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ORIGINAL ARTICLE

Effect of Triple Antibiotic Paste, Double Antibiotic Paste, and Calcium Hydroxide on Antibiotic Resistance of Tet Repressor Proteins, Tetracycline Resistance Gene W, and Tetracycline Resistance Gene Q: A Randomized Controlled Clinical Trial

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ABSTRACT

Objective: To investigate the effect of the use of Calcium Hydroxide ($Ca(OH)_2$), Triple Antibiotic Paste (TAP), and Double Antibiotic Paste (DAP) as intra-canal medicaments on the development of antibiotic resistance by evaluating tetracycline genes. **Methods:** Forty five patients were randomly assigned into 3 groups according to the intracanal medication using a web program (www.randomizer.org), ($Ca(OH)_2$, TAP and DAP). Root canal bacteriological samples were collected before root canal treatment (S1) and after chemo-mechanical procedures (S2). Following root canal shaping procedures, intra-canal medication of the root canals was performed with selected intra-canal medicament. A second appointment was planned for the patients 15 days later. At the second appointment, the medication was removed mechanically and after irrigation of the root canals, the post medication samples (S3) were collected. Bacteriological samples were then investigated for bacterial counts and antibiotic-resistant genes [Tet Repressor proteins (TetR), Tetracycline resistance gene W (TetW) and Tetracycline resistance gene Q (TetQ)] using polymerase chain reaction (PCR), and the data were statistically analyzed. The Friedman, Kruskall-Wallis and chi-square tests were used. These genes were selected as the difference between TAP and DAP is that TAP contains tetracycline and tetracycline resistance is governed by tet genes. **Results:** There was no statistically significant difference among the samples (S1, S2, and S3) in terms of the number of root canals positive for antibiotic resistance genes in both the $Ca(OH)_2$ and DAP groups. However, the number of root canals positive for TetR gene increased significantly (6 [40%] to 12 [86%]) following intra-canal medication with TAP. **Conclusion:** It can be concluded that 15 of days intra-canal medication with TAP causes tetracycline resistance. In contrast, DAP does not cause tetracycline resistance and it has similar antibacterial effectiveness to TAP. The DAP would be the choice of medicament rather than TAP in clinical practice.

Key words: antibiotic resistance, bacterial resistance, $Ca(OH)_2$, Double Antibiotic Paste, endodontics, medicaments, Triple Antibiotic Paste

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INTRODUCTION

The effective elimination of bacteria and bacterial byproducts, which present within the root canal system and are the major cause of endodontic diseases, is required for the periapical repair of both mature and immature teeth.[1] Unfortunately, preparation of the root canals using mechanical instruments and chemical solutions is unable to completely remove bacteria and their byproducts from the root canal of the teeth.[2] Therefore, the use of different intra-canal medicaments such as pure calcium hydroxide ($Ca(OH)_2$) paste and triple or double antibiotic pastes (TAP or DAP) between appointments has been recommended to reduce the bacteria within the root canal system, especially in the case of regenerative endodontic procedures.[3,4]

An ideal intra-canal medicament should be antibacterial, nonirritating, stable in solution and active in the presence of blood and serum, and should have a

prolonged antibacterial activity.[5] Currently, $Ca(OH)_2$ is the most widely used intra-canal medicament which was introduced into dentistry by Hermann in 1930. The mechanism of action of $Ca(OH)_2$ is achieved through the release of hydroxyl ions in an aqueous environment.[6] The alkaline pH of the material provides its antimicrobial activity.[6]

Hoshino and colleagues developed TAP that consists of ciprofloxacin, minocycline and metronidazole,[7] and has been reported to be a successful medicament in controlling the root canal microbiata. DAP is a combination of ciprofloxacin and metronidazole. The minocycline has been removed to overcome the discoloration caused by the TAP. Studies demonstrated that DAP has similar antibacterial effectiveness as the TAP.[8–10]

