

Genotoxicity and hemocompatibility of a novel calcium aluminate-based cement

Rafael FERNÁNDEZ, Carolina BERRUECOS, María Catalina CORTÉS MOTTA, Diego VELÁSQUEZ

ABSTRACT

Objective: The aim of this *in vitro* study was to evaluate the genotoxicity and hemocompatibility of a novel calcium aluminate-based cement, EndoBinder (EB) (Binderware, São Carlos, SP, Brazil) and compare it with Angelus White Mineral Trioxide Aggregate (MTA) (AWMTA) (Angelus, Soluções Odontológicas, Londrina, PR, Brazil).

Methods: For evaluation of genotoxicity, a comet assay was performed with Chinese hamster ovary (CHO) cells that had been grown for 24 h in Dulbecco's Modified Eagle Medium incubated with each of the cements for 24 h at 37°C. DNA percentage in head and Olive tail moment were analyzed. For assessment of hemocompatibility, erythrocyte lysis quantification, and concentration of plasma fibrinogen were determined in human blood samples placed in contact with each of the materials. One way analysis of variance (ANOVA) followed by post hoc Tukey test and Student t-test were used for data analysis of genotoxicity and hemocompatibility, respectively.

Results: Results showed that the genotoxic effects of EB and AWMTA were comparable to that of the negative control, with no statistically significant differences between AWMTA and negative control ($P > 0.05$). Compared to AWMTA, EB showed greater hemolytic potential when placed in direct contact with erythrocytes ($P < 0.05$). Fibrinogen values were low for both materials, with protein concentration being greater in samples exposed to EB than to AWMTA.

Conclusion: Both materials presented a higher hemolytic behaviour compared to what is established by international standards. Fibrinogen formation was low for both materials, and DNA damage induction was not observed in a comet assay.

Keywords: Biocompatibility, blood compatibility, calcium aluminate cement, cytotoxicity, mineral trioxide aggregate

HIGHLIGHTS

- This study investigated the biocompatibility of an aluminate calcium-based cement, EB, and Angelus white MTA, (AWMTA), using genotoxicity (comet assay) and hemocompatibility (hemolysis/fibrinogen quantification) assays.
- Results showed that, with regard to hemocompatibility, both cements—EB and AWMTA—induced high and low hemolysis and fibrinogen levels, respectively.
- None of the endodontic cements showed genotoxic behavior.

INTRODUCTION

Cements used for root end seal during endodontic microsurgery must be biocompatible with periapical tissue to favour healing and treatment success (1, 2). Mineral trioxide aggregate (MTA), is a bioactive material widely used for root end seal, and its osteoconductive and osteoinductive properties have been extensively documented (2, 3). MTA is currently available in two presentations: gray MTA (GMTA) and white MTA (WMTA) (ProRoot MTA, Dentsply

Tulsa, OK, USA). For both presentations, the main components are tricalcium silicate, bismuth oxide, dicalcium silicate, tricalcium aluminate, and calcium sodium dehydrate (2). GMTA has in addition tetracalcium aluminoferrite, which is absent in WMTA. Both formulations are 75 wt% Portland cement, 20 wt% bismuth oxide, and 5 wt% gypsum (2). Additional types of MTA are the Angelus MTAs (AMTA; Angelus, Soluções Odontológicas, Londrina, PR, Brazil) from Brazil, offering both gray (AGMTA) and white (AWMTA) MTAs composed of 80 wt% Portland cement and 20 wt% bismuth oxide (2). Drawbacks of these materials include extended curing time, difficulties in manipulation, discolouration potential of the dental structure, and arsenic release with the exception of AMTA, which does not have an extended setting time (4, 5). However, only noticeable differences have been determined for curing time and particle size, between ProRoot MTA (WMTA, GMTA) and AMTA (AGMTA, AWMTA) (2, 6). Therefore, there is an ongoing effort to develop new materials with optimal mechanical and biocompatibility properties. One of such materials is EndoBinder (EB) (paten-

