Disinfection of Immature Teeth with a Triple Antibiotic Paste

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Abstract

This study assessed the efficacy of a triple antibiotic paste in the disinfection of immature dog teeth with apical periodontitis. The canals were sampled before (S1) and after (S2) irrigation with 1.25% NaOCL and after dressing with a triple antibiotic paste (S3), consisting of metronidazole, ciprofloxacin, and minocycline. At S1, 100% of the samples cultured positive for bacteria with a mean CFU count of 1.7×10^8. At S2, 10% of the samples cultured bacteria-free with a mean CFU count of 1.4×10^4. At S3, 70% of the samples cultured bacteria-free with a mean CFU count of only 26. Reductions in mean CFU counts between S1 and S2 ($p < 0.0001$) as well as between S2 and S3 ($p < 0.0001$) were statistically significant. These results indicate the effectiveness of a triple antibiotic paste in the disinfection of immature teeth with apical periodontitis.

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The immature tooth with apical periodontitis presents numerous challenges that inhibit our ability to provide a predictable long-term treatment outcome. Past efforts have been aimed at eliminating the bacterial challenge and creating an environment conducive to the placement of a root canal filling (1–3). Though these goals have been adequately met, the problem of thin root walls and susceptibility to fracture still remain (4).

Revascularization of necrotic pulp has been considered possible only after traumatic injury to an immature tooth (5, 6). After traumatic injury of an immature tooth a unique set of circumstances exist that favor revascularization. The potential for revascularization appears to directly hinge on this race between bacterial infection of the necrotic (but not infected pulp) and revascularization of the canal space by vital tissue using the ischemic pulp as a matrix. This is evidenced by the significant increase in success rates after soaking the tooth in doxycycline (7) or the application of minocycline before replantation (8).

When an immature root is already infected the potential for revascularization has been thought to be lost. Disinfection with (limited) mechanical instrumentation and irrigation with NaOCL has been proven ineffective (3), therefore we must rely on the placement of a medicament to achieve adequate reduction of intracanal bacteria. The placement of calcium hydroxide, although effective at bacterial reduction (9), fills the space required for revascularization and initiates a hard tissue response by sterile necrosis of the tissue at the apex of the tooth (10).

If our focus were to shift away from the placement of a root filling toward the creation of an environment similar to that of the avulsed immature tooth, revascularization after infection may be possible. For this to occur, disinfection must first be achieved by irrigation and the placement of an intracanal medicament. A mixture of ciprofloxacin, metronidazole, and minocycline has been shown to be very effective in eliminating endodontic pathogens in vitro and in situ (11–13). This mixture has also been demonstrated to be well tolerated by vital pulp tissue (14). After disinfection, the canal should be filled with a resorbable matrix to encourage the in-growth of new tissue. Finally, the coronal access must be sealed to prevent reinfection.

Several reports have recently demonstrated the potential for revascularization after infection if this type of environment is created (15, 16). The aim of this study was to assess the first step in this process; adequate disinfection of immature teeth with an antibiotic paste. We set out to determine the bactericidal efficacy of a triple antibiotic paste consisting of ciprofloxacin, minocycline, and metronidazole in the disinfection of immature dog teeth with apical periodontitis.

Materials and Methods

This research was conducted with the support of The University of North Carolina (UNC) Animal Facility. Before initiation of the study, an application to use live vertebrate animals, including the study protocol, was reviewed and approved by the UNC Institutional Animal Care and Use Committee. Six mongrel dogs approximately 5 months old and weighing approximately 11 kg each were obtained. At this age, the dogs' upper 2nd and 3rd premolars as well as the lower 2nd, 3rd, and 4th premolars are expected to present with incomplete root formation and open apices. Radiographs were taken to confirm incomplete root formation before initiation of the study.

Treatment was carried out during three sessions. The animals were anesthetized during all experimental procedures. Anesthesia was obtained by intravenous adminis-

tration of Pentothal, and Isofluorine maintenance. The animals also received local anesthetic (bupivacaine; 0.5 ml/quadrant) to provide regional nerve block anesthesia.

