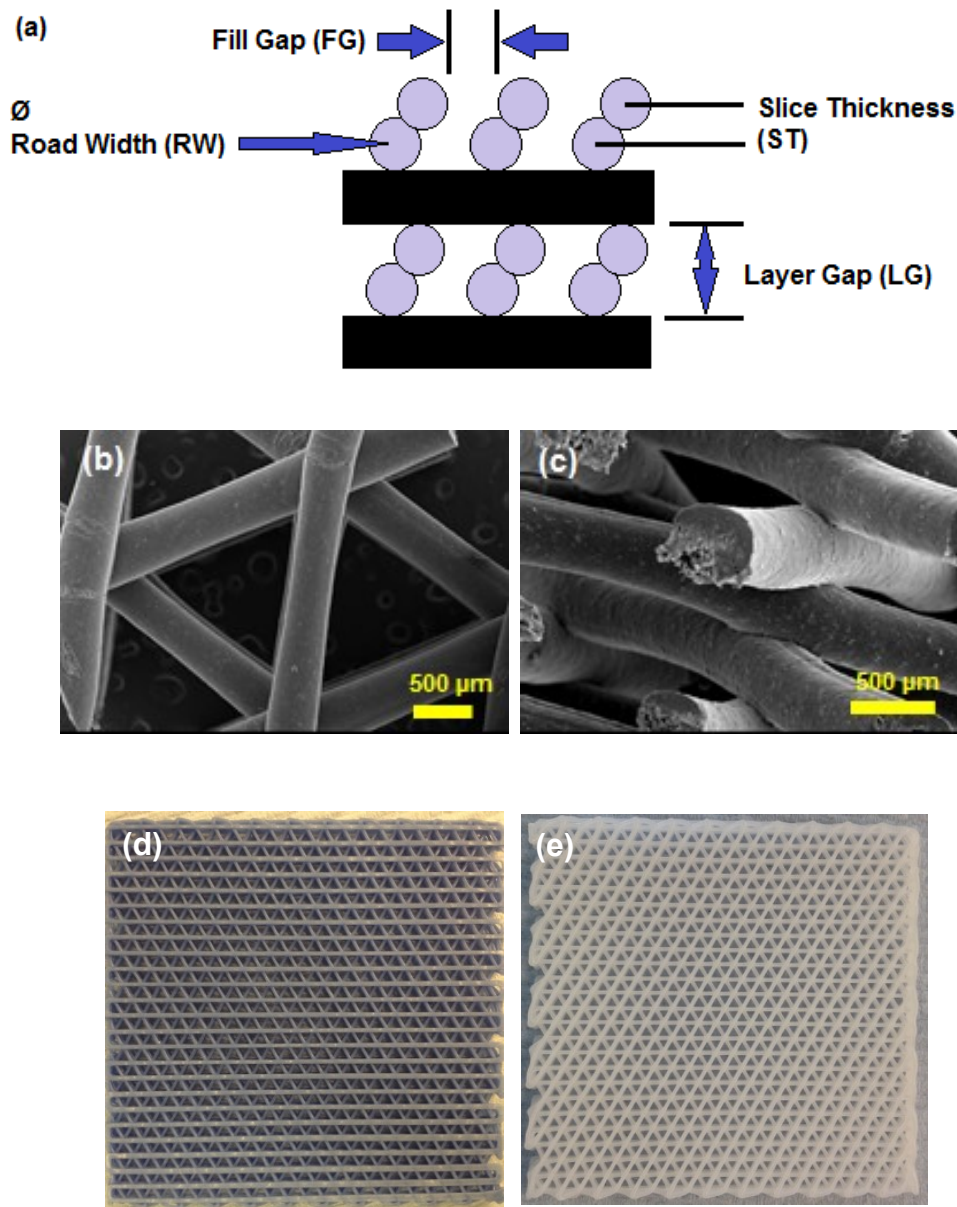


Fig. 6.3: The cross-section of the CoBG/PCL scaffold viewed from XZ-direction of the FDM process, (a) adapted from (318). The 1CoBG/PCL scaffold viewed from the top showing triangular pores (b), and the side view (c). The CoBG/PCL showing purplish discolouration due to the presence of cobalt (d), and the PCL control scaffold (e), both measuring 50 x 50 x 25 mm.

6.3.2 CoBG/PCL scaffolds ion release

6.3.2.1 DMEM ion content following incubation with cell-free CoBG/PCL scaffolds

As described in the previous chapter, incubation of CoBG in cell culture medium resulted in a partial dissolution of the glasses, followed by probable CaP precipitation. These effects were detected by analysing the ion composition in the cell culture media that have been in contact with the glasses. Considering the blending of PCL with 30 wt % of CoBG powder, similar phenomena would be expected following incubation of the CoBG/PCL composites into medium. We assessed the ions that were released or precipitated from/on the glasses by performing ICP-OES studies on scaffolds incubated with DMEM in conditions that would match the cell culture experiments. For this, 500 μ l of culture medium was added to each scaffold in a 48-well plate, and refreshed daily with another 500 μ l of fresh cell culture medium DMEM (DMEM without serum or antibiotic supplements) up to day 13. Therefore, for each time point, the medium samples were in contact with the scaffolds for 24 hours. The ICP-OES measurements were performed on the samples collected on days 1, 2, 5, 7, 11 and 13, and the ionic dissolution profiles are shown in Fig. 6.4.

Calcium (Ca) medium content progressively decreased over time in the presence of all CoBG/PCL samples. A substantial reduction of the calcium concentration was observed from day 5. For the following time points, similar concentrations were measured. No significant differences were observed in regards to the calcium or phosphorous content between the different CoBG glasses. Similarly, the phosphorous concentration slowly decreased from day 1 to day 5, and then became fairly constant from day 5 to day 13. This finding is likely due to the formation of a calcium phosphate (CaP) layer on the scaffold surface, resulting in a decrease of calcium and phosphate in the media (121) due to an initial local dissolution/re-precipitation phenomenon from the CoBG particles contained within the polymer. It has, indeed, been previously shown that entrapment of TCP within such PCL scaffolds triggered CaP layer formation on the material surface upon incubation in simulated body fluid (SBF) within 5 days (445). In our case, the presence of CoBG within the PCL structure seems to have a similar potential.

The silicon (Si) concentration in the culture medium released from the CoBG/PCL scaffolds showed the following pattern: An initial high increase of silicon amount into the medium is observed at day 1, and the amount of silicon released within each 24 hour period decreases over time. By days 11 and 13, the amount of silicon measured in the CoBG/PCL media fell below 10 $\mu\text{g/ml}$. The silicon amount released from the 2CoBG/PCL into the culture medium was almost half the value of the silicon from the 4CoBG/PCL, although the same amount silicon was used during the fabrication of the glass powder. It is hypothesised that this is due to the different cobalt concentration present in the scaffold and indicated that cobalt was taking part in the silicate network (121) causing a decrease in silicon release from the scaffold. In Chapter 5, it was also shown that the silicon release from the 2CoBG powder tended to be lower compared to 4CoBG over a four hour period. Indeed, the silicon release from the 0CoBG, 1CoBG, and 2CoBG was inversely related to the cobalt present in the glass, but not for 4CoBG. As discussed in Chapter 5, higher silicon was released from the 4CoBG powder and also from the 4CoBG/PCL scaffold. We hypothesised that the high cobalt concentration present in the 4CoBG may explain this observation. When more cobalt is available (more than 2 mole% cobalt) in the BG composition, the role of cobalt in replacing a network modifier (calcium) is enhanced. This suggests that cobalt may play a role as network modifier in the CoBG and the effect seen may be due to the compositional design rather than the role of cobalt cation (121).

The release of cobalt from the CoBG/PCL scaffolds within the media followed a decreasing trend from days 1 to 7 and decreased further from days 11 to 13. As expected, the release of cobalt into the culture medium from the scaffold was dependent on the cobalt concentration present within the scaffold. For example, at day 1, the cobalt concentration of the 0CoBG/PCL is 0 ppm (0 μM), for 1CoBG/PCL is 2.27 ppm ($38.54 \pm 13.92 \mu\text{M}$), for 2CoBG/PCL is 2.83 ppm ($48.05 \pm 4.07 \mu\text{M}$), and 4CoBG/PCL is 6.96 ppm ($118.17 \pm 11.88 \mu\text{M}$). According to these ion release patterns, it seems that there is an initial burst release from fast glass dissolution, followed by a slower dissolution of the CoBG from the scaffolds, likely combined with CaP precipitation on the surface. The decrease in ion release over time, with a very low amount released from day 11, may be due to a limited dissolution of the glass in

the centre of the fibres due to limited medium and ion diffusion. It has been previously shown that in an accelerated degradation process using 5M NaOH for up to 7 days, higher release of BG particles was observed within 2 days (105). Importantly, the cobalt released from the scaffold into the surrounding culture medium is within the range for cell survival (258). As expected, there were no differences in ion content of the media in contact with the PCL only scaffolds (control and 100 μM CoCl_2 conditions) over time, indicating that the PCL polymer is not adsorbing/precipitating a significant amount of ions on its surface. The measured cobalt concentration from the 100 μM CoCl_2 control was approximately 5.40 ppm ($91.68 \pm 2.21 \mu\text{M}$).

The controlled release of the cobalt from the CoBG/PCL scaffold highlighted that cobalt release was not compromised by the scaffold fabrication process. For example, after four hours of incubation, the cobalt release from the 2CoBG powder into the surrounding culture medium was estimated to be 31% of the total amount of cobalt present in the glass (as described in Chapter 5), while the cobalt release from the 2CoBG/PCL after 24 hours of incubation was estimated at 2%. In CoBG powder incubated in culture medium (DMEM) at 1.5 mg/ml for four hours, the percentage of the cobalt release for 1CoBG was 32%, 2CoBG was 31%, and 4CoBG was 36%. The incorporation of CoBG powder into the CoBG/PCL scaffold resulted in lower cobalt release due to a lower dissolution of the glass from the scaffold. For instance, at day 1, the 1CoBG/PCL scaffold released about 3% of cobalt, 2CoBG/PCL released 2%, and 4CoBG/PCL released 2%. At day 7, these values decreased to less than 1% for 1Co, 2Co and 4CoBG/PCL, respectively. The calculation for the estimated percentage of cobalt released from the CoBG scaffold was based on the weight of scaffold used in the cell-free ICP-OES assay. The average weight for quadruplicate of each CoBG composition was $13.4 \pm 0.1 \text{ mg}$ (for scaffolds measuring $4 \times 4 \times 2.5 \text{ mm}$) and the volume of media used was 500 μl . From this, only 30 weight per cent (wt %) constituted the CoBG powder, and PCL made up the remaining 70 wt % of the total 0.0134 gram.

In our study, the medium was removed after 24 hours and the ionic concentration was measured, which contributed to the drastic ionic release over time. However, this is not necessarily the case *in vivo*, where the scaffold is implanted and only

retrieved after a certain period of time, if at all, so the cells are probably exposed to the cumulative effect of the ionic release from the scaffold (449). The release of ions from PCL/SrBG scaffold over a week that was also observed in our culture conditions suggested that the incorporation of BG into PCL as a 3D scaffold is beneficial for drug delivery and as a long-term reservoir (105).

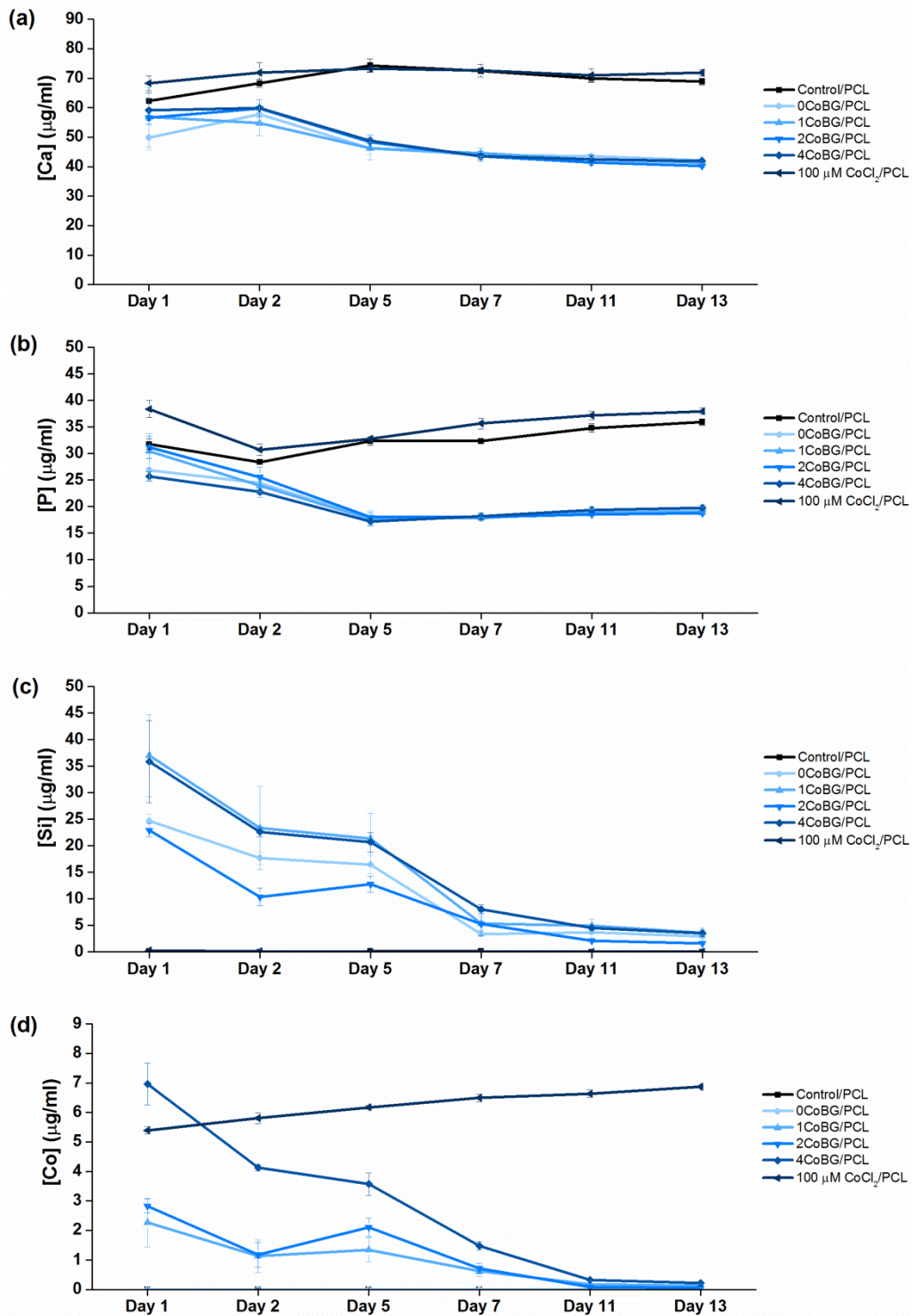


Fig. 6.4: Dissolution ion release profile from CoBG/PCL scaffolds without cells incubated with cell culture medium, DMEM. The DMEM was removed daily, and replaced with fresh medium thereafter. The calcium (Ca) (a) and phosphorus (P) (b) were slowly released from days 1 to 5, and then remained fairly constant up to day 13. The silicon (Si) (c) and cobalt (Co) (d) showed an initial high release from day 1 to 5, and then also remained fairly constant up to day 13.

The cumulated amount of cobalt released and the cumulated percentage of cobalt released from each of the cell-free CoBG/PCL scaffolds are shown in Fig. 6.5a and Fig. 6.5b. The cumulative value was obtained by adding the cumulative value from previous day with the day of observation. The cumulative value was done as a measure to estimate the cobalt release from the scaffold over time. Several assumptions were made in order to estimate the cumulative percentage of cobalt released from the glasses. Although it has not been verified in our study, we first assumed that the percentage of glass into the composite was equal to 30%, which is the percentage initially added into the PCL solution. Furthermore as we described in the materials and method section of this chapter (Section 6.2.3), cell culture medium in contact with the BG was refreshed everyday. The ICP-OES measurements of the conditioned-media were however only performed on specific time points. The cobalt content of the BG-conditioned media obtained on days 3, 4, 6, 8, 9, 10 and 12 had to be estimated. It can be seen that the cumulative cobalt release over 13 days is related to the amount of cobalt present within the scaffold (Fig. 6.5a). The percentage of the cumulative cobalt release from the CoBG/PCL scaffold at day 2 was 5% for 1CoBG/PCL, 3% for 2CoBG/PCL, and 4% for 4CoBG/PCL. At day 13, the percentage of cumulative cobalt release from 1CoBG/PCL scaffold was 15%, 9% for 2CoBG/PCL scaffold and 11% for 4CoBG/PCL scaffold. The 1CoBG/PCL scaffold showed the highest cumulative percentage of cobalt release over 13 days (Fig. 6.5b). The calculated cumulative percentage of cobalt release seems to indicate that only a part of the BG have been dissolved, although a decrease in cobalt and silicon release can be observed for the last days on incubation. As mentioned in Section 6.2.2.2, the PCL pellet was melted before incorporation of the BG powder into the melt, and upon addition of the BG powder, the melt was properly mixed inside the liquefier chamber. We anticipated the BG powder would be evenly distributed in the CoBG/PCL composites, and we found that 45S5 and SrBG powder was homogeneously distributed in the PCL bulk as shown by the micro-computed topography (105). In addition, the presence of BG powder in the PCL bulk created micro-pores on the scaffold surface, which may increase the area for hydrolytic degradation and enhance the degradation of the composites. However, the lower cumulative release of cobalt observed in the current project (only 14% release in 14 days) may be due to surface erosion. Particles on the surface are released first due to slow diffusion of the culture medium into the PCL. The surface erosion of the PCL

will lead to thinning of the CoBG/PCL, which gradually occurs in a slow manner. The remainder of the cobalt is still embedded in the deeper part of the PCL in a very slow degrading polymer. This may be due to a lower accessibility to the BG present in the centre of the PCL fibres because of a limited diffusion of the cell culture medium.

To test this hypothesis, it would have been interesting to dissolve the CoBG/PCL composites on the last day of the experiment to determine the actual amount of cobalt remaining into the structure. Nevertheless, our finding suggested that the cobalt release from the scaffold occurs slowly, and there is probably remaining cobalt within the CoBG/PCL scaffold. This also supports the notion that the CoBG/PCL scaffold is able to act as a reservoir for cobalt.

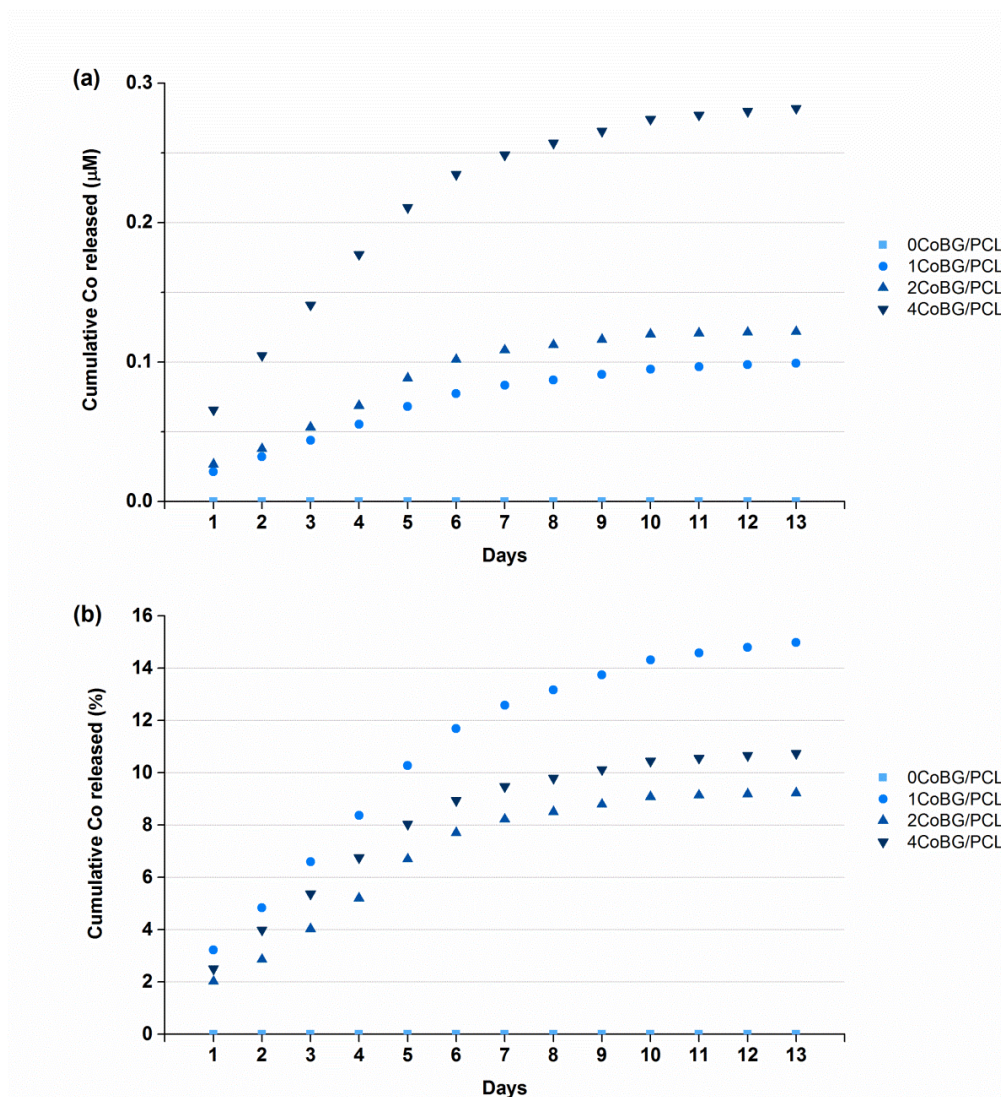


Fig. 6.5: The cumulative of cobalt release was obtained by adding the cumulative value from the previous day with the day of observation (a). The cumulative percentage of cobalt released from the cell-free CoBG/PCL scaffolds in DMEM over time (b). Values for days 3, 4, 6, 8, 9, 10 and 12 were estimated based on the results obtained on the measured time points. The 1CoBG/PCL scaffold showed the highest accumulated cobalt (Co) release over 13 days of incubation.

From the release profile of ionic dissolutions in DMEM, we then decided to incubate the scaffold for 24 hours prior to all subsequent cell experiments to reduce the burst effect of cobalt release from the scaffold. Conditioning the scaffolds with culture medium may also promote CaP deposition on the scaffold surface, stabilise pH and allow biological molecules from the medium to integrate with the CaP layer formed on the scaffold to promote cell attachment.

6.3.2.2 DMEM ion content following incubation with cell-seeded CoBG/PCL scaffolds

For insight into the ionic environment that the cells experience when seeded on the scaffolds, and how their presence may modify the glass dissolution or CaP precipitation from/on the scaffolds, we performed ICP-OES measurements on the culture media that were in contact with the cell-seeded scaffolds.

Prior to seeding cells on the scaffold, the scaffolds were incubated for 24 hours. On the day of the experiment, cells were seeded as described in Section 6.2.4. Then, after 24 hours, the medium was collected when changing it for the Alamar Blue (AB) assay. Thus, for day 1 and 2, the cells were exposed to 24 hours of ionic dissolution from the CoBG/PCL scaffold. The medium was changed at day 4 and 6, and collected again for ICP analysis. However, at day 7, the AB assay was performed for both cells (this corresponds to day 7 of AB assay), and medium was replenished. From day 7, the medium was changed at days 9, 11, and 13, and then at day 14, the cells were subjected to AB assay. The longest exposure of cells adhering on the CoBG/PCL scaffold to the cobalt released was 48 hours.

