J Med Bacteriol. *Vol. 2, No. 3, 4 (2013): pp.* *jmb.tums.ac.ir*

ISMB TUMS

Antimicrobial Effect of Nano-Zinc Oxide and Nano-Chitosan Particles in Dental Composite Used in Orthodontics

Amirhossein Mirhashemi [1], Abbas Bahador [2], Mohammad Zaman Kassaee [3], Ghazaleh Daryakenari [4], Mohammad Sadegh Ahmad Akhoundi [1], Ahmad Sodagar [1]*

[1] *Dental Research Center, Dentistry Research Institute , Department of Orthodontics, Tehran University of Medical Sciences, Tehran, IR Iran*

[2] *Department of Microbiology, School of Medicine, Tehran University of Medical Sciences, Tehran, IR Iran*

[3] *Department of Chemistry, Tarbiat Modarres University, Tehran, IR Iran*

[4] *Department of Esthetic and Restorative Dentistry, Dental School, Babol University of Medical Sciences, Babol, IR Iran*

ARTICLE INFO

Article type:
Original Article

Article history:
Received:
Revised:
Accepted:

Keywords:
Chitosan
Zinc Oxide
Orthodontics

ABSTRACT

***Background*:** Incidence of white spots due to demineralization of enamel and gingival problems is an unacceptable result of orthodontic treatment. Plaque accumulation and bacterial biofilm growth are responsible for these phenomena. The resin-based dental composites used as bonding agents in orthodontics play a major role in mentioned problems. As recent researches assert the antimicrobial effects of chitosan (CS) and zinc oxide (ZnO) nanoparticles (NPs), it seems that adding these nanoparticles to the composite can be beneficial in reducing the number and function of microorganisms. The aim of this study was to evaluate the antimicrobial effects of ZnO-NP and CS-NP-containing orthodontic composite.

***Methods*:** Antibacterial effectiveness of ZnO-NPs and CS-NPs was assessed in four groups against *Streptococcus mutans*, *Streptococcus sanguis* and *Lactobacillus acidophilus* grown both planktonic and as a biofilm on composites. One group as the unmodified control group and three groups consisting of three different concentrations of ZnO-NPs and CS-NPs mixture: 1%, 5% and 10% (1:1 w/w). 10^8 CFU/ml microorganism suspensions were provided with spectrophotometer. Biofilm formation was quantified by viable counts. Disc agar diffusion (DAD) test was carried out to determine antimicrobial effects of nanoparticles by measuring the inhibition diameter on brain heart infusion agar plates. Finally,viable counts of microorganisms on days 3, 15 and 30 were collected for the antimicrobial effects of eluted components from composite discs.

***Results*:** In biofilm formation test, a reduction in bacterial counts was observed with 10% nanoparticle-containing composites compared with their unmodified counterpart. In the DAD test only 10% nanoparticle-containing specimens showed statistically significant inhibition. The only noticeable datain eluted component test was on day 30 for 10% nanoparticle-containing discs, inhibiting *L. acidophilus.*

***Conclusion*:** It seems that a mixture of ZnO-NPs and CS-NPs has induced an antibacterial activity in resin composite; especially in 10% weight concentrations which was significantly higher than other groups.

- ***Please cite this paper as:*** Mirhashemi AH, Bahador A, Kassaee MZ, Daryakenari GH, Ahmad Akhondi MS, Sodagar A. Antimicrobial Effect of Nano-Zinc Oxide and Nano-Chitosan Particles in Dental Composite Used in Orthodontics. *J Med Bacteriol.* 2013; **2** (3, 4): pp.

