

***In vitro* cytotoxicity evaluation of different pulp capping materials: a comparative study**

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Direct pulp capping covers the exposed surface of the pulp to maintain its vitality and preserve its functional and biologic activity. The aim of the present study was to compare the biocompatibility effects of seven different pulp-capping materials *in vitro*: Dycal[®], CalciCur[®], Calcimol LC[®], TheraCal LC[®], ProRoot MTA[®], MTA-Angelus[®], and Biodentine[®]. Using the Transwell insert methodology by Alamar blue test, we evaluated the cytocompatibility of the above mentioned materials towards murine odontoblasts cells (MDPC-23) at three different times (24, 48, and 72 h). For additional control, the cell viability at 72 hours was also assessed by MTT assay. Morphological analysis of murine odontoblasts was assessed by Confocal Laser Scanning Microscope. The results indicate significantly different biocompatibility among materials with different composition. Biodentine[®] and mineral trioxide aggregate (MTA)-based products showed lower cytotoxicity, varying from calcium hydroxide-based materials, which exhibited higher cytotoxicity. Although our findings are limited to *in vitro* conditions, the observation that Biodentine[®] caused a cytotoxic effect similar to MTA suggests that it may be considered an alternative in pulp-capping treatment, as calcium hydroxide-based materials present higher cytotoxic effects.

KEY WORDS: *Alamar blue test; biocompatibility; dental material; MTT test; murine odontoblast*

Direct pulp capping involves the application of a dental material to seal communications between the exposed pulp and the oral cavity (mechanical and carious pulp exposures) in order to protect the dental pulp complex and preserve its vitality (1). Several materials such as calcium hydroxide-based ones and more recently mineral trioxide aggregate (MTA) are commonly used for this purpose (2, 3). Calcium hydroxide is the most popular agent for direct and indirect pulp capping (4, 5). Nevertheless, calcium hydroxide has certain drawbacks, such as poor bonding to dentin, material reabsorption, high solubility, and mechanical instability. In addition, the formation of reparative dentine may not be due to the bioinductive capacity of the material but due to a defence mechanism of the pulp induced by the irritant nature of calcium hydroxide (6, 7). The high pH (12.5) of calcium hydroxide suspensions causes liquefaction necrosis at the surface of the pulp tissue with the formation of a necrotic layer at the material-pulp interface (6). Mineral trioxide aggregate (MTA) cements are therapeutic, endodontic repair calcium silicate materials (7). These materials promote the proliferation/differentiation of human dental pulp cells (8-10) and show calcified tissue-conductive activity (11, 12). Compared to calcium hydroxide materials,

MTA has an enhanced interaction with dental pulp tissue (13) with less pulp inflammation (14, 15). Several new calcium silicate-based materials have recently been developed (16-18) with the aim of improving some MTA drawbacks such as its difficult handling property (19) and long setting time (16). Biodentine[®] (Septodont) is one such material and it is claimed to be used for dental restoration in addition to endodontic indications similar to those of MTA. This agent is characterised by the release of calcium hydroxide in solution (20, 21), which when in contact with tissue fluids forms hydroxyapatite (22-24). As pulp capping materials will be in direct contact with pulp tissue for long periods of time, their biocompatibility is of particular importance. The aim of the present *in vitro* study was therefore to evaluate the possible cytotoxic effects of seven different commercially available pulp-capping materials on murine odontoblasts.

MATERIALS AND METHODS

Dental Materials

Seven pulp-capping materials were selected for this study: Dycal[®] (Dentsply), CalciCur[®] (Voco GmbH), Calcimol LC[®] (Voco GmbH), TheraCal LC[®] (Bisco), ProRoot MTA[®] (Dentsply), MTA-Angelus[®] (Angelus), and

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Biodentine® (Septodont). The components of each pulp-capping material and their manufacturers are reported in Table 1.

