The Effect of Pamidronate on Intervertebral Disc and Endplate

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Abstract

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It has long been known that N-containing bisphosphonates (BPs) can reduce osteoclast number by inducing apoptotic cell death in osteoclasts. As potent inhibitors of bone resorption, they are widely used for the treatment of bone disorders that are due to increased osteoclast activity i.e. postmenopausal osteoporosis, Paget's disease, tumoral bone disease, and particle induced osteolysis around metal implant. BPs also decrease breakdown of type II collagen and exhibit partial chondroprotective effect in a rabbit model of inflammatory arthritis. These findings support the fact that BPs have a chondroprotective effect. Nutritional deprivation caused by sclerosis of vertebral end plate provides, in part, a mechanism of intervertebral disc (IVD) degeneration. The fact that BPs have a powerful antiresorptive effect and chondroprotective effect prompts a research for the sum effect of BPs on IVDs since antiresorptive action can cause hypertrophy of endplate while chondroprotective effect can maintain chondrogenic phenotype of IVDs. Accordingly, the purposes of this experimental

study were, firstly, demonstrate the effect of pamidronate, second generation N-containing BPs, on in vitro metabolism of IVD cells and secondly, the effect of pamidronate on histologic changes of endplate and IVD. Lumbar IVD tissues were obtained from four patients and digested and cultured in three dimensional alginate beads to validate in vitro effect of N-containing second generation pamidronate. Twenty-four mice (C57BL/6) were utilized to test in vivo effect of pamidronate. Various doses of pamidronate (10^{-9}, 10^{-6}, 10^{-3} M) were administered to human IVD cultures. ^{3}H-Thymidine for DNA synthesis and ^{35}S-Sulfate incorporation for proteoglycan synthesis were performed. Reverse transcription polymerase chain reaction for mRNA expression of collagen type I, collagen type II, and aggrecan was done. For in vivo test, high (90ug/kg) and low doses (30ug/kg) of pamidronate were injected intravenously to mice weekly for 5 weeks to 12 weeks. Treatment with normal saline served as control. Hematoxylin-Eosin stain, Safranin-O stain, and TUNEL assay were performed. Histomorphometric data were also collected. Human IVD cell culture in three dimensional alginate beads with various concentrations of pamidronate (10^{-9}, 10^{-6}, 10^{-3} M) showed no significant increase in DNA synthesis compared to control culture. Human IVD cell showed significant increase in proteoglycan synthesis, 40% increase with a dose of 10^{-9} M pamidronate ($p<0.05$), compared to control culture. In densitometric assay of reverse transcription-polymerase chain reaction, human IVD cell cultures showed no statistically significant changes in mRNA expression

of type I collagen, type II collagen, and aggrecan compared to control. There was significant increase in thickness of endplate in animal with high dose pamidronate for 12 weeks after weekly injection. Moreover, there were no significant changes in nucleus and annulus such as necrosis and hyalinization of nucleus, disturbance of circular pattern of annulus, and cleft in nucleus and annulus in low dose and high dose group and in 5 weeks and 12 weeks follow up group, compared to control animals. There was no significant increase in TUNEL positive cells in endplate, nucleus, and annulus of IVD in experimental group compared to control.

In conclusion, pamidronate, N-containing second generation BPs, was safe in vitro metabolism of IVD and stimulated proteoglycan synthesis in low dose, and maintained chondrogenic phenotype. Also long term high dose treatment of pamidronate was also safe to IVD maintaining disc height without evidence of degeneration.

Key words: pamidronate, intervertebral disc, endplate, degeneration

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I. INTRODUCTION

It has long been known that N-containing bisphosphonates (BPs), such as pamidronate, alendronate, and risedronate, can reduce osteoclast number by inducing apoptotic cell death in osteoclasts [1,2,3,4]. Long-term BP administration significantly reduces bone and calcified cartilage resorption through impairment of the structure and bone-resorbing function of osteoclasts, and thereby effectively maintains trabecular bone volume and structure in ovariectomy-induced acute estrogen deficiency in mature rats[5]. Several molecular targets for N-containing BPs have been suggested. N-containing BPs inhibit enzymes of the mevalonate pathway and prevent the biosynthesis of isoprenoid compounds, which are essential for the posttranslational modification of small GTPases. N-containing BPs thus decrease osteoclast function and induce their apoptosis [2, 3]. As potent inhibitors of bone resorption, they are widely used for the treatment of bone disorders that are due to increased osteoclast activity i.e.

postmenopausal osteoporosis, Paget's disease, tumoral bone disease, and particle induced osteolysis around metal implant [6, 7, 8]. Recent evidences demonstrate BPs in therapeutic concentrations are safe for articular chondrocytes in vitro, moreover, BPs i.e., pamidronate and risedronate prevent dexamethasone induced growth retardation and apoptosis of chondrocytes [9]. BPs also decrease breakdown of type II collagen [10] and exhibit partial chondroprotective effect in a rabbit model of inflammatory arthritis [11]. These findings support the fact that BPs have a chondroprotective effect.

Intervertebral disc (IVD) degeneration is characterized in part by a progressive decrease in proteoglycan content leading to dehydration of the nucleus pulposus [12, 13, 14, 15, 16], causing degenerative changes of the spine [17]. Nutritional deprivation caused by end plate sclerosis provides, in part, a mechanism of IVD degeneration [18] Moreover, premature ossification of end plate [19] and nicotine-induced narrowing of vascular lumen renders decrease in oxygen tension[20], leading to IVD degeneration. Therefore, endplate, where vasculature ends and diffusion starts, appears to be a crucial anatomical structure, which maintain the nutritional supply to IVD tissue.

