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Biocompatibility of various root canal filling materials *ex vivo*

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Abstract

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Aim To evaluate the biocompatibility of a resin-based endodontic filler (RealSeal) using the indirect cytotoxicity test.

Methodology Human gingival fibroblasts were cultured *ex vivo*. Pellets of the materials to be tested were incubated for 24, 48, and 72 h at 37 °C under sterile conditions to obtain their eluates. The fibroblasts were exposed to either diluted (50%) or undiluted eluates for 24 h. A culture medium with foetal calf serum was added to the control wells. Cell viability was estimated by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide method. The data concerning cell viability were statistically analyzed using one-way ANOVA test and Bonferroni multiple comparisons test.

Results Eluates obtained after 24 h of incubation with the resin filler did not reduce cellular viability. An increase in cellular viability, as compared with control cells, was observed in the gutta-percha group. The undiluted eluate from the polyether material was cytotoxic, causing an 82 ± 4% decrease in cellular viability. Eluates obtained after 48 h of incubation with the resin filler increased cellular viability, whereas the polyether significantly reduced viability. Gutta-percha did not cause any detectable change. After 72 h of incubation the eluate of the resin filler caused an increase in cellular viability, as did gutta-percha, whereas polyether caused a significant decrease.

Conclusions RealSeal resin filler was nontoxic in this laboratory model. Further investigations are necessary to verify its usefulness in clinical applications.

Keywords: biocompatibility, cytotoxicity test, endodontic filler, fibroblasts cultures, resin sealers.

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Introduction

Root filling materials should be biocompatible, because they can inadvertently extrude beyond the apical foramen and come into contact with the surrounding soft and hard tissues. An irritating material may cause inflammation that delays or prevents the healing process (Pertot *et al.* 1992). Gutta-percha is generally considered a biocompatible material for root fillings (Kawahara *et al.* 1968, Wolfson & Seltzer 1975, Tani-Ishii & Teranaka 2003).

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Many different methods, including both *in vivo* and *ex vivo* tests, have been described for assessing the biocompatibility of dental materials. Yesilsoy *et al.* (1988) advocated injection of the material to be tested directly into the subcutaneous tissues of a test animal. Other studies used implanted Teflon or filled polyethylene tubes in subcutaneous tissues or bone of laboratory animals (Zmener *et al.* 1988, Molloy *et al.* 1992, Pertot *et al.* 1992, Kolokuris *et al.* 1996). The irritant effect of the endodontic materials was then evaluated by histopathological analyses of the tissue response to the implanted material.

Recently, laboratory tests have been used to determine the cytotoxicity of dental materials (Willerhausen *et al.* 2000, Chang & Chou 2001, Szep *et al.* 2003). Such tests offer the opportunity to study toxicity directly or through the release of components of the

material (Willerhausen *et al.* 2000). These tests are also rapid, cheap and reproducible. Cell culture testing methods are frequently more sensitive than *in vivo* assays, but they must be evaluated within the limits of acute toxicity testing (Granchi *et al.* 1995). Permanent cell lines (e.g. HeLa or 3T3 cells) and primary/diploid cells, mainly oral fibroblasts, are used for these experiments (Schmalz 1994, Hauman & Love 2003a). Willerhausen *et al.* (2000) and Al-Nazhan & Spangberg (1990) found that diploid human fibroblasts are an appropriate model for early recognition of possible cytotoxic effects of root filling materials. It has been suggested that diploid fibroblasts more closely resemble *in vivo* situations than do other types of cells (Willerhausen *et al.* 2000, Chang & Chou 2001, Szep *et al.* 2003). In particular, primary cells are characterized by a high degree of differentiation, and even though they are less homogeneous and sensitive than permanent cell lines, their reaction pattern makes them more comparable with the oral mucosa (Schmalz 1994, Weller *et al.* 1997, Tiozzo *et al.* 2003).