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t-PI0704502-6, 2007) (Binderware, São Carlos, SP, Brazil), a calcium aluminate-based endodontic cement that is currently in the experimental phase, and that was developed to keep MTA's clinical properties and applications while improving its negative features (4). EB is primarily composed of Al_2O_3 (68 wt%) and CaO (31 wt%), SiO_2 (0.3–0.8 wt%), MgO (0.4–0.5), and Fe_2O_3 (<0.3) + Bi_2O_3 (20 wt% wt%), to guarantee its radiopacity as required by ISO 6876 standard (4, 7, 8). *In vitro* and *in vivo* studies have previously shown its optimal physical/mechanical properties and favourable cellular response, allowing osteoblastic differentiation to be superior to that achieved with the use of MTA (4, 9). However, other aspects of biocompatibility such as genotoxicity and hemocompatibility have not been reported for EB. To evaluate these properties, the American Dental Association and ISO 7405/10993 standards recommend comet assay to determine DNA damage and hemolysis/fibrinogen quantification to establish hemocompatibility (10–12). Therefore, in this study we have evaluated and comparatively analyzed biocompatibility of aluminate calcium-based cement EB and AWMTA by performing genotoxicity and hemocompatibility assays. The null hypothesis was that there is no difference in genotoxicity and hemocompatibility profile of EB and AWMTA.

MATERIALS AND METHODS

This study was approved by the Institutional Ethics Committee (Minute N°85, code 471) of CES University, Medellín, Colombia; and was developed in accordance with the recommendations for biomedical research stated in the Declaration of Helsinki.

Preparation of cements

Cements used for all assays were EB (Binderware, São Carlos, SP, Brazil) and Angelus white MTA (AWMTA) (Angelus, Soluções Odontológicas, Londrina, PR, Brazil), which were used at a ratio of 0.14 g of powder per one drop of distilled water from the AWMTA dropper bottle, according to the manufacturer's instructions. For each material, 0.1 g was placed into an acrylic mold forming cylinders of 4.2 mm (± 0.2 mm) in height and 0.5 mm in diameter.

Genotoxicity Assay

Cell Culture

One vial of Chinese hamster ovary (CHO) cell line was cultured in four cell culture plates T-75 (MilliporeSigma, St. Louis, MO, USA) with Dulbecco's Modified Eagle Media (DMEM) and Ham's F12 nutrient mixture (MilliporeSigma), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (MilliporeSigma). Cells were cultured for 3 days in a humidified incubator at 37°C, 5% CO_2 , and 95% humidity. Fresh medium was added on a daily basis. Confluent cultures were collected by adding Trypsin-EDTA (0.5%) for 5 min, quenching with 2 mL of complete media, and centrifuged at 1200 rpm for 5 min (Sigma 2-16K centrifuge, Osterode am Harz, Germany). After counting the cells using a Neubauer chamber (MilliporeSigma), they were placed into 24-well plates at a density of 300,000 cells/mL.

Preparation of samples

Fresh cement samples, were prepared, weighed, and immediately placed in direct contact with the cell culture medium (720 μL DMEM/280 μL FBS) (MilliporeSigma), and incubated at 37°C for 24 h. With regard to controls, as a positive control for

genotoxicity, 4-mM H_2O_2 was added to the culture medium, whereas the negative control consisted of the culture medium alone. Eight wells were allocated for each of AWMTA and EB. Four wells served for the control group.

Comet Assay

Two experiments with four wells exposed to the material, and at least 40 comets read in every well were made for alkaline comet assay as previously described by Araldi et al. (12). Briefly, samples of cells subjected to each of the treatments (100 μL) were embedded in a regular 1.5% agarose slide, and immediately covered with a coverslip. Once the agarose had solidified at 4°C, the coverslip was removed and the slides placed for 1 h in 26.7 mL of 89% lysis buffer (2.5 mL NaCl, 100-mM EDTA, 10-mM Tris-HCl, 12 g NaOH/ 1.2%, and 1 g sodium lauroyl sarcosinate /1%; pH 10–10.5) supplemented with 3 mL of dimethyl sulfoxide/10% and 0.3 mL Triton-X100/1%.

