GRANULOCYTE-COLONY STIMULATING FACTOR ENHANCES BONE FRACTURE HEALING

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Abstract

Background

Circulating mesenchymal stem cells contribute to bone repair. Their incorporation in fracture callus is correlated to their bioavailability. In addition, Granulocyte-colony stimulating factor induces the release of vascular and mesenchymal progenitors. We hypothesized that this glycoprotein stimulates fracture healing, and analyzed the effects of its administration at low doses on bone healing.

Methods

27 adult male Sprague-Dawley rats underwent mid-femur osteotomy stabilized by centromedullar pinning. In a post (pre) operative group, rats were subcutaneously injected with 5µg/kg per day of Granulocyte-colony stimulating factor for 5 days after (before) surgery. In a control group, rats were injected with saline solution for 5 days immediately after surgery. A radiographic consolidation score was calculated. At day 35, femurs were studied histologically and underwent biomechanical tests.

Findings

5 weeks after surgery, mean radiographic scores were significantly higher in the Preop group 7.75 (SD 0.42) and in the Postop group 7.67 (SD 0.52) than in the control group 6.75 (SD 0.69). Biomechanical tests showed femur stiffness to be more than three times higher in both the Preop 109.24N/mm (SD 51.86) and Postop groups 100.05N/mm (SD 60.24) than in control 32.01N/mm (SD 15.78). Mean maximal failure force was twice as high in the Preop group 68.66 N (SD 27.78) as in the control group 34.21N (SD 11.79). Histological results indicated a later consolidation process in control than in treated groups.

Interpretation

47 Granulocyte-colony stimulating factor injections strongly stimulated early femur fracture healing,
48 indicating its potential utility in human clinical situations such as programmed osteotomy and
49 fracture.
50 Keywords : Three-point-bending test, Fracture healing, Granulocyte-colony stimulating factor,
51 Circulating mesenchymal progenitor cells, Histology
52

1. Introduction

Fracture consolidation has long been considered a locoregional process involving mesenchymal progenitor cells derived from the tissues damaged by the trauma (local bone marrow, endosteum, bone tissue, periosteum, muscles). Following a series of cellular and molecular event cascades reminiscent of the embryonic stages of skeletal tissue formation, these cell precursors lead to the regeneration of the initially injured tissue. However, the 2000s saw the discovery of circulating osteoprogenitor cells (Kuznetsov et al., 2001; Labat et al., 2000), now known to contribute to the bone formation and repair process (Otsuru et al., 2007; Otsuru et al., 2008). In physiologic state, these cells represent 1 to 2% of circulating mononuclear cells in adults and nearly 10% in adolescents (Eghbali-Fatourechi et al., 2005). They increase in response to osteogenic requirements: in animals, during an ectopic osteogenesis process, they can transiently rise to 80% of circulating mononuclear cells (Otsuru et al., 2008). This cell pool can contribute up to 10% of the osteoblasts present in a fracture consolidation callus (Kumagai et al., 2008) and as much as 50% of the osteocytes present in an ectopic bone regenerate (Otsuru et al., 2008).

Intravenous injections of blood-derived osteoprogenitor cells stimulate fracture repair (Granero-Molto et al., 2009; Wan et al., 2006). Their rate of incorporation into the callus increases proportionally to their serum bioavailability, until it reaches a plateau. Beyond this value, it remains stable regardless of increases in serum level (Granero-Molto et al., 2009). On the other hand, endogenous circulating osteoprogenitor cell bioavailability can be transiently boosted by the pharmacological use of bioactive molecules that trigger the mobilization of their medullary precursors, thereby favoring bone repair (Kumar et al., 2012; Matsumoto et al., 2008; Toupadakis et al., 2013).

G-CSF is a glycoprotein used in human therapeutics for its ability to mobilize medullary hematopoietic stem cells in systemic circulation. G-CSF also induces the mobilization of vascular stem cells (Minamino et al., 2005) and mesenchymal stem cells (Levesque et al., 2007; Tatsumi et al., 2008; Zhdanov et al., 2007), both of which are involved, to varying degrees, in skeletal tissue repair. In rats, there was a significant increase in $CD34^+$ progenitor cells after five consecutive injections of G-CSF (Herrmann et al., 2018). These cells are capable of differentiating into osteogenic as well as vasculogenic lineages (Sidney et al., 2014). Surprisingly, few studies have been published on the use of G-CSF as a skeletal tissue repair or regeneration adjuvant (Kaygusuz et al., 2006; Wu et al., 2008). When administered topically, G-CSF improves neovascularization and osteogenesis, which leads to regeneration of both critical-size bone defects in a rabbit bone resection model (Ishida et al., 2010) and tendon graft in a ligamentoplasty model (Sasaki et al., 2008). When administered parenterally, G-CSF counterbalances the negative effects of NSAIDs on bone healing, probably by stimulating osteogenesis (Kaygusuz et al., 2006). In a rabbit femoral osteonecrosis model, the combined use of G-CSF and Stem Cell Factor cause increased osteoblast activity and improve local revascularization, leading to more effective regeneration of the necrotic bone tissue volume (Wu et al., 2008).

