

Evaluation of antibacterial effect of irrigant solutions (titanium tetra fluoride, green tea, sodium hypochlorite, normal saline) using real-time quantitative – polymerase chain reaction

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ABSTRACT

Background: Removal of bacteria from the pulp system by instrumentation of an infected root canal, will be significantly reduced the number of bacteria, but it is well documented that instrumentation alone can-not clean and kill all bacteria found on the root canal walls. Antibacterial irrigants are needed to kill the remaining microorganisms. The aims of this study was to assess antibacterial effect of titanium tetrafluoride (TiF₄) solution and brewing green tea against root canal bacteria and to compare with sodium hypochlorite and normal saline through microbiological and molecular studies.

Materials and methods: Microbiological study was carried out to determine the concentration of titanium tetrafluoride and brewing green tea at which they exert antibacterial effect against ten swabs that had been taken from necrotic root canals that were incubated aerobically and anaerobically by paper disk diffusion test, while molecular study carried out among Forty children in which the antibacterial effect of titanium tetrafluoride and brewing green tea compared with sodium hypochlorite and normal saline were assessed by real time polymerase chain reaction using SYBR Green.

Results: The microbiological study results showed that TiF₄ achieved maximum antibacterial effect at concentration 5% against aerobic and anaerobic bacteria while green tea exhibited antibacterial effect when brewed for 20 minutes at concentration 100mg/1ml against *staphylococcus aureus*, but not active against other microorganisms like *Escherichia coli* and *streptococcus*. While results of molecular study illustrated that sodium hypochlorite remained the most effective endodontic irrigant solutions followed by titanium tetrafluoride then green tea while normal saline showed no antibacterial effect. Statistically titanium tetrafluoride, green tea and sodium hypochlorite have significant differences compared to normal saline.

Conclusions: This study revealed that, titanium tetra fluoride and brewing green tea can be used as antibacterial irrigant solutions for root canal treatment in children.

Key words: Antibacterial effect, Titanium Tetrafluoride, Green tea, sodium hypochlorite, Normal saline, Real-Time Quantitative – polymerase chain reaction. (J Bagh Coll Dentistry 2015; 27(3):140-145).

INTRODUCTION

Bacteria in a tooth's root canal both initiate and perpetuate periapical inflammatory lesions ⁽¹⁾. Thus, the aim of endodontic therapy is to remove pathogenic bacteria from the pulp system ⁽²⁾.

This is usually accomplished by mechanical preparation along with the use of irrigant solutions.

The complexity of the root canal system, presence of numerous dentinal tubules in the roots, invasion of the tubules by microorganisms, formation of smear layer during instrumentation and presence of dentin as a tissue are the major difficulties in achieving the primary objectives of complete cleaning and shaping of root canal systems ⁽³⁾. The purpose of endodontic irrigation is to facilitate removal of bacteria, debris and necrotic tissue ⁽⁴⁾, especially from areas of the root

canal that have been left unprepared by mechanical instruments ⁽⁵⁾. Since the principal cause of treatment failure is considered to be the residual bacteria in the apical part of the root canal ^(6,7). Endodontic irrigant that possess antibacterial properties have clearly superior effectiveness in bacterial reduction ⁽⁸⁾.

Titanium tetra fluoride is metal fluorides, unlike the commonly used fluorides (e.g., NaF, SnF₂, and APF), has shown to offer greater protection against caries and tooth erosion ⁽⁹⁾. Titanium itself is a nontoxic element, and no side effects have been reported with titanium tetra fluoride ⁽¹⁰⁾. The advantage has been credited to the titanium group present in the compound, which synergizes the effect of fluoride ⁽¹¹⁾. Topical application with high concentration of fluoride may be effective due to the antimicrobial effects of fluoride ⁽¹²⁾.

Green tea is a tea made solely from the leaves of *Camellia sinensis* ⁽¹³⁾. Green tea is unfermented, thus containing the highest concentrations of polyphenols and most likely possessing the greatest antibacterial effect ⁽¹⁴⁾.

