Study of hMSC Proliferation and Differentiation on Mg and Mg-Sr Containing Biphasic β -tricalcium Phosphate and Amorphous Calcium Phosphate Ceramics

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Abstract: Biphasic mixtures of either Mg^{2+} or combined Mg^{2+} and Sr^{2+} cation substituted β -tricalcium phosphate (β -TCP) and amorphous calcium phosphate (ACP) were prepared using a low temperature chemical phosphatizing and hydrolysis reaction approach. Scaffolds prepared using the cation substituted calcium phosphates were capable of supporting similar levels of human mesenchymal stem cell proliferation in comparison to commercially available β -TCP. The concentrations of Mg^{2+} , Sr^{2+} , and PO_4^{3-} released from these scaffolds were also within the ranges desired from previous reports to support both hMSC proliferation and osteogenic

differentiation. Interestingly, hMSCs cultured directly on scaffolds prepared with only Mg^{2+} substituted β -TCP were capable of supporting statistically significantly increased alkaline phosphatase activity, osteopontin, and osteoprotegrin expression in comparison to all compositions containing both Mg^{2+} and Sr^{2+} , and commercially available β -TCP. hMSCs cultured in the presence of scaffold extracts also exhibited similar trends in the expression of osteogenic markers as was observed during direct culture. Therefore, it was concluded that the enhanced differentiation observed was due to the release of bioactive ions rather than the surface microstructure. The role of these ions on transforming growth factor- β and bone morphogenic protein signaling was also evaluated using a PCR array. It was concluded that the release of these ions may support enhanced differentiation through SMAD dependent TGF- β and BMP signaling.

Keywords: human mesenchymal stem cell; substituted tri-calcium phosphate; differentiation

1. Introduction

Calcium phosphates (CaPs) have been widely studied as bioactive components in synthetic bone graft substitutes [1]. Scaffolds prepared using CaP phases such as, dicalcium phosphate dihydrate (DCPD) and β -tricalcium phosphate (β -TCP), have gained increasing interest in hard tissue regeneration due to their resorbability under physiological conditions [2]. Furthermore, the release Ca^{2+} and PO_4^{3-} ions upon degradation may enhance the osteogenic differentiation of both stem and progenitor cells towards mature osteoblasts [3]. In addition to their application as bioactive components in bone graft substitutes, CaPs have also been studied in the form of coatings and as drug delivery vehicles [4-6]. Due to their wide range of applications, the controlled manipulation of both the physical and chemical properties of CaPs, such as particle

size, solubility, roughness, and crystallinity have become of increasing importance to improve the performance of CaP based medical devices.

Ionic substitutions have previously been explored to provide controlled manipulation of the physicochemical properties of CaPs. For example, work by Enderle et al. has shown that the cationic substitution of Ca^{2+} by Mg^{2+} in β -TCP stabilizes β -TCP at temperatures greater than 1100°C , enabling increased sintering and densification [7]. Ionic substitutions may also enhance the biocompatibility of CaP based scaffolds, since mineralized tissues are known to contain small amounts of various elements in addition to Ca and P, such as Mg and Sr, which are believed to play a critical role in regulating both structure and function [8]. Enhanced Mg^{2+} substitution in CaPs is known to stabilize amorphous calcium phosphates (ACPs) which, along with octacalcium phosphate (OCP), is believed to be a precursor to the formation of hydroxyapatite (HA) during mineralization *in vivo* [9, 10]. Interestingly, although the presence of Mg^{2+} in biological apatite has been shown to decrease throughout the calcification process, the exact role of Mg^{2+} in the regulation of bone repair is yet to be determined [8, 11].

In addition to Mg^{2+} , Sr^{2+} substituted CaPs have also gained significant interest due to the capability of Sr^{2+} to support osteoblastic differentiation while simultaneously inhibiting osteoclastogenesis [12]. Work by Yang et al. showed that enhanced *in-vitro* osteogenic differentiation was observed in human mesenchymal stem cells (hMSCs) cultured in the presence of increased Sr^{2+} concentrations through Wnt/ β -catenin signaling. Sr^{2+} substituted HA-collagen composites were also implanted into rat calvarial defects and Sr^{2+} substituted HA was shown to significantly enhance *in-vivo* bone regeneration in comparison to HA [13]. However, increased Sr^{2+} concentrations may lead to rickets and renal failure [14]. Mg^{2+} - Sr^{2+} co-substituted CaPs have also been explored to further mimic native mineralized tissues [15]. However, the

majority of studies performed to date have focused on the synthesis and characterization of these materials rather than evaluating either their *in-vitro* or *in-vivo* biocompatibility.

In the current study, the co-substitution of Mg^{2+} and Sr^{2+} in β -TCP was studied by hydrolyzing DCPD precipitated with Mg^{2+} and Sr^{2+} . The synthesis of Mg^{2+} substituted β -TCP (β -TCMP) from Mg^{2+} substituted DCPD using this approach was previously explored [16]. Upon increasing the Mg^{2+} concentration, increased amorphous content was formed in addition to β -TCMP. Interestingly, β -TCMP prepared with a 50% $\text{Mg}/(\text{Ca}+\text{Mg})$ ratio using this hydrolysis approach also supported significantly increased levels of osteogenic differentiation in a mouse preosteoblast cell line in comparison to scaffolds prepared using commercially available β -TCP [17]. However, the roles of the chemical and physical cues provided by the scaffold were not determined and the mechanism through which these scaffolds supported differentiation was also not studied. In the present study therefore, the influence of Mg^{2+} and Sr^{2+} co-substitution on the phase composition, physicochemical properties, proliferation, and mechanisms through which these scaffolds support the hMSC osteogenic differentiation has been reported.

