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Biocompatibility test with *Artemia salina* for five materials for endodontic use

Ensayo de biocompatibilidad con *Artemia salina* para cinco materiales de uso endodóntico

Leidy Johanna Torres-Avirama [1], Daniela Alexandra Realpe-Urbano [2], Tania Lizeth Guevara –Valencia [3], Gilbert Alfonso-Morales [4], Ingrid Ximena Zamora-Córdoba [5], Carlos Humberto Valencia-Llano [6]

1. Universidad del Valle. Cali, Colombia. Correo: leidy.avirama@correounivalle.edu.co - https://orcid.org/0000-0002-4844-3930
2. Universidad del Valle. Cali, Colombia. Correo: daniela.realpe@correounivalle.edu.co - https://orcid.org/0000-0002-1360-8504
3. Universidad del Valle. Cali, Colombia. Correo: taniaguevara@correounivalle.edu.co - https://orcid.org/0000-0002-5330-5882
4. Universidad del Valle. Cali, Colombia. Correo: gilbert.morales@correounivalle.edu.co - https://orcid,org/0000-0003-2704-8365
5. Universidad del Valle. Cali, Colombia. Correo: ingrid.zamora@correounivalle.edu.co - https://orcid.org/0000-0002-9287-1414
6. Universidad del Valle. Cali, Colombia. Correo: carlos.humberto.valencia@correounivalle.edu.co - https://orcid.org/0000-0003-4892-9786

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Keywords:
Artemia salina; Biocompatibility; Cytotoxicity; Protocols.

ABSTRACT

Introduction: Biocompatibility is a decisive factor in the success of endodontic therapy. **Objective:** To assess the cytotoxicity of five endodontic materials using the *Artemia salina* test. **Method:** An in vitro experimental study was designed, using *Artemia salina*, to evaluate the biocompatibility of Grossman, CaOH, AH-Plus, MTA, and TheraCal-LC cements, with observations at 24 and 48 hours. **Results:** Grossman cement presented 100% toxicity at 24 hours, while the other materials show values lower than 30%, reflecting a biocompatible behavior; however, at 48 hours, CaOH and TheraCal-LC present toxicity higher than 30%, while AH-Plus and MTA continue with values lower than 30%. The data were analyzed with a Generalized Linear Model with binomial error distribution, a deviancy analysis, and Fisher's multiple comparison test with Bonferroni correction. An independent analysis was performed for 24 and 48 hours, under a significance level α=5%; significant differences were found at 48 hours between TheraCal-LC and AH-Plus, and MTA. **Conclusions:** Grossman cement presented a high degree of toxicity, while AH-Plus and MTA showed the highest biocompatibility.

Palabras clave:
Artemia salina; biocompatibilidad; citotoxicidad; protocolos

RESUMEN

Introducción: la biocompatibilidad es un factor decisivo para el éxito de una terapia endodoncica. **Objetivo:** evaluar la citotoxicidad de cinco materiales de uso endodóntico mediante la prueba de *Artemia Salina*. **Método:** se diseñó un estudio experimental *in vitro*, empleando *Artemia salina*, para evaluar la biocompatibilidad de los cementos Grossman, CaOH, AH-Plus, MTA y TheraCal-LC con observaciones a 24 y 48 horas. **Resultados:** el cemento Grossman presentó 100% de toxicidad a las 24 horas, mientras que los demás materiales mostraron valores menores al 30%, lo que indicó un comportamiento biocompatible; sin embargo, a las 48 horas, CaOH y TheraCal-LC presentan una toxicidad mayor al 30%, mientras que AH-Plus y MTA continúan con valores -menores del 30%. Los datos fueron analizados con un Modelo lineal generalizado con distribución de errores binomial, un análisis de devianza, y la prueba de comparación múltiple de Fisher con corrección Bonferroni. Se hizo un análisis independiente para las 24 y 48 horas, bajo un nivel de significancia α=5%, se encontraron diferencias significativas a las 48 horas

entre TheraCal-LC y AH-Plus y MTA. **Conclusiones:** el cemento Grossman presentó un alto grado de toxicidad, mientras que AH-Plus y MTA mostraron la mayor biocompatibilidad.

