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Original Article

Comparison between liraglutide alone and liraglutide in combination with insulin on osteoporotic rats and their effect on bone mineral density

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Abstract

Objectives: To compare the therapeutic efficacy of liraglutide (LRG) single drug combined with insulin (Ins) on osteoporosis in rats and its effect on bone mineral density (BMD). A rat model of diabetes combined with osteoporosis was established. **Methods**: 40 Sprague-Dawley rats were divided into four groups (blank, control, LRG and LRG+Ins). Serum levels of CrossLaps, procollagen type I N propeptide (PINP), alkaline phosphatase (AKP) and osteocalcin (BGP) were detected by ELISA. Blood glucose was measured by its reaction with glucose oxidase. Serum insulin was analyzed by radioimmunology. Bone calcium and phosphorus contents were also recorded. ELISA was used to detect inflammatory factors. Bone mineral density (BMD) measurement was also performed. **Results**: BMD of the control group was significantly lower than that of the other three groups ($p<0.05$) and BMD of the LRG + Ins group was significantly higher than that of the LRG group ($p<0.05$). The inflammatory factors of the control group were significantly higher than those in the other three groups ($p<0.05$). The inflammatory factors were negatively correlated with BMD ($p<0.05$). **Conclusions**: liraglutide in combination with insulin for the treatment of diabetes complicated with osteoporosis can reduce blood glucose *in vivo*, promote production of islet, effectively improve osteoporosis symptoms, increase BMD and reduce the levels of inflammatory factors *in vivo*.

Keywords: Bone Mineral Density, Diabetes, Insulin Therapy, Liraglutide, Osteoporosis

Introduction

Patients with diabetes have an increased risk of fragility fractures and osteoporosis[1,2]. Osteoporosis can cause pain and limitation of physical function and everyday activities in those patients. The main causes are[3]: Deficiency of insulin. High levels of insulin can increase bone mineral density. Islet β cells in the pancreas are stimulated by exogenous or endogenous factors to promote secretion of insulin. Poor control of high blood glucose and elevated blood sugar levels can lead to osmotic diuresis and increase the excretion of magnesium, phosphorus, calcium, etc. High urine glucose can hinder the absorption of magnesium, phosphorus and calcium by the renal tubules and the loss of bone mineral is aggravated. Negative balance of calcium can reduce bone mineral density (BMD), hypomagnesemia can stimulate the secretion of parathyroid hormone, increase bone absorption, resulting in decrease of bone mineral density and osteoporosis[4].

Liraglutide (LRG) is a human glucagon peptide-1 (GLP-1) analog. In recent years, liraglutide effect and action in diabetes (LEAD) series of clinical research results have been published. Among them, in LEAD-3 and its expansion studies, the comparison of liraglutide and glimepiride single drug shows the advantages in efficacy and safety of liraglutide single drug[6]. The study of Sun et al.[7] showed that the trabecular bone mineral density and the expression osteogenesis markers Runx2, ALP, Collagen1, osteocalcin and OPG significantly increased after subcutaneous injection of LRG in rats with spontaneous type II diabetes for 4 weeks. It indicated that liraglutide was likely to promote bone

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growth and improve diabetes-induced osteoporosis, but the precise mechanism needs further investigation. Insulin is the most common type of medication used in type II diabetes treatment. The insulin by external intake to reduce blood glucose can quickly increase the energy and motivation of patients and improve physical strength. Therefore, the treatment of combined insulin is often used[8,9].

In this study, we established a rat model of diabetes-induced osteoporosis and intervened by liraglutide (LRG) in combination with insulin (Ins) so as to investigate and compare the therapeutic efficacy of liraglutide single drug combined with insulin on osteoporosis in rats and its effect on BMD.

Materials and methods

Animals

A total of 40 female Sprague-Dawley (SD) rats aged 6 months were randomly divided into 4 groups (10 rats of each group): blank group, control group, LRG group and LRG+Ins group by number table after adaptive feeding for 1 week.

Main reagents and instruments

Citric acid, sodium citrate, xylene, anhydrous alcohol. Liraglutide (Novo Nordisk Pharmaceutical Co., Ltd.). Human insulin (Jiangsu Wanbang Biochemical Pharmaceutical Group Co., Ltd., SFDA Approval No. S20188O003). F400 fluorescence spectrophotometer (Japan Hitachi). Electronic balance (product of Sartorius, Germany). BWJ-1 single photon BMD detection (product of Zhongchuan Company).

