Journal section: Biomaterials and Bioengineering in Dentistry *doi:10.4317/jced.3.e216*
Publication Types: Research

Effect of the methacrylate-based endodontic sealer Epiphany on rat peritoneal macrophages viability

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Received: 01/02/2011
Accepted: 13/02/2011

Martín-González J, Castellanos-Cosano L, López-Frías FJ, Sánchez-Domínguez B, Calvo-Monroy C, Alonso-Ezpeleta LO, Segura-Egea JJ. Effect of the methacrylate-based endodontic sealer Epiphany on rat peritoneal macrophages viability. J Clin Exp Dent. 2011;3(3):e216-21. http://www.medicinaoral.com/odo/volumenes/v3i3/jcedv3i3p216.pdf

Article Number: 50499 http://www.medicinaoral.com/odo/indice.htm

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Abstract

Objective: To evaluate the effect of the endodontic sealer Epiphany on rat peritoneal macrophages viability.
Materials and methods: Peritoneal macrophages were obtained from Wistar rats and resuspended in RPMI-1640 medium. Undiluted (crude extract) and diluted extracts to 10%, 1%, 0.1%, 0.01%, 0.001% and 0.0001% of Epiphany, AH 26 and AH Plus sealers on RPMI-1640 medium were tested for cytotoxicity to rat peritoneal macrophages using the trypan blue dye exclusion assay. Data were analyzed statistically by the Kruskal-Wallis and Mann-Whitney tests at 5% significance level.
Results: Crude extract of Epiphany killed 51% of cells, but was less cytotoxic that crude extracts of AH Plus and AH 26, which killed 81% and 86% of cells, respectively. Ten-fold dilutions of Epiphany, AH Plus and AH 26 killed 44%, 56%, 62% of macrophages, respectively. A hundred dilution of Epiphany only killed 7% of macrophages, but the same dilution of AH Plus and AH 26 killed 10% and 31% of macrophages, respectively. Lower dilutions of sealer extracts caused minimal cell death as compared to the control groups ($p>0.05$).
Conclusions: The methacrylate-based endodontic sealer Epiphany showed lower cytotoxicity on macrophages than resin-based sealers AH Plus and AH 26. Dilution of elutes of the three materials by tenfold markedly reduced their effects.

Key Words: *Endodontic sealers, Cytotoxicity, Epoxy-resin based materials, Methacrylate based materials, Periapical immune reaction.*

Introduction

Successful endodontic therapy depends on thorough cleaning and shaping followed by obturation of the root canal system. During obturation, root canal sealers serve to fill irregularities between the dentinal walls and the gutta-percha core, act as lubricants, fill lateral or accessory canals, and bond to gutta-percha and dentin. Endodontic sealers must be able to eliminate or minimize the ingress or egress of bacteria and their byproducts (1).

Root filling materials are placed in close contact with the periapical tissues for extended periods of time, and as a result, elutable substances or degradation products from root canal fillings might gain access to surrounding tissues (periodontal ligaments, alveolar bone) through numerous connections, e.g. dentinal tubules, accessory and lateral canals, and apical foramina. The tissue response to these materials may influence the final outcome of the root canal treatment (2). A sealer should not hinder tissue repair, but have a favorable tissue response that promotes healing of the periapical tissues stimulating the reorganization of injured structures. So, it is required that these materials are neither cytotoxic, genotoxic, mutagenic nor associated with any other negative biologic effects.

Previous studies have shown that the biocompatibility of different material classes and products of root canal sealers vary considerably (3-6). AH 26 and AH Plus (Dentsply DeTrey, GmbH, Konstanz, Germany) are two of the most commonly used epoxy resin-based endodontic sealers (7). In the last years, methacrylate based endodontic sealers have been developed. Epiphany (Pentron Clinical Technologies, Wallingford, CT, USA) is a methacrylate based dual curable resin composite sealer used with Resilon, a thermoplastic synthetic polymer (polyester)– based core filling material. It has been suggested that the sealer bonds to both the root canal dentin wall and the Resilon core, forming a "monoblock" system within the root canal (8). It is reasonable to assume that endodontic sealers, following their leakage through dentinal tubules, accessory and lateral canals, and apical foramina, may affect the viability of periradicular macrophages. The aim of this *in vitro* study was to study the effect of the methacrylate based sealer Epiphany on rat peritoneal macrophages viability measured by trypan blue dye exclusion assay.

