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Xylitol Down-Regulates 1α,25-Dihydroxy Vitamin D3-induced Osteoclastogenesis via in Part the Inhibition of RANKL Expression in Osteoblasts

Seung-Ho Ohk[1,3,5], Hyunjoo Jeong[1], Jong-Pill Kim[2], Yun-Jung Yoo[1,4], Jeong-Taeg Seo[1,3,4], Dong-Min Shin[1,4,♣], and Syng-Ill Lee[1,3,4,*]

[1]Department of Oral Biology, [2]Department of Pedodontics, and [3]Brain Korea 21 Project of Dental Sciences, [4]Oral Science Research Center, Yonsei University, Seoul 120-749, [5]Department of Oral Microbiology, Chonnam National University, Gwangju, 501-190, South Korea

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Xylitol is a sugar alcohol with a variety of functions including bactericidal and anticariogenic effects. However, the cellular mechanisms underlying the role of xylitol in bone metabolism are not yet clarified. In our present study, we exploited the physiological role of xylitol on osteoclast differentiation in a co-culture system of osteoblastic and RAW 264.7 cells. Xylitol treatment of these co-cultures reduced the number of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells induced by 10 nM 1α,25(OH)$_2$ D$_3$ in a dose-dependent manner. A cell viability test revealed no marked cellular damage by up to 100 mM of xylitol. Exposure of osteoblastic cells to xylitol decreased RANKL, but not OPG, mRNA expression in the presence of 10^{-8} M 1α,25(OH)$_2$D$_3$ in a dose-dependent manner. Furthermore, bone resorption activity, assessed on bone slices in the co-culture system, was found to be dramatically decreased with increasing xylitol concentrations. RANKL and OPG proteins were assayed by ELISA and the soluble RANKL (sRANKL) concentration was decreased with an increased xylitol concentration. In contrast, OPG was unaltered by any xylitol concentration in this assay. These results indicate that xylitol inhibits 1α,25(OH)$_2$D$_3$-induced osteoclastogenesis by reducing the sRANKL/OPG expression ratio in osteoblastic cells.

Key words: xylitol, bone resorption, osteoclastogenesis, RANKL

*Correspondence to: Syng-Ill Lee, Ph. D. Department of Oral Biology, College of Dentistry, Yonsei University, 134 Shinchon-Dong, Seodaemoon-Gu, Seoul 120-752, Korea, Tel: 82-2-2228-3052, Fax: 82-2-364-1085, e-mail: lsi@yuhs.ac

Introduction

It has been reported that xylitol has a variety of function on cells, such as bactericidal, and anticariogenic effects [1]. Xylitol is a five-carbon natural polyhydric alcohol, which is widely distributed in fruits, berries, and plants. The natural dietary carbohydrate xylitol has been used as a source of energy in infusion therapy and found to act curatively in certain clinical situations. Although this sugar alcohol cannot be metabolized, it is taken up by *Streptococcus mutans* (*S. mutans*) and accumulated as a toxic sugar-phosphate in bacterial cells, resulting in growth inhibition. In addition, xylitol has an anticariogenic effects by inhibiting the glucosyl transferase (GTF) activity [2] which mediates a sucrose-dependant adherence of mutans Streptococci to the tooth surface. Besides the bactericidal effect of xylitol, a conti-

nuous moderate dietary xylitol supplementation leads to increased bone volume and increased bone mineral content in the long bones of aged rats [3-7]. In spite of the extensive xylitol research, no experimental evidence for the cellular mechanism of xylitol on bone metabolism has been suggested.

In general, bone remodeling is regulated by the activity of bone-forming osteoblasts and bone-resorbing osteoclasts. Both osteoblasts and osteoclasts are regulated by a variety of hormones and local factors [8-12]. Osteoblasts stem from mesenchymal stem cells, whereas osteoclasts arise by the differentiation of osteoclast precursors of monocyte/macrophage lineage. Osteoblasts and osteoclasts are required not only for skeletal development, but also for mineral homeostasis and the normal remodeling of bone in adult [13]. An imbalance between bone formation and bone resorption derived from in appropriate RANKL (receptor activator of NF-κB ligand) expression by activated lymphocytes and osteoclasts causes metabolic bone diseases like osteopetrosis and osteoporosis [14,15]. Therefore, osteoblasts and osteoclasts are known to be closely related during the process of remodeling [16-18].

