치의학석사학위논문

Surface characterization and cellular behaviors of zirconia discs immersed in simulated body fluid

Simulated body fluid 에 침전한 지르코니아 디스크 표면분석과 세포반응 연구

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-ABSTRACT-

Surface characterization and cellular behaviors of zirconia discs immersed in simulated body fluid

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Purpose: Zirconia which is a bioinert implant material needs to promote its bioactivity via surface modification for clinical application. This study investigated the effects of HA precipitation on the zirconia surface by immersing zirconia discs in simulated body fluid (SBF), and the proliferation and differentiation of human bone marrow mesenchymal stem cells (hBMMSCs) on the SBF treated zirconia discs.

Materials and methods: Corrected SBF was made at 36.5°C with ion concentrations nearly equal to those of human blood plasma according to the Kokubo's recipe. Eighty Y_2O_3-stabilized tetragonal zirconia polycrystals (Y-TZP) discs were prepared and divided into two groups: (1) test group: SBF treated discs, (2) control group: non-treated discs. Zirconia discs were immersed in SBF for 1, 4, 7 and 14 days at 37°C with initial PH of 7.4, and the HA precipitation was certificated by scanning electron microscope (SEM). For more in-depth validation of HA formation, confocal laser scanning microscopy (CLSM), energy

dispersive X-ray spectroscopy (EDS) and thin film X-ray diffraction (TF-XRD) were conducted on the 7 day-treated discs. hBMMSCs were used for further evaluating cell proliferation, alkaline phosphatase (ALP) activity and osteoblast gene expression on the 7 day-treated zirconia discs. Data were analyzed by student *t* test at a 0.05 level of significance.

Results: The discs showed different surface morphology, and with time increasing, the amount of HA was increased gradually. At 7 days, the HA was formed and uniformly covered on the discs. There was no difference of surface roughness between two groups ($p > 0.05$). Cell proliferation was higher on the SBF treated discs ($p < 0.05$). In the beginning, there was no difference of ALP activity between two groups, but at 9 days, it exhibited higher expression on the SBF treated discs ($p < 0.05$). ALP staining was significant on the SBF treated discs ($p < 0.05$). Gene study revealed that ALP and osteocalcin (OCN) showed higher gene expression in SBF treated discs compared with non-treated discs ($p < 0.05$), on the contrary, type I collagen (COL 1), runt-related transcription factor 2 (Runx2) did not show significant differences between two groups ($p > 0.05$).

Conclusions: The data in this study demonstrated that SBF has the effective role on formation of HA on the zirconia discs. The cell attachment, proliferation and differentiation of the SBF treated zirconia discs were more superior than of the non-treated discs which mean SBF treated zirconia implant has the long term clinical value.

Key words: Zirconia disc, Simulated body fluid (SBF), Hydroxyapatite (HA), Human bone marrow mesenchymal stem cells, Cell proliferation, Osteoblast differentiation

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CONTENTS

Ⅰ. INTRODUCTION

For a relatively long time, pure titanium and titanium alloys have been employed as the best material for dental implants and their efficacy as dental implant materials have been confirmed.[1,2] Titanium surface can achieve direct bonding with bone tissues with a very thin cementum layer in between. This phenomenon was known as osseointegration which was

first introduced by Branemark et al. in 1965[3] and extensively analyzed in other researches. [4,5]

However, one of the main drawback of titanium from the esthetic point of view, is the dark gray color can shine through the thin soft tissue.[6] In case of bad soft tissue condition, the gingiva recession may severe, which leading to the cervical titanium visible. And recently, more and more reports indicated that the titanium considered as a foreign body can cause allergy reactions.[7,8] To resolve the allergy and esthetic problems caused by titanium implants, a ceramic implant was developed inevitably. Bioactive ceramics like sintered hydroxyapatite (HA) used as implant material is the remarkable success due to its bioactivity and osteoconductivity.[9] Whereas, the low strength and fracture toughness of HA ceramics limit the scope of clinical applications.[10]

Zirconia is another ceramic material with adequate biological and mechanical properties for biomedical applications.[11] But, zirconia ceramics are a bioinert material so that do not bond to living bone directly.[12] Hayashi et al. were already did the research about comparing the bone–implant interface shear strength of zirconia and HA using alumina ceramic and stainless steel as reference bioinert materials, and demonstrated that HA had the greatest shear strength among them.[13] So it was hypothesized that if the HA coated onto the zirconia surface, it can not only overcome the strength problem, but also improve the bone bonding process. In vitro, apatite deposition through soaking in simulated body fluid (SBF) has been widely used.[14,15] SBF which was a solution with ion concentrations nearly equal to those of human blood plasma was

first introduced by Kokubo et al.[16] in 1990, since then, in vivo bone bioactivity of various types of materials have been evaluated by apatite formation in SBF. Ti spontaneously deposited HA on the surface in contact with the bottom surface of the polystyrene container after 7 days of soaking in the SBF of Kokubo's recipe.[17] A previous study already published the data about cellular behaviors of SBF soaked Ti and revealed that immersion anodized Ti in modified SBF improved osteoblast responses such as cell proliferation rate and initial cell differentiation.[18] On the contrary, few researches did the extended cellular studies on SBF treated zirconia discs.

The objective of the present study was to evaluate the effects of SBF soaking on HA formation on the zirconia discs and the cytocompatibility of SBF treated zirconia discs compared with non-treated discs. In this study, we adopted zirconia discs together with SBF to examine HA forming ability of zirconia discs soaked in SBF. After obtained HA coated zirconia discs, we used human bone marrow mesenchymal stem cells (hBMMSCs) to investigate cellular proliferation, ALPase activity, and mRNA level of osteoblast-related genes on the SBF treated zirconia discs compared with non-treated zirconia discs.

Ⅱ. MATERIALS AND METHODS

1. Zirconia disc preparation

Eighty Y_2O_3-stabilized tetragonal polycrystals (Y-TZP) zirconia discs with 15 mm diameter and 1 mm thickness were fabricated by cold isostatic press at 200 MPa and then sintered for 2 hours at 1650°C in air. All discs were polished and sandblasted with 50 μm alumina (Al_2O_3) for 1 min to ensure that the discs had similar roughness. Then, the discs were divided into two groups: (1) test group: SBF treated discs, (2) control group: non-treated discs. After surface treatment, the discs were washed in distilled water and alcohol gently, and autoclaved at 120°C for 30 min.

