THE EFFECTS OF PARATHYROID HORMONE ON THE ALVEOLAR BONE OF OVARIECTOMIZED RATS

A Thesis

By

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ABSTRACT

Osteoporosis is a widespread metabolic disease of bone characterized by decreased bone mineral density and reduced quality of bone microarchitecture leading to increased fracture risk and fragility of the bones. Traditionally, the most common therapeutic approach for osteoporosis has been anti-resorptive medications. They promote mineralization by suppressing osteoclastogenesis and osteoclast function, culminating in suppression of bone turnover. However, it has been noted that anti-resorptives such as bisphosphonates cannot rebuild bone structure or bone mass as seen in post-menopausal women. Current treatment modalities also include osteoanabolics. In severe forms of osteoporosis, the use of osteoanabolics such as PTH helps to restore the bone mass through anabolic effects on osteoblasts. PTH has been known to enhance the bone microarchitectural properties by increasing the bone mineral density (BMD), by improving trabecular number and connectivity, and strengthening the resistance to mechanical fracture. Various mechanisms have been proposed to explain how the PTH can improve the quality of bone remodeling in patients with osteoporosis. Most of them are studied in the context of long bones. The molecular mechanism by which parathyroid hormone (PTH) acts in osteoporotic animal models, especially in the maxillary alveolar region have not been fully elucidated. It is important to understand the effects of osteoporosis on the alveolar bone morphology and homeostasis in the context of maxilla as we know it is more porous than mandible, and thus, prone to resorptive changes in osteoporosis. Moreover, understanding this process could also help us explore the role of PTH in other chronic diseases of the alveolar bone such as chronic periodontitis. Our hypothesis was to study the effects of ovariectomy/osteoporosis on the alveolar bone morphology and bone remodeling, and to elucidate the molecular mechanism of PTH treatment on the osteoporotic rats

model. Herein, we used ovariectomized rat model and used the alveolar bone region of the maxillary first molar area as the region of interest.

This is one of the first study to highlight the morphologic, microarchitectural and molecular changes after osteoporosis in a rat maxillary alveolar region and to describe the role of PTH as a therapeutic effect to restore those osteoporotic changes.

DEDICATION

To all the rats sacrificed for this study.

Though various literature have been existent to explore the role of PTH in treatment of osteoporosis, hopefully this study may serve as a foundation for further exploration of its role in the treatment of patient who suffer from the detrimental effects of osteoporosis and its related effects on the alveolar bone.

To my family, my husband and daughter who have been patient throughout my journey of education

To all the future patients

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CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a dissertation committee consisting of Dr. Jian Q. Feng, Dr. Yan Jing of the Biomedical Sciences and Dr. Victoria Woo of the Department of Diagnostic Sciences.

The experiments were mainly carried by Dr. Chunmei Xu and the student in conjunction. The jaw samples were kindly provided by Dr. Sherry Liu in UPENN Medicine,

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NOMENCLATURE

3D	Three dimensional
PTH	Parathyroid hormone
OVX	Ovariectomized
VEH	Vehicle
WT	Wild type
BMD	Bone mineral density
BMC	Bone mineral content
TbN*	Trabecular number
TbS*	Trabecular spaces
BV	Bone volume
TV	Total volume
DMP1	Dentin matrix acid phosphoprotein
MEPE	Matrix extracellular phosphoglycoprotein
MMA	Methyl-methacrylate
RANKL	Receptor activator of nuclear factor kappa-B ligand
SEM	Scanning electron microscopy
H&E	Hematoxylin & Eosin
ROI	Region of Interest
μCT	Micro Computed tomography
Ocys	Osteocytes
Obls	Osteoblasts

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CHAPTER I

INTRODUCTION

Osteoporosis is a widespread metabolic disease of bone characterized by decreased bone mineral density (BMD) and reduced quality of bone microarchitecture leading to increased fracture risk and fragility of the bones. (Zhang & Song, 2020). It shows 30% prevalence in women and 12% in me.(Wright et al., 2014) It is considered one of the leading causes of permanent disability in the ageing population and increases mortality as well as economic burden in both genders equally. (Bryant C. Roberts et al., 2020).

Traditionally, the most commonly used therapeutic approach for osteoporosis has been antiresorptive medications to prevent the risk of vertebral, hip, and other fractures especially in post-menopausal women. (Papapoulos, 2011). These medications are the mainstay treatment as they promote mineralization by suppressing osteoclastogenesis and osteoclast function, culminating in suppression of bone turnover. However, due to limited potency of these medications, it may not be highly efficient in severe forms of osteoporosis. (Rachner, Hofbauer, Gobel, & Tsourdi, 2019). Moreover, it had been noted that although anti-resorptives such as bisphosphonates can improve bone mass density (BMD) due to decrease resorption, they cannot rebuild bone structure or restore bone mass lost as they do not stimulate new bone formation as seen in post-menopausal women. This further limits its role in severe forms of osteoporosis where there has already been significant thinning of trabecular bone. (Augustine & Horwitz, 2014)

Currently approved treatment modalities are classified under anti-resorptives and osteoanabolic. In severe forms of osteoporosis, the use of osteoanabolic modality such as PTH

offers an ideal therapeutic model to stimulate the bone formation without resorption through anabolic effects on osteoblasts. (Marius E. Kraenzlin & Meier, 2011). There are certain FDA-approved anabolic agents in the US such as parathyroid hormone (1-34) (PTH 1-34), or teriparatide and even full length recombinant agents such as parathyroid hormone (PTH 1-84) in certain European countries. (Augustine & Horwitz, 2014)

PTH has been referred to as anti-remodeling agents and have been recommended for osteoporosis patients at high risk for fracture, patients unresponsive to bisphosphonates, and patients with persistent glucocorticoid induced osteoporosis. (Hodsman et al., 2005). PTH has been known to enhance the bone microarchitectural properties by increasing the bone mineral density (BMD), by improving trabecular number and connectivity, and strengthening the resistance to mechanical fracture. (Zhang & Song, 2020).

The mechanism by which parathyroid hormone (PTH) acts is through activation of the PTH receptor 1. Long-term activation of this receptor (as in primary hyperthyroidism) leads to catabolic effect results in bone loss and susceptibility to fracture. On the other hand, an intermittent short activation to PTH leads to an anabolic effect resulting in bone formation, increased bone mass, and improved microarchitecture. (Zhang & Song, 2020). (Hodsman et al., 2005).

Various mechanisms have been proposed to explain how the PTH can improve the quality of bone remodeling in patients with osteoporosis. These mechanisms include:

1) improved bone density through a decrease in the size of the remodeling space; (Hodsman AB, Kisiel M, Fraher LJ, & PH, 2000)
2) preservation of cancellous bone architecture; (Hodsman AB & BM., 1993) (Dempster DW, 2001)

3) a reduction in the number of resorption bone cavities or mechanical stress concentrators that potentiate mechanical failure of the bone; (Hodsman AB et al., 2000)
4) an increase in the amount of bone mineral per unit volume of bone tissue; and (Hodsman AB et al., 2000) (Hodsman AB & BM., 1993)
5) a decrease in cortical porosity of bone (Hodsman AB & BM., 1993).

The major risk factor for osteoporosis in women is estrogen deficiency following menopause that impact the bone homeostasis and inflammatory response. (Recker, Lappe, Davies, & Heaney, 2004). Low levels of estrogen stimulate inflammatory mediators such as IL-1, IL-6 which may further aggravate periodontal disease. Ovariectomized rat models have shown higher expression of IL-6, RANKL, osteoprotegerin (OPG) with a resultant bone resorption.(Luo et al., 2014). PTH is another important hormone for maintaining bone homeostasis . Intermittent PTH have shown to improves bone regeneration and periodontal healing in extraction sites. Hormonal replacement have been effective in improving mandibular BMD and reduce periodontitis thereby suggesting a potential connection.(Wang & McCauley, 2016)

Periodontitis is a chronic multifactorial disease that affects half of the adult population of the world and require long term treatment (Wang & McCauley, 2016). As it is primarily caused by microbial infection, the resultant effect of chronic periodontitis leads to high levels of inflammatory cytokines such as tumor necrosis factor-α (TNF-α), Interleukin-1 &6 (IL-1 &6). These cytokines can cause alveolar bone destruction through up regulation of the receptor activator of nuclear factor-κB ligand (RANKL) (S. J. Hong et al., 2021) . Periodontitis have been linked to various systemic diseases and osteoporosis is one of them. The multifactorial nature and the its bone-resorbing nature brings a highlight to its connection to osteoporosis. Many studies suggest clinical

association of osteoporosis and periodontitis and point to a specific mechanistic relationship between these two chronic bone-resorbing disease (Wang & McCauley, 2016).

While osteoporosis is mainly a systemic disease that affects the cancellous bone and thus results in osteoporotic changes of the alveolar bone, it has been noted that periodontitis initially affects the cortical bone resulting in loss of alveolar bone height. Studies also suggest that systemic osteoporosis can result in low mineral density of alveolar bone that could be a "weak resistance" to various infectious agents in the setting of periodontal disease. (Kribbs, 1990). The osteoporotic change of the alveolar bone may have contributed the premature loss of teeth in periodontitis.

So far the molecular mechanism of osteoporosis in the context of the alveolar bone have not been fully understood. Moreover, given the nature and shared risk factors of osteoporosis with periodontitis (such as age, genetics, hormones, smoking, calcium & vitamin D levels in body etc), there is a need of understanding the molecular mechanism between the two diseases (Genco & Borgnakke, 2013). It is imperative to understand the cause and effect relationship between the two with more longitudinal studies.

CHAPTER II

LITERTURE REVIEW

Osteoporosis is a chronic systemic metabolic disease of bone that is characterized by the resorption and fragility of bones. Osteoporosis is characterized by the occurrence of fragility fractures.(Kraenzlin & Meier, 2011).

Systemic osteoporosis is considered to have a local effect on the dento-alveolar regions as these individuals might develop tooth loss as a result of osteoporotic bone loss. Moreover, a significant decrease in the bone mineral density (BMD) in the jaws of osteoporotic rats, monkey and rabbits have been described. Most of these studies have studied the mandible as the region of interest whereas only few have selected the molar areas of the maxillary jaws in experimental animals. (Bellido et al., 2010).

Two of the major public health problems of the aging population in the world today are osteoporosis and periodontal disease. Various systematic reviews have shown that there is a a positive association between osteoporosis and periodontal disease. It is important to understand the association as both disease process are multifactorial and share the common risk factors. (Dodd & Rowe, 2013).

