Effect of oxytocin receptor antagonist (GSK-221-149-A) on mandibular bone porosity in peri-menopausal rats

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SUMMARY

The period of peri-menopause (PMP) is characterized by hormonal fluctuations that impact the strength and health of bones. Oxytocin (OX), a small peptide known to be present in bone tissue, is the focus of this study. The objective of this research is to gain a better understanding of how OX precisely functions in the remodeling process of the mandibular bone. This understanding is seen as a crucial step in preventing the loss of both cortical and trabecular bone during the PMP. The current findings indicate that OX plays a role in preserving both compact and trabecular bone tissues, enhancing the mineral-to-matrix ratio, and regulating bone markers. Furthermore, it reduces porosity in both cortical and trabecular bone levels. Interestingly, these effects are reversed when an oxytocin receptor antagonist (GSK-221,149-A) is introduced, suggesting that OX's bone-preserving action is primarily mediated through the oxytocin receptor, rather than other mechanisms.

Key words: GSK-221-149-A – Mandibular bone – Estradiol – Peri-menopausal – Oxytocin

INTRODUCTION

The peri-menopause period (PMP) is associated with sex hormone fluctuations affecting strength of skeletal system in females (Marongiu, 2019). The decline in estradiol (E2) levels has a noticeable impact on the activity of bone cells, leading to an overactive state of osteoclasts coupled with reduced activity of osteoblasts (Allan et al., 2010; Nordstrom et al., 2015). This imbalance results in higher bone resorption, which manifests as an increased occurrence of bone fractures. Research findings indicate that the prevalence of hip bone fractures rises significantly in females aged over 50, with up to a 20% increase when compared to middle-aged females (Kim et al., 2020). Since the cortical part of the bone contributes more significantly to its strength than the trabecular part

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(Naqvi et al., 2020), the higher rate of bone fractures with age can be attributed to the diminished presence of orthophosphate ions [(PO4)3-] and carbonate [H2CO3] within the bone cortex. This leads to alterations in the microstructure and thickness of the bone (Akkus et al., 2004; Ahmed et al., 2015; Long et al., 2020; Fañanas-Baquero et al., 2021).

Oxytocin (OX) receptors have been detected in various bone cells, including osteoclasts and osteoblasts (Cheng et al., 2020), and studies have shown that OX can enhance the anabolic activity of these bone cells (Colicci et al., 2002; Tamma et al., 2009; Abbasi et al., 2019). In females during the peri-menopausal period (PMP), there is typically a decrease in estrogen plasma levels, which is reflected in reduced OX levels and the down-regulation of its receptor (Breuil et al., 2011; Colaianni et al., 2014a). There is a clear need to develop alternative therapies that can effectively reduce the prevalence of osteopenia or osteoporosis in females during the PMP (Ferreira et al., 2015). In albino Wistar rats, the peri-menopausal phase, characterized by lower estrogen plasma levels, typically occurs around twenty months of age (Nicola et al., 2016). The primary objective of the current study is to investigate the precise mechanism through which OX operates in the remodeling of the mandibular bone. This research aims to take a crucial step toward preventing the loss of cortical and trabecular bone during the PMP.

MATERIALS AND METHODS

Chemicals and animals

Unless stated otherwise, chemicals and kits were sourced from Sigma-Aldrich (USA). In terms of the animals used, thirty female albino Wistar rats aged twenty months were employed. These rats were individually housed and provided with an ample supply of food and water, maintaining a room temperature of 25°C. Following the protocol outlined by Marcondes et al. (2002), vaginal secretions were examined daily for a period of two weeks. Only rats displaying peri-estropause phases were included in the present research project. The study was conducted in accordance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

Experimental design

The animals were given a two-week period to adapt, after which they were evenly distributed into three groups. Control group (C-group) was treated with normal saline (0.9% NaCl) (1 ml / kg bw / day), intraperitoneal injection (i.p.) only for two days. Oxytocin group (OX-group) was treated with OX (200 µg / Kg bw / day), i.p., only for two days. Retosiban (GSK-221,149-A) group (RS-group), received RS by oral gavage (5 mg / Kg bw / day) for two days. After four weeks, animals were euthanized by injecting sodium pentobarbital i.p., then samples of blood were centrifuged for 20 minutes (10 x 1000 rpm). Plasma was gathered for subsequent analysis. The mandibles were dissected and divided in half using a large pair of scissors. The right half was preserved in saline at -20°C for additional investigations, while the left half was immersed in formalin for fixation.

Histopathological and immunohistochemistry examinations

In accordance with protocol described by Tucker et al. (2016), the mandibular neck was cut into 1cm3 cubes and fixed in 10% formalin (48 hours). Tissue sections (5 µm) were processed and stained with hematoxylin and eosin (H&E) (Tucker et al., 2016). Histopathologists unaware to the aim of our study were consulted for examinations. Non-overlapping fields were analyzed using the software ImageJ 1.24.

Immunohistochemistry was done following the protocol of Ervolino, et al. (2019). Diluted 1ry antibody were used [Goat anti-osterix (Osx) (1:1000), rabbit anti-runt-related transcription factor 2 (RUNX-2) (1:10000), rabbit anti- periostin (PER) (1:10000), goat anti-osteopontin (OPN) (1:100000), goat anti-osteocalcin (OCN) (1:200), rabbit anti-sclerostin (SOST) (1:10000), rabbit anti-bone morphogenetic protein (BMP) (1:200), and goat anti-tartrate-resistant acid phosphatase (TRAP) (1:200)]. Secondary antibodies were alkaline phophatase (Abcam, USA). Scoring was done in accordance with Stringhetta-Garcia et al. (2016) [Negative immunoreactivity (IR) = 0, mild IR = 1, moderate IR = 2 and sever IR = 3].