To date, only two studies have focused on the effects of parenteral administration of G-CSF on fracture healing (Bozlar et al., 2005; Herrmann et al., 2018), both finding that G-CSF accelerated bone repair in rats. However, the doses used in these studies were respectively 2.5 (Bozlar et al., 2005) and 5 times higher (Herrmann et al., 2018) than the recommended dose in human clinical practice with healthy subjects. In fact, for donors providing peripheral blood stem cells for recipients of hematopoietic stem cell transplants, recombinant human G-CSF is generally recommended at a dose of 10µg/kg per day. Even at this dose, side effects are observed, although

when the dose is reduced, the side effects decrease (Lambertini et al., 2014). Furthermore, Bozlar et al. (2005) and Herrmann et al. (2018) chose to work only on emergency clinical applications (e.g fractures, large defects) and did not consider programmed clinical applications (e.g bone lengthening, tumor removal).

In this work, from a perspective of the eventual therapeutic use of GCSF in humans, we investigated the effects of parenteral administration of a 5µg/kg per day dose of G-CSF on fracture consolidation in rats. We also investigated G-CSF administration pre-surgery vs G-CSF administration post-surgery. Pre-surgery administration mirrors the human clinical situation of a programmed osteotomy, and post-surgery administration mirrors a fracture situation.

Materials and Methods

Twenty-seven male *Sprague Dawley* rats (OFA), weighing 500g and twelve weeks old at time of surgery were used in the experiment. The animals were fed a standard diet ad libitum. They were housed singly in cages in temperature-controlled rooms (22°C) having a 12h light cycle. All animal protocols were approved by the University of Aix-Marseille institutional animal care and use committee and the French research ministry (authorization number 02572.02), and performed in the conventional animal house of Marseille Medical Faculty (France).

2.1 Surgical protocol

The surgical model consisted of right femur mid-diaphyseal osteotomy, immediately osteosynthesized by centromedullary pinning. Under general anesthesia consisting of intraperitoneal Ketamine 75 mg/kg and Medetomidine 0.15 mg/kg and under strictly aseptic conditions, the right femur was exposed via a lateral subperiosteal approach. Medial-diaphyseal osteotomy was performed using piezotome. Retrograde centromedullary pinning (2 mm diameter Kirschner wires) was performed by lateral parapatellar arthrotomy. The muscular fascia was closed with separated resorbable stitches and the skin with slow-absorption continuous stitches. Postoperative analgesia and prophylactic antibiotic therapy consisted of an injection of Buprenorphine 0.05 mg/kg and subcutaneous Baytril 10 mg/kg peroperatively, then once per day for 3 days. Rats were followed weekly by radio to check that they were healthy.

2.2 Experimental groups

Rats were randomized to one of three distinct pharmacological procedures. The "Postoperative" group (Postop) with 9 rats were injected subcutaneously with 5µg/kg per day of G-CSF (FILGRASTIM) for 5 days starting from surgery. The "Preoperative" group (Preop) with 9 rats received identical G-CSF injections for 5 days preoperatively. In the Control group, 9 rats were injected subcutaneously for 5 days with a saline solution, starting immediately after surgery. One rat died in the control group upon induction of anesthesia. One rat died in the Postop group being found dead one day after surgery. One rat in the Preop group was sacrificed three days after surgery due to splitting of the scar tissue. No locoregional infections or other complications were observed in the other animals. After 35 days of consolidation, the animals were sacrificed by intraperitoneal lethal injections of Sodium Pentobarbital 100 mg/kg.

2.3 Radiographic analysis

X-rays of the femurs subjected to surgery were taken immediately postoperatively, then at days 7, 21 under general anesthesia and at day 35 after the sacrifice. A radiographic consolidation score (An et al., 1999) was calculated from the analysis of the X-rays by two orthopedic surgeons not involved in the study (Table 1) and who analyzed independently each x-ray according to the radiographic scoring system for fracture healing (An et al., 1999). The final score assigned to each x-ray was the mean of the scores of the two surgeons.

