Effects of human granulocyte-colony stimulating factor on fracture healing in rats

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ABSTRACT

Objective: Granulocyte colony stimulation factor (G-CSF) is generally used to prevent and cure the neutropenia associated with chemotherapy and bone marrow transplantation. In addition to its effects on neutrophil function, G-CSF was found to have the characteristic of modulating the cytokines in the inflammatory response. Then, the question to answer is whether it has any effect on fracture healing and to what extent? In this study, we test the effects of G-CSF on the healing of tibia fracture in a rat model.

Methods: This study was performed at Harran University, Sanliurfa, Turkey between July 2003 and August 2004. Twenty female, healthy Sprague-Dawley rats, weighing between 250 and 300 gm were divided into 2 groups, and their tibiae broken. The rats in the G-CSF group were injected subcutaneous with 25µg/kg/day of recombinant human G-CSF for 7 days, and the ones in the control group with 0.9% sodium chloride. Rats were sacrificed 3 weeks after surgery and then radiological, histological and biomechanical evaluations were performed. Biomechanical tests were performed at the Middle East Technical University, Ankara, Turkey.

Results: The median radiographic scores for the control group were calculated as 4.1, and 6.1 for the G-CSF group (p = 0.016). Cortex remodeling, callus formation, bone union and marrow changes values did not differ significantly ($p > 0.05$). Mechanical parameter (mean max-Load) values for the control group were found to be 24.0 ± 3.0 N, and 241.5 ± 75.7 N for the G-CSF group (p = 0.001).

Conclusions: We found that G-CSF has an important effect on fracture healing. However, this effect requires further study.

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Fracture repair is a result of a cascade of cellular events that leads to bony union of the broken fragments. After a fracture, the space between the ends of broken bones is filled with hematoma rich in pluripotential mesenchymal cells and cytokines. The tissue that forms within this space usually goes through a cascade of events that include cartilage formation, ossification, and remodeling phases. The roles of growth factors,[1,2] ecosanoids,[3] and protooncogenes[4] in fracture healing have been studied. Despite these efforts, the regulatory mechanisms in fracture healing are not clearly understood. Granulocyte colony stimulation factor (G-CSF) is generally used to prevent and cure the neutropenia associated with chemotherapy and bone marrow transplantation.[5-11] In addition to its effects on neutrophil function, G-CSF was found to have the characteristic of modulating the cytokines in the inflammatory response.[5,12,13] Then, the question to answer is whether it has any effect on the fracture healing and to what extent? It is thought that G-CSF is used to shorten the frequency and duration of

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febrile neutropenia in the cases treated with chemotherapy, as malignant diseases may enhance the fracture healing.

Methods. ***Animal and experimental designs.*** The study was performed at Harran University between July 2003 and August 2004. Twenty female, healthy Sprague-Dawley rats, weighing between 250 and 300 gm, were used. The study protocol was reviewed and accepted by the institutional animal care committee. The right limbs were shaved and cleaned by Betadine solution. The right tibia of each rat was exposed via an antero-medial skin incision under ketamine hydrochloride anesthesia. Following the skin incision the midshaft of the tibia was reached by blunt dissection. The shaft was then osteotomized transversally by a Gigli saw taking care to cause minimal soft tissue injury at the fracture site. An additional longitudinal parapatellar incision was made, and a Kirschner wire was introduced to the intramedullary canal of the tibia for fixation. The largest size (0.8 mm) of Kirschner wires was used to fit the distal intramedullary space of the rat tibiae. Following hemostasis, the layers were closed with interrupted sutures. Per operative antimicrobial prophylaxis consisting of 50 mg/kg/day Cefazolin-sodium was administered. The animals were randomly divided into 2 groups: control and G-CSF. The rats in the G-CSF group were injected subcutaneous with 25 µg/kg/day of recombinant human G-CSF for 7 days, and the ones in the control group with 0.9% sodium chloride. The rats were allowed unrestricted weight bearing after recovery from the anesthesia. These animals were kept in individual cages and allowed free access to tap water and a standard pellet diet. The cages were housed in a temperature of 24°C, humid air (55%) and 12 hours of day/night light controlled room. Three animals in the control group and 2 animals in the G-CSF group died of unknown reasons during follow-up, and a surgical wound infection did not develop. The remaining 7 control and 8 G-CSF group rats were sacrificed 3 weeks after surgery. The fractured tibiae and fibulae were removed by careful dissection, followed by resection of the fibulae and the intramedullary Kirschner wires were pulled out by applying small torsional movements.

Radiographs. Radiographs were performed in 2 planes after the animals were killed. All radiographs were randomized and independently scored by 2 orthopedic surgeons, who were unaware of the treatment that the animal had received. Common radiological parameters include the following categories: Periosteal reaction, bone union, and remodeling, which can be semi-quantitated based on a scoring system (**Table 1**).[14] Median radiographic scores were calculated for each group.

