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Leptin Influences Healing in the Sprague Dawley Rat Fracture Model

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Background: Leptin plays a crucial role in bone metabolism, and its level is related to bone callus formation in the fracture repair process. The objective of this study was to evaluate the effect of recombinant leptin on the healing process of femoral fractures in rats.

Material/Methods: Forty-eight male Sprague Dawley (SD) rats with an average body weight of 389 g (range: 376–398 g) and an average age of 10 weeks were included in this animal research, and all rats were randomly divided into two major groups. Then standardized femur fracture models were implemented in all SD rats. Rats in the control group were treated with only 0.5 mL of physiological saline, and rats in the experimental group were treated with recombinant leptin 5 µg/kg/d along with the same 0.5 mL of physiological saline for 42 days intraperitoneally. At the same time, each major group was evenly divided into three parallel subgroups for each parallel bone evaluation separately at the second, fourth, and sixth weeks. Each subgroup included eight rats.

Results: The total radiological evaluation results showed that the healing progress of femoral fracture in the experimental group was superior to that in the control group from the fourth week. At the sixth week, experimental group rats began to present significantly better femoral fracture healing progress than that of the control group rats. Results of biomechanics show the ultimate load (N) and deflection ultimate load (mm) of the experimental group rats was significantly increased compared with that of the control group rats from the fourth week.

Conclusions: Our results suggest that leptin may have a positive effect on SD rat femur fracture healing.

MeSH Keywords: **Femur • Fracture Healing • Leptin • Rats, Sprague-Dawley**

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Background

The possible positive relationship between bone metabolism and leptin level has attracted more and more attention from scientific researchers. Leptin is believed to play a crucial role in bone microstructural alterations and bone metabolism [1,2]. A recent meta-analysis suggested that leptin levels of human serum are positively associated with bone mineral content (BMC) and bone mineral density (BMD) [3]. Leptin has physiological functions such as maintaining bone mass to get better bone quality [4]. Recently, the important relationship between bone mass and leptin has gained popularity, but the role of leptin has not been fully elucidated [5]. Zheng et al. [6] revealed that leptin overexpression in osteoporotic bone marrow stromal cells (BMSCs) enhances the capacity for differentiating into osteoblasts and forming bone-like tissue.

Leptin is known as a polypeptide hormone secreted by the white adipose tissue, which is encoded by the obese gene. Thus, leptin can affect fat tissue size through affecting food intake and energy expenditure [7]. Because bone and adipose tissue are all differentiated from the same MSCs, many recent studies investigated the possible effects of leptin level on bone mass [5,8–11]. Zhang et al. found that the osteogenic differentiation potential and response ability of leptin to BMSCs in postmenopausal women with osteoarthritis and osteoporosis are different [7]. Leptin can increase the differentiation of BMSCs into osteoblasts and meanwhile decrease the differentiation ability of BMSCs into adipocytes [8]. In addition, leptin seems to play a crucial role in the arrangement of the growth plate, including cartilage matrix maturation and chondrocyte differentiation, by secretion along with side secretion mechanisms [9]. At the same time leptin affects the differentiation and proliferation ability of osteoblasts and the coordination of bone development [10]. The effect of leptin on overall bone formation comes not only from acting as a systemic hormone, but also from acting as the local factor in the formation of the vascular tissue of the cartilage [11]. A study by Ducy et al. identified leptin as a potent inhibitor of bone formation that acts through the central nervous system and therefore illustrates the central nature of bone mass control and its disorders [12]. Systemic application of leptin can directly affect osteoblasts and osteoclasts, and reduce the brittleness of the bone [5]. Oheim et al. found that intracerebroventricular application of leptin into the lateral ventricle strongly reduced bone formation and led to a highly significant trabecular bone loss in ewe [13]. Pogoda et al. showed that the central regulation of bone formation is not limited to rodents, but is also found in large animals, providing evidence that bone remodeling in vertebrates is centrally controlled [14]. Some studies report that the relationship between brain injury and fracture healing is linked to leptin [15]. Wei et al. [16] found that the level of leptin was closely related to the formation of bone callus in the process of fracture repair.

Many previous studies focused on the possible effects of serum leptin on angiogenesis, chondrocyte differentiation, and osteoblast differentiation. Our study aimed at verifying whether leptin play a possible positive role in accelerating the healing progress of rat femur fracture.

