BONE REGENERATION WITH CALCIUM SULFATE: EVIDENCE FOR INCREASED ANGIOGENESIS IN RABBITS

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KEY WORDS

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Autologous bone is the preferred bone graft material because it carries proteins as bone-enhancing substrates, minerals, and vital bone cells. Calcium sulfate (CS) is a well-tolerated, biodegradable, osteoconductive bone graft substitute and is a reasonable alternative to autogenous bone graft. Blood vessels are an important component of bone formation and maintenance. The process of vascular induction is called angiogenesis, and it plays a key role in all regenerative processes. Bone tissue differentiation is related to the local presence of blood vessels. One method to evaluate the presence of blood vessels in a tissue is to count the microvessels to evaluate microvessel density (MVD). The aim of the present study was to conduct a comparative evaluation of microvessel density in sites treated with CS and autologous bone in rabbits, with or without e-PTFE nonresorbable membranes (Gore-Tex, Flagstaff, Ariz). Nine New Zealand rabbits, each weighing about 2.5 kg, were used in this experiment. Three 6-mm wide defects were created in each tibial metaphysis. The defects were filled in a random way. The defects of group 1 (3 rabbits) were filled with CS granules (Surgiplaster, Classimplant, Rome, Italy) and covered with e-PTFE membranes. The defects in group 2 (3 rabbits) were filled with CS granules (Surgiplaster). The defects in group 3 (3 rabbits) were filled with autologous bone. A total of 54 defects were filled (18 with CS and e-PTFE membranes, 18 with CS alone, and 18 with autologous bone). No postoperative deaths or complications occurred. All nine animals were sacrificed at 4 weeks. MVD results were as follows: in the first group, 9.88 ± 4.613; in the second group, 7.92 ± 1.998; and in the third group, 5.56 ± 1.895. $P = .000$ was highly significant. Statistically significant differences were found between groups 1 and 3, 1 and 2, and 2 and 3. The presence of more blood vessels in the sites treated with CS could help to explain the good results reported in the literature with the use of CS.

INTRODUCTION

The preferred bone graft material is autologous bone because it carries proteins as bone-enhancing substrates, minerals, and vital bone cells.[1–3] Its main disadvantages are the limited amount of available graft material, an additional surgical site, donor site morbidity, and the requirement of anesthesia for extraoral bone harvesting.[4] Thus, in many instances, it is necessary to use other types of biomaterials. Calcium sulfate (CS) is the simplest alternative and has the longest clinical history as a synthetic bone substitute, having been used for more than 100 years.[5] CS is a well-tolerated, biodegradable, osteoconductive bone graft substitute and is a reasonable alternative to autogenous bone graft for benign bone lesions.[6]

Blood vessels are an important component of bone formation and maintenance.[7] The process of vascular induction is called angiogenesis, and it plays a key role in all regenerative processes.[7,8] Angiogenesis is important in wound healing, inflammatory diseases, and tumors.[9,10] Bone tissue differentiation is related to the local presence of blood vessels. Angiogenesis is increased by the low oxygen tension and high metabolic activity present at injury sites.[8] In these sites, development of a vascular system is needed to deliver oxygen and nutrients and to clear away cellular debris.[7] Angiogenesis is regulated through a complex interplay of molecular signals mediated by growth factors,[7] with extracellular matrix remodeling, endothelial cell migration and proliferation, capillary differentiation, and anastomosis.[9,10]

One method used to evaluate the presence of blood vessels in a tissue is counting the microvessels to evaluate microvessel density (MVD). MVD has been extensively studied in tumors and seems to be correlated with prognosis in several malignant tumors.[11] In a recent study using CS, several blood vessels, without signs of inflammation, were seen throughout the osteoid matrix.[12] The aim of the present study was to conduct a comparative evaluation of MVD in sites treated with CS and autologous bone in rabbits, with and without the use of e-PTFE nonresorbable membranes.

MATERIALS AND METHODS

This study was approved by the ethics committee of our university. Nine New Zealand rabbits, each weighing about 2.5 kg, were used in this experiment. Three 6-mm wide defects were created in each tibial metaphysis (Figure 1). The defects were filled in a random way. The defects of group 1 (3 rabbits) were filled with CS granules (Surgiplaster, Classimplant, Rome, Italy) and covered with e-PTFE membranes (Gore-Tex, Flagstaff, Ariz) (Figure 2). The defects in group 2 (3 rabbits) were filled with CS granules. The defects in group 3 (3 rabbits) were filled with autologous bone (Figure 3). A total of 54 defects were filled (18 with CS and e-PTFE membranes, 18 with CS alone, and 18 with autologous bone). No postoperative deaths or complications occurred. All nine animals were sacrificed at 4 weeks. A total of 54 specimens were retrieved and processed.

