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Hypoxia inhibits mineralization ability of human dental pulp cells treated with TEGDMA but increases cell survival in accordance with the culture time

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ABSTRACT

Objective: To evaluate the cytotoxicity and mineralization effects of TEGDMA in human dental pulp cells (hDPCs) under hypoxic and normoxic culture conditions.
Design: Cell viability was evaluated using XTT assay after incubation periods of 24, 48, or 72 h. The expression of mineralization-related genes (osteonectin, osteopontin, dentin sialophosphoprotein, collagen type 1) and heme oxygenase 1 (HO-1) was assessed by quantitative real-time polymerase chain reaction at 24 and 72 h.
Results: In XTT assay, viability was higher in 0.3, 1, 2, 4, and 5 mM groups in the presence of 21% O_2 after 24 h ($p < 0.05$). Additionally, while 0.3, 1, 2 mM groups had higher cell viability in the presence of 21% O_2 after 48 h ($p < 0.05$), in 3 mM groups cell viability was higher under 3% O_2 than 21% O_2 after both 24 and 48 h ($p < 0.05$). 1–3 mM groups had higher cell viability under 3% O_2 after 72 h ($p < 0.05$). There was no difference between 4 and 5 mM groups with regards to cell viability after 48 or 72 h ($p > 0.05$). In the gene expression study, TEGDMA-treated hDPCs showed lower mineralization potential in the presence of 3% than with 21% O_2 ($p < 0.05$). hDPCs revealed higher HO 1 expression in 0.3 and 1 mM groups under hypoxic than under normoxic conditions after a 72-h time period ($p < 0.001$).
Conclusions: Hypoxic conditions increased cell survival in accordance with the culture period but inhibited the odontoblastic differentiation of hDPCs treated with TEGDMA.

1. Introduction

Triethylene glycol dimethacrylate (TEGDMA) is a major resin monomer and is used extensively in the structure of dentin bonding agents and composite resins. Degradation processes and incomplete polymerization can lead to the release of resin monomers into the oral environment. Direct contact with surrounding pulp tissue or the diffusion of resin monomers through the dentinal tubules may cause hazardous effects on dental pulp tissue, such as cytotoxicity, impaired cellular functions, and pulpal inflammatory responses (Bakopoulou, Papadopoulos, & Garefis, 2009; Galler et al., 2011; Hebling, Giro, & Costa, 1999). Dentin thickness and the severity of caries lesions are important factors in determining the amount of resin monomers interacting with dental pulp tissue (Hamid & Hume, 1997).

Human dental pulp cells (hDPCs) are localized in an environment that is surrounded by hard dentin tissue, and nutritional support for hDPCs comes only through the vessels in root canals. Oxygen is obviously fundamental for the vitality of dental pulp tissue. However, limitations in oxygen sources in dental pulp tissue lead to a lower oxygen tension, equivalent to 3% O_2, in comparison with the 21% O_2 in an ambient air (Yu, Boyd, Cringle, Alder, & Yu, 2002). However, artificial cavities which occur due to access cavities or dental carries provide an access route for the atmospheric oxygen to the pulp tissue and this process increases the oxygen tension at the surface of dental pulp tissue but has

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minimal influence on the deeper pulp (Iida et al., 2010; Yu et al., 2002). Variable factors such as respiratory and cardiac functions, blood volume and haemoglobin, local vascular perfusion and the rate of oxygen consumption may affect the local oxygen tension in the pulp tissue. Yu et al. (2002) suggested that odontoblast layer within the pulp tissue constitutes the region of oxygen consumption. This information reflects that reparative dentinogenesis process may affect the distribution of local oxygen tension. Various studies have explored cell proliferation and differentiation characteristics of dental pulp tissue under almost the same hypoxic conditions (Iida et al., 2010; Sakdee, White, Pagonis, & Hauschka, 2009; Wang, Wei, Ling, Huang, & Gong, 2010). The results revealed that hypoxic conditions increase the cell proliferation capacity of hDPCs while inhibiting their differentiation.

To date, all reported studies regarding the biocompatibility of TEGDMA have been performed in the presence of ambient air, which has a much higher oxygen tension than the natural microenvironment of dental pulp tissue has. In the present study, we tested the hypothesis that hypoxic conditions may alter the cytotoxicity and gene expression profile observed in the presence of TEGDMA on hDPCs. Thus, we compared the results of hypoxic and normoxic conditions on hDPCs in the presence of TEGDMA.

2. Materials and methods

2.1. Cell culture

This study was approved by the Ethics Committee of Gulhane Military Medical Faculty, Turkey. Written informed consent was provided by all participants.

