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EFFECT OF CALCIUM HYDROXIDE AS INTRACANAL MEDICAMENT ON THE APOPTOSIS OF RADICULAR CYST EPITHELIUM

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Επιβλέπων Καθηγητής για την εκπόνηση της Μεταπτυχιακής Διπλωματικής Εργασίας κ. Κερεζούδης Νικόλαος, Αναπληρωτής Καθηγητής

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Στον Νίκο, στον Κωστή και στον Πάνο…

Contents

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Instead of preface

Round, like a circle in a spiral
Like a wheel within a wheel
Never ending or beginning
On an ever-spinning wheel
Like a snowball down a mountain
Or a carnival balloon
Like a carousel that's turning
Running rings around the moon
Like a clock whose hands are sweeping
Past the minutes of its face
And the world is like an apple
Whirling silently in space
Like the circles that you find
In the windmills of your mind
Like a tunnel that you follow
To a tunnel of its own
Down a hollow to a cavern
Where the sun has never shone
Like a door that keeps revolving
In a half-forgotten dream
Or the ripples of a pebble
Someone tosses in a stream
Like a clock whose hands are sweeping
Past the minutes of its face
And the world is like an apple
Whirling silently in space
Like the circles that you find
In the windmills of your mind
Keys that jingle in your pocket
Words that jangle in your head
Why did summer go so quickly?
Was it something that you said?
Lovers walk along a shore
Leave their footprints in the sand
Is the sound of distant drumming
Just the fingers of your hand?
Pictures hanging in a hallway
And the fragment of a song
Half-remembered names and faces
But to whom do they belong?
When you knew that it was over
In the autumn of goodbyes
For a moment you could not recall
The color of his eyes
Round, like a circle in a spiral
Like a wheel within a wheel
Never ending or beginning
On an ever-spinning wheel
As the images unwind
Like the circles that you find
In the windmills of your mind…

Alan & Marilyn Bergman, The windmills of your mind (The Thomas Crown Affair)

RADICULAR CYST

Definition, frequency and types of radicular cysts

A cyst is a pathological lesion consisted of a cavity which contains a liquid or semisolid material and is lined by an epithelium. Periapical (radicular) cysts are inflammatory jaw cysts at the apices of teeth with infected and necrotic pulps (Simon 1980, Nair 2003).

The frequency of periapical cysts among periapical lesions varies between 6% (Sommer 1966) and 55% (Priebe et al. 1954). However, studies with strict histopathologic criteria indicate that the frequency is about 15-32% (Simon 1980, Nair et al. 1996, Ricucci et al. 2006). In the aforementioned studies, periapical cysts were removed *in toto* during extraction; afterwards, a microscopic examination of the whole lesion was carried out, using serial sectioning and step-serial sectioning. The frequency reported in these studies is probably more close to reality since few studies in the literature adopted such methods. In the majority of studies on periapical cysts, only fragments of the lesions are used; however, even if these fragments are epithelialised, the histological diagnosis of "radicular cysts" cannot be safely set because the fragments do not represent the whole lesion. This is one reason why some older studies had recorded an unusually high frequency of periapical lesions: the existence (or not) of epithelium in some parts of the lesion was the only criterion to set the diagnosis of "radicular cyst". (Becconsall-Ryan et al. 2010). Nair et al. (1996) studied 256 periapical lesions using strict criteria and method (periapical cysts were removed *in toto* with tooth extraction, microscopic examination of the whole lesion using serial sectioning and step-serial sectioning) and showed that despite 52% of all lesions presented with epithelium, only 15% of the lesions were cysts. Later, Ricucci et al. (2006) showed that in up to 25% of periapical granulomas epithelial cells are present. However, there was not an apparent cyst formation. It is important to mention that this study used 50 lesions; thus, in spite of the

strict histopathological criteria used –as described above- the frequency of epithelium in periapical granulomas should be carefully assessed.

Two different types of periapical cysts have been described, pocket (or bay) and true cysts. Bay cysts are the ones whose lumen communicates with the root canal (Simon 1980). Nair et al. (1996) suggested that the term "bay cysts" should be replaced with "pocket cyst" because the pouch-like extension of the root canal space into the periapex appears to bear resemblance with a periodontal pocket. This proposal results from the pathogenic, structural, tissue-dynamic, and host-beneficial standpoints of the two lesions. On the other hand, true cysts are the ones whose lumen is completely surrounded with epithelium without any communication with the root canal (Simon 1980). The frequency of pocket and true cysts is 6% and 9% respectively (Nair et al. 1996). There is a general assumption that the greater the age of the cyst, the more likely it is to be a true cyst (Simon 1980, Nair et al. 1996).

Histopathology of radicular cysts

In general, radicular cyst consists of the cystic cavity (lumen), the epithelium, the subepithelial connective tissue (cystic wall) and the collagen capsule that surrounds the whole lesion. The lumen is surrounded by the epithelium and contains exudates, necrotic tissue, cholesterol crystals and erythrocytes in case of bleeding (Ricucci και συν. 2009). The epithelium is characterized as stratified and squamous but the number of layers may vary, usually between 2 and 4. In general, the cyst epithelium is distinguished in basal and suprabasal. Cells located in suprabasal epithelium present a squamous form while those located in basal epithelium present a rather irregular shape (Nair 2006).

Basal stratum cells are separated from the connective tissue with a basement membrane (Lin et al. 2006). Both the epithelium and the connective tissue may present different degrees of inflammation. The existence of inflammatory cells into the epithelium is a common histological finding (Nair 1997, Ricucci et al. 2006). The degree of inflammation as well as the types of inflammatory cells is still a matter of dispute. Early data show a rather mild infiltration by lymphocytes, while the mildest inflammation is charactierized by sporadic infiltration by mononuclear cells and undisrupted basement membrane (Bohne 1990). In contrast, more recent studies report the presence of PMNs; these cells are also able to emigrate through the epithelium into the cyst lumen (Nair 1997, Ricucci et al. 2006). The degree of inflammation seems to be related with the degree of epithelial proliferation (Valderhaug 1972, Nair et al. 1996, Ricucci et al. 2006). In the connective tissue, blood vessels, T- and B-lymphocytes and macrophages are abundant (Torabinejad & Kettering 1985); high rates of lymphocytes and plasma cells, a moderate number of macrophages and mast cells and very low rates of granulocytes have been recorded (Bohne 1990). All the aforementioned chronic condition sometimes (in cases of exacerbation) turns into acute inflammation, manifesting with rather rapid bone resorption and an enlargement of the radiolucent area, and in some cases with symptoms (Nair 1997).

The cystic cavity and cystic fluid

The cystic cavity, or lumen, contains the cystic fluid and is surrounded by the epithelium. In pocket cysts, the lumen communicates with the root canal, while in true cysts there is no such communication (Nair et al. 1996).

The content of the cavity is a lucid liquid with a particular yellowish color (Nair 1999). In case of an acute inflammatory process, the fluid may present with a rather brown

color or purulent or even with a combination of blood and pus. The fluid contains proteins, deceased epithelial cells, PMNs, cholesterol crystals and rarely erythrocytes; cholesterol crystals may comprise for 18-44% of the cystic fluid (Nair et al. 1999). It is possible that cholesterol crystals result from either the deceased erythrocytes of the blood vessels of the cystic wall because of local ischemia or some inflammatory cells that are destroyed or even from lipids with a mechanism similar to that of atherosclerosis (Browne 1971, Nair 2006). Details for cholesterol crystals are given in the next chapters.

The cyst epithelium

Radicular cysts present a stratified unkeratinized epithelium (Bohne 1990). Its width is greater in the part of the cyst close to the apex, as showed by a study of experimental periapical lesions in monkeys; this finding might be attributed to the attachment of the epithelium onto the root surface (Valderhaug 1972). Discontinuities have been observed occasionally in the epithelium (Toller 1966) and may be completely absent in some regions if an acute inflammation is present (Valderhaug 1972).

The epithelium is usually unkeratinized. However, keratinization may be also observed. This is probably due to some kind of metaplasia, especially in chronic lesions that have been in place for a long period of time (Nair 2003). Keratinization is not similar to that of other lesions of the oral cavity, particularly the odontogenic keratocyst: the radicular cyst epithelium may present a varying degree of inflammation, while the polarization of epithelial cells, similar to that observed in odontogenic keratocysts, is absent (Ricucci et al. 2006, Tsuneki et al. 2008).

Sometimes periapical cysts have also been found to be lined partially or predominantly by ciliated columnar or muco-secretory cells of respiratory origin. This finding is rather

rare, below 2% of periapical cysts (3/256 cases in the study of Nair et al. (2002); all concerned maxillary teeth). The origin of ciliated epithelium in radicular cysts is unknown. Since most radicular cysts with ciliated epithelium are located in the maxilla, the involvement of the sinus floor seems more likely (Nair 2003). In the literature, there are 3 different possible mechanisms proposed to explain the presence of ciliated epithelium (Shear 1960):

- emigration of such epithelial cells from the sinus floor (for radicular cysts in the posterior maxilla)
- metaplasia of the ordinary epithelium
- differentiation of pluripotent stem cells of the maxillary bone tissue.

The existence of inflammatory cells into the epithelium is a common histological finding. PMNs have been identified within the epithelium; these cells seem to be capable of emigrating through the epithelium into the cyst lumen (Nair 2006, Ricucci et al. 2006). On the other hand, a mild inflammatory process has also been reported; in this case, lymphocytes are recognized (Bohne 1990). The degree of inflammation seems to be related with the degree of epithelial proliferation (Nair et al. 1996, Ricucci et al. 2006).

Basal cells have an irregular shape (Schulz et al. 2009). It is believed that these cells are able to undergo symmetrical and asymmetrical mitosis in order to replace the suprabasal cells. During asymmetrical mitosis, one cell is divided into two; one of them will be differentiated into a suprabasal cell while the other one will retain the pluripotent features of the parental cell (Lin et al. 2007).

Suprabasal epithelium consists of at least 2 layers, one of which is always the spinous layer; therefore suprabasal epithelial cells have a flat or spinous shape (Nair 2006). Suprabasal cells present a high nuclear/cytoplasmic ratio and many ribosomes. They are connected with each other via desmosomes. In the nucleus, chromatin is mainly packed in the light form of euchromatin; this implies that there is an increased expression of genes. Finally, the number of mitoses is rather low (Bohne 1990).

Hyaline bodies, or Rushton bodies, can also be observed in the epithelium of radicular cysts. Their name results from the researcher that described them in the first place. They are eosinophilic linear, curved or straight, polycyclic, glassy structures (Rushton 1955). They have been correlated exclusively with odontogenic cysts and there is only one case report about their presence in an ameloblastoma in which they were also recognized (Takeda et al. 1985).

Electronic microscopic studies revealed 3 different forms of Rushton bodies (El-Labban 1979):

- the lamellar type, composed of alternating electron dense and electron lucent layers, the outermost layer always being electron dense
- the granular type, composed of amorphous material in which fragments of red blood cells could be seen
- combinations of both types.

The origin of Rushton bodies is unknown. Rushton (1955) believed that they were a keratinized structure produced by epithelial cells. Other researchers tried to find out whether the bodies had a hematogenous origin, but without a clear result (Morgan & Johnson 1974, Browne & Mathews 1985). In spite of this, the fact that they are located exclusively within the epithelium of odontogenic cysts indicates that the epithelium

itself may be strongly implicated in the genesis of Rushton bodies (Jacob 2010). To sum up, all the aforementioned studies tend to the conclusion that Rushton bodies are a secretory product of the cyst epithelium deposited on the surface of particulate matter like cell debris or cholesterol crystals (Jacob 2010).

The cystic wall

The cystic wall consists of connective tissue, while in the periphery a collagen capsule is located; collagen fibers in it are organized in concentric circles (Valderhaug 1972). The connective tissue displays a varying degree of chronic inflammation with many lymphocytes and macrophages (Valderhaug 1972, Torabinejad & Kettering 1985). Fibroblasts are the most common cells. Hemorrhagic infiltrations are also recognized, as well as cholesterol crystals; the latter stimulate a foreign body reaction which results in their being surrounded by foreign-body giant cells (Coleman et al. 1974). Finally, many blood vessels and nerve fibers are located within the connective tissue.

In the literature, there is a growing interest in the possible role of the cholesterol crystals. They seem to cause corrosion of the epithelium (Browne 1971) or foreign body reaction (Spain & Aristizabal 1962, Coleman et al. 1974). Macrophages and foreign-body giant cells cannot alienate the crystals even after a long period of time (Nair et al. 1999). Since these cells are able to secrete inflammatory mediators (mainly IL-1a), it is possible that these cells may also take part in bone resorption (Sjogren et al. 2002). Cholesterol crystals may also be deposited in areas where the epithelium is discontinued and inflammatory cells are gathered (Yamazaki et al. 2004). In the above mentioned study, cholesterol crystals were surrounded by giant cells and inflammatory connective tissue creating in this spot a particular granulomatous tissue (cholesterol granuloma) which penetrated into the lumen through the corroded epithelium.

Immunohistochemistry revealed that this granulomatous tissue contained high levels of perlecan, a proteoglycan sulfate that acts as a receptor for LDL, apoproteins and oxidated LDL (OxLDL). Therefore, it seems that cholesterol is partly composed from the LDL/OxLDL connection and intracellular perlecan. Nevertheless, the existence of perlecan in this tissue is not confirmed in the literature· Tsuneki et al. (2008) found that no perlecan was detected in radicular cysts, in contrast to odontogenic keratocysts.

Bacteria and radicular cysts

Traditionally it was believed that there are no bacteria in radicular cysts and in general in periapical lesions, except for the case of suppuration. Block et al. (1976) confirmed this view, reporting the existence of bacteria only in 1/230 lesions; this study was based on biopsies from periapical lesions. However, recent data disagree with this view. Tronstad et al. (1987) carried out a microbiologic study on periapical lesions from 8 patients and found that bacteria were present in all of them. It is important to state that the sample of this study is small (only 8 lesions), no discrimination between periapical cysts and granulomas was made and more importantly, the authors mention that in some cases a sinus tract was present; this apparently influences the results of the study, since the communication between the oral cavity and the periapical lesion leads inevitably to further microbial colonization from the oral cavity. Despite the limitations of this study, more recent studies agreed on the existence of bacteria in periapical lesions and especially in radicular cysts. Ricucci et al. (2006) studied 50 lesions (20 periapical granulomas, 14 chronic periapical abscesses and 16 radicular cysts) from *in toto* removal of the lesions during extraction, and found that in 18/50 of cases (40%) there were bacteria; all these lesions were either periapical granulomas or cysts. The same researchers, in a more recent study, found even lower frequency of bacteria, indicating

a possible difference according to the existence (or not) of symptoms: bacteria were found in 4/12 symptomatic and 1/12 asymptomatic lesions. (Ricucci et al. 2009). These results are similar to those in the previous study of Nair et al. (1987), who found bacteria in 5/31 lesions. Taken together, bacteria seem to be present in periapical lesions; however, their detection is likely to be influenced by the methodology of each study (Ricucci et al. 2006).

Formation of radicular cyst

The exact mechanism of formation and growth of radicular cysts is not known. It is believed that the epithelial cell rests of Malassez (ERM) which are found both in the healthy periodontal ligament and in periapical granulomas are stimulated to proliferate, thus giving birth to a radicular cyst (Ten Cate 1972). Three different phases of formation and growth have been proposed (Nair 1996 & 2003):

- the ERM are stimulated, in the presence of a chronic periapical inflammation, and begin to proliferate (Ten Cate 1972)
- the cyst cavity is gradually formed. Its formation has been explained by 3 different theories:
    1. the abscess cavity theory: the stimulated ERM create an epithelial tissue that tends to isolate and surround a pre-existing abscess cavity from the rest of the periapical lesion (Ten Cate 1972); however, since the epithelium displays discontinuities, this theory may not be the case (Toller 1966). On the contrary, Nair et al. (2008) showed that the combination of bacteria (the stimuli for histologically-detectable abscess creation) with epithelial cells within Teflon cages implanted in rats resulted in cyst formation; in these

cases, the epithelium had completely surrounded the abscess cavity. This was the only hypothesis-based experimental investigation that resulted in induction of inflammatory cysts. A previous study that tried to investigate cyst formation (Valderhaug 1972) induced apical periodontitis in simian teeth by leaving the root canals of the monkey teeth open to the oral environments and deliberately disregarding any aseptic precautions during the clinical procedures. Indeed, periapical cysts developed in 11 (28%) of the 39 experimented teeth. This study however, contrary to that of Nair et al. (2008), did not provide evidence on the pathogenesis of radicular cysts because it was already known (Kakehashi et al. 1965) that exposure of mammalian teeth to oral environments would lead to the development of apical periodontitis and some of them would be cysts. As a result, the study of Valderhaug concluded that "there is no evidence that cyst formation commences through an epithelial breakdown in the granuloma". Thus, Nair et al. (2008) offer significant evidence to the abscess cavity theory.

2. the nutritional deficiency theory: the ongoing proliferation of the activated ERM leads to a formation of a mass in which centrally-located cells gradually lose access to blood vessels and therefore to nutritional elements; this leads to their necrosis and liquidation as the mass keeps on growing and thus the cyst cavity is formed (Ten Cate 1972). Nevertheless, Valderhaug (1972) could not confirm this theory in his experimental study of creating periapical lesions in monkeys.

3. the merging of epithelial strands theory: the activated ERM proliferate and create strands that eventually merge with each other, forming (in 3

dimensions) a spherical mass; when the tissue isolated within the spherical mass is degenerated, the cyst cavity is formed (Lin et al. 2007). It is important to mention that the results of Nair et al. (2008) (see above) come in contrast with this theory.

- further growth of the cyst; the mechanism for this is unknown. It is believed that it is due to osmosis (Toller 1970), while the role of intracystic pressure has been proposed (Kubota et al. (2004)).

Differential diagnosis between periapical cysts and granulomas

Differential diagnosis has always been a difficult clinical problem. Every now and then, several methods have been proposed for the accurate final diagnosis of these lesions. It is generally acceptable now that the most accurate method to set the final diagnosis is the histological examination following total surgical removal of the lesion. However, such an approach does not allow for conservative healing through endodontic treatment, since the lesion must be surgically removed in order to be histologically examined. Consequently, other methods –preferably non-surgical- are needed to set the final diagnosis.

Pulp necrosis secondary to inflammation may be followed by the development of a periapical lesion, that is, periapical abscess, periapical granuloma or periapical (radicular); all these lesions are a sequel of pulpal necrosis (SPN). However, a variety of other lesions may mimic a SPN, when they develop in a periapical location. As regards the latter, their cause is not related to the pulp; some periapical lesions that are not a sequel of pulp necrosis include developmental cysts (mainly odontogenic keratocysts), fibro-ooseous lesions (mainly central ossifying fibroma) and benign or malignant tumors (ameloblastoma, central giant-cell granuloma, Langerhans cell

histiocytosis, metastatic tumors and so on) (Kontogiannis et al. 2015a). A pulp vitality test is really essential in this stage in order to determine whether a lesion around the apex of a tooth is of pulpal origin or not, since in most lesions not resulting from pulpal infection the pulp of the involved tooth reacts normally to vitality tests. Nevertheless, these tests are not able to distinguish the cause of the lesion by themselves; literature data indicate that even in some lesions not resulting from pulpal necrosis a negative response to vitality tests may be elicited (Dahlkemper et al. 2000).

When periapical lesions are relatively small, they may pass unnoticed and asymptomatic. In this phase, they may be found incidentally with a radiograph taken for an irrelevant reason. However, as their size increases overtime, the following signs may be noted:

- local intraoral swelling in accordance with the location of the apex of the involved tooth. Swelling is more usually noted buccally than lingually (lingual swelling is attributed to lesions resulting from maxillary lateral incisors, palatal roots of molars and rarely of maxillary premolars). The size of the swelling is not related to the final diagnosis.
- fluctuation
- presence of a yellowish fluid after drainage either through the root canal or after incision. In case of inflammation, this fluid may be semi-liquid or purulent.

All these signs are noted in cases of radicular cysts; however, they are not sufficient by themselves to set the final diagnosis, since they are notified only in large lesions.

Radiographic examination

Radiographic examination is carried out with periapical radiographs, conventional or digital, with or without a contrast medium, and cone beam computed tomography (CBCT).

In the periapical radiograph, a radiolucent area surrounding the apex of the responsible tooth is displayed. Its size varies and it may also expand towards the neighboring teeth. Traditionally, a large lesion surrounded by a radiopaque lamina is likely to be a cyst; however, this has not been confirmed in the literature (Siskos 2006).

Some studies have tried to correlate the radiographic size of the lesion with the final diagnosis. According to this assumption, small lesions, below 70mm^2 seem to be mainly periapical granulomas, while larger lesions, above 200mm^2, are more likely to be cysts (Lalonde 1970, White et al. 1994). It is important to keep in mind that periapical radiographs are two-dimensional depictions of three-dimensional structures; thus the evaluation of the "size" parameter is made only in the mesial-distal direction and not in the buccal-lingual one, leading to a possible misinterpretation (Kafle & Gratt 1988).

The application of a contrast medium can be made either through the root canal or through the oral mucosa (in large lesions) before taking a radiograph. Forsberg & Hagglund (1960) used this technique to depict periapical lesions; afterwards, all lesions were surgically removed and histologically examined to confirm their diagnosis. They concluded that cysts display a round and smooth periphery while granulomas have irregular shape and varying radiographic density. However, a later study (Cunningham & Penick 1968) showed that this depiction does not always applies exclusively on cysts. In this same study the contrast medium was applied on 41 periapical lesions related with teeth with pulp necrosis; 37 of them displayed the image described by Forsberg &

Hagglund (1960). However, histologic examination showed that only 11/41 lesions were cysts, with no significant correlation between the type of lesion and the radiographic image. A newer study investigated the use of computerized tomography enhanced with contrast medium for the differential diagnosis between radicular cysts, follicular cysts, odontogenic keratocysts and ameloblastomas (Kakimoto et al. 2013). However, although the contrast medium allowed better assessment of the size of each lesion, no significant correlation between the medium-enhanced image and the histologic diagnosis was established. Thus, the application of a contrast medium does not seem to offer more diagnostic information than the usual periapical radiograph (or even computerized tomography); this is why it is not frequently used in clinical practice.

