Effects of zoledronic acid- and poly (lactide-co-glycolide)-incorporated titanium discs on osteoblasts *in vitro*

치의학석사학위논문

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2009년 8월

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지도교수 곽 재 영

이 논문을 치의학석사 학위논문으로 제출함

2009년 5월

서울대학교 대학원
치의학과 치과보철학 전공

우 정 제

우정제의 치의학석사 학위논문을 인준함

2009년 7월

위 원 장 (인)

부위원장 (인)

위 원 (인)

학위논문 원문제공 서비스에 대한 동의서

본인의 학위논문에 대하여 서울대학교가 아래와 같이 학위논문 저작물을 제공하는 것에 동의합니다.

1. 동의사항

① 본인의 논문을 보존이나 인터넷 등을 통한 온라인 서비스 목적으로 복제할 경우 저작물의 내용을 변경하지 않는 범위 내에서의 복제를 허용합니다.

② 본인의 논문을 디지털화하여 인터넷 등 정보통신망을 통한 논문의 일부 또는 전부의 복제 · 배포 및 전송 시 무료로 제공하는 것에 동의합니다.

2. 개인(저작자)의 의무

본 논문의 저작권을 타인에게 양도하거나 또는 출판을 허락하는 등 동의 내용을 변경하고자 할 때는 소속대학(원)에 공개의 유보 또는 해지를 즉시 통보하겠습니다.

3. 서울대학교의 의무

① 서울대학교는 본 논문을 외부에 제공할 경우 저작권 보호장치(DRM)를 사용하여야 합니다.

② 서울대학교는 본 논문에 대한 공개의 유보나 해지 신청 시 즉시 처리해야 합니다.

논문제목 : Effects of zoledronic acid- and poly (lactide-co-glycolide)-incorporated titanium discs on osteoblasts *in vitro*

학위구분 : ■ 석 사 □ 박 사
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서울대학교총장 귀하

-ABSTRACT-

Effects of zoledronic acid- and poly (lactide-co-glycolide)-incorporated titanium discs on osteoblasts *in vitro*

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BACKGROUND: Biochemical methods are among the most promising for implant surface modifications, and efforts to find new implant coating materials are ongoing. Bisphosphonates have antiresorptive effects and are used as the major drugs for the treatment of bone diseases associated with osteoclast-mediated bone loss. These effects of positive bone balance may be also affected by their effects on osteoblasts. The purpose of this study was to assess the effect of the zoledronate and PLGA coated titanium disc on osteoblastic cell proliferation, differentiation and Osteoprotegerin (OPG) secretion. MATERIALS AND METHODS: Zoledronate was incorporated in an anodized titanium disc coating based on poly(lactide-co-glycolide) (PLGA) in different concentrations (0, 0.1, 1, 10 μM). Control groups were uncoated anodized titanium discs. HOS (human osteogenic sarcoma) cell were cultured for 7 days and analysis was done. We performed direct cell counting and MTS assay for proliferation, ALP assay for differentiation. In addition, Osteoprotegerin (OPG) secretion was analyzed. RESULTS: Results from direct cell counting and MTS assay showed that cell proliferation was increased in a dose dependent manner (up to 181± 9.10%) and its effects were increasing with time. Alkaline phosphatase levels increased in zoledronate treated groups at all concentration (up to 161 ± 6.30%) compared with controls. The amount of OPG in 0.1 μM zoledronate treated groups was significantly higher (140 ± 28.1%) than control groups at day 7. CONCLUSIONS: Zoledronate had no cytotoxic effects on osteoblasts at 0.1 - 10 μM concentration when used with PLGA as an implant disc coating. Bisphosphonates such as zoledronate increased proliferation and differentiation of osteoblasts. In addition, OPG production was stimulated by zoledronate. Our results support the use of bisphosphonates for dental implant surface modifications to enhance bone formation and to prevent peri-implant bone resorption after implant placement.