Modeling and grouping

Forty female SD rats aged 6 months were used for the study. The body mass of the rats was (249.8±56.2) g. Except for the blank (healthy rats) group, phenobarbital (30 mg/Kg) was used for anesthesia in the lower abdomen, and the median incision was performed in the lower abdomen for bilateral ovariectomy. After operation, penicillin (800000 units) was given intramuscular injection for 3 days once a day for preventing wound infection and we observed them closely after surgery. Rats in diabetes induction group (LRG group, LRG+Ins group) were fasted for 12 hours with access to water before modeling. Then the rats were injected with 60 mg/kg STZ (dissolved in sodium citrate buffer solution).

The control group was injected with an equal volume of solvent. After 72 h, fasting blood glucose of rats were tested. The rat model was successfully established with blood glucose above 16.7 mmol/L.

Treatment modality

The drug was administered after the operation for one week. The LRG group was injected with 0.6 mg/kg of liraglutide subcutaneously once a day for 8 weeks. The LRG+Ins group was also administered by subcutaneous injection with liraglutide. The dose was the same as that of the LRG group. At the same time, an additional 2 to 4 IU/d/insulin was added for continuous administration for 8 weeks. The feeding conditions were not changed during the administration.

Detection of indices

After eight weeks, blood was collected from the abdominal aorta. Serum was collected and used to detect CrossLaps, type I PINP, alkaline phosphatase (AKP) and osteocalcin (BGP) levels. The specific operation was carried out according to the instructions of the kit (purchased from Shanghai jinma biotechnology co., LTD., batch number: 20180614). Glucose oxidase method was used to detect blood glucose. Radioimmunology analysis was used to determine serum insulin.

Detection of bone calcium, phosphorus content

The content of calcium in the bone ash solution was determined by a 3510 atomic absorption spectrometric under fuel- rich flame conditions. The absorbance at 600 nm wavelength of the bone ash solution was measured by F400 fluorescence spectrophotometer. The phosphorus content in the sample was calculated by the standard curve method.

Measurement of lumbar vertebra bone mineral density

After 8 weeks of treatment, the weight of the model rats was measured. The rats were injected and anesthetized with phenobarbital (30 mg/kg) in lower abdomen. The lumbar vertebra BMD (in g/cm^2) of rats was measured by dual-energy X-ray absorptiometry (DPX-MD type produced by GE, USA).

Detection of inflammatory factors by ELISA

The blood was withdrawn from the inferior vena cava, the greenhouse was allowed to place for 4 h and centrifuged at 4°C, 3000 r/ min for 10 min. The serum was collected and packed separately. Inflammatory factors TNF-α, IL-6 and IL-1β were detected by ELISA kit. Finally, the absorbance (A) value of each sample at 450 nm wavelength was determined by using enzyme-labelling measuring instrument (to ensure that there was no water droplet on the substrate of the enzyme substrate and no bubbles were formed in the drip hole). After subtracting the A value of the TMB blank color hole from the A value of all standard products and samples, the concentration of standard product was taken as the abscissa, and the A value after zero adjustment as the ordinate to draw the standard curve and calculate the actual concentration of each sample.

Statistical analysis

SPSS18.0 (Boise (Beijing) information technology co., LTD.) was used for statistical analysis. GraphPad Prism 6 was used to draw all the figures in this experiment. Chi-square test was used to compare the counting data. Measurement

Table 1. Comparison of fasting blood glucose and insulin (Ins) levels of rats in different treatment stages.

		Fasting blood glucose (mmol/L)			Ins (mIU/L)
Group	n	At the beginning of the first week	At the end of the fourth week	At the end of the eighth week	At the end of the eighth week
Blank	10	4.29±0.74	5.34±1.27	4.95±0.73	23.26±3.22
Control	10	21.89±2.07	21.44±1.59	21.13±0.69	4.25±3.97
LRG	10	21.64±2.27	11.34±1.71	8.35±0.76	18.04±2.14
LRG+Ins	10	21.96±2.31	9.04±1.34	6.96±0.64	23.81±2.05