CBCT:

The CBCT is an evolved form of computed tomography. Its main advantages are the relatively low radiation dose, the higher resolution and the higher sensitivity in depicting periapical lesions (Stavropoulos & Wenzel 2007). Simon et al. (2006) studied lesions via CBCT whose size was approximately 100mm^2 and evaluated them according to their score in gray-scale (low score was for air or fluid and high score was for bone or soft tissue); they concluded that the reliability of CBCT is really high and even equal to that of histological examination. However, these results were put to doubt by the study of Rosenberg et al. (2010) who found that, in lesions smaller than 25mm^2, the reliability of CBCT in determining the final diagnosis ranged between 51% and 63%.

Cytologic examination:

In a cytologic examination, material aspirated either from the root canal or through the oral mucosa is spread onto microscopic slides, followed by the application of the Papanikolaou staining procedure in order to find epithelial cells that surround the cystic lumen (Whitten 1968, Howell & De la Rosa 1968). Nevertheless, this method is not so specific for diagnosing radicular cysts, because of the following reasons:

- epithelial cells are also spotted in periapical granulomas
- the material aspirated might have been mixed with epithelial cells from the oral cavity when the aspiration is carried out from the oral mucosa.

Electrophoresis:

In this method, proteins obtained from the periapical lesion through aspiration are dispersed into polyacrylamide gel and put into an electric field, which separated them according to their molecular mass. Morse et al. (1973) showed that radicular cysts contain proteins with a greater molecular mass than periapical granulomas. Nevertheless, this method has significant drawbacks, mainly the existence of the appropriate equipment and the inability to retrieve adequate material (in terms of quantity) from the root canal.

Ultrasound real time imaging:

Ultrasound real time imaging can be used to provide information about the content of a cavity in the body; this is why it was proposed to be used for the diagnosis of periapical lesions (Cotti et al. 2002). In ultrasonography, cysts have a low degree of sound, smooth periphery with bony walls surrounding it and contain fluid. Periapical granulomas also have a low degree of sound but its periphery has an irregular shape;

furthermore, there is no cystic fluid but there are many blood vessels (Cotti et al. 2002). Ultrasound imaging usually displays lesions at a smaller size than their real one because of the bony wall that surrounds periapical lesions. This is due to reflection of the ultrasounds by the bone, thus resulting in more difficulty in detecting the lesion (Cotti et al. 2002).

Treatment of radicular cysts

Traditionally, a major talking point in literature is whether radicular cysts can be treated only with conservative endodontic treatment. Given that true cysts have no communication with the infected root canal, it could be inferred that even if bacterial infection is controlled (with endodontic treatment), the cyst will continue to exist since it does not depend on the communication with the canal for its growth. On the other hand, Sjogren et al. (1990) report that about 85% of periapical lesions heal after only conservative endodontic treatment, and this argument cannot be neglected. Therefore, since the discrimination between periapical granulomas, true and pocket radicular cysts is not possible without histological examination, conservative endodontic treatment should always be the first choice of the clinician.

As for the possibility of non-healing of true cysts, Bhaskar (1972) had suggested that, since bacterial control through endodontic treatment would not lead to the resolution of the cyst, the cleaning and shaping should be extended beyond the apical foramen. This would result in destruction of the cyst epithelium, acute inflammatory response and finally destruction of the cystic wall from the PMNs. However, even if cleaning and shaping beyond the apical foramen leads to epithelium destruction (which is not fully accepted yet), it is bound to traumatize periapical tissues (Bender 1972); thus, the approach described by Bhaskar (1972) remains ambiguous.

Healing of radicular cysts

The process of healing includes the same stages and mechanisms as in any other organ. Its main points are the formation of a granular tissue, the destruction of bacteria and necrotic elements by macrophages and finally the regeneration or the repair of periapical tissues. Regeneration differs from repair in that the newly formed tissues are the same as the ones damaged as a result of the periapical lesion. Lin et al. (2009) suggest that recession of the radicular cyst is essential for the onset of periapical healing. There are 2 possible mechanisms for this:

- Cyst recession and bone regeneration at the same time. As osteoblasts begin the formation of the new bone, epithelial cells undergo apoptosis resulting in cyst shrinkage

- Epithelial cells of the cyst undergo apoptosis while the basement membrane degenerates under the influence of matrix metalloproteinases (MMPs). This allows the connective tissue to develop into the lumen; in the end, the cyst epithelium recedes either totally or a few epithelial cells will remain within the periodontal ligament. However, it is not known whether these cells are ERM or differentiated epithelial cells from the former cyst epithelium.

After conservative endodontic treatment, epithelial cells seem to undergo apoptosis. Endodontic treatment results in the elimination of inflammatory elements; blood vessels endothelium, inflammatory cells and fibroblasts of the periapical lesion will also undergo apoptosis (Majno & Joris 1995). Apoptosis takes place without provoking any inflammatory response. Torabinejad (1983) suggested that epithelial cells of radicular cysts possess the ability of foreign material phagocytosis; therefore, epithelial cells may digest antigens from the infected root canal and obtain antigen characteristics.

The destruction of the cyst epithelium takes place via cellular and humoral immunity. The former is carried out via the cytotoxic T lymphocytes (CTL) which destroy virus-infected cells with 2 mechanisms: the secretion of perforin, which creates gaps onto the cell surface, and the activation of Fas, which leads to programmed cell death, known as apoptosis (Alberts et al. 2003). PMNs also express Fas and Fas-L, reinforcing the above-mentioned apoptosis pathway. Loyola et al. (2005) showed that apoptosis in radicular cysts indeed takes places in the epithelium, particularly in sites where the epithelium is hyperplastic rather than atrophic; this was an immunohistochemical study for the detection of Bcl-2 protein, which is important in apoptosis. Suzuki et al. (2005) carried out an immunohistochemical study detecting p53, Bax, Fas, Fs-L, Ki-67 and Caspase-3 proteins in radicular and residual cysts and found that apoptosis takes place in the epithelium of both lesions.

Healing of periapical tissues follows the same mechanisms no matter what kind of treatment has been carried out (conservative or surgical endodontic treatment). The only difference is that healing occurs faster after surgical treatment since the inflammatory elements are removed during the surgical intervention while after conservative endodontic treatment these elements are removed by macrophages which is time consuming (Majno & Joris 2005).

CALCIUM HYDROXIDE ($Ca(OH)_2$)

Properties and mechanism of action

Calcium hydroxide ($Ca(OH)_2$) was used in endodontics in the first place for direct pulp capping (Hermann 1920) and then as an intracanal medicament. The latter consists of pure $Ca(OH)_2$ powder mixed with saline or anesthetic solution. Its molecular weight is 74.08 (Farhad & Mohammadi 2005) and its solubility in water is rather low (1.2 g L^{-1} at 25^o C); furthermore, solubility decreases as temperature rises (Siqueira & Lopes 1999). Low solubility is a clinically significant property: this is why $Ca(OH)_2$ remains for a long period of time before being dissolved by the fluids and exudate from periapical tissues (Spangberg & Haapasalo 2002).

Mixing powder with saline or anesthetic solution results in a creamy paste with pH values between 12.5 and 12.8 (Farhad & Mohammadi 2005). During mixing, the ionization of $Ca(OH)_2$ takes place:

$$Ca(OH)_2 \xrightarrow{H_2O} Ca^{2+} + 2OH^-$$

Since this is an one-way reaction, $Ca(OH)_2$ is considered to be a strong base; however, only 0.2% of the initial $Ca(OH)_2$ placed into the root canal is ionized (Rehman et al. 1996). Hydroxide anions (OH^-) are responsible for further activities of $Ca(OH)_2$.

$Ca(OH)_2$ is also able to react chemically with carbon dioxide (CO_2) in the air, leading to the formation of calcium ($CaCO_3$):

$$Ca(OH)_2 + CO_2 \rightarrow CaCO_3$$

Calcium carbonate eventually results in paste setting. This is the reason why $Ca(OH)_2$ paste must be immediately placed into the canal after mixing. In spite of this, its

alkalinity does not seem to alter even after 30 days after placement (Estrela & Pesce 1996).

The mechanism of action of $Ca(OH)_2$ is due to the activity of the OH^- ions that are produced during ionization. After they have been produced, they diffuse and are able to be active even far from their original source, that is, from the lumen of the root canal (Siqueira & Lopes 1999). From then on, the mechanisms that have been proposed to explain their action are the following (Siqueira & Lopes 1999):

- the negative charge of OH- ions alters the polarity of the charge of the cell membrane of bacteria; this leads to a reversal of the pH values within which the membrane normally functions. As a result, the cell membrane cannot maintain its stability anymore and destabilizes

- protein denaturation, especially for those proteins that are located on the surface of bacteria. After cell membrane destabilization, OH- ions diffuse into the cytoplasm and lead to further denaturation of enzymes which are important to bacterial metabolism (Estrela et al. 1998)

- DNA damage.

In order these mechanism pathways to take place, a constant ionization of $Ca(OH)_2$ is essential, thus it is essential to disperse the powder into a liquid solvent. Athanassiadis et al. (2007) showed that the greater molecular weight the solvent has, the more difficult becomes the ionization. Consequently, water-based solvents are preferred, such as saline (Mohammadi & Dummer 2011). The use of anesthetic solution is also acceptable, although it could be hypothesized that since anesthetics have an acidic pH (between 4 and 5) they might neutralize the alkaline pH of $Ca(OH)_2$. However,

Athanassiadis et al. (2007) showed that, although this reaction indeed happens, it is clinically insignificant; $Ca(OH)_2$ is a strong base while anesthetics are weak acids, therefore the concertation of OH^- ions is by far greater than that of H_3O^+ ions. This means that neutralization of OH^- ions does not have a significant effect on the final pH.

Nowadays, there are $Ca(OH)_2$ pastes that can be directly injected into the root canal. These products also contain organic carriers such as glycerin; however, these solvents have a great molecular weight which, as described above, is not appropriate for a satisfactory ionization of $Ca(OH)_2$ (Athanassiadis et al. 2007). Therefore, mixing powder with saline should be preferred over ready-to-use pastes. Another reason is the fact that organic solvents might leave residues within the root canal, which may hinder a satisfactory obturation (Fava & Saunders 1999).

Antimicrobial activity

The antimicrobial activity of $Ca(OH)_2$ is due to the OH^- therefore this activity remains as long as the pH remains within alkaline ranges (Siqueira & Lopes 1999). Bystrom et al. (1985) showed that the application of $Ca(OH)_2$ led to significantly fewer bacteria residuals within the root canal compared with camphorated paramonochlorophenol. This difference was attributed to the activity of the OH^- ions released by $Ca(OH)_2$. In this study, $Ca(OH)_2$ and camphorated paramonochlorophenol were placed in 35 and 50 root canals respectively, after cleaning and shaping; the evaluation of bacterial levels was made with culture. Stevens & Grossman (1983) had previously indicated the superior activity of $Ca(OH)_2$ compared to camphorated paramonochlorophenol, with similar methodology. They also place emphasis on the fact that the suspension of bacterial growth as a result of $Ca(OH)_2$ is greater when this comes in contact with

bacteria. This argument apparently reinforces the view of Bystrom et al. (1985) about the need for condensation of $Ca(OH)_2$ with paper points.

The minimum time required for $Ca(OH)_2$ to remain within the root canal is 1 week (Sjogren et al. 1991). In this study, this time period was determined as the minimum time needed to get a negative culture. The minimum time of 1 week is also supported by the study of Shuping et al. (2000); in this study, after cleaning and shaping in 42 root canals of teeth with periapical lesions, $Ca(OH)_2$ was placed for 1 week. Both immediately after cleaning and shaping and after 1 week, samples were taken for culture. Cleaning and shaping by itself resulted in a negative culture in 61.9% of the canal, while the application of $Ca(OH)_2$ for 1 week resulted in a negative culture in 92.5% of the canals.

Literature data indicate that *E. faecalis* is resistant to the activity of $Ca(OH)_2$ (Sundqvist et al. 1998, Evans et al. 2002). This resistance ability is due to a proton pump located on the surface of this microbe. This pump allows E. faecalis to absorb protons even in an alkaline environment. In this way, cytoplasmic pH of *E. faecalis* remains acidic even in the presence of $Ca(OH)_2$ (Booth 1985, Evans et al. 2002). Rocas et al. (2004), applied nested-PCR on samples from 80 root canals: 21 from teeth with no periapical lesion, 10 from teeth with necrotic pulps and acute apical periodontitis, 19 from teeth with necrotic pulps and acute apical abscess with swelling and fever, and 30 from retreatments because of periapical lesions. They found that *E. faecalis* was detected only in 18% of cases where endodontic treatment was carried out for the first time; on the contrary, it was detected in 67% of retreatments. The latter frequency of *E. faecalis* lies within the range of *E. faecalis* in retreatments that has been recorded in the literature: 24% (Engstrom 1964) to 77% (Siqueira & Rocas 2004). It is also important

to mention that Rocas et al. (2004) found a statistically significant relationship between the levels of *E. faecalis* and retreatments. This relationship could be attributed to the following factors (Rocas et al. 2004):

- the conditions within an obturated root canal probably cannot suspend the development of *E. faecalis*
- resistance to $Ca(OH)_2$ due to a protein pump (as described above)
- ability of *E. faecalis* to survive without being dependent on other bacteria.

Chlorexidine has been proposed to eradicate *E. faecalis* from the root canals either alone or combined with $Ca(OH)_2$ as some studies recorded better results with their combination (Waltimo et al. 1999, Evans et al. 2003), particularly against *E. faecalis* (Schafer & Bossmann 2005). A major argument against the combination of these 2 medicaments is that chlorhexidine may be neutralized when combined with $Ca(OH)_2$ since the former is active in an acidic pH and the latter creates a highly alkaline pH (Haenni et al. 2003). A detailed description of the interaction between $Ca(OH)_2$ and chlorhexidine is given later.

Antimicrobial activity should also include action against endotoxins. Endotoxins are lipopolysaccharides (LPS) which are found at the cell wall of gram-negative bacteria; they are released during bacterial proliferation or death (Rietschel & Brade 1992). LPS levels seem to be higher in cases of symptomatic teeth, given that more gram-negative bacteria are found in the infected root canals of such teeth (Oliveira et al. 2012). Their role in periapical inflammation is summarized in the following points (Stashenko 1990, Barthel et al. 1997, Sousa et al. 2014):

- activation of monocytes/macrophages for eventual release of pro-inflammatory cytokines (IL-1b, IL-6, CXCL8 or IL-8, TNF-a) and prostaglandins (the latter are related with bone resorption)
- activation of the complement system
- adhesion of leucocytes onto the endothelial cells
- irreversible adhesion of LPS on calcified tissues.

Tanomaru et al. (2003) tried to evaluate the histopathologic reaction of periapical tissues of dogs to *E. coli* LPS, in relation to 4 different irrigation solutions (NaOCl 1%, 2.5%, 5%, chlorexidine 2%) and the application (or not) of $Ca(OH)_2$. They found that none of the above used irrigation solutions was effective against LPS; on the contrary, $Ca(OH)_2$ reduced LPS levels with a statistically significant difference with the irrigation solutions. Jiang et al. (2003) also showed that $Ca(OH)_2$ reduces the LPS-induced differentiation of osteoclasts. Clinically, in terms of LPS, the application of $Ca(OH)_2$ is really essential in cases of infected root canals particularly when symptoms exist (since gram-negative bacteria and LPS are higher in these cases).

A talking point in the literature is the effectiveness of $Ca(OH)_2$ on biofilms. The term biofilm was introduced to designate the thin-layered (sessile) condensations of microbes that may occur on various surface structures in nature (Nair 1987, Svensater & Bergenholtz 2004). Biofilms are firmly adhered on solid surfaces (in case of root canals, these surfaces are the dentin walls). Free-floating bacteria existing in an aqueous environment, the so-called planktonic form of microorganisms, are essential in order a biofilm to be created (Bowden & Hamilton 1998). Biofilm formation on dentin walls bears many resemblances to the formation of the dental plaque. The existence of

biofilms may explain failure of some cases in which a lege artis endodontic treatment has been carried out (therapy-resistant apical periodontitis) (Nair et al. 2005); in this study, after careful cleaning and shaping, copious irrigations and obturations in 1 visit, biofilms remained in 14/16 root canals treated during the study. Biofilms may also be formed in the external surfaces of the root apex; this is the cause of the extra-radicular infection, which is treated only by combining conservative and surgical endodontic treatment. The difficulties in removing biofilms from the root canal successfully during cleaning and shaping are also due to the fact that even the functions of bacteria alter when these are a part of a biofilm. Svensater & Bergenholtz (2004) report that any bacteria of a biofilm appears to be 2-1000 times more resistant than when it is in its planktonic form. Taken together, the use of an intracanal medicament that will be active against biofilms seems to be essential. However, literature data on the effectiveness of $Ca(OH)_2$ on biofilms are not without controversy. Chai et al. (2007) found that $Ca(OH)_2$ was 100% effective against biofilms composed of *E. faecalis*. However, this is an *in vitro* study (therefore the experimental conditions are not the same as in a root canal); moreover, the biofilms used consisted of only one bacterial species, while in infected root canals biofilms consists of many different species (Estrela et al. 2009). Distel et al. (2002) had also previously showed that even in $Ca(OH)_2$-medicated root canals, it is possible that the canal might be colonized in a later time by *E. faecalis* and a *E. faecalis*–based biofilm could be created. Consequently, the effectiveness of $Ca(OH)_2$ on biofilms seems to be dubious, so further investigation on this issue is required.

Last but not least, the role of fungi in root canal microbiology and the effectiveness of $Ca(OH)_2$ (if any) on them has also been studied in the literature. Fungi are usually detected in root canals that have been re-infected after endodontic treatment, as well as in some periapical lesions that do not respond to conventional endodontic treatment; in

contrast, they are very rarely spotted in primary infections. Literature data indicate that 1-17% of all infected root canals may also contain fungi (Siqueira & Sen 2004, Waltimo et al. 2004). *C. albicans* is the most important and most studied fungus; in the oral cavity, it is detected in 35-40% of healthy people (Arendorf & Walker 1980, Lucas 1993) and up to 95% of immunocompromised patients particularly as a result of HIV infection (Dupont et al. 1992). *C. albicans* is resistant to $Ca(OH)_2$. This might be due to the following reasons (Siqueira & Sen 2004):

- the fungus is able to survive within a large pH range, which probably includes the alkaline pH created by $Ca(OH)_2$
- calcium ions that are required for *C. albicans* are provided by the ionization of $Ca(OH)_2$ itself.

In spite of all this, the fact that NaOCl (as the first-choice irrigation solution) is effective against *C. albicans* is an important point to keep in mind for clinical practice. Ferguson et al. (2002) tested the *in vitro* efficacy of NaOCl, chlorexidine, hydrogen peroxide, $Ca(OH)_2$ and camphorated paramonochlorophenol on *C. albicans.* They found that all tested substances were effective against the fungus apart from $Ca(OH)_2$. Furthermore, they found that the minimum inhibitory concentration of NaOCl was particularly low (10μg/ml); thus, its effectiveness against *C. albicans* in the usual concentrations in clinical practice is very high. On the other hand, Siqueira et al. (2003) showed that $Ca(OH)_2$ paste combined with paramonochlorophenol was more effective against *C. albicans* attributing this result to the existence of paramonochlorophenol. Taken together, $Ca(OH)_2$ does not seem to be an effective intracanal medicament against *C. albicans* but the use of NaOCl for irrigation –and if needed, the application of

paramonochlorophenol for intracanal medication- is sufficient enough to eliminate *C. albicans* from infected root canals.

Buffering effect of dentine on $Ca(OH)_2$

The root canal is composed of inorganic and organic components. Hydroxyapatite is the major representative of the former, while pulp tissue, microorganisms and inflammatory exudate, rich in proteins such as albumin (Haapasalo et al. 2007), are the major organic ingredients. Interestingly, few studies have tried to relate the various organic and inorganic compounds with the inactivation of root canal (Haapasalo et al. 2000). Difficulties in designing experiments that will give reliable and comparable data have been some of the greatest challenges. The study of Haapasalo et al. (2000) could be regarded as pioneering in this; they introduced a new dentine powder model for studying the inhibitory effect of dentine on various root canal irrigants and medicaments. They found that in the positive control group (absence of dentine), saturated $Ca(OH)_2$ killed *E. faecalis* cells in a few minutes, whereas with the dentine powder added, no reduction in the bacterial colony-forming units could be measured even after 24 h of incubation with $Ca(OH)_2$; their conclusion was that dentine powder effectively abolished the killing of *E. faecalis* by $Ca(OH)_2$ (Haapasalo et al. 2000). Hydroxyapatite seems to have an effect similar to dentine on $Ca(OH)_2$ (Portenier et al. 2001). The lowest concentration of dentine needed for achieving a buffering effect on $Ca(OH)_2$ was only 1.8% w/v (Portenier et al. 2001). The substantial effect of dentine on the antibacterial activity of $Ca(OH)_2$ can be attributed to the buffering action of dentine against alkali (Wang & Hume 1988).

$Ca(OH)_2$ is used as a thick paste *in vivo*; however, its solubility is low and saturation is achieved in a relatively low concentration of hydroxyl ions. Studies have shown that

buffering by dentine may be behind the reduced antibacterial effect of $Ca(OH)_2$, particularly in the sub-surface layers of the canal walls. It is also possible that deeper in dentine (outside the main root canal), $Ca(OH)_2$ is present as a saturated solution or at even lower concentrations (Haapasalo et al. 2000). Besides dentine, remnants of necrotic pulp tissue as well as inflammatory exudate might affect the antibacterial potential of endodontic disinfectants (Haapasalo et al. 2007).

