

# A Combination of Granulocyte Colony-Stimulating Factor and Stem Cell Factor Ameliorates Steroid-Associated Osteonecrosis in Rabbits

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**ABSTRACT. Objective.** Bone marrow-derived stem cells (BMSC) have been highlighted for the treatment of osteonecrosis (ON) before collapse of the femoral head. In our study, the potential of granulocyte colony-stimulating factor/stem cell factor (G-CSF/SCF)-mobilized BMSC to repair steroid-associated ON was assessed in rabbits.

**Methods.** ON was induced by low-dose lipopolysaccharide and subsequent pulsed high-dose methylprednisolone. Rabbits in the treated group were subjected to subcutaneous injections of G-CSF at a dose of 100 µg/kg and SCF 25 µg/kg per day for 5 days; rabbits in the control group were given saline. Blood samples were collected and serum osteocalcin was detected by ELISA. Radiological analysis was performed by magnetic resonance imaging (MRI). Then bilateral femora and humeri were harvested and processed to paraffin sections and hard-tissue sections for immunohistochemical, histologic, and histomorphometric analysis.

**Results.** The mean number of leukocytes and relative numbers of mononuclear cells increased significantly after mobilization. All rabbits displayed a marked increase in osteocalcin protein expression in response to G-CSF/SCF. MRI scans showed a reactive interface between the necrotic and reparative zones after G-CSF/SCF administration. Quantitative analysis showed that new vessel formation was 3.3-fold greater and vessel density was 2.6-fold greater in the treatment group than the control group. The histologic and histomorphometric analysis revealed that the new bone volume was significantly higher in the G-CSF/SCF group than in the control group at 4 weeks.

**Conclusion.** G-CSF/SCF-induced mobilization of BMSC in the necrotic foci may represent a promising strategy for promoting functional bone repair of early-stage ON. (First Release Sept 15 2008; J Rheumatol 2008;35:2241–8; doi:10.3899/jrheum.071209)

*Key Indexing Terms:*

BONE MARROW-DERIVED STEM CELL      STEM CELL FACTOR      OSTEONECROSIS  
GRANULOCYTE COLONY-STIMULATING FACTOR      MOBILIZATION

Osteonecrosis (ON) is often referred to as ischemic necrosis of bone. Without an adequate blood supply, a serious case of ON can develop that leads to the death of bone tissue. ON usually affects people between 30 and 50 years of age. If untreated, most patients will experience severe pain and limitation in movement within 2 years. Several studies of various treatment modalities have been reported during the past decade; ON of the femoral head remains a difficult therapeutic problem. Although total hip replacement is now used widely to treat ON of the hip, the patients are usually young

and current hip prostheses will not function well for the remaining life expectancy of these patients<sup>1</sup>. Thus, it is necessary to delay or eliminate the need for hip replacement by treating this condition with a method that preserves the femoral head.

Stem cell-based therapies for degenerative disorders and injuries are promising. After tissue injury, hematopoietic and multipotent progenitor cells are mobilized from the bone marrow into the pool of circulating cells. These cells migrate to the site of injury, where they retain a high degree of plasticity and are capable of contributing regenerative progenitor cells to hematopoietic and nonhematopoietic tissues during the early phase of osteonecrosis. Recently, it has been suggested that bone marrow-derived stem cells (BMSC) serve as a valuable source for the regeneration of damaged tissues<sup>2</sup>. Homing and engraftment of BMSC in damaged nonhematopoietic organs, such as vascular tissue<sup>3</sup>, myocardium<sup>4</sup>, and skin<sup>5</sup>, have been observed and were suggested to contribute to the wound-healing process. In principle, these studies have provided the proof of damage repair by the application of BMSC. As ON may be a disease of

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mesenchymal cells, both the number and the activity of the BMSC decrease in patients with necrosis of femoral head and there is also a decrease in the proliferative capacity of the osteoblastic cells in the proximal femur<sup>6,7</sup>. BMSC contain adult stem cells, such as hematopoietic stem cells, mesenchymal stem cells, and endothelial progenitor cells, which might have osteogenic properties implanted into the necrotic lesion and could be of benefit in this condition.

Recently, enhancement of bone repair in the necrotic zone by BMSC has been highlighted for the treatment of ON before collapse of the femoral head<sup>6,8</sup>. Cytokine-induced mobilization of bone marrow stem/progenitor cells in the necrotic foci may represent a promising strategy for replacing necrotic bone. Granulocyte colony-stimulating factor (G-CSF) and stem cell factor (SCF) are hematopoietic factors, and they are involved in proliferation, differentiation, and survival of bone marrow-derived stem and progenitor cells<sup>9,10</sup>. G-CSF in combination with SCF increases the levels of proliferation in the bone marrow prior to mobilization<sup>11</sup>. Further, Orlic, *et al*<sup>12</sup> showed that bone marrow cells, mobilized by administration of G-CSF and SCF to splenectomized mice, home to the infarcted area, differentiate into endothelial and smooth muscle cells, and reduce mortality by forming vascular structures. We assessed the potential of G-CSF/SCF-mobilized BMSC to repair steroid-associated ON in rabbits.