Table 1: Radiographic scoring system for fracture healing (An et al., 1999) **.**

CATEGORY	**SCORE**
Periosteal reaction	

• Full (across the defect)	3
• Moderate	2
• Mild	1
• None	0
Bone union	
• Union	3
• Moderate bridge (> 50%)	2
• Mild Bridge (< 50%)	1
• Non-union	0
Remodeling	
• Full remodeling cortex	2
• Intramedullary canal	1
• No remodeling	0
Maximum total score	8

2.4 Mechanical analysis

Femurs subjected to surgery (6 femurs from control group, 6 femurs from Preop group and 6 from Postop group) and healthy contralateral femurs (6 femurs from control group, 6 femurs from Preop group and 6 from Postop group) underwent destructive mechanical tests. The fixation pin and soft tissue were completely removed before tests.

The fresh material was frozen and stored at -20°C, which does not alter the structure or properties of bone. The samples were slowly thawed at room temperature before preparation and mechanically tested under three-point-bending (Turner et al., 1993) on a testing device (MTS Instron 5566A, INSTRON© Elancourt, France, 1000N load with an accuracy of 0.19%). We used preconditioning at 1N and subsequently imposed a speed of 0.5mm per min.

Force/displacement curves were obtained. Failure force and stiffness of the fracture site were analyzed.

2.5 Histological analysis

Six femurs subjected to surgery underwent histological analysis after 35 days of consolidation (2 from control group, 2 from Postop group, 2 from Preop group). The fixation pin was removed and the fracture site was isolated from the femur and fixed in 4% formaldehyde (Merk Millipore) in 0.01M phosphate buffer saline (Sigma-Aldrich) at pH 7.4 for one week. Following fixation, tissue samples were washed with deionized water and were dehydrated through an ethanol gradient of 60%, 80%, 95%, 100% ethanol, each step lasting 48 hours. Samples were cleared in methylcyclohexan (VWR international) for 48h before infiltration and embedding in Methyl methacrylate (MMA) resin (VWR international). Samples then underwent polymerization in a 28°C water bath for 3 days.

After trimming of the blocks, 5µm thick longitudinal sections were obtained using a microtome (Leica© RM 2265, Wetzlar, Germany) equipped with a D-profile tungsten-carbide knife. The sections were transferred to Superfrost Plus slides and stretched with 70% ethanol. Slides were covered with a plastic film, pressed and dried for 2 days. Before staining, MMA was removed from the sections by immersion in three changes of 2-methoxyethylacetate (Merk Millipore) for 20min each, one change of ethanol 70 for 5min, one change of ethanol 40 for 5min, and then rehydrated in two deionized water baths. The sections were stained with toluidine blue and Von Kossa. They were dehydrated and mounted using a resinous mounting medium (Entellan, Merk Millipore).

2.6 Statistical analyses

Due to the small size of the groups, we chose to use nonparametric tests. The nonparametric Kruskal-Wallis test was used to compare consolidation scores for the three groups (control, Preop, Postop) at each time point (J7, J21, J35). This same test was used to compare stiffness and failure force values for the three groups, both for the contralateral femurs and for the femurs subjected to surgery. The significance level was set at $p<0.05$. For the multiple comparisons, we used the Conover-Iman test with a Bonferroni correction. Statistical analysis was performed using XLSTAT software.

2. Results

3.1 Radiographic results

Radiographic consolidation scores for all time points are presented in table 2. In the peripheral callus, asymmetrically distributed around the fracture site, signs of mineralization were visible from 7 days after surgery. At days 7 and 21, a significant difference was observed between the control group and the Postop group alone ($p = 0.001$ at day 7; $p = 0.001$ at day 21). At day 35, we observed a significant difference between the control group and the Preop group ($p = 0.008$) as well as between the control group and the Postop group ($p = 0.013$). At this date, the osteotomy site remained visible on all femurs, pointing to incomplete remodeling. At each time point, the difference between the two treated groups was not significant ($p = 0.094$ at day 7; $p = 0.046$ at day 21; $p = 0.806$ at day 35).

