

## EXPERIMENTAL STUDY

## Bone biomarkers of ovariectomised rats after leptin therapy

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**Abstract:** *Background and aim:* Under physiological conditions, maintenance of skeletal mass is the result of a tightly coupled process of bone formation and bone resorption. Disease states, osteoporosis included, arise when this delicate balance is disrupted, such as in menopause. The aim of the present work was to study the effect of leptin supplementation on bone metabolism in ovariectomized adult female rats by measuring indices of bone biomarkers.

*Methods:* Forty adult female albino rats were chosen as an animal model for this study and divided into the four equal groups (n=10/group): Group I (control SHAM-operated group) received a single dose of buffer solution i.p. daily for eight weeks. Group II (ovariectomy group) received a single dose of buffer solution i.p. daily for eight weeks. Group III (alendronate group): Ovariectomized rats that received alendronate 0.1 mg/kg body weight i.p. daily for eight weeks. Group IV (leptin group): Ovariectomized rats that received leptin (10 µg/kg body weight) i.p. daily for eight weeks. The obtained serum is required for determination of: Serum osteocalcin, alkaline phosphatase, calcium and phosphorous levels.

*Results:* The obtained data revealed that treatment with alendronate or leptin caused a significant decrease of serum osteocalcin, specific bone alkaline phosphatase and urinary deoxypyridinoline levels compared to ovariectomy group, and there was no significant difference between both drugs.

*Conclusion:* Leptin prevents ovariectomy induced increases in bone turnover in rats. Leptin therapy has a significant effect in treatment of ovariectomy induced osteoporosis in rats (Tab. 1, Ref. 32). Full Text in PDF [www.elis.sk](http://www.elis.sk). Key words: leptin, bone metabolism, ovariectomy, osteoporosis, bone biomarkers.

Bone structure is preserved via a dynamic procedure of bone turnover, a coupled balance between bone resorption and bone formation. Osteoporosis is a metabolic bone disease associated with an unequilibrated bone remodeling due to decreased bone formation or accelerated bone resorption (Saleh and Saleh, 2011). This increases bone fragility, which means that fractures can occur after low-energy traumas (Byun and Lee, 2010). Osteoporosis is three times more common in women than in men, partly because women have a lower peak bone mass and partly because of the estrogen hormonal changes that occur in the menopause (Canalis, 2010).

Remodeling maintains the mechanical integrity of the skeleton by replacing old bone with new (Jackson et al, 2011). It is controlled by several circulating hormones, including parathyroid hormone, 1 $\alpha$ ,25-dihydroxycholecalciferol (calcitriol), gonadal sex hormones and adrenal androgens (dehydroepiandrosterone and androstenedione) (Robling et al, 2006). Several cytokines released in the bone marrow increase the rate of bone turnover. TNF- $\alpha$ , interleukin-1 and interleukin-6, all stimulate bone resorption in vitro and in vivo, and may initiate the bone loss-induced by estrogen deficiency (Weitzmann and Pacific, 2006). Interactions between environmental factors such as dietary intake and physical exercise, as well as between genetic and environmental

factors, might play an important role in the acquisition of bone mineral mass (Deal, 2009).

Leptin, a cytokine-like hormone secreted by adipocytes, is known to regulate food intake but has also emerged as a significant factor in the regulation of bone mass (Cirmanová et al, 2008). Ducy et al (2000) have shown that in leptin-deficient and leptin-receptor-deficient mice there is abnormally high bone mass due to increased bone formation. In this study, it was also shown that an intracerebroventricular infusion of leptin in leptin-deficient mice resulted in bone loss with the bone of the treated animals regaining normal appearance. So, the aim of this study was to assess the possible effect of leptin supplementation compared to alendronate, as the standard treatment of osteoporosis on bone metabolism in ovariectomized adult female rats, by measuring indices of bone resorption and bone formation (bone biomarkers).

