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HEMA modulates the transcription of genes related to oxidative defense, inflammatory response and organization of the ECM in human oral cells

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ABSTRACT

Objectives. 2-Hydroxyethyl methacrylate (HEMA) is a widely used monomer of dental resin composite materials. Incomplete curing of resins leads to elution of HEMA, which may come in contact with different cells in oral tissues. We aimed to analyze the impact of HEMA on the transcription of genes participating in detoxification of oxidative stress, inflammatory response and organization of the extracellular matrix (ECM) using human gingival fibroblasts (HGFs) and human oral keratinocytes (OKF6/TERT2).

Methods. Cells were grown in monolayer cultures and treated with different HEMA concentrations (0.5–10 mM). H33342 and LDH assays were used to determine HEMA-caused cytotoxicity. Quantitative RT-PCR was used to analyze mRNA expression of four genes related to oxidative stress and five genes each related to inflammation and organization of the ECM.

Results. HEMA caused similar concentration-dependent cytotoxicity in fibroblasts and keratinocytes. Analysis of the transcription showed that genes were regulated in both cell types after HEMA treatment. Genes related to defense against oxidative stress were transcriptionally induced, genes related to inflammation were mainly reduced and genes related to the organization of the ECM were differentially modulated.

Significance. We analyzed concurrent and HEMA-dependent differential expression of 14 important genes, which have a special significance for cellular processes that are linked to redox and tissue homeostasis. The results suggest that HEMA has an impact on cellular redox-homeostasis with potential impairment of inflammatory responses and of the organization of the ECM in human gingival fibroblasts and oral keratinocytes as first target cells of eluted HEMA.

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1. Introduction

Composite resins are widely used in dentistry as restorative and adhesive material. These materials are based on the polymerization of organic methacrylate monomers and co-monomers, like 2-hydroxethyl methacrylate (HEMA). It is well documented that unpolymerized resin monomers, like HEMA, are released from dental materials into the oral environment [1,2]. Moreover, it has been shown that hydrophilic monomers can pass intact dentin barriers and are able to diffuse into the pulp at concentrations that may cause cytotoxicity [3,4]. *In vitro* studies revealed that HEMA induces effects like the reduction of cell vitality, induction of apoptosis, inhibition of proliferation and modulation of the immune response toward pathogens [5–7]. HEMA-caused cytotoxicity has frequently been linked to the induction of oxidative stress in form of reactive oxygen species (ROS) in different cell types [8–10]. Additionally, it has been shown that HEMA reduces the amount of the antioxidant glutathione (GSH) in exposed cells [11,12]. Further investigations have shown that the depletion of GSH is a direct consequence of the formation of GSH-HEMA adducts and not of the detoxification of ROS by GSH [10,13]. Therefore, it is widely accepted that ROS, which are produced in cells treated with HEMA, are a consequence of depleted cellular GSH-levels leading to reduced capability of the cells to maintain redox homeostasis [14]. Cells have established various mechanisms to deal with oxidative stress. One of the main mechanisms is the activation of the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) dependent signaling pathway. The activation of the Nrf2 signaling cascade has been shown before in HEMA treated RAW264.7 mouse macrophages [8]. Under normal conditions, Nrf2 is constitutively expressed and rapidly degraded. When cells are exposed to oxidative stress, Nrf2 is stabilized and translocated into the nucleus where Nrf2 activates the transcription of several target genes by binding promoters with antioxidant responsive elements (ARE) [15]. Important redox-regulated genes that are activated by Nrf2 are heme oxygenase 1 (*HO-1*), NAD(P)H quinone dehydrogenase 1 (*NQO1*) and superoxide dismutase 1 (*SOD1*) [16]. HO-1 is involved in the synthesis of the antioxidant bilirubin [17]. Homodimers of NQO1 reduce quinone to hydroquinone *via* the transfer of two electrons to prevent production of the reactive intermediate semiquinone [18]. SOD1 homodimers directly detoxify ROS by the conversion of superoxide to hydrogen peroxide which is subsequently detoxified further to water by catalases [19].

Other studies have shown that HEMA and other methacrylate monomers, like triethyleneglycol dimethacrylate (TEGDMA), influence inflammatory reactions of different cells *in vitro*. Dental pulp mesenchymal stem cells showed an increased release of proinflammatory interleukin-6 and -8 (IL6-, IL-8) when treated with HEMA for 24 h [20]. Di Nisio et al. showed that the mRNA expression of tumor necrosis factor-alpha (*TNF-α*) and prostaglandin-endoperoxide synthase 2 (*PTGS2*) was slightly induced in human gingival fibroblasts (HGFs) when treated with 3 mM HEMA for 24 h. A co-treatment with lipopolysaccharide (LPS) showed an enhancing effect on the expression of both mRNAs while a co-incubation with the antioxidant N-acetylcysteine (NAC) reduced the expression [21]. On the other hand, it was also shown that HEMA and other resin monomers, like TEGDMA, are not able to induce TNF-α expression in human monocytes or murine macrophages and additionally repress LPS induced TNF-α and IL-6 expression [6,22,23]. The cytokine TNF-α and the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) are key regulators of immune reactions with pleiotropic effects that depend on the state of the analyzed cell [24,25].

