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The bone-regenerative properties of Emdogain adsorbed onto poly (D,L-lactic-coglycolic acid)/ calcium phosphate composites in an ectopic and an orthotopic rat model

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Background and Objective: The aim of this study was to evaluate the bone-regenerative properties of Emdogain® in osseous and nonosseous sites.

Material and Methods: For the orthotopic study, unloaded poly(D,L-lactic-coglycolic acid)/calcium phosphate implants, and poly(D,L-lactic-coglycolic acid)/calcium phosphate implants loaded with different concentrations (0.25, 0.50 or 0.80 mg per implant) of enamel matrix derivative (EMD), were inserted into cranial defects of 24 rats. The implantation time was 4 wk. For the ectopic study, 32 implants were placed subcutaneously. The same study period and groups as in the orthotopic study were used. Methods of evaluation consisted of descriptive histology, histomorphometry and an *in vitro* EMD-release study.

Results: In the orthotopic study, new bone formation was most abundant in unloaded implants followed by 0.50-mg EMD composites. Histomorphometric measurements showed 54 ± 15.0% bone ingrowth for unloaded implants, 19 ± 22.5% bone ingrowth for 0.25-mg EMD composites, 40 ± 23.6% bone ingrowth for 0.50-mg EMD composites and 26 ± 17.6% bone ingrowth for 0.80-mg EMD composites. Light microscopic analysis of the subcutaneous sections from the ectopic study revealed no bone formation in any group after 4 wk. The *in vitro* release study showed 60% cumulative EMD release after 4 wk.

Conclusion: Emdogain® is not osteoinductive and is not able to enhance bone healing in combination with an osteoconductive material, such as poly(D,L-lactic-coglycolic acid)/calcium phosphate cement.

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Emdogain® was introduced in 1997 as an alternative approach for periodontal regeneration based on embryonic tooth formation (1,2). It is an extract of porcine enamel matrix, termed 'enamel matrix derivate' (EMD) and thought to induce mesenchymal cells to mimic the events that occur during root development and to stimulate the regeneration of periodontal tissues (i.e. cementum, periodontal ligament and bone). The ability of EMD to enhance bone formation in a nude mouse model was defined as osteopromotive (3) and was suggested to be a result of enhancement of the osteoinductive potential of the graft material by EMD. However, it was also hypothesized that EMD shows osteopromotive characteristics because of the presence of bone growth-stimulating factors and that a threshold concentration of EMD is required to evoke an effect. Although early immunoassay studies could not identify the presence of growth factors in EMD (4), nominal levels of transforming growth factor-β1 (5), as well as of bone morphogenetic protein-2 and bone morphogenetic protein-4, were subsequently detected in an osteoinductive fraction of enamel extracts (6).

Calcium phosphate ceramics are extensively used as a bone substitute in dentistry, orthopaedics and reconstructive surgery because of their biocompatibility and osteoconductivity. Frequently, calcium phosphate ceramics are manufactured and used in the form of granules or prefabricated (porous) blocks, which are unfortunately difficult to apply from a clinical point of view, because of factors such as difficulty of local maintenance of the material, lack of shaping and difficult delivery into the bone defect. These disadvantages have been overcome by the development of calcium phosphate cements, which can be injected into the defect site, shaped according to the defect dimension and set *in situ*. However, depending on the composition of the final set product, the *in vivo* resorption and tissue ingrowth can be slow (7). One approach to overcome this limitation is the creation of a macroporous structure within the finally set calcium phosphate cement. Such a concept has recently been developed in our laboratory. Poly(D,L-lactic-coglycolic acid) microparticles were incorporated in the cement to induce macroporosity (8). Additionally, the inclusion of degradable microparticles provides a method for sustained delivery of bioactive molecules, which enhance the bone healing process (9–11).

In view of the points mentioned above, the objective of this experiment was to investigate the bone-regenerative properties of Emdogain®. We hypothesized that EMD, incorporated in different concentrations into poly (D,L-lactic-coglycolic acid)/calcium phosphate composites, could enhance bone healing as a result of the presence of osteoinductive growth factors in the EMD. To prove this hypothesis, poly (D,L-lactic-coglycolic acid)/calcium phosphate composite implants and EMD-incorporated poly(D,L-lactic-coglycolic acid)/calcium phosphate composite materials were inserted in an orthotopic (cranial) and an ectopic (subcutaneous) location in rats.

Material and methods

Preparation of microparticles

Poly(D,L-lactic-coglycolic acid) microspheres were prepared using a water-in-oil-in-water double-emulsion solvent-evaporation technique. Briefly, 1.4 g of low-molecular-weight poly(D,L-lactic-coglycolic acid) was dissolved in 2 mL of dichloromethane inside a 50-mL PP tube. A 500-mL volume of distilled deionized water (or 8.0/11.2% bovine serum albumin in distilled deionized water) was added while vortexing vigorously for 1 min, subsequently adding 6 mL of a 0.3% polyvinyl alcohol solution. Vortexing was continued for another 1 min. The contents of the 50-mL tube were transferred to a stirred 1000-mL beaker and another 394 mL of 0.3% polyvinyl alcohol was added slowly. This was directly followed by the addition of 400 mL of a 2% isopropylic alcohol solution. The suspension was stirred for 1 h. The spheres were allowed to settle for 15 min and the solution was decanted. The suspension remaining was centrifuged, and the clear solution at the top was decanted. Five millilitres of distilled deionized water was added, the spheres were washed, centrifuged and the solution was aspirated. Finally the spheres were frozen, freeze-dried for 24 h and stored under argon at −20°C.

