Cytotoxicity of substances leached or dissolved from pulp capping materials

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Abstract

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Aim To evaluate the cytotoxic effects of substances leached or dissolved from pulp capping materials on human pulp fibroblasts.

Methodology The substances were applied to cell cultures in conditioned media. The experimental groups were: GI (control; $n = 24$) – cultures treated with fresh medium; GII ($n = 24$) – cultures treated with calcium hydroxide cement; GIII ($n = 24$) – cultures treated with adhesive resin and GIV ($n = 24$) – cultures treated with 37% orthophosphoric acid. The media were conditioned by placing the crude materials in contact with fresh culture medium for 1 h. The cytotoxicity analysis was performed using the Trypan blue dye exclusion assay at times of 0, 6, 12 and 24 h for cell viability assay, and at 1, 3, 5 and 7 days for survival assay. Data were treated by ANOVA ($P < 0.05$) and Tukey's test ($P < 0.05$).

Results GI and II presented similar cell viability and cell growth. GIII and IV exhibited statistically significant lower percentages of cell viability: GIV only at the 0 h experimental time, whereas in GIII this viability markedly diminished reaching values of 10% by 12 h. Cell growth was impaired only in cultures of GIII.

Conclusions Substances dissolved from the adhesive system tested were cytotoxic for human dental pulp fibroblasts in culture, whilst substances leached from calcium hydroxide were biocompatible.

Keywords: adhesive bonding, calcium hydroxide, cell culture, dental pulp, dental pulp capping.

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Introduction

The treatment of the exposed pulp is challenging particularly in the choice of the capping material (Cohen & Burns 1994). Calcium hydroxide, in dry powder, suspension or cement form, has been recommended for this purpose as it exhibits beneficial properties, such as induction of mineralization, low cytotoxicity and high pH (Kitasako *et al.* 2000, Schuurs *et al.* 2000). Its high pH is responsible for inhibition of bacterial growth and the recovery of tissue pH, which is often acidic as a result of the bacterial metabolism (Tarim *et al.* 1998, Medina *et al.* 2002, Murray *et al.* 2002). On the other hand, calcium hydroxide is mechanically weak, and soluble over time (Cox & Suzuki 1994).

The use of bonding systems, which form a hybrid layer with the collagen matrix of dentine, has been suggested as a pulp capping procedure. It promotes an effective seal between the tooth and the restorative material, preventing bacterial leakage (Cox & Suzuki 1994). Some authors observed pulp tissue repairing when bonding systems were tested *in vivo* (Cox *et al.* 1995, Akimoto *et al.* 1998, Cox *et al.* 1999). Conversely, results of other studies showed impairment of the dental pulp repair mechanism when this material was used *in vivo* as a pulp capping agent (Gwinnett &

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Tay 1998; Pameijer & Stanley 1998; Hebling *et al.* 1999, Kiba *et al.* 2000, Trope *et al.* 2002). The controversy over the healing capacity of bonding systems could be assessed with the use of cell culture methods, where the experimental model is more easily controlled (Schmalz 1994, Demarco *et al.* 2001). Moreover, the cell culture model allows various methods of placing the test substances on the cultures (Schedle *et al.* 1998, Huang & Chang 2002, Schmalz *et al.* 2002, Vajrabhaya *et al.* 2003). These aspects become more important when it is known that aggressive substances can be dissolved in the tissue from the nonpolymerized resin monomers (Mantellini *et al.* 2003, Stanislawski *et al.* 2003).

The aim of this study was to compare *in vitro*, possible cytotoxical effects of substances leached or dissolved during pulp capping procedures on human pulp fibroblasts, mimicking the direct pulp capping clinical procedure with a conditioned medium cell culture method.

Material and methods

The toxicity of three substances used in direct pulp capping procedures was measured *in vitro*. The response of human pulp fibroblasts to substances leached from these procedures was analysed. This analysis was based on both an immediate or short-term response and a long-term survival that measures the retention of the self-renewal capacity of the cells. This study was conducted under approval of the Ethical Committee of the School of Dentistry of the University of São Paulo (process no. 191/03).

