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Effects of Two Methacrylic Monomers on Pulp Cells Differentiation Capability: A Preliminary *In Vitro* Study

Federico Barelli-Corbo[1a], Benedetta Acciavatti[1a], Adriana Amalfitano[2], Alessandro Arcovito[2], Cinzia Callà[2], Massimo Cordaro[1] and Giuseppina Nocca[*2,3]

[1]Institute of Dentistry, Università Cattolica del Sacro Cuore, Largo Francesco Vito - 00168 Rome, Italy

[2]Institute of Biochemistry and Clinical Biochemistry, Università Cattolica del Sacro Cuore, Largo Francesco Vito - 00168 Rome, Italy

[3]Istituto di Chimica del Riconoscimento Molecolare, CNR, Largo Francesco Vito - 00168 Rome, Italy

[a]These authors contributed equally to this work

Abstract

Several studies have already demonstrated that the incomplete polymerization of resin-based dental materials causes the release of monomers, which might interact with pulp cells (both fibroblasts and stem cells). Due to possible applications of these cells in regenerative dentistry, the aim of this study was the evaluation *in vitro* cell differentiation after the exposition to sub-cytotoxic concentrations of triethylene glycol dimethacrylate (TEGDMA) and 2-hydroxyethyl methacrylate (HEMA) for 24 h. Our preliminary results seem indicate that HEMA and TEGDMA did not alter the normal differentiation and mineralization process of pulp cells. On the contrary, when dental pulp stem cells were treated with the same monomer concentrations these capabilities were lost. Thus, the use of pulp cells derived from teeth treated with composite resins from applications in regenerative dentistry may be considered.

Introduction

Methyl methacrylate monomers such as triethylene glycol dimethacrylate (TEGDMA) and 2-hydroxyethyl methacrylate (HEMA) are largely present in dental composite resins, whose main function is to make easier the filler incorporation. In clinical conditions, small amounts of uncured monomers are released [1] into the oral cavity and — through dentinal diffusion [2-4] — in pulpal tissues, where they may accumulate reaching millimolar concentrations [3], able to provoke damages [5].

The *in vitro* studies on TEGDMA and HEMA showed that such monomers have cytotoxic activity due to the alteration of lipid metabolism, glutathione concentration, reactive oxygen species (ROS) production, energy metabolism, and mitochondrial activity [6]. All these effects are present also at sub-cytotoxic concentrations (i.e., values able to induce a mortality not higher than 20% with respect to control) of monomers.

It is emerging in the scientific literature a link between the mitochondrial activity and stemness of cells [7]. Therefore, according to the fact that TEGDMA and HEMA are able to affect mitochondrial respiration, the aim of this preliminary study was to analyze the differentiation capability of both human dental pulp cells (HPCs) and of Human Dental Pulp Stem Cells (DPSCs) after the exposition to sub-cytotoxic concentrations of HEMA and TEGDMA for 24 h. This incubation time was chosen because – in clinical applications – the larger amount of monomers was leaked from materials during this period [8].

This aspect is important, not only because pulp cells are responsible of regeneration and repair of the dentin/pulp complex [9], but also because DPSCs could be used in regenerative dentistry, where the stemness capability determination is mandatory.

Materials and Methods

All chemicals and reagents were obtained from Sigma-Aldrich Srl, Milan, Italy, unless otherwise indicated.

Cell culture

HPCs were obtained and cultivated as previously described [6]. Briefly, the tooth pulp tissue

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Correspondence:
Giuseppina Nocca, Institute of Biochemistry and Clinical Biochemistry, Università Cattolica del Sacro Cuore, Largo Francesco Vito, 00168 Rome, Italy, Tel: 0630154215; E-mail: giuseppina.nocca@unicatt.it

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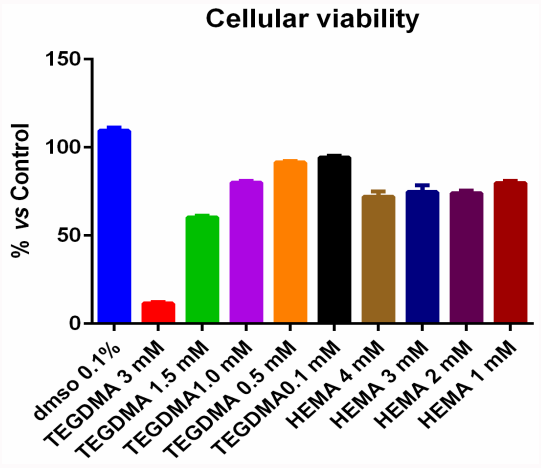

