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ORIGINAL ARTICLE

In Vitro Biocompatibility Assessment of Dental Bonding Agents Containing Colloidal Dispersion of Titanium Dioxide

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Abstract

Purpose: To investigate the direct and indirect cytotoxic effects of two universal dental bonding agents incorporated with titanium oxide colloidal dispersion on a human gingival fibroblast cell.

Materials and Methods: Two commercial dental bonding agents' systems, i.e., Ambar universal (FGM, Brasil) and G-Premio Bond Universal (GC, America) were incorporated with 4% by mass of colloidal dispersion containing titanium oxide (TiO_2) nanoparticles. Human gingival fibroblast cells were used for cytocompatibility analysis. Two cytotoxic assays were used to investigate the cytotoxic activity of four bonding agent groups on the fibroblast-like cells as follows; GA: Ambar Universal (control), GB: Ambar Universal (4% TiO_2 incorporated), GC: G-Premio Bond universal (control), and GD: G-Premio Bond (4% TiO_2 incorporated). Forty bonding agent samples (5 x 1 mm discs) were prepared from the bonding agent groups and used for 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay and 32 discs were used for the High-Content Screening (HCS) assay.

Results: The results from the cytotoxic assays showed a high degree of cytocompatibility for all tested bonding agents. However, the incorporated bonding agent Groups (GB and GD) showed significantly less cytotoxic effects than their controls. Also, groups GC and GD showed significantly higher cytocompatibility than GA and GB.

Conclusion: The incorporation of 4% by mass of colloidal dispersion of titanium oxide nanoparticles significantly enhanced the biocompatibility of the tested universal bonding agents in comparison to their control groups.

Keywords: Universal Bonding Agents; Colloidal Dispersion; Titanium Oxide; Cytocompatibility.

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1. Introduction

Cytotoxic assays are screening assays, utilized to assess the living cell's interaction with a material in a cell culture, including testing the cell viability and their ability to grow. They are in vitro tests for investigating the biocompatibility of a material because they are executed outside a living organism. These tests require the application of the tested materials with an isolated biological system (i.e., cells, enzymes, or some other) [1].

Cytotoxicity tests can be classified into direct or indirect. Direct assays are utilized to test the cell damage mediated by the presence of the tested materials in direct contact with the cells with no barrier [2]. An example of such tests is the multiparametric High Content Screening toxicology (HCS) assay which offers a high degree of accuracy. On the contrary, indirect cytotoxic tests are used to test the toxicologic effect of a substance released from a material, infiltrated through a barrier, that could cause cell damage. Like the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) cytotoxic assay (i.e., a colorimetric assay). It is a sensitive and reliable indicator of the cell's metabolic activity [3].

Regarding the variations in the professional judgment about selecting the number of steps or the bonding agent strategy applied, the manufacturers have launched more versatile bonding agent systems that can be applied using either the etch-and-rinse (i.e., two steps) or self-etch mode (i.e., one or two step) [4, 5]. Such bonding agents are called "Multi-mode Universal" bonding agent systems. However, achieving a tight stable seal between the bonding agent and the dentin substrate is still difficult to achieve because of the hydrolytic degradation together with the action of lytic dentinal matrix enzymes which results in destabilizing the bonding agent bond interface reducing the bond stability [6, 7].

With nanotechnology development, there was a tremendous development in the application of nanoparticles in different fields (i.e., antibacterial materials, electronics, drug delivery, cosmetics, and others) [8]. Titanium dioxide nanoparticles have different physicochemical properties. It is insoluble in water and is highly stable with no toxicity. Recently, TiO_2 nanoparticles have also been utilized in dentistry for their excellent antibacterial activity. They have higher antibacterial activity than chlorhexidine with a high degree of biocompatibility [9].

The concept of TiO_2-containing bonding agents is rather new, and few investigations have explored the physicochemical features of these agents, with even fewer studies on the clinical suitability of such compounds. To date, no investigation has evaluated the cytocompatibility of these bonding agents on human gingival fibroblasts. As such, the purpose of the current in-vitro study is to investigate the cytocompatibility of two universal bonding agents incorporated with a titanium dioxide nanoparticle on a human gingival fibroblast cell line utilizing two cytotoxic assays (MTT and HCS).

