Cent. Eur. J. Med. • 8(4) • 2013 • 523-529
DOI: 10.2478/s11536-013-0171-8

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Central European Journal of **Medicine**

Testing endodontic sealers on human umbilical vein endothelial cells

Research Article

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Received 3 November 2012; Accepted 29 January 2013

Abstract: The toxic potential of the endodontic sealers ingredients, especially the unreacted monomer, that can irritate the periapical tissue and interfere with the healing process, thus having a negative impact on the biocompatibility of the material. The aim of this study was to evaluate the influence of three experimental endodontic sealers on cells viability in vitro. Human umbilical vein endothelial cells (HUVEC) were used. The experiments were done with solid samples and extracts of sealers in artificial saliva and water. The experiments evaluated the cytotoxicity of the residual monomers that resulted from the tested composites. The decrease in cell viability was quantified by colorimetric measurement of formazan. The components of the sealers dissolved in artificial saliva and water were determined by high performance liquid chromatography (HPLC). The HUVEC are a novelty for testing the endodontic sealers biocompatibility, with certain advantages compared to other cell types used in the literature, e.g. HELA cells, fibroblasts. The data showed that cytotoxicity was directly linked with the unreacted monomer - 2-hydroxyethyl methacrylate (HEMA) present in these composites. Two of the three formulations had little or no cytotoxic effect, which makes them suitable for further testing in order to be used in endodontic treatment..

Keywords: *Endodontic Sealers • Cytotoxicity • Monomer Residual • Human Umbilical Vein Endothelial Cells (HUVEC)*

1. Introduction

Sealers could have a significant influence on the apex. The toxic potential of the ingredients can irritate the periapical tissue and can interfere with the healing process [1]. The materials used in endodontics should be carefully chosen, in order to avoid or reduce potential periapical reactions. Ideally, endodontic materials should be biocompatible and have satisfactory physicochemical properties [1].

When using a sealing material in endodontic treatment, the amount of residual monomer should be low, because the unreacted monomer is removed in time from the endodontic location in saliva, thus having a negative impact on the biocompatibility of the material [1]. Residual monomers and oligomers can be extracted by saliva and brought into contact with mucosal tissues. Also, the components can be extracted into dentin and diffuse towards the pulp cavity, where they can cause irritation. This could lead to unexplained treatment failures [1-6]. It is therefore important to test the biological effects and to identify the components that cause these effects, e.g. dissolved components of the dental materials [7-11]. Thus biocompatibility testing should use solid specimens as well as extracts.

The aim of this study was to evaluate the influence of three experimental endodontic sealers in vitro, with focus on the cytotoxic effect of the residual monomers

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resulted from the tested composites. Human umbilical vein endothelial cells (HUVEC) were used. The experiments were done with solid samples and extracts of sealers in artificial saliva and water. The cytotoxic effect was evaluated by a colorimetric method at 24h and 72h of exposure. The components of the endodontic sealers dissolved in water and artificial saliva were determined by high performance liquid chromatography (HPLC). The results showed a direct correlation between the level of the unreacted monomer - 2-hydroxyethyl methacrylate (HEMA) in solution and the cytotoxic effect of each composite.

2. Materials and methods

We have prepared ten different endodontic materials in our laboratory. The endodontic materials used in this experiment (E1, E4, E5) were prepared as a paste, by dispersing in the organic phase the bioactive anorganic fillers. The chemical composition is presented in Table 1.

Table 1. The composition of the endodontic materials employed in the present study.

Endodontic sealer	Organic phase	Inorganic phase	Initiation system
E1	Bis-GMA; PCL; HEMA; UDMA	Glass with barium HA with ZnO	DMEPT POB
E4	Bis-GMA; PCL; HEMA; UDMA	HA with Ag HA with ZnO	DMEPT POB
E5	Bis-GMA; TEGDMA UDMA	Glass with strontium zirconium; HA with ZnO	DMEPT POB

Bis-GMA (produced in our laboratory) - 2,2-bis[4-(2-hydroxy-3-methacryloyloxypropoxy) phenyl]propane; PCL (Є-polycaprolatone-Merck); HEMA (Merck)– 2-hydroxyethyl methacrylate; TEGDMA (Aldrich)-triethylene glycol dimethacrylate; DMEPT (N,N- dimethyl-p-toluide (Merck)); UDMA - 1,6-bis(methacryloxy-2-ethoxycarbonylamino)-2,4,4-trimethylhexane; POB(Merck)- benzoyl peroxide

