DOI: https://doi.org/10.17921/2447-8938.2023v25n4p232-238

Experimental Medications for Using in Endodontics: Cytotoxicity, Genotoxicity and Mutagenesis Analysis

Medicamentos Experimentais para Uso em Endodontia: Análise de Citotoxicidade, Genotoxicidade E Mutagênese

Graziela Garrido Mori[ab]; Thais Akemi Sako[a]; Nathália Evelyn da Silva Machado[b]; Fabíola de Mello Azevedo[c]; Flavia Beltrão Pires[d];Larissa Dragonetti Bertin[d]; Paulo Roberto Goldoni[e]; Rosana Leal do Prado[ab]; Carolina dos Santos Santinoni[ab]; Luciana Prado Maia*[ab]; Gisele Alborghetti Nai[ab]

[a]Universidade do Oeste de São Paulo, Dental School of Presidente Prudente. SP, Brazil.
[b]Universidade do Oeste de São Paulo, Postgraduate Program in Dentistry. SP, Brazil
[c]Universidade do Oeste de São Paulo, Graduated Program in Animal Science. SP, Brazil.
[d]Unopar, Graduate Program in Dentistry. PR, Brazil
[e]Universidade do Oeste de São Paulo, Specialization course in Periodontics. SP, Brazil.
*E-mail: lucianapmaia@gmail.com

Abstract

The objective was to evaluate the cytotoxic, genotoxic and mutagenic properties of two experimental medication in Endodontics. For cytotoxic evaluation, fibroblast and osteoblast cells (1x104 cells/well) were plated and divided into groups conforming to the product added in culture medium: EM1 - 20 µL of experimental medication 1 (EM1); EM2 - 20 µL of experimental medication 2 (EM2); VE - 20 µL of vehicle used in medications; C - without product. The MTT assay was performed at 24, 48 e 72 hours for cytotoxic analysis. For genotoxic and mutagenic evaluation, 42 male rats were used. After 1 and 7 days of tubes containing EM1 or EM2, or empty (NC) were subcutaneously implanted, and after 1 day, a single dose of cyclophosphamide (CY) to be applied, the bone marrow was collected and submitted to comet and micronuclei assay. The significance level of 5% was considered for all statistical analysis. The viability of fibroblasts was <70% to both medications at 24h, and EM1 at 72h; at 72h, the proliferation cells was observed in EM2 (>100%). Both medications were non-cytotoxic to osteoblasts, and the EM2 stimulate the cell proliferation at 72h. The damage frequency of CY was statistically similar to EM1 and different to EM2 ($p<0.05$). The number of micronuclei was insignificant to EM1 and EM2 and no difference to group NC ($p>0.05$). Despite the absence of mutagenesis and non-cytotoxicity to osteoblasts, the EM1 was cytotoxic and genotoxic to fibroblasts. The EM2 was non-genotoxic, non-cytotoxic and non-mutagenic.

Keywords: Cytotoxicity Tests. Genotoxicity Tests. Mutagenicity Tests. Root Canal Medicament.

Resumo

O objetivo foi avaliar as propriedades citotóxicas, genotóxicas e mutagênicas de dois medicamentos experimentais em Endodontia. Para avaliação citotóxica, células fibroblásticas e osteoblásticas (1x104 células/poço) foram plaqueadas e divididas em grupos de acordo com o produto adicionado no meio de cultura: EM1 - 20 µL da medicação experimental 1 (EM1); EM2 - 20 µL da medicação experimental 2 (EM2); VE - 20 µL de veículo utilizado em medicamentos; C – sem produto. O ensaio MTT foi realizado aos 24, 48 e 72 horas para análise citotóxica. Para avaliação genotóxica e mutagênica foram utilizados 42 ratos machos. Após 1 e 7 dias foram implantados por via subcutânea tubos contendo EM1 ou EM2, ou vazios (NC), e após 1 dia, foi aplicada dose única de ciclofosfamida (CY), a medula óssea foi coletada e submetida ao ensaio de cometa e micronúcleos. O nível de significância de 5% foi considerado para todas as análises estatísticas. A viabilidade dos fibroblastos foi <70% para ambas as medicações às 24h e ao EM1 às 72h; às 72h, a proliferação de células foi observada em EM2 (>100%). Ambas as medicações foram não citotóxicas para os osteoblastos, e o EM2 estimulou a proliferação celular às 72h. A frequência de dano do CY foi estatisticamente semelhante ao EM1 e diferente do EM2 ($p<0,05$). O número de micronúcleos foi insignificante para EM1 e EM2 e não houve diferença para o grupo NC ($p>0,05$). Apesar da ausência de mutagênese e não citotoxicidade para osteoblastos, o EM1 foi citotóxico e genotóxico para fibroblastos. O EM2 era não genotóxico, não citotóxico e não mutagênico.

