LETTER TO THE EDITOR

ANTIBACTERIAL EFFICACY AND DRUG-INDUCED TOOTH DISCOLOURATION OF ANTIBIOTIC COMBINATIONS FOR ENDODONTIC REGENERATIVE PROCEDURES

N. MANDRAS[1], J. ROANA[1], V. ALLIZOND[1], D. PASQUALINI[2], P. CROSASSO[3], M. BURLANDO[3], G. BANCHE[1], T. DENISOVA[2], E. BERUTTI[2] and A.M. CUFFINI[1]

[1]*Department of Public Health Sciences and Paediatrics, University of Turin, Turin, Italy;*
[2]*Department of Surgical Sciences, Dental School, Endodontics, University of Turin, Turin, Italy;*
[3]*Department of Pharmacy, AO Città della Salute e della Scienza of Turin, Turin, Italy*

Received January 18, 2013 – Accepted April, 15, 2013

Elimination of microbial contamination from the root canal system is a precondition for successful root canal treatment. Teeth with immature root development, necrotic pulps and apical periodontitis present multiple challenges for successful treatment. Disinfection is achieved by irrigation followed by the placement of an intracanal medicament. A mixture of ciprofloxacin, metronidazole and minocycline (3-MIX S) has been shown to be very effective in eliminating endodontic pathogens *in vitro* and *in vivo*. Among the components of the mixture, minocycline can induce tooth discolouration after long-term oral use. Therefore, the elimination of minocycline from the above-mentioned combination has been suggested to prevent the occasion of this undesirable effect. The aim of this study was to investigate the potential antimicrobial efficacy of alternative antibiotic combinations [3-MIX C (clarithromycin); 3-MIX F (fosfomycin)] against bacteria from infected root canals. An additional objective was to evaluate their discolouration potential as possible alternatives to minocycline-based intracanal medicaments. Our *in vitro* results clearly demonstrated that 3-MIX C and 3-MIX F had a greater antimicrobial activity than 3-MIX S, underlying that clarithromycin still had a higher capacity to kill endodontic pathogens *in vitro* compared to fosfomycin. Both 3-MIX C and 3-MIX F were able to avoid the permanent staining effect of the crown.

Successful endodontic treatment requires the removal of all vital and necrotic pulp tissue, microorganisms and their toxins. Bacteria penetrate more deeply into the tubules of infected teeth and in this location they may be protected from therapeutic antimicrobials in the root canal (1). The young permanent tooth with necrotic pulp tissue requires a complex treatment (2, 3). For years, apexification was the standard treatment. The revascularization/regeneration of non-vital immature permanent teeth might be another treatment option for restoring root development and apical closure (4). Regeneration protocols include the topical use of effective antibiotics with a broad depth of coverage against endodontic pathogens (5). Previous studies demonstrated the efficacy of a triple antibiotic paste which consists of ciprofloxacin, metronidazole and minocycline for the sterilization of infected root dentine (6). However, minocycline can be responsible for the development of an irreversible dentine discolouration with considerable esthetic concerns after long-term oral use; crown

Key words: endodontic treatment, primary teeth, minocycline, triple antibiotic paste

Mailing address: Dr Valeria Allizond,
Department of Public Health Science and Paediatrics,
Via Santena 9,
10126 Turin, Italy
Tel.: +39 011 670 5627
Fax +39 011 670 5648
e-mail: valeria.allizond@unito.it

0394-6320 (2013)

DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

discolouration may appear within 24 hours after application of minocycline (4), or even only one hour after removal of the smear layer and application of triple antibiotic paste with minocycline (7). To prevent this undesirable side effect, the sealing of dentinal tubules of the pulp chamber by a dentine bonding agent has been proposed (8), even if this procedure reduces the overall color change but does not prevent it (4). Therefore, some authors suggested the elimination of minocycline from the original proposed triple antibiotic association (Bimix) in order to eliminate this undesirable side effect (9).

Thus, the primary objective of this study was to investigate the antimicrobial efficacy of alternative antibiotic combinations containing clarithromycin (3-MIX C) and fosfomycin (3-MIX F) against bacteria from infected root canals. Clarithromycin was used because the macrolide has been suggested for non-surgical periodontal therapy in the treatment of chronic periodontitis, and for its therapeutic benefits such as favorable tissue distribution, especially in fibroblasts (10). Fosfomycin was used in order to compare its antimicrobial activity with the literature data (11). An additional objective was to evaluate the discolouration potential of 3-MIX C and 3-MIX F as possible alternatives to minocycline-based intracanal medicaments.

