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Study of cellular toxicity *in vitro* of two resins for orthodontic use

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Abstract. – OBJECTIVE: The objective of this work is to compare cellular toxicity *in vitro* of two resins for orthodontic use: an auto-polymerizable composite and a photo-polymerizable composite.

MATERIALS AND METHODS: Samples were obtained by joining a couple of steel orthodontic brackets by using auto-polymerizing or photo-polymerizing resin. We used a halogen lamp, a mini LED lamp and a fast LED lamp used for orthodontics cure for 40 seconds. The 3T3 Swiss cellular line of fibroblasts was used. The samples obtained were used to determine the cellular toxicity *in vitro* using the Neutral Red Up-take (NRU) and the 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

RESULTS: Toxicity of the extract appraised at a low level at MTT and NRU assays. There were statistically relevant differences between the toxicity induced by the auto-polymerizing material and the toxicity induced by the photo-polymerizing composite material, polymerized with the blue-light lamp ($p < 0.001$) and with the mini LED lamp ($p < 0.05$).

CONCLUSIONS: From the data collected in this study, we can conclude that both resins show a low level of cytotoxicity that, in the case of photochemical polymerizing resin, depends on the characteristics of the lamp.

Key Words:
Orthodontic, Composites, Monomers, Cytotoxicity, Fibroblasts.

Introduction

In recent years brackets replaced bands in orthodontics, although they are only used from the front teeth to the premolars and, consequently, composites have become a very important topic in orthodontics, being the most used adhesive system.

Composites and cements have the following characteristics:

- Optical properties: they contain lithium, barium, strontium or other elements. They absorb X-rays and they can appear radio-opaque (radiodense) to X-rays;
- Mechanical properties: when weight is applied, the structure deforms because of the compression of its connection that can be otherwise pulled or cut;
- Biological properties: exposition time and potentially toxic substance rate are two important clinical factors that determine toxicity. When using these materials on patients, the biological properties that can cause toxicity and sensitivity reactions, both locally and systematically, must be known.

Biocompatibility and properties of the composite resins are linked to the release of monomers and reagents (activators, initiators, stabilizers, inhibitors, etc.) present in the materials.

Researchers[1-4] have detected that monomers, like bisphenol A-diglycidyl-dimethacrylate (Bis-GMA), urethane-dimethacrylate (UDMA), comonomers like triethylene-glycol-dimethacrylate (TEGDMA), 2-hydroxyethyl-methacrylate (HEMA) and initiators, like camphorquinone (CQ), are released by composite resins, glass ionomer cement, and dentinal adhesive.

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Dental monomers can be released in the oral cavity and the tooth/material interface, due to an incomplete polymerization and to the resinous nature of the matrix.

Furthermore, once released, dental monomers can be quickly absorbed by the body, forming intermediate metabolites that can be more toxic than the monomer itself, after its release[5].

Some biological problems, connected to resin-based dental materials (RBDM), prove their apparent biocompatibility.

Some studies have shown potential risks, linked to the monomer release, such as local immunological effects[6], apoptotic reactions[7,8], and inflammatory reactions[9].

Other studies have also demonstrated that RBDM can have a systemic estrogenic effect[10] or they can cause allergic reactions[11] or they can even have a carcinogenic effect[12]. Therefore, when biological materials are used, they need to be as compatible as possible. The most used materials are micro-filled acrylic resins, available in various forms and distinguished by contents, filling, and polymerization (chemical or photoinduced).

Auto-Polymerizable Composites

In orthodontics, auto-polymerizable composite resins are available in two different components: groundwood pulp-catalyst and resin bonding agent-catalyst, that are blended together before using. From that moment, there is a limited time for handling it and for clinical use of the material before the polymerization process begins.

Photo-Polymerizable Composites

The light source provides energy able to interact with photosensitive activators present in the material, causing free radicals formation. Free radicals can open carbon chain double binding in the monomers of the composite, making them available for the polymerization process that explodes in a chain reaction.

Photopolymerization requires a certain quantity of energy and it is produced by the radiant flux by the flow time of the radiant flux itself.

