Evaluation of the genotoxicity and mutagenicity of Ca$_3$SiO$_5$-based cement

Abstract: Ca$_3$SiO$_5$ is new cement based on the composition of Portland that has been developed to have superior physicochemical and biological properties. In a clinical evaluation, the cement did not appear to have cytotoxic properties and allowed for the proliferation of pulp cells and gingival fibroblasts. However, no previous studies have evaluated the genotoxicity or the mutagenicity of Ca$_3$SiO$_5$ in vivo. Therefore, the goal of this study is to evaluate the genotoxic and mutagenic potential of Ca$_3$SiO$_5$-based cement in vivo. Twenty-four male Wistar rats were divided into 3 groups (n = 8). Group A rats received subcutaneous implantation of Ca$_3$SiO$_5$ in the dorsum. Group B rats received a single dose of cyclophosphamide (positive control). Group C rats received subcutaneous implantation of empty tubes in the dorsum (negative control). After 24 hours, all animals were euthanized and the bone marrow of the femurs was collected for use in the comet assay and the micronucleus test. The comet assay revealed that the Ca$_3$SiO$_5$ group had a tail intensity of 23.57 ± 7.70%, the cyclophosphamide group had a tail intensity of 27.43 ± 7.40%, and the negative control group had a tail intensity of 24.75 ± 5.55%. The average number of micronuclei was 6.25 (standard deviation, SD = 3.53) in the Ca$_3$SiO$_5$ group, 9.75 (SD = 2.49) in the cyclophosphamide group, and 0.75 (SD = 1.03) in the negative control group. There was an increase in the micronuclei frequency in the Ca$_3$SiO$_5$ group compared to that of the negative control group (p < 0.05). Our data showed that exposure to the Ca$_3$SiO$_5$-based cement resulted in an increase in the frequency of micronuclei, but no genotoxicity was detected according to the comet assay.

Keywords: silicate cement; genotoxicity; mutagenicity; biomaterial.

Introduction

Biodentine is a recently developed tricalcium silicate cement (Ca$_3$SiO$_5$) based on the composition of Portland cement. The goal of Biodentine creation was to achieve greater physicochemical and biological properties than those of other formulations. This cement is intended to replace damaged dentin, as in cases of pulp capping, perforation repair, retrograde filling, apexification, and temporary coronal sealing.

The product consists of a powder (tricalcium silicate, calcium carbonate and zirconium oxide as a radiopacifier) and a liquid (containing calcium chloride and a water-soluble polymer) that are mixed together to form the cement.

There are a few studies that have investigated the properties of Biodentine. The cement was determined not to be cytotoxic and was thus allowed for...

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use to stimulate the proliferation of pulp cells and gingival fibroblasts. Mori et al. evaluated the in vivo biocompatibility of Biodentine and concluded that this cement was not an irritant when in contact with the subcutaneous tissue of rats. Silva et al. analyzed the Biodentine in contact with cells and demonstrated the cytocompatibility of Biodentine in a 3D cell culture model associated with an in situ root-end filling model.

Several studies have shown that Ca$_3$SiO$_5$ applied directly to the pulp induces the synthesis of reparative dentin, most likely due to changes in the secretion of transforming growth factor (TGF)-beta 1 by the pulp cells. Therefore, Ca$_3$SiO$_5$ is considered to be a good substitute for dentin in restorations and is more resilient to shear force than other cements. In another study, this material was shown to induce an inflammatory response upon initial contact with tissue, but later demonstrated biocompatible acceptance after 2 weeks.

In addition to being biocompatible, an optimal dental material should not be cytotoxic, genotoxic, or carcinogenic. The material should possess biological and chemical stability, mechanical strength, and elasticity and have a suitable coefficient of thermal expansion. Thus, the clinical success of a material depends not only on its physicochemical properties but also on its biological safety.

The comet assay, or single-cell gel electrophoresis (SCGE), was first introduced in 1984 for the direct visualization of DNA damage in single cells. This assay is a valuable method for detecting mutations and defects in the repair of the genetic material of any eukaryotic cell. The micronucleus test is a widely used tool for assessing the mutagenicity of many substances, and this assay provides results that are suitable for statistical analysis. This test is used to evaluate the ability of a substance to break chromosomes (referred to as clastogenicity) or to affect the formation of the mitotic metaphase plate and/or spindle, both of which can lead to the unequal distribution of chromosomes during cellular division.

The micronucleus testing allows for the estimation of the induced amount of chromosome and/or genome mutations, whereas the comet assay permits the direct measure of the DNA-strand breaking capacity of a chemical agent.