Effect of $Ca(OH)_2$ on dentine

The flexural strength of dentine might, in part, depend on an intimate link between two main components of dentine, the inorganic hydroxyapatite crystals and the organic collagenous network. This organic component is composed of proteins and proteoglycans containing phosphate and carboxylate groups (Andreasen et al. 2002). These substances may act as bonding agents between the collagen network and the hydroxyapatite crystals (Andreasen et al. 2002). Rosenberg et al. (2007) measured the effect of $Ca(OH)_2$ on the microtensile fracture strength of teeth and found that it was reduced by almost 50% following 7–84 days of application. White et al. (2002) showed $Ca(OH)_2$ reduced fracture strength by 32%; in this study, bovine dentine was used, maintained in Petri dishes for 5 weeks. A similar reduction of the fracture strength of sheep dentine was also recorded in the study of Andreasen et al. (1989), who found that the percentage of reduction $Ca(OH)_2$ treatment for 1 year was 50%. The mechanism for this phenomenon has been proposed by Kawamoto et al. (2008) in bovine dentine; exposure to $Ca(OH)_2$ paste significantly increased the mean elastic modulus of dentine, thereby making it more prone to fracture. In another *in vitro* study, Grigoratos et al. (2001) also reported that treatment with $Ca(OH)_2$ reduced the flexural strength of human dentine. Andreasen et al. (2002), using sheep dentine, showed that the fracture

strength of $Ca(OH)_2$-filled immature teeth dropped by 50% in approximately 1 year. The same study attributed the frequent reports of fractures of immature teeth filled with $Ca(OH)_2$ for extended periods to this factor. The results of this study were confirmed by Doyon et al. (2005) who examined the resistance of human root dentine to intracanal medication with $Ca(OH)_2$ and found that the fracture resistance of was decreased significantly after 6 months. To sum up, dentine exposed to $Ca(OH)_2$ for an extended period (6 months to 1 year) leads to reduced flexural strength and lower resistance to fracture. This is important particularly in immature teeth whose treatment is a challenge; the concept of apical healing being promoted through the induction of an apical barrier combined with a high pH providing an antibacterial capability remains the ultimate aim (Frank 1966, Heithersay 1975).

$Ca(OH)_2$ and chlorhexidine

Chlorhexidine is a cationic biguanide whose optimal antimicrobial activity is achieved within a pH range of 5.5–7.0 (Athanassiadis et al. 2007). Therefore, it is likely that in a combination of these 2 medicaments, chlorhexidine may be neutralized when combined $Ca(OH)_2$ since the former is active in an acidic pH and the latter creates a highly alkaline pH (Haenni et al. 2003); in this study, agar diffusion tests were applied, and no additional antibacterial effect by mixing $Ca(OH)_2$ powder with 0.5% chlorhexidine could be found. Furthermore, alkalinizing the pH by adding $Ca(OH)_2$ to chlorhexidine will lead to precipitation of chlorhexidine molecules, thereby decreasing its effectiveness (Mohammadi & Abbott 2009).

On the other hand, many studies demonstrate a better antimicrobial effect in combinations of $Ca(OH)_2$ with chlorhexidine. When used as an intracanal medicament, chlorhexidine was more effective than $Ca(OH)_2$ in eliminating *E. faecalis* from inside

dentinal tubules (Athanassiadis et al. 2007). Almyroudi et al. (2002) used various formulations of chlorexidine including a chlorexidine/$Ca(OH)_2$ in a 50 : 50 ratio and found that all of them were effective in eliminating *E. faecalis* from dentinal tubules. The results of this study were later confirmed by Gomes et al. (2006) in bovine dentine and Schafer & Bossmann (2005) in human dentine. In an *in vitro* study using human teeth, Ercan et al. (2006) reported that 2% chlorexidine gel was the most effective agent against *E. faecalis* inside dentinal tubules, followed by a $Ca(OH)_2$/2% chlorhexidine mixture; $Ca(OH)_2$ alone was not effective even after 30 days. In another in vivo study using primary teeth, a 1% CHX-gluconate gel, both with and without $Ca(OH)_2$, was more effective against *E. faecalis* than $Ca(OH)_2$ alone over a 48-h period (Oncag et al. 2006). Schafer & Bossmann (2005) reported that 2% chlorhexidine gluconate was significantly more effective against *E. faecalis* than $Ca(OH)_2$ used alone or a mixture of the two. In an animal study, Lindskog et al. (1998) reported that teeth dressed with chlorexidine for 4 weeks had reduced inflammatory reactions in the periodontium (both apically and marginally) and less root resorption. Taking together into account, all the above results together into account, it seems that by mixing $Ca(OH)_2$ with chlorexidine the antimicrobial activity is increased.

Removal of $Ca(OH)_2$ from canals

$Ca(OH)_2$ placed as a medicament has to be removed before the canal is filled. *In vitro* studies have revealed that remnants of $Ca(OH)_2$ can prevent the penetration of sealers into the tubules (Calt & Serper 1999), hinder the bonding of resin sealers to dentine, increase the apical leakage of root fillings (Kim & Kim 2002) and potentially interact with zinc oxide eugenol sealers and make them brittle and granular (Margelos et al. 1997). Therefore, complete removal of $Ca(OH)_2$ from the root canal before filling is essential. Lambrianidis et al. (1999) evaluated the effectiveness of removing $Ca(OH)_2$

associated with several vehicles from the root canal including normal saline, 3% NaOCl, 3% NaOCl + 17% EDTA as irrigants in combination with hand filing; they found that in 45% of the canal surface area $Ca(OH)_2$ were still identified. They inferred that the amount of $Ca(OH)_2$ powder in the paste did not affect removal, but the vehicle did. Margelos et al. (1997) revealed that using 15% EDTA or NaOCl alone as irrigants was not so helpful a protocol for removing $Ca(OH)_2$ from the root canal, but the combination of these irrigation solutions together with hand instrumentation improved removal. Similar effectiveness of EDTA in $Ca(OH)_2$ removal was also noted by Nandini et al. (2006), and this was related with the vehicle used to prepare $Ca(OH)_2$ paste. Oil-based $Ca(OH)_2$ paste was more difficult to remove than $Ca(OH)_2$ powder mixed with distilled water. Both 17% EDTA and 10% citric acid were found to remove $Ca(OH)_2$ powder mixed with distilled water, whereas 10% citric acid performed better than EDTA in removing an oil-based $Ca(OH)_2$ paste. In another study, Lambrianidis et al. (2006) compared the removal efficiency of $Ca(OH)_2$ mixed with chlorexidine gel, chlorhexidine solution and saline using instrumentation with or without a patency file and irrigation with NaOCl and EDTA solutions. Remnants of medicaments were found in all canals regardless of the experimental material or use of patency filing. When examining the root canal as a whole, $Ca(OH)_2$/chlorexidine gel paste was related to significantly larger amounts of remnants; in contrast, the $Ca(OH)_2$/chlorexidine solution paste was associated with less residue with or without the use of patency filing. The use of patency filing was applied in order to facilitate removal in the apical third (Lambrianidis et al. 2006).

Besides combining irrigation and instrumentation, another method to remove remnants of $Ca(OH)_2$ from the root canal involves ultrasonics. Kenee et al. (2006) assessed the amount of $Ca(OH)_2$ residues in canals after removal with various techniques including

combinations of NaOCl with EDTA irrigation, hand filing, rotary instrumentation, or ultrasonics; they found that no technique removed the $Ca(OH)_2$ entirely. Rotary and ultrasonic techniques, although not very different from each other, removed significantly more $Ca(OH)_2$ than irrigation and hand filing. van der Sluis et al. (2007) combined the use of irrigation with ultrasonics (passive ultrasonic irrigation) to remove a $Ca(OH)_2$ paste from the root canal, using either NaOCl or water as irrigants. Results demonstrated that passive ultrasonic irrigation with 2% NaOCl was more effective in removing $Ca(OH)_2$ paste than NaOCl or water. On the other hand, Balvedi et al. (2010) found that none of the above used ways were efficient in removing intracanal medicaments.

Taken together, $Ca(OH)_2$ paste cannot be completely removed from the root canal walls with the application of the usually used techniques. However, the type of vehicle used, patency filing and combining EDTA and NaOCl with hand instrumentation improves the efficacy of $Ca(OH)_2$ paste removal. Furthermore, ultrasonically-activated irrigations are more efficient in removing $Ca(OH)_2$ remnants than passive irrigations.

Toxicity of $Ca(OH)_2$ in medicaments

Early reports on the outcome of $Ca(OH)_2$ extruded into the periapical region concluded it was well tolerated and was resorbed (Martin & Crabb 1977). However, the periapical response to $Ca(OH)_2$ based on results from other reports seems to be disputed. Spangberg (1969) reported an inflammatory response with inhibition of bone healing 2 weeks after the implantation of $Ca(OH)_2$ into guinea-pig bone; nevertheless, bone remodeling and regeneration took place within 12 weeks of placement. $Ca(OH)_2$ has been reported to have a damaging effect on periodontal tissues when used as an intracanal medicament during root canal treatment (Hauman & Love 2003). Blomlof et al. (1988) observed that $Ca(OH)_2$ could negatively influence marginal soft tissue

healing; they suggested that root canal treatment should be completed before any periodontal therapy, during which cementum is removed. Breault et al. (1995) reported that the use of $Ca(OH)_2$ demonstrated a decreased inhibition of attached human gingival fibroblasts; they proposed that $Ca(OH)_2$ should be avoided as an interim medicament when trying to regenerate or establish new attachment in tissues adjacent to endodontically involved teeth. However, it is important to mention that the decrease noted in this study was not statistically significant. Thong et al. (2001) performed immediate experimental avulsion and immediate implantation, followed by root canal treatment, in monkeys; they applied either $Ca(OH)_2$ or corticosteroid-antibiotic paste (Ledermix) for 8 weeks. Histomorphometric evaluation after sacrificing showed that Ledermix resulted in a higher frequency of normal periodontal ligament compared with $Ca(OH)_2$ (79.6% and 64.6% respectively). Finally, in a recent systematic review, between 1980 and 2013 10 cases of tissue destruction resulting from extrusion have been reported (Olsen et al. 2014). Connective tissue breakdown, damage to blood vessels and nerve lesions (mainly to the inferior alveolar nerve) were found; most of them had been related with injectable $Ca(OH)_2$ products rather than applied with Lentulo spirals.

On the other hand, there are studies that do not agree on the detrimental effect of $Ca(OH)_2$ on the periodontium. Hammarstrom et al. (1986) demonstrated that $Ca(OH)_2$ did not affect the healing of replanted monkey teeth with intact cementum and only temporarily in those undergoing cemental repair. Pissiotis & Spangberg (1990) evaluated mandible bone reactions of pigs to implants of hydroxyapatite, collagen, and $Ca(OH)_2$, alone or in different combinations, over a period of 16 weeks. They did not record any major inflammatory reaction in any of the implant combinations. Hydroxyapatite was not resorbed over the examination periods, but $Ca(OH)_2$ and

collagen implants were partially or totally resorbed and replaced by bony tissue. Wakabayashi et al. (1995) evaluated the effect of a $Ca(OH)_2$ paste dressing on uninstrumented root canal walls and found that it could dissolve the odontoblastic cell layer, but had little effect on predentine. Holland et al. (1998) observed that periodontal healing associated with infected root canals filled with $Ca(OH)_2$ was not prevented 6 months after experimental periodontal surgical injury in dogs. Holland et al. (1999) reported that rat subcutaneous connective tissue reaction to $Ca(OH)_2$ and MTA inside the tubules was tolerable. They observed the formation of calcite granulations, birefringent to polarized light, near the lumen of dentinal tubule in $Ca(OH)_2$ samples. Under these granulations, a bridge of hard tissue was formed which was positive for von Kossa stain (which is used for the detection of calcium, see later for details). In MTA samples, the same granulations was observed, but their number was less than the $Ca(OH)_2$ group. Furthermore, contrary to the $Ca(OH)_2$ group, the calcite granulations were in contact with MTA. Guigand et al. (1999) confirmed the cytocompatibility of $Ca(OH)_2$ and a calcium oxide-based compound.

In summary, it seems that $Ca(OH)_2$ paste is eventually well tolerated by bone tissue. However, its effect on the periodontal tissue is disputed.

$Ca(OH)_2$ and periapical pathology

It is still unclear whether the application of $Ca(OH)_2$ in cases with periapical lesions indeed contributes to their healing. The healing rate of conservative endodontic treatment of teeth with necrotic pulps and apical periodontitis (retreatments excluded) in a single visit or with $Ca(OH)_2$ has been reported to be 96% and 88% respectively with no significant difference (Paredes-Vieyra & Enriquez 2012). On the other hand, the application of $Ca(OH)_2$ in all cases with periapical pathology is carried out on routine basis and even for a long time (more than 2 weeks) in some countries, mainly

Sweden (Bergenholtz et al. 2012). Although there is no strong level of evidence for this, perhaps $Ca(OH)_2$ facilitates healing of large periapical lesions, whose prognosis is not as good as that of small lesions (Matsumoto et al. 1987). A clinical study by Caliskan (2004) showed that the long-term application of $Ca(OH)_2$ for 3 months (with 3-week intervals) resulted in complete healing of large lesions (with a maximum radiographic diameter 7-18mm) in 73.8% of cases and partial healing in an additional 9.5% of cases, all followed-up for 2-10 years. Although there is no control in this study, it clearly demonstrates that $Ca(OH)_2$ in teeth with large periapical lesions can be an alternative treatment to surgical therapy.

Another clinically-established view about the application of $Ca(OH)_2$ is that it should be placed in cases with periapical pathology and exudate in the canal. Indeed, a tooth with constant clear or reddish exudate associated with a large apical radiolucency is a difficult condition to treat. Such a tooth is often asymptomatic, but some degree of sensitivity to percussion and/or palpation of the oral mucosa over the apex may also be noted. If cultured, the drainage will not generally support bacterial growth (Weine 2004). When the pulp chamber is opened during access cavity preparation, a reddish or yellowish fluid discharge may appear, whereas at the succeeding appointment the exudate will be clear. If such a tooth is left opened under a rubber dam for 15–30 min, the exudate will stop (Bence et al. 1982); however, a similar condition will still be present at the next appointment even though canal preparation to an acceptable size has been achieved. This is referred to as a ‘weeping canal’ (Weine 2004). The most appropriate way to halt the exudate discharge in such cases is to dry the canal with sterile paper points and to place $Ca(OH)_2$ paste in the canal. The possible mechanism of action of $Ca(OH)_2$ in these cases is related to its basic pH, which converts the acidic pH of periapical tissues to a more basic environment, as well as its hygroscopic ability

to absorb fluids. Two other theories have also been proposed: (i) the calcifying potential of $Ca(OH)_2$ may start to build up bone in the lesion and (ii) the caustic action of $Ca(OH)_2$ cauterises residual chronically inflamed tissue (Weine 2004).

Other applications of $Ca(OH)_2$ besides intracanal medication

$Ca(OH)_2$ is also used in other ways besides intracanal medication. These include the following (Mohammadi & Dummer 2011):

- Apexification
- Perforations
- Pulp capping
- Pulpotomy
- Root resorption
- Sealers

However, since none of the above applications is related to periapical pathology, further analysis about them is out of the focus of the present thesis.

Hard calcified tissue formation induced by $Ca(OH)_2$

It has been mentioned in the literature that $Ca(OH)_2$ may induce the formation of hard tissue, either dentin-like (in cases of pulp capping and pulpotomy) (Hargreaves & Goodis 2002) or bone-like tissue (in cases of apexification) (Ham et al. 1972, Grossman 1988). Such formation is also noted with other materials, most notably the mineral trioxide aggregate (MTA). A difference between the two is that the tissue induced by $Ca(OH)_2$ displays more tubular-like openings (Cox et al. 1985). When in contact with the periodontal tissues, formation of calcite granulations and a bridge of hard tissue, positive for the von Kossa stain (which is used for the detection of calcium, see later for details), was seen both in $Ca(OH)_2$ and MTA, in rats (Holland et al. 1999). Such similar hard tissue formation may be because of the similarity of the mechanism of

action of MTA and $Ca(OH)_2$; the calcium oxide in the MTA powder is converted into $Ca(OH)_2$ when the paste is prepared with water. Therefore, in contact with periodontal tissues and fluids, both materials would eventually dissociate into calcium and hydroxyl ions. The calcium ions reacting with the carbonic gas of the tissues would originate the calcite granulations, starting the creation of the hard tissue. Close to these granulations, there is accumulation of fibronectin, which allows cellular adhesion and differentiation (Holland et al. 1999).

The aforementioned literature data indicate that calcium ions (resulting in clinical practice from materials such as MTA and $Ca(OH)_2$) may play a role in the hard tissue formation. More recent data also support this view. An et al. (2015) correlated, among others, the presence of calcium ions with mineralization onset on isolated and cultured dental pulp stem cells (DPSCs) for young patients; applied $CaCl_2$ as Ca^{2+} supplementation; alizarin red S staining and reverse transcription-polymerase chain reaction analysis were used to evaluate the onset of mineralization; the cell counting kit-8 and fluorescein isothiocyanate-Annexin V/propidium iodide double-staining method were adopted to detect the proliferation and apoptosis of DPSCs in the growth culture medium. They found that Ca^{2+} accelerated the onset of mineralized matrix nodule formation. The same researchers had previously shown, in a similarly-designed study, that calcium ions from $Ca(OH)_2$ for pulp capping promote osteogenic differentiation and mineralization of human dental pulp cells (An et al. 2012). Consequently, there is evidence to assume that calcium ions resulting from $Ca(OH)_2$ may participate in the formation of hard tissue (dentin-like or bone-like), possibly by stimulating stem cells. Such cells in a periapical lesion are found mainly in the periphery (in the surrounding bone) or even within the lesion itself (Liao et al. 2011).

This is the reason why the fate and the location of calcium ions resulting from $Ca(OH)_2$ is essential to be further studied in the future.

Methods for the histopathological detection of calcium

In a usual hematoxyline-and-eosine-stained (H&E) section from a radicular cyst, calcium is seen as baseophile-like deposits within the lesion (Fig. 1).

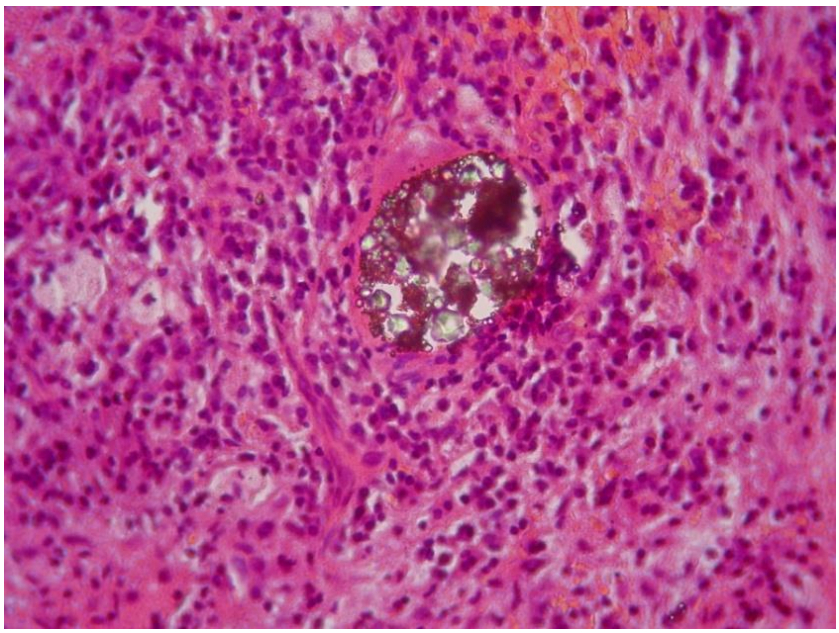


Fig. 1: Calcium ions within the cystic wall of a radicular cyst (baseophile bubble-like formation). H&E, x200.

However, H&E is not considered to be a calcium-specific method for staining. This is why the detection of calcium deposits is carried out by the application of particular histochemical methods. These methods could be roughly classified into two groups (Pearse 1960): methods that are specific for calcium (alizarin red, gypsym method, calcium red, phthalocyanin), and substitution methods for calcium (the von Kossa method).

Alizarin Red methods:

Alizarin dyes are all derivatives of anthraquinone. The first alizarin methods that were applied for the detection of calcium were reported to have a very low sensitivity, while their specifity was not so high either. The most accurate alizarin-based method for calcium detection is the alizarin red S (Dahl 1952). This technique is based on the fact that any difference in the pH results in a different metallic salt that is stained. The variety of the different metals detected with this method as alizarin complexes according to the pH range required for their spotting and the color of the deposits are displayed in Table 1.

Table 1. Solubility and color of alizarin complexes.

Salt	pH range	Color
$CaCl_2$	4.28-6.75	Reddish-orange
$FeCl_2$	5.62-8.05	Deep purple
$Fe(NO_3)_3$	5.73-6.25	Purplish-black
$Ba(C_2H_3O_2)_2$	4.08-8.05	Reddish-orange-magenta
$SrCO_3$	4.5-8.05	Reddish-orange-magenta
$Be(NO_3)_2$	5.73-8.05	Magenta
$CdCl_2$	5.11-5.78	Reddish-orange
$La(C_2H_3O_2)_3$	5.73-8.05	Cherry to light purple
$Pb(C_2H_3O_2)_2$	4.08-4.5	Reddish-orange to purple
$Hg_2(NO_3)_2$	4.5-5.62	Purple

The exact mechanism of staining was presented by Puchtler et al. (1969). They found that salt formation between calcium deposits and alizarin red S was markedly sensitive to pH changes. Contrary to the traditional alizarin methods (which stained better at

very high pH values, around 12), alizarin red S presents the best staining ability at a significantly lower pH, approximately 9 (Dahl 1952).

The Von Kossa technique:

The von Kossa technique (Von Kossa 1901) is a substitution method for the detection of calcium deposits. The main idea behind this technique is the conversion of calcium salts into their silver equivalent. The product of this reaction is subsequently reduced to black metallic silver; this reduction can be carried out using ultra-violet light or by the application of a strong reducing agent (Pearse 1960); calcium deposits are displayed black in the optical microscope. However, Pizzolato & McCrory (1962) showed that none of these two means reported above are necessary for the reduction of the silver equivalent, thus even sunlight is sufficient enough for reduction.

