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Original Research Article

Cytotoxicity of Five Endodontic Sealers in NIH 3T3 Fibroblasts

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Abstract: The objective of this study is to assess the cytotoxic effects of substances leached from five endodontic sealers in time periods of 0 and 24 hours on NIH 3T3 fibroblasts. It was used a medium conditioned by the sealers, subdivided in six groups namely: Control (fresh cell culture medium); SimpliFill (Discus Dental, Culver City, Calif) : EndoREZ® (Utradent Products, Inc); EPIPHANY® (Pentron Clinical Technologies, Wallingford, CT, USA); EPIPHANY® SE **(**Pentron Clinical Technology); AH Plus® ((Dentsply/Caulk, Petrópolis, RJ, Brazil) in intervals of 0 (freshly made) and 24 hours (set form). The obtained data were interpreted by the one-way ANOVA and Tukey test, respectively; with a level of significance of 5% ($p \leq 0.05$). The mean number of viable cells was 0.3208 for the control group, followed by freshly made SimplyFill (0.1818) and its set form (0.1800); freshly made EndoREZ (0.0899) and its set form (00.1465); freshly made Epiphany (0.2138) and its set form (0.1795); freshly made Epiphany SE (0.1505) and its set form (0.1463); and freshly made AH-Plus (0.2480) and its set form (0.2265). Statistical differences were found among the groups ($p \leq 0.05$), with the cell viability decreasing significantly with EndoREZ in its fresh form (p $p \leq 0.01$) and in its set form ($p < 0.05$); followed by EndoREZ in both fresh and set forms (p $p \leq 0.05$), when compared to SimplyFill, Epiphany, AH-plus and the control groups. EndoREZ was the most cytotoxic sealer of all tested. The observed differences among the cytotoxicity concluded that EndoREZ was the most cytotoxic sealer of the five tested in the experiment in time the time intervals of 0 and 24 hours.
Keywords: Endodontics, Fibroblasts, Root Canal Filling Materials.

INTRODUCTION

Endodontic sealers deserve a special attention in Endodontics for the fact of they are inserted directly into the root canal system remaining in continuous direct contact with the periapical tissues for uncertain time periods Therefore, the endodontic obturation should consist of a complete hermetic and resistant sealing of the root canal system with the aid of biocompatible materials which do not interfere in the repairing process and most desirably would stimulated the periapical tissue healing favoring the success of the treatment [1, 2].

Thus; researchers have turned their attention to investigating if endodontic sealing materials have reached a stage which would be classified as satisfactory, considering their biological and physical-chemical properties and their biocompatibility.

It is necessary to remark that freshly made mixtures tend to be more citotoxic than their set forms, so the effect of time along the setting process is important to comprehend cytotoxicity and its effects on the living tissues [3]. Few clinical results include long time control groups to support the advantages of methacrylate resin based sealers, and their merits might only be revealed in the near future [4].

Based on this consideration and on the fact that in the market there have been a great number of sealers available, the aim of the present study was to analyze the cytotoxicity of the endodontic root canal sealers SimpliFill, EndoRez, Epiphany, Epiphany SE and AH-Plus through the cell culture of human 3T3 fibroblast lineage.

MATERIALS AND METHODS

The response of cultured NIH 3T3 fibroblasts induced by substances leached or dissolved from five dental materials was analyzed by an in vitro cell culture method. For this, six groups were established, as follows: **Control** (fresh cell culture medium);

SimpliFill (Discus Dental, Culver City, Calif) : **EndoREZ**® (Utradent Products, Inc); **Epiphany®** (Pentron Clinical Technologies, Wallingford, CT, USA); **Epiphany® SE** (Pentron Clinical Technology); **AH Plus**® ((Dentsply/Caulk, Petrópolis, RJ, Brazil) in intervals of 0 (freshly made sealer) and 24 hours (set form).

Preparation of the tested materials

The cultivation medium (DMEM) was placed individually in contact with the sealers during hardening for the period of 24 hours in an incubator with humid atmosphere and temperature of 37ºC. Following the specifications of the American Society for Tests and Materials (Annual book of standard ASTM, 1992) 0,4g of the sealer was used for each two ml of half DMEM, for the obtention of the conditioned medium.

AH-Plus

Similar amounts of the pastes A and B were mixed (1:1), in agreement with the manufacturer's instructions, on a sterile glass slab with the aid of a metallic spatula equally sterile until the homogenization of the mixture of both pastes was reached. The mixture was then inserted into a Falcon tube, where it received the DMEM, and was left for hardening for the periods of 0 and 24hs.

