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Title: Enhancing osteogenic capabilities of human umbilical cord matrix mesenchymal stem cells on 3D-printed hydroxyapatite/calcium carbonate scaffolds.

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Summary

As the demand for global bone graft surgery increases, there is a growing need for more effective bone graft substitutes. Currently used bone grafting materials at the clinical level show numerous limitations, highlighting the need for producible and more effective bone graft substitutes. Hydroxyapatite/calcium carbonate (HA/CC) is a material similar to natural bone tissue with biodegradability and osteoconductivity, and is, therefore, an ideal replacement of bone grafting. However, the osteogenic capability of HA/CC scaffold has space for improvement. Umbilical cord mesenchymal stem cells (UCMSCs) are expected to be the ideal seed cells for bone tissue engineering due to their widely available source, convenient collection, and similar biological characteristics to bone marrow-derived mesenchymal stem cells (BMSCs), but without the ethical and supply limitations of it. With the growing interest in enhancing the osteogenic capabilities of MSCs, pharmacological approaches were being widely researched. Retinoid acid (RA) is a metabolite of vitamin A. It has been proved that RA signaling plays an important role in development, differentiation and bone metabolism. In this study, we hypothesis that by using RA we can improve osteogenesis of UCMSCs on HA/CC scaffolds. Therefore, the aim of this study is to enhance osteogenic capacity of UCMSCs on HA/CC scaffolds in vitro by supplementing RA in osteogenic medium. We assessed the attachment, proliferation/cytotoxcicity of UCMSCs on HA/CC scaffolds and the effect of RA on osteogenesis of UCMSCs in vitro. Results of Live/Dead assay indicated HA/CC scaffolds possessed high biocompatibility properties evidenced with the high survival rate of UCMSCs (>90%). After 21 days of osteogenic differentiation, the ALP activity and BMP-9 concentration of UCMSCs were increased significantly ($P<0.05$) by RA supplementation respect with control group. In summary, by supplementing proper dose of RA can enhance the osteogenesis capacity of UCMSCs on HA/CC scaffolds in vitro, offering a potential tissue-engineered bone graft substitute for future clinical application.

Table of contents

Table of Figures

Abbreviation

HA	Hydroxyapatite
CC	Calcium carbonate
UCMSCs	Umbilical cord mesenchymal stem cells
BMSCs	Bone marrow-derived mesenchymal stem cells
MSCs	Mesenchymal stem cells
RA	Retinoid acid
ALP	Alkaline phosphatase
BMP	Bone morphogenetic protein
3D	Three-dimensional
FDM	Fused deposition modelling
RNA	Ribonucleic acid
TAZ	Tafazzin
TGF	Transforming growth factor-beta
RAR	Retinoic acid receptors
RAI	Retinoic acid-induced gene
MHC	Major histocompatibility complex
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal bovine serum
pNPP	P-nitrophenyl phosphate
pNP	p-nitrophenol
ELISA	enzyme linked immunosorbent assay
HRP	Horseradish Peroxidase
PBS	phosphate buffer saline
ARS	Alizarin red S
ISCT	International Society for Cellular Therapy
CCK-8	Cell counting kit-8
SEM	Scanning electron microscope

1. Introduction

1.1 Characteristic of bone graft substitutes in tissue engineering

The consistent increase in the prevalence of congenital and lytic bone disorders, infection, trauma, as well as rising life expectancy of global populations have compelled the development of novel bone replacement techniques. Traditionally, bone defects have been addressed by using bone grafts, which may be autografts or allografts (De Long et al., 2007; Ilan & Ladd, 2002). Autografts are generally regarded as the gold standard for repairing deficiencies due to their possession of such features as histocompatibility and osteoconductive potential. However, challenges such as harvesting autografts from specific sites are inherently risky and expensive, hazarding such outcomes as prolonged bleeding and inflammation (Sohn & Oh，2019).

In a bid to overcome such obstacles, research and clinical practice have turned towards bone tissue engineering, and the development of synthetic bone graft substitutes has offered exciting alternatives to traditional grafts. Although synthetic bone graft substitutes are constantly updated, there are still many unsolved problems. The main problem is that the process of material degradation in vivo cannot be perfectly matched with the rate of new bone formation, and the resulting mixture of biomaterial and bone cannot completely reconstruct the bone tissue in physiological state. In addition, the osteoconductivity, osteoinductivity and vascular inductive activity of the material cannot be well balanced, which leads to a serious limitation in the timeliness and regionality of the material for repairing bone defects, and it is often difficult to quickly and effectively reconstruct large bone defects.

The popularity of bone tissue engineering in bone volume replacement mostly arises from its capacity to utilize readily available materials, elimination of the danger of transmitting infections between patients, and relatively low cost. The approach also holds the potential for the fabrication of client-specific designs of implants and

prostheses (Brydone, Meek, & Maclaine, 2010). For the most part, bone tissue engineering is principled in applying naturally occurring or synthetically manufactured scaffolds possessing mechanical, chemical, and biological characteristics comparable to those of native bone extracellular matrix (Gupta, Kumar, Keshav, & Singh, 2015). Their structural and chemical configuration is guided by attempts to confer a set of biological mechanisms integral for grafting, including osteoconduction, osteoinduction, and osteogenesis (Kumar, Fathima, & Vinitha, 2013). Osteogenic lineages and vasculogenic growth factors are seeded into such scaffolds to enhance bone regeneration and repair. Depending on their use, ideal scaffolds should also be biocompatible, non-thrombogenic, anti-inflammatory, and bioactive. Suitably-sized interconnected macro- and micropores should also be incorporated to enhance in vivo osteogenic cell ingrowth. As mentioned, engineered bone scaffolds should be similar in composition to the bone extracellular matrix, which predominantly contains type-1 collagen, nanohydroxyapatite, and carbonated apatite mineralites.

1.2 The application of hydroxyapatite in bone graft

In the search for appropriate natural bone substitutes, one substance that have attracted substantial research and clinical attention are calcium phosphate ceramics, mainly composed of hydroxyapatite, whose chemical structure closely mimics that of the mineral phase of calcified tissues. Calcium phosphate ceramics are synthetic mineral salts prepared using sintering processes at high temperatures to remove water vapor and subsequent molding using high-pressure compression. The forms of this material that are commonly produced include porous and non-porous dense implants and granular porous particles. They have primarily been researched and applied in orthopedic practice as a form of bioabsorbable ceramic possessing remarkable osteoconductivity, biocompatibility and non-immunogenicity (Burg et al.,2000; Yoshikawa and Myoui, 2005; Kattimani et al., 2016).

Hydroxyapatite is a variant of calcium apatite that occurs naturally and which has the formula $Ca_{10}[PO_4]_6[OH]_2$. Comprising up to 50% of bone weight, it has excellent osteoconductive and osteointegrative profiles. However, in its natural form, hydroxyapatite's mechanical structure is similar to that of cancellous bone, which can withstand compressive, a pushing force tends to reduce the size of material after compression loads but is fairly brittle and has poor resistance to tensile forces, a pulling force tends to increase the size of material after tension.

Moreover, the material may undergo up to 40% in situ decrease after several months of implantation. Still, the microporous nature of synthetic versions of the substance allows osteoprogenitor cells to attach, proliferate, and differentiate, in addition to enabling revascularization and ultimate novel bone ingrowth following in vivo implantation (Hart et al.,2017). Despite being significantly limited by its slow degradation rate and mechanical kinetics, hydroxyapatite has several important characteristics that have formed the basis of its application in bone grafting and implant coating procedures.

The principal biological properties make hydroxyapatite similar to natural bone including biocompatibility, low immunogenicity and inflammatory response. It is a porous scaffold with a remarkable resemblance to endogenous bone. Its chemical components contain 50% of natural bone and up to 80% of dentin and enamel. Although its initial mechanical weakness is a notable drawback, its post-integration strength approaches that of natural bone. Moreover, hydroxyapatite forms bonds strongly to newly formed bone by forming a carbonated calcium-deficient epitaxial layer, and after healing it is grossly indistinguishable from an autologous graft (Piaia et al.,2020). As a natural structure composed primarily of calcium and phosphate ions, its immunogenicity and potential to stimulate an inflammatory reaction is low. Additionally, the risk of infection in graft sites using hydroxyapatite is lower than that in sites implanted with allografts or xenografts.

Several chemical and structural properties are also critical to the efficacy of hydroxyapatite in bone grafting and new bone development. The porous nature of hydroxyapatite scaffolds is an important feature in biointegration, particularly due to its role in cell migration and development, which allows the scaffold to facilitate cell stability and consequent vascular development. The concentration, size and interconnectivity of pores in the biomaterial has a notable impact on both bone ingrowth and vascularization, with studies estimating the minimal size of pores for blood vessel formation at 50 micrometers, while osteoid growth requires at least 200 micrometer-wide pores (Saiz, Gremillard, Menendez, Miranda, Gryn, & Tomsia, 2007). As the size of pores increases from less than a micrometer to over 1000 micrometers, various cellular bioactivities become viable in the resulting microenvironment, ranging from interaction with protein compounds to cellular attraction, the directional orientation of ingrowths, increase in mechanical strength, and distortion of the implant's shape and esthetics (Von Doernberg et al. 2006). Porosity also regulates the bone regeneration, osteoconductivity, rate of bone regeneration and equilibrium of novel tissue at the graft site (Hing, 2005). Its surface chemistry, particularly the capacity for minor substitution of calcium ions with magnesium, strontium or other ions, allows for significant boosting of its structure and biocompatibility, and since enhancing cell migration potential is critical to bone growth, this modification is important. Hydroxyapatite also interacts with osteoblasts and external cellular proteins that are necessary for cell migration, and can form a reservoir for cytokines and concentrates of bone morphogenetic proteins (BMPs) from external sources. Overall, the pore structure and other properties of hydroxyapatite allow it to exhibit outstanding biocompatibility and a high affinity for added particles such as drugs and growth proteins, a property that has important applications in tissue bioengineering.

The significant drawbacks of natural hydroxyapatite are its slow rate of biodegradation and inferior mechanical kinetics (Sheikh et al., 2016). As a natural structure, the substance has a mechanical profile similar to that of cancellous bone, which is resistant

to compressive forces but has poor tolerance to tensile forces (Tamimi et al.,2012). As a result, hydroxyapatite was initially restricted to use as a coating for orthopedic and dental implants. The process, which dates as far back as the 1980s, is intended to encourage the fixation of the implant to endogenous bone to prolong the lifetime of metallic implants. In vitro studies demonstrated that hydroxyapatite-coated titanium items had higher osteoblastic activity and induced higher level of collagen than those without the surface layer, resulting in a larger bone-implant contact area (Daugaard et al.,2012). Moreover, the accompanying ingrowth of mineralized tissue translated to better fixation and biocompatibility in dental implants. The application of the hydroxyapatite layer can be achieved using a variety of processes with distinct strengths and disadvantages, with common approaches including plasma spraying, dip coating, electrophoretic plating, biomimetic deposition, and pulsed laser coating. Studies assessing the properties of these coating methods have revealed that stable contact surfaces and biological milieu are better achieved by including both amorphous and crystalline phases in the applied layer. In addition, thinner hydroxyapatite layers on implants have been found to be superior in the induction of local cellular response as compared to thicker layers. Since the mechanical strength of the graft in this case is not dependent on the hydroxyapatite coating, the limitation of slow degradation rates is not of much concern in this application (Dehghanghadikolaei et al.,2019).

The slow biodegradation rate of hydroxyapatite, which is an important disadvantage of the material and a major motivator for the development of composite scaffolds, is dependent on its calcium and phosphate ratio, surface area to volume ratio, and surrounding fluid kinetics, acidity, and temperature. Ideally, a scaffold's degradation rate should approximate the progression of bone replacement by cells in the graft locality. This property is an important consideration in the selection of scaffold material for bone tissue, as well as in ensuring the long-term success of an implanted graft. Degeneration can exert significant influence on the three-dimensional cell formation as well as revascularization of the new tissue, which are critical processes in bone

regeneration. Moreover, the degradation rate has a significant effect on the mechanical features of the newly formed bone, and in the event where the regenerated tissue has poor structural integrity, bone regeneration can fail, especially in such load-bearing engineering applications as spinal fusion implants (Du et al.,1998). Several studies have assessed the degradation of hydroxyapatite, including the biochemical reactions involved and ways of altering the material's degradation speed. Scaffolds of hydroxyapatite may be degraded by acidic dissolution or fragmentation into particles that are subsequently phagocytosed by macrophages (Sheikh et al., 2015). Osteoclasts also play a role in clearing the fragmented material, a finding that reinforces observations of the similarity between hydroxyapatite and natural bone. Some researchers suggest that osteoclastic degradation is a favorable clearing pathway since the mimicry of physiologic bone resorption results in the creation of surfaces ideal for osteoblast colonization and ultimate vascularization. Interestingly, the degree of osteoclastic degradation is dependent on specific qualities of the material, such as the size of crystals and the surface roughness (Hasegawa et al., 2005). Accordingly, macrocrystalline hydroxyapatite does not exhibit surface osteoclastic activity in in vivo implantation, while microcrystalline variants have improved osteoclast-mediated resorption.

Studies examining the biodegradation of hydroxyapatite implants have reported varying findings on the role of osteoclasts and the impact of sintering on the rate of dissolution. In an animal model involving grafting of critically sized artificial defects in pig mandibles, Rumpel et al. (2006) studied the degradation of two forms of hydroxyapatite implants; a crystalline NanoBone produced using a sol-gel procedure, and Bio-Oss, which was prepared by sintering and deproteinization of bovine spongious bone (Rumpel et al. 2006). Five weeks after the operations, both groups of implants had achieved adequate bridging of the mandibular bone defects through the formation of compact, albeit cancellous new bone. Remodelling of the regenerated bone was evident in the form of novel osteons containing sizeable vascular channels in woven bone. The

new bone covered the peripheral parts of the defects, with only minimum quantities of biomaterial remaining (Rumpel et al. 2006). Histological analysis of samples from both groups of implant sites revealed multinucleated giant cells of a similar size and shape to osteoclasts adhering to the bone tissue. The cells also exhibited other characteristics typical of osteoclasts, including morphologic polarization, Tartrate-resistant acid phosphatase activity, and ruffled border reactions. The presence of resorption lacunae on the surface of the regenerated bone further suggested osteoclastic activity (Rumpel et al. 2006).

Irrespective of the role of osteoclasts in the biodegradation process, the development of nanocrystalline hydroxyapatite represents an important step towards overcoming the drawbacks occasioned by the slow dissolution of the material. The advancement has further improved the range of synthetic polymers of hydroxyapatite that can be incorporated into hydroxyapatite. Theoretically, a wide range of polymers can be integrated with nanohydroxyapatite scaffolds to form materials with better biodegradation and other characteristics. However, several basic requirements for optimal dispersion of hydroxyapatite nanocomposites limit the variety of polymers that can be incorporated. For instance, the interfacial strength between the nanoparticles and the complementary monitor should support adhesion between the particles of the two substances. The absence of these adhesive forces can result in the disintegration of the two phases of nanomaterial polymer, adversely affecting the implant's tensile strength. Moreover, as is commonly required of biomaterials intended for implantation, an ideal nanohydroxite polymer should demonstrate adequate wettability. This feature affects the nature and strength of attractive forces between the two polymer surfaces and the adjacent tissues. Wettability is determined by the polarity of particle groups of the polymer, and can render the material either hydrophobic or hydrophilic. The physical properties of nanohydroxyapatite polymers are also affected by the method used to incorporate the constituent substances. Polymer matrices are commonly rendered using two broad techniques, thermomechanical and physico-chemical methods.

Thermomechanical techniques, which include such procedures as compounding and injection moulding, constitute conventional approaches for impregnating porous polymeric matrices with nanoparticles. The physico-chemical methods involve either nanocrystal co-precipitation into such porous matrices or solution of the hydroxyapatite nanocrystals in an appropriate solvent that then serves as a medium for dispersing the particles and adding them into the polymer. (Gupta, Kumar, Keshav, & Singh, 2015). Although both approaches have been shown to produce high-quality polymeric hydroxyapatite frameworks, limitations such as thermal sensitivity of certain polymers, cytotoxicity of some solvents, and gelation rates lower the application of some methods.

The synthesis of hydroxyapatite polymers consisting of varying materials has offered a means for researchers to vary the biophysical characteristics of the ceramic to produce more useful structural variants. As mentioned, the degradation rate is particularly sensitive to alterations in the composition of the construct. The degradation profiles of synthetic nanohydroxyapatite polymers depend on the speed of dissolution of individual components. By striking a balance between fast and slow resorption of polymers, synthetic processes can develop materials that allow for optimum regeneration of bone and remodeling of the new tissue at the graft site. In addition to the nature of the individual compounds, the rate of degradation is also influenced by such characteristics as the size of nanohydroxyapatite crystals and the roughness and topographic structure of the polymer surface. The crystal size is especially important as it determines the material's specific surface area to volume ratio, which can render the polymer easily resorbable if it increases significantly. Another determinant of the rate of degradation in nanohydroxyapatite polymers is the molar ratio of calcium and phosphate in the structure.Along with the advancement in synthetic polymerization and nanocrystal formation techniques, the amount of research published on the characteristics of the resultant polymers has been increasing. A study performed by Dong et al. (2009) examined the rate of resorption and biocompatibility potential of scaffolds of porous hydroxyapatite and polyurethane. The researchers seeded the

scaffolds with bone marrow stromal cells, then implanted them in healthy mice and examine for degradation patterns. They found that while the scaffolds remained nontoxic, the rate of dissolution was higher than that of natural hydroxyapatite. However, in vitro degradation was also observed and shown to result from chemical disintegration of castor oil and subsequent hydrolysis of urethane bonds between the polymer components. Conversely, in vivo degradation was mediated by both polyurethane hydrolysis and digestion of the hydroxyapatite component by monocyte-derived macrophages. A similar experiment conducted by Han et al. utilized a biomimetic nanofibrous polylactide scaffold to which nanohydroxyapatite crystals were added. The scaffold's specific surface area was significantly higher than that of variants without the nanofibrous quality. Due to the higher specific surface area, the nanofibrous scaffold was found to have a higher rate of degradation than typical polymers of polylactide and hydroxyapatite.

The limitations arising from the slow biodegradation rate and mechanical properties of natural hydroxyapatite, along with the need for better control of such qualities as osteoinduction and osteogenesis of implants, have pushed research on other alternatives to bone grafts. Calcium carbonate, which is an abundant naturally occurring substance found in limestone and shells of various organisms, has received significant research attention owing to its mechanical properties, faster degradation rates, and appropriateness for the development of polymer composites (Donate et al., 2019). Calcium carbonate exists naturally in crystalline phases found in calcite, a stable rhombohedral structure, aragonite, which is orthorhombic and metastable, and vaterite, an unstable hexagonal particle (He, Zhang, Yang, Zhu, Tian, & Chen, 2015). Aragonite, which occurs in coral, is often used as a cement component or implant in maxilla-facial surgery, although studies detailing its properties are few. A synthetic aragonite powder has also been developed, but the challenges of fashioning a volumic scaffold made of this material pushes researchers towards animal-origin aragonite extracted from shells of coral-dwelling organisms (Guillemin, Meunier, Dallant, Christel, Pouliquen, & Sedel, 1989; Araiza, Gómez-Morales, Clemente, & Castaño, 1999).). Moreover, pure calcite

materials have several restrictive properties and limited applications independent of hydroxyapatite composite polymers.

Despite the rather limited utility of pure calcium carbonate scaffolds as bone graft materials, several studies have extracted or manufactured such molecules using various techniques and determined their chemical, biological, and physical properties. An in vitro study by Manchau and others (2013) prepared and characterized a calcium carbonate sample using a sintering process. The researchers then assessed the vitality and proliferation rates of osteoblast cells cultured on the macroporous scaffolds of calcium carbonate compared to those cultured in tricalcium phosphate and hydroxyapatite (Monchau, Hivart, Genestie, Chai, Descamps, & Hildebrand, 2013). The viability of cells in the calcium carbonate ceramic remained similar to that in human epithelial cell lines, which was used as a control, but was not significantly different from that of cells cultured in hydroxyapatite and tricalcium phosphate. The proliferation rate of the cultured cells at three days and six days after introduction was, however, higher in hydroxyapatite than in both calcium carbonate and tricalcium phosphate. Considering the absence of dissimilarity in cell viability among the ceramics, this disparity could arise from characteristics that are unrelated to cytotoxicity, such as surface morphology and pore microstructure. In a different rat model study, Vuola and colleagues (1998) compared the compressive strength of implants made of calcium carbonate and compared it to that of hydroxyapatite implants six weeks after implantation. Both implants were derived from the same coral organism, with the hydroxyapatite having been converted from the calcite in a sintering industrial process. Two sets of each type of ceramic were tested, one pre-immersed in bone marrow saline and another undergoing no pre-treatment (Vuola, Taurio, Göransson, & Asko-Seljavaara, 1998). The tensile strength of sets of implants was measured at three, six, and 12 weeks. At three weeks, mild resorption of the coral implants had already occurred, although their overall shapes were intact. At this stage, the tensile strength of the calcium carbonate blocks was significantly higher than those of hydroxyapatite, and

both samples dipped in bone marrow were stronger than the controls that received no pre-treatment. At six and 12 weeks, however, both groups of calcium carbonate had undergone such extensive degradation that tensile strength determination was not feasible (Vuola, Taurio, Göransson, & Asko-Seljavaara, 1998). Conversely, hydroxyapatite blocks retained the tensile strength attained by 3 weeks, with the difference between control and test groups disappearing. While the peak compressive strengths of both types of implants well surpassed that of cancellous bone, none of them approached that of compact bone. The researchers attributed the difference in compressive strength between soaked and dry samples to the role of bone ingrowth in modifying the mechanical properties of ceramic implants. Similar findings had been reported by several other studies utilizing comparable experimental setups (He et al.,2015; Wenisch et al., 2003). Moreover, the experiment demonstrated the difference in degradation rates between the two types of ceramic implants.