Processing of specimens

The specimens were retrieved and stored immediately in 10% buffered formalin and processed to obtain thin ground sections with the Precise 1 Automated System (Assing, Rome, Italy).[13] The specimens were dehydrated in an ascending series of alcohol rinses and embedded in a glycolmethacrylate resin (Technovit 7200 VLC, Kulzer, Wehrheim, Germany). After polymerization, the specimens were sectioned longitudinally along the major axis of the implant with a high-precision diamond disc at about 150 μm and ground down to about 30 μm. Three slides were obtained for each specimen. The slides were stained with acid fuchsin and toluidine blue.

The microvessel count was carried out using 2 techniques. In the first technique, a light microscope (Laborlux S, Leitz, Wetzlar, Germany) was connected to a high-resolution video camera (3CCD, JVC KY-F55B) and interfaced to a monitor and PC (Intel Pentium III 1200 MMX). This optical system was associated with a digitizing pad (Matrix Vision GmbH) and a histometry software package with image-capturing capabilities (Image-Pro Plus 4.5, Media Cybernetics Inc., Immagini & Computer Snc Milano, Italy). In the second technique, an IBAS-AT image analyzer (Kontron, Munich, Germany) with a ×400 magnification was used and the individual microvessel profiles were circled to prevent the duplication or omission of microvessel count. For each case, 10 high-power fields (HPF), corresponding to 1.1 mm^2, were measured. Values were expressed as number of microvessels per mm^2 (microvessel density: MVD). We evaluated only vessels that had a diameter of less than 3 μ, presented a vessel wall thickness of less than 1 μ, and had 1 or more endothelial cells that lined the lumen.