In order to enhance reliability, some modifications of the von Kossa technique have been proposed. The first is the autometallographic development of silver-stained mineral deposits (Danscher 1983); however, no particular increase in sensitivity was reported, since it produces a background similar to the original technique (Rungby et al. 1993). The second is the replacement of silver nitrate to silver lactate as a donor of silver ions for substitution (Rungby et al. 1993); this leads to markedly decreased background, thus enhancing specificity (Rungby et al. 1993). A combination of the autometallographic development with the application of silver lactate is a similar alternative, reducing non-specific reactions (Danscher 1984). Last but not least, uric acid or urates are indeed capable of causing confusion under histochemical conditions (Pizzolato & McCrory 1962); nevertheless, no literature data indicating the presence of these substances in periapical lesions, thus such confusion in the present study is unlikely.

In endodontic research, the von Kossa technique has already been used for the confirmation of hard tissue formation induced by $Ca(OH)_2$, MTA and bioceramics (Holland et al. 1982, Guven et al. 2013, Gomes-Filho et al. 2015), for the effect of these materials on the proliferation and differentiation of stem cells (Guven et al. 2011) and the creation of hard tissue by stem cells induced again by these materials (Du et al. 2013, An et al. 2012a & b, An et al. 2015). In all these studies, the results of the von Kossa technique were undisputed. However, it is important to mention that these studies do not concern a mere identification of calcium deposits (as in conventional histochemistry on paraffin-embedded tissue, such as in the present study), but the confirmation of mineralization in material resulting from cultures (creation of bone-like tissue). In the latter case, the sensitivity of the von Kossa technique is inferior to other methods for detecting mineralization in culture-resluting material, especially electron microscopic analysis, X-ray diffraction, and Fourier transform infrared spectroscopy (Bonewald et al. 2003). As concerns paraffin-embedde periapical lesions, no information about reliability is available; however, taking all the above literature data into account, it could be inferred that the von Kossa technique displays a high accuracy of detecting calcium deposits in periapical lesions.

Other histochemical techniques for calcium: gypsym method and calcium red method:

Gypsum method is a simple technique in which a single drop of 3% H_2SO_4 is applied onto the calcium-containing section. The result is a chemical reaction with calcium, which leads to the formation of gypsum crystals that can be observed with the microscope. Although this method is simple and has a high specifity, it can only detect ionizable forms of calcium; furthermore, in spite of the detection of calcium with this method, the definition of the cytochemical scale is impossible (Pearse 1960).

Kaufmann & Adams (1954) and McGee-Russell (1955) introduced the calcium red method. In this method, a saturated solution of ammonium purpurate in potassium cyanide together with the use of Nuclear fast red dye. Calcium deposits are displayed red. This method works very well in some invertebrate tissues but it is not so reliable when applied to mammalian tissues. Furthermore, it is more time-consuming than the other calcium-detecting methods (Pearse 1960).

Both methods have only rarely been applied in previous studies for the histochemical detection of calcium. Therefore, any further data about their reliability do not exist.

APOPTOSIS

Definition, etymology and general features

The term “apoptosis” refers to a genetically defined process of programmed cell death, which may occur under various conditions and stimuli (Rubin 2001). Although the term had been sporadically used since 1842, it was only in 1972 that the term was purposely used to describe the process of programmed cell death (Kerr et al. 1972). The word “apoptosis” results from the Greek “από” (“by, from, of") and “πτώσις” ("fall").

It is important to differentiate apoptosis from necrosis. Necrosis takes place when environmental signals overcome cellular adaptive response; the result is a massive tissue destruction. Pro-inflammatory products that lead to tissue damage mediate the process of necrosis while at the same time the initial stimulus is reinforced (Blagosklonny 2003, Loro et al. 2003). The most important event in the process of necrosis is the damage to the cell membrane. Destruction of the membrane disrupts the osmotic balance between the cell and its environment; thus, interstitial fluid flows into the cell, causing cell swelling and eventually disintegration (Duvall & Wyllie 1986). In contrast, apoptosis is a genetically programmed cell death mechanism that participates in the homeostatic control of cell population. This mechanism in a fashion opposes that of mitosis (Blagosklonny 2003, Satchell et al. 2003). Cell survival in the tissues depends on continuous arrival of positive signals, for instance the regular supply of oxygen. The lack of such stimuli activates apoptotic pathways to decrease the cellular population (Chu et al. 2002, Blagosklonny 2003, Loro et al. 2003, Renton et al. 2003). Apoptosis occurs in individual or small groups of cells and is initiated by specific signals (see below) which activate intracellular active pathways leading to mitochondrial, cytoskeletal and nuclear changes. Eventually, the cell disintegrates and

its components are engulfed by macrophages. No inflammatory reaction is elicited during this process (Blagosklonny 2003, Loro et al. 2003, Satchell et al. 2003).

Various stimuli and conditions may lead to the onset of apoptosis. These include the following (Rubin 2001):

- morphogenesis, such as during the formation of fingers and toes of the embryo
- normal renewal of cells in healthy tissues
- regulation of immune response, with apoptosis of autoimmune T cells or during maturation of T cells in the thymus
- lack of oxygen and/or hormones and growth factors
- viral infections
- ultraviolet radiation
- neoplastic lesions.

The cells that undergo apoptosis present some particular characteristics. First of all, significant cell shrinkage is noted; nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation, and global mRNA decay also take place (Arends and Wyllie 1991). Furthermore, cells that are in the final stages of apoptosis present phosphatidylserine (Li et al 2003), which acts as a signal for macrophages to engulf the cell; during this procedure, no neighboring cell is affected and no inflammatory reaction takes place (Alberts et al 2003).

Pathways of apoptosis

In general, there are two apoptotic pathways (there is also a caspase-independent pathway, which is secondary and is briefly described later). The first one includes the induction of apoptosis via the activation of Granzyme B, FAS/FAS-L (Suda et al 1993 & 1994) & TNFa (Chen & Goeddel 2002) under the influence of an exogenous stimulus ("extrinsic pathway"). The second one includes the induction of apoptosis via the

activity of cytochrome c (which is released by the mitochondria and is related to a Ca^{2+} ions), thus it could be characterized as an "intrinsic pathway" of apoptosis (Alberts et al 2003, Boron & Boulpaep 2005). Both pathways eventually lead to activation of caspases, which carry out the programmed cell death. A detailed description of the biology of caspases, as well as other apoptosis-inhibiting mechanisms, is given later.

Extrinsic pathway:

The extrinsic pathway is activated when an external signal (TNFa, Fas-L & Granzyme b) interacts with the cell membrane. As regards TNFa, it is a cytokine produced mainly by activated macrophages, and is the major extrinsic mediator of apoptosis. There are two different receptors for TNFa, TNFR1 and TNFR2. The binding of TNFa to TNFR1 has been shown to initiate the apoptotic pathway. After binding the intermediate membrane proteins TNF receptor-associated death domain (TRADD) are activated (Chen & Goeddel 2002).

At the same time, Fas-L binds to Fas, which is a glycoprotein of the cell surface, belonging to the TNF receptor family (Susin et al 1997, Suda et al 1993); the Fas/Fas-L connection results in the activation of Fas-associated death domain protein (FADD), similarly to TRADD.

As far as Granzyme B is concerned, it is a protease that is secreted by cytotoxic T lymphocytes and NK cells, but is has also been found to be expressed in mast cells, basophils and smooth muscle cells (Afonina et al 2010). It is secreted along with the pore forming protein perforin; the latter enables Granzyme B to enter the cytoplasm via the pores created on the cell membrane (Trapani 1996). In addition to apoptosis, it seems to be able to disintegrate fibronectin, a component of the extracellular matrix; fibronectin fractures then may attract neutrophils, thus inducing an inflammatory response (Boivin et al 2009, Afonina et al 2010).

All three signals mentioned above eventually lead to the activation of caspases (Susin et al. 1997). The extrinsic pathway results in the activation of procaspase-8 into its active form, caspase-8 (Chen & Goeddel 2002), which subsequently activates other caspases that will, in the end, carry out the breakdown of the cell. Biology of caspases is described later. The extrinsic pathway of apoptosis is summarized below, in Fig. 2.

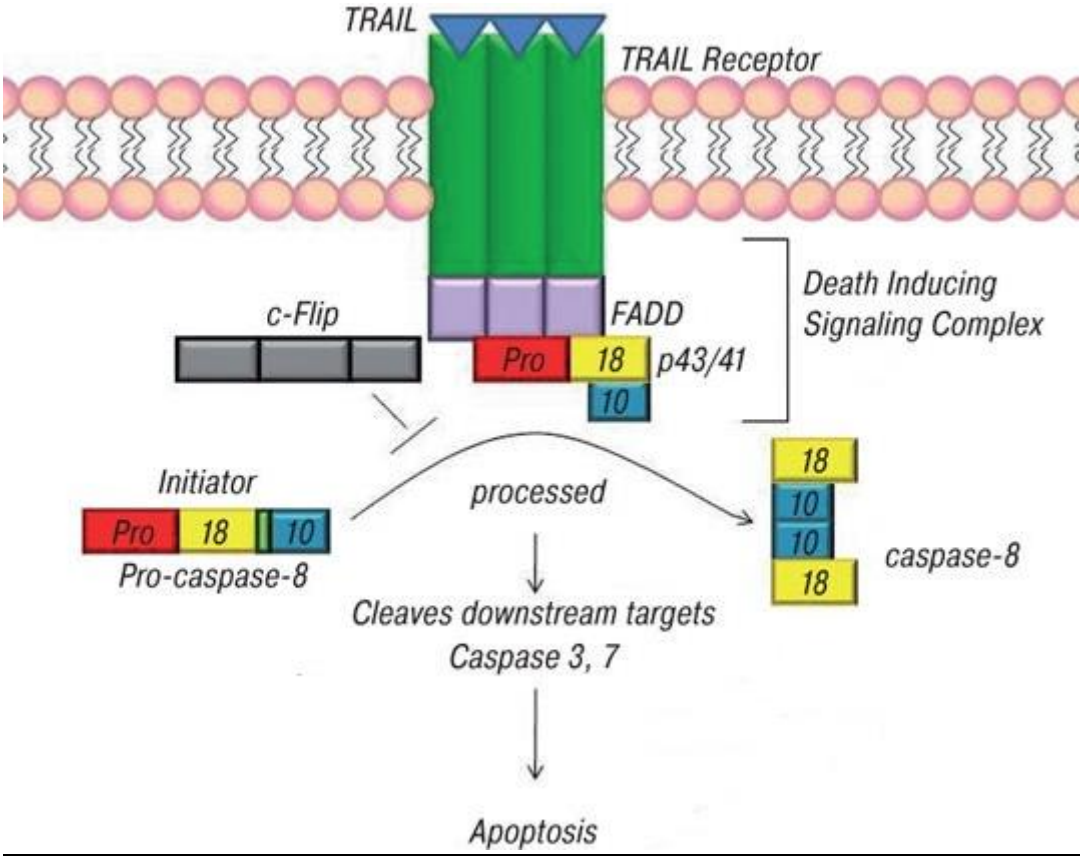


Fig. 2: The extrinsic pathway of apoptosis (Fiandalo & Kyprianou 2012).

Intrinsic pathway:

The intrinsic pathway takes (mainly) place in the mitochondria. In the first phase, the lack of nutritional ingredients forces the cell to consume adenosine triphosphate (ATP) in order to maintain the function of Ca^{2+}-Mg^{2+}-ATPase. This ATPase helps cells to transfer calcium ions from the cytoplasm back into the intercellular space (Moller et al. 1996). Nine types of this ATPase have been recognized; the most critical in the homeostasis of calcium is plasma membrane calcium-transporting ATPase 1

(ATP2B1), which is located on the cell membrane. When the cell has run out of ATP, the ATPase cannot work properly anymore; this leads to an influx of calcium ions from the intercellular space into the cell.

In the second phase, calcium ions flow into the mitochondrion via its voltage-dependent anion channel (VDAC) (Alberts et al 2003, Boron & Boulpaep 2005). Three different types of VDAC have been described, VDAC1, VDAC2 & VDAC3; VDAC1 is the most thoroughly studied and the main responsible for the transfer of calcium ions (Blachly-Dyson & Forte 2001, Hiller et al 2010). This channel is open when the potential of the mitochondrial membrane is below 30mV (positive or negative), but it is very stringent to organic ions (Shoshan-Barmatz & Gincel D 2003); this allows calcium ions to be easily diffused into the mitochondrion. The up-regulation of calcium into the mitochondrion results in the release of cytochrome c, which is also transferred into the cytoplasm via the VDAC1 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 (Taylor et al. 2008).

Cytochrome c is a small hemeprotein found loosely associated with the inner membrane of the mitochondrion. It has the ability to undergo oxidation and reduction, but does not bind oxygen. When it is restricted into the mitochondrion, it seems to be related to the oxidative phosphorylation that normally takes place there (Alberts et al 2003, Boron & Boulpaep 2005); however, when it is released into the cytoplasm -as part of the intrinsic apoptotic pathway- it binds to APAF1 in order to from the apoptosome. The ratio of the two components in a structural study seems to be 1:1 (Acehan et al. 2002). The apoptosome bears a caspase-recruitment domain (CARD); this is the spot where procaspase-9 is recruited and turned into its active form, caspase-9, in order to carry on

the apoptotic process (Yuan et al. 2010). The intrinsic pathway is summarized in Fig. 3.

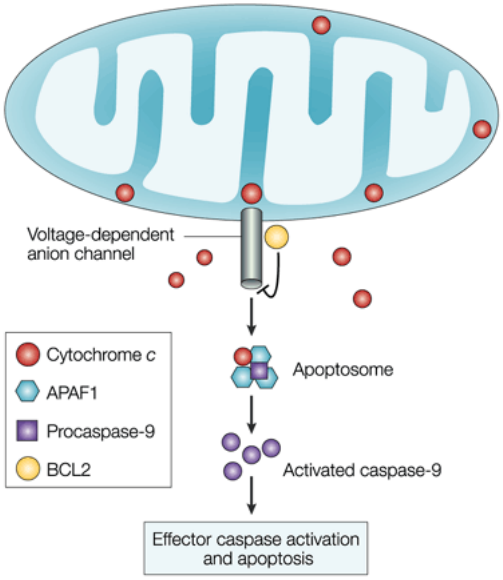


Fig. 3: The intrinsic pathway of apoptosis (Hersey & Zhang 2001).

Caspase-independent apoptotic pathway:

The caspase-independent apoptotic pathway is mediated by AIF (apoptosis-inducing factor) (Susin et al. 1999). It is a flavoprotein that may induce programmed cell death by causing fragmentation of DNA and condensation of chromatin. It is also found inside mitochondria, where it normally acts as a NADH oxidase (Joza et al. 2009).

When mitochondria are damaged, AIF is released from them into the cytoplasm; from there on, it reaches the cell nucleus, where it interacts with DNA as described above. The activity of AIF seems to depend upon the type of cell, the apoptotic insult, and its DNA-binding ability (Hangen et al. 2010). This pathway has not been thoroughly studied yet; information known up to now indicate that it might be complementary to the other two apoptotic pathways.

Biology of caspases

Caspases (cysteine-aspartic proteases or cysteine-dependent aspartate-directed proteases) are cysteine proteinases. In humans, 12 caspases have been recognized. Caspases can be classified into 2 different groups depending on their function (Kaufmann et al. 2008):

- initiator caspases (mainly caspases-2, 8, 9, 10). These are the first that are activated as a result of an apoptotic pathway. Usually, they are found in a pro-form. Once activated, their role is to activate the effector caspases and therefore to set the stage for the final breakdown of the cell
- effector caspases (mainly caspases-3, 6, 7). Once activated by initiator caspases, they begin the degradation of the cell via digestion of structural cytoplasmic proteins, degradation of cellular genetic material and, finally, phagocytosis. Caspase-3 seems to be crucial amongst effector caspases; its activation is regarded as an irreversible step in the caspase cascade pathway (Grutter 2000). Caspase-3 has been recognized as an essential molecule in normal brain development; in apoptosis, it is responsible for chromatin condensation and DNA fragmentation (Porter & Janicke 1999). Elevated levels of a fragment of caspase-3, p17, in the bloodstream is a sign of a recent myocardial infarction (Agosto et al. 2011). It has also been shown that caspase-3 may play a role in embryonic and hematopoietic stem cell differentiation (Abdul-Ghani & Megeney 2008).

The caspase cascade begins as soon as the apoptotic pathways activate an initiator caspase (most usually caspases-8 or 9 depending on the pathway, see above). Once such caspases are activated, they start cleaving either other initiator caspases or (more importantly) effector caspases. This results in a cascade pathway in which caspases

interact with each other, eventually leading to the execution of cell disintegration mainly with degradation of structural cytoplasmic proteins and cellular genetic material. The most critical interactions between caspases are displayed in Fig. 4.

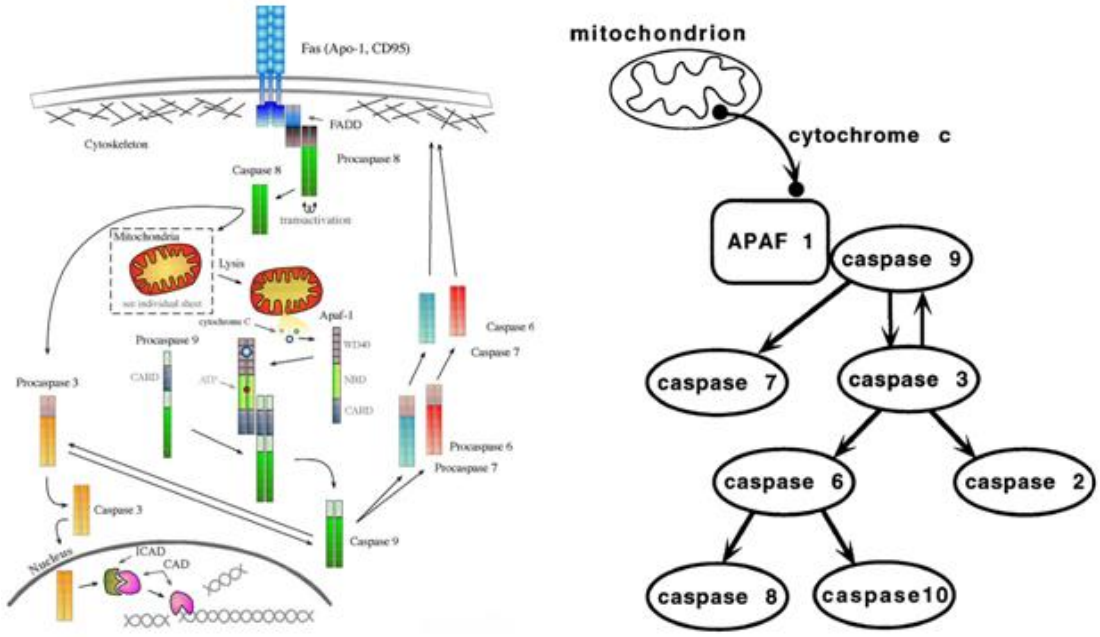


Fig. 4: On the left, the caspase cascade as a result of the apoptotic pathways is shown. On the right, interactions between critical caspases are displayed (modified by Slee et al. 1999).

The role of Bcl-2 family of proteins in apoptosis

The Bcl-2 family of proteins regulate the permeabilization of the mitochondrial outer membrane. Some of its members induce apoptosis (mainly Bax and Bak) or inhibit apoptotis (mainly Bcl-2) (Alberts et al. 2003); the former increase the Ca^{2+} influx into the mitochondrion while the latter decrease it (Martinez-Caballero et al 2005, Kinnally & Antonsson 2007). Furthermore, they seem to be related with the regulation of the release of cytochrome c during the intrinsic pathway of apoptosis (Zamzami et al 1998). BAK1 is located on the outer mitochondrial membrane. It functions as a pro-apoptotic regulator; once activated, it accelerates the opening of VDAC2 (see above). In this way, the release of cytochrome c as a result of the intrinsic pathway of apoptosis is facilitated.

BAX was the first identified pro-apoptotic member of the Bcl-2 protein family (Oltvai et al. 1993). In healthy mammals, it is mainly spotted in the cytoplasm; however, once apoptosis has been inducted, it is located on the outer mitochondrial membrane (Hsu et al. 1997). Once associated with the mitochondrial membrane, its activity appears to be similar to that described above for BAK1, with the exception that BAX is able to induce also the opening of VDAC1 (Shi et al. 2003).

In contrast to BAK1 and BAX, Bcl-2 acts as an anti-apoptotic factor (Cleary et al. 1986). Bcl-2 seems to be crucial in the regulation of apoptosis; mutation of the Bcl-2 gene leads to the formation of an oncogene. In many malignant lesions (lymphoma, chronic lymphocytic leukemia, lung cancer, breast cancer and melanoma) an unstoppable proliferation of malignant cells is recorded; this has been attributed to a malfunctioning Bcl-2 that is not able to inhibit apoptosis of cancer cells (Otake et al 2007).

Bcl-2 might also play a significant role in other diseases and pathologic conditions. Due to the fact that dendritic cells are the most important antigen-presenting cells of the immune system, their activity must be tightly regulated by such mechanisms as apoptosis. Mice that contain dendritic cells which are unable to induce apoptosis obtain autoimmune diseases more frequently than those that have normal dendritic cells; the lifespan of dendritic cells may be related to the anti-apoptotic Bcl-2 (Li et al. 2006). Moreover, schizophrenia is a neurodegenerative disease that may result from an abnormal ratio of pro- and anti-apoptotic factors; there is evidence that this defective apoptosis may result from abnormal expression of Bcl-2 and increased expression of caspase-3 (Glantz et al. 2006).

