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Biocompatibility of Acetazolamide and Its Association with Calcium Hydroxide in Rat Subcutaneous Tissue

Carolina Lazarotto [a], Aline Cristina Batista Rodrigues Johann [a], Everdan Carneiro [a], Patrícia Vida Cassi Bettega [a], Vânia Portela Ditzel Westphalen [a*]

a Graduate Program in Dentistry, School of Life Sciences, Pontifícia Universidade Católica do Paraná-PUCPR; Rua Imaculada Conceição, Prado Velho, Curitiba, Brasil

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**Corresponding author*: Vânia Portela Ditzel Westphalen, Graduate Program in Dentistry, School of Life Sciences, Pontifícia Universidade Católica do Paraná-PUCPR; Curitiba, Paraná; Brazil.

Tel: +55-41 32712548
E-mail: vania.westphalen@pucpr.br

ABSTRACT

Introduction: The aim of this study was to evaluate the biocompatibility of acetazolamide and its association with the calcium hydroxide in rat subcutaneous tissues as an intracanal medication for an avulsed tooth. **Methods and Materials**: Three medications with acetazolamide base were evaluated: group 1 liquid acetazolamide associated with calcium hydroxide powder (LACH); group 2 liquid acetazolamide (LA); and group 3 acetazolamide powder associated with physiological saline (PAPS). The calcium hydroxide associated to physiological saline represented the control group. The medications were implanted in subcutaneous tissues of thirty-nine male rats for 7, 15 and 45 days; after surgery the animals were sacrificed and the sections were stained with hematoxylin and eosin to be evaluated qualitatively or semi-quantitatively with an optical microscope. The inflammation intensity and type of inflammatory cells and the repair process, were assessed. The obtained data were statistically compared through the Kruskal-Wallis test conducted at the 5% level of significance. **Results**: On the seventh day, there was statistically significant difference between PAPS and LA, in relation to the number of neutrophils (P=0.0016). There was a statistically significant difference in the total number of inflammatory cells in PAPS compared to LACH (P=0.0038) on the fifth day. The total number of inflammatory cells from PAPS was significantly higher in relation to LACH (P=0.0038), as well as LA from LACH (P=0.0038) on forty fifth day. A statistically significant reduction in the value of lymphocytes was also observed in LACH (P=0.0072) and LA (P=0.0010) groups in the same period. **Conclusion**: The results of this animal study suggest that the association of the liquid acetazolamide with the calcium hydroxide promoted an inflammation reduction and a faster repair process than in the LA and PAPS groups evaluated in 15 and 45 days.

Keywords: Acetazolamide; Calcium Hydroxide; Root Resorption

Introduction

The indicated therapy for an avulsed tooth is its immediate replantation, because it is a conservative procedure that allows the preservation of the function and of the aesthetics aspect and it delays the necessity of prosthetic works and reduces the psychological impact [1, 2]. Despite its recognized therapeutic values, the immediate replantation is often not done because it involves extensive damages to the dental alveolus.

When the immediate replantation is not possible or when the tooth is not kept in an appropriate storage media [3], the tooth can still be preserved through a procedure known as late replantation. It is characterized by the absence of the minimal conditions for survival of the periodontal ligament cells or, in other words, when the storage mode does not attend nutrition, osmolarity, pH and temperature needs of the cells. When the replantation is performed with the periodontal ligament, whose cells do not present vitality and there are adverse biological prognosis [4].

Root resorption is a common complication in the dental replantation, and this condition is possibly a result of the absence of the periodontal ligament or its part [5-7]. Another hypothesis for the occurrence of root resorption would be the deficiency in cement and

predentine of amelogenin or orosteoprotegerin (OPG) [member of the tumor necrosis factor superfamily (TNF)], whose function is to inhibit resorptive cells [8]. Where an inflammatory reaction occurs or where the bone tissue creeps toward the dental root, being juxtaposed to the radicular surface, establishes ankylosis [9]. Because of the fusion, there will be a reabsorption through substitution, in which the tooth will be replaced by bone [10].