The development of industrial and technological processes for the preparation of polymers of hydroxyapatite and combination with other compounds to manufacture composite scaffold has provided more practical approaches to controlling target properties of implants such as biodegradation rates and mechanical strength. Such compositions can be achieved by modification of traditional sintering procedures or soaking in titration solutions. One such procedure, applied by Yang, Zhong, Zhou, Kundu, Yao, and Cai (2016) entailed synthesizing hydroxyapatite with a controlled calcium and phosphate content from calcium carbonate place in sodium hydrogen phosphate solution. Samples of the resulting material were then treated with a microwave for varying periods, centrifuged, washed with deionized water, and dried. The difference in solubility of calcium carbonate and hydroxyapatite in the alkaline solution evoked an ion exchange reaction involving carbonate and phosphate anions, which was accelerated by the microwave treatment (Yang, Zhong, Zhou, Kundu, Yao, & Cai, 2016). The calcium and phosphate ratio of the resulting composite can be controlled, and this property predicted the degradation time of the composite, which

lays somewhat between the individual degradation rate of the two ceramic implants.

A more novel and promising technique for generating composite scaffolds of various biodegradable compounds is additive manufacturing, which renders three-dimensional (3D) structures with custom biological and chemical properties. Many polymers can be used to fabricate scaffolds for bioengineering using additive manufacturing. Methods such as stereolithography, laser sintering, and fused deposition modelling can be applied to model the material due to their adjustability and macroporous composition. 3D printing based on fused deposition modelling (FDM) has been adopted in many laboratories for regenerative biomaterial modelling, and involves processing of thermoplastic pellets. Composites of hydroxyapatite and calcium carbonate have been prepared and characterized using this technique.

An increasing number of studies is examining the properties of composites of different polymers, including hydroxyapatite and calcium carbonate. In most cases, the biochemical and physical properties of the polymer composites reflect a state between those of the individual components. Chan et al. (2002) demonstrated that adding 9.2% by volume of calcium carbonate to polypropylene raised the substance's impact strength from 55 J/m to 133 J/m. Similarly, Wang et al. (2021) found that the ductility of propylene was improved by the introduction of calcium carbonate particles. The increased hydroxycarbonate formation was attributed to a higher concentration of calcium carbonate, whose faster dissolution can enhance hydroxycarbonate supersaturation. The relevance of these findings lies in the potential applications of hydroxycarbonate as a novel biomaterial as it has effective biocompatibility in various conditions.

In addition to their contribution to the biochemical and physical characteristics of hydroxyapatite polymers, composites containing calcium carbonate are used as transport media for the delivery of growth-promoting molecules and drugs in graft sites.

Collectively, the characteristics of optimal mechanical kinetics, adjusted degradation rate, and macroporous structure allow the material to absorb and slowly release such particles over a prolonged period, while concurrently providing a viable microenvironment for bone-forming cells and growth factors (Verron & Bouler, 2010). This application offers the possibility of conferring the capacity for osteoinduction and osteogenesis to commonly used regenerative bone substitutes.

Fu, Xu, Czernuszka, Triffitt, and Xia (2013) recently performed an experiment to customize the degradation rate of a hydroxyapatite scaffold to allow for optimal stimulation of bone growth. Scaffolds consisting of coralline hydroxyapatite and calcium carbonate were processed by partially generating hydroxyapatite from coral-derived calcium carbonate. In vitro characterization of the scaffolds was then completed before they were subjected to an in vivo test model. Moreover, the composite framework was utilized in a clinical trial assessing their capacity for stimulating bone regeneration (Fu, Xu, Czernuszka, Triffitt, and Xia, 2013). The in vitro models involved culturing of mesenchymal stem cells on the scaffolds or on glass slides, with a group of the samples containing osteogenic media while the other set lacked such an environment (Fu, Xu, Czernuszka, Triffitt, and Xia, 2013). Initially, cells placed on glass slides containing osteogenic factors experienced rapid proliferation. However, at 16 days, the rate of proliferation was comparable for cells placed in osteogenic media and those growing without them. The mesenchymal stem cells growing on composite scaffolds demonstrated a higher level of alkaline phosphatase activity than those on glass slides (Fu, Xu, Czernuszka, Triffitt, and Xia, 2013). As expected, the cells cultured on scaffolds, culturing cells in osteogenic media increased the amount and maturity of collagen deposited on the growth environment. While the researchers did not compare the regenerative potential of the composite frameworks to that of pure hydroxyapatite, the results indicated that the calcium carbonate and hydroxyapatite scaffolds could be used with media containing bone growth promoting proteins and cells to create an osteogenic scaffold. In the study, Fu and colleagues (2013) assessed the osteogenic

properties of the hydroxyapatite and calcium carbonate composite seeded with human-derived mesenchymal stem cells. After treatment with risedronate to slow the degeneration of any bone tissue that would be formed, the stem-cell enriched scaffolds were implanted on the dorsal surface of immunodeficient mice and evaluated 10 weeks later using scanning electron microscopy. The scaffolds lacking stem cells demonstrated minimal formation of fibrous tissue and formed no bone tissue. Conversely, the composite scaffolds seeded with mesenchymal stem cells induced osteogenesis on their surfaces, which was visible on scanning electron microscopy. The composite scaffolds were then used in a clinical trial where bone resection to remove tumors was followed by repair and graft with the composite material. After 4 months, successful bone regeneration was documented in 16 patients. The study represents a promising line of application of composite scaffolds for conferring osteoinduction and osteogenesis in biomaterials.

1.3 Human umbilical cord mesenchymal stem cells: an ideal seed cell for bone tissue engineerin

By merit of its capacity for differentiation into multiple tissue lineages, the fibroblastic cell type referred to as mesenchymal stem cells has attracted substantial attention in tissue engineering and other disciplines. These cells, originally discovered in the bone marrow stroma, have been shown to be present in several adult tissues, including the heart, pancreas, brain, adipose tissue, and tendon (Fitzsimmons, Mazurek, Soos, & Simmons, 2018). While their presence in such a wide anatomical distribution seems to indicate the existence of a ubiquitous stem cell niche in all tissues, bone marrow stem cells and adipose-derived stem cells are the only groups with substantial volumes to allow practical harvesting in the adult (Wan, Shi, Nacamuli, Quarto, Lyons, & Longaker, 2006). In the bone marrow, mesenchymal stem cells typically localize close to the sinusoidal endothelium along with resident hematopoietic stem cells. Besides being osteogenic progenitors, the mesenchymal stem cells also create a niche that is trophic

for hematopoietic stem cells by secreting such factors as angiopoietin 1 and CXC ligand 12. Once isolated, populations of mesenchymal stem cells can be increased exponentially without losing their multipotency and ability to develop fibroblastic colony-forming units (Vater, Kasten, & Stiehler, 2011). The cells manifest a robustly proliferative phenotype while remaining capable of dividing along the three regular lineages. Despite this proliferative trait, human mesenchymal stem cells have a low tendency to accumulate chromosomal or genetic defects that can lead to oncogenic transformation, a feature that is favorable for lowering the risk to patients (Xing, Zhang, Zhong, Ju, Zou, Zhu, & Sun, 2016). By definition, mesenchymal stem cells possess a trilineage differentiation potential consisting of osteogenesis, chondrogenesis, and adipogenesis, all dependent on the factors available in the microenvironment in which the cells are cultured.

Although bone marrow mesenchymal stem cells are well characterized and routinely used for a variety of clinical, biomedical, and research applications, the persistence of several disadvantages plaguing their acquisition and use has necessitated the search for other sources of mesenchymal stem cells. Various embryonic sites have recently become the focus of such research, with the umbilical cord and placenta emerging as important alternative sources of potent proliferative stem cells (Day, Francis, Fu, Pieper, Guy, & Xia, 2018; Michel, Penna, Kochen, & Cheung, 2015).

Human umbilical cord mesenchymal stem cells (UCMSCs) are contained in Wharton's jelly, the principal material in the matrix of human umbilical cord. They express several of the surface molecules identified in adult stem cells, such a CD90, CD105 and CD73 (Dominici, M et al.2006). UCMSCs have been popularized by their advantages over stem cells harvested from other sites. For instance, unlike BMSCs, UCMSCs are readily obtained from human umbilical cord, rendering their use ethically uncomplicated and cheap as the material is regarded as medical waste. The ethical implications of this process are further lessened by the absence of the risk of complications that accompany

bone marrow harvesting, including pain, prolonged hemorrhage, infection, and insufficient sample volumes. Further, considering that over 120 million births occur annually across the globe, UCMSCs are readily available. Some studies have also documented a decrease in the proliferative potential and osteogenic differentiation of mesenchymal stem cells derived from the bone marrow of older patients. Mesenchymal stem cells harvested from the marrow of patients with certain bone diseases have comparable limitations. Conversely, UCMSCs being embryonic in nature, portray a wider multipotency and osteogenic differentiation. They also have an even lower risk of neoplastic transformation, a feature which, coupled with their low immunogenicity, boosts their safety for the recipient. Consequently, UCMSCs have been studied and utilized in many areas, including bone tissue engineering, where studies have documented robust in vitro and in vivo osteogenic potential when the cells are culture in three-dimensional regenerative scaffolds.

Mesenchymal stem cells have been known to release a range of bioactive substances, including cytokines, exosomes, and growth factors. Exosomes are small vesicles secreted from cells that play a role in the mediation of intercellular communication, which may involve the transfer of microRNAs and certain proteins. This secretome is important to the functions of mesenchymal stem cells, which commonly accumulate in damage sites and release factors that encourage angiogenesis, initiate tissue repair, and inhibit inflammation and fibrosis. Umbilical cord mesenchymal stem cells have also been shown to have a similar secretome, including exosome and trophic protein production. Compared to bone marrow mesenchymal stem cells, UCMSCs have been reported to better induce paracrine-mediated differentiation and migration of neural cells and angiogenesis (Maher, Kolieb, Sabik, Abd-Elhalim, El-Serafi, & El-Wazir, 2015). In addition, the cytokines released by these cell populations have been shown to enhance osteoblastic regeneration, proliferation of cells in bone extracellular matrix, as well as bone regeneration (Shen, Yang, Xu, & Zhao, 2019; Wang et al. 2015)). These properties have attracted research into the potential for the use of UCMSCs in ceramic

and other biomaterial scaffolds used as alternatives to bone grafts, a process that further extends the regenerative capacity of such frameworks.

Classically, induction of osteogenic differentiation is done by culturing a confluent monolayer of the mesenchymal stem cells along with dexamethasone, ascorbic acids, and beta-glycerophosphate. These factors are complemented by the addition of BMPs, vitamin D3, and transforming growth factor-beta to enhance osteogenic differentiation. Dexamethasone, which is a regular component of such osteogenic protocols, is a glucocorticoid that has been found to be necessary for the differentiation of stem cells into osteogenic progenitors. Without this supplement, mesenchymal stem cells placed in basal medium do not form mineralized extracellular matrix. The exact mechanism of dexamethasone-induced differentiation of mesenchymal stem cells and regulation of skeletal function is poorly understood, but researchers suggest that it induces transcription effects by binding onto the promotor region of such bone-specific genes as bone sialoprotein. Moreover, dexamethasone has been shown to enhance the expression of certain factors involved in the activation of Runt-related transcription factor-2 (Runx-2), such as integrin alpha-5, it participate in cell-surface mediated signalling. In supraphysiological doses, however, glucocorticoids slow down osteoblastic function, inhibit collagen formation, and are well known to cause osteoporosis. Osteogenic differentiation of mesenchymal stem cells, however, requires at least three weeks of sustained incubation with dexamethasone. In addition to dexamethasone, osteogenic differentiation requires calcium and phosphate ions, which drive bone matrix mineralization. Protocols usually provide these elements through beta-glycerophosphate, whose hydrolysis by ALP release the ions and result in mineralization and lactate generation. Ascorbic acid, another component of regular osteogenic differentiation protocols, is necessary for proline and lysine hydroxylation. Vitamin D3 is also included for optimal osteoblast function through cAMP-dependent calcium ion intake and stimulation of such differentiation factors as Runx-2. In addition to these components, osteogenic differentiation is usually enhanced by the addition of

specific growth factors, including transforming growth factor-beta and BMPs.

At the molecular level, the differentiation of mesenchymal stem cells along the osteogenic lineage is determined by interactions among certain transcription factors and with specific hormones. Among the most well-characterized of these are BMPs and transforming growth factor-beta (TGF-β). TGF-β has 3 isoforms, with TGF-β1 being the most abundant in bone. It regulates cell growth and contributes to bone formation by modulating the formation and degradation of such components as collagen type 1 and other proteins. Although TGF-β1 increases Runx-2 expression, some researchers report that it inhibits late-stage osteoblast differentiation. Bone morphogenetic factors, which also belong to the TGF superfamily, serve as important inducers of osteogenic differentiation. Unlike TGF-β1, BMPs can stimulate the formation of bone in developed tissues. They can also induce the expression of genes regulating bone formation components such as collagen. Runx-2 is an osteogenic growth factor that regulates the expression of a wide range of bone-specific genes, including osterix, osteocalcin, and bone sialoprotein. Along with TGF-β1, BMP-2, and ALP, Runx-2 are considered early markers for mesenchymal stem cell differentiation along osteogenic lineages. Osterix is a zinc-finger-containing protein that controls gene expression in osteoprogenitor cells. It has been shown to be essential for osteoblastic differentiation and bone formation by regulating fundamental genes such as osteocalcin and collagen type 1.

The potential for osteogenic differentiation of human umbilical cord mesenchymal cells has been examined by several studies using regenerative scaffolds of varying compositions. Wang et al. (2010) assessed cell differentiation and formation of a mineral matrix in UCMSCs seeded in nonwoven 3D mesh scaffolds made of polyglycolic acid. The cells were cultured in densities of between 5 × 106 and 50 × 106 cells/mL and placed in an osteogenic medium. Their proliferation, osteoblast formation, and matrix development were then determined at three-week intervals. Based on the expression of ALP activity and a rise in the amount of collagen produced and calcium

entering the extracellular matrix, the researchers recorded significant osteogenic differentiation of the stem cells, which was higher in the samples with a higher density of cells. In addition, the study found an increase in the expression of several genes that are typically associated with osteogenesis, including Runx-2, osteocalcin, and collagen type 1. The researchers concluded that when exposed to signals that enhance bone formation, UCMSCs are capable of differentiating along an osteogenic lineage on an appropriate three-dimensional scaffold, the robustness of the growth being influenced by such factors as the density of cells cultured. In another study, Day, Francis, Fu, Pieper, Guy, and Xia (2018) compared the bone-forming potential of UCMSCs against that of BMSCs, where both cell populations were cultured on a three-dimensional composite scaffold made from coralline hydroxyapatite and calcium carbonate. Brightfield and scanning electron microscopy were used to characterize the multidimensional growth that occurred over a three-week study period. To assess for osteogenesis in the samples, a combination of histochemical techniques and a qPCR scan for common markers such as Runx-2 and collagen type 1 were utilized. On the first day, cells from both populations proliferated and formed bridges between adjacent scaffold granules to develop the structures commonly termed organoids, which then started to form interconnecting bridges by the seventh day. In addition to the proliferation, both cell groups demonstrated considerable deposition of the bone matrix as well as differentiation along osteogenic lineages. However, the osteogenic potential of BMSCs was found to be higher than that of UCMSCs.

The finding of a superior osteogenic potential of BMSCs, marked by a higher ALP activity and Runx-2 expression, despite being contrary to the results of a few studies, is not uncommon in the literature on this subject. In fact, the majority of studies that have compared the osteogenic potential of both cell populations on a common scaffold have reported that BMSCs formed bone at a higher rate than UCMSCs. For instance, Schneider et al. (2010) recorded higher ALP levels in BMSCs cultures compared to UCMSCs, although the UCMSCs showed higher collagen type 1 gene expression.

Zhang et al. (2009), however, reported an interesting deviation from this seeming consensus. While also concluding that more bone matrix was formed in cultures of BMSCs cultures than in populations of mesenchymal stem cells, the study showed that the rate of osteogenic proliferation would be higher if UCMSCs were cultured in monolayer (Zhang et al. 2009). These findings constitute ground for further research assessing the comparative rate of osteoblastic transformation in UCMSCs placed in monolayer then seeded on common 3D scaffolds. Unlike the findings by most researchers, results from a study performed by Todeschi et al. (2015) indicated similar rates of orthotopic bone formation between UCMSCs and BMSCs, an outcome that was supposed to have occurred through recruitment of host osteoprogenitors by the UCMSCs. In an interesting modification of the experimental approach, Chen et al. (2018) exploited the angiogenic property of UCMSCs by coculturing the cell population with endothelial cells derived from human umbilical cord vein, thereby increasing the capacity of the cells to modify the local environment and recruit bone progenitors. The researchers found that in such an in vivo model, the amounts of bone formed by UCMSCs and BMSCs were similar. Overall, while UCMSCs offer several advantages over BMSCs, their potential for forming new bone on regenerative scaffolds may be inferior to that of the BMSCs. However, further research is necessary to conclusively characterize this potential of UCMSCs. In particular, ways of enhancing the potential of these cells to form new bone should be extensively explored in order to realize the possible benefits that they can offer to tissue engineering. As UCMSCs demonstrate increasing potential as an alternative to BMSCs in bone tissue engineering, the search for factors that can increase their potential for differentiation along osteogenic lineages has intensified.

1.4 The potential of retionoic acid in enhancing osteogenesis

Retinoic acid (RA), a metabolite of Vitamin A1 ,play an important role in embryonic development and function maintenance of vital organs in adult (Duester G, 2008). RA

is a vital nutrient most familiar for its role in good vision, is one of the substances that have captured the interest of researchers on this subject. Ingested in the form of carotenoids or retinyl esters, the vitamin is metabolized into its active forms, including all-trans-retinoic acid and 11-cis-retinal. Acting through RA receptors (RAR) that heterodimerize and bind into responsive elements in the genome, RA regulates the expression of several genes (Conaway, Henning & Lerner, 2013). As a result, RA controls many cellular functions, some of which occur in bone tissues. However, a well-known side effect of excessive levels of the compound is higher skeletal fragility. While RA was initially thought to increase the resorption rate of bone tissue, further investigation revealed that its skeletal effects involved both stimulation and inhibition of osteoclast formation (Li et al., 2003; Wang et al., 2019). Studies assessing the influence of RA on bone resorption have unearthed its potential for enhancing the osteogenic capacity of mesenchymal stem cells.

While the mechanism of retinoic acid-induced osteogenesis is not entirely understood, one of the important ways that this effect is affected is modulation of the expression of BMPs through Smad activity. Smads are transcription factors that mediate the bone morphogenetic protein signaling. They are commonly found in the central and peripheral nervous system, where they regulate neuronal cell development from various progenitors in the neural crest (Song, Estrada, & Lyons, 2009). RA enhances the translocation of these mediators to the nucleus of osteogenic progenitors. Of the well-known Smad proteins, RA mostly affects signaling events mediated by Smad 1 and Smad 5 (Liu, Liu, Zhang, Wang, Huang, Yan, 2014). By increasing the activity of these signaling molecules, RA upregulates the expression of BMPs, especially BMP9, thereby increasing osteogenic differentiation of sensitive cells. In addition to regulating bone formation, RA has also been shown to decrease the rate of adipogenic differentiation of mesenchymal stem cells (Li et al. 2015). This effect is thought to occur due to possible cross talk between RA and certain BMPs, which inhibits the differentiation of mesenchymal stem cells along adipogenic cell lineages (Wan,

Shi,Nacamuli, Quarto, Lyons, & Longaker, 2006). All-trans-retinoic may also affect signaling via the Wnt/β-catenin pathway, which also regulates the expression of such factors as BMP-9 (Skillington, Choy, & Derynck, 2002). These observations represent important inroads in unraveling the relationship between RA and bone metabolism and osteogenesis. However, research on the extent of the influence of the compound on the osteogenic potential of mesenchymal stem cells, including its impact on human umbilical cord mesenchymal stem cells seeded in 3D scaffolds, as well as other pathways that mediate this effect, is still necessary.

