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A New Bone-Healing Material: A Hyaluronic Acid-Like Bacterial Exopolysaccharide

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Abstract. Critical size defect (CSD) technique was used to evaluate the bone regeneration capacity of a newly discovered hyaluronic acid-like exopolysaccharide synthesized by a bacteria originating from a deep sea hydrothermal vent. A 5 mm-diameter hole was made on each parietal bone of male rats. The right hole was filled with either a new bacterial exopolysaccharide referenced HE 800 or with collagen used as negative control, while the left hole remained free of any treatment. After 15 days, the holes and surrounding tissues were examined by direct examination, X-ray films, and histological staining. Using HE 800, bone healing was almost complete after only 15 days, with osteoblasts onto lying external bone surfaces and enhancing osteocyte inclusion. Neovascularization was also observed along with an organized trabecular bone. No abnormal bone growth or conjunctival abnormalities were noticed. At the end of the experiment, 95.9% ($\pm$6.2) bone healing ($n = 20$) was observed. Conversely, the collagen-treated animals did not demonstrate significant healing—17.8% ($\pm$18.1).

Key words: Bone healing — Exopolysaccharide — Calvaria — Critical size defects — Rats — HE 800

Several conditions may lead to extensive bone defects in skeleton, and are often associated with severe functional problems. Complete bone regeneration cannot be obtained in critical size osseous defects in the absence of an application of osteogenic or osteoinductive bone material. The best treatment for most bone deficiencies is an autograft that provides bone morphogenetic proteins (BMPs) and a substrate onto which bone-forming cells may attach [1]. Osteoinductive molecules such as BMPs are powerful enhancers of bone healing. Other molecules may be used to heal bone defects: TGF-β_1, heparan sulfates, carboxymethyl-benzylamide-sulphonated dextrans (CMDBS), heparin-binding growth factors (HBGF), or heparan sulphate proteoglycans (HSPG), a major component of the extracellular matrix. These molecules can directly enhance bone healing or protect the endogenous growth factors from proteolysis and modulate their biological activities, HBGFs, or HSPGs. Various bone substitute materials were also proposed, such as allogenous bone matrix, demineralized bone, and natural bone from bovine origin, synthetic hydroxyapatite, bioglass, or bioceramics. Such osteoconductive materials do not directly enhance bone healing, they only act as a support for the bone regeneration.

The efficiency of a new bone-healing material, a newly discovered marine bacterial exopolysaccharide, was evaluated on the restoration of bone integrity for critical size defects performed on the calvaria of Wistar male rats. The efficiency of this high molecular weight polymer was also compared to collagen, used as a control.

Materials and Methods

Animals

Forty Wistar adult male (275–299 g) rats were conditioned for 1 week prior to the experiment. Animals had free access to drinking water and standard laboratory rat pellets and were housed in plastic cages in groups of 2, in a room maintained at a constant temperature of 22°C with 12-hour light-dark cycles. After surgery and prior to sacrifice, animals were kept in individual cages. Twenty animals were treated with the polysaccharide and 20 were treated with collagen [2]. Each animal was considered as its own control.

Bacterial Exopolysaccharide

HE 800, a bacterial exopolysaccharide, was secreted under laboratory conditions by the bacterium *Vibrio diabolicus* originating from deep-sea hydrothermal vents. Detailed procedures for bacterial growth, polysaccharide production, and purification are described elsewhere [3]. HE 800 EPS is a linear high-molecular weight (800 kDa) exopolysaccharide with the following repetitive tetrasaccharidic sequence:

$$\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{Nac-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{A}$$
$$\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{A-}(1\rightarrow 4)\text{-}\alpha\text{-D-Gal}p\text{NAc-}(1\rightarrow$$