After treatment with lysis solution, slides were incubated in alkaline buffer (300-mM NaOH and 1-mM EDTA; pH > 13) for 20 min, and subjected to electrophoresis at 25 V (0.86 V/cm) and 200 mA for 20 min. Slides were then neutralized in 400-mM Tris-HCl (pH 7.5) and fixed in absolute ethanol. Samples were stored at room temperature until being analyzed by fluorescent microscopy (Nikon, Optiphot-2, Tokyo, Japan). Forty comets or nuclei were analyzed from each slide. To minimize DNA damage induced by environmental UV light, all experimental steps were performed under low lighting. The automated analysis system CometScore (Tritek Corp, Sumerduck, VA, USA) software was used to assess DNA damage, taking into account the percentage of DNA in the head and the Olive tail moment. To avoid risk of bias, the principal investigator was blinded for this analysis.

Hemocompatibility

Blood Samples

Blood samples from a consenting healthy volunteer meeting the inclusion criteria were collected in BD vacutainer heparin tubes (Vacutainer 367871, México DF, México) for hemolysis assays, and in BD Vacutainer EDTA tubes (Vacutainer 368171) for fibrinogen quantification assays. To maintain homogeneity, 3 mL of blood was collected for all samples. Blood from a different donor was used for each assay performed on different days.

Hemolysis

Two experiments each with four samples were made for hemolysis percentage determination. Blood was centrifuged, and plasma was removed by aspiration. Red blood cells (RBC) were washed three times with phosphate-buffered saline (PBS) pH 7.4 (MilliporeSigma), centrifuging at 3000 rpm (Biomet Biologics, IN, USA), and 300 μL blood precipitate was diluted in 12 mL of PBS. Then, 600 μL of the latter dilution was placed in direct contact with each of the materials (EB or AWMTA) and was incubated for 1 h at 37°C in 5% CO_2 . Tubes consisting of RBC solutions with or without distilled water, respectively, were used as positive and negative controls. Because cement samples were at the bottom of the tubes, 500 μL of the diluted blood were used for the hemolysis determination, taking care to avoid taking degradation products of the cement samples. Total