2. Materials and Methods

2.1. Synthesis of Mg and Mg-Sr Substituted β -TCP

$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (ACS Reagent $\geq 99.0\%$, Acros Organics), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (ACS Reagent, $\geq 99.0\%$, Acros Organics), and $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$ (ACS Reagent, $\geq 99.0\%$, Sigma Aldrich) were dissolved in 100 ml of deionized water (**Table 1**). This solution was then slowly added under constant stirring to 100 ml of 0.5 M of disodium hydrogen phosphate (Na_2HPO_4 , $\geq 99.0\%$ Sigma-Aldrich) at room temperature. The mixture was stirred for 15 minutes prior to centrifuging and washing the precipitate formed with deionized water. In all cases, the $(\text{Ca}+\text{Mg}+\text{Sr})/\text{P}$ mole ratio was kept

constant at a value of 1. The washed precipitate was then dispersed in 200 ml of deionized water and was boiled with reflux for 2 hours. After refluxing, the powders were once again collected by centrifugation and washed with deionized water prior to drying overnight at 60°C.

2.2. Characterization of Mg and Mg-Sr Substituted β -TCP

X-ray diffraction (XRD) was performed using a Philips X-Pert PRO diffractometer employing Cu K α radiation ($\lambda=1.5406$ Å) with a Si-detector (X'celerator). The X-ray generator was operated at 45 kV and 40 mA at a 2θ range of 10-70° with a step size of 0.0167° and a time per step of 3s. The specific surface area of the particles formed was determined using the multi point Brunauer Emmett Teller (BET, ASAP 2020, Micromeritics) technique. The true density was measured using helium pycnometry (Accupyc II 1340, Micromeritics). Thermogravimetric and differential thermal analyses were performed using a Netzsch STA 409 PC DTA/TGA. These measurements were performed by heating the samples in air to 1000°C at a rate of 10°C min⁻¹. Heat treatments of the as synthesized powder and pressed pellets were performed using a Lindberg box furnace (Lindberg/blue, Riverside MI). Elemental analysis of the as prepared powders was performed using inductively coupled plasma optical emission spectrometry (ICP-OES, iCAP duo 6500, Thermo Scientific). Scanning electron microscopy (SEM, Philips, XL30) and high resolution transmission electron microscopy (HRTEM, JEOL JEM-2100F with Gatan GIF-Tridiem) were also used to observe the particle morphology and crystallinity.

2.3. Human Mesenchymal Stem Cell Maintenance and Culture

Human mesenchymal stem cells (hMSCs) obtained from the normal human bone marrow were purchased from Lonza (Lonza, Allendale, NJ) and were cultured under 37 °C, 5% CO₂, and 95% relative humidity. The cells collected after the third passage were used in all experiments and

were cultured in growth media containing minimum essential media α (MEM α) supplemented with 20% FBS and 1% P/S. Osteogenic differentiation was induced after 7 days of culture using the growth media supplemented with 100 nM dexamethasone, 50 μ M ascorbic acid, and 10 mM β -glycerophosphate.

2.4. MTT Cell Viability

13 mm diameter discs of the as prepared powders were formed by uniaxial pressing (Carver, Wabash, IN) of 0.35 g of powders with an applied load of 2,500 psi followed by heat treatment to 600°C for 4 hours. The cell viability of hMSCs seeded directly on the surface of these disc shaped scaffolds was assessed using the MTT assay (Vybrant MTT Cell Proliferation Assay Kit, Invitrogen, Carlsbad, CA). Water soluble MTT is reduced to insoluble formazan crystals in the living cells. Formazan is then solubilized and its concentration can be determined by measuring optical density at 570 nm. 1 mL of growth media containing 12 mM MTT was added to each well and incubated for 4 hours. A 0.1 g/ml solution of sodium dodecyl sulfate in 0.01 M hydrochloric acid was then added and the samples were incubated for an additional 15 hours. Absorbance was then measured at 570 nm.

2.5. Live/dead Staining

At the various time points of interest, the samples were washed with PBS and were then incubated for 30 minutes with calcein AM and ethidium homodimer-1 diluted in PBS (Invitrogen, Live/dead Staining Kit) at room temperature. After incubation, the samples were once again gently washed with PBS prior to imaging using fluorescence (Olympus CKX41).

2.6. Alkaline Phosphatase Assay

hMSCs were lysed using a lysis buffer following the manufacturer's protocol (CellLytic M, Sigma Aldrich). 170 μL of 1 g L^{-1} p-nitrophenyl phosphate (pNPP) dissolved in 0.2 M tris buffer (SIGMAFASTTM p-Nitrophenyl phosphate tablets, Sigma Aldrich) was added to 30 μL of cell lysate. The samples were then incubated at 37°C for one hour. The reaction was stopped by the addition of 20 μL of 0.3 N NaOH. The concentration of p-nitrophenyl (pNP) in solution after incubation was determined by measuring the absorbance at 405 nm. pNP standards were prepared by diluting pNP in 0.02 N NaOH. Alkaline phosphatase (ALP) activity was also normalized with respect to total protein concentration.

2.7. Osteogenic Protein Expression

Protein expression was quantified using a MAGPIX instrument (Luminex, Austin, TX). A Human Bone Magnetic Bead Panel kit (EMD Millipore, Billerica, Ma) was used according to the manufacturer's protocols. Cell lysates were incubated overnight at 4°C with antibody conjugated magnetic beads. After incubation, the well contents were washed prior to measuring protein concentration using the known standards. The expression of osteoprotegrin (OPG) and osteopontin (OPN) were studied. Measurements were conducted in triplicate for each sample and were normalized with respect to total protein concentration.