2. Corrected simulated body fluid (SBF)preparation and immersing the discs in SBF

Totally forty discs were soaked at 37°C in corrected SBF of kokubo's[19] recipe with ion concentrations nearly equal to those of human blood plasma (Na^+ 142.0, K^+ 5.0, Mg^{2+} 1.5, Ca^{2+} 2.5, Cl^- 147.8, HCO_3^- 4.2 and SO_4^{2-} 0.5, HPO_4^{2-} 1.0 mM)(Table 1). The SBF was prepared by dissolving reagent NaCl, $NaHCO_3$, KCl, $K_2HPO_4 \cdot 3H_2O$, $MgCl_2 \cdot 6H_2O$, $CaCl_2$ and Na_2SO_4 in ultrapure water at 36.5°C little by little, and the solution was buffered at pH = 7.40 using Tris (hydroxymethyl)

aminomethane [$(CH_2OH)_3CNH_2$] (Duksan Pure Chemicals, Korea) and 1 M HCl (Bio-pure Reagents, Canada). The solutions were filtered immediately after preparation (75 mm, NALGENE, Rechester, NY, USA). The discs were kept in 50 ㎖ polypropylene tubes containing 10 ㎖ soaking solution in a humidified atmosphere of 95% air and 5% CO_2 at 37°C with initial PH of 7.4, and the soaking solution was changed every other day. Then, the discs were removed from the SBF after 1, 4, 7 and 14 days, gently washed with ultrapure water, and dried in a clean bench at room temperature.

Table 1. Ion concentrations of Corrected SBF and human blood plasma

	Ion concentration (mM)							
	Na^+	k^+	Mg^{2+}	Ca^{2+}	Cl^-	HCO_3^-	HPO_4^{2-}	SO_4^{2-}
Blood plasma	142.0	5.0	1.5	2.5	103.0	27.0	1.0	0.5
Corrected SBF	142.0	5.0	1.5	2.5	147.8	4.2	1.0	0.5

3. Surface analysis

Surfaces of the soaked discs were coated with a Pt/Pd film and observed under a field emission scanning electron microscope (FE-SEM, Hitachi S-4300, Tokyo, Japan) using an acceleration voltage of 15 kV at three different magnifications (X 20,000, X 5,000, X 1,000). Surface roughness was measured by confocal laser scanning microscopy (CLSM, LSM 5 Pascal, Zeiss, Obercochen, Germany). Energy dispersive X-ray

spectroscopy (EDS, Horiba EX-250, Tokyo, Japan) analysis was also carried out in order to evaluate the chemical composition of the discs. In addition, thin film X-ray diffraction (TF-XRD) measurements for the surface of discs soaked in SBF were carried out on a Bruker D8-Advance X-ray diffractometer (Bruker, Germany) using Cu K α radiation (λ = 0.1514) at a generator voltage of 40 kV and a generator current of 40 mA. The scattering angles (2 θ) were in the range of 20° -40° with a scanning velocity of 0.5° sec^{-1}. All analyzes were performed at three different areas and all experiments were run in triplicate.

4. Mesenchymal stem cell culture

Human bone marrow mesenchymal stem cells (hBMMSCs) were obtained from the Severance Hospital Cell Therapy Center (Seoul, Korea). From passage two to passage five of hBMMSCs were used in this study. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grandisland, NY, USA) medium supplemented with heat-inactivated 10% fetal bovine serum (FBS, Equitech-Bio, Inc., Texas, USA) and 1% Antibiotic-Antimycotic (Gibco, Grandisland, NY, USA) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. The culture medium was renewed every three days. When cells reached confluence, washed with phosphate buffer saline (PBS, Gibco, Grandisland, NY, USA) twice and a trypsin-EDTA solution (0.5 g/ℓ l trypsin and 0.2 g/ℓ EDTA, Gibco, Grandisland, NY, USA) was used to detach cells from the bottom of the culture plates, and replaced on the zirconia discs in 12 well culture

plates at the density of 1 x 10^4 cells/disc/well.

5. Cell proliferation assay

Cell attachment and proliferation on the zirconia discs were assessed by the WST (Water soluble Tetrazolium salts) solution. For the cell proliferation assay, a total of 36 discs, 6 for each group at each time point were used. 1 x 10^4 cells /disc/well were cultured on each disc in 12 well culture plates. After cultured for 1, 4 and 7 days, cell growth inhibition was determined using the EZ-CyTox cell viability assay kit (Daeil Lab Service Co., Seoul, Korea). EZ-CyTox solution (10 μℓ) was added to each cell culture well, and the mixtures were incubated for 1-4 hours at 37°C. Absorbance was then measured using an ELISA reader (Spectra Max 250, Molecular Devices, CA, USA) at 450 nm. Each group consisted of six wells, for which the mean absorbance was considered to be a representative value. A standard curve was assessed to convert optical density (OD) values to the number of viable cells.

The discs with cell seeding were also analyzed by SEM at X 200 and X 1,000 magnifications after cultured 1, 4 and 7 days culturing.

6. Osteogenic differentiation assay

Osteoblastic differentiation was induced by incubation cells in DMEM containing 10% FBS, 1% Antibiotic-Antimycotic, 50 g/mℓ ascorbic acid

(Sigma), 10 mM β-glycerophosphate (Sigma), 100 nM dexamethasone (Sigma) at a density of 1 x 10^4 cells/well in 12 well culture plates for one to two weeks. After the cells reached 60% -70% of confluence in the culture plates or on the discs, changed with differentiation medium, and the medium was changed every three days during osteoblastic differentiation periods. Preliminary experiment had already proved that hBMMSCs had good differentiation ability.

