Biocompatibility of a new pulp capping cement

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Summary

Aim. **The aim of the present study was to evaluate the biocompatibility of a new pulp capping material (Biodentine, Septodont) compared with reference pulp capping materials: Dycal (Dentsply), ProRoot MTA (Dentsply) and MTA-Angelus (Angelus) by using murine odontoblast cell line and Alamar blue and MTT cytotoxicity tests.**
Methods. **The citocompatibility of murine odontoblasts cells (MDPC-23) were evaluated at different times using a 24 Transwell culture plate by Alamar blue test and MTT assay.**
Results. **The results were significantly different among the pulp capping materials tested. Biocompatibility was significant different among materials with different composition.**
Conclusions. **Biodentine and MTA-based products show lower cytotoxicity varying from calcium hydroxide-based material which present higher citotoxicity.**

Key words: biocompatibility, MTT test, murine odontoblast, pulp capping materials.

Introduction

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 an attempt to act as a barrier, protect the dental pulp complex and preserve its vitality (1). Inducing hard tissue formation by pulp cells as an ultimate goal of capping material use is widely accepted (2).
Several materials such as calcium hydroxide-based materials and more recently mineral trioxide aggregate (MTA) are commonly used for this purpose (3,4). Calcium hydroxide is the most popular agent for direct and indirect pulp capping and maintaining pulp vitality, given its ability to release hydroxyl (OH) and calcium (Ca) ions upon dissolution (5,6). Both clinically and histologically it has been found to produce satisfactory results in indirect and direct pulp capping, because it is capable of stimulating the formation of tertiary dentin by the pulp. This is documented by basic research and clinical studies with reported success rates in excess of 80% for direct pulp capping (7,8). Currently, calcium hydroxide products are the best documented and most reliable materials for direct pulp capping and serve as the "gold standard" against which new materials have to be tested (9).
Nevertheless, calcium hydroxide has some drawbacks. Poor bonding to dentin, material reabsorption, high solubility and mechanical instability are among them. In addition, the formation of reparative dentine may not be due to the bioinductive capacity of the material but due to a defense mechanism by the pulp induced by the irritant nature of calcium hydroxide (10, 11); 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 (7).
Dycal (Dentsply) is a self-setting radiopaque calcium hydroxide-based material used both as a pulp capping agent and as a liner under restorations, cements and other base materials. Its toxicity to pulp cells is well documented (12, 13).
Portland cements, commonly named mineral trioxide aggregate (MTA) cements (such as ProRoot MTA, MTA-Angelus, Tech Biosealer and others), are therapeutic, endodontic repair calcium silicate materials introduced at first as a grey cement (14). These materials promote the proliferation/differentiation of human dental pulp cells (15-17) and show calcified tissue-conductive activity with the ability to encourage new hard tissue formation in terms of dentine bridge devel-

opment over the exposed pulp (18,19). Compared to calcium hydroxide materials, MTA has an enhanced interaction with dental pulp tissue (15) with less pulp inflammation and limited pulp tissue necrosis (18, 20).
Several new calcium silicate-based materials have recently been developed (21-23), aiming to improve some MTA drawbacks such as its difficult handling property (24) and long setting time (14). Biodentine (Septodont) is among these materials and it is claimed to be used as a dentine restorative material in addition to endodontic indications similar to those of MTA. This agent is characterized by the release of calcium hydroxide in solution (25, 26), which when in contact with tissue fluids forms hydroxyapatite (27-29).
As pulp capping materials will be in direct contact with pulp tissue for long periods of time, their biocompatibility is of particular importance. Several methods for the determination of biocompatibility of dental materials have been recommended, but the analysis of *in vitro* cellular reactions are generally considered to be the initial approach (30). This allows for the basic biological characterization of a material and for analysis of the underlying cellular mechanisms.
The aim of the present study is to evaluate the biocompatibility of a new pulp capping material (Biodentine, Septodont) compared with reference pulp capping materials: Dycal (Dentsply), ProRoot MTA (Dentsply) and MTA-Angelus (Angelus) by using murine odontoblast cell line and Alamar blue and MTT test.

