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BIOCOMPATIBILITY OF TRICRESOL FORMALIN: A CYTO- AND GENOTOXIC ANALYSIS IN HUMAN MONONUCLEAR CELLS

BIOCOMPATIBILIDADE DO TRICRESOL FORMALINA: UMA ANÁLISE CITO- E GENOTÓXICA EM CÉLULAS MONONUCLEARES HUMANAS

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ABSTRACT

Irrigations solutions and techniques did not show evidences of a complete desinfection of root canal system, in pulpar necrosis. Therefore, the use of intracanal medications between sessions is recommended. Cytotoxicity studys did not show convergence of results about the cytogenotoxic effects of tricresol formalin. The goal of this study was to evaluate the biocompatibility of tricresol formalin on PBMC in culture through cell viability, lipid peroxidation, protein carbonylation and DNA damage assays. Twelve human permanent mandibular incisors were used. After endodontic preparation at 11 and 15 millimeters root lenghts, an intracanal tricresol formalin medication was applied, the pulp chamber sealed and the tooth was positioned on a culture well for periods of 24 and 72 hours. The culture medium containing the cells was used as a negative control and as a positive control hydrogen peroxide was used in the medium. The results was tabulated and analyzed by the 1-way ANOVA, followed by the Tukey test. Statistical significance was set at 5%. Tricresol formalin did not show cytotoxicity at the distance of 15 millimeters and in a 24 hour incubation period ($P<0,05$). At the other distances and times it was considered to be significantly cyotoxic ($P<0,05$). In 24 hours of incubation, DNA damage was not registered in both lenghts ($P <0,05$). After 72 hours, the tested solution induced DNA damage in PBMC($P<0,05$). Therefore, tricresol formalin demonstrated significant cytotoxicity and genotoxicity, except under very specific conditions of low concentration and short exposure time.

Keywords: *Endodontics; Cell Survival; Mutagenicity Tests; DNA Damage; Formaldehyde.*

RESUMO

As técnicas de preparo e soluções irrigadoras não mostram evidências de uma completa desinfecção do sistema de canais radiculares nos casos de necrose pulpar, onde, é recomendado o uso de medicação intracanal entre sessões. Estudos de citotoxicidade não apresentam convergência de resultados quanto ao efeito citotóxico do tricresol formalina. O objetivo deste estudo foi avaliar os efeitos cito-genotóxicos do tricresol formalina em células mononucleadas de sangue periférico em cultura, através da viabilidade celular, peroxidação lipídica, carbonilação de proteínas e dano de DNA. Foram utilizados 12 incisivos inferiores permanentes humanos. Após o preparo endodôntico nos comprimentos radiculares de 11 e 15 milímetros, foi aplicada uma medicação intracanal de tricresol formalina no interior da câmara pulpar, selado a cavidade e

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posicionado o elemento dentário sobre um poço de cultura por períodos de 24 e 72 horas. O meio de cultura contendo as células foi utilizado como controle negativo e como controle positivo foi utilizado 100mM de peróxido de hidrogênio no meio. Os resultados obtidos foram tabulados e analisados pelo teste de variância (ANOVA) de 1 via, seguido pelo teste de Tukey. O nível de significância estatística foi estabelecido em 5%. O tricresol formalina não apresentou citotoxicidade significativa em 24 horas de incubação a uma distância de 15 milímetros do meio de cultura (P <0,05). Nos demais parâmetros de tempo e distância foi significantemente citotóxico (P<0,05). Em 24 horas de incubação, não foram registrados danos de DNA pela solução testada (P<0,05). Após 72 horas o tricresol formalina foi capaz de gerar danos de DNA em CMSP (P<0,05). Portanto, o tricresol formalina demonstrou citotoxicidade e genotoxicidade significativas, exceto em condições muito específicas de baixa concentração e curto tempo de exposição.

Palavras-chave: *Endodontia; Sobrevivência Celular; Testes de Mutagenicidade; Dano ao DNA; Formaldeído.*

1 INTRODUCTION

Even today, mechanical preparation techniques, even when combined with the use of irrigating solutions, do not show efficacy in the complete disinfection of the root canal system in cases of pulpal necrosis. This justifies the use of solutions that act as intracanal medication between sessions (Athanassiadis *et al*., 2015; Vander Wall; Dowson; Shipman, 1972). These solutions must present effective antimicrobial power, and it is also necessary that they be biocompatible with periradicular tissues under clinical use conditions (Lopes; Siqueira Junior, 2004; Vander Wall; Dowson; Shipman, 1972).

Tricresol formalin, composed of 90% formaldehyde and 10% cresol, remains in use in Brazil for endodontic treatments of necrotic teeth and those with periapical lesions (Lopes; Siqueira Junior, 2004). It possesses proven efficacy in its antimicrobial action (Menezes *et al*., 2004; Rosa *et al*., 2002; Silva *et al*., 2012), and its mechanism of action occurs through the release of formaldehyde vapors and the action of cresol - a highly lipophilic component (Milnes, 2008) - on fats, leading to the formation of lysol and the destruction of bacteria (Buckley, 1904).