Bone tissue homogenate Western blotting (W.B.)

W.B. was used in order to estimate alkaline phosphatase (ALP) and TRAP in accordance with protocol described by Peres-Ueno et al. (2020). Briefly, one hundred milligrams of bone tissue were homogenate using Argos Flexi-fuge microcentrifuge (Stellar-scientific, USA) then centrifuged. Supernatants were blotted into polyvinylidene fluoride (PVDF) membranes (Merck, Germany) using Trans-Blot® electrophoretic transfer Cell (BioRad USA). 5% milk solution was used to block PVDF membranes, which were then incubated with the primary antibodies [anti-alkaline phosphatase (ALP) antibody (1:200) and anti-TRAP antibodies (1:400)] (Abcam, UK) for 12 hours. Subsequently, blots were incubated with horseradish peroxidase (HRP) secondary antibodies 1:5000 dilution (Abcam, UK) for 60 min. at 25°C. Bands were imaged using iBright™ Imaging Systems (Thermo-fisher, USA) and quantified were analyzed using the software ImageJ 1.24.

Raman microspectroscopy (RMSS), microtomography (micro-CT) and Dual-energy X-ray absorptiometry (DXA) examinations

Mineral composition of bone tissue was analyzed by chemical analysis technique (Raman microspectroscopy) (CRAIC Technologies, USA) following protocol described by Fernandes et al. (2020). Using SkyScan (Bruker, UK), microtomography examinations were performed (analysis was done for 20 slices in mandibular neck) following protocol described by Stringhetta-Garcia et al. (2017). Bone mineral density (BMD) was assessed by Sunlight MiniOmni™ (Beam-med, USA). The equipment was calibrated to small animals following manufacturer protocol.

Biomechanical compression bending (BCB) examinations

EMIC DL3000 (Instron, Brazil) was used to evaluate of the biomechanical characteristics of the mandible. The mandibular ramus was placed vertically. Compression was applied to mandibular head to evaluate bone stiffness.

Statistical analysis

Data analysis was performed using Statistical Package for Social Sciences (SPSS) software, version 20 (SPSS Inc., USA). To validate the statistical significance of differences between groups, a one-way analysis of variance (ANOVA) was conducted. Post hoc Tukey-Kramer testing was employed for comparing groups. The data were presented as mean ± standard deviation, and a probability value was deemed significant if it was lower than 0.05.

RESULTS

Retosiban and oxytocin administration effect on mandibular neck histopathological and immunohistochemical changes in peri-estropause rats

C-group and OX-group showed healthy tissue. Compact bone was organized as parallel condensed lamellae with minimal thinning surrounding a central trabecular bone area which was formed of branching and anastomosing irregular trabeculae rich in blood vessels. RS-group showed reduction of the bone matrix with an obvious thinning of compact bone in addition to perforations and disconnection of trabecular bone trabeculae (Fig. 1 A-C).

A significant increase of compact bone tissue area in addition to a non-significant change of trabecular bone areas were noticed in OX-group as compared to the control group, while RS-group showed a significant decrease of both compact and trabecular bone areas (by 27% and 50% respectively) as compared to the control (Fig. 1D).

By examination of either compact (Fig. 2) or trabecular (Fig. 3) bone sections stained with anti-Osx, anti-RUNX-2 (plays a cell proliferation regulatory role in cell cycle entry and exit in osteoblasts), anti-PER (member of the matricellular protein family) and anti-BMP antibodies (plays important roles in a wide array of processes during formation and maintenance of bone); OX-group and RS-group showed strong and negative reactions respectively, while C-group showed weak reaction. In bone sections stained with anti-OPN, anti-OCN, anti-SOST and anti-TRAP antibodies, OX-group and RS-group showed weak and strong reactions respectively, while C-group showed weak reaction. Statistical analysis showed that OX-group and RS-group showed significant increase and decrease respectively of Osx, RUNX-2, PER and BMP bone immunoreactivity compared to the control values. OX-group

