Comparative evaluation of the influence of two resin-based restorative materials on the behaviour of progenitor-like cells

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The aim of the present study was to investigate the biological effects of two resin-based restorative dental materials placed in direct contact with cells isolated from palatal connective tissue graft (CTG) and to demonstrate that the harvested palatal CTG contained progenitor cells. Palatal cells were isolated from tissues collected from individuals undergoing periodontal surgeries. Composite resin and compomer discs plated with *ex vivo* expanded cells were used to analyze the cell characteristics by scanning electron microscopy (SEM). The amount of the inorganic filler of the two dental materials was determined by thermogravimetric (TGA) analysis. The composite resin had a relatively smaller amount of inorganic filler and was quite stable above 750 °C in comparison with the glass ionomer-resin composite. Resin composite and compomer materials seemed to have no cytotoxic effect on isolated cells since the cells had grown well on both restorative materials. Over the entire cultivation period, the cells remained undifferentiated and did not change the phenotype, which sustained that they are undifferentiated cell types.

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1. Introduction

When restoring dental cervical lesions, the contemporary restorative techniques are based on the use of advanced materials, which allow the achievement of aesthetic and functional restorations. Composite resins are considered to be the material of choice in restoring cervical lesions because of their excellent aesthetics, elastic modulus similar to that of the dental tissues and because of their ability to bond to enamel and dentin [1-3]. Compomers are alternative materials, combining the composition of composite resins and glass ionomer cements; they are used especially to restore cervical lesions extending onto radicular areas. Their use is based on the excellent biocompatibility, fluoride release, adhesion to tooth structures and visible light curing property, which in turn reduces sensitivity to water during setting [4]. Although modern dental materials have undergone significant improvements in the last years, today methacrylate-based materials still have shortcomings that limit their applications. The organic matrix of dental resin materials has been recognized as a source of compounds that cause a wide variety of adverse biological reactions including cytotoxicity on different cell lines because of the elution of some unpolymerized monomers [5, 6].

Sometimes, composite resins and compomers have to be used to restore cervical radicular lesions associated with gingival recessions. In these particular clinical situations, the restorations must be performed intrasurgically, just before the coverage of gingival recessions with connective tissue graft (CTG) associated with coronally advanced flap (CAF).

Placing gingival grafts on the exposed root surfaces presenting composite resin-based restorations did not impede the complete clinical coverage of gingival recessions [7, 8], but little is known about the nature of the healing in these cases. Histological findings have suggested that epithelial and connective tissues adhere to resin-ionomer restorative materials during the healing process [9], but the effect of resin-based materials on the survival and adherence properties of some cell populations, such as oral progenitor cells, is not well elucidated.

In realizing this study, it was assumed that resin-based restorative materials may release unreacted toxic monomers which may have a toxic effect on progenitor cells from harvested palatal graft.

The main objective of the present *in vitro* study was to investigate the biological effects of two resin-based restorative dental materials placed in direct contact with cells isolated from palatal connective tissue and to demonstrate that the CTG harvested from the palate contains progenitor cells.

2. Experimental

2.1 Preparation of the dental material substrate

Two resin-based restorative dental materials were used to manufacture the specimens used as a substrate for cell growth (Table 1).

Disc-shaped specimens (6 mm in diameter; 1 mm in thickness) of the two tested materials were fabricated by placing the materials in a mould and covering it with a plastic foil. The specimens were light-cured for 40 seconds from one side using a light activation unit (Demi LED Curing Unit, Kerr Corporation, Orange, CA, USA). All discs were sterilized by ethylene oxide gas for 7 hours at 55 ^{0}C followed by degassing for 12 hours.

Table 1. Restorative materials.

Type	Restorative Material	Batch	Manufacturer
Hybrid resin	Herculite® XRV	3528325	Kerr Company Orange, CA, USA
Compomer	Dyract® Extra	1112001478	Densply, DeTray GmbH, Germany

2.2 Characterization of the dental materials by thermogravimetric analysis

The main characteristic of the composite materials is the ratio between the inorganic filler and the organic matrix. The amount of the inorganic filler of the two dental materials was determined by thermogravimetric (TGA) analysis, with Mettler Toledo TGA/ SDTA851 instrument. For this purpose, the disc-shaped specimens were minced into small fragments and samples of 30-35 mg were heated from room temperature to 1000 °C, at a rate of 20 °C/min. The heating was performed in nitrogen atmosphere (35 mL/min) up to 650 °C and in nitrogen and air purging above 650 °C. The residuum amount was determined at 750 °C and 1000 °C.

