Abstract. – OBJECTIVES: The present study assessed the potential role of leptin administration in the protection and intervention against glucocorticoid-induced secondary osteoporosis in female rats.

MATERIALS AND METHODS: For this purpose five groups of female Sprague Dawley rats were classified into: (1) negative control group in which the healthy rats received saline as vehicle, (2) a group orally administered with prednisolone (5 mg kg b.wt.⁻¹) daily for six months (osteoporotic group), (3) a group subcutaneously administered with leptin (400 µg kg b.wt.⁻¹) three times weekly for six months (positive control), (4) a group orally administered with prednisolone daily with simultaneous subcutaneous administration of leptin three times weekly for six months (protective group), and (5) a group orally administered with prednisolone daily for six months then subcutaneously administered with leptin three times weekly for other six months (therapeutic group).

RESULTS: The obtained data revealed that prednisolone administration resulted in significant decrease in serum osteoprotegerin (OPG) level accompanied with significant increase in serum receptor activator of nuclear factor-κB ligand (RANKL) and β₂-microglobulin levels in comparison with the negative control group. Moreover, prednisolone significantly decreased bone mineral density and content of different areas of the right femur bones as compared to the negative control group. Furthermore, administration of leptin with/after stopping prednisolone administration resulted in a marked modulation in the majority of bone biomarkers as well as improvement in bone mineral density and content.

CONCLUSIONS: Leptin provided promising effect on bone through its direct action on bone and matrix mineralization.

Key Words: Prednisolone, Osteoporosis, Leptin, Bone biomarkers, Bone mass.

Introduction

Bone remodeling is a dynamic process in which activated osteoclasts resorb bone and osteoblasts generate a bone matrix that undergoes mineralization. This process repairs microdamages and the microscopic cracks that develop in bone during regular activity-and ensures skeletal strength. A number of local and systemic factors mediate bone cell activity. Systemic regulators include endogenous parathyroid hormone (PTH), vitamin D metabolites, prostaglandins, cortisol, and sex hormones. A number of cytokines and growth factors regulate bone cell function at the local level. For example, bone resorption and formation are tightly orchestrated via the RANK/receptor activator of NF-kappa B ligand (RANKL)/osteoprotegerin (OPG) system. Estrogen deficiency, glucocorticoid use, and immune-mediated conditions lead to an imbalance in the RANKL/OPG ratio, inducing osteoclastogenesis and accelerated bone resorption leading to a reduction in the skeletal mass which is the hallmark of the osteoporosis.

Osteoporosis is a worldwide health problem with a high prevalence. It is a serious metabolic bone disorder that often results in hip fracture and is usually asymptomatic in its initial stages. In this disease, the bone mineral density is reduced, bone microarchitecture is disrupted, and the amount and variety of non-collagenous proteins in bone are altered. Osteoporosis is classified into primary and secondary ones, and glucocorticoid-induced osteoporosis is the most common form of secondary osteoporosis. Glucocorticoid-induced osteoporosis is characterized by decreased bone formation and its pathogenesis is probably different from that of postmenopausal osteoporosis, primary osteoporosis, which is mainly caused by an increased bone resorption.

Glucocorticoids are widely used for treatment of a variety of diseases, including collagen diseases, bronchial asthma and skin diseases. Because of improvements in the outcome of these diseases, the long-term side effects of glucocorticoids, including osteoporosis, have become an
important problem. Glucocorticoids decrease bone formation, suppress osteoblastic functions, inhibit the production of bone matrix components, increase osteoblastic and osteocytic apoptosis, decrease transforming growth factor-β (TGF-β1) type-1 receptor, modulate insulin-like growth factor-I (IGF-I), decrease intestinal calcium absorption and increase calcium excretion. Moreover, glucocorticoids cause secondary hyperparathyroidism and promote hypogonadism through suppressing gonadotropin and inhibiting dehydroepiandrosterone (DHEA) secretion. Furthermore, glucocorticoids may directly promote osteoclastogenesis by increasing receptor activator of NF-κB ligand and colony-stimulating factor-1 (CSF-1) levels, while decreasing osteoprotegerin level.

Leptin is a circulating hormone produced primarily by the adipose tissue. Biological and physiological roles of leptin on bone include elevation of bone size, bone mineral content and density. Moreover, leptin enhances the differentiation of human bone marrow stromal cells (hBMSC) into osteoblasts, inhibits osteoclast generation and attenuates the decrease in bone formation. This effect may be achieved through a direct effect on the expression of OPG and/or RANKL in osteoblasts and marrow stroma cells.

