Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.intl.elsevierhealth.com/journals/jden

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*

ARTICLE INFO

Article history:
Received 10 September 2014
Received in revised form
2 December 2014
Accepted 5 December 2014

Keywords:
Toxicity
Pulp cells
Hydrogen peroxide

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_2DCFDA 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).

http://dx.doi.org/10.1016/j.jdent.2014.12.006
0300-5712/

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_2DCFDA 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_2DCFDA 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_2DCFDA) (Life Technologies, San Francisco, CA, USA) ($n = 6$). The cells were incubated at 37 °C and 5% CO_2 with 5 μM H_2DCFDA 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

total of cells counted in the bright field were calculated. The average value of each well was used for statistical analysis.

2.5. *Thymolphthalein assay*

After 7-day incubation in osteogenic culture medium, cell lysis was performed (0.1% sodium lauryl sulfate; Sigma), and an aliquot was transferred to tubes containing the thymolphthalein monophosphate substrate at 37 °C. After reaction with ALP, released thymolphthalein acquired a purple colour in the presence of sodium carbonate and sodium hydroxide (End point assay; Labtest, Lagoa Santa, MG, Brazil), and absorbance was read at a 590-nm wavelength with the ELISA microplate reader, and converted into U/l by means of a standard curve with known amounts of ALP. Total protein dosage was performed for normalization of ALP, as previously described,[19–21] and absorbance was measured at a 655-nm wavelength with the ELISA microplate reader. The absorbance value obtained was converted into mg/l by a standard protein curve. The final value of ALP was normalized by total protein data, and the ALP activity (U/mg of protein) was transformed into a percentage with the mean value of negative control considered as presenting 100% of ALP activity.

2.6. *Alizarin red staining*

After 7-day incubation, the cells were washed with PBS, fixed in cold 70% ethanol, and then stained with alizarin red dye (40 mM, pH 4.2; Sigma) for 20 min with shaking (VDR Shaker, Biomixer, Ribeirão Preto, SP, Brazil). After aspiration of unincorporated dye, the cells were washed twice with deionized water for the removal of excess stain, and representative images from each group were taken by light microscopy (Olympus BX51, Olympus, Miami, FL, USA). The mineralized nodules were solubilized in 10% cetylpyridinium chloride (Sigma), and absorbance was measured at 570 nm (ELISA microplate reader). The percentage of mineralized nodule deposition was calculated based on the mean value of the control group, which was considered as presenting 100% of staining.

2.7. *Statistical analysis*

To verify the reproducibility of data, two independent experiments for all protocols in this study were performed. Thereafter, data were compiled and subjected to Shapiro–Wilks' and Levene's test for verification of normality and homoscedasticity, respectively. Since data were not normally distributed, they were first subjected to the Kruskal–Wallis test for comparison among the three groups. In the event of a statistical significance, the Mann–Whitney test was then applied to determine which groups were significantly different from each other ($p \leq 0.05$).

3. Results

The results of cell viability are presented in Fig. 1. We observed a significant immediate decrease in cell viability, proportional to the concentration of H_2O_2 applied in contact with HDPCs.

