Effect of leptin on bone metabolism in rat model of traumatic brain injury and femoral fracture

WANG Lei, YUAN Ji-shan*, ZHANG Hong-xi, DING Hua, TANG Xing-guo and WEI Yong-zhong

【Abstract】Objective: To observe serum and callus leptin expression within the setting of fracture and traumatic brain injury (TBI).

Methods: A total of 64 male SD rats were randomized equally into 4 groups: nonoperated group, TBI group, fracture group, and fracture+TBI group. Rats were sacrificed at 2, 4, 8 and 12 weeks after fracture+TBI. Serum leptin was detected using radioimmunoassay, and callus formation was measured radiologically. Callus leptin was analyzed by immunohistochemistry.

Results: Serum leptin levels in the fracture group, TBI group and combined fracture+TBI group were all significantly increased compared with control group at the 2 week time-point (P<0.05). Serum leptin in the combined fracture+TBI group was significantly higher than that in the fracture and TBI groups at 4 and 8 weeks after injury (P<0.05). The percentage of leptin-positive cells in the fracture+TBI callus and callus volume were significantly higher than those in the fracture-only group (P<0.01).

Conclusions: We demonstrated elevated leptin expression within healing bone especially in the first 8 weeks in a rat model of fracture and TBI. A close association exists between leptin levels and the degree of callus formation in fractures.

Key words: Leptin; Brain injuries; Fractures, bone

Clinical and experimental studies have shown enhanced osteogenesis in patients sustaining limb fracture associated with traumatic brain injury (TBI).1,2 Accelerated fracture healing and heterotopic ossifications are well-known phenomena in these patients. Owing to recent findings that bone formation is regulated by leptin through the hypothalamus and the sympathetic nervous system,3,4 it is possible that the disruption of this pathway is a key factor for osteogenesis in TBI.

Several other bone-associated metabolic factors have already been demonstrated an association with this phenomenon. These include predominantly serum factors such as growth hormone (GH), transforming growth factor-β (TGF-β), nerve growth factor (NGF), insulin-like growth factor II (IGF-II), platelet-derived growth factor (PDGF) and interleukin-1 (IL-1) and IL-6.5-7 Few studies have been reported on the changes in serum leptin induced by TBI.

The aim of this study was to measure changes in serum leptin after TBI and femoral fracture and observe any association between leptin levels and the degree of fracture healing. In addition, this report compared the healing response in TBI rats and those without cerebral injury in order to quantify the effect of TBI on bone formation, and the relationship between leptin levels and the quantity of callus formation after TBI. We believe that understanding the role of leptin in bone metabolism and injury may provide further approaches to the treatment of both non-union and heterotopic bone formation.

METHODS

Ethical approval for this study was granted by the ethical board of the Affiliated People’s Hospital of Jiangsu University, China. Adult male SD rats (n=64, 250-290 g, purchased from Shanghai Slaccas Laboratory Animal Co., Ltd, China) were maintained at...
23°C±1°C on a 12-hour light/dark cycle and all animals were housed individually in specific pathogen-free conditions with unlimited access to water and laboratory food and treated strictly in accordance with institutional ethical guidelines. A rat leptin kit (Linco Corporation, USA) with a mouse monoclonal antimyosin-leptin (Sigma, USA) was used for the radioimmunoassay.

Rats were randomized into 4 groups: nonoperated control group, traumatic TBI group, fracture group and fracture+TBI group (n=16 per group). The rats were sacrificed at 2, 4, 8 and 12 weeks respectively after injury. Leptin concentration in serum was detected using radioimmunoassay. Callus formation was monitored with radiographs and callus volumetric analysis was performed. Leptin expression in callus was also analyzed via immunohistochemistry.

**TBI model**

The animals were pre-anesthetized with 2% isoflurane and then given oxygen with 0.75% isoflurane through a fixed face mask on a stereotactic platform. The skull was exposed and craniectomy was made laterally, at the point between the lambda and the bregma, between the central suture and the left temporal ridge using a 6-mm trephine. The exposed dura was subjected to a 5-mm-diameter piston impact of 3.0-mm depth, 4 m/s velocity and 100 msec duration. The scalp was closed without replacement of the bone flap. Anesthesia was discontinued, and the animal was assessed for exclusion criteria (latency of pinna and corneal reflexes and lighting response).

**Femoral fracture model**

Femoral osteotomy and fixation were performed in the same manner as previously reported. Briefly, a transverse osteotomy was made at the midshaft of the femur and intramedullary fixation was performed using a stainless steel wire (1.5 mm in diameter). The fracture fragments were reduced and stabilized. Wires were cut on the surface of the intercondylar groove to avoid restriction of motion of the knee joint. Unrestricted activity was allowed after recovery from anesthesia.