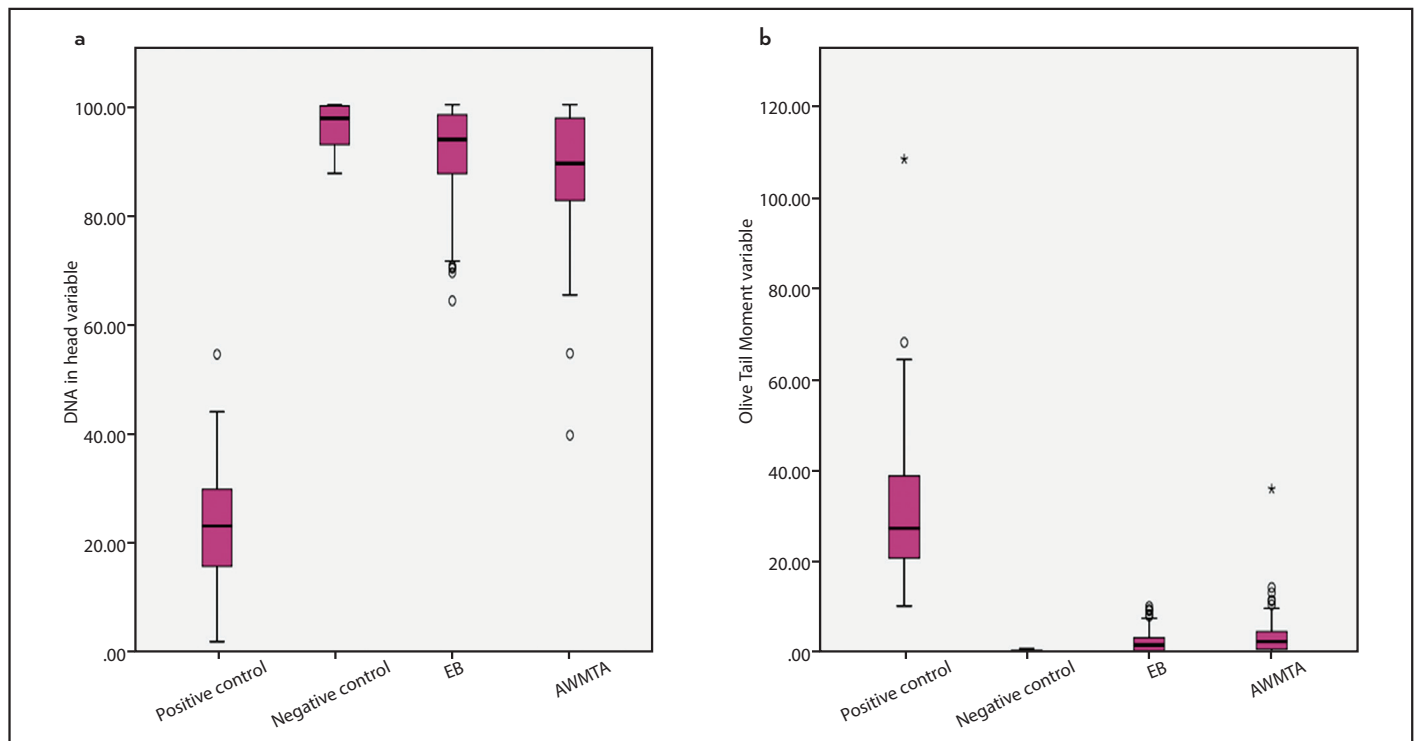


Figure 1. Genotoxic effects (EB AWMTA). (a) DNA in head variable. (b) Olive tail moment variable. Asterisk and circles represent atypical values. In particular, asterisks denote the most remote data

hemoglobin concentration in heparinized blood was quantified with a standard curve using a spectrophotometer (xMarkTM, Bio-Rad, CA, USA) after measuring absorbance at 490 nm; measurements were made in triplicate. Finally, hemolysis percentage was obtained by calculating for each sample the ratio of cell-free hemoglobin to total hemoglobin concentration.

$$\text{Hemolysis (\%)} = 100 \times \frac{(\text{Abs Ab0})}{(\text{Abs100 Abs0})}$$

Fibrinogen Quantification

Two experiments (each containing four samples) were made for fibrinogen quantification. The Human Fibrinogen SimpleStep ELISA® kit (Ref. ab208036, Abcam, Cambridge, MA, USA) was used following manufacturer's instructions. Quantitative fibrinogen measurements was performed in duplicate, interpolating the fibrinogen standard curve and correlating it to the diluted sample.

Two blood samples were collected in EDTA-K2 tubes and centrifuged at 2000 rpm (Biomet Biologics) for 10 min. Blood components were separated because of their density differences. Because of the high sensitivity of the quantification test, plasma was isolated and highly diluted until a volume of 1600 μL and a concentration of 1:10000 were obtained. Subsequently, 100 μL of diluted plasma was exposed to each of the cement samples, and controls were placed on a shaker at 25 rpm for 30 min. After this, 50 μL of sample and 50 μL of the antibody cocktail were added to each of the pretreated kit wells, and placed on an OrbitTM P4 digital shaker at 250 rpm (Labnet International, Inc. NJ, USA) for 1 h at room temperature. Each well was washed three times with 350 μL of Wash Buffer PT 1X (Abcam) with subsequent aspiration and dried. One hundred

microliters of tetramethylbenzidine (TMB) were added to each well, and the plate was placed on shaker at 20 rpm for 10 min. Finally, 100 μL of Stop solution were added to each well. Plate was shaken at 20 rpm for 1 min to mix and take to spectrophotometer (xMarkTM, Bio-Rad, CA, USA) and the absorbance was measured at 450 nm.

Statistical Analysis

Hemocompatibility and genotoxicity assays were performed in duplicates for each of the materials. Statistical analyses were performed using PASW Statistics 21 software (SPSS, Chicago, IL, USA). Normal distribution of data was evaluated by the Kolmogorov-Smirnov test. In the analysis of the hemocompatibility assay, a t-Student test with a 95% confidence interval was used to determine the difference between datasets. For genotoxicity assays, statistical significance was determined by one-way ANOVA followed by a post hoc Tukey test for multiple comparisons.

RESULTS

Genotoxicity

Both materials showed head DNA percentage and Olive tail moment index that were comparable to that of the negative control, indicating a low genotoxic effect because of the direct contact of the materials (Figs. 1 and 2).

