Original Article

Antifungal Efficacy of Green Tea Extract against Candida Albicans Biofilm on Tooth Substrate

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Abstract

Objectives: Biomechanical preparation and irrigation with antimicrobial solutions are necessary to disinfect the root canal space. This in vitro study aimed to examine the antifungal effect of green tea extract on Candida albicans biofilm formed on tooth substrate.

Materials and Methods: Minimum fungicidal concentration (MFC) and minimum inhibitory concentration at which 90% of the isolates were inhibited (MIC90) were studied using green tea extract and sodium hypochlorite with the broth macro-dilution method. Then, anti-candida effects of this extract were tested on tooth substrates of 45 extracted single-canal premolar teeth. After biomechanical cleaning of the root canals, the teeth were sectioned vertically and randomly divided into three groups of 30. All the samples were infected with C. albicans (PTCC 5027) and exposed to the test solutions (sodium hypochlorite, green tea, normal saline) for five, 10 and 15 minutes. Data analyses of the samples were performed using two-way ANOVA.

Results: The average number of microorganisms showed a significant decrease after five, 10 and 15 minutes of exposure to green tea extract and sodium hypochlorite. The average number of C. albicans in green tea extract and sodium hypochlorite groups decreased to 1/3 and 1/2 of the initial values, respectively.

Conclusion: Antifungal activity of green tea extract was time-dependent and its inhibitory action did not decrease significantly over time. It is recommended to consider other properties of green tea such as tissue solubility, impact on dentin structure and use as an intracanal medicament or for smear layer removal in the clinical setting.

Keywords: Biofilms; Candida Albicans; Green Tea Extract Polyphenone E; Root Canal Irrigants

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INTRODUCTION

Successful treatment of root canal infections depends on destroying the causative organisms and preventing re-infection. Irrigating solutions and other intra-canal medicaments are necessary anti-microbial adjuncts to mechanical cleaning. Several studies have shown that vast areas of canal walls, especially in the apical third and in bar- and oval-shaped canals, cannot be efficiently cleaned via

mechanical cleaning. Chemical disinfection is an important cornerstone to achieve a successful outcome; because it can eliminate the bacteria and fungi in dentinal tubules, porosities and branches of the root canal system [1, 2].

During the 1970s, antibiotics were widely used to sterilize the infected root canals; most antibiotic pastes contained an antifungal agent such as nystatin or sodium caprylate. At that time, there was a tendency towards antifungal therapies for the infected root canals but topical antibiotics lost their popularity after which, due to potential emergence of resistant microorganisms and host sensitivity, antifungal property of endodontic disinfectants was given less attention [3, 4].

Over time, various irrigants have been introduced for root canal disinfection. Although sodium hypochlorite solution is commonly used for root canal irrigation, it is toxic to the living tissues and its extrusion from the tooth apex can cause post-treatment pain, swelling and necrosis. Besides, it has a disagreeable smell and taste for patients and its vapor causes eye irritation [5]. Chlorhexidine gluconate solution is also used as an irrigating solution. This solution, which is a bisbiguanide, has amphiphatic and antiseptic effects and is biocompatible. Tooth discoloration and some other side effects such as loss of sense of taste, irritation of oral mucosa, dry mouth and tongue discoloration limit its use as an irrigant [6]. MTAD, which is a combination of tetracycline, citric acid and detergent, was an attempt to achieve a better cleaner, but in vitro studies showed that its efficiency in killing microbes was lower than that of sodium hypochlorite [7].

Due to the increasing rate of antibiotic-resistant microorganisms and the complications caused by synthetic drugs, herbal alternatives have been recently proposed. Generally, herbal formulations are harmless and nontoxic and they have proven to have strong anti-bacterial effects. Anti-bacterial effects of Zataria multiflora Boiss, Triphala, Morinda citrifolia, Satureja khuzistanica jamzad and green tea have been investigated [6-8].

Tea is the second most consumed beverage in the world; it is produced from the leaves and buds of Camellia sinensis. Depending on the manufacturing process, teas are classified into three main categories: non-fermented green tea, semi-fermented Oolong tea and fermented black and red tea.

Green tea contains higher catechins (a type of polyphenol) than black and Oolong tea. Catechins, especially epigallocatechin gallate (EGCG), have shown strong antioxidant properties in vivo and in vitro. Recent human studies have demonstrated that green tea reduces the risk of cardiovascular diseases and some types of cancer, it improves oral health and physiological functions, it has anti-hypertensive effects, it controls body weight and protects from ultraviolet rays of the sun, it increases bone mineral density and has neuroprotective, anti-bacterial and anti-viral properties [7,9].

