

## Effects of HEMA on type I collagen protein in human gingival fibroblasts

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### Abstract

The cytotoxicity of dental composites has been attributed to the release of residual monomers from polymerized adhesive systems due to degradation processes or the incomplete polymerization of materials. 2-Hydroxyethyl methacrylate (HEMA) is one of the major components released from dental adhesives. Cytotoxic effects due to high concentrations of HEMA have already been investigated, but the influence of minor toxic concentrations on specific proteins such as type I collagen has not been studied in depth. The objective of this project was to study the effect of minor toxic concentrations of HEMA on human gingival fibroblasts (HGFs), investigating modification in cell morphology, cell viability, and the influence on type I collagen protein. Primary lines of human gingival fibroblasts were exposed to 3 mmol/L HEMA for different periods of time (24 h, 72 h, 96 h). The cell vitality was determined by MTT assay, and high-resolution scanning electron microscopy analysis was performed to evaluate differences in cell morphology before and after treatment. The presence and localization of type I collagen was determined by immunofluorescence in HGFs treated with HEMA for the same period of time. The vitality of the cells decreased after 72 h of exposure. The HGFs grown in monolayer and observed by field emission in-lens scanning electron microscopy demonstrated a preserved surface morphology after 24 h of treatment, while they showed an altered morphology after 96 h of treatment. Immunofluorescence demonstrated a reduction of type I collagen due to HEMA exposure after 96 h. From these results, we conclude that low concentrations of HEMA can significantly alter the morphology of human gingival fibroblasts and interfere with the presence of type I collagen protein.

**Abbreviations:** HEMA, 2-Hydroxyethyl methacrylate; HGF, human gingival fibroblast; FEISEM, field emission in-lens scanning electron microscopy; TC<sub>50</sub> (concentration responsible for 50% of cell death)

### Introduction

Previous *in vitro* studies have provided evidence of the release of monomers into the oral cavity from dental resin-based restorative material (Hanks et al., 1988; Gerzina and Hume, 1995,

1996; Pashley, 1996; Geurtsen, 1998a, 2000). Leaching from resin may occur during the setting period of the material and later when the resin is degraded. After the hydrolytic degradation of polymers carried out by the tissue, the bacterial

and salivary esterases are able to further hydrolyze polymers over a long period (Freund and Munksgaard, 1990; Munksgaard and Freund, 1990). The amount of the monomer release ranges from micrograms to milligrams (Spagnuolo et al., 2006) and it has been demonstrated to be responsible for many cytotoxic and metabolic events (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., 2000, 2003a,c).

Studies on the degradation of dental biomaterials have confirmed the release of substances such as 2-hydroxyethyl methacrylate (HEMA) and triethyleneglycol dimethacrylate (TEGDMA) from these resins (Goldberg et al., 1994; Gerzina and Hume, 1995; Bouillaguet et al., 1996). HEMA is frequently used in dental bonding resins as a wetting agent. It competes with water for penetration and infiltration into the dentin, and it copolymerizes with other monomers of resin composites (Peutzfeldt, 1997). HEMA has been shown to diffuse rapidly across the dentin toward the pulp which, in turn, could induce hypersensitivity reactions in susceptible individuals (Pashley, 1996).

Previous *in vitro* studies have already shown the cytotoxic effects of HEMA on human fibroblasts and have identified the concentrations that cause 50% toxicity ( $TC_{50}$ ) when compared with controls (Yoshii, 1997; Issa et al., 2004). A  $TC_{50}$  concentration of HEMA ranging from 10  $\mu\text{mol/L}$  to 10  $\text{mmol/L}$  has been demonstrated, mainly by mitochondrial dehydrogenase activity (MTT assay) and lactate dehydrogenase activity (LDH assay). It has been demonstrated that HEMA can induce genotoxicity (Schweickl et al., 2005) and apoptosis (Janke et al., 2003; Mantellini et al., 2003; Engelmann et al., 2004; Spagnuolo et al., 2004; Paranjpe et al., 2005); it interferes with the cell cycle and DNA synthesis (Hanks et al., 1991; Chang et al., 2005), increases the production of reactive oxygen species (ROS) (Chang et al., 2005; Spagnuolo et al., 2006), and induces a strong depletion of intracellular glutathione level even after

