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Assessment of cytotoxic potential of root canal sealers after hardening – an *ex vivo* study

Ocena cytotoksycznego potencjału uszczelniaczy kanałowych po stwardnieniu – badania *ex vivo*

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A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Summary

Aim: The aim of this study was to perform a comparative assessment of the toxic action of root canal sealers currently on the market on human gingival fibroblasts after setting.

Material/Methods: The inserts with an equal quantity of set root canal sealers were transferred into 24-well culture dishes containing human gingival fibroblasts cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS). The dishes with materials were incubated at 37°C, 100% humidity and in an atmosphere of 5% CO_2 for 24 h. The cytotoxic effects of the root canal materials were measured by the mitochondrial succinate dehydrogenase activity in living cells using tetrazolium bromide (MTT assay).

Results: Epiphany and Sealapex exhibited high toxicity towards human gingival fibroblasts – 25.57% ± 0.88 and 27.63 % ± 2.35 respectively (less than 30% live cells in the culture). The remaining materials were characterized by lack of a cytotoxic effect (over 90% of live cells in the culture). None of the preparations exhibited moderate or low toxicity.

Conclusions: The majority of root canal sealers tested after hardening were well tolerated by human gingival fibroblasts. Only two materials were characterized by high toxicity: with methacrylate (Epiphany) and calcium hydroxide (Sealapex).

Key words: root canal sealers • cytotoxicity • MTT assay • succinate dehydrogenase • cell culture

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Introduction

The final stage of endodontic treatment involves closing the root canal tightly by using a basic material – i.e. gutta-percha or Resilon and a sealer. The task of the sealer consists of combining the main filling material with the root canal wall, filling the space between the gutta-percha or between the Resilon and the dentin, as well as ensuring easy sliding to points to introduce them into the root canal more easily [55].

At present, there exist several groups of sealers which are classified according to their chemical composition. The older generation of materials includes zinc oxide eugenol type sealers, calcium hydroxide sealers, epoxy as well as glass ionomer ones. To improve the tightness of root canal fillings, new sealers have been introduced. They contain methacrylate resins or silicon compounds.

Despite the fact that obturation agents should be found only in the root canals, sealers may penetrate the periapical tissues, if the condensation of the filling has not been performed properly, or as a result of anatomical conditions owing to which the material can pass through a broad apical foramen [16]. Due to the long-term contact with the periapical tissues (periodontium, cementum and the alveolar process bone), the materials filling in the root canal should not only possess perfect physicochemical characteristics but should also be characterized by biocompatibility [16,21,23]. A preparation with toxic action may damage the tissue and prevent the healing process of inflamed periapical structures [7].

The results of research using cell cultures show that some sealers may induce metalloproteinase expression in fibroblasts, leading to decomposition of the extracellular matrix of the periapical tissues [48], have a synergistic effect with bacterial toxins (LPS) aggravating inflammatory reactions [28], and hamper the phagocytosis process in bacterial cells due to macrophages [17]. Furthermore, it was proven that the application of some sealers inhibits cellular respiration [29] and fibroblast proliferation [53] and also reduces the activity of alkaline phosphatase – the key enzyme taking part in the process of bone tissue formation [24].

Under clinical conditions, this material is introduced into the root canal immediately after mixing, but even after setting it can have a toxic effect by releasing harmful components during contact with tissue fluid [7,11,26]. Therefore, biocompatibility should be a significant factor affecting the selection of the sealer in endodontic treatment [2].

The authors' earlier research focused on the assessment of toxic action of root canal sealers immediately after mixing on human gingival fibroblasts. Epoxy, methacrylate and zinc oxide eugenol type sealers were characterized by the highest cytotoxic potential [40].

The aim of this study was to perform a comparative assessment of the toxic action of root canal sealers currently on the market on human gingival fibroblasts after setting.

Material and methods

A. Preparation of material samples

The research was conducted using materials presented in Table 1. Endomethasone N, Tubliseal, Sealapex and GuttaFlow were prepared in accordance with the manufacturers' instructions under sterile conditions. The other sealers, placed in two-cannula syringes, were mixed in the dispenser while being pushed out. Immediately after preparation, the materials were put into plastic rings with a size of 5 mm (inner diameter) x 5 mm (height). The rings with the materials were stored at a temperature of 37°C, 5% CO_2 and 95% humidity for 24 hours to ensure their hardening. Next, they were transferred into inserts [5] (manufactured by Nunc) with a surface area of 0.47 cm^2 and a pore diameter of 0.4 µm situated in 24-well culture plates (manufactured by Nunc), containing human gingival fibroblasts. Six samples were prepared for each material. Six wells in each of the 24-well plates with inserts and with no materials added constituted the control.