Certain kinds of signaling molecules, such as, RANKL, osteoprotegerin (OPG) and macrophage colony stimulating factor (M-CSF), expressed by osteoblasts, are involved in osteoclastogenesis. For instance, when osteoblasts/stromal cells are stimulated by osteotropic factors such as parathyroid hormone, RANKL is expressed and induces the differentiation of osteoclast progenitors by binding to the receptor activator of NF-κB (RANK; also known as ODF receptor) [19]. In addition, M-CSF is known to be essential for macrophages to be transformed into osteoclasts, while OPG, a decoy receptor of RANKL, is known to participate in the regulation of osteoclastogenesis [11]. Specifically, OPG, as a member of the tumor necrosis factor receptor (TNFR) family, inhibits the osteoclastogenesis stimulated by $1\alpha,25(OH)_2D_3$, PTH, or IL-11 [17]. Consequently, it is believed that RANKL, M-CSF and OPG, which are expressed by osteoblasts, are associated with osteoclastogenesis, and that osteoblasts play a major role in the bone remodeling process.

Murine RANKL is a 45 kDa, type II transmembrane glycoprotein with 316 amino acids that exists naturally as non-disulfide-linked homotrimer [17]. The molecule has a cytoplasmic domain with 47 amino acids, a transmembrane segment with 23 amino acids, and a extracellular region with 246 amino acids [20]. Soluble RANKL residue with 177 amino acids is generated by the action of metaloprotease cleavage on membrane-bound RANKL. Although both membrane and soluble RANKL are bioactive, the homeostatic form of RANKL might be the membrane-bound form [9], while soluble RANKL might signal underlying pathology [21]. Cells known to express RANKL include odontoblasts and ameloblasts [22,23], osteoblasts, T cell, chrondrocytes, fibroblasts, and skeletal muscle cells [24]. Murine RANKL is active on human cells and shows 85% and 96% amino acid homology to human and rat RANKL, respectively [11]. RANKL binds and signals via a membrane-bound TNF receptor super family member named TRANCE/RANK. RANKL also blinds a naturally occurring 55 kDa soluble receptor antagonist named osteoprotegerin [17].

With respect to osteoclastogenesis, we have focused on the function of xylitol on the osteoblast and osteoclast. Apart from some knowledge of the general functions of xylitol in a whole body as well as bone density and mass, there is no experimental evidence as to whether xylitol is related to osteoclastogenesis at the cellular level. Understanding of xylitol on bone metabolism at the molecular level of osteoblast and osteoclast is necessary. Therefore, we hypothesized that xylitol might concern osteoclastogenesis and bone metabolism with respect to RANKL, OPG, on the osteoblast. Furthermore xylitol might affect osteoclast directly also. To clarify whether xylitol can affect the osteoclastogenesis induced by $1\alpha,25(OH)_2D_3$, we have applied xylitol on an osteoblast/stromal cell co-culture system and RAW 264.7 cells. We have examined the osteoclast differentiation rate and bone resorption activity on the bone slice with xylitol in co-culture system. Also we have compared not only the expression of RANKL and OPG mRNA but also the production of sRANKL and OPG protein.

Materials and Methods

Materials

Routine cell culture media were obtained from GIBCO/BRL (Grand Island, NY). The Tartrate-Resistant Acid Phosphatase Staining Kit was purchased from the Sigma Chemical Co., Ltd. (St. Louis, MO). Trizol was purchased from Invitrogen Corp. (Carlsbad, CA), and the ICR mice were from Samtacho Co., Ltd. (Seoul, Korea). Xylitol was purchased from Borak Corp. (Seoul, Korea). All other chemicals were of the highest grade commercially available. Recombinant murine sRANKL was purchased from KOMA Biotech (Seoul, Korea).

***In vitro* osteoclast formation assay**

The osteoblast formation assay was carried out as previously reported by [25]. Briefly, the osteoblasts were isolated from 1 - 2 day-old newborn mice. 30 - 50 calvariae were digested in 10 ml of an enzyme solution containing 0.2% collagenase (Wako, Japan) and 0.1% dispase (GIBCO/BRL, U.S.A) for 20 minutes at 37°C in a shaking water bath. The supernatant was discarded and 10 ml of the enzyme solution was added. After shaking at 37°C for 20 minutes, the supernatant was collected carefully and transferred to a new tube. This digestion of calvariae by collagenase and dispase was repeated three times. The collected supernatant (30 ml) was placed in a centrifuge at 1,500 ×g for 10 minutes, to collect the osteoblastic cells. Cells were resuspended in α-minimum essential medium (α-MEM) containing 10% fetal bovine serum (FBS) and cultured to confluence in 100 mm culture dishes at a concentration of 1×10^5 cells/dish. The cells were then detached from the culture dishes using trypsin-EDTA, suspended in α-MEM with 10% FBS and used for the co-culture as osteoblastic cells.

