The Potential Role of Leptin Administration in Intervention Against Glucocorticiods-Induced Osteoporosis in Male Rats

Omyma Galal[a], Haytham Mohamed[b], Ahmad Mohammad Abdel-Aleem[c], Sara Salah Abdel-Hameed[b]*, Rehab H. Abdel-Aziz[b]

[a]Department of Medical Physiology, Faculty of Medicine, Assiut University, Assiut71515, Egypt

[b]Department of Medical Physiology, Faculty of Medicine, South Valley University, Qena 83523, Egypt.

[c]Department of Histology, Faculty of Medicine, Al-Azhar University (Assuit Branch), Assuit 71524, Egypt.

Abstract

Background: Glucocorticoids are widely used in the clinical setting. The direct impacts of glucocorticoids on bones involve both transient and early rise in bone resorption and long-term inhibition of bone formation. Leptin enhances the differentiation of human bone marrow stromal cells into osteoblasts, suppresses the generation of osteoclasts and attenuates the reduction in bone formation**.**

Objectives: This study aimed to assess the effects of prednisolone on causing osteoporosis in rats and the effects of leptin on osteoporotic rats**.**

Material and Methods: The study was performed on 21 adult male white albino rats. They were divided equally into; control, osteoporotic and treated groups. The osteoporotic and treated groups were given 5 mg/kg/day oral prednisolone for 3 months. The treated group was given10 µg/kg/day intraperitoneal (IP) leptin for the next 2 months. Blood samples were collected for determination of serum calcium, phosphorous, alkaline phosphatase and osteocalcin levels. Bone of the right femur was used for histopathological examination**.**

Results: The osteoporotic group had a highly significant decrease in serum calcium and phosphorus, in addition to significant increase serum alkaline phosphatase and osteocalcin. Also, there was decrease in cortical bone thickness and number of osteocyte-containing lacunae and increased number of osteocyte-free lacunae in osteoporotic group. Treatment with leptin improved both biochemical and structural changes in prednisolone induced osteoporosis.

Conclusions: Leptin antagonizes the effects of glucocorticiods induced osteoporosis. It normalizes serum calcium and phosphorus. It improves bone mineral density and bone size.

Keywords: Leptin; Osteocalcin; Osteoporosis; Glucocorticoid; Bone mineral density.

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***Correspondence**: sara.salah@ med.svu.edu.eg

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Introduction

Glucocorticoids have been used widely in clinical settings for management of different disorders such as rheumatoid arthritis, asthma and lupus arthritis. However, there are side effects of using these agents, especially at long-term usage. These adverse effects involve lipid metabolism abnormality, glucose metabolic disorder, and fat redistribution and water–sodium retention **(Mitchner et al., 2009)**.

Glucocorticoids induced osteoporosis is one of the most serious complications in long term glucocorticoid treated patients. Long term using of glucocotricoids results in a reduction in mineral density and bone mass leads finally to an increased risk of bone fractures **(DEAL, 2009)**.

The direct impacts of glucocorticoids on bone involve both transient and early increase in resorption of bone, enhancement of bone resorption, and long-term suppression of bone formation, at cellular and tissue levels. Thus, it is necessary to identify medications that could prevent osteoporosis related to glucocorticoid **(Hazavehei et al., 2007)**.

Leptin is a hormone produced primarily by adipose tissue **(Bouret et al., 2015)**. It promotes segregation of bone marrow stromal cells into osteoblasts in human, andsuppresses generation of osteoclast and mitigates the reduction in bone formation **(Mosekilde et al., 2013)**.

Materials and methods

Chemicals and drugs

Calcium, phosphorus, alkaline phosphatase and osteocalcin kits and leptin were purchased from Sky Medical Company, Assiut Egypt. Prednisolone was obtained from Al-Esraa Pharmaceuticals Optima. Cairo, Egypt.

Animals and Experimental design

This study was performed in Physiology Department, Qena Faculty of Medicine, South Valley University, Egypt. Animals were obtained from the animal house of Qena Faculty of Medicine, South Valley University, Egypt. Twenty-one male white albino rats were 170-300 grams weigh and 3 months of age. Rats were housed in clean stainless-steel cages (42 x 21 x 20), natural light/dark cycle in a room infused with air and supplied with free entrance to food and water.

Adaptation took one week for all rats before the experimental study was started.

Experimental procedure was approved by the Institutional Review Board (IRB) of Medical Ethics Committee, Qena Faculty of Medicine, South Valley University. All animal experiments complied with the Arrive guidelines and were performed in agreement with UK.

