치의학박사학위논문

Comparison of the osteogenic potential and fibroblast response of 3 mol% yttria-stabilized tetragonal zirconia polycrystals and niobium oxide containing zirconia discs

Niobium oxide를 포함한 지르코니아와 yttria-stabilized 지르코니아의 골형성능과 연조직 반응에 대한 비교 연구

2018년 2월

서울대학교 대학원

치의과학과 치과보철학 전공

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이 논문을 치의학박사 학위논문으로 제출함

2017 년 10 월

서울대학교 대학원

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AUNG THU HEIN 의 치의과학박사 학위논문을 인준함

2017 년 12 월

위원장 (인)

부위원장 (인)

위 원 (인)

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-ABSTRACT-

Comparison of the osteogenic potential and fibroblast response of 3 mol% yttria-stabilized tetragonal zirconia polycrystals and niobium oxide containing zirconia discs

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Objective: This *in vitro* study evaluated the osteogenic potential and human gingival fibroblast response of 3 mol% yttria-stabilized tetragonal zirconia polycrystals (3Y-TZP) and niobium oxide containing Y-TZPs ((Y, Nb)-TZPs) with specific ratios, namely YN4533 and YN4533/Al20 discs.

Materials and Methods: The surface properties of smooth 3Y-TZP, YN4533, YN4533/Al20 and rough discs (15 mm in diameter, 1 mm thick, 20 discs for each group) were evaluated by confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM). Murine pre-osteoblast MC3T3-E1 cells and human gingival fibroblast cells were cultured on all zirconia discs. Cell attachment was evaluated by CLSM and SEM after twenty four hours of cell culturing. The PicoGreen assay was used to investigate cell proliferation at 1, 4 and 7 days for osteoblasts and 4, 24, 48 and 72 hours for fibroblasts. The mRNA gene expression of alkaline phosphatase (*Alp*) and osteocalcin was measured by real-time RT-PCR at 5, 8 and 11 days. *Alp* activity was also evaluated at the protein level. Moreover, the mRNA gene expressions of type I collagen, integrin α2 and β1 were measured by real-time RT-PCR at 4, 24

and 48 hours. Two-way ANOVA was used for the comparison of the outcomes among the groups. All the data were analyzed at the significance level of 0.05.

Results: Both MC3T3-E1 pre-osteoblasts and human gingival fibroblasts were more widely spread on smooth surfaces than on rough surfaces. Cellular proliferations of both osteoblasts and fibroblasts were higher on smooth surfaces. Fibroblasts were more proliferated on smooth YN4533 and YN4533/Al20 than smooth 3Y-TZP while no significant differences were found in osteoblasts. Osteoblasts were slightly more differentiated on YN4533 and YN4533/Al20 than on 3Y-TZP, considering the results of RT-PCR and *Alp* activity. The mRNA expressions of type I collagen, integrin α2 and β1 were significantly stimulated for (Y, Nb)-TZP groups at 24 hours after seeding.

Conclusions: Within the limitation of this study, (Y, Nb)-TZPs provide appropriate surface condition for osseointegration at the implant level and for peri-implant mucosal sealing at the abutment level. The (Y, Nb)-TZP is anticipated to be a suitable candidate material for ceramic dental implant with a favorable clinical outcome.

Keywords: Dental implant; niobium; zirconia; LTD; osteogenic potential; fibroblast
Student Number: 2015-30784

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I. INTRODUCTION

The replacement of missing teeth with osseointegrated dental implants has become an evidence-based treatment modality and a routine procedure in dentistry for more than four decades. Despite frequent peri-implantitis and other complications, the survival rate for titanium implants is 90-95% over a period up to 20 years.[1] Commercially pure titanium and titanium alloys are materials of choice for dental implants because of their biocompatibility, excellent mechanical properties, and long term clinical success.[2-4]

Despite its great advantages, titanium exhibits grayish discoloration on the peri-implant mucosa and jeopardizes aesthetic outcomes of restoration, especially if there is insufficient soft tissue to mask in the anterior segments.[5,6] Although the prevalence is low (0.6%), titanium allergy can be detected in dental implant patients.[7] Furthermore, titanium might induce hypersensitivity in susceptible patients and can play a critical role in implant failure.[8] Some studies have also reported corrosive behavior that occurs after titanium comes in contact with saliva and fluoride.[9,10] To compensate for the weak points of titanium, many researchers have tried to develop tooth-colored biocompatible ceramic materials for dental implants. Ambitious efforts were made to introduce zirconia ceramics for applications in implant dentistry because of their aesthetic superiority, excellent biocompatibility and mechanical properties.[4,11]

Zirconia is a polymorphic crystal that can be found in three different crystalline phases depending on the temperature: monoclinic (room temperature until 1170℃), tetragonal (1170-

2370℃), and cubic (2370℃ until melting point). The transformation from the tetragonal to the monoclinic phase is associated with a 3-4% localized volume expansion that induces compressive stresses in the compromised areas.[12] The addition of stabilizing oxides like magnesia (MgO), yttria (Y_2O_3), and ceria (CeO_2) prevents this phase transformation and maintains a metastable tetragonal phase at room temperature. Three mol% Y_2O_3-stabilized tetragonal zirconia polycrystals (3Y-TZP) exhibit high strength and toughness as well as tetragonal phase stability at room temperature. 3Y-TZP, which shows superior mechanical properties compared to other ceramics, has been introduced as an alternative to titanium.[13,14] Many studies have compared osseointegration and reported no significant difference between 3Y-TZP and titanium implants.[15-18] *In vitro* studies revealed comparable osteoblast adhesion, proliferation, and differentiation between various types of Y-TZP and sandblasted/acid-etched titanium surfaces.[19,20] Several *in vivo* studies also proved that 3Y-TZP implants undergo osseointegration comparable with that of titanium implants.[21-23]

Despite its excellent mechanical properties and biocompatibility, however, a major shortcoming of zirconia is its inherent accelerated aging and low temperature degradation (LTD). LTD is related to a lattice relaxation process induced by thermally activated oxygen vacancy diffusion.[24] It consists of a spontaneous, slow transformation of the crystals from the tetragonal phase to the monoclinic phase at low temperatures (150-400℃). In a humid environment, this could decrease the strength of the materials and lead to catastrophic failures over time.[25]

Various approaches to eliminate or reduce LTD have included a ceria partially stabilized zirconia/alumina nanostructured composite (NANOZIR),[26,27] alumina-toughened zirconia (ATZ),[28-30] and 3Y-TZP co-doped with niobium oxide (Y, Nb)-TZP.[24,31-34] The resistance of (Y, Nb)-TZP to hydrothermal degradation is attributed primarily to tetragonal phase stability as a result of Y-Nb ordering in the tetragonal phase zirconia (t-ZrO_2) lattice[31] as well as a reduction in the oxygen vacancy concentration in Y-TZP as a result of the substitution of Nb^{5+} for Zr^{4+}.[24,31,35] In order to utilize this advantage of niobium in dental implant treatment, it is important to analyze the osteogenic potential of (Y, Nb)-TZPs because proper osseointegration around the implant body is one of the major criteria for successful implant treatment.[1-4]

Moreover, the longevity and functionality of dental implants are dependent not only on osseointegration around the implant body but also the establishment of a properly functioning soft tissue barrier that protects the underlying hard tissue structures and the implant itself.[36-39] A soft tissue attachment or transmucosal attachment serves as a seal that prevents products in the oral cavity from reaching the bone tissue. Poorly formed gingival connective tissue around the implant allows ease of bacterial invasion that causes inflammation resulting in marginal bone loss and peri-implantitis.[40,41]

