Research Article

Effect of Water-Glass Coating on HA and HA-TCP Samples for MSCs Adhesion, Proliferation, and Differentiation

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Received 15 April 2016; Revised 11 June 2016; Accepted 13 June 2016

Academic Editor: Kailash C. Gupta

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Ca-P and silicon based materials have become very popular as bone tissue engineering materials. In this study, water-glass (also known as sodium silicate glass) was coated on sintered hydroxyapatite (HA) and HA-TCP (TCP stands for tricalcium phosphate) samples and subsequently heat-treated at 600°C for 2 hrs. X-rays diffraction showed the presence of β- and α-TCP phases along with HA in the HA-TCP samples. Samples without coating, with water-glass coating, and heat-treated after water-glass coating were used to observe the adhesion and proliferation response of bone marrow derived-mesenchymal stem cells (MSCs). Cell culture was carried out for 4 hrs, 1 day, and 7 days. Interestingly, all samples showed similar response for cell adhesion and proliferation up to 7-day culture but fibronectin, E-cadherin, and osteogenic differentiation related genes (osteocalcin and osteopontin) were significantly induced in heat-treated water-glass coated HA-TCP samples. A water-glass coating on Ca-P samples was not found to influence the cell proliferation response significantly but activated some extracellular matrix genes and induced osteogenic differentiation in the MSCs.

1. Introduction

Calcium-phosphate (Ca-P) based materials, in either single phase or multiphase, are well known for their bioactivity as a bone substitute [1, 2]. Hydroxyapatite (HA), tricalcium phosphate (TCP), and their different compositions are widely used Ca-P ceramics in the biomedical field [3]. On the other hand, bioactive glasses have been also used in various forms [4]. Herein, presence of silicon (Si) ions was found to influence the bioactivity of other materials in various ways [5]. The combination of Si with Ca and P ions has reported to enhance the apatite formation and affect some cellular functions [6, 7].

Porter et al. found the different types on apatite formation on the HA and Si substituted-HA (Si-HA) granules in a mouse model. However, Si-HA showed rapid bone remodeling than the pure HA [8]. Obata et al. observed the better effect of silica layer on the in vitro proliferation of the osteoblast-like cells (MC3T3-E1) and apatite formation on the titanium surface as compared to hydroxyapatite [9]. Padilla et al. investigated better apatite deposition and proliferation of human osteoblastic-like cell line in the mixture of HA and sol gel glass than the HA alone [10]. Lopes et al. found better bone and implant contact in tibia of rabbits in the case of glass-reinforced-HA as compared to monolithic HA [11]. There are several more studies that showed the better biological response of glass containing Ca-P samples as compared to Ca-P alone [12]. Our research group have also reported the improvement in apatite formation by a water-glass coating on porous Ca-P scaffolds [13].

Human bone marrow-derived-mesenchymal stem cells (MSCs) are the primary cultured cells which have self-replication ability and can differentiate in other types of cells according to surrounding conditions [14, 15]. In addition, extra cellular matrix (ECM) and differentiation related several important genes are marked in MSCs [16, 17]. MSCs are being used in the orthopedic applications because of their differentiation property on the Ca-P ceramic substrates along the osteogenic pathway [18, 19]. Therefore, MSCs are selected as the suitable cellular model for the study of adhesion, proliferation, and differentiation on the ceramic samples.

The aim of current study is to observe the effect of water-glass (WG) coating on Ca-P samples in terms of cell
Table 1: Sample's details and ID.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Sample details</th>
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<tbody>
<tr>
<td>1</td>
<td>HA Pure sintered HA</td>
</tr>
<tr>
<td>2</td>
<td>HA+WG Sintered HA coated with water glass</td>
</tr>
<tr>
<td>3</td>
<td>HA+WG+HT Heat treated water glass coated sintered HA</td>
</tr>
<tr>
<td>4</td>
<td>BCP Sintered HA-TCP (β-TCP + α-TCP) composite</td>
</tr>
<tr>
<td>5</td>
<td>BCP+WG Water glass coated sintered TCP</td>
</tr>
<tr>
<td>6</td>
<td>BCP+WG+HT Heat treated water glass coated sintered TCP</td>
</tr>
</tbody>
</table>

adhesion, proliferation, and differentiation. In this study, water-glass (WG) which is known as sodium-silicate glass was coated on HA and BCP (which is abbreviation used for HA-TCP sample) samples followed by heat treatment. This is a comparative study presenting the different cellular functions influenced by differently treated substrate surfaces.

