The potential osteogenic effects of systemic leptin and insulin administration in streptozotocin-induced diabetic female rats

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ABSTRACT

Objective: To evaluate the effect of leptin administration on some biochemical parameters of bone turnover in diabetic rats using either leptin alone or a combination of leptin and insulin.

Methods: The study was carried out on 32 female Wistar rats supplied by the Medical College animal house at King Khalid Hospital, Riyadh, Kingdom of Saudi Arabia during the period from March to December 2006. Rats were divided into 4 groups (8 rats each), controls, non-treated diabetic, leptin-treated diabetic, and leptin plus insulin-treated diabetic rats. After induction of diabetes by 6 weeks, treatment with leptin either alone or combined with insulin was continued for 2 weeks more. At the end of treatment, serum samples were taken to measure levels of bone alkaline phosphatase (BAP), alkaline phosphatase, osteocalcin, insulin like growth factor-1 (IGF-1), parathyroid hormone (PTH), glucose, creatinine, calcium ions (Ca2+), and phosphorus using enzyme-linked immunoassay (ELISA) and spectrophotometric methods. Body weight and urinary calcium excretion were also measured.

Results: Combined leptin and insulin treatment produced a significant increase of serum BAP and a decrease of urinary calcium and serum glucose as compared to rats treated by leptin only, and a significant increase of BAP, alkaline phosphates, IGF-1, and glucose and a decrease in osteocalcin as compared to control rats. Positive correlations were detected between serum IGF-1 levels and each of BAP, alkaline phosphatase, and osteocalcin in diabetic rats treated by leptin, and those with leptin plus insulin.

Conclusion: Combined leptin plus insulin treatment can offer extra gain of bone formation over leptin treatment alone. Confirmation of these preliminary observations must await careful long-term studies of bone turnover in experimental diabetes.

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As osteoporosis is a major public health problem because of its associated fractures, the identification and evaluation of populations at increased risk of developing osteoporosis are critical to disease prevention and management. Although osteoporosis traditionally has not been listed as a complication of diabetes, patients with either type 1 or type 2 diabetes are among those at increased risk for this disease. However, due to the different pathogenetic mechanisms of type 1 and type 2 diabetes, there is no uniform entity of diabetic osteopathy. In patients with insulin-dependent diabetes mellitus (IDDM), osteopenia/osteoporosis is the result of a lowered bone formation with a predominance of bone resorption over bone formation. The mechanism of bone loss in IDDM is still unknown, although several theories exist based on animal and cellular models that demonstrate the cause/effect relationship between inadequate insulin production and abnormal bone formation.[1] Leptin is now regarded as a multipotent cytokine eliciting indirect, central, and direct, peripheral effects in different tissues and organs such as bone.[2] In the hypothetical framework of a common regulation of bone mass, body weight, and reproduction, it was argued on a genetic basis that leptin may account for the control of bone formation.[3] Failure to demonstrate leptin and leptin receptor expression in bone tissue was interpreted to advocate a role for leptin as a neurogenic stimulus to maintain bone mass at a normal level. The amount of leptin released is related to the amount of body fat present. Overweight people also tend to be less likely to develop

osteoporosis and for a long time it was not known why. Only more recently was a connection between leptin and a reduction in bone mass discovered.[4] Many studies suggest that the rate of bone resorption in diabetes is normal and therefore elevated relative to the decreased rate of bone formation.[5] Studies in diabetic animals show that there is enhanced apoptosis of osteoblastic cells and although there is sufficient production of immature mesenchymal tissue, there is failure to adequately express genes that regulate osteoblast differentiation. Bone cells have receptors for both insulin and insulin growth factor (IGF)-1, and in vitro insulin has been shown to increase proliferation and function of osteoblasts, while insulin deficiency suppresses osteoblastic activity and therefore insulin can modulate bone turnover directly.[6] Diminished expression of IGF-1 or basic fibroblast growth factor may contribute to reduced production of bone matrix, and the increased brittleness of diabetic bone may be due to abnormalities in the microarchitecture.[7] Insulin and insulin like growth factors have an influence on bone metabolism itself. Furthermore, poor glycemic control was found to impair the response of osteoblasts and osteoclasts to one, 25-dihydroxy vitamin D.[8] Streptozotocin-induced diabetic animals can exhibit many of the complications observed in human diabetes including diminished growth factor expression and reduced bone formation.[9] Therefore, this model can be used to study the effect of diabetes on markers of bone metabolism and the possible use of leptin for treatment and prophylaxis of osteoporosis seen in this condition. As osteoporosis is a very common disease and a growing problem worldwide, whether leptin has any role in human bone physiology is an important question. We hypothesized that exogenous leptin administration to diabetic rats might have an osteogenic effect and therefore could be helpful in the treatment and prophylaxis of osteoporosis. We also explored the effect of diabetic control on the response to leptin treatment.

