POSSIBLE NEUROENDOCRINE ROLE FOR OXYTOCIN IN BONE REMODELING

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Objective. The present study was carried out to evaluate the assumption that oxytocin (OT) plays a neuroendocrine role in bone remodeling. For this purpose the changes in serum calcium, serum RANKL and OPG levels were detected in addition to morphological examination of the bone.

Methods. Two regimes for OT administration were used: 1. one group of rats was treated with a high OT dose of 40 μIU/kg b.w. for 6 weeks; 2. second group was treated with a low OT dose of 8 μIU/kg b.w. for a longer period of treatment (12 weeks). To evaluate the possible role of OT in bone remodeling, the changes in serum calcium, serum RANKL (sRANKL – Receptor Activator of Nuclear factor K-ß Ligand) and OPG (Osteoprotegerin) levels were detected and sRANKL/OPG ratio was calculated. To confirm the biochemical data, a histological and ultrastructural study of rat bone samples, before and after injection with oxytocin, was also performed.

Results. In general, the present study shows that intramuscular injection of OT at both concentrations and durations of treatment caused a significant decrease in serum calcium and sRANKL levels and a significant increase in OPG level. The sRANKL/OPG ratio was decreased as well. Morphological observations showed that both OT treatments induced a slight effect on bone remodeling in favor of bone formation.

Conclusion. Oxytocin was found to posses a growth promoting effects on bone. The results also clearly showed that treatment with a high OT dose for a short duration was more effective than the low dose for a longer period of treatment.

Key words: Oxytocin – Bone remodeling – Serum calcium – RANKL – OPG

Osteoporosis "the silent killer" (PARSONS 2005) is considered as a major public health problem. Its incidence is particularly high in postmenopausal women but it can also affect other human groups such as men and patients receiving corticosteroid therapy (GAMBACCINI and VACCA 2004; PARSONS 2005; RAISZ 2005; SIMON 2007). In postnatal life, the skeleton undergoes a continuous remodeling process (HILL 1998; RAISZ 1999; FERNANDEZ-TRESGUERRES-HERNANDEZ-GIL et al., 2006). Bone remodeling is regulated by exogenous and endogenous factors (AMLING et al. 2000). Imbalance between osteoclast and osteoblast activities can arise from a wide variety of hormonal changes or growth factors, resulting in skeletal abnormalities characterized by decreased (osteoporosis) or increased (osteopetrosis) bone mass (RAISZ 1999).

A large body of evidence recently suggests a central neuroendocrine regulatory role in bone remodeling (AMLING et al. 2000). One of the important candidates for this effect is oxytocin (OT) (DURSON et al. 2005). In addition to its well known effects during labor and lactation (IMAMURA et al. 2000; SVENNERSTEN-SJAUNJA and OLSSON 2005), it was reported that OT is implicated in neuroadaptive processes exerting a wide spectrum of central and peripheral effects (JENKINS and NUSSEY 1991; GIMPLE and FAHRENHOLZ 2001). All these effects are mediated by specific cell surface receptors detected in different target tissues (GIMPLE and FAHRENHOLZ 2001).

Recently, functional oxytocin receptors (OTRs) were demonstrated in human osteoblasts as well as osteoclasts (Copland et al. 1999; Colucci et al. 2002; Petersson et al. 2002).

The discovery of RANKL/RANK/OPG system led to a better understanding of osteoclast cell biology and has provided important insights into the pathogenesis of human metabolic bone diseases (Khosla 2001; Stejskal et al. 2001; Hofbauer and Heufelder 2000; Hofbauer and Schoppet 2004). The receptor activator of nuclear factor k-ß ligand (RANKL), a member of the tumor necrosis factor super-family, is expressed by osteoblasts and is necessary for osteoclastogenesis (Zaidi et al. 2002; Hofbauer and Schoppet 2004). RANKL is also expressed by bone-forming osteoblasts, which indicates that bone resorption and formation are coupled (Karsdal et al. 2005). RANKL activates its receptor, receptor activator of nuclear factor k-ß (RANK) which is expressed on osteoclasts and their precursors, thus promoting osteoclast activation through internal signals that include activation of *c-fos*, tumor necrosis factor-associated factor-6 (TRAF-6) and nuclear factor k-ß (NF-k-ß) (Nakagawa et al. 1998). Binding of RANKL to RANK prolongs osteoclast survival by suppressing apoptosis (Suda et al. 1999). The effect of RANKL is blocked by osteoprotegerin (OPG), a glycoprotein, secreted by osteoblasts that acts as a decoy receptor for RANKL (Yasuda et al. 1998). The term osteoprotegerin has been coined for its effects where it protects against bone loss (Simonet et al. 1997). The balance between RANKL and OPG is regulated by cytokines and hormones that determine osteoclast functions. Alteration of the RANKL/OPG ratio is critical in bone pathogenesis and results in increased bone resorption (Hofbauer and Schoppet 2004).

The present study was carried out to evaluate the assumption that OT plays a neuroendocrine role in bone remodeling using changes in serum calcium level, serum RANKL and OPG level in addition to morphological examination of the bone as markers. Most, if not all, of the earlier studies were carried out in an *in vitro* experiment (Copland et al. 1999; Colucci et al. 2002; Petersson et al. 2002). The present study could be that the first to be applied in an *in vivo* model.