Periodontal tissue is mainly inhabited by gingival fibroblasts (GFs) and periodontal ligament fibroblasts (PDLFs), and GFs are involved in the maintenance and production of the gingival connective tissue.[42,43] The formation of a soft tissue barrier between the titanium implant surface and the connective tissue is established and maintained by fibroblasts.[41,44] Fibroblasts were orientated both parallel and perpendicular to the long axis of the implant surface and it was suggested that this fibroblast-rich barrier played an important role in the establishment and maintenance of the soft tissue seal.[45,46]

The adhesive properties of the material used in the abutment part of the implant was the decisive importance for the location of the connective tissue portion of the transmucosal attachment.[41,47] Abutments made of zirconium dioxide (3Y-TZP) allowed for the establishment of a mucosal attachment similar to that occurring at titanium abutments[41,48] but demonstrated limited plaque formation and better esthetics[48]. Soft tissue biological response of zirconia abutments is superior to metal abutments and blood flow in tissue surrounding zirconia abutments is similar to that in soft tissue around natural teeth. Moreover, zirconia abutments could be advantageous for the maintenance of immune function by improving blood circulation.[49]

Researches have already demonstrated that less bacteria plaque accumulation of zirconia than titanium.[48,50-51] From the biological points of view, zirconia demonstrated a low affinity to bacterial plaque, a small amount of inflammatory infiltrate and good soft tissue integration, which might lower the risk of peri-implant diseases.[17] It is important to establish a novel implant and abutment material with better soft tissue seal, more cleanable feature than titanium[39] and little or no LTD property[17].

Our previous studies have shown that sandblasted (Y, Nb)-TZP discs have a similar osteogenic potential to anodized titanium[52] and proper mucosal sealing[53]. However, the optimal combination of the elements to achieve maximal hard and soft tissue responses is still challenging for the development of a new material. In this study, two (Y, Nb)-TZPs were synthesized, with new combinations of niobium oxide and other components, which were denoted as YN4533 and YN4533/Al20. The purpose of this study was to evaluate the osteogenic potential and fibroblast response of these newly combined (Y, Nb)-TZPs, YN4533 and YN4533/Al20, by comparing with that of 3Y-TZP, the most widely used zirconia ceramic in the field of dentistry. The hypothesis underlying this study was that these (Y, Nb)-TZPs would exhibit comparable osteogenic potential and fibroblast response to 3Y-TZP.

II. MATERIALS AND METHODS

1. Specimen preparation

Zirconia discs containing niobium oxide were synthesized according to specific ratios. The overall composition of YN4533 is 92.2 mol% ZrO_2, 4.5 mol% Y_2O_3, and 3.3 mol% Nb_2O_5. YN4533/Al20 discs were prepared with the same mole fractions of YN4533 with an additional 20 vol% of Al_2O_3. YN4533 and YN4533/Al20 were test groups while 3Y-TZP served as a control. 3Y-TZP, YN4533, and YN4533/Al20 disc-shaped green compacts (15 mm in diameter and 1 mm in thickness, 40 discs for each group) were prepared by cold isostatic pressing of the powder mixtures at 200 MPa followed by sintering for 2 hours at 1500℃ for 3Y-TZP, 1450℃ for YN4533, and 1600℃ for YN4533/Al20. The different sintering temperatures were used because the optimum sintering temperature for each material depends on the composition of the specimens to achieve maximum strength without deterioration and based on preliminary studies.[24,31] All zirconia discs were gradually polished and finished with diamond pastes to produce mirror-like surfaces. After polishing, half of the zirconia discs (20 discs in each group) were sandblasted with 50-μm alumina (Al_2O_3) at 2 bar pressure and 90 degrees, perpendicular to the surface for 1 minute to create rough surfaces. Mirror-like smooth surface groups were denoted as 3Y-TZP-M, YN4533-M and YN4533/Al20-M, while sandblasted rough surface groups were denoted as 3Y-TZP-R, YN4533-R and YN4533/Al20-R.

2. Surface roughness and topography

The average surface roughness (R_a) and surface topography were analyzed using a 3 dimensional confocal laser scanning microscope (CLSM; LSM 5 Pascal, Carl Zeiss, Germany). The R_a values represent the mean ± SD resulting from the measurements of 3 samples for each group. Surface morphologies of zirconia discs were observed via a field emission scanning electron microscope (FE-SEM; HITACHI S-4700, Tokyo, Japan).

3. Cell culture and cell attachment observation

Mouse pre-osteoblast MC3T3-E1 and human gingival fibroblast (HGF) cells were purchased from ATCC (Manassas, VA, USA). The osteoblast cells were cultured in α-minimal essential medium (α-MEM, Hyclon) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, and incubated in a humidified atmosphere of 95% air/ 5% CO_2 at 37℃. The osteogenic media included 10 mM β-glycerophosphate and 50 μg/mL ascorbic acid in α-MEM with 10% FBS and 1% penicillin/streptomycin. Another CLSM (LSM700, Carl Zeiss, Germany) and ZEN2011 software (Carl Zeiss, Germany) were used to evaluate cell attachment and morphology. Twenty four hours after seeding onto the zirconia discs, cells attached onto the discs were fixed with 4% formaldehyde. 4'6-diamidino-2-phenylindole (DAPI, Invitrogen, Carlsbad, CA, USA) was used for detection of cell nuclei and Alexa Fluor 568 phalloidin (Invitrogen) was used for detection of the cytoskeleton. Human gingival fibroblast cells were cultured in Dulbecco's Modified Eagle's Medium containing 10% FBS and 1% penicillin/streptomycin and incubated in a humidified atmosphere of 95% air/ 5% CO_2 at 37℃. Twenty four hours after seeding, HGFs morphologies on zirconia discs were observed via the FE-SEM.

4. Cellular proliferation assay and quantitative real-time RT-PCR

Cell proliferation was examined by a PicoGreen assay using the Quant-iT PicoGreen assay kit (Invitrogen) 1, 4 and 7 days for osteoblasts and 4, 24, 48 and 72 hours for fibroblasts after seeding cells on the zirconia discs. Cells adhered to the zirconia discs were washed with PBS and lysed with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) to allow for formation of

DNA samples. Then, 100 μL of the DNA samples was mixed with 100 μL of PicoGreen reagent. Samples were loaded in triplicate and florescence intensity was measured on a microplate reader (Floustar Optima, BMG LABTECH, Germany). Florescence intensity was converted into DNA concentration with a DNA standard curve per the manufacturer's instructions. To evaluate cellular differentiation, osteoblast cells were seeded on the zirconia discs and cultured in osteogenic media, which includes 10 mM β-glycerophosphate and 50 μg/mL ascorbic acid in growth media. Cells were harvested at 5, 8 and 11 days, and RNA was isolated using Trizol lysis reagent (TRIzol Reagent; Invitrogen). Human gingival fibroblast cells were harvested at 4, 24 and 48 hours after seeding and RNA was isolated using QIAzol lysis reagent (QIAGEN, Valencia, CA, USA). The Primescript RT reagent kit (Takara Bio, Shiga, Japan) was used for reverse transcription and then real-time PCR was performed using Takara SYBR premix Ex Taq (Takara Bio, Shiga, Japan) on an Applied Biosystems 7500 Real-Time PCR system (Foster City, CA, USA). All samples were run in triplicate. Alkaline phosphatase (*Alp*) and osteocalcin (*Oc*) gene expressions were analyzed for osteogenic differentiation while type I collagen, integrin α2 and β1 for fibroblast response. The results were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to account for variations in RNA quantitation. The marker genes were synthesized by Integrated DNA technology (IDT; Coralville, IA, USA). *Alp* activity was measured using an ALP kit (Sigma-Aldrich, St. Louis, MO, USA). Osteoblast cells were seeded on the zirconia discs and cultured in osteogenic medium for 10 days. Cells were washed twice with PBS and stained as described by the manufacturer.

5. **Statistical analysis**

All quantitative data are presented as the mean ± SD and each experiment was repeated at least three times. The data analysis was performed with SPSS software (SPSS version 23, IBM, Armonk, NY, USA) using two-way ANOVA-test and Tukey post hoc test. Differences were considered as being significant at $P < 0.05$.