INTRODUCTION

Endodontics, as an area of knowledge, has a primary objective: the elimination or reduction of the number of microorganisms within the space of the root canal and the prevention of a possible infection or reinfection, which is why, in recent years, the development of new technologies, which reach this end, and which, in turn, allow therapeutic paradigms to be broken, in favor of innovation and critical analysis of the environment and resources. This is how from this approach, attention has been directed towards each of the execution phases in endodontics, and one of those aspects consists of the definitive sealing of the root canal, a process that provides biological stability and control over the portals that can generate some imbalance. This hermetic shielding is obtained thanks to the use of a type of agent called: Sealing cement, whose characteristic is to adhere an inert material to biological tissue, limiting the microbiological excursion, capable of inducing or even causing the persistence of a periapical lesion[1].

Throughout history, great interest has been generated in obturation systems and the behavior of sealing cements in conventional endodontic therapy. The search for a luting agent that meets the ideal requirements established by Grossman in 1963 and that also prevents the appearance of any mutagenic or carcinogenic event, or a type of immune reaction on periapical tissues, has resulted in the development of various materials ranging from simple elements such as zinc oxide to more complex compounds such as epoxy resin and bioactive cements[2].

This variety of cementing agents differ in their composition, and it is a characteristic that affects their physical and mechanical properties, as well as the type of interaction with dentin. Zinc oxide-based sealants, the most widely used, have a low resistance to compressive forces and little ability to penetrate the dentinal tubules. On the contrary, epoxy resin-based sealers have a greater penetrating capacity and adhesion to root dentin[3].

Today, there is a vast commercial offer of sealing materials which can be classified according to their chemical nature into seven groups: a) Zinc oxide – eugenol system; b) Based on epoxy resin; c) Based on Silicones; d) MTA system (*Mineral trioxide aggregate*); e) Bioceramic sealants based on the calcium-silicate-phosphate system; f) Based on methacrylate resin; and g) Calcium phosphate-based sealers[4].

The success of conventional endodontic therapy is influenced by the ability of the cementing agent to provide a hermetic and three-dimensional seal of the root canal system and is linked to the type of combination it establishes with the root dentin and a filling material[5].

The most widely used obturator material is gutta-percha, a high molecular weight polymer with two crystallographic forms: Alpha and beta, which occupy most of the canal space, while the sealant fills the interface between the obturator material and the canal wall. These materials must be biocompatible, non-resorbable, and stimulate healing in the periapical area[4,6].

On the other hand, a therapeutic line has been developed, which moves away from conventional endodontic therapy, and whose objective is to promote the permanence of pulp tissue and its recovery and repair called: Vital pulp therapy. This approach is aimed at the interaction of pulp and dentin with a type of luting agent called bioactive cement, whose composition is based on mineral aggregates and can form layers of hydroxyapatite on a surface in vivo, resulting in a phenomenon called: Biomineralization.

Bioactive cements are mainly composed of calcium silicate and calcium aluminate and differ from bioactive glasses because they require water to set and thus adopt a firm, hard, dimensionally stable consistency, which represents a great advantage, given that the lack of expansion or contraction helps to seal the area where living tissue is exposed. Another of its characteristics is its high pH, which imparts an antimicrobial action on planktonic bacteria and yeasts. However, it is insufficient to destroy established biofilms. Another in vivo benefit of these bioactive agents is the release of silicate ions, which benefits osteogenesis, a critical phenomenon for healing injured pulp or periapical tissue and repairing surrounding bone tissue[7].

With the advent of vital pulp therapy, also described as regenerative endodontics, there was a paradigm shift towards new concepts in endodontics, and it was suggested that some materials could stimulate maturation in teeth with open apex, revitalization and recovery of affected pulp tissue, by promoting revascularization and angiogenesis[8].

Regenerative endodontics, defined as an interdisciplinary field that applies the principles of tissue engineering seeking to promote the recovery of affected pulpal and periapical tissues[9], involves the transversal concept of biocompatibility since the interaction with these biological tissues must be established under an atmosphere non-cytotoxic and immunologically compatible[4].

