
HEMA down-regulates procollagen α 1 type I in human gingival fibroblasts

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Abstract: 2-Hydroxyethyl methacrylate (HEMA) can be released from restorative materials and diffused into the tooth pulp over long periods of time. Although cytotoxicity due to high concentrations of monomers has been well studied, little is known about the risk of chronic toxicity resulting from low concentrations. The purpose of the study was to evaluate the effects of a minor toxic concentration of HEMA in the synthesis and expression of procollagen α 1 type I produced by human gingival fibroblasts (HGF). HGF were exposed to 3 mM HEMA from 24 to 96 h. An MTT assay was performed to evaluate cell viability while reverse-transcriptase polymerase chain reaction (RT-PCR), real-time polymerase chain reaction (real-time PCR), and Western-blot analysis were carried out to evalu-

ate the variability in the expression and synthesis of procollagen α 1. Immunofluorescence was performed to detect the protein inside the cells. The results showed that there was a strong reduction of procollagen α 1 type I expression at 72 and 96 h. These findings demonstrate that, even if it does not reduce cell viability, 3 mM HEMA interferes both with the synthesis of the procollagen α 1 type I protein and its mRNA expression, suggesting that normal cell production and activity are modified by HEMA at concentrations below those which cause acute cytotoxicity. © 2008 Wiley Periodicals, Inc. *J Biomed Mater Res* 90A: 256–262, 2009

Key words: biocompatibility; HEMA monomer; human gingival fibroblasts; procollagen alpha 1 type I; PCR

INTRODUCTION

The “silent adhesive revolution” and the recent developments in composite resins changed the approach to restorative dentistry allowing for the extended clinical use of resin-based restorative materials.¹ Currently, resin-based materials are widely used due to their capability of bonding the residual tooth structure,² thus increasing the fracture resistance of the tooth/restoration complex.^{3,4}

Despite extensive use, recent findings indicate adverse biological reactions related to the use of resin-based materials, which may cause skin disease (mainly in dental personnel) due to allergic or irri-

tant dermatitis.^{5,6} Similarly, in cell cultures, acute cytotoxic effects were reported and related to contact with unreacted monomers released from restorative materials in relation to suboptimal curing, aging, water storage, and/or specimen preparation techniques.^{7–9} In fact, the polymerizable matrix contains monomers, which can be released from restorations and eluted in the oral fluids, or diffused over time throughout the residual dentin thickness reaching the tooth pulp.¹⁰

Residual monomers are released from resin-based restorative materials into the oral cavity after polymerization in microgram to milligram amounts.^{11,12} Previous *in vitro* studies have demonstrated that monomer release is higher just after polymerization while it decreases over time.¹³ However, a toxic effect on cell metabolism was found even 160 h after the polymerization.¹⁴

Several recent studies have revealed that released monomers can induce different toxic effects such as tissue inflammation,^{11,14,15} oxidative cell damage (due to the depletion of the intracellular glutathione

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level),^{12,16,17} apoptosis,¹⁸ genotoxic effects,¹⁹ and inhibition of DNA and protein synthesis.²⁰

2-Hydroxyethyl methacrylate (HEMA) is frequently found in aqueous eluates from polymerized dental resinous biomaterials.¹¹ HEMA is one of the most common components of dentin-bonding systems, and its role is pivotal during the dentin impregnation process of the adhesive system due to its high water affinity, which allows HEMA to flow into the collagen network of the dentin organic matrix, thus favoring infiltration and preventing collagen collapse. Because HEMA has a low molecular weight and high hydrophilicity, it can also diffuse throughout the residual dentin and affect the underlying odontoblast vitality, altering cell division and activity.^{14,21}

Several previous studies have examined the cytotoxicity of HEMA on mammalian cell cultures, reporting variable levels of cytotoxicity.^{9,12,20,22,23} However, only very few studies have demonstrated the influence of HEMA on gene expression and the synthesis of specific proteins in gingival and pulp cells.^{24,25}