Trying to prevent or to limit the radicular reabsorption and to promote the repair of the area in case of late replantation, the tooth should be submitted to the radicular surface treatment or to the endodontic treatment [11].

Besides the radicular surface treatment of the avulsed tooth, it is equally important to remove the pulp tissue and to use the intracanal medication. The use of the calcium hydroxide as intracanal medication is widespread and aims to avoid or, at least, to minimize the inflammatory reabsorption [11], because it has an alkaline pH and a bactericidal potential [12], but it does not appear to be effective in the reabsorption through substitution.

As the bone reabsorption is similar to the tooth reabsorption, bone substances used in bone therapies can be effective in the radicular reabsorption through substitution. Acetazolamide is a carbonic anhydrase inhibitor and, consequently a bone resorption inhibitor, so it may be suggested as intracanal medication to avulsed and late replanted teeth [13], and it is also effective against root resorption (8).

Mori *et al.* [14] evaluated and proved the biocompatibility of the powder acetazolamide associated with physiological saline. However, there are no studies which report the biocompatibility of liquid acetazolamide mixed with calcium hydroxide.

The aim of this study was to evaluate the biocompatibility of acetazolamide and its association with calcium hydroxide in rat's subcutaneous tissue.

Materials and Methods

This study was approved by the Committee on Animal Research and Ethics of PUCPR (Pontifical Catolical University of Paraná), in the report number 581. The experiment was carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and in accordance with local laws and regulations governed within that country.

Three acetazolamide-based medications were evaluated, and the calcium hydroxide associated to the physiological saline was use as the control group: group 1; calcium hydroxide powder associated with liquid acetazolamide (LACH), group 2; liquid acetazolamide (LA) and group 3; acetazolamide powder associated with physiological saline (PAPS)

The calcium hydroxide powder and the powder and liquid of acetazolamide were manipulated by Dermo Ervas Comércio de Produtos Químicos Ltda., Curitiba, Paraná, Brasil.

The acetazolamide concentration was 10^{-5} M. The acetazolamide powder was composed of 0.02 g of acetazolamide, with 1% of Nipagin, 0.45 of sodium benzoate and excipient gsp 10 g. And the liquid acetazolamide was composed of 0.02 g of acetazolamide, 0.15 of Nipagim, 0.4% of sodium benzoate and 100 mL of qsp water. The determination of the powder/liquid ratio for the medications was based on the methodology proposed by Estrela *et al.* [15] in 2001.

The tests for the ratio powder/liquid were performed by only one operator. The initial powder portions varied in weight, so there would be handling vices. These were weighted on precision balances, gradually added to 150 µL of the liquid and spatulated until a thick paste, was obtained [16].

The liquid volume determination was established through a pilot study. For the spatulation, a flexible stainless steel spatula was used on a glass plate [16].

Thirty-nine male rats (Rattus norvegicus, Abinus, Wistar), weighing between 180 and 200 gr were used. They were kept in isolation, in cleaned cages and identified according to the groups and periods of study, divided into three rats for group C and twelve rats to the other groups.

The animals were anesthetized with an intramuscular ketamine injection (Dopalen Sespo Indústria e Comércio Ltda., São Paulo, Brazil) and xylazine hydrochloride at a dose of 0.05 mL/100 mg body weight per medication.

Before the subcutaneous implantation of the medications, the animals were submitted to trichotomy in the dorsal region, which was cleaned with 0.12% Chlorhexidine. Two surgical incisions were realized in the midline, measuring 2 cm each, with a blade size 15. The lateral incision of the tissue was gently dissected to the implantation of the medications in the scapular region. The incisions were sutured with 4-0 nylon (Ethicon Johnson & Johnson, São Paulo, SP, Brasil) and removed after 7 days.

After 7, 15 and 45 days, 4 animals from each group and one animal from group C were sacrificed by anesthetic overdose. The tissues containing the medications were removed, fixed in 10% formaldehyde for 48 h and processed to obtain 5 µm-thick slides.

