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Histological Evaluation of Degradable Guided Bone Regeneration Membranes Prepared from Poly(trimethylene carbonate) and Biphasic Calcium Phosphate Composites

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Summary: In oral and maxillofacial surgery, guided bone regeneration using barrier membranes is an important strategy to treat bone defects. The currently used barrier membranes have important disadvantages. Barrier membranes prepared from resorbable poly(trimethylene carbonate) (PTMC) performed as well as collagen barrier membranes. We hypothesized that composite membranes prepared from surface-eroding PTMC and osteoinductive biphasic calcium phosphate (BCP) would enhance bone formation even further. Bicortical critical size defects in the mandibular angle of rats were covered on both sides with the membranes. After 2, 4, and 12 weeks the extent of bone formation in the defects and the soft tissue reaction towards the membranes was examined histologically. At 2 and 4 weeks, the formation of new bone was observed in defects covered with PTMC, PTMC-BCP and Biogide collagen membranes. At 12 weeks, bone defects that were covered with PTMC membranes and control Biogide collagen membranes were fully filled with new formed bone. However, at this time point, defects covered with PTMC-BCP composite membranes had not led to new bone in the defects. Instead a significant tissue reaction, likely to remaining BCP particles, was observed.

Keywords: animal implantation study; biphasic calcium phosphate; composites; degradation; guided bone regeneration; poly(trimethylene carbonate)

Introduction

Guided bone regeneration (GBR) is a surgical technique that is used in clinical practice for the treatment of periodontitis and bone augmentation before placement of dental implants. Different barrier membranes have been used to cover jawbone defects thereby providing space for bone regeneration.[1]

An ideal barrier membrane should possess sufficient rigidity to maintain the necessary space, enhance bone formation, degrade and resorb at an appropriate rate without the formation of detrimental degradation products and it should be easy to use by the clinician. The currently available membranes have important drawbacks including non-degradability and a high risk of membrane exposure, or too rapid loss of mechanical properties and production of acidic degradation products in case of degradable materials.[2]

Composite membranes composed of biodegradable polymers and bone-inducing calcium phosphate granules have drawn much interest.[3] Such composites show increased elastic modulus values and are compatible with osteoblast-like cells and bone marrow-derived mesenchymal stem cells (MSCs).[4] *In vivo* studies have

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demonstrated the potential of calcium phosphate composite membranes in treating jawbone defects.[5]

In a previous study, we assessed the potential of high molecular weight poly (trimethylene carbonate) (PTMC) films as barrier membranes in GBR.[6] This amorphous flexible polymer crosslinks upon sterilization by gamma irradiation, and undergoes enzymatic surface erosion *in vivo* without the formation of acidic degradation products.[7] It has also been shown that biphasic calcium phosphate (BCP) granules prepared by sintering at 1150°C are osteoinductive[8] and can enhance the formation of new bone in orthotopic defects[9]. We hypothesized that composite membranes prepared from surface-eroding PTMC and bone-inducing BCP would allow the enhanced formation of bone in guided bone regeneration. In this study, we investigated the characteristics of such GBR membranes in a previously described rat jawbone model.[10]

Experimental Part

Materials

Polymerization grade 1,3-trimethylene carbonate was obtained from Boehringer Ingelheim, Germany and stannous octoate from Sigma, USA. Both were used as received. Biphasic calcium phosphate ceramic, containing tricalcium phosphate and hydroxyapatite at a ratio of $20 \pm 3\%$ to $80 \pm 3\%$, was provided by Xpand Biotechnology, the Netherlands. The BCP ceramic was sintered at 1150°C for 8 hours and sieved to particle sizes of 45-150 μm. It had a microporosity of 17% and a specific surface of 1.0 $m^2.g^{-1}$.[9] The used solvents were of analytical grade and purchased from Biosolve, the Netherlands.

Collagen membranes (BioGide) were obtained from Geistlich, Switzerland.

Preparation of PTMC-BCP Composite Membranes

PTMC was prepared by ring opening polymerization of trimethylene carbonate under vacuum at 130 °C for 3 days catalyzed by stannous octoate at a concentration of 2×10^{-4} mol per mol of monomer.[11] Proton nuclear magnetic resonance (^{1}H-NMR), gel permeation chromatography and differential scanning calorimetry were used characterize the synthesized polymer as described before.[12] In the polymerization, the monomer conversion was higher than 98%. The polymer was amorphous and had a glass transition temperature of −17°C. Its weight average molecular weight (M_w) was 443000 $g.mol^{-1}$ and its number average molecular weight (M_n) 332000 $g.mol^{-1}$.