### Materials and methods

#### Materials

Rat leptin was purchased from Calbiochem, La Jolla, CA, USA. Alendronate tablets (Fosamax, 10 mg) were brought from Fosamax-Merck Sharp & Dome, Rome, Italy. Osteocalcin ELISA kit was obtained from Biomedical Technologies Inc., Stoughton, Mass., USA. Rat deoxypyridinoline ELISA kit was obtained from My Bio-Source California, USA. Alkaline phosphatase and calcium kits were purchased from Sigma Chemical Co.; St. Louis, MO. Phosphorous kit was obtained from Bio Merieux France. Thio-pental sodium was brought from Sandoz GmbH, Kundl-Austria.

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### Animals model

Forty Sprague-Dawley adult female rats (from Assiut university animal house) weighing 150–200 grams (4–6 months) (at the beginning of the experiment) were used. Rats were housed in boxes, 10 animals each, under standard conditions of temperature and humidity and a 12-hour light/dark cycle. The rats were fed with the standard diet of commercial chow and tap water and left to acclimatize to environment for two weeks prior to inclusion in the experiment. All experiments were performed during the same time of day, between 8 a.m and 2 p.m to avoid variations due to diurnal rhythms. Manipulations during the test were carried out by the same person (Noriyoshi et al, 2000). All of the procedures regarding the care and use of animals and animal experimentation in this study were complied with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

### Procedures

#### Induction of osteoporosis by ovariectomy

A menopausal state in animals can be induced surgically by ovariectomy (OVX). Bilateral OVX was performed using a dorsal approach under i.p. sodium pentobarbital (40 mg/kg body weight) anesthesia (Turkozan et al, 2009). A skin incision (about 0.5–1.0 cm long) was made midway between the last rib and the cervix and about 1 cm lateral to the spinal muscles. A second parallel but shorter incision was made through the muscles under the incised skin area. A high degree of aseptic procedure was maintained throughout the operation. The ovary embedded in fat was withdrawn out and tied with a silk ligature to enclose the whole ovary and to avoid the inclusion of the adjacent fat as much as possible (Tarek and Hala, 2006). The ovary, tied up by the ligature, was cut away and the end of the uterus returned back to the peritoneal cavity. The cut edges of the muscles were sewn with catgut and those of the skin with silk. The ovary of the other side was then similarly removed through a separate incision. The SHAM-operated control rats had their dermal integuments, muscles and peritoneum sectioned, but underwent no excision of the ovaries. All the animals were cared with analgesia and antibiotics for equally after surgery. The success of ovariectomy was confirmed at necropsy by failure to detect ovarian tissue and by observation of marked atrophy of the uterine horns (Li et al, 2002, Turkozan et al, 2009).

#### Animal groupings/treatment

At the 4th day post-surgery, rats were randomized to four groups, each of ten rats. Treatment was not started until the 7th day post-OVX to allow the estrogen levels to become completely depleted (Burguera et al, 2001).

Group I (control group): SHAM-operated rats that received a single dose of phosphate buffer solution (PBS) given intraperitoneally (i.p.) daily for eight weeks.

Group II (OVX group): Rats in which osteoporosis was induced by bilateral OVX and received a single dose of PBS given i.p. daily for eight weeks (Alam et al, 2006).

Group III (alendronate group): Ovariectomized rats that received alendronate tablets (10 mg) suspended in PBS 0.1 mg/kg

body weight i.p. daily for eight weeks (Xiong et al, 2010). The alendronate tablets were used 10 mg once daily – not 70 mg once weekly – to become uniformed with leptin therapy.

Group IV (leptin group): Ovariectomized rats that received exogenous recombinant rat leptin (10 µg/kg body weight) dissolved in PBS (i.p.) daily for eight weeks (Konturek et al, 2001). The rats of all groups were weighed every week during the experiments.