Parbhakar et al. evaluated antibacterial effects of herbal detergents such as triphala, green tea with MTAD and 5% sodium hypochlorite against E. faecalis biofilm in vitro and showed that 5% sodium hypochlorite had maximum antibacterial efficacy; triphala, green tea and MTAD also showed significant antibacterial activity. The researchers expressed that the use of herbal solutions as root canal irrigants may be more useful due to certain undesirable properties of sodium hypochlorite [7].

Hirasawa and Takada evaluated the antifungal activity of green tea (catechin) against different strains of C. albicans in various pH conditions using the broth dilution method and concluded that the antifungal activity of EGCG was weakened in acidic conditions. The MIC90 of EGCG increased by more than 10 folds as the pH decreased from 7.0 to 6.5. They also showed synergic antifungal activity of the combination of EGCG and amphotericin B or fluconazole against antimycotic-susceptible and resistant C. albicans. Antifungal activity of

catechin was correlated with pH, and EGCG in a pH of 6 with a concentration of 2000 mg/L, in a pH of 6.5 with a concentration of 500-1000 mg/L and in a pH of 7 with a concentration of 15.6-250 mg/L inhibited the growth of 90% of the C. albicans colonies [10].

Horiba et al. studied different types of green tea against 24 bacterial strains in vitro and concluded that they were effective on most of them, including obligate anaerobes. The researchers attributed this function to the high content of polyphenols, mainly catechins [11].

Due to easy availability, cost-effectiveness, long shelf life, low toxicity and the lack of microbial resistance, we sought to examine antifungal effects of green tea as an irrigant against biofilms formed by C. albicans on extracted teeth.

MATERIALS AND METHODS

A) Preparation of green tea extract:

Tea leaves were purchased from a tea factory in Ramsar. Polyphenol oxidase (PPO) activity of tea leaves was destroyed by dipping them in boiling water for three minutes. After rinsing and drying, the leaves were ground at room temperature. All reagents and chemicals with analytical grade were purchased from Merck (Darmstadt, Germany) and Sigma-Aldrich (Gillingham, Dorset, UK) companies. Tea extract was prepared as follows:

Ten grams of dry sample were extracted three times using 150 mL of hot distilled water (80°C). After cooling to room temperature and filtration, the extract was exposed to the same volume of chloroform to remove caffeine and pigments. Aqueous phase was separated and extracted twice with the same volume of ethyl acetate. Ethyl acetate phase was removed by rotary evaporation under reduced pressure at 40°C. The concentrated extract was dried and weighed at 40°C under vacuum.

B) Microorganism preparation:

Candida albicans strain (PTCC 5027) was obtained from the Collection Center of Industrial Fungi and Bacteria of Iran. A 0.5 McFarland solution was prepared from the mentioned organism and cultured on Sabouraud dextrose agar using the streak method.

C) MIC90 and MFC determination for green tea extract and sodium hypochlorite:

To determine the minimum inhibitory concentration (MIC), eight test tubes were prepared and 2 mL of green tea extract was poured in tube one, and 1 cc of Sabouraud dextrose broth was poured into tubes two through eight. Then, 1 cc of the first tube contents was transferred to the second tube and after shaking thoroughly, 1 cc of the solution was transferred to the third tube. We continued this to tube eight and then discarded 1 cc of the last tube. Thus, tube one contained undiluted extract (100%), tube two contained the extract diluted to 50% (1/2), tube three contained the extract diluted to 25% (1/4) and tubes four to eight contained extracts diluted to 1/8, 1/16, 1/32, 1/64 and 1/128, respectively; 0.1 mL of Candida suspension (turbidity equivalent to 0.5 McFarland) was added to all test tubes. After 24 hours of incubation at 37°C, loop-full samples from transparent tubes were sub-cultured in order to count Candida colonies and calculate MIC and MFC90 of sodium hypochlorite and green tea extract.

D) Biofilm formation on tooth substrate:

Forty-five extracted single-rooted mandibular premolar teeth with fully formed apices were selected.

To confirm that they had one root canal, radiographs were obtained from mesiodistal and buccolingual dimensions. Debris, plaque and tissue residues were removed and the teeth were stored in saline. To standardize the samples, all teeth were cut by a diamond disc under the cementoenamel junction to obtain 8-mm long samples. Root canals were prepared by the crown down technique using rotary ProTaper files. Apical area was prepared to size F3. At all stages of canal cleaning and shaping, 5.25% sodium hypochlorite was used as an irrigant.