SimpliFill, EndoREZ®, **Epiphany®** and **Epiphany ® SE**

For being a base/catalyst type of sealers, six centimeters of base paste and six centimeters of catalytic paste were placed on a glass plate and with aid of a metallic spatula No.24, were mixed together to incorporate in one another until a homogeneous mixture was obtained. They were manipulated in agreement with the manufacturer's instructions, inserted into a Falcon tube, where they received the DMEM and soon after underwent photopolymerization, which was accomplished with the tip of the photopolimerizer in contact with the external part of the bottom of the Falcon tube where the sealers rested for the establishment of the setting. Each conditioned medium, was then diluted to ten percent (2g/100ml) and applied on the cellular cultures for the periods of 0 and 24hs in the groups mentioned previously.

Cell Culture

Fibroblasts from the NIH lineage was cultured as previously described [5]. Briefly, the cultured medium was the Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and 1% antimycoticantibiotic 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 maintained in an incubator at 37ºC and a humidified 5% CO2 atmosphere. Cultures were supplied with fresh medium every other day. Cells between the fifth and tenth passages were used in all experimental procedures.

Experiments

For the development of the present research the experimental groups were established in time periods of 0 and 24 hours which were previously disposed in plates of cellular cultivation of 96 wells. The first column of the plate was filled entirely (eight wells) with the control group composed for half conditioned of culture. The wells of the following columns were filled until the fourth well. The four other remaining wells of each column were filled with PBS that does not supply MTT reading.

Cell viability analysis

The cell viability was determined by the mitochondrial activity analysis. This analysis was carried out using the MTT-based cytotoxicity assay. The MTT assay involves the conversion of the water soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to insoluble formazan salt. This process will occur only by viable cells. Then, the formazan is solubilized, and the concentration determined by optical density at ≈570 nm. A MTT reduction analysis kit (Vybrant MTT, Molecular Probes, Eugene, OR, USA) was used. Immediately after the end of the assay procedures the absorbance was read in a micro plate reader (Biotrak II, Biochrom Ltd, Eugendorf, Austria) using a 562 nm filter. The absorbance data was transformed into number of viable cells that was used to plot the cell growth curves.

Morphological analysis

The morphology and the distribution of cells were monitored throughout the experimental time. Using phase light microscopy, the relationships between the cells grown in the Petri dishes and the coverslips of all groups were studied. Additionally, the individual morphology of the cells, as well as the presence of both living cells and dead cells was analyzed. Phase photomicrographs were obtained from a Zeiss Axiophot microscope (Carl Zeiss Inc., Oberköchen, Germany).

Statistical analysis

The data were statistically treated with the aid of the Software BioStat 3.0. Such data were initially submitted to test of Kolmogorov - Smirnorv (Lilliefors), revealing compatible behavior with parametric patterns. Data were compared by ANOVA complemented by the Tukey's test ($P \leq 0.05$).

RESULTS

The results of the MTT assay over all the time periods are shown in Figure 1 and collectively represented in TABLE 1. The majority of the sealers

tested showed an initial cytotoxicity that increased along time. AH-Plus was the sealer that was less cytotoxic of all others, especially when it was evaluated as freshly made, and not significantly different from the control. Even when evaluated in set conditions (after 24 hours), AH-Plus still was not statistically different from the control group, although its cytotoxicity increased along time (G10;G11); ($p<0.05$). Fresh Epiphany (G7; G8) was mildly cytotoxic and became more cytotoxic in its set form, but still not statistically significant when compared to the control group ($p<0.05$). SimplyFill (G2 and G3) was mildly cytotoxic although not statistically significant in both fresh and set forms ($p<0.05$); it was the sealer that less changed along time, showing a certain degree of stability, differently from all the other sealers tested in this experiment. On the other hand, EndoREZ was cytotoxic when evaluated in both fresh and set forms, but with one particular feature: its cytotoxicity decreased along time in the setting process, unlike any other sealer tested in this study. Statistically significant differences were found in EndoREZ groups (G4 ; G5) as a freshly made sealer ($p<0.01$) as well as in its set form ($p<0.05$), respectively. Even having the cytotoxicity decreased after 24 hours, it was still cytotoxic. Epiphany SE (G8; G9) was also regarded as cytotoxic since it had statistically significant differences in both fresh and set forms ($P<0.05$), but behaved similarly to the majority of the other sealers in the study by showing its set form more cytotoxic than the fresh one.

The statistical analysis was accomplished with ANOVA test complemented with Tukey´s test. Significant statistical differences were found in the group of freshly made EndoREZ in Tukey´s ($p\leq 0.01$) and in its set form ($p< 0.05$), followed by Epiphany SE in both time periods ($p\leq 0.05$); TABLE 2.

