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1 Title: GRANULOCYTE-COLONY STIMULATING FACTOR ENHANCES BONE
2 FRACTURE HEALING

3

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23

24 **Abstract**

25 *Background*

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

31 *Methods*

32 27 adult male Sprague-Dawley rats underwent mid-femur osteotomy stabilized by centromedullar
33 pinning. In a post (pre) operative group, rats were subcutaneously injected with 5µg/kg per day of
34 Granulocyte-colony stimulating factor for 5 days after (before) surgery. In a control group, rats
35 were injected with saline solution for 5 days immediately after surgery. A radiographic
36 consolidation score was calculated. At day 35, femurs were studied histologically and underwent
37 biomechanical tests.

38 *Findings*

39 5 weeks after surgery, mean radiographic scores were significantly higher in the Preop group
40 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).
41 Biomechanical tests showed femur stiffness to be more than three times higher in both the Preop
42 109.24N/mm (SD 51.86) and Postop groups 100.05N/mm (SD 60.24) than in control
43 32.01N/mm (SD 15.78). Mean maximal failure force was twice as high in the Preop group 68.66
44 N (SD 27.78) as in the control group 34.21N (SD 11.79). Histological results indicated a later
45 consolidation process in control than in treated groups.

46 *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

53 **1. Introduction**

54 Fracture consolidation has long been considered a locoregional process involving mesenchymal
55 progenitor cells derived from the tissues damaged by the trauma (local bone marrow, endosteum,
56 bone tissue, periosteum, muscles). Following a series of cellular and molecular event cascades
57 reminiscent of the embryonic stages of skeletal tissue formation, these cell precursors lead to the
58 regeneration of the initially injured tissue. However, the 2000s saw the discovery of circulating
59 osteoprogenitor cells (Kuznetsov et al., 2001; Labat et al., 2000), now known to contribute to the
60 bone formation and repair process (Otsuru et al., 2007; Otsuru et al., 2008). In physiologic state,
61 these cells represent 1 to 2% of circulating mononuclear cells in adults and nearly 10% in
62 adolescents (Eghbali-Fatourehchi et al., 2005). They increase in response to osteogenic
63 requirements: in animals, during an ectopic osteogenesis process, they can transiently rise to 80%
64 of circulating mononuclear cells (Otsuru et al., 2008). This cell pool can contribute up to 10% of
65 the osteoblasts present in a fracture consolidation callus (Kumagai et al., 2008) and as much as
66 50% of the osteocytes present in an ectopic bone regenerate (Otsuru et al., 2008).

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

75

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

93 To date, only two studies have focused on the effects of parenteral administration of G-CSF on
94 fracture healing (Bozlar et al., 2005; Herrmann et al., 2018), both finding that G-CSF accelerated
95 bone repair in rats. However, the doses used in these studies were respectively 2.5 (Bozlar et al.,
96 2005) and 5 times higher (Herrmann et al., 2018) than the recommended dose in human clinical
97 practice with healthy subjects. In fact, for donors providing peripheral blood stem cells for
98 recipients of hematopoietic stem cell transplants, recombinant human G-CSF is generally
99 recommended at a dose of 10µg/kg per day. Even at this dose, side effects are observed, although

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

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

109

110

111 **Materials and Methods**

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

118

119 **2.1 Surgical protocol**

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

130

131 **2.2 Experimental groups**

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

142

143 **2.3 Radiographic analysis**

144 X-rays of the femurs subjected to surgery were taken immediately postoperatively, then at days 7,
145 21 under general anesthesia and at day 35 after the sacrifice. A radiographic consolidation score
146 (An et al., 1999) was calculated from the analysis of the X-rays by two orthopedic surgeons not
147 involved in the study (Table 1) and who analyzed independently each x-ray according to the
148 radiographic scoring system for fracture healing (An et al., 1999). The final score assigned to
149 each x-ray was the mean of the scores of the two surgeons.