Materials and methods

The following materials were used: Dycal, ProRoot MTA, MTA-Angelus and Biodentine. The components of each material and its manufacturer are reported in Table 1.
Dycal, a two-paste system made of a base paste and a catalyst paste (13), was prepared following the manufacturer's instructions by mixing equal amounts of catalyst paste and base paste. ProRoot MTA and MTA-Angelus, composed of white Portland cement and bismuth oxide (31, 32), were prepared following the manufacturer's instructions. Biodentine consists of a powder in a capsule and liquid in a pipette. The powder was mixed with the liquid in a capsule in the triturator for 30 seconds. Once mixed, Biodentine sets in about 12 minutes. During the setting of the cement calcium hydroxide was formed.

Odontoblast cell line culture condition

The mouse odontoblast cell line (MDPC-23) was kindly provided from Dr Jacques Eduardo (Dept. Cariology, Restorative Sciences, Endodontics; University of Michigan School of Dentistry). Odontoblast-like cell line (MDPC-23) is recommended for application to in vitro studies concerning the biocompatibility of dental materials. Mac Dougall (33), reported that the immortalized mouse odontoblast cell line is positioned in the periphery of the pulp and are the first cells affected by dental materials.
MDPC-23 cells were cultured in DMEM medium (Biowhittaker, Italy) supplemented with 10% fetal bovine serum (FBS), 2% glutamine, 2% sodium pyruvate, 1% amphotericin and 2% (w/v) streptomycin/penicillin at 37 °C in 5% CO_2 atmosphere (34). 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 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) methodology and the immortalized mouse odontoblast cell line MD-

Table 1. Characteristics of tested materials.

Material	Components	LOT	Manufacturer
Dycal	Base paste: (1,3-butylene glycol disalicylate, zinc oxide, calcium phosphate, calcium tungstate, iron oxide pigments). 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
ProRoot MTA	Powder: calcium phosphate, calcium oxide, silica, bismuth oxide. Liquid: distilled water	12001879	Dentsply Tulsa Dental, Johnson City, TN, USA
MTA-Angelus	Powder: potassium oxide, aluminum oxide, sodium oxide, iron oxide, sulfur trioxide, calcium oxide, bismuth oxide, magnesium oxide, potassium sulfate, sodium sulfate, silica. Liquid: distilled water	24120	Angelus, Londrina, PR, Brazil
Biodentine	Powder: tricalcium silicate, dicalcium silicate, calcium carbonate, calcium oxide, iron oxide, zirconium oxide. Liquid: calcium chloride, hydro soluble polymer.	B06562	Septodont, Saint-Maur-des-Fosses, France

PC-23. The advantage of using a non direct contact test for the evaluation of the dental material citotoxicity is related to the fact that cells and materials are usually separated (35).
Cytotoxicity of the four 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 (Sigma) (35). In order to standardize the samples, for the various analyzes and evaluations, the materials were placed on sterile paper disks of 0.5 cm in diameter. All test materials were prepared and mixed under sterile hood following preparation methods recommended by the manufacturer. The excess material was removed using sterile spatula. Dycal, ProRoot MTA, MTA-Angelus and Biodentine were mixed on special glass plates and later will be placed with sterile carrier on paper disks. The Transwell membrane of the inner chamber, filled with the paper disks, was then placed into the lower chamber of the 24 well culture plate each containing at the bottom $5x10^4$ cells and incubated at 37 °C in 5% CO_2 atmosphere for 24 h, 48 h and 72 h, respectively. In order to improve the search, the percentage of vitality of the cells was evaluated in three time intervals: 24, 48 and 72 hours. Some wells were incubated with only tissue culture media (negative control) whereas others with a 10% dilution of 30% H_2O_2 (positive control). The vitality was assessed by Alamar blue test. For a further control, the percentage of vitality of murine odontoblasts, at 72 hours, was also assessed with the MTT assay (bromide 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium). The vitality test to Alamar blue reagent acts as an indicator of cell health, determining the reducing power in order to measure quantitatively 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 hours at 37° C. The degree of fluorescence and the relative values of absorbance were then acquired by reading in a spectrophotometer at a wavelength of 595 nm. The MTT test is a standard colorimetric assay for measuring the activity of enzymes that reduce the MTT to formazan (a salt blue) in the mitochondria, giving the substance a blue/purple color. This reaction is assessed and measured by the spectrophotometric reading of the sample, at a wavelength of 570 nm. Five replicates for each pulp cupping material were used for each experiment performed in duplicate.