2. Materials and Methods

2.1. Preparation and Incorporation of the TiO_2 Nanoparticle's Colloidal Dispersion into the Bonding Agents

To generate a colloidal dispersion of TiO_2 (CAS: 13463-67-7) nanoparticles, the procedures outlined by Al-Abbas *et al.* [10] were followed. According to the mass fraction formula (Equation 1), the produced colloidal dispersion was added at 4% by mass (0.20gm/5gm) to two universal dental bonding agents,

Ambar universal (FGM, Brazil) and G-Premio Bond Universal (GC, America) (Table 1):

Table 1. lists the chemical makeup of the all-purpose bonding agents employed in this study

Bonding agent	Composition
Ambar Universal	Urethane dimethacrylate (CAS: 72869-86-4), Hydroxyethyl methacrylate (CAS: 868-77-9), methacrylate hydrophilic monomers, methacrylate acid (CAS: 79-41-4), monomers, ethanol (CAS: 64-17-5), water (CAS: 7732-18-5), camphorquinone (CAS: 10373-78-1), ethyl 4-dimethylamino-benzoate (CAS: 10287-53-3), surfactant (CAS: 10028-22-5), sodium fluoride (CAS: 7681-49-4)
G-Premio Bond universal	Methacryloyloxydecyl dihydrogyne phosphate (CAS: 85590-00-7), methacryloxyethyl (CAS: 32435-46-4), methacryloyloxyalkyl thiophosphate methacrylate methacrylate monomer (CAS: 3637-26-1), acetone (CAS: 67-64-1), water, initiator, silica (CAS: 7440-21-3)

$$\text{Mass Fraction} = [\text{mass of colloidal dispersion} / \text{Total mass of bonding agent}] \times 100 \quad (1)$$

2.2. Characterization of Nanoparticles

The morphologic characteristics of TiO_2 nanoparticles were studied using a JEM-1000CX II (Jeol, Japan) TEM microscope transmission electron microscope with an accelerating voltage of 120 kV at various magnifications.

Additionally, the microstructure of TiO_2 nanoparticles was studied using a Zeiss LEO SUPRA 25 (Oberkochen, Germany, 2008) Scanning Electron Microscope (SEM).

2.3. Preparation of the Cell Line

Human Gingival Fibroblast cell vials were purchased from Innoprot (Parque Tecnológico de Bizkaia, Spain) which are isolated from healthy human gingiva (Figure 1a) fluorescent microscopy image, (Figure 1b) phase-contrast microscopy image.). These cells were cryopreserved at passage one and delivered frozen. Each vial contains more than 500,000 viable cells [11]. For this study, 25 × 104 gingival fibroblast-like cells were counted by flow cytometry and centrifuged in 5 mL tubes. Afterward, they were incubated at 37 °C with 5% CO_2 in a medium designed for chondrogenic development called Dulbecco's Modified Eagle's Medium (DMEM).

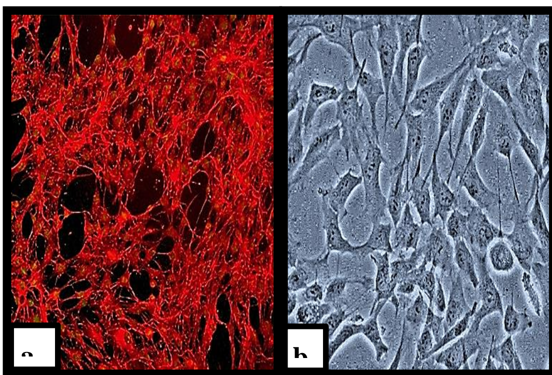

Figure 1. Human gingival fibroblast-like cells: (a) Fluorescent microscopy image, (b) Phase-contrast microscopy image

2.4. Preparation of the Bonding Agent Discs

For this study, 72 Bonding agent discs were prepared utilizing ready-made silicone rubber moulds of 5x1 mm dimensions and 55 μL capacity according to ISO specification 4049-10 in 2009 to be used for cytotoxic screening for both MTT and HCS assays [12]. This was conducted first by filling the bonding agent disc spaces to half, the solvent was then gently evaporated warm air tooth dryer for 20 seconds. Then the remaining halves of the disc spaces were filled, and the solvents were once again evaporated, covered with celluloid strip, and then were light cured using an LED light curing device for 40 seconds. Finally, the bonding agent discs were removed from the mould and verified for thickness and diameter. After that, all discs were polished using #1500-grit silicon carbide papers for 15 seconds. After polishing, all discs were sterilized for 40 minutes using an ultraviolet light directed from both sides by Ultraviolet (UV) lamp.

