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Histological assessment of fracture healing after reduction of the rat femur using two different osteosynthesis methods

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Abstract

Osteosynthesis using Kirschner (K) wires and plates with screws were compared on the same subject in a previous experimental protocol, but none of them led to fracture healing. We designed a new murine study in order to compare this two methods on different subjects in order to obtain histological proofs of which one is better and to see how limited periosteum removal influence fracture healing. Divided in two equal groups, femoral osteotomies were performed on 30 Brown Norway rats, then reduced using plates and screws in the OPS (osteosynthesis using plates and screws) group and using K-wires in the OIKW (osteosynthesis using Kirschner wire) group. The animals underwent clinical, radiological and histological assessment for eight weeks. The quality of the fracture healing was associated with a higher number of osteocytes/microscopic field at eight weeks. The difference between the groups regarding the number of osteocytes inside lacuna was statistically significant (*t*-test for equal variances not assumed, p=0.001), which confirms a mean difference of 32 cells/ microscopic field (mf) with a 95% confidence interval of 15–50 cells/mf. In conclusion, limited periosteum removal did not influence negatively fracture healing. Therefore, we considered that osteosynthesis using plates and screws led to better results compared to fracture fixation using K-wires.

Keywords: fracture healing, osteocytes, periosteum, plates and screws, K-wire.

Introduction

Fracture healing represents a complex processes that mimic parts of the embryological development of the bone framework [1–3]. When there are proper environmental conditions, the bone will regain its initial form after the healing is complete [3]. In order for this to happen, sometimes it is necessary to intervene by means of osteosynthesis materials to create the perfect circumstances for bone regeneration. For choosing the best method, it is crucial for the surgeon to understand the healing process. Therefore, the perfect method will be selected based on the current knowledge regarding the way the reduction technique influence fracture consolidation. It is well known that periosteum plays an important role in this process [4–16], but some of these methods involve limited periosteum removal in order to stimulate bone union. There has always been a struggle between soft tissue preservation and rigid stabilization of the fracture site. Kirschner (K) wires, a technique which preserves the periosteum and osteosynthesis with plates and screws which requires periosteal removal are two of the most often used in order to reduce fractures, especially in hand. Due to ethical principles, histological proofs of the way this methods influence healing is hard to acquire in clinical studies, most of them are based on the clinical, radiological and functional outcomes. There are some experimental studies that simulates different conditions [16–22] in order to study the fracture healing like the lack of parathyroid hormone-related protein [2], the role of pericytes in osteogenesis after periosteal removal [6], the effects of transforming growth factor-beta (TGF-β) in osteogenesis [10], regulation of the osteocyte using sclerostin [12], and like using artificial periosteum [15]. Therefore, we designed and applied an experimental protocol in order to compare this two methods based on the clinical, radiological and histological findings. Our main objective was to study the fracture healing process from the histological point of view for each method and to compare which one is better based on the number of cells involved in bone production and remodeling. The secondary objective was to see if limited periosteum stripping influence significantly bone repair mechanisms and to develop reproducible murine model in order to study different types of osteosynthesis methods.

Materials and Methods

The experimental protocol was applied according to the European Council Directive No. 86/609/24 November 1986, the European Convention on the Protection of Vertebrate Animals (2005) and the Romanian Government Ordinance No. 37/2 February 2002 and with the agreement of the Local Ethics Committee.

Animals

We used 30 mature male Brown Norway rats with ages between 10 and 13 months and with a mean weight of 325 g for the surgical procedures. The animals where divided in two groups: the first group (n=15), where

osteosynthesis with plates and screws (OPS) was performed, and the second group (n=15) where osteosynthesis with intramedullary K-wire (OIKW) was performed. The animals had unlimited access to food and water, being accommodated in individual cages at a constant temperature, with a 12 hour light/dark cycle.

Anesthesia protocol

The induction was performed by inhaling Isoflurane (3.5%) and afterwards a mixture of 0.15 mL Xylazine and 0.4 mL of Ketamine was administered into the thigh muscle. The anesthesia was maintained using 1 mL/kg of Ketamine every half an hour after testing positive to the pain reflex of the tail. We protected the eyes from getting dry using artificial teardrops and avoided hypothermia by working in a warm environment. We administered subcutaneously an antibiotic to prevent infection.

