Australian Endodontic Journal
The Journal of the Australian Society of Endodontology Inc., the Australian and New Zealand Academy of Endodontists and the Asian Pacific Endodontic Confederation

Aust Endod J 2019; 45: 352–356

ORIGINAL RESEARCH

Effect of calcium hydroxide as intracanal medicament on the expression of caspase-9 located within the radicular cyst epithelium

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Keywords
apoptosis, calcium hydroxide, caspases, epithelium, radicular cyst.

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doi: 10.1111/aej.12325

(Accepted for publication 11 October 2018.)

Abstract

Apoptosis (a programmed cell death mechanism) within the radicular cyst epithelium has still not been correlated with any clinical factor. This study aimed to investigate the effect of calcium hydroxide on apoptosis, via the detection of caspase-9. Thirty radicular cysts collected during apicoectomies and stored in paraffin were retrospectively retrieved. Conservative endodontic treatments had been carried out either without (group (a) $n = 14$), or with calcium hydroxide application (group (b), $n = 16$) before obturation. All cysts were immunohistochemically stained for caspase-9 to record apoptosis of the epithelium. Statistical analysis followed. The frequency of caspase-9 immunoreactivity in the cystic epithelium in the two groups was 42.86% and 93.75% of cysts respectively. This difference was statistically significant ($P = 0.04 < 0.05$). In cysts larger than (or equal to) 10 mm, caspase-9 was more frequently expressed. It was found calcium hydroxide appears to enhance the expression of caspase-9, especially in large lesions.

Introduction

Periapical (radicular) cyst is an inflammatory jaw lesion resulting from pulp necrosis. Its lumen is encircled by a cystic wall consisting of connective tissue lined by stratified squamous epithelium (1). Its pathogenesis is associated with the epithelial cell rests of Malassez (ERM) that under various stimuli initiating during periapical inflammation proliferate (2) and form the epithelial lining of the cyst (1). The epithelium is crucial both for growth and recession of radicular cysts. For instance all three theories proposed for cyst formation, that is the 'abscess cavity' theory, the 'nutritional deficiency' theory and the 'merging of epithelial strands' theory, place emphasis on the role of the epithelium (2–4). Moreover, epithelial dissolution has been regarded as a prerequisite for cyst shrinkage and periapical healing, with apoptosis of the epithelial cells suggested as a possible mechanism (5).

Apoptosis is defined as a programmed cell death mechanism that participates in the homeostatic control of cell population; the cell disintegrates and its components are engulfed by macrophages with no inflammatory reaction occurring (6). Two main apoptotic pathways have been described (7): extrinsic and intrinsic. Both pathways lead to the activation of proteases which eventually carry out cell disintegration and are called caspases (6). The extrinsic pathway results in the activation of caspase-8 in response to external stimuli. The intrinsic pathway leads to the activation of caspase-9 as a result of influx of calcium ions from the inter-cellular space into the cell. These two molecules subsequently activate other molecules that eventually carry out cell disintegration (6). Anti-apoptotic factors are activated as well, for example Bcl-2 protein regulatory factor. Apoptosis is also present in radicular cysts (8–13). Most studies on this issue focus on the immunohistochemical expression of Bcl-2 and caspase-3. Nevertheless, no study has correlated the immunoexpression of an apoptotic molecule with the use of any endodontic technique or medicament utilised in everyday clinical practice.

Traditionally, in cases of teeth with necrotic pulps and periapical lesions, calcium hydroxide ($Ca(OH)_2$) is used

as an intracanal medicament (14–16). It is hypothesised $Ca(OH)_2$ may provide calcium ions which are essential in the onset of the intrinsic pathway of apoptosis. Thus, the aim of this study was to investigate the effect of $Ca(OH)_2$ as intracanal medicament on the apoptosis of radicular cyst epithelium, via the immunohistochemical detection of caspase-9 within epithelial cells.