Preparation of poly(D,L-lactic-coglycolic acid)/calcium phosphate implants

Implants were made by adding low-molecular-weight poly(D,L-lactic-coglycolic acid) microspheres (Purasorb®; Purac, Gorinchem, the Netherlands) to calcium phosphate cement (Calcibon®; Biomet Merck, Darmstadt, Germany), so that a weight ratio of 20 : 80 [poly(D,L-lactic-coglycolic acid)/calcium phosphate cement] was achieved. The chemical composition of the calcium phosphate cement was 62.5% α-tri-calcium phosphate, 26.8% $CaHPO_4$, 8.9% $CaCO_3$ and 1.8% precipitated hydroxyapatite. An aqueous solution of 1% Na_2HPO_4 was used as a liquid component with a liquid/powder ratio of 0.35 mL/g. The cement powder was sterilized by gamma radiation with 25 kGy (Isotron B.V., Ede, the Netherlands) and the cement liquid was filter-sterilized (0.2 μm filter). Calcium phosphate cement powder, provided with poly(D,L-lactic-coglycolic acid) microparticles, was added to a 2-mL plastic syringe. Then, 1% Na_2HPO_4 was applied to the mixture, which was shaken vigorously for 30 s using a Silamat® mixing apparatus (Vivadent, Schaan, Liechtenstein). After mixing, the cement was immediately injected into a round mould to ensure a standardized shape of the samples. In this way, implants in the form of discs were created with a diameter of 8 mm and a height of 2 mm. These discs were removed from the molds after setting of the cement. The average weight of the samples was 140 mg.

Adsorption of EMD on poly(D,L-lactic-coglycolic acid) microspheres

Thirty milligrams of Emdogain® (BIORA AB, Malmö, Sweden) was dissolved in 800 μL of 0.1% acetic acid and added to 1.0 g of microspheres,

after which the EMD was freeze-dried onto the microspheres.

Preparation of EMD/poly(D,L-lactic-coglycolic acid)/calcium phosphate cement implants

Poly(D,L-lactic-coglycolic acid)/calcium phosphate cement implants provided with EMD were made by adding a fraction of EMD-adsorbed poly(D,L-lactic-coglycolic acid) microspheres to a fraction of as-prepared poly(D,L-lactic-coglycolic acid) microspheres and subsequent mixing of both fractions through calcium phosphate cement powder. By adding different amounts of EMD-adsorbed microspheres, implants with different concentrations of EMD were obtained (i.e. 0.25, 0.50 or 0.80 mg per implant). For the final shaping and setting of the implants, the same procedure was followed, as described above, for non-loaded cement implants.

Surgical procedure

The loaded and unloaded composites were inserted orthotopically and ectopically in rats for 4 wk.

Orthotopic implants — For the orthotopic implantation, 24 healthy skeletally mature male Wistar rats, with an average weight of 250 g, were used. National guidelines for the care and use of laboratory animals were observed.

In total, 24 implants were inserted into the parietal cranial bone of the rats, resulting in four different groups of rats:

- group A: unloaded poly(D,L-lactic-coglycolic acid)/calcium phosphate composites ($n = 6$);
- group B: poly(D,L-lactic-coglycolic acid)/calcium phosphate composite loaded with 0.25 mg of EMD per implant ($n = 6$);
- group C: poly(D,L-lactic-coglycolic acid)/calcium phosphate composite loaded with 0.5 mg of EMD per implant ($n = 6$); and
- group D: poly(D,L-lactic-coglycolic acid)/calcium phosphate composite loaded with 0.8 mg of EMD per implant ($n = 6$).

For installation of the implants, full-thickness, critical-sized cranial defects with a diameter of 8 mm were created (12). Therefore, anaesthesia was induced with 4% isoflurane and maintained with 2% isoflurane and 0.4% N_2O, 0.4% O_2 by nonrebreather mask and monitored to ensure that an appropriate level of anaesthesia was achieved and maintained for surgery. The animals were premedicated by an intramuscular injection of fentanyl (2.7 mL/kg) to reduce the operative pain, and a subcutaneous injection of buprenorphine (150 μg/kg) was applied to reduce the postoperative pain.

Before surgery, the dorsal part of the rat cranium was shaved and swabbed with iodine. A median sagittal incision, extending from the nasofrontal area to the occipital protuberance, was made and soft tissues were sharp dissected to visualize the cranial periosteum. The periosteum was then undermined and reflected, exposing the parietal bones. A hollow trephine bur (ACE Dental Implant System, Brockton, MA, USA), with an outer diameter of 8 mm, in a dental hand piece was used to create a full-thickness defect in the dorsal part of the parietal bone. Although the defect included the sagittal suture, care was taken not to damage the dura mater or to puncture the superior sagittal sinus. After insertion of the various materials, the periosteum and overlying skin were closed in separate layers with 5-0 and 4-0 Vicryl resorbable sutures.