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 the buffer Buffer-TES, 250 ml of 10 mM solution of the fluorescent dye PSVue TM480 were added per well, in order to detect the presence of apoptotic cells present in the culture. Apoptosis is defined as programmed, physiological cell death and plays an important role in tissue homeostasis. 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 (36). PSVue reagents are a family of fluorescent probes containing a bis($zinc^{2+}$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. The plate was kept under gentle agitation for 2 hours at room temperature. After 2 hours, the solution of PSVue has been eliminated and the washing of the plate has been 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 confocal laser scanning microscope (CLSM) (37).

Results

Cytotoxicity tests

Figure 1 shows the results obtained with the Alamar blue test at 24, 48 and 72 hours.
The results obtained to 24 hours show that the higher percentage of cell vitality is found in Biodentine (106%), which shows an average of even greater compared to the negative control cells, while Dycal (8.6%) is the material that presents the lowest values, so as to become the minimum value of the range of vitality of the pulp-capping materials tested in research. ProRoot MTA and MTA-Angelus both show good percentage of vitality, which amounted to 95% and 93.6% respectively.
In the assessment performed at 48 hours, dissimilar results emerge between the various materials. Some of them show an improvement of the percentage of vitality; MTA-Angelus equals the number of cells of the negative control and Biodentine presents a cell vitality greater than 13% compared to control. Contrariwise Dycal and ProRoot MTA show a deterioration of the data.
In the assessment at 72 hours, the analysis of the samples show a worst general behavior of the materials which leads to a decrease in the percentage of vitality and in the average number of cells. The only exceptions are Biodentine and Dycal. Biodentine is the material with the best percentage compared to the negative control, thus demonstrating a marked biocompatibility. The average number of cells remains stable compared to the previous assessment made at 48 hours with a percentage which stabilizes at 114%. Dycal demonstrates a slight increase in the number of cells (6%), with a substantially cytotoxic behavior. ProRoot MTA and MTA-Angelus prove to have a slight negative trend, but with good percentage of vitality that are stabilized on 71% for both materials.
Figure 2 shows the results of the vitality tests performed with the MTT assay at 72 hours. The MTT test confirmed the percentage ratios between the various

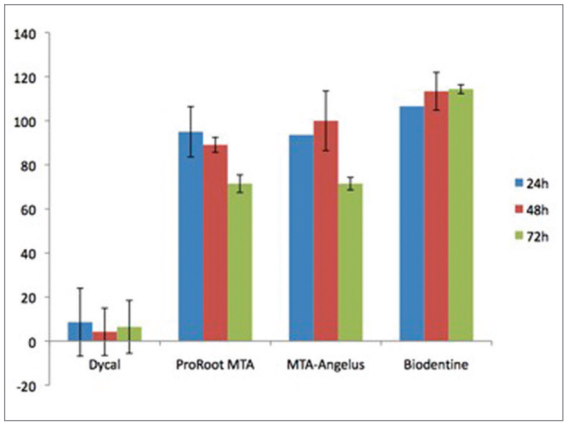

Figure 1. Alamar blue test results at 24, 48 and 72 hours.

