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Assessment of 10-MDP and GPDM monomers on viability and inflammatory response in human dental pulp stem cells

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ABSTRACT

Objectives: to assess the cytotoxicity of the following functional monomers used in dental adhesives: 10-Methacryloyloxydecyl dihydrogen phosphate (10-MDP) and glycerol phosphate dimethacrylate (GPDM), and their effect on cytokine release from human dental pulp stem cells (hDPSCs).

Methods: The hDPSCs cells were isolated from the dental pulp of extracted human third molars. The functional monomers, 10-MDP and GPDM, were diluted in dimethyl sulfoxide (DMSO) at concentrations ranging from 1 to 4 mM. Cells not exposed to the compounds served as controls. The hDPSCs were seeded into 96-well plates and incubated for 48 h. Subsequently, the cells were exposed to 10-MDP and GPDM for 24 h. Then, the culture medium was removed, the mitochondrial metabolism was evaluated using the MTT assay, while cell death analyzed by flow cytometry. Cytokine release (IL-1β, IL-6, IL-8, IL-10, TNF-α) was analyzed by the MAGPIX. The data were analyzed using one-way ANOVA and Tukey's test.

Results: 10-MDP demonstrated significant toxicity to hDPSCs, reaching the IC50 at 3 mM. However, its impact on cytokine release was minimal, resulting only in IL-6 and IL-8 levels. GPDM exhibited lower toxicity, even at 4 mM, but induced an increase in IL-1β release and a reduction in IL-6, IL-8, and IL-10 levels, with no effect on TNF-α. Despite the MTT assay results indicating cytotoxicity, the cell death was low for both functional monomers.

Significance: 10-MDP exhibited significant toxicity to hDPSCs, unlike GPDM, however, both monomers resulted in minimal cell death. 10-MDP had a minor impact on cytokine release, whereas GPDM demonstrated a potential to trigger an inflammatory reaction, particularly in the short term.

1. Introduction

Functional monomers play a crucial role in restorative dentistry, serving to enhance the chemical bonding between dental resin — whether used as restorative or luting composite — and the enamel/dentin substrate [1]. Initially introduced through self-etching adhesives, these monomers have significantly advanced dental materials. Their development has paved the way for self-adhesive cements and versatile universal adhesives, which can be applied using both etch-and-rinse and self-etching techniques [2,3].

Over the years, various functional monomers have been proposed and utilized, including 10-Methacryloyloxydecyl dihydrogen phosphate (10-MDP), glycerol phosphate dimethacrylate (GPDM), 2-(methacryloyloxyethyl)phenyl hydrogenphosphate (Phenyl-P), and 4-[2-(methacryloyloxy)ethoxycarbonyl]phthalic acid (4-MET), among others [1,4,5]. Currently, 10-MDP and GPDM are the most widely used monomers in the market, with 10-MDP recognized as the most effective functional monomer in dental materials [4,5]. Both 10-MDP and GPDM effectively promote chemical bonding to metal, zirconia, and dental substrates [4–7], making them extensively used in silanes, cements, and adhesives.

Previous studies have demonstrated that adhesives can exhibit high toxicity to dental pulp cells [8–12]. This toxicity may be attributed to various components, such as solvents [8], the presence and

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concentration of monomers like hydroxyethyl methacrylate (HEMA) [8, 13], bisphenol A-glycidyl methacrylate (BisGMA) [14], and components from the initiation system [15,16].

Equally important as the toxicity to cells are the consequences arising from this condition. When damaged or negatively stimulated by a toxic compound, cells can initiate a cascade of signaling events that modulate the tissue response. One significant reaction that can be triggered is inflammation. The inflammatory process is coordinated by a series of molecules released following a noxious stimulus, with cytokines being the primary mediators responsible for initiating and controlling inflammation in tissues [17,18]. Understanding the behavior of cells in contact with various agents present in dental resins is crucial for developing new materials that are both highly efficient and safe for oral tissues.

