Original Article

Leptin-deficiency eradicates the positive effect of traumatic brain injury on bone healing: histological analyses in a combined trauma mouse model

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Abstract

Introduction: The combination of traumatic brain injury (TBI) and long-bone fracture leads to increased formation of callus and mineral density in wild-type (WT) mice. However, this effect was not detected radiologically in leptin-deficient mice. Due to the complex interactions between hormonal and bone metabolism and the important role of leptin in this setting, our aim was to investigate morphologic properties and the tissue composition in the fracture callus comparing WT and leptin-deficient mice. **Methods:** Female C57/Black6N mice (n=36) and leptin deficient *ob/ob* mice (n=36) each were assigned to two groups (fracture Fx/combined trauma Fx/TBI). Femoral osteotomy was stabilized with external fixator, TBI was induced with controlled cortical impact injury. After sacrifice of the animals, femora were harvested, cryofixated, and 7 µm slices were prepared. Staining was performed adhering to Movat's Pentachrome protocol. Histomorphometric analysis, quantifying percentage of mineralized bone area, and a semi-quantitative evaluation of bone bridging were performed. **Results:** Leptin deficient mice showed a higher rate of non-union after osteotomy, less callus formation in the osteotomy gap, and unexpected bone and cartilage formation independent of the osteotomy region. **Discussion:** Leptin plays an important role in fracture healing and bone formation. Without Leptin, the positive effect of TBI on fracture healing ceases. The comprehension of the underlying pathophysiological process could sign important for novel strategies in stimulation of fracture healing.

Keywords: Fracture Healing, Bone, Brain, Mouse

Introduction

In the last decade, the discussion about metabolic interactions between brain and bone has gained new impetus. The long stated clinical observation of exuberant callus formation in patients with long-bone fractures and concomitant traumatic brain injury (TBI) - first described by Calandriello et al. in 1964[1], and confirmed by several other work groups later on[2-6] - was transferred to different animal experimental settings. In 2005, the first review on the topic by Morley et al. concluded that the question whether there is a positive interaction between TBI and fracture healing could not be answered at that time[7]. The scientific update, published ten years later by Hofman et al., observed a consensus of the scientific community over the positive effect of TBI on fracture healing[8]. Most recently, a newly established standardized animal model in mice combining TBI and externally fixated femoral osteotomy allowed for detailed radiographic and biomechanical investigations on the *in vivo* effect of TBI on bone healing[9]. Additionally, an experimental study in 138 wild-type (WT) mice showed an increased callus volume and higher bone density in mice with femoral fracture and concomitant TBI, leading to superior biomechanical testing results compared to fracture alone[10]. Although it seems
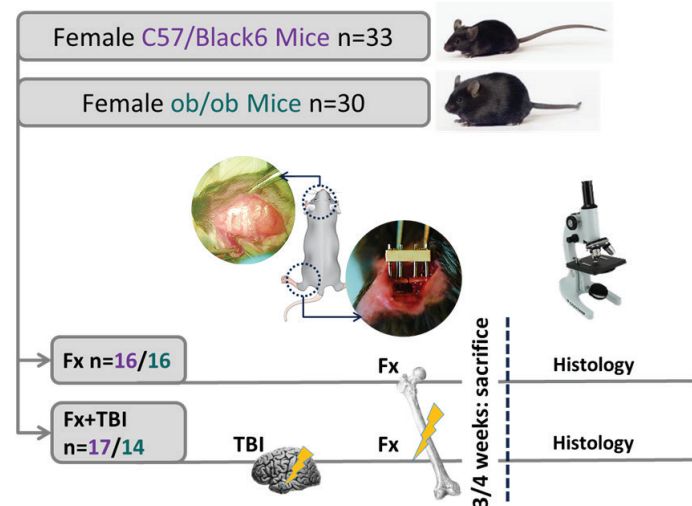
Figure 1. Experimental set-up. 36 WT-mice and 36 leptin-deficient mice were divided into 2 subgroups: fracture-only group and combined trauma group. Mice were sacrificed after 3 or 4 weeks, allowing for histological analysis. (Images of mice used with kind permission of Janvier Labs).