Rats were randomly categorized into three groups; each group involved seven rats.

Group I (Control group): Rats were given distilled water orally.

Group II (Osteoporotic; prednisolone treated group): Rats were given prednisolone (5 mg/kg/) orally for 3 months**(Chen et al., 2017).**

Group III (Leptin treated group): Rats were given prednisolone in the same dose and duration as group II then, they were injected with IP leptin(10 µg/kg/day) for the next 2 months**(Abdel-Sater, & Mansour. 2012)**

At the end of the experiment, rats were weighed with a top loader weighing balance (Model D0030, A&D Company Limited, USA).Thiopental sodium (50 mg/kg) was used for anesthetizing the rats. Rats were sacrificed by decapitation. Blood samples were collected by cardiac puncture and centrifuged at 2000 rpm for 15 minutes (min) within 30 min of blood collection to determine serum calcium, phosphorous, alkaline phosphatase and osteocalcin levels. Bone of right femur was carefully cleaned, for histopathological studies.

Histopathological Examination

Bone of right femur was used for histological screening. Samples were 10%

formalin fixed; paraffin embedded and cut into 5 μm tissue sections. Tissue slides were stained by hematoxylin and eosin (H&E). Images were analyzed by Optimas (Media Cybernetics, 1998 version 6.21.19); where three parameters were examined and assessed: thickness of cortical bone, counting osteocyte lacunae and nuclei and counting the number of all osteocyte lacunae with or without nuclei.

Statistical Analysis

All data were collected, tabulated and statistically analyzed using SPSS 22.0 for windows (SPSS Inc., Chicago, IL, USA) & MedCalc 13 for windows (MedCalc Software ,Ostend, Belgium).All statistical comparisons were two tailed with p-value ≤ 0.05 was considered significant, p<0.001 was considered highly significant.

Results

Effect of prednisone on serum levels of calcium, phosphorus, alkaline phosphatase and osteocalcin

Comparing osteoporotic group with control group, it showed significant decrease (p<0.05) in the mean values of serum calcium (6.60±1.25 Vs. 10.29±0.38 mg/dL), and serum phosphorus (5.21± 1.43 Vs. 7.39±1.45 mg/dL) and significant increase (p<0.05) in the mean values of serum alkaline phosphatase (196.43±45.57 Vs. 99.14±20.76 IU/L) and serum osteocalcin(2.21±0.32 Vs. 1.38±0.34 ng/mL).These results are shown in tables(1-4).

Effect of leptin on serum calcium, phosphorus, alkaline phosphatase and osteocalcin levels

When comparing leptin treated group, with other groups, it showed significant increase (p<0.05) in the mean values of serum calcium (9.11± 0.89 mg/dL), serum phosphorus (7.27± 1.54 mg/dL) and significant decrease (p<0.05) in the mean value of serum serum alkaline phosphatase (131.86±27.06 IU/L) and osteocalcin(1.28± 0.25 ng/mL). These results were shown in (**Table 1,2,3**,and **4**).

Difference between leptin treated and control groups

Leptin treated group, when compared with control group, showed no significant statistical difference (p>0.05) in the mean values of serum calcium, phosphorus, alkaline phosphatase orosteocalcin(9.11± 0.89 Vs.10.29±0.38 mg/dL,7.27± 1.54 Vs.7.39±1.45 mg/dL,131.86± 27.06 Vs. 99.14±20.76 IU/L and 1.28±0.25 Vs. 1.38± 0.34 ng/mL respectively). These results were shown in (**Table 1,2,3**,and **4**).

Effect of leptin on osteoporosis

When comparing leptin treated group, with osteoporotic group, there was a significant increase (p<0.05) in the mean values of serum calcium and phosphorus (9.11± 0.89 Vs.6.60± 1.25 mg/dL,7.27± 1.54 Vs.5.21± 1.43 mg/dL) and a significant decrease (p<0.05) in the mean value of alkaline phosphatase and osteocalcin(131.86± 27.06 Vs.196.43± 45.57 IU/L and 1.28± 0.25 Vs. 2.21± 0.32 ng/mL). These results were shown in (**Table 1,2,3**,and **4**).

