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The formation of tertiary dentin after pulp capping with a calcium phosphate cement, loaded with PLGA microparticles containing TGF-β1

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Abstract: The aim of the current study was to evaluate the effect of a calcium phosphate material equipped with poly (lactic-*co*-glycolic acid) microspheres for pulp capping, and to measure the dentin bridge formation, when using various concentrations of transforming growth factor (TGF) β1. Preset samples were made (2 mm diameter; 2 mm height), containing 0 (controls), 20, or 400 ng TGF-β1. These were placed in goat incisors. Incisors capped with glass-ionomer cement only were used as negative controls. Twelve weeks after pulp capping, the incisors were retrieved, processed for histology, and graded on basis of tertiary dentin formation. The results showed that new dentin formation was seen in all samples, except the negative controls. The histological grading indicated significant differences between the samples loaded with high amount of TGF-β1 versus the three other groups ($p < 0.05$). In conclusion, our study demonstrated that the composite with 400 ng TGF-β1 was able to trigger resident stem cells in the pulp to differentiate into odontoblast-like cells and to induce the formation of tertiary dentin. The material might be a good candidate for vital pulp therapy. Production and manipulation methods could be improved for follow-up studies. © 2007 Wiley Periodicals, Inc. J Biomed Mater Res 85A: 439–444, 2008

Key words: dental materials; pulp capping; calcium phosphate cement; transforming growth factor β1

INTRODUCTION

Although application of calcium hydroxide is a treatment option to encourage hard tissue bridging after dental pulp capping, the material is not able to really induce new tissue formation.[1] It has been suggested that tissue engineering techniques can offer a solution for this problem.[2] One approach is the development of a conductive scaffold that releases morphogenetic signals as required to induce the resident stem cells as present in the dental pulp to regenerate the new tooth tissue.

Transforming growth factor βs (TGF-βs) are a group of proteins that play a role in embryonal development, cellular differentiation, hormone secretion, and immune function. TGF-β has three highly similar isoforms, namely, TGF-β1, TGF-β2, and TGF-β3. In human teeth, odontoblasts express all three isoforms of TGF-β. However, only TGF-β1 becomes sequestered within the matrix.[3]

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During tooth formation, TGF-β1 is at first highly expressed in the epithelium. Subsequently, this high expression shifts to the related mesenchyme.[4] This suggests that TGF-β1 plays an important role during tooth formation. It has been proven that TGF-β1 can directly induce the differentiation of odontoblast-like cells, and upregulate the secretion of matrix components in the dentin-pulp complex.[5] The dentinogenic activity of EDTA-dissolved dentin is hampered when the material is preincubated with an anti-TGF-β1 antibody.[6] When TGF-β1 is applied on tooth slices, which are cultured *in vitro*, increased cell proliferation was observed in the subodontoblastic layer, and in the underlying pulp.[7] Consequently, it seems that TGF-β1 might be a good candidate to apply when regenerating the dentin-pulp complex, for instance after pulp capping.

An alternative for calcium hydroxide as pulp-capping material is self-setting calcium phosphate cement. Self-setting calcium phosphate materials have been widely used for orthopaedic treatment, because they are highly biocompatible, and have the potential to stimulate osteogenesis. Their injectability and setting/cohesion properties add more value to such materials. It has been proven that self-setting calcium phosphate cements show similar biocompat-

ibility to pure calcium hydroxide. Also the effect in dentin bridging, when used as a direct pulp capping agent, is comparable between both materials.[8]

On basis of the above-mentioned, it can be hypothesized that the biological properties of the cement can be further optimized by bringing hydrosoluble polymers (like polylactic-glycoloc acid) into the calcium phosphate, which subsequently can act as vehicles for the delivery of signal molecules that stimulate the differentiation of naturally present stem cells into odontoblasts, and thus results in a biological means of regeneration through enhanced tertiary dentin formation. The degradable microparticles can offer a long-term release of the bioactive molecules, but the particles will also account for porosities, after polymer degradation, which can provide the necessary space for tissue ingrowth.

Therefore, the aim of the current study was to evaluate the effect of a calcium phosphate/hydrosoluble polymer composite for pulp capping, and to measure the dentin bridge formation, when using various concentrations of TGF-β1.