III. RESULTS

1. Surface analysis of 3Y-TZP and new (Y,Nb)-TZP discs

The average roughness values (R_a) and topographies of all zirconia discs under the 3 dimensional CLSM are shown in Figure 1. The R_a values of the mirror-like surface of 3Y-TZP, YN4533, and YN4533/Al20 were 0.09 ± 0.01 μm, 0.09 ± 0.01 μm, and 0.08 ± 0.02 μm, respectively. The surface roughness of the mirror-like surface discs was similar. To increase roughness, zirconia discs were sandblasted with alumina particles. After sandblasting, the roughness of all zirconia discs increased significantly. As a result, the R_a values of the rough surfaces of 3Y-TZP, YN4533, and YN4533/Al20 were 0.62 ± 0.05 μm, 0.72 ± 0.04 μm and 0.71 ± 0.07 μm, respectively. Although there was no significant difference between the rough surface discs ($P > 0.05$), slightly higher R_a values were noted for the (Y, Nb)-TZP discs.

The surface morphologies of zirconia discs were analyzed by using scanning electron microscopy (SEM) (Figure 2). Mirror-like zirconia surfaces showed a smooth and fine dotted pattern, which is assumed to be from the process of sintering. After sandblasting with alumina particles, all zirconia discs exhibited irregular rough patterns. The surface morphologies of 3Y-TZP and (Y, Nb)-TZP discs did not differ significantly and were in good agreement with their R_a values (Figure 1).

2. Cell attachment and morphology analysis

Figure 3 shows MC3T3-E1 pre-osteoblast cells after twenty four hours of culture on the mirror-like and the rough surfaces of zirconia discs. The cells on the mirror-like surface of all zirconia discs showed regular size and morphology and a widely spread cytoskeleton. However, cells on the rough surface exhibited some morphologic irregularities, with a thin cytoskeleton and a less-stretched appearance on all the 3Y-TZP and new (Y, Nb)-TZP discs. Both the newly modified discs displayed good cell attachment similar to 3Y-TZP, and cell to cell contacts were observed on all zirconia discs regardless of surface roughness. Figure 4 shows human gingival fibroblast cells after twenty four hours of culture on the mirror-like surface and the rough surface of zirconia discs. It was found that smooth zirconia surfaces favored fibroblast cells attachment than rough zirconia surfaces.

3. Cellular proliferation assay by PicoGreen

A PicoGreen assay was performed to examine cellular proliferation. Figure 5 shows osteoblast proliferation on the zirconia discs for 1, 4 and 7 days. Cells proliferated well on all zirconia discs and the proliferation rate increased as time went on. Mirror-like surfaces showed higher cell proliferation than rough surfaces, and cell proliferation was highest at day 7. This indicated that MC3T3-E1 cells proliferated well on the smooth zirconia surface. Significant differences were found only between day 4 of 3Y-TZP mirror and YN4533 rough surface groups ($P < 0.05$) and day 7 of 3Y-TZP mirror and YN4533/Al20 rough surface groups ($P < 0.05$). There was no statistically significant difference between cells grown on 3Y-TZP, YN4533, and YN4533/Al20, within the same surface roughness groups ($P > 0.05$). Figure 6 shows fibroblast proliferation on the zirconia discs for 4, 24, 48 and 72 hours. All smooth (Y, Nb)-TZP discs showed significantly higher proliferation when compared with smooth 3Y-TZP discs except 4 and 24 hours results of YN4533-M group ($P < 0.05$). YN4533/Al20-M groups exhibited significant increased proliferation at all experiment time points ($P < 0.05$). Significant decreased proliferation was noted in YN4533/Al20-R at 72 hour ($P < 0.05$).

4. Cellular differentiation by real-time RT-PCR

Quantitative real-time RT-PCR was performed to evaluate mRNA expression levels after 5, 8 and 11 days of culture. Figure 7 (A and B) show the mRNA expression patterns of *Alp* and *Oc*, which are marker genes of osteogenic differentiation. Although the morphology of cells cultured on the rough surface appeared smaller and less stretched, cell differentiation between smooth and rough (Y, Nb)-TZP discs did not differ significantly. Gene expression levels of (Y, Nb)-TZP discs were not influenced by surface roughness, however rough 3Y-TZP discs showed more cellular differentiation than smooth 3Y-TZP discs. Significant differences were found when compared with the 3Y-TZP-M surfaces. Both mirror and rough (Y, Nb)-TZP discs showed significant *Alp* expression at all experiment days except day 5 of YN4533-M compared to that of 3Y-TZP-M, while significant *Oc* levels were seen at all experiment days. Moreover, *Alp* gene expression level of both mirror and rough (Y, Nb)-TZP discs showed significantly higher than that of both mirror and rough 3Y-TZP discs at experiment day 8 ($P < 0.05$), while osteocalcin level showed significantly higher at experiment day 5 ($P < 0.05$). Osteoblast

differentiation patterns on (Y, Nb)-TZP discs were similar to or slightly higher than that of 3Y-TZP. *Alp* staining was also performed to confirm the differentiation capacity of modified zirconia. Cells were stained at differentiation day 10. As shown in Figure 8, (Y, Nb)-TZP discs had a higher differentiation capacity than 3Y-TZP, regardless of surface roughness. Although there were no significant differences in *Alp* and *Oc* gene expression levels, more differentiated osteoblasts tended to be found on (Y, Nb)-TZP in the *Alp* activity results at the protein level to those on 3Y-TZP. Quantitative real-time RT-PCR was also performed to evaluate mRNA expression levels of HGF cells after 4, 24 and 48 hours of culture. Figure 9 (A, B and C) shows the mRNA expression patterns of Type I collagen, Integrin α2 and Integrin β1. The mRNA expressions of type I collagen, integrin α2 and β1 were significantly stimulated for (Y, Nb)-TZP groups at 24 hours after seeding ($P < 0.05$). Type I collagen levels of YN4533-M and YN4533/Al20-R groups at four hours and YN4533-M and YN4533/Al20-M groups at 24 hours were significantly higher than 3Y-TZP-M ($P < 0.05$). Although YN4533/Al20-M group showed significantly reduced integrin α2 and β1 at four hours, all smooth and rough (Y, Nb)-TZP discs exhibited significantly higher levels than 3Y-TZP-M group at 24 hours ($P < 0.05$). However, the integrin β1 level on the (Y, Nb)-TZPs significantly decreased at 48 hours when compared with that on 3Y-TZP-M ($P < 0.05$).

IV. DISCUSSION

Modified zirconia newly combined with yttrium, niobium, and aluminum oxides were developed in this study to overcome the drawbacks of 3Y-TZP. Several researchers have already shown that niobium has higher biocompatibility and osteoconductivity than titanium.[54-56] Other previous studies revealed that the LTD phenomenon in zirconia was substantially reduced by the addition of Nb_2O_5.[31-33,57,58] In order to utilize this advantage of niobium in dental implant treatment, we analyzed the osteogenic potential of (Y, Nb)-TZP and compared with that of the most widely used zirconia ceramics, 3Y-TZP.