Odontoblast cell line culture condition

The mouse odontoblast cell line (MDPC-23) was kindly provided by Dr Jacques Eduardo (Dept. Cariology, Restorative Sciences, Endodontics; University of Michigan School of Dentistry, USA). MDPC-23 cells were cultured in DMEM medium (Biowhittaker, Italy) supplemented with 10 % foetal bovine serum (FBS), 2 % glutamine, 2 % sodium pyruvate, 1 % amphotericin, and 1 % (w/v) streptomycin/penicillin at 37 °C in 5 % CO₂ atmosphere. The cells were routinely detached using a trypsin-EDTA solution for 2 minutes at 37 °C, and resuspended in DMEM medium. For the cytotoxicity tests, MDPC-23 cells were deposited in the lower chamber of the 24-well culture plate and left for 4 hours at 37 °C before any experiment.

Cytotoxicity tests

Cytotoxicity tests were performed with the Transwell insert (Sigma-Aldrich, St. Louis, MO, USA) methodology (25). The cytotoxicity of the seven pulp-capping materials was assessed with MDPC-23 cells grown in the lower

chamber of a 24-mm diameter Transwell plate with a 0.3 mm pore size polycarbonate membrane. Each pulp-capping material (300 µL) was mixed following manufacturer's instructions onto the Transwell membrane of the inner chamber. The Transwell membrane of the inner chamber containing the pulp capping materials was then placed into the lower chamber of the 24-well culture plate containing at the bottom 5x10⁴ cells/well and incubated at 37 °C in 5 % CO₂ atmosphere for 24, 48, and 72 h, respectively. Some wells were incubated with only tissue culture media (negative control) whereas others with a 10 % dilution of 30 % H₂O₂ for 72 h (positive control). At the end of each incubation time, the cell viability was performed with Alamar blue test at 24, 48, and 72 h. The results were presented as percentage of cell viability referred at cells incubated in the absence of pulp capping materials set at 100 %. The MDPC-23 treated with H₂O₂ did not show cell viability (data not shown). Five replicates for each pulp cupping material were used for each experiment performed in duplicate. The vitality test to Alamar blue reagent acts as an indicator of cell health, determining the reducing power in order to quantitatively measure the proliferative capacity; the reagent was added in a ratio of 1:10 to the cell culture and then the cells were kept in the incubator for 3-4

Table 1 *Characteristic of tested materials*

Material	Components	LOT	Manufacturer
Dycal®	Two-paste system made of a base paste (1,3-butylene glycol disalicylate, zinc oxide, calcium phosphate, calcium tungstate, iron oxide pigments) and a catalyst paste (calcium hydroxide, N-ethyl-o/p-toluene sulphonamide, zinc oxide, titanium oxide, zinc stearate, iron oxide pigments)	120717	Dentsply Tulsa Dental, Johnson City, TN, USA
Calcicur®	Water-based calcium dihydroxide paste	1246209	Voco GmbH, Cuxhaven, Germany
Calcimol LC®	Light-curing radiopaque one-component material containing urethane dimethacrylate resin, calcium dihydroxide, dimethylaminoethyl-methacrylate, TEGDMA	1244494	Voco GmbH, Cuxhaven, Germany
Theracal LC®	Light-curing, resin-modified calcium silicate filled liner single paste containing CaO, calcium silicate particles (type III Portland cement), Sr glass, fumed silica, barium sulphate, barium zirconate and resin containing Bis-GMA and PEGDMA	1200012524	Bisco Inc, Schamburg, IL, USA
ProRoot MTA®	Powder containing calcium phosphate, calcium oxide, silica, bismuth oxide.	12001879	Dentsply Tulsa Dental, Johnson City, TN, USA
MTA-Angelus®	Powder containing type Portland cement, bismuth oxide, tricalcium silicate, dicalcium silicate, tricalcium aluminate tetracalcium aluminoferrite	24120	Angelus, Londrina, PR, Brazil
Biodentine®	Powder containing tricalcium silicate, calcium carbonate and zirconium oxide. Liquid containing water, calcium chloride (accelerator) and modified polycarboxylate	B06562	Septodont, Saint-Maur-des-Fosses, France