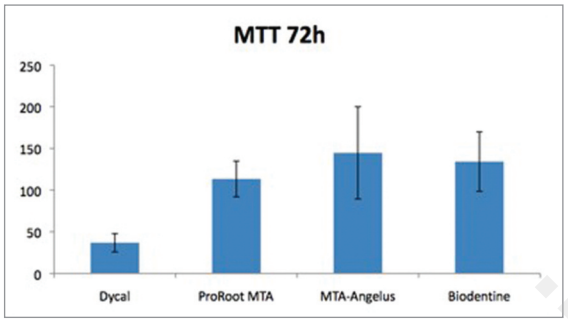

Figure 2. Results of the vitality test performed with the MTT assay at 72 hours.

materials and between the materials and the positive/negative controls determined with the Alamar blue test.. MTA Angelus shows the best percentage of vitality at all. In general even if the relationships between the various materials are similar, there was a slight increase in the mean number of cells.

Confocal Laser Scanning Microscope (CLSM) images

After staining with PSVueTM480 and Hoechst 33342 dyes, the morphological structure of the cells in culture was observed with CLSM. The use of fluorescent dye PSVueTM480 shows the presence of apoptotic cells. The Hoechst 33342 dye acts by binding to the DNA of viable cells and coloring the nucleus in blue. Figure 3 shows the negative control observations (preparation containing only the culture medium) while Figure 4 shows the positive control observations (prepared with hydrogen peroxide added to the culture medium). As clearly shown, H_2O_2 is very cytotoxic and the cells stained with PSVue480™ reagent are completely fluorescent in green (Fig. 3). In absence of any type of materials, the cells were not green fluorescent (Fig. 4) but we could see the nuclei stained with Hoechst. Figures 5 - 8 show the images acquired for each material. These images obtained after incubation with different pulp capping materials confirmed the cytotoxicity tests results: a few cells were observed in the presence of Dycal, indicating an high level of citotoxicity (Fig. 5) whereas ProRoot MTA (Fig. 6), MTA-Angelus (Fig. 7) and Biodentine (Fig. 8) did not seem to be cytotoxic.

Statistical analysis

As reported in Table 2, after 24 hours the amount of cells present in contact with MTA-Angelus is not sta-

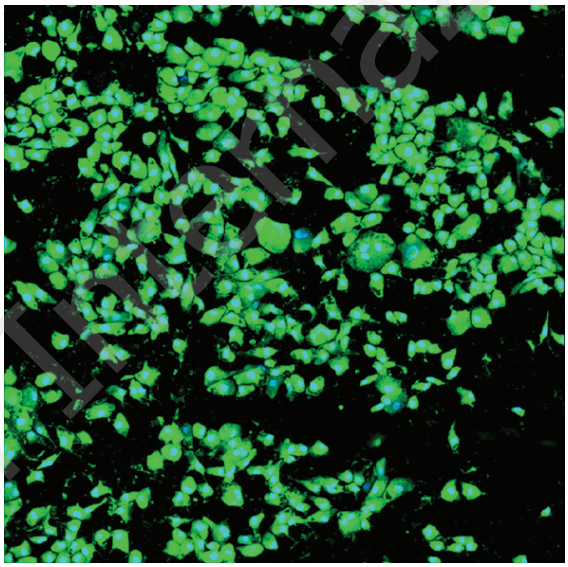

Figure 3. CLSM images of apoptosis assay in the transwell wells prepared with a 30% solution of hydrogen peroxide (positive control).

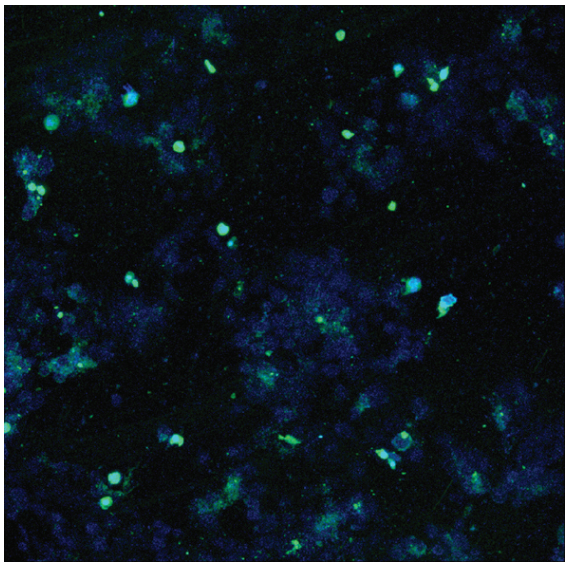

Figure 4. CLSM images of apoptosis assay in presence of culture medium only (negative control).