Femoral and tibial bone marrow cells were collected from 4-week-old mice. The tibiae and femora were removed and dissected free of adhering tissues. The bone ends were removed and the marrow cavities were flushed by slowly injecting media at one end using a 25-gauge needle. The calvariae and bone marrow cells collected were washed and used in the co-culture. Mouse calvarial cells (1×10^4 cells/well) were co-cultured with bone marrow cells (1×10^5 cells/well) in α-MEM containing 10% FBS in 48-well plates (Corning Inc., Corning, NY). The culture volume was made up to 400 μl per well with α-MEM supplemented with 10% FBS, in the presence of $1\alpha,25(OH)_2D_3$ (10^{-8} M), without or with xylitol (1, 10, 30, 50 or 100 mM). All cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2 in atmosphere. After incubation for 4 days, the cells were subjected to tartrate-resistant acid phosphatase (TRAP, an osteoclast marker enzyme) staining. *In vitro* formation assay of osteoclast was repeated four times.

Viability test

The MTT (3-4,5-dimethlthiazol-2-yl-)-2,5-diphenyltetrazolium bromide) test is based on the principle that tetrazolium salts are reduced by reducing mitochondrial enzymes (succinate, dehydrogenase), which allows the toxicity of viable cells and the level of cellular differentiation to be measured. MTT was dissolved in phosphate-buffered saline (PBS) at 5 mg/ml and filtered to remove any insoluble residue. MTT solution was added directly to the assay plates. The cells were subsequently incubated for an additional 4 hours at 37°C. The purple formazan crystals that formed were dissolved in DMSO, and the plates were read on a spectrophotometer at 570 nm.

Bone resorption activity assay (Pit formation assay)

Osteoblastic cells obtained from the calvariae of newborn ICR mouse and bone marrow cells obtained from the tibiae and femora of male ICR mouse were co-cultured in α-MEM in calcium phosphate apatite-coated 24-well plate, (OAAS plate, Oscotec Inc., Korea) at 2×10^5 cells/0.8 ml/well and 2×10^6 cells/0.8 ml/well, respectively. The cells were cultured for 4 days at 37°C in a humidified 5% CO_2 atmosphere. Then the cells were treated with 10 nM $1\alpha,25(OH)_2D_3$ and xylitol with different concentrations. Cultures were maintained for 4 days. The medium in each well was replaced with the respective fresh medium with $1\alpha,25(OH)_2D_3$ (10^{-8} M) and xylitol. The experiments were performed four times. At the end of culture, attached cells were removed from the plate by abrasion with 4% sodium hypochloride solution (Sigma, St. Louis, MO). Images of pit were acquired with a digital camera attached to a microscope at x100 magnification, and total areas of resorption pits were analyzed by the Meta Morph program (Molecular Devices, LLC., CA).

Reverse Transcriptase-PCR

The expressions of RANKL, OPG, and β-actin were evaluated by RT-PCR using total RNA isolated from murine osteoblastic cells. Total RNA was isolated using Trizol reagent. The primers used were : for RANKL (750 bp), 5'-ATCAGAAGACAGCACTCACT-3' (forward), 5'-ATCTAGGACATCCATGCTAATGTTC-3' (reverse); for OPG (636 bp), 5'-TGAG TGTGAGGAAGGGCGTTA C-3' (forward), 5'-TTCCTCGTTCTCTCAATCTC-3' (reverse) and for β-actin (366 bp), 5'-GGACTCCTATGGTGGGTGACGAGG-3' (forward), and 5'-GGGAGAGCATAGCCCTCGTAGAT-3' (reverse).