2.8. Indirect Cell Culture

hMSCs were cultured in the presence of Mg^{2+} and Sr^{2+} substituted β -TCP pellet extracts using transwell membrane inserts. The membranes used in the study had a pore size of 0.4 μm and a nominal pore density of 4,000,000 pores cm^{-2} (Transwell PET, Corning). Sintered dense disc shaped scaffolds were placed on transwell inserts and hMSCs were cultured in the lower chamber on tissue culture plastic. The influence of the scaffold extracts containing various Ca^{2+} ,

Mg²⁺, Sr²⁺, and PO₄³⁻ concentrations on the proliferation and osteogenic differentiation of hMSCs in the absence of physical contact with dense cationic substituted β -TCP pellets was also studied.

2.9. Transforming Growth Factor- β and Bone Morphogenic Protein PCR Array

After 21 days of culture in osteogenic media directly on scaffolds prepared with varying Mg²⁺ and Sr²⁺ content, the RNA was collected using a NucleoSpin RNA II kit (Macherey Nagel, Bethlehem, Pa) according to the manufacturer's protocol. The RNA concentration was determined by measuring the absorbance at 260 and 280 nm, respectively, using a BioRad SmartSpec spectrophotometer. Reverse transcription was performed using the RT² First Strand Kit (Qiagen, Germantown, MD). cDNA was then combined with SYBR Green (RT² SYBR Green qPCR Mastermix, Qiagen) and added to 96 well plates preloaded with primers relevant to TGF- β and BMP signaling (TGF- β /BMP Signaling Pathway PCR Array, Qiagen). The markers with cycle threshold (C_t) values of less than 30 were reported. The data collected was analyzed using the software provided by the PCR Array manufacturer.

2.10. Statistical Analysis

All experiments were performed with a minimum of three repeats for each condition. The mean and standard error were reported. One way analysis of variance (ANOVA) was used to determine the statistical significance. The Tukey-Kramer test was used for all pairwise comparisons. A p value < 0.05 was considered significant.

3. Results and Discussion

3.1. Characterization of Mg and Mg-Sr Substituted β -TCP

3.1.1. Phase Composition

X-ray diffraction was used to determine the crystallinity and phase composition of powders precipitated with varying Mg^{2+} and Sr^{2+} concentrations after refluxing (**Figure 1**). The patterns observed for all the compositions closely resembled that of β -TCP (JCPDS 09-0169). A slight peak shift to lower 2θ values was observed for samples prepared with increased Sr^{2+} concentrations in comparison to β -TCP (**Figure 1 i**) and **ii**). The observed peak shift is likely due to an increase in lattice parameter as a result of the ionic substitution of Ca^{2+} (99 pm) by the larger Sr^{2+} ions (113 pm) [15]. An increase in full width half maxima (FWHM) was also observed upon increasing Mg^{2+} concentration and reducing Sr^{2+} concentration.

Previous work has shown that the maximum amount of Mg^{2+} that can be substituted into the β -TCP crystal structure is roughly 14 mol. % [7, 18]. This is primarily due to the large difference in ionic radii between the Mg^{2+} and Ca^{2+} ions [8, 19]. In contrast, although larger than Ca^{2+} , Sr^{2+} is known to have an increased solubility in β -TCP due to less of a discrepancy in ionic radii with Ca^{2+} . Previous reports have found that up to 80 atomic % Sr^{2+} can be substituted for Ca^{2+} in the β -TCP structure thereby resulting in a linear enlargement of the unit cell [20, 21]. As a result of the increase in FWHM, the presence of amorphous phases could not be confirmed for compositions prepared with increased Mg^{2+} concentrations. However, for all the conditions studied, β -TCP was the only crystalline phase detected.

3.1.2. Elemental Composition

Elemental analysis was performed on the refluxed powders using ICP to determine the mol. % of Mg^{2+} and Sr^{2+} retained after precipitating, refluxing, and washing steps (**Table 2**). For all of the conditions synthesized with Sr^{2+} , the mol. % of Sr^{2+} measured after refluxing was similar to the mol. % of Sr^{2+} used in the initial precipitation step. However, the mol. % of Mg^{2+} measured

using ICP was much lower than the mol. % of Mg^{2+} used during synthesis for all conditions. Interestingly, although β -TCP was the only crystalline phase detected using X-ray diffraction (**Figure 1**), the mol. % of Mg^{2+} measured in some cases exceeded the maximum amount of Mg^{2+} that can be substituted for Ca^{2+} in the β -TCP crystal structure [7, 18]. In addition, the (Ca+Mg+Sr)/P ratio measured using ICP was much lower than 1.5, corresponding to that of β -TCP, for all the conditions studied.

3.1.3. Thermal Stability

The presence of excess Mg^{2+} and the lower than expected (Ca+Mg+Sr)/P ratio suggests that in addition to cationic substituted β -TCP, amorphous phases may also be formed similar to our earlier work on Mg substituted β -TCP [17]. Therefore, thermal analysis was performed on the refluxed powders to further study the phase composition and thermal stability (**Figure 2**). A weight loss was observed for samples prepared with 50% Mg and 35-15% Mg-Sr while heating to 1000°C in air. A greater than 10% weight loss was observed for the samples prepared with 50% Mg while those prepared with 35-15% Mg-Sr experienced a near 5% weight loss. In both cases, an endothermic peak near 150°C was also observed. Similar to the observed weight loss, samples prepared with 50% Mg had a more intense endothermic peak in comparison to those prepared with 35-15% Mg-Sr. The endothermic peak near 150°C is believed to correspond to the evaporation of moisture adsorbed to the surface of powder samples. The observed change in weight is also believed to be due to the evaporation of the adsorbed moisture since the majority of weight loss occurred at temperatures below 200°C for both compositions shown.

