UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

FERNANDA SANDES DE LUCENA

Influence of the addition of TiO_2 nanotubes on the biocompatibility of Y-TZP ceramics and resin-based materials

Influência da adição de nanotubos de TiO_2 na biocompatibilidade de cerâmicas Y-TZP e de materiais a base de resina

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FOLHA DE APROVAÇÃO

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Abstract

ABSTRACT

Influence of the addition of TiO_2 nanotubes on the biocompatibility of Y-TZP ceramics and resin-based materials

The aim of this *in vitro* study was to evaluate the biocompatibility of yttrium-stabilized tetragonal zirconia polycrystal (Y-TZP), a resin-based cement (RelyX™ Ultimate) and a 10-MDP-based adhesive (Single Bond Universal) modified or not by titanium nanotubes (TiO_2), by means of the MTT cell viability test and Crystal Violet. For this purpose, disks of 13 mm in diameter per 2 mm is thickness of pre-sintered Y-TZP zirconia (IPS e.max ZirCAD) were obtained. The resin-cement and a 10-MDP adhesive disks were obtained through a metal mold with the same dimensions. For Y-TZP, the incorporation of TiO_2 nanotubes occured before sinterization, while for the resin-based materials 0.3wt% of nanotubes were added to the uncured materials. The specimens were divided into 8 groups (n = 8). The in vitro evaluation was carried out by means of tests in which fibroblast line NIH 3T3 cells were placed into indirect contact with these materials. For cell viability were made MTT assay tests and Crystal Violet in duplicate and after 24, 48 and 72 hours the absorbance levels were analyzed by spectrophotometry Elisa reader. The data obtained were submitted to two-way ANOVA, followed by Tukey test ($\alpha = 0.05$). In the period of 72 the highest increases of absorbance happened for the groups Y-TZP without TiO_2 nanotubes and adhesive with TiO_2 nanotubes when compared to the other groups. In general, the incorporation of nanotubes into these materials did not interfere with cell viability in both tests.

Keywords: Adhesive. Biocompatibility. TiO_2 Nanotubes. Resin Cement. Zirconia.

Resumo

RESUMO

Influência da adição de nanotubos de TiO_2 na biocompatibilidade de cerâmicas Y-TZP e materiais à base de resina

O objetivo deste estudo *in vitro* foi avaliar a biocompatibilidade de uma zircônia tetragonal policristalina estabilizada por ítrio (Y-TZP), um cimento à base de resina (RelyX ™ Ultimate) e um adesivo à base de 10-MDP (Single Bond Universal) modificados ou não por nanotubos de titânio (TiO_2), por meio dos testes de viabilidade celular MTT e Cristal Violeta. Para este fim, foram obtidos discos de 13 mm x 2 mm de zircônia Y-TZP (IPS e.max ZirCAD). Os discos de cimento resinoso e adesivo com 10-MDP foram obtidos através de um molde metálico com as mesmas dimensões. Para Y-TZP, a incorporação de nanotubos de TiO_2 ocorreu antes da sinterização, enquanto para os materiais à base de resina, 0,3% em peso de nanotubos foram adicionados antes da fotopolimerização. Os espécimes foram divididos em 8 grupos (n = 8). A avaliação *in vitro* foi feita através de testes nos quais as células da linhagem de fibroblastos NIH 3T3 foram colocadas em contato indireto com estes materiais. Para a viabilidade celular foram realizados MTT e Cristal Violeta em duplicata e após 24, 48 e 72 horas a absorbância foi analisada por espectrofotometria em leitora Elisa. Os dados obtidos foram submetidos a ANOVA a dois critérios, seguido do teste de Tukey ($\alpha = 0,05$). Os resultados mostraram que em 24 e 48 hs todos os materiais mostraram-se biocompatíveis. No período de 72 hs os maiores aumentos de absorbância aconteceram para os grupos Y-TZP sem nanotubos de TiO_2 e adesivo com nanotubos de TiO_2 comparados aos demais grupos. De modo geral, a incorporação de nanotubos a estes materiais não interferiu na viabilidade celular em ambos os testes.

Palavras-chave: Adesivo. Biocompatibilidade. Cimento Resinoso. Nanotubos de TiO_2. Zircônia.

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1 Introduction

1 INTRODUCTION

TiO_2 materials when presented in nanoscale have shown remarkable antimicrobial properties when incorporated in dental cements (ELSAKA et al., 2011; POOSTI et al., 2013). This is probably due to their small size that allows penetration into cell membranes, altering intracellular processes and thus resulting in a higher reactivity and antimicrobial activity. But the analysis of direct and indirect cell viability is critical in materials that are directly in contact with the oral environment. Through cell culture one can observe qualitative and quantitative information, cell changes or inhibiting the formation of cell colonies. Furthermore, whenever a new material with biological interaction is synthesized, their cytotoxicity is to be evaluated, especially in the case of nanoparticles, since large surface area relative to volume can lead to a high chemical reactivity (CALLISTER Jr. and RETHWISCH, 2013).