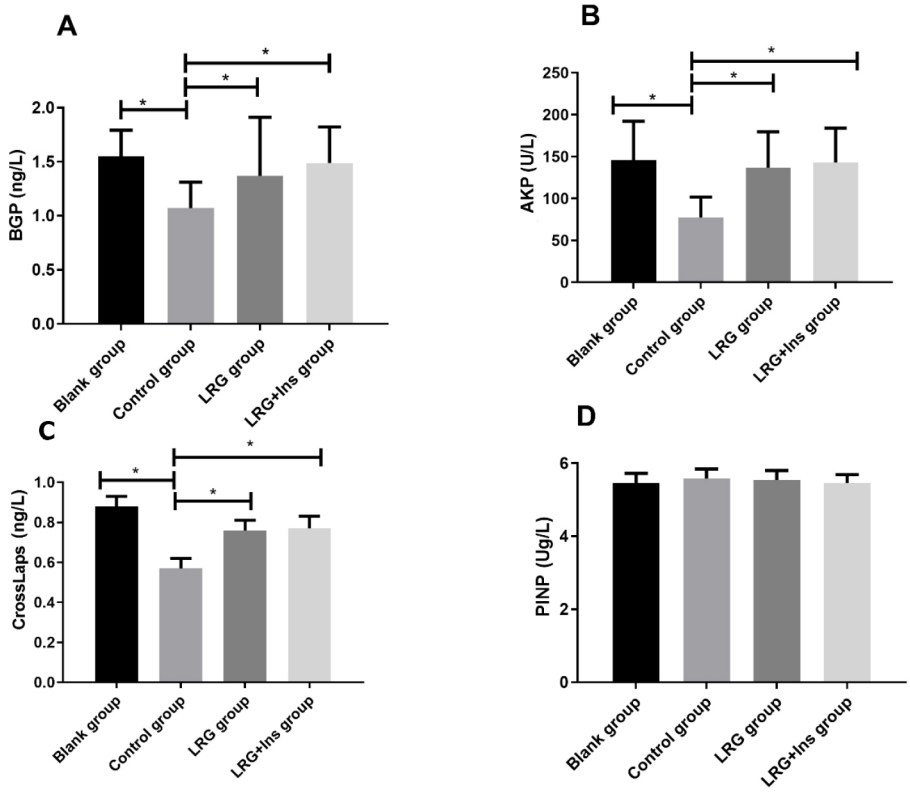

Figure 1. Comparison of serum BGP, AKP, PINP and CrossLaps of rats in each group. A. The Osteocalcin (BGP) in the blank group and the treatment group was significantly higher than that in the control group. B. The alkaline phosphatase (AKP) in the blank group and the treatment group was significantly higher than that in the control group. C. The CrossLaps in the blank group and the treatment group was significantly higher than that in the control group. D. There was no statistical difference in the PINP levels in the serum of the four groups. * indicates p<0.05.

data were expressed by mean number ± standard deviation. T test was used for analysis between groups. Variance analysis was used to compare multiple groups. Pearson correlation analysis was used to analyze the relationship among variables. p<0.05 was considered statistically significant.

Results

Serum biochemical indexes

After treatment, the levels of BGP, AKP, CrossLaps and PINP in the serum of the four rats groups were detected. The levels of BGP, AKP and CrossLaps in the blank group, LRG group and LRG+Ins group were higher than those in the control group (all p<0.05). There was no statistical significance of PINP in the serum of 4 groups (all p>0.05) (Figure 1).

Detection of blood glucose and insulin

Compared with rats in 4 groups, at the beginning of the first week of treatment, at the end of the fourth week, at the end of the eighth week, the fasting blood glucose were