Apoptosis in radicular cysts

Literature data indicate that apoptosis indeed takes place in radicular cysts (Loyola et al. 2005, Suzuki et al. 2005, Loreto et al. 2013). There is evidence that apoptosis is not restricted in the cyst epithelium, but may be also present in the cystic wall, particularly in endothelial cells (Loreto et al. 2013). As mentioned before, since cyst regression is a prerequisite for periapical restitution, the possible scenarios for cyst healing after conservative endodontic treatment are the following (Lin et al. 2009): simultaneous bone regeneration (starting from the periphery) and cyst recession (via apoptosis of epithelial cells, resulting in cyst shrinkage), or apoptosis of the epithelium followed by degeneration of the basement membrane under the influence of matrix metalloproteinases (MMPs); this allows the connective tissue to develop into the lumen and eventually leading to partial or total recession of the epithelium (see above, "Healing of radicular cysts", for details). Although there is no sufficient experimental data to confirm these scenarios, it is evident that apoptosis of the cyst epithelium has a prominent role in the recession of radicular cysts, being crucial for successful healing. This is why most research carried out is about apoptosis in the epithelium.

In most research on apoptosis in the cyst epithelium the expression of Bcl-2 is studied. As mentioned above, Bcl-2 is regarded as an apoptosis-inhibitory factor; thus, a significant expression of this molecule could be correlated with limited apoptosis. Indeed, Piatelli et al. (1998) carried out an immunohistochemical study about the expression of Bcl-2 in human radicular cysts, dentigerous cysts and odontogenic keratocysts; they found that all radicular and dentigerous cysts were Bcl-2-negative while all keratocysts were Bcl-2-positive. They concluded that keratocysts present a high proliferative potential while radicular cysts and dentigerous cysts show a significant degree of apoptosis. The same conclusion was also drawn by Soluk Tekkesin

et al. (2012); studying 20 odontogenic keratocysts, 20 ameloblastomas and 20 radicular cysts, they found that Bcl-2 levels in the epithelium were significantly lower in radicular cysts while Bax levels were significantly higher. Loyola et al. (2005) also relied on Bcl-2 expression in their study on apoptosis in radicular cysts; they found that apoptosis takes place in all cystic epithelia but more extensively in atrophic epithelium. They tried to correlate this finding with low levels of Bcl-2 but they found no statistically significant difference. It is also important to say that cells that were undergoing apoptosis were identified only in the hematoxyline-and-eosine-stained sections without any more sensitive method (i.e. identification of caspase levels) which could be more accurately be correlated with Bcl-2 levels. Such a comparison is made in the study of Suzuki et al. (2005). In this study, the immunohistochemical expression of Bax, Bcl-2, caspase-3, Fas and Fas-L was searched in 19 radicular and 5 residual cysts. The pattern of expression was the following: Bax & Bcl2 were expressed in the basal layer, caspase-3 in the superficial epithelial layers. This pattern was recorded both in radicular and in residual cysts. However, staining was more intense for all molecules in radicular cysts, and particularly in cysts with a thick epithelium (more than 10 cell layers). The latter difference was statistically significant; therefore, the expression of these molecules in higher when the epithelium is thick (more than 10 cell layers). Fas and Fas-L was limited in lining epithelium of both lesion types. The same results as regards the pattern of caspase-3 and Bcl-2 were found in the study of Martins et al. (2011) in 17 radicular and 9 dentigerous cysts; they also found a positive correlation between the expression of caspase-3 and the thickness of the epithelium; the thicker the epithelium, the higher the caspase-3 expression. More recently, Loreto et al. (2013) carried out an immunohistochemical study of the expression of TNF, death domain (see

above) and caspase-3 in 5 radicular cysts. They found that all three were expressed in the epithelium, although a significantly stronger staining for TNF was shown.

Taking all information above into account, it seems clear that apoptosis indeed takes place in the cyst epithelium. Nevertheless, all research carried out concerns immunohistochemistry. No study has tried to correlate the expression of all (or some) of these molecules with any clinical factor that could possibly regulate their expression. For instance, there is no evidence on whether clinical conditions such as apical extrusion of debris during cleaning and shaping of the root canal or extrusion of filling materials into the radicular cyst can up- or down-regulate the expression of some of these molecules. More importantly, the role of calcium hydroxide (if any) in apoptosis has also not been investigated. This is interesting because, besides the well-known properties of calcium hydroxide (derived mainly from the activity of OH^-), literature data show that calcium ions are involved in the intrinsic apoptotic pathway (see above). It would be very interesting if calcium ions (derived from $Ca(OH)_2$ for intracanal medication) could be indeed correlated with one or more molecules involved in apoptosis.

EXPERIMENTAL PART

AIM

The aim of the present 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

The present study was based on radicular that had been surgically removed during apicoectomies carried out mostly in the post-graduate Endodontics Clinic from January 1993 to September 2016 (Fig. 5 & 6) and subsequently diagnosed at the Department of Oral Pathology after histopathologic examination.

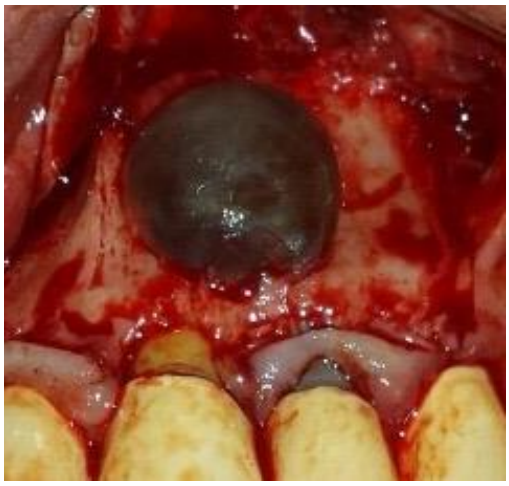


Fig. 5. A large cyst-like periapical lesion related with the left central incisor.

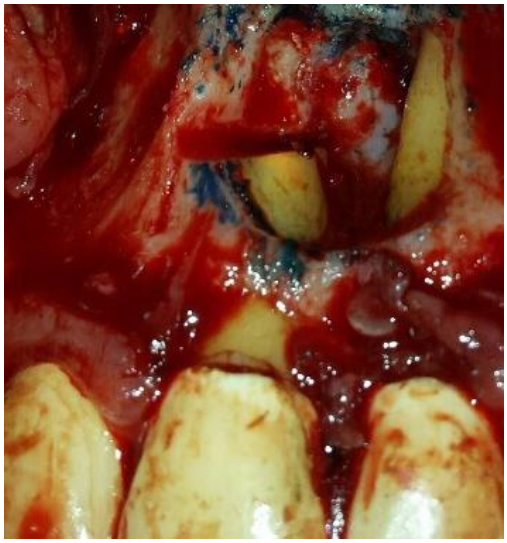


Fig. 6. Immediately after the surgical excision of the lesion and the apicoectomy of the previous case.

Information collected from both the clinical history of each patient and the biopsy report concerned the following:

- Gender and age of patients
- Size of each cyst, expressed by its maximum diameter, measured immediately after surgical removal
- if $Ca(OH)_2$ had been applied as intracanal medicament before the surgical treatment, as well as the period of time of $Ca(OH)_2$ application (in cases where applied)

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).

The main criteria for the selection of the study material were:

1. A histologically confirmed periapical cyst
2. Information about the application (or not) of $Ca(OH)_2$ as described above
3. A follow-up period of up to 12 months after conservative treatment
4. Adequate tissue to new sections showing cystic epithelium
5. No decalcification during histological preparation and storage of the specimens

New 5μm-thick sections stained with hematoxyline and eosine from all cysts that had been collected were observed under a light microscope. The purpose of this observation was to confirm the presence of a squamous epithelium around the lumen of all cysts. From an initial pool of eighty-four (84) cases, eventually forty-two (42) of them fulfilling these criteria were forwarded firstly for histochemistry and subsequently for immunohistochemistry.

Histochemical staining

Histochemical staining consisted of the application of the von Kossa technique on new 3-4μm-thick undecalcified sections from the specimens in order to detect any calcium deposits within the cysts.

The staining protocol was as follows:

- Deparaffinizization of paraffin sections and hydration to water.
- Rinsing in several changes of distilled water.
- Incubation of sections with 1% silver lactate solution in a clear glass coplin jar placed under ultraviolet light for 20 minutes.
- Rinsing in several changes of distilled water.
- Removal of un-reacted silver with 5% sodium thiosulfate for 5 minutes.
- Rinsing in distilled water.
- Counterstain with nuclear fast red for 5 minutes.
- Rinsing in distilled water.
- Dehydration through graded alcohol and clear in xylene.
- Coverslip and mounting.

After performing the von Kossa technique, calcium deposits are stained black, nuclei are stained red, and cytoplasms are stained pink. Other negative structures are stained green.

Positive control for the von Kossa technique may be any paraffin-embedded, calcium-containing tissues, mainly undecalcified bone (Sheehan & Hrapchak 1980). Thus, in the present study, rat healthy bone tissue was used as positive control.

Upon completion of this procedure, the final classification of the specimens into three groups -based on clinical, histological and histochemical criteria- was made:

1. lesions in which conservative endodontic treatment had been carried out in a single visit. (n=14)
2. lesions in which $Ca(OH)_2$ had been applied as intracanal medicament for various time periods before the surgical treatment, but no calcium deposits were spotted with the von Kossa staining (n=12)

lesions in which $Ca(OH)_2$ had been applied as intracanal medicament before the surgical treatment, with calcium deposits being spotted with the von Kossa staining (n=16), essentially corresponding to $Ca(OH)_2$ extrusion.

Immunohistochemical staining

A rabbit polyclonal caspase-9 antibody (LAP6/Ab-4, Thermo Fisher Scientific Inc. (Waltham, MA, USA)) was used for immunohistochemistry. Immunohistochemistry was applied in 3-4μm-thick sections from the cysts included in the present study. The staining protocol was as follows:

- Deparaffinization with successive placement of the sections in xylole heated at 60˚C for 1min, xylole in room temperature for 10min and 5min.
- Hydration with successive placement of the sections in 100% alcohol for 10min, 100% alcohol for 5min, 96% alcohol for 3min, 80% alcohol for 3min, 70% alcohol for 3min, 50% alcohol for 3min.
- Rinsing with phosphate-buffered saline (PBS; Ml07; Bio-Optica, Milano, Italy).
- Pre-treatment with Epitope Retrieval Solution 1 (ER1) (citrate based pH 6.0) at 90°C for 1 hour.

- Peroxidase blocking for 10 min using the Bond Polymer Refine Detection Kit DC9800 (Leica Microsystems GmbH).
- Rinsing in distilled water.
- Rinsing with phosphate-buffered saline (PBS; Ml07; Bio-Optica, Milano, Italy).
- Incubation with the primary antibody for 1 hour at room temperature. The antibody was used at a 1:80 dilution.
- Rinsing with phosphate-buffered saline (PBS; Ml07; Bio-Optica, Milano, Italy).
- Application of the LP polymer (UltraVision LP Detection System: HRP Polymer, Thermo Fisher Scientific Inc., Waltham, MA, USA); first, the Enhancer was applied for 10min followed by the application of the HRP Polymer for 15min.
- Staining with diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich Corp, St Louis, MO) chromogen for 5min
- Rinsing in tap water.
- Counterstain with Mayer's hematoxylin (AR106/Artisan; Dako North America, Inc, Carpinteria, CA) for 45 seconds.
- Rinsing with phosphate-buffered saline (PBS; Ml07; Bio-Optica, Milano, Italy).
- Dehydration with successive placement of the sections for 3min in 80%, 96% and 100% alcohol.
- Successive placement of the sections in xylole for 5min and 10min.
- Coverslip and mounting.

Positive controls for immunohistochemistry were Jurkat cells (immortalized line of human T lymphocytes) and human tonsils (Li et al. 1997, Hakem et al. 1998).

Substitution of the antibody with non-immune serum of the same species served as negative control.

Evaluation

Evaluation included the assessment of both the von Kossa staining and the immunohistochemistry. Both evaluations were the result of a consensus made by two independent observers that were previously calibrated with similar histochemical and immunohistochemical images.

Because the number of specimens used in the present study was not large enough, it was more appropriate to evaluate both the von Kossa technique and immunohistochemistry with a universal qualitative-focused method, as follows:

1. The evaluation of the von Kossa technique was carried out under a double-headed light microscope at x400 magnification:
    - grade “0” corresponds to absence of calcium deposits
    - grade “1” corresponds to presence of calcium deposits
2. The evaluation of the immunohistochemistry was carried out under a double-headed light microscope at x400 magnification:
    - grade “0” corresponds to absence of epithelial cells being positive
    - grade “1” corresponds to presence of epithelial cells being positive

In the evaluation of the von Kossa staining, positive structures with a trabecular shape at the periphery of the cystic wall were ignored as they represented bone trabeculae. Furthermore, no effort was made to record caspase-9 immunoreactivity in relation with particular epithelial strata.

Statistical analysis

Data were analyzed with the x^2 test and Fisher's exact test. The former test is applied when all observed frequencies are above 1 and at least 80% of them are above 5; if these requirements are not fulfilled, the latter test was carried out. $P < 0.05$ was considered as level of significance for both tests. All analyses were carried out with SPSS Statistics 18.0 software (SPSS, Inc., Chicago, IL, USA) software. The following comparisons were made:

- application of $Ca(OH)_2$ and expression of caspase-9 in the cyst epithelium
- existence of calcium deposits (corresponding to $Ca(OH)_2$ extrusion) within the cysts and expression of caspase-9 in the cyst epithelium
- time of application of $Ca(OH)_2$ and expression of caspase-9 in the cyst epithelium

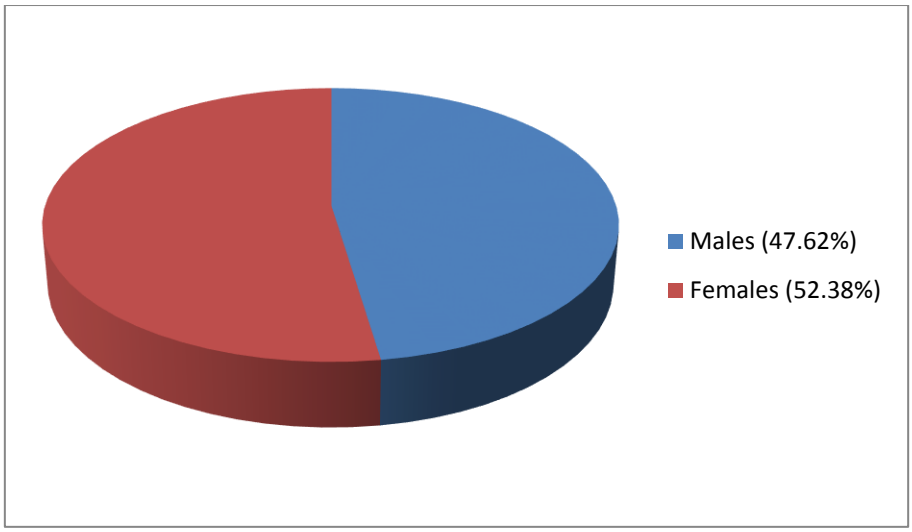
RESULTS

Epidemiological results:

Forty-two (42) lesions fulfilled all clinical, histological and histochemical criteria mentioned above and were included in the present study. According to data from the clinical histories of the post-graduate Endodontics clinic, in 14 cases no $Ca(OH)_2$ had been applied as intracanal medicament (group 1), in 12 cases despite that $Ca(OH)_2$ had been applied into the root canal, no calcium deposits were spotted with the von Kossa staining (group 2) and finaly in 16 cases $Ca(OH)_2$ had been applied with calcium deposits being spotted with the von Kossa staining (group 3).

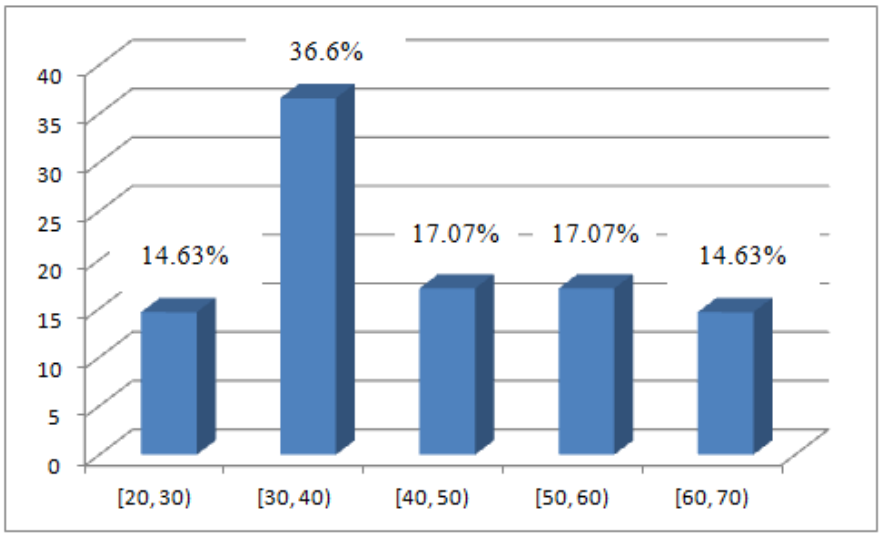
Overall, 20 lesions (47.62%) concerned male patients and 22 lesions concerned female patients (52.38%). The distribution of lesions according to the gender of the patients is displayed in Table 2.

Table 2. Distribution of lesions according to the gender of the patients.



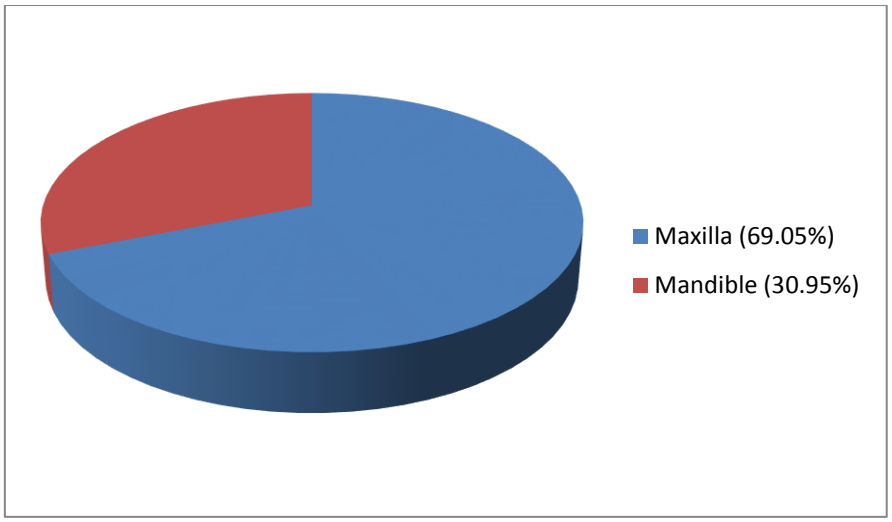
Information about the age of the patients was available for 37/42 lesions. The average age was 42.38±13.78 years. The distribution of patients according to the different age groups is displayed in Table 3.

Table 3. Distribution of lesions according to the age of the patients.



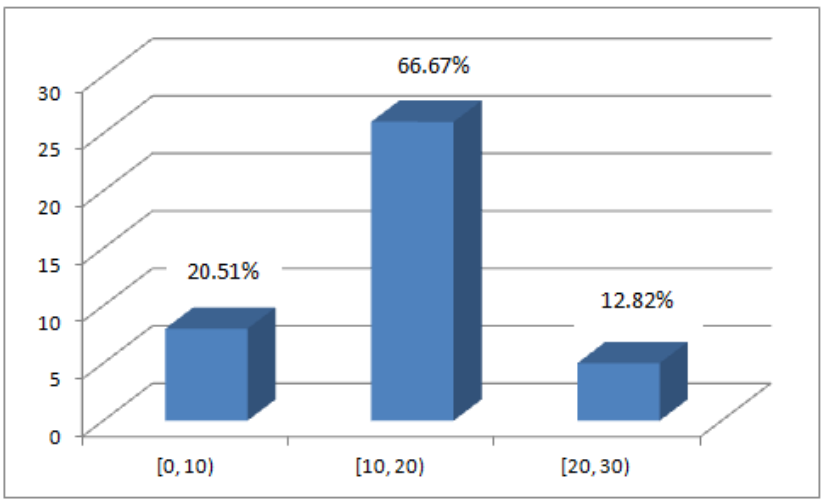
As regards the location of the lesions, 29 of them (69.05%) was spotted in the maxilla and 13 (30.95%) in the mandible. The distribution according to the location of the lesions is displayed in Table 4.

Table 4. Distribution of lesions according to their location.



Information about the size (expressed by the maximum diameter) was available for 39/42 lesions. The average maximum diameter was 13.33±5.41mm. The distribution according to the size of the lesions is displayed in Table 5.

Table 5. Distribution of lesions according to their size (expressed by the maximum diameter).



The particular features of each subgroup are displayed in Table 6 for group 1, in Table 7 for group 2 and in Table 8 for group 3.

Table 6. Clinical, histological, histochemical (Von Kossa staining) and immunohistochemical (IHC) features of the lesions of group 1 (lesions in which $Ca(OH)_2$ had not been applied as intracanal medicament).

Sample number	**Age**	**Gender**	**Location**	**Maximum diameter (mm)**	**Foreign material (if spotted with H&E)**	**Von Kossa staining**	**IHC**
04-514	25	male	maxilla	10	not spotted[1]	0[2]	0[3]
04-549	45	male	maxilla	30	not spotted	0	1
04-780	67	male	mandible	7	not spotted	0	0
04-882	42	female	maxilla	5	not spotted	0	0
04-905	38	female	maxilla	10	not spotted	0	1
05-145	50	female	mandible	n/a[4]	not spotted	0	0
09-0864	56	female	maxilla	10	not spotted	0	0
09-794	30	male	maxilla	15	not spotted	0	1
10-0163	37	female	maxilla	15	not spotted	0	0
10-0442	37	female	mandible	13	not spotted	0	1
11-0397	60	female	maxilla	7	not spotted	0	0
12-0445	50	female	maxilla	10	not spotted	0	1
13-247	47	male	mandible	18	not spotted	0	1
13-258	n/a	male	maxilla	22	not spotted	0	0

[1]when spotted, a “baseophile-like foreign material” corresponded to calcium ions, which was consistent to $Ca(OH)_2$ extrusion.