very short times of exposure (Volk et al., 2006). Many of these data have shown the cytotoxic effects of HEMA at concentrations near the  $TC_{50}$  value while very few studies have demonstrated HEMA influence in cells exposed to minor toxic concentrations (Costa et al., 1999; Bouillaguet et al., 2000) and the effect of these monomers on the expression of specific proteins (About et al., 2002). Furthermore, the influence of dental materials on the morphology of cells exposed to minor toxic concentrations is not well known or understood. To this end, we suggest high-resolution scanning electron microscopy (FEISEM) as a powerful complementary tool in the evaluation of the toxicity of biomaterials. FEISEM is field emission in lens scanning electron microscopy, which allows the observation of biological samples at high resolution, even without metal coating (Rizzoli et al., 1994; Gobbi et al., 1999; Falconi et al., 2006).

The purpose of this study was to evaluate the morphological changes on human gingival fibroblasts exposed to a minor toxic concentration of HEMA for different periods of time, and to combine these data with the potential effect of HEMA on the expression of type I collagen protein. Our null hypothesis is that HEMA is not cytotoxic at the concentration we tested on human fibroblasts.

We tested a minor toxic concentration of HEMA for prolonged periods of time to simulate the constant release of unreacted resin monomers from dental adhesives over time (Hanks et al., 1988; Bouillaguet et al., 1996; Costa et al., 1999).

## Materials and methods

### *Culture of human gingival fibroblasts*

Human gingival fibroblasts (HGFs) were obtained from biopsies of gingival tissue from healthy normal volunteers. The biopsies were taken during routine oral surgery and we obtained informed consent from the donors according to the guidelines of the National Bioethics Committee. The specimens were rinsed three

times in  $1\times$  phosphate-buffered saline solution (PBS), minced into small pieces and cultured in Dulbecco's Modified Eagle's Medium (DMEM)/F12, containing 10% fetal bovine serum (FBS), 1% penicillin and streptomycin and 1% fungizone, and were cultivated in 25 and 75 cm<sup>2</sup> flasks. All cells were maintained at 37°C in a humidified atmosphere of 5% (v/v) CO<sub>2</sub>.

#### *MTT assay*

To determine the minor toxic concentrations of HEMA on HGF primary culture we first carried out an MTT assay, testing different concentrations of the monomer.

The cell viability of the HGFs was analyzed 24 h after the treatment using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (*In Vitro* Toxicology Assay kit MTT based, Sigma, St. Louis, MO, USA). Cells ( $10^5$  per well) were seeded into each well of a 6-well culture plate with DMEM containing 10% FBS, 1% penicillin and streptomycin and 1% fungizone. After 24 h, the medium was changed to a fresh one containing 1 mmol/L, 3 mmol/L, 5 mmol/L, 7 mmol/L, 10 mmol/L of HEMA and all samples were left for 24 h. After incubation, the cells were washed with PBS and the medium was changed to a new one containing 0.5 mg/ml MTT in DMEM and left for 2 h. The cells and the dye were then solubilized in solvent solution and optical density was read at 570 nm. All readings were performed with an ND-1000 NanoDrop Spectrophotometer (NanoDrop Technologies, Rockland, DE, USA).

A further MTT assay was carried out to test the vitality of the HGFs incubated with 3 mmol/L HEMA for different periods of time. We performed the second MTT assay following the conditions described above except that, after 24 h of seeding, the HGFs were incubated with fresh medium containing 3 mmol/L of HEMA and were exposed for 24 h, 72 h, and 96 h. Both MTT assays were performed in three independent experiments.