The change in the paradigm that regenerative endodontics implied led to the investigation of the effect on structural strength and to reassess the degree of biocompatibility of the luting agents traditionally used, which generated the need for more studies on the new materials. Within the registered results of some investigations, it has been found that prolonged exposure to calcium hydroxide increases the possibility of root fracture[8,10], high concentrations of antibiotic paste have a cytotoxic effect on the remaining stem cells of the apical papilla[8,11]; and that zinc oxide-eugenol- based pastes can release eugenol, a highly cytotoxic component.

Taking into account that biocompatibility is a decisive factor for the success of endodontic therapy, whether conventional or as vital pulp therapy, this research aims to evaluate the *in vitro cytotoxicity* of five of the most commonly used sealant materials in endodontic treatments: by the *Artemia salina* test.

METHOD

Design of investigation

In vitro, experimental study with invertebrate animal models is helpful in research studies that can be inferred from vertebrates. A preliminary *in vitro* assay prioritizes only the best chemicals for further vertebrate testing. To develop this research, the toxicity of five materials used in endodontics was evaluated using the *Artemia salina test*, a type of crustacean commonly known as "sea monkey." The *Artemia salina* test was proposed in 1956 as a bioassay[12]. However, it was in the 1980s that the Artemia reference center proposed its standardization as an acute toxicity test to determine the lethal dose 50 (LD50) in *Artemia salina* larvae in stages 2 and 3 at 24 hours and 48 hours[13]. Since then, the method has been widely used to study the compatibility/toxicity of different materials[12].

Instruments and procedures

Based on the studies carried out by Rotini *et al*[14], Abushaala[15], and Pecoraro *et al*[16] to carry out the test, one gram of *Artemia salina eggs* (*Brine shrimp eggs*, *Brine shrimp direct*, Ogden, UT, USA), in 4 liters of artificial seawater solution from the relationship: 30 grams of sea salt/liter of distilled water.

For the hatching of the eggs, the seawater solution with the eggs was deposited in an artemisia manufactured according to the protocol and the description of the equipment used by Rotini *et al*[14]; this equipment is made up of a series of concentric rings that act as barriers to guarantee that only healthy nauplii reach a central ring where they are captured for the experiment.

The materials selected for the study were: Grossman/Eugenol cement (Proquident, Medellín, Colombia), a material used as a sealing agent; Calcium Hydroxide (CaOH) paste (Dycal® Dentsply Sirona, Milford, DE, USA), used as an agent for indirect pulp capping in vital therapies; AH-Plus cement (Dentsply DeTrey GmbH, Konstanz, Germany), used as a sealing cement for obturation in conventional therapies; MTA Angelus Blanco (Angelus, Londrina-PR-Brazil), used as a direct and indirect pulp capping agent in vital therapies; and TheraCal -LC (Bisco, Schaumburg, USA), also with application as direct or indirect pulp capping.

The materials were prepared at 24 °C, following each manufacturer's instructions for their preparation. Based on the studies by Abushaala et al[15], five samples of each material with a weight of 10 mg were taken, using a RADWAG WAS 100/X analytical balance.

For the experiment, 6-well cell culture boxes (Corning, Termo Fischer Waltham, MS, USA) were used (Figure 1); ten 48-hour nauplii and a sample of each material with a weight of 10 mg were deposited in each well; enzymatic soap (Bonzyme, Laboratorios Eufar, Bogotá, Colombia) was used as a positive control, in order to obtain toxicity data and

verify the sensitivity of the larvae[17], before the toxic residues left by this material[18], and as a negative control solution of seawater with crustaceans.