The common risk factors are smoking habits, alcohol consumption, diabetes, and socioeconomic status. It has been shown that decreased BMD may be related to periodontal tissue destruction. Previous studies have emphasized that postmenopausal women with osteoporosis are susceptible to dental plaque formation and subsequent chronic periodontitis. The assessment of the severity of periodontitis have been associated with pathologic bone fractures using radio-

morphometric measurements, such as the mandibular cortical index. This proves an association between periodontitis and osteoporosis/fractures. (S.-J. Hong et al., 2021).

In an osteoporotic rabbit model that was induced after ovariectomy and glucocortocid administration , there was a significant decline in BMD, mineral content and calcium content and this was especially pertaining in the pre-alveolar region of mandible. There was also a differential pattern of bone loss seen in the OVX models. (Jilka, 2007).

Various recombinant human parathyroid hormone (PTH) analogues, either the full-length PTH1–84 or the shortened PTH1–34, which is also known as teriparatide have been used for the treatment of osteoporosis. (Kraenzlin & Meier, 2011).

However, studies have shown that both teriparatide and PTH 1-84 stimulate bone resorption as well as bone formation, both have reduced efficacy after a certain duration of therapy, and they can be inconvenient to administer as daily subcutaneous injections.(Augustine & Horwitz, 2014).

Short-term intermittent exposure by PTH treatment causes the shift of the osteogenic progenitor cells from the proliferation to the differentiation stage and exit from the cell cycle. This further leads to differentiation of osteoblasts and help in the anabolic process of bone formation.(Aggarwal & Zavras, 2012).

Micro CT or micro-computed-tomography, (μ-CT) is a recently developed non-destructive method to image and quantify trabecular bone. It can both quantify and qualify the trabecular architecture and help to understand the mechanical properties of bone and to study trabecular bone remodeling. The principle is based on a compact fan-beam type tomograph that can work in spiral scanning or multi-slice mode. It is housed with an X-ray tube and a CCD-array as a detector. Samples with various size diameters can be measured with high spatial resolution. 3D stereological indices are extracted according to the standard definitions used in histomorphometry. Various

measurements are employed to "calibrate" lower-dose, lower-resolution images in vivo to nondestructively assess unprocessed surgical bone biopsy specimens that remain intact for mechanical or histological testing. (Rüegsegger, Koller, & Müller, 1996)

The ovariectomized rat, is often a widely used preclinical model for studying postmenopausal bone loss that mimics osteoporosis of bone tissue in the hip and spine. It can also be used to study the mineral content and structural changes in alveolar bone to develop drug and/or therapeutic strategies to prevent various systemic and metabolic disorders causing tooth loss. (Johnston & Ward, 2015).

We can compare both the cortical bone as well as the trabecular bone of the alveolar process. Bouxsein et al. highlighted four parameters (i.e., BV/TV, TbTh, TbSp, and TbN) in describing trabecular bone microarchitecture and four parameters (TtAr, CtAr, CtTh, and Cr.Ar/Tt.Ar) must in describing cortical bone morphology. In case, of measuring the bone volume by means of BV/TV, previous studies show significant decrease in OVX. (Bouxsein et al., 2010).

Various studies highlight the role of selecting a region of interest (ROI). Studies have investigated short and longer-term changes in mandibular morphometry of rats ovariectomized at 26 weeks and studied 52 weeks later using μ-CT at a resolution of 20 μm. The ROI selected was the interradicular septum of the first molar. However, not many studies involving the alveolar region of maxillary region have been conducted. It is to be noted that maxillary alveolar bone are quite porous and could be the first one to undergo resorption and/or destruction during various systemic and local diseases. (Johnston & Ward, 2015).

Various genes have been implicated as a marker for early osteocytes bone formation and important. Most important ones along with regulators of mineralizaton and phosphate homeostasis such as DMP1, MEPE, or PHEX.

Studies carried out by inducing osteoporosis in rabbit models with the help of ovariectomy and glucocorticoid administration reveal a significant decrease in bone mineral density, bone mineral content, and calcium content with a particular susceptibility to the osteoporotic pre-alveolar region of the mandible, though there is also a global bone mineral density loss in the jaw bone (Jilka, 2007).

These differences in the pattern of bone loss can be contributed to the mixed densitometric composition of the various regions of maxilla and mandible bone. As osteoporosis adversely affects the dental bone composition, density, and strength, long-term intermittent PTH use would be of particular interest from the perspective of improving dental outcomes. Questions also remain about the use of the bone-remodeling mechanism of PTH action in the management of ankylosis, fractures, implant procedures and so on.

CHAPTER III

AIMS

It has been known that osteoporosis affects the bone remodeling in patients and different experimental animal models. However, the effects of osteoporosis on the alveolar bone morphology and homeostasis are often overlooked. Although the role of PTH both independently and as a treatment modality for osteoporotic bone has been established in the setting of long bones, it has been rarely studied in alveolar bone. This raises the question of whether PTH may have a role in bone remodeling in the alveolar bone.

The molecular mechanism underlying the effects of osteoporosis on the alveolar region has been not yet fully understood. An ovariectomized rat model can be used as a standard model as previous studies have shown that experimental periodontitis in an ovariectomy mouse model aggravated the disease process by inducing bone loss. Does osteoporosis serve as a nidus of increased risk for periodontal disease owing to its low BMD and reduced microarchitecture of alveolar bone? Or on the contrary could periodontal disease be an early sign of osteoporosis? As the architectural and BMD changes associated with osteoporosis in the alveolar bone have not been explained, our study provides a deep understanding of the architectural changes and elucidate the molecular mechanisms of the osteoporotic changes.

Historically, the central dogma surrounding osteoporotic mouse model is that ovariectomized rats (OVX) will have an increase in osteoclastic activity leading to more bone resorption. The process of bone formation has always been attributed to the actions of osteoblasts. However, emerging data shows that osteocytes (Ocys) and not osteoblasts (Obls) are the key

regulators of bone formation. They are the active cells for bone mineralization and regulate mechanical strain reactions as well as osteoclast formation. Moreover, they are also thought to play roles in regulating bone homeostasis and adaptive bone remodeling. They are regarded as key players for maintaining a connection to the nutritional demands through their lacunae, around which bone formation occurs. (Ke Wang et al., 2021).

Thus, when OVX-experimental mouse models are created we expect to observe a higher number of Ocys and an increase in the Ocys-canaliculi in the molars of the OVX rats compared to control rats. Moreover, in the setting of OXV-rats treated with PTH, we expected an improved bone phenotype along with enhanced bone functions. Hence, our hypothesis is that Ocys play a key role in the onset of OVX-induced bone defects of the alveolar bone and that these defects can be partially restored by applications of PTH.

SPECIFIC AIMS

The purposes of this study are to:

1) study the effects of ovariectomy/osteoporosis on the alveolar bone morphology and bone remodeling, and
2) examine the effects of PTH treatment on the OVX-rats compared to controls on bone phenotype, mineral content, ultrastructural morphology, dendritic number, osteoclast quantification, and osteocytes phenotype.

CHAPTER IV

MATERIALS AND METHODS:

Experiments/ Methods of Investigation:

1. X-rays
2. uCT
3. Triple labeling (G-2,R-5,Y-12,G-19)
4. SEM
    a. Back scatter
    b. Acid etch
5. Histology
    a. H&E
    b. Masson's trichome
    c. Picrosirius red (polarizing)
6. Immunostains
    a. DMP-1
    b. MEPE

Rats, Ovariectomized Vs WT; PTH/VEH treatment

This study represented a collaboration between Dr. Jerry Feng's laboratory in the Department of Biomedical Sciences and Oral and Maxillofacial Pathology in the Department of Diagnostic Sciences. Animal models of Sprague Dawley female rats (N=5/treatment group), at 16 weeks (4 months) of age, will be randomly assigned a unique number or identifier. Then ovariectomy will be performed in the ovariectomized (OVX) group whereas no surgery would be performed on the controls (WT). The rats in the OVX groups and WT groups would be then continued for a further period of 12 weeks (3 months) to establish the effects of ovariectomy in the OVX rats. After a total of 28 weeks (7 months0, each of the OVX and WT groups would then receive either a vehicle (VEH) or PTH treatment. PTH treatment will be administered intermittently by subcutaneous injections of 20 μg/kg/day five times a week for 3 weeks in both the WT and OVX groups. All VEH groups will receive subcutaneous isotonic saline (0.9%, W/V) injections. Following the same injection schedules, PTH will be administered in rats in the PTH groups. For carrying out the labeling experiments, all experimental animals will further receive calcein (green, G), cyan (blue, B), alizarin red (red, R), calcein (green, G) fluorochrome injections during the 3-week PTH or VEH treatment period in the order of G-B-R-G on days -2 (G), 5(B), 12(R), 19(G) (initiation of PTH or VEH on day 0). Finally, the experimental animals will be euthanized after further 3 weeks of either PTH or VEH treatment. After the mice were euthanized, the upper right jaws were fixed in 70% ethanol and used for radiographs, μCT, and SEM examination. The upper left jaws were fixed in freshly prepared 4% paraformaldehyde in phosphate-buffered saline (pH 7.4), decalcified in EDTA, and embedded in paraffin using standard histological procedures. The areas for further study would be obtained from the molar region of the maxillary alveolar bone. All experiments would be conducted jointly by the PhD student in

Jerry Feng's laboratory and the MS oral pathology student. The histopathology experiments will be reviewed by the MS oral pathology resident and thesis advisor. All experiments conducted and analyses will be further reviewed by Dr. Jerry Feng, Dr. Yan Jing, and Dr. Victoria Woo.

