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# Biodentine™ is cytocompatible with human primary osteoblasts

**Miriam Zaccaro SCELZA**[a]
**Joyce Costa NASCIMENTO**[b]
**Licinio Esmeraldo da SILVA**[c]
**Vinícius Shott GAMEIRO**[d]
**Gustavo DE DEUS**[e]
**Gutemberg ALVES**[f]

[a]Universidade Federal Fluminense – UFF, Department of Endodontics, Laboratory of Experimental Cell Culture, Niteroi, RJ, Brazil.

[b]Universidade Federal Fluminense – UFF, Post Graduation Program on Dentistry, Niteroi, RJ, Brazil.

[c]Universidade Federal Fluminense – UFF, Department of Statistics, Niteroi, RJ, Brazil.

[d]Universidade Federal Fluminense – UFF, Hospital Universitário Antônio Pedro, Department of General Surgery, Niteroi, RJ, Brazil.

[e]Universidade Federal Fluminense – UFF, Department of Endodontics, Niteroi, RJ, Brazil.

[f]Universidade Federal Fluminense – UFF, Department Cell and Molecular Biology, Niteroi, RJ, Brazil.

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**Corresponding Author:**
Gutemberg Alves
E-mail: gutopepe@yahoo.com.br

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**Abstract:** Calcium silicate-based materials have been widely studied due to their resemblance to, and similar applicability of, mineral trioxide aggregate (MTA). Among these, Biodentine™ (BD) was specifically designed as a "dentin replacement" material for applications such as root perforations, apexification, treatment of resorptive lesions, and as a retrograde filling material. The present study aimed to assess the *in vitro* response of human primary osteoblasts to BD using MTA Angelus™ as a reference material, by simultaneously analyzing three different cell viability parameters, namely mitochondrial activity, membrane integrity, and cell density. BD and MTA extracts were prepared by incubation on culture media for 24 h or 42 days after mixing. Primary human osteoblasts were exposed to extracts for 24 h, at 37°C with 5% $CO_2$, and cell viability was evaluated by the XTT, NRU, and CVDE assays. Both materials induced cell viability levels higher than 70% when extracted for 24 h. However, when cells were exposed to extracts with increased conditioning times, MTA presented significant cytotoxic effects ($p < 0.05$) in comparison to the control and MTA at 24 h. After 42 days, the XTT assay identified a significant reduction in cell viability by BD when compared to the control ($p<0.05$), despite the fact that levels above the 70% viability cutoff were attained for biocompatible materials. It can be concluded that BD is cytocompatible with human primary osteoblasts, indicating its adequacy in direct contact with bone tissues.

**Keywords:** Osteoblasts; Materials Testing; Dentin, *In Vitro* Techniques

## Introduction

Calcium silicate-based materials have been widely studied due to their resemblance to, and similar applicability of, Mineral Trioxide Aggregate (MTA). Among such materials, Biodentine™ (BD, Septodont, Saint Maur des Fossés, France),[1] was specifically designed as a "dentin replacement" material for applications such as root perforations, apexification, treatment of resorptive lesions, as well as a retrograde filling material in endodontic surgery.[2] Biodentine™ is composed of a mixed powder and liquid system: the powder contains tricalcium silicate (main component), calcium carbonate (as filling material), zirconium oxide (as radiopacifier), with traces of dicalcium silicate, calcium oxide, and iron oxide; the liquid phase of BD consists of a water-soluble polymer solution (water-reducing agent), using calcium chloride to decrease the setting time.[3]

Biodentine was described as a bioactive dentin substitute with apatite formation after immersion in phosphate solution,[4] as the elemental uptake of Ca and Si into root canal dentin was found to be more prominent for Biodentine than for MTA.[5] Even though tricalcium silicate appears to be a common component of both MTA and Biodentine, X-ray diffractometry of unhydrated cements revealed that Biodentine consists of a triclinic form of tricalcium silicate, while MTA consists of the monoclinic form.[6] In comparison to other similar cements, Biodentine contains 30%-50% alumina (while Portland has less than 5%),[7] which produces an acid-resistant feature that may be useful in infection-laden sites of the human body, where the pH value drops significantly. Moreover, due to its biological properties as acid-resistant cement, it can be used as a root repair material.[2]