Regarding the biocompatibility of this medication, studies have sought to evaluate the cyto- and genotoxic potential of its components, particularly formaldehyde, which have presented divergent results. Furthermore, the conditions of use in clinical practice were often not considered as parameters for conducting the studies, leading to varied and non-standardized methodologies.

Peripheral Blood Mononuclear Cells (PBMCs) have been applied for decades as biomarkers of cyto- and genotoxic effects, serving as an appropriate instrument for cytogenotoxicity assays. PBMCs are capable of reflecting recent damage, since, being abundant in the bloodstream, they can be exposed to any mutagenic agent (Maluf, 2011). In this context, the objective of this study was to evaluate the potential cyto-genotoxic effects of tricresol formalin on cultured PBMCs, through cell viability, lipid peroxidation, protein carbonylation, and DNA damage assays, utilizing a methodology that seeks to resemble the clinical use of this medication at two distinct distances and two different time periods.

The objective of this study was to evaluate the potential cyto-genotoxic effects of tricresol formalin on Peripheral Blood Mononuclear Cells (*in vitro* culture), considering the usage conditions recommended by Buckley (Buckley, 1904). Specifically, the study aimed to compare the cytogenotoxic effects of the substance at different distances from the culture medium (11 mm and 15 mm), as well as to analyze its action across distinct exposure periods, characterized as acute (24 hours of incubation) and chronic (72 hours of incubation).

2 METHODOLOGY

2.1 ETHICAL CONSIDERATIONS

This study was approved by the Research Ethics Committee of the Federal University of Santa Maria (CAAE number 61012816.1.0000.5346).

2.2 STUDY DESIGN, DATA COLLECTION, AND TEST GROUPS COMPOSITION

Twelve (12) extracted human permanent mandibular incisors were donated by the Human Permanent Teeth Bank of the Federal University of Santa Maria (BDPH-UFSM) and used in this study. The evaluation of the cyto-genotoxic potential was performed at two distances from the tricresol formalin (11 and 15 millimeters) to the PBMC culture and at two time points: acute (24 hours of incubation) and chronic (72 hours of incubation). The study groups were defined according to Chart 1.

Coronal access and chemo-mechanical preparation of the teeth were performed at the Endodontics Clinic of the Dentistry Course at the Federal University of Santa Maria, by a single, experienced Endodontics operator. The toxicity assays were carried out at the Cell Culture Laboratory of the Franciscan University Center, under the guidance of Prof. Dr. Michelle Rorato Sagrillo, professor of Medical Genetics and Human Genetics and researcher at the aforementioned institution. The tests were performed in triplicate for each dental element.

Chart 1 - Organization of the Study Groups.

Groups	Number of dental elements (n)	Root length (millimeters)	Intracanal medication	Period in culture medium (hours)
I	3	11	Tricresol Formalin	24
II	3	11	Tricresol Formalin	72
III	3	15	Tricresol Formalin	24
IV	3	15	Tricresol Formalin	72
V	-	-	Positive Control (C +) - Hydrogen Peroxide	24
VI	-	-	Positive Control (C +) - Hydrogen Peroxide	72
VII	-	-	Negative control (C-)	24
VIII	-	-	Negative control (C-)	72

Source: Construction of the Authors.

2.3 SIZING, PREPARATION, AND POSITIONING OF SPECIMENS

The specimens were standardized to the root lengths of the research groups, 11 and 15 millimeters, considering the average root length of mandibular incisors (Camargo *et al.,* 2013) and the average far-reaching action of tricresol formalin (Holland *et al*., 1978). To obtain the distances to be tested, the lengths of the sample teeth were measured with a caliper in the apico-incisal direction, receiving markings with the aid of a mechanical pencil on all dental surfaces at distances of 17 or 21 millimeters (root length + 6 mm coronally), according to the pre-established test group. Subsequently, a cut was made with the aid of a diamond disc (American Burs, Brazil) and the incisal portion was eliminated from the previously made marking, creating an incisal surface.