The culture medium from DPSC and hMSC on the CoBG/PCL scaffold showed that the calcium concentration present in the culture medium decreased from day 1 to 6 and then remained fairly constant from day 6 to 13, while the calcium level in both controls remain unchanged from day 1 to 14 (Fig. 6.6a and Fig. 6.7a). The calcium concentration present in the culture medium from the CoBG/PCL scaffolds did not show a drastic reduction. This is may be due to the formation of CaP on the scaffold surface. As reported previously (22), the constant level of calcium in the culture medium is supported by the decrease of phosphorus concentration, since phosphorus is involved in the CaP formation. In our experiment, the phosphorus concentration in the culture medium was higher from day 2 to day 6 when compared to day 1 for all the conditions, even the control ones. This indicated that these differences in phosphorous concentration may be due to the presence of phosphorous based compounds in the FBS or as a result of cell activity (Fig. 6.6b and Fig. 6.7b). The effect of CoBG incorporation within the composite on the phosphorous concentration in this culture system can be evaluated by comparing the

phosphorous content of the BG/PCL composite-conditioned media to the controls for each time point. The amount of phosphorous in the CoBG/PCL composites tended to be generally lower than the one present in the control samples. This is consistent with the reduction of calcium amount observed in presence of the composite materials and would support the hypothesis of the formation of a CaP layer in the glass-containing samples.

For both cell types, the silicon level in the culture medium conditioned with the CoBG/PCL samples remained fairly constant during the assessed period until it decreased slightly at day 13 (Fig. 6.6c and Fig. 6.7c), suggesting the silicon was released slowly into the culture medium compared to its release from BGs incubated with SBF (314).

In culture medium with both cell types, overall the cobalt release increased from day 1 to 6, and was dependent on the amount of cobalt present in the scaffold (Fig. 6.6d and Fig. 6.7d). 4CoBG/PCL released the highest amount of cobalt compared to other scaffolds, but the AB assay showed no effect on cell viability, indicating the concentration released was non-toxic. From day 6 to 13, the cobalt level in the culture medium decreased, with the exception of 4CoBG/PCL incubated with hMSC which showed a decrease in cobalt release only from day 11. As expected, no cobalt was detected from the control or 0CoBG/PCL scaffold, while the cobalt level in 100 μM CoCl_2 containing medium remained constant. At day 6, the DPSC were exposed to approximately 2.48 ppm ($42.11 \pm 2.89 \mu\text{M}$) cobalt concentration when seeded on 1CoBG/PCL, 3.29 ppm ($55.86 \pm 31.92 \mu\text{M}$) from 2CoBG/PCL, and approximately 7.53 ppm ($127.84 \pm 13.41 \mu\text{M}$) on 4CoBG/PCL. At the same time point, hMSC were exposed to approximately 2.32 ppm ($39.39 \pm 5.43 \mu\text{M}$) cobalt when seeded on 1CoBG/PCL, 2.83 ppm ($48.05 \pm 42.11 \mu\text{M}$) from 2CoBG/PCL, and approximately 4.30 ppm ($73.01 \pm 21.73 \mu\text{M}$) from 4CoBG/PCL. Thus, DPSC and hMSC were continuously exposed to safe dose of cobalt concentration release from the scaffold into the surrounding culture medium (258). Although the concentrations measured here are in the medium, it is possible that the local concentration of cobalt at the vicinity of the scaffolds would be different. Considering the wide pores of the scaffolds, however, diffusion limitations are not anticipated, unlike other materials with smaller and more confined pores (105, 437).

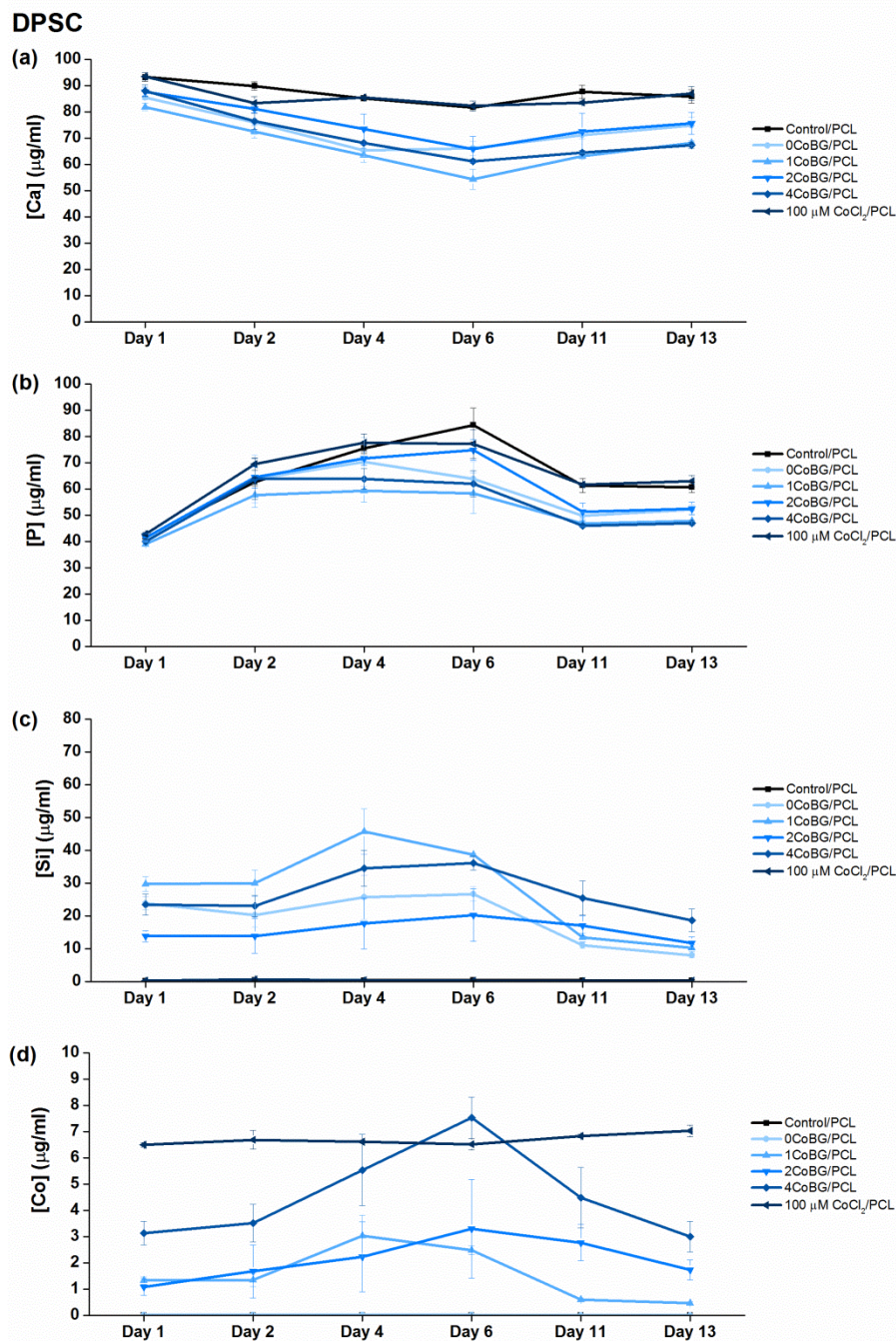


Fig. 6.6: Dissolution ion release profile from CoBG/PCL scaffold cultured with DPSC, and incubated with cell culture medium, DMEM. There was a slow reduction of calcium (Ca) concentration (a) until day 6, and it remained fairly constant thereafter. The phosphorus (P) showed an increasing concentration (b) until day 6 with a slight reduction at days 11 and 13. The silicon (Si) concentration remained fairly constant (c). The cobalt (Co) showed an increasing concentration until day 6, and gradually decreased up to day 13 (d).

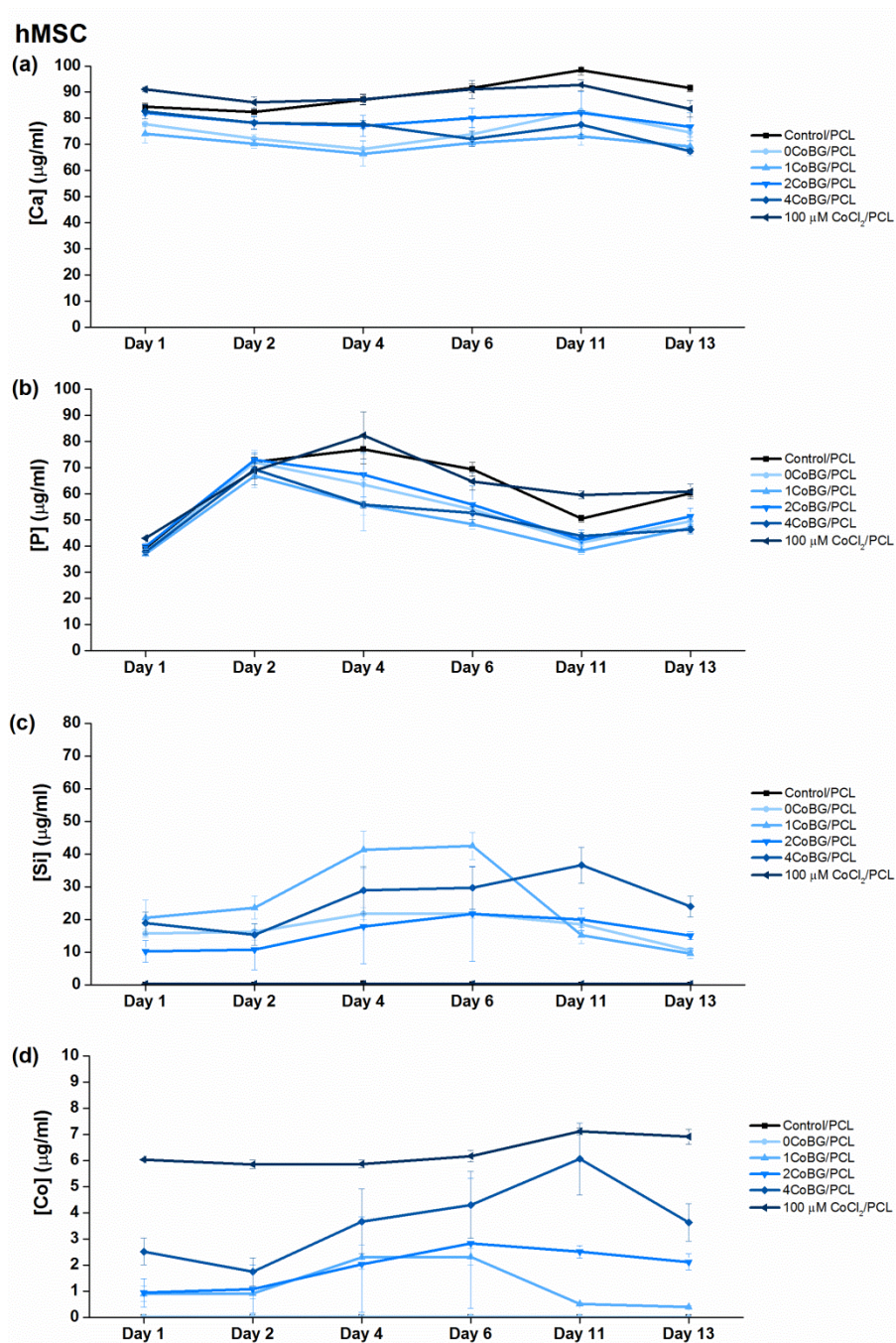


Fig. 6.7: Dissolution ion release profile from CoBG/PCL scaffold cultured with hMSC, and incubated with cell culture medium, DMEM. The calcium (Ca) concentration (a) remained fairly constant. The phosphorus (P) had a higher concentration (b) at day 2, which gradually decreased at day 11. The silicon (Si) concentration remained fairly constant (c). There was an increasing cobalt (Co) concentration (d) until day 6, which gradually decreased up to day 13.

6.3.3 The effects of hypoxia-mimicking BG scaffolds on cell adhesion, viability and proliferation

6.3.3.1 Determining the seeding methods

Considering the high porosity of the scaffold, with pores higher than 500 μm , we anticipated challenges in cell seeding. Accordingly, several methods were compared.

Based on a literature search, many of the scaffolds that have similar design as our CoBG/PCL scaffold use a static seeding method for *in vitro* purposes (105, 446, 448). This may be due to the high porosity and large interconnecting pores that make it difficult to use a dynamic seeding method. Nonetheless, seeding cells onto a porous scaffold remains a challenge, which is especially true if low numbers of cells are used, since sufficient cell numbers are needed before implantation (450). However, it was also shown that for *in vivo* applications, scaffolds should have high porosity to allow better cell penetration to enhance vascularisation (451), and our CoBG/PCL scaffold meets that requirement. Nevertheless, a dynamic seeding method may be suitable for scaffold preparation if the scaffold is designated for cell-based scaffold therapy prior to *in vivo* implantation. It may also be suitable if cells can infuse into the scaffold using a bioreactor before implantation, or when the scaffold is fabricated with these challenges in mind, for example using fibres with different porosities and diameters (40, 442, 450, 452).

For our CoBG/PCL scaffold, we first determined the volume of medium that can be retained within each scaffold. Addition of 10, 20 or 30 μl of cell suspension on top of the scaffolds was tested. For the volumes of 20 and 30 μl , most of the cells passed through the scaffold, and many cells attached to the base of the well plates, while 10 μl of medium could be retained by the scaffolds (data not shown). We then tried two seeding methods for the scaffold: the rotator (dynamic) and static (direct placement of cells on the scaffold) methods.

The static seeding method allowed a higher number of cells to attach compared to the rotator method, although there were significant variations between each scaffold (Fig. 6.8), and some cells were lost when transferring the scaffolds from tubes to well

plates. We then decided to use the static seeding method for the adhesion assay to ensure cells would adhere to the scaffold. Further improvements to this method were tested and used for the following cell studies: after seeding the 10 μ l of cell suspension onto the scaffolds, the samples were left undisturbed for 1 hour prior to the addition of cell culture medium into each well in order to allow pre-adhesion of the cells. The results from the adhesion assay confirmed that the static method was suitable for our scaffold (Fig. 6.9).

A few studies suggested leaving the seeded cells undisturbed for 2 to 3 hours before addition of culture medium (317, 453). However, since we were using a small volume for cell seeding (10 μ l), we opted to leave the scaffold undisturbed for 1 hour. It was previously shown that the cell seeding efficiency using static surface seeding, orbital seeding, centrifuge, injection seeding and dynamic seeding methods did not create any statistical differences in the cell metabolic activity, as measured by an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay (454).

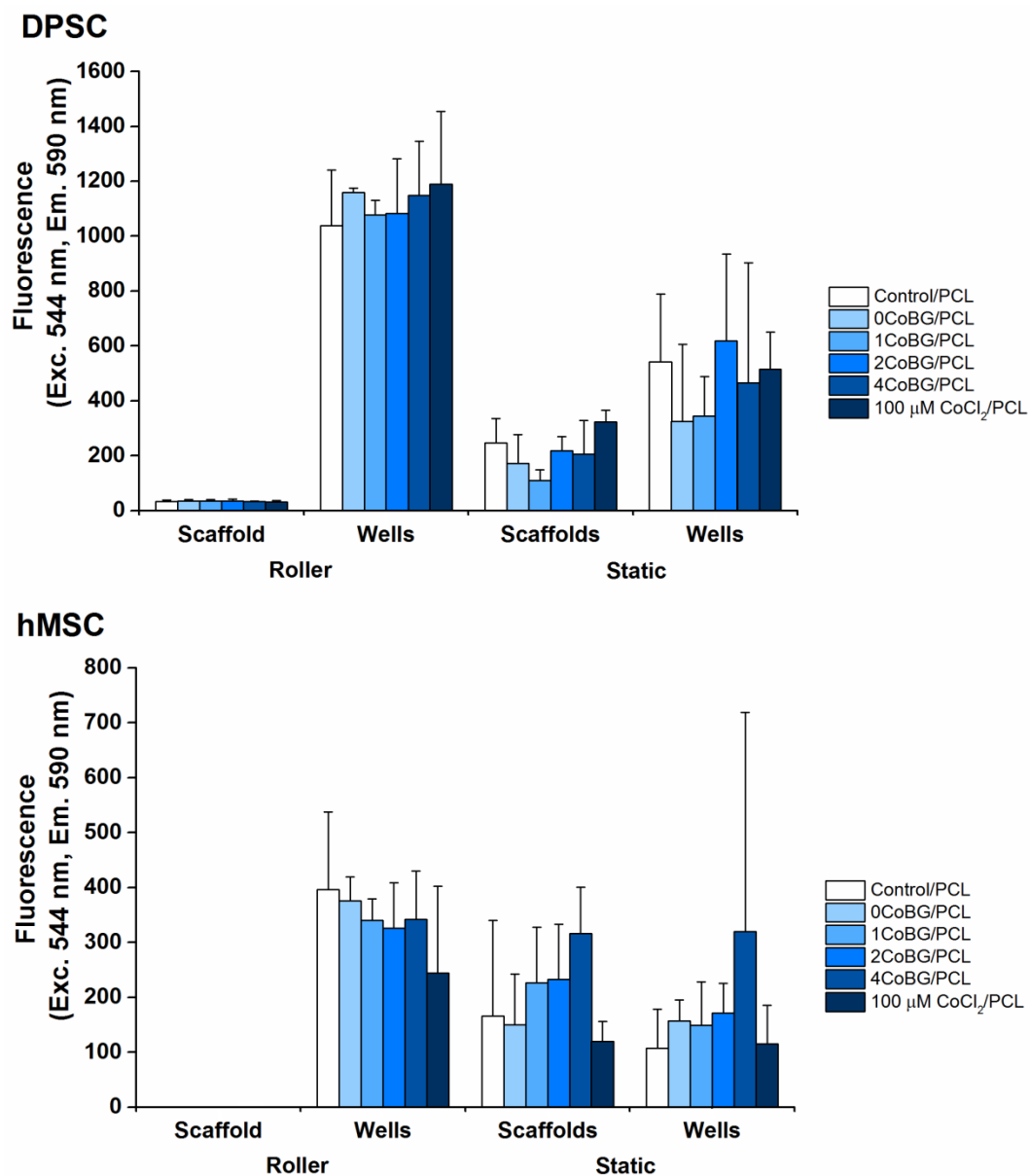


Fig. 6.8: Seeding method: Static seeding method enabled many cells to attach to the CoBG/PCL scaffold compared to the roller method for DPSC and hMSC.

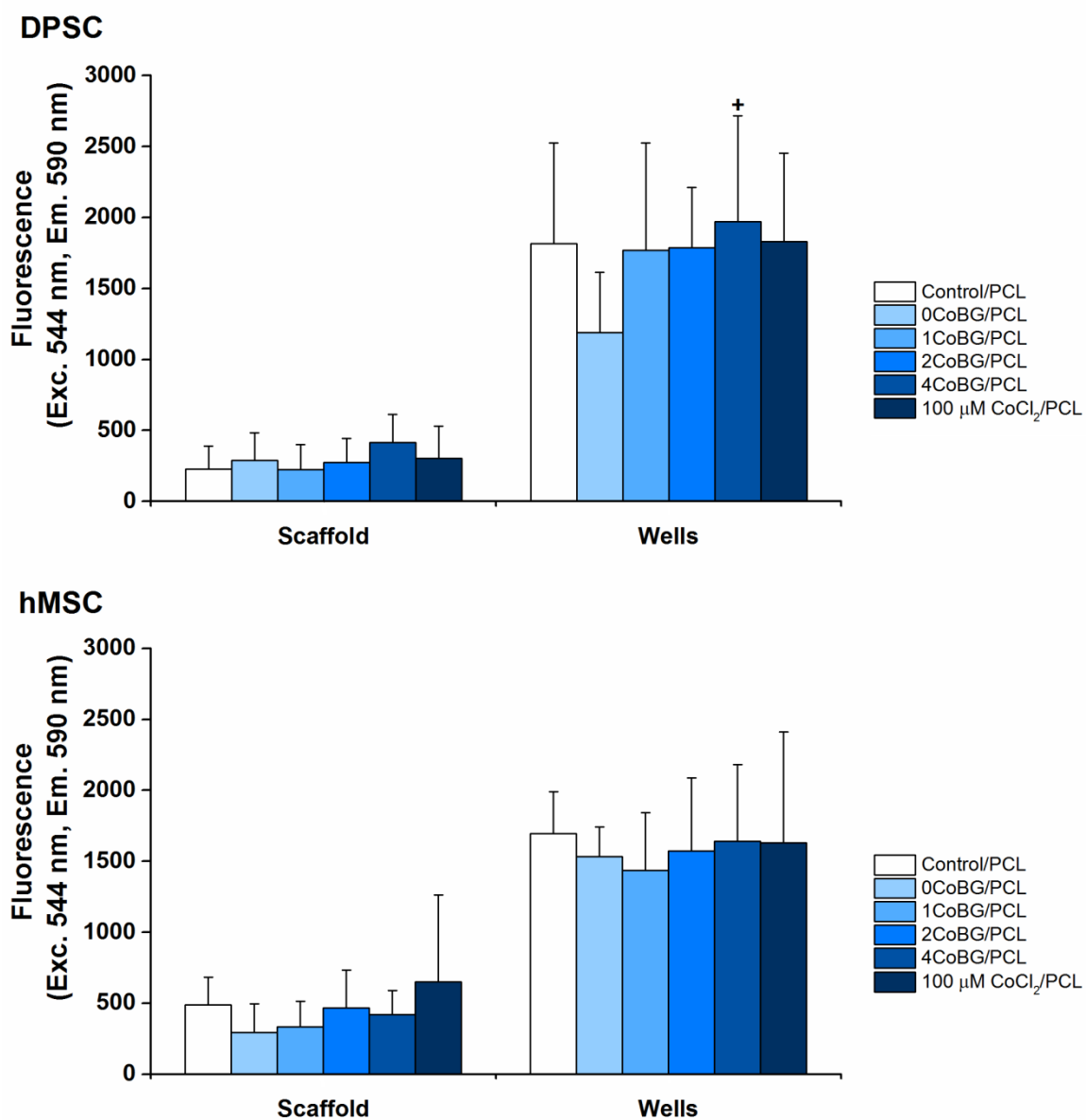


Fig. 6.9: Adhesion assay: Cells (DPSC and hMSC) had adhered to the CoBG/PCL scaffolds two hours after seeding, which was determined with an adhesion assay. * denotes a significant difference of the marked bar compared to the control at the same time point. + denotes a significant difference of the marked bar compared to 0Co at the same time point.

6.3.3.2 Cell metabolic activity on CoBG/PCL scaffolds over time

The effect of CoBG/PCL scaffolds on DPSC and hMSC metabolic activity was assessed using an Alamar Blue (AB) assay. The CoBG/PCL scaffolds were pre-incubated for 24 hours before seeding.

6.3.3.2.1 DPSC metabolic activity on CoBG/CPL scaffolds

Based on the AB assay, the DPSC were able to proliferate on the scaffold and showed an increasing metabolic activity – that is often considered proportional to be number of cells - up to day 14 on all CoBG/PCL scaffolds and the positive control of 100 μ M CoCl₂-containing culture medium, with no statistical differences between the samples. This result suggested that the level of cobalt released from the CoBG/PCL scaffolds into the surrounding culture medium may be within the physiological range for cell culture (258).

Since variations in cell number on each scaffold following adhesion could be measured (as seen in Fig. 6.10), we decided to normalise data to Day 1 and also to assess the viability of cells seeded per scaffold each day. For example, in DPSC seeded on 2CoBG/PCL scaffold, at day 2 the cell viability was 126.4%, and at day 7, it had increased to 619.1%.

Indeed, such differences in adhesion could mask the changes of cell number over time on the scaffolds, so representing the results as percentage compared to day 1 (Fig. 6.11) and individually for each scaffold appeared more appropriate than plotting the absolute values. Indeed, the variability of the number of cells attached to each scaffold and how many were retained during the AB assay contributed to the variations measured in cell metabolic activity.