2.3 Collection and transport of the tissue samples

Tissue samples were collected from 3 individuals aged of 19-29 years, undergoing surgical procedures in order to cover gingival recessions, at the Periodontology Department of "Iuliu Hatieganu" University. The following entry criteria had to be satisfied for a patient to be enrolled into the study: age $\geq$ 18 years, no relevant systemic diseases, full-mouth plaque score [10] $\leq$ 30 %, smoking $\leq$ 10 cigarettes/day, presence of at least one Miller Class I or II [11] buccal gingival recession(s) $\geq$ 2 mm requiring CTG associated with CAF as surgical approach.

After the enrolment of the patients, the study protocol and the procedural details were explained and written informed consents were obtained from all subjects. In obtaining the informed consent and conducting the research, the study adhered to the principles outlined in the Declaration of Helsinki on experimentation involving human subjects. The study was approved by the Ethical Board of "Iuliu Hatieganu" University (No.505/2011).

The connective tissue grafts, 1.5 to 2 mm in thickness, were harvested from the palate, and 2 to 3 mm of the full-thickness CTG (including adipose tissue) were cut off and transferred in sterile plastic tubes (Nunc) containing transport medium: Dubelcco's Modified Eagle Medium DMEM 1X (Sigma-Aldrich) supplemented with Fetal Calf Serum 10 % FCS (Sigma-Aldrich) and 1 % Antibiotic/Antimycotic (Gibco). The samples were transported to the tissue culture laboratory immediately after sampling.

2.4 Culture of presumed progenitor-like cells

The tissue samples were processed using special culture medium in order to favour the development of only undifferentiated cellular lines. The methodology is detailed elsewhere [12, Roman *et al*. unpublished data].

2.5 Cell culture on discs

Palatal progenitor-like cells from passage number 4 were used for all experiments. At the 4th passage, trypsinization (trypsine + EDTA 1:4) of the culture was performed. Once unicellular suspension obtained, the quantification of the total number of cells was realized. A quantity of 10^4 cells from the cellular suspension was cultivated on every disc and the discs were placed in multi-compartmented culture plates (Nunc) of 2 cm diameter, in 500 μL culture medium. The medium was replaced after 48 hours. The plates were incubated for 72 hours in order to obtain individualized cell clusters.

The cultivated cells from each patient were seeded on four discs, two for each material.

2.6 SEM observation

The resin-based specimens plated with cells were used to analyze the cell morphology and adherence characteristics by scanning electron microscopy (SEM). The number of the attached cells on each disc was also evaluated.

The discs were carefully removed from the culture medium, rinsed with Phosphate Buffered Saline PBS 1X (pH 7.2) to eliminate unattached cells, immediately immersed in 2.7 % glutaraldehyde in PBS 1X for 60 minutes and rinsed with PBS. The discs with adherent cells to the disc surfaces were air-dried for 30 minutes. The specimens were attached with double side carbon sticky tabs and sputter-coated with platinum/palladium in a 7 nm layer using an AGAR Automatic Sputter-Coater. Samples were examined with a JEOL JSM 5510LV

Scanning Electron Microscope. Because the flattened appearance of the cells and in order to visualize the cell morphology the samples were tilted up to 60°.

2.7 Statistical analysis.

Statistical analysis was conducted with SPSS software 15.0. Means and standard deviation values for cell populations grown on six discs prepared from each test material were calculated. Comparisons were done with the Mann-Whitney test, because data were not normally distributed. The level of significance was $p < 0.05$.

3. Results

Two trade mark dental materials were used in this study in order to investigate both the behaviour of the progenitor-like cells in contact with them and the influence of their composition on the cell growth. The tested materials and their main characteristics are presented in Table 2.

Herculite® XRV is a fine hybrid composite resin that consists in a methacryilic acid ester based matrix filled with inorganic particles with different dimensions i.e. micrometric glass grains and nanosized particles of silicon dioxide. The inorganic filler possesses an average particle dimension of 0.6 μm and it amounts to 79 wt. % [13].

Dyract®Extra is a compomer i.e. a combination between composite resins and glass-ionomers, and possesses fluoride releasing properties. This material consists of a polyacid modified composite resin filled with fine particles of glass, highly dispersed silica and strontium fluoride. The filler amount is of 73 wt. % and the average particle size is about 0.8 μm [14].

