

## SHORT COMMUNICATION

# Effect of Systemic Administration of Granulocyte-Colony Stimulating Factor on Rate of Fracture Healing of Bone Defect in Goats as Animal Model

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## ABSTRACT

Granulocyte-colony stimulating factor (G-CSF) serves as an important cytokine in haematopoiesis; released at both physiological and pathological conditions by a range of cells. We hypothesized that the systemic administration of G-CSF would produce an accelerated fracture-healing rate in non-union bone defects; thus, potentially leading to useful clinical applications. Ten male adult Katjang goats, weighing about 15-26 kilograms were randomly chosen and a tibial bone defect was induced in each animal. The defect was maintained by internal fixation with a titanium plate and reinforced by an external fiberglass cast. Post-operative radiographs were performed twice weekly and radiographic assessments were performed by evaluating the bridging and union measurements through a validated method. In the treatment group, the time for bridging and union exhibited statistically significant differences when compared with a control group. The outcomes of the present study establishing a notion that administration of G-CSF besides inducing haematopoiesis, promotes healing of fractures and non-union bone defects as well.

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## INTRODUCTION

The biology of bone fracture healing has been well described and involves a cascade of cellular and biochemical events which eventually lead to the complete restoration of the structure and function of the bone (1). The restorative ability of bone demonstrates its amazing regenerative potential in comparison to other tissues and organs in the body. This unique characteristic however, is a time consuming process and occasionally, impaired healing occurs in 5-10% of fractures, resulting in delayed or non-union (2). Bone is known to heal by either direct or indirect methods. Direct bone healing occurs when anatomical reduction of fracture fragments and rigid stabilization are achieved, whereas indirect bone healing occurs in conditions when these conditions are not fulfilled (1). More advanced studies, however, are refining our understanding on the precise

nature and specific components of these processes. Attention to the specific components involved in these physiological processes has come to the fore in recent times as researchers aim to optimize these processes to accelerate the rate of fracture healing. Key research has recently been focused on cell-based therapies and tissue engineering modalities to enhance bone fracture healing. It has been well described that mesenchymal stem cells are key cellular components involved in bone fracture healing (1). Focus has therefore been made with particular emphasis on CD34+ cells, which play significant roles in bone fracture healing. This cohort of cells contains osteoblast precursor cells (OPCs), endothelial progenitor cells (EPCs) and hematopoietic stem cells (HSCs), which orchestrate key functions in fracture healing. These cells were identified in both the bone marrow and in the peripheral blood albeit in low concentrations (3–8).

Higher concentrations of the above-mentioned cells are required to enhance the process of fracture healing. Mobilization of these cells from the bone marrow to peripheral blood is usually triggered by tissue ischemia

and cytokines such as granulocyte-colony stimulating factor in normal circumstances (4). The primary function of G-CSF is to escalate the number of peripheral blood neutrophils via inducing the expansion and maturation of respective progenitor cells in bone marrow (9). In addition, it also increases donor-derived mesenchymal stromal cell proliferation in the bone marrow and peripheral blood (9). These features have vital roles in the supply and delivery of cells needed for fracture healing.

The use of bone defect in large animal models has been reviewed. Critical size defects (CSD) has been defined as a bone defect of larger than 1.5 to 2 times of the diameter of the diaphysis of the long bone in which failure of spontaneous healing of critical bone defects may lead to non-union (10,11). With key emphasis given to the role of G-CSF in fracture healing, various studies have been done to assess the advantages that it may offer in fracture healing, especially in the area of fracture that may lead to a non-union (4,5). Therefore, a novel strategy to provoke bone healing using G-CSF using goat as animal model induced with bone defect has been envisioned. In this study using a Caprine model, a 1.2cm tibial non-critical bone defect was induced and maintained by internal fixation with a locking titanium plate, followed by external fiberglass cast. We aimed to determine if systemic administration of G-CSF would produce an accelerated fracture-healing rate in bone defects in goats. These results could be extrapolated for the use in human medicine and in other veterinary patients.