* *Corresponding Authors:* Ahmad Sodagar, PhD., Dental Research Center, Dentistry Research Institute, Department of Orthodontics, Tehran University of Medical Sciences, Tehran, IR Iran, *Tel:* +98 21 88351162, *E-mail:* sodagara@tums.ac.ir

Introduction

Bonding, due to aesthetics and simplicity of use, has become the common method for bracket attachment on dental enamel since 1980. However *in vitro* (1, 2) and *in vivo* (3) studies for resin filler-based dental composite has been reported to cause more bacterial plaque accumulation, including *Streptococcus mutans*, than either other dental materials or dental tissues such as enamel. The presence of brackets along with the resin used for bonding effects the self-cleansing contour of the tooth and provides a platform for microbial plaque to accumulate (4-6). Studies show, three weeks after placement of band and bracket in the oral cavity of a person with normal hygiene habits, mature cariogenic plaque is detectable around the resin while at the same time the plaque present around the gingival margin is only primary with very little cariogenic potential (5). Evidently orthodontic treatments remain in the oral cavity for a while making enamel demineralization an even more important concerns for the dental community.

S. mutans is one the most common microorganisms found in cariogenic plaque on dental hard tissues. In the pH below, 5.5 this microorganism has anaerobic activity, which produces organic acids. A similar microorganism that is common in cariogenic plaque is *Lactobacillus acidophilus* which helps the demineralization of dentinal tissues. On the other hand, the microorganism found in non-cariogenic plaque at pH equal to 5.5 is *Streptococcus sanguis* and its presence in the plaque is an indication of low cariogenic activity of the plaque (7).

Several methods have been used to inhibit biofilm growth that contributes to dental caries (8-11). One example is composites with chlorhexidine, which successfully inhibited microbial growth, but were only effective over a short period of time due to high solubility of the antibiotic (12, 13). Some metal oxides, such as magnesium, zinc, silver and calcium, are proven to have antimicrobial characteristics (11, 14, 15). This seems to even enhance when the oxides are in nano scales (16). Zinc oxide has shown anti *S. mutans* properties which will been hanced with higher concentrations (17). Nano zinc oxide particles as a 10% weight filler in dental composite have proven to reduce the microorganism count by 80% in the plaque formed around a composite restoration (18).

A widely used material due to its biodegradable, nontoxic, non-antigenic, and biocompatible properties is a biopolymer isolated from shellfish, crab and shrimp, called chitosan, which is reported to exhibit numerous health-related beneficial effects, including strong antimicrobial (19, 20) and antioxidative activities in food (21). Because nano-sized composites (nanoparticles, nanomaterials) are expected to be more effective in penetrating and disrupting bacterial cell membranes, nanochitosans are effective against a variety of organisms, and if added silversalts or other functional antimicrobial agents to chitosannanoparticle composites, antimicrobial activity enhances (21-25).

This study examines the inhibition of *S. mutans*, *S. sanguis* and *L. acidophilus* biofilms and planktonic cultures by ZnO-NPs and CS-NPs blended with orthodontic composite.

Materials and Methods

Preparation of nanoparticles

The chitosan which was purchased from ACROS Organic (UK) with low molecular weight (1 - 3 kDa), prepared as nanoparticles using water and 1% acetic acid and mixed vigorously with sodium triphosphate followed by centrifuging for 30 minutes, in the biochemical laboratory of Tarbiat Modarres University, Tehran, Iran. The sediment gathered was then rinsed several times and frozen. The powder wasready for use after grinding the frozen substrate. 38 nanometer ZnO-NPs were prepared in Iran's Nuclear Science and Tech laboratory.

Preparation of modified composites

To achieve dental composite containing 10% nanoparticles,64 mg nano powder (containing equal amount of nanoparticles) was blended into 576 mg Transbond XT (3M Unitek, USA) composite, using a mixing spatula on a glass slab in a semi-dark environment, until a uniform consistency was achieved. 200 mg of the 10% w/w blended composite was then mixed with 200 mg the original composite to obtain 5% w/w containing composite; similarly, 40 mg of 10% composite was blended with 360 mg original composite for the 1% w/w composite.

Preparation of bacterial suspensions

S. mutans ATCC 25175, *S. sanguis* ATCC 10556 and *L. acidophilus* ATCC 4356 were supplied (Industrial fungi & bacteria, Centre for Scientific and Industrial Research of Iran) in lipophilised form. *S. mutans*, *S. sanguis* were grown in brain heart infusion broth (BHI; Difco, Sparks, MD, USA) at 37°C in a 5% CO_2 atmosphere until the cells reached the mid-logarithmic phase (OD_{600nm} = 0.2). *L. acidophilus* was grown in BHI at 37°C under anaerobic conditions until the cells reached the mid-logarithmic phase (OD_{600nm} = 1). 10^8CFU/ml microorganism suspensions were prepared by spectrophotometer for determining the antimicrobial effect of ZnO-NPs and CS-NPs (22).