Material and Methods

The research was carried out in the laboratory animal experimental center after the approval of the hospital ethics committee (Shanghai, China). The animal model was strictly in accordance with the guidelines of the Shanghai Laboratory Animal Center and the policy on animal use in Shanghai Tenth People's Hospital. This animal research protocol was approved by Animal Care and Welfare Committee of Tongji University. Forty-eight male Sprague Dawley rats provided by Shanghai SLAC Laboratory Animal Inc. with the standard of GB14924.2-GB14924.6, with an average body weight of 389 g (range: 376–398 g) and an average age of 10 weeks, were equally and randomly divided into two main groups. Control group rats were treated with only 0.5 mL of physiological saline intraperitoneally every day. Experimental group rats were treated with recombinant leptin 5 µg/kg/d (Rat Recombinant Leptin, ProSpec, Rehovot, Israel) along with the same 0.5 mL of physiological saline intraperitoneally every day. Treatment began from the day of surgery and was repeated daily at the same time for a total course of 42 days. At the same time, each major group of rats was evenly divided into three parallel subgroups (n=8 rats) for each parallel evaluation at the second, fourth, and sixth weeks after operation. We monitored all rats' conditions every day and recorded their weight.

Femur fracture model

All rats were reared at the specific pathogen-free laboratory animal experimental center, Shanghai Tenth People's Hospital. After the adaptation period with free access to water and regular food for one week, all rats were for fasted four hours before surgery. The operation was performed under anesthesia, and the method was intraperitoneal injection of 1% sodium pentobarbital (50 mg/kg of body weight). The rats were placed in a supine position, and 10% iodine solution was used to scrub the skin over the right femur preoperatively and postoperatively. All efforts were made to minimize their suffering.

A 1.0 mm stainless-steel rod was threaded directly into the rat's right femur medullary cavity through its skin and patellar ligament over its knee joint and finally advanced up to its femur distal end. The rat's femur was exposed by a longitudinal median skin incision directly over its femur. Three holes were made at the right angles of its femur midshaft by using a drill. Light manual bending broke its femur gently while

the stainless-steel rod was held in the right place of the femur medullary canal. Then the skin incision was closed with a 3-0 absorbable traction suture. All the operations were carried out by the same surgeon. After anesthesia, the rats were allowed to have whole weight bearing and perform unrestricted movement. Preventative antibiotics were administered by intramuscular injection to all rats preoperatively and postoperatively. The rats were given free access to water and normal laboratory chow 6 hours after the operation.

Serum leptin level was analyzed using available enzyme-linked immunoassay (ELISA) kit (Rat leptin ELISA kit, catalog no: SK00050-08) at the fourth and sixth weeks. Anesthetized rats were killed by cervical dislocation. The rat right leg was amputated from the hip joint. The rat femurs were stripped from their soft tissues and finally stored for radiological evaluation and biomechanical testing.

Radiological evaluation

Radiological evaluation with the Lane-Sandhu scoring (LSS) system was used for bone formation in fracture healing progress [17]. The callus diameter of X-ray (mm) and volume of fracture callus (mm³) parameters were evaluated by two separate observers in a blinded manner according to the standard lateral radiographs that were taken after anesthesia. Serial micro-computed tomography (CT) scans of these femur specimens were carried out under a micro-CT scanner with protocols. The bone volume fraction (BV/TV) parameter was calculated, then the BMD was calibrated by using the attenuation coefficient of two hydroxyapatite phantoms with defined BMD of 0.25 g/cm³ and 0.75 g/cm³. After the micro-CT scan, tissue plugs corresponding to the volumes of interest (VOIs) of femur plateaus were further processed for histological analysis. Serial sections were made into 5 mm thicknesses, and microscopy of callus with tetracycline bone double-labeling fluorescence was done. Fracture regions were observed under fluorescence microscopy. Evaluations were conducted by an experienced expert blinded to the findings from X-ray and micro-CT. Mineral apposition rate (MAR) was assessed by Image-Pro Plus version 5.0. Briefly, at least five sections from each sample were stained for analysis. For each section, five areas were measured.

Biomechanical and RT-PCR testing

All femora from rats were stored in separate sealed plastic bags -20°C after the radiological evaluation until one day before biomechanical testing. All femora was removed from the freezer and allowed to thaw overnight in the original sealed plastic bags before biomechanical testing. Biomechanical tests were performed in a configuration that simulated the mechanical axis of the rat femur. The actuator moved downward

Figure 1. Weight change of Sprague Dawley rats in the two groups.

at a rate of 1 mm/s until failure occurred. The fracture position and fracture load were recorded. The ultimate load (N) and deflection ultimate load (mm) parameters were evaluated by an experienced expert blinded to the findings from radiological evaluation according to the curves of position and fracture load. Real-time polymerase chain reaction (PCR) was then performed to measure associated mRNA expression levels from fracture callus relative to GAPDH mRNA expression with the ABI PRISM® 7500 Sequence Detection System (Applied Biosystem, Foster City, California, USA) using SYBR Green Master Mix (Toyobo Co., Ltd.).

Statistical evaluation

All research results were expressed as means $\pm$ standard deviation (SD). All available data were analyzed by SPSS statistical software version 21.0 as appropriate. Comparisons between the two groups were analyzed by using the t-test. The Mann-Whitney U test was used for the analysis of non-normal distribution research data. A difference of $P < 0.05$ was usually considered to be statistically significant.