[2]The Von Kossa staining was evaluated with “0” for absence and “1” for presence of calcium ions.

[3]IHC results were evaluated with “0” for absence and “1” for presence of of caspase-9 immunoreactivity within the cystic epithelium.

[4]not available information

Table 7. Clinical, histological, histochemical (Von Kossa staining) and immunohistochemical (IHC) features of the lesions of group 2 (lesions in which $Ca(OH)_2$ had been applied as intracanal medicament but no calcium ions consistent to $Ca(OH)_2$ extrusion were spotted histochemically).

Sample number	**Age**	**Gender**	**Location**	**Time of $Ca(OH)_2$ application (weeks)**	**Maximum diameter (mm)**	**Foreign material (if spotted with H&E)**	**Von Kossa staining**	**IHC**
00-71	11	male	mandible	12	20	not spotted	0	0
01-0012	47	female	maxilla	2	9	not spotted	0	0
01-169	53	female	maxilla	3	20	not spotted	0	0
04-887	n/a	male	mandible	4	10	not spotted	0	0
10-0780	60	female	maxilla	1	n/a	baseophile-like material	0	1
10-0815	40	male	mandible	1	15	baseophile-like material	0	1
10-0834	34	male	maxilla	2	15	baseophile-like material	0	1
10-0977	32	male	maxilla	12	15	not spotted	0	1
11-0099	29	male	mandible	3	15	not spotted	0	1
12-0359	25	female	maxilla	3	5	not spotted	0	0

14-0324	24	female	maxilla	8	15	not spotted	0	0
14-0805	n/a	female	maxilla	4	18	not spotted	0	1

Table 8. Clinical, histological, histochemical (Von Kossa staining) and immunohistochemical (IHC) features of the lesions of group 3 (lesions in which $Ca(OH)_2$ had been applied as intracanal medicament, with sufficient calcium ions consistent to $Ca(OH)_2$ extrusion being spotted histochemically).

Sample number	**Age**	**Gender**	**Location**	**Time of $Ca(OH)_2$ application (weeks)**	**Maximum diameter (mm)**	**Foreign material (if spotted with H&E)**	**Von Kossa staining**	**IHC**
01-973	n/a	male	maxilla	1	10	baseophile-like material	1	1
04-780	67	male	maxilla	2	7	baseophile-like material	1	0
04-875	32	female	maxilla	2	n/a	not spotted	1	1
05-0236	55	female	mandible	3	18	not spotted	1	1
08-319	36	female	maxilla	2	15	baseophile-like material	1	1
08-650	15	female	maxilla	3	8	baseophile-like material	1	1
08-987	35	male	mandible	1	10	baseophile-like material	1	1

09-0636	54	female	mandible	4	12	baseophile-like material	1	1
09-0731	n/a	female	mandible	2	10	baseophile-like material	1	1
10-0254	41	male	maxilla	2	15	baseophile-like material	1	1
10-0265	61	male	mandible	2	14	baseophile-like material	1	1
10-0343	40	female	maxilla	1	12	baseophile-like material	1	1
10-0505	37	female	maxilla	4	10	baseophile-like material	1	1
10-0714	61	male	maxilla	2	25	baseophile-like material	1	1
16-795	54	male	maxilla	12	20	not spotted	1	1
16-965	35	male	maxilla	8	13	baseophile-like material	1	1

Histochemistry (Von Kossa):

Positive control for the von Kossa technique was rat healthy bone tissue that was stained black (Sheehan & Hrapchak 1980) (Fig. 7). Nearby connective tissue was entirely negative.

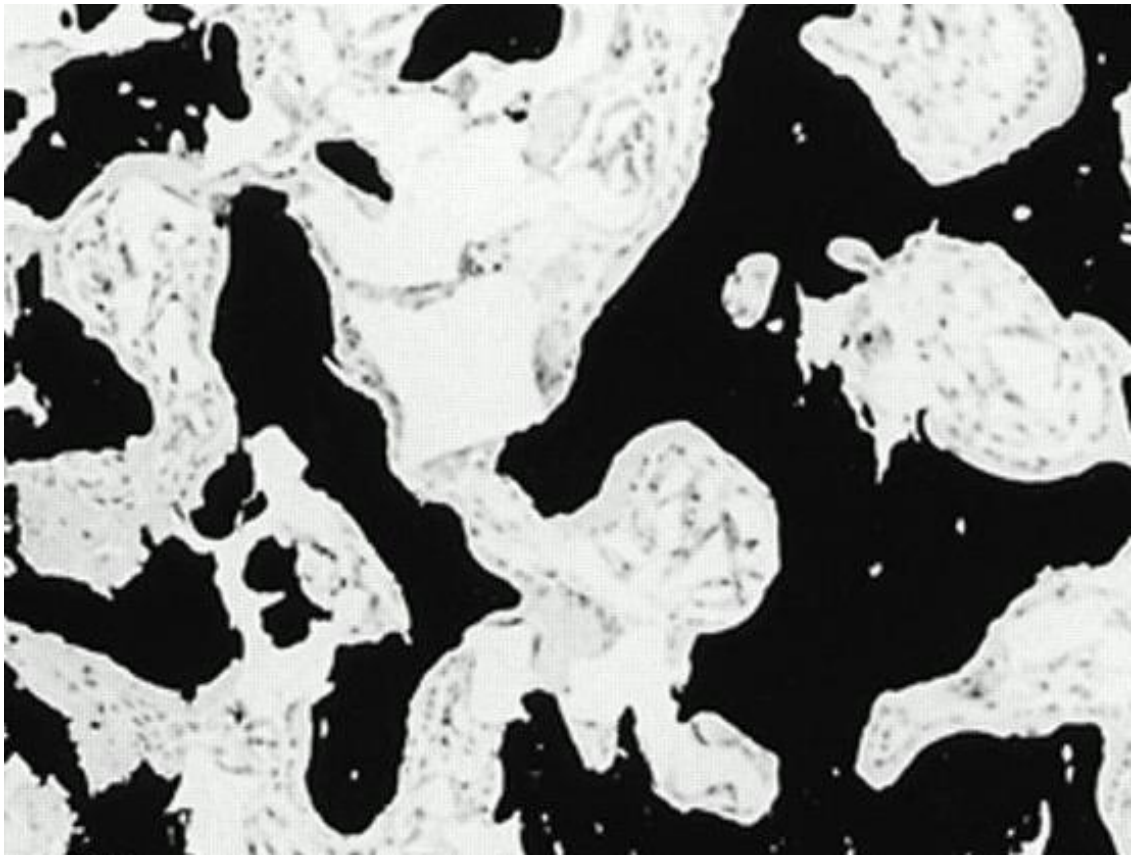


Fig. 7. Healthy rat bone tissue, which was used as positive control. Positive bone tissue is stained black. Von Kossa staining, x100.

In group 1, no lesion presented with baseophile-like deposits in the hematoxyline and eosine staining; the Von Kossa technique was subsequently negative for all cysts of this group. Thus, the existence of foreign material consistent with calcium deposits could not be confirmed.

In group 2, three specimens from the total of 12 cysts (25%) presented with baseophile-like deposits which were initially recognized in the hematoxyline and eosine staining; however, the von Kossa technique did not confirm the existence of foreign material consistent with calcium deposits. Since calcium deposits could not be ultimately spotted in this group, there was no adequate (clinical, histological and histochemical) evidence that histological $Ca(OH)_2$ extrusion had taken place.

In group 3, 13/16 cysts (81.25%) presented with baseophile-like deposits which were initially recognized in the hematoxyline and eosine staining. The von Kossa technique confirmed the existence of foreign material consistent with calcium deposits in all cysts (16/16) of this group. Therefore, there was adequate (clinical, histological and histochemical) evidence to confirm that $Ca(OH)_2$ extrusion had taken place in the lesions of this group. In 11/16 cysts, the deposits were spotted within the connective tissue of the cystic wall (Fig. 8a & 8b), in 3/16 the deposits were found within the cyst epithelium (Fig. 9a & 9b) and in 2/16 scattered into the cyst lumen (Fig. 10a & 10b).

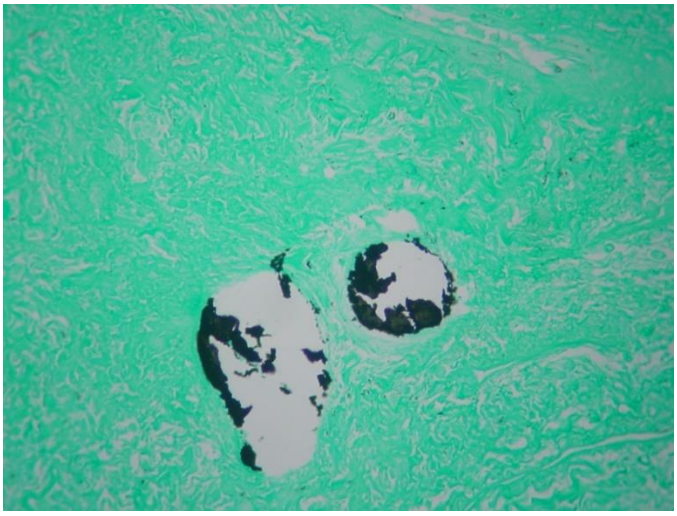


Fig. 8a. Calcium deposits within the connective tissue of the cystic wall. They are displayed as black-stained deposits within bubble-like formations. Von Kossa staining, x400.

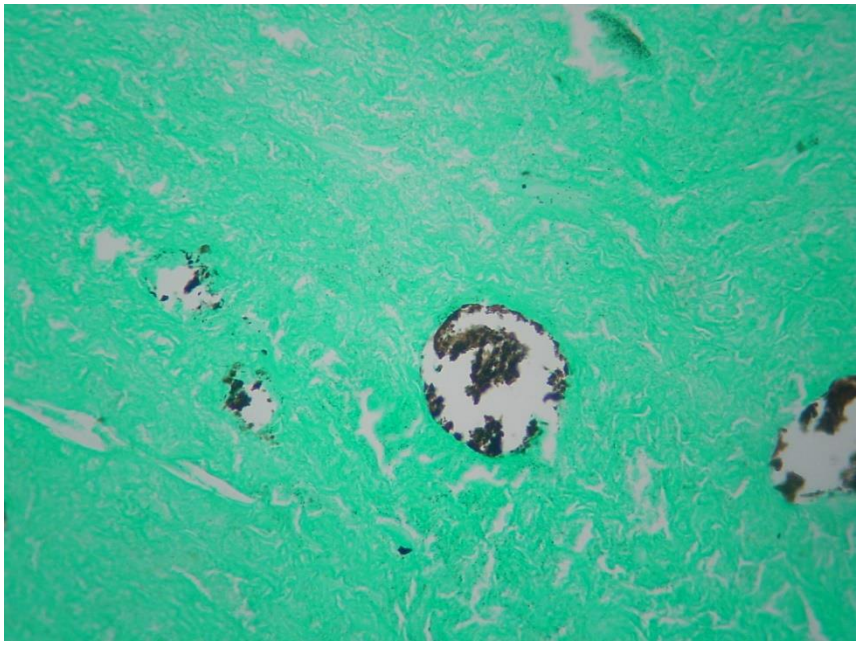


Fig. 8b. Calcium deposits within the connective tissue of the cystic wall. They are displayed as black-stained deposits within bubble-like formations. Von Kossa staining, x400.

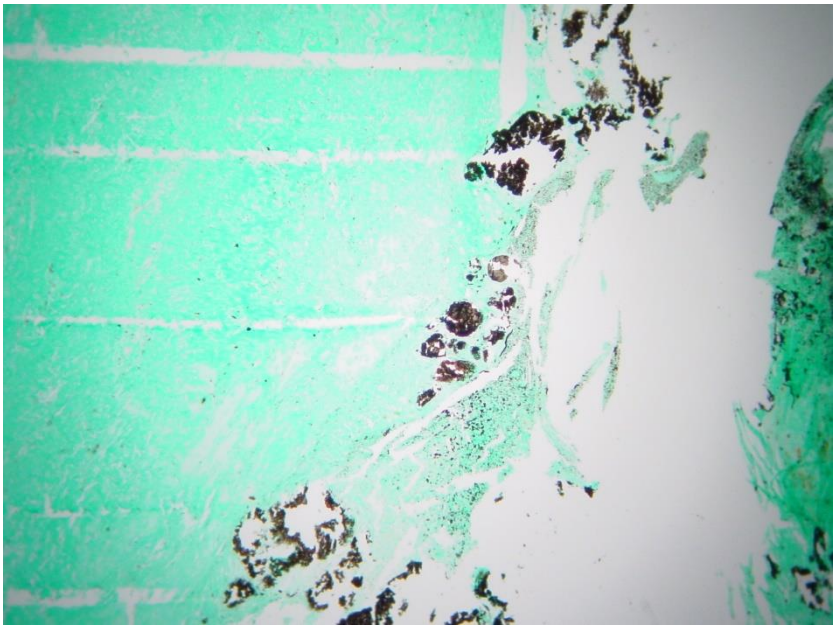


Fig. 9a. Calcium deposits within the cystic epithelium. They are displayed as black-stained deposits within bubble-like formations. Von Kossa staining, x200.

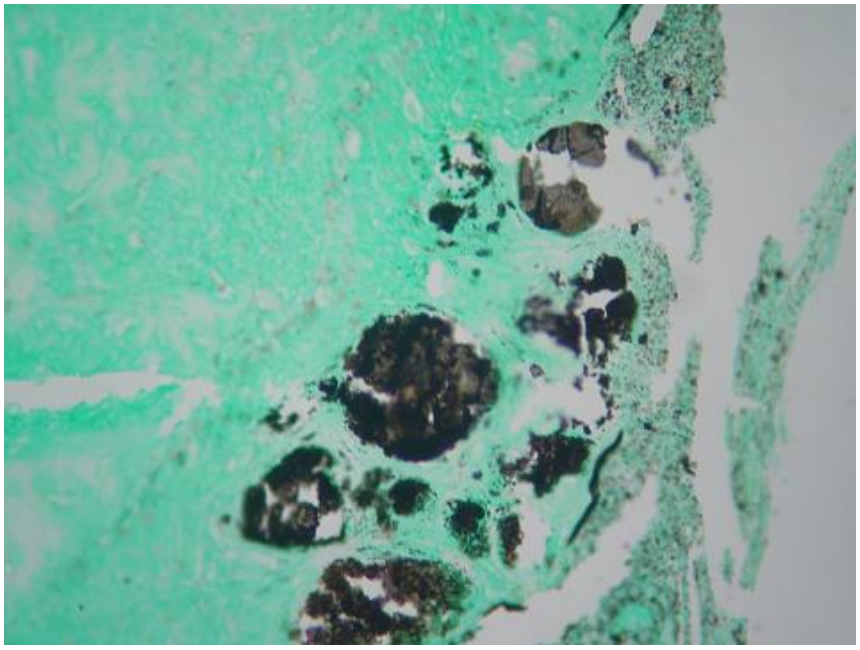


Fig. 9b. Calcium deposits within the cystic epithelium. They are displayed as black-stained deposits within bubble-like formations. Von Kossa staining, x400.

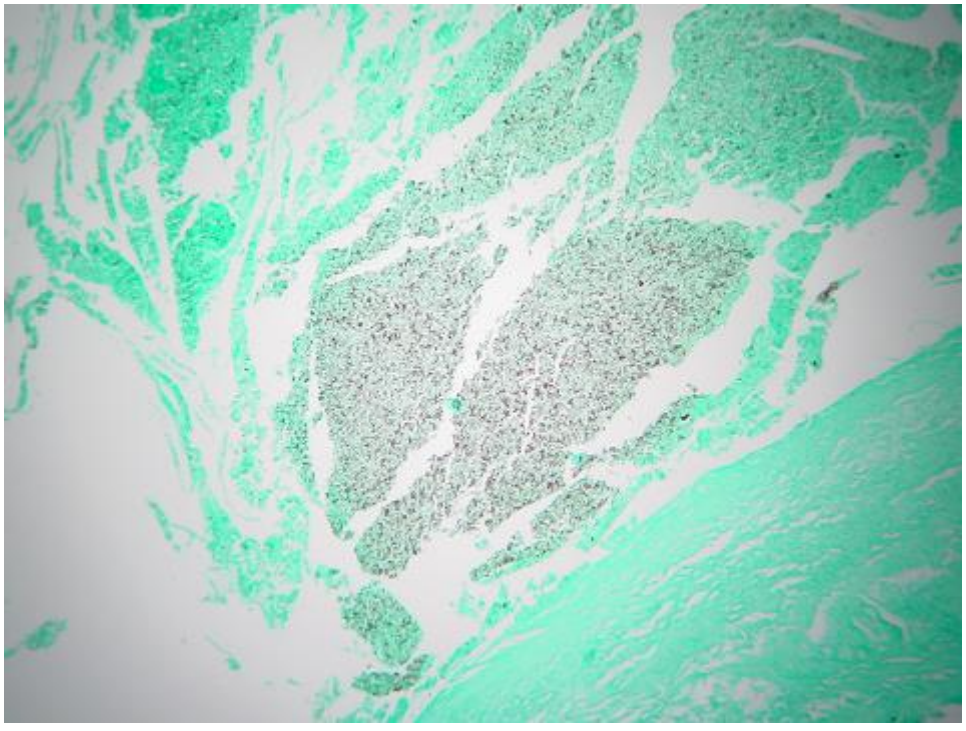


Fig. 10a. Calcium deposits within the cyst lumen. They are displayed as black-stained deposits within bubble-like formations. Von Kossa staining, x200.

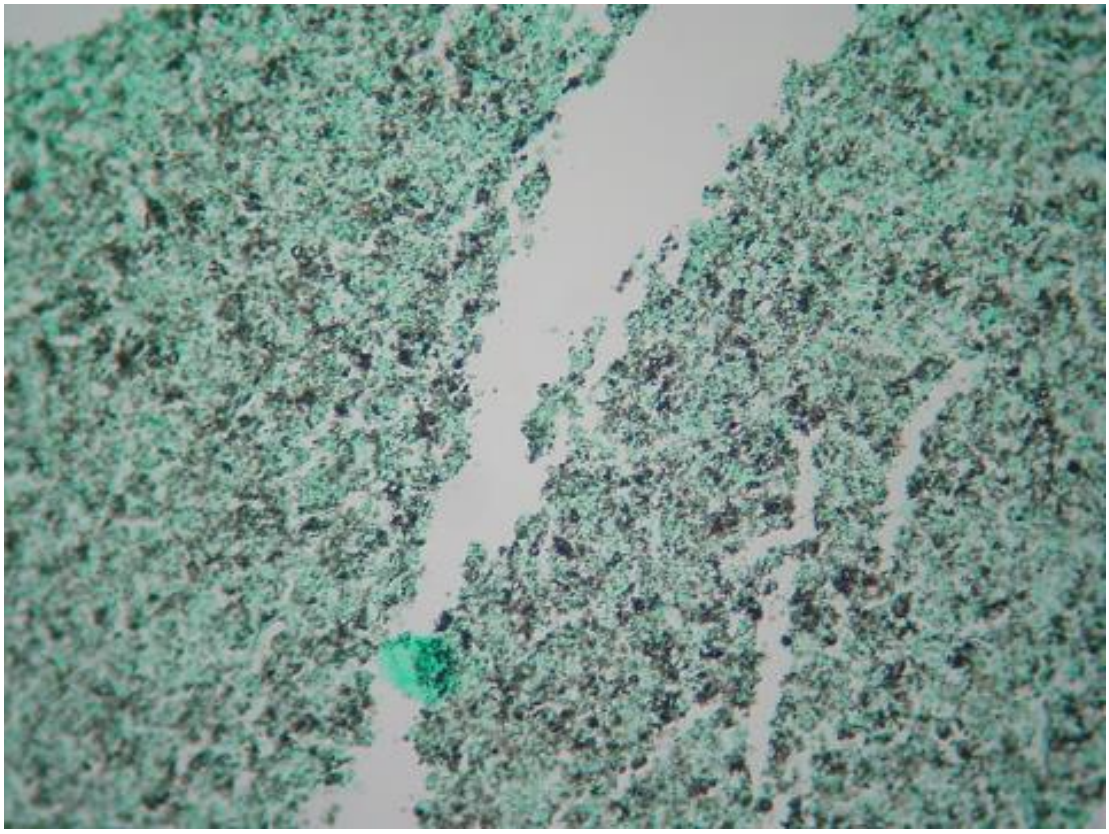


Fig. 10b. Calcium deposits within the cyst lumen. They are displayed as black-stained deposits within bubble-like formations. Von Kossa staining, x400.

Immunohistochemistry results:

Positive controls for immunohistochemistry were lymphatic cells of human tonsils (Fig. 11a & 11b) and Jurkat cells (Fig. 12a & 12b) (Li et al. 1997, Hakem et al. 1998). The selected antibody for caspase-9 showed strong nuclear immunoreactivity in both cell types, while no other nearby tissue reacted.

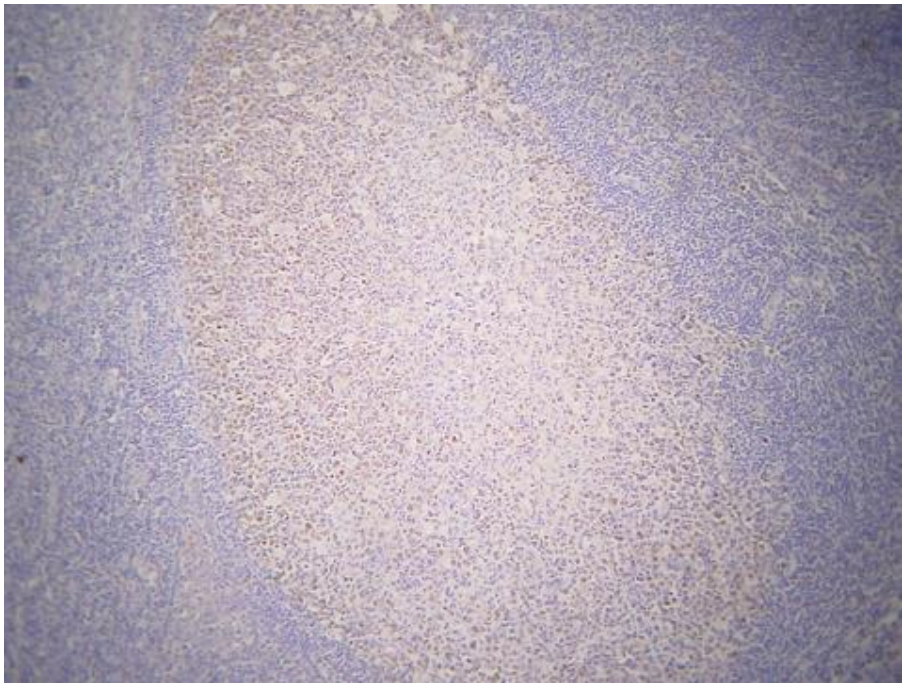


Fig. 11a. Lymph node within a human tonsil, which was used as external positive control. Positive cells are stained brown. Immunohistochemistry, x200.

