Down-regulation of 1α,25-dihydroxyvitaminD_3- induced osteoclastogenesis in response to xylitol in mouse

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Jong- Pil Kim

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Abstract

Down-regulation of 1α,25-dihydroxyvitaminD_3-induced osteoclastogenesis in response to xylitol in mouse

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(Directed by Prof. Jong-Gap Lee, DDS, Ph. D)

Xylitol has a variety of functions to cells, such as bacteriostatic, and anticariogenic effects. However, understanding of cellular mechanism for the role of xylitol on bone metabolism remains to be solved. In this study, the physiological role of xylitol in osteoclastogenesis was exploited in a co-culture system and RAW 264.7 cell. Xylitol reduced the number of tartrate-resistant acid phosphatase (TRAP) positive multinucleated cells. The number of multinucleated osteoclast cell not only in co-culture system but also in RAW 264.7 cell exposured to medium containing 1, 10, 30, 50, and 100 mM of xylitol was reduced in a dose dependent manner. Bone resorption activity, which was performed on the bone slice in co-culture system, was dramatically decreased as xylitol concentration increasing. According to the viability test, there was no cellular damage at up to 100

mM of xylitol. In order to investigate the mechanism by which xylitol inhibits osteoclastogenesis, the mRNA expressions of receptor activator of NF-κB ligand (RANKL) and osteoprotegerin (OPG) were analyzed by RT-PCR. Exposure of osteoblastic cells to a medium containing of xylitol reduced RANKL mRNA expression induced by 10^{-8}M 1α, 25$(OH)_2D_3$ in a dose-dependent manner. In addition, RANKL and OPG protein were also analyzed with ELISA using anti-RANKL and anti-OPG antibody. The expression of RANKL was decreased with the increase of xylitol concentration. The amount of OPG was slightly increased in the presence of xylitol, but there was no statistically significant difference. Nonetheless these results imply that xylitol inhibits osteoclast differentiation by reducing RANKL/OPG ratio in osteoblast. Also, we found that the phosphorylation of c-Jun NH_2-terminal Kinase (JNK) and Extracellular Signal-regulated Kinase(ERK), which regulates the activity of various transcriptional factors, was reduced by xylitol treatment in RAW 264.7 cells.

Conclusively, these findings suggest that xylitol down-regulates the 1α,25$(OH)_2D_3$- induced osteoclastogenesis via partly modulation of RANKL/ RANK/ OPG regulatory axis and the decrement of phosphorylation of MAPK in preosteoclast.

Key words: 1α,25$(OH)_2D_3$, xylitol, bone resorption, osteoclastogenesis, RANKL, JNK, ERK

I. Introduction

It has been reported that xylitol has a variety of functions to cells, such as bacteriostatic and anticariogenic effects. Xylitol is a five carbon natural polyalcohol, which is widely distributed in fruits, berries, and plants. This sugar alcohol is taken up via constitutive phsphotransferase system-Fructose (PTS-Fru) but it cannot be metabolized to an energy source by *streptococcus mutans* (*S. mutans*) and accumulated as a toxic xylitol-phosphate in the cell. Expulsion of dephosphorylated xylitol from the bacterial cell results in an energy-consuming futile cycle which, together with a harmful intracellular accumulation of xylitol phosphate, resulting in growth inhibition. Due to the accumulation, xylitol induces biological responses including the emergence of xylitol-insensitive populations. Glucosyl transferase (GTF) activity that mediates sucrose-dependant adherence of *S. mutans* to the tooth surface is essential for the cariogenicity of these microorganisms, and contributes significantly to the exopolysaccharide component of the dental-plaque matrix. xylitol inhibits GTF (Wunder D. *et al* 1999). Hrimech M. suggest that xylitol is a strong metabolic inhibitor that disturbs protein synthesis and reduces the expression of heat shock protein (HSP) -70 (Dnak-like) and HSP-60 (Groel-like) proteins in the wild type xylitol-sensitive *S.mutans,* but not in the xylitol-resistant natural strain. The natural dietary carbohydrate xylitol has been

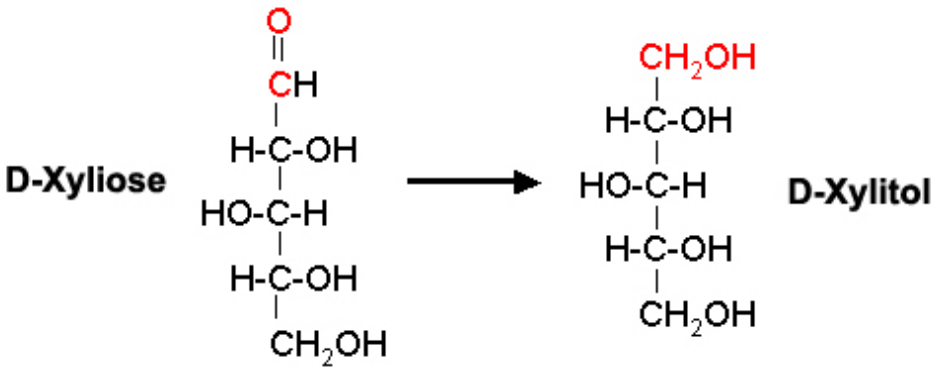


Fig 1. Chemical nature of xylitol. Xylitol is a unique linear five-carbon polyalcohol, which is widely distributed in nature. Xylitol can be also produced by many industrial methods from xylose**.**

used as a source of energy in infusion therapy and found to act curatively in certain clinical situations and used as diabetic diet. In the human body, 5 to 15g of xylitol is formed as an intermediate of the mammalian carbohydrate metabolism daily.

With respect to the role of xylitol on bone, it has shown that moderate dietary xylitol supplementation protects against weakening of bone biomechanical properties in experiment of postmenopausal osteoporosis. A continuous moderate dietary xylitol supplementation leads to increased bone volume and increased bone mineral content in the long bones of aged rats (Matilla *et al.* 1998, 1999, 2001, 2002, Svanberg *et al.* 1993, 1994, 1997). In spite of extensive xylitol research, no experimental evidences for the cellular mechanism of xylitol on bone cells are emerged on the horizon.