Inflammatory processes are frequently associated with a reorganization of the extracellular matrix (ECM) to facilitate an appropriate response of immune cells [26]. For example, periodontal disease and gingivitis are characterized by inflammatory reactions that lead to a progressive breakdown of the periodontal tissue with collagen I (COL1A1) and collagen IV (COL4A1) as the main components of the ECM [27,28]. Among others, the matrix metallopeptidase 9 (MMP9) plays a major role in the degradation of the ECM in periodontal disease [29]. Tissue inhibitors of metalloproteinases (TIMPs) are counterparts of activated MMPs and inhibit MMPs by binding to their catalytic sites [30]. Integrins are membrane proteins that regulate the adhesion of cells to the ECM and integrate signals from the ECM to the cytoplasm. For example, integrin beta 3 (ITGB3) is known to bind RGD sequences from molecules of the ECM and also participates in ROS signaling in cancer [31,32]. However, little is known about the impact of HEMA on the organization of the ECM. Human gingival fibroblasts showed a reduced expression of COL1A1 protein when treated with HEMA [33]. And HEMA caused a reduction of the expression and activity of MMP2 and MMP9 in dental pulp and odontoblast-like cells [34].

Here, we describe concurrent effects of HEMA on the regulation of three different, but interconnected cellular pathways evolutionary evolved to protect cellular viability and to maintain redox and tissue homeostasis: defense against oxidative stress, inflammation and maintenance of the ECM. We selected 14 representative genes of these pathways because of their prominent role in according processes or since they were shown to be affected by HEMA in different cellular models and studies before, as described above. For that, we used oral gingival fibroblasts and keratinocytes as first target cells of eluted HEMA to evaluate if the expression of particular candidate genes was modified. We hypothesized that HEMA may induce an altered mRNA-expression of genes participating in the detoxification of oxidative stress, inflammatory responses and organization of the ECM. We used cytotoxicity assays to determine appropriate HEMA-concentrations and quantitative reverse transcription PCR (qRT-PCR) analysis to quantify mRNA-expression.

2. Material and methods

2.1. Cell cultures

Cultures of primary human gingival fibroblasts (HGFs) were established from biopsies of the healthy gingiva of a permanent molar. Informed consent was obtained from tissue donors according to the guidelines of the Institutional Review Board. Extracted cells were cultivated in

Dulbecco's modified Eagle's medium with 4.5 g/L glucose, 25 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethane sulfonic acid (HEPES), 4 mM L-glutamine, 3.7 g/L $NaHCO_3$, 100 U/mL penicillin, 100 μg/mL streptomycin and 2.5 μg/mL amphotericin B, supplemented with 10% fetal bovine serum (FBS, all from Biochrom KG, Berlin, Germany) at 37 °C and 10% CO_2 in a humidified atmosphere. Medium was exchanged three times a week and cells were grown to confluency. Confluent monolayers were detached by trypsin/ethylenediaminetetraacetic acid (EDTA, 0.25% trypsin, 0.02% EDTA; GIBCO/Invitrogen, Darmstadt, Germany) and seeded in new culture flasks. Passages 6 to 10 were used for the experiments.

The immortalized oral keratinocyte cell line OKF6/TERT2 was provided by Dr. J. Rheinwald (Havard University). Immortalization was achieved by ectopic expression of the catalytic subunit of telomerases hTERT and a defect in the $p16^{INK4a}$ expression. Immortalized OKF6/TERT2 cells retain normal growth and differentiation characteristics [35]. OKF6/TERT2 cells were cultured in keratinocyte serum-free medium (ker-sfm) supplemented with 25 μg/mL bovine pituitary extract (BPE), 0.2 ng/mL epidermal growth factor (EGF, all from GIBCO/Invitrogen, Darmstadt, Germany), 0.3 mM $CaCl_2$, 100 U/mL penicillin, 100 μg/mL streptomycin and 2.5 μg/ml amphotericin B. For passaging, cells were detached by trypsin/EDTA solution (0.125% trypsin, 0.01% EDTA). Trypsin was inactivated using Dulbecco's modified Eagle medium/F-12 medium (DMEM/F-12, Biochrom KG, Berlin, Germany) supplemented with 10% FBS. OKF6/TERT2 cells for experiments were grown in medium with a higher nutrient concentration when certain confluency was reached (HD-ker): 1:1 mixture of DMEM/F-12 and ker-sfm supplemented with 25 μg/mL BPE, 0.2 ng/mL EGF, 0.2 mM $CaCl_2$, 0.75 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 2.5 μg/mL amphotericin B. OKF6/TERT2 cells were cultivated at 37 °C and 5% CO_2 in a humidified atmosphere at all stages.

Both cell cultures were regularly tested for mycoplasma contamination with a mycoplasma detection kit (Venor GeM, Minerva Biolabs, Berlin, Germany).

2.2. *Preparation of HEMA*

The co-monomer 2-hydroxyethyl methacrylate (HEMA) was provided by VOCO (Cuxhaven, Germany). 400-fold concentrated stock solutions of HEMA were prepared in ethanol. Stock solutions were freshly diluted 1:400 in the corresponding growth medium prior to each experiment to obtain the treatment solutions. The final concentration of ethanol was 0.25% for all HEMA concentrations. Cells incubated with fresh growth medium containing 0.25% ethanol (C1) and fresh growth medium without ethanol (C2) served as controls.

2.3. *H33342 assay*

The DNA-intercalating fluorescence dye Hoechst 33342 (H33342, Sigma, Taufkirchen, Germany) was used to determine the cytotoxicity of HEMA in oral keratinocytes and human gingival fibroblasts after 24 h. HEMA containing solutions for treatments were prepared in the respective growth media of both cell types as described above (HD-Ker or DMEM). The final concentrations of HEMA ranged from 0.25 mM to 10 mM. Solvent controls (C1) with 0.25% ethanol and cell-type specific medium controls were included in all experiments. Gingival fibroblasts and oral keratinocytes were seeded in 96-well plates at a density of 1×10^4 cells/well for HGFs and 2×10^4 cells/well for OKF6/TERT2 cells. After a growth period of 24 h, cells were treated with medium containing HEMA or respective control media. After 24 h, media were removed and growth medium with H33342 was added with a final concentration of 10 μg/mL. After incubating for 30 min at 37 °C, cells were washed with PBS to remove excess dye and supplied with fresh PBS for the measurement. The fluorescence of the cultures was recorded using an FLx800 microplate reader (excitation/emission wavelength = 360 nm/460 nm, BioTec, Neifahrn, Germany). Each assay was performed at least 3 times independently with 6 technical replicates each. Cytotoxicity was calculated relative to the untreated control C2 (% of C2).

