



In Vitro Assessments of Antimicrobial Potential and Cytotoxicity Activity of an Orthodontic Adhesive Doped with Nano-Graphene Oxide

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Abstract

Introduction: Formation of white spots and initial carious lesions are the most important complications of fixed orthodontic treatment. Preparation of orthodontic adhesives containing antimicrobial agents might be a practical solution for the prevention of the mentioned defects.

Aim: The current study aims to assess the antimicrobial and cytotoxicity effects of a conventional orthodontic adhesive containing different concentrations of nano-graphene oxide (N-GO).

Materials and methods: 50 Transbond XT orthodontic adhesive discs containing 0, 1, 2, 5, and 10% N-GO were prepared and sterilized by 25 kGy Gamma irradiation. After determination of cytotoxicity potential of modified orthodontic adhesive on human gingival fibroblast (HGF) cells, antimicrobial effects of the modified orthodontic adhesive against *Streptococcus mutans* in the preformed cariogenic biofilms was investigated using eluted components from composite discs by comparing the viable counts of bacteria after 3, 7, 15, 30, and 60 days of the aging process in artificial saliva.

Results: Based on the results, there was no cytotoxic effects of modified orthodontic adhesive on HGF cells ($p > 0.05$). Transbond XT orthodontic adhesive containing 5 and 10 wt% N-GO reduced considerably the mean total viable counts of *S. mutans* up to 30 days ($p < 0.05$). However, at 60 days, only 10 wt% N-GO could statistically decrease the colony-forming unit (CFU)/mL of test microorganisms. Antimicrobial activity of eluted components from modified orthodontic adhesive discs against *S. mutans* was in line with the concentration of N-GO.

Conclusions: At 5% and 10% concentrations, a modified orthodontic adhesive containing N-GO has a significant antimicrobial activity against *S. mutans* in cariogenic biofilms.

Keywords

antimicrobial agents, composite resins, eluted component test, nano-graphene oxide, orthodontics, *Streptococcus mutans*

Abbreviations

N-GO: nano-graphene oxide;

CFU: colony-forming unit;

WSL: white spot lesion;

NPs: nanoparticles;

S. mutans: *Streptococcus mutans*;

BHI: brain heart infusion;

SD: standard deviation;

ZnO: zinc oxide

INTRODUCTION

Enamel decalcification or a white spot lesion (WSL) adjacent to fixed orthodontic appliances are the most common complications (up to 96%) of orthodontic treatment which may increase the cost and increase the time of patient visits.¹ The WSLs occur due to the microbial biofilm/plaque formation found to arise in patients who undergo fixed orthodontic treatment as compared to non-orthodontic subjects.² The fixed orthodontic appliances can play a role as a constraint in the patient's ability to maintain good oral hygiene, decrease the cleansing function of saliva, and reduce remineralization of the enamel surface. Thus, to address these important issues, different antimicrobial agents such as cationic curcumin doped zinc oxide nanoparticles were incorporated into the orthodontic adhesives.^{3,4}

Deliberate embedding of nanoparticles (NPs) in dental materials including resin-based composites and application of nanomaterials for improvement in dentistry has been much lauded and rapidly gaining significant importance. Inorganic NPs, including nano-graphene oxide (N-GO), are the most commonly used as antimicrobial agents, which are more encouraging.⁵ Previous studies have demonstrated that N-GO is effective against *Streptococcus mutans*, a major causative agent of WSL and dental carries.⁶

Ever since the observation of the antimicrobial and anti-biofilm activities of N-GO in 2010, the interest in antimicrobial N-GO materials is rapidly growing. It has been shown that the sharp edges of N-GO can vigorously rupture and puncture the microbial membrane via physico-chemical processes (a.k.a. "nanoknife" mechanism) resulting in microbial cells death.⁷ N-GO containing materials show great promise for inhibition of microbial proliferation and combating microbial infections including dental caries due to its intrinsic antimicrobial and antibiofilm activities of N-GO.⁸

AIM

Consequently, the current experimental study aimed to assess the antimicrobial activity of a conventional orthodontic composite resin blended with N-GO against *S. mutans* in the preformed cariogenic biofilms.