Given these considerations, functional monomers present in self-etch and universal adhesives applied to deep dentin can diffuse through dentinal tubules, reaching the pulp chamber and potentially causing a tissue reaction. Previous studies have demonstrated that 10-MDP can negatively influence the differentiation of immortalized dental pulp cells into odontoblasts, increasing the gene expression of certain inflammatory mediators, although the cytokine release by these cells was not evaluated [19]. Additionally, there is a lack of information regarding the influence of 10-MDP and GPDM on the toxicity and cytokine release from human dental pulp stem cells (hDPSCs).

Therefore, this study analyzed the effect of different concentrations of the functional monomers 10-MDP and GPDM, used in resin dental materials, on mitochondrial metabolism, cell death, and the release of specific inflammatory mediators by hDPSCs. The hypotheses of the present study were a) the functional monomers evaluated would be toxic to hDPSCs, and b) the cytokine release would be influenced by the distinct monomers analyzed.

2. Materials and methods

2.1. Cell culture

Human dental pulp stem cells (hDPSCs) were obtained from freshly extracted human third molar from one 20-year-old subject (Ethics Committee Approval from the Paulista University - CAAE 26617119.0.0000.5512). The teeth were extracted, and the coronal portion was sectioned at the cement-enamel junction using a sterilized diamond disc with water cooling. Pulp tissue was subsequently removed using a dentin excavator and placed in a culture medium. hDPSCs were isolated via enzymatic digestion using collagenase type 1 (3 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) and dispase (Gibco BRL, Carlsbad, CA, USA). Following a two-hour incubation at 37 °C, the resultant cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, SIGMA Chemical Co., St. Louis, MO, USA) supplemented with 10 % fetal bovine serum (FBS, Cultilab, Campinas, SP, Brazil), 100 IU/mL penicillin, 100 mg/mL streptomycin, and 2 mmol/L glutamine (GIBCO, Grand Island, NY, USA) at 37 °C in a 5 % CO_2 atmosphere until reaching the desired cell number for experimental procedures. hDPCs from passages 5 to 7 were utilized for all experiments. Cells were characterized based on the relative expression levels of specific cell surface markers. Positive markers included CD105/AP and CD166/PE, while negative markers included CD34/FITC and CD45/PerCP (BD Biosciences, San Diego, CA, USA), confirming the hDPSCs phenotype [15].

2.2. Reagents

The functional monomers evaluated in the present study were: 10-Methacryloyloxydecyl dihydrogen phosphate (10-MDP) and glycerol phosphate dimethacrylate (GPDM) provided by Yller Biomateriais (Pelotas, RS, Brazil).

2.3. Preparation of solutions and cells exposure

The functional monomers were diluted in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) so that the DMSO concentration never exceeded 0.1 %. The concentrations of the monomers (10-MDP - 1, 1.25, 1.5, 2.5, 2.75, 3 mM; GPDM 1, 1.25, 1.5, 2.5, 2.75, 3, 3.5, and 4 mM) were selected based on a pilot study.

hDPSCs were seeded at a density of 10×10^4 cells in 24-well plates and cultured in DMEM in a humidified incubator with 5 % CO_2 and 95 % air at 37 °C for 48 h. After this incubation period, cells from the experimental groups were exposed to different concentrations of 10-MDP and GPDM for 24 h. The control group consisted of hDPSCs cultured in standard medium under the same conditions and for the same duration as the experimental groups. A culture medium containing low and high concentrations of DMSO, used for dilution, served as a control for mitochondrial metabolism evaluation.

2.4. Mitochondrial metabolism (MTT Assay)

To assess the mitochondrial activity of hDPSCs following treatments, the MTT (3-(4,5-Dimethylthiazol-2-yl)−2,5-Diphenyltetrazolium Bromide) assay (InvitrogenTM reagents - Thermo Fisher, United Kingdom) was conducted. This assay measures succinic dehydrogenase (SDH) activity via the methyl tetrazolium reaction in both experimental and control groups ($n = 6$), as previously described in various studies [12,15, 20–22]. Three independent experiments were performed, with six replicates for each group on each experiment, to verify the reproducibility of the results.

Absorbance readings were taken at 570 nm using the Epoch microplate reader (Biotek Instruments, Winooski, VT, USA). Absorbance values were converted into percentages relative to the control group (DMEM), which was set at 100 %.