#### Collection of samples

At the end of the experimental period, all animals were fasted overnight and separated and the urine of each animal was collected for measurement of deoxypyridinoline (DPD) during the 24-hour period before slaughtering. Animal was anaesthetized with thio-pental sodium 40 mg/kg intraperitoneally. Blood was collected from carotid artery after sacrificing the animal. Serum supernatant was separated by centrifugation at 3000 cycles / min. and kept in deep freeze at –70 °C for future use.

#### Determination of serum calcium and phosphorous levels

Serum calcium and phosphorous levels were measured by colorimetric method (Brown, 2010).

#### Determination of bone turnover biomarkers

Due to their high specification and sensitivity in clinical tests, biochemical markers are considered to be useful tools for estimating the rate of osteoporosis related to bone loss, as well as for monitoring the effectiveness of anti-resorptive therapies in osteoporosis (Stavropoulou et al, 2005). Bone formation was assessed by measuring serum osteocalcin and bone-specific alkaline phosphatase (BSALP), whereas bone resorption was assessed by measuring the DPD. DPD is a cross linked collagen in the bone that is excreted in the urine with collagen by osteoclastic activity (Byun and Lee, 2010).

#### Bone formation biomarkers

##### Determination of serum osteocalcin levels

This assay is based on the competitive binding of soluble osteocalcin in serum to a monoclonal antibody raised against human osteocalcin. Biotinylated osteocalcin (100 µL) was added to a sterile 96-well streptavidin-coated microtiter plate and incubated for 30 min at room temperature. After three washes with washing solution, duplicate 20 µL serum samples were pipetted into the wells, followed by 150 µL of osteocalcin primary monoclonal antibody (diluted with primary incubation buffer at a volumetric ratio of 1:100). The mixtures were incubated for 60 min at room temperature on a microtiter mixing apparatus (300 rpm). The secondary antibody was added (100 µL per well) after washing the wells three times with washing solution. The incubation time was 60 min, followed by washing and the addition of chromogenic substrate solution (tetramethylbenzidine in an acidic solution) for 15 min in the dark. The reaction was stopped by the addition of 100 µL of stopping solution (0.18 M H<sub>2</sub>SO<sub>4</sub>) and the absorbance was measured at 450 nm with a 650 nm filter as reference (Stavropoulou et al, 2005, Byun and Lee, 2010).

**Tab. 1. Effects of leptin administration on serum osteocalcin, alkaline phosphatase, calcium and phosphorous levels in normal and ovariectomized rats.**

Parameters	Control group	OVX group	Alendronate group	Leptin group
Final body weight (g)	270.2±4.54***	320.3±5.247	300.2±5.2	275.3±9.5***
Final uterine weight (g)	2.32±0.11***	0.51±0.044	1.33±0.16***	1.23±0.16**
Osteocalcin (ng/ml)	8.060±0.6709***	12.56±0.4587	8.203±0.6379***	9.986±0.6678***
BSALP (IU%)	2.56±0.3***	6.08±0.26	3.81±0.4**	3.9±0.4**
DPD (nmol/mmol creatinin)	151.3±11.49***	219.6±9.36	157.5±10.9**	168±9.45**
Calcium (mg%)	10.05± 0.360***	5.618±0.517	9.76±0.189***	5.13±0.32
Phosphorous (mg%)	4.357±0.236	5.271±0.291	4.314±0.256	5.7±0.33

Data are the means ± SEM of 10 observations. \* p<0.05 (significant), \*\* p<0.01 (highly significant), \*\*\* p<0.001 (extremely significant), compared to the OVX group

#### Determination of serum bone specific alkaline phosphatase

Serum BSALP was measured by colorimetric method. The samples were pretreated by incubating 300 µl of serum with 30 µl Triton X (20 g/l) for 30 min at 37 °C. An aqueous solution of wheat germ lectin (300 µl Sigma-9640, 5 g/l in distilled water) was then added, and the samples were mixed and incubated for 30 min at 37 °C. After centrifugation at 2000 g for 10 min. BSALP was measured in the supernatant after precipitation with wheat germ lectin. The intra-assay CV was 5.1 % and the inter-assay CV 6.8 % at a mean value of 244 µl (Alam et al, 2006).