MATERIALS AND METHODS

Preparation of capping agents

Poly(lactic-co-glycolic acid) PLGA microspheres were prepared by a double-emulsion-solvent-evaporation technique (w/o/w).[9] In brief, 1.0 g of PLGA was dissolved in 4 mL of dichloromethane (DCM). Then, 500 μL of MilliQ and 6 mL of a 0.3% poly(vinyl alcohol) (PVA) solution were added while vigorously vortexing. Subsequently, 394 mL of 0.3% PVA and 400 mL of a 2% isopropylic alcohol (IPA) solution were slowly added. The formed particles were allowed to settle and the supernatant was discarded. Finally, the particles were lyophilized, and stored in Argon at −20°C.

Shortly before use, PLGA microparticles were submerged in (0.1% Bovine serum albumin/phosphate-buffered saline solution; BSA/PBS) solutions with different concentrations of TGF-β1, lyophilized overnight and stored at −20°C until the next day. The density of TGF-β1 was calculated according to the amount of PLGA particles, in order to reach final concentrations of 20 or 400 ng per capping material. Also, CaP cement/ PLGA composite samples with no added growth factor were made.

The CaP cement used was composed of 62.5% α-tricalcium phosphate (α-TCP), 26.8% dicalcium phosphate (DCPA), 3.9% $CaCO_3$, and 1.8% hydroxyapatite (HAP), and sterilized by γ-irradiation. A 80/20 (w/w) CaP cement/PLGA composite was prepared, that is, 2 mg PLGA/ TGF-β1 microparticles was added to 8 mg of CaP cement. The PLGA/CaP powder was mixed with 4 μL of 1% Na_2HPO_4 to create a paste. After vigorously stirring, the prepared mixture was injected into cylindrical moulds of 2 mm in diameter and 2 mm in depth under aseptic conditions. All preset implants were dried overnight and then stored at −20°C until use in the animal study. Finally, several samples of each group were fixed and analyzed by scanning electron microscopy (SEM).

Animal model and pulp capping procedure

Incisors of healthy mature female Saanen goats (age = 3 ± 1 years) were chosen as experimental model. Approval for the study was obtained from the Radboud University Nijmegen Animal Ethical Committee (RU-DEC-2005-030). Further, only goats already involved in other experiments were used. The placement of the pulp capping cement was performed under general anaesthesia, induced by an intravenous injection of Pentobarbital and maintained by Isoflurane 2%–4%. In order to reduce the possibility of infection, antibiotics (Albipen®) were given during the operation (3 mL/50 kg s.c), one day after the operation (7.5 mL/50 kg s.c), and 3 days after the operation (7.5 mL/50 kg s.c). All goats received analgesic (Finadyne®) for 2 days postoperatively.

Before pulp capping, the oral cavity, and especially the tooth surface, was cleaned with povidone-iodine. Then, cavities of 2.1 mm in diameter and 2.5 mm in depth were prepared through the lingual surface of the tooth with a sterile ball-shaped bur mounted on a conventional dental hand-piece. During cavity preparation, sterile 0.9% saline was used for cooling and removal of debris. After punctuation of the dental pulp, haemostasis was controlled by a sterile cotton pellet. The incisors were divided into four different groups, and samples were available in sixfold (n = 6) according to Table I. After installation of the various composite materials, all cavities were closed with glass-ionomer cement. Incisors capped with glass-ionomer cement only were used as negative control.

Histological analysis

Twelve weeks after pulp capping, the goats were sacrificed. The mandibles including incisors were surgically dissected en bloc, divided through the mid-line, and fixed in 10% buffered formalin solution for 1 week. Subsequently, the biopsies were demineralized in Formical-2000 (Decal Chemical; Congers, NY) for 3 weeks at 4°C with continuous stirring. After dehydration, the demineralized samples were embedded in paraffin. Buccal-labial serial sections of 7 μm were cut. The sections through the exposure sites were selected, stained with hematoxylin-eosin, and evaluated using a light microscope.

Five sections from the centre of each sample were assessed. Digitized photomicrographs were made, and the samples were graded in a blinded manner by three independent observers, according to the criteria described in

TABLE I
Study Groups in the Experiment

Group	Description	Capping Materials	Number
1	TGF-β1 (high dose)	PLGA/CaP cement + TGF-β1 (400 ng/tooth)	6
2	TGF-β1 (low dose)	PLGA/CaP cement + TGF-β1 (20 ng/tooth)	6
3	PLGA/ CaP control	PLGA/CaP cement + 0.1% BSA	6
4	Negative control	None	6

TABLE II
Histological Grading Scale

Dentin Bridge Formation	Score
No visible hard tissue formation beneath or around the exposed area	0
Initial signs of hard tissue formation beneath or around the exposed area	1
Incomplete bridge-like hard tissue formation beneath or around the exposed area	2
Complete bridge formation beneath or around the exposed area	3

Table II. All data were analyzed by an analysis of variance (ANOVA) and *post hoc* Tukey testing.