2.4. *Gene expression analyzes including LDH assay*

Four HEMA concentrations (0.5 mM, 2.5 mM, 5 mM and 10 mM) were selected for analyzing gene expression after 24 h-treatment by qRT-PCR. HEMA was prepared in the respective growth media of both cell types as described above (HD-Ker or DMEM). Solvent (C1) and untreated (C2) controls were included in every experiment. Gingival fibroblasts and oral keratinocytes were seeded in 6-well plates at a density of 2×10^5 cells/well for HGFs and 5×10^5 cells/well for oral keratinocytes. After a growth period of 24 h, cells were treated with medium containing HEMA or the respective control media for 24 h. Subsequently, aliquots for LDH assays were taken from cell culture supernatants and remaining medium was removed. Cells were washed with PBS and total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The kit was used according to manufacturer's instructions. A DNase digestions step was carried out during the isolation (DNase Set, Qiagen, Hilden, Germany). The concentration and the 260/280 ratio of eluted RNA was measured with a Synergy H1 plate reader using a Take3 micro-volume plate (BioTek, Winooski, USA). The integrity of isolated RNA was checked by agarose gel electrophoresis. 1 μg of total RNA was used for cDNA synthesis using QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). cDNA synthesis was carried out according to the manual. Experiments were repeated three times independently.

Lactate dehydrogenase (LDH) assays were carried out using the cell culture supernatant of treated cells to evaluate the reproducibility of treatments and to explore the nature of cytotoxic effects that were observed in the Hoechst assays in more detail (Cytotoxicity detection kit [LDH], Roche, Basel, Switzerland). The kit was used according to manufacturer's instructions. The absorbance of samples was measured at 490 nm with a SpectraMax 250 (MWG Biotech, Ebersberg, Germany). Results of the LDH assay were calculated relative to the supernatants of cells that were grown in respective growth media and lysed with 1% Triton X-100 (Sigma, Taufkirchen, Germany) in growth medium.

2.5. *Quantitative reverse-transcription polymerase chain reaction*

Quantitative analysis of gene expression was carried out by quantitative reverse transcription PCR (qRT-PCR) with the Rotor-Gene Q system (Qiagen, Hilden, Germany). QuantiTect SYBR Green PCR Kits and QuantiTect Primer Assays (supplemental file A) were used to set up the qRT-PCR reactions (both Qiagen, Hilden, Germany). The cycling conditions for qRT-PCR reactions were chosen according to the manual of the QuantiTect SYBR Green PCR Kit for a two-step PCR. The initial denaturation step of 95 °C for 5 min was followed by 40 cycles of 5 s at 95 °C for denaturation and 60 °C for 10 s for primer annealing and extension. After the amplification cycles were completed a standard melting curve was performed to validate the specificity of the primer systems. qRT-PCRs were carried out in technical duplicates. LinRegPCR was used to process raw data and to calculate average PCR amplification efficiencies of primer systems from amplification plots [36]. The expression of six candidate housekeeping genes was measured in each experiment (18S ribosomal RNA [*18S rRNA*], Actin beta [*ACTB*], Beta-2-microglobulin [*B2M*], Glyceraldehyde-3-phosphate dehydrogenase [*GAPDH*], Succinate dehydrogenase complex, subunit A, flavoprotein [*SDHA2*] and Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta [*YWHAZ*]). Gene expression of target genes was normalized to the two most stable housekeeping genes identified by geNorm [37]. Housekeeping genes were identified for both cell lines independently: *B2M* and *YWHAZ* for oral keratinocytes and *18S rRNA* and *YWHAZ* for gingival fibroblasts. Normalized ratios between the untreated control (C2) and treated samples were calculated according to the efficiency adjusted delta delta ct method [38].

2.6. *Statistics*

Experiments were carried out at least three times independently and the data were calculated relative to untreated controls (C2) and are expressed as means ± SD. Significance levels of the H33342 assay, the LDH assay and qRT-qPCR analysis were calculated relative to respective solvent controls (C1) using one way ANOVA. The normalized fold changes of qRT-qPCR analysis were log2 transformed to enable statistical testing. In all cases Dunnett's *post-hoc* tests were carried out to account for multiple testing.

3. Results

3.1. *Cytotoxicity*

The H33342 assay was used to determine cytotoxicity of HEMA (0.25–10 mM) in human gingival fibroblasts and oral keratinocytes after a treatment for 24 h. HEMA showed a concentration dependent cytotoxicity in both cell types (Fig. 1). The cell number of HGFs treated with HEMA showed a reduction down to 42.7% ± 5.7% relative to the untreated control (C2) at the highest concentration of 10 mM HEMA. The number of OKF6/TERT2 cells was reduced down to 57.3% ± 7.0% relative to the untreated control (C2) when treated with 10 mM HEMA. 2.5 mM HEMA was the lowest concentration at which significant cytotoxic effects could be observed in both cell types (HGF: 73.8% ± 8.5%; OKF6/TERT2: 81.1% ± 5.2%). Four HEMA concentrations, covering the whole range of cytotoxicity, were chosen for gene expression analysis: 0.5 mM, 2.5 mM, 5 mM and 10 mM HEMA.