#### Bone resorption biomarker

##### Determination of Urinary Deoxypyridinoline Levels

DPD was measured by a competitive immunoassay using a rat monoclonal anti-DPD antibody. The urinary creatinine level was used to adjust DPD values for variations in urine volume. The results were expressed as nanomoles of DPD per millimole of creatinine to avoid the possible influence of the glomerular filtration rate (Byun and Lee, 2010).

#### Statistical analysis

Statistical analysis was done using the computer software program prism (Comshare's version of a decision support system = DSS) version 3.3. Data were expressed as the means ± SEM. Statistical significance for data was determined using the one-way analysis of variance (ANOVA) with Bonferroni's Multiple Comparison Test to find the inter-group significance. p<0.05 was considered statistically significant.

## Results

The mean values for body weight, uterine weight, osteocalcin, BSALP, DPD, calcium and phosphorous measurements in the control and the OVX groups and 60 days after the treatment with either alendronate or leptin were shown in the Table 1.

#### Body and uterine weights

The final body weight of the OVX group was significantly higher than that of the sham group (p<0.001). The OVX-induced body weight gain was significantly inhibited by leptin (p<0.001). As expected, the final uterus weight was significantly (p<0.001) reduced in the OVX group as compared to the sham group, indicating that estrogen deficiency resulted in the atrophy of the uterus of OVX rats. Leptin administration prevented the loss of uterine

weight compared to the OVX group (p<0.01), but still resulted in a lower uterine weight than the sham group (Tab. 1).

#### Serum Calcium and Phosphorous Levels

The present data showed a significant decrease in serum calcium levels (p<0.001) in the OVX rats when compared to the control rats. The results in the Table 1 revealed that ovariectomized rats treated with alendronate demonstrated a significant increase (p<0.001) of serum Ca levels accompanied with a non-significant decrease (p>0.05) in serum p level when compared to the ovariectomized rats. Ovariectomized rats treated with leptin showed a non-significant decrease (p>0.05) in serum Ca level accompanied with a non-significant increase (p>0.05) in serum p level when compared to the ovariectomized rats.

#### Bone turnover biomarkers

After 60 days, the levels of serum osteocalcin, serum BSALP and urinary DPD had significantly increased in the OVX group as compared to the groups I (p<0.001), III and IV (p<0.01). However, no significant differences in the serum levels BSALP were observed in the groups I, III and IV (p>0.05).

## Discussion

#### Body and Uterine Weights

As seen in many studies, ovariectomized rats have significantly higher body weights compared to sham-operated rats due to fat deposition caused by estrogen deficiency (Zhao et al, 2011). Studies have noted that increased body weight provides an additional stimulus for bone neoformation, serving as a partial protection against the osteopenia that occurs in long bones from supporting body weight (Notomi et al, 2003). In this study, as expected, the final uterus weight was significantly reduced in the OVX group as compared to the sham group, indicating that estrogen deficiency resulted in the atrophy of the uterus of OVX rats.

Leptin administration prevented the loss of uterine weight compared to the OVX group but it still resulted in a lower uterine weight than the sham group. Leptin directly stimulates gonadotropin secretion of the anterior pituitary gland. The mechanism whereby leptin directly stimulates gonadotroph function is not known. There is reason to believe that the effects on hypothalamic GnRH release could be mediated by neuropeptide Y (Baldock et al, 2006). Increased uterine weight in leptin-treated rats seems to occur as a result of proliferative responses to increased ovarian estrogen production.

*Serum Calcium and Phosphorous Levels*

Serum calcium level was significantly decreased while serum phosphorus was non significantly increased in the OVX group as compared to the control group. In this study, hypocalcemia associated with ovariectomy was due to decrease of intestinal calcium absorption and increase of renal excretion of calcium due to estrogen deficiency (Saleh and Saleh, 2011). Estrogen deficiency was associated with changes in expression of many proteins involved in distal tubule calcium reabsorption (Lee et al, 2007).