**Tissue preparation and histologic analysis**

After sacrifice, fractured femurs from rats were dissected and carefully cleaned of muscle around the fracture callus to preserve callus integrity. Calluses from weeks 2, 4, 8 and 12 after fracture were then fixed in 4% paraformaldehyde solution, the intramedullary wire was removed, and the specimen was decalcified before paraffin embedding. Sagittal sections (5-7 μm) were made through the fracture site and mounted on Fisherbrand Superfrost/Plus slides (Fisher Scientific, USA). Sections were stained with hematoxylin-eosin for histological evaluation of healing at 2, 4, 8 and 12 weeks after fracture.

**Immunohistochemistry staining of leptin**

Tissue sections were deparaffinized with xylene, and then rehydrated through a series of decreasing ethanol concentrations. Sections were washed in phosphate buffered saline with 0.1% Triton X-100 (Sigma, USA) and endogenous peroxidase was blocked using a solution of 30% methanol and 3% H₂O₂ for 30 minutes. The tissue sections were pretreated with 10% normal rabbit serum diluted in phosphate buffered saline (blocking buffer) for 30 minutes. The primary antibody was rat antihuman leptin monoclonal antibody (Sigma, USA), which was diluted 1:200 in blocking buffer. Sections were incubated in diluted primary antibody for 1 hour at room temperature and then washed three times with phosphate buffered saline containing 0.1% Triton X-100 for 5 minutes each time. The secondary antibody used was rat anti-mouse polyclonal antibody (R&D Systems, USA) diluted 1:1000 in blocking buffer. After three washes, the tissue sections were incubated in diluted secondary antibody for 30 minutes at room temperature and then washed three times in phosphate buffered saline with 0.1% Triton X-100 for 5 minutes each time. Sections were washed with water, counterstained with hematoxylin, dehydrated, cleared with 100% xylene, and mounted for microscopic examination with an Olympus BH-2 microscope (Olympus Corporation, USA). Negative controls were stained as above but without primary antibody.

**Radioimmunoassay of leptin**

By radioimmunoassay, a fixed concentration of labeled tracer antigen (Linco Research Inc., USA) was incubated with dilution of 125I-labeled rat leptin antiserum such that the concentration of antigen binding sites on the antibody was limited. If unlabeled antigen (testing rat serum) was added to this system, there was competition between labeled tracer and unlabeled antigen for the limited antibody. Then antibody-bound tracer can be separated from unbound tracer, and finally, an instrument (DFM-96 radioimmunity-counter, Nanjing Medical
Measurement of callus volume

Perkins volume formula was used as a measure of volume of the fracture callus in experimental rats and control rats at 2, 4, 8 and 12 weeks after fracture. Anterior-posterior radiographs of fractures were taken for all rats and the volume of callus was calculated using Perkins volume formula: 

$$2 \pi \cdot R_1 \cdot (R_2 - R_1) \cdot L$$

(where \(R_1=\)femur radius; \(R_2=\)callus radius; \(L=\)length of callus).

Statistical analysis

We used the SPSS 12.0 statistical analysis software package for data analysis. Mean and standard deviation of the mean of positively stained cells and the serum level of leptin were evaluated. One-way ANOVA and Student-Newman-Keuls tests were used in this study. \(P\) values below 0.05 were considered statistically significant.

RESULTS

Serum levels of leptin

The serum levels of leptin in the TBI group, fracture group and fracture+TBI group was significantly increased compared with control group at 2 weeks after injury (\(P<0.05\)). The rate of increase in concentration of serum leptin in the fracture+TBI group was higher than other treatment groups at 4 weeks after operation (\(P<0.01\)). The concentration of serum leptin in the fracture+TBI group continued to be significantly greater than that in other treatment groups at 8 weeks after injury (\(P<0.05\)). This difference was not statistically significant at 12 weeks (\(P>0.05\), Table 1).

Radiological analysis

At 2 weeks after operation, the evidence of callus formation was found at the fracture site in the fracture+TBI group, whereas callus formation in the fracture group was minimal. This difference, however, did not reach statistical significance (Table 2).

At 4 weeks after operation, callus formation was more rapid in the fracture+TBI group and the volume of callus formation in fracture+TBI group was found to be significantly greater than fracture group. At 8 weeks, the volume of callus in the fracture+TBI group continued to be significantly larger than the fracture group (\(P<0.01\), Figure 1).