The sections were stained with hematoxylin and eosin and analyzed under an optical microscope (OLYMPUS® Medusa BX40, Melville, NY, USA), under 400× magnification. Then the following items were evaluated: *a)* the inflammation intensity and the type of inflammatory cells (including the multinucleated giant cells); *b)* necrosis; *c)* abscess; *d)* the repair process (characterizes by angiogenesis and fibroblasts); and *e)* the presence of materials.

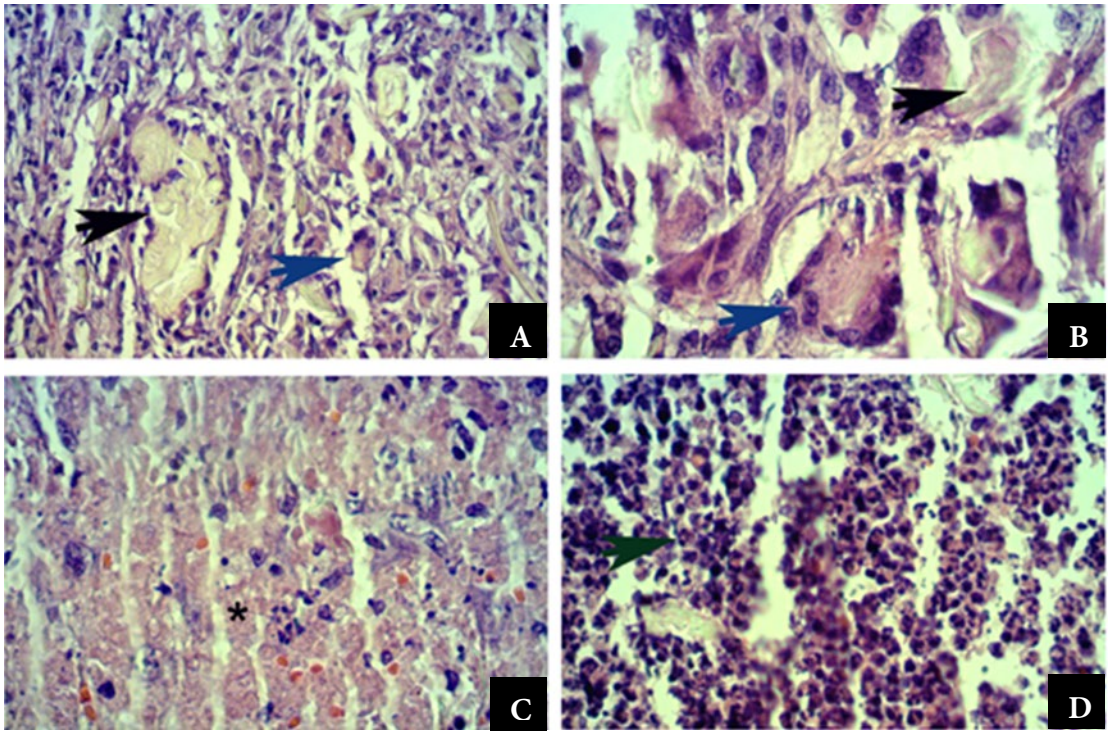

Figure 1. A and B) Material (Black arrow) surrounded by multinucleated giant cells (blue arrow); C) Necrosis areas (asterisk); D) Abscess area, revealing several neutrophils (green arrow); (H&E, 200× and 400× magnification)

Under the same microscope and with the same magnification, the neutrophils, the lymphocytes and the macrophages were semi-quantitatively analyzed. The criteria of this assessment were based on morphological aspects. The polymorphic form of the cores identified the neutrophils. The cells with a small nucleus, round, hyperchromatic and exhibiting scant cytoplasm were considered lymphocytes. The macrophages, on the other hand, had a large, hyperchromatic and an oval or bean form nucleus, with a larger amount of cytoplasm [17]. A blind-exam was realized by an experienced examiner, who gave the following scores to the inflammatory answers [18]: a) Grade 0; absent or less than 5 inflammatory cells; b) Grade 1 (discrete): from 5 to 25 cells; c) Grade 2 (moderate): from 25 to 125 cells; and d) Grade 3 (severe): more than 125 cells. So, the score for each inflammatory cell and for the total score (grouping all cells) was obtained, considering the average in 10 consecutive fields.

