In vitro biocompatibility study of zirconia/alumina composites

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지르코니아/알루미나 복합 세라믹의 시험관내 생체적합성에 관한 연구

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치과용 임플란트의 세라믹 지대주를 위해 개발된 지르코니아 함유 세라믹 시편의 생체 적합성을 평가하기 위하여 시험관내 세포 독성을 검사를 시행하였다. L929 섬유모세포를 37℃, 90% 습도, 5% CO2 및 95% 공기의 조건을 유지하는 세포 배양기에서 배양하여 실험에 사용하였다. 배양 2일, 4일, 6일 마다 시편을 넣은 배양 접시 내의 전체 세포 수와 생존 세포 수를 세어 세포 증식과 세포 생존율 검사를 시행하였다. millipore filter test를 이용하여 succinate dehydrogenase 효소 활성을 검사하였으며, 세포막 투과성의 변화를 관찰하기 위해 agar overlay test를 시행하였다. 음성 대조군은 시편을 사용하지 않았으며, 양성 대조군은 시편과 같은 크기의 구리를 사용하여, 다음과 같은 결과를 얻었다.

1. 세포 증식과 세포 생존율 검사에서는 지르코니아 함유 세라믹을 넣은 실험군과 음성 대조군 모두에서 시간이 경과함에 따라 세포가 증식하는 양상을 보였다. 세포 생존율 검사에서도 실험군과 음성 대조군이 유사한 결과를 나타내었다.
2. millipore filter test에서는 실험 시편 모두에서 염색 정도의 변화가 없이 음성 대조군과 동일한 결과를 나타냈다. 반면에 구리 시편을 넣은 양성 대조군에서는 중등도의 세포 독성을 나타냈다.
3. agar overlay test에서도 시편을 넣지 않은 음성 대조군에서는 세포 성장에 변화가 나타나지 않았으며, 실험군에서도 시편 주위로 탈색이 관찰되지 않아서 음성대조군과 같은 결과를 나타냈다. 양성 대조군에서는 심한 세포 독성을 나타내었다.
4. 실험결과, 치과용 임플란트의 세라믹 지대주를 위해 개발된 지르코니아 함유 세라믹 시편은 시험관내 세포 독성을 나타내지 않았다.

주요어 ; 세라믹 지대주, 지르코니아, 세포 독성, 세포 배양

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I. INTRODUCTION

As the clinical application of osseointegrated implant for single tooth replacement and partially edentulous patients is increased, the interest in the esthetics of implant-supported prostheses is growing.[1] Therefore, the ceramic abutment, which has high strength, esthetics and biocompatibility, replacing the existing titanium abutment, was introduced with the development of bioceramic materials. The possibility of abutment preparation following the gingival contour of anterior teeth, the tooth-like shade, the prevention of metal showing through thin gingiva, the prevention of exposure of titanium abutment surface in the case of gingival recession caused by peri-implant bone loss, are the advantages of the ceramic abutment.[2,3,4] Also, by using ceramic instead of the existing titanium for dental implant abutment, other additional advantages can be obtained. Ingber et al.[5] studied the possible injuries of generated heat in intraoral abutment preparation to the interface of bone and fixture and they showed that ceramic abutment has a significant lower possibility of heat damage than existing titanium due to lower thermal conductivity. Moreover, Marzouk[6] reported that high density of zirconia ceramic allowed the use of ultrasonic scaler without changes in surface quality in contrast to titanium.

Recently, the ceramic abutment(Ceradapt) fabricated by using sintered alumina[5] showed a clinical success rate of 97.2% according to the Andersson's 2-year study[7]. In general, aluminum oxide has high biocompatibility and wear resistance. However, fracture of alumina can be occurred because of the low fracture toughness and low tensile strength. In addition, its resistance to stress concentration and mechanical impact in service is low due to microstructural flaws.[8]

Zirconia is used for fabrication of ball head for total hip replacement in orthopaedic surgery.[9] The advantages of zirconia, compared to the alumina, are low Young's modulus, high strength and fracture toughness.[10] The high fracutre toughness, similar to that of steel, is due to the energy-absorption property, which originates from martensitic transformation of tetragonal particles to monoclinic ones. The ivory color of zirconia ceramics, used as dental materials, resembles the color of natural teeth. In addition, zirconia ceramic is highly radiopaque and is easy to prepare its superstructure.[11]

Alumina and zirconia are generally known as bio-inert materials with biocompatibility.[12] However, zirconia can show different cell reactions, depending on its composition and surface form, reacting cell line, contained amount of impurities, and its manufacturing process, and when they are used as medical devices,

the biocompatibility test of the product itself should be carried out[13-17].

