

Original Article

Impaired fracture healing with high non-union rates remains irreversible after traumatic brain injury in leptin-deficient mice

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Abstract

Patients with traumatic brain injury (TBI) and long-bone fractures can show increased callus formation. This effect has already been reproduced in wild-type (wt) mice. However, the mechanisms remain poorly understood. Leptin is significantly increased following TBI, while its role in bone healing remains unclear. The aim of this study was to evaluate fracture healing in leptin-deficient ob/ob mice and to measure any possible impact of TBI on callus formation. 138 female, 12 weeks old, ob/ob mice were divided into four groups: Control, fracture, TBI and combined trauma. Osteotomies were stabilized with an external fixator; TBI was induced with Controlled Cortical Impact Injury. Callus bridging was weekly evaluated with *in vivo* micro-CT. Biomechanical testing was performed *ex vivo*. Micro-CT showed high non-union rates after three and four weeks in the fracture and combined trauma group. No differences were observed in callus volume, density and biomechanical properties at any time point. This study shows that bony bridging is impaired in the present leptin-deficient trauma model. Furthermore, the phenomenon of increased callus formation after TBI could not be reproduced in ob/ob mice, as in wt mice. Our findings suggest that the increased callus formation after TBI may be dependent on leptin signaling.

Keywords: Fracture Healing, Traumatic Brain Injury, Tbi, Leptin, Ob/ob

Introduction

Increased callus formation can be surprisingly observed in patients suffering from traumatic brain injury (TBI) and concomitant long-bone fractures¹⁻⁵. Pre-clinical models in large and small animals reproduced this effect both radiographically and biomechanically^{6-9,10}. A novel *in vivo* trauma model in mice¹⁰ that combines a femoral osteotomy with a controlled cortical impact injury of the brain was

also able to simulate this effect in wild-type (wt) mice¹¹. Nevertheless, the underlying pathophysiologic mechanisms remain unknown to date. Identifying these mechanisms could help develop new strategies for the treatment of osteoporosis, non-unions and large bone defects. Previous studies focused on identifying humoral factors that are increased after TBI, and which are considered serum markers of bone growth, such as alkaline phosphatase, Runx2 and serine protease 7³, stromal cell-derived factor-1¹² or calcitonin gene-related peptide⁶. However, none of these factors has yet proven to be the primary causative factor of the increased callus formation after TBI, but rather seem to be secondary to upstream processes.

Leptin is a 16 kDa adipocyte-derived hormone that was initially found to primarily control energy and food intake^{13,14} and is now recognized as a pleiotropic hormone¹⁵. Besides adipocytes, leptin is also expressed in several other tissues such as the placenta¹⁶, the stomach¹⁷, the brain and pituitary gland¹⁸, the yellow bone marrow¹⁹ and

The authors have no conflict of interest.

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Edited by: F. Rauch

Accepted 25 April 2017



is involved in the regulation of multiple organ systems such as insulin homeostasis, reproduction, immune function and brain development²⁰. Leptin-deficient (*ob/ob*) mice lack the leptin gene and have a phenotype of hyperglycemia, hyperinsulinemia, a metabolic syndrome²¹ and obesity²². Interestingly, leptin has also turned out to be an important factor in the regulation of bone metabolism. Leptin was initially thought to be a central inhibitor of bone formation, as *ob/ob* mice showed increased bone volume and infusion of leptin into the brain resulted to a decrease of bone volume to the levels of *wt* mice²³. The picture, however, turned out to be more complex, as other studies showed that *ob/ob* mice did not seem to produce more bone in general but rather tend to have site-specific differences in bone volume, as well as, decreased biomechanical strength properties compared to *wt* mice²⁴⁻²⁷. *In vivo* studies showed that peripheral injection of leptin, either intraperitoneally, or subcutaneously in *ob/ob* mice significantly increased bone mass and osteoblast activity^{20,26}. Therefore, a dual mechanism was suggested (central and peripheral), where leptin decreases bone growth through the central nervous system, while acting peripherally as a promoter of bone growth^{26,28}. However, central leptin gene therapy in *ob/ob* mice resulted in increased bone formation to the levels of *wt* mice^{26,27}. Thereby, an alternative view is that leptin promotes bone growth directly through peripheral, as well as, indirectly through central mechanisms in which the peripheral actions are deemed superior²⁶.

