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Collagen-hydroxyapatite composite enhances regeneration of calvaria bone defects in young rats but postpones the regeneration of calvaria bone in aged rats

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Abstract The potential differences in bone repair of calvaria defects treated with a collagen sponge (HELISTAT®) or a collagen-hydroxyapatite composite (HEALOS®) in young and aged rats were evaluated at 8 weeks after surgery. A histomorphometric analysis of new bone formation and an evaluation of angiogenesis, mast cell, and eosinophil local infiltration were performed. Evaluation showed that HELISTAT® induced a similar amount of new bone in both young and aged rats. However this occurred to a lesser degree than in young rats treated with HEALOS®. The largest number of blood vessels was present in the defects of aged rats treated with HEALOS®, and the number of mast cells was highest in the defects treated with HELISTAT® in both young and aged rats. Eosinophils were present to the greatest extent in defects treated with HEALOS® in comparison to defects treated with HELISTAT® in both young and aged rats. Collagen-hydroxyapatite composite (HEALOS®) enhances calvarial bone repair more than collagen sponge alone (HELISTAT®) in young rats but not in aged rats at 8 weeks after surgery. HEALOS® appears to induce a more intense inflammatory response than HELISTAT® especially in aged rats.

1 Introduction

The major extracellular matrix components of bone are collagen type I and a substituted hydroxyapatite (HA). Therefore, these two components have been extensively studied as biomaterials of choice to facilitate bone repair and regeneration. Justification for the widespread use of collagen is attributed to its low immune response, low toxicity, and its ability to promote cellular growth and attachment [1]. Collagen is used in a variety of physical forms such as sheets, sponges, powders, and fleeces for soft tissue augmentation, burn and wound dressing, or as haemostatic agents [1]. HA have been used as implants in bone implantation for many years. However, HA is not bioabsorbable, and has a low rate of substitution, as a result loosening or breaking of HA implants often occurs in vivo [2, 3]. Both collagen and HA have been reported to enhance osteoblast differentiation [4], but together, they were shown to accelerate osteogenesis [5]. As a consequence, hydroxyapatite-collagen composites may have the potential to mimic and replace skeletal bones [6]. Thus, combining both collagen and HA should be advantageous over other biomaterials to induce and facilitate bone formation.

Two commercially available collagen scaffolds, HELISTAT® (Integra Life Sciences Corp., Plainsboro, NJ) and HEALOS® (DePuy Spine, Inc., Raynham, MA), were used in this study to treat bone defects of rat calvaria. The HELISTAT® is used in surgical procedures to help control bleeding. It controls bleeding within 2–5 min. The HEALOS® is intended for use in filling bony voids or gaps of the skeletal system (the extremities, spine and pelvis) that are not intrinsic to the stability of the bony structure. These defects may be surgically created osseous defects or osseous defects resulting from traumatic injury to the bone.

The addition of collagen to a ceramic structure can provide many additional advantages to surgical applications such as shape control, spatial adaptation, increased particle and defect wall adhesion, and the capability to favor clot formation and stabilization [7]. A tissue response to an implanted biomaterial is influenced by many factors including age and site of implantation, and as such is

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mainly a wound healing process. Wound healing is a highly ordered series of events that includes hemostasis, inflammatory cell infiltration, tissue regrowth, and remodeling. It is well known that there is an age-related decline in the rate of repair.

Mast cells are found in the majority of the organs and tissues of the body, particularly in association with connective tissue structures such as blood vessels, lymphatic vessels and nerves [8]. The mast cell reaches the tissues from the bone marrow through the systemic circulation as an undifferentiated progenitor cell, which acquires its morphological and functional characteristics peripherally. To date, mast cells are recognized to be involved both in inflammation, and wound healing. The cells are attracted to and invade the local site of injury at different time points during healing, whether in the acute or in the later phases. Mast cells control the key events in wound healing including deposition of temporary connective tissues and subsequent remodeling of the matrix support [9, 10].