Keywords: Zoledronic acid, Osteoblast, Bisphosphonate, poly(lactide-co-glycolide) (PLGA), Osteoprotegerin (OPG), implant coating

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CONTENTS

Ⅰ. INTRODUCTION

Dental implant surface modifications are evolving rapidly to enhance surface bone formation and to increase the predictability of implant therapy.[1] Biochemical methods are among the most promising for surface modifications, and efforts to find new implant coating materials are ongoing.

Two recent studies have demonstrated the positive effects of bisphosphonate on bone formation.[2,3] Bisphosphonates have high affinity for mineral surfaces, and are subsequently internalized by osteoclasts where they inhibit various biochemical processes, reducing bone resorption and increasing bone density.[4,5] Bisphosphonates are one of the most potent classes of antiresorptive agents used in the treatment of bone disorders.[10-13] Oral bisphosphonates such as alendronate sodium and risedronate are the treatment of choice for bone diseases associated with excessive resorption in adults such as Paget's disease of bone, myeloma, bone metastasis, and osteoporosis. Stronger forms of bisphosphonate such as zoledronic acid have been used intravenously to treat osseous metastases and primary bone tumors such as multiple myelomas.

While most studies have focused on the primary action of bisphosphonates in osteoclasts, there is increasing evidence that bisphosphonates also interact with osteoblasts,[4,5,13,14] but the mechanism of action of bisphosphonates

in either role is not completely understood. Recent studies have shown that bisphosphonates also affect proliferation and differentiation, expression and secretion of cytokines, extra cellular matrix protein production, and OPG and RANKL secretion.[15] These effects on positive bone balance may be responsible for the therapeutic effects of bisphosphonates in various bone diseases.

Bisphosphonates have potential side effects including osteonecrosis of the jaw, which may result from cessation of bone remodeling or inhibition of capillary neoangiogenesis.[31] The clinical delivery of bisphosphonate is typically systemic and intravenous, and unwanted side effects such as and osteonecrosis of the jaw have been reported.[6] Jaw osteonecrosis has been associated with systemic use of bisphosphonate,[6] and therefore local application of bisphosphonates using a drug carrier is recommended to avoid this and similar complications.

Biodegradable polymers such as PLGA (poly lactide-co-glycolide) have been extensively investigated in animal studies as vehicles for sustained, localized delivery of relevant medications and are commonly used in human patients for orthopedic applications such as resorbable sutures, bone implants and screws, in graft materials for artificial organs, as supporting scaffolds in tissue engineering, and in contraceptive implants.[7,8,16,32] These polymers undergo hydrolysis upon implantation into the body, forming biologically compatible and metabolizable moieties (lactic acid and glycolic acid) that are eventually removed from the body by the citric acid cycle.[7] There are numerous techniques for manufacturing drug loaded biodegradable PLGA devices.[17]

Osteoprotegerin (OPG), a novel member of the tumor necrosis factor receptor (TNFR) superfamily, is a protein naturally secreted by osteoblasts. OPG, which is secreted by osteoblastic lineage cells, acts as a soluble receptor antagonist for RANKL, an osteoclast differentiating factor that is necessary for osteoclast formation and activation.[9,10,18] Administration of OPG inhibits osteoclastogenesis and associated bone resorption, and blocks pathological increase in osteoclast numbers and activities.[19] OPG has been shown to block ovariectomy-associated bone loss in rats and osteoclast differentiation from precursor cells in a dose-dependent manner *in vitro*.[18,24] The RANKL:OPG ratio is a key factor in the regulation of osteoclastogenesis and osteoclastic activity.[20] Various osteotropic drugs have been demonstrated to modulate the production of OPG and RANKL from osteoblasts.

In this study, we assessed the effects of bisphosphonates delivered via PLGA coated titanium discs on osteoblastic cell proliferation and differentiation, as well as their influence on OPG secretion.