Relative RT-PCR was performed to measure gene expression of RANKL, OPG, and β-actin mRNAs. Polymerase chain reactions were performed on a T gradient 96 PCR machine (Biometra Co., Gottingen, Germany) using 1~2 ng of cDNA, 5 pmoles of each oligonucleotide primer, 200 μM of each dNTP, 1 unit of Taq Polymerase (Applierd Biosystems, CA, USA) and 10 x Taq polymerase buffer in a 50 μl vo-

lume. The PCR program initially started with a 95°C denaturation for 5 min, followed by 25 to 35 cycles of 95°C/1 min, T_a/1 min, 72°C/1 min (T_a, annealing temperature; 45.3°C for RANKL, 47.9°C for OPG, and 58°C for β-actin). Linear amplification range for each gene was tested on the adjusted cDNA. The less expressed transcripts of RANKL and OPG required 35 cycles of PCR for detection. For β-actin, 25 cycles of PCR was performed, respectively. Densitometry values were measured at each cycle sampling using the TINA software (University of Manchester, Manchester. U.K.). RT-PCR values are presented as a ratio of the specified gene's signal in the selected linear amplification cycle divided by the β- actin positive control signal.

ELISA

Quantikine® M murine Mouse RANK Ligand kit (R & D systems Inc., Minneapolis, IN) was used to analyze RANKL protein. Briefly, mRANKL standard was diluted in Calibrator Diluent RD6-12 solution to make final concentration of 0, 31.2, 62.5, 125, 250, 500, 1000, and 2000 pg/ml. Assay Diluent RD1W and standards (50 μl each) were added to each well and incubated for 2 hours at room temperature. Each well was aspirated and washed, repeating the process four times for a total of five washes. mRANKL conjugate (100 μl) was added to each well and incubated for 2 hours at room temperature. Washing was repeated as described above. Substrate solution (100 μl) was added to each well and incubated for 30 minutes at room temperature in dark room. Stop Solution (100 μl) was added to each well and mixed by gentle tapping. Then the enzyme reaction yields a blue product that turns yellow. The intensity of the color of each well was determined within 30 minutes, using a microplate reader at 450 nm.

Data analysis and statistics

The results are expressed as the mean ± S.E.M. The statistical significances of differences between the groups were determined using the one-way ANOVA test. In statistical tests, the p value < 0.05 was considered to be significant.

Results

Xylitol inhibits 1α,25(OH)$_2$D$_3$-induced osteoclast formation

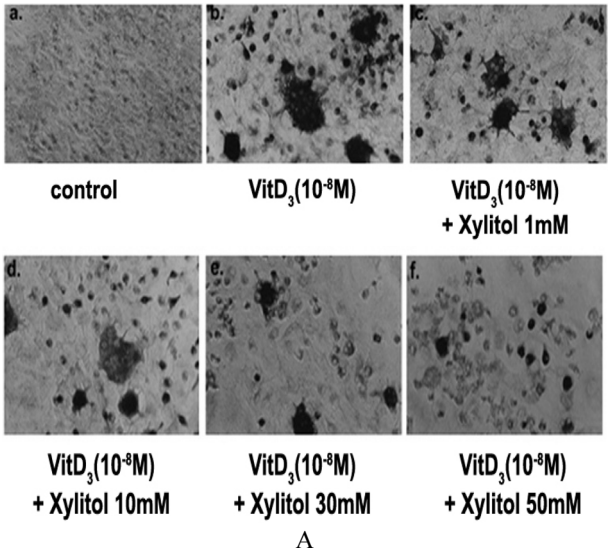
Osteoclastogenesis was induced by 1α,25(OH)$_2$D$_3$ in osteoblastic cells/bone marrow co-culture. To clarify the role of xylitol on bone metabolism, 1, 10, 30, 50, or 100 mM of xylitol were added to co-cultures and incubated at 37°C for 4 days. When 10 nM of 1α,25(OH)$_2$D$_3$ was added to the co-culture TRAP positive multinucleated cells were formed, whereas no TRAP positive cells were detected in media only. In the presence of xylitol, 1α,25(OH)$_2$D$_3$-induced osteoclast differentiation was reduced (Fig. 1A). The addition of 1, 10, 30, 50 or 100 mM of xylitol reduced the number of TRAP positive multinucleated cells up to about 35% in 50 mM of xylitol (Fig. 1B). However, it might be possible that xylitol could cause cell damage directly without interrupting the normal maturation of osteoclasts. To confirm possibility, we have carried out a viability test. As shown in Fig. 1C, xylitol did not show any remarkable toxic effect when treated with xylitol at up to 50 mM. These results suggest that the effect of xylitol on bone metabolism was not caused by its direct toxic effect upon the cells.