MATERIALS AND METHODS

Patients

In the present study 29 healthy subjects (19 females and 10 males, 18-78 years of age) diagnosed with pulp necrosis with or without apical periodontitis (acute or chronic) were recruited at the Department of Surgical Sciences, Dental School, University of Turin. All subjects were informed on an individual basis about the purpose of the study and gave their written consent. The patients' medical and dental status data were collected. For each patient, pulpal and periradicular status were assessed through vitality thermal and electric pulp tests (Diagnostic Unit, Sybron, Orange, CA, USA), palpation and percussion. Periapical X-ray examination was performed (Planmeca Intra - Helsinki, Finland) using Rinn XCP devices (Rinn Corp, Elgin Ill). After local anaesthesia with 2% mepivacaine with adrenaline 1:100.000 and isolation of the tooth with a rubber dam, the access cavity was made.

Samples

Bacterial samples used in this study were collected from necrotic non-carious permanent teeth and handled under strict anaerobic conditions in a special chamber with controlled atmosphere and temperature, taking into account those precautions necessary for avoiding contamination. The samples were taken with sterile paper cones, maintained for about 20 seconds in the necrotic root canal, before irrigation with 5% NaOCl. The paper cones were placed without introducing air in an anaerobic sterile transport tube containing 2 ml of an anaerobic transport media (Port-A-Cul™, Becton Dickinson, Italy) (12). The samples were sent to the Department of Public Health Sciences and Paediatrics (Microbiology Laboratory), University of Turin, and analyzed within one hour after the sample was taken.

Antibiotics

Metronidazole (VAGILEN®, Alfa Wassermann, Italy), ciprofloxacin (IBAXICIN®, IBI, Istituto Biochimico Italiano Giovanni Lorenzini, Italy), minocycline (MINOCIN®, Wyeth Lederle, Italy) and clarithromycin (MACLADIN®, Menarini Diagnostics, Italy) were used. Fosfomycin was employed as fosfomycin salified with trometamol (fosfomycin tromethamine; MONURIL®, Zambon Group, Italy).

Preparation of 3-MIXs for microbiological analysis

The enteric coating of metronidazole, ciprofloxacin, minocycline and clarithromycin was removed. The tablets were pulverized using a mortar and pestle. The powdered antibiotics (fosfomycin included) were stored and sealed separately in airtight containers not exposed to moisture or light.

Clarithromycin, fosfomycin, minocycline and ciprofloxacin concentrations used in 3-MIXs were obtained from minimal inhibition concentration (MIC) value on *Enterococcus faecalis* ATCC 29212 determined by broth microdiluition method, while the metronidazole concentration used resulted from MIC value recommended by the CLSI in document M100-S21 (13) for *Enterococcus spp. Enterococcus faecalis* was chosen as the test species because it is present in the infected root canal and capable of invading the dentine tubules.

Powdered antibiotics were added to thyoglycollate medium enriched with vitamin K1 and hemin agar (TGA, Becton Dickinson) plates to obtain: a mixture of ciprofloxacin (2 μg/ml), metronidazole (8 μg/ml) and minocycline (4 μg/ml): 3-MIX S; a mixture of ciprofloxacin (2 μg/ml), metronidazole (8 μg/ml) and fosfomycin (64 μg/ml): 3-MIX F; a mixture of ciprofloxacin (2 μg/ml), metronidazole (8 μg/ml) and clarithromycin (2 μg/ml): 3-MIX C; a mixture of ciprofloxacin (2 μg/ml) and metronidazole (8 μg/ml): 2- MIX.