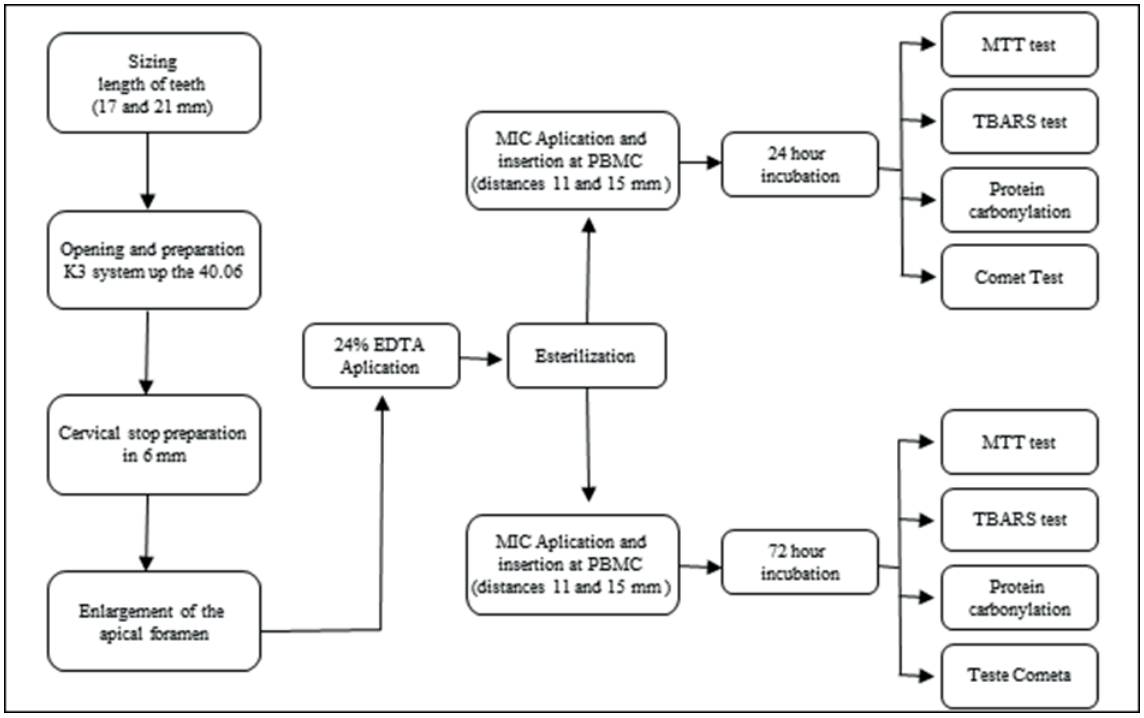
Coronal access was electively positioned at the most central portion of the incisal surface, performed with a spherical diamond bur 1014 (KG Sorensen, Brazil), and the outline shape was given using a multi-bladed tapered bur with an inactive tip, Endo Z (Microdont, São Paulo, Brazil), coupled to a high-speed handpiece (Kavo, Brazil), under constant irrigation. Root canal preparation was performed with the K3 continuous rotary system (Sybron Endo, USA) up to instrument 40.06 at a working length of 1 mm short of the apical foramen. With each instrument change, the canals were irrigated with 2 mL of 2.5% Sodium Hypochlorite solution (Nova Derme, Santa Maria, RS, Brazil), using a 5 mL syringe (Ultradent Products Inc., South Jordan, Utah, USA) and a 30 G Navi Tip (Ultradent Products Inc., South Jordan, Utah, USA) calibrated to 2 mm short of the working length with up-and-down movements of 2 to 3 mm amplitude. Simultaneously, aspiration was performed with a metal suction cannula (Golgran, Brazil). Apical patency was maintained with each instrument change by introducing a #10 K-file (Dentsply Maillefer, Brazil) up to the apical foramen. Largo drills II, III, and IV were measured and marked with a rubber stop at 6 millimeters, with the aid of a millimeter ruler, so that when used in increasing order of diameter, a shoulder was created which served as a stopper where the “cotton pellet” was accommodated. The apical foramen was enlarged with a manual #10 K-file (Dentsply Maillefer, Brazil), inserted in the apico-incisal direction, through the foramen, up to a distance of 15 millimeters, in order to standardize the medication exit orifice to a diameter of 0.40 millimeters.

After preparation, 24% Trisodium EDTA gel (Maquira, Brazil) was applied for 3 minutes under constant agitation, and the teeth were subjected to autoclaving (134ºC, 6 cycle, for one hour) for proper disinfection.

To optimize the handling of the *ex vivo* teeth, the specimens were immobilized by inserting them into custom containers fabricated from Eppendorf tubes (Eppendorf, Brazil) with a 10-millimeter diameter, stabilized by a metallic frame made of 1-millimeter diameter orthodontic wire (Morelli, Brazil). The containers were positioned so that their lower portion was located about 1 millimeter away from the cell culture. The teeth were positioned inside the Eppendorf tube, where their apex

passed 2 millimeters beyond its lower portion, with the apical region of the dental element immersed in the cell culture medium. The aforementioned steps are illustrated in a schematic flowchart in Figure 1.

Figure 1 - Flowchart for Sizing, Preparation, and Positioning of Specimens for Toxicity Assays



Source: Construction of the Authors.

2.4 TOXICITY ASSAYS

To evaluate *in vitro* cytotoxicity, the following tests were used: MTT assay, lipid peroxidation (TBARS), protein carbonylation, and the Comet assay to verify the genotoxicity of the compound.

Blood Collection for Toxicological Assays: Peripheral blood samples were obtained from three discarded samples from the Clinical Analysis School Laboratory of the Franciscan University Center, under approval by the Institutional Human Research Ethics Committee (CAAE: 31211214.4.0000.5306) with an absence of identification data. The samples were obtained through venipuncture using Vacutainer® tubes with heparin, which were used to separate the PBMCs for subsequent treatments in cell culture.