2.5. MTT Assay

2.5.1. Samples Distribution

Forty bonding agent discs were prepared as described above from the control and 4% TiO_2 (Sigma-Aldrich, MO, USA) with a purity of ≥ 99% being incorporated into bonding agents and distributed as follows:

Group A: 10 bonding agent discs (Ambar Universal) were immersed in 6 ml of Dulbecco's Modified Eagle Medium (DMEM) for 72 hours in an incubator at 37Co (Control).

Group B: 10 bonding agent discs (4% TiO_2 Ambar Universal) were immersed in 6 ml DMEM for 72 hours in an incubator at 37Co.

Group C: 10 bonding agent discs (G-Premio Bond Universal) were immersed in 6 ml DMEM for 72 hours in an incubator at 37Co (Control).

Group D: 10 bonding agent discs (4% incorporated TiO_2 G-Premio Bond Universal) were immersed in 6 ml DMEM for 72 hours in an incubator at 37Co.

At the end of the 72-hour immersion period, the bonding agent discs were carefully removed from the DMEM media leaving the culture media containing

the bonding agent extracts to be used for MTT screening assay.

2.5.2. Cultivation of Test Cells

Human gingival Fibroblast cells were cultivated and activated in Roswell Park Memorial Institute (RPMI) 1640 culture media (Sigma, Irvine, CA, UK) with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin in an incubator with 5% CO_2 at 37 °C atmosphere. After that, the cells were detached using 0.25% trypsin and aliquots of separated cells were then sub-cultured [13].

2.5.3. MTT Assay Procedure

On 96-well Costar® plates (Corning, USA), human gingival fibroblast cells were cultivated at a density of 1.8x104 cells per microliter and 0.44x104 cells per well. The cultivated cells are separated after 1, 2, and 3 days using 0.05% trypsin before being counted in a Neubauer chamber to determine their proliferation index. The plates were then covered with sterile parafilm, gently stirred, and incubated for 24 hours at 37 °C, 5% CO_2 with 200 µl of the culture media containing the bonding agent eluents (extracts) after that. Then, 80 L of DMEM medium and 12 L of MTT solution (8 mg/mL in PBS) were applied to each well, respectively. For three hours, cells were incubated at 37 °C, 5% CO_2, and 95% air humidity. The 110 L dimethyl sulfoxide (CAS: 7-68-5) solvent solution was then added in place of the MTT solution after 3 hours. After that, the plate was incubated at room temperature for a further 15 minutes. Following that, the optical density of the wells was measured using a spectrophotometer at a wavelength of 590 nm to determine the vitality of the cells. To assure accuracy, the experiment was conducted twice under identical conditions.

2.6. Multiparametric Cytotoxicity Assay: High-Content Screening (HCS)

2.6.1. Sample Preparation and Distribution

Thirty-two bonding agent discs were prepared and divided into four groups:

Group A: 8 bonding agent discs (Ambar Universal) were cultivated in direct contact with the cells (Control).

Group B: 8 bonding agent discs (4% TiO_2 Ambar Universal) were cultivated in direct contact with the cells.

Group C: 8 bonding agent discs (G-Premio Bond Universal) were cultivated in direct contact with the cells (Control).

Group D: 8 bonding agent discs (4% incorporated TiO_2 G-Premio Bond Universal) were cultivated in direct contact with the cells.

For this assay, a negative control group of untreated cells was utilized to compare the bonding agent's cytotoxic activity results with it.

2.6.2. HCS Protocol

Human gingival fibroblast cells were seeded at a density of 1.5x104 cells/mL per well (32 wells, 8 wells per group). Then the bonding agent discs were directly cultured in contact with the cells (one disc in each well) and incubated at 37 °C and 5% CO2 for 1 day. While the negative control was seeded as untreated cells in the culture media. After 1 day, 50 microliters of HCS cell viability. Staining solutions were added for each well. The cells were then incubated at 37°C for 30 min.