Surgical procedures

We performed a 1.5 cm incision on the external side of the thigh along the line of the skin projection of the femur and dissected down to the femur covered by the periosteum, between the inferior side of the tensor fasciae latae muscle and the superior side of the femural biceps. The osteotomy was performed from the proximal to distal side, from anterior to posterior, using 0.15 mm thick Sabo Sagittal Electric Saw™ powered by Stryker Cordless Driver 3™. After the osteotomy was performed, the fractures were reduced using four-hole standard titanium plates and screws (measuring 13×1.2×0.6 mm) with four self-tapping (1.2×6 mm) for 15 rats of the OPS group. The periosteum was removed circumferentially at the osteotomy site for the length of 1.5 mm and also the length of the plate on the side where it was placed. In the OIKW group, the femur fracture was reduced using a 25×1.6 mm intramedullary K-wire. After reducing the fractures, the surgical wound was sutured in anatomical layers using 5-0 Vycril. Local ointment was applied. We performed a total number of 30 osteotomies and fracture reductions.

Clinical, radiological and histological assessment

The animals were assessed on daily basis for eight weeks. At eight weeks, a video recording was made in order to capture the reestablished function of the operated limb and the mobility at climbing an oblique 60º grid. Also, the mobility of the limb was assessed while swimming in a transparent water tank, this way we eliminated the axial compression forces at the fracture site.

Plain radiographs of the fracture site were taken immediately after surgery, at two, four and eight weeks using a fixed radiography (RAD) system with an output of 60 kV, 4 mAs and exposure time of 0.1 ms (Optima™ XR646 GE Healthcare autoRAD system). It was noted if the fracture site was totally visible, partial visible (there was limited contact between one or two cortices), and absent.

After eight weeks, the operated femurs were harvested under general anesthesia by means of disarticulation. The animals were euthanized after harvesting. The plates and screws were removed, but the K-wire was left inside the medullar cavity. The bones were immediately immersed in 10% buffered neutral formalin, for 48 hours at room temperature, and then decalcified using 10% ethylenediaminetetraacetic acid (EDTA) for 28 days, at 37^0C temperature and a pH 7, the solution being changed daily (Figure 1). Each femur harvested was sectioned in 5 mm slices, then automatically processed (using a Leica ASP6025 tissue processor) for paraffin embedding; paraffin blocks were sectioned using Accu-Cut™ SRM 200 rotary microtome in order to obtain anterior, middle and posterior sets of three longitudinal 4 μm sections at 100 μm distance from each other. The sections were assessed using a compound microscope Leica DM750 equipped with a Leica ICC 50 W high-definition video camera after specific staining (Masson's trichrome, Gömöri, Van Gieson, Hematoxylin–Eosin). All tissue fragments were globally assessed for microscopic lesions. The presence of new bone formation, necrosis and cartilage was noted. The osteocytes inside the lacunae were numbered by two independent examiners using Image Pro Plus 7 AMS software (Media Cybernetics Inc., Marlow, Buckinghamshire, UK) on a rectangular microscopic field measuring 340×250 μm under 400× magnification. The data collected was analyzed using Microsoft Excel 2016 and IBM SPSS Statistics 23. The Shapiro–Wilk test was used to check for the normal distribution of the values. To see the degree of understanding and correlation between the two examiners, we used Kendall's W and Pearson's tests. To see if there is any difference between the two groups regarding the number of cells, we used t-test for equal variances not assumed after Levene's test was applied to see which t-test should be used.

Results

Clinical assessment

In the OPS group, the operated limb was used as it full potential after six hours from surgery, showing great stability and support at the fracture site. During the climbing test and the swimming test inside the transparent water tank, both hind limbs were used equally, with no noticeable difference between them. One rat presented with wound dehiscence and infections because of self-injury after 31 days and was excluded from the study. The femur of this rat was histologically assessed.

In the OIKW group, the operated limb regains its function after 72 hours, but not at its full potential. The full support of the operated limb was seen at a mean time of 30 days. During climbing and swimming tests at eight weeks, the mobility of the hind limbs was the same. One rat was excluded from the study after gaining a vicious position of the operated limb (external rotation and retraction) at 28 days.