hours at 37 °C. The degree of fluorescence and the relative values of absorbance were then acquired by reading in a spectrophotometer (BioRad Laboratories, Hercules, CA, USA) at a wavelength of 595 nm. For further control, the percentage of murine odontoblast vitality, at 72 hours, was also assessed with the MTT assay [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)] (Sigma-Aldrich, St. Louis, MO, USA). The MTT test is a standard colorimetric assay for measuring the activity of enzymes that reduce MTT to formazan (a salt blue) in the mitochondria, giving the substance a blue/purple colour. This reaction is assessed and measured by the spectrophotometric reading of the sample, at a wavelength of 570 nm by a microplate reader (BioRad Laboratories, Hercules, CA, USA).

Confocal Laser Scanning Microscope (CLSM)

Once performed, the cytotoxicity test of the different materials, the Transwell inserts was removed and the land was eliminated from the culture plate. After washing the slides with Buffer-*TES*, 250 mL of 10 mmol L⁻¹ solution of the fluorescent dye PSVue480™ was added per well, in order to detect the presence of apoptotic cells present in culture. The loss of plasma membrane asymmetry is an early event in apoptosis, independent of cell type, resulting in the exposure of phosphatidylserine (PS) residues at the outer plasma membrane leaflet (26). PSVue reagents are a family of fluorescent probes containing a bis (zinc²⁺-dipicolylamine) group (Zn-DPA), a motif that has been found to bind with high affinity to surfaces enriched with anionic phospholipids, especially phosphatidylserine (PS) exposed on cell membranes. After 2 hours, the solution of PSVue was eliminated and the washing of the plate was carried out with abundant Buffer-*TES*. The next step involved the addition of the dye Hoechst 33342, affine to DNA for viable cells. After 15 minutes, the images were acquired using CLSM (Carl Zeiss AG, Jena, Germany).

Statistical analysis

For each pulp capping material, the mean and standard deviation were calculated. Due to the fact that the variance in the population is unknown and the number of observations per material is less than 30 we applied the T-test, after assuming the equality of the variances. The null hypothesis was that there were no differences in the number of vital cells among the pulp capping materials tested for $\alpha=0.05$. The analysis was applied for the data obtained at each time. T test for paired data was conducted to investigate the differences in the number of vital cells after 72 hours for each material. The base assumption to build the analysis was that the vitality should become lower in time; and this is the alternative hypothesis which guided this analysis for $\alpha=0.05$. $H_0: \mu_d \geq 0$ & $H_1: \mu_d < 0$, where μ_d represents the mean of the differences as shown in Table 2. The analysis was

conducted with Stata/SE 12.0 for Mac (StataCorp, College Station, TX, USA).

RESULTS

Cytotoxicity tests

Alamar blue test and MTT assay results are reported in Figure 1. With regard to Alamar blue test (A), Biodentine® showed the highest percentage of cell biocompatibility if compared to the other pulp capping materials. Biodentine® did not show difference in cell viability at the three incubation times whereas MTA-Angelus® and ProRoot MTA® (70 %) were less biocompatible at 72 h; Calcicur® showed a discrete cell biocompatibility (50-60 %) whereas Calcimol LC® and TheraCal LC® were quite cytotoxic but only at the longest incubation time (72 h). Dycal® showed the highest cytotoxic effect (10 % cell viability) among the pulp capping materials independently of the culture times. MTT test (B) confirmed the percentage ratios between the various materials and between the materials and the positive/negative controls determined with the Alamar blue test. MTA-Angelus® showed the best percentage of vitality of all. In general, even though the relationships between the various materials were similar, there was a slight increase in the mean number of cells.

