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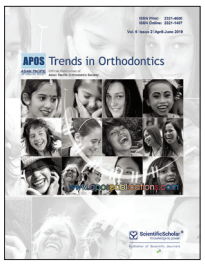

Original Article

Genotoxicity and cytotoxicity of lingual bonded retainers coated with silver and titanium dioxide nanoparticles – A randomized controlled trial

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ABSTRACT

Objectives: The popularity of fixed retainers is also challenged by the heightened chance of plaque and calculus accumulation which can lead to carious lesions, gingival inflammation, and periodontal disease. Nanotechnology has been used to coat the surface of orthodontic appliances to control the biofilm with promising results although the information on toxicity is limited. This study aimed to evaluate the genotoxicity and cytotoxicity of lingual bonded retainers coated with silver (Ag) and titanium dioxide nanoparticles and compare them with conventional uncoated fixed retainers.

Material and Methods: 60 subjects of which 45 patients requiring retainers were randomly allocated to three experimental groups of 15 each (retainers coated with Ag, TiO_2 nanoparticles, and uncoated retainers, respectively). A fourth group of 15 patients not orthodontically treated was taken as baseline control. Oral mucosal scrapings from the tongue were taken at three time intervals: At debonding (T_0), at 3 months (T_1), and at 6 months (T_2). The smears were stained with Papanicolaou stain and studied under a light microscope for micronuclei (MN).

Results: All three experimental groups showed an increase in MN counts over a period of 6 months. Group I showed the highest count at T_1 and this was significantly greater than Group 3. Group 2 showed the highest count at T_2 but this was not significantly different from the other groups.

Conclusion: Lingual bonded retainers coated with Ag and TiO_2 nanoparticles are biocompatible and can be used clinically.

Keywords: Genotoxicity, Cytotoxicity, Nanocoating, Lingual bonded retainers, nanocoating, Silver nanocoating

INTRODUCTION

With retention posing an ongoing challenge, fixed retainers have become extremely popular especially since Zachrisson in 1977 proposed the use of multi-stranded wires bonded canine-to-canine retainers.[1] Besides being esthetic and not requiring patient cooperation, there is evidence that using a fixed bonded retainer reduces the chances of lower labial segment relapse.[2]

The downside is an increased chance of plaque and calculus accumulation which can lead to the formation of carious lesions, gingival inflammation, and periodontal disease.[3] Mechanical

methods to remove plaque and antimicrobials to control the microflora can be used; however, these methods depend on patient compliance. In recent times, nanoparticles have been explored as coatings on orthodontic appliances or incorporated into bonding materials such as cement and composites. They have proved effective against various microorganisms thereby controlling the formation and composition of the oral biofilm.[4]

TiO_2 is a popular nanomaterial due to its high photocatalytic activity resulting in the organic degradation processes and also due to its low cost, chemical stability, and resistance to photo corrosion.[5] Silver (Ag) is a strong disinfectant, having a broad bactericidal spectrum with the ability to cause changes in the bacterial cell membrane leading to cell death.[6]

Although these nanomaterials have antibacterial action on the oral biofilm, the potential cytotoxicity of these materials on normal cells must be considered. Potential hazards are inflammation, necrosis, reactive oxygen species, and apoptosis. Nanoparticles used in the oral cavity can be absorbed and their small size allows them to be transported to other sites in the body.[7] The increased cytotoxicity of smaller particles is attributed to the "Trojan horse effect."[7] A study on human periodontal fibroblasts has proved that Ag nanoparticles of size <20 nm are more cytotoxic than the size of 80–100 nm.[8] Similarly, it has been demonstrated that the cytotoxicity of TiO_2 nanoparticles also differs with size, with 5 nm proving more cytotoxic than 32 nm.[9]

The study of cytotoxicity of TiO_2, Ag, and other nanoparticles has been restricted to *in vitro* and cell line studies.[10-13]