Separation of Lymphocytes and Monocytes: PBMCs separation occurred through density gradient centrifugation (Histopaque® - 1077), and a concentration of $2x10^5$ cells was obtained by counting in a Neubauer chamber using 0.4% Trypan blue.

Cell Viability Assay (MTT): Cell viability was analyzed according to Mosman (1983). Cytotoxic activity in PBMCs was evaluated by the colorimetric method, whose principle is based on the reduction of 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) into a dark purple product, MTT-formazan, by the mitochondrial enzyme tetrazolium-succinate-dehydrogenase.

Since conversion occurs only in viable cells, the decrease in the absorbance of the tests relative to the negative control indicates cell death. The experiment was performed in triplicate in a 96-well ELISA plate with the samples and controls as previously described. The plate remained in an incubator at 37 °C with 5% CO_2, for 24 and 72 hours, with samples analyzed in a spectrophotometer at 570 nm. The experiment was performed in triplicate, and the results were expressed as a percentage of the control.

Lipid Peroxidation (TBARS): The determination of lipid peroxidation was evaluated by determining thiobarbituric acid reactive substances (TBARS) according to the method described by Ohkawa *et al.* (Ohkawa; Ohishi; Yagi, 1979). After cell treatment, an aliquot of the cells was mixed with a reaction medium containing 2-thiobarbituric acid (TBA) (0.8%) and incubated at 95 °C for 1 hour. Absorbances were measured at a wavelength of 532 nm in a spectrophotometer.

Protein Carbonylation: Oxidative damage to proteins was measured by determining the formation of carbonyl groups based on the reaction with dinitrophenylhydrazine as previously described by Levine et al. (Levine *et al.,* 1990). After homogenizing the samples in carbonyl buffer (120 mM KCl, 30 mM KH_2PO_4), they were centrifuged at 7000 g for 15 min at 4 °C and lysed in 20% TCA. After lysis, the samples were centrifuged again at 14000 g for 5 min. The pellet was resuspended in 100 µL of 0.2 M NaOH, and the samples were incubated for 1 hour at room temperature with 2 M HCl (1:3) supplemented with 10 mM DNPT. After the incubation period, 100 µL of 20% TCA was added, and the samples were centrifuged at 14000 g for 3 min. The pellet was washed with 500 µL of ethanol-ethyl acetate. The samples were incubated for 30 min at 60 °C, and centrifuged again at 14000 g for 3 min. The carbonyl formation content was determined spectrophotometrically at 370 nm.

Comet Assay: The Comet assay was performed according to Singh and collaborators (1988) (Singh et al., 1988), modified by García and collaborators in 2004 (García *et al.,* 2004). This test has high sensitivity and makes it possible to quantify the levels of DNA single-strand breaks.

The isolated PBMCs, suspended in Low Melting agarose, were deposited onto a glass slide previously covered with a layer of 1.5% agarose. The material was immersed in lysis solution (89 mL of lysis solution to 10 mL of Dimethyl sulfoxide and 1 mL of Triton X-100) to remove the cell membranes and cytoplasm. Subsequently, the slides were incubated in alkaline electrophoresis buffer (300 mM NaOH and 1 mM EDTA in distilled water) and subjected to electrophoresis for about 30 minutes, at 25V and 300mA. Subsequently, neutralization, fixation, and staining processes were performed so that the genetic material could be analyzed.

The analysis of each slide was performed under an optical microscope, and the cells were classified according to the image shape into four damage classes proposed by García and collaborators (García *et al*., 2004), ranging from 0 (no damage) to 4 (maximum damage), also including the classification of cellular apoptosis.

2.5 TREATMENTS

The proposed treatment consisted of applying tricresol formalin using the technique recommended by Buckley (Buckley, 1904) - and still utilized in clinical practice today.

For standardization, sterile cotton pellets (autoclaved at 134ºC, cycle 6, for 1 hour) compatible with the fabricated "cervical stop" were used. These were soaked in 20 microliters of tricresol formalin (Iodontosul, Brazil) with the aid of an electronic pipette (BioPet, Brazil). The excess substance was removed from the assembly using sterile gauze until it left no residue mark on an absorbent paper measuring 20 millimeters in width by 20 millimeters in length, estimating a final solution volume of 5 microliters. This volume is reported as non-toxic to periapical tissues under use conditions in a previous study by Wesley *et al*. (1970).

The cotton pellet containing the treatment was placed at the entrance of the root canal, at the level of the most apical portion of the "cervical stop," and immediately sealed at its superior extremity with a 1-millimeter-long layer of zinc oxide-based cement (CaiTHEC, Brazil) and subsequently with a 3-millimeter-long layer of glass ionomer cement (DFL, Brazil) following the manufacturer's recommended application protocol.

Immediately thereafter, the specimens were positioned as described above and immersed in the culture medium. They were then kept incubated, for the test periods, in a humidified incubator at 95% air/5% CO_2 at 37ºC.

The culture medium containing the cells was used as a negative control, and the culture medium supplemented with 100 mM hydrogen peroxide was used as a positive control.