X-ray Radiography and Micro-computed Tomography

Radiographs of bone samples were taken using a Faxitron model MX-20 Specimen Radiography System with a digital camera (Faxitron X-Ray Corp., Lincolnshire, IL, USA). Maxillary alveolar bone μCT analysis was performed at the first molar region by Scanco μCT35 (μCT35; Scanco Medical AG, Bassersdorf, Switzerland). Serial tomographic imaging was done at an energy level of 55 kV and intensity of 145 μA. Bone volume vs total volume at the midshaft was calculated and used for comparison of the samples. (P, B, & R, 1996) (Jing et al., 2017) (Feng et al., 2006)

Backscattered scanning electron microscopy (SEM), acid-etched SEM

The freshly isolated jaw bones were fixed in 4% paraformaldehyde solution at room temperature for 48 hours. The tissue specimens were dehydrated in ascending concentrations of ethanol (from 70% to 100%), embedded in methyl-methacrylate (MMA, Buehler, Lake Bluff, IL). The MMA embedded samples were cut and the surface polished using 1 μm and 0.3 μm alumina alpha micropolish II solution (Buehler), followed by acid etching with 37% phosphoric acid for 2 to 10 seconds, 5% sodium hydrochloride for 5 minutes and then coated by gold (for acid-etched

SEM imaging). For backscattered SEM, samples were coated by carbon after polish. Samples were scanned by a FEI/Philips XL30 field-emission environmental SEM (Hillsboro, OR, USA) as previously described. (Feng et al., 2006)

Fluorochrome labeling of the mineralization front

Double fluorescence labeling to visualize bone mineralization active front, was performed. For carrying out the labeling experiments, all experimental animals will further receive calcein (green, G), cyan (blue, B), alizarin red (red, R), calcein (green, G) fluorochrome injections during the 3-week PTH or VEH treatment period in the order of G-B-R-G on days -2 (G), 5(B), 12(R), 19(G) (initiation of PTH or VEH on day 0). Briefly, mice were first injected intraperitoneally with calcein green (5 mg/kg), followed by injection of an alizarin red label (5 mg/kg i.p.; Sigma-Aldrich, St Louis, MO, USA). Mice were euthanized 48 hours after injection of the second label, and the bones were removed and fixed in 70% ethanol for 48 hours. The specimens were dehydrated through a graded series of ethanol (70% to 100%) and embedded in methyl methacrylate (MMA) without prior decalcification. The 50-mm non-decalcified samples from these animals were photographed using a Nikon PCM-2000 confocal microscope coupled with an Eclipse E-800 upright microscope (Nikon Instruments, Melville, NY, USA) for fluorochrome labeling or in combination with 4,6-diamidino-2-phenylindole (DAPI) staining of nuclei of osteocytes. (Feng et al., 2006)

Microscopic Sample preparation and immuno-histochemistry

After the mice were euthanized, the upper right jaws were fixed in 70% ethanol and used for radiographs, μCT, and SEM examination. The upper left jaws were fixed in freshly prepared 4% paraformaldehyde in phosphate-buffered saline (pH 7.4), decalcified in EDTA, and embedded in paraffin using standard histological procedures. After decalcification, the tissue blocks were cut into 5-μm-thick serial sections and mounted on glass slides and dried. The sections were used for histological stains like H&E, Masson's trichrome, Picrosirius red, and immunohistochemistry DMP-1 (dentin matrix protein-1) and MEPE (Matrix extracellular phosphoglycoprotein). The concentrations of the primary antibodies for the immunohistochemistry are: rabbit polyclonal anti-DMP1(1:400, generously provided by Dr. Chunlin Qin from Baylor College of Dentistry; rabbit polyclonal anti-MEPE (1:100, LF-155, a gift from Dr. Larry W. Fisher, NIDCR/NIH).

After deparaffinization and rehydration, the sections were immersed in 3% H_2O_2 to quench endogenous peroxidase and further digested with 1 mg/ml trypsin for 30 min at 37 °C. Sections were then blocked with 1% bovine serum albumin containing 1% serum at room temperature for 2 h. The primary antibodies were added to the sections and incubated overnight at 4 °C. After washing, the sections were coated with biotinylated second antibody (Vector Laboratories, Burlingame, CA) at a dilution of 1:200 and then incubated at room temperature for 60 min. The sections were washed again and incubated with the ABC reagent (Vector Laboratories) at room temperature for 60 min. The 3,3′-diaminobenzidine substrate was used to visualize immunoreaction sites. Sections were counterstained with hematoxylin and mounted on glass slides. Negative controls were obtained by substituting the primary antibody with normal serum or normal IGG. (Jing et al., 2017) (Feng et al., 2006)

Methods of analysis

The analysis involving bone cells, osteons, etc. will be analyzed both quantitively and qualitatively with the assistance of Image J.

Control tissue sections will be processed along with experimental tissue on each slide to facilitate appropriate calibration of the Image J software. All data are reported as mean values ± SEM. Three representative high-power fields (40x) per case will be evaluated using image analysis, The Kruskal–Wallis test was used to detect any significant differences among samples. The Mann–Whitney U test (post hoc test) was used to compare differences between the OVX and WT group. Significance level is defined as follows for all analyses performed: $*p < 0.05$; $**p < 0.01$.

I. CONSORTIUM/CONTRACTUAL ARRANGEMENTS

N/A

II. COMPLIANCE

This project involves tissues from experimental mouse models. Application for IRB approval was in compliance with the university regulations.

CHAPTER V

RESULTS & DISCUSSION

RESULT 1: PTH leads to increase in bone mineral density of alveolar bone of WT rats

Here we showed that the treatment of PTH in the adult female rats improved the bone mineral density even in WT rats (Figure 2A) . This change can be clearly see in μ-CT cross-sectional analysis than in the simple X-rays (Figure 2C). μ-CT has now become the "gold standard" for evaluation of bone morphology and microarchitecture in experimental animals like rats. μ-CT provides high resolution 3D imaging information that can't be obtained by any other non-destructive technology. It can be used to study the interior structure of both material and biological samples without having to cut the samples. It allows us direct 3D measurements of trabecular morphology, trabecular thickness and separation than with standard histologic evaluations. (Feldkamp, Goldstein, Parfitt, Jesion, & Kleerekoper, 1989). Bone mineral density (BMD) is measured in terms of Bone volume/ total volume. Here the trabecular bone micro architecture of the alveolar region is measured in terms of BV/TV, TbSp, and TbN between the control and PTH treated groups. Our results show that BV/TV ratio and BMD are slightly increased in the OTH group (Figure 2B). Heat map image of the same cross -sectional μ-CT analysis at the first molar region show a slight increase in the mineral density of the PTH treated WT rats (Figure 2D)

Moreover, the BMD is also significantly increased in the PTH treated WT rats although there is no statistical significance in the increase in bone trabecular number in the PTH treated group.

3D image of the trabecular bone microarchitecture showing the alveolar bone in between the five roots of a maxillary first molar which uses a digital method to arbitrarily remove all the tooth roots in the sections and the bone between two roots is the alveolar bone and represents our region of interest (ROI) (Figure 2E)

RESULT 2: OVX mice have reduced bone mineral density and compromised micro-architecture which can be improved by PTH treatment as it leads to a significant increase in bone volume, trabecular number, and trabecular space in OVX rats

X-ray analysis in the OVX group showed reduced mineral density from simple observations. (Figure 3A). The trabecular bone micro architecture of the alveolar region is measured in terms of BV/TV, TbSp, and TbN and BMD. In OVX rats model, the bone volume is small, it is more porous and compared with the control group, the ovariectomized group showed a significant decrease in BV/TV and TbN, at the same time increase in trabecular spaces (TbSp). Previous studies have shown a significant decline in the BMD of OVX animal models than in the control group. (Bellido et al., 2010) In an ovariectomized rat model that received intermittent PTH therapy for 12 weeks, the μ-CT analysis from the mandible and femoral head showed reduced trabecular bone microarchitecture, bone volume fraction and trabecular thickness in the OVX group than in the healthy group. However, after PTH treatment, it was almost restored back to those in the healthy group. (Jing et al., 2017)

Here in the cross sectional sliced μ-CT image of OVX rats (Figure 3C), we can observe that the trabeculae are widely spaced and seem porous. We can observe that there is significant reduction in the BMD in the OVX rats which seems to be restored with OPTH treatment (Figure 3B). OVX causes bone volume reduction side by side (Figure 3B), However, the use of PTH has been shown to increased bone mass, volume and density to some extent. This can be further illustrated by the heat map image of the same section on the right (Figure 3D). 3D image of the trabecular bone

microarchitecture showing the alveolar bone in between the five roots of a maxillary first molar shows a more porous bone in the OVX group that is restored with PTH treatment (Figure 3E). Studies have shown that post-ovariectomy the interradicular septum expanded forming a large marrow space. They seem as if the trabeculae were sparse and appear floated in the bone marrow. However, in the control group the trabeculae seem interconnected and for a clear network. (Luo et al., 2014)

RESULT 3: PTH treatment results in increased active bone deposition as indicated by triple labeling

Confocal microscopy images of fluorochrome labeling shows areas of new bone formation with active mineralization fronts (Figure 4A). Both the brightness of the labelling index and distance between the red and green line means the active mineral deposit. Even at a lower magnification the labelling index shows that the intensity is higher in the treated group, i.e, PTH-WT and PTH-OVX groups (Figure 4B). The control bone in WT shows three discrete lines of fluorescent label, reflecting the active mineralization fronts at the time of injection. However, in the OVX group, there is lack of distinct lines showing there is lack of active bone deposition (Figure 4B,C). Past studies have demonstrated that confocal laser scanning microscopic image of the interradicular septum in control experimental animals showed large areas on bone (in green label) regular and normal sized marrow space whereas that of OVX group showed an irregular and wide marrow space, and few areas of bone volume (green label areas) (Ejiri et al., 2008).

RESULT 4: PTH treatment increases bone matrix and resistance to acid treatment

Osteocytes are contained within its own lacuna and connected to each other by an intricate network of canaliculi within the bone matrix. Recently scanning electron microscope (SEM) following a standard acid etched surface has emerged as a standard tool to visualize the cellular network and their processes (Kubek, Gattone, & Allen, 2010). The acid treatment in acid etch SEM depends on the mineral density of bone matrix. If mineral content of a bone is low, acid can easily etch the surface thereby exposing the osteocytes with their network of canaliculi (Kubek et al., 2010). Compared to the WT group we can see that the bone matrix in PTH treated group still remains (Figures 5A,B &C) which signifies that the matrix mineral is better and resistant to acid treatment. The yellow arrows on the left side images (Figure 5A,B,C) of the VEH treated rats indicate the number of osteocytes that are exposed after acid etching on the WT whereas the red arrows on the right indicate the resistant bone minerals resistant to acid etching in PTH treated rats, thus resulting in more matrix but few number of osteocytes that are exposed. After acid treatment we can see more number of osteocytes and its cell processes exposed in WT group (Figure 5A, left panel), whereas in the PTH treated group we can see the mineral matrix that are resistant to acid treatment (Figure 5A, right panel). In contrast, in the OVX group we can clearly see an increased cells and its processes (Figure 5B, left panel) which signifies that more bone was lost to acid etching as it is more porous, and has less BMD. However, on the right side (Figure 5B, right panel, Figure 5C) we observe that there are more areas of bone matrix that are still left in the PTH treated group. This signifies that the mineral content in the PTH-OVX group is better and quite resistant to acid treatment. In the highest magnification image (Figure 5C, right panel) osteocytes and its

canalicular processes are clearly evident and also a large area of bone matrix that is remaining even after the acid treatment in the PTH-OVX group.

This clearly demonstrates that the bone matrix improves in PTH treatment whereas it is less resistant in the OVX group.