The fact that BPs have a powerful antiresorptive effect and chondroprotective effect prompts a research for the sum effect of BPs on IVDs, since antiresorptive action can cause hypertrophy of endplate while chondroprotective effect can maintain chondrogenic phenotype of IVDs. There have been several

reports of the effect of BPs on chondrocytes and articular cartilage [9, 10, 11]. Nevertheless, the effect of BPs on IVDs in vitro and in vivo was not thoroughly elucidated before. Especially, long-term treatment of BPs for postmenopausal osteoporosis more than ten years [21] renders an important question of how IVD and end plate react to BPs in long term.

Thus, we hypothesized that BPs, powerful antiresorptive, can cause hypertrophy or sclerosis of endplate, leading degeneration of IVD, while, BPs can protect chondrogenic phenotype by inhibiting breakdown of type II collagen and apoptosis of IVD cells which have chondrocyte like characteristics.

Accordingly, the purposes of this experimental study were, firstly, demonstrate the effect of pamidronate, second generation N-containing BPs, on in vitro metabolism of IVD cells and secondly, the effect of pamidronate on histologic changes of endplate and IVD.

II. MATERIALS AND METHODS

All of the experimental protocols were approved by the human subjects Institutional Review Board of the institution, and Animal Experimental Committee of the institution.

1. Experimental design

To test the effect of pamidronate on IVD cells in vitro, human IVD cells were

utilized. Various concentrations (10^{-9}, 10^{-6}, 10^{-3} M) of pamidronate were administered to the three dimensional alginate bead cultures of IVD cells. Then DNA and proteoglycan synthesis were measured and mRNA expressions of type I collagen, type II collagen, and aggrecan were analyzed by reverse transcription-polymerase chain reaction.

To test in vivo effect of pamidronate on endplate and IVD, 24 mice (C56BL/6) were utilized. Each experimental animal was allocated to low dose (30ug/kg) and high dose (90ug/kg) pamidronate groups and also to groups of 5 weeks and 12 weeks follow up after pamidronate injection. Tail vein of experimental animal was utilized for once a week intravenous injection of pamidronate. Then animals were killed for histologic examination i.e. Hematoxylin-Eosin stain, Safranin-O stain, and TUNEL assay for apoptosis. Histomorphometric data i.e., thickness of end plate, growth plate, and IVD were also collected using image analyzer.

Table 1. Experimental design of in vivo pamidronate treatment

	Pamidronate vs. Saline			
	Low dose*	Saline control	High dose**	Saline control
Duration of treatment†				
5 weeks	3	3	3	3
12 weeks	3	3	3	3

* 30ug/kg of pamidronate per animal

** 90 ug/kg of pamidronate per animal

† weekly injection of pamidronate

2. Patient data and tissue acquisition procedures

Lumbar IVD tissues were obtained from four patients (age range: 34 to 46 years) during surgical disc procedures. Classification of the IVD of each patient as grade of degeneration was performed based on magnetic resonance images of each disc as described in the literature [22]. Grade III and IV degenerations were included in this study to minimize the effect of degeneration grades on the expression of phenotype and matrix synthesis. An attempt was made by the operating surgeon to carefully obtain tissue from the central aspect of the disc to optimize harvest of only the nucleus pulposus and transitional zone. Herniated disc material was strictly excluded from current study. The disc tissue specimens were washed with Hank's balanced salt solution (HBSS, Invitrogen, Grand Island, NY, USA) to remove blood and bodily fluid contaminants, and were then transported in sterile HBSS to the laboratory, less than 20 minutes following surgical removal.

3. Experimental animal

Twenty four mice (C57BL/6, SamDako, Korea) were utilized. Experimental design was summarized in Table 1. Three were allocated for low dose pamidronate with 5 weeks follow up and three were for high dose with 5 weeks follow up, three were

for low dose with 12 weeks follow up, and finally three were for high dose with 12 weeks follow up.

4. Intervertebral disc cell culture

Any obvious granulation tissue, dense outer annulus, and cartilaginous endplate were removed carefully from the disc tissue specimens. Disc cells were then isolated from the inner annulus and nucleus pulposus as described before [23]. Briefly the dissected specimens were minced with a scalpel into pieces of approximately two cubic millimeters in volume. Disc tissues from the nucleus pulposus and inner annulus were digested for 60 minutes at 37° C under gentle agitation in a medium composed of equal parts of Dulbecco's Modified Eagle Medium and Ham's F-12 medium (DMEM/F12, Invitrogen, Grand Island, NY, USA) containing 5% heat-inactivated fetal bovine serum (FBS, Invitrogen, Grand Island, NY, USA) with 0.2% pronase (Sigma, St. Louis, MO, USA) and 0.004% deoxyribonuclease II type IV (DNase, Sigma, St. Louis, MO, USA). The tissue was then washed 3 times with DMEM/F12 and digested overnight under the same conditions, except that the pronase was replaced with bacterial 0.02% collagenase type II (Worthington Biochemical Corp., Lakewood, NJ, USA). Cells were filtered through a sterile nylon mesh filter (pore size: 75um) and then were counted in a haemocytometer and plated in 24 well plates (Nunc, Naperville,

IL, USA) at a density of approximately 5 x 105 cells/ml. Primary cultures were sustained for 2 to 3 weeks in DMEM/F12 containing 10% FBS, 1% v/v penicillin and streptomycin (all antibiotics from Invitrogen) in a 5% CO_2 incubator with humidity. Culture medium was changed twice a week. Cell viability was determined by trypan blue exclusion test [24].