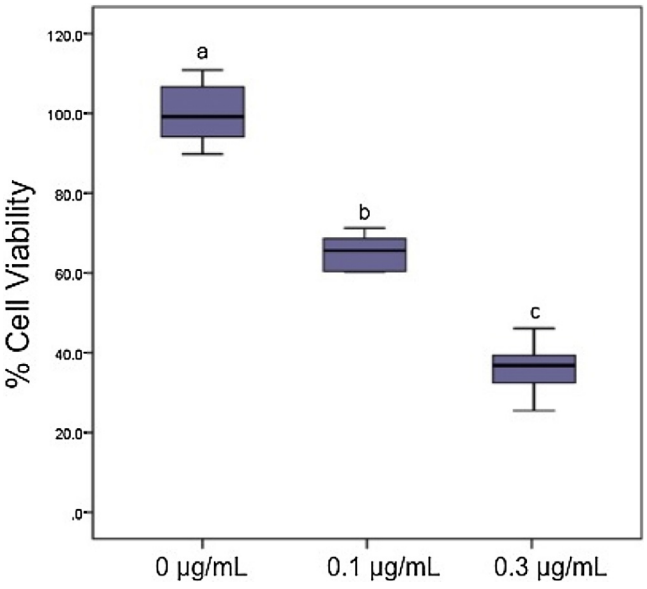

Fig. 1 – Box-whisker plot of MTT assay. Vertical axis represents percentage of cell viability, which represents the mixed culture of pulp cells containing a subpopulation of HDPCs, and horizontal axis represents the concentrations of H_2O_2 in experimental groups. Upper and lower limits of boxes represent, respectively, the 25th and 75th percentiles, and the horizontal line represents the median. Upper and lower lines indicate the maximum and minimum values, respectively. Since groups were not statistically similar (Kruskal–Wallis, $p < 0.05$), pairwise comparison is indicated by letters. Groups identified by distinct letters are statistically different (Mann–Whitney, $p < 0.05$).

Significant reduction of about 34.4% and 63% in cell viability ($p \leq 0.05$) was observed for 0.1 μg/ml and 0.3 μg/ml H_2O_2 solutions, respectively. The number of cells under oxidative stress significantly different from negative control ($p \leq 0.05$), and was also proportional to the H_2O_2 concentration in the solution (Fig. 2B). No fluorescence was observed in cells of the negative control, whereas 59.4% and 82.8% of cells showed positive fluorescence for the H_2DCFDA probe after contact with the 0.1 and 0.3 μg/ml H_2O_2 solutions, respectively. In the images obtained by fluorescence microscopy (Fig. 2A), we observed that the intensity of fluorescence in the cells was also proportional to the H_2O_2 concentration in the solutions. Significant reductions ($p \leq 0.05$) in ALP activity occurred when the cells were exposed to 0.1 μg/ml (82.2%) and 0.3 μg/ml (89.9%) of H_2O_2, compared with negative controls (Fig. 3C). The percentage of alizarin red staining was strongly reduced ($p \leq 0.05$) in H_2O_2-treated groups in comparison with negative controls. With the negative controls considered as presenting 100% of positive stain, the alizarin red staining in the HDPCs was reduced by 93% and 95% when they were exposed to 0.3 μg/ml and 0.1 μg/ml H_2O_2 solutions, respectively (Fig. 3B). The images obtained by light microscopy revealed the presence of mineralized nodules in the negative control group only (Fig. 3A).