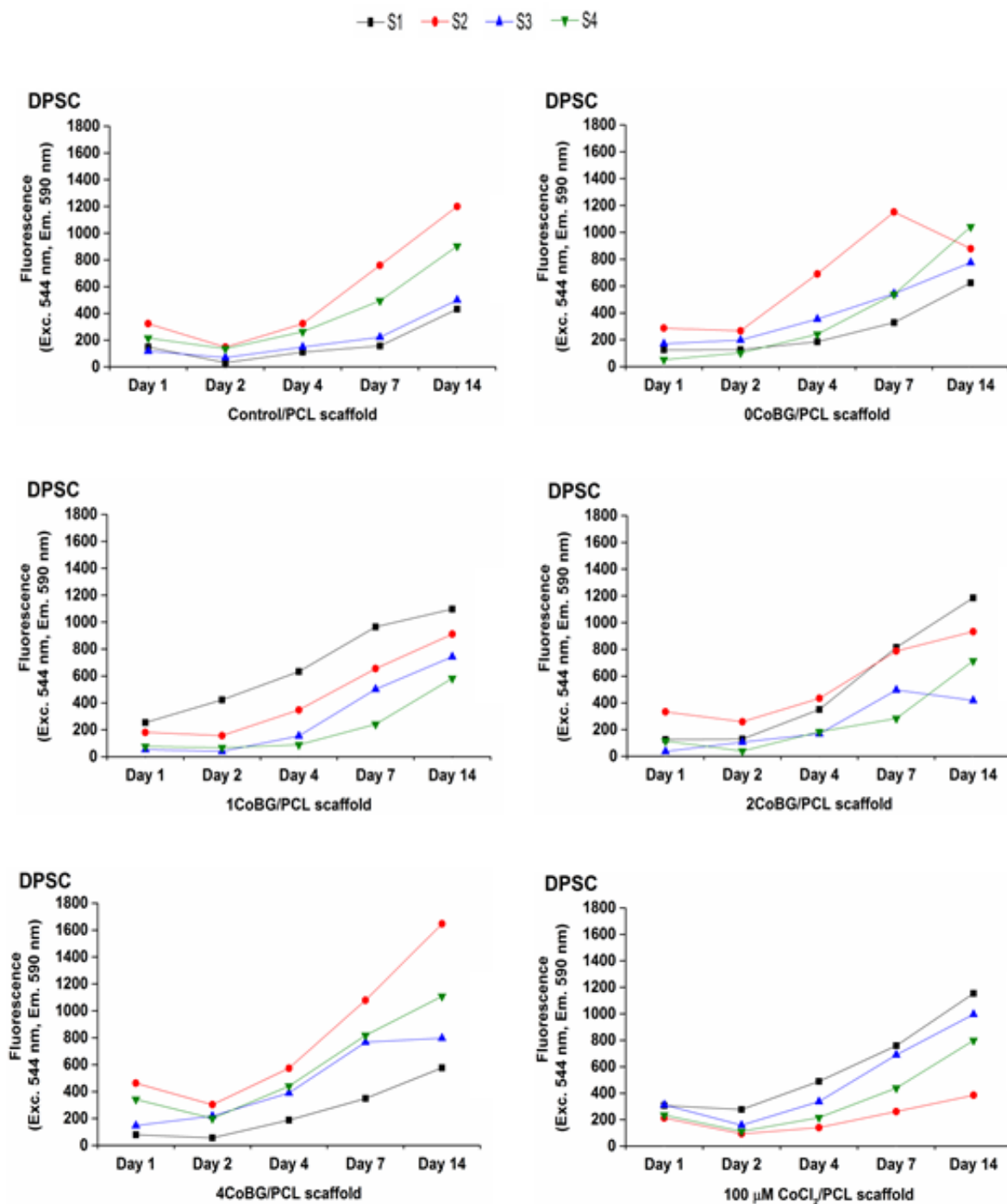


Fig. 6.10: Alamar Blue assay: DPSC showed increasing viability on the scaffolds up to day 14. The scaffolds were prepared in quadruplicates and labelled as S1 for replicate 1, S2 for replicate 2, S3 for replicate 3, and S4 for replicate 4. The variations observed between scaffold replicates contributed to the wide variation in viability.

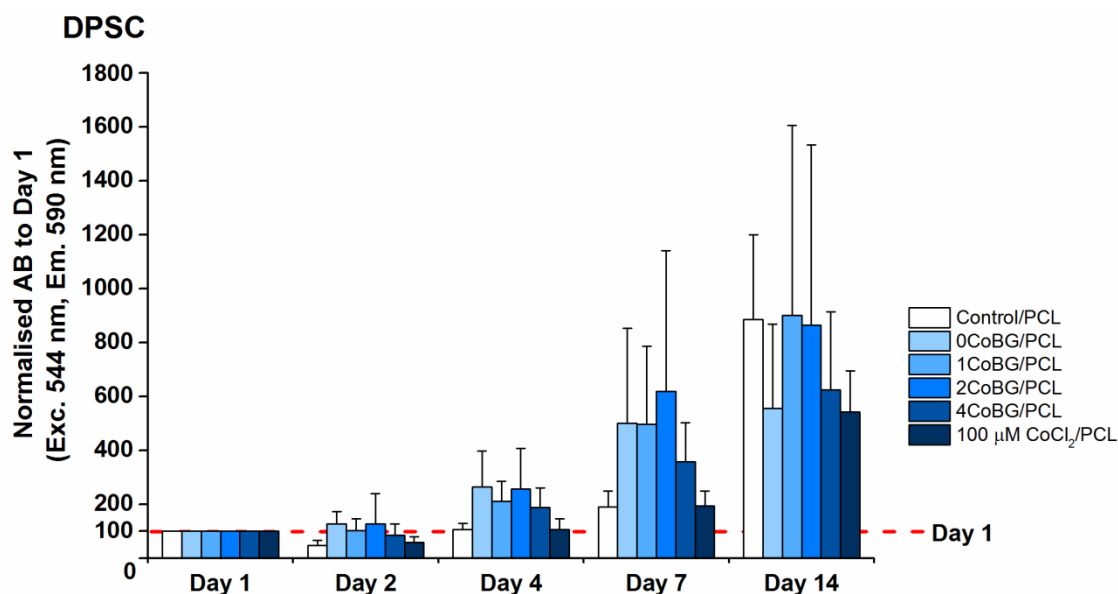


Fig. 6.11: Alamar Blue assay: DPSC progressively showed increased viability from day 2 to day 14 when compared to day 1. There were no statistically significant differences.

Each scaffold was able to retain cells and allow continued cell growth from days 1 to 14. As shown in Fig. 6.10, the cells attached on the CoBG/PCL scaffolds appeared to have almost the same viability as those cultured on control and 100 μ M CoCl₂ PCL samples. These results demonstrated that the presence of CoBG (up to 4% cobalt substitution) within the PCL material was not strongly affecting viability or proliferation of DPSC. The significant increase in cell metabolic activity on all scaffolds was observed as early as day 4. We have shown previously that the CoBG/PCL was still releasing cobalt at that time, confirming that the amount of cobalt released from the samples were not detrimental for cell survival. In general, the results from the DPSC data showed that the CoBG/PCL scaffolds with all cobalt concentrations (1Co, 2Co and 4Co) were able to support DPSC proliferation and maintain cell viability up to day 14.

When dental pulp cells (DPC) from human third molars were subjected to normoxic (20% O₂) and hypoxic (3% O₂) conditions and cultured up to 14 days, the cell number was significantly higher in the hypoxia, which gradually increased from day 0 to 14, compared to normoxia (425). Further to that, the authors showed that DPC in hypoxia expressed higher levels of STRO-1. In the hypoxia group, the STRO-1 positive DPC were more concentrated in a larger group of cells, which correlated to cell proliferation. This suggests that hypoxia enhanced cell proliferation. In addition, DPC subjected to hypoxic (5% O₂) conditions showed higher viability compared to DPC in normoxic (20% O₂) conditions, suggesting that hypoxia enhanced proliferation (455).

6.3.3.2.2 hMSC metabolic activity on CoBG/CPL scaffolds

For hMSC, a decrease in metabolic activity was observed from days 1 to 14 for all conditions (Fig. 6.12). It was unexpected to see that viability was lower in the control and 100 µM CoCl₂-containing medium, but might be explained by the variations in cell number on each scaffold. When the results were normalised to day 1 (Fig. 6.13), the viability on 0Co, 1Co, 2Co and 4CoBG/PCL scaffolds increased from day 2 to 4, but subsequently decreased at days 7 and 14, except for cells seeded on the 0CoBG/PCL scaffold. For example, in hMSC seeded on 4CoBG/PCL, the viability decreased from 188.4% at day 4 to 68.1% at day 14. At day 7, there were no significant differences. At day 14, hMSC seeded on the 0CoBG/PCL scaffold showed significantly higher viability compared to the control and 100 µM CoCl₂-containing medium.

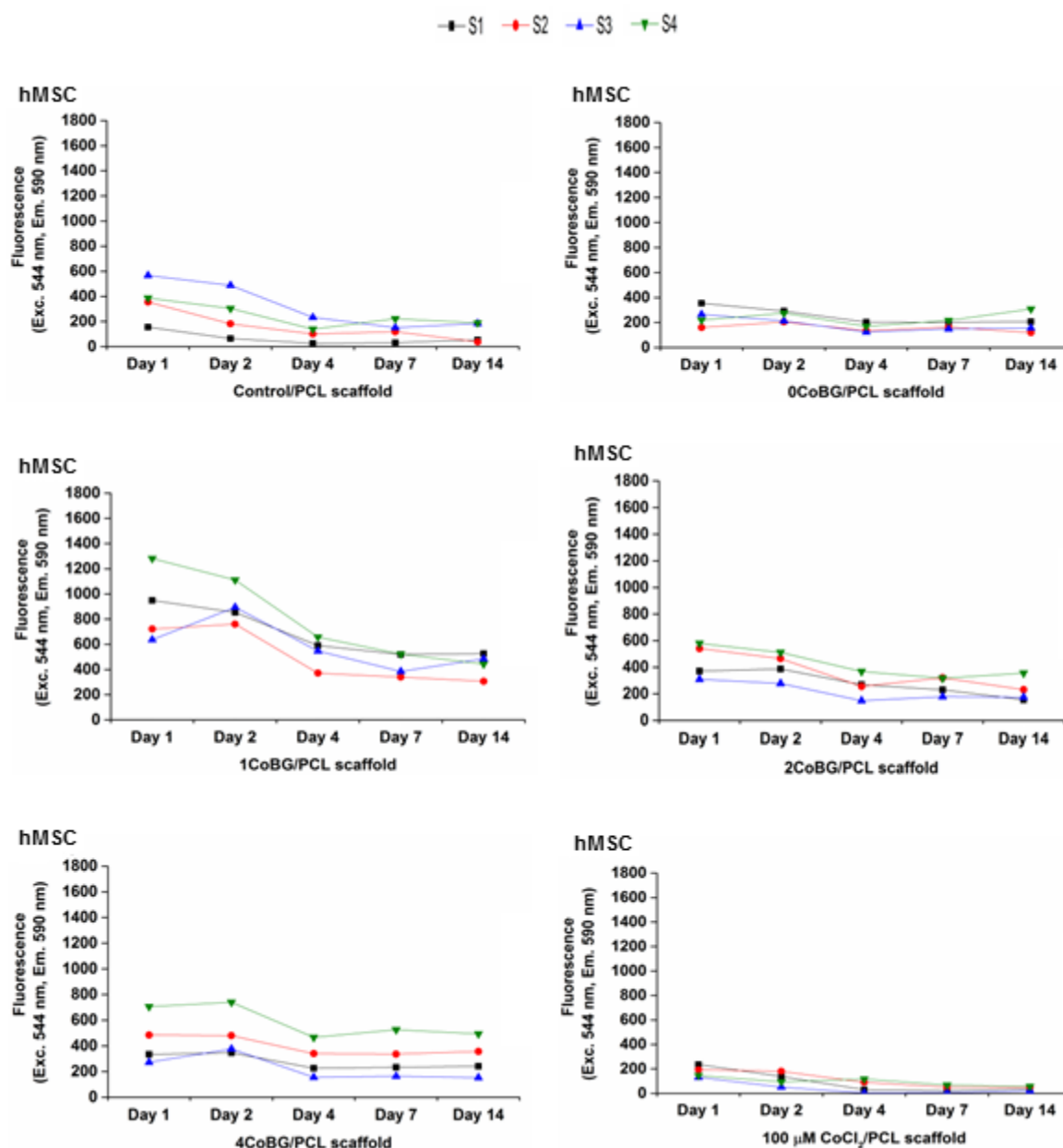


Fig. 6.12: Alamar Blue assay: hMSC on all CoBG/PCL scaffolds maintained continued growth up to day 14 compared to controls. The scaffolds were prepared in quadruplicates and labelled as S1 for replicate 1, S2 for replicate 2, S3 for replicate 3, and S4 for replicate 4. The variations observed between scaffold replicates contributed to the wide variation in viability.

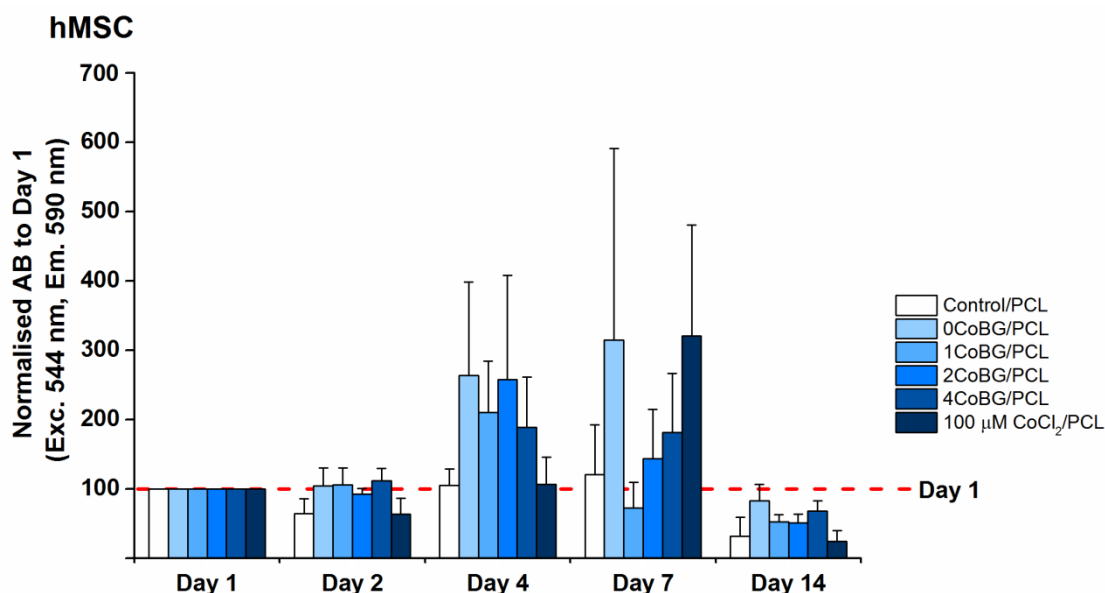


Fig. 6.13: Alamar Blue assay: hMSC viability was higher at day 4 and decreased at day 14. There were no statistically significant differences.

When AB activity was analysed on each scaffold ($N=4$), as shown in Fig. 6.12, hMSC seeded on scaffolds displayed a different trend from DPSC (Fig. 6.10). Cells seeded on the PCL scaffolds showed a reduction in cell activity from day 1 to 4, but then it remained constant from days 4 to 14 (Fig. 6.12). The first week of incubation (days 1 to 7) was possibly a period of acclimatisation of hMSC to the CoBG/PCL scaffolds. Considering that all conditions, including control PCL, had a similar effect on viability, it was likely unrelated to the presence of the CoBG.

As previously shown for DPSC, there was variability in the number of hMSC that adhered on the scaffolds. Such variability could also be observed between different scaffold types. As an example, the number of hMSC that adhered on the 1CoBG/PCL scaffolds was higher than on PCL only scaffolds, although, such results do not appear to be reproducible over various independent experiments.

As reported previously, a higher cobalt concentration affects cell viability (24). Wu *et al* fabricated mesoporous BGs containing 2% and 5% cobalt (substitution in molar ratio with calcium) namely 2Co-MBG and 5Co-MBG *via* a polymeric sponge method and assessed human BMSC cell proliferation using an MTT assay. Cells seeded on 5Co-MBG had lower proliferation compared to 2Co-MBG and MBG scaffolds at day 7, suggesting that higher cobalt concentration reduces BMSC cell viability (24).

The DPSC and hMSC showed different responses when cultured on the CoBG/PCL scaffolds, which might be related to their embryonic development. As described in Chapter 4, DPSC originate from dental pulp within the pulp chamber, which mainly consists of fibrous tissue, while hMSC are isolated from bone marrow aspirates containing mainly hematopoietic cells (4). The low viability observed for hMSC may also be due to cells dying or being lost during the changing of the plate for the AB reading. The scaffolds were transferred to new 48-well plates for AB assessment to ensure that only the cells attached on the scaffold were measured, after which the scaffolds were returned to the original plate. A study conducted to assess cell seeding efficiency on porous poly(lactide-co-glycolide) (PLGA) scaffolds advocated changing well plates after 3 to 6 days in culture to prevent cell migration from the bottom of the plate to the scaffold (454). But in our case, we allowed the cells and scaffolds to stay together until the end of the experiment. This may have resulted in some cells from the base of the well plate migrating into the scaffold.

In a recent study, PCL fabricated with BAG particles, (PCL/BAG, particle size $\leq 50 \mu\text{m}$) did not significantly enhance human gingival fibroblast proliferation when compared to PCL scaffolds. The BAG incorporation into the PCL structures increased the pH of the cell culture medium, which may slow down cell proliferation, but 10 wt % BAG incorporation into the PCL scaffold was low enough to stimulate proliferation (443). In our study, we did not check the pH of the medium from the cultured cells before sending it for ICP-OES assessment. The media volume was low (500 μl /well) which may have decreased after 2 days of incubation. However, we tried to stabilise the pH of the medium before adding the medium to the well plates by incubating the supplemented DMEM in an incubator at 37°C overnight. Furthermore, it was shown that the pH of the culture medium incubated with

PCL/45S5 (10% w/v) and PCL/SrBG (10% w/v) did not significantly change even in longer incubation time (105).

Another point to consider is the weight ratio of the BG powder to the PCL. In our study, we incorporated 30 wt % of the BG powder for fabrication of the CoBG/PCL scaffold, which did not negatively affect viability, meaning the cobalt release was within the safe physiological limit to cells (258). In general, CoBG addition into 3D PCL scaffold did not significantly affect cell viability.

6.3.3.3 Cell morphology on CoBG/PCL scaffolds

6.3.3.3.1 DPSC morphology on CoBG/PCL scaffolds

We examined the DPSC attachment on the scaffolds. Cells were found attaching to the junctions of connecting struts compared to the single struts alone (Fig. 6.14). The control PCL showed a smooth surface with micro pits (Fig. 6.14a) compared to the cells attaching on the struts of all CoBG/PCL scaffold (Fig. 6.14b-e), which were shown to have a rougher surface that may be attributed to the BG particles being released to the surface.

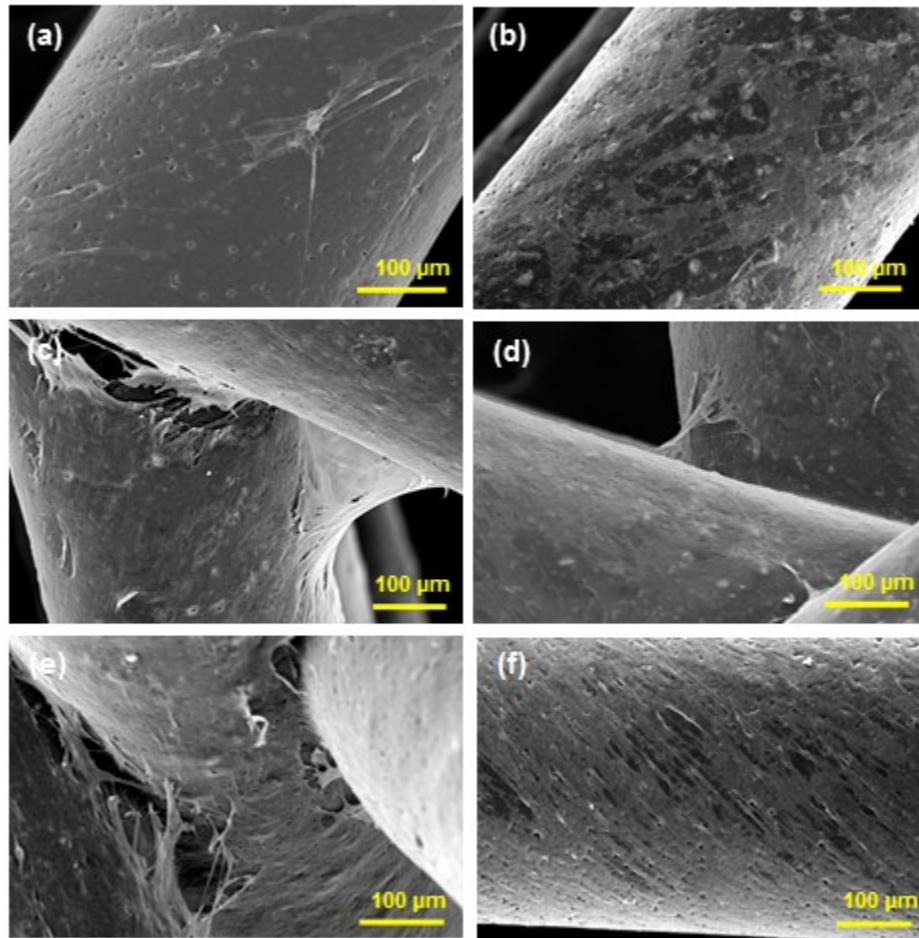


Fig. 6.14: DPSC on CoBG/PCL scaffold at day 7. Many cells attaching to the struts (a, b, f) and also the interconnecting the struts (c, d, e) as seen on the SEM images of DPSC on PCL (a), 0CoBG/PCL (b), 1CoBG/PCL (c), 2CoBG/PCL (d), 4CoBG/PCL (e), and the positive control cell culture medium containing 100 μ M CoCl₂ using PCL only scaffold (f).

Confocal images of actin staining supported the observation of DPSC attaching on the scaffold (Fig. 6.15), and the cells can be seen wrapping around the bars of the scaffold and appear to be well spread.

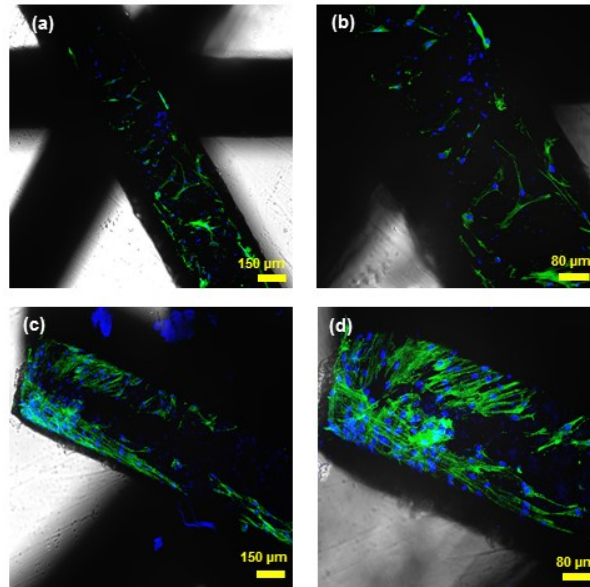


Fig. 6.15: DPSC seeded on 0CoBG/PCL scaffold (a, b) and on 2CoBG/PCL (c, d) with actin and DAPI staining. Many cells can be observed attaching to the bar of 2CoBG/PCL compared to 0CoBG/PCL at day 4 of incubation. The image was viewed using confocal microscopy with a bright field illumination overlay.

6.3.3.3.2 hMSC morphology on CoBG/PCL scaffolds

The SEM images of hMSC are shown in Fig. 6.16. The hMSC were found scattered on the PCL control scaffold, and also on the CoBG/PCL scaffolds. Cells were found attaching to the struts and also the interconnecting struts.

