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In vitro antimicrobial effects of green tea, microwaving, cold boiled water, and chlorhexidine on *Streptococcus mutans* and *Candida albicans* on silicone pacifiers

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ABSTRACT

Background: This study aimed to compare the antimicrobial effects of green tea, microwaving, cold boiled water, and chlorhexidine (CHX) on *Streptococcus mutans* and *Candida albicans* on silicone pacifiers.

Materials and Methods: In this *in vitro* experimental study, 60 equal-size samples of silicone pacifiers were cut, ultraviolet sterilized, and randomly divided into two groups (n = 30) for immersion in 0.5 McFarland standard suspension of *S. mutans* and *C. albicans*. The samples in each group were then randomly divided into five subgroups (n = 6) for disinfection with 0.12% CHX, cold boiled water, green tea, microwaving for 7 min, and distilled water. The sample suspensions were cultured on blood agar (for *S. mutans*) and Sabouraud dextrose agar (for *C. albicans*) and incubated. The number of colonies was counted after 24 and 48 h. Data were analyzed using the Kruskal–Wallis and Mann–Whitney tests ($P < 0.05$).

Results: At 24 and 48 h, the *S. mutans* colony count was the lowest in CHX and green tea subgroups followed by microwave, cold boiled water, and distilled water subgroups ($P < 0.05$).

Conclusion: CHX and green tea can significantly decrease the *S. mutans* and *C. albicans* colony count on silicone pacifiers.

Key Words: *Candida albicans,* chlorhexidine, microwaves, pacifiers, *Streptococcus mutans*

INTRODUCTION

Pacifiers are extensively used in today's world due to their calming and relaxing effect, improving the sleep quality and decreasing the risk of sudden death syndrome in infants during their first 6 months of life.[1-3] The pacifier tip is in constant contact with the oral normal flora and saliva, and can become contaminated and serve as a route of infection transmission to infants.[4,5] In older children, bacteria can lead to biofilm formation and subsequent development of dental plaque, which can lead to dental caries.[6] Children who use pacifiers for

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longer periods of time often have a higher risk of such conditions.[6,7] Furthermore, the use of pacifier can be associated with middle ear infection in children,[8] dental caries,[9-11] fungal infections,[12] viral infections,[13] asthma,[14] and autoimmune diseases.[15]

Early childhood caries (ECC) is among the most common chronic diseases of childhood. Despite the worldwide reduction in the prevalence of dental caries, the rate of ECC is still high and it is one of the concerns of the World Health Organization.[16]

Streptococcus mutans is the main culprit responsible for the development of ECCs,[17] and evidence shows that it is dominantly present on silicone pacifiers.[18]

Candida albicans is responsible for thrush in infants and children.[12] A previous study reported 80% contamination of pacifiers with *C. albicans*.[6] Another study on infants over 8 months of age showed that the use of pacifiers had a significant relationship with higher prevalence of oral fungal infections, and *C. albicans* was the most commonly isolated species.[12]

Despite the extensive use of pacifiers, studies on ideal methods for disinfection of pacifiers are limited. In general, pacifiers are not disinfected after each time of use and are usually rinsed with water and dried.[19,20]

Chlorhexidine (CHX) is the gold standard antibacterial mouthwash.[21] CHX mouthwash is capable of decreasing dental plaque and pathogenic microorganisms such as *S. mutans*.[22,23] On the other hand, evidence shows that green tea[24] and microwaving[11] affect *S. mutans* and *C. albicans*. Green tea exerts its cariostatic effects by inhibiting bacterial proliferation, preventing bacterial adhesion to the enamel, and inhibiting the bacterial glycosyltransferase and amylase.[25,26] On the other hand, evidence shows that microwave energy can enhance thermal[27] and nonthermal[28] structural changes in the cell wall of microorganisms. Considering the availability of green tea, boiled water, and microwave and gap of information regarding their disinfecting efficacy for silicone pacifiers, this *in vitro* study aimed to assess the antimicrobial effects of green tea, microwaving, cold boiled water, and CHX on *S. mutans* and *C. albicans* on silicone pacifiers.

MATERIALS AND METHODS

This study has been supported by a grant from Isfahan University of Medical Sciences, Isfahan, Iran NO: (398115). This *in vitro* experimental study was conducted on 60 equal-size samples of silicone pacifiers. Sample size was calculated to be 6 in each group considering alpha = 0.05, minimum difference of 1.6, and study power of 80%.

