



## Original Research Article

# Evaluation of cytotoxicity of copper oxide and zinc oxide hybrid nanocoated orthodontic brackets

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## ABSTRACT

**Background:** Using a combination of two nanoparticles for coating stainless orthodontic brackets might alter their potential cytotoxicity. The study investigated the cytotoxicity of brackets nanocoated with copper oxide, zinc oxide and a combination of both the particles.

**Materials and Methods:** Stainless steel orthodontic brackets (Ormco Mini -Diamond series 0.22" slot, MBT prescription) (ORMCO CORP Glendora, California, USA) (n= 31 in each group) were coated with nanoparticles of copper oxide, zinc oxide and a combination of copper oxide –zinc oxide using a spray pyrolysis method. The brackets was assessed for cytotoxicity in mouse fibroblast (L929) using MTT assay with a standard control and a group of uncoated brackets for comparison. The optical density and percentage of cell viability of the 5 groups were compared with ANOVA and Post hoc Tuckey HSD. P value  $\leq 0.05$  was considered as statistically significant.

**Results:** The three groups of coated brackets and the uncoated brackets exhibited significantly lesser percentage of cell viability than the control group. The percentage of cell viability in all the four groups was greater than 70%. Zinc oxide and copper oxide nanocoated brackets exhibited lesser cell viability than the combination group and the uncoated brackets.

**Conclusion:** Brackets coated with combination of copper oxide and zinc oxide nanoparticles exhibited lesser cytotoxicity than the brackets coated with copper oxide or zinc oxide nanoparticles.

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## 1. Introduction

Coating of orthodontic bands, brackets and archwires with nanoparticles possessing antimicrobial and solid lubricant properties have been extensively investigated in the past decade. Nanocoated orthodontic attachments demonstrated antibacterial properties and studies have proven that nanocoating could considerably reduce the friction generated during sliding of brackets along archwires.<sup>1–9</sup> Using a combination of two nanoparticles as a hybrid coating method improved the antibacterial properties and may also reduce the adverse effects of the individual

particles.<sup>9–11</sup> Copper oxide and zinc oxide nanoparticle coated stainless-steel orthodontic brackets reduced the count of streptococcus mutans in cell culture and a hybrid nanocoating of these metal oxides obtained by mixing them in the ratio of 1:1 by weight was reported to have better antibacterial property than their individual coatings or when mixed with silver nanoparticles.<sup>9–11</sup>

Orthodontic brackets, bands, archwires, ligatures, solder joints, composite resins, mini-implants, and clear aligners are often examined for their toxicity to determine whether they release ingredients that can be harmful to cells.<sup>12–18</sup> The concerns about release of metal ions from the metallic orthodontic brackets have received a lot of attention.<sup>19–21</sup> Various testing methods were used to use to investigate

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the quantity of ions released from components of fixed orthodontic appliance and its potential cytotoxic effects. Amini et al. evaluated the release of nickel into the saliva of patients undergoing fixed orthodontic treatment using an atomic absorption spectrometry to compare it with their siblings of same gender who has not received any orthodontic appliances. They concluded that the components of fixed orthodontic appliances leach metal leading to an increase in their salivary concentration.<sup>19</sup>

Huang et al. evaluated the effect of recycling on the biocorrosion of orthodontic brackets stored in artificial saliva and buffers of various pH. The concentration of nickel, chromium, iron, and manganese released was detected by atomic absorption spectrometry and they confirmed that recycling increases the biocorrosion but the total ions released over a period of 12 weeks did not exceed the recommended daily dietary intake.<sup>20</sup> Kuhta et al. evaluated the effect of the composition of the archwire, the pH of artificial saliva and the duration of immersion on the release of six different type of ions including copper and zinc. They concluded that the ions released were sufficient to cause delayed hypersensitivity reactions and should be considered while selecting type of appliance and archwires in patients with hypersensitivity or compromised oral hygiene.<sup>21</sup>