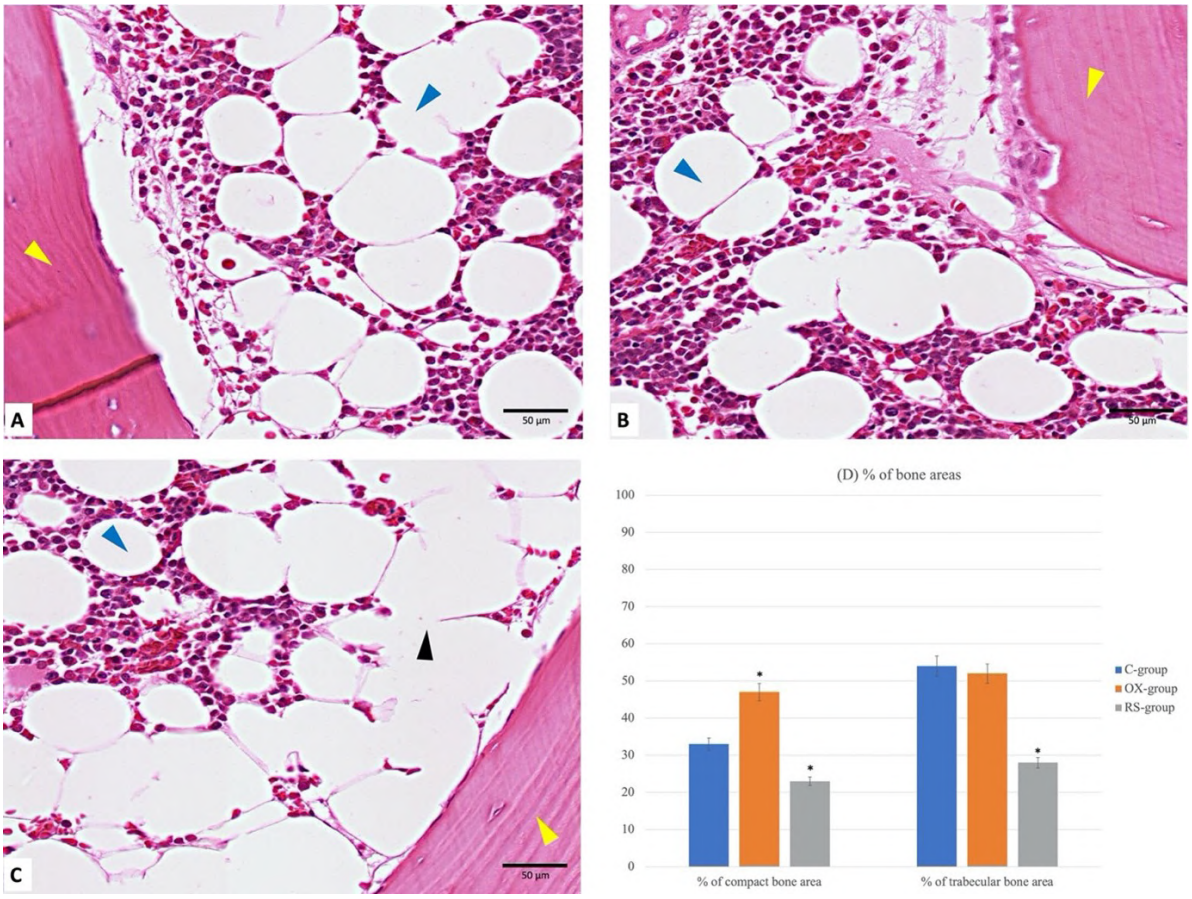

Fig. 1.- (A-C) Mandibular neck tissue stained with hematoxylin and eosin (X 1000), (n=20). Scale bars = 50 µm. C-group (A) and OX-group (B) showed normal bone histological architecture. Compact bone was organized as parallel condensed lamellae with minimal thinning surrounding a central trabecular bone area which is formed of branching and anastomosing irregular trabeculae rich in blood vessels. RS-group (C) showed reduction of the bone matrix with an obvious thinning of compact bone in addition to perforations and disconnection of trabecular bone trabeculae. (Note: Yellow arrow = compact bone, Blue arrow = trabecular bone, Black arrow = disconnected trabeculae). **(D)** Represents % of compact and trabecular bone areas. * Significant ($p < 0.05$) difference in comparison to C-group. Data are expressed in mean ± standard deviation and probability value is considered significant if <0.05, (n=20).

showed non-significant difference of OPN, OCN, SOST and TRAP bone immunoreactivity. A significant increase of bone immunoreactivity was noticed in RS-group compared to C-group (Fig. 4 A-B).

Retosiban and oxytocin administration effect on mandibular bone turnover markers in peri-estropause rats

A significant increase of ALP activity in addition to a significant decrease of TRAP activity were recorded in OX-group as compared to the control values. RS-group showed a significant decrease of ALP activity in addition to a significant increase of TRAP activity as compared to C-group (Fig. 5 A-C).

Retosiban and oxytocin administration effect on mineral / matrix ratio in peri-estropause rats

OX-group presented a significant increase of bone-mineral/matrix ratio in addition to a significant drop of B-type carbonate as compared to the control values. RS-group showed a significant drop of both bone-mineral/matrix ratio and crystallinity, in addition to a significant rise of B-type carbonate values as compared to the control values (Fig. 6 A-C).

Retosiban and oxytocin administration effect on cortical bone area and % of trabecular bone volume in peri-estropause rats

OX-group presented a significant increase of the area of cortical bone and % of trabecular bone volume with a significant drop of % of cortical porosity and number of trabeculae as compared to control values. On the other hand, RS-group showed a significant decrease of % of trabecular bone volume and cortical bone area in addition to a significant rise % of cortical porosity as compared to control values (Fig. 7 A-D).