Among these intra-canal medicaments, $Ca(OH)_2$ displays a weaker antibacterial activity compared to antibiotic pastes.[10,11] However, antibiotic pastes have disadvantages such as high toxicity,[12] crown discoloration,[13] and interference with the release of dentin growth factors.[14] Moreover, the most important concern about antibiotic pastes is that they may cause resistance of antibiotic. Therefore, there are ongoing researches on more effective and safer alternatives to antibiotic pastes.[15–17] For this reason, Simittiset et al. evaluated the bactericidal efficiacy of a new formulated antibiotic paste and reported that the antibacterial activity of this new product was comparable to the currently used antibiotic paste.[15] Similarly, Thakur et al. compared the antimicrobial efficacy of currently used antibiotic pastes with cefixime-based triple antibiotic paste and reported greatest antimicrobial effectiveness for the new product.[17]

According to the World Health Organization, one of the biggest threats to global health is antibiotic resistance and one which can affect anyone, in any country, of any age. Although antibiotic resistance occurs naturally or spontaneously through mutation,[18] the process is accelerated by the antibiotic misuse and overuse.[19] In the last decade, there has been an increase in the absolute number and the proportion of microbial pathogens resistant to multiple antibiotics.[20] It is believed that the completion of prescribed antibiotic courses, even after resolution of symptoms, prevents the development of antibiotic resistance.[21] However, clinical studies in the early 2000s demonstrated that shorter antibiotic usage is also as effective as longer courses of therapy.[22] Currently, American Association of Endodontics suggests 1 to 4 weeks antibiotic paste medication for regenerative endodontic procedures.[23] Since the antibiotic therapy with a longer duration increases the emergence of antibiotic resistance,[22,24,25] antibiotic pastes used for root canal medication may cause antibiotic resistance.[26]

Tetracycline is a widely-used broad-spectrum antibiotic which has been used to treat infectious diseases for more than half a century.[27,28] However, the number of isolates that are resistant to tetracycline has increased over the years.[27] To date, no studies have investigated whether or not intra-canal antibiotic therapy causes tetracycline resistance. Therefore, the aim of the present study was to evaluate the effect of the use of $Ca(OH)_2$, TAP, and DAP as root canal medicaments on the development of antibiotic resistance. The null hypothesis was that none of the medicaments used in the study cause the development of tetracycline resistance.

METHODS

The sample size was calculated by using the GPower software program (Franz Faul, University of Kiel, Germany). Data from any previous studies could not be used for the sample size calculation as there were none investigating the effect of the use of $Ca(OH)_2$, TAP, and DAP as intra-canal medicaments on the development of bacterial resistance. Therefore, a pilot study was conducted and the data obtained through the pilot study was used for the calculation. A total of 9 patients were included to the pilot study and the samples obtained from the patients were analyzed for tetracycline resistant genes [Tet Repressor proteins (TetR), Tetracycline resistance gene W (TetW) and Tetracycline resistance gene Q (TetQ)]. The data indicated that a total of 30 patients were sufficient with an alpha error of = 0.05, an effect size of 0.816, and an actual power of 0.952. Forty-five participants were included in the present clinical study, considering patients lost during follow-up. The clinical trial registration number is TCTR20220118002.

The ethical approval was obtained from the Dental School Ethical Committee of the University of A (decision no. 2021-27), and all the patients were requested for signing an informed consent form prior to treatment. The study patients were randomly divided into three groups using a web program (www.randomizer.org). The study was gender-neutral study.

Healthy patients (American Society of Anesthesiology [ASA] I) of ages $\geq$ 18 and $\leq$ 65 without any allergy to any antibiotics were included. Mature incisor, canine, and premolar teeth with a diagnosis of asymptomatic apical periodontitis, with a periapical index score of 3, 4, or 5,[29] a pocket depth of <3 mm and no previous root canal treatment were included. Necrotic teeth with single or two root canals were included. In teeth with 2 root canals, the root canal with periapical lesion was considered as the included root canal. Only necrotic teeth with periapical lesions were included based on the fact that the root canals had to

be infected. The exclusion criteria of the study were determined as follows: participants who had treated with antibiotics within one month; participants with a ASA classification of II, III or IV; teeth with crown destruction that prevented isolation of rubber dam properly; and the existence of external or internal root resorption.

