Biocompatibility Testing on Cell Culture of some Root Canal Sealers used in Endodontics

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Abstract

Cleaning and shaping of the root canal, followed by a complete seal are an important steps in root canal treatment. The aim of this study was a comparative evaluation of biocompatibility of some endodontic root-end filling materials on cultures of primary human osteoblasts (OBL) and dental follicle derived adult mesenchymal stem cells (DF MSCs). The differentiation process was evaluated with alamar blue viability assay and by immunocytochemical staining for bone markers (osteopontin, alkaline phosphates). The samples were examined using a Zeiss Axiovert microscope by reversed phase fluorescence and image capture was performed using a MRC camera. Tested materials proved to develop a degree of toxicity that occurs especially when osteoblasts after 2 weeks, with higher extent for AH Plus material. A very good biocompatibility with stem cells was observed for Acroseal especially after 9 days of cultivation, with an increased proliferation rate of DF MSCs.

Keywords: biocompatibility, sealer, mineral trioxide aggregate, AH Plus, Acroseal

1. Introduction

The tested materials have been developed to provide a complete seal, especially in the apical part of the root canal. The presence of mineral trioxide aggregate in MTA Fillapex (Angelus, Londrina, PR, Brazil) provides the predictable results and healing response, sealing off all pathways between the root canal system and surrounding tissues. AH Plus (De Trey Dentsply, Konstanz, Germany) is an improved paste-paste material based on epoxy-amine resins with excellent apical sealing. Acroseal (Septodont, France) has an advanced epoxy matrix containing calcium hydroxide which creates a high intra-canal pH during final obturation that helps to improve periapical healing. Their properties have been extensively studied and tested, because sealers used in endodontics are placed in proximity of periodontium, so endodontic sealers have to be non-toxic and biocompatible. The purpose of the study was a comparative
2. Materials and methods

Preparation of substrates with root-end filling materials

The test materials were mixed according to the manufacturer’s instructions and placed as thin layers with the help of cell scrapers onto the surface of 2-well chamber slides. Each probe was performed in triplicate. Samples were dried and after 24 hours, the samples were sterilized with ethylene oxide gas.

Obtaining of Human Osteoblastic Cells

Osteoblast-like cells were isolated from bone fragments obtained during surgery arthroplasty in the Orthopedic Clinic Cluj-Napoca, after obtaining the signed informed consent from the patient. Briefly, bone fragments were mechanically and enzymatically processed, and isolated cells and bone explants were cultured in Dulbecco’s modified Eagle’s medium (DMEM)/F-12HAM (Sigma Aldrich) containing 20% fetal bovine serum (FBS), 2mM L-glutamine, 1% antibiotics, 1% non-essential amino acids (NEA) (all reagents from Sigma) 6–8 weeks, until cell confluence was reached. Cells were characterized for bone cells’ markers osteopontin and osteonectin by immunocytochemical staining [1]. For biocompatibility and differentiation experiments isolated osteoblasts were used after six\textsuperscript{th} passage.

Isolation of mesenchymal stem cells from the dental follicle

Fragments from dental follicles (DF) removed from completely intrabone impacted third molar/canine were harvested from patients free of infections after signing the agreement. The DF fragments were processed mechanically and enzymatically and a monolayer suspension was obtained by filtration with 70µm Filcons meshes. The cultivation medium consisted of DMEM high glucose/F-12HAM (Sigma Aldrich) containing 15% FBS, 2mM L-glutamine, 1% antibiotics, 1% NEA, 55µM beta-mercaptoethanol, 1mM sodium pyruvate (Sigma). After 3 days of cultivation adherent confluent cells monolayers were detached by trypsinisation and subcultivated, with changing the medium at 2-3 days. The cells were characterized for stem cells markers by immunocytochemical staining and flow-cytometry and showed a strong positivity for SSEA-4, Oct3/4, Nanog, CD44, D90, CD 73 and weak positivity for CD105 and CD49e. Cells did not express CD 45, CD 117 and CD34. RT-PCR analysis showed that isolated cells expressed Oct3/4, CXCR4, SCF (stem cell factor), CBF\beta, Thy-1, Tie-2, vimentin, nanog, and HLA-ABC. These cells didn’t express hTERT, Sox-2, c-kit and HLA-DR\alpha.