RESULTS

Cement preparation

After mixing with Na_2HPO_4, the PLGA/CaP cement was easy to inject into the molds. After hardening, several samples from each group were inspected with SEM microscopy, which indicated that the PLGA microparticles were spherically shaped with a diameter ranging from 20 to 50 μm. The surface of the microparticles appeared smooth, while only occasionally small pores were found on the surface of particles. The distribution of PLGA microparticles within cement was uniform. The addition of growth factors had no obvious influence as the morphology or distribution of microparticles for all three PLGA/CaP cements showed a similar appearance upon qualitative inspection (Fig. 1).

Animal study

All goats appeared to be in good health throughout the experimental period. No weight loss was observed. No fracture or other damage was found in any of the tested incisors. All incisors were retrieved complete with surrounding tissues.

Light microscopy showed no mass infiltration of inflammatory cells in any of the samples. Only occasionally, inflammatory cells were detected in the pulp near the treated sites. No distinct tissue degradation was observed in any of the samples. Notice-
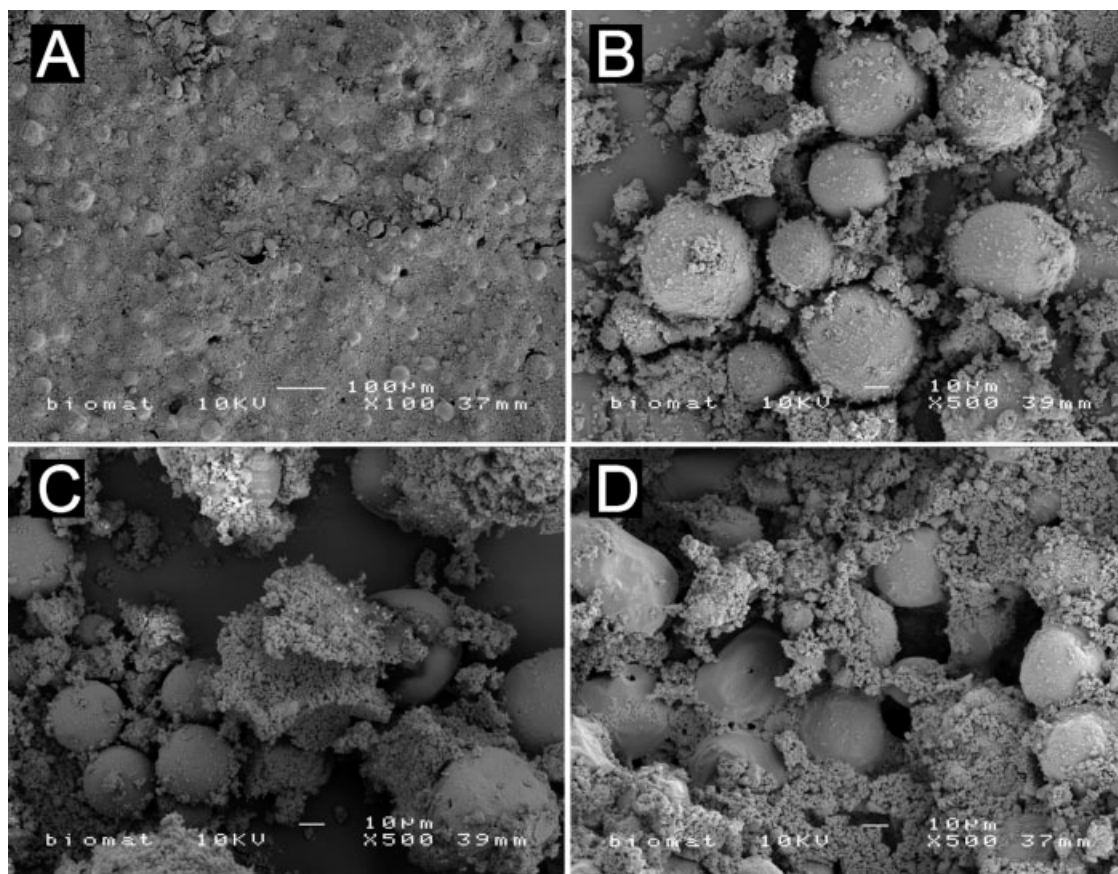

