International Endodontic Journal

doi:10.1111/j.1365-2591.2010.01720.x

The cytotoxic effects of resin-based sealers on dental pulp stem cells

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Abstract

Trubiani O, Caputi S, Di Iorio D, D'Amario M, Paludi M, Giancola R, Di Nardo Di Maio F, De Angelis F, D'Arcangelo C. The cytotoxic effects of resin-based sealers on dental pulp stem cells. *International Endodontic Journal*, **43**, 646–653, 2010.

Aim To evaluate the effect of four current resin-based adhesives on expanded *ex vivo* human dental pulp mesenchymal stem cells (DP-MSCs).

Methodology Dental pulp mesenchymal stem cells were derived from dental pulps of ten donors. After *in vitro* isolation, dental pulp stem cells were analysed using flow cytometry. The immunophenotype of DP-MSCs disclosed the homogeneous expression of the mesenchymal-related antigens CD29, CD44, CD73, CD90, CD105, CD166. DP-MSCs were exposed to four different commercially available bonding systems (CMF Bond, Prime&Bond NT, Clearfil S[3] Bond, XP Bond), and after 24, 48 and 72 h of incubation the morphological features and the cell growth were analysed. Moreover, the cell viability was evaluated at the same times by MTT assay. Data were statistically analysed using a two-way ANOVA and Holm–Sidak method (α set at 0.05).

Results Significant differences were observed between the four groups when comparing DP-MSCs appearance. DP-MSCs survived and proliferated without inhibition in the presence of CMF Bond adhesive. On the contrary, microscopic evaluation of the other three groups revealed extensive cytotoxic effects from the dentine bonding agents. The MTT assay revealed no statistically significant differences in cell viability after 72 h between the control group and CMF Bond group. All the other experimental groups had statistically lower optical density values.

Conclusions CMF Bond adhesive allowed human dental pulp stem cells to survive and proliferate. All of the other dentine bonding agents had extensive cytotoxic effects.

Keywords: bonding systems, dental pulp, mesenchymal stem cells.

Received 19 June 2009; accepted 4 March 2010

Introduction

The increasing use of resin-based adhesive materials in dentistry has raised questions of their cytotoxicity with oral and dental tissues (Abebe *et al.* 2005). Hanks *et al.* (1991) reported that most adhesive system and composite resin components, such as bis glycidyl methacrylate (bis-GMA), methane dimethacrylate (UDMA), triethyleneglycol dimethacrylate (TEG-DMA), camphoroquinone, 2-hydroxyethyl methacrylate (HEMA), and others, are cytotoxic when in direct contact with fibroblasts. Moreover, it has been demonstrated that methyl methacrylate, HEMA, TEGDMA, and dimethylaminoethyl methacrylate relaxed the isolated rat aorta (Cehreli *et al.* 2000, Abebe *et al.* 2003). Different resin components have also shown to be bioactive in the vasculature (Onur *et al.* 2000, Abebe *et al.* 2005). Kitasako *et al.* (1999) concluded that three different dentine bonding agents (DBA)

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applied to exposed monkey tooth pulps provoked slight inflammatory reactions and the deposition of dentine bridging over time, without demonstrable bacterial leakage. Costa *et al.* (2000a) pointed out that DBA application on animal teeth exhibited specific pulp reactions that could not be directly extrapolated to humans. Several studies revealed inflammatory changes and necrotic zones on exposed human pulps directly capped with various DBAs (Hebling *et al.* 1999, Scarano *et al.* 2003, Sübay & Demirci 2005). Studies in humans have demonstrated mild to severe inflammatory pulp reactions to resin-containing restorative materials, leading in many cases to cell apoptosis, followed by severe pulp alteration (Goldberg 2008).