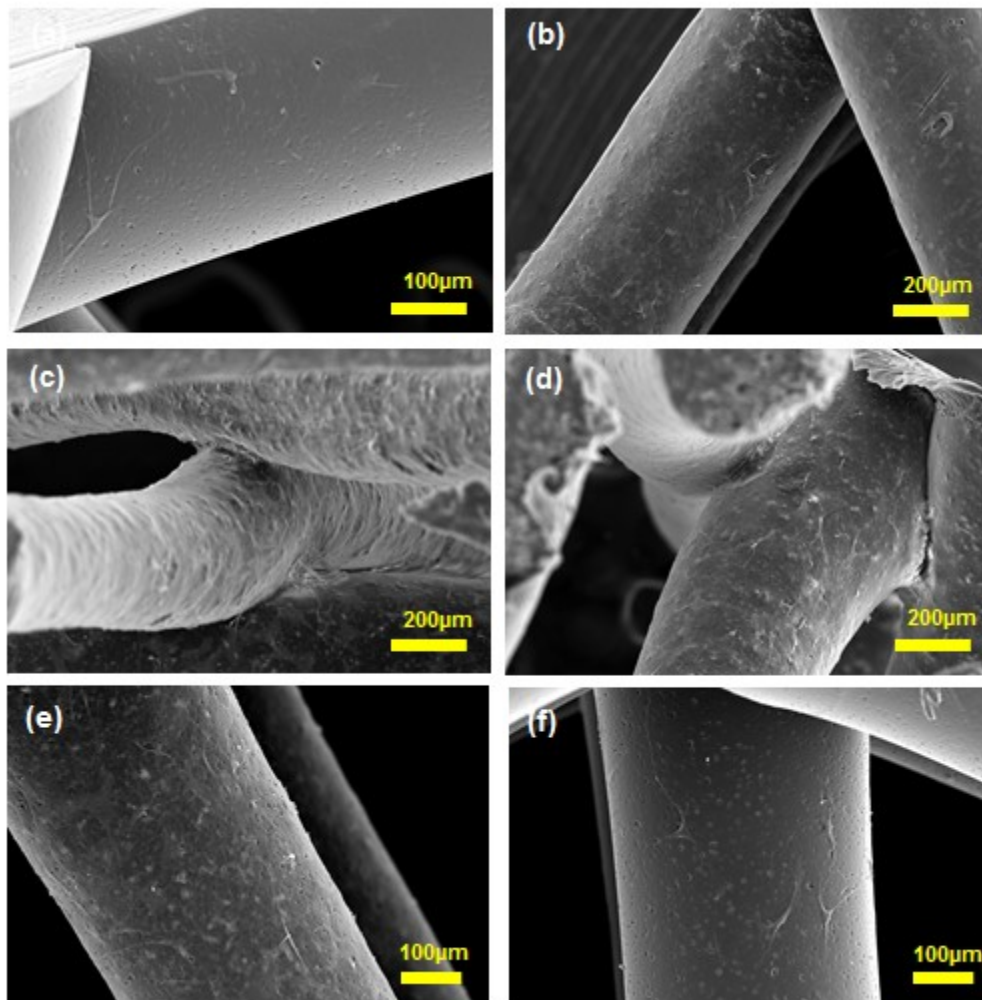


Fig. 6.16: hMSC on CoBG/PCL scaffold at day 7. Fewer cells were present on the strut bar (a, b, e, f) and the interconnecting struts (c, d) seen on the SEM images of hMSC on PCL (a), 0CoBG/PCL (b), 1CoBG/PCL (c), 2CoBG/PCL (d), 4CoBG/PCL (e), and the positive control cell culture medium containing 100 μM CoCl_2 using PCL only scaffolds (f).

The confocal images of the actin staining (Fig. 6.17) were presented as supporting information for SEM images. The cells were spread, covering wider areas of the struts on the scaffold. It was shown that in PCL only scaffolds, cells could be found on the surface of the struts (308), and in PCL/SrBG and PCL/45S5 scaffold, cells were found on the bar surface and also in the interconnecting pores (105). Cells attachment on the scaffold may be enhanced by the micropits created by the NaOH treatment and probably also by the space previously occupied by the BG particles because their dissolution created micropits that enhance cell attachment (105, 437).

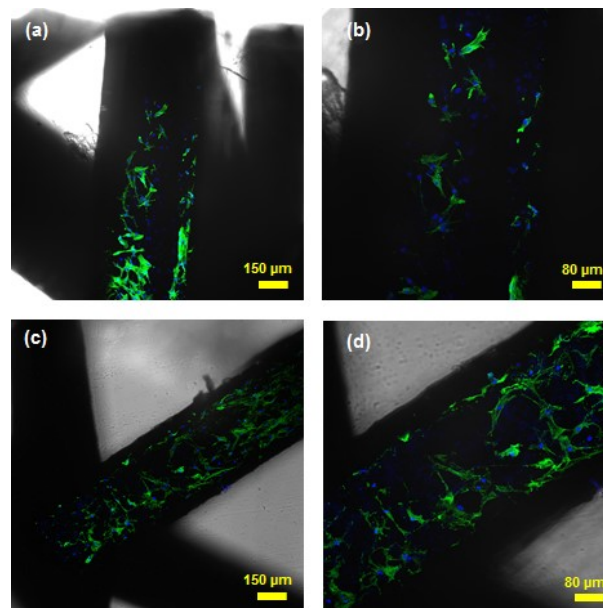


Fig. 6.17: hMSC seeded on a 0CoBG/PCL scaffold (a, b) and also on a 2CoBG/PCL (c, d) using actin and DAPI staining. Many cells can be observed attaching to the bar of 2CoBG/PCL compared to 0CoBG/PCL at day 4 of incubation. The image was viewed using confocal microscopy with a bright field illumination overlay.

The images from the confocal microscopy showed that both cell types (DPSC and hMSC) are spread and follow the shaped of the struts. It was difficult to achieve even cell distribution on the scaffold because of the large interconnecting pores. This problem could be overcome by using hydrogels as carriers for cells (456), or by treating the PCL scaffold surfaces using polymeric layer containing arginine-glycine-aspartic acid (RGD-PCL) for enhanced attachment (457). However, these methods need proper investigation since in our CoBG/PCL scaffold we already knew that the

cobalt is being released rapidly within 24 hours, which then decreases in 7 days. Thus, we may not be able to see the effect of cobalt on the cells if their contact with the scaffold is delayed. Although our scaffolds have large interconnecting pores, we understand that the presence of pores is crucial for cell migration involved in bone formation, and for allowing transfer of nutrients and blood supply to the site of repair (reviewed in (451)).

6.3.4 The hypoxia-mimicking BG scaffold effects on activation of hypoxia pathway and subsequent hypoxia response

In Chapter 5, it was shown that the conditioned medium from the 2CoBG powder was able to stimulate the hypoxia pathway in both dental stem cells, which led to the release of VEGF. In this chapter, the effect of cobalt on the cells was assessed using qPCR for the detection of *HIF1A* mRNA, and the release of VEGF from both cells was measured to assess the activation of the hypoxia pathway and the subsequent hypoxic response.

6.3.4.1 *HIF1A* mRNA expression of cells cultured on the CoBG/PCL scaffolds

For DPSC, although a slight increase in *HIF1A* mRNA expression was detected after 24 hours on the CoBG/PCL scaffolds compared to 8 hours, there was no significant difference. Furthermore, the large error bars highlighted that the *HIF1A* expression was highly variable between different scaffold replicates, making it difficult to highlight the differences observed in this test.

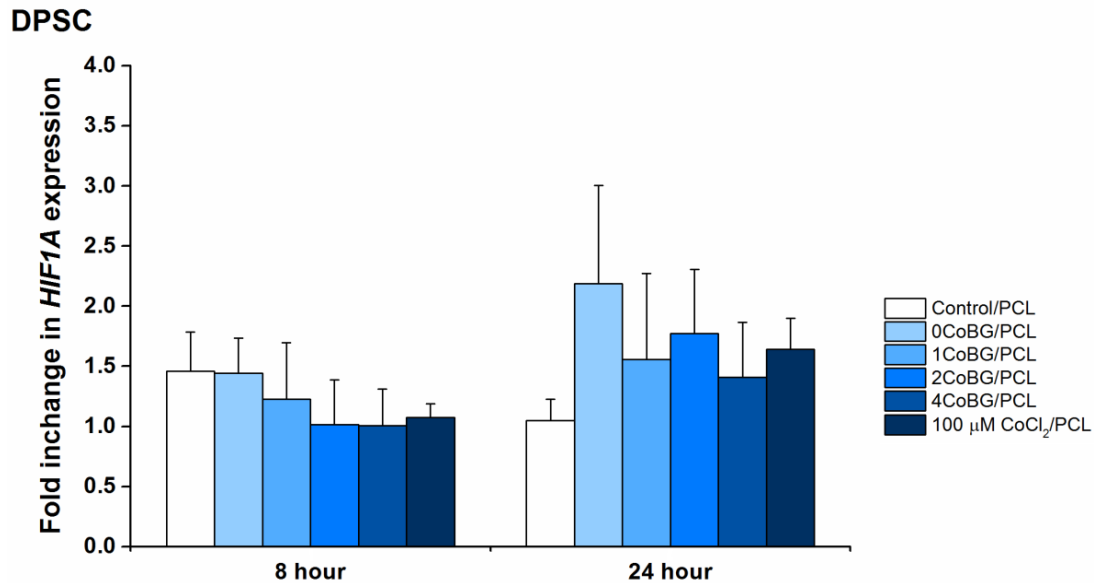


Fig. 6.18: *HIF1A* mRNA expression from DPSC cultured on CoBG/PCL scaffolds. There were no statistically significant differences.

The slight increase in *HIF1A* mRNA expression that was detected after 24 hours was also observed in DPSC exposed to cobalt-containing BG-conditioned medium and 100 μ M CoCl₂-containing medium in Chapter 5 (Fig. 5.7). The result for DPSC is different from that observed in hMSC (discussed below). DPSC were harvested from dental pulp tissue, which is situated in a closed dental pulp chamber, and receives its sole nutrient supply from the apical vessels, which enters the tooth through the apical foramen (as mentioned in Chapter 2). This makes DPSC accustomed to reduced oxygen partial pressure, making the effect of hypoxia-mimicking from the cobalt released from the CoBG/PCL less of a stimulus.

For hMSC, there were more differences in *HIF1A* expression. A strong decreasing trend in expression was found in hMSC seeded on Co-containing BG scaffolds and in the positive control (100 μ M CoCl₂) after 8 hours of incubation (Fig. 6.19). After 24 hours, cells seeded on 1Co, 2Co and 4CoBG/PCL scaffolds, and also in 100 μ M CoCl₂-containing medium showed significantly lower *HIF1A* expression compared to 0CoBG/PCL scaffolds. hMSC seeded on 0CoBG/PCL (control BG) expressed the highest *HIF1A* expression compared to other CoBG/PCL scaffolds. In another study, human bone marrow stromal cells seeded on mesoporous BG containing molar

substitutions of 2% and 5% cobalt (Co-MBG) scaffold for 1 and 7 days, had HIF-1 α protein expression dependent on cobalt concentration (24).

hMSC

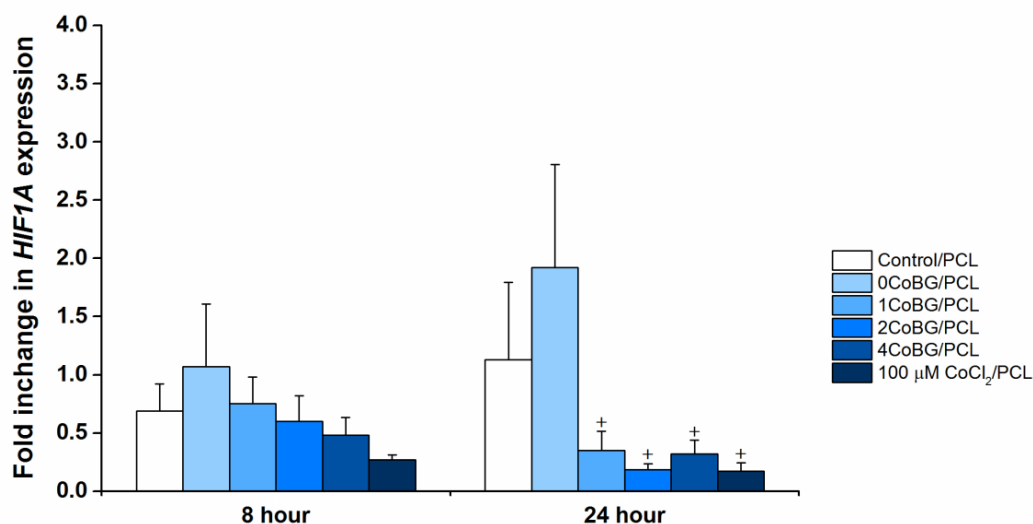


Fig. 6.19: *HIF1A* gene expression from hMSC cultured on CoBG/PCL scaffolds. Strong reduced of HIF1A expression was observed at 24 hours on hMSC seeded on the 1Co, 2Co and 4CoBG/PCL scaffold. * denotes a significant difference of the marked bar compared to the control at the same time point. + denotes a significant difference of the marked bar compared to 0Co at the same time point.

An important result to consider is the *HIF1A* expression in cells seeded on the cobalt containing-BG/PCL scaffolds (1Co, 2Co and 4CoBG/PCL scaffolds) and in 100 μ M CoCl₂-containing medium. As explained in Chapter 5 (Section 5.3.3), while an increase of HIF-1 α protein level can be detected due to its stabilisation leading to nuclear localisation, the mRNA amount of *HIF1A* has been reported to decrease during the first 24 hours following hypoxia pathway activation. The decrease of *HIF1A* mRNA expression observed in this experiment in the presence of cobalt is in accordance with such previous reports (117) and would indirectly indicate an activation of the HIF-1 pathway, as shown in the previous chapter. The reduction of *HIF1A* mRNA expression after 24 hours observed in this study may occur as an adaptation to the stabilised HIF-1 α protein content, which may indicate a feedback control mechanism (117). Cobalt exposed to endothelial cells resulted in more

pronounced reduction of *HIF1A* mRNA expression in the course of 48 hours compared to hypoxia stimulation (117). The exposure of Hep3B to 75 μM CoCl_2 showed that *HIF1A* RNAs reached a peak at four hours and decline after eight hours, however continuous exposure up to 16 hours showed increased *HIF1A* expression (110). This trend is observable in the *HIF1A* expression from cells on 2CoBG/PCL scaffolds and cells from the 100 μM CoCl_2 group.

As shown by the ICP-OES data in Fig. 6.5 (for DPSC), at day 1, the cobalt concentration of the 2CoBG/PCL is approximately 1.08 ppm ($18.34 \pm 5.43 \mu\text{M}$), and the 100 μM CoCl_2 medium is approximately 6.49 ppm ($110.19 \pm 1.36 \mu\text{M}$), while the cobalt from 4CoBG/PCL is approximately 3.13 ppm ($53.14 \pm 7.64 \mu\text{M}$).

For hMSC, the ICP-OES data in Fig. 6.6 showed that the cobalt concentration of the 2CoBG/PCL is approximately 0.94 ppm ($15.96 \pm 9.17 \mu\text{M}$) and the 100 μM CoCl_2 medium is approximately 6.03 ppm ($102.38 \pm 0.68 \mu\text{M}$), while the cobalt concentration from 4CoBG/PCL is 2.51 ppm ($42.61 \pm 8.66 \mu\text{M}$). The *HIF1A* expression was nearly the same in both DPSC and hMSC seeded on 2CoBG/PCL as that in 100 μM CoCl_2 -containing medium. This may be due to the similar cobalt concentration present within the cell surroundings that trigger this effect. The controlled release of cobalt from the CoBG/PCL scaffold led to the differences observed in *HIF1A* gene expression, suggesting that the CoBG/PCL scaffold is suitable for triggering the hypoxic response that will then stimulate the angiogenesis needed during the early stages of healing.

Another interesting finding was that DPSC and hMSC seeded on 0CoBG/PCL scaffolds (control BG) expressed the highest *HIF1A* gene expression after 24 hours compared to other CoBG/PCL scaffolds. While these results were not significantly different for any of the independent experiments, such findings were found for all of our *HIF1A* mRNA expression experiments using 0CoBG/PCL and also 0Co BG-conditioned media (in the previous Chapter 5). This trend seems to be reproducible, and it would be interesting to repeat the experiment to evaluate its statistical significance. This increased *HIF1A* mRNA expression may be due to an effect of BG itself on DPSC and hMSC proliferation. It was previously shown that osteoblasts seeded on 45S5 BG proliferated and apoptosis increased (148). As a response to

cell proliferation, the apoptotic process may have triggered the hypoxia pathway, which led to the higher expression of *HIF1A* in cells seeded on 0CoBG/PCL scaffold observed in this study.

6.3.4.2 VEGF release from cells cultured on the CoBG/PCL scaffolds

The hypoxic response was assessed by the release of VEGF from both DPSC and hMSC into the surrounding medium.

For DPSC, the VEGF level from DPSC cultured on the scaffolds decreased from day 2 to day 6 (Fig. 6.20a). As seen from the large error bars, the release was highly varied between scaffold replicates. It was therefore impossible to highlight differences between the various conditions that were studied.

A DNA assay was performed to normalise the VEGF level and to confirm that no cells were lost during the incubation period. As shown in Fig. 6.20b, no cells were lost and there was an increasing number of cells present on the scaffolds, despite 48 hours of exposure to the cobalt released from the CoBG/PCL into the surrounding culture medium. In this experiment, the number of cells attached on both the 2CoBG/PCL and 4CoBG/PCL scaffolds was significantly higher than the control and also higher than 0CoBG/PCL for the 4CoBG/PCL. These differences in initial cell number persisted up to day 6 for the 4CoBG/PCL scaffold. This result emphasises the importance of normalizing VEGF released to DNA quantity in order to evaluate the effects of CoBG incorporation within the materials. It also indicates that a similar seeding efficiency on the scaffolds should be obtained for future experiments, such as the migration assay described in the Section 6.2.4. For this experiment on the DPSC, however, normalisation of the VEGF release to the total DNA present from each well did not reveal any differences between the scaffolds. The decrease in VEGF release per cell however was even more emphasised in this graph (Fig. 20c).

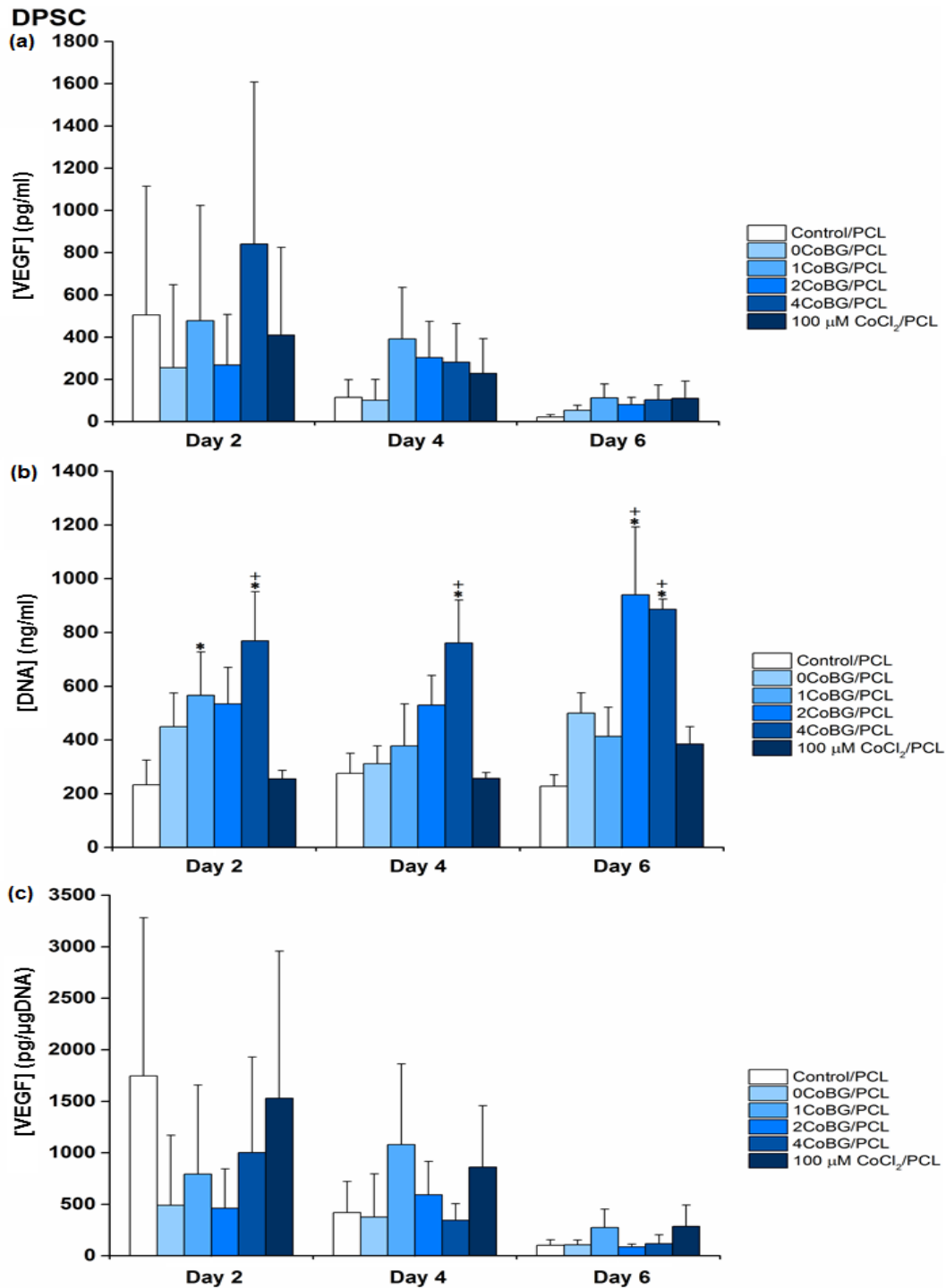


Fig. 6.20: VEGF release from DPSC cultured on CoBG/PCL scaffolds (a), total DNA assay of DPSC seeded on CoBG/PCL scaffolds (b), the amount of VEGF release normalised to total DNA of DPSC (b). Increasing cell number from day 2 to day 6 was observed for DPSC seeded on the CoBG/PCL scaffolds. * denotes a significant difference of the marked bar compared to the control at the same time point. + denotes a significant difference of the marked bar compared to 0Co at the same time point.

For hMSC, their VEGF secretion was fairly constant from days 2 to 6 (Fig. 6.21a). At day 2, VEGF levels from cells seeded on the 2CoBG/PCL and 100 μ M CoCl₂-containing medium was statistically higher than those cells seeded on the control and 0CoBG/PCL. VEGF from cells seeded on the 2CoBG/PCL remained statistically higher than from cells seeded on the control and 0CoBG/PCL scaffolds at days 4 and 6. The VEGF levels from cells seeded on 2Co, 4CoBG/PCL and 100 μ M CoCl₂-containing medium were statistically higher than VEGF levels from cells seeded on control PCL at day 6. When compared with the control, the VEGF level was higher in all CoBG/PCL scaffolds, suggesting the cobalt within the CoBG/PCL scaffolds had successfully triggered cells to release VEGF.

A DNA assay was performed to normalise the VEGF level and to confirm that no cells were lost during the incubation period (Fig. 21b). No significant differences between samples or time points were observed. These results are in accordance with the data obtained from the AB assay. These findings also suggest that the CoBG/PCL scaffolds were able to retain cells, while releasing cobalt into the surrounding medium causing cells to secrete VEGF.

Normalisation of VEGF to total DNA assay showed that at day 2, the cells present in 100 μ M CoCl₂-containing medium had released the highest level of VEGF compared to the control and 0CoBG/PCL (Fig. 21c). Wide variations were nonetheless observed amongst the cells on each scaffold replicate. At days 4 and 6, no statistical differences were observed for the VEGF release from cells seeded on the scaffolds. Nevertheless, cells seeded on the 2CoBG/PCL and 4CoBG/PCL scaffolds seem to release a higher amount of VEGF into the medium compared to other CoBG/PCL scaffolds, while cells seeded with 100 μ M CoCl₂-containing medium still released the highest level of VEGF compared to other groups.