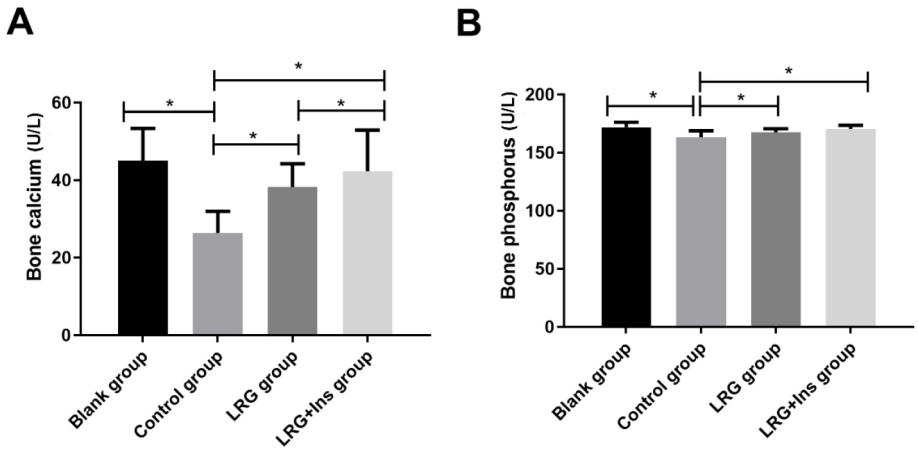

Figure 2. Comparison of bone phosphorus and bone calcium levels of rats in each group. A. The levels of bone calcium of rats *in vivo* of the blank group and the treatment group were significantly higher than that in the control group and the levels of bone calcium of rats *in vivo* of the LRG+Ins group were significantly higher than that of the LRG group. B. The levels of bone phosphorus of rats *in vivo* of the blank group and the treatment group were significantly higher than that in the control group. *p<0.05.

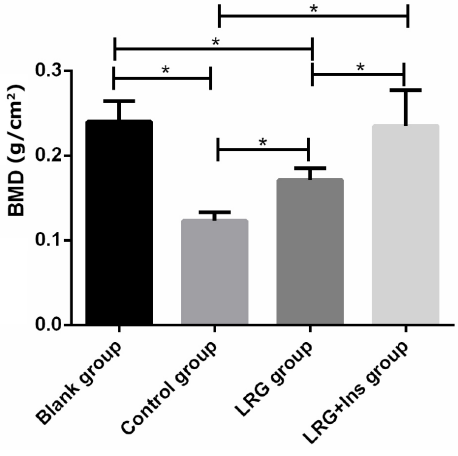

Figure 3. Comparison of BMD between groups. BMD of rats in the blank group and the treatment group were significantly higher than those in the control group. BMD of rats *in vivo* of the LRG+Ins group were significantly higher than that in the LRG group. There was no significant difference in BMD of rats *in vivo* between the LRG+Ins group and the blank group. * p<0.05.

compared with the levels of INS after the end of the treatment. At the beginning of the first week, the fasting blood glucose of the control group, LRG group and LRG+Ins group was significantly lower than that of the blank group (all p<0.01). At the end of the fourth week, the fasting blood glucose of the treatment group was significantly higher than that of the control group after 4 weeks of treatment (all p<0.01) and the fasting blood glucose of the LRG+Ins group was significantly higher than that of the LRG group (all p<0.05). At the end of the eighth week, the fasting blood glucose of the treatment group was significantly higher than that of the control group (all p<0.01) and the fasting blood glucose of the LRG+Ins group was significantly higher than that of the LRG group (all p<0.05). After the end of treatment, Ins levels *in vivo* of the treatment group were significantly higher than those in the control group (all p<0.01). Insulin levels *in vivo* of the LRG+Ins group were significantly higher than those in the LRG group (all p<0.05) and there was no statistical difference with the blank group (all p>0.05). (Table 1).

Comparison of bone phosphorus and bone calcium levels of rats in each group

After treatment, the levels of bone calcium and bone phosphorus in the treatment group were significantly higher than those in the control group (all p<0.05). The levels of bone calcium of rats *in vivo* of LRG+Ins group were significantly higher than those in LRG group (all p<0.05). There were no significant differences between the LRG+Ins group and the blank group in the levels of bone calcium and bone phosphorus of rats *in vivo* (all p>0.05) (Figure 2).

Comparison of lumbar bone mineral density levels in each group

After treatment, BMD of rats in each group were examined and compared. It was found that BMD of rats in the treatment group were significantly higher than those in the control group (all p<0.05). BMD of rats *in vivo* of LRG+Ins group were significantly higher than those in LRG group (all p<0.05). There was no significant difference in BMD of rats *in vivo* between the LRG+Ins group and the blank group (all p>0.05) (Figure 3).