Optical density for *L. acidophilus* in 600 nm is 1, (OD = 1); which is 10^7cells per ml. This density was then diluted ten times and inoculated on BHI (brain heart infusion) agar. OD = 0.2 for the other two organisms was equivalent to 10^8 cells per ml.

Composite disc preparation

5 mm diameter standard ring-shaped molds were placed between glass slides, for attaining a smooth surface, after being filled with composite. Visible light cure (470 nm) was applied for 30 seconds (Bluphase ® 16i, Ivoclar Vivadent AG, Australia). Discs were cured another 10 seconds after application of a thin layer of bonding.

All specimens (n = 162), were sterilized in Iran's Nuclear Science and Tech gamma radiation center with 25 kGy dosage.

Biofilm inhibition

Three day biofilm were generated on composite discs (n = 90) using 24-well plates. Each well was inoculated with adjusted bacterial inoculum. Biofilm was grown at 37°C (2 mL BHI containing 0.5% sucrose), and media was changed every 24 hours. At the end of the third day, each disc was rinsed with phosphate buffered saline (PBS) to remove loosely attached bacteria and planktonic bacteria. To count the colony forming units (CFUs) responsible for biofilm formation, specimens were sonicated in sterile saline and then vortexed in PBS with 3 mm glass beads. CFU/ml of the microorganism present in the suspension was counted with drop-plate method using serial

dilution in microtiter plates. This and all the following tests were repeated three times for each microorganism (18).

Disc Agar Diffusion test (DAD)

Antibacterial activity of discs via diffusion of ZnO-NPs and CS-NPs was examined by this test. Composite discs (n = 36) were placed, 2 cm apart, on BHI agar plates, which were inoculated with a 200 µL bacterial solution (~10^8 CFU/mL) by a sterile swap. After 48 hour incubation, at 37°C, the bacterial growth zone of inhibition was measured (18).

Antibacterial properties of eluted components

Antibacterial activity of possible eluted components from nanoparticle comprising composite discs was also evaluated. Composite discs (n = 36) were placed in tubes containing 5 ml BHI media. 5 ml media was then removed from the tubes on days 3, 15 and 30 and placed in other 15 ml plastic tubes; tubes were inoculated with 50 µl of bacterial culture (final ~ 25 × 10^5 CFU in 5 mL media) and shaken at 300 rpm rate in 37°C for 24 hours. The remaining CFU of bacteria was then counted (18).

Statistical evaluation of data

Multiple statistical tests were used to analyze the data. One-way ANOVA was first used for biofilm inhibition test, followed by Tukey HSD test. Colony diversity was analyzed with homogeneity of variances test.

Kruskal-Wallis test was used to analyze data attained from DAD test. Two-way ANOVA was first used to analyze day × concentration relation in eluted components test. For those groups with significant difference one-way ANOVA was used.

Results

Biofilm inhibition test

Mature biofilms on four different composite groups were recorded after 72 hours. Counts of viable bacteria are shown in *table* 1 for the following groups: three nanoparticle containing composite discs with concentrations of 1%, 5% and 10% (by weight) and one unmodified group as the control group. All tests were carried out three times for each group. No significant difference was found among the specimens in one group, but they were meaningful between different groups.

S. mutans colonies were meaningfully lowered for 5 and 10% groups ($p < 0.005$); While for *S. sanguis*, colonies were meaningfully lowered for the three different NPconcentrations (all groups $p < 0.007$).

Only 10% NP containing composites ($p < 0.002$) resulted in statistically meaningful decrease for *L. acidophilus*. None of the above microorganism colonies demonstrated a significant diversity in colony counts.

