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Additive effects of TEGDMA and hydrogenperoxide on the cellular glutathione content of human gingival fibroblasts

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ABSTRACT

Objectives. Only few data are available about cytotoxic effects of leachable dental resin compounds in combination with hydrogen peroxide (H_2O_2) segregated from dental bleaching agents. Therefore, the purpose of this study was to evaluate the effects of various concentrations of triethylene-glycol dimethacrylate (TEGDMA) and H_2O_2 on intracellular glutathione levels (GSH) and viability of human gingival fibroblasts (HGF) that are primary target cells of cytotoxic actions of these substances.

Methods. HGF were grown in 96-well plates for 24 h, treated with various concentrations of TEGDMA (0.5–5.0 mM) for 24 h and subsequently for 90 min with 0.2 mM H_2O_2 or culture medium (control). The relative intracellular GSH concentration was determined using a fluorescence assay with monobromobimane. Readings were normalized to cell numbers, which were determined by a propidium iodide assay. Data were statistically analyzed by t-test and ANOVA with Tukey's post test. A significance level of $p < 0.05$ was used.

Results. Exposure to TEGDMA reduced the viability of HGF at concentrations $\geq$1.0 mM. TEGDMA induced a decrease of the GSH pool in a concentration-dependent manner ($p < 0.05$). The depletion of GSH was correlated with a reduction of viability ($p < 0.05$) and the total cell number. Furthermore, a significant decrease of the intracellular GSH content was found when cells were exposed to TEGDMA in combination with H_2O_2, compared to experiments without H_2O_2.

Significance. We conclude from our findings that TEGDMA and H_2O_2 have additive adverse effects on GSH metabolism and cell viability.

1. Introduction

Many patients, specifically adolescents and young adults, frequently use peroxide-based bleaching agents to lighten their teeth. The active ingredient within these products is primarily H_2O_2, which is either directly applied or liberated from carbamide peroxide (CP) through an intra-oral chemical reaction. The concentration of H_2O_2 in bleaching materials varies significantly between 3.4 and 6.8% in home bleaching agents and up to 35% in office bleaching agents [1,2]. Wattanapayungkul et al. [3] determined that during a 1-h-period a concentration of 20 mM H_2O_2 was created in saliva from a 10% CP gel. McMillan et al. [4] found a maximum concentration of 2 mM H_2O_2, which was released from a 6.5% H_2O_2–gel during a period of 60 min. H_2O_2 is slowly degraded in saliva. In fact, after 40 min, only 25% of the initially applied peroxide is disintegrated [5]. Likewise, the CP concentration only gradually declines at the tooth surface. After a period of 1 h, 70% of the initial concentration was

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still present [3], and after 2 h 50% of the initial concentration remained [6]. These data indicate that oral cells are exposed to high H_2O_2 concentrations during bleaching therapies, which comprise a period of several weeks or even longer. H_2O_2 readily penetrates cell membranes and generates various reactive oxygen species (ROS), such as the hydroxyl radical ($^{\bullet}OH$). This radical is highly reactive and is considered to be the most toxic ROS causing genetic instability [7].

The application of tooth-colored esthetic filling materials, specifically composite resins, increased dramatically during the past decade equivalent to the use of bleaching agents. The most important composite comonomer, triethylene-glycol dimethacrylate (TEGDMA), was frequently found in aqueous eluates from various products [8–10]. TEGDMA can easily penetrate cell membranes and subsequently react with intracellular molecules and structures [11]. TEGDMA reveals a high cytotoxicity [12,13], moderate genotoxic potency at low 'subtoxic' concentrations [14–16], and interferes with various metabolic pathways. Recently, it has been shown that non-toxic concentrations of TEGDMA cause a depletion of the important intracellular antioxidant glutathione (GSH, L-γ-glutamyl-L-cysteinyl-glycine) within the very short period of 90 min to 4 h [17,18]. This reaction may subsequently cause severe cellular alterations in different cell types [13,17,19–21]. It was found, for instance, that the reduction of antioxidant GSH due to TEGDMA caused a significant increase of the endogenous H_2O_2 concentration [11,16]. The significance of these data is that a persistent exposure of cells and tissues to low concentrations of water-leachable TEGDMA may have chronic negative effects, which may cause dysfunction or disease in time. In addition, GSH-depleted cells will be more susceptible to subsequent injuries from other toxic xenobiotics, for instance H_2O_2.

