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The Effects of Parathyroid Hormone and Bisphosphonate on the Cartilage and Subchondral Bone of the Temporomandibular Joint

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Abstract:

Introduction: Parathyroid hormone increases bone strength primarily by stimulating bone formation, whereas Bisphosphonate, an antiresorptive drug, reduce bone resorption by blocking osteoclast activity. Here we examined the effects of alendronate (ALN), a type of bisphosphonate, and we also examine the effects of parathyroid hormone (PTH), which has an anabolic effect and increases bone remodeling when treated intermittently (I-PTH). I-PTH treatment leads to increased bone volume fraction, tissue density and trabecular thickness of the subchondral bone. I-PTH treatment also increases proteoglycan synthesis with a concomitant increase in mineralization of the Mandibular Condyle Cartilage (MCC). We hypothesize that combined treatment of I-PTH + ALN will have a chondroprotective effect on the cartilage of the Temporomandibular Joint (TMJ) **Methods:** Mice were divided into 4 groups; a control group, a PTH group, an ALN group, and a combined PTH and ALN group. **Results:** ALN combined with PTH, and alendronate alone, caused an increase in bone volume. I-PTH or ALN used individually increased cartilage thickness and cell proliferation while the combined treatment decreased this effect. **Conclusion:** The use of combined treatment of ALN and I-PTH showed a decrease in cell proliferation with an increase in bone volume but there was no evidence of synergy between parathyroid hormone and alendronate. Bone

formation increased as expected in the parathyroid hormone group but not in the combination therapy group.

Keywords: Mandibular Condylar Cartilage, Alendronate, I-PTH, Osteoporosis.

Introduction and Literature Review:

The Mandibular condyle cartilage (MCC) is a secondary cartilage, which develops from the periosteum and has four distinct zones: the fibrous layer, the polymorphic cell layer, the pre-hypertrophic cell zone, and the hypertrophic cell layer (1). Beneath the hypertrophic cell layer is the subchondral bone, which closely interacts with the condylar cartilage in maintaining the physiological functions of the temporomandibular joint (TMJ). A physiological relationship (biological and mechanical) occurs between the subchondral bone and the MCC (2).

PTH acts through binding the PTH receptor (PTHR1). PTH is expressed in the MCC, in the bone, and in the kidney and plays a vital role in calcium and phosphate homeostasis. PTH signaling contributes to bone and cartilage growth and remodeling (3-8) and has differential effects on bone depending on the mode of administration. Continuous exposure leads to catabolic bone resorption, whereas I-PTH leads to anabolic effects in the bone (5-8). I-PTH treatment leads to increased bone volume fraction, increased tissue density, and increased trabecular thickness of the subchondral bone. I-PTH treatment also leads to an increase in proteoglycan distribution with an increase in the mineralization of the MCC (9).

Bisphosphonate drugs such as Alendronate (ALN) have been used to restore the balance between bone resorption and bone formation (10,11). ALN is a nitrogen-containing bisphosphonate which binds to bone surfaces and inhibits bone resorption by osteoclasts, possibly through inhibition of the mevalonate pathway. ALN is effective and generally well tolerated in the treatment of both sexes. ALN is used for treating osteoporosis in postmenopausal women and corticosteroid-induced osteoporosis, as well as for the prevention of osteoporosis in premenopausal women. Many studies have shown that bisphosphonates can affect osteoclast mediated bone resorption in a variety of ways

that include effects on osteoclast recruitment, differentiation, and resorptive activity (10). Bisphosphonates may also affect osteoclast morphology and cause osteoclast apoptosis *in vitro* (10).

Current osteoporosis treatments improve bone mass by increasing net bone formation. Anti-resorptive drugs such as bisphosphonates block osteoclast activity, while anabolic agents such as I-PTH treatment increase bone remodeling largely by inducing formation (11). We hypothesize that combined treatment with I-PTH + bisphosphonate (ALN) will have a chondroprotective effect on the cartilage of the TMJ (12).

Materials and Methods:

Twenty-four 3-week-old triple transgenic mice (Col1a1 X Col2a1 X Col10a1) on a CD-1 background were used for this study. The Col1a1-GFP reporter is driven by a 3.6-kb fragment of the rat type 1 collagen promoter that is strongly expressed in the bone and the MCC. The Col2a1-GFP reporter is driven by a 1-kb fragment of the type 2 collagen promoter that is expressed in the prehypertrophic zone of the MCC. The Col10a1-RFP is fused to mCherry fluorescent protein and is expressed in the hypertrophic zone of the MCC. Transgenic mice with each reporter were crossed to generate the triple transgenic Col3.6-green X Col2-blue X Cola10-red mice used in this study.

