

THE EFFECTS OF HYALURONIC ACID, CALCIUM HYDROXIDE, AND DENTIN ADHESIVE ON RAT ODONTOBLASTS AND FIBROBLASTS

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The aim of this study was to investigate the effects and efficiency of pulp capping preparations based on hyaluronic acid, calcium hydroxide, and dentin adhesive on the pulp tissue of Sprague-Dawley rats. The rats were killed and extracted teeth sectioned transversely through the pulp. The slices were placed in a RPMI 1640 cell culture medium supplemented with 10 % foetal calf serum. During 14 days of cultivation cultures were treated with preparations that contained hyaluronic acid (Gengigel Prof[®]), and calcium hydroxide (ApexCal[®]), or with dentin adhesive (Excite[®]). Cellularity and viability of fibroblasts and odontoblasts was analysed using a haemocytometer. Hyaluronic acid proved most efficient and the least toxic for direct pulp capping. Even though calcium hydroxide and dentin adhesive demonstrated a higher degree of cytotoxicity, their effects were still acceptable in terms of biocompatibility.

KEY WORDS: *direct pulp capping, fibroblast proliferation, odontoblastic proliferation*

Maintaining the vitality of dental pulp has been one of the fundamental concepts of dental treatment for a long time. If the pulp chamber is exposed, it is usually treated by direct capping. The procedure relies on the ability of the dentin-pulp complex to repair dentin (1-5).

The dental pulp is a highly vascular connective tissue. Generally, pulp cells can be divided into formative and protective cells. Formative cells are odontoblasts and fibroblasts, and protective cells are macrophages, lymphocytes, mastocytes, lymphoid cells, pericytes, and undifferentiated cells (1, 6). Odontoblasts are responsible for dentinogenesis. They are post-mitotic cells which, once differentiated, can no longer divide and can only be replenished from cells from the subodontoblastic layer (7). Fibroblasts

are the most common pulp cells. They are responsible for secreting ground substance and collagen (1).

Products most often used for pulp capping are calcium hydroxide-based materials, glass ionomer cements, adhesive systems, and mineral trioxide aggregates (8-11).

Calcium hydroxide is the most common material for direct capping of exposed pulp (8). Due to its alkalinity, it has a bactericidal effect and stimulates the formation of reparative dentin. It causes sterile necrosis of the pulp tissue (2). Its main drawbacks are the risk of dystrophic calcification, of discontinuous dentin bridge with the so called "tunnel defects", of overaggressive caustic effect on the pulp, and of internal root resorption (12).

The main advantage of dentin adhesives is they bond with the dentin tightly to minimise bacterial penetration into the damaged pulp (13). *In vitro* and *in vivo* studies (14-19) have demonstrated that the cytotoxic effects of adhesives depend on the amount of non-polymerised resin monomers. Many components of the adhesive systems and resin composites such as bisphenol A-glycidyl methacrylate (Bis-GMA), urethane dimethacrylate (UDMA), triethylene glycol dimethacrylate (TEGDMA), 2-hydroxyethyl methacrylate (HEMA), and camphorquinone are cytotoxic to directly exposed mammalian fibroblasts (20-22).

Since neither calcium hydroxide nor adhesives can fully preserve the vitality of the exposed pulp, new materials are studied to replace them (21, 23, 24). Hyaluronic acid (HA) has recently emerged as a material of choice in preserving the vitality of the pulp. It is a natural mucopolysaccharide, carbohydrate polymer from the group of glycosaminoglycans. HA is synthesised on the cytoplasmic surface of plasma membranes (25, 26) and is common in humans and other vertebrates. It is the major component of the inner-cell cement, of the capillary wall, and of the extracellular matrix of the connective tissue (27). Deposition of HA significantly increases during development, morphogenesis, wound repair and regeneration, malignancy, and inflammation (26, 28, 29).

The aim of this study was to investigate the effects of HA on the proliferation of odontoblasts and fibroblasts and to assess its potential as a material for direct pulp capping in humans. For comparison, we also assessed the effects of calcium hydroxide and of one of commonly used dentin adhesives.