DAD test

In all the repeated tests, only the 10% group had a significant diameter of bacterial growth inhibition for the three microorganisms; While for 1 and 5 percent groups the inhibition diameter was zero and similar to control group. The results are illustrated in table 1.

Table1. Microorganism inhibition diameter (millimeter) by nanoparticles diffusion

Microorganism	Nanoparticle percent	N	Minimum	Maximum	Mean	Std. Deviation
Streptococcus mutans	10%	3	7	8	7.33	0.577
Streptococcus sanguis	10%	3	6	8	6.67	1.155
Lactobacillus acidophilus	10%	3	5	7	6	1

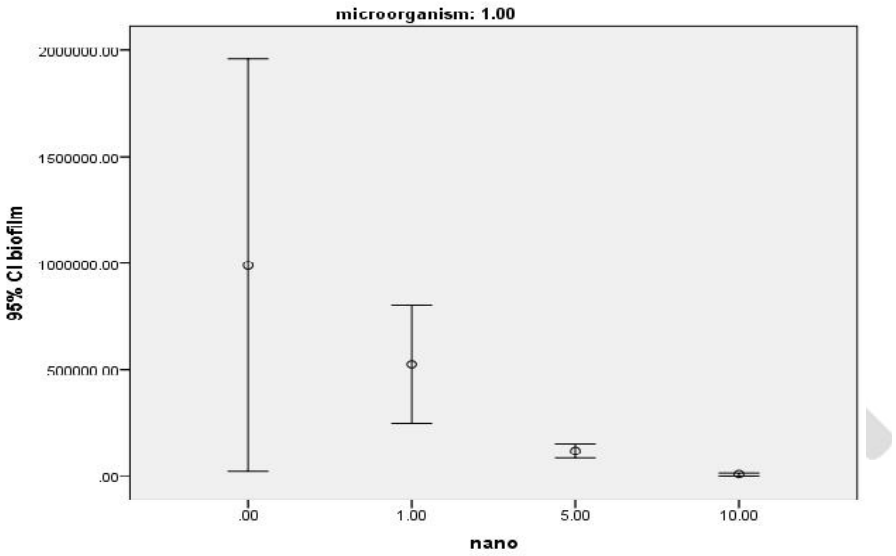

Figure 1.Viable counts of *Streptococcus mutans* biofilms on the following composites: 0%, 1%, 5% and 10% nanoparticles-containing composite discs

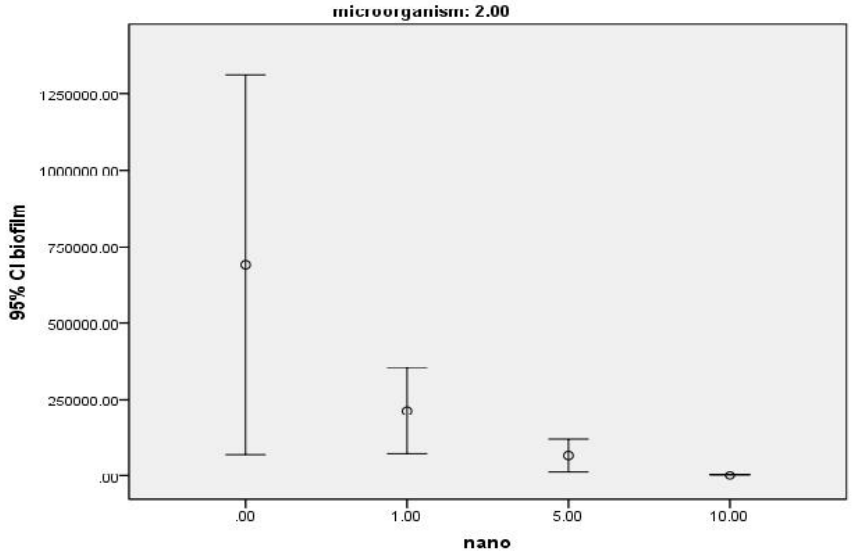

Figure 2. Viable counts of *Streptococcus sanguis* biofilms on the following composites: 0%, 1%, 5% and 10% nanoparticles-containing composite discs