CLSM

In Figure 2, the CLSM images representative of MDPC-23 cells are reported. As clearly shown, in the negative control (A) we can see the nuclei stained in blue with Hoechst, which is used to stain live cells. H₂O₂ is very cytotoxic and the cells were stained in green fluorescent with PSVue480™ reagent (B). The CLSM images obtained after incubation with different pulp capping materials confirmed the cytotoxicity results: Biodentine® (I), MTA-Angelus® (H) and ProRoot MTA® (G) were not cytotoxic, whereas Calcicur® (D) showed some cells fluorescent in green; Calcimol LC® (E) and TheraCal LC® (F) were very slightly cytotoxic if compared to the negative control (A); a few cells were observed in the presence of Dycal®, indicating a high level of cell cytotoxicity (C).

Statistical analysis

Results regarding statistical analysis are reported in Tables 2-4. As for Alamar blue test (Table 2 and 3), after 24 hours there were no differences in the number of vital cells among ProRoot MTA®, MTA-Angelus®, Biodentine® and the negative control ($P>0.05$). These results changed uniformly after 48 hours and maintained no significant differences among the materials. After 72 hours, all three materials differed from the negative control, in particular ProRoot MTA® and MTA-Angelus® gained a similar and lower number of vital cells than the control, respectively,

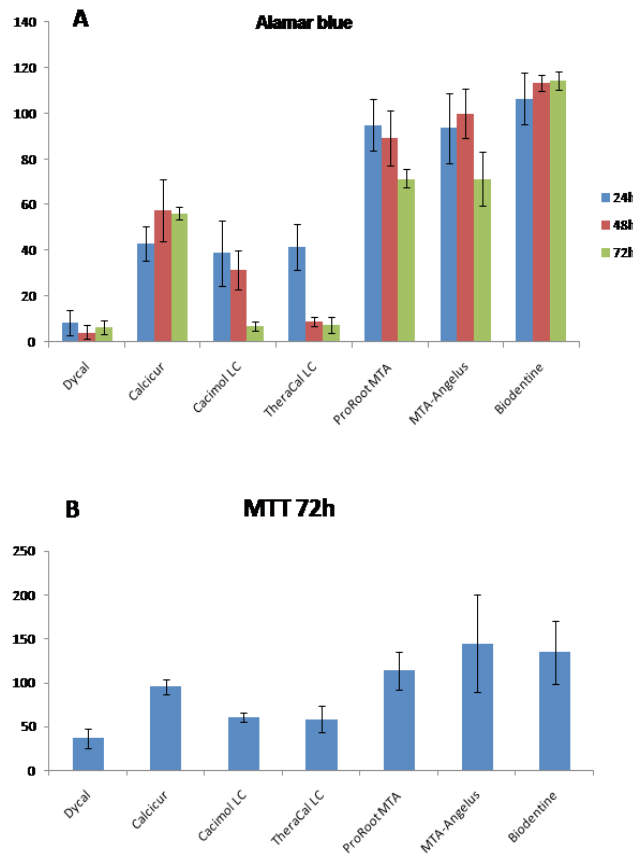


Figure 1 MDPC-23 cells viability of the different pulp capping materials by using Transwell method. The cell viability was assessed with Alamar blue (A) and MMT (B) tests. Y-axis-percentage of cell viability referred at cells incubated in the absence of pulp capping materials set at 100 %