Although it is known that nanomaterials have been widely used in dentistry, the addition of titanium dioxide (TiO_2) nanotubes to zirconia and resin-based materials, such as dental adhesives and resin cements still need further studies. These nanostructures have a high surface area relative to volume and have a variety of applications due to its chemical stability, non-cytotoxicity and high refractive index (ZHANG et al., 2015). The addition of TiO_2 nanostructures to resin-based materials has shown promissing results related to flexural strength, hardness, degree of conversion and the antibacterial properties (ARRUDA, 2015; POOSTI et al., 2013; SUN et al., 2011; TONELO 2016; XIA et al., 2008). However, it is important to address the preservation of the biocompatibility of the material before the clinical use.

Biocompatibility is defined as the ability of a material to perform with an appropriate host response in a specific application (WILLIAMS, 1987). To study the biocompatibility of certain materials *in vitro* models are used that are very effective to analyze cell behavior when in contact with these materials, avoiding complications and interference occurring *in vivo* models. In the assessment of tissue responses materials used in dental implants, the major cell types used are fibroblasts and epithelial cells, especially animal origin (JOSSET et al., 1999). Cytotoxicity assays are performed with extracts of the material and indirectly assess the biocompatibility of the materials, through interaction with the cell culture. These tests also determine the toxic concentration of the tested materials and their effects on the morphology of the cells and their growth, cell loss and degree of enzymatic activity in a specific cell type. In other words, this test sets the biological behavior of the material and its components (ATA et al., 2009; ATT et al., 2009; KEYS et al., 2012).

Due to the increasing demand for esthetic dentistry metal free restorations have become more frequent. In this context, dental ceramics had their clinical use established because of its properties as excellent aesthetic (optical characteristics simulating the natural appearance of the teeth), compressive strength, high chemical stability, similar thermal expansion coefficient to the tooth structure and biocompatibility (KELLY et al., 1996).

Polycrystalline ceramics are those which have no vitreous content and they must have the regular crystalline arrangement of atoms for composing the structure, making this type of ceramic harder and tougher than the glass-based ceramic (KELLY, 2008). This group has a ceramic fine-grained crystal structure that is responsible for strength and fracture toughness, however, is limited to translucency. Among these ceramics there are alumina and zirconia (GRACIS et al., 2015).

The use of zirconia has been widely spread in dental materials, and especially with regard to ceramic restorations because of its good chemical properties, dimensional stability,

strength, hardness, good aesthetics, low plaque accumulation and excellent biocompatibility (SASAKI et al., 2015; SIARAMPI et al., 2014). Zirconia, besides being present in some ceramic systems to strengthen them, can also be used as a tetragonal polycrystalline zirconia reinforced by ítrea (Y-TZP), where yttrium oxide (Y_2O_3) is associated with a pure form of Zirconia to stabilize the cubic or tetragonal crystals at room temperature. This stabilization of the zirconia crystals in the tetragonal phase makes the Y-TZP presents better mechanical properties such that high fracture toughness and flexural strength compared to other ceramic systems (GUAZZATO et al., 2004; MIYAZAKI et al., 2013).

It is known that, to ensure the clinical success of a ceramic restoration, adequate union is required between the ceramic and resin cement used (BARATTO et al., 2015). As for the cementation process of dental ceramics, the glass ceramics, such as lithium disilicate, have large amount of silica in its composition, so the etching with hydrofluoric acid (4 to 9.5%) has been an effective surface treatment, causing an increase in the roughness and the mechanical cement imbrication (BLATZ et al., 2003). Despite excellent properties Y-TZP ceramics are acid-resistant due to their high crystalline content having resistance to etching with hydrofluoric acid, hindering the adhesive process and hence interfering with cementation (AMARAL et al., 2008; THOMPSON et al., 2011; TZANAKAKIS et al., 2016), which makes the cementing of zirconia still critical. The infiltration of TiO_2 nanotubes into zirconia to increase roughness and create bonding areas on the surface of Y-TPZ ceramics is a hypohtesis curretly under study.

In order to simplify the bonding procedures to both dental substrates and indirect restorations while reducing the clinical time, new bonding agents were developed, the universal system. This designation is due to the fact that this system can be used as total-etch, self-etch and selective-etch adhesive. In addition to the different possible application forms, this system is also capable of promoting adhesion to different substrates such as the tooth,

ceramics, resins and metal. Some studies have shown promising results of universal bonding agents, such as the most commonly used Single Bond Universal adhesive including effective zirconia bonding (KIM et al., 2015).