What makes leptin interesting to look into on the matter of increased callus formation after TBI, is the fact that levels of leptin in serum and cerebrospinal fluid (CSF) are significantly increased following TBI^{7,29-31}. Given its stimulating effect on bone growth and direct relation to TBI, leptin may be an important primary factor responsible for the exuberant callus formation seen after TBI. To test this hypothesis we analyzed radiographically and biomechanically the impact of TBI on fracture healing in the absence of leptin with the use of a combined trauma model¹¹.

Methods

Animals and experimental setup

Ten week old, female B6.V-Lep-*ob*/JRj mice (n=138) were purchased from Janvier Labs (Saint Berthevin, France). The animals were housed under conditions of controlled temperature (20±2°C) and humidity with a 12 h light/dark cycle and food and water *ad libitum*. Prior to study inclusion, all animals were kept for one week in the laboratory premises in order to allow acclimatization. Animals were housed in a standard type III cage ("Euronorm"). All experiments were carried out according to the policies and principles established by the Animal Welfare Act, the National Institutes of Health Guide for Care and Use of Laboratory Animals, and the National Animal Welfare Guidelines. The study was approved by the local legal representative animal rights protection authorities (G 0009/12).

Animals were randomly subdivided into four groups:

control (n=28), fracture (n=37), TBI (n=35) and fracture + TBI (n=38). Group sizes were different because of different mortality rates between the groups¹⁰.

Anesthesia for all operations was conducted and maintained using a gas mixture of 1.5-2% isoflurane (FORENE, Abbott, Wiesbaden, Germany), 0.3% O₂ and 0.5% N₂O. Perioperative single-shot antibiotic prophylaxis was performed by subcutaneous injection of 0.02 ml clindamycin and analgesia by 0.1 mg/kg of body weight buprenorphine (TEMGESIC, Reckitt Benckiser, Mannheim, Germany). Operations for the different groups were randomized and were conducted between 08.00 a.m. to 02.00 p.m.

Animals in the fracture group were operated at the left femur using a standardized open osteotomy model³². An external fixator (MouseExFix, Research Implant System, RIS, Davos, Switzerland) was mounted on the bones for stabilization. Afterwards, a diaphyseal osteotomy was performed with a 0.7 mm Gigli-wire saw. This relatively large bone defect size was chosen in order to be able to analyze bone healing over a longer time course. Animals in the TBI group were operated at the left parietotemporal cortex using the standardized controlled cortical impact injury (CCI) model for mice³³⁻³⁵. Further pain reduction was achieved by preoperative subcutaneous infiltration of the skin incision site with 1 ml per kg body weight bupivacaine (Bupivacain-RPR-Actavis, Actavis GmbH & Co. KG, Munich, Germany). After skin incision, craniotomy was performed using a 1.2 mm ball cutter to open a 7x7 mm bone window. Special care was taken not to damage the dura mater in order to simulate a closed TBI. CCI was then performed with a 5x5 mm impactor tip at an impact angle of 45°, a contusion depth of 0.25 mm, an impact velocity of 3.5 m/s and a contact time of 150 ms. For the combined trauma model both methods were combined, as previously reported¹⁰.

In vivo micro CT analyses of the osteotomy gaps were performed once a week on all animals in the fracture and combined trauma group. After three and four weeks the animals were sacrificed and biomechanical testing was performed *ex vivo*.

Micro-CT analysis

Longitudinal *in vivo* micro-CT analyses were performed at an isotropic voxel size of 15 microns (Scanco vivaCT 40, Scanco Medical, Brüttisellen, Switzerland; 55 kV, 145 µA, 381 ms integration time). For the elimination of movement artifacts during the scanning procedure the mice were sedated with an intraperitoneal injection of 0.3 mg/kg body weight medetomidin (DORMITOR, Orion Pharma, Bad Homburg, Germany) and 60 mg/kg body weight ketamin (KETAMIN, Actavis, Munich, Germany). The volume of interest (VOI) comprised of 70 slices including the entire fracture gap and adjacent cortical bone structures. Micro-CT scans were evaluated in terms of callus volume (mm³) and callus density (mgHa/mm³) (Scanco Software IPL, version 5.15, Scanco Medical, Brüttisellen, Switzerland). The lowest threshold for callus tissue was 244 (equaling 576.3 mg hydroxylapatite/