The objective of this study was to evaluate the osteoconductive potential of two different collagen biomaterials, produced in the presence or absence of HA, in young and aged rats using a critical-size calvarial defect model. Quantitative and qualitative evaluation of new bone formation, mast cell and eosinophil infiltration, angiogenesis, and scaffold degradation was assessed.

2 Materials and methods

The HELISTAT® (Integra Life Sciences Corp., Plainsboro, NJ) is an absorbable collagen haemostatic sponge produced from bovine deep flexor (Achilles) tendon. No data from the HELISTAT® manufacturer is available concerning the pore size. The HEALOS® (DePuy Spine, Inc., Raynham, MA) is an absorbable mineralized collagen. The HEALOS® is an osteoconductive matrix constructed of cross-linked collagen fibers that are fully coated with hydroxyapatite. The principal components of HEALOS® are type I bovine collagen and hydroxyapatite [$Ca_{10}(PO_4)6(OH)_2$]. The scaffold contains approximately 30% mineral by weight with pore sizes of 4–200 μm. The HEALOS® manufacturer does not define the exact source of the bovine type I collagen.

2.1 Experimental design

Eight Sprague–Dawley young rats (male, 12 weeks old, ~350 g) and eight Sprague-Dawley aged rats (male, 56 weeks old, ~800 g) were used. The rats were kept under uniform conditions for a period of least 1 week before commencement of the experiment. Free access to water and standard pelleted food was provided throughout the experiment. The experiment was approved by the Research Ethics Committee of Karolinska University—Huddinge Hospital in accordance with the policy on humane care and use of laboratory animals.

The rats were anaesthetized using isoflurane inhalation (4% induction, 2–3% maintenance). The rat's head was shaved and washed with iodine solution and an incision was made in the sagital plane across the cranium. The skin and underlying tissues including the temporal muscle were detached to expose the calvarial bone. An 8-mm complete circular defect was created on the left parietal region using a saline-cooled trephine drill (Fig. 1). The defect was covered with a disk of mineralized collagen HEALOS® (8-mm diameter, 2.5 mm thickness) or with a disk of collagen sponge HELISTAT® (8-mm diameter, 3.5 mm thickness). The incisions were closed with single sutures in two layers.

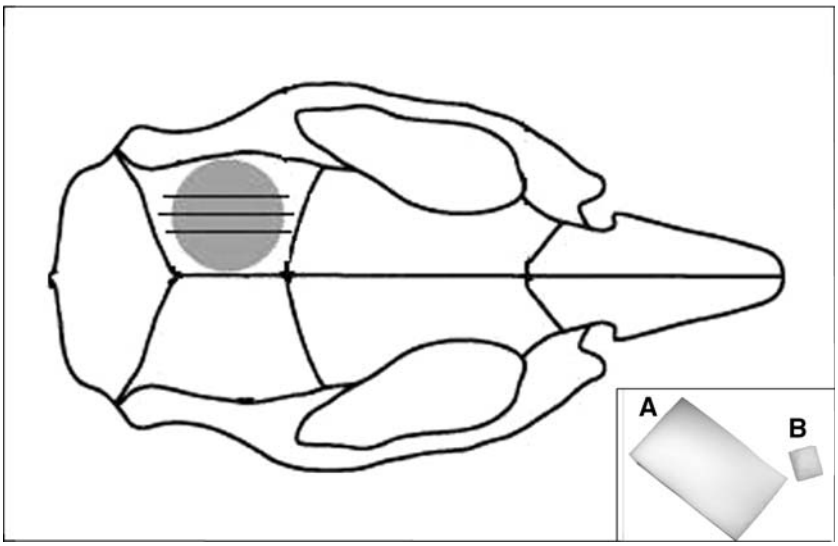

Fig. 1 Schematic drawing of the parietal bone defect in rat calvaria, and general views of collagen scaffolds (**a**) HELISTAT®, (**b**) HEALOS®. An 8-mm complete circular defect was created in the left parietal bone. Five-μm serial sections were prepared parallel to the sagittal line. Three central sections from each specimen stained with HE were used for the measurement of new bone areas, whereas two central sections from each specimen stained with Giemsa-Wright were used for the counting of blood vessels, mast cells and eosinophils

Four rats from each age group were treated with HELISTAT® while another four rats from each age group were treated with HEALOS®.