In this in vitro study, NIH3T3 line of mouse fibroblast cells were used to assess the biological behavior of these cells when in contact with tetragonal polycrystalline zirconia partially stabilized by yttria (Y-TZP), a resin-based cement and a 10-MDP-containing universal adhesive system incorporated by titanium dioxide nanotubes (TiO_2).

2 Article

2 ARTICLE

Article – The article presented in this Dissertation was written according to the Biomaterials instructions and guidelines for article submission

Influence of the addition of TiO_2 nanotubes on the biocompatibility of Y-TZP ceramics and resin-based materials

1. Introduction

Nanotechnology is the understanding and control of matter at dimensions of roughly 1 to 100 nanometers, where unique phenomena enable novel applications and has been applied in science, medicine and more recently in dentistry. In dentistry, nanoparticles have been incorporated into resin-based materials to improve or modify their properties such as surface smoothness, aesthetics, degree of conversion, strength, and wear resistance [1,2,3,4]. The nanostructures incorporated into the materials may present different morphologies. Among them nanotube oxides such as titanium dioxide (TiO_2) nanotubes have been of special interest due to the tubular structure and their large surface area, which provides greater chemical stability [5].

A possibilty that is currently under evaluation is the incorporation of TiO_2 nanotubes to a dental cement (RelyX Ultimate Adhesive resin cement, 3M ESPE Dental Products, St. Paul, MN) and a MDP-based adhesive (Single Bond Universal adhesive, 3M ESPE, St. Paul, MN, USA), with the objective of improving the chemical and mechanical properties of these materials used for luting procedures of dental ceramics to the dental substrate and also the incorporation of these nanotubes to the surface of a polycrystalline ceramic (IPS e.max ZirCAD, Ivoclar Vivadent, Schaan, Liechtenstein), in order to create more binding sites of superficial bonding and to improve the adhesion of this material. However, it is important to address the preservation of the biocompatibility of the material is modified by the addition of nanotubes.

Polycrystalline ceramics, such as yttria-stabilized tetragonal zirconia polycrystal (Y-TZP) are well known due their properties such as high strength and fracture toughness and good biocompatibility and have been applied successfully as framework materials for dental

restorations and oral implant abutments [6,7]. Despite the excellent mechanical properties, an important factor for the longevity of the ceramic restorations is the cementation process [8]. However, those ceramics have no silica and vitreous phase in their composition, which affects the procedures of conventional adhesive cementation as the application of hydrofluoric acid and silane agent. Because of that several alternatives have been used with the objective of improving the adhesion to zirconia, such as: sandblasting with aluminum oxide for surface roughening [9], silicoating [10], Er: YAG laser [11] and liner application for zirconia [12]. However, none of these procedures showed long-term clinical success.

Although zirconia restorations can be cemented with conventional cements, it is more suitable to use resin cements due to better marginal sealing, fracture resistance after cementation, and good retention [13]. 10-metacryloxydecyl dihydrogen phosphate (MDP) has been developed, which consists of an acid phosphated monomer for binding to metal oxides and which has been widely used for zirconia binding [14,15], this monomer has been used in the composition of adhesives and cements and the phosphate terminal group can react with the hydroxyl group (-OH) on the surface of the zirconia forming chemical bonds between oxygen and zirconium [16], showing promising results of bond strength [17].

Titanium dioxide nanotubes have been studied as reinforcement of polymeric materials due to their ease of incorporation into the resin matrix and they give these materials excellent mechanical properties when incorporated such as flexural strenght, hardness, modulus of elasticity and degree of conversion [18]. Although TiO_2 nanotubes showed good biocompatibility and even good antimicrobial properties when incorporated into dental cements and TiO_2 nanotubes incorporated into resin composites can significantly reduce bacterial growth compared with conventional composites, one concern is the cytotoxicity of this material in nanoescale [19,20], since these materials can penetrate cell membranes, interfering with intracellular activities, due to its reduced size.

This study was performed to define attachment and growth behavior of fibroblasts cells cell line NIH 3T3 cultured on zirconia, on a resin-based material and a 10-MDP adhesive incorporated by TiO_2 nanotubes by MTT assay, Crystal Violet and Scanning Electron Microscopy (SEM).

2. Materials and Methods

2.1 Zirconia samples preparation

For this study, slices obtained from tetragonal zirconia stabilized by yttrium oxide (Y-TZP) blocks were used, IPS e.max ZirCAD (Ivoclar Vivadent, Schaan, Liechtenstein). The zirconia blocks were machined and reduced to obtain blocks with circular section 13 mm in diameter. These blocks were sectioned in a cutting machine (Isomet 1000, Buehler, LakeBluff, IL, USA) with a diamond double faced diamond disk (Extec Dia disco, Wafer blade 5 "x. 015x1 / 2, Extec Corp., Enfield, CT, USA) under an water irrigation system for obtaining 2-mm-thick circular specimens.