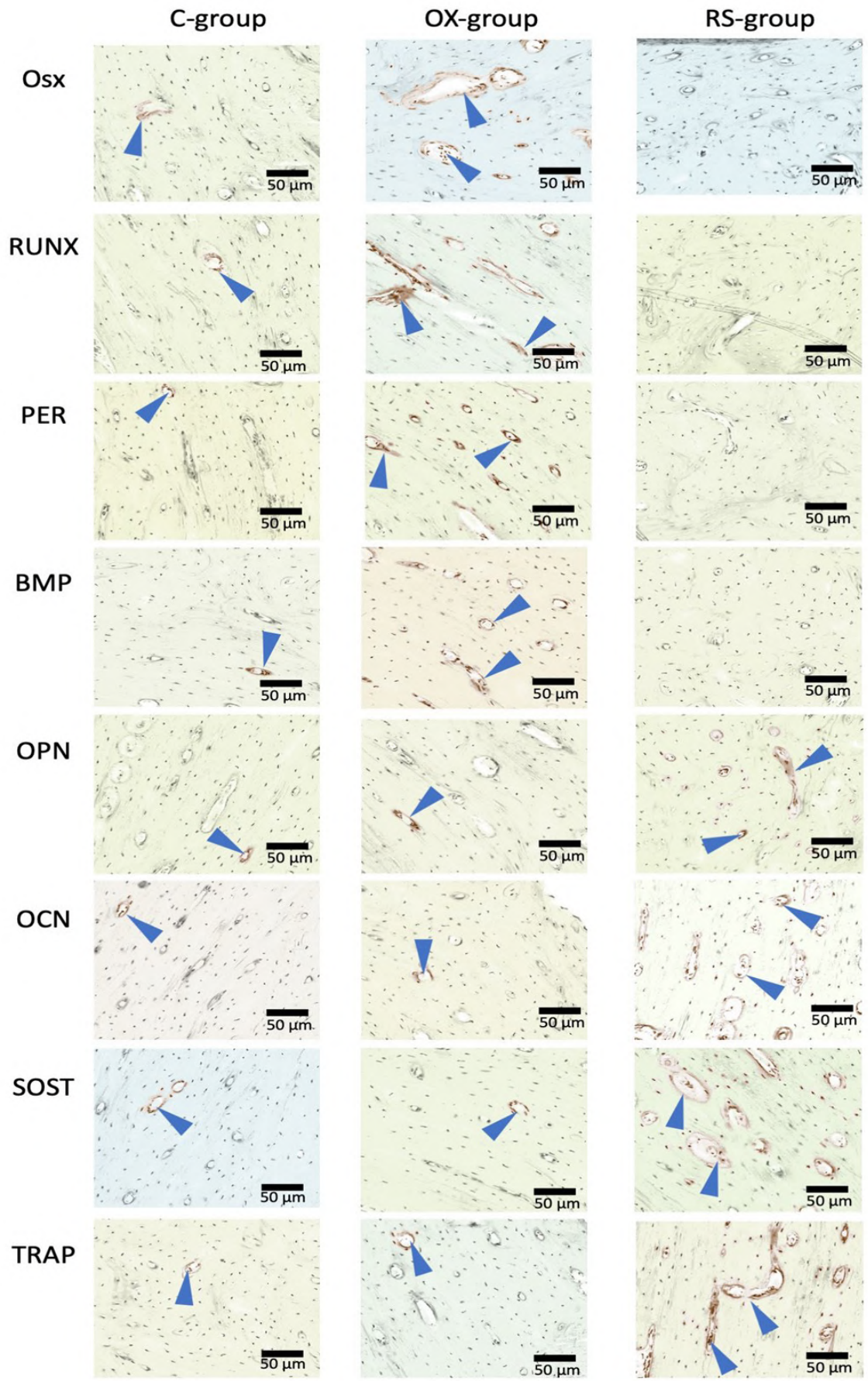

Fig. 2.- Mandibular neck compact bone stained immunohistochemically (X 1000), (n=20). Scale bars = 50 µm. By examination of compact bone sections stained with anti-Osx, anti-RUNX-2, anti-PER and anti-BMP antibodies; OX-group and RS-group showed strong and negative reactions respectively while C-group showed weak reaction. By examining compact bone sections stained with anti-OPN, anti-OCN, anti-SOST and anti-TRAP antibodies; OX-group and RS-group showed weak and strong reactions respectively while C-group showed weak reaction (Note: Immune-positive areas are labelled with blue arrows).