2.2 Tissue preparation

All rats were euthanized by CO_2 inhalation at 8 weeks after surgery. Calvaria bone was surgically removed, decalcified for ten days in 20% formic acid solution and embedded in paraffin; 5-μm serial sections were prepared parallel to the sagittal line (Fig. 1), and were placed on glass slides. Sections were stained with hematoxylin–eosin (HE) for assessment of general morphology and osteogenesis, or with Giemsa-Wright for evaluation of angiogenesis, eosinophil and mast cell infiltration (blood vessels stained deep blue, eosinophil granules—red-pink, and mast cells—purple). The most central region of each defect was identified and subject to histological and histometrical analysis.

Three central sections from each specimen stained with HE were used for the measurement of new bone areas using image-analysis software (Adobe Photoshop® CS2, Adobe Systems Incorporated, San Jose, California, and ImageJ, National Institutes of Health, USA). Furthermore, two central sections from each specimen stained with Giemsa-Wright were used for the counting of blood vessels, mast cells and eosinophils. All these components were counted twice in a blinded fashion and were expressed as the number per tissue section. The data was statistically analyzed using the Mann-Whitney U-test, and significance was defined as $p < 0.05$.

3 Results

Rats survived well the post-operative period, with the exception of one aged rat treated with HELISTAT®, which died at 6 weeks after surgery for unknown reasons.

3.1 Osteogenesis

Intramembranous bone formation was induced by both collagen sponge HELISTAT® and mineralized collagen HEALOS®. Islands of bone tissue occupied the former defect area (Fig. 2). However complete bone repair did not occur in any of the rats. We discovered that HELISTAT® induced more new bone in the young rats than in the aged rats (Fig. 3), although this was not statistically significant. The HEALOS® treated young rats formed a statistically significant larger amount of new bone than the HEALOS® treated aged rats and the HELISTAT® treated young rats (Fig. 3). The smallest amount of new bone was formed in aged rats treated with HEALOS®.

Over the duration of the experiment the collagen sponge HELISTAT® was almost completely reabsorbed (Fig. 4), although traces of collagen sponge were detected in both aged and young rats. A few giant multinuclear cells were found attached to the remnants of the collagen sponge. In comparison to the collagen sponge HELISTAT®, many remnants of mineralized collagen HEALOS® were present in young rats, but particularly so in the aged rats (Fig. 5). The remaining biomaterial made tight contact with the newly formed bone. Furthermore, a large number of giant