Disks were submitted to finishing and polishing. For finishing, the samples were attached to a blade with heated godiva bats (Lysanda, São Paulo, SP, Brazil) for use of sandpaper silicon carbide (Carbimet, Buehler, Lake Bluff, IL, USA), granulation #800 and #1200 in polishing machine (Metallographic Polishing Machine AROPOL 2V, AROTEC, Cotia, SP, Brazil). The polishing was performed with fine-grained felt discs associated with diamond solution of 1 μm (Buehler, Lake Bluff, IL, USA), also for polishing.

Subsequently, the samples were placed in ultrasound (USC 750 - Unique Group, Indaiatuba, SP, Brazil), immersed in deionized water during 5 minutes.

2.2 Synthesis and addition of TiO_2 nanotubes to the materials

The TiO_2 nanotubes were synthesized from the 10g of commercial mixture of TiO_2 anatase powder (Aldrich, 99%) and 120 ml of alkaline solution of NaOH (10M), as described by Arruda et al. (2015) (Arruda et al., 2015). The mixture was maintained at 120˚C for 24 hours in a Teflon container. This container was heated by glycerin bath using a heating mantle as heat source. The whole process of synthesis was carried out at ambient atmospheric pressure.

After 24 hours alkaline treatment of commercial TiO_2 powder, alkaline NaOH solution and TiO_2 powder were washed with deionized water and hydrochloric acid (HCl) (0.1 M) sequentially and repeatedly up to pH 7 is achieved. Finally, the solution was passed through a drying process at 200 °C for 24 hours in air atmosphere to eliminate the liquid part and obtaining the final powder and nanotubes with a diameter of approximate 10 nm length and 200 nm, respectively.

The TiO_2 nanotubes were mixed with absolute etanol in a 50% concentration to obtain a paste. This paste was actively applied by rubbing with a microbrush for adhesives on the surface of the ceramic disk to obtain a uniform thin layer. Then, light jets at a distance of 15 cm were used to evaporate all ethanol. Nanotubes excess was removed with a dry microbrush. As a control, the nanotubes were not applied. The ceramics were syntherized at a suitable oven cycle following the recommended by the manufacturer.

2.3 Resin cement and adhesive samples preparation

The specimens of resin cement (RelyX Ultimate Adhesive, 3M ESPE Dental Products, St. Paul, MN) and adhesive (Scotchbond Universal adhesive, 3M ESPE Dental Products, St. Paul, MN) were obtained with a metalic circular mould with 13 mm in diameter and 2 mm in thickness for standardizing specimen size. Before curing, the TiO_2 nanotubes were

incorporated to the resin cement and adhesive in weight percentage of 0,3%. The surface of the matrix was covered with a polyester strip, then the cement and adhesive were light cured with an 1000mW/cm^2 (Valo, Ultradent).

Table 1. Materials used in the present study

Material	Manufacturer	Composition
IPS e.max ZirCAD	Ivoclar Vivadent, Schaan, Liechtenstein	ZrO2□□, HfO2, Al2O3, Y2O3 and other oxides.
RelyX Ultimate (Adhesive resin cement)	3M ESPE Dental Products, St. Paul, MN	10-Methacryloxydecyl dihydrogen phosphate (MDP) Dimethacrylate resins. HEMA. Vitrebond™ copolymer. Filler. Ethanol. Water. Initiators. Silane.
Scotchbond Universal (Universal adhesive)	3M ESPE Dental Products, St. Paul, MN	MDP phosphate monomer. Dimethacrylate resins. HEMA. Vitrebond. Copolymer. Filler. Ethanol. Water. Initiators. Silane.

2.4 Surface characterization

To characterization stage of ceramic surfaces using Scanning Electron Microscopy (SEM) the ceramic surfaces were evaluated ceramics with and without the application of TiO_2 nanotubes. The images were generated through the Scanning Electron Microscope (SEM) by variable pressure APEX Express (APEX Corporation, Delmont, PA, USA).

Biocompatibility

For biocompatibility evaluation through testing of cell culture, cell viability, MTT, Crystal Violet and SEM divided ceramic surfaces were used in eight groups (n = 8): 1) Y-TZP with application of nanotubes; 2) Y-TZP without application of nanotubes; 3) Y-TZP with application of nanotubes and Single Bond Universal adhesive; 4) Y-TZP without application of nanotubes and Single Bond Universal adhesive; 5) RelyX Ultimate Adhesive Resin Cement disks with 0,3% of nanotubes; 6) RelyX Ultimate Adhesive Resin Cement disks without nanotubes; 7) Single Bond Universal adhesive system with 0,3% of nanotubes; 8) Single Bond Universal adhesive system without nanotubes.

