치의학석사학위논문

Osteogenic potential of the modified zirconia based bioceramics: in vitro study

지르코니아의 조성 및 표면처리에 따른 조골세포 분화능에 대한 연구

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-ABSTRACT-

Osteogenic potential of the modified zirconia based bioceramics: in vitro study

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(Directed by Professor **Jung-Suk Han**, DDS, MS, PhD)

Objective: The aim of this study was to evaluate the osteogenic effect of zirconia modified with Tantalum oxide (Ta_2O_5) and Niobium oxide (Nb_2O_5) for preventing low temperature degradation which is a major problem described in previous studies and to evaluate possibility of using zirconia as an implant material instead of titanium.

Materials and Methods: The surface properties were evaluated by Confocal Laser Scanning Microscopy (Zeiss, Germany) and Scanning Electron Microscopy (SEM). Osteoblastic MC3T3-E1 cells were cultured on Ti-m (machined Ti), Ti-a (anodized titanium), (Y, Ta)-TZP (sandblasted withAl_2O_3 particles), (Y, Nb)-TZP (sandblasted with Al_2O_3 particles) discs. Cell attachment evaluation was performed by Confocal Laser Scanning Microscopy after 24 hours of cell culturing. The PicoGreen assay with the Quant-iT PicoGreen assay kit (Invitrogen Ltd., Paisley,

UK) was used to investigate cell proliferation at 1, 3 and 7 days. The mRNA expressions of ALP (alkaline phosphatase) and OC (osteocalcin) were measured by real time PCR at 3, 7 and 10 days in the osteogenic media.

Results: The average roughness values (Ra) of surface modified Ti and zirconia specimens were different. Both Ti and Zr surface showed favorable cell attachment reaction after 24 hours culture regardless of surface roughness. Cell proliferation on the smooth surface (Ti-m) was higher than those of rough surfaces Ti-a, (Y, Ta)-TZP and (Y, Nb)-TZP. Osteogenic response on the rough surfaces Ti-a, (Y, Ta)-TZP and (Y, Nb)-TZP was higher than that of smooth surface (Ti-m). Cells on the (Y, Ta)-TZP and (Y, Nb)-TZP discs showed similar osteogenic potential with Ti-a.

Conclusions: New (Y, Nb)-TZP and (Y, Ta)-TZP composites have similar osteogenic potential with titanium in cell culture experiment and may be suitable for implant fixture material. Further in vivo research is demanded to confirm biocompatibility of new zirconia composites.

Keywords: Dental implant, Zirconia, Titanium, Osteoblast, Bone healing, Biocompatibility
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I.INTRODUCTION

Different kind of biomaterials has been used to dental implants. Among them, commercially pure titanium has becomechoice of the implant material with excellent mechanical properties and biocompatibility [1-5]. However, its grey color sometimes raised esthetic problems when the quantity of soft tissue was not enough to mask it at the gingival area or bone and soft tissue resorption were anticipated [6]. Additionally, allergic reactions and sensitivities of titanium have been reported [7]. To solve problems of the allergic reaction and esthetics caused by titanium implants, a ceramic implant was developed as a viable alternative. Alumina and zirconia are of increasing popularity because of their light transmittance quality and color [8]. However, fracture resistance of alumina is inferior to those of titanium or zirconia [9, 10]. Therefore, zirconia has recently attracted significant interest because of its improved biocompatibility, high strength and fracture toughness and translucency which make it an ideal candidate for use in esthetic restorations and implant abutment and fixture [11, 12]. There were several studies on bone response to zirconia. One study performed by Sennerby et al. demonstrated comparable bone implant contact result in rabbit study [11]. One of the most undesirable complications in implant dentistry is the fracture of the fixture. It happened both titanium and 3Y-TZP implant [13].