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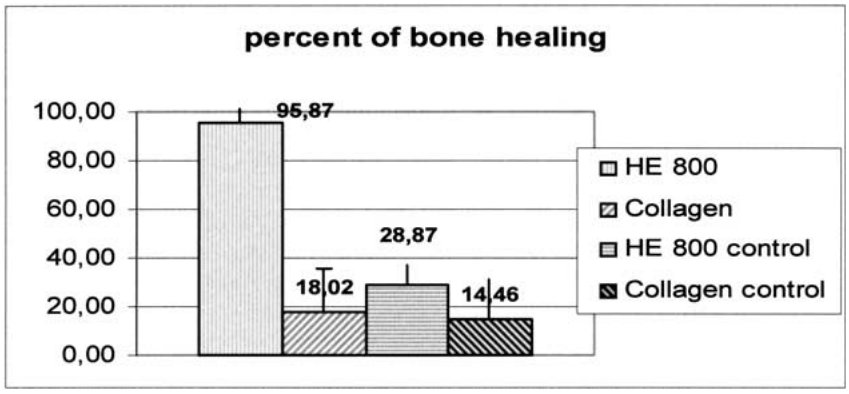

Fig. 1. Percentage of bone healing.

Anesthesia and Surgical Procedures

After achieving suitable sedation using subcutaneous Robinul (0.01 mg/100 g) (Vétoquinol, Lure France), the anesthesia was intramuscularly performed with Kétamine (0.1 ml/100 g) (Imalgène, Mérial, Lyon, France) and intraperitoneal Nesdonal 0.1 ml/100 g) (Specia, Rhone Poulenc Rorer, Montrouge, France). The skin overlying the calvaria was shaved and swabbed before and after surgery with a 10% povidone iodine solution (Betadine, Asta Medica, Mérignac, France). A 20-mm midline incision was made from the posterior nuchal line to the middle of the nasal bone. The periosteum was reflected laterally. A 5-mm diameter full-thickness skull defect was created in each parietal bone at the same distance from the midsagittal suture using a slow-speed dental bur (801.104.014 Komet, France) mounted on a dental handpiece under copious sterile physiological saline solution irrigation [4–7]. Care was taken to avoid underlying dura-mater injury. The periosteum was carefully positioned under the wound using resorbable suture material (Vicryl, Johnson & Johnson Intl, Brussels, Belgium). The skin was closed with a nonresorbable suture (Ercylene II, Sherwood Medical, St Louis, USA).

Implantation with Polysaccharide

Either the polysaccharide HE 800 or collagen was implanted inside the bone defect created into the right parietal bone while the defect created on the left parietal bone was kept free of any polymer. Twenty rats were treated with the bacterial exopolysaccharide and the 20 remaining with collagen. The polysaccharide (1 mg) was carefully positioned inside the bone defect; this resulted in instantaneous formation of a gel that stopped bleeding and created a coagulum. Extreme care was taken to prevent the polysaccharides from spreading out of the cavities during surgical site closure.

Tissue Processing and Histology

Animals were sacrificed after 15 days using 2-ml intraperitoneal pentobarbital (Dolethal, Vétoquinol, Lure France). The area of the craniotomy was removed along with surrounding normal bone and tissues, rinsed in a physiological saline solution, and placed in a fresh 10% buffered formaldehyde-glutaraldehyde solution for 48 hours. Each calvaria was identified, photographed, and X-ray analyzed to localize the holes followed by demineralization in a 10% formic acid solution for 24 hours, and finally bisected through themidsagittal suture to separate the two parietal bones. Each sample was cut through the middle of the hole using X-ray localization. Serial 5-μm thickness sections were cut from the center of the defect and prepared for histological staining with Hemalin-eosin, Masson Trichrom stain, or Red Sirius staining.

Histological Evaluation

The histological sections were examined and quantitatively evaluated by bone histomorphometry. The percentage of closure of the bone defect was calculated directly from digitized slide pictures as the ratio between the margin of the newly formed bone and the initial bone defect [5]. Statistical Student's t tests were performed for all experiments.

Results

After surgery, all animals recovered well and the expected increase in weight during the postsurgical period was then recorded. Neither side effects, such as paralysis, convulsions, respiratory distress, or signs of pain nor any clinical signs of wound infections were observed. Skin tissues were in fair condition and scars were not proliferative.