The experiments were made by obtaining ZnO and Ag nanoparticles, through sol-gel method, followed by thermal treatments. The started materials was CaO, H_3PO_4 (Aldrich) for synthesized HAP and ZnO (Aldrich) for zinc sol. Different amounts of metal oxide powders prepared through sol-gel method were added into the above solution to control the weight ratio of HAP/ZnO and Ag such as 2:1; 2:1. The mixed sols were submitted at heating treatment at 120ºC, respectively 400ºC. The chemical composition for glasses and the condition for synthesis in our laboratory are: 45% SiO_2, 10% Al_2O_3, 17% B2O3, 20% BaO, 8% NaF–CaF_2; 40% SiO_2, 12% Al_2O_3, 7% B_2O_3, 25% SrO; 8% ZrO_2, 8% NaF–CaF_2 obtained through the conventional melting method at 1350°C. These tests were performed on solid specimens with dimensions of 0.8 mmx6 mm and on extracts in water and artificial saliva for 21 days at 37°C.

2.1 Biomaterials bioassay

To obtain the conditioned saliva (ARTISIAL sol) and water stock solutions, the endodontic materials were incubated in saliva or water for 21 days, at 37°C (0,6 g solid sample/2ml artificial saliva or water). Solutions were used to make increasing dilutions (0%, 3.125%, 6.25%, 12.5% 25%, 50%) in HUVEC medium immediately before the experiments.

Cell source: HUVEC (European Collection of Cell Cultures, Porton Down, Salisbury, UK) cultures were cultivated in standard medium: RPMI, supplemented with 10% fetal calf serum (FCS), gentamicin 50 µg/ml, amphotericin 100µg/ml (Biochrom AG, Germany). Were used cell cultures in the 23rd to 26th passages.

2.2 Cytotoxicity assay

The cells were seeded at a density of 104/well in ELISA 96 wells micro titration flat bottom plaques (TPP, Switzerland) and allowed to settle down for 24hours. Then cells were exposed either to a solid composite sample (0.6 g), completely submerged in medium or to different dilutions of conditioned saliva or water (as described above) for 24 and 72 hours at 37°C and 5% CO_2 in humidified atmosphere. The samples were then discarded. Untreated cultures exposed to medium were used as controls. Cytotoxicity of the composites was evaluated by colorimetric measurement of formazan, a coloured compound generated by viable cells using CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega Corporation, Madison, USA). Briefly, HUVEC cultures were exposed to 20 µl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetra-zolium, inner salt (MTS) /phenazine metosulphate (PMS) mixture (2ml:100 µl) in 100 µl fresh medium/well, for 2 hours, then the optical density values were tested at absorbance of 490 nm by an ELISA plate reader (Tecan, Switzerland). Cytotoxicity was evaluated as % of untreated controls.

2.3 Residual monomer analysis

Conditioned saliva and water samples prepared as described were used for analysis. The extracted residual monomer was identified and measured using a chromatograph HPLC (ABLE JASCO), after a calibration curve. A Lichrosorb RP 18 column 5U, 25x0, 46 cm at a wavelength of 280 nm was used. The solid phase used (RP18) was preconditioned with 5 ml acetonitrile (Merck), and then with 5 ml of water after the sample were introduced. The components of interest (2,2-bis[4-(2-hydroxy-3-methacryloyloxypropoxy)phenyl]propane or Bis-GMA, triethylene glycol dimethacrylate-Aldrich or TEGDMA, 1,6-bis(methacryloxy-2-ethoxycarbonylamino)-2,4,4-trimethyl hexane or UDMA, 2-hydroxyethyl methacrylate

or HEMA and polycaprolatone-Merck or PCL) were retained on the solid phase eluted with 5 ml acetonitrile (Merck).

2.4 Statistical method

All the experiments were conducted in triplicate. Data were analysed using the paired Student TEST, results were considered significant for p≤0.05. Statistical package Origin Pro 8 SRO (Origin Lab Corporation 2007, Northampton MA 01060, USA) was used for data analyses.

3. Results

3.1 Cytotoxicity of the solid endodontic sealers samples

Control cultures showed proliferation, as expected (p=7.23E-3) (Figure 1). The cells viability significantly decreased after exposure to the tested endodontic sealers at 24 hours, E1 (p=1.81E-3), E4 (p=1.69E-2) and E5 (p=1.18E-2) and 72 hours E1 (p=2.46E-2), E4 (p=1.6E-2), E5 (p=7.66E-3).