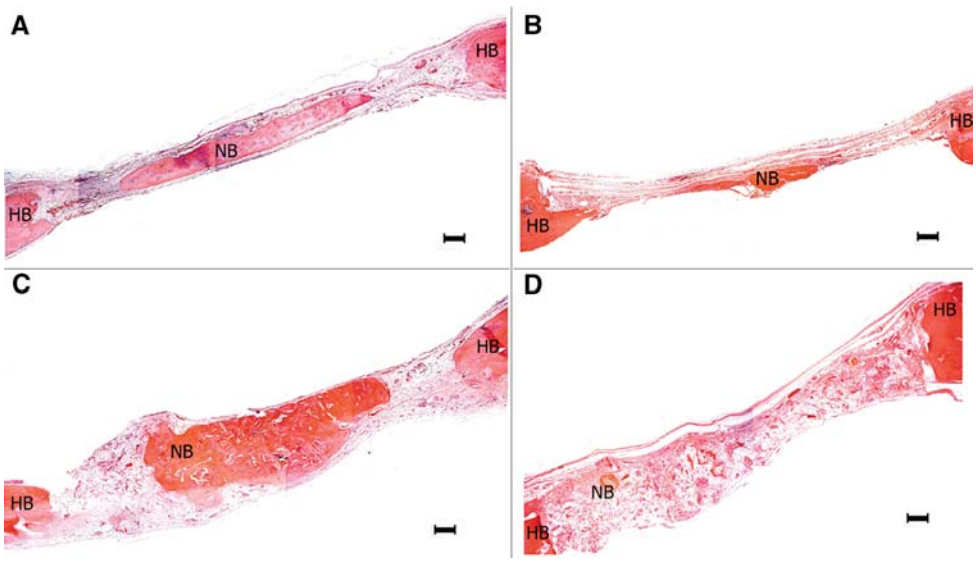

Fig. 2 Histological images stained with HE, illustrating the general morphological aspect of the calvarial defect repair. Intramembranous bone formation was induced by both HELISTAT® and HEALOS®. Islands of bone tissue occupied the defect area. However no complete bone repair has occurred in any of the rats. (**a**, **b**) Treated with HELISTAT®. (**c**, **d**) Treated with HEALOS®. (**a**, **c**) Young rats. (**b**, **d**) Aged rats. NB, new bone; HB, host calvarial bone. Bars—200 μm

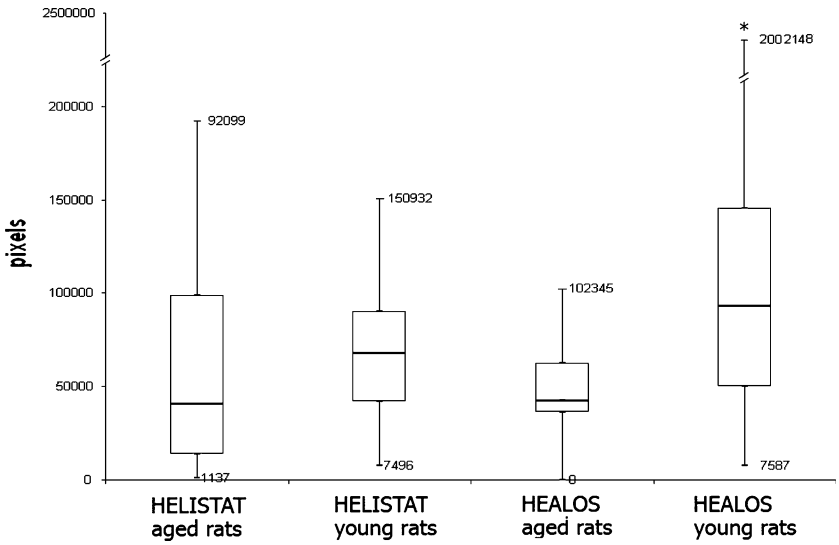

Fig. 3 Box plots (maximum, third quartile, median, first quartile, minimum) of the regenerated bone area (pixels per tissue section) in different experimental groups. Twelve tissue sections from each experimental group were analyzed with the exception of the HELISTAT® treated aged rats from which nine sections were analyzed. The HEALOS® treated young rats formed a statistically significant larger amount of new bone than HEALOS® treated aged rats and HELISTAT® treated young rats. Significant levels are marked with *