1. Study Design

Mice were divided into 4 groups: (1) Control group: saline was injected subcutaneously daily for 10 days (n = 6); (2) PTH group: PTH [1–34] (60μg/kg body weight, Prospec, East Brunswick, NJ, USA) was injected subcutaneously daily for 10 days (n = 6); (3) Alendronate group: alendronate (alendronate sodium trihydrate, 50μg/kg, Sigma Aldrich, St. Louis, MO) was injected subcutaneously every three days for 10 days (ALN, n = 6); (4) Combined PTH and ALN group:

subcutaneous injections of PTH were given daily and subcutaneous injections of ALN were given every 3 days (PTH+ALN, n = 6). All the mice were injected with alizarin complexone (3 μg/kg body weight) on the 7th day and calcein (3 μg/kg body weight) on the 9th day. The mice were subsequently injected with EdU (5-ethnyl-2'-deoxyuridine, Life Technologies, Grand Island, NY, USA) (30 mg/kg body weight) 48 hours and 24 hours prior to euthanization. All the animals in the control and experimental groups were healthy and gained weight during the entire duration of the study. All of the mice were sacrificed on day 10.

2. Tissue Preparation and Histology Sectioning

After sacrificing, the mandibles were dissected by cutting the muscular attachment without removal of the cartilage of the condyle. The MCC along with subchondral bone were fixed in 10% formalin for 24 hours, placed in 30% sucrose in PBS overnight, then embedded in cryomedium (Thermo Shandon, Pittsburgh, PA, USA) using disposable base molds (Thermo Shandon, Pittsburgh, PA, USA). The medial surfaces of the samples were embedded parallel to the floor and against the base of the mold. Specimens were stored at -20 °C until sectioning with a Leica cryostat (Nussloch, Germany). Transfer of frozen sagittal sections of the condyles (5–7 μm) to slides was completed with a tape transfer method.

3. Morphological Measurement

MX20 Radiography System (Faxitron X-Ray LLC, Lincolnshire, IL, USA) was used to image the mandibles at 26 Kv for 5 seconds. The parameters of interest were: 1) mandibular length (condylion to incisor process) 2) condylar head length (perpendicular distance between condylion to a line traced from the sigmoid notch to the deepest point of the concavity of the mandibular ramus), and 3) condylar head width (anterioposterior distance of the condylar articular surface). Measurements

were performed using Digimizer® Image software (MedCalc Software, Mariakerke, Belgium). Each parameter was measured in triplicate and the average was calculated.

4. Micro-CT

The MCC and subchondral bone of condyles were analyzed using micro-computerized tomography (micro-CT) (SCANCO Medical AG, Brüttisellen, Switzerland) at 55kV and 145μA, with a voxel size of 6 μm. The samples (n = 6 per group) were scanned in 70% alcohol and serial tomographic projections were acquired at 1000 projections per rotation at 300,000 μs. An automated algorithm using local threshold segmented the reconstructed grey scale images to distinguish calcified tissue from non-calcified tissue. The mushroom shaped head of the condyle was the region of interest. Bone volume fraction (BVF (%)), trabecular thickness (Tb.Th (μm)), trabecular spacing (Tb.Sp (μm)) and tissue density (mg/ccm HA) were assessed.

5. Histomorphometry, Histological Staining, and Immunohistochemistry

Our histological sections were stained and analyzed as previously described. The 5–7μm MCC and subchondral sagittal sections adhered to glass slides through the entire processes of staining and imaging. The sections were first imaged for fluorescent signals Col1a1 (green), Col2a1 (blue) and Col10a1 (red) and the bone labels alizarin complexone (red) and calcein (green). Baseline imaging of the sections was performed with an observer ZI fluorescent microscope (Carl Zeiss, Thornwood, NY, USA) using a yellow fluorescent protein filter (eYFP, Chroma Cat 49003ET, EX: 500/20, EM: 535/30), a cyan fluorescent protein filter (CFP, Chroma Cat 49001ET, EX: 436/20, EM: 480/40), and a RFPcherry filter that was also used for detecting alizarin complexone staining (mCherry, Chroma Cat 49009ET, EX: 560/40, EM: 630/75). Subsequently, the coverslip was removed by soaking slides in PBS, and slides were stained for EdU (Life Technologies, Grand Island, NY,

USA) then imaged. Sections were then stained for Tartrate Resistant Acid Phosphatase (TRAP) using ELF97 (Life Tech, Waltham, MA, USA), generating a yellow fluorescent signal. Afterwards, the coverslip was removed and stained for alkaline phosphatase activity using a fluorescent fast red substrate (Sigma, St. Louis, MO, USA), and for cell nuclei using DAPI (Thermo Fisher Scientific, Waltham, MA, USA) then reimaged. Finally, the slide was rinsed in distilled water then stained with Toluidine Blue (TB) and reimaged using bright field microscopy to examine the proteoglycans of the cartilage. We also performed Safranin-O staining, NovaUltra™ von Kossa-Alcian Blue staining (IHC WORLD, Woodstock, MD, USA) and fluorescent TUNEL (R&D systems, Minneapolis, MN, USA). Immunohistochemistry for SMAD158 (EMD Millipore, Billerica, MA, USA), VEGF (Vascular Endothelial Growth Factor, ABCAM, Cambridge, MA, USA), PRG4, HIF1alpha, MMP13, ADAMST4, Sox9, Elk1, RUNX2 and BMP2 were performed.

