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Please cite this article EFFECT OF HYDROXYAPATITE AND GROWTH FACTORS ON REPARATIVE DENTIN FORMATION IN THE THERAPY OF INJURED PULP


UDC:

DOI: https://doi.org/10.2298/VSP190115032V

When the final article is assigned to volumes/issues of the Journal, the Article in Press version will be removed and the final version appear in the associated published volumes/issues of the Journal. The date the article was made available online first will be carried over.
EFFECT OF HYDROXYAPATITE AND GROWTH FACTORS ON REPARATIVE DENTIN FORMATION IN THE THERAPY OF INJURED PULP

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Running title:
Effect of hydroxyapatite and growth factors on dentin.
Abstract

Background/Aim. The studies of hydroxyapatite (HAp) and growth factors as the materials used for direct pulp capping have produced conflicting results for both the issue of inflammatory response and the issue of calcified bridge formation. Calcium hydroxyapatite/poly(lactide-co-glycolide) is a bioresorbable polymer with demonstrated good characteristics as the carrier for bone morphogenetic protein (BMP) necessary in bone tissue regeneration. The role of growth factors in dental tissue reparation (in both reactionary and reparative dentinogenesis) represents the new foundation and provides a different approach to dental pulp treatment. Growth factors – TGF-beta 1 – directly induce morphological and functional differentiation of neodontoblasts. The aim of this experimental study was to investigate the effect of calcium hydroxyapatite/poly(lactide-co-glycolide) HAp/PLGA and growth factors (TGF-β1) in the formation of a calcified tissue – dentine bridge – on the teeth of our experimental model. Methods. Rodent (rabbit) teeth were used as the animal model. After the trepanation of pulp space with sterile steel drills, the pulp was capped with calcium hydroxyapatite/poly(lactide-co-glycolide) HAp/PLGA (experimental group I; n=60); calcium hydroxyapatite/poly(lactide-co-glycolide) HAp/PLGA combined with TGF-β1 growth factor (experimental group II; n=60), and there was a control group of intact teeth (n=20). The experiment was performed in general anesthesia. The animals were kept alive for 1, 3 and 6 months. The extracted teeth were adequately prepared for scanning electron microscopy. Results. Scanning electron microscopy (SEM) demonstrated that the number of teeth with calcified tissue in the form of dental bridges in the HAp/PLGA+TGF-β1 group was statistically significantly greater 6 months (66.67%) than 3 months after the treatment (26.67%), at the statistical significance level of p<0.05. Conclusion. Direct pulp capping covers the artificially exposed dental pulp and makes possible the formation of a dentine bridge (a tubular structure composed of reparative dentine) in the period of 3 months.

Key words: TGF-β1, HAp/PLGA, reparative dentine, pulp capping, rabbits.

Apstrakt

Uvod/Cilj. Studije o hidroksiapatitu (HAp) i faktorima rasta kao materijalima za direktno prekrivanje pulpe daju potpuno oprečne rezultate i po pitanju zapaljenog odgovora i po pitanju formiranja kalcifikovanog mosta. Kalcijum hidroksiapatit poly(lactid-co-glikolid) je bioresoribilni polimer koji je pokazao dobra svojstva kao nosač koštanog morfogenog proteina (BMP) neophodnog za regeneraciju koštanog tkiva. Uloga faktora rasta u reparaciji zubnih tkiva (bilo da se radi o raktivnoj ili reparativnoj dentinogenezi) daje nove osnove i drugačiji pristup tretmanu pulpe. Faktori rasta – TGF-β direktno indukuju morfošku i funkcionalnu diferencijaciju neodontoblata. Cilj ove eksperimentalne studije

Ključne reči: 
TGF-β1 + HAp/PLGA, dentinski most, prekrivanje pulpe, kunići.

Introduction

Direct pulp capping (DPC), as one of the essential endodontic modalities, is often used as a therapeutic procedure for dental pulp vitality preservation. It is usually defined as a treatment on exposed pulp tissue, where the pulp wound is covered (capped) with materials which stimulate reparative dentine formation. Since the capping material comes into direct contact with pulp tissue, it plays a key role in this treatment.