At elevated temperatures, a sharp exothermic peak was observed between 700-750°C in the DSC curves collected from samples prepared with 50% Mg and 35-15% Mg-Sr. Similar to the trends

observed in weight loss and the intensity of the endothermic peak due to the evaporation of moisture, the intensity of the exothermic peak observed was much greater for samples prepared with 50% Mg. This exothermic peak is believed to correspond to the crystallization of an amorphous phase which was not previously detected using X-ray diffraction. The increased peak intensity for the samples prepared with 50% Mg also suggests that increased amorphous content which may be formed in comparison to samples prepared with 35-15% Mg-Sr similar to the observation in the Mg substituted β -TCP[17]. Further analysis using X-ray diffraction confirmed the formation of pyrophosphates after heat treating the refluxed powders to temperatures greater than 750°C (data not shown). Unconverted DCPD is known to form calcium pyrophosphate upon heat treatment to elevated temperatures [22, 23]. However, after heat treatment to 600°C, β -TCP remained the only crystalline phase detected (**Figure 3**).

3.1.4. Particle Size and Density

Thermal analysis confirmed that in addition to poorly crystalline cationic substituted β -TCP, a secondary amorphous phase was formed for all of the conditions described in **Table 1**. Therefore, it can be construed that the presence of Sr^{2+} in addition to Mg^{2+} did not result in the formation for single phase cationic substituted β -TCP. High resolution transmission electron microscopy (HRTEM) was used to further study the crystallinity and to observe the particle size after heat treatment to 600°C (**Figure 4**). The representative low magnification image shown in **Figure 4 a)** of samples prepared using 35-15% Mg-Sr illustrates the formation of 40-50 nm particles after heat treatment. A higher magnification image with a Fast Fourier Transform diffraction pattern (FFT-DP) inset collected from region marked 'x' indicates the formation of primarily crystalline material (**Figure 4 b)**). **Figure 4 c)** illustrates a similar representative low magnification image of samples prepared with 50% Mg. A slight increase in particle size after

heat treatment in comparison to samples prepared with 35-15% Mg-Sr was observed. In contrast to samples prepared with 35-15% Mg-Sr, high magnification images of samples prepared using 50% Mg also indicated the formation of both crystalline and amorphous materials (**Figure 4 d** and the FFT-DP insets collected from regions marked as 'x' and 'A', respectively)). This data confirms the formation of increased amorphous content for samples prepared with 50% Mg and suggests that these samples may have been sintered to a higher degree after heat treatment to 600°C.

BET and helium pycnometry were used to further study the influence of composition on particle size and density after precipitating, refluxing, and heat treatment (**Table 3**). After precipitating, the samples prepared with increased Sr^{2+} concentrations had increased surface area. Upon refluxing, surface area further increased at least five-fold for all conditions. Samples prepared with 50% Mg however, had the largest surface area after refluxing. In contrast to the as-prepared state, upon refluxing, the increase in surface area was observed to be lower with increasing Sr^{2+} content. In all cases however, as expected, surface area decreased after heat treatment to 600°C due to an increase in the particle size, as expected. Similar to HRTEM images, BET results also indicate an increased particle size for samples prepared with 50% Mg in comparison to 35-15% Mg-Sr after sintering. The true density after heat treatment to 600°C was also measured. Increasing Sr^{2+} concentration resulted in an increase in true density while the opposite trend was observed upon increasing Mg^{2+} concentration. The reduced density observed upon increasing Mg^{2+} concentration may be due to a reduction in mass and lattice parameters as a result of the smaller Mg^{2+} ions substituting for the larger Ca^{2+} ions in the β -TCP crystal lattice or the formation of increased amounts of an amorphous phase, previously confirmed using DSC and HRTEM (**Figures 2 and 4**).

From this experimental data it can be concluded that biphasic mixtures of cationic substituted β -TCP and amorphous calcium phosphates were formed for all conditions similar to our earlier reports on Mg substituted β -TCP[17]. The majority of the Sr^{2+} added during synthesis was retained in the product formed after refluxing, while large amounts of Mg^{2+} were lost. X-ray diffraction indicated that β -TCP remained the only crystalline phase formed after heat treatment at temperatures of up to 600°C. The differences in composition were also observed to vary the particle size and the degree of sintering. Due to the varying crystallinity and particle size, the surface properties and rate at which Ca^{2+} , Mg^{2+} , Sr^{2+} , and PO_4^{3-} ions are released due to dissolution from the cationic substituted β -TCP will vary upon exposure to physiological solutions [24, 25] as reported and explained in the following sections. Interestingly both surface properties and ionic concentrations are known to influence the cell proliferation and differentiation [26, 27]. Therefore, by varying the composition during the synthesis, β -TCP with tailored properties for improved cytocompatibility can be synthesized as demonstrated in this work.