Genotoxicity is the ability of an agent to exert adverse effects on the cell's genetic material whereas cytotoxicity is the ability of an agent to be virulent to living cells.[14] The micronuclei (MN) index is considered one of the standard cytogenetic endpoints and biomarkers in genetic toxicology. [15,16]

MN are extranuclear cytoplasmic bodies that have their origin from the acentric chromosome fragments, acentric chromatid fragments, or whole chromosomes that fail to be included in the daughter nuclei at the completion of telophase during the process of mitosis because they failed to attach properly with the spindle during segregation process in anaphase.[17]

Objectives

Thus, this study was conceived to evaluate the genotoxicity and cytotoxicity of multistranded lingual bonded retainers coated with Ag and TiO_2 nanoparticles in an *in vivo* environment and compare them with conventional uncoated fixed retainers using the MN assay.

MATERIAL AND METHODS

Trial design

This was a randomized parallel study design with an allocation ratio of 1:1.

Participants

The study was conducted in the Department of Orthodontics, Faculty of Dental Sciences, Sri Ramachandra University, Chennai with approval from the Institutional Ethics Board: CSP/16/SEP/51/281.

Patients with permanent dentition who had completed fixed orthodontic treatment with all teeth present in the lower anterior segment and clinically healthy oral mucosa were included in the study. The age of the patients ranged from 14 to 25 years. Patients who had a history of smoking, oral or systemic diseases, or under any medications or supplements were excluded from the study.

Enrollment

The sample size was calculated using the alpha significance level of 0.05 with a power of 80 as per the study of Natarajan M *et al*.[15] A total number of 60 subjects were enrolled in the study. Among them, 45 patients who had completed fixed orthodontic treatment and required retainers were recruited into three groups that received intervention. A fourth group of 15 patients (within the same age group) who were not orthodontically treated made up the baseline control [Figure 1].

Randomization

Randomization was done using the computer-generated program, i.e., www.random.com and an allocation sequence was generated to distribute the intervention methods equally. Patients in the experimental groups were randomly divided into 3 groups of 15 patients each [Table 1].

Blinding

The participants were blinded throughout the study period.

Sample preparation

45 wires of 10 cm length of commercially available 0.017-inch diameter coaxial multi-stranded wire (Rabbit Force Multistranded Retainer, Libral Traders Pvt., Ltd.) were used to prepare the fixed retainers. Of these, 15 samples were coated with Ag nanoparticles, 15 samples were coated with nitrogen (N)-doped TiO_2 nanoparticles, and 15 samples were uncoated.

