Connective tissue responses to calcium hydroxide-based root canal medicaments

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Abstract

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Aim The objective of the present study was to evaluate the tissue inflammatory response induced by calcium hydroxide pastes, with or without paramonochlorophenol and camphor.

Methodology Isogenic BALB/c mice were inoculated into the subcutaneous tissue with either 0.1 mL of a suspension of Calen, Calen with camphorated paramonochlorophenol, Calen with paramonochlorophenol, Calasept paste or phosphate-buffered saline (control). After 6, 12 and 24 h and 2, 3, 5, 7 and 15 days, three animals in each group were sacrificed and the excised lesions processed for histopathological evaluation of the inflammatory response. Events monitored and graded included the assessment of vascular congestion, oedema, haemorrhage, inflammatory infiltrate, necrosis and tissue repair.

Results The pastes induced an inflammatory response at every observation period, although the intensity, duration and extension of inflammation varied. Calen paste always produced an initial short-term inflammatory response whilst the other pastes produced extended reactions. All pastes allowed repair to take place by the end of the experimental period, although the speed of this process varied between the materials. Calen presented the best biocompatibility; the phenolic compound caused greater tissue response, which was even more severe in the absence of camphor. Calasept paste was damaging and the repair process slower.

Conclusions All calcium hydroxide formulations caused an inflammatory response. The severity and longevity of the responses varied between pastes as a result of the various antiseptic agents. Although irritating, repair was apparent with all formulations.

Keywords: antibacterial dressing, biocompatibility, calcium hydroxide.

Introduction

In spite of the known biocompatibility of calcium hydroxide and the high degree of success obtained with its use, it is known that the association of calcium hydroxide with different vehicles can interfere with the ionic dissociation of the product (Anthony et al. 1982, Staehele et al. 1989, Simon et al. 1995, Beltes et al. 1996), its antiseptic properties (Ferraresi 1990, Alencar et al. 1997), tissue compatibility and the capacity to induce mineralized tissue (Holland et al. 1977, Leonardo et al. 1993a).

The association of camphorated paramonochlorophenol with calcium hydroxide, suggested by Kaiser (1964) and used by Frank (1966), has currently been proposed by Leonardo et al. (1993a), who showed higher flow and penetrability of this mixture in the root dentine in vitro. Furthermore, this combination induced the formation of calcium paramonochlorophenate, a substance responsible for the controlled release of the medication inside the root canals, thus...
increasing its time of action, improving some physical properties of the paste and consequently favouring the repair process.

At low concentrations, phenols show reduced toxicity when in contact with live tissue (Harrison & Madonia 1971) whilst their bactericidal effect is maintained (Harrison & Madonia 1970), thus being able to activate cell functions and to stimulate fibroblast activity (Tsukamoto et al. 1989). With respect to the mechanism of action of phenolic compounds in the inflammatory response, the findings of Azuma et al. (1986) suggest that the inhibition of leucocyte chemotaxis may be involved in this process, and that one of the anti-inflammatory activities of phenolic compounds may be the inhibition of the production of free oxygen radicals by neutrophils.

On the other hand, although the association of camphorated paramonochlorophenol with calcium hydroxide provides satisfactory results in terms of physicochemical aspects (Leonardo et al. 1993b) and of apical and periapical repair of human teeth (Frank 1966), studies on its biological behaviour are scarce in the literature, and consequently the ideal formulation for the clinical use of calcium hydroxide continues to be a subject of investigation.

Therefore, the inflammatory response induced by the injection of different calcium hydroxide pastes, with or without paramonochlorophenol and camphor, into mouse subcutaneous connective tissue after different periods was evaluated.

**Materials and methods**

A total of 120 isogenic female BALB/c mice, 68 weeks old, weighing 15–20 g, were used. The animals were divided into five experimental groups of 24 animals each and injected with the following substances – group I: Calen (S.S. White Artigos Dentários, Rio de Janeiro, RJ, Brazil; 2.5 g calcium hydroxide p.a., 0.5 g zinc oxide p.a., 0.05 g colophony and 1.75 mL polyethylene glycol 400); group II: Calen + camphorated paramonochlorophenol (2.5 g calcium hydroxide p.a., 0.5 g zinc oxide p.a., 0.05 g colophony, 1.75 mL polyethylene glycol 400 and 0.15 mL camphorated paramonochlorophenol); group III: Calen + paramonochlorophenol (2.5 g calcium hydroxide p.a., 0.5 g zinc oxide p.a., 0.05 g colophony, 1.75 mL polyethylene glycol 400 and 0.04 g p-monochlorophenol); group IV: Calasept (Scania Dental AB, Knivsta, Sweden); group V (control): phosphate-buffered saline (PBS) containing 7.2 g NaCl, 1.38 g monobasic sodium phosphate and 6.96 g dibasic potassium phosphate.