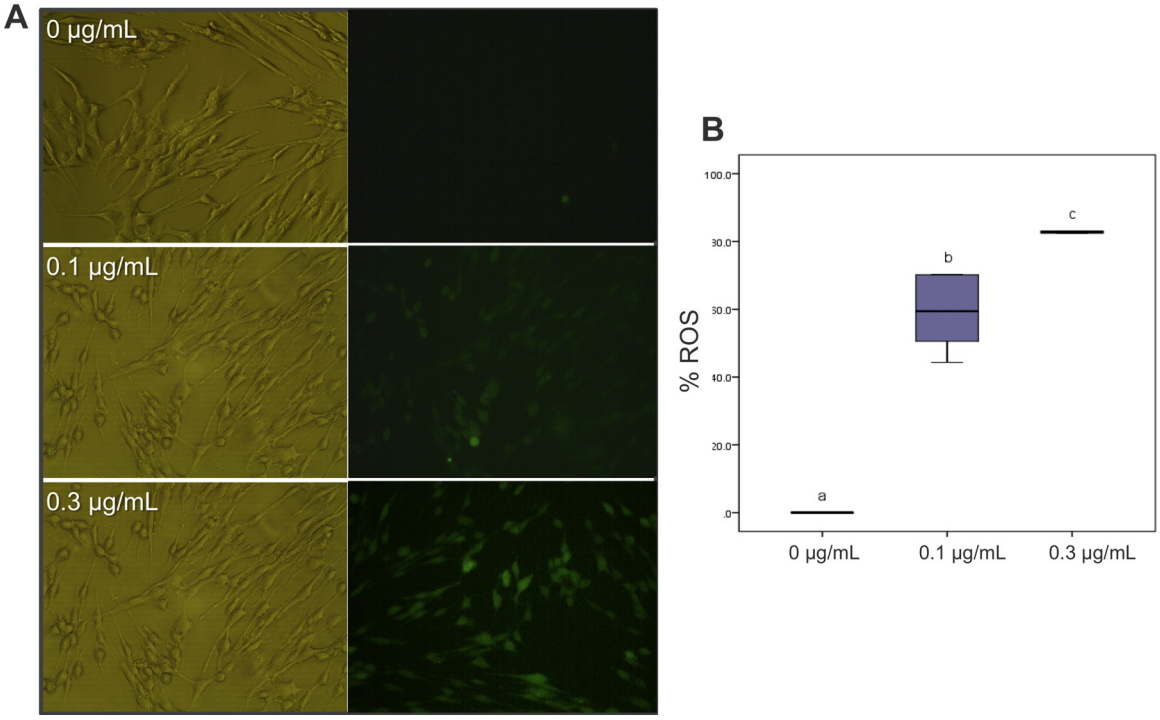

Fig. 2 – H_2DCFDA assay. (A) Images obtained by fluorescence microscopy of HDPCs stained with H_2DCFDA (20×) for each H_2O_2 concentration tested. For each group, the left image represents the bright field and the right image is the same area under fluorescence. Cells with positive stain for H_2DCFDA are marked in green, indicating that they are under oxidative stress. (B) Box-whisker plot of ROS percentage. Since groups were not statistically similar (Kruskal–Wallis, $p < 0.05$), pairwise comparison is indicated by letters. Groups identified by distinct letters are statistically different (Mann–Whitney, $p < 0.05$).

4. Discussion

In the present investigation, the long-term effect of H_2O_2 mediating cytotoxicity on the HDPCs regenerative potential through time was assessed. It is known that dental pulp stem cells are essential for the homeostasis of pulp–dentine complex, especially in clinical situations in which the odontoblasts are killed by toxic components capable of diffusing across dentine to reach the pulpal space. Then, stem cells from the Howl's layer are recruited to differentiate into odontoblast-like cells which perform the deposition and mineralization of collagen-rich dentine matrix.[2] However, previous in vivo studies carried out in lower human incisors demonstrated that both odontoblasts and pulpal stem cells are negatively affected by in-office tooth bleaching therapy.[10,11] In vitro studies using extracted human incisors and third molars showed that an amount of 0.1–0.6 μg/ml of H_2O_2 can reach the pulp chamber after applying bleaching gels with high concentrations of H_2O_2 on enamel.[16,17] Additionally, it was reported that the amount of H_2O_2 capable of diffusing through enamel/dentine discs simulating the thickness of human maxillary incisors (3.5 mm) causes toxicity to odontoblast-like cells and human dental pulp cells. The intensity of this toxic effect is proportional to the H_2O_2 concentration in contact with cells.[13–15] On the other hand, HDPCs exposed to 0.1–0.3 μg/ml of H_2O_2 were able to proliferate significantly 3 days thereafter.[15] Therefore, the odontoblastic differentiation and mineralization potential of HDPCs, which are directly related with the regenerative potential of pulp tissue, were assessed in this study.