Experimental groups

Four groups were established:
GroupI (GI): Control (fresh cell culture medium).
GroupII (GII): Calcium hydroxide cement (Life, Kerr, Orange, CA, USA).
GroupIII (GIII): One bottle adhesive resin (Single Bond, 3M ESPE, St Paul, MN, USA).
GroupIV (GIV): 37% orthophosphoric acid in gel form (Dentsply/Caulk, Petròpolis, RJ, Brazil).

Preparation of the substances

Calcium hydroxide cement (GII) was prepared according to the manufacturers' instructions, on a sterile glass plate and with a size 24 spatula. The mixed cement was applied to the bottom of a 50 mL centrifuge tube. The adhesive resin was applied to the bottom of the centrifuge tube and light cured with a 600 mW cm^{-2} halogen light (Dabi Atlante, Ribeirão Preto, SP, Brazil) for 20 s. The acid (group IV), in a gel form, was placed in the bottom of the tube.

Culture medium conditioning

In order to obtain conditioned media (e.g. media containing either the leached substances from the calcium hydroxide cement or the dissolved substances from adhesive resin and acid), the test tubes containing the capping materials were filled with Dulbecco's modified Eagle culture medium (DMEM; Sigma, St Louis, MO, USA). Conditioning was carried out for 1 h, at 37 °C, using 0.2 g of each substance per millilitre of fresh medium, according to the American Society for Testing and Materials (1992). These media were placed in contact with the substances during its setting (GII), polymerization (GIII) or only contact (GIV). In the group III, light polymerization was performed after filling the tube with fresh medium, allowing dissolution of substances before and during this procedure. The stock conditioned media obtained during this step were diluted (10%) and then applied into the cell cultures.

Cell culture

The cells were cultured as described previously (Garcia *et al.* 2003). Briefly, the FP5 cell line, fibroblasts derived from a human third molar germ was used. These cells were cultured in DMEM, supplemented by 10% foetal bovine serum (Cultilab, Campinas, SP, Brazil) and 1% antimycotic-antibiotic solution (10 000 units of penicillin, 10 mg of streptomycin and 25 μg of amphotericin B per mL in 0.9% sodium chloride; Sigma). The cells were kept in an incubator at 37 °C and a humidified 5% CO_2 atmosphere. Cultures were supplied with fresh medium every other day. Cells between the fifth and 10th passages were used in all experimental procedures.

Cell viability and cell survival assays

After cell culture, the cells were plated on 35 mm diameter culture dishes ($n = 96$). For the cell viability (short-term) assay, 1.8×10^3 cells per dish were plated and for the cell growth assay (long-term), 1×10^3 cells per dish were plated.

The viability (short-term) and growth (long-term) curves were obtained as previously described (Freshney

2000, Scelza *et al.* 2001). Briefly, cell counts were determined by counting the viable cells in a haemocytometer, using the Trypan blue dye exclusion assay. The number of viable cells harvested from each dish was obtained by the following mathematical equation: $UC \times D \times 10^4/nSQ$, where UC, unstained cell count (viable cells); *D*, the dilution of cell suspension; and nSQ, number of counted squares of the haemocytometer. The viability percentage of the cell population of each dish was obtained by the following mathematical equation: $UC/TC \times 100$, where UC, unstained cell count (viable cells); and TC, total cell count (stained plus unstained cells).

It is important to emphasize that, in GIV, the conditioned medium was kept in contact with the cells for 15 s and then replaced by fresh DMEM, mimicking the clinical use of this substance.

Statistical analysis

Each data point corresponded to the mean ± standard error of the mean (SEM) of either cell count or percentage of cell viability from three dishes. The data were compared by ANOVA ($P < 0.05$), with Tukey's *post hoc* test ($P < 0.05$).

Results

Cell viability assay

The mean cell viability in the short-term assay was 86.46% for GI, 77.24% for GII and 67.18% for GIV. GIII presented marked cell viability decrease, reaching values of 10% within 12 h. GIV, despite its stable cell viability approaching those of GI and II, showed statistically lower cell viability at the first experimental time (0 h). The viability curves are shown in Fig. 1.