The study was initiated in May 2021. A strictly aseptic technique was used during the collection of samples from root canals. An oral rinse was performed by using 0.12% chlorhexidine, and the plaque was removed by supra-gingival scaling, followed by cleansing with pumice. Following rubber-dam isolation, clamp, rubber dam and the crown were disinfected using 30% H_2O_2 and 2.5% NaOCl for 30 s. Subsequently, the effect of the NaOCl was deactivated with 5% sodium thiosulfate. Sterile round burs were used to perform access cavity preparation, and the cavity walls disinfected using 30% H_2O_2 and 2.5% NaOCl for 30 s to obtain sterility control samples. The pulp chamber was covered with a sterile cotton pellet to prevent the leakage and then the first sterility control samples were obtained from the surface of crown, access cavity walls, clamp and rubber dam using sterile paper points. The samples were then stored in Tris-EDTA buffer solution in Eppendorf tubes. The tubes were stored at -80ºC until the qPCR test. The samples were then investigated for bacterial existence in the qPCR analysis, and only teeth with negative sterile samples were included in the study.

Following the access cavity preparation, the working length was determined using an apex locator (Raypex 6; VDW, Munich, Germany). A gently root canal preparation was performed with a #15 K-file and the microbial samples (S1) were obtained using three sterile paper points (Dentsply SironaMaillefer, Ballaigues, Switzerland). The paper points were introduced into the root canals to the level of WL. After keeping the paper points in position for 30 s, they were removed and placed in Eppendorf tubes with Tris-EDTA buffer solution. During the sampling procedure, it was avoided to contact the paper points with the access cavity walls. The paper points were then stored in Tris-EDTA buffer solution in Eppendorf tubes. The sample was taken from the root canal with periapical lesion in teeth with 2 root canals.

All the root canal preparations were completed using Reciproc (R25, R40, or R50) instruments according to the manufacturer's instructions at full working length. During preparation of the root canals, irrigation was performed with 1 mL of 1% NaOCl (Chloraxid, Cerkamed, Poland) between three pecking motions of the file, and the root canals were finally irrigated with 5 mL of 10 % citric acid followed by 5 mL of 1% NaOCl, 2 mL of 0.5% sodium thiosulfate, and distilled water respectively. Second samples (S2) were obtained as described earlier, and then the root canals were dried by using paper points. Finally, intra-canal medication of the root canals was performed with selected intra-canal medicament.

$Ca(OH)_2$: 1 mL saline with 1 g $Ca(OH)_2$ powder.
TAP: 1 mL saline with 1 g Triple Antibiotic powder (doxycycline, metronidazole, and ciprofloxacin). The TAP used in the present study was including doxycycline instead of minocycline, and saline instead of Macrogold and Prophylene glycol which is different with the TAP formula provided by Takushige et al.[30]
DAP: 1 mL saline with 1 g Double Antibiotic powder (metronidazole and ciprofloxacin).

K-files were used to place medicaments into the root canals at a level 1mm short of the working length. Care was taken to place all of the prepared medicament (1mg/mL) into the root canal to ensure that the amount of medicament introduced was equal in all samples. Additionally, K-files were were touched all of the root canal walls to ensure the uniform distribution of the medicament in the root canals. The canal orifices were covered with sterile Teflon pellets and Cavit-G (3M ESPE, Seefeld, Germany) was used to seal access cavities. A second appointment was planned for the patients 15 days later.

During the second visit, following the removal of the temporary restoration, the operative field was disinfected and second sterility control samples were obtained as described previously. Five mL of distilled water was used for irrigation of the root canals and a 25 or 35 size K-type files was gently used to remove the medication mechanically. After irrigation of the root canals with 5 mL of 17% EDTA followed by 5 mL of distilled water, post-medication samples (S3) were obtained using the sampling method described previously. Finally, a cold lateral compaction technique was used for filling of all the root canals, and permanent restorations were performed. Bacteriological samples were then investigated for bacterial counts and antibiotic-resistant genes [Tet Repressor proteins (TetR), Tetracycline resistance gene W (TetW) and Tetracycline resistance gene Q (TetQ)] using polymerase chain reaction (PCR). These genes were selected as the difference between TAP and DAP is that TAP contains tetracycline and tetracycline resistance is governed by tet genes. All the root canal treatment procedures were performed by the same author.