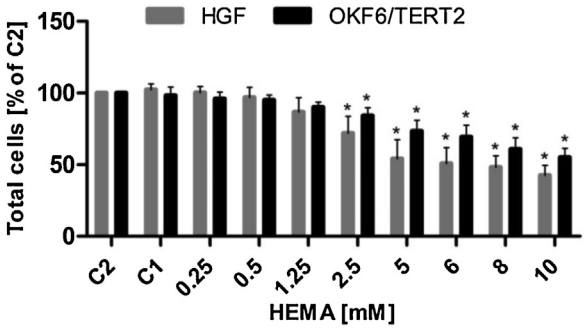

Fig. 1 – HEMA-induced cytotoxicity in HGFs and OKF6/TERT2 cells by H33342 assay. Cells were treated with 0.25–10 mM HEMA for 24 h. Total cell numbers were assessed by H33342 assay. Data are shown as means ± SD of at least three independent experiments. C1 = solvent control (0.25% EtOH), C2 = untreated control. Asterisks above the error bars indicate significant differences (P < 0.05) within cell lines in comparison to C1 (ANOVA, Dunnett's *post-hoc* test).

The LDH assay was used for simultaneous evaluation of cytotoxic effects in experiments that were carried out to analyze gene expression in HGFs and OKF6/TERT2 cells induced by 0.5–10 mM HEMA. At lower HEMA concentrations (0.5 mM and 2.5 mM) no significant increase of released LDH relative to the solvent control (C1; HGFs: 2.1% ± 0.1%; OKF6/TERT2

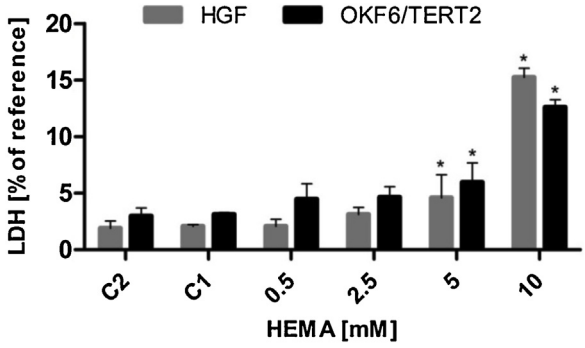

Fig. 2 – HEMA-induced cytotoxicity in HGFs and OKF6/TERT2 cells by LDH assay. Cells were treated with the indicated concentrations of HEMA for 24 h. Enzymatic activity of released LDH was measured from the supernatant of treated cells and quantified relative to untreated reference cells which were lysed by 1% Triton X-100. The data are shown as means ± SD of three independent experiments. C1 = solvent control (0.25% EtOH), C2 = untreated control. Asterisks above the error bars indicate significant differences (P < 0.05) within cell lines in comparison to the according C1 (ANOVA, Dunnett's *post-hoc* test).

cells: 3.2% ± 0.1%) could be observed in both cell types (Fig. 2). At 5 mM HEMA, gingival fibroblasts released 4.6% ± 1.6% and oral keratinocytes 6.0% ± 1.4% LDH relative to the according reference cells while the relative amount of released LDH in the according solvent controls was 2.1% ± 0.1% for HGFs and 3.2% ± 0.1% for OKF6/TERT2 cells. At 10 mM the relative amount of released LDH increased markedly to 15.2% ± 0.7% for gingival fibroblasts and 12.6% ± 0.5% for oral keratinocytes.

3.2. *Gene expression analysis*

3.2.1. *Genes related to oxidative stress*

The transcription of four genes that are primarily linked to oxidative stress was analyzed in human gingival fibroblasts and oral keratinocytes after a 24 h exposure to different HEMA concentrations (0.5–10 mM; *Nrf2, HO-1, NQO1, SOD1*, Fig. 3(a). HGFs and OKF6/TERT2 cells exhibited a concentration dependent induction of the analyzed genes related to oxidative stress. Especially the mRNA expression of *HO-1* and *NQO1* was significantly induced in both cell types at all HEMA concentrations. Transcription of *HO-1* was increased up to 26.9 ± 2.6 fold of the untreated control at 10 mM HEMA in HGFs and even up to 120.5 ± 12.1 fold in OKF6/TERT2 cells. *NQO1* expression showed a relative induction up to 2.7 ± 0.2 fold at 5 mM HEMA in HGFs and up to 9.3 ± 1.6 fold at 10 mM in OKF6/TERT2 cells. Also the expression of *SOD1* was slightly, but significantly increased at 5 mM (1.5 ± 0.3 fold) and 10 mM HEMA (1.6 ± 0.3 fold) in HGFs and additionally at 2.5 mM HEMA (1.5 ± 0.1 fold) in oral keratinocytes in comparison to the solvent control. The highest increase of the *SOD1* transcription in keratinocytes was observed at 10 mM HEMA (2.1 ± 0.3 fold). The amount of mRNA of the transcription factor *Nrf2* in OKF6/TERT2 cells was significantly increased when treated with 10 mM HEMA to 1.9 ± 0.3 fold of the untreated control at 10 mM HEMA. However, no significant upregulation of *Nrf2* could be overserved in HGFs.