Palavras-chave: *Testes de Citotoxicidade. Testes de Genotoxicidade. Testes de Mutagenicidade. Medicamento para Canal Radicular.*

1 Introduction

One of the aims of intracanal medications is to collaborate with the control of microorganisms present in the root canal system in teeth with pulp necrosis[1,3]. In many clinical cases, especially in secondary or persistent infection, *Enterococcus faecalis (E. faecalis)* is present and organized in biofilms[3-5], being their elimination essential to clinical repair and establishes patient health[1,3].

Calcium hydroxide is the foremost intracanal medication used in the majority of endodontic cases[2], but it has been showing ineffective on *E. faecalis* biofilm[3,6]. Therefore, the efficient chemical-mechanical preparation with copious use of antimicrobial intracanal irrigants, as 2.5%-6% sodium hypochlorite or 2% chlorhexidine[7-10], and the study of new intracanal medications or biomaterials are crucial to clinical success[11-13].

Parhizhar et al.[12], in 2018, reviewed the literature and observed that the triple antibiotic paste (TAP) has been effective to eliminate diverse types of microorganisms. This paste is composed of an association of ciprofloxacin, metronidazole, and minocycline, and despite the antimicrobial effect, it permitted the development of resistant bacterial

strains and tooth discolouration.

Considering these aspects above, two new experimental medications, for applying in Endodontics as root canal medicament, were proposed. These new experimental medications are composed by an association of two antibiotics (metronidazole and one derived of tetracycline - doxycycline or minocycline, and one anti-septic (chlorhexidine) since all components have action on *E. faecalis*[7-9,14-17]. Machado et al.[13] demonstrated that both experimental medications were effective against *E. faecalis* biofilms, proving the antimicrobial action of these medications. Mendes et al.[11] evaluated the tooth discolouration and concluded that experimental medication containing doxycycline did not promote the tooth staining in opposite to experimental medication containing minocycline.

Those data could suggest the use of experimental medication in the endodontic clinic. However, studies to determine the biological properties are essential since the medications have contact with tissue adjacent to the root (periodontal ligament and bone)[18]. Having studied about those aspects, the present study was proposed. Thus, the objective of this study was to evaluate the cytotoxicity, genotoxicity and mutagenicity properties of both cited experimental medication.

2 Materials and Methods

2.1 Experimental medications

The experimental medications were idealized by Goldoni and, these were similar that those used by Mendes et al.[11] and Machado et al.[13]. The experimental medication 1 was composed of 0.2% chlorhexidine, 2.5% metronidazole and 2% doxycycline (Pharmacotechnical Formulas, Tupã, SP, Brazil), and the experimental medication 2 was composed of 0.2% chlorhexidine, 2.5% metronidazole, and 2% minocycline (Pharmacotechnical Formulas, Tupã, SP, Brazil).

2.2 Cytotoxicity Analysis

The cells used in this study were fibroblasts (L929 fibroblast, Mouse conjunctive tissue – ATCC CCL-1 NCTC - Instituto Adolfo Luiz – SP, Brazil) and osteoblast (OFCOLII, Mus musculus, Mouse, Balb/C - Bank of Cells from Rio de Janeiro – BCRJ, RJ, Brazil). Fibroblast cells were cultivated in growth media (GM) Minimum Essential Media (MEM) (GibcoTM – Invitrogen Corporation, Grand Island, USA) supplemented with 5% fetal bovine serum (FBS) (Cultilab, Brazil) and 1% antibiotic and antimycotic (Gibco, USA). Osteoblast cells were cultivated in GM Dulbecco Minimum Essential Media DMEM (GibcoTM – Invitrogen Corporation, Grand Island, USA) supplemented with 5% fetal bovine serum (FBS) (Cultilab, Brazil) and 1% antibiotic and antimycotic (Gibco, USA). Both cultures were kept at 37 °C, in a humidified atmosphere containing 5% CO2 and 95% air. After sub-confluence (80%), cultures were subcultured and plated in 96-well culture plates.

To evaluate in vitro cytotoxicity, the used protocol was based on the standards established by International Organization for Standardization (ISO 10993-5)[19]. For this, 96-well plates (Fisher Scientific, Pittsburg, PA, USA) were used, and four experimental groups for each type of cell (fibroblast and osteoblast) were defined: group EM1 (experimental medication 1) - 1x104 cells/well with standard culture medium without antibiotic plus 20 µL of experimental medication 1; group EM2 (experimental medication 2) - 1x104 cells/well with standard culture medium without antibiotic plus 20 µL of experimental medication 2; group VE (vehicle) - 1x104 cells/well with standard culture medium without antibiotic plus 20 µL of vehicle used in the experimental medications; group C (control) - 1x104 cells/well with standard culture medium without antibiotic.

The plates were incubated at 37 °C, 5% CO2, and the Thiazolyl blue tetrazolium bromide (MTT) assay was performed at 24, 48 e 72 hours. Aliquots of MTT at 5 mg/ml in phosphate-buffered saline solution (PBS; Gibco) were prepared and the primary cultures were then incubated with 10% solution in culture media for 4 hours at 37ºC, in a humidified atmosphere containing 5% CO2. In sequence, 100 µL of dimethyl sulfoxide solution (DMSO) was added to each well under stirring for 5 minutes for complete solubilization of the precipitate formed. Aliquots of 150 µL were changed to 96-well culture plates to colourimetric in a spectrophotometer (SpectraCount - Packard Instrument Company, USA) using a wavelength of 570 nm. Each experiment was made in triplicate.