It was determined, as expected, that the reduction in HDPCs viability was H_2O_2-concentration-dependent. Fluorescence analysis with the H_2DCFDA probe demonstrated that the remaining cells were under oxidative stress. The intensity of the oxidative stress was also directly related to the concentration of H_2O_2 in contact with cells. The H_2O_2-mediated reduction in pulp cell viability has been related to two basic mechanisms: (1) the direct contact of H_2O_2 by-products with cell membranes, causing disruption and, consequently, cell death; and (2) the induction of oxidative stress conditions due to H_2O_2 diffusion through cell membranes.[15,22,23] In the physiological state, a balance exists between the production of ROS (reactive oxygen species) and their neutralization by antioxidant systems. Oxidative stress occurs when this balance is disturbed. Depending on the intensity of the oxidative stress, the cell components undergo severe oxidative damage, ultimately compromising cell viability. It has been reported that the ROS accumulated during oxidative stress are transient due to their high reactivity, leading to oxidative damage of indispensable biomolecules such as proteins, lipids, and nucleic acids.[22,24]

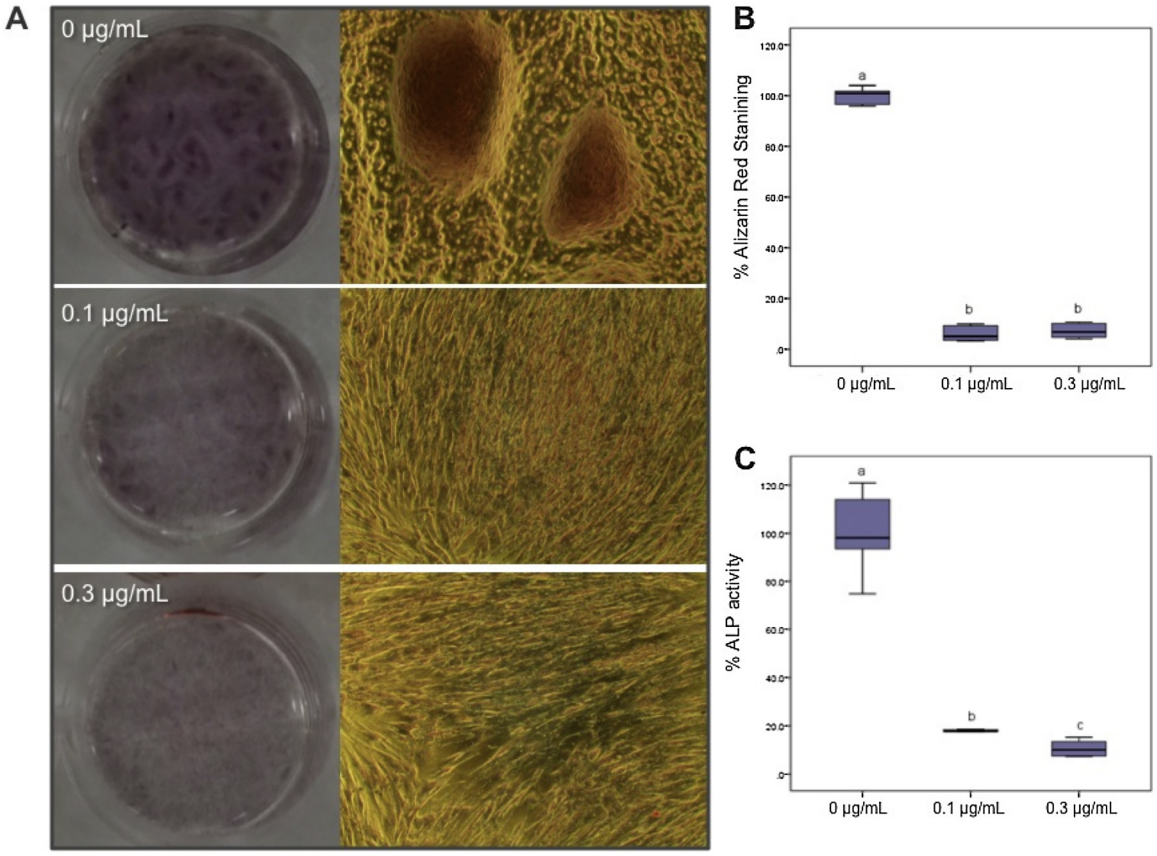

Fig. 3 – Alizarin red assay. (A) Panel of mineralized nodule deposition for each H_2O_2 concentration tested. For each group, the left image represents a digital photograph of the well, and at right is a light-microscopic (40×) image from a representative area of the well. Absence of nodule deposition was observed for the H_2O_2-treated groups. (B, C) Box-whisker plot of alizarin red and ALP activity percentage, respectively. Since groups were not statistically similar (Kruskal–Wallis, $p < 0.05$), pairwise comparison is indicated by letters. Groups identified by distinct letters are statistically different (Mann–Whitney, $p < 0.05$).