Bone resorption activity assay (pit formation assay)

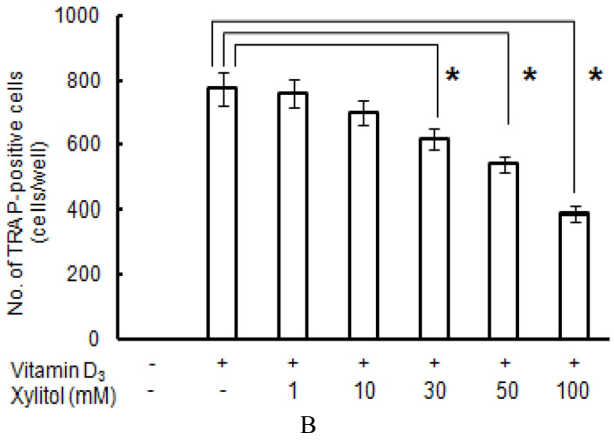
We have measured resorbed bone lacuna and sum up on each bone slice. The addition of 10 mM of 1α,25(OH)$_2$D$_3$ effectively caused the formation of lacuna on bone slices whereas no resorption lacuna has been observed without 1α,25(OH)$_2$D$_3$-induction. However, the resorbed area was gradually decreased as the concentration of xylitol was increased up to 50 mM (Fig. 2A). At 50 mM of xylitol about 80% of bone resorption area has been decreased. The average areas of resorption pit were measured and depicted in Fig. 2B. This indicated that xylitol might be effective on osteoclast activation and function.

Xylitol caused changes in mRNA expression of RANKL

As shown in Fig. 3, the expressions of RANKL and OPG mRNA in osteoblasts were monitored by RT-PCR in the presence or absence of xylitol. As the xylitol concentration in the co-culture medium was increased, the 1α,25(OH)$_2$D$_3$-induced expression of RANKL mRNA was decreased (Fig. 3A). RANKL mRNA expression in osteoblasts was inversely proportional to xylitol concentration. On the other hand, the expression of OPG mRNA was not changed regardless of xylitol concentration. These findings indicate that xylitol inhibits osteoclast differentiation by down-regulating the expression of RANKL. The ratio of RANKL to OPG mRNA in osteoblast is illustrated in Fig. 3B. As the xylitol concentration was



A



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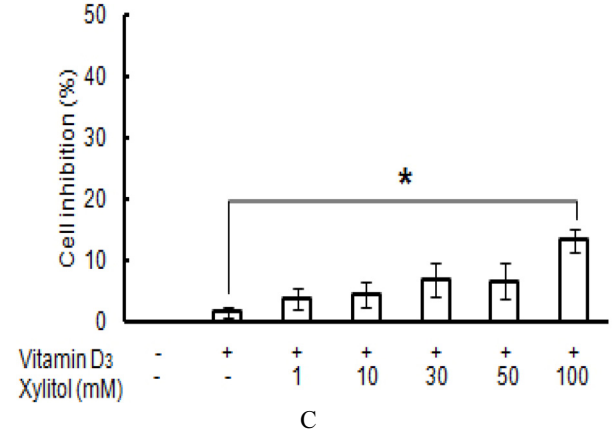
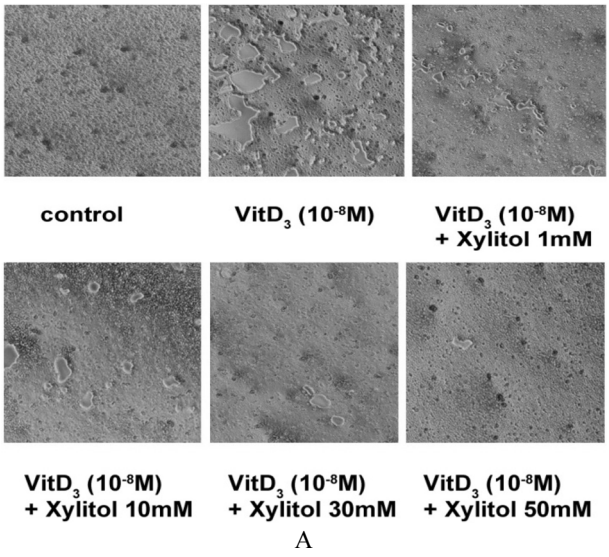


C

Fig. 1. Inhibition of osteoclast differentiation by xylitol. (A), In the presence of xylitol, 1α,25(OH)$_2$D$_3$-induced osteoclast differentiation was reduced (×200). (B), TRAP-positive multinucleated cells containing three or more nuclei were counted as osteoclasts. (C), MTT test. The statistical significance of differences between the groups was determined using the one-way ANOVA test. (*), In all statistical tests, a p value < 0.05 was considered to be statistically significant. Each data was shown in mean ± SEM of four cultures.