There is a lack of studies in the literature evaluating the genotoxic and mutagenic potential of Ca$_3$SiO$_5$ in vivo. Therefore, the goal of this study was to evaluate the effects of Ca$_3$SiO$_5$ using the micronucleus test and the comet assay in vivo.

**Methodology**

**Approval by the Research Ethics Committee**

This study was approved by the Ethics Committee on Animal Use at the University of Oeste Paulista (CEUA - UNOESTE) (Protocol 1450).

**Animal Protocol**

This study was conducted with 24 8-week-old male rats (Rattus norvegicus albinus, Wistar) weighing 170-210 g. The rats were kept in individual cages and were maintained in a controlled temperature of 25±2°C, a relative humidity of 50±15%, and a normal photoperiod (12-12 h light-dark cycle). All animals received solid feed, except during the first twelve preoperative hours, and water ad libitum.

To perform the surgery, the animals were anesthetized with an intramuscular injection of ketamine hydrochloride (Dopalen Sespo Indústria e Comércio Ltda, Jacareí, SP, Brazil) and xylazine hydrochloride (Anasedan Agribrands do Brasil Ltda., Jacareí, SP, Brazil) at a dose of 0.05 mL/100 g body weight for each drug. After this, the animals were divided into 3 groups:

a. **Group A**: experimental group (n = 8): Polypropylene tubes containing Ca$_3$SiO$_5$ measuring 2 mm in diameter x 10 mm in length were subcutaneously implanted into the dorsum. After preparing the Ca$_3$SiO$_5$ (Biodentine™, Septodont, St-Maur-des-Fossés, France) according to the manufacturer’s recommendations, the matrix was filled with this material. After the initial curing of the material, the specimen was introduced into the subcutaneous tissue.

b. **Group B**: positive control (n = 8): A single dose of cyclophosphamide (Genuxal, Baxter Oncology GmbH, Halle/Westfalen, Germany) (50 mg/kg) was subcutaneously injected on the first day of the experiment.

c. **Group C**: negative control (n = 8): Polypropylene tubes measuring 2 mm in diameter x 10 mm in length were subcutaneously implanted into the dorsum.
The number of animals per group (n = 8) was based on the study of Hayashi et al., which established a minimum of 4 animals per study group.

Prior to the subcutaneous implantation of the tubes, the animals from groups A and C were subjected to trichotomy and antiseptic with 0.12% chlorhexidine gluconate (PerioGard®, Colgate Palmolive, São Paulo, SP, Brazil). Thereafter, the dorsal region was cleaned with gauze that had been moistened with saline solution to remove any chlorhexidine residue.

One surgical incision was made in the median region of the dorsum with a 15 blade (Embramac Import Export, São Paulo, Brazil). Lateral to the incision, the cutaneous tissue was pinched, and the tissue was dissected using blunt-ended scissors to introduce the tubes. The incisions were sutured using 4-0 nylon (Ethicon Johnson & Johnson, São Paulo, SP, Brazil).

All animals were euthanized 24 hours after the administration of Ca₃SiO₅, as recommended by Hayashi et al. Euthanasia was performed using CO₂ inhalation in a gas chamber (Indústria Beiramar, São Paulo, Brazil). Immediately after euthanasia, both femurs were removed from each animal and sectioned at both ends to collect the bone marrow.

### Comet assay

Material from the bone marrow of the right femur from each rat was collected. The number of cells per mL was determined by placing 10 µL of the solution of bone marrow cells into a Neubauer chamber and applying the formula C = [(n / 5) x 25] / 10⁻⁴ (C = cells / ml; n = average number of cells in each square of the diagonal from the top to the bottom of the Neubauer chamber; 5 = number of squares diagonally; and 25 = total number of squares).

To analyze the cell viability, a solution of 10 µL of bone marrow cells was mixed with 10 µL of Trypan Blue (Thermo Scientific, South Logan, Utah - USA). A total of 200 cells per group were counted. The minimum viability detected was 81%. The minimum viability recommended for the comet assay is 75%, so no samples were excluded.

The alkaline version (pH > 13) of the comet assay was conducted according to the method described by Singh et al.