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Sodium hypochlorite had proven to be an effective solution for the chemomechanical preparation of root canal because of its antimicrobial activity and tissue dissolving ability⁽¹⁵⁾.

Sterile normal saline is the most biocompatible irrigant solution, because it is inactive with minimum effect on the periapical tissue⁽¹⁶⁾.

The aims of this study to evaluate the antibacterial effect of TiF₄ and green tea solution when used as endodontic irrigant and to compare with the NaOCl and normal saline.

MATERIALS AND METHODS

1-Patient Selection

The sample was selected from a pool of patients attending College of Dentistry, University of Baghdad, specialist health dental Center in Al-Ma'moon and specialist dental Center in Al-Ameria. The study was divided into two parts. The microbiological study included 10 patients of both sexes having necrotic pulp. In the second part, molecular study was conducted on 40 patients have necrotic pulp. In both study groups, patients were healthy and did not receive antibiotic treatment during the previous one month and age range was from 9-12 years. All selected teeth are maxillary central incisors with necrotic pulp, lack of response to pulp vitality test by using the ethyle chloride, asymptomatic, had not received previous root canal treatment and had radiographic evidence of closed apex with or without periapical lesion.

2-preparation of solution

Titanium tetra fluoride solution prepared by dissolving TiF₄ powder in deionized water with varying concentration (1%, 2%, 3%, 4%, 5 %).

The green tea was brewed at 90°C with varying concentrations of crushed dried tea leaves (40, 60, 80, 100, 140 mg/ml) and varying brewing times (10, 20, 30 min). Left to cool at room temperature. Green tea solution was filtered by filter paper (No.1). The resulted extract was kept in closed container in refrigerator.

3- Microbiological Examination

Ten children aged (9-12) years, require root canal treatment were participated in this study. They were divided into two groups: group I (n=5) tested with varying concentration of titanium tetra fluoride solution, group II (n=5) tested with varying concentration of green tea.

After isolation of each tooth with rubber dam, the crown and the surrounding rubber dam were disinfected by 2.5% NaOCl for 30s. The

disinfectant was air dried before access was gained to the pulp chamber and root canal⁽¹⁷⁾.

Access opening was established without water spray, instead, as a coolant, sterile saline was dripped from a sterile disposable syringe. Proper unrestricted access opening was established in the crown of the tooth by high speed hand piece with round bur No.14 to ensure complete access to the canal wall in order to acquire adequate debridement then the pulp canal was extirpated by barbed broach, swabs were taken from root canals by sterile paper points⁽¹⁸⁾.

Paper points were immersed in the root canals of maxillary central incisors with necrotic pulps left for three minutes then immediately inserted to nutrient broth and thioglycollate broth and incubated for 72 hours at 37°C⁽¹⁹⁾. The swabs streaked on MacConkey agar and blood agar then incubated aerobically and anaerobically by using anaerobic jar with gas pack for 24 hours at 37°C⁽²⁰⁾.

Identification of microorganisms by colony morphology of bacteria, Gram's stain and biochemical test was done⁽²¹⁾.

The antimicrobial properties of titanium fluoride and green tea were tested using paper disk diffusion test. Nutrient agar plates were inoculated by spreading a lawn of the root canal culture across each plate. Small paper disks were infused with the test solution by soaking in the solution for approximately 5 minutes. The disks were then placed onto the inoculated petri dishes, control disks were also prepared by soaking in the distilled water. The plates were incubated aerobically and an aerobically at 37°C for 48 hours. After incubation the zones of inhibition were measured using a ruler⁽²²⁾.

4-Endodontic sample collection

In the clinical trial, forty teeth were prepared in the same procedure of swabbing in microbiological study for root canals sample collection for molecular study in which paper point was immerse in the canal and left for 3 minutes then immediately insert to an eppendorf tube containing 200 µL of tris-EDTA buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, and pH 8).