Recently, the identification of putative dental stem cell populations, capable of regenerating organized tooth structures, has stimulated interest into the potential use of post-natal stem cell-based therapies for treating damage caused by trauma, cancer, caries and periodontal disease (Gronthos *et al.* 2000, Murray *et al.* 2007). The dental pulp is a viable source of attainable cells with a possible potential for cell transplantation therapies. This indicates the presence of a cell population in the dental pulp, with neuronal differentiation capacity (Nosrat *et al.* 2004) and having the ability, after induction, to differentiate into mesenchymal tissue. This has the potential to offer a novel approach for regenerative medicine (Gronthos *et al.* 2000, Batouli *et al.* 2003, Trubiani *et al.* 2006).

The purpose of this *in vitro* study was to evaluate the biological response of four current resin-based adhesives on expanded *ex vivo* human mesenchymal stem cells obtained from dental pulp tissues (DP-MSCs).

Materials and methods

Cell culture

Ten sound human pre-molar teeth, scheduled to be removed for orthodontic purposes, were selected from disease-free patients ranging from 14 to 16 years of age. The local ethics committee approved the experimental protocol, and the guardians of the patients signed consent forms. Each patient was pre-treated for a week with professional dental hygiene. Extracted teeth were rinsed four times in phosphate buffered saline containing penicillin and streptomycin. Subsequently, the pulps were exposed using a cylindrical diamond bur (314, Ø ISO 014, L.8.0 mm; Intensiv, Grancia, Switzerland) mounted on a high-speed handpiece (Bora L; Bien-Air, Bienne, Switzerland) with water-spray cooling. Pulps were then removed with a sterile excavator, cut into small pieces and then cultured in MSCM medium (Cambrex Co., Walkersville, MD, USA) according to the manufacturer's indications (Trubiani *et al.* 2007b, 2008a,b). After 20 days of culture, numerous cells forming colonies (CFU-F) migrated from the explants. The adherent cells, which were 80–90% confluent, as determined by phase contrast microscopy, were subsequently isolated using 0.1% trypsin solution and plated in tissue culture polystyrene flasks at 5×10^3 cells cm^{-2}. Primary cultures of DP-MSCs mainly consisted of bipolar fibroblastoid cells colonies, which after sub-cultivation proliferated with a population doubling time of 48 h, were used for all experiments (Trubiani *et al.* 2007a).

Flow cytometry analysis

Mesenchymal stem cells were washed in PBS and subsequently resuspended in PBS with saturating concentrations (1 : 100 dilution) of anti-human antibodies fluorescein isothiocyanate conjugated (HLA-Dr, CD2, CD3, CD7, CD44, CD45, CD73, CD90) and CD14, CD29, CD33, CD34, CD68, CD105, CD166, S-100 phycoerythrin conjugated, for 30 min at 4 °C. Labelled cells were acquired and analysed using a FACStar-plus flow cytometry system running CellQuest software (Becton-Dickinson, Mountain View, CA, USA). All reagents were obtained from Becton Dickinson.

Surfaces/scaffold

Four commercially available bonding systems (Table 1) were tested. Two drops of each adhesive were applied separately into pre-fabricated moulds measuring 5 × 2 mm, gently air-dried for 5–10 s and light-cured for 40 s (L.E. Demetron I; Sybron/Kerr, Orange, CA, USA, with a 1200 mW cm^{-2} output). The materials were treated with 1% penicillin/streptomycin for 1 h and washed with PBS. DP-MSCs were then seeded onto adhesives and cultured in MSCM medium for different time periods.

Cell viability assay and light microscopy study

To distinguish the living cells after 24, 48 and 72 h, the control samples and the cells incubated with the different adhesive were stained with trypan bleu and analysed by an inverted light microscope (Leica DMIL; Leica Microsystems S.p.A, Milan, Italy). To determine

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Table 1 Information on adhesives tested