2.5. Cell death analysis through flow cytometry

To assess potential induction of cell death, hDPSCs exposed to the two tested concentration (1 and 3 mM) of each functional monomer were analyzed using flow cytometry with the nucleic acid dye 7-Amino-Actinomycin (7AAD/PE) and phospholipid-binding protein Annexin V (Annexin V/FITC) (BD Pharmingen™, San Jose, CA, USA), following the manufacturer's instructions. Cell populations were categorized into four groups and quantified using a BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with BD Accuri C6 Sampler software (BD Biosciences, USA). Non-apoptotic cells were identified and sorted into the lower left quadrant.

Three independent experiments were performed, with six replicates for each group on each experiment, to verify the reproducibility of the results.

2.6. Quantification of cytokines released from hDPCs in culture

To investigate early inflammatory responses following exposure of hDPSCs to functional monomer concentrations, levels of cytokine release were analyzed. Three concentrations of each component ($n = 6$) were selected (1, 1.5 and 3 mM), and the levels of IL-1β, IL-6, IL-8, IL-10, and TNF-α were measured after 24 h in technical triplicate using Milliplex Map assays (Cat # HCYTOMAG-60K, Millipore Corporation, Billerica, MA, USA) according to the manufacturer's instructions. Samples were diluted in PBS +5 % Tween buffer, vortexed for 30 s prior to analysis, and centrifuged. Supernatants (25 μL) were then added to 96-well plates containing cytokine-specific immunomagnetic beads. Subsequently, secondary antibodies and substrate were applied, and the plates were analyzed on the MAGPIX platform (Millipore Corporation, Billerica, Massachusetts, USA). Concentrations of each cytokine in the samples were estimated from standard curves using Xponent® software (Millipore Corporation, Billerica, MA, USA), and mean concentrations

were calculated and expressed as pg/mL. Three independent experiments were performed, with six replicates for each group on each experiment, to verify the reproducibility of the results.

2.7. Statistical analyses

Prior to statistical analysis, data were assessed for normality and homogeneity of variance. Mitochondrial metabolism and cytokine release were analyzed using one-way ANOVA, as each functional monomer's results were not compared directly. Tukey's test was applied when appropriate. Statistical analyses and graphical representation of the results were performed using GraphPad Prism 7.0 (GraphPad Software Inc. Boston, MA, USA), with a significance level set at 5 %.

3. Results

3.1. 10-MDP and GPDM – mitochondrial metabolism and cell death

In terms of cellular viability, it is noteworthy that the functional monomer 10-MDP exerted a pronounced influence on mitochondrial metabolism in hDPSCs (Fig. 1). While 1 mM of this monomer did not induce a significant change in metabolic activity, a notable reduction of