Hemolysis

Both materials presented high percentages of hemolysis when in contact with blood in situ, according to the international standard. Comparing results of both cements, a greater quantity was detected for EB compared to AWMTA; however, these differences were not statistically significant ($P > 0.05$) (Table 1).

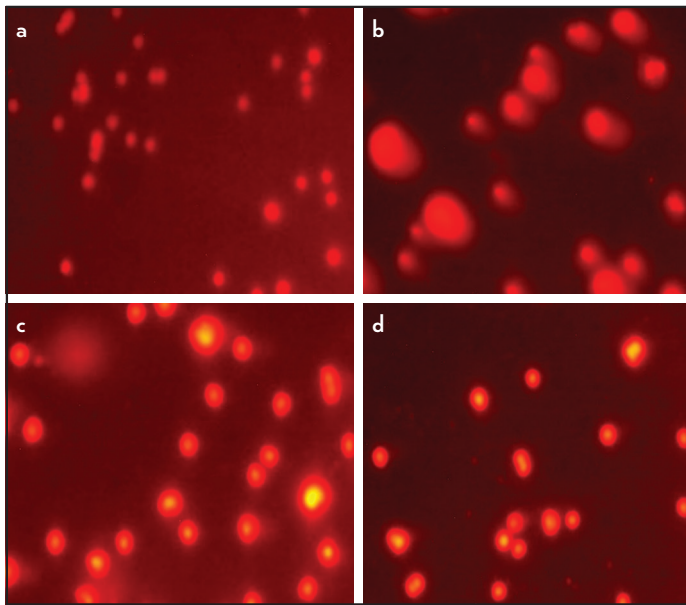


Figure 2. Shows micrographic images of nuclei from Chinese hamster ovary (CHO) cells after running the comet assay, where halos or DNA degradation can be seen in the inset B. (a) Negative control. (b) Positive control (H_2O_2). (c) EB. (d) AWMTA

TABLE 1. Percent of hemolysis (EB AWMTA)

Material	n	Mean (%)	Standard Deviation	P value
EB	12	13.22	13.58	0.35
AWMTA	12	8.64	9.99	

TABLE 2. Fibrinogen quantification

	Material	n	Mean	Standard Deviation	P value
Fibrinogen (ng/ml)	EB	6	0.30	0.34	0.10
	AWMTA	6	0.04	0.09	

Fibrinogen Quantification

Compared to AWMTA, samples exposed to EB showed an increased quantity of fibrinogen; however, these results were not statistically significant ($P>0.05$) (Table 2).

DISCUSSION

Cements used for retrograde filling in endodontic microsurgery come into direct contact with periapical tissues. Therefore, sealing and biocompatibility properties must be optimal to obtain successful results (1). In this *in vitro* study, we evaluated genotoxicity, hemolysis, and fibrinogen quantification of EB and AWMTA cements. On the basis of results presented in this study, the null hypotheses regarding these three aspects were not rejected.

The specific time of 1 h of exposure was decided to limit the possible hemolysis because of prolonged *in vitro* culture periods and the potential coagulation of blood that may have an influence in the spectrophotometrical analysis. Because both materials have setting times of around 10 min (13), it may happen that during this time some histological reactions may be expected because of the interaction of non-set material with

blood in periapical tissues. Further studies should be performed with kinetic assays, in which samples will be analyzed at intervals between 1 min and 1 h.

With regard to genotoxicity, we used the comet assay. This assay is a fast and sensitive test to determine DNA damage by fluorescent microscopy, and its advantages and applications in the field of toxicology have been reported previously (12). For cell culture, CHO-K1 cells were used for genotoxicity assessment. These epithelial-like cells are a well-established model available in ATCC. This cell line is recommended for several *in vitro* assays, particularly for a comet assay. The latter evaluates DNA damage within the cells exposed to the culture medium with the material diluent. For this assay, some aspects, such as cell membrane composition, nuclear envelope composition, and the presence of nuclear DNA, must be considered. Cell membrane and nuclear envelope of CHO-K1 cells are composed of a phospholipid bilayer similar to that in fibroblasts or other cell types from connective tissue such as osteoblastic or periodontal ligament cells. On the other hand, for *in vitro* assays, according to international standards, it is more advisable to use established cell lines rather than stem cells or human cells derived directly from primary cultures, because their proliferation rate might be compromised and may also add false positives due to senescence (14).