Figure 1: Evaluation of sub-cytotoxic concentrations. HPCs were treated with different concentrations of monomers for 24 h. The viability was determined by MTT; results were reported as percentage of viable cells versus control ± SD. Data are the mean of 5 different experiments performed in sextuplicates.

was cut and incubated in phosphate buffered saline (PBS), containing type I collagenase (3 mg/mL) and dispase (4 mg/mL), for 60 min at 37°C. The cells were plated in tissue culture flasks with Dulbecco's modified Eagles' medium (DMEM). HPCs were not used beyond the fifth passage.

DPSCs were isolated by magnetic beads (dynabeads, Life Technologies Italia Fil. Monza MB, Italy) as reported in Gronthos et al. [10]. In our study monoclonal antibodies were used versus three specific mesenchymal stem cell associated markers (STRO-1, CD146/MUC-18 – expressed on stem cell membrane and CD45 – not expressed on these cells). Magnetic beads are conjugated with monoclonal antibodies against IgM.

Preparation of methacrylate solutions

Stock dimethyl sulfoxide (DMSO) solutions of TEGDMA (from 0.1 mol/L to 3.0 mol/L), were prepared immediately before use. A final concentration of DMSO (0.1% v/v) was utilized in all samples because — as shown by preliminary studies — it did not induce any alterations in the parameters under study. Dulbecco Modified Eagle Medium (DMEM) containing the monomers was then added to the exponentially growing HPCs at the following final concentrations: TEGDMA (3.0, 1.5, 1.0, 0.5, and 0.1 mmol/L). HEMA was added purely to the medium, because this monomer is hydrophilic, to reach a final concentration ranging from 1.0 mmol/L to 4.0 mmol/L.

Determination of HEMA and TEGDMA sub-cytotoxic concentrations

Sub-cytotoxic concentrations of TEGDMA and HEMA monomers were determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay [11]. HPCs and DPCSs were seeded in a 96-well tissue culture dish at 10.000 cells/well and, after 24 h of incubation, DMEM was removed and the cell monolayer was incubated with the above indicated monomer concentrations for 24 h. DMEM was replaced by a solution of MTT (0.5 mg/mL, 100 µL/well) in PBS, and the cells were incubated at 37°C for 1 h in a 5% CO_2 atmosphere. The MTT solution was replaced with DMSO (100 µL/well) and gently swirled for 10 min. The optical density was measured by a plate reader at 540 nm (Packard Spectracount, Packard BioScience, Meriden, CT, USA). The results were expressed as the percentages of untreated cultures. Each experiment was performed

