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Toxicity analysis of ocular prosthesis acrylic resin with or without pigment incorporation in human conjunctival cell line

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ABSTRACT

The aim of this study was to evaluate the influence of pigment incorporation on the cytotoxicity of ocular prosthesis N1 color acrylic resin. Nine samples were manufactured by heat-polymerization in water bath and divided into 3 groups: acrylic resin without pigment incorporation (group R), acrylic resin with pigment incorporation (group RP), and acrylic pigment (group P). Eluates formed after 72 h of sample immersion in medium were incubated with conjunctival cell line (Chang conjunctival cells) for 72 h. The negative control group consisted in medium without samples (group C). The cytotoxic effect from the eluates was evaluated using MTT assay (cell proliferation), ELISA assay (quantification of IL1β, IL6, TNF α and CCL3/MIP1α) and RT-PCR assay (mRNA expression of COL IV, TGF β and MMP9). Data were submitted to ANOVA with Bonferroni post-tests ($p < 0.05$). All groups were considered non-cytotoxic based on cell proliferation. However, resin with pigment incorporation showed significant IL6 quantity increase. Resin without pigment incorporation exhibited higher mRNA expression of COL IV, MMP9 and TGF β, however it was also observed for the negative control group. The materials exhibited divergent biological behavior. Despite the pigment incorporation that resulted in an increase of IL6, no cytotoxicity was observed based on cell proliferation.

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1. Introduction

The ocular prosthesis is commonly used in anophthalmic patients and is generally made of N1 acrylic resin, which mimics the patient sclera, and colorless acrylic resin, responsible for the prosthesis characterization covering. The acrylic resin is an option of choice due to its handling and adjustment, satisfactory aesthetics and low cost (Goiato et al., 2012, 2014; Andreotti et al., 2014; Fernandes et al., 2009a).

Acrylic pigments with different colors can be incorporated into acrylic resin for artificial sclera, aiming to simulate the natural sclera of the patient. Moreover, the artificial iris is positioned and red silk fibers may also be incorporated to mimic blood vessels. Then, a thin layer of a colorless resin is used to cover the artificial iris and silk fibers, providing a natural appearance to the prosthesis (Goiato et al., 2010a, 2014).

When the liquid, which consists of a methyl methacrylate (MMA) monomer, is mixed with the powder, an MMA polymer, there is a polymerization reaction that consists of the conversion of monomers into polymers, optimizing the physical properties of the material (Bural et al., 2011a). If this reaction is incomplete, residual MMA monomers and other chemicals that can be toxic can be released. Examples of such products are benzoic acid, formaldehyde, and methacrylic acid, among others (Bural et al., 2011a,b; Chaves et al., 2012; Tay et al., 2012; Att et al., 2009; Fernandes et al., 2009b).

Aesthetics, durability and proper adaptation are some of the essential requirements of the ocular prosthesis. However, biocompatibility is a critical characteristic for the success of the treatment and can be evaluated through *in vitro* cytotoxicity tests, such as the method of cell cultures, which has relatively simple performance and reproduction conditions (Goiato et al., 2010a; Fernandes et al., 2009b; Borra et al., 2009; Jorge et al., 2007; Saravi et al., 2012).

Preferably, primary cells or cell lines, which are closest to the target organ, should be used (Jorge et al., 2007), the use of the cell line from human conjunctiva (Wong Kilbourne derivative of Chang conjunctiva) has been widely reported in *in vitro* studies of ophthalmic products (Clouzeau et al., 2012; Ayaki et al., 2011a,b, 2012). Therefore, this cell line can be used for the assessment of the cytotoxicity of materials used for ocular prosthesis confection, since its support tissue is the

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conjunctiva, a thin membrane responsible for eye protection (Barisani-Asenbauer et al., 2013; Willoughby et al., 2010).

The biocompatibility of the ocular prosthesis was not previously evaluated, however the necessity to know this property is real and important to guarantee its secure clinical use in patients. Therefore, this study aimed to evaluate the influence of pigment incorporation on the cytotoxicity of ocular prosthesis N1 color acrylic resin. This evaluation was performed through the analysis of the cell proliferation by MTT assay, and the production of proinflammatory cytokines and extracellular matrix proteins by a human conjunctival cell line.

The null hypothesis is that the N1 color acrylic resin, with or without acrylic pigment, and the isolated acrylic pigment do not produce toxic effects on the cell line studied.

2. Materials and methods

The number of samples was determined based on previous studies (Bural et al., 2011a; Jorge et al., 2004; Retamoso et al., 2014), and based on the results of a pilot study. A power analysis was performed to determine the number of specimens required for the study, aiming to provide sufficient power (over 95%). Therefore, 3 samples were used.

Nine samples of materials used for making ocular prosthesis were manufactured by heat-polymerization in water bath (Table 1). These samples were divided into 3 groups (Saravi et al., 2012): acrylic resin without pigment incorporation (R, resin), acrylic resin with pigment incorporation (RP, resin with pigment), and acrylic pigment (P, pigment).

The samples were 10 mm in diameter and 3 mm in thickness (Monteiro et al., 2012) and were manufactured through auto-polymerized resin samples, previously obtained from a metallic matrix, which were included in flasks (Artigos Odontológicos Clássico Ltda, Sao Paulo, Brazil) by using type IV dental stone (Durone, Dentsply Ind e Com Ltda, Rio de Janeiro, Brazil) and extra-hard laboratory silicon (Zetalabor, Zhermack, Rovigo, Italy), for embedding the molds. Then, the flasks were opened and the molds were obtained after the samples' removal (Goiato et al., 2010a,b).