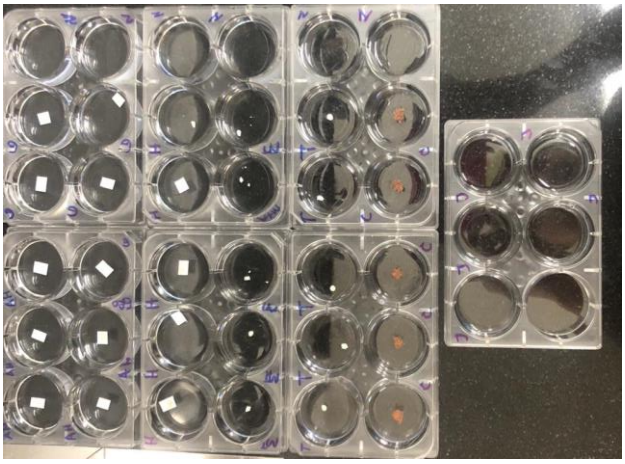

Figure 1. Distribution of materials in culture boxes with six wells.

All samples and controls were replicated five times, making observations at 24 and 48 hours. To determine toxicity, the following formula was used[19,20]:

$$Mortality\% = \frac{Number\ of\ Dead\ AS}{Number\ of\ initial\ AS} \text{x} 100$$

The test is performed with second instar larvae (nauplii), considered toxic, the extract that induces mortality greater than or equal to 30%[21,22]. Artemia *salina* that is immobile for 10 seconds is counted as dead, and nauplii that are observed to be mobile are considered alive[16]. The test is valid if less than 10% of the negative control nauplii are immobile[14,19].

The reviews at 24 and 48 hours were performed with the help of a *Thomas Scientific stereoscopic microscope* (Swedesboro, New Jersey, USA) equipped with a Motic Camera (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA).

In the data analysis, the mortality counts were recorded in proportions using a generalized linear model with binomial distribution. An analysis of deviance associated with the previous model was performed to assess whether each group had a significant effect on the mortality rate. Fisher's multiple comparison test was performed with Bonferroni correction[23] to determine which groups showed statistical differences. The previous analysis was used independently for the 24-hour and 48-hour time instants, under a significance level of α=5%. The R Statistical Software, version 4.1.2, was used.

Declaration on ethical aspects

Artemia salina larvae, an invertebrate organism, were used; the procedures recommended in the scientific literature were followed[14-19]. The study was reviewed and authorized by the Animal Research Ethics Committee of the Universidad del Valle through CEAS code 002-021.

RESULTS

Twenty-four hours after implantation, a visual inspection of the wells was carried out using stereoscopic microscopy at different magnifications (1X, 2X, 3X, 4X), observing a variable number of live larvae swimming and in contact with the materials, as well as dead larvae (Figure 2).

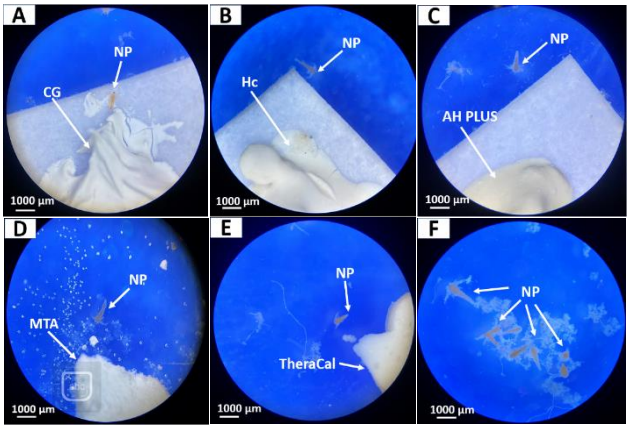

Figure 2. Materials cultured with nauplii at 24 hours. Stereoscopic microscope, images at 4X. A: Grossman cement. B: Calcium hydroxide (CaOH). C: AH-Plus. D: MTA. E: TheraCal -LC. F: Positive control.

The first observation of the wells at 24 h showed that most of the larvae were found alive, with a mortality rate between 10% and 18%; however, a mortality of 26% was evidenced in the CaOH group and 100% in the positive control group and the Grossman cement group. Mortality greater than 30% was evidenced at 48 hours in the CaOH and TheraCal-LC group. The AH-Plus and MTA materials groups had the lowest mortality rate at 48 h of observation (Table 1).

Table 1. Percentage (%) of dead *Artemia salina larvae mortality* at 24 and 48 hours.