2. Materials and Methods

Commercially available hydroxyapatite (HA), dicalcium phosphate dehydrate (DCPD), and Ca(OH)₂ were used as raw materials to prepare samples. In order to fabricate the HA-TCP (BCP) samples, 100 grams DCPD, 100 grams HA, and 21 grams Ca(OH)₂ were mixed by wet ball milling in ethanol medium for 1 day. Further, mixed powder was dried in oven at 80°C temperature. Green pallets of 15 mm diameter of HA and above mixture were prepared at 45 MPa pressure and all samples were sintered at 1250°C temperature for 2 hrs with the 5°C/min heating rate [20].

All sintered samples of HA and HA-TCP were cleaned in ethanol by ultrasonication and then dried and coated with 10 vol% water-glass by spin coater at 200 rpm. One set of water-glass coated samples was heat-treated at 600°C for 2 hrs. Description of samples used in current study is written in Table 1. This study is based on comparisons of cell behavior on six different sets of samples; (1) HA, (2) HA with WG coating, (3) HA with WG coating and heat treatment, (4) BCP, (5) BCP with WG coating, and (6) BCP with WG coating and heat treatment.

3. Characterizations

Phase analysis of all the samples was conducted by X-ray diffractometer (XRD, Rigaku) on 40 KV and 30 mA current with 1 deg/min scan rate in the 2-theta range of 25°-60° and recorded XRD patterns were analyzed by using the XPert HighScore Plus Software. In order to quantify the phase composition from the XRD patterns, Rietveld mode associated with XPert HighScore Plus software was used.

4. Cell Culture

We have previously described that human bone marrow derived-mesenchymal stem cells were isolated from the aspirated iliac crest of patients undergoing nonemergency orthopedic surgery through an Internal Review Board approved protocol at Yeungnam University Hospital using a previous method [20, 21]. The experimental cells were cultured in the culture media that is DMEM medium supplemented with 10% FBS (FBS; Gibco-BRL, Rockville, MD) and 1% antibiotic-antimycotic (Gibco) at 37°C with 5% CO₂. In this study, the second passages cells were used for the experiments. For the cell culture, all samples were sterilized by ethylene oxide gas. Prior to cell incubation, all samples were placed in the culture media for 4 hrs for media adaptation and then MSCs were seeded in the fresh culture media.

4.1. Immunofluorescence Analysis. In order to observe the adhesion and proliferation of MSCs, all samples were seeded with a cell density of 3 × 10⁵/cm² and incubated for 4 hrs, 1 day, and 7 days at 37°C in biological incubator. To image the cells under fluorescence microscopy, all incubated samples were washed three times with 1x PBS followed by fixation with 3.7% formaldehyde for 20 min. Further fixed cells were washed and permeabilized with 0.1% Triton X-100 for 10 min and background blocking was done by incubation for 30 min in a 0.1% bovine serum albumin (BSA) solution. Subsequently, all samples were stained with a 1:40 dilution fluorescein-conjugated phalloidin (Invitrogen, Carlsbad, CA, 200 U/mL) solution for 1 hr for the staining of actin fibers. After that, nuclei of the cells were stained by using a 100 nM 4′-diamidino-2-phenylindole (DAPI, Sigma) solution. Finally, washed samples were mounted using anti-fluorescence-fade medium (1,4-Diazabicyclo[2.2.2]octane, DABCO, Sigma) and observed under fluorescence microscopy (Leica DM6000B, Germany). The detailed protocol of cell staining has been also described in our previous work [20]. For the quantification of the cell adhesion and proliferation on the different samples, minimum of five images of the nuclei of the cells were taken at different areas of the samples and the number of cells/unit area for each sample was plotted in Figure 3.

