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Biology and cytotoxicity of dental materials: an *in vitro* study

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Abstract

Objective: The purpose of the experiment was to determine the degree of biocompatibility of a sealer (RO, laboratory made product) dental material in terms of cytotoxicity and animal tests. *Materials and Methods*: In the present study, the biological compatibility of eight experimental composite materials was examined by *in vitro* methods. The bio-composites used for the cytotoxicity test were placed into direct contact with normal human fibroblasts in a cell-culture dish. After fibroblast bioassay was performed, a duplicate sample of biomaterial was placed in each well, and then the fibroblasts were incubated for 48 hours at 37^{0}C and 5% carbon dioxide. Local reactions after the implantation of the material regarding preclinical evaluation have been carried out within the Biobase Laboratory of the "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania. The biocompatibility was studied using the tolerance test by the subcutaneous and intramuscular implantation of the cured specimens. *Results*: The sealant C3 scored the highest value to the cell viability. The results of the present study showed that different dental materials had different effects on cells. The resin monomer TEGDMA, present in the sealer's composition, increased the amount of intracellular reactive oxygen species. Resin-based composites are cytotoxic before polymerization and immediately thereafter, whereas already set specimens cause almost no reaction. The test of tolerance showed that the composite materials do not contain any toxic, irritant substances or destructive ones for the living cells or tissues. *Conclusions*: The tests with experimental composite materials revealed that they are not cytotoxic for the living cells, in all versions of the materials used. All the samples of composite materials have maintained their integrity during the experiment, allowing the testing together with the embedded cells, which proved good viability, so they are suitable for dentistry use.

Keywords: fibroblasts, cytotoxicity, biocompatibility, viability, animal tests.

Introduction

Restorative materials may cause different reactions in the oral soft tissues such as gingiva.

It is not very clear today how much of the *in vivo* observed cytotoxicity is caused either by the restorative materials or by bacterial plaque that accumulates on teeth and restorations [1].

Cements exhibit some cytotoxicity in the freshly set state, but it decreases substantially in time. The buffering and protein-binding effects of saliva appear to mitigate against the cytotoxic effects [2].

Composites are initially very cytotoxic in *in vitro* tests of direct contact with fibroblasts. The cytotoxicity seems to be, in the early phase, from the not-polymerized components in the air-inhibited layer that leach out from the materials [3]. Other in vitro studies, which have "aged" the composites in artificial saliva for up to six weeks, have shown that the toxicity diminishes in some materials but remains high for others [4].

Usually, for *in vitro* toxicity tests, some cells are plated in a well of a cell-culture dish where they attach, forming the so-called test system. The material to be tested is then placed in this test system. If the material is not cytotoxic, the cells will remain attached to the well and will proliferate with time. If the material is cytotoxic, the cells may stop growing, exhibit cytopathic features or detach from the well. If the material is a solid, then the density (number of cells per unit area) of cells may be assessed at different distances from the material, and a "zone" of inhibited cell growth may be described [5, 6]. Cell density can be assessed qualitatively, semi-quantitatively, or quantitatively.

Substances such as Teflon can be used as negative (non-cytotoxic) controls, whereas materials such as plasticized polyvinyl chloride can be used as positive (cytotoxic) controls. Control materials should be well defined and commercially available to facilitate comparisons among testing laboratories.

In vitro studies looking for the "immune function" or other tissue reactions took place over the time. Their *in vivo* significance is yet to be ascertained, but many are trying to reduce the number of animal tests required in order to assess the biocompatibility or toxicity of a material. These assays measure cytokine production by lymphocytes and macrophages, lymphocyte proliferation, chemotaxis, or T-cell resetting to sheep red blood cells [7].

Animal tests for biocompatibility are usually used in

mammals such as mice, rats, hamsters, or guinea pigs, although many types of animals have been used. Animal tests are distinct from usage tests (which are also often done in animals) where the material is not placed in the animal with regard to its final use. The use of an animal allows many complex interactions between the material and the biological functioning system that may occur. Next to animal-based usage tests, different cell culture techniques have been described to test the biological properties of dental restorative materials [8]. Data from these tests can provide information on basic biological properties (*e.g.*, the toxicity of components of a material and the influence of different setting conditions) [9].