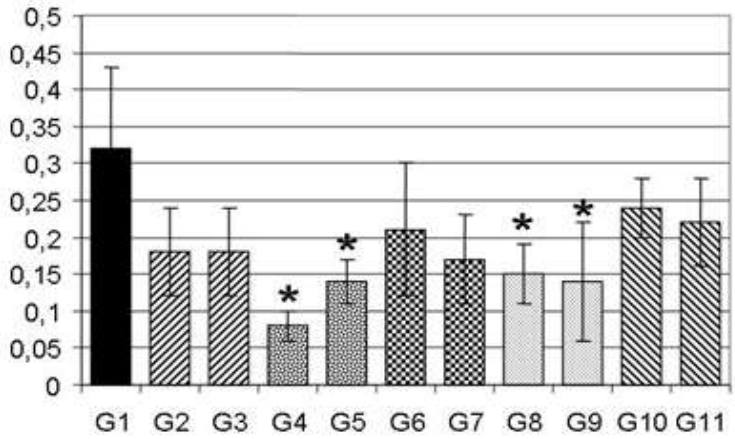

Fig-1: Graphic representation averages of the mitochondrial activity of the fibroblasts treated by the substances liberated by endodontic sealers in the period of 0 and 24hs of the conditioning of the cell culture medium.

G1- control; G2-SimpliFill Time 0; G3- SimpliFill 24Hs; G4- EndoRez Time 0; G5 -EndoRez 24Hs; G6- Epiphany Time 0; G7- Epiphany 24Hs; G8- Epiphany SE Time 0; G9- Epiphany SE 24Hs; G10- AH-Plus Time 0; G11- AH-Plus 24Hs.

Table 1: Absorbance data of all experimental groups

G1-Control	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11
0.239	0.149	0.135	0.099	0.175	0.121	0.123	0.131	0.247	0.183	0.192
0.215	0.111	0.148	0.125	0.142	0.148	0.183	0.216	0.133	0.275	0.319
0.388	0.255	0.278	0.078	0.097	0.314	0.139	0.138	0.043	0.245	0.175
0.229	0.212	0.159	0.057	0.172	0.272	0.273	0.117	0.162	0.289	0.220

Absorbance data: G1- control; G2-SimpliFill Time 0; G3- SimpliFill 24Hs; G4- EndoRez Time 0; G5 -EndoRez 24Hs; G6- Epiphany Time 0; G7- Epiphany 24Hs; G8- Epiphany SE Time 0; G9- Epiphany SE 24Hs; G10- AH-Plus Time 0; G11- AH-Plus 24Hs.

Table 2: ANOVA

Variation Sources	**GL**	**SQ**	**QM**
Treatments	10	0.215	0.022
Error	37	0.213	0.006
F=	37.396		
P=	0.0018		

Tukey´s critical value = $p\leq 0.05$

DISCUSSION

Endodontic sealers have the characteristic of being prepared and put in the root canal during the obturation, which can be accomplished using many techniques available. Nevertheless, no matter which technique has been chosen, sealers are immediately inserted into the root canal which implies in freshly prepared samples and their behavior along time into the setting process. The freshly made sealers correspond to the Time 0 interval. Some of the sealers may have their degree of citotoxicity altered, launching substances directly in contact with the surrounding tissues as time goes by. When this happens, any cytotoxic degradation product may gain access to, and damage surrounding tissues. As a result, it is fundamental that root canal sealers be biocompatible, being the least irritant possible [5].

Therefore every sealer must have their biocompatibility tested with *in vitro* and *in vivo* experiments before any clinical use [6]. However, before testing them in a living organisms and in order to provide a ranking of toxicity for such materials, it is crucial these sealers be tested by *in vitro* tests firstly [7]. In this study, the materials were used both in fresh and set states in order to investigate the effect of setting process in cytotoxicity of experimental material as it has stated in former studies such as lodiene *et al*. [8] and merdad *et al* [9]. The results of this study show that the freshly made samples of all five materials showed differente degrees of citotoxicity in absolute numbers. Despite the common assumption that toxicity decreases over time [9, 10], results of our study show that after 24 hours, cell viability values reduce for almost all samples, except for EndoREZ.

AH-Plus is an epoxy resin sealer whose one of the main characteristics is being hydrophobic, because of the presence of bisphenol A and F in their composition. Studies on its biocompatibility are not concordant and show cytotoxicity indexes ranging from mild to severe [11-13]. Another study [14] compared Epiphany, AH-Plus and EndoREZ and found the best scores of biocompatibility with Epiphany, against AH-Plus and EndoREZ. Our results disagree with this previous study and showed that the citotoxicity of AH-Plus can be considered as mild, when compared to the Control group, as it was the less citotoxic sealer of all tested on NIH fibroblasts. SimpliFill remained stable as freshly made and after 24 hours, while the second most cytotoxic sealer was Epiphany SE, followed by its predecessor, Epiphany.