Radiological assessment

In the OPS group, 12 rats had at least two cortices united after two weeks so the fracture site was partial visible, becoming absent after eight weeks. No external callus was revealed during this period of time. In two of the cases, the fracture site was partial visible at eight weeks and one rat was excluded from the OPS group due to plate dislocation (Figure 2).

In the OIKW group, no cortices were united with a totally visible fracture site at two weeks in five rats. At eight weeks, there were four cases of incomplete consolidation of the fracture site and one case was excluded from the study after revealing two fracture sites with intermediary bone segment (Figure 3). Callus formation accompanied by a periosteal reaction (linear elevation and mineralization of the periosteum) was only seen in OIKW group.

At eight weeks, more than 75% of the OPS group revealed the absence of the fracture site, while in the OIKW group only 66% (Table 1).

Table 1 – ***Radiological visibility of the fracture site***

Study group	Radiological assessment of the fracture site								
	Absent			*Partial visible*			*Totally visible*		
	2 W	*4 W*	*8 W*	*2 W*	*4 W*	*8 W*	*2 W*	*4 W*	*8 W*
OPS (*n*)	0	2	12	12	12	2	3	0	0
OIKW (*n*)	0	0	10	10	14	4	5	0	0

W: Weeks; *n*: Number of rats; OPS: Osteosynthesis using plates and screws; OIKW: Osteosynthesis using Kirschner wire.

Histological assessment

All the samples revealed areas of new bone formation in the periphery of fracture foci (Figures 4–7). In the OPS group, where limited periosteum stripping was performed, the fracture site was healed by means of direct bone repair without the presence of cartilage. The slides revealed extensive remodeling of lamellar bone, Haversian canals and blood vessels with trabecular areas of new bone including osteoblasts and forming intertrabecular cavities occupied by inflammatory cells, osteoclasts, osteoblasts and fibroblasts. New bone trabeculae were surrounded by numerous osteoblast with normal morphology. In the OIKW group, the bone healing occurred by means of endochondral ossification with the presence of hyaline cartilage (Figure 8) mixed with periosteal bony callus. Intertrabecular cavities were also hypercellular, including rests of granulation tissue, inflammatory cells and fibroblasts. Healing in these tissue fragments was indirect type with extensive cartilage formation and secondary endochondral ossification. Number of osteoblasts surrounding bone trabeculae was reduced comparing with the previous group. The histomorphometry study revealed a mean of 65 osteocytes/microscopic field (mf), with a median of 59 cells/mf and a standard deviation of 28 cells/mf, in the OPS group. In the OIKW group, the osteocytes mean was 33 cells/mf with a median of 32 cells/mf and a standard deviations of 10 cells/mf (Figure 9). The difference between the groups regarding the number of osteocytes inside lacuna was statistically significant (*t*-test for equal variances not assumed, p=0.001), which confirms a mean difference of 32 cells/mf with a 95% confidence interval of 15–50 cells/mf; deviation of 10 cells/mf. The counting was performed by two independent examiners with a high-grade correlation between their results (Pearson's test, p<0.001).

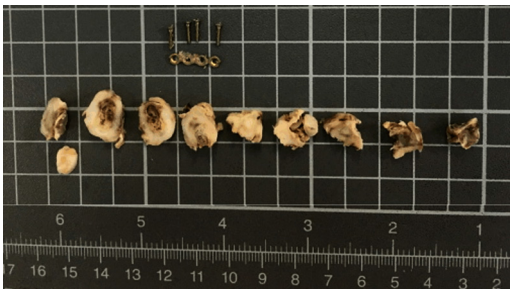

Figure 1 – ***The macroscopic aspect of serial sections through one femur reduced with plates and screws after demineralization process using EDTA.***

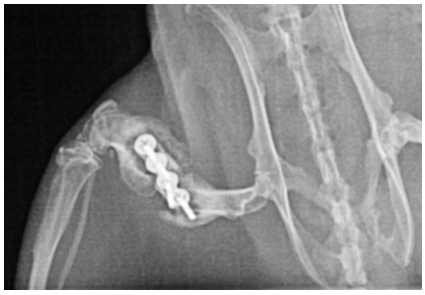

Figure 2 – ***Plain radiographs at four weeks revealing plate dislocation from the proximal bone segment.***