RESULT 5: PTH treatment do not cause any change in the cortical bone morphology in rats

When we looked at the bone morphology of the cortical areas in the maxillary molar region of rats, there was not much morphological differences noted in the cortical area and the supporting areas of the maxillary first molar area of the experimental rats (Figure 6A,B,C,D). It has been explained in some previous studies that the most earliest and common effect of the osteoporosis are mainly evident in the alveolar bone in the trabecular region than in the cortical bone. It has been studied that the BMD, bone mineral content (BMC) and calcium content in the ash from peri-alveolar region of the osteoporotic animal was markedly reduced that influenced the tooth loss process. PTH treatment to such models completely reversed the BMD, BMC and calcium content, especially in the peri-alveolar region. (Bellido et al., 2010). Previous study in a PTH treated osteoporotic model did not show any difference in the osteocytes to influence the bone tissue in the cortical bone. The authors stated that the ROI was a rat cortical bone as it lacks secondary osteonal remodeling minimizing the chance of any influence by the therapeutic effects of PTH. Their study showed that the positive effects of PTH and anti-resorptive agents on cortical bone in small experimental animals are not of enough significance and are less robust than trabecular alveolar bone. (Brouwers, van Rietbergen, Huiskes, & Ito, 2009) (Yano, Yamada, Konda, Shiozaki, & Inoue, 2014) (Arita et al., 2004)

On the contrary, few studies were able to publish results on the recovery of the lost mineral content (BMC) and bone thickness to the sham levels after PTH treatment in ovariectomized rats but failed to demonstrate any pronounced recovery of the trabecular BMC and total cross-sectional area in the trabecular are. However, their study included the data from the rat lumbar vertebrae and not from the trabecular region of the alveolar region of the jaws. (Arita et al., 2004)

RESULT 6: PTH treatment leads to the onset of osteon-like structure + bone volume increase in OVX rats

Histologically, primary and secondary osteons form as basic structural elements of compact bone. It is generally known that aged rats and mice lack true Haversian cortical bone remodeling under physiological conditions but not cancellous bone remodeling activity. Therefore, there are some secondary osteons can be observed in their long bones (mainly near endosteal border). (Duranova et al., 2014). Individual osteon formation often reflects patterns of bone formation, thus histomorphological analyses provides an important information about the bone reorganization process in various situations. Histomorphological changes of primary and secondary osteons can be seen in osteoporosis as well.

In the present study see the differences in osteon-like formations in the OVX rat model are noted. At lower magnification, the overall bone volume seems to be reduced in the OVX group (Figure 7A & 7C, 3rd panel). whereas it is restored in the PTH-OVX group almost as in the WT (Figure 7A fourth panel). Moreover, at higher magnification (Figure 7B & 7D ,4th panel). we can compare the well-formed osteon-like structural formation with concentric ring like structures around Haversian canals in the PTH treated group. There are many Haversian canals which are osteon-like or "tree-rim like" structures in PTH treated group compared with OVX. But the morphology in the OVX group are more cellular and lack well-formed osteon-like structures. Formation of so called osteon-like structures which are more thicker and prominent supports our previous morphological analysis in H&E slides that there is an overall bone volume and quality bone restoration after PTH treatment after osteoporosis.

One study have highlighted the difference in histomorphology in the intracortical canal networks with irregular canals reminiscent for resorption spaces in ovariectomized 16-month old rats, when compared to intact canals morphology in 16-month old control rat. Their study also showed the development of haversian canal like morphology in elderly rats over 16 months of age along with resorption cavity and irregular osteonal canals in the femoral cortex. They suggested that the elderly OVX model over 16 months' age could be used as a suitable methods to evaluate the cortical porosity via the morphological features. (Lee, Kim, Shin, Koh, & Song, 2020)

RESULT 7: PTH treatment leads to more homogenous and thick bundles of collagen and well-formed osteon like structures of the alveolar bone structure

When Picrosirius red staining is observed through polarized microscopy ,the collagen fibers can be seen in shades of green , yellow and green (Figure 8A). An intense red staining indicates well formed, homogenous and highly packed thick collagen (Type I mainly) whereas a heterogenous red-yellow-green staining indicates diminished packaging and thickness of Type I collagen. Often than not, no picrosirius red staining is observed within non-mineralized bone tissue (such as osteoid) surrounding blood vessels. (Kühnisch et al., 2014). In the present study, if we compare the area of the periodontal ligament area (PDL) in PTH treated WT and OVX, we can see the thicker collagen fibers in the PTH treated group (Figure 8 A&B, 2nd and 4th panel). The intensity of the red staining in WT controls and that of PTH treated OVX group are almost comparable as there are areas of homogenous red staining which indicate highly packed and thick collagen in the treated group (Figure 7A&B, 2nd and 4th panel). At the same time, in the OVX group, we see heterogenous red-yellow staining which is indicative of diminished packaging of collagen fibers and thickness of bone collagen. Not only the PDL fibers are thicker but they are also arranged in a more regular fashion in the PTH group supporting the evidence that the PDL area is more organized in the PTH group (Figure 8A&B, 1st and 2nd panel).

The Picrosirius red staining in the alveolar bone area showed similar results. In the alveolar bone region of OVX group we see heterogenous red-yellow staining which is indicative of diminished packaging of collagen fibers and thickness of bone collagen (Figure 8 C&D, 3rd panel). The intensity of the red staining in WT controls and that of PTH treated OVX group are almost

comparable as there are areas of homogenous red staining which indicate highly packed and thick collagen in the treated group (Figure 8 C&D, 2nd and 4th panel).

Previous study have shown that a difference in the intensity of red fibers reflect the collagen fibers organization, intense red staining with minimal yellow and green indicate stronger structural organization of the bone matrix and well-formed collagen type I fibers. However, a yellowish and green refraction in polarized light means inhibition of the biosynthesis of collagen by osteoblasts and osteocytes with modeling osteoporosis with a tendency to increase the content of collagen fibers type III and reduction part of collagen fiber type I. This adverse changes in the structural organization of the bone matrix could increase the risk of fractures, and is a sign of degenerative/osteoporotic changes (Maltseva, 2016).

RESULT 8: PTH leads to increase in osteocytes and DMP1 expression in rats whereas PTH leads to decreased MEPE expression in OVX rats

DMP1 is a bone dentin non collagenous matrix proteins. DMP1 is highly expressed in osteocytes and has a key role for osteocytes formation and phosphate homeostasis in mineral metabolism. Dentin matrix protein 1 (DMP-1) is expressed predominantly in odontoblasts in tooth and osteocytes in bone. (Lu et al., 2011).

Here, at low power we can see that OVX-with treated PTH (Figure 9 A, 4th panel) has strong intensity of DMP1 staining while there is a stronger and more expression of DMP1- in PTH group of WT as well (Figure 9 A, 2nd panel) . Even at low power we can see that OVX-with treated PTH has strong intensity of DMP1 staining (Figure 9 A, 4th panel) . OVX with PTH treated has cells which has strong DMP 1 in cell body and matrix(Figure 9 B, 4th panel) .

This further supports the previous observation that active bone formation does take place in the PTH treated OVX rats while activation of osteocytes is reduced in osteoporotic rat model.

Previous study shows that PHEX and DMP1 control a common pathway regulating bone mineralization and FGF23 production, where DMP1 play a major role in the activation of the FGFR signaling in osteocytes for bone formation (Martin et al., 2011)

MEPE is a member of acid phosphoproteins which is expressed in teeth and bone. MEPE mRNA is highly and selectively expressed in mineralised matrix embedded osteocytes and MEPE protein is localised along dendritic processes. Normally a strong MEPE expression means that bone formation is not taking place, its expression has an inhibitory effect. The hypothetical role of MEPE could be mineralisation and mineral removal within the canalicular wall and within the

osteocyte lacunae, since MEPE knockout mice have been shown to have accelerated mineralisation.

MEPE immunoreactivity is seen both intracellularly, on the wall of osteocyte lacunae and in the cell processes. Here in both the control groups (Figure 9 C&D, 1st & 3rd panel) , we can see there is strong MEPE expression whereas the PTH treatment groups the MEPE expression is lost (Figure 9 C&D, 1st & 4th panel) . This suggesting that bone mineralization is improved in the PTH treated group than the controls. (Figure 9 C& D). (Rowe et al., 2004)

CHAPTER VI

SUMMARY AND CONCLUSION

OVX (Ovariectomized rat models) can be used as an osteoporotic model to study the changes in alveolar bone, where the bone mass density, bone volume, and bone microarchitecture are decreased significantly.

The role of PTH in osteoporosis is only effective in small, intermittent doses where is produces an anabolic effect. This effect helps in formation and differentiation of osteoblasts from the pre-osteoblasts and promotes survival. (Figure 10)

On one hand the OVX model showed decrease in osteon formation, decrease in osteocytes, reduced BMD, reduced BMC, decreased bone volume and a heterogenous and haphazard formation of collagen fibers. The underlying molecular mechanism is reduced expression of bone forming proteins such as DMP1 while over expression of inhibitors of bone formation such as MEPE (Figure 10).

PTH treatment in the osteoporotic models rescues the lost quantitative and qualitative characteristics of alveolar bone, with improved bone quality, restore quantity and improved alveolar bone microarchitecture. The osteon-like structures which serves are well formed and increased in number along with increase in osteocytes. There is overall resistance to bone resorbing factors while inducing new bone formation with improved mineral content and bone mineral density. Together all these mechanisms lead to anabolic effect in chronic diseases such as osteoporosis (Figure 10).

This study model (Figure 10) can be used as a basis for understanding the role of PTH treatment in compromised osteoporotic alveolar bone microarchitecture in periodontal health and disease and can serve as a setting stone in exploring various implications of PTH treatment in various bone pathologies such as ankylosis of the tooth, metabolic bone diseases of the alveolar bone and even efficient osseointegration in implant placement in the alveolar bone of osteoporotic patients.

This is the first study to highlight the architectural and molecular changes with PTH treatment in alveolar bone and serves as a platform to further expand the scope of treating various chronic debilitating diseases of the alveolar bone.