The membranes were prepared as previously described.[6] The PTMC was dissolved in chloroform at a concentration of 5 $g.100\,ml^{-1}$, and the BCP granules (2.5 $g.100\,ml^{-1}$) were dispersed in the solution by magnetic stirring. The homogeneous dispersion was then rapidly precipitated into a five-fold excess of ethanol 100%. The PTMC-BCP precipitate was dried under vacuum at room temperature until constant weight.

Composite PTMC and BCP membranes, 8 mm in diameter and 0.3 mm in thickness, were produced by compression molding the precipitate at 140 °C using a Carver model 3851-0 laboratory press (Carver Inc., USA).[12] The composite membranes were sealed under vacuum and sterilized by gamma irradiation (25 kGy) at Isotron BV, Ede, The Netherlands. This procedure leads to cross-linking of the PTMC, thereby forming a flexible and elastic network.[12]

Surgical Procedures

All procedures on animals were done according to international standards on animal welfare and were approved by the Animal Research Committee of the University Medical Center of Groningen.

Thirty six Sprague-Dawley rats, between 12 and 16 weeks old weighing between 325 and 400 g were used in this study. The rats were anaesthetized using isofluranenitrous-oxygen gas. A peri-angular incision was made to expose the left mandibular angle. Bicortical bone defects with a

diameter of 5 mm were drilled with a trephine.[10] The defects were covered on both the buccal and the lingual side with PTMC membranes, the PTMC-BCP composite membrane and with BioGide collagen membranes also 8 mm in diameter which were used as a control. None of the membranes were fixed to the bone tissue. The wounds were closed layer-by-layer using resorbable sutures (Vicryl Rapide 4-0, Ethicon, USA). A single dose of Temgesic (0.05 mg.kg^{-1}) was administered for pain relief immediately after the operation, and the animals were provided with standard laboratory food.

Follow-up was at 2 weeks, 4 weeks and at 12 weeks post-surgery. At each follow-up moment four animals per material group were sacrificed by intracardial injection of an overdose of pentobarbital under isoflurane-nitrous-oxygen inhalation anaesthesia. The left mandibles were retrieved and fixated in neutralized 4% paraformaldehyde solution.

Histological Evaluation

All explanted samples were decalcified and dehydrated in a graded series of ethanol solutions, and then embedded in glycol methacrylate (GMA). Sections of 2 μm thickness were cut perpendicularly to the defects. The sections were stained with toluidin blue or with toluidin blue/basic fuchsin as counterstain, using standard protocols.

The sections were examined using a Leica DMR (Germany) microscope, and were graded using a semi-quantitative histological grading scale (Table 1) that was also used in an earlier study.[11] Of each sample two sections were blindly analyzed by two investigators. Each section was scored independently, only whole numbers were given to the sections. In this scale, highest scores correspond to best results. For the ordinal data collected in our study, the Mann-Whitney U rank sum test was used to statistically evaluate differences between the 3 membrane groups.

Results

Clinical Observations

All rats recovered uneventfully, the rats showed interest in food shortly after the surgery and maintained a normal body weight. One animal from the two weeks PTMC-BCP composite membrane group was excluded from the study because of fracture at the site of the defect.

Table 1.
Semi-quantitative histological grading scale.

Bone formation in defect	
Mature bone and differentiation of bone marrow	4
Bone or osteoid formation	3
Fibrous connective tissue: collagen fibers at defect site	2
Fibrous connective tissue: cellular and vascular components	1
Cannot be evaluated because of infection or other factors not necessarily related to the material	0
Space maintaining properties of membrane	
No contact between membranes at defect site, bone formation in between	4
No contact between membranes at defect site, connective tissue in between	3
Contact between membranes at defect site, bone formation present	2
Contact between membranes at defect site, connective tissue in between	1
Cannot be evaluated because of degradation or absence of the material	0
Soft tissue response to membrane	
Fibrous, mature, not dense, resembling connective or at tissue in the non-injured regions	4
Shows blood vessels and young fibroblasts, few macrophages and giant cells are present	3
Shows macrophages and other inflammatory cells in abundance, but connective tissue components between	2
Dense and exclusively of inflammatory type	1
Cannot be evaluated because of infection or other factors not necessarily related to the material	0

Light Microscopical Observations

No suppuration or sequestra and no signs of resorption of bone adjacent to the defects could be seen by light microscopy.