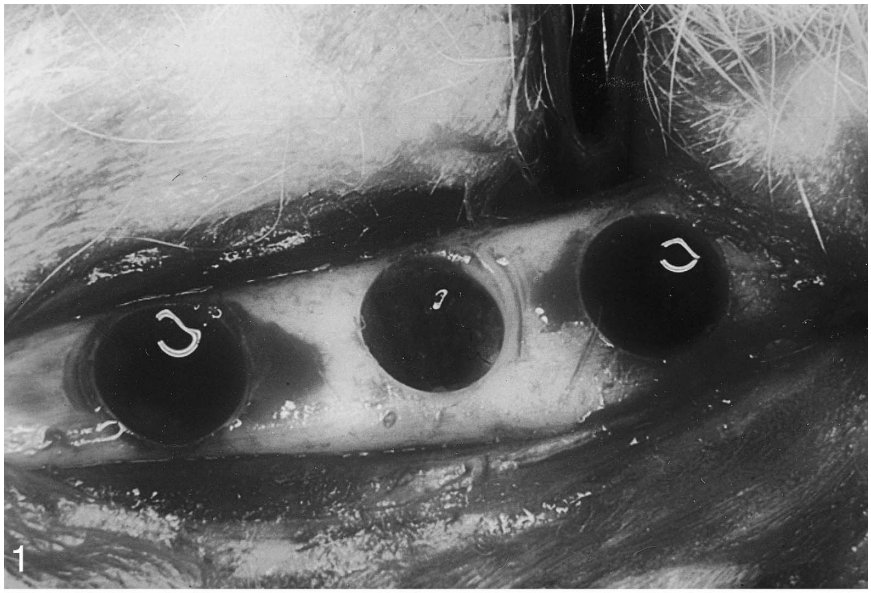
Statistical evaluation

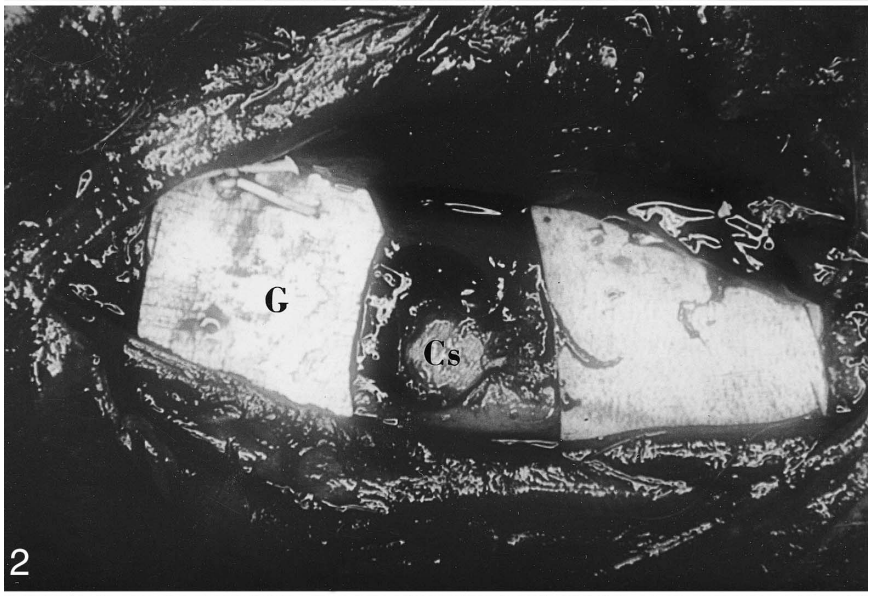
Differences in the 3 groups were evaluated using analysis of variance (ANOVA). The significance of the differences observed was evaluated using the Student-Neuman-Keuls test for multiple comparisons. The number of microvessels was expressed as a mean ± standard deviation and standard error. Statistically significant differences were set at $P < .05$.

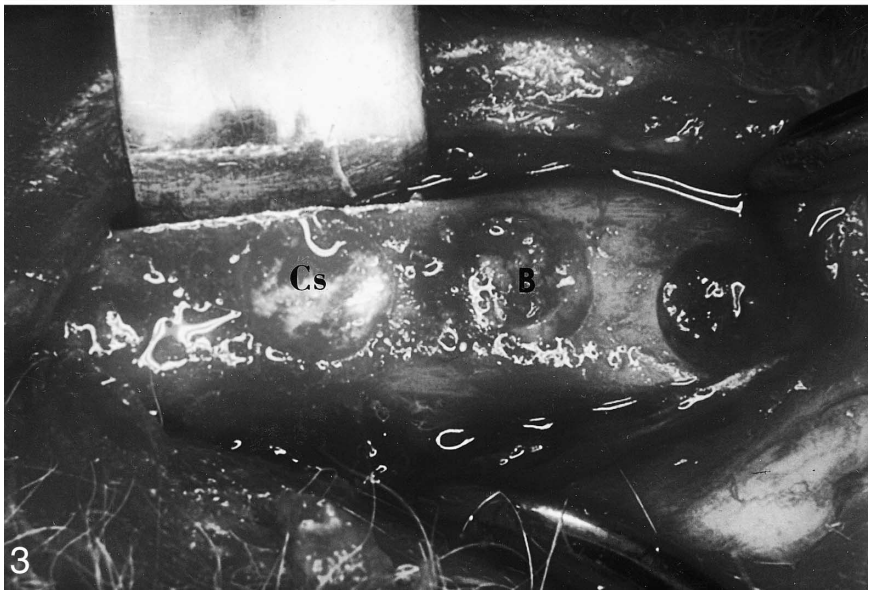
RESULTS

Microvessels were present in all specimens. In group 1 (defects filled with CS granules and covered with e-PTFE membranes), the mean number of microvessels was 9.88 ± 4.613 (Figure 4). In group 2 (defects filled with CS granules), the mean number of microvessels was 7.92 ± 1.998 (Figure 5). In group 3 (defects filled with autologous bone), the mean number of microvessels was 5.56 ± 1.895 (Figure 6).

In Table 1, the number of blood







vessels is indicated, and in Table 2, the mean values with standard deviation and standard error are indicated. The finding that $P = .000$ was highly significant. Statistically significant differences were found between groups 1 and 3, 1 and 2, and 2 and 3.

Discussion

Angiogenesis is the process by which new blood vessels sprout from established vessels.[14] An increased expression of the mediators of the inflammation is present in inflamed tissues, and many of these factors can promote angiogenesis.[14] Osteogenesis in skeletal regeneration has close links to the revascularization of the differentiating tissues. Regenerating tissues have higher metabolic needs and thus require a dense capillary network during repair.[7] Bone is, moreover, a rich source of angiogenic growth factors such as FGF and TGFβ, as well as osteogenic growth factors such as BMP.[7]

Initially, macrophages and monocytes migrate to the injured region and release angiogenic growth factors.[7] Furthermore, surgical vascularized bone grafts are better retained than nonvascularized tissue.[15] A reduction in bone blood and microvascular defects in the sinusoidal compartment has been reported in osteoporotic bone as well as in aplastic anemia.[16] Blood vessels are an often overlooked but important event in the process of bone remodeling.[17] Remodeling tissues have higher metabolic needs.[7]