#### *Cell morphology analysis with FEISEM*

For FEISEM analysis, the HGFs were seeded on silica wafers placed in the bottom of each well of a 6-well culture plate. For each treatment two silica wafers were prepared and the entire experiment was performed three times. After 24 h of growth, the cells were exposed to 3 mmol/L HEMA for 24 h, 72 h, and 96 h. At the end of the treatment, the cells were postfixed in a solution of 2% glutaraldehyde in PBS, for 1 h at room temperature (RT). After washing in the same buffer, the samples were immersed in a solution of 1% OsO<sub>4</sub> in PBS for 30 min at RT. The specimens were dehydrated in an ethanol series (70%, 90%, 100%) and critical-point dried (CPD 030, Balzers, Lichtenstein), and coated with platinum metal using a sputter (MED 010 Balzers, Liechtenstein). Finally, the samples were observed using a field emission in lens scanning electron microscope (JSM 890, Jeol LTD, Tokyo, Japan), at 7 kV accelerating voltage and  $1 \times 10^{-11}$  A probe current.

#### *Immunofluorescence for type I collagen*

The HGFs were grown on cover glasses and treated with HEMA 3 mmol/L for 24 h, 72 h, and 96 h. For each treatment, two cover glasses were prepared and the entire experiment was performed for three times. The samples were washed three times in PBS and fixed with 4% formalin–0.1% Triton for 20 min at 4°C. After rinsing in PBS three times for 5 min each, the samples were blocked in 1% dry milk in PBS (Carnation natural non fat dry milk, Carnation Company, Los Angeles, CA, USA) for 30 min at RT and were then incubated with anti-type I collagen antibody (Sigma) diluted to 1:100 in blocking reagent at 37°C for 1 h. After three washes in PBS for 10 min each, the samples were incubated with CY<sub>3</sub>-conjugated anti-mouse IgG antibody (Sigma) diluted to 1:2000 in blocking reagent at 37°C for 1 h. Finally, the slides were washed three times in PBS and then mounted in VECTASHIELD mounting medium with 4',6-diamidino-2-phenylindole

(DAPI) (Vector Laboratories, Burlingame, CA, USA). The slides were observed using a fluorescence microscope (Nikon Eclipse E800, Tokyo, Japan).

#### Controls for type I collagen immunofluorescence

The samples fixed with 4% formalin–0.1% Triton were incubated with only the secondary antibody to check the presence of a nonspecific interaction between the antibody and the free aldehyde groups of fixative. All the samples showed a low spread fluorescence signal (data not shown). A further control was the incubation of samples fixed with 4% formalin–0.1% Triton with an isotype-matched irrelevant antibody on the HGFs under the same conditions as previously described. The results demonstrated no contrast between the two different conditions.

#### Statistical analysis

The data on MTT activity are presented as mean ( $\pm$ SD) of triplicate experiments. Statistical differences in MTT activity were assessed by one-way ANOVA ( $p < 0.05$ ) and Dunnett's multiple comparison test ( $p < 0.001$ ). Statistical analysis of the MTT data was performed with GRAPH PAD PRISM 3.0 software (San Diego, CA, USA).

## Results

#### MTT assay

Figure 1(a) shows HGF viability expressed as percentage relative to HGFs not exposed to HEMA (0 mmol/L). The toxicity of HEMA depends on its concentration. Under our experimental conditions, the  $TC_{50}$  (concentration responsible for 50% of cell death) was 5.83 mmol/L. We performed the subsequent experiments with the concentration of 3 mmol/L, which corresponded to a cell viability of 78%. The HGFs were exposed to 3 mmol/L HEMA for 0 h, 24 h, 72 h, and