Numerous studies have demonstrated that HDPCs are highly sensitive to H_2O_2 damage. The same amount of H_2O_2 able to reduce almost 100% of the viability of cultured HDPCs causes no significant reduction in the viability of osteoblasts, fibroblasts, and odontoblast-like cells.[13,15,25–27] This result seems to be associated with a more intense oxidative stress generated in human pulp cells in comparison with other cell lineages.[15,27] In the present study, treatment of HDPCs with 0.1 and 0.3 µg/ml H_2O_2 significantly affected the expression of odontoblastic markers. This result was related to the intense oxidative stress generated immediately after H_2O_2 came into contact with cultured cells. Alkaline phosphatase (ALP) is involved with the initial phase of dentine matrix biomineralization. According to Goldberg et al.,[1] ALP promotes dephosphorylation of extracellular matrix proteins, providing inorganic phosphate. In the present study, the HDPCs of the control group showed ALP activity and deposition of mineralized nodules after 7-day incubation. These data are in agreement with those from a previous study by Zanini et al.,[28] who observed ALP activity during the odontoblast maturation process associated with secretory activity. Conversely, cells in contact with H_2O_2 presented dramatic reductions in ALP activity and the absence of mineralization nodule deposition. Thus, one can conclude that toxic concentrations of H_2O_2 in contact with pulp cells for a relatively short-term period (30 min) can drastically alter the phenotypic characteristic related to the odontoblastic differentiation and deposition of mineralized matrix. A recent study also demonstrated that exposition of pulp cells to H_2O_2 for long periods (12 days) resulted in intense oxidative stress generation associated to low cell viability, down expression of antioxidant molecules and odontogenic markers, impairing the odontoblastic differentiation.[29]

However, in the present investigation, odontoblastic differentiation capability was evaluated over a 7-day period. Some in vitro studies have demonstrated that numerous cells are required for biomineralization.[28,30] Therefore, since reduced numbers of cultured HDPCs remained viable in the wells immediately after contact with H_2O_2 solutions, one can speculate that longer periods of incubation would be necessary for the cells to reach confluence and deposit mineralized matrix, as observed in the negative control group. Additionally, in spite of the reduction in ALP activity compared with that of the negative controls, the HDPCs still maintained this activity after 7-day exposure to H_2O_2 solutions. The potential of human pulp cells exposed to similar concentrations of H_2O_2

to overcome oxidative stress and proliferate after 72-h exposure was recently demonstrated by Soares et al.[15] The authors also demonstrated that pulp cells exposed to H_2O_2 presented immediate morphological alterations, which was recovered with time. Therefore, one may speculate that pulp cells not lethally damaged by H_2O_2 have an inherent ability to regulate oxidative stress, recovering their viability and functions over time. However, in spite of pulp cells can recover from damage caused by oxidative stress and proliferate 3 days after H_2O_2 exposure,[15] it was demonstrated in the present investigation that the regenerative potential of these cells is significantly altered even 7 days later. Therefore, it may be suggested that longer periods than used in the study are necessary to determine if pulp cells can definitively recover from damage caused by toxic concentrations of H_2O_2. However, this is a specific focus for future studies.