2.6 STATISTICAL ANALYSIS

The obtained results were tabulated and analyzed using one-way Analysis of Variance (ANOVA), followed by the Tukey test. The level of statistical significance was established at 5% ($\alpha = 0.05$). The analyses were performed using the SPSS Statistics 18 Software (SPSS, Illinois, United States).

3 RESULTS

In *in vitro* cyto- and genotoxicity tests, cytotoxicity was evaluated by the MTT, lipid peroxidation (TBARS), and protein carbonylation assays, and genotoxicity of the compound was verified by the Comet assay. The obtained results are presented in Figures 2 to 9.

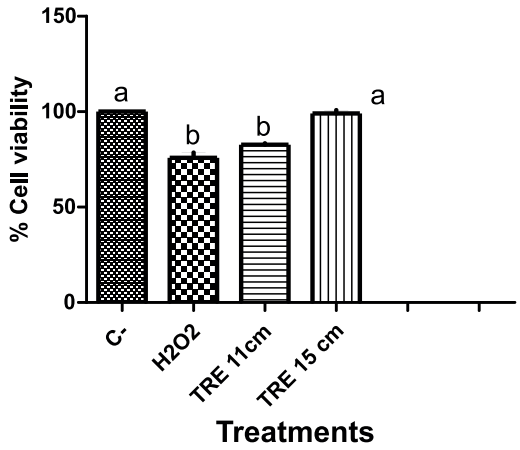
Tricresol formalin, after 24 hours of incubation, significantly decreased cell viability to 82.73% at the 11-millimeter distance, whereas it was not considered cytotoxic at the 15-millimeter distance, where it maintained 99.07% cell viability after treatment ($P < 0.05$) (Figure 2). In the 72-hour

incubation period, both distances significantly decreased cell viability to 56.23% and 58.87% at 11 and 15 millimeters, respectively ($P < 0.05$) (Figure 3).

At neither distance nor incubation period did tricresol formalin present alterations in the levels of lipid peroxidation (TBARS) or protein carbonylation (Figures 4-7). The results of both assays were considered significantly different ($P < 0.05$) compared to the negative control.

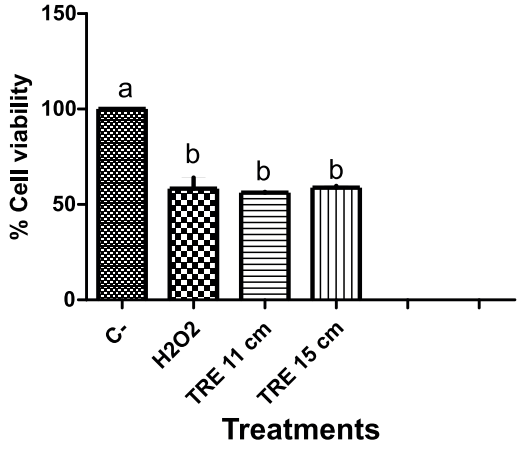
After 24 hours of incubation, tricresol formalin did not cause DNA damage in PBMCs at either distance verified by the Comet assay ($P < 0.05$) (Figure 8). However, after 72 hours of incubation, the assays revealed that the compound, at both 11 and 15 millimeters of distance, presented a DNA damage index considered significant ($P < 0.05$) (Figure 9).

Figure 2 - Cell Viability Assay (MTT) after 24 hours of incubation



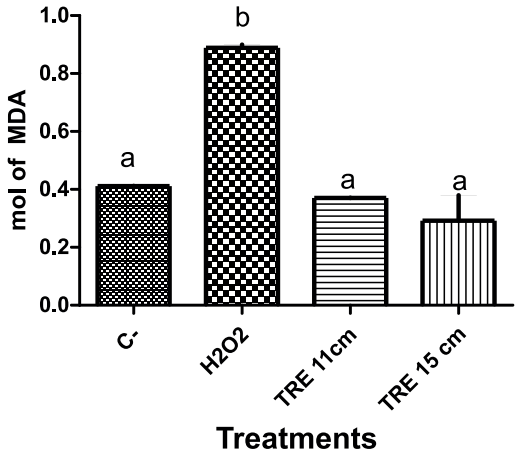
MTT assay after 24 hours of incubation. Results expressed as a percentage of the negative control (100%). Negative control (C-): cells in culture medium; Positive control (C+): cells with H_2O_2. Data expressed as mean ± standard deviation (S.D.). Means followed by the same letter do not differ statistically from each other. Analyses were performed by one-way analysis of variance (ANOVA), followed by the Tukey test. Values with $P < 0.05$ were considered statistically significant.