Biocompatibility of the most commonly used root canal filling materials has been widely analysed. Pascon & Spangberg (1990) demonstrated that although pure gutta-percha has good biocompatibility, various brands of endodontic gutta-percha were toxic, because of the leakage of metal ions. The composition of gutta-percha for endodontic use is not provided by any manufacturer and is substantially different from pure gutta-percha. Usually the major component of a gutta-percha point is zinc-oxide, with only about 20% of its composition being gutta-percha (Gurgel-Filho *et al.* 2003, Hauman & Love 2003b). Finding an alternative material to gutta-percha for endodontic use may therefore be desirable. A new soft resin endodontic obturation system has been recently introduced (RealSeal, SybronEndo, Orange, CA, USA). The manufacturer claims that it adheres to root canal walls ensuring hermetic root fillings. A variety of resin-based sealers have been studied and have shown promising results in terms of biocompatibility (Pascon *et al.* 1991, Molloy *et al.* 1992, Azar *et al.* 2000, Schwarze *et al.* 2002a,b, Bouillaguet *et al.* 2004). Schwarze *et al.* (2002a) evaluated the cellular compatibility of five endodontic resin sealers in the first 24 h of setting. They found that the eluates of the resin sealers tested did not have a cytotoxic potency on human periodontal fibroblasts at 5 min after mixing. Molloy *et al.* (1992) examined the biocompatibilty in rat connective tissue of two resin root canal sealers with four frequently used sealers. After 60 days implantation in rat tissue, all materials were well tolerated. These results suggest that such materials have acceptable biocompatibility.

The aim of the present study was to assess the potential cytotoxicity of a resin endodontic filler (RealSeal), which could be used *in lieu* of gutta-percha. This root canal filler offers potential advantages when compared with gutta-percha, such as adhesion to canal walls (Leonard *et al.* 1996, Economides *et al.* 2004, Gogos *et al.* 2004) with consequent better coronal and apical seal (Imai & Komabayashi 2003, Shipper *et al.* 2004); furthermore, it allows a more rapid, easier three-dimensional filling of the root canal system.

Materials and methods

Cell culture of human gingival fibroblasts

Human gingival tissues were obtained (with informed consent) from a healthy patient, who was undergoing gingivectomy of the molar region, at the Department of Dental Sciences, University of Bologna, Italy.

Immediately after removal, the tissues (0.2–5 mm size and 1–3 mm depth) were placed in a 'Collection Medium' composed of Hanks' Balanced Salt Solution (HBSS), 250 U/mL penicillin, 0.25 mg/mL streptomycin, 0.05 mg/mL gentamycin, and 0.0025 mg/mL amphotheracin B. With the aid of an optical microscope, the epithelial layer was detached mechanically using a thin scalpel under sterile conditions.

The sub-epithelial specimens were finely minced and plated in tissue culture flasks (25 cm^2) with a thin layer of Dulbecco's Modification of Eagles' Medium (DMEM), supplemented with 50% foetal calf serum (FCS), 2 mM L-glutamine, 1 mM Na pyruvate and antibiotics (see 'Collection Medium') at 37 °C in humidified atmosphere, 95% air and 5% CO_2. All the aforementioned products were from Gibco (Grand Island, NY, USA). The culture medium was gradually increased over the following 3–7 days to 7 mL in 25 cm^2 flask (Falcon, BD Biosciences, Milan, Italy). Fibroblasts started moving from the explants within 2 weeks. They were trypsinized and passaged once they covered at least 50% of the flask surface. After the first passage, the gingival fibroblasts, (300 000 cells seeded in 25 cm^2 flask), were routinely cultured in DMEM supplemented with 10% FCS (Gibco), 50 UI/ml penicillin, 50 μg/ml streptomycin, 2 Mm L-glutamine (Gibco) and 1 mM Na pyruvate (Gibco), at 37 °C in humidified atmosphere, 95% air and 5% CO_2 (Tiozzo Costa *et al.* 1988, Quaglino *et al.* 2000). The gingival fibroblast cultures

reached confluence in 7 days and were then subcultured (split 1 : 3) until the start of the experiment. The gingival fibroblasts cultures were used down to the fifth passage.

Measurements of cytotoxicity

This study was carried out according to 'A practical Guide to ISO 10993 - Part 5 Biocompatibility of Medical Devices-Test for Cytotoxicity: *in vitro* Methods' (1998).

The indirect test of cytotoxicity was performed following the methods described by Lang & Mertens (1990) and Sydiskis & Gerhardt (1993), with some modifications (Tiozzo *et al.* 2003).

