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Assessment of cytotoxicity of clear aligners coated with zinc oxide nanoparticles

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ABSTRACT

Background: Coating with nanoparticles can have clinical benefits like anti-bacterial activity and therefore an improvement in oral hygiene. However, the toxicity of these coatings is not known. The aim was to assess and compare the viability of human gingival fibroblasts (HGFs) of clear aligners coated with zinc oxide nanoparticles of different sizes.

Methods: Zinc oxide nanoparticles of 30 nm, 60 nm & 90 nm were sputter coated on five aligner samples and were compared with 5 uncoated samples. The thickness of the coating was standardized at 100 nm. The cytotoxicity on HGFs was assessed at day 0, 7 and 14 using assay ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]).

Results: The coated aligners exhibited "slight-cytotoxicity" on day 7 and day 14 compared to the uncoated aligners which were categorized as "no cytotoxicity". Though the cell viability decreased in both the coated and uncoated aligners from day 0 to day 7 to day 14, this decrease was statistically significant for the coated aligners only. ($p < 0.05$) The greater cytotoxicity observed for the coated aligners was statistically significant only on day 14 ($p < 0.001$). Within the coated aligners, the 90 nm coating showed the least cytotoxicity, when compared with 60 nm and 30 nm at the time points measured, but this difference was significant only between the 90 nm and 60 nm group on day 14.

Conclusion: The mean decrease in cell viability for the uncoated aligner within the "no cytotoxicity range" while it was within the "slight cytotoxicity range" for the coated aligner. An increase in particle size demonstrated lesser cytotoxicity. ,.Zinc oxide coated aligners should be used with caution after day 7 even though there is only slight toxicity.

1. Introduction

Clear aligners are the preferred appliances for patients concerned with aesthetics.[1] Clear aligner therapy also results in white spot lesions which continues to be a problem for orthodontic patients and the contemporary orthodontist.[2] While these lesions are managed by various methods, nano coatings which have been used on orthodontic brackets[3] arch-wires[4] and adhesives[5] have shown promise by exhibiting anti-microbial properties. Clear aligners are now gaining in acceptance within the clinical fraternity and the general population. With advancements in scanning accuracy, it is now possible to plan for more accurate and precise treatment.[6]

Clear aligners are in close proximity with the teeth and the oral mucosa. They have shown slight cytotoxicity under experimental conditions and the thermoforming process increases the cytotoxicity.[7] The increased use of nanoparticles has also raised a concern regarding their cytotoxicity· Cytotoxicity depends on the type of nanoparticle, particle size, concentration of the nanoparticles used and their route of administration.[8,9] In a comparison between zinc, copper and iron oxide nanoparticles at low concentrations, zinc oxide exhibited higher cell viability[10] and hence should be the focus of further research.

Previously, two studies have assessed clear aligners coated with nanoparticles and demonstrated significant antimicrobial properties.[11,12] However, the particle size of the nano coatings and their influence on the biological safety were not assessed. Hence the aim was to evaluate the cell viability as an indicator of the possible cytotoxicity of clear aligner materials coated with zinc oxide nanoparticles of various sizes by assessing the cell viability human gingival fibroblasts (HGFs).

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2. Materials and Methods

Ethical approval from the University's Institutional Ethics Committee was obtained [Number: CSP/21/JUL/96/393]. A prospective invitro study was designed to evaluate the cytotoxicity of zinc oxide coated aligners on human gingival fibroblasts. The study was conducted for a period of fourteen days. The inclusion criteria were 'as received' Invisalign® aligners (Align Technology, San Jose, California, USA), wherein the labial aspect was intact. The exclusion criteria were aligners with attachments on central incisors, aligners fabricated for incisors with morphological abnormalities and distorted or used aligners.

Based on the data from Martina et al.,[7] who reported cell viability difference of 18 ± 1 between aligner groups and a power of 80 % and a significance level of 0.05, the sample required was 5 samples per group. With the control group consisting of uncoated aligners, a cell viability difference of 20 % between the coated and the uncoated aligners was considered significant.

The samples were divided into: (1).

- Group 1: Aligners coated with 30 nm zinc oxide nanoparticles
- Group 2: Aligners coated with 60 nm zinc oxide nanoparticles
- Group 3: Aligners coated with 90 nm zinc oxide nanoparticles
- Group 4: Uncoated aligner (Control)

Aligner segments corresponding to the flat anatomical region of the labial surface of the maxillary central incisors were cut vertically in 9 mm × 2.5 mm dimensions.[13] All the aligner samples were exposed to similar conditions prior to the radio frequency magnetron sputtering process which was used to coat the nanoparticles on the tooth facing surface of the aligner.sample. The samples were checked for their cell viability on HGFs at day 0, 7 and 14.**Sample Preparation:** Zinc Oxide coating (ZnO) was achieved in a cathodic sputtering unit (Alcatel SCM 450-Alcatel –Lucent, Paris, France) which was equipped with a radio-frequency generator operating at 13.56 MHz. ZnO target of 99.9 % purity fixed on an equilibrated magnetron cathode was used. The deposition temperature used for sputter coating was standardized at 200^0 C. In order to vary the zinc oxide nano-particle size, the sputtering power was varied.[14] ZnO nanocoating of 100 nm thickness was coated. To check the validity and uniformity of the coating, a contact profilometer was used (DektakXT Profiler, Karlsruhe, Germany). One sample was chosen at random from each of the coated groups and the thickness was measured at three points and an average was calculated. To calculate the particle size, to confirm the uniformity and view the surface topography, all the coated samples were subjected to FE-SEM analysis (Tescan VEGA-3,Brno, Czech Republic).

