Cytotoxicity of Experimental Resin Composites on Mesenchymal Stem Cells Isolated from Two Oral Sources

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Abstract: Resin composite materials that are used to restore tooth cervical lesions associated with gingival recessions can hamper healing after root coverage surgeries. This study evaluates the in vitro cytotoxic effect of five resin composites (two commercial and three experimental) on oral mesenchymal stem cells (MSCs) and the persistence of stemness properties in high passage MSCs. Sorption and solubility tests were made for all materials. MSCs were isolated from re-entry palatal and periodontal granulation tissues and were characterized and cultured on composite discs. Cytotoxicity of the materials was evaluated by the Alamar Blue viability test, by Paul Karl Horan (PKH) labeling, and by immunocytochemical staining for actin. Water and saliva sorption and solubility data revealed that two of the experimental materials behaved comparable with the marketed resin composites. The Alamar Blue viability test shows that both cell lines grew well on composite discs that seemed to induce no apparent toxic effects. No signs of disruption of cytoskeleton organization was seen. Experimental resin composites can be recommended for further investigation for obtaining approval for use. The standard minimal criteria were fulfilled for high passage MSCs. Palatal tissue regains its regenerative properties in terms of MSC presence in the re-entry area after 6 months of healing.

Key words: resin composite, cytotoxicity, leakage, stem cells

INTRODUCTION

Resin composite materials are frequently used to restore tooth loss located in the cervical part of the tooth [root-crown cervical lesions (RCCL)] associated with gingival recessions and they can contact the surrounding gingival tissues when a combined restorative surgical treatment of these lesions is required. In this particular clinical situation, resin composites are used just before root coverage surgeries to restore the coronal part of tooth loss. The remaining lesion located on the root is then surgically covered, frequently with a palatal connective tissue graft (CTG) plus a coronally repositioned flap (CAF) because of positive clinical outcomes associated with this approach (Tonetti & Jepsen, 2014; Zuhr et al., 2014) and possible intrinsic regenerative potential provided by the presence of mesenchymal stem cells (MSCs) in the transplanted tissue (Roman et al., 2012, 2013; Páll et al., 2015).

Composite restorative materials represent one of the many successes of modern biomaterial research, as they replace tissues in both appearance and function. However, some drawbacks are still associated with their use such as polymerization shrinkage and leakage effect. As with other dental materials used in the oral cavity, resin composites are not inert and can have hazardous effects on gingival and other local cells due to eluted components (Geurtsen, 2000; Krifka et al., 2013; Salehi et al., 2015). The cytotoxicity of dental composite materials has been attributed to the release of residual monomers as a result of incomplete polymerization reaction, or to the by-products of resin degradation processes (Goldberg, 2008). Well-cured resin composites are less cytotoxic than those with a lower degree of conversion. The majority of unreacted monomers leached from dental resin-based composites are extracted within 1 week (Caughman et al., 1991; Salehi et al., 2015), thus explaining...
the negative effect of composites on the viability of undifferentiated pulp cells in culture diminished after 7 days (Ferracane, 1994). Dental composites themselves are unlikely to be a chronic source of products inducing cytotoxic effects. Monomers released from dental composites can induce local biological effects. Composites have equivalent potency in terms of reducing the viability of undifferentiated pulp cells in culture (Ferracane, 1994). Resin monomers like triethylene glycol dimethacrylate (TEGDMA) or hydroxyethyl methacrylate-induced cytotoxicity via apoptosis in pulp cells and gingival cells and had genotoxic or mutagenic effects. Moreover, monomers inhibited lipopolysaccharide-induced cytokine production in macrophages (Krifka et al., 2013) and induced inflammation when applied in direct contact with dental pulp tissue (Murray et al., 2002; Modena et al., 2009). Low levels of TEGDMA and of chemically related molecules inhibited or delayed odontogenic differentiation and dentin mineralization processes with serious consequences in physiological dentin repair or developmental processes of permanent teeth (About et al., 2002; Bakopoulou et al., 2011, 2012).

The healing of root surgical coverage may be hampered by eluted components from the freshly placed resin composites restoring RCCL during simultaneous reconstructive surgical approaches. As response to resin components differs among cell types (Costa et al., 1999; Geurtsen, 2000; Taira et al., 2000) it seems important to thoroughly investigate the influence of resinous materials using cells isolated from different sources.

Composite resins are composed of three distinct phases, each with its own role in dictating the properties of materials: the polymerizable resin, the filler, and the filler–resin interface. Each component of resin composites represents an opportunity for improvements in the overall material and is the target of continuous research. Having in view the drawbacks associated with resin composite materials, in the present study three experimental composite resins were designed by a research team in the ICCRR Laboratory. The present study evaluates the in vitro cytotoxic effects of five resin composites (two commercial products and three experimental materials), in relation with some of their properties, on MSCs isolated from two previously described oral sources (Roman et al., 2013; Páll et al., 2015). It was presumed that resin composites have a harmful effect on these cells, thus reducing the regenerative potential of CTGs during surgical root coverage associated with restorative therapy. The study also investigated the persistence of stemness properties in high passage MSCs used for the experiment by analyzing surface antigen expression and their differentiation potential.