For the image analysis, only well-defined comet structures were considered. Variables were chosen according to their complementary information, because DNA head percentage measurements take indirectly in account the presence of DNA in the tail, but do not include tail length. Olive tail moment represents the percentage of DNA present in the tail as a product of the distance between the centers of the mass of the head and the tail regions. Results for both variables were accurate due to their complementarity (15). Our results showed that both materials (EB and AWMTA) demonstrated an effect comparable to that of negative controls. This was in agreement with previous studies in which AMTA and ProRoot MTA were used, and no DNA damage was reported in lymphocytes or other analyzed cell types upon 1, 4, and 24 h of exposure (16-18). As for EB, an important finding was that the Olive tail moment did not show a statistical difference compared to the negative control, suggesting that this may be a successful treatment in retrograde fillings done with this cement. However, due to lack of previous scientific evidence regarding EB genotoxicity, we were unable to establish additional comparisons.

Evaluating biomaterial hemocompatibility is relevant because once the retrograde filling material is placed in the apical cavity, an initial direct contact with blood and periapical tissues is established, activating the coagulation cascade and a host inflammatory response as defense mechanisms (19). Thus, hemocompatibility can be assessed by determining hemolytic potential and quantifying fibrinogen (20). Ideally, a biomaterial with optimal hemocompatibility should have a low level of hemolysis, because otherwise it may lead to rupturing of erythrocyte membranes and the subsequent release of hemoglobin (21). Notably, both materials tested in this study, showed hemolysis levels of 13.2% and 8.5%, respectively, which is

above the maximum level for biomaterials recommended by ISO 10993 standards for *in vitro* biological evaluation of medical devices (19). This finding is relevant to the present study, because scientific evidence of the hemolytic potential of these two materials has not been previously reported. Other studies have evaluated the hemolytic potential of individual components of these cements such as alumina (Al₂O₃) and calcium oxide (CaO). In those studies, high concentrations of alumina were found to favour hemolysis (22, 23), which may explain our observations. Furthermore, changes in osmotic pressure, pH values above 8.5, and presence of metals as a consequence of peroxidation may alter erythrocyte membrane (24-27). These observations may partly explain the high hemolysis levels caused by EB and AWMTA, because both cements contain metallic oxides, and develop high alkaline levels *in situ*.

With regard to fibrinogen quantification in our study, compared to negative control, all plasma samples that were in contact for 30 min with both cements showed low fibrinogen levels. Several hypotheses may help explain this. First, it is expected that once a biomaterial is in contact with blood, a layer of plasma proteins is adsorbed on its surface, a biological response is elicited, and protein detachment may not take place. Other hypothesis is that having a porous material may induce entrapment of this protein within the structure, making it hard to quantify. AMTA has been reported to have a 28% porosity with a pore size of 3 µm (28-30). Although this information is not currently available for EB, for other cements composed of calcium aluminate, a porosity of 18% and pore size of 0.25 µm, have been previously reported (31). Moreover, compared to MTA, a lower surface porosity has been reported for EB by quantitative analysis with scanning electron microscopy, which was mainly attributed to the balance between phases rich in Al₂O₃ and CaCO₃ found in EB (32). These last statements and the fact that fibrinogen molecular size is approximately 0.04 µm (33) could justify these results, (0.3 ng/mL for EB and 0.04 ng/mL for AWMTA). An important argument to consider is the low levels of fibrinogen synthesis because of the absence of coagulating potential of the material.

Other aspects of hemocompatibility not addressed in the current study, such as platelet activation and adhesion, or surface characteristics of materials, may have also influenced our results because after the initial adsorption there is a constant exchange of free proteins that reaches a steady state after 2 h, which should be addressed in future studies (19).

CONCLUSION

Our results showed that with regard to hemolysis, both EB and AWMTA presented a higher hemolytic behaviour than the established in the international standard (5%). Fibrinogen formation levels were low for both materials and induction of DNA damage was not observed.

Disclosures

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