3.2.2. *Genes related to inflammation*

The impact of HEMA on inflammatory processes in gingival fibroblasts and OKF6/TERT2 cells was analyzed based on the transcription of five genes that are related to inflammatory responses (*NF-κB1, TNF-α, IL-6, IL-8* and *PTGS2*; Fig. 3(b). Except for *IL-8* and *PTGS2*, all genes showed a concentration dependent reduction of the expression in HGFs and oral keratinocytes after a treatment with HEMA for 24 h in comparison to the controls. Especially the mRNA expression of *IL-6* was markedly reduced in comparison to the according solvent control in both cell types. In HGFs the amount of *IL-6* mRNA was reduced by a factor of 13.7 ± 7.9 when treated with 5 mM HEMA and by a factor of 36.8 ± 7.0 at 10 mM HEMA in OKF6/TERT2 cells. The amount of *IL-8* mRNA was not significantly affected in HGFs. However, HEMA induced a 7.3 ± 2.9 fold reduction relative to the untreated control at 10 mM in oral keratinocytes. *PTGS2*-expression affected by HEMA showed a similar pattern in OKF6/TERT2 cells. *PTGS2*-mRNA was significantly increased when treated with 2.5 mM and 5 mM HEMA (5.6 ± 1.1 fold) but, in contrast to HGFs, was significantly reduced in OKF6/TERT2 cells when treated with 10 mM HEMA (2.4 ± 0.7 fold). Both genes, *IL-8* and *PTGS2*, differed in their transcriptional regulation at the highest concentration of HEMA when compared to lower concentrations in oral keratinocytes. In contrast, the expression of *PTGS2* was induced in HGFs at 2.5 mM, 5 mM and 10 mM HEMA in comparison to the untreated control with the highest increase at 5 mM HEMA by a factor of 3.8 ± 1.1. HEMA induced minor but significant reductions of the mRNA expression of the analyzed subunit of the NF-κB complex, *NF-κB1*, in both cell types when treated with 2.5 mM, 5 mM and 10 mM HEMA. In HGFs, *NF-κB1* expression was decreased 1.6 ± 0.03 fold at 10 mM HEMA relative to the untreated control and by a factor of 2.3 ± 0.3 in oral keratinocytes. The mRNA amount of *TNF-α* showed a reduction in OKF6/TERT2 cells at 2.5 mM, 5 mM and 10 mM HEMA. The strongest reduction could be observed at 10 mM HEMA with a decrease of 18.9 ± 11.0 fold relative to the untreated control. However, no transcription of *TNF-α* could be detected in gingival fibroblasts.

3.2.3. *Genes related to the extracellular matrix*

To determine the effects of HEMA on genes coding for proteins of the ECM, four genes that participate in the organization of the ECM (*COL1A, COL4A, MMP9* and *TIMP1*; Fig. 3(c)) and one gene that plays a role in the integration of signals from the ECM and the regulation of cell adhesion (*ITGB3*) were analyzed. Transcripts of *COL1A1* showed a significant reduction of the abundance in gingival fibroblasts at 2.5 mM, 5 mM and 10 mM HEMA but could not be detected in oral keratinocytes. Minimal *COL1A1* expression in HGFs was observed at 5 mM HEMA with a 2.3 ± 0.7 fold reduction relative to the untreated control. The expression of *COL4A1* showed no significant reduction in both cell types but tendencies for a down regulation could be observed at higher HEMA concentrations when compared to the according untreated control. Expression of *MMP9* mRNA could not be detected in HGFs and the mRNA amount of the analyzed tissue inhibitor of metalloproteinases, *TIMP1*, was not regulated after HEMA treatment of gingival fibroblasts. On the contrary, the expression of both genes was altered in oral keratinocytes when treated with HEMA. The expression of *MMP9* was significantly reduced at 2.5 mM (3.2 ± 1.2 fold) and 5 mM (3.1 ± 1.9) HEMA, but showed no significant regulation at 10 mM HEMA. Accordingly, the analyzed antagonist of MMPs, *TIMP1*, was transcriptionally induced in keratinocytes at 2.5 mM, 5 mM and 10 mM HEMA with a maximum induction of 4.3 ± 0.8 relative to the untreated control at 5 mM HEMA. Also the expression of *ITGB3* was only affected in OKF6/TERT2 cells and showed significant increases at 2.5 mM and 5 mM HEMA when compared to the solvent control. However, no significant regulation was observed at 10 mM HEMA.

It is noteworthy, that a number of genes related to inflammation and to the organization of the ECM showed an inverted (*IL-8* and *PTGS2*) or markedly reduced (*MMP9* and *ITGB3*) regulation of the mRNA expression at 10 mM HEMA compared to lower HEMA concentrations in oral keratinocytes but not in gingival fibroblasts. Genes encoding for proteins of the cellular antioxidant machinery did not show such an effect at 10 mM HEMA in OKF6/TERT2 cells.

4. Discussion

In the present study, we analyzed the influence of the dental co-monomer HEMA on adaptive cell responses of human gingival fibroblasts and oral keratinocytes based on gene