Cell Culture: Human gingival fibroblasts were cultured in Dulbecco's minimum modified essential medium (DMEM) supplemented with 10 % fetal bovine serum and 1 % antibiotic antimycotic solution (penicillin-streptomycin). The cells were incubated under standard cell culture conditions (37 °C, 100 % humidity, 95 % air, and 5 % CO2).

Cell Viability Assessment: MTT assay[15] [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] was used to evaluate cell viability. Human gingival fibroblasts were planted into 96-well flat-bottomed, tissue culture plates, with a density of 105 cells/well. After incubation for 24 h, the aligner samples were placed in each well and incubated after 24 h. The medium was then replaced with 100 μL/well of the MTT solution (5 mg/mL). The cells were then incubated for an additional 4 h at 37 °C in a 5 % CO2 atmosphere. Hundred μL/well of dimethyl sulfoxide was added after removing the solution and the plates were swirled gently for 10 min. A spectrophotometer at 570 nm was used to measure the optical density of each well immediately. The optical density of the cells cultured in the DMEM medium without any clear aligner material was used as a reference. Three independent experiments were performed in triplicate.

Vannet's[16] formula was used to calculate the cell viability:

$$\text{Percentage Viability} = \frac{\textit{Optical density of Test}}{\textit{Optical density of Control}} * 100$$

Cell viability was scored according to the classification of Ahrari et al.[17] and is as follows.

- More than 90 percent cell viability: no cytotoxicity
- 60–90 percent cell viability: slight cytotoxicity
- 30–59 percent cell viability: moderate cytotoxicity
- Less than 30 percent viability: severe cytotoxicity

2.1. Statistical analysis

This data was subject to statistical analysis with IBM SPSS statistics software 21.0 Version. (IBM, Chicago, USA). Differences between mean values were determined by one-way analysis of variance (ANOVA) with Bonferroni post-hoc test. The level of significance was set as $p < 0.05$.

3. Results

SEM images taken on day 0, 7 and 14 days depicted uniformly distributed cells on the aligner surface. On day 0, the mean percentage of viable cells was similar and cytotoxicity was not exhibited by the coated and the uncoated groups. On day 7 & day 14, the coated aligners had a lesser percentage of viable cells when compared to the uncoated aligner group and also exhibited "slight cytotoxicity", while the uncoated aligners did not exhibit any cytotoxicity.

Among the coated aligners, the 90 nm group showed the least toxicity while the 60 nm group showed the highest cytotoxicity. Intra-group comparison revealed a statistically significant difference ($p < 0.05$) between the means of the 30,60 and 90 nm coated aligner groups on day 0, 7 & 14 (Table 1). Within the 30 nm group, a statistically significant difference ($p = 0.03$) was found between day 0 and day 14, within the 60 nm group, a statistically significant difference ($p = 0.02$) was found between day 0 and day 14 and the within the 90 nm group, a significant difference was found between day 7 & day 14 ($p = 0.02$) and day 0 & day 14 ($p = 0.04$) (Table 2)). From day 0–7, day 7 to day 14 and day 0 to day 14, there was an increase in the cytotoxicity for all the groups. On day 0 and day 7, there was no statistically significant difference ($p > 0.05$) between the coated and the uncoated groups (Table 2), while there was a significant difference ($p < 0.01$) on day 14. The coated aligners exhibited greater cytotoxicity than the uncoated sample and was statistically significant.

4. Discussion

Several factors affect the cytotoxicity of these coatings, such as the type of nanoparticle used, the concentration and the particle size used for coating.[18] Zinc oxide was used in this study due to its proven antimicrobial properties and high transparency levels.[4] While there are multiple advantages of zinc oxide nano coatings, the biggest disadvantage is its cytotoxicity which may damage cell membranes by perforating them.[18] Smaller sized nanoparticles are more cytotoxic than larger ones.[19] Small nanoparticles possess a high surface area relative to their total mass, which increases their chance to interact with surrounding biomolecules and triggers adverse responses. Hence the effect of three different particle sizes i.e. 30 nm, 60 nm and 90 nm was assessed in this study. The recommended duration for aligner change is currently 7 or 14 days.[20] Hence, the cytotoxicity was evaluated for a period of two weeks. The samples were analysed for toxicity on day 0, 7 and 14.

