Histological Study of Pulp Response to Different Durations of Topical Triple Antibiotic Medicament

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A Thesis

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by

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ABSTRACT

Objectives: The aim of this study was to investigate the impact of topical triple antibiotics application duration (3, 5, and 7 days) on disinfection of intentionally infected ferrets' canine teeth by histological examination of pulp response.

Methods: Four ferret canine teeth in each group were intentionally infected under general anesthesia with *Enterococcus fecalis* for 6 weeks. Teeth were assigned into four groups: control (no antibiotics); 3 days of triple antibiotic application; 5 days of triple antibiotic application; and 7 days of triple antibiotic application. Topical triple antibiotics ointment (metronidazole, ciprofloxacin, and minocycline) was applied on all teeth except the control group, which were extracted. All treated teeth were extracted 4 weeks following antibiotics removal. Tooth samples were prepared for histologic assessment of inflammatory cell response intensity and type, tissue necrosis, and bacterial presence. Periodontal ligament (PDL) thickness was assessed using initial, post-infection, and post-treatment radiographs.

Results: Data were analyzed with the Generalized Estimating Equations. Control group showed statistically significantly higher inflammatory cell response intensity ($p = 0.0125$) and an acute type of inflammatory cells ($p < 0.001$) compared to 3-days, 5-days, and 7-days groups. In contrast, there were no statistically significant differences between 3-days, 5-days, and 7-days groups in terms of inflammatory cell response intensity and type. There was no statistically significant difference in tissue necrosis and PDL thickness between any of the study groups.

Conclusions: We can conclude that application of topical triple antibiotics on pulpally involved ferrets' teeth show a tendency to promote healing by decreasing the inflammation

intensity and formation of fibrous pulp tissue. There was no significant difference between the longer and shorter treatment groups, which suggests that shorter application duration may be as suitable as the longer duration in treating pulpally involved teeth.

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Dental Infection and Inflammation

Dental pulp is the deepest part of the tooth that is confined within the rigid structure of enamel, dentin, and cementum.[1] It is derived from the embryonic neural crest cells (ectomesenchyme) that proliferate to form the dental papilla which gives rise to the mature dental pulp.[1] Dental pulp resembles an embryonic connective tissue with the presence of specialized cells called odontoblasts.[1] Odontoblasts are dentin-forming cells and they have the ability to form dentin throughout life.[1] Dental pulp plays an important function in the defense mechanism of the tooth especially against an irritation.[1] It is also equipped with other cellular components, nerves, and microcirculation[1]. T-lymphocytes, dendritic cells and macrophages are the main immune cells present in normal pulp.[1] Both dendritic cells and macrophages are antigen-presenting cells that present foreign antigens to T-lymphocytes.[1] Pulp inflammation is a physiological process that occurs after irritation to the pulp and periradicular tissue.[1,2] Pulp irritants could be microbial, traumatic, chemical or iatrogenic.[1] Bacterial irritation is the most common cause of pulp and periapical disease.[1] Microorganisms that cause dental caries often penetrate deep enough into the tooth through dentinal tubules thus causing carious exposure[3] (Figure 1). Such microorganisms (mainly mutants streptococci, lactobacilli and actinomyces)[4] are considered microbial irritants to the pulpal tissue and will lead to infection and subsequently inflammation of pulp and periradicular tissues[2] (Figure 2). This can happen before microorganisms in the carious lesion have made contact with the pulp tissue.[1,2] The presence of these microorganisms and their by-products transmitted through permeable dentin will lead to chronic inflammation,

primarily demonstrated by local infiltration of mononuclear leukocytes including macrophages, lymphocytes, and plasma cells[5,6] (Figure 3). Furthermore, the presence of these microorganisms will activate an immune response through antigen presenting cells.[7] Dendritic cells, which function as antigen presenting cells in the dental pulp, present the antigenic protein from the microorganisms and their by-products to T helper lymphocytes in lymph nodes to differentiate into memory T lymphocytes and migrate to pulp tissue.[7,8] When the carious lesion progresses and pulpal exposure occurs, the pulpal tissue will be infiltrated by secondary invaders from dental plaque that will provoke acute inflammation.[2] Acute inflammation contains mainly polymorphonuclear leukocytes (PMNs) that form a liquefaction necrosis areas at the site of pulp exposure9 (Figure 3). When inflammation persists, damage caused by bacteria and their by-products will diffuse causing extensive damage that will spread, thus causing an inflammatory periapical lesions.[10] Studies have shown that in the absence of bacterial contamination, no periradicular pathosis will develop.[11, 12] Thus bacteria play an essential role in the development of pulpal and periapical lesions.

When pulpal infection occurs, the response will vary according to the severity, intensity and duration of the irritant.[8] The early inflammatory response is characterized by the presence of focal aggregation of chronic inflammatory cells, such as macrophages, lymphocytes, and plasma cells.[1,5,6] Specialized immunocompetent dendritic cells have also been identified.[13]

Unlike other parts of the human body connective tissue, normal and healthy pulp lack mast cells, but are found in inflamed pulps.[14] As the infection progresses, and due to the fact that pulp is surrounded by rigid tissues that limit filtration of fluids in the pulp, an acute inflammatory reaction occurs which is characterized primarily by the presence of

PMNs.[8,9] In advanced stages, there will be marked cellular infiltration and abscess formation that might cause pulpal necrosis.[8]

Odontoblasts are also affected in the development of pulp inflammation presented by disruption of the odontoblastic layer in the form of morphologic changes, along with reduction in the size and number of their cell bodies.[1] These changes appear even before the appearance of inflammatory changes in the pulp.[1] The odontoblasts are supplied with nutrients through the capillary network in the pulp tissue.[1] The capillaries along with the venules form the pulp microcirculation.[1] Pulp microcirculation is affected by pulp inflammation through capillary dilation, which comprises the early sign of the acute inflammation.[1] This inflammation causes an increase in tissue fluids in the pulp, which in turn increases the pressure that collapses the venules and results in vascular stasis and localized cell death.[1]

It is sometimes claimed that the pulpal reaction of primary teeth to irritation is different than the reaction of permanent teeth to pulpal irritation due to thinner layer of dentin and the wide opening of the pulpal foramen during all stages of development of the primary teeth.[15] It is also believed that primary teeth become inflamed more easily, degenerate more readily, react less favorably to irritations and have lower rate of healing than pulps of permanent teeth.[15] In contrast, Cohen and Massler[15] found that the pattern of reaction of primary teeth followed closely the reaction of permanent teeth to dental caries and they heal as well as the permanent teeth pulps.[15]

Treatment of Dental Infection

Treatment of pulpally infected primary teeth is very important to prevent the progression of infection to the radicular tissues that might damage the developing dentition.

Treatment of pulpally involved primary teeth depends on the extension of the inflammatory reaction, in addition to other factors, such as the restorability of the tooth, tooth root resorption, the child's behavior and the presence of any other medical problems.[16,17] Vital pulpotomy is a treatment technique used when inflammation is confined to the coronal part of the pulp without the presence of radicular pathology.[17] It involves the removal of the infected coronal pulp tissue, adjacent to the carious exposure, followed by treating the radicular pulp with a medicament to allow for healing, repair, and physiological root resorption, which continues until the tooth exfoliates[16] (Figure 4). When inflammation extends to the radicular pulp, and signs and symptoms such as swelling, fistula, periapical or interradicular radiolucency develop, irreversible pulpitis or pulp necrosis is evident.[17] Clinically, irreversible pulpitis or pulp necrosis is recognized when hemorrhage from radicular roots is not controlled with application of damp cotton pellets for several minutes during the pulpotomy procedure, or the presence of suppuration or purulence. In these situations pulpotomy is contraindicated and pulpectomy, which is the complete removal of the inflamed and non-vital pulp tissue from the coronal and radicular root canal space or tooth extraction will be the treatment of choice[16] (Figure 5). Pulpectomy procedure involves cleaning the canals with files and disinfection with irrigants such as sodium hypochlorite. After cleaning the canals, a resorbable canal obturating material is used. The ideal pulpectomy canal obturating material should have the following properties: 1) resorb at a rate similar to the root structure, 2) has antiseptic properties, 3) non- inflammatory and non-irritating to the underlying permanent tooth germ, 4) radiopaque for visualization on radiographs, and 5) easy to insert, and easy to remove if necessary.[18] The most widely used materials are non-reinforced zinc oxide

eugenol, Iodoform based paste (KRI Paste), or Idoform with calcium hydroxide mix (Vitapex®). Zinc oxide and eugenol (ZOE) paste were the first root canal filling materials recommended for primary teeth, as described by Sweet in 1930.[19] ZOE paste fails to meet many of the ideal obturating material criteria. The paste shows slow rate of resorption.[20] When the paste is forced beyond the primary root apex, its hardness might increase the risk of deflection of erupting succedaneous teeth.[21] It also has limited antibacterial action.[22] On the other hand, Vitapex was shown to have nearly ideal obturating material properties.[23] Mortazavi and Mesbahi reported 100% overall success rate (clinical and radiographic) of Vitapex pulpectomy compared to 78% success rate for ZOE.[19]

Pulpectomy is considered a difficult procedure in primary dentition due to the complexity of root canals, proximity to developing tooth bud, and difficulty in finding a material that undergoes resorption rate similar to the tooth structure.[16] While it has been reported that pulpectomy may have a decreased success rate, Coll and Sadrian[21] showed that the most important pre-operative predictor to pulpectomy success rate is the amount of primary tooth root resorption. The study also showed that teeth with more than 1mm of root resorption had decreased success rate (23%) compared to non-resorbed roots.[21]

Maintaining a primary dentition free from disease is important to prevent damage to the developing dentition and to preserve teeth and their surrounding tissue, hence preserving arch integrity.[17,24] For these reasons, researchers have been looking for new methodologies, such as topical triple antibiotic treatment, that will disinfect and preserve the vitality of the pulpal tissue.