## MATERIALS AND METHODS

### Ethics

The study protocol was approved by the Institutional Animal Care and Use Committee (UPM/IACUC/AUP-R027/2016), Universiti Putra Malaysia. The domestic goats were purchased from a local farmer and kept in clean individual pens with proper fenestrated flooring, adequate ventilation and fed freshly cut grass and pellets twice daily. Clean water was available in mechanical water dispensers at all times.

### Groups

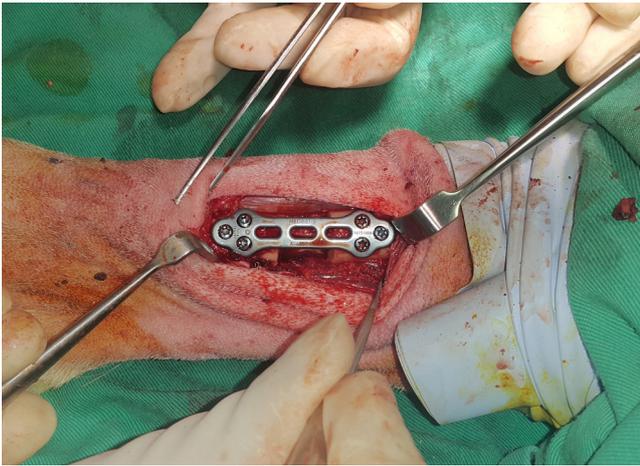
Ten, 12-14 months old intact male Katjang goats (*Capra aegagrus*), weighing from 15-26 kilograms, underwent surgery to create on each animal a non-critical bone defect of 1.2cm of the right tibia. In this experiment, two groups were studied: Group A (Control group, n=5) was not given any additional treatment post-operatively; while group B (G-CSF group, n=5) was given a dose of G-CSF each (5 mcg/kg/day, subcutaneous injection) on post-operative days 1, 2 and 3.

### Surgical procedure

For the orthopaedic surgical procedure, the goat was induced with a combination of ketamine hydrochloride

(2.2mg/kg, Ilium Ketamil, Troy Laboratories, Australia) and diazepam (0.05mg/kg, Diapine Injection, Atlantic Laboratories, Thailand) intravenously. Anaesthesia was maintained with 3-5% isoflurane via endotracheal tube with positive pressure ventilation of 100% oxygen. Normal saline at maintenance rate was administered intravenously throughout the procedure. For intra-operative anaesthesia, the goat received an epidural using lidocaine hydrochloride (0.2 mg/kg, Xylocaine 2%, Astra Zeneca).

The goat was positioned in right lateral recumbency with the left hind limb flexed and adducted. The medial and lateral sides of the right hind limb, up to the level of the caudal ventral abdomen were clipped and surgically prepared. An Esmarch bandage was applied from the fetlock to the stifle region to exsanguinate blood from the operating field. A 4-5cm longitudinal skin incision was made along the anterior-medial aspect of the right tibia. Soft tissue was dissected in layers down to the tibial bone to expose the periosteum. The periosteum layer enveloping the bone was carefully incised longitudinally with #15 size scalpel blade and elevated off to expose the underlying bone. A 2.5mm metatarsal locking plate with 6 holes 2.8mm Medartis APTUS locking plate (Medartis®, Basel, Switzerland) was placed and adjusted on the exposed tibial bone, to follow the contour of the bone. The proximal and distal screw holes as well as the intended bony non-critical defect of 1.2cm between the proximal and distal holes of the plate (Fig. 1) were marked using a surgical marker (Secureline®, Aspen Surgical). The plate was then temporarily locked into position by placement of three proximal and three distal 2.5 mm locking screws. These screws however, were not locked at this stage as they were to be removed, except the screw at the index hole at the proximal part of the plate. The plate was then swiveled proximally; to allow creation of the bone defect using an electrical bone saw. Proximal and distal near complete cuts 1.2cm apart, were induced on the tibia under saline rinse. The locking plate was repositioned on the previously drilled holes and the screws were then used to secure the plate to the bone. After all the screws were locked, a 3mm osteotome was used to wedge out the near complete fractured tibia by gentle tapping. Once the intended fractured bone was removed from the central space beneath the plate, the fractured bone gap was rinsed with saline. The periosteal layer was sutured using 3/0 or 4/0 Vicryl (Ethicon, Somerville, NJ), simple interrupted pattern, to cover the bone and bone gap. The subcuticular wound was then sutured using 3/0 Vicryl routinely, while skin closure was done using Dafilon 3-0 sutures (Ethicon, Somerville, NJ). A layer of Opsite® (Smith & Nephew Inc., Massachusetts) wound dressing was applied on the surgical wound. The whole right hind leg was bandaged with multiple layers of soft cotton roll before the Esmarch tourniquet was released and then removed. Following removal of the tourniquet, the whole right hind leg was placed in a Nemoa™