The current study aimed to evaluate the potential role of leptin as an alternative hormone for prevention and intervention against prednisolone-induced secondary osteoporosis in female rats.

Materials and Methods

Experimental Design

Forty adult female Sprague Dawley rats (130-150 g) were obtained from the Animal House Colony of National Research Centre, Cairo, Egypt. The animals were housed in plastic cages at room temperature (25±2°C) under a 12 hr dark-light cycle. They were provided with tap water and balanced diet ad libitum and allowed to acclimate for two weeks to housing conditions. They were divided into five groups each comprised 8 rats, as follows: group I, the negative control group in which the healthy rats received saline as vehicle; group II, was orally administered with prednisolone daily with simultaneous subcutaneous administration with 400 µg kg b. w. of leptin three times weekly for six months (positive control group); group IV, was orally administered with 5 mg kg b. w. of prednisolone daily with simultaneous subcutaneous administration with 400 µg kg b. w. of leptin three times weekly for six months (protective group) and group V, was orally administered with 5 mg kg b. w. of prednisolone daily for six months then subcutaneously administered with 400 µg kg b. w. of leptin three times weekly for other six months (therapeutic group).

Sample Collections

At the end of the experimental period, blood samples from fasting rats were withdrawn from retro-orbital venous plexus under diethylether anaesthesia in dry clean centrifuge tubes and left to clot. Then, the blood samples were centrifuged at 3000 rpm for 15 min at 4°C where the clear sera were separated and immediately stored at −20°C in clean plastic Eppendorf until analyses. The animals were then rapidly sacrificed and the right femurs were harvested. Each right femur bone was carefully cleaned, length and weight were recorded and then stored in formalin buffer 10% for dual energy X-ray absorptiometry (DEXA). Bone mineral density (BMD) and bone mineral content (BMC) of each right femur were measured by DEXA using Norland XR46, version 3.9.6/2.3.1 instrument (Norland X-R-46 version 3.9.6, Peachtree City, GA, USA) equipped with dedicated software for small animal measurements. This technique provided an integrated measure of right femur proximal, distal and total areas.

Analytical Determinations

Serum OPG and RANKL levels were determined by enzyme linked immunosorbent assay (ELISA) technique using R&D Elisa (Sorin Biomedica, Eti-System, Denlay Instruments Ltd, England) kit as described by O’Brien et al and Teng et al respectively. While, serum β2-microglobulin level was assayed by ELISA procedure using International Immuno-Diagnostics kit (Orgentec Diagnostika GmbH, Mainz, Germany) as described by Crisp et al.

Statistical Analysis

Results were statistically analyzed using the method of Saunders and Trapp to determine the significance between the different investigated groups using SPSS version 15 (Chicago, IL, USA). One-way ANOVA test of significance was used to evaluate the difference between more
than two groups. This procedure performs one way analyses of variance. It also tests for equality of variances between levels. The F test with the associated p-value, was used to test the differences among the means. The F test assumes that the within group variances are the same for all groups. Also, receiver operating characteristic (ROC) curves were calculated to evaluate classification and prediction models for decision support, diagnosis, and prognosis. ROC analysis investigates the accuracy of a model’s ability to separate positive from negative cases and the results are independent of the prevalence of positive cases in the study population. It is especially useful in evaluating predictive models or other tests that produce output values over a continuous range, since it captures the trade-off between sensitivity and specificity over that range.

The results were expressed as mean ± SD. Comparison between groups was carried by ANOVA analysis. Comparisons were done against negative control and osteoporotic groups.

### Results

The data in Table I indicated the effect of leptin administration on serum osteoprotegerin (OPG), Receptor Activator of Nuclear factor-κB Ligand (RANKL) and beta 2-microglobulin (β₂-microglobulin) levels in glucocorticoid-induced secondary osteoporosis in female rats. Administration of prednisolone caused significant decrease in serum OPG level associated with significant increase in serum RANKL and β₂-microglobulin levels in comparison with the negative control group. While, the positive control group showed significant increase in serum OPG level and insignificant change in the other two parameters as compared with the negative control group. Moreover, leptin administration for the protection or intervention against osteoporosis revealed significant increase in serum OPG level with a concomitant significant decrease in serum RANKL and β₂-microglobulin levels as compared with the untreated osteoporotic group.