B. Preparation of cell culture

Human gingival fibroblasts with adherent properties (adherent permanent cell line) (ATCC CRL-2014HGF-1, manufactured by LGC Promochem) grew in Falcon containers (growth surface area 75 cm^2) on the DMEM substrate (Dulbecco's Modified Eagle's Medium) with the addition of 10% fetal bovine serum (FBS) at a temperature of 37°C, 5% CO_2 and 95% humidity. After the stage of confluent growth was obtained, cells were separated using 0.25% trypsin solution with addition of 0.53 mM EDTA. The activity of the enzyme was inhibited by adding the substrate from 10% FBS. The cellular suspension was diluted in fresh substrate, inoculated in 24-well plates and incubated for 24 hours.

C. Cytotoxicity assessment

The assessment of cytotoxicity of the materials towards human gingival fibroblasts was assessed by means of the MTT test [3,10,11,20,31,38,47,50]. This method enables determination of cell viability and proliferation on the basis of mitochondrial activity of succinate dehydroge-

nase. In live cells this enzyme causes reduction of the yellow tetrazolium salt MTT-3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyltetrazolium bromide to violet formazan. The dye content is measured in the absorption spectrophotometer. The amount of formazan is directly proportional to the number of live cells in the culture. Low cell viability corresponds to low activity of the enzyme and, at the same time, a low content of violet formazan and reduced absorbance value.

Culture plates with cells and the materials used were incubated at a temperature of 37°C, 5% CO_2 and 95% humidity for 24 h. After this time, inserts with materials were removed, placed in 1 ml of 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at a concentration of 0.5 mg/ml and incubated for 2 hours under the conditions specified above without access to light. Next, fluid from the culture was aspirated, 1 ml of isopropanol acidified with hydrochloric acid (0.04 mol L^{-1}) was employed and the solution obtained was stirred for a short time to dissolve formazan crystals. The absorbance was measured by means of a two-bundle absorption spectrophotometer Lambda EZ 201(manufactured by Perkin Elmer) at 560 nm wavelength.

Cell viability was calculated according to the following formula [52]:

(Absorbance of the tested sample/absorbance of the control sample) x 100%.

The criteria specified by Dahl et al. were adopted for the assessment of the cytotoxicity [quoted after 20]

No cytotoxicity – cell viability compared to the control > 90%

Low cytotoxicity – cell viability compared to the control 60%-90%

Moderate cytotoxicity – cell viability compared to the control 30%-59%

High cytotoxicity – cell viability compared to the control < 30%

The results obtained were analysed statistically using one-way analysis of variance (ANOVA) and they were compared in a complementary manner by means of Tukey's HSD post-hoc test using the Statistica 8.0 software (manufactured by StatSoft). The adopted significance level was $p < 0.05$.

Results

The results are presented in Table 2. From amongst the sealers used, only two sealers, Epiphany and Sealapex, exhibited high toxicity towards human gingival fibroblasts: 25.57% ± 0.88 and 27.63 % ± 2.35 respectively (less than 30% live cells in the culture). The remaining materials were characterized by lack of a cytotoxic effect (over 90% of live cells in the culture). None of the preparations exhibited moderate or low toxicity.

The one-way ANOVA showed significant differences in results between those materials with the highest cytotoxicity (Epiphany, Sealapex) and the other sealers ($p < 0.001$). No significant differences were observed between preparations characterized by lack of a cytotoxic effect or between Epiphany and Sealapex ($p > 0.05$).

Discussion

Research using cell cultures is a common method of testing materials before introducing them into clinical use. Such tests make it possible to perform simple, reproducible experiments controlled under laboratory conditions, comparing numerous agents under the same conditions. Human gingival fibroblasts, apart from other cells, are often used in such tests [3,20,27,35,47,51].

Despite the fact that gingival and periodontal fibroblasts are phenotypically different, according to Guertsen et al. [21] there are no significant differences in the sensitivity of different types of cells to harmful substances released from sealers during the short-term (24-hour) toxicity assessment.