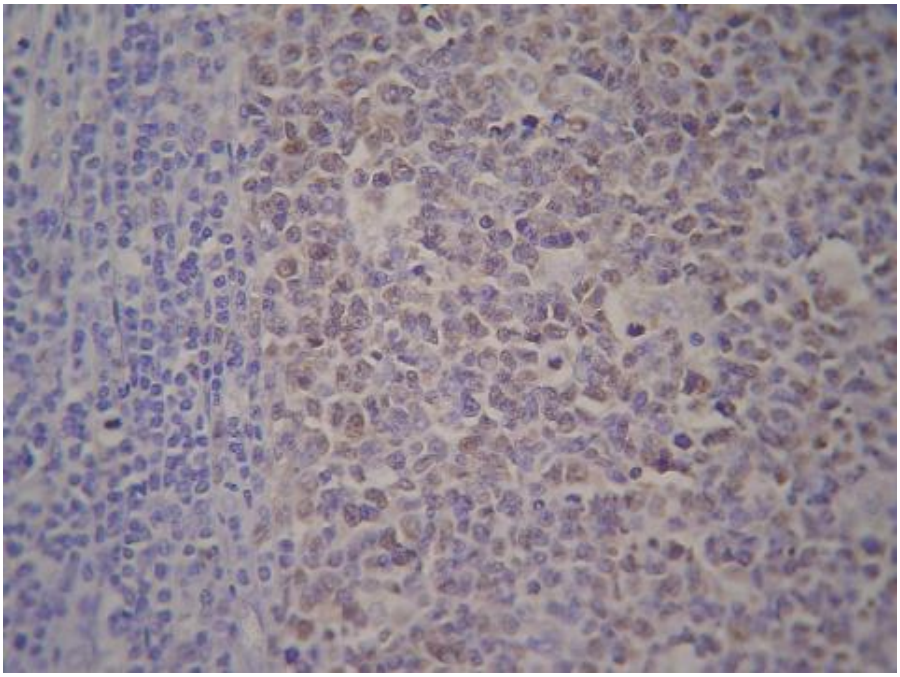


Fig. 11b. Lymph cells within a human tonsil, immunoreactive for caspase-9 (nuclear staining). Positive cells are stained brown. Immunohistochemistry, x400.

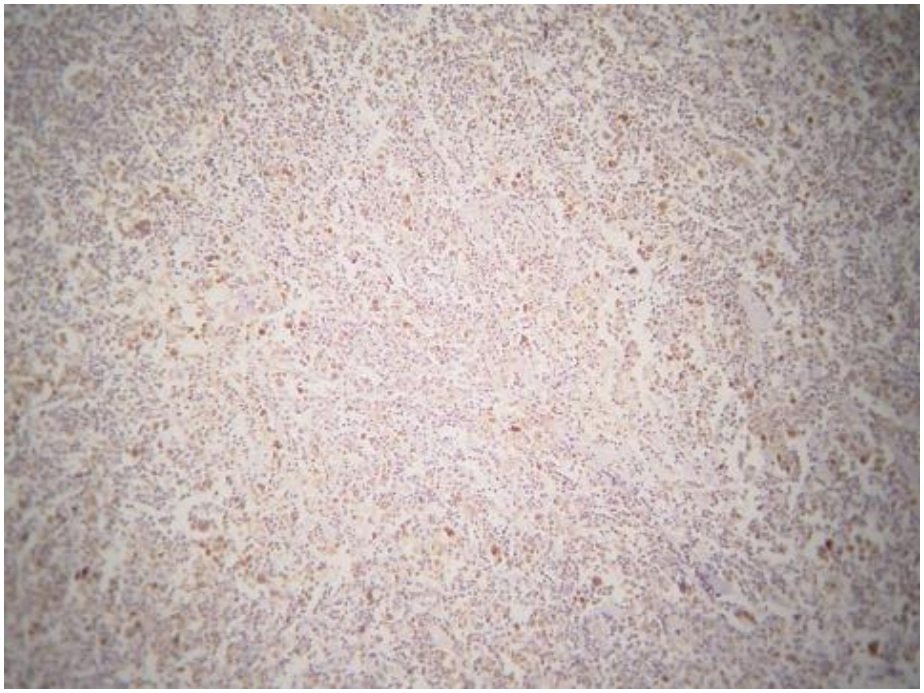


Fig. 12a. Jurkat cells, which were used as external positive control (nuclear staining). Positive cells are stained brown. Immunohistochemistry, x200.

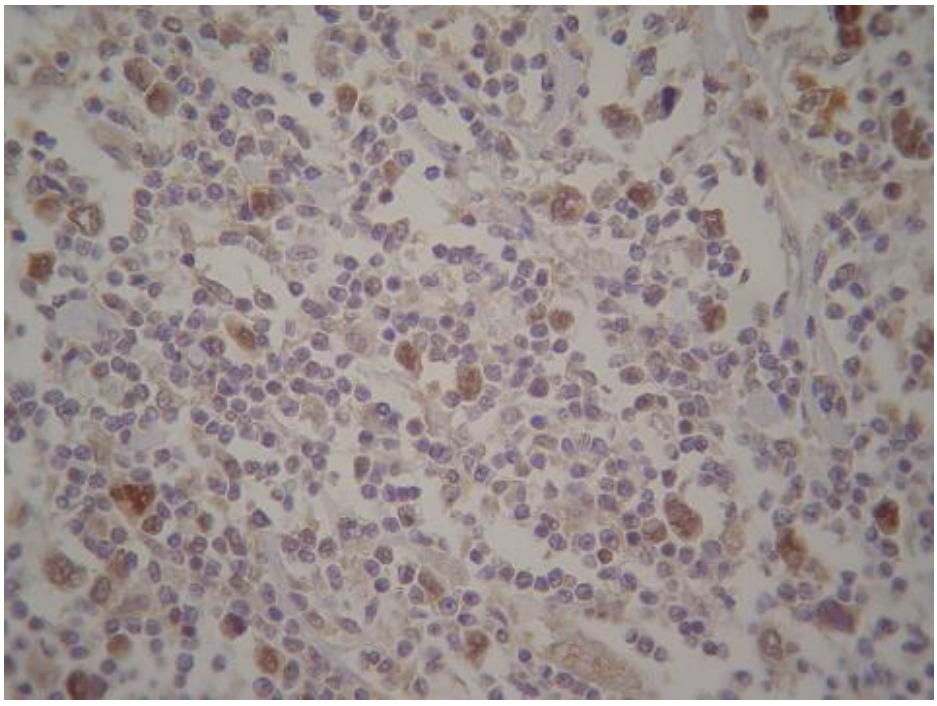
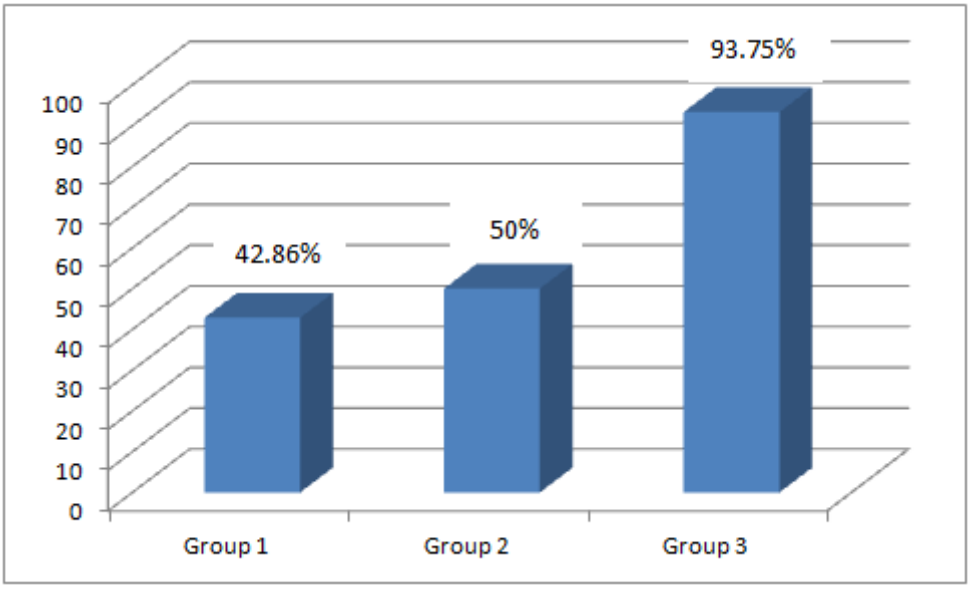


Fig. 12b. Jurkat cells immunoreactive for caspase-9 (nuclear staining). Positive cells are stained brown. Immunohistochemistry, x400.

Similar to the control, a nuclear staining was recorded in epithelial cells. In group 1, 6/14 cysts (42.86%) showed caspase-9 immunoreactivity in the cystic epithelium. The frequency of caspase-9 reactivity in groups 2 & 3 was 6/12 (50%) & 15/16 (93.75%) respectively (Table 10).

Table 10. Distribution of caspase-9 immunoreactivity among the three groups.



The distribution of caspase-9 in the cystic epithelium according to the application (or not) of $Ca(OH)_2$ is displayed in Table 11. Chi square test showed a significant difference ($p<5\%$) in caspase-9 expression. Similarly, a significant difference ($p<5\%$) was recorded as regards the existence of caspase-9 immunoreactivity among the 3 groups (Table 12). This difference was also recorded via the Fisher's exact test when comparisons were made solely between groups 1 & 3 (Table 13) and between groups 2 & 3 (Table 14). No difference was found between groups 1 & 2. Taken together, these results indicate that the application of $Ca(OH)_2$ favors the expression of caspase-9, particularly when $Ca(OH)_2$ is extruded into the lesion.

Table 11. 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	8	6
	Yes	7	21

Table 12. Distribution of caspase-9 in the cystic epithelium among the 3 groups.

	Caspase-9 immunoreactivity	
	Negative (“0”)	Positive (“1”)
Group 1	8	6
Group 2	6	6
Group 3	1	15

Table 13. Distribution of caspase-9 in the cystic epithelium between groups 1 & 3.

	Caspase-9 immunoreactivity	
	Negative (“0”)	Positive (“1”)
Group 1 (no $Ca(OH)_2$ application)	8	6
Group 3 (histological extrusion of applied $Ca(OH)_2$)	1	15

Table 14. Distribution of caspase-9 in the cystic epithelium between groups 2 & 3.

	Caspase-9 immunoreactivity	
	Negative (“0”)	Positive (“1”)
Group 2 ($Ca(OH)_2$ application without histological extrusion)	6	6
Group 3 (histological extrusion of applied $Ca(OH)_2$)	1	15

Representative images of caspase-9 immunoreactive epithelial cells are displayed in Fig. 13 to 18.

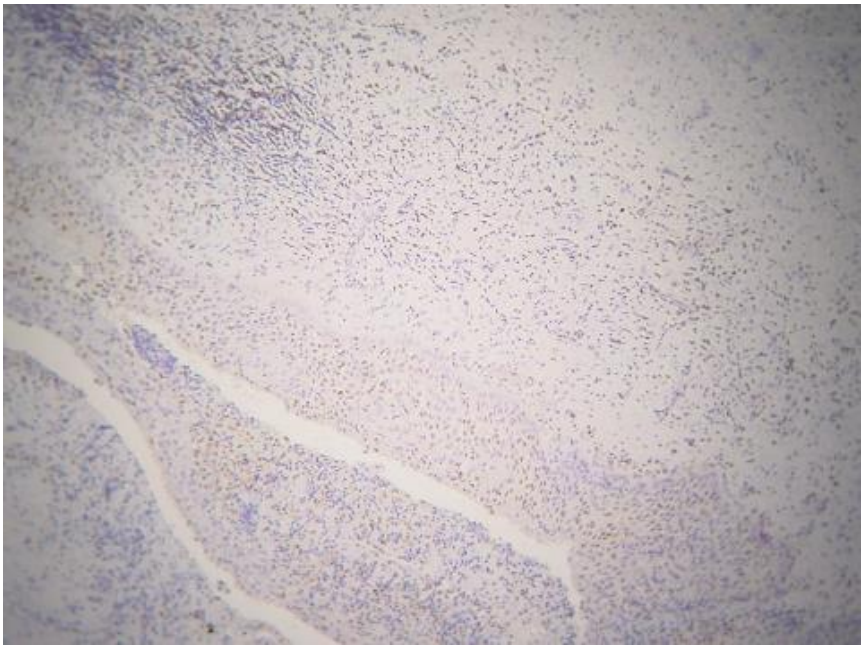


Fig. 13. Caspase-9 immunoreactivity in the cystic epithelium. Positive epithelial cells are stained brown (nuclear staining). Immunohistochemistry, x200.

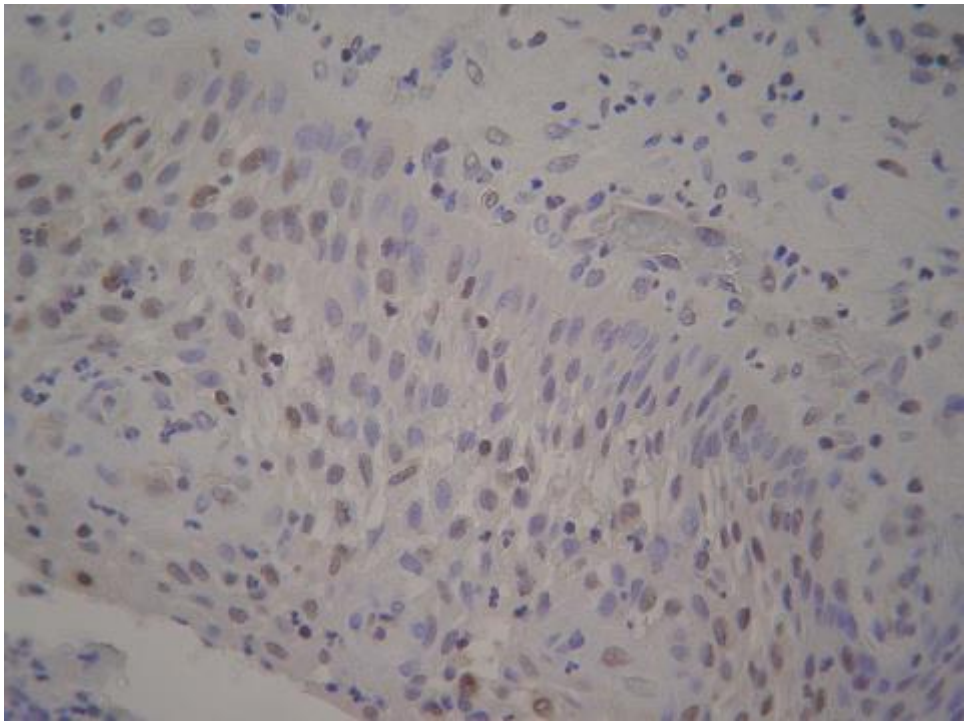


Fig. 14. Caspase-9 immunoreactivity in the cystic epithelium of the previous lesion. Positive epithelial cells are stained brown (nuclear staining). Immunohistochemistry, x400.

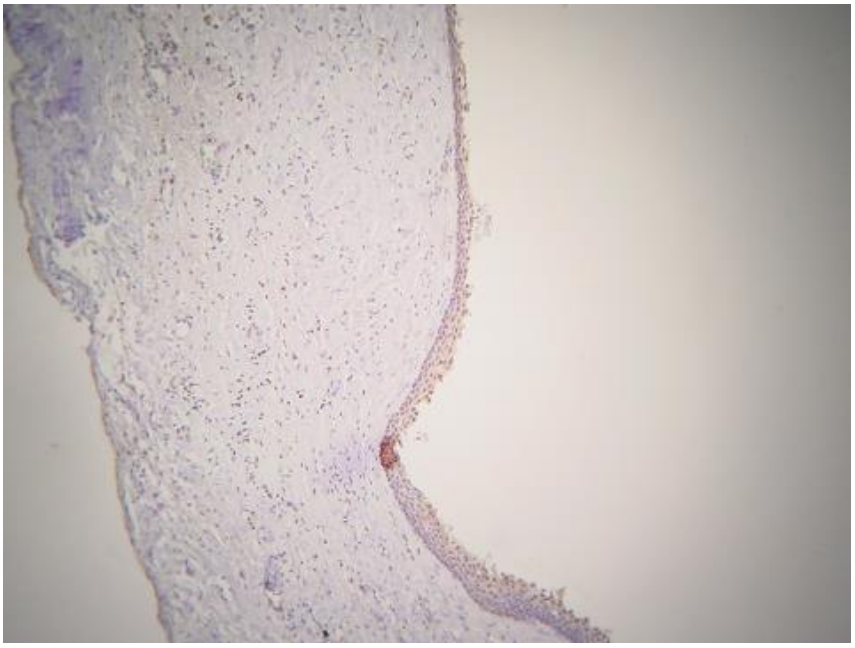


Fig. 15. Caspase-9 immunoreactivity in the cystic epithelium. Positive epithelial cells are stained brown (nuclear staining). Immunohistochemistry, x200.

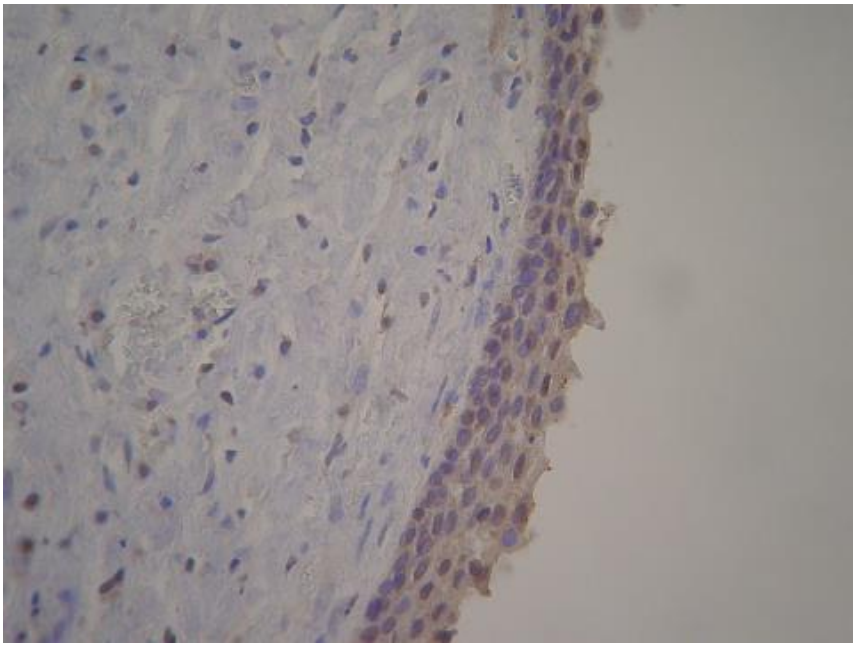


Fig. 16. Caspase-9 immunoreactivity in the cystic epithelium of the previous lesion. Positive epithelial cells are stained brown (nuclear staining). Immunohistochemistry, x400.

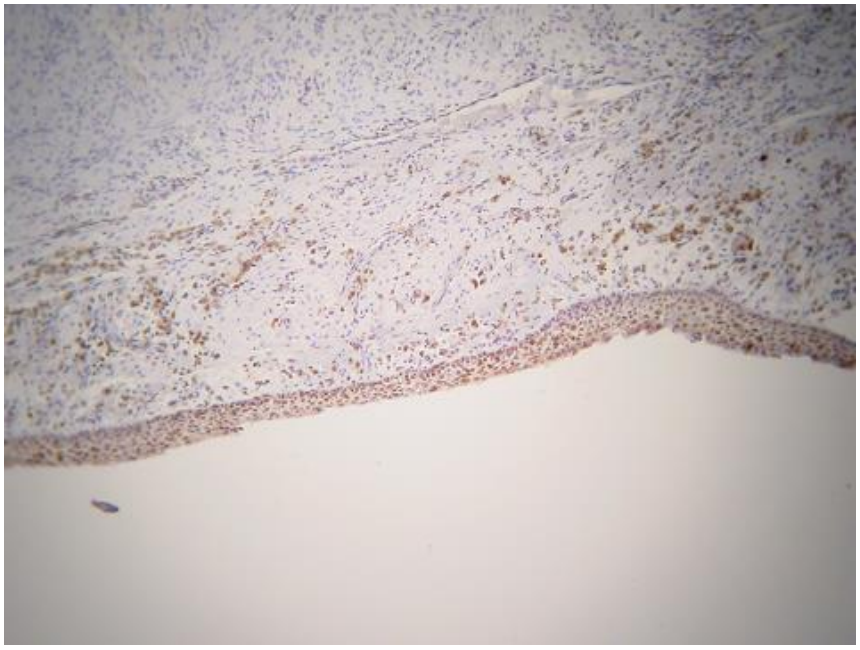


Fig. 17. Caspase-9 immunoreactivity in the cystic epithelium of the previous case. Positive epithelial cells are stained brown (nuclear staining).Immunohistochemistry, x200.