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APPENDIX A

FIGURES

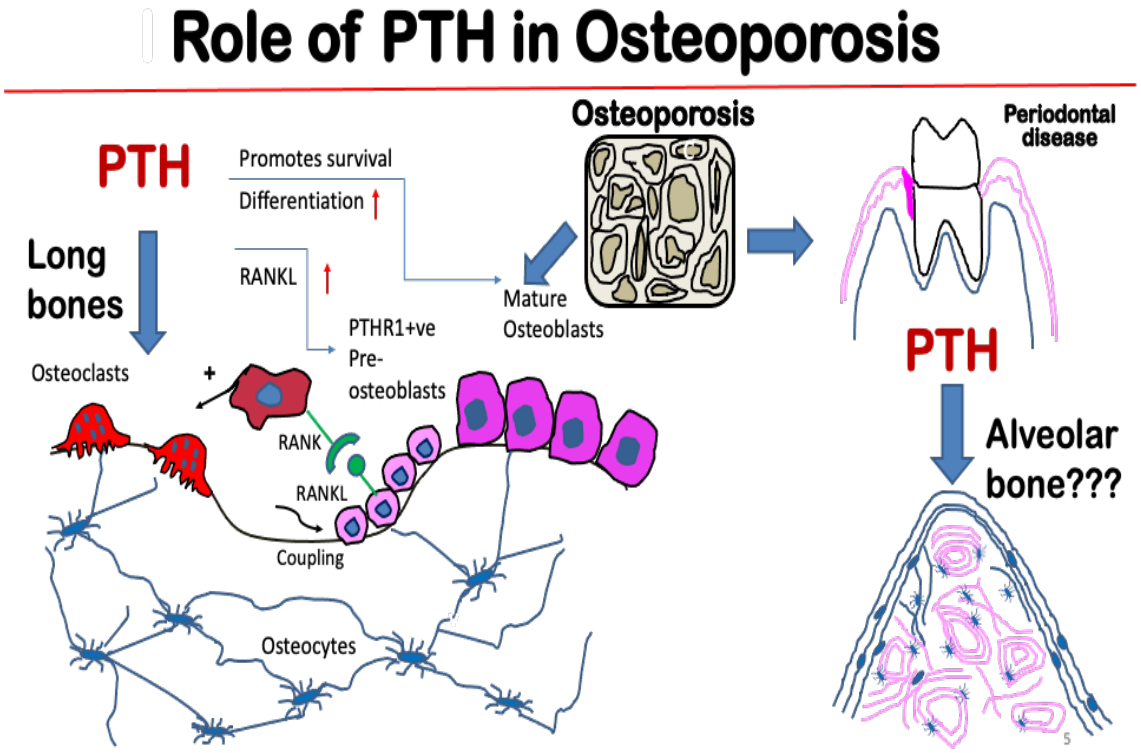

Figure 1: Schematic representation of the proposed mechanism of the osteoanabolic role of PTH

Under normal circumstances, when the calcium level in the body is low, the PTH has a catabolic effect on the bone. It causes RANKL activation and activation of osteoclasts which then resorb the bone and cause release of calcium from the bone into the blood to maintain its level. However, recently the anabolic effects of PTH in a low intermittent dose have been described, where the PTH is able to help the differentiation and survival of pre-osteoblasts into osteoblasts. PTH promotes survival, increased differentiation of the pre-osteoblasts into mature osteoblasts and thus increases bone formation. This anabolic effect of PTH is mostly attributed to a low dose in an intermittent pattern and is an established therapeutic modality for treating severe osteoporosis, especially in women who do not respond to anti-resorptives. On the other hand, chronic periodontitis is a chronic bone-resorbing disease that share some common risk factors with osteoporosis. Many studies have shown an association between the two. However, the underlying molecular mechanism of PTH in the alveolar bone of an ovariectomized rat have not been elucidated, most in the maxillary alveolar area, especially where the bone is more porous than any other place. Also the mechanism of association and PTH treatment in the context of various chronic diseases of the maxillary alveolar bone such as periodontal disease have not been elucidated.

Figure 2: PTH leads to increase in bone mineral density of alveolar bone of WT rats

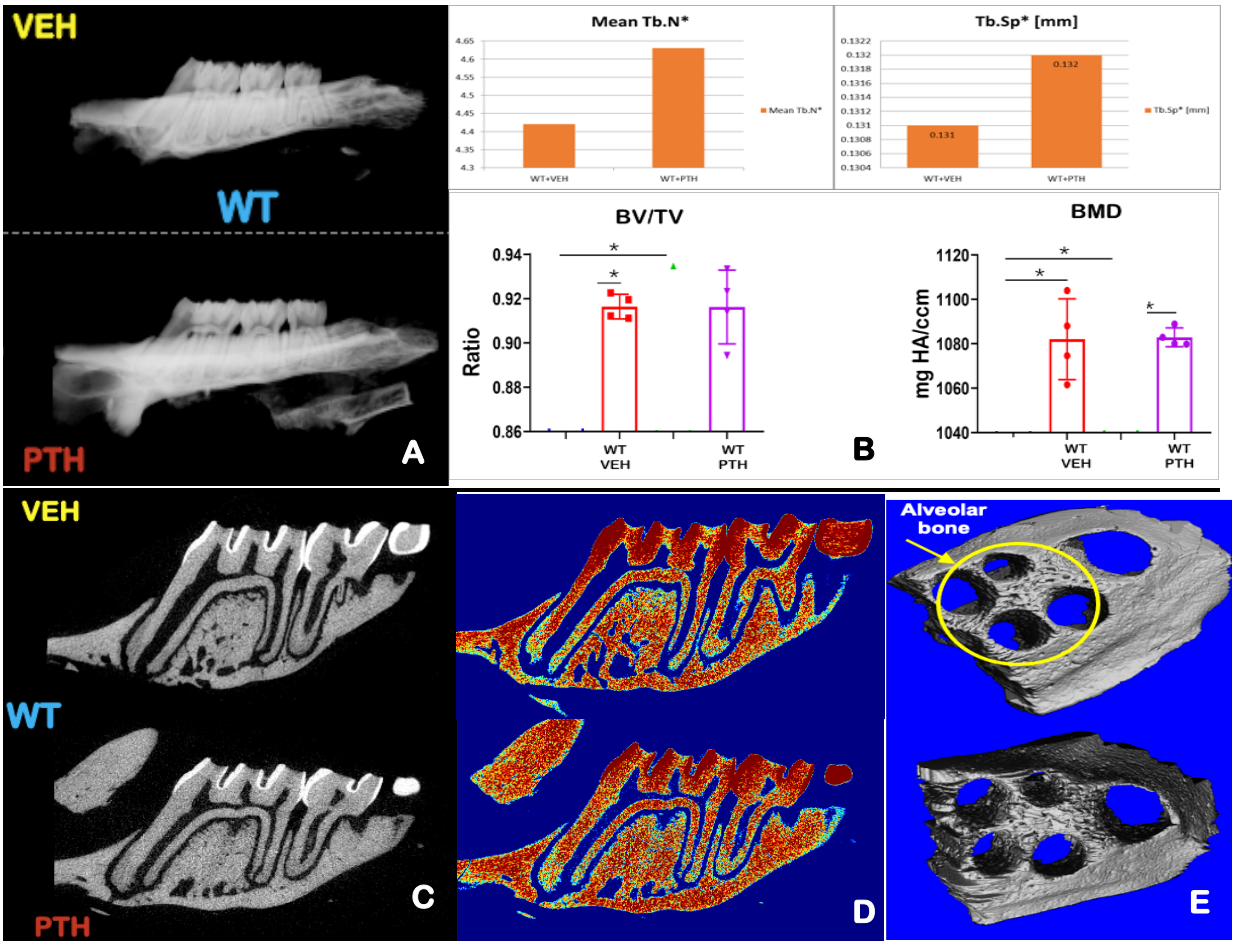

Fig 2: (A) Simple X-ray imaging of the cross section of the first maxillary molars show an increase in the radiodensity of the PTH treated rats in the WT group. (B) Statistical analysis showing the significant difference in BMD and BV/TV using . (C) The μ-CT image of the maxillary first molar shows more mineral density in the PTH treated group which is shown with a slight intense red heat map. Red intensity means more mineralized matrix in heat map analysis. (D) Cross section micro CT 3D image of the molar region of the maxilla. (E) 3D image of the trabecular bone microarchitecture showing the alveolar bone of the WT group and PTH treated group

Figure 3: PTH leads to a significant increase in bone mineral density of alveolar bone of OVX rats

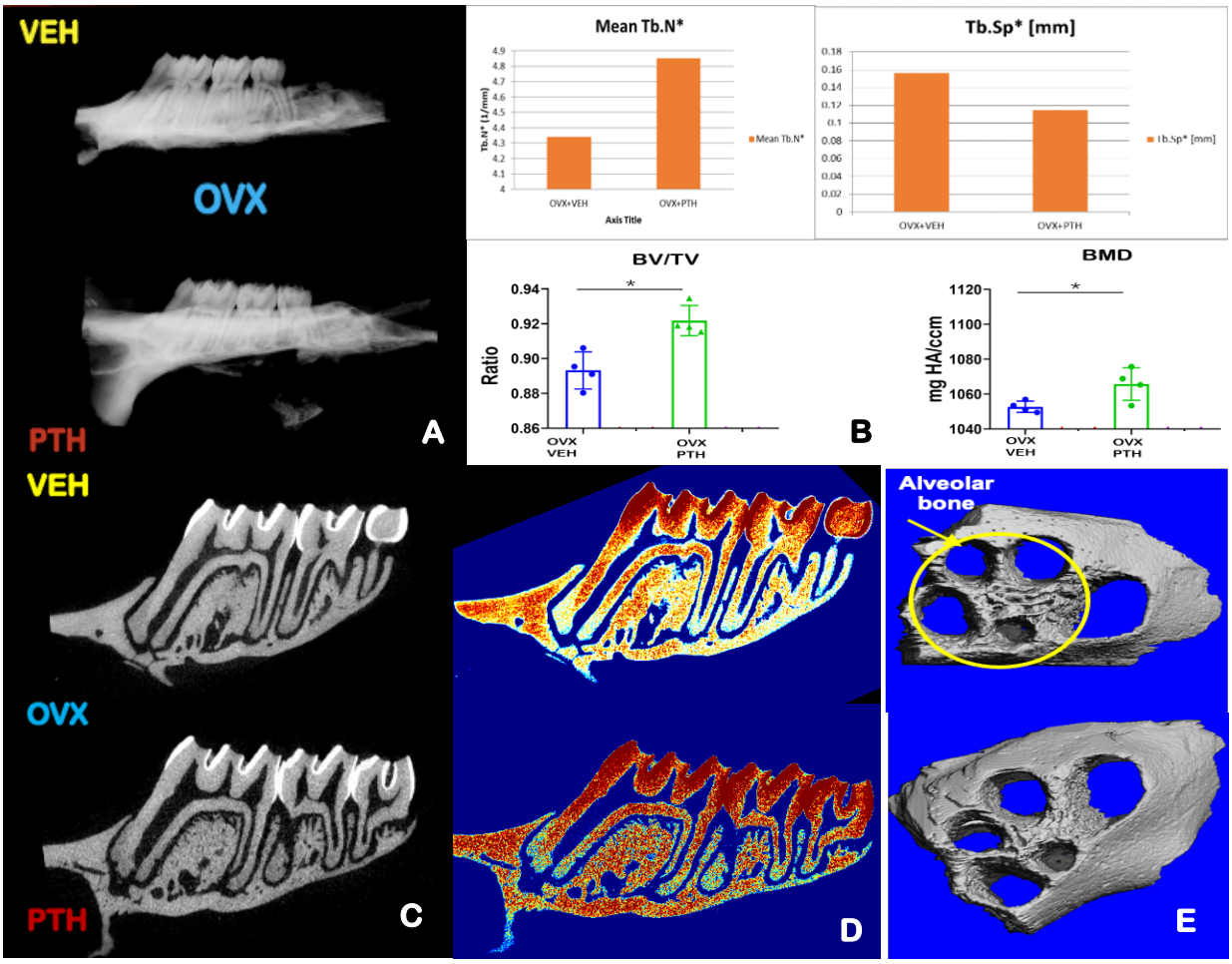

Fig 3:(A) Simple X-ray imaging of the cross section of the first maxillary molars show an increase in the radiodensity of the PTH-OVX group. This points towards increased mineral density. (B) Statistical analysis shows an overall increase in both the bone volume and the bone mass density (BMD). Mean trabecular number show a significant increase whereas the trabecular space also show a significant decrease in case of PTH-OVX rats. (D) The u-CT image of the maxillary first molar shows more mineral density in the PTH treated OVX group. (E) There is a significant intensity of red signal in the heat map data. Red intensity means more mineralized matrix in heat map analysis. In this image the mineral density in PTH-OVX group is intense red as compared to the non-treated VEH-OVX group