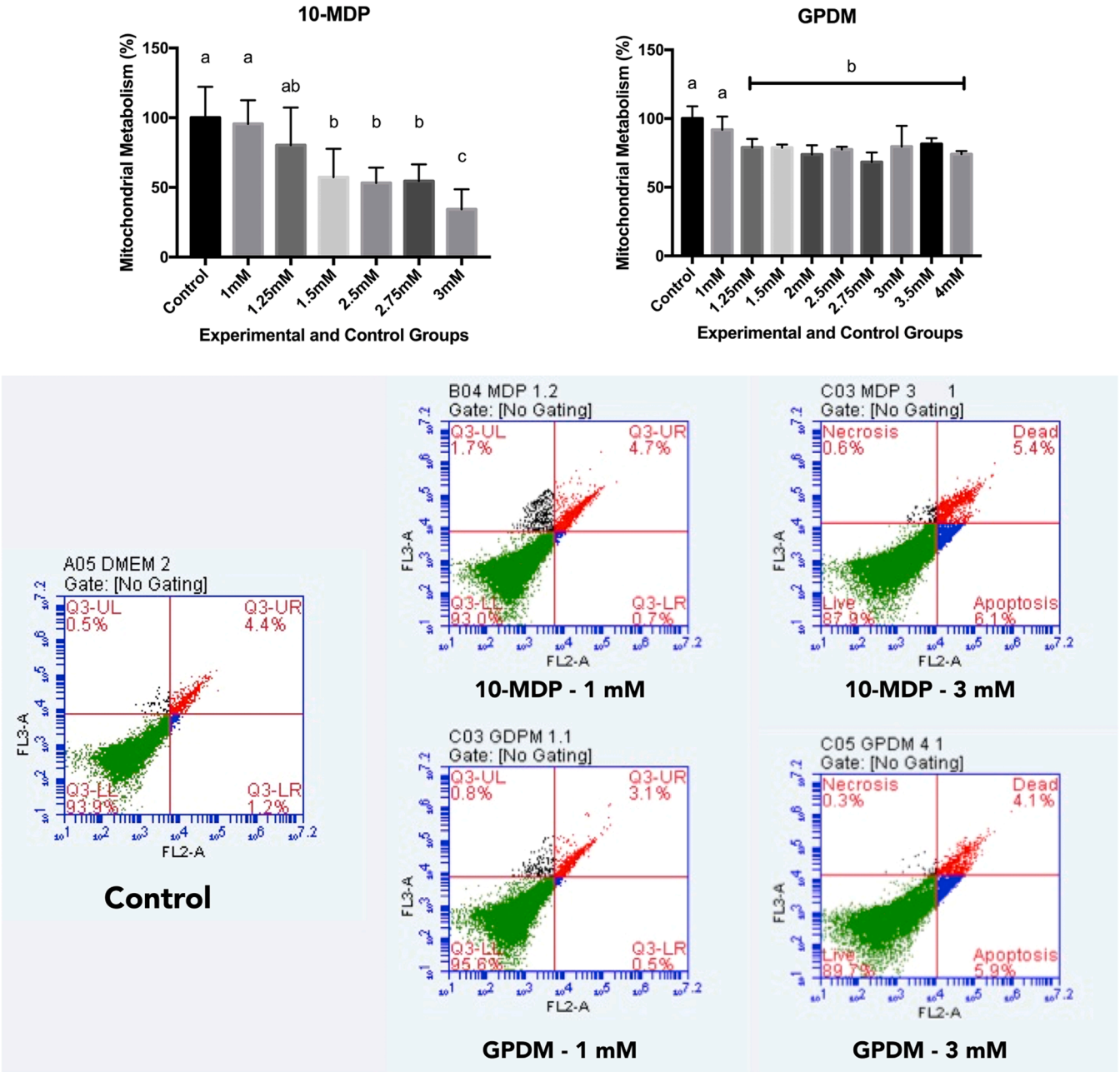

Fig. 1. Mitochondrial metabolism and flow cytometry of the hDPSCs after exposure to 10-MDP and GPDM. For 10-MDP, mitochondrial metabolism began to decrease at 1.25 mM, reaching the half-maximal inhibitory concentration (IC50) at 3 mM. For GPDM, a concentration of 1.25 mM also caused a statistically significant reduction in cell metabolism compared to the control. However, this reduction remained consistent across other tested concentrations, with similar results observed even at an increased concentration of 4 mM. Neither of the treatments triggered a significant cell death compared to the control group. The non-apoptotic cells are sorted into the lower left (LL) quadrant. The data for MTT assay are presented relative to the control group (hDPSCs treated with DMEM) as mean and standard error (SE) of percentage of reduction, considering the control group as 100 %. Distinct letters indicate significant difference. (ANOVA-one way and Tukey test, $p < .05$). ANOVA, analysis of variance; hDPSCs, human dental pulp stem cells; DMEM, Dulbecco's Modified Eagle Medium.

25 % was observed starting from 1.25 mM. Concentrations ranging from 1.5 to 2.75 mM resulted in an approximate 50 % reduction, being significantly lower than control and 1 mM concentration. At 3 mM, a substantial decrease (65 %) in mitochondrial metabolism of pulp cells was noted, which was statistically significant compared to lower concentrations and control.

