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Ultrastructural changes in human gingival fibroblasts after exposure to 2-hydroxy-ethyl methacrylate

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Abstract

Polymerized resin-based materials are successfully utilized in medical applications. One drawback is the release of monomers from the matrix due to an incomplete polymerization or degradation processes. Released monomers can diffuse in the systemic circulation and induce adverse effects to biological tissues. Although there are many hypotheses about the induction of cell death by resin monomers, the underlying mechanisms are still under discussion. The aim of the study was to investigate the morphological modifications in human gingival fibroblasts exposed to 2-hydroxy-ethyl methacrylate (HEMA) to better elucidate the mechanism of cell death induced by resin monomers. Primary cultures of gingival fibroblasts were exposed to 3mM HEMA for 24 h, 72 h, 96 h. Morphological investigations were performed by scanning and transmission electron microscopy, while western blot for caspase-3 was carried out to verify apoptosis. Electron microscopy images showed deep changes in the cell surface and cytoplasm after 72 h and 96 h of HEMA treatment. Autophagic vesicles were easily observed just after 24 h. Cleaved caspase-3 was detected after 72 h of treatment. These findings suggest that resin based materials induced cell death by the cooperation of apoptosis and autophagy mechanisms. The understanding of these mechanisms will lead to the development of smart biomaterials without or with low adverse effects.

Key words

Biocompatibility, HEMA, human gingival fibroblasts, apoptosis, autophagy.

Key to abbreviations:

HEMA: 2-hydroxy-ethyl methacrylate
PHEMA: poly(2-hydroxyethyl) methacrylate
BIS-GMA: bis-glycidyl methacrylate
BPA: bis-phenol A,
TEGDMA: tri-ethyl-glycol dimethacrylate
TEM: transmission electron microscopi
FEISEM: field emission in lens scanning electron microscopy
HGFs: Primary human gingival fibroblasts

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Introduction

The monomer 2-hydroxy-ethyl methacrylate (HEMA) has multiple uses as a synthetic polymer, *i.e.* poly(2-hydroxyethyl) methacrylate (PHEMA), which is successfully utilized in medical applications such as soft contact lenses, drug delivery scaffolds, hydrogels in biomedical engineering, artificial cornea, potential substrate for artificial skin, rhinoplastry, drug delivery systems, and bone composite materials (Ahmad et al., 2003; Oh and Matyjaszewski, 2006).

HEMA is also widely utilized in dental practice as a common component of bonding resins. It has an hydrophilic portion that allows the molecule to penetrate deeply into dentinal tubules, thus enhancing micromechanical or chemical bonding with dental matrix (Qvist et al., 1977). Monomers such as HEMA, bis-glycidyl methacrylate (BIS-GMA), bis-phenol A (BPA), tri-ethyl-glycol dimethacrylate (TEGDMA) can be released after polymerization into the oral cavity or can diffuse through the dentin into the pulp space (Gerzina and Hume, 1996; Geurtsen, 1998) inducing adverse effects (Hanks et al., 1992; Gerzina and Hume, 1996). Incomplete polymerization or mechanical and chemical degradation processes can cause residual monomer release (Geurtsen, 2000). Released monomers have been found in saliva, dentin and pulp after placement of resin based restorative materials (Bouillaguet et al., 1996) and consequent tissue inflammation, cell necrosis, local immunological reactions have been demonstrated (Jontell et al., 1995; Costa et al., 2000, 2003).

Over the past 20 years the cytotoxic effects of resin based restorative material have been assessed by cell culture studies. Resin monomers such as HEMA can induce oxidative stress and apoptosis in primary cell cultures or immortalized cell lines (Lefeuvre et al., 2005; Spagnuolo et al., 2004, 2006), in turn responsible for inhibitory effects on DNA synthesis and total protein content (Hanks et al., 1991) followed by mutagenic effects (Schweikl et al., 2006).