Figure 1 shows that at the 2 and 4 week time points remainders of the three different membranes could still be recognized. At the 12 week time point the PTMC- and the Biogide collagen membranes could not be seen because of degradation and resorption. Upon explantation of the composite PTMC-BCP membranes at this time point, remnants of the polymer embedded in a thick layer of connective tissue containing macrophages and foreign-body giant cells was observed (Figure 1). The presence of foreign body giant cells is most evident at higher magnifications. Figure 2 already shows the presence of foreign body giant cells in the tissue surrounding remnants of the PTMC-BCP composite after 4 weeks. Foreign body giant cells were not found near or in contact with the bone surface. As the tissue sections were decalcified, the ceramic BCP granules could not be seen in the images.

For all the different membranes, signs of new bone formation were seen at 2 weeks. At 4 weeks new bone formation was seen from the ends to the center of defects covered with PTMC membranes and collagen membranes. In the defects covered with PTMC-BCP membranes abundant bone formation was observed at this time point. Most defects were fully bridged with *de novo* bone tissue. The jawbones seemed healed, with only a narrowing of the width of the bone at the site where the defect was created. However, after 12 weeks the defects that had been covered with the PTMC-BCP composite membranes were filled with connective tissue containing polymeric remnants of the membranes. The new formed bone could not be seen anymore. At 12 weeks, the defects that were covered with PTMC membranes or Biogide collagen membranes were almost completely filled with relatively mature bone tissue. Figure 1 illustrates this sequence of events.

Semi-Quantitative Histological Grading

The semi-quantitative results of the histological grading of the sections of the tissue treated with the different membranes are shown in Figure 3.

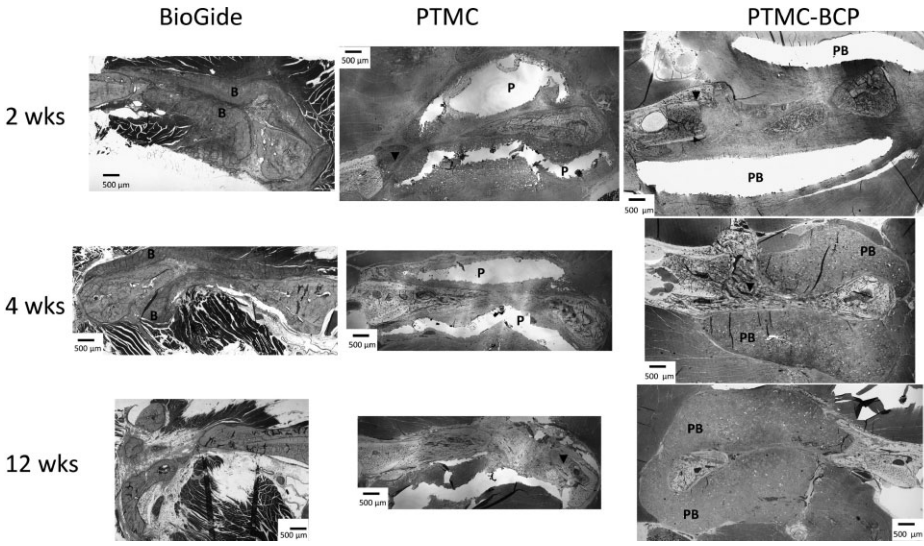

Figure 1.
Light microscopical observations of mandibular defects covered with BioGide collagen, PTMC or PTMC-BCP membranes for 2, 4 and 12 weeks. (B) BioGide; (P) PTMC membrane; (PB) PTMC-BCP membranes; (▼) newly formed bone; (▲) thickening of the tissue.