Tissue repair requires the development of a vascular system at the injury site to clear away the cellular debris and to provide important components of bone formation and maintenance.[7] The formation of blood

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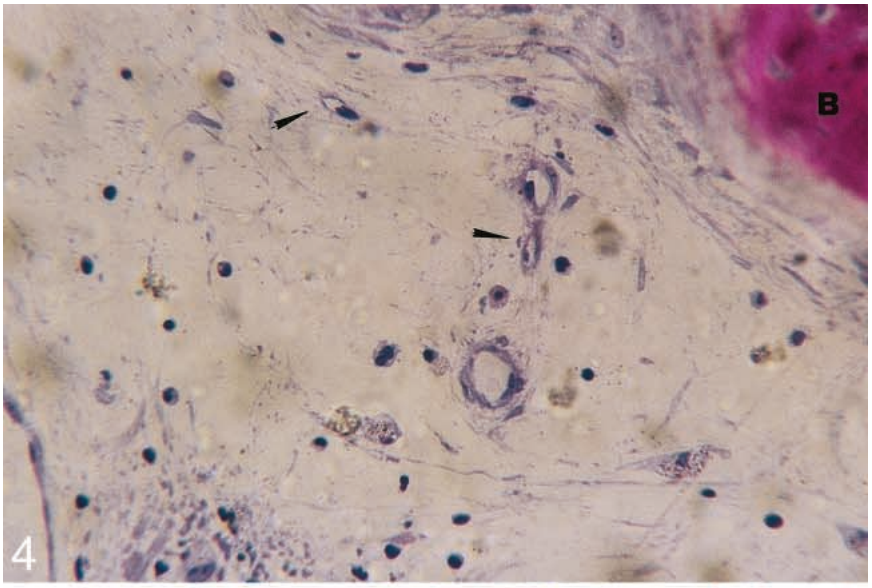
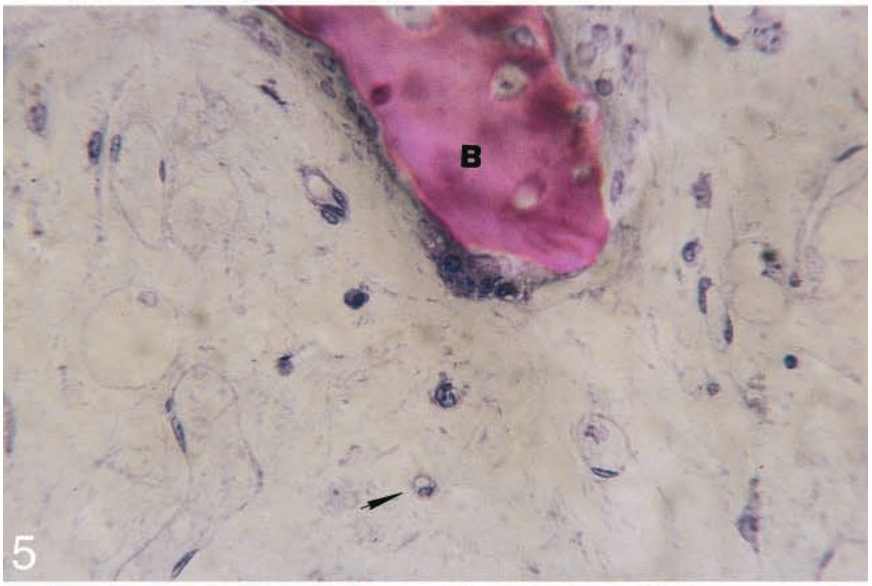
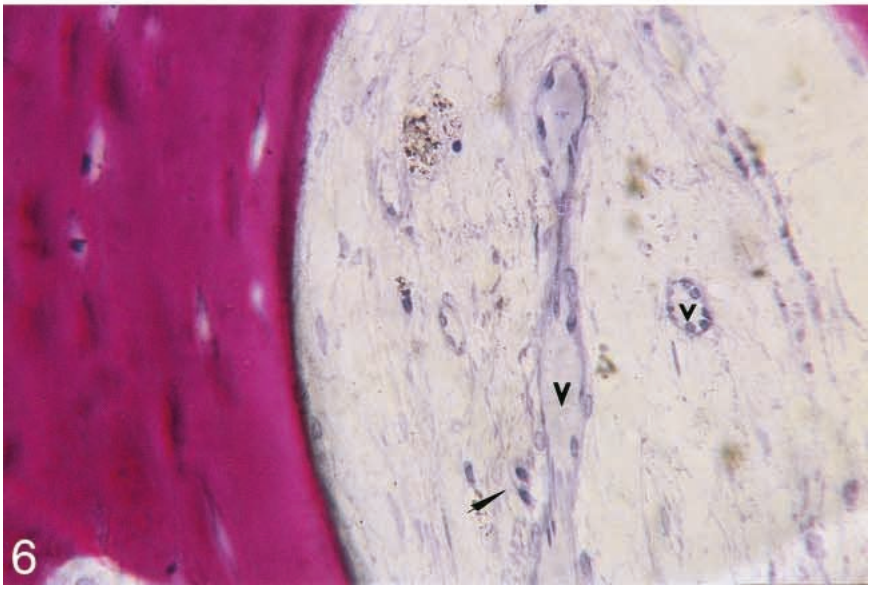
Figures 1–3. Figure 1. Three 6-mm wide defects were created in the tibial metaphysis. Figure 2. These 3 defects were filled with calcium sulfate (CS) granules, and 2 of them were covered with e-PTFE membranes (G). Figure 3. In this tibial metaphysis, 1 defect was filled with CS and 1 with autologous bone (B).