Figure 1. Scanning electron micrographs of samples before use; (A) ×100 overview of PLGA/CaP cement (B) higher magnification of the microparticles at ×500 (C) cement and microparticles loaded with the high low of TGF-β1, ×500 (D) cement and microparticles loaded with the high dose of TGF-β1, ×500.
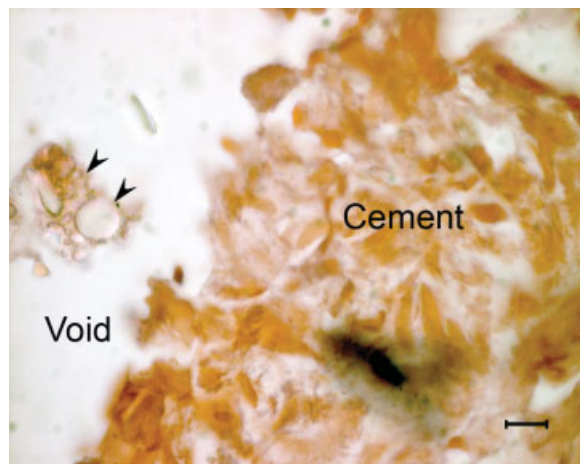
Figure 2. Remnants of the PLGA/CaP cement in a control sample. Arrow indicates nondegraded polymer particles (bar: 10 μm). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
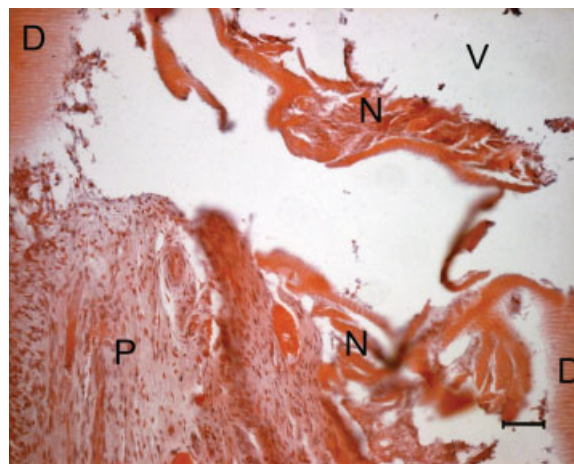

Figure 3. The formation of tertiary dentin in a sample with the high concentration TGF-β1. P, pulp tissue; D, existing dentin; N, new formed dentin; V, void due to histological preparation (bar: 25 μm). [Color figure can be viewed in the online issue, which is available at www. interscience.wiley.com.]

ably, in the sections of the negative control samples (receiving glass ionomer cement only) the soft tissue of the pulp was no longer in contact with the surrounding dentin, but had detached over the largest part. This detached area extended to more than half of the length of the pulp. Furthermore, for the teeth provided with the various CaP/PLGA cements, it was observed that the PLGA microparticles had not fully degraded after 12 weeks. Nondegraded polymer particles were found in most of the remaining capping materials (Fig. 2).

In all sections, it seemed that tissue had lost its original integrity during the histological preparation. Although all tissues were present in the samples, often between tissue layers empty voids were observed. Still, new dentin formation was clearly present in the samples (Fig. 3). Occasionally, a dentin-like tubular structure was observed in the newly formed reparative dentin. Complete dentin-bridge was found only in one of the samples capped by PLGA/CaP cement with the high amount of TGF-β1. No hard tissue formation at all was found in any of the negative control incisors capped directly with glass-ionomer cement. The histological grading scores for all four groups are presented in Table III.

Statistical analysis indicated significant differences between the samples loaded with high amount of TGF-β1 versus the three other groups ($p < 0.05$). No difference ($p > 0.05$) existed between the samples loaded with low amount of TGF-β1 and the samples loaded with PLGA/CaP cement without added growth factor. However, when the data of the low concentration and nonloaded cement group were pooled, they showed a significant difference ($p < 0.05$) compared to the negative control group using glass ionomer cement only.

DISCUSSION

The objective of this study was to evaluate the effect of a calcium phosphate/ hydrosoluble polymer composite for pulp capping, and to measure the dentin bridge formation, when using various concentrations of TGF-β1. Results showed two evident effects. First, the used PLGA/CaP composite enhanced tertiary dentin formation, and second, this process was enhanced considerably by the addition of 400 ng of TGF-β1.