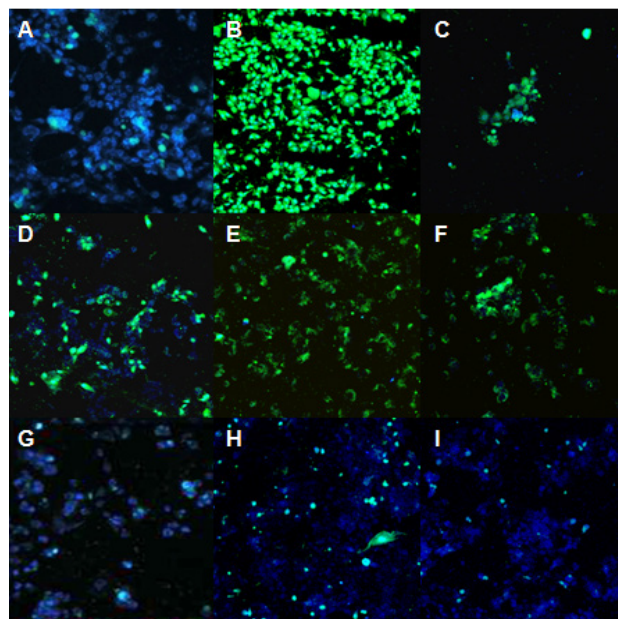


Figure 2 CLSM images of apoptosis assay. MDPC-23 cells were cultured in the absence (A) or in the presence of: H₂O₂ (B), Dycal[®] (C), Calcicur[®] (D), Calcimol LC[®] (E), TheraCal LC[®] (F), ProRoot MTA[®] (G), MTA-Angelus[®] (H), and Biodentine[®] (I)

Table 2 Mean±standard deviation of the number of vital cells for each material tested with Alamar blue

Material	24 h	48 h	72 h
Dycal®	43000±2326 ^c	22000±5740 ⁱ	30000±1868 ⁿ
Calcicur®	215000±16257 ^b	300000±40580 ^e	262000±7454 ^m
Calcimol LC®	195000±27777 ^b	165000±14107 ^b	33000±2152 ⁿ
TheraCal LC®	208000±20529 ^b	48000±8196 ^j	35000±1193 ⁿ
ProRoot MTA®	475000±53675 ^a	465000±55629 ^f	333000±33157 ^k
MTA-Angelus®	468000±72158 ^a	522000±56089 ^f	333000±59216 ^k
Biodentine®	533000±60897 ^a	592000±20182 ^f	533000±42179 ^l
Negative control	500000±0 ^a	522000±0 ^f	466000±0 ^o
Positive control*	37000±2738 ^c	25000±1850 ⁱ	18000±1332 ^p

Means with the same superscript letters are not significantly different ($P>0.05$)

* cells exposed to a 10 % dilution of 30 % H₂O₂ for 72 hours

while Biodentine® showed no decrease, as confirmed by the analysis for paired data. Calcicur®, Calcimol LC® and TheraCal LC® showed similar results after 24 hours ($P>0.05$); however, the number of vital cells varied significantly after 48 hours for Calcicur® and for TheraCal LC®. The analysis for paired data showed similar results after 72 hours for Calcimol LC® and TheraCal LC®, while Calcicur® maintained a higher number of vital cells. After 24 and 48 hours no significant differences were maintained between Dycal® and the positive control ($P>0.05$), while after 72 hours a lower biocompatibility was registered for the positive control ($P<0.05$). When the MTT test (Table 4) was applied no significant differences were recorded among ProRoot MTA®, MTA-Angelus®, Biodentine®, and the negative control ($P>0.05$). Lower values in the percentage of vitality were obtained with the remaining materials; in particular Calcimol LC® and TheraCal LC® maintained similar results ($P>0.05$) lower than Calcicur® ($P<0.05$) and higher than Dycal® ($P<0.05$).

DISCUSSION

In the last decade, many experimental and clinical studies have been carried out to develop and test new dental materials endowed with safe biocompatibility and anti-infective properties (26-28). The material used should provide an appropriate host response. This means that the tissues that come in contact with the materials should not show any toxic, irritating, inflammatory, allergic, genotoxic or carcinogenic response (29). Pulp capping materials should act as a barrier that protects the vitality of the entire pulp tissue by covering the minimal exposed tissue and by preventing further endodontic treatments. This study evaluated the cytocompatibility of the assayed materials towards a murine odontoblast cell line (MDPC-23). Although a recent study by Moodley et al. (30) reported that human pulp cell-lines showed higher sensitivity than murine 3T3 cell-lines, many authors have recently used murine odontoblasts for cytocompatibility evaluations of dental materials (31, 32). Studies using odontoblast-like