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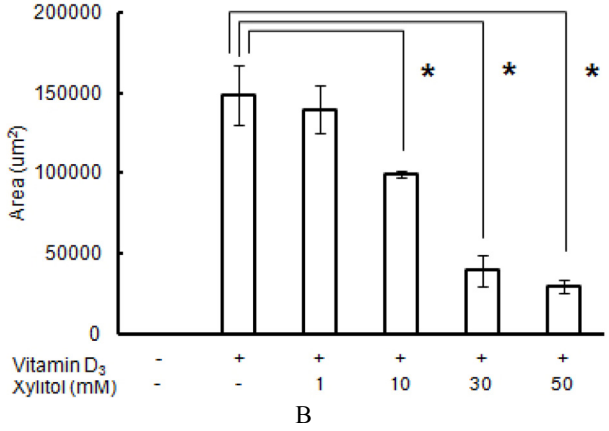

B

Fig. 2. Effects of xylitol treatment on resorption pit formation. (A), Resorbed lacuna on the OAAS plates were photographed the microscope (x100). (B), Total resorption area per well measured by image analyzer and graphed. (*), A p value < 0.05 was considered to be statistically significant.

increased, the ratio of RANKL to OPG mRNA decreased, which means RANKL and OPG, which are closely linked to osteoclastogenesis.

In addition, RANKL and OPG proteins were also analyzed with ELISA using anti-RANKL antibody (Fig. 4A). RANKL protein was decreased with the increase of xylitol concentration. However, the addition of xylitol did not change the amount of OPG protein which is consistent with OPG mRNA data (Fig. 4B). Consequently, xylitol inhibited 1α,25(OH)$_2$D$_3$-induced RANKL mRNA and protein, and led to alter osteoclastogenesis. In addition, such changes of signaling molecules were dependent on the xylitol concentration.

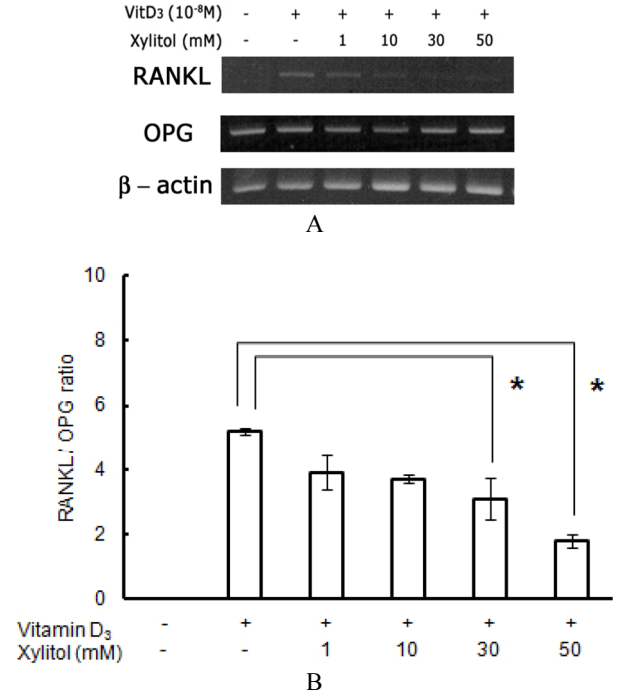

Fig. 3. Effects of xylitol on mRNA expression of RANKL and OPG in osteoblastic cells. (A), Various concentrations of xylitol were added to the mouse calvarial osteoblast culture with 10 nM of $1\alpha,25(OH)_2D_3$. After incubation for 4 days, total RNA was then extracted from osteoblasts, and the expressions of RANKL and OPG mRNAs were analyzed by RT-PCR products. (B), The expression of RANKL mRNA compared with OPG mRNA. The results were expressed as the means ± SEM of four experiments. (*), A p value < 0.05 was considered to be statistically significant.