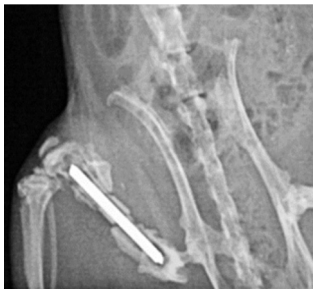

Figure 3 – ***Plain radiographs at four weeks revealing two fracture sites with intermediary bone segment.***

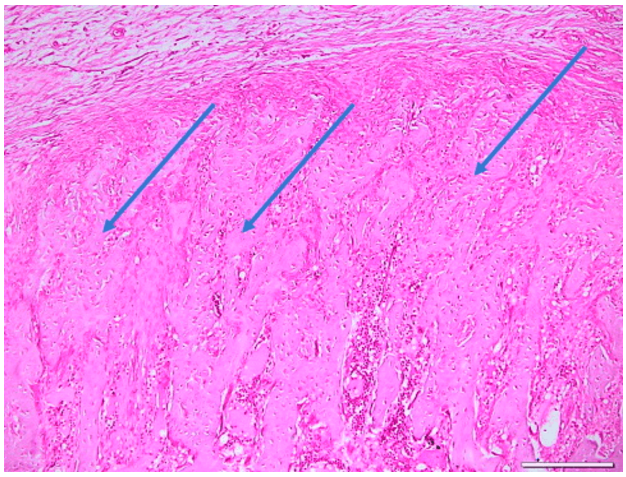

Figure 4 – ***Micrograph of longitudinal section through the rat fractured femur (HE staining, ×100) revealing hypercellular bone trabeculae surrounded by hypertrophic osteoblasts (blue arrows).***

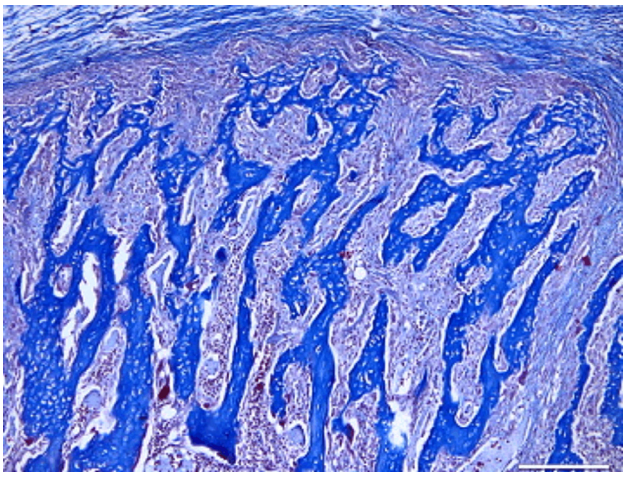

Figure 5 – ***Micrograph of longitudinal section through the rat fractured femur (Masson's trichrome staining, ×100) revealing new formed bone trabeculae and the hypercellular spaces between them. No hyaline cartilage was identified in the areas of bone healing (no endochondral ossification).***

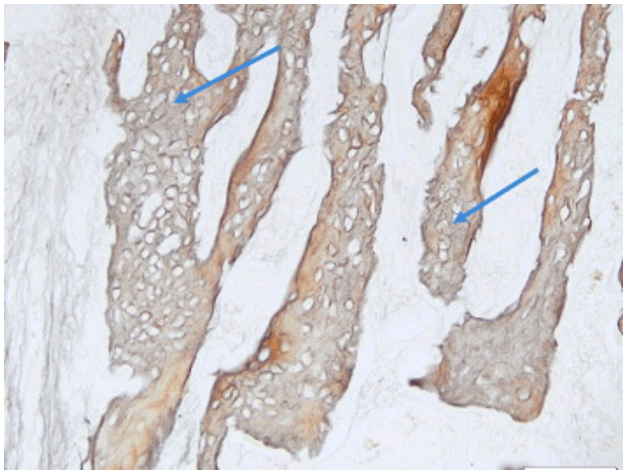

Figure 6 – ***Micrograph of longitudinal section through the rat fractured femur (Gömöri staining, ×200) revealing mesenchymal ossification – new formed bone trabeculae composed of lamellae and osteocytes cavities (blue arrows).***

