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A STEREOLOGICAL ANALYSIS OF THE HETEROTOPIC OSTEOGENESIS INDUCED BY ALLOGENIC BONE MATRIX GRAFTS IN RAT SUBCUTANEOUS TISSUE

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ABSTRACT

The subcutaneous cellular and tissue alterations that occur during heterotopic osteogenesis induced by a demineralized bone matrix graft in rats were studied morphometrically. Diaphysis segments obtained from 42 rat femurs were cleaned and demineralized with 0.6 M HCl. Each segment was filled with a blood clot and implanted into the back of rats (1 segment/rat). Histological specimens were collected 2, 5, 7, 14, 21, 28 and 49 days after implantation (6 rats/period) and processed after demineralization. Qualitative and morphometric analysis of the histological sections showed that the external blood clots and those filling the medullary canal were replaced with connective tissue within 2-5 days post-implantation. After 14 days, 11.2% and 0.5% of the implanted matrix was replaced with areas of resorption and hyaline cartilage, respectively. After 21 days, 13.7%, 2.4% and 2.7% of the graft matrix showed areas of resorption, cartilage tissue and bone tissue, respectively. At the end of the 49-day study period, 20.2%, 7.8% and 14.5% of the matrix was replaced with areas of resorption, bone tissue and hematopoietic tissue, respectively. Bone induction in rat subcutaneous tissue occurred only at the site of allogenic matrix implantation. The quantity of neoformed cartilage, bone and myeloid tissue depended on the volume of matrix implanted and on the microenvironmental conditions, and was regulated temporally by a cascade of events mediated by bone morphogenetic proteins (BMPs) presents in the graft. These results also show that morphometric methods may be useful for assessing osteoinduction at ectopic sites.

Key words: Allogenic bone graft, ectopic osteogenesis, morphometry, rat

INTRODUCTION

Injured bone tissue has a high potential for spontaneous regeneration [3]. The pioneering discovery by Urist [27] that allogenic organic bone matrix can induce the formation of new bone tissue at heterotopic sites allowed the cellular and molecular basis of this regeneration to be established. Organic bone matrix is a reservoir of various growth factors (PDGF, EGF, IGF and TGF) and of morphogens known as bone morphogenetic proteins which are the molecular mediators of bone differentiation, maintenance and repair [24].

The implantation of allogenic demineralized bone matrix in rat subcutaneous tissue leads to a series of biochemical and morphogenetic events that culminates in the formation of new bone at the site of implantation as a result of endochondral ossification [20]. Since this allogenic matrix is not a vital tissue, it will gradually be resorbed and replaced with new bone tissue. In this case, the transplanted tissue serves as a framework for new bone formation and also exerts a stimulating action on bone cell differentiation through the release of bone morphogens present in the tissue during the process of resorption [21]. This phenomenon currently provides the basis for the increasing use of allogenic bone matrix obtained from bone banks and of xenogenic matrix in the treatment of perennial bone lesions, especially in the cranio-maxillo-facial region.

The osteoinductive ability of allogenic bone matrix varies with donor age, physiology and pharmacological status, as well as with a variety of other factors related to the processing and sterilization protocols used by different bone banks [4]. In the last decade, rat and nude mice ectopic models have been used to evaluate the osteoinductive potential of commercial demineralized bone allografts [4,7,8].

Although the cellular events that occur during ectopic osteogenesis in subcutaneous tissue are well established, no morphometric studies that better

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quantify the bone formation have been done. The aim of this study was to use morphometric methods to evaluate the time course of graft matrix disappearance and to quantify the amount of new cartilage and bone matrix formed in relation to the process of ectopic ossification induced by the allogenic matrix.

MATERIAL AND METHODS

Seventy male Wistar rats 60 days old and weighing about 180 g were obtained from the Central Animal House of Bauru Dental School and received standard pelleted rodent Purina chow and water *ad libitum* throughout the experiment. The rats were divided into two groups: group I consisted of 28 rats that provided the femurs used for the preparation of the demineralized bone matrix (allogenic graft) and group II consisted of 42 rats that were surgically implanted with a graft in the dorsal subcutaneous tissue and sacrificed 2, 5, 7, 14, 21, 28 and 49 days after implantation (6 rats/period).