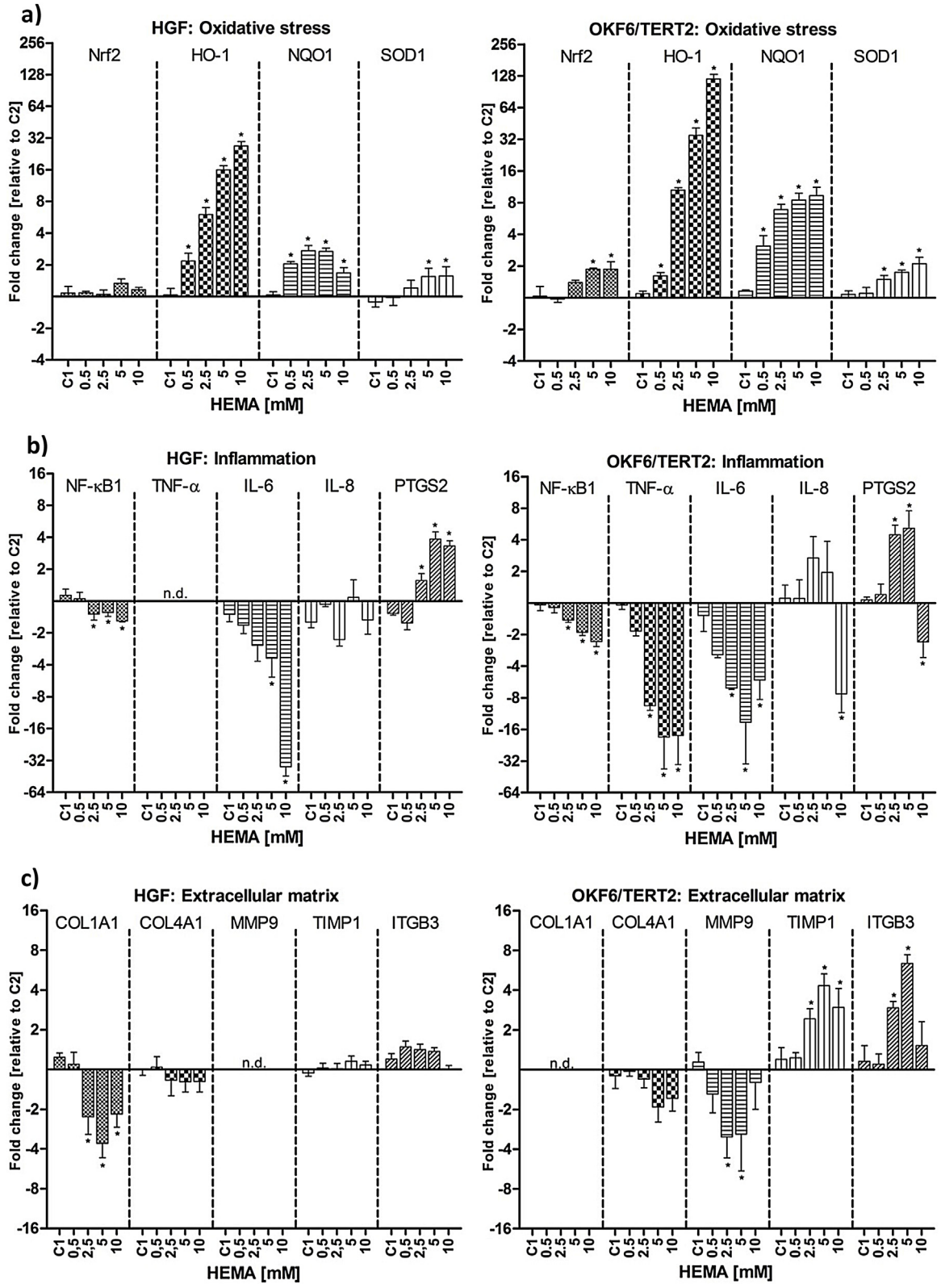

Fig. 3 – HEMA-induced alterations in gene expression of HGFs and OKF6/TERT2 cells. Cells were treated with the indicated concentrations of HEMA for 24 h. Gene expression was analyzed by qRT-PCR. (a) Genes related to oxidative stress, (b) genes related to inflammation, (c) genes related to the ECM, C1 = solvent control (0.25% EtOH), C2 = untreated medium control, n.d. = not detectable. Gene expression was normalized to the expression of untreated control cells (not shown) and two stable reference genes (*18s rRNA* and *YWHAZ* for HGFs and *B2M* and *YWHAZ* for OKF6/TERT2 cells). The data are shown as means ± SD of log2-transformed fold changes of three independent experiments. For a better clearness, log2 values at the Y-axis were transformed to linear values. Asterisks above the error bars indicate significant differences ($P < 0.05$) in comparison to the according solvent control C1 (0.25% EtOH; ANOVA, Dunnett's *post-hoc* test).

expression analysis of 14 genes from three processes using qRT-PCR. The measurement of mRNA-levels is a suitable approach to generate such an overview since the dynamics of protein abundances are mostly reflected by fold-changes of according mRNAs [39,40]. The data is of particular interest since we provide valuable data concerning the material-modulated cellular metabolism and its potential significance for redox and tissue homeostasis. Both cell types are one of the first 'target' cells for effects of components released from resinous filling materials. Our results showed that HEMA modulates the cellular metabolism involving cellular redox homeostasis and the composition of the extra cellular matrix with major inflammatory components. Most of the analyzed transcripts were highly modulated by HEMA-treatment in both cell types. Interestingly, a number of genes encoding for proteins related to inflammation (*IL-8* and *PTGS2*) and proteins of the ECM (*MMP9* and *ITGB3*) showed a markedly different regulation of the mRNA expression at 10 mM HEMA compared to the lower HEMA concentrations in oral keratinocytes.