Suspensions of each calcium hydroxide paste were prepared in PBS at a concentration of 1 mg paste per 0.1 mL PBS. The pH of the suspensions to be injected was as follows: Calen, −1.21; Calen + camphorated paramonochlorophenol, −12.0; Calen + PBS, 12.0; Calasept, −12.1.

After disinfection, the animals of each group were injected into ventral subcutaneous connective tissue, in the right and left sides, with 0.1 mL of the suspension (groups I, II, III and IV) or PBS (group V).

The animals were kept at an environmental temperature (23±2°C) with free access to water and food. The ethical guidelines from the NIH Guide for care and use of laboratory animals were followed.

After periods of 6, 12 and 24 h and 2, 3, 5, 7 and 15 days, three animals per group and period were sacrificed by ethyl ether asphyxia. The skin was incised, spread apart and everted and the lesions were excised (3–8 mm³ volume) and fixed for 24 h in phosphate-buffered formalin. After fixation, the left and right lesions were submitted to histological processing, with the lesions from one side being embedded in paraffin, cut into serial 5 μm thick sections and stained with haematoxylin and eosin (H&E). The lesions from the other side were embedded in plastic resin (glycolmethacrylate, GMA), cut into serial 2 μm thick sections and stained by an H&E-like technique, as described by Abreu et al. (1993).

Histopathological analysis was performed with a light microscope based on the following events of the inflammatory response occurring in the subcutaneous connective tissue: vascular congestion, oedema, haemorrhage, inflammatory cell infiltrate (cellularity and type of cells), tissue lesion (necrosis) and tissue repair (fibrosis). These events were classified as absent (0), mild (1), moderate (2) or intense (3) according to subjective qualiquantitative criteria.

**Results**

There was an absence of histopathological alterations at the site of inoculation of PBS alone (control), but all pastes induced an inflammatory response at the different observation periods. The events of the inflammatory response were identified and graded and a variability in the intensity, duration and extension of these events was observed amongst the pastes analysed (Fig. 1).

In response to Calen paste, intense congestion (Fig. 1A) and oedema (Fig. 1B) and haemorrhage of
moderate intensity were observed near the injected material after 6 h (Fig. 2B). A leucocyte infiltrate with moderate amounts of neutrophils was observed (Fig. 2A). There was a progressive reduction in congestion and oedema which were no longer observed after the third day. The neutrophil infiltrate (Fig. 1C) also decreased within the course of the observation period, being moderate at 24 h, remaining mild up to the fifth day and no longer being observed after the seventh day. Mononuclear cells (Fig. 1D) started to increase at 12 h, with a large number of these cells being observed on the fifth day. In the course of the experimental period, the injected material was surrounded by an aggregate of phagocytic mononuclear cells of different size and shape which were identified as macrophages, epithelioid cells and multinucleate giant cells. This lesion was generally delineated by a capsule of fibrous tissue, consisting of a foreign body granuloma (Fig. 2C), which was clearly visible after 7 days and no longer observed after 15 days. On the surface of the inoculation site, there was an extensive area of necrosis (Figs 1E, 2A & B).
which was only observed up to the first day when a small area of ulceration delimited by granulation tissue occurred (Fig. 2B). The proliferation of fibrous and vascular structures within the granulation tissue was more intense on the seventh day, coinciding with the healing process of the ulcer which was repaired completely on the 15th day (Fig. 2D).

At the site of inoculation of Calen paste + camphorated paramonochlorophenol, congestion (Fig. 1A), oedema (Fig. 1B) and a leucocyte infiltrate (Figs 1C & D) were observed after 6 h. Congestion was intense and remained for up to 24 h. The oedema, which was mild after 6 h, became moderate after 12 h and was observed up to the third day. The inflammatory cell infiltrate was of mild intensity (Fig. 3A) and consisted of mononuclear and polymorphonuclear cells (Fig. 3B). A progressive difference in the migration kinetics was observed between polymorphonuclear and mononuclear cells (Figs 1C & D). The increase in the number of neutrophils, considered to be intense at 12 and 24 h, became moderate after 2, 3 and 5 days. In contrast, the number of mononuclear cells, although observed in smaller quantities throughout the experimental period, showed an increase after 12 h which

Figure 2  Histopathological findings at the site of inoculation of Calen paste. (A) Superficial necrosis and a polymorphonuclear leucocyte infiltrate (×200). (B) Superficial necrosis (arrow), fibrosis and vascular congestion (×200). (C) Foreign body granuloma delimited by a fibrous capsule (arrow, ×250). (D) Regeneration of the epidermis and repair of the dermis (arrow, ×200). Staining: H&E-like.
immediately regressed and remained of mild intensity up to the end of the experiment, with the most pronounced decrease being observed during the 7- and 15-day periods. A foreign body granuloma (Fig. 3D) involving the injected material was visible on the seventh day and was characterized by a mononuclear cell aggregate (Fig. 3C) including macrophages and multinucleate giant cells. Necrosis induced by this paste included the entire region overlying the injection site and was present up to the third observation day (Fig. 3A), followed by a progressive decrease in intensity to a mild pattern observed on the third day. During this period, an ulcerated lesion with the sides and base consisting of granulation tissue was clearly visible. After 15 days, repair on the surface of the epidermis was complete.