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 (Burgess *et al.* 1999; Fuller *et al.* 1998; Kong *et al.* 1999; Lacey *et al.* 1998). 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 (Raise, 1998). An imbalance between bone formation and

bone resorption derived from inappropriate receptor activator of NF-κB ligand (RANKL) expression by activated lymphocytes and osteoclasts causes metabolic bone diseases like osteopetrosis and osteoporosis (Aubin, 1998). Therefore, osteoblasts and osteoclasts are known to be closely related each other during the process of bone remodeling (Suda *et al.* 1995; Takahashi *et al.* 1999; Tsukii *et al.* 1998). Certain kinds of signaling molecules, such as RANKL, osteoprotegerin (OPG) and macrophage colony stimulating factor (M-CSF), expressed by osteoblasts, are involved in osteoclastogenesis and in osteoclast development. For instance, when osteoblasts/stromal cells are stimulated by osteotropic factors such as $1\alpha,25(OH)_2D_3$, RANKL is expressed and induces the differentiation of osteoclast progenitors by binding to the receptor activator of NF-κB (RANK) (Jimi *et al.* 1999). 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 (Lacey *et al.* 1998). Specifically, OPG, as a member of the tumor necrosis factor receptor (TNFR) family, inhibits the osteoclastogenesis stimulated by 1α, $25(OH)_2D_3$, or PTH (Takahashi *et al.* 1999). Consequently, it is believed that RANKL, M-CSF and OPG, which are expressed by osteoblasts, are associated with osteoclastogenesis, and osteoblasts are a major factor in the bone remodeling process.

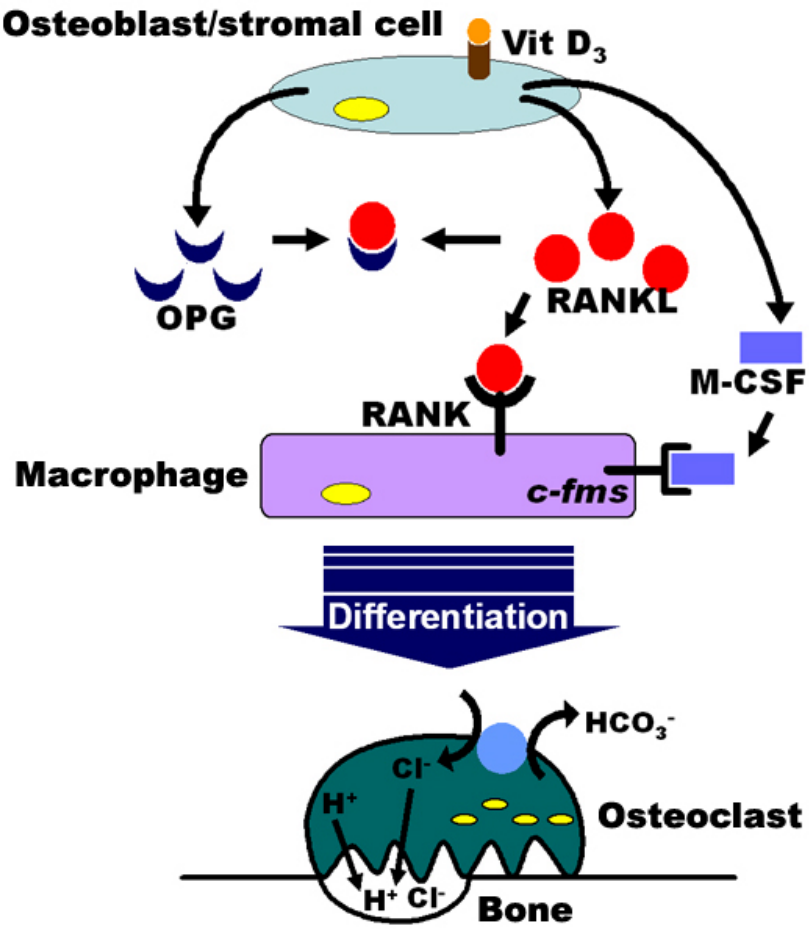


Fig 2. **Schematic regulation mechanism of osteoclastogenesis and bone resorption**. Stromal cells and osteoblasts express RANKL and M-CSF, which are up regulated by osteoclastogenic molecules such as $1\alpha,25(OH)_2D_3$. $1\alpha,25(OH)_2D_3$ also blunts expression of OPG. RANKL and M-CSF, interacting their receptors on monocyte-macrophage cells, induce osteoclast differenciation, a process inhibited by OPG. The differenciated osteoclast polarized on the bone surface. After formation of the ruffled membrane, the osteoclast acidifies an extracellular microenviroment by means of proton pump. Intracellular pH is maintained by HCO_3^-/Cl^- exchange.

Mouse RANKL is a 45kDa, 316 amino acid type II transmembrane glycoproteine that exists naturally as nondisulfide-linked homotrimer (Kong Y *et al*, 2000). The molecule has a 47 amino acid cytoplasmic domaine, a 23 amino acid transmembrane segment, and a 246 amino acid extracellular region (Kodaira K *et al*, 1999). Within its extracellular region, it contains a 159 amino acid TNF domain. 177 amino acid residue soluble RANKL is generated by metaloprotease cleavage of membrane-bound RANKL (Lacey D *et al*, 1998). Although both membrane and soluble RANKL are bioactive, the homeostatic form of RANKL may be the membrane-bound form (Fuller K *et al*, 1998), while soluble RANKL may signal underlying pathology (Udagawa *et al* 2000). Cells known to express RANKL include odontoblasts and ameloblasts (Rani CS, Wise GE *et al* 2000), osteoblasts, T cell, chrondrocytes, fibroblasts, and skeletal muscle cells (Kartsogiannis *et al* 1999). Mouse RANKL is active on human cells. Mouse RANKL shows 85% and 96% amino acid identity to human and rat RANKL respectively (Lacey et al, 1998).

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 evidences as to whether xylitol is related to osteoclastogenesis at the cellular level. Understanding of xylitol on bone

metabolism is necessary at the molecular level of osteoblast and osteoclast. Therefore, we hypothesized that xylitol might concern osteoclastogenesis and bone metabolism with respect to RANKL, OPG in the osteoblast. Furthermore xylitol may affect osteoclast directly. To investigate how xylitol affect the osteoclastogenesis induced by $1\alpha,25(OH)_2D_3$, and by sRANKL we applied an osteoblast/stromal cell co-culture system and RAW 264.7 cells. We examined the osteoclast differentiation rate and pit formation activity on the bone slice with xylitol in co-culture system. Also we compared not only RANKL and OPG mRNA expression profiles but also sRANKL and OPG which exocytosised in osteoblast. Finally we investigated TRAP positive multinucleated cell and the change of phosphorylated JNK (p-JNK) and ERK (p-ERK) in RAW 264.7 cell.

II. Materials and Methods

1. Materials

Routine cell culture media was 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 supported from Borak corp. (Seoul, Korea). All other chemicals were of the highest grade commercially available.