Material	24h (%)	48h (%)	Total
Grossman cement	100	100	100
Calcium hydroxide (CaOH)	26	40	40
AH Plus	12	26	26
MTA Angelus white	12	28	28
TheraCal LC	18	58	56
Negative Control	5	23	23
Positive Control	100	100	100

The test data were recorded in Excel sheets and analyzed in the R Statistical Software, version 4.1.2. When performing the deviance analysis associated with the generalized linear model with a binomial distribution, no statistically significant differences were identified in the mortality rate between the groups at 24 hours of observation (Deviance = 9.09, df=4, p-value=0.059) (Figure 3). These results were corroborated by the Fisher test with Bonferroni correction (p>0.05).

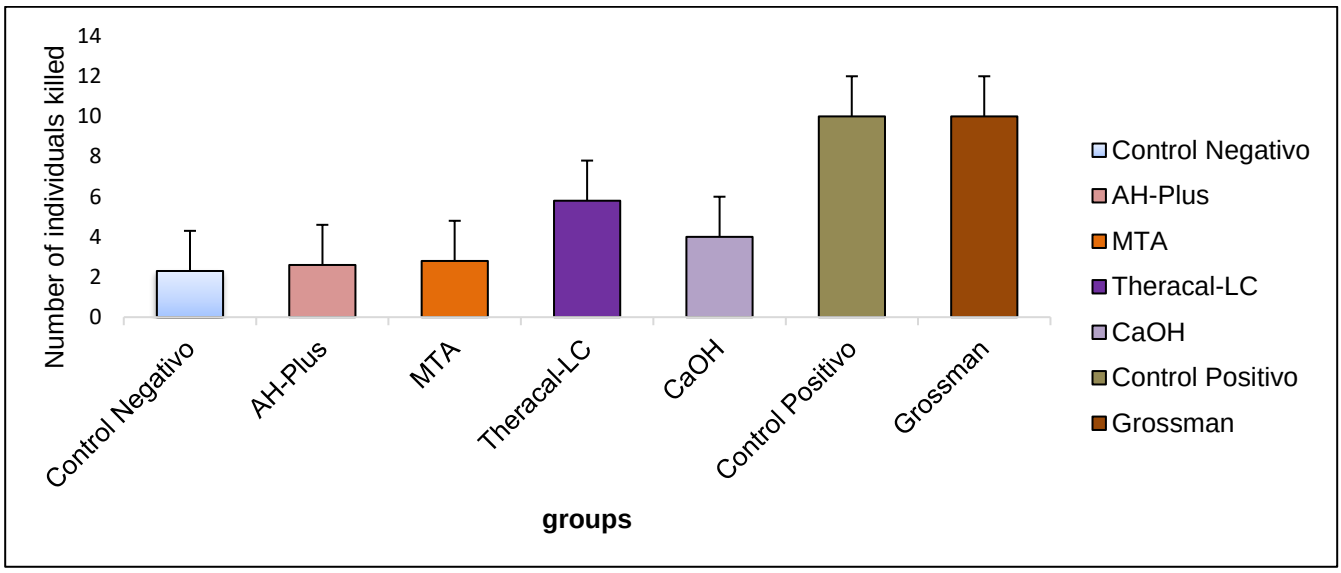

Figure 3. Number of dead individuals for *Artemia salina* after 24 h (Deviance =9.09, df=4, p-value=0.059).

After 48 hours of observation, when performing the deviance analysis associated with the generalized linear model with Binomial distribution, statistically significant differences were identified concerning the mortality rate between the different groups (Deviance =17.30, df=4, p-value=0.002). See Figure 4. The results of the Fisher test with Bonferroni correction suggest that there are significant differences between the Theracal -LC group and the MTA (p-value=0.029), AH-Plus (p-value=0.015), and negative control (p-value=0.0075). There were no significant differences between the other groups (p-value>0.05).