Figure 3 - Cell Viability Assay (MTT) after 72 hours of incubation



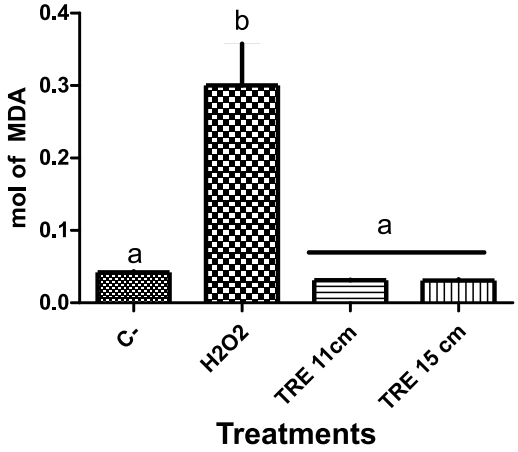
MTT assay after 72 hours of incubation. Results expressed as a percentage of the negative control (100%). Negative control (C-): cells in culture medium; Positive control (C+): cells with H_2O_2. Data expressed as mean ± standard deviation (S.D.). Means followed by the same letter do not differ statistically from each other. Analyses were performed by one-way analysis of variance (ANOVA), followed by the Tukey test. Values with $P < 0.05$ were considered statistically significant.

Figure 4 - Lipid Peroxidation Assay (TBARS) after 24 hours of incubation



TBARS assay after 24 hours of incubation. Results expressed in mol of MDA. Negative control (C-): cells in culture medium; Positive control (C+): cells with H_2O_2. Data expressed as mean ± standard deviation (S.D.). Means followed by the same letter do not differ statistically from each other. Analyses were performed by one-way analysis of variance (ANOVA), followed by the Tukey test. Values with $P < 0.05$ were considered statistically significant.

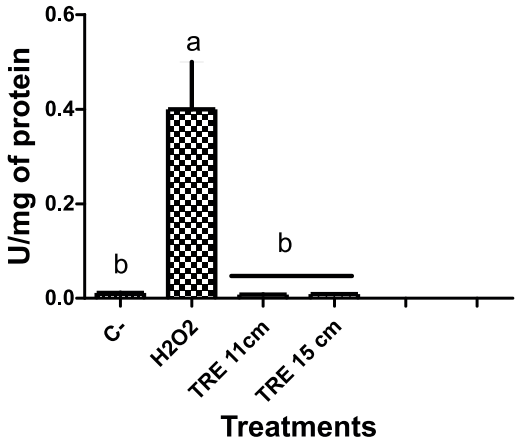
Figure 5 - Lipid Peroxidation Assay (TBARS) after 72 hours of incubation



TBARS assay after 72 hours of incubation. Results expressed in mol of MDA. Negative control (C-): cells in culture medium; Positive control (C+): cells with H_2O_2. Data expressed as mean ± standard deviation (S.D.). Means followed by the same letter do not differ statistically from each other.

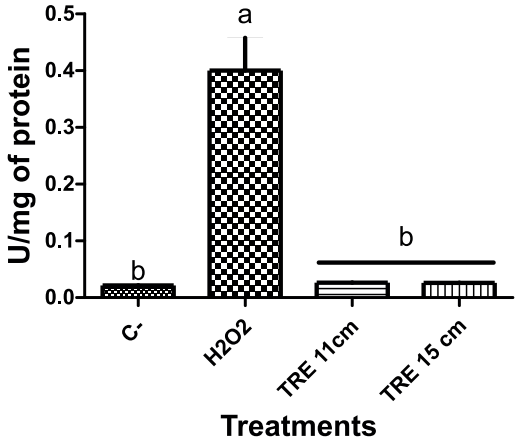
Analyses were performed by one-way analysis of variance (ANOVA), followed by the Tukey test. Values with $P < 0.05$ were considered statistically significant.

Figure 6 - Protein Carbonylation Assay after 24 hours of incubation.



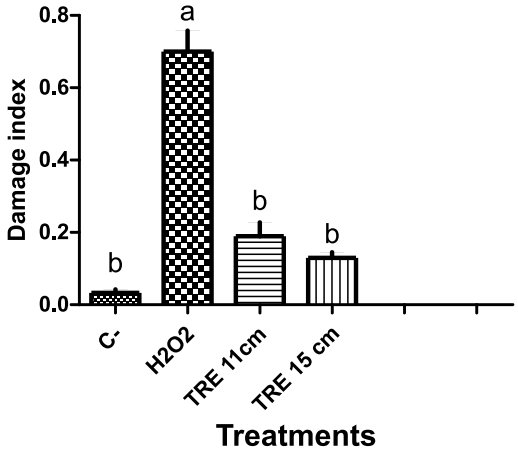
Protein carbonylation assay after 24 hours of incubation. Results expressed in units per mg of protein. Negative control (C-): cells in culture medium; Positive control (C+): cells with H_2O_2. Data expressed as mean ± standard deviation (S.D.). Means followed by the same letter do not differ statistically from each other. Analyses were performed by one-way analysis of variance (ANOVA), followed by the Tukey test. Values with $P < 0.05$ were considered statistically significant.

Figure 7 - Protein Carbonylation Assay after 72 hours of incubation.