2. *In vitro* osteoclast formation assay

The osteoblast formation assay was carried out as previously reported by Choi *et al*. (2001). Briefly, the osteoblasts were isolated from 1~2 day-old newborn mice. 3~10 calvariae were digested in 10 ㎖ 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 ㎖ 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 ㎖) 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 x 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 flushed by slowly injecting media at one end using a 25-gauge needle. The calvaria and bone marrow cells collected were washed and used in the co-culture. Mouse calvarial cells (1 x 10^4 cells/well) were co-cultured with bone marrow cells (1 x 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 $\mu\ell$ per well with α-MEM supplemented with 10% FBS, in the presence of 1α,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. We counted TRAP positive multinucleated(more than three) cells as osteoclast. *In vitro* formation assay of osteoclast was repeated four times.

3. 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 ㎎/㎖ 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.

4. 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 (Oscotec, korea) according to the method of Eijiro *et al.* and Youngnim *et al.* with a slight modification. Briefly, the osteoblastic cells and bone marrow cells were resuspended in complete α-MEM medium and plated into a calcium phosphate apatite-coated plate, OAAS at 2 x 10^5 cells/0.8 ㎖/well and 2 x 10^6 cells/0.8 ㎖/well, respectively.

The cells were cultured for 4 days at 37˚C in a humidified 5% CO_2 atmosphere. Then the cells treated with 10 nM 1α,25$(OH)_2D_3$ (10^{-8} M) and xylitol by different concentration, respectively. Cultures were maintained for 4 days. The medium in each well was replaced with the respective fresh completive medium and 1α,25$(OH)_2D_3$(10^{-8} M) and xylitol. The experiments were performed four times. After termination of culture, attached cells were removed from the plate by abrasion with 4% sodium hypochloride solution (Sigma). We got images of pit with a digital camera attached to a microscope at x100 magnification, and the Image Meta Morph program analyzed total resorption pits. The data were presented as means ± SD of four times sample.

5. RT-PCR (Reverse Transcriptase-Polymerase Chain Reaction)

RT-PCR experiment consists of RNA isolation, cDNA polymerization, and PCR ampulification. 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, chloform, and isoprophanol. The primers used for RANKL (750 bp), 5'-ATCAGAAGACAGCACTCACT-3' (forward), 5'-ATCTAGGACATCCATGCT AATGTTC-3' (reverse); for OPG (636 bp), 5'-TGAG TGTGAGGA AGGGCGTTAC-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 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 ㎕ volume. The PCR program initially started with a 95°C denaturation for 5 min, followed by 25 to 38 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 and β-actin, 30 and 25 cycles of PCR was performed, respectively. The PCR samples were electrophoresed on 1 % agarose gels in TAE buffer. The gels were stained with ethidium bromide (10 ㎍/㎖) and photographed on top of a 280 nm UV light box. The quantity and base pair size of the PCR generated DNA fragments were estimated relative to DNA ladder standards. 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.

6. ELISA (Enzyme Linked Immuno-Solbent Assay)

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/㎖. Assay Diluent RD1W and standards (50 ㎕ each) were added to each well and incubated for 2 hours at room temperature. A plate layout is provided to record standards and samples assayed. Each well was aspirated and washed, repeating the process four times for a total of five washes. mRANKL conjugate (100 ㎕) was added to each well and incubated for 2 hours at room temperature. Washing was repeated as described above. Substrate solution (100 ㎕) was added to each well and incubated for 30 minutes at room temperature in dark room. Stop Solution (100 ㎕) 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.

7. Western blot of MAP kinase in RAW 264.7 cell

Protein extracts were prepared from RAW 264.7 cells as follows. RAW 264.7 cells were washed with ice-cold PBS and then lysed by adding Tris-HCl , NaCl, and EDTA buffer (1% NP-40, 10 mM of Tris-HCl [pH 7.8] , 150 mM of NaCl, 1 mM of EDTA, 2 mM of Na_3VO_4, 10 mM of NaF, 10 ㎍/㎖ of aprotinin, 10 ㎍/㎖ of leupeptine, and 10 ㎍/㎖ of PMSF. The lysates were clarified by centrifugation at 15,000 rpm for 20 minutes. An equal amont of protein was subjected 10% sodium deoxyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred electrophoretically onto a nitrocellulose membrane. The membrane was incubated in a blocking solution containing 5% non-fat milk at room temperature for 1 hour and washed 1xTBE solution containing 0.1% of Tween-20 (TBST). The membrane was probed with 1:1000 dilution of anti-phospho ERK (New England Biolabs.) or anti-phospho JNK (new England Biolabs.) at 4℃ for overnight. The same membrane followed by secondary antibody coupled with horseradish peroxidase (Santa Cruz Biotecnology). Immunoreactive proteins were visualized by enhanced chemiluminescence (ECL) western blotting detection reagents (Amercham, Alington Heights, IL, USA) following the procedure recommended by the supplier.

