Randomized controlled trial

A comparative assessment of enamel mineral content and Streptococcus mutans population between conventional composites and composites containing nano amorphous calcium phosphate in fixed orthodontic patients: a split-mouth randomized clinical trial

Arezoo Jahanbin¹, Fahimeh Farzanegan², Mohammad Atai³, Saeed Amel Jamehdar⁴, Parvaneh Golfakhrabadi⁵ and Hooman Shafaee²

¹Dental Research Center, Department of Orthodontics, School of Dentistry, Mashhad University of Medical Sciences, Iran, ²Oral & Maxillofacial Diseases Research Center, Department of Orthodontics, School of Dentistry, Mashhad University of Medical Sciences, Iran, ³Iran Polymer and Petrochemical Institute, Tehran, Iran, ⁴Antimicrobial Resistance Research Center, Avicenna Research Institute, Mashhad University of Medical Sciences, Iran, and ⁵Department of Orthodontics, School of Dentistry, Golestan University of Medical Sciences, Gorgan, Iran

Correspondence to: Fahimeh Farzanegan, Department of Orthodontics, School of Dentistry, Mashhad Dental School, Vakilabad Boulevard, Azadi Square, Mashhd, Iran. E-mail: farzaneganf@gmail.com

Summary

Objectives: The aim of this ‘split-mouth design’ trial was to evaluate the effect of the nano amorphous calcium phosphate (NACP) containing composite on enamel mineral contents and streptococcus mutans population in fixed orthodontic patients.

Design, setting, participants, and intervention: Randomized, prospective, single-center controlled trial. Twenty-four patients between the ages of 13–18 years participated in this study. The control and test sides were randomly selected by a coin toss (1:1 ratio). On the control side orthodontic brackets were bonded on the buccal surfaces of upper premolars and laterals using an orthodontic composite (Transbond XT), and on the study side NACP-containing composite was used. Outcome measures were the mineral content around the brackets and S. mutans count. The later were calculated in the plaque around the brackets by real-time PCR at 3 months, and 6 months after the initiation of treatment. All stages of the study were blind using coding system. Paired t-test and repeated measurements were used for data analysis.

Results: In the third and sixth month, the bacterial population was significantly lower in the study side than the control side (P = 0.01 and 0.000). The mineral content of the study side was significantly higher than the controls, 6 months after bracket bonding (P = 0.004). There were no significant differences between the premolars and lateral teeth for all measurements.

Limitations: This research was performed in a single-center by one experienced clinician.

Conclusion: NACP-containing composites have the potential to inhibit mineral content loss and S. mutans colonization around orthodontic brackets during fixed orthodontic treatments.

Trial registration: This trial was not registered.

Protocol: The protocol was not published before trial commencement.
Introduction

Enamel decalcification and white spot lesions can occur whenever microbial biofilm is retained on the enamel surface for a long time. Despite improvement in orthodontic materials and techniques, demineralization of enamel around the brackets remains a problem during orthodontic treatment that can jeopardize ideal esthetic goals. Up to now, preventive regimens including fluoride agents, mouth rinses, and varnishes have been suggested. All of these products require patient cooperation and they fail to prevent total decalcification. Therefore, finding new procedures that do not depend on patient cooperation is essential (1–4).

The best material to bond orthodontic brackets is resin composite. However, resin composites do not hinder plaque formation and enamel demineralization (5). Hence, many studies are being done to develop a new antibacterial composite to reduce risk of enamel decalcification (6–8). One promising component that can release calcium and phosphate to reduce enamel decalcification is amorphous calcium phosphate (ACP) (9, 10). However, a main drawback of ACP is poor mechanical properties (11). Recently, in restorative dentistry, a resin composite containing ACP nanoparticles [nano amorphous calcium phosphate (NACP)] was developed that releases high levels of Ca and PO₄, and its mechanical properties are nearly 2-fold better than previous ACP composites (9, 12). When pH decreases and the risk of decalcification increases, the amount of Ca and PO₄ released from the NACP composite significantly increases. Moreover, composites containing NACP have a unique potential to neutralize lactic acid, while conventional composites maintain the pH level (13).

Because prevention is highly preferable than treatment in the management of white spot lesions, the aim of this study was to evaluate the effect of the containing NACP orthodontic composite on enamel mineral contents and the Streptococcus mutans population during fixed orthodontic treatment.