Cell death analysis using flow cytometry (Fig. 1) revealed that exposure to 1 mM did not differ from the control group (DMEM). However, exposure to 3 mM of MDP resulted in a significant increase of 6 % in cellular apoptosis, totaling approximately 12 % cell death, contrasting with the 6 % observed in the control.

The monomer GPDM caused a less pronounced negative impact on mitochondrial metabolism in pulp cells than observed for 10-MDP. Despite observing a reduction in metabolism, this reduction occurred uniformly across all evaluated concentrations (between 1–4 mM) with approximately a 20 % decrease (Fig. 1). Cell death following exposure to 1 mM was similar to that of the control group (Fig. 1), becoming more pronounced when cells were exposed to 4 mM of GPDM (around 10.5 %).

3.2. 10-MDP – cytokine release

The analysis of inflammatory mediators by hDPSCs demonstrated that 10-MDP exerted minimal influence on the IL-1β, IL-10, and TNF-α release, compared to the control group (Fig. 2). Although the highest levels of release were from the proinflammatory cytokines IL-6 and IL-8, the reductions in those secretion were observed being more pronounced when cells were exposed to 3 mM of 10-MDP.

3.3. GPDM – cytokine release

Regarding inflammatory mediators, GPDM increased the IL-1β secretion levels while the IL-10 levels were decreased after exposure to all three evaluated concentrations. For IL-6, GPDM reduced its release from cells exposed to 1 and 3 mM of the monomer. Additionally, GPDM decreased the release of IL-8 at concentrations of 2 and 3 mM, being this pro-inflammatory cytokine the most expressed amongst all the inflammatory markers evaluated (~20 times more) (Fig. 3). GPDM had no influence on the release of TNF-α at any of the evaluated concentrations.

4. Discussion

With the advancements of adhesive systems, numerous new components have been added to their formulations, with functional monomers being notable examples. Although these monomers have been present since the development of self-etch adhesive systems, the popularity of multi-mode or universal adhesive systems has led to an increased use of these monomers. These agents are crucial because they chemically bond with mineralized dental tissues [1,4], yielding excellent results when applied to dentin. Their use is essential for ensuring that universal adhesives can effectively perform in the self-etch mode. However, the application of universal adhesives on deep dentin, whether through total-etch or the self-etch technique, may allow functional monomers to reach the pulp, which is potentially harmful to this tissue.

The results demonstrated that the cell toxicity of the evaluated functional monomers (10-MDP and GPDM) is concentration-dependent. The monomer 10-MDP reduced the mitochondrial metabolism of hDPSCs in 20 % at a concentration of 1.25 mM. This impairment in mitochondrial metabolism was more pronounced at a concentration of 3 mM, resulting in a reduction of more than 50 %. Similarly, GPDM monomer at 1.25 mM also negatively impacted mitochondrial metabolism, causing an approximately 20 % reduction. Aiming to identify a more toxic dose for GPDM, the concentration was increased up to 4 mM.

However, even at this higher dose, the 20 % reduction of mitochondrial metabolism with GPDM remained constant, in contrast to 10-MDP, which caused a 50 % reduction in cell metabolism at 3 mM. This suggests a lower influence of GPDM on hDPSCs. Despite this, the first hypothesis that these functional monomers would be toxic to hDPSCs was accepted.