**Materials and Methods**

**Preparation of Experimental Composite Materials**

Three experimental composite resins were prepared in the laboratories of RaIaU University in Chemistry (ICCRR, Cluj-Napoca, Romania). One experimental material named PM was a mix of 2,2-bis[3-(2’-hydroxy-3’-methacryloxypropoxy)phenyl]propane (Bis-GMA) (synthesized in ICCRR Laboratory), urethane dimethacrylate (UDMA) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), polycaprolactone diol (PCL) (Sigma-Aldrich Chemie GmbH), and TEGDMA (Sigma-Aldrich Chemie GmbH), in which a complex filler (78 wt%) containing hydroxylapatite zirconium (HA-Zr) particles of 0.01–0.35 μm and 5–8 nm (synthesized in an ICCRR Laboratory), silica (Degussa, Hanau-Wolfgang, Germany), and barium oxide-based glass filler of 0.1–0.35 μm (synthesized in ICCRR Laboratory) was incorporated. The second experimental material named P14M was formed by a Bis-GMA, UDMA, and TEGDMA-based resin (79 wt% filled with silica particles and barium oxide and barium fluoride glass fillers of 0.01–0.035 μm and 2–6 nm (synthesized in the ICCRR Laboratory). The third experimental material named P2S had the same organic composition as the second material, but a different filler composition (80 wt%) based on HA-Zr, quartz (Romcuart SRL, Uricani, Romania), and silica.

The complex filler was obtained as a mass through the conventional melting method in the ICCRR Laboratory. Surface treatment of all fillers was made with silane coupling agent, γ-methacryloxypropyltrimethoxysilane (A174) (Sigma-Aldrich Chemie GmbH), which acts as a sort of intermediate that bonds organic materials to inorganic ones. All experimental composite resins contain camphorquinone (0.5% relative to the liquid mixture)/amine (1%) as initiator/activator system.

Using a magnetic stirrer, the monomers and photo-activators were mixed (in the absence of blue light) until a uniform consistency was achieved. The experimental composites PM, P14M, and P2S were prepared as mono pastes by dispersing the silanized bioactive inorganic fillers in the organic phase.

Control dental composites were represented by two well-known marketed products recommended for the restoration of RCCL [G-anial Anterior® (Ge), GC EUROPE N.V., Leuven, Belgium; Enamel plus HRI® (En), Micerium S.p.A, Avegno, GE, Italy]. Ge is a UDMA-based resin containing dimethacrylate co-monomers, filled with pre-polymerized silica, strontium, and lanthanide fluoride containing fillers of 16–17 μm, silica >100 nm, and fumed silica <100 nm (manufacturer brochure).

The composition of En as provided by the manufacturer is a complex monomer mixture formed by diurethane dimethacrylate, Bis-GMA, 1,4-butandiol dimethacrylate and a filler (75 wt%) formed by glass particles with a mean size of 0.7 μm and highly dispersed silicon dioxide with a mean particle size of 0.04 μm (manufacturer brochure).

**Measurement of Water Sorption and Solubility of Resin Composites**

These physical tests, water sorption and solubility, were made in order to observe the stability of the experimental materials and to compare it with that of commercially available ones. Degradation of the organic matrix of resin
composite materials greatly depends on the conversion rate of C = C bonds, as an increased polymerization rate and network density reduce solubility and water diffusion through the matrix.

Disc-shaped composite specimens with standard dimensions (15 mm in diameter, 1 mm in thickness, \( n = 8 \) per composite type) were polymerized in accordance with the manufacturer’s instructions for 20 s from both sides in a teflon mold. A polyester film was placed onto the surface of the mold and pressed with a plate glass in order to remove the excess material. Light curing was carried out with a Woodpecker Curing Light LED-B lamp (Guilin Woodpecker Medical Instrument Co. Ltd., Guilin, China).

Water sorption and solubility of composite materials were measured according to ISO 4049/2000 and ADA Specification No. 27 (ADA Division of Science & ADA Council on Scientific Affairs, 2003). Before the initial weighing, all samples were placed in a desiccator (DURAN; DURAN Produkts GmbH & Co. KG, Mainz, Germany) at 37°C for 24 h, stored in another desiccator at 23°C and weighed (Partner 220 mg; Partner Corporation, Bucharest, Romania) until a constant mass was obtained \( (m_1) \).

Four specimens of each material were grouped into group A and were immersed in glass tubes (25 mL, SIMAX; SIMAX, Sázava Czech Republic) with 10 mL distilled water, and the other four specimens representing group B, in glass tubes with artificial saliva—prepared using AFNOR S90-701 formula (Grosogolat et al., 1999) (ICCR Laboratory). The specimens were maintained at 37°C for 3 months. Specimens were dried to remove excess water and weighed daily for the first 7 days and then on days 14, 21, 60, and 90 \( (m_2) \). After 3 months of immersion, the samples were kept in a desiccator until getting a constant weight \( (m_3) \). The values of water sorption and solubility were calculated for each disc as follows: sorption \( = m_2 - m_3/V \) and solubility \( = m_1 - m_3/V \), where \( m_1 \), \( m_2 \) are the masses of the specimen before and after immersion in water, respectively and \( V \) the specimen’s volume.