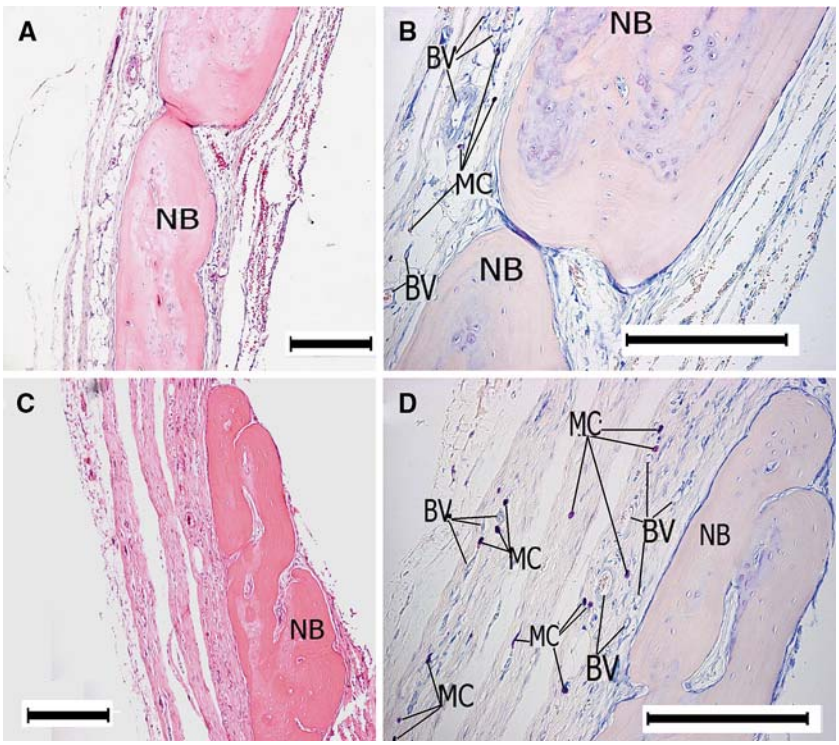

Fig. 4 Histological images taken from the central area of calvarial defects treated with HELISTAT®. The HELISTAT® was almost completely reabsorbed in both young and aged rats. The calvarial defects of rats treated with HELISTAT® exhibited a greater number of mast cells than those treated with HEALOS®. (**a**, **b**) Young rats. (**c**, **d**) Aged rats. (**a**, **c**) Stained with HE. (**b**, **d**) Stained with Giemsa-Wright. NB, new bone; BV, blood vessels; MC, mast cells. Bars—200 μm

multinuclear cells were also attached to the residual biomaterial.

3.2 Angiogenesis, mast cell and eosinophil infiltration

The number of blood vessels present in the calvarial defect of aged rats treated with HEALOS® was significantly higher in comparison to young rats treated with HEALOS® or rats treated with HELISTAT® (Fig. 6).

Mast cells were mainly present at the epicranial site of the calvarial defect and more often located in the vicinity of blood vessels. The number of mast cells found in the calvarial defect of aged rats treated with HELISTAT® was significantly higher than in aged rats treated with HEALOS® but not significantly higher than in young rats treated with HELISTAT® (Fig. 6).

Eosinophils were detected in the calvarial defects treated with HELISTAT® in both young and aged rats, but only to a small extent. In comparison, the remnants of