In the selection of materials for vital pulp therapy, the following material properties should be sought: antibacterial action, ability to induce mineralization, adequate sealing of the pulp space in order to prevent the entry of bacteria from the mouth cavity. Some studies have investigated the use of biomaterials such as hydroxyapatite in dental pulp treatments within the technique of direct pulp capping or amputation of the crown portion of the dental pulp. The results suggested that their use in the observation period of 3 months speeds up the process of healing, i.e. the formation of dentine bridge and continued dental root growth. Hydroxyapatite (HAp) is one of the most frequently used calcium phosphate bioceramics with osteoconductive properties. Since its structure is similar to bone minerals, it is capable of forming direct bonds with the bone tissue. HAp has got several clear advantages: it is well accepted and incorporable into the host bone, but it also provides a solid base for new bone growth. Its biocompatibility is excellent, and its surface layer has a key role in the formation, growth and maintenance of the tissue/biomaterial bond. Moreover, it does not contain any proteins and consequently does not induce any allergic reactions or immune system responses. On the other hand, HAp has very poor mechanical properties.

Synthetic hydroxyapatite belongs to the group of non-resorbable ceramic biomaterials. It could perhaps successfully replace bone tissue, facilitate new bone formation and exert an osteoconductive effect.

Poly(lactide-co-glycolide) (PLGA) is a copolymer of lactide and glycolide registered by the Food and Drug Administration (FDA) as a material which can be used in medicine and pharmacy, and it belongs to the class of biodegradable and biocompatible polymers.
Pulp capping with HAp-based materials requires the use of a mechanically more resilient material over the medicament and only then a better quality of the dentine-HAp interface becomes prominent. Novel insights in the role of growth factors in dental tissue reparation, in both reactive and reparative dentinogenesis, could represent the basis of different pulp treatment.

Growth factors are biological mediators which regulate key processes in tissue reparation, including cell proliferation, differentiation, extracellular matrix synthesis and angiogenesis.

There have been attempts to use hydroxyapatite and growth factors for the same purposes, although with a low success rate. A number of these investigations are still ongoing and attract much attention.

TGF-β1 is a member of the superfamily of homologous disulfide-bound homodynamic proteins, regulating proliferation and differentiation of normal and transforming cells. Human TGF-β1 is a 25.0 kDa protein which contains 2 identical polypeptide chains of 112 amino acids interconnected by one disulfid bond.

Growth factors are present in the dentin matrix and can play an important role in mediating pulp responses to an injury or restorative procedure. Since they may be released during the process of tooth decay, this could represent the basis of a novel biological approach to dental tissue reparation.

The aim of this paper was to investigate dentinogenetic effectiveness of bioactive materials calcium hydroxyapatite/poly(lactide-co-glycolide) HAp/PLGA and growth factor TGF-β1 in reparative dentine formation in the cases of injured pulp during the standard procedure of dental pulp capping.

Methods

The experimental study took place at the Institute of Biomedical Research, Faculty of Medicine in Niš, and in the Faculty of Medicine in Priština, temporarily seated in Kosovska Mitrovica, with the approval of the Ethics Committee of the Faculty of Medicine, number: 05-603/1 of 2011.

In the experiment five types chinchilla rabbits were included, 6 months old and of mean weight of 3-4 kg. The animals were anesthetized by intramuscular Zoletil 100 administration (Virbac S.A. ere avenue-2065 m-L.I.D. 06516 Carros, France) at a dose of 10 mg/kg of body weight and Ketalar (1-4,5 mg/kg body weight). After the induction of anesthesia and placement of cofferdam rubber insulation, the teeth were cleaned using 70% ethanol. Small cavities were created on the occlusal surfaces of the teeth with small round drills. The cavities were washed with saline for the removal of debris created during cavity preparation. After the trepanation of pulp space, the lesions were covered with biomaterial and growth factor and cavities were definitively closed with glass-ionomer cement (GJC) and amalgam. For the purpose of this study, we used calcium hydroxyapatite/poly(lactide-co-glycolide) HAp/PLGA and autogenic growth factor-beta (TGF-β1).

