Original Study

ANTI STREPTOCOCCUS MUTANS PROPERTY OF ARGININE IN FLUORIDE AND NON-FLUORIDE CONTAINING FLOWABLE COMPOSITE RESIN

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ABSTRACT

Aim: This study was designed to evaluate the antimicrobial properties of flowable composites containing various weight percentages of arginine against *Streptococcus mutans* and synergistic effect of fluoride and arginine on Streptococcus mutans.

Materials & Method: In this study four groups of non-fluoridated flowable composite (Grandio Flow, Voco) containing 0, 1, 2 and 3% w/w arginine (Merck), and four groups of fluoridated flowable composites (Wave, SDI) with the same weight percents of arginine was prepared and antimicrobial properties of them were examined using direct contact test.

Results: The results showed that with increasing arginine concentration, the growth rate of bacteria decreased, which was significant in both groups of non-fluorided and fluorided resin composites.

Conclusion: Fluoride groups in all concentrations of arginine, the growth of bacteria was lower than that of non-fluorided groups, which was significant only in groups containing 2% and 3% arginine. Moreover arginine showed a synergistic effect with fluoride in suppressing *S.mutans*.

Introduction

Dental caries, known as tooth decay, is considered as one of the most prevalent infectious and chronic diseases. In recent years, an alarming increase has been observed in childhood dental caries, especially in deprived populations.[1] Despite the efforts made to prevent dental caries in recent years (including the use of fluoride compounds), dental caries remain as a public health problem. As it has been since ancient times, the carious lesions occur when the acid products are synthesized by bacteria due to the carbohydrate fermentation and thus dental demineralization.[2]

Among the bacterial flora found in oral cavity, the mutans streptococci species play a key role in the initiation of dental caries.[3] *Streptococcus mutans* is a facultative anaerobic gram-positive cocci bacterium that is colonized on the tooth immediately after its growth, and produces acid by fermenting simple carbohydrates such as sucrose. The growth of *S. mutans* in acidic environment is one of its specific features, which is associated with caries formation.[4]

Free arginine is a natural human salivary component secreted at a mean concentration of 50 μM.[5] This amino acid is a substrate to produce ammonia in microbial arginine deiminase system that can cope with the acid accumulation in the biofilm.[6] Many bacteria, such as streptococci, lactobacilli and spirochetes, convert arginine into ornithine, ammonia and CO_2.[7] Ammonia can produce an alkaline environment that has a positive effect on the balance between demineralization and remineralization.[8] As a result, the ability of biofilms to produce ammonia can be an important factor in preventing decay.[9]

Currently, the majority of dental caries are restored due to beauty issues with tooth-colored restorative materials and composite resins. Studies have shown that microbial plaque is formed mostly on the composite resin surface compared to amalgam and enamel. Recurrent caries have been introduced as the most common reasons for failure of composite restorations.[10] The recurrent caries often occur in the interface between the restoration and cavity walls. The tooth structure is demineralized due to acid-producing bacteria such as *S. mutans* in the presence of fermentable carbohydrates. As a result, an antibacterial restorative material can be effective in preventing recurrence of caries.[11] Due to the lack of antimicrobial activity in composite resins, many efforts have been made to combine them with antibacterial agents such as chlorhexidine, silver nanoparticles, chitosan and ursolic acid.[12-15]

Since the potential of oral bacteria to produce ammonia as a factor in preventing dental caries has many evidences in studies, also because more microbial plaques are formed on composite resin surfaces compared to other restorative materials, the use of arginine in composites can be effective in preventing recurrent caries. In a single study aimed at investigating the effect of arginine and fluoride on oral bacteria in 2015, the synergistic effect of these two agents has been observed on *S. mutans*.[9] Therefore, this study was designed to evaluate the antimicrobial properties of flowable composites containing various weight percents of arginine against *S. mutans* that plays a major role in dental caries. Moreover, the synergistic effect of fluoride and arginine was studied against *S. mutans* by adding arginine to fluoridated flowable composites.

Materials & Method

Preparation of composite specimens

This experimental study consisted of four groups of non-fluoridated flowable composite (Grandio Flow, Voco) containing 0, 1, 2 and 3% arginine w/w (L-Arginine, Merck), and four groups of fluoridated flowable composites (Wave, SDI) with the same weight percents of arginine. For

more precision, each sample was prepared three times, resulting in a total of 24 microtubes containing flowable composite. Table 1 shows the composition of resin-based composites used in this study.