The in vitro cytotoxicity tests were performed to test biocompatibility of zirconia/alumina composites using cell growth and survival test, millipore filter test and agar overlay test.

Ⅱ. MATERIALS AND METHOD

The L929 mouse fibroblast (ATCC, USA) was used in this in vitro study. Cells were cultured in minimum essential medium(MEM) (Gibco, USA) supplemented with 10% horse serum (Gibco BRL, USA), penicillin (100unit/ml), streptomycin (100unit/ml) and fungizone (0.3㎍/ml). Incubators (New Brunswick Scientific, USA) were maintained at 37℃, 90% humidity, 5% CO_2 and 95% air. Culture medium was replaced every two days.

The test specimen used for this study is a newly developed zirconia/alumina composites. This specimens were prepared by mixing a 80 vol% tetragonal ZrO_2 and 20 vol% Al_2O_3. CeO_2 and Nb_2O_5 were added to stabilize ZrO_2 solid solution, and Fe_2O_3 was added to give the light yellow color. Copper was used as a positive control materials, and no specimen was used a negative control.

1. Cell growth and cell survival test

Culture medium containing 5×10^4 L929 fibroblast cell was put in the petri dish in diameter 60mm and test specimens (diameter 13mm × thickness 1mm) were placed in the center of each petri dish. After 2, 4, and 6 days of culture, ten dishes were tested. The medium in the dishes was aspirated, and dishes were rinsed with phosphate buffered saline(PBS) solution. Cells were detached with trypsin (0.05%) plus EDTA (0.53mM) in Ca, Mg-free PBS solution and stained with trypan blue. The number of total cells and vital cells were determined by a hemocytometer, and the ratio of vital cells to the total cells was calculated. Statistical analysis has been calculated by Mann-Whitney Test, considered signigicant when $P < 0.05$.

2. Millipore filter test

Millipore filter disk (pore size 0.45㎛, 47mm in diameter, Whartman, USA) was placed in the petri dishes in diameter 60mm. Culture medium containing 1.8×10^6 L929 fibroblast cell was added and incubated for 48 hour. MEM agar solution containing 1.5% bacto-agar (Difoo, USA) was poured into another petri dish, then medium was allowed to solidify at room temperature. The millipore filters with cell monolayers attached were placed on the agar medium, cell side down. The three experimental specimens (diameter 7mm × thickness 1mm) were paced on top of the filter and incubated for 2 hours. Negative control had no specimens and positive control used copper with same size. After 2 hours incubation, the specimens were removed carefully and filter disk gently loosened from agar layer and rinsed with PBS solution. And the filter was incubated for cytochemical demonstration of succinate dehydrogenase(SDH) activity.

Cell response was evaluated by examining appearance of cell-material contact area, according to the scoring system presented by Wenberg et al.[18]

score	interpretation of cytotoxicity	appearance of cell-material contact area
0 ;	(no cytotoxicity),	no difference in staining intensity compared with control area
1 ;	(mild cytotoxicity),	a zone of reduced staining intensity , or an unstained zone <7mm wide
2 ;	(moderate cytotoxicity),	an unstained zone 7-11mm wide
3 ;	(severe cytotoxicity),	an unstained zone >11mm wide

3. Agar overlay test

Culture medium containing 9×10^6 L929 fibroblast cell was put in the petri dish in diameter 60mm. After 24 hours of culture, the liquid medium was aspirated and replaced by MEM agar solution containing 1.5% bacto-agar (Difoo, USA), and allowed to solidify at room temperature for 30 minutes. Cells were stained with vital dye neutral red and specimens were placed onto the agar layer. Its toxicity was evaluated after 24 hours of culture. Ten test specimens (diameter 13mm × thickness 1mm) were examined and copper was used as a positive control, and no specimens as a negative control.