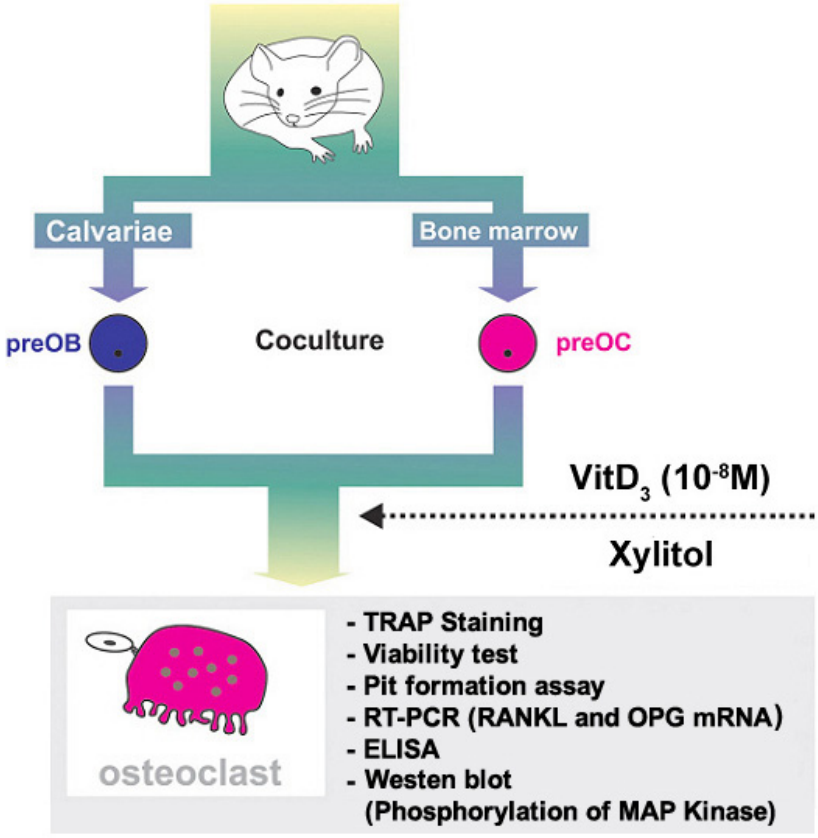


Fig 3. Schematic diagram of experimental materials and methods. Mouse calbarial cells (1X 10^4 cells/well) were co-cultured with bone marrow cells (1X10^5 cells/well) in α-MEM containing 10 % fetal calf serum in 48-well plates. All cultures were maintained at 37 ℃ in a humidified atmosphere of 5 % CO_2 in air. After 4 days, 1α,25$(OH)_2D_3$ and xylitol was added for comparison. After treatment, we counted tartrate-resistant acid phosphatase (TRAP, a marker enzyme of osteoclast) positive cells. We preceded cell viability test, pit formation assay on the bone slice in co-culture system. Also the expressions of RANKL and OPG mRNA and protein in osteoblasts were monitored by RT-PCR and ELISA in the presence or absence of xylitol. The changes in MAP kinase phosphorylation in RAW 264.7 cells were examined by western blot.

8. Data analysis and statistics

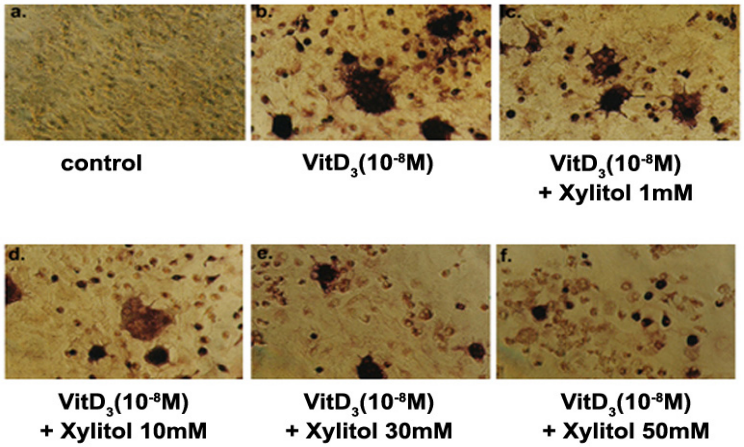
The results are expressed as the mean ± S.E.M. The statistical significances of differences between the groups were determined using the Kruskal-Wallis and Bonferroni's test. In statistical tests, the p value < 0.05 was considered to be significant.

III. Results

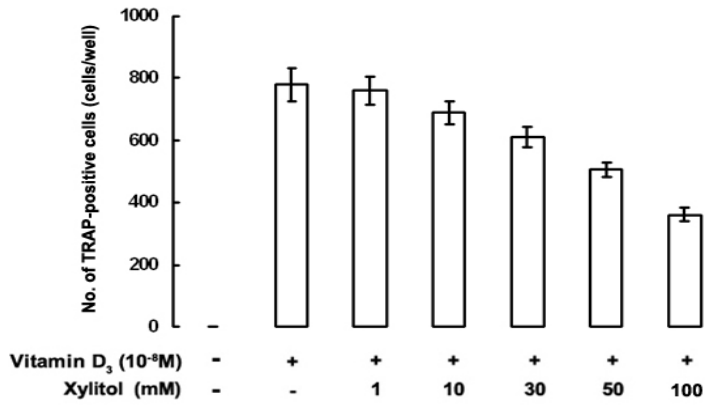
1. Xylitol inhibits 1α,25(OH)$_2$D$_3$-induced osteoclast formation

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 to investigate osteoclast differentiation. 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. 4A). 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 50mM of xylitol (Fig. 4B). However, it might be possible that xylitol causes cell damage directly without interrupting the normal maturation of osteoclasts. To this possibility, we used a viability test. As shown in Fig. 4C, xylitol did not show a toxic effect. These results suggest that the effect of xylitol on bone metabolism was caused by its some effect or changing of activity, not by its direct toxic effect upon the cells.

A.


a.
b.
c.
control
VitD3(10-8M)
VitD3(10-8M)
+ Xylitol 1mM
d.
e.
f.
VitD3(10-8M)
+ Xylitol 10mM
VitD3(10-8M)
+ Xylitol 30mM
VitD3(10-8M)
+ Xylitol 50mM

B.


No. of TRAP-positive cells (cells/well)
1000
800
600
400
200
0
Vitamin D3 (10-8M) - + + + + + +
Xylitol (mM) - - 1 10 30 50 100

C.

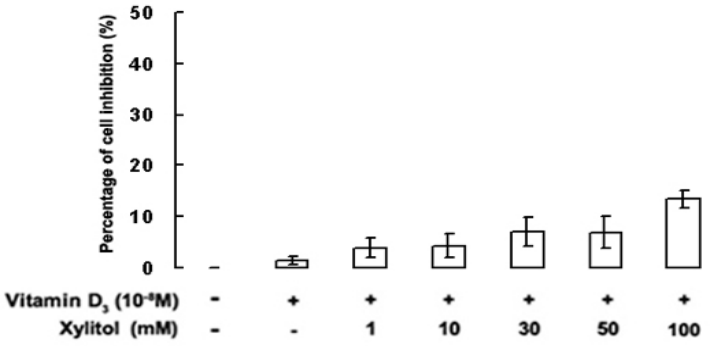

Percentage of cell inhibition (%)
50
40
30
20
10
0
Vitamin D3 (10-8M) - + + + + + +
Xylitol (mM) - - 1 10 30 50 100

Fig. 4. Inhibition of osteoclast differentiation by xylitol (A) In the presence of xylitol, $1\alpha,25(OH)_2D_3$-induced osteoclast differentiation was reduced (x200). (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. 100mM of xylitol showed a high cell inhibition rate. 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.