Subjects and methods

Trial design

This was a randomized controlled clinical trial approved by the research ethic committee of the Mashhad University of Medical Sciences in Iran. The study took place at the orthodontic clinic of its dental school in 2014, and the study protocol was registered with the research chancellor of the Mashhad University of Medical Sciences (No. 920225).

Participants, eligibility, and setting

Twenty-four female patients (13–18 years old, mean age: 15.5 years) who presented at the orthodontic clinic and required fixed orthodontic treatment participated in this study. Inclusion criteria included: upper second premolars and upper lateral incisors of the participants to be free of macroscopic enamel cracks, caries, or enamel hypoplasia. Exclusion criteria included: patients with drug related xerostomia, systemic and active periodontal disease, active caries on at least one tooth, patients in whom the bracket of their upper second premolars or upper lateral incisors was debonded during orthodontic treatment.

After primary case selection (by first author) a detailed document including patient medical and dental history was filled out and a signed informed consent was obtained from each patient, parent, or guardian. No changes to methods after trial commencement occurred.

Intervention

Nanoparticle production

The spray drying technique was used to produce NACP. To prepare solution 1 Ca(NO₃)₂·4H₂O and polyethylene glycol (PEG, MW 10000) were dissolved in distilled water, and for solution 2 (NH₄)₂HPO₄ was dissolved in distilled water. Later, solution 2 was added to solution 1 drop-by-drop at 5°C. Calcium phosphate was deposited and the particles were separated by centrifuge. X-ray diffraction was used to confirm morphology of nanoparticles and the size of NACP was checked by a transmission electron microscopy. The average size of the NACP was 116 nm (ranging from 81 to 130nm) (Figures 1 and 2).

Pilot study

To evaluate the effect of the NACP on the shear bond strength (SBS) of Transbond XT and to determine the appropriate concentration of NACP in the composite a pilot study was designed. Twenty premolars (without any decay or restorations) were selected, mounted in acrylic molds, and then randomly divided into four groups. To bond orthodontic brackets the following were used: the first group was composed of Transbond XT (3M Unitek, Monrovia, California, USA) containing 1.5% NACP, in the second group was Transbond XT containing 2.5%, in the third group was Transbond XT containing 5% NACP, and in the...
fourth group was Transbond XT without the NACP (control). The SBS of all four composites was measured by an Instron Universal Testing Machine (Rauenstein, S No: 2213/R17 Germany). The mean values for SBS showed no difference among the groups (Table 1); therefore, Transbond XT containing 5% NACP was selected for investigation.

**Randomization method**

For randomization, in each patient the control and test sides were randomly selected by a coin toss (allocation ratio = 1:1).

**Main study**

For enamel preparation, 37% phosphoric acid gel (Ultra-Etch, Ultradent Product Inc., Utah, USA) was applied for 30 seconds, then washed for 30 seconds, and dried for 20 seconds. On the control side, after enamel preparation Transbond XT liquid primer (3M Unitek) was applied onto the tooth surface in a thin layer. Transbond XT adhesive (3M Unitek) was used immediately for bonding. On the test side Transbond XT containing 5% NACP was used for bonding. Curing was performed by a LED light curing device (Ortholux, 3M Unitek) for 10 seconds from mesial and distal directions individually.

**Blinding**

The operator who performed bracket bonding was blinded to the study so that he did not know on which side the brackets were bonded with which composite. At this stage, a code was given to study's coding system submitted the reports.

**Outcomes**

The primary outcome was the count of the *S.mutans* population around the lateral incisor and second premolar’s brackets. The secondary outcome was the enamel mineral content of the lateral and second premolar teeth in both groups.