The purpose of this study was to assay the mRNA expression and the synthesis of the procollagen $\alpha 1$ type I protein in primary cultures of human gingival fibroblasts (HGF) after exposure to HEMA for different periods of time. The effect of HEMA on cell viability of a primary culture of HGFs was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and on procollagen $\alpha 1$ type I synthesis using immunofluorescence and Western-blot analysis. A reverse transcriptase PCR (RT-PCR) and a relative quantitative real-time PCR allowed the analysis of possible effects on the expression of the gene *COL1A1*, which produces the procollagen $\alpha 1$ type I mRNA (precursor of collagen type I protein). The null hypotheses tested were (1) HEMA has no effect on the expression of procollagen $\alpha 1$ type I mRNA, and its synthesized protein in primary cultures of HGF and (2) the effect of HEMA is not related to exposure time.

MATERIALS AND METHODS

Culture of human gingival fibroblasts

HGF was obtained from fragments of healthy marginal gingival tissue from the retromolar area taken during surgical extraction of impacted third molars. Signed informed consent was obtained from the donors according to a protocol approved by the University of Bologna. The tissue fragments were immediately placed in Dulbecco's modified Eagle's medium (DMEM)/F12 for at least 1 h, rinsed three times in phosphate-buffered saline solution (PBS), minced into small tissue pieces, and cultured in DMEM/

F12, containing 10% fetal bovine serum (FBS), 1 \times penicillin and streptomycin, 1 \times fungizone. Cells were maintained at 37°C in a humidified atmosphere of 5% (v/v) CO₂. Cultured HGF of passage 4–8 were used for these studies.

MTT assay

The cell viability of HGFs was measured by an MTT test. Cells were seeded into a 96-well culture plate with DMEM containing 10% FBS, 1 \times penicillin and streptomycin, and 1 \times fungizone in a density of 10⁴ cells/well. After 24 h, the medium was changed to a fresh one containing 3 mM HEMA (previously dissolved in absolute ethanol). We chose to test 3 mM HEMA according to the previous studies in which it was demonstrated that 3 mM HEMA was responsible of a reduction of cell viability lower than 50%.²⁶

The specimens were exposed to 3 mM HEMA for 0, 24, 72, and 96 h. After incubation, the cells were washed with PBS, and the medium was changed to 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 (0.1N HCl in Isopropanol), and the optical density was read at 570 nm. All readings were carried out using an ND-1000 NanoDrop[®] Spectrophotometer (NanoDrop Technologies, Rockland, DE). The MTT assay was performed in three independent experiments, three replicate wells for each experimental point.

Protein extraction and Western-blot analysis

HGF, incubated with 3 mM HEMA for 0, 24, 72, and 96 h, was trypsinized after treatment and centrifuged at 1250 rpm for 10 min at 4°C. The pellets were lysed with a radioimmuno precipitation assay (RIPA) modified buffer containing a protease inhibitor cocktail (Sigma Aldrich, Saint Louis, Missouri) and then centrifuged at 14,000 rpm for 10 min at 4°C. A protein assay as described by Bradford²⁷ was performed to quantify the amount of proteins obtained in each sample. Then, 20 μ g of total protein for each sample was separated on 8% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and then electrophoretically transferred into a nitrocellulose membrane using a wet-blotting apparatus (Mini Tank Electroblothing System, Owl, Portsmouth, UK). The membranes were saturated in 5% dry milk (Carnation natural nonfat dry milk, Carnation Company, LA) in Tris buffer solution/0.1% Tween 20 (blocking reagent) for 2 h at room temperature (RT) and then incubated with antiprocollagen $\alpha 1$ type I antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or anti- β tubulin antibody (Sigma Aldrich) diluted 1:10,000 in blocking reagent for 1 h at 37°C followed by horseradish peroxidase-conjugated anti-goat IgG antibody (Santa Cruz Biotechnology) for antiprocollagen antibody and horseradish peroxidase-conjugated anti-mouse IgG antibody (Sigma Aldrich) for anti- β tubulin antibody. Both antibodies were diluted 1:80,000 in blocking reagent for 1 h at 37°C. Bands were visualized with the chemiluminescence detection system (ECL plus, Amersham Biosciences, Little Chalfont

Buckinghamshire, UK). Images were obtained by Image Station 2000R (Kodak, NY, USA).