Results

Surgery and ELISA results

Open transverse femur fractures were created in the experimental rat, and the fracture healing was typical secondary fracture healing. No complications occurred during anesthesia or intraperitoneal injection. Postoperative limping was only seen during the first week; then, all rats could use their fixed extremities in the normal fashion. Also, no wound infection or complications occurred. No rat died in the whole experiment. The weight change of the Sprague Dawley rats is shown in Figure 1. No significant difference was observed between the two major groups until the sixth week, so the effect of weight

B

C

Figure 2. (A) Radiological images showing different degrees of union in the two groups. (B) Callus diameter of X-ray (mm) in the experimental group was larger than that in the control group. (C) Volume of fracture callus (mm³) in the experimental group was larger than that in the control group.

Figure 3. (A) Bone volume fraction (BV/TV) in the experimental group was larger than that in the control group from the fourth week ($P<0.05$). (B) Bone mineral density (BMD) of the callus in the experimental group was significantly increased compared with that in control group from the fourth week ($P<0.05$).

on the biomechanical results can be ignored. Serum leptin levels were significantly different ($P<0.01$) between the leptin (5.97 ± 0.23 ng/mL) and control (2.42 ± 0.16 ng/mL) groups at the fourth week. At the sixth week, serum leptin also was significantly different ($P<0.05$) between the leptin (4.65 ± 0.46 ng/mL) and control (2.27 ± 0.06 ng/mL) groups. Therefore, treatment with recombinant leptin 5 μ g/kg/d intraperitoneally can increase the serum leptin level in rats.

Radiological results

Different degrees of fracture union progress in both groups of rats can be seen in Figure 2. At the first week a holonomic fracture line could be seen in both major groups. At the second week the fracture lines in the experimental group rats began to disappear. However, no significant difference was observed between both major groups at the first and second weeks, respectively. At the fourth and sixth weeks, a significant difference was observed between both major groups: the progress of fracture healing in the experimental group rats were better than that of the control group rats ($P=0.034$ and $P=0.002$, respectively) (Figure 2A). The callus diameter of X-ray (mm) in the experimental group was larger than that in the control group, and it was significantly different between the two groups at the sixth week ($P<0.05$) (Figure 2B). The volume of fracture callus (mm^3) in the experimental group was larger than that in the control group, and it was significantly different between the two groups from the fourth week ($P<0.05$) (Figure 2C). In both major groups, no significant difference was observed between the second, fourth, or sixth week in all parallel subgroups. Result of micro-CT scan showed that leptin treatment induced a larger and more matured callus than that in the control group, the cortical thickness of the callus significantly exceeded that in the control group, the healing processes were earlier than those in the control group, and the quality of the callus was improved. The BV/TVs of the experimental group rats were larger than those of the control

group rats from the fourth week ($P<0.05$) (Figure 3A). The BMD of rat callus in the experimental group was significantly increased compared with that in the control group from the fourth week ($P<0.05$) (Figure 3B). Also, microscopy of callus with tetracycline bone double-labeling fluorescence showed that the growth distance of new bone was significantly different between the groups at the sixth week (Figure 4A), and histomorphology showed that the MAR of fractured femora was significantly different between the groups from the fourth week ($P<0.05$) (Figure 4B). Also, the MAR of non-fractured femora was significantly different between the groups at the sixth week ($P<0.05$) (Figure 4C). The results suggest that leptin has a positive influence on osteoblast activity.

Biomechanical and RT-PCR results

Results of biomechanics show the ultimate load (N) of the experimental group rats was significantly increased compared with that of the control group rats at the fourth and sixth weeks ($P<0.05$) (Figure 5A). The deflection ultimate load (mm) was significantly different at the fourth and sixth weeks, too ($P<0.05$) (Figure 5B). As presented in Figure 5, rats treated with leptin exhibited significantly increased Vegfa (Figure 5C) and HIF1A (Figure 5D) expression at the measured time points.

Discussion

With the development of the world's population and technology, the frequency and severity of bone injury are increasing. It is a serious medical problem for both doctors and patients. The research literature on fracture healing is also rapidly increasing. [18–21]. A study by Beil et al. [22] indicated that the formation of bone has great significance in the various stages of fracture healing. Compared with normal wild-type mice, ob/ob and db/db mice have a twofold increase in bone formation, which can significantly accelerate the bone fracture

Figure 4. (A) Microscopy of callus with tetracycline bone double-labeling fluorescence showed that the growth distance of new bone was significantly different between the groups at the sixth week. (B) Histomorphology showed the mineral apposition rate (MAR) of fractured femora between the groups was significantly difference from the fourth week ($P<0.05$). (C) MAR of non-fractured femora was significantly different at the sixth week ($P<0.05$).

healing process in the ob/ob and db/db mice. Therefore, increasing bone formation is an important way to improve fracture healing. Fracture healing is a process of wound healing, which is similar to bone growth and development, including the interaction between cells, extracellular matrix, and growth factors. Bone progenitor cells, vascular cells, inflammatory cells, and osteoclasts play a crucial role in the repair process at the cellular level. Similarly, growth factors, proinflammatory cytokines, angiogenic factors, and proosteogenic factors also play a crucial role in the bone repair progress at the molecular level [23,24].

