



Antibacterial Effects of Orthodontic Primer Harboring Chitosan Nanoparticles against the Multispecies Biofilm of Cariogenic Bacteria in a Rat Model

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Abstract

Introduction: Microbial biofilm accumulation around orthodontic brackets and composite is a common complication of fixed orthodontic treatment. This study assessed the antibacterial effects of orthodontic primer containing chitosan nanoparticles (CNPs) against the multispecies biofilm of cariogenic bacteria in a rat model.

Materials and methods: Transbond XT orthodontic primer containing 0%, 1%, 5%, and 10% CNPs was experimentally prepared. The Wistar rats were randomly divided into four groups (n=7) of control (0% CNPs), 1%, 5% and 10% CNPs. The oral cavities of the rats were infected with cariogenic bacteria. After anesthetizing the rats, 1 drop (10 µL) of primer with different concentrations of CNPs was applied to their central incisor and light-cured for 20 seconds. Transbond XT orthodontic adhesive (2 × 2 mm) was applied on the primer. Another drop (10 µL) of primer was applied and light-cured for 40 seconds. The number of *Streptococcus mutans*, *Streptococcus sanguinis*, and *Lactobacillus acidophilus* colonies in the saliva of rats was quantified at 24 hours, 4 days and 7 days.

Results: Adding 1% ($p=0.005$), 5% ($p<0.001$) and 10% ($p<0.001$) of CNPs to orthodontic primer significantly reduced the *S. mutans* colony count at 24 hours compared with the control group. At 24 hours, the mean *S. sanguinis* colony counts in the 5% ($p=0.04$) and 10% ($p=0.02$) CNP groups were significantly lower than that in the control group. Also, at 4 and 7 days, the mean colony counts in the 5% and 10% CNP groups were significantly lower than that in the control group ($p<0.05$). At 24 hours and 4 days, the mean *L. acidophilus* colony count in the 10% CNP group was significantly lower than that in the control group ($p<0.05$). At 7 days, rats with failed adhesive showed a significantly higher count of all three bacteria compared with rats with adhesive ($p<0.05$).

Conclusions: The addition of 5% CNPs to orthodontic primer significantly decreased the colony count of cariogenic bacteria in rats.

Keywords

cariogenic bacteria, chitosan, composite, nanoparticles, orthodontic adhesive, primer, rat

INTRODUCTION

Enamel demineralization of surfaces adjacent to or under orthodontic appliances is a common complication of fixed orthodontic treatment.¹ The majority of orthodontic patients have at least one mild white spot lesion (WLS) on their teeth.² A previous study reported that 38% and 46% of patients who received fixed orthodontic treatment for 6 months and one year, respectively, had mild to severe WLSs.^{1,2}

Microbial biofilm accumulation around orthodontic brackets and composite is a common finding.³ The surface properties of composite enhance plaque accumulation as well. On the other hand, oral hygiene control is much more difficult for orthodontic patients and requires high patient cooperation. Thus, antibacterial agents are increasingly used to prevent WLSs in orthodontic patients. For instance, composites containing nano-fillers providing sustained release of fluoride over a long period were introduced to the market.⁴ Many attempts have been made to prepare composites with antimicrobial and antiplaque properties. Nano-filled composites have minimum adhesion to cariogenic bacteria such as *Streptococcus mutans*.⁵ Addition of chlorhexidine to dental composites has also shown antibacterial effects; these effects, however, proved to be short term.⁶ Antimicrobial orthodontic cements and primers containing silver nanoparticles have also been designed, which can efficiently prevent bacterial growth and proliferation around fixed orthodontic appliances.⁷ Adding nanoparticles to composite is another effective method to decrease the microbial plaque accumulation around restorations and orthodontic brackets. The addition of polyethyleneimine nanoparticles to composite not only confers biocompatibility but also decreases the count of cariogenic bacteria.⁸ Chitosan is another product with optimal antiplaque and antimicrobial properties.^{9,10} Chitosan is a natural, non-toxic polymer prepared by the de-N-acetylation of chitin, which is the main constituent of the hard shell of crustaceans. Evidence shows that chitosan nanoparticles (CNPs) have antimicrobial properties.¹¹ A previous study also showed that surfaces coated with chitosan have efficient anti-biofilm properties.¹²