After that, the plate was incubated at room temperature for 20 minutes. 100 microliters of 1x wash buffer were then added to each well after the fixation solution had been gently aspirated. Following the addition of a blocking buffer, 50 L of primary antibody solution was added to each well, and the mixture was incubated at room temperature for 60 minutes in a light-protected environment. Following the aspiration of the main antibody solution, the plate was washed three times with 100 l of 1 wash buffer per well [14].

After removing the wash buffer, 100 microliters of 1X permeabilization buffer per well were added, and the wells were then incubated at room temperature in the dark for 10 minutes. The plate was then rinsed twice with 100 microliters of 1X wash buffer added to each well before the permeabilization buffer was aspirated. The plate was sealed. The plates were sealed and stored in the dark at 4°C for 1 day. Finally, the

plates are then inserted into the HCS automated fluorescence microscopy for simultaneous readout of several parameters; including the viable cells count, total nuclear intensity, and cell membrane permeability [14].

3. Results

3.1. Electron Microscopy Evaluation

To evaluate the structural features of TiO_2 nanoparticles, we adopted both transmission and scanning electron microscopy (TEM/SEM) techniques, with the former providing details regarding the size and dispersion pattern of nanoparticles and the latter providing a visualization of the surface characteristics of these nanoparticles. As can be viewed in Figure 2, TiO_2 nanoparticles were mostly observed as subspherical particles and polyhedrons, with a size ranging from 7 to 26 nm, and an increased tendency for aggregation in smaller sizes. Additionally, TiO_2 nanoparticles were also examined using scanning electron microscopy, the images of which can be viewed in Figure 3.

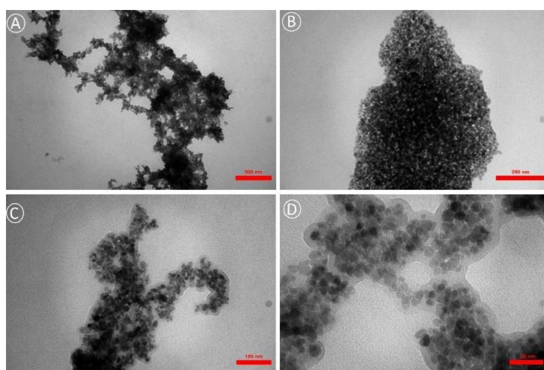

Figure 2. TEM images of the colloidal dispersion of TiO_2 nanoparticles at (A) 5,000x, (B) 12,500x, (C) 25,000x, and (D) 50,000x magnification

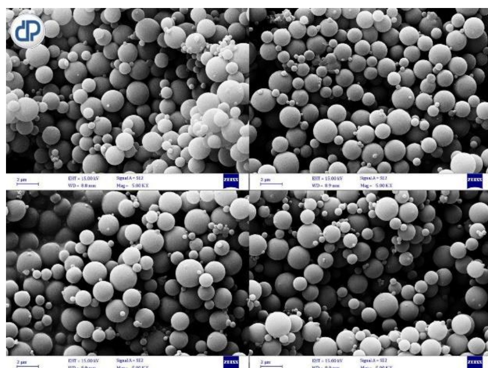

Figure 3. Scanning Electron Microscopy (SEM) images of the colloidal dispersion of TiO_2 nanoparticles

3.2. Results of MTT Assay

3.2.1. Descriptive Statistics

The results of the descriptive statistics for the viability of human gingival fibroblast cells in response to the bonding agent extracts of 72h immersion time are presented in Table 2 and Figure 4. The Data were expressed as means, standard deviation, and standard errors.

Table 2, and Figure 4 showed that the 4% TiO_2 incorporated bonding agents have much higher cell viability mean values than their control groups. The 4% TiO_2 incorporated G-Premio bond showed better cell viability than the 4% TiO_2 incorporated Ambar Universal bonding agents. The Ambar Universal (control) showed the highest cytotoxic effect (lowest cell viability).