Leptin administration caused non-significant decrease of serum Ca<sup>++</sup> level compared to the ovariectomized rats. Leptin has two opposing effects on calcium metabolism. It has been linked to increased calcium excretion in the kidney. Loss of leptin function contributes to aberrant regulation of renal 25-hydroxyvitamin D3 metabolism by increasing the gene expression of renal 1 $\alpha$ -hydroxylase, which catalyzes 1, 25-dihydroxyvitamin D3 [1, 25(OH) 2D3] synthesis and thus elevates serum 1, 25(OH) 2D3 concentrations (Matsunuma and Horiuchi, 2007). Increased of calcium level in ovariectomized rats after administration of leptin, was due to increased ovarian estrogen production.

*Bone Turnover Biomarkers*

In the present study, the obtained data revealed that the induction of osteoporosis by ovariectomy caused significant increase in skeletal remodeling, as was evidenced by the increased levels of the bone turnover markers, osteocalcin, specific bone alkaline phosphatase and deoxypyridinoline. This elevation of serum osteocalcin concentration in OVX-group indicates an increase in bone turnover, which would result in bone loss so that an imbalance would exist between bone resorption and bone formation (Byun and Lee, 2010).

Leptin prevented the increased rate of bone formation in an effort to restore the disturbed balance of bone turnover. The consequent effect of leptin on bone formation was reflected in the increased levels of circulating osteocalcin observed after ovariectomy.

Plasma alkaline phosphatase was significantly increased in the OVX-rats, indicating an increased osteoblastic activity on bone formation (Alam et al, 2006).

Treatment with alendronate or leptin caused significant decrease of serum alkaline phosphatase and significant increase of serum calcium levels compared with OVX group, but there was no significant difference between both drugs.

With respect to the elevated urinary DPD level in ovariectomized rats, it is well known that bone turnover is increased by ovariectomy and the activity of osteoclasts is higher than that of osteoblasts, hence osteoporosis develops (Hassan et al, 2010). In osteoporosis there was a bone turnover with the resorption exceeding the formation (Li et al, 2002).

Leptin decreases both bone formation and resorption. These decreases are reflected in reductions in serum osteocalcin and BSALP concentrations and in urinary DPD levels. This suggests that leptin prevents OVX-induced increases in bone turnover in rats. Leptin also suppressed the increased body weight of OVX rats and returned it to the sham levels, significantly increased uterine weight compared to OVX rats.

The protective effect of leptin may be due to its peripheral and central action. Peripherally, leptin interacts with bone marrow stromal cells, osteoblasts, and osteoclasts to increase bone mass (Jackson et al, 2011). Leptin increases both expression of osteogenic genes in stromal cells (van Marken et al, 2009) and osteoblast proliferation and decreases osteoclastogenesis (Cirmanová et al, 2008). Centrally, leptin inhibits bone formation by binding to its receptors located in hypothalamus and thereby activating the sympathetic nervous system (SNS), which requires the adrenergic  $\beta$ 2 receptors (Adrb2) expressed in osteoblasts (He et al, 2011). Downstream of Adrb2, leptin signaling activates molecular clock genes that regulate osteoblast proliferation and hence bone formation (Sato et al, 2007). Leptin also inhibits bone resorption by the stimulation of sympathetic nervous system and CART (cocaine amphetamine regulated transcript) (Elefteriou et al, 2005). In addition to the sympathetic nervous system, leptin also interacts with various hypothalamic neuropeptides, such as neuropeptide Y (Baldock et al, 2006), neuromedin U (Sato et al, 2007) and/or serotonin (Yadav et al, 2006), which might modulate the effects of leptin on bone.

**Conclusions**

The results obtained in the present study provide evidence that leptin prevents OVX-induced increases in metabolic bone turnover in rats. Leptin chronic administration has a significant effect in bone formation and bone resorption biomarkers of ovariectomy induced-osteoporosis in rats.

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