Cell Culture

Mouse fibroblast cell line NIH 3T3 was cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS). For the expansion, the cells were trypsinized with EDTA (1 mM) and trypsin (0.25%), and then stored for 5 min in an oven at 37 °C, followed by inactivation of trypsin with medium containing FBS. After centrifugation at 500g for 10 min, the pellet was resuspended in DMEM 10% FBS and cultured in bottles at a density of 0.5 x 104 cells/cm^2. For experiments, aliquots were thawed in DMEM medium containing 10% FBS and cultured as described above.

Cell viability

The *in vitro* tests for the analysis of cytotoxicity by the MTT reduction method by crystal violet incorporation and were conducted in accordance with ISO 10993-5 (ISO 2009) standard. Previously, ceramic discs were weighed to evaluate the amount to be set in each medium. With the materials previously sterilized, for every 1g of material, 10 ml of DMEM medium were used to prepare the extract and this solution was incubated at 37 °C for 48 h before being placed in culture plates to achieve the desired confluency.

The plating cell was performed in 96-microwell plates at a density of 2 x 103 cells/well in DMEM with 10% FBS in contact with extracts of the material previously prepared. In each microwell plate 8 of each group was filled with diluted extracts at 50%.

For *in vitro* evaluation of cytotoxicitythe MTT reduction test and crystal violet were chosen. Both were made in duplicate in periods of 24, 48 and 72 h after plating. The wells as a positive control (C 10%) received the addition of DMEM medium with 10% FBS corresponding to 100% cell viability. In the wells for negative control (C 1%) was added DMEM and Phenol 1% to indicate toxicity.

MTT assay

The aim of colorimetric assay of reduction of MTT (3- (4,5-dimethylthiazol-2-yl) - 2,5-diphenyltetrazolium bromide) is to analyze the cellular mitochondrial activity (Mosmann, 1983). For the test with MTT a yellowish salt, soluble in water, is reduced by the activity of dehydrogenase enzyme in a compound called formazan, and insoluble purple coloration. This reduction only occurs in living cells.

In each well, after removing the medium and washing with PBS (Phosphate Buffer Solution) 110 uL of a solution containing 0.5 mg MTT / ml were added and the plates were wrapped in foil for protection against light and stored in oven for 4 h at 37 ° C and 5% CO_2. After removal of the supernatant and discard the solution 200 uL of dimethyl sulfoxide (DMSO) were added in each well. Cytotoxicity was assessed by spectrophotometric absorbance measured by Elisa reader apparatus (Fluostar Optima, BMG Labtech, Offenburg, Germany) at a wavelength of 570 nm.

Through the intensity of this color in microwell plates used in these assays containing cells treated with different dilutions of the extract of each material it was possible to analyze whether or not cell death. When there is cell death staining become clearer since

incorporation occurs less crystal violet and MTT. Otherwise, microwells where there are living cells, the staining is more intense.

Crystal Violet

This test assesses the cell density by means of DNA stained with crystal violet dye.

The medium was removed from the microwell, which was washed with PBS. It was add 100% methanol and shall await-10 min. The methanol was removed and then the cells were stained with 0.2% crystal violet diluted in 2% ethanol, waiting for 3 min. The dye was removed and the wells were washed again with PBS. It was added sodium citrate 0.05 mol/l in 50% ethanol, acting for 10 min. Then, reading was performed of the optical densities of the microwell ELISA reader at wavelength of 570 nm.

Statistical analysis

The data obtained in indirect viability were expressed as percentages of total viable cells in each well calculated relative to the positive control group (C 10%). The percentage of indirect cell viability was calculated using the following formula:

$$\% \textbf{ cell viability } = \frac{\textbf{Sample absorbance x100}}{\textbf{absorbance of the positive control}}$$

The mean and standard deviation of percentage values for MTT and crystal violet assays were statistically analyzed by two-way ANOVA followed by Tukey considering materials and periods as independant variables. Statistical differences were considered significant for $\alpha = 5\%$.

3. Results

3.1 Cell Viability

The quantitative analysis was carried out so that the data obtained were processed in percentage according to positive control, considered 100%. Thus the percentage of the absorbance of the groups were statistically compared to each other within each period were also assessed and differences within each group between the periods 24, 48 and 72 hours.

Mean values and standard deviations for the MTT assay are presented in Table 2. There were significant differences between materials ($p < 0.00001$) and periods ($p < 0.00001$). The interaction effect was also significant ($p < 0.00001$). At 24 h all materials were considered biocompatible with the MTT results showing no difference when compared with the positive control ($p > 0.05$), while, as expected, almost no cell viability occurred for the negative control. The same occurred at 48 h. At 72 h there was an overall increase in the absorbance means, with groups zirconia without TiO_2 and adhesive with TiO_2 showing increased cell viability when compared with the other groups.