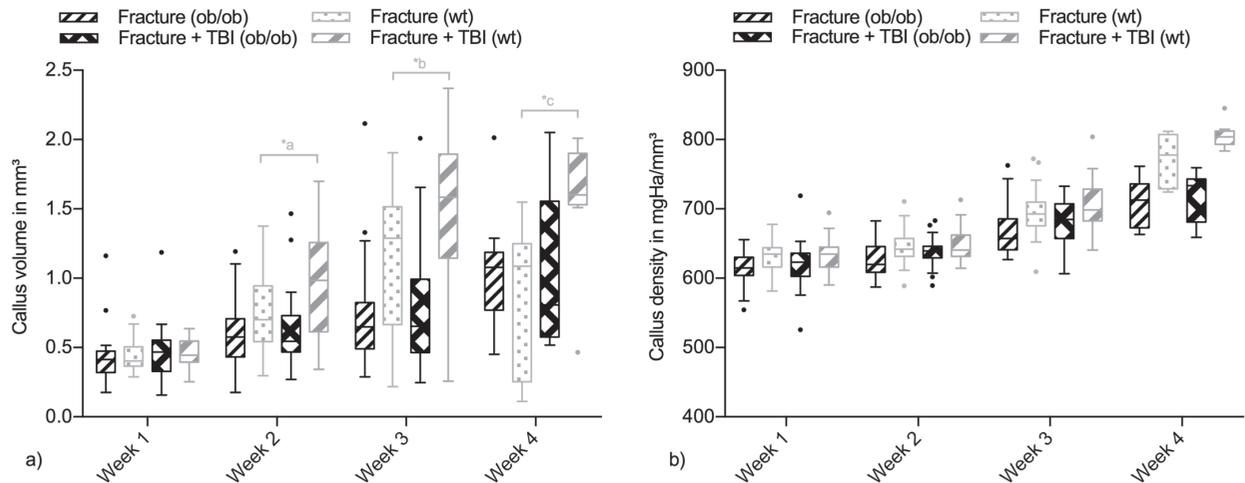


Figure 1. Box plots (interquartile range) of (a) callus volume (mm³) and (b) callus density (mgHa/mm³) displayed for both groups (Fracture and TBI+Fracture) and both mouse strains (ob/ob and wt). Differences between both groups of ob/ob mice in callus volume and callus density were not significant at any time point. Note the significant increase of callus volume in wt mice with an additional TBI (*a p=0.02, *b p=0.04, *c p<0.01)11. Also, there was no decrease in callus volume after three weeks in ob/ob mice as is normally expected in bone healing. Significance level p<0.05, Mann-Whitney U test. Figures displayed in grey already published¹¹.

cm³) and was determined using a histogram on 20 micro-CT series. By segmentation of the cortical bone and callus tissue using a semi-automatic drawing tool, callus tissue was analyzed excluding cortical structures.

Furthermore, callus bridging was evaluated using a micro-CT scoring system³⁶. Image stacks of all scanned specimens from week three and four were 2-D reconstructed using Scanco Software and screened in two perpendicular views (sagittal and frontal reconstructions). Two independent observers rated the callus bridging according to four categories: A: complete bridging (four cortices bridged by callus), B: incomplete bridging (one to three cortices bridged by callus), C: no bridging (presence of callus but no cortex bridging at all), D: non-union (rounding of cortex, and almost no callus formation).

Biomechanical analysis

Biomechanical testing (maximum torque, maximum stiffness) was performed on eight mice after three weeks and on six mice after four weeks in each group. The left femora were harvested *post mortem* and the external fixator was dismantled. The right femora were also harvested in the fracture and combined trauma group to report the torque and stiffness values of the fractured bones as percentage of the contralateral healthy femora. Bones were tested directly after harvesting. Testing was conducted using a test bench system (Bose ElectroForce System TestBench, Friedrichsdorf, Germany), which was controlled with WinTest software (ElectroForce System Group, Minnesota, USA). Bones were fixed at the femoral head and femoral condyles

in two aluminum cylinders filled with a two-component resin based on methyl methacrylate (MMA) (Technovit 3040, Heraeus Kulzer GmbH, Wehrheim, Germany). Torque testing was performed at constant axial preloading and at a median rotation speed of 0.6 °/s until failure of the bones. Testing results were extracted to Excel (Microsoft Corporation, Redmond, USA) to determine maximum torque. Maximum stiffness was determined by calculating the gradient from the linear part of the torsion/angle curve.