currently evident that there is a positive connection between TBI and fracture healing, the molecular pathways that lead to the above described effects remain still unclear. Two major fields of mechanisms crystallize out of the numerous possible pathways and factors involved in the process: mesenchymal stem cells and their signaling pathways[11-14], and hormones like leptin or CGRP (calcitonin gene-related peptide)[15], which in turn are influenced by a variety of humoral factors such as cytokines or growth factors, proteins, and enzymes. Leptin, the so-called "satiety hormone" originating from the leptin (*ob*) gene and first described by Friedman in the 1990s, plays a major role in obesity research[16], and has gained attention over the last years also in bone metabolism. Depending on its mode of action, leptin is assumed to influence bone metabolism either pro-osteogenic (peripheral pathway) via promotion of differentiation of osteoblasts to osteocytes and bone mineralization[15,17-19] or anti-osteogenic (central pathway) via the hypothalamic-sympathetic axis[20]; more recent research suggests that the strict division of central and peripheral effects of leptin does not always meet the complex homeostatic mechanisms[21-23]. Up to now, the exact metabolic pathways of leptin have not been deciphered and its role in bone metabolism is subject to ongoing research[16]. One animal model widely used in experimental research on the topic is the leptin-deficient mouse, lacking the obese gene (*ob/ob*). These mice, weighing three times more than average WT mice, show all signs of a metabolic syndrome. In terms of skeletal characteristics, they show reduced bone mass, reduced bone formation[18,24], and reduced longitudinal growth[25], but also reduced biomechanical bone quality[26]. A recent study was able to show that bone formation and bony bridging in leptin-deficient mice is impaired radiologically and

biomechanically in a trauma model of femoral osteotomy and external fixation[27], and that bone healing did not improve in the combination of osteotomy with TBI, as was the case in WT mice[9,10]. Due to the complex interactions between hormonal and bone metabolism and the important role of leptin in this setting, our aim was to investigate the histomorphologic properties and the tissue composition in the fracture callus comparing WT and leptin-deficient mice, with and without concomitant traumatic brain injury.

Materials and methods

Animal care and perioperative management

All experiments were approved by the local legal representative animal rights protection authorities (G 0009/12) and were performed adherent to the policies and principles established by the Animal Welfare Act (Federal Law Gazette I, p.1094) and the National Institutes of Health Guide for Care and Use of Laboratory Animals[28]. Female C57/Black6N mice (Charles River, Sulzfeld, Germany, n=36, age: 12 weeks, body weight: 22±3 g) and B6.V-Lep-ob/JRj mice (Janvier, Saint Berthevin, France, n=36, age: 10-12 weeks, body weight: 50±5 g) were kept in standardized cages with a twelve-hours light-darkness cycle with controlled temperature of 20±2°C. Access to food and water was *ad libitum*. The animals were kept in the laboratory premises for at least one week prior to inclusion in the study, in order to allow for acclimatization and minimize stress, and were kept in stable groups throughout the experiment. Anesthesia for all surgical procedures was induced using isoflurane 1.6 vol % (FORENE, Abbot, Wiesbaden, Germany) in a mixture

of N_2O/O_2 in spontaneously breathing animals. A heating pad (37°C) used in all surgical procedures prevented hypothermia. Perioperative antibiotic prophylaxis was performed by subcutaneous injection of Clindamycin (0.02 ml). Subcutaneous application of buprenorphine 0.1 mg/kg body weight (TEMGESIC, Reckitt Benckiser, Mannheim, Germany) ensured sufficient analgesia. Additionally, Tramadol 25 mg/l (TRAMAL, Grünenthal, Aachen, Germany) was added to the drinking water (8 drops/250 ml of water) for three days postoperatively.

Experimental design and surgical procedures

Both strains of mice (WT and leptin-deficient) were assigned to two groups, respectively: fracture (Fx) group: WT n=18, leptin-deficient n=18; combined-trauma (Fx/TBI) group: WT n=18, leptin-deficient n=18. (Figure 1) A standardized femoral osteotomy model stabilized with an external fixator was used[29,30]. A 2 cm lateral longitudinal incision of the skin allowed for a mid-diaphyseal approach to the femur. Dissection of the fascia lata was followed by blunt preparation of Musc. vastus lateralis and Musc. biceps femoris, carefully sparing the sciatic nerve. The external fixator (MouseExFix, RIsystem, Davos, Switzerland) was mounted strictly parallel to the femur, positioning the pins perpendicularly to the longitudinal femoral axis and cortical surface. After rigid fixation, a 0.70 mm osteotomy was performed between both middle pins using a Gigli wire saw (RISystem, Davos, Switzerland). Wound closure was performed with Ethilon 5-0 suture (Ethicon, Johnson&Johnson, Norderstedt, Germany).