**Figure 1: A tibial bone defect was maintained using a 2.5mm metatarsal locking plate with 6 holes (Medartis®) that was placed and adjusted on the exposed tibial bone.** The plate was temporarily locked into position by placement of three proximal and three distal 2.5 mm locking screws. The plate was then swiveled proximally; to allow creation of the 1.2cm bone defect using an electrical bone saw. The locking plate was repositioned on the previously drilled holes and the screws were then used to secure the plate to the bone and a 3mm osteotome was used to wedge out the near complete fractured tibia by gentle tapping.

Cast (T&L Co. Ltd, Korea). The cast was applied from the distal tip of the hoof to beyond the proximal part of the stifle. The hock was slightly flexed to prevent the cast from slipping down. Once the fiberglass cast had hardened, the goat was recovered from anaesthesia.

#### Post-operative care

The goat was maintained with analgesia and anti-inflammatory of tramadol hydrochloride (5mg/kg, SID/BID, for 3 days, subcutaneously, Analab Injection), Flunixin Meglumine (2.2mg/kg, SID, for 3 days, intramuscularly, Flumine) and antibiotic of penicillin streptomycin (1ml per 25kg, for 5 days, intramuscularly, Pen & Strep).

Additional treatment protocol was added for group B (G-CSF group, n=5), in which doses of G-CSF or Filgrastim (NEUPOGEN®, Amgen®) of 5 mcg/kg/day on post-operative days 1, 2 and 3 was administered subcutaneously.

#### Assessment of fracture healing

Radiological assessments were performed twice weekly until evidence of bone union was observed. Anterior posterior (AP) and lateral (L) views of the fractured tibia were taken. Radiographic criteria interpreted by Morshed (12) and adapted for this study as described: i) bridging: when there was soft callus seen bridging 2 cortices on both views (AP and L), ii) union: when the medulla was filled with soft callus bridging the fracture ends. Two orthopaedic surgeons viewed the radiographs and by consensus allocated scores between 1 to 3 depending

on the visibility of a fracture line and morphology of the healing bone on radiographs. Radiographs with a visible fracture line and absence of callus were given a score of 1. Radiographs with a visible fracture line but with soft callus trabeculae were allocated a score of 2. A score of 3 was allocated to radiographs with an absence of any fracture lines accompanied by well-healed cortical trabeculae.

#### Analysis of results

In view of the non-parametric distribution of the data, the median time (in weeks) to bridging and the median time (in weeks) to union were used instead of the mean time for both these parameters. The data collected were analyzed using a non-parametric test (Mann-Whitney U test) using a statistical software (Statistical Package for Social Sciences, SPSS® version 22.0). Significant differences were observed when p-values were less than 0.05.