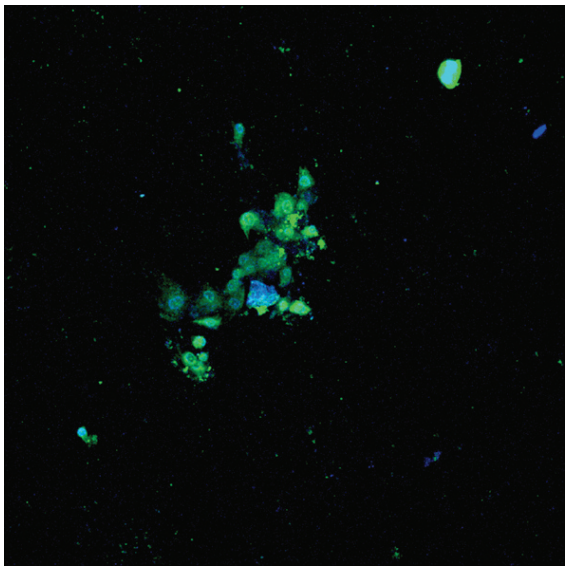

Figure 5. CLSM images of apoptosis assay in the transwell wells prepared with Dycal.

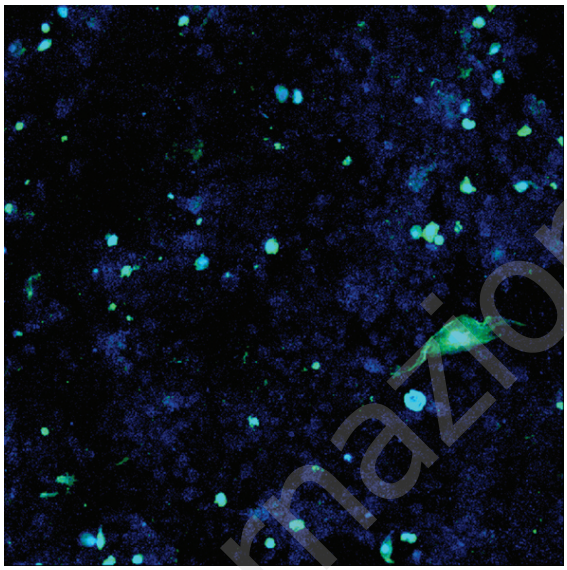

Figure 7. CLSM images of apoptosis assay in the transwell wells prepared with MTA-Angelus.

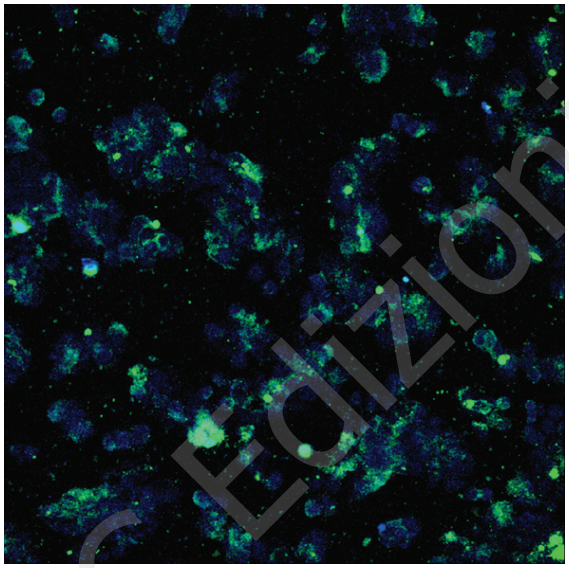

Figure 6. CLSM images of apoptosis assay in the transwell wells prepared with ProRoot MTA.