MATERIALS AND METHODS**Synthesis of N-GO**

N-GO was prepared using Hummers method.⁹ Briefly, flake graphite (10 g), KMnO_4 (6 g), K_2FeO_4 (4 g), and H_3BO_3 (0.01 g) (all purchased from Merck, Darmstadt, Germany) were dissolved in H_2SO_4 (100 mL) (Merck, Darmstadt, Germany) by keeping them in cool water bath (5°C). Then, KMnO_4 (5 g) was added and incubated into a water bath (35°C, for 3 h). The volume of the suspension was increased up to 400 mL by adding dH_2O_2 and incubated at 98°C for 3 hours. After adding 30 wt% H_2O_2 (12 mL) (Merck, Darmstadt, Germany) to the solution, it was centrifuged (10000 rpm; 20 min). Then residuals were washed with HCl (5%) and dH_2O_2 repeatedly and dried at 60°C.

Characterization of N-GO

Field emission scanning electron microscopy (FESEM; Zeiss, Sigma VP, Germany, 15 kV accelerating voltage) with ImageJ program was used to determine the morphology of N-GO.

Modified orthodontic adhesives samples preparation

Orthodontic adhesive Transbond XT (3 M Unitek, Monrovia, CA) and Transbond XT supplemented with different concentration of N-GO (0, 1, 2, 5, and 10%) were used as the original and the test materials, respectively. The orthodontic adhesive was prepared using a mixing spatula on a glass slab in a moderately dark room until a uniform consistency was achieved. FESEM was used to determine the uniform consistency of modified adhesive. Metal molds (5 mm in diameter and 1 mm thick) were used to make disc-shaped orthodontic adhesive samples containing different concentrations of N-GO (**Fig. 1**) based on a previous study.⁴

Cytotoxic effects of N-GO on normal human gingival fibroblast cell line

Human gingival fibroblast cells (HGF, CELL No. IBRC C10459) were obtained from the National Center for Biological Genetic Resources of Iran. The cell line was cultured

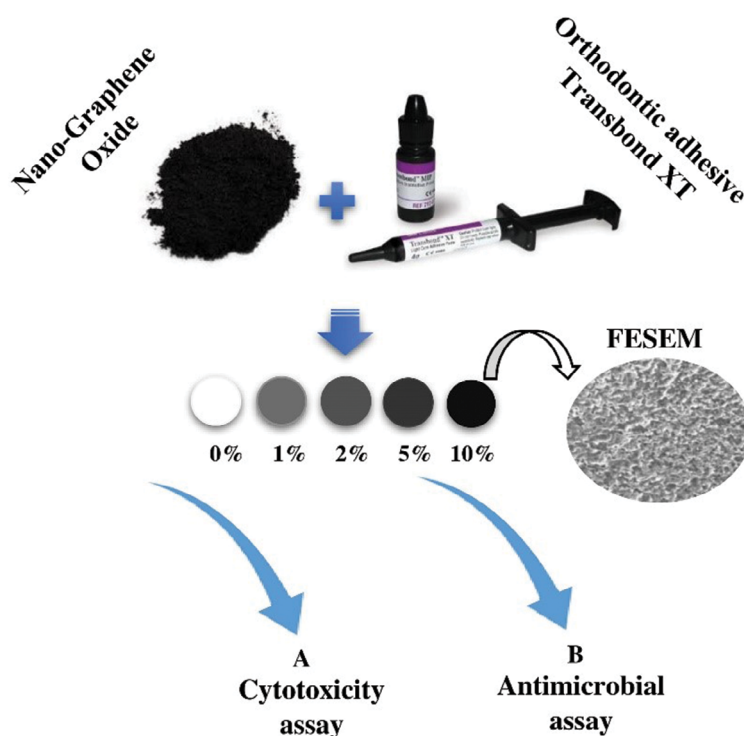


Figure 1. Sample preparation of modified orthodontic adhesives using N-GO.