Pellets of the three test materials were incubated in 60 mm diameter Petri dishes (Falcon, BD Biosciences, Milan, Italy) in 5 mL of culture medium without foetal calf serum for 24, 48, and 72 h at 37 °C under sterile conditions. The use of culture medium without serum was adopted to avoid possible interaction of substances released by the test materials with the serum components (Tiozzo *et al.* 2003). At the end of the incubation, the soluble extracts or eluates of these materials were collected in sterile tubes and enriched with 10% foetal calf serum.

Human gingival fibroblasts were plated at 20×10^3 cells per well in 24-well plates (Falcon, BD Biosciences, Milano, Italy) in 2 mL of culture medium. When the gingival fibroblasts cultures were at sub-confluence, the medium was removed. The cell monolayer was washed with phosphate buffered saline (PBS) and exposed to 1 mL of culture medium with 10% FCS (control) and to 1 mL of diluted (50%) or undiluted (100%) extracts for 24 h. Each concentration was tested in quadruplicate wells, as were the controls. At the end of the treatment, cellular viability was estimated by 3-[4,5-dimethyl thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann 1983).

MTT test

The MTT (Sigma, St Louis, MO, USA) is a water-soluble tetrazolium dye which produces a yellowish solution when dissolved in culture medium or in saline solutions. Only live cells will reduce it to a purple formazan product insoluble in aqueous solutions (Mosmann 1983). MTT viability test is based on the amount of formazan generated and consequently is directly proportional to the number of viable cells. The MTT assay is an indirect marker for cytotoxicity.

After 24 h in culture, both in the absence of and in the presence of undiluted and diluted extracts, respectively, the medium was removed and 2 mL of growth medium with 100 μL of MTT (5 mg/mL in PBS) were added to the cultures. Subsequently, the cells were incubated at 37 °C for 3 h in a humidified atmosphere (95% air and 5% CO_2). At the end of the incubation, 2 mL of dimethyl sulphoxide (DMSO) (Sigma) were added to each well to dissolve purple crystals of formazan. The coloured solution was measured by spectrophotometer at a wavelength of 540 nm to evaluate the optical density, which directly correlates with the number of viable cells. Reported values are the mean of four measurements and are expressed as percentages of the control values. Results were statistically analyzed with the one-way ANOVA test, Bonferroni multiple comparisons test and linear regression correlation test.

Results

Figure 1 shows the effect on cellular viability of 24 h diluted and undiluted eluates of Obtura, RealSeal and Permadyne Penta L, as determined by the MTT test.

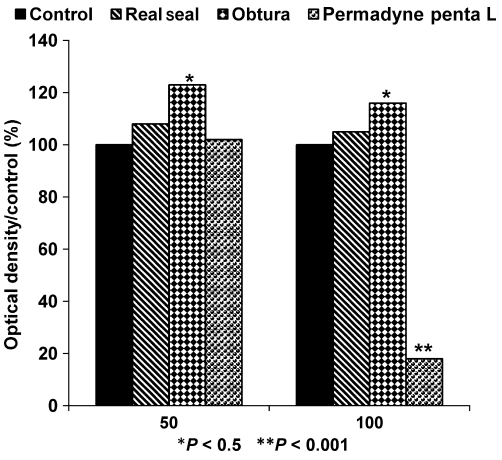

Figure 1 Effect of diluted (50%) and undiluted (100%) eluates of RealSeal, Obtura and Permadyne Penta L, obtained after 24 h of incubation, on human gingival fibroblasts viability, evaluated by MTT test. The data are expressed as a percentage of optical density compared with the untreated cells. The 50% diluted and undiluted eluates of Obtura and the undiluted eluates of Permadyne Penta L show a statistically significant difference in comparison to the control. $^*P < 0.05$; $^{**}P < 0.001$ One-way ANOVA Test and Bonferroni multiple comparisons test.