Ziconia exists in three phases, monoclinic (M), cubic (C) and tetragonal (T) depending on temperature. M-phase is fragile at room temperature, therefore it should be stabilized by formation solid solutions to prevent tetragonal (T) to monoclinic (M) phase transformation for its technical application [14, 15]. Yittria

(Y_2O_3) is used for general stabilizer for maintaining T-phase ZrO_2. Y_2O_3-stabilized tetragonal zirconia polycrystals (Y-TZP) have properties of high strength and toughness and biocompatibility and proper biological response at bone/zirconia interaction are similar with titanium [11, 16, 17], therefore, Y-TZP have been considered as potential titanium alternative. However, structural instability by low temperature degradation (LTD, often referred as 'aging') due to the tetragonal (T) to monoclinic (M) phase transformation in the moisture or stress conditions is strong drawback of zirconia. Until 2001, because transformation rate is the highestaround 250℃, it was considered negligible factor in in vivo temperature (37℃) [18], but several failures in the use of hip prosthesis were reported clinically[19, 20]. Therefore, many efforts to inhibit phase transformation by LTD in the low temperature have been tried, among them, LTD is avoided by including stabilizers, niobium oxide (Nb_2O_5) [21, 22] or tantalum oxide (Ta_2O_5) [23]. Unlike Y_2O_3, the alloying of Ta_2O_5 or Nb_2O_5 raises T-M transformation temperatures, which contributes to the increase in the fracture toughness of Y-TZP[24, 25]. Interactions at the cell–material interface play an important role in determining the success of the implant by assessing the processes of cell adhesion, proliferation and differentiation on the material surface [26].

In this background, we have examined the biological behavior of osteoblasts on roughened pure Ti, (Y, Ta)-TZP and (Y, Ta)-TZP disks. Our study has focused on comparing the adhesion, proliferation and differentiation of mouse calvarial osteoblasts (MC3T3-E1) on them. The purpose of present study is to evaluate

osteogenic potential in the 3Y-TZP co-doped with Ta_2O_5 or Nb_2O_5 which compensate the structural defect in vivo usage.

II.MATERIALS AND METHODS

Specimen preparation

Pure titanium specimens were prepared in disc shape (25 mm in diameter and 1 mm in thickness) through machining (Ti-m, Ti-machined) and treated by anodizing (Ti-a, Ti-anodizing) (OnePlant System, Warrantec Co., Ltd, Korea). Y-TZP powders mixed with 4 mol% of Nb_2O_5 ((Y, Nb)-TZP) or 6.5 mol% of Ta_2O_5 ((Y, Ta)-YZP) were used the starting materials in this study. Disc shaped green compacts (15 mm in diameter and 1 mm in thickness) were prepared by cold isostatic press at 200 MPa and then sintered for 2h at 1650℃ in air. All zirconia discs were gradually polished and finished with diamond pasts to acquire mirror like surface. After polishing, (Y, Ta)-TZP and(Y, Nb)-TZP were sandblasted with 50 μm alumina (Al_2O_3) with 1 bar and 2 bar for 1 min to make a similar rough surface respectively.

Surface roughness assessment

The average surface roughness (Ra) and surface topography were measured using a confocal laser microscope (Zeiss, Germany). Surface morphology of specimens was observed by a scanning electron microscope (JEOL, Japan) after sputter coating with platinum (Pt).

Cell culture

Mouse pre-osteoblast MC3T3-E1 cells were purchased from ATCC (Manassas, VA,USA) and were cultured on the Ti-m, Ti-a, (Y, Ta)-TZP, (Y, Nb)-TZP discs. Discs were cultured in α-minimal essential medium (α-MEM) which contains10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Osteogenic media includes 10mMβ-glycerophosphate and 50 ug/mL ascorbic acid.

Cell attachment analysis

Confocal microscopy observation was performed after 24 hours of cell culturing. Cells on the discs were fixed in 4 % formaldehyde and 4', 6-diamidino-2-phenylindole (DAPI, Invitrogen) was used for detection cell nucleus, and Alexa Fluor 488® phalloidin (Invitrogen) was used for detection of cytoskeleton. Fluorescence was visualized with a Carl Zeiss LSM700 microscope and analyzed with ZEN2011 software (Carl Zeiss, Oberkochen, Germany).

