Evaluation of cytotoxicity and pH changes generated by various dental pulp capping materials — an in vitro study

Elzbieta Luczaj-Cepowicz¹, Grazyna Marczuk-Kolada¹, Małgorzata Pawinska², Marta Obidzinska¹, Adam Holownia³

¹Department of Pedodontics, Medical University of Bialystok, Poland
²Department of Integrated Dentistry, Medical University of Bialystok, Poland
³Department of Clinical Pharmacology, Medical University of Bialystok, Poland

Abstract

Introduction. Various materials are used in direct dental pulp capping method. Their biocompatibility and alkalizing abilities are of primary importance affecting therapeutic effects. The aim of this study was to evaluate and compare the cytotoxicity of various pulp-capping materials on human gingival fibroblasts and investigate the pH changes induced by these materials.

Material and methods. Human gingival fibroblasts were cultured with nine direct pulp materials using culture plate inserts. The cytotoxic effects were recorded by using an MTT-based colorimetric assay after 3 and 24 h. In the second part of the experiment, the materials were inserted in dialysis tubes and transferred into plastic vials containing deionized water. The changes of the medium pH were measured after 3 and 24 h.

Results. We showed differences in cell viability of gingival fibroblasts after varied time of exposition for the tested materials. Cell viability after 24 h increased for Dycal, Biopulp, and Calciopro, and decreased for Calcipulp, Angelus, Angelus White, and ProRoot Regular. Cell viability for ProRoot and Life did not change. Non-setting calcium hydroxide preparations followed by the MTA group and setting calcium hydroxide materials produced the highest pH. All the tested materials significantly increased pH (p < 0.0001) at 24 h.

Conclusions. Currently used pulp capping materials varied in their cytotoxicity relative to human gingival fibroblasts and their alkalizing capacities. Since most likely pH does not affect the viability of cultured cells, further investigations are required to determine physicochemical properties of these materials and the biological activity of the dental pulp. (Folia Histochemica et Cytobiologica 2017, Vol. 55, No. 2, 86–93)

Key words: gingival fibroblasts; cytotoxicity; dental pulp capping materials; pH
showed failures due to the porosity of the produced dentinal bridge, poor adherence to the dentin and low sealing ability [6].

Hence, there have been recent attempts to develop more effective materials. One of these materials is mineral trioxide aggregate (MTA), which appears to have better effects and has proved more beneficial than previously used materials. It has been demonstrated that MTA had better sealing capability and led to less pulpal inflammation and more predictable dentin bridge formation than CH [5].

Biocompatibility of direct pulp capping materials is of prime importance since they will be in close contact with dental pulp for long periods of time [2]. The American Dental Association and International Standards Organization Committee have encouraged the use of ex vivo methods [7]. To determine cytotoxicity, various methods have been recommended, including MTT-based colorimetric assay. The method is a rapid and simple test which identifies only living cells what allows measuring cell cytotoxicity and proliferation [8, 9]. Since the therapeutic effects of direct pulp capping materials are related to their alkalinizing abilities, it is important to define the pH values of commercial products. Hydroxide ions act by stimulating alkaline phosphatase, which plays a key role in the mineralization of dentin and bone [10].

The aim of this study was to: 1) evaluate and compare the cytotoxicity of various pulp-capping materials on human gingival fibroblasts mimicking the clinical procedure, and 2) investigate the pH changes produced by these materials.

Material and methods

Cell cultures. Human gingival fibroblasts (adherent permanent cell line ATCC® CRL-2014HGF-1) (LGC Promochem, Warsaw, Poland) grew in Falcon flasks (75 cm² growth area) in the DMEM (Dulbecco’s Modified Eagles Medium) medium (Gibco, Warsaw, Poland) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 U/mL streptomycin (all from Gibco), at 37°C, 5% CO₂ and 95% humidity. When confluent growth was obtained, the cells were incubated without light in the conditions described above for 3 h (six plates) and for 24 h (next six plates). Six wells with inserts without any material constituted the control both for the 3-hour as well as 24-hour experiment.