Although many data are available about the toxicity and the signaling pathways involved in the cell death induced by exposition to resin monomers, there are really a few investigations about the morphological modifications of the cells induced by exposition to polymerized resin-based materials. An ultra-morphological study, by electron microscopy, represents a powerful tool in evaluating the structural and functional alterations of cellular components in normal and pathological conditions, giving information on the bio-energetic status of the cell. Furthermore, electron microscopy has a pivotal role in the determination of different types of programmed cell death (Condello et al., 2013).

The aim of this study was to evaluate the ultra-morphological modifications of gingival fibroblasts after treatment with 3 mmol/L HEMA. According to our previous studies (Falconi et al., 2007) 3 mmol/L of HEMA induces a slight reduction of cell viability in human gingival fibroblasts treated for 24 h.. We tested 3 mmol/L HEMA in primary cell cultures of human gingival fibroblasts (HGF) after exposition for 24 h, 72 h, 96 h. The morphological analysis was performed by transmission electron microscopy (TEM) and high resolution scanning electron microscopy (FEISEM). Western blot for caspase-3 protein was carried out to demonstrate apoptotic death in cells treated with the resin monomer.

Materials and methods

Human gingival fibroblasts primary culture

Primary human gingival fibroblasts (HGFs) were established as described by Falconi et al. (2007). Briefly, HGFs were cultured from biopsies of healthy gingiva of permanent molars. Informed consent was obtained from the donors. The biopsies were stored at 4 °C for 24 h in DMEM/F12 culture medium (Life Technologies, Monza, Italy) supplemented with 1% penicillin and 1% streptomycin. The tissue samples were fragmented in small pieces, placed into tissue culture Petri dishes and grown in DMEM/F12 culture medium with 4.5 g/L glucose, 1% penicillin and 1% streptomycin, supplemented with 10% fetal calf serum (FCS) at 37 ◦C and 5% CO_2. When outgrowth of cells was observed, the medium was replaced twice weekly until cells reached confluence. Cells were detached from the substrate by a brief treatment with trypsin (Sigma Aldrich, St. Luis, MO) and cultured in 75 cm^2 tissue flasks until confluent monolayers were re-obtained.

Treatment of cells with HEMA

Stock solutions (1 mol/L) of HEMA were prepared in absolute ethanol and freshly diluted in DMEM prior to each experiment. HGFs from passages number 6 to 12 were seeded in 75 cm^2 tissue flasks and allowed to grow for 24 h. Then cells were washed with DMEM and exposed to HEMA for 24, 72, 96 h at concentration of 3 mmol/L. According to our previous results, 3 mmol/L HEMA after 96 h of treatment was responsible for a 40% reduction in cell viability (Falconi et al., 2007). Control cultures were incubated with medium containing 0.2% absolute ethanol for the same time periods.

Preparation of HGFs for FEISEM analysis

HGF cells were seeded on silicon chips in DMEM medium supplemented with 10% fetal calf serum for 24 h. The medium was then changed with a fresh one containing 3 mmol/L HEMA and exposed for 24, 72 and 96 hrs. Following each treatment, silicon chips were washed with phosphate buffer saline (PBS) and fixed with 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.4, for 2 h at 4 °C. After washing in cacodylate buffer, specimens were post fixed with a solution of 1% osmium tetroxide in 0.1 mol/L cacodilate buffer for 1 h at room temperature (RT), subsequently dehydrated in an increasing ethanol series and critical point dried (critical point dryer CPD 030, Bal-Tec, Lichtenstein). Samples were platinum metal coated using a sputter (MED 010, Balzers, Liechtenstein). The analysis was carried out with a FEISEM Jeol JSM 890 (Jeol, Tokyo, Japan) at 7 kV accelerating voltage and 1 x 10^{-11} A probe current.

Preparation of HGFs for TEM analysis

HGFs cultured in 75 cm^2 tissue flasks with DMEM medium were exposed to HEMA 3 mmol/L for 0, 24, 72, and 96 h, then detached from the substrate by a

brief treatment with trypsin and centrifuged at 1000 rpm for 10 minutes. Cell pellets were fixed with 2% glutaraldehyde in PBS for 1hr at 4°C and then post fixed in 1% OsO_4 for 1 hr at RT. Following a brief wash in PBS, cell pellets were dehydrated in an increasing ethanol series and embedded in Epoxy resin (Sigma Aldrich). Samples were sectioned in a Reichert Jung FC 4/E (Leica, Wien, Austria) ultramicrotome. Sections of 90 nm were mounted on grids and stained with uranyl acetate and lead citrate for 10 min at RT. The analysis was carried out by Philips CM10 TEM (FEI Company, Eindhoven, The Netherlands) and the images were digitally captured by SIS Megaview III CCD camera (FEI Company).

Protein extraction and western blot analysis

At each experimental point, the cell pellets were lysed for 30 min using a cell extraction buffer (Invitrogen, Life Technologies, Monza, Italy) supplemented with a protease inhibitor cocktail (Sigma Aldrich), 1mmol/L phenylmethylsulfonyl fluoride (PMSF) and 0.15% β-mercaptoethanol (Fluka-Sigma Aldrich). The samples were centrifuged at 14.000 rpm for 10 min a 4 °C and the total protein amount was assayed using Breadford reagent (Sigma Aldrich).

Twenty μg of total protein were resolved on NuPAGE® SDS-PAGE pre-cast Gels (4-12%) (Invitrogen), and proteins were transferred to a nitrocellulose membrane (GE Healthcare Europe, Milan, Italy), blocked with 2.5% bovine serum albumin and 2.5% no fat dry milk (Sigma Aldrich), and immunolabeled with anti-caspase-3, 1:1000 in blocking reagent (Cell Signaling Technology, Danvers, MA) and anti-β-tubulin 1:10000 in blocking reagent (Sigma Aldrich) for $2^1/_2$ h at RT. Specific secondary antibodies, horseradish peroxidase-conjugated (Sigma Aldrich), were diluted 1:80000 in TBS buffer for $1^1/_2$ h at RT.

The bands were visualized by an ECL Advanced TM Western blotting detection kit (GE Healthcare Europe) and the images were recorded using a Kodak digital image station (Eastman Kodak, Rochester, NY).

Results

FEISEM analysis

After 24 h of HEMA treatment, cells still showed a typical fibroblast morphology (Fig. 1A), with cell surface covered by several small cytoplasmic protusions. At the end of 72 h of HEMA treatment, the cell shape was drastically changed. Cells shifted from a fibroblastic morphology to a round shaped one (Fig. 1B). Several microvilli covering the cell surface and numerous cytoplasmic filaments arising from the cell bodies and allowing cell adhesion to the substrate were observed (Fig. 1B). After 96 h of HEMA treatment, HGFs showed a round morphology with the surface covered by several microvilli and protruding vesicles, presumably corresponding to apoptotic blebbing (Fig. 1C).

Control HGFs showed a fibroblastic morphology, with several cytoplasmic filaments which allow the adhesion of the cell to the substrate and interconnection with adjacent cells (Fig. 1D).