4.2. Reverse Transcription Polymerase Chain Reaction (RT-PCR). As previously we described that to measure the expression levels of the ECM genes, the total RNA was isolated from human bone marrow derived-mesenchymal stem cells grown on the substrates used in current study, in 100 μL of TRIzol® Reagent (Ambion by Life Technologies, Gaithersburg, MD) reagent [20]. 20 μL of chloroform was mixed with the sample and the solution was incubated for 2 min at room temperature. The samples were then centrifuged at 12,000 rpm for 15 min at 4°C. Next, 50 μL of the upper aqueous phase was carefully transferred to a fresh tube and the RNA was precipitated by mixing with 50 μL isopropyl alcohol. The solution was incubated for 1 hour at −20°C and centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was removed and the RNA pellet was washed once with 70% ethanol. The RNA pellet was air-dried and dissolved in diethylpyrocarbonate-treated water (Amresco, USA). The cDNA was synthesized using an oligo (dT) primer (Genotech, South Korea) and M-MLV reverse transcriptase (Promega, Fitchburg, WI) following the manufacturer's
Table 2: Primers and PCR conditions.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense (5’-3’)</th>
<th>Antisense (5’-3’)</th>
<th>Annealing/cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibronectin (FN)</td>
<td>AGT TCA GGG TTC CTG GAA</td>
<td>TGC CAC TGT TCT CCT ACG TG</td>
<td>56°C/25</td>
</tr>
<tr>
<td>E-cadherin (E-cad)</td>
<td>CAA GGA CAG CCT TCT TTT CG</td>
<td>TGG ACT TCA GGG TCA CTT TG</td>
<td>57°C/38</td>
</tr>
<tr>
<td>Osteocalcin (OC)</td>
<td>CCC TCA CAC TCG CCG TAT</td>
<td>TCA GCC AAC TCG TCA CAG TCC</td>
<td>59°C/30</td>
</tr>
<tr>
<td>Osteopontin (OPN)</td>
<td>CCA AGT AAG TCC AAC GAA AG</td>
<td>GTG GAT GTG TCT TCG TGT A</td>
<td>57°C/30</td>
</tr>
<tr>
<td>α-smooth muscle actin (α-SMA)</td>
<td>ACT GTG TTA TGT AGC TCT GGA C</td>
<td>ACA ATG GAA GGC CCG GCC TCT TC</td>
<td>57°C/25</td>
</tr>
<tr>
<td>GAPDH</td>
<td>AGG TCG GAG TCA ACG GAT TTG</td>
<td>GTG ATG GCA TGG ACT GTG GT</td>
<td>58°C/25</td>
</tr>
</tbody>
</table>

Figure 1: XRD analysis of the water-glass coated Ca-P samples.

Figure 2 presents the fluorescence microscopy images of MSCs, stained with DAPI and FITC-conjugated phalloidin, seeded on WG coated Ca-P samples for 4 hrs, 1 day, and 7 days. Herein, 4 hrs culture was done to observe the adhesion property of cells on these samples and all samples showed good cell adhesion. After 1 day all attached cells spread properly on the surface. Further, cells on all samples proliferated well and almost reached confluence after 7-day incubation.

Figure 3 shows the number of cells/unit area on each sample after 4 hrs, 1 day, and 7-day incubation. After culture, the attached cells were stained with DAPI, the cell nuclei were counted manually, and the number of cells/unit areas was plotted for the each sample. Average cell density of 1-day incubation is decreased as compared to 4 hrs but there is no statistically significant difference in data. The 4 hrs cell incubation shows the initial attachment of cells which is not very strong on ceramic sample. It is possible that seeding solution contains unhealthy cells as well and those were also initially attached on the sample surface but died later on. Although all the samples showed similar cell adhesion and proliferation after 7-day culture WG coated HA and BCP samples showed slightly higher but not statistically significant difference in cell density as compared to noncoated samples and coated-heat-treated samples.