2. pit formation assay

We measured bone lacuna resorbed at each bone slice. There was no resorption lacuna on bone slice without 1α,25 $(OH)_2D_3$-induction. On the other hand, 1α,25 $(OH)_2D_3$ (10^{-8} M) induced many bone resorption pits and we could find multiple lacuna, which was used as a control. But in the experimental group, resorption pits were incrementally decreased as concentration of xylitol was increasing up to 50 mM (Fig. 5A). Total resorption area was also decreased by xylitol (Fig. 5B)

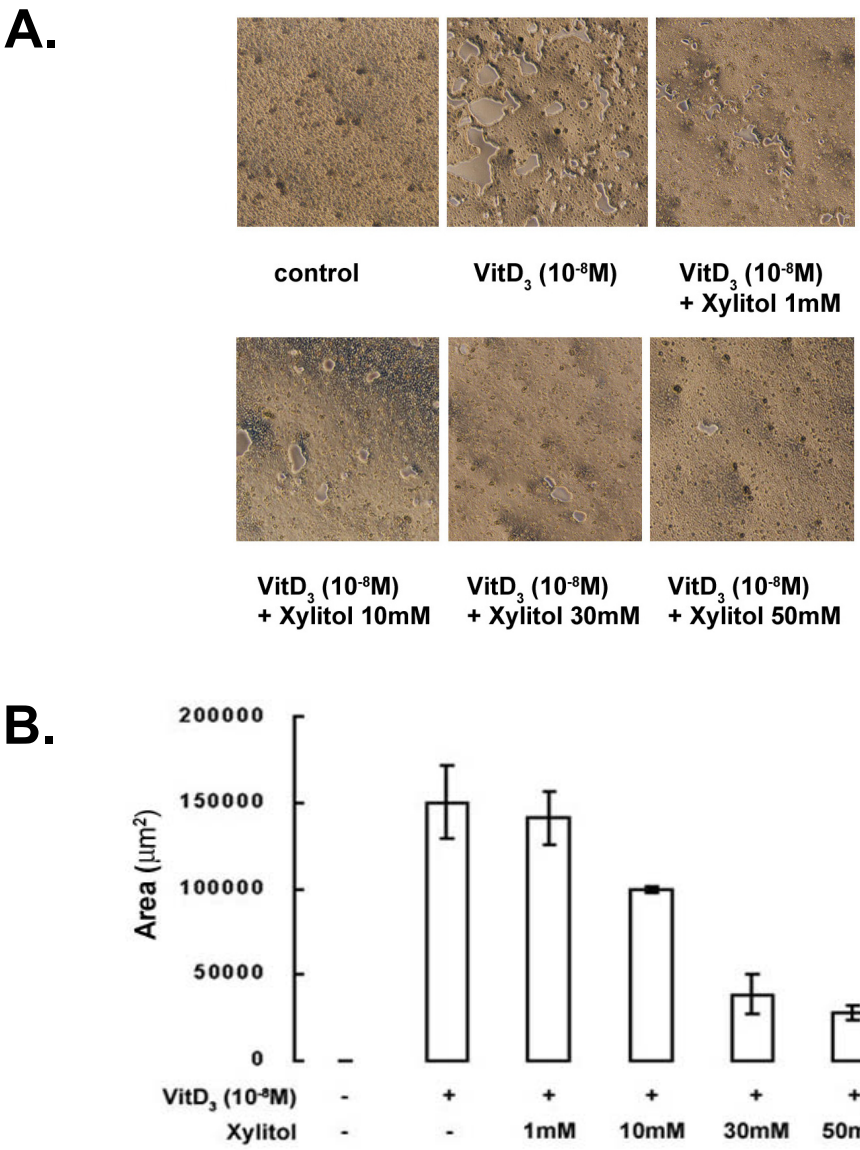


Fig. 5. Effects of Xylitol treatment on resorption pit formation (A), Resorbed lacunae on the OAAS plates were photographed the microscope (x100). (B), Total resorption area per well measured by image analyzer and graphed.

3. Xylitol caused changes in RANKL and OPG expression.

As shown in Fig. 6, the expressions of RANKL and OPG mRNA in osteoblasts were monitored by RT-PCR in the presence and absence of xylitol. As the xylitol concentrations in the cell culture medium were increased, the $1\alpha,25(OH)_2D_3$-induced expression of RANKL mRNA was down-regulated (Fig. 6A). RANKL mRNA expression in osteoblasts was inversely proportionate 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 Figure 6B. As the xylitol concentration was increased, the ratio of RANKL to OPG mRNA decreased, which means RANKL and OPG, which are closely linked to osteoclastogenesis.

RANKL and OPG proteins were also analyzed with ELISA using anti-RANKL antibody (Fig. 7A). RANKL protein was decreased with the increase of xylitol concentration. On the other hand, the addition of xylitol did not change the OPG protein quantitatively, which is consistent with OPG mRNA data (Fig. 7B). Consequently, xylitol inhibited RANKL mRNA and protein, and led to altered osteoclastogenesis. In addition, such changes of signaling molecules were dependent on the xylitol concentration.