Histopathological results

a. Cortical bone thickness (in micrometers):

By comparing cortical thickness in all groups there was a significant difference between control group and osteoporotic group while the difference between control group and leptin treated group is non-significant. Also there was a significant difference between osteoporotic group and leptin treated group (**Table 5, Fig. 1**).

b. Number of osteocyte containing lacunae (per HPF):

Comparing osteocyte number in all groups, there was a significant difference between control group and osteoporotic group. On the other side, there was a non-significant difference between osteoporotic and leptin treated group (**Table 5, Fig. 1**).

c. Number of osteocyte-free lacunae (per HPF):

Comparing osteocyte-free lacunae in all groups, there was a strong significant difference between control group and osteoporotic group. However, there was a non-significant difference between osteoporotic and leptin treated group (**Table 5, Fig. 1**).

Table 1. Comparison between the three studied groups regarding calcium level

Variables		*Group (1) Control group (no.= 7)*	*Group (2) Osteoporotic group (no.= 7)*	*Group (3) Leptin treated group (no.= 7)*	**P-value**
Calcium (mg /dl)	**Mean± SD**	10.29± 0.38	6.60± 1.25	9.11± 0.89	**<0.001** P_{1-2}**<0.001** P_{1-3}= 0.279 P_{2-3}= **0.024**
	Median	10.30	6.90	9.10	
	IQR	9.90 – 10.70	5.10- 7.80	8.10- 10.0	
	Minimum	9.80	4.80	7.90	
	Maximum	10.80	8.00	10.30	

SD: standard deviation, p≤0.05 considered significant, SD: deviation, IQ: interquartile range, Kruskal-Wallis test for independent samples.

Table 2. Comparison between the three studied groups regarding phosphorus level

Variables		*Group (1) Control group (no.= 7)*	*Group (2) Osteoporotic group (no.= 7)*	*Group (3) Leptin treatedgroup (no.= 7)*	**P-value**
Phosphorus (mg /dl)	**Mean± SD**	7.39± 1.45	5.21± 1.43	7.27± 1.54	**<0.021** P_{1-2}**= 0.039** P_{1-3}= 1.00 P_{2-3}= 0.053
	Median	7.0	5.03	7.01	
	IQR	6.30 – 8.0	4.09- 6.02	6.0- 8.07	
	Minimum	6.0	3.90	5.04	
	Maximum	10.20	8.01	9.80	

SD: standard deviation, p≤0.05 considered significant, SD: deviation, IQ: interquartile range, Kruskal-Wallis test for independent samples, analysis done by One Way ANOVA Test

Table 3. Comparison between the three studied groups regarding alkaline phosphatase level

Variables		*Group (1) Control group (no.= 7)*	*Group (2) Osteoporotic group (no.= 7)*	*Group (3) Leptin treated group (no.= 7)*	**P-value**
Alkaline phosphatase (IU/L)	**Mean± SD**	99.14± 20.76	196.43± 45.57	131.86± 27.06	**<0.001** P_{1-2}**<0.001** P_{1-3}= 0.237 P_{2-3}= **0.005**
	Median	98.0	185.0	125.0	
	IQR	83.0 – 117.0	153.0- 220.0	112.0- 163.0	
	Minimum	67.0	150.0	103.0	
	Maximum	125.0	283.0	175.0	

SD: standard deviation, p≤0.05 considered significant, SD: deviation, IQ: interquartile range, Kruskal-Wallis test for independent samplesanalysis done by One Way ANOVA Test

Table 4. Comparison between the three studied groups regarding osteocalcin level

Variables		*Group (1) Control group (no.= 7)*	*Group (2) Osteoporotic group (no.= 7)*	*Group (3) Leptin treated group (no.= 7)*	**P-value**
Osteocalcin(ng/ml)	**Mean± SD**	1.38± 0.34	2.21± 0.32	1.28± 0.25	**<0.001** P_{1-2}**<0.001** P_{1-3}= 1.00 P_{2-3}**<0.001**
	Median	1.32	2.09	1.25	
	IQR	0.98 – 1.68	2.01- 2.48	1.01- 1.051	
	Minimum	0.96	1.76	0.99	
	Maximum	1.79	2.67	1.70	

SD: standard deviation, p≤0.05 considered significant, SD: deviation, IQ: interquartile range, Kruskal-Wallis test for independent samples, analysis done by One Way ANOVA Test