Alamar Blue viability assay

Cell viability and proliferation was evaluated with Alamar blue test. Alamar blue contains a non-fluorescent dye-resazurine which is reduced to fluorescent resorufin in metabolically
active cells. 4 x 10^5 osteoblasts and DF MSCs were suspended in 2 ml of complete medium and seeded on surface of endodontic root-end filling materials created substrates in 2-wells chamber slides. Control samples were cultivated on plastic surfaces. After 48 hours and 5, 9 and 14 days of cultivation, the medium was changed and 100 μl of Alamar blue (Invitrogen) was added in 900 μl complete medium in each well. After 1 hour of incubation at 37° C in the dark, the medium was transferred to another 12-well plate and fluorescence intensity was measured using a BioTek Synergy 2 plate reader (excitation 540 nm, emission 620 nm).

**Vital fluorescent labeling of cells**

A membrane fluorescent technology was used to highlight the cell culture onto substrate surfaces. Fluorescent labeling of osteoblasts and DF stem cells was performed using PKH26 Red Fluorescent Cell Linker Kits (Sigma-Aldrich). Viable cells incorporate a yellow orange fluorescent dye (PKH26) into lipid regions of the cell membrane, and this staining ensures maintenance of fluorescence of live cells for a longer period of time. Briefly, after trypsinization, 1x10^6 were washed twice with PBS by centrifugation at 1000 rpm, 5 min and the cell pellet was resuspended in a mixture of 1ml Diluent C and 1ml of Dye Solution containing 4μl of PKH26/ml. After 5 min the staining was stopped by adding 2 ml of complete medium containing 10% FBS and cells were centrifugated for 10 min at 1000 rpm. Another two washing steps were performed with 10 ml of complete medium. After counting the cells, 7x10^4 cells/well were seeded in 2-well chamber slides (Nunc Thermoscientific) in complete medium. The samples were examined using a Zeiss Axiovert microscope by reversed phase fluorescence using filters at 546 nm and image capture was performed using a MRC camera. The cells were quantified at different time intervals by counting in 3 different microscopic fields, through random selection: after 45 min for cell adhesion, 24 hours, 5 and 8 days.

**Statistical analysis**

Cell viability data were analyzed using GraphPad Prism 5 statistics program. We compare the control group with each endodontic root-end filling materials substrate group with one-way ANOVA Tukey’s Multiple Comparison Test with setting the statistical significance at p < 0.05.

**Results**

In fluorescence microscopy, stained cells with PKH26 were visualized at different intervals of time. After 45 minutes of cell seeding on substrate and on control plastic surfaces was observed that the adhesion process was not complete, even in cells grown on plastic surfaces, these having a rounded shape. The number of DF stem cells was higher on substrates than osteoblasts (Fig. 1).

After 24 hours the adhesion process was completed for cells cultivated on plastic surface. Cultivated cells on substrates showed still a rounded shape, which suggest that those materials do not allow the cells to adhere to their surfaces (Fig. 2). This aspect of osteoblasts and DF stem cells was maintained also after 5 days in the case of AH Plus and MTA substrate. Only Acroseal substrate showed that the DF stem cells proliferate and spread on substrate surface. The control cells partially lost their fluorescent staining due to increased proliferation (Fig. 3). Similar aspects regarding the cell number and morphology were observed after 8 days of cultivation (Fig. 4).
**Figure 1** - Images captured in fluorescence of PKH26 stained osteoblasts (OBL) and dental follicles (DF) stem cells 45 min after seeding on substrate surfaces. Graphical aspect of counted cells.

**Figure 2** - Images captured in fluorescence of PKH26 stained osteoblasts (OBL) and dental follicles (DF) stem cells after 24 hours of cultivation on substrate surfaces. Graphical aspect of counted cells.
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Figure 3 - Images captured in fluorescence of PKH26 stained osteoblasts (OBL) and dental follicles (DF) stem cells after 5 days of cultivation on substrate surfaces.

Figure 4 - Images captured in fluorescence of PKH26 stained osteoblasts (OBL) and dental follicles (DF) stem cells after 8 days of cultivation on substrate surfaces.