Standard strain *S. mutans* (ATCC 35668) and standard strain *C. albicans* (ATCC 10231) were obtained from the Pasteur Institute in lyophilized form, and 1 mL of them was incubated with Trypticase soy broth at 37°C for 5 h according to the instructions. Next, 100 µL of the culture medium was transferred to blood agar and Sabouraud dextrose agar and cultured, followed by 24 h of incubation (VEVOR Lab Incubator, China) at 37°C.

Next, 3–4 colonies were transferred from the 24-h primary culture to a sterile falcon tube containing sterile saline using a swab to obtain a suspension with 0.5 McFarland standard concentration. This suspension approximately contained 1.5×10^8 colony-forming units per milliliter (CFUs/mL) of *S. mutans* and $1–5 \times 10^6$ CFUs/mL of *C. albicans*.[29]

Thirty silicone pacifiers (Camro, Iran) were unpacked under sterile conditions. The pacifiers were cut into equal-size (15.5 × 6.38 × 12.44 inches) samples under sterile conditions, and each side of each sample was ultraviolet sterilized (Aduro U-Clean, Germany) for 30 min. Next, the samples were randomly divided into two groups (n = 30). The samples in Group 1 were immersed in 0.5 McFarland standard suspension of *S. mutans* while the samples in Group 2 were immersed in 0.5 McFarland standard suspension of *C. albicans* in test tubes for 5 min. They were then immersed in sterile phosphate-buffered saline to eliminate unattached bacteria and fungi. Next, each group was randomly divided into five subgroups (n = 6) for disinfection as follows:

- Subgroup 1: In this subgroup, 0.12% CHX (Perio Aid, Iran) was sprayed on the samples four times and they were then separately placed in sterile containers at room temperature for 1 h in order for the CHX to exert its antimicrobial effect. They were then rinsed with sterile distilled water for 2 s to remove excess solution from the surface[11,30,31]
- Subgroup 2: The samples were immersed in cold boiled water for 15 min and they were then rinsed with sterile distilled water for 2 s[30]
- Subgroup 3: We immersed pacifiers in cold boiled water for 15 min[11] and microwaved them for 7 min at 800 W power. The samples were microwaved (Vitek, Russia) at 800 W power for

7 min in sterile containers. They were placed vertically in the microwave distant from each other. They were then allowed to cool down and were rinsed with sterile distilled water for 2 s[11]

- Subgroup 4: First, 25 g of green tea (Ahmad, Iran) was poured into 1 L of boiling water at 100°C. The container was then capped and allowed to reach room temperature. The tea solution was then sprayed on the samples four times. They were then rinsed with sterile distilled water for 2 s[32]
- Subgroup 5 (control): Distilled water was sprayed on the samples four times and they were rinsed with sterile distilled water for 2 s.[31]

After disinfection, the samples were separately placed in beakers containing 2 mL of 1% sterile phosphate-buffered saline and vortexed for 2 min to detach the bacteria and fungi. Next, 0.1, 0.01, and 0.001 dilutions of the first suspension were prepared, and 0.1 mL of each dilution was added to blood agar by a sampler to assess the proliferation of *S. mutans* and to Sabouraud dextrose agar to assess the proliferation of *C. albicans*. Three repetitions were performed for each sample. The plates were incubated at 37°C (and 5% CO_2 for *S. mutans*) for 48 h. The number of colonies was counted after 24 and 48 h using a colony counter.

All sides of the pacifiers were analyzed, and the sessile colonies/biofilms of mass spectrometry (MS) adhered to latex's surface (based on colony morphology) were counted by a blinded examiner under aseptic conditions, using a stereomicroscope (Nikon, Tokyo, Japan) with reflected light.

The number of colonies/biofilms of MS on the surface of latex after microbial culture was counted and expressed according to a 4-point scoring system: 0 for no MS colonies/biofilms or no bacterial growth, 1 for 1–20 colonies/biofilms of MS, 2 for 21–50 colonies/biofilms of MS, and 3 for >50 colonies/biofilms of MS, which includes intense bacterial growth with confluent colonies, not allowing an accurate counting.