There are ample evidences that the increased surface roughness of commercially pure titanium results in higher bone-to-implant contact ratios and removal torque values, or faster osseointegration. This principle is the same for zirconia surfaces. However, it is difficult to

modify a dense, hard zirconia surface to achieve sufficient roughness and this may adversely affect its mechanical strength. The sandblasting technique is the most commonly used technique to increase surface roughness of zirconia.[59] In this study, zirconia discs were sandblasted with alumina particles (Al_2O_3). Albrektsson and Wennerberg classified implants into four different categories depending on their surface roughness (R_a): smooth (R_a < 0.5 μm), minimally rough (R_a between 0.5 and 1.0 μm), moderately rough (R_a between 1.0 and 2.0 μm), and rough (R_a > 2.0 μm).[60] Most currently used titanium implants have a moderately rough surface to facilitate osseointegration.[60,61] Several studies revealed that zirconia, (3Y-TZP) and titanium implants have comparable biocompatibility and osseointegration.[15-18] Although the average roughness values of niobium oxide containing zirconia discs were less than that of current titanium implants, they have a comparable osteogenic potential to titanium.[52]

We found that MC3T3-E1 and HGF cells attach more weakly to rough surfaces than to smooth ones, and this was consistent with the cell morphologies on these two surfaces (Figures 2, 3 and 4). Cellular proliferation was predominant on the mirror-like surfaces (Figure 5 and 6). However, there was no significant difference in cellular proliferation between the experimental and control groups, regardless that the surfaces of those groups were smooth or rough (Figure 5). Cell proliferation rates increased as time went on and highest at day 7 for all zirconia discs. Significant differences were found when compared with the 3Y-TZP-M surfaces, between day 4 of 3Y-TZP-M and YN4533-R surface and day 7 of 3Y-TZP-M and YN4533/Al20-R surface groups. These results are in agreement with a previous study that showed that cells on polished surfaces proliferated more rapidly than those on the rough surfaces,[52] but was not consistent with another study that stated that cell proliferation was significantly greater on rough zirconia surfaces than on smooth surfaces.[62] The sample discs used in this study were minimally rough, while samples from Taniguchi's study were moderately rough.[62] When performing zirconia surface roughing, it is important to achieve the minimum effective roughness without jeopardizing the mechanical properties. In our study, cell morphology and cellular proliferation were associated with and influenced by the surface roughness of zirconia discs. For HGF cells, all smooth (Y, Nb)-TZP discs showed significantly higher proliferation than smooth 3Y-TZP discs except 4 and 24 hours results of YN4533-M

group. YN4533/Al20-M groups exhibited significant increased proliferation at all experiment time points (Figure 6).

Cell differentiation of 3Y-TZP increased with surface roughness. However, although the differentiation patterns of all (Y, Nb)-TZP discs were increased, their osteogenic responses were not influenced by surface roughness. Statistically significant differences were found when compared with 3Y-TZP-M discs. Moreover, *Alp* gene expression level of both mirror and rough (Y, Nb)-TZP discs showed significantly higher than that of both mirror and rough 3Y-TZP discs at experiment day 8, while osteocalcin level showed significantly higher at experiment day 5 (Figure 7). This indicated that (Y, Nb)-TZP have comparable osteogenic potential to 3Y-TZP discs. On the basic of the available data from systematic reviews, osseointegration of 3Y-TZP implants might be comparable to that of titanium implants, however, they are prone to low temperature degradation.[15-18] Our tested bioceramics, a new (Y, Nb)-TZP, has the potential to solve this problem. Bosshardt[18] stated that yttria-stabilized zirconia can be toughened by adding alumina and our study revealed that addition of 20 vol% Al_2O_3 into YN4533 does not affect its osteogenic potential. It was important to note that although osteocalcin levels of new (Y, Nb)-TZP discs increased as time went on, *Alp* activities decreased at day 11. In addition to RT-PCR, we also performed *Alp* staining to confirm the osteogenic potential of modified zirconia (Figure 8). *Alp* staining showed that the osteogenic potential of all zirconia discs increased with surface roughness. *Alp* staining also revealed that new niobium oxide containing zirconia discs have superior osteogenic potential compared to 3Y-TZP, which are biomaterials that have been widely used and already proven for medical and dental restorations.[63,64]

Type I collagen is mainly produced from gingival fibroblasts, osteoblasts and periodontal ligament cells.[65,66] It is an important factor of gingival connective tissue and contributes to rapid periodontal tissue regeneration and maintenance of tissue architecture.[67.68] In this study, the mRNA levels of type I collagen were significantly higher on smooth (Y, Nb)-TZP discs at 24 hours. Interestingly, the mRNA levels of integrin α2 and β1 showed almost similar patterns at each time points and significantly higher on (Y, Nb)-TZP groups than 3Y-TZP-M at 24 hours (Figure 9 A, B and C). These integrin subunits were identified in the periodontal tissue regeneration and they regulate cellular functions, cell proliferation, adhesion, shape, and differentiation.[68] Both α1β1 and α2β1 integrins are cell surface receptors for type I

collagen in fibroblasts, and acts as a positive regulator of type I collagen gene expression.[69] Type I collagen mRNA expressions of (Y, Nb)-TZPs were high at 24 hours and then decreased at 48 hours, and it was similar in integrin α2 and β1. The results of this study support the fact that integrin α2 and β1 gene expressions are closely related with type I collagen gene expression and these results are in agreement with the previous study.[53] Although cellular proliferation rates depend on surface roughness of the zirconia discs, mRNA gene expressions were not influenced by the surface roughness.

The results of this study indicate that (Y, Nb)-TZP has significant potential for use as an implant biomaterial. Niobium oxide, which is contained in new modified zirconia discs, has shown excellent biocompatibility and osteogenic potential.[37,39] Besides, oxygen ions in niobium oxide may stabilize the tetragonal structure, resulting in enhanced crack resistance and biaxial strength in addition to resistance to LTD. This study revealed that new (Y, Nb)-TZP discs, YN4533 and YN4533/Al20 have comparable or superior osteogenic response to 3Y-TZP and favorable soft tissue response when considering alternative titanium bioceramic implants. However, further *in vivo* studies might be necessary to confirm good osteogenic potential, proper peri-implant soft tissue integration, and the mechanical strength of (Y, Nb)-TZP bioceramics (YN4533 and YN4533/Al20) in the form of implant fixtures. Limitation of this study is no evaluation on tendency of moisture related LTD in new (Y, Nb)-TZP. And titanium as well as 3Y-TZP needs to be included in further studies as another control.

V. CONCLUSION

Within the limitation of this study, (Y, Nb)-TZPs provide appropriate surface condition for osseointegration at the implant level and for peri-implant mucosal sealing at the abutment level. (Y, Nb)-TZPs are expected to be a suitable candidate for ceramic dental implants with favorable clinical outcomes.

-FIGURES-

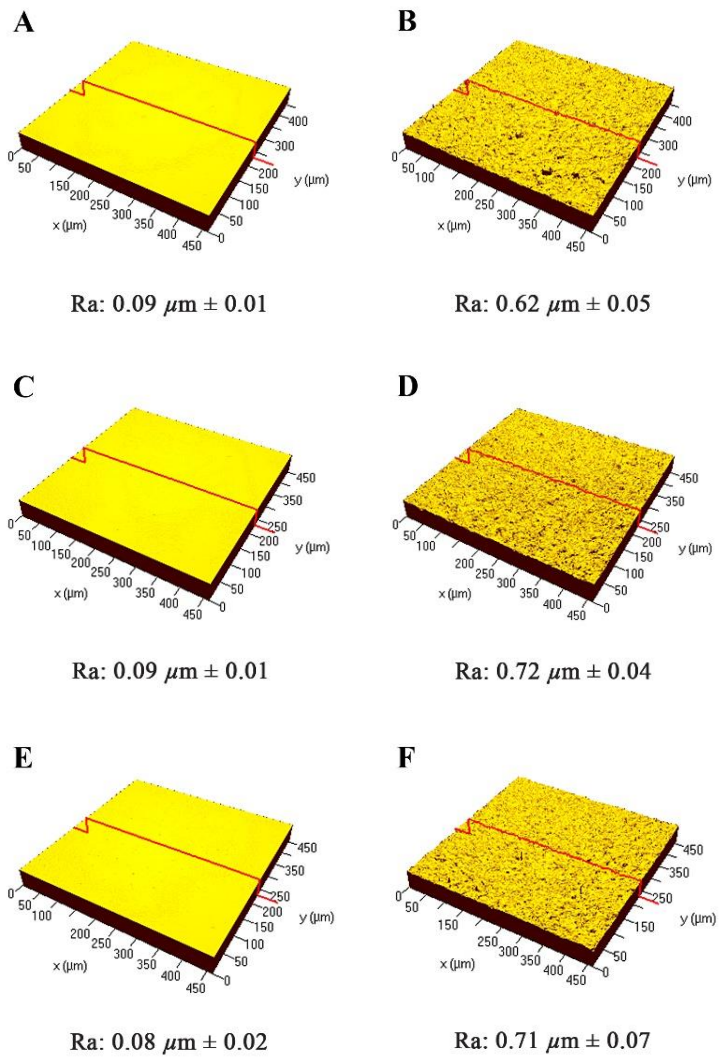


Figure 1. 3-D confocal laser scanning microscopy (3D-CLSM) images show the roughness R_a values of zirconia discs. (A) 3Y-TZP-M, (B) 3Y-TZP-R, (C) YN4533-M, (D) YN4533-R, (E) YN4533/Al20-M, (F) YN4533/Al20-R. M represented for mirror-like surface and R represented for rough surface.