Figure 4: PTH treatment results in increased active bone deposition

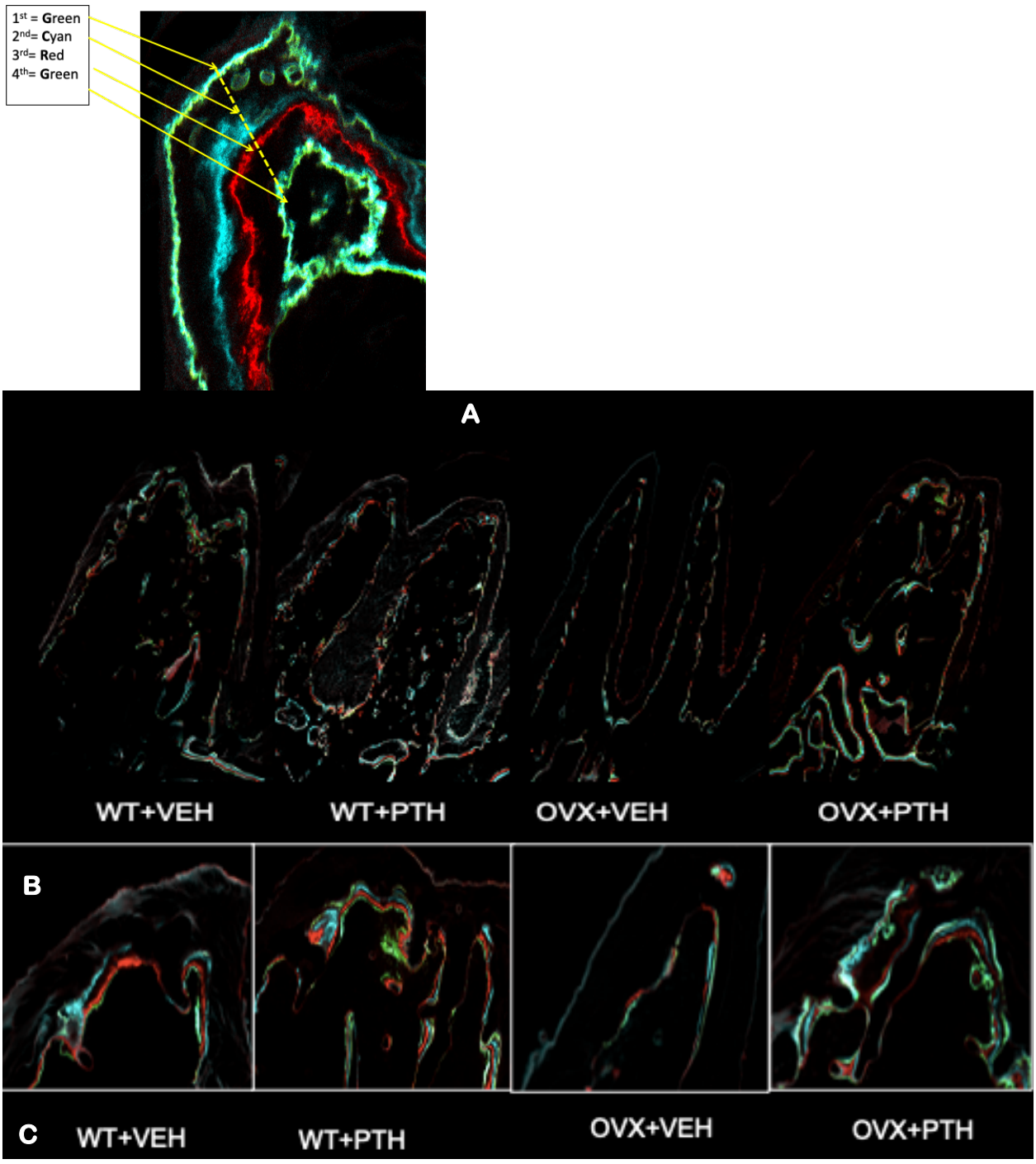

Fig 4: Confocal microscopy using the triple labeling. (A) Various layers of the labeling index showing the labeling dye used. (B) Low magnification showing active mineral deposit in the OVX-PTH. (C) Higher magnification of the same

Figure 5: PTH increases bone matrix and increases resistance to acid treatment

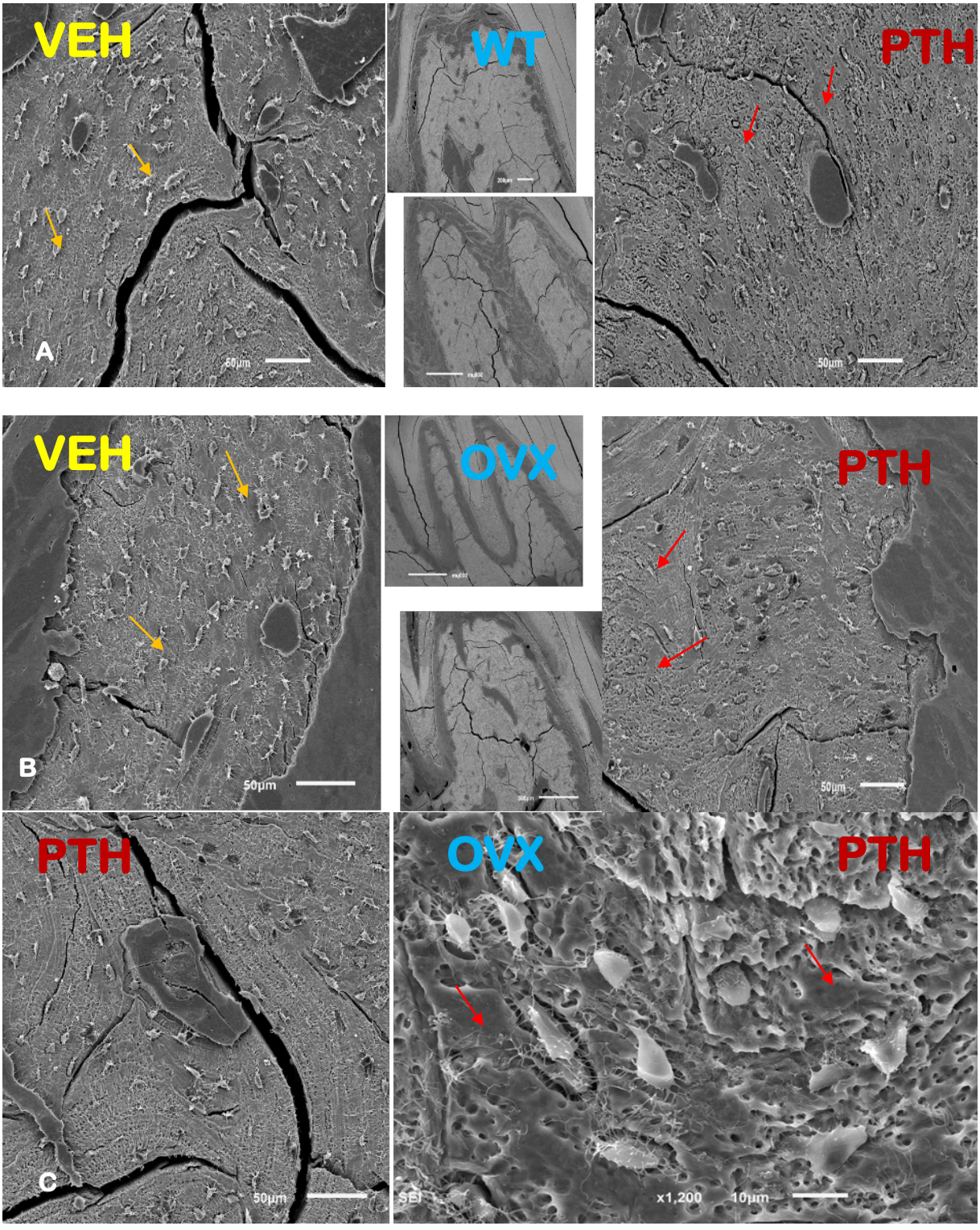

Fig 5: SEM analysis of the alveolar bone at the first molar region. (A) Low power magnification of the alveolar bone after 15 s of acid treatment in the WT group x500. (B) Low power magnification of the alveolar bone after 15 s of acid treatment in the OVX group x500 (C) Higher magnification of the OVX group with VEH and PTH treatment. Right panel shows magnified view of the osteocytes with their cell processes and the larger areas of mineral matrix of the bone that is resistant to acid treatment in the PTH-OVX group