The aim of the first treatment session was to infect the pulps of five randomly selected premolars per dog, for a total of 30 teeth. The pulp chambers were accessed with a #4 round carbide bur in a high-speed handpiece with irrigation. Plaque was obtained from the cervical area of each dog's mandibular first molar with a small spoon excavator and mixed in a dapping dish with sterile saline. A cotton pellet was then soaked in the mixture for approximately 30 s and then placed into each access cavity. This procedure was repeated for each individual dog. The access was then closed with Cavit (ESPE, Norristown, PA). At 6 wk, each premolar was radiographed to confirm the development of apical periodontitis (see Fig. 1).

At the second treatment session each tooth was isolated with a rubber dam. The tooth and adjacent dam were cleaned with 30% hydrogen peroxide until no bubbling of the peroxide occurred. If difficulty occurred in attaining a bubble-free environment, Oraseal (Ultradent Products Inc., South Jordan, UT) was placed around the neck of the tooth and the process repeated. All surfaces were then coated with tincture iodine and allowed to dry. The temporary restoration was removed with sterile burs in a high-speed handpiece. Sterile saline was then used to flush any debris from the pulp chamber. Sterile cotton pellets were then used to dry the chamber before the placement of 0.02 ml of dental transport fluid (DTF) (Anaerobe Systems, Morgan Hill, CA) into the mesial canal of each premolar with a sterile tuberculin syringe. The canal was then agitated with a #20 sterile stainless steel Hedstom file. After removal of the file, the DTF in the canal was soaked up and transferred to the DTF bottle with sterile xfine paper points (Mynol, Block Drug Corp, Jersey City, NJ) placed to the estimated working length. This constituted the first sample (S1).

Each canal was then irrigated slowly with 10 ml of 1.25% NaOCL. The canals were then flushed with sterile saline and dried with sterile paper points. Each canal was then flushed with 2 ml of 5% sodium thiosulfate to neutralize the NaOCL. The canals were then flushed with sterile saline and dried with sterile paper points. Approximately 0.02 ml of DTF was placed into the mesial canals with a sterile tuberculin syringe. The canal contents were then agitated with a #20 sterile stainless steel Hedstrom file. The DTF was then absorbed with sterile paper points and transferred to the DTF sample bottle. This constituted the second sample (S2).

Each canal was then flushed with 2 ml of sterile saline and dried with sterile paper points. A triple antibiotic paste consisting of cipro-

Figure 1. Radiographic confirmation of apical periodontitis.

floxacin, metronidazole, and minocycline at a concentration of 20 mg of each antibiotic per ml was then inserted into each canal with a sterile Lentulo spiral filler (Caulk, Milford, DE). The access was then cleaned with sterile cotton pellets followed by the placement of IRM (Caulk) for the coronal seal. The antibiotics were left in the canal for a period of 2 wk.

At the third treatment session, the teeth were isolated with a rubber dam as described previously. The coronal seal was removed with sterile high-speed burs followed by flushing of the chamber with sterile saline. Each canal was then flushed with 10 ml of sterile saline. The canals were then dried with sterile paper points. Approximately 0.02 ml of DTF was then placed into the mesial canals with a tuberculin syringe. A #20 sterile stainless steel Hedstrom file was then inserted to agitate the canal contents. After removal of the Hedstrom file, xfine sterile paperpoints were used to absorb the DTF and then placed into the DTF bottle. This constituted the third sample (S3).

Six additional premolars diagnosed with a normal healthy pulp and no radiographic signs of apical periodontitis were accessed and sampled identically to the test group. These served as controls to verify the effectiveness and asepsis of the sampling technique.

The laboratory procedures were performed by the University of North Carolina Dental Microbiology Laboratory (a CLIA certified laboratory). All samples were immediately transferred to the laboratory for processing. The sample containing vials were agitated with a vortex, at a power setting of 4 for 30 s, before aliquot disbursement. The samples were plated on various media including, Sheep Blood Agar (SBA), Chocolate agar (CHOC), and Anaerobic Sheep Blood Agar (ANASBA) using a Model D Spiral Plater (Spiral Systems Inc., Cincinnati, OH) to deliver 49.2 μl of sample while accomplishing 2.3 log dilutions on the various agar plates. The SBA and CHOC plates were incubated for up to 3 days at 37°C in an ambient or 5% CO_2 atmosphere, respectively. The ANASBA were incubated for 5 to 7 days in a Coy anaerobic chamber in an atmosphere of 5% CO_2, 85% N_2, and 10% H_2. Growth was measured quantitatively by the direct counting of colonies and calculations specific to the spiral plate grid system. The spiral plater deposits a known volume of sample as it traces across specific areas of the plate, or grids. Once the colonies are counted in each grid, a dilution factor for each grid (specified by the manufacturer) can be used to determine the total bacterial count in the original sample.