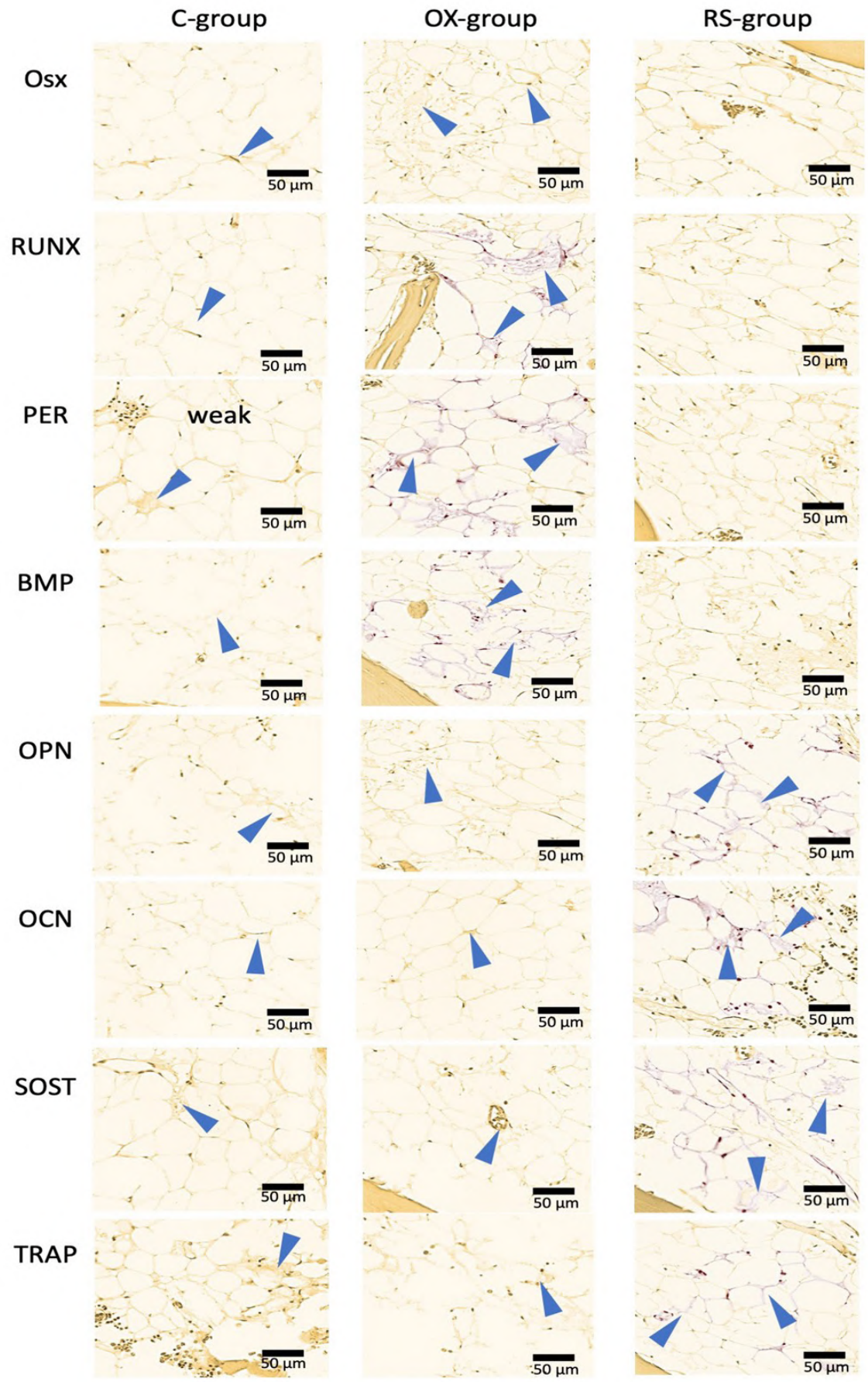

Fig. 3.- Mandibular neck trabecular bone stained immunohistochemically (X 1000), (n=20). Scale bars = 50 µm. By examination of trabecular bone sections stained with anti-Osx, anti-RUNX-2, anti-PER and anti-BMP antibodies; OX-group and RS-group showed strong and negative reactions respectively while C-group showed weak reaction. By examining of trabecular bone sections stained with anti-OPN, anti-OCN, anti-SOST and anti-TRAP antibodies; OX-group and RS-group showed weak and strong reactions respectively while C-group showed weak reaction (Note: Immune-positive areas are labelled with blue arrows).

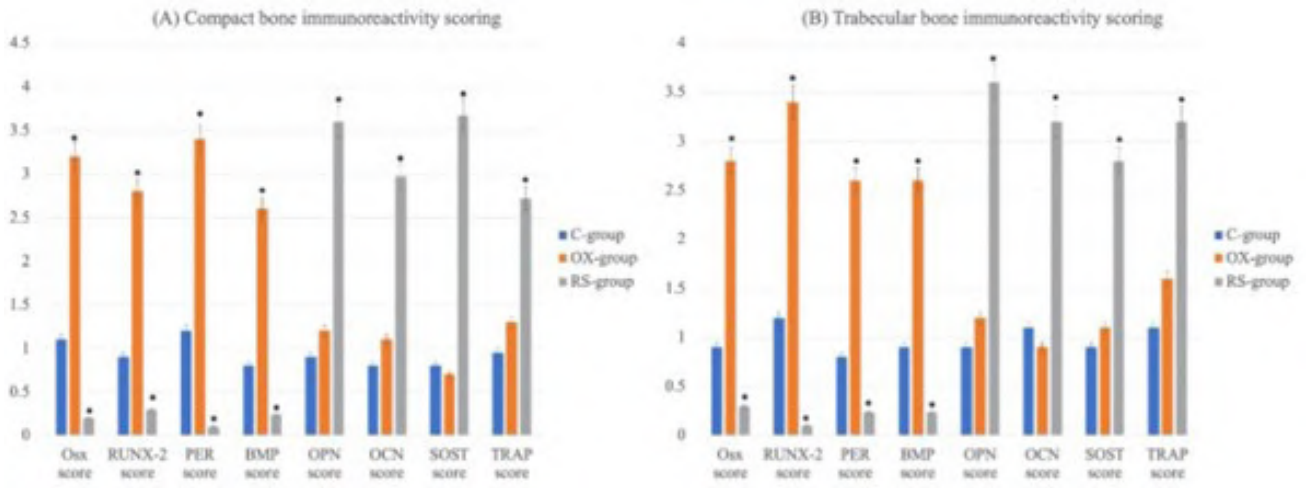

Fig. 4.- Effect of retosiban and oxytocin administration on mandibular neck compact **(A)** and trabecular **(B)** bone immunoreactivity in peri-estropause rats. Statistical analysis showed that OX-group and RS-group showed significant ($p < 0.05$) increase and decrease (of Osx, RUNX-2, PER and BMP bone immunoreactivity) respectively as compared to C-group. OX-group showed non-significant difference (of OPN, OCN, SOST and TRAP bone immunoreactivity) as compared to C-group, while RS-group showed significant ($p < 0.05$) increase (of bone immunoreactivity) as compared to C-group. * Significant ($p < 0.05$) difference in comparison to C-group. Data are expressed in mean ± standard deviation and probability value is considered significant if <0.05, (n=20).