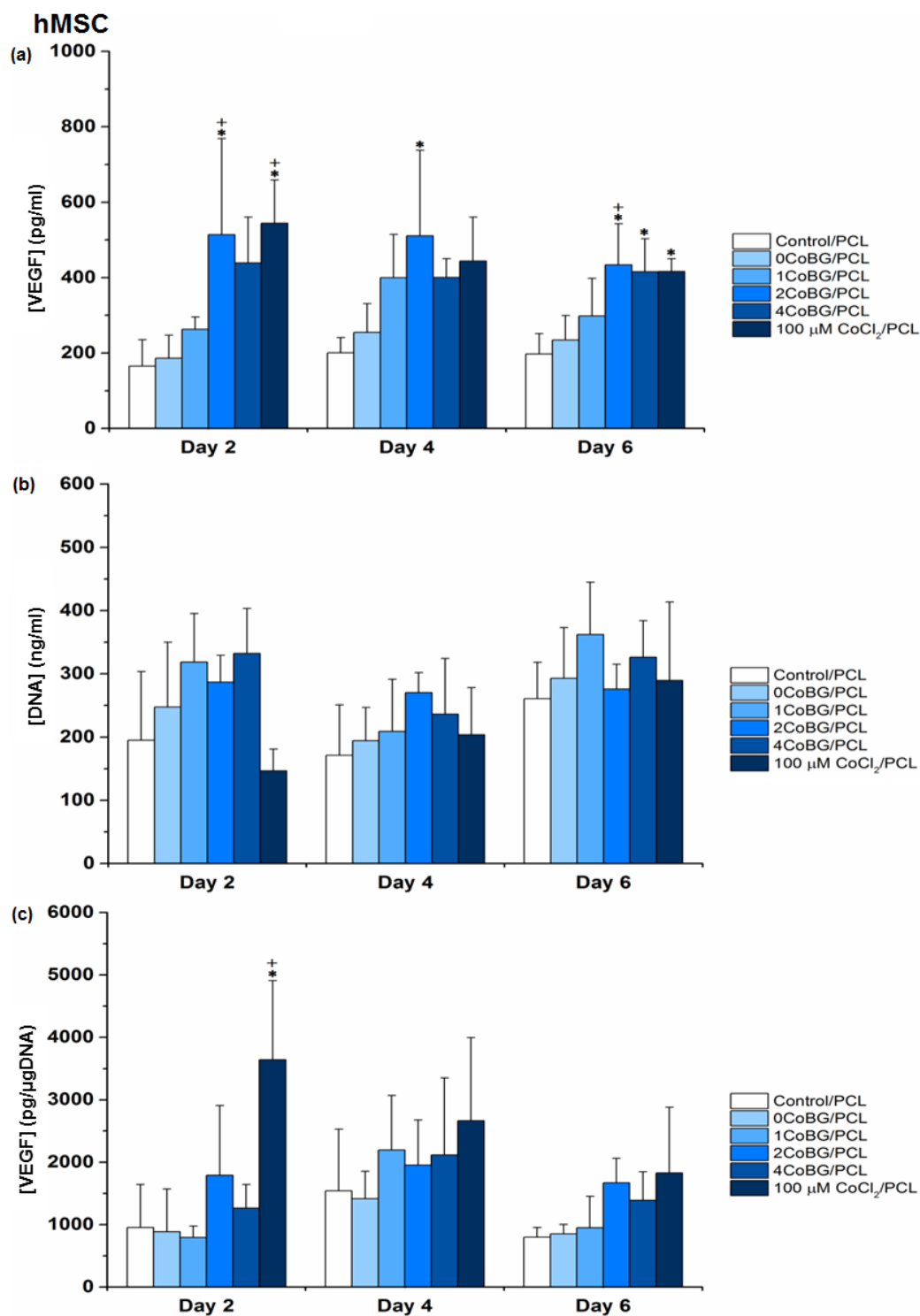


Fig. 6.21: VEGF release from hMSC cultured on CoBG/PCL scaffolds (a), total DNA assay of hMSC seeded on CoBG/PCL scaffolds (b), the amount of VEGF release normalised to total DNA (c). Increasing cell number from day 2 to day 6 was observed for hMSC seeded on the CoBG/PCL scaffolds (b). * denotes a significant difference of the marked bar compared to the control at the same time point. + denotes a significant difference of the marked bar compared to 0Co at the same time point.

Rat PDLSC exposed to hypoxia (2% O₂) showed significantly higher VEGF level compared to the control group at day 1 and 2, and as expected, the VEGF level decreased from day 1 to 3. In PDLSC, VEGF is upregulated during hypoxia, which promotes cell proliferation and differentiation in an autocrine manner (245). VEGF, being a potent mediator for vascular permeability, has been detected in the healthy dental pulp (247) and also dentin matrix (458), which may explain the substantial amount of VEGF release observed in the present study. Nevertheless, the results from the control PCL scaffold showed that VEGF release is lower than that produced from cells seeded on CoBG/PCL scaffolds. Thus, the effects are due to the cobalt released from the CoBG/PCL scaffolds that activated the hypoxia pathway and triggered the hypoxic responses. In addition to the hypoxic effect of the cobalt, other mechanisms may be involved in maintaining the higher level of VEGF seen from cells seeded on all CoBG/PCL scaffolds.

As shown in Chapter 5, the cobalt-containing BG-conditioned medium lead to HIF-1 α stabilisation, which allowed for HIF-1 α translocation into the nucleus and led to a reduction in *HIF1A* expression upon dimerisation of HIF-1 α with HIF-1 β . This subsequently triggered a hypoxic response, ie. VEGF release from the cells. Using the CoBG/PCL scaffold, we were also able to demonstrate an almost similar trend: a reduction in *HIF1A* mRNA expression supported by the release of VEGF. The VEGF released can then trigger angiogenesis, which is important for the regeneration and repair of dental tissues.

In general, the results suggest the CoBG/PCL scaffolds as a hypoxia-mimicking agent have succeeded in initiating both DPSC and hMSC to stimulate the hypoxia pathway, and subsequently triggered the hypoxic response by releasing VEGF into the surroundings. The DNA assay from the same cell lysates did not show a decrease in cell number, which further suggested the cells seeded on the CoBG/PCL maintained viability and continued proliferating. The hypoxia-mimicking effect of the CoBG/PCL on these cells then caused them to release VEGF.

6.3.5 The hypoxia-mimicking BG scaffold effects on *in vitro* angiogenesis and promoting healing and repair

One of the ways to achieve a vascularised scaffold is by incorporating growth factors such as VEGF, FGF or PDGF into the scaffold (459), or by coculture of cells with endothelial cells (EC), for example DPSC with HUVEC (422), PDLSC with HUVEC (460), and hMSC with HUVEC (461).

In our study, cobalt incorporation into CoBG/PCL scaffolds is able to mimic hypoxia, causing the cells cultured on them to release VEGF, which could promote vascularisation. Tooth regeneration as a whole organ (5, 462) is the ultimate aim in dental tissue engineering, but problems exist in terms of providing adequate nutrient supply to the tooth following implantation due to the need to regenerate the networks of vessels and nerves (463). Environmental conditions surrounding the cells or scaffolds *in vitro* and *in vivo* also affect cell behaviour, and nutrients and oxygen supply is crucial for cells undergoing osteogenic differentiation and for cell survival (225).

The CoBG/PCL scaffolds are capable of mimicking the hypoxic response of DPSC and hMSC, and of activating the hypoxia pathway based on *HIF1A* gene expression and VEGF secretion. Thus, in this section, we analysed the potential of the CoBG/PCL scaffolds to promote *in vitro* angiogenesis, which could improve healing and repair in tissue engineering and regenerative medicine.

6.3.5.1 Endothelial cell migration in response of cell-seeded CoBG/PCL scaffold conditioned media

For the migration assay, only four conditions were tested: control, 0CoBG/PCL (control BG), 2CoBG/PCL and 100 μ M CoCl_2 -containing medium. The cells (DPSC and hMSC) were seeded on the CoBG/PCL scaffolds and incubated for 48 hours in serum-free DMEM to prepare the cell-conditioned medium (cell-conditioned DMEM). The cobalt present within the scaffold will leach out to create a hypoxia-mimicking environment and cause the cells to release VEGF, a chemoattractant for EC. Then, these VEGF containing media (cell-conditioned DMEM) were tested on HUVEC in a migration assay. As a control for the potential direct influence of the cobalt released from the scaffold on EC migration, media incubated with the same protocol with cell-

free scaffolds were prepared (cell-free DMEM). In that case, only cobalt was present in the medium that had leached out from the CoBG/PCL scaffolds. The VEGF level from the cell-conditioned medium was assessed and compared with cell-free DMEM by an ELISA in a 96-well plate pre-coated with a monoclonal antibody specific for VEGF A₁₆₅.

When DPSC cell-conditioned DMEM was used as a chemoattractant for HUVEC in the migration assay, no significant difference was observed between samples or between cell-free and cell-conditioned DMEM. HUVEC showed an almost similar pattern of migration between the scaffolds and conditions (Fig. 6.22). In cell-conditioned DMEM, only 82.59% of HUVEC migrated towards the chemoattractant when exposed to 2CoBG/PCL cell-conditioned medium, and 85.31% HUVEC migrated in the 2CoBG/PCL cell-free DMEM. Higher HUVEC migration was observed in 0CoBG/PCL cell-conditioned DMEM, which was 95.88% compared to 69.96% migration in the 0CoBG/PCL cell-free DMEM.

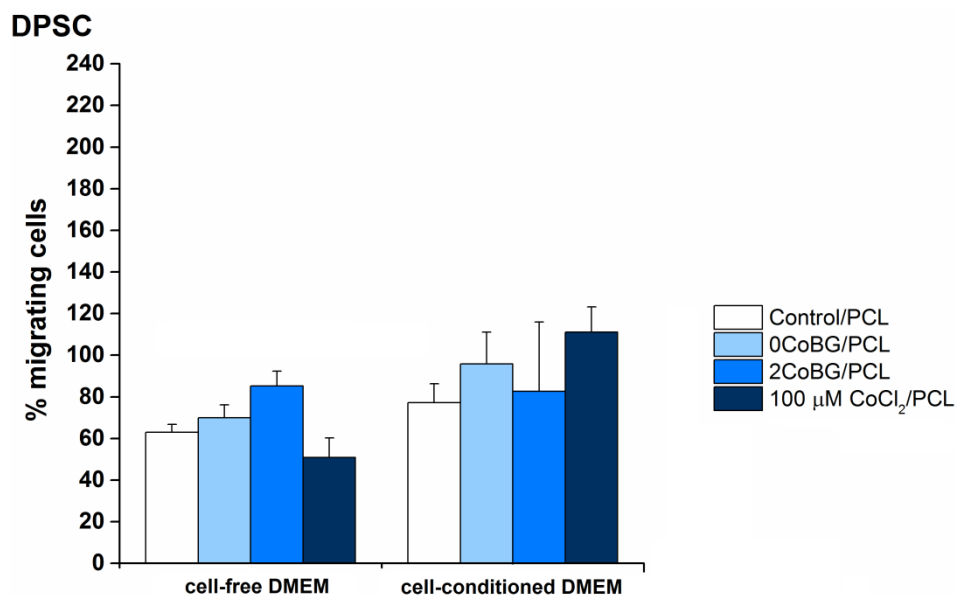


Fig. 6.22: Migration assay performed to assess the effect of hypoxia-mimicking BG scaffolds on HUVEC. In 2CoBG/PCL cell-free DMEM, higher HUVEC migration was observed compared to the control, 0CoBG/PCL and 100 µM CoCl₂-containing medium. There were no statistically significant differences.

In hMSC cell-conditioned DMEM, although not statistically significant due to high sample variability, a slightly higher percentage of HUVEC migrated towards the medium prepared from the 2CoBG/PCL cell-conditioned DMEM (Fig. 6.23). The 100 μ M CoCl₂-containing cell-conditioned DMEM also may cause HUVEC to migrate towards the chemoattractant compared to the control group. While it is not possible from this experiment to conclude that 2CoBG/PCL can trigger EC migration *via* the secretion of angiogenic factors from hMSC, the results obtained are promising and warrant further investigation.

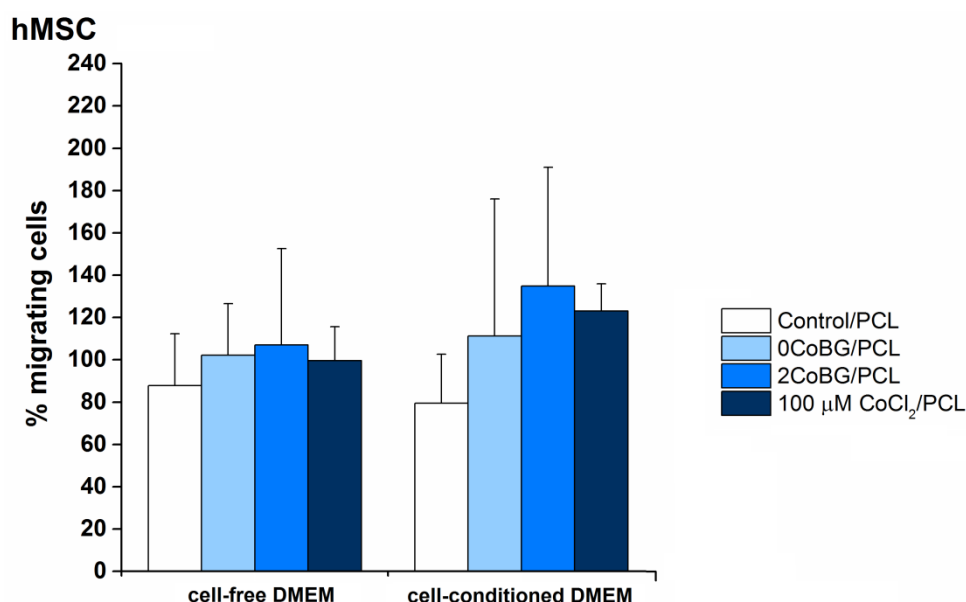


Fig. 6.23: Migration assay performed to assess the effect of hypoxia-mimicking BG scaffolds on HUVEC cells. In 2CoBG/PCL cell-free DMEM and 2CoBG/PCL cell-conditioned DMEM, higher HUVEC migration was observed compared to the control, 0CoBG/PCL and 100 μ M CoCl₂-containing medium. There were no statistically significant differences.

When we assessed the VEGF level in both cell-free DMEM and cell-conditioned DMEM from DPSC, the results showed that the VEGF level in scaffolds from the cell-free DMEM group was low and almost undetectable (< 25 pg/ml is the detection limit). However, the VEGF level in cell-conditioned DMEM from cells seeded on the 2CoBG/PCL was significantly higher compared to the control and 0CoBG/PCL, confirming our initial results that 2CoBG/PCL scaffolds may trigger VEGF secretion (Fig 6.24). Even though DPSC seeded on the 2CoBG/PCL scaffold resulted in the release of VEGF in culture medium, this did not promote HUVEC migration towards the chemoattractant. The concentration of VEGF released into the 2CoBG/PCL cell-conditioned DMEM medium after 48 hours in serum-free medium (in Fig. 6.24) was 138.93 pg/ml. This value was lower compared to when DPSC were seeded on 2CoBG/PCL scaffolds using serum-supplemented media, where after 48 hours of seeding (Day 2), the VEGF release was 268.18 pg/ml (Fig. 6.20).

The addition of supplements to the culture medium, such as foetal bovine serum (FBS) and bovine serum albumin (BSA), may also affect the cell response. It was shown that using 20% FBS as the chemoattractant, 40 to 60% of the loaded HUVEC will migrate towards the lower surface of the transwell insert membrane after four hours, and when BSA is used, less than 1% of cells migrated (464). We observed that HUVEC migration was more than 50% in all samples, and factors other than VEGF were clearly involved. There are many known factors involved in signalling (464), and changes in cytosolic free calcium ($[Ca^{2+}]$), and intracellular pH (pH_i) are also important. In the case of calcium it is known that BGs release calcium (as shown in Fig. 6.5 and 6.6) into the culture medium, and the presence of cobalt in the BG may lead to a change in medium pH. The potential influence of such factors in HUVEC migration confirm the relevance of using cell-free conditioned-media as controls in our experiments. However, we did not observe any significant changes in the cell-free conditioned media when compared to the control medium condition.

It was shown that the VEGF level in DPSC exposed to hypoxic conditions was higher (~ 250 pg/ml) compared to normoxic conditions (~ 50 pg/ml). No differences were detected in fibroblast growth factor (FGF), another important factor in vessel formation, suggesting that VEGF plays more of a role than FGF in promoting

angiogenic responses in this system (260). Furthermore, using a HIF-1 α inhibitor did not reduce VEGF expression in DPSC to baseline levels, suggesting that other factors are involved in hypoxia-induced VEGF release (260).

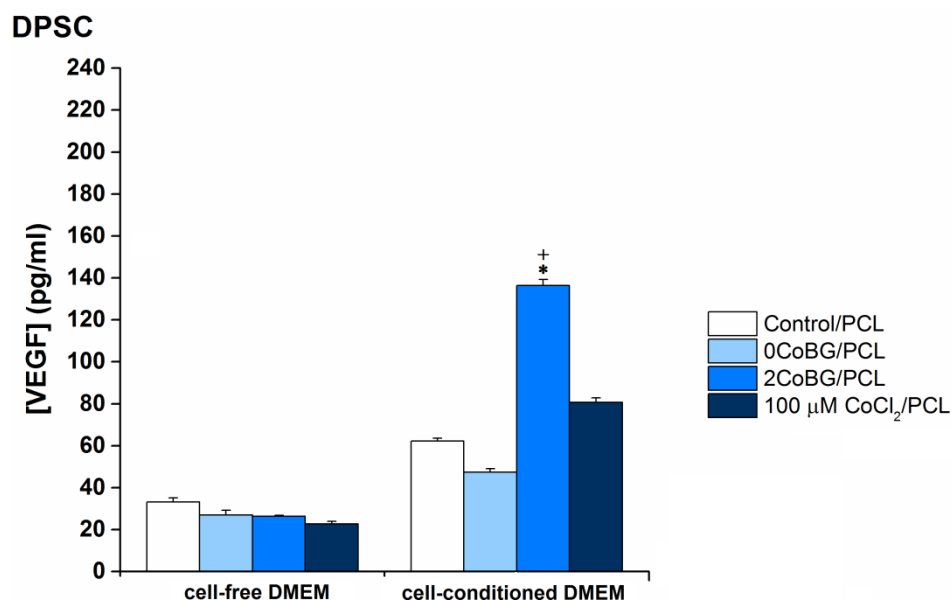


Fig. 6.24: The VEGF as a chemoattractant for HUVEC migration performed to assess the effect of hypoxia-mimicking BG scaffolds on HUVEC cells. The VEGF level was higher in 2CoBG/PCL cell-conditioned DMEM compared to the control, 0CoBG/PCL and 100 μ M CoCl₂-containing medium. * denotes a significant difference of the marked bar compared to the control at the same time point. + denotes a significant difference of the marked bar compared to 0Co at the same time point.

When we assessed the VEGF level from the cell-conditioned DMEM group from hMSC, cells seeded on the 2CoBG/PCL scaffold from the cell-conditioned DMEM group released the highest VEGF level compared to the control and 0CoBG/PCL (Fig. 6.25). As shown by the migration assay, HUVEC seemed to be slightly more attracted towards the 2CoBG/PCL cell-conditioned DMEM medium when compared to other conditions. This suggests that the cobalt released from the 2CoBG/PCL scaffold may have successfully mimicked hypoxia for the hMSC, which responded by releasing VEGF into the surrounding media.

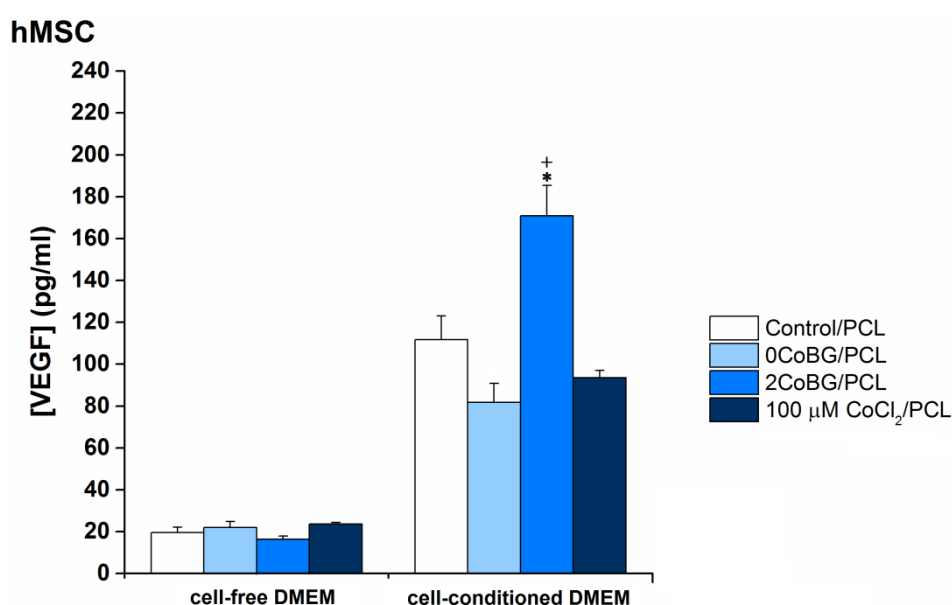


Fig. 6.25: The VEGF as a chemoattractant for HUVEC migration performed to assess the effect of hypoxia-mimicking BG scaffolds on HUVEC. The VEGF level was higher in 2CoBG/PCL cell-conditioned DMEM compared to the control, 0CoBG/PCL and 100 μ M CoCl₂-containing medium. * denotes a significant difference of the marked bar compared to the control at the same time point. + denotes a significant difference of the marked bar compared to 0Co at the same time point.

The VEGF concentration measured in the cell-free DMEM group was negligible as expected. The amount of VEGF released from the hMSC seeded in 2CoBG/PCL cell-conditioned DMEM medium after 48 hours in serum-free medium (Fig. 6.25) was 170.9 pg/ml. This concentration appeared was slightly higher than the amount

secreted from the DPSC (138.93 pg/ml) in the same conditions, which may explain why HUVEC responded more to 2CoBG/PCL cell-conditioned DMEM from the hMSC than from the DPSC. A similarly high secretion of VEGF from hMSC in response to cobalt has been previously shown (24, 265). It is also interesting to note that the basal level of VEGF released from hMSC cell-conditioned DMEM control medium is rather high in the conditions of the study. This may also contribute to the low differences observed with regards to the EC migration.

A recent study assessed the effect of hMSC conditioned medium on HUVEC (465). hMSC and HUVEC incubated in serum-free EGM were subjected to normoxia (21% O₂) and hypoxia (1% O₂) for 48 hours, and these media were used in a transwell migration assay. After 24 hours, the highest number of HUVEC migrating towards the chemoattractant occurred in conditioned medium from MSC exposed to hypoxia (MSC-CdM^{HYP}) compared to the conditioned medium from MSC exposed to normoxia (MSC-CdM^{NOR}) and conditioned medium from HUVEC exposed to hypoxia (HUVEC-CdM^{HYP}) (465). VEGF level assessed by ELISA was significantly higher in MSC-CdM^{HYP} (1340 ± 250 pg/ml) compared to both MSC-CdM^{NOR} (630 ± 140 pg/ml) and HUVEC-CdM^{HYP} (less than 100 pg/ml), suggesting that MSC-CdM promotes HUVEC migration and hypoxia enhances VEGF secretion from MSC (465).

Surprisingly, the VEGF secretion from cells seeded in the 100 µM CoCl₂-containing medium (positive control) was low compared to that from cells seeded on 2CoBG/PCL scaffolds in the cell-conditioned DMEM group (Fig. 6.24 and Fig.6.25). This result is consistent with the lack of strong differences in HUVEC migration observed in these conditions.