Table 2. Composition of the tested materials as found in the literature, and the filler content as estimated by TGA measurements

Material	Main components*	Filler load*		Filler Particle* size	Filler load, TGA residuum
		Weight	Volume		
Herculite® XRV	Resin matrix: Bis-GMA, UDMA, TEGDMA Inorganic filler: barium-alumino–sodium silicate glass, pyrogenic silicon dioxide	79 %	59 %	Av. 0.6 μm	69.0 %/750 °C 68.5 %/1000 °C
Dyract® Extra	Resin matrix: UDMA, TCB, TEGDMA Inorganic filler: strontium-alumino-sodium fluoro-phosphor-silicate glass, highly dispersed silicon dioxide, strontium fluoride	73 %	47 %	Av. 0.8 μm	75.0 %/750 °C 73.0 %/1000 °C

**Composition according to references* [13,15] *for Herculite XRV and* [14,16,17] *for Dyract® Extra; Bis-GMA = Bisphenol glycidyl dimethacrylate; UDMA = Urethane dimethacrylate; TEGDMA = Triethylene glycol methacrylate, and TCB = Di-ester of 2-hydroxyethyl di-methacrylate with butane tetracarboxylic acid*

In order to determine the amount of filler particles, the two dental materials used as substrate for cell growth were investigated by thermal analysis. The thermal behaviour of the specimens of Herculite ®XRV and Dyract® Extra was in agreement with the literature data [18]. TGA investigation was utilized to accurately measure the variation of the specimen mass as temperature was increased and it allowed to monitor the thermal degradation of the resin and to evaluate the residuum amount left after organics removal. The most obvious difference in the weight loss profile of the two composite materials was the residue amount i.e. the quantity of the material remnant after all organic component had been volatilized, due to the air purging stage. The amount of TGA residuum at 750 °C reflected the weight proportion of the inorganic filler present in each type of restorative material (Table 2). The hybrid material had a relatively smaller amount of inorganic filler (about 69 % after ignition), in comparison with the glass ionomer-resin composite (about 75 % after ignition). One could also note that, the hybrid material was quite stable above 750 °C, whereas the compomer lost about 2 % in weight, in relation with the specific inorganic filler.

The ability of palatal-derived cells to form adherent clonogenic cell clusters of fibroblast-like morphology, similar to those recorded for different mesenchymal stem-cell populations, was shown by the formation of about 170-single colonies, generated from 10^4 single cells cultured at low density. These colony-forming cell populations were termed progenitor-like cells.

The progenitor-like cell morphology and growth on the substrate of Herculite® XRV and Dyract® Extra were investigated by scanning electron microscopy (Fig. 1, Fig. 2).

SEM images revealed that the progenitor-like cells grew well onto the tested materials (Fig 1a, Fig. 2a). The texture of Dyract® Extra substrate was rather uniform and smooth, in comparison with the rougher surface with irregular particles of Herculite®XRV substrate (Fig 1b, Fig 2b). However, the density of the progenitor-like cells seem to be higher on Dyract® Extra specimens than on Herculite® XRV specimens, probably in relation with the relatively higher filler amount. Moreover, the dimension of the cells grown on Dyract® Extra substrate seem higher than those grown on Herculite® XRV (average 310 μm *vs* average 170 μm).

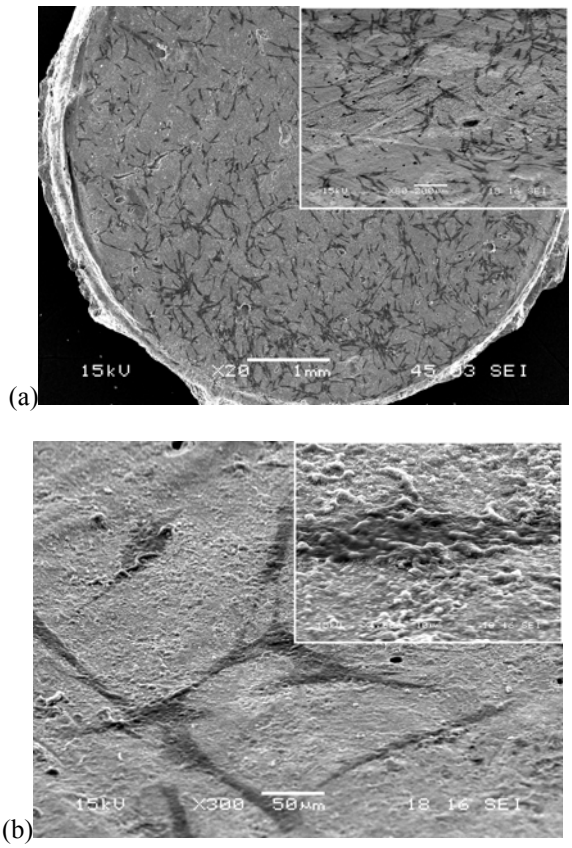

Fig. 1. Cell growth and morphology on Herculite® XRV specimens: (a) Cell density (20 ×magnification) and cell morphology (inset picture, 80 × magnification), (b) Cell morphology (300×magnification) and substrate structure (inset picture, 750 × magnification).