Porphyromonas gingivalis (strain A7436), a bacterium with known susceptibility to ciprofloxacin, metronidazole, and minocycline, was added to a phosphate buffered solution to create a concentration of 1 McFarland standard. From this concentration a 1:2 dilution was performed. There were 100 ul of bacteria then spread-plated as a lawn to the surface of 100 mm^2 ANSBA plates. After creation of the bacterial lawns, control plates were developed to determine characteristic zones of inhibition for the antibiotics used in this study. Serial dilutions of known concentrations of each antibiotic, alone and in combination, were spot plated on the spread plates of *P. gingivalis* to determine the endpoint of inhibition of bacterial growth. Known concentrations of antibiotic were then spiral-plated on spread plates to determine characteristic zones of inhibition for each antibiotic concentration. The bacterial lawns were allowed to develop by incubation at 37°C in the anaerobic chamber for 72 h. The zones of inhibition were then recorded.

To test for residual antibiotic activity that may interfere with the sampling results, a model D spiral plater was used to deliver a second 49.2 μl of each sample taken at (S3) to *P. gingivalis* spread plates. Residual antibiotic activity was then determined by the presence or absence of zones of inhibition on the developed lawns of bacteria. If present, the location and size of the zone of inhibition was correlated with the control plates, to determine the approximate concentration of residual antibiotic. As a further control, 49.2 ul of sterile DTF was also

delivered to bacterial lawns to determine if there was any inhibition of bacterial growth caused by the sampling fluid alone.

Differences were assessed between three time points (S1-S2, S1-S3, and S2-S3). A log transformation of each CFU count was performed to normalize the data before statistical evaluation because of the high range of bacterial counts. A paired t test was then used to determine the difference in mean bacterial counts between samples. A McNemar test was then performed to compare the proportion of cases that cultured bacteria between samples.

Results

Thirty test premolars and six control teeth were included in this study. No bacterial growth was observed from any samples taken from the teeth serving as controls. The CFU values for each test premolar are listed in Table 1.

Bacteria were found initially in all 30 test samples. The mean bacterial count at the initial sample (S1) was 1.33×10^8 with a range of 7.90×10^3 to too many to count ($>10^9$). The median number was 1.80×10^5. The mean $\log_{10}$ value was 5.49 ± 1.47 and the median $\log_{10}$ value was 5.26.

At (S2) bacteria were found in 27 of the 30 (90%) samples with a mean count of 1.89×10^4 and a range of 0 to 2.00×10^5. The median bacterial count was 1.10×10^3. The mean $\log_{10}$ value was 2.96 ± 1.38 and the median $\log_{10}$ value was 3.10.

At (S3) bacteria were found in 9 of 30 samples (30%) with a mean count of 26 CFU and a range of 0 to 243. The median bacterial count was 0. The mean $\log_{10}$ value was 0.54 ± 0.86 and the median $\log_{10}$ value was 0.

The paired t test showed a highly significant difference in mean bacterial count between S1 and S2 ($p < 0.0001$) and between S1 and S3 ($p < 0.0001$). A significant difference in mean bacterial count was also found between S2 and S3 ($p < 0.0001$). The McNemar test showed no significant difference ($p = 0.0756$) between the proportion of teeth that sampled positive for bacteria in S1 (100%) compared to S2 (90%). However, there was a highly significant difference in the proportion of teeth sampling positive for bacteria between S1 (100%) and S3 (30%) ($p < 0.0001$) as well S2 (90%) and S3 (30%) ($p < 0.0001$).