The teeth were divided into three groups:

1. Experimental group (n=60), composed of the left lower jaw teeth, where calcium hydroxyapatite/poly(lactide-co-glycolide) HAp/PLGA biomaterial was applied.
2. Experimental group (n=60), composed of the left upper jaw teeth, where calcium hydroxyapatite/poly(lactide-co-glycolide) HAp/PLGA was applied in combination with TGF-β1; calcium hydroxyapatite/poly(lactide-co-glycolide) HAp/PLGA biomaterial served as a carrier, 80:20 (0.5 g), manufactured by ITN SANU, Belgrade (Figure 1).
3. Intact left upper jaw teeth and right lower jaw teeth served as our control group (n=20).

After this phase of the study, our animals were kept alive for 1, 3 and 6 months, and after these periods they were sacrificed with a lethal dose of ketalar. Jaw bones were disarticulated and each tooth was individually extracted. Material preparation involved tooth storage in sterile saline at 4°C without any fixation agents.

Occlusal surfaces of dental crowns, 2-3 mm thick, were cut in a circular manner using the finest diamond fissure bur. Dental roots were cut longitudinally using separating discs, producing longitudinal separation into the oral and vestibular surfaces. In order to eliminate superficial debris produced by cutting, the samples were washed in distilled water and dried with compressed air. Using the separation pliers, occlusal surfaces were separated first, and then the roots were longitudinally separated along the already prepared grooves. Each half of the sample was placed onto an appropriate mount; the samples thus fixed were gold vapour treated in a vacuum evaporator and viewed under scanning electron microscopy JEOL-JCM-5300.

The entry and tabular data representation were done using the MS Office Excel 2007 software package, and calculations were performed using the SPSS, 15.0 version. The results of the statistical analysis were presented in tables.

The differences in the observed parameters both between the groups and within them, in different intervals of time, were established using the Mantel-Haenszel \(\chi^2\)-test or Fisher’s test of exact probability of the null hypothesis (when some of the expected frequencies was less than 5).

**Results**

In total, 140 teeth of 5 sacrificed experimental animals (rabbits) were used in the study. Table 1 presents the total number of treated teeth in the experimental and control groups.

As the measure of effect of the studied material types and treatment modalities we tried to observe the formation of new hard dental tissue – dentine bridges (reparative dentine) in the studied specimens. This parameter was monitored using scanning electron microscopy 1, 3 and 6 months after the treatment.

The presence of dentine bridges in the experimental groups with direct pulp capping (DPP) and in the control group (SEM) is presented in Table 2.

Comparing the number of teeth among the studied groups, it was established that the number of teeth with formed dentine bridges in the HAp/PLGA+TGF-\(\beta\)1 group was statistically significantly greater 6 months (66.67%) than 3 months after the treatment (26.67%), at the statistical significance level of \(p<0.05\).

Comparing the number of teeth among the studied groups in the same time intervals, it was found that 3 months after the treatment the number of teeth with dentine bridges in the TGF-\(\beta\)1+HAp/PLGA group was statistically significantly greater than in the control group (\(p<0.01\)). Six months after the treatment, the number of teeth with dentine bridges in the TGF-\(\beta\)1+HAp/PLGA group (66.67%) was statistically significantly greater than among controls at an even higher level of statistical significance (\(p<0.001\)). In the same period, comparing the TGF-\(\beta\)1+HAp/PLGA and HAp/PLGA groups 6 months after the treatment, it was established that the number of teeth with dentine bridges in the TGF-\(\beta\)1+HAp/PLGA group was twice the number observed in the HAp/PLGA group (66.67% vs 33.00%), but a statistically significant difference was not established due to a small sample size.
Results of the SEM analysis

HAp/PLGA and HAp/PLGA + TGF-β1 – observation period of one month
The results of SEM analysis with direct pulp capping after the application of HAp/PLGA showed the formation of fibrodentine of atubular structure (Figure 2), and after TGF-β1 and HAp/PLGA application showed a regular structure of reparative dentine from the cavum towards the periphery (Figure 3).

HAp/PLGA and HAp/PLGA + TGF-β1 – observation period of three months
The results of SEM analysis with direct pulp capping after the application of HAp/PLGA revealed the formation of fibrodentine of atubular structure (Figure 4), and after TGF-β1 and HAp/PLGA application showed the presence of newly formed dentine with numerous channels and dentine bridge formation – tubular dentine (Figure 5).