A 10-mL (≈ 10,000 cells) sample of the suspension of bone marrow cells was embedded in 120 mL of 0.5% agarose (Invitrogen, Carlsbad, CA, USA) with a low melting point and spread on a microscope slide coated with normal 1.5% agarose (Invitrogen, Carlsbad, CA, USA). Then, the slides were immersed overnight in a lysis solution [2.5 M NaCl, 100 mM EDTA (ethylenediaminetetraacetic acid; Invitrogen, Carlsbad, CA, USA), 10 mM Tris (Invitrogen, Carlsbad, CA, USA), 1% salt sodium N-lauryl sarcosinate pH 10 (Sigma-Aldrich Co. LLC., USA) with 1% Triton X-100 (JT Baker, Phillipsburg, NJ, USA) and 10% DMSO (Dimethylsulfoxide; Mallinckrodt, Phillisburg, NJ, USA)] at 4°C. After this incubation, the slides were immersed in PBS (phosphate-buffered saline - Sigma-Aldrich Co. LLC.; EUA) without Ca++ or Mg++ for 5 minutes for homogenization of the agarose. Next, the cells were exposed to an alkaline buffer [1 mM EDTA (Invitrogen, Carlsbad, CA, USA) and 300 mM NaOH, pH 13.4 (Merck, USA)] at 4°C for 40 minutes. Electrophoresis was performed in the same solution at 4°C for 30 minutes at 25 V and 300 mA. After electrophoresis, the slides were neutralized (0.4 M Tris-HCl, pH 7.5), fixed in absolute ethanol, and stored. Subsequently, the slides were stained with 50 mL of SYBR Green (1:10,000) (Invitrogen, Carlsbad, CA, USA) and analyzed with a fluorescence microscope coupled to an image analysis system (Comet Assay IV; Perceptive Instruments, USA). Two slides were analyzed per sample, representing 100 cells (50 nucleoids per slide), at a magnification of 400x. The parameter used for the analysis was the average of the “tail intensity”. According to the manufacturer (Perceptive Instruments, USA), “tail intensity” is defined as the percentage of DNA in the comet tail. This parameter reflects the level of DNA damage in each cell. Of the nine parameters provided by the software, this parameter is recommended by international guidelines.

As a positive control for each run of electrophoresis, we used lymphocytes that were isolated from healthy volunteers and then treated with 100 µM hydrogen peroxide for 30 minutes to induce DNA damage.

### Micronucleus test

Bone marrow samples were collected from the left femur of each rat, and two sample slides were
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Prepared per animal¹⁷. The slides were stained with Giemsa stain (Dolles, São Paulo, Brazil). Two thousand polychromatic erythrocytes (1000 per slide) were counted for each animal at a magnification of 400x using an optical microscope to determine the number of micronuclei¹⁷. Micronuclei were defined as structures with probable halos surrounding the nuclear membrane and a volume of less than one-third of the diameter of the associated nuclei. The staining intensity of the micronuclei was similar to the staining intensity of the associated nuclei, and both structures were observed in the same focal plane²². The slide analysis was performed in a blinded manner by a single individual (GAL), and the slides were reviewed by a second individual (GAN). The results obtained by both individuals were in agreement (Kaplan = 0.9).

Statistical analysis

For the comet assay, an analysis of variance test with one criterion (one-way ANOVA) was performed. The frequency of micronuclei was evaluated by Tukey’s test. The statistical tests were performed using SPSS 12.0 software, and the significance level was 5%.

Results

Comet assay

The samples from the negative control group and the group treated with Ca₃SiO₅ had lower levels of DNA damage (p < 0.05) compared to those of the positive control group. There was no statistically significant difference in the levels of DNA damage between the negative control group and the group treated with Ca₃SiO₅ (p > 0.05) (Table, Figure 1).

Micronucleus test

There was an increase in the frequency of micronuclei in the group exposed to Ca₃SiO₅ compared to that of the negative control group (p < 0.05) (Table 1). There were significant differences in the number of micronuclei among the positive control group, the group exposed to Ca₃SiO₅, and the negative control group (p < 0.0001) (Figure 2).

Table. Level of DNA damage (tail intensity %) and frequency of micronuclei in bone marrow cells treated with Ca₃SiO₅ or cyclophosphamide and in untreated cells (negative control).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Comet assay</th>
<th>Micronucleus Test</th>
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<tr>
<td></td>
<td>Level of DNA damage</td>
<td>Mean (standard deviation)</td>
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<tr>
<td></td>
<td>(Tail intensity %) – SBs*</td>
<td></td>
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<tr>
<td>Ca₃SiO₅</td>
<td>23.57 ± 7.70b</td>
<td>6.25 (±3.53)b</td>
</tr>
<tr>
<td>Cyclophosphamide*</td>
<td>27.43 ± 7.40a</td>
<td>9.75 (±2.49)a</td>
</tr>
<tr>
<td>Negative control</td>
<td>24.75 ± 5.55b</td>
<td>0.75 (±1.03)c</td>
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*SBs: strand breaks. *Cyclophosphamide: positive control. Results with different letters differ significantly (p < 0.05).