Preparation of capping material and cell treatment. The nine materials included in this study were: MTA-Angelus white (Angelus Ind. De Productos Odontologicos LTDA, Londrina-Parana, Brazil), MTA-Angelus (Angelus Ind. De Productos Odontologicos LTDA, Londrina-Parana, Brazil), ProRoot (Densply Tulsa Dental Specialities, Johnson City, TN, USA), ProRoot Regular (Densply Tulsa Dental Specialities, Johnson City, USA), Biopulp (Chema-Elektromet, Rzeszów, Poland), Calciro (lege artis Pharma GmbH + Co., Dettenhausen, Germany), Calcipulpe (Septodont, Saint Maur des Fossés, France), Dycal (Dentsply De Tray GmbH, Konstanz, Germany) and Life (Kerr Italia S.r.l., Salerno, Italy). The main components of each material are described in Table 1. The materials were mixed directly before the test, according to the manufacturer’s instructions under aseptic conditions, except for Calcipulpe which was packaged in a syringe with no preparation needed.

The prepared materials were applied into plastic rings of 5 × 5 mm (inner diameter × height). The rings with the materials were placed in inserts (Nunc GmbH & Co. KG, Wiesbaden, Germany) of a surface area of 0.47 cm² and a pore diameter of 0.4 µm, which were located in 24-well culture plates (Nunc GmbH & Co. KG) containing human gingival fibroblasts. Twelve samples were prepared for each material. The culture plates with cells and applied materials in a freshly mixed state were incubated at 37°C, 5% CO₂ and 95% humidity for 3 h (six plates) and for 24 h (next six plates). Six wells with inserts without any material constituted the control both for the 3-hour as well as 24-hour experiment.

Cytotoxicity assay. The cytotoxicity of the studied materials was evaluated with the MTT test. It is an indirect method determining cell viability and proliferation on the basis of mitochondrial succinate dehydrogenase activity. In live cells, this enzyme reduces a yellow tetrazole salt, 3-(4,5-di- methyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide, to formazan precipitating as insoluble grey-purple crystals. The intensity of the solution color after dissolving the crystals, measured by spectrophotometer, is a measure of cell viability. For low cell survival, low enzymatic activity is found resulting in low content of purple formazan and lower optical density values [11].

After 3 h and 24 h of cell incubation with capping materials, the inserts with the materials were removed and 1 mL of medium containing 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was added to each well at a concentration of 0.5 mg/mL, and the plates were incubated without light in the conditions described above for 3 h. Afterwards, the fluid was aspirated from the culture and 1 mL of isopropanol acidified with hydrochloric acid was added. To dissolve the formazan crystals, the obtained solution was stirred for a short time. The optical density (OD) was measured with a double-beam absorption spectrophotometer, Lambda EZ 2001 (Perkin Elmer, Waltham, MA, USA), at the 560 nm wavelength. Cell viability was calculated using the following formula: [Mean OD of test group/Mean OD of control group] × 100%.

The evaluation of cell viability was scored according to the method of da Silva et al. [12]. If cell viability exceeded
90%, the material was deemed non-cytotoxic. For cell viability at the 60–90% range, the material was regarded as slightly cytotoxic. For cell viability at the 30–59% range, the material was regarded as moderately cytotoxic. For cell viability below 30%, the material was considered severely cytotoxic.

**pH assay.** All materials were prepared as mentioned above. Shortly thereafter, 0.1 g of each material was placed into dialysis tubes (Sigma-Aldrich Chemie, Steinheim, Germany) and transferred into separate plastic vials containing 20 mL of deionized water. A total of 6 samples were used for each material. The vials were hermetically sealed and kept in an incubator at 37°C.

Before each measurement, the vials were shaken for 5 sec to ensure uniform hydroxyl ion distribution. The pH values were recorded immediately after immersion (baseline) and after 3 and 24 h with a pH-meter (ISE 710A, Orion Research Inc., Boston, MA, USA), previously calibrated with solutions of known pH [13–15]. Each sample was measured twice and the mean value was recorded. The experiment was performed in static conditions (without changing the deionized water) [15]. The pH of the deionized water in which an empty tube without capping materials was immersed (control) was 6.9 at all study periods [16].

**Statistical analysis.** All statistical analyses were performed using the Statistica 8.0 (StatSoft, Cracow, Poland) software package. One-way analysis of variance, ANOVA, for independent samples was applied to compare cytotoxicity and pH of the materials at each time point. If the difference was significant, individual comparisons were performed using Tukey’s multiple comparisons test. The level of significance was set at $p < 0.05$.