Material and Methods

Forty four young adult male albino rats (150-200 g body weight) were used in the present study. Animals were raised in the facilities of the Medical Research Institute, Alexandria University, Egypt. They were housed in plastic cages (5 or 6 per cage) under constant temperature (22±2 °C) and a light:dark cycle of 12:12. Food and drinking water were available ad libitum. Animals were divided into three groups. Animals of group I were subdivided into four subgroups (six animals each): 1. subgroup IA received no treatment and sacrificed after six weeks. 2. subgroup IA' received no treatment and sacrificed after 12 weeks; 3. subgroup IB received a daily intramuscular injection of the vehicle, dimethylsulphoxide (DMSO) for six weeks; subgroup IB' received the same treatment for 12 weeks. The original DMSO solution (95 %) was diluted (1:10) with bi-distilled water and was used as a vehicle for OT (Paquin et al., 2002). Animals of group II (10 animals) were treated with a daily intramuscular injection of OT at a dose of 40 µIU/kg body weight for six weeks (Ciriello et al., 1998) and group III (10 animals) were treated with OT at a dose of 8 µIU/kg body weight for twelve weeks (Xie et al. 2003). Oxytocin and its solvent (DMSO) were purchased from Sigma Chemical Co.(St. Louis, Mo, USA).

After the laps of six or twelve weeks of treatment, the body weight of experimental animals ranged between 180-230 g and 200-250 g, respectively, and blood samples were collected by cardiac puncture. Blood was allowed to clot and serum was separated and immediately frozen in 1.5 ml eppendorph tubes at -20 ^{0}C until assayed.

Determination of serum calcium level. Serum calcium was determined using the method reported by Bamelt et al. (1973) employing methyl thymol blue. The formed complex (methyl thymol blue complex) was spectrophotometrically read using Pharmacia LKB ultra spec III at λ=610 nm.

Measurement of OPG level. Serum OPG level was determined according to the method of Chen et al. (2001) employing a quantitative sandwich type of enzyme linked immunoassay (ELISA) technique using kits purchased from Biomedica Co., Vienna, Austria.

Measurement of serum RANKL level and RANKL/OPG ratio. Soluble form of RANKL (sRANKL) level was determined in serum according to the method of Hawa et al. (2003) employing two-steps sandwich type of ELISA technique using kits purchaised from Biomedica Co., Vienna, Austria. sRANKL/OPG ratio was calculated by dividing the sRANKL value by the OPG value for each experimental animal. The mean of the sum of all readings was then obtained.

Bone histology and ultrastructure. Bone specimens of distal femur were divided into small pieces and fixed in formalin-glutaraldehyde mixture ($F_4 G_1$) in phosphate buffer solution (pH 7.2) at 4 °C for three hours. Specimens were then postfixed in 2 % osmium tetroxide (OsO_4) in the same buffer at 4 °C for two hours. Fixed tissues were decalcified in a mixture of 4% ethylenediamine-tetra-acetic acid (EDTA) and 6 % glutaraldehyde (1:1) for at least 21 days. Semithin sections were stained with 1 % toluidin blue for histological observations. For ultrastructural studies, ultrathin sections were double stained with uranyl acetate for 20 min and counter-stained with lead citrate for 5 min (Reynolds 1963). Examination was carried out using Jeol-100CX electron microscope,

Statistical evaluation. Data are presented as means ± SEM and were statistically analyzed with two ways ANOVA followed by LSD for multiple comparisons.

Results

Biochemical results. In all studied control groups, no effect was detected by administration of the solvent (DMSO) on any of the studied parameters.

Generally, OT treatment induced a significant effect on all of the studied parameters. It induced a significant decrease in serum calcium level from 10.92 ± 0.83 mg/dl in untreated control rats (group IA) to 4.99 ± 0.06 mg/dl ($p<0.001$) in rats treated with OT at a dose of 40 μIU/kg b.w. for 6 weeks (group II) (Fig. 1). Serum calcium level was also decreased from 11.00 ± 0.40 mg/dl in untreated control rats (group IA') to 7.58 ± 0.12 mg/dl ($p<0.05$) in rats treated with OT at a dose of 8 μIU/kg b.w. (group III) (Fig. 1). On the contrary, OT treatment induced a significant increase in serum OPG level from 2.61 ± 0.28 pmol/l in untreated controls to 5.89 ± 0.21 pmol/l ($p<0.001$) in rats treated with 40 μIU/kg b.w. for 6 weeks (Fig. 2). Serum OPG level was also increased from 2.55 ± 0.17 pmol/l in untreated controls to 4.07 ± 0.20 pmol/l ($p<0.001$) in rats treated with OT at a dose of 8 μIU/kg b.w. for 12 weeks (Fig. 2). As in the case of serum calcium level, OT treatment induced a significant decrease in sRANKL level from 7.60±0.42 pmol/l in untreated control rats to 4.28 ± 0.24 pmol/l ($p<0.05$) in rats treated with 40 μIU/Kg b.w. for 6 weeks (Fig. 3). sRANKL level was also decreased from 7.47 ± 0.38 pmol/l in untreated control rats to 5.59 ± 0.32 pmol/l ($p<0.01$) in rats treated with OT at a dose of