2.3 Genotoxicity and Mutagenesis Analysis

This study was based on and adapted from a previous study by Nai et al.[20]. Forty-two male rats (Rattus, Norvegicus, Albinus, Wistar), 90-days-old, and weighing 180-200 g (the research was approved by the Institutional Review Board for Animal Experimentation – Protocol n.3980) were used in this study. The animals were maintained in the individual home cage, which was monitored and cleaned daily. Water and solid food were provided ad libitum during the experiment, except for food at twelve hours before the surgical interventions.

For surgical interventions, the animals were anaesthetized with ketamine hydrochloride (Sespo Indústria e Comércio Ltda, Paulínia, SP, Brazil) and xylazine hydrochloride (Sespo Indústria e Comércio Ltda, Paulínia, SP, Brazil), by intraperitoneal injection, and they were divided into four experimental groups: group EM1 (n=12) - the dorsal region of each rat was trichotomyzed, antiseptic was performed with 2% chlorhexidine (FGM Produtos Odontológicos, Joinville, Santa Catarina, Brazil), and the one sterile polypropylene tubes with 2 mm in diameter x 10 mm in length containing experimental medication 1 was subcutaneously implanted after the incision and dissection of subcutaneous tissue. The incision was sutured using 5-0 nylon (Ethicon Johnson &

Johnson, São Paulo, SP, Brazil); group EM2 (n=12) - similar to group EM1, except for the experimental medication, since in this group, the experimental medication 2 was used; group NC (n=12) - similar to group EM1, except for the experimental medication, since in this group, the empty tubes were used (negative control); and, group CY (n=6) - the animals received a single dose of cyclophosphamide (Genuxal, Baxter Oncology GmbH, Halle/Westfalen, Germany) (50 mg/kg), by intraperitoneal injection.

After 1 and 7 days, six rats of groups EM1, EM2 and NC, respectively, were euthanized with an excessive dose of anaesthetic. Rats of group CY was euthanized after 1 day[21]. In sequence, the femurs were removed and sectioned to collect the bone marrow, which was submitted to Comet and Micronucleus assays.

The evaluation of DNA damage by Comet assay was done as described by Scherer and Strohschoen[22]. Slides were placed in an electrophoresis chamber, covered with buffer, and incubated. Electrophoresis was performed in alkaline conditions (pH>13) at 25 V and a current of 300 mA for one hour. After, the samples were neutralized with Tris buffer, the slides were placed in a fixative solution and then washed. The samples were stained in a silver solution at 37 °C. After they were washed and placed in a stop solution for five minutes and washed again. After this process, slides were covered with a coverslip for analysis. So, 100 cells were analyzed, and the damage index may vary from 0 to 4. The damage frequency was determined as the percentual of cells with a tail, independent of their length[23]. A blind examiner performed the analysis.

For micronuclei analysis, slides were stained with Giemsa stain (Dolles, São Paulo, Brazil) and there was determined the number of micronuclei was present in 2000 polychromatic erythrocytes for each animal. Thus, a blind examiner counted 1000 per slide (2 slides for each animal) using the optical microscopic (400X magnification)[21]. The micronuclei were determined as the structures surrounding the nuclear membrane and a volume of less than one-third of the diameter of the associated nuclei; furthermore, the staining intensity of the micronuclei was similar to nuclei cell, and both structures were observed in the same focal plane21.