Topical Triple Antibiotic

Various combinations of antibiotics have been shown to be effective in disinfecting carious dentin and infected pulp tissue.[25-27] Both *in vitro*[25,26] and *in situ*[27] studies have shown effectiveness of the triple antibiotic in disinfecting root dentin. Metronidazole is a bactericidal antibiotic against oral obligate anaerobes[28] and against bacteria isolated from infected necrotic pulp tissues.[29] Metronidazole by itself did not eliminate all bacteria from carious dentin *in vitro*[30] indicating the need for combining antibiotics to completely disinfect carious dentin. Sato *et al.*[31] and Hoshino *et al.*[26] found that the combination of metronidazole, ciprofloxacin, and minocycline can sterilize carious lesions, infected pulpal tissue, and infected root dentin.

Topical triple antibiotic consisting of metronidazole, ciprofloxacin, and minocycline have been shown to be successful treatment for necrotic immature permanent teeth.[32-38] In this situation, the topical antibiotic will disinfect the infected pulp, which will stimulate revascularization through the apical vital tissue. Disinfection with triple antibiotic has gained much interest. Its effectiveness has also been demonstrated in multiple studies as a procedure to disinfect pulpotomized primary teeth with signs of radicular infection that would otherwise be pulpectomized or extracted.[39-42] Repair of the damaged tissue can be expected and prognosis is improved if the tissue is disinfected and an option of pulpectomy or extraction could be excluded.

Animal studies[43-45], human studies[32,33], and case reports[34-38] of permanent teeth reported different durations of triple antibiotic application, which ranged from one week to 11 weeks, while others had applied it permanently. On the other hand, studies on primary teeth did not specify the duration of triple antibiotic application.[39-42,46] To date, there is no guideline for triple antibiotic application duration, but as suggested by Sato *et al.* a

shorter duration of antibiotic application, such as one to two days, would be able to penetrate dentin, be effective to disinfect, and be safe to prevent the development of drug resistance.[25,27]

Takushige *et al.*[42] studied the clinical outcome of triple antibiotic application on 87primary teeth with periradicular lesion. Clinical evaluation was measured in short- (10 days) and long-term (mean 680 days). Cases showed improvement in clinical symptoms after a single application of the triple antibiotic. That study demonstrated the possibility of successfully treating primary teeth with periradicular lesion by topical application of triple antibiotic. Prabhakar *et al.*[41] carried out a study to evaluate the clinical and radiographic success of endodontic treatment of infected primary teeth with a combination of antibiotic. Evaluation of teeth was done at one month, 6 months and 12 months. It was concluded that endodontic treatment of infected primary teeth with antibacterial mix showed good clinical and radiographic success, but cases treated with the removal of coronal and accessible radicular pulp tissue showed better results compared to teeth treated by removing coronal pulp only. Nakornchai *et al.* further studied triple antibiotic combination.[39] The study compared the clinical and radiographic success of endodontic treatment of pulpally involved primary teeth with triple antibiotic to the conventional endodontic treatment with pulpectomy and Vitapex application. Results suggested effectiveness of treatment withtriple antibiotic. Pinky *et al.*[40], in 2011, studied the effectiveness of combination antibioticsin endodontic treatment of primary teeth. Two different antibiotic combinations were compared in this study. Both groups showed clinical and radiographic success with no statistically significant difference between the two groups. Recently, in 2012, Trairatvorakl and Detsomboonrat[47] conducted a study to evaluate the clinical and radiographic success rate of triple antibiotic in non-

instrumented primary mandibular molars. The study showed good clinical success (75%) but the overall success rate, defined as both clinical and radiographic success, was 36.7% at 24-27 months. However, the duration of antibiotic application was either not mentioned in any of these studies or it was applied permanently.

Few histological studies have been conducted to investigate the effectiveness of combination of antibiotics in pulpal tissue. Wang *et al.*[44] investigated the type of tissue grown into the pulp space of intentionally infected immature permanent dogs' teeth after treatment with triple antibiotic. Animals were sacrificed after 3 months and teeth were examined histologically. Only one tooth showed partial survival of pulp tissue identified by the presence of odontoblasts along the dentinal wall of one side of the canal space. Other teeth showed the presence of cementum like tissue with presence of healing and regenerated tissue and inflammatory infiltrates. Bone-like tissue was found scattered in the canal space of some teeth. All these finding explained the increase of length and thickness of roots after treatment with triple antibiotic.

Windley *et al.*[45] assessed the efficacy of a triple antibiotic paste in the disinfection of immature dogs' teeth with apical periodontitis. A statistically significant reduction in bacterial count was found after treatment with triple antibiotic paste for two weeks. The results of that study demonstrated the effectiveness of triple antibiotic paste in the disinfection of immature teeth with apical periodontitis.

Evaluation of pulpally involved teeth treated with a combination of triple antibiotics, as a pulp dressing material, has not yet been evaluated histologically. The availability of human samples for histological evaluation is difficult to obtain, and an animal model is therefore needed for such an investigation. It was found that ferret's canine teeth could be

used for regenerative endodontic investigations.[48] Ferret's teeth have been used in multiple endodontic studies.[49-51] The ferret is a moderate size animal that is more similar as an animal model to humans than rats and rabbits, and it is less subjected to ethical concerns compared to cats, dogs, and primates.[52] The ferret has four canine teeth in which the size and root canal anatomy are suitable for endodontic procedures.[53] It was found that the most appropriate time to conduct an endodontic study that needs an open apex is when the ferret is between 50 and 90 days of age (Figure 6).[48] For these reasons, our study was performed on ferret's canine teeth to investigate the impact of topical triple antibiotic application duration on disinfection.

It is important to investigate the effect of the triple antibiotic treatment on pulp of infected teeth due to the limited information available on the histological outcome of teeth with radicular infection treated with pulpotomy and triple antibiotic. Due to the need for finding a new treatment modality that would help save more primary teeth and avoid undesirable complications that might arise with pulpectomy and extraction, this study investigated the best duration of triple antibiotic application, as there is a lack of knowledge in that aspect.

SPECIFIC AIMS AND HYPOTHESIS

The aim of this study is to investigate the impact of topical triple antibiotic application duration (3, 5, and 7 days) on disinfection success using intentionally infected ferrets' canine teeth. The degree of disinfection was assessed by histological examination of the pulp response measured by the level of inflammatory cell response, hard tissue formation, tissue necrosis, and bacterial infiltration; coupled with radiographic examination of periodontal ligament (PDL) thickness.

Hypothesis:

There is no difference in the pulp response between short (3 days) and long (7 days) duration of triple antibiotic application in terms of:

- Level of inflammatory cell response
- Level of hard tissue formation
- Level of tissue necrosis
- Level of bacterial infiltration
- Periodontal ligament (PDL) thickness

RESEARCH DESIGN AND METHODS

A. Animals and Sample Size Calculation

Seventy days old male ferrets were used in this study. An animal protocol was approved by Tufts Medical Center Institutional Animal Care and Use Committee (IACUC). Based on other studies,[7,54] nQuery (Version 7.0) was used to determine the sample size needed for α=0.05 and power=90%, with medians of inflammatory cell response for the 4 different treatment groups ranging from 1.0 to 2.5 and with a common SD=0.5. It was determined that N=4 teeth in each tested group, and therefore, four permanent canine teeth from each ferret were used in this study. Teeth in each ferret were divided into four groups (Figure 7):

- Group I: control (no antibiotic)
- Group II: 3 days of triple antibiotic application
- Group III: 5 days of triple antibiotic application
- Group IV: 7 days of triple antibiotic application

B. Methodology

Six dental surgeries were performed on each animal (Figure 8).

- First surgery: intentional tooth infection
- Second surgery: application of the triple antibiotic (6 weeks after the first surgery)
- Third, fourth, and fifth surgeries: to remove the antibiotic on days 3, 5, and 7 after application of the antibiotic.
- Sixth surgery: teeth extraction (4 weeks after the fifth surgery)

1. Intentional infection procedure

The animals underwent general anesthesia with Ketamine and Xylazine. Induction was performed using a mask and 3-4% isoflurane in O_2 for intubation. Intubation was performed per standard operating procedures using an endotracheal tube, laryngoscope, and lidocaine splash when needed. The general anesthetic intra-operative agent was isoflurane in O_2 titrated to effect. Minimal amount of isoflurane gas was used to reach the needed surgical anesthetic plane. Local anesthesia was established using < 2 mg/kg Bupivicaine injected into the buccal gingiva as an infiltration around the involved tooth. Pre-operative radiographs of maxillary and mandibular canine teeth were taken using bisecting angle technique with size 1 periapical film (40 mm x 24 mm) placed parallel to the tooth and using an X-ray machine with a round cone on an average angulation of 45º to the occlusal plane. All dental pulps of the ferrets' canine teeth in this experiment were mechanically exposed by removing approximately 4 mm of tooth structure from each tooth using a high-speed handpiece with water coolant and round bur until pulp is exposed. Standard composite bonding procedure was performed per manufacturer instructions in the following order: (1) enamel and dentin were etched with 37% phosphoric acid for 15 seconds; (2) rinse for 15 second; (3) Optibond^{TM} Solo Plus adhesive was applied on cavity walls, air dried for 3 seconds then light cured for 5 seconds.

After culturing *Enterococcus faecalis* in brain-heart infusion (BHI) broth at 37^{o}C, 0.01 mL (1.5 x 10^8 colony forming units (CFUs)/mL) of the bacterial suspension was placed on the pulp tissue and access was sealed with composite resin restoration. Approximately 3 weeks later, radiographs were taken to confirm apical periodontitis. The infection was not evident on the radiographs and it was decided to wait another 3 weeks until the infection could be confirmed on the radiographs. During that period, it was noticed that all ferrets were losing

some of their composite resin restorations due to their heavy chewing habit of their cages bars. An amendment to the animal protocol was submitted to IACUC to remove all composite resin restoration and keeping their dental pulps exposed to oral cavity in order to get equal opportunity of infection. The amendment was approved by IACUC and all composite resin restorations were removed. Dental pulps of all ferrets were open for one week. When apical periodontitis was evident, around 6 weeks after the intentional infection, and confirmed by radiography, standard pulpotomy procedure was performed, except for the control canine, which was extracted and placed immediately in 10% buffered formaldehyde.

