

Antimicrobial activity and biocompatibility of a calcium hydroxide and aloe vera-based intracanal medication

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• **Conflicts of interest:** none declared.

ABSTRACT

Objective: to investigate the antimicrobial activity and biocompatibility of the calcium hydroxide [Ca(OH)₂] + propylene glycol (PG) + aloe vera (AV) paste in comparison with other pastes used as intracanal medication. **Material and Methods:** there was evaluated 3 intracanal medication based on calcium hydroxide and propylene glycol, varying only the third component. In group I, the third component was camphorated paramonochlorophenol (CPMC); in group II, chlorhexidine (CHX); and in group III, aloe vera. The antimicrobial activity was analyzed through the direct contact of the intracanal medication pastes with strains of *Enterococcus faecalis*; *Kocuria rhizophila*; *Pseudomonas aeruginosa* and *Candida albicans*. The biocompatibility was evaluated in subcutaneous tissue of rats during experimental 7, 21 and 63 days. **Results:** it was observed that the Group II showed the best results regarding antimicrobial activity, followed by group III and I. The Ca(OH)₂ + PG + AV paste was considered biocompatible since it presented discrete fibrosis and suggestive histological characteristics of normal healing after 63 days, approaching the control group. **Conclusion:** the association of Ca (OH)₂ + PG + AV showed antibacterial activity and adequate biocompatibility when compared with commonly pastes used as intracanal medication.

Keywords: Alo vera; Intracanal medication; Calcium hydroxide.

Introduction

The objective of endodontic treatments is to control infection and avoid the reinfection of the root canal system (RCS).¹ However, chemical-mechanical preparations may not be able to promote complete cleansing of the RCS due to its anatomic complexity.² In order to overcome this limitation, intracanal medication has been used as a subordinate therapy in endodontic treatments after preparing the RCS.^{3,4}

Calcium hydroxide [Ca(OH)₂] is the most used intracanal medication in the endodontic treatment of teeth that present chronic apical periodontitis because of its ability to neutralize bacterial endotoxins and stimulate apical and periapical restoration.⁵

Several substances, such as propylene glycol (PG), camphorated paramonochlorophenol (CPMC), glycerin, and chlorhexidine (CHX), have been suggested as vehicles to improve hydroxyl ion dissociation and increase the antimicrobial ability of Ca(OH)₂.⁶⁻⁹

According to Silveira *et al.*,⁹ propylene glycol is an adequate vehicle for Ca(OH)₂ due to its antimicrobial potential associated with the ability to control pH variation and more effective liberation of calcium ions. Moreover, Simon *et al.*,¹⁰ observed that only 15 seconds were needed for the association between Ca(OH)₂ and PG to eliminate microorganisms found in the RCS.

Aloe vera (AV), also known as *babosa*,¹¹ is a natural medication that presents anti-inflammatory activity, the ability to restore tissues,¹¹ the ability to form a mineralized barrier, and antimicrobial activity.¹² Considering the beneficial properties of AV, the objective of the present study was to investigate the antimicrobial activity and biocompatibility of the association of Ca(OH)₂ + PG + AV in comparison to other pastes used as intracanal medication.

Material and Methods

Preparation of Medicated Pastes

Calcium hydroxide-based medicated pastes were compounded in order to evaluate *in vitro* antimicrobial potential and tissue response in the subcutaneous tissue of rats.

The pastes analyzed consisted of calcium hydroxide (Biodinâmica, Ibiporã, Brazil), propylene glycol (Cavalieri Compounding Pharmacy, Juiz de Fora, Brazil), and a third component that varied among groups:

- Group I (GI) – Camphorated paramonochlorophenol (Biodinâmica, Ibiporã, Brazil);
- Group II (GII) – Chlorhexidine solution 2% (Cavalieri Pharmacy, Juiz de Fora, Brazil);
- Group III (GIII) – Lyophilized aloe vera (Freeze-Dried), *Aloe barbadensis* Miller, at a concentration of 200:1 (Synthon Especialidades Químicas Ltda., Sorocaba, Brazil).

The amount of powder of each component was measured using a spoon with a volume of 0.13 cm³. In the case of liquid substances, the volume measurement of one drop (0.05 ml) was adopted. Five compounding procedures were performed based on the expected consistency of a tooth paste. Finally, the following proportion was established for each substance: one measure of Ca(OH)₂ and AV, and one drop of CHX and CPMC. Regarding PG, one drop was used in groups I and II, and two drops were used in Group III.¹³ The total mass of each medicated paste was 0.1311, 0.1188, and 0.1744 grams for groups I, II, and III, respectively. The proportions of the substances used in the medicated pastes are described in Table 1.