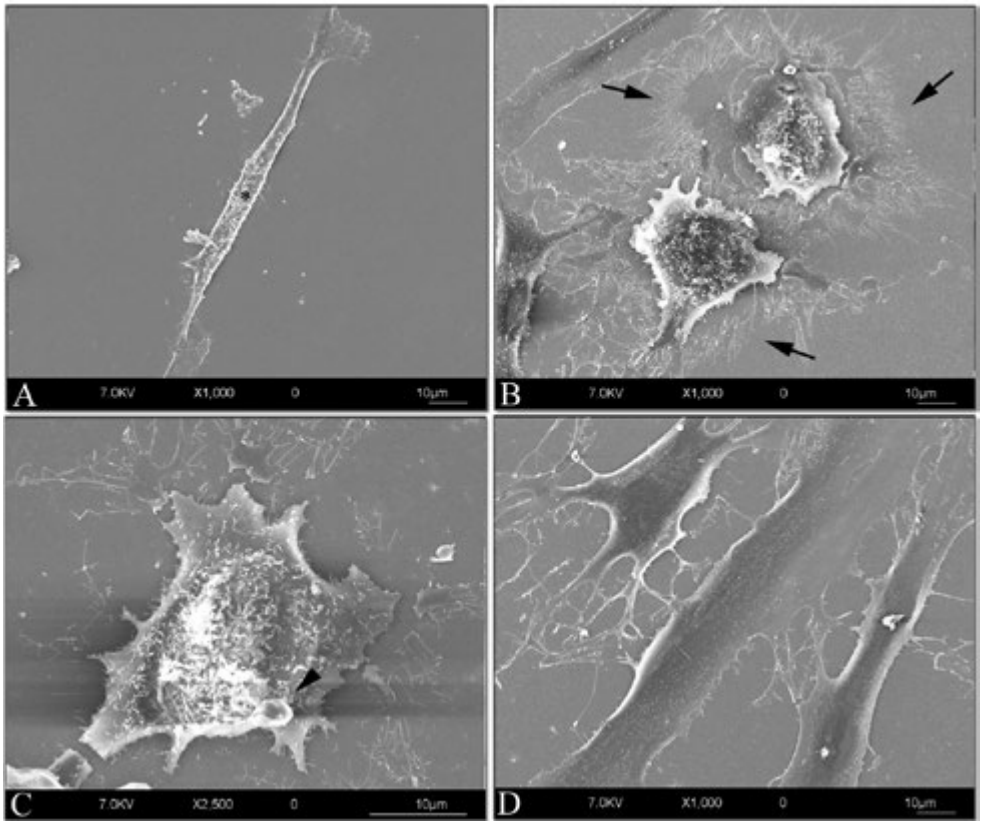

Figure 1 – Highe resolution, field-emission-in-lens SEM images of human gingival fibroblasts after HEMA exposition for (A) 24 hr; (B) 72 h; (C) 96 h; (D) untreated cells. asterisk: cytoplasmic protusions; arrows: cytoplasmic filaments; arrowhead: cell blebbing.

TEM analysis

Human gingival fibroblasts treated with HEMA for 24 h still showed a well preserved morphology (Fig. 2A). The cell nucleus with the nuclear envelope was easily detected (Fig. 2A). The cytoplasm was characterized by several mitochondria and rough endoplasmic reticulum (REG) (Fig. 2B). Many vesicles expressive of autophagy were also detected in the cytoplasm (Fig. 2B). After 72 h of HEMA exposure, cells showed a still well preserved nucleus (Fig. 2C), while the cytoplasm showed several vacuoles and autophagy vesicles (Fig. 2D). Mitochondria with dilated inner membranes (Fig. 2D, insert) and rough endoplasmic reticulum with enlarged membranes were detected (Fig. 2D). At the end of HEMA treatment (96 h) HGFs morphology was deeply changed. Nuclei were characterized by condensed areas connected with apoptotic chromatin degradation (Fig. 3A and insert). Cytoplasm showed several large and small vacuoles and numerous vesicles expressive of autophagy (Fig. 3B).