Materials and methods

This study was approved by the Ethics Committee of the Dental School of the National and Kapodistrian University of Athens (protocol number 297, 24/06/2016).

This study was based on periapical lesions that had been surgically removed during apicoectomies carried out mostly at the post-graduate Endodontics Clinic from January 1993 to September 2016. Surgical specimens had been fixed in 4% buffered formalin immediately after excision, for 24–48 h, and embedded in Paraplast (R) at 59°C. Five micron thick tissue section had been cut from each specimen and stained with haematoxylin and eosin to establish the final diagnosis. All lesions had been subsequently submitted to the Department of Oral Pathology for histopathologic examination, diagnosed as radicular cysts presenting with a squamous epithelium surrounding the lumen of the cystic lesion, and stored for future research. For this study, a new 5-μm-thick section was cut from each embedded lesion (for immunohistochemistry, as described below).

Information that was collected from both the clinical history of each patient and the biopsy report concerned the gender and age of patients, the size of each cyst (expressed by its maximum diameter, measured immediately after surgical removal) and whether $Ca(OH)_2$ had been applied as intracanal medicament before the surgical treatment (if so, the period of time during $Ca(OH)_2$ had been applied was recorded). All cases had been followed up for 6–12 months after completion of conservative treatments (regardless of whether $Ca(OH)_2$ had been applied during treatment or not). All apicoectomies were carried out after this follow-up period, if there was not healing; after excision of the lesions and root-end resections, SuperEBA (Harry J Bosworth Co, Skokie, IL, USA) was used as retrofilling material.

Thirty (30) cysts were obtained with determined criteria, mainly the existence of a squamous epithelium and whether or not $Ca(OH)_2$ had been applied as intracanal medicament before the surgical treatment. They were classified into two groups: (i) lesions where $Ca(OH)_2$ had not been applied at all during conservative endodontic treatment ($n = 14$), (ii) lesions in which $Ca(OH)_2$ had been applied as intracanal medicament before the surgical treatment ($n = 16$). All cysts were subsequently forwarded for immunohistochemistry.

Immunohistochemistry for caspase-9 was performed in all 30 cysts with a standard avidin-biotin-peroxidase technique utilizing a rabbit polyclonal antibody (dilution 1:80, LAP6/Ab-4, Thermo Fisher Scientific Inc., Waltham, MA, USA), after pre-treatment with Epitope Retrieval Solution 1 (ER1) (citrate based pH 6.0) at 90°C for 1 h. The reaction product was visualised with diaminobenzidine tetrahydrochloride (DAB) and counterstain was performed with Mayer's haematoxylin. Positive control was human tonsils & Jurkat cells (Fig. 1a,b). Substitution of the antibody with non-immune serum of the same species served as negative control.

Evaluation was carried by two observers through a double-headed light microscope at x200 magnification. Cases were considered as negative (grade '0') or positive (grade '1'). The evaluation was restricted exclusively on the expression of caspase-9 within the cystic epithelium; all other positive cells were ignored.

Data were analysed with Fisher's exact test (significance level at .05) carried out with SPSS Statistics 18.0 software (SPSS, Inc., Chicago, IL, USA) software.

Results

Sixteen (16) of 30 cysts (53.33%) appeared in male patients and 14 in female patients (46.67%); the average age was 42.38 ± 13.78 years; 21 cysts (70%) were located in the maxilla and 9 (30%) in the mandible. The average maximum diameter was 13.33 ± 5.41 mm; 12 cysts (40%) were smaller than 10 mm and 18 cysts (60%) were larger than 10 mm.

In 14 of 30 cases $Ca(OH)_2$ was not applied at all during conservative endodontic treatment (group 1). In the other 16 of 30 cases $Ca(OH)_2$ was applied as an intracanal medicament for various periods.