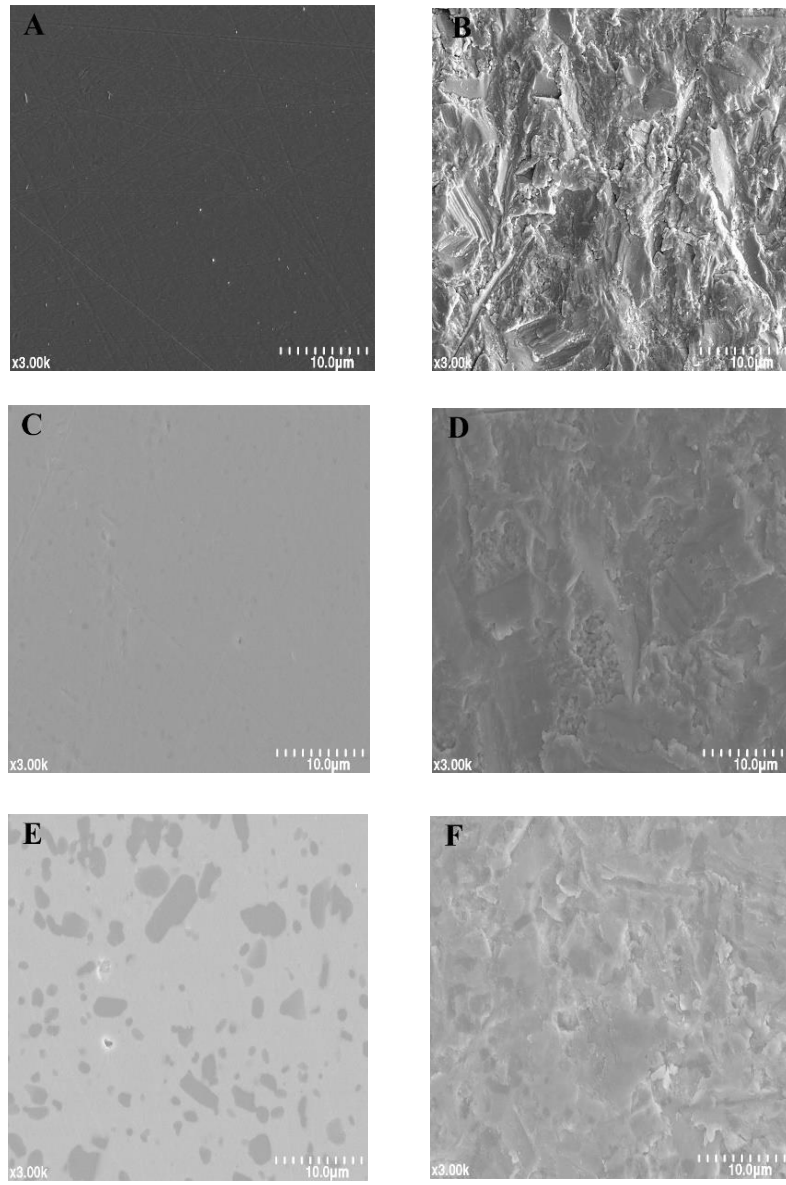


Figure 2. Scanning electron microscopy (SEM) images of zirconia discs. (A) 3Y-TZP-M, (B) 3Y-TZP-R, (C) YN4533-M, (D) YN4533-R, (E) YN4533/Al20-M, (F) YN4533/Al20-R. X3000 magnification. M represented for mirror-like surface and R represented for rough surface.

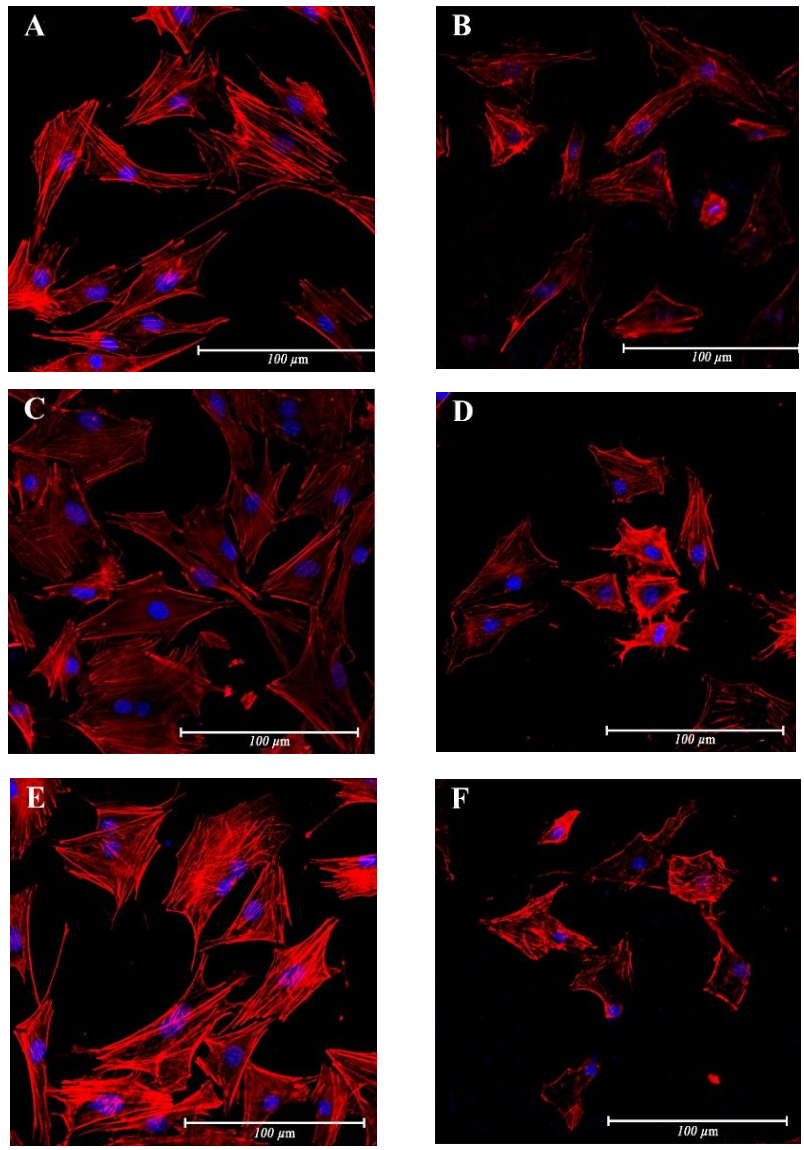


Figure 3. Microscope observation 24 hours after MC3T3-E1 cells were seeded onto the zirconia discs. (A) 3Y-TZP-M, (B) 3Y-TZP-R, (C) YN4533-M, (D) YN4533-R, (E) YN4533/Al20-M, (F) YN4533/Al20-R. Original magnification is X200 and the scale bar is 100 μm. M represented for mirror-like surface and R represented for rough surface.