The results in Table II demonstrated the effect of leptin administration on bone mineral density of proximal (BMD- proximal), distal (BMD- distal) and total (BMD- total) areas of femur bones in glucocorticoid-induced secondary osteoporosis in female rats. The untreated osteoporotic group showed significant decrease in BMD of the three measured areas in comparison with the negative control group. While, the positive control group showed insignificant increase in BMD of proximal, distal and total areas of rats femur bones in comparison with the negative control one. However, leptin administration for the protection from osteoporosis caused significant increase in BMD of the three areas as compared with the untreated osteoporotic group. Meanwhile, the therapeutic group showed insignificant increase in BMD of proximal area and significant increase in BMD of distal and total areas in comparison with the untreated one.

Table III represented the effect of leptin administration on bone mineral content of proximal (BMC- proximal), distal (BMC- distal) and total (BMC- total) areas of femur bones in glucocorticoid-induced secondary osteoporosis in female rats. Prednisolone administration produced significant decrease in BMC of proximal, distal and total areas in comparison with the negative control group. Moreover, the positive control group showed significant increase in BMC of all areas in comparison with the untreated osteoporotic group.

### Table I. Statistical analyses for serum OPG, RANKL and β₂-microglobulin levels in all groups (number of rats = 8/group).

<table>
<thead>
<tr>
<th>Parameters/Groups</th>
<th>OPG (ng mL⁻¹)</th>
<th>RANKL (pg mL⁻¹)</th>
<th>β₂-microglobulin (µg mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>2.91 ± 0.52</td>
<td>61.78 ± 11.08</td>
<td>0.17 ± 0.02</td>
</tr>
<tr>
<td>Osteoporotic</td>
<td>1.20 ± 0.27a</td>
<td>162.71 ± 16.94a</td>
<td>0.26 ± 0.02</td>
</tr>
<tr>
<td>Positive control</td>
<td>3.41 ± 0.70a</td>
<td>53.81 ± 9.88</td>
<td>0.18 ± 0.02</td>
</tr>
<tr>
<td>Protective</td>
<td>2.11 ± 0.34b</td>
<td>95.73 ± 10.67b</td>
<td>0.18 ± 0.01b</td>
</tr>
<tr>
<td>Therapeutic</td>
<td>1.78 ± 0.24b</td>
<td>107.29 ± 6.32b</td>
<td>0.19 ± 0.02</td>
</tr>
</tbody>
</table>

*Significant change at p > 0.05 in comparison with the negative control group. **Significant change at p > 0.05 in comparison with the osteoporotic group.
Potential role of leptin against glucocorticoid-induced secondary osteoporosis in adult female rats

In order to define the discriminating cutoff values for each parameter, receiver operating characteristic (ROC) curves were done by using the osteoporotic vs. normal levels. From these curves, the cut-off value of each parameter was calculated (according to its sensitivity and specificity). The data were expressed as “area under curve” (AUC), significance ($p$), sensitivity % and specificity %. Chi-square test (or $\chi^2$-test) analyses was then carried out in order to calculate the % of rats that reverted to the normal level for each parameter.

The OPG, RANKL and $\beta_2$-microglobulin cut-off values were 1.7 ng mL$^{-1}$, 110.2 pg mL$^{-1}$ and 0.215 µg mL$^{-1}$ respectively with 100% sensitivity and 100% specificity ($p < 0.001$). The positive control and the protective groups gave 100 and 87.5% normal OPG respectively and 100% normal RANKL and $\beta_2$-microglobulin. While, the therapeutic group gave 50, 75 and 87.5% normal OPG, RANKL and $\beta_2$-microglobulin respectively (Table IV). The improvement in the levels of these biomarkers were highest in the positive control and the protective groups followed by the therapeutic one.

The BMD of proximal, distal and total areas of female rats femur bones cut-off values were 92.75, 88.70 and 93.70 mg cm$^{-2}$ respectively with 100% sensitivity and 100% specificity ($p < 0.001$). The positive control group gave 100% normal BMD of all areas. While, the protective and the therapeutic groups gave 33.3 and 16.66 % normal BMD-proximal, 66.67% normal BMD-distal as well as 16.66 % normal BMD-total respectively (Table V). The improvement in BMD of the measured areas was highest in the positive control group.

Moreover, the BMC of proximal and distal areas of female rats femur bones cut-off values were 43.9 and 50.10 mg respectively with 100% sensitivity and 83% specificity ($p<0.01$). While, the BMC of total area of rats femur bones cut-off value was 222.50 mg with 100% sensitivity and 100% specificity ($p < 0.001$). The positive control group gave 83.3% normal BMC-proximal and 100% normal BMC-distal and total areas. Meanwhile, the protective and the therapeutic groups gave 83.3% normal BMC in all areas (Table VI). The improvement in BMC was highest in the positive control followed by the protective and the therapeutic groups.