5. Incorporation of isolated cells into alginate beads

The preparation of IVD cells in alginate beads was performed as described elsewhere [24]. Briefly, isolated cells from primary culture with trysinization were resuspended in sterile 0.15M NaCl containing 1.2% low-viscosity alginate (Sigma, St. Louis, MO, USA) at a density of one million cells per milliliter, and then slowly expressed through a 22 gauge needle in a drop-wise fashion into 102mM CaCl2 solutions. After gelation, the beads were allowed to polymerize further for a period of 10 minutes in the CaCl2 solution. After wash in 10 volume of 0.15M NaCl and 3 washes in 10 volumes of DMEM/F12 medium. The beads were finally placed in complete culture medium. Ten beads were cultured in each well of a 24-well plate.

6. Depolymerization of alginate bead

To remove cells from the alginate bead, wells were rinsed twice with 0.15M NaCl with gentle pipetting into the well. The rinse solution was incubated for 1 minute and was aspirated off. Three times the volume of alginate in dissolving buffer (55mM sodium citrate and 0.15M NaCl) was added to the wells and plates were incubated at 37 °C for 10 minutes with shaking.

7. Reverse transcription-polymerase chain reaction for collagen type I, collagen type II, and aggrecan

Total cellular RNA was isolated using the TRIzol reagent (Invitrogen, Grand Island, NY, USA). cDNA was synthesized from 3ug total RNA using M-MLV reverse transcriptase (Promega, Medison, WI, USA) with the oligo(dT)priming method in a 50ul reaction mixture. 2ul aliquots were amplified in a 20ul reaction mixture that contained 1U Taq DNA polymerase (Bioneer, Korea), 250uM of each dNTP, 10mM Tris-HCl(pH9.0), 40mM KCl, and 1.5mM MgCl2. The same reaction profile was used for all primer sets: an initial denaturation at 94° for 1 minute, followed by 25 cycles of: 94° for 5 seconds; 47-50° for 5 seconds; and 72° for 30 seconds; and an additional 2 min extension step at 72° after the last cycle. Amplification reactions specific for the following cDNAs were performed: β-actin, collagen type I alpha, collagen type II and aggrecan. Primer sequence of each cDNA was listed on Table 2. PCR products (5ul) were analysed by electrophoresis

in 2 % agarose gels, and detected by staining with ethidium bromide. The intensity of the products was quantified using the BioImage Visage 110 system (BioRad, Hercules, CA, USA).

Table 2. Sequences of primers for reverse transcription polymerase chain reaction for collagen type I, collagen type II, and aggrecan.

Target gene	Forward primer (5'-3')	Reverse primer (3'- 5')
Collagen I	CCT GTC TGC TTC CTG TTA AC	AGA GAT GAA TGC AAA GGA AA
Collagen II	CAG GAC CAA AGG GAC AGA AA	TTG GTC CTT GCA TTA CTC CC
Aggrecan	GAA TCT AGC AGT GAG ACG TC	CTG CAG CAG TTG ATT CTG AT
β- actin	GGC GGA CTA TGA CTT AGT TG	AAA CAA CAA TGT GCA ATC AA

8. DNA synthesis

Chondrocyte proliferation after 48 hours was determined by a ^{3}H- thymidine incorporation method [25]. All experiments were done in quadruplicate. Results were expressed as counts per minute (cpm).

9. Newly synthesized proteoglycan

Cultures were administered with fresh serumless medium containing ^{35}S-sulfate (final concentration 20 uCi/ml). After a period of 4 hours, cultures with

labeling medium were extracted at 4° C for 48 hours by addition of an equal volume of 8M guanidine hydrochloride, 20mM EDTA and a mixture of proteinase inhibitors (Sigma, St. Louis, MO, USA). For quantitative evaluation of ^{35}S-sulfate labeled proteoglycans, aliquots of the stored extracts were eluted on Sephadex G-25M in PD 10 columns (Amersham Pharmarsia, Uppsala, Sweden) under dissociative conditions. The radioactivities of the newly synthesized proteoglycans were measured by scintillation counting (Packard #1900 TR, Mariden, CT, USA). All data was normalized by ^{3}H thymidine incorporation, which was measured at the end of culture as described elsewhere.

11. Histochemical stain

After completion of the 5- or 12-week drug treatment, all experimental and control mice were killed with CO_2 gas inhalation. The vertebral column was removed en bloc, embadded in paraffin, and cut into sagital section. The sections were decalcified and stained with hematoxylin-eosin (H&E) or safranin-O. The H&E-stained tissues were used to analyze the structure of the intervertebral disc and end plate. The safranin-O was used to quantify the content of proteoglycan in cells constituting the disc.

12. Histomorphometric assay

From the midline section of IVD, thickness of endplate cartilage, thickness of growth plate next to endplate, IVD height, IVD surface area were measured and analyzed.

13. Apoptosis measured by end labeling of fragmented DNA

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate- biotin nick end labeling (TUNEL) was used to detect the fragmented DNA known to be associated with apoptotic cell death [26]. End labeling was performed by use of the Apoptag Plus *in situ* apoptosis detection kit (ONCOR, Gaithersburg, MD, USA). Tissue sections were exposed to an antidigoxigenin antibody conjugated with fluorescein isothiocyanate and viewed by epi-fluorescence microscopy (Nikon E800 M, Tokyo, Japan). Counter-staining was performed with propidium iodide. In general, the assay was performed according to the manufacturer's instructions with minor modifications to optimize staining. Negative controls included tissue that had not been treated with the terminal transferase.