Cell proliferation assay

The PicoGreen assay was performed using the Quant-iT PicoGreen assay kit (Invitrogen Ltd., Paisley, UK) at 1, 3 and 7 days. MC3T3-E1 cells on the discs were washed with PBS and lysed using TE buffer (10 mMTris-HCl, 1 mM EDTA, pH 7.5). The DNA content was determined by mixing 100 μL of PicoGreen reagent and 100 μL of DNA sample. Samples were loaded in triplicate and florescence intensity was measured on a GloMax-Multi Detection System machine (Promega,

Madison, WI). Values are represented mean±SD of three independent measurements.

Cell differentiation Assay

Reverse transcription PCR and quantitative Real-Time PCR

RNA was isolated at 3, 7 and 10 days, using QIAzollysis reagent (QIAGEN, Valencia, CA,USA).The Primescript^{TM}RT reagent kit for reverse transcription was purchased from TAKARA(Takara Bio, JAPAN).Quantitative real-time PCR was performed with the primer sets, type I collagen, alkaline phosphatase (ALP) and osteocalcin (OC) as previously described [27]. Quantitative real-time PCR for murine ALP and OC were performed with the primer sets, ALP (forward)5'-GGCTACATTGGTCTTGAGCTTTT-3',ALP (reverse)5'-CCAACTCTTTTGTGCCAGAGA-3', and OC (forward) 5'-CTGACAAAGCCTTCATGTCCAA-3', OC (reverse) 5'-GCGCCGGAGTCTGTTCACTA-3'by using Takara SYBR premix Ex Taq (Takara Bio, JAPAN) on Applied Biosystems 7500 Real Time PCR system (Foster city, CA). PCR primers were synthesized by Integrated DNA technology (*IDT*; Coralville, IA). All samples were run in duplicate, and the relative levels of mRNA expression level were normalized to those of glyceraldehyde-3-phosphatedehydrogenase (Gapdh).

Statistical Analysis

All quantitative data are presented as the mean and standard deviation (± SD). Each experiment was performed at least three times, and the results from one representative experiment are shown. Significant differences were analyzed using Student's t-test. A value of $p < 0.05$ was considered as statistically significant.

III.RESULTS

Surface topography of the substrates

The average roughness value (Ra) of specimens of each groupwas shown in Fig 1. Ra of (Y, Nb)-TZP sandblastedgroup (0.819 μm ± 0.05 μm) was significantly higher thanmirror plished (Y, Nb)-TZP group (0.092 μm± 0.001 μm), and (Y, Ta)-TZP sandblasted group was significantly higher (0.880 μm± 0.06μm) than mirror polished(Y, Ta)–TZP group (0.096 μm± 0.001 μm).

Topography of the different surfaces was revealed by SEM as shown in Fig 2. Sandblasted (Y, Nb)-TZP and (Y, Ta)-TZP specimens demonstrated rough surface because of the impact of sandblast particles. On the other hand, Ti-a surface showed porous oxide layer compared with the Ti-m surfaces. At high magnification, many dark spot can be seen on mirror polished (Y, Nb)-TZP and (Y, Ta)-TZP surfaces.

Cell attachment analysis

Twenty four hours after cell culturing, confocal laser scanning images of MC3T3 – E1 cells showed that cells adhered on both titanium and zirconia surfaces (Fig.3). Well spread polygon shape cytoplasm and distinct nucleus were observed on both titanium and zirconia specimens regardless of surface morphology.

Cell proliferation assay by Picogreen

The results of proliferation assay were shown in Fig 4, at different time periods of 1, 3and 7 days. 1 day after culturing, concentration of DNA was not statistically different among all groups of surfaces. However, cell proliferation was significantly higher on Ti-m group than those of zirconia groups after 3 and 7 days culture period. Other three groups, Ti-a group, (Y, Nb)-TZP and (Y, Ta)–TZP group showed no statistically significant difference in cell proliferation up to 7 days ($P<0.05$).