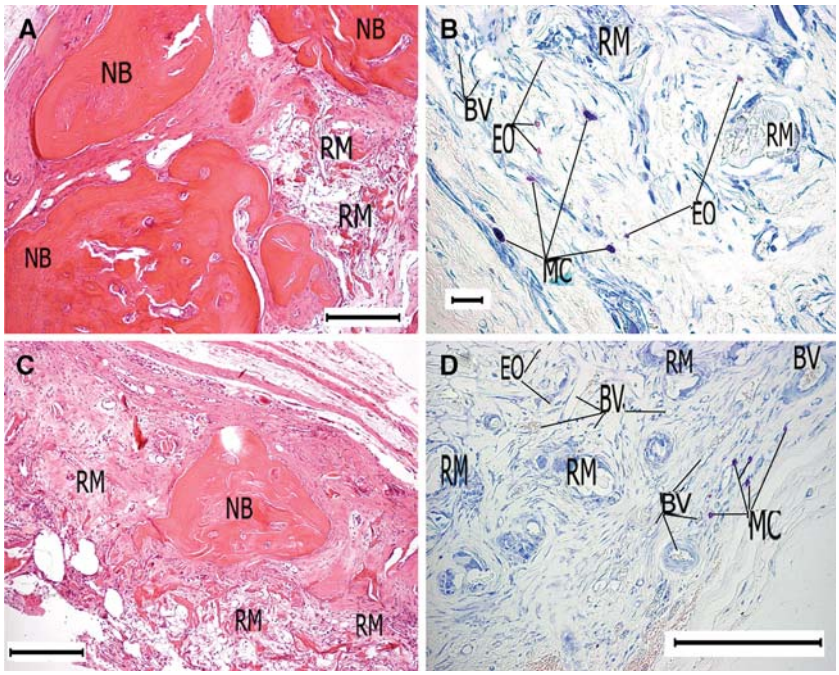

Fig. 5 Histological images taken from the central area of calvarial defects treated with HEALOS®. Many remnants of mineralized collagen HEALOS® were present in young rats, but particularly so in the aged rats. The remnants of HEALOS® material were infiltrated with eosinophils. (**a**, **b**) Young rats. (**c**, **d**) Aged rats. (**a**, **c**) Stained with HE. (**b**, **d**) Stained with Giemsa-Wright. NB, new bone; BV, blood vessels; MC, mast cells; EO, eosinophils; RM, residual biomaterial. Bars—200 μm (**a**, **c**, **d**), and 20 μm (**b**)

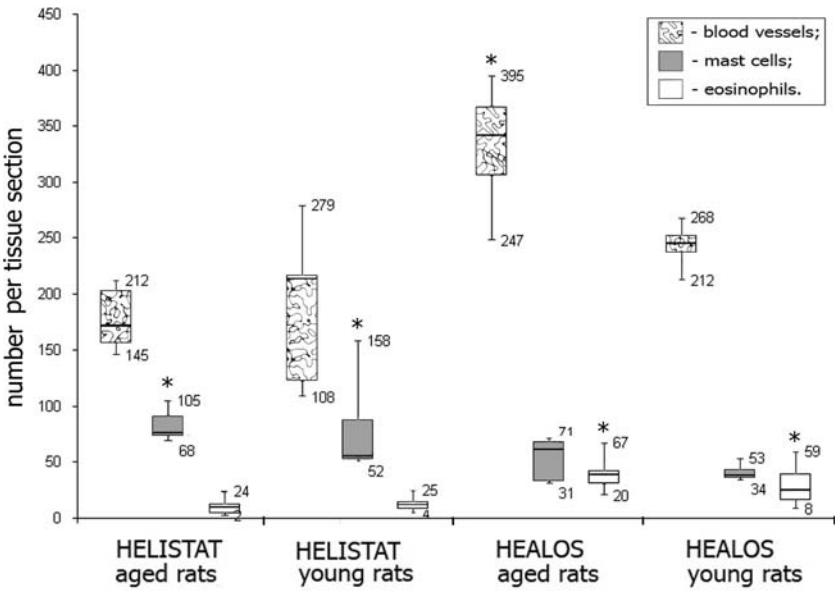

Fig. 6 Box plots (maximum, third quartile, median, first quartile, minimum) of the numbers of blood vessels, mast cells and eosinophils per tissue section in different experimental groups. Twelve tissue sections from each experimental group were analyzed with the exception of the HELISTAT® treated aged rats from which nine sections were analyzed. The number of blood vessels was the highest in the HEALOS® treated calvarial defect of aged rats, as well as the number of eosinophils. The highest number of mast cells was found in the HELISAT® treated calvarial defect of aged rats. Significant levels are marked with *

HEALOS® material were infiltrated with eosinophils to a greater degree (Fig. 6).

4 Discussion

The objective of this study was to evaluate the osteogenic potential and the biocompatibility of two different collagen biomaterials, produced in the presence or absence of HA, in young and aged rats using a critical-size calvarial defect model.