S. mutans, *S. sanguinis* and *Lactobacillus acidophilus* are the most common cariogenic microorganisms in oral microbiome.¹³ *L. acidophilus* is mainly responsible for progression of enamel caries into dentin and deeper areas, and the count of this microorganism is correlated with the amount of fermentable carbohydrate. *S. sanguinis* is associated with dental plaque biofilm and is among the first bacteria colonizing the oral cavity. It helps the attachment of other microorganisms and plays a key role in biofilm maturation in the oral cavity.^{14,15}

Considering the optimal antibacterial properties of chitosan and the need for preparation of orthodontic primers and adhesives with antibacterial properties, this study aimed to assess the antibacterial effect of orthodontic primer containing CNPs against the multispecies biofilm of cari-

ogenic bacteria namely *S. mutans*, *S. sanguinis* and *L. acidophilus* in rats. We hypothesize in the present study that there is a statistically significant difference between the antibacterial activity of the orthodontic primer containing CNPs and the original orthodontic primer against the multispecies biofilm of cariogenic bacteria in a rat model. Under the null hypothesis, this difference is insignificant.

MATERIALS AND METHODS

This animal study evaluated 36 Wistar rats initially weighing 150-200 g. The study was approved by the Ethics Committee of Tehran University of Medical Sciences (IR.TUMS.DENTISTRY.REC.1396.2773) and was conducted in accordance with the Declaration of Helsinki with regard to the guidelines for care and use of laboratory animals.

Sample size was calculated to be 7 rats in four groups (a total of 28) according to previous studies.^{13,16} A pilot study was performed to assess the effect of 0%, 1%, 5%, and 10% concentrations of CNPs against the cariogenic bacteria in 8 rats in 4 groups (n=2). These concentrations were selected according to previous studies.¹³

Preparation of CNPs

In order to prepare the appropriate stock solution of CNPs in accordance with the study of Govindarajan et al.¹⁷, 50 mg of chitosan powder (Merck, Darmstadt, Germany) was taken in 20 mL of deionized distilled water. Then 1 mL of 1% acetic acid solution (Merck KGaA, Darmstadt, Germany) was added dropwise to a solution that was shaken in a magnetic stirrer to create a homogeneous CNPs solution. The CNPs solution was lyophilized and used for further experiments. The morphological analysis of CNPs was performed using scanning electron microscopy (SEM). In addition, the particle size and ζ -potential of the prepared CNPs were determined by dynamic light scattering (DLS) technique.

Preparation of orthodontic primer

Transbond XT orthodontic primer (3M Unitek, Monrovia, CA, USA)¹⁴ was used to prepare the experimental orthodontic primers containing 0%, 1%, 5%, and 10% CNPs. For this purpose, first, one drop of Transbond XT orthodontic primer (50 μ m) was weighed by a digital scale with 0.001 mg accuracy (weighing 0.05 g). A 10-100 μ m sampler was used to standardize the drops. In order to prepare 20 drops of each experimental primer (50 μ m each) for each group, 1 g of primer was mixed with 0.01, 0.05, and 0.1 g of CNPs for the control (no CNPs), 1% CNP, 5% CNP, and 10% CNP groups, respectively using a small spatula. More dispersing of nanomaterials into the primer preform was performed by applying an ultrasonic bath for 30 minutes. The prepared experimental primers were transferred into microtubes. The microtubes were then covered with aluminum wraps to prevent contact with air and exposure to light.

Preparation of rats

Forty-eight Wistar rats (150-200 g; Pasteur Institute, Tehran, Iran) were housed one rat per cage, (22–25°C and 12 h light/dark cycles) under sanitary conditions, with access to sanitized food and water. Rats were acclimated to room conditions for 7 days prior to each experiment.