Statistical analysis

To compare the average score of inflammatory cells according to group and time, the nonparametric Kruskal-Wallis test was used, conducted at the 5% level of significance. When the Kruskal-Wallis test indicated differences among groups (three groups), time (three different times) and among group×time (nine treatments), the nonparametrical Kruscal-Wallis multiple comparison test was used, at 5% level of significance, seeking to detect where the differences occurred.

Results

Quantitative analyses

On the seventh day, there was statistically significant difference between PAPS and LA, in relation to the number of neutrophils (P=0.0016). There was a statistically significant difference in the total number of inflammatory cells in PAPS compared to LACH (P=0.0038) on the fifth day. The total number of inflammatory cells from PAPS was significantly higher in relation to LACH (P=0.0038), as well as LA from LACH (P=0.0038) on forty fifth day. A statistically significant reduction in the value of lymphocytes was also observed in LACH (P=0.0072) and LA (P=0.0010) groups in the same period.

Qualitative analyses

In the histopathological evaluation, in all groups and in all times, the presence of material surrounded by multinucleated giant cells, was observed (Figures 1A and B).

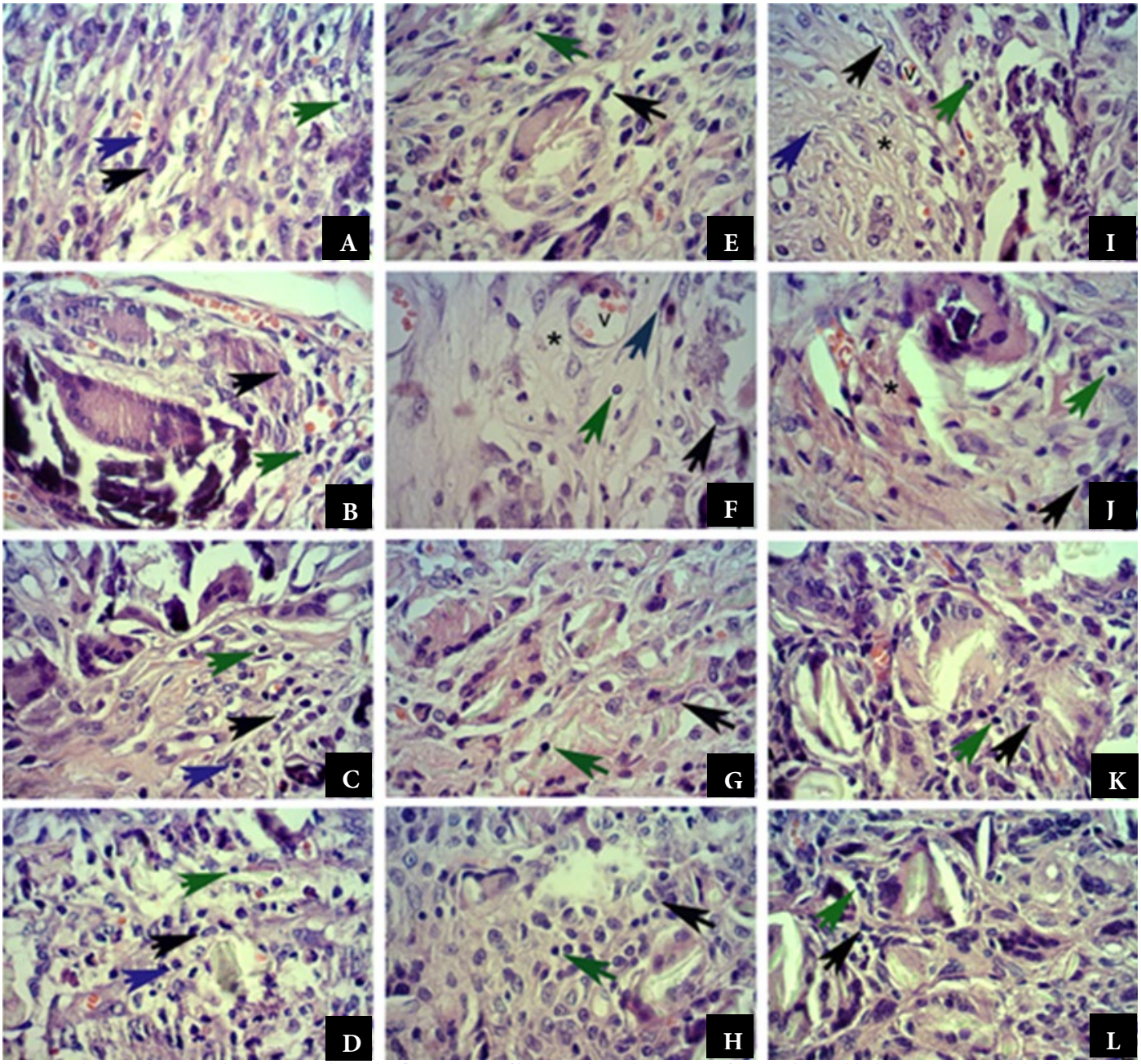

Figure 2. *A)* Seven-day control group, a moderated inflammatory infiltrate, with predominance of macrophages (black arrow), neutrophils (blue Arrow) and lymphocytes (green arrow); *B)* Seven-day group LACH, an inflammatory infiltrate with intensity similar to control group; *C)* Seven-day group LA, an inflammatory infiltrate with equivalent intensity; *D)* Seven-day group PAPS, when compared to control group, an inflammatory infiltrate of higher intensity was observed. In 15 days the results were; *E)* Fifteen-day control group, in comparison with the control group of seven days, an inflammatory infiltrate of similar intensity, but with a predominance of macrophages (black arrow) and lymphocytes (green arrow); *F)* Fifteen-day group LACH: a lower intensity inflammatory process and a more advanced repair process with the proliferation of fibroblasts (blue arrow), collagen fibers deposition (asterisk) and angiogenesis (letter v) when compared with the control group; *G)* Fifteen-day group LA, inflammatory infiltrate of higher intensity when compared with control group was observed. *H)* Fifteen-day group PAPS, inflammatory infiltrate of higher intensity when compared with control group