2.4 Statistical analysis

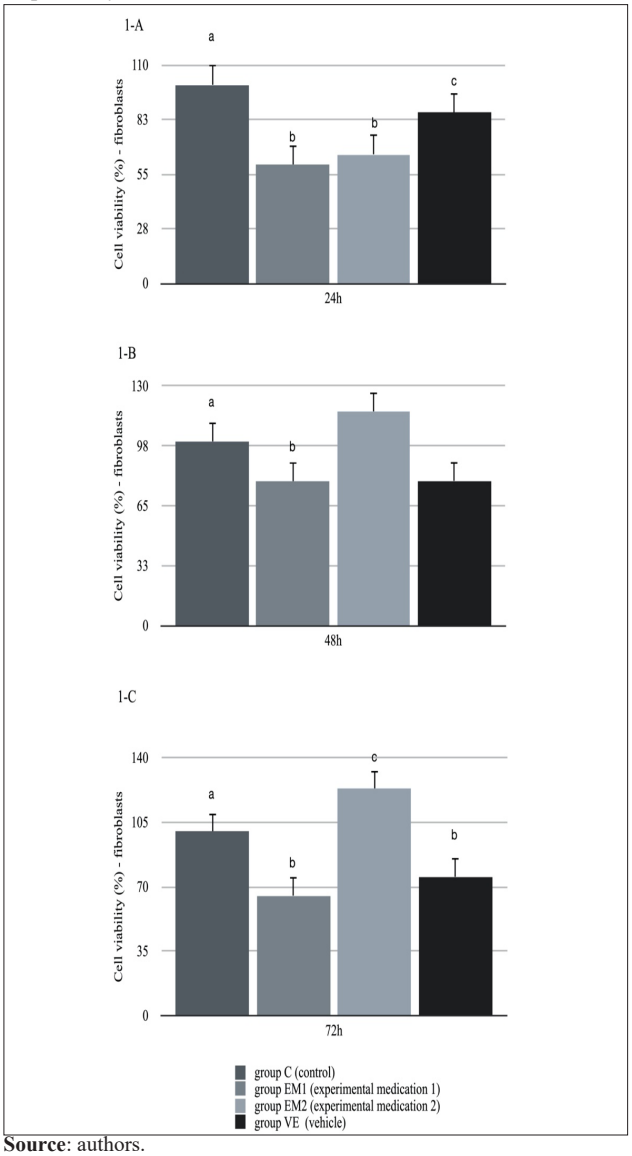
After verifying the assumptions of normality and homoscedasticity, for data referring to cytotoxicity, damage frequency and the Micronucleus test, the Kruskal-Wallis test was used, with Dunn's post hoc test. For the Comet assay, the data were arranged statistically employing a two-way analysis of variance, with Tukey's multiple comparison test. The tests were performed with the software R [25], and the significance level of 5% was considered for all analyzes.

3 Results and Discussion

3.1 Cytotoxicity Analysis

In fibroblast culture, the experimental medications presented a considerable reduction of viability cell at 24 hours since this was inferior of 70%, and the data were statistically significant difference to group C ($p<0.05$). Even without a statistically significant difference to group C at 48h, the experimental medication 1 (group EM1) had a cytotoxicity effect because the viability cell percentual was inferior of 70% at 72 hours (Figure 1-A). At 48 and 72 hours, the experimental medication 2 (group EM2) presented viability and proliferation cell considering the expansion of percentual cell and absence of statistically significant difference to group C (Figure 1-B and 1-C). The vehicle was a statistically significant difference to group C in all experimental times, but the viability cell percentual remained up of 70%, indicating the absence of cytotoxicity.

Figure 1 - Viability percentual of fibroblasts by experimental groups and periods, and statistically significant difference between those ($p<0.05$). In 1-A, 1-B and 1-C, different letters indicate statistically significant difference at 24h, 48h and 72h, respectively

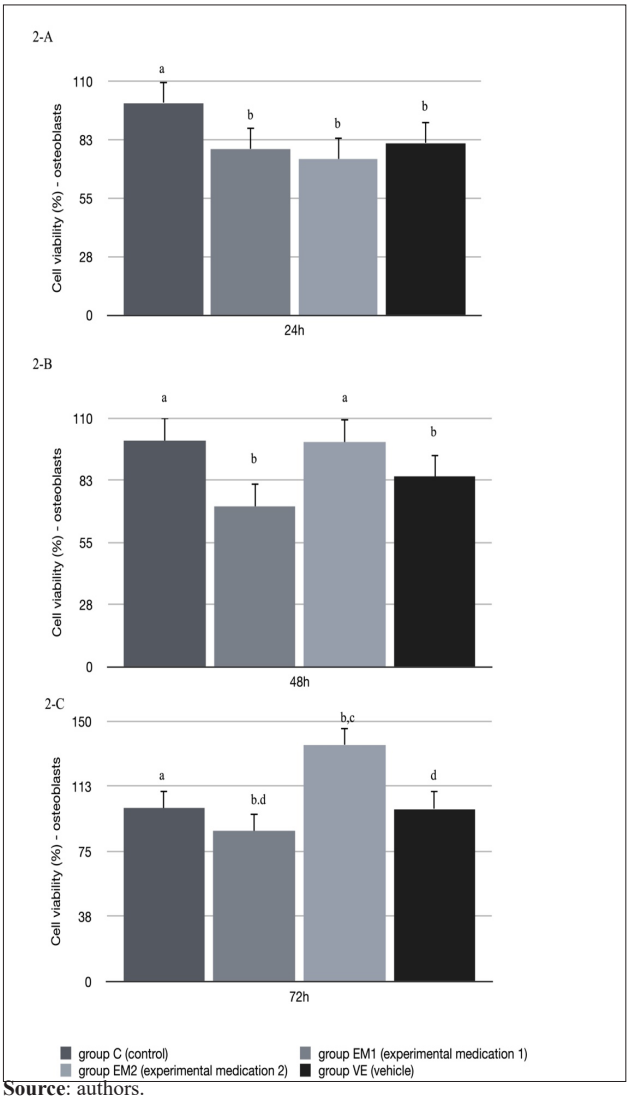


Source: authors.