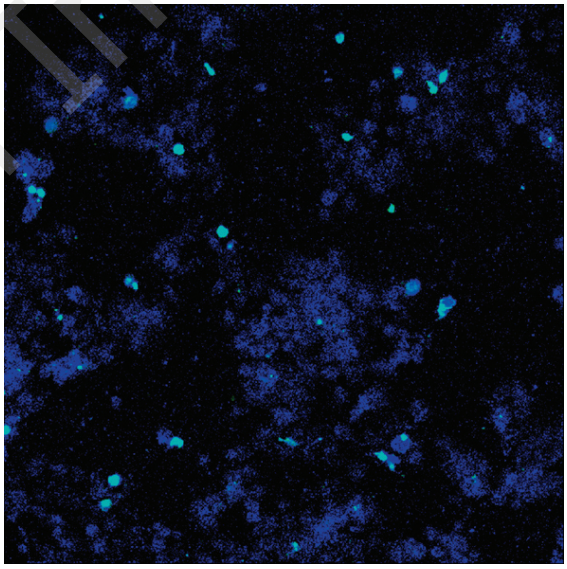

Figure 8. CLSM images of apoptosis assay in the transwell wells prepared with Biodentine.

Table 2. Mean ± standard deviation of Bonferroni post-hoc test of the different values of cell viability. Different superscript letters indicate a statistically significant difference (P <0.001). The same superscript letter indicates a not statistically significant difference (P> 0.001).

Materials	24 h	48 h	72 h
Negative control	500000 ± 0^{a}	522000 ± 0^{d}	466000 ± 0^{g}
Positive control (H_2O_2)	37000 ± 2738^{b}	25000 ± 1850^{e}	18000 ± 1332^{i}
Dycal	43000 ± 2326^{c}	22000 ± 5740^{e}	30000 ± 1868^{l}
ProRoot MTA	475000 ± 53675^{a}	465000 ± 55629^{d}	333000 ± 33157^{h}
MTA-Angelus	468000 ± 72158^{a}	522000 ± 56089^{d}	333000 ± 59216^{h}
Biodentine	533000 ± 60897^{a}	592000 ± 20182^{d}	533000 ± 42179^{f}

tistically different from the amount of cells present in contact with Biodentine, ProRoot MTA and the negative control (P> 0001). The lowest values after 24 h is provided by Dycal and the positive control.
After 48 hours no statistically significant difference is demonstrated between MTA-Angelus, Biodentine, ProRoot MTA and the negative control (P> 0.001). No statistically significant difference is also present between Dycal and the positive control (P> 0.001), where the number of live cells is significantly lower compared to the samples of the remaining materials (P <0.001).
After 72 hours Biodentine shows a significantly greater number of viable cells compared to all other materials tested (P <0.001), MTA-Angelus and ProRoot MTA show values not significantly different between them (P> 0.001) and the negative control has retained intermediate values. Finally in the positive control samples the lower number of cells was found (P <0.001).
Table 3 shows the values obtained with the MTT test. There are no statistically significant differences among ProRoot MTA, MTA-Angelus, Biodentine and the negative control (P>0.001). Dycal shows significant lower values (P<0.001).