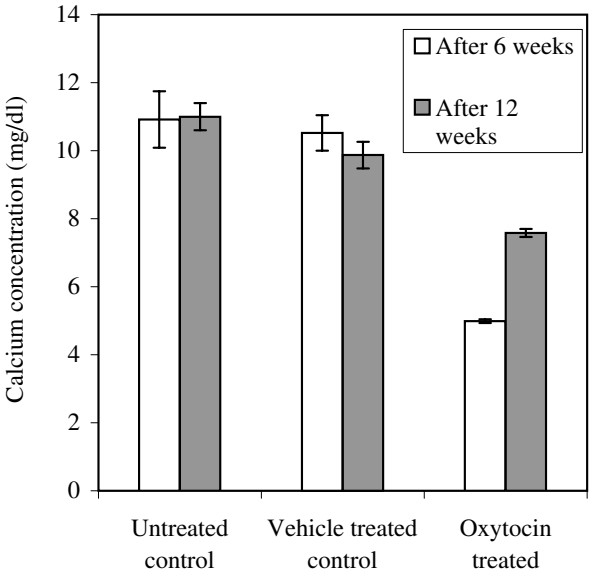

Fig. 1 Serum calcium level (mg/dl) in untreated control (IA, IA'), vehicle treated control (IB, IB') and oxytocin treated rats (40 μIU/kg b.w.) for six weeks (II) and oxytocin treated rats (8 μIU/kg b.w.) for twelve weeks (III). * P < 0.05 vs. their respective controls, ** P < 0.001 vs. their respective controls.

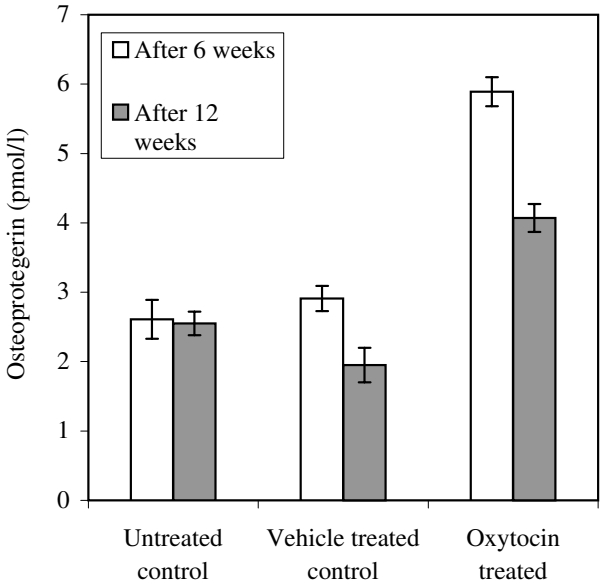

Fig. 2 Serum calcium level (mg/dl) in untreated control (IA, IA'), vehicle treated control (IB, IB') and oxytocin treated rats (40 μIU/kg b.w.) for six weeks (II) and oxytocin treated rats (8 μIU/kg b.w.) for twelve weeks (III). * P < 0.05 vs. their respective controls, ** P < 0.001 vs. their respective controls.

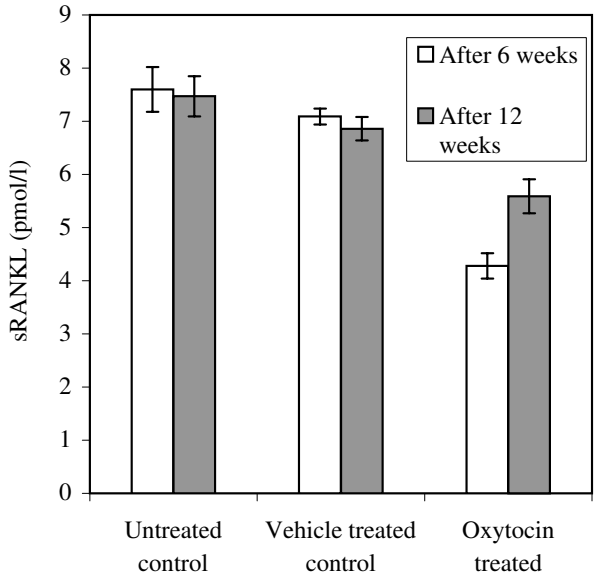

Fig. 3 Serum RANKL level (pmol/l) in untreated control, vehicle treated control and oxytocin treated rats (40 μIU/kg b.w.) for six weeks (white columns) and oxytocin treated rats (8 μIU/kg b.w.) for twelve weeks (gray columns). * P < 0.05 vs. their respective controls.

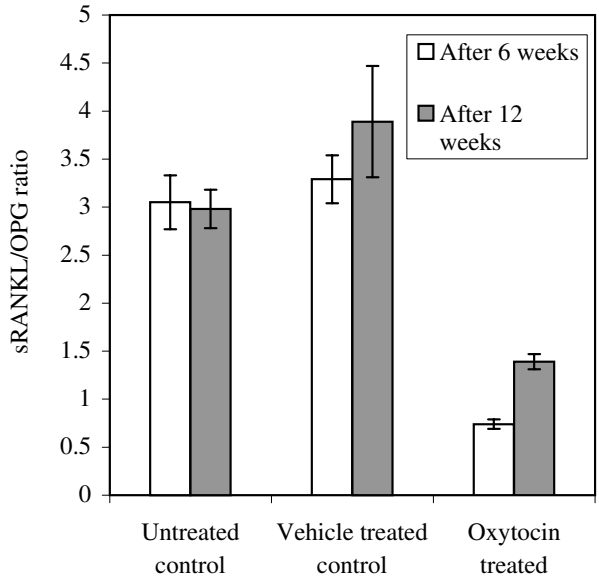

Fig. 4 sRANKL/OPG ratio in untreated control (IA, IA'), vehicle treated control (IB, IB') and oxytocin treated rats (40 μIU/kg b.w.) for six weeks (II) and oxytocin treated rats (8 μIU/kg b.w.) for twelve weeks (III). * P < 0.05 vs. their respective controls, ** P < 0.001 vs. their respective controls.