Materials and Methods

Collection of rat peritoneal macrophages

The Ethics Committee approved the study. Peritoneal macrophages were elicited from Wistar rats as described previously (5). Briefly, each rat was injected intraperitoneally with 5 ml of sterile 6% sodium caseinate. Animals were killed after 4 days by decapitation and the peritoneal cavity was washed with 10 ml of cold 0.9% NaCl (Sigma Chemical, St. Louis, MO, USA). After a 2 min massage, the cell exudate was removed with a syringe and centrifuged for 10 min at 250 x g at 4°C. The contaminating red blood cells were lysed with cold 0.2% NaCl. The remaining cells were then washed with 0.9% NaCl by centrifugation, resuspended in RPMI-1640 medium (Sigma Chemical, St. Louis, MO, USA), counted, adjusted in the same medium at 2-4 x 10^6 macrophages/ml and used immediately for experiments. Mean cells per rat varied from 20-30 x $l0^6$, of which 85% to 95% were macrophages by morphological criteria in Giemsa and Papanicolaou staining techniques.

Endodontic sealer extracts preparation

Table 1 shows the tested materials and their compositions.

Product	Material type	Manufacturer	Composition
AH 26	Epoxi-resin based	Dentsply DeTrey GmbH, Konstanz, Germany	**Component A** Silver powder Bismuth oxide Hexamethylenetetramine Titan (IV)-oxide **Component B** Epoxybisphenol (liquid)
AH PLUS	Epoxi-resin based	Dentsply DeTrey GmbH, Konstanz, Germany	**Component A** Epoxy resins Calcium tungstate Zirconium oxide Silica Iron oxide pigments **Component B** Amines Calcium tungstate Zirconium oxide Silica Silicone oil
Epiphany	Multi-methacrylates	Pentron Clinical Technologies, LLC, Wallingford, CT, USA	**Sealer**: Bis-GMA, ethoxylated Bis-GMA UDMA, hydrophilic monomers **Thinning resin**: EBPADMA resins with photo initiator, amines, stabilizer.

Table 1.

The sealers were mixed according to the manufacturer's instructions under aseptic conditions. One gram of each of the mixed materials was then dispensed into one well of a 6-well tissue culture plate. The materials were covered with 10 mL of sterile RPMI-1640 medium and eluted for 1 week at 37ºC. After 1 week, the plates were removed from the incubator and the elutes were centrifuged at 750 x g for 1 min to remove any solid particles. These elutes (crude extracts) were then used for cytotoxicity testing by trypan blue (Sigma Chemical, St Louis, MO, USA) dye exclusion assay. Serial dilutions from the crude extract (100%) were made to obtain dilutions of 10%, 1%, 0.1%, 0.01%, 0.001%, and 0.0001% in RPMI medium. Media alone stored under the same conditions were used as negative controls.

Assay of macrophages viability

Aliquots of 180 µl of cell suspension, adjusted in RPMI 1640 medium at 2-4 x 10^6 cells/ml, were dispensed in Eppendorf tubes. Crude extract or dilutions of endodontic sealer, AH 26, AH Plus or Epiphany, was added (20 µl). Medium alone (20 µl) was added instead of endodontic sealer to control samples. To test the effect of crude extracts of the endodontic sealers, cell suspension was adjusted in undiluted extracts at 2-4 x 10^6.

After incubation for 15 min at 37ºC in a humidified atmosphere of 5% CO2, the reaction was stopped and the effect of the different materials on rat peritoneal macrophages was evaluated by 0.2% (final concentration) trypan blue dye exclusion analysis. Blue coloured cells were considered to be nonviable. Briefly, the cell number was