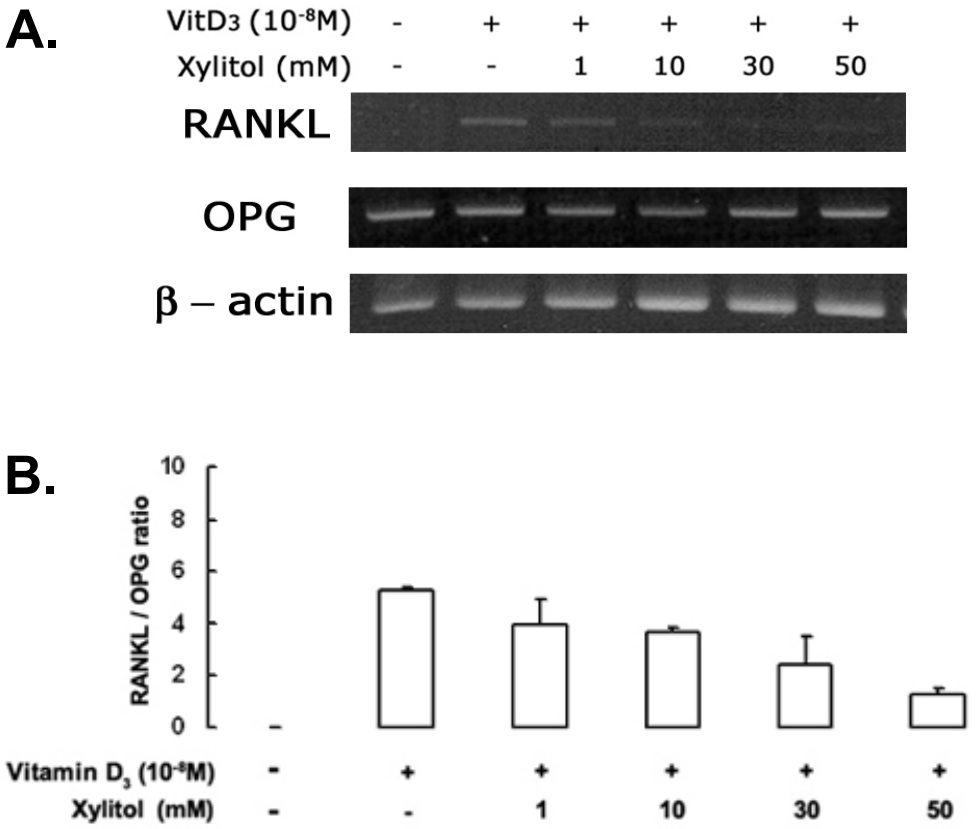


Fig. 6. Effects of Xylitol on mRNA expression of RANKL and OPG in osteoblasts. (A), Various concentrations of Xylitol were added to the mouse calvarial osteoblasts culture with 1α,25$(OH)_2D_3$ (10^{-8} M). After incubation for 4 days, total RNA was then extracted from osteoblasts, and the expression of RANKL and OPG mRNAs was analyzed by RT-PCR products for RANKL and OPG were 750bp and 636bp, respectively. (B), The expression of RANKL mRNA compared with OPG mRNA. The results were expressed as the means $\pm$SEM of four experiments.

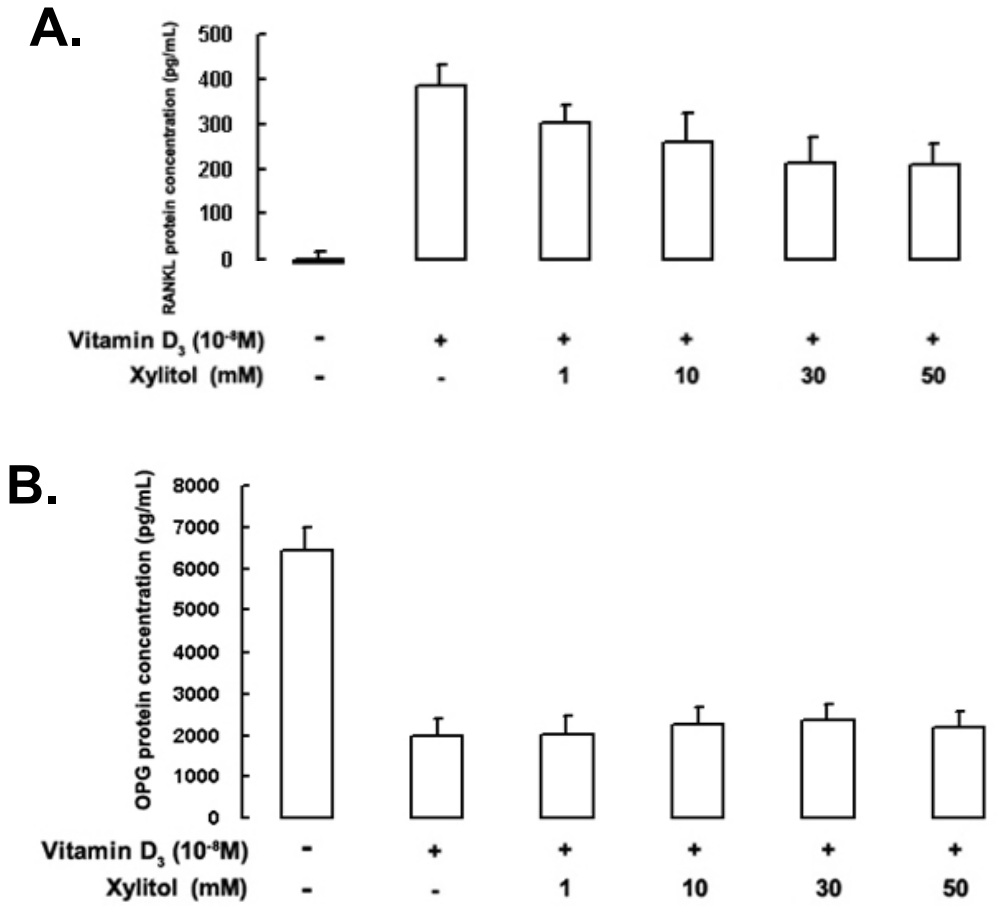
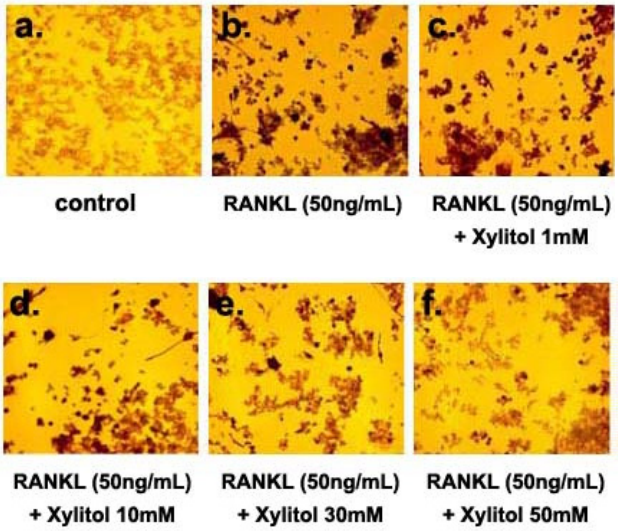


Fig. 7. Expression of RANKL and OPG in mouse calvarial osteoblasts. (A), Protein analysis using ELISA showed that xylitol inhibited the expression of RANKL. (B), On the other hand OPG level were increased slightly in osteoblasts stimulated by xylitol, but it was not statistically significant. The results were expressed as the means ± SEM of four experiments.

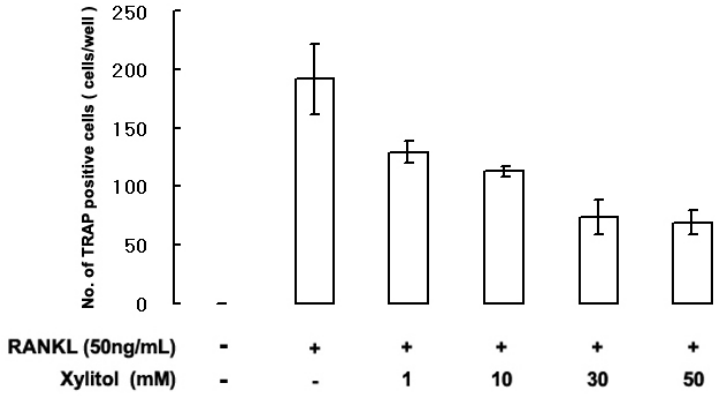
4. Xylitol inhibits sRANKL-induced osteoclastogenesis.