Microbiological analysis. All samples were vortexed

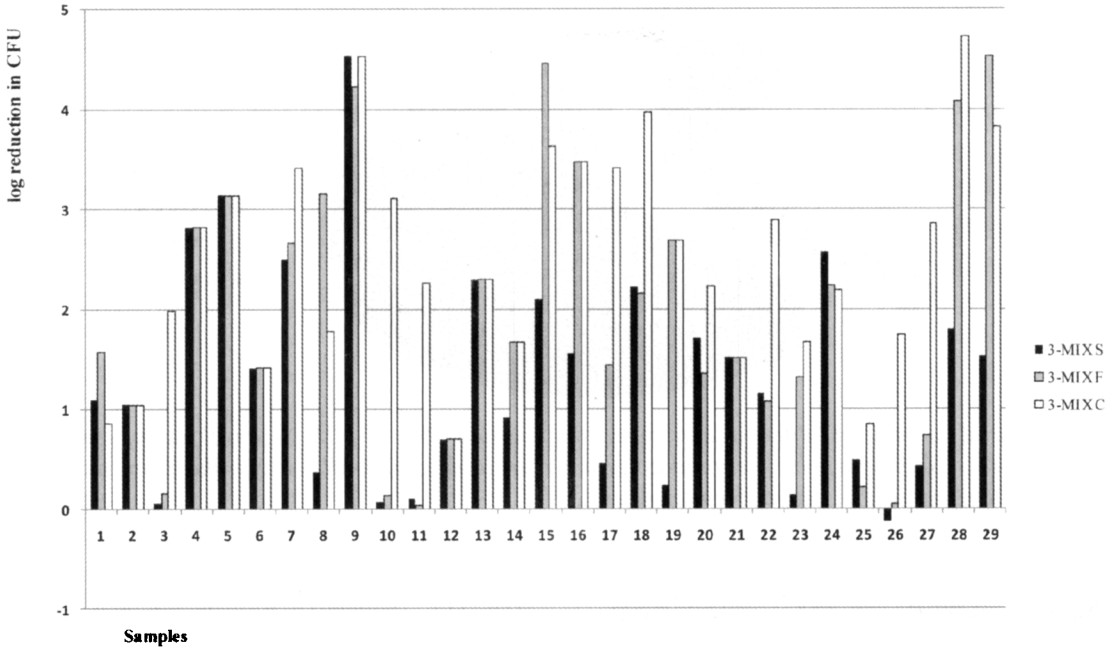

Fig. 1. *Comparison between Log reduction in CFU of 3-MIX S, 3-MIX F and 3-MIX C [log_{10}(CFU control)/(CFU 3-MIX)]. 3-MIX S(ciprofloxacin, metronidazole and minocycline); 3-MIX F (ciprofloxacin, metronidazole and fosfomycin); 3-MIX C(ciprofloxacin, metronidazole and clarithromycin).*