Since the oral microbial flora of the rats is different from that of humans, the rats had to become oral germ-free first based on the previous study.¹⁸ After that, the samples were obtained from the oral cavity of the rats and cultured on brain heart infusion (BHI) agar (Merck, Germany) to ensure that the rats were oral germ-free. Next, a suspension of three cariogenic bacteria was prepared¹⁹ including *S. mutans* (ATCC 25175) in an amount of 3×10^8 colony forming units per milliliter (CFUs/mL), *S. sanguinis* (ATCC 10556) in an amount of 3×10^8 CFUs/mL and *L. acidophilus* (ATCC 4356) in an amount of 3×10^9 CFUs/mL. The oral cavity of the rats was inoculated with this suspension using a swab.²⁰ The process of infecting the oral cavity of the rats with the bacterial suspension continued three consecutive days using a swab. After 24 hours, saliva samples were collected from the rats and cultured on BHI broth (Merck, Germany). The samples were then cultured on Mitis Salivarius-Mutans valinomycin agar, modified medium 10-sucrose agar, and Man Rogosa and Sharpe-clindamycin ciprofloxacin agar to determine the presence of *S. mutans*, *S. sanguinis*, and *L. acidophilus*, respectively.^{21,22}

The rats with confirmed colonization of all three cariogenic bacteria in their oral cavity were selected for the next phase of the study. A total of 28 rats that met this condition were randomly divided into 4 groups (n=7).

Application of orthodontic primer and adhesive

The rats were first anesthetized by intraperitoneal injection of ketamine and xylazine using a 1-mL syringe. The amount of anesthetic agent required for each rat was found to be 50 mg/kg ketamine and 6 mg/kg xylazine based on their weight.²³ The rats were then fixed on a wooden board, their maxillary central incisor was dried with a cotton pellet, etched with 37% phosphoric acid for 40 seconds²⁴ and cleaned with a moist cotton pellet. The tooth was then rinsed and dried. Care was taken not to allow saliva contamination.²⁵ Next, 10 µL of primer was applied on the central incisor and cured for 20 seconds. A thin layer of adhesive (Transbond XT; 3M Unitek, Monrovia, CA, USA) measuring 2×2 mm was then applied to the labial and proximal surfaces of the tooth and spread to form a very thin layer. Next, another 10 µL of primer was applied over the adhesive and cured for 40 seconds. Mandibular central incisors were shortened by 2 mm in order to prevent trauma from occlusion. The steps of the orthodontic adhesive with combined primer and adhesive are shown in Fig. 1. The status of adhesive was evaluated after 24 hours, 4 days and 7 days, and saliva samples were collected from all rats at the de-

signed time points. The samples were immersed in BHI broth and the number of colonies was counted after culture (as explained earlier). At day 14, no adhesive remained in the oral cavity of the rats.

Statistical analysis

One-way ANOVA was used to compare the CFU/mL of colonies at each time point. An independent sample t-test was used to compare the count of bacteria on day 7 in rats with failed adhesives. Tukey's post hoc test was used for pairwise comparisons. Data were analyzed using SPSS version 23.0 (SPSS Inc., IL, USA) at 0.05 level of significance.

RESULTS

As shown in Fig. 2, the CNPs are nano-sized particles, approximately 50-90 nm in diameter, with uniform shapes. The results of DLS showed that the mean diameter and polydispersity of nanoliposomes were 75 nm and 0.45, respectively, and the ζ-potential of CNPs was -10.2 mV. In general, these results confirm the successful synthesis of CNPs.