Cell differentiation assay

For investigating osteoblastic differentiation, real time PCR was performed at 3, 7 and 10 days after culturing. The mRNA expressions of osteoblast differentiation marker genes, type I collagen (Fig. 5A), ALP (Fig. 5B) and OC (Fig. 5C) showed well differentiation patterns.

The ALP expression level of Ti-a and(Y, Nb)-TZP group showed significantly higher than Ti-m and (Y, Ta)-TZP group after 3 and 7 days culture. There was no significant difference between Ti-a and (Y, Nb) - TZP group. All three groups with rough surfaces showed more active ALP expression than that of Ti-m group. (Y, Ta)-TZP group showed slightly less ALP expression among three roughened groups.

OC expression was continuously increased up to 10 days in all groups. Especially, Ti-a and (Y, Ta)-TZP group showed remarkable OC expression after 7 days culture period (Fig 5C).

IV. DISCUSSION

There has been an attempt to develop tooth colored implant fixtures to fulfill patient's and clinician's demand for restoring esthetics and function of loss of teeth. Zirconia based material was investigated and developed to utilize as an implant fixture. Among them, 3Y-TZP and alumina roughened zirconia were the materials which have high strength and fracture toughness due to its transformation toughening characteristics. However, innate low temperature degradation of zirconia has been a problem for a long term success of implant fixture. Recently fracture of small diameter zirconia implant fixture made with 3Y-TZP was reported clinically. Even though 3Y-TZP has superior esthetic properties to Ti implant, low temperature degradation phenomenon of this material still needed to be solved for

long term stability as an implant fixture. Therefore, several attempts were tried to overcome LTD of zirconia. Development of (Y, Nb)-TZP with free of LTD was one of the examples [6] of this kind of effort.

One of the factors that cause the zirconia implants to induce stronger and faster bone responses is the surface roughening. The surface roughened zirconia implants showed superior bone response than machined surface implants in histomorphometrical assay [11, 16, 17]. Surface characteristics of implant materials such as topography, surface chemistry and energy are important in the initial phase of cell interactions and the quality of this stage will influence the capacity of cell proliferation and differentiation [26]. The Ra of (Y ,Nb)-TZP and (Y, Ta)-TZP sandblasted group was significantly higher than machined group.

Twenty four hours after cell culturing, confocal laser scanning images of MC3T3–E1 cells showed that the cells properly adhered on both titanium and zirconia surfaces (Fig.3).

At first day of cell culturing, DNA concentration was not significantly different between all groups of surfaces. However, at 3 and 7 days after cell culturing, cell proliferation was significantly highest in Ti-m group (Fig 4). On the other hands, osteoblast differentiation is predominant in the rough surfaces Ti-a, (Y, Nb)-TZP and (Y, Ta)-TZP and they showed well differentiated gene expression patterns (Fig. 5).The ALP activity increased at 7 day and dropped at 10 days after culturing.

We found that no difference in OC mRNA expression during the culture period up to 3 days in all groups. However, the OC expression level at 7 days, cells on Ti-a specimen expressed significantly higher than others. At 10 days, OC expression was higher on Ti-a and (Y, Ta)-TZP discs than others (Fig.5).

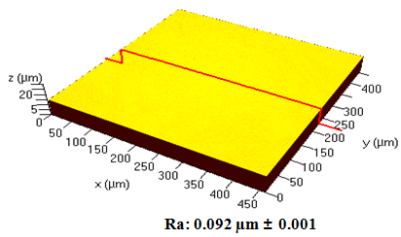
The present study results show that the overall cell response to pure titanium and new zirconia composite materials (Y, Nb)-TZP and (Y, Ta)-TZP was comparable. It proved previous study about new zirconia composite ceramic which showed similar biological response of osteoblast like cells during a short time cell culture period [6]. At the same time, previous studies have shown that osseointegration behavior of zirconia implants is as good as titanium implant [17, 28, 29]. Further study needs to confirm our results on biomechanical and histological aspects and the long-term stability of these new zirconia composition materials.