Osteoclastogenesis was induced by sRANKL in RAW 264.7 cell culture. 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 5 days to investigate osteoclast differentiation. When 50 ng/㎖ of sRANKL 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, sRANKL-induced osteoclast differentiation was reduced (Fig. 8A,B). However, it might be possible that xylitol causes cell damage directly without interrupting the normal maturation of osteoclasts. For this possibility, we used a viability test. As shown in Fig. 8C, xylitol did not show a toxic effect.

A.


a.
b.
c.
control
RANKL (50ng/mL)
RANKL (50ng/mL)
+ Xylitol 1mM
d.
e.
f.
RANKL (50ng/mL)
+ Xylitol 10mM
RANKL (50ng/mL)
+ Xylitol 30mM
RANKL (50ng/mL)
+ Xylitol 50mM

B.


No. of TRAP positive cells (cells/well)
250
200
150
100
50
0
RANKL (50ng/mL) - + + + + +
Xylitol (mM) - - 1 10 30 50

C.

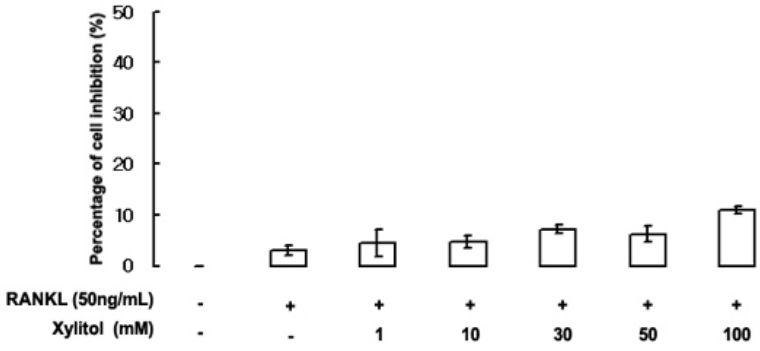

Percentage of cell inhibition (%)
50
40
30
20
10
0
RANKL (50ng/mL) - + + + + + +
Xylitol (mM) - - 1 10 30 50 100

Fig. 8. Inhibition of sRANKL-induced RAW 264.7 cell differentiation by Xylitol. (A), In the presence of xylitol, sRANKL-induced osteoclast differentiation was reduced (x100). (B) TRAP-positive multinucleated cells containing three or more nuclei were counted as osteoclasts. (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.

5. Phosphorylation of ERK and JNK was suppressed by xylitol in RAW 264.7

We have demonstrated how xylitol is linked to the RANKL mRNA and protein expression in osteoblastic cell. Because mitogen activated protein (MAP) kinase in general, is preferentially activated by cytokines and cellular stress and plays a key role in regulating the activity of various transcriptional factors. The changes in MAP kinase phosphorylation in RAW 264.7 cells were examined to investigate whether those were inhibited by xylitol or not. It was resulted in the inhibition of ERK and JNK phosphorylation in a dose dependent manner. (Fig.9). These findings suggest that the xylitol is closely associated with osteoclast fusion via ERK and JNK phosphorylation.

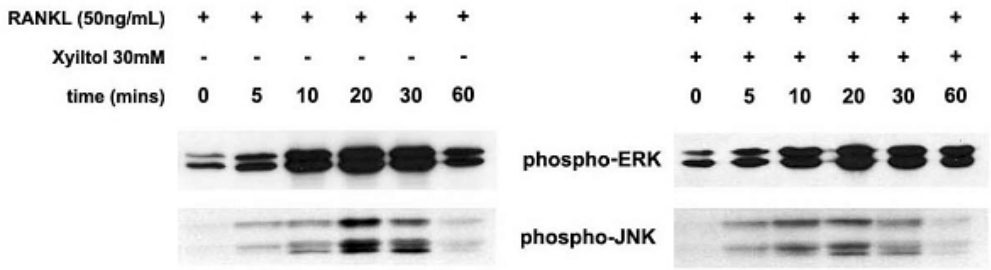


Fig. 9. sRANKL- induced activation of MAP kinase. RAW 264.7 cells (2x 10^6cells/culture) were treated with 50ng/㎖ sRANKL and 30mM of xylitol for the indicated periods of time, and the cell lysed. Cell lysates were detected by Western blot analysis using specific antibody to phosphorylated JNK and ERK.

IV. Discussion

On this study, the effects of xylitol on osteoclastogenesis in osteoblast-osteoclast co-culture system and differentiation of RAW 264.7 into osteoclast-like cells were investigated. As mentioned earlier, xylitol affects the bone metabolism, leading to the changes in RANKL-dependent osteoclastogenesis. This finding first confirms that the xylitol acts as a modulator that might involve in RANKL-dependent osteoclastogenesis. In co-culture system, xylitol acts as a signal for various cellular functions. Interestingly, we found that xylitol inhibited $1\alpha,25(OH)_2D_3$-induced osteoclastogenesis. Nevertheless, it could be argued that the inhibition of osteoclastogenesis by xylitol might not due to the physiological intervention of xylitol in the normal process of osteoclastogenesis but due to the cell damage. To rule out the possibility that xylitol causes non-physiological cell damage, an MTT viability test was performed. The test showed that xylitol(under 50 mM) 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. Since osteoclast differentiation is mediated by several factors, such as RANKL, OPG and M-CSF (Burgess *et al.* 1999; Fuller *et al.* 1998; Kong *et al.* 1999; Lacey *et al.* 1998), We used an osteoblast/stromal cell co-culture system to evaluate whether alterations affect the mRNA expression profiles of RANKL

induced by 1α,25$(OH)_2D_3$, was down-regulated upon increasing the xylitol concentration, and the expression of OPG mRNA was not changed significantly (Fig. 6). In addition, the expression of RANKL was decreased with xylitol concentration in the process of 1α,25$(OH)_2D_3$-induced osteoclastogenesis, being in consistency with the decrease in RANKL mRNA expression (Fig. 7A). On the other hand, OPG protein was slightly increased with xylitol treatment but it was not statistically significant (Fig. 7B). On the basis of such findings, inhibition mechanism of osteoclastogenesis by xylitol might be associated with modulating RANKL, not OPG expression in osteoblasts. In this study, differenciation of RAW 264.7 was reduced via suppressing p-JNK, p-ERK as the xylitol concentration was increasing (Fig. 9). Then, how could xylitol affect on osteoblast and osteoclast? Our previous study showed that high concentration of sucrose reduced expression of RANKL mRNA via cell volume shrinkage of osteoblast due to the hypertonisity. But concentration of xylitol under 50 mM used in this study may be considered insufficient to bring out hypertonic shrinkage. Then, probability that we can imagine is direct effects on membrane receptors or physiologic process of cell, which may be possible through membrane transportation as in *S. mutans*. If osteoblast and osteoclast have transporter something like phosphorylation transferase system of fructose (PTS-Fru) or similar one, absorbed xylitol-