Fig. 3 shows the *S. mutans*, *S. sanguinis*, and *L. acidophilus* colony counts in the saliva of rats following exposure to orthodontic primer containing 0%, 1%, 5%, and 10% CNPs for 24 hours, 4 and 7 days. One-way ANOVA showed a significant difference in *S. mutans* colony count in presence of different concentrations of CNPs at 24 hours ($p < 0.001$), 4 days ($p = 0.001$) and 7 days ($p = 0.001$). According to the finding in Fig. 3a, 37.3% ($p = 0.005$), 85.3% ($p < 0.001$) and 98.5% ($p < 0.001$) reduction was shown in *S. mutans* colony count following exposure to 1%, 5% and 10% concentrations of CNPs, respectively compared to the control group at 24 hours. The colony counts of *S. mutans* in presence of 1% CNPs were significantly higher than that in the presence of 5% ($p < 0.001$) and 10% ($p < 0.001$) CNPs. At 4 days, 45% ($p = 0.008$), 83.7% ($p = 0.004$) and 96.1% ($p = 0.001$) reduction in *S. mutans* colony count was observed in the presence of 1%, 5%, and 10% CNPs, respectively compared with the control group (Fig. 3a). Also, 66.2% ($p = 0.026$) and 96.0% ($p = 0.001$) reduction in *S. mutans* was displayed in the presence of 5% and 10% CNPs, respectively compared to the control group at 7 days.

Results of one-way ANOVA analysis showed a significant difference in *S. sanguinis* colony count in presence of different concentrations of CNPs at 24 hours ($p = 0.008$), 4 days ($p = 0.026$) and 7 days ($p = 0.012$). A significant reduction was seen in *S. sanguinis* colony count following exposure to 5% and 10% concentrations of CNPs after 24 hours, 4 and 7 days compared with the control group (Fig. 3b). However, the mean colony counts in the presence of 10% CNPs was significantly lower than that in the presence of 1% CNPs at 4 and 7 days ($p = 0.011$).

A significant difference in *L. acidophilus* count in the presence of 1%, 5%, and 10% CNPs at 24 hours ($p < 0.05$) but no significant difference was observed in the presen-