3.2. In-Vitro Cytocompatibility

3.2.1. Cell Proliferation

To evaluate the cytocompatibility of cation substituted β -TCP, the powders formed after heat refluxing were uni-axially pressed and heat treated to 600°C in air forming dense pellets of crystalline cation substituted β -TCP and amorphous cation substituted calcium phosphate. The heat treated scaffolds were rinsed with ethanol and dried under ultraviolet light prior to seeding human mesenchymal stem cells (hMSCs) cells directly on their surfaces. hMSC proliferation on Mg^{2+} and Sr^{2+} containing scaffolds in comparison to commercially available β -TCP was assessed after 3 and 9 days using the MTT assay (**Figure 5 a**)). After 3 days of culture, the scaffolds

prepared with 35-15% Mg-Sr supported increased hMSC proliferation in comparison to other cationic substituted β -TCPs. In addition, it should be noted that a similar level of proliferation was observed in comparison to commercially available β -TCP. However, following 9 days similar levels of proliferation were obtained for all the conditions in comparison to β -TCP.

Live/dead staining as well as SEM imaging were used to observe cell morphology after 3 days of culture in growth media (**Figure 5 b**)). hMSCs cultured on the pellets prepared with 35-15% Mg-Sr had a similar live cell density in comparison to hMSCs cultured on commercially available β -TCP (**Figure 5 b**) i) and ii)). However, a slightly increased number of dead cells were also observed on scaffolds prepared using 35-15% Mg-Sr in comparison to those cultured on β -TCP. The samples were then fixed using 2.5% glutaraldehyde and were subjected to alcohol dehydration prior to imaging using SEM (**Figure 5 b**) iii) and iv)). In both cases, hMSC morphology was observed to be broad rather than elongated and the cells were also observed to be evenly dispersed throughout the surface of the substrate.

The results obtained thus suggest that at earlier time points the increased Sr^{2+} and reduced Mg^{2+} content in samples prepared with 35-15% Mg-Sr β -TCP supports increased proliferation in comparison to all other conditions. In previous work studying Sr^{2+} substituted CaP cements, a similar level of hMSC proliferation was observed for cells cultured in growth media without osteogenic supplementation containing no Sr^{2+} and media containing up to 1 mM Sr^{2+} [27]. With respect to Mg^{2+} , the addition of up to 10 mM MgSO_4 resulted in a similar level of human bone marrow stromal cell (hBMSC) proliferation in both growth media and osteogenic media [11]. Therefore, it can be construed that hMSCs are capable of tolerating much greater Mg^{2+} rather than Sr^{2+} concentrations. However, the concentration of these ions in culture media is yet to be determined. Nonetheless, after longer periods of culture as shown by the results presented herein,

the scaffolds prepared with both increased Mg^{2+} and Sr^{2+} concentrations were at least equally capable of supporting hMSC proliferation in comparison to commercially available β -TCP.

3.2.2. Ionic Concentrations in Culture Media

To evaluate the role of ionic concentrations on cell proliferation, the concentrations of Sr^{2+} , Mg^{2+} , Ca^{2+} , and PO_4^{3-} in culture media were measured throughout the experiment using ICP (**Figure 6**). The culture media was collected on the days it was changed and diluted in a pH 7.4 tris-HCl buffer solution. Known standards were similarly diluted and used to generate a standard curve. The Ca^{2+} concentration measured from the β -TCP scaffolds was initially increased in comparison to all other groups and remained relatively constant at approximately 1.2 mM throughout the 9 day period (**Figure 6 a**). The Ca^{2+} concentration for all other groups gradually increased throughout the proliferation experiment and was comparable to that of β -TCP after approximately 7 days. The measured basal concentration of Ca^{2+} in the culture media was 1.7 mM. This data suggests that a greater amount of mineral from the media was therefore deposited on the cation substituted β -TCP scaffolds at earlier time points in comparison to β -TCP. The increased surface modification at earlier time points may explain the reduced proliferation observed for most of the cation substituted β -TCP conditions (**Figure 5 a**).

Sr^{2+} concentration was observed to constantly decrease throughout the experiment (**Figure 6 b**). Scaffolds prepared with increased amounts of Sr^{2+} also released increased amounts of Sr^{2+} into the surrounding culture media. Based on previous reports, it can be concluded that the amount of Sr^{2+} released into the culture is non-toxic to hMSCs [27]. Similar trends in the ionic concentrations of both Mg^{2+} and PO_4^{3-} were observed throughout the experiment (**Figure 6 c** and d)). The basal concentrations of Mg^{2+} and PO_4^{3-} in culture media were measured to be 0.8

and 0.9 mM, respectively. Increased Mg^{2+} and PO_4^{3-} concentrations were measured for samples prepared with increased Mg^{2+} concentrations. This is most likely due to the formation of increased amounts of a highly soluble Mg^{2+} rich amorphous calcium phosphate phase upon increasing the Mg^{2+} concentration during synthesis, previously confirmed using thermal analysis (**Figure 2**).

It can thus be concluded that the concentrations of the ions released into the culture media are non-toxic to hMSCs. However, their role in supporting the differentiation of hMSCs towards mature osteoblasts, or bone forming cells, is yet to be determined. In previous work, Sr^{2+} concentrations between 0.01 and 0.1 mM were found to support increased hMSC alkaline phosphatase expression [27]. Concentrations of Mg^{2+} between 5 and 10 mM were found to support the increased formation of mineral in comparison to hMSCs cultured in basal concentrations of Mg^{2+} [11]. In addition to the increased Sr^{2+} and Mg^{2+} concentrations compared to β -TCP, increased PO_4^{3-} concentrations were also detected. Recent work studying the role of PO_4^{3-} ions on osteogenic differentiation found that the growth media containing 5 mM PO_4^{3-} and no osteogenic supplementation was able to support comparable levels of OCN, a mature osteoblast marker, expression in comparison to hMSCs cultured in growth media containing osteogenic supplementation [28]. Therefore, it can be concluded that based on the reported literature the amounts of these ions released into the culture media are within the range required to support enhanced osteogenic differentiation.