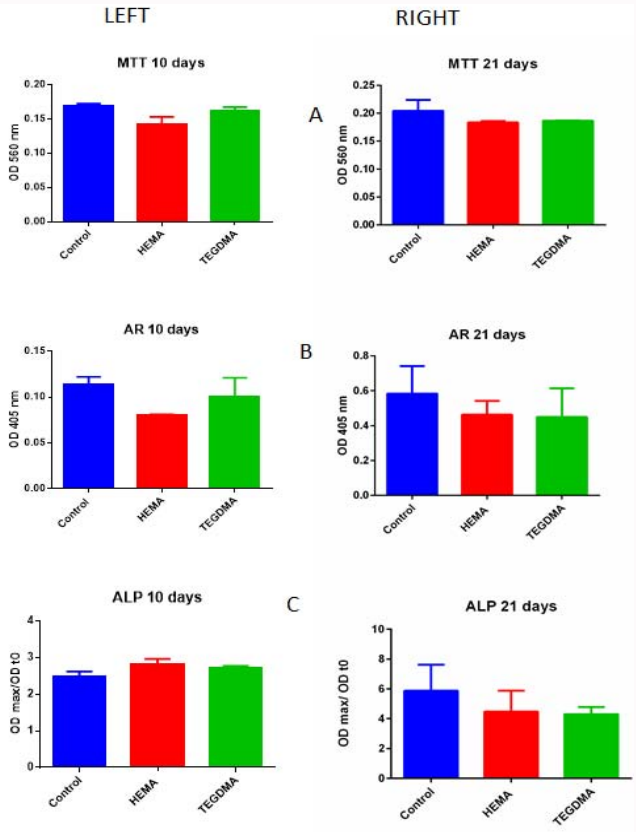

Figure 2: Left Panel: A) MTT values after 10 days of incubation in osteogenic medium in presence of 1 mmol/L HEMA or 0.1 mmol/L TEGDMA. B) AR assay C) ALP activity. Each value represents the mean ±SD for almost 3 independent experiments. Right Panel: A) MTT values after 21 days of incubation in osteogenic medium in presence of 1 mmol/L HEMA or 0.1 mmol/L TEGDMA. B) AR assay C) ALP activity. Each value represents the mean ± SD for almost 3 independent experiments.

five times in sextuplicate. On the basis of the obtained results the highest concentration of each monomer inducing a decrease of succinate dehydrogenase (SDH) activity — less than 20% compared to control — was determined and used in all the following experiments [12].

Treatment of HPCs and DPCSs

Cells were seeded onto six well tissue culture plates (2×10^5 cells/well) and cultured to sub-confluent monolayer for 72 h. Then the cells were incubated with HEMA (sub-cytotoxic value), or TEGDMA (sub-cytotoxic value), or DMEM (control) for 24 h. After this period, the medium was removed from all wells and replaced with DMEM supplemented with 10 mmol/L β-glycerol phosphate (β-GP), 0.05 mg/mL ascorbic acid (AA) and containing 10 nmol/L dexamethasone (DXM) (osteogenic medium). Medium was changed every 3 days. HPCs and DPSCs (both control and treated cells) were analyzed for cell proliferation and the osteogenic phenotype was assessed on the basis of changes in alkaline phosphatase (ALP) activity and in Ca^{++}-content evidenced by Alizarin red assay with different incubation periods (days 10 and 21).

Alkaline phosphatase assay

HPCs and DPSCs (both control and treated cells) were rinsed with PBS, re-suspended in Tris/glycine/Triton buffer pH 10.5 (50 mmol/L Tris, 100 mmol/L glycine, and 0.1% Triton X-100), and lysed by sonication on ice (twice, 15 s). The lysates were centrifuged (13,000

rpm, 5 min, 4°C) and a small amount of the obtained supernatants (10 µL) was mixed with 500 µL of p-nitrophenylphosphate (PNPP) substrate (8 mg/mL) and incubated at 37°C for 30 min Thus 0.1 N NaOH (3 mL) were added to each specimen to stop the reaction. The absorbance of new-formed p-nitrophenol (PNP) - contained in each well - was determined using an automatic microplate photometer (PackardSpectracount™, Packard BioScience Company, Meriden, USA) at a wavelength of 410 nm. The Optical Density (OD) values were normalized against protein concentration of the lysates [13]. Three different experiments (n=3) were performed in sextuplicate. Results are expressed as ratio between OD max and OD at initial time (t0).

Alizarin red assay

HPCs and DPSCs (both control and treated cells) were fixed in 10% formaldehyde for 15 min and stained with a solution of alizarin red (1%) for 10 min. The cells were washed four times with distilled water and the obtained red precipitate was solubilized in acetic acid (10%). The OD of solution was evaluated at 405 nm with an automatic microplate photometer (PackardSpectracount™, Packard BioScience Company, Meriden, USA) [14]. Three different experiments (n=3) were performed in sextuplicate.

Statistical analysis

Each value represents the average of (at least) three different experiments in quadruplicate. All results are expressed as mean Standard Deviation (SD). The group of means were compared by analysis of variance (ANOVA) followed by a multiple comparison of means by Student-Newman-Keuls; if necessary, comparison of means by Student's t test was used. $p<0.05$ was considered significant.

Results

Determination of sub-cytotoxic concentrations in HPCs and DPSCs

As expected, both monomers are able to cause a decrease of HPCs vitality as observed by MTT (Figure 1); similar results were obtained for DPSCs (data not shown). On the basis of the obtained results, the highest concentration of each monomer inducing a decrease of cell numbers less than 20% was selected: TEGDMA (0.1 mmol/L), HEMA (1.0 mmol/L). In literature, concentrations of methacrylic monomers with sub-cytotoxic effect are not unique. In fact, this value is influenced by the different cell populations as well as by the inter-individual variability associated with the use of primary cultures.