## Discussion

Osteoporosis is a metabolic bone disease that is characterized by reduced bone mass and deterioration of bone microarchitecture$^{29}$. The present

### Table II. Statistical analysis for BMD in proximal, distal and total areas of rats femur bones in all groups (number of rats=8/group).

<table>
<thead>
<tr>
<th>Parameters/Groups</th>
<th>BMD-proximal (mg cm$^{-2}$)</th>
<th>BMD-distal (mg cm$^{-2}$)</th>
<th>BMD-total (mg cm$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>108.5 ± 13.3</td>
<td>109.1 ± 17.1</td>
<td>109.2 ± 13.8</td>
</tr>
<tr>
<td>Osteoporotic</td>
<td>77.5 ± 5.6$^a$</td>
<td>76.3 ± 5.6a</td>
<td>78.1 ± 6.2$^a$</td>
</tr>
<tr>
<td>Positive control</td>
<td>110.5 ± 13.5</td>
<td>110.6 ± 17.5</td>
<td>110.9 ± 15.1</td>
</tr>
<tr>
<td>Protective</td>
<td>94.4 ± 16.7$^b$</td>
<td>96.1 ± 16.6$^b$</td>
<td>95.0 ± 16.9$^b$</td>
</tr>
<tr>
<td>Therapeutic</td>
<td>90.5 ± 12.5</td>
<td>95.3 ± 16.6$^b$</td>
<td>93.1 ± 11.4$^b$</td>
</tr>
</tbody>
</table>

$^a$Significant change at $p > 0.05$ in comparison with the negative control group. $^b$Significant change at $p > 0.05$ in comparison with the osteoporotic group.

### Table III. Statistical analysis for BMC in proximal, distal and total areas of rats femur bones in all groups (number of rats=8/group).

<table>
<thead>
<tr>
<th>Parameters/Groups</th>
<th>BMC-proximal (mg)</th>
<th>BMC-distal (mg)</th>
<th>BMC-total (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>43.9 ± 8.8</td>
<td>68.2 ± 10.1</td>
<td>275.6 ± 26.3</td>
</tr>
<tr>
<td>Osteoporotic</td>
<td>30.4 ± 3.4$^a$</td>
<td>47.4 ± 6.5$^a$</td>
<td>196.7 ± 22.3$^a$</td>
</tr>
<tr>
<td>Positive control</td>
<td>45.9 ± 7.3</td>
<td>70.6 ± 8.8</td>
<td>279.1 ± 19.5</td>
</tr>
<tr>
<td>Protective</td>
<td>39.6 ± 6.7$^b$</td>
<td>65.0 ± 11.6$^b$</td>
<td>250.6 ± 33.9$^b$</td>
</tr>
<tr>
<td>Therapeutic</td>
<td>38.5 ± 5.8$^b$</td>
<td>63.0 ± 13.5$^b$</td>
<td>242.0 ± 22.8$^b$</td>
</tr>
</tbody>
</table>

$^a$Significant change at $p > 0.05$ in comparison with the negative control group. $^b$Significant change at $p > 0.05$ in comparison with the osteoporotic group.
study showed that treatment with prednisolone resulted in significant reduction in serum OPG level. This finding was supported by that of Vidal et al21 who reported that glucocorticoids reduce OPG mRNA level and reduce production of OPG from osteoblasts and/or marrow stroma cells. While, the leptin administration produced a significant increase in the serum OPG level in prednisolone-treated rats. Leptin has a direct effect on the osteoblast cells stimulating OPG expression22.

Moreover, the present study revealed that the treatment with prednisolone caused a significant elevation in the serum RANKL level. This result agreed with that of Mclaughlin et al23 who reported that glucocorticoids promote osteoclastogenesis via increasing RANKL and decreasing the OPG expression. Leptin administration caused a significant decrease in serum RANKL level in the prednisolone-treated rats. Leptin has a direct effect on the osteoblast cells stimulating OPG expression22.