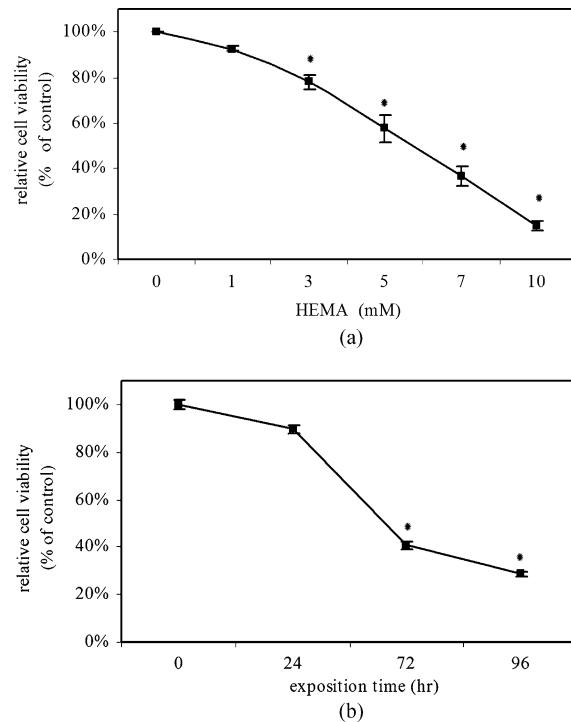
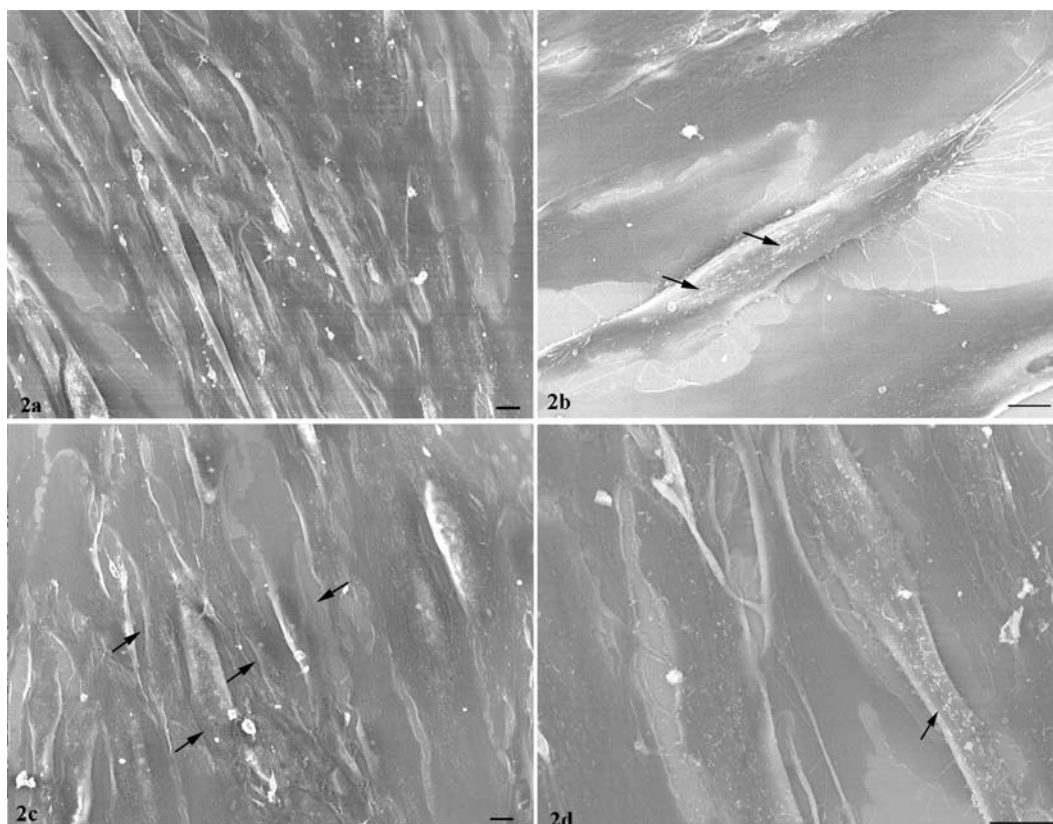


Figure 1. (a) Cytotoxic effect of HEMA on the viability of HGFs. The cells were exposed to various concentrations of the monomer for 24 h and the viability was determined by MTT assay. (b) Cytotoxic effect of HEMA on the viability of HGFs exposed to 3 mmol/L of the monomer for 0 h, 24 h, 72 h, and 96 h. At 72 h of treatment there is a decrease in cell viability. The data represent the mean ( $\pm$ SD) of triplicate experiments per condition and are expressed as a percentage of the control value. \*Statistically significant differences between groups ( $p < 0.001$ ).