Ectopic implants — For the ectopic implantation, eight male Wistar rats of 100 g were used. Each rat received four implants subcutaneously, according to a randomization scheme. In total, 32 implants were placed, as follows:

- group A: unloaded poly(D,L-lactic-coglycolic acid)/calcium phosphate composites ($n = 8$);
- group B: poly(D,L-lactic-coglycolic acid)/calcium phosphate composites loaded with 0.25 mg of EMD per implant ($n = 8$);
- group C: poly(D,L-lactic-coglycolic acid)/calcium phosphate composites loaded with 0.5 mg of EMD per implant ($n = 8$); and
- group D: poly(D,L-lactic-coglycolic acid)/calcium phosphate composites loaded with 0.8 mg of EMD per implant ($n = 8$).

Surgery was performed under general inhalation anesthesia with a combination of 2% isoflurane, 0.4% N_2O and 0.4% O_2. The composites were subcutaneously implanted into the back of the animals. Four small longitudinal incisions were made through the full thickness of the skin on both sides of the vertebral column. Lateral to the incisions, a subcutaneous pocket was created using blunt dissection. Subsequently, one implant was inserted into each pocket. Finally, the skin was closed using Agraven suture material.

Histological preparation

The animals were killed using an overdose of carbon oxide.

For the orthotopic implants, the skin was dissected and the defect sites were removed, along with the surrounding bone and soft tissues, and fixed in 10% neutral formalin for 1 wk. The specimens were left undecalcified and were embedded in polymethylmethacrylate. After polymerization, sections were prepared using a modified diamond-blade sawing microtome technique (Leica Microsystems GmbH, Wetzlar, Germany). The sections were 10 μm thick and were stained with methylene blue and basic fuchsin.

For the ectopic study, implants with their surrounding tissue were retrieved, fixed in 10% neutral formalin for 1 wk and then embedded in polymethylmethacrylate. The procedure for sawing and staining was the same as that described above.

The light microscopical evaluation of all samples (from both the orthotopic and ectopic studies) was performed using an optical microscope (Leica BV, Rijswijk, the Netherlands) and consisted of a complete morphological description of the tissue response to the different implants.

Histomorphometry

Histomorphometrical analysis was performed only for the cranial implants. Measurements were carried out on three digitalized sections per cranial specimen to quantify bone ingrowth within the defect. For this purpose, a Leica Qwin Proimage

analysis system (Leica BV) was used and sections were digitized at low magnification (2.5×). The newly formed bone, as defined by its woven structure and location, was marked in an interactive manner and the computer measured its length in mm. Another parameter to determine was the total length of the defect [i.e. the area (in mm) between the defect borders that has to be filled with newly formed bone]. From these two data, the length of the bone ingrowth, in per cent, was calculated. The results presented are based on the average of these measurements.

In vitro EMD release test

To determine the release of EMD out of the poly(D,L-lactic-coglycolic acid)/calcium phosphate implants, a release test was carried out. Therefore, a sample of each EMD-loaded implant group was put into a 15-mL tube with 1 mL of Milli Q and the tube and its contents were incubated in a water bath (37°C) to mimic the conditions in the rat body. After 1, 3 and 5 h, and 1, 2, 4, 7, 14 and 28 d, a 1-mL sample of Milli Q was taken from the tubes and replaced with 1 mL of fresh Milli Q. The sample was put into a 15-mL tube and freeze-dried for 1 d. Subsequently, it was dissolved in 200 μL of MilliQ. In this way, a five-fold higher concentration of EMD was achieved, which facilitated the final analysis. To determine the drug release per week and the cumulative release, the concentration of the release medium was measured by high-performance liquid chromatography, using a reverse-phase column (Atlantis®; Waters Corp., Milford, MA, USA). A 30:70 mixture of acetonitrile/water was used with phase containing 10% 0.1 M formic acid. Before each analysis, samples were filtered through Acrodisk® filters.

Statistical analysis

For statistical analysis the GRAPHPAD INSTAT program (GraphPad Software, San Diego, CA, USA) was used. One-way analysis of variance was performed on the data obtained from the histomorphometric analyses. Analysis of variance assumes that the data are sampled from populations with identical standard deviations. This assumption was tested using the method of Bartlett. Furthermore, analysis of variance assumes that the data are sampled from populations that follow Gaussian distributions. This assumption was tested using the method of Kolmogorov & Smirnov. In addition, a Tukey–Kramer Multiple Comparisons Test was performed. Differences were considered to be significant when $p < 0.05$.

Results

All animals had an uneventful recovery. Their weight remained stable and increased during the implantation period. No infection occurred during that time.

Cranial implants

Light microscopic examination after 4 wk showed the presence of newly formed bone and no complete closure of the defects in any of the experimental groups. The bone ingrowth was initiated mainly from the dura side and defect edges, whereas no bone formation from the periosteal side was observed. Only a layer of connective tissue was present on the top of the composites that penetrated into the macropores as created by the degraded poly(D,L-lactic-coglycolic acid) particles close to the surface. There was no difference in thickness and vascularization of this connective tissue layer between the groups.

Newly formed bone was most abundant for unloaded implants and implants loaded with 0.50 mg of EMD. Cranial defects provided with 0.25 mg of EMD-loaded implants showed minor bone ingrowth. Poly(D,L-lactic-coglycolic acid)/calcium phosphate implants with 0.80 mg of EMD induced bone formation similar to that of the 0.25-mg EMD implants (Fig. 1). The newly formed bone was in direct contact with the surface of both loaded and unloaded composites without any fibrous tissue interface (Fig. 2A). Evidently, bone apposition was guided over the surface of the poly(D,L-lactic-coglycolic acid)/calcium phosphate implants (Fig. 2B).