The extent of decolorization of the red-stained cell layer under and around the specimens was examined. The Zone index measures the clear zone where cells do not stain with neutral red. The Lysis index measures the extent of cell lysis within the clear zone. And Response index may be determined by assigning a fraction with the Zone index and the Lysis index.[19,20]

Response index(zone index / lysis index)	
no cytotoxic reaction	0/0
mild cytotoxic reaction	1/1-1/5, 2/1
moderate cytotoxic reaction	2/2-2/5, 3/1-3/5, 4/1-4/3
strong cytotoxic reaction	4/4, 4/5, 5/1-5/5

Ⅲ. RESULTS

1. Cell growth and cell survival test

As shown in Table 1 and Fig. 1, cell proliferation appeared as time went by, in both test groups with zirconia/alumina composites specimens and negative control groups. In positive control, which is cultured in copper specimens, there were no surviving cells observed from the second day after culture. (Fig. 1)

In the cell survival test, which is the ratio of total cell numbers to vital cell numbers, there were no dead cells observed on the second day after culture, and therefore both test groups and negative control groups showed survival rate of 100%. On the fourth day after

Zone index	Description of zone
0	no detectable zone around or under specimen
1	no detectable zone around or under specimen
2	zone extended less than 0.5 ㎝ beyond specimen
3	zone extended greater than 1.0 ㎝ beyond specimen but does
4	zone extended greater than 1.0 ㎝ beyond specimen but does not involve entire dish
5	zone involves entire dish
Lysis index	**Description of zone**
0	no observable cytotoxicity
1	less than 20 % of zone affected
2	20 % to 39 % of zone affected
3	40 % to 59 % of zone affected
4	60 % to 79 % of zone affected
5	greater than 80 % of zone affected

Table 1. cell growth test

group / days	2 days	4 days	6 days
Test	13.1 ± 4.0	81.1 ± 13.3	372.6 ± 37.6 *
Negative	15.7 ± 1.6	83.7 ± 11.9	405.1 ± 20.9 *

number of cells ; (Mean ± SD) × 10^4

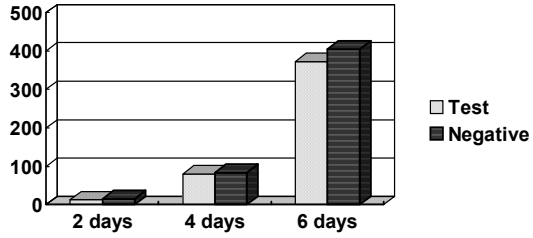

Fig. 1. cell growth test

Table 2. cell survival test

group / days	2 days	4 days	6 days
Test	100.0	97.9 ± 9.0	98.9 ± 3.5 *
Negative	100.0	98.0 ± 9.3	99.2 ± 1.8 *

(Mean ± SD) %

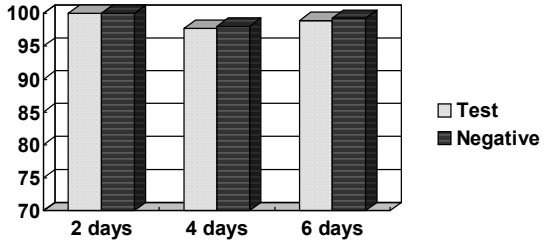

Fig. 2. cell survival test

culture, the survival rate were 97.71% and 97.89% respectively. On the sixth day, it showed a higher survival rate of 98.86% and 99.19%. But a significant difference was found between the two groups on the sixth days. (Table 2, Fig. 2)

2. Millipore filter test

After the examination of changes in the cell metabolism of specimens by observing SDH activity, the results in all the ten test specimens appeared to be the same as in negative control, without any change in staining. On the contrary, the positive control filled with copper specimens, showed severe cytotoxicity. (Table 3)

3. Agar overlay test

In negative control, without specimens, there was no change in cell growth, and in test groups there was no decolorization around the specimens such that it recorded zone index 0 and lysis index 0. Also, it is evaluated that there is no cytotoxicity in this test. Positive control registered a response index of 4/4 and showed strong cytotoxicity. (Table 4)

Table 3. millipore-filter test of L929 fibroblast

group	response index	cytotoxicity
Test	0	non-cytotoxicity
Negative	0	non-cytotoxicity
Positive	2	cytotoxicity