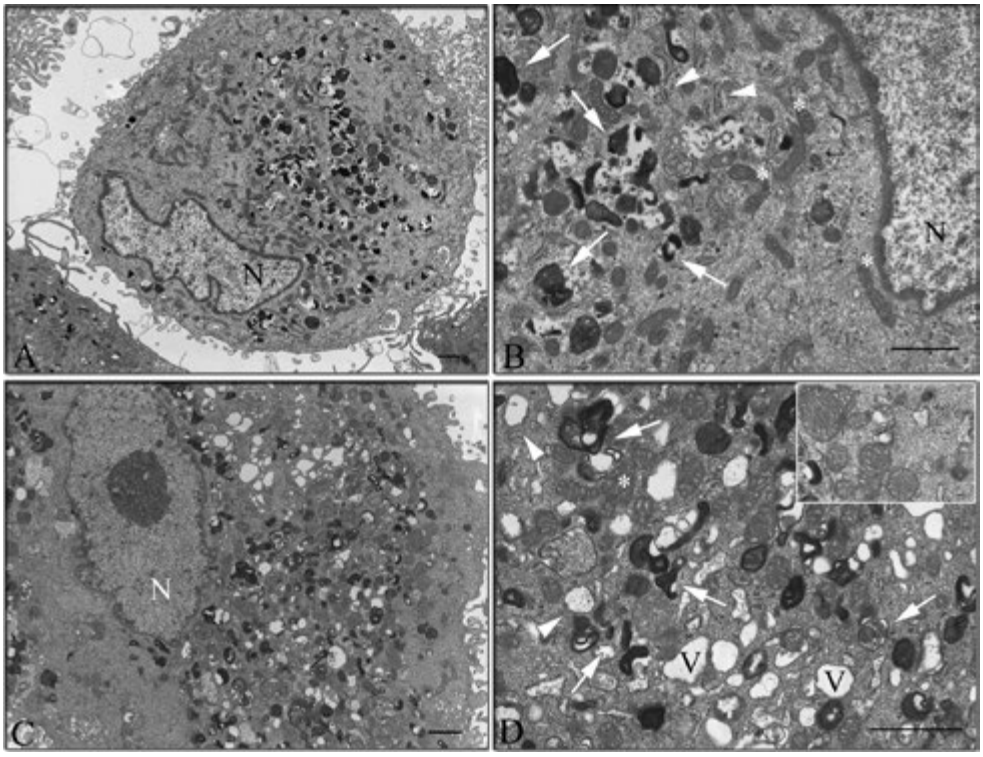

Figure 2 – TEM image of human gingival fibroblasts treated with HEMA. A: cells treated for 24 h; bar: 1m. B: Detail of the cytoplasm in which autophagic vesicles were already observed; bar: 1 µm. C: Cells exposed to HEMA for 72 h; bar: 1 µm. D: Detail of a cell exposed to HEMA for 72 h: in the cytoplasm several vacuoles and autophagic vesicles were shown and mitochondria showed dilatation in the inner membrane; bar: 1 µm; inset, bar: 100 nm. Asterisks: mitochondria; arrowheads: rough endoplasmic reticulum; arrows: autophagy vesicles; N: nucleus; V: vacuoles.

Control HGFs showed a well preserved morphology of both nucleus and cytoplasm (Fig. 3C). The rough endoplasmic reticulum was generally well developed indicating intense protein synthesis (Fig. 3D).

Western blot analysis for caspase -3

To verify if HEMA treatment induced apoptosis a western blot analysis for caspase-3 was carried out. Results showed the presence of a faint signal corresponding to the active fragment of caspase-3 (Fig. 4) on samples exposed to HEMA for 72 h, suggesting the activation of the apoptotic pathway.