Group	Adhesive	Manufacturer	Batch number	Composition	Classification
CMF	CMF Bond (bonding)	Saremco Dental AG, Rebstein, Switzerland	00020105	Bimethacrylate miscela, inorganic fillers, stabilizers (free of HEMA and TEGDMA)	Total-etch 3 steps
PB	Prime&Bond NT	Dentsply DeTrey GmbH, Konstanz, Germany	0510000814	Di- and Trimethacrylate resins, PENTA, Nanofillers-Amorphous Silicon Dioxide, Photoinitiators, Stabilizers, Cetylamine hydrofluoride, Acetone	Total-etch
CL	Clearfil S^3 Bond	Kuraray Medical Inc., Okayama, Japan	00050B	2-Hydroxyethyl methacrylate, bis-phenol A diglycidylmethacrylate, 10-methacryloyloxydecyl dihydrogen phosphate, silanated colloidal silica, dl-camphorquinone, ethyl alcohol, water	Self-etch
XP	XP Bond	Dentsply DeTrey GmbH	0604001288	Carboxylic acid modified dimethacrylate, phosphoric acid modified acrylate resin, urethane dimethacrylate, TEGDMA, HEMA, butylated benzenediol, ethyl-4-dimethylaminobenzoate, camphorquinone, functionalized amorphous silica, t-butanol	Total-etch

HEMA, 2-hydroxyethyl methacrylate; TEGDMA, triethyleneglycol dimethacrylate; PENTA, dipentaerythritol penta acrylate mono-phosphate.

adhesive-induced cytotoxicity in DP-MSCs, the cells were cultured in the absence (control test) or in the presence of one of the four tested bonding systems for 24, 48 and 72 h. Moreover, cell viability was evaluated by MTT assay. Briefly, the cells were seeded into flat-bottom 96-well plates (10^4 per well) in 100-μL medium. After 24-h incubation for attachment, the medium was replaced with fresh medium for different time intervals (24, 48 and 72 h). At the designated time, 10 μL of MTT were added to each well and incubated for 3 h. Absorbance at 570 nm was measured with a reference wavelength of 630 nm. The cytotoxicity experiment was conducted independently in duplicate with twelve replicates for each experiment ($n = 12$). Mean absorbance values were calculated in each group at 24, 48 and 72 h. The effect of the different bonding agent and the time interval upon cell viability were analysed statistically by a two-way ANOVA. Multiple comparisons were performed according to the Holm–Sidak method. The level of α was set at 0.05 in all tests.

Osteogenesis induction

For osteogenesis induction, the DP-MSCs were maintained in culture for 24 h in MSCM medium at 37 °C, in a humidified atmosphere of 5% CO_2. Subsequently, the osteogenic differentiation was induced for 4 weeks by replacing the MSCM medium with osteogenesis induction medium, a-MEM medium supplemented with 10% FBS, 10 mmol L^{-1} b-glycerophosphate, 0.2 mmol L^{-1} ascorbic acid, 1028 M dexamethasone. In all samples the medium was changed every 3 days. Controls consisted of DP-MSCs grown without osteogenic medium.

Mineralization assay

Mineralization in DP-MSCs cultures induced to osteogensis was determined by staining using alizarin red S. Cells were allowed to grown in differentiation media for 28 days. Plates were washed three times with PBS (pH 7.4), then stained with 0.5% alizarin red S in H_2O, pH 4.0, for 1 h at room temperature. After staining, the cultures were washed three times with H_2O followed by 70% ethanol.

Results

The immunophenotype of DP-MSCs evaluated by flow cytometry showed a homogeneous expression (>95%) of mesenchymal-related antigens CD29, CD44, CD73, CD90, CD105, CD166 and the absence (≤2% positive) of hematopoietic stem cell markers CD14, CD34, CD45 (Fig. 1). These cells were also negative for the presence of HLADr, CD2, CD3, CD7, CD15, CD26, CD33 and CD38 (data not shown). To evaluate the intrinsic capacity of self-renewal and ability to regenerate tissues of the mesenchymal lineage an osteogenic differentiation of DP-MSCs was induced. After 4 week of induc-

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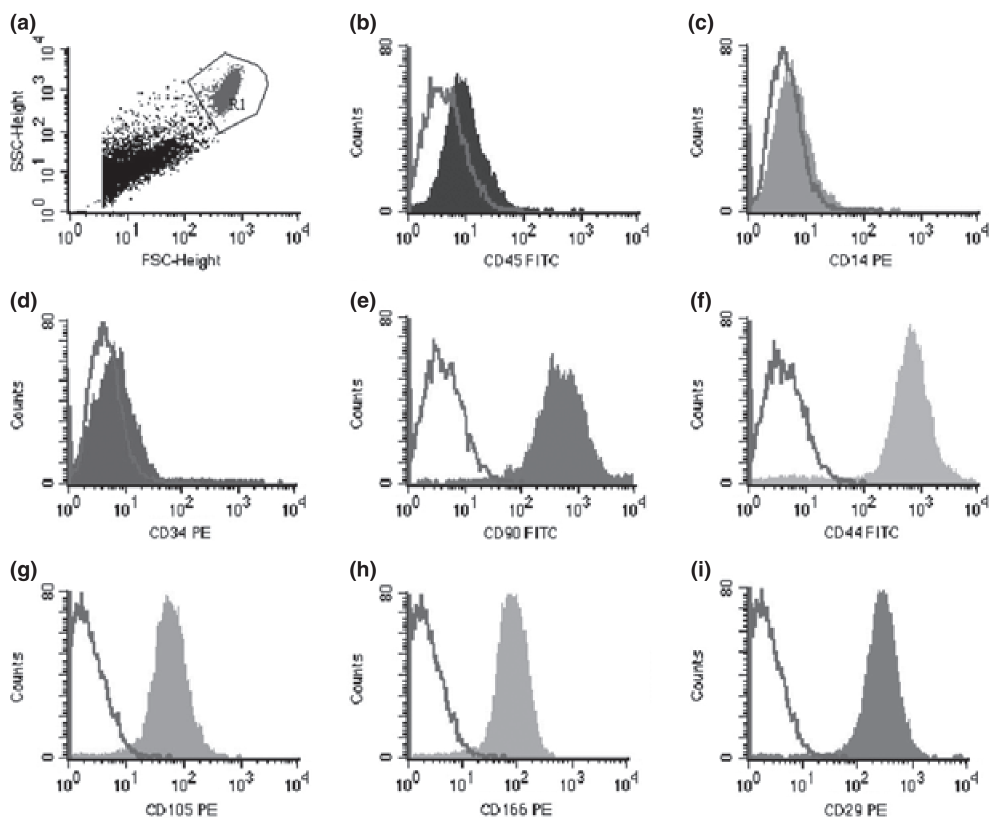

Figure 1 Flow cytometry of the mesenchymal-related antigens in dental pulp stem cells (DP-SCs). Section (a) shows a representative flow cytometry plot of DP-MSCs reflecting cytoplasmic granularity and large cell size. Sections (b–d) show the negativity expression of CD45, CD34, CD14 antigens. The other sections show the positivity expression of mesenchymal-related antigens.

tion, a mineralized matrix evidenced by alizarin red staining was evident (Fig. 2a,b). Light microscopy evaluation of *ex vivo* expanded DP-MSCs at a second passage revealed that a majority of cells with fibroblast-like morphology had dispersed onto the surface forming colonies (Fig. 3a). At a higher magnification, light microscopic analysis of DP-MSCs primary cultures demonstrated a morphologically homogeneous fibroblast-like appearance with a stellate shape and long cytoplasmic processes that had made contact with neighbouring cells. The cells adhered to each other. The elliptical nuclei demonstrated abundant euchromatin, indicative of an active gene transcription (Fig. 3b).

Significant differences were observed when comparing DP-MSCs appearance in the four test groups. Morphological analysis of DP-MSCs seeded on CMF Bond adhesive stained with the trypan blue revealed that some of the cell colonies were to closely adhere to

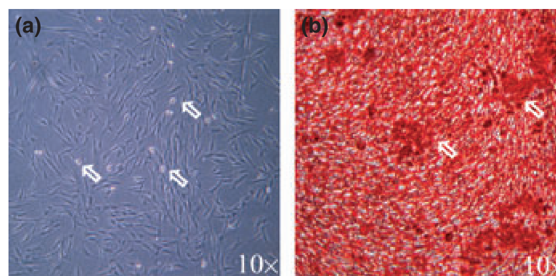

Figure 2 (a) Light microscopy photomicrograph of primary cultures of dental pulp mesenchymal stem cells (DP-MSCs) at the second passage consisting of adherent cells showing a morphological homogeneous fibroblast-like appearance with a stellate shape and long cytoplasmic processes (arrows); (b) The ability of DP-MSCs to differentiate versus osteoblasts after 28 days of culture in the presence of the osteoinductive medium is indicated by calcium deposition with Alizarin Red staining (arrows).

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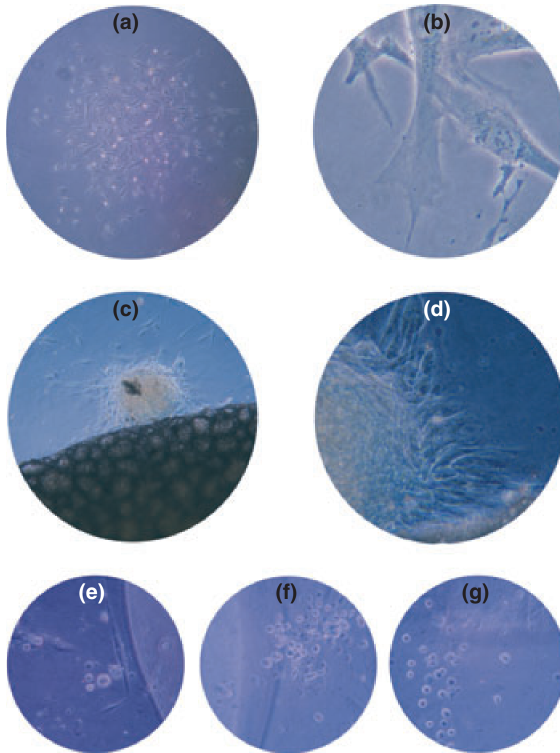

Figure 3 Micrograph of DPM-SCs expanded *ex vivo*, forming colonies, observed under inverted microscope, showing fibroblastoid-like morphology (original magnification: 10×); (b) Dental pulp mesenchymal stem cells (DP-MSCs) showing stellate shape and long cytoplasmic processes. The cells seem to adhere to each other. The elliptical nuclei show abundant euchromatin (original magnification: 25×); (c) Representative micrograph of DP-MSCs seeded on CMF Bond adhesive. Many living DP-MSCs are evident. Cell colony seems to closely adhere to substrate (original magnification: 10×); (d) Representative micrograph of DP-MSCs seeded on CMF Bond adhesive, at higher magnification. An elaborate form of attachment to adhesive with cellular bridging is detectable. DP-MSCs are not stained by trypan blue exclusion test (original magnification: 25×); (e) Representative micrograph of DP-MSCs seeded on Prime&Bond NT. The cells show remarkable morphological changes and trypan blue test results positive; (f) DP-MSCs seeded on Clearfil S^3 Bond. The few cells result positive to trypan blue test (original magnification: 25×); (g) DP-MSCs seeded on XP Bond. The cells appear with roundish shape showing inability to adhere to substrate (original magnification: 25×).

the substrate (Fig. 3c). No signs of biomaterial degradation were observed and the scaffolds appeared to supply sufficient support to the cell structure. In particular, an elaborate form of attachment to matrices with cellular bridging was detectable also at 48 h of incubation.

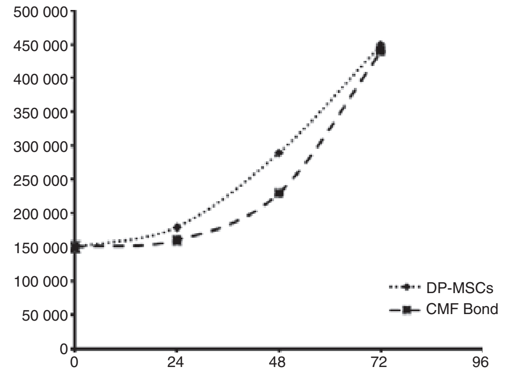

Figure 4 Proliferation rate and viability as determined by the trypan blue exclusion test. Dental pulp mesenchymal stem cells (DP-MSCs) display an increase in cell growth starting at 0 h. The growth profile exhibited by the DP-MSCs in presence of CMF Bond adhesive is interesting: adhesive presence increases cell growth commencing only after 24 h. The *Y* axis shows cell number and *X* axis shows culture time. The data are representative of four separate experiments.