Depending on the intensity of immune cell recruitment during inflammation, intense tissue damage may take place, since these cells release proteolytic enzymes such as metalloproteinases, which break down the extracellular matrix components and cellular contacts.[31] This histological event, characterized by inflammatory reactions associated with partial tissue necrosis and the absence of tertiary dentine deposition, was also reported by de Souza Costa et al.[10] after applying a bleaching gel with a high concentration of H_2O_2 to human sound teeth. According to Cooper et al.,[31] there is a fine balance between inflammatory mediator dose and pulp tissue regeneration. When low dosages of pro-inflammatory cytokines are applied to pulp cells for short periods, up-regulation of odontoblastic markers is observed.[31,32] However, pulp cells exposed to these cytokines, or high concentrations thereof, for long periods can result in impaired odontoblastic marker expression as well as no mineralized matrix deposition.[33–35] Similarly, some studies have demonstrated that exposure of cultured cells to low concentrations of H_2O_2 (0.1–0.3 mmol/l) increased the deposition of calcified nodules and induced the over-expression of proteins related to odontogenic differentiation,[26,36] which was related to heme oxygenase-1 (HO-1) over-expression.[26] The over-expression of HO-1 by odontoblasts was also observed in an in vivo study where pre-molars were subjected to a low-concentration bleaching gel (3.5% H_2O_2).[37] Additionally, Lee et al.[29] demonstrated that the peroxisome proliferator-activated receptor gamma (PPARγ), a transcriptional factor belonging to the ligand-activated nuclear receptor superfamily, plays a role in the odontogenic differentiation capability of human dental pulp cells undergo oxidative stress mediated by H_2O_2. When pulp cells were treated with a toxic concentration of H_2O_2, intense cell dead (around 60%) was observed associated to down-regulation of odontoblastic markers gene expression; however, PPARγ overexpressed cells survived more than 75% when exposed to the same condition and highly expressed odontoblastic differentiation markers. According to the authors, PPARγ promotes ROS removal by increasing the antioxidant system activity. Analysis of these data demonstrates that pulp cells have an inherent mechanism of cytoprotection against H_2O_2-induced cytotoxicity, which depends on the H_2O_2 concentration applied to them. Therefore, further studies aimed to prevent or reduce the diffusion of H_2O_2 in concentrations high enough to interfere with pulp cell functions are needed. This scientific approach will certainly drive the development of further tooth-bleaching protocols capable of providing satisfactory aesthetic outcome without damage to the pulp–dentine complex.

5. Conclusions

It was concluded that toxic concentrations of H_2O_2 cause intense oxidative stress to HDPCs, interfering with their phenotypic function relative to the odontoblastic differentiation ability.

Conflict of interest

The authors have no conflict of interest.

Acknowledgments

The authors acknowledge FAPESP (grant # 2013/23520-0), CNPq (grant # 301029/2010-1), and FUNDUNESP (grant # 0024/021/13-PROPE-CDC) for financial support.

REFERENCES

1. Goldberg M, Kulkarni AB, Young M, Boskey A. Dentin: structure, composition and mineralization. *Frontiers in Biosciences* 2011;**3**:711–35.
2. Goldberg M, Smith AJ. Cells and extracellular matrices of dentin and pulp: a biological basis for repair and tissue engineering. *Critical Reviews in Oral Biology and Medicine* 2004;**15**:13–27.
3. Couve E, Osorio R, Schmachtenberg O. The amazing odontoblast: activity, autophagy, and aging. *Journal of Dental Research* 2013;**92**:765–72.
4. Simon SR, Berdal A, Cooper PR, Lumley PJ, Tomson PL, Smith AJ. Dentin-pulp complex regeneration: from lab to clinic. *Advances in Dental Research* 2011;**23**:340–5.
5. Hebling J, Giro EM, Costa CA. Human pulp response after an adhesive system application in deep cavities. *Journal of Dentistry* 1999;**27**:557–64.
6. Bakopoulou A, Leyhausen G, Volk J, Tsiftsoglou A, Garefis P, Koidis P, *et al*. Effects of HEMA and TEDGMA on the in vitro odontogenic differentiation potential of human pulp stem/progenitor cells derived from deciduous teeth. *Dental Materials* 2011;**27**:608–17.
7. Krifka S, Spagnuolo G, Schmalz G, Schweikl H. A review of adaptive mechanisms in cell responses towards oxidative stress caused by dental resin monomers. *Biomaterials* 2013;**34**:4555–63.
8. Costa CA, Giro EM, do Nascimento AB, Teixeira HM, Hebling J. Short-term evaluation of the pulpo-dentin complex response to a resin-modified glass-ionomer cement and a bonding agent applied in deep cavities. *Dental Materials* 2003;**19**:739–46.
9. Costa CA, Ribeiro AP, Giro EM, Randall RC, Hebling J. Pulp response after application of two resin modified glass ionomer cements (RMGICs) in deep cavities of prepared human teeth. *Dental Materials* 2011;**27**:e158–70.