8 μIU/kg b.w. for 12 weeks (Fig. 3). The sRANKL/OPG ratio was significantly decreased as well from 3.05 ± 0.28 in untreated controls to 0.74 ± 0.05 ($p<0.001$) in rats treated with high OT dose for 6 weeks (Fig. 4). Similarly, sRANKL/OPG ratio was decreased from 2.98 ± 0.20 in untreated controls to 1.39 ± 0.08 ($p<0.05$) in rats treated with the low OT dose for 12 weeks (Fig. 4).

These results clearly show that the high OT dose is more effective than the low OT dose on all studied parameters.

Histological and ultrastructural results. Histological examination of decalcified distal femur from control rats displayed the normal features of bone morphology. The mineralized bone matrix appeared as a dark stained layer (Fig. 5) the inner surface of which is covered by a layer of flat or cuboidal osteoblasts arranged in sheets forming the so called endosteum (Fig. 5). These cells appear separated from the mineralized matrix by a narrow light stained band which represent the osteoid or non-mineralized matrix (Fig. 5). Within the mineralized matrix, osteocytes are present housed in lacunae (Fig. 5). Bone resorbing cells, osteoclasts, usually act by forming shallow resorption cavities "Howships lacunae". The existence of these cavities is a sign of osteoclastic existence and activity (Fig. 5). These cells are presenting adjacent to the mineralized matrix and separated from it by a clear zone or an interface (Fig. 5, inset). Ultrastructurally, each lacuna houses a single osteocyte that virtually fills its space (Fig. 6). Osteocytes are oval in shape (Fig. 6), the nucleus is characterized by peripheral heterochromatin (Fig. 6) and the cytoplasm contains numerous lysosomes (Fig. 6). The clear area around the cell is occupied by un-mineralized matrix in which collagen fibers are visible (Fig. 6). The adjacent osteocytes communicate with one another by cytoplasmic processes which lie in narrow channels called canaliculi (Fig. 6). Normal osteoblasts with prominent nucleus were also observed (Fig. 7). The cytoplasm contains rough endoplasmic reticulum, Golgi complex and lysosomes (Fig. 7). In rare specimens, active osteoclasts were observed (Fig. 8) with the distinctive ruffled border.

Oxytocin administration induced moderate changes in bone histology. As control, the mineralized bone is covered by a row of osteoblasts (Fig. 9). Reversal or cement lines are present as dark stained lines within the mineralized bone. They are more obvious in OT treated groups than in the control groups (Fig. 9, inset). Mineralized bone matrix contains osteocytes with faintly stained cytoplasm (Fig. 9). Ultrastructurally, the

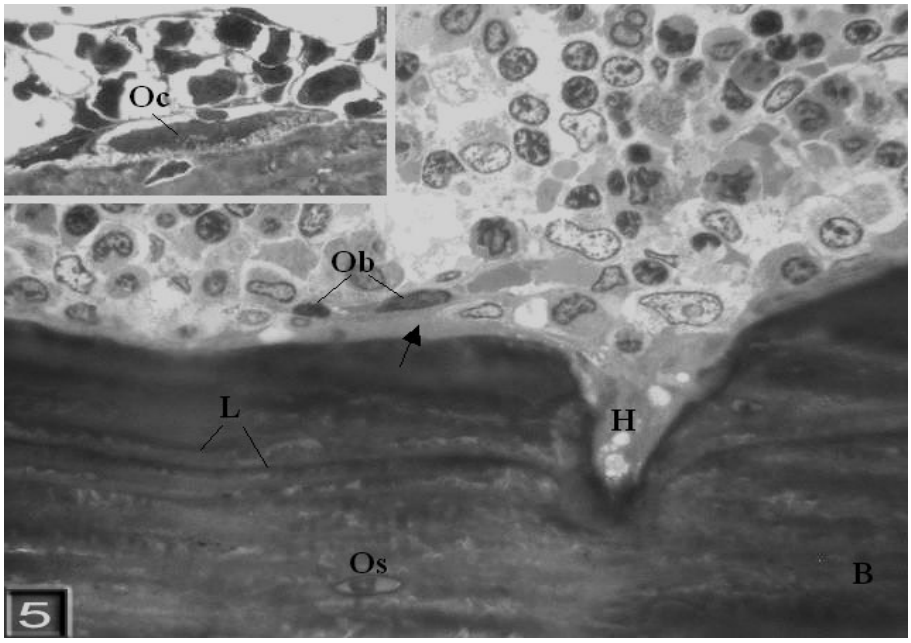

Figure 5: A photomicrograph of a 5µm thick section through the femur bone of an untreated control rat. Mineralized bone (B) is dark stained and covered by a light stained layer, osteoid (arrow). The osteoid is lined by a row of osteoblasts (Ob). Osteocytes (Os) are present deep within the mineralized bone. Reversal lines (L) mark the extent of bone remodeling. Howship's lacuna (H), a depression from the bone surface, resorbed by osteoclasts (X1000). Inset: **An osteoclast (Oc) is present in a shallow resorption cavity. Its nucleus is polarized away from the bone surface and the secretary granules are adjacent to the clear zone or interface between the osteoclast and the mineralized bone (X1000).**