Ⅱ. MATERIALS AND METHODS

1. Anodized titanium disc preparation

Titanium discs 1 mm in thickness and 23 mm in diameter were ultrasonically degreased in trichlorethylene for 20 min, followed by soaking in 99% ethanol for two cycles of 20 min each followed by soaking in distilled water overnight. The surfaces of the pre-treated discs were then subjected to anodic oxidation treatment at 300 V in an aqueous electrolytic solution of 0.02 M calcium glycerophosphate and 0.15 M calcium acetate, resulting in the production of anodized surfaces. All procedures were done at room temperature, and the total time for anodization of each disc was 3 min. The samples were dried and sterilized by ethylene oxide gas prior to cell culture experiments.

2. Cell culture

Osteoblast-like human osteogenic sarcoma cells (HOS, KCLB, Seoul, Korea) were used in this study. Cells were cultured in RPMI medium with 10% FBS (fetal bovine serum), 0.5% penicillin (10,000U) and streptomycin (10,000 ㎍/ml). Cell culture dishes, each containing one titanium disc, were 35 mm in diameter. Each dish was seeded with 2 ml of 1×10^{-4} cell/ml of cell culture.

3. Disc coating

Zometa (Novartis Pharma AG, Basel, Switzerland) was used for zoledronic acid coating and PLGA (PURAC Biochem BV, Gorinchem, Holland) was chosen as the drug carrier. PLGA (120 mg) was dissolved in 10 ml methyl acetate at room temperature and the resulting solution was passed through a sterile filter (DISMIC, 0.45 ㎛, ADVANTEC, Japan). Zometa was dissolved in methyl acetate and mixed with PLGA solution to obtain the desired concentration. To coat the discs, 300 ㎕ of zoledronate was dropped on the sterilized anodized titanium discs at concentrations of 0, 0.1, 1 and 10 ㎛M and dried under laminar air flow conditions. Uncoated anodized titanium discs were used for controls.

Titanium discs were divided into 5 groups and coated with the solution as follows:

- Group 1: Anodized at 300V
- Group 2: Anodized at 300V and then coated with 300 ㎕ PLGA solution
- Group 3: Anodized at 300 V and then coated with 300 ㎕ [PLGA/zoledronate(0.1 ㎛M)] solution
- Group 4: Anodized at 300 V and then coated with 300 ㎕ [PLGA/zoledronate(1 ㎛M)] solution
- Group 5: Anodized at 300 V and then coated with 300 ㎕ [PLGA/zoledronate(10 ㎛M)] solution

4. FE-SEM (Field Emission Scanning Electron Microscopy)

Anodized, PLGA coated, PLGA and zoledronate coated titanium discs were prepared for surface analysis. Samples were mounted on metal stubs and coated with gold using a sputter coater (POLARON SC 7620, VG Microtech, England) for surface characterization. The samples were observed by FE-SEM (Hitachi S-4700, Tokyo, Japan) at 15kV accelerating voltage.

5. Direct cell counting

After one, three, and seven days of culture, cells were washed with PBS (phosphate buffered saline) and isolated from culture dishes by trypsinization. After centrifugation at 1200rpm, 5 min at E-tube, cell number and viability were determined using a hemocytometer and trypan blue dye exclusion test. Direct cell counts were performed in eight cultures (n=8).

6. MTS (Methylthiazole sulfate) assay

After one, three, and seven days of culture, cells were washed, isolated and centrifuged as mentioned above. An MTS assay was performed according to the manufacturer's protocol (CellTiter 96™ AQueous NonRadioactive Cell Proliferation Assay, Charbonnières, France). 20 ㎕ of MTS solution was added to each well of a 96-well plate containing cells in 100 ㎕ of culture medium and incubated for 3 hr. After incubation, color changes were measured at 490 nm using a 96-well plate reader (PowerWave 340, Bio-Tek, Vermont, USA).

Measurements were performed in duplicate and repeated in eight cultures (n=16).

7. ALP (Alkaline phosphatase) assay

Cells were plated in culture dishes containing titanium discs, and alkaline phosphatase (ALP) activity was assayed after one, three, and seven days of culture utilizing the conversion of a colorless p-nitrophenyl phosphate to a colored p-nitrophenol. Color changes were measured spectrometrically at 405 nm, and the amount of enzyme released by cells was quantified by comparison with a standard curve. ALP levels were divided through entire protein production of cells in each culture. Measurements were performed in duplicate and repeated in five cultures (n=10).