Mobeen et al. evaluated the release of copper and zinc ions from copper-oxide and zinc- oxide nanoparticles coated orthodontic brackets in artificial saliva using an atomic absorption spectrometry. The findings concluded that leaching of zinc from the zincoxide nanocoated brackets was greater than that of copper from the copper oxide nanocoated brackets but the amount of both zinc and copper ions released over a period of 28 days was well below the levels that are toxic to humans.<sup>22</sup>

Jacoby et al. assessed the in-vitro cytotoxicity of orthodontic bands with and without silver solder in keratinocytes, fibroblasts and kidney epithelial cells using MTT assay and concluded that the cell lines showed decreased viability after exposure to the extracts from bands with solder joints.<sup>12</sup> Retamoso et al. evaluated the cytotoxicity of metal, nickel free metal, ceramic and polycarbonate brackets in mouse fibroblast (NIH/3T3) using a MTT assay and a microplate reader that recorded the optical density at 570nm and concluded that nickel-free brackets had better biocompatibility and polycarbonate brackets were cytotoxic.<sup>14</sup> Bracket identification dyes displayed cytotoxicity at higher concentrations when evaluated with MTT assay and a real-time cell analysis system.<sup>18</sup>

Ersoz et al. assessed the biocompatibility of three different light cure orthodontic resins using a real time cell analysis system in human gingival fibroblasts (HGF) and found that two of the composite resins tested low HGF index when exposed to the resin elutes for a longer

period of time.<sup>16</sup> Bahrami et al. evaluated the cell viability of human gingival fibroblasts exposed to orthodontic bands coated with silver or zinc oxide nanoparticles and concluded that the zinc oxide coated bands had the highest biocompatibility.<sup>23</sup>

Even though the antibacterial and frictional properties of nanocoated orthodontic brackets have been investigated extensively, the hybrid coating using a combination of two nanoparticles and their cytotoxicity, which might be presumed to be less toxic than the individual materials, have not been previously examined. The present study, therefore evaluated the cytotoxicity of orthodontic brackets nanocoated with copper oxide, zinc oxide and a combination of both nanoparticles in L929 mouse fibroblasts using an MTT assay.

## 2. Materials and Methods

This study design was approved by Institutional Review Board and Institutional Ethical Committee of SRM Dental College, Ramapuram, Chennai with an approval number of SRMDC/IRB/2023/PhD/NO.156. The sample size determination was performed using N-Master software (V2.0) and with a power of 80% and alpha error of 5%, the sample size arrived was 31 in each group. There were four groups, Group I brackets were coated with nanoparticles of copper oxide, Group II with nanoparticles of zinc oxide, Group III with a hybrid coating comprising a combination of copper oxide and zinc oxide and Group IV of uncoated stainless steel orthodontic brackets.

In total 136 Ormco Mini -Diamond series 0.22" slot, MBT prescription, stainless steel orthodontic brackets (ORMCO (ORMCO CORP Glendora, California, USA) were used in the study. Group I, II & III were allocated with 35 brackets and group IV with 31 brackets. The group I, II and III brackets were coated with nanoparticles of copper oxide, zinc oxide and a hybrid of copper oxide -zinc oxide using a spray pyrolysis method. Zinc oxide nanoparticles with an average size of 40 nm and copper oxide nanoparticles with an average particle size of 45 nm and 99.9% purity were procured (Ultrananotech, Bangalore, India).

The brackets to be coated were cleaned with deionized water and ethanol at 80° C for 30 minutes to get rid of the oxidized layer over the surface. The spray pyrolysis method was used to coat the brackets with the nano particles to achieve uniform coating using the spray pyrolysis equipment (Ho-TH-04, Holmark -optomechtronics Ltd, Kochi, kerala, India) The solution for the coating was prepared by diluting 0.3gms of zinc oxide nanoparticle powder in isopropanol solution for 1.5 hours and stirring well in an ultrasonic bath.<sup>22</sup> The distance between the spray nozzle and face of the bracket was maintained at 15cm and a thin film of zinc oxide nanoparticle was precipitated over the brackets as a uniform coating of 100 nm thickness

at 150°C temperature and 0.5 Pa pressure. A similar method was used for coating the orthodontic brackets with copper oxide nanoparticles. The hybrid coating of copper oxide–zinc oxide was obtained by mixing the two nanoparticles in the ratio of 1:1 by weight.<sup>9</sup> Four coated brackets from each group were randomly selected and examined under SEM at different magnification to confirm the uniformity of coating and the particle size (Figures 1, 2 and 3). The rest 31 brackets were used for assessing the cytotoxicity.