Histomorphological observation

The histologic appearance of the fracture callus was different between the fracture group and fracture+TBI group. At 2 weeks, fracture callus from the fracture+TBI group showed a well organized callus with areas of newly formed fibrous callus, calcified cartilage, chondrocytes and hypertrophic chondrocytes. At 4 and 8 weeks, the fracture+TBI group demonstrated more mature trabecular bone formation and larger external callus compared with the fracture group (Figure 2). At 12 weeks, lamellar bone in fracture+TBI group also had thicker appearance compared with fracture-only group.

Immunohistochemistry analysis

Throughout the process of fracture healing, products of leptin can be detected immunohistochemically in many cells around the fracture site including osteoblasts, fibroblasts, mesenchymal cells and cartilage cells. Using our staining technique, cells with light-brown staining particles in the cytoplasm were regarded as positive. Positive cells were counted under 400 times magnification. The number of positive cells was then standardized to the same counting area.

At 2 weeks after operation, a greater percentage of positive cells were found in fracture+TBI group compared with the fracture group; however, this did not reach statistical significance. At 4, 8 and 12 weeks, the per-
centages of positive cells in callus for fracture+TBI group were significantly higher than that of the fracture alone group \((P<0.05, \text{ Table } 3 \& \text{ Figure } 3)\). This difference peaked at 8 weeks and decreased at 12 weeks.

**Figure 2.** X-ray radiographs of fractured femur in fracture and fracture+TBI groups. At 4 weeks after fracture, the fracture line was visible in both groups (arrow). The callus became dense and callus formation at the fracture site in fracture+TBI group (B) occurred more rapidly than those of the fracture group (A). The volume of callus in the former group was significantly larger than the latter group. At 8 weeks the size of callus in both groups was increased, with fracture+TBI group (D) again significantly larger than fracture alone group (C).

**Table 1.** Changes in serum levels of leptin at different phases in 4 groups \((\bar{x}\pm s, \text{ ng/ml})\)

<table>
<thead>
<tr>
<th>Groups</th>
<th>1 w ((n=6))</th>
<th>2 w ((n=6))</th>
<th>4 w ((n=6))</th>
<th>8 w ((n=6))</th>
<th>12 w ((n=4))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonoperation</td>
<td>1.22(\pm)0.07</td>
<td>1.26(\pm)0.05</td>
<td>1.43(\pm)0.27*</td>
<td>1.30(\pm)0.15*</td>
<td>1.31(\pm)0.06</td>
</tr>
<tr>
<td>Fracture</td>
<td>1.25(\pm)0.06</td>
<td>1.67(\pm)0.07*</td>
<td>2.31(\pm)0.13*</td>
<td>2.20(\pm)0.09*</td>
<td>3.55(\pm)0.09</td>
</tr>
<tr>
<td>TBI</td>
<td>1.23(\pm)0.34</td>
<td>1.74(\pm)0.13*</td>
<td>3.23(\pm)0.09*</td>
<td>2.84(\pm)0.22*</td>
<td>1.47(\pm)0.19</td>
</tr>
<tr>
<td>Fracture+TBI</td>
<td>1.30(\pm)0.05</td>
<td>1.81(\pm)0.11*</td>
<td>5.03(\pm)0.35*</td>
<td>3.55(\pm)0.46*</td>
<td>1.56(\pm)0.16</td>
</tr>
</tbody>
</table>

\*\(P<0.05\), compared with control group; ★\(P<0.05\) compared with fracture+TBI group; ▲\(P<0.01\) compared with fracture+TBI group.

**Figure 3.** Immunohistochemical staining for leptin in the fracture group at 4 weeks (A) and 8 weeks (B), and the TBI+fracture group at 4 weeks (C) and 8 weeks (D). Note the general increased amount of leptin staining at both time points in the TBI+fracture group.

**Table 2.** Volume of fracture callus in fracture and fracture+TBI groups \((\bar{x}\pm s, \text{ mm}^3)\)

<table>
<thead>
<tr>
<th>Groups</th>
<th>2 w ((n=4))</th>
<th>4 w ((n=4))</th>
<th>8 w ((n=4))</th>
<th>12 w ((n=4))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fracture</td>
<td>37.12(\pm)2.86</td>
<td>70.23(\pm)7.19*</td>
<td>115.72(\pm)10.50*</td>
<td>67.59(\pm)7.55</td>
</tr>
<tr>
<td>Fracture+TBI</td>
<td>40.05(\pm)2.30</td>
<td>122.74(\pm)9.65*</td>
<td>182.12(\pm)10.69*</td>
<td>79.42(\pm)14.46</td>
</tr>
</tbody>
</table>

*\(P<0.05\), compared with fracture+TBI group.