8. Osteoprotegerin (OPG) protein measurement

The amount of OPG in the supernatant secreted by osteoblast-like cells was measured after three and seven days of culture. Medium was harvested from cultured cells and centrifuged to remove debris. OPG protein measurement was performed using an OPG ELISA Kit (Biovender, Czech Republic) according to the manufacturer's protocol in 96-well plates. Optical densities were read using an ELISA microplate reader at 450 nm. Total protein content in all samples was determined by bicinchoninic acid (BCA) assay (BCA Protein Assay Reagent Kit, PIERCE) before ELISA. Bovine serum albumin (BSA, 2 mg/mL) was used to create a standard curve. Results were divided through entire protein production of cells in each culture. Measurements were repeated in eight cultures (n=8).

9. Statistical analysis

The results are presented as a percentage over controls. The mean and standard deviations were obtained from sums of each data. Data were analyzed by one-way ANOVA and post-hoc Bonferroni and Tukey's tests. The Kruskall-Wallis test was also used for non-parametric analysis. *P*-values less than 0.05 were considered significant.

Ⅲ. RESULTS

1. Characteristics of implant surfaces

Anodized titanium disc surfaces were observed by SEM. Disc surfaces were uniformly porous and composed of small craters. In test groups, titanium discs were coated with smooth, nonporous PLGA nanospheres and microspheres (Fig. 1).

2. Cell proliferation

Zoledronate and PLGA coating resulted in a significant dose-dependent increase in cell proliferation that was also associated with time. Peak effects were observed at 10 μM, and seven days after drug introduction cell proliferation was increased by 82% over control (Table 1, Fig. 2).

To further investigate these findings, we studied the effects of zoledronate by MTS assay. Cell proliferation increased in a dose-dependent manner in zoledronate and PLGA coated groups over controls, and peak effects were also observed at the 10 μM concentration (Table 2, Fig. 3).

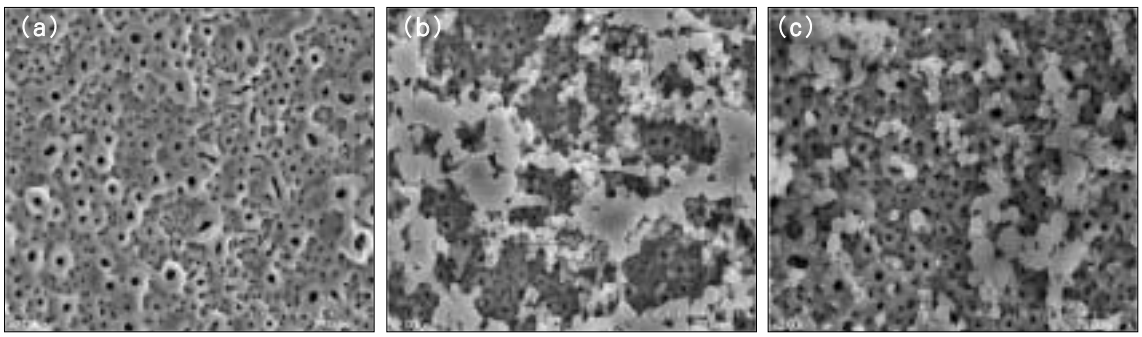

Fig. 1. SEM surface morphology of Ti disc surfaces (15 KV, 2.00 KX).
(a) anodized surface, (b) PLGA coated surface, (c) PLGA and ZOL coated surface.