6-1. Alkaline phosphatase (ALP) staining and activity assay

After being induced on the SBF treated discs and non-treated discs for 14 days (1 x 10^4 cells /disc/well), the cells with disc were fixed by fixation solution (citrate, aceton, 4% paraformaldehyde) for 30 seconds, and rinsed with distilled water twice. Then the cells/disc constructs were incubated in staining solution (Alkaline phosphatase staining kit, Sigma-Aldrich) in dark at room temperature for 15 min according to the manufacturer's instruction. ALP positive cells were stained pink and after the discs dried completely, we scanned them. Then, the percentage of stained area was measured using Photoshop (Photoshop CS6, Adobe Systems Inc., San Jose, CA, USA).

For ALP activity assay, a total of 36 discs, 6 for each group at each time point were used. After culturing 1 x 10^4 cells/disc/well hBMMSCs in osteoblastic differentiation medium for 3, 6 and 9 days, the ALP activity assay was measured. Briefly, the cells were rinsed with PBS twice, lysed with 400 μℓ of PRO-PREP™ Protein Extraction Solution (iNtRON

Biotechnology, Seoul, Korea) at 4°C for 10 min, lysate was collected by scraper and centrifuged at 13,000 rpm for 5 min at 4°C. The supernatants were analyzed using an ALP assay kit (Wako Pure Chemical industries, Ltd., Osaka, Japan) according to the guidelines set by their manufacturer. The absorbance was read at 405 nm in a micro plate spectrometer and ALP levels were normalized to the amount of total protein. Protein concentration was determined with a bicinchoninic acid (BCA) protein assay kit (Pierce Chemical, Rockford, IL, USA) with bovine serum albumin (BSA) as the standard. The enzyme activity was expressed as nanomoles of *p*-nitrophenol produced per minute per microgram of protein.

6-2. Real time reverse transcriptase-polymerase chain reaction (RT-PCR)

1 x 10^4 /disc /well hBMMSCs were cultured on two kinds of zirconia discs in 12 well culture plates. Real time RT-PCR was performed to determine the gene mRNA expression levels in osteoblast cells for one reference gene: HPRT, and four target genes: alkaline phosphatase (ALP), type I collagen (COL 1), runt-related transcription factor 2 (Runx2) and osteocalcin(OCN).

Total RNA was isolated from cell samples after 14 days of osteoblast differentiation culture using Trizol® reagent (Invitrogen, Carlsbad, CA, USA) and prepared using an RNeasy Mini kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. Total RNA was

quantified using a Nanodrop 2000 Spectrohotometer (ThermoScientific Nanodrop Technologies, Wilmington, DE, USA). 2 μg of total RNA from each sample was used for generating first-strand complementary DNA (cDNA) using reverse transcriptase (Superscript II Pre amplification System, Invitrogen, Carlsbad, CA, USA).

The following human specific primers were used (Table 2):

Table 2. Gene list and primer sequences that have been used in RT-PCR

Gene name	Primer sequences (F = forward; R = reverse)
HPRT	(F): 5' GACCAGTCAACAGGGGACAT 3', (R): 5' CCTGACCAAGGAAAGCAAAG 3';
ALP	(F): 5' GGACATGCAGTACGAGCTGA 3', (R): 5' GCAGTGAAGGGCTTCTTGTC 3';
COL 1	(F): 5' CTGACCTTCCTGCGCCTGATGTCC 3', (R): 5' GTCTGGGGCACCAACGTCCAAGGG 3';
Runx2	(F): 5' CGCATTCCTCATCCCAGTAT 3', (R): 5' GACTGGCGGGGTGTAAGTAA 3';
OCN	(F): 5' GTGCAGAGTCCAGCAAAGGT 3'; (R): 5' TCAGCCAACTCGTCACAGTC 3'.

All reactions were performed in an ABI 7300 Sequence Detection System. (Applied Biosystems, Foster City, CA, USA) using SYBR green (Invitrogen) detection. The PCR was set up in a 20 μℓ reaction volume containing 10 μℓ SYBR green, 2 μℓ of cDNA, 0.4 μℓ of the forward and

reverse specific primers and 7.6 μℓ nuclease free water. The optimized cycling conditions were as follows: initial denaturation at 95℃ for 5 min, followed by 40 cycles of denaturation at 95℃ for 20 seconds, primer annealing at 56℃ for 30 seconds, and extension at 72℃ for 20 seconds. Relative levels of each primer were normalized to HPRT.

7. Statistical analysis

All data were expressed as mean±SD. Differences between the groups were statistically analyzed by student *t* test analysis (SPSS Statistics 19.0 software, IBM Inc, Armonk, NY, USA). Differences with a *p* value less than 0.05 were considered statistically significant.

Ⅲ. RESULTS

1. Surface characterization of the discs

Zirconia discs showed HA forming ability in SBF, and after soaking for various periods, it formed different amount of apatites. As the figure 1 shows that on first soaking day, it almost formed none of apatite, and at 4 days, it just formed a little. But at 7 days, these apatites were observed grown, developing sharp needle-like morphology and apparently covered the entire surface. Thereafter, at 14 days, it formed superimposed layer. The SEM images reflected that 7 day-soaking was sufficient.

CLSM was conducted to analyze the surface roughness. Figure 2 shows 3D images of surface of microstructure and figure 3 shows statistical results of surface roughness related parameters. Based on the figure 3 data, the Ra value for the non-treated discs was 0.4380±0.0823 μm, the SBF treated discs was 0.4913±0.0058 μm; the Sa value for the non-treated discs was 0.4780±0.0082 μm, the SBF treated discs was 0.5377±0.02578 μm; the Sdr value for the non-treated was 0.2150%±0.0080%, the SBF treated discs was 0.2113%±0.0146%, there were no significant differences of Ra, Sa and Sdr between the two groups ($p > 0.05$).

As table 3 and figure 4 showing, EDS exhibited HA deposited on the zirconia discs after 7 day-soaking in SBF. The Ca/P ratio for SBF treated group was 1.65, which was very close to the stoichiometric

composition ratio of 1.67 for HA (Ca10(PO4)6(OH)2) (Table 3). Comparison of EDS spectra taken from the surfaces of the non-treated and SBF treated surfaces, revealed Ca and P peaks appeared after soaking in SBF for 7 days (Fig. 4). This change can be considered as the evidence for the formation of calcium phosphate layer.