All teeth were then sectioned vertically. Samples were randomly divided into three 30-member groups (A, B, C) and placed in test tubes and autoclave-sterilized. We added 3 mL of the culture medium (Sabouraud dextrose broth) to the tubes containing the samples and inoculated them with 0.5 mL of Candida suspension (turbidity equivalent to 0.5 McFarland) and then incubated them at 37°C for 96 hours. To prevent the accumulation of toxic materials and nutrient shortage, the culture medium was refreshed every 24 hours. After 96 hours, the liquid culture medium was removed and we added 3 mL of green tea extract to each tube in group A; 3 mL of sodium hypochlorite was added to each tube in group B and 3 mL of saline was added to each tube in group C. After five, 10 and 15 minutes, the tubes' contents were evacuated and the samples were washed with 5 mL of distilled water. For qualitative analysis, a sterile swab was rubbed on each tooth surface and cultured on the Sabouraud dextrose agar plates. Inoculated plates were incubated at 37 °C for 48 hours for growth investigation. For quantitative analysis, 2 mL of sterile saline was added to the remaining tubes, and after severe vortexing (about one minute), the resulting solution was pour-plated in Sabouraud dextrose agar plates and after 48 hours of incubation at 37°C, the number of colonies was counted as colony forming units per milliliter (CFU/mL) [7]. Data analysis of the samples was performed using two-way ANOVA.

RESULTS

Table 1 shows MIC90 and MFC of the tested solutions against C. albicans. MIC90 of green tea extract was 0.625 mg/mL after 24 hours and its MFC was 1.25 mg/mL after 24 hours.

The statistical results showed that the effects of material and time and the interaction effect of material and time were all significant (all Ps<0.01, Table 2). Analysis of the effect of material on the number of colonies (Table 3) indicated that the average number of colonies after five, 10 and 15 minutes of contact with green tea extract decreased dramatically (P<0.01). In the sodium hypochlorite group, reduction in the average number of colonies during five, 10 and 15 minutes was significant (P<0.01). In other words, within 15 minutes of exposure to C. albicans, green tea reduced the average number of colonies to one-third of the initial value. However, in the sodium hypochlorite group, this reduction was half the initial value. Comparison of the mean number of colonies in nine groups using Duncan's post-hoc test showed that the lowest number of colonies was grown in sodium hypochlorite and then in green tea extract group after 15 minutes of exposure (P<0.01); the difference between these experimental groups was not statistically significant (P>0.05). The highest number of colonies was grown in saline at five, 10 and 15 minutes of exposure. There was no significant difference over time in these groups (P>0.05, Table 4). In terms of antifungal activity and reduction in number of colonies, sodium hypochlorite after 10 minutes of exposure (mean=100) ranked second and sodium hypochlorite after five minutes of exposure (mean=150) and green tea extract after 10 minutes of exposure (mean=150) ranked third. Green tea extract after five minutes of exposure (mean=230) only had a superior antifungal activity to saline.

DISCUSSION

This in vitro study evaluated the antifungal efficacy of green tea as a root canal irrigant against C. albicans biofilm formed on tooth substrates.

Table 1. MIC90 and MFC of tested solutions against C. albicans

Solution	MFC mg/mL	MIC90 mg/mL
Green tea extract (1%)	1.25	0.625
Sodium hypochlorite (5%)	2.5	1.25

Based on preliminary results, MIC of green tea was 0.625 mg/mL and we accordingly used 1% green tea as an irrigant. In our study, antifungal activity of green tea was found to be time-dependent and its inhibitory action increased over time.

Within 15 minutes of exposure of C. albicans to green tea, the average number of colonies reduced to one-third of the initial mean value, while this reduction in sodium hypochlorite was one-half. Thus, shelf life of green tea was longer than that of sodium hypochlorite. Green tea contains many polyphenols particularly flavonoids. Catechins are the main flavonoids found in green tea. This plant also contains gallic acid and fluoride.

Anti-bacterial and anti-viral activities of catechins against different viral and bacterial pathogens have been previously demonstrated [9]. Parbhakar et al, and Horiba et al. proved satisfactory anti-bacterial effects of green tea [7,11]. Hirasawa and Takada also confirmed the inhibitory effects of green tea on C. albicans [10]. Okubo et al. reported that 2.5% black tea extract completely inhibited Trichophyton mentagrophytes and Trichophyton rubrum, but its 10% extract had no effects on C. albicans [12]. The important point in the methodology of the aforementioned studies is direct contact of microorganisms with different concentrations of the disinfectant at various pH values (in the culture medium not the teeth).