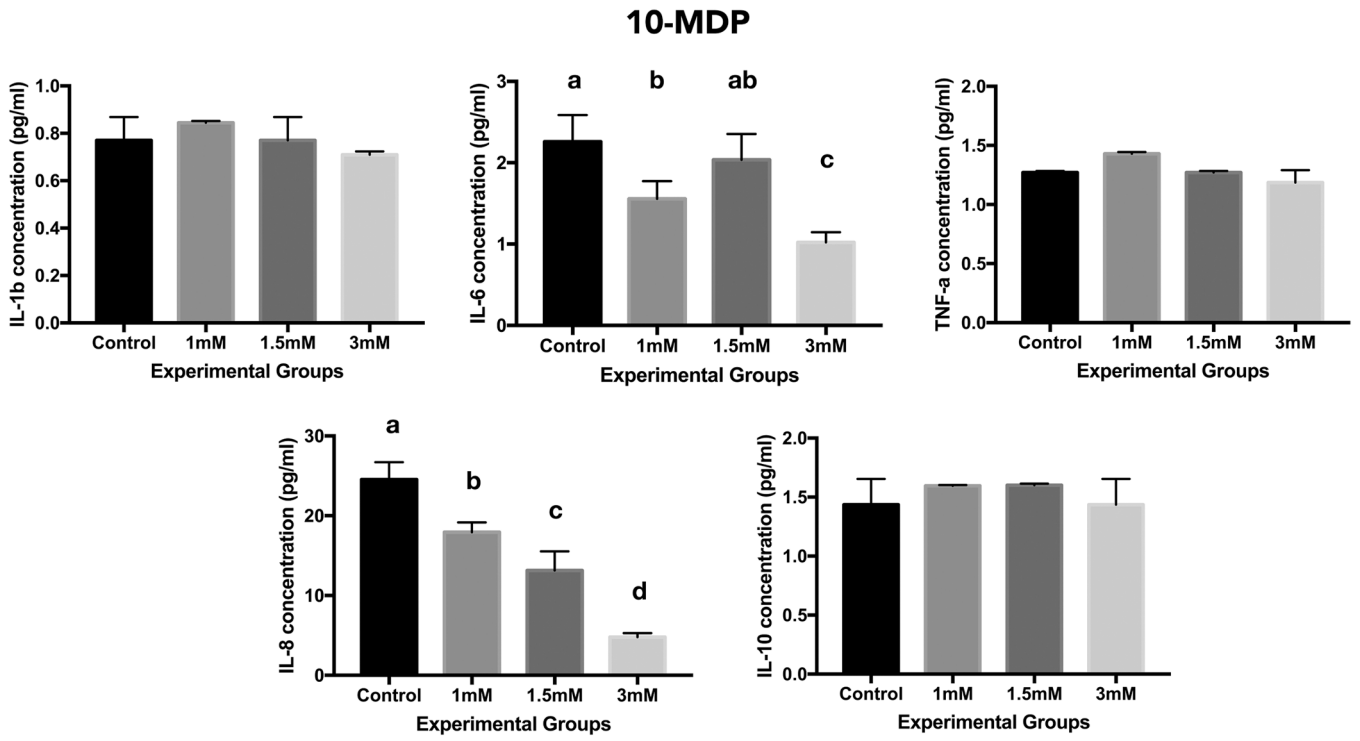

Fig. 2. Cytokine release profile by hDPSCs after immediate exposure to different concentrations of 10-MDP. Distinct letters indicate significant difference. (ANOVA one-way and Tukey test, $p < .05$). ANOVA, analysis of variance; hDPSCs, human dental pulp stem cells; DMEM, Dulbecco's Modified Eagle Medium; IL-1b, Interleukin 1 Beta; IL-6, Interleukin 6; IL-8, Interleukin 8; TNF-α, Tumor necrosis factor; IL-10, Interleukin 10.