The resulting cells presented an extremely elongated-shaped morphology and long cytoplasmatic prolongations originating from their membrane. The prolongations showed numerous attachment areas to the substrate, for both materials (Fig.1b and 2b). This morphology could indicate an improved adherence of the cells to the substrate.

From a biological point of view, it was noticed that the palatal isolated cells attached after isolation on culture plates and did not changed their phenotype after multiple passages conserving in the same time their clonogenic capacity. Moreover, during long term cultivation, no signs of culture degeneration/senescence or spontaneous differentiation were observed. The parameters resulted from the statistical analysis of the number of cells grown on six substrates prepared from each of the two types of restorative materials are revealed in Table 3. The mean number of the progenitor-like cells is considerably higher on Dyract® Extra specimens than on Herculite® XRV substrates, but the p-value of 0.29 indicates that this result is not significant at any acceptable level, due to the important intra-group variation.

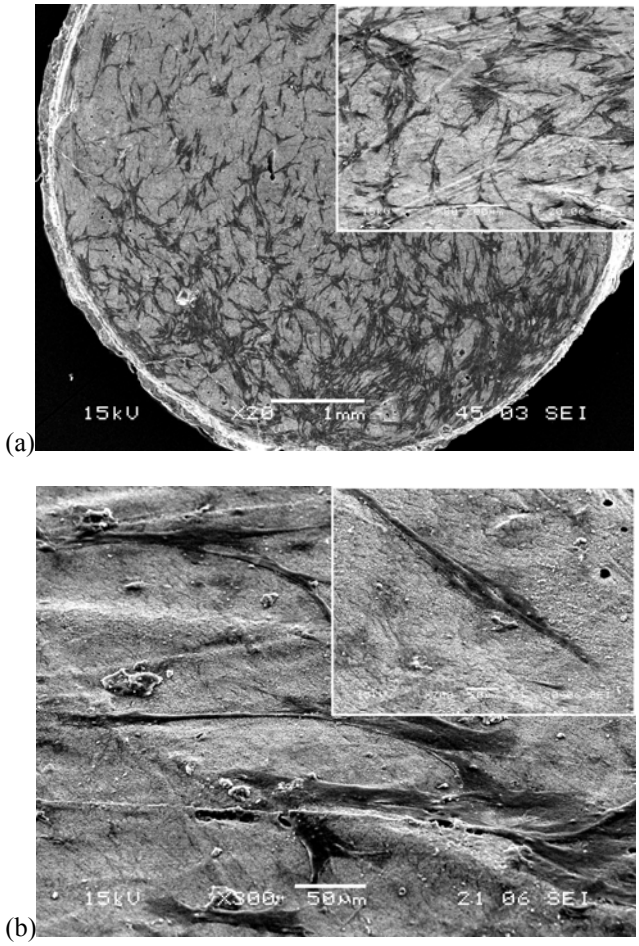

Fig. 2.Cell growth and morphology on Dyract® Extra specimens: (a) Cell density (20 × magnification) and cell morphology (inset picture, 80 × magnification), (b) Cell morphology (300 × magnification) and substrate structure (inset picture, 700 × magnification)

Table 3. Statistical analysis parameters determined for the number of cells attached on the examined disc: mean value (Mean), standard deviation (SD), standard error (SE), 95 % confidence interval (CI), minimum (Min), maximum (Max) and significance level (p)

Restorative material	N	Mean	SD	SE	CI		Min	Max	p
Dyract® Extra	6	1307.00	940.76	384.06	319.73	2294.27	0	2521	0.29
Herculite® XRV	6	814.50	506.27	206.69	283.20	1345.80	214	1506	

4. Discussions

This research investigated whether the two studied restorative materials have a potentially toxic effect on palatal progenitor-like cells. In the same time, the behaviour of the isolated cells when placed on the surface of composite resin and compomer disc substrates was monitored.