HAp/PLGA and HAp/PLGA + TGF-β1 – observation period of six months
The results of SEM analysis with direct pulp capping after the application of HAp/PLGA showed the presence of dentine bridges, amorphous fibrodentine, dentine bridges with large hydroxyapatite crystals, fibrodentine rich in bioactive proteins (Figure 6), and after TGF-β1 and HAp/PLGA application indicated the formation of dentine bridges with tubular structure, reparative dentine, calcified dentine, calcified Tomes’ fibers and calcified pulp within dental roots (Figure 7). Figure 8 shows regular dentine in an intact tooth.

Discussion

Direct pulp capping is a therapeutic procedure of tooth vitality preservation, whereby adequate materials are applied onto the exposed pulp tissue in order to stimulate reparative dentine formation. Since the material used for direct pulp capping comes into a direct contact with pulp tissue, it therefore plays a key role in this kind of treatment.

Our study dealt with the advantages of application of hydroxyapatite calcium hydroxyapatite/poly(lactide-coglycolide) HAp/PLGA alone, or combined with growth factors. A high biological potential of the pulp, including optimal conditions for the tissue function, with adequate vascularization and absence of inflammation, was the primary criterion in the interpretation of obtained results. The tested materials were applied in accordance with the manufacturers’ manuals. Hydroxyapatite powder was mixed with saline in order to obtain the consistency easy to manipulate with.

In in vitro and in vivo studies, biocompatibility of calcium hydroxyapatite/poly(lactide-coglycolide), composite BC/PLGA and its effect on dental pulp cells have been demonstrated. Histological analysis has demonstrated the presence of cellular infiltration and dentine bridge formation after 60 days. In all studied groups, fibroblast development and growth, and survival of macrophages were identified.

In recent years, growth factors and their role in the initiation of reparatory processes in pulp injury have attracted much attention, what was in part the subject of our study as well. These bioactive molecules promote proliferation and differentiation of cells, matrix synthesis and angiogenesis. Growth factors are necessary in tissue regeneration and has an important role in inducing angiogenesis, i.e. oxygenation and supply of nutrients, essential for biological functioning.

Numerous authors have identified various difficulties in clinical manipulation, application and retention of the materials at the site of application. As the potentially suitable growth factor carriers, calcium-phosphate-based materials have been suggested, as those in our
study, the porous structure of which enables gradual release and diffusion of growth factors. In our study, HAP was shown to be a good growth factor carrier.

Since synthetic biomaterials have been shown to be successful in the restoration of bone tissue, with their well known biocompatibility and bioconductivity, the intention in this study was to investigate the use of hydroxyapatite as a synthetic biomaterial and growth factors in direct pulp capping.

The results obtained by Tziafas et al. have shown that the use of some of the growth factors, especially TGF-β, stimulates odontoblast differentiation and leads to the release of endogenous growth factors contained in the organic dentine matrix, which further stimulates dentinogenesis.

The results of the study by Popović Bajić have shown a highest regularity in the organization of deeper pulp layers in the zones of the thickest dentine bridges, but also a farthest deviation from the statement concerning DDP thrombocyte rich fibrin (PRF) in animals compared to other materials. In their study, they proved the formation of calcified tissue in the pulp in all the samples, which partly agreed with our investigation. However, it is still unclear which growth factor concentration in PRF is optimal for the processes of reparation and regeneration.

Hebling et al. have demonstrated in all their cases dentine bridge formation with direct pulp capping using autogenous growth factors combined with a hydroxyapatite-based material. In two observation periods increased dentine bridge thickness was noticed, although without statistically significant differences compared to the groups where hydroxyapatite alone was applied. In this study, similar results were obtained: in a shorter observation period dentine bridge formation occurred more rapidly and more regularly in the samples in which hydroxyapatite combined with growth factors was applied, although without any statistically significant differences. At the end of 12-months observation period, the results were the same for both HAP-treated samples and those treated with HAP combined with thrombocyte-rich plasma, suggesting that growth factors produced more rapid healing, i.e. dentine bridge formation, which agreed with our own results.