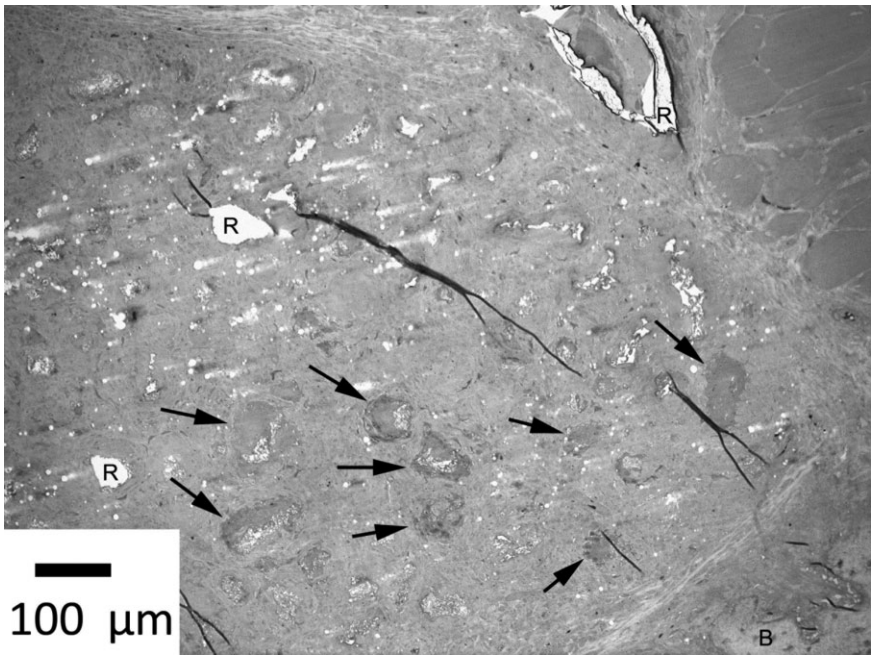

Figure 2.
Histological picture of remnants of a PTMC-BCP membrane after 4 weeks of implantation, stained with toluidin blue. The arrows indicate foreign body giant cells; B = bone; R = remnants of the membrane.

Regarding the extent of new bone formation, at 2 and at 4 weeks the different materials seem to perform similarly indicating that the bone regeneration process was the same in all experimental animal groups. Noteworthy is the relatively large standard deviations in the bone formation values of animals treated with Biogide collagen. At 12 weeks the amount of new bone present in defects treated with the PTMC-BCP composite is much lower than in defects treated with PTMC or the Biogide collagen control.

The ability to maintain space for bone regeneration was found to be higher for the PTMC- and PTMC-BCP composite membrane than for the Biogide collagen membrane. At 12 weeks space maintenance could not be observed due to degradation of the membranes.

The extent of the soft response to the membranes was found to be most favorable for the Biogide collagen membranes at all the time points. The reaction of the soft tissue to the degraded PTMC-BCP composite at 12 weeks is remarkable, and much less favorable than that to the Biogide collagen- or PTMC membrane.

Discussion

Despite significant interest in the development of degradable composite membranes, relatively few studies have been carried out to verify their suitability for GBR. Experiments using proper defect animal models are required. In this study, we set out to evaluate the suitability of composites prepared from surface-eroding PTMC polymer and osteoinductive BCP ceramic particles for use as GBR membranes using a previously described critical mandibular defect model in the rat.[10].

The space maintaining properties of barrier membranes is a determinant factor in allowing bone formation.[1] Although collagen membranes lose their mechanical strength and rigidity very rapidly *in vivo*, their use still allows the formation of bony