The role of RA in osteogenesis, including its influence on the bone-forming capacity of mesenchymal stem cells, has been investigated in several studies. Cowan et al. (2005) determined how the addition of recombinant BMP-2 and RA affected bone formation in a mouse model. The researchers harvested osteoblasts, bone marrow stem cells, and adipose-derived stromal cells from mice and seeded them in polyglycolic acid scaffolds. The cells were exposed to BMP-2 and RA in an ex-vivo setup for four weeks, then implanted into standardized calvarial bone defects (Cowan et al, 2005). Samples of the grafted areas were collected and examined fortnightly for up to 12 weeks. By two weeks, the cells had formed complete ridges across the defects, with osteoclasts localizing to the site. The rate of osteogenesis was significantly higher in areas implanted with samples that had been treated with RA and bone morphogenetic protein-2. While the optimal strategy for in vitro priming of mesenchymal stem cells for osteogenic transformation may yet be undiscovered, the results from this and other studies suggested that RA treatment and exposure to recombinant osteotropic factors such as BMP-2 was one method of achieving this outcome. In a similar experiment, Cruz, Cardozo, Magini, and Simoes (2019) examined the impact of RA on the osteogenic capacity of adipose-derived stem cells. The setup involved treatment of samples of the stem cells cultured in osteogenic media with BMP-2 alone, RA alone, or a combination of BMP-2 and RA, then assessing ALP activity every other day (Cruz, Cardozo, Magini, & Simões, 2019). Determination of ALP activity was done using r-nitrophenol

detection, while von Kossa staining enabled assessment of extracellular matrix mineralization. In addition, the researchers assessed the presence of such markers as osteocalcin and osteonectin using qPCR, while the levels of factors such as Smad and BMP-4 were determined using western blotting. The findings were that the highest alkaline phosphate activity was recorded after a week in samples that were only stimulated using RA. Moreover, treatment of the stem cells with a mixture of RA and BMP-2 resulted in the highest expression of phosphorylated Smad proteins after seven days. This treatment also enhanced the expression of both osteonectin and osteocalcin. This study reinforced previous suggestions that the effect of RA on bone differentiation of mesenchymal stem cells is based on the upregulation of BMPs and Smad factors. This observation had also been made by Adams et al. (2003), who suggested that RA increased osteogenic activity by enhancing BMP signaling and consequently stimulating the formation of collagen type-X. Other researchers such as Liu et al. (2014) documented an increase in bone-forming activity in stem cells treated with RA, but attributed this event to the compound's effect on the Wnt/β-catenin pathway (Liu et al., 2014). A different mechanism of action was suggested by Zhang et al. (2017), who attributed the osteogenic enhancement by RA to stimulation of collagen formation driven via the BMP-2-Wnt-Runx-1 pathway.

An interesting development in the literature on the osteogenic impact of RA is the surfacing of reports that the vitamin has a negative effect on the capacity of stem cells to differentiate along osteogenic lines. For instance, Sheng et al. (2010) and Zhang et al. (2014) found that RA increased the duration of BMP signaling. The authors proposed that RA promoted the engagement of phosphorylated Smad-1 with ubiquitin E3 ligase, causing ubiquitination and breakdown of phosphorylated Smad 1, which eventually downregulated BMP and Smad signaling (Sheng et al. 2010). Similarly, Hoffman et al. (2006) reported an antagonistic interaction between BMPs and RA signaling. Their study aimed to assess the mechanism of BMP regulation of chondrogenesis. They identified a RA formation gene, Aldh1a2, as a principal target of BMP regulation of RA

signaling (Hoffman et al. 2006). By attenuating the gene's expression, BMPs inhibited chondrogenesis in mesenchymal stem cells. In another study, Jin et al. (2020) reported that downregulation of RA-induced gene 3 (RAI3) is an important event in osteogenesis. The authors documented decreased expression of the gene in stem cells that had undergone osteogenic differentiation. In addition, they demonstrated that knockout of the gene in in vivo models resulted in enhanced osteogenic activity (Jin et al. 2020). While no conclusive explanation has been given for these contradictory findings, an important variable that is discordant across the different studies is the type of cell being evaluated (Zhang et al. 2020). The researchers in these investigations assessed signaling pathways and osteogenic potentials using different cell types, including chondrocytes, preadipocytes, and osteosarcoma cells (He, 2012). Therefore, a possible reason for the variations in results and conclusions is that the effect of RA is cell-specific. Overall, the literature on how RA influences bone formation is characterized by gaps and inconclusive findings. Consequently, further research is still necessary in this sphere, particularly one involving the specific impact of RA on the bone-forming activity of mesenchymal stem cells, as well as the interaction of these factors with contemporary three-dimensional composite scaffolds.

1.5 Current approach of scaffolds carrying MSCs in bone repairing

As the prevalence of bone trauma and skeletal disorders increases across the globe, the need for alternative means of filling skeletal defects and restoring function becomes more acute. Despite vigorous research effort in this area, the mainstream solution to the problem in the current clinical scenario is the replacement of bone tissue with bone grafts, which may be derived from the patient (autologous) or donated by another individual (allogeneic). While autologous grafts are presently considered the gold standard in orthopedic and dental bone defect repair, they carry several disadvantages that often prove overwhelming for many patients (Laurencin, Khan, & El-Amin, 2006). To begin with, the patients who need bone replacement are typically inappropriate

sources of the grafts themselves. This situation arises in such conditions as osteoporosis, which is a systemic disorder causing bone degeneration in several sites at a time, such that finding places for harvesting autologous grafts for patients is a challenging goal. In addition, the option of autologous transplantation of bone may not be practical for patients bearing large defects due to the limited availability of bone for grafting from the patient's skeletal tissues. Bone grafting is also burdened by the risk of pain and morbidity following the operation. These challenges initially pushed researchers and practitioners to utilize allogenic grafts, mainly derived from cadavers (Cancedda, Dozin, Giannoni, & Quarto, 2003). However, these too had several drawbacks, including a high immunoreactivity that resulted in frequent tissue rejection and unsatisfactory host integration. Over time the morbidity and scarcity problems plaguing transplanted bone grafts have resulted in the emergence of tissue engineering as a highly promising source of readily available, safe, and optimally functioning bone replacement material.

Techniques of engineering bone and cartilage tissue have emerged as attractive alternatives to bone grafting. In addition to utilizing synthetic materials that were not constrained by unavailability, these techniques provide means of controlling design parameters such that the construct so developed mimics the characteristics of the diseased site. This allowance for optimization of the characteristic of engineered products makes it potentially possible to generate constructs whose efficiency in replacing diseased or lost bone exceeds that of allogeneic or autologous grafts. However, fashioning these parameters to meet such a specific goal has turned out to be an arduous challenge, one that constitutes the foundation of the attempts to enhance the osteogenic potential of scaffolds of hydroxyapatite and calcium carbonate by seeding them with mesenchymal stem cells.

In a bid to increase the regenerative potential of synthetic materials, tissue engineering techniques have relied on developing a combination of biomaterials and cells. The biomaterial in such models serves as a scaffold that attempts to reproduce the

environment that exists in the natural bone tissue as well as encourage appropriate cross-talk between cells and the matrix. The biomaterial also constitutes the primary housing for cells and factors seeded in the system, providing appropriate signals for the development of desired phenotypic properties. Since it serves as the main interface with native tissue, the scaffold should not be toxic to cells, should not elicit an immune reaction, and should be biocompatible. While several scaffolds, including HA/CC composites, have been able to meet these criteria, their capacity for either forming novel bone or inducing its formation from osteoblasts in the local vicinity has been modest. This limitation has been the main motivation for seeding the scaffolds with mesenchymal stem cells or factors that not only encourage bone proliferation in the model, but also initiates its formation via osteogenic differentiation.

As mentioned, scaffolds based on hydroxyapatite represent a promising alternative to natural bone grafting. These ceramic materials bear several of the prerequisite parameters targeted by tissue engineering processes (Koëter, Tigchelaar, Farla, Driessen, van Kampen, & Buma, 2009). The physical and biological characteristics that make hydroxyapatite a good candidate for this role have been discussed in this report. From a tissue engineering perspective, the scaffolds constitute the main mechanical component of synthetic bone constructs, with the aim being to approximate the structural properties of natural bone as closely as possible. Having a chemical composition that is naturally comparable to that of bone, hydroxyapatite was readily seen as a good material to meet this need. These chemical similarities with skeletal tissues resulted in hydroxyapatite being osteoconductive and osteoinductive, two of the parameters that are critical to compatibility with human tissues and to host integration of a foreign implant. Hydroxyapatite, owing to its chemical similarity to bone, has been shown to be biocompatible and subject to the vital modification of natural bone that is usually performed by osteoclasts. As a result, the substance can be biodegraded in situ, albeit at a rate that is too slow to allow optimal long-term remodeling and structural function (Vago, Plotquin, Bunin, Sinelnikov, Atar, & Itzhak, 2002). This problem, as

discussed, has been addressed by the fashioning of composite scaffolds that incorporate materials such as calcium carbonate, whose much faster biodegradation rates boost the response of the construct to native morphogenetic factors. Studies have demonstrated that hydroxyapatite-based constructs undergo good integration with hard as well as soft connective tissues. Moreover, its porous microstructure enables vascular capillary and other vessel ingrowth, allowing not only nutrient and oxygen perfusion, but also the investment of host cells that aid in integration and remodeling.

Ceramic composites represent a promising alternative to the traditional autologous or allogeneic grafts in the repair of bone injuries, but the beneficial features of most constructs do not extend beyond osteoconductivity and biocompatibility. In essence, these bioengineered alternatives are devoid of the ability to induce or originate the formation of novel bone, an event that is critical to the natural repair of bone defects in the body. To supplement these characteristics of 3D scaffolds, an array of regenerative factors and cells are often added to them as part of the transplantation process. Mesenchymal stem cells have emerged as a go-to solution for this need, providing a means for readily generating bone tissue that bridges the composite scaffolds and increases the rate of defect repair. Overall, these shortcomings underlie the need to enhance the osteogenic potential of both HA/CC scaffolds and the UCMSCs seeded in the matrix.

The addition of cells and growth factors aims to remodel the local environment and drive bone regeneration, essentially serving as the dynamic phase of the synthetic system. While their influence on the 3D models is not uniform and is not fully understood, their introduction is motivated by the hope that they parallel the functionality of natural bone tissue, make the model more responsive to remodeling forces that are essential for host integration, and promote optimal functioning of the adjacent natural tissue. Despite these attempts to improve the properties of engineered scaffolds, entirely successful models are yet to be developed. Such a regenerative

framework would be able to meet the key parameters guiding engineering techniques. A basic characteristic of such a synthetic alternative should be the ability to serve as a morphogenic signal driving osteoinduction (Michel, Penna, Kochen, & Cheung, 2015). Moreover, it should possess responsive host cells capable of detecting and responding to signals for morphogenesis, serve as a framework where the cells added can proliferate and remodel, and be transplanted next to a vascular bed in the host tissue. Moreover, an important prerequisite for a functional synthetic regenerative scaffold is the osteobiologic capacity to match the native tissue's bulk profile and functional status. Moreover, it should be modifiable by the relevant morphogenetic cells and factors, a trait that allows for osteoconduction, interaction with local tissue, as well as osteoinduction of cells from the local environment.

The major approach adopted for enhancing the osteogenic potential of synthetic scaffolds is the seeding of undifferentiated stem cells. Depending on their source, the ability of these cells to form different tissues varies from pluripotency, where any adult and embryonic cell can be developed, to unipotency, where the progenies can only end up as one type of cell (Diao, Ma, Cui, & Zhong, 2009). Due to the ease with which stem cells can be expanded in common culture media, they have been extensively utilized in bioengineering research and practice. This widespread adoption is also driven by the versatility of application of the stem cells owing to their capacity to grow into various cell types, as well as their capacity to induce alterations in the local environment by releasing growth factors and by responding to cytokines.

Despite the apparent availability of a large variety of stem cell options that can be added to synthetic scaffolds to confer regenerative potential, frameworks that are relevant in clinical practice have been found to favor multipotent stem cells derived from adult tissue cites to the pluripotent embryonic stem cells and the variant of stem cells that can be induced to differentiate (induced pluripotent stem cells). This tendency has been reported by several studies despite the induced and embryonic stem cells having a wider

repertoire of terminal cell fates, being more capable of prolonged self-renewal, and sustaining the potential for pluripotency for longer than the multipotent adult stem cells (Langenbach et al., 2010). However, the mesenchymal stem cells are thought to have a higher tendency to undergo neoplastic transformation and develop teratomas or other tumors following prolonged cell division. The utility of pluripotent stem cells in synthetic scaffolds can also be derailed by their capacity to be immunologically incompatible with the adult host tissue. Moreover, induced pluripotent stem cells have commonly been reported to possess epigenetic memory accrued from lineages into which they have differentiated in the past, a property that can lower the efficiency of induction along a desired lineage.

Adult stem cells, whose presence in the human body has been known for a while now, can be harvested from a variety of sites, with the most commonly utilized source being the bone marrow. Owing to their lack of some of the molecules central in antigenicity, such as MHC I, these cells are virtually non-immunogenic, a trait that has boosted their popularity in tissue engineering. Adult stem cells have also been reported to suppress host immune response, an effect whose mechanism of action remains poorly understood. Their application in synthetic regenerative scaffolds is further supported by their capacity to form specific bone cell types, which makes their osteogenic differentiation potential superior to that of embryonic and induced pluripotent stem cells. However, prolonged culturing of adult mesenchymal stem cells can result in terminal differentiation or lead to senescence arising from progressive telomeric shortening. Moreover, as discussed before, the widespread application of adult mesenchymal stem cells such as those harvested from the bone marrow is limited by the pain and potential morbidity accompanying their collection. Moreover, these niches only provided small amounts of mesenchymal stem cells. These challenges have encouraged research on the osteogenic capacity of embryonic stem cells, particularly those harvested from umbilical cord blood. While several studies have reiterated the presence of the aforementioned disadvantages in these cells, some have reported superior or similar

osteogenic differentiation capacities as those of adult stem cells. This inconsistency suggests an inadequate understanding of the properties of these cells, which is unsurprising given their relatively recent introduction in tissue engineering research. Consequently, attempts to understand the events moderating bone regeneration in these cells, their behavior in synthetic scaffolds, and ways of enhancing their osteogenic potential are ongoing.

The research into the process of scaffold-based bone regeneration has further identified the potential for human umbilical cord mesenchymal stem cells to replace bone marrow-derived mesenchymal stem cells as the osteogenic cells in 3D scaffolds. UCMSCs offer several advantages to the process, including more relaxed ethical implications, easier harvesting, ready availability, and robust proliferation (Fu et al.,2010; Can & Karahuseyinoglu, 2007). Therefore, the combination of composite 3D scaffolds made of such biomaterials as hydroxyapatite and calcium carbonate, onto which UCMSCs and bone growth factors are seeded, constitutes an artificial regenerative framework that is the best synthetic alternative to bone grafting. The model so-formed not only offers the traditional benefits of bioengineered tissues such as osteoconductivity and ready availability, but also boasts of an osteogenic capacity that approaches that of natural bone. Overall, this goal forms the principal driving force for efforts to enhance the osteogenic capacity of stem cell-containing 3D scaffolds.

As efforts to capitalize on the benefits of UCMSCs by using them to confer superior host integration and remodeling potential to synthetic scaffolds increase, several studies have investigated the performance of these stem cells in such systems. The research has ranged from investigations of the osteogenic differentiation rate and phenotypic characteristics of the cells to their bone-forming activity in several synthetic scaffolds, including HA/CC (Honsawek, Dhitiseith, & Phupong, 2006; Zhang et al. 2009; Wang, Singh, Bonewald, & Detamore, 2009). Consistently, the umbilical cord stem cells have been reported to have notable advantages of bone marrow cells for use in this role,

including a simpler and safer harvesting process, higher proliferation rate, capacity for prolonged expansion in culture without diminishing differentiation potential, as well as low immunogenicity in clinical use (Kargozar et al., 2016; Panepucci et al., 2008). One such study directly compared the osteogenic capacity of human umbilical cord stem cells with that of bone marrow stem cells on a hydroxyapatite-calcium carbonate scaffold. In the study, Day, Francis, Guy, and Xia (2018) used scanning electron microscopy to characterize the 3D cultures of both cell populations, noting the formation of organoids that encircled the hydroxyapatite-calcium carbonate granules by the first day on both stem cell populations. By 21 days, both stem cell populations had undergone deposition of matrix and differentiation along osteogenic cell lines. However, the results showed that compared to UCMSCs, BMSCs have a superior capacity to form bone on the HA/CC scaffold. Another study of this group applied HA/CC scaffold carrying UCMSCs in vitro and in a rat model. Significant weight loss of HA/CC scaffolds was noted at successive time points respect with hydroxyapatite. Furthermore, HA/CC supported ALP activity of mesenchymal stem cells was proved, again indicated the biocompatibility, biodegradable, nontoxic of HA/CC scaffold and the potential of UCMSCs in osteogenecity in vitro. However, after implantation in rat, no bone formation was observed after 6 weeks. (Shi et al.,2020). Therefore, the osteogenic capacity of this model need to be enhanced. They recommended further research to determine ways of optimizing the osteogenicity of UCMSCs on such scaffolds in order to realize their potential in this application.

1.6 Aim of study

Above all, hydroxyapatite is a biomaterial with osteoconductivity, biocompatibility and non-immunogenicity, similar to the natural bone tissue. A significant drawback of HA is the slow degradation rate. By adding calcium carbonate, the degradation rate of the scaffolds can be custumised which make the hydroxyapatite/calcium carbonate scaffold a suitable replacement for bone grafts. However, it’s osteogenic capability remains to be improved. In this study, we hypothesis that by using RA we can improve

osteogenesis of UCMSCs on HA/CC scaffolds. The aim of this study is to enhance osteogenic capacity of UCMSCs on HA/CC scaffolds in vitro by supplementing RA in osteogenic medium. The objectives of this study are to assess viability/cytotoxicity of UCMSCs on HA/CC scaffolds and to evaluate the effect of RA on osteogenesis of this model in vitro.

2.Materials and methods

2.1 Materials, chemicals and supplies

Umbilical cord samples for stem cell isolation were provided by Singleton Hospital. The institutional research ethics was approved by the South West Wales Research Ethics Committee (**REC reference: 11/WA/0040**). HA/CC scaffolds were provided by Dr. ZhiDao Xia's group of Swansea University Institute of Life Science. Dulbecco's Modified Eagle Medium (DMEM) ,penicillin / streptomycin , Fetal bovine serum (FBS) , phosphate buffered saline (PBS) and osteogenic differentiation kit were purchased from Gibco Life Technologies. CCK-8 reagent, Live/Dead assay kit were purchased from Sigma-Aldrich. ALP biochemical quantification kit was purchased from Abcam and BMP-9 ELISA kit was purchased from R&D systems. Unless stated otherwise, all other chemicals were purchased from Thermo Fisher Scientific or Sigma-Aldrich. All cell / tissue culture wares were purchased from CELLSTAR® or Corning, Inc

2.2 Cord dissection

Collected samples of umbilical cord (Catalog Number: AUK1424, AUK 1420, AUK 1415), around 10cm in length, were placed in a 150mm petri dish washed with DMEM/F12 media (Gibco, Thermo Fisher Scientific, USA) to remove blood clots and keep the sample moist. By using autoclaved forceps and a scalpel, the UC sample was cut longitudinally along the whole length, exposing the Wharton's jelly and blood

vessels. One vein and two arteries were carefully removed by using the autoclaved surgical tools and placed in a separate petri dish ready for destruction. The remaining tissue was washed with media to remove remaining blood and then processed into 1-3mm^3 particles by using a scalpel. The tissue particles were introduced into a T-25 (25cm^2) tissue culture flask (CELLSTAR®; Greiner bio-one, Germany), ensuring equal distribution of tissue samples across the surface area. After that, 2ml of fetal bovine serum (FBS, GibcoTM; Thermo Fisher Scientific, USA) was carefully added to a T-25 tissue culture flask to wet the tissue particles. The T-25 tissue culture flask was reintroduced into the incubator. After 24 hours, 3ml of growth media (DMEM/F12, 10% FBS and 1% penicillin-streptomycin (Life Technology, Thermo Fisher Scientific, USA)) were gently added into the flask. Culture media was changed every 3-4 days.