The sterile sampling fluid resulted in no inhibition of bacterial growth. Of the 30 (S3) samples tested for residual antibiotic activity, four were positive. All four samples were found to have a residual antibiotic concentration of approximately 0.002 mg/ml. Two of the four samples showed no growth of bacteria at (S3). One of the samples found to contain residual antibiotic displayed the highest number of bacterial CFU (243) of all 30 samples taken at (S3).

Discussion

We consider that predictable revascularization of immature teeth with apical periodontitis would be possible if three challenges can be met: (a) Disinfection of the canal; (b) placement of a matrix in the canal for tissue in-growth; and (c) a bacterial tight seal of the access opening. This study is designed to test the first of these challenges.

In mature teeth, disinfection is carried out with a combination of instrumentation, irrigation, and the placement of an intracanal medicament. Though complete disinfection is not achieved through mechanical instrumentation alone (17), it is a very important step in the disinfection process, resulting in an approximate 20 to 40% reduction in

TABLE 1. CFU count for each sample and residual antibiotic concentration at S3

Sample #	S1	S2	S3	S3 Residual Antibiotic (RA)
1	1.7×10^5	2.2×10^2	0	0
2	9.3×10^3	1.1×10^4	61	0
3	7.9×10^3	3.7×10^3	0	0
4	8.1×10^4	7.1×10^2	0	0
5	1.0×10^9	1.0×10^4	0	0
6	1.0×10^9	1.6×10^5	0	0
7	1.9×10^5	4.5×10^3	0	0
8	1.9×10^4	7.3×10^2	0	0
9	1.0×10^9	2.0×10^5	121	0
10	2.3×10^5	4.0×10^1	162	0
11	3.2×10^4	2.8×10^3	20	0
12	4.7×10^4	0	0	0.002 mg/ml
13	1.0×10^9	1.3×10^5	0	0
14	2.2×10^5	2.2×10^3	0	0
15	2.0×10^4	2.2×10^4	0	0
16	1.9×10^5	1.1×10^3	40	0
17	1.8×10^5	1.5×10^3	101	0
18	1.9×10^5	1.1×10^3	0	0
19	2.6×10^5	6.0×10^2	0	0
20	2.4×10^5	2.0×10^1	0	0
21	4.5×10^5	3.7×10^3	20	0
22	6.0×10^5	5.6×10^3	0	0
23	1.0×10^5	1.1×10^3	0	0
24	9.0×10^4	2.1×10^3	0	0
25	8.9×10^4	1.0×10^3	0	0
26	2.2×10^5	2.4×10^2	0	0
27	2.3×10^5	0	0	0
28	4.5×10^4	0	0	0.002 mg/ml
29	2.6×10^4	6.0×10^1	243	0.002 mg/ml
30	3.8×10^4	4.0×10^1	40	0.002 mg/ml

S1: post infection sample.

S2: post irrigation with 1.25% NaOCL.

S3: post antibiotic dressing sample.

S3 (RA): sampling fluid showing bacterial growth in the laboratory.

bacterial counts. In immature teeth, mechanical instrumentation is either impossible or held to a minimum, depending upon the degree of root development

The infection of the root canal system is considered to be a polymicrobial infection, consisting of both aerobic and anerobic bacteria (18, 19). Because of the complexity of the root canal infection it is unlikely that any single antibiotic could result in effective sterilization of the canal. More likely a combination would be needed to address the diverse flora encountered. A combination of antibiotics would also decrease the likelihood of the development of resistant bacterial strains. The combination that appears to be most promising consists of metronidazole, ciprofloxacin, and minocycline. Sato et al. (12) investigated this drug combination in vitro and found it to be very effective in the sterilization of carious lesions, necrotic pulps, and infected root dentin of deciduous teeth. Hoshino et al. (11) performed an in vitro study testing the antibacterial efficacy of these drugs alone and in combination against the bacteria of infected dentin, infected pulps, and periapical lesions. Alone, none of the drugs resulted in complete elimination of bacteria. However, in combination, these drugs were able to consistently sterilize all samples. An in situ study by Sato et al. (13) found that this drug combination was very effective in killing bacteria in the deep layers of root canal dentin. This same antibiotic combination was used in the case report by Iwaya et al. (15) as well as in a recent clinical report by Banchs and Trope (16) that demonstrated disinfection and revascularization of an immature tooth with apical periodontitis.