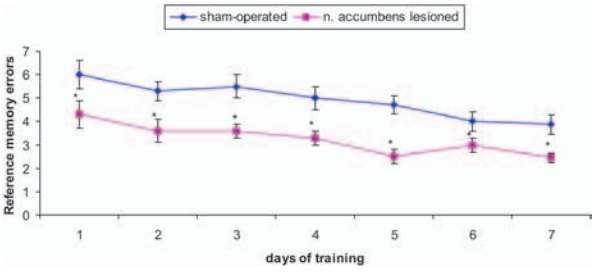

Figure 1. Cell cytotoxicity after exposure of the HUVEC cultures to solid samples of biomaterials for 24, respective 72 hours (results are shown as % of untreated controls). Panel is representative of three independent experiments, * represents the value of p, respective *=p<0.05; **= p<0.005; *** =0.05<p>0.005; p ≤0.05 was considered significant.

3.2 Cytotoxicity of the endodontic sealers conditioned saliva

The HUVEC cultures exposed to artificial saliva experienced a dose dependent decrease in cell viability (Figure 2 (a)). This effect was significant at 24 hours for 12.5% (p=2.42E-3), respective for all the concentrations used at 72 hours (p≤4.83E-2) compared to untreated controls. However, cell viability decrease was not significantly correlated with time exposure.

As seen in Figure 2 panels (b), (c), (d), the conditioned saliva determined a dose dependent decrease in cell viability compared to saliva. This effect was time dependent, only for the small concentrations (3.12% and 6.25%) of biomaterials E1 and E5.

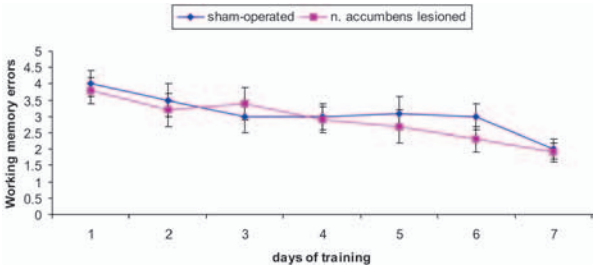

Figure 2. Cell cytotoxicity after exposure of the HUVEC cultures to artificial saliva (a) respective conditioned saliva with biomaterial E1 (b), E4 (c), and E5 (d) for 24 and 72 hours (results are shown as % of untreated controls). Panels are representative of three independent experiments; p ≤0.05 was considered significant.

E1 determined a significant cytotoxic effect at 24 hours for high concentrations (p=1.495E-2 for 25%; p=3.02E-04 for 50%) (Figure 2 (b)). At 72 hours, all concentrations induced a cytotoxic effect; not significant for 3.12% (p=6.79E-2) and significant for the higher concentrations (p ≤3.31E-3). E4 significantly decreased cell viability at 24 hours (p≤5.61E-2) and 72 hours (p≤2.03E-2) (Figure 2 (c)). E5 showed a cytotoxic effect at 24 hours, not significant for 3.12% (p=0.18) and 6.25%, (p=5.6E-2) and significant for the higher concentrations (p≤2.65E-3) (Figure 2 (d)). At 72 hours the same effect was observed for all the concentrations used (p≤2.38E-2).

3.3 Cytotoxicity of the endodontic sealers in conditioned water

HUVEC cultures exposed to water showed a dose dependent decrease in cell viability (Figure 3 (a)). This effect was significant at 24 hours, for 6.25% (p=5.9E-03); 12.5% (p≤4.72E-2) and 50% (p≤4.79E-2) concentrations. At 72 hours the decrease was not significant, compared to untreated controls. Although viability is improved with time exposure to water, this finding was not significant.

As seen in Figure 3 panels (b), (c), (d), the conditioned water determined a dose but not time dependent decrease in cell viability compared to water.

E1 determined a significant cytotoxic effect at 24 hours for concentrations exceeding 6.25% (p≤4.02E-3) (Figure 3 (b)). At 72 hours, this effect was significant (p≤2.267E-2) for the concentrations higher than 3.12% (p=7.77E-1). E4 decreased the cell viability at 24 hours although not significant for 3.12% (p=2.53E-1) and 6.25% (p=11.3E-01), but significant for the higher concentrations (p≤1.45E-2) (Figure 3 (c)). At 72 hours, this effect was significant for all the concentrations used (p≤1.98E-2). E5 significantly decreased viability (p≤4.32E-2) at 24 hours for concentrations exceeding

6.25% (p=1.97E-1) (Figure 3 (d)). At 72 hours, this effect was significant (p≤2.07E-2) for concentrations higher than 3.12% (p=2.53E-1).