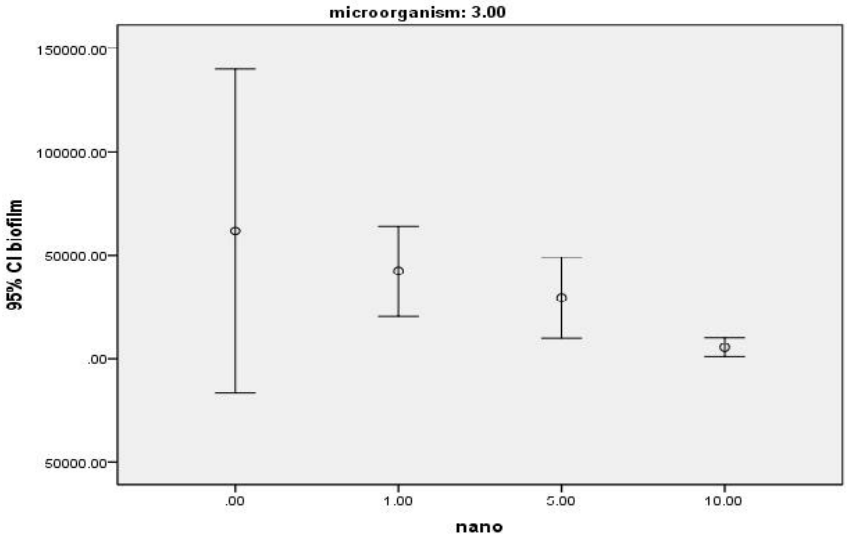

Figure 3. Viable counts of *Lactobacillus acidophilus* biofilms on the following composites: 0%, 1%, 5% and 10% nanoparticles-containing composite discs

Eluted components test

For *S. mutans* no significant difference was found for any of the groups in any day (p = 0.299). Statistically meaningful differences were found for *S. sanguis* (p = 0.045) and *L. acidophilus* (p = 0.024) so each of the categories were analyzed by one-way ANOVA, which showed no significant difference for *S. sanguis*on different days and was only statistically significant for 10% *L. acidophilus* on day 30 (p = 0.039) as illustrated in figure 6.

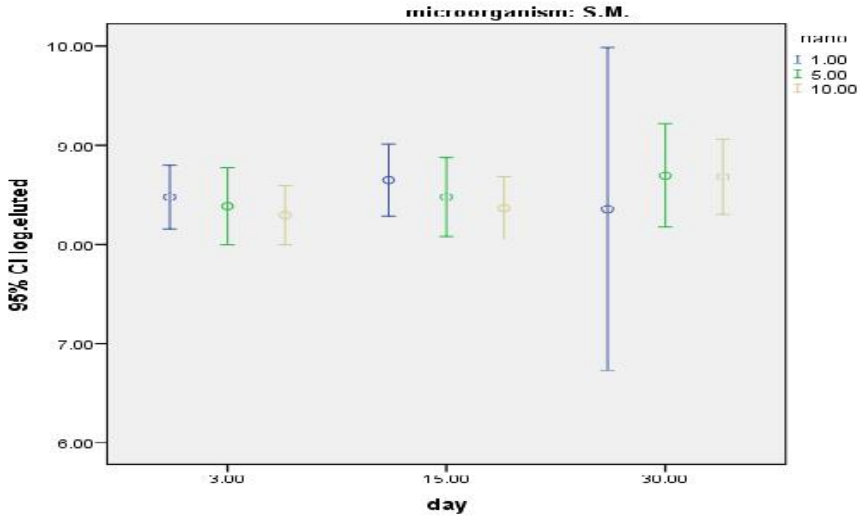

Figure 4. Viable counts of *Streptococcus mutans* colonies on days 3, 15 and 30 on the following composites: 1%, 5% and 10% nanoparticles-containing composite discs