2.3 Umbilical cord mesenchymal stem cells (UCMSCs) subculture

After around 14 days, the tissue particles were carefully removed from the T-25 culture flask and exposing the adherent cells underneath, the confluence was around 70%. UCMSCs (P0) were washed with 2ml of phosphate buffer saline (PBS) (GibcoTM; Thermo Fisher Scientific, USA). 3 ml of Trypsin was added to the flask to detach the UCMSCs from the surface of the flask, after which the cells were introduced to theincubator for 5 minutes. Detachment of the cells was confirmed using light microscopy imaging, and the trypsinization was stopped by adding 3 ml of culture media into the culture flask. The solution of cells, Trypsin and culture media was transferred to sterile 25ml universal centrifuge tubes and centrifuged at 150*g* for 5 minutes. The supernatant was removed and the remaining pellet of cells was resuspended by adding 1ml of culture media and flicking the tube. To count the cells, 10µl of the cell suspension was added to a 1.5ml Eppendorf followed by 10µl of trypan blue solution (Corning Inc, USA). 10µl of this solution was then added to a cell counting slide (Bio-Rad Laboratories, USA) and counted using an automated cell counter (Bio-Rad Laboratories, USA). Sterile T-75 (75cm^2) cell culture flasks

(CELLSTAR®; Greiner bio-one, Germany) were reseeded with UCMSCs (P1) at a density of 2×10^5 cells per flask, followed by 9ml of fresh culture media. Culture media was replaced every 3 days. At around 70% confluence, UCMSCs were passaged again. For cryofeezing, the culture media was removed and the attaching cells were trypsinized. Trypsinization was then neutralized by adding 3 ml of culture media. Cells were counted at this time, and the cell suspension was centrifuged at 150*g* for 5 minutes to form a pellet. The cells were resuspended in prepared freezing media (90% FBS and 10% dimethyl sulfoxide (DMSO), the density of cells was around 1×10^6. The cells in freezing media were transferred to cryovials (Greiner bio-one, Germany), which were stored in liquid nitrogen for future use.

2.4 Cell authentication by Flow Cytometry

Cells were detached by using 3 ml of Trypsin, and trypsinization was neutralized by adding 3 ml of culture media. Live cells were rate tested by Trypan blue and cell counted by cell counter (live cells proportion over 90%). The cell suspension was centrifuged at 150*g* for 5 minutes, the supernatant was removed, and some FACS buffer (PBS, 0.05% sodium azide and 0.2% bovine serum albumin (BSA, Sigma-Aldrich, Merck life science, UK)) was added to resuspend the cells, the concentration was around 1×10^6 cells/ml. 5µl of primary labeled antibodies (CD45, CD34, HLA-DR, CD73, CD105, CD90, CD14, CD11b, IgG1 eFluor450, IgG1 APC, IgG1 PE, IgG1 FITC (Sigma-Aldrich, Merck life science, UK)) were added to their corresponding labeled round bottom polystyrene tube, followed with 100µl of cell suspension. 12 tubes of cell suspension with antibodies added and one unstained tube with cell suspension only were left on ice and in darkness for 30 minutes. 3 ml of FACS buffer was added in each tube after 30 minutes and recentrifuged at 266*g* for 7 minutes. The supernatant of each tube was tipped off, and cells were resuspended by 100µl of FACS fix buffer and gently flicking the tubes. All tubes were analyzed by Novocyte flow cytometer (ACEA bioscience, USA).

2.5 Effect of different concentration of RA on cell growth

To assess the appropriate dose of RA supplement, three groups of UCMSCs with different concentrations of RA (0uM, 20uM, 200uM) were set for further experiments. Cells were seeded at a density of 5×10^3 in each well of 96-well plates (CELLSTAR, Greiner bio-one, Germany) with roughly 15 HA/CC particles placed. After 24 h and 72 h of incubation, cells were gently washed using PBS to remove detached cells. Subsequently, according to the manufacturer's instructions, 10µl of CCK-8 reagent (Sigma-Aldrich, Merck life science, UK) and 190µl of growth media were added in each well, and the plate was transferred to an incubator. After one hour of incubation, the optical density (OD) of each well was measured at 450 nm by using a microplate reader (Serial Number:415-1387, BMG Labtech, Germany).

2.6 Preparation of Hydroxyapatite/Calcium Carbonate particles and cell seeding

HA / CC scaffolds were crushed with a mortar and pestle and subsequently sieved through a 300 µm sieve followed by a 200 µm sieve to capture only 200–300 µm particles. The sieved HA/CC particles were autoclaved in a small glass container and transferred in a sterile 25ml universal centrifuge tube (Greiner bio-one, Germany). A few fresh culture media was then added to the tube to let particles soak in it for 24 hours before use.

2.7 Proliferation and cytotoxicity on scaffolds (Live/Dead Assay)

To determine the cytotoxicity of HA/CC particles and assess the proliferation of UCMSCs on HA/CC particles, cells were seeded as previous described (Section 3.7). After 72 h of incubation, growth mediawas removed from each well, cells were gently washed 3 times with PBS, then 100 µl of PBS was added to each well. Subsequently,

2µl of Calcein-AM (5ug/ml, Sigma-Aldrich, UK), 5µl of propidium iodide (PI, 5ug/ml, Sigma-Aldrich, UK) and 5µl of Hoechst 33342 (10ug/ml, Abcam, UK) were added in each well. Plates were gently shacked to ensure that the dyes evenly covered the cells. Plates were then transferred in a 5% CO_2 incubator at 37°C for 20 minutes. Following the incubation, the plates were imaged and analyzed by confocal microscope (Zeiss, Germany) (Fluorescence filter and laser line set: FITC at 488nm, Texas Red at 545nm and DAPI at 461).

2.8 Osteogenic differentiation

An osteogenic differentiation kit (A10072-01, GibcoTM; Thermo Fisher Scientific, USA) was used for differentiation. According to manufacturer's instructions, pure osteogenic differentiation medium was configured with 90% osteocytes differentiation basal medium and 10% osteogenic supplement (containing dexamethasone (0.01 µM), β-glycerophosphate (0.1 M), ascorbic acid (0.5 mM), 10% FBS and 1% antibiotics (P/S)). 200µM RA supplemented osteogenic differentiation medium was made from 2µl RA stock (100mM) in every 10ml of pure osteogenic differentiation medium. 20µM RA supplemented osteogenic differentiation medium was configured by 10% 200µM RA supplemented osteogenic differentiation medium and 90% pure osteogenic differentiation medium. For osteogenic differentiation assays, cells were seeded at a density of 5×10^3 onto HA/CC particles and cultured in growth media for 3 days. After 3 days, samples were divided into three groups (0RA, 20µM RA supplemented, and 200µM RA supplemented), growth media was replaced by a corresponding osteogenic differentiation medium. Osteogenic differentiation medium being changed every 3days. Samples being imaged and analyzed at Day 1, Day 7, Day 14 and Day 21.

2.9 Scanning electron microscope (SEM)

To assess the 3D culture of hUCMSCs on HA/CC particles, samples were analyzed at

Day 7 and Day 14 after osteogenic differentiation. At the scheduled time, the osteogenic differentiation medium was removed, cells were gently washed 3 times with PBS and then fixed by treatment with 4% glutaraldehyde (Sigma-Aldrich, UK) for 20 minutes. Cells were then gradually dehydrated using sequentially higher concentrations of ethanol (70%, 80%, 90%, 95%, and 100%) for 10 minutes each. Samples were then further dehydrated in 50% hexamethyldisilazane (Sigma-Aldrich, UK) diluted with ethanol for 10 minutes, followed by 100% hexamethyldisilazane for 10 minutes. Samples were then left in a fume hood to let evaporate overnight. The next day, dried samples were carefully collected and stuck on 9mm carbon tabs (agar scientific, UK). Samples were then coated with 5nm of chromium by a sputter coater (Quorum, model: Q150T ES, UK). Finally, samples were analyzed by an S-4800 II SEM (Hitachi, Hitachi Limited, Japan).

2.10 Alkaline phosphatase biochemical quantification

Alkaline phosphatase (ALP) activity of UCMSCs on HA/CC particles was assayed according to the manufacturer's protocol (ab83369, Abcam, UK). Cells at a density of 5×10^3 /well were seeded onto HA/CC particles in osteogenic differentiation medium supplemented with different concentrations of RA (0RA, 20μM RA, and 200μM RA). After 1 day, 7 days, 14 days and 21 days of osteogenic differentiation, samples with attached cells were washed 2 times by cold PBS. Cells were then scraped and lysed in ALP assay buffer on ice for 2 hours with shaking. Lysate samples were then transferred in labeled Eppendorfs and centrifuged in a cold microcentrifuge (Eppendorf, Germany) at 20379*g* for 15 minutes. After centrifugation, the supernatant was collected in new labeled Eppendorfs. For ALP assays, 80μl per well of each lysate was transferred to 96 well plates and made up to 200μl with assay buffer. 5mM P-nitrophenyl phosphate (pNPP) solution was made by reconstituting 2 supplied pNPP tablet in 5.4ml of assay buffer. 1mM pNPP standard was prepared by diluting 40μl of 5mM pNPP solution, after which a standard curve was set up with known concentrations of pNPP. To start the

reaction, 50µl of 5mM pNPP solution was added to all samples and mixed thoroughly by pipetting up and down, 10µl of enzyme solution was then added to the standard wells. The plate was wrapped in foil and incubated for 60 minutes at 25°C in a hybridization oven/shaker (Stuart Scientific, UK). After 60 minutes of incubation, 20µl of stop solution was added to each well. Finally, ALP was quantified using a microplate reader (BMG LabTech, Germany) at an OD of 405 nm. ALP activity (µmol/min/min or U/mL) was then calculated from the following equation:

$$\text{ALP Activity} = (B/\triangle T \times V) \times D$$

Where B is the amount of p-nitrophenol (pNP) in the sample well, calculated from the standard curve; $\triangle$T is the reaction time; V is the original sample volume added to each sample well and D is the sample dilution factor.

2.11 BMP-9 ELISA

To assess the BMP-9 concentration, BMP-9 ELISA was assayed following the manufacturer's protocol of Human BMP-9 Duoset ELISA Kit (DY3209, R&D Systems, UK). Working capture antibody solution was configured by diluting 50µl capture antibody in 6ml PBS. Sample wells of a 96 well plate were coated with 50µl of this solution, the plate was then sealed and incubated at room temperature overnight. The following day the plate was aspirated and washed 3 times with 200µl wash buffer. The plate was banged on absorbent tissue to ensure that all excess liquid was removed from each well between washes. The plates were blocked by adding 150µl of reagent diluent to each well, followed by an hour of incubation. After incubation, 50µl of all samples (Day 1, Day 7, Day 14 and Day 21 in reagent diluent) was added to their respective wells. 50µl of known standard (in reagent diluent) was also added to their respective wells. The plate was then incubated in a hybridization oven/shaker at room temperature for 2 hours. The known standard was serially diluted in order to construct a standard curve. After incubation, the plate was washed 3 times as previously done and 50µl of

the diluted detection antibody (100µl of supplied detection antibody solution in 5.9ml reagent diluent) was added to each well. The plate was then recovered and further incubated in a shaker for 2 hours at room temperature. Following incubation, the plate was washed 3 more times with 200µl wash buffer. 50µl of working concentration streptavidin-HRP (150µl of supplied streptavidin-HRP in 5.85ml of reagent diluent) was added to each well, and the plate was incubated for another 20 minutes. The plate was again aspirated and washed 3 times, followed by the addition of 50µl of substrate solution to each well, avoiding placing the plate in direct light. Roughly 20 minutes later, 50µl of stop solution was added to each well to stop the reaction. A microplate reader set to 450nm was used to analyze the optical density of the standards, samples and the blank.

2.12 Alizarin Red S quantification

To determine the extracellular calcium deposits in each sample wells, ARS solution (Sigma-Aldrich, UK) was diluted in half from the working concentration of 5mM to construct a standard curve. At Day 1, Day 7 and Day 14, the osteogenic differentiation medium was removed from each well and gently washed 3 times with PBS. Subsequently, 50µl of ARS solution was added to sample wells, and the plates were incubated at room temperature for 20 minutes. At the desired time, ARS solution was removed and the wells were washed with PBS 3 times with shaking. 100µl of 10% acetic acid (Sigma-Aldrich, UK) was then added to each well for ARS stain extraction, after which the plates were placed at room temperature for 30 minutes. After 30 minutes, 100µl of 10% ammonium hydroxide (Sigma-Aldrich, UK) was added to each well to neutralize the reaction and reintroduce the ARS stain. Plates were then read by a microplate reader for colorimetric detection at 405 nm.

2.13 Statistics

All the experiments in this project were repeated at least 3 times. Statistics SPSS 25.0 software (IBM, USA) was used to analyze the data involved in the study. ImageJ software (NIH, USA) was used to process images in this project. Differences between the ALP activity and BMP-9 concentration between the 0RA supplemented sample group and 20uMRA supplemented sample group were defined by one-way ANOVA and LSD t-test. Differences were considered statistically significant when $P < 0.05$.

3.Results

3.1 Cell culture and morphological observation

Early morphological change in the UCMSCs (P1) were observed using light microscopy. After one day of incubation (Figure 1A), most of the cells were short fusiform or spindle-shaped. After 2 days of incubation (Figure 1B), the cells displaying in flat polygons and fibroblast-like, and the number of cells increased. On the fifth day of culture (Figure 1C) the number of cells increased significantly, covering the bottom of the T-75 flask. The cells displaying aggregation and swirling or radiating shaped. Figure 1D shows the out-growth of primary UCMSCs (P0) under human umbilical cord pieces, which indicates the time to harvest primary cells.

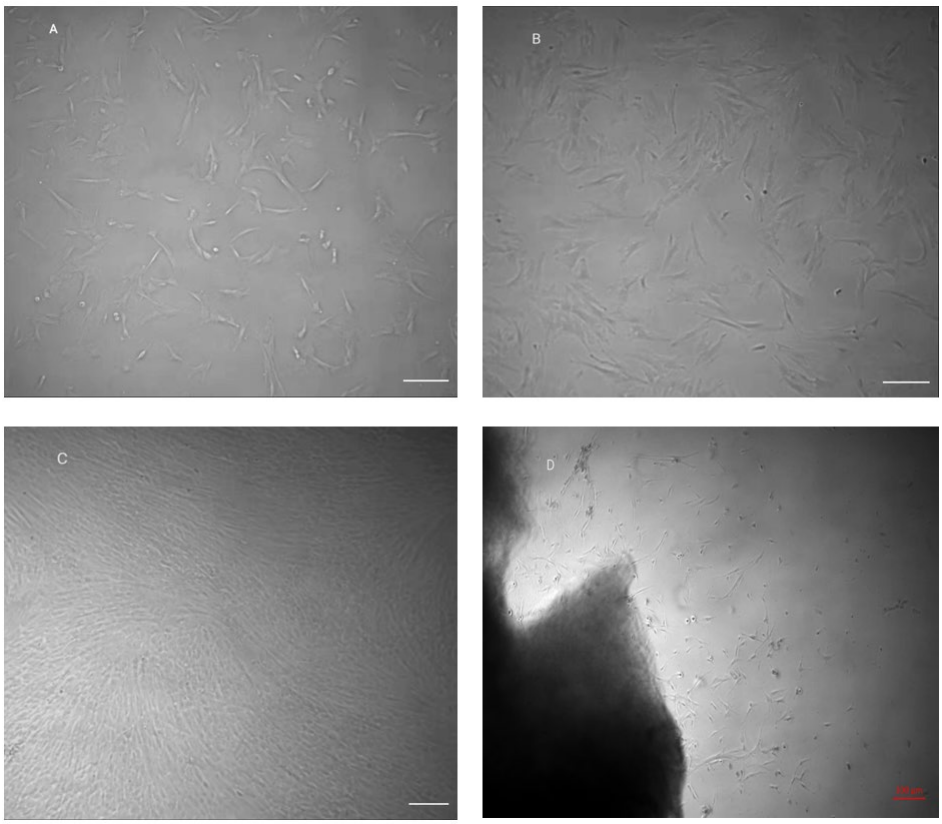
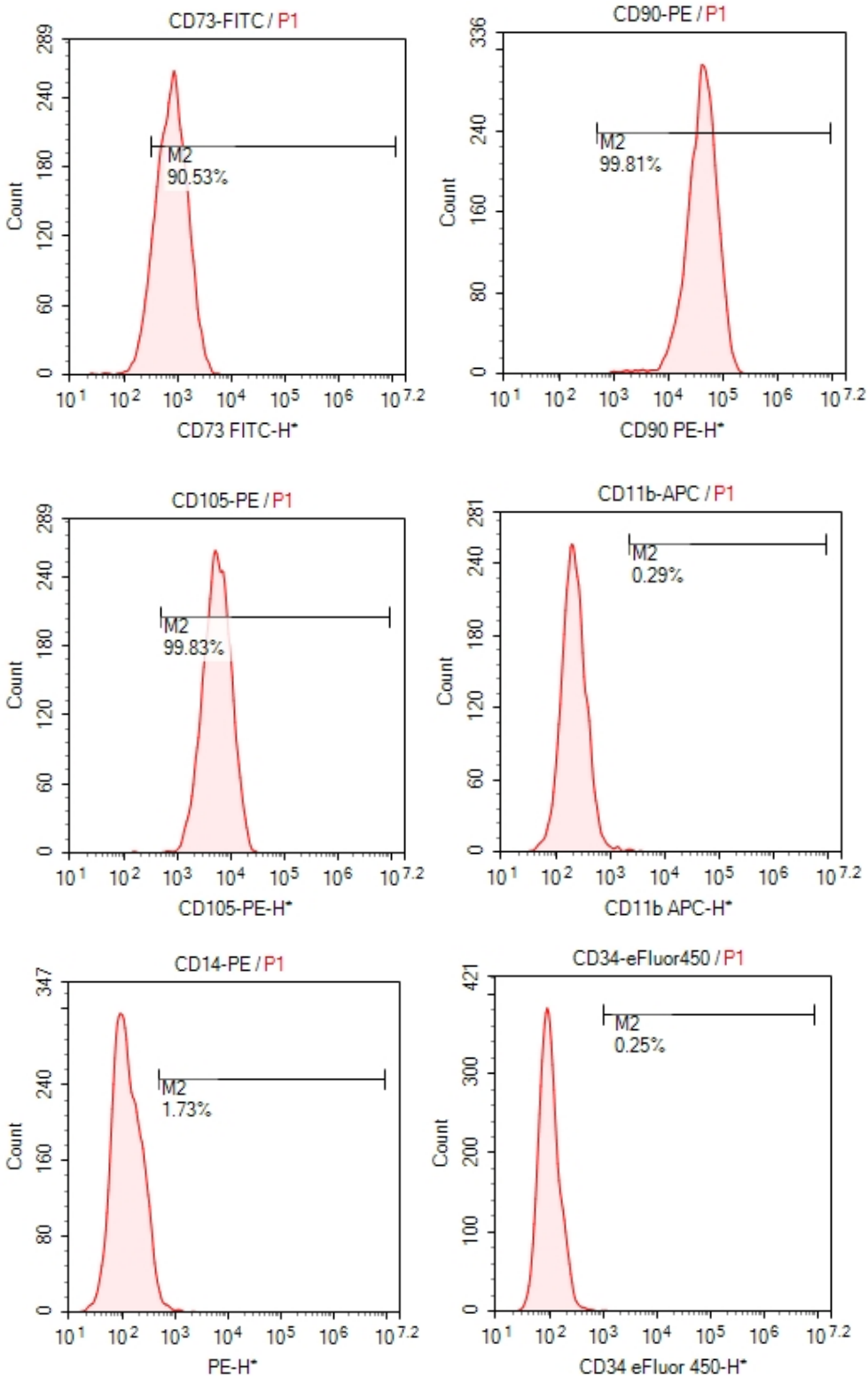
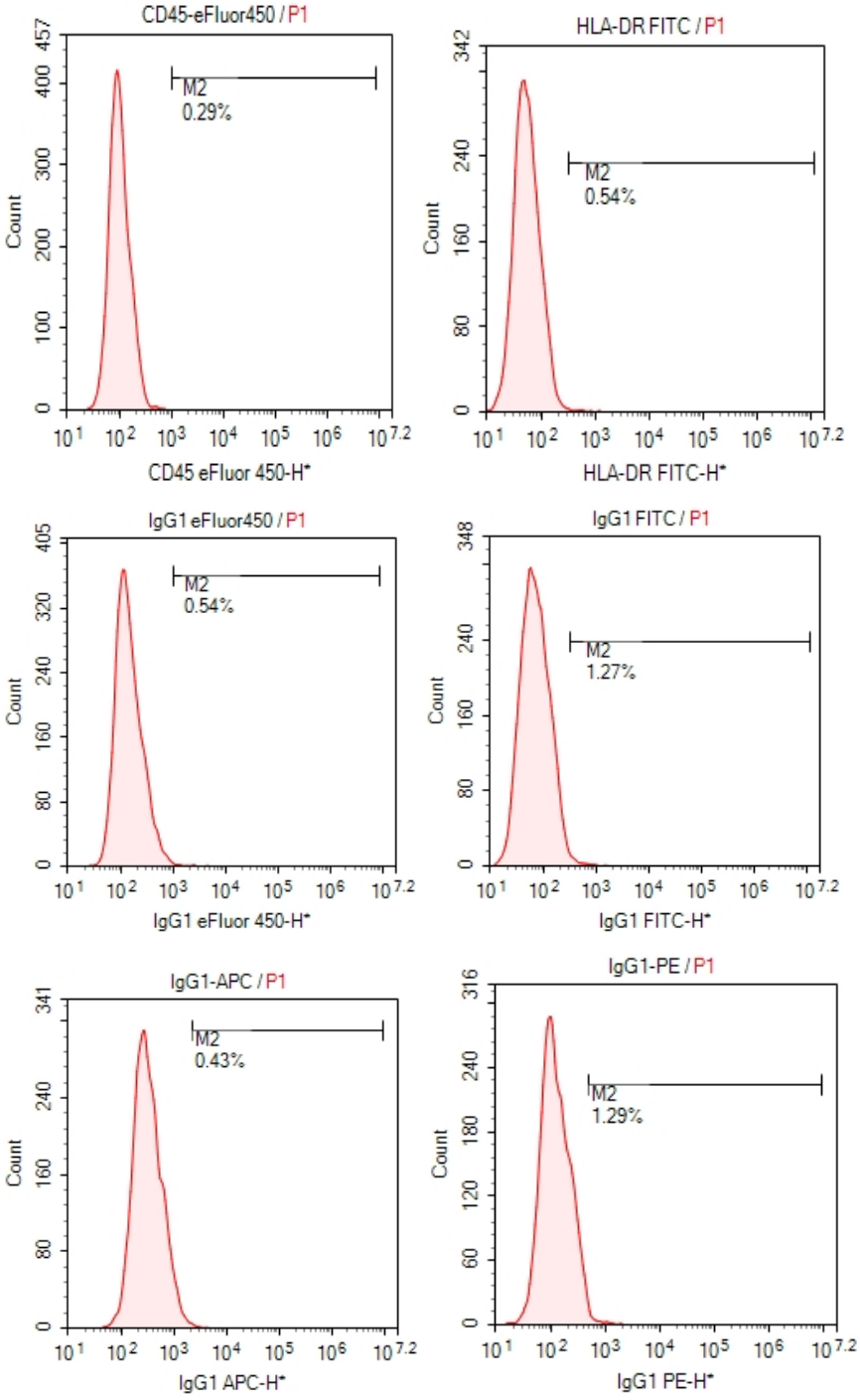