was observed. This intensity was also observed when we compared this group with the LACH; *I)* Fifteen-day control group, when compared with the control group of 15 days, a lower intensity inflammatory process, with the presence of macrophages (black arrow) and lymphocytes (green arrow), higher proliferation of fibroblasts (blue arrow) and higher collagen fibers deposition (asterisk) and angiogenesis (letter v); *J)* Fifteen-day group LACH, when compared with the 45 days control group, an inflammatory infiltrate of similar intensity, with higher deposition of collagen fibers; *K)* Fifteen-day group LA, in comparison with control group, a higher inflammatory infiltrate. A higher infiltrate is also observed when we compare this group with the LACH; *L)* Fifteen-day group PAPS, comparing with control group, a higher inflammation intensity. This higher intensity is also observed when we compare this group with the LACH (H&E, 400× magnification)

Seven days

The histopathological evaluation of control group indicated a moderated inflammatory infiltrate, with predominance of macrophages and with the presence of neutrophils and lymphocytes (Figure 2A). The necrosis areas (Figure 1C) and the abscess areas (Figure 1D) were verified. The LACH group compared with group C showed a similar intensity of inflammatory infiltrate (Figure 2B). The necrosis and abscess areas were verified in two patterns. The LA group showed an intensity of inflammatory infiltrate similar to group C (Figure 2C). No necrosis or abscess area was observed. The PAPS group revealed an inflammatory infiltrate more intense than the control group (Figure 2D).

Fifteen days

When the control group of 15 days was compared with the control group of 7 days, we could observe an inflammatory infiltrate of similar intensity, with the predominance of macrophages and lymphocytes (Figure 2E). Contrary to control group of seven days, we noticed the absence of abscess and necrosis. When compared with control group of 15 days, the LACH group revealed a lower inflammatory infiltrate and a more advanced repair process, with the proliferation of fibroblasts, collagen fibers deposition and angiogenesis (Figure 2F). In one case, however, there was necrosis and abscess. The LA group revealed similar results to control group, according to the inflammation intensity (Figure 2G), except for two cases, in which the inflammatory processes happened with lower intensity. The PAPS group revealed, compared to control group, an inflammatory infiltrate of higher intensity (Figure 2H).