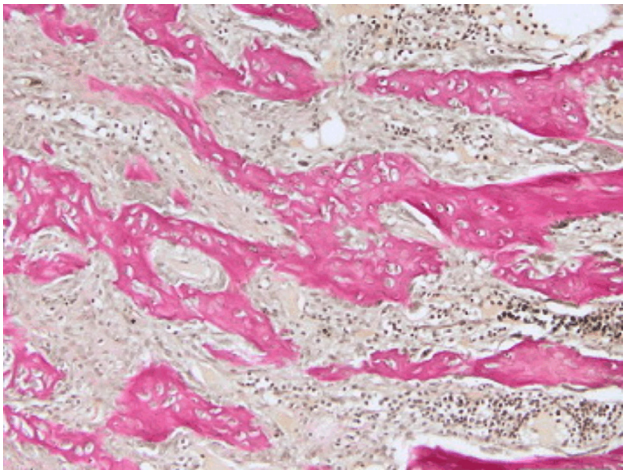

Figure 7 – ***Micrograph of longitudinal section through the rat fractured femur in the OPS group (van Gieson staining, ×200) revealing mesenchymal ossification – unequal new formed trabecular bone with numerous osteocytes.***

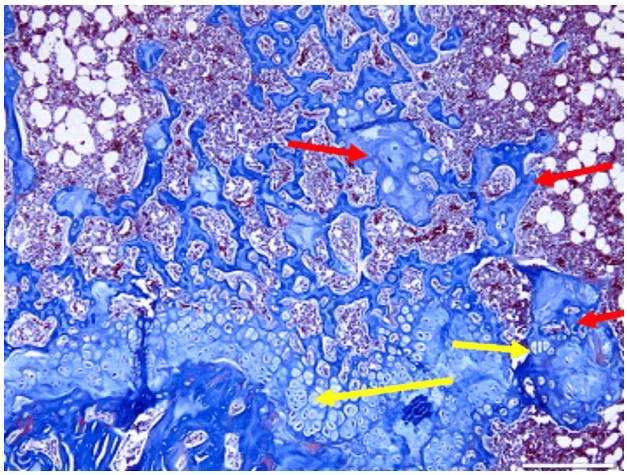

Figure 8 – ***Micrograph of longitudinal section through the rat fractured femur in the OIKW group (Masson's trichrome staining, ×200) revealing hyaline cartilage (red arrows) and new bone tissue (yellow arrows) that includes lesser osteocytes than in previous image.***

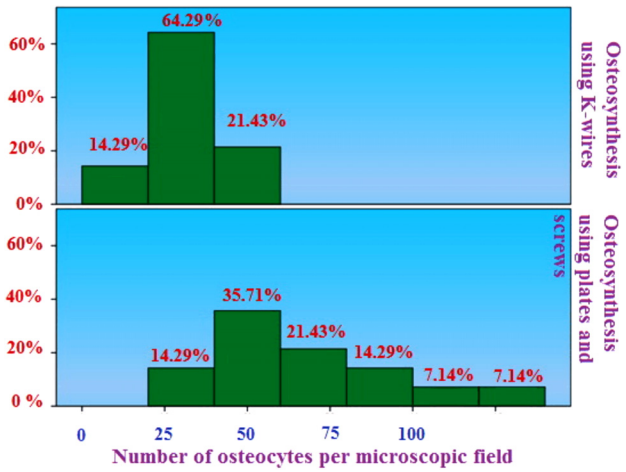

Figure 9 – ***Percentage distribution according to the number of osteocytes per microscopic field for each group.***

Discussion

Osteosynthesis using plates and screws has several advantages. Therefore, in this study we relied mainly on the redistribution of the mechanical forces along the fracture site and on the stable reduction of the fracture. Osteosynthesis using K-wires is associated with the existence of macro-movements at the fracture site, which determine an endochondral ossification process with a cartilaginous intermediary stage. The cell source, in this case, is represented mainly by the internal layer of the periosteum, endosteum and bone marrow. In the OPS group, the macro-movements at the fracture site are canceled, only the micro-movements are presented due to the elasticity of the material they are built of, but this are stimulants for bone regeneration. In this murine model, no compression at the fracture site was desired. Most of the osteoprogenitor cells migrated from the Haversian systems and a small part from the medullar cavity. In this type of bone healing, no intermediary cartilaginous stage was present; therefore, the mesenchymal cells differentiated directly into osteoblasts, which then started producing bone anarchic lamellaes, perpendicular or parallel oriented, according to the distance between bone fragments.