Using the bacterial exopolysaccharide, a direct examination showed an important neo-angiogenesis into both the control and the experimental sites. The bone defects were macroscopically closed for the treated holes. All animals showed complete bone healing (95.9% ± 6.2), while the left hole control healed about 28.9% (±8.4) (Fig. 1). The histology showed no fibrous connective tissue (Fig. 2). The regenerated bone consisted of a woven bone showing a beginning of cortical bone. A closer magnification showed old lamellar bone edge and a new woven bone along with the presence of cortical bone. Numerous blood vessels and osteocytes were observed at the end of the 15-day experiment. No gap was observed between old and new bone and, for some rats, a high amount of new bone surrounding the old bone (Fig. 3) was also noticed. Red Sirius coloration indicated a collagen fiber organization and orientation (Fig. 4) parallel to the surfaces when observed with polarized light (Fig. 5). Conversely, collagen-treated samples healed (18.1% ± 17.8) and defects on the control side showed similar bone healing (14.5% ± 16.0) (Fig. 1). An inflammatory process was also visible after 15 days collagen use. The Red Sirius coloration showed collagen fibers orientation and bone defect colonization by a connective fibrous tissue (Fig.

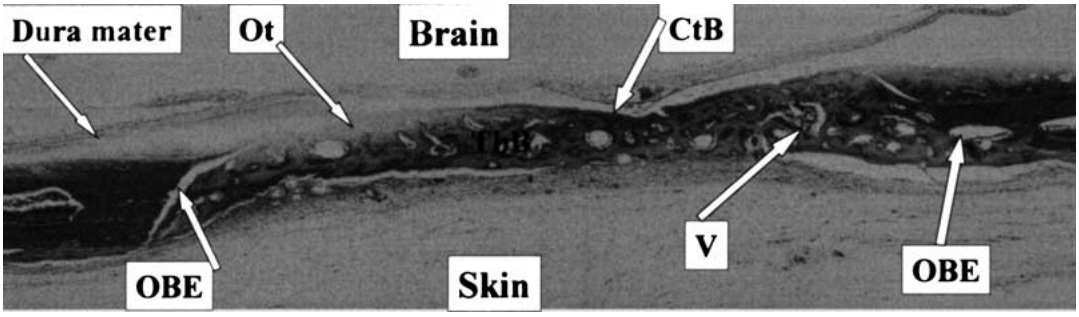

Fig. 2. Closed critical size defect after 15 days (×4). Old bone edge (OBE), trabecular bone (TbB), cortical bone (CtB), blood vessel (V), osteocytes (Ot).