For induction of TBI, we used the standardized model of controlled cortical impact injury (CCII)[31,32]. Animals were mounted on a stereotactic device (Stoelting, Wood Dale, Illinois/USA). A sagittal and temporal incision of the skin was followed by skin mobilization and preparation of the left temporal muscle. After craniotomy of the parietotemporal region with a micro drill, a 7x7 mm bone window was lifted, carefully sparing the dura mater. TBI was induced in a standardized manner with a pneumatic impactor (penetration depth 0.25 mm, impact velocity 3.5 m/s, contact duration 150 ms). The preserved piece of cranial bone was repositioned and fixed with dental cement (Hoffmann, Berlin, Germany) afterwards in order to simulate a closed brain injury. Wound closure was performed as described above. In the combined-trauma group, both injuries were sequentially combined with initial induction of CCI followed by femoral osteotomy.

Histological sample preparation

The animals of each group were sacrificed three (n=12) and four weeks (n=6) postoperatively. The femora were harvested and prepared with the external fixator *in situ*, leaving a layer of soft tissue around the osteotomy site to avoid any damage to the callus. The femora were kept in paraformaldehyde (PFA) 4% over night at 4°C and then incubated in glucose solution of crescent concentrations (10%, 20%, 30% for 24 hours each). The femora were then placed in an embedding mold, using the external fixator to ensure standardized alignment of the femora in the blocks, and covered with embedding medium (SCEM Embedding Medium, Section Lab Co Ltd., Hiroshima, Japan), removing the fixator after medium hardened. The embedding mold was dipped into a beaker with cooled hexane [C6H14] (n-Hexan>95, Carl Roth GmbH&CoKG, Karlsruhe, Germany), the beaker being surrounded by acetone (Acetone 3221, SIGMA ALDRICH, Steinheim, Germany) and dry ice in a cooling tank. When the medium had hardened, the blocks were separately wrapped and stored at -80°C. Longitudinal 7 µm sections from the blocks were cut with a cryotome (Leica CM3050S, Leica Microsystems, Nussloch, Germany), and mounted onto microscope slides using cryofilm (Cryofilm type II C, Section Lab Co Ltd., Hiroshima, Japan). Sections dried at room temperature and were stored at -80°C.

Histological staining and preparation for histomorphometry

Histological staining was performed using Movat's Pentachrome Stain. Briefly, the sections were successively dipped in solutions of Alcian blue (8GS, Chroma, 1A288, Hamburg, Germany), Weigert's hematoxylin (Merck 4302 and Merck 3943, Darmstadt, Germany), Brilliant Crocein/Acid Fuchsin (Brilliant Crocein R, Chroma 1B109, Hamburg, Germany and Acid Fuchsin, Merck 7629, Darmstadt, Germany), 5% Phosphotungstic acid PTA (Chroma 3D092, Hamburg, Germany), and Saffron du Gâtinais (Chroma 5A394, Hamburg, Germany). Finally, sections were fixed and cover slides were mounted with Vitro-Clud (Langenbrinck, Emmendingen, Germany). In preparation for histomorphometric analysis, digital mosaic photographs were taken of the stained slices using the Zeiss Axioskop 40 and the computer program AxioVision (both Carl Zeiss MicroImaging, Göttingen, Germany). A scale of 2mm was introduced, enabling analysis by the evaluation software.

Histomorphometric analysis and semi-quantitative analysis

Histomorphometric analysis was performed using the software KS Run (KS Run 400 Version 3.0, Carl Zeiss Vision, Eching, Germany), quantifying percentages of different tissue types. After manual identification and indication of different tissue types, the program calculated color pixel areas in square millimeters (mm^2). A semi-quantitative evaluation of bone bridging was performed adapting and using a score system initially introduced for MicroCT scans by Mehta et al.[33]. Two independent blinded reviewers classified the status of bone healing according to the following: A= complete bridging (all four cortices bridged by callus), B= incomplete bridging (two to three cortices bridged by callus), C= no bridging (callus present, but no bridging visible), and D= non-union (rounded cortices, minimal presence of callus). In case of differences, a third reviewer was involved.

