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Original Article

Effects of the Incorporation of Alpha-Tocopherol as Antioxidant on Biological and Physicochemical Properties of Calcium Hydroxide Associated with Bioactive Vehicle

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Abstract

Aim: The aim of this study to evaluate the effects of 10% alpha-tocopherol (10AT) or 20% alpha-tocopherol (20AT) incorporation on biological compatibility, pH, and calcium release of calcium hydroxide (CH) paste associated with 2% chlorhexidine digluconate (CHX). **Materials and Methods:** Four groups were evaluated: CH, CH-CHX, CH-CHX-10AT, and CH-CHX-20AT. For biological compatibility test, polyethylene tubes containing several pastes were implanted in Wistar rats' subcutaneous tissue (n = 28). After 48 h and 7, 14, and 21 days postimplantation, the specimens were removed and subjected to histologic and histomorphometric analysis. The number of inflammatory cells was evaluated. For pH and calcium release analysis, the pastes were placed individually (n = 10) in plastic tubes and immersed in deionized water. The calcium release and pH changes were evaluated in 24 and 48 h and 7, 14, and 21 days. All data were submitted to Kruskal–Wallis test ($\alpha = 0.05$). **Results:** Concerning biological compatibility, all materials shown a similar decidual response ($P > 0.05$). In the first hours, there was as increase in the number of inflammatory cells, inducing an expressive inflammatory response. After 14 days, inflammation reaction decreased and collagen fiber was organized for the tested pastes ($P = 0.05$). The pH analysis of the groups maintained the same relationship during the different periods evaluated: the CH and CH-CHX groups showed higher values and were similar to each other ($P > 0.05$), followed by the CH-CHX-10AT and CH-CHX-20 AT groups. Regarding the amount of calcium ions, in the initial (24 hours) and final (21 days) periods, the groups did not present differences between them ($P > 0.05$). **Conclusion:** The 10AT or 20AT, as an antioxidant agent, incorporation to CH and 2% CHX paste negatively affected biological and physicochemical properties.

Keywords: Antioxidants, calcium hydroxide, calcium release, chlorhexidine, subcutaneous tissue

Introduction

The intracanal medication aims to favor healing after endodontic therapy and to reduce the bacterial load resistant to root canal biochemical preparation.[1] Peculiarly, endodontic microbiota is extremely complex, presenting a variety of microbial profiles which are dependent on the environmental nutrition and oxygen conditions.[2]

Despite the number of substances indicated to be used inside the root canal, calcium hydroxide (CH) is still widely used due to its satisfactory antimicrobial activity and neutralizing action of bacteria residues, such as endotoxins.[3,4] However, it is known that in secondary infection of root canals, where there is the prevalence of atypical microorganisms, such as *Enterococcus faecalis*, its antimicrobial activity is limited.[2,5,6] The addition of CH with various bioactive vehicles has been proposed, to improve antimicrobial action over these microorganisms, such as the camphorated paramonochlorophenol and the 2% chlorhexidine digluconate (CHX), and/or with different vehicles, such as glycerin, polyethylene glycol, propylene glycol, water, or saline solution.[7,8]

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Chlorhexidine is a bisbiguanide composite with satisfactory antimicrobial activity on facultative anaerobic bacteria.[9] Its association with CH has been recommended to offer a synergistic action between these substances.[10] However, CHX degradation releases toxic and potentially carcinogenic substances, raising doubts about its value as intracanal dressing.[9] Parachloroaniline (PCA) is considered the most important of chlorhexidine degradation product[9,11] associated with high oxidative free radical (reactive oxygen species [ROS]) presence.[12,13] However, this combination has a positive effect, and this medication develops an acute inflammatory response, presenting a poor decidual compatibility.[14]

In this way, if the residual substance that would presumably be toxic is not present, other substances may also participate to trigger this acute inflammation, suggesting ROS participation.[13,14] Therefore, it is interesting to evaluate an association between CH and CHX with antioxidant substances, which can inhibit ROS. Several antioxidants are reported in the literature. Among them, sodium ascorbate and alpha-tocopherol (AT) are those which are indicated for dentistry application and are widely used in tooth whitening.[15,16] Sodium ascorbate is derived from ascorbic acid, whereas AT is a lipidic fraction of Vitamin E, being largely used in the cosmetic industry and as oxygen peroxide neutralizing after dental bleaching,[17,18] and does not cause tooth discoloration.[15]

Taking this information into consideration, we thought to evaluate the composition of an intracanal medicament containing CH, 2% CHX, and AT, possibly resulting in a substance with antimicrobial action. However, these suppositions are still unknown, which would be the ideal AT concentration to clinical use. Furthermore, there is no information about which would be the effects of this antioxidant on pH and calcium release of the CH + 2% CHX paste.