The importance of maintaining the vitality of the dental pulp is widely accepted. The possibility of exposed pulps to heal and form reparative dentin has been confirmed by numerous researchers.[10,11] However, vital pulp capping is known to have a very variable prognosis.[12,13] Numerous factors may influence the healing process of the dental pulp, including the condition of the pulp itself, the restorative manipulation, the applied capping material, and so forth. Amongst these, the selection of an appropriate capping material is one of the essential factors. The most commonly used materials for dental pulp capping are various forms of calcium hydroxide, and the odontogenetic effect of such materials has

TABLE III
Average Scores of the Histological Grading of Six Samples by the 3 Observers

Group	Score (±SD)
TGF-β1 (high dose)	1.72 (±0.85)
TGF-β1 (low dose)	0.89 (±0.50)
PLGA/CaP control	0.78 (±0.50)
Negative control	0.06 (±0.14)

been confirmed in many studies. One previous study proved that self-setting calcium phosphate cements show similar biocompatibility to pure calcium hydroxide. Also the effects, when used as a direct pulp-capping agent, are comparable between both materials.[8] This was the rationale for using CaP material in the current study.

It should be noticed that the degradation ability of a solid calcium phosphate cement is fairly low, especially when applied in tooth cavity, which is a comparatively entrapped environment with limited blood supply. Low degradation rates thus could have a hampering effect on new tissue ingrowth and replacement. In our study, we therefore opted for a cement previously validated in bone regeneration studies, which was equipped with degradable PLGA particles into the calcium phosphate matrix.

Noticeably, the nonloaded cement/polymer composite was beneficial when compared to using glass ionomer cement only. Further bioactive properties were introduced in the cement by the addition of a growth factor. The odontogenic properties of TGF-β1 have been proven in previous studies, which indicated that TGF-β1 indeed is an appropriate candidate to apply when regenerating the dentin-pulp complex, for instance after pulp capping. However, the appropriate concentration for TGF-β1 (or any other growth factor) to support pulp stem cell differentiation remains difficult to determine. There is no previous literature on the application of TGF in this model. According to two previous reports,[14,15] two distinct concentrations of 20 or 400 ng of TGF-β1 were chosen for the current study, although the Tziafas study used a different release system/animal model, and the Goldberg study used another growth factor. Still, the formation of tertiary dentin in our study supported the possibility to use PLGA/CaP cement loaded with TGF-β1 for dental capping in a dose-dependent way. The amount of new regenerated dentin in the samples loaded with the higher dose of TGF-β1 was superior to the other study groups. However, the samples capped with lower dose of TGF-β1 (20 ng/tooth) were not effective and illustrated similar dentin formation as the samples capped with PLGA/CaP cement without TGF-β1. In this, it could very well be that the degradation of the polymer was insufficient to release the 20 ng dose of the growth factor.

An intriguing observation was the presence of microparticles at the end of the study. Previous implantation experiments in bone and soft tissue showed that the polymer microparticles degrade relatively fast, which allowed tissue ingrowth in the emptied porosity.[16,17] In our study however, even after 12 weeks microparticles were often seen to have remained undegraded. The small volume and limited blood supply of the dental pulp might be the reason for this phenomenon. This would implicate that the cement we used, with optimal properties for bone ingrowth, should be further adapted to the specific application into the pulp.

A final noticeable histological observation was the existence of empty spaces in most of the samples, where tissue layers were no longer connected. These might have been caused by a (too) high demineralization speed of the Formical, which caused artifacts disrupting the integrity of the specimens. Some open spaces in the tissue might also have partially been caused by the use of our pre-fabricated material. Since the drilling hole will always be slightly larger than the sample itself it is almost impossible to avoid a gap between the capping material and the tooth. For clinical application, an injectable cement would be optimal. However, with a size of only 2 mm in diameter and 2–3 mm in height it is hard to maintain the stability of prepared materials, and especially difficult to control the accurate concentration of growth factor. Therefore, we chose the prefabricated method to prepare the capping PLGA/CaP cement with same dose of TGF-β1 in the entire batch, in order to achieve more reliable and comparable results. Future studies should elaborate on the injectable concept to greater extent.

In conclusion, our study demonstrated that the prefabricated PLGA/CaP cement loaded with 400 ng of TGF-β1 was able to trigger resident stem cells in the pulp to differentiate into odontoblast-like cells and to induce the formation of tertiary dentin. The material might be a good candidate for vital pulp therapy. Production and manipulation methods could be improved for follow-up studies.

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