To create conditions similar to the clinical situations which occur if the root canal is filled properly, the materials inserted are not in direct contact with cells, but on semi-permeable membrane of the inserts. The use of inserts makes it possible to track changes in cell cultures while freshly prepared sealers are hardening. This reflects clinical conditions, as the material is introduced into the canal immediately after mixing [5]. At the same time, the risk of mechanical damage to the cells by the material is eliminated and it is possible to assess the chemical harmfulness of the material.

While testing the unfavourable effects of various compounds on cell cultures, cell viability may be evaluated using morphological methods which enable determination of the size, shape and appearance of cell organelles [13,20,51]. Cell viability can also be determined by means of techniques evaluating the integrity of the cell membrane – release of ^{51}Cr, lactic acid dehydrogenase – [5,25] or determining proliferative abilities [21,35,42,51]. At present, the method of measuring mitochondrial activity of succinic acid dehydrogenase which informs about normal function of cells is used [10,11,20,23,31,38,47,50]. This technique is not complicated, but it is sufficiently accurate and makes it possible to obtain results quickly. Additionally, the possibility of assessing the functional condition of cells is a further advantage of this method, even without proliferation [46].

In this study, two sealers – with methacrylate (Epiphany) and calcium hydroxide (Sealapex) – were characterized by marked toxicity (> 30% cell viability).

Epiphany is a dual-cured (self-cured and light-cured) resin with a matrix of Bis-GMA (bisphenol-glycidyl methacrylate), ethoxylated-bis-GMA, UDMA (urethane dimethacrylate) and hydrophilic difunctional methacrylates, and fillers of calcium hydroxide, barium sulphate, barium glass and silica. This material can set completely without light-induced polymerization, within 30 minutes, under precisely defined conditions – at the temperature of the human body and under anaerobic conditions. With oxygen access, the bonding reaction is considerably extended, and an unpolymerized monomeric layer remains on the surface [36]. In the study, the sealer was mixed with air access, and it was not polymerized by means of the light of a polymerization lamp, so its toxic activity was probably connected with leaching of the residual monomer from the material. Beriat et al. [6] proved that the degree of Epiphany sealer conversion (transformation of monomers into polymers by changing double bonds between carbon atoms into single ones) activated by polymerization lamps was only 60% after 2 weeks following exposure to light. Moreover, it was shown that hydrophilic resins easily absorb water from the environment, which leads to plasticisation of the organic matrix, to leaching inorganic molecules and gradual degradation of the material [20,54]. Incomplete conversion of resin and their solubility in tissue fluids seem to be important factors affecting Epiphany toxicity [19,32,43,54]. Additionally, it can be derived from research conducted by Camargo et al. [13] that the exposure of pulp fibroblasts to extracts obtained from hardened Epiphany increases the formation of free oxygen radicals in cells, thus contributing to the generation of oxidative stress and impairment of their function.

There is no consensus among researchers as to the length of time of Epiphany toxicity. Some of them claim that hardened material is less harmful than freshly prepared material [27,31,33,50]. Others think that the sealer has a toxic effect on cell cultures, in the form of both paste and hard material [11,20]. It has been reported in numerous publications that the toxicity of polymethacrylate sealers increases together with a rise in their concentration in the substrate, while extending the cell exposure time to the material [2,13,22,26,45]. Brackett et al. [11] performed a long-term (6-week) assessment of the influence of root canal sealers of fibroblasts and observed high toxicity of Epiphany and other polymethacrylate preparations (RealSeal, InnoEndo) throughout the experimental period.

Sousa et al. [49], on the other hand, implanted AH Plus, EndoRez and Epiphany into the mandibular bone of guinea pigs and found that the latter was the only agent characterized by biocompatibility during the 12 weeks of the experiment. The authors assigned the beneficial effect to the calcium hydroxide content and the alkaline reaction stimulating the process of bone tissue healing.

Epiphany is introduced into the root canal after prior primer application. It has been found in numerous studies that the primer is more cytotoxic than the sealer itself, as it contains the HEMA resin, which inhibits the phases of the cellular cycle [4,10,27,33]. On the other hand, experiments consisting of the implantation of the material into the rat subcutaneous tissue showed that Epiphany polymerized by means of light was characterized by the highest damaging potential without prior primer application. According to the authors, the use of primer reduces the risk of the release of harmful components from the sealer [18].