Biodentine is reported as presenting better biological properties than other tricalcium silicate cements such as MTA. A previous study has shown that the dynamic interaction of Biodentine with the dentin and pulp tissue interface stimulates pulp cell recruitment and differentiation, upregulates transformation factors (gene expression), and promotes dentinogenesis.[7] Other studies have shown that Biodentine was non-cytotoxic and non-genotoxic to pulp and gingival fibroblasts,[8] as well as to murine fibroblasts cultivated on a 3D model.[9] However, regarding the use of Biodentine in procedures such as root repair, involving contact with surrounding bone-related cells, its cytocompatibility remains to be evaluated. Therefore, in the present study, the purpose was to assess the *in vitro* response of human primary osteoblasts to Biodentine, employing a widely studied material (MTA Londrina, PR, Brazil) as a reference material, by simultaneously analyzing three different cell viability parameters, namely (i) mitochondrial activity, (ii) membrane integrity, and (iii) cell proliferation after indirect exposure to both materials.

## Material and methods

This work is part of a project approved by the Research Ethics Committee of the Antonio Pedro Hospital/Fluminense Federal University. No human beings or animal subjects were directly involved. The methodology was based on international standards for the evaluation of biocompatibility of dental materials (ISO 7405:2008),[10] including however a multiparametric cytotoxicity approach that investigates different cell viability parameters (cell density, metabolism, and membrane integrity), which contribute to a wider detection of toxicity.[11]

### Sample preparation

Biodentine™ (BD) (Septodont, Saint Maur des Fossés, France)[1] and MTA (Angelus, Londrina, PR, Brazil) were prepared as described in the manufacturers' instructions (Table 1). Test samples consisting of conditioned media were obtained according to ISO 7405:2008.[10] Briefly, 0.2 g of each tested material (BD or MTA) was mixed with 1 mL of serum-free culture media (D-MEM, Gibco, Cergy-Pontoise, France) and incubated for either 24 h or 42 days at 37 °C in a moisture chamber. Conditioned media containing latex fragments or dense polystyrene (PS) beads produced under the same conditions were employed as positive and negative controls for cytotoxicity, respectively.

Biocompatibility was assessed *in vitro* according to ISO 7405:2008[10] for the evaluation of dental materials, using a multiparametric assay kit (In-Cytotox, Xenometrix, Germany), which evaluates three different cell viability parameters sequentially on the same cell culture.

### Cell culture

Primary human osteoblasts (hOB) at the second passage were obtained from the collection of the Clinical Research Unit-HUAP and grown in cell culture bottles (Corning, NY, USA) in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Cergy-Pontoise, France) in the presence of 10% Fetal Bovine Serum (Gibco, Cergy-Pontoise, France), without antibiotics, at 37 °C in a 5% $CO_2$ atmosphere, until confluence was reached. Cells were then trypsinized and subcultured for the cell viability assays.

For the cell viability assay, hOB cells were subcultured at 37 °C / 5% $CO_2$ onto 96-well culture plates (Corning, NY, USA) at an initial cell density of 10,000 cells per well for 24 h, in order to achieve an 80% confluence. Subsequently, the culture media were removed by plate inversion and each well

**Table 1.** Description of the tested materials, according to manufacturers.

| Product and manufacturer | Composition | Brief preparation mode |
|---|---|---|
| MTA Angelus ™ | $SiO_2$ $K_2O$, $AL_2O_3$, $Na_2O$, $Fe_2O_3$, $SO_3$, CaO, | - |
| (Angelus PR Brazil) | $Bi_2O_2$, MgO, crystalline silica, CaO, $KSO_4$, $NaSO_4$ | The components are combined by mixing the powder into distilled water. |
| Biodentine™ | Tri-calcium Silicate (C3S), Di-calcium Silicate (C2S) | |
| (Septodont, Saint-Maur-des-Fosses,France) | Calcium Carbonate and Oxide, Iron Oxide | The components are combined by mixing the powder into Liquid (Calcium chloride, Hydrosoluble polymer) |
| | Zirconium Oxide, Calcium chloride, Hydrosoluble polymer | |

received one of the conditioned media described previously (BD, MTA, positive and negative controls), being incubated for another 24 hours (as stated in ISO 7405:2008). One additional group (experimental control) was exposed only to the culture medium. Each condition was tested with three biological and five technical replicates.