**Preparation of the Dental Material Substrate for Cell Growth**

The five above-described resin-based restorative dental materials were used to manufacture the specimens for cell growth. Disc-shaped specimens (6 mm in diameter; 1 mm in thickness; \( n = 19 \) per composite type) of each of the five tested materials were fabricated by placing the material in a mold and covering it with a plastic foil. The specimens were light cured for 20 s from both sides using a light activation unit (Demi LED Curing Unit; Kerr Corporation, Orange, CA, USA). All discs were sterilized using ethylene oxide gas for 2 h at 56°C followed by degassing for 12 h.

**Sample Collection and Cells Isolation**

The study was performed according to a protocol approved by the Ethical Board of the Iuliu Hațieganu University (359/13.10.2014). The samples used to isolate MSCs had been collected from patients undergoing periodontal surgical treatments in the Periodontology Department of Iuliu Hațieganu University of Medicine and Pharmacy, Cluj-Napoca. In obtaining informed consent and conducting the research, the study adhered to the principles outlined in the Declaration of Helsinki on experimentation involving human subjects. The experiments using stem cells were done according to EU and national laws.

MSCs were isolated from two oral sources. One newly identified MSC source was represented by a connective tissue collected during a re-entry surgery from the palatal premolar region that was already used as a donor site 6 months before; the tissue was harvested using the single incision technique (Hürzeler & Weng, 1999). The patient was a 24-year-old man treated with CAF and a CTG in order to cover a Miller class I gingival recession (Miller, 1985). The other sample was collected from the periodontal granulation tissue during a pocket reduction surgery, as previously described (Päll et al., 2015), from a 38-year-old female patient diagnosed with chronic periodontitis.

The samples were collected in sterile vials containing αMinimum Essential Medium (αMEM) supplemented with 1% antibiotic-antimycotic (100×) (Gibco Life Technologies, Paisley, UK) and were immediately transported to the laboratory.

Cells were isolated using the explant method following a protocol detailed elsewhere (Päll et al., 2015). In brief, the pieces were washed with sterile phosphate saline supplemented with 1% antibiotic-antimycotic minced into 1–2 mm pieces and added to T25 flasks pretreated with fetal bovine serum (FBS) for 20 min. The propagation medium was represented by Dulbecco’s Modified Eagle’s Medium/Nutrient F-12 Ham (DMEM/F12; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal calf serum (EuroClone, Pero, MI, Italy), 2 mM glutamine, 1% non-essential amino acids (NEAA) (Sigma-Aldrich), 1 mM sodium pyruvate, and 55 mM β-mercaptoethanol. The cultures were maintained in a humidified atmosphere with 5% CO2 at 37°C. After 5 days of culture, the medium was replaced and tissue pieces were removed. Both palatal re-entry-derived cells (reMSCs) and granulation periodontal tissue-derived cells (gtMSCs) were grown until confluence (70–80%) and then sub-cultured (1:2).

**Cell Characterization**

*Immunophenotype Analysis Using Flow Cytometry*

For this experiment cells at the 14th passage of culture were used. Some surface antigens of MSCs isolated from both sources were analyzed using a standard protocol (Roman et al., 2013; Päll et al., 2015). In brief, the cells were immunolabeled for 20 min at 4°C with monoclonal antibodies against CD105, CD73, CD90, CD44, CD49f, CD34/45, Human Leukocyte Antigen - antigen D Related (HLA-DR), and CD79a (BD Biosciences, San Jose, CA, USA) following the manufacturer’s recommendations. The prepared samples were analyzed using a FACS Canto II flow cytometer and DIVA software (BD Biosciences). Data from 10,000 events were recorded.
Trilineage Mesenchymal Differentiation

Chondrogenic differentiation. gtMSCs and reMSCs were trypsinized at passage 14 and seeded into 24-well plates at a cell density of 1 x 10^5 cells/well in 1.5 mL complete standard stem cell medium. When cultures arrived at confluence, the medium was changed to a serum-free chondrogenic one consisting of DMEM high glucose/F12 medium, 1% antibiotics, 2 mM l-glutamine, 1% NEAA, 1 mM sodium pyruvate, 1% insulin, transferrin, selenium 100 μg/mL (Invitrogen, Carlsbad, CA, USA), 10 mM dexamethasone, 50 ng/mL ascorbic acid, 10 ng/mL transforming growth factor-β3 (all reagents from Sigma-Aldrich Chemie GmbH). The medium was changed every 3 days. After 10 days of culture in chondrogenic medium, cells were fixed with 4% paraformaldehyde for immunocytostaining and Alizarin Blue staining.

Osteogenic differentiation. MSCs were plated as described above. Osteogenic differentiation medium consisted of DMEM high glucose/F12 (ratio 1:1) supplemented with 10% FBS, 1% antibodies, 1% NEAA, 2 mM l-glutamine, 10 μM dexamethasone, 10 mM β-glycerol phosphate, 50 μg/mL ascorbic acid, 10 ng/mL bone morphogenetic protein-2 (BMP-2). The concentration of BMP-2 was reduced progressively at each change of medium every 3 days (10 ng/mL, 5 ng/mL, 0), with complete removal of BMP-2 after 8 days, to allow initiation of the mineralization process. After 10 days of culture in osteogenic medium, cells were fixed with 4% paraformaldehyde for immunocytostaining and Alizarin Red staining.