6. Histological Analysis and Quantification

We used the Osteoarthritis Research Society International (OARSI) for the histopathologic grading of mouse cartilage degeneration (by Safranin-O staining). Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA, USA) was used to quantify the green, blue and red pixels within the MCC to count Col1a1, Col2a1 and Col10a1 expression in sagittal sections of condyles. Percentages were obtained by dividing the number of green, blue and red pixels over the total number of pixels of the entire MCC. Similarly, we examined TRAP activity in the MCC and subchondral bone by counting the number of yellow pixels and dividing by the total number of pixels in the subchondral bone region. Cell proliferation was quantified with the percentage of EdU positive pixels over DAPI positive pixels in the proliferative zone of the MCC. Alkaline phosphatase distance mapping was analyzed with Digimizer® Image software, and measurements were performed from the outer

cellular layer of the MCC to the tidemark (in six different locations in the entire MCC). Finally, Toluidine Blue stained area was evaluated using Digimizer® Image software.

Results:

Our micro-CT analyses of the mandibular condyle compared subchondral bone between groups. We found an increase in bone volume fraction and trabecular bone thickness in mice treated with ALN combined with I-PTH, versus treatment with ALN alone. Trabecular spacing was found to be decreased in all experiment groups compared to the control group as a side effect to the increase bone volume and trabecular thickness (Fig.1).

TRAP staining showed increased bone remodeling with I-PTH and ALN treatment in the subchondral area. Under normal circumstances TRAP is highly expressed by osteoclasts (13). Compared to the control, all treatment groups showed an increase in osteoclast activity (Fig.2). ALN had the highest increase in osteoclast activity (Fig.2)

I-PTH increases the population of Col I 3.6 positive cells and ALN decreases this effect. Histology showed an increase in YFP positive (green cells) as compared to control with I-PTH but when combined with ALN or ALN alone caused a statistically significant decreased of these cells (Fig.3).

I-PTH and ALN increases Col II positive cells with ALN with higher increase followed by I-PTH and ALN combined and PTH alone (Fig.4).

I-PTH decreases Col X positive cells, while Alendronate increases Col X positive cells. Histology showed a statistically significant difference between groups. Alendronate had the highest increase in Col X cell production but the combination of ALN and I-PTH, or I-PTH alone showed decreased Col X expression (Fig. 5).

I-PTH and ALN, treated individually, increase cartilage thickness while the combined treatment decreases this effect. (Fig 6)

I-PTH and ALN, treated individually, increased cell proliferation as shown by Edu staining, while the combined treatment decreased this effect (Fig 7)

Discussion:

With the advance of osteoporosis treatment and the develop of new drugs ,the attention by dentists to the side effects of such drugs on the mandibular condyle cartilage has increased. It is well known the use of intermittent PTH and bisphosphates for osteoporosis treatment in post menopause females but it's effects on MCC when combined is not well known. Although parathyroid hormone therapy increases both bone formation and bone resorption, bone formation is increased preferentially over resorption, at least initially. The bisphosphonate alendronate has also been shown to increase bone mineral density and reduce the risk of fracture, but its mechanism of action differs from that of parathyroid hormone; it preferentially suppresses bone resorption over bone formation .(20,21)

Alendronate (alendronic acid) is a nitrogen-containing bisphosphonate which binds to bone surfaces and inhibits bone resorption by osteoclasts. Oral alendronate 5 or 10 mg/day produces sustained increases in bone mineral density (BMD) (14). In randomized, comparative studies, alendronate 10 mg/day was as effective at increasing BMD as conjugated estrogens at 0.625 mg/day,(15). Alendronate is effective and generally well tolerated in the treatment of women or men with primary (including postmenopausal) or corticosteroid-induced osteoporosis. The drug has been associated with upper GI tract adverse events, although the extent to which alendronate is responsible for these events has not been clearly established (14). PTH is primarily regarded as a