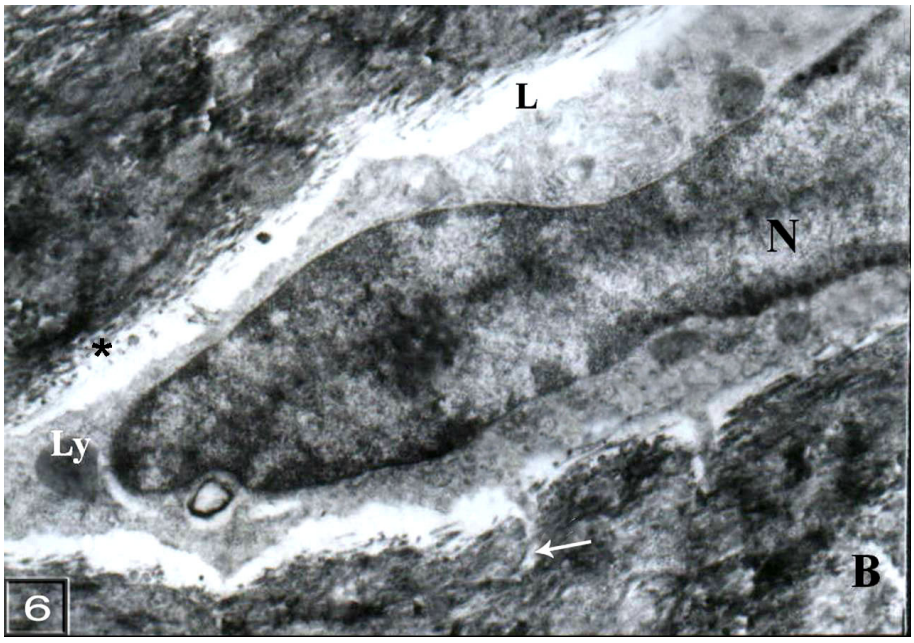

Figure 6: Electron micrograph of an osteocyte of a control rat present in its lacuna (L) within the electron dense mineralized bone (B). The osteocyte contains a prominent nucleus (N). Lysosomes (Ly) are also evident. The clear area around the cell represents the unmineralized matrix. The cell process is seen extending into a canaliculus (arrow) (X10000).

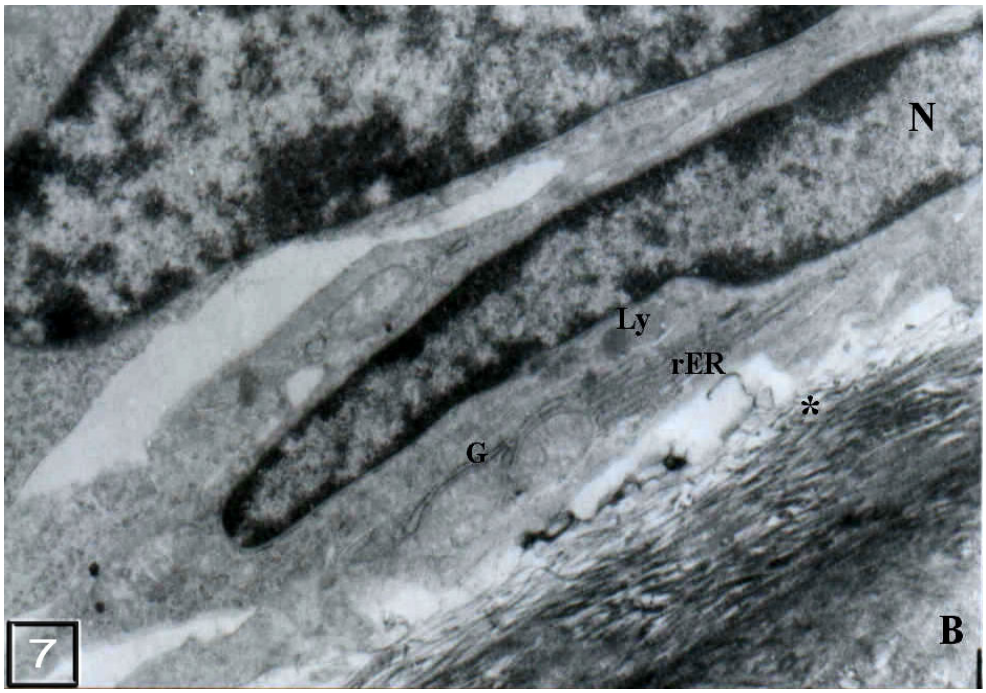

Figure 7: Electron micrograph of an osteoblast of a control rat, displaying a prominent nucleus (N), lysosomes (Ly) and rough endoplasmic reticulum (rER). The clear zone between osteoblast and mineralized bone (B) represent unmineralized matrix in which collagen fibers are faintly visible (X7500).