10. de Souza Costa CA, Riehl H, Kina JF, Sacono NT, Hebling J. Human pulp responses to in-office tooth bleaching. *Oral Surgery Oral Medicine Oral Pathology Oral Radiology and Endodontics* 2010;**109**:e59–64.
11. Roderjan DA, Stanislawczuk R, Hebling J, de Souza Costa CA, Soares DG, Reis A, *et al.* Histopathological features of dental pulp tissue from bleached mandibular incisors. *Journal of Materials Science and Engineering B* 2014;**6**:178–85.
12. Cintra LT, Benetti F, da Silva Facundo AC, Ferreira LL, Gomes-Filho JE, Ervolino E, *et al.* The number of bleaching sessions influences pulp tissue damage in rat teeth. *Journal of Endodontics* 2013;**39**:1576–80.
13. Duque CC, Soares DG, Basso FG, Hebling J, de Souza Costa CA. Bleaching effectiveness, hydrogen peroxide diffusion, and cytotoxicity of a chemically activated bleaching gel. *Clinical Oral Investigations* 2014;**18**:1631–7.
14. Soares DG, Ribeiro AP, da Silveira Vargas F, Hebling J, de Souza Costa CA. Efficacy and cytotoxicity of a bleaching gel after short application times on dental enamel. *Clinical Oral Investigations* 2013;**17**:1901–9.
15. Soares DG, Basso FG, Hebling J, de Souza Costa CA. Concentrations of and application protocols for hydrogen peroxide bleaching gels: effects on pulp cell viability and whitening efficacy. *Journal of Dentistry* 2014;**42**:185–98.
16. Gökay O, Yilmaz F, Akin S, Tunçbìlek M, Ertan R. Penetration of the pulp chamber by bleaching agents in teeth restored with various restorative materials. *Journal of Endodontics* 2000;**26**:92–4.
17. Camargo SE, Valera MC, Camargo CH, Gasparoto Mancini MN, Menezes MM. Penetration of 38% hydrogen peroxide into the pulp chamber in bovine and human teeth submitted to office bleach technique. *Journal of Endodontics* 2007;**33**:1074–7.
18. Kerkis I, Kerkis A, Dozortsev D, Stukart-Parsons GC, Gomes Massironi SM, Pereira LV, *et al.* Isolation and characterization of a population of immature dental pulp stem cells expressing OCT-4 and other embryonic stem cell markers. *Cells Tissues and Organs* 2006;**184**:105–16.
19. Lizier NF, Kerkis A, Gomes CM, Hebling J, Oliveira CF, Caplan AI, *et al.* Scaling-up of dental pulp stem cells isolated from multiple niches. *PLoS ONE* 2012;**7**:e39885.
20. Basso FG, Pansani TN, Oliveira CF, Turrioni AP, Soares DG, Hebling J, *et al.* Cytotoxic effects of zoledronic acid on human epithelial cells and gingival fibroblasts. *Brazilian Dental Journal* 2013;**24**:551–8.
21. Soares DG, Basso FG, Pontes EC, Garcia LF, Hebling J, de Souza Costa CA. Effective tooth-bleaching protocols capable of reducing H_2O_2 diffusion through enamel and dentine. *Journal of Dentistry* 2014;**42**:351–8.
22. Cecarini V, Gee J, Fioretti E, Amici M, Angeletti M, Eleuteri AM, *et al.* Protein oxidation and cellular homeostasis: emphasis on metabolism. *Biochimica et Biophysica Acta* 2007;**1773**:93–104.
23. Markowitz K. Pretty painful: why does tooth bleaching hurt. *Medical Hypotheses* 2010;**74**:835–40.