150

151

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

CATEGORY	SCORE
<i>Periosteal reaction</i>	

<ul style="list-style-type: none"> • Full (across the defect) • Moderate • Mild • None 	3 2 1 0
<p><i>Bone union</i></p> <ul style="list-style-type: none"> • Union • Moderate bridge (> 50%) • Mild Bridge (< 50%) • Non-union 	3 2 1 0
<p><i>Remodeling</i></p> <ul style="list-style-type: none"> • Full remodeling cortex • Intramedullary canal • No remodeling 	2 1 0
<p>Maximum total score</p>	8

153

154 **2.4 Mechanical analysis**

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

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

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

166

167 **2.5 Histological analysis**

168 Six femurs subjected to surgery underwent histological analysis after 35 days of consolidation (2
169 from control group, 2 from Postop group, 2 from Preop group). The fixation pin was removed
170 and the fracture site was isolated from the femur and fixed in 4% formaldehyde (Merk Millipore)
171 in 0.01M phosphate buffer saline (Sigma-Aldrich) at pH 7.4 for one week. Following fixation,
172 tissue samples were washed with deionized water and were dehydrated through an ethanol
173 gradient of 60%, 80%, 95%, 100% ethanol, each step lasting 48 hours. Samples were cleared in
174 methylcyclohexan (VWR international) for 48h before infiltration and embedding in Methyl
175 methacrylate (MMA) resin (VWR international). Samples then underwent polymerization in a
176 28°C water bath for 3 days.

177 After trimming of the blocks, 5µm thick longitudinal sections were obtained using a microtome
178 (Leica© RM 2265, Wetzlar, Germany) equipped with a D-profile tungsten-carbide knife. The
179 sections were transferred to Superfrost Plus slides and stretched with 70% ethanol. Slides were
180 covered with a plastic film, pressed and dried for 2 days. Before staining, MMA was removed
181 from the sections by immersion in three changes of 2-methoxyethylacetate (Merk Millipore) for
182 20min each, one change of ethanol 70 for 5min, one change of ethanol 40 for 5min, and then
183 rehydrated in two deionized water baths. The sections were stained with toluidine blue and Von
184 Kossa. They were dehydrated and mounted using a resinous mounting medium (Entellan, Merk
185 Millipore).

186

187 **2.6 Statistical analyses**

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

195

196 2. Results

197 3.1 Radiographic results

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

207

208 **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) *

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

210

211 **3.2 Mechanical results**

212 5 weeks after surgery, force/displacement curves were obtained for 18 healed femurs from the
 213 three experimental groups and for 18 control femurs obtained from their contralateral femurs.

214 Although the femurs systematically fractured at the level of the bony callus during the 3-point

215 bending tests, two types of failure profile were observed. The control group femurs experienced

216 ductile failure, while the preop and postop groups femurs experienced brittle failure, like the

217 contralateral group femurs (Figure 1). The contralateral femurs did not display any significant

218 difference in mean stiffness ($p = 0.24$) or in maximum failure force ($p = 0.17$), irrespective of

219 origin (control, Preop and Postop). Comparing the 3 experimental groups via the Kruskal-Wallis

220 test, we found a significant difference in stiffness ($p = 0.011$) and maximal failure force ($p =$

221 0.024). Concerning stiffness, the multiple comparisons showed that the control group

222 significantly differed both from the Preop group ($p = 0.002$) and from the Postop group ($p =$

223 0.003). Mean callus stiffness was slightly more than three times higher in the Preop 109.24

224 N/mm (SD 51.86) and in the Postop groups 100.05 N/mm (SD 60.24) than in the control group

225 32.01 N/mm (SD 15.78).

226 Concerning maximal failure force, the multiple comparisons showed that the control group

227 significantly differed from the Preop group ($p = 0.004$) but no difference from the Postop group

228 was observed ($p = 0.053$). Mean maximal failure force was twice as high in the Preop group

229 68.66 N (SD 27.78) as in the control group 34.21N (SD 11.79). No significant difference was

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

232

233

234 **Table 3. Mechanical results.**

235

	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)

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

237

238 **3.3 Histological results**

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

244 No difference was observed between the Preop and the Postop G-CSF-treated groups. Neither of
245 the groups showed any evidence of endochondral ossification. There was complete bony union.