The statistical analysis indicated differences in the factors time, material and in their interactions. According to the material factor, the adhesive resin was significantly more cytotoxic when compared with other groups, and the acid (GIV) was more cytotoxic than GI and II, in an intermediate biocompatibility level.

Cell survival assay

The growth curves are shown in Fig. 2. All cell cultures, excluding those of GIII, had a continuous growth rate from the first to the third day, when they became stable with a cell number of approximately 3×10^3 cells per dish. GIII cultures (adhesive resin) had marked cell number decreases 24 h after contact with the conditioned medium, reaching values of less than 100 viable cells per dish. In GIV (acid) the growth rate was continuous, with the number of cells lower than those observed in GI and II at the first and third experimental times, but with no statistical significance.

The statistical analysis showed differences only according to the material factor. The adhesive had significantly fewer cell numbers than the other groups ($P = 0.00$).

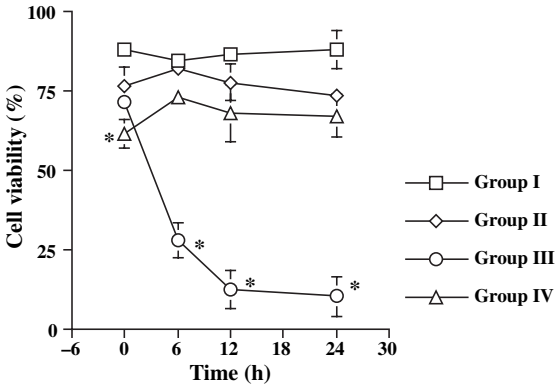

Figure 1 Viability curves, in percentage, of FP5 cells of the short-term assay (*significantly smaller than the other groups at the same experimental time).

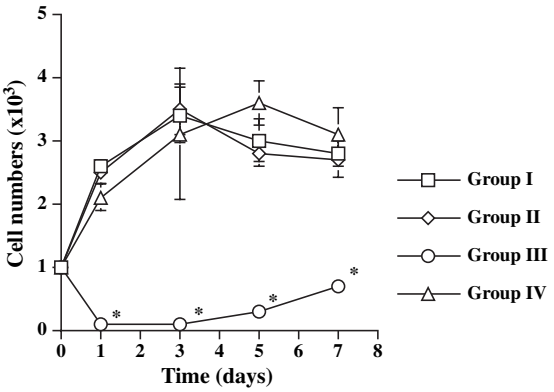

Figure 2 Growth curves of FP5 cells. Groups I, II and IV present similar cell growth. Growth of group III cultures was significantly smaller than the other groups (*).

Discussion

The analysis of the effects on cell viability and cell growth of putative substances leached or dissolved from

pulp capping materials showed that, at least during the polymerization of the bonding system, these substances are present and lead to cell death and to loss of proliferation capacity of cells in culture.

Most studies on the cytotoxicity of dental materials were developed by using samples of test materials already cured or set (Demarco *et al.* 2001, Huang & Chang 2002). In others, the materials were applied on dentine disks and were placed indirectly in contact with the cells (Schmalz *et al.* 2002, Vajrabhaya *et al.* 2003). Since these cited techniques are very good to study other effects (e.g. indirect pulp capping), they do not take into account the effect of putative leaching or dissolved substances released from the dental materials during their setting or curing, because they were lost before making contact with the cells. For this reason, the use of conditioned media is indicated for analysing substances leached or dissolved during setting or polymerization. Hence, this method is especially important when direct pulp capping materials are studied, since these are directly applied to the pulp connective tissue in a humid environment in clinical practice. When in contact with moist pulp tissue, the setting or curing processes could be delayed or prevented, allowing the leaching or dissolution of substances that could influence pulp tissue healing. Using the conditioned media technique, the clinical situation is mimicked and the polymerization or setting of the materials is carried out in a humid environment, making the study more clinically relevant.