regulator of calcium homeostasis but it has also been shown to enhance bone-remodeling (16). I-PTH administration exerts an anabolic effect on subchondral bone modeling by increasing the activity and number of osteoblasts, as indicated by an increase in the number of Col1a1 cells in the I-PTH group when compared to the control (8). Current osteoporosis treatments include anti-catabolic agents, which reduce bone resorption (16,17)], and anabolic agents, which increase bone formation (18). However, due to coupling of bone resorption and formation, drugs that inhibit resorption often also inhibit formation, and those that increase formation also increase resorption, thereby limiting their potential benefits (16,19). Recent data suggest that intermittent parathyroid hormone (PTH) treatment, currently the only FDA-approved anabolic agent, may increase bone formation partly through a modeling-based mechanism (bone formation without prior resorption). We hypothesized that adding ALN to PTH treatment would inhibit bone resorption while maintaining the elevated bone formation activities induced by PTH treatment, thus resulting in an additive, beneficial effect on trabecular bone and will have a chondroprotective effect on the cartilage of the TMJ. The current therapeutic protocol for the treatment of osteoporosis involves parathyroid hormone (1–84) injections at 100 μg daily [NPS Pharmaceuticals]) or alendronate ,10 mg daily [Fosamax, Merck], with the addition of calcium carbonate (500 mg of elemental calcium [Tums, Smith Kline Beecham]), and a multivitamin containing 400 IU of vitamin D (Rugby Laboratories). Our study simulated in a mouse models the current treatment protocol without the supplemental calcium and vitamin D. By stimulating bone formation and inhibiting bone resorption simultaneously, combination therapy might be more effective than therapy with parathyroid hormone or alendronate alone. We hypothesized that, as compared with parathyroid hormone therapy alone, combination therapy with parathyroid hormone and alendronate would induce larger increases in bone mineral density, preserve the increase in bone formation, and minimize increases

in bone resorption and increase or preserve the mandibular cartilage. However, Dennis M. Black, Ph.D at al. on a randomized clinical trial found that taken together, the changes in areal and volumetric bone mineral density, cortical volume, and the levels of biochemical markers of bone turnover had little evidence that this combination is better than either drug alone. Overall, the changes that were induced by parathyroid hormone therapy in cortical and trabecular bone were not seen with combination therapy or with alendronate monotherapy, which suggests that combination therapy alters the distinct effects of parathyroid hormone on bone. A recent study of combination alendronate and parathyroid hormone (1–34) (40 μg) therapy in men, initiated after 6 months of alendronate monotherapy, showed that the increases in bone mineral density over 24 months of combination therapy were less than those observed over 24 months of parathyroid hormone monotherapy (23). On another study with 238 post menopause women found that bone formation increased markedly in the parathyroid hormone group but not in the combination therapy group and bone resorption decreased in the combination-therapy group and the alendronate group(23).

Our study confirmed an increase in bone volume mineral and trabecular thickness in iPTH groups and in the combination group compared to ALN alone , evidencing that the anabolic effect of iPTH on bone does not suffer evident interference by ALN and the TRAP staining showed increased osteoclasts in all groups but specially in the ALN alone group. The increase in osteoclast activity is typical of the bone remodeling process. ALN showed highest activity of osteoclasts probably due to the inhibitory effects of the nitrogencontaining bisphosphates on osteoclasts ,result from their inhibition of farnesyl pyrophosphate synthase (FPPS), a key branch-point enzyme in the mevalonate pathway. FPPS generates isoprenoid lipids used for the posttranslational modification of small GTP-binding proteins essential for osteoclast function, thus showing increased number of potentially non-active osteoclasts.

Conclusions:

In summary this study showed that the use of Alendronate combined with I-PTH, and well as the use of Alendronate alone increased the bone volume of the MCC. When used alone I-PTH increased the proliferation of Col I positive cells while Alendronate alone decreased the number of Col 1 positive cells. When combined I-PTH and Alendronate increased the number of Col II positive cells.I-PTH decreases expression of Col X positive cells ploriferation and Alendronate increases Col X cell ploriferation.
In conclusion treatment with I-PTH alone or Alendronate alone increase cartilage thickness and cell proliferation. The combination of both I-PTH and Alendronate decreases this effect .