in Dulbecco's Modified Eagle Medium (DMEM) medium (Gibco, Germany) supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/mL penicillin, 100 µg/mL streptomycin, and 100 µg/mL amphotericin B (all purchased from Sigma-Aldrich, Steinheim, Germany). Cells were grown at 37°C with 5% CO₂/95% air in a humidified incubator. The culture medium was changed every three days. In the two subculture, HGF cells with 1×10⁵ cells/mL were seeded in a flat-bottomed 96-well cell culture microplate (JET BIO-FIL[®], Jet Bio-Filtration Co., Ltd, and Guangzhou, China) and allowed to attach for 24 h in a humidified incubator at 37°C in the presence of 5% CO₂. After 24 h of incubation, HGF cells were treated with different concentrations of N-GO (0, 1, 2, 5, and 10%). Finally, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay was used to determine the cytotoxicity effects of N-GO on HGF cells and cell viability was measured at 570 nm using a spectrophotometric microplate reader (BioTek Elx 808, USA).¹⁰

Microorganism and growth conditions

Streptococcus mutans (ATCC 35668) was grown in brain heart infusion (BHI) broth (Merck, Darmstadt, Germany) under capnophilic atmosphere (aerobic plus 5% CO₂) at 37°C for 48 h. To assay the antimicrobial efficacy of the modified orthodontic adhesive doped with N-GO by an eluted component test against *S. mutans*, the cariogenic biofilms were prepared as described previously.⁴

Eluted component test

This assay was used to evaluate the antimicrobial effect of N-GO released from modified orthodontic adhesive samples according to a previous study.¹¹ Following gamma-irradiation sterilization, the 50 discs with the different concentrations of N-GO were placed in tubes containing 1 mL of sterile artificial saliva by 0.22-micron filter (0.453 g CaCl₂·2H₂O, 0.2 g KCl, 0.0025 g Na₂S·9H₂O, 0.2 g NaCl, 0.345 g NaH₂PO₄·2H₂O, 0.5 g urea in 1000 ml of distilled water; pH 7) at 37°C for up to 60 days in an aging process.⁴ After 3, 7, 15, 30, and 60 days, 100 µL of the content of the tubes were transferred to the separate wells of the 96-well microtiter plates containing the preformed cariogenic biofilm as described previously.⁴ The microplates were then shaken in a shaking incubator with 120 rpm in an capnophilic atmosphere for 24 h at 37°C. The wells were then sonicated under ultrasonic conditions with an ultrasonic power of 100 W and a frequency of 30 kHz for 15 s. The obtained microbial suspension was serially diluted and cultured in BHI agar. The *S. mutans* colony counts were determined as mentioned in the previous study.¹²

Statistical analysis

Statistical analysis was performed using SPSS version 22.0 (SPSS Inc., Chicago, IL, USA). Tukey's post hoc analysis was used for the pairwise comparisons of the experimental groups. The significance level was set at $p < 0.05$.

RESULTS

Confirmation of synthesized N-GO

The morphology of N-GO was confirmed by FESEM analysis. As demonstrated in **Fig. 2a**, N-GO was a nano-sized particle around 10 nm in diameter with the spherical morphology and acceptable distribution of particles. Also, uniform consistency of modified adhesive is shown in **Fig. 2b**.

Cell viability assay

The cytotoxicity of HGF cells non-targeted and HGF cells-targeting N-GO was evaluated. The results of MTT assay showed that there was no significant cytotoxic effects against HGF cells ($p > 0.05$), which suggested that N-GO had no effect on eukaryotic cells (**Fig. 3**).

Eluted component assay

Table 1 shows the mean \pm SD of CFU/mL values of the groups tested. The highest CFU/mL was found in the control group (6.23 ± 0.07) and the least CFU/mL was found with 10 wt% N-GO after 15 days (2.56 ± 0.14), with the difference being statistically significant ($p = 0.001$). As shown in **Table 1**, a significant difference in the mean CFU/mL of the modified composite and conventional (control) groups was observed ($p < 0.05$). The modified composite adhesive groups containing 5 and 10 wt% N-GO showed high antimicrobial activity compared to the conventional composite adhesive as controls ($p < 0.05$). According to the results in **Table 1**, concentrations of 5 and 10 wt% N-GO could significantly reduce the counts of viable microorganisms (CFU/mL) up to 30 days ($p < 0.05$). Despite the higher antimicrobial activity in the 10 wt% N-GO group compared to 5 wt% N-GO, the difference was not significant ($p > 0.05$). However, at 60 days, only 10 wt% N-GO could consider-