PCR analysis
Genomic DNA isolation of the clinical samples were carried out using the Promega WizardR Genomic DNA Purification Kit following the protocol recommended by the manufacturer. To quantify the total bacterial level, 16S rRNA gene region-specific primers were used. Root canal isolates were analyzed for the presence of three tetracycline resistance genes (TetR, TetW and TetQ). QuantiTect SYBR® Green PCR Kits (Qiagen, Courtaboeuf, France) was used to perform the qPCR amplification in a 20 µL of total reaction volume. The

qPCR primers used for quantification of the bacteria and for the tetracycline resistance gene detection were as follows: 5′-ACTACGTGCCAGCAGCC-3′, 5′-GGACTACCAGGGTATCTAATCC-3′ for Universal 16S rRNA gene and TetQ gene: 5′-TTATACTTCCTCCGGCATCG-3′, 5′-ATCGGTTCGAGAATGTCCAC-3′, TetW gene: 5′-GAGAGCCTGCTATATGCCAGC-3′, 5′-GGGCGTATCCACAATGTTAAC-3′, and TetR gene: 5′-ACAACCCGTAAACTCGCC-3′, 5′-TTCCAATACGCAACCTAAAG-3′. The real-time PCR Cycling (Rotor-Gene Q, Qiagen, Germany) conditions included: initial denaturation; 95 °C for 15 minutes, and: 95 °C for 15 seconds, 30 s for annealing (59 °C) and 72 °C for 30 seconds (40 repeats each). Sample, standard and control measurements were taken in triplicate. The determination of the number of bacterial was performed using standard graphics according to cycle threshold values. The analysis of the was performed using GraphPad Prism Software (GraphPad Software, San Diego, CA, USA.

Statistical analysis

The bacterial count calculation in the samples (S1, S2, and S3) was performed using the Poisson regression analysis with the help of Software of GraphPad Prism (GraphPad SoftwareInc.; San Diego, CA, USA). The data normality was tested using Shapiro-Wilk test. As the data were not normally distributed, non-parametric tests were used during the statistical analyses. For this reason, intragroup analysis in terms of the reduction of total bacteria counts among the S1, S2, and S3 samples was performed using the Friedman test as the data was including repeated measures. For intergroup analysis, the comparison of the age data and the number of bacteria in the samples (S1, S2 and S3) with regard to the $Ca(OH)_2$ paste, TAP, and DAP groups was analyzed by using Kruskal-Wallis H test which is able to conduct pairwise comparisons like a post-hoc test. The chi-square test was used to analyze the gender and teeth number data, and the number of root canals positive for the antibiotic-resistant genes TetR, TetW, and TetQ in the three samples with regard to the $Ca(OH)_2$ paste, DAP, and TAP groups. The level of significance was set as 95% for all the statistical tests. Patients with missing data were not included and any correction was not applied during the statistical tests. The sample size was determined as described above.

RESULTS

A total of 45 participants were included. One sample in the TAP group could not be analyzed as the tube was cracked during centrifuge procedure. Therefore, 44 patients were evaluated in total (Figure 1). The difference among the groups in terms of demographic data (age, gender, or teeth number,) was statistically insignificant ($p > 0.05$) (Table 1). The actual P values