The research studies on the possible relationship between bone metabolism and body fat are rapidly increasing. Perhaps the effect of fat on bone mass is through central and endocrine regulation. The cytokines secreted by fat cells, such as hormones like leptin, are important factors in the functional connectivity of bone and adipose tissue [25–27]. In a recent

study, Khan et al. expressed the belief that leptin expression was in a unique stage of the process of fracture healing, the lack of leptin leads to delayed fracture healing, and local application of leptin can accelerate fracture healing [28]. Some studies showed the interesting result that leptin can accelerate fracture healing after traumatic brain injury [29].

The experimental studies showed that femoral fractures, traumatic brain injury, or spinal cord injury could activate the secretion of endogenous leptin; then, the serum leptin level would increase immediately. Higher serum leptin levels are associated with the increased capacity for bone formation in the fracture site [30,31]. In addition, leptin in the serum can induce the osteogenic differentiation of myeloid progenitor cells in bone marrow, promote the proliferation progress of osteoblasts, and promote the mineralization capacity of bone at the fracture site peripherally [8,26,30,31]. A study by Kerimoglu et al. [32] found that leptin seems to have a significant dose-dependent

Figure 5. (A) Ultimate load (N) in the experimental group was significantly increased compared with that in the control group at the fourth and sixth weeks ($P<0.05$). (B) Deflection ultimate load (mm) was significantly different at the fourth and sixth weeks ($P<0.05$). (C) Relative mRNA expression levels of Vegfa. (D) Relative mRNA expression levels of HIF1A were determined by quantitative polymerase chain reaction.

positive effect on the healing progress of tibial fracture in rats. The weaknesses of the study by Kerimoglu et al. include not determining serum leptin levels and not performing biomechanical testing of the callus. We found that exogenous leptin in daily use could maintain a high level of serum leptin, so we observed better fracture healing progress in the exogenous leptin group rats in this study (Figure 1).

In a study by Wang et al. [30], the rats with highest levels of serum leptin had the highest callus formation. Similarly, in this study, rats receiving exogenous leptin had better fracture healing. Some research studies indicated that leptin could inhibit the differentiation of BMSCs into adipocytes and promoted the differentiation of BMSCs into osteoblasts [8,10]. Leptin plays a promoting role in fracture healing progress at the cellular level. In addition, leptin can be combined with the receptor on osteoblasts directly to increase the growth of osteoblasts and the extent of bone mineralization [26]. The process of fracture healing can be accelerated by increasing osteoblast differentiation and promoting chondrocyte differentiation.

Angiogenesis in the process of fracture healing provides nutrients, cells and biological media, and waste disposal environment. Angiogenesis plays a crucial role in the process of

osteogenesis [33,34]. These are all the natural stages of the fracture healing progress. A study by Bouloumie et al. [35] overemphasized that leptin is a potent regulator for angiogenesis. Also, *in vivo* study showed that leptin has an activation effect on migration of vascular endothelial cells and angiogenesis. In this study, leptin treatment could accelerate the progress of fracture healing. Therefore, we believe that the effect of leptin on angiogenesis may play a crucial role in the process of fracture healing.

Although the specific mechanism of how leptin promotes fracture healing progress is not clear, we believe that the positive effects of leptin in regulating angiogenesis in the cartilage into bone process, promoting effects on osteogenic and chondrogenic differentiation and growth, and promoting effects on bone metabolism eventually led to this result. One research study supports the idea that leptin receptor-deficient diabetic (db/db) mice had impaired function of bone regeneration after birth [36]. Also, leptin receptor-expressing mesenchymal stem cells are the main source of bone formation in adult bone marrow [37].

In this study, we observed that leptin had a positive effect on the progress of fracture healing, based on the radiological

evaluation results. The radiological outcome data from two groups were obtained without interruption and showed the applicability of this method. The weaknesses of the study included no measurement of serum leptin concentration in the rats and not clearly understanding which pathway of bone metabolism and bone remodeling is affected by leptin.

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Conclusions

To sum up, intraperitoneal injection application of exogenous leptin has a positive effect on the healing progress of femoral fractures in rats. We conclude that leptin can promote the formation of callus and finally promote the fracture healing.

Disclosure

None.