Immunofluorescence for procollagen $\alpha 1$ type I

HGF was grown in a monolayer on cover glasses and treated with 3 mM HEMA for 0, 24, 72, and 96 h. Two cover glasses were prepared for each treatment, and the entire experiment was performed three times. The samples were washed three times in PBS and fixed with 4% formalin/0.1% Triton X100 in PBS for 20 min at 4°C. After a brief rinsing, the samples were blocked in 1% dry milk in PBS (Carnation natural nonfat dry milk, Carnation Company, Los Angeles, USA) for 30 min at RT and then incubated with antiprocollagen $\alpha 1$ type I antibody (Santa Cruz Biotechnology) diluted to 1:400 in blocking reagent at 37°C for 1 h. After three washes in PBS for 10 min each, the samples were incubated with CY₃-conjugated anti-goat 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 under a fluorescence microscope (Nikon Eclipse E800, Tokyo, Japan).

Reverse-transcriptase polymerase chain reaction

HGF cultured under the same conditions as previously described were exposed to 3 mM HEMA for 0, 24, 72, and 96 h. After each treatment, total RNA was extracted from the cultured cells using the RNeasy[®] Mini Kit (Qiagen, Germantown, MD), according to the manufacturer's instructions. To remove the presence of genomic DNA, a DNase I digestion step was performed (RNase-Free DNase Set, Qiagen) during the isolation procedure. An ND-1000 NanoDrop Spectrophotometer (NanoDrop Technologies, Rockland, DE) was used to quantify the concentration and quality of isolated total RNA by calculating the ratio of absorbance of the samples at 260 nm versus 280 nm. One microgram total RNA was reverse transcribed into cDNA with the aid of the GeneAmp[®] Gold RNA PCR Core Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. One microliter of reverse-transcribed cDNA was then amplified by PCR reaction with specific primers for human collagen $\alpha 1$ type I gene (*COL1A1*) (MWG Biotech, Ebersberg, Germany) and for human glyceraldehyde-3-phosphate dehydrogenase gene (*hGAPDH*) (MWG Biotech, Ebersberg, Germany), which was used as a housekeeping gene for amplification control during PCR assay. The primer sequences were designed by Primer Express[®] Software (Applied Biosystems) as follows: *COL1A1* (target size, 468 bp) sense, 5'-ATTCCAG TTCGAGTATGGCGG-3'; antisense, 5'-GTTGCTTGCTG TTTCCGGGT-3'; *hGAPDH* (target size, 1041 bp) sense, 5'-GTCAGTGGTGGACCTGACCT-3', antisense, 5'-AGGGGT CTACATGGCAACTG-3'. RT-PCR products were analyzed by electrophoresis on 2% agarose gel stained with ethidium bromide, and the gel images were acquired under ultraviolet light by Image Station 2000R (Kodak, NY). The data presented is representative of three independent experiments.

Real-time PCR assay

HGF exposed to 3 mM HEMA for the same periods of time was processed for total RNA extraction (RNeasy Mini Kit, Qiagen). To remove the presence of genomic DNA, a DNase I digestion step (RNase-Free DNase Set, Qiagen) was performed during the isolation procedure. Total RNA (1 μ g) was reverse transcribed into cDNA using a GeneAmp Gold RNA PCR Core Kit (Applied Biosystems) and then amplified by real-time PCR. One microliter cDNA was combined with 1 \times TaqMan[®] Universal Master Mix (Applied Biosystems) and 1 \times TaqMan predeveloped probe (Applied Biosystems) specific for *COL1A1* (target gene) or 1 \times TaqMan predeveloped probe (Applied Biosystems) specific for *hGAPDH* (housekeeping gene). To reduce the error of a failed or suboptimal PCR reaction, each sample was run in duplicate. The amplification of *COL1A1* and of *hGAPDH* cDNA was performed simultaneously in a 96-well plate using the ABI-PRISM 7300 Sequence Detection System (Applied Biosystems). The relative quantification of the *COL1A1* expression gene normalized to *hGAPDH* and relative to the sample treated for 96 h (calibrator) was determined by the comparative C_T method (Applied Biosystems, User bulletin no. 2. Relative quantification of gene expression; 1997). The data presented is representative of three independent experiments.