The coating thickness was standardized to 100 nm to ensure antibacterial efficiency.[21] Thickness greater than 100 nm could also affect the optical properties of the aligners. The cell staining images showed that the cells adhering to both the coated and uncoated aligners retained

Table-1
Assessment of cell viability within different groups on day 0, 7 and 14 of observation. (NS – Not significant).

Groups	Cell viability (%) on different observation days			Significance (P value)	Comparison between different observation days (P value)		
	Day 0	Day 7	Day 14		0 Vs. 7	0 Vs. 14	7 Vs. 14
Group 1	97.1 ± 1.3	78.3 ± 12.3	66.6 ± 6.6	0.04	0.29	0.03	0.22
Group 2	96.5 ± 2.5	77.5 ± 11.3	62.4 ± 5.6	0.01	0.22	0.02	0.14
Group 3	95.1 ± 3.8	81.5 ± 0.3	75.0 ± 0.8	0.03	0.08	0.04	0.02
Group 4	98.1 ± 0.2	95.5 ± 0.7	93.6 ± 1.0	0.06	NS	NS	NS

Table-2
Assessment of cell viability between different groups on day 0, 7 and 14 of observation.

Observation Day	Cell viability (%) in different groups				Significance (P value)	Comparison between different group (P value)					
	Group 1	Group 2	Group 3	Group 4		Group 3 Vs 2	Group 3 Vs 1	Group 3 Vs 4	Group 2 Vs 1	Group 2 Vs 4	Group 1Vs 4
Day 0	97.1 ± 1.3	96.5 ± 2.5	95.1 ± 3.8	98.1 ± 0.3	0.52	NS	NS	NS	NS	NS	NS
Day 7	78.3 ± 12.3	77.5 ± 11.3	81.5 ± 0.3	95.5 ± 0.8	0.9	NS	NS	NS	NS	NS	NS
Day 14	66.6 ± 6.6	62.4 ± 5.6	75.0 ± 0.8	93.7 ± 1.1	<0.00	0.04	0.3	0.01	1	<0.00	<0.00

their viability. There was no occurrence of dead cells. The SEM images also revealed that the cells formed a confluent layer on the surface of the aligners which was more evident on day 14 for both the coated and uncoated aligner groups indicating slight toxicity.

The MTT assay is a type of colorimetric assay. This determines the cell viability through determination of mitochondrial function of cells.[22] MTT assay is considered to be the gold standard for determination of cell viability and proliferation[23] and hence was used in this study. This was performed on human gingival fibroblast cell lines, because this is one of the most abundant in the oral cavity and is clinically exposed to potential toxic effects of orthodontic appliances during treatment.[24] Liu et al.[25] hypothesized that the toxicity of ZnO NPs was due to then accretion of autophagosomes while Singh S[26] reported Zinc oxide nano particles caused toxicity by promoting zinc release, the assembly of reactive oxygen species (ROS) and mechanical damages caused by physical contact between the cell and the nano particle.

The results of this study showed that, the coated aligners showed lesser cell viability and therefore higher cytotoxicity compared to uncoated aligners. This was in accordance with the Prach et al.[19] based on non-orthodontic materials. Our findings were contradictory to the findings of Baek et al.[9] who found that lesser dimension (20 nm) zinc oxide nanoparticles were more cytotoxic compared to larger (70 nm) particles. This could be because of the different cell lines used for assessment of cytotoxicity, with previous studies demonstrating a cell type-specific toxicity profiles.[27,28]

Controlling the ZnO nanoparticle size is essential to achieve best bactericidal response, and ZnO-NPs with smaller size showed highest antibacterial activity.[15] Anita et al.[11] demonstrated anti-microbial efficacy of zinc oxide coated aligners with particle size ranging from 61.59 to 82.03 nm against *S mutans*. Zinc oxide coated aligners exhibited higher cytotoxicity compared to the uncoated aligners, though it was slight. Since there was no statistically significant difference in their cell viability up to 7 days and as there was increased toxicity at the 14-day timepoint, a 7-day protocol could prove advantageous. This protocol involving a new aligner on day 7 could reinforce their antimicrobial property as well as be less cytotoxic. • Zinc oxide coated aligners should be used with caution even though there is only slight toxicity. Further research is required for its use up to 14 days.

Limitations: The major limitation is the in vitro design with the results being obtained under stationary conditions. Dynamic in vivo conditions involving the masticatory cycle and intra oral ageing could cause leeching of the zinc ions leading to variations in the results obtained. Hence future studies evaluating different thickness and varying concentrations of zinc oxide should be performed in in vivo conditions to assess toxicity. While only HGFs were assessed in this study, the effects on epithelial cells warrant further research.

5. Conclusions

- The mean decrease in cell viability for the uncoated aligner was within the “no cytotoxicity range”
- The mean decrease in cell viability for all the coated aligners was within the “slight cytotoxicity range”
- The particle size had an effect on cytotoxicity and this effect was greater on day 14 than on day 7
- Zinc oxide coated aligners should be used with caution even though there is only slight toxicity

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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