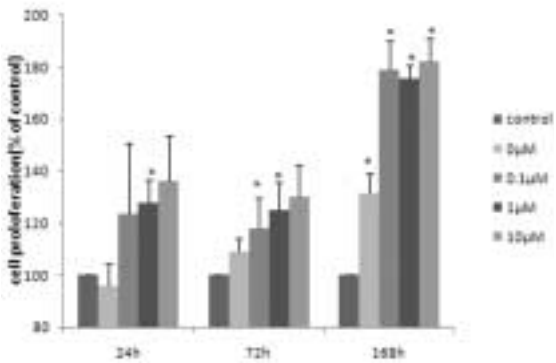

Fig. 2. Proliferation of cells treated with zoledronate at different concentrations by direct cell counting. (mean + SD, n = 8, $^{*}P < 0.05$ compared to control).

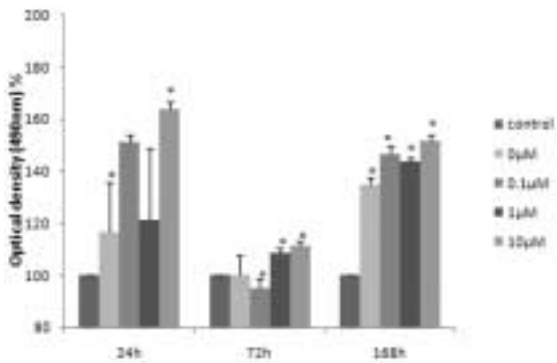

Fig. 3. Proliferation of cells treated with different concentrations of zoledronate by MTS colorimetric assay. (mean + SD, n = 16, $^{*}P < 0.05$ compared to control).

Table 1. Results of direct cell counting

	24 h	72 h	168 h
Group 1	2.94 ± 0.42	8.31 ± 0.65	24.6 ± 1.85
Group 2	2.81 ± 0.26	9.06 ± 0.42	32.4 ± 1.85
Group 3	3.63 ± 0.52	9.81 ± 1.03	44.1 ± 2.24
Group 4	3.75 ± 0.27	10.4 ± 0.92	43.2 ± 1.31
Group 5	4.00 ± 0.80	10.8 ± 0.96	44.8 ± 2.80

Data are presented as mean ± SD ($\times 10^4$, n = 8).

Table 2. MTS assay optical densities at 490 nm

	24 h	72 h	168 h
Group 1	0.140 ± 0.004	0.321 ± 0.080	0.496 ± 0.025
Group 2	0.163 ± 0.027	0.322 ± 0.024	0.673 ± 0.013
Group 3	0.212 ± 0.004	0.306 ± 0.012	0.731 ± 0.016
Group 4	0.171 ± 0.380	0.349 ± 0.006	0.716 ± 0.007
Group 5	0.230 ± 0.004	0.357 ± 0.006	0.731 ± 0.014

Data are presented as mean ± SD (n = 16).

3. Alkaline phosphatase (ALP) synthesis

ALP secreted by osteoblasts is an early sign of osteoblastic differentiation in culture. We observed that ALP levels increased in zoledronate treated groups at all concentrations compared with controls (Table 3, Fig. 4).

4. OPG synthesis

Osteoblasts treated with zoledronate and PLGA coated discs for seven days showed a significant increase in OPG production at a concentration of 0.1μM in comparison to total protein production. The amount of OPG in the zoledronate treated groups was significantly higher ($p<0.05$) than in the PLGA-only coated group at day 7. OPG production by zoledronate treated osteoblasts was higher than control group at day 3, but this difference was not statistically significant (Table 4, Fig. 5).