In osteoblast culture, besides the statistically significant

difference of group EM1 with group C in all experimental periods (Figure 2), this group presented an absence of cytotoxicity effect because the viability cell percentual remained up to 70%. The experimental medication 2 (group EM2) presented viability cell in all experimental periods considering 70% of percentual minimum to its parameter (Figure 2); this group was a statistically significant difference to group C ($p<0.05$) at 24 and 72h, being that the latter a considerable proliferation was observed in relation of other experimental groups. The vehicle was a statistically significant difference to group C at 24 and 48h, but the viability cell percentual remained up of 70%, indicating the absence of cytotoxicity.

Figure 2 - Viability percentual of osteoblasts by experimental groups and periods, and statistically significant difference between those ($p<0.05$). In 2-A and 2-B, different letters indicate statistically significant difference at 24h and 48h, respectively; in 2-C, [a] with the difference of [b] and [c] with the difference of [d] at 72h.



Source: authors.

3.2 Genotoxicity and Mutagenesis Analyses

The details of DNA damage frequency by Comet assay can be observed in Table 1. The experimental medication 1 (group EM1) exhibited a similar damage frequency with group CY at 1 day and a different damage index with group EM2 (experimental medication 2) and NC (negative control) ($p<0.05$), independence the experimental periods. The experimental medication 2 (group EM2) performed a parallel index with group NC (negative control) in all experimental periods, and it was the difference to group CY. Details to each score of Comet assay and experimental groups and periods can be observed in Table 2. These data confirmed the absence of genotoxicity to EM2.

Table 1 - Mean (in percentual) and standard deviation (SD) of the damage frequency by comet assay according to experimental groups and periods

	1 day		7 days	
	Mean	**SD**	**Mean**	**SD**
Group EM1 (experimental medication 1)	67.00[a]	7.56	67.83[a]	6.73
Group EM2 (experimental medication 2)	53.67[b]	7.89	51.00[b]	6,54
Group NC (negative control)	55.00[b]	7.08	52.00[b]	8.06
Group CY (positive control)	79.40[a]	7.61	-	-

Different lowercase letters represent statistical difference in the column ($p<0.05$)

Source: research data.

Table 2 - Minimum and maximum number of each comet assay score according to experimental groups and periods

		1 day	7 days
Score 0	Group EM1 Group EM2 Group NC Group CY	0-10 0-20[a] 0-12[a] 9-24[b]	0-2[a] 2-10[b] 0-19[b]
Score 1	Group EM1 Group EM2 Group NC Group CY	0-28 10-26 16-34 7-16	3-11 10-26 17-31
Score 2	Group EM1 Group EM2 Group NC Group CY	0-10 6-12[a] 4-13 1-30[b]	7-8 9-17 5-12
Score 3	Group EM1 Group EM2 Group NC Group CY	0-10 3-13 3-6 1-17	9-13[a] 3-10[b] 3-10
Score 4	Group EM1 Group EM2 Group NC Group CY	5-50 2-19 2-9 0-6	19-25[a] 1-14[b] 2-15

Different lowercase letters represent statistical difference in the column to same score and same periods ($p<0.05$). Group EM1 = experimental medication 1; Group EM2 (experimental medication 2); Group NC (negative control); Group CY (positive control).

Source: research data.

The use of experimental medication 1 and 2 (groups EM1 and EM2) promoted an insignificant occurrence of micronuclei in all experimental periods without a statically difference to NC ($p<0.05$). Both medications showed different to group CY evidencing the absence of mutagenesis capacity (Table 3).

Table 3 - Minimum and maximum number of micronuclei according to experimental groups and periods

	1 day	7 days
Group EM1 (experimental medication 1)	0-0[a]	0-1[a]
Group EM2 (experimental medication 2)	0-2[a]	0-7[a]
Group NC (negative control)	0-1[a]	0-1[a]
Group CY (positive control)	6-11[b]	

Different lowercase letters represent statistical difference in the column ($p<0.05$)

Source: research data.

Evaluation of biocompatibility, cytotoxicity, genotoxicity and mutagenicity contribute considerably to indicate new medications and biomaterials in endodontics clinica[5,18,26,27]. The cytotoxic, genotoxic and mutagenesis assays provide initial data to use these medications[26,27] since these analyses demonstrate destructive effects, which can reduce the viability and growth of cells[28] or genetic damages link to some diseases including cancer[29-31]. Reflecting on these last parameters, the ideal biomaterial or medication must be no-cytotoxic and does not have genotoxic and mutagenic potential[26,27,32].

We chose the criteria established by the International Organization for Standardization (ISO 10993-5)[19] for analysis of cytotoxicity. A cytotoxic assay is a primary form for evaluating initial aspects of biological properties, and it should be associated with other assays to an assertive analysis[28]. Then, for expanding the biologic evaluation, we decided to include two analyses following the comet and micronuclei assays for genotoxicity and mutagenicity evaluation, respectively. These assays can measure DNA damage or genomic instability in safe and sensitive form, and when they are used in association permit results are more substantial[20,31,33-36].