#### Termination of subject

For the control group, sacrifice of the animals was performed at 16 weeks, while for the test group sacrifice was at 8 weeks post-surgery. For the treatment group, accelerated fracture-healing rate in bone defects were seen in the weekly radiographic series, hence the sacrifice time was decided to be performed earlier than the control group, once the bone has shown complete union. Upon sacrifice, followed by dissection, the plates and screws were removed. The healed tibiae were sectioned in half in the mid-sagittal plane using an electric bone saw. The bone sections were harvested and immersed in phosphate buffered formalin, subjected to decalcification (10% formic acid) and then processed for paraffin embedding.

### RESULTS AND DISCUSSION

#### Radiographic Union in tibial bone defect

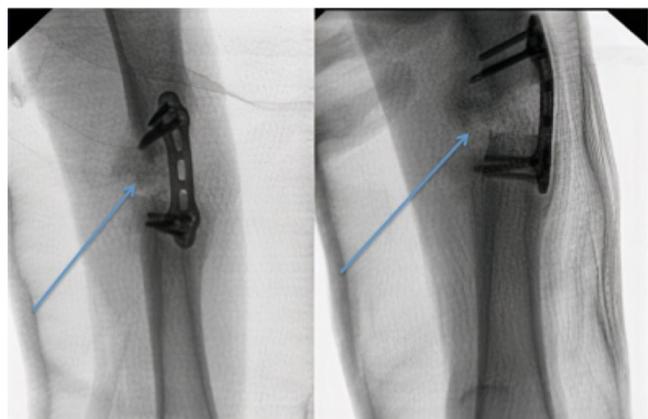
In the control group, the fixed fractured tibiae were subject to weekly radiographic assessment for 13 weeks. Soft callus formation started at 7–9 days post induction of tibial bone defect. Soft callus was observed to develop from the distal fractured end towards the proximal end of the lateral cortex, for which the median time to bridging was 5.5 weeks (interquartile range of 1.25 weeks). Following bridging, callus progressively formed in the medullary canal filling up the bone gap and complete union was observed at 12 weeks (interquartile range of 2.5 weeks) post-operatively. In the test group (G-CSF group), significant improvements were noted. The median time to bridging was 3 weeks (interquartile range of 0.25 weeks) while the union time was 6.5 weeks (interquartile range of 4.5 weeks) (Table I).

The Mann-Whitney U test demonstrated a significant difference between the control group and G-CSF group in relation to “bridging” (p-value of 0.008) and “union” (p-value of 0.016) times. These findings support the

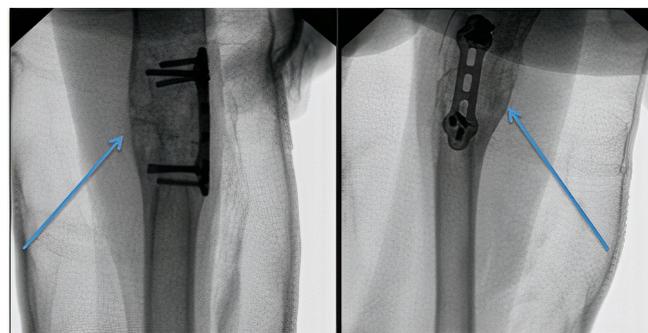
**Table I: Median radiological bridging and union time in tibial bone defect in goats for the control and treated with Granulocyte-Colony Stimulating Factor groups.**

Group	Median time to bridging (Weeks)	Median time to union (Weeks)
Control	5.50 (IQR 1.25)	12.0 (IQR 2.50)
G-CSF	3.00 (IQR 0.25)	6.50 (IQR 4.50)

notion that when G-CSF is administered systemically, the fracture-healing process in bone defects is enhanced. The selected radiographic images to demonstrate the progression of the soft callus in bone healing of the goat tibia studied are presented in Fig. 2 and 3.