phosphate can not metabolized and it may have toxic effect on bone cells like *S. mutans*. But this is only my hypothesis and also it may be untrue. Therefore 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 inhibits not only RANKL synthesis in osteoblastic cells but also osteoclast function (pit formation) in $1\alpha,25(OH)_2D_3$–induced osteoclastogenesis. Also xylitol inhibited RAW 264.7 cell differentiation via the suppression of phosphorylation of JNK and ERK, which regulate the activity of various transcriptional factors.

Further studies are needed to clarify the exact target and inhibition mechanism of xylitol.

V. Conclusion

Xylitol down-regulated $1\alpha,25(OH)_2D_3$–induced osteoclastogensis in co-culture system via reduction of RANKL mRNA expression and RANKL synthesis. Also xylitol directly inhibited sRANKL-induced preosteoclastic cell fusion and activation via suppresion of phosphorylation of MAP kinase(p-JNK, p-ERK) in RAW 264.7 cells.

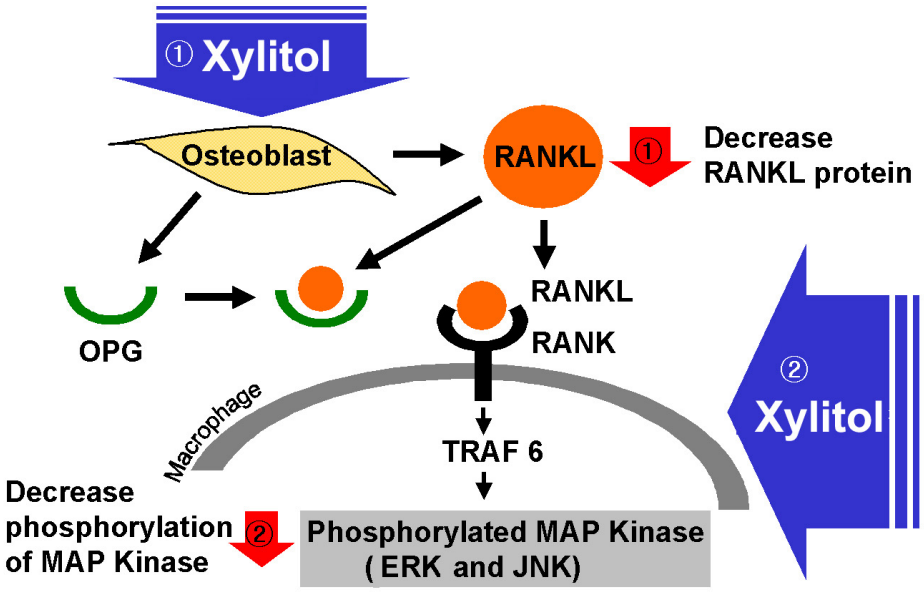


Fig. 10. Hypothetical regulation mechanism of xylitol in bone cells. Xylitol down-regulated 1α,25$(OH)_2D_3$–induced osteoclastogensis in co-culture system via reduction of RANKL mRNA expression and RANKL synthesis. Also xylitol directly inhibited osteoclast cell differentiation and activation via suppresion of phospolylation of MAP kinase in RAW 264.7 cells.

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국문요약

자일리톨에 의한 1α,25(OH)$_2$D$_3$-induced osteoclastogenesis 억제 효과

연세대학교 대학원 치의학과

김 종 필

자일리톨은 식물에 존재하는 5탄당의 당알콜으로 항 우식 효과 등 여러 가지 다양한 기능을 가지고 있다. 이전의 연구에서 자일리톨이 골 흡수를 감소시키고 골 강도를 증가 시킨다는 논문들이 발표 되었으나 분자 생물학적인 기전은 아직 보고된 바가 없었다.

본 연구는 활성형 비타민 D$_3$나 sRANKL을 공존 배양계와 RAW 264.7에 투여하여 파골세포 분화를 유도한 후에 자일리톨 1, 10, 30, 50, 그리고 100mM을 각각 투여하여 대조군과 비교 분석하였다. 먼저, 형성된 TRAP양성 파골세포 수를 계수한 결과 공존 배양계와 RAW 264.7 모두에서 자일리톨 농도 증가에 비례하여 핵이 3개 이상인 파골세포 수가 점차 감소하였다. 또한 bone slice상에서 골 흡수 능력을 비교한 결과에서도 감소하였다. 세포 독성능 검사 결과 자일리톨 50mM 이하에서는 독성이 없었다.

자일리톨의 파골세포분화 억제 기전을 규명하기 위하여 조골세포에서 골흡수 기전의 중요 Cytokine인 골흡수 촉진인자 (RANKL)와 골흡수 억제인자(OPG)의 mRNA 양(by RT-PCR)과, 최종 생산된 단백질의

양(by ELISA)을 측정하였다. 그 결과 자일리톨 농도가 증가 함에 따라 RANKL 및 mRNA 양이 점차적으로 감소하였다. 반면에 OPG 에는 변화가 없었다. 자일리톨이 조골세포 뿐 아니라 파골세포에도 직접 작용하는지 알아 보고자 RAW 264.7을 이용하여 파골 세포로 분화된 다핵세포 수를 계수한 결과, 그 숫자 또한 자일리톨 농도가 증가 함에 따라 점차 감소하였다. RAW264.7에서 세포내 신호전달 물질 중 하나인 JNK와 ERK를 Western blot을 이용하여 조사 하였다. 자일리톨을 투여한 결과 활성형인 p-JNK와 p-ERK가 대조군에 비해서 감소하였다.

결론적으로 자일리톨은 조골세포에서 골 흡수 촉진인자인 RANKL의 발현을 억제하고 파골세포 내에서 신호 전달 물질인 JNK와 ERK의 활성을 감소 시킴으로써 파골세포 분화를 억제하였다.

핵심되는 말 : 자일리톨, 골흡수, 1α, 25(OH)$_2$D$_3$, osteoclastogenesis, RANKL, JNK, ERK