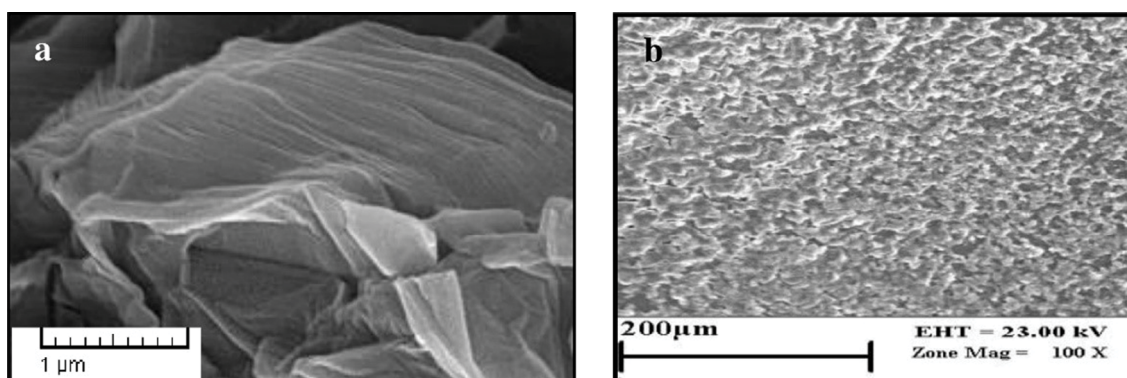


Figure 2. a) SEM image of the synthesized N-GO (1000 \times magnification [scale bar represents 1 μ m]); b) Uniform consistency of modified adhesive (1000 \times magnification [scale bar represents 200 μ m]).

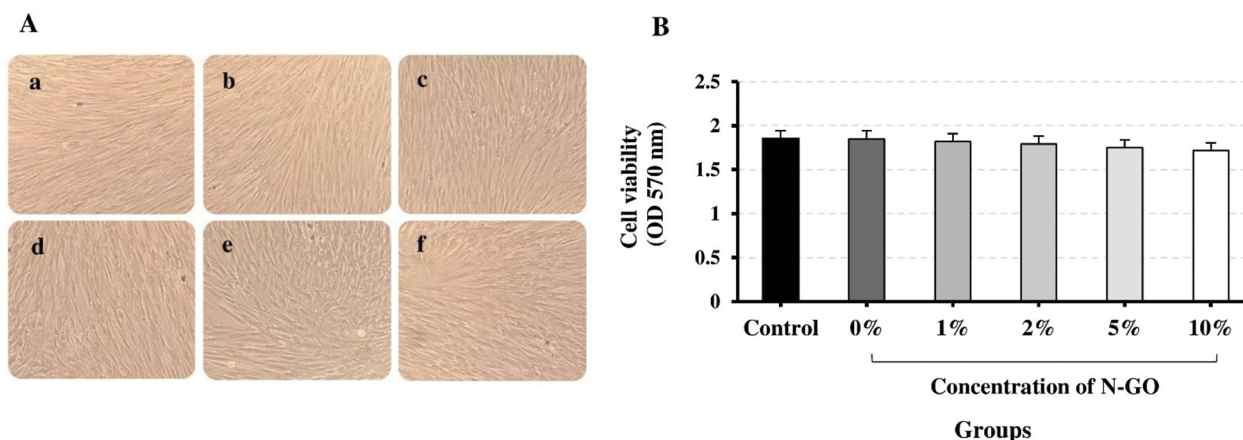


Figure 3. Cell viability of treated HGF cell by N-GO. **A)** Inverted micrograph of HGF cells at a final magnification of $\times 40$; a) Control (HGF cell without treatment); b) 0% N-GO; c) 1% N-GO; d) 2% N-GO; e) 5% N-GO; f) 10% N-GO. **B)** Cell viability at 570 nm (in percent).