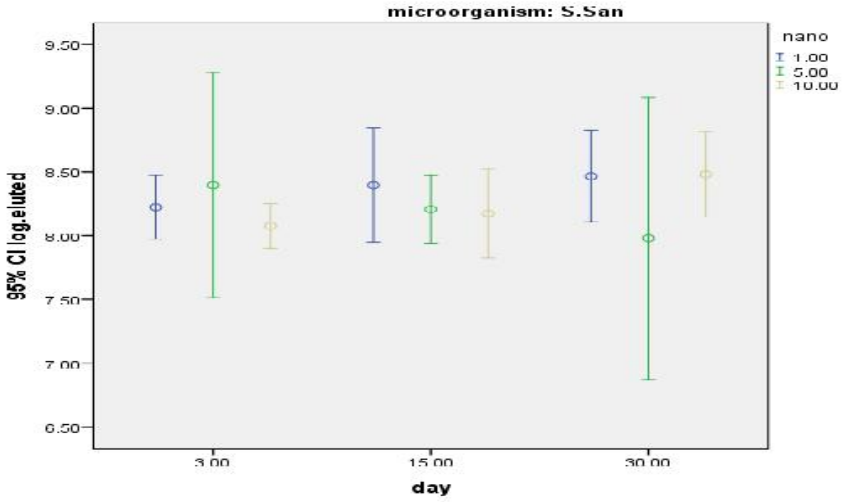

Figure 5. Viable counts of *Streptococcus sanguis* colonies on days 3, 15 and 30 on the following composites: 1%, 5% and 10% nanoparticles-containing composite discs

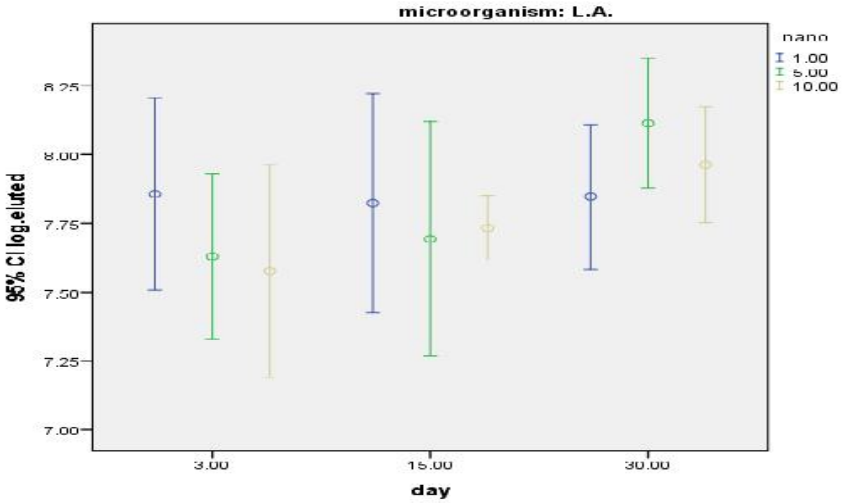

Figure 6. Viable counts of *Lactobacillus acidophilus* colonies on days 3, 15 and 30 on the following composites: 1%, 5% and 10% nanoparticles-containing composite discs

Discussion

To date many variable sized particles are used as resin adhesive fillers to induce antibacterial properties. Alt, *et al* concluded that nano silver particles have antimicrobial effect against refractory bacteria (23). Ahn, *et al* also proved that nano silver and nano silica filler containing composites prevent enamel demineralization around orthodontic brackets (24). Beyth *et al* showed the antibacterial effects of polyethylenimine nanoparticles in resin composite on a variety of microorganisms in long-term (2). Another study illustrated the higher antimicrobial properties of 1% weight nano titanium oxide containing composite compared to the control group (25).

The antibacterial effect of ZnO-NPs and CS-NPs as fillers in orthodontic resin composite was evaluated in this study. Previous studies were carried out successfully demonstrating antibacterial effects and biofilm growth inhibition by ZnO-NPs in composite (18, 26). Fernandes *et al* and Friedman *et al* both showed the antibacterial characteristics of the chitosan particles, concluding that this effect enhances by lower molecular weight. Friedman discussed that the nano-sized particles are more likely to penetrate the bacterial membranes thus enabling them to be more destructive in lower concentrations (21, 27). Another study used the antibacterial effects of CS-NPs in combination with nano silver particle, against microorganisms commonly found in food. What questions the application of the latter combination with composite is the color modification caused by silver which defies the esthetic purposes of composites (19).