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FIG. 1

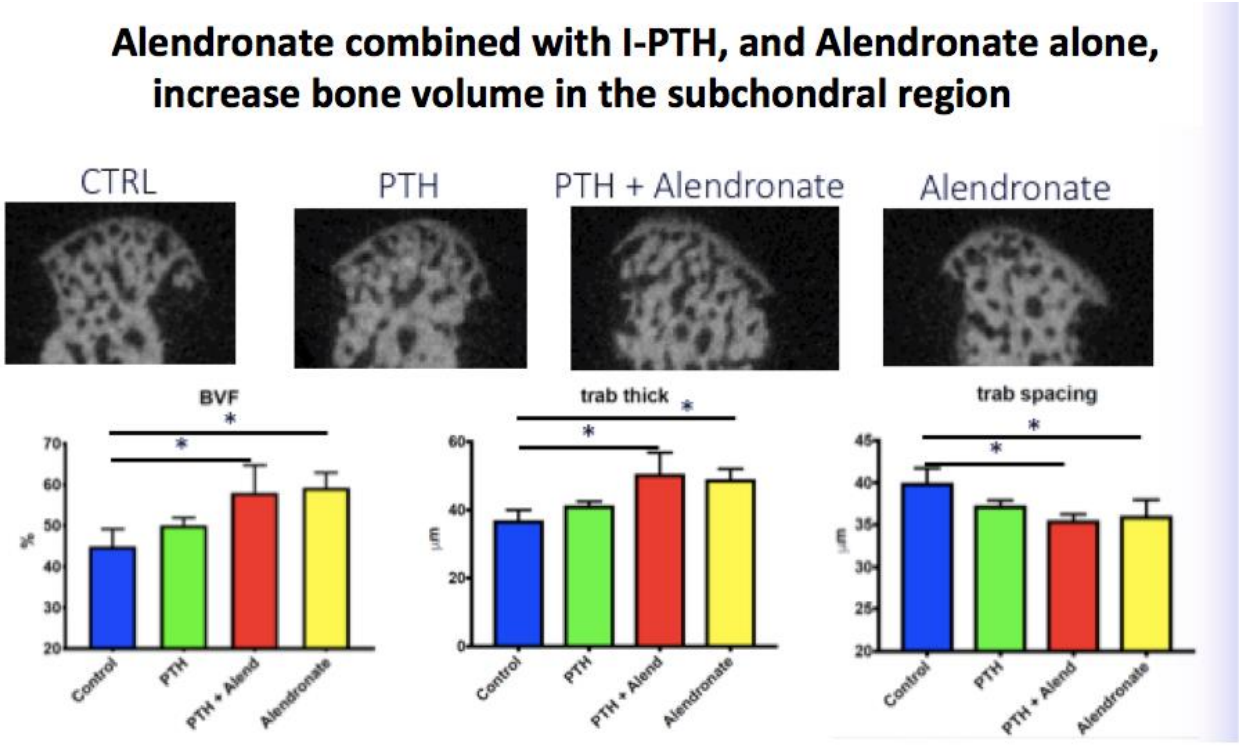
Alendronate combined with I-PTH, and Alendronate alone, increase bone volume in the subchondral region
CTRL
PTH
PTH + Alendronate
Alendronate
BVF
%
trab thick
μm
trab spacing
μm
Control
PTH
PTH + Alend
Alendronate

FIG.2

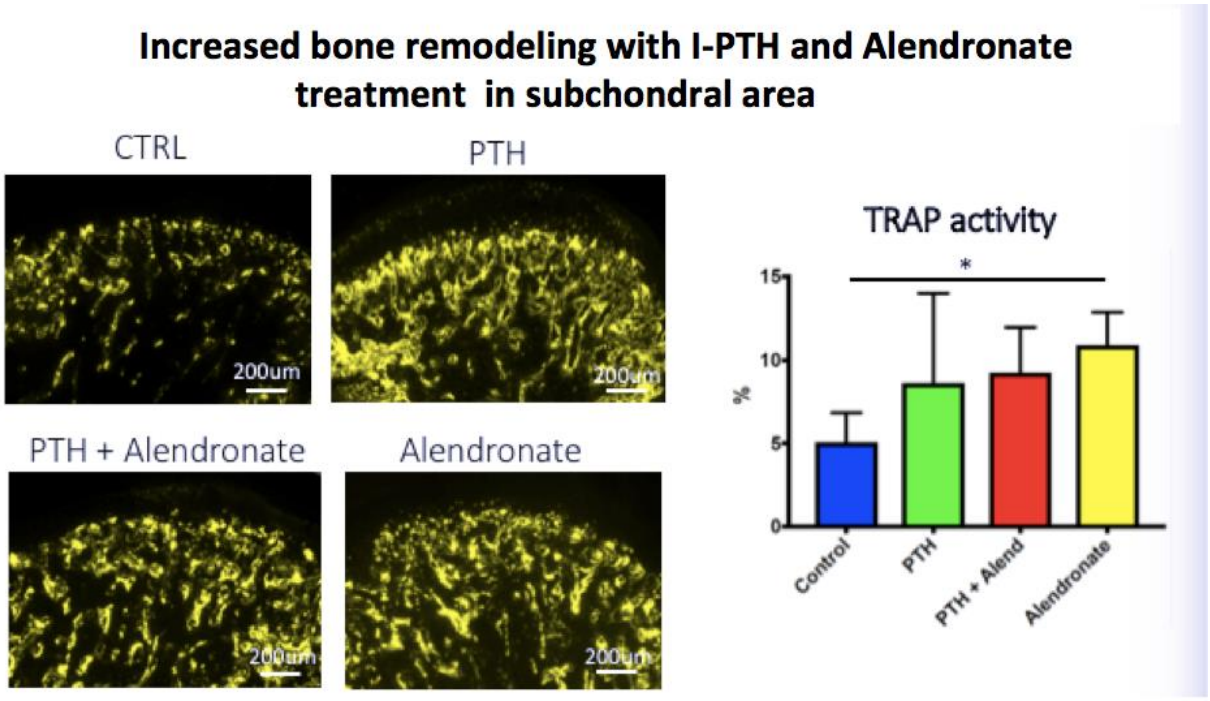
Increased bone remodeling with I-PTH and Alendronate treatment in subchondral area
CTRL
PTH
PTH + Alendronate
Alendronate
200um
TRAP activity
*
%
15
10
5
0
Control
PTH
PTH + Alend
Alendronate