Table 2. MTT cell viability results (%) at 72 h immersion time

Groups	Immersion period	Mean	Std. Deviation	Std. Error
GA	72hours	57.89	0.421	1.422
GB	72hours	77.52	1.424	1.242
GC	72hours	75.51	0.632	0.575
GD	72hours	84.54	0.827	0.425

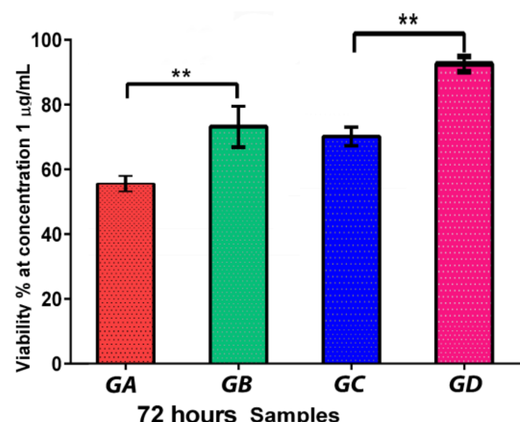

Figure 4. Graph showing the means of cell viability (%) in response to the bonding agent extracts at three 72-hour immersion times

3.2.2. Inferential Statistics (MTT Assay)

An unpaired t-test was performed to determine the significance of differences in the cell's viability mean values ($p \leq 0.05$) between the bonding agent groups. The test showed highly significant differences between groups (Table 3).

Table 3. Independent samples t-test to assess the significance of variations in cells' mean viability between the 4% TiO_2 included bonding agents and the control groups

Groups	P value (t-test)	Significance
GA vs GB	0.010	**HS**
GC vs GD	0.002	**HS**
GA vs GC	0.001	**HS**
GB vs GD	0.001	**HS**

3.3. Results of HCS Assay

The results of viable cell parameters (Figure 5, a: viable cell count, b: mitochondrial membrane potential, c: nuclear strength, d: cell membrane permeability) for all groups are demonstrated in the fluorescence images of cells in direct contact with the bonding agent discs.

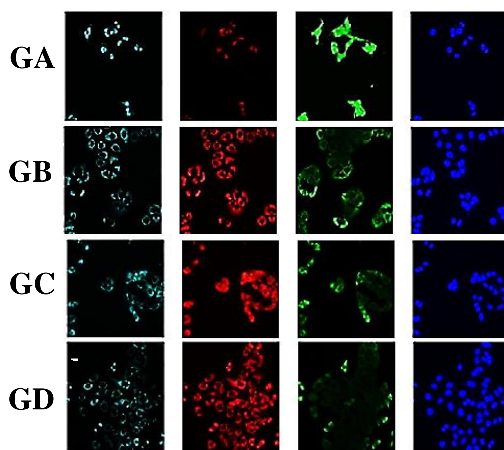

Figure 5. HCS fluorescence images for gingival fibroblast cells in direct contact with the bonding agent discs for the four groups showing different cells viable parameters (a: viable cell count for group A, a: viable cell count for group B, b: viable cell count for group B, a: viable cell count for group C, d: viable cell count for group D

3.3.1. HCS Assay Descriptive Statistics

The results of the descriptive statistics of viable cell count for the tested bonding agent groups and the untreated control are shown in (Table 4) and (Figure 6). The table showed a much higher viable cell count for the 4% TiO_2 incorporated bonding agent groups than the control bonding agent groups. G-Premio bond showed better viable cell count than Ambar universal bonding agent groups. Amber universal (control) showed the lowest viable cell count.

Table 4. The Viable Cell Count of cells in response for the tested groups and the negative control of the untreated cells

	Negative Control (Untreated)	GA	GB	GC	GD
Mean	2833	2578	2784	3248	3449
Std. Deviation	144.1	297.2	110.8	148.6	121.28
Std. Error of Mean	120.5	240.7	112.7	174.5	181.44

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Std. Error of Mean	120.5	240.7	112.7	174.5	181.44

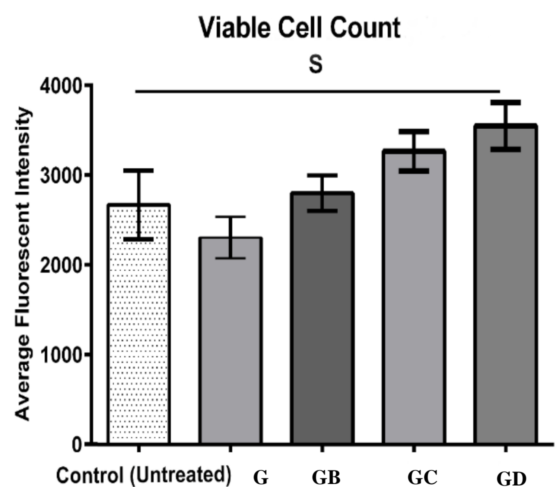

Figure 6. Graph showing viable cell count for the tested bonding agent groups in comparison to the control untreated cells

3.3.2. Inferential Statistics

The results of the inferential statistic for testing the significance of the difference in the viable cell count

between the tested bonding agent groups and the untreated control are presented in Table 5. The independent t-test showed significantly higher difference between the groups.