2. Pulpotomy with triple antibiotic Procedure

a) Preparation of triple antibiotic

On the day of the procedure, 20 mg of each drug powder, namely Metronidazole (Metronidazole, U.S. Pharmacopeia, Rockville, MD), Ciprofloxacin (Ciprofloxacin HCL, Santa Cruz Biotechnology, Santa Cruz, CA), and Minocycline (Minocycline Hydrochloride, MP Biomedicals LLC, Solon, OH) in a weight ratio of (1:1:1) were mixed together. After that, the powder was mixed with 1mL of liquid consisting of macrogol and propylene glycol (1:1 ratio by volume) to form an ointment. Unused mix was discarded at the end of the procedure.

b) Pulpotomy and triple antibiotic application

The animals underwent general anesthesia. A No. 9 Ivory clamp was used to hold the rubber dam in place and to isolate the operative field. Chlorhexidine gluconate was used to disinfect the operative field. Using a high-speed handpiece with water coolant and round bur, the same area that was previously opened was widened. Pulpotomy was performed using a sharp spoon

excavator to remove the coronal pulp tissue and cavity was irrigated with 2.5% sodium hypochlorite (NaOCl). Standard composite bonding procedure was performed per manufacturer instructions. Care was taken not to apply any of the bonding materials on the exposed pulp tissue. The exposed pulp tissue was then covered with the triple antibiotic mix. The cavities were sealed with Teflon disc, followed by composite resin restoration. Experimental animals' teeth were reopened either on day 3, 5, or 7 days after treatment depending on the group assignment, and the triple antibiotic was washed away with sterile saline. After performing standard composite bonding, cavities were closed with Teflon disc then sealed with Mineral Trioxide Aggregate (ProRoot® MTA. Tulsa Dental Products, Tulsa, OK, USA) and composite resin restoration.

Four weeks after the final restoration, teeth were extracted, under general anesthesia, for histological studies. Local anesthesia was established using < 2 mg/kg Bupivicaine injected into the buccal gingiva as an infiltration around the involved tooth. A straight elevator was used to luxate the tooth then the tooth was extracted as a one piece using forceps. Gel foam was placed in all extraction sockets and suturing was done with 5-0 PDS absorbable suture to control bleeding. Radiographs were taken of all groups before extraction to assess the thickness of periodontal ligament.

After each surgery, the animals were kept warm and continuously monitored post-operatively to ensure recovery from anesthesia. The first 72 hours post-surgery, animals were monitored three times daily for signs of pain or distress. Thereafter they were monitored daily (not including weekends and holidays) and weighed 3 times per week. The first 72 hours post-surgery, the animals were given buprenorphine subcutaneously (0.01-0.05 mg/kg, twice a day then as needed thereafter). Following the last surgery

(extractions), animals were fed carnivore care liquid diet to decrease any possible pain associated with chewing solid food.

3. Histology

Teeth samples were fixed at 4°C in 10% formaldehyde made in phosphate buffered saline (PBS, containing in mM: 145 NaCl, 7.3 Na_2HPO_4, and 2.7 NaH_2PO_4 at pH 7.2). After trying the optimum decalcification period on two ferret's teeth it was found that 48 hours was enough to decalcify the teeth. Samples were then decalcified in hydrochloric acid (VWR® Rapid Decalcifier) for 48 hours until decalcification was complete. Then the teeth were cut horizontally in 1:2 ratios with the root being the longest part. The root was then cut in half in a bucco-lingual direction, and the coronal section was cut in half as well in mesio- distal direction as shown (Figure 9). Next, teeth were dehydrated in ascending alcohol grade solutions of 50%, 70%, 95%, then 100% followed by 1:1 xylene:paraffin embedding then finally embedded in paraffin wax. Serial sections of 5 μm thickness were prepared using a microtome. Sections were made and placed on gelatin-coated slides. Sections were deparaffinized followed by hematoxylin and eosin staining for analysis using a light microscope. In addition, Gram staining was used for bacterial detection. MiPACS® software was used to view the radiographs and measure periodontal ligament thickness, which is the black radiolucent line surrounding a tooth root. Measurement was performed on each radiograph obtained from all teeth. Five random points (mesial, mesioapical, apical, distoapical, and distal) were selected from each tooth radiograph. Linear measurement tool was used to measure the radiographic PDL thickness.

C. Outcome Measures (dependent variables)

A board certified oral pathologist blinded to the treatment groups carried out microscopic examinations. The pulp response was measured according to the criteria used by Rutherford and Gu[55], and Koliniotou Koumpia and Tziafas[54], and are summarized in Tables 1 through 4. The following parameters were evaluated:

- Inflammatory cell response intensity: scored from 0 to 3 (Table 1)
- Inflammatory cell response type: scored from 0 to 2 (Table 2)
- Tissue necrosis: scored from 0 to 2 (Table 3)
- Bacterial infiltration: scored 0 or 1 (Table 4)

Four slides were selected from each tooth sample. Using a 40x magnification of a light microscope, five random areas were selected and the histological parameters were scored. Radiographic measurement of periodontal ligament thickness was performed in five random areas by a blinded examiner to the treatment groups.

D. Data Presentation and Statistical Analyses

Medians and interquartile ranges (IQRs) of inflammatory cell response intensity, tissue necrosis, inflammatory cell response type outcome measures were reported, while PDL thickness is continuous outcome measure, thus means and standard deviations (SDs) were reported. All outcome measures are considered correlated, thus Generalized Estimating Equations (GEEs) were used to estimate the average effect of time on the study sample. Any p- values less than 0.05 were considered statistically significant. All analyses were performed using SAS, version 9.2 (SAS Institute, Cary, NC).

RESULTS

Association between different days of triple antibiotic application and inflammation intensity, inflammation type and tissue necrosis

The control group showed statistically significantly higher levels of inflammation intensity and an acute type of inflammation when comparing the inflammatory cell response intensity ($p = 0.0125$) and inflammatory cell response type ($p < 0.001$) of all four groups. After omitting the control group to check for differences between the different days of treatment, there was no statistically significant difference in terms of inflammatory cell response intensity ($p = 0.5437$) or inflammatory cell response type ($p = 0.9999$) among the different durations. Furthermore, when comparing tissue necrosis level, there was no statistically significant difference between all groups as well as between the active treatment groups ($p = 0.4047$ and $p = 0.5887$ respectively) (Table 5).

Inflammatory cell response intensity

Various levels of inflammation were observed in teeth of control group. The mildest type of reaction was a mild to moderate inflammation in focal areas of the coronal part of the pulp, with healthy radicular pulp and well-organized odontoblastic layer. Another tooth showed severe acute inflammation. While, the other two teeth were completely necrotic (Figure 10) with some focal areas of sever inflammation. Inflammatory cell response intensity median in this group was 2 with IQR (0.0, 2.5).

Two out of four teeth in the 3-days group exhibited areas of mild chronic inflammation, one of these two teeth showed hyperemia as well. The other two teeth in this group showed moderate and severe separate focal areas of inflammation with healthy pulp apically. The

calculated inflammatory cell response intensity was 0 with IQR (0.0, 1.0).

Pulpal response in the 5-days group was mostly confined coronally in the area closer to the capping material. Two teeth showed no signs of inflammatory cell infiltrate, except for coronal hyperemia in one of these teeth. The other two teeth in this group showed coronal areas of hyperemia, and mild to moderate inflammation. Inflammatory cell response intensity median in this group was 0 with IQR (0.0, 0.0).

One tooth in the 7-days group showed no signs of inflammation; vital pulp observed but became more fibrous as progressing apically. However, mild to moderate coronal inflammatory cell infiltrate was observed in two teeth with healthy radicular pulp. Coronal and apical linear areas of moderate to severe inflammation were observed on the peripheries of another tooth. . Inflammatory cell response intensity median was 0 with IQR (0.0, 0.5)

Inflammatory cell response type

Inflammatory cell response type of the control group were varied from mixed acute and chronic focal areas of inflammation, which was composed of plasma cells, lymphocytes and neutrophils, to an acute coronal inflammation and hyperemia with some areas of chronic radicular inflammation (Figure 11). Inflammatory cell response type median was 0 with IQR (0.0,1.0).

On the other hand, inflammatory cell response type in the 3-days group was either chronic inflammatory cells or a mixed acute and chronic inflammation (Figure 12). Inflammatory cell response type median of this group was 2 with IQR (2.0, 2.0). Moreover, inflammatory cells response type of the 5-days group was mainly chronic inflammatory cells infiltrate (Figure13) defined by the presence of plasma cells. The Inflammatory cell response type

median of this group was 2 with IQR (2.0, 2.0). Additionally, the 7-days group also showed chronic inflammatory cells response type. Inflammatory cell response type median of this group was 2 with IQR (2.0, 2.0).

Tissue necrosis

Two teeth in the control group showed complete necrosis. This group median was 0.5 with IQR (0.0, 2.0). On the other hand, one tooth in each of 3-days, 5-days, and 7days groups showed coronal areas of partial necrosis. Tissue necrosis medians of each of the 3-days, 5-days and 7-days groups were 0 with IQR (0.0, 1.0).

Bacterial Presence

Histologic examination of slides stained with gram stain showed no detectable bacteria in the pulp space, the axial floor, along the cavity walls or within the cut dentinal tubules in any specimen of any group in this study.

Radiographic PDL thickness

Radiographic interpretation of teeth showed continued root development and periodontal ligament maturation despite the ongoing infection. Means of initial, post infection, and post treatment radiographic PDL thickness of all study groups were not statistically significant. (p-values = 0.8389, 0.7233, and 0.3922 respectively). Similarly, after excluding the control group, there was no statistically significantly difference between the 3-days, 5-day, and 7-days groups (p-values = 0.6582, 0.5165, 0.3922 respectively). Means of radiographic PDL thickness are summarized in Table 6.

DISCUSSION

Our study found statistically significant differences between the treated and untreated groups (control, and 3-days, 5-days and 7-days treatment groups) in terms of inflammatory cell response intensity and type. Inflammation intensity was the highest in the control group and it was decreased following application of the triple antibiotic treatment. The median of the inflammation intensity in the control group was 2, which is interpreted as moderate inflammation according to the histologic criteria followed in our study. All other groups' medians of inflammation intensity scored 0, which corresponds to absent or very few inflammatory cells. On the other hand, inflammatory cell response type median of the control group was 0, which was described as predominately acute reaction characterized by a presence of polymorphonuclear leukocytes (PMN), while all other treatment groups medians were 2, which means that it is predominately chronic pulp reaction characterized by presence of mononuclear leukocytes. However, after excluding the control group there was no statistically significant difference between the three treatments groups (3, 5, and 7 days). This suggests that the longer duration of antibiotic application is not significantly better than the shorter duration of antibiotic application. Our study did not find any significant difference in term of tissue necrosis among the groups.