14. Statistics

One-way analysis of variance with Fisher's protected LSD post-hoc test was performed to test difference in densitometric data and ^{3}H-thymidine

incorporation, ^{35}S-sulfate labeled proteoglycan. Significance level was set as $p<0.05$.

III. RESULTS

1. In vitro effect of pamidronate in human IVD cells

A. DNA synthesis

Human IVD cell culture in three dimensional alginate beads with various concentrations of pamidronate (10^{-9}, 10^{-6}, 10^{-3} M) showed no significant increase in DNA synthesis compared to control culture without pamidronate. (Figure 1)

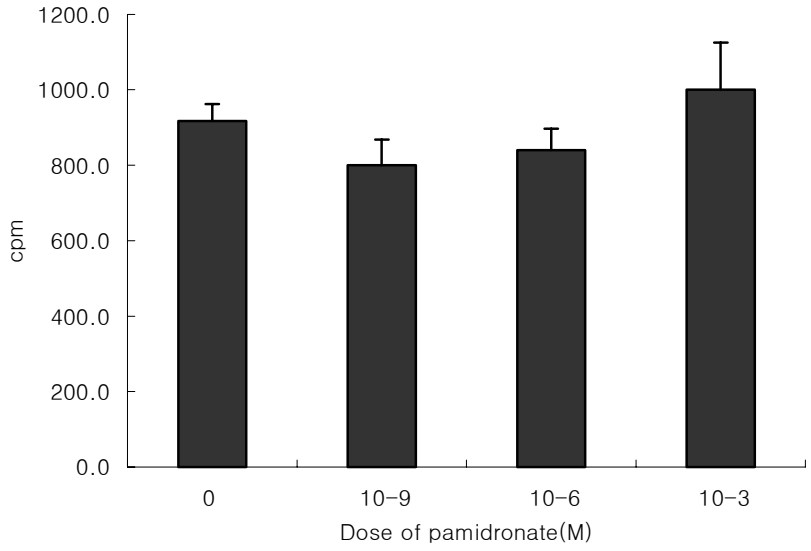


Figure 1. Human intervertebral disc cells were cultured in three dimensional alginate beads with various concentration of pamidronate (10^{-9}, 10^{-6}, 10^{-3} M). DNA synthesis was analyzed with ^{3}H-Thymidine incorporation. Cultures with pamidronate showed no significant change in DNA synthesis compared to cultures without pamidronate ($P>0.05$)

. B. Proteoglycan synthesis

Human IVD cell culture in three dimensional alginate beads with low dose of pamidronate (10^{-9} M) showed significant increase in proteoglycan synthesis, 40% increase with a dose of 10^{-9} M pamidronate ($p<0.05$), compared to control culture without pamidronate. In contrast, cultures with high dose of pamidronate (10^{-6} and 10^{-3} M) demonstrated no significant increase in proteoglycan synthesis compared to control culture (Figure 2).

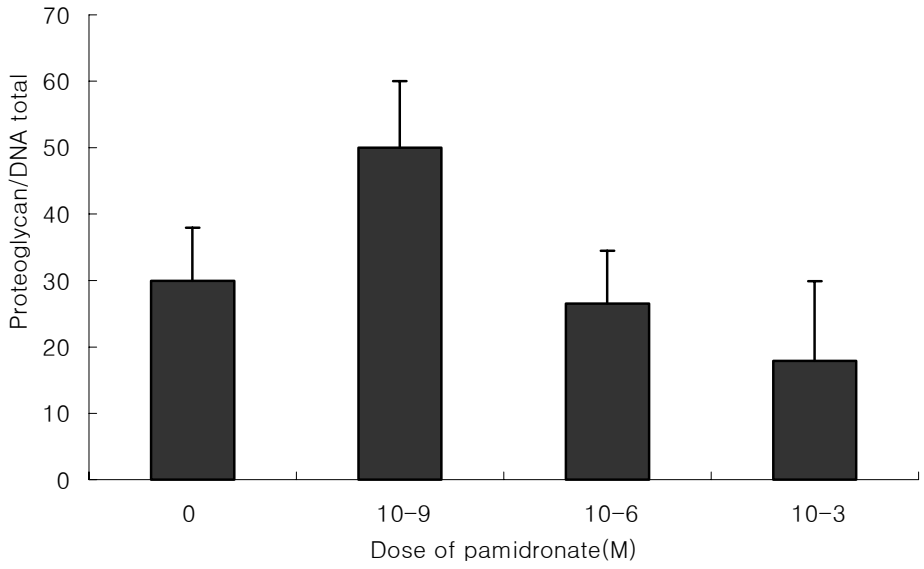


Figure 2. Human intervertebral disc cells were cultured in three dimensional alginate beads with various concentration of pamidronate (10^{-9}, 10^{-6}, 10^{-3} M). Proteoglycan synthesis was analyzed with ^{35}S-Sulfate incorporation. Cultures with low dose of pamidronate (10^{-9} M) demonstrated significant increase in proteoglycan synthesis, 40% increase with a dose of 10^{-9} M pamidronate ($p<0.05$), compared to control culture without pamidronate. Cultures with high dose of pamidronate (10^{-6} and 10^{-3} M) demonstrated no significant increase in proteoglycan synthesis compared to control culture.