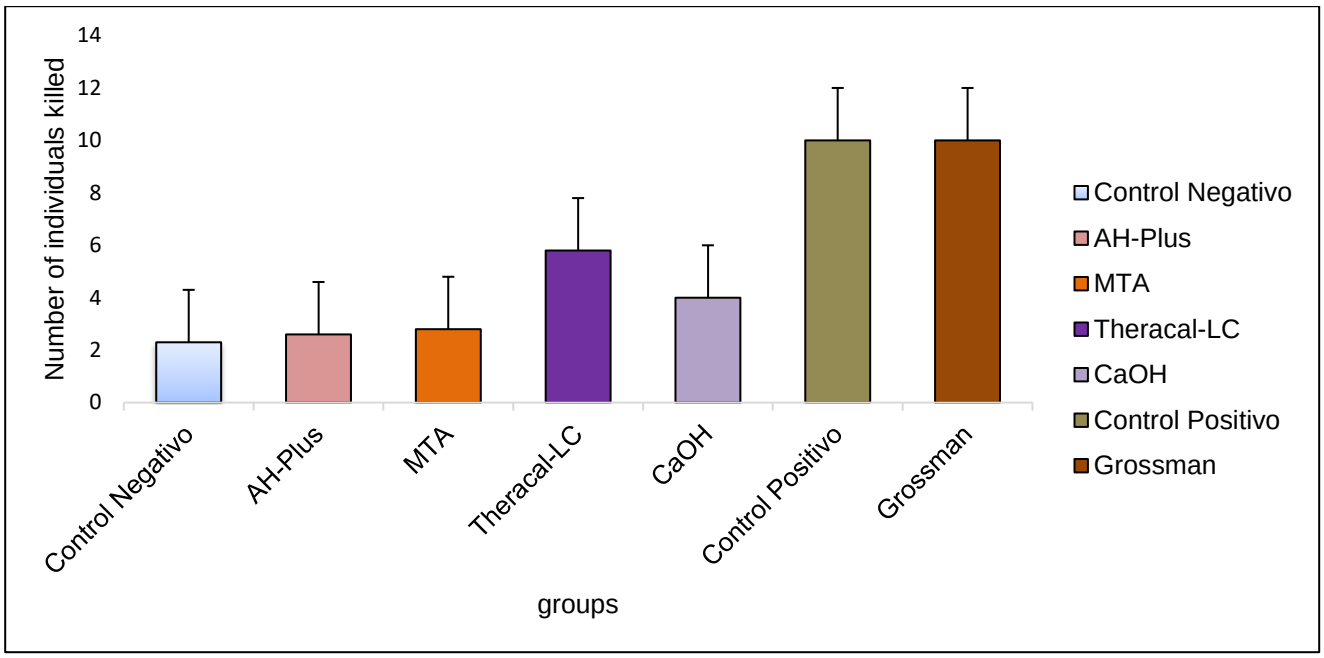

Figure 4. Number of dead individuals for *Artemia salina* after 48 hours (Deviance=17.30, df =4, p-value=0.002).

The test with *Artemia Salina* showed that, except Grossman cement, most of the cement behaved as compatible, showing low toxicity at 24 hours; however, at 48 hours, both CaOH and TheraCal-LC presented percentages of toxicity above 30%, which would indicate that they are no longer compatible[21]. The AH-Plus and MTA materials had very similar behavior at 24 and 48 hours, maintaining a mortality rate of less than 30%; the negative control group had an increase in mortality from 5% at 24 hours to 23% at 48 hours, which is explained by the depletion of reserve nutritional resources since in the experiment the larvae are not fed.

In general, except for Grossman cement, all materials showed low toxicity at 24 hours, with AH-Plus and MTA showing the highest biocompatibility. In the observation at 48 hours, while the AH-Plus and MTA cements continued with mortality percentages below 30%, comparable to the negative control, the CaOH and TheraCal-LC had mortality percentages much higher than those of the control, negative and above 30%, which makes them non-biocompatible for this observation period.

DISCUSSION

In recent years, the objective of many laboratories related to the study of biocompatibility has been to develop and use new bioassay procedures, trying to promote cheap methods, easy to use and that generate statistically reliable results. Currently, mortality assays with *Artemia salina* are widely used in cytotoxicity tests on dental materials or endodontic bioactive materials since they provide an initial overview of the cytotoxicity of a material without the need for a significant investment in infrastructure; this indication of the cytotoxicity of a material allows the researcher to discriminate samples in order to carry out more specific tests such as molecular biology or cell cultures[24,25].