Statistical analysis

The data about MTT activity were presented as the mean (\pm SD) of triplicate experiments. Statistical differences in MTT activity and real-time PCR data were assessed by one-way ANOVA ($p < 0.05$) and Dunnett's multiple comparison test ($p < 0.05$). The statistical analysis was performed with GRAPH PAD PRISM 4.0 software (San Diego, CA).

Controls

Each of the above-described analyses were also performed on the HGF exposed only to HEMA solvent (the final dilution of ethanol was 0.2%) to assay its possible influence on the expression and synthesis of the procollagen $\alpha 1$ type I (control 1).

A negative control for the immunofluorescence technique was also prepared (control 2). Control specimens for procollagen $\alpha 1$ type I protein immunofluorescence were prepared with the same HGF fixed with 4% formalin/0.1% Triton X-100, incubated only with the secondary antibody to avoid a nonspecific interaction between the antibody and the free aldehyde groups of the fixative. An additional check was done, incubating the HGF fixed with 4% formalin/0.1% Triton X-100 with an isotype-matched irrelevant antibody under the same experimental conditions.

RESULTS

An MTT assay of primary cultures of HGF exposed to 3 mM HEMA for 0, 24, 72, and 96 h is shown in Figure 1. Interestingly, the relative cell via-

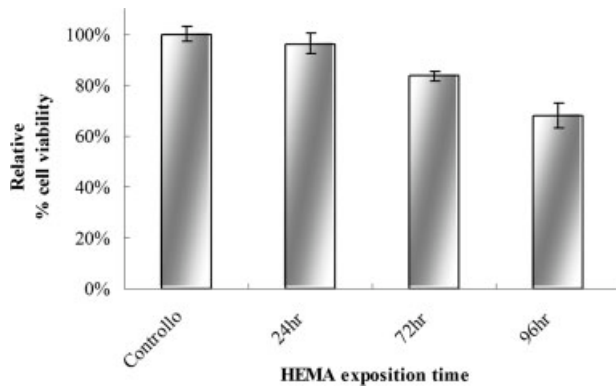


Figure 1. Cell viability of HGF exposed to 3 mM HEMA for 0, 24, 72, and 96 h. During the treatment, there was a mild reduction of cell viability. After 96 h of incubation with HEMA, the cells still showed a high value of cell viability. The data represent the mean (\pm SD) of triplicate experiments per condition and are expressed as a percentage of the control value. The MTT data were statistically analyzed by one way ANOVA followed by the Dunnett test. There were no statistically significant differences between the groups ($p > 0.05$).

bility was still high even after 96 h of 3 mM HEMA exposure.

Western-blot analysis showed a significant decrease of the procollagen α 1 type I protein in specimens exposed for 24 h to 3 mM HEMA, while the protein was absent in specimens treated for longer periods (i.e., 72 and 96 h; Fig. 2)

Immunofluorescence for procollagen α 1 type I protein showed positive labeling characterized by small clusters organized around the cell nucleus of the HGF [Fig. 3(a)]. The labeling pattern was strongly reduced in HGFs after 24 h exposure [Fig. 3(b)] and almost vanished in samples exposed for a prolonged time [i.e., for 72 h as shown in Fig. 3(c) and for 96 h as shown in Fig. 3(d)].

RT-PCR to assay the influence of HEMA on the expression of procollagen α 1 type I mRNA in HGF yielded a reduction of *COL1A1* gene expression in samples treated for 24 h [Fig. 4(a)], while a stronger decrease was detectable in HGF exposed to 3 mM HEMA for 72 and 96 h.

To correlate the data obtained using RT-PCR, a relative quantitative real-time PCR to evaluate the relative amounts of *COL1A1* mRNA in HGF exposed to 3 mM HEMA was carried out. Figure 4(b) shows levels of *COL1A1* mRNA in HGF after HEMA treatment revealing a decrease of 96% when compared with the untreated cells (calibrator) after only 24 h of HEMA exposure. A total reduction (100%) was found in samples exposed to HEMA for 72 and 96 h.