The results showed that even though the cobalt present in the scaffold mimicked hypoxia, and the DPSC and hMSC responded by releasing VEGF, no difference in HUVEC migration was observed. This may be explained in part due to: (a) the concentration of VEGF (170.9 pg/ml from hMSC, Fig. 6.25) into the cell-conditioned DMEM medium that was insufficient for chemotaxis; and (b) a culture medium devoid of any cell growth supplements. In HUVEC growth medium, EGM-2, the presence of cocktails of endothelial cell growth supplements, such as 10 ng/ml basic fibroblast growth factor (bFGF), 5 ng/ml endothelial growth factor (EGF), and 0.5 ng/ml VEGF,

may also drive the HUVEC migration. As mentioned previously, HUVEC exposed to hypoxia cultured in HUVEC-CdM^{HYP} failed to show migration when the VEGF was almost undetectable (less than 100 pg/ml), thus, a threshold amount of VEGF is needed to trigger migration (465).

Using DMEM to prepare the chemoattractant may also have led to reduced migration of HUVEC, since they are normally cultured in EGM-2 media with many growth factors and supplements to maintain viability. Bidarra *et al.* (461) showed that for a coculture system between HUVEC and MSC, the suitable media for HUVEC was Iscove Modified Dulbecco Medium (IMDM) rather than combination of DMEM with Medium 199 (M199) or DMEM alone, while MSC preferred DMEM plus M199 compared to DMEM alone or M199 and IMDM alone. However, we used DMEM to provide a clearer picture of the CoBG effects on DPSC and hMSC. The hypoxia-mimicking effect of the cobalt present in the CoBG/PCL scaffolds had triggered DPSC and hMSC to release VEGF into the surrounding culture medium, where cells seeded on 2CoBG/PCL scaffold released the highest amount of VEGF in the cell-conditioned DMEM group (Fig. 6.24 and 6.25). Furthermore, the media to be used for the coculture system needs to be optimized for both types of cells to ensure reliable results are obtained (466). In angiogenesis, besides VEGF, other factors have been identified as the 'potential positive regulators', including acidic and basic fibroblast growth factors (aFGF and bFGF), transforming growth factors α and β (TGF- α and - β), and epidermal growth factor (EGF) (reviewed in (467)).

While not fully conclusive, these findings suggest that CoBG/PCL scaffolds may be a suitable material for use in tissue repair. During healing and repair, vessel formation is crucial and we have shown that CoBG/PCL scaffolds can mimic hypoxia to promote DPSC and hMSC to release VEGF. In turn, this will promote vessel formation *via* the HIF/VEGF pathway.

6.3.5.2 ALP activity of cells seeded on CoBG/PCL scaffolds

In hard tissues such as bone, the healing and repair process occur together with vessel formation. As new bone is deposited, blood vessel formation is crucial to ensure nutrients are supplied to the new tissue, which is achieved by transplantation

of scaffold-cell mixtures, scaffolds only or cells only (225, 227, 459). One of the sign of new bone formation is the alkaline phosphatase activity (ALP). Studies have shown that HIFs may play different roles in osteogenesis (reviewed in (225)). HIF-1 α promotes osteogenesis and angiogenesis through the HIF/VEGF pathway *in vivo* (251), while contradicting results were obtained *in vitro* from osteoblast cells harvested from mice lacking HIF-1 α (Δ HIF-1 α) where the ALP activity and nodule formation from Δ HIF-1 α osteoblasts was comparable to control osteoblasts (226). Nevertheless, the CoBG/PCL fabricated in our study are intended for use as scaffolds to promote angiogenesis, healing and repair in areas devoid of blood vessels. Bone formation requires angiogenesis, and mediators such as CoBG/PCL scaffolds may also stimulate bone regeneration (251). We assessed the role of CoBG/PCL scaffolds in promoting repair and healing by assessing the ALP activity in DPSC and hMSC when cultured on the CoBG/PCL scaffolds in osteogenic inductive medium. Although the incubation period was short (up to day 7), the results provide initial data for further studies using the CoBG/PCL scaffold.

6.3.5.2.1 ALP activity of DPSC seeded on CoBG/PCL scaffolds

For DPSC, their incubation on the CoBG/PCL scaffolds in osteogenic medium showed no differences in term of total ALP activity at days 3 or 7 (Fig. 6.26a). Nevertheless, when we normalised the ALP activity to total DNA, we observed that the ALP activity of cells cultured on all of the CoBG/PCL scaffolds were significantly lower than control PCL at days 3 and 7 (Fig. 6.26b). At day 3, the ALP activity was significantly higher in cells cultured with 100 μ M CoCl₂-containing osteogenic medium compared to cells cultured on 0CoBG/PCL. However, at day 7, the ALP activity of cells cultured on control PCL was the highest. No differences between the CoBG/PCL groups were observed. A previous study has similarly shown a dose-dependent effect of ALP activity related to hypoxia (468). Dog pulp tissue was subjected to normoxia (20% O₂) and hypoxia (2% O₂) for 1 to 3 days and a group was subjected to reoxygenation after 24 hours. Following that, ALP activity was statistically higher in the hypoxia group compared to the control. The ALP activity decreased from day 1 to 3 (468), which is also observable in our results (Fig. 6.26b).

We then analysed the total DNA content from each well corresponding to the ALP assay in order to perform the normalisation of the ALP activity. Based on the total

DNA assay, at day 3, all CoBG/PCL scaffolds used in this experiment had a statistically higher cell number compared to the control, with 2CoBG/PCL showing the highest cell number of the CoBG/PCL scaffolds (Fig. 6.27). Cell number was significantly lower in 100 μ M CoCl₂ containing osteogenic medium compared to the 0CoBG/PCL. As shown previously in Fig.20b, the presence of cobalt did not affect cell number when DPSC were seeded on the CoBG/PCL scaffolds in normal culture medium. The CoBG, although minimally promoting ALP activity, still allows cells to proliferate. At day 7, the highest cells number was found on the 4CoBG/PCL scaffold compared to other scaffolds.

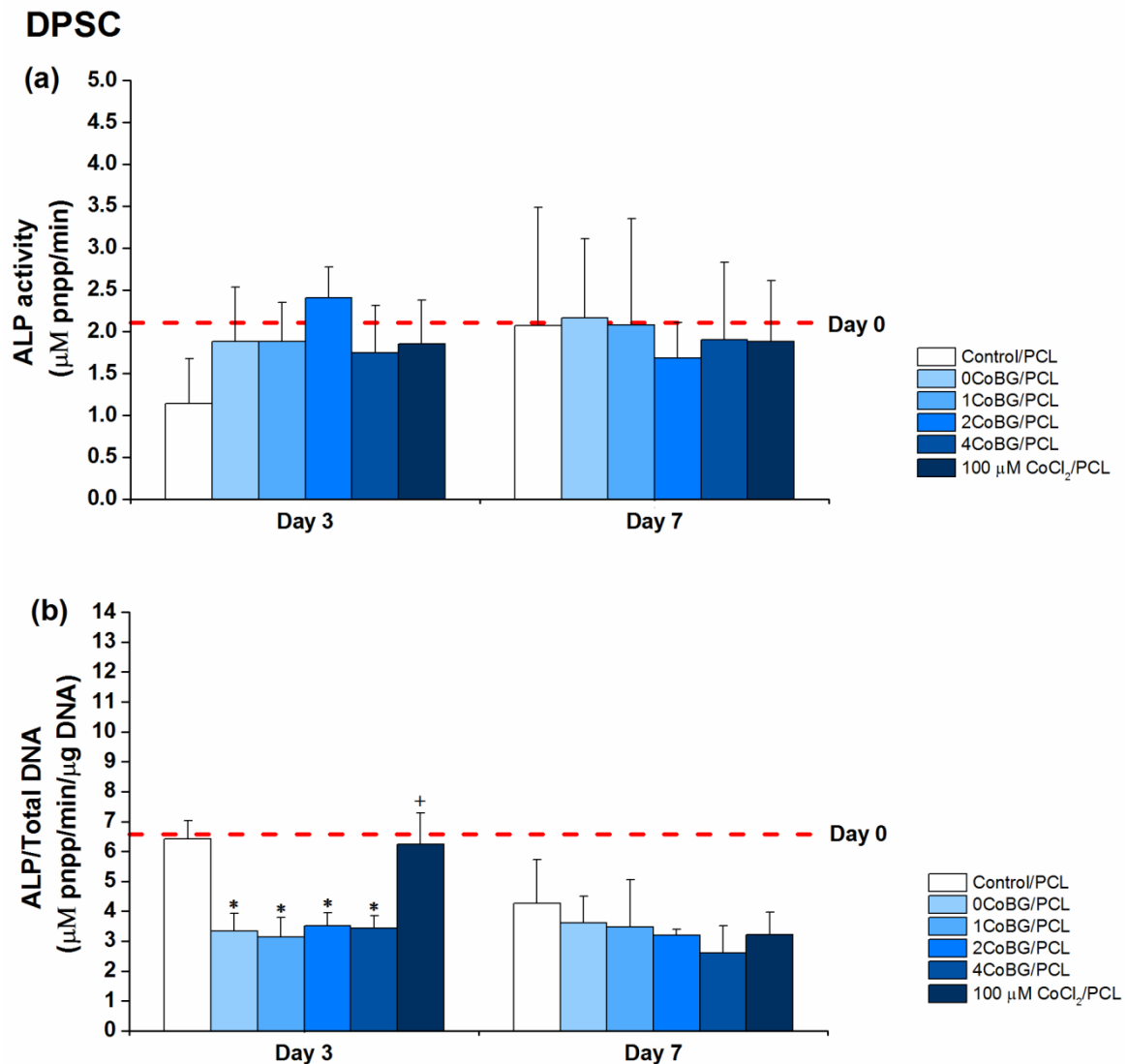


Fig. 6.26: Total ALP activity (a), and normalised ALP activity to total DNA of DPSC (b) in osteogenic medium. At day 3, lower ALP activity observed in DPSC seeded on the CoBG/PCL scaffolds compared to the control and 100 $\mu\text{M CoCl}_2$ -containing medium (b). Day 0 is taken after 2 hours of seeding on control PCL scaffolds using osteogenic medium during the transfer to a new well-plate. * denotes a significant difference of the marked bar compared to the control at the same time point. + denotes a significant difference of the marked bar compared to 0Co at the same time point.

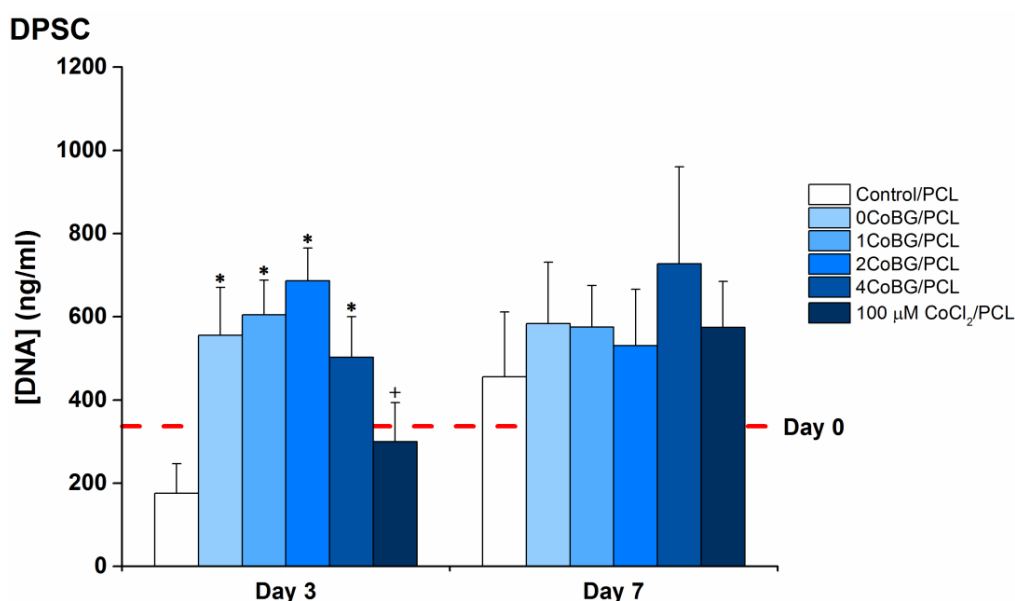


Fig. 6.27: Total DNA of DPSC per well when induced in osteogenic medium. Cell number was higher on cells seeded on CoBG/PCL scaffolds. Day 0 is taken after 2 hours of seeding on control PCL scaffolds using osteogenic medium during the transfer to a new well-plate. * denotes a significant difference of the marked bar compared to the control at the same time point. + denotes a significant difference of the marked bar compared to 0Co at the same time point.

To date, no literature is known regarding DPSC response to cobalt-containing BG. Hypoxia resulted in different cell responses to osteogenic differentiation, especially during early stages, which may be dependent on the cell type (245), and human tissues also showed different O₂ concentration (reviewed in (469)). The oxygen concentration used to stimulate hypoxia in dental related stem cells or tissues varies, as mentioned previously. 2% O₂ stimulated higher ALP release from rat PDLSC (245), and dog pulp cells (468), 5% O₂ stimulated higher ALP release from human DPC (455), while others reported low ALP activity in 5% O₂ for human osteoblastic PDL (470), and in 1, 3, 5 and 10% O₂ for human bone marrow stromal cells (223).

Contrary to the results obtained from our study, DPC subjected to hypoxia (5% O₂) showed higher cell viability compared to DPC in normoxia (20% O₂), and when these cells were induced by osteogenic medium, higher mineralisation was detected by

Von Kossa staining and higher expression of genes involved in mineralisation was detected in hypoxic DPC (455). The authors suggested that hypoxia enhanced calcium phosphate formation by DPC *in vitro*. In a different study (245), rat periodontal ligament stem cells (PDLSC) were subjected to hypoxia (2% O₂) for 24 hours followed by re-oxygenation. The PDLSC exposed to hypoxia showed significantly higher ALP activity than the control group at day 3, and no significant difference was detected for the ALP activity between the hypoxia group with or without re-oxygenation. Their findings also supported the hypothesis that different oxygen tension results in different ALP activity, which is also dependent on the type of cells and species used for assessment (245). The effects might occur when cells respond to hypoxia by increasing their mitochondrial function as a protective measure to overcome DNA damage related to low oxygen supply (455, 469). Nevertheless, the effect seen in DPSC from our study probably involved a different mechanism of cell survival. In our study, we were not able to distinguish the effect of cobalt incorporation in the BG on DPSC ALP activity. Although a reduction of ALP activity was observed on the CoBG/PCL composites, it was not statistically different from the non-cobalt containing scaffolds (control 0CoBG/PCL), which already had an effect on the ALP activity of these cells.

6.3.5.2.2 ALP activity of hMSC seeded on CoBG/PCL scaffolds

We then assessed the effect of CoBG/PCL scaffolds on the osteogenic induction of hMSC. For hMSC, induction by osteogenic medium showed that at day 3, the ALP activity was lowest in cells seeded on PCL scaffolds in osteogenic medium containing 100 µM CoCl₂ compared to cells seeded on the control PCL scaffolds (Fig. 6.28a). The ALP activity of cells seeded on all CoBG/PCL scaffolds was dependent on the cobalt concentration present within the scaffold. Cells seeded on 0CoBG/PCL had the highest ALP activity, followed by 1Co, 2Co and 4CoBG/PCL scaffolds. The highest ALP activity was observed in cells seeded on control PCL.

At day 7, cells seeded on the control PCL scaffold maintained a high ALP activity. Cells seeded on 0CoBG/PCL scaffolds showed the highest ALP activity compared to the control and other scaffolds. We also observed that, at day 7, the initial ALP activity in cells cultured on the CoBG/PCL scaffolds was dependent on the cobalt concentration compared to the control and 0CoBG/PCL. A decreasing trend of ALP

activity was observed with the increasing amount of cobalt in the glass. The ALP activity of cells seeded on scaffolds in osteogenic medium containing 100 μM CoCl_2 was also significantly lower than the control and 0CoBG/PCL.

The ALP activity normalised to total DNA is shown in Fig. 6.28b with similar trends observed in Fig. 6.28a. The ALP activity from cells seeded on the CoBG/PCL scaffolds tended to show a dose-dependent response the cobalt concentration present within the scaffold. The findings strengthened our hypothesis that cobalt present within the scaffold had mimicked a hypoxic environment for hMSC, leading to a decrease in ALP activity in accordance with the study reported previously (223). Human bone marrow stromal cells isolated from adult marrow (MIAMI) cells were subjected to osteogenic differentiation and exposed to hypoxia (3% O_2) for 3, 7, 10 and 15 days (223). The ALP activity was lower in MIAMI cells exposed to hypoxia, even with osteogenic induction, compared to cells in control medium and normoxia, although *HIF1A* expression was upregulated after 2 hours exposure to hypoxia. Further analysis of the MIAMI cells using flow cytometry for stage-specific embryonic antigen 4 (SSEA-4) showed that the hypoxia maintained the MIAMI in an undifferentiated state (223) suggesting that low pO_2 or hypoxia with less than 3% O_2 increases proliferation, maintains cells in an undifferentiated state, and may promote self-renewal. Although this warrants further investigation, the lower ALP activity despite the osteogenic induction observed in our study may be partly related to the hypoxia effect produced by the cobalt release into the culture medium by the CoBG/PCL scaffold. This finding suggests that cobalt addition to the BG creates a hypoxia-mimicking environment that maintains cells in undifferentiated state (223). The low ALP activity was also observed in DPSC, Fig. 6.26.

Another factor that may reduce ALP production in hypoxia is impaired mitochondrial function, resulting in cells switching to glycolysis for obtaining energy to ensure survival (471). Human MSC were induced in osteogenic medium and cultured under normoxia (21% O_2) or hypoxia (1% O_2) for three weeks, and results showed that ALP activity and ALP staining was low in hypoxia (471). Further analysis of these cells also revealed low mitochondrial respiratory enzymes, suggesting that hypoxia hinders osteogenic induction of hMSC. When 50 or 100 μM CoCl_2 was used to mimic

hypoxia after the cells had been induced in osteogenic medium, the ALP activity was also low, comparable to cells in hypoxia, suggesting that hypoxia led to a decrease in ALP activity (471), which is in accordance with our study.

It is furthermore important to note that even though an effect of the cobalt released from the scaffolds (or added to the medium in the 100 μM CoCl_2 condition) reduced ALP activity in hMSC, the presence of the glass by itself also decreased the enzymatic activity, as seen in the 0CoBG/PCL samples. A similar effect was observed for DPSC. This result may be due to the lower calcium and phosphorus concentrations available in the CoBG/PCL media compared to the control DMEM as observed from the ICP-OES result (Fig. 6.6 and 6.7).

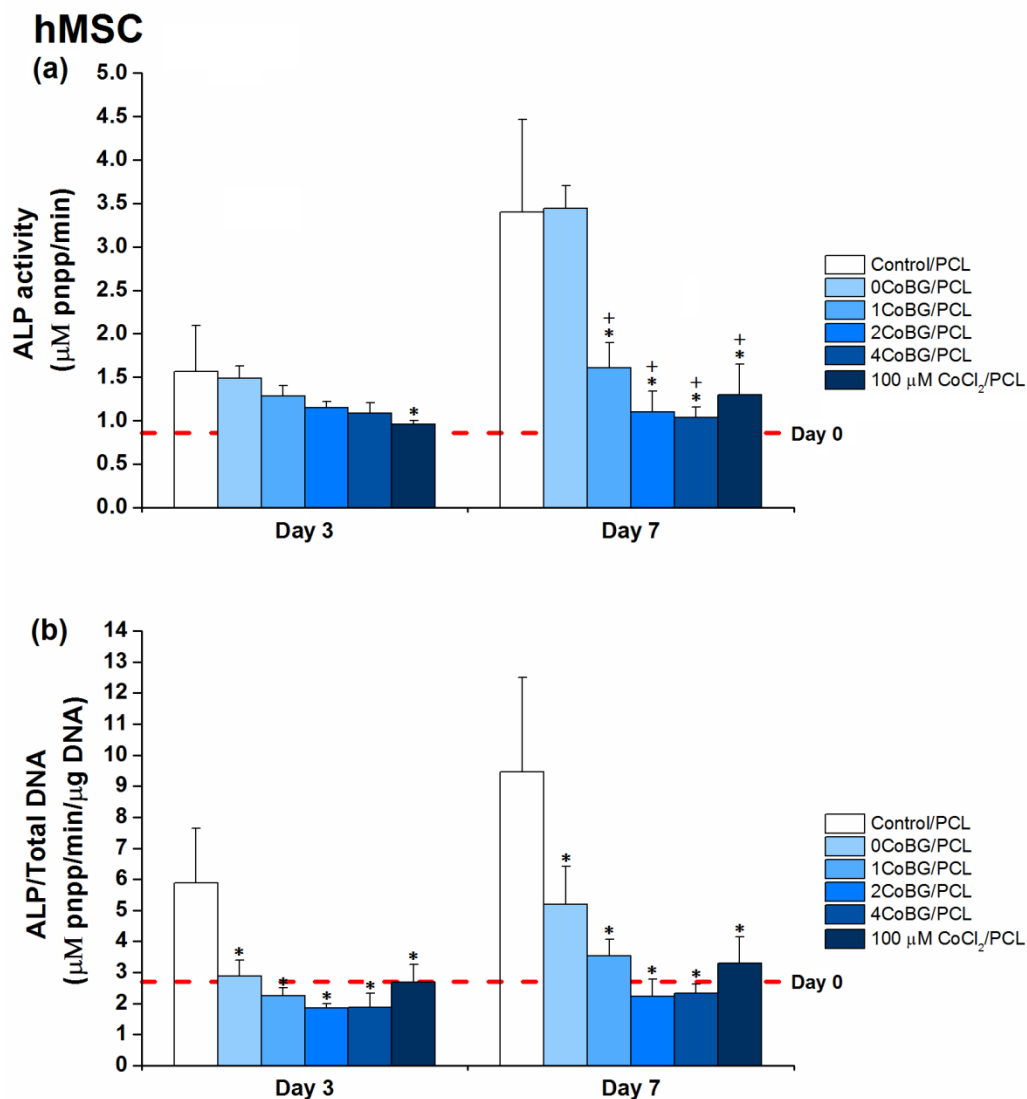


Fig. 6.28: Total ALP activity (a), and normalised ALP activity to total DNA (b) of hMSC in osteogenic medium. At day 3, lower ALP activity was observed in hMSC seeded on the CoBG/PCL scaffolds compared to the control and 100 $\mu\text{M CoCl}_2$ -containing medium (a). Day 0 is taken after 2 hours of seeding on control PCL scaffolds using osteogenic medium during the transfer to a new well-plate. * denotes a significant difference of the marked bar compared to the control at the same time point. + denotes a significant difference of the marked bar compared to 0Co at the same time point.

We then analysed the total DNA present from the same cell lysates. At day 3, cell number was significantly higher on all CoBG/PCL scaffolds compared to the control PCL (Fig. 6.29). Control PCL had the lowest cell number at day 3 and 7. The highest cell number was found on 0CoBG/PCL scaffold at day 7, which is significant compared to the control PCL. Although the number of cells present on all cobalt-containing CoBG/PCL scaffolds was slightly lower at day 7 compared to day 3, the scaffolds were able to retain cells, and the cell number was slightly higher compared to control PCL and 100 μ M CoCl₂-containing osteogenic medium. As shown in previously in Fig.21b, the presence of cobalt did not affect cell number when hMSC were seeded on the CoBG/PCL scaffolds in normal culture medium.