When we compared the 45 days control group with the one of 15 days, we could verify an inflammatory process of lower intensity, a higher proliferation of fibroblasts, a higher collagen fibers deposition and also angiogenesis (Figure 2I). Similar to control group of 15 days, necrosis and abscesses were not observed. In the LACH group, compared with the 45-day control group, a similar intensity of inflammation, but with a higher deposition of the collagen fibers was observed (Figure 2J). In two blades, unlike control group, necrosis was observed and, in one blade only, also abscess areas were detected. The LA group, in comparison with control group, revealed a higher inflammatory infiltrate (Figure 2K). The absence of necrosis and abscesses was verified. The PAPS group presented, in comparison with control group, a higher intensity of inflammation (Figure 2L). The absence of necrosis and abscess was verified.

In 15 days, a lower score of the total cells was attributed to the group LACH when compared with the PAPS. In 45 days this difference remained. A lower score of the total cells was observed when we compared the group LACH with the LA (Table 1).

Table 1. Mean (SD) and nonparametric multiple comparisons test of Kruskal-Wallis for the count of inflammatory cells in rats treated with LACH, LA and PAPS, in 7, 15 and 45 days

Group/days	LACH (group1)	LA (group 2)	PAPS (group 3)	Dunn test (*P*-value)		
				LACH×LA	LACH×PAPS	LA×PAPS
7 days						
Total	2.25 (0.50)	2.50 (0.58)	3.00 (0.00)	NA	NA	NA
Neutrophils [a]	0.25 (0.50)	0.00 (0.00)	1.50 (0.58)	NA	NA	0.0016
Lymphocytes[a]	1.75 (0.50)	1.00 (0.00)	1.25 (0.50)	NA	NA	NA
Macrophages[b]	2.00 (0.00)	2.00 (0.00)	2.25 (0.50)	NA	NA	NA
15 days						
Total	2.00 (0.00)	2.50 (0.58)	3.00 (0.00)	NA	0.0038	NA
Neutrophils[a]	0.00 (0.00)	0.00 (0.00)	0.25 (0.50)	NA	NA	NA
Lymphocytes[a]	2.00 (0.00)	1.50 (0.58)	1.50 (0.58)	NA	NA	NA
Macrophages[b]	1.50 (0.58)	2.00 (0.00)	2.00 (0.00)	NA	NA	NA
45 days						
Total	2.00 (0.00)	3.00 (0.00)	3.00 (0.00)	0.0038	0.0038	NA
Neutrophils[a]	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	NA	NA	NA
Lymphocytes[a]	1.75 (0.50)	1.50 (0.58)	3.00 (0.00)	NA	0.0072	0.0010
Macrophages[b]	2.00 (0.00)	2.00 (0.00)	1.97 (0.29)	NA	NA	NA

Kruskal-Wallis [a] P<0.05; [b] P>0.05; NA=it is not applied, once the nonparametric; Group 1: Calcium hydroxide powder associated with liquid acetazolamide (LACH); Group 2: Liquid acetazolamide (LA); Group 3: acetazolamide powder associated to physiological saline (PAPS)

Discussion

The acetazolamide is a substance known for being a strong inhibitor of the carbonic anhydras [19-21], an enzyme found in osteo/odontoclasts. This enzyme catalyzes the reaction between carbonic acid and water, resulting in the formation of hydrogen ions [22], which are responsible for the pH decrease of the reabsorption gap [20, 21]. The acidic pH, in its turn, promotes the liberation and the action of other enzymes, which will contribute to the reabsorption process [22, 23]. So, the inhibition of carbonic anhydrase will result in a decrease of the pH and there will be no reabsorption.