Controls

Control 1 specimens showed a constant cell viability, mRNA expression, and protein synthesis (data

not shown), suggesting no influence of the solvent. A check of the immunofluorescence procedure (control 2) showed a low spread fluorescence signal (data not shown).

DISCUSSION

HEMA is a commonly used constituent of dental restorative materials, and it ranges from 30 to 55% in dental adhesives.^{11,28} Because the current concept of dental adhesion relies on the substitution of the dentin mineral phase by the substrate impregnation with the adhesive system, the use of high percentages of HEMA in the bonding agent allows high diffusivity of the comonomer blends into the a very hydrophilic substrate, that is, the dentin collagen meshwork. Thus, HEMA is of utmost importance in ensuring proper infiltration and formation of the so-called hybrid layer, which determines the stability of the bonded interface over time.^{29,30}

Other than in dentistry, HEMA is widely used in ophthalmology³¹ and drug delivery.³² Despite the extensive use of HEMA on biomedical materials, its biocompatibility and biological safety are still a matter of study. Because several studies have demonstrated that HEMA and other monomers are rapidly released,^{11,13} an awareness of HEMA-related adverse biological effects is fundamental.

The results of this study revealed that at the concentration of 3 mM HEMA tested, the monomer is nontoxic for primary HGF as revealed by the MTT assay (Fig. 1). In fact, after 96 h of exposure, a reduction to only 60% in cell viability was observed. Previous reports revealed variable and contradictory data regarding the amount of HEMA responsible for a 50% cell death rate. This variability has been attributed to the different cell lines tested and the various conditions under which the experiments were conducted.^{17,33} Furthermore, Moharamzaadeh²³ demonstrated that there was a significant difference in the cytotoxic concentration values (TC50) among the same type of primary cell cultures obtained from dif-

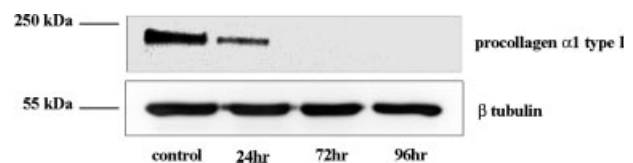


Figure 2. Western-blot analysis for procollagen α 1 type I in HGF exposed to 3 mM HEMA for 0, 24, 72, and 96 h. There was a strong reduction of the protein signal after 24 h of treatment, while the signal was absent in samples treated for 72 and 96 h. Twenty micrograms of total protein was loaded per lane. Bands were visualized using the ECL method. β -Tubulin represents the loading control.