The results indicate that the Grossman cement used in this research presented a percentage of cytotoxicity of 100% at 24 hours and agrees with what has been reported in the literature, indicating that, despite the modifications made, the material continues to present biocompatibility problems.

The literature indicates that zinc oxide is the primary compound in Grossman cement, followed by eugenol, which acts as an activator. This zinc oxide-eugenol system has an antibacterial effect [25] but is also attributed to an inflammatory effect that can lead to necrosis. The explanation is related to the release of residues of the Eugenol component[26], a phenolic derivative obtained from clove[27]. It is important to highlight that zinc oxide-eugenol-based cements are considered a standard in conventional endodontic therapy, and this is due to the large number of reported procedures in which they were used, currently the initial cement formulas. Grossman's

have been modified by including other components to reduce their cytotoxicity[25,26].

Calcium hydroxide is also widely used in dentistry, considered for several decades as the ideal material for direct and indirect coating in vital therapies, thanks to its ability to stimulate reparative processes, induce dentin mineralization and have anti-inflammatory and antibacterial properties.[28] However, some studies have shown that calcium hydroxide particles have a cytotoxic effect *in vitro* and that direct contact with pulp tissue generates an inflammatory response, possibly due to its high alkalinity[29]. This investigation showed that this material had a relatively high percentage of mortality, which makes its biocompatibility questionable.

The AH-Plus sealing agent, mainly made up of an aminoepoxy resin, showed high biocompatibility, despite its chemical nature, which is responsible for a potential cytotoxic effect due to the release of aldehydes[30].

In the case of MTA cement, a calcium silicate cement derived from Portland cement, the scientific literature has reported bioactive, antimicrobial, and biocompatibility properties[31], and some studies have reported low cytotoxicity[32,33]. Despite this, its cytotoxic potential continues to be questioned[34], mainly due to the presence of two highly toxic components: Salicylate resin and resin and silica diluent[30]. This investigation showed a low percentage of cytotoxicity, which agrees with what has been reported in the literature.

TheraCal-LC is a modified calcium silicate resin. In vitro studies have found a possible dose-dependent cytotoxic effect attributable to the release of monomers35; In an in vivo study in a canine model, this product showed low biocompatibility35; In the study carried out with Artemia salina, this material presented a relatively high percentage of mortality, but below 30% at 24 hours.

The literature review shows that the five materials evaluated present some cytotoxic potential when studied using specialized in vitro tests such as the metabolic activity assay (MTT). The results of this investigation using the Artemia salina test allow us to confirm these findings.

It can be concluded that, of the five materials studied, Grossman cement had the worst performance, with 100% mortality at 24 hours, while MTA Angelus white had the lowest percentage, close to that of the negative control group. Calcium Hydroxide and TheraCal-LC showed biocompatibility at 24 hours, but the percentage of mortality increased remarkably at 48 hours. The biocompatibility test with Artemia salina is valid for the in vivo study of biomaterials for endodontic use.

With this research, it is hoped to offer the scientific community, in the short term, a standardized protocol for the biocompatibility test with Artemia salina; in the medium and long term, it is expected that this research will be accepted by different researchers, thus contributing to biocompatibility studies of new biomaterials. Research with Artemia salina does not have environmental or legal restrictions since they are not protected or invasive species; they are generally used to feed aquarium fish; The solutions used are based on sodium chloride in concentrations that allow the survival of these organisms. It does not impact the environment since the waste generated is not polluting, and the protocols indicate strict and coordinated management for waste collection during the experiments.

The study has limitations; the Artemia salina test is carried out with invertebrates. Therefore, it should be considered as a preliminary biocompatibility test; in the future, cytotoxicity tests with cells should be performed.

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DECLARATION ON CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHORS' CONTRIBUTION

First, second and third author: Fieldwork and correction of the manuscript.
Fourth author: Drafting, statistical analysis, and correction of the manuscript.
Fifth author: Drafting of the manuscript.

Sixth author: Drafting, monitoring, and approval.

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