Both cell types exhibited concentration-dependent cytotoxic effects when treated with HEMA in two different assays that were applied; the LDH and H33342 assay. H33342 was used to determine the effective concentration for a 50% (EC_{50}) growth reduction due to HEMA, whereas the LDH assay was simultaneously performed with supernatants of gene expression experiments to monitor the cytotoxicity of HEMA on cells that were subsequently harvested for isolation of total RNA and to monitor the reproducibility of the treatment. Overall, the LDH assay showed lower HEMA cytotoxicity in comparison to the Hoechst33342 assay. In the LDH assay, no significant cytotoxicity could be detected at 2.5 mM HEMA. Additionally, at 10 mM HEMA the values are 12.6% for OKF6/TERT2 cells and 15.2% for HGFs relative to the maximum control in the LDH assay, while the cell number detected by the H33342 assay was around 50% of the untreated control. The assays are based on different operational principles. The LDH assay measures the amount of released LDH from existing cells while the H33342 assay is a measure of the total cell number. It has previously been described that HEMA induces apoptosis and cell cycle arrest, leading to reduced proliferation [5,7,41,42]. These findings might explain the differences observed between both assays since the pure cell number measured by H33342 assay is a combined measure of both, apoptosis and reduced proliferation, while the LDH assay only measures released LDH from existing cells. Based on the results of the H33342 assay, four concentrations were chosen for gene expression analysis covering the whole range of cytotoxicity: 0.5 mM HEMA showed no significant cytotoxic effects in both cell types, 2.5 mM and 5 mM showed low to moderate cytotoxic effects and 10 mM HEMA reduced the total cell number to approximately 50% of untreated control cells (EC_{50}). The cytotoxic potential of HEMA on HGFs has been described previously and showed a notably lower EC_{50} value of 2.38 mM. However, in this study the cytotoxic potential was analyzed after an incubation of 48 h so that HEMA could affect the viability of cells over a longer period [43]. To evaluate HEMA concentrations, which might be relevant for the clinical situation, we used findings of published analytical studies addressing the release of components from resin-based dental materials. Leachable amounts of HEMA were calculated based upon experiments using test specimens under defined laboratory conditions [2]. A meta-analysis of 22 studies showed that up to 475 nmol/mm^2 HEMA can be released from dental resins within 24 h after polymerization. Therefore, the potential average release of HEMA from only one large incisal restoration with an average surface area of 117 mm^2 can be calculated to reach 55 mmol after 24 h [2]. In dependence of the degree of dilution and the number of fillings it may be speculated that concentrations of 10 mM HEMA can be present in the close vicinity of the filling due to accumulation in the surrounding tissues. When the restorations are not cured sufficiently or when the restoration is located below the gingival level, resulting concentrations might even be higher locally. Similar HEMA concentrations (up to 8 mM) were calculated by Noda et al., for the pulp following a direct pulp-capping procedure [44].

We investigated if the transcription of genes of the defense machinery against oxidative stress is induced when HGFs and OKF6/TERT2 cells were treated with HEMA. We observed significant inductions of the analyzed genes in both cell types. The activity of Nrf2 is mainly regulated by the translocation of Nrf2 into the nucleus [15]. However, here we show that the transcription of *Nrf2* itself was induced upon HEMA treatment in OKF6/TERT2 cells. The potential of Nrf2 to enhance its own transcription by a positive feedback loop has been described previously [45,46]. This mechanism might be an additional layer of regulation to enhance the protection toward ROS/RNS generated by the activity of HEMA in OKF6/TER2 cells through an increased protein content of Nrf2 that could translate into an increase of nucleus-located Nrf2 if the signaling cascade was activated. However, transcription of *Nrf2* was not significantly induced in HGFs. We also analyzed three ARE containing target genes of Nrf2 that play a role in the detoxification of ROS (*HO-1, NQO1* and *SOD1*) [16]. All three genes were strongly induced in both cell types, which leads to the conclusion that the redox homeostasis was impaired even at the lowest HEMA concentration applied (0.5 mM) and that the Nrf2 signaling cascade was presumably induced. The increase of *SOD1* transcription indicates an enhanced generation of superoxide as a consequence of HEMA treatments. The transcriptional induction of *HO-1* and *NQO1* was notably higher in OKF6/TERT2 cells compared to HGFs. Potentially, this is a consequence of the increased expression of *Nrf2* mRNA in oral keratinocyte indicating a more pronounced demand for redox regulating proteins in HEMA treated oral keratinocytes in comparison to HGFs.

Concurrent with affecting the regulation of redox-regulating proteins HEMA modulates the immune status in OKF6/TERT2 cells and HGFs on the level of mRNA expression of pro-inflammatory genes. To our knowledge, the presented results show for the first time that the base level transcription of pro-inflammatory cytokines and *NF-κB1* was reduced in both cell types when treated with HEMA. *NF-κB1* and *IL-6* transcription was repressed in both cell types while *TNF-α* and *IL-8* transcription was repressed only in OKF6/TERT2 cells. The relationship between the key regulators of immune reactions NF-κB and TNF-α is highly complex. The response of cells toward TNF-α is specific and depends on various factors, like the presence of certain receptors and other downstream components [25]. Also the NF-κB complex can induce or repress genes in dependence of the processing of precursor proteins

