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# Effect of hydrogen-peroxide-mediated oxidative stress on human dental pulp cells

*Diana Gabriela Soares* [a], *Fernanda Gonçalves Basso* [b], *Josimeri Hebling* [c], *Carlos Alberto de Souza Costa* [b,*]

[a] *Department of Dental Materials and Prosthodontics, Univ. Estadual Paulista – UNESP, Araraquara School of Dentistry, Araraquara, SP, Brazil*

[b] *Department of Physiology and Pathology, Univ. Estadual Paulista – UNESP, Araraquara School of Dentistry, Araraquara, SP, Brazil*

[c] *Department of Orthodontics and Pediatric Dentistry, Univ. Estadual Paulista – UNESP, Araraquara School of Dentistry, Araraquara, SP, Brazil*

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ABSTRACT

*Objectives:* To evaluate the effect of the oxidative stress on human dental pulp cells (HDPCs) promoted by toxic concentrations of hydrogen peroxide ($H_2O_2$) on its odontoblastic differentiation capability through time.

*Methods:* HDPCs were exposed to two different concentrations of $H_2O_2$ (0.1 and 0.3 μg/ml) for 30 min. Thereafter, cell viability (MTT assay) and oxidative stress generation ($H_2$DCFDA fluorescence assay) were immediately evaluated. Data were compared with those for alkaline phosphatase (ALP) activity (thymolphthalein assay) and mineralized nodule deposition (alizarin red) by HDPCs cultured for 7 days in osteogenic medium.

*Results:* A significant reduction in cell viability and oxidative stress generation occurred in the $H_2O_2$-treated cells when compared with negative controls (no treatment), in a concentration-dependent fashion. Seven days after $H_2O_2$ treatment, the cells showed significant reduction in ALP activity compared with negative control and no mineralized nodule deposition.

*Conclusion:* Both concentrations of $H_2O_2$ were toxic to the cells, causing intense cellular oxidative stress, which interfered with the odontogenic differentiation capability of the HDPCs.

*Clinical significance:* The intense oxidative stress on HDPCs mediated by $H_2O_2$ at toxic concentrations promotes intense reduction on odontoblastic differentiation capability in a 7-day evaluation period, which may alter the initial pulp healing capability in the in vivo situation.



* *Corresponding author at*: Department of Physiology and Pathology, Univ. Estadual Paulista – UNESP, Araraquara School of Dentistry, Humaitá Street, 1680, Araraquara 14801-903, SP, Brazil. Tel.: +55 16 3301 6477; fax: +55 16 3301 6488.
E-mail address: casouzac@foar.unesp.br (C.A. de Souza Costa).

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## 1. Introduction

Dental pulp is a highly specialized connective tissue almost completely enclosed by dentine, which is deposited by odontoblasts. These long-lived pulp cells are organized in a layer at the dentine-pulp interface, where a continuous collagen-rich dentine matrix is maintained for the entire life of the tooth.[1] It is known that the pulp–dentine complex responds to external pathological stimuli through deposition and mineralization of tertiary dentin.[2] Following relatively mild tooth injury, odontoblasts are up-regulated to secrete and mineralize a tubular reactionary dentine. The main objectives of this process are to: (1) keep the pulp tissue away from noxious stimuli; and (2) reduce the diffusion of toxic components released from dental materials or microorganisms into the pulp space, thus protecting the underlying cells and maintaining pulp vitality.[3] However, high-intensity injury can kill odontoblasts, compromising the homeostasis and vitality of the pulp-dentine complex. In this specific situation, dental pulp stem cells found on pulp tissue are recruited, then differentiated into odontoblast-like cells able to secrete and mineralize the underlying reparative dentine matrix.[4] Previous studies have demonstrated that, in addition to causing toxic effects to odontoblasts,[5] components released from dental materials can also inhibit the odontoblastic differentiation of pulp stem cells.[6,7] In clinical circumstances, this inhibitory effect may interfere with pulp healing and cause persistent inflammatory reactions as well as inner dentine resorption.[8,9]