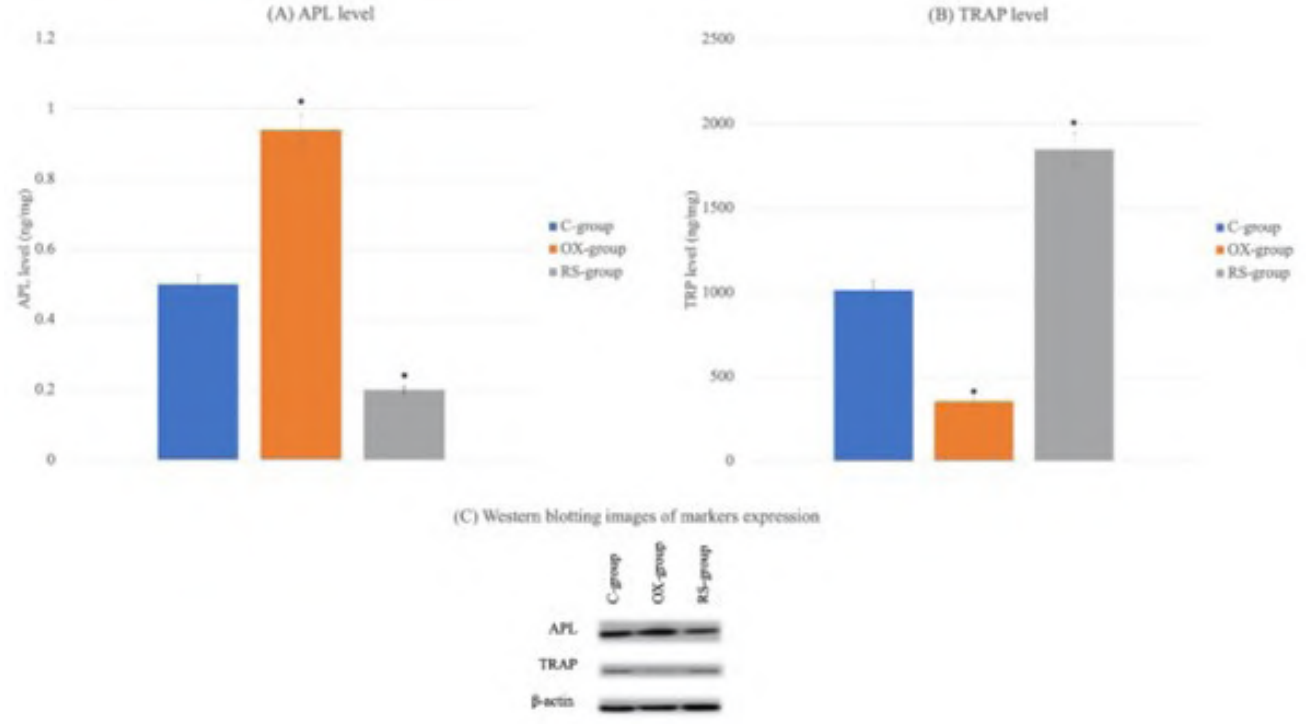

Fig. 5.- Effect of retosiban and oxytocin administration on mandibular bone APL **(A)** and TRAP **(B)** levels in peri-estropause rats. OX-group showed a significant increase of ALP activity in addition to a significant decrease of TRAP activity as compared to C-group. RS-group showed a significant decrease of ALP activity in addition to a significant increase of TRAP activity as compared to C-group. **(C)** Represents Western blot images of markers expression. * Significant ($p < 0.05$) difference in comparison to C-group. Data are expressed in mean ± standard deviation and probability value is considered significant if <0.05, (n=20).

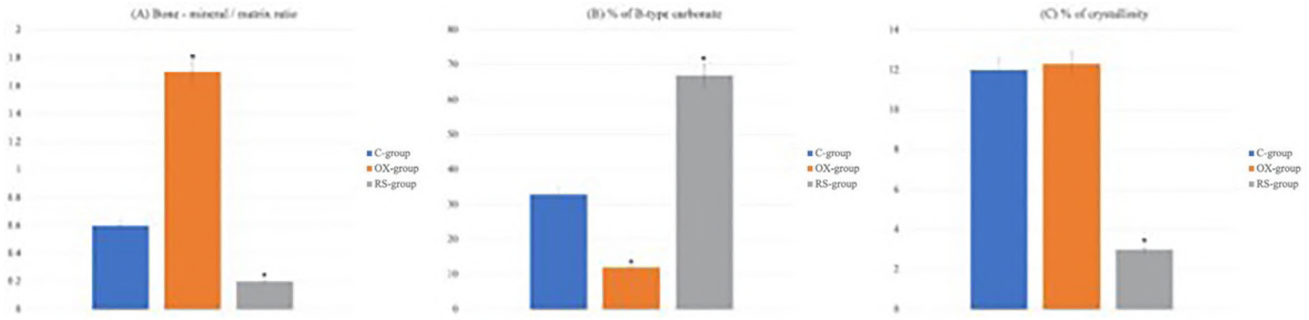

Fig. 6.- Effect of retosiban and oxytocin administration on mineral / matrix ratio in peri-estropause rats. OX-group showed a significant ($p < 0.05$) increase of bone - mineral / matrix ratio **(A)** in addition to a significant ($p < 0.05$) decrease of B-type carbonate **(B)** with non-significant change of crystallinity **(C)** as compared to C-group. On the other hand, RS-group showed a significant ($p < 0.05$) decrease of both bone - mineral / matrix ratio and crystallinity in addition to a significant ($p < 0.05$) increase of B-type carbonate as compared to C-group. * Significant ($p < 0.05$) difference in comparison to C-group. Data are expressed in mean ± standard deviation and probability value is considered significant if <0.05, (n=20).

