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Analysis of *Aloe vera* cytotoxicity and genotoxicity associated with endodontic medication and laser photobiomodulation☆

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ABSTRACT

This study aims to evaluate, *in vitro*, the effect of *Aloe vera* associated with endodontic medication, with or without laser photobiomodulation (FTL) irradiation in FP6 human pulp fibroblasts. The materials were divided into eight groups: CTR - control; CL - FTL alone; AA - *Aloe vera* with distilled water; AL - *Aloe vera* with distilled water and FTL; HA - calcium hydroxide P.A. with distilled water; HL - calcium hydroxide P.A. with distilled water and FTL; HAA - calcium hydroxide P.A. with *Aloe vera* and distilled water; HAL - calcium hydroxide P.A. with *Aloe vera*, distilled water, and FTL. The cytotoxicity was evaluated by MTT assay at 24, 48, and 72 h and the genotoxicity by micronucleus test assay. This study was performed in triplicate. Data obtained in both tests were statistically analyzed by ANOVA and Tukey's tests ($p \leq 0.05$). Group AA presented high genotoxicity and low cytotoxicity. After 24, 48, and 72 h, the group HAA significantly reduced the cell viability. Interaction with FTL showed slightly increase cell viability after 24 and 48 h in groups CL and HL ($p < 0.001$), despite the high genotoxicity in group CL and low genotoxicity in group HL. Group AL showed higher cell survival rate at 72 h ($p < 0.05$) and high genotoxicity ($p < 0.001$). It was concluded that *Aloe vera* allowed higher cell viability in human pulp fibroblasts in the presence of calcium hydroxide or with FTL separately, but genotoxicity increased in these associations.

1. Introduction

The main objectives of root canal therapy are removal of pathologic pulp, cleaning, disinfection of contaminated root canals, shaping and obturation of the root canal system in three dimensions to prevent re-infection [1]. Hence, the professionals must know the complexity of the root canal system (RCS) of teeth with apical periodontitis and periapical radiolucency, to choose the better technical in these cases, to associate substances and medications that disinfect dentinal tubules, and to promote repair of periapical tissues [2].

The presence of microorganisms in areas with a difficult instrument access such as isthmus, ramifications, lateral canals, apical accessories and delta canals, and deep into dentinal tubules can interfere in the reparation process, thus requiring medication in between sessions to reach these areas [3].

Calcium hydroxide, antiseptics, and antibiotics are the most used medications in endodontic practice [4]. These medications can eliminate bacteria that survival to biomechanical preparation, reduce periradicular inflammation, neutralize toxic products, and stimulate repair by mineralized tissue [5].

Cytotoxicity is a destructive effect of some material on the cells [6], and genotoxicity is a detection of genetic material damages such as DNA and chromosome breakage, genetic mutation, and change in the repair ability of DNA [7]. In order to justify its applicability, intracanal medication should present high antimicrobial activity and low cytotoxicity and genotoxicity, aiming to achieve a potential for eliminating bacteria without damaging periapical tissues [8,9].

Laser photobiomodulation is another resource used in dental

☆ Conflicts of interest: none.

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practice, acting as analgesic, anti-inflammatory, anti-edematous, and helping the tissue repair process [10]. The mechanisms of laser therapy irradiation include an increase of local microcirculation, angiogenesis, vasodilation, and inhibition of inflammatory mediators such as prostaglandins. Laser irradiation can avoid initiating the arachidonic acid cascade on damaged tissues, decreasing prostaglandin production [11].

It has been performed studies of new natural products with repair abilities aiming to support and accelerate the endodontic treatment in teeth with apical periodontitis and periapical radiolucency. The *Aloe vera* plant popularly known as "babosa" features among these products, keeping interesting properties to endodontic [12] such as anti-inflammatory and antibacterial action, as well as tissue repair stimulation [13]. Therefore, this study aims to analyze the cytotoxicity and genotoxicity of *Aloe vera* associated with endodontic medication with or without laser photobiomodulation irradiation in human pulp fibroblasts at 24, 48, and 72 h.