Based on the XRD, we detected that the identical phase of HA peaks at 25°-27°, 31°-34° and near 40°, which were very similar to HA peaks (Fig. 5).

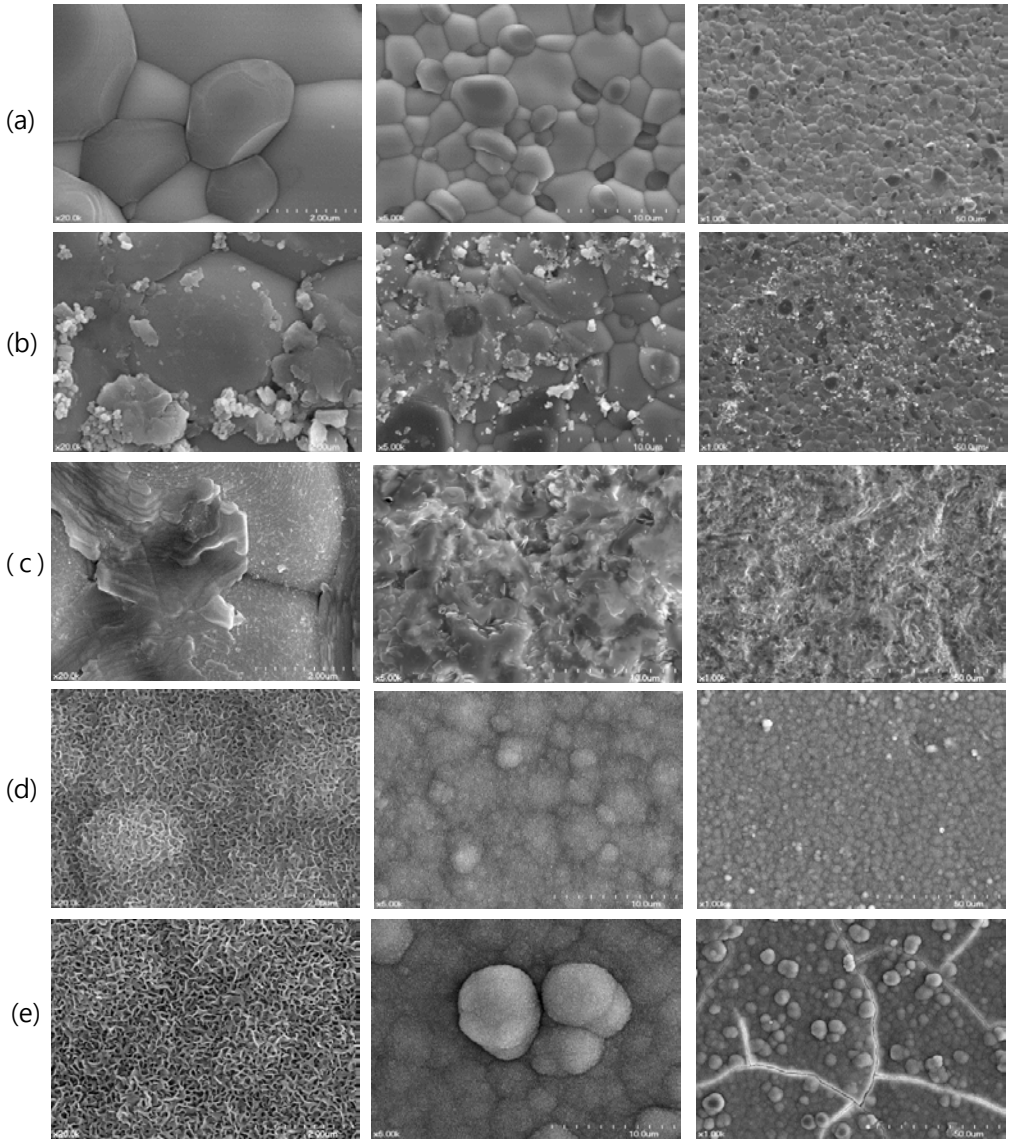
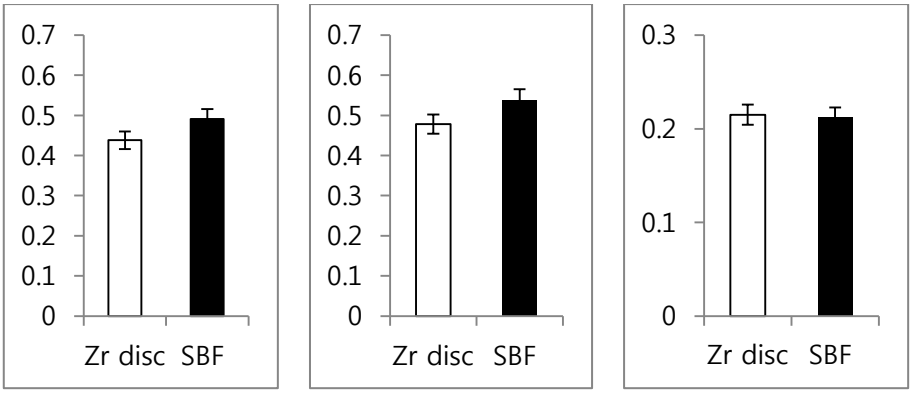


Fig. 1. SEM images of the surface of zirconia discs with deposited HA after 1, 4, 7 and 14 days of soaking at 37°C in SBF with initial PH of 7.4 (every row, left to right, x 20,000, x 5,000, x 1,000 magnification). (a) Control group: non-treated zirconia disc; (b) zirconia disc after soaking 1 day; (c) zirconia disc after soaking 4 days; (d) zirconia disc after soaking 7 days; (e) zirconia disc after soaking 14 days.

(a) (b)

Fig. 2. 3D images of surface of microstructure with dimensional range of 50 μm by CLSM. (a) is control group: non-treated zirconia disc; (b) is test group: 7 day-treated zirconia disc.



(a) Ra (b) Sa (c) Sdr

Fig. 3. Surface roughness related parameters obtained by CLSM. (a) Ra: Surface roughness, (b) Sa: arithmetic mean height deviation from a mean plane and (c) Sdr: developed interfacial area ratio. No statistical difference was observed between non-treated group and 7 day-treated group on any measurement scale ($p > 0.05$; the graph bar represents mean ± SD).