The present study aimed to evaluate the effects of the experimental addition of AT, at 10% and 20% concentration (in mass), in CH and a bioactive vehicle (2% CHX) paste, through biological compatibility test, pH values, and calcium release in 24 and 48 h and 7, 14, and 21 days. The null hypothesis is that the addition of the 10% or 20% AT (10AT or 20AT, respectively) in CH and 2% CHX paste offers beneficial effects.

Materials and Methods

All experimental protocols involving animals conformed to procedures described in the Guiding Principles for the Use of Laboratory Animals and the study approved by the Animal Committee of Sagrado Coração University, Bauru, Sao Paulo, Brazil (protocol # 180/10).

Groups evaluated

CH paste used in the present study (Calen; SS White, São Paulo, SP, Brazil) contains CH (49.77%), zinc oxide, colophony, and polyethylene glycol 400. The following groups were organized according to paste composition:

- CH (CH paste): Only CH paste (Calen; SS White, São Paulo, SP, Brazil)
- CH-CHX (CH with 2% chlorhexidine paste): The paste was prepared with 1 mL of 20% CHX in 100 mg CH paste (Calen; SS White, São Paulo, SP, Brazil)
- CH-CHX-10AT (CH with 2% chlorhexidine paste and 10AT): 90 mg of CH-CHX added with 10 mg of AT (Nutrifarm, São Paulo, SP, Brazil)
- CH-CHX-20AT (CH with 2% chlorhexidine paste and 20AT): 90 mg of CH-CHX added with 20 mg of AT (Nutrifarm, São Paulo, SP, Brazil).

Biological compatibility

Surgical procedure

Twenty-eight Albinus Wistar male rats, 3 month-years old, average weight of 300 g, were used in the present study. For the surgical procedure, the animals were anesthetized with the association of 10% ketamine (Ketamina; Agener, São Paulo, SP, Brazil) and 2% xylazine (Virbaxyl 2%; Virbac, São Paulo, SP, Brazil) in the recommended dose throughout intramuscular administration in the left tight.

Trichotomy of the dorsal area was carried out previously to the longitudinal incision (approximately 10 mm length) in the skin of each animal for divulsion and exposure of the subcutaneous tissue. After, polyethylene tubes (100 mm length × 1.2 mm diameter) with one of the extremities closed with gutta-percha (Dentsply, Petrópolis, RJ, Brazil) were filled with the experimental materials to be initially inserted in the right cervical quarter, clockwise direction, so that the open extremity containing the material was positioned opposite to the other tube. Each animal received four tubes (total of 28 tubes for each group). The incisions were sutured with 4-0 mononylon (Ethicon; Johnson and Johnson, São Paulo, SP, Brazil).

Histological and histomorphometric analysis

After 48 h and 7, 14, and 21 days, the animals were euthanized with an overdose of anesthetic drugs, and the specimens were retrieved and immersed in buffered 10% formalin for 24 h. Longitudinal 6.0 μm slices were obtained, stained with hematoxylin and eosin, and analyzed under light microscopy considering subcutaneous tissue repair, such as granulation tissue, inflammation, and fibrosis. For histomorphometric analysis, two 20x fields were selected surrounding the material positioned in one extremity of polyethylene tube. The images were taken with a light microscope (Eclipse 80i; Nikon Instruments, Melville, NY, USA). Neutrophils, mononuclear leukocytes, and multinucleated giant cells were counted using Image-Pro Plus 5.1.2 software (Media Cybernetics, Rockville, MD, USA). The arithmetic average from the histomorphometry was obtained from the evaluated fields.

pH analysis

Forty acrylic teeth, upper central incisor shaped, presenting 21 mm of length and root canal with 1.2 mm in diameter, were

used for this test. Each group was composed of 10 teeth, and after their root canals were filled with the prepared pastes, they were sealed with gutta-percha, immediately immersed in 10 mL of distilled water (pH = 6.9), individually positioned into properly sealed plastic recipients, and placed into a dry-heat oven (Fanem, São Paulo, SP, Brazil) in a fixed temperature of 37°C during all the experimental period. pH analysis performed at 24 and 48 h and 7, 14, and 21 days was made using a calibrated pH meter (Quimis, Diadema, SP, Brazil) directly in the water where the teeth were immersed at the room temperature of 25°C. After this step, the teeth were immersed again in 10 mL of distilled water.