2. Materials and Methods

The entire research method was performed according to the guidelines of the International Organization for Standardization (ISO) 10993-5:2009 [14] and in triplicate. The cell bank of the Laboratory of Cell Biology of the São Paulo State University (UNESP), Campus São José, Brazil, provided the human pulp fibroblasts (FP6) lineage used in the research.

2.1. Division of Experimental Groups

The intracanal medication used was calcium hydroxide P.A (Biodinâmica Química e Farmacêutica LTDA, PR, Brazil) associated with *Aloe vera*, with or without laser photobiomodulation irradiation. The vehicle selected for this experiment was distilled water (Eurofarma Laboratorios LTDA, SP, Brazil), due to its biologically inactive property. Table 1 shows the groups description and the concentrations of the intracanal medications used in this study.

FTL: † laser photobiomodulation.

2.2. Preparation of Extracts

The *Aloe vera* leaves were obtained in the state of Sergipe, identified by voucher number ASE-37.261. A parenchyma scraping provided the gel, which was filtered, stored in sterile collector, wrapped in plastic film, and kept between − 18° to − 25° until freezing. Then, the sample was lyophilized. The *Aloe vera* pastes were manipulated in sterile Becker, stored in Falcon tubes protected with aluminum paper, and maintained at room temperature for 24 h. The same tube received 5 mL of DMEM culture medium supplemented with 10% SBF and penicillin/streptomycin; and incubated in 5% CO_2 at 37 °C, for 24 h.

Table 1
Description of groups and the concentrations of the intracanal medications.

Groups	Substances	Concentrations
CTR [Control)	Fibroblast culture medium	200 µL/well
CL	FTL †	–
AA	*Aloe vera*	4%
	Distilled water	
AL	*Aloe vera*	4%
	Distilled water	
	FTL †	
HA	Calcium hydroxide P.A.	90.9%
	Distilled water	
HL	Calcium hydroxide P.A.	90.9%
	Distilled water	
	FTL †	
HAA	Calcium hydroxide P.A.	16.39%
	Aloe vera	3.27%
	Distilled water	
HAL	Calcium hydroxide P.A.	16.39%
	Aloe vera	3.27%
	Distilled water	
	FTL †	

Table 2
Irradiation parameters for groups CL, AL, HL, and HAL.

Irradiation parameters	
Emission mode (CW)	Continuous
Length	ʎ660 nm
Active medium	InGaAlP
Laser optical power (output)	10 mW
Laser optical power [(nput)	40 mW
Beam area	4 mm^2
Power density (PD)	1 mW/cm^2
Energy density (ED)	3 J/cm^2
Irradiation time (by session)	3 s
Total energy per session (by well)	0.12 J
Beam divergence perpendicular to the junction	17°
Tip angle	50°

The specimens were produced with standardized shape and volume for all groups. Hence, the pastes were inserted in 5-mm diameter and 2-mm thick sterile polyethylene tubes [14]. A humid atmosphere conditioned the calcium hydroxide pastes at 37 °C with 5% CO_2 for 24 h so that all specimens would begin setting. After this period, still inside the polyethylene tubes, the medication was sterilized by ultraviolet radiation in the laminar flow chamber for 1 h to avoid contamination [15]. The *Eppendorf* tubes received 1 mL of DMEM culture medium supplemented with 10% SBF and penicillin/streptomycin, and were stored at 37 °C and 5% CO_2 for 24 h for the active products of the intracanal medication could release through the culture medium, producing eluates [8,15].

2.3. Parameters for Laser Irradiation

Laser irradiation was performed by a Twin-laser device (MMOptics™, Equipamentos Ltda., São Carlos, São Paulo, Brazil). The device was registered at the Brazilian National Health Surveillance Agency (ANVISA) under # 80051420007 and certified by the Brazilian Institute of Metrology, Standardization, and Industrial Quality (INMETRO) under # NCC 2756/05. The plates were irradiated 1 h after wells contacting eluates, and again with a 6-hour interval [16]. Table 2 describes irradiation parameters.