Figure 5 - Graphical aspect of viability data Alamar blue test (fluorescence units) of osteoblasts and DF stem cells cultivated on substrates surfaces (legend: OBL-osteoblasts grey columns, FD stem cells- red columns)
Testing the cells’ viability with Alamar Blue showed a constantly growing proliferation rate of control cells cultivated on plastic surfaces in the first nine days. The cells’ number and viability was declining after 14 days. In all tested materials cell viability was lower than in controls but with some variations depending on endodontic root-end filling materials substrate and cell type. An increased toxicity was observed for AH Plus substrate in the first 48 hours of cultivation of osteoblasts (decrease of cell viability compared to control samples, statistically significant) toxicity maintained also after 5 days. After 9 and 14 days, the viability of osteoblasts cells grown on all tested materials was lower than control cells (Fig. 5). A different behavior was observed in the case of DF MSCs cultivated on endodontic root-end filling materials substrate. Acro seal material showed a higher biocompatibility with DF stem cells, by comparison with control cells and with cells cultivated on MTA and AH Plus. In the first 5 days the stem cells cultivated on Acro seal material were comparable with control cells, but after 9 and 14 days the cultures exhibited an increase of proliferation rate even when were compared with control samples. MTA material showed the greatest toxicity on DF stem cells.

3. Discussion

Primary cell strains derived from living tissues are necessary for sensitivity testing, measure proliferation, viability, and cytotoxicity to revealed the response of cells in cultures with different tested materials. In our study was selected osteoblast-like cells isolated from bone and fragments from dental follicles removed from human intrabone impacted third molar or canine, in order to simulate the clinical environment. The tested sealers showed different toxicity, especially MTA Fillapex and AH Plus. Garrido et. al [2] evaluated the cytotoxic effect of a new Copaiba oil-based root canal sealer, Endofill and AH Plus on osteoblast-like Osteo-1 cells and showed a decreased cellular viability considerably for AH Plus, with statistical significance compared with the other tested sealers. The sensitivity of toxicity depended on the materials tested and the cell culture system used. Thus, the use of both permanent and primary cells is recommended for screening of the cytotoxic effects of root canal sealers. Use of calcium hydroxide-based material as a root canal sealer initially may result in a more favorable response to periradicular tissues [3]. The resin-based sealers (AH Plus) caused a dose-dependent increase in genotoxicity, but no such effect was seen with the calcium hydroxide-based sealer. The highest level of DNA damage was induced by the resin-based sealers [4]. On the other hand, in a recent meta-analysis whose aim was to determine the relative toxicity of commonly used root canal sealers like zinc oxide eugenol, calcium hydroxide, and resin-based sealers, the authors found that calcium hydroxide sealer and zinc oxide eugenol were significantly biotoxic as compared to resin-based sealers after 3 days [5]. The cytotoxicity of MTA has been investigated in several studies. In a study analyzed bone tissue reactions to MTA Fillapex compared with an epoxy resin-based material after 7, 30, and 90 days, the authors found that MTA Fillapex scored significantly higher for neutrophils at 7 days than at 90, comparing with AH Plus. The presence of mineral trioxide aggregate in MTA Fillapex composition did not improve the bone tissue repair, but MTA-like sealers and epoxy resin-based sealers provided a re-establishment of the original bone tissue structure a decreased inflammatory response over time [6]. In a study evaluating the cytotoxicity, radiopacity, pH, and flow of MTA Fillapex and AH Plus, MTA Fillapex proved to be more cytotoxic than AH Plus, a different result compared with our study [7]. Moreover, another study found that MTA Fillapex reduced the number of cells attached to the surface whilst AH Plus may promote better attachment to human tooth germ stem cells [8].
Similar to our results, it was revealed that mineral trioxide based materials can cause significantly lower proliferation rates especially for the osteoblasts [9]. Acroseal showed a very good biocompatibility with DF stem cells. The result is not in agreement with study done by Eldeniz et al [10] where Acroseal was found to be strongly cytotoxic. The source of the toxicity might be because of the presence of amines in the epoxy base of this material. In another study done by Gambarini et al [11], Acroseal showed only mild cytotoxic effects.

4. Conclusions

Endodontic root-end filling tested materials proved to develop a degree of toxicity that occurs especially when osteoblasts after 2 weeks, with higher extent for AH Plus material. The osteoblasts seem to be more sensitive to tested materials than FD stem cells. A very good biocompatibility with stem cells was observed for Acroseal especially after 9 days of cultivation, with an increased proliferation rate of DF stem cells.

References