Neuronal differentiation. For differentiation into neuronal progenitors we used a two-stage differentiation protocol adapted from Tamagawa et al. (2008). MSCs were seeded in 24-well plates as mentioned before. After 24 h, when cells adhered and began to proliferate, the medium was changed with the first neuronal induction medium containing two growth factors, epidermal growth factor and basic fibroblast growth factor (Sigma-Aldrich), and two specific commercial neuronal supplements (N2 and B27 from Invitrogen). For supporting a better cell viability, in the second phase after 48 h a modified medium was used consisting of a basal neuronal medium (DMEM high glucose/F12 supplemented with 1% N2 and 2% B27) associated with 10 mM nicotinamide (Sigma-Aldrich), 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich), and 10 ng/mL nerve growth factor (Sigma-Aldrich). This medium was changed every 3 days. After 10 days of culture in neuronal medium, cells were fixed with 4% paraformaldehyde for immunocytostaining.

Immunocytochemical staining. Cells were fixed with 4% paraformaldehyde and cell membrane permeabilization was performed with 0.1% Triton X-100 solution. Blocking of non-specific binding of antibodies was done with 10% bovine serum albumin for 15 min. Samples were incubated overnight with the primary antibodies. The primary antibodies used were anti-collagen 2A (Santa Cruz Biotechnology, Dallas, Texas, USA) (dilution of 1:50) for chondrogenic differentiation; glial fibrillary acidic protein (GFAP) [rabbit anti-human IgG (Sigma) diluted at 1:200 ratio], p-nestin (Santa Cruz Biotechnology) (dilution of 1:50), and anti-neurofilaments (NF) [rabbit anti-human IgG (Sigma) diluted at 1:200 ratio] for neuronal differentiation; anti-osteopontin and alkaline phosphatase (ALP; Santa Cruz Biotechnology) (dilution of 1:50) for osteogenic differentiation. At the end of the incubation period, the samples were incubated for another 45 min with secondary antibodies conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (Santa Cruz Biotechnology) at a dilution corresponding to primary antibodies. Each step of staining was followed by three washes with phosphate buffered saline (PBS). Finally, Hoechst solution (Sigma) was added for nuclear staining. Samples were then analyzed by fluorescence with a Zeiss Axiovert D1 inverted microscope (Zeiss GmbH, Jena, Germany) using filters at 488, 546, and 346 nm and the images were captured with a MRM AxioCam CCD camera (Carl Zeiss Micro Imaging GmbH, Jena, Deutschland).

Specific cytochemical staining

Alizarin Blue staining. Cells fixed with 4% paraformaldehyde were washed 3× with PBS, and the samples were incubated for 30 min with Alizarin Blue (1% Alizarin Blue 8 GX in 3% glacial acetic acid solution adjusted to pH 2.5), followed by extensive washing with PBS. Samples were examined by optical microscopy. Mucopolysaccharides were colored blue, nuclei pink, and cytoplasm pale pink.

Alizarin Red staining. Alizarin Red was used to determine the presence of calcium deposition in the extracellular matrix of bone. MSCs fixed with 4% paraformaldehyde were washed 5× with deionized water and incubated for 10 min at room temperature with 2% Alizarin Red solution (pH 4.1). After intensive washing of samples with PBS to remove the excess staining solution, cells were observed using optical microscopy.

Cell Culture on Discs and Biocompatibility Tests

reMSCs and gtMSCs at passage 14 were used. When the cell monolayers were subconfluent, the cells were detached from culture flasks by trypsinization (trypsin + ethylenediaminetetraacetic acid (EDTA) 1:4), counted by a hemocytometer (Isolab Laborgeräte GmbH, Wertheim, Germany) and cell suspensions were adjusted.

Alamar Blue Viability Test

Cell adhesion and viability were investigated using the Alamar Blue test. Cells were seeded onto the surface of composite discs placed in Nunc 24-well plates (Thermo Fisher Scientific, Waltham, MA, USA) at a concentration of 8 x 10^4 cells/well in 1.5 mL complete medium/well.

The Alamar Blue test, which is used mainly as a test of cell viability, is based on the resazurin property (7-hydroxy-3H-phenoxazin-3-one-10-oxide, a blue dye, weakly...
fluorescent) to be converted to resorufin (red fluorescence) in metabolically active cells, through a reduction mechanism (Van Tonder et al., 2015). Alamar Blue is a nontoxic dye that allows study of the cell response without cell sacrifice during the test procedure (Vega-Avila & Pugsley, 2011). In our experiment, cell adhesion capacity was determined after 21 h of seeding of MSCs onto composite discs. After the completion of cultivation time, the medium was discarded and 900 µL medium + 100 µL Alamar Blue (Thermo Fisher Scientific) was added to each well. Each determination was performed in triplicate, and controls consisted of cells cultivated on plastic surfaces. The plates were incubated for 1 h at 37°C in the dark. Then, medium aliquots were transferred to another 96-well plate and fluorescence intensity was measured using a BioTek Synergy 2 plate reader (excitation 540 nm, emission 620 nm; BioTek, Winooski, VT, USA). Cell viability and proliferation were evaluated in the same way after 3 and 7 days of cultivation onto substrate surfaces.