**Microbial examination**

In this study, *S.mutans* count in the plaque around the brackets was calculated by the real-time PCR technique. Patients were advised to not eat, drink, or brush their teeth 2 hours before sampling. A blinded operator performed the sampling using a sterile swap before enamel preparation for bonding from the enamel surface near the gingival margin of the upper second premolars and lateral incisors. The swap was transferred to a microtube containing 200 µl phosphate buffer sterile. The microtubes were immediately transferred to a microbiology lab at 4°C. First, 100 µl of each sample was cultured on Mitis Salivarius Agar medium and the other 100 µl was washed for 30 seconds, and dried for 20 seconds. On the control side, after enamel preparation Transbond XT liquid primer (3M Unitek) was applied onto the tooth surface in a thin layer. Transbond XT adhesive (3M Unitek) was used immediately for bonding. On the test side Transbond XT containing 5% NACP was used for bonding. Curing was performed by a LED light curing device (Ortholux, 3M Unitek) for 10 seconds from mesial and distal directions individually.

**Determination of enamel mineral content**

To determine the enamel mineral content of the upper lateral incisors and second premolars the Vista Cam IX (Durr Dental, Bietigheim-Bissingen, Germany) was used (Figure 3). Vista Cam measures the enamel mineral content based on fluorescence characteristics and has the ability to specify a number in a range of 0–3 for each point on the enamel image. Figure 4 shows that the larger number indicates less enamel mineral content. Measurements were performed at 8 points around the brackets, before bonding (T0), 3 months (T1), and 6 months (T2) afterward, in both control and test sides (Figure 3).

**Sample size calculation**

The calculated sample size for each group was based on a significance level of 0.05 and 80 per cent power to detect a clinically meaningful difference of 25 000 (standard deviation = 30 000) of the *S.mutans* count in the plaque. The power analysis showed that at maintained at −20°C for molecular studies. Furthermore, sampling was performed 3 months (T1) and 6 months (T2) after the bonding of the brackets. Sampling was performed by third author who was blinded to the study.

In the various real-time PCR studies of *S.mutans*, different gene sequences were used. In this study, the gene sequence of glycosyltransferase-1 (gtfb-1) was selected. DNA of the *S.mutans* was extracted using a RIBO-sorbt kit (Amplisense, Moscow, Russia) as per instructions. Extracted DNA was maintained at −20°C. The PCR test was performed based on the thermal instructions of the thermocycle device, and to control infection the PCR test was performed in association with a negative control. The PCR products were electrophoresed in agarose gel. To quantify the *S.mutans*, real-time PCR (TAKARA Kit, Clontech Labs Inc., Mountain Views, California, USA) was performed at 0 day, 3 months and 6 months.

**Table 1. Comparison of shear bond strength (MPa) of orthodontic brackets bonded with different concentration of NACP in composite (pilot study).**

<table>
<thead>
<tr>
<th>Group (%)</th>
<th>N</th>
<th>Mean</th>
<th>SD</th>
<th>F</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>5</td>
<td>12.52</td>
<td>0.42071</td>
<td>0.08</td>
<td>0.971</td>
</tr>
<tr>
<td>2.5</td>
<td>5</td>
<td>12.48</td>
<td>0.42071</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>12.58</td>
<td>0.35637</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 (control)</td>
<td>5</td>
<td>12.46</td>
<td>0.48270</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
least 11 patients would be required. To compensate for dropouts in the follow-up study, 25 patients were enrolled in this study.

**Statistical analysis**

Data were analyzed by the paired t-test and repeated measurements analysis of variance (RMANOVA) using SPSS software (SPSS Inc., Chicago, Illinois, USA). Statistical significance was set at the 0.05 probability level.

**Results**

**Participant flow**

Four of the 24 participants were excluded because they missed their follow-up appointments at the determinate times, and one person was excluded from the study because her lateral incisor’s bracket was deboned during treatment. Therefore, 40 upper second premolars and 40 upper lateral incisors (20 teeth in control group and 20 teeth in test group) in the remaining patients (20 patients) were evaluated at the beginning of treatment, 3 months, and 6 months (Figure 5). Kolmogorov–Smirnov test showed that all data had normal distribution ($P > 0.05$).

**Primary outcome**

**Antibacterial effect**

The paired t-test showed, regardless of the tooth type after 3 months (T1) and 6 months (T2) of treatment the $S. mutans$ population in the control side was significantly greater than the test side ($P = 0.014$ and 0.000, respectively) (Table 2). These differences were true for the lateral and second premolar. The mean differences also showed that the difference was clinically significant (Tables 6 and 7). But, the RMANOVA test showed that the bacterial population changes were not significant during the time course for lateral incisors and second premolars in both the control and test sides (Tables 2–4).