The suggestion of the acetazolamide as a substance that could inhibit the reabsorption appeared in 2002 [11]. This solution was used in the surface treatment of avulsed teeth that were late replanted. The results showed the occurrence of radicular reabsorption, which probably happened due the time of contact of the acetazolamide with the surface, which was only 20 min.

Mori *et al.* [13] evaluated the use of acetazolamide as an intracanal curative using this solution in avulsed rat teeth, which were late replanted, based on the hypothesis that acetazolamide should remain for longer periods to exert its effective action. The results indicated absence of radicular reabsorption in the experimental periods of 15, 30 and 60 days, when compared to the group that had the canals filled with calcium hydroxide, where the inflammatory reabsorption was observed.

In 2009, Mori *et al.* [14] evaluated the biocompatibility of the acetazolamide powder added to physiological saline and propylene glycol in rats' subcutaneous tissues, to facilitate the use of this medication as intracanal curative. They found that acetazolamide powder in physiological saline was considered biocompatible, as well as the control while the acetazolamide associated to the propylene glycol irritated the subcutaneous tissue of the rats and, therefore, its use is not indicated. In this study, it was verified that the association of acetazolamide with calcium hydroxide presented more favorable results than in the groups evaluated in the study by Mori *et al.* [14].

In our study acetazolamide powder added to calcium hydroxide was used for the very first time, considering that calcium hydroxide is the most common intracanal medication in endodontics due its antimicrobial [12] and anti-reabsorption properties [13]. The purpose of this study was to associate the acetazolamide and calcium hydroxide properties, and we found that this association promoted less inflammation and a faster repair process, verified by: reduction of the inflamed cell quantity and acceleration of the repair processes (represent through a higher number of fibroblasts and through the collagen fibers deposition) when compared with the control and with the PAPS (in 15 days); higher collagen fiber deposition when compared with the control and with the PAPS (in 45 days), and lower number of inflammatory cells compared with the LA and with the PAPS (being this due to an alteration in the lymphocytes quantity) (in 45 days).

The acetazolamide powder added to the liquid acetazolamide was not evaluated in this study because Souza *et al.* [16], assessed the physical-chemical properties of these drugs *in vitro* and observed that this association did not present any benefit, it was the most problematic group in the radicular dentin alteration and showed a slightly acidic pH, with statistically significant differences when compared with the association of the liquid acetazolamide and the calcium hydroxide. Souza *et al.* [16], concluded that the acetazolamide did not alter the properties of the calcium hydroxide. The calcium hydroxide medication associated to the physiological saline and to the acetazolamide kept the same alkaline medium in 7, 14, 30, 45, 60 and 120 days. The author suggested that the association of calcium hydroxide with acetazolamide presented the best results and indicated its use in late replantation of avulsed teeth in terms of physical-chemical properties. These findings reinforce the benefits of this association, whose favorable results were also observed in this study.

The implantation of materials in the connective tissue of small animals is considered an appropriate way to evaluate their biocompatibility. To be considered biocompatible, the material must present low toxicity with little or no promotion of inflammation [24]. In these tests, the control must be proved to be nontoxic. The combination of the acetazolamide with the calcium hydroxide in the current study revealed lower inflammation and a faster repair process than the acetazolamide associated with the physiological saline. It was also verified that the association of the calcium hydroxide with the physiological saline, a well-known medication routinely used as intracanal curative, caused more inflammations and a slower repair process than the association of the acetazolamide with the calcium hydroxide.

Other studies about the association of acetazolamide with calcium hydroxide is suggested, since this work demonstrated a promissory future to this association.

Conclusion

Considering the obtained results and the aims of this study, it is possible to conclude that the association of liquid acetazolamide with calcium hydroxide powder promoted a lower inflammation and a faster repair process in the groups evaluated in 15 and 45 days.

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The authors declare that there is no conflict of interest (professional, financial and direct and indirect benefits) that may influence the results of the research and the responsibility of the authors for the content of the manuscript. They have contributed significantly to the conception or design; the acquisition, analysis, or interpretation of data for the work and are in agreement with the content of the manuscript.

Conflict of Interest: 'None declared'.

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