Methods. The present study was carried on 32 female Wistar rats supplied by the Medical College Animal House at King Khalid Hospital, Riyadh, Saudi Arabia during the period from March to December, 2006. The initial weight of rats was between 206-296 gm. The animals were housed in stainless steel cages at room temperature (±25°C) and with a 12 hours light cycle. Normal rat chow and water were allowed ad libitum. Institution and national guide for the care and use of laboratory animals was followed. The rats were divided into 4 groups (8 rats each): group I included control rats that received no treatment, group II included streptozotocin induced diabetic rats that received no medication (STZ), group III included STZ induced diabetic rats that received leptin (STZ+L), and group IV included STZ induced diabetic rats that received leptin and insulin (STZ+L+I). Diabetes was induced by a single intraperitoneal injection of freshly prepared streptozotocin (Sigma Chemical Co, St. Louis, Missouri, USA) dissolved in 0.1 mol/L citrate buffer (pH 4.5) at a dose of 65 mg/kg body weight.[10] The normal control rats received an equivalent dose of buffer. Animals with blood glucose level higher than 16 mmol/l 3 days after STZ injections were used. Rat recombinant leptin (Sigma Aldrich, Inc, Saint Louis, Missouri, USA) was administered subcutaneously, starting 6 weeks after induction of diabetes, at a dose of 100 μg/kg/day for 2 weeks.[11] It is a 147 amino acid recombinant protein (product No. L5037) and is expressed in *Escherichia coli*. It is highly purified and biologically active. Long acting insulin (ultralente) (Novo Nordisk A/S. 2880 Bagsvaerd, Denmark) was administered to diabetic rats subcutaneously at a dose of 3-4 U/day[10] immediately after induction of diabetes and continued up to the end of the study (for 8 weeks), namely, until the time of killing. The dosage of insulin was adjusted on the basis of daily serum glucose determination to maintain serum glucose concentration below 13 mmol/l during the course of the experiment. At the end of the treatment, blood samples and 2-hour urine samples were collected. Blood samples were centrifuged; serum samples were frozen within one hour and stored under identical conditions, and then assayed for the levels of the following parameters: 1. Markers of bone formation: - bone specific alkaline phosphatase (BAP) using Metra BAP immunoassay (Quidel Co, San Diego, USA).[12] Alkaline phosphates by colorimetric method (catalogue No. AP542, Randox Laboratories Ltd, United Kingdom).[13] Osteocalcin by immunoenzymetric assay using HOST-EASIA kit (catalogue No. KAP1381, BioSource Europe S.A., Belgium).[14] Insulin like growth factor-1 by enzyme linked immunosorbent assay, catalogue No. KAPB2010, BioSource Europe S.A., Belgium.[15] 2. Marker of bone resorption as urinary excretion of calcium. 3. Other parameters included serum levels of: Parathyroid hormone (PTH) by enzyme amplifier sensitivity immunoassay (EASIA) using hPTH-EASIA kit (catalogue No. KAP1481, BioSource Europe S.A., Belgium).[16] Calcium, phosphorous and creatinine by colorimetric method (Spectrophotometer, Shimadzu, UV, 1201, Japan) and glucose by oxidase method.[13] 4. Body weight measurement at the start of the study and 8 weeks after that.