Table 5. Comparison between the three studied groups regarding histopathological results

a.Cortical bone thickness(in micrometers)			
	Control group	**Osteoporotic group**	**leptin Treated group**
Average	347.7	273.5	312.5
Standard deviation	± 6.324	± 6.426	± 11.87
P value between:	▪ Control and osteoporotic ▪ Control and treated ▪ Osteoporosis and treated		*** < 0.001 * <0.05 **< 0.01
b. Number of osteocyte-containing lacunae (per high power field)			
	Control group	**Osteoporotic group**	**leptin Treated group**
Average	85.56	38.14	50.67
Standard deviation	± 2.231	± 3.719	± 2.836
Pvaluebetween:	▪ Control and osteoporotic ▪ Control and treated ▪ Osteoporosis and treated		*** < 0.001 *** < 0.001 * <0.05
c.Number of osteocyte-free lacunae (per high power field)			
	Control group	**Osteoporotic group**	**leptin Treated group**
Average	13.78	62.00	48.83
Standard deviation	± 2.235	± 3.723	± 2.522
P value between:	▪ Control and osteoporotic ▪ Control and treated ▪ Osteoporosis and osteoporotic		*** < 0.001 *** < 0.001 * <0.05

Average, standard deviation and p value of: Cortical bone thickness measured from periosteum to endosteum (in micrometer). Number of osteocyte-containing lacunae counted per HPF. Number of osteocyte-free lacunae counted per HPF. The following parameters were measured by computerized image analysis using ImageJ (version1.51w, National Institute of Health, USA), then by Graph Pad Prism version 5.04, 2010 to perform the statistical analysis and obtaining the average, standard deviation and p value in each group.

Discussion

Endogenous glucocorticoids have an important role in bone homeostasis. Osteoporosis induced by glucocorticoids is one of the adverse events of chronic use of glucocorticoids. Up to 40% of patients on long-term glucocorticoids develop bone loss leading to fractures **(Hardy et al., 2018).**

We had succeeded in inducing osteoporosis in rats by administering prednisolone in our study.

There was a significant decrease in serum calcium and phosphorus in the osteoporotic group because prednisolone decreases the absorption of calcium from the intestine by decreasing the synthesis of calcium-binding protein and depleting mitochondrial ATP. Also, prednisolone increases urinary calcium loss **(Thakor et al., 2022).**Hypocalcemiaoccurs when glucocorticoids are prescribed in high doses for a short period or low doses for a prolonged time. Hypocalcemia leads to the development of secondary hyperparathyroidism **(Bhadada and Rao, 2021)**.Parathormone increases renal losses of phosphate by increasing the amiloride-sensitive Na^+/H^+

exchange activity in the renal proximal tubule brush border vesicles and decreasing the Na^+ gradient-dependent phosphate uptake, resulting in increased acid secretion and phosphaturia **(Hall et al.,2022).**

There was a significant increase in serum osteocalcin and alkaline phosphatase in the osteoporotic group. This can be explained by the fact that prednisolone alters the homeostasis of bone metabolism quickly. Bone mineral density can decrease by as much as 12% within the first year of steroid use **(Xavier et al., 2021).**

Also, when bone mineral density is low, static osteoblasts are prompted to active osteoblasts. This results in bone-like tissue that cannot be mineralized and osteoblasts cannot be transformed into osteocytes. Osteoblasts proliferate in feedback and synthesize large amounts of bone alkaline phosphatase so the serum total alkaline phosphatase increases significantly in the osteoporotic group **(Shu et al., 2022).**

Using of glucocorticoids causes elevations of osteoclast activity followed by delayed and continued reduction in osteoblast activity and bone mass **(Lane, 2019**).This was confirmed by histopathological examination of the osteoporotic group of animals.