Table 5. Comparisons of the significance of differences ($p \leq 0.05$) of Viable Cell Counts between the negative control (Untreated) with the 4% TiO_2 incorporated and control (non-incorporated bonding agents), and between the four bonding agent groups

Groups	independent t-test	Significance
Control (Untreated) vs. GA	0.006	**HS**
Control (Untreated) vs. GB	0.002	**HS**
Control (Untreated) vs. GC	0.000	**HS**
Control (Untreated) vs. GD	0.000	**HS**
GA vs. GB	0.001	**HS**
GC vs. GD	0.003	**HS**
GA vs. GC	0.001	**HS**
GB vs. GD	0.000	**HS**

4. Discussion

can provide the most reliable results regarding the biocompatibility of different materials, and for ethical purposes, cytotoxicity assays are mostly conducted on cell line cultures [15].

Many in vitro tests were described in the literature for investigating the cytotoxic effects of dental bonding agent systems. Basically, The MTT is the most common assay mainly because it is a rapid, convenient, inexpensive, repeatable, and reliable assay. On the other hand, the HCS assay can provide more reliable information on the cell viability by testing different cell viability parameters [16].

Since the composition of the commercial dental bonding agents will probably be changed after the colloidal dispersion of the TiO_2 nanoparticles was incorporated, two cytotoxic assays (MTT, HCS) were conducted in this study to investigate their cytotoxic effects in comparison to their non-incorporated control bonding agents on human gingival fibroblast cells.

The present study showed that all the tested bonding agent groups have metabolic effects on gingival fibroblast cells and that the 4% TiO_2 incorporated bonding agents showed significantly higher cell viability than the control non-incorporated bonding agents.

The study results are probably correlated to the action of the incorporated TiO_2 nanoparticles. Titanium and its alloys are commonly used biomaterials for the synthesis of clinical and dental implants mainly because of their excellent biocompatibility [17]. In addition, the studies further showed a correlation between the antibacterial properties and biocompatibility of titanium dioxide nanoparticles [18]. This is because TiO_2 has a low electrical conductivity which is related to the low electrochemical oxidation rate leading to the creation of a thin oxide layer reducing the level of oxidative stress and the release of reactive oxygen species which are the most cytotoxic [19].

In addition, the study revealed the significantly higher cytotoxicity of the Ambar universal bonding agent when compared to G-Premio bond universal which is probably attributed to the differences in their chemical composition. The Ambar universal contains in their composition (Hydroxyethyl)methacrylate (HEMA) and Urethane-dimethacrylate (UDMA) monomers in their chemical structure, while G-Premio bond Universal is not. HEMA can be easily disintegrated in aqueous environments which is related to its low molecular weight because of the hydroxyl group which can affect the cell's viability through increasing the levels of reactive oxygen species [20].

The MTT study results are also supported by the results of the HSC assay which revealed a high degree of viable cell count which is supported by the fluorescence images for the cells in direct contact with the tested bonding agents. The assay revealed a significantly higher viable cell count for the incorporated than the non-incorporated bonding agent groups.

5. Conclusion

In comparison to control groups, the universal bonding agents' cytocompatibility was dramatically improved by the 4% mass incorporation of titanium oxide nanoparticles. In comparison to the G-Premio bond universal bonding agent, the Ambar bond universal demonstrated noticeably increased cytotoxicity.

Our findings confirm that TiO_2-containing bonding agents confer good biocompatibility, and are well-tolerated by human gingival fibroblasts, which represent the entire oral cavity in terms of cytotoxicity. Based on

these findings, bonding agents containing TiO_2 up to 4% of their mass could be considered for use in clinical practice. Still, further investigations on this subject matter are warranted.

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