## MATERIALS AND METHODS

All experimental procedures adhered to the recommendations of Experimental Animal Center of Tongji Medical College and the U.S. Department of Health for the care and use of laboratory animals, and were approved by the Ethics Committee of Tongji Medical College.

**Establishment of ON model and treatment protocol.** Sixty-four 28-week-old male New Zealand white rabbits with body weight of 3.5–4.5 kg were housed at the Experimental Animal Center of the investigators' hospital and received a standard laboratory diet and water *ad libitum*. An ON model of the femoral head was established according to the modified inductive protocols<sup>13</sup>. In brief, 2 injections of 10 µg/kg body weight of lipopolysaccharide (LPS; Sigma) were given intravenously, and then 3 injections of 20 mg/kg body weight of methylprednisolone (MPS; Pfizer) were given intramuscularly, at a time interval of 24 h. Four out of 64 rabbits died within 4 days after last injection of MPS. The remaining rabbits were randomly divided into 2 groups: treated and control. Six weeks after the last injection of MPS, the treated group (n = 40) were subjected to subcutaneous injections of G-CSF at a dose of 100 µg/kg and SCF at a dose of 25 µg/kg per day for 5 days; and the control group (n = 20) were given isodose physiological saline.

**Laboratory analysis.** After 5 days' treatment with G-CSF/SCF, peripheral blood samples were collected from the auricular arteries for laboratory analysis under sterile conditions. The total numbers of leukocytes (white blood cells, WBC) and the relative numbers of mononuclear cells (MNC) were determined in each collection. Automated blood counts were performed with a Sysmex XE-2100 counter (Sysmex, Kobe, Japan). MNC purity was determined by microscopic differential counts.

**Osteocalcin (OCN) assays.** We collected blood samples from the rabbits into plastic tubes and centrifuged them immediately afterward to obtain serum before and 1, 2, 3, and 4 weeks after the last treatment of G-CSF/SCF. The serum was divided into aliquots and frozen at -30°C until use. OCN, a sensitive biochemical marker of bone formation, was meas-

ured in the serum of the rabbits using the enzyme linked immunosorbent assay (ELISA) kit. Serum levels of OCN were measured according to the manufacturers' instructions (Boster).

**Radiological analysis.** Magnetic resonance imaging (MRI) was performed for bilateral proximal and distal femora and humeri in each group at 6 weeks after the last injection of MPS and 4 weeks after G-CSF/SCF administration, using a 1.5 T superconducting system (Siemens Magnetom Vision, Erlangen, Germany). Preliminary sagittal and oblique axial images were obtained to define the femoral longitudinal axis. Imaging measures were as follows: T1-weighted MRI images [T1W, repetition time (TR)/echo time (TE) = 490/14 ms], T2-weighted MRI images [T2W, TR/TE = 2632/96 ms], a section thickness of 3.0 mm, intersection gap of 1 mm, and imaging matrix of 512 × 512. Changes in the femora were observed and noted.

**Autopsy and sample preparation.** All the animals were euthanized at 4 weeks after treatment with G-CSF/SCF or saline, and bilateral proximal one-thirds and distal condyles of both femora and humeri were harvested. After fixing in 10% neutral formalin, the extirpated bone samples were either decalcified in 0.5 M EDTA, pH 7.4, or embedded in methylmethacrylate and processed for hard-tissue sections. Decalcified bones were embedded in paraffin and sectioned at 5 µm, and hard-tissue samples were sectioned at 10 µm. These sections were prepared for the following examinations.

**Evaluation of ON.** Whole areas of the proximal one-third and distal condyle of both femora and humeri, a total of 8 regions, were histopathologically examined for the presence of ON. The paraffin sections from bone samples were stained with H&E. For each group, the frequency of ON, its location, the number of necrotic foci, the size of the ON area, and its histology were examined. All rabbits that had at least one osteonecrotic lesion out of 8 areas examined were considered to be rabbits with ON (ON + rabbits), while those with no osteonecrotic lesions were considered rabbits without ON (ON - rabbits).