**Cell Membrane Fluorescent Labeling**

MSCs were fluorescently marked using a lipophilic dye PKH26 Red Fluorescent Cell Linker Kits (Sigma-Aldrich). This staining ensures visualization of marked live cells in cultures for a long period. The staining protocol consisted of two washes with PBS of $1 \times 10^6$ cells by centrifugation at 1,000 rpm for 5 min. The cell pellet was resuspended in 1 mL Diluent C and 1 mL of Dye Solution (containing 4 µL of PKH26/mL), followed by gentle pipetting for a rapid and homogenous mixing of cells with the dye. The staining was stopped after 5 min by adding 2 mL of complete medium containing 10% FBS and cells were centrifuged for 10 min at 1,000 rpm. Another two washing steps were performed with 10 mL of complete medium, and then the cells were counted, resuspended in complete medium, and seeded in 24-well plates on the surface of composite discs. After 21 h adherent cells were visualized by fluorescence microscopy with a Zeiss Axiosvert D1 microscope, using filters at 546 nm. Image capture of PKH26-stained MSCs was also performed after 21 h, 3, and 7 days of cultivation onto composite discs.

**Immunocytochemical Staining for Actin**

This test was done to observe organization of the cell cytoskeleton as a further insight in the materials’ biocompatibility. After 3 days of cultivation of reMSCs and gtMSCs on composite discs in 24-well plates, a fixation and permeabilization protocol was performed: cells were washed three times with PBS and fixed with 4% paraformaldehyde solution in PBS followed by a permeabilization step with 0.1% Triton X-100 in PBS for 20 min at room temperature. Each step was followed by three washes with PBS. For visualization of filamentous actin, cells were stained with phalloidin conjugated with TRITC (Sigma-Aldrich) at a dilution of 1:50 in PBS. The nuclei were stained with an antifade medium containing 4,6-diamidino-2-phenylindole (Santa Cruz Biotechnology). Samples were examined using a Zeiss Axiosvert microscope by reversed-phase fluorescence using 546 and 340/360 nm filters.

**Statistical Analysis**

Two-way mixed analysis of variance (ANOVA) was used in order to compare the relative efficiency (sorption and solubility in water and saliva, and Alamar Blue tests) of different dental materials over time. Our design included a within-subjects factor, Time (with three levels of analysis—7, 14, and 90 days for sorption and solubility, and 21, 72 h, and 7 days for Alamar Blue test) and a between-subjects factor, Material type (with five levels of analysis—Ge, En, P2S, P14M, and PM). Pairwise differences between material types were analyzed using one-way ANOVA, followed by Bonferroni’s post hoc comparison. All statistical tests were considered to be significant at $\alpha = 0.05$. Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad, La Jolla, CA, USA).

**RESULTS**

**Sorption and Solubility of Resin Composites**

Descriptive statistics, mean (SD) of measures of sorption and solubility in water and saliva of different material types at three times are given in Supplementary Table 1.

Statistical analysis of the data for sorption in water revealed a significant two-way interaction between Time and Material types ($p = 0.001$). On day 7, the effect of the between-subject factor Material type was significant ($p = 0.001$), and all the post hoc pairwise comparisons between material types were found to be significant ($p = 0.001$), except P14M versus Ge ($0.055$). Statistical analysis performed for data collected on day 14 revealed a significant main effect for Material type ($p = 0.001$), all post hoc comparisons were found to be significant excepting P2S versus Ge ($p = 0.161$). The same was found to be true for data collected on day 90, a significant main effect of Material type and a nonsignificant pairwise comparison for P2S versus Ge ($p = 0.079$), all other comparisons being found to be significant ($p = 0.001$) (Fig. 1a).

Statistical results of measurements of solubility in water showed a significant two-way interaction between Time and Material types ($p = 0.001$). Analysis of data collected on the 1st day of the study indicated a significant main effect for Material type ($p = 0.001$). Post hoc comparisons on day 7 were significant for all pairs of materials, except P14M versus Ge ($p = 0.981$), P2S versus Ge ($p = 0.976$), and P2S versus P14M ($p = 0.977$). On day 14, the main effect of Material type was significant ($p = 0.001$), the only insignificant pairwise
comparison was P2S versus Ge (\(p = 0.539\)). On day 90, there were two insignificant pairwise comparisons, namely P2S versus Ge (\(p = 0.565\)) and PM versus P14M (\(p = 0.108\)), all other comparisons being significant (\(p = 0.001\)) (Fig. 1b).

Sorption in saliva measures showed a significant two-way interaction between Time and Material types (\(p = 0.001\)), meaning that differences between material sorption in saliva do not reflect the same pattern in time. On day 7, we found a significant main effect for Material type, but this main effect reflected only differences between PM versus other Materials (P2S, P14M, Ge, and En), all other pairwise comparisons were insignificant. The same pattern of results was identified on days 14 and 90, only comparisons of other materials with PM were statistically significant (\(p = 0.001\)) (Fig. 1c).