Generally, there was a tendency towards healing and tissue repair following treatment, which exhibited fibrotic pulp. Fibroblasts are considered pulp-forming cells. They increase in number and size when there is a decrease in pulp cellular content. Fibrotic pulp tissue was mainly noticeable in 7-days treatment group. Furthermore, the type of inflammatory cells in the control group was mixed acute and chronic inflammatory cells, which turned to chronic type of inflammatory cells in the 5-days and 7-days groups. Although a tendency towards

healing was observed, it was not statistically significant when compared between 3-days, 5-days, and 7-days group. This might be attributed to the short observation period after washing out the antibiotics, which might suggest the need for prolonged periods of observation for the healing process to continue.

Focal areas of calcification (Figure 14) were observed in the 5-days and 7-days treatment groups. These areas of calcification were detected only in the coronal part of the tooth. There is histologic evidence that these areas are dentin like calcifications (reparative dentin) intermixed with dystrophic calcification. These areas of calcification might be reparative in response to pulp irritation or as part of a hard tissue bridge formation stimulated by the triple antibiotic to seal the exposure site. It is worth mentioning that only in the treatment groups (3-day, 5-days, and 7-days groups) was there pulp reaction to a foreign material, probably the triple antibiotic medicament. This reaction can be described as a foreign body fibrous connective tissue response, characterized by edematous areas surrounded by fibrous septae (Figure 15). It is not clear if this is a favorable reaction to the material, or the pulp was undergoing necrosis. This reaction might explain the high clinical success of teeth treated with triple antibiotic in the beginning, where clinical symptoms are relieved as presented in other studies[39-42] then the drop in overall success rate at the time of 24-27 months follow up as explained by Trairatvorakul and Detsomboonrat.[47] Another study with longer observational period would be beneficial to better characterize this kind of reaction and its effect on pulp tissue.

In our study, no bacteria were detected in the pulp space, the axial floor, or along the cavity walls and within the cut dentinal tubules in any of the slides in any group. Pulp inflammation in all groups seems to exist in the absence of detectable bacteria. Although bacteria were

injected into the pulp tissue and should be detected at least in the control group where no antibiotics applied, processing the teeth for histology is a very harsh procedure where bacteria might be lost with the use of strong acids during decalcification. In addition, one of the limitations of gram staining is that it requires a sufficiently large number of potential pathogens to be present in order to be detected.[56] Moreover, it was thought that animals were fed food containing antibiotics, which might help the animals immune system to fight against and eliminate the injected bacteria. A diet description was checked and it showed that it did not contain any type of antibiotics. Therefore, the ferret's diet did not contribute to this finding. Furthermore, although a standardized inoculum was used for pulpal infection, total exposure to bacteria may be variable as cavities were not sealed, and pulp was exposed to bacteria in the oral cavity. The absence of a restoration to seal the pulpal chamber may have contributed to the absence of bacteria.

Radiographic PDL thickness was not statistically different between control, 3-days, 5-days, and 7-days groups. Similarly, there was no statistical significant difference when comparing the treatment groups only (3-days, 5-days, and 7-days groups). This is not consistent with the histologic observation of the teeth, where pulpal responses varied from mild inflammation to necrotic pulps. This finding had been also observed in a study by Green *et al*[57] where radiographically normal teeth showed different histologic signs of inflammation in root canal treated teeth from cadavers. However, longer infection periods are suggested to demonstrate noticeable radiographic increases in PDL thickness, which will also help assess and better understand radiographic changes after treatment.

After intentionally inducing infection in all ferrets teeth, and detecting infection radiographically in at least one tooth of each animal, it was decided to proceed with the

treatment assuming that infection has occurred in all teeth. Overall, there was no evidence of radiographic pulp infection in five teeth of three ferrets. At least one of these teeth falls in each of the study groups. The histologic outcome of these teeth varied from mild inflammation to necrosis. Only one tooth in the 7-days group did not show any radiographic evidence of infection and displayed healthy pulp histologically. This might have affected the study outcome since this tooth fell in the 7-days group and the histologic observation of non-inflamed pulp might not be attributed to the triple antibiotic treatment

By showing a tendency towards healing with topical triple antibiotic medicament in pulpotomized teeth, this study can be the base for future, broader studies with larger sample size and longer duration of observation, which may have the potential to lead to new treatment modalities. This will facilitate the treatment of pulpally involved primary teeth after conducting human studies. Another advantage of this new treatment modality would be the reduced chair side time in comparison to the current treatment options of pulpally involved primary teeth (single-step or a two-steps pulpectomy procedure), and it can also be performed in teeth that show advanced physiologic root resorption. Moreover, this methodology is considered superior to extraction, since the infection is treated and the tooth is free from disease, it will serves as a natural space maintainer.

The present study had several limitations that could have affected the study outcomes. In order to be able to start with a consistent infection, a longer observation period after injecting bacteria to stimulate pulp infection and subsequent inflammation is suggested. Furthermore, It was difficult to conclude that all dental pulps of ferrets' teeth were undergoing healing process due to the observed foreign body reaction to the pulp capping material. A longer observation period is needed to better understand the pulp healing process after treatment

with the topical triple antibiotic medicament. Additionally, it should be taken into consideration that generalizing animal studies to humans is not suitable and data obtained from animal studies should be interpreted with caution.

CONCLUSIONS

Based on the results of our study, we conclude that the application of topical triple antibiotic namely, metronidazole, ciprofloxacin, and minocycline on pulpally involved ferrets' teeth as a pulp capping material showed a tendency to promote healing by decreasing inflammation intensity and formation of fibrous pulp tissue. There was no significant difference between the longer (7-days) and shorter (3-days) treatment groups, which suggests that shorter application duration may be as suitable as the longer duration in treating pulpally involved teeth. Moreover, it seems necessary to study the foreign body reaction to the capping material observed in the treated teeth to better understand the fate of the pulp tissue. It appears that treatment of pulpally involved teeth with topical triple antibiotic as a pulp capping material is very promising; however, further investigations are necessary to better determine the healing potential.

APPENDIX A

TABLE 1: INFLAMMATORY CELL RESPONSE INTENSITY WITH REPRESENTATIVE PICTURES OF INTENSITY LEVELS

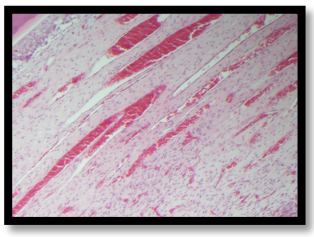
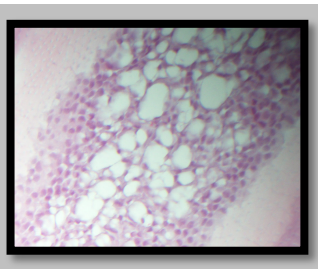
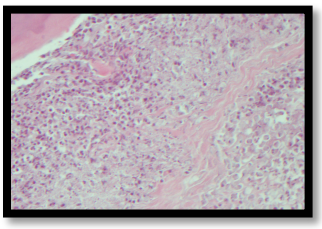
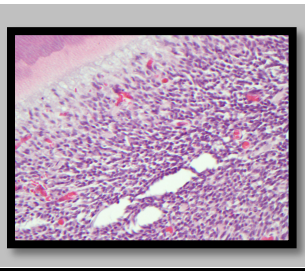
Inflammatory cell response intensity		
Score	*Description*	
0	Absent or very few inflammatory cells	
1	Mild inflammation	
2	Moderate inflammation	
3	Severe inflammation	

TABLE 2: INFLAMMATORY CELL RESPONSE TYPE[55]

Inflammatory cell response type	
Score	*Description*
0	Predominately acute reaction characterized by a predominance of polymorphonuclear leukocytes (PMN)
1	Mixed acute and chronic reaction characterized by approximately equal numbers of PMN and mononuclear leukocytes
2	Predominately chronic reaction characterized by a predominance of mononuclear leukocytes

TABLE 3: TISSUE NECROSIS [54]

Tissue necrosis	
Score	*Description*
0	No necrosis: Presence of complete tissue organization in the radicular pulp
1	Partial necrosis: Half or more of the radicular pulp degenerated
2	Complete necrosis: Complete tissue degeneration of the radicular pulp

TABLE 4: BACTERIAL INFILTRATION[54]

Bacterial infiltration	
Score	*Description*
0	Absence of bacteria in the pulp space, or the axial floor, or along the cavity walls and within the cut dentinal tubules
1	Presence of bacteria in the pulp space, or the axial floor, or along the cavity walls and within the cut dentinal tubules

TABLE 5: INFLAMMATION INTENSITY, INFLAMMATION TYPE, AND TISSUE NECROSIS OUTCOMES BY DAYS OF TREATMENT GROUPS

	Control		3 Days		5 Days		7 Days			
	Median	**IQR**	**Median**	**IQR**	**Median**	**IQR**	**Median**	**IQR**	**p-value[1]**	**p-value[2]**
Inflammation Intensity	2.0	(0.0, 2.5)	0.0	(0.0 1.0)	0.0	(0.0, 0.0)	0.0	(0.0, 0.5)	0.0125	0.5437
Inflammation Type	0.0	(0.0, 1.0)	2.0	(2.0, 2.0)	2.0	(2.0, 2.0)	2.0	(2.0, 2.0)	<0.001	0.9999
Tissue Necrosis	0.5	(0.0, 2.0)	0.0	(0.0, 1.0)	0.0	(0.0, 1.0)	0.0	(0.0, 1.0)	0.4047	0.5887

[1] p-value from GEE, comparing all 4 groups
[2] p-value from GEE comparing Day 3, 5 and 7