Preparation of the allogenic demineralized bone matrix

The femurs removed from group I rats were cleaned, stored at -18ºC, demineralized in 0.6 M HCl (under radiographic control), neutralized in 0.9% saline, and stored in 70% ethanol for three days [31]. A cylindrical block measuring 8 mm in length by 3mm in outer diameter (mean weight of 36 ± 0.94 mg) was obtained from each demineralized femur.

Surgical procedures for matrix implantation

Under general anesthesia following an intraperitoneal injection of ketamine/xylazine (Agribrands do Brasil Ltda, Paulínia, SP, Brazil), all rats in group II underwent a dorsal trichotomy and vigorous disinfection of the region with iodophor alcohol. A contralateral incision was then made in the dorsal skin of the rat with a nº. 15 surgical knife and subcutaneous pouches were created by blunt dissection. The cylindrical block of allogenic graft soaked in blood obtained by cardiac puncture was then implanted in the pocket formed. The skin was subsequently closed with no. 4 Ethicon sutures (Johnson & Johnson, São José dos Campos, SP, Brazil).

Histological procedures

The rats were sacrificed with an overdose of anesthetic on days 2, 7, 14, 21, 28 and 49 after implantation, and the implanted graft along with the surrounding tissue was collected and fixed in 10% formalin in phosphate buffer for one week. After demineralization in formic acid and sodium citrate [11], the specimens were dehydrated in ethanol, cleared in xylene and embedded in Histosec-Merck (paraffin + synthetic resin) melted at 60ºC. Alternating sections 5 μm thick were stained with hematoxylin-eosin (HE).

Stereological analysis

The volume density (Vvi) or the fraction of graft volume occupied by the demineralized bone matrix, the area of resorption, cartilage tissue, bone tissue and myeloid tissue were determined with a digitized image system consisting of a Zeiss Axioskop 2 microscope, a Sony CCD-IRIS-RGB camera and Kontron KS-300 software (Kontron Electronic GmbH) installed on an IBM computer. Using a 100X immersion objective, images from 25 histological fields per rats, selected by systematic randomization, were captured. In these images, the area occupied by each type of structure (Ai) and the total area analyzed (A) were determined. The density of the area of each type of structure (AAi), which corresponded to its volume density (Vvi), was calculated according to the relation AAi = Vvi = Ai/A [36].

Statistical analysis

The data were tested for normality using the Kolmogorov-Smirnov test and comparisons between groups were done by analysis of variance (ANOVA) and pairwise multiple comparison procedures (Student-Newman-Keuls test) using Sigma Stat software (Jadel Corporation, Chicago, IL, USA), with the level of significance set at 1% and 5%. Volume densities were compared after arcsin transformation of the original data.

RESULTS

Postoperative follow-up showed no signs of infection in the surgical area in any of the rats. Some rats presented moderate edema that disappeared completely a few days later with no suppuration or opening of the incision.

Morphological results

At two days post-surgery, the graft was surrounded by blood clots and by an acute inflammatory infiltrate containing a large number of polymorphonuclear neutrophils; the old medullary canal was also occupied by blood clots. Within the graft matrix, the spaces corresponding to the nutrient canals of old bone were occupied by cells of the surrounding inflammatory infiltrate. Five days after implantation (Fig. 1A,B), the internal part of the graft matrix was histologically similar to that observed after two days. However, on the external surface, fibrous connective tissue had begun to form a capsule rich in cells and with collagen fibers arranged in parallel to the graft surface. Fibrin nets and many scattered blood cells were still seen in the old medullary canal, as well as a mass of fibroblast-like cells close to the newly formed blood vessels. After seven days, the matrix was surrounded by a thick fibrous capsule rich in blood capillaries and elongated fibroblast-like cells organized in layers parallel to the matrix. The external graft surface already showed areas of resorption containing numerous cells with a fibroblast-like morphology and the old medullary canal was occupied by richly vascularized and cellularized loose connective tissue. At 14 days post-implantation (Figs. 2A-D), numerous areas of mononuclear cell-mediated resorption were observed on the external and internal