Table 3. Ca/P ratio of two groups obtained by EDS

	Control group	SBF treated group
Ca/P ratio	–	1.65

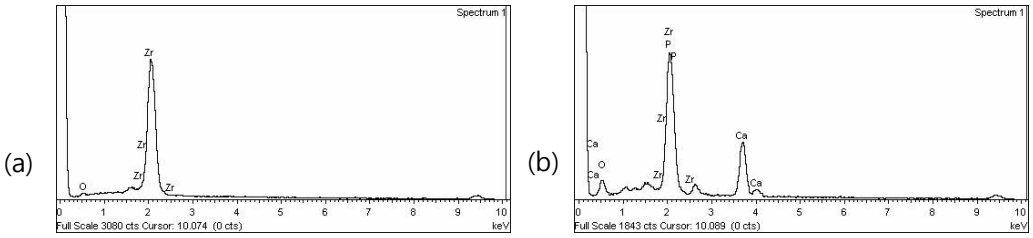


Fig. 4. EDS patterns of deposited HA on the SBF treated zirconia disc compared with the non-treated disc. (a) is control group: non-treated zirconia disc; (b) is test group: 7 day-treated zirconia disc. The pattern shows that after SBF soaking, the Ca and P appeared which means that HA deposited on the disc.

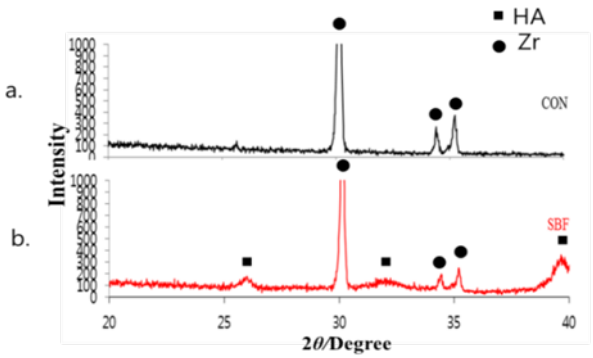


Fig. 5. XRD patterns of deposited HA on SBF treated zirconia disc compared with non-treated zirconia disc. (a) is control group: non-treated zirconia disc; (b) is test group: 7 day-treated zirconia disc. The pattern shows that after SBF soaking, the HA deposited on the disc.

2. Surface morphology of the discs seeding with cells

After hBMMSCs cultured on two kinds of zirconia discs for 1, 4, 7, three different days, SEM was carried out to show cell attached morphology and cell number. On the first day, it does not show clear difference. On 4 days, the SBF treated group has a little bigger cell number, and on 7 days, more spread cells were clearly observed on SBF treated discs than non-treated discs (Fig. 6).

3. Proliferation of MSCs on the discs

The cells cultured on the SBF treated zirconia discs exhibited significant higher proliferation rate than non-treated discs on 1, 4 and 7 days ($p < 0.05$) (Fig. 7). At the first day, the proliferation rates of non-treated discs were 0.0464±0.0028 and the SBF treated discs were 0.0543±0.0052, the SBF treated discs showed just a little higher proliferation rates than the non-treated discs ($p < 0.05$). After four days, the proliferation rates of non-treated discs were 0.1179±0.0051, and the SBF treated discs were 0.1248±0.0068, the differences were statistically significant ($p < 0.05$), although the gap was so small that not be clearly seen in the graph indeed. At the last day, the proliferation rates of non-treated discs were 0.4180±0.0130, and the SBF treated discs were 0.4496±0.0080, the differences were progressively increased and clearly larger than before ($p < 0.05$).

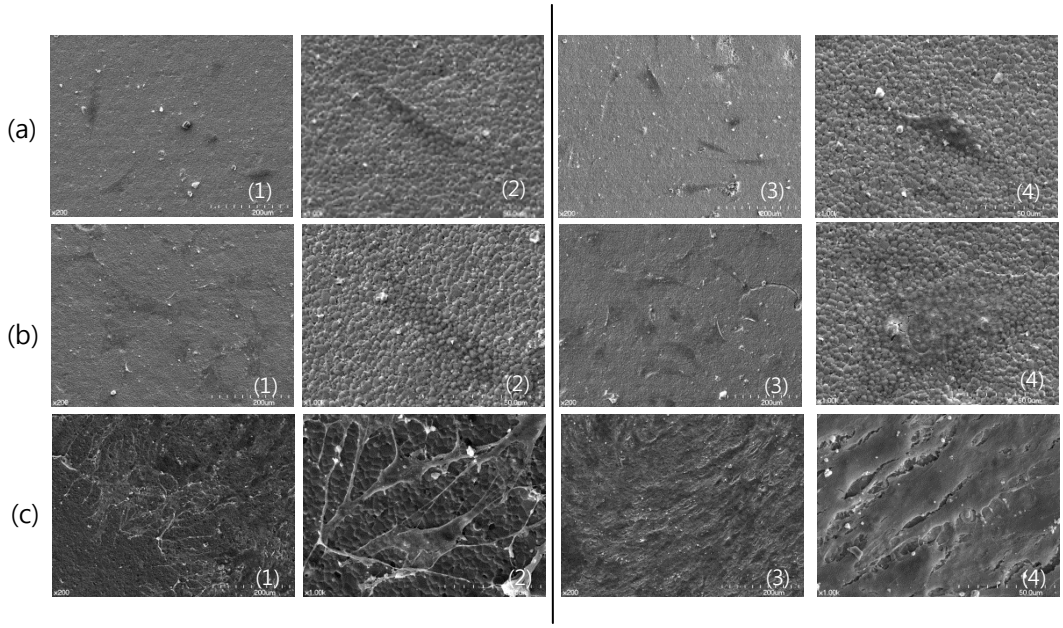


Fig. 6. SEM images showing the morphology of hBMMSCs seeded on the SBF treated zirconia discs and non-treated zirconia discs after three different times: (a) after 1 day of cells seeded on the zirconia discs; (b) after 4 days of cells seeded on the zirconia discs; (c) after 7 days of cells seeded on the zirconia discs ((1), (2) are non-treated zirconia discs and (3), (4) are 7-day SBF treated zirconia discs; (1)(3) are x 200 magnification and (2)(4) are x 1,000 magnification).