Statistical analysis

Continuous variables were expressed as means ± standard deviation (SD), whereas categorical variables were expressed
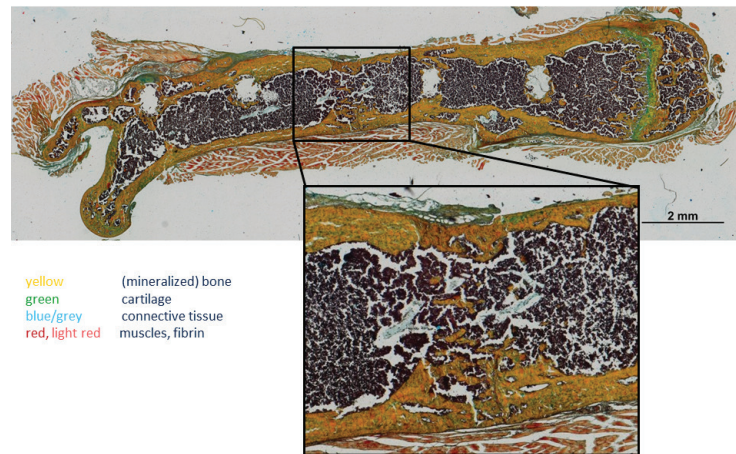
Figure 2. Movat's Pentachrome Stain of the femoral bone of a fracture-only group WT mouse (cryosection). Mineralized bone is stained yellow, cartilage green, connective tissue in blue or grey, and muscles are stained in red or light red. Focus of evaluation is on fracture gap.
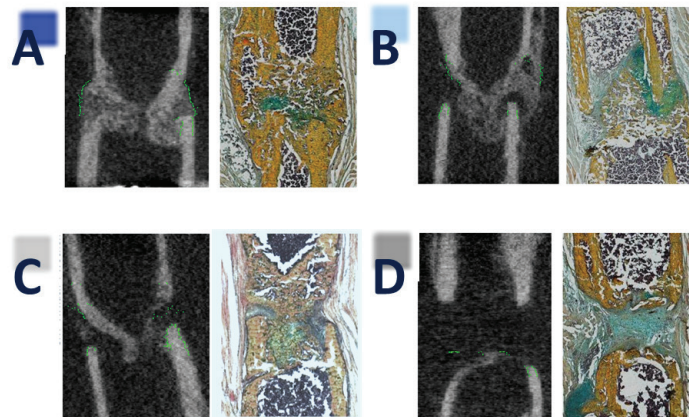
Figure 3. Semi-quantitative evaluation of bone bridging, adapting and using a score system initially introduced for MicroCT scans. Examples shown from WT mice. A = complete bridging, B = incomplete bridging, C = no bridging, and D = non-union.

as percentages (%). For non-parametric variables the Mann-Whitney test was implemented. Differences were considered statistically significant if the null hypothesis could be rejected with >95% confidence (p<0.05).

Results

Histological staining and semi-quantitative analysis

Figure 2 exemplarily illustrates the results of Movat's Pentachrome Stain of the femoral bone of a fracture-only group WT mouse. See Figure 3 for exemplary illustration of semi-quantitative evaluation of bone bridging, adapting and using a score system initially introduced for MicroCT scans by Mehta et al.[33].
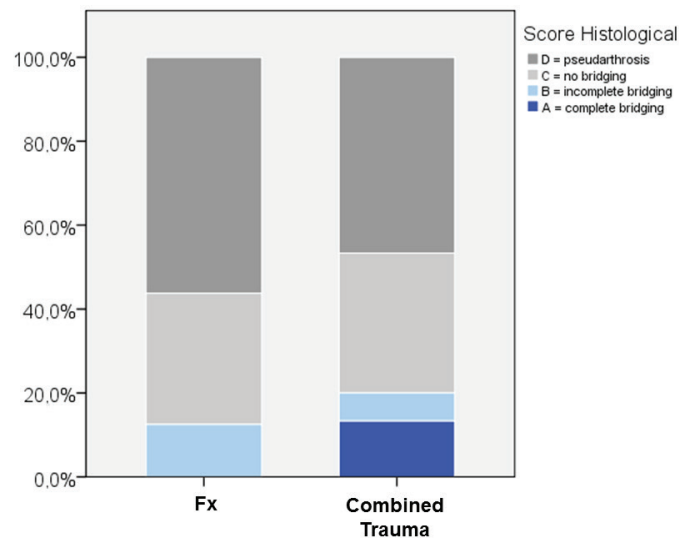
Semi-quantitative analysis

Most WT mice in the Fx (fracture) group showed a high rate of bridging at all time points (69% of mice were scored A= complete bridging, 6% were scored B= incomplete bridging). In WT mice, combined trauma group showed a lower rate of delayed healing (12% scored D) than Fx group (25% scored D). Almost 90% of leptin-deficient mice in the Fx group showed no bridging (31% were scored C) or delayed healing (56% were scored D). These results did not reach statistical significance (Figure 4).