The canals in each group were irrigated with 5ml of specific irrigation solution (10 canals with 5% TiF₄, 10 canals with 10% green tea, 10 canals with 5% NaOCl and 10 canals with 0.9% normal saline) with disposable syringe (23-gauge needle).

Each canal was immerse with irrigant solution for three minutes then another sterile paper point was insert inside the canal of tooth for three minutes then immediately insert to an eppendorf

tube containing 200 μ L of tris-EDTA buffer and vortexed for 60s. The eppendorf tubes were transferred on dry ice and stored at (-20°C) immediately. Collection lasted almost 3 months and the time from the sampling procedure until the processing of the DNA ranged between three and three and half months.

5-Real-time quantitative-polymerase chain reaction

Extraction of genomic DNA from gram negative and positive bacteria by using Exiprep™ bacteria genomic DNA kit (BIONEER). The deep frozen pre- and post-preparation sample were thawed on ice and dispersed by vortexing for 1 minute. DNA was extracted and purified with ExiPrep™ 16 plus DX Automated Nucleic Acid Extraction System (according to the manufacturer's instructions). By using this extraction method, DNA from both gram-positive and Gram-negative bacteria was retrieved with no apparent discrimination against either bacterial group. The extracted DNA was quantified in a spectrophotometer at 260 nm and stored at -20°C until required.

A real-time PCR procedure was used for relative quantification of root canal bacteria (gram positive and negative). Quantification was performed using SYBR green method and one universal 16S rDNA primer. Primers were designed from highly conserved regions of the 16sDNA gene sequence of Salmonella enterica (GenBank accession no. U90316)⁽²³⁾: forward primer EuF:5'CTGTCGTCAGCTCGTGTGTG-3', reverse primer EuR:5'CGTAAGGGCCATGAT GACTT -3', amplifying 157 bp (according to Salmonella enterica position).

Amplification and detection of DNA by RTQ-PCR was performed with the aid of the sequence detection system (Exicycler™ 96, BIONEER-Korea) using optical grade 96-well plates. In each run, four negative controls nuclease free water as template were used. All samples were analysed using AccuPower® Greenstar Qpcr PreMix (BIONEER). Samples were run in duplicate in a total volume of 20 μ l.

Final reactions contained (10 pmol L) 1 μ L of each primer, 5 μ L of template DNA and DEPC-distal water adjust to 20 μ L. The temperature profiles were as follows: SYBR Green: denaturation 94 °C for 5 min; 40 cycles: 94 °C for 20 sec, stringent annealing at 64 °C for 20 sec, and elongation at 72 °C for 20 sec. Melting curve analysis was performed to assess reaction specificity. After reaction is completed, data analysis performed. DNA was calculated by determining the threshold cycle (C_T), the number

of PCR cycles required for the fluorescence to exceed a threshold value significantly higher than the background fluorescence.

RESULTS

The results of the microbiological study, indicated that the titanium tetra fluoride at concentration 5% produced same zone of inhibition against both aerobic and anaerobic bacteria with mean zones of inhibition 17.2 mm higher than other concentration (1%, 2%, 3% and 4%). This is shown in Figure (1).

While green tea showed that the most effective concentration was 100 mg/mL with an average zone of inhibition of 20.4 mm and also most effective brewing time was 20 minutes. However, for the 30 minutes, the concentration 140 mg/mL was effective with an average zone of inhibition of 13 mm. It was also found that at brewing time 10 minutes, green tea proved ineffective in preventing the growth of bacteria, with all five concentrations exhibiting no zones of inhibition (Figure 2).

Green tea at concentration of 100 mg / mL that brewed for twenty minutes affects only on one type of bacteria isolated from infected root canal that is *staph. aureus* with mean zone of inhibition 20.4 mm while other types of bacteria isolated from the same root canals such as *E. coli* and *streptococcus* unaffected versa show growth rather than inhibition. According to ANOVA test the results show high significant difference in sensitivity of *staph. arueus* to green tea compared to the *Streptococcus* and *E. coli* (P value = 0.001) (Table 1).