MATERIALS AND METHODS

Materials

In this study we tested three preparations used in dental medicine: (1) calcium hydroxide preparation (ApexCal[®], Ivoclar Vivadent, Liechtenstein), which is composed of calcium hydroxide, bismuth carbonate, polyethylene glycol, glycerine, and water; (2) dentin adhesive (Excite[®], Ivoclar Vivadent, Liechtenstein), which is composed of phosphonic acid acrylate, hydroxyethyl methacrylate, dimethacrylate, highly dispersed silica, ethanol, catalysts, stabilizers, and fluoride; and (3) hyaluronic acid gingival gel (Gengigel

Prof[®], Ricerfarma Srl, Italy), which contains purified water, xylitol, cellulose gum, alcohol, sodium hyaluronate, PEG-40 hydrogenated castor oil, polyvinyl alcohol, polycarbophil, and dichlorobenzyl alcohol.

Methods

The study was performed on the cells isolated from two male and two female adult Sprague-Dawley rats, weighing 200 g to 400 g. The animals were euthanised with CO₂ in a glass air-tight chamber (Tlos, Croatia). The incisor teeth were extracted and immediately immersed in cold (3 °C to 5 °C) buffer containing 140 mmol L⁻¹ NaCl (Kemika, Croatia), 3 mmol L⁻¹ KCl (Kemika, Croatia), 1 mmol L⁻¹ CaCl₂ (Kemika, Croatia), 1 mmol L⁻¹ MgCl₂ (Kemika, Croatia), and 10 mmol L⁻¹ HEPES (Sigma-Aldrich, USA) whose pH was 7.3 to 7.4 (adjusted with NaOH). After all the connective tissue, bone and blood that adhered to the outer surface of the tooth were removed carefully with a small piece of sterile gauze. The crown was then sliced transversally through the pulp using an ISOMET low-speed saw (Buehler, Lake Bluff, USA) at 100 rpm. The slices were then collected with a forceps one by one and incubated for 15 min at 35 °C in an extracellular solution supplemented with 3 mg mL⁻¹ collagenase and 0.25 mg mL⁻¹ protease (Sigma-Aldrich, USA). After incubation, the suspension was centrifuged at 600 rpm for 10 min, the supernatant removed, and the remaining slices and isolated cells cultured in plastic, 25 cm² Falcon culture flasks with a vented cap (BD Biosciences, USA). Into each flask we added 5 mL RPMI 1640 (Gibco, UK), 1000 IU of streptomycin, 1000 IU of penicillin, 25 µg mL⁻¹ of chloramphenicol (Sigma-Aldrich, USA) and 1.4 mL of fetal bovine serum (Gibco, UK). In total, four cell cultures were cultivated. In order for the cells to adjust to *in vitro* conditions, the treatment started four days after the cultivation began. We added 2 mm² of polymerised dentin adhesive (Excite[®]) into the first culture, 2 mm² of solid calcium hydroxide preparation (ApexCal[®]) into the second, 0.1 mL of HA preparation (Gengigel Prof[®]) into the third, while the fourth culture served as control. The cultures were kept at 37 °C in an atmosphere of 5 % CO₂. Every four days the cultures were transferred into sterile plastic test tubes with plastic caps (Greiner Bio-One, Germany) and centrifuged at 600 rpm for 10 minutes. Supernatant was removed to 1 mL, the sediment of cells was resuspended and put back into the culture of the same nutritive value as the initial culture. After each change

of the nutritive medium, the cultures were re-treated with the capping preparations. The treatment lasted 14 days, after which the cultures were centrifuged at 600 rpm for 10 minutes, and supernatant removed to 1 mL. The sediment was resuspended and cell number and viability assessed.