Bone morphometry. Seven tibiae (3 from the control group, 4 from the G-CSF group) were prepared for histological examination. The specimens were fixed in 10% neutral buffered formaldehyde for 2 days and than decalcified with 1.5% aqueous hydrochloric acid for 3 days. The tibiae and fibulae were separated and embedded in paraffin that allows obtaining 5 micron sections from each block and stained with hematoxylin-eosin. The degree of fracture healing was determined by light microscopic examination according to a histological scoring system. Common histological parameters include the following categories: callus formation, bone union, marrow changes, and cortex remodeling, which can be semi-quantitated based on a scoring system (**Table 1**).[14] The grading was carried out blindly without knowledge of which treatment had been given. Median fracture healing scores were calculated for each group.

Mechanical testing. Eight tibiae (4 from each group) were prepared for the mechanical test. The control and G-CSF groups were numbered so as to keep the biomechanical measurements blind. The tibias were kept frozen at –20°C for further analysis. Prior to the tests, the tibias were placed in humid medium and kept there for 4 hours until they thawed to room temperature. The distal and proximal parts of the tibiae were cut to obtain a better adjustment to the 3-point bending fixture. The tibiae were

Table 1 - Radiographic and histological scoring systems for fracture healing.

Radiographic scoring system		Histological scoring system	
Category	**Score**	**Category**	**Score**
Periosteal reaction		***Callus formation***	
Full (across the defect)	3	Full (across the defect)	3
Moderate	2	Moderate	2
Mild	1	Mild	1
None	0	None	0
Bone union		***Bone union***	
Union	3	Full bone bridge (union)	3
Moderate bridge (>50%)	2	Moderate bridge (>50%)	2
Mild bridge (<50%)	1	Mild bridge (<50%)	1
Non-union	0	No new bone in the fracture line (non-union)	0
Remodeling		***Marrow changes***	
Full remodeling cortex	3	Adult type fatty marrow	4
Intramedullary canal	1	2/3 replaced by new tissue	3
No remodeling	0	1/3 replaced by new tissue	2
Maximum total score	**8**	Fibrous tissue	1
		Red	0
		Cortex remodeling	
		Full remodeling cortex	2
		Intramedullary canal	1
		No remodeling	0
		Maximum total score	**12**

Table 2 - Result of mean radiological evaluation score and mechanical parameters.

Groups	Experimental group	Control group	Significance level
Radiological evaluation score (XP-Total) Mean ± SD	6.1 ± 1.2	4.1 ± 1.5	p = 0.0.16*
Mechanical parameter (Max-Load) Mean (N) ± SD	241.5 ± 75.7	24 ± 3	p = 0.001*
*Non-parametric Mann-Whitney U-test			

Table 3 - Result of histological evaluation according to histological scoring system for fracture healing in experimental group.

Histological evaluation of fracture healing categories	Control group (n = 3) X±SD	Experimental group (n = 4) X±SD	*P*-value*
Callus formation	2.75 ± 0.5	2.65 ± 0.51	>0.05
Bone union	1.25 ± 0.5	1.87 ± 0.64	0.05
Marrow changes	3 ± 0.81	3 ± 0.75	>0.05
Cortex remodeling	0.25 ± 0.5	0.25 ± 0.46	>0.05
*Non-parametric Mann-Whitney U-test			

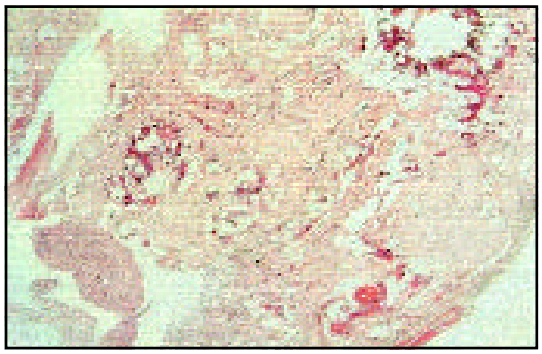

Figure 1 - Histological incomplete union in a rat from the control group at the 21st day (Hematoxylin & Eosin x 25).