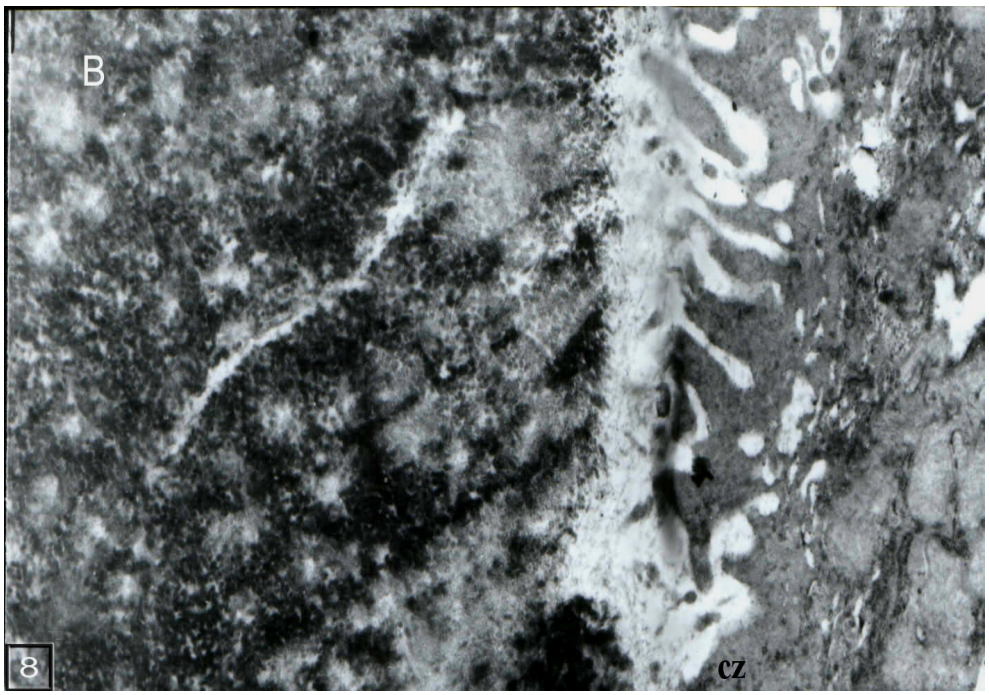

Figure 8: Electron micrograph of a portion of an osteoclast of a control rat at the bone surface undergoing resorption. Calcified bone appears electron dense at the left side of the micrograph. The right side of the micrograph is occupied by the cytoplasm of an osteoclast. Extending from top to bottom, the upper portion of the osteoclast, is the ruffled border. A clear zone (cz) is also observed beside the ruffled border (X10000).

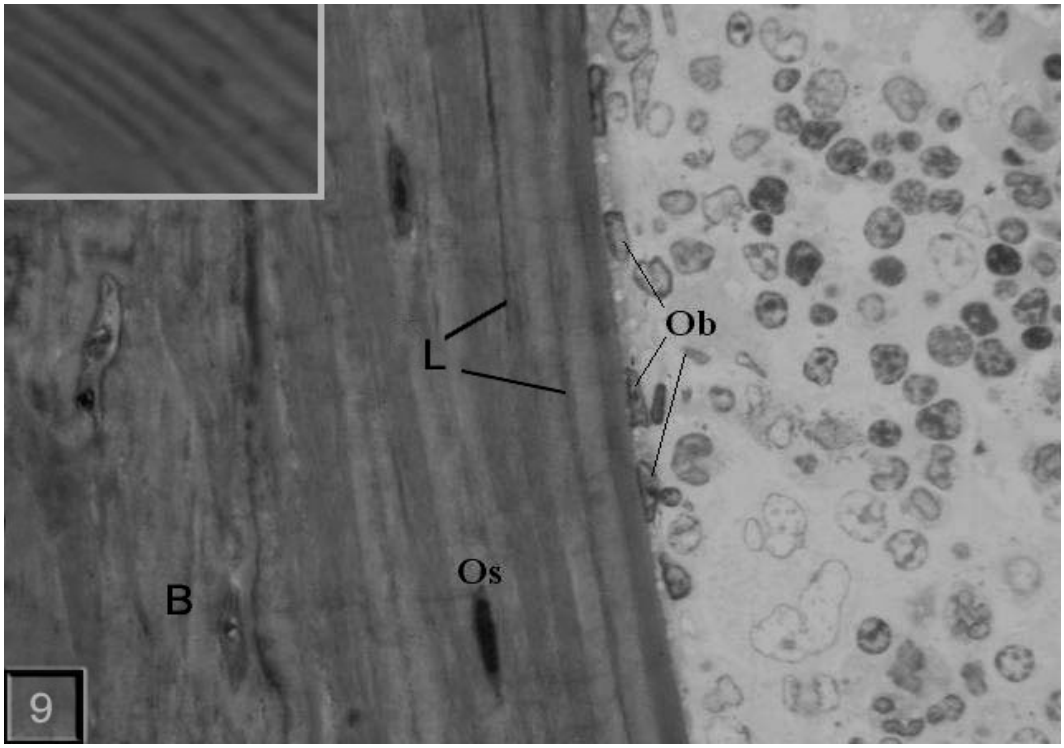

Figure 9: Photomicrograph of a 5µm thick section through the femur bone of a rat treated with a daily injection of 8µIU oxytocin/Kg body weight for twelve weeks. Osteocytes (Os) are present deep within the mineralized bone. Dark stained reversal lines (L) mark the extent of bone remodeling. The mineralized bone lined with a row of osteoblasts (Ob) (X1000). Inset: **Large number of dark stained reversal lines present deep within the bone matrix (X400).**