At higher magnification, the newly formed bone revealed thin trabeculae and large marrow spaces filled with fat tissue and haematopoietic cells. The newly formed bone had a woven structure without preferred orientation of the collagen fibers in the bone matrix and an abundance of osteoblasts and small blood vessels. Occasionally, some multinucleated inflammatory-like cells were seen in contact with the cement, but they did not occur specifically for loaded or unloaded implants.

All implants were maintained in the cranial defects during the implantation time and kept their shape and stability.

Polymethylmethacrylate samples showed complete maintenance of integrity of the undecalcified composites. They revealed very limited degradation of the poly(D,L-lactic-coglycolic acid) microparticles, especially in the middle of the composites. At the space left by the degraded microparticles, which was mainly at the dura and periosteal surface of the implants, fibrous tissue was observed inside the resulting microporosity. In the center of the composites, no degradation at all of the poly(D,L-lactic-coglycolic acid) was seen (Fig. 3).

Histomorphometry

Histomorphometric measurements confirmed the subjective evaluation. Bone formation was most abundant for unloaded implants (54 ± 15.0% bone ingrowth) and lowest for the 0.25-mg EMD implants (19 ± 22.5% bone ingrowth). For the 0.50-mg EMD implants, the bone ingrowth was 40 ± 23.6%, whereas for the 0.80-mg implants, it was 26 ± 17.6%. Statistical analyses identified no significant differences ($p > 0.05$) in the amount of the newly formed bone among the EMD groups. There was a statistically significant difference ($p < 0.05$) only between the unloaded implants and those loaded with 0.25 mg of EMD in favour of the unloaded implants (Fig. 4B).

Ectopic implantation

Light microscopic analysis of the subcutaneous sections revealed no bone

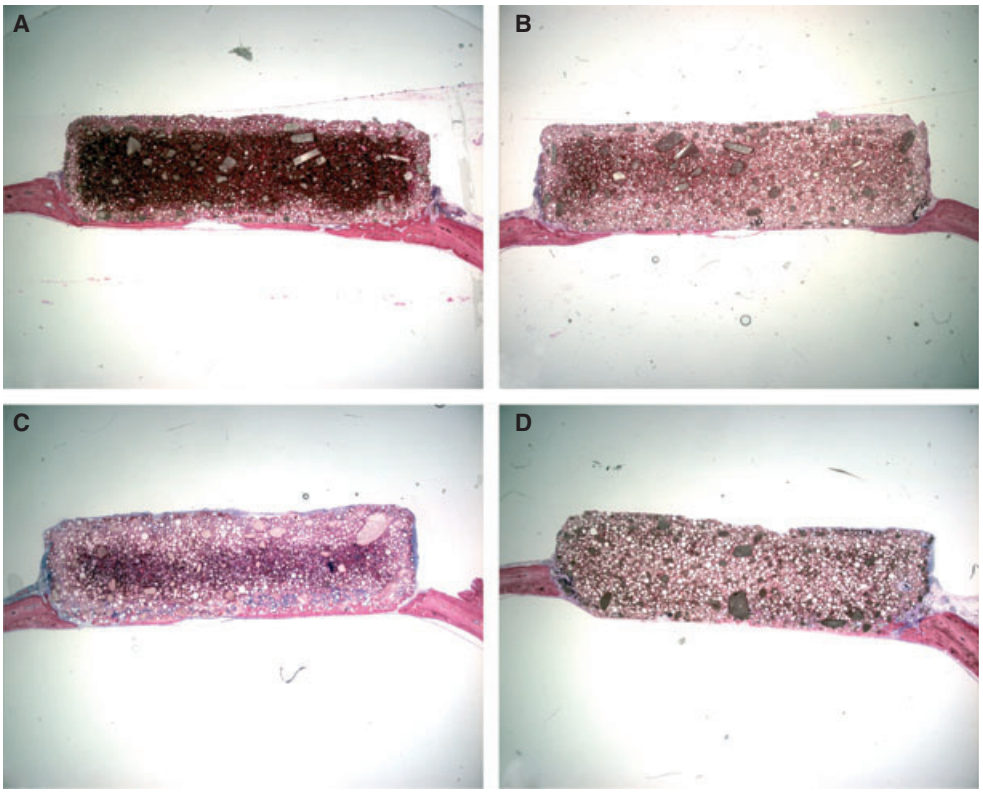

Fig. 1. Light micrographs of polymethylmethacrylate-embedded specimens showing bone ingrowth into cranial defects (0.6× magnification). (A) Unloaded implants; (B) implants loaded with 0.25 mg of enamel matrix derivative (EMD); (C) implants loaded with 0.5 mg of EMD; and (D) implants loaded with 0.8 mg of EMD.

formation in any group after an implantation period of 4 wk. Only a layer of highly vascularized loose connective tissue was present around the composites, which penetrated into the macropores of the poly(D,L-lactic-co-glycolic acid) particles most close to the implant surface (Fig. 5). There was no apparent difference in the thickness of this fibrous capsule or in vascularization among the various implant groups. Occasionally, some multinucleated inflammatory-like cells were observed at the surface of all implant groups.

All implants maintained their integrity and shape during implantation, and only limited degradation of the poly(D,L-lactic-coglycolic acid) particles was observed, as characterized by the absence of tissue structures in the macroporosity.

In vitro EMD-release study

The results of the *in vitro* EMD kinetics are depicted in Fig. 6. An initial burst release of ≈10% of the total dose of EMD was observed at 1-, 3- and 5-h time-points for all EMD-loaded implant groups. Subsequently, a sustained-release profile was observed during the first week of incubation. Thereafter, the release increased rapidly (Fig. 6A) and was calculated to be ≈35% per week. On day 28, 60% of the total amount of EMD loaded in the poly(D,L-lactic-coglycolic acid)/calcium phosphate implants was released into the medium (Fig. 6B).

Discussion

The present study failed to support the hypothesis that EMD can enhance bone regeneration when applied in combination with an osteoconductive material orthotopically and induces bone formation ectopically. On the contrary, less bone formation was observed compared with the poly (D, L-lactic-coglycolic acid)/calcium phosphate composites alone in the critical-sized cranial defects. Nevertheless, 0.50 mg of EMD-loaded implants tended to result in more bone ingrowth than 0.25- and 0.80-mg EMD-loaded implants. This finding is in agreement with the *in vitro* study of Yoneda *et al.* (13), in which the effect of EMD on osteoblastic cells was evaluated. They demonstrated that EMD affected the cell proliferation, alkaline phosphatase activity and mineralized nodule formation in a concentration-dependent manner, whereas the most favorable concentration of EMD was reported to be 0.50 mg. On the other hand, in the *in vivo* part of the same study, in which EMD-loaded collagen pellets were inserted into 3.8 mm full-thickness cranial defects for an implantation period of 2 wk, the released EMD stimulated new bone formation, which does not corroborate our observations. This discrepancy may be a result of the fact that in the *in vivo* experiment of Yoneda *et al.*, smaller defects (3.8 vs. 8 mm in diameter), shorter implantation periods (2 vs. 4 wk) and a higher concentration of EMD (1 vs. 0.25, 0.50

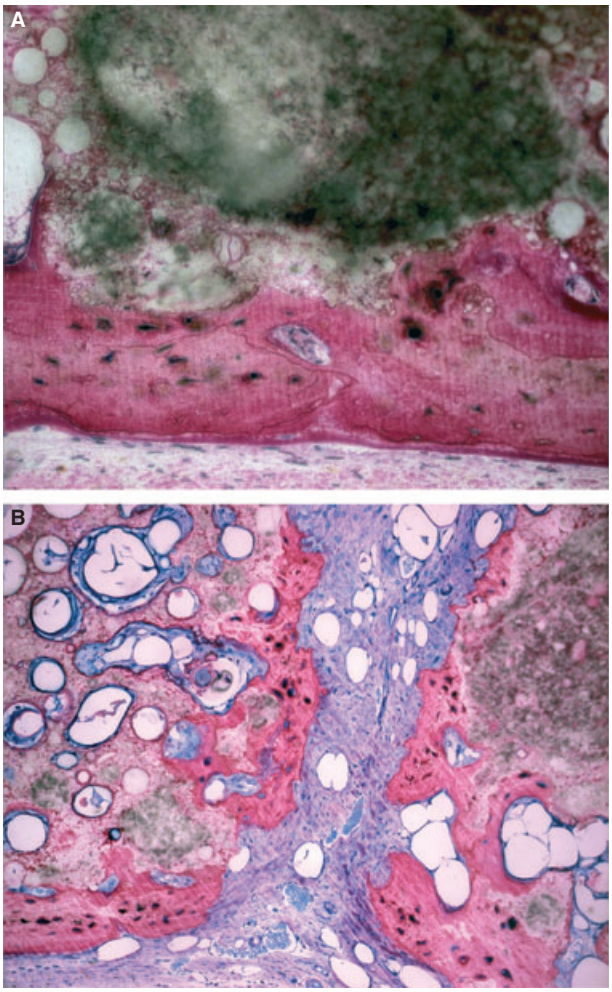

Fig. 2. Histological sections of polymethylmethacrylate-embedded specimens. Guided bone formation is visible, showing direct contact of cement with newly formed bone, without any sign of a fibrous tissue interface. (A) At the side of the dura (40× magnification); and (B) within a crack of an implant (20× magnification).

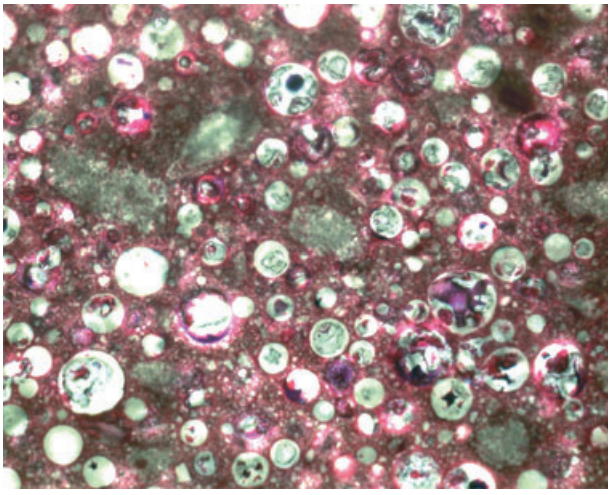

Fig. 3. Light micrograph showing the presence of nondegraded poly(D,L-lactic-coglycolic acid) microparticles in the centre of the composites (20× magnification).

or 0.80 mg) were used, which might have resulted in better outcomes. Moreover, a different delivery vehicle [collagen pellet vs. poly(D,L-lactic-co-glycolic acid)/calcium phosphate cement] was used, and although the EMD-release profile was not reported, it might also have affected the study results. Furthermore, it should be noted that Yoneda *et al.* included an empty defect as control group, whereas we used poly(D,L-lactic-coglycolic acid)/calcium phosphate implants as a control, which have already demonstrated excellent osteogenic capacity.

In our study, 0.80 mg of EMD-loaded composites caused less bone ingrowth than the composites loaded with 0.50 mg of EMD (40 vs. 27% bone formation). One explanation for this might be that there is an optimal dose of action of EMD, which when exceeded or not reached results in an inhibitory effect. This has already been observed in an *in vitro* study with EMD and periodontal ligament cells (14). In this study, the viability of periodontal ligament cells was observed to be negatively affected by higher doses of EMD over time, whereas lower doses elicited no change when compared with control cultures.

The conclusion of the present study, that EMD does not provide an additional stimulus for bone formation, is supported by the findings of Donos *et al.* (15,16). In one of their studies, cranial defects with a diameter of 5 mm were treated with barrier membranes and/or EMD, and demineralized bovine bone matrix + EMD vs. a combination of a barrier, EMD and demineralized bovine bone matrix, for 4 mo. The combined use of the barriers with EMD did not significantly enhance bone healing. Therefore, the authors even suggested that the use of EMD for the purpose of generating new bone in clinical situations should be questioned. The same is true for the possible osteoinductive nature of EMD. Previous studies of Boyan *et al.* (3) and Yoneda *et al.* (13), suggested that EMD does not have bone morphogenetic protein-like osteoinductive activity. Our investigation confirmed this observation, as no bone formation was found when EMD composites

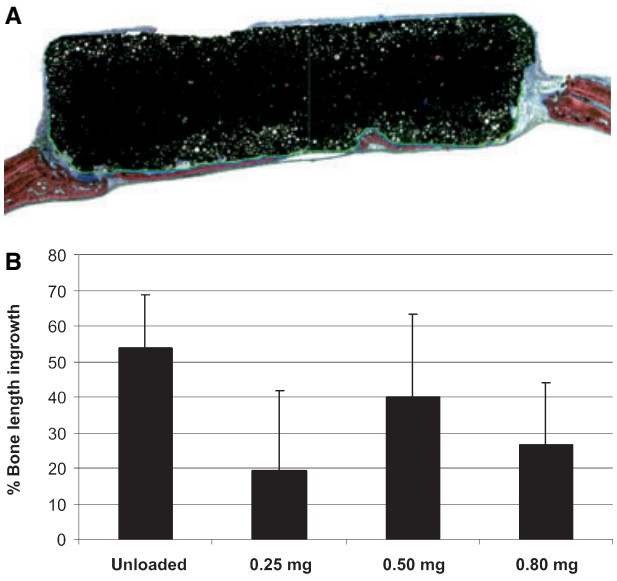

Fig. 4. (A) Histomorphometric measurements of the bone length ingrowth; the total length of the defect is marked in green and the length of the newly formed bone is marked in blue. (B) Histomorphometric measurements of bone length ingrowth (in percentage) for all groups. Unloaded, unloaded implants; 0.25 mg, implants loaded with 0.25 mg of enamel matrix derivative (EMD); 0.5 mg, implants loaded with 0.5 mg of EMD; and 0.8 mg, implants loaded with 0.8 mg of EMD.

were implanted subcutaneously for 4 wk.

Delivery systems are of great importance when growth factors are used to enhance wound healing (17,18). Considering our histological findings [i.e. the lack of a bone formation-enhancing effect of the EMD-loaded poly(D,L-lactic-coglycolic acid)/calcium phosphate implants], it can be assumed that the present delivery system of EMD was not appropriate. In view of this, an *in vitro* release study was performed to obtain information about the release kinetics of EMD. Our findings correspond to the release profiles of other growth factors from the same calcium phosphate cement (9,10). In our study, the initial burst release of 10% was caused by the release of EMD from the surface of the scaffold. Afterwards, the release was sustained until day 7, when low-molecular-weight poly(D,L-lactic-coglycolic acid) particles started to erode. By day 14, the erosion of the poly(D,L-lactic-coglycolic

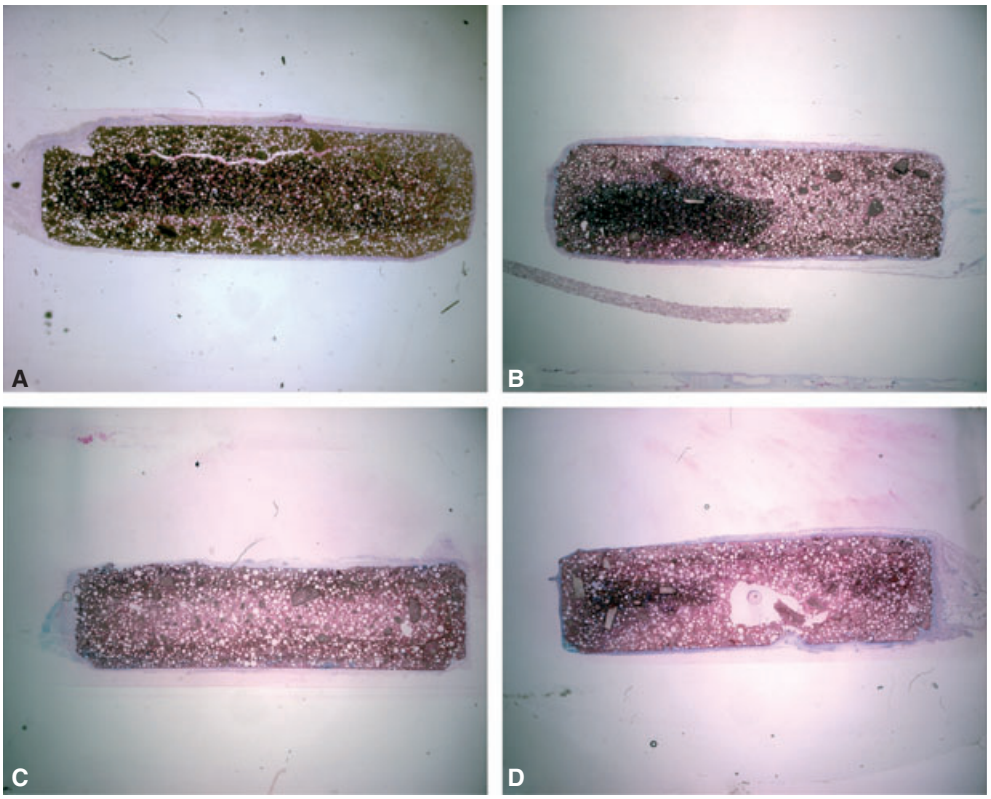

Fig. 5. Fibrous tissue capsule around the subcutaneous implants/polymethylmethacrylate samples (basic fuchsin, 0.6× magnification). (A) Unloaded implants; (B) implants loaded with 0.25 mg of enamel matrix derivative (EMD); (C) implants loaded with 0.5 mg of EMD; and (D) implants loaded with 0.8 mg of EMD.

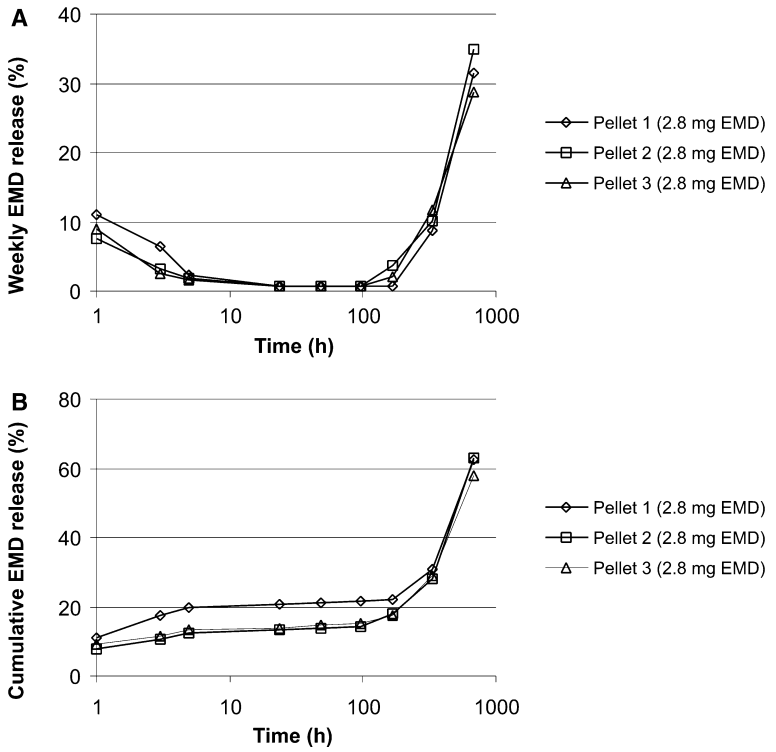

Fig. 6. Results of the *in vitro* enamel matrix derivative (EMD)-release study. (A) Release of EMD per week. (B) Total (cumulative) release of EMD.

acid) particles was complete, which resulted in a fast increase in the EMD-release profile. After 4 wk, 60% of the total amount of EMD present in the poly(D,L-lactic-coglycolic acid)/calcium phosphate composites was released in the medium and it seemed that this increasing pattern continued. However, *in vivo* release patterns are known to differ from the *in vitro* situation with a lower release profile *in vivo* compared with *in vitro* (9,10). Besides that, calcium phosphate cement has been reported to exhibit a high protein-binding affinity (19). As a consequence, an interaction of EMD with calcium phosphate cement may have occurred, which might have hampered additionally the final release of EMD. Therefore, it cannot be excluded that the released EMD failed to reach the dose required to evoke an effect. However, further investigations with other carrier materials or higher concentrations of EMD, as well as *in vivo* release assays of EMD, are required to prove this hypothesis.

The current results confirmed again the excellent biocompatibility and osteoconductivity of poly(D,L-lactic-coglycolic acid)/calcium phosphate cement (20,21). Although the material was applied in a preset condition, defects with unloaded poly(D,L-lactic-coglycolic acid)/calcium phosphate composites showed the most abundant bone formation. In all cranial specimens, direct contact between the newly formed bone and the composites was found, whereas a few multinucleated inflammatory cells were seen on the cement surface.

Conclusion

In summary, we conclude that in the current study, Emdogain® was found to lack osteoinductive properties and was not able to enhance bone healing in combination with an osteoconductive poly(D,L-lactic-coglycolic acid)/calcium phosphate material. However, the limited release pattern of EMD over the 4 wk period of this study should be taken into account. Therefore, as the exact mechanism of action of EMD is still unknown, further investigations are required.

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References

1. Hammarstrom L. Enamel matrix, cementum development and regeneration. *J Clin Periodontol* 1997;**24:**658–668.
2. Heijl L. Periodontal regeneration with enamel matrix derivate in one human experimental defect. A case report. *J Clin Periodontol* 1997;**24:**693–696.
3. Boyan BD, Weesner TC, Lohmann CH *et al*. porcine Fetal Enamel matrix derivate enhances bone formation induced by demineralized freeze dreied bone allograft *in vivo*. *J Periodontol* 2000;**71:** 1278–1286.
4. Gestrelius S, Andersson C, Lidstrom D. *In vitro* studies on periodontal ligament cells and enamel matrix derivate. *J Clin Periodontol* 1997;**24:**685–692.
5. Kawase T, Okuda K, Momose M, Kato Y, Yoshie H, Burns DM. Enamel matrix derivate (Emdogain®) rapidly stimulates phosphorylation of the MAP kinase family and nuclear accumulation of smad2 in both oral epithelial and fibroblastic human cells. *J Periodont Res* 2001;**36:**367–376.
6. Iwata T, Morotome Y, Tanabe T, Fukae M, Ishikawa I, Oida S. Noggin blocks osteoinductve activity of porcine enamel extracts. *J Dent Res* 2002;**81:**387–391.
7. del Real RP, Wolke JG, Vallet-Regi M, Jansen JA. new method to produce macropores in calcium phosphate cements. *Biomaterials* 2002;**23:**3673–3680.
8. Ruhe PQ, Hedberg EL, Padron NT, Spauwen PH, Jansen JA, Mikos AG. Biocompatibility and degradation of poly (DL-lactic-co-glycolic acid) /calcium-phosphate cement composites. *J Biomed Mater Res A* 2005;**74:**533–544.
9. Ruhe PQ, Hedberg EL, Padron NT, Spauwen PH, Jansen JA, Mikos AG. rh BMP-2 release from injectable poly (DL-lactic-co-glycolic acid) /calcium-phosphate composites. *J Bone Joint Surg Am* 2003;**85(A** Suppl. 3)**:**75–81.
10. Ruhe PQ, Boerman OC, Russel FG, Spauwen PH, Mikos AG, Jansen JA. Controlled release of rhBMP-2 loaded poly (DL-lactic-co-glycolic acid)/calcium phosphate cement composites *in vivo*. *J Control Release* 2005;**106:**162–171.

11. Ruhe PQ, Hedberg-Dirk EL, Padron NT, Spauwen PH, Jansen JA, Mikos AG. Porous poly (DL-lactic-co-glycolic acid)/calcium phosphate cement composite for reconstruction of bone defects. *Tissue Eng* 2006;**12:**789–800.
12. Schmitz JP, Hollinger JO. The critical size defect as an experimental model for craniomandibular nonunion. *Clin Orthop Relat Res* 1986;**205:**299–308.
13. Yoneda S, Itoh D, Kuroda S *et al*. The effects of enamel matrix derivate (EMD) on osteoblastic cells in culture and bone regeneration on a rat skull defect. *J Periodont Res* 2003;**38:**333–342.
14. Davenport DR, Mailhot JM, Wataha JC, Billman MA, Sharawy MM, Shrout MK. Effects of enamel matrix protein application on the viability, proliferation, and attachment of human periodontal ligament fibroblasts to diseased root surfaces *in vitro*. *J Clin Periodontol* 2003;**30:**125–131.
15. Donos N, Lang NP, Karoussis IK, Bosshardt D, Tonetti M, Kostopoulos L. The effect of GBR in combination with deproteinized bovine bone mineral and/or enamel matrix proteins on the healing of critical-size defects. *Clin Oral Impl Res* 2004;**15:**101–111.
16. Donos N, Bosshardt D, Lang N *et al*. Bone formation by enamel matrix proteins and xenografts: an experimental study in rat ramus. *Clin Oral Implants Res* 2005;**16:**140–146.
17. Hedner E, Linde A. Efficacy of bone morphogenetic protein (BMP) with osteopromotive membranes – an experimental study in rat mandibular defects. *Eur J Oral Sci* 1995;**103:**236–241.
18. Puolakkainen PA, Twardzik DR, Ranchalis JE, Pankey SC, Reed MJ, Gombotz WR. The enhancement in wound healing by transforming growth factor-b1 (TGF-b1) depends on the topic delivery system. *J Surg Res* 1995;**58:**321–329.
19. Blom EJ, Klein-Nulend J, Wolke JG, van Waas MA, Driessens FC, Burger EH. Transforming growth factor-beta1 incorporation in a calcium phosphate bone cement: material properties and release characteristics. *J Biomed Mater Res* 2002;**59:**265–272.
20. del Real RP, Ooms E, Wolke JG, Vallet-Regi M, Jansen JA. *In vivo* bone response to porous calcium phosphate cement. *J Biomed Mater Res A* 2003;**65:**30–36.
21. Ruhe PQ, Kroese-Deutman HC, Wolke JG, Spauwen PH, Jansen JA. Bone inductive properties of rhPMP-2 loaded porous calcium phosphate cement implants in cranial defects in rabbits. *Biomaterials* 2004;**25:**2123–2132.