Both of the restorative materials used as substrate for cell growth were obtained by light curing from methacrylate ester monomers, mixed with glass- and silica-based inorganic fillers. The compomer Dyract® Extra contain additional "ion-leachable glass" powder as found in glass-ionomer materials and special monomers with characteristic structure [19].

The completeness of polymerization of these materials is a major concern, because due to steric reasons the polymerization process fails to incorporate all available monomer molecules into polymer chains. The unreacted portion may be leached out of the material in the oral environment, exerting cytotoxic effects. The less the resin composite is cured the larger quantities of unreacted components are eluted [20]. Increased irradiation periods succeeded in reducing the toxicity of the respective resin composite specimens [21]. In the present study, the material discs were realized in standardized conditions in order to increase the polymerization rates of the materials, as well as to simulate the clinical conditions. The following protocol was respected: curing under foil protection for diminishing the inhibitory effect of the oxygen [22, 23], the use of light shades and the use of 40 s curing time [5]. The light curing from one side applied in this study is a more close simulation of the oral cavity conditions. The discs were not finished because of the difficulty to perform this step intrasurgically.

Even if these polymerization conditions are fulfilled, a tight contact of the composite-resin-based substrates with progenitor-like cells might have a cytotoxic effect because both of the investigated materials contain TEGDMA in their organic matrix as a diluent added to decrease the viscosity and to increase their workability. TEGDMA is the main component released from cured dental composites [24, 25], having a cytotoxic effect on fibroblasts [26, 27], an increased genotoxicity [28] and a deleterious effect on reparative processes [29]. The elution of unreacted monomers contained in resin-based materials could be a main component of the adverse effects on progenitor cells [29, 30]. On the other hand, the saliva may dilute the uncured components from the freshly placed restorations [5] diminishing their toxic effect. But when the CTG is placed intrasurgically immediately over the fresh cured restoration, the "rinsing" effect of the saliva does not take place anymore.

As determined by SEM assessments, resin composite and compomer materials seemed to have no cytotoxic effect on palatal progenitor-like cells since the cells grew well on both restorative materials. Moreover, the SEM analysis had shown that palatal progenitor-like cells had a morphology consistent with that of attached cells, which means that the two restorative materials would not impede the development of a new attachment apparatus on previously exposed root surfaces treated with CTG associated with CAF. These results are opposed to recent findings revealing the cytotoxic effect of resin-based materials on stem cells [31, 32].

Over the entire cultivation period, the palatal isolated cells remained undifferentiated and did not change the phenotype, conserving in the same time the clonogenic capacity, which sustained their undifferentiated character. Further immuno-phenotypic characterization is needed in order to specifically define the obtained cells or to demonstrate their stem-like affiliation. The full characterization of the isolated palatal cells will be the subject of another report. So, the cells in the present study were named progenitor-like cells until more certain results better characterize their denomination.

When covering gingival recessions with CTG associated with CAF, the exact nature of the attachment obtained at the grafted tissue-root interface is not well elucidated [33-35]. The clinical situation is furthermore complicated by the concomitant presence of cervical lesions associated with gingival recessions, which demands parallel restorative and periodontal surgical approaches in order to restore the dental and periodontal loss. As other adipose tissues in the body the palatal CTG may contain progenitor-like cells and in our opinion, the transfer of these palatal progenitor-like cells onto the root surfaces during root coverage surgeries may favour the regeneration of the periodontal lost tissues.

Using progenitor cells is actually one of the more promising tissue engineering techniques that may be employed to reconstruct periodontal lost tissues [36]. The use of strict surgical root coverage techniques and restorative protocols may increase the chance to develop the healing processes in which the palatal progenitor-like cells participate.

Study limitation. In this study, the small size of the samples jeopardized the obtaining of firm conclusions. On the other hand, the very complex clinical and laboratory protocols made the development of the research difficult. These preliminary results are the beginning of a set of experiments on this theme.

5. Conclusions

The resin composite (Herculite® XRV) and compomer (Dyract® Extra) materials investigated in this study had no cytotoxic effect on isolated palatal progenitor-like cells. Both materials stimulated the cells growth and development and no statistical differences were recorded. Connective tissue graft harvested from the palate contained progenitor-like cells, which were isolated and expanded *ex vivo,* resulting cells with undifferentiated characteristics. Palatal tissues may represent an alternative and easily accessible source of specific progenitor-like cells which are compatible with patient-specific tissues and may be used to sustain the regeneration of the periodontal tissues but also of the other injured tissues of the body of mesenchymal and nonmesenchymal origin.

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