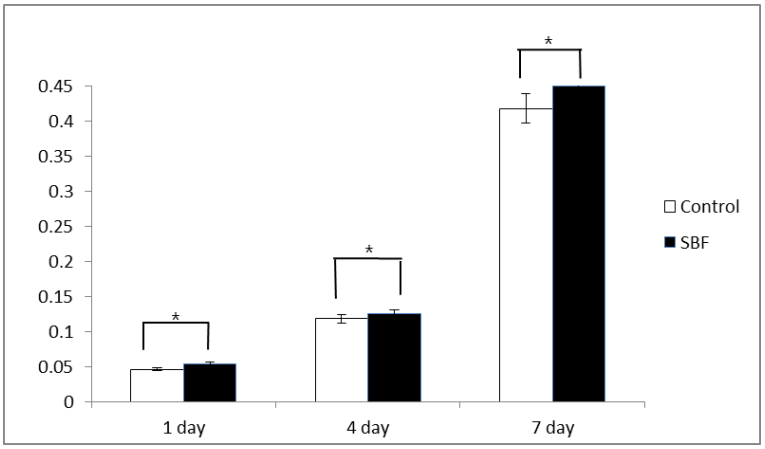


Fig. 7. Proliferation assay of hBMMSCs cultured on 7 day-treated discs and non-treated zirconia discs. hBMMSCs viability was measured by EZ-CyTox cell viability assay kit at 1, 4 and 7 days. The values are mean ± SD of three independent experiments. There were significant differences of cell viability between two groups at each time point ($*p < 0.05$; the graph bar represents mean ± SD).

4. Osteogenic differentiation assay

4-1. ALP synthesis by hBMMSCs on the discs

Figure 8 (a) shows that both the two kinds of discs were stained pink, however, the areas stained pink were obviously bigger and uniformly covered on the SBF treated disc compared with non-treated disc. The percentage of the stained area in the non-treated discs was 15.1467% ± 0.6573%, and in the SBF treated discs was 23.5367% ± 3.1512% ($p < 0.05$).

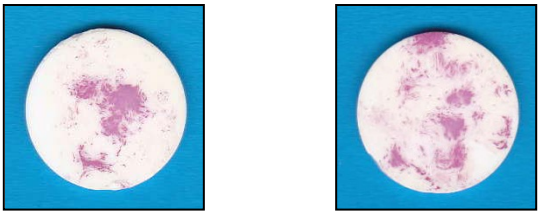
At figure 8 (b), there was no significant difference of ALP activity between the two kinds of discs at 3 days and 6 days, the exact ALP protein level for the non-treated discs at 3 days was 0.0547±0.0091, the SBF treated discs was 0.0907±0.0156 ($p > 0.05$), and the ALP protein level for the non-treated discs at 6 days was 0.1011±0.0170, the SBF treated discs was 0.1275±0.0506 ($p > 0.05$). However, at 9 days, the protein level of SBF treated discs was increased markedly than the control. At 9 days, the exact protein level of non-treated discs was 0.1358±0.0434, and the SBF treated discs was 0.3685±0.0871, the data obviously exhibited the statistically significant difference between the two compared groups ($p < 0.05$).

4-2. Osteoblast gene expression

Gene expression of osteoblast differentiation markers like alkaline phosphatase (ALP), type I collagen (COL1), runt-related transcription factor 2 (Runx2) and osteocalcin (OCN) at 14 days following mineral induction were investigated by real-time RT-PCR (Fig. 9). When compared to the control, the expression level of all tested genes was increased in the SBF treated group. Among them, the ALP and OCN expression levels were dramatically increased in mineral induced cells on the SBF treated zirconia discs. The extent of ALP expression of SBF treated was 2.3849±0.1923 and the OCN expression was 1.6614±0.0099, which revealed significant difference ($p < 0.05$). Although, the expression of COL 1 and Runx2 genes were slightly increased in SBF

treated discs, each corresponding expression level was as follows: COL 1 was 1.0617±0.1470 and Runx2 was 1.0980±0.6941, they did not reach statistical significance ($p > 0.05$).

(a)



Non-treated SBF-treated

(b)

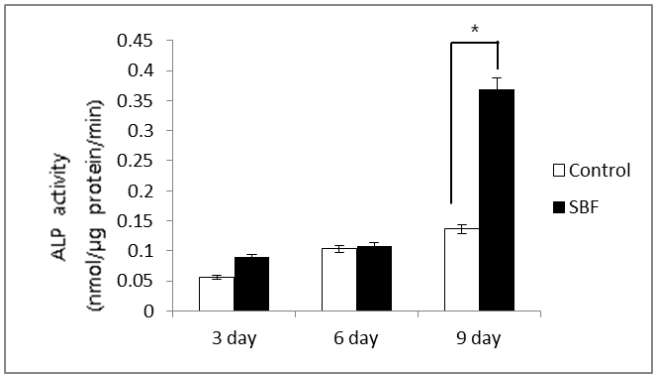


Fig. 8. ALP staining and time course of ALP activity assay of hBMMSCs cultured on 7 day-treated and non-treated zirconia discs. 1 x 10^4 cells were plated on each disc. The released 4-nitrophenylate was determined photometrically at 405 nm and related to protein concentration of the lysates. (a) Scan images of ALP staining on the discs. ALP expression of hBMMSCs cultured either on SBF treated zirconia disc or non-treated zirconia disc for 14 days was stained by

pink. (b) Quantitative ALP activity of hBMMSCs cultured either on 7 day-treated zirconia disc or non-treated zirconia disc was determined at 3, 6 and 9 days after seeding. The values are mean ± SD of three independent experiments. There was no difference between two kinds of discs at 3 days and 6 days, however, at 9 days, the result indicated that there was significant difference of ALPase activity (*$p < 0.05$; the graph bar represents mean ± SD).