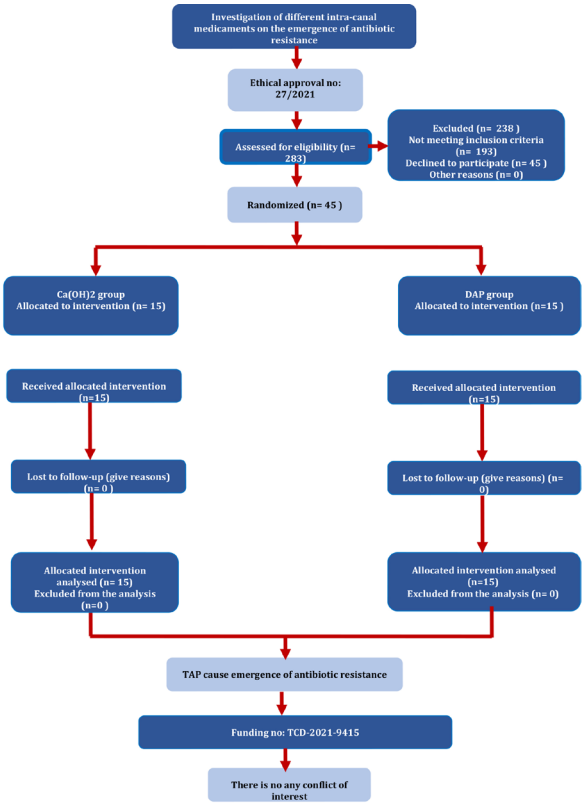

Figure 1. PRIRATE 2020 Flowchart.

Table 1. Distribution of patients according to age, gender, and tooth number.

	$Ca(OH)_2$	DAP	TAP	p
n	15	15	14	
Mean age	31 ± 12	38.5 ± 13	32 ± 11.3	**0.252**
Gender				**0.631**
Female	7	8	9	
Male	8	7	5	
Tooth number				**0.569**
#11	1	0	1	
#12	4	1	2	
#14	1	0	2	
#15	0	0	1	
#21	0	1	2	
#22	2	2	1	
#23	2	4	1	
#24	0	0	1	
#25	1	0	0	
#33	0	1	1	
#34	0	1	0	
#35	1	0	0	
#41	0	0	1	
#43	0	1	0	
#44	2	1	0	
#45	1	3	1	

Table 2. The number of total bacteria according to the groups.

		$Ca(OH)_2$	DAP	TAP	p
	Mean	$6.6^{A} \times 10^7$	$6.5^{A} \times 10^7$	$6.3^{A} \times 10^7$	0.267
S1	Median	6.6×10^7	6.4×10^7	6.1×10^7	
	Range	$5.8 \times 10^7 – 7.5 \times 10^7$	$5.6 \times 10^7 – 7.9 \times 10^7$	5.5×10^7 - 7.2×10^7	
	Mean	$4.2^{B} \times 10^3$	$4.2^{B} \times 10^3$	$3.9^{B} \times 10^3$	0.226
S2	Median	4.1×10^3	4×10^3	3.8×10^3	
	Range	$3.2 \times 10^3 – 5.3 \times 10^3$	$3.6 \times 10^3 – 5.7 \times 10^3$	2.8×10^3- 5.6×10^3	
	Mean	$1.9^{Ca} \times 10^2$	$1.3^{Cab} \times 10^2$	$0.9^{Cb} \times 10^2$	**0.011**
S3	Median	2×10^2	1.2×10^2	0.9×10^2	
	Range	$0.1 \times 10^2 – 3.1 \times 10^2$	0.2×10^2 - 3×10^2	0.2×10^2 - 2×10^2	
	p	**0.000**	**0.000**	**0.000**	

Within the same row (intergroup comparison of Ca(OH)2, DAP and TAP), values with the same letters were not statistically different at p = 0.05
Within the same column (intragroup comparison of Ca(OH)2, DAP or TAP), values with the same letters were not statistically different at p = 0.05, S1; before preparation, S2; after preparation, S3; after medication, Ca(OH)2; Calcium Hydroxide, DAP; Double antibiotic paste, TAP; Triple antibiotic paste

of the age, gender and teeth number for the $Ca(OH)_2$, DAP and TAP groups were 0,252, 0,631, and 0,569, respectively. The mean age and standard deviation values for the Ca(OH)2, DAP and TAP groups were 31 ± 12, 38.5 ± 13, and 32 ± 11.3, respectively.

Bacterial removal

Intragroup analysis was performed by using Friedman test and showed that the number of bacteria was significantly reduced in S1 to S2 and in S2 to S3 in all groups (p = 0.000) (Table 2). According to the intergroup analysis which was performed by using Kruskal-Wallis test, it was found that the difference among the groups was statistically insignificant when comparing the number of bacteria in S1 (p = 0.267) and S2 (p = 0.226). However, the difference among the groups in terms of the bacterial count following intra-canal medication (S3) was statistically significant (p = 0.011). In the TAP group, significantly fewer bacterial counts were detected compared with the $Ca(OH)_2$ group. The difference between the TAP and DAP groups in terms of the number of bacteria was not statistically significant.