V.CONCLUSION

New (Y, Nb)-TZP and (Y, Ta)-TZP composites have similar osteogenic potential with titanium in the cell culture experiment and may be suitable for implant fixture material. Further in vivo researches are demanded to confirm biocompatibility result of composition and surface modified zirconia.

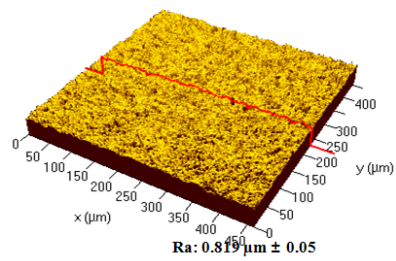
FIGURES

A. (Y ,Nb)-TZP



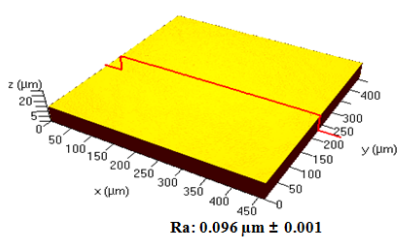
Ra: 0.092 µm ± 0.001

B. (Y,Nb)-TZP
+ sandblasting (50 um, 2bar)



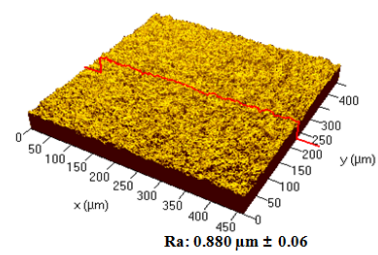
Ra: 0.819 µm ± 0.05

C. (Y,Ta)-TZP



Ra: 0.096 µm ± 0.001

D. (Y,Ta)-TZP
+ sandblasting (50 um, 1bar)



Ra: 0.880 µm ± 0.06

Figure1.Three-dimensional image showing the microtopography and roughness (Ra) of the examined substrate surfaces (50 µm x 50 µm). A. (Y, Nb)-TZP, B.(Y, Nb)-TZP sandblasted, C. (Y, Ta)-TZP, D. (Y, Ta)-TZP sandblasted