Calcium ion release analysis

Calcium release analysis (in mg/L) was measured using an atomic absorption spectrophotometer (AA6800; Shimadzu, Tokyo, Japan). The teeth remained submerged in the distilled water. A lanthanum nitrate solution at 2 mL was added to the 8 mL of each sample.

Statistical analysis

To histological and histomorphometric analysis and calcium release evaluation, the data were submitted to Kruskal–Wallis and Dunn tests ($\alpha = 0.05$). To pH analysis, the data were submitted to ANOVA one-way and Tukey's tests ($\alpha = 0.05$).

Results

Histological analysis

After 48 h, CH showed an intense acute inflammation with a diffuse neutrophilic infiltration. At day 7, a highly vascularized granulation tissue infiltrated by mononuclear leukocytes could be seen. At day 14, inflammation persisted, but now presenting foreign body multinucleated giant cells (GCs). On the other side, at day 21, inflammation decreased and collagen fibers were organized next to the material [Figure 1a-d]. CH-CHX showed after 48 h, acute inflammatory reaction permeated by tissue degeneration. However, after 7 days, granulation tissue was predominantly infiltrated by mononuclear leukocytes and eventual GCs, persisting at 14 and 21 days [Figure 1e-h]. CH-CHX-10AT showed that, in 48 h, tissue reaction was similar to CH-CHX. In 7 days, highly vascularized granulation tissue was observed, presenting sparse deposition of collagen fibers and mononuclear leukocytes, associated with degenerated tissue. In 14 and 21 days, the inflammatory condition was similar, now with the presence of CG [Figure 2a-d]. CH-CHX-20AT showed in 48 h, a severe inflammation with an exuberant neutrophilic infiltration and tissue degeneration. However, in 7 days, mononuclear leukocytes were predominant among a highly vascularized tissue next to the material, which persisted in the following periods along with a disorganized extracellular matrix [Figure 2e-h]. There are no differences among groups in the same period evaluation ($P > 0.05$).

Histomorphometric analysis

The existence of neutrophils, monocytes, and GCs considering each experimental period did not differ among the groups.

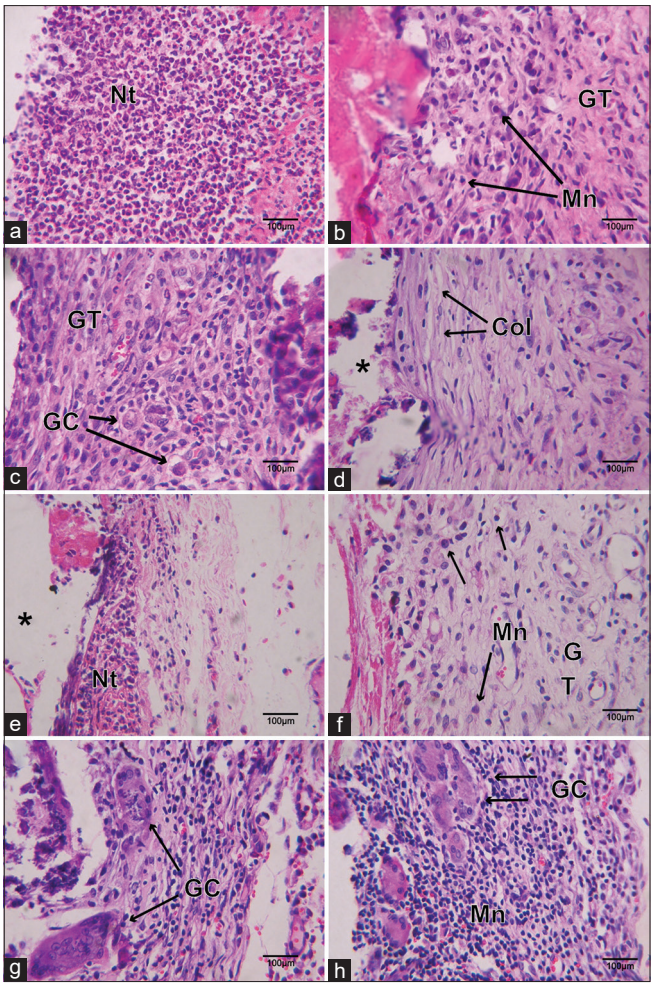

Figure 1: CH paste: (a) 48 h; (b) 7 days; (c) 14 days; (d) 21 days. CH-CHX paste: (e) 48 h; (f) 7 days; (g) 14 days; (h) 21 days. CH: Calcium hydroxide paste, CH-CHX: Calcium hydroxide with 2% chlorhexidine paste