The photo-polymerizing light must have a wavelength between 400 and 500 nm (blue light) because the photo-initiator (camphorquinone/tertiary ammine), present in most composites, is sensitive to wavelengths near 470 nm. There are also other photo-initiators (light-activated) that are specifically sensitive to other wavelengths, always in the indicated span. Light sources used for photoinitiation are halogen, LED or plasma.

Purpose of the Work

This work aims to compare and contrast cellular toxicity *in vitro* of two resins used in orthodontics: one auto-polymerizable (Orthocryl, Dentaurum, Ispringen, Germany) and another one photo-polymerizable composite (Transbond XT Unitek, 3M, Maplewood, MN, USA). This was accomplished by cytotoxicity assay 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT) on the murine cellular line 3T3 Swiss.

Materials and Methods

Cells and Treatments

The 3T3 Swiss cellular line of fibroblasts was grown in incubator at an atmosphere with 5% CO_2 at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) with hepes (10 mM), glucose (1 g/L), NaHCO3 (3,7 g/L), penicillin (100 U/ml), streptomycin (100 µg/ml) and 10% Fetal calf serum (FCS).

The samples were obtained by joining a couple of steel orthodontic brackets (Sweden & Martina, Due Carrare, PD, Italy) by using auto-polymerizing resin following producers' instructions or by using the photopolymerizing resin following the producers' instructions and using different lamps.

We used a halogen lamp (Blue-light pro, Mectron, Loreto, AN, Italy) for an illumination period of 40 seconds, a mini LED lamp (Mini LED, Acteon, Mérignac, France) and a fast LED lamp (Ortholux, 3M, Maplewood, MN, USA) used for orthodontics cure also for 40 seconds.

Samples obtained following these premises were used to determine the cellular toxicity *in vitro* using the Neutral Red Uptake (NRU) and the MTT assay.

Toxicity of the Eluates After 24 Hours

Every sample was immersed in 1 ml of DMEM and left *in situ* for 24 hours, at a temperature of 37°C. At the same time, 10.000 3T3 Swiss fibroblasts were sowed in each well of a 96 well plate and put into a culture for 24 hours, up to the formation of a monomolecular layer.

After incubation, 200 µL of DMEM, containing what had been released by the composite resin to the cellular monomolecular layer, were added.

After the other 24 hours, the cellular viability was judged with the MTT assay and the NRU (Figure 1).

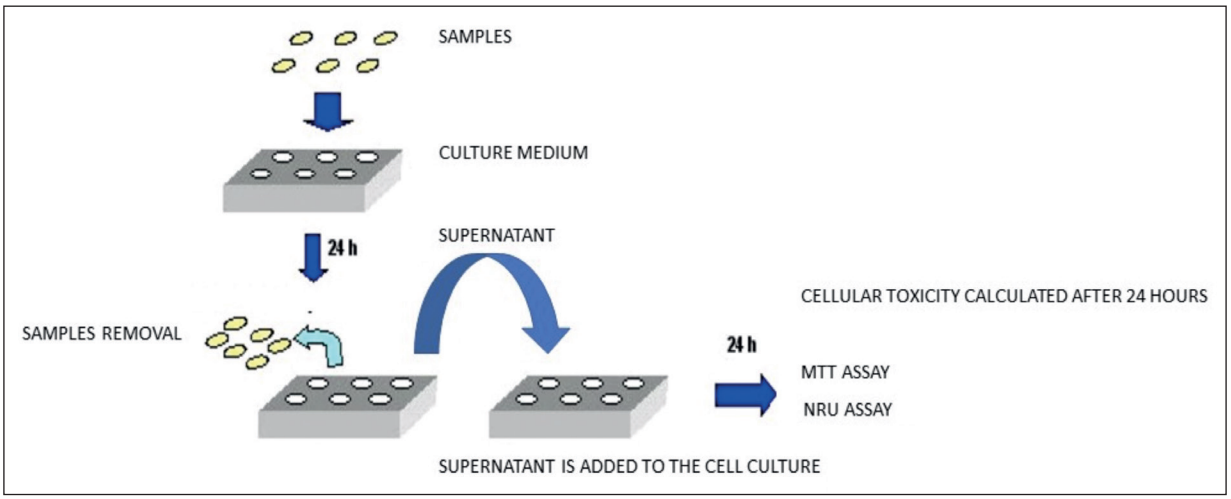

Figure 1. MTT and NRU assays performing procedure.