**Secondary outcome**

**Enamel mineral content loss**

Paired t-test showed, regardless of the tooth type, there was no significant difference in enamel mineral content at 3 months (T1) after treatment between the two sides, while there was significant difference after 6 months (T2) ($P = 0.004$) (Table 5). RMANOVA test showed in the test side there were significant changes in enamel mineral content between T0 and T2, while in the control side there was significant changes between T0, T1, and T2 during the time course.

In addition, the paired t-test showed in the lateral and upper second premolar there was no significant difference in enamel mineral content at T1 between test and control sides, while there was significant difference at T2 between the two sides ($P = 0.011$ and 0.003, respectively) (Tables 3 and 4). Also, RMANOVA results were showed in Tables 6 and 7.

**Discussion**

Poor oral hygiene leads to enamel decalcification around and under the brackets during orthodontic treatment. Therefore, preventive procedures are very important (1–4). The use of nanoparticles in resin composites has been taken into consideration by researchers for bonding of orthodontic brackets (4, 14–17). In the present study, the efficacy of the NACP-containing composite has been examined. The results of the study show that the NACP-containing composite has a suitable antibacterial effect on $S. mutans$. Also, reduction in enamel mineral content was diminished by this composite during orthodontic treatment.

$Streptococcus mutans$ are the main factor in the development of dental caries. Nano-Ag, ZnO, and Au are among the nanoparticles that are used against cariogenic bacteria. Nano-Ag particles interfere with DNA synthesis and prevent bacterial activity (18, 19). Nano Au has poor antibacterial efficacy and is expensive (20, 21). In our study, the addition of the NACP to the composite caused significant reduction in the $S. mutans$ population around the brackets when compared to the control side at 3 months and 6 months after the initiation of orthodontic treatment. Moreau et al. (13) reported that the NACP significantly hindered growth of the $S. mutans$ in medium. Also, Cheng et al. (5) observed that the NACP in combination with Nano-Ag had suitable antibacterial characteristics. But, Melo et al. (22) reported, although the NACP nanocomposite released Ca and P ions and hadenamel mineral loss around the NACP nanocomposite, it was much less than the control. There was no significant difference in the biofilm CFU between the NACP composite and control composite. Their study was done on palatal devices containing bovine enamel slabs with cavities and measurement was performed at 14 days. The difference of the methods of the microbial examination could be the reason for the difference of the results of their study with our study.

Increasing pH by releasing calcium and phosphate ions is the mechanism of the NACP in reducing the risk of caries development (13, 23). Moreu et al. (13) observed that NACP-containing composites progressively increased the pH of the lactic acid solution. One of the advantages of the NACP compared to Nano-Ag is the color stability of the NACP. Also, Nano-Ag is only bactericidal, while the NACP is bactericidal and able to remineralize caries by releasing calcium and phosphate ions (13, 24).

Uysal et al. (24) reported that ACP was able to release calcium and phosphate ions and facilitate enamel remineralization. In previous studies, the antibacterial effect of the NACP was not examined for a long term (5, 25). In the present study, in the sixth month...
Table 2. Comparison of the *Streptococcus mutans* population between test and control sides during the time course.

<table>
<thead>
<tr>
<th>Time</th>
<th>N</th>
<th>Test side Mean ± SD</th>
<th>95% Confidence interval</th>
<th>Control side Mean ± SD</th>
<th>95% Confidence interval</th>
<th>Difference Mean ± SD</th>
<th>95% Confidence interval</th>
<th>Paired t-test</th>
<th>T</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lower</td>
<td>Upper</td>
<td>Lower</td>
<td>Upper</td>
<td>Lower</td>
<td>Upper</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3rd Month (T1)</td>
<td>40</td>
<td>63871.5 ± 30283.8</td>
<td>77414.38</td>
<td>80886.8 ± 30143.7</td>
<td>94366.77</td>
<td>17015.3 ± 42737</td>
<td>12349.8</td>
<td>2.518</td>
<td>0.014*</td>
<td></td>
</tr>
<tr>
<td>6th Month (T2)</td>
<td>40</td>
<td>56269.4 ± 32831.4</td>
<td>70946.92</td>
<td>83427.1 ± 33394.5</td>
<td>98361.7</td>
<td>27157.7 ± 46826.71</td>
<td>41965.6</td>
<td>3.668</td>
<td>0.000*</td>
<td></td>
</tr>
</tbody>
</table>

RMANOVA: in test side there was no significant differences—in control side there was no significant differences.  
*Changes were significant.