Substance	Dilution	Viable cells (%)	*p* (*vs* control)
Control	----	89.7 ± 5.6%	
AH 26	0.0001%	89.4 ± 7.3%	> 0.05
AH 26	0.001%	90.6 ± 6.6%	> 0.05
AH 26	0.01%	85.2 ± 7.9%	> 0.05
AH 26	0.1%	86.2 ± 5.1%	> 0.05
AH 26	1%	68.8 ± 7.1%	< 0.05
AH 26	10%	38.2 ± 8.9%	< 0.01
AH 26	100%	13.9 ± 9.4%	< 0.01
AH Plus	0.0001%	93.6 ± 5.8%	> 0.05
AH Plus	0.001%	94.2 ± 7.6%	> 0.05
AH Plus	0.01%	88.2 ± 6.4%	> 0.05
AH Plus	0.1%	86.2 ± 5.1%	> 0.05
AH Plus	1%	89.6 ± 8.1%	> 0.05
AH Plus	10%	43.7 ± 6.9%	< 0.01
AH Plus	100%	19.3 ± 8.6%	< 0.01
Epiphany	0.0001%	96.3 ± 8.6%	> 0.05
Epiphany	0.001%	92.1 ± 7.2%	> 0.05
Epiphany	0.01%	94.7 ± 6.4%	> 0.05
Epiphany	0.1%	95.9 ± 7.8%	> 0.05
Epiphany	1%	92.8 ± 6.3%	> 0.05
Epiphany	10%	56.2 ± 4.8%	< 0.01
Epiphany	100%	48.6 ± 5.8%	< 0.01

Table 2. *Effect of the methacrylate based sealer Epiphany and the epoxy-resin based sealers AH 26 and AH Plus on rat peritoneal macrophages viability by trypan blue dye exclusion assay expressed as percentage of viable cells. Values are the mean ± SEM of eight separate experiments performed in triplicate.*

determined by counting the viable cells in a hemocytometer. The percentage of viable cells from each well after incubation with material extracts was obtained by applying the following equation: % viable cells = (VC/TC) X 100, where VC = viable cells counted and TC = total cells counted (stained plus unstained cells).

Statistical analysis

All values were expressed as the mean ± SEM of the number of experiments (eight), performed in triplicate. The difference between the control and experimental groups was analyzed statistically by the Kruskal-Wallis and Mann-Whitney tests (SPSS version 10.0; SPSS Inc. Chicago, IL, USA). A p-value <0.05 was considered statistically significant.

Results

The effect of the methacrylate based endodontic sealer Epiphany and the epoxy-resin sealers AH 26 and AH Plus on rat peritoneal macrophages viability by trypan blue dye exclusion assay are shown in Table 2 expressed as percentage of viable cells. Crude and 10% extracts of the three sealers reduced significantly cell viability when compared to the control groups ($p<0.05$). The extract of AH 26 at 1% also caused a significant reduction of macrophages viability ($p < 0.05$). Crude extracts of Epiphany, AH Plus and AH 26 killed 51%, 81%, and 86% of cells, respectively. Ten-fold dilutions of Epiphany, AH Plus and AH 26 and killed 44%, 56% and 62% of macrophages, respectively. A hundred dilution of Epiphany killed 7% of cells, but the same dilution of AH Plus and AH 26 killed 10% and 31% of macrophages, respectively. Lower dilutions of sealer extracts caused minimal cell death as compared to the control groups ($p>0.05$).

Discussion

Extrusion of endodontic sealers beyond the apical foramen is undesirable because involve direct contact of the sealer with periapical tissues altering the inflammatory and reparative processes. This makes relevant to study the effects of the sealers on immune cells and their cytotoxicity: to avoid unwanted side effects following the use of canal sealers, which is important for the clinical outcome, only materials exerting minimum deleterious effects on living cells should be used(4).

Root canal sealers cytotoxicity can be studied in vitro by means of several tests, being trypan blue dye exclusion assay chosen for the present study because it is easy to perform and allows for distinguishing non-viable from viable cells by microscopic analysis (9). Trypan blue staining of non-viable cells is a common procedure used in cell culture research, and it relies on the premise that vital cells will not allow the stain to penetrate through cell membranes (10). The accuracy of trypan blue in distinguishing vital and non-vital cells has been verified using scanning electron microscopic (11). These authors showed that cells permeated by the stain (non-vital cells) presented disruption of organelles, whereas vital cells presented integrity of membranes and organelles, as confirmed by ultra structural analysis. Trypan blue dye exclusion assay has been used by others investigators to evaluate the cytotoxic effects of different dental materials and endodontics sealers (12).