3.2.3. Osteogenic Protein Expression

After demonstrating that CaP scaffolds prepared with varying concentrations of Mg^{2+} and Sr^{2+} were capable of supporting hMSC proliferation and showed minimal cytotoxicity in comparison

to commercially available β -TCP, their capability to also support the differentiation of hMSCs towards an osteogenic lineage was studied. hMSCs were cultured for up to 7 days in growth media, allowing them to become near fully confluent. Osteogenic differentiation was then induced by culturing in media supplemented with β -glycerophosphate, ascorbic acid, and dexamethasone for up to 21 days. Alkaline phosphatase (ALP) activity and the intracellular expression of osteogenic proteins, such as osteopontin (OPN) and osteoprotegrin (OPG) were used to study the hMSC differentiation towards mature osteoblasts.

ALP is an enzyme secreted during osteogenic differentiation which cleaves PO_4^{3-} from the surrounding molecules providing the increased PO_4^{3-} concentrations necessary for osteoblast progenitor cells to mineralize their extracellular matrix [29]. ALP activity was determined after 14 and 21 days of culture in osteogenic media and was normalized with respect to the total protein concentration (**Figure 7**). At both time points, hMSCs cultured on scaffolds prepared with 50% Mg and no Sr^{2+} expressed increased ALP activity in comparison to cells cultured on all Mg-Sr containing compositions. Between the two time points, a slight decrease in ALP activity was observed for most conditions. Interestingly, a near three-fold increase in ALP activity was observed for hMSCs cultured on samples prepared with 50% Mg in comparison to those cultured on commercially available β -TCP at both the time points studied.

The intracellular expression of two osteogenic proteins, OPN and OPG, were also analyzed after 21 days of culture in osteogenic media using a magnetic bead kit. OPN is a secreted protein which regulates attachment to extracellular matrix thus modulating the resorption of mineralized tissues [30]. OPN is considered to be a potent inhibitor of mineralization and is expressed at the initial stages of differentiation [31]. In addition to its role in bone remodeling, elevated OPN levels have been observed during wound healing and inflammation [32]. OPG is a RANKL

antagonist secreted by osteoblasts and other cell types which has been shown to inhibit the formation of osteoclasts through the RANK-RANKL-OPG signaling pathway [33]. OPG deficient knockout mice have been shown to develop osteoporosis due an increased number of osteoclasts [34]. The expression of OPN and OPG in hMSCs cultured on scaffolds prepared using β -TCP and the two conditions synthesized with the maximum concentrations of Mg^{2+} and Sr^{2+} were reported (**Figure 8 a**)).

After 21 days, similar to ALP activity, the OPN and OPG expression of hMSCs cultured on samples prepared with 50% Mg was elevated in comparison to all other groups (**Figure 8 a**)). In summary, samples prepared with 50% Mg and no Sr supported increased levels of osteogenic markers in comparison to all other Mg and Mg-Sr containing compositions as well as commercially available β -TCP. Interestingly, although increased Sr^{2+} concentrations have been shown to enhance osteogenic differentiation, in the current study the incorporation of Sr^{2+} did not appear to facilitate increased hMSC differentiation in comparison to samples prepared with 50% Mg. It has been previously shown that increasing the Mg^{2+} concentration in the synthesis results in the formation of increased amorphous content [9]. While amorphous CaP phases are generally considered to be more reactive and more soluble than their crystalline counterparts, the influence of the amorphous phase on the physical and chemical cues provided by the scaffold to support osteogenic differentiation remains largely unknown.

3.2.4. Indirect Cell Culture

In order to study the role of the physical and chemical cues provided by cation substituted β -TCP scaffolds prepared with varying Mg^{2+} and Sr^{2+} concentrations on supporting hMSC osteogenic differentiation independently, transwell membrane inserts were also used. Accordingly, hMSCs

were cultured on the tissue culture plastic for the duration of the experiment while cation substituted β -TCP scaffolds were suspended on permeable membranes supports. The use of transwell membranes enabled hMSCs to be exposed to dissolved Ca^{2+} , Mg^{2+} , Sr^{2+} , and PO_4^{3-} concentration profiles generated from the sintered substituted CaP substrates identical to the previous experiments while eliminating the physical contact with sintered dense CaP substrates. The intracellular expression of OPG and OPN were once again used to study the hMSC differentiation after 21 days (**Figure 8 b**). In the absence of any physical contact with the sintered scaffold surfaces, similar trends in the expression of OPN and OPG were observed. Therefore, this data suggests that the release of Mg^{2+} , Sr^{2+} , and PO_4^{3-} ions from these scaffolds plays a more influential role in providing the signaling necessary to support differentiation rather than the exposure of hMSCs directly to the scaffold surface. The result therefore invalidates the role of surface composition and microstructural effects contributing to inducing osteogenic differentiation of hMSCs.