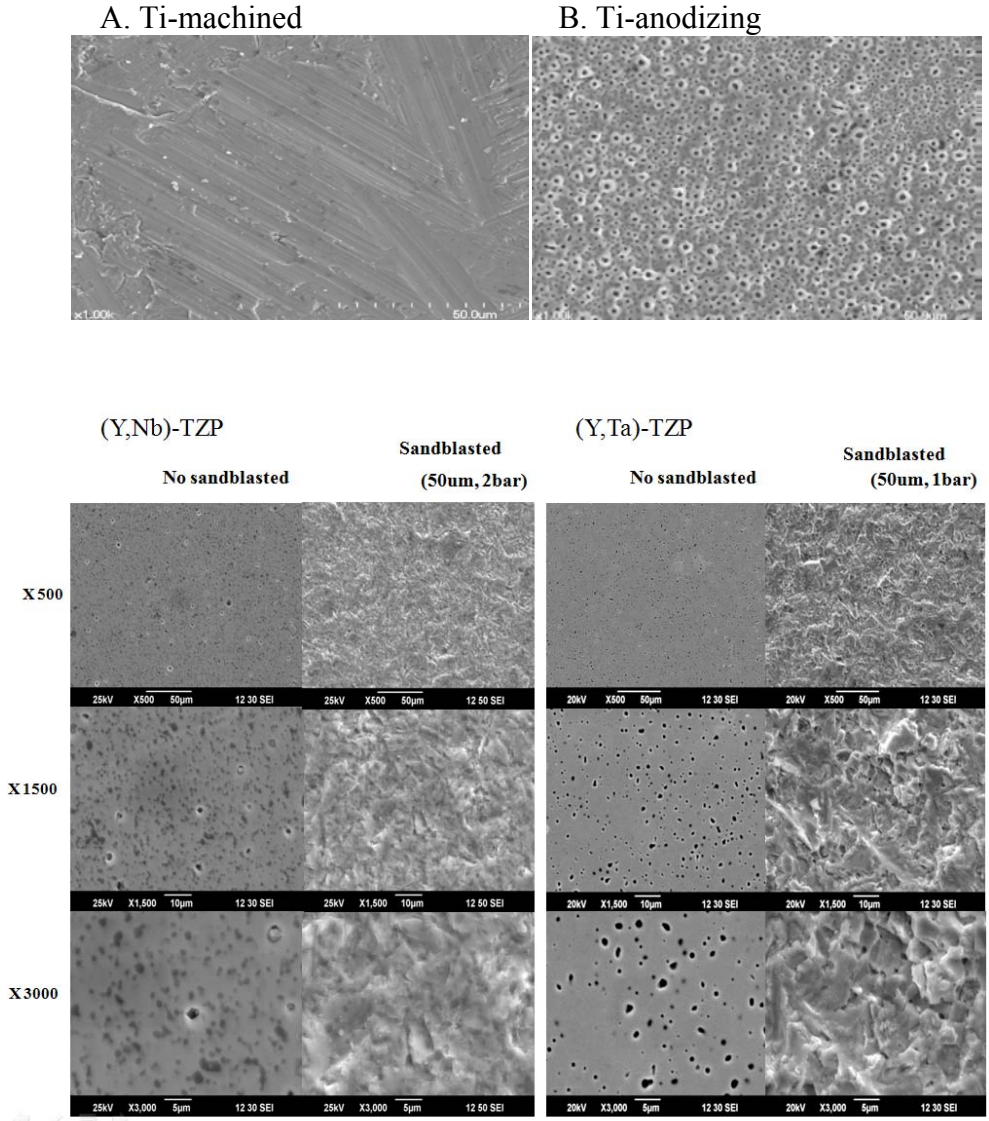


Figure 2.SEM images of Ti-machined, Ti-anodizing, smooth and sandblasted (Y, Nb)-TZP and (Y, Ta)-TZP surface. Irregular surface morphology was observed after sandblasting