The desired application per well was 3 J/cm^2. It was standardized the design of experiments and developed a laser-adapted support for placing the plates to be irradiated. Knowing that the distance between the laser and the application surface is crucial, the distance between the laser beam and the cells was standardized. It was intercalated the wells to avoid energy overload due to the spreading characteristics of laser irradiation. A dark mask made of perforated matte cardboard was placed on the plate according to the location of wells, exposure only the area to be irradiated. After laser irradiations, the plates were incubated again at 37 °C in humid atmosphere with 5% CO_2 [16].

2.4. Cytotoxicity Test

Human pulp fibroblasts (FP6) were cultivated in DMEM (LGC Biotecnologia, Cotia, Brazil) supplemented with 10% SBF (Invitrogen, New York, USA) at 37 °C and 5% CO_2. Therefore, 8×10^3 cells were cultivated with 4 mL of cell medium for 24 h at 37 °C in atmosphere of 5% CO_2, in plates of 96 wells (Prolab, São Paulo, SP, Brazil). The culture medium present in the wells of the plates where fibroblasts adhered was removed and added 200 µL of the eluate of medication tested to each well. After, the eluates were removed and added 100 µL of MTT (3-(4,5-dimethylthiazol-2-il)-2,5-diphenyltetrazolium bromide) (Life Technologies, Carlsbad, USA) reagent solution, in each experimental