MTT Assay

The MTT assay was executed following the procedure described by Wataha et al[13]: 20 µl of MTT was dissolved in phosphate-buffered saline (PBS), at a concentration of 5 mg/ml. The solution was added to the culture medium and, after a 4 hourlong incubation at 37°C, the intracellular formazan crystals produced were solubilized with a muriatic acid solution in isopropanol.

The absorbance of the solution in each well was determined by using an automatic exposure meter for microplates (Packard Spectracount, Packard BioScience Company, Meriden, CT, USA) at the wavelength of 570 nm. The NRU was executed according to Borenfreund and Puerner[14].

A water solution of neutral red (0,4%) was added until it reached a concentration of 50 µg/ml. Everything was placed in an incubator at 37°C for 4 hours, then, the supernatant was removed. The neutral red captured by the viable cells was solubilized with 200 µL of a solution, made of ethanol at 50% and acetic acid at 1%. An automatic photometer for microplates with a wavelength of 540 nm was used to calculate the optical density (OD) of each well.

For each experiment, realized in quadruple copies and repeated for three times, the cellular toxicity was calculated through the equation described by Hashieh et al[15].

Statistical Analysis

All the values were expressed as mean and standard error of mean (SEM). The means groups were compared through a variance analysis (ANOVA), followed by a multiple means comparison through the Student-Newman-Keuls method. Following the *t*-Student method of means comparison, $p < 0.05$ was considered statistically significant.

Results

Toxicity of the extracts appraised through the MTT assay (Figure 2A and 2B): both materials showed slight toxicity (inferior to 20%) without significative rate differences.

Extracts toxicity appraised through the NRU assay (Figure 3A, 3B, and 3C): both materials showed toxicity between 30% and 50% (depending on the lamp used for the polymerization) (Figure 3A and 3B). There were statistically relevant differences between the toxicity induced by the auto-polymerizing material and the toxicity induced by the photopolymerizing composite material polymerized with the blue-light lamp ($p < 0.001$) and with the mini LED lamp ($p < 0.05$) (Figure 3C).

Discussion

This study evaluated the cytotoxicity of two resins for orthodontic use: a chemically polymerized resin and a photochemically polymerized one.

The cytotoxicity assays were characterized by three factors[16]:

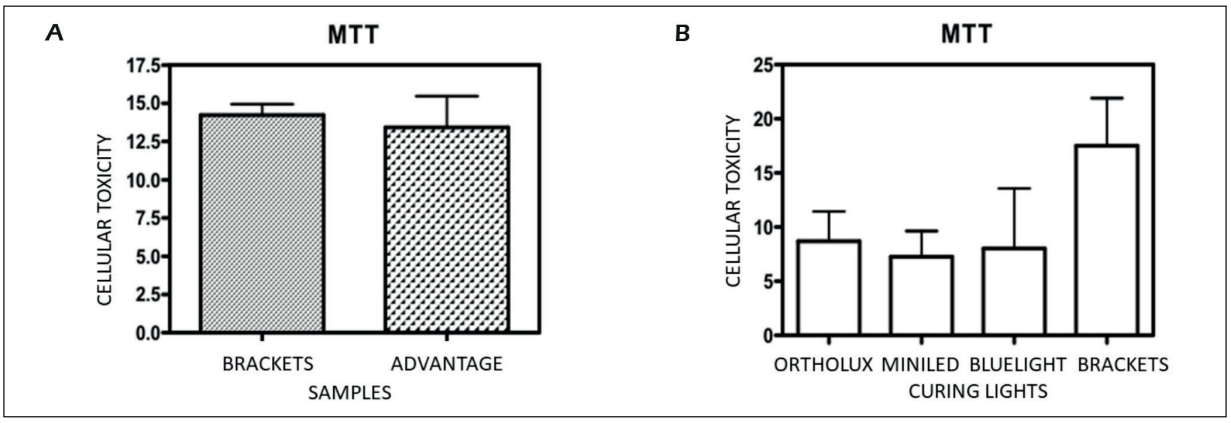

Figure 2. Toxicity induced by auto-polymerizable resin and by orthodontic brackets (**A**). Toxicity induced by photo-polymerizable resin in different photo-polymerization conditions, and toxicity induced by brackets (**B**).