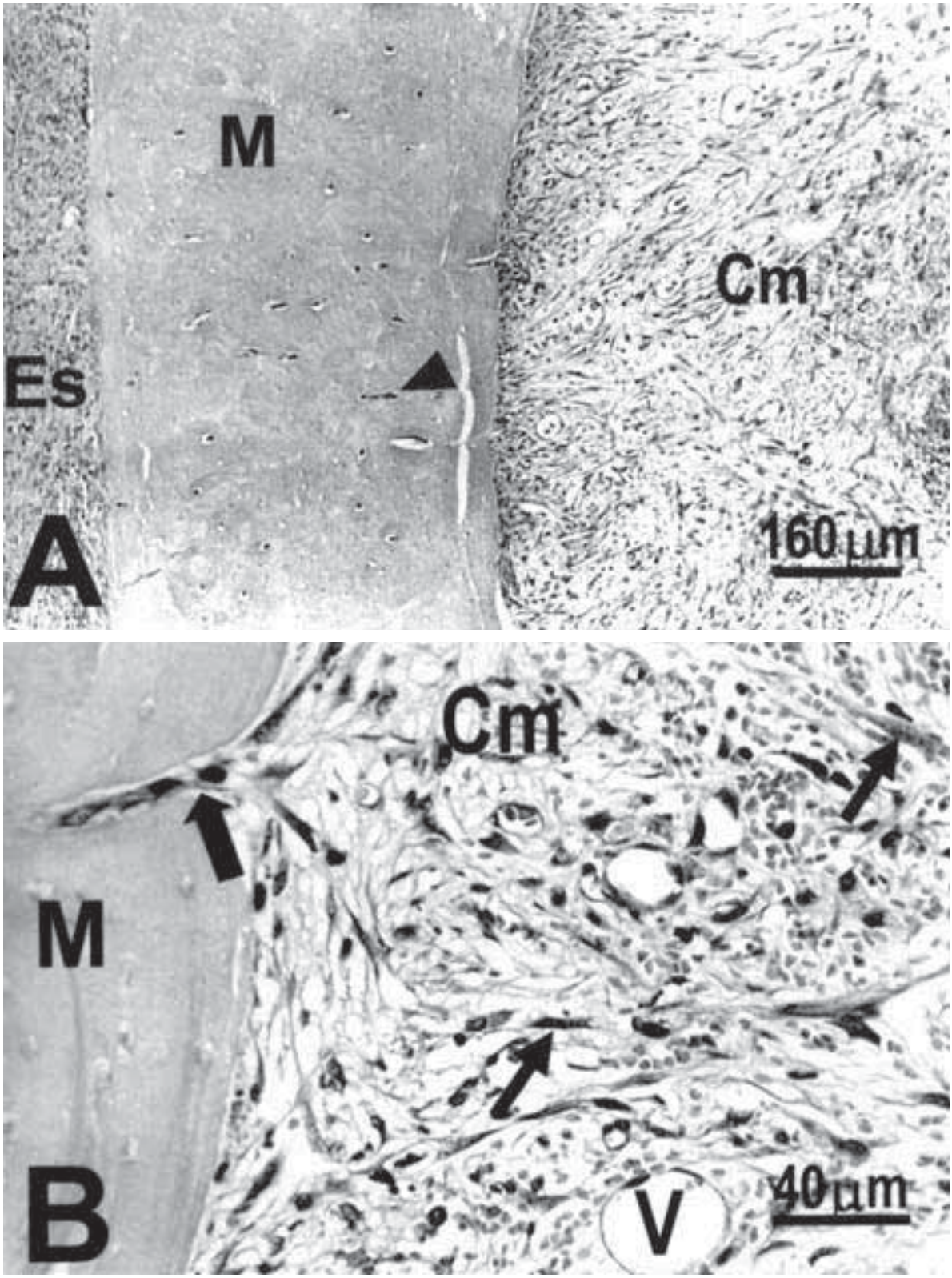

Figure 1. Five days after implantation of an allogenic graft. **A)** Panoramic view of the bone matrix (**M**) showing the old nutrient canals (**arrowhead**) occupied by mononuclear cells, the external surface (**Es**) surrounded by a capsule of connective tissue and the old medullary canal (**Cm**). **B)** Detail of the old medullary canal (**Cm**) filled with connective tissue rich in cells with a fibroblast-like morphology (**thin arrows**), capillaries (**V**) and remains of blood clots. Fibroblast-like cells are also present in the bone matrix canal (**thick arrow**), HE.