Statistics

Statistical analysis was performed with SPSS 20 (SPSS Inc. Chicago, Illinois, USA) and GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, USA). The Mann-Whitney U test was used to compare asymmetric continuous variables between two groups and the Wilcoxon Signed Rank Test for paired non-parametric data. The Bonferroni correction for multiple comparisons was applied, if needed. Categorical variables are presented as percentages. Differences of categorical variables between groups were analyzed by Fisher's exact test. A p value of <0.05 was considered significant for all tests.

Results

Micro-CT

Micro-CT analysis showed that both callus volume and callus density steadily increased over the period of four weeks in the fracture and combined trauma group (Figure 1). In the fracture group callus volume (p<0.001) and callus density (p=0.017) were significantly increased in week 2

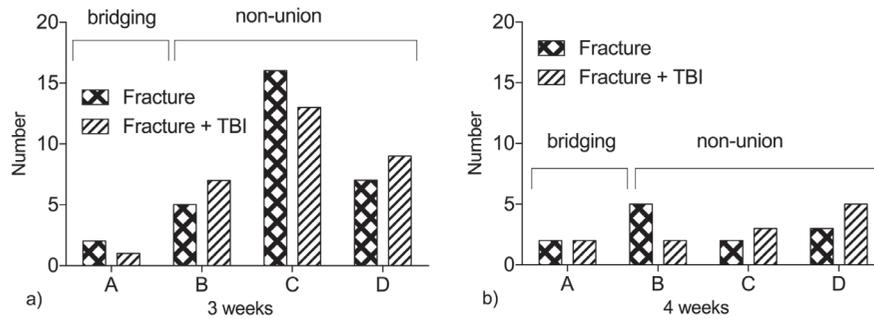


Figure 2. Bar graphs showing the results of the micro-CT score on bony bridging after three (a) and four (b) weeks. A: complete bridging (four cortices bridged by callus), B: incomplete bridging (one to three cortices bridged by callus), C: no bridging (presence of callus but no cortex bridging at all), D: non-union (rounding of cortex, and almost no callus formation). Differences between both groups (Fracture and TBI+Fracture) regarding bridging vs. non-union were not significant at three and four weeks (Fisher's exact test).