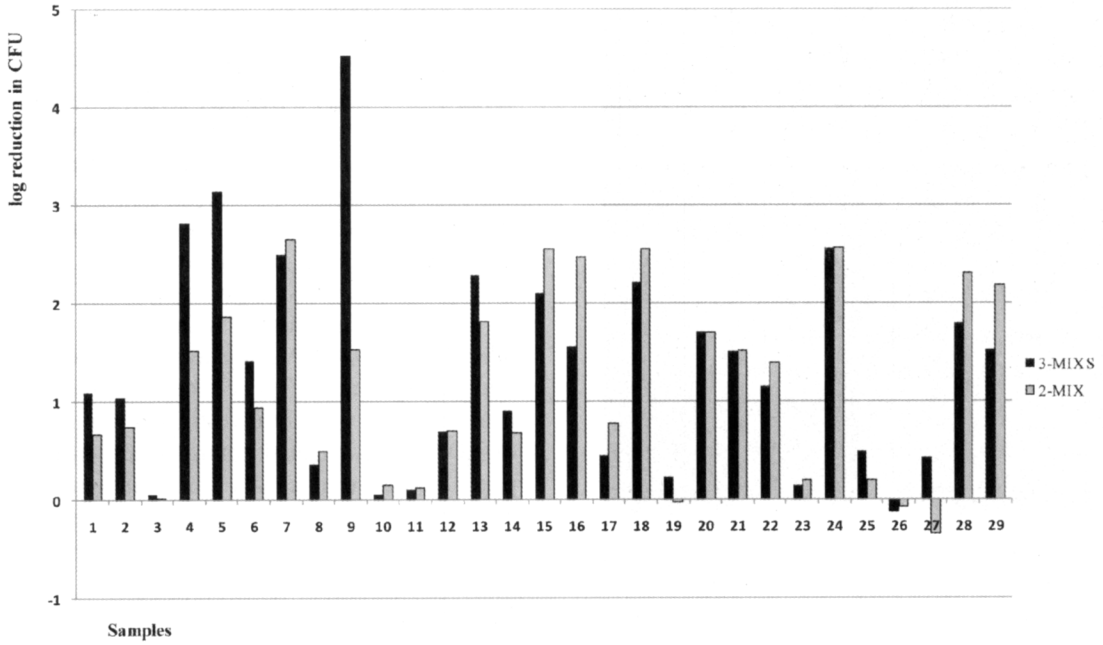

Fig. 2. *Comparison between Log reduction in CFU of 3-MIX S and 2-MIX [log_{10}(CFU control)/(CFU 3-MIX)]. 3-MIX S(ciprofloxacin, metronidazole and minocycline);2-MIX(ciprofloxacin and metronidazole).*

for 30 seconds, diluted 1:10 in sterile distilled water and spread on TGA control plates for initial colony forming unit (CFU) determination and on plates containing the mixed drugs to quantify the number of anaerobic bacterial strains grown in presence or absence of the different drug combinations: 3-MIX S, 3-MIX F, 3-MIX C and 2-MIX. All plates were incubated at 37°C for 3-4 days under anaerobic conditions in an anaerobic system (Anaerocult IS; Merck, Bracco, Italy). All cultures were kept for at least 2 weeks but examined for growth every 3 days. The microbial counts were reported as CFUs/ml (14). Survival fractions were calculated from each sample taking into

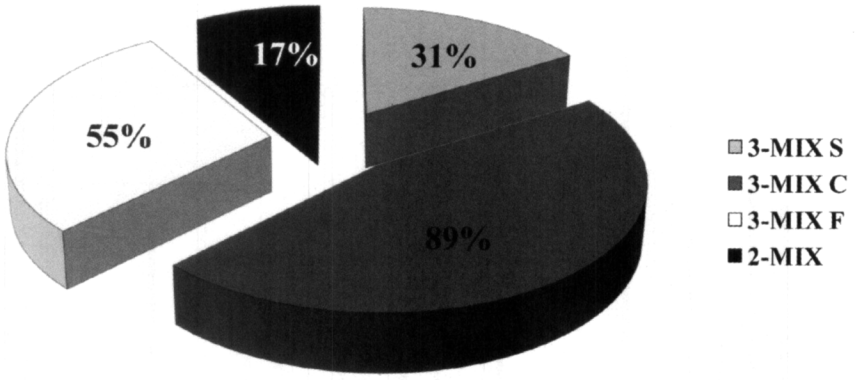

Fig. 3. *Antibacterial activity of different antibiotic combinations (%). 3-MIX S(ciprofloxacin, metronidazole and minocycline); 3-MIX C(ciprofloxacin, metronidazole and clarithromycin);3-MIX F(ciprofloxacin, metronidazole and fosfomycin); 2-MIX(ciprofloxacin and metronidazole).*

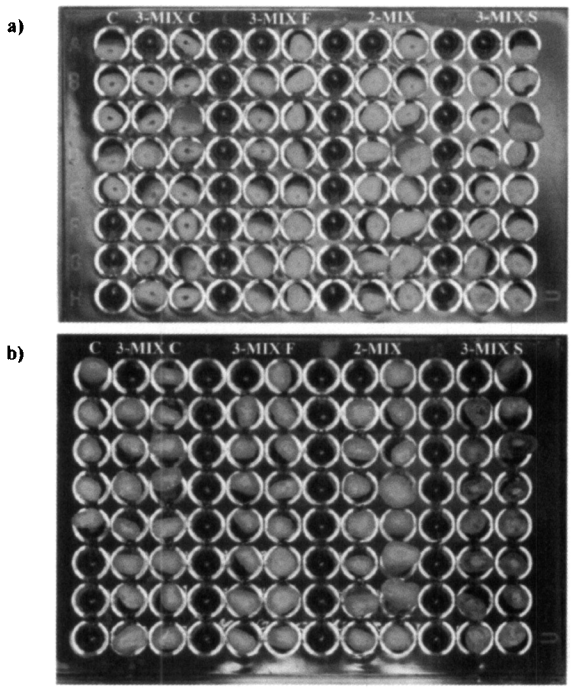

Fig. 4. *Experimental set up for the discolouration test before (**a**) and after 3 weeks ofincubation (**b**). C, control group; 3-MIX C(ciprofloxacin, metronidazole and clarithromycin); 3-MIX F(ciprofloxacin, metronidazole andfosfomycin); 2-MIX(ciprofloxacin andmetronidazole); 3-MIX S (ciprofloxacin, metronidazole and minocycline).*

account its initial bacterial load.