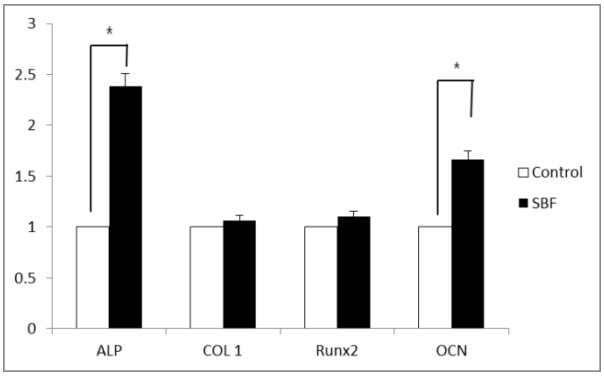


Fig. 9. Expression levels of the osteogenic genes were measured by the real time RT-PCR on 7 day-treated and non-treated zirconia discs, and normalized to that of the HPRT. Osteoblast related genes like alkaline phosphatase (ALP), type I collagen (COL 1), runt-related transcription factor 2 (Runx2), osteocalcin(OCN) were tested. Among these genes, only ALP and OCN expression were statistically significant (*$p < 0.05$; the graph bar represents mean ± SD).

Ⅳ. DISCUSSION

Bone integration is the prerequisite for the success of dental implants, and requires cell attachment, proliferation and differentiation. Therefore, the implant material must have the capability of osseointegration and long term stability. Although, titanium was widely used in dental implant for several years, with its side effects like esthetic problem and allergy reactions made it no longer the preferred material in dentistry. In dentistry, ceramic gradually become alternative material that can overcome such problems.[20]

Our research mainly focused on zirconia, which has biocompatibility, osseointegration properties and tooth-like color.[10] Zirconia can be organized three different forms: monoclinic (M), cubic(C), and tetragonal (T). To accelerate the stability, other metallic oxides like MgO, CaO, or Y_2O_3 can be mixed with zirconia. Y_2O_3-stabilized tetragonal zirconia polycrystals (Y-TZP), we adopted in this study, is the most studied combination with great mechanical properties.[11] However, zirconia could not bond to living bone directly, so it is necessary to surface modification to stimulate bone-implant shear strength. HA has been coated on titanium surface for better osseointegration[21], and produced acceptable long-term survival rate.[22,23] HA-doped zirconia was already recognized as osteoconductive and durable biomaterials and expected to be used in extended application fields.[24] One of the techniques used for obtaining HA coatings is a biomimetic deposition which is a method that a bone-

like apatite layer is formed through soaking the substrate in simulated body fluid (SBF) under physiological conditions of temperature (37°C) and PH (7.4).[16,19] SBF which can mimic the typical ion concentrations in body fluid was widely used in previous researches to confirm materials' bioactivity.[25,26] SBF with ion concentrations nearly equal to those of human blood plasma could induce HA formation on zirconia gel,[27] but none of research used the SBF soaked zirconia discs to do the cell proliferation assay and cell differentiation assay on its surface.

In this study, in the beginning, we soaked zirconia discs in SBF for different time periods to prove the HA forming ability on zirconia discs and determine how long was the enough time for soaking. The SBF used in present study was corrected SBF. Since Kokubo firstly introduced conventional SBF in 1990,[16] many researches attempted to revise the ingredient concentrations because the conventional SBF was a solution highly supersaturated so it was not easy to prepare. [28-30] The SBFs were subjected to round robin test in many research institutes, and finally, the authority recipe for preparing corrected SBF was refined and published in 2006.[19] Therefore, we prepared corrected SBF according to that recipe. About soaking periods, there were also a wide range of views. Gou et al. did the research about soaking the dicalcium silicate ceramic in SBF and demonstrated that a crystalline layer thought to be HA covered the whole surface after 3 days of soaking.[9] Sintered HA was also found to have apatite forming ability on its surface in SBF from 3 hours up to 120 hours.[31] HA was also deposited on Ti surface after 7 days soaking in SBF. [17] The island like apatite was appeared on zirconia gel after

immersed in SBF for 14 days.[27] In our research, we tested 1, 4, 7 and 14 days, and from the SEM images (Fig. 1), we confirmed that HA deposited on zirconia surface after soaking in SBF. At day 7, HA covered the whole surface of discs, so we thought 7 days were enough time for soaking. That's why we used 7 day-treated zirconia discs in following experiments.

Surface roughness is very important parameter for achieving stable implant fixation.[32] CLSM usually used to measure surface roughness. Our CLSM results demonstrated that there was no significant difference between the two kinds of discs in surface roughness which means that HA uniformly covered the whole surface so that it did not affect the surface roughness. EDS was frequently used to determine surface composition. In this study, we also used EDS to analyze surface conditions. After SBF soaking, the Ca and P ions were appeared which confirmed that the HA was formed on its surface (Fig. 4). The Ca/P atomic ratio obtained by EDS for SBF treated group was 1.65 which is very similar to 1.67 for HA ($Ca_{10}(PO4)6(OH)_2$).[33] Pandey et al. conducted XRD in his research and claimed that the two main characteristic peaks of biological apatite were 26° and 32°.[34] Other researches for XRD patterns of bonelike apatite indicated that the HA peaks appeared near 26°, 32° and 40°.[15,35] From our XRD pattern (Fig. 5), we easily found that the different peaks existed at 25°-27°, 31°-34° and near 40° which were just equivalent to HA peaks. Indeed, in SBF, zirconia grains act as nucleation site and promote biomimetic growth of calcium phosphate minerals.[36] The release of sodium or calcium ions into

the body fluid may cause the formation of Zr−OH groups on the surfaces of zirconia ceramics because of the ion−exchange reaction with the hydronium ion present in the body fluid.[27]

SEM observation (Fig. 6) shows that cells cultured on the SBF treated surfaces formed multi cellular layers completely covering the surfaces and proliferated while those cultured on non−treated discs exhibited lower proliferation rate. EZ−CyTox cell viability assay, a fast, stable and sensitive to determine the cell viability or cell proliferation, was conducted in this study. The cells cultured on SBF treated discs exhibited higher cell proliferation compared with non−treated zirconia discs which indicate that HA deposited on the zirconia surface is favorable for cell attachment and growth. ALP staining and ALP activity pattern correlated well with nodule formation in osteoblast cells.[37] Bellows et al. demonstrated that ALP played a crucial role in the differentiation and mineralization of cells with osteoblastic phenotypes.[38] In our study, at first, the cells cultured on SBF treated displayed no difference of ALP activity, but with time increasing, at 9 days, it appeared significant differences. Taken together, SBF treated zirconia discs were efficacious in inducing cell proliferation and ALP activity of hBMMSCs.