Langeland K and Cotton WR [10] proposed in the FDI standard, which was adopted as the ISO Technical Report 7405 in 1984, the following sequence:

1. Initial tests (cytotoxicity, systemic toxicity, mutagenicity);

2. Secondary tests (sensitization, implantation tests, mucosal irritation);

3. Usage tests.

In both concepts, a dental restorative material should be subjected to these three steps in the given sequence from the simple to the complicated test method, from in vitro to animal tests, and from preclinical to clinical testing on humans. Among the large number of newly developed materials, only those which successfully passed the first level should be further tested at the second level, and, finally, the best of these in the third [11].

The purpose of the experiment was to determine the degree of biocompatibility of a sealer (RO, laboratory made product) dental material in terms of cytotoxicity and animal tests.

Materials and Methods

Eight sealers containing organic and inorganic phase were tested. For photochemical initiation system, we used Camphorquinone and Dimethylaminoethyl Methacrylate and our polymerization inhibitor was the Tertbutylhydroxytoluene. Their chemical composition is presented in Table 1.

Table 1 – ***The composition of the sealer materials for the present study***

Composites Code	Organic phase	% wt	Inorganic phase
C2	Bis-GMA-TEGDMA	70	Glasses with strontium and zirconium, Colloidal silica
C3	Bis-GMA-TEGDMA	72–73	Glasses with strontium and zirconium, Colloidal silica, Quartz
C4	Bis-GMA-TEGDMA	71	Glasses with barium, Quartz, Colloidal silica
C5	Bis-GMA-TEGDMA	75	Glasses with zinc, Colloidal silica, Quartz
C6	Bis-GMA-TEGDMA	70	Glasses with barium, Colloidal silica
C7	Bis-GMA-TEGDMA	73	Quartz, Colloidal silica
C8	Bis-GMA-TEGDMA	75	Glasses with zinc, Quartz

Bis-GMA: 2,2-bis[4-(2-hydroxy-3-methacryloyloxypropoxy)phenyl] propane (produced in ICCRR Laboratory); TEGDMA: triethylene glycol dimethacrylate (Aldrich).

The samples used for the cytotoxicity test were polymerized in a Teflon mould sized 2×2×8 mm. The bio-composites, which are solid materials sterilized by exposure to ultraviolet radiation, were placed into direct contact with normal human fibroblasts in a cell-culture dish. Cell source: normal human fibroblasts, which were obtained from skin biopsies taken from healthy volunteers. Primary cultures of dermal fibroblasts were obtained by using Dulbecco's modified Eagle's medium (DMEM), supplemented with fetal calf serum and antibiotics. The fibroblasts were then seeded at a density of 50×10^3 cells/well in a 24-multiwell plate in complete fibroblast medium and incubated for 24 hours at 37^0C and 5% CO_2. After fibroblast bioassay was done, a duplicate sample of biomaterial was placed in each well, and then the fibroblasts were incubated for 48 hours at 37^0C and 5% CO_2. To determine the cell number, the cultures were then washed twice with phosphate buffered saline, treated with 0.025% Trypsin and 0.05% EDTA, incubated for 10 minutes at 37^0C and finally 1 mL of fibroblast medium was added. The cultures were then centrifuged at 1100 g for 5 minutes, the supernatant was discarded and the pellet was again suspended in 1 mL PBS. After that, the cells were treated with 0.5% Trypan Blue to assess the viability, and numbered using both an inverted microscope and an improved Neubauer hemocytometer. The viability result was obtained by reporting the number of viable cells (cells which do not capture the dye) to the total number of cells:

Viability = (normal cells/total number of cells) × 100

Local reactions after the implantation of the material regarding preclinical evaluation have been carried out within the Biobase Laboratory of the "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania. The biological properties of the experimental dental composites were evaluated in according to ISO 10993 standards. The biocompatibility was studied using the test of tolerance by the subcutaneous and intramuscular implantation of the cured specimens (Wistar rats). The sealant C3 scored the highest value to the cell viability.