Preparation of 3-MIXs for discolouration test. The same amount of each powdered drug (1:1:1) was mixed together. The mixed drugs were then combined with macrogol and propylene glycol (Vidhyasom Co., Ltd, Bangkok, Thailand) to form an ointment. Unused 3-MIXs were discarded.

Discolouration test. Sixty-five root canals of extracted human single-root permanent teeth with a fully formed apex that had not undergone prior endodontic treatment were used. Teeth were sectioned at the cement-enamel junction and the crown was eliminated. After debriding the root surface, specimens were immersed in a 5% solution of NaOCl (Niclor 5; OGNA, Muggiò, Italy) for 1 h and then stored in saline solution until preparation. Each root canal was preflared using PathFile (Dentsply Maillefer, Ballaigues, Switzerland) and then shaped using ProTaper S1-S2-F1-F2-F3-F4-F5 (Dentsply Maillefer) at the working length. Irrigation was performed with a 30-gauge needle syringe using 33 ml of 5% NaOCl at 37°C and alternating with 10 ml of 10% EDTA (Tubuliclean, OGNA); the total irrigation time was 10 min per specimen. The effect of smear layer on root discolouration was not investigated through the adoption of EDTA non-irrigated control teeth. After drying with paper points, root canals were randomly divided into 5 assigned groups and brought into contact with the different antibiotic associations for 3 weeks.

Statistical analysis

A statistical analysis was performed on the collected data. Kolmogorov-Smirnov test for normality was used to analyze data, which were normally distributed. To compare the CFU counts in all groups a one-way analysis of variance model (ANOVA) was used. Correction for multiple comparisons with Bonferroni's test was applied to the significance levels presented. The level of statistical significance was set at $P<0.05$. All statistical analyses were performed using the SPSS for Windows 17.0 software package (SPSS, Inc. Chicago, IL, USA).

RESULTS

Data relative to the addition of different antibiotic combinations to 29 necrotic pulp samples showed that the initial infectious burden varied widely among single samples with a mean value of $5.48x10^4$ CFU/ml (range $5.3x10^5$ to 5; median $1.28x10^3$). This variation was probably caused by differences in the internal anatomy and geometry of the individual root canal systems and the duration of the infections. The addition of 3-MIX S, 3-MIX F, 3-MIX C, 2-MIX to TGA agar plates significantly decreased the bacterial detection: the mean microbial load was reduced to $7.45x10^2$ (range $8.39x10^3$ to 1; $1.3x10^2$ median) in the presence of 3-MIX S, to $3.27x10^2$ (range $2.24x10^3$ to 1; 5 median) with 3-MIX F, to $1.06x10^2$ (range $2.35x10^3$ to 1; 1 median) in the presence of 3-MIX C and to $5.33x10^2$ (range $2.56x10^3$ to 1; $2.4x10^2$ median) with 2-MIX.

The percentage of bacterial reduction in 3-MIX C and 3-MIX F (99.49% and 97.31% median, respectively) was greater than that achieved in 3-MIX S and 2-MIX (92.95% and 88.46% median, respectively) according to the bacterial load, the difference among groups being statistically significant ($P<0.05$).

Even the values for individual log reductions reveal a pronounced bactericidal activity of 3-MIX C and 3-MIX F compared to 3-MIX S and 2-MIX (Fig. 1-2). The bactericidal activity of each mix is best described by analyzing the action on the entire population of subjects (Fig. 3): this was significantly greater in 3-MIX C (89%) and 3-MIX F (55%) compared with that observed in 3-MIX S (31%) and 2-MIX (17%), showing a microbicidal activity of 3-MIX C approximately 3-4 times higher than that observed in 3-MIX S and in 2-MIX, respectively.