24. Sinha K, Das J, Pal PB, Sil PC. Oxidative stress: the mitochondria-dependent and mitochondria-independent pathways of apoptosis. *Archives of Toxicology* 2013;**87**:1157–80.
25. Lee DH, Lim BS, Lee YK, Yang HC. Effects of hydrogen peroxide (H_2O_2) on alkaline phosphatase activity and matrix mineralization of odontoblast and osteoblast cell lines. *Cell Biology and Toxicology* 2006;**22**:39–46.
26. Min KS, Lee HJ, Kim SH, Lee SK, Kim HR, Pae HO, *et al.* Hydrogen peroxide induces heme oxygenase-1 and dentin sialophosphoprotein mRNA in human pulp cells. *Journal of Endodontics* 2008;**34**:983–9.
27. Zhu T, Lim BS, Park HC, Son KM, Yang HC. Effects of the iron-chelating agent deferoxamine on triethylene glycol dimethacrylate, 2-hydroxylethyl methacrylate, hydrogen peroxide-induced cytotoxicity. *Journal of Biomedical Materials Research Part B: Applied Biomaterials* 2012;**100**:197–205.
28. Zanini M, Sautier JM, Berdal A, Simon S. Biodentine induces immortalized murine pulp cell differentiation into odontoblast-like cells and stimulates biomineralization. *Journal of Endodontics* 2012;**38**:1220–6.
29. Lee YH, Kang YM, Heo MJ, Kim GE, Bhattarai G, Lee NH, *et al.* The survival role of peroxisome proliferator-activated receptor gamma induces odontoblast differentiation against oxidative stress in human dental pulp cells. *Journal of Endodontics* 2013;**39**:236–41.
30. Coyac BR, Chicatun F, Hoac B, Nelea V, Chaussain C, Nazhat SN, *et al.* Mineralization of dense collagen hydrogel scaffolds by human pulp cells. *Journal of Dental Research* 2013;**92**:648–54.
31. Cooper PR, Takahashi Y, Graham LW, Simon S, Imazato S, Smith AJ. Inflammation-regeneration interplay in the dentine–pulp complex. *Journal of Dentistry* 2010;**38**:687–97.
32. Min KS, Kwon YY, Lee HJ, Lee SK, Kang KH, Lee SK, *et al.* Effects of proinflammatory cytokines on the expression of mineralization markers and heme oxygenase-1 in human pulp cells. *Journal of Endodontics* 2006;**32**:39–43.
33. Paula-Silva FW, Ghosh A, Silva LA, Kapila YL. TNF-alpha promotes an odontoblastic phenotype in dental pulp cells. *Journal of Dental Research* 2009;**88**:339–44.
34. Alongi DJ, Yamaza T, Song Y, Fouad AF, Romberg EE, Shi S, *et al.* Stem/progenitor cells from inflamed human dental pulp retain tissue regeneration potential. *Regenerative Medicine* 2010;**5**:617–31.
35. Spoto G, Fioroni M, Rubini C, Tripodi D, Di Stilio M, Piattelli A. Alkaline phosphatase activity in normal and inflamed dental pulps. *Journal of Endodontics* 2001;**27**:180–2.
36. Matsui S, Takahashi C, Tsujimoto Y, Matsushima K. Stimulatory effects of low-concentration reactive oxygen species on calcification ability of human dental pulp cells. *Journal of Endodontics* 2009;**35**:67–72.
37. Anderson DG, Chiego Jr DJ, Glickman GN, McCauley LK. A clinical assessment of the effects of 10% carbamide peroxide gel on human pulp tissue. *Journal of Endodontics* 1999;**25**:247–50.