Solubility measurements showed a nonadditive relation between Material and Time types (\(p = 0.001\)), the pattern of differences being different in time. Post hoc analysis showed very similar results to those found for sorption, only pairwise comparisons with PM were found to be statistically significant (Fig. 1d).

**Characterization of MSCs**

The standard minimal criteria suggested by the International Society for Cellular Therapy such as adherence to plastic, specific surface antigen makeup, and multipotent differentiation potential were fulfilled in the present experiment.

**Immunophenotype Analysis**

The expression of markers associated with reMSCs and gtMSCs was evaluated by immunophenotyping and results of the analyses revealed a similar expression pattern, but the level of antigen expression indicated some differences. In agreement with minimal criteria requirements, both cell lines showed positivity for CD105, CD73, and CD90 and a negative expression for CD34, CD45, HLA-DR, and CD79a. The cells were also positive for two other surface markers, CD44 and CD49f (Fig. 2; Supplementary Table 2).

**Supplementary Table 2**

Supplementary Table 2 can be found online. Please visit journals.cambridge.org/jid_MAM.
Figure 2. Flow cytometry analysis of (a) re-entry-derived mesenchymal stem cells (reMSCs) and (b) granulation periodontal tissue-derived mesenchymal stem cells (gtMSCs) at passage 14. PE, phycoerythrin; FSC-A, Forward Scatter A.
Trilineage Mesenchymal Differentiation

The differentiation capacity of the isolated cells was evaluated by culturing the cells in chondrogenic, osteogenic, and neurogenic induction media. Exposure of reMSCs and gtMSCs to chondrogenic differentiation medium induced changes in cell morphology during 10 days of the experiment, from a characteristic fibroblast-like appearance of stem cells to a rounded shape and multicellular cluster formation (Figs. 3a, 3b). These multicellular clusters included rich mucopolysaccharide deposits as observed in Alcian Blue staining. reMSCs showed a more intense coloration for Alcian Blue (Figs. 3c, 3d).

Immunocytostaining showed a weak positive expression of type II collagen after 10 days of chondrogenic differentiation for both MSC lines (Figs. 3e, 3f).

In the first stage of neuronal differentiation, under the influence of growth factors, the cells maintained their proliferation rate, but showed important changes in morphology, with a rounded shape and a characteristic cell body retraction. In the second stage, cell numbers decreased and cell morphology changed with the appearance of multiple elongated dendritic spines and establishment of intercellular connections (Figs. 4a, 4b). Moreover, some cells lost their adhesion capacity and formed multicellular aggregates, presumably representing neurospheres (Fig. 4a).

After 10 days of cultivation in selective neuronal medium, both MSC-derived cell lines expressed in different degrees neuronal antigens (GFAP, nestin, and NF), suggesting their differentiation into neuronal progenitors, with a higher positivity for NF (Figs. 4c–4h).

Induction of osteogenic differentiation of MSCs was associated with an increased proliferation rate (possibly under the influence of BMP-2) and morphological changes (Figs. 5a, 5b). Calcium deposits were visualized by Alizarin Red staining after 10 days of cultivation, with increased amounts localized in ossification nodules (Figs. 5c, 5d).

Figure 3. Mesenchymal stem cells after 10 days of chondrogenic differentiation. a,b: Morphological aspects in phase contrast microscopy (Obx10); (c,d) specific Alcian Blue staining (arrows) (Obx20); (e,f) immunocytochemical staining for collagen 2A (FITC—green) and Hoechst (nuclei—blue) (Obx63). (a, c, e) reMSC-derived cells; (b, d, f) gtMSC-derived cells.
Immunocytochemical staining for bone markers showed positivity for non-collagenic phosphoprotein osteopontin (OP) marked with FITC, which was more intensely expressed in ossification nodules derived from reMSCs in comparison with gtMSCs (Figs. 5e, 5f). A much weaker staining was observed for ALP (Figs. 5g, 5h).

**Biocompatibility Tests**

**Alamar Blue Viability Test**

In order to assess cell viability as a measure of the materials’ biocompatibility after seeding on composite discs, staining with Alamar Blue was performed and the results were evaluated at...
different time intervals. Descriptive statistics, fluorescence units/cell viability is given in Supplementary Table 3.

**Supplementary Table 3**
Supplementary Table 3 can be found online. Please visit journals.cambridge.org/jid_MAM.

Statistical analysis of the data representing fluorescence unit count revealed a significant two-way interaction between Time and Material types ($p = 0.001$). For reMSC data collected after 21 and 72 h the effect of Material type was not significant. For data collected on day 7, a significant main effect of Material type was found. Pairwise comparisons were

Figure 5. Mesenchymal stem cells after 10 days of osteogenic differentiation. a,b: Morphological aspects in phase contrast microscopy (Obx10); c,d) specific Alizarin Red staining of ossification nodules (arrows) (Obx40); e–h) immunocytochemical staining osteopontin (FITC—green) and alkaline phosphatase (FITC—green), and counterstained for nuclei with Hoechst (blue) (Obx63). a, c, e, g) reMSC-derived cells; b, d, f, h) gtMSC-derived cells.
found to be statistically significant for Ge versus P2S, Ge versus PM, and Ge versus P14M \((p < 0.05)\), all other comparisons were not significant (Fig. 6a).