To date, only few studies have reported age-related differences in bone repair in calvarial defects. Gosain et al. [11] studied the contribution of the dura, the pericranium, and the adjacent calvarial bone in the process of calvarial regeneration in both mature and immature rabbits after creation of bilateral, 100-mm^2, parietal defects. They used one time point (12 weeks after surgery), and performed histometric analysis to quantitate the area of the original bone defect, new bone formation, and new bone density. Bone formation was quantified separately both at the periphery and in the center of the defects. The analysis

revealed that bone regeneration was incomplete in all groups over the 12 week study period. Dural bone production (center of the defects) was significantly greater in immature compared with mature animals. Within a mouse model for calvarial healing, Aalami et al. [12] made nonsuture-associated parietal defects 3, 4, and 5 mm in diameter in both juvenile (6-day-old) and adult (60-day-old) mice. The study used one time point at 8 weeks after surgery. Bone repair was analyzed radiographically and histologically, and the percentage of healing was quantified using an image software analysis of calvarial radiographs. The authors' analysis revealed that the calvarial defects in juvenile mice healed to a significantly greater degree than that of the adult mice. All three defect sizes (3, 4, and 5 mm) were found to be critical in the adult, whereas significant healing was seen regardless of the size of the defect in juvenile mice. The findings of both research groups were in accordance with our results; juvenile animals were able to repair their calvarial defects to a greater extent than the adult animals. However, the reason for such a difference is still unclear.

There are several studies reporting that the proliferating capacity and the differentiation potential of the osteoprogenitor cells are age dependent. Cowan et al. [13] investigated cellular and molecular differences between primary osteoblasts derived from juvenile (2-day-old) and adult (60-day-old) rat calvaria. They found that juvenile primary osteoblast cultures contained a larger number of immature cells, while adult primary osteoblast cultures included mostly mature cells. Moreover, they showed that juvenile osteoblasts proliferated and attached to culture dishes significantly faster than adult osteoblasts. One reason they found was that juvenile osteoblasts produced and organized cytoskeletal and extracellular matrix proteins necessary for increased attachment, proliferation, and signaling to a greater degree than adult osteoblasts. Bellow et al. [14] compared the number, proliferation and differentiation capacities, and the self-renewal ability of the osteoprogenitor cells derived from lumbar vertebras of aged (17–26-month-old) and young (1.5-month-old) female rats using limiting dilution analyses and continuous subculture experiments. They showed that the osteoprogenitors from the aged rats had a reduced capacity for self-renewal in vitro, which would translate into a reduced number of osteoblasts and a decrease in bone formation in aged animals.

In the present study, an 8-mm circular defect was created in parietal bone, which does not heal, spontaneously in young rats neither in adult rats. Smaller defects can heal spontaneously in young rats at 8 weeks after surgery making such defects unsuitable for the age-related evaluation of biomaterials. Larger defects would include interosseous suture making such defects difficult to compare.

The collagen sponge HELISTAT® showed a similar degree of bone formation in young and aged rats. Repair of the calvarial defects with HEALOS® significantly enhanced bone formation only in young rats but not in aged rats at 8 weeks after surgery. Pryor et al. [15] demonstrated in rats complete defect closure of 6-mm calvarial defect for some sites receiving absorbable collagen sponge at 8 weeks after surgery. In our study, the rat calvarial defect was too large for a complete bone repair with the collagen sponge.