Table 2. Two way ANOVA for different groups

Source	Type III Sum of Squares	df	Mean Square	F	P value
Material	736361.111	2	368180.556	2.987E3	.000
Time	43027.778	2	21513.889	174.516	.000
Material * time	30222.222	4	7555.556	61.289	.000
Error	4438.000	36	123.278		
Total	2981063.000	45			

Table 3. The mean number of colonies in CFU/mL (mean ± SD) in different groups

Material	Time			P value*
	5 min.	10 min.	15 min.	
Green tea extract	230 ± 15.1	150 ± 18.7	80 ± 14.1	P<0.01
Sodium hypochlorite	150 ± 15.8	100 ± 12.3	70 ± 12.3	P<0.01
Normal saline	400 ± 7.1	390 ± 10	405 ± 8.7	P>0.05
P value**	P<0.01	P<0.01	P<0.01	

P value*: One-way ANOVA to compare microorganisms at the three different time points
P value**: One-way ANOVA to compare microorganisms among the three different groups

Table 4. Duncan test for comparison of the mean number of colonies in the nine groups

Material	Time	Mean ± SD
Green tea extract	5	230^{b} ± 15.1
	10	150^{c} ± 18.7
	15	80^{e} ± 14.1
Sodium hypochlorite	5	150^{c} ± 15.8
	10	100^{d} ± 12.3
	15	70^{e} ± 12.3
Normal saline	5	400^{a} ± 7.1
	10	390^{a} ± 10
	15	405^{a} ± 8.7

In our study, C. albicans was grown in the form of biofilm, which is an imitation of intraoral conditions. On the other hand, given the loss of antimicrobial activity of irrigants such as chlorhexidine, sodium hypochlorite and potassium iodide/iodine in the presence of dentin [1], the antifungal efficacy of this substance on tooth substrate examined the inhibitory role of dentin against this natural irrigant. It has been proven that the ability to make biofilm and organize the biofilm structure is affected by the chemical nature of the substrate. An experimental work performed on polycarbonate or glass substrates would not be real proof of the microorganism-substrate interactions [7,13]. Therefore, in our study, biofilm was formed on tooth substrate.

In conditions where microorganisms can grow as biofilm, changes in genetic and metabolic processes prevent penetration and performance of antimicrobial agents in the biofilm matrix. Cells in biofilm gain antibiotic resistance up to 1500 times the rate in planktonic cells [10]. As a result, examination of the efficacy of an antimicrobial irrigant on planktonic cells will not reflect its efficacy under in vivo conditions. We also studied antifungal activity of different irrigants on the biofilm media in order to simulate intraoral conditions. The results of studies on antibacterial activity of catechins against phytopathogenic bacteria were consistent with studies on C. albicans [14,15]. Ikigai et al. investigated the mechanism of bactericidal efficacy of catechins and suggested that catechins operate mainly by damaging the bacterial membrane. Antibacterial activity of catechins is mainly related to gallic acid and hydroxyl groups. By inducing rapid leakage of small molecules, catechin entraps them in the intra-liposomal space and accumulates liposomes [16]. Toyoshima et al, also evaluated the mechanism of action of green tea catechin exposed to T. mentagrophytes using an electron microscope and suggested that catechin causes lysis of hyphae and conidia by attacking the cell membrane [17]. Sodium hypochlorite, which is a strong irrigant, has a broad-spectrum anti-microbial activity and destroys proteins and amino acids by releasing free chlorine [3].

Since C. albicans has been isolated from primary endodontic infections, especially in cases of treatment failure from infected root canals, it is of particular importance to disinfect the infected canals from this microorganism. Therefore, our study used C. albicans to test the antifungal effectiveness of irrigating solutions. Candida albicans is a pleomorphic microorganism that can grow in different forms such as germ tubes, yeasts, pseudo- and true-hyphae and chlamydospores depending on the environmental conditions. Morphological and physiological characteristics of C. albicans change quickly by phenotypic switching [1]. Sen et al. showed that C. albicans can grow in various forms in the root canal walls and penetrate into dentinal tubules. This microorganism can also use dentin as a food source [4]. The idea of potential healing effects of herbs has long been considered, but recently it has regained importance and attention. It has been proven that green tea polyphenols are safe and composed of active elements that have physiological effects in addition to anti-oxidant and anti-inflammatory effects [7, 9] and they may have additional beneficial effects compared to common root canal irrigants. In addition, green tea is a very desirable chelating agent [7]. Easy access, cost-effectiveness, long shelf life, low toxicity and lack of antimicrobial resistance are the main benefits of natural alternatives, which have been reported to date [7].

CONCLUSION

The use of green tea as an endodontic irrigant can be helpful, since it is a biocompatible antioxidant and lacks the serious risks associated with the use of sodium hypochlorite. Preclinical and clinical trials are recommended for evaluating its safety and biocompatibility before its use as an intracanal irrigating solution in the clinical setting.

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