Table 2 - Mean values and standard deviations for the MTT assay. Similar lowercase superscript letters means no significant differences ($p < 0.05$). The symbol * highlights increases in cell viability at 72 h.

Group	Nanotubes	24 h	48 h	72 h
Adhesive	With	90.38 ± 9.6^{b}	93.20 ± 8.3^{b}	$141.08 \pm 13.5^{ef*}$
	Without	77.64 ± 5^{b}	68.47 ± 8.2^{ab}	128.13 ± 17.6^{def}
Y-TZP	With	89.18 ± 5.9^{b}	128.26 ± 6^{b}	98 ± 31^{cd}
	Without	114.4 ± 8.2^{b}	100.27 ± 9.5^{b}	$150.35 \pm 13.9^{f*}$
Y-TZP + adhesive	With	88.46 ± 6.8^{b}	109.78 ± 8.1^{b}	98.77 ± 27.5^{c}
	Without	100.96 ± 7.4^{b}	119.29 ± 9^{b}	119.57 ± 35^{cde}
Cement	With	90.14 ± 8.1^{b}	96.46 ± 5.3^{b}	96.63 ± 30.6^{c}
	Without	85.81 ± 8.9^{b}	103.53 ± 9.4^{b}	101.12 ± 32.9^{cd}

Mean values and standard deviations for the crystal violet are presented in Table 3. There were significant differences between materials ($p < 0.00001$) and periods ($p < 0.00001$). The interaction effect was also significant ($p < 0.00001$). The highest cell density was verified at 72 h period in the groups Y-TZP and adhesive without TiO_2 nanotubes and Y-TZP without TiO_2 nanotubes. Meanwhile, the lowest, as expected, was found in the group adhesive with and without TiO_2, in the first period, 24 h.

Table 3 - Mean values and standard deviations for the crystal violet assay. Similar lowercase superscript letters means no significant differences ($p < 0.05$).

Group	Nanotubes	24 h	48 h	72 h
Adhesive	With	59.02 ± 2.2^{ab}	104.02 ± 33.6^{cdefgh}	74.71 ± 12.3^{efghi}
	Without	84.16 ± 11^{ab}	74.04 ± 19.5^{bcdef}	68.89 ± 17^{defgh}
Y-TZP	With	153.47 ± 7^{abcd}	57.71 ± 9.8^{cdefg}	111.07 ± 15.8^{jk}
	Without	136.11 ± 4.7^{abcd}	156.37 ± 43^{ghijk}	129.97 ± 32.5^{jk}
Y-TZP + adhesive	With	$0,192 \pm 6.8^{abcdef}$	102.46 ± 12.2^{abcdef}	119.17 ± 14.1^{hijk}
	Without	133.33 ± 5.2^{ab}	72.93 ± 7.6^{bcdef}	144.74 ± 21.5^{hijk}
Cement	With	144.44 ± 6.6^{abcde}	121.02 ± 9.3^{fghi}	106.96 ± 23.3^{ghijk}
	Without	139.58 ± 7.5^{abcd}	148.76 ± 28^{ghij}	109.37 ± 20.9^{ghijk}

3.2 **Scanning Eletronic Microscopy (SEM)**

SEM images were used to evaluate the surface characterization of the materials and are presented below. Figures 1, 2, 3 and 4 show the difference between the surfaces of the diferente materials of this study incorporated or not by TiO_2 nanotubes. Figure 1 shows Y-TZP without the incorporation of nanotubes, whose surface is smooth and polished and, with the incorporation of nanotubes, in which we can see the nanotubular structures on the whole surface of the zirconia showing that the incorporation of the TiO_2 nanotubes modified the surface of the material. The same occurs in Figure 2, but there is also the presence of the adhesive.

Figure 3 (resin-based cement) doesn't show the nanostructures the same way that it appears in the other materials, the surfaces with and without nanotubes appear similar. While in Figure 4 (10-MDP adhesive) there is also a large difference between the surfaces due to TiO_2 nanotubes incorporation.