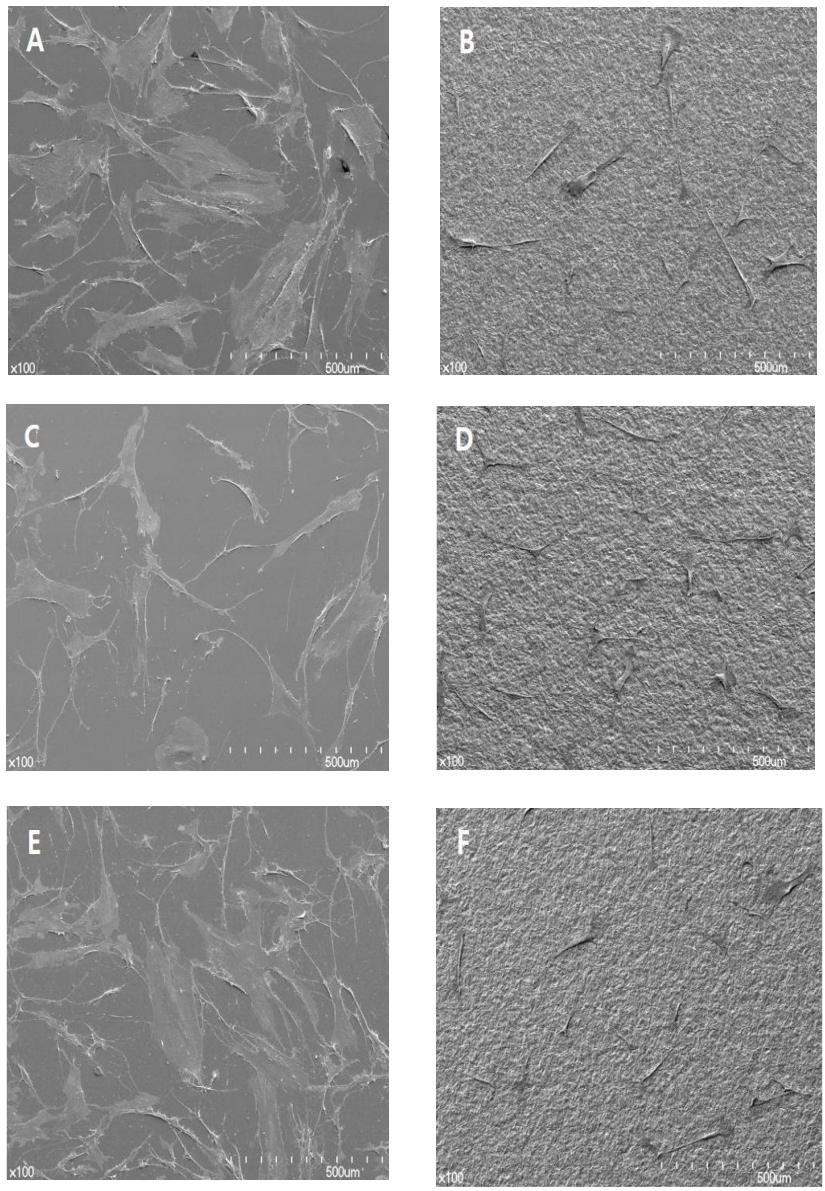


Figure 4. Microscope observation 24 hours after HGF cells were seeded onto the zirconia discs. (A) 3Y-TZP-M, (B) 3Y-TZP-R, (C) YN4533-M, (D) YN4533-R, (E) YN4533/Al20-M, (F) YN4533/Al20-R. Original magnification is X100 and the scale bar is 500 μm. M represented for mirror-like surface and R represented for rough surface.

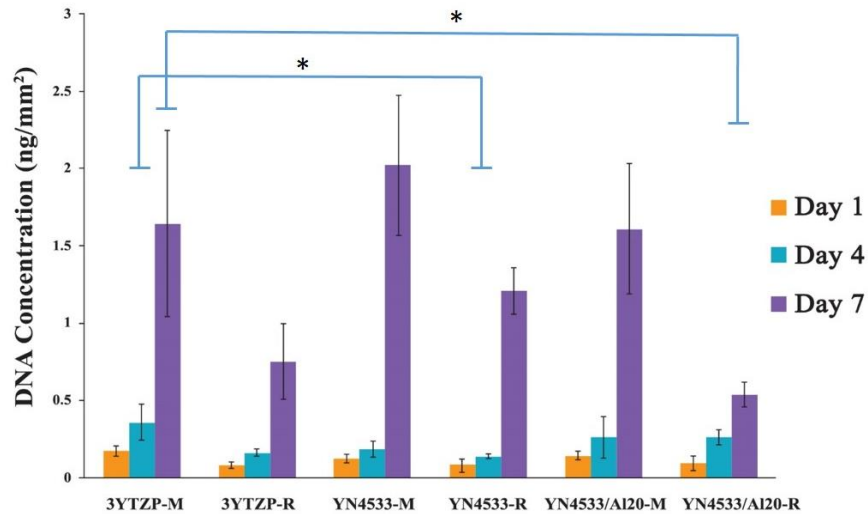


Figure 5. Cellular proliferation (PicoGreen assay) of MC3T3-E1 on the zirconia discs at days 1, 4, and 7. Data are expressed as the mean ± standard deviation. Significant differences (*) were denoted by Tukey and two-way analysis of variance tests at $P < 0.05$.

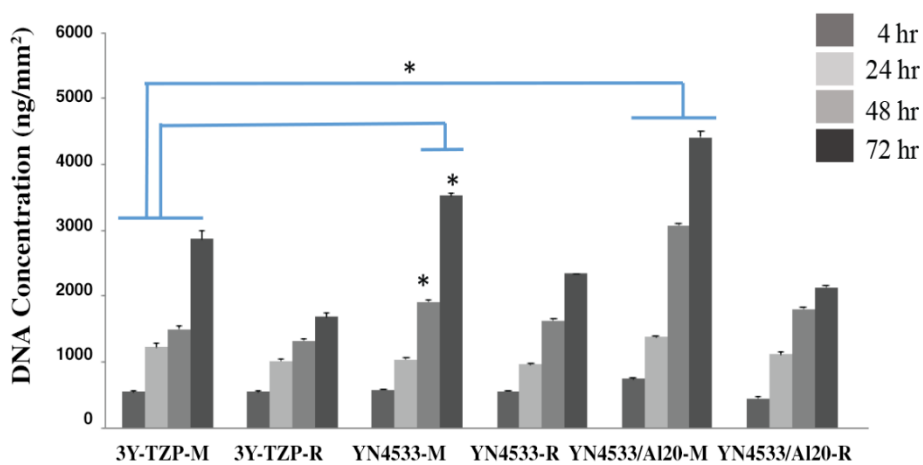


Figure 6. Cellular proliferation (PicoGreen assay) of HGF on the zirconia discs at 4, 24, 48 and 72 hours. Data are expressed as the mean ± standard deviation. Significant differences (*) were denoted by Tukey and two-way analysis of variance tests at $P < 0.05$.