TABLE 6: MEANS (IN MM) OF PDL THICKNESS OF DIFFERENT TREATMENT GROUPS

	Control		3 Days		5 Days		7 Days			
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**p-value[1]**	**p-value[2]**
PDL thickness (initial)	0.188	0.092	0.200	0.059	0.202	0.091	0.184	0.047	0.8389	0.6582
PDL thickness (infection)	0.185	0.063	0.168	0.058	0.189	0.077	0.176	0.073	0.7233	0.5165
PDL thickness (final)	_	_	0.147	0.035	0.180	0.158	0.163	0.128	0.3922	0.3922

[1]p-value from GEE, comparing all 4 groups
[2] p-value from GEE comparing Day 3, 5 and 7

APPENDIX B

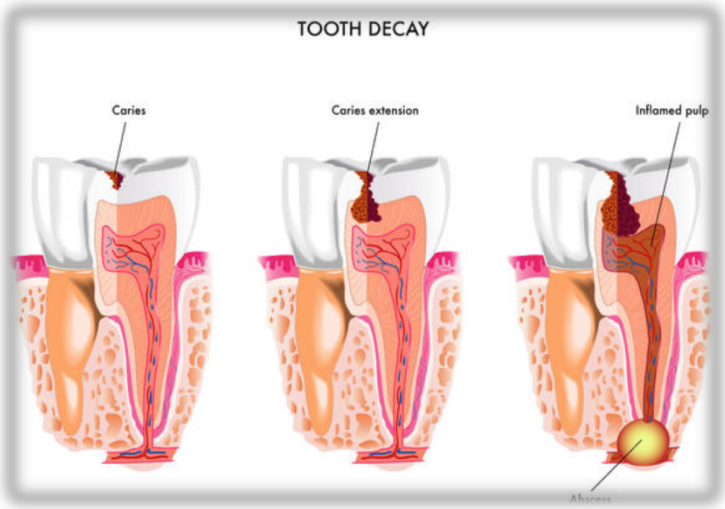


Figure 1 Progression of carious lesion causing carious exposure. (From www. healthtap.com)

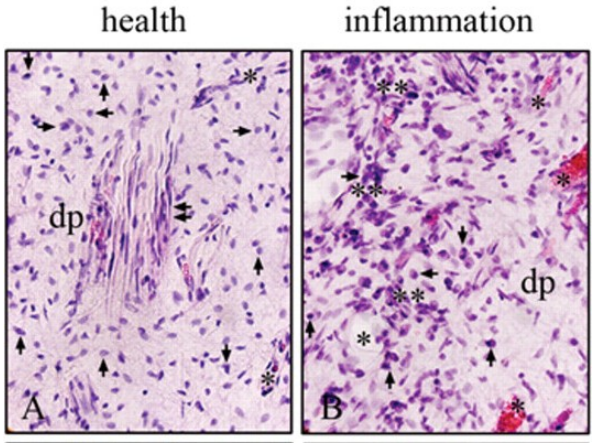


Figure 2 Healthy and inflamed dental pulp in Hematoxylin and Eosin staining. (FromKorkmaz *et al*. [58])

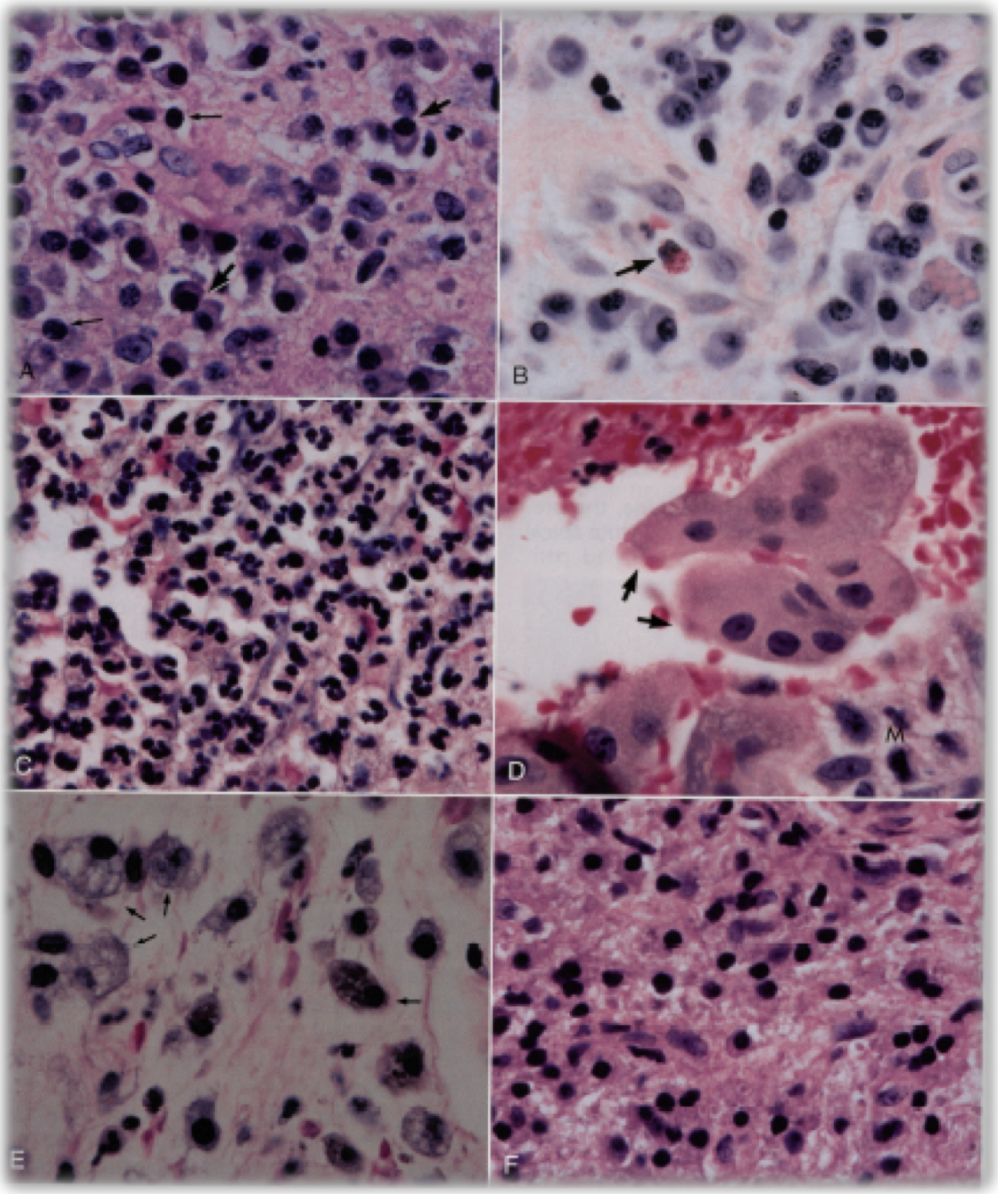


Figure 3 Pulp inflammatory cells. A: Lymphocytes (small arrows). Plasma cells (largearrows) have eccentric nucleus. B: Eosinophil with bilobed nucleus..C: Polymorphonuclear leukocytes (PMNs) have multilobed nuclei. D: Giant cells (arrows) with multiple nuclei and Macrophages (M) with lighter stained nuclei. E: Macrophages. F: Lymphocytes with their densely basophilic nuclei. (From Endodontics: Principles and Practice[59])

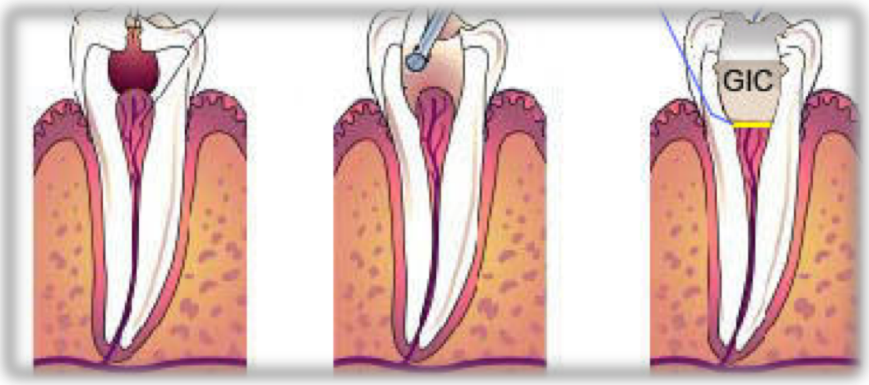


Figure 4 Pulpotomy procedure. Coronal pulp tissue removed and the remaining radicular pulp treated with medicament. (From www.comwoodlandhillspharmacy.com)

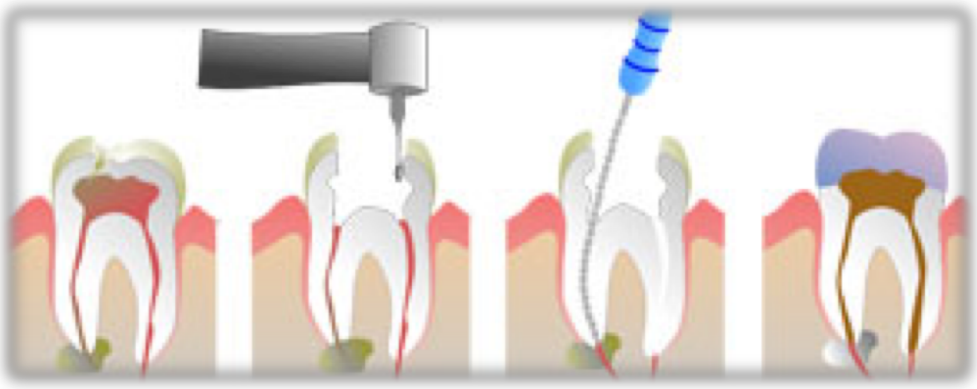


Figure 5 Pulpectomy procedure. Coronal and radicular pulp tissue removed and pulp space is filled with medicament. (From www.dentalfind.com)

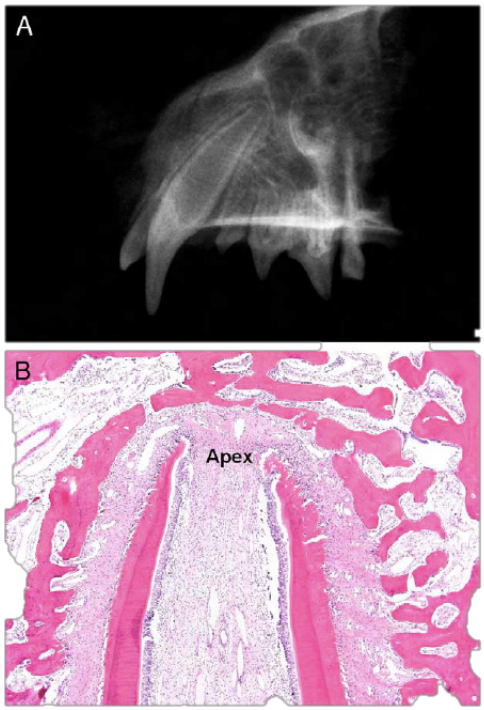


Figure 6 A:Radiographic Image of ferret canine tooth at age 90 days with open apex.