Contrary to the rapid increase in clinical use of resin-based filling materials as well as bleaching agents, very little is known about the chemical–biological interactions of the highly reactive substances generated during a bleaching therapy with oral cells and tissues. Therefore, it was the objective of this study to investigate the hypothesis that TEGDMA and H_2O_2 in combination will have additive cytotoxic effects on oral human cells. Primary human gingival fibroblasts were selected as important target cells of adverse reactions due to both substances. The relative intracellular GSH concentration was determined as parameter for the individual and combined cytotoxic activity of TEGDMA and GSH.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), HEPES, penicillin, streptomycin, amphotericin, and fetal calf serum (FCS) were purchased from Biochrom (Berlin, Germany), $NaHCO_3$ from Riedel de Haën (Seelze, Germany), and trypsin/EDTA from Sigma (Taufkirchen, Germany). triethylene-glycol dimethacrylate (TEGDMA) was a gift from VOCO (Cuxhaven, Germany). H_2O_2 (30%) was purchased from Merck (Darmstadt, Germany), monobromobimane (MBBr) and Nonidet P-40 were purchased from Fluka (Seelze, Germany), propidiumiodide (PI) and dimethylsulfoxide (DMSO) from Sigma (Taufkirchen, Germany), and Hank's balanced salt solution (HBSS) from GIBCO BRL (Karlsruhe, Germany).

2.2. Cell culture

Primary human gingival fibroblasts (HGF) were cultured from biopsies of healthy gingiva of permanent molars. Informed consent was obtained from the donors according to the guidelines of the Institutional Review Board.

The biopsies were stored at 4 °C for 24 h at most in HBSS supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), and amphotericin (2.5 μg/mL) prior to amplification. The tissue samples were placed into 25-cm^2 tissue culture flasks and grown in DMEM culture medium with 4.5 g/L glucose, 10 mM HEPES, $NaHCO_3$ (3.7 g/L), penicillin (100 U/mL), and streptomycin (100 μg/mL), supplemented with 10% fetal calf serum (FCS) at 37 °C and 10% CO_2. When outgrowth of cells was observed, the medium was replaced twice weekly until cells reached confluency. Cells were detached from the substrate by a brief treatment with trypsin/EDTA (0.25% trypsin, 0.02% EDTA) and cultured in 75-cm^2 tissue flasks until confluent monolayers were re-obtained. Early passages were frozen in liquid nitrogen.

All cultures were routinely tested for mycoplasma contamination by means of the mycoplasma detection kit Venor GeM (Minerva Biolabs, Berlin, Germany).

2.3. Treatment of cells with TEGDMA and H_2O_2

Stock solutions (200×) of TEGDMA were prepared in DMSO and freshly diluted in DMEM prior to each experiment. HGF from passages number 6 to 12 were seeded in 96-well plates (1×10^4 cells/well) and allowed to grow for 24 h. Then cells were washed with DMEM and exposed to TEGDMA for 24 h at concentrations between 0.5 and 5 mM. After washing, TEGDMA-treated cells were incubated for another 90 min with 0.2 mM H_2O_2 and medium, but without TEGDMA. Control cultures were incubated during the initial 24 h with medium containing 0.5% DMSO. After washing control cells were either exposed to medium containing 0.2 mM H_2O_2 or to medium containing 0.5% DMSO for additional 90 min.

2.4. Glutathione assay

The relative intracellular GSH concentrations were determined using an assay with monobromobimane (MBBr) in microtiter plates as described previously [19]. Briefly, medium was removed from the wells after treatment of the cells. Monolayers were washed with HBSS. Then, MBBr in HBSS was added and after 35 min in the darkness, fluorescence intensity of the MBBr-GSH adduct was measured at the excitation wavelength (Ex) of 360 nm and the emission wavelength (Em) of 460 nm. Subsequently, PI was added for 20 min and the fluorescence (F_{PI}) of this stain was measured at Ex 530 nm/Em 645 nm. By adding the surfactant Nonidet P-40 for 20 min, all vital cells were lyzed and the fluorescence (F_{max}) was read again to determine the total cell number per well (PI stains only DNA of non-vital cells). Then MBBr-readings were normalized to cell numbers based on F_{max} in the presence of the surfactant Non-