During the pouring of DMEM on the adhesive resin, the surface layer of this material was dissolved in the medium, mimicking the bonding layer in direct contact with the dental pulp that probably permeates this tissue. In a pilot test, the crude conditioned media proved to be highly cytotoxic. Thus, it was decided to work with this media diluted to 10%, in order to make this comparative study possible. This dilution is appropriate, since in pulp tissue, the cell numbers are higher than the number of cells in a culture dish. At the same time, blood and lymphatic vessels are present in living tissue, diluting the substances. Even with the diluted media, the present results, both in short and long-term assays, indicated a high cytotoxicity of the adhesive resin group when compared with the other groups. These data were important in order to validate the method used, since many histological studies agree that the use of adhesive resins as direct pulp capping agents can lead to irreversible inflammation and necrosis of the pulp (Gwinnett & Tay 1998, Hebling *et al.* 1999, Kiba *et al.* 2000, Trope *et al.* 2002).

The media conditioned with calcium hydroxide was not able to change cell behaviour, since no statistical differences in both cell viability and cell growth were observed between the control and calcium hydroxide cement groups. The calcium hydroxide cement increased the pH of the medium at the initial moment of the study, observed by the colour change of the DMEM, which has phenol red in its composition, a pH indicator. However, probably because of the buffer action of the sodium bicarbonate of the medium, this effect disappeared and at the end of the conditioning procedure the pH returned to neutral. The results of the present study agree with data found in the literature, which demonstrated the biocompatibility of calcium hydroxide both in cement and dry powder forms (Tarim *et al.* 1998, Kitasako *et al.* 2000, Medina *et al.* 2002, Murray *et al.* 2002, Trope *et al.* 2002).

The 37% orthophosphoric acid was used in this study not as a pulp capping agent, but as part of a pulp capping procedure, the hybridization. Initially, it was expected that the acid could have a high cytotoxic effect, as previously reported (Pameijer & Stanley 1998). However, this substance was aggressive only at the initial contact with cells as reflected in the data observed in the short-term assay. This low cytotoxicity could be the result of the short contact time with the cells, that allowed them to recover and maintain their proliferative capacity, as observed in the long-term assay. However, this result is relevant, because in the clinical procedure, acid is kept in contact with the pulp or dentine for no more than 15 s.

Conclusions

Substances dissolved from the adhesive system tested were cytotoxic for human dental pulp fibroblasts in culture, whilst those leached from calcium hydroxide cement were biocompatible.

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References

Akimoto N, Momoi Y, Kohno A *et al.* (1998) Biocompatibility of clearfil liner bond 2 and clearfil AP-X system on nonexposed and exposed primate teeth. *Quintessence International* **29**, 177–88.

American Society for Testing and Materials (1992) *Annual Book of ASTM Standards: Medical Devices*. West Conshohocken, USA: ASTM.

Cohen S, Burns RC (1994) *Pathways of the Pulp*, 6th edn. St Louis, MO: Mosby.

Cox CF, Hafez AA, Akimoto N, Otsuki M, Mills JC (1999) Biological basis for clinical success: pulp protection and the tooth-restoration interface. *Practical Periodontics and Aesthetic Dentistry* **11**, 819–26.

Cox CF, Suzuki S, Suzuki SH (1995) Biocompatibility of dental adhesives. *Journal of California Dental Association* **23**, 35–41.

Cox CF, Suzuki S (1994) Re-evaluating pulp protection: calcium hydroxide liners vs. cohesive hybridization. *Journal of American Dental Association 1994* **125**, 823–31.

Demarco FF, Tarquínio SBC, Jaeger MMM, Araújo VC, Matson E (2001) Pulp response and citotoxicity evaluation of 2 dentin bonding agents. *Quintessence International* **32**, 211–20.

Freshney RI (2000) *Culture of Animal Cells: A Manual of Basic Technique*, 4th edn. Indianapolis, IN: Wiley-Liss.