**Assessment of regional angiogenesis and vasculogenesis.** In order to examine newly formed vessels surrounding the necrotic zone, paraffin sections from the animals in each group were examined using standard immunohistochemical techniques for proliferating cell nuclear antigen (PCNA; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and immunoperoxidase staining. Newly formed vessel density was expressed as the number of PCNA+ endothelial cells per mm<sup>2</sup> under light microscopy. Immunofluorescence was performed for observation of vasculogenesis and vessel counting. The sections were stained with anti-factor VIII polyclonal antibody (Dako) and Cy3-linked anti-mouse IgG (Sigma). Sections were viewed using a fluoroscope (Olympus, Tokyo, Japan). Vessel density was expressed as the number of factor VIII+ endothelial cells per mm<sup>2</sup> under a fluoroscope.

**Assessment of bone regeneration around the necrotic zone.** Histological analysis was performed to observe the osteonecrotic changes and repair processes in the femoral heads at 4 weeks after treatment. The hard-tissue sections were stained with toluidine blue and observed and photographed under light microscope to visualize histological difference between individuals. Histomorphometric analysis was performed with image-analysis software (Image; U.S. National Institutes of Health). The new bone volume was measured as the percentage of new bone/total area of tissue. Osteoid tissue was excluded from new bone calculations. A pathologist, blinded to the treatment conditions, evaluated all sections.

**Statistical analysis.** Statistical analyses involved Student's t-test or 1-way analysis of variance followed by analysis using SPSS software. Data (mean ± SEM) were considered statistically significant at a value of p < 0.05.

## RESULTS

**Bone marrow stem cell mobilization.** Both G-CSF and SCF are powerful agents for bone marrow stem cell mobilization, and the combination of them educates a synergistic effect. In our study, the number of WBC and the percentage of MNC

were significantly enhanced in the treatment group after mobilization with G-CSF and SCF, compared with the control group (Table 1).

**MRI scanning.** Six weeks after the last injection of MPS, MRI findings showed irregular low signal on T1-weighted images (Figure 1A) and irregular low or high signal on T2-weighted images in the proximal and distal femoras, which indicated early-stage ON. In the control group, 4 weeks later, sequential MRI investigations yielded increasing hyper-intensity signals on T1-weighted images (Figure 1B), with punctiform hypo-intense signals on T2-weighted images, which showed inflammatory effusion and invaded fibrous repair tissue. In the treated group, the lesion was a ring-like or band-like hypo-intense signal around the necrotic zone in T1- and T2-weighted sequences (Figure 1C), which indicated marked increase in bone formation.

**Histologic evaluation of ON.** Histologically, ON lesions

showed an accumulation of bone marrow cell debris, bone trabeculae demonstrating empty lacunae and/or ghost nuclei in the lacunae, and an increase in the fat cells of the bone marrow (Figure 2A, 2B). The incidence of ON, in addition, was 85% (34/40) in the treated group and 90% (18/20) in the control group (Table 2). The necrotic areas of the femoral and humeral bones were seen mainly in the metaphysis.

**G-CSF/SCF treatment stimulating OCN secretion in serum.** In order to evaluate the effects of G-CSF/SCF treatment on bone formation, we examined the levels of serum OCN, a specific biochemical marker of bone formation. The release of OCN was a marker of osteoblast activity. Surprisingly, OCN levels were significantly increased in G-CSF/SCF-treated rabbits compared with control rabbits. These data indicated that G-CSF/SCF acted to promote bone formation (Figure 3).

**Increase in neovascularization and vessel density.** Since

Table 1. Changes of white blood cells (WBC) and mononuclear cells (MNC) before and after mobilization in each group. Data are mean  $\pm$  SD.

Group	Before Mobilization		After Mobilization	
	WBC ( $\times 10^9/l$ )	MNC, %	WBC ( $\times 10^9/l$ )	MNC, %
Control	8.16 $\pm$ 0.71	16.42 $\pm$ 4.86	11.78 $\pm$ 2.32*	17.66 $\pm$ 3.24
G-CSF/SCF	8.24 $\pm$ 0.36	16.86 $\pm$ 5.42	17.26 $\pm$ 6.24 <sup>†</sup>	36.48 $\pm$ 6.26 <sup>†</sup>

Compared to before mobilization, \*  $p < 0.05$ ; compared to control group, <sup>†</sup>  $p < 0.01$ . G-CSF/SCF: granulocyte colony-stimulating factor/stem cell factor.