Table 2 : Radiographic score

	Control group	**Preop group**	**Postop group**

D + 7 postop	0.75 (0.27)	1.50 (0.84)	2.33 (1.13) *
D + 21 post op	3.83 (0.82)	4.83 (0.75)	5.75 (0.82) *
D + 35 postop	6.75 (0.69)	7.75 (0.42) *	7.67 (0.52) *

Values are Mean (SD). D: day of surgery. ***** Significant by comparison with the control group.

3.2 Mechanical results

5 weeks after surgery, force/displacement curves were obtained for 18 healed femurs from the three experimental groups and for 18 control femurs obtained from their contralateral femurs. Although the femurs systematically fractured at the level of the bony callus during the 3-point bending tests, two types of failure profile were observed. The control group femurs experienced ductile failure, while the preop and postop groups femurs experienced brittle failure, like the contralateral group femurs (Figure 1). The contralateral femurs did not display any significant difference in mean stiffness ($p = 0.24$) or in maximum failure force ($p = 0.17$), irrespective of origin (control, Preop and Postop). Comparing the 3 experimental groups via the Kruskall-Wallis test, we found a significant difference in stiffness ($p = 0.011$) and maximal failure force ($p = 0.024$). Concerning stiffness, the multiple comparisons showed that the control group significantly differed both from the Preop group ($p= 0.002$) and from the Postop group ($p = 0.003$). Mean callus stiffness was slightly more than three times higher in the Preop 109.24 N/mm (SD 51.86) and in the Postop groups 100.05 N/mm (SD 60.24) than in the control group 32.01 N/mm (SD 15.78).

Concerning maximal failure force, the multiple comparisons showed that the control group significantly differed from the Preop group ($p= 0.004$) but no difference from the Postop group was observed ($p = 0.053$). Mean maximal failure force was twice as high in the Preop group 68.66 N (SD 27.78) as in the control group 34.21N (SD 11.79). No significant difference was

observed between Preop and Postop groups neither for stiffness (p = 0.884) nor for failure force (p = 0.217).

Table 3. Mechanical results.

	Contralateral Femurs		Operated femurs	
	Stiffness (N/mm)	Max failure force (N)	Stiffness (N/mm)	Max failure force (N)
Control Group	327.03 (16.71)	191.38 (27.96)	32.01 (15.78)	34.21 (11.79)
Preop Group	380.75 (123.20)	169.53 (28.65)	109.24 (51.86)*	68.66 (27.78) *
Postop Group	386.19 (61.65)	169.14 (19.20)	100.05 (60.24) *	51.65 (16.75)

Values are Mean (SD). * Significant by comparison with the control group.

3.3 Histological results

In the control group, a fibrous tissue joined the two cortices, while incomplete bony union was observed in the periosteal callus (Fig 2a). The latter was composed of centrally located uncalcified and calcified cartilage adjacent to newly woven bone, indicating endochondral ossification (Fig 3a). At the periphery, the periosteal callus consisted of woven and lamellar bone (Fig 2a).

No difference was observed between the Preop and the Postop G-CSF-treated groups. Neither of the groups showed any evidence of endochondral ossification. There was complete bony union. The osteotomy gap was filled with anastomosed bone trabeculae in the periosteal region of the callus (Figs 2b and 2c) as well as between the cortices (Fig 3b). In the internal and periosteal

calluses, signs of bone formation and bone resorption indicated that remodeling of regenerated bone was occurring. Bone trabeculae showed on their surface osteoid tissue apposed to rows of osteoblasts and osteoclasts that resorbed bone (Fig 4).

3. **Discussion and conclusion**

The concomitant mobilization of angiogenic (Minamino et al., 2005) and mesenchymal stem cells (Wan et al., 2006; Levesque et al., 2007; Tatsumi et al., 2008) by G-CSF suggests its potential contribution to skeletal tissue repair. The purpose of this study was to evaluate the effect of low doses of G-CSF on the consolidation processes in fractures treated by open surgery, mirroring two human clinical situations: programmed osteotomy (Preop group) and fracture (Postop group). Our study showed that a 5µg/kg per day dose is sufficient to stimulate fracture repair processes. Five weeks after surgery, femurs from both treatment groups showed stimulation of osteotomy site consolidation stemming from systemic G-CSF administration. The efficacy of G-CSF in improving bone repair process was previously demonstrated at higher doses in rats, by Herrmann et al. (2018) and Bozlar et al. (2005). In the latter study, in a rat model of tibia fracture, the authors observed a 10-fold increase in callus mechanical strength three weeks after surgery in animals treated with a 25µg/kg per day G-CSF injection. In our study, maximal failure force was twice as high in the Preop group as in the control group, and callus stiffness slightly more than tripled in both G-CSF groups compared to control. Since our mean failure force values obtained on healthy bone were similar to those reported in the literature for rats of the same genetic origin, age and sex (Utvåg et al., 1998a; Utvåg et al., 1998b, Utvåg et al., 2001), methodological issues can be excluded. The difference in mechanical values is probably related to the dose of G-CSF administered. Moreover, we did not observe any impairment of