Regarding statistical results of cell count for gtMSCs, a significant main effect of Material type and a significant pairwise comparison between Ge versus P2S \((p < 0.05)\) were found only for day 7. Global statistical tests and \textit{post hoc} comparisons for data collected at 21 and 72 h were not significant (Fig. 6b).

**Cell Membrane Fluorescent Labeling**

The PKH analysis showed presence of the cells on discs after 21 h for both cell lines. An obvious development during the entire observation period could be remarked for reMSCs; a more discrete presence of gtMSCs was recorded (Fig. 7).

**F-Actin Cytoskeleton Organization**

Overall, 3-day MSC cultures on discs showed, after F-actin cytoskeleton and nucleus staining, well-spread cells with an elongated morphology and a well-developed F-actin skeleton as well as a prominent nucleus. A scarce expression of reMSCs grown on PM discs was observed (Fig. 8).

**DISCUSSION**

This study investigates the influence of some biomaterials on the development of oral MSCs in response to the interfering effects of local and general factors. Three experimental resin composites were designed and characterized for their mechanical properties as well as for physical-chemical (water sorption and solubility) and surface characteristics. Besides conventional biocompatibility tests, the biological acceptability of these materials was monitored by observing their cytotoxic effect on MSCs in order to improve their composition and to further develop activities for their clinical implementation.

The restorative therapy of RCCL, just before performing surgical coverage of gingival recessions, complicates the clinical approach of this type of lesion (Santamaria et al., 2010; Zucchelli et al., 2011). These combined defects may have different prognosis regarding soft tissue coverage rate due to complex associated factors including the influence of the restorative materials contacting the surgical area, which may increase plaque accumulation or may interfere by eluted molecules.

Composite resins are considered materials which direct the course of dental therapy by interaction with living systems (Williams, 2009). Elaboration of dental composites in the present study had in view not only their use to esthetically restore the structural integrity of teeth affected by carious or non-carious lesions, but also their influence on environmental tissues for choosing the material providing the best properties.

Physical-chemical properties and clinical performance of composite restorative materials, as well as their biocompatibility, depend on adequate polymerization of resin monomers (Salehi et al., 2015). The availability of leachable molecules in restorations depends on the degree of monomer conversion and on the stability of polymer networking. If the polymerization conversion rate can be at some degree controlled by a strict clinical protocol thus decreasing monomer loss, the stability of the network influences the intrinsic property of the resin composite. In our study, the resinous phase composed of Bis-GMA, UDMA, and the diluting monomer TEGDMA for P2S and P14M materials and also of PCL for PM material. Filler composition was represented by a complex mixture of inorganic compounds of nano- and micro-dimensions for inducing good mechanical properties and reducing polymerization shrinkage, thermal expansion, and water sorption (Chen, 2010) as...
well as for providing optimal translucency, opalescence, radiopacity, and surface characteristics (Mitra et al., 2003; Ilie & Hickel, 2009). The fillers of experimental resins contained quartz, which is known to induce excellent esthetics and durability, but as it lacks radiopacity; barium glass and zirconium (HA-Zr) were added to provide radiopacity (Ronald et al., 2012). However, because of their hardness, composites containing quartz and zirconium fillers wear out opposing enamel significantly more than composites containing microfillers or barium glass (Albers, 2001).

The filler of P14M experimental material contained fluoridated particles—barium fluoride—in order to improve the safety of restorations in cervical areas because it was reported that dental composites might release fluoride ions (Wiegand et al., 2007) thus inducing a cariostatic effect (Duggal et al., 1991). Even an exposure to minimal amounts of fluoride with levels of 0.095–0.190 ppm fluoride in saliva can be sufficient for cariostasis (Zero et al., 1992).

Water sorption and solubility—two important parameters of resin composites—were investigated in the

**Figure 7.** Cell adhesion and proliferation on composite discs as revealed by PKH labeling of mesenchymal stem cells at different time points: (a, b) 21h; (c, d) 72h; (e, f) 7 days (Obx20). Ge, G-ænial<sup>®</sup>; En, Enamel plus HRi<sup>®</sup>; PM, P14M, P2S, experimental resin composites; reMSCs, re-entry-derived mesenchymal stem cells; gtMSCs, granulation periodontal tissue-derived mesenchymal stem cells.
present research. Water sorption may reduce mechanical properties and wear resistance (Sideridou et al., 2008), and water absorbed by the polymer matrix could cause hydrolysis of the organic component and filler–matrix debonding (Chang et al., 2012) thus influencing the longevity and the biocompatibility of the restorations (Hahnel et al., 2012). In our research, 7-day water sorption data revealed the best significant result for En, which was maintained over the entire observation period, followed by P14M and Ge that behaved similarly. At 14 and 90 days, no significant difference in sorption values was recorded for Ge and P2S, whereas P14M and PM were associated with the highest sorption parameters in comparison with the other materials. As for the water solubility test, again En had statistically the lowest solubility followed by Ge and P2S material, which behaved in the same manner over the entire experiment. The solubility for P14M was not statistically different from that of Ge and P2S at 7 days, but showed a worsening pattern at 14 and 90 days so that, at the final moment, it was not significantly different from the solubility value of the PM material. Sorption and solubility values in saliva showed an improved pattern for two experimental materials—PM and P2S—which was not significantly different from that of commercial resin composites.