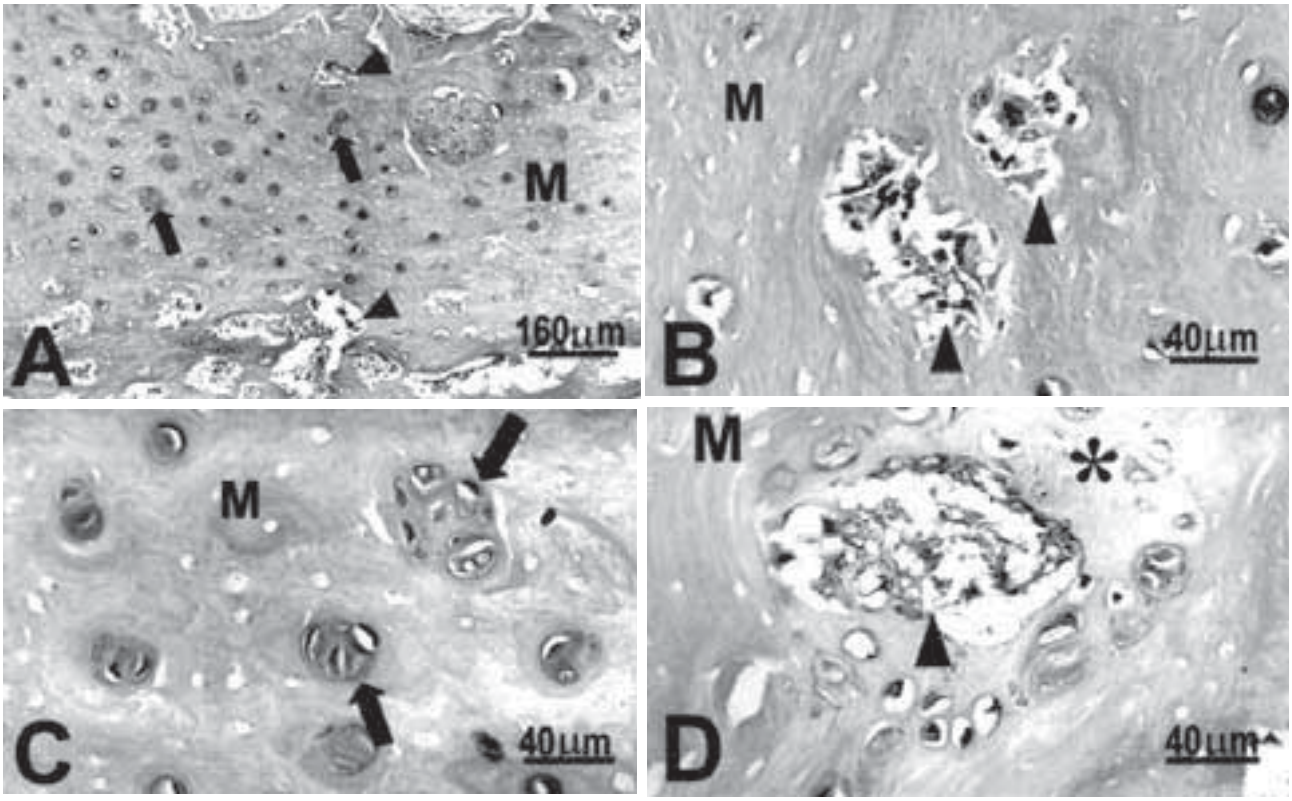

Figure 2. Fourteen days after implantation of an allogenic graft. **A)** Panoramic view of the bone matrix (**M**) showing areas of matrix reabsorption (**arrowheads**) and numerous old nutrient canals (**large arrows**) filled with chondrocyte-like cells. **B)** Detail of matrix (**M**) showing reabsorption by mononuclear cells (**arrowheads**). **C)** Detail of the small islets of chondrocytes in old nutrient canals (**arrows**), and **D)** Reabsorption areas (**arrowhead**) of neoformated cartilage tissue (*****), HE.

surfaces and within the graft matrix. Intensely basophilic, chondrocyte-like cells were seen within the nutrient canals of old bone, and some areas of cartilaginous extracellular matrix exhibited coronary isogenous groups, hypertrophied chondrocytes and reabsorption of calcified matrix. The old medullary canal was filled with loose connective tissue. At 21 days post-surgery (Fig. 3A,B), the implanted matrix showed a large increase in the area of resorption. Newly formed bone matrix covering the resorbed matrix surface was observed in various areas of resorption; in these cases, the space of the resorption area was occupied by red myeloid tissue. Between 28 and 49 days post-implantation, the histological picture was characterized by the gradual disappearance of cartilaginous tissue and increase in the areas of resorption and an increase in newly formed bone and myeloid tissue.

Morphometric results

The volume density results obtained for the components involved in the process of graft resorption and in new bone formation during the different periods are shown in Figure 4.