Same antibacterial effects were resulted in this study while the color of the composite resin was not changed drastically and the combination of the two NPs made an enhancement of the antibacterial effect using a lower percent of each of the NPs. 1%, 5% and 10% concentrations of NPs were used in this study for purpose of comparing the new combination with the previously studied documents. Gold standard composite Transbond XT was used as the resin cement.

Our results show that ZnO-NPs and CS-NPs cause inhibition of bacterial biofilm which is more prominent with higher NP concentrations. The 1% group was almost similar to the control group while the 5% group significantly reduced *S. mutans* and *S. sanguis* and all three organisms were inhibited significantly by the 10% group. The diameter of inhibition by diffusion was only meaningful with the 10% group ranging 5-8 mm for the three organisms in this study. This is supposedly caused by the addition of CS-NPs because the studies on ZnO-NPs solely showed zero diameter of inhibition by diffusion even in 10% concentrations, although the choice of microorganisms was different (18). *L. acidophilus* was the only bacteria increased significantlyon day 30 for the eluted components test which shows similar results in the present combination of NPs compared to ZnO-NPs used alone, since the latter showed no significant difference on any day for any concentrations as well (18).

For other NPs such as TiO_2, the biofilm inhibition was similar in the 1% group, but for the DAD test, 1% TiO_2 proved more antibacterial inhibition (25).

Conclusion

Our results showed that the combination of ZnO-NPs and CS-NPs has induced an antibacterial activity in resin composite; especially in 10% weight concentrations which was significantly higher than other groups. More studies are required however, to determine the longevity of this effect and to investigate the mechanical modifications of adding NPs as filler to dental composite.

Acknowledgments

The authors would like to thank Dr Mohammad Javad Kharazi Fard for his contribution in performing the statistical analysis of this study.

This research is approved and supported by the research center of Tehran University of Medical Sciences (research code: 4973). The authors report no conflicts of interest and they alone are responsible for the content and writing of this paper.

Conflict of interest

None declared conflicts of interest.

References

1. Zalkind MM, Keisar O, Ever-Hadani P, *et al.* Accumulation of Streptococcus mutans on light-cured composites and amalgam: an in vitro study. *J Esthet Dent* 1998; **10** (4): 187-90.
2. Beyth N, Houri-Haddad Y, Baraness-Hadar L, *et al*. Surface antimicrobial activity and biocompatibility of incorporated polyethylenimine nanoparticles. *Biomaterials* 2008; **29** (31): 4157-63.
3. Gunyakti N, Gur G, Misirligil A. [In vivo adhesion of Streptococcus mutans on amalgam and composite restorative materials]. *Ankara Univ Hekim Fak Derg* 1990; **17** (1): 83-6.
4. Chin MY, Busscher HJ, Evans R, *et al*. Early biofilm formation and the effects of antimicrobial agents on orthodontic bonding materials in a parallel plate flow chamber. *Eur J Orthod* 2006; **28** (1): 1-7.
5. Sukontapatipark W, el-Agroudi MA, Selliseth NJ, *et al*. Bacterial colonization associated with fixed orthodontic appliances. A scanning electron microscopy study. *Eur J Orthod* 2001; **23** (5): 475-84.
6. Tufekci E, Dixon JS, Gunsolley JC, *et al*. Prevalence of white spot lesions during orthodontic treatment with fixed appliances. *Angle Orthod* 2011; **81** (2): 206-10.
7. Roberson TM. Sturvedant's art and science of operative dentistry. 1. 5th ed 2006. chap 2: 65-77.
8. Imazato S, Ebi N, Takahashi Y, *et al*. Antibacterial activity of bactericide-immobilized filler for resin-based restoratives. *Biomaterials* 2003; **24** (20): 3605-9.
9. Yamamoto K, Ohashi S, Aono M, *et al*. Antibacterial activity of silver ions implanted in SiO2 filler on oral streptococci. *Dent Mater* 1996; **12** (4): 227-9.
10. Syafiuddin T, Hisamitsu H, Toko T, *et al*. In vitro inhibition of caries around a resin composite restoration containing antibacterial filler. *Biomaterials* 1997; **18** (15): 1051-7.
11. Sodagar A, Bahador A, Khalil S, *et al*. The effect of TiO2 and SiO2 nanoparticles on flexural strength of poly (methyl methacrylate) acrylic resins. *J prosth res* 2013; **57** (1): 15-9.
12. Leung D, Spratt DA, Pratten J, *et al.* Chlorhexidine-releasing methacrylate dental composite materials. *Biomaterials* 2005; **26** (34): 7145-53.
13. Jedrychowski JR, Caputo AA, Kerper S. Antibacterial and mechanical properties of restorative materials combined with chlorhexidines. *J Oral Rehabil* 1983; **10** (5): 373-81.
14. Sawai J. Quantitative evaluation of antibacterial activities of metallic oxide powders (ZnO, MgO and CaO)