## Multiparametric *in vitro* assay

Biocompatibility was assessed *in vitro* using a multiparametric assay kit (In-Cytotox, Xenometrix, Germany), which evaluates three different cell viability parameters sequentially on the same cell culture, as described below.

### Mitochondrial dehydrogenase activity

After exposure of hOB to each conditioned medium, mitochondrial dehydrogenase activity was measured by the XTT assay. This test is based on the ability of mitochondrial enzymes from metabolically active cells to reduce 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) molecules to a water-soluble formazan salt, detectable by its Optical Density (O.D) at 480 nm, as measured by a UV-Vis microplate reader (Synergy II, Bio Tek, Winooski, VT, USA).

### Membrane integrity

The same cells subjected to the XTT test were washed with a washing solution provided with the commercial kit (containing phosphate buffer in saline solution) and assayed with the Neutral Red Uptake (NRU) test, which determines the levels of viable cells through their membrane integrity. NR is incorporated through endocytosis and accumulates preferably in the lysosomes of membrane-intact viable cells. After 3 h of exposure to the dye, the cells were fixed and the NR was extracted and measured by the OD of the supernatant at 540 nm, which is directly related to the proportion of viable cells.

### Cell Density

Cell density was measured in each well according to the Crystal Violet Dye Exclusion Assay (CVDE). After performing the NRU test, the cells were washed in PBS, exposed to crystal violet dye for 10 minutes, washed four times with PBS, and the dye extracted with an extraction solution (containing methanol and acetic acid). The OD at 540 nm was directly related to the amount of DNA and, therefore, to the density of cells in each well.

## Statistical analysis

The normality of the data was assessed by the Shapiro-Wilk test. The differences across groups and experimental times were analyzed by the non-parametric Kruskal-Wallis test, while Dunn's post-test was used to compare all groups. The F statistics, at a significance level a = 0.05, indicated the factors and their interactions accountable for the statistically significant differences. The GraphPad Prism 5 (GraphPad, La Jolla, CA, USA) software and Excel were used as statistical support.

## Results

Figure 1 shows cell viability measured by XTT (A), NRU (B), and CVDE (C) assays after exposure to Biodentine or MTA extracts for 24 h or 42 days, and expressed as percentage of control (cells exposed to unconditioned medium). Both positive and negative controls behaved as expected (high and low cytotoxicity, respectively), indicating the adequacy of the extraction/exposure protocol.

As illustrated inpanels A, B, and C, both Biodentine and MTA presented cell metabolism, integrity, and cell density levels statistically equivalent to those of the control group during the first 24 hours, as measured by the three parameters. Even though the mean cell survival measured by the NRU assay (Figure 1B) apparently attains lower levels for MTA, as compared to Biodentine, no statistical significance was found. However, when cells were exposed to extracts with an increased conditioning time (42 days), MTA presented significant cytotoxic effects ($p<0.05$) in comparison to the control and MTA at 24 hours, with cell viability levels lower than 50% of the control in the three tested parameters. There was no statistical difference for Biodentine between 24 hours and 42 days, even though the XTT assay identified a 20% decrease in cell survival at 42 days when compared to the control ($p<0.05$). Nevertheless, cell survival remained above the 70% cutoff for biocompatible materials in such conditions.[10]