In WT mice, combined trauma group showed a lower rate of delayed healing (12% scored D) than Fx group (25% scored D). In leptin-deficient mice, this was not true: combined
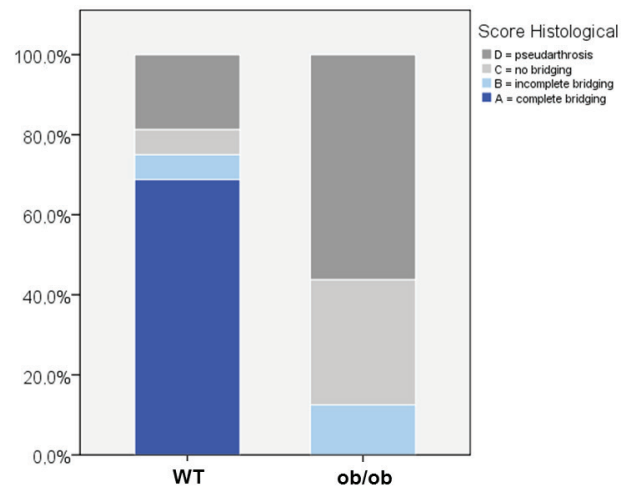
Figure 4. Semi-quantitative evaluation comparing WT and leptin-deficient mice of fracture-only group. WT mice showed a high rate of complete bridging, while more than 80% of leptin-deficient mice showed no bridging or non-union.


Figure 5. Semi-quantitative evaluation comparing fracture-only group and combined trauma group in leptin-deficient mice. Additional TBI did not increase the rate of bridging in leptin deficient animals.

trauma group showed a comparable low rate of bridging (33% scored C= no bridging) or non-union (47% scored D) to the fracture group (31% C and 56% D, see above) (Figures 4 and 5) and no improvement in rate of bridging. The bridging score distribution in leptin deficient mice comparing fracture-only group and combined trauma group was almost the same in both groups. In fracture-only group, none out of 16 mice scored A; in combined trauma group two out of 16 mice scored A (Figure 5). Differences were distinct already at the time point of three weeks after trauma, so that we combined both time points here in order to draw a clear picture.