Statistical analysis. Results were expressed as mean ± standard deviation (mean ± SD). The significance of the difference between the values from different groups was determined using one way analysis of variance (ANOVA) (F-test) combined with post-ANOVA

Tukey-Kramer test for multiple comparisons. Pearson correlations between different parameters were carried out. A level of $p<0.05$ was being defined as statistically significant. The statistical software used during the study period was SPSS program.

Results. Serum specific alkaline phosphatase (BAP) levels were significantly lower in non-treated STZ rats as compared to controls, leptin-treated rats, and leptin+insulin-treated rats. Moreover, leptin+insulin-treated rats had higher BAP levels as compared to controls and rats treated by leptin alone. Also, leptin+insulin-treated STZ had the highest serum alkaline phosphatase levels as compared to controls and non-treated rats. Leptin treatment increased serum alkaline phosphatase levels (while non significant) as compared to controls. While non-treated, leptin-treated, and leptin+insulin-treated STZ rats had significantly lower serum osteocalcin levels as compared to controls, however, leptin-treated and leptin+insulin-treated rats showed a significant increase of serum osteocalcin levels as compared to non treated STZ rats. No significant changes could be found between leptin-treated and leptin+insulin-treated rats. The decrease of serum level of IGF-1 was evident in non-treated STZ rats as compared to controls, leptin-treated and leptin+insulin-treated rats. Moreover, IGF-level was significantly higher in leptin+insulin-treated rats as compared to controls. While no significant changes could be detected in leptin-treated rats as compared to controls and leptin+insulin-treated rats. The increase of urinary calcium (Ca2+) level was highest in non-treated rats as compared to controls, leptin-treated, and leptin+insulin-treated rats. Comparing non-treated, leptin-treated, and leptin+insulin-treated rats to each other, leptin+insulin-treated rats have the lowest values of urinary Ca2+, which was significantly decreased as compared to rats treated by leptin only, while the change of urinary Ca2+ excretion was insignificant when comparing leptin+insulin-treated rats to controls. A significant decrease of serum PTH levels was observed in non treated STZ rats as compared to each of controls, leptin+insulin-treated rats, and leptin-treated rats. No significant changes were found in STZ-treated rats either by leptin alone or combined leptin and insulin as compared to each other or to controls. Non-treated STZ rats have the highest serum glucose level as compared to controls, leptin-treated STZ rats, and leptin+insulin-treated STZ rats. Moreover, the serum glucose level in leptin-treated STZ rats is higher as compared to controls and leptin+insulin-treated STZ rats. Comparing leptin+insulin-treated STZ rats to controls, serum glucose was significantly higher. The mean values of serum creatinine were not changed significantly in non-treated STZ rats, or rats treated by leptin or combined leptin and insulin as compared to controls. While treated STZ rats showed a significant increase of mean value of serum Ca2+ level as compared to control rats, leptin+insulin-treated rats, and leptin-treated rats, no significant changes could be observed between leptin-treated and leptin+insulin-treated rats as compared to each other, or to controls. The serum level of phosphorus was significantly higher in non-treated rats as compared to controls, leptin-treated, and leptin+insulin-treated rats. While no significant changes were found in leptin-treated or leptin+insulin-treated rats as compared to each other or to controls. At the start of the study, non treated-STZ, leptin-treated and leptin+insulin-treated rats had higher initial body weigh as compared to controls. However, one month after the study, STZ rats, whether non treated or treated by leptin or leptin+insulin, showed a decrease of their body weight (although non significant for each group separately as compared to their initial body weight) (**Table 1**).

Correlations (Pearson r) between the measured parameters in all the studies groups. Positive correlations were detected between serum IGF-1 levels and each of BAP (r=0.7671, p=0.0263), alkaline phosphatase (r=0.7780, p=0.023), and osteocalcin (r=0.7040, p=0.0499) in STZ rats treated by leptin (group III), or leptin+insulin (group IV), where for BAP, r=0.9050, p=0.002), for alkaline phosphatase, r=0.7681, p=0.026 and osteocalcin, r=0.8726, p=0.0047). Moreover, a negative correlation was detected between IGF and urinary calcium only in group II (r=-0.7505, p=0.0319. No other significant correlations were found between the other different studied parameters.