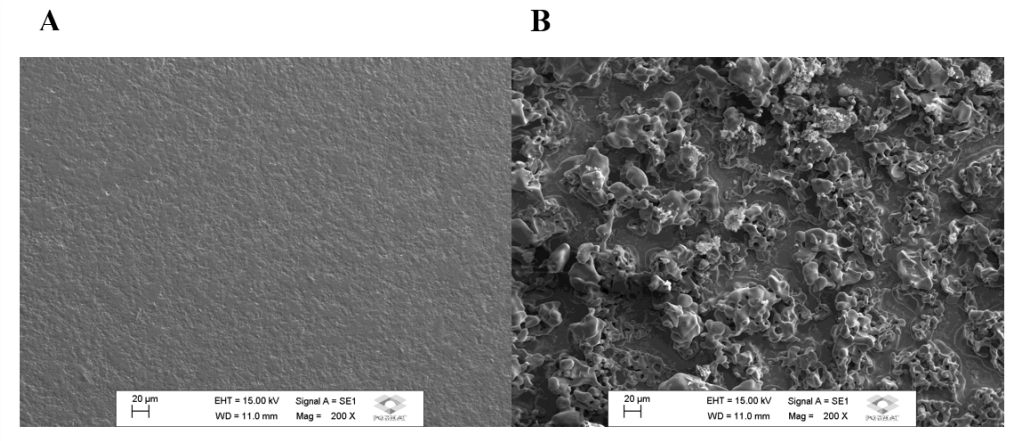

Fig. 1 - Surface characterization of two different specimens of Y-TZP. A) Surface morphology of Y-TZP without the incorporation of TiO_2 nanotubes using SEM (x200 magnification). B) Surface morphology of Y-TZP with the incorporation of TiO_2 nanotubes, showing the surface modification.

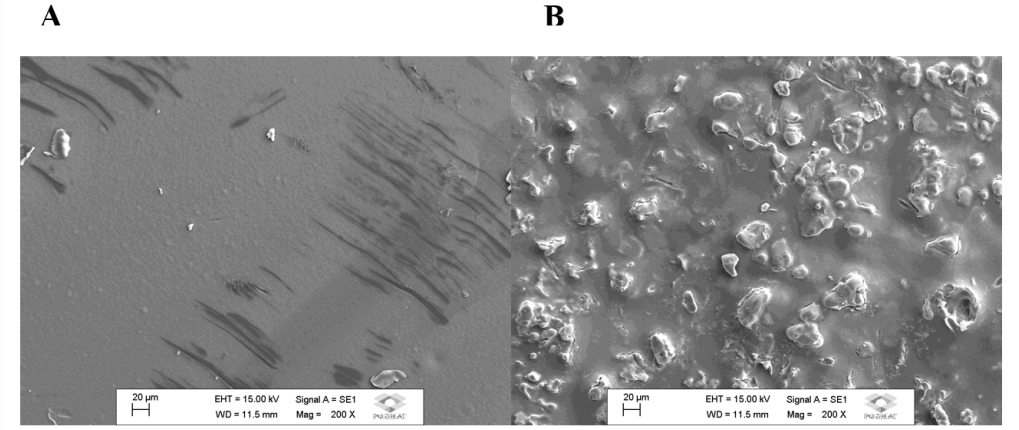

Fig. 2 - Surface characterization of two different specimens of adhesive. A) Surface morphology of Y-TZP and adhesive without the incorporation of of TiO_2 nanotube using SEM (x200 magnification). B) Surface morphology of Y-TZP and adhesive with the incorporation of TiO_2 nanotubes.

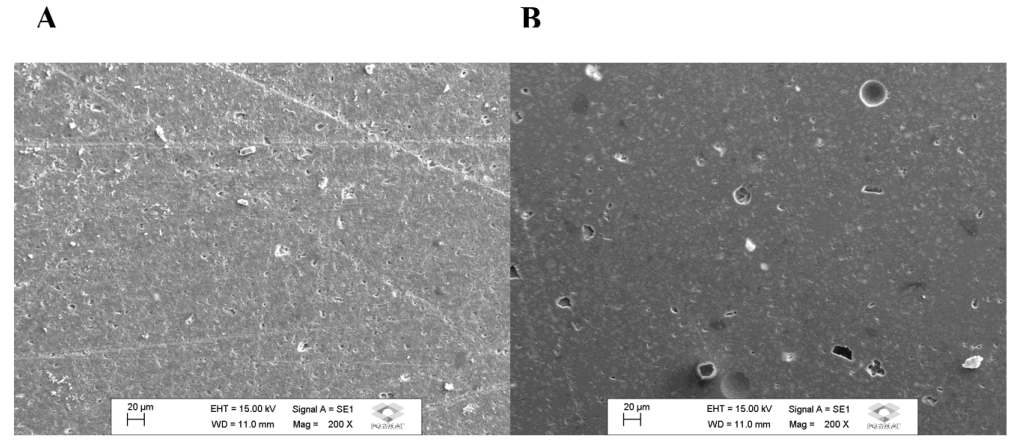

Fig. 3 A) Surface morphology of resin cement RelyX Ultimate without the incorporation of TiO_2 nanotubes (x200 magnification). B) Surface morphology of RelyX Ultimate with the incorporation of of TiO_2 nanotubes.