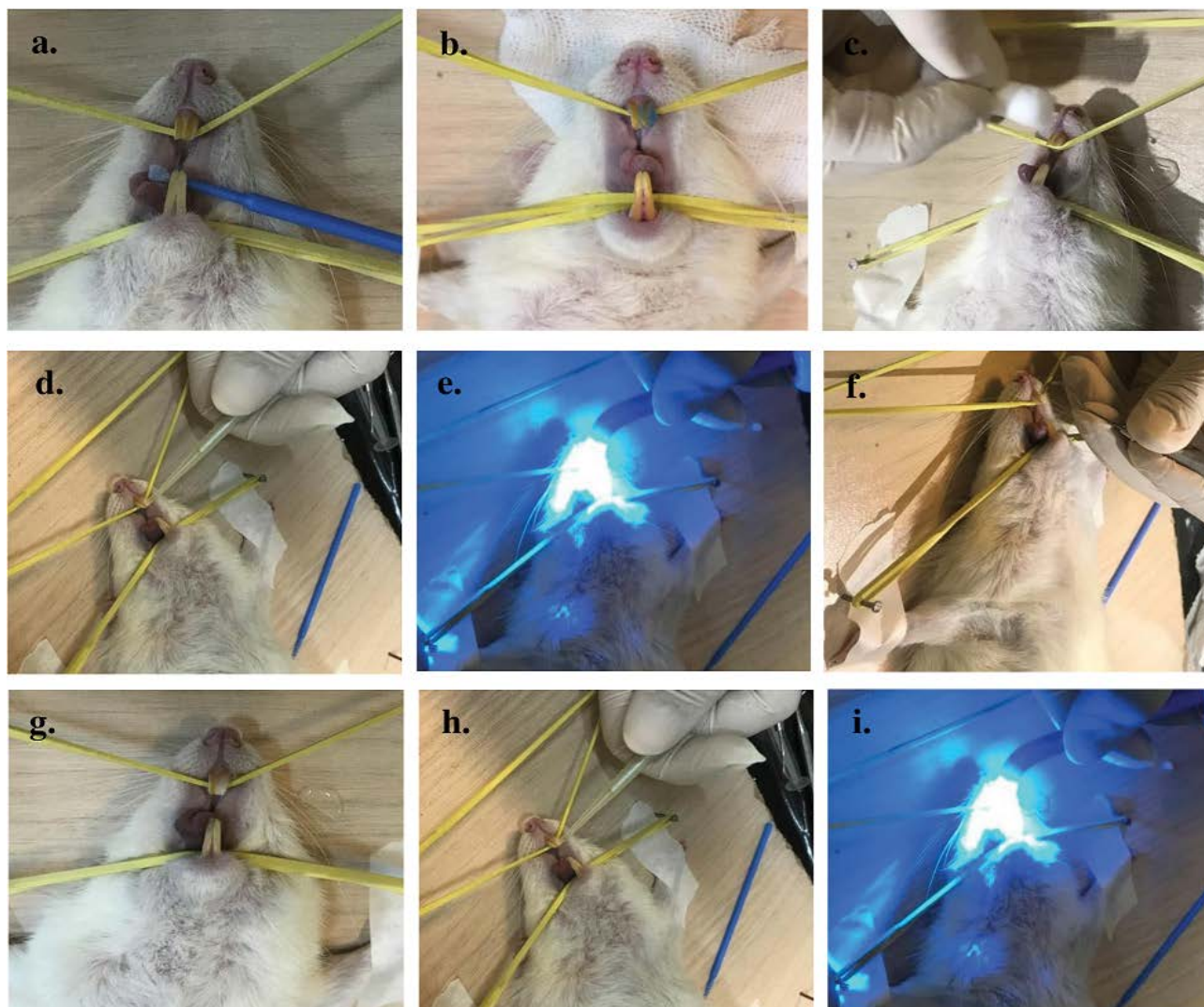


Figure 1. The steps of the orthodontic adhesive with combined primer and adhesive. a) Fix the rat; b) Etching; c) Washing and drying; d) Adding the primer; e) Curing the primer; f and g) Adding the adhesive; h) Adding the primer; i) Curing the primer.

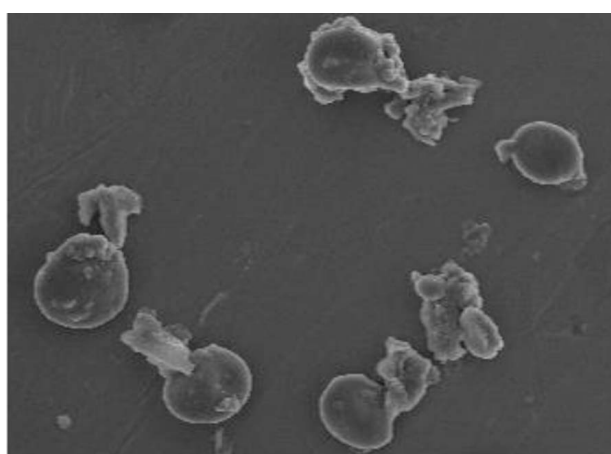


Figure 2. SEM image of synthesized CNPs at $\times 6000$ magnification (scale bar represents 100 nm). The CNPs in 50-90 nm in diameter with uniform shapes were confirmed the successful synthesis of CNPs.

ce of 5%, and 10% CNPs at 4 days ($p > 0.05$) and 7 days ($p > 0.05$). As shown in **Fig. 3c**, there was 98.2% ($p = 0.012$), 99.1% ($p = 0.001$), and 95.7% ($p = 0.012$) reduction in *L. acidophilus* count in the presence of 10% CNPs at 24 hours, 4 and 7 days, respectively compared with the control group. The difference between 1% CNP and 10% CNP groups was also significant at all examined times ($p = 0.011$).

A comparison of colony counts between the two groups of successful and failed adhesives at 7 days was shown in **Table 1**. At 7 days, rats with failed adhesives had significantly higher mean count of *S. mutans* ($p < 0.001$), *S. sanguinis* ($p = 0.001$) and *L. acidophilus* ($p = 0.003$) compared with rats with successful adhesives (**Table 1**).