Figure 1. Morphological analysis of UCMSCs in different days of culture. Morphological analysis of UCMSCs in different days of culture using a light microscope at 4x objective magnification. Scale bar =50 µm. (Figure 1 A. B and C), Scale bar =100 µm. (Figure 1D)

3.2 Characterization by flow cytometry

Cultured cells were characterized in accordance with the criteria set by the International Society for Cellular Therapy (ISCT) (Dominici et al., 2006). All cells were single stained with antibodies against surface antigens.


CD73-FITC / P1
M2
90.53%
Count
CD73 FITC-H*
CD90-PE / P1
M2
99.81%
Count
CD90 PE-H*
CD105-PE / P1
M2
99.83%
Count
CD105-PE-H*
CD11b-APC / P1
M2
0.29%
Count
CD11b APC-H*
CD14-PE / P1
M2
1.73%
Count
PE-H*
CD34-eFluor450 / P1
M2
0.25%
Count
CD34 eFluor 450-H*


CD45-eFluor450 / P1
M2
0.29%
Count
CD45 eFluor 450-H*
HLA-DR FITC / P1
M2
0.54%
Count
HLA-DR FITC-H*
IgG1 eFluor450 / P1
M2
0.54%
Count
IgG1 eFluor 450-H*
IgG1 FITC / P1
M2
1.27%
Count
IgG1 FITC-H*
IgG1-APC / P1
M2
0.43%
Count
IgG1 APC-H*
IgG1-PE / P1
M2
1.29%
Count
IgG1 PE-H*

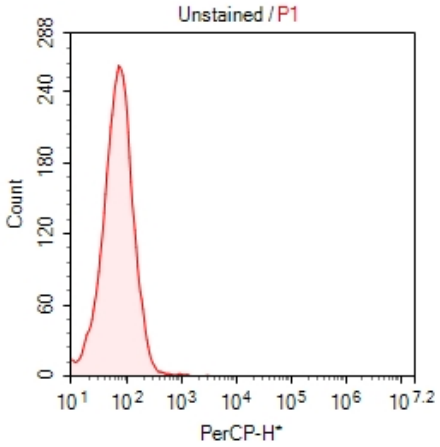


Figure 2.Flow cytometry. The horizontal axis represents the fluorescence channel, the vertical axis represents the number of cells collected in the channel, and the in-gate data shows the fluorescence characteristics of cell subpopulations to detect surface antigen expression. Cells exhibit positive expression for CD73, CD105 and CD90 and negative expression of CD34, CD45, CD14, CD19 and HLA-DR, which indicated the cultured cells were UCMSCs (Dominici et al.,2006).

3.3 Population doubling of UCMSCs

Data of population doubling（Figure 3）was collected from cells of three independent umbilical cord samples that were all incubated at 37°C and a 5% CO^2 condition, detached using trypsin and counted using an automatic cell counter and trypan blue staining.

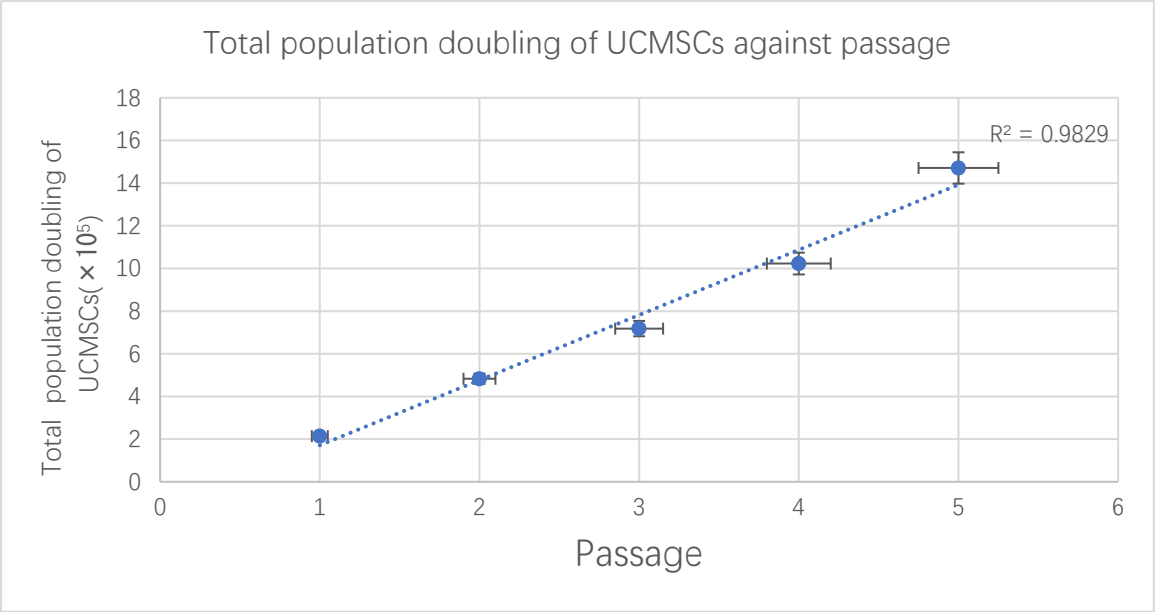


Figure 3. Total population doubling of UCMSCs against the passage(n=3). A scatter graph represents the result of the total population doubling of UCMSCs against the passage. The passage process took place after every 3 days. The coefficient of determination R^2= 0.9829.

3.4 Viability of UCMSCs against passage

The viability of UCMSCs against passage was assessed using a CCK-8 cell counting kit. Statistics SPSS 25.0 was used to process the data, student–Newman–Keuls method was used to compare the viability of UCMSCs between passages and there was no significant difference between groups ($P > 0.05$)(Figure 4). For this project, the P3-P5 of UCMSCs that can be passaged stably were used in future experiments.

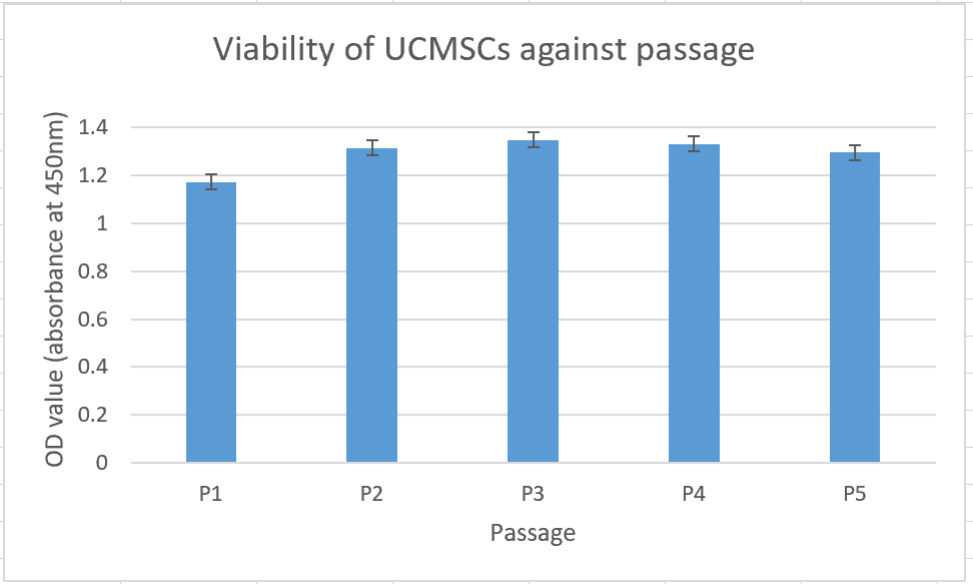


Figure 4. Viability of UCMSCs against passage. Viability of UCMSCs against passage. There was no significant difference in the viability of UCMSCs between each passage ($P > 0.05$).

3.5 Dose-response of RA

Different concentrations of RA were supplemented to samples to assess the effect of RA on cell growth. After 24h and 72h of incubation, a CCK-8 assay was performed and the OD values of samples were recorded (Figure 5.1 and Figure 5.2). Paired student's t-tests were used to analyze the differences in each group compared to the control (0RA). No significant differences were shown in RA supplemented groups compared to the control after 24h of incubation ($P > 0.05$). However, after 72h of incubation, the 200μM of RA supplemented group showed significant difference compared to the control ($P < 0.05$). 0.2μM, 2μM and 20μM of RA were considered safe doses for supplementation; The addition of 200μM of RA has shown a cytotoxic and inhibitory effect on cell growth. To enhance osteogenesis, the highest safe dose, 20μM of RA, was considered the ideal dose for supplementation in future experiments.

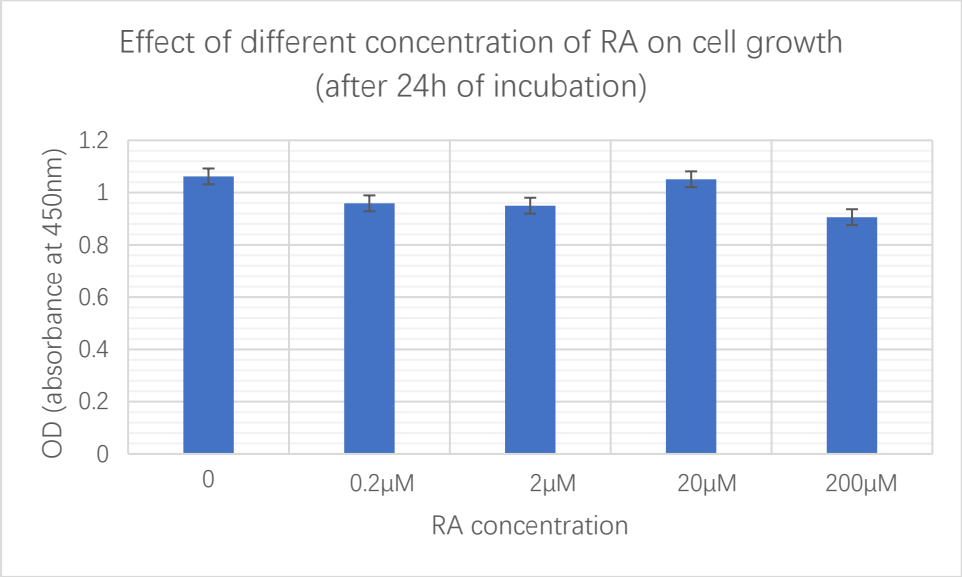


Figure 5. Effect of different concentrations of RA on cell growth. Figure 5.1 Effect of different concentrations of RA on cell growth after 24h of incubation. There were no significant differences between RA supplemented groups and the control ($P > 0.05$).

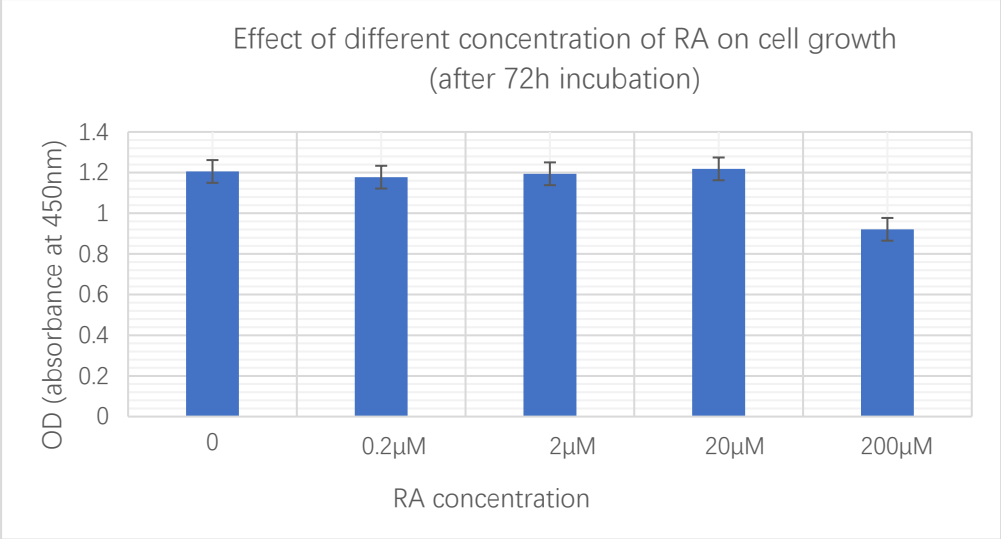


Figure 5.2 Effect of different concentrations of RA on cell growth after 72h of incubation. There were no significant differences of 0.2μM, 2μM, and 20μM RA supplemented groups with respect to the control ($P > 0.05$), while 200uM RA supplemented group has a significant difference compared with the control ($P < 0.05$).

3.6 Proliferation and cytotoxicity assay

After 3 days of incubation, a Live/Dead assay was performed on the samples. Three fields of view in each sample were randomly selected to take images for counting live and dead cells and statistical analysis. The live cell proportion of the cell-only group (group A, Figure 6A), the cells on HA/CC particles group (group B, Figure 6B) and the 20μM RA treated cells on HA/CC particles group (group C, Figure 6C) were more than 90%, and there was no significant difference between groups ($P > 0.05$). It was demonstrated that the UCMSCs can develop and proliferate normally on HA/CC particles and the none-cytotoxicity of the particles. A greater number of dead cells can be observed in 200μM RA treated cells on HA/CC particles group (group D, Figure 6D), which again proved the cytotoxicity of this concentration compared to group B. However, through the morphological observation of cells in group D, the obvious effect of RA on inducing differentiation should be noticed.

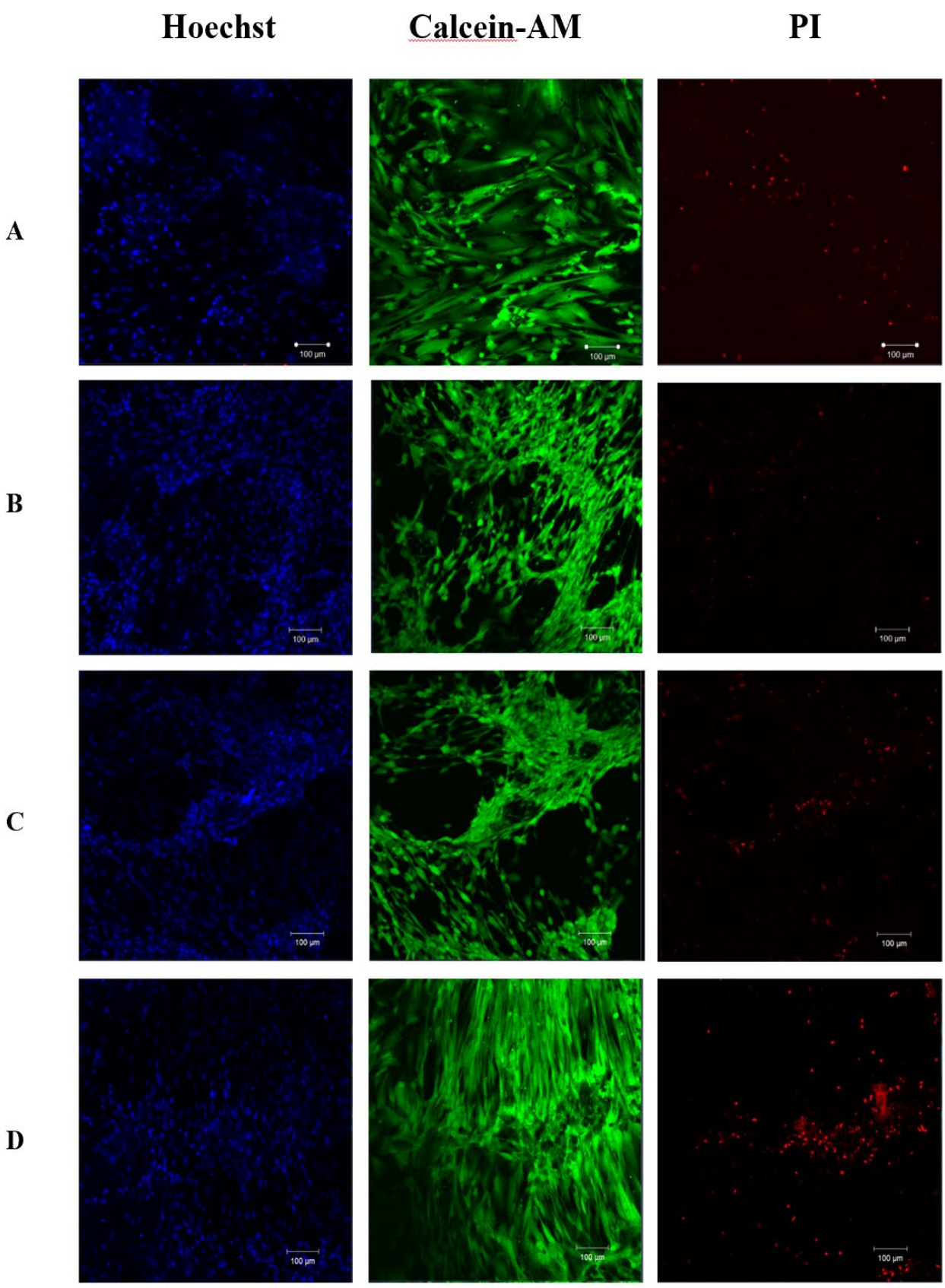

Hoechst
Calcein-AM
PI
A
B
C
D
100 μm

Figure 6. Cell proliferation on HA/CC particles and cytotoxicity assay.
(A) cells only, (B) Cells on HA/CC particles, (C) Cells on HA/CC particles with 20μM RA supplemented in culture medium, (D) Cells on HA/CC particles with 200μM RA supplemented in culture medium. The nuclei of cells labelled with blue fluorescence, the live cell labelled with green fluorescence, while the dead cell labelled with red fluorescence. All images were taken at 20x objective magnification and scale bar = 100 μm.