Xylitol inhibits RANKL-induced osteoclastogenesis.

To clarify the role of xylitol on bone metabolism, 1, 10, 30, 50, or 100 mM of xylitol were added to cultures and incubated at 37°C for 6 days to investigate osteoclast differentiation. Osteoclastogenesis was induced by RANKL in RAW

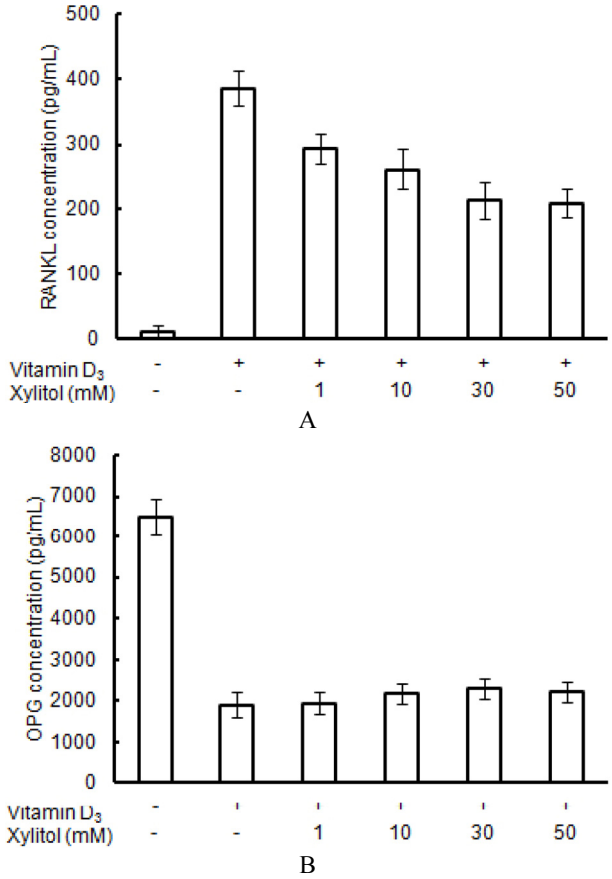

Fig. 4. Expression of RANKL and OPG protein in mouse calvarial osteoblastic cells. (A), Protein analysis using ELISA showed that xylitol inhibited the expression of sRANKL. (B), OPG level were slightly increased in osteoblasts stimulated by xylitol, but it was not statistically significant. All data were expressed as the means ± SEM of four experiments.

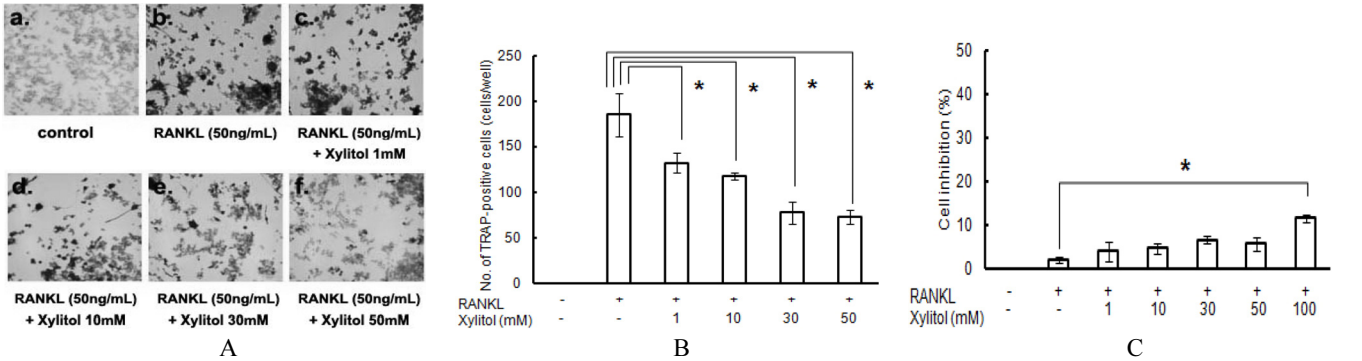

Fig. 5. Inhibition of sRANKL-induced RAW 264.7 cell differentiation by xylitol. (A), RANKL-induced osteoclast differentiation was reduced in the presence of xylitol (×100). (B), TRAP-positive multinucleated cells containing three or more nuclei were counted. (C), MTT test. (*), In all statistical tests, a p value < 0.05 was considered to be statistically significant. Each data was shown in mean ± SEM of four cultures.