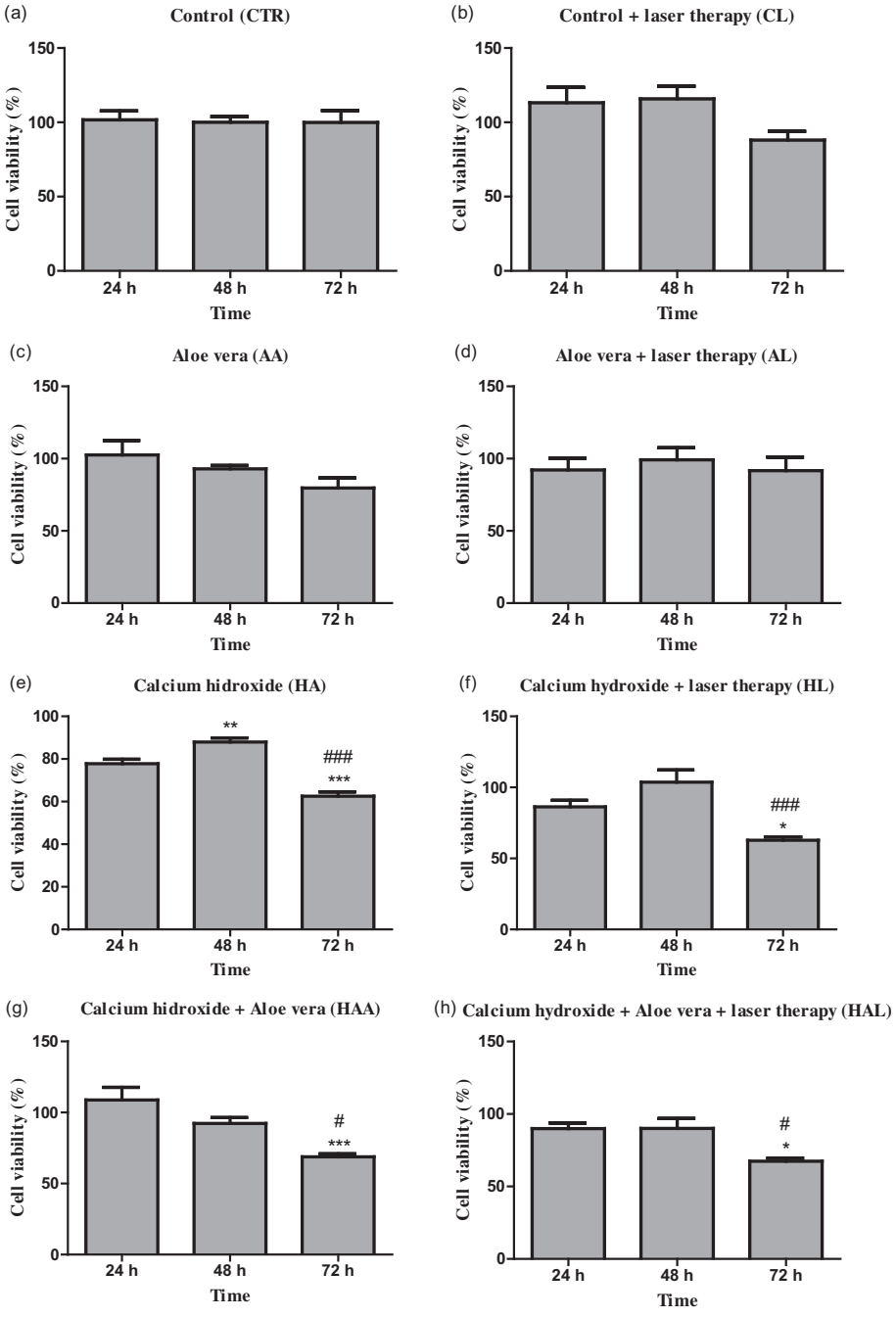

Fig. 1. Determination of percentage of cell viability of each group in relation to the control group at 24 h, 48 h and 72 h. Data expressed as mean ± mean standard deviation. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, significantly different from 24 h; # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ significantly different from 48 h (ANOVA and Tukey's post-hoc test).

period and the plates were incubated for 1 h. Then, the MTT solution was removed and added DMSO (Dimethyl Sulfoxide) for 10 min. The optical density of culture plates was measured in the spectrophotometer (Cambrex ELx808cse) at 570-nm wavelength, obtaining data by Gen5 Data Analysis Software (BioTek U.S. - World Headquarters, USA), with 10-s agitation protocol followed by the reading.

2.5. Genotoxicity Test

Genotoxicity was evaluated by the micronucleus test (FluoroShield with DAPI). For this, the human pulp fibroblasts (FP6) (2×10^4) were cultivated with 1 mL of DMEM supplemented with 10% SBF for 24 h at 37 °C in atmosphere of 5% CO_2, in plates of 24 wells (Prolab, São Paulo, SP, Brazil). Cells were exposed to eluates for 24 h, according to experimental groups. Next, the supernatant was discarded and performed washes with buffered saline solution (free from calcium and magnesium PBS-CMF) for removing non-viable cells. Then, the cells were fixed with 10% formaldehyde for 10 min. After washing, it was added 200 µL of PBS and a drop of FluoroShield with DAPI. The plate was agitated in orbital table (Solab, Piracicaba, São Paulo, Brazil) for 5 min under light protection. The micronuclei were analyzed in fluorescence microscope (100 ×), with potential assessment of 2.000 cells/plate per concentration, in at least three independent experiments.

2.6. Statistical Analysis

Cytotoxicity was measured by adding the absolute values of optical density obtained in the spectrophotometer and by calculating the mean of intracanal medications at each experimental period. The genotoxicity analysis was performed by fluorescence microscopy counting only mononucleate cells presenting micronuclei, mitosis and apoptotic cells. Data obtained in both tests were statistically analyzed by ANOVA