3.7 Effect of RA on the concentration of BMP-9

After 1 day, 7 days, 14 days and 21 days of culture in osteogenic differentiation medium with different concentration of RA, BMP-9 Elisa was performed to determine the BMP-9 concentration. One-way ANOVA and LSD -test were used for statistical analysis. The 20μM RA treated group showed a significant increase in BMP-9 expression after osteogenic differentiation, compared to the 0RA control group ($P < 0.05$), while no significant differences were found between the 200μM RA treated group and control group ($P > 0.05$). The results (Figure 7) indicate that an appropriate dose of RA can promote BMP-9 expression and enhance osteogenic differentiation.

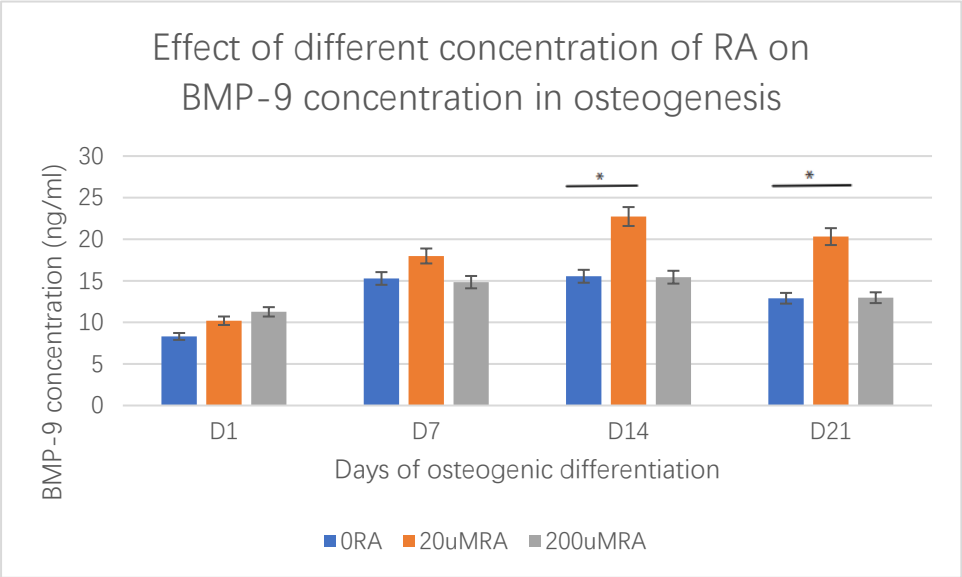


Figure 7.Effect of different concentration of RA on BMP-9 concentration in osteogenesis. The bar chart presenting the results obtained from the BMP-9 ELISA, depicting the changes in BMP-9 concentration after 1,7,14 and 21days of osteogenic differentiation of three different groups: cells grown in regular osteogenic medium, cells supplemented with 20μM of RA in osteogenic medium and cells supplemented with 200μM of RA in osteogenic medium. All cells were grown on HA/CC particles under the same conditions.

3.8 ALP biochemical quantification

The enhanced osteogenic capacity of UCMSCs on HA/CC particles was confirmed by ALP assay. At desired time points (day 1, 7, 14 and 21) of culture in osteogenic differentiation medium with different concentration of RA supplementation, an ALP assay was performed. The ALP activity of UCMSCs seeded on HA/CC particles is shown in Figure 8. The LSD -test was used for statistical analysis. The ALP activity in 20μM RA treated and 200uM RA treated groups increased from Day 1 compared with the control group. The ALP activity of the 20μM RA treated group increased significantly compared to the control group ($P < 0.05$), and the ALP activity has been rising throughout the whole osteogenic differentiation process. There was no significant difference between the 200μM RA treated group and control group ($P > 0.05$), which proved RA at the concentration of 200μM has an inhibitory effect on differentiating UCMSCs, and 20μM RA is an ideal concentration to enhance the osteogenesis of UCMSCs on HA/CC particles.

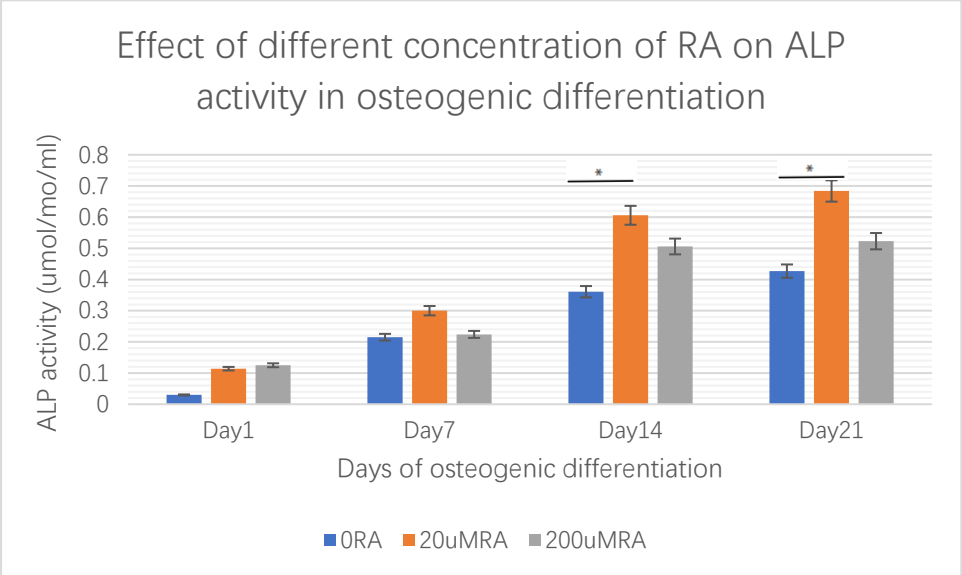


Figure 8. Effect of different concentration of RA on ALP activity in osteogenic differentiation. The diagram presenting the effect of different concentration of RA on ALP activity in osteogenic differentiation, depicting the changes in ALP activity through 21- days of osteogenic differentiation. All cells were grown on HA/CC particles under the same conditions.

3.9 Alizarin red S staining and quantification

To assess the effect of RA dose on ARS concentration during osteogenic differentiation, alizarin red S kits were used to stain the extracellular calcium deposits. After 14 days of osteogenic differentiation, 0RA, 20μMRA and 200μMRA supplemented samples were stained with ARS solution and imaged by light microscope (Figure 9A, B and C). To correlate microscope with quantification, at Day 1, Day 7 and Day 14 of osteogenesis, ARS stained samples were extracted and measured at 405nm by microplate reader. The results are as shown in Figure 10. Interestingly, over time the surface of the particles becomes smoother, and due to the increasing cell deposition and density of the extracellular matrix, which bound the HA/CC particles together forming a larger conglomerate, the staining was darker than surrounding area.

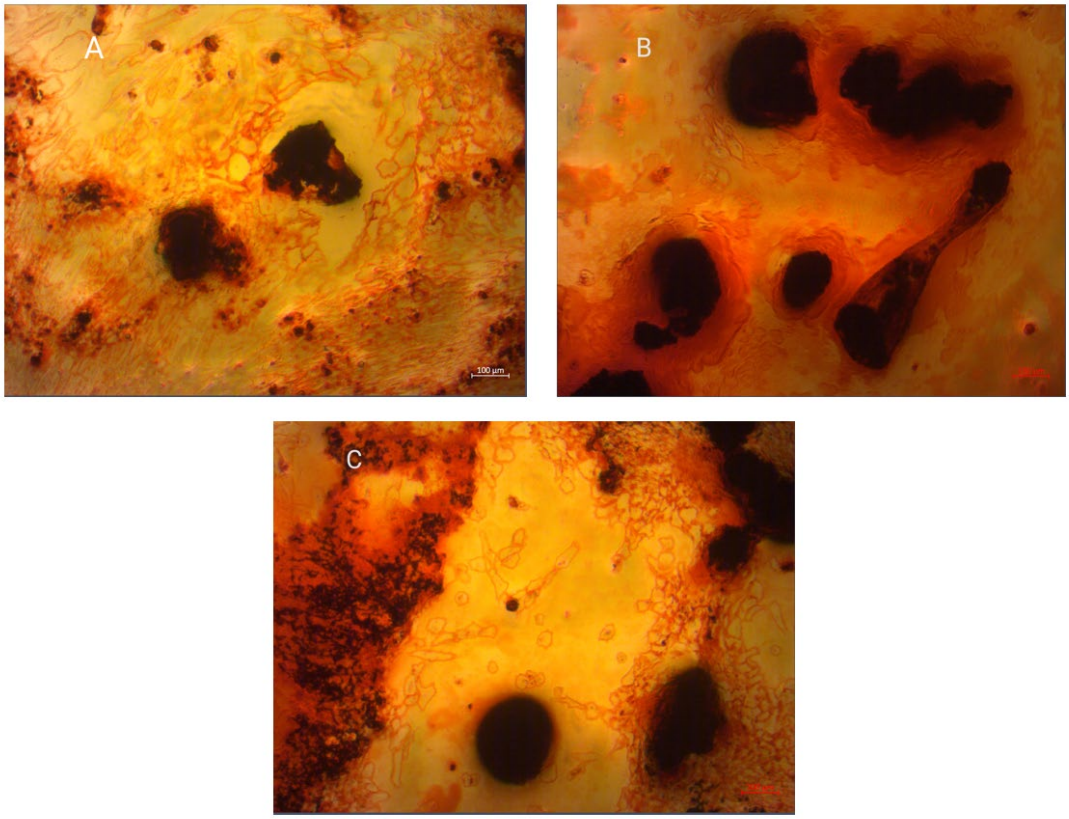


Figure 9. Alizarin red S staining. Figure 9(A) UCMSCs on HA/CC particles in regular osteogenic medium. (B) UCMSCs with 20μM RA treated on HA/CC particles. (C) UCMSCs with 200μM RA treated on HA/CC particles. Calcium nodular deposits can be observed in both groups. All images were taken at 4x objective magnification and scale bar = 100 μm.

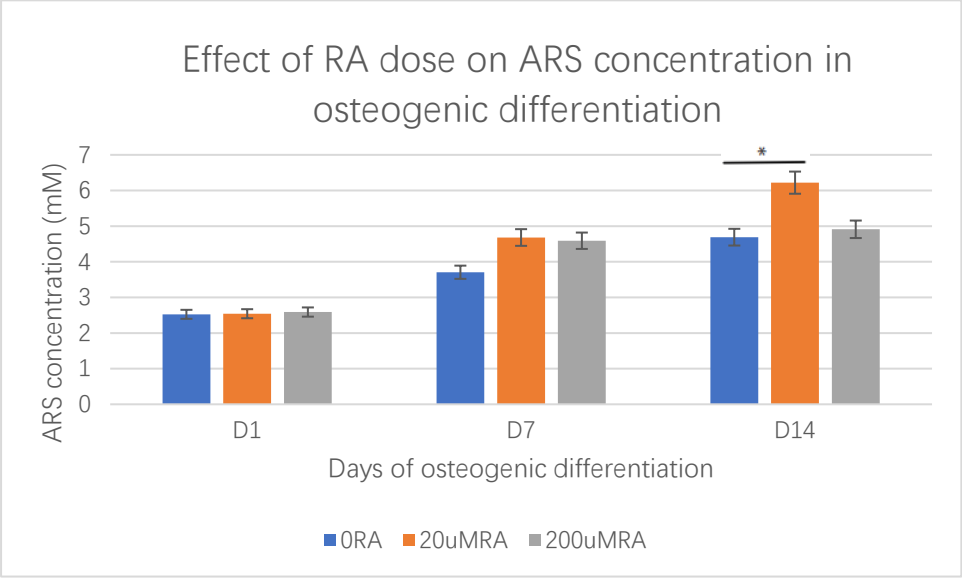


Figure 10. Effect of RA dose on ARS concentration in osteogenic differentiation. The concentration of ARS in RA treated groups increased on Day 7. On Day 14, the ARS concentration of the 20μM RA supplemented group increased significantly compared to the control group ($P < 0.05$). No significance was shown between the 200μM RA treated group and control group ($P > 0.05$). The results indicated that UCMSCs treated with 20μM RA produced higher levels of extracellular calcium deposits in the osteogenic differentiation process compared with other groups.

3.10 SEM observation

After 14 days of differentiation, Samples were dehydrated and stuck on 9mm carbon tabs and were then coated with 5nm of chromium. Cell attachment and bone-like tissue formation of each sample were analyzed by an S-4800 II SEM (Figure 11.). UCMSCs were induced to bone-like tissue, and the groups supplemented with RA appeared to have increased cell attachment.

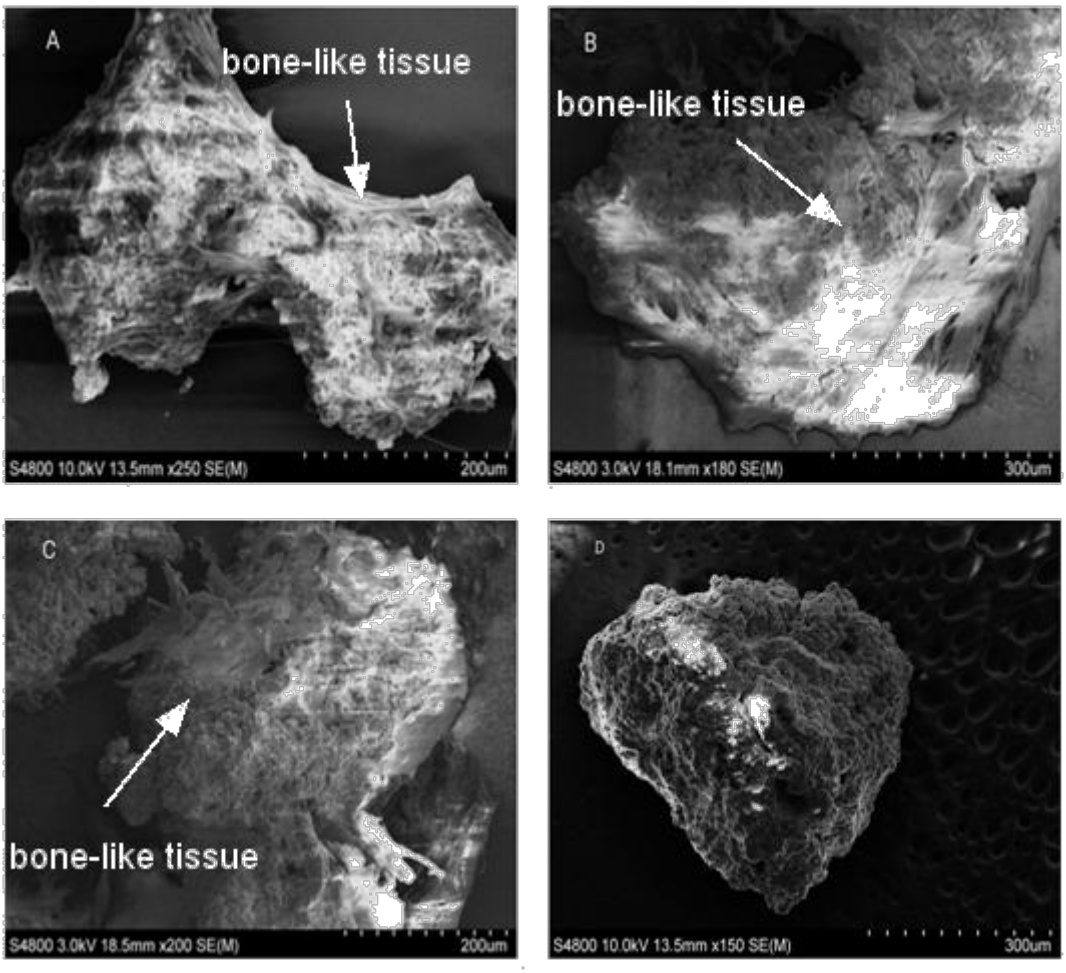


Figure 11. Cell attachment by SEM. Figure 11. (A) 0RA treated UCMSCs on HA/CC particles. (B) 20μM RA treated cells on HA/CC particles. (C) 200uM RA treated cells on HA/CC particles. (D) HA/CC scaffold particles only. SEM images were taken on the 14th day of the osteogenic differentiation process.

4.Discussion

This study has demonstrated that the use of a composite made up of hydroxyapatite and calcium carbonate is an ideal scaffold for bone engineering. The composite utilized in the study demonstrated superior biocompatibility and lower immunogenicity compared to alternatives like allografts and autografts and also had better physical properties compared with other synthetic options. The incorporation of calcium carbonate into the matrix of the scaffold improved its physical properties, making it more suitable for use as a bone substitute or as an implant.

We conducted an experiment to analyze the difference in bone cell growth on a surface with cells only (the control) and one that is coated with HA/CC particles. No significant difference was noted in the rate of cell growth in the two samples. However, it was observed that as time progressed the surface of the HA/CC group became increasingly smoother. The difference is indicative of a higher cell density and increased extracellular matrix deposition in the HA/CC particles group compared to the control group. HA/CC scaffolds, therefore, present numerous advantages over traditional surfaces such as hydroxyapatite alone.

The findings of the study have also indicated that the HA/CC environment makes higher infiltration of extracellular matrix, that contribute to the density of the scaffold. The particles of HA/CC combine with these cells to form a larger than normal conglomerate that affects the rate at which cells like osteoclasts as well as vascular regenerations integrate into the host's bone matrix (ISHIKAWA, 2019).

The presence of a suitable environment provided by the composite HA/CC scaffold requires further configuration to improve osteogenesis. Seeding the scaffold with undifferentiated stem cells is one way of enhancing its osteogenic potential. Several options exist for the possible extraction and use of undifferentiated stem cells on the synthetic scaffold, including multipotent stem cells from adult tissues to pluripotent embryonic stem cells. This study looked at the use of UCMSCs as osteogenic cells in the HA/CC scaffold.

In bone engineering, the common practice involves extracting pluripotent cells from the bone marrow of the individual, with most clinical frameworks leaning towards this approach. The action is justified by the tendency of the multipotent cells derived from adult tissues to provide a good option in transplants because they can easily be expanded in common culture medium. Stem cells derived from bone marrow also have the advantage of lacking molecules such as MHC-1, making them non-immunogenic and thus a very popular option in bone transplants that require additional cells to stimulate osteogenic activity (Chen et al., 2020). Adult stem cells also have the advantage of lowering the immune response of the host to the implant, although the exact mechanism of immunosuppression is yet to be fully understood. The use of BMSCs is also widespread because of its capacity to differentiate into specific bone cell types, making their osteogenic capacity greater than that found in embryonic stem cells, which have a wider range of potential terminal cell types after differentiation raising the risk of telomeric shortening.

However, the use of adult stem cells has limitations that made it necessary to consider UCMSCs as a viable alternative. The first major drawback of adult stem cells is that the act of harvesting BMSCs is a painful procedure with the potential of comorbidities such as extraction site infection and hemorrhage. This drawback limits the number of times one can extract samples from adult sites. Another issue with using adult stem cells

is that prolonged culturing of adult mesenchymal stem cells can lead to senescence that adversely impacts the structural integrity and makeup of a scaffold. These fundamental shortcomings of adult site mesenchymal stem cells encouraged the researchers to explore the use of UCMSCs as an osteogenic stimulant in HA/CC composite scaffolds in bone implants.

The use of UCMSCs is a demonstration of the need to enhance the formation of new bone cells by seeding pluripotent cells on implants. The choice of using the UCMSCs over adult stem cells from other regions of the body was driven by the advantages that UCMSCs offer. One of the advantages of using UCMSCs over BMSCs is the simpler and safer method of harvesting (Day, Francis, Fu, Pieper, Guy, & Xia, 2018; Michel, Penna, Kochen, & Cheung, 2015). The cord extraction process is painless and has little ethical implication since it is a widely available source of undifferentiated pluripotent stem cells that can induce osteogenic activities and characteristics to the synthetic implant. There are numerous live births across the globe, with an annual average of 120 million births giving a wide pool of UCMSCs available for use.

The use of UCMSCs is also beneficial since it displays a higher proliferation rate compared to bone marrow mesenchymal stem cells. The UCMCSs used to stimulate osteogenesis in the experiment showed superior differentiation when incorporated into the HA/CC matrix. UCMCSs provide a large pool of viable pluripotent stem cells that have the potential of differentiating into a wider range of terminal cells compared to those derived from the bone marrow. There is a wider multipotency and potential for osteogenic differentiation when it comes to using UCMSCs instead of bone marrow mesenchymal stem cells. The cells derived from adult sites may be limited by systemic diseases affecting the bone and bone marrow structure, such as lymphomas and hemolytic anemias. There is also markedly reduced osteogenic and proliferative potential in bone marrow extracted from older patients compared to that found in UCMSCs. The embryonic nature of mesenchymal cells derived from the umbilical cord

does not face the shortcomings of adult bone marrow cells and are thus more proliferative.