Figure 1. Levels of damage to bone marrow cells. (A) strand breaks (SBs) in Ca₃SiO₅-treated cells; (B) SBs in cyclophosphamide-treated cells (positive control); (C) SBs in negative control cells.
Discussion

In the present study, according to results from the comet assay, the negative control group and the group treated with Ca₃SiO₅ had similar levels of DNA damage, and both groups had lower levels of DNA damage compared to those of the positive control group (p < 0.05). These results were different in the micronucleus test. There was an increase in the frequency of micronuclei in the group treated with Ca₃SiO₅ compared to that of the negative control group (p < 0.05), although it was lower than the number of micronuclei in the positive control group (p < 0.0001).

A study by Laurent et al.⁵ evaluated the genotoxicity and cytotoxicity of Ca₃SiO₅ in addition to its effects on specific functions in target cells. These authors showed that the mortality rate of cells exposed to this cement was similar to that of cells exposed to other biocompatible materials. Furthermore, the Ames test performed with lymphocytes and fibroblasts from human dental pulp showed no evidence of mutagenicity associated with this new material, and this finding was supported by our results of the micronucleus test and the comet assay. In the present study, we also did not find evidence of mutagenicity in the comet assay, but we found evidence of mutagenicity in the micronucleus test.

In vivo testing to evaluate the genotoxic and mutagenic potential of a product is important because it may reveal effects that are not observed in in vitro tests. An in vivo test can assess the metabolic and pharmacodynamic effects of a product and can help evaluate the influence of gut microbiota on the substance.²³,²⁴ Therefore, we decided to conduct an in vivo evaluation of the genotoxic and mutagenic properties of Ca₃SiO₅. Our results also demonstrate the importance of performing both in vitro and in vivo tests to better evaluate the genotoxic and mutagenic potential of new products.

The mutagenicity and genotoxicity of MTA (mineral trioxide aggregate), a cement with a composition and formulation similar to that of Ca₃SiO₅, has been evaluated by several authors. MTA showed no mutagenic or genotoxic effects according to results from the micronucleus test²⁵ and the comet assay²⁶. MTA and Ca₃SiO₅ cement differ in their specific chemical formulations. In particular, MTA requires only the addition of distilled water for hardening. The increased frequency of micronuclei found in the Ca₃SiO₅ group in our study may be related to a chemical compound present in the powder and/or in the liquid component of Ca₃SiO₅ that is not present in MTA. One study demonstrated that polycarboxylate (Aqualox®) had a mutagenic effect in an assay using S. typhimurium TA 98 and TA 1535²⁷. This finding supports the hypothesis that the increased frequency of micronuclei observed after exposure to Ca₃SiO₅ may be related to compounds in the liquid component, such as polycarboxylate.

![Figure 2. Polychromatic erythrocyte with micronucleus (arrow) in an animal exposed to Ca₃SiO₅ (Giemsa staining, 1000x magnification).](image)
Micronuclei are strongly indicative of chromosomal aberrations. However, micronuclei formation is not the only means by which a chemical exposure can induce mutagenicity or genotoxicity. The comet assay evaluates DNA damages and DNA repair capacity at the single-cell level. The different tests for mutagenicity and genotoxicity evaluate various potential DNA damage that may result from a chemical or radioactive agent. Therefore, more than one test should be performed to assess if DNA damage is present, and if so, to determine which particular damages were caused by a chemical or radioactive agent. The comet assay and the micronucleus test are broadly applied tests to check for genotoxic effects. This prompted us to utilize these specific tests for evaluating Ca₃SiO₅ genotoxicity and mutagenicity in this study.

The present study suggests that the mutagenic effect of Ca₃SiO₅ is clastogenic or aneugenic because these defects are detected by the micronucleus test, but not by the comet assay.

Further in vivo studies are needed to evaluate the mutagenic effects of exposure to different doses of Ca₃SiO₅. Furthermore, it would be of interest to determine whether exposure to either the powder or the liquid component or exposure to Ca₃SiO₅ prepared with distilled water is associated with an increased frequency of micronucleus formation.

**Conclusion**

Our data showed that exposure to Ca₃SiO₅-based cement was associated with an increased frequency of micronucleus formation compared to the negative control, although the micronucleus frequency was lower than that of the positive control. However, the Ca₃SiO₅-based cement showed no genotoxic effect when the comet assay was used to evaluate the bone marrow cells of rats after in vivo exposure.

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**References**