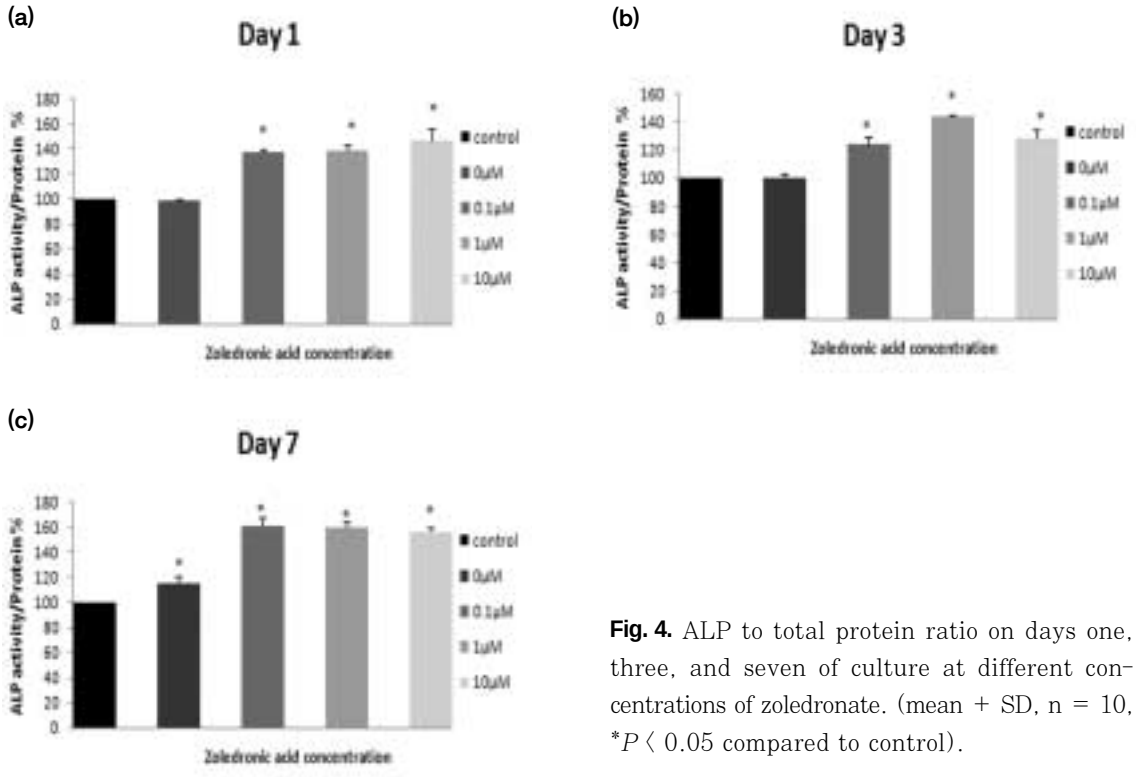

Fig. 4. ALP to total protein ratio on days one, three, and seven of culture at different concentrations of zoledronate. (mean + SD, n = 10, $^{*}P < 0.05$ compared to control).

Table 3. Alkaline phosphatase activity

	24 h	72 h	168 h
Group 1	0.148 ± 0.025	0.851 ± 0.007	0.947 ± 0.053
Group 2	0.145 ± 0.002	0.857 ± 0.040	1.096 ± 0.052
Group 3	0.216 ± 0.014	1.091 ± 0.051	1.484 ± 0.026
Group 4	0.205 ± 0.006	1.229 ± 0.009	1.514 ± 0.040
Group 5	0.202 ± 0.004	1.058 ± 0.044	1.525 ± 0.060

Alkaline phosphatase activity is normalized to total protein at each time period. Data are presented at mean±SD (n=10).