The previous use of HEALOS® has mainly been in combination with recombinant human growth/differentiation factor-5 or with bone marrow cells for experimental spinal fusion [16], which has showed similar results to those reported with autologus bone grafts. In the current investigation, a large amount of residual biomaterial remained in the calvarial defects treated with HEALOS® at the completion of the experiment, particularly so in the aged rats. A longer time period (12–16 weeks) following surgery may be helpful in order to establish the complete reabsorption time for HEALOS®. We did expect that HEALOS® would be almost completely reabsorbed at 8 weeks after surgery. The longer reabsorption time may be attributable to an increased resistance of HEALOS® to collagenase. Wu et al. [17] analyzed uncrosslinked collagen and HA-collagen gel beads treated with collagenase in vitro. The study demonstrated that HA-containing gel beads were less prone to collagenase digestion and degraded more slowly than collagen gel beads. The increased resistance of the composite material to degradation was explained through the potential competition of the HA for the collagenase cleavage sites, or through the absorption of some collagenase to the HA surface.

Implantation of a biomaterial initiates a tissue response similar to that of a foreign body reaction, starting with an acute inflammation. Persistence of an inflammatory stimulus leads to chronic inflammation, which is characterized by the presence of macrophages, giant multinuclear cells, lymphocytes and plasma cells together with the proliferation of blood vessels and the formation of connective tissue. Thus, the degree of vascularity seen within the tissues surrounding an implant may be a function of the degree of inflammation [18]. Moreover, a study by Ashcroft et al. [19] reported a delay but overall increase in angiogenesis during the acute healing of wounds in aged mice compared to their young counterparts; thereby suggesting that angiogenesis is not a rate limiting factor in age-related wound repair. During the course of the current study an extensive inflammatory response was observed in rats treated with HEALOS® compared with rats treated with HELISTAT®. The local inflammatory response was even more apparent in aged rats treated with HEALOS® than in young rats treated with HEALOS®. Furthermore

the number of blood vessels was found to be the highest in HEALOS® treated aged rats together with the largest number of eosinophils. The extensive inflammatory response may have significantly hindered bone repair in the aged rats treated with HEALOS® at 8 weeks after surgery. However, later on, after complete reabsorption of implanted material and resolution of inflammation the bone regeneration may be also enhanced in aged rats treated with HEALOS®. It also appears that calvarial osteoblasts and osteoclasts may have a different activity in response to the local inflammatory stimuli in juvenile rats compare to aged rats.

It seems that HA particles from HEALOS® have caused the extensive local inflammatory response in rats. This finding is supported by Laquerriere et al. [20] who showed that the interaction between HA particles and human monocytes lead to the release of inflammatory mediators such as cytokines and metalloproteinases. Furthermore, Grandjean-Laquerriere et al. [21] found that the production of TNF-alfa by macrophages exposed to HA particles was partially toll-like receptor-4 (TLR4) dependent. They suggested that TLR4 could be considered as an interesting protein that could modulate the inflammatory reaction of macrophages exposed to HA particles.

The level of mast cell and eosinophil infiltration at the site of the calvarial defects treated with collagen biomaterials was not reported before. At sites of tissue repair and fibrosis the mast cells are always found in increased number, and the presence of these cells in connective tissues has been linked to the development of fibrosis through the production of cytokines and growth factors, such as histamine, heparin, tryptase, fibroblast growth factor-2, tumor necrosis factor-α and transforming growth factor-β [22]. In the rat calvarial defect model, the number of mast cells was significantly higher in defects treated with HELISTAT® compared to those treated with HEALOS® in both young and aged rats. This may suggest that the collagen sponge alone (HELISTAT®) attracts more mast cells to the site of implantation making the tissue healing prone to fibrosis, whereas collagen-HA composite (HEALOS®) attracts more eosinophils therefore increasing the risk of allergic reaction. Our results indicate that a proper influx modulation (by specific drugs) of mast cells and eosinophils into the bone defect area may increase the rate of bone repair. More research is needed to prove that concept.

In conclusion, the collagen-HA composite (HEALOS®) enhances calvarial bone repair more than collagen sponge alone (HELISTAT®) in young rats but not in aged rats at 8 weeks after surgery. The collagen-HA composite appeared to induce a more intense inflammatory response than collagen alone especially in aged rats.

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