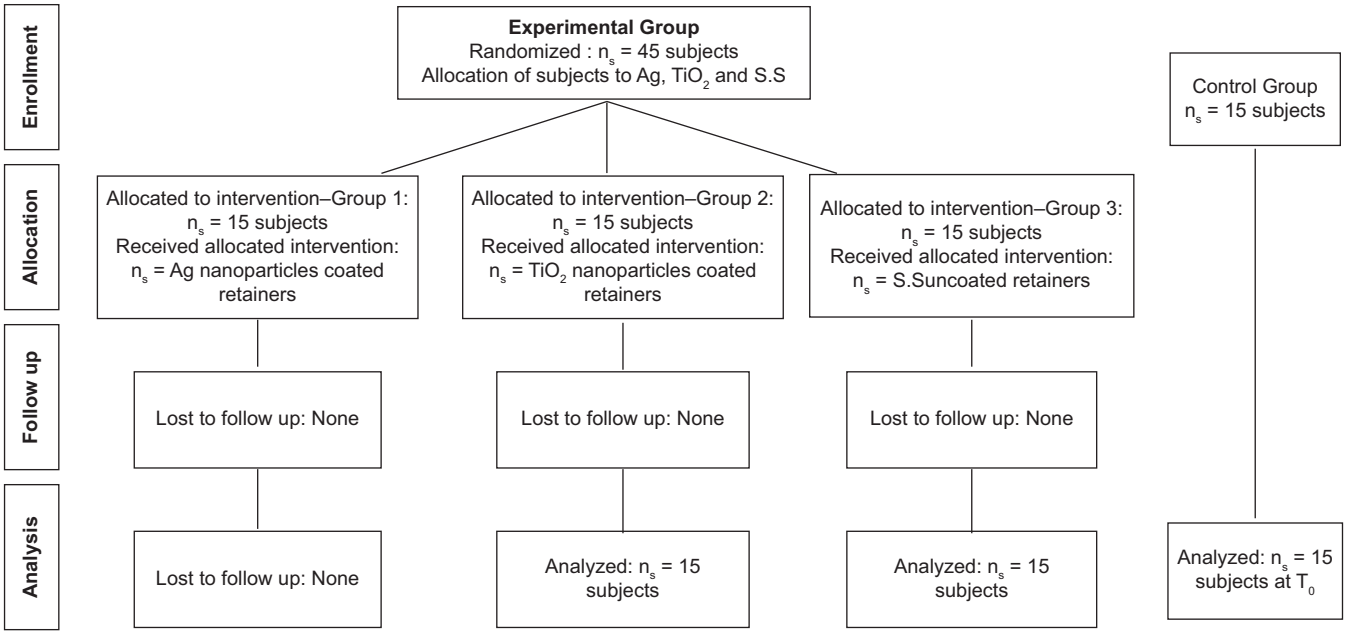

Figure 1: Consort flow chart. Ag: Silver, TiO_2: Titanium dioxide, S.S: Stainless steel

Table 1: Participant allocation into various groups.

Groups	Allocated Intervention
Group 1	Lingual bonded retainer coated with Ag nanoparticles
Group 2	Lingual bonded retainer coated with TiO_2 nanoparticles
Group 3	Uncoated stainless steel (S.S) lingual bonded retainer
Group 4	Untreated controls at T_0

Ag: Silver, TiO_2: Titanium dioxide

Preparation of retainer wires coated with N-doped TiO_2

Surface coating of coaxial multi-stranded retainer wires with N-Doped TiO_2 was carried out by the Radio Frequency (RF) magnetron sputtering (Anelva Sputtering Unit Model SPF-332H) method. The wires were coated with TiO_2-N of 32 nm size and 99.99% purity and then cooled to room temperature and annealed in a Nitrogen atmosphere at 450°C in a muffle furnace (Sastha Scientific, Bangalore). After annealing, the coated wires were analyzed under a Scanning Electron Microscope, and a film thickness of 50–80 nm TiO_2 was observed. X-ray diffractionometer analysis was done to ensure that TiO_2 existed in the anatase phase. The wires were then activated in a chamber by visible light (100 W) for 24 h before placing them into the oral cavity.[10]

Surface coating of coaxial multistranded retainer wire with Ag nanoparticles was carried out similarly. The wires were coated with a particle size of 80–100 nm, 99.99% purity, and thickness in the range of 60–80 nm of Ag.

Bonding of fixed retainers

After oral prophylaxis, etching of enamel was done with 37% phosphoric acid gel (d-tech® gel), followed by rinsing with water and drying. Primer (Meta P& Bond) was applied and cured using a Quartz Tungsten Halogen light unit (QLH75 Dentsply) for 10 s/tooth. The retainers were bonded from canine to canine using conventional orthodontic flowable adhesive (Meta Biomed). The adhesive was light cured for 40 s/tooth.

Oral mucosal cell sampling

The cells were collected from each individual using a sterile cement spatula. The blunt end of the spatula was used to perform the motion 2–3 times with a firm force until sufficient material was collected on the edges of the spatula. The end of the spatula was placed on the glass slide and smeared in a single sweep unidirectionally to obtain a perfect smear without clubbing or folding of squamous cells. For the experimental groups, the samples were collected in 3 time periods.