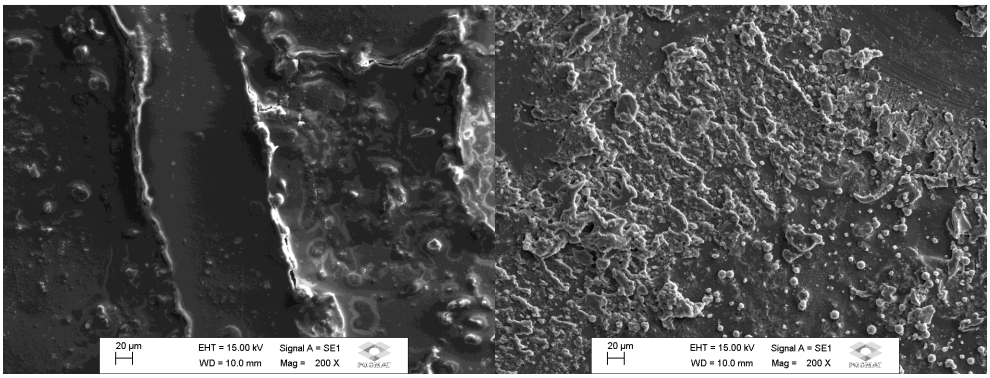

Fig. 4 - A) Surface morphology of Single Bond Universal adhesive without the incorporation of TiO_2 nanotubes using Scanning Electron Microscopy (SEM) x200 magnification. B) Surface morphology of Single Bond Universal adhesive with the incorporation of TiO_2 nanotubes.

4. Discussion

Biological compatibility is one of the most important properties of dental materials. The final result of prosthodontics and aesthetic procedures depends on the tissue response to these materials. In the present study, the cytotoxicity of Y-TZP zirconia, a resin cement and an MDP-containing adhesive incorporated by TiO_2 nanotubes were evaluated, employing and *in vitro* methodological strategy with cell viability tests and the use of mouse fibroblast cell line.

The cellular viability of this study was determined by MTT assay, which is based on the ability of the mitochondrial enzymes of the living cells in the conversion of the MTT salt (yellow) to crystal formation and, through violet crystal, which is a test based on the ability Of viable cell DNA in capturing the pigment (violet) [21]. These in vitro tests have many advantages like simplicity, low costs, can be carried out in a reduced time and offer more controllable research conditions, among them, the concentration and maintenance of the pH. In addition, in vitro models avoid animal use and possible ethical implications.

Despite the advantages, the in vitro method presents some limitations, including the susceptibility of contamination of the cells, short life of the cells due to the saturation of the

culture medium according to the cellular expansion, besides the impossibility to identify the quantity and the type of dead cells. Although those *in vitro* studies are important for evaluation of the performance of dental materials, it should be noted that generalization of *in vitro* results to the clinical setting has numerous limitations because in the laboratory, materials are applied conveniently and the confounding factors can be controlled.

Zirconium dioxide has been highly suitable for dental use because of its properties like high low cytotoxicity and reduction of bacterial adhesion with low corrosion potential [22]. Yttria-stabilized tetragonal zirconia polycrystal (Y-TZP) in combinations with nanotubes structures has also been reported as an acceptable material for biomedical applications, showning no biocompatibility modifications [23]. Y-TZP groups, as expected, showed good biocompatibility and, at 72 h period there was an increase in the absorbance mean for zirconia without TiO_2 group.

Resin-based materials have a source of compounds that can cause adverse biological reactions due to its organic matrix and incomplete polymerization and the cytotoxicity depends of the concentration of those components, such as the monomers HEMA and TEGDMA [22-25]. This study showed that the group of adhesive incorporated by TiO_2 had increased cell viability when compared with the other groups by MTT assay.

Besides the good biocompatibility results of MTT assay in this study and also Crystal Violet, some studies have shown that those nanoparticles such as titanium nanotubes can can enter into the circulatory system and be harmful to lungs, liver, spleen and bones, they can also contribute to heart or respiratory diseases and even lead to the development of carcinomas [26], nevertheless the mechanisms of TiO_2 nanotubes that are responsible for these effects are not completely understood and further research is required to elucidate them. These data may be applied in future studies to modify the surface of the materials to promote better adhesion and sprouting of cells. However, future studies for evaluation of cell

morphology and PCR are necessary for a better understanding of the mechanism of TiO_2 nanotubes when incorporated into materials.

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3 Discussion

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4 Conclusion

4 CONCLUSION

According to the results of MTT and Crystal Violet assays of this study, the addition of TiO_2 nanotubes in the tested materials did not affect the cell viability.

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