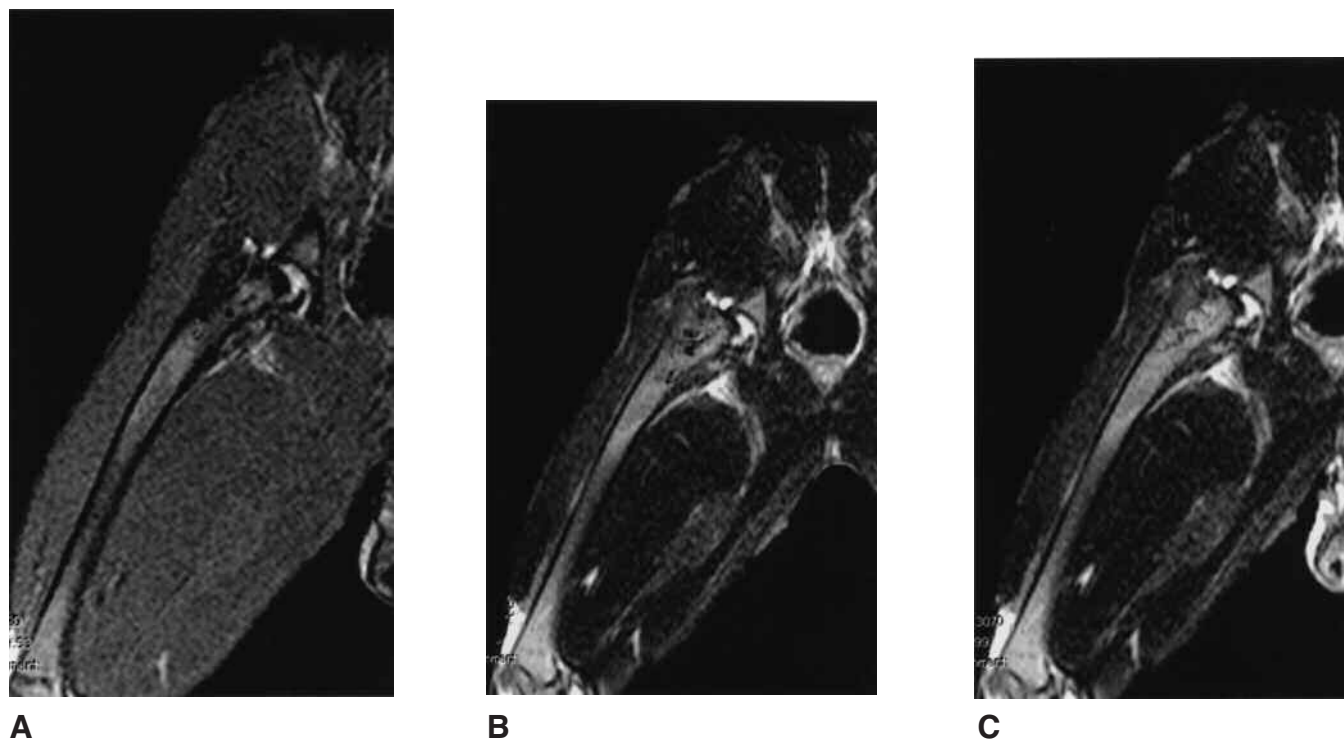
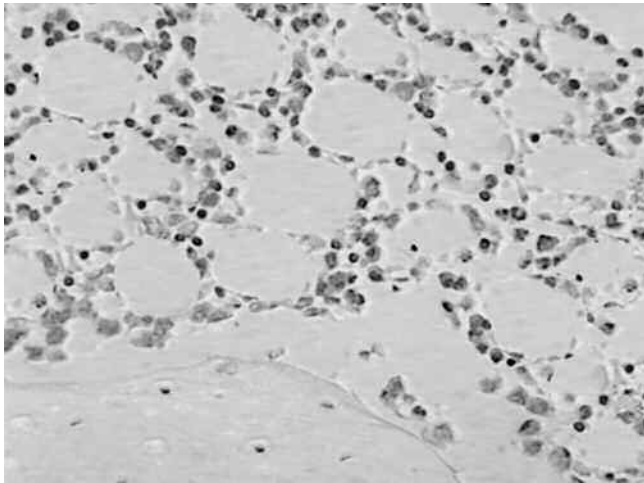
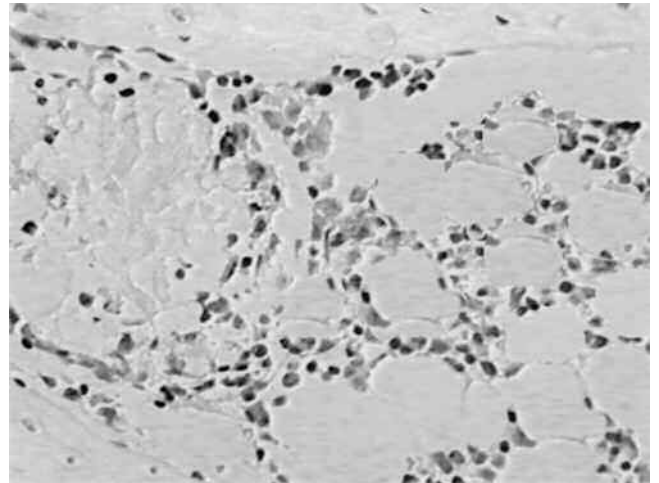


Figure 1. Sequential T1-weighted coronal MRI images of the osteonecrotic proximal femur at 6 weeks after lipopolysaccharide-methylprednisolone (LPS-MPS) administration (A), and then at 4 weeks after saline administration (B) and at 4 weeks after G-CSF/SCF intervention (C).