3.2.5. TGF- β and BMP Signaling PCR Array

In the data reported, scaffolds prepared with Mg^{2+} and no Sr^{2+} supported increased differentiation in comparison to all other conditions, including commercially available β -TCP. The capability of these scaffolds to support increased differentiation in comparison to β -TCP was determined to be due to the release of sufficient amounts of Mg^{2+} and PO_4^{3-} ions resulting in concentrations within the desired range for both ions previously reported to stimulate hMSC differentiation. To study the influence of Mg^{2+} and PO_4^{3-} on the mechanisms involved in regulating osteogenic differentiation, a TGF- β and bone morphogenetic protein (BMP) signaling PCR array was used. All three forms of TGF- β are found in bone tissue and both osteoblasts and osteoclasts are capable of synthesizing TGF- β [35]. In contrast to TGF- β s, BMPs are capable of

stimulating ectopic bone formation and are expressed in multiple mineralized tissues at various stages throughout development of bone tissue [36]. Through the activation of TGF- β and BMP receptors, the regulatory SMADs are phosphorylated and together with SMAD4, a co-SMAD, the complex formed then translocates to the nucleus [37]. SMAD4 serves to stabilize the SMAD complex and interacts with several critical transcription factors. In addition to SMAD dependent TGF- β and BMP signaling, TGF- β and BMP proteins can also enhance differentiation through SMAD independent signaling using the mitogen activated protein kinase (MAPK) pathway [38, 39].

Based on the above knowledge, to study the influence of the ions on osteogenic differentiation, RNA was collected from hMSCs cultured directly on the scaffolds prepared with β -TCP and 50% Mg β -TCP after 21 days in osteogenic media. The gene expression of the cells cultured on the scaffolds prepared with 50% Mg β -TCP was then normalized with respect to those cultured on scaffolds prepared with β -TCP alone. Therefore, the data was reported as fold change with respect to β -TCP. The markers with C_t values of less than 30 for both β -TCP and 50% Mg β -TCP were reported. In **Figure 9 a)** the gene expression of several BMPs and BMP receptors is illustrated. The gene expression of two of the three BMP proteins was upregulated by greater than 1.5 fold in comparison to hMSCs cultured on β -TCP while all three of the BMP receptors illustrated were upregulated by greater than 1.5 fold. The gene expression of TGF- β and its receptors is reported in **Figure 9 b)**. None of the TGF- β family proteins had a gene expression greater than 1.5 fold in comparison to β -TCP while the gene expression for two of the three receptors exceeded a 1.5 fold increase. The gene expression of the SMAD proteins is illustrated in **Figure 9 c)**. SMAD4, the co-SMAD, which is critical for SMAD dependent TGF- β and BMP signaling, was upregulated by greater than 1.5 fold. In **Figure 9 d)** the gene expression of the

ECM components and transcription factors included in the PCR array with C_t values of less than 30 and either up or downregulated by greater than 50% are illustrated. The upregulation of several key ECM components, such as COL1A1 and OCN, once again suggests that hMSCs cultured on scaffolds prepared with 50% Mg β -TCP undergo increased levels of differentiation in comparison to those cultured on β -TCP.

The greater than 50% increase in the gene expression of both TGF- β and BMP receptors and near two fold upregulation of SMAD4 suggests that hMSCs cultured on scaffolds prepared with 50% Mg β -TCP undergo osteogenic differentiation through SMAD dependent TGF- β and BMP signaling. Commercially available β -TCP, which is known for its excellent biocompatibility and has been demonstrated to possess osteoinductive properties, was used as the control [40]. Therefore, very few markers were observed to be greater than two fold upregulated in comparison to β -TCP for the 50%Mg substituted β -TCP. A further analysis of the interaction network between the markers which were upregulated by greater than two fold in comparison to β -TCP was performed using the Ingenuity Pathway Analysis (**Figure 10**).

From the data illustrated, several interactions were observed between the markers upregulated by greater than two fold in comparison to β -TCP and both CREB and ERK1/2. Interestingly, in previous work studying the differentiation of human adipose derived stem cells in the presence of the extracts of a Ca, Mg, and Si containing ceramic, it was observed that ERK signaling could facilitate the enhanced differentiation observed [41]. Similar to the current work, this effect was also observed to be concentration dependent. In the current study however, the increased Mg^{2+} and PO_4^{3-} ion concentrations stimulated increased levels of osteogenic differentiation. With respect to PO_4^{3-} ions, the mechanisms by which the cellular uptake of PO_4^{3-} ions are regulated and their roles in supporting osteogenic differentiation have only recently been reported [28].

However, the role of Mg^{2+} in regulating this process in the presence of PO_4^{3-} ions is yet to be determined. Therefore, further work is required to evaluate the role of the individual ions released in the current system on supporting osteogenic differentiation and to determine which of the two is more influential.

4. Conclusions

Biphasic mixtures of Mg^{2+} and Sr^{2+} co-substituted β -TCP and an amorphous phase were formed using a low temperature chemical synthesis approach. All the compositions reported support similar levels of hMSC proliferation in comparison to commercially available β -TCP. Significantly increased levels of ALP, OPN, and OPG were measured from hMSCs cultured directly on samples prepared with 50% Mg and no Sr^{2+} in comparison to all other groups, including those prepared with increased Sr^{2+} concentrations. In the absence of physical contact with scaffold surfaces while maintaining similar ionic concentrations, similar trends in differentiation were also observed. Therefore, it was concluded that the release of increased amounts of Mg^{2+} and PO_4^{3-} ions supported increased hMSC osteogenic differentiation rather than the materials surface microstructure and grain size or grain boundary effects. The influence of these ions on TGF- β and BMP signaling was also evaluated using a TGF- β and BMP PCR array. From this data it was concluded that the scaffolds prepared with 50% Mg β -TCP may support increased differentiation in comparison to commercially available β -TCP through the SMAD dependent TGF- β and BMP signaling pathway. More concrete research is clearly warranted although the influence of Mg^{2+} and PO_4^{3-} in the synthesized substituted TCP scaffold is clearly demonstrated.