246 The osteotomy gap was filled with anastomosed bone trabeculae in the periosteal region of the
247 callus (Figs 2b and 2c) as well as between the cortices (Fig 3b). In the internal and periosteal

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

251

252 **3. Discussion and conclusion**

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

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

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

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

295 Our therapeutic approach, derived from regenerative medicine, is founded on the well-
296 documented contribution from circulating progenitor cells to many tissue repair processes.
297 Stimulation of bone repair processes is related to the increased bioavailability of circulating
298 vascular and mesenchymal progenitor cells at the neo-osteogenesis site (Kumar et al., 2012;
299 Matsumoto et al., 2008; Toupadakis et al., 2013; Pignolo et al., 2011). Their incorporation into
300 the skeletal tissue repair site contributes to many steps in the cascade of cellular and molecular
301 events of the endochondral osteogenesis processes. During initial stages, these cells modulate the
302 local and systemic inflammatory response (Iguchi et al., 1991) and induce local emission of
303 cytokines such as BMP-2 that initiate bone tissue repair processes (Granero-Molto et al., 2009).
304 During a later phase, vascular invasion of the cartilage matrix is promoted by angiogenesis and
305 vasculogenesis mechanisms. The latter are supported by the influx and proliferation of circulating
306 angiogenic progenitor cells of medullary origin (Matsumoto et al., 2008; Minamino et al., 2005),
307 which depend on their serum level (Lee et al., 2008). This neoangiogenesis allows the influx of
308 circulating osteogenic progenitor cells, which are incorporated into the callus undergoing
309 ossification. Their incorporation is also dependent upon their serum level (Granero-Molto et al.,
310 2009). Now, according to Herrmann et al. (2016), the bioavailability of vascular and osteogenic
311 progenitor cells such as CD34+ significantly increased after five consecutive injections of G-CSF
312 in rats. In addition, in patients with femoral and tibial nonunion, it has been observed
313 radiographically that fracture healing improved when G-CSF-mobilized CD34+ cells were
314 transplanted to non-union sites (Kuroda et al., 2014). Thus, the stimulation of fracture repair by
315 systemically administered G-CSF observed in our study could result from G-CSF-induced
316 medullary mobilization of vascular and mesenchymal progenitor cells in the vascular
317 compartment, leading to increased incorporation at the tissue repair site. Nevertheless, the
318 possibility that G-CSF improves consolidation by other mechanisms cannot be ruled out. Froberg

319 et al. (1999) showed that G-CSF induces an increase in blood markers of bone formation such as
320 osteocalcin and bone-specific alkaline phosphatase, which suggests that G-CSF stimulates
321 osteoblastic activity. In addition, the effects of G-CSF could also be related to an increase in
322 some cytokines. In an in vitro model, Kaygusuz et al. (2006) showed that G-CSF, while raising
323 the TGF β ₁, has a positive effect on fracture healing and Czekanska et al. (2014) that stimulation
324 of mesenchymal stem cells by G-CSF results in an upregulation of mRNA expression of BMP2,
325 which is involved in bone formation.

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

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

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

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

365

366

367

368

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373

374

375 **Reference**

- 376 An YH, Friedman RJ, Draughn RA. Animal models of fracture or osteotomy. In: An YH,
377 Friedman RJ, CRC press. Animal models in orthopaedic research. 1999. pp 197-217.
- 378 Bozlar M, Aslan B, Kalaci A, Baktiroglu L, Yanat AN, Tasci A. Effects of human granulocyte-
379 colony stimulating factor on fracture healing in rats. Saudi Med J. 2005; 26:1250-4.
- 380 Czekanska EM, Ralphs JR, Alini M, Stoddart MJ. Enhancing inflammatory and chemotactic
381 signals to regulate bone regeneration. Eur Cell Mater. 2014;28:320-334.
- 382 Eghbali-Fatourehchi GZ, Lamsam J, Fraser D, Nagel D, Riggs BL, Khosla S. Circulating
383 osteoblast-lineage cells in humans. N Engl J Med. 2005 ;352: 1959-1966.
- 384 Froberg MK, Garg UC, Stroncek DF, Geis M, McCullough J, Brown DM. Changes in serum
385 osteocalcin and bone-specific alkaline phosphatase are associated with bone pain in donors
386 receiving granulocyte-colony-stimulating factor for peripheral blood stem and progenitor cell
387 collection. Transfusion. 1999;39(4): 410-4.
- 388 Granero-Molto F, Weis JA, Miga MI, Landis B, Myers TJ, O'Rear L, et al. Regenerative effects
389 of transplanted mesenchymal stem cells in fracture healing. Stem Cells. 2009;27: 1887-1898.
- 390 Herrmann M, Zeiter S, Eberli U, Hildebrand M, Camenisch K, Menzel U, et al. Five Days
391 Granulocyte Colony-Stimulating Factor Treatment Increases Bone Formation and Reduces Gap
392 Size of a Rat Segmental Bone Defect: A Pilot Study. Front Bioeng Biotechnol. 2018; Feb 12;6:5.
- 393 Iguchi K, Inoue S, Kumar A. Effect of recombinant human granulocyte colony-stimulating factor
394 administration in normal and experimentally infected newborn rats. Exp Hematol. 1991;19: 352-
395 8.