1. Cellular culture
2. Cell/material contact
3. Final parameter to judge (it variates depending on the nature of the examined material).

In this investigation, 3T3 Swiss fibroblasts were used because they are recommended by the International Standard Organization (ISO)[17] among the cellular lines for *in vitro* studies of dental materials.

The cell-material contact used was mediated with eluate because it requires only a set of samples for each multiple measure, but above all, since the evaluated materials were for orthodontic use, the contact between cells and oral cavity was mediated by saliva.

The different cytotoxicity assays value different parameters, all influenced in different ways, depending on the chemical nature of the components of the material.

We decided to use two assays because this study concerns the composite resins, made of materials of various chemical nature, in particular NRU, more sensitive to lipophilic substances, and MTT, more sensitive to hydrophilic substances, instead.

The NRU assay is used to evaluate the toxicity of lipophilic substances because it evaluates the cell wall integrity: the live cells, incubated in presence of Neutral Red, capture and withhold dyestuff; on the contrary, the cells with a damaged membrane cannot retain the dye after the washing and fixation procedures[18].

On the other hand, hydrophilic substances do not damage cell walls, but can interact with intracellular enzymes. For this reason, their effects can be evaluated with functional assays like MTT[19].

This study was based on the capacity of the succinate dehydrogenase enzymes of live cells to

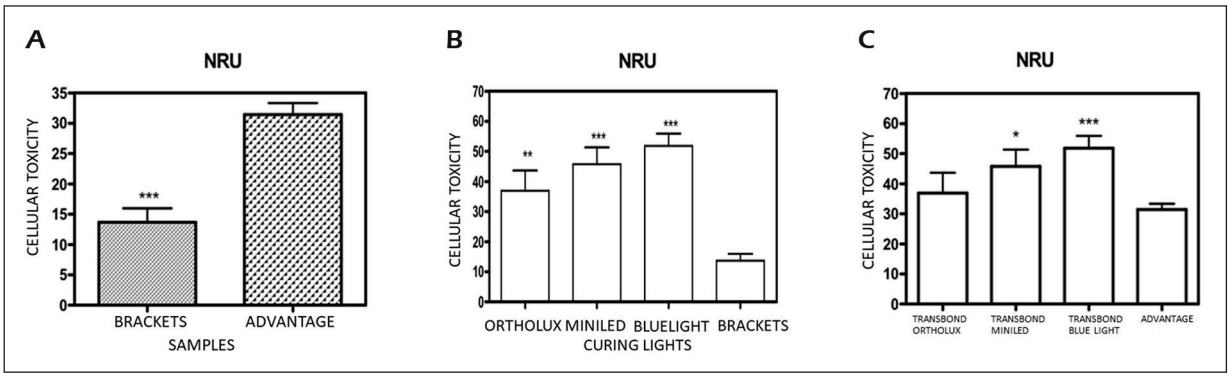

Figure 3. Toxicity induced by auto-polymerizable resin and by orthodontic brackets (**A**). Toxicity induced by photo-polymerizable resin in different photo-polymerization conditions, and toxicity induced by brackets (**B**). Toxicity induced by both resins (**C**) (*$p < 0.05$ *vs.* auto-polymerizable, **$p < 0.01$ *vs.* auto-polymerizable, ***$p < 0.001$ *vs.* auto-polymerizable).

transform the soluble salt bromide of 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazole into insoluble formazan that precipitates inside the cells.

As expected, due to the preponderancy of lipo-soluble substances in both the resins, the NRU assay turned out to be more sensitive than the MTT assay and, for this reason, the evaluation between the two samples was made only for the NRU assay.

A comparison between the results, obtained through different conditions of polymerization applied to the same material, was made for both the assays. Besides, the higher sensibility of the assay RNU was evident in this kind of analysis.

Conclusions

In line with the data collected, both resins show a low level of cytotoxicity that, in the case of photochemical polymerizing resin, depend on the lamp's characteristics.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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