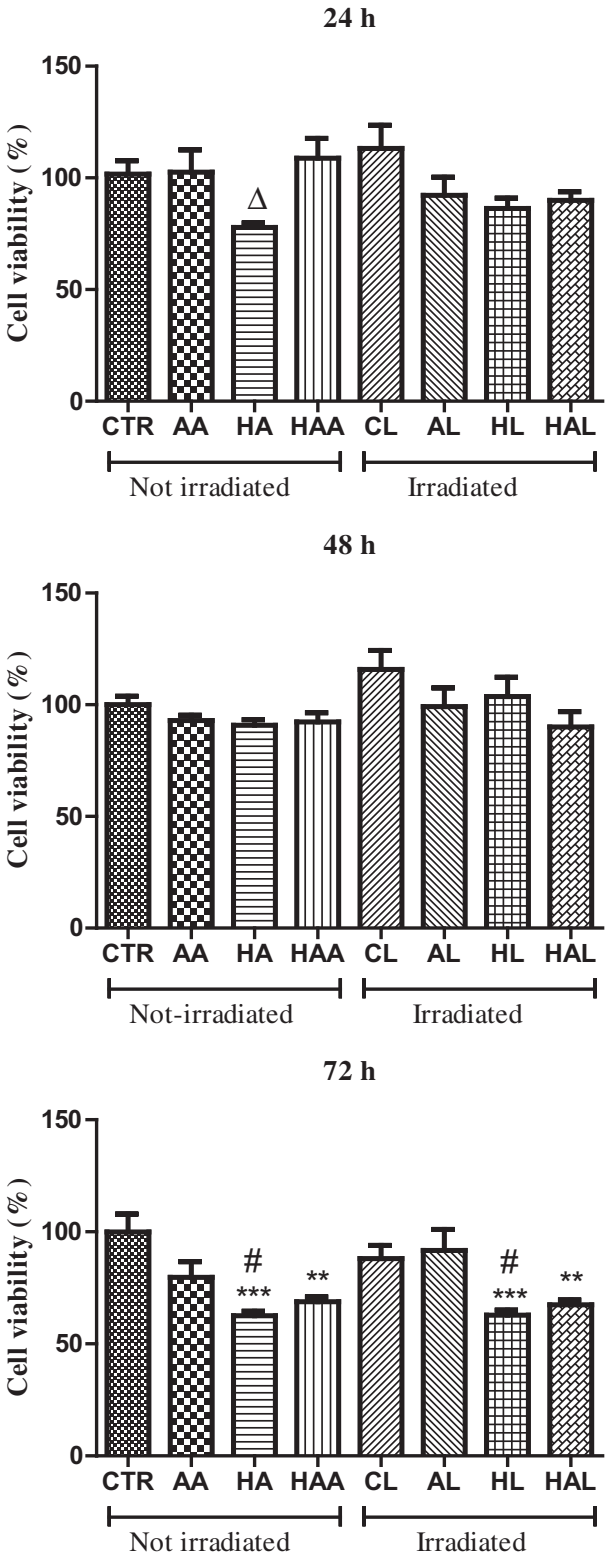

Fig. 2. Determination of percentage of cell viability relative to the mean of the control group after 24, 48, and 72 h. Data expressed as mean ± mean standard deviation. $\Delta p < 0.05$, significantly different from CL; *** $p < 0.001$, and ** $p < 0.01$, significantly different from CTR; # $p < 0.05$, significantly different from AL (ANOVA and Tukey's post-hoc test).

complemented by Tukey's test, at 5% significance ($p \leq 0.05$), with the statistical software GraphPad Prism 5.0.

3. Results

The cytotoxicity results, by means of the percentage of cell viability, are shown in Fig. 1. All tested medications showed different cell viability depending on experimental period. At 48 h, calcium hydroxide (HA) increased cell viability compared to 24 h ($p < 0.01$), and considerably decreased it at 72 h compared to 24 h and 48 h ($p < 0.001$) (Fig. 1-e).

At 72 h, group HAA decreased cell viability in relation to 48 h ($p < 0.05$) and presented significantly reduce compared to 24 h ($p < 0.001$) (Fig. 1-g). The FTL in groups HL and HAL showed a considerable difference between 48 h and 72 h ($p < 0.001$ and $p < 0.05$, respectively), and both groups decreased cell viability after 24 h ($p < 0.05$) (Fig. 1-f and h).

The group treated with laser alone (CL) had the highest cell survival rate at 24 h, with mean of 113.13%. The calcium hydroxide (HA) presented the lowest cell survival rate at 24 h (77.81%) with significant difference only from group CL ($p < 0.05$). The laser alone group (CL) showed higher cell survival rate at 48 h, with mean of 115.85% of cell viability. After 48 h, the group HAL showed the lowest cell viability, with 90.07% (Fig. 2).

The group treated with *Aloe vera* and FTL (AL) showed higher cell survival rate at 72 h, with mean of 91.71% and statistical difference from groups HA and HL ($p < 0.05$). Group HL showed the lowest cell survival rate at 72 h, with mean of 62.56% and significant difference from the control group (CTR) ($p < 0.001$). At 72 h, groups HA and HL showed significant statistical difference ($p < 0.001$) from CTR (Fig. 2).