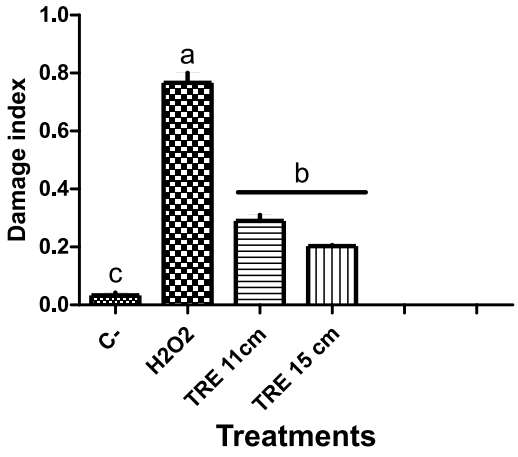
Protein carbonylation assay after 72 hours of incubation. Results expressed in units per mg of protein. Negative control (C-): cells in culture medium; Positive control (C+): cells with H_2O_2. Data expressed as mean ± standard deviation (S.D.). Means followed by the same letter do not differ statistically from each other. Analyses were performed by one-way analysis of variance (ANOVA), followed by the Tukey test. Values with $P < 0.05$ were considered statistically significant.

Figure 8 - Comet Assay after 24 hours of incubation.



Comet assay after 24 hours of incubation. Results expressed in DNA damage index. Negative control (C-): cells in culture medium; Positive control (C+): cells with H_2O_2. Data expressed as mean ± standard deviation (S.D.). Means followed by the same letter do not differ statistically from each other. Analyses were performed by one-way analysis of variance (ANOVA), followed by the Tukey test. Values with $P < 0.05$ were considered statistically significant.

Figure 9 - Comet Assay after 72 hours of incubation.



Comet assay after 72 hours of incubation. Results expressed in DNA damage index. Negative control (C-): cells in culture medium; Positive control (C+): cells with H_2O_2. Data expressed as mean ± standard deviation (S.D.). Means followed by the same letter do not differ statistically from each other. Analyses were performed by one-way analysis of variance (ANOVA), followed by the Tukey test. Values with $P < 0.05$ were considered statistically significant.

4 DISCUSSION

Tricresol formalin has been used in endodontics since the 20th century (Ribeiro *et al*., 2004; Silva *et al.,* 2012) and is indicated in cases of pulpal necrosis with the presence of periapical lesion and in emergency procedures (Lopes; Siqueira Junior, 2004). Studies have been conducted regarding this drug and its components; however, controversy still exists regarding its biological compatibility under clinical use conditions (Athanassiadis *et al*., 2015; Goldmacher; Thilly, 1983; Kreiger; Garry, 1983; Natarajan *et al*., 1983).

Formaldehyde-based solutions, such as tricresol formalin, have been reported as potentially cyto- and genotoxic solutions due to the release of substances through their vaporization (Athanassiadis *et al.,* 2015; Ribeiro; Marques; Salvadori, 2004; Tai *et al*., 2002). No single experiment is capable of determining the true biological response of biomaterials (Hauman; Love, 2003; Leirskar; Helgeland, 1981); however, despite their limitations, laboratory tests are suitable for verifying mechanisms involved in biocompatibility (Leirskar; Helgeland, 1981). Therefore, this study performed four *in vitro* tests related to biological compatibility, providing data on the safety of using tricresol formalin as an intracanal medication.

The cells used were Peripheral Blood Mononuclear Cells (lymphocytes and monocytes). PBMCs have been applied for decades in cyto- and genotoxicity studies, as they are biomarkers of primary damage (Ciapetti *et al.,* 1993; Maluf, 2011). Furthermore, considering that solutions used as intracanal medication can diffuse into the bloodstream by penetrating the dentinal tubules, periodontium, and ramifications of the root canal system (Bartelstone, 1951; Vander Wall; Dowson; Shipman, 1972), these cells are capable of representing the damage these drugs may cause to humans.

The MTT assay is a test that expresses biomaterial cytotoxicity by measuring the cell viability of a sample (Ciapetti *et al*., 1993). Cell viability was verified by exposing the cultured PBMCs at 11 and 15 millimeters distance to the tested solution for periods of 24 and 72 hours. Our findings show that tricresol formalin presented no significant cytotoxicity only at the 15-millimeter distance from the culture medium and in a 24-hour incubation period. In the other parameters, the drug presented significant cytotoxicity ($P < 0.05$). However, the MTT test cannot be considered in isolation as a parameter to define whether a material is biocompatible or not, as it represents only one aspect of biological compatibility (Peters, 2013). The data found can be justified by the presence of formaldehyde - a component of tricresol formalin - and its release in vapor form, which is considered a cytotoxic and mutagenic material, showing significant cytotoxicity in previous studies that used lymphocytes and Chinese hamster ovary cells (CHO cells) (Goldmacher; Thilly, 1983; Kreiger; Garry, 1983; Natarajan *et al*., 1983).

Lipid peroxidation is damage to the cell membrane resulting from oxidative stress (Saghiri *et al.,* 2011). The oxidative stress induction test (TBARS) is based on the reaction of thiobarbituric acid

with the decomposition products of hydroperoxides. In the TBARS test, tricresol formalin did not alter lipid peroxidation levels at either incubation period or distance ($P < 0.05$). This data contrasts with the results of the MTT test. This may be explained by the repair in the balance of oxidation-reduction reactions, and the damage to the lipid layers not being sufficient to cause cell death, only affecting the biological mechanism of the cells.