**A**

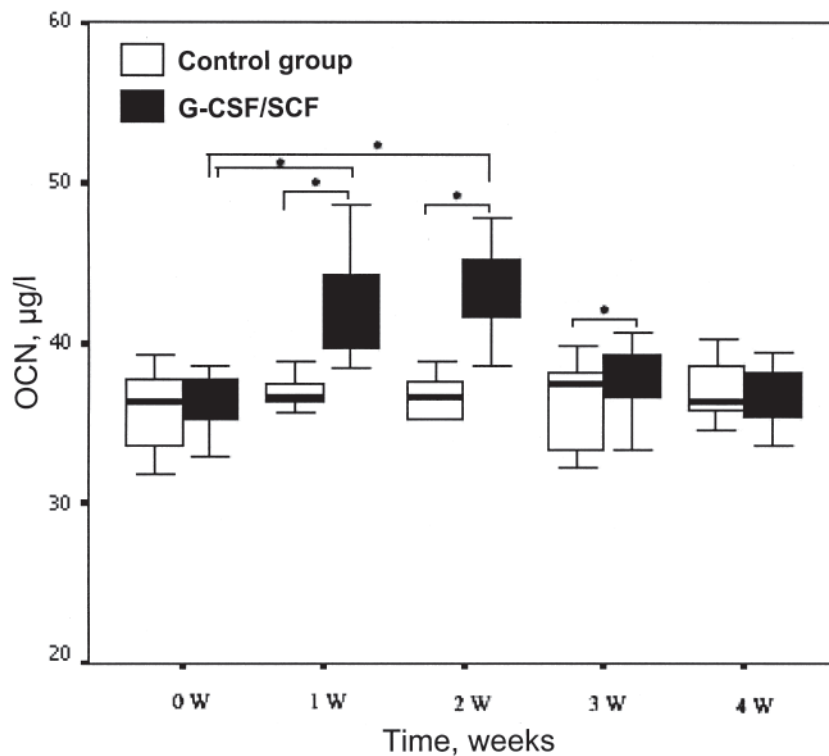


**B**

*Figure 2.* Histologic features of osteonecrosis in the LPS-MPS-treated rabbits. (A, B) There were a larger number of inhomogeneous and enlarged marrow fat cells. Massive trabeculae had been absorbed and the bone trabeculae showed empty lacunae or pyknotic nuclei of osteocytes (H&E stain, original magnification  $\times 400$ ).

*Table 2.* Prevalence and location of osteonecrosis (ON).

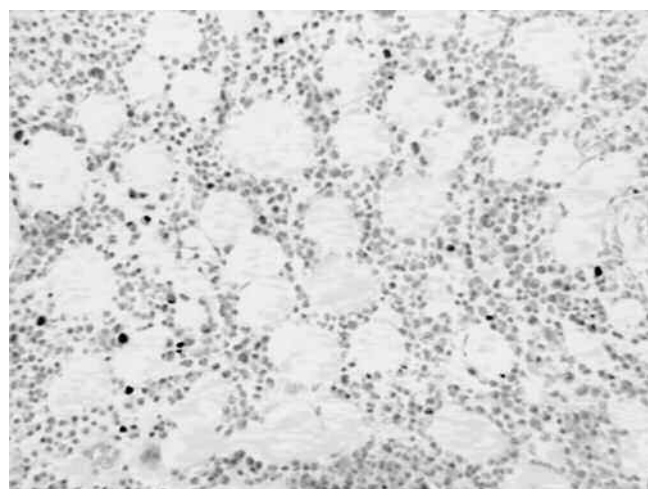
Group	n (%)	No. of ON Lesions			
		Proximal Femora	Distal Femora	Proximal Humeri	Distal Humeri
Treated	34/40 (85)	25	10	7	3
Control	18/20 (90)	14	6	4	1