capillaries precedes the formation of new bone, and the perivascular loose connective tissue that accompanies the proliferating capillaries represents the source of osteoprogenitor cells.[18] This process of vascular induction is termed angiogenesis[7]. A close spatial correlation between angiogenesis and bone formation has been shown.[18] Angiogenesis is thus the development of a microvascular network that is critical for the development, remodeling, and healing of most tissues, including bone.[9,10,19]

In endochondral bone formation, the avascular cartilage that precedes bone may produce antiangiogenic factors, whereas the appearance of osteoblasts coincides with blood vessel invasion. Autologous bone seems to be not only a passive filling material but also can act through osteogenic growth factors and seems to contain vital osteoprogenitor cells[2]. Surgical grade CS pellets seem to be a convenient, safe, and readily available bone graft substitute that yields consistently successful results.[20] CS can function as a resorbable barrier and as a resorbable space filler.[21] CS pellets plus decompression bone produced a bone regeneration equivalent to autologous crestal bone in a majority of patients with spinal fusion, and CS may be a viable alternative to autologous bone.[22] In 21 out of 23 patients with a benign bone lesion grafted with CS, bone repair 1 year postoperatively occurred at a rate of between 76% and 100%.[6] Moreover, implants of CS were the osteoconductive equals of autologous iliac crest bone/marrow preparations.[23] Ricci et

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FIGURES 4–6. FIGURE 4. Defect filled with calcium sulfate (CS) and covered with e-PTFE membrane. Newly formed vessels (arrows) accompanied by loose perivascular connective tissue are observable in contact with the new bone (B). FIGURE 5. Defect filled with CS. Some blood vessels (arrow) are found. B = bone. FIGURE 6. Defect filled with autologous bone. Preexisting blood vessels (v) and newly formed vessels (arrow) are visible. Toluidine blue and acid fuchsin (magnification ×40).

TABLE 1

Number of blood vessels

Group 1	Group 2	Group 3
12	8	1
15	5	7
16	4	5
8	7	7
12	8	6
8	8	2
5	5	8
10	7	3
13	8	7
10	12	5
6	8	5
5	5	6
5	7	4
21	8	7
21	5	3
8	9	5
5	10	6
7	9	4
8	8	5
12	10	7
8	9	8
5	9	7
7	8	6
8	10	7
12	11	8

TABLE 2

Number of microvessels

	Group 1	Group 2	Group 3
Mean	9.88	7.92	5.56
Standard deviation	4.613	1.998	1.895
Standard error	0.9225	0.3997	0.3789

al[24] found that CS acts as more than a simple space filler that prevents soft tissue ingrowth; in fact, as it dissolved, CS left behind a consistent latticework of an hydroxyapatite calcium-phosphate mineral, which was stable in the long term and acted as an osteoconductive trellis for new bone formation.

Our results show that the positive results of CS in bone regeneration reported in the literature may be due not only to the fact that CS mimics the mineral phase of bone, resorbs at the rate of bone formation, provides a structural support, prevents the ingrowth of fibrous tissue,[25] allows an earlier ingress of osteoprogenitor cells, and provides a barrier effect limiting the ingrowth of connective tissue from adjacent periosteum,[26] but also to the fact that it seems to stimulate the formation of blood vessels, which has been found to be extremely important in bone formation. Thus, our findings can add angiogenesis as a further action of CS, leading to a better understanding of all the factors that improve osteogenic processes. Moreover, the significance of our results after 4 weeks suggests that the advantages of CS occur at an early time, which is of primary relevance for bone regeneration purposes. Further research will be carried out to investigate what would happen at different time intervals.

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