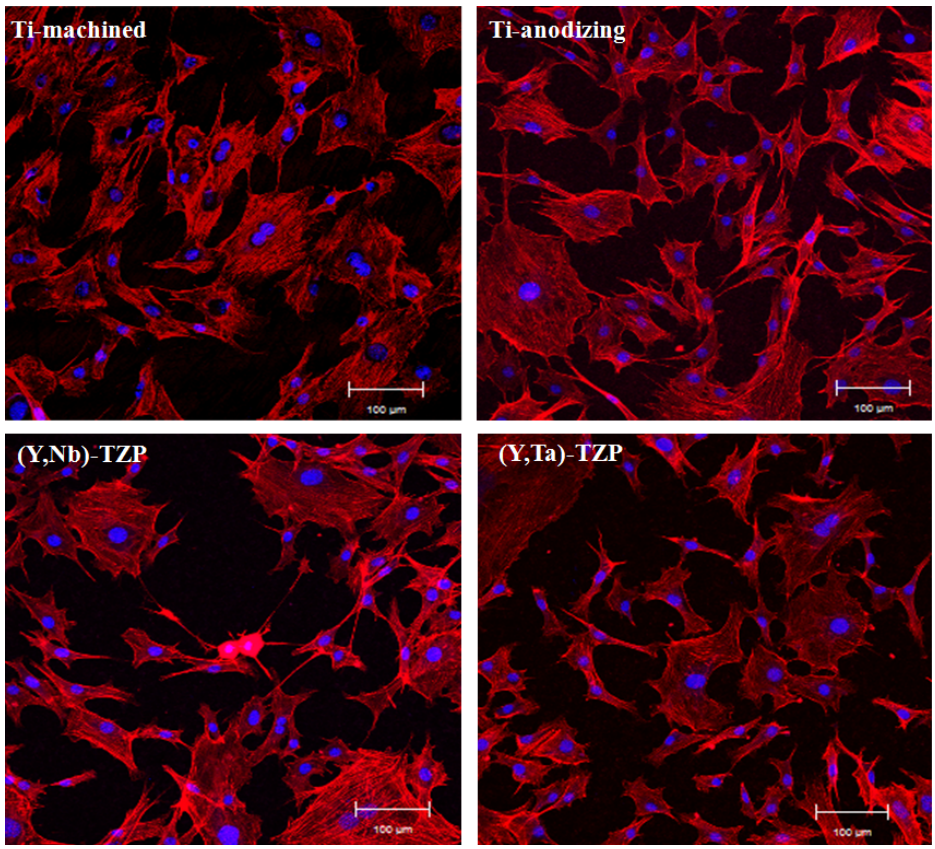


Figure 3.Confocal microscopic images of MC3T3-E1 cell attachment on the 24 hours after cell culture.4', 6-diamidino-2-phenylindole (DAPI) was used for detection cell nucleus and rhodaminephalloidin was used for detection of cytoskeleton.

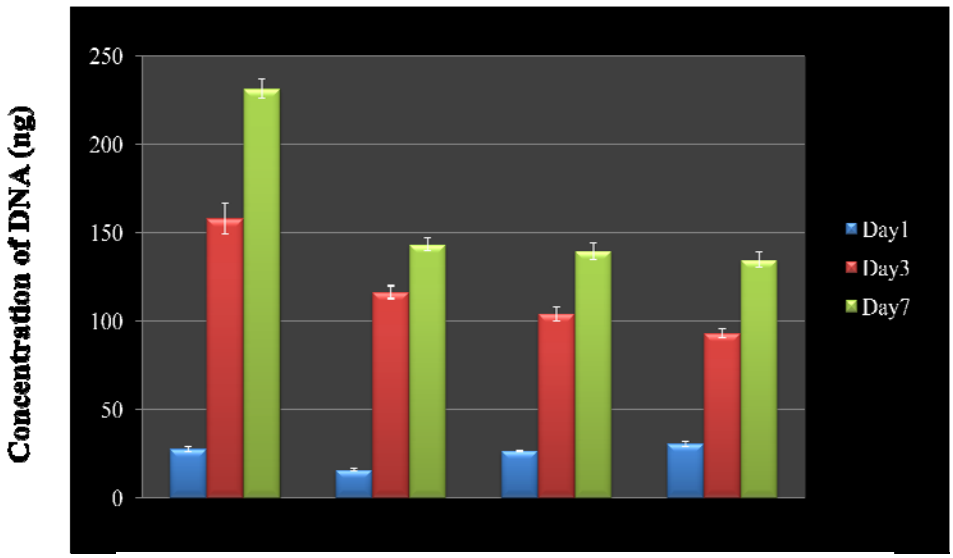


Figure 4.Cell proliferation assay (Picogreen) of MC3T3-E1 cells seeded on the Ti and Zr- discs at days 1, 3 and 7. Data are expressed as the mean ± S.D of three independent experiments.