In the present study, we evaluated two new experimental medications for applying in Endodontics. The components of these medications included 0.2% chlorhexidine, 2.5% metronidazole and 2% tetracycline derivate in addition to a viscous vehicle. So, the difference of experimental medications was the tetracycline derivative, since the doxycycline was used in experimental medication 1 (EM1) and minocycline in experimental medication 2 (EM2).

According to our data, the EM1 was cytotoxic to fibroblast cells since the viability of these cells was inferior 70% in this group at 24 and 72h, and the EM2 was not cytotoxic to fibroblast cells, including the increase of proliferation cell at 72h (Figure 1). Both medications were no-cytotoxic to osteoblasts; however, it was possible to notice the difference between EM1 and EM2 since the latter stimulates the cell proliferation at 72h (>100%).

Since that difference between medications is the tetracycline derivatives as explained above, probably, the difference in the behaviour of both medications could be attributed to this factor. Park[37], in 2012, observed in his study that doxycycline and minocycline are no-cytotoxic to osteoblast. And, Ma et al.[38] observed the proliferation of osteoblasts and increasement of alveolar crest height in the periodontitis model when the minocycline was used. These results can explain our data since both medications were no-cytotoxicy to osteoblasts and EM2 contains minocycline improved osteoblast proliferation.

Considering the genotoxic potential, which was evaluated in this study by Comet assay, EM1 was genotoxic instead of EM2. The Comet assay is a reliable method to determine DNA damage, and it is used routinely[20.23.35]. We could infer that the genotoxic potential was caused by chlorhexidine used as a component in medications. Ribeiro et al.[39] identified the genotoxic potential of chlorhexidine at concentrations varying from 0.01% to 1.00% on cells. And, in 2008, Ribeiro [29] confirmed this statement and highlighted that continuous exposures increase the risk. Barbin et al.[40], in 2013, demonstrated that 2% chlorhexidine converts itself in molecules as para-chloroaniline and reactive oxygen species (ROS), substances known as genotoxic[40,41]; and, the reduction of its concentration can collaborate to decrease this side effect[40,42]. Hence, interpreting these studies, the concentration in experimental medications used in our present was 0.2%. Furthermore, we deduced that 0.2% chlorhexidine was not responsible for the genotoxic potential of EM1 because a similar concentration of chlorhexidine was used in EM2, too. Probably, ionization reactions, the solubility of components or acid-base balance[43] that occurred in EM1 can be induced this undesirable effect.

Despite EM1 had genotoxic potential, the mutagenisis was not observed to this medication (Table 3). In according of Sommer et al.[36], in 2020, some DNA damage can repair and the fail or damage does not transfer during cell divides. So, we believe that it can occurred with EM1 since that the number of micronuclei was insignificant in this group. In relation to EM2, it was possible to establish the absence of mutagenesis capacity because these data were not different from the group NC.

Considering these results and antimicrobial action against *E. faecalis*[13], it is possible to infer the efficacy of EM2 to use in the endodontic clinic. However, this medication promoted the discolouration of the tooth[11], indicating the necessity of changes in its formula or attention in the cleaning of the tooth crown when it will be used.

4 Conclusion

Despite the absence of mutagenesis and non-cytotoxicity to osteoblasts of EM1, this medication was cytotoxic and genotoxic to fibroblasts. The EM2 was non-genotoxic, non-cytotoxic and non-mutagenic to osteoblasts and fibroblasts.

References

1. Vera J, Siqueira JF Jr, Ricucci D, Loghin S, Fernández N, Flores B, et al. One-versus two-visit endodontic treatment of teeth with apical periodontitis: a histobacteriologic study. J Endod 2012;38:1040-52. doi: https://doi.org/10.1016/j.

joen.2012.04.010.

2. Mohammadi Z, Dummer PM. Properties and applications of calcium hydroxide in endodontics and dental traumatology. Int Endod J 2011;44:697-730. doi: https://doi.org/10.1111/j.1365-2591.2011.01886.x

3. Neelakantan P, Romero M, Vera J, Daood U, Khan AU, Yan A, et al. Biofilms in endodontics: current status and future directions. Int J Mol Sci 2017;18:1748. doi: https://doi.org/10.3390/ijms18081748.

4. Zhang C, Du J, Peng Z. Correlation between Enterococcus faecalis and persistent intrarradicular infection compared with primary intraradicular infection: a systematic review. J Endod 2015;41:1207-13. doi: https://doi.org/10.1016/j.joen.2015.04.008.

5. Jacob VP, Paião LI, da Silva ACG, Magario MKW, Kaneko TY, Martins CM, et al. Antimicrobial action of NeoMTA Plus on mono-and dual-species biofilms of *Enterococcus faecalis* and Candida albicans: an in vitro study. Arch Oral Biol 2020;120:104925. doi: https://doi.org/10.1016/j.archoralbio.2020.104925

6. Upadya M, Shrestha A, Kishen A. Role of efflux pump inhibitors on the antibiofilm efficacy of calcium hydroxide, chitosan nanoparticles, and light-activated disinfection. J Endod 2011;37:142-6. doi: https://doi.org/10.1016/j.joen.2011.06.017.