Discussion and conclusion

Pulp capping materials should act as a barrier which protects the vitality of the entire pulp tissue by covering the minimal exposed tissue and by preventing from further endodontic treatments. Due to this fact the material used should provide an appropriate host response; this means that tissues that come into contact with the materials do not show any toxic, irritating, inflammatory, allergic, genotoxic, or carcinogenic action (38).
In the present study Dycal demonstrates the lower rates of vitality and a strong cytotoxic capability. Dycal has shown the lowest mean number of cells in the colorimetric assay performed with Alamar blue, with assessments at 24, 48 and 72 hours, and in the MTT assay at 72 hours. The low percentage of vitality of Dycal occurs already in the first 24 hours, manifesting small variations of 1-2 percentage points to the various measurement intervals. These results confirm the conclusions of others Authors (39, 40) on the non-complete biocompatibility of calcium hydroxide-based materials: the protective effect of these materials towards the pulp is not complete. Calcium hydroxide has an important action in protecting the pulp from thermal, mechanical and microbiological stimuli (5, 6) because of its antibacterial action and its property of stimulating sclerotic an reparative dentin formation. In clinical practice, the presence of hard tissue barrier after capping can be considered an asset, since it provides natural protection against the infiltration of bacteria and chemical products. However, the importance of calcified hard tissue barrier formation after capping has been challenged by other studies, which have shown multiple tunnel defects and cell inclusions in bridges following pulp capping with calcium hydroxide (41). This may lead to leakage and bacteria penetration into pulp tissue unlike the permanent seal produced by bonding agents. Furthermore it is equally demonstrated that, due to the alkalinity of its pH, calcium hydroxide induces cytotoxicity, causing the formation of a layer of coagulation necrosis, when it is in direct contact with the dental pulp (7). For both these reasons calcium hydroxide do not seem the eligible material to be used in case of exposed pulp tissue.
Very different results were obtained from the analysis of the MTA-based materials (ProRoot MTA and MTA-Angelus). Both materials to the evaluation of the 72 hours, with Alamar blue test and MTT assay, have reported excellent percentage of vitality, detected in a range that goes from 71% to 95% of vitality and in some samples the results were even assimilated to the negative control. This significant difference between the values of vitality of calcium hydroxide and MTA is clearly due to the structural difference of the two basic components and due to the various physiological and biochemical reactions induced on tissues. It has been demonstrated that MTA has the ability to induce the formation of a bridge of hard tissue of greater thickness compared to the bridge established in presence of calcium hydroxide, also managing to cause less inflammation in the adjacent tissues (18, 20). The dental pulp also contains progenitor cells and stem cells, which can proliferate and differentiate into odontoblasts forming dentin; Guven and Cehreli (42) reported that, probably, MTA is able to facilitate these cellular changes by inducing the secretion of morphogenetic proteins and growth factors such as BMP-2 and TGF-β1.
In the present study Biodentine proved to be the more biocompatible material. Biodentine, in measurements made at 24, 48 and 72 hours, reported percentage of vitality above the negative control. After 24 hours it recorded values equal to 106%, rose to 113% and 114% in the two subsequent analysis. Biodentine is a new bioactive cement based on calcium silicate for pulp capping, derivation of bioengineering, with anti-inflammatory behavior (43), different from the classic materials based on calcium silicate, such as Portland cement. The technology behind the manufacturing process of the active bio-silicate, the main constituent of Biodentine, removes the metallic impu-

Table 3. Mean ± standard deviation of Bonferroni post-hoc test of the different values of cell viability. Different superscript letters indicate a statistically significant difference (P <0.001). The same superscript letter indicates a not statistically significant difference (P> 0.001).

Materials	MTT
Negative control	124074 ± 0[a]
Dycal	45741 ± 5040[b]
ProRoot MTA	140741 ± 30098[a]
MTA-Angelus	179444 ± 99142[a]
Biodentine	166481 ± 59317[a]

rities which are present in other cements (44). The setting reaction involves the hydration of tricalcium silicate, the production of a calcium silicate-based gel and calcium hydroxide, which in contact with phosphate ions, it is able to create precipitated similar to hydroxyapatite.
Considering the interface between dentin and Biodentine with confocal microscopy, Atmeh et al. (45) showed that microstructural changes occur in this area with an increased content of carbonate at the dentin interface. These observations suggest how the intertubular spread produced by Biodentine hydration lead to the creation of a hybrid layer (46). Furthermore, histological evaluations carried out on samples prepared with Biodentine have demonstrated the ability of the material to induce the differentiation of odontoblasts starting from pulp progenitor cells, forming a mineralizing matrix with the characteristics of dentin (47).
In conclusion Biodentine has shown to be the material with the best qualities and characteristics, that are the basis of biocompatibility. Because of the lower cytotoxicity and the higher bio-inductive ability, Biodentine can be considered an ideal cement for pulp-capping. Nevertheless, especially for the new generation materials, further studies must be started to demonstrate the clinical efficacy and illustrate the actual mechanisms of action, both *in vitro* and *in vivo*.

Acknowledgments

Nothing to declare.

Conflict of interest statement

The authors of this study have no conflict of interest to disclose.

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