Positive reaction for caspase-9 appears as strong, nuclear, brown-coloured staining (Fig. 1c,d). The frequency of caspase-9 immunoreactivity in the cystic epithelium in the two groups was 6/14 cysts (42.86%) and 15/16 (93.75%) respectively (Table 1); this difference was statistically significant (chi square test, $P = 0.04$).

Interestingly, in 5 of 16 cysts in which $Ca(OH)_2$ had applied, basophile, bubble-like formations of exogenous material was identified during histopathological examination; the biopsy reports of these cases mentioned it as 'calcium deposits of exogenous origin', probably corresponding to extrusion of $Ca(OH)_2$ into the cysts.

In cysts larger than (or equal to) 10 mm, caspase-9 was more frequently expressed, irrespective of whether $Ca(OH)_2$ had been applied or not (Table 2). This difference

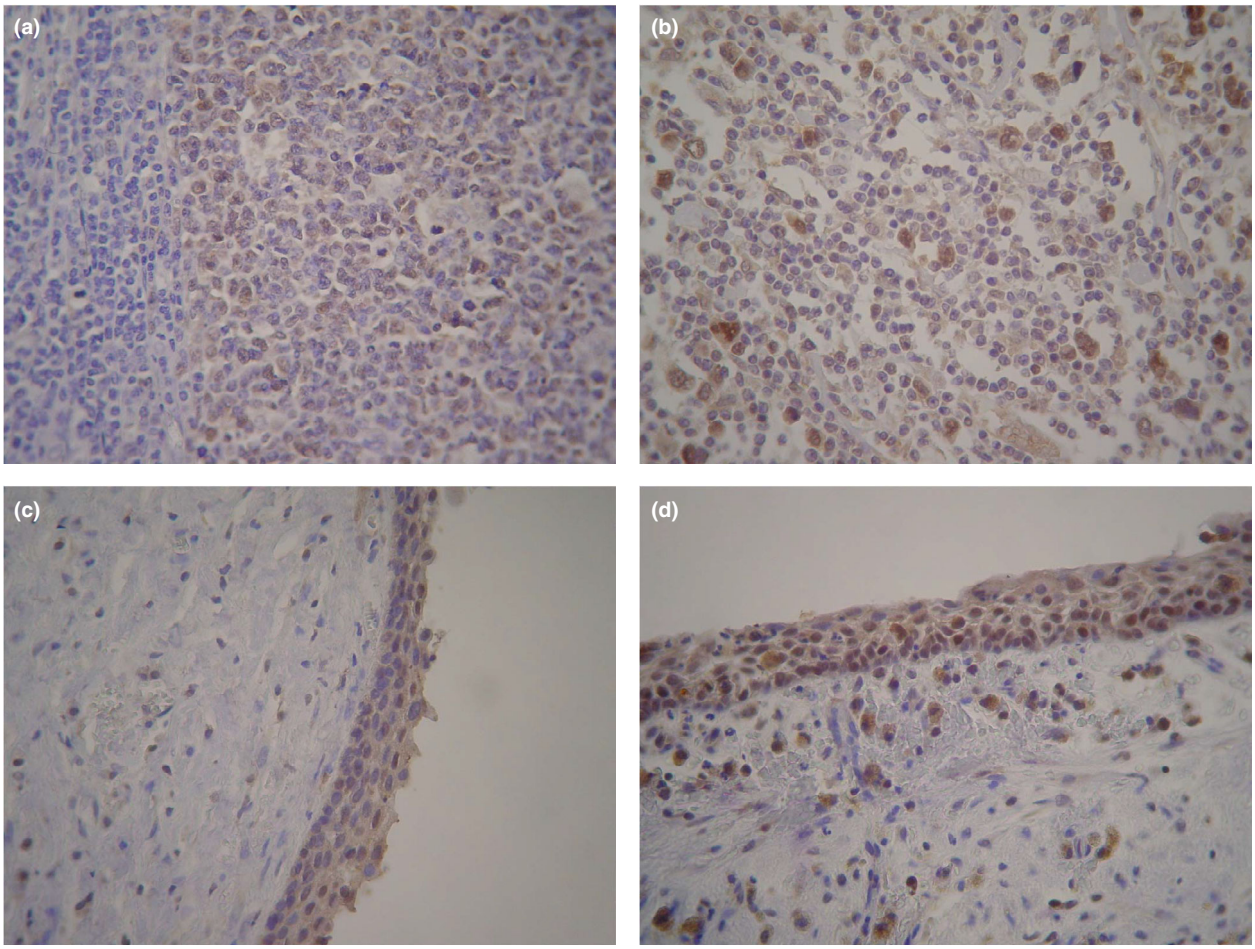

Figure 1 (a) Lymph cells within a human tonsil, displaying nuclear immunoreactivity for caspase-9. Positive cells are stained brown (immunohistochemistry, x200). (b) Jurkat cells displaying nuclear immunoreactivity for caspase-9. Positive cells are stained brown (immunohistochemistry, x200). (c & d) Caspase-9 nuclear immunoreactivity in the cystic epithelium. Positive epithelial cells are stained brown (immunohistochemistry, x200).

Table 1 Distribution of caspase-9 in the cystic epithelium according to the application (or not) of $Ca(OH)_2$

		Caspase-9 immunoreactivity	
		Negative ('0')	Positive ('1')
$Ca(OH)_2$ application	No (group 1)	8	6
	Yes (group 2)	1	15

according to the size of the lesions was statistically significant (Fisher's exact test, $P = 0.004$).

Table 2 Distribution of caspase-9 immunoreactivity according to the size of the lesions

	Size of cysts	
	Less than 10 mm	More than (or equal to) 10 mm
Negative ('0')	9	5
Positive ('1')	3	13

Discussion

In this study, the application of $Ca(OH)_2$ was associated with more intense expression of caspase-9 in the cystic epithelium.