The light microscopic evaluation of the other three groups (PB, CL, XP) demonstrated extensive cytotoxic effects from DBAs. DP-MSCs demonstrated distinct morphological changes and trypan blue test resulted positive (Fig. 3e–g). Many cells appeared to display a spherical shape indicating an inability to adhere to substrate with likely changes to the cytoskeleton rearrangement and the adhesion molecules. No differences in cell death and cell morphology at different incubation times were present, indicating that biological damage occurred at precocious incubation times. Cell growth evaluated after 24, 48 and 72 h of DP-MSCs incubation with CMF Bond adhesive by trypan blue test is shown in Fig. 4.

The MTT assay revealed no statistically significant differences after 72 h between the control group and CMF group viability. All the other experimental groups (PB, CL, XP) had statistically lower optical density values (Table 2).

Discussion

Laboratory studies have been recommended to evaluate the cytotoxicity of endodontic and restorative materials. Several experimental models, using established cell lines as well as primary cells cultures, are now available for screening purposes. In addition,

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Table 2 Mean absorbance values (standard deviations) as determined by MTT assay in the experimental groups after 24, 48 and 72 h

		Group				
		Control	CMF	PB	CL	XP
Time	24 h	0.106^{c}_{1} (0.030)	0.069^{c}_{2} (0.026)	0.009^{a}_{3} (0.007)	0.006^{a}_{3} (0.004)	0.004^{a}_{3} (0.004)
	48 h	0.120^{b}_{1} (0.032)	0.094^{b}_{2} (0.020)	0.008^{a}_{3} (0.006)	0.007^{a}_{3} (0.004)	0.006^{a}_{3} (0.004)
	72 h	0.163^{a}_{1} (0.023)	0.157^{a}_{1} (0.021)	0.005^{a}_{2} (0.005)	0.005^{a}_{2} (0.004)	0.006^{a}_{2} (0.004)

Same superscripted letters indicate no statistically significant differences amongst levels of factor 'Time'. Same numbers in pedex indicate no statistically significant differences amongst levels of factor 'Group'.

implementation of distinct target cells and mechanistically different end-point markers has been suggested for practical cytotoxicity testing of dental materials (Messer & Lucas 1999). In addition to permanent cell line fibroblasts, predictively *in vitro* assays that using murine and human progenitor cells considerably aids in the estimating the toxic effects of different chemicals (Souza *et al.* 2006).

This study assessed the cytotoxicity of four adhesive systems using DP-MSCs. Many protocols exist to isolate and expand DP-MSCs, which invariable have subtle and occasionally quite substantial differences. Furthermore, many laboratories have isolated SCs from a variety of tissue with similar properties (Zuck *et al.* 2001, Bühring *et al.* 2009). These varied tissue sources and cell preparation raises the question of whether these cells are similar in terms of biological properties and experimental outcomes, especially in the context of cell therapy. For this purpose, the Mesenchymal and Tissue Stem Cell Committee of the ISCT proposes three criteria to define human SCs for pre-clinical studies: adherence to plastic, specific surface antigen expression and multipotent differentiation potential (Dominici *et al.* 2006). The DP-MSCs used in this study had strong adhesion to plastic surface in standard culture condition; furthermore, more than 90% of the cell population was positive for mesenchymal-related antigens CD29, CD44, CD73, CD90, CD105, CD166 and lacked the expression of hematopoietic markers CD14, CD34, CD45 HLADr, CD2, CD3, CD7, CD15, CD26, CD33 and CD38, and then the multipotent differentiative capacity of these cells have been demonstrated (Trubiani *et al.* 2007a).