Table 1. Number of colony-forming units (CFU) per milliliter of *S. mutans* (mean \pm SD) in the different treatment groups compared with the control group in eluted component test

Days	Concentration (%)	<i>S. mutans</i>	
		Mean \pm SD* of Log ₁₀ CFU/mL	P value
	Control	6.23 \pm 0.07	-
3	1	5.88 \pm 0.38	0.122
	2	5.53 \pm 0.40	0.071
	5	2.97 \pm 0.09	0.001*
	10	2.65 \pm 0.09	0.001*
7	1	5.50 \pm 0.41	0.068
	2	5.26 \pm 0.50	0.064
	5	3.05 \pm 0.15	0.001*
	10	2.83 \pm 0.12	0.000*
15	1	5.18 \pm 0.77	0.141
	2	4.83 \pm 0.48	0.055
	5	3.01 \pm 0.12	0.000*
	10	2.56 \pm 0.14	0.000*
30	1	5.36 \pm 0.50	0.072
	2	4.65 \pm 0.80	0.070
	5	3.19 \pm 0.13	0.002*
	10	2.97 \pm 0.13	0.001*
60	1	5.77 \pm 0.36	0.125
	2	5.31 \pm 0.38	0.064
	5	4.57 \pm 0.59	0.051
	10	2.84 \pm 0.09	0.000*

*SD: standard deviation, * $p < 0.05$

ably reduce the number of *S. mutans* ($p < 0.05$). According to Tukey's post hoc analysis, no significant difference was observed between 1 and 2 wt% N-GO in all days examined ($p > 0.05$). Analysis of the data of eluted component test for assessment of microbial count reduction at five different time points by two-way ANOVA showed that the interaction effect of 5 and 10 wt% N-GO concentration and time was significant for *S. mutans* ($p = 0.01$). The effect of concentration of 1 and 2 wt% N-GO was not significant all test time points for *S. mutans* ($p = 0.796$).

DISCUSSION

To avoid enamel decalcification or WSL adjacent to fixed orthodontic appliances and maintain treatment success during fixed orthodontic treatment, modified orthodontic adhesive containing antimicrobial agents are used to resist the accumulation and formation of biofilm of cariogenic bacteria including *S. mutans*, followed by white spot lesions and the risk of tooth decay around the brackets. Thus, adequate control of oral cariogenic bacteria has a critical role in successful orthodontic treatments.⁶

In the current study, we assessed the effect of incorporation of N-GO on antimicrobial effects of the orthodontic adhesive. N-GO has an intrinsic antimicrobial effect, minimum cytotoxicity, high biocompatibility, and gray colour which are less likely to alter its esthetic.¹² The nano-scale of GO was used because it improved the antimicrobial, physical, and mechanical properties as well as higher solubility, better pass through the microbial cell envelope, excellent biocompatibility and greater efficacy in the lower dose compared to GO.¹³

The results of the current study revealed that adding up to 10% wt. N-GO, the antimicrobial effect of Transbond XT composite against *S. mutans* changes significantly in comparison with the control group. This clinically important property of N-GO incorporated modified orthodontic adhesive is in line with the study conducted by Sodagar et al.¹⁴; their study revealed that the antimicrobial effect of modified orthodontic adhesive containing 10% CurNPs was significantly higher than that in the control group (original orthodontic adhesive); however, they used CurNPs only instead of N-GO for preparation of the modified orthodontic adhesive with antimicrobial properties.

Our findings are consistent with a recent Sodagar et al. study¹⁵ showing that Transbond XT composite discs containing 5% and 10% silver/hydroxyapatite nanoparticles can inhibit biofilm formation of test cariogenic strains (*S. mutans*, *Lactobacillus acidophilus*, and *S. sanguinis*) at its surface through the time. The results of current eluted component assay presenting continuity of antimicrobial activity indicated a significant decrease of the colony count of *S. mutans* for 5 and 10% N-GO groups. Similar results were shown in recent studies^{11,14} that assessed the antimicrobial effect of the combination of TiO₂, chitosan/zinc oxide, and curcumin nanoparticles into original orthodontics adhesive. Their results revealed a significant reduction in *S. mutans*, *L. acidophilus*, and *S. sanguinis* bacterial colony counts only 30 days following the exposure to orthodontics adhesive containing test nanoparticles, irrespective of the concentration of NPs, which indicates poor diffusion and low solubility of NPs in an aqueous environment.