FIG.3

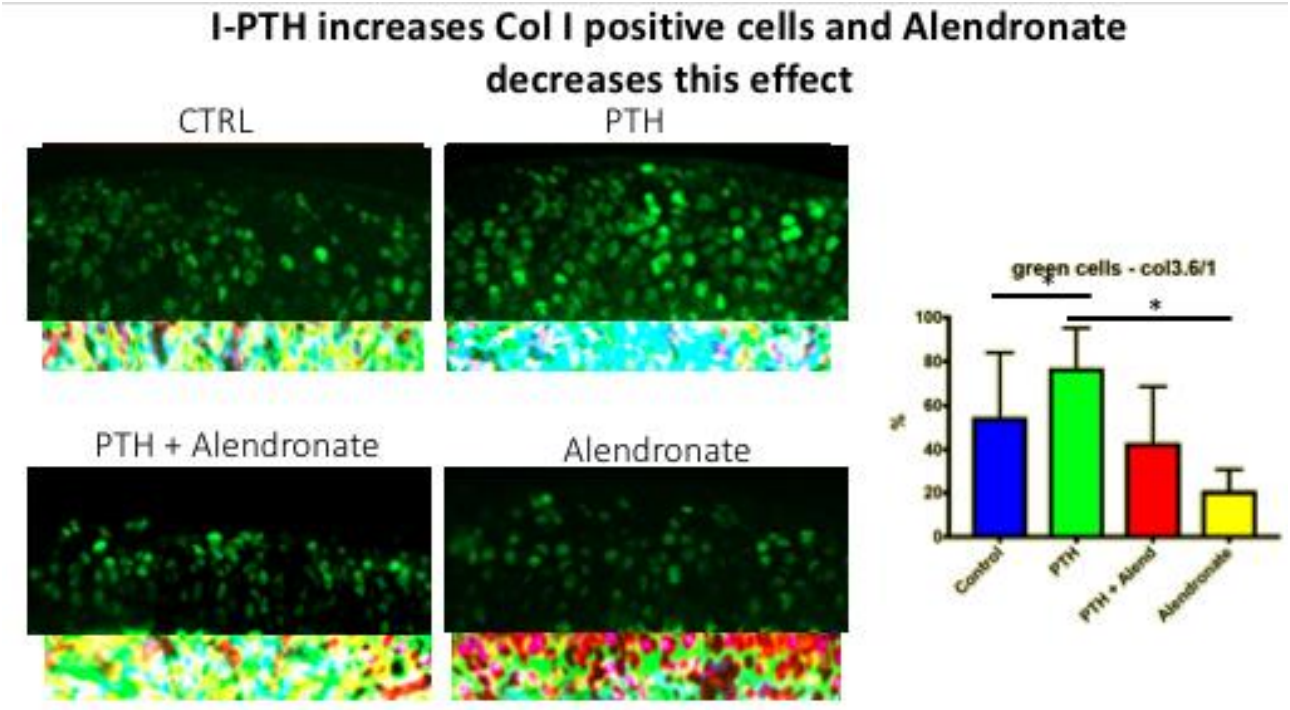
I-PTH increases Col I positive cells and Alendronate decreases this effect
CTRL
PTH
PTH + Alendronate
Alendronate
green cells - col3.6/1
%
100
80
60
40
20
0
Control
PTH
PTH + Alend
Alendronate
*
*