Complete reliance on UCMSC, however, is not without its minor shortcomings when compared with the use of BMSCs. One of the shortcomings is that there is evidence that BMSCs have a superior capacity to form bone tissue on a HA/CC composite, even though both showed similar osteogenic capacity. This research, however, was based on the use of UCMSCs because of its easy availability, its abundance, and the safe harvesting technique required to obtain the stem cells compared to bone marrow. The proliferation rate of UCMSCs on HA/CC particles is comparable to when it is on a plain surface. UCMSCs are pluripotent cells that can differentiate into a wide array of terminal cells when placed under the right conditions. The study involved a CCK-8 assay, which using WST-8 produces a water-soluble formazan dye upon bioreduction in the presence of an electron carrier, 1-Methoxy PMS. CCK-8 allows sensitive colorimetric assays for the determination of the number of viable cells in the proliferation and cytotoxicity assays. The detection sensitivity is higher than any other tetrazolium salts such as MTT, XTT or MTS. It looked at how UCMSCs differed in growth when mixed with HA/CC particles and that placed on a plain surface with no additional particles. There was no significant difference in the cell growth in the two instances. HA/CC particles, therefore, do not offer additional proliferation properties for UCMSCs with the rate of growth similar to the control.

A possible explanation for the phenomenon of the similar proliferation rate is that UCMSCs proliferation is mainly driven by other factors, such as exposure to dexamethasone, as well as interaction with certain hormones and transcription factors. Traditionally, differentiation in bone substitutes is done when a confluent layer of the mesenchymal cells is cultured with dexamethasone, ascorbic acid, and beta-glycerophosphate. These three factors are further complemented by the presence of bone proteins, TGF-beta, and vitamin D3. The presence of dexamethasone might be the

biggest determinant of osteogenic differentiation, thus explaining the similar rate of proliferation between the UCMSC in the control and the cells in contact with HA/CC particles. Dexamethasone is a necessary glucocorticoid in initiating osteogenic differentiation from undifferentiated stem cells. A complete absence of dexamethasone for UCMSCs in medium will fail to mineralize and form the extracellular matrix. Even though the exact manner in which dexamethasone achieves this action remains poorly understood, there are many viable hypotheses. One such explanation could be that the glucocorticoid induces transcription effects by attaching to the promoter region of genes that are unique to bones like bone sialoprotein. Dexamethasone is also closely linked to enhancing the expression of factors that are responsible for the activation of Runx-2. The presence of dexamethasone in appropriate amounts in both samples of UCMSCs will lead to an almost equal proliferation rate. However, it is important to note that excessively high levels of dexamethasone counter the action of osteoblasts and inhibit the formation of collagen. Dexamethasone levels higher than physiologically recommended levels cause osteoporosis that weakens bone's structure.

Other critical components present in both the control and the HA/CC particles that can explain the growth pattern are calcium and phosphate ions. Calcium and phosphate are critical components in the structure of bones and are the main electrolytes in the skeletal tissue of the human body. The equivalent presence of these two critical ions in both the control and HA/CC particles would explain the similarity seen in its growth pattern. Calcium and phosphate ions are provided in normal protocols through the addition of beta-glycerophosphate, which releases the ions gradually as its structure is hydrolyzed by ALP. The presence of these two ions determines the rate of proliferation of UCMSCs in both the control medium and those placed with HA/CC particles.

The presence of equal exposure to ascorbic acid in the experiment is another determinant for the equal proliferation rate observed. Ascorbic acid is a common component in protocols for the stimulation of osteogenic activity in bone transplants—

ascorbic acid functions by facilitating the hydrolysis of lysine and proline. Exposure of UCMSCs to all these factors could be the reason why proliferation rates were similar in cells exposed to HA/CC particles and those without any exposure at all.

The notable difference between UCMSCs exposed to HA/CC particles and the control group is a smoother surface in the HA/CC group. Increasing cell density is one of the main factors that makes HA/CC composite an attractive alternative in bone replacement. The smooth appearance of UCMSCs once it is exposed to HA/CC is attributed to an increased deposition rate of extracellular matrix that contributes to its overall density. The UCMSCs bind with the HA/CC particles to form a conglomerate that is bigger than UCMSCs alone (Trávníčková & Bačáková, 2018).re The structure of the HA/CC composite scaffolds provides an environment that encourages the invasion of osteogenic cells and revascularization, which increases bone density (Wang et al., 2021). The semi-porous nature of HA/CC scaffolds in bone transplants encourages the ingrowth of novel bone with a density that mirrors that of the native bone structure. As mentioned earlier in this study, HA/CC scaffolds contain the biological and physical features that make them a suitable synthetic alternative.The results did not produce any evidence pointing to the cytotoxicity of HA/CC particles.

In the study, it was demonstrated that cells can develop and proliferate normally on HA/CC particles. There was no discernible difference in the proportion of live cells in the HA/CC sample and the control. The HA/CC composite scaffold provides an ideal environment for the proliferation of all cell lineages without an increased risk of death. This is attributable to the low immunogenicity of the material as well as its close structural and biological similarity with human bone. Hydroxyapatite, one of the components of the HA/CC scaffold, has a surprisingly close resemblance to endogenous bones. The materials' chemical structure has 50% of what is contained in natural bone with a 80% similarity to enamel and dentine. This makes it less likely to cause inflammation or elicit an immune response as seen with other less biocompatible

options for bone transplants. The material also creates a strong bond with the surrounding endogenous bones by forming a carbonated epitaxial layer that is free of calcium (Mozafari, Banijamali, Baino, Kargozar, & Hill, 2019). There is little knowledge on the use of calcium carbonate scaffolds used in their pure form as a synthetic orthopedic implant. However, it shares the same characteristics of biocompatibility and low immunogenicity in-vivo as that of hydroxyapatite. The nature of these two materials contributes to the lack of cytotoxicity in the HA/CC scaffold. The findings showed that the proportion of dead cells was higher in the sample that was treated with 200μMRA compared with the 20μMRAand the 0RA samples. The higher cytotoxicity, in this case, can be attributed to high levels of RA, which is significantly higher than what is physiologically normal. The cytotoxicity, therefore, can be attributed to high RA levels that are dangerous to differentiating mesenchymal stem cells of the umbilical cord on the HA/CC composite.

The HA/CC scaffold, therefore, encourages the proliferation of UCMSCs but not significantly more than other factors and is not cytotoxic. UCMSCs are a rich source of pluripotent cells that can play a big role in inducing osteogenic capabilities in synthetic implants. However, the undifferentiated cells have to be cultured with dexamethasone, ascorbic acid and beta-glycerophosphate, which play a bigger role in the growth rate of osteoblasts and eventual novel bone structure in implants. Dexamethasone is critical for the formation of a mineralized extracellular matrix because it induces transcription factors like hormone promoters and Runx-2. The ascorbic acid is important because it is responsible for the hydroxylation of proline and lysine, while beta-glycerophosphates provide the differentiating matrix with calcium and phosphate ions. Calcium and phosphate ions are crucial components of bone and a requirement for osteogenic activity. A notable difference seen with the use of the HA/CC scaffold is an increase in density. The semi-porous nature of HA/CC encourages the biological invasion of surrounding bone structure with an increase of the underlying bulk and density. There is no evidence of HA/CC cytotoxicity, with the synthetic scaffold providing an ideal

environment for the growth and sustainability of the new cells formed as a result of differentiation.

A look at the microscopic features of the HA/CC particles with UCMSC reveals how the surface of the synthetic scaffold encourages osteogenic activity. The findings show that under SEM there was normal cell growth on HA/CC particles. It was observed that the medium provided a stable environment that encouraged more than 90% of cells to grow, with only a small proportion of less than 10% dead cells during the process. The proportion of live cells observed in the 0RA and 20μMRA groups were greater than 90%. HA/CC scaffolds provide a 3D structure that allows for the proliferation of cells in its osteogenic lineage, and the particles interact with UCMSCs as well as other osteogenic promoting factors such as dexamethasone, ascorbic acid, beta-glycerophosphate, and vitamin D3 to form new osteocytes. Microscopic analysis of the scaffold shows proliferation of the cells, osteoblast formation, and the growth of the extracellular matrix that gives the implant its density.

In-vivo models show that the HA/CC composites with UCMSCs placed in an osteogenic differentiation environment encourage ingrowth of blood vessels and the formation of extracellular matrix that closely resembles that of naturally occurring bone after a period of time. The initial appearance of the scaffold resembles cancellous bone because of the presence of micro-pores that makes the HA/CC scaffold brittle at the start. The calcium carbonate plays a role in increasing its tensile strength. The micro-pores serve to attract the migration of newly formed osteoblasts into the scaffold as well as revascularization of the new implant. The deposition of extracellular matrix and collagen was notably higher in implants that use the HA/CC composite scaffolds. There was also a higher degree of calcium found in the extracellular matrix of the HA/CC scaffold that was coated with UCMSCs since the structure encouraged infiltration of the same after ALP hydrolyzed the beta-glycerophosphate into its constituent calcium and phosphate ions.

The proliferation of UCMSCs along the osteogenic line took part in a step-wise manner as observed under SEM. Initially, the cells of the UCMSCs in all the samples proliferated and formed bridges with neighboring HA/CC granules and create structures that are known as organoids. Eventually, the organoids went further to form interconnecting bridges between them that further solidifying the structural integrity of the scaffold. With the addition of the extracellular matrix, after 7 days the microscopic appearance of the HA/CC and UCMSCs scaffold closely resembled that seen in natural bone structures.The amount of UCMSCs deposited on HA/CC scaffolds is another factor that determines the rate of differentiation in the synthetic structure. In samples where a lot of UCMSCs has been incorporated, it was noted that the differentiating cells were more and provided more density to the structure. UCMSCs provide a large pool of undifferentiated cells that, under the right osteogenic conditions, can lead to the formation of osteoblasts and other features of natural bone. The more UCMSC present in the implant, the denser the osteogenic cells and activities become. An earlier analysis of the SEM of the samples studied showed differences in densities that can be attributed to differences in the amount of UCMSCs used.

An assay of the different scaffolds revealed that the dead cells made up less than 10% of the total matrix. The dead cells can be attributed to experimental shortcomings and human error that fails to precisely replicate the in-vivo environment that a normal implant would be located. The low proportion of cell death is indicative of the advantage of using UCMSCs on HA/CC scaffolds. Unlike adult stem cells whose differentiation capacity might be hindered by an underlying pathology or due to its limited amount, UCMSCs provide researchers with a large pool of pluripotent embryonic cells with a much bigger capacity to differentiate into terminal cells.

Overall, the microscopic appearance of UCMSCs on HA/CC scaffolds shows features of osteogenic activity when placed in the optimum environment (ISHIKAWA, 2019).

A large proportion of the cells differentiate and grow under these conditions in a process where bridges are formed between HA/CC molecules and UCMSC cells to form organoids which form further bridges between them. Under SEM observation, the complex develops into a structure that closely resembles natural bone with infiltration of calcium and deposition of extracellular matrix into the porous synthetic scaffold. The characterization of the complex is different depending on the time of observation because of the ongoing osteogenic differentiation process.

Osteogenic differentiation in the samples studied showed that UCMSCs on HA/CC scaffolds can form new bone cells under the right condition. Osteogenic differentiation of UCMSCs is driven by the presence of osteogenic factors, hormones, and the ability to attract bone progenitors. UCMSCs are a large pool of pluripotent and undifferentiated cells that are capable of transforming into any terminal cell type. The UCMSCs have intrinsic properties that make them suitable for osteogenic differentiation, which includes the presence of CD90 and CD73 markers that are similar to those found in the mesenchymal cells of adult bone marrow (Liu et al., 2020). The USMCS also produces secretomes like exosome and trophic proteins that play a role in the mediation of intracellular communication, which is critical in differentiation. The intracellular communication may involve the transfer of certain proteins and microRNAs that encourage actions like the formation of new blood vessels, tissue repair, and inhibiting the formation of inflammation and fibrosis in the HA/CC complex. It was also noted that the UCMSCs coated with HA/CC scaffolds produced cytokines that are responsible for improving the osteoblastic regeneration, regeneration of bones, and proliferation of novel bone cells in the extracellular matrix. The embryonic nature of UCMSCs means that they have high pluripotency and can easily transform into osteogenic cells compared with other alternatives.

As mentioned earlier, osteogenic differentiation is driven by the presence of promoting factors including dexamethasone, ascorbic acid, and beta-glycerophosphate. The

dexamethasone works to convert the pluripotent stem cells present in the umbilical cord into osteogenic progenitors (Parate, Zhang, Hui, & Toh, 2016). Dexamethasone raises the expression of factors such as integrin alpha-5 and TAZ, which function to increase the expression of Runx-2 (Ma et al., 2020). The role of dexamethasone is central in the osteogenic differentiation of UCMSCs. The role of ascorbic acid has also been well elaborated. The acid is useful in the hydroxylation of lysine and proline that is further incorporated in the final bone-like matrix that is formed (Prakash et al., 2020). The beta-glycerophosphate provides calcium and phosphate ions that are critical components in bone formation. The compound is broken down into its constituting elements of calcium and phosphate ions through hydrolysis by alkaline phosphate. The role of the released ions is to encourage the mineralization of the extracellular matrix, as well as the generation of lactate. These factors give the complex better bone density and further encourages the mimicking of normal bone physiology.

Osteogenic proliferation is further complemented by the presence of BMPs, vitamin D3 and TGF-beta (Ramesh, Moratti, & Dias, 2017). Morphogenetic proteins enhance osteogenic differentiation even in developed tissues. The most common are the BMPs, which not only stimulate osteogenetic differentiation but also induce the expression of genes that control the formation of crucial components of bones, like collagen and matrices. Vitamin D3 is another factor that complements the osteogenic proliferation process. The role of the vitamin is to optimize the function of osteoblast cells by inducing cAMP-dependent calcium ion intake into the composite to mineralize and strengthen the extracellular matrix that is meant to improve the density and tensile strength of the newly formed bone structure (Yap & Toh, 2016). TGF-β is another critical promoter of osteogenic proliferation in UCMSCs. TGF-β occurs in three isoforms. However, the most abundant and physiologically important in bone formation is TGF-β1. TGF-β1 is a regulator of the growth of cells and plays an important role in the formation and degradation of collagen type 1 and other related proteins found in bones. TGF-β1 increases the expression of Runx-2, which is an important factor in

osteoblast activity and found in all bones. TGF-β1, however, has been shown to inhibit the late-stage differentiation of osteoblasts into mature bone cells and is only useful in the initial stages of osteogenic differentiation of cells. Runx-2 is an early indicator of osteogenic differentiation in mesenchymal cells and shows the impending formation of bone cells from undifferentiated ones (Wang et al., 2010). Runx-2 is a growth factor that plays a critical role in the expression of genes that are specific to bone tissue and include osteocalcin, osterix, and bone sialoproteins. Osterix produces an important zinc-finger containing protein that regulates the genetic expression of osteoprogenitor cells with a role in the formation of bones and the rate of osteogenic differentiation. Osteocalcin, on the other hand, is a gene that plays a mediating role in the formation of collagen type 1, which improves the stability and strength of the implanted bone. The presence of bone sialoproteins in the HA/CC scaffolds is a promoter of osteoblast formation from multipotent stem cells. Runx-2, therefore, is an important marker indicating the start and continuation of osteogenic differentiation of the UCMSCs placed on the HA/CC scaffolds.

This study conducted using UCMSCs on the HA/CC particles, it was noted that osteogenic differentiation was also affected by the presence of the composite and the amount of UCMSCs available for proliferation (Yea, Bae, Kim, Cho, & Jo, 2020). The research initially showed that UCMSCs deposited along with HA/CC particles differentiated faster than the control samples that did not have the HA/CC particles. The finding points to the importance of having an environment that structurally and chemically resembles that of indigenous bones. The HA/CC scaffolds provide an ideal environment that favors osteogenic proliferation due to their close resemblance to bone. Hydroxyapatite, as one of the components of the scaffold, has a 50% structural resemblance to natural bone and a much higher 80% similarity with enamel and dentine. Osteogenic differentiation is markedly increased in the environment created by the HA/CC scaffold compared with the control surface. The amount of UCMSCs available for differentiation played an equally important role. In samples where HA/CC and

UCMSCs were both used, the HA/CC scaffolds with the higher concentration of undifferentiated stem cells appeared thicker and smoother. Under microscopic observation, it was seen that over time the scaffold with the thick layer of UCMSC had an increase in cell density and deposition of extracellular matrix. The larger pool of undifferentiated cells on the scaffold provided more opportunities for successful osteogenic differentiation leading to the creation of a denser alternative to allografts and autografts. An interplay of the environment provided by the HA/CC composite, which closely resembles that of actual bone and the presence of a large pool of UCMSCs favor osteogenic differentiation and proliferation.

RA plays an important role in the osteogenic differentiation process in bone transplants using HA/CC scaffolds. The experiment that was conducted involved the use of HA/CC scaffolds with similar traits and amounts of UCMSCs, and the only difference was the amount of RA included in each of the samples. In one of the scaffolds no RA was added, and it was thus labelled 0RA. In a second one the scaffold was treated with 20μMRA, and in the final scaffold, 200μMRA was added. The three different scaffolds were then observed for osteogenic differentiation with interesting findings on how RA affects cell growth and death, as well as the effects it had on parameters such as ALP and BMP expressions in the final synthetic bone replacement candidate.

It was noted that RA increased the rate of differentiation of the UCMSCs up to a certain extent, after which its role becomes cytotoxic. The three HA/CC scaffolds were analyzed to understand the impact that RA has on cell proliferation and death. 0RA displayed a high differentiation rate, while the 20μMRA showed a statistically similar rate of cell growth. The proportion of the cells that were alive in both of these samples were more than 90% of the total. However, in the sample with 200μMRA there was a much higher proportion of dead cells, amounting to more than 10% of the total. The findings show that RA is an important component in encouraging osteogenic differentiation in scaffolds but at low and safe doses (Manokawinchoke, Osathanon,

Egusa, & Pavasant, 2016). Higher doses of RA trends to have a cytotoxic effect on the differentiating cells.

The impact of RA seen in the study is a reflection of established literature on the skeletal effects of the compound. RA is an important nutrient that is best known for its role in promoting good vision but whose skeletal effects have also raised the interests of scholars across the globe (Ahmed et al., 2019). The substance is initially digested in the form of carotenoids and retinyl esters before it is broken down into its active components, which include 11-cis-retinal and RA. The RA binds to the RA receptor (RAR), leading to the heterodimerization of the responsive elements of the cell genome (Chen & Li, 2016). The active components of RA, therefore, play a great role in the expression of several genes seen in cell structure. RA acts on several cells and tissues, including those found in bone tissues (Jacobsen & Craft, 2019). RA has been well documented in having a role in osteogenesis by promoting the resorption of bones tissue. However, this effect is limited by the level of exposure since it also has a tendency to cause skeletal fragility at high doses. RA has both a promoting and inhibitory effect on the formation of osteoclasts in bones. In mesenchymal stem cells, RA can act as a promoter of osteogenesis and improve the rate at which new bone cells are formed from the pool of undifferentiated cells. In the experiment conducted in the study, the 20μMRA scaffold displayed a high rate of osteogenic differentiation after exposure to RA that was proportionally similar to that of the control scaffold. At the extreme dose of 200μMRA it was evident that the compound had a cytotoxic effect on the UCMSCs, with more than 10% of the cells formed through osteogenic differentiation dying.

The mechanisms through which RA promotes osteogenic differentiation remains a mystery. One theory behind the osteogenic role of RA is that it has an impact on the expression of BMPs by regulating Smad activity. Smads are found in the central and peripheral nervous systems and act as transcription factors that regulate the signaling of bone morphogenic proteins. Smads also modulate neuronal cell development from

the progenitors available in the neural crest (Song, Estrada, & Lyons, 2009). The active components of RA increase the translocation of these mediators into the nucleus of the osteogenic precursor cells. The RA works on the signaling events mediated by Smad-1 and Smad 5 more than all other such proteins. RA, therefore, increases the activity and expression of these Smad proteins, which in turn increases the activity of BMPs with particular emphasis on BMP-9. The increased expression of these transcription factors, in turn, lead to osteogenic differentiation of sensitive cells. RA also promotes osteogenic differentiation through the inhibition of the formation of adipogenic cells. Pluripotent cells have the potential of differentiating into any cell lineage, and the environment that surrounds them plays a critical role in the terminal fate of these cells. RA is favored in promoting osteogenic differentiation because its action inhibits the formation of adipogenic cells from the mesenchymal cells presents. Adipogenic tissue formation is favored by the environment that the HA/CC particles provide with a risk of forming these cells, which would grossly affect the integrity and functionality of the HA/CC scaffolds as a substitute for bone transplantation (Mondal & Pal, 2019). RA manages to suppress adipogenesis due to the crosstalk that occurs between its active components and bone-forming proteins, creating an environment that is hostile to the growth and proliferation of adipose progenitor cells. The actions of RA on the Wnt/β-catenin pathway affects the expression of key genes and factors that are required for osteogenic differentiation. The role of RA in promoting osteogenesis is one that inspires extensive research. The findings of the study, however, aligns with the general understanding that RA plays a role in osteogenesis differentiation with an excess of the same inhibiting the production of osteoblasts from mesenchymal stem cells.