The volume density of the implanted matrix declined from 100% to 57% ($p<0.01$) during the period from 5 to 49 days post-surgery, while the volume density of the resorption areas increased from 3% to 21% ($p<0.01$) between 7 and 49 days after implantation. The volume density of cartilage tissue showed a biphasic evolution. During the first phase, from 14 to 21 days post-implantation, there was an increase from 1.5% to 4% ($p<0.01$), while during the second phase (from day 21 to day 49), this tissue almost completely disappeared to reach a volume density of 0.08% at the end of the period. In contrast, the volume density of bone tissue increased from 3% to 8% ($p<0.01$) between 21 and 49 days postsurgery. This increase coincided with the decline in the volume fraction occupied by cartilage tissue.

DISCUSSION

Since the pioneering discovery of Urist [27,32] in the 1960s, numerous studies have demonstrated the osteoinductive capacity of allogenic bone matrix grafts at heterotopic and orthotopic sites in laboratory

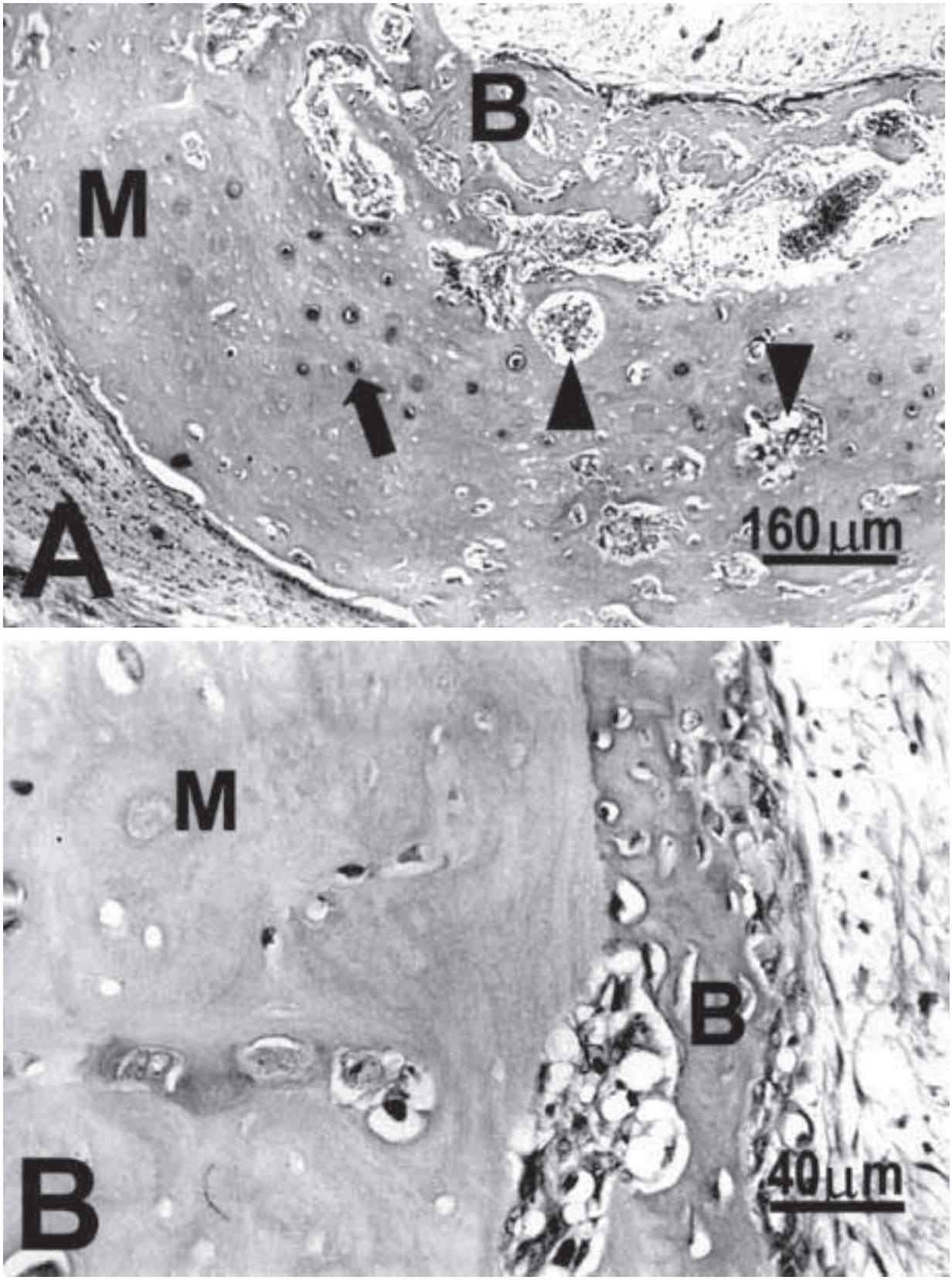

Figure 3. Twenty one days after implantation of an allogenic graft. **A)** Panoramic view of bone matrix (**M**) showing large areas of matrix reabsorption (**arrowheads**), a few nutrient canals (**large arrow**) filled with chondrocyte-like cells, and neoformed bone tissue (**B**). **B)** Detail of new bone (**B**) inside the matrix (**M**), HE.

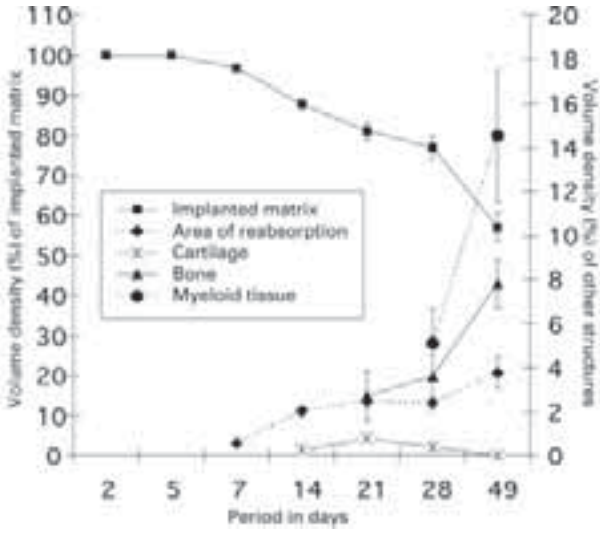

Figure 4. The volume density (%) of implanted matrix, area of reabsorption, cartilage, bone and myeloid tissue during the post-implantation periods. The points are the mean ± SEM of six rats.

animals [13,33,35]. This inductive capacity of bone matrix is due to the presence of soluble matrix proteins known as bone morphogenetic proteins (BMPs) or osteoinductive proteins (OP), which show a high capacity to stimulate the morphogenetic phase of osteogenesis, leading to the cytodifferentiation of osteoblasts and new bone formation [2,10,26,29].