96 h. Figure 1(b) shows a reduction in cell viability at 72 h and 96 h of exposure. After 96 h of exposure, cell viability is reduced to 35%; thus 3 mmol/L HEMA with 96 h of treatment is cytotoxic.

#### HGF analysis by FEISEM

To evaluate the effects of HEMA on the cell morphology we observed the surface of HGFs grown in the presence of 3 mmol/L HEMA for 0 h, 24 h, 72 h, and 96 h. The analysis was performed using FEISEM. Figure 2a shows HGFs grown in monolayer without any treatment. The cells were always



**Figure 2.** FEISEM images showing HGFs after HEMA treatment. (a) HGFs without any treatment. The area of silica support is covered by a high number of cells ( $\times 1000$ ; bar =  $10\ \mu\text{m}$ ). (b) FEISEM image of fibroblasts showing the typical shape. Short cells processes cover the surface of the fibroblasts (arrow) ( $\times 2000$ ; bar =  $10\ \mu\text{m}$ ). (c) HGFs after 24 h of HEMA treatment. The image shows the typical fibroblastic morphology comparable to untreated cells (arrows) ( $\times 1000$ ; bar =  $10\ \mu\text{m}$ ). (d) At higher magnification, the cells still have the fibroblastic shape and many processes on the surface (arrow) ( $\times 3000$ ; bar =  $10\ \mu\text{m}$ ).

close to confluence and, at higher magnification, gradual narrowing of shape toward both ends is observed, which corresponds to the typical morphology of fibroblasts (Figure 2b). The surface of the cells was covered by short cell processes (Figure 2b).

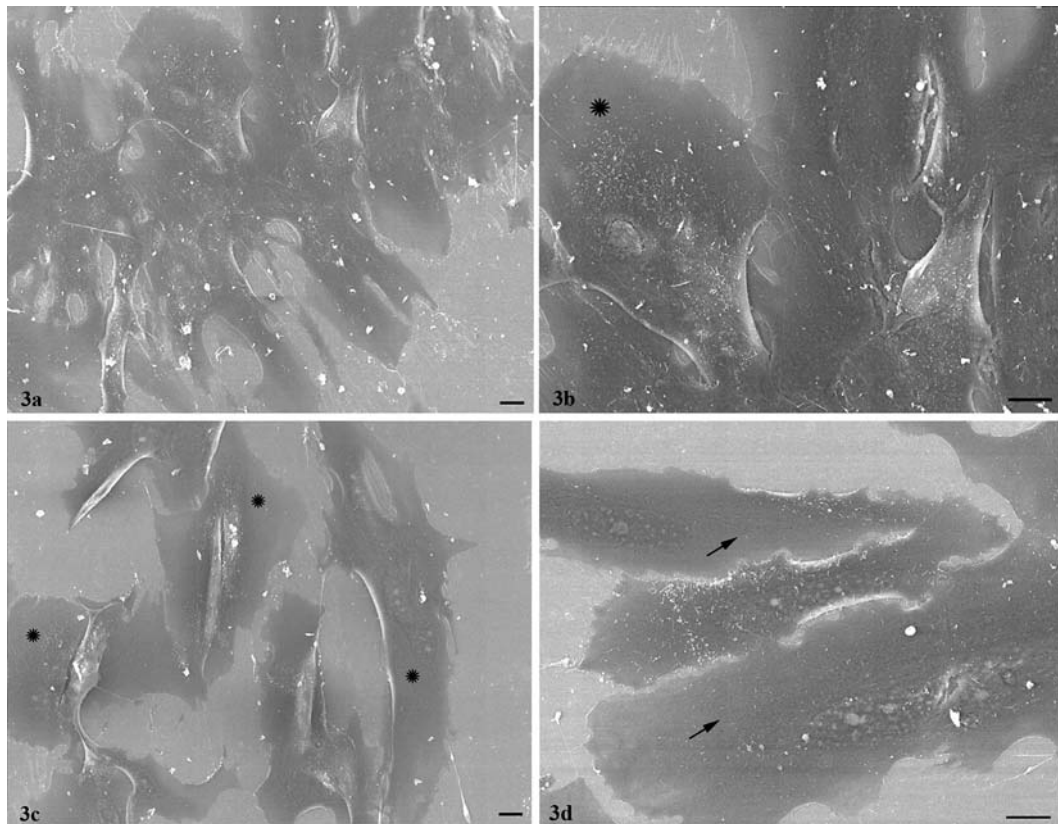
After 24 h of HEMA treatment, HGFs showed a morphology comparable to the untreated cells (Figure 2c, 2d). At higher magnification, the cells still had fibroblastic shape (Figure 2d).