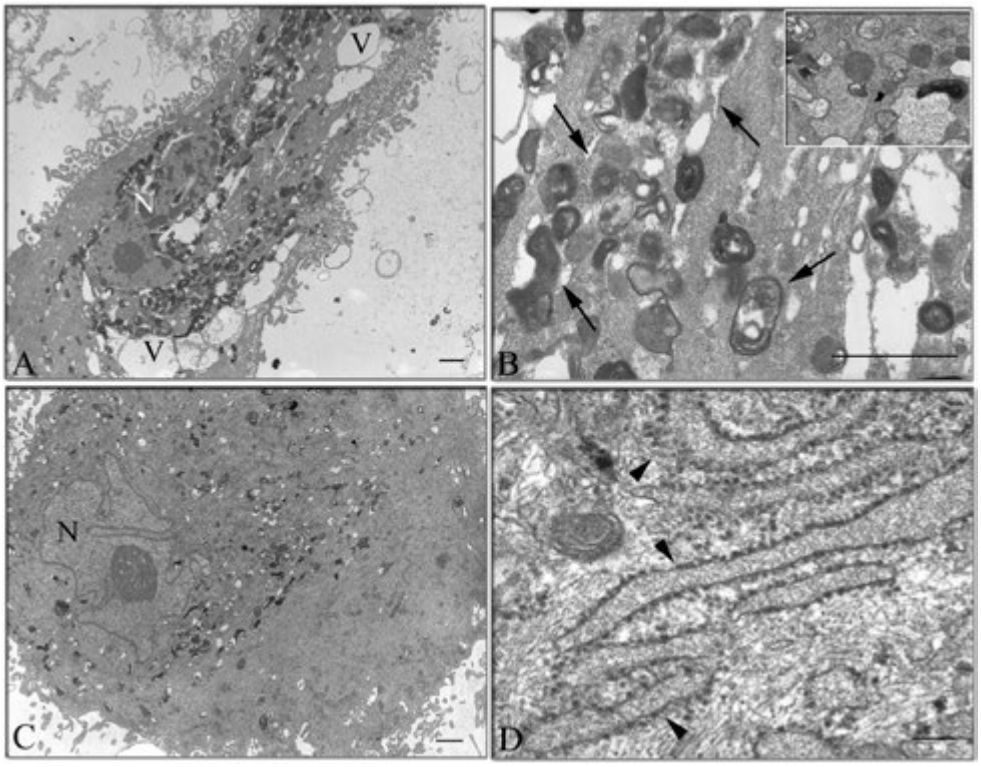

Figure 3 – TEM image of human gingival fibroblasts treated with HEMA for 96 h (A, B) or untreated (C, B). A: Overview of treated cells; bar: 1 µm. B: Detail of the cytoplasm in which autophagy vesicles were clearly detected; bar: 100 nm. C: Overview of untreated cells; bar: 1 µm. D: Detail of well-developed rough endoplasmic reticulum; bar: 100 nm. Arrowhead: rough endoplasmic reticulum; arrows: autophagy vesicles; N: nucleus; V: vacuoles.

Discussion

The clinical performance of HEMA-based materials depends on the degree of polymerization which, however, is never complete. Previous results have shown that, after polymerization, the monomer elution from cured material is about 1.5–2.5% of its total weight (Kaga et al., 2001). It is known that these eluted substances can cause adverse local and systemic effects including allergic reactions (Goon et al., 2008). In vitro studies revealed mutagenic, teratogenic and genotoxic effects of components of resin based materials (Schwengberg et al., 2005; Schweikl et al., 2006; Di Pietro et al., 2008; Urcan et al., 2010). Further results indicate that HEMA can affect osteoblastic proliferation and differentiation, and mineralization (Imazato et al., 2009).

Although there are many data about the cytotoxicity of methacrylate based materials and the signal pathways involved in stress and cell death conditions (Krifka et al., 2013), a few studies investigated the morphological and ultrastructural changes in cells exposed to resin monomers. The aim of the present work was to investigate

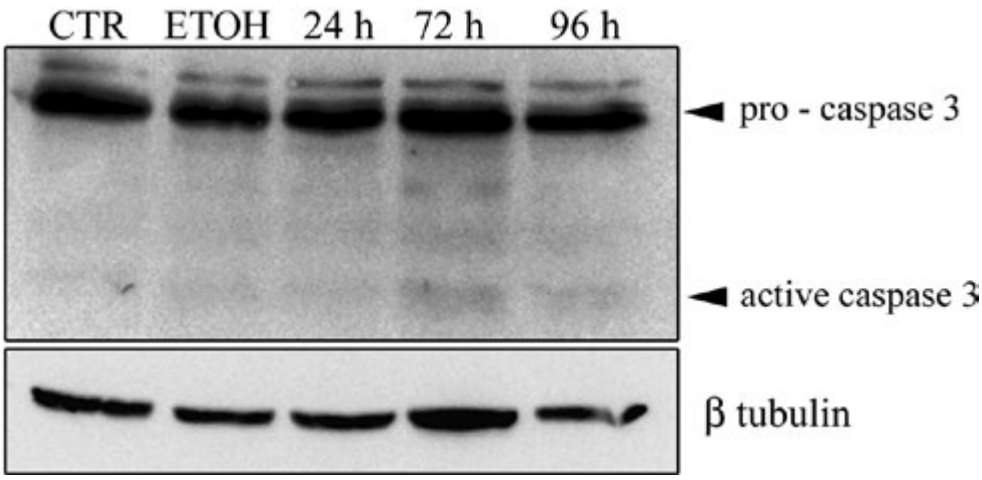

Figure 4 – Western blot for caspase-3. A faint band corresponding to active caspase-3 fragment is detectable in human gingival fibroblasts treated with HEMA for 72 h. β tubulin represents loading control.

the ultrastructure of HGFs exposed to HEMA for a period of time ranging from 24 h to 96 h. The obtained ultrastructural data, combined with molecular biology data and scientific literature, helped to elucidate the mechanism of action of resin monomers in inducing cell death.