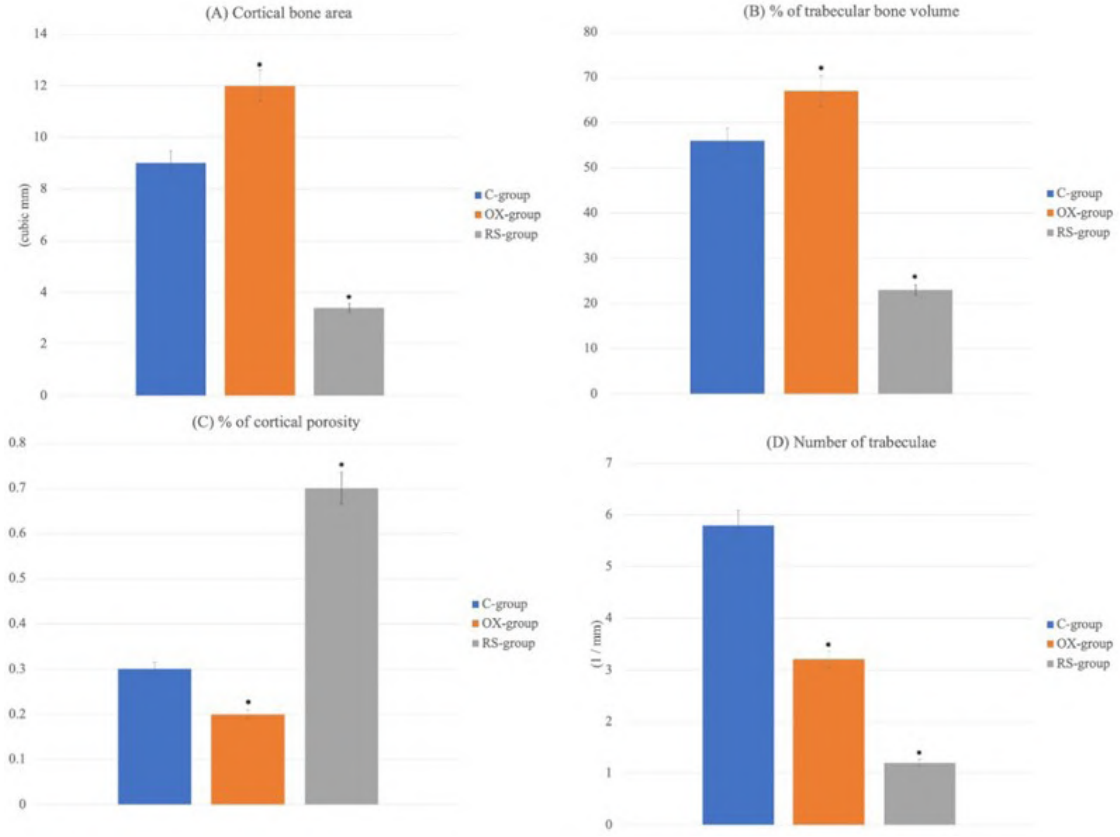

Fig. 7.- Effect of retosiban and oxytocin administration on cortical bone area and % of trabecular bone volume in peri-estropause rats. Microtomography results showed that; OX-group represents a significant ($p < 0.05$) increase of cortical bone area **(A)** and % of trabecular bone volume **(B)** in addition to a significant ($p < 0.05$) decrease of % of cortical porosity **(C)** and number of trabeculae **(D)** as compared to C-group. On the other hand, RS-group showed a significant ($p < 0.05$) decrease of cortical bone area and % of trabecular bone volume in addition to a significant ($p < 0.05$) increase % of cortical porosity as compared to C-group. * Significant ($p < 0.05$) difference in comparison to C-group. Data are expressed in mean ± standard deviation and probability value is considered significant if <0.05, (n=20).

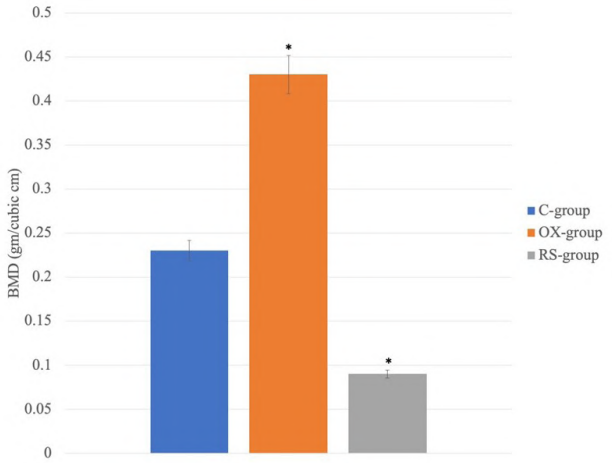

Fig. 8.- Effect of retosiban and oxytocin administration on BMD in peri-estropause rats. OX-group showed a significant ($p < 0.05$) increase of BMD as compared to C-group. On the other hand, RS-group showed a significant ($p < 0.05$) decrease of BMD as compared to C-group. * Significant ($p < 0.05$) difference in comparison to C-group. Data are expressed in mean ± standard deviation and probability value is considered significant if <0.05, (n=20).