Table 1. Proportion (%) of each of the component substances of medicated pastes tested

Proportion (%) [*]	Ca(OH) ₂	Aloe vera	CHX	PMCC	PEG
Group I	43,93%	-----	-----	31,65%	24,40%
Group II	30,63%	-----	24,57%	-----	26,93%
Group III	33,02%	30,27%	-----	-----	36,69%

* Proportioned in relation to the total weight of each medicated pastes used

Antimicrobial Activity of the Medicated Pastes

Antimicrobial activity was assessed using the direct contact method between medicated pastes and the following strains:

- *Enterococcus faecalis* (ATCC 51299);
- *Kocuria rhizophila* (ATCC 9341);
- *Pseudomonas aeruginosa* (wild) of clinical origin, isolated in the Clinical Analysis Laboratory of the University Hospital of the Universidade Federal de Juiz de Fora - HU/UFJF;
- *Candida albicans* (ATCC 10231).

Microbial suspension was prepared for each strain in a sterile saline solution (sodium chloride – NaCl 9.0 g/l), with 25% transmittance, using a spectrophotometer (Libra S12, Biochrom, Denmark). Standardized microbial suspension underwent serial dilution with the sterile saline solution.

After the incubation period (24 h for bacteria and 48 h for fungi), colony forming units (CFU) were counted in a Tryptone Soy Agar (TSA) culture medium for bacteria and in a Sabouraud Dextrose Agar (SDA) culture medium for fungi, in order to obtain the concentration of 2×10^3 CFU/ml in each tube.

A negative control was prepared using either 1.5 ml of Mueller-Hinton Broth (MHB) sterile for *Pseudomonas aeruginosae* and *Kocuria rhizophila*, or 1.5 ml of Brain Heart Infusion Broth (BHI) sterile for *Enterococcus faecalis* (*E. faecalis*), or 1.5 ml of Sabouraud Dextrose Broth (SDB) sterile for *Candida albicans*. A positive control was prepared using 1.5 ml of inoculated MHB / BHI / SDB and 1 spoon measurement ($v = 0.13 \text{ cm}^3$) of each medicated paste. In order to confirm that the medicated pastes were adequately diffused in the culture medium, pH test strips (Merck Química Brasil, São Paulo, Brazil) were used to measure the pH of the media before and after the incubation period.

Tubes were incubated in an aerobic environment at 37°C for 24 h for bacterial analysis, and at 25°C for 48 h for *Candida albicans*. Due to the natural turbidity of the medicated pastes tested, presence or absence of antimicrobial activity was confirmed after further incubation with 20 µl of the content of the test tubes in 4 ml of culture medium for each microorganism tested. Culture medium turbidity indicated microorganism growth in the presence of the tested paste. The absence of turbidity in the culture medium indicated antimicrobial activity of the paste. The entire procedure was performed in triplicate.

Biocompatibility Study

The present study was approved by the Ethics (No. 064/2010). A total of 18 male Wistar rats were used. These specimens were all

young adults (3 to 4 months of age) and weighed approximately 275 g. Animals were randomly divided into three groups ($n = 6$)¹⁴ according to experimental periods of 7, 21, and 63 days.¹⁵ The tissue inflammatory reaction of Group I ($\text{Ca(OH)}_2 + \text{PG} + \text{CPMC}$), Group II ($\text{Ca(OH)}_2 + \text{PG} + \text{CHX}$), Group III ($\text{Ca(OH)}_2 + \text{PG} + \text{AV}$), and the control group (CG) (empty tube) was evaluated.

Surgical Procedure and Preparation of Polyethylene Tubes

Animals were anesthetized with 8 µL/100 g of ketamine and 4 µL/100 g of xylazine hydrochloride 2% (Virbac do Brasil Indústria e Comércio Ltda, Brazil). Specimens underwent trichotomy and antisepsis on their backs. Two 1 cm-long incisions were performed on each animal, standardly located 2 cm away from the spine¹⁶. Blunt-tip scissors were used to perform subcutaneous tissue divulsion. The scissors were introduced approximately 20 mm in the subcutaneous tissue towards the skull, in the surgical cavities of the scapular region, and towards the tail, in the surgical cavities of the pelvic region.