Tables 1 and 2 show the median, minimum, and maximum values of neutrophils and mononuclear leukocytes, respectively. There are no differences among groups in the same period evaluation ($P > 0.05$).

pH analysis

After 24 h, CH and CH-CHX presented the highest pH in comparison to the other groups ($P < 0.05$). CH-CHX-10AT presented higher pH values than CH-CHX-20AT ($P < 0.05$). After 48 h and 7 and 14 days, CH and CH-CHX presented similar pH values ($P > 0.05$) but higher than CH-CHX-10AT and Ch-CHX-20AT ($P < 0.05$). There is no difference between CH-CHX-10AT and CH-CHX-20AT ($P > 0.05$). After 21 days, CH and CH-CHX presented similar pH values ($P > 0.05$) but higher than other associations. CH-CHX-10AT presented a higher pH value than CH-CHX-20AT. Table 3 shows the pH arithmetic average and standard deviation presented by the different groups in the analyzed periods.

Calcium ion release analysis

In 24 h and 21 days, the several pastes presented similar calcium release values ($P > 0.05$). In 48 h and 7 days, CH presented

Table 1: Median (M), minimum (min), and maximum (max) values of neutrophils in the different experimental periods

	48 h		7 days		14 days		21 days	
	Median	Minimum-maximum	Median	Minimum-maximum	Median	Minimum-maximum	Median	Minimum-maximum
G1	209	80-368.5	2.5	0-190	1	0-18	0.5	0-7
G2	154	67-246.5	0	0-19.5	2	0-11.5	2.5	0-6
G3	121.5	10.5-354.5	0	0-14	1.5	0-14	2	0-3.5
G4	173.5	23.5-393	0	0-21.5	1.5	0-3	3	0-3

No significant difference was detected among the groups considering the same period (P>0.05). G1 – Calcium hydroxide, G2 – Calcium hydroxide + 2% chlorhexidine digluconate, G3 – Calcium hydroxide + 2% chlorhexidine digluconate + 10% alpha-tocopherol, G4 – Calcium hydroxide + 2% chlorhexidine digluconate + 20% alpha-tocopherol

Table 2: Median (M), minimum (min), and maximum (max) values of mononuclear leukocytes in the different experimental periods

	48 h		7 days		14 days		21 days	
	Median	Minimum-maximum	Median	Minimum-maximum	Median	Minimum-maximum	Median	Minimum-maximum
G1	51.5	24.5-71.5	146	77-200	151	56-269	177	127-280
G2	48.5	23-91.5	111	61.5-135.5	166	63-230	234.1	52-348
G3	65.7	15.5-155	84.7	22.5-211	302	141-432	143	63.5-205
G4	51	18.5-164	160	104-214.5	235.5	97-343	171	113-269

No significant difference was detected among the groups considering the same period (P>0.05). G1 – Calcium hydroxide, G2 – Calcium hydroxide + 2% chlorhexidine digluconate, G3 – Calcium hydroxide + 2% chlorhexidine digluconate + 10% alpha-tocopherol, G4 – Calcium hydroxide + 2% chlorhexidine digluconate + 20% alpha-tocopherol

Table 3: Median and standard deviation of pH values shown by groups in different periods analyzed

	G1	G2	G3	G4
24 h	11.73 (0.05)^{a}	11.68 (0.04)^{a}	11.58 (0.04)^{b}	11.50 (0.09)^{c}
48 h	11.21 (0.05)^{a}	11.29 (0.06)^{a}	11.12 (0.04)^{b}	11.08 (0.09)^{b}
7 days	11.42 (0.03)^{a}	11.46 (0.04)^{a}	11.36 (0.03)^{b}	11.34 (0.05)^{b}
14 days	11.40 (0.08)^{a}	11.41 (0.05)^{a}	11.27 (0.03)^{b}	11.22 (0.03)^{b}
21 days	10.77 (0.06)^{ab}	10.80 (0.01)^{a}	10.70 (0.04)^{bc}	10.64 (0.09)^{c}
28 days	9.83 (0.08)^{ab}	9.88 (0.05)^{a}	9.77 (0.05)^{bc}	9.73 (0.09)^{c}