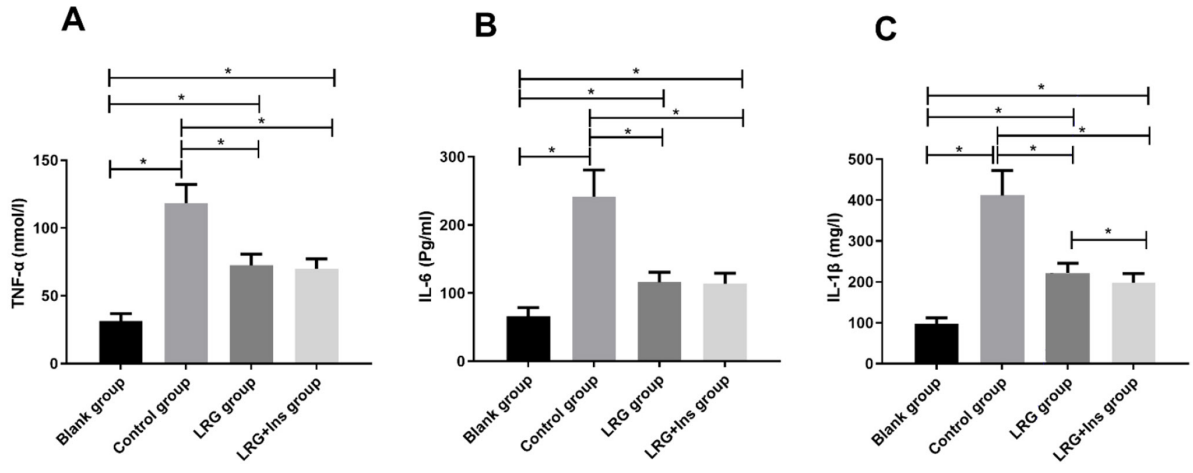

Figure 4. Comparison of serum inflammatory factors levels in each group. A. The levels of TNF-α in the blank group and the treatment group was significantly lower than that in the control group. The levels of TNF-α in the treatment group was statistically different from that in the blank group. B. The levels of IL-6 in the blank group and the treatment group were significantly lower than that in the control group. The levels of IL-6 in the treatment group were statistically different from that in the blank group. C. The levels of IL-1β in the blank group and the treatment group was significantly lower than that in the control group. The levels of IL-1β in the treatment group was statistically different from that in the blank group. When IL-1β in the LRG+Ins group was lower than that in the LRG group, there were statistically significant differences. * indicates p<0.05.

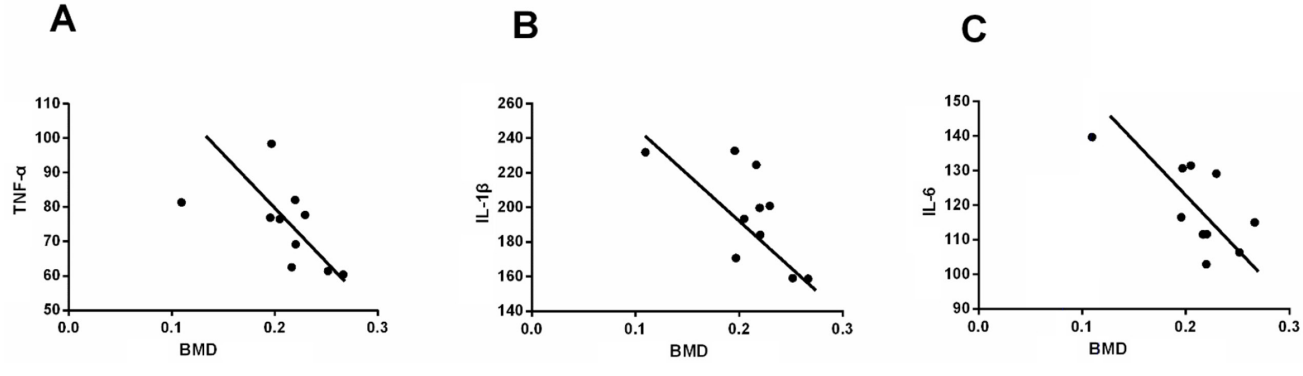

Figure 5. Correlation between BMD and TNF-α, IL-1β, IL-6. A. Correlation analysis of TNF-α level with BMD, r=-0.6354, negative correlation. B. Correlation analysis of IL-6 level with BMD, r=-0.6354, negative correlation. C. Correlation analysis of IL-1β level with BMD, r=-0.6621, negative correlation. The correlation was p<0.05.

Comparison of serum inflammatory factors levels of rats in each group

After detecting the inflammatory factors in serum, the levels of TNF-α, IL-6 and IL-1β in the treatment group was significantly lower than that in the control group (all p<0.05). The levels of TNF-α, IL-6, IL-1β in the treatment group were statistically different from those in the blank group (all p<0.05). When IL-1β in the LRG+Ins group was lower than that in the LRG group, there were statistically significant differences (all p<0.05) (Figure 4).