Antibiotic resistance

The number of root canals positive for antibiotic resistance genes (TetR, TetW, and TetQ) was evaluated by using chi-square test (Table 3). In the DAP and $Ca(OH)_2$ groups, while there were 4 TetR gene positive root canals in S1 and S2, the number of root canals positive for the TetR gene decreased to 3 following medication of the root canal with either DAP or $Ca(OH)_2$ (S3). The difference among the samples (S1, S2, and S3) was not statistically significant when comparing the number of root canals positive for antibiotic resistance genes in both the $Ca(OH)_2$ and DAP groups (p = 0.887). However, an increase in the number of root canals positive for the TetR gene was observed following intra-canal medication involving TAP (S3). There were 6 root canals (40%) positive for the TetR gene in S1 and S2. Following intra-canal medication, 12 samples (86%) were positive for the TetR gene in S3. The difference among the S1, S2, and S3 was statistically significant (p = 0.030). In other words, while the TAP medication results in the emergence of antibiotic resistance, $Ca(OH)_2$ and DAP medication did not cause any antibiotic resistance.

When the TetW gene in the $Ca(OH)_2$ group was evaluated, 4, 4, and 3 root canals were found to be positive for the TetW gene in S1, S2, and S3, respectively. The difference among the samples (S1, S2, and S3) were not statistically different (p = 0.887). In the DAP group, the number of root canals positive for the TetW gene was 5 in all the samples (S1, S2, and S3). In the TAP group, 5 root canals were positive for the TetW gene in S1, and this did not change following root canal preparation (S2). However, after the medication of the root canals with TAP, the number of TetW gene positive root canals was increased (7 root canals). The difference among the S1, S2, and S3samples was not statistically significant (p = 0.673).

In the $Ca(OH)_2$ and DAP groups, 3 root canals were positive for the TetQ gene in S1, and neither preparation (S2) nor medication of the root canals (S3) changed the number of positive root canals. Similarly, in the TAP group, there were 7 root canals positive for the TetQ gene in S1, while the number of positive root canals did not change in either S2 or S3.

Table 3. The number of root canals positive for resistant genes.

	S1	S2	S3	p
TetR				
$Ca(OH)_2$	4/15 (27)	4/15 (27)	3/15 (20)	0.887
DAP	4/15 (27)	4/15 (27)	3/15 (20)	0.887
TAP	6[a]/14 (43)	6[a]/14 (43)	12[b]/14 (86)	**0.030**
TetW				
$Ca(OH)_2$	4/15 (27)	4/15 (27)	3/15 (20)	0.887
DAP	5/15 (33)	5/15 (33)	5/15 (33)	-
TAP	5/14 (36)	5/14 (36)	7/14 (50)	0.673
TetQ				
$Ca(OH)_2$	3/15 (20)	3/15 (20)	3/15 (20)	-
DAP	3/15 (20)	3/15 (20)	3/15 (20)	-
TAP	7/14 (50)	7/14 (50)	7/14 (50)	-

The number of cases with a positive result/ number of cases (%) Within the same row, values with the same letters were not statistically different at p = 0.05, S1; before preparation, S2; after preparation, S3; after medication, Ca(OH)2; Calcium Hydroxide, DAP; Double antibiotic paste, TAP; Triple antibiotic paste, TetR; Tet Repressor proteins, TetW; Tetracycline resistance gene W, TetQ; Tetracycline resistance gene Q

DISCUSSION

Antimicrobial resistance is a global concern and leads to prolonged hospital stays, higher medical costs, and an increase in the mortality. It causes approximately 700,000 deaths per year worldwide. Treatment of numerous diseases such as tuberculosis, pneumonia, gonorrhea, foodborne diseases, and blood poisoning are becoming harder because of antibiotic resistance.