^{a,b,c}Different letters in the same line indicate statistical differences (P<0,05). No significant difference was detected among the groups considering the same period (P>0.05). G1 – Calcium hydroxide, G2 – Calcium hydroxide + 2% chlorhexidine digluconate, G3 – Calcium hydroxide + 2% chlorhexidine digluconate + 10% alpha-tocopherol, G4 – Calcium hydroxide + 2% chlorhexidine digluconate + 20% alpha-tocopherol

higher calcium release than other CH pastes ($P < 0.05$). There are no differences among CH-CHX, CH-CHX-10AT, and CH-CHX-20AT ($P > 0.05$). In 14 days, CH, CH-CHX, and CH-CHX-10AT presented similar calcium release ($P > 0.05$) but higher than CH-CHX-20AT. Table 4 presents the median, minimum, and maximum values of calcium release by the different groups in the analyzed periods.

Discussion

In the present study, the addition of the 10AT or 20AT did not have beneficial effects on biological compatibility, hydrogenionic potential, and calcium release of the CH with 2% CHX paste. Therefore, the null hypothesis was rejected. Our study presented divergent results of the previous research that evaluated the effects of AT when used as an antioxidant agent in dental practice.[19,20]

CH concentration in a medication exerts a direct effect on its biological properties, being considered ideal at 50% and 60% when associated with an aqueous vehicle or 35% when associated with methyl-cellulose.[21] Therefore, a decrease in its concentration, when agreed with CHX and/or an antioxidant (AT), can justify tissue degeneration, as observed in the initial periods (48 h and 7 days), mainly in CH-CHX-10AT and CH-CHX-20AT. However, after 14 or 21 days, the amount of the neutrophils, mononuclear leukocytes, and giant cells was similar among several composition CH pastes. However, exceptionally, in CH-CHX-10AT and CH-CHX-20AT, we observe a small incidence of degenerated tissue near the experimental pastes. This degeneration reaction can also be related to tissue reaction caused by the tocopherol excipients used in the present study, since it is composed of 400 mg of the active ingredient added to conservatives, such as methylparaben, which is toxic to the tissues.[22,23]

Histomorphometry analysis revealed a direct relationship between the reduction in the number of inflammatory cells and the time evaluation regardless of CH composition paste. The presence of PCA in the CH-added CHX paste is almost inexistent but being detected mainly the oxidative radicals (ROS).[12] Since the presence of the inflammatory cells was similar among the groups and periods, it is possible that AT had no effect on ROS in agreement with Chapple *et al*. who observed the noninterference of this antioxidant on the extracellular release of ROS by TLR-stimulated cells.[24]

On the other side, Vitamin E is constituted by four tocopherols and four tocotrienols, being the AT the predominant form of this

Table 4: Median, maximum, and minimum values of calcium release (mg/L) shown by groups in different periods analyzed

Period	Values of calcium release	G1	G2	G3	G4
24 h	Median	30.9[a]	21.8[a]	21.5[a]	22.2[a]
	Minimum-maximum	21.6-52.2	19.4-77.7	17.3-41.6	13.6-36.2
48 h	Median	24.8[a]	11.6[b]	10.6[b]	12.6[b]
	Minimum-maximum	18.9-49.7	6.11-15.8	8.8-17.8	11.6-14.8
7 days	Median	19.3[a]	15.8[ab]	10.6[bc]	10.1[c]
	Minimum-maximum	11.6-22.6	8.9-19.1	8.8-17.1	9.0-13.2
14 days	Median	13.5[b]	41.5[a]	33.3[a]	27.5[a]
	Minimum-maximum	10.1-17.6	12.7-49.1	15.1-34.9	14.4-37.2
21 days	Median	5.6[a]	6.1[a]	2.7[a]	3.7[a]
	Minimum-maximum	2.3-10.2	2.3-10.5	1.9-5.8	1.7-8.4
28 days	Median	2.4[a]	4.2[a]	2.2[a]	2.1[a]
	Minimum-maximum	1.1-7.2	1.1-7.3	1.1-3.6	0.3-6.2

[a,b,c]Different letters in the same line indicate statistical differences l (P<0.05). No significant difference was detected among the groups considering the same period (P>0.05). G1 – Calcium hydroxide, G2 – Calcium hydroxide + 2% chlorhexidine digluconate, G3 – Calcium hydroxide + 2% chlorhexidine digluconate + 10% alpha-tocopherol, G4 – Calcium hydroxide + 2% chlorhexidine digluconate+20% alpha-tocopherol