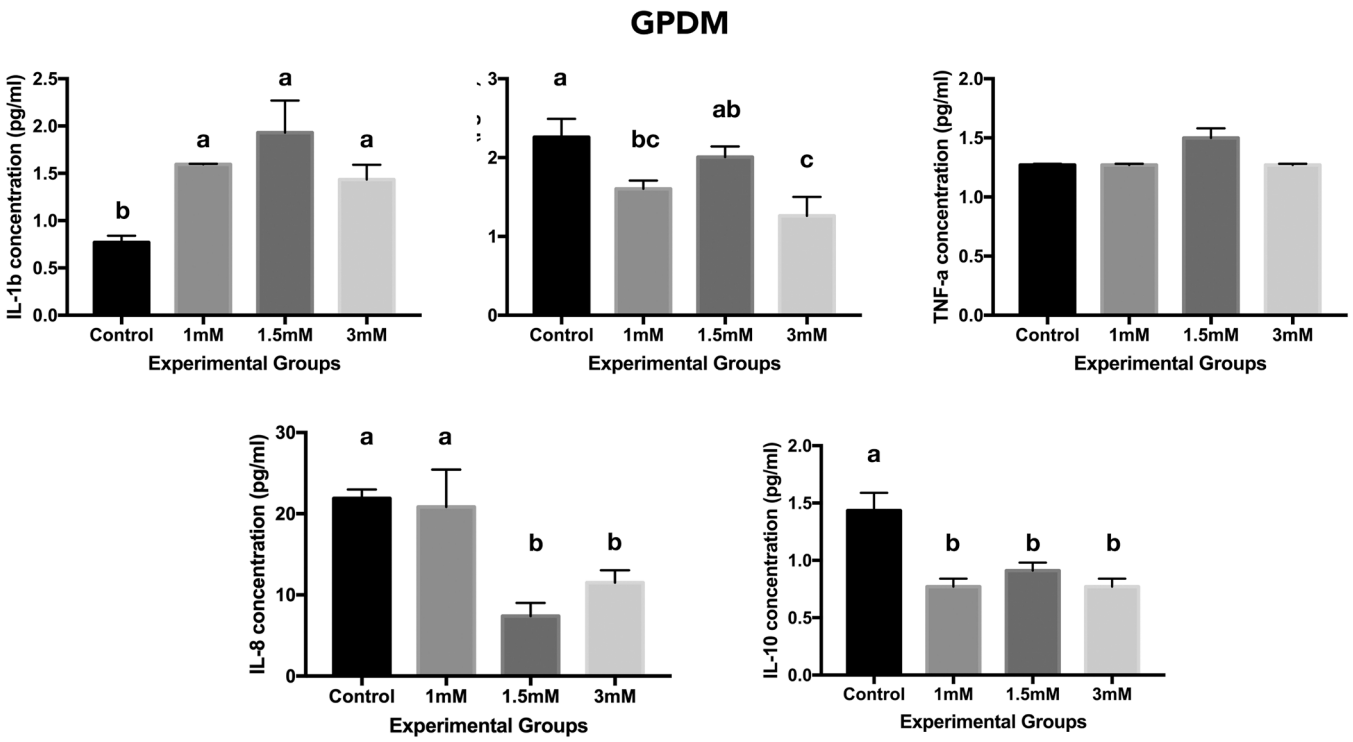

Fig. 3. Cytokine release profile by hDPSCs after immediate exposure to different concentrations of GPDM. Distinct letters indicate significant difference. (ANOVA one-way and Tukey test, $p < .05$). ANOVA, analysis of variance; hDPSCs, human dental pulp stem cells; DMEM, Dulbecco's Modified Eagle Medium; IL-1b, Interleukin 1 Beta; IL-6, Interleukin 6; IL-8, Interleukin 8; TNF-α, Tumor necrosis factor; IL-10, Interleukin 10.

To further investigate the effects on cell death, two concentrations (low and high) were evaluated using flow cytometry to observe cell death under different conditions and determine the type of cell death (necrosis or apoptosis) induced by the monomers. The different concentrations were selected to determine whether a low concentration, with minimal impact on mitochondrial metabolism, and a high concentration, with a more pronounced effect, could induce cell death and to assess the mechanism by which this occurs, either through necrosis or apoptosis. It was observed that, despite a significant reduction in mitochondrial metabolism using 3 mM of the 10-MDP monomer, cell death was modest (around 12 %), primarily due to apoptosis. Similar values were observed for the GPDM monomer (around 11 %). Thus, it can be inferred that the toxicity of the evaluated functional monomers is low at the tested concentrations, which is extremely important for components widely used in adhesive systems. The discrepancy between the results of mitochondrial metabolism analysis and cell death, particularly for 10-MDP, may be due to the limited cellular damage caused by the agent, allowing a decrease in the cell's mitochondrial metabolism after contact with some concentrations of monomers, without leading to an increase in the cell death.