DISCUSSION

This study assessed the antibacterial effects of orthodontic primer containing CNPs against the multispecies biofilm of cariogenic bacteria in rat model. The results showed that at

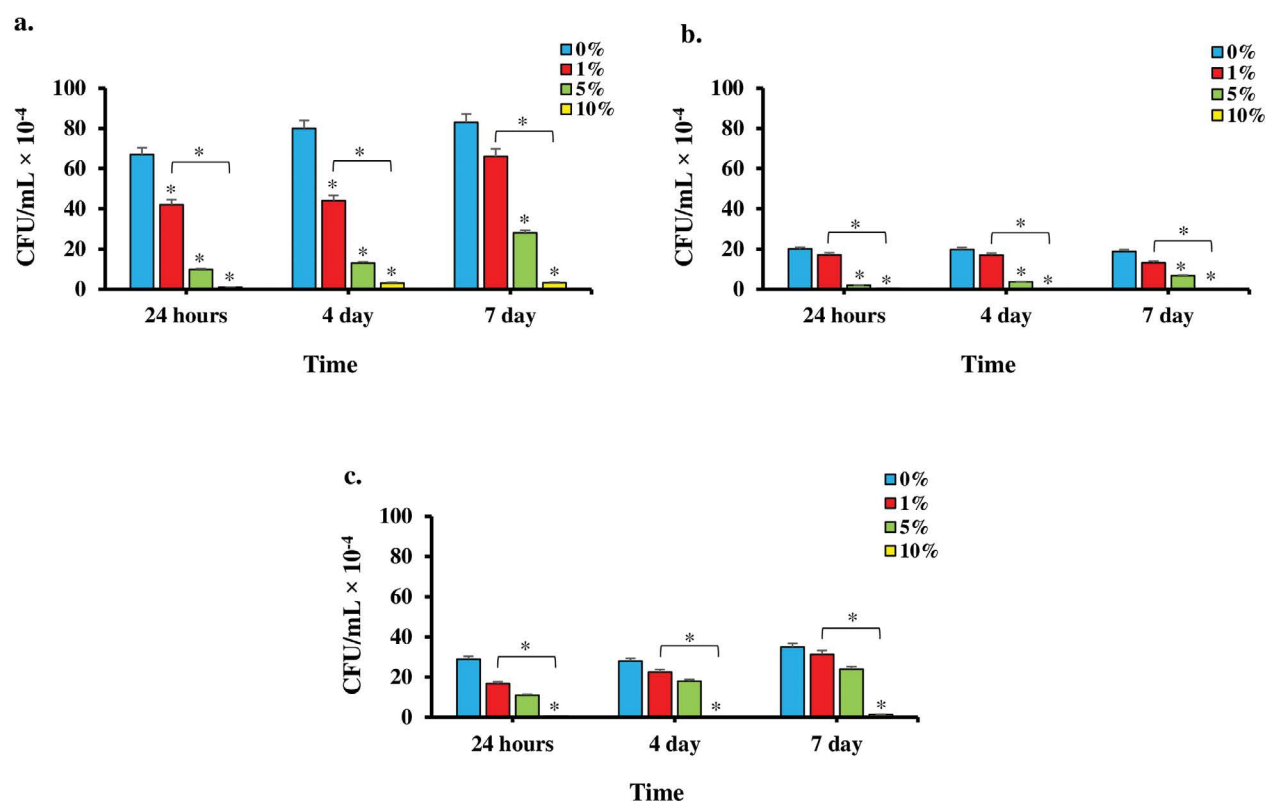


Figure 3. Microbial colony count (CFUs/mL) in the saliva of rats following exposure to orthodontic primer containing 0%, 1%, 5%, and 10% CNPs for 24 hours, 4 and 7 days (n=7). a) *S. mutans*, b) *S. sanguinis*, c) *L. acidophilus*. Orthodontic primers containing 10% CNPs were effective against *S. mutans*, *S. sanguinis* and *L. acidophilus* up to 7 days in rat model.