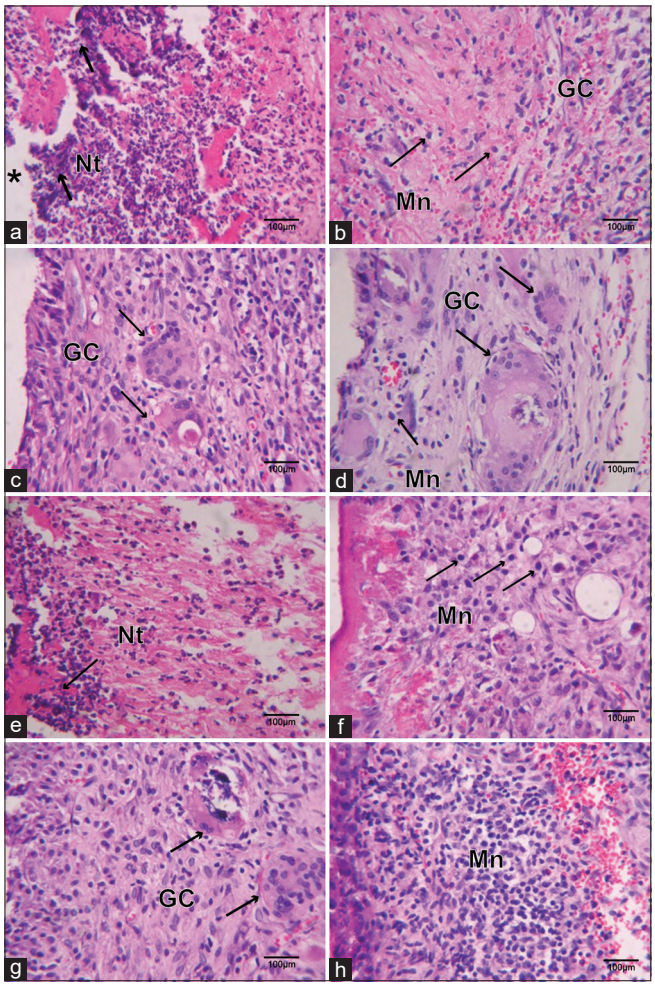

Figure 2: CH-CHX-10AT paste: (a) 48 h; (b) 7 days; (c) 14 days; (d) 21 days. CH-CHX-20AT paste: (e) 48 h; (f) 7 days; (g) 14 days; (h) 21 days. CH-CHX-10AT or CH-CHX-20AT, calcium hydroxide with 2% chlorhexidine paste and 10% or 20%, respectively, alpha-tocopherol calcium hydroxide paste

vitamin. However, γ-tocopherol, δ-tocopherol, and γ-tocotrienol forms present higher antioxidant and anti-inflammatory properties than AT, since scavenging reactive nitrogen species inhibit cyclooxygenase- and 5-lipoxygenase-catalyzed eicosanoids and suppress pro-inflammatory signaling such as NF-κB and STAT3/ 6.[25] It is presumed that we obtained these results due to antioxidant effects which have been minimized, because in the present study only, the AT form was added to the CH-CHX paste, which resulted in similar histomorphometric values, despite the period evaluated.

Hydrogenionic potential of the medication used showed a different behavior in each analyzed period. At the end of the experimental periods, medication with 20% of AT presented the lowest pH value than the other compositions, possibly due to the oil characteristic of the compound, since the chemical structure of the vehicle interferes with the physicochemical properties of the medication containing CH, such as the pH and calcium release.[25-31]

Considering calcium release, several variations occurred among the groups during the periods. However, in 21 days, all groups were similar in agreement with Duarte *et al*.[30] In this way, facing the results taken from the performed methods, it is possible to verify that AT incorporation into the medication containing CH and 2% CHX is not necessary and that the eventual free radicals released due to this association did not interfere in its biological behavior.

It is important to emphasize that the results were obtained by analyzing the medication containing the CH associated with the 2% CHX, as indicated by Zehnder.[9] Further analyses should be made to evaluate the effects of antioxidant incorporation, such as AT and its analogs, in the medication containing higher concentrations of CHX, as described by previous studies.[26]

Conclusion

Notwithstanding the limitations of this study, it can be concluded that 10AT or 20AT, as an antioxidant agent,

incorporation to CH and 2% CHX paste negatively affected biological and physicochemical properties.

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Conflicts of interest

There are no conflicts of interest.

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