Regarding cytokine release, the monomers exhibited different influences on hDPSCs. In a similar approach to that used for assessing cell death, three different concentrations (low – 1 mM, intermediate – 1.5 mM, high – 3 mM) were chosen to evaluate cytokine release. This was done to determine whether varying effects on mitochondrial metabolism could influence cytokine secretion. The 10-MDP monomer did not cause any changes in the release of IL-1β, TNF-α, and IL-10. However, it led to a reduction in the release of IL-8 and IL-6, with this effect being more pronounced at a concentration of 3 mM. Thus, the second hypothesis that the monomers would modulate the cytokine release was also accepted. The IL-8 protein, encoded by the Interleukin-8 (*IL-8*) gene, is an essential component of the CXC chemokine family and plays a pivotal role in the inflammatory response [23]. IL-8 is instrumental in inducing chemotaxis, which directs the migration of cells to sites of inflammation, and it facilitates the activation and migration of neutrophils [24]. Notably, elevated levels of IL-8 have been observed in inflamed dental pulps compared to normal pulps, according to previous studies [25,26]. In the early stages of inflammation, IL-8 production is crucial. Conversely, the pro-inflammatory cytokine IL-6 contributes to tissue degradation by increasing matrix metalloproteinases (MMPs) levels and promoting the differentiation of mature B lymphocytes into plasma cells [23].

A previous study demonstrated that the gene expression of some cytokines (IL-1β, IL-6, IL-8, TNF-α) can be increased following exposure of dental pulp cells to 100 μM of 10-MDP [19]. The differences between the results of the present study and those reported in the previous study is attributable to the methodologies employed. In the previous study, the gene expression of some inflammatory mediators was assessed by means of RT-PCR following exposure to 10-MDP, whereas in the present study, the release of cytokines was evaluated after exposure to the functional monomers 10-MDP and GPDM. Additionally, the previous study observed the mRNA levels of those cytokines, which is the intermediate molecule, while in this study it was investigated the secretion levels of the protein itself, which is the final molecule. It is important to highlight that there are several regulatory mechanisms responsible for adjusting protein translation levels according to biological demand. Therefore, caution is necessary when comparing distinct studies that investigate molecules at different biological functional levels.

Despite the reduced influence on the mitochondrial metabolism of hDPSCs and the minimal cell death observed even after exposure to the highest concentrations of GPDM, this monomer resulted in greater modulation of the release of the evaluated interleukins. Although there was a slight reduction in IL-6 release, GPDM led to an increased release of the pro-inflammatory cytokines IL-1β, an important cytokine for initiating the inflammatory cascade [23]. Additionally, GPDM caused a decrease in the release of IL-10, an anti-inflammatory mediator that reduces the production of pro-inflammatory cytokines, particularly IL-6 and IL-8 [27,28]. This suggests that diffusion of this monomer could lead to more significant inflammation in dental pulp compared to 10-MDP exposure, despite its lower toxicity to pulp cells.

In the present study, the results obtained after exposure to the different functional monomers were not directly compared. Both tested functional monomers are commercially used, and the primary focus of this study was to evaluate their effects on hDPSCs and their influence on the release of inflammatory mediators, rather than to determine which monomer is safer for use. The selection of each monomer for use in dental resins involves various considerations, including adhesion to different substrates (enamel, dentin, metals and zirconia), interaction with other monomers during polymer formation, cost, and safety for oral tissues. By presenting the distinct cellular reactions triggered by each monomer, this study aims to assist manufacturers in selecting the optimal option, considering all relevant characteristics required for each application.

It is widely acknowledged that the results of in vitro studies cannot be directly extrapolated to clinical practice. However, the findings of the present study serve as a guide for future in vivo studies on the application of agents containing the evaluated monomers in teeth with very deep cavities and consequently thin dentin barriers. These results help explain observed phenomena and recommend the best materials for use.

5. Conclusions

The present study demonstrated that the toxicity of the widely used functional monomers 10-MDP and GPDM is concentration-dependent. Specifically, 10-MDP significantly reduced mitochondrial metabolism in hDPSCs to a greater extent compared to GPDM. However, both monomers induced low and similar levels of cell death, indicating relatively low toxicity at the tested concentrations. In contrast, GPDM had a more pronounced effect on the release of evaluated cytokines, particularly IL-1β, IL-6, IL-8, and IL-10.

Declaration of Competing Interest

The authors declare no potential conflicts of interest concerning the authorship or publication of this work. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001.

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