C. Expression of collagen type I, collagen type II, and aggrecan mRNA

In densitometric assay of reverse transcription-polymerase chain reaction, human IVD cell culture in three dimensional alginate beads with various dose of pamidronate (10^{-9}, 10^{-6}, 10^{-3} M) showed no statistically significant changes in mRNA expression of type I collagen, type II collagen, and aggrecan compared to control.(Figure 3,4,5)

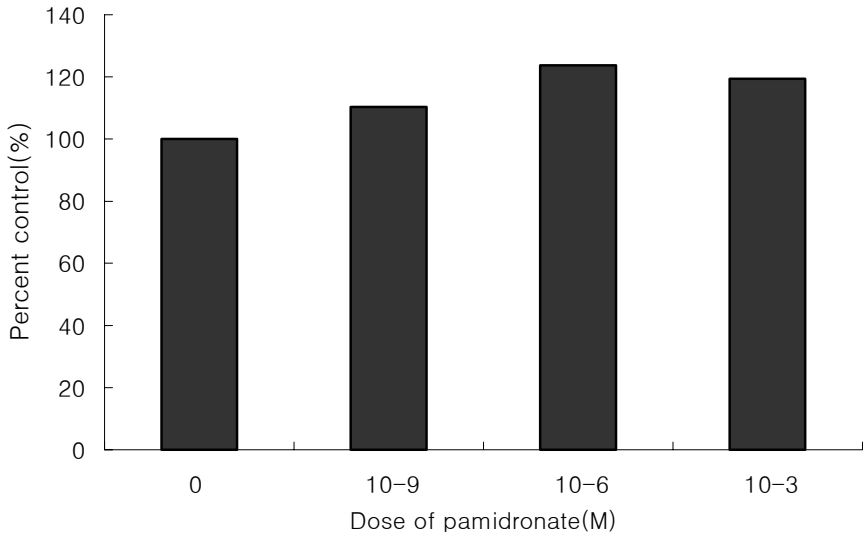


Figure 3. In densitometric assay of reverse transcription-polymerase chain reaction, human intervertebral disc cell cultures in three dimensional alginate beads with various dose of pamidronate (10^{-9}, 10^{-6}, 10^{-3} M) showed no statistically significant changes in mRNA expression of type I collagen compared to control. mRNA expression was normalized by ß-actin mRNA expression.

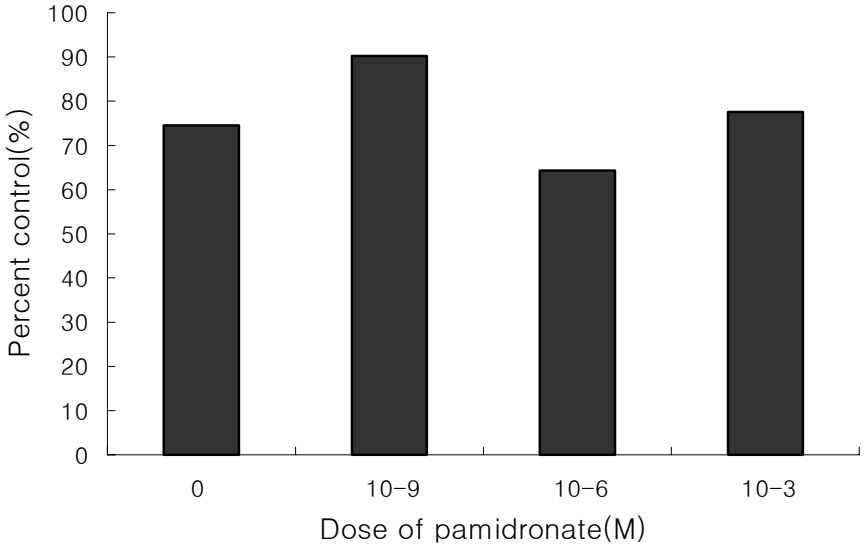


Figure 4. In densitometric assay of reverse transcription-polymerase chain reaction, human intervertebral disc cell cultures in three dimensional alginate beads with various dose of pamidronate (10^{-9}, 10^{-6}, 10^{-3} M) showed no statistically significant changes in mRNA expression of type II collagen compared to control. mRNA expression was normalized by ß-actin mRNA expression.