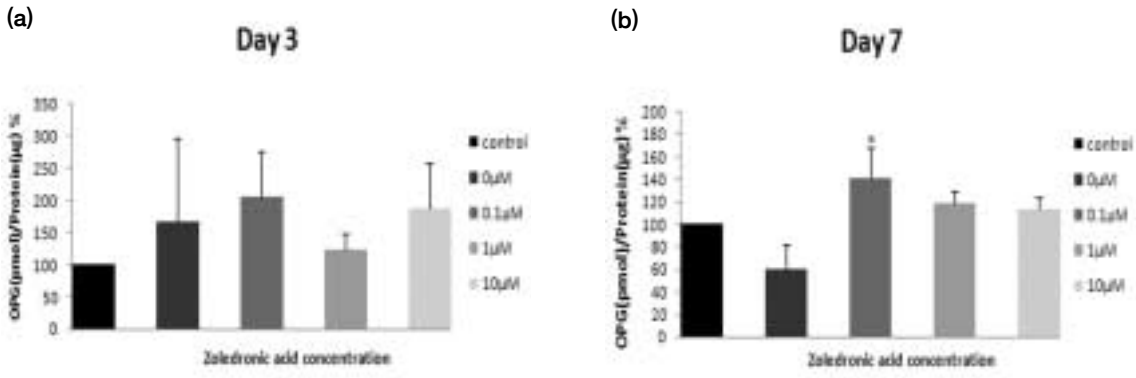

Fig. 5. OPG to total protein ratio on days 3 and 7 of culture with different concentrations of zoledoronate. (mean + SD, n = 5, $^{*}P < 0.05$ compared to control).

Table 4. OPG to total protein ratio

	72 h	168 h
Group 1	0.031 ± 0.014	0.589 ± 0.133
Group 2	0.051 ± 0.040	0.355 ± 0.133
Group 3	0.057 ± 0.022	0.665 ± 0.064
Group 4	0.038 ± 0.008	0.697 ± 0.060
Group 5	0.063 ± 0.022	0.827 ± 0.166

Osteoprotegerin production was normalized to total protein production at each time period. Data are presented as mean ± SD (n = 5).

Ⅳ. DISCUSSION

In this study, a modified cold coating technique[8], which is simple, experimentally proven, and eliminates the risk of heat degeneration, was used to incorporate bisphosphonate into titanium discs. We found that zoledronate, thought to be the most potent bisphosphonate, stimulated OPG production from osteoblasts, with the peak effects occurring at concentrations of 0.1 μM zoledronate. These results agree with previously published reports that zoledronate at low dose increases OPG production from primary human osteoblasts (hOB).[21] Similarly, zoledronate enhanced OPG gene expression by hOB in a dose-dependent fashion with a maximum effect at 0.01 μM after 72 hours.[26] Higher concentrations of zoledronate, however, decreased OPG secretion.[26] We believe that the slow release of zoledronate from PLGA particles may increase OPG secretion in high concentrations of zoledronate in our study, although OPG secretion was observed to decrease when similar concentrations of zoledronate was administered directly in other studies.[21,26]

We assessed cell proliferation by direct cell counting and MTS cell proliferation assay. Cell proliferation in all test groups was increased compared to controls in a dose-dependent manner. The peak proliferative effect of zoledronate occurred at 10 μM zoledronate for all time periods. Treatment with 10 μM zoledronate caused 36%, 30%, and 81% increases in osteoblast number at days 1, 3, and 7, respectively. At day 3 the effect on the increase of proliferation over controls was diminished, which may be explained by the release mechanism of the drug. Approximately 50% of the incorporated drug is released within the first two days of culture, and following this initial peak, further release takes place at a constant rate.[8] The results of a number of recent *in vitro* and *in vivo* studies indicate that bisphosphonates promote osteoblastic proliferation and bone for-

mation.[2,22-26] However, one *in vitro* study reported that aminophosphonates cause osteoblast apoptosis at micromolar concentrations, which may inhibit bone formation,[28] and another study determined that treatment with zoledronate reduced osteoblast numbers at 0.1 μM concentrations and was strongly toxic at 10 μM concentrations.[29] In our study, bisphosphonate showed no cytotoxicity at 0.1 μM - 10 μM concentrations. However, experiments using a wider range of drug concentrations are necessary to identify the concentrations that result in either cytotoxicity or maximum proliferative effects.

We assessed early osteoblast differentiation using an ALP assay, and the results were normalized by comparison with the total protein concentration. All test groups showed a higher ratio of alkaline phosphatase activity to total protein than controls, about 25% to 60% at all time periods. These data strongly support the stimulating effects of bisphosphonates on osteoblastic differentiation and confirm the results of previously published reports indicating that bisphosphonate enhances the differentiation of cultured osteoblasts in mature bone forming cells.[30] According to the results of other studies, the induction of osteoblast differentiation following bisphosphonate treatment appears to be donor- and drug-type dependent.[27] Contrary to our results, one study reported that bisphosphonate inhibit osteoblast differentiation at nanomolar concentration[28], and zoledronate cause up to 78%, 98% and 〉99% inhibition of alkaline phosphatase activity at concentrations of 0.1 μM, 1 μM, and 10 μM.[29] The stimulating effects of zoldedronate on osteoblastic differentiation may be partially responsible for the increase in OPG production observed in our study.