by conductimetric assay. *J MicrobMeth* 2003; **54** (2): 177-82.
15. Jones N, Ray B, Ranjit KT, *et al*. Antibacterial activity of ZnO nanoparticle suspensions on a broad spectrum of microorganisms. *FEMS Microbiol Lett* 2008; **279** (1): 71-6.
16. Hernandez-Sierra JF, Ruiz F, Pena DC, *et al*. The antimicrobial sensitivity of Streptococcus mutans to nanoparticles of silver, zinc oxide, and gold. *Nanomedicine* 2008; **4** (3): 237-40.
17. Spencer CG, Campbell PM, Buschang PH, *et al*. Antimicrobial effects of zinc oxide in an orthodontic bonding agent. *Angle Orthod* 2009; **79** (2): 317-22.
18. Aydin Sevinc B, Hanley L. Antibacterial activity of dental composites containing zinc oxide nanoparticles. *J biomed mater res Part B, Applied biomater* 2010; **94** (1): 22-31.
19. Pinto RJ, Fernandes SC, Freire CS, *et al*. Antibacterial activity of optically transparent nanocomposite films based on chitosan or its derivatives and silver nanoparticles. *Carbohydr Res* 2011; **348**: 77-83.
20. Uysal T, Akkurt MD, Amasyali M, *et al*. Does a chitosan-containing dentifrice prevent demineralization around orthodontic brackets? *Angle Orthod* 2011; **81** (2): 319-25.
21. Friedman M, Juneja VK. Review of antimicrobial and antioxidative activities of chitosans in food. *J Food Prot* 2010; **73** (9): 1737-61.
22. Yang YJ, Chuang CC, Yang HB, *et al*. Lactobacillus acidophilus ameliorates H. pylori-induced gastric inflammation by inactivating the Smad7 and NFkappaB pathways. *BMC microbiology* 2012; **12**: 38.
23. Alt V, Bechert T, Steinrucke P, *et al*. An in vitro assessment of the antibacterial properties and cytotoxicity of nanoparticulate silver bone cement. *Biomaterials* 2004; **25** (18): 4383-91.
24. Ahn S, Vang MS, Yang HS, *et al*. Histologic evaluation and removal torque analysis of nano- and microtreated titanium implants in the dogs. *J adv prosthod* 2009; **1** (2): 75-84.
25. Poosti M, Ramazanzadeh B, Zebarjad M, *et al*. Shear bond strength and antibacterial effects of orthodontic composite containing TiO2 nanoparticles. *Eur J Orthod* 2012; **35** (5): 676-9.
26. Tavassoli Hojati S, Alaghemand H, Hamze F, *et al*. Antibacterial, physical and mechanical properties of flowable resin composites containing zinc oxide nanoparticles. *Dent Mater* 2013; **29** (5): 495-505.
27. Fernandes JC, Tavaria FK, Soares JC, *et al*. Antimicrobial effects of chitosans and chitooligosaccharides, upon Staphylococcus aureus and Escherichia coli, in food model systems. *Food Microbiol*. 2008; **25** (7): 922-8.