**Results**

The cytotoxicity of the tested materials after 3 and 24 h of incubation is presented in Table 2. After 3 h of incubation, Angelus White and ProRoot exhibited no cytotoxicity. ProRoot Regular, Calcipulp, Life and Dycal demonstrated slight cytotoxicity.
Table 2. Cytotoxic effects of dental pulp capping materials on human gingival fibroblasts

<table>
<thead>
<tr>
<th>Material</th>
<th>Survival rate % (SD)</th>
<th>3-hour experiment</th>
<th>24-hour experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP</td>
<td>86.28 (2.23)</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>CR</td>
<td>39.81 (1.97)</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>B</td>
<td>25.69 (0.84)</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>GPMTA</td>
<td>74.94 (2.25)</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>GAMTA</td>
<td>52.00 (4.01)</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>WPMTA</td>
<td>93.17 (2.53)</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>WAMTA</td>
<td>94.93 (4.10)</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>L</td>
<td>83.73 (2.18)</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>D</td>
<td>72.35 (2.12)</td>
<td>**</td>
<td>**</td>
</tr>
</tbody>
</table>

Statistically significant differences between cell viability of gingival fibroblasts after varied times of exposition of the tested materials: 3-hour experiment: *p < 0.001; **p < 0.0001 and 24-hour experiment: *p < 0.05; **p < 0.0001 based on Tukey’s multiple comparisons test.

and Calcipro — a moderate one, and Biopulp was severely cytotoxic. After 24 hours ProRoot and Dycal were non-cytotoxic. Slight cytotoxicity was observed for Angelus White, Calcipulpe, Life, Calcipro, and moderate one for Angelus, ProRoot Regular and Biopulp. After 24 h of incubation with gingival fibroblasts cultures none of the materials demonstrated severe cytotoxicity.

The differences between cell viability of gingival fibroblasts after various times of exposition to the tested materials are presented in Figure 1. Dycal (p < 0.01), Biopulp and Calcipro (p < 0.001) significantly increased cell viability after 24 h. The viability of cells was significantly decreased for cells incubated with Calcipulpe, Angelus (p < 0.001), Angelus White and ProRoot Regular (p < 0.001). There were no significant cell viability differences for ProRoot and Life throughout the entire experiment (p > 0.05).

The pH profile of all the tested materials at 3 and 24 h is shown in Table 3. The pH of non-setting calcium hydroxide materials at 3 h varied from 11.88 to 11.99, and at 24 h from 12.21 to 12.31. The values for MTA preparations at 3 h varied from 11.20 to 11.69, and at 24 h from 11.50 to 11.87, while the pH of setting calcium hydroxide materials at 3 h ranged from 10.15 to 10.47, and at 24 h from 10.88 to 11.09. Statistically significant differences between particular materials are presented in Table 3.

The pH of all materials tended to significantly increase (p < 0.0001) at 24 h (Fig. 2).

Discussion

In this study, we evaluated the cytotoxicity and pH variation of materials used in direct contact with pulp tissue, and provided novel information on some of the
Figure 1. Mean cell viability of human gingival fibroblasts after 3 or 24 hours of exposition to all tested dental capping materials. The statistically significant differences between cell viability of gingival fibroblasts after varied times of exposition for the tested materials as compared to control cells (100% viability): *p < 0.01; **p < 0.001; ***p < 0.0001.

Table 3. Mean and standard deviation pH values at the two incubation times for all tested materials

<table>
<thead>
<tr>
<th>Material</th>
<th>pH (SD)</th>
<th>Time (h)</th>
<th>3</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP</td>
<td>11.99 (0.06)</td>
<td>12.21 (0.03)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CR</td>
<td>11.89 (0.17)</td>
<td>12.31 (0.10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>11.88 (0.02)</td>
<td>12.25 (0.02)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GPMTA</td>
<td>11.69 (0.14)</td>
<td>11.87 (0.12)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAMTA</td>
<td>11.40 (0.17)</td>
<td>11.67 (0.12)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WPMTA</td>
<td>11.58 (0.13)</td>
<td>11.76 (0.13)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WAMTA</td>
<td>11.20 (0.17)</td>
<td>11.50 (0.14)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L</td>
<td>10.47 (0.22)</td>
<td>11.09 (0.12)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>10.15 (0.30)</td>
<td>10.88 (0.23)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3-hour experiment

CP

CR

B

GPMTA

GAMTA

WPMTA

WAMTA

L

D

24-hour experiment

Statistically significant differences of pH values between particular materials in the 3-hour experiment: *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 and 24-hour experiment: *p < 0.05; **p < 0.001; ***p < 0.0001 based on Tukey’s multiple comparisons test.
biological and chemical properties of these materials. Cell culture studies of dental materials have shown previously that cell response to the material depends on various factors such as cell line and experimental period, material state, the use of inserts or material extracts [1, 2, 5].