Medicated pastes were introduced in previously autoclaved 1.5-mm wide and 10-mm long polyethylene tubes (Abbott Laboratories, Brazil) with one sealed extremity¹⁶. A polyethylene tube containing one of the three types of medication paste evaluated was introduced in each surgical cavity. An empty tube was introduced in the fourth cavity as a control. Skin was sutured using a silk thread (3-0, Johnson & Johnson Produtos Profissionais, Ltda., SP, Brazil).

By the end of each experimental period, animals underwent euthanasia by anesthetic overdose. The areas corresponding to where the polyethylene tubes were inserted were obtained through excisional removal, encompassing the areas adjacent to the edges and the entire associated scar tissue, with a 10-mm safety margin.¹⁷

Histopathological and Histomorphometric Evaluation

After removing the material, samples were fixed in a buffered formaldehyde solution 10% for a minimum period of 24 hours. The material was then included in paraffin and a 4-µm thick microtomy was performed, producing slides that were stained with hematoxylin and eosin. An optical microscope (Hallbergmoos, Zeiss, Germany) was used to thoroughly analyze each slide, using magnifications of 250 and 400 times. Images of the slides were captured using a digital camera coupled to the optical microscopic.

The software Axiovision® version 4.5 was used to perform morphometric analyses of the captured images. Number of leukocytes, fibroblastic cells, and blood vessels were quantitatively analyzed. Presence of giant cells and degree of fibrosis were qualitatively analyzed. Fibrosing was classified according to severity: (0) absence of fibrosing; (1) mild, with individualized collagen fibers, similar to regular conjunctive tissue; (2) moderate, with individualized collagen fibers, but with alternated areas of eosinophilic extracellular matrix; (3) intense, with collagen fibers with no individualization within an eosinophilic extracellular matrix.^{17,18}

Statistical Analysis

Data was tabulated in spreadsheets using the software Excel (Windows XP, Microsoft, USA). The software Statistical Package for Social Sciences (SPSS) version 23.0 (Chicago, IL, USA) was used to perform statistical analyses. The ANOVA and Scheffe post hoc tests were used to compare each quantitative analysis group (leukocyte, fibroblastic cell, and blood vessel counting). The level of significance adopted was 95% ($p < 0.05$). Tissue fibrosing results, which were expressed in scores, were described qualitatively.

Results

Antimicrobial Activity of Medicated Pastes

The qualitative analysis of the results showed that Group I [$\text{Ca}(\text{OH})_2$ + PG + CPMC] presented antibacterial activity against *Enterococcus faecalis*. Group II [$\text{Ca}(\text{OH})_2$ + PG + CHX] inhibited all microorganisms tested, except *Pseudomonas aeruginosa*. Finally, Group III [$\text{Ca}(\text{OH})_2$ + PG + AV] presented antibacterial activity against *Pseudomonas aeruginosa* and *Enterococcus faecalis* (Table 2).

Table 2. Antimicrobial activity of medicated pastes

	<i>Pseudomonas aeruginosa</i>	<i>Kocuria rizophila</i>	<i>Enterococcus faecalis</i>	<i>Candida albicans</i>
Group I	R	R	S	R
Group II	R	S	S	S
Group III	S	R	S	R

(S)-Sensitive; (R)-resistant to evaluated microorganisms

Biocompatibility

Analysis of the Inflammatory Infiltrate

Leukocytes were found in statistically significant ($p < 0.05$) higher concentrations in groups II and III when compared to Group I and to the control group.

The microscopic aspect of the region where polyethylene tubes were open and allowing contact between the subcutaneous tissue of the rats and each group of medicated pastes was assessed over a period of 7 days (magnification of 400 times). In the control group, mild to moderate fibrosing was observed with accentuated presence of fibroblasts, and moderate presence of leukocytes and small-caliber blood vessels. Group I presented inflammatory infiltrate, a large population of fibroblastic cells, and neoformed blood vessels. Group II presented moderate thicker fibrosing, a remarkable presence of leukocytes, and a discrete presence of fibroblasts and giant cells. Finally, Group III presented individualized collagen fibers and inflammatory infiltrate (Figure 1- A7,B7,C7,D7).