FIG.4

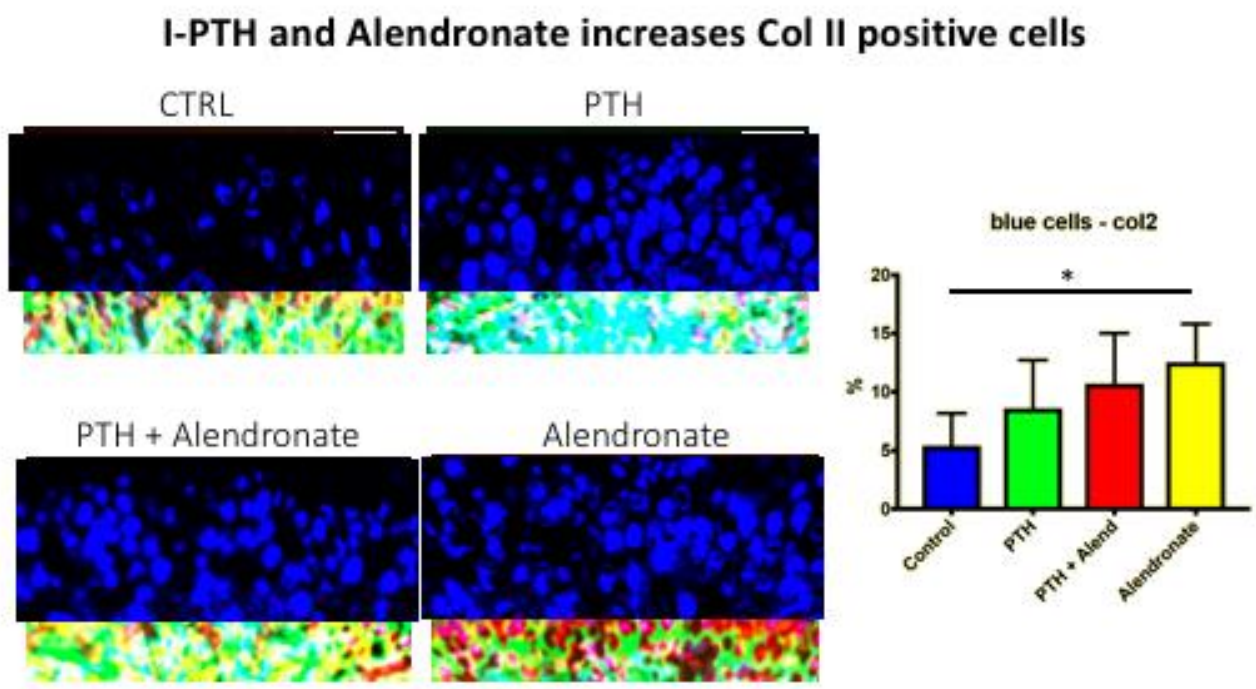
I-PTH and Alendronate increases Col II positive cells
CTRL
PTH
PTH + Alendronate
Alendronate
blue cells - col2
%
Control
PTH
PTH + Alend
Alendronate

FIG 5

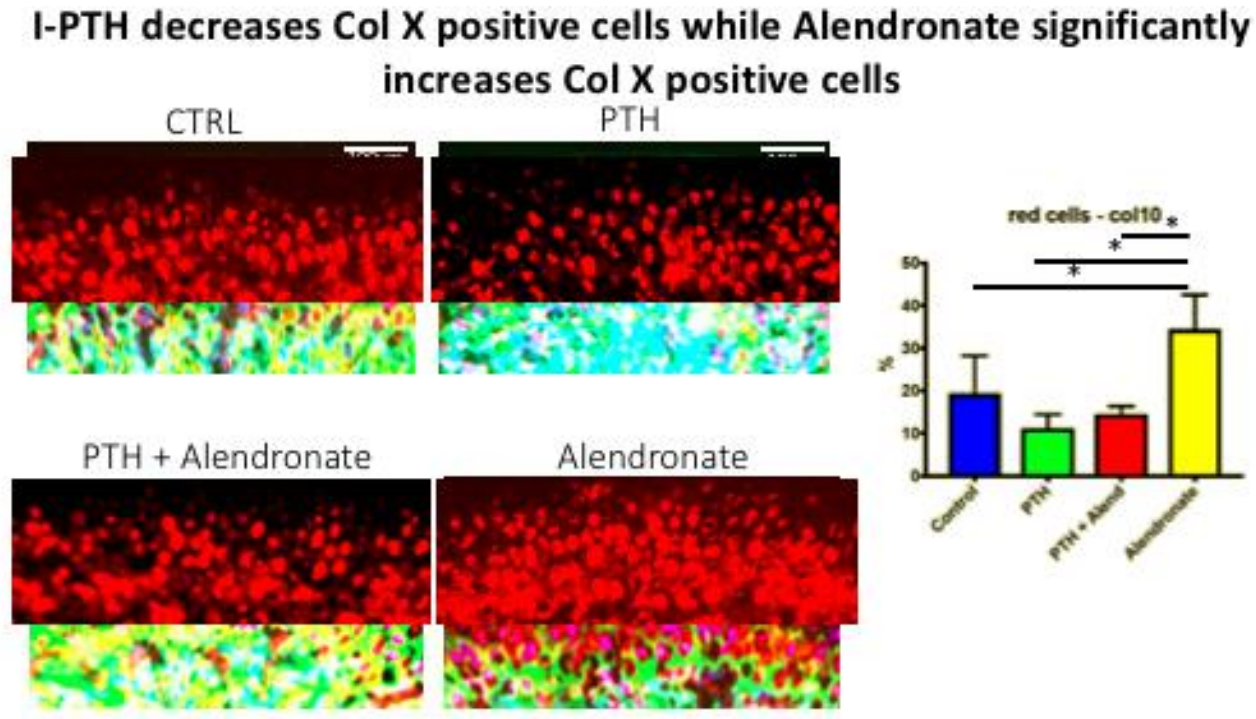
I-PTH decreases Col X positive cells while Alendronate significantly increases Col X positive cells
CTRL
PTH
PTH + Alendronate
Alendronate
red cells - col10
%
Control
PTH
PTH + Alend
Alendronate