The experimental set-up for the discolouration test before (a) and after 3 weeks of incubation (b) is reported in Fig. 4: from the time of drug placement, a dark greenish brown shade appeared in only 3-MIX S samples containing minocycline. In the other 3 groups and in the control group, the colour of the roots remained unchanged.

DISCUSSION

Regenerative endodontic procedures can lead to the development of a nearly fully matured root, with normal thickness and length (15, 16). Traditional apexification procedures with calcium hydroxide to promote the formation of a calcified barrier or, alternatively, the creation of an artificial apical barrier with mineral trioxide aggregate, are predictable and successful (17). However, they do not allow complete development of the root (15) and the long-term calcium hydroxide therapy might negatively affect the mechanical properties of dentine (18). Regenerative procedures require high level disinfection of the root canal system spaces. Antimicrobial efficacy of irrigant solutions may be decreased by the presence on root canal walls of the smear layer produced during root canal instrumentation, composed of dentine, odontoblastic processes, nonspecific inorganic contaminants, and microorganisms (19) that may inhibit the adherence of implanted dental pulp stem cells, potentially causing the failure of regenerative endodontic treatment (20). The removal of the smear layer also allows the direct contact of 3-MIX to the root walls, which significantly influences disinfection and risk of discolouration (7). However, regenerative procedures still lack standardization of treatment protocols, with intracanal medicaments, and irrigants (2, 15). At the moment to remove the bacterial constituents harbored in the root canals and deepest dentine layers, a 3-mix paste of metronidazole, ciprofloxacin, and minocycline is largely used and its bactericidal efficacy and penetration through dentine from prepared root canals have been clearly demonstrated *in vitro* (6, 11). However, minocycline has been reported to cause tooth discolouration after long-term oral use by binding to calcium ions via chelation to form an insoluble complex (4, 21). The literature reports many attempts to solve this problem but without success (4, 11, 22). In our study, the bactericidal efficacy and the ability to prevent crown discolouration by 3-MIX C or 3-MIX F were compared. The antimicrobial activity of both 3-MIX F and 3-MIX C was more effective than that of 3-MIX S and 2-MIX: 3-MIX F showed a good efficacy confirming what has been reported by other authors (11); 3-MIX C induced a bacterial load significantly decreased compared to 3-MIX F. Furthermore, the bactericidal activity of each mix is best described by analyzing the action on the entire

population of subjects where 3-MIX C exhibited a microbicidal activity approximately 3-4 times superior to that observed in 3-MIX S and in 2-MIX and 2 times to that detected with 3-MIX F, probably related to clarithromycin's better antimicrobial properties. The most interesting result is that a severe discolouration only occurred in the 3-MIX S samples: discolouration by the tetracycline family is thought to be a photoinitiated reaction (4); in our experimental conditions, the tooth samples became dark after minocycline treatment despite a lack of sunlight. No significant differences in terms of the surface darkness of the samples were observed among all the other groups, 2-MIX included, confirming that minocycline is the cause of coronal discolouration. To overcome the issue of staining, both 3-MIX F or 3-MIX C would be preferred as alternative.

The following conclusions can be drawn: the bactericidal effect of 3-MIX C and 3-MIX F was significantly superior to that of 3-MIX S and 2-MIX, against anaerobic microorganisms isolated from necrotic permanent teeth; 3-MIX C shows a higher capacity to kill endodontic pathogens *in vitro* compared to 3-MIX F; both 3-MIX C and 3-MIX F were able to avoid the permanent staining effect of the crown.

Within the limitations of this study, the results obtained *in vitro* show that 3-MIX C is highly encouraging as a feasible alternative in the pulp treatment of irreversibly infected or necrotic permanent teeth. Clarithromycin antimicrobial substantivity, the property of a substance to bind to the soft and/or hard tissue walls of the pocket, will be further assessed to suggest its clinical use as an endodontic antimicrobial medicament.