Passariello et al.¹⁶ reported a significant induction in antimicrobial following the addition of benzalkonium chloride, zinc oxide, and chlorhexidine. In a dose-dependent manner, their results on the intensity and duration of antibacterial activity of different adhesives used in orthodontics were inconsistent with the study we conducted. Because using the aging process in the artificial saliva in our study, contrary to the study of Passariello et al.¹⁶, the obtained data of our study are closer to in vivo and clinical situation. The influence of oral in vivo situations on this effect cannot be caused based on the acquired data of these in vitro assessments. However, in contrast to their results, the test strain of streptococci (*S. mutans*), in the current study, were more susceptible to modified orthodontic adhesive containing N-GO than their test material. Passariello et al.¹⁶, in tests assessing the residual inhibitory effect of the modified orthodontic adhesive, during 180 day of

rinsing with phosphate buffer saline, on cariogenic bacterial biofilm, showed that from days 30 and 60 onwards, light-cured adhesive, glass ionomer, and light-cured resin-modified glass ionomer +23% ZnO-enriched test materials were colonized by test strains, although glass ionomer +10% chlorhexidine + fluoride was not colonized by test microorganisms, even following the rinsing after 180 days.

In the current study, despite applying the ageing process of modified test material and the use of artificial saliva, in interpreting the results, it should be highlighted that the study design did not cover all variability of the physico-chemical conditions of the oral environment including complex oral microbiome that may affect the antimicrobial effectiveness of the test materials in vivo. Colour stability and polymerization shrinkage of adding N-GO on orthodontics adhesive could be considered in the future.¹⁷ In spite of the discussed limitations of the current study, the data offer sufficient information to support significant antimicrobial effect against *S. mutans*, which will be amended clinician confidence in the selection of modified materials that can be arbitrated companionably with different clinical application. Consequently, further clinical trial study is required to confirm that modified orthodontic adhesive containing N-GO is related to a decreased incidence of WSL and incipient caries.

CONCLUSIONS

We present evidence that 5% and 10% wt. N-GO can serve as an orthodontic adhesive additive with antimicrobial effect for controlling *S. mutans* growth in cariogenic biofilms. However, low-solubility of N-GO remains a major drawback.

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Ethics approval and consent to participate

This study was approved by the Ethics Commission of IR.NIMAD.REC.1397,101, 25.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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In Vitro оценка антимикробного потенциала и цитотоксической активности ортодонтического адгезива, легированного наноксидом графена

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Резюме

Введение: Образование белых пятен и начальные кариозные поражения являются серьёзнейшими осложнениями лечения постоянными ортодонтическими аппаратами. Приготовление ортодонтических адгезивов, содержащих антимикробные вещества, может быть практическим решением для предотвращения вышеупомянутых дефектов.

Цель: Настоящее исследование направлено на оценку антимикробного и цитотоксического действия обычного ортодонтического адгезива, содержащего различные концентрации наночастиц оксида графена (N-GO).

Материалы и методы: 50 ортодонтических адгезивных дисков Transbond XT, содержащих 0, 1, 2, 5 и 10% N-GO, были приготовлены и стерилизованы облучением гамма-лучами при 25 kGy. После определения цитотоксического потенциала модифицированного ортодонтического адгезива на клетках фибробластов десны человека (ФДЧ) было исследовано антимикробное действие модифицированного ортодонтического адгезива против *Streptococcus mutans* в приготовленных кариесогенных биоплёнках с помощью элюируемых компонентов из композитных дисков путём сравнения числа жизнеспособных бактерий на 3, 7, 15, 30 и 60 день процесса старения в искусственной слюне.

Результаты: На основании полученных результатов можно сказать об отсутствии цитотоксического действия модифицированного ортодонтического адгезива на клетки ФДЧ ($p > 0.05$). Ортодонтический адгезив Transbond XT, содержащий 5 и 10 wt% N-GO, значительно снижал среднюю общую жизнеспособную численность *S. mutans* на 30 дней ($p < 0.05$). Но на 60-й день только 10 wt% N-GO могли статистически значимо снизить колониеобразующие единицы (CFU)/mL исследуемых микроорганизмов. Антимикробная активность элюированных компонентов дисков с модифицированным адгезивом в отношении *S. mutans* соответствовала концентрации N-GO.

Заключение: В концентрациях 5% и 10% модифицированный ортодонтический адгезив, содержащий N-GO, обладает значительной антимикробной активностью в отношении *S. mutans* в кариесогенных биоплёнках.

Ключевые слова

противомикробные агенты, композитные смолы, тест элюируемых компонентов, наноксид графена, ортодонтия, *Streptococcus mutans*