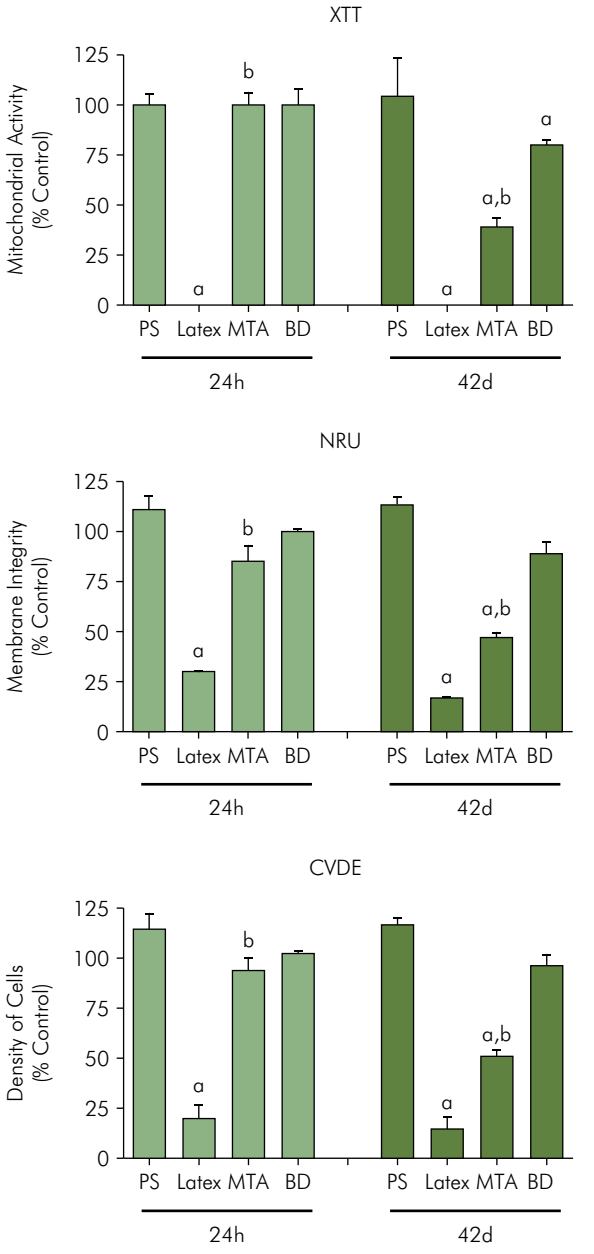


**Figure 1.** Cytotoxicity assay. Cytotoxic effects of test materials (Biodentine - BD and MTA Angelus) on human osteoblasts by XTT (A), Neutral Red Uptake (NRU) (B) or Crystal Violet Dye Exclusion (CVDE) (C) assays, expressed as a percentage of the control (cells exposed to extract media). Bars indicate mean ± standard deviation. The letter (a) indicates significant difference from the control group ($p < 0.05$). The letter (b) indicates significant difference between experimental times ($p < 0.05$).

## Discussion

Biodentine™ is a dentin substitute for use in pulp capping treatment, which may find several applications in endodontics. Therefore, the evaluation of its biocompatibility has been considered of great relevance in several different *in vitro* and *in vivo* models.[8,9,12,13,14,15] Actually, studies have assessed the *in vitro* cytocompatibility of Biodentine using immortalized cell lineages of different tissues or mammal origins, including three-dimensional spheroid pulp cell culture[15] or osteosarcoma cells,[12,13] with interesting results pointing to the biocompatibility of this material, indicating its capacity to induce proliferation and mineralization *in vitro*.[13] Nevertheless, these results still require some level of extrapolation either from animal-to-human models, whose limitations have been constantly discussed in light of the 21st century toxicology paradigms,[16] or extrapolation from aneuploid/immortalized cell lineages to the unaltered primary human cells found in healthy

tissues. In this regard, studies[17,18] have confirmed that, even though many similarities might be found between human primary cells and the main studied osteoblast-like cell lines, such as MC3T3, MG63, or SAOS2, differences in their response to biomaterials point to the importance of primary cell studies. In this scenario, the purpose of the present study was to demonstrate the cytocompatibility of Biodentine with primary human osteoblasts, by employing a standardized model and assessing three different *in vitro* methodological parameters.

The present work investigated cells of osteoblastic origin since it focused on the link between the fields of endodontics and dental traumatology. In this way, the exposure of human osteoblastic cells might shed some light on those situations in which Biodentine is in direct contact with the periodontal ligament. Indeed, these cells are more related to the root canal system. The relevance of the use of a satisfactory cell model employing human primary cells for *in vitro* studies of endodontic materials has been already pointed out previously.[19] It is important to note that the human primary osteoblast-like cells employed in this work also presented reliable and predictable results in previous studies on endodontic materials.[11,20] Finally, primary cells are also considered to provide a better simulation of *in vivo* events,[21] resembling more closely the behavior of cells in their tissue environment than transformed cell lines.[22]

Cell viability was evaluated with an interesting three-step multiparametric model, where three different parameters are evaluated sequentially in the same samples: (A) mitochondrial metabolism and respiratory toxicity, (B) lysosomal integrity and membrane permeability, and (C) the presence of DNA and cell proliferation. This method increases the chance of detection of cytotoxic effects, allows the correlation of different parameters, and sometimes provides evidence for the mechanisms of toxicity.[11] In order to assess the adequacy of the extraction methodology, latex fragments were employed as a positive control of well-known toxicity, and polystyrene beads were used as a recognized biocompatible material (negative control). The results show that both controls behaved as expected, since polystyrene presented lower viability levels similar to those of the experimental control (cells exposed only to the culture medium), and latex promoted high levels of cytotoxicity. As previously described for latex, mitochondrial activity is more impacted than cell density and membrane integrity, even though all parameters remained under 25% of cell survival when compared to the control.[23]