Table (2) represent the result of Real Time PCR include mean of C_T value (threshold cycle) for different irrigant solutions before and after treatment. The result show that the mean value of C_T after treatment increased for groups treated with titanium tetra fluoride, green tea and sodium hypochlorite indicate succeeded in significantly reducing the number of bacterial taxa, only group treated with normal saline show decrease in mean C_T value that indicate increase significantly in number of bacteria. The result of ANOVA test demonstrated significant difference between group irrigated with normal saline and other three groups (TiF₄, green tea and NaOCl). No significant differences were found between group irrigated with 5% TiF₄, group irrigated with 100 mg/ml green tea and group irrigated with 5% Sodium hypochlorite.

Table 1: Comparison of the antibacterial effect of green tea among different bacteria of root canals

Bacterial isolated	Zone of Inhibition (Mean)	P value
Staphylococcus aureus	20.4	0.001 (HS)
Streptococcus	0	
E. coli	0	

Table 2: Multiple comparisons of antibacterial effects of different irrigation solutions before and after treatment

Groups	Mean ± S.E.		P-value
	Pre-treatment	Post-treatment	
TiF4	22.46 ± 0.27A	25.34 ± 0.47A	0.01
Green tea	22.94 ± 0.23A	25.16 ± 0.77A	0.01
NaOCL	23.16 ± 0.28A	26.06 ± 0.75A	0.01
Normal saline	23.07 ± 0.44A	21.51 ± 0.88B	0.05

Different Letters: Significant difference ($P \leq 0.05$) between means of columns

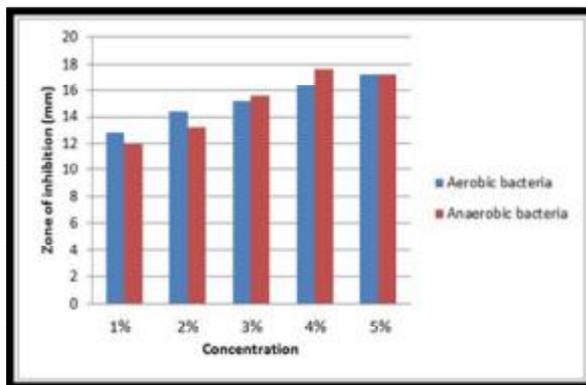


Figure 1: Zones of inhibition for different titanium tetra fluoride concentration

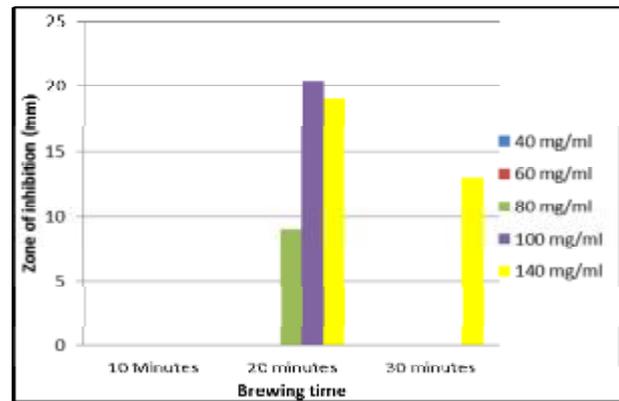


Figure 2: Zones of inhibition for different green tea concentration and brewing time's

DISCUSSION

The results of the study indicated that TiF4 showed antimicrobial effect when used as root canal irrigant at different concentration, but the best antibacterial effect was noticed at concentration of 5% due to the fact that TiF4 contain fluoride in their structures and used higher concentration of TiF4 mean higher concentration of fluoride and fluoride at high concentration have antibacterial effect⁽¹³⁾. Fluorine ions released from fluoride can affect bacterial metabolism as an enzyme inhibitor. At the lower external pH provided by low PH of TiF4 solution, fluoride diffuses into bacteria in the form of HF (a weak acid), because of a higher internal pH of cells than external more HF diffuses inside the bacterial cell and HF dissociates into H + and F - .This continued diffusion and dissociation leads to the accumulation of fluoride in the cell and the acidification (accumulation of H+) of the cytoplasm. The result is a reduction in both the proton gradient and the enzyme activity that induced effective inhibitors of bacteria⁽²⁴⁾.