Table 4. Agar overlay test of L929 fibroblast

group	zone index	lysis index	response index	cytotoxicity
Test	0	0	0	0
Negative	0	0	0	0
Positive	4	4	4/4	severe

IV. DISCUSSION

For clinical use of biomaterials, biologic stability must be established, next to physical and mechanical properties of the products. Several criteria are presented for methods to evaluate the biologic stability of dental materials.[21] Generally, in vitro model

provides an ideal system for studying cell behaviors of the materials and can be carefully controlled under strict laboratory conditions, and they are free from the influence of the many variables inherent in an animal models.[22,23] In general, cell line is used for in-vitro cell growth, instead of primary cell, which decreases variability among laboratories, as in using inbred animal strains in in vivo studies, and which can obtain reproducibility. L929 mouse fibroblast cell has been used most extensively for testing biomaterials and even in this study, this cell line was used. This resembles to the in vivo situation, where implant abutment is used. In addition, fibroblast is one of the early cells to populate in a healing wound and is often the major cell in the tissue that attach to implanted medical devices.[22,24]

In this study, in-vitro cytotoxicity test of zirconia/alumina composites, which are fabricated to use as ceramic abutments of implant, was performed. The results of L929 fibroblast growth test showed increased cell numbers, during second, fourth and sixth day of culture. Considering physical trauma caused by specimens which can be shown in the initial stage of culture, considering the difference in initial cell numbers which can occur while spreading cells on petri dish and considering the absence of cell growth in positive control filled with copper, it seems that the cell growth is not inhibited by the specimens itself, although a significant difference exists to the negative control without any specimens on the sixth day after culture. Moreover, with the cell survival rate higher than 95% during the whole culture period, it can be concluded that these ceramic specimens don't show toxicity to L929 fibroblasts.

In the millipore filter test, which is to examine the influence of specimens on cell metabolism, the results indicated that test groups don't have cytotoxicity as well as negative control groups. Changes in cell function, due to the cytotoxicity of specimens, occur before changes such as cell lysis. Cytotoxicity can be evaluated by the decreased activity of enzymes in cell-specimens contact sites.[18] Dehydrogenase is an important enzyme in catalyzing reaction of the Krebs cycle of cell. Succinic dehydrogenase, as soluble iron flavoprotein, which is used in this test, catalyzes reversible oxidation from succinic acid to fumaric acid. This enzyme activity obtains hydrogen from substrates and pass it to tetrazolium. When hydrogen is added, tetrazolium changes from colorlessness to purple-blue formazan pigment and indicates the location of the enzyme activity.[25,26] Pore size of the filter can also influence the cell growth. Yesilsoy reported that optimal cell growth is obtained in 0.45 micrometer pore size filter.[27]

In agar overlay test, which was performed to observe the changes in cell membrane permeability, there was no cytotoxicity shown by specimens. This method is to examine the cytotoxicity of components, that can be diffused from specimens to the cells by agar layer and not by direct cell-specimens contact.[28] Mohammad et al.[20] used this method for cytotoxicity evaluation of root canal sealers and claimed this to be a condition near clinical endodontic situation, because direct cell contact is not necessary. Likewise, diffusable components of ceramic abutment materials can influence non-keratinized sulcular epithelium by gingival fluid.

The results from observing petri dish by LM indicated that L929 fibroblast attached to zirconia specimens surface during culture period for cell growth. Cell attachment of specimens is deeply related to the formation of biologic barrier when used as an implant abutment. Tissue attachment which surrounds the implant is also important in maintenance of osseointegration.[29,30,31] This suggested that the possibility of mucosal seal around this specimens.

V. CONCLUSION

The biocompatibility of zirconia/alumina composites, developed for ceramic abutment of dental implant, was evaluated by means of in vitro cytotoxicity test. Cell growth and survival test, millipore filter test, and agar overlay test were performed. In cell growth and

survival test, the results were similar to negative control, which is cultured without specimens. Also in millipore filter test and agar overlay test, specimens didn't show any cytotoxicity. This suggests that newly developed zirconia/alumina composites demonstrated no cytotoxicity on L929 fibroblast, even though in vivo study and further studies are needed.

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