396 Ishida K, Matsumoto T, Sasaki K, Mifune Y, Tei K, Kubo S, et al. Bone regeneration properties
397 of granulocyte colony-stimulating factor via neovascularization and osteogenesis. *Tissue Eng*
398 *Part A*. 2010;16: 3271-3284.

399 Kaygusuz MA, Turan CC, Aydin NE, Temel I, Firat S, Bulut T, et al. The effects of G-CSF and
400 naproxen sodium on the serum TGF-beta1 level and fracture healing in rat tibias. *Life Sci*.
401 2006;80: 67-73.

402 Kumagai K, Vasanji A, Drazba JA, Butler RS, Muschler GF. Circulating cells with osteogenic
403 potential are physiologically mobilized into the fracture healing site in the parabiotic mice model.
404 *J Orthop Res*. 2008;26: 165-175.

405 Kumar S, Ponnazhagan S. Mobilization of bone marrow mesenchymal stem cells in vivo
406 augments bone healing in a mouse model of segmental bone defect. *Bone*. 2012 ;50: 1012-8.

407 Kuroda R, Matsumoto T, Niikura T, Kawakami Y, Fukui T, Lee SY et al. Local transplantation
408 of granulocyte colony stimulating factor-mobilized CD34+ cells for patients with femoral and
409 tibial nonunion: pilot clinical trial. *Stem Cells Transl Med*. 2014;3(1): 128-34.

410 Kuroda Y, Dezawa M. Mesenchymal stem cells and their subpopulation, pluripotent muscle cells,
411 in basic research and regenerative medicine. *Anat Rec (Hoboken)*. 2014;297: 98-11

412 Kuznetsov SA, Mankani MH, Gronthos S, Bianco P, Robey PG. Circulating skeletal stem cells. *J*
413 *Cell Biol*. 2001;153: 1133-1140.

414 Labat ML, Milhaud G, Pouchelet M, Boireau P. On the track of a human circulating
415 mesenchymal stem cell of neural crest origin. *Biomed Pharmacother*. 2000;54: 146-162.

416 Lambertini M, Del Mastro L, Bellodi A, Pronzato P. The five "Ws" for bone pain due to the
417 administration of granulocyte-colony stimulating factors (G-CSFs). *Crit Rev Oncol Hematol*.
418 2014;89(1): 112-128.

419 Lee DY, Cho TJ, Kim JA, Lee HR, Yoo WJ, Chung CY, et al. Mobilization of endothelial
420 progenitor cells in fracture healing and distraction osteogenesis. *Bone*. 2008;42: 932-941.

421 Levesque JP, Winkler IG, Larsen SR, Rasko JE. 2007. Mobilization of bone marrow-derived
422 progenitors. *Handb Exp Pharmacol*. 2007;180: 3-36.

423 Matsumoto T, Kuroda R, Mifune Y, Kawamoto A, Shoji T, Miwa M, et al. Circulating
424 endothelial/skeletal progenitor cells for bone regeneration and healing. *Bone*. 2008;43: 434-9.

425 Minamino K, Adachi Y, Okigaki M, Ito H, Togawa Y, Fujita K, et al. Macrophage colony-
426 stimulating factor (M-CSF), as well as granulocyte colony-stimulating factor (G-CSF),
427 accelerates neovascularization. *Stem Cells*. 2005;23: 347-354.

428 Otsuru S, Tamai K, Yamazaki T, Yoshikawa H, Kaneda Y. Bone marrow-derived osteoblast
429 progenitor cells in circulating blood contribute to ectopic bone formation in mice. *Biochem*
430 *Biophys Res Commun*. 2007;354: 453-48.

431 Otsuru S, Tamai K, Yamazaki T, Yoshikawa H, Kaneda Y. Circulating bone marrow-derived
432 osteoblast progenitor cells are recruited to the bone-forming site by the CXCR4/stromal cell-
433 derived factor-1 pathway. *Stem Cells*. 2008;26: 223-234.