Effects of HEMA and TEGDMA on differentiation parameters

HPCs: None of the two used methacrylic monomers has altered the differential capability of the HPCs (Figure 2 left and right panels). In fact, after 10 or 21 days of incubation in osteogenic medium, the values of ALP activity and AR assay of HEMA or TEGDMA treated cells did not show any statistical differences respect to control cells.

DPSCs: In DPSCs, both methacrylic monomers used altered the differentiation capability (Figure 3 left and right panels) with an interesting time-edge mechanism. In fact, after 21 days of incubation in osteogenic medium, the values of ALP activity and AR assay of HEMA or TEGDMA treated cells were reduced respect to control cells in a statistically relevant percentage while this same inhibiting effect is not present in the initial phase (10 days).

Discussion

In this study, we induced HPCs and DPSCs (previously treated with sub-cytotoxic concentration of HEMA and TEGDMA for 24 h) to differentiate into osteoblast-like cells using an osteogenic medium containing β-GP, AA and DXM. AA is responsible of the correct structure of collagen, β-GP supports the ions necessary for mineralization and dexamethasone increases the synthesis of proteins present in extracellular matrix [15].

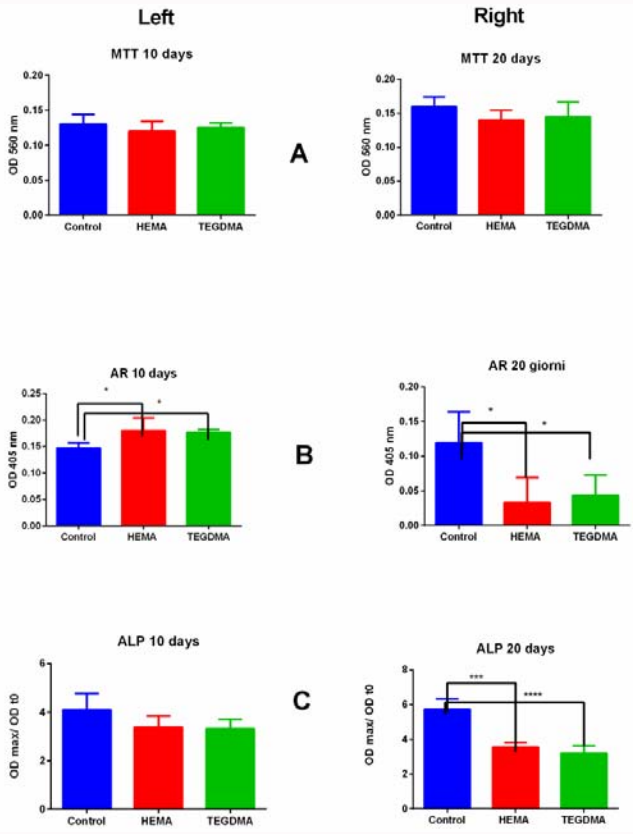

Figure 3: Left Panel: A) MTT values after 10 days of incubation in osteogenic medium in presence of 1 mmol/L HEMA or 0.1 mmol/L TEGDMA. B) AR assay C) ALP activity. Each value represents the mean ± SD for almost 3 independent experiments. Right Panel: A) MTT values after 21 days of incubation in osteogenic medium in presence of 1 mmol/L HEMA or 0.1 mmol/L TEGDMA. B) AR assay C) ALP activity. Each value represents the mean ± SD for almost 3 independent experiments. *p<0.05; ***p<0.001; ****p<0.0001.

These preliminary results seem indicate that HEMA and TEGDMA did not alter the normal differentiation and mineralization process of pulp cells. On the contrary, when dental pulp stem cells were treated with the same monomer concentrations these capabilities were lost.

The causes of the different behavior observed between DPSCs and HPCs could be multifactorial. However, one aspect is particularly intriguing: the link between stemness and mitochondrial functionality [7], because a reduction of the latter could compromise the cellular differentiation capability. With regard to the time-edge mechanism shown by the DPSCs, it is possible to hypothesize that the oxygen consumption rate of stem cells increases during differentiation process thus leading to the obtained results.

Conclusion

Based on these preliminary results, the use of pulp cells derived from teeth treated with composite resins from applications in regenerative dentistry may be considered.

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