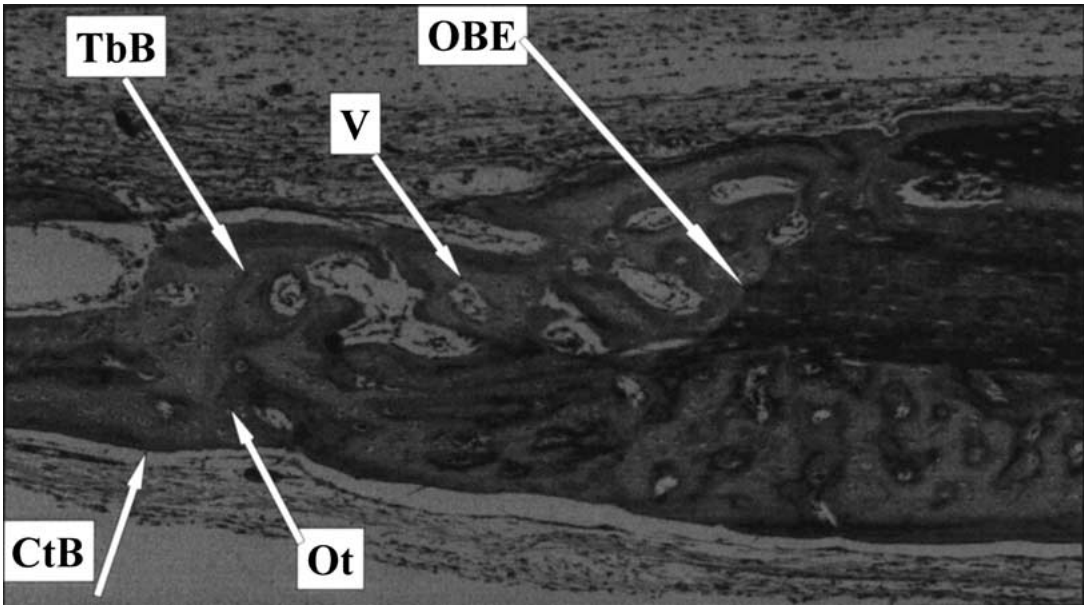

Fig. 3. Closed critical size defect after 15 days (×10) (Masson Trichrom). Old bone edge (OBE), trabecular bone (TbB), cortical bone (CtB), blood vessel (V), osteocytes (Ot).

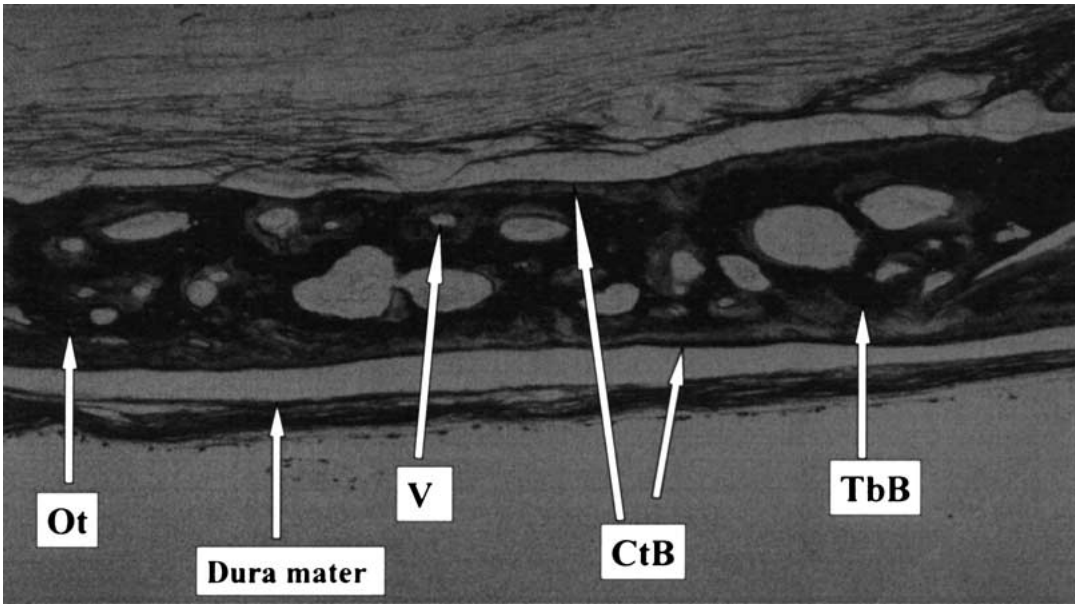

Fig. 4. Red Sirius coloration of a healed HE 800-treated defect showing a perfect histologically structured bone (×10).