Several in vitro and in vivo studies have demonstrated that professional tooth bleaching, an aesthetic procedure widely performed in clinical offices, causes intense damage to pulp cells.[10–15] This kind of operative procedure is based on the oxidative action of hydrogen peroxide ($H_2O_2$) in the organic phase of dentine. However, it is known that $H_2O_2$ can diffuse through enamel and dentine to gain access to pulp space only a few minutes after the application of the bleaching gel to the tooth surface.[16,17] In a current study, Soares et al.[15] demonstrated that high concentrations of $H_2O_2$ can cause oxidative stress to human dental pulp cells (HDPCs), interfering with the proliferative capacity of this cell type in short-term evaluations. However, the effects of this oxidative molecule on the odontogenic differentiation ability of HDPCs, are unclear. Therefore, the aim of the present study was to evaluate the effects of the oxidative stress generated by different concentrations of $H_2O_2$ on HDPCs odontoblastic differentiation.

## 2. Materials and methods

### 2.1. Cell culture

HDPCs were obtained by enzymatic digestion of pulp tissue from freshly impacted third molar surgically extracted and donated by a young patient (Proc. no. 13/11; Ethics Committee of Araraquara School of Dentistry, SP, Brazil). The pulp tissue was incubated with type II collagenase (200 units/ml; Worthington Biochemical Corporation, Lakewood, NJ, USA) for 24 h at 37 °C and 5% $CO_2$. Thereafter, the cells were subcultured in complete DMEM (Dulbecco's Modified Eagle Medium; supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mmol/l glutamine; Gibco, Grand Island, NY, USA) and 10% heat-inactivated FBS (Foetal Bovine Serum; Gibco). Cells at passage #3 were used in this experiment.[18] For experimental analysis, the cells were seeded on 24-well plates to 80% confluence (60,000 cells/well; 24 h).

### 2.2. Experimental procedure

To simulate a professional tooth bleaching treatment in which a bleaching agent is applied on enamel for 30 min,[10] in this study the HDPCs were exposed for the same time (30 min at 37 °C and 5% $CO_2$) to solutions containing 0.1 μg/ml and 0.3 μg/ml of $H_2O_2$ (Labsinth, Diadema, SP, Brazil) diluted in fresh DMEM (without FBS). The concentrations of $H_2O_2$ used in this study were based on a previous investigation in which the authors observed that the HDPCs had its viability significantly reduced immediately after exposure (65–83%); however, the remaining cells exhibited short-term proliferative capability,[15] allowing for long-term analysis of odontoblastic differentiation. Fresh DMEM was used in negative control. Immediately after cell contact with the $H_2O_2$ solutions, the viability (MTT assay) and oxidative stress ($H_2$DCFDA fluorescence assay) were assessed. For odontoblastic differentiation analysis, the cells were incubated in complete osteogenic medium (DMEM + heat-inactivated FBS supplemented with 10 nmol/l β-glycerophosphate and 50 μg/ml sodium ascorbate; Sigma) for 7 days (the medium was changed daily). Thereafter, ALP activity (thymolphthalein assay) and mineralized nodule deposition (alizarin red) were analyzed.

### 2.3. MTT assay

Immediately after the contact time, the culture medium was aspirated, the cell washed with 1 ml of PBS, and then the cells ($n = 6$) were incubated with MTT solution (5 mg/ml; Sigma), diluted in DMEM (1:10), at 37 °C and 5% $CO_2$ for 4 h. Next, the formazan crystals formed in the viable cells were dissolved in acidified isopropanol, and the absorbance was measured in an ELISA reader at 570 nm (Tp Reader, Thermoplate, Nanshan District, Shenzhen, China). The mean absorbance value of the negative control group was considered 100% of cell viability, and the percentage cell viability for each experimental group was calculated based on this parameter.

### 2.4. $H_2$DCFDA assay

Oxidative stress was measured immediately after the contact with the test solutions by means of a cell-permeant fluorescence probe, 2′,7′-dichlorodihydrofluorescein diacetate ($H_2$DCFDA) (Life Technologies, San Francisco, CA, USA) ($n = 6$). The cells were incubated at 37 °C and 5% $CO_2$ with 5 μM $H_2$DCFDA for 30 min prior to incubation with the $H_2O_2$ solutions. Immediately after contact with $H_2O_2$, the culture medium was aspirated and cells washed with PBS (1 ml), in order to remove dead cells detached from the bottom of the well. The cells were then analyzed by fluorescence microscopy with the FITC filter. Images of 4 fields from each sample were captured, and the percentages of cells with positive fluorescence related to the