As described above, calcium ions play a decisive role in the intrinsic pathway of apoptosis. Under physiological conditions, the levels of the intercellular calcium are 4-5 times greater than that of the intracellular; this homeostasis of calcium levels is regulated by a Ca^{2+}-Mg^{2+}-ATPase which exports calcium from inside the cell to the inter-cellular space by consuming adenosine triphosphate (ATP) (6). In a lack of nutritional ingredients, the cell exhausts its ATP deposits in order to maintain the function of this ATPase. When the cell has run out of ATP, the ATPase cannot function properly; this leads to an uncontrolled influx of calcium ions from the intercellular space into the cell. The ions eventually flow into the

mitochondrion via its voltage-dependent anion channel (VDAC) (6). The up-regulation of calcium into the mitochondrion results in the release of cytochrome c, which is also transferred into the cytoplasm via the VDAC channel. In the cytoplasm, cytochrome c binds to a cytoplasmic protein called APAF1 and forms the apoptosome; the latter activates procaspase-9 into its active form, caspase-9, which subsequently activates other caspases that will, in the end, carry out the breakdown of the cell (17). Caspase-9 is the first protease to be activated in the intrinsic pathway of apoptosis as a result of influx of calcium ions, which might result from $Ca(OH)_2$ as the latter dissolves gradually into calcium and OH^- ions. Thus, the expression of caspase-9 in the epithelium is important; if such a pro-apoptotic molecule is highly expressed, this might mean that apoptosis is favoured, consequently there would be a tendency for destruction of the epithelial cells. This may be crucial in understanding the mechanisms of the healing of radicular cysts.

Apoptosis in the epithelium of periapical lesions has been confirmed by the expression of Bcl-2 or caspase-3 (8–12). The expression of caspase-9 has been studied only once, in 20 epithelialised lesions of periapical and gingival origin, showing strong immunoreactivity in high levels in both lesion types (18). This study did not clarify whether the 'periapical lesions' included true radicular cysts or periapical granulomas with epithelial cells. However, the structure of the epithelium reported, the description of the spots positive for caspase-9 and the immunohistochemical images provided leads to the inference that the term 'periapical lesions' probably referred to radicular cysts. Nevertheless, no study correlated the expression of these molecules with any factor that regulates them.