T_0-Immediately after debonding of fixed orthodontic appliances subsequent to oral prophylaxis.

T_1-3 months after retainer placement

T_2-6 months after retainer placement.

Samples were collected from untreated participants and compared to the $T_{0\ sample}$s.

Slide preparation

The sample obtained was smeared onto the center of a clean glass slide and the smears were immediately fixed in absolute alcohol (Isopropyl alcohol - 100%) for a period of 20–30 min. Then, the slides were hydrated with distilled water and stained using the Papanicolaou (PAP) method according to the standard protocol (PAP, 1942).[18]

The staining technique results in the blue-black appearance of nuclei and the blue-green appearance of cytoplasm. The keratinizing cells have a pinkish-orange hue.

The slides were observed at 40× magnification under the light microscope (Lawrence and Mayo XSZ-N107T). Cells were observed using high-power magnification in 10 fields in a zigzag fashion to determine the presence of MN [Figure 3].

Evaluation of MN

MN was identified according to the standard protocol[19] and to fulfill the following characteristics:

- Round, smooth perimeter suggesting a membrane
- Less than a third of the diameter of the associated nucleus, but large enough to discern the shape and color
- Staining intensity similar to that of the nucleus
- The same focal plane as the nucleus
- No overlap with, or bridge to, the nucleus.
- No overlap of the cells.[19]

The number of MN in all the groups was determined and subjected to statistical analysis.

Statistical analysis

The collected data were analyzed with IBM, the Statistical Package for the Social Sciences statistics software 23.0 Version. To describe the data descriptive statistics, mean and standard deviation were used. To find the significant difference within the groups, at different time intervals, the Friedman test was used followed by the Wilcoxon signed-rank test. To find the significant difference between the groups, the Kruskal–Wallis test followed by the Mann–Whitney U-test was used for the intergroup comparison ($P < 0.05$).

RESULTS

Outcomes

Intragroup comparisons

In group 1, the MN count increased from T_0 to T_1 and decreased from T_1 to T_2. The overall increase from T_0 to T_2 was not statistically significant.

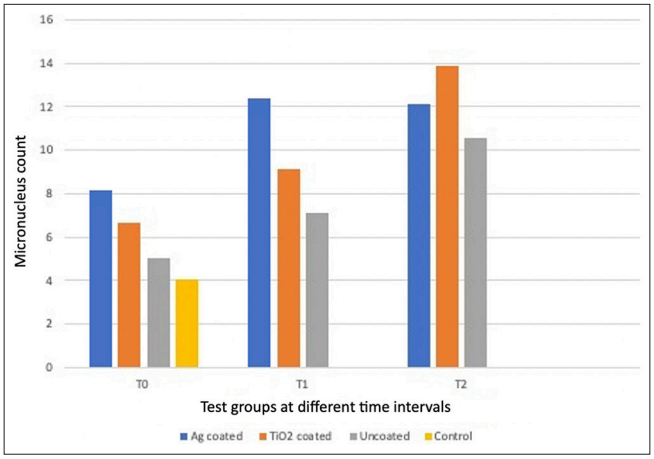

Figure 2: Comparison of micronuclei count between groups at different time intervals.

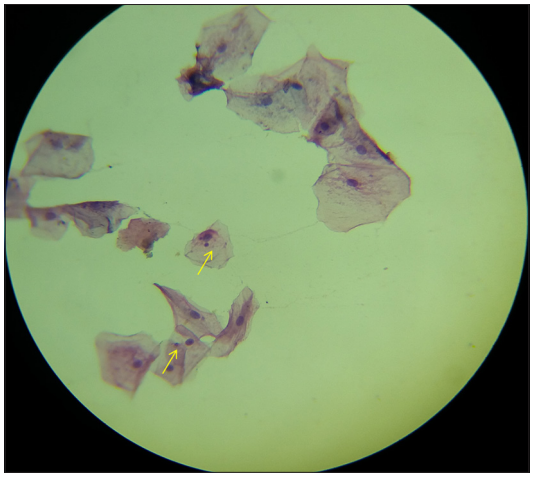

Figure 3: Micronuclei stained with papanicolaou (PAP) highlighted in high-power magnified field.