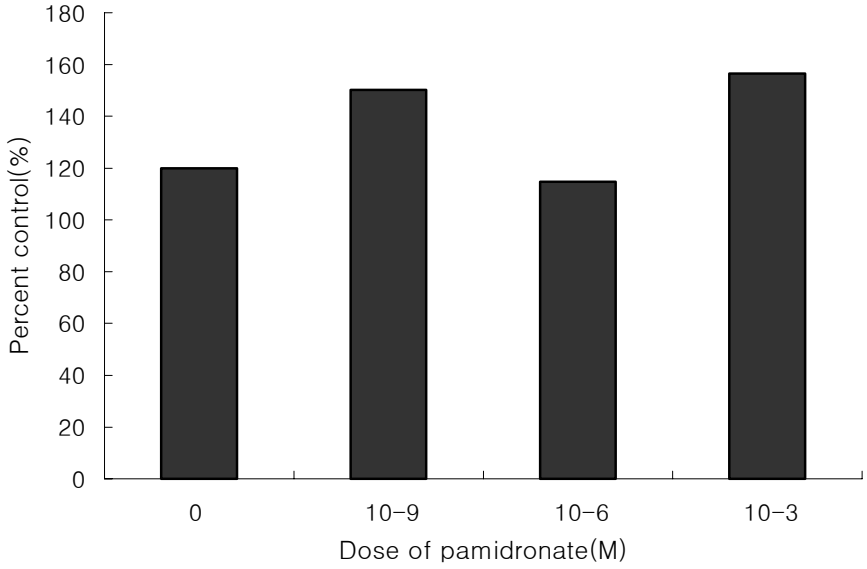


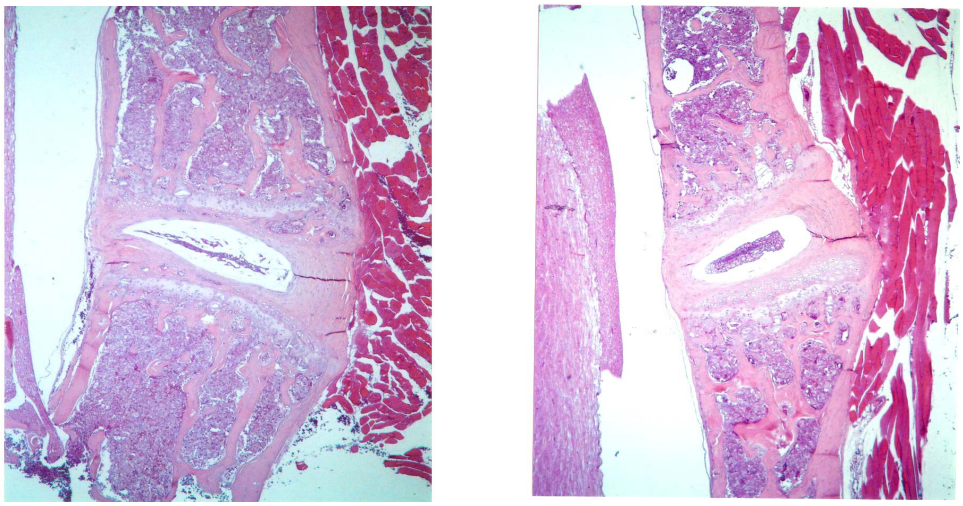
Figure 5. In densitometric assay of reverse transcription-polymerase chain reaction, human intervertebral disc cell cultures in three dimensional alginate beads with various dose of pamidronate (10^{-9}, 10^{-6}, 10^{-3} M) showed no statistically significant changes in mRNA expression of aggrecan compared to control. mRNA expression was normalized by ß-actin mRNA expression.

2. In vivo effect of pamidronate in experimental animal

A. Histochemical stains

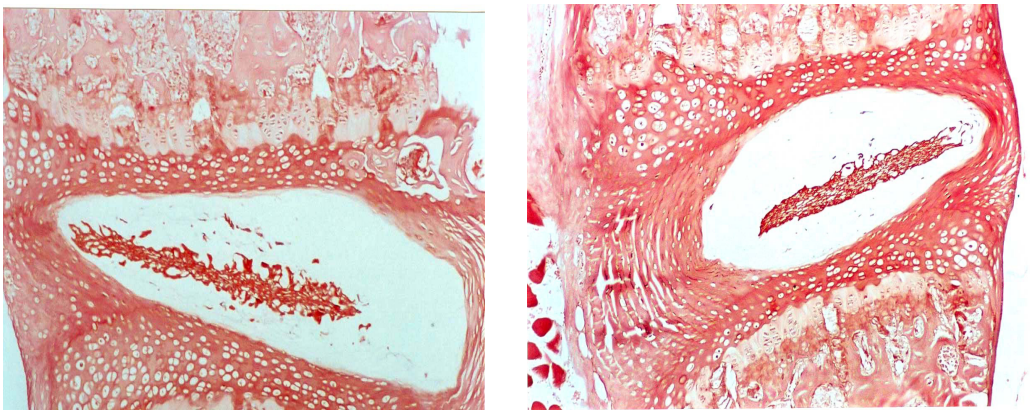
There were no significant changes in thickness of endplate and IVD height in mice with low and high dose of pamidronate (30 and 90 ug/kg) 5 weeks after weekly injection. However, there was significant increase in thickness of

endplate in animal with high dose pamidronate for 12 weeks after weekly injection. Moreover, there were no significant changes in nucleus and annulus such as necrosis and hyalinization of nucleus, disturbance of circular pattern of annulus, and cleft in nucleus and annulus in low dose and high dose group and in 5 weeks and 12 weeks follow up group, compared to control animals (Figure 6, 7).



Control 90ug/kg Pamidronate

Figure 6. Intervertebral disc of mice with weekly injection of pamidronate (90ug/kg) for 12 weeks showed increase in thickness of endplate, maintenance of intervertebral disc height, no perivascular calcification in the endplate. (Hematoxylin–Eosin stain, Original magnification x40)



Control 90ug/kg Pamidronate

Figure 7. Intervertebral disc of mice with weekly injection of pamidronate (90ug/kg) for 12 weeks showed increase in thickness of endplate, maintenance of intervertebral disc height, no perivascular calcification in the endplate. (Safranin-O stain, Original magnification x100)

B. Histomorphometric assay

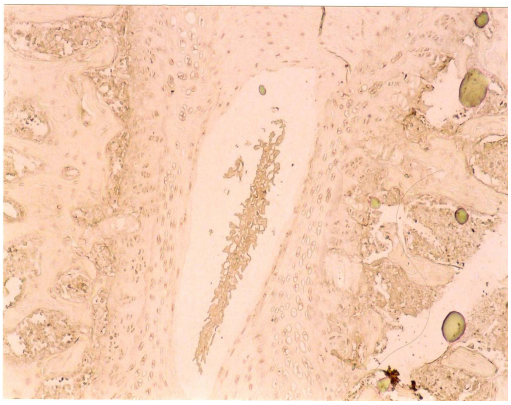
After 12 weeks of weekly high dose pamidronate injection, thickness of endplate (103 ± 16 nm) increased compared to control (91 ± 6 nm) ($p<0.05$) while thickness of growth plate remains unchanged. However, IVD height and surface area did not significantly differ from those of control (Table 3).