This multiparametric methodology confirmed that Biodentine is cytocompatible with human bone cells, even when challenged with long-time extractions of possible toxicants. It is not easy to compare the results of different cell culture experiments due to the numerous variations in experimental conditions, such as cell type, cell material contact method, and exposure time. According to the methods reported in ISO 7405:2008[10] for the *in vitro* evaluation of dental materials, there are basically two approaches to the *in vitro* evaluation of the cytotoxicity of such type of cements, either with the material being placed directly in contact with cells, or by analyzing the material liquid extract after its incubation in physiological medium.[24,25] In the current study, the quantitative indirect exposure test usually recommended in the above-mentioned international standard for material testing[10] was chosen, including longer extraction time (42 days). Previous studies reported that the time of extraction can impact on the level of cytotoxicity presented by dental materials and medical devices.[26,27] The shorter extraction time of 24h corresponds to the early biological impact that might characterize the setting process and its adverse effects. The extraction time of 42 days, on the other hand, assesses the hypothesis that cumulative effects may arise from further release of toxic components several days after contact with biological medium. It is important to notice that the extraction protocol limited the material to the same liquid volume for 42 days. Even though it is able to detect a cumulative release, this extraction does not perfectly emulate the possible removal of toxic agents by diffusion to surrounding tissues and blood vessels. In this regard, recent studies have offered suggestions on how to simulate this process, to some extent, including the continuous washing of test material during longer extraction times.[28] Therefore, it is possible that the reduction in cell metabolism induced by Biodentine at 42 days, detected in the present study by the XTT assay, might be related to an overestimation induced by the restrictive culture conditions of the assay.

Similarly to the present results, a previous study[12] employing human MG63 osteosarcoma cells in direct contact with calcium silicate materials described high biocompatibility for both Biodentine and MTA after a 24-hour exposure. However, after 3 or 5 days of growth on the cement surfaces, both materials induced a reduction in cell viability and were rated as slightly cytotoxic. Even though those results might appear in contradiction with the present findings for Biodentine, where no cytotoxicity was identified, it is important to notice that the present work ruled out the possible release of toxicants into biological media by Biodentine even at longer extraction times, but did not report surface-related effects such as those identified in the previous study.[12] Another recent work[13] has shown dose-dependent effects on cell metabolism, as measured by a MTT assay, for both MTA Plus (Avalon) and Biodentine with Saos-2 osteosarcoma cells, while at the same time both materials induced cell proliferation and mineralization, reinforcing the relevance of assessing different biocompatibility parameters, such as cell density/proliferation (CVDE) and mitochondrial metabolism (XTT). In fact, in spite of the differences in the cell model (tumoral versus primary bone cells), these results are complementary to the understanding of bone cell response to Biodentine. In this sense, it is important to notice that our results are in accordance with *in vivo* animal studies reporting the absence of long-term negative biological responses to Biodentine,[29,30] as well as with clinical evidence of its efficiency in long-term assessments after pulpotomy.[14]

Therefore, the present results reinforce the need to perform cytotoxicity assessments of dental materials including primary human cell models for safe extrapolation of results to the clinical environment. However, one of the main limitations of this study lies in the fact that it focused only on cytotoxicity, an important factor that, however, represents only the first step in the assessment of material biocompatibility. Therefore, these results should be complementary to further *in vivo* and clinical studies, necessary to confirm the adequacy and advantages of the use of Biodentine in diverse applications, such as an indirect pulp-capping material.[31] In fact, further *in vitro* assessments may also be performed, aiming to contribute to the understanding of the underlying mechanisms of Biodentine biocompatibility in its broader sense, including the use of the human primary cell model in the investigation of cell proliferation, differentiation, and mineralization, along with the study of protein expression and release of cytokines and growth factors, as well as other *in vitro* assays for assessment of bone cell behavior.

## Conclusion

The results indicate that Biodentine™ is non-cytotoxic to human primary osteoblasts, suggesting its safety for applications involving contact with adjacent bone tissue.

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