The cytotoxicity of epoxy resin-based sealers has been previously studied using various cell types (12-15), as well as the cytotoxicity of Epiphany (16, 17). However, few investigations have been developed to study the effect of these endodontic sealers on macrophages (18, 19). Macrophages play an essential role in the immune response of the host to inflammatory and infectious processes, as well as in the reparative process. At the level of periapical tissues macrophages, by phagocytosis and antigen presentation, have a central function in the repair of chronic apical periodontitis. Moreover, macrophage plays an essential role in periapical lesion development by acting as antigen-presenting cell to memory T lymphocytes (20) and is the predominant cells type in periapical chronic inflammatory processes (21). The results of the present study, demonstrating that AH 26 and AH Plus decreased rat peritoneal macrophages viability, are in good accordance with previous findings showing that resin-based endodontic sealers suppressed substrate adherence capacity and phagocytosis of mouse peritoneal macrophages (4).

In the present study, crude extracts of Epiphany, AH Plus and AH 26 tested cytotoxic in rat peritoneal macrophages, reducing significantly the viability of rat peritoneal macrophages, killing 51%, 81% and 86% of cells, respectively. Correa *et al*. (9), using the same technique and TPH-cells, reported similar results for AH Plus. Donadio *et al*. (1) have studied the cytotoxicity of RealSeal sealer, another commercially available form of methacrylate based endodontic sealer, in L929 mouse fibroblasts *in vitro*, comparing it with traditional AH 26. Their results show that freshly mixed RealSeal sealer have lower cytotoxicity than AH 26 sealer.

Miletić *et al*. (14) reported high cytotoxicity of AH 26 and AH Plus after determining the number of viable cells using a light microscopy, but did not find mutagenicity for AH26 and AH Plus on human lymphocytes in highly controlled conditions in vitro. Cohen *et al.* (22) demonstrated the cytotoxic effect of both AH 26 and AH Plus using the agar diffusion test. Similar results were obtained by Huang *et al*. (7) in cultures of rat hepatocytes. The cytotoxicity of AH Plus when tested using an in vitro culture of human gingival fibroblasts was no longer detectable after 4 hr, whereas the cytotoxic effect of AH 26 remained at a high level until 5 wk (23).

AH Plus showed a lower effect on cell viability than AH 26. Other properties and advantages of AH Plus are that it is eugenol and paraformaldehyde free, has a rapid setting time, higher radiopacity, improved removability,

lower solubility and an acceptable biocompatibility (7, 24, 25). Moreover, AH 26 shows oestrogenic effect *in vitro* whereas AH Plus did not have oestrogenic effect (26).

The present study shows that Epiphany has lower cytotoxic effect on macrophages viability than resin-based sealers. In accordance with these results, cytotoxicity on HeLa cell of freshly mixed Epiphany sealer has been found to be lower than that of freshly mixed AH-Plus (27). Moreover, Epiphany has been found to present better intraosseous biocompatibility than AH Plus (28).

On the contrary, several studies have found disparate results. Lodiené et al. (13) compared the toxicity of methacrylate resin-based root canal sealers with sealers based on epoxy resin and silicone using the MTT assay ([3-4,5-dimethyl thiazol-2-yl]-2,5-diphenyl tetrazolium bromide succinate), which tests for mitochondrial enzyme activity. These authors found that AH Plus were moderately cytotoxic. The in vitro cytotoxicity of AH-plus and Epiphany has been measured using L929 mouse fibroblasts, osteoblastic cells (ROS) 17/2.8 rat osteoblasts, and MC3T3-E1 mouse osteoblasts (29). After 12 weeks of immersion in saline, AH Plus exhibited the cellular succinate dehydrogenase (CSD) activity above control, but Epiphany reduced CSD by 28% ($p < 0.05$). However, histopathological analysis has demonstrated similar satisfactory results in periapical repair after root canal filling with both AH Plus and Epiphany (30).

Conclusions

Based on the results of this study, it can be concluded that the methacrylate-based endodontic sealer Epiphany showed lower cytotoxicity on macrophages than resin-based sealers. Both AH 26 and AH Plus crude extracts reduced significantly cell viability of macrophages, but AH Plus was less cytotoxic than AH 26. Dilution of elutes of the three materials by tenfold markedly reduced their effects. Root canal obturation must be performed carefully to avoid the leakage of endodontic sealers to periapical tissues, because they could reduce macrophage viability modulating the repair mechanisms and inflammatory reactions in periradicular tissues.

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