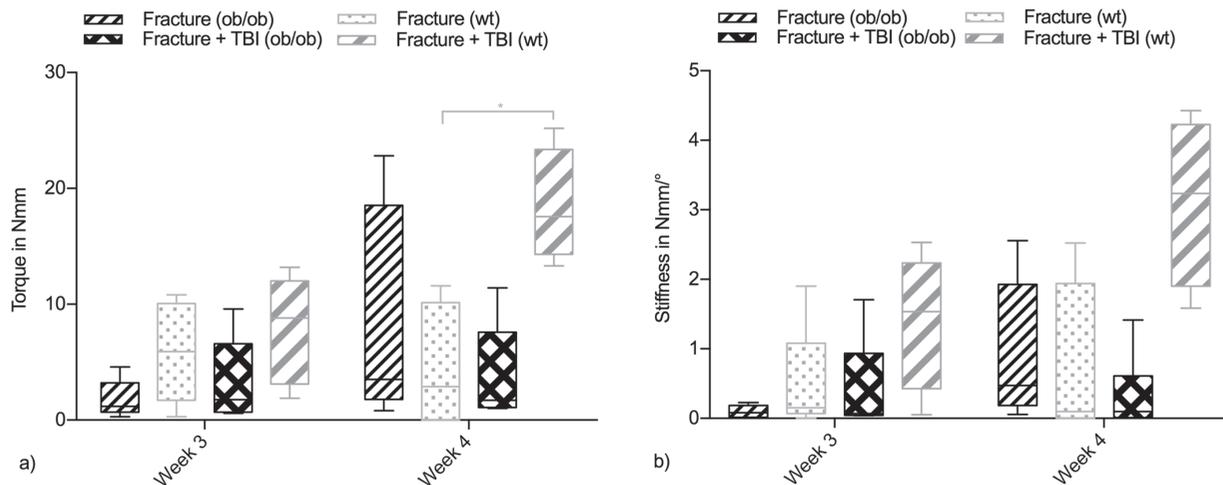


Figure 3. Box plots (interquartile range) of (a) max. torque (Nm) and (b) max. stiffness (Nm/°) of both groups (Fracture and TBI+Fracture) and both mouse strains (ob/ob and wt). There were no significant differences between both groups after 3 and 4 weeks in ob/ob mice. In week 4 the combined trauma group in wt mice had significantly higher torque than the Fracture group ($p=0.03$). Significance level $p<0.05$, Mann-Whitney U test. Figures displayed in grey taken from previous study¹¹.

compared to week 1. Similarly, in the combined trauma group callus volume ($p<0.001$) and callus density ($p=0.001$) were significantly increased in week 2 compared to week 1. No significant differences in callus volume and density between the fracture and combined trauma group could be detected at any time point.

Comparing the results from this study on ob/ob mice with our previous study on wt mice we could see that wt mice with an isolated fracture had significantly more callus volume after two ($p=0.03$) and three weeks ($p<0.01$)¹¹ than ob/ob mice in the present study. After four weeks there was no statistically significant difference between wt and ob/ob mice because wt mice showed a decrease in callus volume, as it would be

expected in physiological bone healing in the course of callus remodeling³⁷. In this context callus density increased in week 4 in wt mice as a result of the remodeling process and was significantly higher ($p<0.01$) than in ob/ob mice (Figure 1).

The evaluation of the callus bridging using the micro-CT scoring system showed that after three weeks only 6.6% of the mice in the fracture group and 3.3% of the mice in the combined trauma group had completed bony bridging (category A). After four weeks 16.6% of the mice in both groups were category A (complete bridging). Given this, micro-CT revealed non-union rates of 93.4% and 96.7% after three weeks in the fracture and combined trauma group, respectively. After four weeks both groups had non-

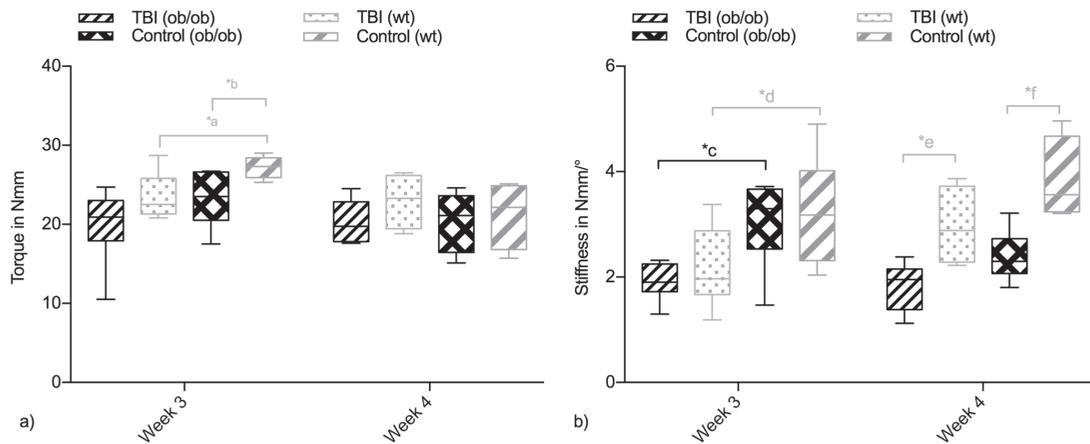


Figure 4. Box plots (interquartile range) of (a) max. torque (Nm) and (b) stiffness (Nm/°) between the control and TBI group of both mouse strains (ob/ob and wt). After three weeks torque was significantly higher in the Control group compared to the TBI group in wt mice (*a $p=0.02$). The Control group in wt mice had also significantly higher torque after three weeks than the Control group in ob/ob mice (*b $p=0.02$). The Control groups of both mouse strains (ob/ob and wt) had significantly higher stiffness than the corresponding TBI groups after three weeks (*c $p<0.01$, *d $p=0.04$). After four weeks, the TBI and Control group of wt mice had significantly higher stiffness than the TBI and Control group of ob/ob mice (*e $p=0.02$, *f $p=0.02$). Significance level $p<0.05$, Mann-Whitney U test. Figures displayed in grey taken from previous study¹¹.

union rates of 83.4%. Differences between the fracture and combined trauma group regarding completed fracture bridging (category A) and incomplete fracture bridging (category B-D) were statistically non-significant after three and four weeks (Figure 2).