Calen paste + paramonochlorophenol induced congestion, oedema and a leucocyte infiltrate. The initially mild congestion became intense after 12 h and was followed by a discrete reduction within 24 h up to the second day (Fig. 1A). After an elevation on the third day, there was a progressive regression which was of mild intensity after 5 days and which disappeared during the following days. The oedema, which was of

Figure 3 Histopathological findings at the site of inoculation of Calen paste + camphorated paramonochlorophenol. (A) Superficial and extensive necrosis reaching a deep level, inflammatory infiltrate and oedema (×250). (B) Inflammatory infiltrate with predominance of mononuclear cells (×300). (C) Macrophage aggregate with some multinucleate giant cells (arrow, ×500). (D) Superficial necrosis, partial repair, neutrophil and mononuclear cell infiltrate and granuloma (arrow) at a deep level (×250). Staining: H&E-like.
mild intensity during the first period, became moderate during the following 12 h and was no longer observed 24 h after injection. On the second observation day, a mild oedema was again observed which became intense on the third day and gradually regressed until its disappearance on the seventh day (Fig. 1B). The leucocyte infiltrate consisting of neutrophils (Fig. 1C) and mononuclear cells (Fig. 1D) was present throughout the observation period, varying in terms of intensity and the proportion of these cells, being of moderate intensity after 6 h and becoming intense after 12 h. Subsequently, there was a variation in the number of neutrophils, with a moderate infiltrate being observed after 2 days and an intense infiltrate after 3 days. After 5 and 7 days, the intensity was moderate, becoming mild after 15 days. Necrosis, which was observed throughout the experimental period (Fig. 1E), varied in terms of intensity. During the 6 h period, necrosis involved the entire overlying tissue at the injection site (Figs 4A & B), presenting a discrete reduction after 24 h and remaining unchanged up to the third day. From the fifth observation day onwards, necrosis was mild. Ulceration associated with necrosis was still observed and was characterized by delimitation by granulation tissue of a loose pattern and associated with oedema and a neutrophil infiltrate (Fig. 4C). This pattern remained up to the seventh day. On the 15th day, resolution of the ulcer was observed but without regeneration of the epidermis (Fig. 4D), with the presence of material generally surrounded by mononuclear cells, few giant cells and some neutrophils, and without definition of the foreign body granuloma, thus conferring a pattern of aggregated macrophages with neutrophilic exudation on this residual lesion.

At the site of inoculation of the Calasept paste, intense vascular congestion (Fig. 1A) was initially (6 h) observed associated with an oedema (Fig. 1B) of moderate intensity and a cellular infiltrate consisting of a small number of mononuclear cells (Fig. 1D) and a moderate number of polymorphonuclear cells (Fig. 1C). Congestion was moderate up to the seventh day and was no longer observed on the 15th day (Figs 5B & D). The oedema remained moderate up to the fifth day, becoming mild on the seventh day, and was absent on the 15th day. A neutrophil infiltrate was observed throughout the experimental period, being of predominantly moderate intensity (Figs 5A & C) up to the seventh day and of mild intensity on the 15th day. Regarding mononuclear cells, the infiltrate became moderate during the 24 h period up to the fifth day and remained mild up to the 15th day. Necrosis, which was present throughout the observation period, was initially superficial (Fig. 5A), reaching deep levels on the third day when it was associated with ulceration. Partial repair was observed from the fifth day on, associated with a leucocyte infiltrate. On the 15th day, partial repair was associated with inflammatory mononuclear cells, giant cells and neutrophils.

**Discussion**

Isogenic BALB/c mice were used in the present experiment since these animals generated in the laboratory are genetically similar, and thus present a homogeneous response pattern to the same stimulus, avoiding the inconvenience of testing all pastes in the same animal, a procedure performed when rats are used as experimental animals. As a consequence, the results obtained with this methodology can be considered to be of great reliability since there is no interference from genetic factors.