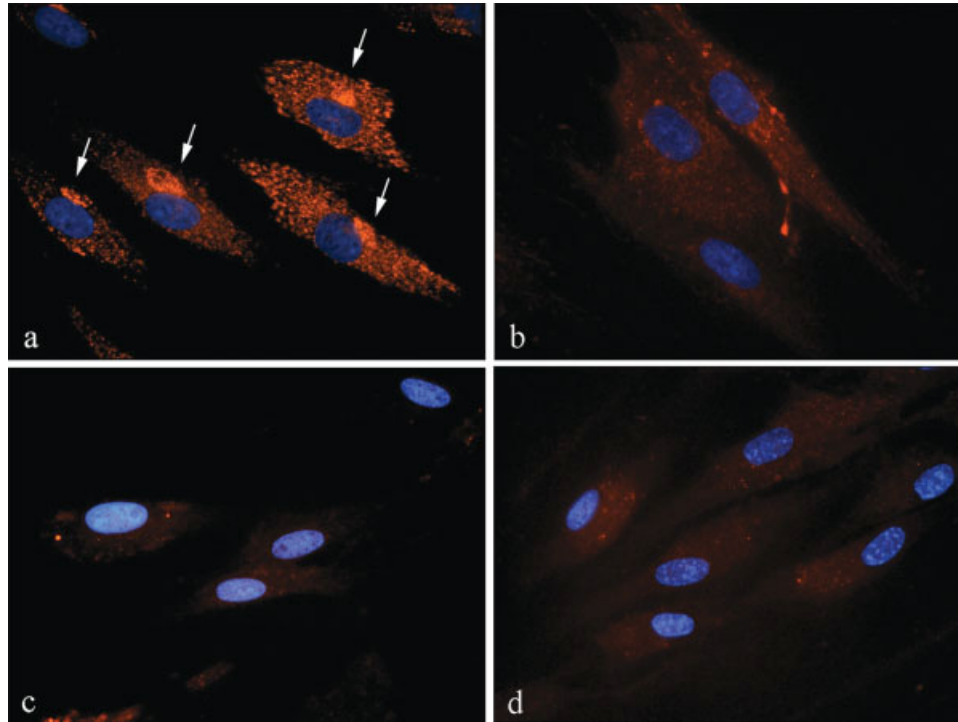


Figure 3. Immunocytochemical localization of procollagen $\alpha 1$ type I in HGF treated with HEMA at different periods of time. CY3-conjugated anti-mouse IgG antibody was used to detect the localization of the protein. All samples were counterstained with DAPI. (a) HGF without any treatment. A CY3 signal was localized in one area around the cell nucleus (arrows) (600 \times). (b) HGF after 24 h of HEMA treatment. The fluorescence signal was reduced and the clusters around the nucleus disappeared (600 \times). HGF after 72 h (c) and 96 h (d) of HEMA treatment. In both images, the signal of procollagen $\alpha 1$ type I was strongly reduced (600 \times). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

ferent donors. The author suggested that this variability was strictly connected to the different intrinsic cell susceptibility to the monomer.

As the amounts of HEMA contained in commercially available products are still unknown, it is difficult to compare these *in vitro* findings with an *in vivo* situation. Nevertheless, several studies have reported HEMA concentrations released by different polymerized bonding agents as ranging from millimolar to nanomolar.^{13,34}

The cytotoxicity and the genotoxicity of HEMA have been examined in a vast number of previous investigations in which the HEMA concentration responsible for 50% of the cell death rate were analyzed. On the contrary, few data are available on the influence and the effect of HEMA if tested at subcytotoxic concentrations as reported in our experimental set-up. In fact, despite the finding that 3 mM HEMA is not directly toxic to HGF, this study pointed out that HEMA interferes with the presence and localization of the procollagen $\alpha 1$ type I protein as confirmed by the correlative Western-blot analysis, immunofluorescence, RT-PCR, and real-time PCR assays performed on HGF exposed to 3 mM HEMA for 0, 24, 72, and 96 h (Figs. 2–4).

Western-blot analysis showed a protein reduction in HGF treated for 24 h and a complete protein absence in HGF treated for 72 and 96 h. This finding was confirmed by means of immunofluorescence revealing a specific labeling pattern of the procollagen $\alpha 1$ type I protein located just outside the nucleus in the untreated samples. This typical protein localization (i.e., close to the nucleus) is related to the presence of a rough endoplasmic reticulum in proximity of the nuclear membrane, that is, the protein right after its synthesis is transported into the reticulum where it is further processed.³⁵ Conversely, after exposure to HEMA for 24 h, the procollagen $\alpha 1$ type I protein showed a reduced and diffuse-labeling pattern spreads in the cytoplasm. The labeling signal was almost absent after 72 and 96 h of exposure (Fig. 3). Our data are in agreement with About and colleagues²⁴ who demonstrated a reduction of the collagen type I protein and an inhibition of odontoblast differentiation in human pulp fibroblasts if exposed to different resin monomers for 4 weeks.

The reduction of the protein signal could be due to a direct interaction between the HEMA and the procollagen $\alpha 1$ type I protein or due to the interference between the HEMA and the expression of pro-