Garcia JM, Martins MD, Jaeger RG, Marques MM (2003) Immunolocalization of bone extracellular matrix proteins (type I collagen, osteonectin and bone sialoprotein) in human dental pulp and cultured pulp cells. *International Endodontic Journal* **36**, 404–10.

Gwinnett AJ, Tay FR (1998) Early and intermediate time response of the dental pulp to an acid etch technique in vivo. *American Journal of Dentistry* **11**, S35–44.

Hebling J, Giro EM, Costa CA (1999) Biocompatibility of an adhesive system applied to exposed human dental pulp. *Journal of Endodontics* **25**, 676–82.

Huang FM, Chang YC (2002) Citotoxicity of dentin bonding agents on human pulp cells in vitro. *International Endodontic Journal* **35**, 905–9.

Kiba H, Hayakawa T, Nakanuma K, Yamazaki M, Yamamoto H (2000) Pulpal reactions to two experimental bonding systems for pulp capping procedures. *Journal of Oral Science* **42**, 69–74.

Kitasako Y, Shibata S, Pereira PN, Tagami J (2000) Short-term dentin bridging of mechanically-exposed pulps capped with adhesive resin systems. *Operative Dentistry* **25**, 155–62.

Mantellini MG, Botero TM, Yaman P, Dennison JB, Hanks CT, Nör JE (2003) Adhesive resin induces apoptosis and cell-cycle arrest of pulp cells. *Journal of Dental Research* **82**, 592–6.

Medina VO III, Shinkai K, Shirono M, Tanaka N, Katoh Y (2002) Histopathologic study on pulp response to single-bottle and self-etching adhesive systems. *Operative Dentistry* **27**, 330–42.

Murray PE, Kitasako Y, Tagami J, Windsor LJ, Smith AJ (2002) Hierarchy variables correlated to odontoblast-like cell numbers following pulp capping. *Journal of Dentistry* **30**, 297–304.

Pameijer CH, Stanley HR (1998) The disastrous effects of the ''total etch'' technique in vital pulp capping in primates. *American Journal of Dentistry* **11**, S45–54.

Scelza MF, Daniel RL, Santos EM, Jaeger MM (2001) Cytotoxic effects of 10% citric acid and EDTA-T used as root canal irrigants: an in vitro analysis. *Journal of Endodontics* **27**, 741–3.

Schedle A, Franz A, Rausch-Fan X *et al.* (1998) Cytotoxic effects of dental composites, adhesive substances, compomers and cements. *Dental Materials* **14**, 429–40.

Schmalz G, Schuster U, Koch A, Schweikl H (2002) Citotoxicity of low pH dentin-bonding agents in a dentin barrier test in vitro. *Journal of Endodontics* **28**, 188–92.

Schmalz G (1994) Use of cell cultures for citotoxicity testing of dental materials: advantages and limitations. *Journal of Dentistry* **22**, S6–11.

Schuurs AH, Gruythuysen RJ, Wesselink PR (2000) Pulp capping with adhesive resin-based composite vs. calcium hydroxide: a review. *Endodontics and Dental Traumatology* **16**, 240–50.

Stanislawski L, Lefeuvre M, Bourd K, Soheili-Majd E, Goldberg M, Périanin A (2003) TEGMA-induced citotoxicity in human fibroblasts is asoosciated with early and drastic glutathione depletion with subsequent production of oxygen reactive species. *Journal of Biomedical Materials Research* **66A**, 476–82.

Tarim B, Hafez AA, Suzuki SH, Suzuki S, Cox CF (1998) Biocompatibility of Optibond and XR-Bond adhesive systems in nonhuman primate teeth. *International Journal of Periodontics and Restorative Dentistry* **18**, 86–99.

Trope M, McDougal R, Levin L, May KN Jr, Swift EJ Jr (2002) Capping the inflamed pulp under different clinical conditions. *Journal of Esthetics and Restorative Dentistry* **14**, 349–57.

Vajrabhaya L, Pasasuk A, Harnirattisai C (2003) Cytotoxicity evaluation of single component dentin bonding agents. *Operative Dentistry* **28**, 440–4.