In a general way, AH-Plus, EndoREZ and Epiphany have been demonstrated to show an initial inflammatory reaction that decreases over time [15-17]. In this study, this could not be observed for the sealers tested unless for EndoREZ, which was the only sealer of all those tested that behaved accordingly, although even after 24 hours when its toxicity had decreased, it could still be considered very toxic.

Nevertheless, statistically significant differences were only present in EndoREZ and Epiphany SE in Tukey´s test. None of the sealers were more cytotoxic then EndoREZ, whose cytotoxicity has been attributed to the presence of urethane dimethacrylate (UDMA), a known toxic agent, in the structure of this sealer [18]. As for Epiphany and Epiphany SE, the cytotoxicity might be explained by the high resin content of this sealer, resins which consist of bisphenol A-glycidyl methacrylate (Bis-GMA), ethoxylated Bis-GMA, UDMA, and hydrophilic difunctional methacrylates [19, 20]. It is also possible that degradation causes leaching of monomers and filler particles, resulting in cytotoxicity of this sealer.

Methacrylate based sealers have been agressively promoted as sealers able to create monoblocks within the root canal space that could be defined as a mass of different materials and interfaces: solid materials (gutta-percha or special resin points) compacted with the sealers that would theoretically create perfectly gap free canals, with no voids, that would enhance seal and improve fracture resistance to endodontic treated teeth [21, 22]. Many are the qualities explored in their marketing strategies. An example of this is Epiphany Root Canal Sealer, which is the main representative of the third generation of endodontic sealers. As for what concerns biocompatibility, Epiphany in both freshly mixed and set conditions showed a severe to moderate cytotoxic effect according to one study [23], and its cytotoxicity actually increased with time, posing significant cytotoxic risks [24, 25]. Our results are in agreement with them. The toxicity of Epiphany might be explained by the presence of unpolymerized hydrophilic monomers (such as HEMA) that can easily diffuse into the cell-culture medium [26] and elicit significant toxicity [27].

Despite of the good results as for what concerns radiopacity, an expected and desired endodontic radiographic feature, EndoREZ has been interpreted as well-tolerated by connective tissues by some studies [28, 29] and also well tolerated by bone tissue [30]. It is also described to have minimal cytotoxic effects, when freshly mixed or even after setting. Such findings were not supported by Bouillaguet *et al* [31] and Scarparo *et al* [32]. Their results indicated that EndoREZ had a more intense and longer-lasting inflammation in subcutaneous connective tissue of rats than AH Plus sealer. Moreover, the mentioned authors found that EndoREZ became more cytotoxic with increased exposure time to the cell culture medium, in agreement with our results.

Thermoplastic resin-coated gutta-percha cone is recommended for use with the EndoREZ system [33]. Nevertheless, with the great amount of techniques available, and with the fact that these sealers are photopolymerized inside the root canal, it is very possible that overflowing might happen, especially when dealing with thermoplasticized gutta-percha whose melting process often favors trespasses of the material mass into the periapical area, especially rests of unpolymerized sealers exerting a great potential inflammation in the area. Contact of extruded unpolymerized sealers might result in irritation of the periradicular tissues and in delayed wound healing [34, 35]. Consequently, extrusion of a methacrylate resin–based sealer through the periapical foramen would create an uncured surface layer for extended time periods [36, 37]. This might alter the toxicity profile of resin-based sealers because more incompletely polymerized, toxic monomers are present in the exposed sealer. Forty percent of the sealer remained unpolymerized despite a post-curing time of as long as 2 weeks in vitro [38]. Therefore, thermal and chemical irritation would be a natural and expectable clinical outcome of endodontic treatments with such sealers. With all of such situations mentioned above, it is of extreme importance the due and careful choice of the most appropriate sealer to be used according to each specific clinical situation, preponderating as for the risk of overflow versus the benefit that each specific sealer may offer as well.

According to the results herein, an overflow of any methacrylate based sealers would cause periapical irritation, especially EndoREZ and Epiphany SE in the universe of this study.

CONCLUSION

The observed differences among the cytotoxicity of SimpliFill, EndoREZ, Epiphany, Epiphany SE and AH Plus, reached a significant level for EndoREZ as the most cytotoxic sealer of all tested in the experiment. Additional *in vivo* studies are proposed to confirm these *ex vivo* results.

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