biomechanical behavior in the femurs not subjected to surgery, even though prolonged medullary progenitor cell mobilization is known to lead to deterioration of bone tissue (Wu et al., 2008; Toupadakis et al., 2012). This result is not surprising in view of the short period of injection with G-CSF.

Histological analysis corroborated the biomechanical results. In G-CSF treated animals, we observed the complete replacement of endochondral cartilage by bone trabeculae, evidence that the process of remodeling is underway. In the control group animals, however, we observed the persistence of cartilaginous tissue in the process of ossification, which indicates a later consolidation process than in the treated group. The difference between control group and treated groups in terms of tissue type within the callus probably explains why ductile failure was observed in the control animals' bone and brittle failure in animals treated with G-CSF.

In human clinical practice, G-CSF is administered both to patients with chronic neutropenia or cancer and to donors providing peripheral blood stem cells for recipients of hematopoietic stem cell transplants. In healthy donors, G-CSF is recommended at the dose of 10 µg/kg administered once daily, and although well tolerated, it induces side effects. In a large prospective study, Pulsipher et al. (2014) reported that some rare life-threatening events occurred after G-CSF administration in healthy donors and that no fatalities occurred. In addition, treated donors showed no evidence of increased risk for cancer, autoimmune illness and stroke. The most common adverse event associated with G-CSF administration in healthy donors is bone and musculoskeletal pain (Lambertini et al., 2014; Tigue et al., 2007). To prevent and treat this pain, nonsteroidal anti-inflammatory agents are commonly used as a first-line treatment, and dose reductions are considered as second-line therapy (Lambertini et al., 2014). Thus, with a view to using G-CSF treatment as a fracture repair adjuvant in humans, we chose to test a low dose of G-CSF.

Our therapeutic approach, derived from regenerative medicine, is founded on the well-documented contribution from circulating progenitor cells to many tissue repair processes. Stimulation of bone repair processes is related to the increased bioavailability of circulating vascular and mesenchymal progenitor cells at the neo-osteogenesis site (Kumar et al., 2012; Matsumoto et al., 2008; Toupadakis et al., 2013; Pignolo et al., 2011). Their incorporation into the skeletal tissue repair site contributes to many steps in the cascade of cellular and molecular events of the endochondral osteogenesis processes. During initial stages, these cells modulate the local and systemic inflammatory response (Iguchi et al., 1991) and induce local emission of cytokines such as BMP-2 that initiate bone tissue repair processes (Granero-Molto et al., 2009). During a later phase, vascular invasion of the cartilage matrix is promoted by angiogenesis and vasculogenesis mechanisms. The latter are supported by the influx and proliferation of circulating angiogenic progenitor cells of medullary origin (Matsumoto et al., 2008; Minamino et al., 2005), which depend on their serum level (Lee et al., 2008). This neoangiogenesis allows the influx of circulating osteogenic progenitor cells, which are incorporated into the callus undergoing ossification. Their incorporation is also dependent upon their serum level (Granero-Molto et al., 2009). Now, according to Herrmann et al. (2016), the bioavailability of vascular and osteogenic progenitor cells such as CD34+ significantly increased after five consecutive injections of G-CSF in rats. In addition, in patients with femoral and tibial nonunion, it has been observed radiographically that fracture healing improved when G-CSF-mobilized CD34+ cells were transplanted to non-union sites (Kuroda et al., 2014). Thus, the stimulation of fracture repair by systemically administered G-CSF observed in our study could result from G-CSF-induced medullary mobilization of vascular and mesenchymal progenitor cells in the vascular compartment, leading to increased incorporation at the tissue repair site. Nevertheless, the possibility that G-CSF improves consolidation by other mechanisms cannot be ruled out. Froberg

et al. (1999) showed that G-CSF induces an increase in blood markers of bone formation such as osteocalcin and bone-specific alkaline phosphatase, which suggests that G-CSF stimulates osteoblastic activity. In addition, the effects of G-CSF could also be related to an increase in some cytokines. In an in vitro model, Kaygusuz et al. (2006) showed that G-CSF, while raising the $TGF\beta_1$, has a positive effect on fracture healing and Czekanska et al. (2014) that stimulation of mesenchymal stem cells by G-CSF results in an upregulation of mRNA expression of BMP2, which is involved in bone formation.