After 72 h of HEMA exposure, the number of HGFs was moderately reduced (Figure 3a). Cells lost the fibroblastic morphology and appeared to lack a regular shape (Figure 3b).

After 96 h of HEMA exposure, there was a notable reduction in the number of cells (Figure 3c) and the fibroblastic morphology had almost disappeared. Indeed, the cells appeared to have an irregular shape and to have lost the majority of cell processes on the surface (Figure 3d).

#### *Immunofluorescence for type I collagen*

To test the interference of HEMA with the production of fibroblastic proteins, immunostaining was carried out for type I collagen in HGFs with and without HEMA exposure. Samples without any treatment showed type I collagen organized in



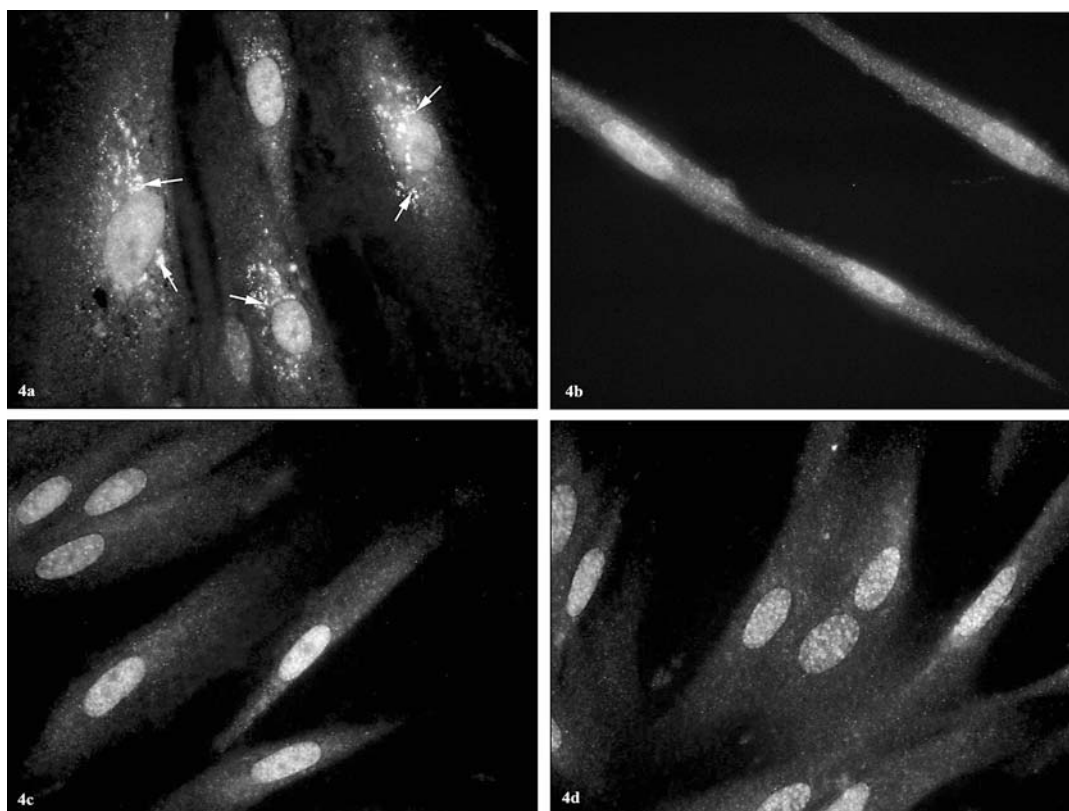
*Figure 3.* FEISEM images showing HGFs after HEMA treatment. (a) HGFs after 72 h of HEMA exposure. The number of cells is slightly reduced ( $\times 1000$ ; bar = 10  $\mu\text{m}$ ). (b) HGFs appear with an irregular shape (\*) ( $\times 2000$ ; bar = 10  $\mu\text{m}$ ). (c) HGFs after 96 h of HEMA exposure. There is a strong reduction in the number of cells and the fibroblastic morphology almost disappears (\*) ( $\times 1000$ ; bar = 10  $\mu\text{m}$ ). (d) Cells show few short processes on the surface (arrows) ( $\times 2000$ ; bar = 10  $\mu\text{m}$ ).