Chronic glucocorticoid therapy is associated with low BMD with evidence of low bone turnover and high prevalence of fractures26. The explanation of decreased BMD due to prednisolone administration is the induction of apoptosis for both osteoblasts and osteocytes, leading to suppression of bone formation and stimulation of osteoclastogenesis which may be mediated through increasing β₂-microglobulin24. While, the lowering effect of leptin on β₂-microglobulin is attributed to its inhibitory action on osteoclasts25, where it acts as an inhibitor of bone resorption. Leptin has been shown to play a role on bone both in vitro and in vivo. The administration of leptin in vitro induced the expression of leptin receptors on stromal cells, and inhibition of the differentiation of osteoclasts leading to the inhibition of osteoclastogenesis25.

### Table IV
Cross tabulation showing statistical analysis (chi-square tests “χ²”) of OPG, RANKL and β₂-microglobulin ordinariness in the different groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>OPG (ng mL⁻¹)</th>
<th>RANKL (pg mL⁻¹)</th>
<th>β₂-microglobulin (µg mL⁻¹)</th>
<th>χ²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&gt; 1.7</td>
<td>≤ 1.7</td>
<td>≥ 110.2</td>
<td>≤ 0.215</td>
</tr>
<tr>
<td>Negative control</td>
<td>8</td>
<td>0</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Osteoporotic</td>
<td>0</td>
<td>8</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Positive control</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Protective</td>
<td>7</td>
<td>1</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Therapeutic</td>
<td>4</td>
<td>4</td>
<td>6</td>
<td>2</td>
</tr>
</tbody>
</table>

### Table V
Cross tabulation showing statistical analysis (chi-square tests “χ²”) of BMD-proximal, distal and total areas of rats femur bones ordinariness in the different groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>BMD-proximal (mg cm⁻²)</th>
<th>BMD-distal (mg cm⁻²)</th>
<th>BMD-total (mg cm⁻²)</th>
<th>χ²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&gt; 92.8</td>
<td>≤ 92.8</td>
<td>&gt; 88.7</td>
<td>≤ 88.7</td>
</tr>
<tr>
<td>Negative control</td>
<td>6</td>
<td>0</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Osteoporotic</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Positive control</td>
<td>6</td>
<td>0</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Protective</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Therapeutic</td>
<td>1</td>
<td>5</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>
Potential role of leptin against glucocorticoid-induced secondary osteoporosis in adult female rats

It has been shown that leptin has direct effects on bone (anabolic). Leptin increased proliferation of isolated fetal rat osteoblasts comparably with IGF-1, and these cells expressed the signaling form of the leptin receptor. The increase in IGF-1 level by leptin (25%) was confirmed in this study (data not shown). The study of Thomas et al29 on human marrow stromal cell line indicated that leptin promoted differentiation into an osteoblast phenotype and increased synthesis of bone matrix proteins such as type I collagen and osteocalcin. The level of osteocalcin was increased (60%) in the groups administered with leptin (data not shown). Moreover, Burguera et al14 found that leptin increases osteoprotegerin levels and decreases RANK ligand levels in human marrow stromal cells. This event is supported by Maor et al30 who found that leptin directly promotes the growth of chondrocytes. These are the possible mechanisms by which leptin could increase BMD in the present study.

Also, the current study revealed that chronic prednisolone administration produced significant decrease in BMC of right femur bones. Studies of Canalis31 and Koczwoska et al12 stated that chronic glucocorticoid therapy has a direct negative effect on BMC with concomitant decrease in bone matrix available for mineralization. Steroid therapy also increases the risk for long bone fractures (proximal femoral epiphysis, radius)33.

Leptin administration to osteoporotic rats resulted in a significant increase in BMC of right femur. Jürimäe and Jürimäe34 reported that leptin has strong positive effect on BMC and BMD values. The effect of leptin on chondrocyte gene expression, cell cycle, apoptosis and matrix mineralization were assessed using primary chondrocyte culture35. In primary chondrocyte cultures, the matrix mineralization in mice chondrocytes becomes strong with leptin administration, so Kishida et al35 suggested that peripheral leptin signaling plays an essential role in endochondral ossification at the growth plate.

Conclusions

The beneficial effects of leptin in modulating the majority of bone markers and improving bone mineral density and bone mineral content in glucocorticoid-treated animals are mostly mediated by its anabolic and/or antiresorptive effects. Based on the current results, we recommend leptin as a potent alternative hormone for preserving bone tissue against secondary osteoporosis induced by glucocorticoid therapy.

References


15) O'Brien EA, Williams JHH, Marshal MJ. Osteoprotegerin is produced when prostaglandin synthesis is inhibited causing osteoclasts to detach from the surface of mouse parietal bone and attach to the endocapillar membrane. Bone 2001; 28: 208-214.