Oxidative damage to proteins, carbonylation, was measured by the formation of carbonyl groups based on the reaction with dinitrophenylhydrazine as previously described by Levine *et al*. (1990). Our results indicated no significant alteration in protein carbonylation levels ($P < 0.05$). The findings suggest that tricresol formalin, under this methodology, did not generate sufficient damage to cause cell death due to an increase in free radicals in the medium from increased protein carbonylation levels. No studies were found in the literature regarding tricresol formalin or its components that evaluated oxidative stress in proteins or lipids under the conditions proposed by the methodology applied in this study.

The Comet assay is a standard, easily executed method for evaluating DNA damage with high sensitivity (García *et al*., 2004). Genotoxicity tests are essential for obtaining data on genetic damage and can be important indicators of carcinogenesis, as these tests are capable of measuring an initial tumor process (Ribeiro, 2008). In this study, no DNA damage was registered by the tested solution at the 11 and 15 millimeter distances after 24 hours of incubation ($P < 0.05$). After 72 hours, tricresol formalin was capable of generating DNA damage in PBMCs at both distances by the Comet assay ($P < 0.05$). Our data after 24 hours corroborate results found in previous studies by Ribeiro (2004 and 2006), Nishimura (2008), and Ramos (2008). Specifically, the data measured after 72 hours show that tricresol formalin can induce DNA damage, reinforcing the recommendation of Bernabé; Holland; Souza (1972), who histologically evaluated the responses of periapical tissues to tricresol formalin in dogs and concluded that the compound is not indicated to remain in the pulp chamber for more than two days.

In vitro tests are essential for understanding the effects of biomaterials widely used in Dentistry. Although they have great sensitivity and are important markers for clinical conduct, they are limited in simulating the conditions of use of the tested materials. In the human body, cyto- and genotoxic effects may present differently, since cells have repair capacity, the substrate where the intracanal medication is applied is colonized by bacteria and other organisms, and the diffusion of vapors through periapical tissues may occur quite differently than in a cell culture medium (Botton *et al.,* 2016; Pires *et al.,* 2016; Vander Wall; Dowson; Shipman, 1972). Additionally, Mohorn *et al.* (1971) verified that the effect of positive and negative pressure in the periapical region of a necrotic tooth can affect the movement of the drug through the root canal system and apical foramen.

Furthermore, human beings produce endogenous formaldehyde as part of their cellular metabolism. Human cells are equipped to deal with this exposure through multiple oxidation mechanisms,

aiming to incorporate it as a biological macromolecule (Milnes, 2008). Therefore, additional *in vitro* and *in vivo* animal studies are necessary to reproduce the clinical use conditions of formaldehyde-based substances and to verify, in addition to the toxic effects of the compound, the reparative effects of cells post-treatment.

The results of this study, which point to the significant cito-genotoxicity of tricresol formalin depending on the exposure time and distance, reinforce the consolidated trend in recent scientific literature regarding the urgent need to replace conventional agents containing formaldehyde. Formaldehyde is a recognized cytotoxic and mutagenic agent, and the recording of DNA damage in PBMCs after 72 hours echoes the warnings from systematic reviews regarding its biological risks. In this context, the frontier of nanobiotechnology emerges as a promising solution, given that recent studies,such as Barcellos *et al*., 2021, have demonstrated encouraging results by incorporating tricresol formalin into nanostructured formulations. Such works indicate that this approach drastically mitigates the cito- and genotoxic effects in *in vitro* assays while simultaneously enhancing its antimicrobial efficacy, proving particularly important in combating resistant microorganisms. This innovation represents a robust advancement in the development of new endodontic products, aiming to reconcile high effectiveness with improved biocompatibility and establish a new standard of treatment.

5 CONCLUSION

This study performed four *in vitro* tests related to biological compatibility, providing safety data concerning the use of tricresol formalin as an intracanal medication. We concluded that tricresol formalin did not show significant cytotoxicity at a distance of 15 millimeters from the culture medium after 24 hours of incubation. However, in the remaining time (72 hours) and distance (11 mm) parameters evaluated, the compound was significantly cytotoxic. Regarding genotoxicity, no DNA damage was recorded by the tested solution at 24 hours of incubation, but, after 72 hours, tricresol formalin was capable of generating DNA damage in PBMCs. Therefore, tricresol formalin demonstrated significant cytotoxicity and genotoxicity, except under very specific conditions of low concentration and short exposure time.

The limitations of this study involve the *in vitro* model, which restricts extrapolation to the *in vivo* context, the use of PBMCs (a systemic, non-local model), and the absence of precise quantification of the released Tricresol Formalin concentration. For further investigation, future research should compare the cito-genotoxicity of the compound with modern medications, such as Calcium Hydroxide and chlorhexidine, quantify its concentration-dependent release, and employ more tissue-specific cell lines for better simulation of local effects

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