**Figure 2: Radiographic images demonstrating bridging at the fracture gap with soft callus (indicated with arrows) at 6 weeks post-surgery in a goat tibia from the control group**



**Figure 3: Radiographic images demonstrating union at the fracture gap with soft callus (indicated with arrows) at 7 weeks post-surgery in a goat tibia from the G-CSF group**

The results of this study indicated that when a bone defect of 1.2cm was created, the median time to bridging was 5.5 weeks in the control group and 3 weeks in the G-CSF treated group (Table II). In addition, the median time to union was 12 weeks in the control group as compared to 6.5 weeks in the test group, thus supporting the hypothesis that systemic administration of G-CSF hastened fracture healing in bone defects.

The findings of this study agree with another study that explored the potential used of low doses of G-CSF (5 days subcutaneous injection of 5 µg/kg/day), on bone regeneration in rat model of distraction osteogenesis (13).

**Table II: Summary of time periods for bridging and union in tibial bone defect in goats for the control and treated with Granulocyte-Colony Stimulating Factor groups.**

Goat	Group	Bridging time (Weeks)	Union time (Weeks)
1	Control Group	6.0	10.0
2	Control Group	5.0	13.0
3	Control Group	6.0	9.0
4	Control Group	6.5	11.5
5	Control Group	5.5	13.0
6	G-CSF Group	3.0	8.0
7	G-CSF Group	3.0	11.0
8	G-CSF Group	3.0	6.5
9	G-CSF Group	3.0	5.5
10	G-CSF Group	2.5	4.5

Contrary to other studies in rats with induced segmental bone defect model (14) and delayed bone union model (15), a dose of 50 µg/kg/day was given up to 5 days to achieve accelerated bone healing in treatment group when compared to the control group. Another study in rats was using a dose of 25 µg/kg/day, up to 7 days post-operatively to achieve similar results (16).

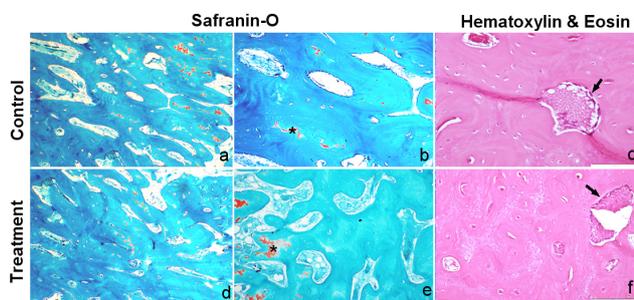
In comparison to our study, the G-CSF (at 5 µg/kg/day) was given up to 3 days only, as our decision was supported by a pilot study in Katjang goats in which results administration of G-CSF daily for up to three days showed a consistent elevation of white blood cell counts over a period of 2-days which would provide a window of access to harvest peripheral blood stem cells for various clinical uses (17). This observation is also supported by another study in normal rats, where neutrophil levels increased during the first day of treatment and returned to normal values approximately 2 days (48 hours) after the last treatment, and then the neutrophil counts does not peak any further (18). Even though the duration of the use of G-CSF was reduced in this study, we still achieve acceleration of bone healing when compared to the control group.

It is worth to note that in another study in rats, G-CSF was administered 5 days pre-operatively and was compared with the result of G-CSF given 5 days post-operatively to accelerate femur fracture. For that study the radiological, mechanical, and histological results were reported to be similar (19).

In this study, the decision to terminate the subjects was based on radiological scoring evaluation. Once the hard callus formation had progressed and the calcified cartilage was replaced with woven bone, the goats were sacrificed, and the fractured site was harvested for histological observation. For the control group, the animals were sacrificed at 16 weeks based on complete union of bone on radiographic assessment. For the

treatment group with G-CSF, accelerated fracture-healing rate in bone defects were seen in the weekly radiographic series, hence the sacrifice time was decided to be performed earlier at 8 weeks, earlier than the control group.

G-CSF promotes histological union in tibial bone defect. In all the goats, the healed tibiae showed a well-developed layer of compact bone, normal trabecular bone and bone marrow with no signs suggestive of pathological processes such as inflammation or infection in both groups. On Safranin-O staining, cartilage islands surrounding the remodeling bone were seen and on H & E staining, the osteoblasts appeared flattened and inactive in all bone samples, indication that the bone has healed (Fig. 4). In the control group woven bone was replaced by compact bone by 16 weeks, while in the test group this was achieved by 8 weeks.