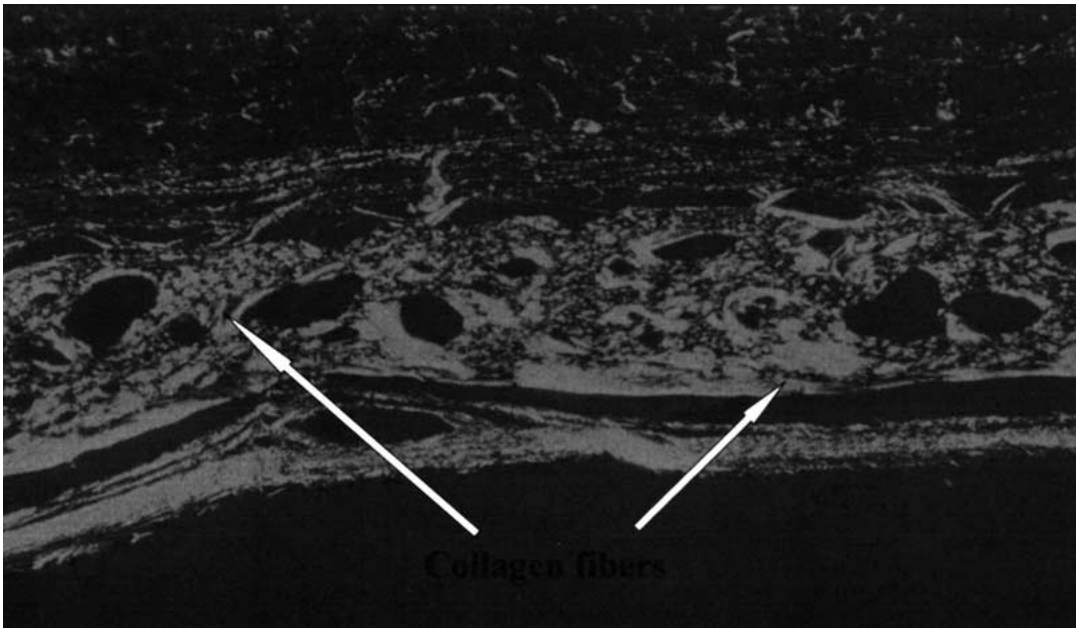

Fig. 5. Red Sirius coloration with polarized light (×10) (HE 800-treated defect). Collagen fibers are parallel to bone surfaces.

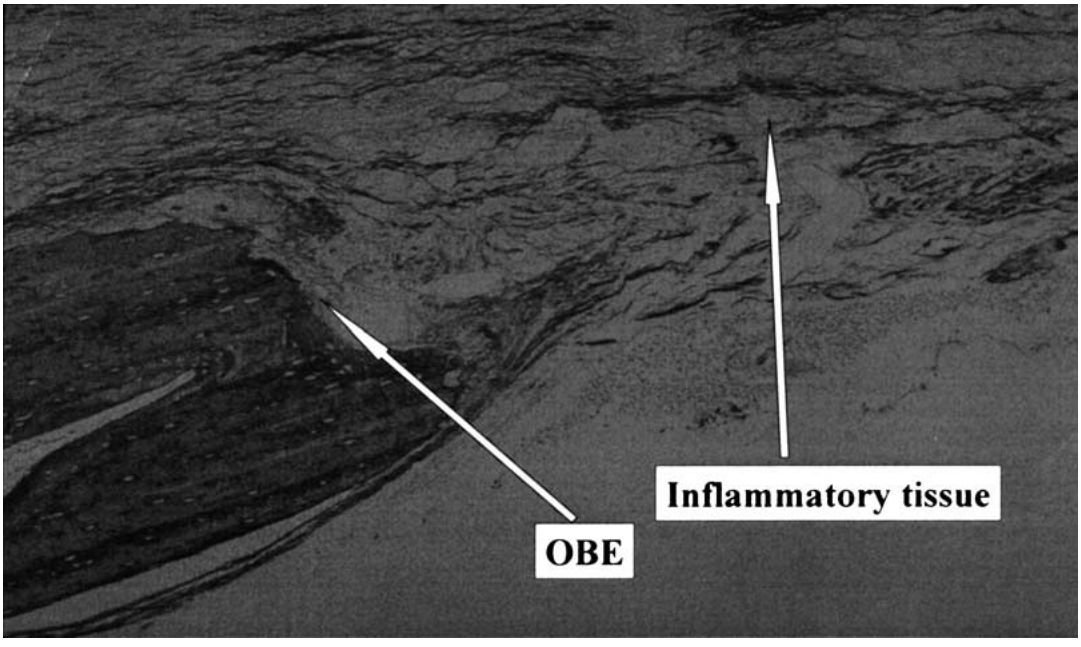

Fig. 6. Red Sirius coloration of a collagen-treated calvaria (×10) showing collagen fibers orientation and lack of bone healing.