The acrylic resin (R) and the isolated acrylic pigment (P) were proportioned and mixed according to the manufacturer's instruction and positioned into the molds contained in the flasks. For the RP group, the incorporation of acrylic pigment was performed during the resin mixing. For this, the acrylic resin and pigment were properly weighed on a precision digital scale (BEL Equipamentos Analítico, Sao Paulo, Brazil) and the pigment was equivalent to 7% of the acrylic resin weight (Goiato et al., 2013).

After the materials' insertion in the molds contained in the flasks, a counter-flask was positioned and raised in a hydraulic bench press with a 1.250 kgf weight for 2 min (Goiato et al., 2012). Posteriorly, polymerization was executed according to the manufacturer's instructions, initiated with bench polymerization, immersion of the flask in water (room temperature), mild heating for 30 min, no heating for 30 min and boiling for one hour. After these periods, the samples were removed from the flasks and a samples' finishing was performed with a Maxi-Cut abrasive drill (Vicking, Sao Paulo, Brazil).

An additional specimen of each group was used for the analysis of the surface chemical composition. This analysis was performed on small volumes (on the order of 1 μm^3), through energy-dispersive spectroscopy (EDS).

For the cytotoxicity analysis, three samples from each group were placed into a sterile vial with 10 mL of Medium 199 (Gibco, New York, United States) supplemented with 10% fetal bovine serum (FBS) and incubated at 37 °C for 72 h. During this period, substances were leached for the medium. Then, the eluates were filtered through 0.22 μm filters (Millex, Millipore, Darmstadt, Germany) for sterilization (Att et al., 2009; Jorge et al., 2007; Saravi et al., 2012; International Organization for Standardization [homepage], 2009).

To evaluate the possible toxic effect of substances released by the groups, the cell culture of the human conjunctiva cell line (Wong Kilbourne derivative of Chang conjunctival cell line, clone 1-5c-4) was the selected method. This cell line was obtained from the American Type Culture Collection (CCL-20.2, Virginia, United States). The cells were expanded in flasks with Medium 199. The medium was supplemented with 10% FBS, 10 μg/mL penicillin, 10 μg/mL streptomycin, 10 μg/mL gentamicin and 250 μg/mL fungizone. The cells were incubated with 5% CO_2 and controlled humidity at 37 °C (Clouzeau et al., 2012; da Silva et al., 2016).

The cells were expanded until cell suspensions of 5×10^4 cells/mL were achieved, predetermined by a pilot study. In a 24 well plate, 1 mL of this suspension was pipetted into each well and after 24 h of incubation in a humidified atmosphere (5% CO_2 and controlled humidity at 37 °C in an incubator), the medium was replaced by 500 μL of eluates from different groups. Negative control (group C, control) consisted in culture medium with 10% FBS and without samples (Att et al., 2009; Clouzeau et al., 2012). Medium with Tween 20 (Sigma-Aldrich, Missouri, United States) served as a positive control (group T, Tween). The plate was incubated for 72 h with the same incubation and temperature conditions determined for generation of the eluates.

The culture medium was discarded and 500 μL of Medium 199 without FBS and with 0.5 mg/mL MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) was pipetted into each well. The plate was incubated in the same cited conditions for 4 h (Att et al., 2009; Jorge et al., 2007; Gonçalves et al., 2008). Later, the culture medium was removed and the intracellular formazan was released by solubilization with 1 mL of isopropanol per well. The plate was agitated for 5 min and the absorbance (570 nm) was measured, in triplicate, using a UV–visible spectrophotometer (SpectraMax 190, Molecular Devices, California, United States) (Att et al., 2009; Saravi et al., 2012; Gonçalves et al., 2008).

For the Enzime-linked immune-absorbent assay (ELISA) (DuoSet ELISA Development Systems, R&D System, Minnesota, United States), the cell-free supernatants were collected after 72 h of eluate exposition to the cells and the quantification of interleukin 1β (IL1β), interleukin 6 (IL6), tumor necrosis factor α (TNF α) and, macrophage inflammatory protein 1α (CCL3/MIP1α) were performed in triplicate (da Silva et al., 2016; Oliveira and Santos, 2011; Trubiani et al., 2012) by using a total volume of 100 μL of the supernatant, according to the manufacturer's recommendations.

The real time reverse transcription-polymerase chain reaction (RT-PCR) was performed for quantitative analysis of gene expression for type IV collagen (COL IV) (COL4A3BP: Hs00178621_m1), matrix metalloproteinase 9 (MMP9) (MMMP9: Hs00234579_m1) and transforming growth factor β (TGF β) (TGFB1: Hs0099133_m1) (da Silva et al., 2016; Oliveira and Santos, 2011), except for the P group.

The P group was not evaluated in the present study, since the acrylic pigment could be incorporated into the resin during the manufacture of the ocular prosthesis, though its use does not occur in an isolated form.

Table 1
Material, commercial brand and chemical composition of the groups.

Material	Commercial brand	Chemical composition
Acrylic resin Powder	N1 acrylic resin (Artigos Odontológicos Clássico Ltda, Sao Paulo, Brazil)	MMA polymer, dibuthylftalato, ethyl acrylate, pigments
Acrylic pigment Powder	Poli-Côr (color R2) (Artigos Odontológicos Clássico Ltda, Sao Paulo, Brazil)	MMA polymer, dibuthylftalato, ethyl acrylate, around 1.5% of various organic and inorganic pigments
Acrylic resin Liquid	Clássico (Artigos Odontológicos Clássico Ltda, Sao Paulo, Brazil)	MMA monomer, topanol

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