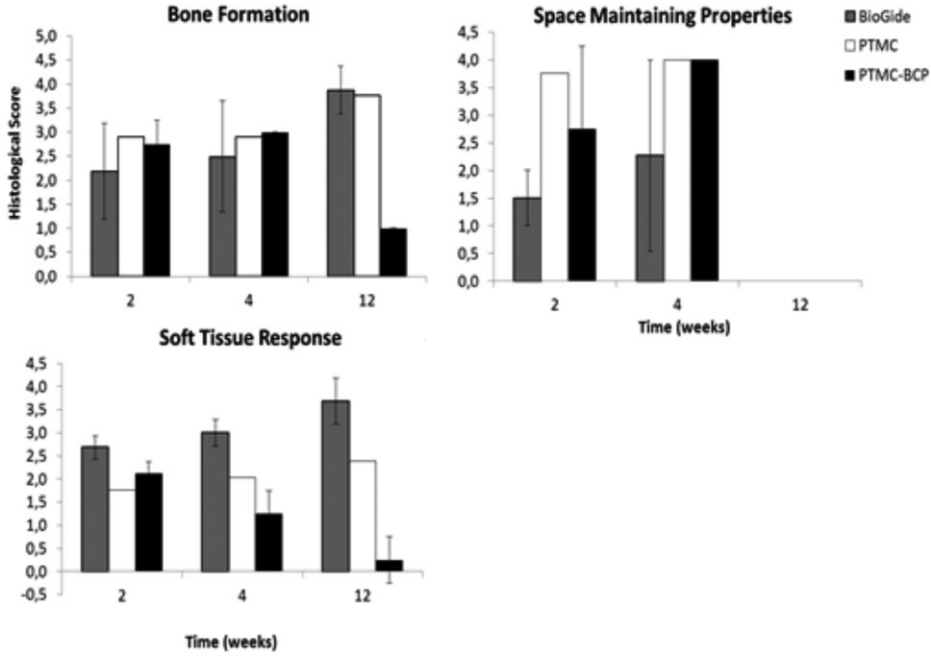

Figure 3.
Semi-quantitative histological grading of sections of bicortical rat jawbone defects that were covered with BioGide collagen, PTMC or PTMC-BCP membranes at 2, 4 and 12 weeks. The error bars indicate the standard deviation of the mean, all scorings in the PTMC group were equal and the standard deviation equaled 0. The Mann-Whitney U rank sum test was used for statistical analysis.

islets between the membranes. Bone formation between the hydrophilic collagen membranes is possibly caused by the adherence of the membranes to adjacent bone tissue and the release of bone-inducing degradation products like peptides.[13] It has been observed that the success of the GBR procedure using collagen membranes greatly varies between surgeons. This can be related to their operating skills.

Compared to the collagen membranes, the hydrophobic PTMC and the PTMC-BCP composite membranes maintain their mechanical properties for longer times much longer times *in vivo*. As a result the necessary space is provided to allow for the formation of abundant new bone that was observed at 4 weeks. Although the rigidity of the PTMC membrane is lower than that of the PTMC-BCP composite membrane,[14] it apparently is sufficient to maintain space adequately. At 12 weeks the extent of new bone formation in defects treated with PTMC membranes was very good and comparable to that in defects treated with Biogide collagen membranes. The PTMC-BCP composite membranes performed significantly worse and more foreign body giant cells were present.

The surface erosion process of PTMC *in vivo*,[15] will lead to liberation of BCP particles from the PTMC-BCP composite and roughening of the surface of the membrane. In the rat model used, the membranes were not fixed to the bone and shifting of the position of the membranes could have occurred as a result of masticatory forces and other forces exerted by muscles from physiological activities.[16] In addition to this, the diameter of the membranes (8 mm) was small in comparison to the diameter of the defects (5 mm) and invagination of the composite membranes into the defect might have been difficult to prevent.

The reaction of the soft tissue to the degrading PTMC-BCP composite membranes can result from the degradation process of PTMC that results in the liberation of BCP particles. As the polymer matrix degrades and resorbs *in vivo*, phagocytic cells including macrophages are attracted to conduct the degradation at the interface between the membrane and the surrounding soft tissue. While PTMC membranes were found to be fully resorbed and replaced by normal soft tissue after 12 weeks implantation,[11] a pronounced reaction of the tissue near the degrading PTMC-BCP composite membrane was obvious. At this time point the tissue reaction is most likely towards the BCP granules which degrade only very slowly.[8] (In the histology images these particles cannot be observed, as the coupes were decalcified prior to embedding. Micro-CT imaging, however, did show the presence of BCP granules in the soft tissue). In the animal model we used, mechanical irritation resulting from forces exerted by muscles on tissue surrounding the particles could have intensified the reaction of the tissue.

Conclusion

In this study we confirmed that to be able to successfully regenerate bone in defects, the membranes used to cover the defects need to have sufficient space maintaining properties. With regard to bone formation in a rat jawbone defect, synthetic degradable and resorbable membranes prepared from poly(trimethylene carbonate) performed very well. Abundant bone formation, comparable to that when collagen Biogide membranes were used, was observed. In the model used, composite membranes prepared from PTMC and osteoinductive biphasic calcium phosphate particles did not prove to be advantageous and much reduced bone formation was observed. Although this needs to be investigated in more detail, this can be due to reaction of the tissue towards the slowly degrading particles mechanical irritation as a result of mechanical irritation.

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