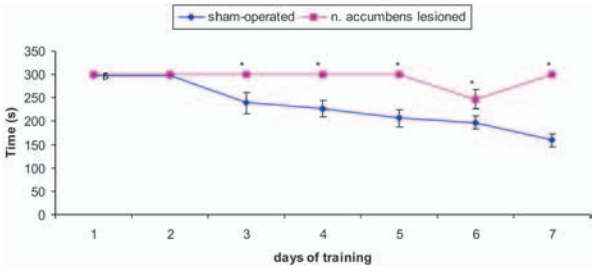

Figure 3. Cell cytotoxicity after exposure of the HUVEC cultures to distilled water (a) respective conditioned water with biomaterial E1 (b), E4 (c) and E5 (d) for 24 and 72 hours (results are shown as % of untreated controls). Panels are representative of three independent experiments; p ≤0.05 was considered significant.

3.3 Residual monomer quantification

Using HPLC method, the only residual monomer identified was HEMA, with the largest amount for E4 sealing material. There is the possibility of TEGDMA monomer existence too, but in our study it was not identified.

The resulting concentration in μL of HEMA solution is presented. The results of three samples investigated of biomaterial conditioned water and artificial saliva are showed in Table 2. The curve correlation factor is r=0.9988 and the right equation Y=0.24802X+1.47411.

Table 2. Results of residual monomers.

	HEMA-peack 1 m
E1-water	-
E4- water	3,50
E5- water	-
E1-artificial saliva	3.28
E4- artificial saliva	15.90
E5- artificial saliva	10.14

4. Discussion

The dental materials used in our study have original formulations and are not commercially available, being produced by us. The tested experimental endodontic sealers, E1 and E5, had little or no cytotoxic effect on HUVEC cultures in vitro (with HEMA, the only residual monomer identified), when used in small concentrations.

There are several studies on the standardization of cytotoxicity tests and new standard reference materials useful for evaluating the effectiveness and safety of biomaterials [12]. We chose to test the cytotoxicity of the endodontic materials of interest on the HUVEC cells. The HUVEC cultures were previously used for drugs and biomedical materials cytotoxicity evaluation (investigation of the effects of cyclosporine (CsA) on apoptosis, assessment of the effectiveness of Paclitaxel-loaded stents by immobilization with a biodegradable polymer) [13-14], but they are a novelty for the endodontic sealers biocompatibility testing. However, they posses certain advantages compared to other cell types used in the literature, e.g. HELA cells, fibroblasts [15]. HUVEC are human, normal, endothelial cells, which are directly involved in all the inflammatory, allergic reactions and tumor outgrowths. Our results following the in vitro testing of endodontic sealers on HUVEC cultures proved that they can be successfully used for this purpose.

With advances in etch-&-rinse and self-etching adhesive technologies [7,16], there is increasing interest in the use of low viscosity, methacrylate resin-based composites as sealers for root filling materials in endodontics [3,4,17]. The release of unreacted monomers from resin composites may stimulate the growth of bacteria [18] and promote allergic or cytotoxic reactions to intraoral tissues [18]. Diffusion of water into resin matrices may result in the deterioration of the physical/mechanical properties, decreasing the life expectancy of the interfaces by hydrolysis and microcrack formation [19,20]. Water ingress may, however, have related beneficial effects [19,20].

Currently, endodontic materials based on various formulas such as epoxy resin, calcium hydroxide and zinc oxide-eugenol are available. Unfortunately, several studies have shown that most commercially available materials can cause mild to severe inflammatory changes in apical tissues, leading to bone destruction [21-24]. Endodontic sealants may also affect periradiculare tissues by direct contact in cases of overfilling or extracted components. They can be released into the surrounding tissues by the dentin tube and channels from the apical level [21-24].

In our study, the solid biomaterial samples induced cytotoxicity diminished with time exposure, especially for E4 and E5. This is consistent with the fact that after an initial cytotoxic effect of the composite, the cells had the ability to adapt and started to proliferate, despite the presence of the biomaterial. The mechanical trauma generated by the solid sample application initially inhibited cell proliferation and induced apoptosis, thus generating a lower number of viable cells. We used a large sample, (0.6 g), in order to imitate a clinical situation, thus the dose of the residual monomers solved in the medium surrounding the cells was important.