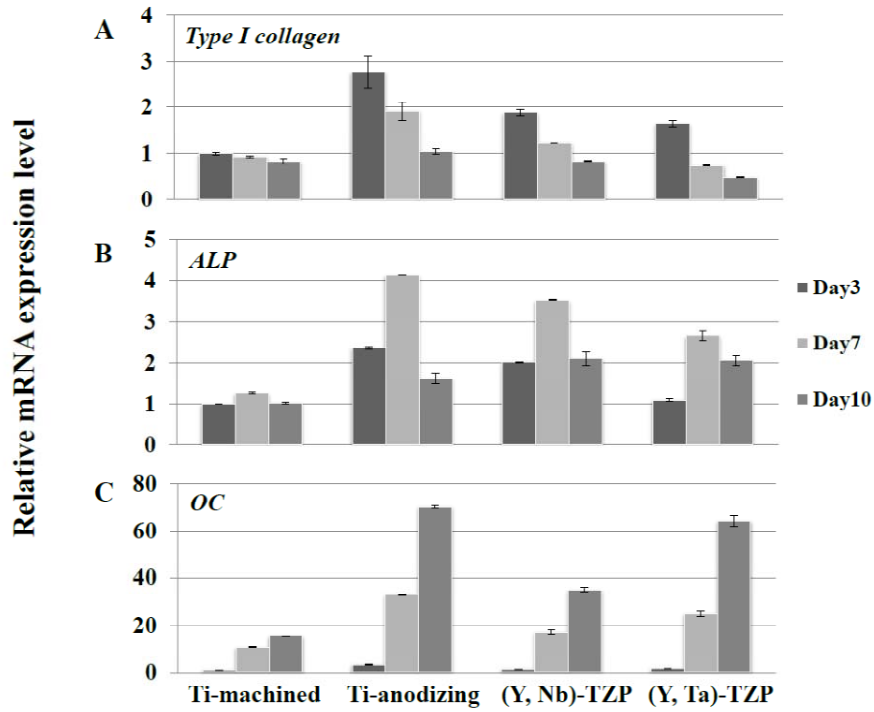


Figure 5. RT- PCR of MC3T3-E1 cells at 3, 7 and 10 days after cultured on Ti-machined (Ti-m), Ti-anodized (Ti-a), (Y, Nb)-TZP and (Y, Ta)-TZP specimens in ostegenic media

국문초록

지르코니아의 조성 및 표면처리에 따른 조골세포의 분화능에 대한 연구

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게렐마

연구목적

티타늄 중심의 치과용 임플란트 연구를 벗어나 지르코니아를임플란트 재료로 사용하기 위한 많은 노력들이 이루어지고 있다. 본 연구에서는 기존 연구결과에서 제시된 지르코니아의 가장 큰 문제점인 저온열화에 의한 강도 저하를 막고 안정성을 부여하기 위해 Nb oxide 및 Ta oxide 을 첨가하여 개발한 지르코니아의 생체적합성을 확인하여 임플란트고정체로서 티타늄을 대신할 지르코니아의사용가능성을 알아보고자 한다.

연구대상 및 방법

저온열화를 방지하기 위해 조성을 변화시킨 이트리아 부분 안정화 지르코니아(Y,Nb)-TZP 및 (Y,Ta)-TZP 를 제작하였다. 50um 크기의 알루미나입자를 이용한 샌드블라스팅으로 표면의 거칠기를 형성하고 SEM 및 3-D image topography 관찰을 통해 표면의 조도를 측정하였다. 기존의 방법으로 표면 처리된 티타늄과 거칠게 한 지르코니아 표면에 MC3T3-E1 조골세포주를 배양하여, confocal laser scanning microscopy 관찰을 통해 세포 부착 능을 확인하고, picogreen assay 로 세포의 증식능을 확인하였으며, Real time PCR 을 통해 조골세포 분화 마커유전자들 (ALP, OC) 의 발현 패턴을 분석함으로써 지르코니아에서의 조골세포 반응을 티타늄과 비교 분석하였다.

결 과

티타늄 및 지르코니아에서 표면처리에 따른 세포 부착 능에는 큰 차이가 없었으나, 세포 증식능은 재료의 종류에 관계없이 매끈한 면에서 우수하였다. 조골세포의 분화능은 대체적으로거친 면의 티타늄에서 우수하였는데, 이는 지르코니아에서도 같은 결과를 보였다.

결론

지르코니아는 티타늄과 비슷한 정도의 조골세포 반응을 보임으로써 현재 널리 사용되고 있는 치과용 임플란트의 재료인 티타늄을 대체할 수 있는

재료로써 지르코니아의 가능성을 보여준다.이러한 연구결과를 바탕으로 임플란트 재료로써 최적화 및 실용화되기 위해서는 HA coating 등을 통한 조골세포 분화능의 향상 및 동물실험 등 더 많은 연구가 필요함을 제시해준다.

주요어: 치과임플란트, 지르코니아, 티타늄, 조골세포, 저온열화, 생체적합성

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