Numerous studies have stressed the importance of adequate cavity sealing after the therapy and prevention of superimposed bacterial contamination, which was provided for in this study by the application of glass ionomer cement and amalgam for cavity sealing.

Formation, quality and thickness of the calcified bridge, presence of inflammatory cells and pulp tissue preservation are all important indicators of tissue response in the therapy with direct pulp capping. According to Accorinte et al. these parameters have been considered as relevant and used for histological assessment of treatment success with the nanomaterials tested in this study. The formation of dentine bridge at the interface of pulp and material used for direct capping is an issue for further discussion, since a dentine bridge is not necessarily the sign of dental pulp preservation (health). The presence of dentine bridges may be interpreted as a sign of healing or as an reaction (response) to irritation. In this study, it was interpreted as a sign of biocompatibility and bioactivity of the material, which agrees with other authors’ opinions. Dentine matrix is not just a scaffold serving to mobilize and support the development of mineralized tissue; it is also the pool of growth factors excreted by odontoblasts and pulp fibroblasts. These growth factors hypothetically produce signals for proliferation, differentiation and recruitment of pulp cells at the sites of injured pulp tissue and initiate tissue regeneration.

In all the samples in our study in the periods of 1, 3 and 6 months after treatment, a thin layer of calcified tissue – dentine bridge – was observed. These follow-up intervals
matched those in the studies in which hard tissue formation was observed as early as 2 weeks after the treatment.\(^{28,33}\)

Finally, it should be mentioned that these findings are the result of response of a healthy dental pulp, without any inflammation, and that the performance of these materials in the presence of inflamed pulp will be assessed in further studies. Some authors have reported the absence of dentine bridges, while others report well calcified bridges after the period of three months.\(^{43,44}\)

In the study by Nowicka et al.,\(^{45}\) it has been histologically confirmed that Biodentin applied as direct pulp capping of intact teeth planned for extraction, leads to the formation calcified tissue and dentine bridges in 50% of samples after the period of 6 weeks.

**Conclusion**

Based on all of the above, a conclusion may be drawn that calcium hydroxyapatite/poly(lactide-co-glycolide) HAp/PLGA combined with TGF-β1 yields better results after both shorter observation periods (3 months) and longer periods of time (6 months) compared to HAp/PLGA alone, which has been demonstrated to be a good growth factor carrier.

It is reasonable to consider, with all the necessary precautions, the clinical use of growth factors, especially TGF-β, which has been reported to be able to induce differentiation of the second generation of odontoblast cells.

TGF-β directly induces morphological and functional differentiation of neo-odontoblasts. However, clinical use of TGF-β may lead to the „doubling“ of its unchanged positive action. Clinical use of TGF-β involves also paying special attention to the means of molecule transport, response dosing and control of the degree of reparation processes, molecule half-life and possible immune reactions.

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Fig. 1 – Calcium hydroxyapatite biomaterial.

Fig. 2 – HAp/PLGA – atubular irregular dentine (fibrodentine).
Fig. 3 – TGF-β1 and HAp/PLGA – regular structure of reparative dentine at the periphery.

Fig. 4 – HAp/PLGA – a detail of the dentine bridge – fibrodentine with atubular structure.

Fig. 5 - TGF-β1 and HAp/PLGA – dentine bridge – tubular dentine.

Fig. 6 – HAp/PLGA. a) dentine bridge (amorphous dentine), b) dentine bridge structure - amorphous fibrodentine.
Fig. 7 – TGF-β1 and HAp/PLGA - 1. dentine bridge with tubular structure; 2. portion of the calcified pulp within dental root canal.

Fig. 8 – Regular dentine (an intact tooth).

Table 1
Total number of treated teeth in the experimental and control group.

<table>
<thead>
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<th>Number of the animal</th>
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<td>I</td>
<td>4</td>
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<td>III</td>
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<td>IV</td>
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<tr>
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<tr>
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<tr>
<td>TGF-β1 + HAp/PLGA</td>
<td>60</td>
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<tr>
<td>HAp/PLGA</td>
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* p<0.01 vs. Control group  
† p<0.05 vs. The same group one month after the treatment  
‡ p<0.001 vs. Control group

Revised on February 26, 2019.  
Accepted March 1, 2019.  
Online First March, 2019.