The results obtained with the Calen paste demonstrate that this paste is clearly biocompatible. The relevant fact was that with Calen paste neutrophilia only occurred up to 48 h, whereas with Calen + camphorated parachlorophenol, Calen + PBS and Calasept an elevated number of neutrophils was observed up to the fifth, seventh and seventh days, respectively. With the Calen paste, vascular congestion occurred only up to 48 h, whereas with the other pastes this event was extended for longer periods, persisting with Calasept paste up to the seventh day. Another relevant aspect was that with Calen paste the granulation tissue present on the seventh day was organized, with complete tissue repair being observed on the 15th day.

Calcium hydroxide in an aqueous vehicle supplemented with some blood salts was introduced on the specialized market in 1972 under the name Calasept, the qualitative composition of which corresponds to that of Calxyl paste (Bintakys & Holts 1982). In the present study, although Calasept paste presented satisfactory results after 15 days when compared with the other pastes, it showed inflammatory events such as high leucocyte exudation and tissue necrosis of greater intensity and for longer periods of time as well as the worst pattern of tissue repair later. Furthermore, Leonardo et al. (1993a), studying the teeth of dogs with incomplete rizogenesis and chronic periapical reactions, observed the formation of mineralized barriers which were more compact and occurred in a larger number with the Calen paste than with the Calasept paste. The latter also presented a greater inflammatory infiltrate.
The reduced aggression against tissue caused by pastes containing polyethylene glycol 400 as a vehicle may be a result of the \( \text{OH}^- \) ion present in these pastes. After the ionization reaction, the \( \text{OH}^- \) ion does not remain free for a long period since it combines (neutralization reaction) with the \( \text{H}^+ \) ion released during the ionization of polyethylene glycol 400 in water, whereas in the Calasept paste the \( \text{OH}^- \) ion deriving from the ionization of calcium hydroxide is not neutralized, as after this reaction there is no free \( \text{H}^+ \) in the solution.

Although Calasept is the most soluble paste, it can be classified as the most aggressive since it principally showed the highest leucocyte exudation, the largest tissue necrosis and the smallest number of mononuclear cells observed throughout the experimental period. In spite of the good results reported by other investigators who studied Calasept paste in humans and animals (Ghose et al. 1987, Mitomo 1987), in the present study Calasept showed a moderate and late inflammatory reaction.

**Figure 4** Histopathological findings at the site of inoculation of Calen paste + paramonochlorophenol. (A) Superficial necrosis extending to a deep level and an intense neutrophil infiltrate (×160). (B) Neutrophil infiltrate and necrosis at a deep level (×280). (C) Oedema (×200). (D) Fibrosis with oedematous dissociation of matrix collagen, congestion and neutrophil infiltrate (×480). Staining: H&E-like.
Several studies in the literature have reported an elevated ability to attack live tissue provoked by PBS and camphorated paramonochlorophenol (Harrison & Madonia 1971, Spangberg et al. 1979). In the present study, the presence of paramonochlorophenol and camphorated paramonochlorophenol in the pastes used also altered the pattern of tissue response. Tissue irritability provoked by the addition of phenolic compounds, even at low concentrations, was evident in the present study; these pastes provoked an inflammatory reaction of higher magnitude and longer duration, inducing vascular congestion for longer periods, and higher neutrophilia and tissue necrosis of greater extension and longer duration when compared with Calen paste. Although all pastes presented satisfactory results at the end of the experiment, they made the repair process difficult.

A comparison of Calen + camphorated paramonochlorophenol and Calen + paramonochlorophenol showed a better tissue response to the former. The addition of camphor to PMC provided better tissue

Figure 5 Histopathological findings at the site of inoculation of Calasept paste. (A) Superficial and extensive necrosis reaching a deep level associated with an inflammatory cell infiltrate (×200). (B) Intense vascular congestion (×200). (C) Intense inflammatory cell infiltrate consisting of neutrophils and mononuclear cells (×280). (D) Necrosis and vascular congestion (×200). Staining: H&E-like.
compatibility and reduced aggression. Calen paste + PMC presented a response pattern similar to that of Calasept. It can be noted that, although the duration of the stimulus caused by Calasept was short, the stimulus was of great intensity, whereas the attack caused by Calen + PMC, although of less intensity, persisted for a longer period. The perpetuation of the inflammation together with the necrotic material delayed the repair process.

Since the basic component of all pastes analysed is calcium hydroxide, the variation in the tissue response observed may be attributed to the substances incorporated into the pastes. The comparison of the tissue responses evaluated here led to a conclusion that Calen paste alone is the most biocompatible, followed by responses evaluated here. The variation in the tissue response delayed the repair process.

PMC presented a response pattern similar to that of Calasept. It can be noted that, although the duration of the stimulus caused by Calasept was short, the stimulus was of great intensity, whereas the attack caused by Calen + PMC, although of less intensity, persisted for a longer period. The perpetuation of the inflammation together with the necrotic material delayed the repair process.

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