**Table 3.** The mean percentage of positive cells in callus of fracture and fracture+TBI groups \((\bar{x}\pm s)\)

<table>
<thead>
<tr>
<th>Groups</th>
<th>2 w ((n=4))</th>
<th>4 w ((n=4))</th>
<th>8 w ((n=4))</th>
<th>12 w ((n=4))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fracture</td>
<td>8.5(\pm)2.65</td>
<td>13.75(\pm)2.50*</td>
<td>21.25(\pm)2.88*</td>
<td>14.25(\pm)2.98*</td>
</tr>
<tr>
<td>Fracture+TBI</td>
<td>11.75(\pm)5.12</td>
<td>21.25(\pm)4.35*</td>
<td>33.75(\pm)5.17</td>
<td>20.75(\pm)2.06</td>
</tr>
</tbody>
</table>

*\(P<0.05\), compared with fracture+TBI group.

**DISCUSSION**

Leptin, a circulating protein product of Ob gene, is initially discovered as a satiety factor regulating food intake and energy expenditure.\(^9\) It is produced primarily by adipocytes and acts on the hypothalamus, but important non-adipocytic, extra-hypothalamic pathways also exist.\(^10,11\) More recently the role of leptin in bone metabolism has been identified\(^12\) and is undergoing intensive characterization, especially in the context of
Secondly, a pulmonary inflammatory response contributes to elevated leptin levels in the fracture-only condition from the second week after operation. In regard to elevated leptin in the fracture-only condition there are several explanations. Firstly, the hypermetabolic response from injury generally results in mobilization of more free fatty acids, which leads to leptin elevation by neuroendocrine feedback regulation. Secondly, a pulmonary inflammatory response contributes to elevated leptin levels in the fracture-only condition from the second week after operation. In regard to elevated leptin in the fracture-only condition there are several explanations. Firstly, the hypermetabolic response from injury generally results in mobilization of more free fatty acids, which leads to leptin elevation by neuroendocrine feedback regulation.

In demonstrating a possible relationship between leptin levels and fracture healing in the setting of TBI, our study has a number of limitations. Firstly, the study design only allows the observation of leptin and fracture-healing associations, and not a direct causal link. Secondly, longer-term observation is needed to fully evaluate the remodeling process of fracture callus. Thirdly, our findings may not accurately represent clinical practice given our animal model, and also the use of localized, surgically produced lesions clearly lacks the additional deceleration and complexity involved in the majority of patients with femoral fractures and TBI.

In spite of these limitations, our findings demonstrate a number of important trends in the level and time course of serum leptin within the context of trauma, and its association with fracture healing.

Serum leptin in TBI, fracture and fracture+TBI groups was significantly increased compared with the control group from the second week after operation. In regard to elevated leptin levels in the fracture-only condition there are several explanations. Firstly, the hypermetabolic response from injury generally results in mobilization of more free fatty acids, which leads to leptin elevation by neuroendocrine feedback regulation. Secondly, a pulmonary inflammatory response contributes to elevated leptin levels in the fracture-only condition from the second week after operation. In regard to elevated leptin in the fracture-only condition there are several explanations. Firstly, the hypermetabolic response from injury generally results in mobilization of more free fatty acids, which leads to leptin elevation by neuroendocrine feedback regulation.

By acting on the hypothalamus and increasing both sympathetic output and β 2-adrenergic receptors on the surface of osteoblasts, leptin exerts an anti-osteogenic effect. Peripherally acting leptin, however, has an opposite effect by promoting bone mineralization and osteoblast to osteocyte differentiation. In the physiological resting state, these two opposing actions of leptin are in balance.

The overall control of leptin action is not well understood, especially in the setting of fracture where there is significant metabolic disturbance and inflammatory reaction. A link with this is the stress response of the neuroendocrine system where several cytokines and hormonal factors such as insulin and glucocorticoids have been shown to alter serum leptin levels. Notably, IL-1, a key cytokine in tissue injury and inflammation is found to increase leptin levels and this inflammatory pathway is likely to be involved in our findings of raised leptin in trauma.

In the physiological resting state, these two opposing actions of leptin are in balance. Notably, IL-1, a key cytokine in tissue injury and inflammation is found to increase leptin levels and this inflammatory pathway is likely to be involved in our findings of raised leptin in trauma.

Serum leptin in TBI, fracture and fracture+TBI groups was significantly increased compared with the control group from the second week after operation. In regard to elevated leptin levels in the fracture-only condition there are several explanations. Firstly, the hypermetabolic response from injury generally results in mobilization of more free fatty acids, which leads to leptin elevation by neuroendocrine feedback regulation. Secondly, a pulmonary inflammatory response contributes to elevated leptin levels in the fracture-only condition from the second week after operation. In regard to elevated leptin in the fracture-only condition there are several explanations. Firstly, the hypermetabolic response from injury generally results in mobilization of more free fatty acids, which leads to leptin elevation by neuroendocrine feedback regulation.