The *Aloe vera* with FTL (AL) stimulated higher cell division, followed by CL, HAL e HA. Regarding to the micronucleus test, group AL presented higher genotoxicity and significant results compared to group CTR ($p < 0.001$). Similarly, group CL showed high genotoxicity, followed by groups HAA and AA. Group HL exhibited low genotoxicity compared to AL ($p < 0.001$). The number of apoptotic cells was higher in groups AA and HAL. However, the other groups presented a slightly higher mean than CTR, except for group HAA, which showed lower number of apoptotic cells (Figs. 3 and 4).

4. Discussion

This research presented that using FTL decreased cell viability in the association with *Aloe vera* and standard endodontic medication. However, FTL stimulated higher cell proliferation when used alone and associated with only one intracanal medication. Laser irradiation had no influence in the genotoxicity of *Aloe vera* and calcium hydroxide association. Groups HAA and HAL presented low micronucleus formation, showing that FTL did not interfere with the genotoxicity of the combined medications. However, we found that the presence of *Aloe vera* increased gene mutation compared to isolated calcium hydroxide, achieving a lower biocompatibility index.

In this study, the pulp fibroblasts were chosen once they grow easily and fast, and are located in areas susceptible to the effects of intracanal medications and their degradation products, as periapical tissues and periodontal ligaments. Also, these cells are the main producers of collagen tissue and therefore actively participate in the tissue repair process [17,18].

Calcium hydroxide is the gold-standard material for the treatment of root canals, which feature antimicrobial activity, mechanical blockage to avoid canal reinfection, limitation of root resorption, induction of mineralized tissue, and promotion of periapical and adjacent tissue repair [9,19] with secondary anti-inflammatory action. This intracanal medication presented no genotoxicity but showed cytotoxicity, also observed in other studies [20,21]; however, some authors disagree with these findings [22,23]. This inconsonance may be related to the

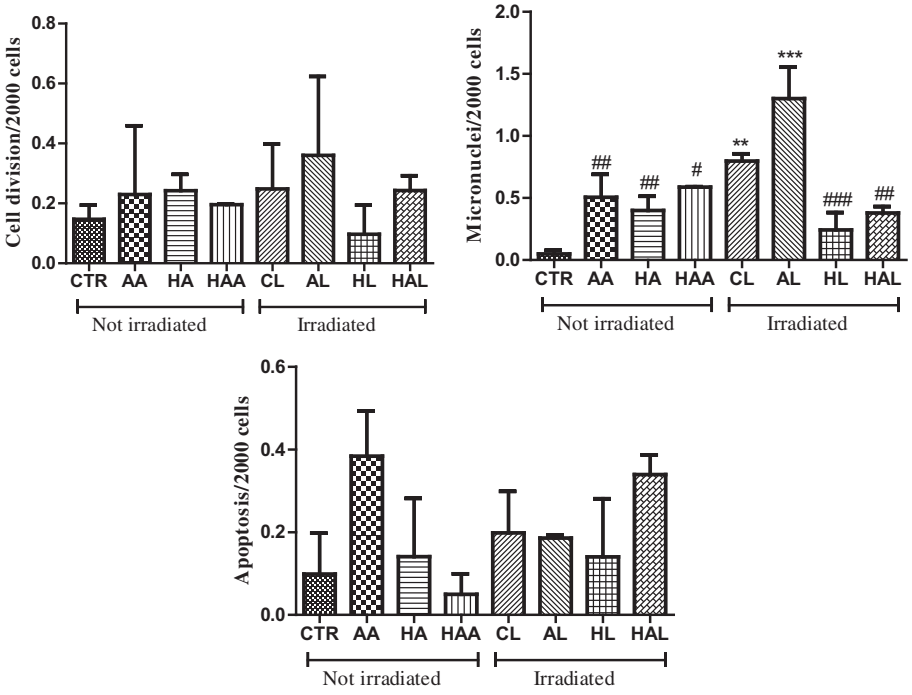

Fig. 3. Determination of the mean number of consistent changes with genotoxicity in the different groups, after 24 h. Data expressed as mean ± mean standard error. *** $p < 0.001$, and ** $p < 0.01$, significantly different from CTR; ### p < 0.001, ## p < 0.01, and # $p < 0.05$, significantly different from AL (ANOVA and Tukey's post-hoc test).