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6. References

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Table Captions

Table 1. The amounts by weight of Ca, Mg, and Sr containing precursors used in the precipitation of Mg and Mg-Sr doped $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$.

Table 2. Elemental analysis of as prepared Mg and Mg-Sr containing β -TCP.

Table 3. The surface area and true density of Mg and Mg-Sr containing β -TCP after precipitating, refluxing, and heat treatment to 600°C .

Figure Captions

Figure 1. X-ray diffraction data collected from as prepared i) β -TCP (JCPDS 09-0169), ii) 35-15%, iii) 40-10%, iv) 45-5%, and 50% Mg and Mg-Sr β -TCP.

Figure 2. DTA-TGA data collected from samples prepared with 50% Mg and 35-15% Mg-Sr β -TCP while heating in air at a rate of $10^\circ\text{C min}^{-1}$ up to 1000°C .

Figure 3. X-ray diffraction data collected from i) β -TCP (JCPDS 09-0169), ii) 35-15%, iii) 40-10%, iv) 45-5%, and 50% Mg and Mg-Sr β -TCP heat treated to 600°C .

Figure 4. HRTEM images 35-15% Mg-Sr β -TCP after heat treatment to 600°C at a magnification a) 6,000X and b) 40,000X (x represents the area from which FFT DP was collected and 50% Mg β -TCP after heat treatment to 600°C at a magnification of c) 6,000X and d) 40,000X (x represents the crystalline and A represents the amorphous region from which FFT DPs were collected).

Figure 5. a) Cell viability assessed using the MTT assay and b) live/dead staining of hMSCs seeded on scaffolds prepared with i) 35-15% Mg-Sr β -TCP and ii) β -TCP after 3 days and SEM images of hMSCs after 3 days on samples prepared using iii) 35-15% Mg-Sr β -TCP and iv) β -TCP.

Figure 6. The ionic concentrations of a) Ca^{2+} , b) Sr^{2+} , c) Mg^{2+} , and d) PO_4^{3-} in culture media throughout the experiment.

Figure 7. ALP activity measured from hMSCs after culturing for 14 and 21 days in osteogenic media on β -TCP prepared with various amounts of Mg and Mg-Sr. A p value of < 0.05 was considered significant.

Figure 8. The intracellular expression of osteopontin (OPN) and osteoprotegerin (OPG) of hMSCs cultured for 21 days in osteogenic media a) directly and b) indirectly on β -TCP prepared with various amounts of Mg and Mg-Sr. A p value of < 0.05 was considered significant.

Figure 9. The gene expression of a) BMP and its receptors b) TGF- β and its receptors, c) SMAD proteins, and d) the ECM components and transcription factors either up or downregulated by

more than 50% cultured on 50% Mg β -TCP in comparison to commercially available β -TCP. The markers with C_t values of less than 30 were reported.

Figure 10. The interaction of genes in the Cellular Development network analyzed using Ingenuity Pathway Analysis. The red filled shapes correspond to the up-regulated genes while the blank ones were not found in the input list. The genes with a fold change greater than or equal to 2 in comparison to β -TCP were included in the analysis.

Mg-Sr	CaCl ₂ •2H ₂ O (g)	MgCl ₂ •6H ₂ O (g)	SrCl ₂ •6H ₂ O (g)
35-15%	3.68	3.56	2.00
40-10%	3.68	4.07	1.33
45-5%	3.68	4.57	0.67
50%	3.68	5.08	-

Table 1. The amounts by weight of Ca, Mg, and Sr containing precursors used in the precipitation of Mg and Mg-Sr doped CaHPO₄•2H₂O.

	Sr/(Ca+Mg+Sr)	Mg/(Ca+Mg+Sr)	(Ca+Mg+Sr)/P
35-15%	19.0 ± 0.1%	12.9 ± 0.2%	1.36 ± 0.01
40-10%	13.4 ± 0.2%	17.2 ± 0.2%	1.34 ± 0.01
45-5%	7.0 ± 0.3%	22.4 ± 2.4%	1.33 ± 0.01
50%	-	27.9 ± 1.3%	1.33 ± 0.02

Table 2. The elemental analysis of Mg and Mg-Sr containing β-TCP. The data reported was collected from 3 independent batches synthesized for each composition.

	BET Surface Area after precipitating (m ² /g)	BET Surface Area after Refluxing (m ² /g)	BET Surface Area after Heat Treatment (m ² /g)	True Density after Heat Treatment (g/cm ³)
35-15%	43.37	115.20	17.98	3.28 ± 0.01
40-10%	34.68	185.09	14.12	3.21 ± 0.02
45-5%	24.29	184.11	15.96	3.14 ± 0.01
50%	28.84	207.96	9.21	3.00 ± 0.01

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Figure 3. X-ray diffraction data collected from i) β -TCP (JCPDS 09-0169), ii) 35-15%, iii) 40-10%, iv) 45-5%, and 50% Mg and Mg-Sr β -TCP heat treated to 600°C for 4 hours.

Figure 4. HRTEM images 35-15% Mg-Sr β -TCP after heat treatment to 600°C at a magnification a) 6,000X and b) 40,000X ('x' represents the area from which FFT DP was collected) and 50% Mg β -TCP after heat treatment to 600°C at a magnification of c) 6,000X and d) 40,000X ('x' represents the crystalline and 'A' represents the amorphous region from which FFT DPs were collected).

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