During the root canal treatment, the $Ca(OH)_2$, TAP or DAP can be used for the elimination of bacteria inside the root canal. For this reason, these medicaments are placed into the root canal and kept there for 1 week to 1 month. During these periods, the medicaments may cause antibiotic resistance development. Because of the importance of the antibiotic resistance, the present study aimed to evaluate the effect of intra-canal antibiotic therapy on the development of antibiotic resistance. This is the first study evaluating the effect of different medicaments on the emergence of antibiotic resistance and revealed a significant result showed that intra-canal medication with TAP results in the development of antibiotic resistance. This is an important finding which may affect the clinical use of intracanal medicaments. The null hypothesis was that none of the medicaments used in the study cause the development of tetracycline resistance. Thus, the null hypothesis was rejected.

Complete elimination of the bacteria from the root canal space following chemo-mechanical preparation is a challenge due to the anatomical complexities of the root canal system.[10] Achieving negative microbiological culture in the root canal system is essential for apical repair, since it has been demonstrated that bacteria present at the time of the obturation of the root canal negatively affects the success of the root canal treatment.[31] Therefore, numerous techniques have been proposed for complete elimination of the bacteria within the root canal system, such as irrigation activation techniques,[32] usage of the heated irrigation solutions,[33] and intra-canal medication with different medicaments.[2]

The $Ca(OH)_2$, TAP and DAP are the most widely used intra-canal medicaments in endodontics. Although there are some concerns that the antibiotic paste usage as intra-canal medicament causes antibiotic resistance, there is no study investigating the effect of different intra-canal medicaments on the emergence of antibiotic resistance. Therefore, the present study is the first comparing different medicaments in terms of the antibiotic resistance emergence. Because of the lack of the studies evaluating the effect of these medicaments in the emergence of antibiotic resistance, a direct comparison could not be done between the present study and the previous ones.

Tetracycline resistance is governed by tet genes. The tetracycline antibiotic was discovered approximately 75 years ago and became the first class of broad-spectrum antibiotics.[34] Therefore, tetracyclines have found wide use in the clinic and agriculture. The fact that many tetracyclines can be taken orally and have a wide spectrum make them an excellent therapeutic agent. However, the widespread use of tetracyclines in agriculture and medicine has led to the development of tetracycline resistance in microorganisms that were previously sensitive to this antibiotic.[35] Resistance to tetracyclines was first reported in 1953 and spread rapidly as a result of resistance genes being carried by mobile elements such as plasmids and transposons.[36] Like most antibiotics, resistance to tetracyclines can occur through a number of mechanisms. These include active exit from the cell, production of ribosomal protection proteins, decreased drug permeability, target mutation, and enzymatic degradation of antibiotics. Large-scale new studies are needed to better understand these mechanisms. Especially, prospective studies with higher sample size needed to reveal the effect of intracanal medicaments on the emergence of antibiotic resistance. Consquently, in the present study, the presence of TetR, TetW, and TetQ genes in root canals was evaluated. TetR family members regulate approximately 85 proteins that control genes, and the products of these genes are involved in multidrug resistance.[37] TetW and TetQ genes encode ribosomal protection proteins that provide tetracycline resistance by abolishing the inhibitory effect of tetracycline on protein synthesis.[38,39] According to the findings of the present study, 15 days of intra-canal medication with

TAP caused a 100% increase (6 to 12) in the number of TetR positive cases. Thus, it can be clearly stated that intra-canal medication with TAP results in the development of bacterial resistance to tetracycline.

Previous studies reported 2 weeks of intra-canal medication with TAP is effective for the disinfection of the root canals.[40–42] Moreover, according to the American Association of Endodontics, the duration of the medication with TAP can be extended for up to 4 weeks.[23] However, numerous studies reported that antibiotic therapy with a shorter duration decreases the emergence of antibiotic resistance.[22,24,25] Therefore, the result of the present study can be explained by the fact that a relatively longer duration (2 weeks) of intra-canal medication with TAP would result in antibiotic resistance development. There is an illogical dogma that the completion of prescribed antibiotic courses, even after resolution of symptoms, prevents the development of antibiotic resistance.[21] However, there is no evidence that taking antibiotics after the resolution of symptoms reduces the development of antibiotic resistance.[43] Numerous studies have reported that 7 days of intra-canal medication with TAP is effective for the disinfection of root canals.[8,9,44] It is generally believed that it takes a long time for bacteria to mutate and evolve resistance and it requires a series of mutation. However, the evolution of a bacteria to develop resistance to antibiotics has a frightening speed. A previous study showed that evolution can occur very quickly.[45] It has been demonstrated that the level of resistance increased dramatically in just 20 days.[45] Moreover, resistance level may increase 1000 fold over 11 days.[45] Therefore, 7 days of application of TAP can be considered for root canal disinfection to reduce the emergence of antibiotic resistance.