Various methods, in vitro and in vivo, are described for the evaluation of the dental materials' biocompatibility. The in vitro methods are simple, rapid, reproducible and cost-effective. The established cell lines, which were used in this experiment, are more sensitive and provide more reproducible results than primary cell cultures [17, 18]. The possibility of introducing experimental variations of the in vitro test systems allows evaluating the cytotoxicity of dental materials. The assay adapted in the present study used the tetrazolium salt MTT to measure mitochondrial dehydrogenase activity. There are no uniform standards for cytotoxicity assays on cell cultures. We used the insert system for testing the effects of the capping materials since they are applied in the cells' medium without interfering directly with the cells which resembles the in vivo situation.

Our research scheme largely reflects clinical conditions. The materials were tested as freshly mixed materials at two different exposure times — 3 and 24 h — to observe their effects on cell survival. Most of the studies concern materials after setting [1, 7–9, 19, 20]. Only Keiser et al. evaluated the cytotoxicity of freshly mixed and set grey experimental MTA after 24 h of exposure to the two varying concentrations. They observed that the lower tested concentration resulted in increased cell viability than the higher one [21].

In the current study, we showed differences in cell viability of gingival fibroblasts after varied times of exposition (3 and 24 h) to the tested materials. Cell viability after 24 h significantly increased for Dycal, Biopulp and Calcipro, and significantly decreased for Calcipulpe, Angelus, Angelus White and ProRoot Regular. Cell viability for ProRoot and Life remained statistically equivalent throughout the entire experiment.

From non-setting calcium hydroxide materials, only Calcipulpe showed an increase in cytotoxicity over time. The other two materials, Biopulp and Calcipro, significantly reduced cytotoxicity. Unlike Calcipulpe, the latter two materials require being mixed just before use, which may result in difficulties maintaining accurate proportions and may affect the discussed parameter. Although similar in composition, setting calcium hydroxide materials had varied effects. After 24 h of observation, Life reduced cell viability in culture, while Dycal significantly increased it. This might have been caused by different components of both materials (Table 1).

MTA is available as gray and white. Not only do the two differ in composition, but some studies have shown differences in their properties [22, 23]. There is no consensus among authors in relation to the cytotoxicity of white and gray MTA. According to Al Anezi et al. [22] there were no differences in cytotoxicity between gray and white ProRoot. In our experiment, we demonstrated significant differences between two kinds of MTA, at 3 and 24 h. Gray forms of MTA demonstrated significantly greater cytotoxicity than white ones at both experimental periods. This is presumably caused by increased content of
arsenic in gray MTA, as was confirmed by Bramante et al. [23]. Arsenic is well known as an inhibiting factor of enzyme activity, also of succinate dehydrogenase which was assessed in the MTT test.

The pH range of a material is an essential physical attribute, which is associated with pulpal response [24]. All testing materials used in our study had alkaline pH. Based on the obtained results, the tested materials were divided into 3 groups in terms of their pH value. The highest pH was produced by non-setting calcium hydroxide preparations followed by the MTA group and setting calcium hydroxide materials. Numerous investigations referring to pulp capping material pH are available in the literature. However, there are no studies comparing non-setting and setting calcium hydroxide materials and also MTA cements in a single experiment. Authors using a similar methodology to ours observed the greatest release of OH ions from non-setting calcium hydroxide materials [25], which is confirmed by our results. The fast alkalizing effects of these materials is due to the direct contact of the calcium hydroxide with the environment, leading to instantaneous immediate release of hydroxyl ions [25]. On the other hand, setting materials are consisted of two pastes, which following curing process produce calcium-alkyl-salicylate chelate. Hydroxyl ions are chemically bound and are incapable to be released immediately after application [26].

The release of calcium and hydroxyl ions is the result of the formation of calcium hydroxide in materials on the basis of MTA [27]. In the present study, four MTA cements still released hydroxyl ions and maintained elevated pH, which corroborate the findings of other authors [28, 29].

Under the conditions of our study, final conclusions cannot be formulated. Currently used pulp capping materials varied in their cytotoxicity relative to human gingival fibroblast and their alkalizing abilities. Since most likely pH does not affect the viability of cultured cells, further investigations are required to determine physicochemical properties of these materials and the biological activity of the dental pulp.

Conflict of interest

The authors deny any conflicts of interest.

References