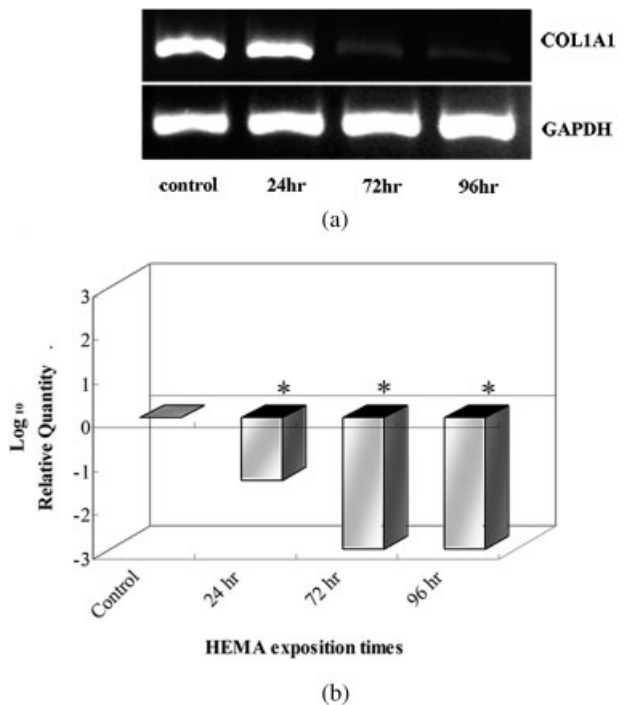


Figure 4. (a) COL1A1 mRNA expression evaluated by RT-PCR amplification. The expression was high in the untreated sample (control), while it was reduced in the sample exposed to 3 mM HEMA for 24 h. After 72 and 96 h of exposure, the expression of COL1A1 mRNA was drastically reduced. hGAPDH was used as a RT-PCR control assay. (b) Relative quantification of COL1A1 mRNA in HGF exposed for 0, 24, 72, and 96 h to 3 mM HEMA. All the samples treated showed a significantly reduced expression when compared with the untreated sample (control). After 24 h of HEMA exposure, there was a reduction of 96% while, after 72 and 96 h of HEMA exposure, the COL1A1 mRNA expression was reduced about 100%. hGAPDH was used as a housekeeping gene for all the samples. The relative amounts were calculated using the ddCt method and statistically analyzed by one way ANOVA followed by the Dunnett's test. * indicates statistically significant differences between the groups ($p < 0.05$ vs. control).

collagen $\alpha 1$ type I mRNA. Nishiyama and colleagues³⁶ hypothesized direct interaction between HEMA and the dentin collagen fibrils, which was responsible for higher bond strength.³⁷ This interaction could similarly reduce the binding between the procollagen $\alpha 1$ type I and the specific antibody, and it could thus be responsible for decreased labeling signal.

The reduced protein level observed after HEMA exposure could be a consequence of reduced mRNA expression. To detect the possible influence of HEMA in the transcription process, a RT-PCR followed by relative quantitative real-time PCR were performed. Both techniques showed reduced mRNA in HGF exposed to the monomer for 24 h, and an almost complete absence in HGF exposed for 72 and

96 h. To our knowledge, no previous data are available regarding the effects of HEMA on the transcription of mRNA specific for extracellular matrix proteins. Reduced mRNA could be due to the direct interaction of HEMA with DNA. Indeed, Dearfield and colleagues³⁸ suggested that monomers such as HEMA and triethyleneglycol dimethacrylate (TEG-DMA) may cause partial deletions in the genome of mammalian cells by direct binding to DNA. This condition could induce genotoxicity.^{33,39} On the contrary, it was hypothesized that oxidative stress was involved in the mutagenicity of TEG-DMA and HEMA, because they were found to produce radical oxygen species,^{17,19,40} which are mutagenic. Interestingly, these data were obtained at HEMA concentrations higher than 3 mM, which induced apoptosis via radical oxygen species generation. In cells treated with different concentrations of HEMA, only 20% were detected as apoptotic.³³ All these reports support the hypothesis that direct interaction occurs between HEMA and DNA, thus influencing the transcription process instead of inducing radical oxygen species generation.

CONCLUSION

In conclusion, the null hypotheses tested were rejected because HEMA reduced the expression of procollagen $\alpha 1$ type I mRNA and its synthesized protein in primary cultures of HGF, and its effect is strongly related to exposure time. Moreover, this effect was observable at a subtoxic concentration of HEMA. Further investigations are currently taking place to evaluate all the biological risks of resin-based materials to the dentin-pulp complex. In fact, monomers at subcytotoxic concentrations could modify cell pathways such as metabolism, dentinogenesis, and tissue repair and could result in cell sufferance which, until now has been underestimated.

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