434 Pignolo RJ, Kassem M. Circulating osteogenic cells: implications for injury, repair, and
435 regeneration. *J Bone Miner Res*. 2011;26: 1685-1693.

436 Pulsipher MA, Chitphakdithai P, Logan BR, Navarro WH, Levine JE, Miller JP et al. Lower risk
437 for serious adverse events and no increased risk for cancer after PBSC vs BM donation. *Blood*.
438 2014;123(23): 3655-63.

439 Sasaki K, Kuroda R, Ishida K, Kubo S, Matsumoto T, Mifune Y, et al. Enhancement of tendon-
440 bone osteointegration of anterior cruciate ligament graft using granulocyte colony-stimulating
441 factor. *Am J Sports Med*. 2008;36: 1519-1527.

442 Sidney LE, Branch MJ, Dunphy SE, Dua HS, Hopkinson A. Concise review: evidence for CD34
443 as a common marker for diverse progenitors. *Stem Cells*. 2014;32(6):1380-9.

444 Tatsumi K, Otani H, Sato D, Enoki C, Iwasaka T, Imamura H, et al. Granulocyte-colony
445 stimulating factor increases donor mesenchymal stem cells in bone marrow and their
446 mobilization into peripheral circulation but does not repair dystrophic heart after bone marrow
447 transplantation. *Circ J*. 2008;72: 1351-8.

448 Tighe CC, McKoy JM, Evens AM, Trifilio SM, Tallman MS, Bennett CL. Granulocyte-colony
449 stimulating factor administration to healthy individuals and persons with chronic neutropenia or
450 cancer: an overview of safety considerations from the Research on Adverse Drug Events and
451 Reports project. *Bone Marrow Transplant*. 2007; 40: 185-192.

452 Toupadakis CA, Granick JL, Sagy M, Wong A, Ghassemi E, Chung DJ, et al. Mobilization of
453 endogenous stem cell populations enhances fracture healing in a murine femoral fracture model.
454 *Cytotherapy*. 2013;15: 1136-1147.

455 Toupadakis CA, Wong A, Genetos DC, Chung DJ, Muruges D, Anderson MJ, et al. Long-term
456 administration of AMD3100, an antagonist of SDF-1/CXCR4 signaling, alters fracture repair. *J*
457 *Orthop Res*. 2012;30: 1853-9.

458 Turner CH, Burr DB. Basic biomechanical measurements of bone: a tutorial. *Bone*. 1993;14:
459 595-608.

460 Utvåg SE, Grundnes O, Reikerås O, Graded exchange reaming and nailing of non-unions.
461 Strength and mineralisation in rat femoral bone, *Arch Orthop Trauma Surg*. 1998;118: 1-6.

462 Utvåg SE, Korsnes L, Rindal DB, Reikerås O. Influence of flexible nailing in the later phase of
463 fracture healing: strength and mineralization in rat femora, *J Orthop Sci*. 2001;6: 576-584.

464 Utvåg SE, Reikerås O. Effects of nail rigidity on fracture healing. Strength and mineralisation in
465 rat femoral bone, *Arch Orthop Trauma Surg*. 1998;118: 7-13.

466 Wan C, He Q, Li G. Allogenic peripheral blood derived mesenchymal stem cells (MSCs)
467 enhance bone regeneration in rabbit ulna critical-sized bone defect model. *J Orthop Res.* 2006;24:
468 610-8.

469 Wu YD, Chien CH, Chao YJ, Hamrick MW, Hill WD, Yu JC, et al. Granulocyte colony-
470 stimulating factor administration alters femoral biomechanical properties in C57BL/6 mice. *J*
471 *Biomed Mater Res A.* 2008;87: 972-9.

472 Wu X, Yang S, Duan D, Liu X, Zhang Y, Wang J, et al. A combination of granulocyte colony-
473 stimulating factor and stem cell factor ameliorates steroid-associated osteonecrosis in rabbits. *J*
474 *Rheumatol.* 2008;35: 2241-8.

475 Zhdanov VV, Stavrova LA, Dygai AM, Goldberg ED. Mechanism of mobilization of
476 mesenchymal stem cell under the effect of granulocyte colony-stimulating factor. *Bull Exp Biol*
477 *Med.* 2007;144: 151-3.

478

Figure legends

479

480

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

483

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

490

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

498

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

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