Table 3 Differences between the number of vital cells after 72 hours (μ_{72}) and the number of vital cells after 24 hours (μ_{24}) calculated for each material. μ_d represents the mean of the differences. Means with the same superscript letters are not significantly different ($P>0.05$)

Material	24 h	72 h	$\mu_d = \mu_{72} - \mu_{24}$
Dycal®	43000±2326 ^c	30000±1868 ⁿ	-13000
Calcicur®	215000±16257 ^b	262000±7454 ^m	47000
Calcimol LC®	195000±27777 ^b	33000±2152 ⁿ	-162000
TheraCal LC®	208000±20529 ^b	35000±1193 ⁿ	-173000
ProRoot MTA®	475000±53675 ^a	333000±33157 ^k	-142000
MTA-Angelus®	468000±72158 ^a	333000±59216 ^k	-135000
Biodentine®	533000±60897 ^a	533000±42179 ^l	0
Negative control	500000±0 ^a	466000±0 ^o	-34000
Positive control	37000±2738 ^c	18000±1332 ^p	-19000
μ_d			-80111

$\mu_d = \mu_{72} - \mu_{24}$

* cells exposed to a 10 % dilution of 30 % H₂O₂ for 72 hours

Table 4 Percentage of vitality registered for each material with the MTT test after 72 hours. Percentages with the same superscript letters are not significantly different ($P > 0.05$)

Material	MTT (%)
Dycal®	9.15 ^s
Calcicur®	21.56 ^t
Calcimol LC®	15.04 ^r
TheraCal LC®	14.41 ^r
ProRoot MTA®	28.15 ^q
MTA-Angelus®	35.88 ^q
Biodentine	33.27 ^q
Negative control	26.82 ^q

cells are important because odontoblasts make up the layer of cells that line the periphery of the pulp and are the first cells affected by substances that reach the pulp chamber via transdental diffusion (33). In this *in vitro* study, cell viability was recorded at three different times (24, 48, and 72 h) using the Transwell insert methodology by Alamar blue test. In accordance with our previous studies and for further control, the percentage of cell vitality was also assessed with the MTT assay at the end of the incubation period (72 hours) (34). The Transwell insert methodology is a non-direct contact test. The advantage of using a non-direct contact test for the evaluation of dental material cytotoxicity has to do with the fact that cells and materials are usually separated (25). Even though all of the materials that we tested are already available on the market and have passed safety tests, our purpose was to evaluate which material may be preferred in terms of cytotoxic effects in clinical practice use. Our results indicate certain considerable negative effects after the application of each of the materials tested, except Biodentine®, on the culture plate. As shown in Figure 1, the decrease in the number of cells in the culture plate is sizable for calcium hydroxide-based materials. Dycal® demonstrate lower rates of vitality and a strong cytotoxic capability. The remnant calcium hydroxide-based materials (Calcicur®, Calcimol LC®) showed a reduction in the percentage of cell viability after 72 h, suggesting a different rate of biocompatibility long-term. Although Cavalcanti et al. (35) reported lesser cytotoxic effects for calcium hydroxide compared to adhesive pulp-capping materials our results confirm the conclusions of other authors (36-38) regarding the non-complete biocompatibility of calcium hydroxide-based materials. Calcium hydroxide is important in protecting the pulp from thermal, mechanical, and microbiological stimuli (4) because of its antibacterial action and its property of stimulating sclerotic and reparative dentin formation. However, multiple tunnel defects and cell inclusions in bridges following pulp capping with calcium hydroxide have been demonstrated (36). This may lead to leakage and bacteria penetration into pulp tissue. Furthermore, it has equally been demonstrated that,