FIG 6

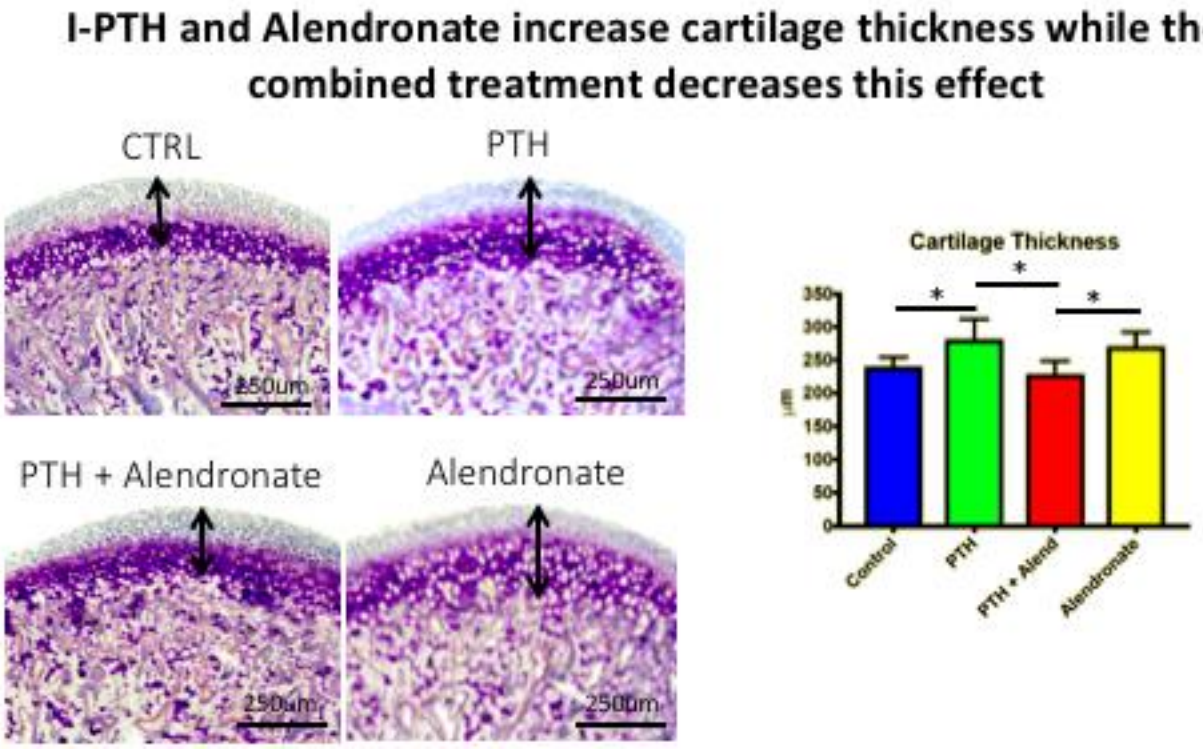
I-PTH and Alendronate increase cartilage thickness while the combined treatment decreases this effect
CTRL
PTH
PTH + Alendronate
Alendronate
250um
250um
250um
250um
Cartilage Thickness
*
*
*
350
300
250
200
150
100
50
0
Control
PTH
PTH + Alend
Alendronate

FIG 7

I-PTH and Alendronate increase cell proliferation while the combined treatment decreases this effect