Figure 6: PTH treatment has no effect on the cortical bone area of OVX rats

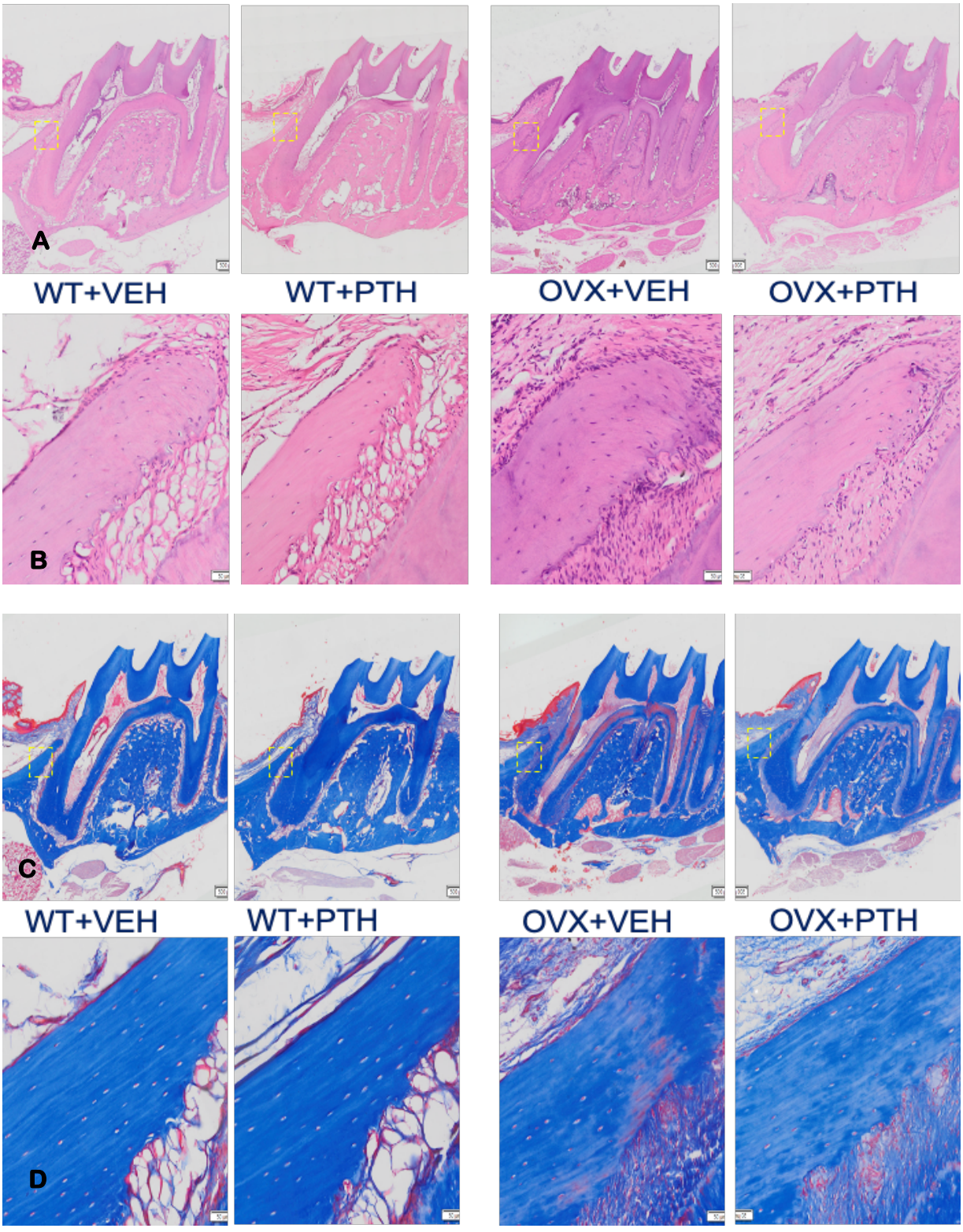

Fig 6: HE and Masson's trichome staining of the cortical bone area. (A) Low magnification of the HE staining of the cortical region of the alveolar bone region x100. (B) High power magnification HE staining of the cortical region of the alveolar bone region x400 (C) Low magnification of the Masson trichome staining of the alveolar bone region x100. (D) High power magnification of the Masson's staining in the cortical region of alveolar bone after x400

Figure 7: PTH treatment improves the osteon-like structures in alveolar bone of OVX rats

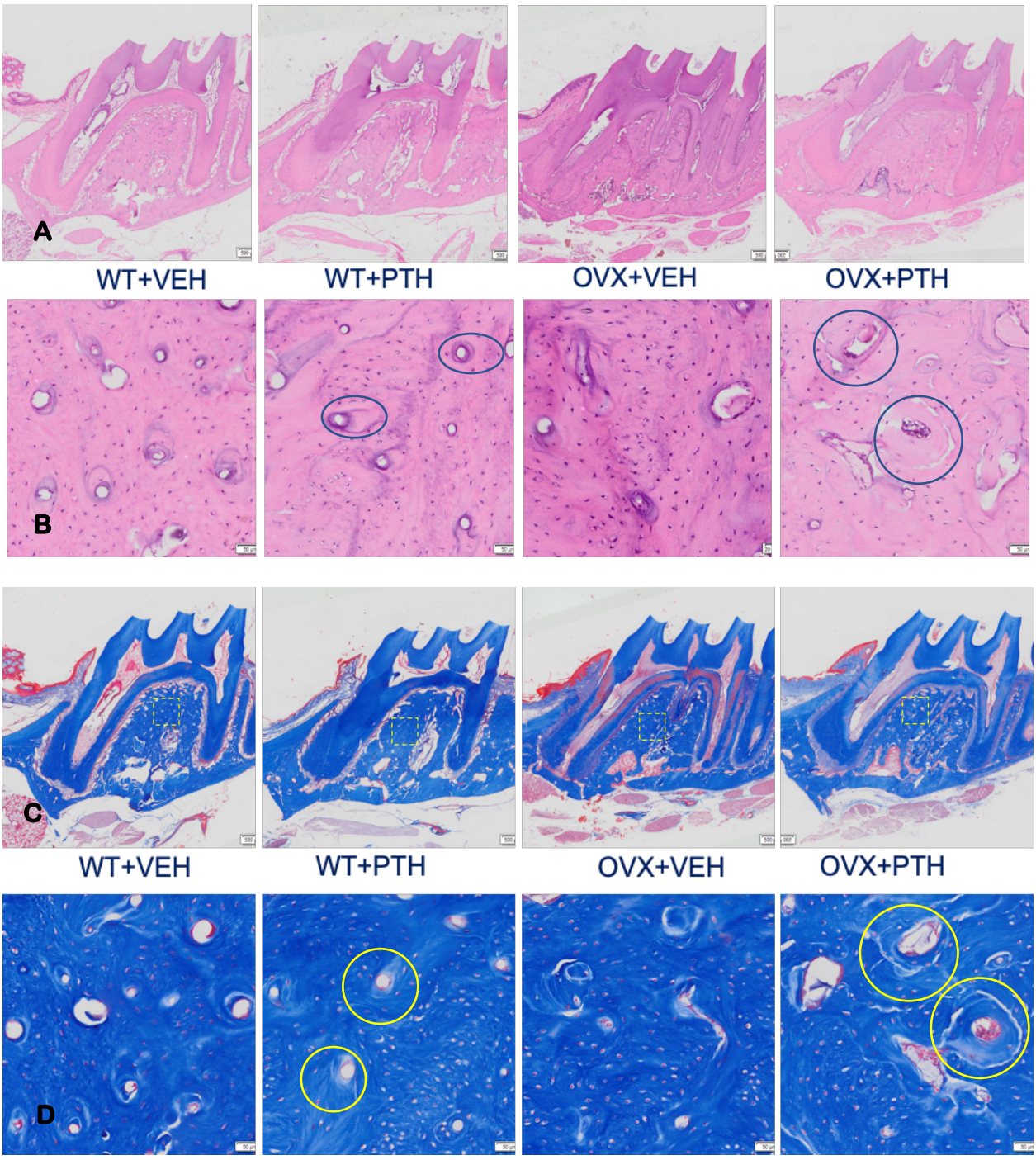

Fig 7: HE and Masson's trichome staining of the alveolar bone area. (A) Low magnification of the HE staining of the alveolar bone region x100. (B) High power magnification HE staining of the of the alveolar bone region x400 (C) Low magnification of the Masson trichome staining of the alveolar bone region x100. (D) High power magnification of the Masson's staining of the alveolar bone after x400

Figure 8: PTH treatment improves the collagen fibers, leads to more homogenous and thick bundles of collagen and well-formed osteon like structures of the alveolar bone structure

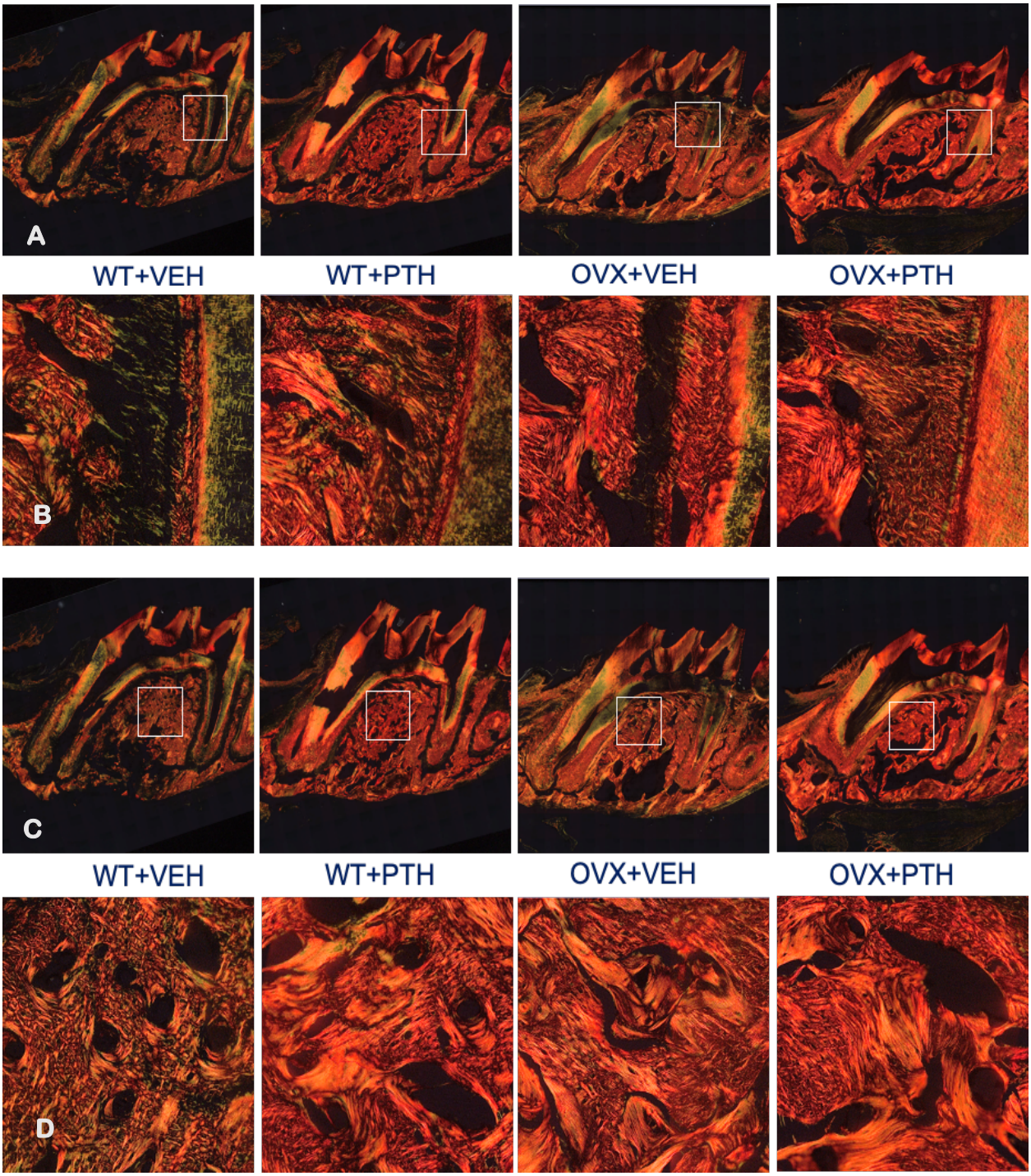

Fig 8: Picrosirius red staining with polarized light (A) Picrosirius red staining of the cortical bone area. Low magnification of the PDL bone region x100. (B) High power magnification of the PDL region x400 (C) Picrosirius red staining of the alveolar bone area x100. (D) High power magnification of the Picrosirius red staining in the alveolar bone area x400