Notably, IL-1, a key cytokine in tissue injury and inflammation is found to increase leptin levels and this inflammatory pathway is likely to be involved in our findings of raised leptin in trauma.

In the physiological resting state, these two opposing actions of leptin are in balance. Notably, IL-1, a key cytokine in tissue injury and inflammation is found to increase leptin levels and this inflammatory pathway is likely to be involved in our findings of raised leptin in trauma.

Serum leptin in TBI, fracture and fracture+TBI groups was significantly increased compared with the control group from the second week after operation. In regard to elevated leptin levels in the fracture-only condition there are several explanations. Firstly, the hypermetabolic response from injury generally results in mobilization of more free fatty acids, which leads to leptin elevation by neuroendocrine feedback regulation. Secondly, a pulmonary inflammatory response contributes to elevated leptin levels in the fracture-only condition from the second week after operation. In regard to elevated leptin in the fracture-only condition there are several explanations. Firstly, the hypermetabolic response from injury generally results in mobilization of more free fatty acids, which leads to leptin elevation by neuroendocrine feedback regulation.

An interesting finding in the fracture+TBI group, which mimics the injuries and recovery response of the multi-trauma patient, was a significantly higher leptin level than other treatment groups at time points of 4 and 8 weeks. The number of leptin-positive cells and volume of callus in the fracture+TBI group were also significantly greater than those in fracture group. These results support the concept that injury to the hypothalamus may result in additional, peripheral secretion of leptin and bone regeneration through loss of sympathetic inhibitory control. After TBI, direct damages include diffuse axonal injury, contusions, and intracranial hematomas. Pathophysiologic finding are also amplified by secondary or delayed events including hypoxia, hypotension, ischemia, edema, increased intracranial pressure, all of which will lead to hypothalamic injury and dysfunction. Singleton et al found that mild brain injury in mice resulted in early apoptotic neuronal death in the thalamus and cortex which was implemented as a point of biomechanical vulnerability. Hypothalamic damage may also impair the anti-osteogenic effects of leptin acting through this site, contributing to the enhanced bone formation observed in the fracture+TBI group. Brain and hypothalamic injury, a main focus of our study, is more likely to result in increased sympathetic outflow in the acute period, but it is evident from our data that with the passage of time, the peripheral effect of increasing leptin may outweigh any potential sympathetic inhibition of leptin and bone formation.

The delay in leptin increase until week 4 after operation may be related to the acute postinjury stress...
response, and specifically early increases in sympathetic outflow. Dysautonomia and 'sympathetic storms' are well known to occur after TBI, and adrenergic stimulation acts to downregulate leptin expression and secretion by adipocytes. Leptin may also be low at an early stage because of fasting conditions in the injured patient or animal. After an approximate period of 3 months, features of dysautonomia such as heart rate variability begin to resolve and at this point it is possible that sympathetic inhibition of both leptin and osteogenesis gives way to pro-osteogenic effects and leptin increase noted in both TBI and specifically lesions of the hypothalamus. It is possible that an abatement of sympathetic instability occurred earlier in our animal model (2 to 4 weeks) given that the brain injury inflicted was a tightly regulated, focused procedure compared with the often complex TBI sustained by human patients.

The peak in callus formation demonstrated at 8 weeks with subsequent reduction, corresponds with the known process of callus resorption, most likely by osteoclasts, facilitating consolidation and true bony healing. Around this time point, we observed concomitantly reduced leptin levels, which may allow for a net callus resorption in the later stages of healing.

In conclusion, leptin levels increase more significantly after combined TBI and fracture, which may be related to hypothalamic damage and reduced inhibition of peripheral leptin secretion by the mechanisms discussed above. These high leptin levels are associated with increased callus formation in femoral fractures and also increased leptin expression in the fracture callus. Leptin does not reach a maximum level until 4 weeks after injury, and this may relate to early inhibition by sympathetic activity seen in TBI. In future studies on leptin in TBI and fracture healing, it will be important to identify the temporal relationships between serum leptin, and physical signs of sympathetic activity. Furthermore, it would be beneficial to perform a study including a leptin-knockout group to more accurately determine the effect of leptin on callus formation in TBI.

Acknowledgements

The authors would like to thank LI Yu-hua and WANG Fang for histological preparation, and CAO Xiao-jian, SUN Si-xin, and SUN Yu for their assistance with the animal model.

REFERENCES