7. Estrela C, Silva JA, de Alencar AH, Leles CR, Decurcio DA. Efficacy of sodium hypochlorite and chlorhexidine against Enterococcus faecalis- a systematic review. J App Oral Sci 2008;16:364-8. doi: https://doi.org/10.1590/S1678-77572008000600002.

8. Mohammadi Z, Abbott PV (2009) The properties and applications of chlorhexidine in endodontics. J Endod 42: 288-302. doi: https://doi.org/10.1111/j.1365-2591.2008.01540.x.

9. Gomes BP, Vianna ME, Zaia AA, Almeida JF, Souza-Filho FJ, Ferraz CC. Chlorhexidine in Endodontics. Braz Dent J 2013;24. doi: https://doi.org/10.1590/0103-6440201302188.

10. Ruksakiet K, Hanák L, Farkas N, Hegyi P, Sadaeng W, Czumbel LM. et al. Antimicrobial Efficacy of Chlorhexidine and Sodium Hypochlorite in Root Canal Disinfection: a systematic review and meta-analysis of randomized controlled trials. J Endod 2020;1032-41. doi: https://doi.org/10.1016/j.joen.2020.05.002.

11. Mendes LO, Machado NE, Goldoni PR, Mori GG. Evaluation of tooth discoloration after the use of experimental medication as root canal dressings: an in vitro study. Minerva Stomatol 2018;67:210-6. doi: https://doi.org/10.23736/s0026-4970.18.04150-x.

12. Parhizkar A, Nojehdehian H, Asgary S. Triple antibiotic paste: momentous roles and applications in endodontics: a review. Restor Dent Endod 2018;43:e28. doi: https://doi.org/10.5395/rde.2018.43.e28.

13. Machado NES, Mendes LO, Goldoni PR, Mori GG In vitro study of the antimicrobial action of experimental intracanal medications on *Enterococcus faecalis* biofilm. Dent Press Endod 2020;10:43-8. doi: https://doi.org/10.14436/2358-2545.10.3.043-048.oar.

14. Somayaji K, Acharya SR, Bairy I, Prakash PY, Rao MS, Ballal NV. In vitro scanning electron microscopic study on the effect of doxycycline and vancomycin on Enterococcal induced biofilm. Iran Endod J 2010;5:53-8. doi: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3487500/.

15. Bhangdia MB, Nandlal B, Vijaykumar GS, Kulkarni PK, Shanbhog R. Clinical evaluation of sustained-release metronidazole gel versus metronidazole solution as an intracanal medicament in abscessed primary molars. Eur Arch Paediatr Dent 2014;15:19-26. doi: https://doi.org/10.1007/s40368-013-0047-2.

16. Marickar RF, Geetha RV, Neelakantan P. Efficacy of contemporary and novel intracanal medicaments against *Enterococcus faecalis*. J Clin Pediatr Dent 2014;39:47-50. doi: https://doi.org/10.17796/jcpd.39.1.wmw9768314h56666.

17. Tagelsir A, Yassen GH, Gomez GF, Gregory RL. Effect of antimicrobials used in regenerative endodontic procedures on 3-week-old *Enterococcus faecalis* biofilm. J Endod 2015;42:258-62. doi: https://doi.org/10.1016/j.joen.2015.09.023.

18. Fonseca DA, Paula AB, Marto CM, Coelho A, Paulo S, Martinho JP, et al. Biocompatibility of root canal sealers: a systematic review of in vitro and in vivo studies. Materials (Basel) 2019;12:4113. doi: https://doi.org/10.3390/ma12244113.

19. ISO 10993-5. Biological evaluation of medical devices: Part 5: Tests for in vitro cytotoxicity. ISO; 2009.

20. Nai GA, Logar GA, Mori GG, Teixeira LM, Silva BC, Moraes AE, et al. Evaluation of the genotoxicity and mutagenicity of Ca3SiO5-based cement. Braz Oral Res 2016;30:e97. doi: https://doi.org/10.1590/1807-3107BOR-2016.vol30.0097.

21. MacGregor JT, Heddle JA, Hite M, Margolin BH, Ramel C, Salamone MF, et al. Guidelines for the conduct of micronucleus assays in mammalian bone marrow erythrocytes. Mutat Res 1987;189:103-12. doi: https://doi.org/10.1016/0165-1218(87)90016-4.

22. Scherer K, Strohschoen AAG. Padronização do teste cometa para análise de genotoxicidade como atividade de ensino para graduação na área da saúde. Rev Dest Acad 2013;5: 49-60.

23. Villela IV, de Oliveira IM, da Silva J, Henriques JA. DNA damage and repair in haemolymph cells of golden mussel (*Limnoperna fortunei*) exposed to environmental contaminants. Mutat Res 2006;605:78-86. doi: https://doi.org/10.1016/j.mrgentox.2006.02.006.

24. Tolbert PE, Shy CM, Allen JW. Micronuclei and other nuclear anomalies in buccal smears: methods development. Mutat Res 1991;271:69-77. doi: https://doi.org/10.1016/0165-1161(92)90033-i.