264.7 cell culture. When 50 ng/ml of RANKL was added to the RAW 264.7 cell culture, TRAP positive multinucleated cells were formed whereas no TRAP positive cells were detected in media only. In the presence of xylitol, RANKL-induced osteoclast differentiation was reduced (Fig. 5A). The addition of 1, 10, 30, 50 or 100 mM of xylitol reduced the number of TRAP positive multinucleated cells (Fig. 5B). However, it might be possible that xylitol causes cell damage directly without interrupting the normal maturation of osteoclasts. To confirm this possibility, we have carried out a viability test. As shown in Fig. 5C, xylitol did not show any toxic effect at up to 100 mM.

Discussion

In this study, the effects of xylitol on osteoclastogenesis in osteoblast-osteoclast co-culture system and differentiation of RAW264.7 into osteoclast-like cells were investigated. As mentioned earlier, we found xylitol affects the bone metabolism, leading to the changes in $1\alpha,25(OH)_2D_3$-induced osteoclastogenesis. Interestingly, xylitol inhibited the $1\alpha,25(OH)_2D_3$-induced osteoclastogenesis (by up to 65 % of the control) in co-culture system (Fig. 1). Although xylitol inhibited $1\alpha,25(OH)_2D_3$-induced osteoclastogenesis, it could be argued that such an inhibition of osteoclastogenesis might not due to the physiological intervention of xylitol in the normal process of osteoclastogenesis, but the cell damage non-physiologically. To rule out the possibility that xylitol might cause non-physiological cell damage we have performed the MTT test. The test showed that xylitol did not exert any harmful effect upon the cells in this co-culture system, which suggests that xylitol inhibits the formation of TRAP positive cells, without a toxic effect upon the cells.

Here, we raised the question about how xylitol triggers the down-regulation of $1\alpha,25(OH)_2D_3$-induced osteoclastogenesis. Since osteoclast differentiation is mediated by critical signal molecules, such as RANKL, OPG and M-CSF [8-11], we used an osteoblast/stromal cell co-culture system to evaluate whether xylitol alter the $1\alpha,25(OH)_2D_3$-induced osteoclast differentiation in terms of the expression profiles of RANKL and OPG mRNA. The expression of RANKL in osteoblastic cells by the treatment of $1\alpha,25(OH)_2D_3$ was down-regulated upon increasing the xylitol concentration, and the expression of OPG mRNA was not changed significantly (Fig. 3). In addition, the expression of sRANKL was decreased with xylitol concentration in the process of $1\alpha,25(OH)_2D_3$-induced osteoclastogenesis, being in consistency with the decrease in RANKL mRNA expression (Fig. 4A). On the other hand, OPG protein was slightly increased with xylitol treatment but it was not statistically significant (Fig. 4B). From the findings described above, such an inhibitory mechanism of osteoclastogenesis by xylitol might be associated with modulating RANKL, not OPG expression in osteoblasts. Previous study showed that Bumethnide, NaKCl cotransmitter inhibitor, reduced expression of RANKL via cell volume shrinkage of osteoblast due to the hyperosmolarity [26]. Molarity of xylitol under 50 mM used in this study may not be considered enough to explain effect of xylitol. Since, it might be insufficient to cause the hyperosmotic shrinkage. Then, probability which we can speculate is direct effect on inner cell structure or physiologic process, which is possible through membrane transportation as in *S. mutans*. If osteoblast and osteoclast have transporter like phosphorylation transferase system of fructose (PTS-Fru) or similar one, absorbed xylitol-phosphate cannot be metabolized and it may have toxic effect on bone cells like in *S. mutans*. However, understanding of the exact nature of down-regulation of osteoclastogenesis by xylitol requires further studies at the level of osteoblast and osteoclast.

In summary, we have provided the first evidence that xylitol inhibit not only RANKL protein synthesis in osteoblastic cells but also osteoclast function (bone resorption activity) in $1\alpha,25(OH)_2D_3$-induced osteoclastogenesis. Xylitol down-regulated osteoclastogenesis in $1\alpha,25(OH)_2D_3$-induced osteoclastogensis in co-culture system via reduction of RANKL mRNA expression and sRANKL synthesis.

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