Biocompatibility Study of Several Esthetic Dental Restorative Materials

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Abstract

Biocompatibility of three materials used in restorative dentistry were investigated in vitro. The aim of this study was a comparative evaluation of biocompatibility of several restorative materials on cultures of primary human osteoblasts (OBL) and HFL (human lung fibroblasts) cells. The differentiation process was evaluated with alamar blue viability assay and by immunocytochemical staining. The samples were examined using a Zeiss Axiovert microscope by reversed phase fluorescence and image capture was performed using a MRC camera. A glass ionomer cement (Ketac Molar, 3M ESPE), a light cured universal microhybrid composite material (Fitek Z250, 3M ESPE) and a total etch adhesive (Adper Single Bond 2 Adhesive, 3M ESPE) were used for analysis. The results of this study have shown that none of these materials exerted increased toxicity, explained by the low capacity of these materials to allow cell adhesion. Only HFL cells grown on Adper Single Bond 2 showed some degree of adhesion to the substrate.

Keywords: biocompatibility, cell cultures, cell viability

1. Introduction

The restoration of dental cavity requires a material capable of integrating with its biological environment. An ideal material should be biocompatible with dental tissues and create a local environment that is conducive to pulpal healing. Their properties have been extensively studied and tested; nevertheless, there is few data in the literature reference relating to their longer-term effect on the oral mucosa. Therefore, this was an extra reason to search out for testing the biocompatibility of these restorative materials used in our personal research to benchmark the biological response and their eventual irritant potential. The evaluation of biocompatibility and cytotoxicity of any dental material is as important as the assessment of its physical properties (De Souza et al. [1]).

The aim of this study was a comparative evaluation of biocompatibility of several restorative materials on cultures of primary human osteoblasts (OBL) and HFL (human lung fibroblasts) cells. The differentiation process was evaluated with alamar blue viability assay and by immunocytochemical staining. The samples were examined using a Zeiss Axiovert microscope by reversed phase fluorescence and image capture was performed using a MRC camera. Ketac Molar Easymix (3M ESPE, St. Paul, MN, USA) is a glass ionomer restorative
material designed for the lateral tooth area. It is a conventional, metal-free glass ionomer cement which is widely used for conservative, minimally invasive restorations using Atraumatic Restorative Treatment (ART) technique. His main indications are: class I restorations, fissure sealing, primary teeth restorations, semi-permanent Class II restorations, core build-ups. Also, it can be used as linings under composite fillings of cavity classes I and II and cavity class V fillings when aesthetic aspects do not play a major role. All these indications are a result of his improved mechanical properties: excellent strength and marginal integrity, radiopacity, and packable consistency (Rodrigues et al. [2]). Ketac Molar allows fluoride release and recharge to take place, enabling remineralization the adjacent tooth substrate. 3MTM Filtek™ Z250 Universal Restorative (3 M ESPE, St. Paul, MN, USA) is a light cured universal microhybrid composite, used both for anterior and posterior restorations: direct restorations, sandwich technique with glass ionomer resin material, core buildup, splinting, indirect anterior and posterior restorations including inlays, onlays and veneers. Composite Filtek Z 250 (3M ESPE) is a very good material for this type of restorations because of its natural esthetics, reduced shrinkage for less post-operative sensitivity, low wear, high fracture toughness, radiopacity, excellent marginal adaptation and polishability (Lien et al. [3]). Adper™ Single Bond 2 Adhesive (3 M ESPE, St. Paul, MN, USA) is a total etch, visible-light activated dental bonding agent. Adper Single Bond Adhesive is indicated for direct light-cured restorative material as well as for the treatment of cervical sensitivity (Munoz et al. [4]). Adper Single Bond Plus Adhesive may also be used for bonding laboratory fabricated composite or porcelain veneers when used with RelyX™ Veneer Cement and for bonding amalgam and other indirect applications when used with RelyX™ ARC adhesive resin cement (Vaz et al. [5]).

2. Materials and methods

Materials entered in the study were tested for biocompatibility using two cell lines: HFL cells (Human Lung Fibroblast) and primary human osteoblasts (Tomuleasa et al. [6]).

• **Cell culture**: Human fibroblasts and osteoblasts, stored in nitrogen at -196 °C, were thawed rapidly in 7 ml of pre-warmed medium at 37 °C and centrifuged at 1000 rpm for 5 min. The culture medium was DMEM high glucose / F-12 HAM with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 µg / ml streptomycin, 2 mM L-Glutamine, 1% non-essential aminoacids (NEA) (all reagents were purchased from Sigma Aldrich). The cells were seeded in 25cm² Cole flasks and cultivated until reached subconfluence at 37°C in humidified atmosphere.

• **Substrates** were created according to manufacturer prospects: powder substrate were solubilised in diluents in the ratio of 1: 1 (v / v) followed by another dilution of 1: 3 with absolute ethanol. The substrates solutions were added in 96-well plates (each 50 µl/well). After evaporation of the alcohol and solidification of substrates, the plates were sterilized by exposure to UV for 3 hours. For immunocytochemical staining we used 4-well chamber slides and substrates were created using the same procedure, applying 100 µl of substrate solution/well.