The results demonstrate that the green tea brewed for 10 minutes was ineffective in killing the bacteria. The tea brewed for 30 minutes was effective in killing the bacterial cultures, but was not as efficient as the tea brewed for 20 minutes.

The longer the green tea leaves were infused in the hot water, the greater the breakdown of the antibacterial polyphenolic compounds, rendering the tea less effective.

In this study green tea at concentration 100mg/ml and brewing time 20 minutes inhibited the growth of *staphylococcus aureus* isolated from swabs of infected root canals and ineffective against other bacteria isolated from same root canals like *E. coli* and *Streptococcus*.

Green tea's effectiveness as an antimicrobial agent can in part be attributed to its low degree of fermentation. During the fermentation process, catechins such as epigallocatechin gallate (EGCG) are destroyed, reducing the tea's antimicrobial properties. The antibacterial activity also is due to inhibition of bacterial enzyme gyrase by binding to ATP B sub unit⁽²⁵⁾.

Real Time PCR was used to compare antibacterial activity of 5%TiF4 and 10% green tea with that of 5% sodium hypochlorite and0.9% normal saline.

Table (2) show that group irrigated with TiF4 produced increased in C_T value compared to C_T value before irrigation indicate successful in reducing the bacteria count because reactions with lower C_T values contain more of the gene of interest since they took less time to amplify. In

the same way, samples with a higher C_T contain less of the gene of interest⁽²⁶⁾.

There are two possible explanations for antibacterial mechanism of the titanium tetra fluoride. On one hand, the action of the fluorine ions could be responsible for this mechanism; on the other hand, the action of the metal-fluoride complexes are also responsible for fluoride inhibition of proton-translocating F-ATPases and are thought to act by simulating phosphate to form complexes with ADP at the reaction centers of the enzymes^(13,27).

Herbal products such as green tea have been used in dental practice and have become more popular today due to their high antimicrobial activity, biocompatibility, anti-inflammatory and anti-oxidant properties⁽²⁸⁾.

The results show that group irrigated with green tea produced increased in C_T value in comparison to C_T value before irrigation which indicates successful reduction in the bacterial number (Table 2).

Antibacterial properties of green tea have been associated with the polyphenol catechin fractions which constitute up to 30% of solid green tea leave^(29,30). EGCG is the most abundant of these catechins, comprising about 50% of the catechin pool. It had been shown that catechin components of green tea and particularly (EGCG), epigallocatechin and epicatechin-3-gallate, which are all catechin derivatives having a galloyl moiety linked by an ester linkage, constitute the most important antibacterial agents in green tea⁽³¹⁾.

The group irrigated with sodium hypochlorite produced increased in C_T value compared to C_T value before irrigation indicates successful in reduction the number of bacteria, NaOCl still the most frequently used root canal irrigant, the antibacterial effect of 5% sodium hypochlorite could be attributed to the irreversible inactivation of the bacterial essential enzymatic site through the action of hydroxyl ions and the chloramination process. Sodium hypochlorite when come in contact with organic tissue releases chlorine that could combine with protein amino group to form chloramine which exerts its antimicrobial action through interfering with cellular metabolism, thus NaOCl has powerful germicidal properties⁽³²⁾.

The result of this study showed that normal saline had no antibacterial effect against root canal bacteria. It produced decrease in C_T value (Table 2), this indicate increased in bacterial count. This increase in bacteria can be explained by washing action of saline may cause extrusion

of bacteria from periapical lesion to root canal system.

Under the conditions of this study TiF4 solution can be used as an endodontic irrigant since it possess an excellent antibacterial action similar to sodium hypochlorite. Also green tea can be used as an endodontic irrigant because it show an acceptable antibacterial effect and non-toxic because it is natural agent.

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الخلاصة

المقدمة: ان إزالة البكتيريا المرضية بصوره ميكانيكيه في علاج قنوات الجذور المصابه بالالتهابات قد يسبب انخفاض في عدد كبير من البكتيريا ، ولكن استعمال الطريقة الميكانيكيه لوحدها لا تعمل على تنظيف قناة الجذر المصابه و قتل جميع البكتيريا الموجوده على جدران القناة لذلك تستدعي الحاجه إلى استعمال محاليل اروانيه مضاده للبكتريا لقتل الكائنات الحيه الدقيقة المتبقية. ان الهدف من هذه الدراسه هو تقييم تأثير محلول التيتانيوم رباعي الفلورايد ومغلي الشاي الأخضر كمضاد للبكتريا عند استعماله كمحاليل اروانيه لقنوات جذور الاسنان وذلك بمقارنتها مع هايبيوكلوريت الصوديوم والمحلول المغذي من خلال دراسة الاحياء المجهرية و الجزيئية.

المواد وطرائق العمل: اجريت الدراسة الاحياء المجهرية لتحديد تركيز التيتانيوم رباعي الفلورايد ومغلي الشاي الأخضر التي يكون لها تأثير مضاده للبكتريا ضد عشرة مسحات مأخوذة من قنوات جذور الاسنان المصابه حضنت هوانيا و لاهوانيا باستخدام طريقة ورقة قرص اختبار الانتشار. اما الدراسة الجزيئية اجريت على اربعين طفل حيث تم تقييم تأثير المحاليل الاروانيه على البكتريا باستعمال محلول التيتانيوم رباعي الفلورايد ومغلي الشاي الأخضر مقارنة مع هايبيوكلوريت الصوديوم والمحلول المغذي بواسطة الوقت الحقيقي الكمي لسلسله تفاعل البلمر باستخدام SYBR الأخضر.

النتائج: أظهرت نتائج دراسة الاحياء المجهرية بأن التيتانيوم رباعي الفلورايد بتركيز 5٪ يحقق تأثير مضاد للبكتيريا الهوائية واللاهوائية بينما الشاي الأخضر أظهر تأثيرا مضاد لبكتريا *staphylococcus aureus* عندما اغلي لمدة 20 دقيقة و بتركيز 100ملغم/مللتر ولكن ليست فعاله على الكائنات الحيه الدقيقة الأخرى مثل *Escherichia coli and streptococcus*. أظهرت نتائج الدراسه الجزيئية، ان محلول هايبيوكلوريت الصوديوم هو الأكثر فعالية يليه التيتانيوم رباعي الفلورايد ثم الشاي الأخضر في حين أظهرت الدراسه ان المحلول المغذي ليس له أي تأثير مضاد للبكتريا . كما بينت الدراسه الإحصائية ان هناك فرق معنوي عند مقارنة التيتانيوم رباعي الفلورايد , الشاي الأخضر و هايبيوكلوريت الصوديوم مع المحلول المغذي.

الاستنتاج: اعتمادا على نتائج هذه الدراسه فقد تبين ان التيتانيوم رباعي الفلورايد ومغلي الشاي الأخضر يمكن استخدامه كمحلول ارواني في مجال علاج قنوات الجذور للأطفال.

الكلمات الرئيسية: تأثير مضاد للبكتريا، التيتانيوم رباعي الفلورايد، الشاي الأخضر، هايبيوكلوريت الصوديوم، المحلول المغذي، الوقت الحقيقي الكمي -سلسله تفاعل البلمر.