Cell counting

A cover slip was attached to the Bürker-Türk chamber. Pasteur's pipette with a pointed tip was used to put a drop of cell suspension along the rim of a cover slip next to the engraved net. Cell count included all cells in four corner squares and those found on its two neighbouring sides. Square dimensions were 1 mm x 1 mm and 0.1 mm high. This makes the volume of 0.1 mm³ (0.1 µL). In order to calculate the number of cells per millilitre of a culture, cell counts were multiplied by 10,000. Odontoblasts and fibroblasts were counted separately. Cellularity was determined for each culture in triplicate and presented as an arithmetic mean and standard deviation.

Viability assessment

Fifty microlitres of cell suspension was placed on a microscope slide and dyed with 50 µL of solution

that contained acridine orange (100 mg mL⁻¹; Sigma-Aldrich, USA) and ethidium bromide (100 µg mL⁻¹; Sigma-Aldrich, USA). Specimens were then covered with a cover slip and analysed with an epifluorescent microscope Olympus AX70 (Olympus, Japan). The analysis included 50 fibroblasts and 50 odontoblasts for each culture. Differences in staining made it possible to count apoptotic cells (condensed chromatin was visible) and necrotic cells (red fluorescent cells) (30). Viability assessment was repeated for each culture in triplicate and presented as an arithmetic mean and standard deviation.

Statistical analysis

The findings were analysed using the chi-square test. The level of $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

In comparison with the control culture, the number of fibroblasts and odontoblasts was greater in the culture treated with HA and smaller in cultures treated with calcium hydroxide and dentin adhesive, but not significantly (Table 1).

Table 1 Cell count in cultures by dental capping material.

Cell type	Treatment			
	Control	Hyaluronic acid (Gengigel Prof®)	Dentin adhesive (Excite®)	Calcium hydroxide (ApexCal®)
Fibroblasts	200.7±14.0	226.0±23.5	176.7±18.1	163.7±12.6*
Odontoblasts	92.7±6.6	113.3±13.9	79.0±17.5	78.7±14.5

Data are expressed as mean cell number per milliliter of culture ± SD
 * $p < 0.05$

Table 2 Cell viability in culture by dental capping material

Cell type	Viability	Treatment			
		Control	Hyaluronic acid (Gengigel Prof®)	Dentin adhesive (Excite®)	Calcium hydroxide (ApexCal®)
Fibroblasts	Viable	46.3±2.1	41.3±1.2	36.3±3.8*	32.3±3.1*
	Apoptosis	3.3±1.5	6.3±1.2	10.7±2.1*	14.0±1.7*
	Necrosis	0.3±0.6	2.3±1.2	3.0±1.7	3.7±4.7
Odontoblasts	Viable	45.3±1.5	44.3±3.2	33.0±1.7**	37.3±1.2
	Apoptosis	4.7±1.5	4.3±2.1	12.0±2.6**	10.3±1.5
	Necrosis	0.0±0.0	1.3±1.2	5.0±2.0	2.7±1.5

Data are expressed as % of cells ± SD
 * $p < 0.05$
 ** $p < 0.01$

Viability was also higher in the culture treated with HA and smaller in those treated with calcium hydroxide and dentin adhesive (Table 2).

In contrast, the number of apoptotic and necrotic cells was smaller in the culture treated with HA than in control. Cultures treated with dentin adhesive had a greater number of odontoblasts affected by apoptosis and necrosis, and those treated with calcium hydroxide had a greater number of fibroblasts affected by apoptosis and necrosis.

Our results demonstrate high therapeutic efficiency of HA in direct pulp capping. They also confirm the efficiency of calcium hydroxide and dentin adhesives (12, 31). The mechanism by which calcium hydroxide initiates the reparative process is unclear. Tronstadt et al. (32) suggest that free hydroxyl ions raise pH, which stimulates mineralisation (32). In that way calcium hydroxide neutralises the acidic reaction of the inflamed tissue, which is characteristic of caries-affected dentin (33). The first effect of calcium hydroxide on exposed pulp is the development of a superficial, three-layer necrosis (2, 34). This could explain the large number of apoptotic and necrotic cells found in our study. The mesenchymal and endothelial pulp cells adjoining the necrosis zone start to coagulate. Before mitosis they synthesised nuclear DNA, which resulted with an increased number of cells. Another result is the intensified synthesis of collagen at the periphery of the cells (35-37). When the pulp is protected from irritation, new odontoblasts can differentiate (2). Calcium hydroxide has a dentinogenic effect only when it is applied at the periphery of the pulp. It has been proven that placing calcium hydroxide in the central part of the pulp does not lead to the formation of the hard dentin tissue. It seems that the ability of calcium hydroxide to stimulate dentinogenesis is closely related to the presence of dentin and odontoblasts (38).