Table 3. Comparison of the *Streptococcus mutans* population around the brackets of lateral incisors between test and control sides.

<table>
<thead>
<tr>
<th>Time</th>
<th>N</th>
<th>Test side Mean ± SD</th>
<th>95% Confidence interval</th>
<th>Control side Mean ± SD</th>
<th>95% Confidence interval</th>
<th>Difference Mean ± SD</th>
<th>95% Confidence interval</th>
<th>Paired t-test</th>
<th>T</th>
<th>P value</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>Lower</td>
<td>Upper</td>
<td>Lower</td>
<td>Upper</td>
<td>Lower</td>
<td>Upper</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3rd Month (T1)</td>
<td>20</td>
<td>61095.6 ± 31846.3</td>
<td>75337.4</td>
<td>78818.0 ± 30632.2</td>
<td>92517.46</td>
<td>17722.4 ± 18225.9</td>
<td>26141.6</td>
<td>4.21</td>
<td>0.001*</td>
<td></td>
</tr>
<tr>
<td>6th Month (T2)</td>
<td>20</td>
<td>53686.1 ± 33381.4</td>
<td>68614.89</td>
<td>81517.6 ± 35008.9</td>
<td>97173.98</td>
<td>27831.5 ± 23352.1</td>
<td>38274.8</td>
<td>5.33</td>
<td>0.001*</td>
<td></td>
</tr>
</tbody>
</table>

RMANOVA: in test side there was no significant differences—in control side there was no significant differences.  
*Changes were significant.
### Table 4. Comparison of the *Streptococcus mutans* population around the bracket of second premolars between test and control sides.

<table>
<thead>
<tr>
<th>Time</th>
<th>Test side</th>
<th></th>
<th>Control side</th>
<th></th>
<th>Difference</th>
<th></th>
<th>Paired t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Mean ± SD</td>
<td>95% Confidence interval</td>
<td>Lower</td>
<td>Upper</td>
<td>Mean ± SD</td>
<td>95% Confidence interval</td>
</tr>
<tr>
<td>3rd Month (T1)</td>
<td>20</td>
<td>65403.7 ± 29354.6</td>
<td>52275.65, 78532.35</td>
<td>81731.5 ± 30640.4</td>
<td>68027.96, 95434.04</td>
<td>16327.8 ± 18627.6</td>
<td>7997.3, 24658.3</td>
</tr>
<tr>
<td>6th Month (T2)</td>
<td>20</td>
<td>58610.9 ± 31880.7</td>
<td>44352.95, 72869.05</td>
<td>83595.2 ± 34035.0</td>
<td>68387.04, 98802.96</td>
<td>24984.3 ± 20653.1</td>
<td>14747.9, 34220.6</td>
</tr>
</tbody>
</table>

RMANOVA: in test side there was no significant differences—in control side there was no significant differences.  
* Changes were significant.

### Table 5. Comparison of enamel mineral content between test and control sides during the time course.

<table>
<thead>
<tr>
<th>Time</th>
<th>Test side</th>
<th></th>
<th>Control side</th>
<th></th>
<th>Difference</th>
<th></th>
<th>Paired t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Mean ± SD</td>
<td>95% Confidence interval</td>
<td>Lower</td>
<td>Upper</td>
<td>Mean ± SD</td>
<td>95% Confidence interval</td>
</tr>
<tr>
<td>3rd Month (T1)</td>
<td>40</td>
<td>0.757 ± 0.119</td>
<td>0.70, 0.81</td>
<td>0.780 ± 0.120</td>
<td>0.72, 0.83</td>
<td>0.023 ± 0.173</td>
<td>−0.0318, 0.0778</td>
</tr>
<tr>
<td>6th Month (T2)</td>
<td>40</td>
<td>0.820 ± 0.122</td>
<td>0.76, 0.87</td>
<td>0.905 ± 0.129</td>
<td>0.84, 0.96</td>
<td>0.085 ± 0.178</td>
<td>0.0285, 0.1415</td>
</tr>
</tbody>
</table>

RMANOVA: in test side T0/T2*—in control side T0/T1*, T0/T2*, and T1/T2*.  
* Changes were significant.
### Table 6. Comparison of enamel mineral content between test and control sides during the time course around the brackets of lateral incisors.