Dental pulp tissues were obtained from the molars of five healthy patients undergoing orthodontic treatments. The extracted molars were kept in phosphate-buffered saline solution (Biological Industries, Kibbutz Beit Haemek, Israel) containing 100 U/mL penicillin and 100 μg/mL streptomycin (Biological Industries). After they were transferred to the laboratory, extracted molars were cut horizontally at 1 mm below the cementoenamel junction. The pulp tissues were gently separated from the crown and root and placed in a 100-mm Petri dish. The pulp tissues were cut into small pieces with a blade and cultured in Dulbecco's modified Eagle's medium (DMEM; Biological Industries) containing 10% fetal bovine serum (FBS; Biological Industries), 100 U/mL penicillin, and 100 μg/mL streptomycin (Biological Industries). Tissue cultures were maintained in a humidified atmosphere of 5% CO_2 at 37 °C. Cells from the fifth passage were used for the experiments and cultured for 24 h before they were used in the test.

2.2. XTT assay

The XTT assay is useful for determining cellular proliferation and viability by spectrophotometric quantification. The assay is used to measure cell proliferation in response to growth factors, cytokines, and nutrients based on the conversion of the yellow tetrazolium salt 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) to an orange formazan dye by metabolically active and viable cells. Cells were seeded in 96-well plates at 2×10^4 cells/well and incubated for 24 h in a humidified atmosphere of 5% CO_2 at 37 °C to allow attachment of the cells. They were then transferred to normoxic or hypoxic conditions; 21% O_2 + 5% CO_2 was designated as a normoxic, and 3% O_2 + 5% CO_2 as a hypoxic condition. Six study groups were prepared for the different concentrations of TEGDMA (Sigma Chemical Company, St. Louis, MO, USA): 0.3, 1–5 mM. Untreated cell cultures were used as a control group. The cell cultures were exposed to serial dilutions of the test material. After incubation periods of 24, 48, and 72 h under normoxic or hypoxic conditions, 50 μL (0.3 mg/mL) of XTT labeling mixture (Cell Proliferation Kit II; Roche, Mannheim, Germany) was added to each well, followed by incubation for 4 h in a humidified atmosphere of 5% CO_2 at 37 °C. The absorbance of the metabolized medium was measured at 450 nm using a microplate reader. The cell viability of each test group was calculated as a percentage of the control group. Each experimental group consisted of 18 samples.

2.3. Quantitative real-time PCR (qRT-PCR) analysis

Cells were seeded in 6-well plates at 1×10^6 cells/well and incubated for 24 h in a humidified atmosphere of 5% CO_2 at 37 °C ($n = 3$ per experimental group). The cell cultures were exposed to serial dilutions of TEGDMA (0.3, 1, and 3 mM) for 24- and 72-h incubation periods under normoxic and hypoxic conditions. Total cellular RNA was extracted from cultured hDPCs using an RNA isolation kit (High Pure RNA Isolation Kit, Roche, Germany). cDNA was synthesized from 10 ng of total RNA using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche). The cDNA obtained was used as a template for PCR.

The odontogenic responses of the hDPCs were assessed by analyzing specific markers: osteonectin (ON), osteopontin (OPN), dentin sialophosphoprotein (DSPP), collagen type 1 (COL1A1), and heme oxygenase 1 (HO-1). β-Actin (ACTB) was used as a housekeeping gene to normalize RNA expression. Probe–primer pairs for target genes were purchased from Roche Diagnostic as RealTime ready assays (ON 103218, OPN 101170, DSPP 139816, COL1A1 100861, HO-1 110977, and ACTB 101125). Primer sequences are listed in Table 1. Real-time PCR was performed

TABLE 1
Primer sequence list in qRT-PCR.

Target Gene	Primer sequence (5′-3′)	Annealing Temperature (°C)
ON	F: 5′-TTGATGATGGTGCAGAGGAA-3′ R: 5′-CTTGCCGTGTTTGCAGTG-3′	**60**
OPN	F:5′- CGCAGACCTGACATCCAGTA −3′ R:5′- GGCTGTCCCAATCAGAAGG −3′	
DSPP	F:5′- TGGAAAAGACAGTAGTAATAACAGCAA −3′ R:5′- TCTTCTTTCCCATGGTCCTG −3′	
COL1A1	F:5′- AGGTGAAGCAGGCAAACCT −3′ R:5′- CTCGCCAGGGAAACCTCT −3′	
HO-1	F:5′- CAGTCAGGCAGAGGGTGATAG −3′ F:5′- CCTGCAACTCCTCAAAGAGC-3′	
ß-Actin	F:5′- GGCCAGGTCATCACCATT −3′ R:5′- GGATGCCACAGGACTCCAT −3′	