Results

The international standards complied as ISO 10993 describes the methodology in testing medical devices and materials for biocompatibility and an *in vitro* test for cytotoxicity is recommended as a basic requirement, prior to the commencement of other advanced tests [12, 13]. Cytotoxicity testing has gained much importance in the assessment of biocompatibility in dental material research [14–16]. ISO 10993 also describes that cell lines other than established and commercially available may be used in *in vitro* testing for cytotoxicity, if they can lead to the same or more relevant results. Table 2 presents the cell validity percentage on the tested sealer samples.

If the material is not cytotoxic, the cells will remain attached and will proliferate in time. If the material is cytotoxic, the cells stop their proliferation showing cytopathologic features or they simply detach from the vessel. In Figure 1, we have yield of fibroblasts treated

with different types of biomaterials. The results of the present study showed that different dental materials had different effects on cells.

Table 2 – ***Fibroblast yield and viability***

	Fibroblast yield/well × 10^3	Viability [%]
Untreated control 1	63	98.41
Sample 2	53.5	96.26
Sample 3	58.5	96.58
Sample 4	51	92.15
Sample 5	53	93.39
Sample 6	56.5	92.92
Sample 7	49	93.87
Sample 8	55.5	92.79

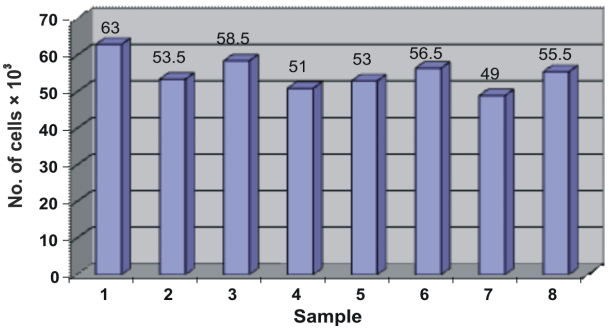


Figure 1 – ***Yield of fibroblasts treated with different types of biomaterials.***

The resin monomer TEGDMA, present in the sealer's composition, increased the amount of intracellular reactive oxygen species.

Figure 2 shows the results for the viability of the cells treated with different types of biomaterials. Resin-based composites are cytotoxic before polymerization and immediately after, whereas already set specimens cause almost no reaction.

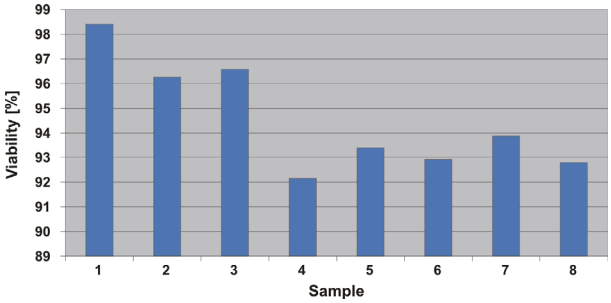
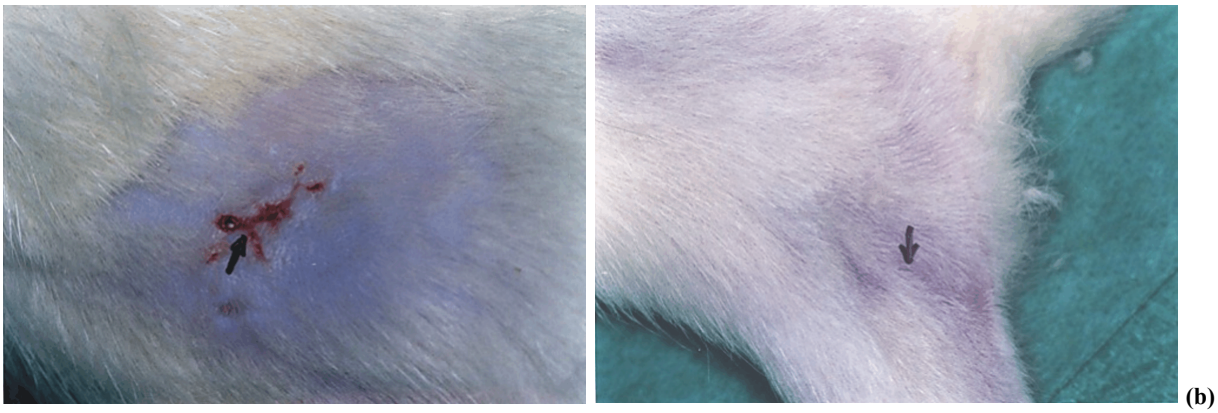