In contrast, wt mice, as shown in our previously published study, with a combined trauma showed union rates of 79% compared to 59% in the fracture-only group after three weeks. After four weeks the combined trauma group had 89% unions compared to 60% in the fracture group¹¹.

Biomechanical testing

Biomechanical analysis did not show significant differences between the fracture and combined trauma group regarding maximum torque and stiffness of the left femora after three and four weeks. In wt mice the combined trauma group showed a significantly increased torque in week 4 compared to the fracture-only group (Figure 3)¹¹.

To evaluate if TBI changes the biomechanical properties of bones of ob/ob mice, biomechanical testing was also performed on the left femora in the control and TBI group. After three weeks stiffness was significantly higher in the control group than in the TBI group ($p=0.009$) whereas after four weeks there was no significant difference (Figure 4b). Regarding maximum torque after three and four weeks there were no significant differences between control and TBI group. In wt mice the control group had significantly increased torque as well as stiffness after three weeks compared to the TBI group (Figure 4)¹¹.

In order to see if bone quality in ob/ob mice was already

affected before fracture we compared biomechanical torque and stiffness of both mouse strains. After three weeks femora of wt mice in the control group had significantly higher torque and after four weeks significantly higher stiffness than the control group of ob/ob mice (Figure 4)¹¹.

Discussion

The nature of the interaction between TBI and fracture healing remains an open question. While the phenomenon has been reproduced in animal models, the underlying pathophysiological mechanisms still need to be elucidated. Leptin, as a pleiotropic hormone of the homeostasis, makes a strong candidate over its effect on bone metabolism, especially in the presence of TBI. Under this scope, we were able to show in this study that: 1) ob/ob mice showed impaired fracture healing after a femoral osteotomy and 2) do not respond with increased callus formation or improved biomechanical strength after TBI, in contrast with wild-type animals. To our knowledge, this is the first study to look at the impact of TBI on fracture healing in ob/ob mice.

Fracture healing in ob/ob mice is a newly emerged topic that has not been analyzed extensively so far. Khan et al. also found that leptin-deficient mice had significantly decreased fracture healing compared to wt mice³⁸. In their study, femora of ob/ob and wt mice were fractured in an open manner using customized bone scissors and stabilization was performed by intramedullary nailing. Additionally, recombinant leptin was locally administered into the fracture gap of ob/ob mice. Micro-CT scans showed that ob/ob mice had significantly larger callus volume than wt mice. Nevertheless, histologic

analysis revealed that the callus in ob/ob mice consisted mainly of hypertrophic chondrocytes, thus indicating a delay in endochondral bone formation in these mice, whereas callus in wt mice predominantly consisted of mature bone at the same time point. When recombinant leptin was administered into the fracture gap, callus volume and callus maturity of ob/ob mice were similar to those of wt mice. Turner et al. also reported that ob/ob mice are less effective in replacing calcified cartilage with mature bone, probably because of decreased osteoclast activity²⁶.

Interestingly, Beil et al. reported improved fracture healing of ob/ob mice compared to wt mice³⁹. In the same ob/ob mouse strain as in our study, closed femoral fractures were stabilized by intramedullary nailing. After 21 days callus from ob/ob mice was biomechanical superior with significantly increased force to failure and bending stiffness compared to callus from wt mice after three-point bending. Additionally, the histomorphometric analysis showed significantly increased periosteal callus area in ob/ob and db/db mice than in wt mice.