6). Statistical comparison between the HE 800 and collagen-treated animals was highly significant ($P < 10^{-6}$).

Discussion

When bone loss exceeds critical dimension [7, 8], a spontaneous healing is usually not expected [2, 9]. Osteoinductive proteins like BMP2 and BMP7 can be used to induce ectopic bone repair [1, 10–13]. These materials induce a regeneration ad-integrum of the lost tissue according to a mechanism of cellular activation by proteins or peptides originating from the surrounding tissues [2, 14]. Carboxy-methylbenzylamides dextran sulfate (sulfonates) (CMDBS) and heparan sulfates [15–17] have also been proposed as stabilizing growth factors in operative sites by protecting them from enzymatic degradation. However, these polymers required a vector, usually collagen [17], and their efficiency is dose, molecular weight, and degree of sulfation dependent. By

using these modified polysaccharides, complete healing can be observed within 1 month for 3–4 mm diameter holes produced in rat calvaria [6, 18–20].

The repeating unit of the HE 800 consists of 50% uronic acids such as glucuronic acid and 50% of aminosugars such as N-acetyl galactosamine and N-acetyl glucosamine; this composition is similar to both other well-known biologically active glycosaminoglycans (GAGs) and to the extracellular matrix. Both of these bioactive molecule polysaccharides and the extracellular matrix are known to play a major role in the first healing steps [21–24] and during bone or injury healing [25].

The percentage of closure obtained in the control sites is slightly superior to those observed in previous works [16, 26]. HE 800 enhanced osteoblast adhesion, proliferation, and later mineralization in a medium without ascorbic acid and L glutamine in 5 days [27]. This polysaccharide did not induce any inflammatory reactions and the material was no longer present after 15 days. The new woven bone formed in 15 days was well structured with oriented collagen fibers and osteoblast cells covering the bone surfaces and the presence of osteocytes. This new bone was histologically normal and the new vascularization was significant. No proliferative fibrous tissue was noticed.

The high molecular weight of this bacterial polysaccharide (800 kDa) may play an important role in the binding of growth factors and their protection by reducing enzymatic degradation [16, 17, 26, 28] and forming a large and physically stable coagulum. The efficiency of this polysaccharide could also be related to the fact that this polymer greatly enhanced osteoblast adhesion and phenotypic expression. This bacterial exopolysaccharide forms an attractive extracellular matrix for the direct adhesion of osteoblasts, osteoprogenitor cells, and pericytes and may protect the growth and hormonal factors associated with the healing process. The high binding capacity of calcium by this polysaccharide could also be another important parameter in its efficiency to induce such fast bone healing [29]. An increase in the calcium concentration in the matrix then provides the correct extracellular conductive environment prior to initial coagulum formation followed by mineralization.

The promising results obtained with the HE 800 polysaccharide are the combination of factors such as osteoblast adhesion [27], proliferative process, and a protection of growth factor from degradation. A pharmaceutical effect on bone healing can also be hypothesized as suggested by significant differences ($P < 10^{-3}$) in bone healing on the control sides of HE 800 and collagen-treated animals ($28.9 \pm 8.4/14.5 \pm 16$).

Conclusion

HE 800, a high molecular weight linear bacterial exopolysaccharide, appears to be a strong bone-healing material. Introducing this new glycosylaminoglycan-like polysaccharide in critical size defects in bone induces a nearly complete healing within no longer than 2 weeks. In addition, the anatomy of the defect with trabecular and cortical structure is totally restored. Several factors could contribute to this process including the chemistry of the polysaccharide (similar to other biologically active molecules), its property to encourage osteoblastic cell adhesion, and its high binding capacity of alkaline-earth cations such as calcium. Such results are encouraging, and additional studies are in progress to help understand the role of this polysaccharide in the healing process and its systemic activity.

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