and the formation and modification of heterodimers from different NF-κB subunits [24]. In our system, the transcriptional reduction of *NF-κB1* and *TNF-α* in oral keratinocytes and *NF-κB1* in gingival fibroblasts was accompanied by a reduction of *IL-6* and *IL-8* mRNA in OKF6/TERT2 cells and *IL-6* mRNA in HGFs. *IL-6* and *IL-8* are mainly characterized as pro-inflammatory cytokines and their expression was found to be associated with different inflammatory diseases of oral tissues [47,48]. Taken together, these results suggest that HEMA modulates the immune status of HGFs and oral keratinocytes *in vitro* on the mRNA level. The transcriptional reduction of *NF-κB1, TNF-α, IL-6* and *IL-8* in a situation when the immune system is not challenged might lead to a reduced immune response when cells are threatened by pathogens. Such effects have been shown for murine macrophages *in vitro* when co-treated with TEGDMA and LPS [6,49]. TEGDMA is another methacrylate and co-monomer that elutes from dental materials and causes similar effects as HEMA, *e.g.* cell cycle arrest, cytotoxicity or depletion of intracellular GSH [42,50]. Within the group of genes related to inflammation, only the expression of *PTGS2* was significantly induced in both cell types when treated with 2.5 mM and 5 mM HEMA, although *PTGS2* is known to be a target gene of Nf-κB, like *TNF-α*, *IL-6* and *IL-8* [51]. It has been shown previously that PTGS2 or the final product of the enzymatic reaction of PTGS2, prostaglandin 2, was induced in murine macrophages [52] and also in HGFs when treated with HEMA [21]. The differential regulation of *NF-κB1, TNF-α, IL-6* and *IL-8* on the one side and *PTGS2* on the other side, might be explained by the differential regulation by various transcription factors in addition to Nf-κB. For instance, the promoter of *PTGS2* contains two distinct NF-κB response elements and is additionally regulated by the transcription factors AP1, CRE, HIF, SP1 and STAT [53]. Interestingly, *PTGS2* was transcriptionally repressed in OKF6/TERT2 cells when treated with 10 mM HEMA. The transcriptional repression of genes related to inflammation is potentially a consequence of the enzymatic activity of HO-1. It has been described previously that a lack of HO-1 results in hypersensitive inflammatory reactions [54,55]. On the other hand, overexpression of HO-1 reduced inflammatory responses caused by LPS treatment in macrophages [56]. Byproducts of the HO-1 reaction are carbon monoxide and free Fe^{2+} [57]. Free Fe^{2+} induces the expression of ferritin and activates ATPase iron pumps, which leads to a depletion of free Fe^{2+} overall [58,59]. It was shown that carbon monoxide and the depletion of free Fe^{2+} could mimic the suppression of inflammatory reactions by HO-1 in endothelial cells by repressing the activity of NF-κB [60]. Anti-inflammatory reactions toward carbon monoxide and free Fe^{2+} have been described also in other systems and have been reviewed recently [61,62]. Therefore, the transcriptional down-regulation of *PTGS2* and *IL-8* in OKF6/TERT2 cells at 10 mM HEMA might be a consequence of an excessive induction of *HO-1* mRNA by a factor of about 120 relative to the untreated control. If this transcriptional induction translates into active HO-1 protein, the resulting amount of heme degradation and its byproducts might have led to a profound repression of the immune response including the down regulation of *PTGS2* and *IL-8* at 10 mM HEMA. This might indirectly affect the transcriptional modulation of *MMP9* and *ITGB3* as well, since the transcription of both genes was significantly modulated only at lower HEMA concentrations (2.5 mM and 5 mM). However, we have to be aware of the fact that we analyzed mRNA expression after a 24 h treatment of HEMA. Therefore, we cannot rule out that genes were differentially regulated post-transcriptionally or that analyzed genes were transcriptionally induced at an earlier time point.

Oxidative stress and inflammation are known to influence the organization of the ECM. Therefore, we also analyzed alterations of the transcription of important ECM components caused by HEMA in oral keratinocytes and gingival fibroblasts. Among the analyzed genes, only the transcription of *COL1A1* was significantly regulated (reduced) in gingival fibroblasts when treated with HEMA concentrations above 0.5 mM. This finding is in line with results from the literature. It has been described previously that HEMA reduces the amount of collagen type 1 protein in HGFs [33]. A decrease of the *COL1A1* mRNA in HGFs upon HEMA treatment has also been shown before and indicates that the reduction of collagen 1 protein is at least partially regulated on the mRNA level [63]. In oral keratinocytes, the transcriptional response toward the HEMA treatment was different compared to HGFs. The transcription of *COL4A1* was also not affected in comparison to the control while *MMP9*, *TIMP1* and *ITGB3* were significantly regulated in oral keratinocytes when treated with HEMA. The transcriptional induction of *TIMP1* and the repression of *MMP9* are in accordance. Both observations indicate a reduction of the MMP9 activity, since TIMP1 is an inhibitor of MMP9 [64]. The transcriptional repression of *MMP9* is in line with the observed repression of *TNF-α* mRNA in oral keratinocytes since *TNF-α* regulates the expression of MMP9 [65]. Additionally, we showed for the first time that the transcription of the integrin *ITGB3* was reduced in OKF6/TERT2 cells when treated with HEMA. Integrins act as heterodimers and participate in cell adhesion to the ECM and facilitate signal perception from the ECM [31]. Although we cannot deduce the specific function of ITGB3 in oral keratinocytes from our data, the transcriptional repression of *ITGB3* together with the regulation of *MMP9* and *TIMP1* indicates a modulation of the integrin signature of oral keratinocytes and the ECM in general as consequence of the HEMA treatment.

Taken together, our analysis showed alterations in mRNA abundances of genes related to three important and interconnected metabolic pathways that are crucial for the maintenance of redox and tissue homeostasis in response to HEMA in HGFs and OKF6/TERT2 cells. Our results demonstrate that even non-cytotoxic concentrations of HEMA (0.5 mM) induce gene expression of oxidative stress related genes in human gingival fibroblasts and keratinocytes while low to moderate cytotoxic concentrations additionally affect mRNA expression of genes related to inflammation and organization of the ECM. Higher HEMA concentrations potentially induce more pronounced oxidative stress that causes an overload of oxidative cell defense systems resulting in cell death and in modifications of inflammatory pathways and metabolic pathways modifying the ECM. Furthermore, our results at 10 mM HEMA indicate that increased concentrations of byproducts from reactions of antioxidant enzymes modulate the expression of genes related to inflammation and the organization of the ECM in addition to the direct modulations caused by oxidative stress. The data presented in this article are valuable for

estimating effects of HEMA on cells of the oral mucosa and for that risk assessment of dental resinous materials. The causal links between the different metabolic pathways affected by HEMA and the transition of alterations in mRNA levels into active proteins will be analyzed in the future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.dental.2019.01.011.

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