Composite	Resin Composition	Filler weight	Manufacture (LOT NO.)
Grandio flow	Bis- GMA,TEGDMA, HEDMA	80.2% Nanohybrides	VOCO GmbH, Cuxhaven, Germany (1630376)
Wave SDI	Multifunctional Methylacrylic Ester	65% Nanoparticles	SDI Dental Limited, Dublin, Ireland (151043)

Table 1: Composition of resin-based composites used in the study

In this study, 500-µl microtubes were used for bacteriological tests to make direct contact with bacteria floating in fluid medium and resin, and to prevent possible contamination during their adjacency. To create a more contact surface of the bacterial medium with composites inside the microtube, the microtubes mold with putty and a Teflon specimen, smaller than a microtube were made on the putty.

In order to achieve the desired weight percent, a large volume of flowable composite was mixed with the highest weight percent of arginine, stirred for two minutes with a plastic spatula on a glass plate under the hood and in sterile conditions without light, and was placed on a vibrator for 2 minutes to obtain a homogeneous mixture. Subsequent groups were prepared with lower weight percents by accurately calculating the flowable composite weight and arginine percentage by adding flowable composite to the previous prepared sample.

Preparation of microtubes containing flowable composite

Before preparation, the microtubes were sterilized in the autoclave. Then, 200 µl of each flowable composite group was injected into the microtubes using an insulin syringe. The Teflon model was introduced into the microtube to spread composites at all surfaces and then its polymerization was carried out using light curing device (Bluephase C5-Ivoclar Vivadent). Thus, a certain volume of microtubes was occupied by a flowable composite (200 µl) and 300 ml remained in the chamber for 0.5 McFarland standard solution of *S. mutans*.

Laboratory experiments

Direct contact testing was performed to evaluate the antibacterial properties of the free surfaces of arginine-containing flowable composite. Then, 10 µl of microbial suspension equivalent to 0.5 McFarland *S. mutans* ATCC 35668 (1.5×10^8 CFU/ml) prepared from the Pasteur Institute of Iran was poured in the free space of the microtubes. The microtubes were incubated for one hour in sterile conditions. After complete evaporation of the solution, 300 µl of Brain-heart infusion (BHI) medium was added to each of the specimens. The lid of the containers was completely closed, incubation was carried out at 37°C for 24 hours, and then 10 µl of the solution in each of the microtubes was cultured on blood agar medium. The plates were incubated at 37°C for 24 hours and the appeared colonies were counted by colony counting methods. For a positive control group, a microtube without composite was prepared, but in conditions similar to the specimens. The bacteria were cultured inside it at the times similar to the samples.

Since the arginine solubility in water is 14.87 g/100 ml, it is expected that the arginine will be released from the composite composition in the oral environment over time. In order to determine the stability of the arginine effect on the composite, another culture was prepared again from the microtubes after one month, and the number of grown colonies was counted. During this time, the microtubes were incubated at 37°C and their contents were washed daily with artificial saliva (Hypozalix-Biocodex).

Since the baseline number of bacteria present in each of the microtubes and the volume of the solution are clear, the reduction in the number of bacteria represents the antimicrobial effect.

The data obtained from this research were analyzed by a statistician in SPSS version 23 software using Wilcoxon, Mann-Whitney and Kruskal wallis tests.

Results

The Kolmogorov-Smirnov (K-S) test was used to analyze the data distribution in each experimental group. The test showed that all of the raw data obtained in the studied groups had non-normal distribution.

Table 2 shows the mean, standard deviation and median of the bacteria count grown after 24 hours on the surface of the groups in the direct contact test.

According to this table, the bacterial growth rate was reduced by increasing the concentration of arginine, which was significant in both groups of composite resins with and without fluoride ($p<0.05$).