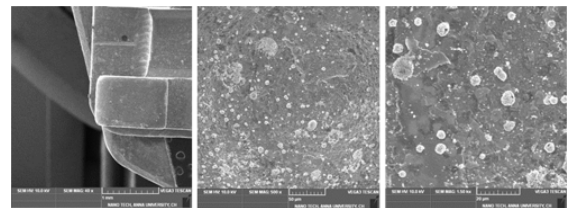
The cytotoxicity was evaluated as per the specifications mentioned in the ISO10993-5 norm and as used in the previous studies.<sup>14,18,24–27</sup> The mouse fibroblast cell line (L929) used in the cytotoxicity evaluation was procured from the National Centre for Cell Science, Pune, India. The Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich Chemie, Germany) was used to culture the cells. The medium was supplemented with 1% penicillin, streptomycin and amphotericin B to prevent bacterial and fungal growth. Fetal bovine serum (10%) and L-glutamine was added to provide nutrition to the growing cells. The cells were incubated in humidified carbon dioxide incubator with 5% Carbon dioxide and 95% humidity at 37°C for 2 days. Once 70% confluence was reached, the confluent cells were detached using 0.025% trypsin and 0.05% EDTA. The cell suspension was shared to the flasks in a laminar flow cabinet equipped with ultraviolet light sterilization and centrifugation followed by pipetting until the target concentration of  $2.5 \times 10^6$  cell/mL was reached.

The percentage of viable fibroblasts was assessed using the colorimetric MTT assay, which measured the ability of succinate dehydrogenase, present in their mitochondria to reduce the yellow colour tetrazolium dye [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide] to purple colour formazan. The cells were seeded into 4 groups of 31 well plates and incubated. After the cells attained confluency, the autoclaved sterile brackets were placed in the cell plates and incubated at 37°C, in 5 per cent CO<sub>2</sub> -in-air for a period of 2 days. Culture medium with cells and without brackets were also incubated under the same conditions which served as control. Following the incubation, 200 µl of MTT dye for every ml of culture was added to each well and the plates were incubated at 37°C, and 5 per cent CO<sub>2</sub> in air for a period of 4 hours. The MTT solution was prepared by dissolving 5mg of the dye in 1 ml of Phosphate buffered saline PBS and filtered through a 0.2 µm filter. After incubation, 300 µl dimethyl sulfoxide was added to each culture well incubated for 30 min to lyse the cell and obtain a homogenous colour. The solution was centrifuged for 2 min to sediment the cells and 100 µl from each well will be transferred to a new plate before measuring the optical density. The experiments were carried out in triplicates to increase the credibility of the results.

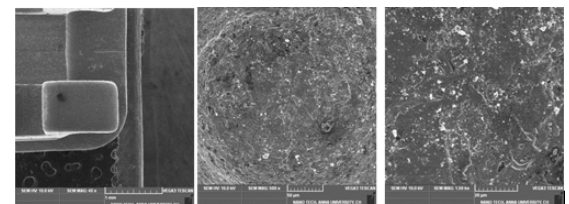
This assay is based on the capability of metabolically active fibroblast cells to reduce the yellow colored MTT

salt to purple formazan crystals. The intensity of the purple colour obtained is proportional to the quantity of the viable cells and is measured as the degree of absorbance or optical density (OD) at 570 nm using an enzyme-linked immunosorbent assay reader (U2000, Hitachi, and Tokyo, Japan). The cell viability is calculated as the ratio of the optical densities of the experimental wells to that of the control wells.