Figure 2 – ***Viability of the cells treated with different types of biomaterials.***

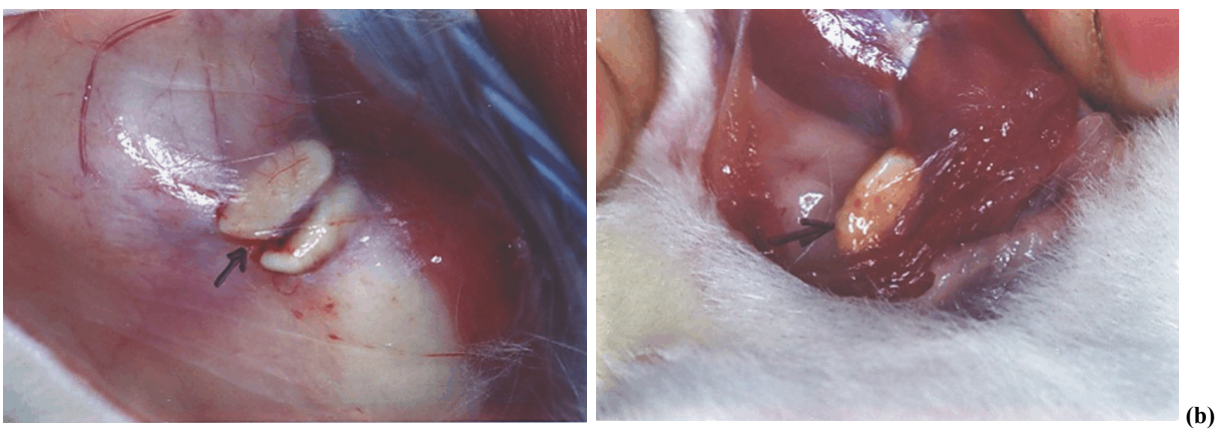
Biological tests

Placing the material was easily performed and the subjects showed no post-op complications. No loss was recorded. For all animals, irrespective of the species and/or inoculation method, the follow-up was favorable and the surgical wound healed with a crust scar. Upon 21 days, in case of the rat, at the implant areas (subcutaneous, intramuscular) we noticed a linear or irregular scar, covered or not with a fragmented crust undergoing scaling. At the skin wound area, we had no inflammatory or septic phenomena or any irritation/ rejection (Figures 3–5).



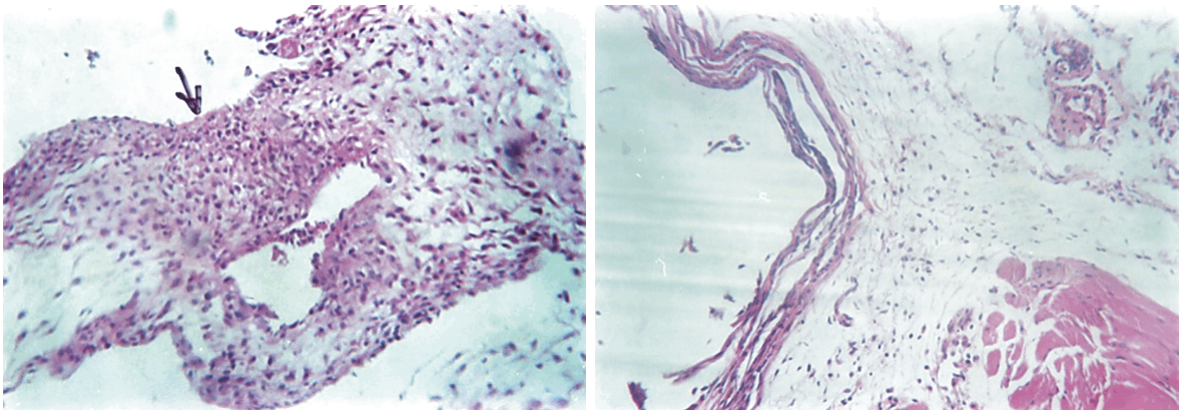
(a) **(b)**

Figure 3 – ***(a) Skin wound with irregular scar, with pieces of crust upon 21 days after subcutaneous implant (rat); (b) Skin wound completely healed, no crust on the inter-muscular implant in case of a rat after 21 days after implant.***