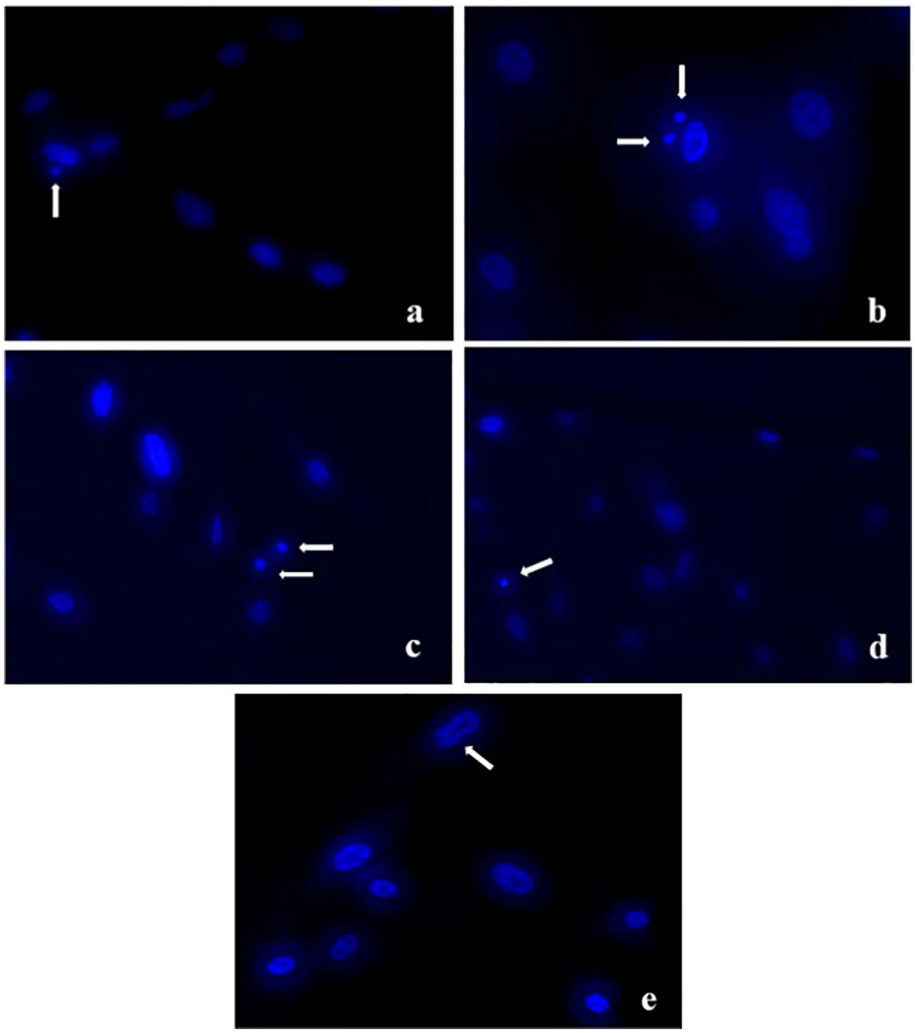

Fig. 4. a, b: Image of micronuclei in fluorescence microscope at 100 × magnification, at 24 h; c, d: Image of cells with nuclear fragmentation by apoptosis in fluorescence microscope at 100 × magnification, at 24 h; e: Image of cell division in fluorescence microscope at 100 × magnification, at 24 h.

different protocols among researches, such as the rate between the surface area of specimens and the volume of culture medium defined by the ISO 10993-5:2009 [14], which varies from 0.5–6.0 cm^2/mL.

The direct action of calcium hydroxide in the cell promotes physical and chemical changes, glycoprotein cleavage, protein denaturation, and formation of necrosis areas with fast crystal precipitation, causing calcium and phosphorus ion depositions [24], as well as the formation of coagulation necrosis foci of dystrophic calcification in subcutaneous tissues of rats [25].