In group 2, the MN count increased sequentially from T_0 to T_1 to T_2. The increase was statistically significant from T_0 to T_2 and from T_1 to T_2.

In group 3, the MN count increased sequentially from T_0 to T_1 to T_2. The increase was statistically significant overall from T_0 to T_2 and T_1 to T_2 [Tables 2 and 3].

Intergroup comparison

At both T_0 and T_1, the MN count was highest in group 1 followed by group 2 and group 3. All experimental groups showed a greater MN count as compared to the baseline control at T_0 but this was not significant [Figure 2]. At T_1, there was a significant difference between group 1 and group 3. At T_2, the MN count was greatest in Group 2 followed by Group 1 and Group 3 but this was not statistically significant [Table 4].

Table 2: Descriptive statistics of the various groups.

Time period	Groups	*n*	Mean	Standard deviation	Mean ranks	Chi-square	*P*-value
T_0	Ag	15	8.13	6.896	33.77	3.360	0.339
	TiO_2	15	6.67	5.052	34.57		
	S.S	15	5.00	3.162	29.43		
	Control	15	4.07	1.751	24.23		
T_1	Ag	15	12.40	5.742	30.53	9.521	0.009*
	TiO2	15	9.13	3.204	22.67		
	S.S	15	7.13	3.248	15.80		
T_2	Ag	15	12.13	4.719	23.47	2.893	0.235
	TiO2	15	13.87	5.927	26.80		
	S.S	15	10.53	1.922	18.73		

S.S: Stainless steel, Ag: Silver, TiO_2: Titanium dioxide

Table 3: Intragroup comparison at different time intervals.

Groups	Friedman test	Wilcoxon signed-rank test		
	T_0-T_2	T_0 versus T_1	T_1 versus T_2	T_0 versus T_2
Group 1	0.169	0.102	0.155	0.925
Group 2	0.002*	0.083	0.006*	0.003*
Group 3	0.001*	0.131	0.001*	0.009*

*Statistically significant difference at P<0.05

Table 4: Intergroup comparison using Mann–Whitney test.

Time period	Ag and TiO_2	Ag and S.S	TiO_2 and S.S
T_0	0.870	0.412	0.436
T_1	0.056	0.004*	0.089
T_2	0.512	0.345	0.089

S.S: Stainless steel, Ag: Silver, TiO_2: Titanium dioxide

Harms

The participants in the study did not present with any allergic or adverse reactions during the course of the study.

DISCUSSION

Fixed retention brings associated challenges of maintaining oral hygiene and increased plaque and microbial accumulation on the tooth surface.[4]

The application of nanocoatings on orthodontic appliances has been widely explored and found effective but most studies have been restricted to *in vitro* situations without evaluating the toxicity. This study sought to evaluate the genotoxicity and cytotoxicity of multistranded lingual bonded retainers coated with Ag and TiO_2 nanoparticles in an *in vivo* environment.

The photocatalytic activity of TiO_2 when exposed to ultraviolet (UV) light has been employed since TiO_2 has a wide bandgap of 3.2 eV, where its absorption edge occurs below 400 nm (UV region) and only a small fraction of solar spectrum is absorbed.[20] However, since exposure to UV light has its downsides, doping using non-metal ion elements has been favored to reduce the optical gap of TiO_2 to visible light. Nitrogen has gained popularity and it has been suggested by Asahi *et al.*[21] that visible light of <500 nm would be sufficient to activate N-doped TiO_2. Since its efficacy has been proven in clinical settings, we chose N-doping to activate TiO_2 under visible light.[20] Since the anatase phase has more photocatalytic activity and minimal cytotoxic effects as compared to the rutile phase, we chose to coat our wires with the anatase phase of TiO_2.[10]