REFERENCES

1. Fouad AF. The microbial challenge to pulp regeneration. Adv Dent Res 2011; 23:285-9.
2. Hargreaves KM, Giesler T, Henry M, Wang Y. Regeneration potential of the young permanent tooth: what does the future hold? J Endod 2008; 34:51-6.
3. da Silva LA, Nelson-Filho P, da Silva RA, Flores DS, Heilborn C, Johnson JD, Cohenca N. Revascularization and periapical repair after endodontic treatment using apical negative pressure irrigation versus conventional irrigation plus triantibiotic intracanal dressing in dogs' teeth with apical periodontitis. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2010; 109:779-87.
4. Kim JH, Kim Y, Shin SJ, Park JW, Jung IY. Tooth discolouration of immature permanent incisor associated with triple antibiotic therapy: a case report. J Endod 2010; 36:1086-91.
5. Fouad AF. Are antibiotics effective for endodontic pain? An evidence-based review. Endodontic Topics 2002; 3:52-66.
6. Hoshino E, Kurihara-Ando N, Sato I, Uematsu H, Sato M, Kota K, Iwaku M. *In-vitro* antibacterial susceptibility of bacteria taken from infected root dentine to a mixture of ciprofloxacin, metronidazole and minocycline. Int Endod J 1996; 29:125-30.
7. Ahmed HMA, Abbott PV. Discolouration potential of endodontic procedures and materials: a review. Int Endod J 2012; 45:883-97.
8. Reynolds K, Johnson JD, Cohenca N. Pulp revascularization of necrotic bilateral bicuspids using a modified novel technique to eliminate potential coronal discolouration: a case report. Int Endod J 2009; 42:84-92.
9. Akgun OM, Altun C, Guven G. Use of triple antibiotic paste as a disinfectant for a traumatized immature tooth with a periapical lesion: a case report. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2009; 108:62-5.
10. Agarwal E, Pradeep AR, Pavan B, Savitha BN. Efficacy of local drug delivery of 0.5% clarithromycin gel as an adjunct to non-surgical periodontal therapy in the treatment of chronic periodontitis subjects among smokers – a randomized controlled clinical trial. J Periodontol 2012; 9:1155-63.
11. Sato I, Kurihara NA, Kota K, Iwaku M, Hoshino E. Sterilization of infected root-canal dentine by topical application of a mixture of ciprofloxacin, metronidazole and minocycline in situ. Int Endod J 1996; 29:118-24.
12. Pasqualini D, Cuffini AM, Scotti N, Mandras N, Scalas D, Pera F, Berutti E. Comparative evaluation of the antimicrobial efficacy of a 5% sodium hypochlorite subsonic-activated solution. J Endod 2010; 36:1358-60.
13. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial

susceptibility testing; twenty-first informational supplement. Document M100-S21. 2011 Wayne, PA: CLSI.

14. Banche G, Roana J, Mandras N, et al. Microbial adherence on various intraoral suture materials in patients undergoing dental surgery. J Oral Maxillofac Surg 2007; 65:1503-7.
15. Bose R, Nummikoski P, Hargreaves K. A retrospective evaluation of radiographic outcomes in immature teeth with necrotic root canal systems treated with regenerative endodontic procedures. J Endod 2009; 35:1343-9.
16. Murray PE, Garcia-Godoy F, Hargreaves KM. Regenerative endodontics: a review of current status and a call for action. J Endod 2007; 33:377-90.
17. Witherspoon DE, Small JC, Regan JD, Nunn M. Retrospective analysis of open apex teeth obturated with mineral trioxide aggregate. J Endod 2008; 34:1171-6.
18. Andreasen JO, Farik B, Munksgaard EC. Long-term calcium hydroxide as a root canal dressing may increase risk of root fracture. Dent Traumatol 2002; 18:134-7.
19. Czonstkowsky M, Wilson EG, Holstein FA. The smear layer in endodontics. Dent Clin North Am 1990; 34:13-25.
20. Trevino EG, Patwardhan AN, Henry MA, Perry G, Dybdal-Hargreaves N, Hargreaves KM, Diogenes A. Effect of irrigants on the survival of human stem cells of the apical papilla in a platelet-rich plasma scaffold in human root tips. J Endod 2011; 37:1109-15.
21. Kumar A, Kumar V, Singh J, Hooda A, Dutta S. Drug-induced discolouration of teeth: an updated review. Clin. Pediatr 2012; 51:181-5.
22. Trope M. Treatment of the immature tooth with a non-vital pulp and apical periodontitis. Dent Clin North Am 2010; 54:313-24.