Discussion. The ability of leptin administration either alone or combined with insulin to improve bone metabolism was previously postulated by Steppan et al.[17] Another study showed that intraperitoneally infused leptin prevented disuse-induced reduction of bone mineral density.[18] Our data show that non treated STZ rats had a significant decrease in serum levels of BAP, osteocalcin, IGF-1, and PTH, while urinary calcium excretion and serum glucose, calcium, and phosphorus levels were increased as compared to controls. Administration of leptin to STZ rats was effective in increasing serum levels of markers of bone formation (BAP, osteocalcin, IGF-1) and decreasing those of bone resorption as manifested by decreasing urinary calcium excretion and serum levels of calcium and phosphorus as compared to non-treated STZ rats. Combined leptin and insulin treatment of STZ rats was more effective than leptin treatment alone in improving serum levels of BAP and decreasing urinary Ca2+ excretion and serum glucose. This improvement of markers of bone

metabolism in response to leptin treatment was not accompanied by impairment of renal function as evident by the normal serum creatinine levels seen in leptin-treated rats (either alone or combined with insulin) as compared to controls. Supporting the hypothesis that leptin may have positive effects on bone metabolism, the study by Oguch et al[19] has shown that there is a negative correlation between leptin level and levels of markers of bone resorption. They concluded that leptin might play a role in human fetal bone resorption with the overall effect of increasing bone mass. However, our findings are in contradiction to another study, which reported that intracerebroventricular infusion of leptin caused bone loss in leptin-deficient mice. The researchers postulated that leptin is a potent inhibitor of bone formation acting through the central nervous system.[20] The findings of the present study would suggest that leptin has a protective peripheral skeletal effect, and it may be that overall leptin effects on bone metabolism result from a balance between negative central effects and positive direct peripheral effects, depending on serum leptin levels or blood-brain barrier permeability. Blain et al[21] reported that leptin level was positively correlated with PTH, and a marker of bone turnover. The influence of leptin on bone metabolism through the CNS is postulated to be independent of the effect of IGF-1, PTH, and kidney function. Our findings of improvement in the high serum glucose level and increasing IGF-1 level in leptin-treated diabetic rats, which is potentiated by combined insulin treatment are in accordance with the study of Liu et al[22] who found that 4 weeks treatment of female genetically obese (ob/ob) mice with leptin corrected high serum glucose and insulin levels and increased IGF-1 level and osteoblast number and activity while, osteoclast numbers were unaffected. These results confirm earlier reports that leptin treatment normalizes the metabolic state of ob/ob mice and provide the novel finding that the metabolic

Table 1 - Mean values ± SD of the measured parameters in controls (Group I), streptozotocin diabetic rats (STZ) rats (Group II), leptin-treated STZ rats (Group III), and leptin+insulin-treated STZ rats (Group I).