<table>
<thead>
<tr>
<th>Time</th>
<th>Test side</th>
<th>Control side</th>
<th>Difference</th>
<th>Paired t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Mean ± SD</td>
<td>95% Confidence interval</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>3rd Month (T1)</td>
<td>20</td>
<td>0.78 ± 0.092</td>
<td>0.74 - 0.82</td>
<td>0.76 ± 0.135</td>
</tr>
<tr>
<td>6th Month (T2)</td>
<td>20</td>
<td>0.84 ± 0.119</td>
<td>0.78 - 0.89</td>
<td>0.89 ± 0.144</td>
</tr>
</tbody>
</table>

RMANOVA: in test side T0/T1* and T0/T2*—in control side T0/T1*, T0/T2*, and T1/T2*.
*Changes were significant.

### Table 7. Comparison of enamel mineral content between test and control sides during the time course around the brackets of upper second premolars.

<table>
<thead>
<tr>
<th>Time</th>
<th>Test side</th>
<th>Control side</th>
<th>Difference</th>
<th>Paired t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Mean ± SD</td>
<td>95% Confidence interval</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>3rd Month (T1)</td>
<td>20</td>
<td>0.75 ± 0.131</td>
<td>0.70 - 0.81</td>
<td>0.79 ± 0.125</td>
</tr>
<tr>
<td>6th Month (T2)</td>
<td>20</td>
<td>0.81 ± 0.130</td>
<td>0.75 - 0.87</td>
<td>0.92 ± 0.115</td>
</tr>
</tbody>
</table>

RMANOVA: in test side T0/T1* and T0/T2*—in control side T0/T1*, T0/T2*, and T1/T2*.
*Changes were significant.
examination it has become evident that the NACP preserved its antibacterial characteristics during thistem. In our study, it was observed that the enamel mineral content around the brackets that were bonded by the NACP-containing composite was significantly higher than the control side. Despite the fact that the enamel mineral content was reduced in both sides during the time course, this reduction in the test group was less than the control side. Melo et al. (22) reported that the NACP composites significantly reduced the extent of enamel caries in a human in situ model. Skr tic et al. (11) observed that the ACP-containing resin composite was able to restore 71% of decalcified enamel mineral content. Uysal et al. (24) also reported that the NACP efficiently prevented enamel mineral content reduction. In our study there were no significant differences between the lateral teeth and premolar teeth in the enamel mineral content and bacterial population.

In the evaluation of white spot lesions, different image parameters such as light, the distance between lens and sample, and its angle with the surface should be the same, which was adhered to in our study. Also, a Vista cam was used to measure enamel mineral content, which is a repeatable and non-invasive method. Studies of Uysal et al. (24), Featherstone et al. (26), Twetman et al. (27), and Pascotto et al. (17) showed mineral content loss in the cervical part was greater than the occlusal part. Therefore, in our study the enamel mineral content around the bracket was measured at 8 points and the mean of the measurements was reported.

In our study, the spray drying technique was used to produce the NACP and the size of the NACP was 116 nm, which was similar to previous studies (13, 14, 28, 29). It should be noted that one concern about nanoparticle addition to the composite is the effect of different particles on mechanical properties such as SBS (25). In our study, before conducting the trial, the effect of different concentrations of the NACP on the SBS of the composite was evaluated and it was shown that there was no significant difference between the different concentrations of nanoparticles and conventional composites.

Harms
No serious harm was observed during the entire treatment process.

Limitations
The real-time PCR is an expensive procedure therefore in this study only accumulation of bacteria around the upper lateral incisors’ and second premolars’ brackets have been measured.

Generalization
The generalizability of the results might be limited because of this research was performed in a single-center by one experienced clinician.

Conclusion
The NACP-containing bonding agent was able to reduce the amount of cariogenic bacteria around the bracket and also this composite was able to prevent enamel mineral content reduction over a long term.

Funding
This study was supported by the research chancellor of the Mashhad University of Medical Sciences.

References