These discoveries have resulted in the use of allogenic bone matrix previously subjected to special demineralization and preparation procedures to completely eliminate matrix cells and antigenic determinants, as graft material for inducing new bone formation in permanent bone defects or defects that are difficult to repair [12,30,31]. Despite major advances in the molecular biology of bone regeneration, this alternative treatment continues to be widely used, especially in dentistry. Experimental osteoinduction at heterotopic sites has been used not only to evaluate the osteogenic potential of bone matrix as graft material but also as a model for studying osteogenesis, including the factors involved in new bone formation and in the regulation of bone metabolism (see reviews by Reddi [19,20]). The genes for bone morphogenetic proteins have been cloned and recombinant proteins have been used to explore the mechanism of action in ectopic models [16,18].

The cellular events that occur during heterotopic osteogenesis induced by allogenic demineralized bone matrix in rat subcutaneous tissue have been studied in detail by Reddi and Anderson [21]. In their study, histological analysis revealed invasion of the area by an inflammatory infiltrate consisting of polymorphonuclear neutrophils on the first day after implantation of the particulate matrix. After 3-4 days, fibroblast-like cells were already observed around and within the graft canals. Chondrocyte differentiation had started by day 5, with a marked increase in the number of chondrocytes being observed 7-8 days after graft implantation. Neovascularization, hypertrophy and calcification of the hyaline cartilage formed started on day 9 post-implantation. On day 10, there was generalized chondrolysis and the presence of bone matrix-producing osteoblasts. The histological picture 12 days post-implantation was characterized by the almost complete disappearance of cartilage and the beginning of bone remodeling. Foci of myeloid tissue were seen as early as the end of 16 day.

Although studies such as that above have described the morphological and molecular events involved in the osteogenesis induced by allogenic grafts in subcutaneous tissue, there was no stereological assessment of these events.