Figure 9: PTH leads to increase in osteocytes and DMP1 expression in rats whereas PTH leads to decreased MEPE expression in OVX rats

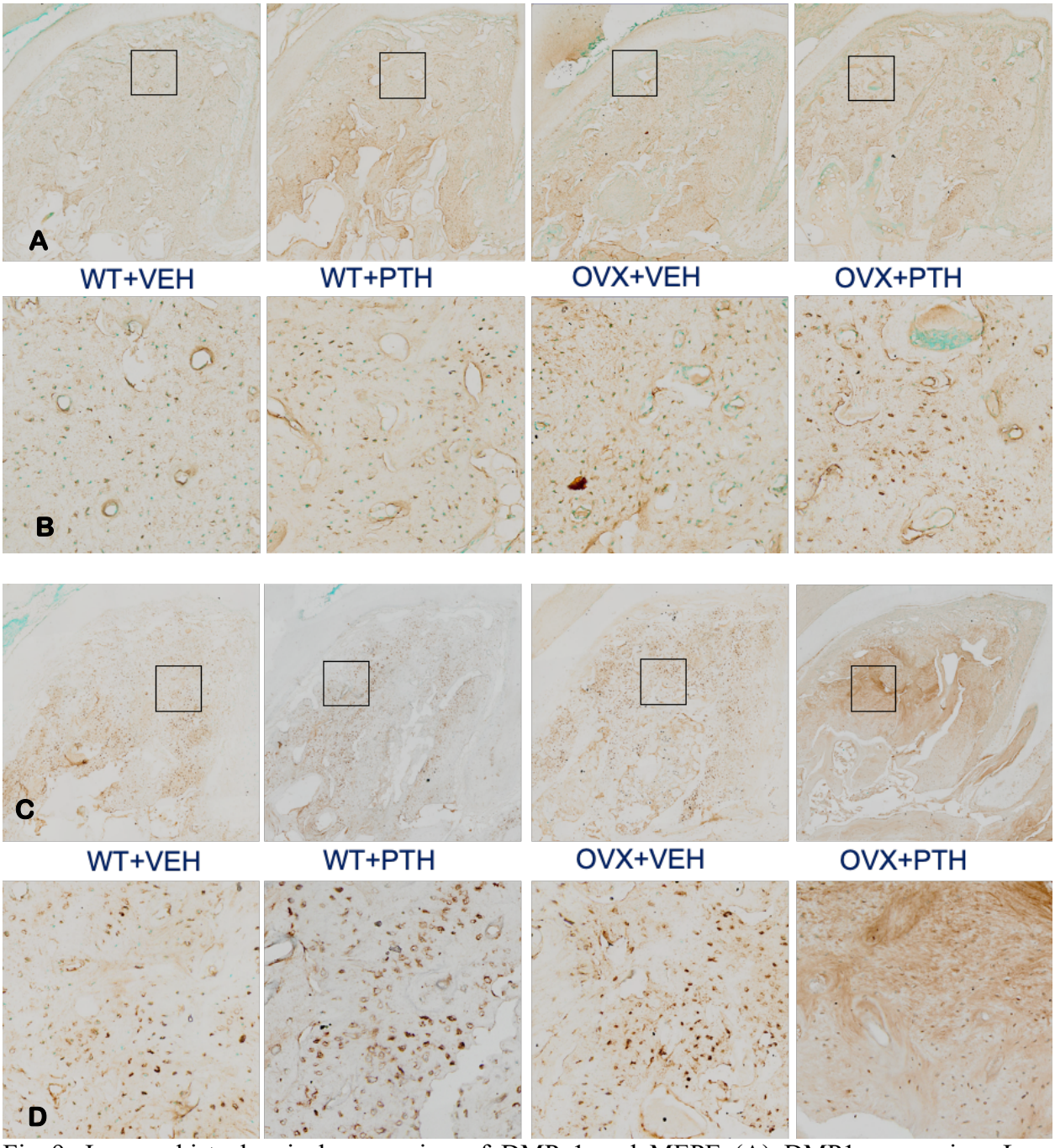

Fig 9: Immunohistochemical expression of DMP 1 and MEPE (A) DMP1 expression. Low magnification of the alveolar bone region x100. (B) DMP1 expression, High power magnification of the region x400 (C) MEPE expression. Low magnification of the alveolar bone region x100. (D) MEPE expression, High power magnification of the region x400

Summary:

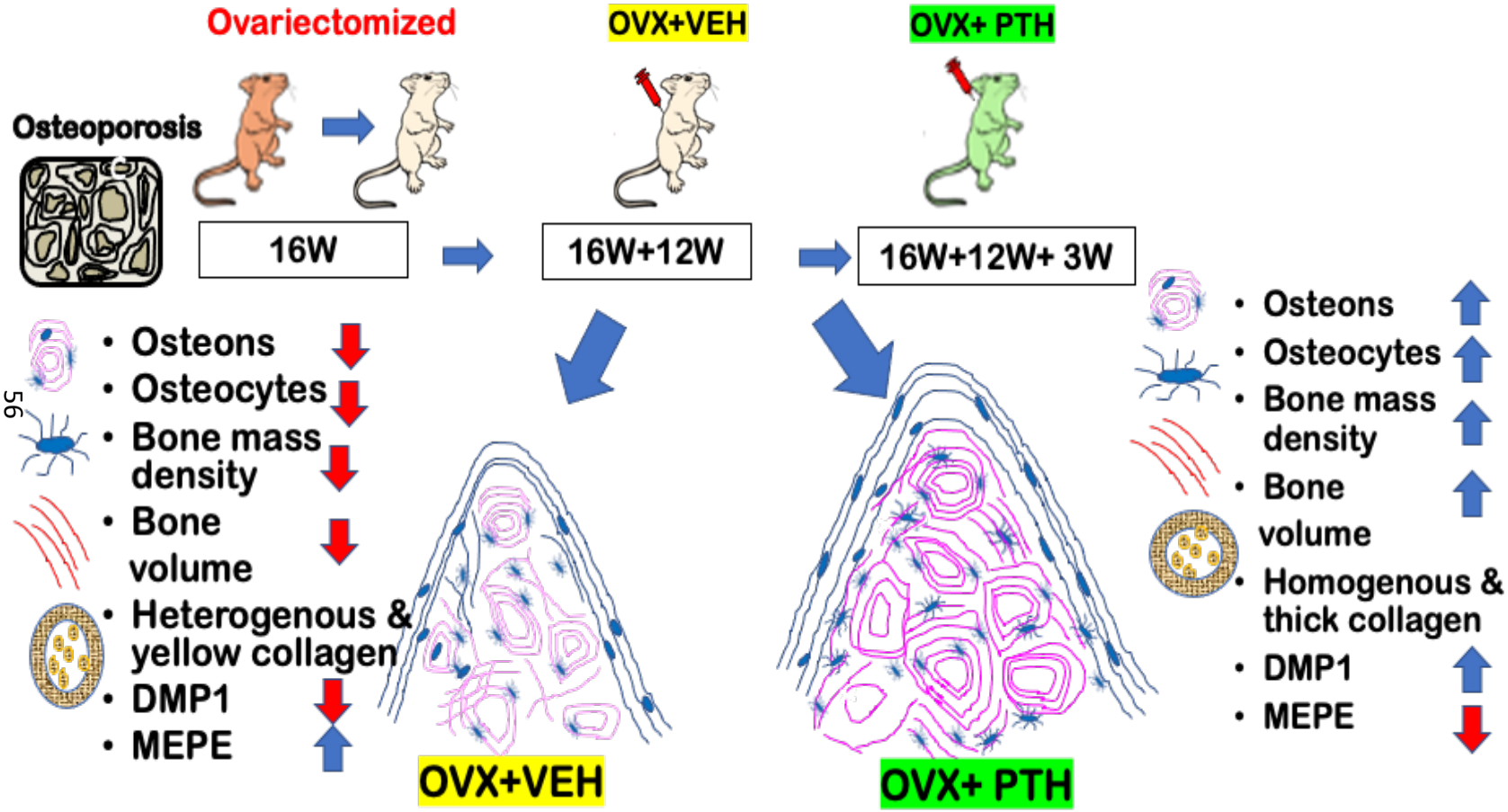
Ovariectomized
OVX+VEH
OVX+ PTH
Osteoporosis
16W
16W+12W
16W+12W+ 3W
• Osteons
• Osteocytes
• Bone mass density
• Bone volume
• Heterogenous & yellow collagen
• DMP1
• MEPE
OVX+VEH
OVX+ PTH
• Osteons
• Osteocytes
• Bone mass density
• Bone volume
• Homogenous & thick collagen
• DMP1
• MEPE

Figure 10: Schematic diagram showing the role of PTH in osteoporosis and periodontal disease

OVX (Ovariectomized rat models) can be used as an osteoporotic model to study the changes in alveolar bone, where the bone mass density, bone volume, and bone microarchitecture are decreased significantly. On one hand the OVX model showed decrease in osteon formation, decrease in osteocytes, reduced BMD, reduced BMC, decreased bone volume and a heterogenous and haphazard formation of collagen fibers. The underlying molecular mechanism is reduced expression of bone forming proteins such as DMP1 while over expression of inhibitors of bone formation such as MEPE. PTH treatment in the osteoporotic models rescues the lost quantitative and qualitative characteristics of alveolar bone, with improved bone quality, restore quantity and improved alveolar bone microarchitecture.