Within 21 days, the control group presented mild fibrosing, a higher population of fibroblastic cells and moderate inflammatory infiltrate. Group I presented mild inflammatory infiltrate when compared to the previous assessment, higher population of fibroblastic cells, and a fewer blood vessels. Group II presented moderate thicker fibrosing, lower inflammatory infiltrate, higher population of fibroblastic cells, and the absence of giant cells. Finally, Group III presented mild to moderate fibrosing, moderate presence of leukocytes and fibroblasts, fewer blood vessels, and the absence of giant cells.

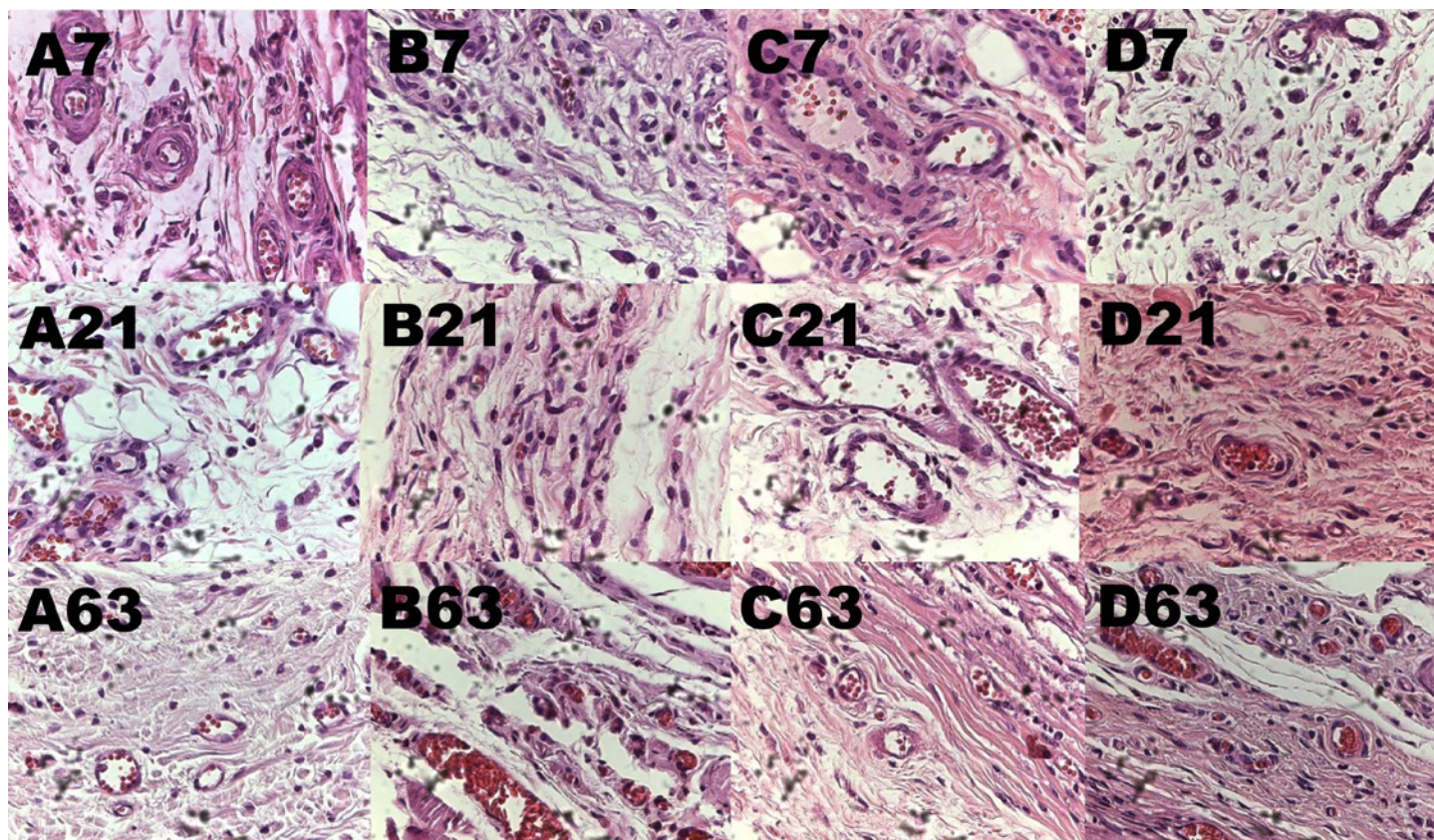


Figure 1. Microscopic aspect (magnification of 400 times) of the region where polyethylene tubes were open and allowing contact between the subcutaneous tissue of the rats and groups: A – control group; B – group I; C – group II e D – group III, after 7 (A7, B7, C7 e D7), 21 (A21, B21, C21 e D21) e 63 days (A63, B63, C63 e D63)