mineralized matrix appeared light due to the presence of collagen which is still in the process of calcification (Fig. 10). Presence of numerous randomly oriented rays of deposited minerals in the mineralized bone was evident (Fig. 10). Dark droplets that represent mineral deposition were also observed in the matrix (Fig. 10). Adjacent cells are connected to each other by long cytoplasmic processes extending through the canaliculi; these were more visible in the treated groups than in the control ones (Fig. 10). As in the control groups, osteoblasts appeared with their characteristic features (Fig. 11). The surface of the osteoblast shows numerous cytoplasmic processes, particularly on the side facing the existing osteoid (Fig. 11). Neighboring oseoblasts on bone surface are connected by gap junctions (Fig. 11).

Discussion

The results of the present study showed that OT treatment decreased serum calcium, sRANKL and sRANKL/OPG ratio and increased serum OPG levels. Moreover, OT stimulated osteoblast proliferation and increased the rate of bone remodeling with a net gain of bone. All of the studied parameters were more affected by the high OT dose for a short period of treatment rather than by the low OT dose for a long period of treatment, i.e. OT effect on bone remodeling is dose dependant rather than time dependent.

Some studies suggested that neuroendocrine mechanisms may affect the bone metabolism either directly or indirectly (Amling et al. 2000; Xing et al. 2005). Among the different factors suggested to affect bone remodeling is the hormone oxytocin. Functional OTRs have been found in human osteoblast and osteoclast (Copland et al. 1999; Colucci et al. 2002; Petersson et al. 2002; Durson et al. 2005). Colucci et al. (2002) showed for the first time the expression of OTRs in human osteoclasts. They also found that the expression of OTRs in pre-osteoclasts is lower as compared with mature osteoclasts, indicating that OTR expression levels could be related to the different stages of osteoclast differentiation. In addition, they showed that the receptors are functional indicating that OT could

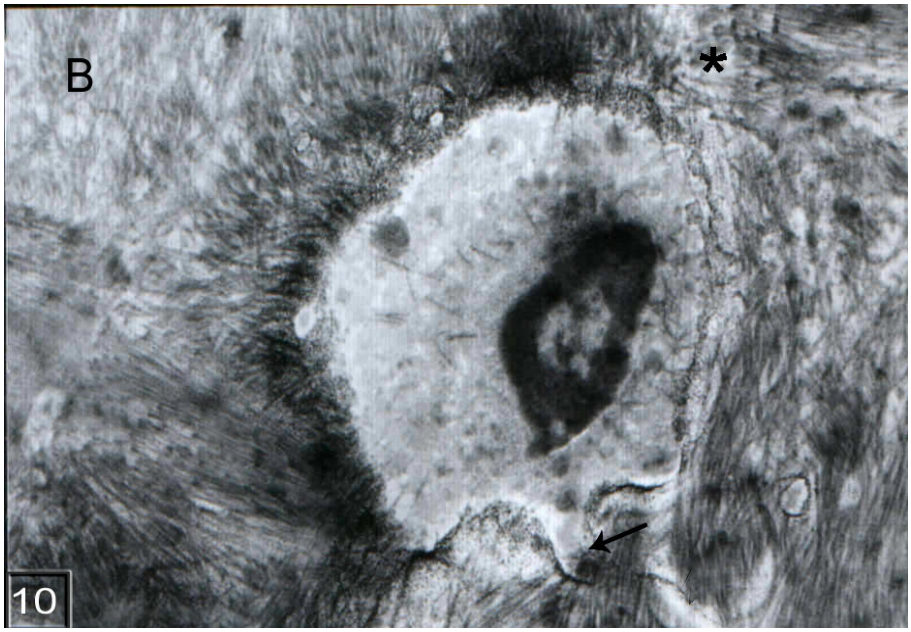

Figure 10: Electron micrograph of an osteocyte of a rat treated with daily injection of 8µIU oxytocin/Kg body weight for twelve weeks. Note that the osteocyte is present in its lacuna, within the mineralized bone (B). The cell process could be observed extending into a canaliculus (arrow). The black droplets present in the matrix represent mineral deposition (X10000).