Ⅴ. CONCLUSIONS

1. Zoledronate had no cytotoxic effects on osteoblasts at 0.1 - 10 μM concentrations when used with PLGA as an implant disc coating.
2. Bisphosphonates such as zoledronate increased proliferation of osteoblasts (81 ± 9.10% at 7 days of culture) and stimulate differentiation (61 ± 6.30% increase in ALP activity).
3. OPG production from osteoblasts was also stimulated by zoledronate.
4. Our results support that zoledronates incorporated in PLGA have anabolic effects on osteoblasts.
5. Our results support the use of bisphosphonates for dental implant surface modifications to enhance bone formation and to prevent peri-implant bone resorption after implant placement.

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국문초록

Zoledronic acid와 poly (lactide-co-glycolide) 코팅이 티타늄 디스크에서 조골세포에 미치는 영향

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우 정 제

1. 목적

현재 임플란트 표면을 개선하는 노력이 시도되고 있으며 그 중 하나가 생화학적 방법이다. Bisphosphonate는 골 흡수를 억제하는 약물로 파골세포에 의한 골흡수와 관련된 골 질환의 치료에 주로 사용되고 있다. 하지만, 이러한 긍정적인 골 반응은 bisphosphonate가 파골세포 뿐만 아니라 조골세포에도 작용한 결과라는 주장이 제기되고 있다. 본 연구에서는 PLGA와 zoledronate로 코팅한 티타늄 디스크가 조골세포의 증식과 분화, osteoprotegerin (OPG) 분비에 미치는 영향을 평가하고자 하였다.

2. 재료 및 방법

PLGA를 이용해 zoledronate를 양극 산화 티타늄 디스크에 0.1 μM, 1 μM, 10 μM의 농도로 코팅하였다. 대조군은 코팅하지 않은 양극 산화 티타늄 디스크와 PLGA만 코팅한 티타늄 디스크였다. 코팅된 디스크에 HOS (human osteogenic sarcoma) cell을 7일간 배양하여 분석을 시행하였다. 양극 산화 티타늄 표면 분석을 위해 주사전자 현미경 (SEM)으로 관찰하였다. 세포 증식을 평가하기 위해 Direct cell counting과 MTS assay를 시행하였으며, 세포 분화를 평가하기 위해 ALP assay를 시행하였다. 또한 osteoprotegerin (OPG) 분비 역시 평가하였다.

3. 결과

Direct cell counting과 MTS assay 결과, 세포 증식은 zoledronate의 농도에 비례해서 증가하였으며 (최대 181 ± 9.10%) 시간이 지나면서 효과가 증가하는 경향을 보였다. Alkaline phosphatase 역시 zoledronate로 처리한 모든 실험군에서 대조군에 비해 더 높은 수치 (최대 161 ± 6.30%)를 나타냈다. OPG의 양은 세포 배양 7일 후, zoledronate (0.1 μM)로 처리한 실험군에서 대조군보다 통계적으로 유의성 있게 높은 값(140 ± 28.1%)을 보였다.

4. 결론

이 실험으로, zoledronate와 같은 bisphosphonate는 조골세포의 증식과 분화를 활성화시키며, OPG 분비를 촉진시킨다는 사실을 확인할 수 있었다. 이번 실험 결과는 bisphosphonate를 임플란트 주위의 골형성을 개선하기 위한 임플란트 표면처리에 사용할 수 있다는 가능성을 제시하였다.

주요어: 졸레드론산 (zoledronic acid), 조골세포, PLGA, 비스포스포네이트, Osteoprotegerin (OPG), 임플란트 코팅

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