small clusters around the cell nucleus (Figure 4a). This labeling pattern was reduced in cells after 24 h of exposure (Figure 4b) and almost disappeared in samples after 72 h (Figure 4c) and 96 h (Figure 4d) of exposure.

We obtained the same results in HGFs grown in monolayer on silica support, immunolabeled for type I collagen and processed for FEISEM observation. In this case, a secondary gold-conjugated antibody was utilized as a detection system and, even when the HGFs showed a modified morphology due to the detergent/fixation step, the gold signal was specifically inside the cells and decreased during the period of treatment (data not shown).

## Discussion

HEMA is one of the main components in dental restorative materials and it is also used in other fields such as ophthalmology for the production of contact lenses (Goda et al., 2006; Lord et al., 2006) and in drug delivery and tissue engineering (Mei et al., 2005). Owing to its multiple applications, the biocompatibility of HEMA has been widely tested for many years in order to evaluate its cytotoxic effects. Because results regarding HEMA biocompatibility are sometimes contradictory and confusing, it is necessary to develop investigations based on a great variety of experimental



*Figure 4.* Immunocytochemical localization of type I collagen in HGFs treated with HEMA for different periods of time. CY3-conjugated anti-mouse IgG antibody was used to detect the localization of type I collagen. All samples were counterstained with DAPI. (a) HGFs without any treatment. CY3 signal localizes around the cell nucleus appearing as small clusters (arrow) ( $\times 600$ ). (b) HGFs after 24 h of HEMA treatment. The fluorescence signal is reduced and the clusters around the nucleus disappear ( $\times 600$ ). (c, d) HGFs after 72 h (c) and 96 h (d) of HEMA treatment. In both images, the signal of type I collagen is strongly reduced ( $\times 600$ ).

parameters to produce a better understanding of the biocompatibility of resin monomers (Schmalz, 1997).

In the present study, the effects of minor toxic concentrations of HEMA on human gingival fibroblasts were assessed by MTT assay, high-resolution scanning electron microscopy and immunofluorescence microscopy. MTT assay is currently used in biocompatibility tests to evaluate cell viability (Costa et al., 1999, 2003c; Yoshii, 1997), while high-resolution scanning electron microscopy and immunofluorescence microscopy are seldom taken into consideration (Costa et al., 1999, 2003b).

There is evidence that the expression of cytotoxicity can vary significantly depending on the type of cells used (Hanks et al., 1991; Wataha et al., 1994). We chose human gingival fibroblasts because in the oral cavity they are in close proximity to restorative dental materials. A wide range of toxic concentrations for resin monomers has been reported. Moharamzadeh and colleagues (2007) demonstrated that the lethal concentration of a specific dental material can vary with different cell lines and between the same types of cells obtained from different donors.