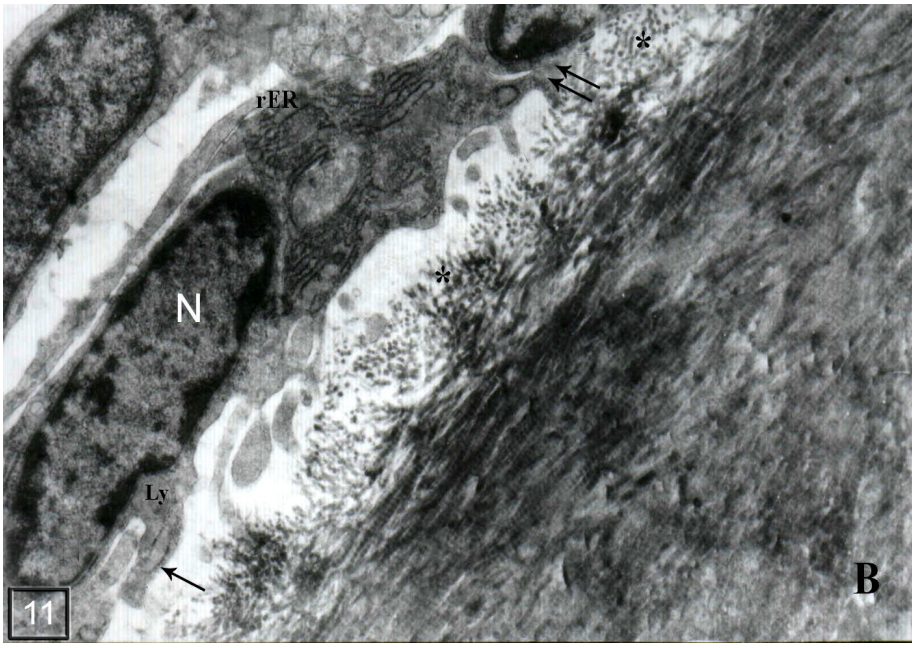

Figure 11: Electron micrograph of an osteoblast of a rat treated with daily injection of 8µIU oxytocin/Kg body weight for twelve weeks. The nucleus (N), lysosomes (Ly) and rough endoplasmic reticulum (rER) are evident. The unmineralized matrix present between osteoblas and mineralized bone (B), in which collagen fibers are faintly visible (*). The cell surface shows numerous cytoplasmic processes (^), particularly on the face in contact with existing osteoid. Double arrows (^^) indicate the junction between cells. (X7500).

be involved in the control of osteoclast formation during the early stages of their differentiation.

The significant decrease in serum calcium level after OT treatment could be explained by an increased deposition of calcium in the form of inorganic salts (crystalline hydroxyapatite $Ca_{10}(PO_4)_6 H_2O$) in bone. This is in accordance with Bus-Solati et al. (2001) who stated that OTR activation in rat and mouse leads to an increase in intracellular calcium coupled to a stimulatory effect on cell growth. In humans, Colucci et al. (2002) found that intracellular calcium concentration in response to 10^{-6} M OT is increased and is dependent upon the release from intracellular calcium stores. Recently, Colucci et al. (2004) reported that OTR stimulation elicited an intracellular calcium increase in both bone cell types (osteoblasts and osteoclasts).

The data reported in the present study also showed that OT has growth promoting effects on bone, that could be mediated through the OTR, and OT decreases RANKL production and stimulates OPG secretion. This stimulatory effect of OT on serum OPG synthesis suggests that this protein may be involved in the up regulated anabolic activity observed in bone histology detected in this study. It could be concluded that the over expression of OPG in OT treated rats increases osteoblastic activity over the osteoclastic activity and this may explain the imbalance in bone remodeling in favor of bone formation. Uemura et al. (2002) concluded that the fall in OPG levels after delivery may be partially connected with the marked acceleration of bone resorption after delivery. Grigorie et al. (2003) found that the relationship between circulating OPG levels and osteoporosis in postmenopausal women is controversial. Szulc (2001) found that OPG serum levels are positively correlated with age and concluded that OPG may act as an important paracrine mediator of bone metabolism in elderly men. This could represent a possible protective mechanism against bone loss. Seidel et al. (2001) stated that low OPG levels appear to be a marker of bone disease. Eghali-Fatourechi et al. (2003) found that expression of RANKL by bone marrow cells contributes to the increased bone resorption in early postmenopausal women and the up regulation of RANKL on bone marrow cells is an important determinant of increased bone resorption induced by estrogen deficiency.

Copland et al. (1999) concluded that OT can influence some of the osteoblast derived factors that are involved in bone metabolism. Earlier, Cheng and Rosenthal (1995) stated that it is possible that IGF-1 which is a potent stimulator of osteoblasts proliferation might be involved in the growth promoting effect of OT on human osteoblastic cells. Colucci et al. (2004) found that functional OTRs present in human osteoblasts stimulate prostaglandin E_2 (PGE_2) synthesis; PGE_2 plays a key role in achieving a positive bone balance. These findings are in agreement with the results of Ohmichi et al. (1995) who found that OT stimulates PGE_2 synthesis through pathway that could involve increase in Ca^{2+}. Latter, Petersson et al. (2002) reported that OT decreases production of IL-6 from human osteoblastic cells, which is involved in osteoclast formation (Roodman 1999). They speculated that OT may have a net effect of decreasing osteoclastogensis and thereby bone resorption. Worth to mention that the positive effect of estrogen on bone formation in women after menopause has been suggested to be mediated through an estrogen-included decrease in IL-6 production (Manalogas 1998).

Oxytocin influence bone metabolism in humans during certain physiological conditions, during pregnancy and lactation and after menopause, and it may have a role in the recovery of bone loss that occurs during pregnancy and lactation (Karlsson et al. 2005). It is known that the rate of bone loss increases after menopause due to the decrease in estrogen levels (Riggs et al. 1998). Also, plasma OT level decreases after menopause and several effects of OT are potentiated by estrogens. Thus, the decrease in OT levels may also contribute to the increased bone loss after menopause (Durson et al. 2005; Parsons 2005).

In conclusion, since OT affects bone metabolism, its serum level could be used as a biochemical marker for the prediction and monitoring of osteoporosis. Moreover, OT agonists may be used as a new treatment modality for osteoporosis. As OT treatment induces lowering in serum calcium level, it could be recommended to monitor blood calcium level during OT treatment or the use of calcium supplement along with OT treatment.

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