Data were analyzed using SPSS version 24 (SPSS Inc., IL, USA). The Kolmogorov–Smirnov test was used to assess the normal distribution of data. Since data were not normally distributed, comparisons were carried out using the nonparametric Kruskal–Wallis test. Pairwise comparisons were performed using the Mann–Whitney test. The level of significance was set at 0.05.

RESULTS

Table 1 presents the mean count of *S. mutans* colonies in the five subgroups at 24 h. According to the Kruskal–Wallis test, the *S. mutans* colony count was significantly different in the five subgroups at 24 h after culture ($P < 0.001$). The Mann–Whitney test was applied for pairwise comparisons of the subgroups [Table 2] which showed the following order in terms of *S. mutans* colony count at 24 h: CHX = green tea < microwave group = cold boiled water < distilled water ($P < 0.05$).

Table 1 also presents the mean count of *S. mutans* colonies in the five subgroups at 48 h. According to the Kruskal–Wallis test, the *S. mutans* colony count was significantly different in the five subgroups at 48 h after culture ($P < 0.001$). The Mann–Whitney test was applied for pairwise comparisons of the subgroups [Table 2] which showed the following order in terms of *S. mutans*

Table 1: Mean count of Streptococcus mutans colonies in the five subgroups at 24 and 48 h (CFUs/mL)

Time	Group	Mean (CFUs/mL)	SD	Median	*P*
24 h	Green tea	5.4	2.8	0	<0.001
	Microwave	4.65	13.8	20	
	CHX	0.9	0.4	0	
	Cold boiled water	65.4	13.8	20	
	Distilled water	107.4	25.4	40	
48 h	Green tea	7.2	4.2	0	<0.001
	Microwave	86.5	16.1	30	
	CHX	0.9	0.4	0	
	Cold boiled water	62.2	13.6	20	
	Distilled water	158.3	30.9	95	

CHX: Chlorhexidine; SD: Standard deviation; CFU: Colony-forming units

Table 2: Pairwise comparisons of the subgroups in terms of Streptococcus mutans colony count at 24 and 48 h after culture

Groups	*P* value at 24 h	*P* value at 48 h
Green tea and microwave	0.03	0.009
Green tea and CHX	0.59	0.56
Green tea and cold boiled water	0.03	0.01
Green tea and distilled water	<0.001	<0.001
Microwave and CHX	0.01	0.004
Microwave and cold boiled water	1	0.85
Microwave and distilled water	0.02	0.02
CHX and cold boiled water	0.01	0.01
CHX and distilled water	<0.001	<0.001
Cold boiled water and distilled water	0.02	0.01

CHX: Chlorhexidine

colony count at 48 h: CHX = green tea < cold boiled water = microwave < distilled water ($P < 0.05$).

Table 3 presents the mean count of *C. albicans* colonies in the five subgroups at 24 h. According to the Kruskal–Wallis test, the *C. albicans* colony count was significantly different in the five subgroups at 24 h after culture ($P = 0.02$). The Mann–Whitney test was applied for pairwise comparisons of the subgroups [Table 4] which showed the following order in terms of *C. albicans* colony count at 24 h: CHX < green tea < microwave = distilled water < cold boiled water ($P < 0.05$).

Table 3 also presents the mean count of *C. albicans* colonies in the five groups at 48 h. According to the Kruskal–Wallis test, the *C. albicans* colony count was significantly different in the five subgroups at 48 h after culture ($P = 0.04$). The Mann–Whitney test was applied for pairwise comparisons of the subgroups [Table 4] which showed the following order in terms of *C. albicans* colony count at 48 h: CHX < green tea = microwave < distilled water = cold boiled water ($P < 0.05$).

Table 3: Mean count of Candida albicans colonies in the five groups at 24 and 48 h (CFUs/mL)

Time	Group	Mean (CFUs/mL)	SD	Median	*P*
24 h	Green tea	7.6	4.3	0	0.02
	Microwave	12.2	4.9	0	
	CHX	0	0	0	
	Cold boiled water	20	5.3	0	
	Distilled water	14.4	4.9	0	
48 h	Green tea	9.4	5.3	0	0.04
	Microwave	13.1	5.2	0	
	CHX	0.4	0.2	0	
	Cold boiled water	20	7.1	0	
	Distilled water	17.4	5.9	0	

CHX: Chlorhexidine; SD: Standard deviation; CFU: Colony-forming units

Table 4: Pairwise comparisons of the subgroups in terms of Candida albicans colony count at 24 and 48 h after culture