B: Histological view of 90 days old ferret. (From Torabinejad *et al*.[48])

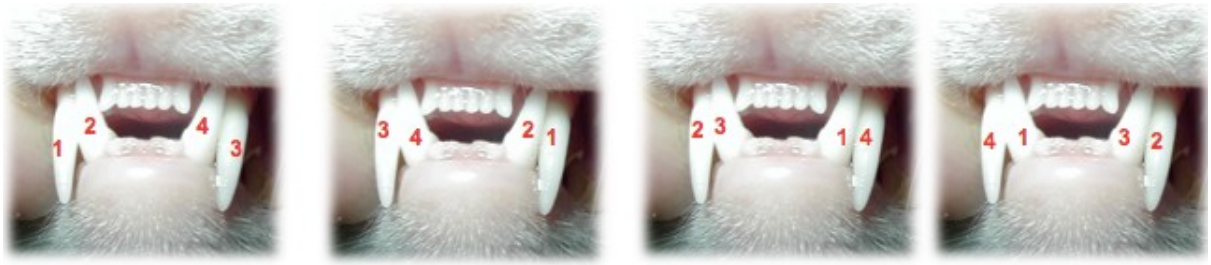


Figure 7 Teeth in each ferret were divided into four treatment groups

(1: control, 2: 3-days group, 3: 5-days group, and 4: 7-days group)