25. R Core Team. R: A language and environment for statistical computing. Vienna: R Foundation for Statistical Computing; 2017.

26. Hauman CH, Love RM Biocompatibility of dental materials used in contemporary endodontic therapy: a review. Part 1. Intracanal drugs and substances. Int Endod J 2003;36:75-85. doi: https://doi.org/10.1046/j.1365-2591.2003.00631.x.

27. Carvalho NC, Guedes SAG, Albuquerque-Júnior RLC, de Albuquerque DS, de Souza Araújo AA, Paranhos LR, et al. Analysis of *Aloe vera* cytotoxicity and genotoxicity associated with endodontic medication and laser photobiomodulation. J Photochem Photobio B 2018;178:348354. doi: https://doi.org/10.1016/j.jphotobiol.2017.11.027.

28. Peters OA. Research that matters: biocompatibility and cytotoxicity screening. Int Endod J 2013; 46:195-7. doi: https://doi.org/10.1111/iej.12047.

29. Ribeiro DA Do endodontic compounds induce genetic damage? A comprehensive review. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2008;105:251-6. doi: https://doi.org/10.1016/j.tripleo.2007.07.045.

30. Tubbs A, Nussenzweig A. Endogenous DNA damage as a source of genomic instability in cancer. Cell 2017;168:644-56. doi: https://doi.org/10.1016/j.cell.2017.01.002.

31. May JE, Donaldson C, Gynn L, Morse HR. Chemotherapy-induced genotoxic damage to bone marrow cells: long-term implications. Mutagenesis 2018;33:241-51. doi: https://doi.org/10.1093/mutage/gey014.

32. Dos Santos Costa FM, Fernandes MH, Batistuzzo de Medeiros SR. Genotoxicity of root canal sealers: a literature review. Clin Oral Investig 2020;24:3347-62. doi: https://doi.org/10.1007/s00784-020-03478-z.

33. Araldi RP, de Melo TC, Mendes TB, de Sá Júnior PL, Nozima BH, Ito ET, et al. Using the comet and micronucleus assays for genotoxicity studies: a review. Biomed Pharmacother 2015;72:74-82. doi: https://doi.org/10.1016/j.biopha.2015.04.004.

34. Ciğerci İH, Ali MM, Kaygısız ŞY, Kaya B, Liman R. Genotoxic Assessment of Different Sizes of Iron Oxide Nanoparticles and Ionic Iron in Earthworm (Eisenia hortensis) Coelomocytes by Comet Assay and Micronucleus Test. Bull Environ Contam Toxicol 2018;101:105-9. doi: https://doi.org/10.1007/s00128-018-2364-y.

35. Møller P. The comet assay: ready for 30 more years. Mutagenesis 2018;333:1-7. doi: https://doi.org/10.1093/mutage/gex046.

36. Sommer S, Buraczewska I, Kruszewski M. Micronucleus Assay: the state of art, and future directions. Inter J Mol Sci 2020;21:1534. doi: https://doi.org/10.3390/ijms21041534.

37. Park JB. Low dose of doxycyline promotes early differentiation of preosteoblasts by partially regulating the expression of estrogen receptors. J Surg Res 2012;178:737-42. doi: https://doi.org/10.1016/j.jss.2012.03.072.

38. Ma Y, Song J, Almassri HNS, Zhang D, Zhang T, Cheng Y, et al. Minocycline-loaded PLGA electrospun membrane prevents alveolar bone loss in experimental peridontitis. Drug Deliv 2020;27:151-60. doi: https://dx.doi.org/10.1080/10717544.2019.1709921.

39. Ribeiro DA, Scolastici C, De Lima PL, Marques ME, Salvadori DM. Genotoxicity of antimicrobial endodontic compounds by single cell gel (comet) assay in Chinese hamster ovary (CHO) cells. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2005;99:637-40. doi: https://doi.org/10.1016/j.tripleo.2004.07.010.

40. Barbin LE, Estrela C, Guedes DF, Spanó JC, Sousa-Neto MD, Pécora JD. Detection of para-chloroaniline, reactive oxygen species, and 1-chloro-4-nitrobenzene in high concentrations of chlorhexidine and in a mixture of chlorhexidine and calcium hydroxide. J Endod 2013;39:664-8. doi: https://doi.org/10.1016/j.joen.2012.10.018.

41. Liou GY, Storz P. Reactive oxygen species in cancer. Free Rad Res 2010;44:479-96. doi: https://doi.org/10.3109/10715761003667554.

42. Câmara De Bem SH, Estrela C, Guedes DF, Sousa-Neto MD, Pécora JD. Determination of chemical components derived from 2% chlorhexidine gel degradation using gas chromatography-mass spectrometry. Acta Odontol Scand 2014;72: 630-8. doi: https://doi.org/10.3109/00016357.2014.880941.

43. Newton DV. Drug incompatibility chemistry. Am J Health Syst Pharm 2009;66:348-57. doi: https://doi.org/10.2146/ajhp080059.