Exposure of the cells to the endodontic sealer conditioned saliva and water reduced the cell viability in a dose, though not time dependent, manner. In these ex-

periments we compared the effects on cell viability determined by the composite materials with that of the solvents: saliva or water in order to minimize their role in inducing these effects. The water and saliva contributed to the cytotoxic effect immediately after being added to the cells, by modification of the osmotic pressure of the medium. These are both hypotonic solutions that affect the membrane integrity. When cells are exposed to low osmotic pressure solutions, water enters the cells, the membrane swallows and can rupture, especially in the cells that suffered a loss of membrane elasticity, leading to cell death [25]. However, the other cells maintained a good viability rate, especially with longer exposures, thus allowing the cells to recover. This is sustained by our findings, indicating a dose dependent reduction of cell viability exerted by both water and saliva. Longer exposures to the solvent decreased the cytotoxic effect.

The biomaterial conditioned solutions decreased the cell viability in a dose dependent manner for all the three experimental sealers tested. However, the highest concentrations used 25% and 50%, induced a very strong cytotoxic effect and diminished the cell population by half. Exposure to the modification of the osmotic pressure, due to the solvent, combined with the cytotoxicity of the material itself increased the cell damage. Our data showed a consistent similarity in the cell viability with that of the solvent when cells were treated with low concentration (3.125%) for both E1 and E5 conditioned saliva and water. This shows that the E1 and E5 endodontic sealers were not toxic for the HUVEC cultures in this concentration.

The residual monomer analysis results showed that the largest percentage of residual monomer in all the investigated composites is due to the unreacted monomer HEMA. The small amount of extracted residual monomer from E1 and E5 compared to E4 composite materials is explained by a higher reactivity of the first two monomers, leading to an advanced copolymerisation.

The chromatograms presented no significant differences between the samples extracted in distilled water. It is, however possible that the monomer was volatilized, thus leading to a reduction in the monomer release in water. For the E4 composite we found large amounts of residual monomer in two areas of extraction.

Our results showed a stronger cytotoxic effect induced by the E4 composite. These data are consistent with the residual monomer findings, thus showing that cytotoxicity is directly linked with the unreacted monomer HEMA. Moreover, it determined a dose, but not time dependent decrease of cell viability. The lack of time correlation can be explained by the osmotic pressure modifications due to the solvent.

In several studies, uncured bonding materials were tested in direct contact to the culture cells. Materials were tested either diluted or undiluted without light curing. Several studies reported severe cell damage caused by undiluted materials [26,27], which decreased with increasing dilutions of the materials [27-28]. These findings were concordant with our study. Uncured materials were more cytotoxic than polymerized materials [29-30]. In the present study, bonding substances were cured in conjunction with resin-composites in aerobic conditions where the bonding substance cure could have been affected by air inhibition.

Components of dental materials have been tested individually as single substances and have been shown to be cytotoxic [29] or to modulate cellular function [30]. Kaga et al. compared the cytotoxicity of cured materials, uncured materials and two major components, HEMA and TEGDMA [31]. Materials were applied in glass cylinders with indirect cell contact. HEMA contributed mostly to the cytotoxicity, which is consistent with our findings. The clinically acceptable values for the release of residual monomer in composite restorations are in the range 1-3% [32]. In our study, HEMA release from the undiluted extracted samples ranged from 3.28 µL/mL to 15.90 µL/mL. The results vary from material to material, which may be due to the monomer concentration in the system, method of polymerization, storage time and method of evaluation. Our in vitro study showed that the investigated dental experimental composite materials E1 and E5 are biocompatible when used in small quantities, while the E4 formulation had a high cytotoxic effect. This effect was correlated with the concentration of the residual monomer, HEMA, but not with time exposure.

These experiments may be considered advantageous in dentistry, due to opening of a new perspective for testing experimental and commercial endodontic materials using HUVEC cultures. However, this is an imperfect model, due to the fact that in the in vivo conditions, the blood and saliva flow washes and dilutes the toxic substances from the treated area, thus avoiding the accumulation of the residual monomers. In vivo studies are needed to complete the current findings.

5. Conclusion

This study sustains the major role of the residual monomer HEMA, freed by saliva from endodontic sealers, in inducing the decrease in cell viability, and thus the toxic oral effects. Reducing the concentration of the residual monomer may lead to a better endodontic sealer formulation with a less cytotoxic effect. Further research will be done to improve the composition of the sealers. Following the current data, E1 and E5 composites may be successfully used for the endodontic therapy.

Acknowledgments

Funding from the Romanian Ministry of Education and Research, National project PNII no: 72-190/ 2008, is gratefully acknowledged.

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