After a period of 63 days, the control group presented mild fibrosing, reduced cellularity, and plentiful blood vessels. Group I presented pseudocapsular organization, less inflammatory infiltrate, higher population of fibroblastic cells, and higher-caliber blood vessels. Group II presented moderate to intense fibrosing and well-individualized fibroblastic cells. Group III presented mild fibrosing, less inflammatory infiltrate when compared to previous periods, plentiful blood vessels, and absence of giant cells.

In sum, according to the microscopic aspect of the subcutaneous tissue, a higher cell population was observed in the 7-day period in relation to the periods of 21 and 63 days ($p < 0.05$). No statistically significant difference was observed between these last couple of periods ($p > 0.05$) (Figure 2). Giant cells were observed only in groups II and III during the 7-day period. The degree of tissue fibrosis around the pastes decreased as days passed, which was also observed for the control group by the end of the experiment.

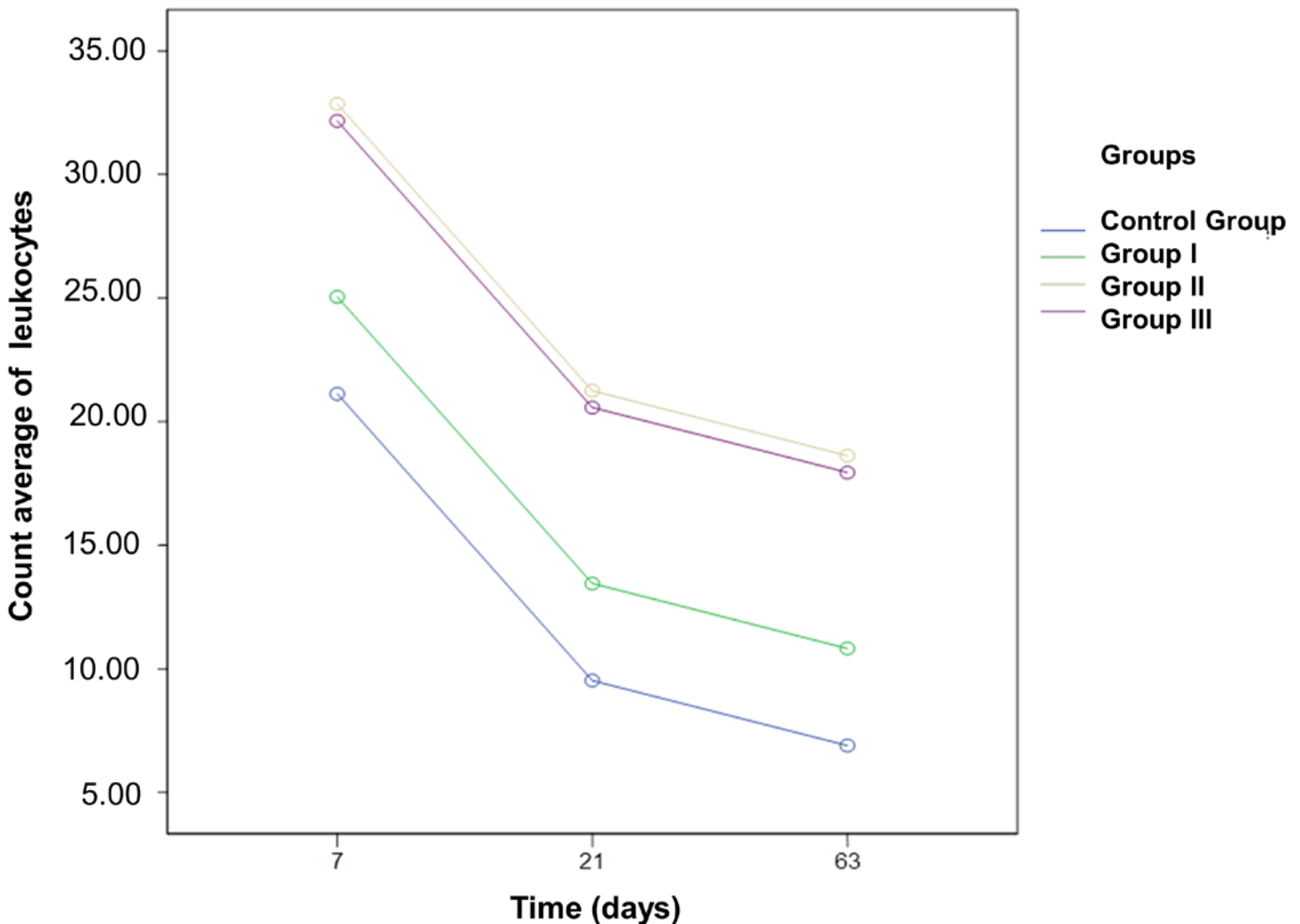


Figure 2. Leukocytes quantification in the reaction area over the time

Fibroblastic Cell Analysis

The statistical evaluation of fibroblastic cell counting showed that the smallest population of this type of cell was observed in Group I and in the control group, with no statistical difference ($p > 0.05$). On the other hand, groups II and III presented the highest values, also without a statistically significant difference ($p > 0.05$) between them. Group III was the only group that presented a statistically significant difference in relation to the control group ($p < 0.05$). In this group, a higher cell population was observed in relation to the control group.

Cell population was observed to increase over the periods of time analyzed (7, 21, and 63 days) in all groups (Figure 3), with no statistically significant difference between them ($p = 0.140$).