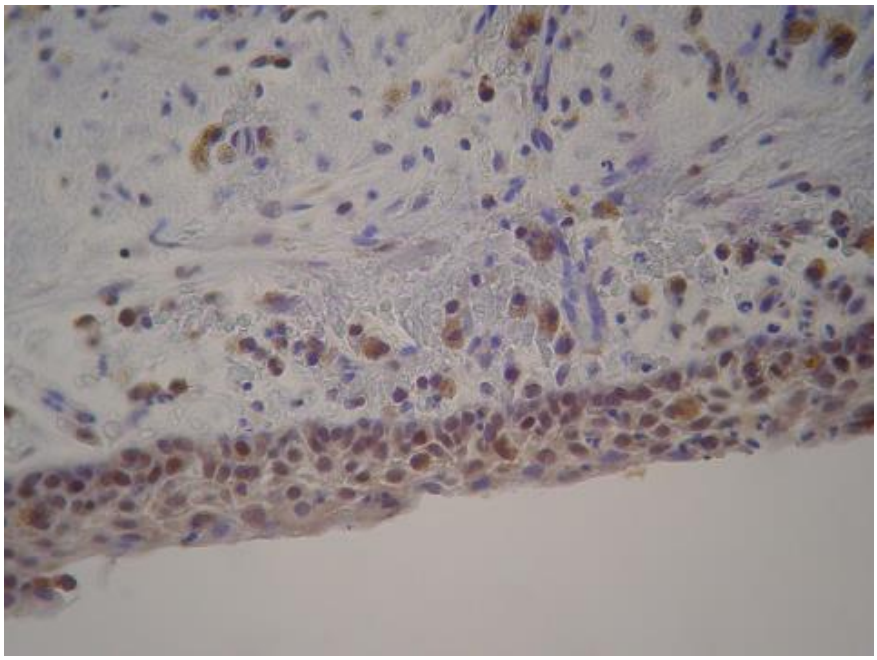


Fig. 18. Caspase-9 immunoreactivity in the cystic epithelium of the previous lesion. Positive epithelial cells are stained brown (nuclear staining). Immunohistochemistry, x400.

As mentioned before, information about the size of the cysts was available in 39/42 cases. Table 16 displays the distribution of those 39 cysts according to their caspase-9 immunoreactivity. The vast majority of cysts (22/39, 56.41%) concerned large cysts (more than or equal to 10mm) that were positive for caspase-9. Fisher's exact test indicated that this difference was statistically significant ($p<5\%$). This means that bigger cysts are significantly more likely to express caspase-9. Thus, in large cysts (more than or equal to 10mm) apoptosis is significantly favored.

Table 16. Distribution of caspase-9 immunoreactivity according to the size of the lesions (only in 39/42 cysts information about size was available).

		Size of cysts	
		Less than 10mm	More than (or equal to) 10mm
Caspase-9 immunoreactivity	Negative ("0")	7	9
	Positive ("1")	1	22

Last but not least, this study also examined any significant effect of the time of application of $Ca(OH)_2$ on the expression of caspase-9. The potential correlation between time and caspase-9 expression was searched only in group 3 ($Ca(OH)_2$ extrusion). This distribution is displayed in Table 17. Fisher's exact test showed no significant difference; thus, the time of application of $Ca(OH)_2$ is not significantly correlated to the expression of caspase-9.

Table 17. Caspase-9 immunoreactivity in the positive lesions of group 3 ($Ca(OH)_2$ extrusion) according to the time of application of $Ca(OH)_2$.

		Caspase-9 immunoreactivity	
		Negative ("0")	Positive ("1")
Time of $Ca(OH)_2$ application (weeks)	1	0	3
	2	1	6
	3	0	3
	4 or more	0	2

DISCUSSION

The present study aimed to investigate whether the application of $Ca(OH)_2$ as intracanal medicament promotes the expression of caspase-9 within the cyst epithelium of radicular cysts. The results indicate that there seems to be such a correlation, especially in cases where $Ca(OH)_2$ has been extruded into the lesion.

Based on the clinical data of the analyzed cases (gender & age of patients, location), the selected sample can be considered as indeed representative, as the clinical features of the present study are similar to previous data on a similar clinicopathological study (Kontogiannis et al. 2015b), and this was the reason why they were recorded.

Cyst regression is a prerequisite for healing of the periapical tissues and it has been proposed that this may be carried out either via simultaneous bone regeneration and cyst shrinkage, or via apoptosis (Lin et al. 2009). Available data indicate that apoptosis

takes place in radicular cysts (Loyola et al. 2005, Suzuki et al. 2005, Loreto et al. 2013), and there is evidence that it is present both in the cystic epithelium and in the cystic wall (Loreto et al. 2013). The caspase-dependent pathway is the most common mechanism of apoptosis (Hangen et al. 2010). This pathway includes initiator and effector caspases (Kaufmann et al. 2008); the former are activated in the first phase of apoptosis and they subsequently activate the latter, which initiate the breakdown of the cell (Slee et al. 1999). Caspase-9 is an initiator caspase; once activated, it activates the effector caspases and therefore sets the stage for the final breakdown of the cell (Alberts et al. 2003). Caspase-9 is activated in the mitochondria (Taylor et al. 2008) as a result of up-regulation of calcium ions into the cell (Boron & Boulpaep 2005). Since calcium ions participate in the activation of caspase-9 it would be interesting to investigate whether calcium ions extruded into the periapical tissues during endodontic therapy might influence the expression of caspase-9, and therefore propagate the initiation of apoptosis of epithelial cells.

Since now, it is not known whether the immunohistochemical expression of a molecule related with apoptosis, such as caspase-9, is influenced by the extruded $Ca(OH)_2$ used as intracanal medicament in clinical endodontics. The results of the present study showed that the application of $Ca(OH)_2$ in the root canal increased the expression of caspase-9 in the cystic epithelium; this was more frequent when $Ca(OH)_2$ was extruded into the lesion. These results are consistent to those of Leone et al. (2012), who investigated the immunoexpression of caspases-9 in 20 epithelialized lesions of periapical and gingival origin. Caspase-9 was expressed in high levels in both lesion types, and was present in both basal and suprabasal epithelial cells, being more intense in the gingival rather than in periapical lesions, indicating a higher regression potential in the periodontal tissues. This study did not clarify whether the “periapical lesions”

included true radicular cysts or periapical granulomas with epithelialization. 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 referrred to radicular cysts.

The present results indicate that when extrusion of $Ca(OH)_2$ is present, caspase-9 expression in the cyst epithelium is significantly accelerated. An interesting finding of the present study was that caspase-9 expression was found even in cases where no $Ca(OH)_2$ had been applied, since in all these cases a single-visit endodontic therapy had been carried out. However, a significantly greater caspase-9 expression was found in cases where $Ca(OH)_2$ had been applied as intracanal medicament, and in those with extrusion was even greater. It is highly probable that von Kossa-positive structures represent $Ca(OH)_2$ extrusion. On the other hand, since they were not found in all cases where $Ca(OH)_2$ was applied as intracanal medicament, it is not definite that the absence of von Kossa-positive structures excludes presence of calcium deposits. It could be speculated that in some cases calcium deposits could be present in other sites of the cyst, thus they were not microscopic observation; dissolution of the deposits during the period between conservative and surgical endodontic therapy could also take place.

On the other hand, the results of the present study clearly showed that the greatest expression of caspase-9 took place when $Ca(OH)_2$ was present into the lesion. Therefore, the question is whether $Ca(OH)_2$ should be intentionally extruded into the lesion, in order to accelerate the apoptotic pathway in which caspase-9 participates and perhaps lead to regression of the lesion. The study of Caliskan & Sen (1996) investigated the effect of $Ca(OH)_2$ extrusion on the healing of periapical lesions. In this study, 172 teeth with periapical lesions were included. In all of them, after cleaning and

shaping, a mixture of pure $Ca(OH)_2$ with barium sulfate (which adds radiopacity) with glycerin as a medium, was placed in the root canal for 1 week. However, in 58/172 teeth (10 with lesions smaller than 5mm, 28 with lesions 6-10mm and 18 with lesions 11-15mm radiographically), the mixture was intentionally extruded. All cases were followed up for up to 5 years, in order to record whether complete healing had occurred. They showed that in lesions smaller than 5mm, complete healing is similar without and with extrusion (88.7% and 80%, respectively). However, as size increases, healing is more frequent when $Ca(OH)_2$ is extruded, particularly for lesions larger than 11mm: complete healing without and with extrusion was recorded in 61.1% and 75% of lesions respectively. The present study correlates well with these results. First, the majority of the cases of studies were lesions larger than 10mm. Second, a correlation between extrusion and apoptosis was found in the present study, and apoptosis proved to be more intense in large cysts. Therefore, combining the results of Caliskan & Sen (1996) with our results, it seems that large lesions might have a greater potential to heal via the mechanism of apoptosis when $Ca(OH)_2$ is extruded. Furthermore, in all cases of Caliskan & Sen (1996), $Ca(OH)_2$ was applied for a short period of time, while in the present study it was applied for up to 12 weeks, and in other studies even longer (Siskos 2006). All these data clearly indicate that the duration of $Ca(OH)_2$ application and the time of the follow-up play a very important role in the effect of $Ca(OH)_2$ extrusion on periapical healing. In the present study, no significant correlation was found between the time of application of $Ca(OH)_2$ and caspase-9 expression, thus the role of time was not confirmed; however, this might be attributed to the relatively low number of cases available. Future investigation -possibly with a larger sample size- could shed more light on the role of time on healing following $Ca(OH)_2$ extrusion.

The adverse effects of $Ca(OH)_2$ extrusion have also been discussed in the literature. In a recent systematic review, between 1980 and 2013 10 cases of tissue destruction resulting from extrusion have been reported (Olsen et al. 2014). Connective tissue breakdown, damage to blood vessels and nerve lesions (mainly to the inferior alveolar nerve) were found; most of them had been related with injectable $Ca(OH)_2$ products rather than applied with Lentulo spirals. On the other hand, early studies suggest that $Ca(OH)_2$ extrusion positively affects periapical healing, due to its anti-inflammatory activity, the neutralization of acid products and the activation of the alkaline phosphatase (Souza et al. 1989). Furthermore, it might cause destruction of the epithelium near the site of the extrusion, thereby allowing connective tissue invagination into the lesion (Canalda Sahli 1988). More recent studies also support the view that $Ca(OH)_2$ extrusion is well tolerated by periapical tissues, especially the bone. De Moor & De Witte (2002) presented 11 cases of periapical lesions in which unintentional extrusion of 2 ready-to-use $Ca(OH)_2$ products (Reogan-Rapid (Vivadent, Schaan, Liechtenstein) and Calxyl (Otto and Co., Frankfurt, Germany)) had taken place. Medication was carried out for 3-11 months in all teeth. All of them showed complete healing. However, a period of more than 6 months was needed in all of them in order healing to be recorded, probably because of the extrusion. Furthermore, in 5/11 cases, where Reogan-Rapid had been applied, remnants of the extruded material remained despite the fact that periapical healing had indeed occurred. It is important to mention, however, that Reogan-Rapid contains barium sulfate (for radiopacity) while Calxyl does not; barium sulfate has been reported not to be readily resorbable compared to calcium hydroxide (Webber et al. 1981). Thus, the image of non-resorbed material may concern solely barium sulfate while calcium hydroxide may have been resorbed (Caliskan & Sen 1996). Resorption was recorded by Caliskan (2004), when $Ca(OH)_2$

was unintentionally extruded into the periapical lesion; although only partial resorption was recorded, complete healing was evident in 5/6 cases.

The size of a periapical lesion has always been a matter of concern for two reasons: first, whether it may determine the type of the lesion (cyst or granuloma) and second, whether it influences the therapeutic approach and healing. Lesions radiographically larger than 200mm^2 are cysts (Lalonde 1970) and most granulomas are not radiographically larger than 70mm^2 (White et al. 1994). Furthermore, large lesions are chronic lesions (Simon 1994) and periapical cysts need more time to develop than granulomas (Valderhaug 1972). This eventually leads to the assumption that large, chronic lesions that fulfill certain clinical criteria (painless swelling, fluctuation, aspiration of a yellowish fluid, divergence of the root of the involved and nearby teeth, large size as described) are probably cysts (Siskos 2006). More recently, Caliskan et al. (2016) found that in lesions larger than 10mm the probability of a radicular cyst is approximately 3.7 times greater than in lesions smaller than 10mm. Therefore, size might be an indicator for the existence of a radicular cyst. This should be taken into account for the theuraputic approach of large lesions. It has been proposed that the size of the lesion does not influence healing (Orstavik 1996, Sjogren et al. 1997, Huumonen & Orstavik 2013). In contrast, other studies reject this view (Matsumoto et al. 1987, Weiger et al. 2000, Caliskan 2004, Siskos 2006, Caliskan et al. 2016), proposing that large lesions might be more difficult to heal than small ones. This proposal is based on evidence that supports the fact that larger periapical lesions tend to be associated with higher bacterial counts (Sundqvist 1976) and more prevalent biofilm formation (Ricucci & Siqueira 2010). Thus in large lesions the disinfection of the root canal of the involved tooth is more difficult (Wenteler et al. 2015). As regards the therapeutic approach, it has been suggested (Nair 1998) that oral surgeons generally hold the view

that cysts do not heal and have to be removed by surgery; this view is more widespread in cases of large lesions (Nair et al. 1993). On the other hand, available data suggest that such lesions may heal even with conservative endodontic treatment (Matsumoto et al. 1987, Caliskan & Sen 1996, Caliskan 2004, Siskos 2006). 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 (defined with the same cut-off used by Caliskan et al. (2016)) as well. Indeed, the present study showed that in large cysts (more than or equal to 10mm) caspase-9 is highly expressed.

In the present study, no case was medicated for more than 12 weeks. However, it has been shown that the time of application might be an important parameter in periapical healing. Caliskan (2004) showed that the long-term application of $Ca(OH)_2$ for 3 months (with 3-week intervals) resulted in complete healing of large lesions (with a maximum radiographic diameter 7-18mm) in 73.8% of cases and partial healing in an additional 9.5% of cases, all followed-up for 2-10 years. Siskos (2006) selected 62 large periapical lesions in which a clinical diagnosis of radicular cyst was established based on the aforementioned clinical criteria (painless swelling, fluctuation, aspiration of a yellowish fluid, divergence of the root of the involved and nearby teeth, large size) and applied $Ca(OH)_2$ for 12-26 months (similar to the traditional apexification protocol). Complete healing occurred in 85% of the lesions treated. The remaining 15% underwent periapical surgery; in all these cases, a histological diagnosis of radicular cyst was established (thus confirming the initial diagnosis based on the clinical criteria). A similar approach is also applied in Sweden (Bergenholtz et al. 2012); the application of $Ca(OH)_2$ in all cases with periapical pathology is carried out on routine basis and even for a long time (more than 2 weeks). This approach was also adopted in the clinical management of the cases of the present study which were medicated with $Ca(OH)_2$.

The present study showed that $Ca(OH)_2$, when used as intracanal medicament, up-regulates the expression of caspase-9; this is more intense when the medicament is extruded into the lesion and especially in large lesions. Taking the above mentioned literature data into account, it seems that $Ca(OH)_2$ should be applied in cases with periapical lesions, even for a longer period of time (Siskos 2006, Bergenholtz et al. 2012). In large lesions, the prolonged application might be combined with extrusion to facilitate healing (Caliskan & Sen 1996, Caliskan 2004), since extruded calcium ions highly enhance the expression of caspase-9 and therefore apoptosis. The latter seems to be an important mechanism for regression of the lesion.

CONCLUSION

Calcium hydroxide up-regulates the expression of caspase-9 and therefore apoptosis. This is more intense when it is extruded into the lesion and in particular in large lesions.

SUMMARY

Introduction: Apoptosis is a genetically-determined programmed cell death. Two basic apoptotic pathways exist; one of them seems to be regulated by calcium ions, resulting in the activation of caspase-9. Although apoptosis indeed takes place in the radicular cyst epithelium, and calcium ions might be present in the lesion as exogenous material via extrusion, it has not been possible to be correlated with any clinical factor.

Aim: To investigate the effect of calcium hydroxide ($Ca(OH)_2$) as intracanal medicament on the apoptosis of radicular cyst epithelium, via the immunohistochemical detection of caspase-9.

Materials & methods: 42 paraffin-embedded radicular cysts were retrospectively collected. Information about gender and age of the patients, the size of each cyst (expressed by its maximum diameter) and whether $Ca(OH)_2$ had been applied as intracanal medicament before the surgical treatment were collected from the clinical histories. 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. Sections from all cysts were stained with the von Kossa technique for the detection of exogenous calcium deposits. Three groups were formed: (a) single-visit treatments with no calcium deposits (n=14), (b) multiple-visit treatments without calcium deposits (n=12), (c) multiple-visit treatments with calcium deposits, corresponding to hydroxide extrusion (n=16). All cysts were then immunohistochemically stained for caspase-9 to record apoptosis of the epithelium. Statistical analysis followed.

Results: The frequency of caspase-9 immunoreactivity in the cystic epithelium in the three groups was 42.86%, 50% & 93.75% of cysts, respectively. In the two hydroxide

groups, immunoreactivity was significantly more frequent ($p=0.04<0.05$), particularly in the group with extrusion ($p=0.007<0.05$). In cysts larger than (or equal to) 10mm, caspase-9 was more frequently expressed, irrespective of whether $Ca(OH)_2$ had been applied or not ($p=0.004<0.05$).

Conclusion: Calcium hydroxide up-regulates the expression of caspase-9 and therefore apoptosis; this is more intense when it is extruded into the lesion and especially in large lesions.

GREEK SUMMARY (ΠΕΡΙΛΗΨΗ)

Εισαγωγή: Η απόπτωση είναι ένας, γενετικά καθορισμένος, προγραμματισμένος κυτταρικός θάνατος. Δυο βασικές οδοί απόπτωσης υπάρχουν. Ένας από αυτούς φαίνεται πως ρυθμίζεται από τα ιόντα ασβεστίου, και καταλήγει στην ενεργοποίηση της κασπάσης 9. Παρόλο που η απόπτωση όντως λαμβάνει χώρα στο επιθήλιο των ακρορριζικών κύστεων, και είναι δυνατό να υπάρχουν ιόντα ασβεστίου από εξωγενές υλικό που προωθήθηκε εντός της βλάβης, δεν έχει καταστεί δυνατό να συσχετιστεί η απόπτωση με κάποιο παράγοντα που εμπλέκεται στην κλινική πράξη.

Σκοπός: Να διερευνηθεί η επίδραση του υδροξειδίου ασβεστίου ως ενδορριζικό φάρμακο στην απόπτωση του επιθηλίου των ακρορριζικών κύστεων, μέσω της έκφρασης της κασπάσης 9.

Υλικά & μέθοδος: 42 ακρορριζικές κύστεις εγκιβωτισμένες σε παραφίνη συλλέχθηκαν αναδρομικά από το αρχείο του Εργαστηρίου Στοματολογίας. Πληροφορίες που συγκεντρώθηκαν από το κλινικό ιστορικό κάθε ασθενή αφορούσε το φύλο, την ηλικία, το μέγεθος κάθε βλάβης (εκφραζόμενο ως μέγιστη διάμετρος) και το κατά πόσον είχε τοποθετηθεί υδροξείδιο ασβεστίου ως ενδορριζικό φάρμακο κατά τη συντηρητική ενδοδοντική θεραπεία. Όλα τα περιστατικά είχαν επανεξεταστεί σε διάστημα 6-12 μήνες μετά τη συντηρητική θεραπεία (ανεξάρτητα από το αν είχε τοποθετηθεί υδροξείδιο ασβεστίου ή όχι). Όλες οι ακρορριζεκτομές έλαβαν χώρα μετά το παραπάνω διάστημα. Τομές από όλες τις βλάβες χρώσθηκαν με την τεχνική von Kossa για τον εντοπισμό εναποθέσεων ασβεστίου. Οι βλάβες χωρίστηκαν σε 3 ομάδες: (a) βλάβες όπου έγινε ενδοδοντική θεραπεία σε 1 συνεδρία, και ιστολογικά δε βρέθηκαν εναποθέσεις ασβεστίου (n=14), (b) βλάβες όπου τοποθετήθηκε υδροξείδιο ασβεστίου μεταξύ των συνεδριών αλλά ιστολογικά δε βρέθηκαν εναποθέσεις

ασβεστίου (n=12), και (c) βλάβες όπου τοποθετήθηκε υδροξείδιο ασβεστίου μεταξύ των συνεδριών και παρατηρήθηκαν ιστολογικά εναποθέσεις ασβεστίου, κάτι που πρακτικά σήμαινε έξοδο υδροξειδίου ασβεστίου εντός της βλάβης. Όλες οι βλάβες μελετήθηκαν στη συνέχεια για την ανοσοϊστοχημική έκφραση της κασπάσης 9 στο επιθήλιο. Ακολούθησε στατιστική ανάλυση.

Αποτελέσματα: Η συχνότητα έκφρασης της κασπάσης 9 στις 3 ομάδες βλαβών παρατηρήθηκε στο 42,86%, 50% και 93,75% των βλαβών, αντίστοιχα. Στις δυο ομάδες που είχε τοποθετηθεί υδροξείδιο ασβεστίου κατά τη συντηρητική θεραπεία, η διαφορά στη θετικότητα της κασπάσης 9 στο επιθήλιο ήταν στατιστικά σημαντική ($p=0,007<0,05$). Σε κύστεις μεγαλύτερες από 10mm, η κασπάση 9 εκφραζόταν πιο συχνά από μικρότερες κύστεις ($p=0,004<0,05$), ανεξάρτητα αν είχε τοποθετηθεί υδροξείδιο ασβεστίου ή όχι.

Συμπέρασμα: Το υδροξείδιο ασβεστίου ενισχύει την έκφραση της κασπάσης 9 και κατ' επέκταση την απόπτωση. Το φαινόμενο είναι πιο έντονο σε έξοδο υδροξειδίου ασβεστίου εντός της κύστης καθώς και σε κύστεις μεγάλου μεγέθους.

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