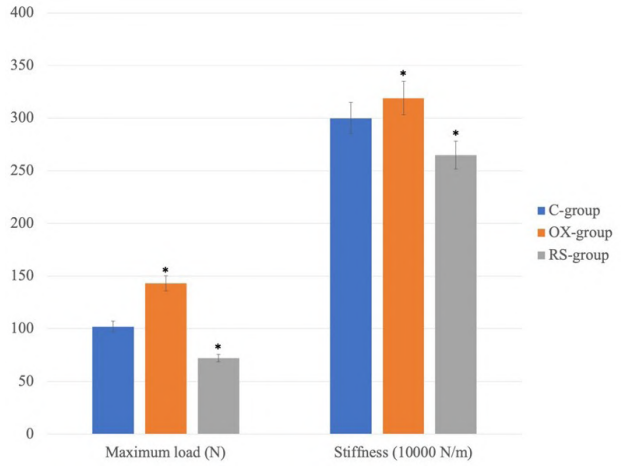

Fig. 9.- Effect of retosiban and oxytocin administration on mandibular bone stiffness in peri-estropause rats. OX-group showed a significant ($p < 0.05$) increase of maximum load and stiffness as compared to C-group. On the other hand, RS-group showed a significant ($p < 0.05$) decrease of same parameters as compared to C-group. * Significant ($p < 0.05$) difference in comparison to C-group. Data are expressed in mean ± standard deviation and probability value is considered significant if <0.05, (n=20).

Retosiban and oxytocin administration effect on BMD in peri-estropause rats

OX and RS groups showed a significant rise and drop of BMD respectively as compared to the control values (Fig. 8).

Retosiban and oxytocin administration effect on mandibular bone stiffness in peri-estropause rats

EMIC DL3000 (Instron, Brazil) was used in order to evaluate the biomechanical characteristics of the mandible. OX-group showed a significant rise of maximum load and stuffiness as compared to control group. On the other hand, RS-group showed a significant drop of same parameters as compared to control group (Fig. 9).

DISCUSSION

Marongiu (1) linked the reduction in bone stiffness during the peri-menopausal period (evidenced by an increased susceptibility to fractures) to the fluctuations in sex hormone levels. In addition to OX's direct impact on bone, the connection between OX and steroid sex hormones, particularly estrogen, was elucidated by Colaianni et al. (2011). They reported that OX's influence on bone in response to estrogen operates through an autocrine loop, where OX is locally released from bone marrow osteoblasts via a non-genomic, Erk-dependent pathway. Furthermore, OX serves as a mediator for estrogen's effects on bone by influencing the expression of oxytocin receptors through genomic mechanisms. Di Benedetto et al. (2014) outlined the mechanism by which OX operates on its own receptors (OTR) in the skeletal system, involving translocation to the nucleus of osteoblasts. This translocation process is the primary mechanism responsible for osteoblast differentiation.

The current study explored the impact of OX on bone during the peri-estropause period. In the OX-treated group, there was an observed increase in ALP activity, which is an osteoblastic marker, along with a significant decrease in TRAP activity, a marker associated with osteoclasts. These findings align with the research by Naylor and Eastell (2012). Conversely, the RS-group exhibited the opposite trend in these parameters. Colli et al. (2012) noted that OX's peripheral effects in older rats were linked to substantial increases in ALP and osteocalcin, suggesting enhanced osteogenesis coupled with reduced bone resorption activity following OX administration. Furthermore, Colaianni et al. (2014b) pointed out that oxytocin's influence on skeletal tissue is also mediated by its impact on osteoclasts. This finding is consistent with Nicola et al. (2016) research, which highlighted the altered activity of both osteoblasts and

osteoclasts during the perimenopausal period.

In present results, OX-group showed a strong immunohistochemical reaction of bone sections indicating increased expression of Osx, RUNX-2. These are osteogenesis markers indicating osteoblast differentiation as reported by Tamma et al. (2009), in addition to PER and BMP proteins, while RS-group showed negative reactions. This comes in the same context as, Stringhetta-Garcia et al. (2016) who noticed the ability of PER protein to interact with collagen (type I), resulting in better bone quality. Fernandes et al. (2020) reported the role of PER in osteogenesis, mineralization which results in better bone quality and thickness reflected directly on bone strength. Kim et al. (2015) reported the role of down-regulated PER in bony fractures of postmenopausal women, in addition to the peripheral role of OX in a greater osteoblastic activity. Santos et al. (2018) reported the in vitro effect of OX in activating bone marrow derived mesenchymal stem to differentiate into osteoblasts. In the same line, Tamma et al. (2009) reported that OX induced osteoblast development toward a mineralizing phenotype through increasing of bone morphogenetic protein2 (Bmp-2). On the other hand, they added that despite OX stimulate osteoclastogenesis, it was found that OX treatment can inhibits bone resorption of mature osteoclasts.

Following the examination using Raman microspectroscopy, the OX-treated group exhibited an increase in the mineral-to-matrix ratio, along with a reduction in carbonate (B-type), aligning with findings from other authors (Bi et al., 2011; Khalid et al., 2018; Burr, 2019). Ge et al. (2019) also reported that OX was observed to stimulate mineral formation and the deposition of Ca2+.

In the current study, OX-group represented an increase of cortical and trabecular bones in addition to a decrease of % of cortical porosity and number of trabeculae, which was reflected as an increased bone stiffness as reported by Kazakia et al. (2013). According to Vilayphiou et al. (2016), it is commonly observed that aging leads to heightened porosity and reduced strength in bones. They also noted that evaluating cortical porosity can serve as a predictive measure for future fractures.

In conclusion, Oxytocin plays a crucial role in enhancing bone density and mineralization in both compact and trabecular bone by promoting osteoblast differentiation and regulating bone turnover markers. Additionally, it contributes to improved bone strength. These effects were negated by Retosiban (GSK-221,149-A), suggesting that Oxytocin's anabolic impact on bone in peri-estropause rats is most likely mediated through oxytocin receptor mechanisms rather than other pathways.

STATEMENT OF ETHICS

The Research and Ethics Committee, Kafrelsheikh University, Egypt approved the study protocol (MKSU 65-2-14).

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