There are studies that show the role of the periosteum and other factors in bone healing [2–13], but few of them reveals the influence on bone healing of the periosteum stripping [4, 14, 15]. In our experimental study, limited periosteum removal was associated with a larger cell population compared to periosteum preservation. Therefore, other cell sources were able to compensate the osteoprogenitor cells that were removed along with the limited part of the periosteum.

A recent experimental study revealed the effects of the soft tissue surrounding the fracture site of 30 Wistar rats' tibias. The biomechanical tests at eight weeks showed no major differences between avascular bone segment, vascular bone segment and simple fracture, meaning that the osteoprogenitor source was represented by the

endosteum and bone marrow [16]. When compression between bone fragments at the fracture site is achieved, any communication between the cortical bone and the medullar cavity or periosteum are closed, therefore, the healing is accomplished by the cells of the Haversian system, with a prolonged healing period in special conditions (renal impairment, diabetes mellitus, malnutrition, important metabolic acidosis, severe anemia, etc.) [17–21].

Another study reported a murine model of fracture non-union due to poor vascularization after performing femoral periosteum removal and bone cavity reaming. After 65 days, no consolidation of the fracture site was found. However, the histological assessment revealed signs of osteoclast activity, which could represent the draft of the beginning of a bone healing process, despite the absence of a solid mesenchymal cell source and adequate blood supply [22].

In our study, we remove the periosteum only at the level of the fracture site and on the surface corresponding to the plate. Therefore, the medullar blood supply was not harmed, as it could have been done during intra-medullar fixation using K-wire.

Circumferential periosteum removal at the fracture site was reported in other studies [23–25]. We chose this method in order to protect the rest of the periosteum from avulsion during osteotomy using the electric saw.

During fracture consolidation, in order for the lamellar bone to be produced, the cellular component is well represented by the proliferation of the osteoblast. Afterwards, the remodeling phase is represented by a decreasing cell population and by intense production of a dense and well mineralized collagen matrix, mechanism impaired in special conditions (*e.g.*, renal insufficiency) [26, 27].

Periosteum removal is characterized by two consequences: losing a vascular network in the affected area and losing an important source of mesenchymal cells, which are present in the inner layer. Theoretically, this should have a negative impact on fracture healing, but in this experimental study, the results showed better healing despite periosteum removal.

The mean of cells/mf was higher in the OPS group, which demonstrates the existence of a valuable osteoprogenitor cells source more capable of restoring the injured bone to its initial form, compared to OIKW group, where the mean was approximately 33. The decrease in blood supply due to the limited periosteum removal and its vascular network is theoretically associated with a degree of hypoxia at the fracture site that would determine the mesenchymal cells to differentiate into chondrocytes in order to produce cartilage. However, in the OPS group, no cartilage was present during histological assessment, only in the group where reduction was achieved using K-wires. It is well known that only the periosteal mesenchymal cells have the ability to become either chondroblasts, either osteoblasts, but in our study, due to rigid fixation in OPS group, no mesenchymal cells could penetrate inside the fracture site.

Therefore, according to this experimental study, removing of a small area of periosteum in order to obtain a stable fixation of the fracture site using plates and screws did not influence negatively the fracture healing. In fact, rigid fixation of the fracture favors and accelerates the healing process compared to less stable methods like using K-wires.

Conclusions

The results of this study demonstrate that osteosynthesis with plates and screws after limited periosteum stripping lead to a better fracture healing according to the histological findings with a larger amount of osteocytes embedded in the material produced by the osteoblasts. This experimental protocol can be easily applied in order to study other effects of periosteum removal or other factors that could influence bone healing using different osteosynthesis methods.

Conflict of interests

The authors declare no conflict of interests.

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