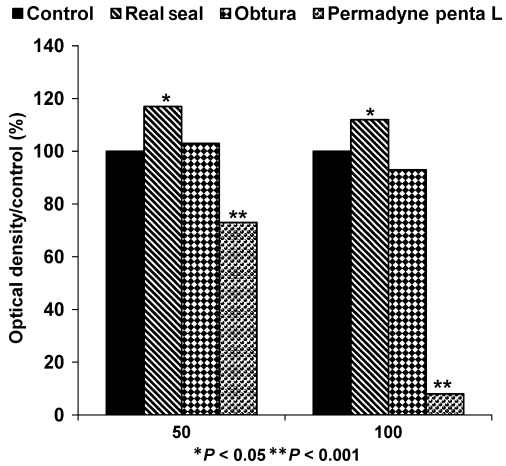

Figure 2 Effect of diluted (50%) and undiluted (100%) eluates of RealSeal, Obtura and Permadyne Penta L, obtained after 48 h of incubation, on human gingival fibroblasts viability, evaluated by MTT test. The data are expressed as a percentage of optical density compared with the untreated cells. The 50% diluted and undiluted eluates of RealSeal and Permadyne Penta L show a statistically significant difference in comparison to the control. $^{*}P < 0.05$; $^{**}P < 0.001$ One-way ANOVA Test and Bonferroni multiple comparisons test.

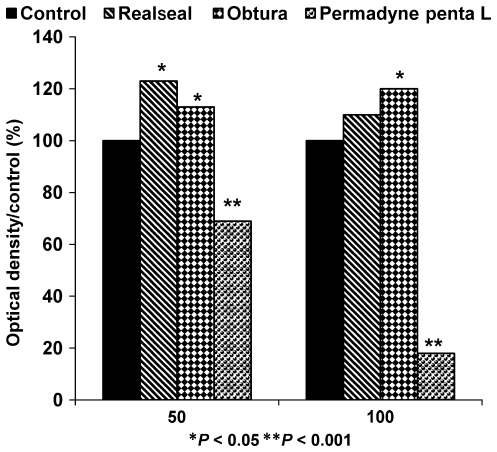

Figure 3 Effect of diluted (50%) and undiluted (100%) eluates of RealSeal, Obtura and Permadyne Penta L, obtained after 72 h of incubation, on human gingival fibroblasts viability, evaluated by MTT test. The data are expressed as a percentage of optical density compared with the untreated cells. The 50% diluted eluates of the three materials tested and the undiluted eluates of Obtura and Permadyne Penta L show a statistically significant difference in comparison to the control. $^{*}P < 0.05$; $^{**}P < 0.001$ One-way ANOVA Test and Bonferroni multiple comparisons test.

The 24 h diluted (50%) and undiluted eluates of RealSeal did not induce any significant alteration of the viability of fibroblasts.

In the 24 h diluted and undiluted eluates of Obtura gutta-percha, an increase in optical density of 23% ± 5% and 16% ± 7%, respectively, was observed, as compared with the control cultures. In both cases, the difference was statistically significant ($P < 0.05$).

As has been found previously (Tiozzo *et al.* 2003), the 24 h undiluted eluate of Permadyne Penta L was cytotoxic, causing a clear decrease in cellular viability (82% ± 4%), with a statistically significant difference ($P = 0.001$) compared to the control.

Figure 2 shows the effect on cellular viability of the 48 h eluates. The undiluted and the 50% diluted eluates of RealSeal caused an increase in optical density in comparison to control cells (12% ± 4% and 17% ± 0.5 respectively). The effect in both cases was statistically significant ($P < 0.05$).

The diluted and undiluted eluates of Obtura gutta-percha did not induce any significant alterations to cellular viability. The diluted (50%) and undiluted eluates of Permadyne Penta L reduced cellular viability by 27% ± 9 and 92% ± 1% respectively. The decrease of cellular viability was statistically significant in both cases when compared with the control group ($P = 0.001$).

The human gingival fibroblasts were exposed to undiluted and diluted (50%) eluates obtained after 72 h of incubation of RealSeal, Obtura, and Permadyne Penta L (Fig. 3). The diluted and undiluted eluates of RealSeal continued to positively affect cellular viability: increases of 23% ± 4% and 10% ± 0.6%, respectively, were observed, with a statistically significant difference between the diluted eluate and the control ($P < 0.05$). Comparable effects were obtained after the incubation of diluted and undiluted eluates of Obtura: increases of 13% ± 0.1% and 20% ± 5%, respectively, were recorded. In each case, the effect on cell viability was statistically significant when compared with the control ($P < 0.05$).

The 50% diluted and undiluted eluates of Permadyne Penta L clearly affected cellular viability, causing a marked decrease in both cases: 31% ± 0.8% for the 50% diluted and 82% ± 0.7% for the undiluted eluate. The difference was always statistically significant in comparison with the control group ($P = 0.001$).