Histomorphometric analysis

Histomorphometric analyses of WT mice showed higher mineralized bone density (values as percentages) in the combined trauma group compared to the Fx group from the third postoperative week (combined trauma group 0.46, Fx group 0.42), while mineralized bone area was comparable. At four weeks postoperatively, mineralized bone density was 1.3 times higher in combined trauma group than in Fx group (combined trauma group: 0.62, Fx group: 0.47). These results did not reach statistical significance, however. Comparison
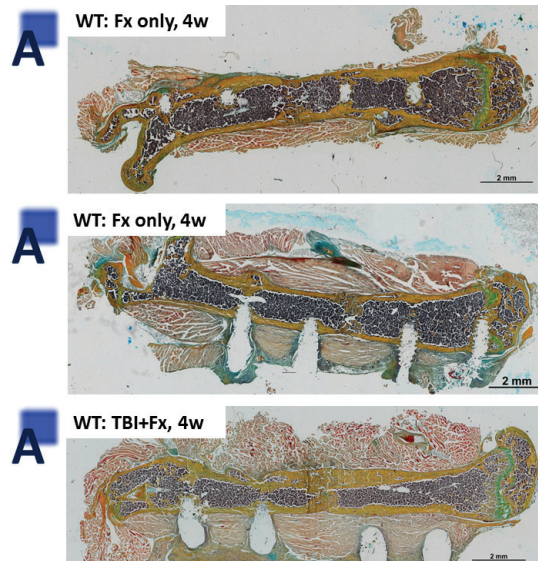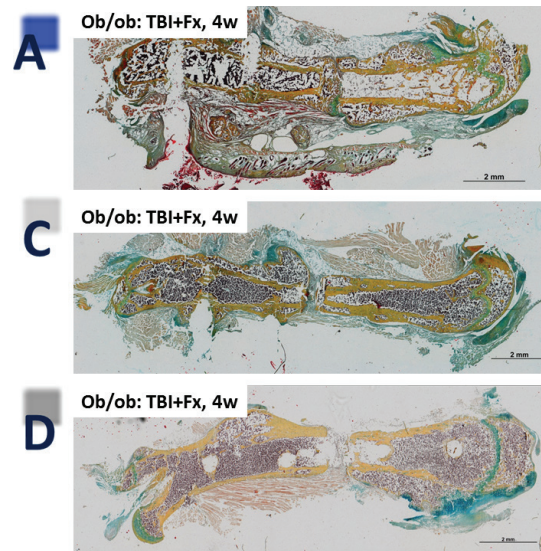
Figure 6. Movat's Pentachrome Stain of representative images of mouse femora of WT mice. Above and middle: WT mice of Fx only group 4 weeks after osteotomy. There is a fully bridged fracture gap with mineralized bone and signs of beginning recanalization, indicating ongoing remodeling. Below: WT mouse of combined trauma group 4 weeks after osteotomy. Fracture gap is fully bridged as well with more voluminous mineralized bone than in Fx only group.
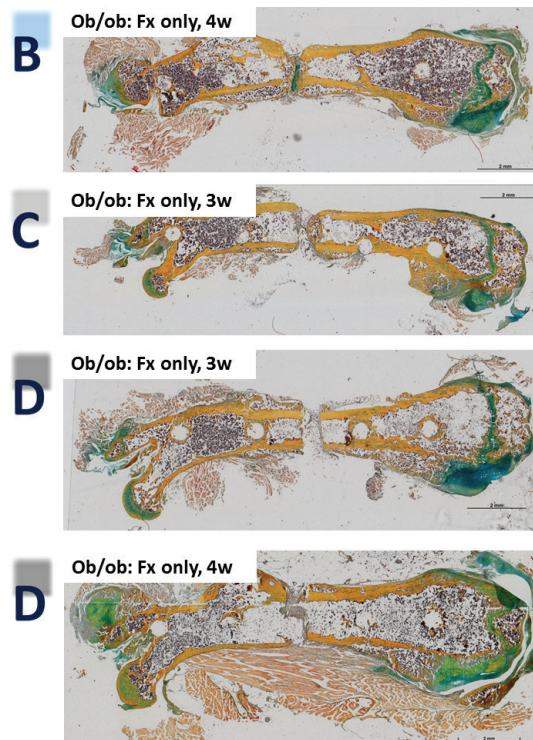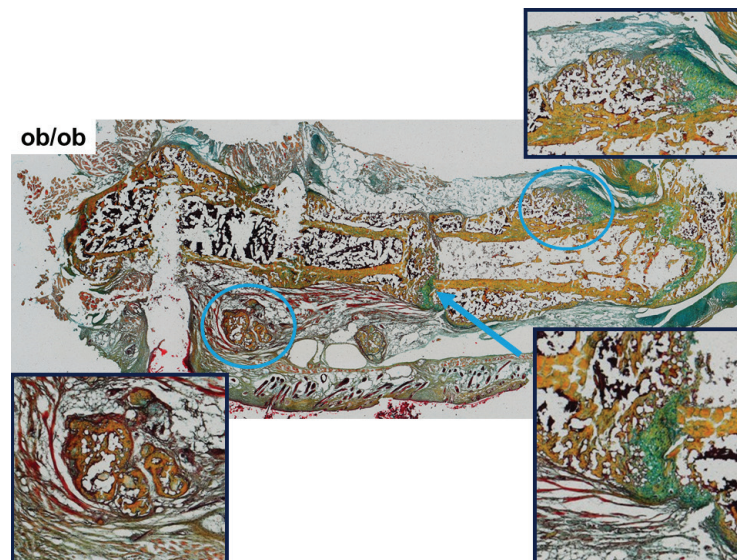

Figure 7. Movat's Pentachrome Stain of representative images of mouse femora of leptin deficient mice of Fx only group, 3 or 4 weeks after osteotomy. None of the specimen scored A (complete bridging). Note the specimen that scored D: there is connective tissue in the fracture gap and rounded cortices, indicating non-union.

Figure 8. Movat's Pentachrome Stain of representative images of mouse femora of leptin deficient mice of combined trauma group 4 weeks after osteotomy. Two out of 16 mice scored A None of the specimen was classified A (complete bridging), the rest scored B, C, or D.