As for *Aloe vera*, it stimulates healing through antibodies production and removes free radicals produced by neutrophils. Different from steroids, the anti-inflammatory properties of this plant block inflammation and stimulate fibroblast growth, accelerating the healing process [26]. It stands out that the seeding area and the form of cultivating the plant may change its chemical composition. Acemannan is the main polysaccharide extracted from the *Aloe vera* gel and can promote cell proliferation of dental pulp and differentiation and mineralization of the extracellular matrix [27,28].

In this study, *Aloe vera* showed low cytotoxicity, probably because of the presence of catalase enzyme, which converts antioxidant hydrogen peroxide (H_2O_2) into water and oxygen, suppressing the generation of these free radicals and improving cell efficacy and conservation, as well as hindering lipid peroxidation, in agreement with Fox et al. [29]. Thus, the presence of *Aloe vera* antioxidants is necessary to inhibit the generation of free radicals, minimizing cell damages [30]. However, this plant increased the frequency of genetic changes in fibroblasts, expressing chromosome damages by micronuclei formation [31]. These micronuclei are major genotoxicity biomarkers, considering they are fragments of genetic material separated from the nucleus, formed over the telophase of mitosis or meiosis because of chromosome damages suffered by the exposure to external agents [32].

In another *in vitro* experiment, concentrations from 2 to 16 mg/mL of acemannan significantly increased the proliferation of gingival fibroblasts and stimulated the secretion of keratinocytes-1 growth factor (KGF-1) and vascular endothelial growth factor (VEGF), as well as type-I collagen. All these substances have a direct connection to healing once they play important roles such as tissue re-epithelialization, formation of blood vessels and connective tissue [28].

According to previous studies regard to FTL, Loevschall et al. [33] showed that the energy density with stimulant effects in the synthesis of deoxyribonucleic acid (DNA) increased fibroblast proliferation [33,34]. Moreover, the ISO 10993-5:2009 [14] instructs that the parameter of laser irradiation used is related to the visible wavelength, which is absorbed by mitochondria photoreceptors, resulting in photochemical effects that trigger a cascade of metabolic events and finally result in biomodulation [35].

Interaction with FTL showed a tendency to increase cell viability after the experimental periods of 24 and 48 h in groups CL and HL, probably because it increased ATP concentration, stimulating fibroblast proliferation and consequently releasing cell growth factors (PDGF, IGF-I, TGF) [36]. However, there was a low cell viability at 72 h, which shows an increase of cytotoxicity throughout time, considering that laser application in the first hours of the experiment. Nevertheless, group HL and CL presented the lowest and highest genotoxicity, respectively.

Aloe vera (AA) presented high genotoxicity and its association with FTL (AL) increased even more. The calcium hydroxide present in group HAA promoted high cytotoxicity, especially at 72 h, maybe because of its solubility and increase of pH promote enzyme denaturation and cell membrane destruction, leading to cell death [24]; however, it showed no genotoxicity.

The associations of *Aloe vera* with FTL aim to facilitate the use of calcium hydroxide, to enable ion diffusion through dentinal tubules, and to improve its biocompatibility [37]. However, it is clear that the natural environment presents several intrinsic factors in the root canal system, which an *in vitro* study may not reproduce, such as temperature, inflammatory exudate, several pathogens, necrosis cells, and the response of each organism. Therefore, an *in vivo* study is required to gather more data for including a new intracanal medication in endodontic treatment.

However, our research points to the relevance of using an appropriate cell line to study the cytotoxicity and genotoxicity of endodontic materials, since the clinical use of intracanal medications involves direct contact with the pulp and periradicular tissues.

In addition, we emphasize the importance of an intracanal medication with active and viscous vehicle, conferred by the mucilaginous properties of *Aloe vera*. In this way, the beneficial qualities of this plant, when associated with calcium hydroxide or FTL, may improve apical and periapical repair, once *Aloe vera* allowed higher cell viability in human pulp fibroblasts.

5. Conclusions

The association of *Aloe vera* with calcium hydroxide or FTL promoted higher cell viability in human pulp fibroblasts, but the genotoxicity increased. However, calcium hydroxide presented high cytotoxicity for pulp fibroblasts, and FTL alone showed higher cell proliferation. Therefore, *Aloe vera* could be an alternative for association with intracanal medication or FTL during the endodontic therapy.

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