Correlation between bone mineral density and inflammatory factors

The data of LRG+Ins group were analyzed and the levels of TNF-α, IL-6 and IL-1β was negatively correlated with BMD (all p<0.05) (Figure 5).

Discussion

Celiac disease is associated with reduced BMD and reversible secondary osteoporosis

Diabetes-induced osteoporosis is a secondary osteoporosis. In recent years, more and more studies have reported that diabetes is often associated with abnormal bone metabolism, and low bone mineral density. Elderly women lose bone faster than men and are more prone to fractures. Once fracture occurs in patients, the disability rate and mortality rate are high, which seriously affects the quality of life. Therefore, it is of great significance to further explore the pathogenesis of diabetes-induced osteoporosis and study the effectiveness of drugs[10,11].

The GLP-1 analogue, liraglutide, is a glucose-dependent incretin. Studies have shown that the risk of fracture in individuals treated with LRG was significantly reduced[12]. LEAD project study[13] indicated that GLP-1 could reduce the body weight of diabetic patients. The effect of reducing body weight is more obvious with the increase of BMI in patients and is more effective than glimepiride, metformin and so on. Weight control has become an important part of diabetes treatment[14]. The LEAD-3 expansion study confirmed the long-term safety and efficacy of liraglutide single drug in patients with early type II diabetes. It had the advantage of reducing weight and the risk of hypoglycemia and provided strong evidence for clinical applications[15]. The results of this study showed that in the rat model of diabetes-induced osteoporosis treated with liraglutide single drug, except for the type I PINP, other indicators were significantly improved compared with the control group (all $p<0.05$). This showed the same results as the previous study. Liraglutide single drug can effectively reduce concentration of blood lipid and secretion of insulin.

In previous studies, glucagon-like peptide-1 analogue and insulin combination therapy had been pointed out[16]. The results of Seino[17] confirmed the efficacy and safety of adding liraglutide to insulin therapy. The results of the combined treatment group showed that LRG was combined with Ins in the treatment of diabetes-induced osteoporosis. After the period of 8 weeks that the drug was being administered, alkaline phosphatase, osteocalcin, crosslinks, blood glucose levels of rats *in vivo* were significantly higher in the LRG single drug group and the levels of insulin approached the normal level after the end of treatment. Under the stability of blood glucose and insulin levels, osteoporosis was controlled and bone phosphorus and bone calcium levels were controlled *in vivo* of rats. There was no significant change in bone phosphorus level and single drug group in the combination therapy group (all $p>0.05$). The levels of bone calcium were significantly higher than that of the single drug group (all $p<0.05$). The BMD in the LRG combined with Ins group were significantly higher than that in the LRG single drug group (all $p<0.05$). The results indicated that liraglutide can increase the levels of bone calcium, bone phosphorus, increase cell concentration, increase bone mineral density and promote the formation of new trabecular bone[18]. This indicates that liraglutide has a certain preventive and therapeutic effect on diabetes complicated with osteoporosis. At the same time, the combination of insulin can enhance the effect of liraglutide in the treatment of osteoporosis, which is consistent with the conclusion of Seino Y[17]. In addition, the study showed that in patients with postmenopausal osteoporosis, serum IL-1β, IL-6 was released in large quantities and the expression of osteocalcin was significantly decreased[19]. The expression of inflammatory factors TNF-α, IL-6 and IL-1β was significantly increased in ovariectomy rat model and negatively correlated with bone mineral density[20]. It was indicated that the inflammatory response plays an important role in the deterioration of diabetes complicated with osteoporosis. The results of this study showed that liraglutide can reduce the expression of TNF-α, IL-6 and IL-1β and effectively inhibit the inflammatory reaction *in vivo* of rats. It is preliminarily proved that liraglutide can improve the diabetes complicated with osteoporosis and this effect is at least partially accompanied by changes in the inflammatory response. It can also be indicated from the results that combined insulin therapy has little effect on the levels of inflammatory factors.

In summary, liraglutide in compination with insulin for the treatment of diabetes complicated with osteoporosis can reduce blood glucose *in vivo*, promote production of islet, increase bone mineral density and reduce the levels of inflammatory factors *in vivo*.

Authors' contributions

KC, RW and MC conceived and designed the study, and drafted the manuscript. KC, BM, XY and DS collected, analyzed and interpreted the experimental data. KC revised the manuscript for important intellectual content. KC wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval

The study was approved by the Ethics Committee of Fenghua People's Hospital.

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