(a) **(b)**

Figure 4 – ***(a) The body of the implant fixed and encapsulated in the subcutaneous connective tissue after 21 days in case of the rat; (b) Encapsulation and fixing the implant in the inter-muscular connective tissue after 21 days (rat).***



(a) **(b)**

Figure 5 – ***(a) Connective proliferation of young tissue (neoformation) around the body of the implant, after 21 days in case of the rat (HE stain, ×200); (b) Fibro-vascular connective reaction which envelops the body of the implant: rat intermuscular area after 21 days (HE stain, ×200).***

Between the muscular fibers, the implant is fixed and encapsulated in a connective proliferation started from the interstitial connective tissue. The surface of the implant is isolated by a connective-vascular tissue of neoformation, with a lymphohistiocytic infiltrated liquid of moderate intensity (Figure 5, a and b). Around the body of the implant, we noticed a fibrous tissue with fibers placed concentrically underneath, up to the muscular fibers, where a well-vascularized young connective tissue is developed. After the surgery, the wound healed with no complications under the crust, and after 21 days, the healing is complete.

Discussion

In the present study, the biological compatibility of eight experimental composite materials was examined by *in vitro* methods. Visual examination showed similar density of cells adherent to each material. The living cells seemed to adhere and attained a normal (polygonal) morphology, when seeded on all eighth materials.

Fibroblasts are cells found in all tissues of the human body, including the oral cavity. In sample No. 4, the fibroblasts showed a lower viability (92.15) and inhibition of proliferation. In samples No. 2, 4, 5 and 8, the fibroblasts continued to proliferate after the biomaterial was added to the well. The different cyto-toxicity of the materials tested could be related to the different kind of ingredients, the interactions between them and the degree of resin polymerization. It is known that oxygen acts as an inhibitor of monomer polymerization. It has also been reported that unfilled resin cured in room air has a significantly greater thickness of polymerization-inhibited material than the resin cured in an argon atmosphere [17, 18]. The inhibition layer thickness varies across dentin adhesives and depends on the type and combination of monomers existing in each product. In addition, an aqueous environment may interfere with the polymerization of resinous materials [19].

These findings can be related with other reports on the induction of oxidative stress caused by TEGDMA and other compounds of many resin-based dental restorative materials like HEMA (Hydroxyethyl Methacrylate) or common photosensitizes [20–24]. From our findings, it is difficult to reach any conclusion concerning reactive oxygen species production related to a specific compound. Yet, there is evidence that reactive oxygen species generated by monomers like TEGDMA and HEMA can effectively interfere with cellular signal transduction networks regulating cell survival pathways [24].

Consequently, a relatively high amount of non-reacted co-monomers may be released from dental adhesives. Leachable monomers induce the production of intracellular reactive oxidative species that can be generated in both healthy and diseased tissues [25]. The results provide also technical support that the biomaterials tested were not toxic for the dermal fibroblasts [26].

The implant, both in the sub-cutaneous area and in the inter-muscular one, did not change position and did not cause sensitivity or mobility reactions. Locally, when touching the area, the presence of the unabsorbed implant is felt as a non-painful nodule, fixed by connective proliferation, by means of a capsule, which isolates the product. The examination of the area showed that the tissue included the material and isolated it, proving the fact that it did not contain irritating components, which could lead to rejection or elimination of the material. The microscopic histological examination confirms the fact that after 21 days the implanted material is included in the structure of the tissue by a specific inflammatory granulomatous process. All the changes observed in the contact area of the implant body with host tissues proved that it does not contain any toxic, irritant substances or destructive ones for the living cells or tissues.

The microscopic histological examination confirms that after 21 days the implanted material is included in the tissue's structure by a specific inflammatory granulomatous process. All the changes in the implant body site area proved that the materials used in this study do not contain any toxic or irritant substances for the living cells or tissues.

Conclusions

The tests with experimental composite materials revealed that they are not cytotoxic for the living cells,

in all versions of the materials used. All the samples of composite materials have maintained their integrity during the experiment, allowing the testing together with the embedded cells, which proved good viability. Therefore, they could be suitable for use in dentistry.

Acknowledgments

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