Regarding the good outcomes in sorption and solubility tests associated with P2S material in comparison with the weakest ones associated with PM material, it can be assumed that this may be due to the presence of polycaprolactone in PM material as the sole major difference in the two experimental materials’ composition. Polycaprolactone may be degraded through a hydrolytic mechanism by oral microorganisms as well as by hydrolysis, thus inducing an instability of the network (Deng et al., 2010).

In the present experiment, no signs of cytotoxicity of resin composites on MSCs were highlighted. The Alamar Blue viability test revealed that both cell lines grew well on composite discs. No statistical differences were recorded to be associated with composite resins regarding MSC development except for reMSC after 7 days when Ge seemed more biologically attractive than all the experimental materials and for gtMSCs at 72 h, when P2S seemed to be more biocompatible than Ge. This behavior superposes with that recorded in water and solubility tests. This may not be attributed to the cytotoxic effects of Ge as reMSCs grew well in relation with this material, but rather to some impaired properties of MSCs isolated from granulation tissues in comparison with MSCs isolated from healthy sites (Alongi et al., 2010; Liao et al., 2011; Yazid et al., 2014; Páll et al., 2015).

Cytotoxicity of composite resin materials was indirectly evaluated upon the observation of the exposed cultures to composite discs by PKH cell labeling and F-actin cytoskeleton staining. As PKH cell labeling images revealed, development of MSCs on PM material seemed impaired in comparison with the other materials. No signs of disruption of cytoskeleton organization was seen (excepting for gtMSCs at 72 h, when P2S seemed to be more biocompatible than Ge). This behavior superposes with that recorded in water and solubility tests. This may not be attributed to the cytotoxic effects of Ge as reMSCs grew well in relation with this material, but rather to some impaired properties of MSCs isolated from granulation tissues in comparison with MSCs isolated from healthy sites (Alongi et al., 2010; Liao et al., 2011; Yazid et al., 2014; Páll et al., 2015).

Limitations of the present study include no cytotoxicity tests after storage of the discs in culture medium in order to analyze the leakage of residual monomers or analysis of the eluted filler particles in relation with MSCs.

The persistence of stemness properties in high passage MSCs used for this experiment was also demonstrated. Both reMSCs and gtMSCs were fully characterized at passage 4 (Roman et al., unpublished data; Pall et al., 2015). At passage 14, the putative MSCs were plastic adherent when maintained in standard culture conditions and exhibited an antigen makeup specific for MSCs (Roman et al., 2013;
Dominici et al., 2006; Latif et al., 2007; Orciani et al., 2010; Páll et al., 2015). At passage 14, both cell lines showed a more intense expression of the positive markers, recording >99.3%, in comparison with the markers expressed by MSCs at passage 4, as our previous works described (Roman et al., 2013; Páll et al., 2015). A dramatic increase in marker expression from passage 4 to passage 14 was recorded for gtMSC, possibly due to the fact that at the 4th passage MSCs maintained some heterogeneity. The present data addressed one of the shortcomings associated with a previous study (Páll et al., 2015) that did not analyze the antigenic makeup of MSCs at different passages. The present data demonstrated that not only did MSCs maintain their stemness potential during passages, but they became more homogenous in culture.

Our study demonstrated the capacity for trilineage mesenchymal differentiation for MSCs isolated from both oral sources. Chondrogenic differentiation was highlighted by a positive expression of type II collagen. The weak positive expression observed for both differentiated MSC cultures can be explained by the short time of cultivation in the present experiment, as the synthesis of higher amounts of collagen needs up to 21 days (Yoon et al., 2015).

After cultivating in selective neuronal medium, specific neuronal cell markers (GFAP, nestin, and NF) were highlighted. The intermediate class VI filament nestin is expressed by many types of dividing cells during development of the central nervous system, especially by neuroepithelial stem cells. Upon differentiation, nestin becomes down-regulated and is replaced by tissue-specific intermediate filament proteins, such as NF and GFAP, which could explain the higher positivity observed for NF in our specimens (Laser-Azogui et al., 2015).

The positive immunocytochemical staining of the differentiated cells for bone markers (OP, ALP) sustained the differentiation toward osteoblastic lineage. Osteogenesis is associated with a sequential expression of markers such as runt-related transcription factor 2 (RUNX2), followed by ALP, OP, osteocalcin, and osteonectin (Golub et al., 1992). OP is important for the initiation of bone mineralization. Although other genes such as the one for osteocalcin are associated with a sequential expression of markers such as OP, ALP, osteocalcin, and osteonectin (Golub et al., 1992).

Palatal tissue regained its regenerative properties in terms of MSC presence in the re-entry area after 6 months of healing and can be regarded as an available source of progenitors to be used.

CONCLUSIONS

The sorption, solubility, and cytotoxicity tests on experimental resin composites provided positive results, which recommended them for further investigations in order to be used in restorative procedures directly contacting the gingival tissues.

Biocompatibility tests using MSCs may be useful tools to monitor biological acceptability of resin composite materials in order to better understand their clinical properties and eventually to improve their composition.

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