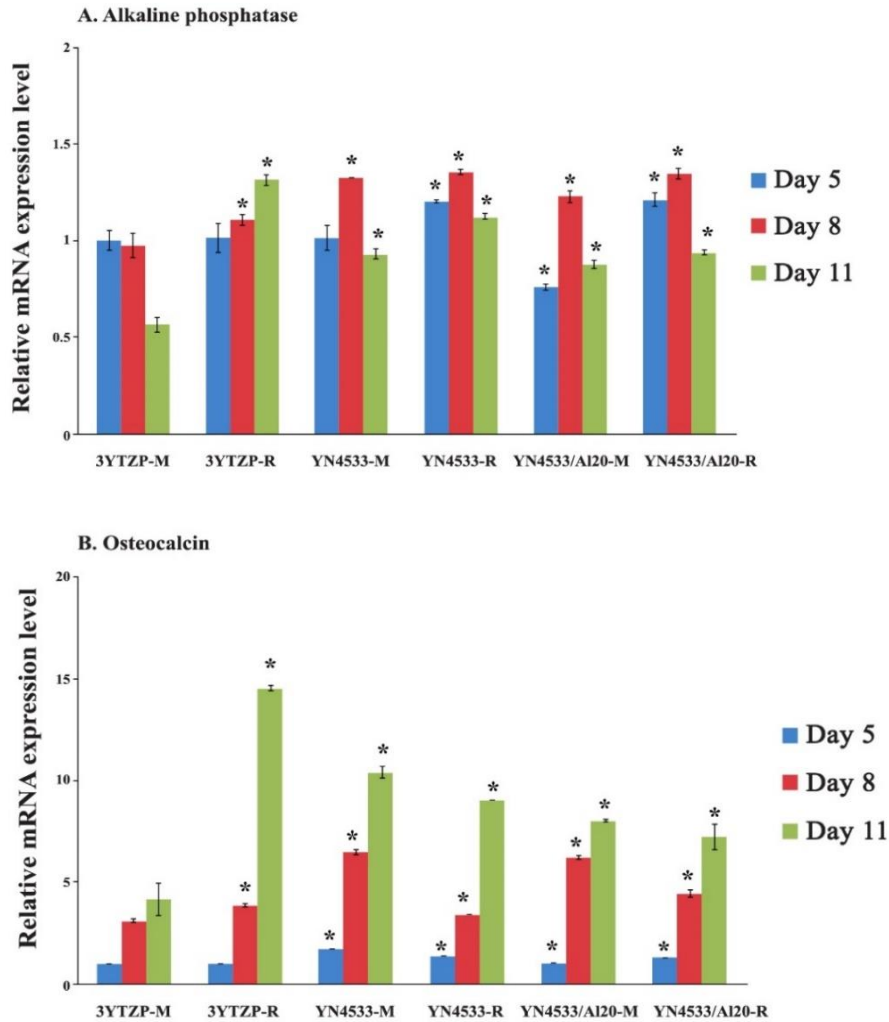


Figure 7. Real-time RT-PCR analysis of MC3T3-E1 cells on the zirconia discs after 5, 8 and 11 days of culture in osteogenic medium for both (A) Alkaline phosphatase (*Alp*) and (B) Osteocalcin (*Oc*). Data are expressed as the mean ± standard deviation. Significant differences (*) were evaluated using Tukey and two-way analysis of variance tests at $P < 0.05$. Significant differences were found when compared with 3Y-TZP-M.

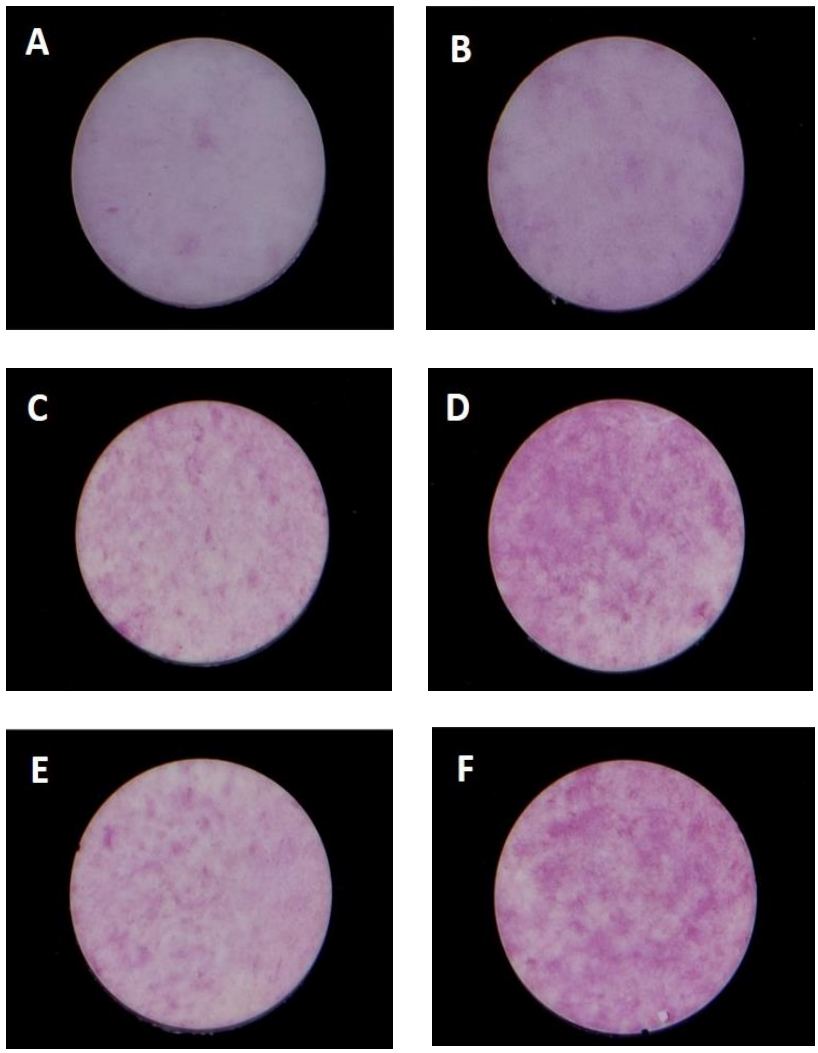
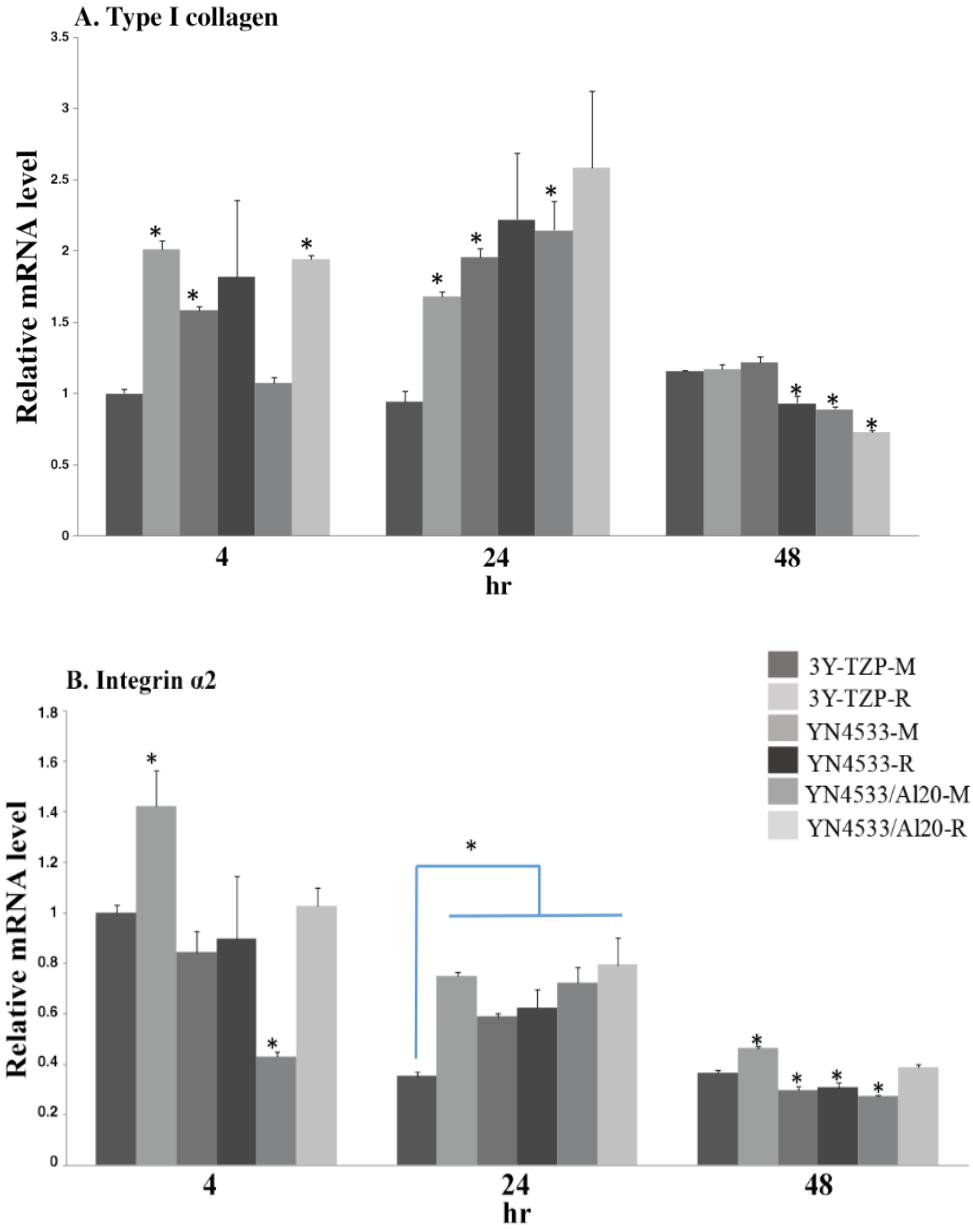