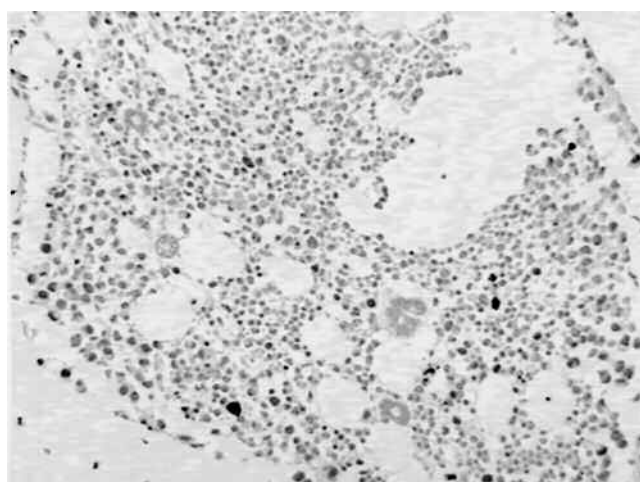
*Figure 3.* Granulocyte colony-stimulating factor/stem cell factor (G-CSF/SCF) treatment stimulating bone formation. Serum osteocalcin (OCN;  $\mu\text{g/l}$ ) was measured by ELISA. Samples were measured in triplicate. Values represent means  $\pm$  SEM. \*Statistically significant difference,  $p < 0.05$ .

PCNA-positive vessels were deemed to be newly formed, regional angiogenesis in the necrotic region was measured in the femoral tissue sections using immunohistochemical techniques. Examination of high-magnification views of PCNA-stained samples revealed a relatively lower density of newly formed capillary structures in the control group (Figure 4A) compared with the treatment group (Figure 4B).

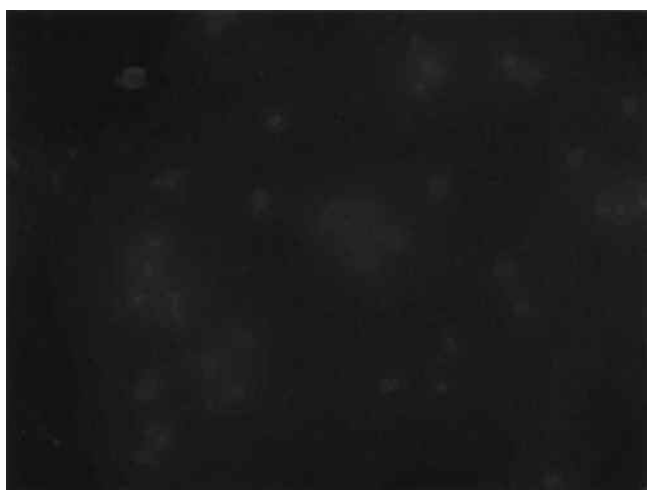
Quantitative analysis showed that new vessel formation in the treatment group was 3.3-fold greater than in the control group (Figure 4E). To determine the vessel density and vasculogenesis, the necrotic regions from the femoral samples of the control (Figure 4C) and the treatment (Figure 4D) groups were stained for factor VIII. Analysis of these data showed that the vessel density in each group based on the



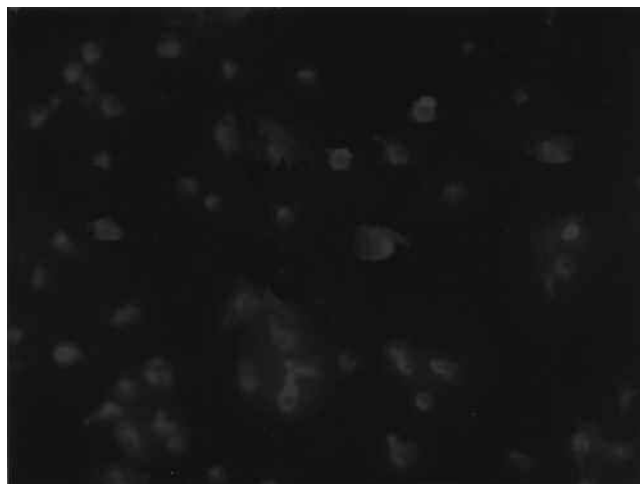
A



B



C



D

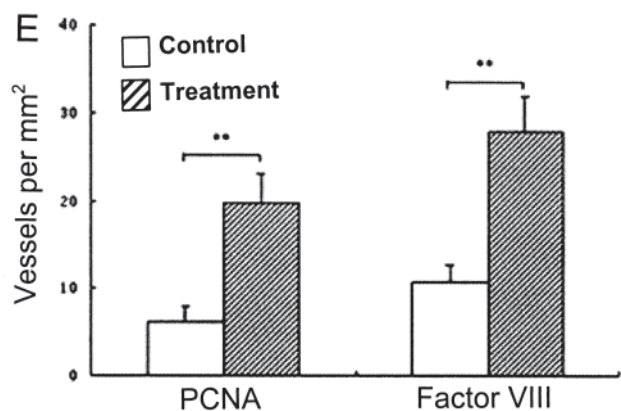


Figure 4. Assessment of regional angiogenesis and vasculogenesis. Newly formed vessels stained with anti-proliferating cell nuclear antigen (PCNA) in the control group (A) and the treatment group (B) (original magnification  $\times 400$ ). Vessel density revealed by staining with anti-factor VIII antibody in the control group (C) and the treatment group (D). E: Quantitative analysis of vessel density by staining with PCNA and factor VIII in control and treatment groups.  $**p < 0.01$ .

number of factor VIII+ endothelial cells correlated with the new vessel densities determined by PCNA staining. The vessel density in the treatment group was 2.6-fold higher than in the control group (Figure 4E).