Ag nanoparticles have a broad bactericidal spectrum and have been used to coat orthodontic appliances including fixed and removable retainers demonstrating strong anti-bacterial effects.[22,23]

In our study, the coating of the retainer wires was done using a magnetron sputtering machine since this technique has certain advantages such as a strong and uniform coating and high hydrophilicity.[24] While previous studies have used buccal mucosal cells to evaluate genotoxicity, in this study, samples were collected from the tip of the tongue due to its direct contact with the lingual surface of the bonded retainer. MN assay is considered a simple, sensitive, and non-invasive method for evaluating DNA damage, proliferation of basal cells, and cell death. The presence of MN is indicative of chromosomal abnormalities that include breakage of chromosomes and subsequent damage to the DNA. This test is generally performed as a reliable screening test for the presence of genotoxic compounds.[25]

A recent study concluded that PAP stain is the preferred method for detecting MN and this method was used. It encompasses a fixative that has the potential to demarcate the cell boundaries clearly so that the MN is visible in the transparent cytoplasm.[26]

Since orthodontic appliances can also contribute to genotoxicity, we used an untreated control to mitigate the residual influence

of fixed appliances for baseline comparison.[14] At T_0, all three experimental groups and the untreated control group showed some presence of MN. Although the latter displayed the least MN count compared to the experimental group, the difference was not statistically significant.

At T_1, all three experimental groups showed an increase in the MN count, with group 1 (Ag) coated retainers showing the highest count and this was significant when compared to the uncoated retainers.

At T_2, there was a further increase in all the groups except group 1, which showed a small and insignificant decrease. There was no significant difference between the three groups.

It appears that Ag nanoparticles were more cytotoxic in the initial periods which reduced over the 3–6-month period. Although TiO_2-coated retainers showed a significant increase in the MN over 6 months, this was not significant when compared to the other groups since the uncoated samples group also showed an increase in the number of MN. This could probably be attributed to the inherent cytotoxicity of the metal and mechanical irritation caused by the retainer in contact with the tongue.[14] Although both Ag and TiO_2 nanoparticles showed a significant increase over a period, the result was no different when compared to the uncoated stainless steel retainer group.

Despite statistical significance reflected at some time intervals, the increase in MN count from T_0 to T_2 was not high and leveled off by 6 months. The lack of clinically significant genotoxicity and cytotoxicity when compared with the uncoated retainer group advocates the usage of Ag and TiO_2 nanoparticles in orthodontic practice.

Limitations

This study evaluated the cytotoxicity based on the premise that nanocoatings remain viable on the surface of the retainers over 6 months which was not confirmed. The presence of fixed appliances in the mouth can contribute to genotoxicity which is generally reversed. This was mitigated by including an untreated sample.

Generalizability

This is the first study that has reported on the cytotoxicity and genotoxicity of retainers coated with Ag and TiO_2 nanoparticles over a period of 6 months. Although the coated retainers did show an increased MN response as compared to non-coated retainers, this was not remarkably significant both statistically and clinically.

CONCLUSION

This study was done to evaluate and compare the genotoxicity and cytotoxicity of lingual bonded retainers coated with Ag and TiO_2 nanoparticles.

The following conclusions were drawn from the study.

- Ag and TiO_2 nanoparticles coated retainers showed a significant increase in MN count over a period of 6 months although this increase was no different when compared to uncoated stainless-steel retainers.

Thus, orthodontic lingual bonded retainers coated with Ag and TiO_2 nanoparticles are biocompatible and can be used clinically.

Ethical approval: The research/study was approved by the Institutional Review Board at Sri Ramachandra Institute of Higher Education and Research, number CSP/16/SEP/51/281, dated September 2016.

Declaration of patient consent: The authors certify that they have obtained all appropriate patient consent.

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