The 20μMRA samples displayed an increased tendency to differentiate along the osteogenic progenitor line when it was combined with other factors such as BMP as well as the environment created by the HA/CC composite. The findings are a reflection of the importance of creating an environment that resembles that of the bone as closely as possible. The interaction between RA and BMP-9 is one of the reasons that

osteogenic differentiation was markedly increased in 20μMRA samples. An appropriate amount of RA mixed with BMP-9 had a positive effect on the formation of osteoclasts from the UCMSCs that were in use. The interplay of these factors led to a greater rate of cell differentiation in the 20μMRA compared with the 0RA and 200μMRA scaffolds. The RA had a better interaction with the scaffold and expressed
a higher degree of BMP-9 and osteogenic differentiation when it was at 20uMRA compared with the higher concentration and the control group. Other than the BMP-9 and its influence on the action of RA in influencing osteogenic differentiation, it is also important to mention the impact that HA/CC on the influence of RA. The HA/CC composite provided an environment that favored the maximal expression of RA's influence on the osteogenetic differentiation of cells. The 20μMRA coated scaffold displayed a high rate of osteogenic differentiation partly because of the environment that favored such differentiation. There was enhanced expression of osteocalcin and osteonectin,indicative of a higher propensity to form bone tissue on the scaffold. An increase was seen in the overall bone-forming activities on the scaffold with increased expression of collagen formation, osteoclast invasion of the scaffold, and a greater capacity to enable revascularization of the scaffold in-vivo.

Interestingly, it was noted that exposing UCMSCs and HA/CC composite to extremely high levels of RA displays a lower propensity of osteogenic differentiation. The results for the 200μMRA scaffold showed that the high RA concentration negatively impacted osteogenic differentiation of mesenchymal stem cells. The sample showed a higher proportion of dead cells from the microscope observation. The 200μMRA also expressed few osteogenic factors, ALP and BMP-9 expression compared with that seen in the 0RA and 20μMRA scaffolds. The findings show how differences in concentration of RA can have conflicting and opposite effects on osteogenic differentiation of UCMSCs. The negative effect of high concentrations of RA has been attributed to several causes. One of the more widely accepted reason is that high RA levels increase the duration of BMP signaling. This extension negatively affected the formation of

osteoclasts in such an environment. On a molecular level, it is believed that RA increases the interaction of phosphorylated Smad 1 and ubiquitin E3 ligase. The interaction leads to the ubiquitination and the eventual breakdown of the Smad-1, which is phosphorylated which further downregulates the expression and signaling of BMP ad Smad (Zhang et al., 2018). An antagonistic relationship exists between BMPs and RA signaling. In the process of chondrogenesis the gene Aldh1a2, a constituent of RA, acts as a primary target for BMP's regulation of RA signaling. Changing the gene's expression has the effect of causing BMPs to inhibit chondrogenesis that is seen in UCMSCs.

Another explanation for the lower rate of osteogenic differentiation in the 200μMRA compared with the other samples is the failure to induce the RA13 gene. The RA13 gene is induced by the presence of high levels of RA, and its downregulation is key to the osteogenic differentiation process. It has been well documented that the gene is markedly reduced in cells that have undergone osteogenic differentiation. In developed bones RAI3 genes are not present at all in-vivo, indicating the minimal role that it plays in the osteogenic and bone-forming process. An interplay of all the factors mentioned above could explain the cytotoxic effect of excessive RA on UCMSC on the HA/CC scaffold.

Overall, the role of RA in the osteogenic differentiation of mesenchymal stem cells can be divided into two. The first is that in moderate amounts, RA is a promoter of osteogenic differentiation in pluripotent stem cells. RA achieves this feat by promoting expression of Smad proteins, as well as BMPs that increase the likelihood of mesenchymal cells differentiating into bone tissue that can be used in bone transplantation. The 0RA and 20μMRA scaffolds used in the experiment showed almost similar osteogenic differentiation patterns and differentiation rates. However, the 20□MRA scaffold displayed faster differentiation of its cells as well as an increase in the expression of collagen formation compared to the 0RA control group. Ironically,

high levels of RA have been shown to have a cytotoxic and inhibitory effect on the osteogenic differentiation of mesenchymal stem cells. The 200μMRA scaffold used in the study displayed a slower rate of osteogenic differentiation with a higher proportion of dead cells compared to other groups. The high concentration of RA in UCMSCs works to counter the osteogenic differentiation process. Reasons behind this phenomenon include the extension of BMP signaling time by the active components of RA, the ubiquitination of Smad-1 proteins, and its role in inducing the expression of RAI3 genes whose downregulation is a critical step in the bone-forming process. The use of RA, therefore, in osteogenic differentiation of UCMSC should be measured and well quantified to ensure that osteogenic differentiation is achieved without compromising the integrity of the process.

The experiment conducted involving the 0RA, 20μMRA, and 200μMRA treated scaffolds were observed for 21 days to understand the osteogenic process in all the samples. During this period, regular measurements of ALP expression were taken from the three study samples, and a comparison was made. The results show that after 21 days of osteogenic differentiation, the ALP activity of the 20μMRA group was significantly higher compared with the control group as well as the 200μMRA group. In the 20μMRA group, there was a steady rise in ALP concentration from the first day, with the peak seen on day 21 with close to 0.684 umol/min/ml expressed from the scaffold. The control scaffold with 0RA experienced a slower but steady rise in ALP expression as the days passed, reaching a peak value of 0.42 umol/min/ml on the 21st day of the experiment. The ALP quantification of the 0RA group was the slowest in rising and lowest at the end of the experiment compared with those that were treated with RA. The 200μMRA sample, on the other hand, also saw a steady rise in the expression of ALP activity but at a slower rate compared with the 20μMRA sample. ALP activity for the 200μMRA sample reached a peak of 0.52 umol/min/ml on the 21st day of the experiment.

The interaction between the scaffold, the UCMSCs and the differing RA treatment of the 3 experimental samples could explain the difference in ALP expression. The initial observation was that ALP activity was steadily increasing in the three experimental samples showing osteogenic differentiation in all the samples. ALP activity is a good indicator for bone formation since it plays a critical role in the expression of BMPs and other factors that are critical to the formation of mature bone cells and tissues. All 3 of the experimental samples displayed the capacity to differentiate into osteoclasts and osteoblasts from the UCMSCs samples used. The osteogenic differentiation that is common across all samples could be attributed to the HA/CC particles and the use of UCMSCs as the source of progenitor cells. The HA/CC particles used in the experiment provide a conducive environment for the growth of bone cells. The composite takes advantage of the benefits of its constituting members to create a structure that closely resembles human bones, can withstand both tensile and compressive forces, as well as provide a porous structure that allows for the invasion of blood vessels and collagen formation. ALP activity also increased across the board for the 3 experimental samples because of the use of UCMSCs as the progenitor for differentiation. UCMSCs provide a large pool of embryonic pluripotent stem cells with the ability to differentiate into numerous terminal cells. Placing the UCMSCs on the HA/CC scaffold stimulates osteogenic differentiation of the mesenchymal cells present, given that the surrounding environment is one that resembles bone tissue. The use of UCMSCs favors osteogenesis because of their easy availability and potential for differentiation, and their embryonic nature means that they are not limited by disease processes that might affect stem cells derived from adult tissues. Osteogenic differentiation is happening across all three samples because of these common underlying conditions, hence the increase in ALP activity for all groups in the experiment.

The 20μMRA sample displayed superior ALP activity compared to both the 0RA and the 200μMRA samples. The 20μMRA sample, therefore, displayed a superior rate of osteogenic differentiation compared to the other samples indicating that RA plays a role

in improving the bone formation process. RA used in moderate amounts is a promoter of faster osteogenic differentiation in pluripotent stem cells. The mechanisms behind this phenomenon have been mentioned earlier and include the role that it plays in promoting bone formation, its suppression of adipose cells formation, as well as osteoclast and osteonectin expression. The 20μMRA sample has the appropriate amount of RA needed to encourage optimal bone formation compared to the other samples. RA is believed to act alongside other BMPs to promote the differentiation of UCMSCs to osteoclasts and osteoblasts. The RA enhances the activities of BMPs on the undifferentiated cells, thereby initiating osteogenic differentiation faster than the control group. Secondly, the RA used in the 20μMRA sample encouraged ALP activity by inhibiting the formation of adipose cells that is a common occurrence in such a pool of undifferentiated stem cells. Naturally, UCMSC has the potential of differentiating into any terminal cell line in the human body. UCMSCs left on a HA/CC scaffold can form numerous types of cells with adipogenic differentiation. RA plays the critical role of inhibiting the UCMSCs from differentiating along the adipogenic line but rather develop into bone cells. The increased ALP activity for the 20μMRA can also be explained by the markedly increased expression of markers such as osteocalcin and osteonectin that is commonly seen with the use of RA in bone formation. The RA present in the sample further encourages osteogenic differentiation by increasing the expression of these important markers that further drive the process of bone formation. The markers indicate a higher rate of osteoblast formation in the HA/CC composite compared with the other samples. Other than the markers, microscopic observation of the scaffold showed a greater rate of collagen formation and deposition in the sample, further illustrating the promoting action of the 20μMRA dose on osteogenic differentiation of the mesenchymal stem cells. The findings from the study show that 20μMRA is a safe dose of RA that should be used in the process of promoting osteogenic differentiation in HA/CC scaffolds in the pursuit of the most suitable synthetic bone option available for transplantation. The addition of 0RA sample, RA significantly improves osteogenic differentiation in UCMSCs samples.

Another interesting observation made from the findings of the experiment was the difference in ALP activity seen between the 0RA and the 200μMRA samples. A look at this study on the effects of excessive RA on osteogenic differentiation would indicate that it would be the 200μMRA that had the lowest level of ALP activity. However, the results showed a different phenomenon, with the ALP activity of the 200μMRA sample being higher than that seen in the control 0RA sample but lower than that of the 20μMRA sample. This anomaly could indicate that the cytotoxic effect of excessive RA may take a while to act on the maturing cells. One possible explanation could be the effect that high concentrations of RA on the signaling of BMPs. The high levels of RA lengthen the signaling time for BMPs in mesenchymal stem cells leading to a sustained period of osteogenic differentiation before the cytotoxic effect of the same is observed. Another reason for the sustained increase in ALP activity for the scaffold with 200μMRA treatment could be the lack of ubiquitin E3 ligase in the in-vitro experimental setup. High concentrations of RA in-vivo tend to promote the engagement of Smad-1 factors and ubiquitin E3 ligase that eventually leads to the phosphorylation of Smad-1 and the downregulation of BMP and Smad signaling. However, since the experiment was conducted in-vitro within the controlled environment created by the HA/CC scaffolds, the stimulation of the ubiquitin E3 ligase failed to occur, and this allowed for the continuous growth of osteogenic cells in the experiment (Sethmann, Luft, & Kleebe, 2018). Similarly, the continuous increase in ALP activity for the 200μMRA can also be attributed to the absence of RAI3 in the HA/CC scaffold. One of the more established reasons for high RA levels leading to a decline in osteogenic differentiation is the expression of RAI3 genes. The suppression of these genes is a critical part of the bone-forming process with no traces of the genes found in established bone tissue. In the controlled in-vitro environment of the HA/CC scaffold, there is a smaller chance of RAI3 gene induction compared to the normal sequence of events seen in actual bones. The process of downregulating RAI3 genes is therefore not needed in the experiment described in this study which is one of the reasons for the observed

increase in ALP activity.

Finally, the 0RA group recorded increasing levels of ALP activity in the 21 days the samples were observed. The rate of ALP activity for 0RA was lower than that of both the 20μMRA and 200μMRA samples. The ALP activity reached its peak on day 21 at a value of 0.427 umol/min/ml. The control group had no RA treatment and is a reflection of the normal growth pattern of UCMSC placed on HA/CC composites to promote the process of osteogenic differentiation. The ALP activity for the 0RA scaffold is much slower than the others treated with RA, meaning that normal bone growth pattern is strongly promoted by the presence of RA. Since all other factors in the samples remained constant except for the presence of RA in the matrix, it seems RA promotes ALP activity in the process of osteogenic differentiation of mesenchymal stem cells. However, there is no direct evidence that RA promotes ALP activity, some other studies suggest that RA synergistically promotes ALP activity (Sun et al.,2020. Weng et al.,2019). The rate of ALP activity almost doubles when 20μMRA is used to treat the HA/CC composite containing UCMSCs. RA is believed to enhance bone formation in-vivo.

The BMP-9 concentration of three groups of scaffolds, 0RA, 20μMRA, and 200μMRA, were measured serially during the 21-day experiment. It was noted that the BMP-9 concentration levels were markedly higher in the experimental group of 20μMRA compared with that seen in the control group of 0RA and the ones observed in the 200μMRA sample. The rise was significant in the first 7 days, with the peak in all three groups seen on the 14th day of the experiment before a reduction was noted in all of them. The 0RA sample saw a rise in BMP-9 concentrations from a value of 8.308 ng/ml on the first day to a high of 15.544 ng/ml on day 14 before eventually falling back down to 12.9 ng/ml on day 21. The 20μMRA sample had an initial BMP-9 concentration of 10.196 ng/ml on day one, and this value rose to a high of 22.73 ng/ml on the 14th day of the experiment. The 200μMRA sample had the highest first day values of the three

at 11.267 ng/ml on day 1 and peaked at 15.434 on day 14 of the experiment before falling to a value of 12.968 on the Day 21 experiment. The differing BMP-9 expressed in the three samples are a reflection of the effect that RA has on the action of BMPs in the process of osteogenic differentiation.

There are different types of BMPs in the human body that play a critical role in the formation of bones (Wang et al., 2019). BMPs belong to the transforming growth factor-beta superfamily and act by stimulating bone formation in both undifferentiated cells as well as in established tissues (Oryan, Alidadi, Moshiri, & Bigham-Sadegh, 2014). The action is driven by the ability of the proteins to induce genes that are important in the regulation of bone components such as collagen. BMPs are also important in the regulation of some bone-specific genes such as osterix, bone sialoprotein, and osteocalcin that are needed to form the bone cells and the structure surrounding the same. The normal range of BMPs expressed in the body depends on its type and the sex of the individual. In the study, BMPs and their levels were noted from different donors to provide a baseline to compare the three experimental scaffolds with differing RA levels. BMP-2 was seen at 22.1+/-12.1 with a normal range of 1.90 to 43.3ng. BMP-4 stood at 5.45 +/- 2.05ng with a normal range of 2.28 to 10.3ng. BMP-7 concentrations were 85.1 +/- 34.6 ng with a normal range of 28.2 to 174.4 ng. An analysis of the three types of BMPs revealed that BMP-7 was more abundant in the adult's samples compared with the other two types. There was also a gender correlation of BMP-7 and BMP-2, with females displaying 52% more BMP-7 and 72% more BMP-2 than males. However, none of these three BMPs were measured in the study. Rather, BMP-9 was preferred as RA was chosen to enchance the osteogenesis and RA can promote BMP-9 expression. The preference was driven by the fact that the osteogenic differentiation under in-vitro conditions using UCMSCs. The findings show that the BMP-9 expressed in the study correlated to the normal BMPs range seen in normal bone matrix.

The BMP-9 concentrations on day 1 reflect the impact that RA has on osteogenic

differentiation. The BMP-9 concentration of the 0RA sample was the lowest initially, with the 200μMRA being the highest. One possible reason for this observation is the impact that RA has on differentiation (Osathanon, Manokawinchoke, Egusa, & Pavasant, 2017). The control sample with 0RA is not influenced by RA, and its BMP concentrations reflect that seen in a normal surface. The 200μMRA sample has the highest concentration of BMP-9 at this initial stage because the high levels of RA present promote osteogenic differentiation at a faster rate compared with the other two samples. The impact of the RA on the undifferentiated stem cells is almost instant, leading to faster induction of BMPs along with other TGF-beta in the three samples (Sun et al., 2020). The 20μMRA scaffold shows an increased BMP-9 expression compared to the control but less than the 200μMRA one because of its lower concentration and slower induction.

On day 7, the 20μMRA scaffold surpasses those of the control and the 200μMRA samples. The 20μMRA scaffold has the safest amount of RA and is thus able to express higher BMP-9 concentrations compared to the other 2 scaffolds. The higher expression of BMP-9 in the 20μMRA sample at this time reflects the underlying impact that the BMP has on the UCMSC on the HA/CC. The RA present in the sample increases Smad activity which in turn increases the expression of BMP-9 in the experimental samples. The active component of RA, RA, increases the translocation of the BMP-9 into the nucleus of the progenitor cells of osteogenesis. The RA in the scaffold influences the signaling events mediated by both Smad-1 and Smad 5, further enhancing the production of BMP-9 in these sensitive cells. The active component of RA also acts on the Wnt/β-catenin pathway, which is a critical factor in the expression of BMP-9. The 0RA scaffold on the 7th day showed a higher degree of BMP-9 expression compared to the 200μMRA, which was leading on day 1. At this point, the inhibitory effects of high concentrations of RA are starting to manifest, leading to a slower rate of osteogenic differentiation in these cells compared to those seen in the 20μMRA. 0RA is still differentiating at a reasonable rate, with BMP-9 expression slightly higher than that

seen in the 200μMRA group.

Day 14 was the peak day from BMP-9 expression in all of the three experimental scaffolds. The 20μMRA scaffold had the highest concentrations of BMP-9 in the experiment, while the 0RA and 200μMRA have almost similar peaks in concentration levels. RA at the safe dose of 20μMRA has a profound impact on osteogenic differentiation of UCMSCs in sensitive cells. The levels of BMP-9 concentration seen in the 20μMRA samples on day 14 is reflective of the normal reference range found in-vivo. At the same time, the similarity in the peak levels of both the 0RA and the 200μMRA scaffolds reflects the maximum BMP-9 concentrations seen without the effect of RA. The 200μMRA has markedly increased RA acting on its cells. After 14 days of differentiation, the inhibitory effects of RA are seen and it does not continue rising like it is seen in the 20μMRA sample. At this stage of differentiation, the high RA levels play a minimal role in furthering the differentiation process and does not play a role in the further expression of BMP-9 proteins along with other factors. On day 21, a decline in all the experimental scaffolds is seen with 0RA and 200μMRA samples displaying similar levels of BMP-9 expression. At this stage of osteogenic differentiation, BMP-9 expression is on the decline as a larger portion of the UCMSC has now undergone differentiation into osteoclasts and osteoblasts. Fewer BMP-9 are required by the scaffolds to form new bone cells since the pool of pluripotent cells has declined at this stage. The decline reflects the findings seen on day 14 with the 0RA and the 200μMRA lowering at similar rates since the impact of RA in the 200μMRA sample is inconsequential to further differentiation. However, the BMP-9 concentration remains higher for the 20μMRA sample due to its superior impact on the osteogenic differentiation of cells.

Overall, the findings of this study have demonstrated the ability of RA to improve the osteogenic differentiation of UCMSC. The sample with the 20μMRA displayed a greater propensity to induce differentiation of cells, has a markedly higher ALP activity

level and produces BMP-9 at concentrations that closely mimic those seen in normal demineralized bone matrix. It is therefore reasonable to postulate that RA can be used in the induction of bone formation in-vivo as well as acting as a key ingredient in osteogenic differentiation of synthetic scaffolds. Ideal concentrations of RA can be used in the creation of HA/CC scaffolds to further improve the osteogenic differentiation of UCMSCs.

5.Future work

The combination of hydroxyapatite and calcium carbonate has good mechanical strength and biocompatibility. The organic combination of this scaffold material and mesenchymal stem cells has been widely used in bone tissue engineering. For human umbilical cord mesenchymal stem cells, it is relatively easy to obtain, but studies have shown that its osteogenic capabilities are not as good as bone marrow-derived mesenchymal stem cells. Therefore how to make UCMSCs more effective proliferation and osteogenic differentiation is one of the issues that need to be studied in the future. This study proved that RA can enhance the osteogenic capability of umbilical cord mesenchymal stem cells by stimulating the expression of BMP-9. In the future, gravimetric method and longitudinal compression test should be performed to test the porosity and the mechanical properties of HA/CC scaffold. Identification of osteoblasts after osteogenic differentiation of this model should be tested. The main mechanisms through which RA promotes osteogenic differentiation need to be explored and further optimise of the experiment is needed to assessing the effectiveness of enhancement by RA in vitro. Moreover, in vivo experiments are needed to determine the enhanced osteogenic capabilities and biodegradation of this model before it can be successfully to gain clinical benefit of patients.

6.References

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