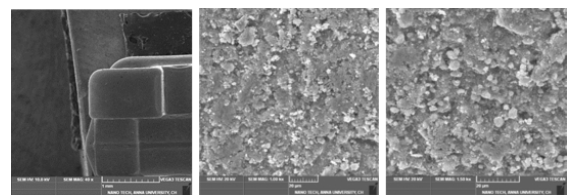
The mean optical density and the percentage of cell viability was calculated (Table 1) (Figures 4 and 5). Intergroup comparison of optical densities and percentage of cell viability was done with ANOVA and Post hoc Tuckey HSD (Tables 2, 3, 4 and 5). A  $P \leq 0.05$  was considered statistically significant.



**Figure 1:** Scanning electronmicroscopic images of copper oxide nanocoated brackets



**Figure 2:** Scanning electronmicroscopic images of Zinc oxide nano coated brackets



**Figure 3:** Scanning electronmicroscopic images of copper oxide-Zinc oxide nanocoated brackets

### 3. Results

The mean optical density from the MTT assay of the standard control was greater than the three experimental groups and the uncoated bracket group ( $1.35 \pm .023$ ) ( $p$  value= 0.00) (Tables 1, 2 and 3) (Figure 4). The lowest optical density was observed in the zinc oxide nanocoated

**Table 1:** Optical density and % of Cell viability values obtained from MTT assay of Group I (copper oxide nanocoated brackets), Group II (Zinc oxide nanocoated brackets), Group III (Combination of copper oxide –Zinc oxide nanocoated brackets), & Group IV (uncoated Brackets) samples and the standard control.

Groups (n=31)	Optical density		% of cell viability	
	Mean	SD	Mean	SD
Control	1.35	.023	100.00	.000
Group I	1.041	.12	77.45	8.183
Group II	.99	.082	73.61	6.438
Group III	1.16	.079	85.27	5.732
Group IV	1.20	.066	88.91	4.866

**Table 2:** ANOVA to compare the optical density obtained from MTT assay of Group I (copper oxide nanocoated brackets), Group II (Zinc oxide nanocoated brackets), Group III (Combination of copper oxide –Zinc oxide nanocoated brackets), & Group IV (uncoated Brackets) samples and the standard control.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.426	4	.607	96.671	.000
Within Groups	.941	150	.006		
Total	3.367	154			

**Table 3:** Post hoc Tuckey HSD to compare the optical density obtained from MTT assay of Group I (copper oxide nanocoated brackets), Group II (Zinc oxide nanocoated brackets), Group III (Combination of copper oxide –Zinc oxide nanocoated brackets), & Group IV (uncoated Brackets) samples and the standard control.

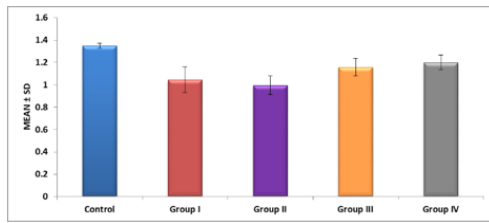
	Mean Difference	Std. Error	Sig.
Control vs Group I	.307161*	.020120	.000
Control vs Group II	.355710*	.020120	.000
Control vs Group III	.192645*	.020120	.000
Control vs Group IV	.150097*	.020120	.000
Group I vs Group II	.048548	.020120	.117
Group I vs Group III	-.114516*	.020120	.000
Group I vs Group IV	-.157065*	.020120	.000
Group II vs Group III	-.163065*	.020120	.000
Group II vs Group IV	-.205613*	.020120	.000
Group III vs Group IV	-.042548	.020120	.219

**Table 4:** ANOVA to compare the % of cell viability obtained from MTT assay of Group I (Group I (copper oxide nanocoated brackets), Group II (Zinc oxide nanocoated brackets), Group III (Combination of copper oxide –Zinc oxide nanocoated brackets), & Group IV (uncoated Brackets) samples and the standard control.