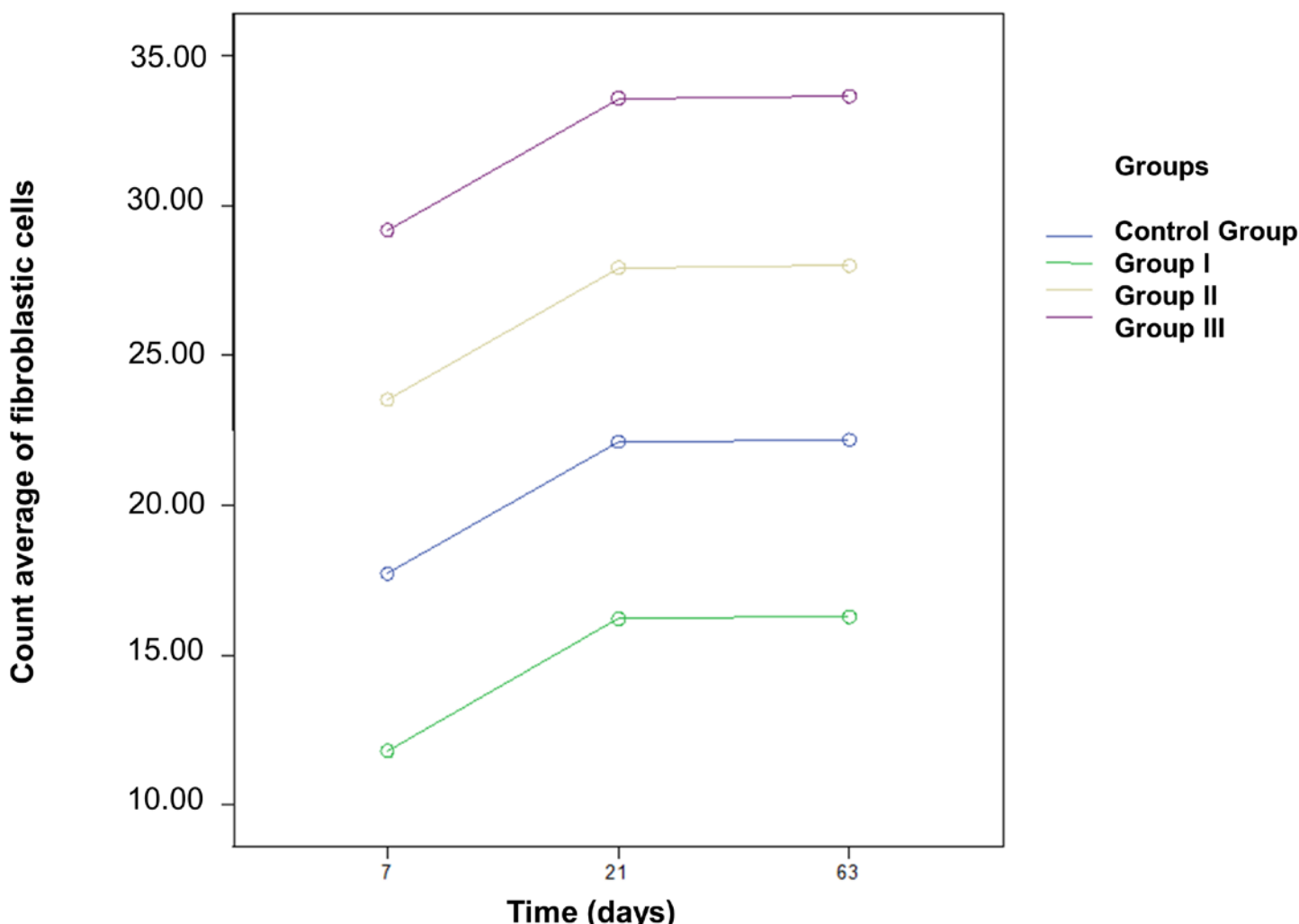


Figure 3. Fibroblastic cells in the reaction area over the time

Angiogenesis

Regarding the number of blood vessels found in the reactive areas, no statistically significant difference was observed between groups ($p > 0.05$). A higher number of vessels was found over the 7-day period (Figure 1 – A7 through D7) when compared to the 21-day and the 63-day periods (Figure 1 – A21 through D21, and A63 through D63, respectively). After 21 and 63 days, the number of blood vessels decreased in all experimental groups ($p < 0.05$). No statistically significant differences were observed between the periods of 21 and 63 days ($p > 0.05$).

Evaluation of Extracellular Matrix Density

The control group presented over the 7-day and 21-day periods mild to moderate fibrosing, decreasing to mild fibrosing by the end of the period of 63 days. Although groups I and III presented higher scores, they also showed a similar decreasing trend as that of the control group. In this case, a score of 2 predominated over the 7-day and 21-day periods, characterizing moderate fibrosing, and by the end of the 63-day period a decrease in fibrosing was observed, characterizing only mild fibrosing.

Moderate fibrosing was observed in Group II (predominant score of 2) during the 7-day period. However, unlike the other groups in the 21-day and 63-day periods, fibrosing was characterized as moderate to intense, with the predominance of score 3.

Discussion

The use of natural extracts as intracanal medication has been proposed due to their anti-inflammatory and antimicrobial properties.¹⁹⁻²⁵

Antimicrobial Activity

The direct contact test was used in the present study to evaluate antimicrobial activity. This test can promote better conditions to allow paste formulation diffusion when compared to the agar diffusion test.²⁶ However, this methodology still presents limitations regarding the transposition of results to clinical reality.²⁷