To the best of our knowledge, this is the first study trying to correlate an apoptotic pathway with a clinical factor, namely $Ca(OH)_2$. When the latter is applied, caspase-9 expression in the cyst epithelium is significantly accelerated. Therefore, the question that arises is why all these cases were forwarded for apicoectomy since they proved to have a healing potential (as recorded via the expression of caspase-9). This paradox can be attributed to the effect of the time of application of $Ca(OH)_2$, which could not be studied adequately due to the relatively small number of cases. In this study, it was applied usually up to 4 weeks, with a couple of cases up to 12 weeks. In other studies, it was applied for 1 week (15) or more than 2 weeks (19), whereas in another study application for 3 months, with 3-week intervals, resulted in complete healing of large (7–18 mm) lesions in 73.8% of cases and partial healing in an additional 9.5% of cases after 2–10 years (16). Consequently, combining these results with this study, it seems possible that if all cases of this study had been medicated with $Ca(OH)_2$ on a long-term basis (perhaps similar to a tooth with an open apex), they might may have healed and possibly no need for surgical intervention.

It is important to note that in the aforementioned studies on periapical healing it was impossible to know whether the lesions were indeed radicular cysts, since all these studies were clinical in nature. Conversely, all these studies concerned mainly large lesions. Large lesions are chronic lesions (20) and periapical cysts need more time to develop than granulomas (21). Furthermore, a recent study (22) found that in lesions larger than 10 mm the probability of a radicular cyst is approximately 3.7 times greater than in lesions smaller than 10 mm. Therefore, it seems highly likely that large, chronic lesions are probably cysts, thus size might be an indicator for the existence of a radicular cyst. This means that, since most of the studies mentioned above were based on large lesions, it seems probable that many (or most) of them could be radicular cysts. Moreover, since size seems to be an indicator for the existence of a radicular cyst, it would be interesting to see whether there is any difference in caspase-9 expression in large cysts as well. Indeed, this study showed that in large cysts (more than or equal to 10 mm) caspase-9 is highly expressed.

In five cases, $Ca(OH)_2$ was histologically extruded into the lesions. This phenomenon has been discussed in the literature. A previous study (15) found that in radiographically detected periapical lesions complete healing was seen in 75% and 61.1%, after intentional extrusion or not, respectively, in a 5-year follow-up period, when the lesions were >11 mm in maximum diameter. During application, it is possible that extrusion of $Ca(OH)_2$ into the periapical tissues might occur, the material being resorbed in most cases over time (16,23–25). Although adverse effects of extrusion has also been described (connective tissue breakdown, damage to blood vessels and to the inferior alveolar nerve), only 10 such cases were detected in a recent systematic review evaluating the last 33 years (26).

Conclusion

$Ca(OH)_2$ may possibly up-regulate the expression of caspase-9; this is more intense in large lesions. However, the effect of time of application might be important, thus further research on this issue is essential. Given the limitations of this study, it seems that $Ca(OH)_2$ should be applied in cases with periapical lesions, possibly for a longer period of time. Any possible effect of extrusion of $Ca(OH)_2$ into the periapical lesion needs to be also discussed in future studies.

Acknowledgements

This study was adapted from a thesis submitted by Taxiarchis G. Kontogiannis in partial fulfilment of the

requirements for the MSc degree in Endodontics at the School of Dentistry of the National and Kapodistrian University of Athens. The authors would like to thank Dr. Panos Panopoulos, Professor and Director of the Department of Endodontics, for his valuable comments and evaluation of this study.

Authorship declaration

All authors have contributed significantly and all authors are in agreement with the manuscript.

Disclosure statement

The authors deny any conflict of interest related to this study.

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