**Formation of new bone surrounding the necrotic zone.** In the control group, normal marrow had been replaced by fibrotic marrow, and a large number of osteocyte-filled lacunae had been replaced by empty lacunae. Continuity of trabecular bone appeared to be compromised, with fewer continuous trabeculae seen on the cross-sections, and marked resorption across trabeculae, and pronounced resorption was observed at intersections of trabecular plates (Figure 5A). The histologic observations suggested that G-CSF/SCF had potential to regenerate bone and repair the ON. In the G-CSF/SCF group, the reparative response resulted in progressive development of a reactive margin, or interface, between the dead zone and adjacent viable tissues (Figure 5B, 5C). During the repair process of ON, primitive mesenchymal cells and capillaries proliferated and invaded dead trabecular bone, differentiated into osteoblasts, and laid down new living bone surrounding dead trabecular bone, which was later remodeled. Histomorphometric analysis revealed that the new bone volume was significantly higher in the G-CSF/SCF group than in control group at 4 weeks (Table 3;  $p < 0.01$ ).

## DISCUSSION

Recent advances in understanding of the pathophysiology of ON suggest that a decrease in the mesenchymal stem cell pool of the proximal femur and a decrease in the osteoblastic cell proliferation rate might not provide enough osteoblasts to meet the needs of bone remodeling in the early stage of the disease<sup>14</sup>. Bone marrow is a rich source of stem and progenitor cells that can be mobilized to ischemic sites. This capacity may reflect the activities of multiple stem cells, such as hematopoietic stem cells, mesenchymal stem cells, and marrow multipotent stem cells. The effec-

tiveness of bone marrow cells may be related to the mobilized stem cells endowed with osteogenic properties, arising from an increase in the supply of such cells to the femoral head via treatment with G-CSF/SCF.

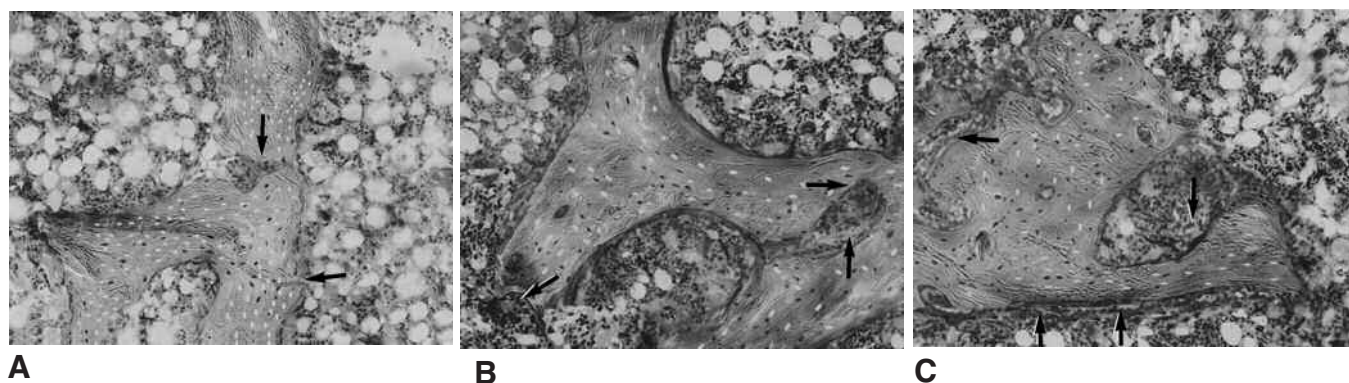
MRI, a highly sensitive and specific technique, has become the tool of choice for early and accurate detection of ON<sup>15</sup>. MRI provides excellent soft-tissue contrast, and allows images to be produced in virtually any plane. In the majority of cases, the basic pattern of ON consists of a zone of decreased signal intensity on T1- and T2-weighted images. The low-intensity zone may be homogeneous or heterogeneous, with a speckling of high-intensity against the low-intensity background on T1- and T2-weighted images. Previous studies have shown that MRI changes may not be evident despite histologic evidence of ON<sup>16,17</sup>. In our study, MRI provided a picture of the area affected and the bone-rebuilding process of rabbits with ON. Microradiographs of the bone sections showed that the necrotic segment consisted of a delicate and regular open-work of bone, whose uniform appearance was at odds with the heterogeneous MRI signal pattern. The necrotic segment was separated from the living bone by a remodeling zone, which perfectly matched the low-intensity band seen on MRI scans.