Table 1. Colony count (CFUs/mL) of cariogenic bacteria between the two groups of rats with successful and failed adhesives at 7 days using independent sample t-test

Bacteria	Restoration status	Number	Mean \pm SD ^c	p value
<i>S. mutans</i>	FR ^a	16	$6.98 \times 10^5 \pm 0.44$	< 0.001
	SR ^b	12	$1.26 \times 10^5 \pm 0.17$	
<i>S. sanguinis</i>	FR	16	$1.55 \times 10^5 \pm 0.13$	0.001
	SR	12	$2.47 \times 10^4 \pm 0.35$	
<i>L. acidophilus</i>	FR	16	$3.15 \times 10^5 \pm 0.25$	0.003
	SR	12	$5.82 \times 10^4 \pm 0.11$	

^a failed restoration; ^b successful restoration; ^c standard deviation

24 hours, 4 days and 7 days, by an increase in the concentration of CNPs from 1% to 10%, the number of *S. mutans*, *S. sanguinis* and *L. acidophilus* colonies decreased; this finding indicates that the antimicrobial effects of CNPs were dose-dependent. However, the number of *S. sanguinis* colonies in the presence of 1% concentration was higher than that of the control group, but not significantly higher. Also, by an increase in time from 24 hours to 7 days, the number of *S. mutans* and *L. acidophilus* colonies increased. However, the number of *S. sanguinis* colonies decreased, which can indicate successful colonization of *S. mutans* and its successful competition with *Lactobacillus*. It should be noted, though, that the bacterial count depends on a number

of factors such as the consumed foods and drinks, oral habits, method of sampling and processing of samples. Also, this increase in colony count over time (from 24 hours to 7 days) may indicate that the antimicrobial properties of CNPs decrease over time, which may be due to decreased release of CNPs over time; this finding was in line with the results of Sodagar et al.¹³ However, this increase in colony count was significantly lower compared with the control group.

The present study results showed that the addition of CNPs significantly decreased the *S. mutans* and *S. sanguinis* colony counts compared with the application of primer without CNPs. Also, the reduction in colony count incre-

ased with the increase of CNPs concentration. This finding was in line with that of Sodagar et al.¹³ that showed that the addition of 10% CNPs decreased the *S. mutans* and *S. sanguinis* colony counts. Similar results have been reported with the use of zinc oxide nanoparticles²⁶, TiO₂ nanoparticles²⁷, and zinc-oxide-CNPs.²⁸

S. mutans and *S. sanguinis* are in balance in the oral cavity and the presence of *S. sanguinis* can decrease the count of *S. mutans*.²⁹ Considering the reduction in colony count of *S. sanguinis* in this study following the addition of CNPs, it can be concluded that CNPs decrease both *S. mutans* and *S. sanguinis* colony counts and this reduction in colony count was not selective. However, since the magnitude of reduction of *S. mutans* colony count was greater than the magnitude of reduction of *S. sanguinis* colony count, the ultimate result would be favourable. In line with our findings, Sodagar et al.¹³ also reported that the reduction in *S. mutans* count was greater than the reduction in *S. sanguinis* count. Kim and Shin³⁰ evaluated the antibacterial effects of a composite resin containing CNPs with low, moderate and high molecular weight on *S. mutans* and reported results similar to the results of this study. Mirhashemi et al.²⁸ evaluated the antimicrobial effects of 10% CNPs in combination with zinc oxide nanoparticles and showed that zinc oxide-CNPs inhibited microbial biofilm of *S. mutans*, *S. sanguis*, and *L. acidophilus*. This effect was magnified by an increase in the concentration of CNPs.²⁸ In another study, Rajabnia et al.¹⁷ reported that sealants containing 2% to 5% CNPs inhibited *S. mutans* and this inhibition increased by an increase in the concentration of CNPs.