Figure 8. *Alp* staining 10 days after cells were seeded on the zirconia discs and cultured in osteogenic medium. (A) 3Y-TZP-M, (B) 3Y-TZP-R, (C) YN4533-M, (D) YN4533-R, (E) YN4533/Al20-M, (F) YN4533/Al20-R. M represented for mirror-like surface and R represented for rough surface.


A. Type I collagen
Relative mRNA level
3.5
3
2.5
2
1.5
1
0.5
0
4
24
48
hr
B. Integrin α2
Relative mRNA level
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
4
24
48
hr
3Y-TZP-M
3Y-TZP-R
YN4533-M
YN4533-R
YN4533/Al20-M
YN4533/Al20-R

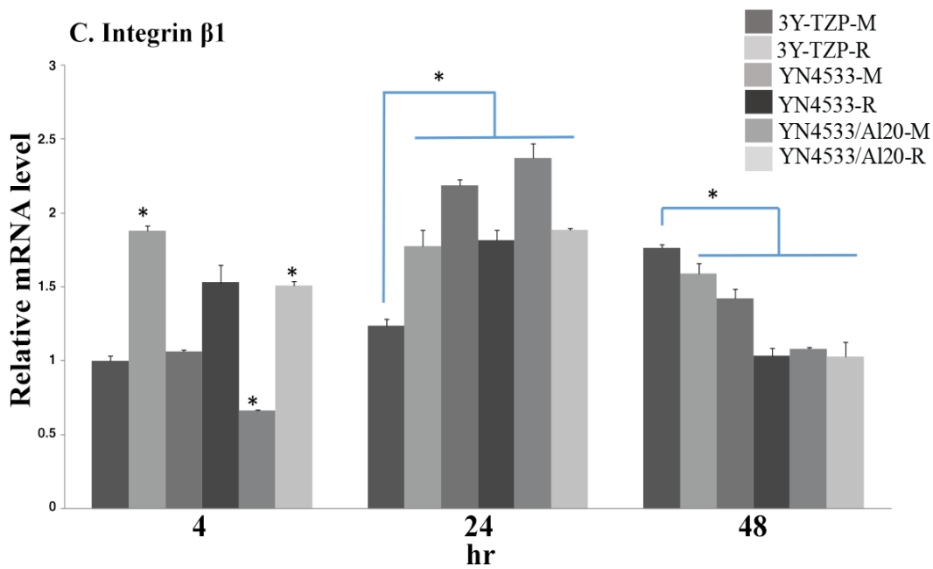


Figure 9. Real-time RT-PCR analysis of HGF cells on the zirconia discs after 4, 24 and 48 hours of culture (A) Type I collagen (B) Integrin α2 and (C) Integrin β1. Data are expressed as the mean ± standard deviation. Significant differences (*) were evaluated using Tukey and two-way analysis of variance tests at $P < 0.05$. Significant differences were found when compared with 3Y-TZP-M.

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국문초록

Niobium oxide 를 포함한 지르코니아와 yttria-stabilized 지르코니아의 골형성능과 연조직 반응에 대한 비교 연구

서울대학교 대학원 치의과학과 치과보철학

(지도교수: 한중석)

엉투헤인

연구목적

본 *in vitro* 연구는 3 mol% yttria-stabilized Tetragonal Zirconia Polycrystals (3Y-TZP) 과 niobium oxide 를 포함하는 특정 성분 구성의 (Y, Nb)-TZP 인 YN4533 과 YN4533/Al20 디스크의 골형성 잠재력과 human gingival fibroblast 반응을 평가하고자 하고자 한다.

연구대상 및 방법

매끄러운 3Y-TZP, YNY533 및 YNY533Al20 디스크와 거친 디스크(직경 15mm, 두께 1mm, 각 그룹 20 개)의 표면 특성을 confocal laser scanning microscopy (CLSM)과 scanning electron microscopy (SEM)을 이용하여 분석하였다. Murine pre-osteoblast MC3T3-E1 cell 과 human gingival fibroblast cell 을 모든 지르코이나 디스크에서 배양하였다. 배양 24 시간 후, CLSM 과 SEM 을 이용하여 세포 부착을 평가하였다. PicoGreen assay 를 통해 osteoblast 에 대하여 1, 4 그리고 7 일 후, fibroblast 에 대하여 4, 24, 48 그리고 72 시간 후에 세포 증식능을 평가하였다. Real time RT-PCR 을 이용하여 5, 8 그리고 11 일 후에

alkaline phosphatase (*Alp*)와 osteocalcin 의 mRNA 유전자 발현을 평가하였다. *Alp* activity 는 단백질 수준에서도 평가하였다. 또한, real time RT-PCR 을 이용하여 4, 24 그리고 48 시간 후에 type I collagen, integrin α2, β1 의 mRNA 유전자 발현을 평가하였다. 그룹 간의 결과를 피교하기 위하여 two-way ANOVA 을 이용하였다. 0.05 의 significance level 에서 모든 데이터를 분석하였다.

결 과

MC3T3-E1 pre-osteoblasts 와 human gingival fibroblast 모두 거친 표면보다 매끄러운 표면에서 더 넓게 분포되었다. Osteoblast 와 fibroblast 모두 세포 증식능은 매끄러운 표면에서 높게 관찰되었고, 3Y-TZP, YN4533 그리고 YN4533/Al20 사이에서는 유의한 차이가 없었다. RT-PCR 과 alp activity 의 결과를 통해, Osteoblast 의 분화는 YN4533 과 YN4533/Al20 이 3Y-TZP 보다 약간 더 높은 것으로 관찰되었다. type I collagen, integrin α2, β1 의 mRNA 유전자 발현은 24 시간 후에 (Y, Nb)-TZP 에서 상당히 자극을 받은 것으로 관찰되었다.

결 론

이 연구의 한계 내에서, (Y, Nb)-TZP 는 implant level 에서의 osseointegration 과 abutment level 에서의 peri-implant mucosal sealing 을 위한 적절한 표면 조건을 나타낸다. (Y, Nb)-TZP 는 ceramic dental implant 에서 좋은 임상 결과를 나타내는 적절한 재료가 될 것으로 예상된다.

주요어: 치과임플란트, 니오비움, 지르코니아, 저온열화, 골형성성능, 섬유아세포

학번: 2015-30784