Parameters	Group I (controls)	Group II (STZ rats)	Group III (leptin+STZ rats)	Group IV (leptin+insulin+ STZ rats)	F value
Serum BAP (U/l)	81.9 ± 5.489	38.169 ± 1.520* $p<0.05$†	83.808 ± 5.524	91.886 ± 5.261* $p<0.05$¶	207.4
Serum alkaline phosphates (U/l)	296.41 ± 53.636	301.85 ± 45.074	331.57 ± 49.798	396.55 ± 55.315* $p<0.01$**	6.474
Serum osteocalcin (ng/ml)	3.304 ± 0.647	1.056 ± 0.069* $p<0.001$†	2.535 ± 0.327* $p<0.01$§	2.656 ± 0.194* $p<0.01$††	50.836
Serum IGF-1 (ng/ml)	159.5 ± 1.921	124.24 ± 4.883* $p<0.001$†	164.504 ± 10.788	172.826 ± 8.287* $p<0.01$††	68.953
Urinary calcium (mmol/l)	1.447 ± 0.333	5.014 ± 1.212* $p<0.01$†	3.607 ± 0.707* $p<0.05$□	2.508 ± 0.6596	29.678
Serum PTH (pg/ml)	14.139 ± 1.541	6.396±1.643* $p<0.01$†	14.050 ± 5.513	16.055 ± 5.700	8.613
Serum glucose (mmol/l)	7.839 ± 0.480	31.389 ± 3.245* $p<0.001$†	19.651 ± 1.699* $p<0.001$□	11.543 ± 1.248* $p<0.01$††	228.96
Serum creatinine (µmol/l)	199.91 ± 9.659	198.89 ± 9.374	199.04 ± 8.866	195.96 ± 11.961	0.2356
Serum calcium (mmol/l)	2.373 ± 0.423	3.737 ± 0.458* $p<0.01$†	2.634 ± 0.625	2.501 ± 0.606	10.959
Serum phosphorous (mmol/l)	2.198 ± 0.341	3.605 ± 0.606* $p<0.001$†	2.511 ± 0.349	2.376 ± 0.254	19.241
Change of body weight (%)	8.976 ± 3.689	-9.674 ± 3.082* $p<0.001$‡	-6.594 ± 6.342* $p<0.001$§	-5.976 ± 3.601* $p<0.001$††	29.267

*Significant changes, †Group II versus Groups I, III, IV, ‡Group II versus Group I, §Group III versus Group I, □Group III versus Groups I, IV, ¶Group IV versus Groups I, III,**Group IV versus Groups I, II, ††Group IV versus Group I.
BAP - bone alkaline phosphatase, IGF-1 - insulin-like growth factor 1, PTH - parathyroid hormone,

normalization is accompanied by dramatic increase in bone formation. Serum levels of osteocalcin, a protein synthesized by mature osteoblasts, are regarded as a sensitive and specific marker of osteoblastic activity and the rate of bone formation. Hence, the finding of the present work of reduced serum osteocalcin in diabetic rats suggests that reduced osteoblast activity and bone turnover may be responsible for the osteoporosis that could be seen in diabetic patients. Evidence of a stimulatory effect of leptin on serum osteocalcin levels was apparent following leptin administration either alone or combined with insulin. This is in accordance with the study of Goldstone et al[23] who found that leptin administration increased osteocalcin level in male ob/ob mice and prevented its fall during starvation in normal male mice. Leptin influences the release of growth hormone and other agents that accelerate bone growth.[24] Likewise, leptin administration improves the body's response to insulin and insulin like growth factors. These may be the mechanism whereby leptin indirectly shifts the homeostasis balance in favor of bone formation instead of bone resorption with overall effect of increasing bone mass.[25] The present study clearly confirms the existence of low IGF-1, osteocalcin concentrations and other markers of bone formation in non-treated STZ rats that could be corrected by reasonably good diabetic control. It was reported that bone loss is greater in patients with poorly controlled diabetes than in those whose diabetes is in good control, and here diminished expression of IGF-1 may contribute to reduced production of bone matrix due to increased concentration of cytokines associated with inflammation, microvascular complications, and reduced blood flow to bone.[26] The importance of IGF-1 in bone remodeling is supported by many studies, which have proved a correlation between expression of IGF-1 in cells of the osteoblasts lineage and indices of bone formation and resorption.[27] The possible explanation for the increase in bone formation markers seen in insulin-treated diabetic rats is that insulin may produce a decline in the production of IGF binding protein-1, leading to an increase of IGF-1 that may stimulate the proliferation of osteoblasts.[28] The present study demonstrates a positive correlation between serum IGF-1 levels and indicators of bone formation (BAP, alkaline phosphatase, and osteocalcin) in all rats treated by either leptin alone or leptin+insulin.

It could be concluded that combined leptin and insulin treatment can offer extra gain of bone formation over leptin treatment alone. Confirmation of these preliminary observations must await careful long-term studies of bone turnover in experimental diabetes.

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