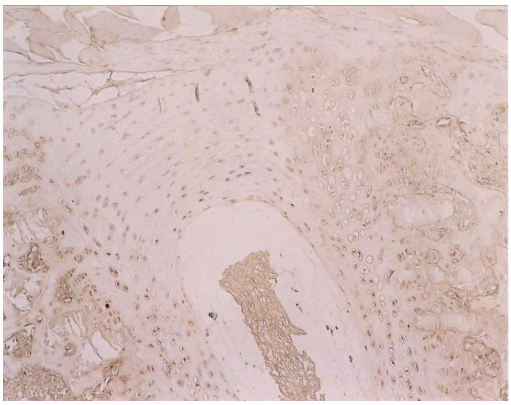
Table 3. Histomorphometric data of intervertebral disc

	5weeks			12weeks		
Dose of Pamidronate(ug/kg)	0	30	90	0	30	90
Endplate cartilage thickness	127±9	123±9	131±13	91±6	92±10	103±16
Growth plate thickness	67±5	84±6	89±4	64±5	61±6	61±4

C. TUNEL assay

In experimental animals with high dose of pamidronate (90ug/kg) and 12 weeks follow up, there were no significant increase in TUNEL positive cells in endplate, nucleus, and annulus of IVD compared to control. (Figure 8)





Control 90ug/kg Pamidronate

Figure 8. Intervertebral disc of mice with weekly injection of pamidronate (90ug/kg) for 12 weeks showed no significant increase in TUNEL positive cells in nucleus, annulus, and endplate. (TUNEL stain, Original magnification x100)

IV. DISCUSSION

BPs have profound effect on skeletal tissue since they have inherent affinity to hydroxyapatite. Fundamental effect of BPs on skeletal tissue is inhibition of osteoclastic bone resorption by increasing apoptosis and decreasing cellular function of osteoclast, leading to maintenance of trabecular and cortical bone volume [1,2]. Hence BPs have been utilized in various skeletal conditions due to increased osteoclastic activity including postmenopausal osteoporosis, steroid

induced osteoporosis, Paget's disease, tumoral bone disease, and particle induced bone resorption around metal implant [6, 7, 8]. Recently, non-skeletal effects of BPs have been investigated in various tissues i.e., articular cartilage, chondrocyte [5, 7, 9, 10, 11]. With regard to non-skeletal effect of BPs, it appears to be beneficial in terms of non toxicity, metabolism, and maintenance of its phenotype.

IVDs are known to express chondrogenic phenotype and have similar functions like articular cartilage. IVDs gain access to nutritional supply through diffusion from vertebral endplate, since IVDs are essentially avascular tissue separated by systemic circulation. Antiresorptive effect of BPs may cause the hypertrophy of endplate leading to nutritional deprivation to IVDs. On the other hand, BPs were known to have chondroprotective effect by inhibiting breakdown of type II collagen and maintenance of chondrogenic phenotype. Net effect of BPs on IVD, whether protective or hazardous, should be elucidated since clinical application of BPs was rapidly increasing in preventing and treating osteoporosis [6].

In this experimental study, in vitro and in vivo effects of pamidronate (second generation N-containing BPs) were investigated using human IVD cells and experimental animals. Pamidronate appears to stimulate proteoglycan synthesis of human IVD cells in low concentration while render no significant effect in high dose of pamidronate. Pamidronate demonstrated minimal cellular proliferative effect as measured by DNA synthesis. Expressions of matrix component mRNA i.e.,

type I collagen, type II collagen, and aggrecan in culture with pamidronate showed no significant changes compared to control, implicating pamidronate appears to maintain chondrogenic phenotypes, not down-regulate expressions of chondrogenic phenotype. In vivo trial using experimental animal, pamidronate did not cause degeneration of IVD, while induce hypertrophy of endplate. Increased thickness of endplate may render partial inhibition of diffusion, nevertheless, maintenance of vascular bud and chondroprotective effect of pamidronate provide net positive influence on IVD in chondroprotective way. Furthermore, in TUNEL assay, animals treated with pamidronate showed minimal apoptotic response justifying hypertrophy of endplate caused by pamidronate did not affect IVD metabolism and its survival. Taken together, pamidronate in various concentrations proved to be safe for intervertebral disc metabolism in vivo and in vitro in accordance with previous researches on chondrocyte and articular cartilage [9-11]. Although pamidronate affects vertebral endplate causing hypertrophy, it does not cause any structural change or degeneration in IVD in given dose and follow up duration. In nicotine induced IVD degeneration, reduction of vascular buds and perivascualr calcification of endplate resulted in degeneration of IVD i.e. necrosis, hyaline degeneration, decreased disc height, and disruption of overlapping lamina of annulus fibrosus with cleft and separation. In contrast to nicotine induced IVD degeneration, animal with high dose of pamidronate with 8 weeks treatment showed no pathologic findings in

endplate except hypertrophy. Moreover, vascular buds and cellular density of endplate appear to be maintained, in addition, nucleus and annulus showed no signs of degeneration, maintaining disc height, disc surface area, no disruption of circular lamina of annulus, no cleft, nor separation.