The formation of new blood vessels is required for the development and repair of all tissues, and is particularly

*Table 3.* Comparisons of new bone volume between the G-CSF/SCF treated and control groups after 4 weeks.

Group	New Bone Volume (percentage of new bone/total area of tissue)
Control	3.19 ± 0.92
G-CSF/SCF	6.74 ± 1.31*

Compared to control group, \* $p < 0.01$ . G-CSF/SCF: granulocyte colony-stimulating factor/stem cell factor.



*Figure 5.* Toluidine blue-stained sections of rabbit necrotic femoral samples 4 weeks after treatment. A: Representative photomicrograph from the control group showed massive trabeculae had been absorbed and become empty (arrows), surrounded by more marrow fat cells with increased size dominantly occupying marrow space. B and C: Photomicrographs showing development of new bone in the treatment group, 4 weeks after administration of G-CSF/SCF. During the repair process of osteonecrosis, primitive mesenchymal cells invade dead trabecular bone, differentiate into osteoblasts, and lay down new living bone (arrows) to surround dead trabecular bone.

important for bone formation and remodeling. Endothelial progenitor cells have been proposed to circulate in adult organisms and to be recruited and incorporated into sites of physiological and pathological neovascularization<sup>18,19</sup>. Therefore, new therapeutic approaches to promote angiogenesis have evolved when it is suggested that the infusion of circulating bone marrow-derived stem or endothelial progenitor cells may improve blood-flow recovery in various ischemic models<sup>10,20</sup>. In the necrotic area, a proliferation of fibrous tissue and vascular ingrowth was noted in the dead trabeculae. G-CSF/SCF treatment not only enhanced angiogenesis but also incorporated into new vessels. These effects significantly increased the number of newly formed vessels in the G-CSF/SCF group compared with the control group. The significantly increased capillary networks could support the demands of “at-risk” tissue adjacent to the necrotic zone, which may have prevented deleterious destruction of the bone and improved bone renovation.

Bone formation is an important physiological process in regulating bone remodeling. During repair of dead compact bone, resorption of necrotic bone precedes the formation of new bone. In order to replace dead compact bone, it has to be resorbed first, before new bone can be laid down<sup>21-23</sup>. Assessment of the repair process of the necrotic femoral head was conducted by histological and histomorphometric analysis at Week 4 post-administration. G-CSF/SCF treatment enhanced osteogenic activity in the femoral heads of the ON rabbits. On histology slides, there was characterization of the repair process of ON, primitive mesenchymal cells had invaded necrotic trabecular bone, differentiated into osteoblasts, and laid down new living bone covering dead trabeculae, which created the characteristic appearance of “creeping substitution.” Histomorphometric analysis revealed that the new bone volume was significantly greater in the G-CSF/SCF group than the control group. G-CSF/SCF ultimately led to an increase of bone volume, which was consistent with the observed increase in OCN protein expression in response to G-CSF/SCF. Serum OCN is a late marker of calcification in osteoblastogenesis and is present in the later stages of skeletal bone formation<sup>24</sup>. OCN is exclusively produced by osteoblasts, and the release of OCN is a specific marker of osteoblast activity and bone formation rate. It may be involved in regulation of osteoblast function, regulation of bone turnover, and/or mineralization<sup>25</sup>. Repair of ON could be accelerated accordingly, thus providing a potential method for therapy of ON.

Unfortunately, it was clear from our study that the LPS+MPS injection model of ON produced patchy areas of ON, but these features were not always seen in the femoral head. For instance, 62.5% (25/40) of animals developed ON in the proximal femur and 25% (10/40) in the distal femur, in the treated group. So we failed to analyze BMSC mobilization in rabbits with ON in a quantitative way following G-CSF/SCF administration. In addition, we could not measure

the volume of the necrosis using contiguous MRI scans<sup>26</sup>. Because of subtle variations in the magnetic field, geometrical distortions may occur in MRI and the limits of the lesion (appearing at the proximal and distal femora and humeri) may be difficult to outline precisely.

Our study showed the feasibility of BMSC mobilization induced by G-CSF/SCF in early-stage ON in rabbits. We could consider that the mobilized BMSC following G-CSF/SCF administration might favor the homing of immature cells in the necrotic zone where they might possibly contribute to tissue regeneration (osteogenesis and angiogenesis). This promising therapeutic approach might modify the treatment of early-stage ON of the femoral head, and might avoid progression of disease to the stage of subchondral fracture (stage III) and reduce the need for total hip replacement.

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