**Figure 4: Histology assessment of bone samples control (4a, b and c) and treated group with GCSF (4d, e and f).** 4a, b, d and e: showed healed tibial bone by the presence of the cartilage island surrounding the remodeling bone on Safranin-O staining (40x and 100x, asterisk). 4c and f: On Hematoxylin & Eosin staining of all the bone section, the osteoblasts appeared inactive (black arrow) (200x), indication that the bone has healed. Histological findings of the goat tibial sample supported that the bone defect for the treated group with G-CSF had healed well at 8 weeks, while in the control group the bone defect took 16 weeks to completely heal.

### Caprine as animal model for orthopaedic study

The preferred large animal for pre-clinical testing prior to human trials has commonly involved the adult Ovine model because it offers the advantage of possessing a similar body weight to an adult human. The Caprine model, however possesses long bones of suitable lengths and diameters for testing implants and prostheses for orthopaedic use (20,21). The tibia was chosen because it is a weight bearing long bone of suitable dimensions for orthopaedic studies (20). In orthopaedic research models, bone defects are induced to assist the development of favourable simulation protocols. In order for us to compare the healing rates, a non-critical defect was induced to create a large bone defect that would not be beyond potential bone regeneration (21). Low plate profile fixation was made possible in this experiment as the chosen site of osteotomy was a flat surface on the medial side, at the junction of the middle and distal third of the tibia. A low-profile titanium plate

was preferred to enable skin and periosteal coverage over the surgical site. A minimum purchase of six cortices was deemed stable and a locking mechanism would provide added stability. Thus, the 2.5mm metatarsal locking plate with 6 holes 2.8mm Medartis APTUS locking plate (Medartis®, Basel, Switzerland) was chosen.

An interesting point to note is that the callus started forming at the lateral cortex (the cortex that is away from the plate) from the distal fracture fragment to the proximal. We feel this may be due to the stress riser under the plate preventing bone formation, as well as the plate holding the fracture ends apart. This could be further elaborated; when compression was applied by internal fixation on the medial cortex, the gap in the lateral cortex was approximated by callus formation that was induced by strain (22). The amount of strain within and around the fracture gap adjusted the amount of callus produced and the presence of fibroblast and chemotactic factors drew the ends closer (23).

To further enhance stabilization of the internal fixator of choice, fiberglass casting of the whole right hind leg (hoof to stifle) was applied prior to recovery from anaesthesia. Reinforcement of the fiberglass cast proximal to the stifle with plaster of Paris and flexion of the hock ensured the cast would not slip down. Rolling and smoothing the proximal edges of the cast prevented chaffing of the skin.

In this study, only G-CSF is being tested to promote fracture healing. Among factors that can impair bone healing include inappropriate mechanical stabilization, infection, impaired blood supply, advanced age, comorbid diseases, hormone and nutrition status, pharmacological therapy and genetic variations (24). For our study, all of these factors are made constant, and we can assure that these factors did not affect the fracture healing.

Based on the successful outcome of this study using goats as animal models, the treatment strategy could also be used in ruminant practice to accelerate bone healing in clinical setting. As long as the bone are aligned and immobilized, even with a gap up to 1.2cm, without any other complications, the bone will still able to form callus and heal. It would be ideal to prolong the duration of the study up to 6-12 months to allow further assessment on the consolidation phase of the healing bone. However, financial constraint and availability of man power would become a limitation.

### CONCLUSION

In this study, we have proved that the bone healing in goats could be significantly accelerated with the use of GCSF subcutaneously. The encouraging results from this study using goat as an animal model, could add to the treatment armamentarium in orthopaedics,

both in medical and veterinary fields. In orthopedics, its potential use may be in scenarios requiring expedited healing, for example high-performance athletes or in instances where union is delayed.

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