Groups	*P* value at 24 h	*P* value at 48 h
Green tea and microwave	0.04	0.21
Green tea and CHX	0.03	0.03
Green tea and cold boiled water	0.02	0.008
Green tea and distilled water	0.005	0.01
Microwave and CHX	0.009	0.02
Microwave and cold boiled water	0.03	0.01
Microwave and distilled water	0.88	0.03
CHX and cold boiled water	0.004	0.002
CHX and distilled water	0.007	0.004
Cold boiled water and distilled water	0.04	0.46

CHX: Chlorhexidine

DISCUSSION

Considering the need for a safe technique for disinfection of pacifiers and the gap of knowledge regarding the antimicrobial efficacy of green tea for this purpose, this study compared the antimicrobial effects of green tea, microwaving, cold boiled water, and CHX on *S. mutans* and *C. albicans* on silicone pacifiers. The results showed that the *S. mutans* colony count at 24 and 48 h after culture was not significantly different in the CHX and green tea groups and the value in these two groups was lower than that in the microwave and cold boiled water groups (with no significant difference between the latter two). Thus, the parents may be advised to use green tea as their first choice to decrease *S. mutans* count on silicone pacifiers. Microwaving and use of cold boiled water are the next best choices.

Anand *et al*.[33] reported that the effects of neem, garlic, green tea, and 0.2% CHX on *S. mutans* on the surface of toothbrushes were the same and higher than that of CHX, which was in line with our findings. Sato *et al*.[34] assessed the effect of CHX and distilled water alone on *S. mutans* and reported results similar to ours. Komiyama *et al*.[35] and Nelson-Filho *et al*.[36] assessed the effect of CHX on *S. mutans* on toothbrushes and reported results in line with ours. da Silva *et al*.[18] evaluated the effect of immersion of pacifiers in boiled water and microwaving on *S. mutans* count on the surface of pacifiers and concluded that microwaving was more effective for disinfection of pacifiers, which was different from our findings. This difference between their results and ours may be due to different methodologies since they immersed the pacifiers in boiled water for 5–10 min and microwaved them for 5 min while we immersed them in cold boiled water for 15 min and microwaved them for 7 min at 800 W power.

Our results revealed that *C. albicans* colony count in the CHX and green tea groups was significantly lower than that in other groups at 24 and 48 h. At 48 h, the number of *C. albicans* colonies in the microwave group was higher than that in distilled water and similar to that in the green tea group. The *C. albicans* colony count in cold boiled water after 24 and 48 h was significantly higher than that in other groups. These findings may be due to the fact that *C. albicans*

takes a longer time to proliferate in the culture medium and the results are stabilized if the colonies are counted 72 h after culture.

da Silva *et al.*[18] showed that microwaving for 5 min was more effective for disinfection of pacifiers than using boiling water for 5–10 min, which was in agreement with our results. Komiyama *et al.*[35] reported that CHX was the most effective for elimination of *C. albicans* on toothbrushes; their results were in accordance with our findings. Molepo and Molaudzi[11] concluded that microwaving was more effective for elimination of *C. albicans* on pacifiers than CHX, which was different from our findings. This difference may be attributed to the different methodologies. Molepo and Molaudzi[11] sprayed the pacifiers with CHX three times while we sprayed the pacifiers with CHX four times and we allowed it to remain there for 1 h. It was then rinsed with distilled water for 2 s. The current results showed that CHX and green tea significantly decreased the number of *S. mutans* and *C. albicans* colonies and were significantly more effective than microwaving and use of cold boiled water. Considering the availability of green tea, it can be used for disinfection of pacifiers.

This study had an *in vitro* design, which limits the generalization of results to the clinical setting. Future studies are required to assess the minimum inhibitory concentration of green tea. Furthermore, the antimicrobial efficacy of different materials should be evaluated at 72 h and 1 week after the culture of *C. albicans* to obtain more accurate results. Frequency of spraying of CHX and different disinfecting agents available in the market for this purpose should also be evaluated in future studies.

CONCLUSION

Within the limitations of this *in vitro* study, the results showed that CHX and green tea can significantly decrease the *S. mutans* and *C. albicans* colony count on silicone pacifiers.

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Conflicts of interest
The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or nonfinancial in this article.

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