The microorganisms used as antimicrobial activity indicators were strains of *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Candida albicans*, and *Kocuria rizophila*, selected because of their association with either primary or persistent endodontic infections.²⁸

All groups presented antimicrobial activity against *E. faecalis*. This finding corroborates various studies^{20,22,23,29} that have shown acceptable bactericide effects among various natural extracts and Ca(OH)_2 associated with CPMC and chlorhexidine against *E. faecalis*.

After evaluating the antibacterial activity of Ca(OH)_2 associated with various vehicles, such as propylene glycol, distilled water, chlorhexidine, and CPMC, Silveira *et al.*⁹ observed that all groups were able to eliminate microorganisms, similar to what was also observed in the present study.

Bhardwaj *et al.*¹⁹ evaluated the antimicrobial activity of three natural extracts and chlorhexidine 2%. They observed that chlorhexidine eliminated 100% of microorganisms, followed by the natural extracts. This result agrees with the findings of the present study, where Group II [Ca(OH)_2 + PG + CHX] showed better results when compared to the other groups, since it inhibited all microorganisms tested, with the exception of *Pseudomonas aeruginosa*.

Lima *et al.*²⁹ analyzed the antimicrobial activity of the Calen paste (S.S. White, Rio de Janeiro, RJ, Brazil), associated with other vehicles, against *E. faecalis*. Their results showed that Calen + CPMC, and Calen + CHX were effective in eliminating *E. faecalis*, with no statistical difference between them. This finding corroborates the present study, considering that all groups of paste analyzed were able to eliminate *E. faecalis*.