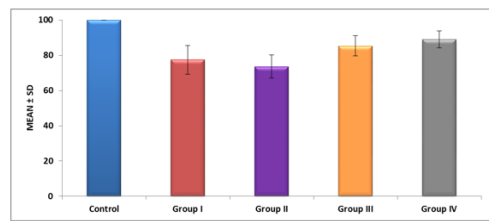
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	13243.148	4	3310.787	100.362	.000
Within Groups	4948.268	150	32.988		
Total	18191.415	154			

**Table 5:** Post hoc Tuckey HSD to compare the % of cell viability obtained from MTT assay of Group I (copper oxide nanocoated brackets), Group II (Zinc oxide nanocoated brackets), Group III (Combination of copper oxide –cinc oxide nanocoated brackets), & Group IV (uncoated Brackets) samples and the standard control.

	Mean Difference	Std. Error	Sig.
Control vs Group I	22.554*	1.459	.000
Control vs Group II	26.394*	1.459	.000
Control vs Group III	14.732*	1.459	.000
Control vs Group IV	11.093*	1.459	.000
Group I vs Group II	3.840	1.459	.070
Group I vs Group III	-7.822*	1.459	.000
Group I vs Group IV	-11.461*	1.459	.000
Group II vs Group III	-11.662*	1.459	.000
Group II vs Group IV	-15.300*	1.459	.000
Group III vs Group IV	-3.639	1.459	.097



**Figure 4:** Mean optical density values obtained from the MTT assay of Group I (copper oxide nanocoated brackets), Group II (Zinc oxide nanocoated brackets), Group III (Combination of copper oxide –Zinc oxide nanocoated brackets), & Group IV (uncoated Brackets) samples with the standard control



**Figure 5:** Percentage Cellviability values obtained from MTT assay of Group I (copper oxide nano coated brackets), Group II (Zinc oxide nanocoated brackets), Group III (Combination of Copper oxide –Zinc oxide nanocoated brackets), & Group IV (uncoated Brackets) samples and the standard control.

group ( $.99 \pm .082$ ) (Table 1). The mean value of copper oxide group was slightly higher but was not significantly different from that of the zinc group ( $1.041 \pm .12.$ ) ( $p$  value= 0.00) (Tables 1, 2 and 3).

The nanocoated brackets exhibited toxic effects at different levels on the fibroblasts when compared with the control group and the uncoated bracket. Cells exposed to copper oxide & zinc oxide nanocoated brackets resulted in lower percentage of cell viability ( $73.61 \pm 6.438$ ) ( $77.45 \pm 8.183$ ) (Tables 1, 4 and 5), (Figure 5). The greatest value of optical density and cell viability was observed in copper oxide –zinc oxide hybrid nano coated brackets which exhibited toxicity levels similar to that of uncoated brackets. ( $88.91 \pm 4.866.$ ) ( $85.27 \pm 5.732$ ) ( $p$  value= 0.00) (Tables 1, 2, 3, 4 and 5).

#### 4. Discussion

Cytotoxicity assays estimate the quantity of cells that are viable and thereby provide a measure of cell death caused by the direct contact of the material or their eluates.<sup>28</sup> Cytotoxicity refers to the chain of events which results in functional and structural damage of a cell after exposure to a substance.<sup>29</sup> The apparent cytotoxicity of a material can be significantly affected by the cell line used for the assay and hence the choice of cells remains a crucial factor.<sup>30</sup> Different cell lines including human gingival

fibroblasts, keratinocytes, human endothelial cells, mouse fibroblasts, osteogenic precursor cells from mice and HeLa cells are used to assess the cytotoxicity of nanoparticles in dental field. The mouse fibroblast (L-929) is a permanent cell line with good reproducibility and more sensitivity to toxic effects than human fibroblasts.<sup>27,31</sup> Hence in the current study, the mouse fibroblast (L-929) cell line was used to evaluate the cytotoxicity of orthodontic brackets coated with nano particles of copper oxide and zinc oxide.