due to the alkalinity of its pH, calcium hydroxide induces the formation of a layer of coagulation necrosis, when it is in direct contact with the dental pulp (6). TheraCal LC® showed a dramatic decline in the percentage of cell viability at 72 h, so as to be comparable to calcium hydroxide-based materials. This result is quite surprising as the main constituent of TheraCal LC® is Portland cement, whose biocompatibility has been demonstrated in several studies (39, 40). Very different results were obtained from the analysis of the MTA-based materials (ProRoot MTA® and MTA-Angelus®). Both materials reported an excellent percentage of vitality with Alamar blue test and MTT assay after 72 h. Compared to calcium hydroxide, MTA has the ability to induce the formation of a bridge of hard tissue of greater thickness, also managing to cause less inflammation with limited pulp tissue necrosis (less caustic effect) shortly after its application (41, 42). In this study, the *in vitro* analysis regarding biocompatibility showed the best percentage of for Biodentine®'s cell viability. During the 72 hours of application of Biodentine® on the culture plate, the modifications that occurred underlined the positive trend of mitochondrial activity. Considering the interface between dentin and Biodentine® with confocal microscopy, an increased content of carbonate and the creation of a hybrid layer was demonstrated (43, 44). Furthermore, Biodentine® showed the ability to induce the differentiation of odontoblasts starting from pulp progenitor cells, forming a mineralizing matrix with the characteristics of dentin (45). In accordance with Novicka et al. (46) and Zhou et al. (47), the present study underlined that Biodentine® caused a cytotoxic effect similar to that by MTA and that it may be considered an alternative to MTA in pulp-capping treatment, differing from calcium hydroxide-based materials, which exhibit higher cytotoxicity.

Studies such as this are important since they are designed to find, among the available dental materials, those that show the highest degree of biocompatibility. This *in vitro* study reported significant differences in the biocompatibility effects of the seven tested materials. In particular, calcium-hydroxide based ones demonstrated important cytotoxic activity on murine odontoblasts. Although these findings are limited to an *in vitro* model system and cannot be directly extrapolated to *in vivo* situations, they point to the necessity of taking precautions in applying such materials in clinical practice, as they might cause potential irritations and cellular damage.

Conflict of interest statement

The authors deny any conflict of interest.

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Procjena citotoksičnosti materijala za prekrivanje zubne pulpe u uvjetima *in vitro*: usporedno istraživanje

Direktnim prekrivanjem zubne pulpe štiti se njena izložena površina kako bi se zadržala vitalnost te očuvala funkcionalna i biološka aktivnost. Cilj ovoga istraživanja bio je usporediti biokompatibilnost sedam različitih materijala za prekrivanje zubne pulpe u uvjetima *in vitro*: Dycal[®], Calcicur[®], Calcimol LC[®], TheraCal LC[®], ProRoot MTA[®], MTA-Angelus[®] i Biodentine[®]. Primjenom metode *Transwell insert*[®] i testa preživljenja stanica s bojom Alamar Blue[®] procijenili smo citokompatibilnost navedenih materijala na staničnim kulturama mišjih odontoblasta (MDPC-23) izlaganima ispitivanim materijalima u tri različita vremena (24, 48 i 72 h). Radi dodatne kontrole, nakon 72 sata proveden je i MTT-test za procjenu vijabilnosti stanica. Morfološka analiza mišjih odontoblasta provedena je konfokalnom laserskom skenirajućom mikroskopijom. Rezultati upućuju na značajno različitu biokompatibilnost među materijalima drugačijeg sastava. Materijal Biodentine[®] te proizvodi zasnovani na mineral trioksid-agregatu (MTA) pokazali su nižu citotoksičnost, bitno se razlikujući od materijala zasnovanih na kalcij hidroksidu, koji su bili više citotoksični. Iako je naše istraživanje ograničeno *in vitro* uvjetima u kojima je provedeno, naš pronalazak da je Biodentine[®] uzrokovao citotoksičan učinak sličan MTA-u upućuje na to da bi se mogao koristiti kao alternativa u prekrivanju zubne pulpe, jer je poznato kako materijali zasnovani na kalcij hidroksidu iskazuju veće citotoksične učinke.

KLJUČNE RIJEČI: biokompatibilnost; mišji odontoblasti; MTT-test; test preživljenja stanica s bojom Alamar Blue[®]; zubne ispune