Figure 9. Movat's pentachrome stain of leptin-deficient mouse femoral bone 4 weeks after osteotomy with callus and cartilage in the fracture gap, but also cartilage independent of the osteotomy. There is callus formation without connection to the femoral bone, and exuberant callus formation alongside the diaphysis, forming some sort of "pseudo-lumen", a lumen filled with bone marrow.

of fracture gaps of WT mice and leptin-deficient mice who received femoral osteotomy only (Fx group) showed WT mice to have significantly higher mineralized bone density (WT Fx group 3 weeks 0.42, 4 weeks 0.47; *ob/ob* Fx group 3 weeks 0.18, 4 weeks 0.3) and mineralized bone area (WT Fx group 3 weeks 0.35 mm^2, 4 weeks 0.27 mm^2; *ob/ob* Fx group 3 weeks

0.11 mm^2, 4 weeks 0.17 mm^2) than leptin-deficient mice at three and four weeks postoperatively (p=0.005). The same applied to combined trauma group; WT mice had significantly higher mineralized bone density (WT combined trauma group 3 weeks 0.46, 4 weeks 0.62; *ob/ob* combined trauma group 3 weeks 0.23, 4 weeks 0.42) and mineralized bone area (WT

combined trauma group 3 weeks 0.34 mm^2, 4 weeks 0.42 mm^2; *ob/ob* combined trauma group 3 weeks 0.14 mm^2, 4 weeks 0.24 mm^2) than leptin-deficient mice at both time points (p=0.002). TBI as a variable did not cause differences in bone healing parameters in leptin-deficient animals: *ob/ob* mice did not show any significant differences between the Fx group and the combined trauma group regarding mineralized bone density and mineralized bone area.

Descriptive (qualitative) evaluation of osteotomy gaps and overall bone formation underlines the above stated differences between WT mice and leptin-deficient mice. Figure 6 shows the femoral bone of WT mice 4 weeks after osteotomy. Note the fully bridged fracture gap with yellow stained bone indicating mineralization both cortically and in the callus area. Signs of beginning recanalization are identifiable by the dark blue stained bone marrow, indicating ongoing re-formation of bone and gradual remodeling to "normal" bone. This is different in the leptin-deficient mouse (Figures 7 and 8), where we only see connective tissue (bright blue staining) in the fracture gap, rounded cortices, and almost no callus at all in the fracture gap. This indicates delayed healing, or as we postulate, non-union, for it is not probable that further bone formation would take place in the area of osteotomy.

Interestingly, many of the leptin-deficient mice did not show bone formation at the fracture gap site, but in areas not associated with the osteotomy site. Figure 9 shows the femoral bone of a leptin-deficient mouse four weeks after osteotomy. Here, we see callus and cartilage in the fracture gap, but also cartilage not connected to the area of the osteotomy (blue arrow and circles). We can see callus formation without connection to the femoral bone, and we can see exuberant callus formation alongside the diaphysis, forming some sort of pseudo-lumen, a lumen filled with bone marrow.

Discussion

In a combined trauma mouse model, we analyzed the histological features of bone healing comparing wild type mice and leptin-deficient mice. Leptin deficient mice showed a higher rate of non-union after osteotomy, only little callus formation in the osteotomy gap, and unexpected bone and cartilage formation independent of the osteotomy. Concomitant TBI did not have a positive effect on fracture healing in leptin deficient mice. The histomorphometric results underline previous radiological findings[27], while the descriptive histology reveals interesting characteristics of bone formation in leptin deficient mice that to our knowledge have not yet been reported.

First, our results add evidence in favor of the hypothesis that leptin deficient mice show compromised bone healing after osteotomy, confirming the critical role of leptin for bone formation. While WT mice of the Fx group showed a high rate of bridging, almost 90% of leptin-deficient mice in the Fx group showed no bridging or non-union. Comparison of fracture gaps of WT mice and leptin-deficient mice who received femoral osteotomy only (Fx group) showed WT mice to have significantly higher 2D bone density and mineralized bone area than leptin-deficient mice. These results match the radiological findings of Graef et al., who described similar findings using the ABCD-score by Mehta et al. Still, we face inconclusive results in literature, especially when looking at mouse trauma models in leptin deficient animals. The study by Beil et al.[34] in 2011 reports increased periostal callus formation and earlier mineralization in leptin deficient mice (*db/db* as well as *ob/ob*) in histomorphometric analysis. On the other hand, Rőszer et al.[35] described a compromised bone regeneration in leptin-deficient (*db/db*) mice. Khan et al.[36] also reported compromised bone regeneration in leptin deficient mice; although larger callus volume was seen radiologically (MicroCT), the callus did not consist of mineralized, mature bone as in WT mice of the comparison group, but of hypertrophic chondrocytes. Turner et al.[37] underline this observation by stating reduced osteoclast activity in *ob/ob* mice, resulting in less effective mineralization and replacement of calcified cartilage by mature bone. Certain limitations apply when comparing our work with the above mentioned studies. While Khan et al. used the same strain as we did (*ob/ob* mice) for their experiments, Beil et al. used *db/db* as well as *ob/ob* mice, and Rőszer et al. investigated bone healing in leptin-deficient mice lacking the leptin receptor (*db/db*) rather than the leptin gene itself (*ob/ob*). Differences in mouse strain of course imply a variety of different pathways to be taken into account for possible explanation and interpretation of the observed phenomena. The fracture model is another important factor; while Beil et al. and Khan et al. used intramedullary nailing, resulting in a dynamic fixation and therefore explaining larger callus formation, we used a model with external fixation not allowing for rotational or axial instability, mainly healing by endosteal bone formation with small callus formation[30,38]. Another important point is that our model reduces artifacts on the direct fracture site, supposedly allowing for reproducible and more exact evaluation.