Although some very encouraging findings have been published (39, 40), it seems that dental adhesives may be too toxic for direct pulp capping (14-19, 41), as due to their water solubility they gradually release potentially toxic resin components: HEMA and TEGDMA. Both of them are capable to induce apoptosis or necrosis in normal human primary cells. Spagnuolo et al. (42) found time- and concentration-dependent apoptotic effects of TEGDMA on human pulp fibroblasts and predominantly apoptotic effects of HEMA on human skin fibroblasts (43). Their cytotoxicity depends on dentin permeability and duration of perfusion. The dentin with high permeability

allows greater penetration of dentin adhesive and/or its components (40).

Our results on cell count in the culture treated with dentin adhesive have shown a drop in the number of fibroblasts and odontoblasts compared to the control culture. Cell viability was also lower. The number of apoptotic and necrotic cells was higher and odontoblasts were more affected than fibroblasts. However, the cytotoxicity of the dentin adhesive observed on fibroblasts and odontoblasts was lower than that of calcium hydroxide.

Hyaluronic acid seems to stimulate tissue vascularization, probably through direct action on endothelial cells (44, 45). Indirectly, it creates conditions favoring mineralisation. It stimulates migration of different cell types into the injured area and their proliferation and differentiation into odontoblast-like cells (46). We have shown that the culture treated with HA had an even greater number of odontoblasts and fibroblasts than the control culture, which indicates a great potential of this material for creating reparative dentin. In cultures treated with HA we found a smaller proportion of apoptotic and necrotic cells. Viability analysis has also shown that HA is less toxic and therefore more biocompatible with pulp than dentin adhesive and calcium hydroxide.

Taken together, our results show that all tested materials have a satisfactory degree of biocompatibility. However, as HA was more efficient than either of the traditionally used materials, its administration for direct pulp capping could be advised, especially because it is also safer to use.

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Sažetak

UTJECAJ HIJALURONSKE KISELINE, KALCIJEVA HIDROKSIDA I DENTINSKIH ADHEZIVA NA ODONTOBLASTE I FIBROBLASTE ŠTAKORA

Cilj ovog rada bio je istražiti djelovanje preparata na bazi hijaluronske kiseline i kalcijeva hidroksida te dentinskog adheziva na pulpno tkivo Sprague-Dawley štakora u svrhu procjene učinkovitosti navedenih materijala kod direktnog prekrivanja pulpe. Izvađeni zubi transverzalno su podijeljeni kroz pulpu. Naresci su uzgajani u RPMI 1640 staničnom mediju obogaćenom s 10 % fetalnoga telećeg seruma u plastičnim bočicama za staničnu kulturu. Kulture su tijekom 14 dana tretirane preparatima s hijaluronskom kiselinom (Gengigel Prof®), kalcijevim hidroksidom (ApexCal®) i dentinskim adhezivom (Excite®). Nakon 14 dana pristupilo se analizi staničnosti i vijabilnosti s pomoću hemocitometra. Iako su preparati na bazi kalcijeva hidroksida i dentinski adheziv pokazali nešto viši stupanj citotoksičnosti, dobiveni su rezultati u granicama biokompatibilnosti. Primjena preparata na bazi hijaluronske kiseline postigla je najbolje rezultate te se ovaj materijal pokazao najboljim za direktno prekrivanje pulpe između tri ispitivana preparata.

KLJUČNE RIJEČI: *direktno prekrivanje pulpe, proliferacija fibroblasta, proliferacija odontoblasta*

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