According to the finding of the current study, neither $Ca(OH)_2$ nor DAP caused tetracycline resistance. The reason for this finding would be that neither of the medicaments include tetracycline. Although factors such as mutation,[46] and environment[47] are also effective in the development of antibiotic resistance, the emergence of antibiotic resistance is primarily due to the overuse and misuse of antibiotics.[48] Previous studies demonstrated that DAP is as effective as TAP in reducing intra-canal bacteria without any significant difference.[8–10] Additionally, according to the findings of the current study, TAP, and DAP have similar antibacterial effectiveness. Moreover, none of the medicaments provided bacteria-free root canals. Therefore, DAP would be the choice of a medicament for root canal disinfection in the case of persistent infections as it has similar antibacterial effectiveness to TAP and does not cause tetracycline resistance. Interestingly, one patient in the $Ca(OH)_2$ group became negative for the TetR gene following intra-canal medication. However, the difference was statistically insignificant.

In the present study, Tetracycline resistance genes including TetR, TetW, and TetQ were examined. These genes were also determined in the $Ca(OH)_2$ and DAP groups in the preoperative (S1) and postoperative samples (S2 and S3) meaning that Tetracycline resistant microorganisms were present in the root canals which did not medicated with an antibiotic including Tetracycline. However, the number of root canals positive for these genes did not increase with either the $Ca(OH)_2$ or DAP medication. Thus, it can be asserted that these medications do not cause the emergence of tetracycline resistance.

A sterile root canal could not be achieved following medication with TAP, DAP or $Ca(OH)_2$ in all samples. These finding is in accordance with the previous studies evaluating the antibacterial effect of the selected medicaments.[49,50] To achieve negative root canals for bacteria, the antibacterial efficacy of these medicaments should be increased or searching for new medicaments with stronger antibacterial effectiveness should continue.

In the present study, TAP with a concentration of 1g/mL was used for intra-canal medication. This is a relatively high concentration when compared with the concentration of 1mg/ml recommended by the regenerative procedure guidelines of the American Association of Endodontics. However, Sabrah et al.[9] reported that TAP with a concentration of 1gr/mL provides significant residual antibacterial effectiveness up to 14 days. Additionally, Reyhani et al.[40] demonstrated that TAP with 1g/mL concentration can completely remove Enterococcus faecalis biofilms, whereas TAP with 1mg/mL concentration cannot. Since the lower level of antibiotic concentrations than the minimum inhibitory concentration contributes to the emergence of antibiotic resistance,[51-53] TAP with a concentration of 1g/mL was preferred in the present study.

In the present study, there were positive cases of tetracycline resistance in all the medication groups. This can be explained by the worldwide overuse of tetracycline. It has been reported that the overuse of antibiotics leads to the spread of antibiotic resistance.[54] However, this factor could not be standardized in the present study.

One of the limitations of the present study would be that different size of apical enlargement performed for the root canals included. Because more apical enlargement may result in better irrigation penetration, and thus more cleaning and less bacterial count. Another limitation would be that immune systems are variable between individuals which may affect the development of antibiotic resistance. However, it is impossible to standardize all the variables especially in a clinical study.

The aim of the present study was to elaborate on the increased resistance to tetracyclines observed in the root canal system due to the use of certain medicaments. These findings are confirming existing knowledge about gene induction and transfer in bacteria in the root canal system. However, emergence of antibiotic resistance in the root canal system caused by intra canal medications should be investigated in future studies.

CONCLUSION

Within the limitations of the present study, it can be concluded that 15 days intra-canal medication with TAP causes tetracycline resistance. In contrast, DAP does not cause tetracycline resistance and it has similar antibacterial effectiveness to TAP. Therefore, intra-canal medication with DAP can be considered for root canal disinfection.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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