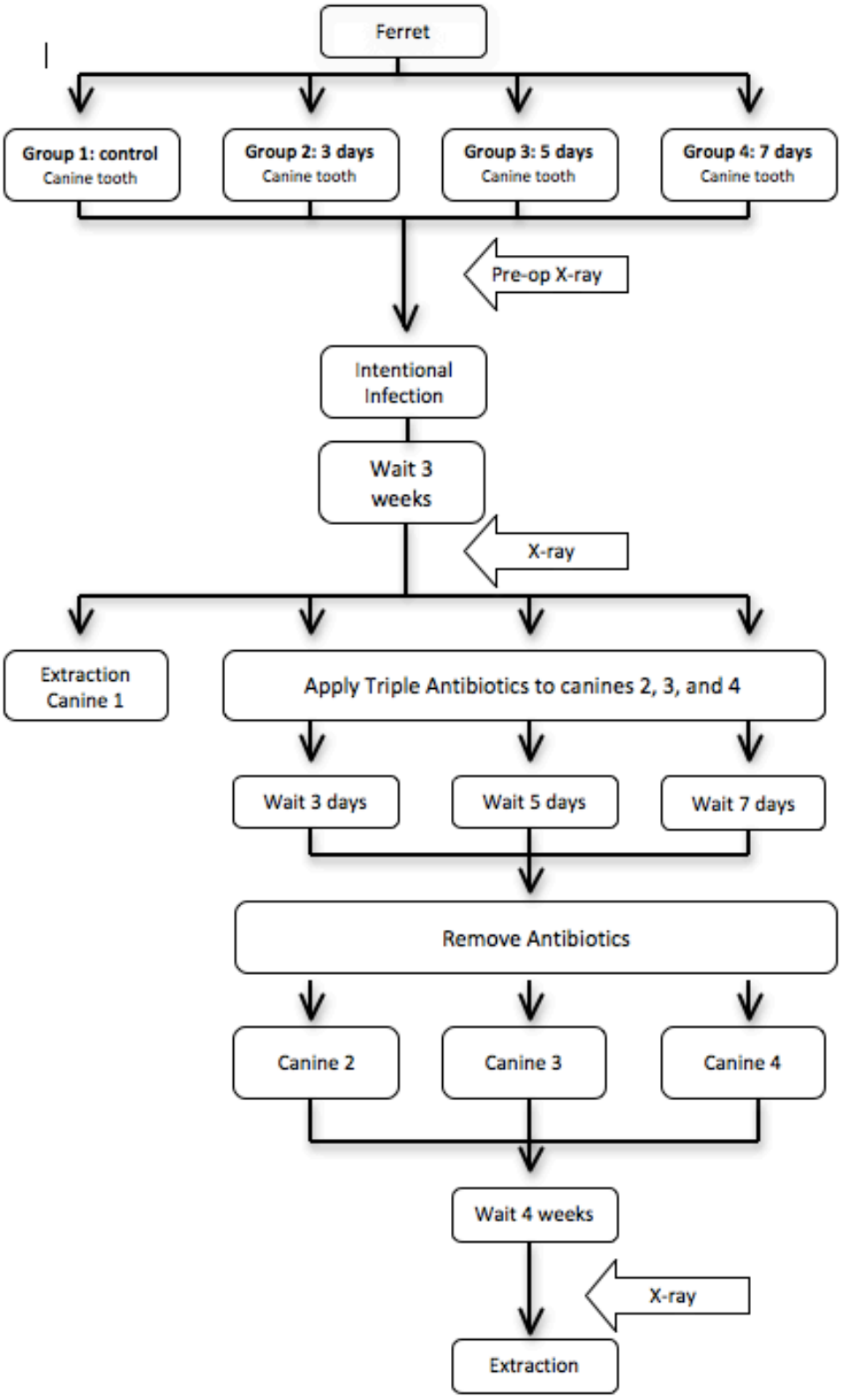


Figure 8 Flow chart of the experimental design

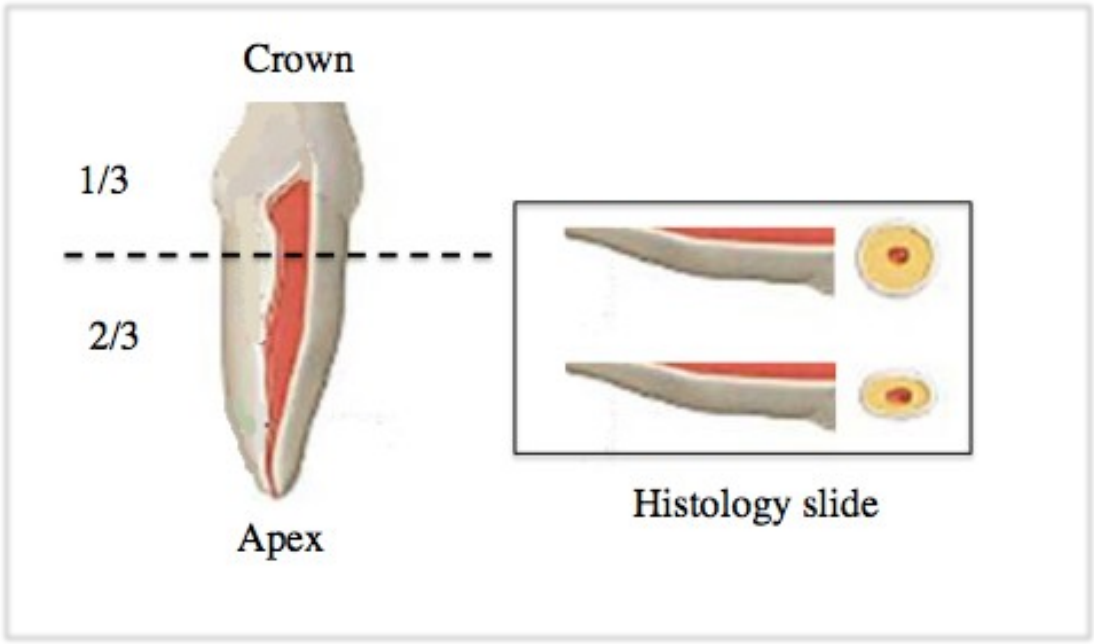


Figure 9 Schematic of a tooth sample preparation for histology processing

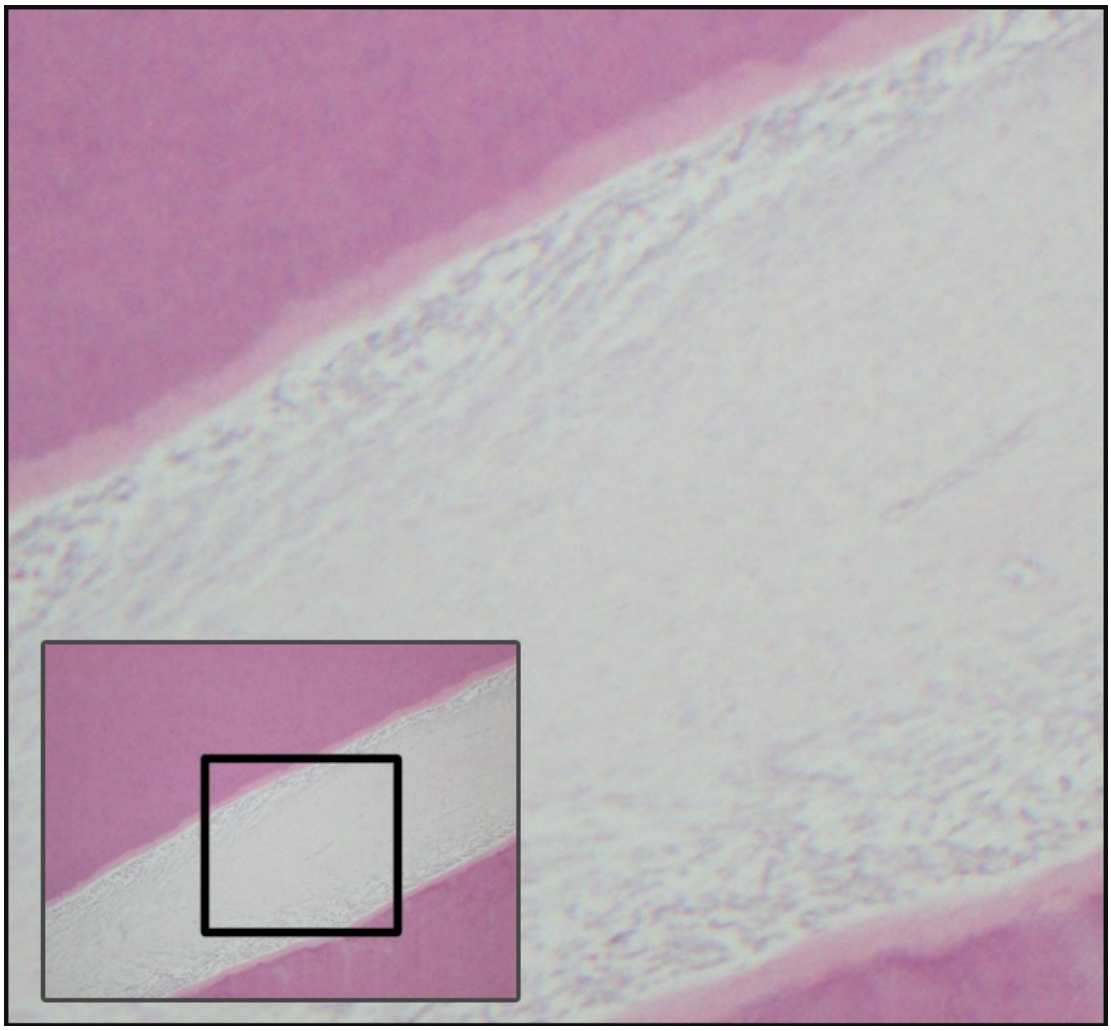


Figure 10 Light micrograph of H&E stained tooth in control group showing pulp necrosis. Medium power (40X) magnification. **Inset:** lower magnification (10X) of the same field with a box showing the close up view location.

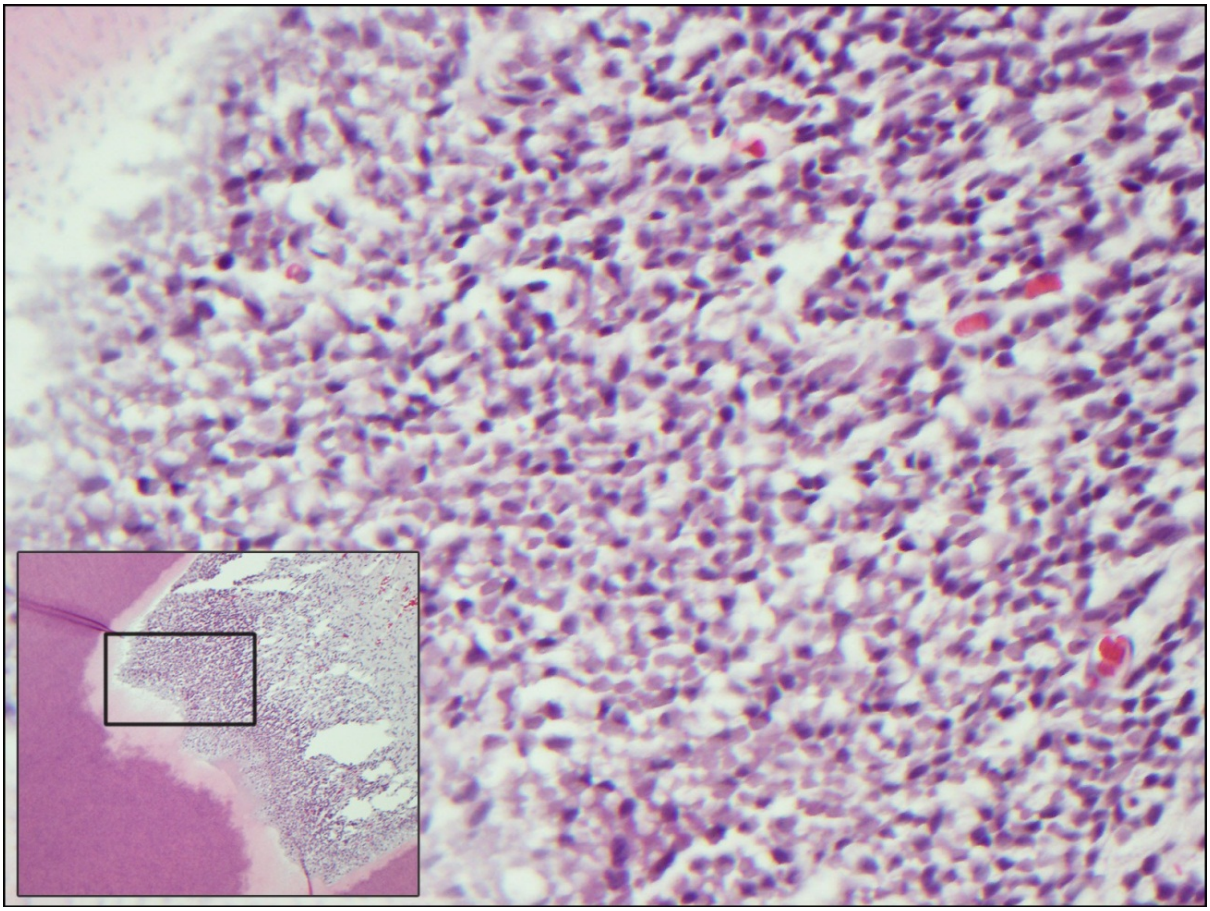


Figure 11 Light micrograph of H&E stained tooth in control group showing chronic severe inflammation. Medium power (40X) magnification. **Inset:** lower magnification (10X) of the same field with a box showing the close up view location.

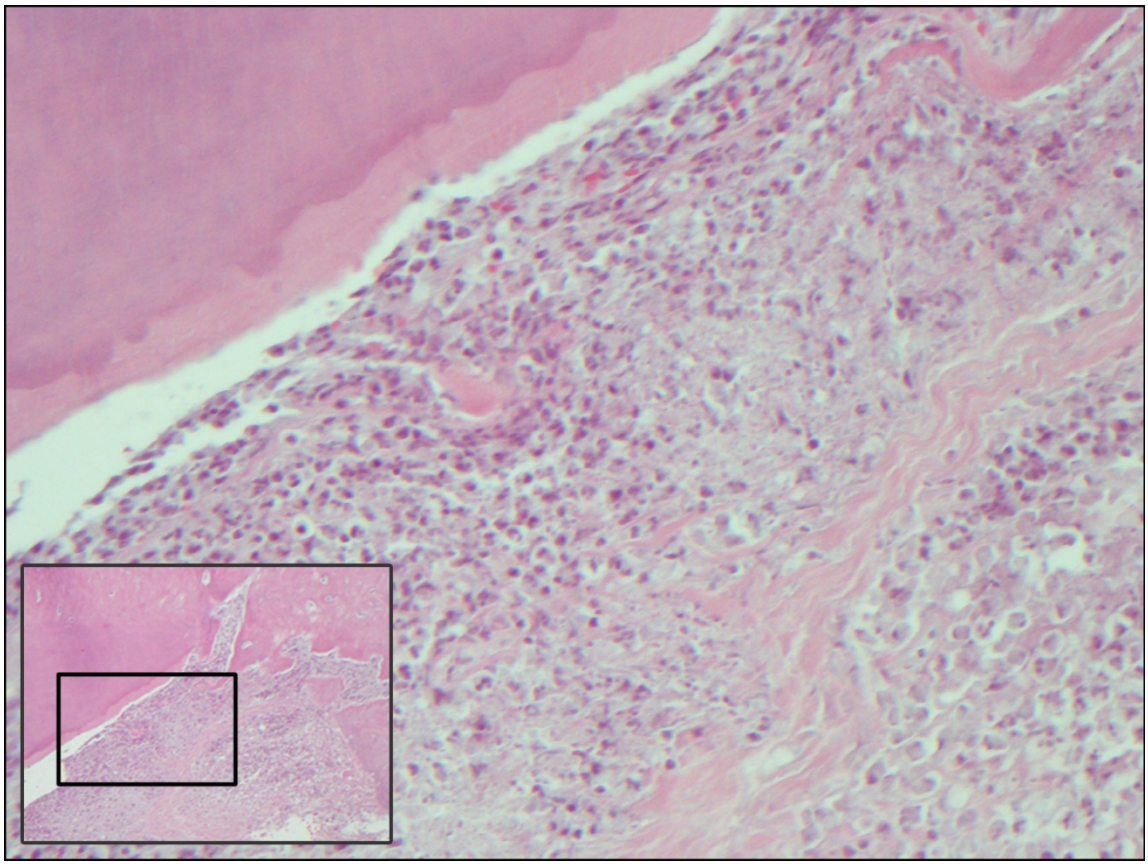


Figure 12 Light micrograph of H&E stained tooth in 3-days group showing moderate mixed acute and chronic inflammation. Medium power (40X) magnification. **Inset:** lower magnification (10X) of the same field with a box showing the close up view location.