Reichl et al. (2012) demonstrated that unpolymerized TEGDMA and HEMA remain chemically and physically unchanged and can leach up to 30 days. Geurtsen et al. (1998) found cytotoxic concentrations for TEGDMA, up to 0.26 mmol/L, and for HEMA, up to 2.5 mmol/L, in HPF. Reichl et al. (2008) indicated EC_{50} cytotoxic concentrations for HGF to be 3.7 mmol/L TEGDMA and 11.9 mmol/L HEMA.

Previous data demonstrated that 3 mmol/L of HEMA induced a downregulation of collagen type I protein (Falconi et al., 2007, 2010; Teti et al., 2009) and an up-regulation of tenascin-C protein (Zago et al., 2008), evidence of cell damage in gingival and pulp fibroblasts exposed to HEMA up to 2 weeks.

FEISEM analysis showed that HGFs lost the spindle shaped morphology and assumed a round shaped one in the course of HEMA exposition. The cell surface was characterized by blebbing, a morphological feature connected with apoptosis and oxidative stress (Condello et al., 2013).

TEM analysis confirmed these data. After 96 h of HEMA treatment, the nucleus showed several condensed areas suggesting chromatin fragmentation, a morphological feature of apoptotic death (Condello et al., 2013). The cytoplasm demonstrated heavy changes in mitochondria, with dilatation of the inner membranes, and in the rough endoplasmic reticulum, with a loss of ribosomes on the external surface and enlargement of membranes. All these morphological changes are in keeping with damages induced by oxidative stress (Condello et al., 2013). Furthermore, TEM analysis showed several autophagic vesicles in the cytoplasm after the treatment, suggesting an attempt of the cells to survive during HEMA exposition, followed by cell death (Eskelinen, 2005; Condello et al., 2013).

It has been widely demonstrated that methacrylate based materials induce oxidative stress generating high levels of reactive oxygen species (Lee et al., 2006; Spagnuolo et al., 2006; Krifka et al., 2012) followed by the onset of apoptosis (Spagnuolo et al., 2004; Samuelsen et al., 2007; Krifka et al., 2010, 2012).

Recent studies suggest that autophagy may represent a general cellular and tissue response to oxidative stress (Ryter and Choi, 2013). Autophagy is a well-known stress-induced cell survival mechanism. Under oxidative stress, large amounts of reactive oxygen species oxidize proteins and affect their functions. Autophagy is able to degrade these oxidized proteins (Huang et al., 2011), allowing cell to survive. When oxidative stress reaches a level beyond the control of cellular protective mechanisms, cell death will occur through necrosis, apoptosis, or autophagic cell death (Huang et al., 2011). It could be supposed that methacrylate based materials induce cell death by a combination of autophagic and apoptotic cell death. In particular, resin monomers induce oxidative stress which initially triggers autophagy as a mechanism of survival; however, in the presence of high reactive oxygen species levels, autophagy shifts from a mechanism of survival to a mechanism of cell death.

TEM analysis showed chromatin fragmentation in HGFs exposed to HEMA, compatible with apoptoic death, while the cytoplasm of cells was characterized by several autophagic vesicles, suggesting the participation of both cell mechanisms of death. Western blot results showed a faint signal of cleaved caspase-3, corresponding to the active fragment of the protein executing apoptosis.

In conclusion, our study provides insight into the morphological damages induced by exposition to resin based materials, major components of biomaterials for medical devices. The ultrastructural analysis allowed to detect morphological details connected with mechanisms of cell death such as apoptosis and autophagy, whose signalling pathways are still under discussion. The understanding of these mechanism will lead to the development of smart biomaterials without or with low adverse effects.

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