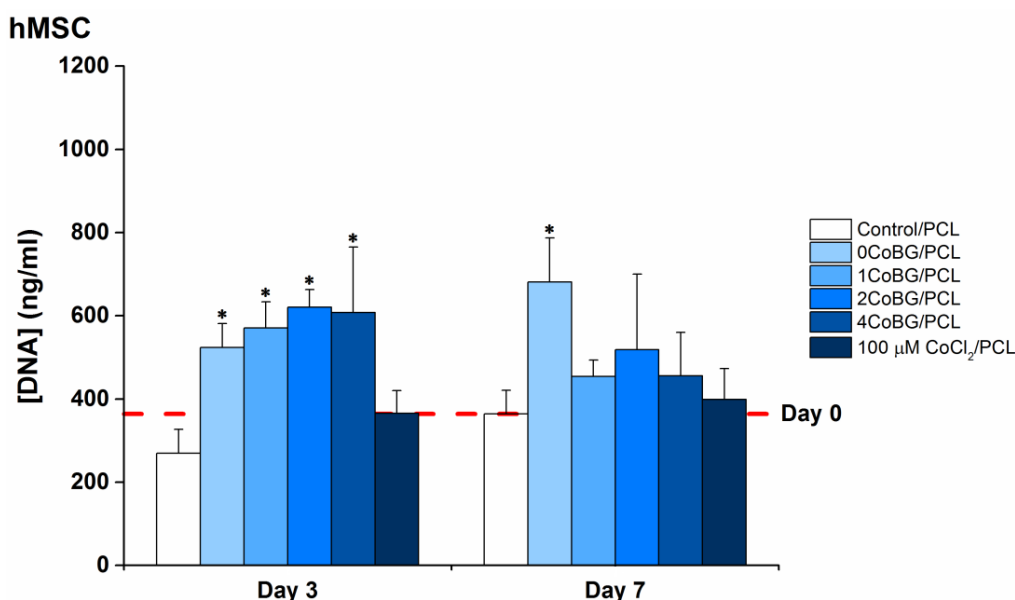


Fig. 6.29: Total DNA of hMSC per well when induced in osteogenic medium. Cell number was higher on cells seeded on CoBG/PCL scaffolds compared to the control and 100 μ M CoCl₂-containing medium. Day 0 is taken after 2 hours of seeding on control PCL scaffolds using osteogenic medium during the transfer to a new well-plate. * denotes a significant difference of the marked bar compared to the control at the same time point. + denotes a significant difference of the marked bar compared to 0Co at the same time point.

As shown in ICP-OES analysis, the cobalt release into the culture medium persisted up to day 7 and subsequently decreased (both ICP-OES from DPSC and hMSC). Therefore, we anticipated that the seven day period would be suitable to assess the

effect of CoBG/PCL for promoting osteogenesis. We hypothesised that a longer incubation period with CoBG/PCL will be masked by the effect of BG, since once cobalt has leached out from the scaffolds, other ions (calcium, sodium and silicon) present within the scaffold will also have an effect on cell behaviour (22, 148). As shown by the ALP activity assay, the low activity in both DPSC and hMSC seeded on the CoBG/PCL scaffolds did not hinder the VEGF production from these cells, which is important since vascularisation is crucial during the early stages of bone formation in adult life, especially during the healing process (115). This role can be met by the CoBG/PCL scaffold when used as a scaffold in tissue repair.

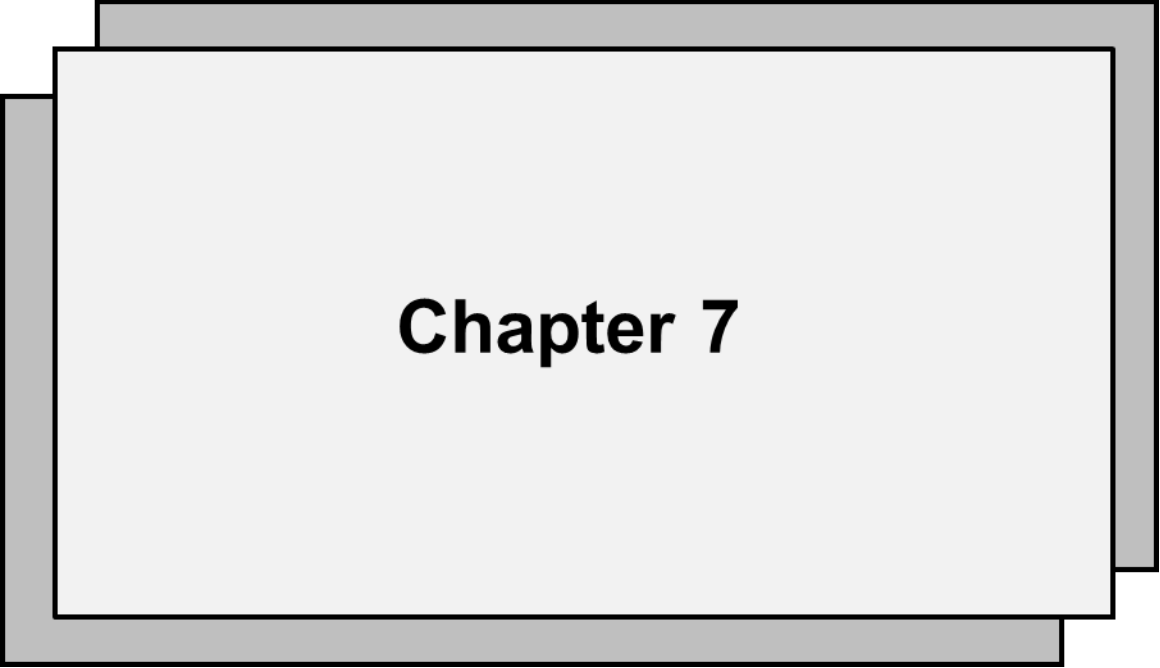
As mentioned previously, the cobalt from the CoBG/PCL scaffold showed a fast dissolution profile, and still released substantial cobalt until day 13. This is important to trigger the HIF-1 pathway and subsequently activate VEGF release for vessel formation and repair. HIF-1 α peaks within four to six hours, and decreases in 24 hours, and a high HIF-1 α level is important to trigger VEGF expression during the early phases following injury which coincide with VEGF expression *in vitro* (165). The *in vitro* response, however, is known to be different from *in vivo*. HIF-1 α expression was observed from the day of surgery, and it peaked at day 10 and subsequently decreased. However, VEGF expression was low from day 0 to day 10, peaked at day 20, and decreased by day 40 (472). As mentioned previously, the percentage of cobalt release from the cell-free CoBG/PCL scaffold is low when compared to cobalt release from the BG powder. This is beneficial since it helps to reduce the risk of Co²⁺ toxicity, and if the CoBG/PCL scaffold were to be implanted *in vivo*, the cobalt will be gradually absorbed or washed out from the circulation, and the CoBG/PCL scaffold can be used as a reservoir for providing continued cobalt release with the hope to activate the HIF-1 pathway and subsequent hypoxic responses. Thus, the CoBG/PCL scaffolds show great potential to be used in promoting healing and repair in dental tissue engineering and regeneration.

6.4 Conclusion

In this chapter, cobalt-containing BG powder was incorporated with PCL for the fabrication of CoBG/PCL scaffolds. The 30 weight per cent incorporation of CoBG powder to the scaffold fabrication did not affect the physical features such as the large interconnecting pores and higher porosity. The CoBG/PCL scaffolds were incubated in DMEM culture medium without cells and subjected to ICP-OES. Our initial results showed that incubation of the scaffolds into the DMEM produced a similar trend of cobalt release into the culture medium as that seen when using CoBG powder. We removed the medium daily from the CoBG/PCL scaffold in the initial assessment to examine whether the scaffold fabrication process affected cobalt dissolution from the CoBG/PCL. Our results showed the scaffold fabrication process did not compromise the cobalt released. When the cobalt release in each day during the incubation period was cumulated, approximately 14% of the cobalt was released from the scaffold in the 13 day period. These values are estimates but show that only a small part of the BG in the CoBG/PCL scaffold has been dissolved. The remaining cobalt remains trapped in the PCL bulk, which may be due to the slow PCL degradation process. This finding suggests the CoBG/PCL scaffold can act as reservoir for released cobalt, which is important for the activation of the hypoxia pathway. The effects of these CoBG/PCL scaffolds were assessed on two cell types: dental pulp stem cells and human mesenchymal stromal cells. The CoBG/PCL scaffolds were found to not affect cell viability and were able to mimic hypoxia and stimulate downstream hypoxic responses to release VEGF, a potent angiogenic agent. This was shown by the lower *HIF1A* mRNA expression after 8 hours. After hMSCs were seeded on the CoBG/PCL scaffold for 24 hours, there were indications that HIF-1 α had dimerized with HIF-1 β , and subsequently triggered the hypoxia pathway through the release of VEGF. We then assessed the potential of the CoBG/PCL for *in vitro* angiogenesis through a migration assay, and also assayed the production of ALP following osteogenic induction to examine the role in promoting repair. Although lower HUVEC migration was observed, the cells seeded on the 2CoBG/PCL scaffold released the highest VEGF, and showed the highest HUVEC migration, with the VEGF released from the cells becoming the chemoattractant for HUVEC migration. Osteogenic induction for cells seeded on the CoBG/PCL scaffold failed to show significant ALP activity. The ALP activity tended to reduce with an

increasing incubation period. Hence, we hypothesised the cobalt released from the CoBG/PCL scaffolds may have succeeded in mimicking hypoxia, which led to a low ALP activity despite the osteogenic induction. Indeed, the cobalt effect from the CoBG/PCL scaffolds in mimicking hypoxia has been shown to maintain the 'stemness' of the cells resulting in a lower differentiation potential without affecting the cells' proliferative capacity. Our results showed the CoBG/PCL scaffold has great potential to be used in the regeneration and repair of bone and dental related structures. The controlled release of cobalt from these scaffolds can be tailored for individual needs, and the large interconnecting pores are also an important property of the scaffold. The next step would be more *in vitro* testing, which will hopefully move on to *in vivo* testing.

In conclusion, although further optimisation is needed to improve the cell seeding efficiency, along with additional *in vitro* investigations (eg. the effect of the CoBG/PCL scaffold on other hypoxia target genes and comparison with actual hypoxia), the CoBG/PCL scaffolds presented in this chapter possess the potential to be used in the regeneration and repair of bone and dental related structures.



Chapter 7

Chapter 7

Conclusions

Our body undergoes changes as we age, including decreasing bone density, and a lower capability to heal fractured bones. An ageing human tooth will have a narrower pulp canal due to deposition of secondary dentin, and diminished blood supply. The increasingly ageing population is in part due to the advancement in research and healthcare, thus, there is a demand for more complex and sophisticated treatment options. Bone fractures and osteoporosis are common in the elderly. Dental problems such as tooth wear and hypersensitivity have also become a major concern in this age group. The demand for aesthetic and cosmetic dentistry among the adolescents is also growing, and this group are also prone to traumatic or sports injuries where fractured teeth or exposed dentin-pulp complex creates a major challenge to ensure the dental pulp remains vital. One of the biggest challenges in developing materials for tissue repair and regeneration is the production of a material that enables the host tissue to respond favourably to enhance tissue longevity. In this thesis, bioactive glasses (BG) were chosen as a suitable material due to their biocompatibility and bioactivity. The strontium-substituted BG provided the additional benefit of stimulating bone cells, and the cobalt-containing BG acted as a hypoxia-mimicking agent, which helps to stimulate angiogenesis *in vitro*.

In the preliminary experiments, the dissolution products of strontium-substituted BG (SrBG) were used on bone modifying cells, Saos-2 osteoblasts, and RAW264.7 monocytes that can differentiate into osteoclasts upon addition of RANKL. Two different concentrations of BG powder to culture medium ratio were used for DMEM (6.0 mg/ml) and RPMI (1.5 mg/ml). The different particle size of the BG powder affected the ionic dissolution profiles following incubation in culture medium DMEM and RPMI. However, our results showed the strontium released from the SrBG was dependent on the strontium concentration in the SrBG. Larger particles tend to release lower strontium in the culture medium, but our results also showed the strontium release after 4 hours in the culture medium was below 1 mM $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$ (a positive control) This is lower than the concentration normally used for patients

receiving strontium ranelate to treat osteoporosis (352). In Saos-2 cells, the ALP activity increased steadily from days 3 to 14 but then decreased at day 21, which is dependent on the strontium concentration in the SrBG. The DNA assay showed increasing proliferation up to day 21 in all strontium-containing BG except the 0SrBG. The ALP activity of 0SrBG was the highest at day 21. Our finding may suggest that lower strontium concentration or control BG without the addition of strontium helps promote Saos-2 proliferation. For RAW264.7 cells, the TRAP activity (which represents osteoclast differentiation and bone resorbing activity) was highest at days 2 and 4 before decreasing at day 6. Again, the DNA assay showed increasing cell proliferation at days 2 to 6. This suggests that in the presence of strontium, osteoclast activity is lower but it did not prevent the cells from proliferating. These results demonstrated the potential for SrBG to be used as a bone substitute in the regeneration of hard tissue in the field of dental pulp tissue engineering. In view of these findings, dental stem cells from the permanent tooth (DPSC) and from human exfoliated deciduous teeth (SHED) were isolated from the permanent and deciduous teeth as previously described (4, 59). Immunohistochemistry was used to confirm the presence of stem cells in the dental pulp. DPSC and SHED were then seeded on the 10SrBG (10% strontium substitution for calcium) discs, and the results showed that SrBG discs promoted cell attachment, and allowed proliferation. The 10SrBG was chosen because the low dose was found to be beneficial in the treatment of osteoporosis (20), and the slow release of strontium with tailored concentrations showed great potential to be used in regenerative dentistry, especially in hard tissue engineering of oral and dental related tissues and structures.

Dental pulp is known to develop in an environment with low oxygen tension, and procedures such as drilling of cavities for carious treatment creates a traumatic injury that reduces blood flow and changes intra-pulpal pressure, which may lead to pulp necrosis. Furthermore, dental extraction procedures, especially in teeth with pre-existing problems such as abscesses, or when patients have medical conditions requiring dental treatment, such as patients on bisphosphonate therapy that may cause osteonecrosis of the jaw, require carefully developed therapies. Thus, the use of cobalt-containing BG as a hypoxia-mimicking agent may be advantageous for the repair and regeneration of dental related tissues. For this, the investigation of the

potential of CoBG in dental tissue engineering was carried out. In the first part, the dissolution products of CoBG were assessed on DPSC and SHED. The second part involved the incorporation of CoBG powder into polycaprolactone (PCL) for the development of a 3D scaffold in collaboration with Professor Dietmar Hutmacher's group. The CoBG developed by a member in the group was previously optimised for *in vitro* studies (121). It has since resulted in the development and characterisation of more cobalt-containing BGs for use as biomaterials in the field of tissue engineering and regenerative medicine (24, 122).

When 1.5 mg/ml CoBG powder in DMEM culture medium was subjected to ICP-OES, it was found the cobalt release from the BG was dependent on the cobalt concentration present in the BG powder. In addition, its release could be tailored. The dissolution products of CoBG at a concentration of 1.5 mg/ml from CoBG with low cobalt concentrations of 0Co, 1Co and 2CoBG (0%, 1% and 2% cobalt substitution for calcium in mole %) did not strongly affect DPSC or SHED cell viability, and proliferation. The 2% cobalt substitution for calcium (2CoBG) conditioned medium was able to activate the HIF-1 pathway in the dental stem cells through HIF-1 α stabilisation as shown by the higher HIF-1 α nuclear extracts after 8 hours. This was further supported by the lower *HIF1A* mRNA expression at 8 and 24 hours, which suggested that upon translocation into the nucleus, HIF-1 α dimerised with HIF-1 β and bound to the hypoxia-responsive element on target genes. As in our case, it is shown by the release of VEGF upon exposure of these dental stem cells to the hypoxia-mimicking effect of the CoBG. The CoBG has succeeded in mimicking hypoxia in normoxic environment. However, as mentioned previously, mimicking hypoxia using cobalt may result in different responses from different cell types because the response to hypoxia is cell type-specific.

The ionic dissolution products from CoBG powder showed great potential for dental pulp tissue engineering. To further investigate its potential, the CoBG powder was incorporated with a biodegradable polymer, polycaprolactone (PCL), to make a 3D CoBG/PCL scaffold for potential clinical use in surgery. BG powder has been widely used in dentistry – it is commonly mixed with body fluids or blood prior to implantation. However, using BG as powder may be problematic in surgery because the correct powder-to-body fluid ratio needed to be used is difficult to determine to

stabilise the powder in surgical site. For this, additional support is needed such as the use of membrane or suitable flap design to ensure the BG powder stays in place. Thus, the use of a 3D scaffold may help to overcome these problems. The 3D CoBG/PCL scaffolds were seeded with DPSC and human mesenchymal derived stem cells from the bone marrow (hMSC). The scaffold was fabricated by our collaborator Professor Dietmar Hutmacher from Queensland University of Technology (QUT) using the fused deposition modelling method (FDM) with 70-75% porosity and pores size ranging from 0.2 to 2.0 mm (105, 308). We opted to use the 0-60-120° lay-down pattern for our scaffold. Indeed, the 0-60-120° lay-down has been shown to possess the highest compressive strength in the x-axis compared to the PCL only scaffolds (443). Due to the large porosity, initial difficulties were faced, particularly regarding cell seeding in order to get the maximum cell number on the scaffold. However, this problem was solved by adopting a static seeding method for DPSC and hMSC. The scaffold was fabricated with 30 weight percent (wt %) of CoBG powder, a value made possible because the CoBG powder is having particle size less than 20 μm , and this is the first reported study to use this formulation. However, we understand that the BG powder incorporation into the polymer requires further optimisation. Previous studies used particle size ranging from 38 to 50 μm with 10 to 20 wt % BG powder (105, 443) and 10 to 50 mass % beta-tricalcium phosphate (β -TCP) powder (particle size 3 to 5 μm) (473) incorporated into the polymer scaffold. The ICP-OES data showed that cell-free CoBG/PCL scaffolds were able to release cobalt ranging from 1.84% of cobalt for 1CoBG/PCL, 1.15% cobalt for 2CoBG/PCL and 1.42% cobalt for 4CoBG/PCL at day 1 upon immersion in 500 μl DMEM culture medium. We found it intriguing that the cumulative cobalt release throughout the 13-day incubation period was only 14%. This may be due to the cobalt remaining embedded in the PCL bulk, while the slow PCL polymer backbone degradation allows continued cobalt release at low concentration. Low cobalt release helps to reduce cobalt toxicity and the incorporation of cobalt into CoBG/PCL allows for the use of these scaffolds as a reservoir for continued cobalt release that remains within a safe range. The cobalt release from the CoBG/PCL did not negatively affect the DPSC and hMSC viability, and the highest cobalt concentration (4 mole % of cobalt incorporation into the CoBG powder) allowed proliferation on this scaffold for up to 14 days. This is expected, since the concentration of cobalt from the

CoBG/PCL within the culture medium is lower than with the CoBG-conditioned medium.

The CoBG/PCL scaffolds, particularly the 2CoBG/PCL scaffold, successfully triggered both DPSC and HMSC to activate the HIF-1 pathway and release VEGF. Our observation that the cobalt release from the CoBG/PCL had successfully activated the HIF-1 pathway through *HIF1A* mRNA expression and release of VEGF was also seen in Chapter 5 when we used CoBG powder. VEGF is one example of the activation of the hypoxia pathway and indicates that the hypoxia-mimicking CoBG/PCL scaffolds have potential to be used in regenerative dentistry. Although both DPSC and hMSC were able to release VEGF when seeded on the CoBG/PCL scaffold and cultured in serum-free media, the VEGF release failed to induce HUVEC migration towards the chemoattractant. Factors other than VEGF may have contributed to these observations, for example, the use of correct media for HUVEC and the addition of growth factors cocktail in HUVEC's EBM growth media. When both DPSC and hMSC were induced in osteogenic media, the ALP activity was lower compared to cells seeded in control medium and control BG (0CoBG/PCL). This may suggest that the hypoxia-mimicking effect of the CoBG/PCL scaffold retains these cells in an undifferentiated state, which is why they failed to show higher ALP activity. However, the high cell number based on the DNA assay from the same DPSC and hMSC cell lysates showed that these cells were growing and attaching on the CoBG/PCL scaffold, suggesting that the cobalt release in the osteogenic medium did not affect cell number.

Further studies are needed in order to meet the full potential of these hypoxia-mimicking CoBG/PCL scaffolds. The incorporation of a higher percentage of CoBG powder into the polymer is suggested. For example 50 wt % with particle size ≤ 20 μm would achieve a higher cobalt release from the CoBG/PCL scaffold. The seeding method may also be improved by the use of hydrogel to hold the cells in place or a surface treatment of the CoBG/PCL struts to enhance cell attachment, and the use of a CoBG/PCL scaffold during the migration assay with cell-free and cell-seeded scaffolds. In addition, the cellular morphology and attachment on the CoBG/PCL scaffold in relation to the scaffold struts, bars and interconnecting struts is also an

interesting subject to look into, especially how the cells responded morphologically to various cobalt concentrations within the CoBG/PCL scaffold. Finally, an *in vivo* study of the CoBG/PCL scaffold, particularly the effect of cobalt release from the scaffold to the surrounding tissue, would be interesting.

In this thesis, the potential of strontium-containing BG and cobalt-containing BG were assessed *in vitro*. Although further studies are needed to understand the dental stem cells responses to these SrBG and CoBGs, these investigations provide an insight into the potential of SrBG and CoBG in regenerative dentistry. The SrBG has the potential to be used in restorative and preventive regimes. Strontium in dental materials may help to retard caries progression and allow the tooth be retained in the oral cavity. The development of 3D scaffolds from the SrBG and CoBG may help its use in surgical applications, and strontium and cobalt as SrBG and CoBG in 3D scaffolds as bone substitutes may help to improve hard tissue regeneration, especially in fractured or traumatised bone. The strontium may stimulate bone cell differentiation and the cobalt may promote angiogenesis by mimicking hypoxia in the vicinity of traumatised area. Thus, SrBG and CoBG have great potential to be used in dental tissue engineering.

Based on the project carried out in this thesis, a few suggestions for future work are listed below:

- The assessment of differentiation of cells towards osteogenic lineage, particularly when using the strontium-containing BG-conditioned medium. Quantitative assays such as Alizarin Red or Von Kossa staining should be used to assess nodule formation.
- The addition of controls such as bone mineralising agent are beneficial for the *in vitro* assessment of bone formation or resorption when using osteoblasts or osteoclasts.
- The ionic dissolution from the BG discs should be measured. The discs are usually prepared with variable diameters and thicknesses. Measuring the weight of each disc prior to seeding with cells may help determine the ionic release from the BG disc upon immersion in culture medium.

- An *in vitro* investigation using the cobalt-containing BG in mimicking hypoxia is needed to examine the metabolic switch to anaerobic metabolism due to hypoxia. This may include measuring lactate dehydrogenase activity and reactive oxygen species (ROS) formation. The use of actual hypoxia as control (1-5% oxygen) may also provide useful information when assessing the CoBG hypoxia-mimicking effect.
- Optimisation of the BG-powder-to-PCL ratio is needed to ensure cobalt release from the CoBG/PCL scaffold remains within therapeutic range.
- Besides using the SrBG and CoBG in powder forms, incorporation of the BG powder into thin film, membrane, gels, paste, or injectable suspension is desirable, while fabrication into shaped-3D objects such as plates, screws, and tooth implants may also help deliver these beneficial ions to the site of injury for tissue regeneration.
- Finally, once suitable *in vitro* investigations have been carried out, *in vivo* studies are necessary, first on small lab animals before clinical studies using human patients.