Metronidazole is a nitroimidazole compound that exhibits a broad spectrum of activity against protozoa and anaerobic bacteria. Known for its strong antibacterial activity against anaerobic cocci, as well as gram-negative and gram-positive bacilli it has been used both systemically and topically in the treatment of periodontal disease. Metronidazole readily permeates bacterial cell membranes. It then binds to the DNA, disrupting its helical structure, and leads to very rapid cell death. In a study by Roche and Yoshimori (20) the in vitro activity of metronidazole against clinical isolates from odontogenic abscesses was investigated. It was concluded that metronidazole has excellent activity against anaerobes isolated from odontogenic abscesses but has no activity against aerobes. It was further suggested that no single antimicrobial agent can be used appropriately for the treatment of mixed infections. Thus a combination of medicaments must be considered. Knowing the concern over the potential for the development of resistant flora when using certain topical antibiotics, Slots (21) advocated the use of metonidazole because of the unlikelyhood of inducing bacterial resistance.

Tetracyclines, which include doxycycline and minocycline, are a group of bacteriostatic antimicrobials. They have a broad spectrum of activity against both gram-positive and gram-negative microorganisms. Tetracyclines are effective against most spirochaetes, and many anaerobic and facultative bacteria. The tetracyclines gain access to bacterial cells by passive diffusion through the outer membrane followed by active transport through the inner membrane. They then act by inhibiting protein synthesis on the surfaces of ribosomes. Minocycline is a semisynthetic derivative of tetracycline with a similar spectrum of activity. It is available in many topical forms ranging from gel mixtures to sustained release microspheres, and has also been used extensively in periodontal therapy.

Ciprofloxacin, a synthetic floroquinolone, has a bactericidal mode of action. It acts through the inhibition of DNA gyrase, resulting in degradation of the DNA by exonucleases. This bactericidal activity persists not only during the multiplication phase, but also during the resting phase of the bacterium. Ciprofloxacin has very potent activity against gram-negative pathogens but very limited activity against gram-positive bacteria. Most anaerobic bacteria are resistant to ciprofloxacin, therefore, it is often combined with metronidazole in the treatment of mixed

infections. Side effects of ciprofloxacin have been reported, however, Black et al. (22) found the drug to be clinically safe when applied in low doses. When applied as an intra-canal medicament in low doses, adverse systemic side effects should be minimized.

Typically the sensitivity of bacteria to antibiotics is determined as the minimum inhibitory concentration (MIC). Because of the diversity and complexity of the endodontic flora it is impossible to determine the MIC for all of the bacterial species. Hoshino et al. (11) suggested that the MIC method may not be suitable for determining whether combinations of drugs can kill all the bacteria in such a flora. Hoshino et al. (11) determined that a combination of ciprofloxacin, metronidazole, and minocycline at a concentration of 25 μg each per milliliter of paste was able to sterilize infected root dentin in vitro. Sato et al. (13) found that this combination at 50 μg of each antibiotic per milliliter was sufficient to sterilize infected root dentin in situ. However, it is questioned whether this concentration would be adequate in vivo. The in vivo environment of a young tooth presents many challenges to the disinfection of the root canal. The open apex allows the potential for periapically derived fluid to have a washing-out effect on the antibiotic paste.

It was also demonstrated by Portenier et al. (23) that dentin itself can have an inhibitory effect on the bactericidal activity of intra-canal medicaments. Thus a decision was made to use metronidazole, ciprofloxacin, and minocycline in a thick paste at a concentration of 20 mg of each drug per milliliter to counter these potential effects.

Six teeth without caries or any signs of apical pathology were included as controls. These teeth were vital upon access and sampled at each stage (S1–S3). All samples were negative for bacteria at each stage. This control group supports the effectiveness of the sampling technique.