Second, our results show that TBI did not have a positive effect on bone healing in leptin deficient mice, as was previously shown for WT mice radiographically and biomechanically. We were able to reproduce these results histomorphometrically, as WT mice showed higher mineralized bone density and higher mineralized bone area in the combined trauma group compared to the Fx group. The histological evaluation confirmed the tissue composition associated with a remodeling bone healing phase. The fact that these results did not reach statistical significance probably is to be attributed to changing from a three-dimensional (MicroCT) to a two-dimensional (histomorphometrics) evaluation method. Considering the results contextually, they enable a better understanding of the bone healing modification under TBI influence. As to the positive effect of TBI on fracture healing in WT mice, we did not observe this effect in our histological analysis of leptin deficient mice: the bridging score distribution comparing Fx group and combined trauma group was almost

the same in both groups. Furthermore, the two groups did not show any significant differences regarding histologically evaluated mineralized bone density and mineralized bone area. These findings support the assumption that leptin plays an important role in the interaction between brain and bone in the context of TBI and accelerated fracture healing. This is in line e.g. with the findings of Yan et al.[39], who reported stronger expression of leptin in the brain and elevated levels of leptin in serum and cerebrospinal fluid in rabbits with TBI than in sham-operated animals, again indicating the central role of this hormone.

Third, descriptive (qualitative) evaluation of osteotomy gaps and overall bone formation showed interesting differences between WT and leptin-deficient mice not yet described in literature. While WT mice showed good bridging of fracture gaps with mineralized bone as well as signs of ongoing remodeling (like beginning recanalization) as expected, leptin deficient mice showed bone formation almost everywhere *but in the fracture gap*. We observed bone tissue without any connection to the fracture gap, for instance parallel to the femoral bone that we named "pseudo-lumen"; but also without any connection to the femoral bone at all. Another interesting finding was the formation of cartilage independent of the osteotomy. Lacking leptin during the process following a bone injury seems to lead to uncontrolled tissue formation. Leptin might be important for the bone healing process in view of directing bone formation to the injury site rather than the periphery of the bone.

Here again, we need to discuss different factors possibly affecting our results or influencing healing processes in our mouse model. As to the characteristics of mice during the experimental phase *in vivo*, we did not see differences regarding postoperative mortality or in complications like wound infections. The quality of our cryosections is comparable to paraffin sections when evaluating tissues related to bone and bone formation. We chose the external fixator in our osteotomy model in order to achieve maximum stability and thus enabling a bone healing process without large callus formation but preferential endogenous ossification. Although leptin-deficient mice weigh thrice as much as WT mice, the biomechanic impact is neglectable in comparison with the stiffness of the fixator construct in relation to the murine bone, so that in our opinion this is not likely to be the reason for the characteristic bone formation in leptin deficient mice. Furthermore, the fact that other workgroups did not find increased bone formation in fracture gaps of leptin-deficient mice with rotational and axial instability due to intramedullary nailing leads us to the assumption that a proposed instability is not the predominant cause for the described uncontrolled callus formation not connected to the osteotomy gap in leptin deficient mice.

In conclusion, leptin plays an important role in fracture healing and bone formation. Without leptin, the positive effect of TBI on fracture healing ceases. The comprehension of the underlying pathophysiological process could sign important for novel strategies in stimulation of fracture healing.

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