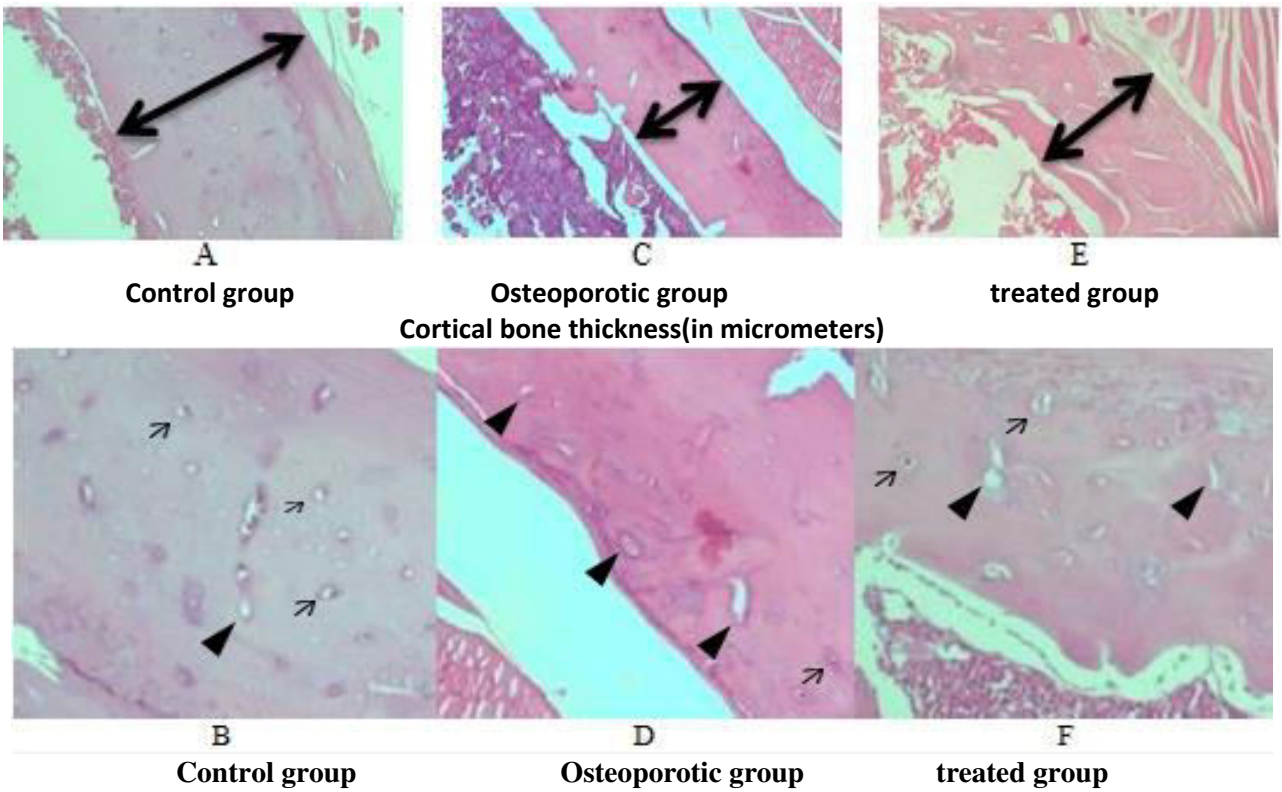

Fig.1.Photomicrographs of femur sections from various rat groups stained by H&E. Images (A & B) represent control group. Image (A) showed normal cortical bone thickness, while image (B) show numerous osteocytes with prominent nuclei "arrow" and few osteocyte-free lacunae "arrow head". Images (C & D) represent osteoporotic group. Image (C) showed reduced cortical bone thickness, wide resorbing area, while image (D) showed many osteocyte lacunae devoid of nuclei "arrow head". Images (E &F) represent leptin treated group. Image (E) showed some increase in cortical bone thickness, while image (F) showed some osteocyte lacunae filled with nuclei "arrow". Number of osteocyte-containing lacunae "arrow" and osteocyte-free lacunae "arrow head" per high power field(HPF; x200).

In leptin treated group of animals, there was a significant increase in serum calcium and phosphorus because leptin inhibits glucocorticoids and cortisol levels through the hypothalamic-pituitary-adrenal axis **(Arfusoet al., 2021).**

In addition, there was a significant decrease in serum alkaline phosphatase and osteocalcin in leptin treated group of animals because leptin administration increases bone mineral density by inhibition of glucocorticoids and cortisol levels induced decreased bone mass **(Arfuso et al., 2021)**.**Hoang et al (2017)**noted that parathormone was increased in parathyroid explants on exposure to leptin and reduced with leptin receptor inhibition. Systemic administration of leptin causes parathormone-estrogenic-like bone formation **(George, 2017).** Furthermore, leptin may also improve bone growth by activation of fibroblast growth factor-23, inhibiting serotonin synthesis and decreasing serotonergic receptors. leptin also increases osteoprotegerin levels and activates the RANK ligand-binding receptor, which may lead to reduced osteoclast activity (**Wang al., 2020).**This is confirmed by histopathological examination.

CONCLUSION:

Great results of leptin in normalizing the majority of bones and improving bone volume in sample of animals suffering from osteoporosis due to predinslone therapy, as well as modifying the majority of bone markers were confirmed by the current study. So, taking leptin with high doses with glucocorticoids to antagonize their side effects was recommended.

List of abbreviation: IP; intraperitoneal, IRB; Institutional Review Board, min;minutes, H&E; hematoxylin and eosin, high power field (HPF).

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