Arginine Percentage / Groups	0 Mean SD (Median)	1 Mean SD (Median)	2 Mean SD (Median)	3 Mean SD (Median)
Non-fluoridated	$65\times10^6 \pm 35.3\times10^6$ $(65\times10^6)^{A,a}$	$19.3\times10^6 \pm 17.9\times10^6$ $(10^6)^{A,ab}$	$3\times10^4 \pm 2\times10^4$ $(3\times10^4)^{A,ab}$	$17\times10^2 \pm 15\times10^2$ $(9\times10^2)^{A,b}$
Fluoridated	$53.5\times10^4 \pm 40.1\times10^4$ $(30\times10^4)^{A,a}$	$1.1\times10^4 \pm 7.9\times10^3$ $(8\times10^3)^{A,ab}$	68 ± 75 $(54)^{B,b}$	0 ± 0 $(0)^{B,b}$

* Groups that share a superscribe uppercase letter were not significantly different within a row

* Groups that share a superscribe lowercase letter were not significantly different within a column

Table 2: The mean, standard deviation and median of the bacteria count grown at the baseline

Also, in fluoridated groups in all concentrations of arginine, the bacterial growth was lower than that of non-fluoridated groups. However, this difference was significant only in groups containing 2% and 3% arginine, but no significant difference was observed in other concentrations.

This table shows that the number of bacteria in all groups was lower than the control group (without arginine and without fluoride).

The direct contact test was repeated on samples after a month of daily washing of samples with artificial saliva. Table 3 presents the mean, standard deviation and median of the bacteria count grown in each group.

Arginine Percentage / Groups	0 Mean ± SD (Median)	1 Mean ± SD (Median)	2 Mean ± SD (Median)	3 Mean ± SD (Median)
Non-fluoridated	$45 \times 10^6 \pm 49.4 \times 10^6$ $(45 \times 10^6)^{A,a}$	$86.6 \times 10^6 \pm 15.2 \times 10^6$ $(9 \times 10^6)^{A,ab}$	$11.3 \times 10^4 \pm 7.7 \times 10^4$ $(9 \times 10^4)^{A,ab}$	52 ± 43 $(58)^{A,b}$
Fluoridated	$43.3 \times 10^6 \pm 41.6 \times 10^6$ $(30 \times 10^6)^{A,a}$	$3.3 \times 10^6 \pm 2.5 \times 10^6$ $(3 \times 10^6)^{A,ab}$	$20 \times 10^2 \pm 10 \times 10^2$ $(22 \times 10^2)^{B,b}$	0 ± 0 $(0)^{B,b}$

* Groups that share a superscribe uppercase letter were not significantly different within a row

* Groups that share a superscribe lowercase letter were not significantly different within a column

Table 3: The mean, standard deviation and median of the bacteria count grown in one month.

This table also shows that the growth rate of bacteria decreases with increasing arginine concentration. This decrease was significant in both groups of composite resins with and without fluoride ($p<0.05$).

Also in fluoridated groups at all arginine concentrations, the growth of bacteria was lower than that of non-fluoridated groups, which was significant only in groups containing 2% and 3% arginine, but no significant difference was observed in other concentrations.

In comparing 24 hours and 1 month with Wilcoxon test, no significant difference was found among any of the groups.

A pairwise comparison of arginine percentages using Wilcoxon test at both times suggests that the difference in the bacteria count is significant only between 3% and 0% of arginine concentrations in the non-fluoridated composites, and was not significant in pairwise comparison of other concentrations. However, the difference between the concentrations of 2% and 3% with 0% was significant in the fluoridated composite.

Discussion

In this study, the effects of fluoridated and non-fluoridated flowable composites were investigated against *S. mutans* after adding arginine at concentrations of 0%, 1%, 2% and 3% by weight. The results of the direct contact test showed that arginine-containing flowable composite in both non-fluoridated and fluoridated groups significantly prevented the growth of *S. mutans* compared to the control group without arginine; the growth of bacteria was reduced by increasing the concentration of arginine.

This study employed the direct contact testing to evaluate the antibacterial properties of arginine-containing composites. This test is used to investigate the antibacterial properties of low solubility solid materials in which the bacteria are in direct contact with the sample tested under controlled conditions, and it is possible to count the number of grown bacteria.

The addition of arginine to composite was conducted for the first time in this study and no studies have investigated so far the anti- *S. mutans* properties in arginine-containing composites. In the study, the number of grown bacteria was reduced by adding arginine weight percentages. Statistical analyses demonstrated that the difference in the bacteria count in non-fluoridated composite in the group containing 3% arginine with 0% arginine was significant. However, in the fluoridated composite, this difference was significant in the groups containing 2% and 3% arginine in the arginine-free group.