Direct comparison of our study with the work of Khan et al. and Beil et al. is possible to a certain extent. In our study we used an open fracture model with a fracture gap of 0.7 mm and an external fixator producing a rigid fracture fixation. The large osteotomy gap used in our study might indeed be responsible for the poor union rates of the ob/ob mice, as the commonly used fracture gap for rodent models according to Histing et al. is about 20% the size of the bone diameter, equaling 0.4 mm in our mice⁴⁰. However, in the previously published study by Locher et al. union-rates of 60% were observed at three and four weeks despite the osteotomy gap of 0.7 mm¹¹. Beil et al. and Khan et al. on the other hand used fracture models without a bone defect. In both studies, stabilization was achieved by intramedullary nailing using a Kirschner wire. This fixation technique, however, does not provide axial and rotational stability resulting in a dynamic rather than rigid fracture fixation⁴⁰. Because ob/ob mice have a body weight that can be four times higher than in wt mice²², we can assume that interfragmentary movement (IFM) in ob/ob mice was higher than in wt mice in both studies. Increased IFM can lead to hypertrophic callus production and may therefore be responsible for the increased callus volume observed in both studies⁴¹⁻⁴³. In contrast, the external fixator used in our study provides rigid fracture stabilization despite the body weight differences of ob/ob and wt mice^{40,44}.

Our results suggesting that ob/ob mice have impaired fracture healing also seem to be reasonable on a cellular level. Osteoblasts and mesenchymal stem cells express the long form of the leptin receptor (OB-Rb) which performs signal transduction^{20,45,46}. Application of leptin in cell cultures led to an increase in osteoblast proliferation, collagen synthesis, mineralization and enhanced differentiation of human marrow stromal cells to osteoblasts^{47,48}. Furthermore, central and peripheral injection of leptin in ob/ob mice increased osteoblast activity and bone mass^{20,26-28}.

Furthermore we report that ob/ob mice did not respond

to with increased callus volume, as this is the case in wt-mice. Given that leptin serum and CSF levels are significantly increased after TBI, the increased callus formation seen after TBI could therefore be dependant on leptin signaling^{7,29-31}. Testing this hypothesis with a mouse model deprived of leptin may raise two concerns. First, leptin does not only seem to be necessary for physiological bone metabolism but is also believed to be important for the physiological course of fracture healing^{38,49,50}. Therefore, testing whether TBI is able to increase an already deteriorated callus formation may be controversial. However, as we were able to show in this study, ob/ob mice with an isolated fracture showed a significant increase of callus volume already after two weeks.

Second, due to the variety of endocrinologic, metabolic and neuronal pathways that leptin influences ob/ob mice have a complex phenotype of pathological bone metabolism and delayed fracture healing that does not fully allow to ascribe observations made on this model to direct effects of leptin⁵¹. For instance, ob/ob mice suffer from type II diabetes, a metabolic syndrome, hypogonadism and hypercortisolism all of which are known to reduce bony mass and quality, and may therefore be responsible for the poor fracture healing, too^{22,52-55}. Whether bone quality, mass, and biomechanical strength of ob/ob mice is reduced in comparison to wt mice is still subject to ongoing debate. Comparing previous data in wt mice with the data of this study on ob/ob mice we saw that wt mice had stronger biomechanical testing parameters in the control group indicating that bone quality in ob/ob mice could have been already reduced prior to the femoral osteotomy¹¹. Ealey et al. could also demonstrate that ob/ob mice had significantly decreased biomechanical strength while Ducy et al. and Reimer et al. did not find significant differences between ob/ob and wt mice^{23,24,56}.

To confirm these observations made here future studies should address the problems that arise with the complex phenotype of ob/ob mice. This could for instance be achieved by systemic application of leptin which has already be shown to reverse the diabetic phenotype in ob/ob mice and improve bone metabolism^{20,26,57}. But it remains to be tested if it reverses the poor fracture healing in ob/ob mice, too. Systemic injection of leptin furthermore promises to deliver identical limited concentrations of leptin in both groups (fracture vs. combined trauma) which would allow for testing if TBI fails to increase callus formation despite the re-adjustment of metabolic disorders in ob/ob mice.

Despite these limitations our study is a first step in identifying leptin as a possible amplifying mediator on fracture healing after TBI, as at its absence, even in the complexity of its effects on the whole organism, the effect of increased callus formation was not observed.

Source of funding

The present study has been funded by the German Research Society (DFG, project TS 303/1-1).

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