The current results showed that 10% CNPs caused maximum inhibition of *S. mutans* and *S. sanguinis*; 5% and 10% concentrations had no significant difference with each other at any time point. Also, it should be noted that the use of CNPs may affect physical and mechanical properties such as bond strength. Thus, 5% concentration seems to be the ideal concentration of CNPs to be added to the primer to have optimal inhibitory effects on *S. mutans* and *S. sanguinis*. The results showed that 1% and 5% concentrations of CNPs had no significant effect on *L. acidophilus* colony count but 10% concentration of CNPs significantly decreased the *L. acidophilus* colony count. No significant difference was found between the orthodontic primers containing 5% and 10% concentrations of CNPs against *L. acidophilus*. The result of the current study revealed that 10% concentration of CNPs showed significant inhibition of *L. acidophilus* compared with the control group. Thus, 10% concentration of CNPs is recommended for inhibition of *L. acidophilus*. Our results at 7 days showed that the addition of CNPs decreased the mechanical properties of the adhesive and increased the frequency of debonding. Rats with failed adhesives showed significantly higher count of all three cariogenic bacteria compared with rats with successful adhesives. By increasing in concentration of CNPs, the frequency of failures increased, which highlighted a reduction in the bond strength by increasing of the concentration of CNPs. Similarly, Sodagar et al.¹³ found that

the addition of CNPs did not cause a significant change in bond strength but an increase in concentration of CNPs from 1% to 10% decreased the bond strength, although not significantly. Akhavan et al.³¹ reported that by an increase in concentration of silver nanoparticles and hydroxyapatite from 1% to 10%, the bond strength decreased. Further studies over longer periods of time and on other concentrations of CNPs are needed to better elucidate this topic.

CONCLUSIONS

Orthodontic primers containing 10% CNPs were effective against *S. mutans*, *S. sanguinis* and *L. acidophilus* up to 7 days in a rat model. Considering the competition of bacteria in the oral cavity, orthodontic primers containing 5% concentration of CNPs seem to be effective against *S. mutans* and *S. sanguinis* while 10% concentration of CNPs is suitable for inhibition of *L. acidophilus*.

Conflicts of Interest

The authors have no conflicts of interest to declare for this study.

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Антибактериальные эффекты ортодонтического праймера, содержащего наночастицы хитозана, в отношении многовидовой биоплёнки кариесогенных бактерий на модели крыс

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Резюме

Введение: Накопление биоплёнки вокруг ортодонтических скоб и композитов является частым осложнением несъёмных ортодонтических аппаратов. В этом исследовании оценивали антибактериальный эффект ортодонтического праймера, содержащего наночастицы хитозана (НЧХ), против биоплёнки различных типов кариесогенных бактерий на модели крыс.

Материалы и методы: Ортодонтический праймер Transbond XT, содержащий 0%, 1%, 5% и 10% НЧХ, был приготовлен экспериментально. Крысы линии Wistar были разделены случайным образом на 4 группы (n=7) – контрольную (0% НЧХ), 1%, 5% и 10% НЧХ. Ротовые полости крыс были инфицированы кариесогенными бактериями. После анестезии крыс 1 каплю (10 µL) праймера с различными концентрациями НЧХ наносили на их центральный резец и фотополимеризовали в течение 20 секунд. Ортодонтический клей Transbond XT (2×2 мм) был нанесён на праймер. Была нанесена ещё одна капля (10 µL) праймера, которая фотополимеризовалась в течение 40 секунд. Количество колоний *Streptococcus mutans*, *Streptococcus sanguinis* и *Lactobacillus acidophilus* в слюне крыс количественно определяли через 24 часа, 4 дня и 7 дней.

Результаты: Добавление 1% (p=0.005), 5% (p<0.001) и 10% (p<0.001) НЧХ ортодонтического праймера значительно уменьшило количество колоний *S. mutans* через 24 часа по сравнению с контрольной группой. Через 24 часа среднее количество колоний *S. sanguinis* в группах 5% (p=0.04) и 10% (p=0.02) было значительно ниже, чем в контрольной группе (p<0.05). На 24-ом часу и на 4 день среднее количество в колонии *L. acidophilus* в группе 10% НЧХ было значительно ниже, чем в контрольной группе (p<0.05). На 7 день у крыс с нарушенным адгезивом наблюдалось значительно большее количество всех трёх бактерий по сравнению с крысами с адгезивом (p<0.05).

Заключение: Добавление 5% НЧХ к ортодонтическому праймеру значительно снизило количество кариесогенных бактерий у крыс.

Ключевые слова

кариесогенные бактерии, хитозан, композит, наночастицы, ортодонтический клей, праймер, крыса