All treatment teeth sampled at S1 were positive for bacteria. The mean counts were similar but on average slightly higher than previous studies performed on teeth with complete root development. This may be a result of the ease of sampling in a tooth with a very large canal space or it may be that this environment is more conducive to higher bacterial growth. The mean CFU in the initial sample was 1.33×10^8 with a range of 7.9×10^3 to too many to count. The mean $\log_{10}$ value was 5.49. Orstavik et al. (24) reported a mean $\log_{10}$ value of 4.8. Dalton et al. (25) had a mean $\log_{10}$ value of 4.6 with a mean bacterial CFU count of 1.4×10^6. Shuping et al. (26) found a mean CFU count of 5.58×10^6 with a mean $\log_{10}$ value of 5.51.

The S2 sample showed a moderate reduction in mean bacterial counts, however, only 10% (3 of 30) of the samples were bacteria-free (Table 1). The mean bacterial count was 1.89×10^4 with a range of 0 to 2.00×10^5. The median bacterial count was 1.10×10^3. With NaOCL, the volume of irrigant is the key to its effectiveness. In large canals with open apices irrigation is very simple; however, 10 ml of NaOCL was still unable to produce an environment that was consistently free of bacteria.

Of the 30 treatment teeth sampled at S3, 70% (21 of 30) cultured negative for bacteria (Table 1). The 2 wk application of this antibiotic paste resulted in a highly significant reduction in bacterial counts. There was a mean count of only 26 CFU and a range of 0 to 243. The median bacterial count was 0. The mean $\log_{10}$ value was 0.54. There was a significant reduction in mean $\log_{10}$ values with each progression of sampling. The bacterial counts found at S3 are extremely low and as such their clinical significance is unknown. However, the fact that 70% of the teeth cultured negative after one application of this paste without any mechanical instrumentation being performed is in and of itself a very promising finding.

The use of medicaments or irrigants with antibacterial activity before anaerobic sampling has the potential to result in false negative cultures. When employing NaOCL irrigation, it is necessary to rinse with sodium thiosulfate to neutralize its effect before bacterial sampling.

After medication of the canal with calcium hydroxide, citric acid is often used as a rinse to counteract any lingering effects of the calcium hydroxide. In this study, no chemicals were used to neutralize the antibiotic paste before bacterial sampling. It was felt that the limited activity of the antibiotics, as well as the washing-out effect by the periapically derived fluid and the extensive irrigation with sterile saline would together make chemical neutralization unnecessary. However, tests were performed to determine if there was any residual antibiotic activity that could potentially interfere with the results obtained at (S3).

A model D spiral plater was used to deliver a second 49.2 μl of each sample taken at (S3) to lawns of bacteria with known susceptibility to each antibiotic alone and in combination. Residual antibiotic activity was then determined by the presence or absence of zones of inhibition on the lawns of bacteria. If present, the location and size of the zone of inhibition was correlated with control plates, to determine the approximate concentration of residual antibiotic. Of the 30 samples taken at (S3), four were positive for residual antibiotic. All four samples were found to have a residual antibiotic concentration of approximately 0.002 mg/ml. Two of the four samples with residual antibiotic showed no growth of bacteria at (S3). One of the samples found to contain residual antibiotic displayed the highest number of bacterial counts (243) of all 30 samples taken at (S3). From this information we consider the residual antibiotic activity to be insignificant. It appears that this concentration is well below the threshold necessary to result in a false negative culture. In fact, the highest bacterial count at (S3) was obtained from a sample with known residual activity.

In conclusion, a statistically significant reduction in bacteria, cultured from infected immature dog teeth, was found following the irrigation and antibiotic paste protocol used in this study. Of the 30 samples that cultured bacteria before treatment, 90% remained positive following irrigation with 10 ml of 1.25% NaOCL. However, this dropped to 30% following the application of the triple antibiotic paste for 2 wk. Though the irrigation protocol alone resulted in only 10% of the samples culturing bacteria-free, the mean bacterial counts were significantly reduced from 1.33×10^8 to 1.89×10^4. The bacterial counts were further reduced to a mean of only 26 following the application of the triple antibiotic paste for 2 wk. Based on the residual antibiotic controls, it appears that there was no residual antibiotic activity that interfered with the culturing process, thus the results obtained in this study should be considered valid. The results of this experiment demonstrate the effectiveness this triple antibiotic paste combination in the disinfection of immature teeth with apical periodontitis.

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