Since the differentiation to osteoblast was expected, we examined the gene expression of osteoblast specific markers like ALP, COL 1, Runx2 and OCN by real time RT−PCR. ALP activity and expression of OCN were used as indicators of osteoblastic activity. COL 1 is considered to be a very important extracellular matrix protein in osteoblast

proliferation and differentiation. The levels of COL 1 and OCN mRNA and ALP activity were previously studied in osteoblastic cells under mechanical strain.[39,40] Recently, it was established that Cbfa1/Runx2, a member of the runt family of transcription factors, was a key regulator of osteoblast recruitment and differentiation from mesenchymal stem cells. Cbfa1-binding motifs exist in the promoter region of osteoblast phenotype-related genes encoding OCN, COL 1 and regulate their expression.[39] Higher expression of these genes seems to be related with higher mineralization. In present study, although all genes revealed higher expression in the SBF treated group than the control group (Fig. 9), only the ALP and OCN markers expressed statistically significant.

Although there is a need to do the *in vivo* animal studies to confirm the outcomes in this study, this study fully demonstrated that SBF soaking was effective for cell proliferation and osteoblastic differentiation so that it may be clinically available for the improvement of bone osseointegration in the following implantation.

Ⅴ. CONCLUSIONS

HA is formed on the surface of zirconia disc after it is soaked in SBF for a period of time. Human bone marrow mesenchymal stem cells are observed to proliferate and differentiate in good states on the SBF treated zirconia discs compared with non-treated discs. Our results suggest that the HA produced by this method is bioactive and cytocompatible and could induce high osteoconductivity and osteoinductivity of zirconia. So it is to be expected that using zirconia implant with SBF treated for dental implantology could improve the osseointegration and osteoconduction process in humans.

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국문초록

Simulated body fluid 에 침전한 지르코니아 디스크 표면분석과 세포반응 연구

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목적: 지르코니아는 bioinert 한 쎄라믹 재료이기 때문에 치과임상에서 임플란트 재료로 응용이 되려면 표면처리를 거쳐 지르코니아의 생체 활성을 증가시키는 것이 필요하다. 본 연구의 목적은 지르코니아 디스크를 simulated body fluid (SBF) 용액에 침전시켰을 때 지르코니아 디스크 표면에서 hydroxyapatite (HA) 의 형성 능력평가, 및 SBF 처리를 한 디스크 표면에서 사람 골수 간엽줄기세포의 생장 능력과 분화 능력을 분석하는 것이다.

재료 및 방법: 본 실험에서는 Kokubo 의 recipe 에 따라 36.5℃ 에서 사람 혈장농도와 비슷한 이온 농도를 가진 corrected SBF 를 제조하였다. 80 개의 Y_2O_3–stabilized tetragonal zirconia polycrystals (Y-TZP) 지르코니아 디스크를 제조하여 아래와 같은 두 개 그룹으로 나누어 실험을 진행하였다.

(1) Test group: SBF 처리한 디스크; (2) Control group: 아무 처리를 하지 않은 디스크. 지르코니아 디스크를 PH 가 7.4 인 SBF 용액에 1 일, 4 일, 7 일과 14 일, 부동한 시간 동안 37℃ 에서 침전시키고 HA 의 생성형상을 표면 형태를 통하여 분석하였다. HA 의 생성을 더한층 증명하고자, 7 일간 SBF 처리를 거친 디스크 표면에서 표면 거칠기, 표면 조성, 표면 결정화 분석 실험을 진행하였다. 세포적합성 측정을 위하여 사람 골수 간엽줄기세포를 7 일간 SBF 처리를 거친 디스크 표면에서 배양하여 cell proliferation, alkaline phosphatase (ALP) staining 및 activity assay 와 osteoblast 유전자 발현 능력을 비교 분석하였다. 모든 데이터는 0.05 의 유의수준에서 student *t* test 통계방법을 사용하여 분석하였다.

결과: 부동한 처리 시간 후, 디스크는 부동한 표면 형태를 나타냈고, SBF 처리 시간이 길어짐에 따라 더 많은 HA 가 생성되었다. 7 일간 SBF 처리를 거친 디스크 표면에서 HA 가 생성되었고, 균일하게 깔려있었다. 표면 거칠기에는 두 가지 디스크 간에 유의미한 차이가 없었다 ($p > 0.05$). Cell proliferation 은 SBF 처리 그룹에서 더 좋은 결과를 보여주었다 ($p < 0.05$). ALP assay 결과 처음 에는 큰 차이를 보이지 않다가 9 일차에 확연하게 큰 차이를 보였다 ($p < 0.05$). ALP staining 결과 SBF 처리 그룹에서 더 좋은 staining 결과를 보여주었다 ($p < 0.05$). 유전자 발현 능력 측정 결과 ALP 와 osteocalcin (OCN)은 SBF 처리한 그룹에서 통계학적으로 유의미한 높은 발현율을 ($p < 0.05$) 보이는 반면에 type Ⅰ collagen (COL 1), runt-related transcription factor 2 (Runx2)는 통계학적으로 유의미한 차이를 보이지 않았다 ($p > 0.05$).

결론: 지르코니아 디스크는 SBF 용액에서 HA 형성 능력을 갖고 있다. Cell attachment, proliferation 과 differentiation 실험 결과 모두 SBF 처리한 디스크에서 아무 처리를 하지 않은 디스크에 비해 뚜렷한 증가 추세를 보이고

있다. 이는 SBF 처리를 거친 지르코니아가 치과용 임플란트로 이용될 수 있다는 가능성을 제시해주고 있다.

주요어: 지르코니아 디스크, Simulated body fluid (SBF), Hydroxyapatite (HA), 사람 골수 간엽줄기세포, Cell proliferation, Osteoblast differentiation

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