Sealapex was another material exhibiting a very strong toxic effect towards a fibroblast culture. The results obtained correspond to the results obtained by the majority of authors, who emphasize that the cytotoxic potential of this material increases after it has hardened [14,21,27,45]. Sealapex decomposes quickly in a humid environment. This instability in aqueous solutions may be the reason for leaching harmful components from the material after mixing [23,27], in particular, salicylate resins. It was found that there existed a significant relationship between the toxicity of the preparation and the salicylates it contained [quoted according to 14]. Moreover, evidence was presented that Sealapex may have a synergistic effect with bacterial liposaccharides, stimulating the release of cyclogenase 2 – an enzyme participating in the inflammatory process of periapical tissues – by macrophages present in the inflammation zone.

Despite belonging to the same group of materials – being another calcium hydroxide sealer – Apexit did not reduce cell viability in the culture, which was also confirmed by Guertsen et al. [21]. Opinions on Apexit action in the literature are not unambiguous. According to Bojar et al. [9], this material is characterized by insignificant toxicity (over 80% cell viability), and in research using experimental animals it is even characterized by biocompatibility [7]. However, Eldeniz et al. [20] point to the problem of Apexit being highly toxic towards human gingival fibroblasts and mouse fibroblasts L929, obtained directly after binding the material and 7 days after the hardening process. Discrepancies in the result may be caused by a different preparatory technique and sample examination (direct contact method). These factors have a significant influence on the results of *in vitro* tests.

No toxic effects (< 90% cell viability in the culture) were also observed in hard samples of the other sealers under analysis: epoxide (AH Plus Jet), zinc oxide eugenol (Endomethasone N, Tubliseal) and silicon ones (RoekoSeal Automix, GuttaFlow).

The majority of authors emphasize that AH Plus has toxic action only immediately after mixing and during the setting reaction [3,23,31,33,38,44,56]. AH Plus toxicity is attributed to the transient release of formaldehyde, which is a side product of the reaction initiating the bonding process of the material and, to a lesser extent, to amines added to the preparation to accelerate polymerization

[15]. The formaldehyde release decreases during 8 hours following the paste preparation at a temperature of 37°C [30], and the cytotoxic potential of the material is reduced with time [26,41].

The problem of the harmfulness of zinc oxide eugenol sealers is mostly associated with the eugenol content, which is frequently mentioned in the literature [23,48,56]. Our own research has not confirmed this phenomenon, as both Endomethasone N and Tubliseal proved non-toxic after hardening. Similar results regarding Endomethasone N were obtained by Bojar et al. [9]. Chang et al. [14], on the other hand, reported that Tubliseal was indeed characterized by some cytotoxicity towards periodontal fibroblasts, but it caused a transient increase in the succinate dehydrogenase in fibroblasts. In the authors' opinion, it only shows the possibility of existence of a mechanism of adaptation to some irritant factors.

In the experiments described in this study, high (100%) cell viability was also found for siloxane sealers (RSA, GuttaFlow). The results of our own research are similar to those presented by other authors [9,20,31,34,38,50,56]. Al-Awadhi et al. [1] observed that, after exposure to RSA, the number of live osteoblasts in culture plates increases. Some authors claim that GuttaFlow is slightly more toxic than RSA, probably due to the addition of silver as a preserving agent [20]. Brackett et al. [11] noted that GuttaFlow initially inhibited mitochondrial activity of mouse L929 fibroblasts in direct contact; however, the toxicity of the material decreased with time.

The present research and the research performed by other authors shows that the cytotoxicity of the majority of materials is not a permanent characteristic and it decreases after they have hardened or with time [10,23,27,31,47,50], as fresh components of freshly prepared sealers have a higher diffusion ability than the hardened ones [12]. However, the harmful effect of root canal fillings, even if it lasts for a short time, may induce temporary inflammatory reactions in periapical tissues [39] and inhibit the healing process [33]. It is likely that materials exhibiting high toxicity towards cells in *in vitro* cultures will also have a negative influence on tissues under *in vivo* conditions. This extrapolation, however, is considerably simplified, as it does not take into account all factors that may affect the maintenance of the proper conditions of the apical periodontal tissue in a live organism. The results of *in vitro* tests present a general picture of the effect of endodontic materials, and they are useful in explaining their behaviour under clinical conditions.

Conclusions

The majority of root canal sealers tested after hardening were well tolerated by human gingival fibroblasts. Only two materials were characterized by high toxicity: with methacrylate (Epiphany) and calcium hydroxide (Sealapex).

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