Cytotoxicity evaluation of a material can be performed on an extract of the test sample or the sample can be directly used. Majority of the previous studies evaluating the toxicity of orthodontic brackets have used the eluates obtained at different time interval.<sup>27</sup> In the current study the brackets were used directly placed in the cell culture to observe the effects on the culture cells. Quantitative methods were used by measuring the reduction of the visible dye and measuring the colour change as the optical density of the medium as they are more reliable than the qualitative methods.<sup>27</sup>

Coating the stainless steel orthodontic brackets with copper and zinc nanoparticles improves the antimicrobial properties and reduce the frictional resistance but their potential cytotoxicity is a major concern. Studies have reported the toxicity of copper and zinc nanoparticles but they are not adequate to understand their cytotoxic potential completely as it is influenced by many factors.<sup>32</sup> Copper and zinc are micronutrients required for normal functioning of the body but when they exceed the tolerance level, they can cause toxic effects in the respiratory tract, nervous, excretory system or gastrointestinal tract based on the portal of entry.<sup>32</sup> Kim et al. demonstrated that laser generated ultra-pure copper nanoparticles possess moderate cytotoxicity to human cells in a cell-dependent manner.<sup>33</sup> The nanoparticles have the tendency to be more toxic than the copper microparticles as they can penetrate the body through skin contact, inhalation, and ingestion and smaller particles at higher concentration exerted maximum toxic effects on the cell viability.<sup>34,35</sup> The dose and size dependent cytotoxicity of copper nano particles have been reported by several studies.<sup>34–38</sup> Zinc oxide nanoparticles produced oxidative stress and exhibited size and dose dependent cytotoxicity in type II alveolar epithelial cells, lung epithelial cells, hepatic cells, skin fibroblasts and astrocytes.<sup>39–44</sup> Hence it became important to assess the cytotoxicity of nanocoated brackets. In the current study zinc oxide nanoparticles with an average size of 40 nm and copper oxide nanoparticles with an average particle size of 45 nm was used to coat the brackets.

The cytotoxicity of brackets coated with the combination of copper oxide and zinc oxide nano particles was noted to be similar to the uncoated brackets and lesser than the copper and zinc oxide coated brackets. This may be attributed to their dose dependent cytotoxic nature and the reduction in quantity of the particles used in the combination

coating as discussed by Zeidan et al. who evaluated the antibacterial effect of a combination of silver and zinc oxide nanoparticles coating of orthodontic brackets.<sup>10</sup> The uncoated brackets exhibited a certain level of reduction in the cell viability which may be due to the release of nickel, chromium, iron and other ions. This is in agreement with the previous studies published in the literature.<sup>12,14,19,21</sup>

The zinc oxide group exhibited the least cell viability and this may be due to the greater leaching tendency of the zinc ions from the coatings as confirmed by Mobeen et al. who noted that the quantity of zinc leached was greater than of copper from the respective coated brackets.<sup>22</sup> They noted that the quantity of zinc and copper ions leached were well below the levels that can elicit systemic toxicity from ingestion in humans until 28th day of immersion.<sup>22</sup> Materials that exhibit cell viability lesser than 70% of the cell viability of the positive control are considered to possess potent cytotoxic activity and cannot be used safely in patients.<sup>27</sup> MTT assay is a sensitive assay with an excellent linearity up to  $10^6$  cells per well and even a small change in metabolic activity of the cells can generate a large variation in the findings, allowing one to detect cell stress upon exposure to a toxic agent even in the absence of cell death.<sup>45</sup> The percentage of cell viability as calculated from the MTT assay for all the four group of brackets evaluated were greater than 70% of that of the positive control. This suggests that both zinc and copper oxide nanocoated stainless steel brackets can be used safely in patients to reduce the incidence of enamel decalcification and resistance during sliding.

## 5. Conclusion

Within the limitations of the study, it can be concluded that the nanocoating of copper and zinc oxide nanoparticles did not increase the toxicity levels of the stainless steel brackets beyond the critical levels above which the materials are considered potentially cytotoxic. The hybrid coating with a combination of copper and zinc oxide nanoparticles mixed in the ratio of 1:1 resulted in reduced cytotoxicity than separate coating of the same nano materials. Their toxicity levels were as low as that of uncoated brackets.

## 6. Source of Funding

None.

## 7. Conflict of Interest

None.

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