Group III [Ca(OH)_2 + PG + AV] presented antibacterial activity against *Pseudomonas aeruginosa* and *Enterococcus faecalis*, which corroborates the results found by Abbaszadegan *et al.*²⁰. These authors evaluated the antimicrobial effectiveness of various natural extracts and a calcium hydroxide paste. They found that aloe vera was able to eliminate *E. faecalis* when used as intracanal medication.

Biocompatibility

In the present study, the biocompatibility of each paste was evaluated through subcutaneous implantation of polyethylene tubes in rats. This method is the most commonly applied to analyze biocompatibility in preliminary in vivo studies. In addition, the use of rats (*Rattus norvegicus albinus*, Holtzman) provides safer treatment and relevant results over a short period of time due to the accelerated metabolism of these animals.^{30,31}

The results of leukocyte quantification showed that groups II and III presented a higher population of leukocytes when compared to the other groups. In addition, cell concentration was higher by the end of the 7-day period, in relation to the 21-day and 63-day periods, for all groups. Over time, cells mature and polymorphonuclear leukocytes are replaced by mononuclear leukocytes. These cells, in turn, release several growth factors that are responsible for the proliferation of fibroblastic cells and blood vessels, contributing towards tissue regeneration.³² This process seems to have been reflected in the fibroblastic cell count, considering the increase in cell population observed in relation to the time elapsed in all groups.

No difference was observed between groups regarding blood vessel count. This suggests that none of the medicated pastes interfered in the process of angiogenesis of reactive tissues. However, a higher number of vessels were observed by the end of the 7-day period when compared to the 21-day and the 63-day periods ($p < 0.05$). This reduction can be explained by the increase in vessel caliber over time. Tissue revascularization observed in all groups showed favorable tissue reaction, meaning that the animals were in a healthy biological condition.³¹

The control group presented the lowest scores of fibrosing after 63 days ($p < 0.05$). Group II [Ca(OH)_2 + PG + CHX] presented the highest degrees of fibrosis in relation to the other groups. This can be explained by the toxic effect of chlorhexidine on gum fibroblasts, endothelial cells, and osteoblastic alveolar cells.¹⁵

Silva *et al.*¹⁵ showed that the use of paste composed by Ca(OH)_2 + CHX 2% caused severe infiltrate, suggesting persistent residual aggression of the tested material, even after 63 days of its implantation. In addition, Madena *et al.*³³ observed that the association of Ca(OH)_2 + CHX 0.4% caused higher inflammatory response when compared to the other tested groups [*Casearia sylvestris* + PG, and Ca(OH)_2 + PG]. These results corroborate those found in the present study, which showed greater inflammatory infiltrate in Group II.

Group III [Ca(OH)_2 + PG + AV] presented similar results to Group I [Ca(OH)_2 + PG + CPMC], which represents the composition of a paste that is widely used as intracanal medication.⁶⁻⁹ This result was due to the fact that the gel extracted from AV leaves stimulates fibroblastic cell growth, thus favoring the healing process.⁷

All groups presented higher degree of tissue fibrosing after 7 days when compared to the control group. After 21 days, the inflammatory reaction of the tissues analyzed was between moderate and mild, which was closer to the condition of the control by the end of the experiment. These findings corroborate the studies by Silva *et al.*,¹⁵ Scarparo *et al.*,¹⁶ Mattos *et al.*,¹⁷ and Garcia *et al.*,³¹ who observed that experimental groups presented higher tissue fibrosing within the initial period analyzed when compared to the control group. They also observed that over time fibrosing became milder, confirming the biocompatibility of the experimental group.

Despite the limitations of the present study, the paste tested associating Ca(OH)_2 + PG + AV presented antimicrobial ability and adequate biocompatibility. However, more studies should be conducted in order to validate its use in clinical practice.

Conclusion

The association of calcium hydroxide, propylene glycol, and aloe vera presented antibacterial activity and good biocompatibility when compared to pastes used as intracanal medication.

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Mini Curriculum and Author's Contribution

1. Liza Porcaro de Bretas – DDS. Contribution: experimental design and performed the experiments.
2. Carolina Oliveira de Lima – DDS and MSc. Contribution: analysis and interpretation of data and wrote the manuscript.
3. Nadia Rezende Barbosa Raposo – PhD. Contribution: antimicrobial evaluation.
4. Beatriz Julião Vieira Aarestrup – PhD. Contribution: biocompatibility evaluation.
5. Maíra do Prado – DDS and PhD. Contribution: general discussion and final approval of the version to be published.
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