Appendices

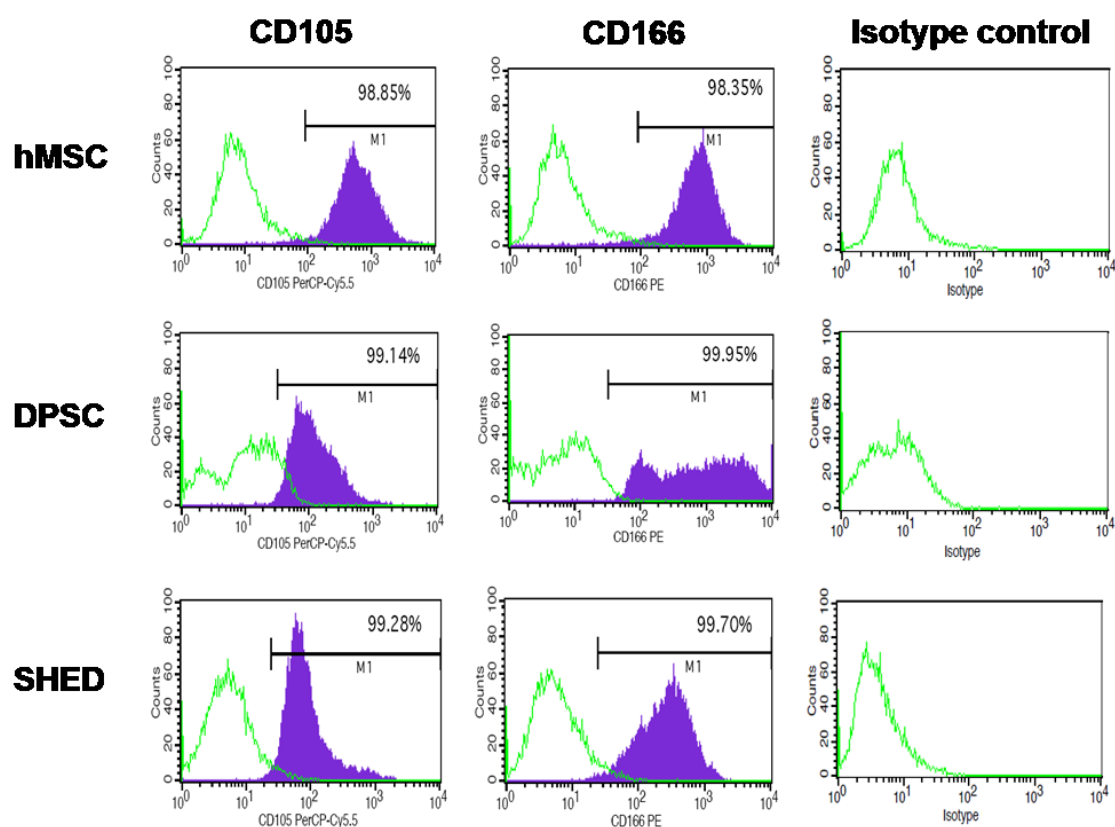
Appendix 4.1

Characterisation of the stem cells from the dental pulp of human teeth using flow cytometry

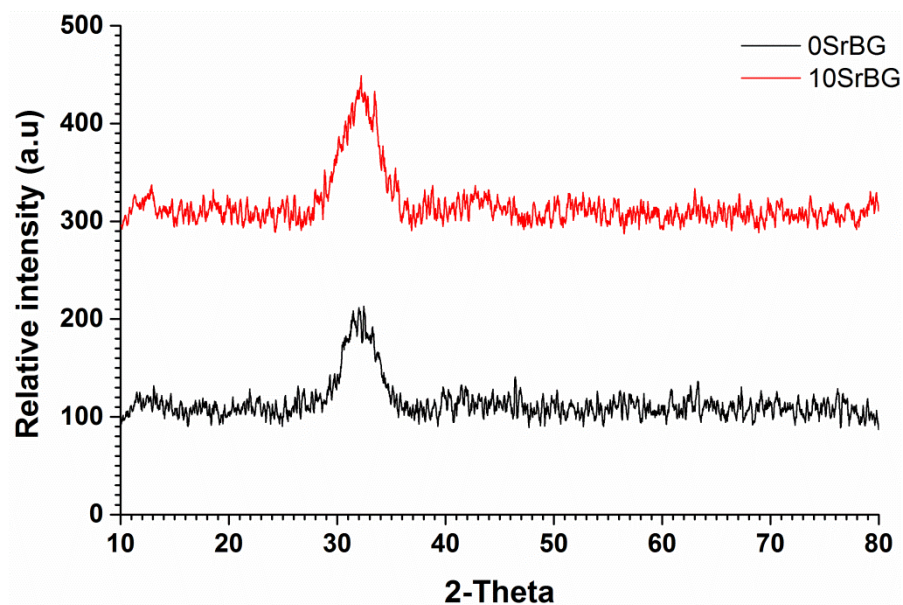
The cultures of DPSC and SHED were washed twice with sterile PBS and dissociated using cell dissociation solution non-enzymatic (C5914, Sigma Aldrich, UK) for 30 minutes at 37°C and inactivated using sterile PBS containing 2% (v/v) fetal bovine serum (FBS, GIBCO, USA). The cells colonies were dissociated into single-cell suspensions by trituration, centrifuged, resuspended with 2% (v/v) FBS in PBS, and filtered using a 70 µm cell strainer (BD Falcon, USA) prior to counting using trypan blue exclusion criteria to identify viable cells. The pulp stem cells suspensions (DPSC and SHED) were resuspended at a concentration of 3×10^5 cells in 500 µL PBS with 2% (v/v) FBS and incubated for 30 minutes at 4°C in the dark with PerCP-Cy5.5 or PE-conjugated antibodies as follows: anti-CD166-PE, and anti-CD105-PerCP-Cy5.5. The samples were analysed on a flow cytometer machine FACSCalibur (BD Biosciences, USA).

The percentage of positive cells was determined as percentage of cells with higher fluorescence intensity than the upper 0.5% isotype immunoglobulin control and the classification criteria is as followed: <10% no expression, 11-40% low expression, 41-70% moderate expression and >71% high expression (390). In the flow cytometric analysis, DPSC and SHED showed high expression for the following adhesion molecules; CD105 (monoclonal mouse anti-human Endoglin) (BD BioSciences, USA), and CD166 (monoclonal mouse anti-human ALCAM) (BD BioSciences, USA).

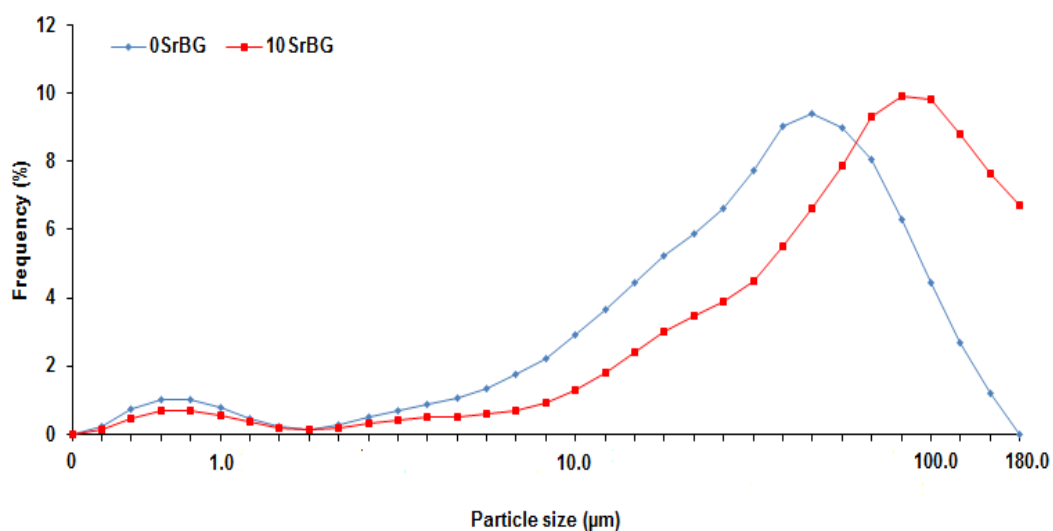
For a negative control, the pulp stem cells suspensions of DPSC and SHED were resuspended at a concentration of 1×10^6 cells in 500 µl PBS and incubated for 30 min at 4°C in the dark with PerCP-Cy5.5 or PE-conjugated-isotype-matched control antibodies.



Supporting information regarding the flow cytometry analysis for DPSC, SHED, and hMSC performed at Universiti Sains Malaysia.

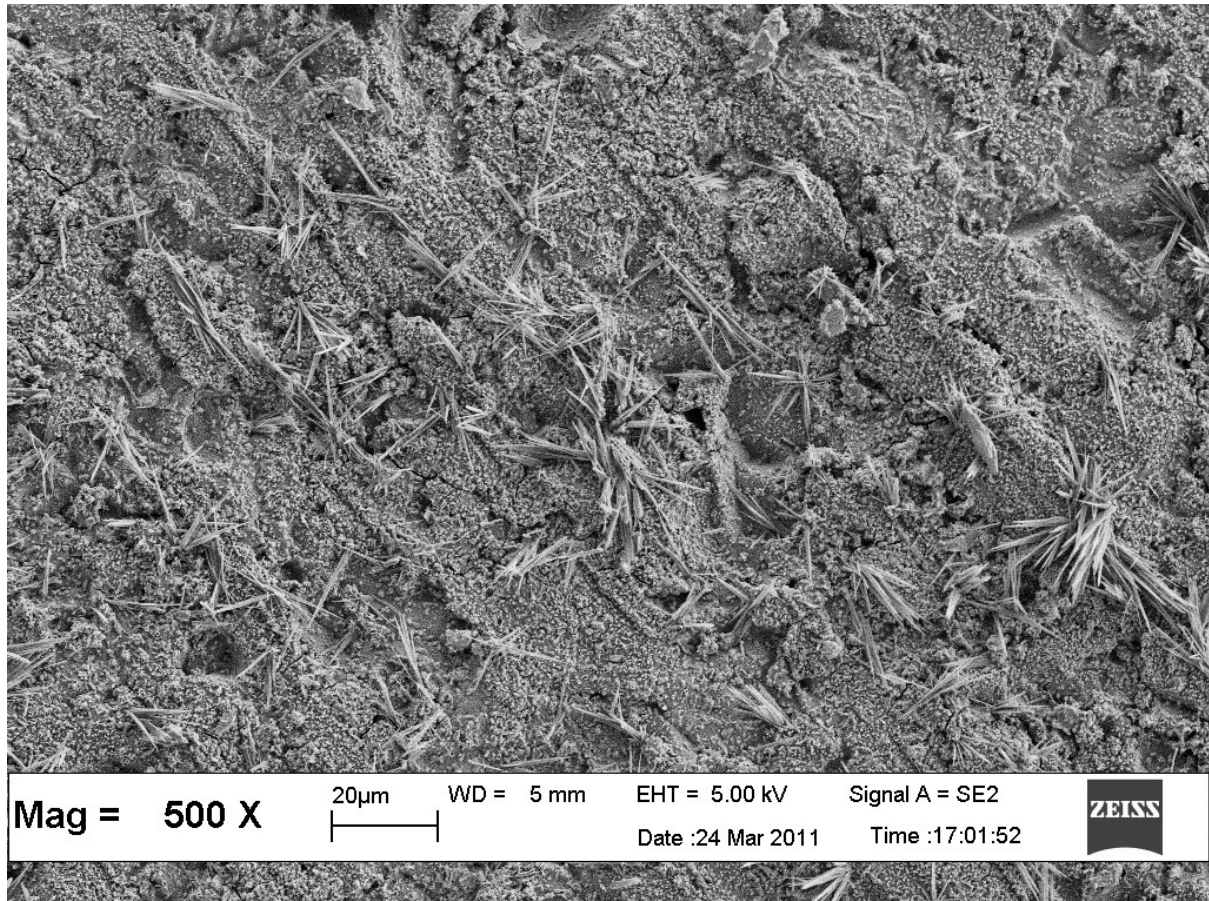
Appendix 4.2

Supporting information regarding the amorphous structure of 0SrBG and 10SrBG powder determined by XRD analysis performed at Universiti Sains Malaysia.

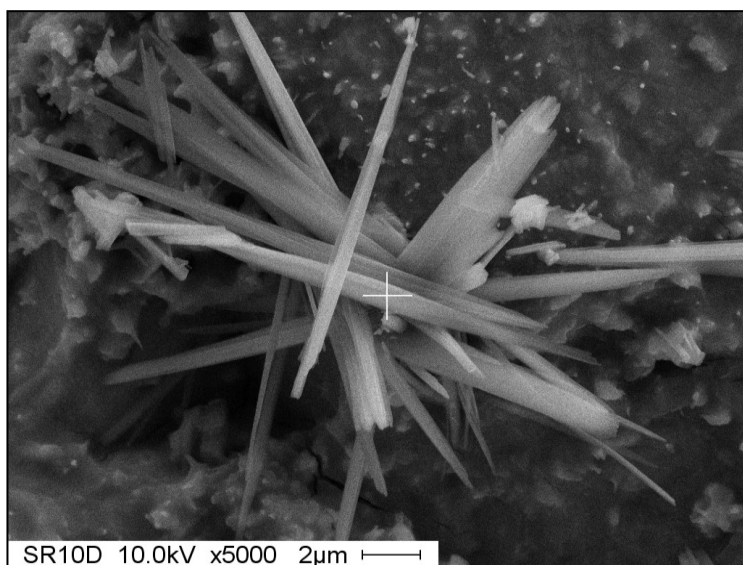
Appendix 4.3

Supporting information for particle size distribution for 0SrBG and 10SrBG powder performed using particle size analyser (Malvern Instruments) at Universiti Sains Malaysia.

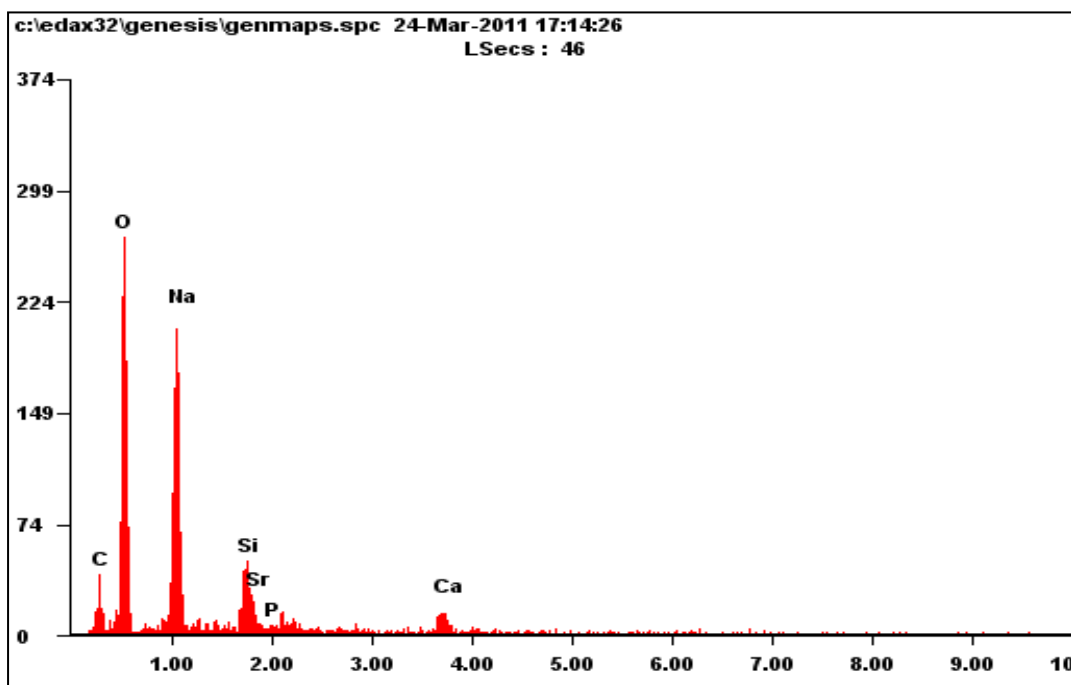
Appendix 4.4

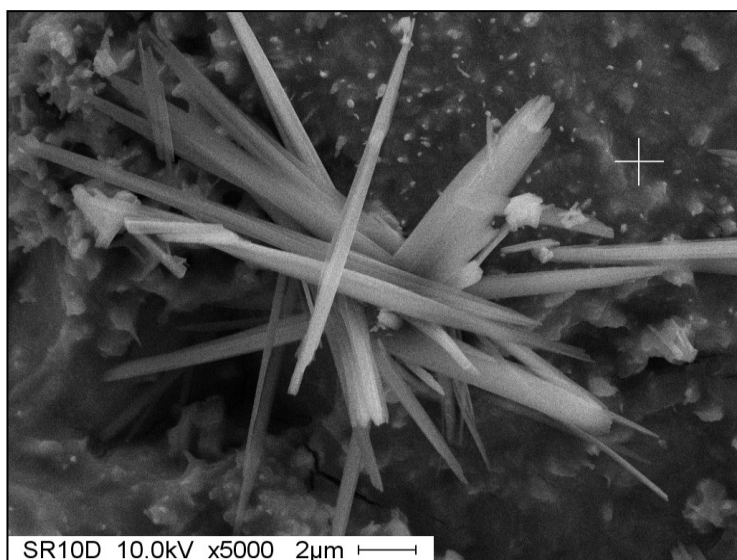


The scanning electron microscopy view of 10SrBG disc. The scanning was performed by Mr. Abdul Rashid Selamat at Universiti Sains Malaysia.

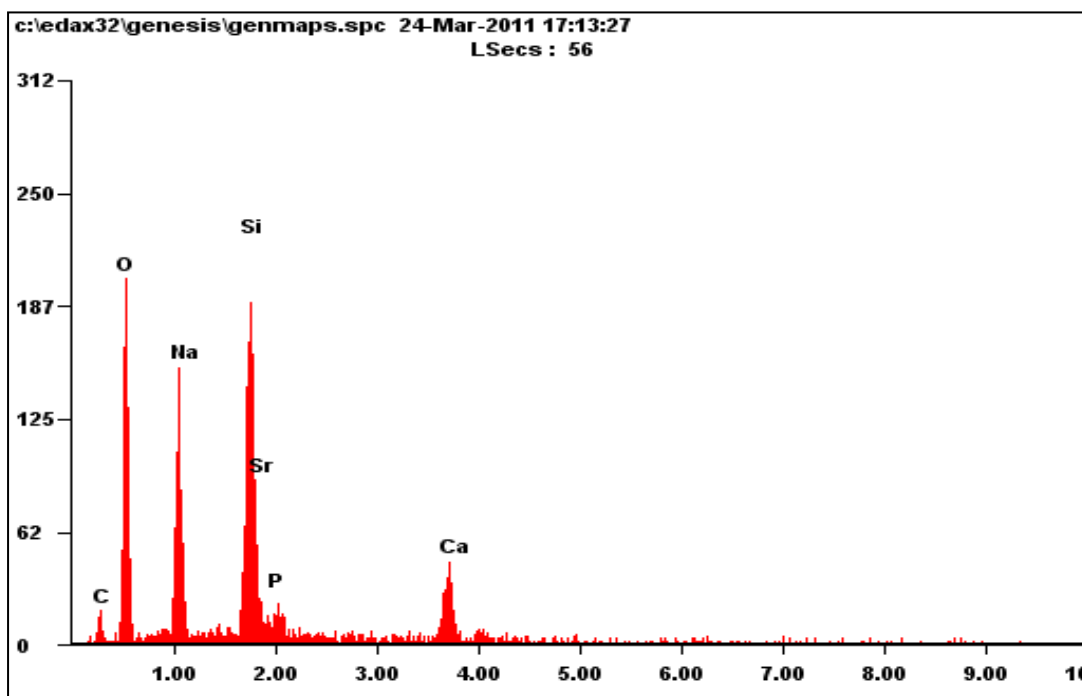


<i>Element</i>	<i>Wt%</i>	<i>At%</i>
C K	12.47	19.67
O K	40.76	48.27
NaK	26.51	21.84
SiK	07.93	05.35
SrL	04.14	00.90
P K	00.72	00.44
CaK	07.47	03.53





<i>Element</i>	<i>Wt%</i>	<i>At%</i>
C K	07.21	13.62
O K	28.51	40.46
NaK	12.31	12.15
SiK	25.06	20.25
SrL	07.58	01.96
P K	03.65	02.68
CaK	15.69	08.88



Appendix 6.1

Scaffold fabrication

Bioactive glass powder (control/ CoBG) with particles size of $\leq 20\mu\text{m}$ were incorporated into the PCL bulk by fast precipitation into excess ethanol. Briefly, 10% w/v PCL solution was prepared by dissolving PCL pellet (3.0 mm, CAPA 6500) in chloroform (100% concentration, MERCK) at room temperature. Then, 30 wt % of BG relative to the PCL mass was added into the PCL solution and stirred until a homogenous mixture was achieved. The PCL/BG solution mixture was then precipitated into 5-fold excess of 100% ethanol (MERCK). The solid PCL/BG composite was then isolated and air dried. All scaffolds were fabricated with melt extrusion based additive manufacturing technology using an in-house screw-based extrusion system (317). Briefly, composite material (CoBG/PCL) was loaded into the material reservoir and melted at 95°C for PCL and 110°C for composite material. As pressure is applied, molten material moved towards the rotating screw chamber and extruded through the nozzle tip aided by the rotational movement of the screw. All scaffolds were designed with a lay-down pattern of 0-60-120°, filament gap of 2 mm and slice thickness of 0.35 mm. Scaffolds of 50 × 50 × 2.5 mm were fabricated. All scaffolds were fabricated using 21G nozzle. Ambient temperature in the lab was maintained at 25 ± 2°C (Parameters used as shown in table below).

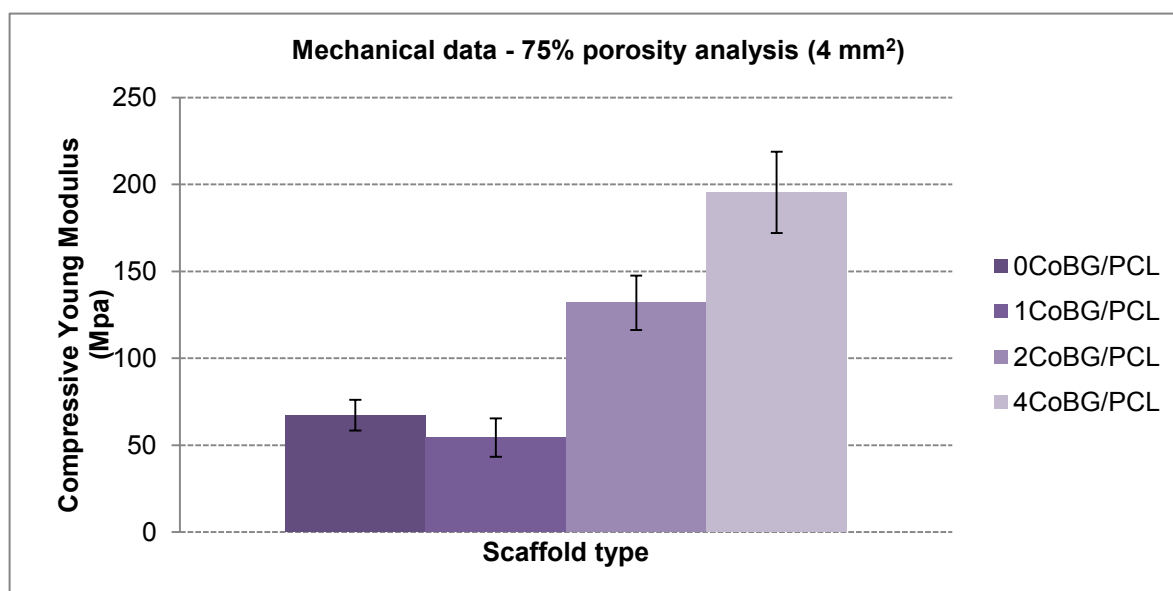
Parameters used for scaffold fabrication	
Lay-down pattern	0-60-120°
Reservoir temperature	70°C
Reservoir pressure	6 bar
Screw velocity	30 rpm
Extrusion temperature	
PCL	95°C
PCL/CoBG	110°C
Scanning velocity	8 mm/s
Nozzle/platform distance	0.2 mm
Nozzle size	21G
Filament gap	2.0 mm

The CoBG/PCL scaffolds were prepared by Professor Dietmar Hutmacher's group from Queensland University of Technology (QUT), Australia.

Appendix 6.2

Mechanical properties of the CoBG/PCL scaffold

Mechanical compression tests were conducted using an Instron 5848 microtester fitted with a 500N load cell (Instron, Australia). Scaffolds (n=8) were subjected to 10% compression at a rate of 1 mm min^{-1} and the compressive Young's modulus of the scaffolds was determined using the initial linear portion of the stress versus strain data. The test was conducted on the scaffold having 75% porosity.



The mechanical properties of the CoBG/PCL scaffolds were performed by Professor Dietmar Hutmacher's group from Queensland University of Technology (QUT), Australia.

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