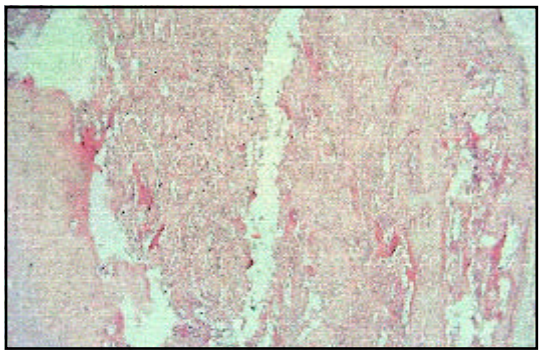

Figure 2 - Histological incomplete union in a rat from the G-CSF group at the 21st day (Hematoxylin & Eosin x 25).

placed on the 3-point bending configuration on the Lloyd LS500 material testing machine (Southampton, UK). The 500-N load cell was used for load detection, and the sampling rate was 4.0 Hz. The loading speed was 2 mm/min, and the load was applied at the mid-span in the anteroposterior direction, with a span length of 40 mm. All the bones were kept in a humid medium during the tests. The load-deflection curves were stored in the computer to be processed later to obtain fracture load values.

All values were analyzed using a Sigmastat program (SPSS-10, SPSS Inc., Chicago, IL, USA.). The differences between experimental and control group were compared by using Mann-Whitney U-test and the level of significance was set at $p<0.05$.

Results.

Radiographs. Median radiographic scores for the control group were calculated as 4.1, and 6.1 for the G-CSF group. There was a statistically significant difference between the radiographic scores of the control and G-CSF groups ($p=0.016$) (**Table 2**).

Bone morphometry. Cortex remodelling, callus formation, bone union and marrow changes values did not differ significantly ($p>0.05$) (**Table 3**).

Histological incomplete union in a rat from the control group and the G-CSF group at the 21st day are shown in **Figures 1 & 2**.

Mechanical tests. Our results showed that the application of G-CSF resulted in an increase in the fracture load values of the fractured bone over those of the control group (**Table 2**). There was a statistically significant difference between the corresponding fracture load values of the control and G-CSF groups ($p=0.001$).

Discussion.

Osteoblastic bone formation and osteoclastic bone resorption dictate the delicate balance that leads to a homeostatically appropriate bone volume in the fracture healing. Two fundamental mechanisms have been suggested for the regulation of this coupling (namely, bone formation and resorption): Systemic regulation by hormones (for example, calcitonin, parathyroid hormone) and regulation by local bone growth factors.[15] Bone matrix contains numerous growth factors, including bone morphogenetic proteins, transforming growth factor-β, insulin-like growth factors I and II, platelet-derived growth factors and acidic and basic fibroblast growth factor. Various osteoblastic culture models, as well as in vitro experimental and clinical models, have revealed that these growth factors influence cellular proliferation, differentiation, chemotaxis, and protein synthesis.[1,2,15-21] Chemical mediators orchestrate acute inflammation in fracture healing, similar to all tissue healing processes that result from injury. The process is complex and

redundant, with several protein factors inducing and inhibiting cell differentiation and new bone growth. Nearly all of the known mediators are beckoned from multiple locations and have multiple functions.[15] We believe that several factors, other than those mentioned, are involved in fracture healing, and we planned this study to investigate the effect of G-CSF in the fracture healing of rats.

Granulocyte-colony stimulating factor is a human glycoprotein hormone, which acts primarily to stimulate proliferation, differentiation and activation of committed progenitor cells of the neutrophil granulocyte lineage into functionally mature neutrophils.[5,12,13,19,22-26] These cells are also further stimulated by G-CSF to show enhanced activity at infection and inflammation.[6-13] Granulation tissue is revealed at the inflammatory phase of fracture healing, and this process provides the modulation of cells flowing to the capillary lumen, necessary for healing. New cell revelation starts by activation of precursor cells. Some of these new cells organize and differentiate to vessels, connective tissues, fibroblasts, chondroblasts, osteoclasts and osteoblasts. The G-CSF also affects neutrophil differentiation, growing and stimulation to all differentiated precursor cells to increase functional capacity.[9,25,27,28] The G-CSF is the mainstay of treatment, and evidence exists that G-CSF may promote angiogenesis. Increased angiogenesis may result from a direct effect of G-CSF on endothelial cells or may be an indirect effect from increased neutrophils.[11] Obviously differences were found in the biomechanical and radiological study of fracture healing between the G-CSF and control group.

In conclusion, we found that, G-CSF affects the fracture healing positively by increasing hematoma and angiogenesis at the inflammatory phase and increases bone mass at the reparation phase of fracture healing. The administration of 25 µ/kg/day G-CSF given subcutaneously accelerated fracture healing in a healthy animal model by: Accelerating the proliferation and differentiation of osteo-progenitor cells in the callus. Stimulation of angiogenesis in fracture healing, resulting in advancing the blood supply at the fracture site. Advancing the hematoma at the fracture site. Stimulating the organization of trabecular bone, and activating mineralization of the matrix. We found that G-CSF has an important effect on fracture healing, however, the effect of G-CSF on fracture healing needs further study.

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