In the present study in which relatively large femur diaphysis pieces were used as graft material, the first five days after implantation were marked by blood clot resorption and by an initial acute inflammatory reaction followed by vascular and cellular invasion. According to Reddi [22] and Kale *et al.* [9], this inflammatory reaction characterized by the recruitment, activation and interaction between polymorphonuclear leukocytes and the graft matrix represents a critical factor for activation of the cascade of events leading to osteogenesis. For this reason, some investigators have questioned the use of anti-inflammatory drugs such as indomethacin and corticoids during the initial post-implantation period since these drugs reduce the initial inflammatory response and, consequently, the migration and proliferation of undifferentiated mesenchymal cells essential for osteoinduction and osteoblast differentiation [6,14,17,22]. In the present study, none of the rats required anti-inflammatory drugs or antibiotics.

The acute inflammation had practically disappeared by day 7 post-implantation and was replaced with cell- and blood vessel-rich connective tissue, which was more fibrous outside and looser inside the medullary canal, and closely resembled the mesenchymal tissue observed by others [22,27, 28,32,]. With respect to the graft, only 3% of its total volume had been resorbed by mononuclear cells. This resorption, which became more intense after the first

week post-implantation, was always associated with the presence of mononuclear cells and the absence of multinucleate giant cells. Morphologically, these cells showed fibroblast characteristics upon light microscopy. Fibroblasts have a high capacity to secrete matrix metalloproteinase-1 (MMP-1), a potent collagenase able to degrade structured collagen I and other MMPs that act as gelatinases during collagen turnover [23].

At 14 days post-implantation, the areas of external and internal resorption already occupied 11% of the graft matrix volume, i.e., a 2.6-fold increase compared to the previous period. This period was marked by the presence of hyaline cartilage filling the old matrix nutrient canals, with practically no resorption on the internal surface; this cartilage occupied 2% of the graft volume. In relation to the formation of this tissue, BMPs present in the bone matrix can induce the formation of new bone by endochondral ossification. This formation of new heterotopic bone in subcutaneous or intramuscular connective tissue stimulated by BMPs mimics bone development during embryonic and fetal life [5,24].

The BMPs are intimately bound to collagen and other binding proteins such as noggin and chordin of demineralized extracellular matrix. These soluble morphogens act in the cells through an autocrine or paracrine mechanism, binding to two types of transmembrane serine/threonine kinase receptors, that transduce the signal to the nucleus through Smad proteins. After translocation to the cell nucleus, Smad proteins regulate the transcription of target genes binding directly to DNA, interacting with other DNA binding proteins, and recruiting transcriptional co-activators or co-repressors, with specific effects in various cell types (for reviews see [1,34]). In addition, the signals mediated by Smad proteins are also positively or negatively controlled by cross-talk with other hormone, growth factor or cytokine signaling pathways, thereby modulating the biological actions of BMPs [15]. Thus, the inflammatory response results in by-products that interact with BMPs and affect their biological potential [25].

The quantity of hyaline cartilage was maximal by day 21 post-implantation and occupied 4% of the graft volume. After this period, the amount of cartilage tissue and the number of chondrocytes declined drastically, and had almost completely disappeared by day 49. The peak level of cartilaginous tissue seen on day 21, was accompanied by a relatively large amount of newly formed bone tissue close to the resorption areas; this tissue occupied 3% of the graft volume. During subsequent periods, concomitant with the disappearance of hyaline cartilage and enlargement of the resorption areas, the amount of bone tissue increased significantly by 2.86-fold to occupy 8% of the graft volume after 49 days.

By day 28, relatively large amounts of myeloid tissue were present in areas of new bone formation. This tissue occupied 5% of the graft volume, but increased significantly to 14% by day 49 post-implantation.

One unexpected finding was the absence of cartilage formation in the old medullary canal during throughout the experiment. In addition, the presence of bone and myeloid tissue at the resorbed internal surface of the graft matrix and the connective tissue filling the old medullary canal was seen as early as 14 days post-implantation, indicating that the osteogenesis resulted from intramembranous ossification with no previous formation of hyaline cartilage.

The present results indicate that bone formation in rat subcutaneous tissue occurred only at the site of allogenic matrix implantation and that the quantity of cartilage, bone and myeloid tissue formed depended on the volume of matrix implanted and on the micro-environmental conditions. This bone formation was limited temporally by a cascade of events mediated by BMPs. These findings also show that morphometric methods may be useful for measuring osteinduction at ectopic sites.

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