The rate of incorporation of circulating progenitor cells into the fracture callus is time- and dose-dependent (Granero-Molto et al., 2009). These cells can interact both qualitatively and quantitatively at various stages and times in the osteogenesis process (Iguchi et al., 1991; Lee et al., 2008). We therefore modulated the bioavailability peak of circulating progenitor cells in order to make it overlap with various times and stages in the bone consolidation process.

We compared two different chronological sequences, one in which the adjuvant treatment preceded the surgical procedure and one in which it followed the surgery. Five weeks after surgery, no significant difference was observed between the Preop and the Postop groups, neither in callus mechanical behavior nor in the biological structure of the regenerated bone at the fracture site. However, mechanical tests are structural analysis, so we have no information about the callus undergoing ossification and its microstructure. Yet the mechanical characteristics of the callus are governed by its morphology, volume and material characteristics, in particular its degree of mineralization and remodeling. Moreover, only the consolidation score of the Postop group differs from that of the control group at days 7 and 21, indicating potential differences in degree of mineralization between the two treated groups. This difference in consolidation score disappears at day 35. This suggests that, despite the similar radiological profile and mechanical behavior of the explants from the two treated groups observed five weeks after surgery, the

pharmacological effect of G-CSF may have stimulated different stages in the endochondral ossification process.

During skeletal tissue regeneration, systemic cell mechanisms participate in tissue repair. Medullary mesenchymal and vascular progenitor cells are mobilized in systemic circulation, then captured at the tissue repair site, where they participate in the various stages of the cellular and molecular events leading to the restoration of injured tissue (Kuroda et al., 2014). G-CSF increases the bioavailability of these circulating progenitor cells. However, we measured neither the kinetics, nor the intensity of blood mobilization of progenitor cells. The correlation between the pharmacological increase in progenitor cell bioavailability and fracture repair stages will be studied in future experimental work. It would be of interest to reevaluate treatment duration and starting time for G-CSF injections relative to the biological phases of the endochondral osteogenesis process. Finally, the stimulation mediated by G-CSF can be considered either in terms of consolidation speed and functional recovery, or in terms of final fracture site strength. In the present study, we chose to study the radiographical, mechanical and histological characteristics of the callus before completion of consolidation and bone remodeling. It would be interesting to follow consolidation callus kinetics to completion. This would reveal whether the final consolidation product is obtained earlier, and whether it is mechanically more efficient in the G-CSF treated groups than in the control group after callus remodeling. Despite its limitations, this study demonstrates that a low-dose, short-term systemic G-CSF adjuvant treatment stimulates fracture repair, indicating its potential utility in human clinical situations such as programmed osteotomy and fracture. The therapeutic modalities of this emerging "regenerative medicine" strategy remain to be determined.

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Figure legends

Fig 1. Examples of **force/displacement curves for femurs from the control, preop, postop groups and for contralateral femurs.**

Fig 2. **Low magnification of the fracture calluses 5 weeks after the osteotomy in control (a), preop (b) and postop group (c).** Sections were stained with Von Kossa and counterstained with toluidine blue. (a) Note the fibrous tissue (*) between the cortices (C). Near the site of the osteotomy, the periosteal callus (P) is composed of bony and cartilaginous tissue. (b et c) Bony bridge (arrowheads) joins the fractured ends in the internal and in the periosteal callus regions. Scale bar 2mm.

Fig 3: **Micrograph of section stained with Von Kossa and counterstained with toluidine blue** from the control group (a) and from the Postop group (b) 5 weeks after osteotomy**.** (a) Around the fracture gap, the reparative process occurs by endochondral ossification. New bone formation (arrow). Capillary invasion (*). Hypertrophic chondrocyte embedded in calcified cartilage (arrowhead). Uncalcified cartilage (UC). Scale bar 100µm. (b) A network of anastomosed trabeculae (arrows) is observed between the cortices (C). In this specimen, trabeculae are composed mainly of mature bone. Scale bar 500µm.

Fig 4: **Micrograph of section stained with toluidine blue from the postop group** 5 weeks after osteotomy**.** On either side of bone trabeculae, the osteoclasts (arrowheads) and osteoblasts that deposit the osteoid (arrows) are observed. Scale bar 50µm.

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