Study by Nascimento *et al*. in 2014 with the aim of evaluating the effect of arginine on oral biofilm communities indicated that the use of non-fluoridated toothpaste containing 1.5% arginine twice a day for 4 weeks changed the microbial profile of people with caries toward more healthy microbial population. It was more similar to the microbial profile of non-decay individuals. They concluded that compounds containing arginine had an anti-decay effect due to increased activity of the arginine deiminase system and the potential for changing the microbial composition of the mouth toward a more favorable profile.[16]

Koopman *et al* in 2017 studied changes in oral ecosystems caused by the use of toothpaste with 8% arginine and concluded that arginolytic capacity was increased and metabolic activity of sucrose was reduced, in fact, in the presence of arginine in toothpaste. In addition, they found that the salivary microbial composition goes towards a healthy ecology using this toothpaste.[17]

Chakrabory and Burne in 2017 examined the effects of arginine on growth, virulence gene expression and stress tolerance by *S. mutans*. They indicated that arginine had a negative effect on the growth and potential of pathogenic mutations. In our study, it was also shown that the count of *S. mutans* was decreased with increasing the arginine percentage.[18]

In this study, the pairwise comparison revealed that the bacterial growth was lower in fluoridated group at all arginine concentrations compared to the non-fluoridated group; however, this difference was significant only in groups containing 2% and 3% arginine, but no significant difference was observed in other concentrations.

In studies on antibacterial effect of fluoride, there are conflicting results about the direct antibacterial effect of this substance.

RomShi and Raina *et al*. in 2017 conducted a randomized clinical trial to compare the antibacterial efficacy of 0.5% sodium fluoride impregnated miswak and plain iswak sticks on *S. mutans*. They concluded that the use of fluoridated miswak sticks for 6 days caused statistically significant reduction in *S. mutans* count compared to the baseline count in the saliva. However, there was no significant difference in anti-bacterial effect of fluoridated miswak sticks and plain miswak sticks on *S. mutans* count.[19] In our study, the difference in *S. mutans* count between the two composites with and without fluoride in 0% and 1% arginine was not significant.

In addition, Cheng *et al*. in 2012 found that the biofilms formed on non-fluoridated composites had greater metabolic activity and colony count. The fluoridated composites somewhat showed a decrease in biofilm growth and colony count compared to non-fluoridated composites, and stated that fluoride ion inhibits metabolic pathways such as the bacterial fermentation pathway to produce lactic acid.[20]

In our study, in the groups containing 2% and 3% arginine, the difference in the number of bacteria grown on the composites with and without fluoride was significant.

In the present study, the difference in antibacterial activity of fluoridated and non-fluoridated composites in different percentages of arginine was compared and it was shown that this difference was significant only in 2% and 3% arginine. However, there was no significant difference at a lower concentration of 1 % and without arginine samples, due to the synergism of fluoride and arginine, also mentioned in the study of Zheng X. *et al*. in 2015. Zheng X. examined combinatorial effects of arginine and fluoride on oral bacteria (*S. mutans*, *S. sanguinis* and *Porphyromonas gingivalis*) in planktonic and biofilm cultures, and concluded that arginine has a synergistic effect with fluoride in suppressing acidogenic *S. mutans* in both cultures, but *S. sanguinis* was increased in multispecies biofilms.[9]

Since the arginine solubility in water is 14.87 g/100 ml, another culture was prepared again from the microtubes after one month in order to determine the stability of the arginine effect on the composite and the number of cultured colonies was counted. The results of colony count showed that the number of grown bacteria was increased in all groups though this difference was not significant. Given that no investigations similar to this study have been done so far, it is recommended to consider longer periods to check the time effect.

Conclusion

- The flowable composite containing 1% to 3% by weight of arginine in the direct contact test showed anti-mutans property; the number of bacteria was significantly decreased with increasing the arginine concentration.
- The fluoridated flowable composites had more anti-mutant properties compared to the non-fluoridated composites, which was significant in 2% and 3% arginine percentages.
- There was no decrease over time in the anti- mutans effect of composites containing different percentages of arginine.

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