To determine the interaction between the cells and the materials, extracellular matrix (ECM)-related genes expression, including fibronectin (FN) (Figure 4(a)), E-cadherin (E-cad) (Figure 4(b)), and α-smooth muscle actin (α-SMA) (Figure 4(c)), were investigated after 7-day culture. Fibronectin binds the extracellular matrix components and plays key role in adhesion dependent cell growth, proliferation, signaling event, and differentiation. FN expression was induced significantly in the cells grown on the BCP+WG+HT sample but not significantly different than the BCP+WG. On the other hand, HA, HA+WG, and HA+WG+HT showed no statistically significant difference in cell density as compared to noncoated samples and coated-heat-treated samples.

5. Results and Discussion

Figure 1 shows the XRD analysis of water-glass coated HA and BCP samples. XRD peaks of sintered HA commensurate well with the peaks of standard HA (JCS code 157481). However, water-glass coated and heat treatment of water-glass coated samples at 600°C did not reveal presence of any addition phase along with HA. The critical analysis of XRD patterns of the BCP sample showed the presence of β-TCP and α-TCP along with the HA phase. The quantification of these three phases was obtained by X’Pert HighScore operated in Rietveld mode which showed 56% HA, 25% β-TCP, and 19% α-TCP phases in the BCP samples. In addition, the effect of heat treatment of water-glass coating on BCP samples was similar to HA. All sintered HA and BCP samples are nearly fully densified.

Figure 2 presents the fluorescence microscopy images of MSCs, stained with DAPI and FITC-conjugated phalloidin, seeded on WG coated Ca-P samples for 4 hrs, 1 day, and 7 days. Herein, 4 hrs culture was done to observe the adhesion property of cells on these samples and all samples showed good cell adhesion. After 1 day all attached cells spread properly on the surface. Further, cells on all samples proliferated well and almost reached confluence after 7-day incubation.

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Figure 2: Fluorescence microscopy images of MSCs cultured on different water-glass coated Ca-P samples for 4 hrs, 1 day, and 7 days. Scale bar = 100μm.
The current study presents the effect of water-glass coating on Ca-P substrates in terms of adhesion, proliferation, and ECM- and osteogenic differentiation-related gene expression of MSCs. The cell adhesion and proliferation showed no statistical significant difference between the samples regardless of water-glass coating. On the other hand, BCP+WG+HT showed the significant ECM- and osteogenic differentiation-related gene expression (FN, E-cad, OC, and OPN) while remaining similar to other samples in case of α-SMA. In addition, all water-glass coated-BCP samples showed higher values of gene expressions than the water-glass coated-HA samples because of the faster dissolution of BCP as compared to HA, in the culture media. Therefore, it can be considered as the released Si ions (from water-glass coating) in the culture media along with Ca and P ions (from BCP samples) can activate the several genes and induce the osteogenic differentiation in the MSCs.

6. Conclusions

This study describes the effect of a water-glass coating on Ca-P substrates in terms of adhesion, proliferation, and ECM- and osteogenic differentiation-related gene expression of MSCs. The cell adhesion and proliferation showed no statistical significant difference between the samples regardless of water-glass coating. On the other hand, BCP+WG+HT showed the significant ECM- and osteogenic differentiation-related gene expression (FN, E-cad, OC, and OPN) while remaining similar to other samples in case of α-SMA. In addition, all water-glass coated-BCP samples showed higher values of gene expressions than the water-glass coated-HA samples because of the faster dissolution of BCP as compared to HA, in the culture media. Therefore, it can be considered as the released Si ions (from water-glass coating) in the culture media along with Ca and P ions (from BCP samples) can activate the several genes and induce the osteogenic differentiation in the MSCs.

Competing Interests

The authors do not have conflict of interests regarding the current work.
Figure 4: ECM-related gene expressions of MSCs when cultured on water-glass coated Ca-P samples for 7 days. The values are expressed in mean ± SE. (*) denotes a significant difference (p < 0.05) between the samples as compared to HA for n = 3.
Figure 5: Osteogenic differentiation related gene expressions of MSCs when cultured on water-glass coated Ca-P samples for 7 days. The values are expressed in mean ± SE. (*) denotes a significant difference (p < 0.05) between the samples as compared to HA for n = 3.

Acknowledgments

This study was supported by a grant from the National Research Foundation of Korea, Ministry of Education, Science and Technology, Korean Government (Grant no. NRF-2015R1D1A1A01056602) and a Yeungnam University research grant, 2015.

References