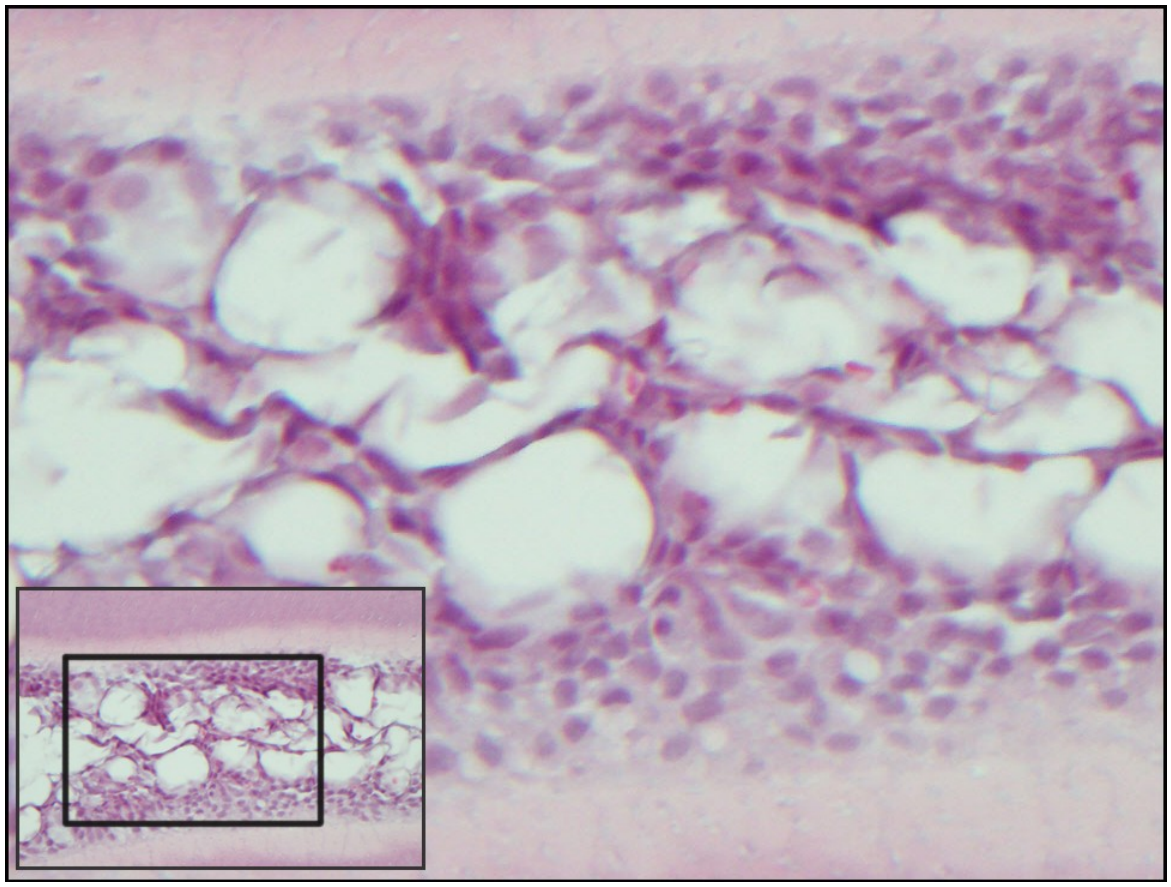


Figure 13 Light micrograph of H&E stained tooth in 5-days group showing mild chronic inflammation. Medium power (40X) magnification. **Inset:** Medium power (40X) magnification of the same field with a box showing the close up view location.

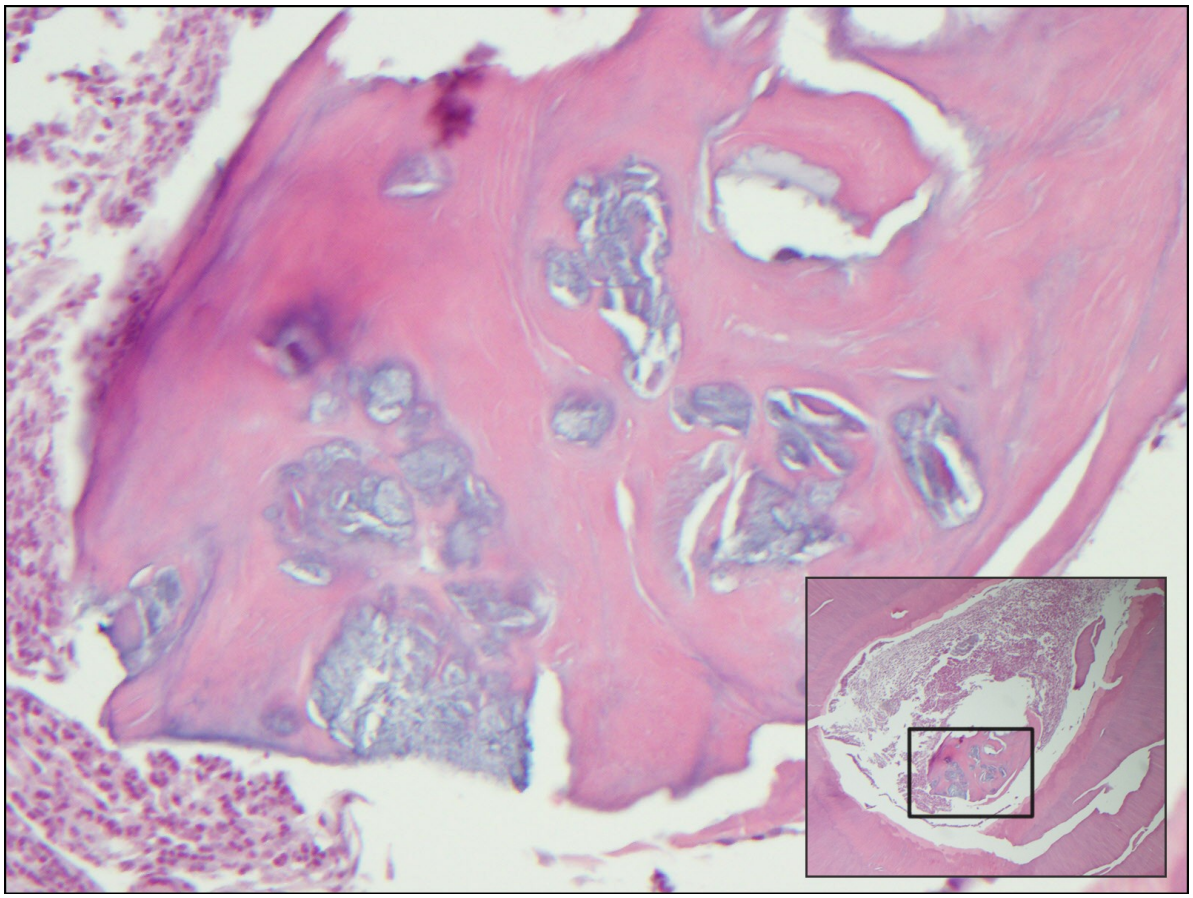


Figure 14 Light micrograph of H&E stained tooth in 5-days group showing areas of calcification in the coronal part of a tooth. Medium power (40X) magnification. **Inset**: lower magnification (10X) of the same field with a box showing the close up view location.

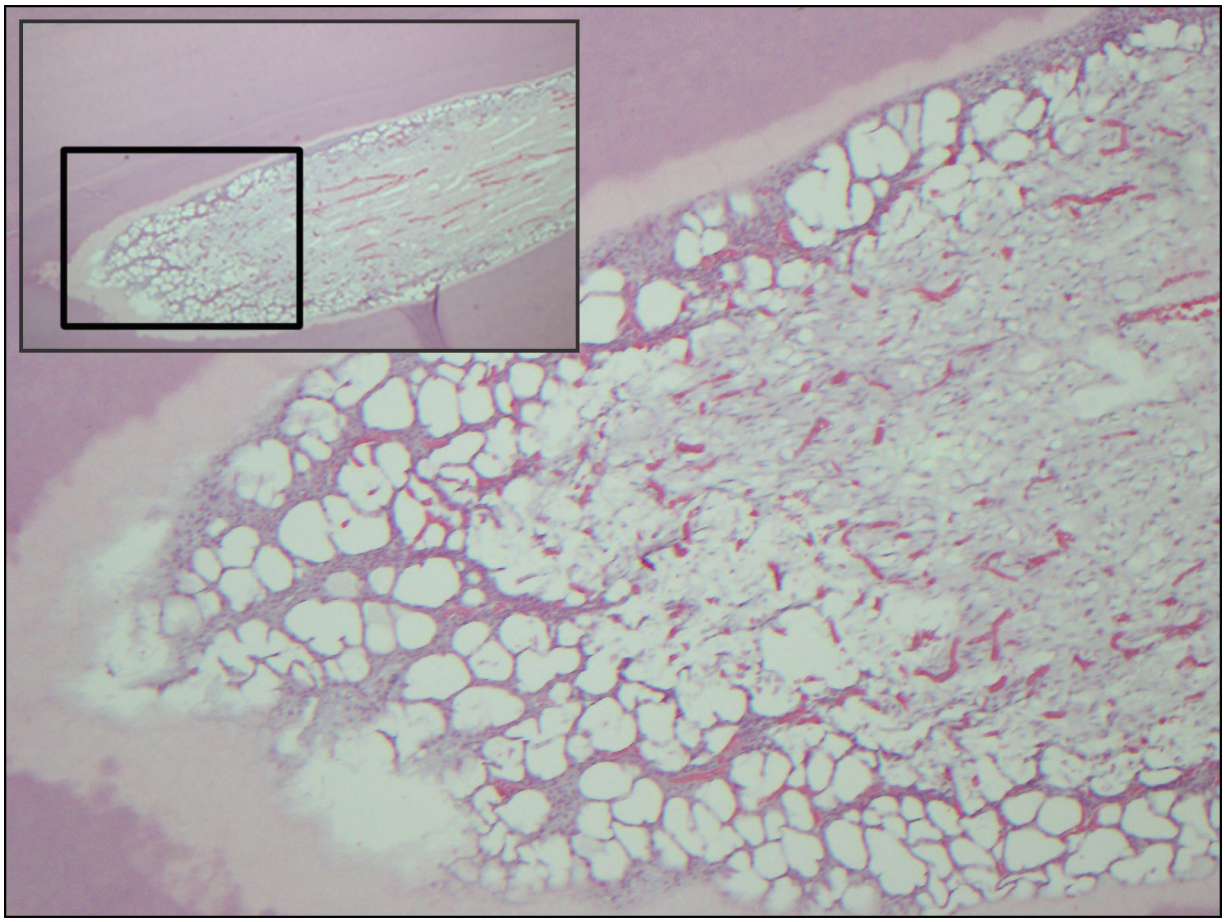


Figure 15 Light micrograph of H&E stained tooth in 7-days group showing a reaction to the medicament along the dentinal wall. Medium power (40X) magnification. **Inset:** lower magnification (10X) of the same field with a box showing the close up view location.

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