Discussion

Recently, a new soft resin canal filling system has been introduced as an alternative to gutta-percha in root

canal treatment (RealSeal, Sybron Dental Specialities, Orange, CA, USA). The main component of this system is a soft resin called Resilon, which is a bondable material made of polyester polymers. It contains fillers and radio-opaque substances in a soft resin matrix (Barnett & Trope 2004). The manufacturer claims that this new filling system has good handling and working properties, resists leakage better than gutta-percha, is as retrievable and is inert. Eluates of the resin sealer tested did not show any cytotoxic potency on human periodontal fibroblasts.

In the present study, the cytotoxicity of three different dental materials was examined according to 'The International Organization for Standardization, ISO 10993 (1998). The study used MTT test to evaluate the effects of different root canal filling materials on the viability of gingival fibroblasts grown *in vitro*.

In agreement with the literature (Spangberg 1969, Molyvdas *et al.* 1989), the present findings revealed that gutta-percha (Obtura) is nontoxic for endodontic use. It not only modified cellular viability but also in some cases increased optical density. However, it must be emphasized that only pure gutta-percha can be considered absolutely biocompatible. Pascon & Spangberg (1990) reported that some commercially available gutta-percha points were highly cytotoxic because of the substances added to the base material, particularly zinc, and its leakage into the tissues. The authors also found that the toxic effect is time dependent: Ultrafil and Obtura, the two brands recommended for thermoplastic injection, were nontoxic at 4 h cell/material contact, but became toxic at 24 h. Azar *et al.* (2000) evaluated the *ex vivo* cytotoxicity on fibroblasts of an epoxy resin used as a root canal sealer, AH-plus, in comparison with two well-known sealers (AH26 and zinc oxide-eugenol). They found that its cytotoxicity was confined to the early period of the experiment and was no longer detectable, 4 h after mixing. The authors suggested that because AH-plus improves biocompatibility, resulting in milder *in vivo* inflammatory responses in the periradicular area and less post-operative symptoms, the use of this sealer has potential advantages over zinc-oxide eugenol sealers.

The polyether was used in this study because it is a well-documented cytotoxic substance (Tiozzo *et al.* 2003, 2004). The cell inhibition caused by Permadyne Penta L eluate is strongly correlated with its dilution ($P = 0.001$, linear regression correlation test). After 48 and 72 h of incubation, a significant cell inhibition had already started with the 50% concentration obtained eluates and was even more evident with the undiluted eluates. Similar results were obtained in previous studies (Tiozzo *et al.* 2003, 2004). The new resin-based material did not negatively influence cellular viability but, surprisingly, in most cases it caused a significant increase in the optical density. Moreover, a statistically significant difference between the effect on cellular viability caused by Obtura and RealSeal eluates ($P > 0.05$, one-way ANOVA test, Bonferroni multiple comparisons test) was not found, with the exception of the undiluted eluates obtained after 48 h of incubation. These findings, therefore, suggest that the biocompatibility of RealSeal, when considered in terms of cytotoxicity on human fibroblasts, is similar or even better than that of Obtura gutta-percha and, consequently, RealSeal can be considered for use in endodontic therapy.

Any root canal filling material should be in permanent contact with the vital tissues of the periradicular area for as long as the tooth is in place. Long-term prediction of biocompatibility for RealSeal and Obtura is supported by the absence of cell inhibition even after 72 h of incubation. The effect of the 50% diluted eluates of RealSeal and the undiluted eluates of Obtura on cell viability is not time dependent, so the hypothesis is that the absence of any cytotoxic effect is not dependent on the time in which the material remains in contact with the vital tissues. However, the undiluted eluates of RealSeal had a positive effect on cell viability that increased with time. The hypothesis is that these materials are stable, do not release toxic substances and will maintain this tendency for even longer incubation periods. These findings are relevant, because the root canal filler remains in permanent contact with periradicular tissues, and its long-term biocompatibility could affect the clinical behaviour and survival rate of any endodontic treatment.

Although this study can be considered as a short-term test for acute toxicity on human gingival fibroblasts, these promising results suggest that in the near future the use of resin-based materials as root canal filling could be recommended.

Conclusion

The results indicate that RealSeal resin-based endodontic filler has, within a period of 72 h, the same cytotoxic potency of dental gutta-percha, appearing as a suitable material for the sealing of root canals.

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