Because we had developed our own primary cell culture of human gingival fibroblasts, we decided

to test different concentrations of HEMA in order to identify which were the non-toxic concentrations. We tested concentrations ranging from 1 mmol/L to 10 mmol/L and performed an MTT assay to check cell viability. Under our experimental conditions, the  $TC_{50}$  concentration was 5.8 mmol/L; thus, we chose the minor toxic concentration of 3 mmol/L in order to investigate the risk of toxicity resulting from this low concentration. Previous studies have reported different  $TC_{50}$  values of HEMA compared to ours (Ratanasathien et al., 1995; Geurtesen et al., 1998; Bouillaguet et al., 2000). Rakich and colleagues (1998) demonstrated a  $TC_{50}$  value of 10 mmol/L for HEMA after 24 h of treatment in THP-1 monocyte-macrophages, while another study reported a  $TC_{50}$  of HEMA of 3.6 mmol/L in 3T3 fibroblasts (Bouillaguet et al., 1996). This difference has been suggested to be related to different sensitivities between different cell types (Bouillaguet et al., 2000). However, the degree of toxicity obtained under our experimental conditions was similar to those reported by other studies (Moharamzadeh et al., 2007; Issa et al., 2004).

According to Spagnuolo et al. (2006) the release of HEMA from polymerized dental adhesives ranges from 1.5 mmol/L to 8 mmol/L. Leaching of resin monomers is high in the first 24 h and the most toxic effects occur during this time. However, a continued release of resin based materials occurs for longer periods (Çetingüç et al., 2007; Gerzina et al., 1996). Our purpose was to simulate the effects of this release *in vitro* in order to study the cell morphology and cell activity. A second MTT assay was carried out on HGFs exposed to 3 mmol/L of HEMA for different periods of time ranging from 0 h to 96 h. The cell viability starts to decrease after 72 h of exposure, which means that 3 mmol/L of HEMA is able to reduce cell proliferation after this time. Our data are in agreement with previous studies in which it was shown that the toxicity of HEMA generally increases with time of exposure (Bouillaguet et al., 1996, 2000).

HGF morphology observed using FEISEM after HEMA exposure showed mild modification of

the cell surface after 72 h, while the fibroblastic shape clearly changed into an irregular one after 96 h, also with a reduction in the number of cell processes that normally covered the cell surface in untreated cells. There are not many studies of modification of cell morphology after exposure to dental materials. Costa et al. (1999, 2003b) demonstrated a stronger decrease in the number of cells attached to the substrate and a more dramatic change in the shape of an immortalized odontoblast cell line (MDPC-23). However, the authors tested three different commercial adhesive systems in which HEMA was one of the monomers but the concentration was not known. Other authors have reported a concentration of HEMA in commercial adhesive systems ranging from 4.09 mol/L and 2.60 mol/L (Çetingüç et al., 2007; Costa et al., 1999), definitely higher than 3 mmol/L.

We performed immunofluorescence staining for type I collagen protein and a strong reduction of the fluorescence signal was observed after 96 h. Our data are in agreement with previous results showing that, when resin monomers were added to pulp cell culture, they affect the protein expression of type I collagen, osteonectin and dentin sialoprotein (DSP) (About et al., 2005).

Nishiyama et al. (2002, 2003) examined the characteristics of adsorption of HEMA to collagen and suggested a direct interaction between the ester carbonyl group of HEMA and the undissociated carboxylic acid of the collagen. This interaction could be responsible for a modification in the three-dimensional structure of the type I collagen leading to a reduced interaction of the primary antibody with the specific epitope.

The cytotoxic effects of those monomer concentrations normally released by resin composites (Spagnuolo et al., 2006) are not well known because the majority of studies are based on testing concentrations responsible for cell death. For a complete understanding of all the possible cytotoxic effects, it is necessary to test minor toxic concentrations for longer periods corresponding to the exposure time of dental materials in contact

with tissues of the oral cavity. These conditions may not induce cell death but, instead, an underestimated degree of damage.

Our results demonstrate that 3 mmol/L HEMA does not induce cell death but causes a modification in the surface morphology of HGFs and a decrease in the type I collagen signal. These alterations could be a consequence of altered cell activity which is responsible for cell damage.

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