The results of our study correspond with the growing evidence that despite their apoptotic effects on osteoclasts and macrophages, BPs appear to have opposite effects on other cells. It has been shown that BPs can enhance the differentiation and bone forming activities of osteoblasts[27] and also it has been demonstrated that BPs prevent steroid induced apoptosis of osteoblasts in vitro and in vivo[28]. Furthermore, recent results have clearly demonstrated that BPs, in addition to inhibiting osteoclast mediated bone resorption, can exert marked proapoptotic and antiproliferative effects on tumor cells, especially when combined with other standard antineoplastic therapy [7].

V. CONCLUSION

Pamidronate, N-containing second generation BPs, was safe in metabolism of IVD in vitro and stimulated proteoglycan synthesis in low concentration, and maintained chondrogenic phenotype. Also long term high dose treatment of pamidronate in experimental animal was also safe in IVD, maintaining disc height without evidence of degeneration.

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Abstract (in Korean)

척추 추간판과 종판에 있어서 pamidronate의 효과

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N-containing bisphosphonate 는 파골세포의 세포 고사(apoptosis)를 유도하여 파골세포의 숫자를 감소하는 약제로 널리 알려져 왔다. bisphosphonate 는 골 흡수를 억제하는 강력한 억제제로서, 폐경 후 생기는 골다공증, Paget 병, 골종양, 그리고 금속성 이식물를 사용하였을 때 발생하는 osteolysis 등의 파골세포 기능 항진으로 인한 경우에 널리 이용되고 있다. 또한 관절염에 있어서는 type 2 collagen 의 분해를 억제하고, 부분적으로 연골을 보호하는 기능도 있음이 알려져 왔다. 척추 종판의 경화는 추간판으로의 영양 결손을 유발하며, 이는 추간판 퇴행의 원인이 된다. Bisphosphonate 가 강한 골 흡수 억제능과 연골 보호능이 있다는 사실을 종합하였을 때, 이러한 약제의 골 흡수 억제능으로 인해 척추 종판의 비후가 유발될 수 있고 한편으로는 연골 보호능으로 인한 추간판의 연골세포 유사 표현형이 유지될 수 있으리라 예상된다. 따라서 본 연구에서는 시험관내에서 bisphosphonate 가 추간판세포의 대사에 미치는 영향을 살펴보고, 조직학적으로 척추 종판과 추간판에 미치는 영향을 살펴보았다.

추간판 조직은 4 명의 환자로부터 획득하였으며, 단계적 효소처리를 하였으며, 2 세대 bisphosphonate 인 pamidronate 의 영향을 시험관내에서 확인하고자, 추간판세포를 alginate bead 를 이용한 삼차원 배양을 실시하였다. 삼차원 배양 중인 추간판 세포에 pamidronate 의 농도가 10^{-9}, 10^{-6}, 10^{-3}M 이 되도록 각각 처리한 후, 세포 증식에 따른 DNA 합성을 알아보기 위해 [^{3}H]-Thymidine incorporation 을 실시하였고, 신생 proteoglycan 합성을 확인하고자 [^{35}S]-Sulfate incorporation 을 실시하였다. Collagen type 1, collagen type 2, 그리고 aggrecan 유전자에 대한 mRNA 발현을 확인하고자 RT-PCR 을 시행 하였다. 조직학적으로 pamidronate 의 척추체에 미치는 영향을 확인하고자, 24 마리의 C57 BL/6 mouse 를 이용하여, pamidronate 를 매주 정맥 주사하였으며, 실험군은 대조군, 저용량(30ug/kg), 고용량(90ug/kg)군으로 나누고, 투여 시작 후 5 주와 12 주에 각각 쥐를 희생하여 척추체를 획득하였다. 획득한 조직은 H&E 염색, Safranin-O 염색 그리고 TUNEL assay 를 실시하였고, 형태계측학적 자료는 Image analyzing system 을 이용하여 분석하였다.

시험관 내에서 pamidronate 를 추간판세포에 농도별로 처리한 결과, 대조군과 비교하여 세포증식에 있어서 의미 있는 변화는 관찰되지 않았으나, 신생 proteoglycan 의 합성은 대조군과 비교하여 저농도인 10^{-9}M 의 농도에서는 40%의 증가를 보였다. RT-PCR 에 대한 densitometric assay 에서는, 대조군과 비교해서 type 1 collagen, type 2 collagen 그리고 aggrecan 의 mRNA 발현이 통계상으로 의미 있는

결과는 관찰되지 않았다. 12 주 동안 매주 과용량의 pamidronate 를 투여한 실험동물에서 종판의 두께는 증가하였으나, 대조군과 비교하여 5 주와 12 주째 저용량과 고용량 군에서의 수핵부의 괴사나 섬유륜부의 이상, 추간판 전체의 균열과 같은 특이한 변화는 없었다. TUNEL assay 결과에서도 고사된 세포에 대한 양성 반응은 관찰 할 수 없었다.

결론적으로, 제 2 세대 bisphosphonate 인 pamidronate 는 시험관 내에서 추간판세포에 대해서는 비교적 안전하며, 저용량 처리시에는 proteoglycan 합성이 촉진되었고, 세포표현형의 변화도 없음을 확인하였다. 실험동물에서도 pamidronate 를 장기간 과용량 투여하였을 시 퇴행의 변화 없이 추간판 높이가 안정되게 유지되었다.

핵심되는말: 파미드로네이트, 추간판, 종판, 퇴행