Cytotoxic effects of the materials were correlated with DP-MSCs reaction when seeded on cured adhesives in relation to the baseline value using the trypan blue Dye Exclusion Test at 24, 48 and 72 h incubation times. Trypan blue is a vital stain used to selectively colour dead tissues or cells. Significant differences were observed when comparing the four test groups. DP-MSCs remained viable without inhibition in the CMF group. The results obtained using trypan blue revealed that CMF adhesive induced a slowing down of the growth until 24 h of culture. Subsequently, restoration of cell growth was evident: after 72 h of incubation, the number of proliferating cells in the presence of CMF Bond adhesive was the same as the control cells (Fig. 4). Similar results were obtained using MTT assay performed at each point of culture (Table 2).

The MTT method was developed originally by Mosmann (1983) and is simple, accurate and gives reproducible results. The key component is 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide or MTT. This product is of yellowish colour in solution. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, leading to the formation of purple crystals, which are insoluble in aqueous solutions. The crystals are redissolved in acidified isopropanol, and the resulting purple solution is measured spectrophotometrically. An increase or decrease in cell number results in a concomitant change in the amount of formazan formed, indicating the degree of cytotoxicity caused by the oxidative stress of test material. MTT assay offered the opportunity to evaluate the extensive cytotoxic effects evident for the other DBAs (PB, CL, XP groups). In these groups DP-MSCs were altered morphologically, and the trypan blue test was positive at all incubation times.

These different cytotoxic effects could be explained when considering that CMF Bond adhesive (Saremco Dental AG) is free of HEMA and TEGDMA. However, the results of the CMF group do not indicate absence of any negative effect on the differentiation potential of the stem cell population, particularly in terms of dentine-pulp regeneration. Further studies are needed to clarify this issue.

The data of the present investigation are in accordance with other studies (Hanks *et al.*1991, Mantellini *et al.* 2006, Falconi *et al.* 2007, Goldberg 2008), which reported that some adhesive systems and composite resins components, such as TEG-DMA, HEMA, bis-GMA, UDMA, are a likely cause of cellular stress and

have definite local cytotoxic effects. HEMA is frequently used in dental bonding resins as a wetting agent. It competes with water for penetration and infiltration into dentine, and it copolymerizes with other resin composite monomers (Peutzfeldt 1997). HEMA has been shown to diffuse rapidly across dentine towards the pulp which, in turn, could induce hypersensitivity reactions in susceptible individuals (Pashley 1996).

Intense debate concerning the cytotoxicity of resin components after application to both unexposed and exposed pulp has taken place (Hebling *et al.* 1999, Scarano *et al.* 2003, Sübay & Demirci 2005). The cytotoxic effects of monomers have been reported in several *in vitro* studies (Hanks *et al.* 1991, Hashieh *et al.* 1999, Costa *et al.* 2000b, Abebe *et al.* 2003, Falconi *et al.* 2007), whereas with regard to the medical literature a persistent chronic inflammatory reaction is present after implantation of polymeric materials in patients subjected to a treatment for organic disorders (Konovsky *et al.* 1991). Studies on the degradation of dental biomaterials have confirmed the release of substances like HEMA and TEGDMA from these resins (Gerzina & Hume 1995, Bouillaguet *et al.* 1996). The amount of monomer release ranges from micrograms to milligrams (Spagnuolo *et al.* 2006) and is responsible for many cytotoxic (Spagnuolo *et al.* 2008) and metabolic conditions (such as tooth sensitivity) (Unemori *et al.* 2001), local immunological effects (Jontell *et al.*1995), and chronic inflammatory reactions of human pulp (Costa *et al.* 2003).

Conclusions

The co-monomer-free adhesive allowed mesenchymal stem cells derived from human dental pulp to survive and proliferate. These results are particularly important because the pulp tissue seems to harbour a rare population of high, proliferating stem cells with the ability to regenerate a dentine-pulp structure *in vivo* and with a self-renewal capacity (Gronthos *et al.* 2000).

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