For biocompatibility tests, after 3 days of cultivation, while the cells have adapted to the new environment, fibroblast cells and osteoblasts were detached from the plates with 0.25% trypsin EDTA for 5 min. Trypsin was then inactivated by the addition of 5 ml of medium with 10% FCS, and the cells were centrifuged for 5 min at 1000 rpm. The supernatant was discarded and cells were counted using a Thoma chamber. The cell suspension was adjusted and 2X10⁴ cells/well. Controls consisted of cells grown under the same conditions without substrate. For immunocytochemical stains were seeded 40 000 cells / well in 4-well chamber slides.
Alamar Blue test is used mainly as a test of cell viability. Resazurin, which is a non-fluorescent dye, is converted into resorufin (red fluorescence) through a reduction mechanism in metabolically active cells. The fluorescence intensity is directly proportional to the number of viable cells. 2 X10^4 cells/well were suspended in 200μl complete medium and seeded on the surface of tested sterilized substrates placed in 96-well plates. Each determination was performed in triplicate. After 24 hours of cultivation, the medium was changed and 10μl of Alamar Blue solution (Invitrogen) was added in 100μl of culture medium/well. The plates were incubated for 1h at 37 °C in the dark. The medium was transferred to another plate for reading, and the intensity of staining was measured using a BioTek Synergy 2 plate reader at absorption to 570nm.

Immunocytochemical staining protocol: the expression of some specific proteins in fibroblasts and osteoblasts and the rearrangement appearance of filamentous actin fibers was revealed immunocytochemistry. After 5 days of culture, cells growing on chamber slides were washed 3 times with PBS and fixed with 4% paraformaldehyde (PFA). For F actin staining a membrane permeabilization step was applied with exposure for 15 min at room temperature with 0.1% Triton X100, followed by 3 washes with PBS. To block unspecific binding of the monoclonal antibodies, the cells were exposed 20 min to a solution of blocking 10% BSA (bovine serum albumin) in PBS. Osteoblastic cells were labeled with primary antibody against osteopontin (OP), and for fibroblasts primary antibody against CD 90 (Santa Cruz Biotecnologies) and incubated overnight at 4 °C followed by 3 washes with PBS. Cells were then incubated with secondary antibodies labeled with FITC (goat antimouse IgG1) for 45 min at room temperature in the dark, followed by washing 3 times with PBS. To visualize cytoskeletal actin fibers was used phalloidin marked with TRITC. DAPI staining of nuclei was done by using a mounting medium containing DAPI stain. Immunolabeled cells were visualized with a reverse phase Zeiss Axiovert microscope with epifluorescence using appropriate filter for each fluorochrome: FITC 488 nm, 546nm for TRITC and 346 for DAPI. Images were capture with a CCD camera AxioCam MRC and analyzed with image analysis software Axiovision Rel 4.6.

Statistical analysis: absorbance data obtained from alamar blue tests were analyzed with Graph Pad Prism 5 software, using One-way ANOVA followed by Dunnett Multiple Comparison Test (p value was set at p <0.05).

3. Results and discussion

Three materials were tested: Ketac Molar Easymix (noted with I), Adper SingleBond 2 (II) and Filtek Z 250 (III). Cells’ adherence to substrates surface after 2 hours was almost similar to the control samples (cells grown on plastic), with no statistically significant difference. (Fig.1A and B) Both fibroblasts cells and osteoblasts were dramatically affected by cultivation on substrates, as alamar blue results showed a strong decrease of cell proliferation after 3 and 5 days of cultivation. These data were confirmed by fluorescence microscopy of immunocytochemically stained cells grown for 5 days on substrates. Control cells (HFL cells and osteoblasts) expressed a typical distribution of actin filaments and positivity for specific markers (CD90 for HFL cells and osteopontin for osteoblasts). Cells grown on substrates were identified by staining nuclei with DAPI, observing that cell density was very low, with an exception for HFL cells cultivated on Adper Sigle bond 2. Also, staining for actin filaments was negative (Fig. 2 A and B) (Beriat et al. [7]), (Kierklo et al. [8]), (Porto et al. [9]).
Figure 1. Alamar blue tests graphical aspect of data obtained for osteoblasts (A) and for HFL fibroblasts cells (B) cultivated on substrates’ surfaces. Alamar Blue viability test shows a decrease in viability and proliferation of fibroblasts grown on substrates after 3 and 5 days compared to control cells grown on plastic surface.

Figure 2. Fluorescence microscopy of HFL cells (upper panel-A) marked with CD 90 FITC (green), actin F TRITC (red) and DAPI staining of nuclei (blue) and for osteoblasts cells and the appearance of osteoblasts (lower panel-B) stained for Osteopontin FITC, actin F TRITC and DAPI.
4. Conclusions

Cells grown on tested materials in the first 2 hours showed similar results to those grown on standard plastic surfaces, suggesting that these substrates do not exert increased toxicity. However, the fact that the cells number dropped drastically when cells were cultivated on substrates for 3 to 5 days can be explained by the low capacity of these materials to allow cell adhesion. The protocol for immunocytochemical requires numerous washes, and the poor adhered cells to the substrate cells will be removed during the staining procedure. Only HFL cells grown on Adper Single Bond 2 showed some degree of adhesion to the substrate.

References: