A COMPARATIVE STUDY ON THE CYTOTOXIC EFFECT OF DIFFERENT CONCENTRATIONS OF HYDROGEN PEROXIDE AND CARBAMIDE PEROXIDE ON THE SURVIVAL OF HUMAN DENTAL PULP STEM CELLS IN VITRO

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Abstract. Background: Tooth discoloration due to different causes (systemic or acquired) is one of the major issues that has involved the dentists to itself. up to now, dental bleaching has been reported as the most conservative method of treating teeth discoloration. In most bleaching methods, hydrogen peroxide and carbamide peroxide derivatives are used at different concentrations. Considering that these materials are oxidative materials, in this study, we are trying to evaluate and compare the toxicity of different concentrations of these two materials on dental pulp stem cells. **Materials and methods:** Human dental pulp stem cells (DPSCs) were extracted and cultured, and they were treated with 50, 100, 180, 250 and 300 μM concentrations of hydrogen peroxide and 1.9, 0.95, 0.71, 0.47 and 0.24 mg/ml concentrations of carbamide peroxide. Metabolic activity of the cells was evaluated by MTT test. **Objective:** The purpose of this study was to evaluate and compare the cytotoxic effects of various concentrations of hydrogen peroxide and carbamide peroxide on the survival of human dental pulp stem cells. **Results:** The results showed that viability of treated cells with hydrogen peroxide and carbamide peroxide has decreased at high concentrations. IC50 was at 180 μM concentration from HP and 1.9 mg concentration of CP, which was statistically significant ($P<0.05$). **Conclusion:** The results of our studies showed that these two materials affect the growth of stem cells in a dose-dependent manner. These materials, at low concentrations, cause a resistance to oxidative stress, however, at high concentrations, they have toxic effects on cell growth. The fatal effect of hydrogen peroxide on cells is much more than the effect of carbamide peroxide on the same cell line. It can be concluded that carbamide peroxide has less side effects than hydrogen peroxide and its use at a lower dose compared to hydrogen peroxide can be a good method for tooth bleaching.

Keywords: Stem cells, Dental pulp, Apoptosis, Hydrogen peroxide, Carbamide peroxide

Introduction. Teeth bleaching is a simple and very effective way to whiten the color of the dental tissue, which is a good therapeutic option to enhance the beauty of the teeth during the past 100 years (1, 2). A wide range of bleaching agents, including hydrogen peroxide and carbamide peroxide are used in this field (3, 4). Although, these materials have whitening properties, but they are also among the strongest oxidizing agents. One of the most important complications that these materials have on the teeth is in the cellular structure, which causes pulp sensitivity and inflammation (5). Dental pulp is a soft and vital tissue that is a source of stem cells (6). Stem cells are primary cells that under physiological or laboratory stimulation turn into cells with a specific function such as muscle, heart and skin cells and so on (7). There are two major categories of stem cells: Embryonic stem cell and Adult stem cells. The embryonic stem cells are pluripotent cells, which means they can produce differentiated cells of all body tissues (8). The adult stem cells are unipotent cells, which means that adult stem cells can produce differentiated cells belonging to the tissue in which they are located, and they are responsible for restoration and reconstruction (9). One of the applications of stem cells in clinical dentistry is the reconstruction of teeth and face and body tissues (10). Stem cells of dental origin can definitely produce dental tissues. The Use of stem cells can be effective in treatment of loss or damages to the bone or teeth and oral and dental cancers. In teeth, the stem-cell niche is formed in certain anatomical places, such as dental pulp. Dental pulp stem cells are adult cells found in deciduous teeth (children's teeth in the ages of 5-12) and permanent teeth of adults (11). The human dental pulp stem cells, due to the potential for differentiation to multiple cell lines, are considered as a good source for in vitro studies on stem cells.

Hydrogen peroxide is a common material for teeth whitening that is used at concentrations of 5% to 35%, and by being decomposed by enzymes, produces anion superoxide and free radicals (12). Carbamide peroxide is a material used in dentistry for bleaching and disinfecting teeth. The concentrations used are 10% to 35%, which in the mouth, decomposes to 7% urea and 3% hydrogen peroxide (13).

A study by Soares et al. (2015) was conducted on evaluating the early and late response of dental pulp stem cells to bleaching strategies. The results indicated that the concentration of 35% of this material reduces the cell survival by 93-97% and alters the morphology of the cells. However, gels containing 8% and 10% hydrogen peroxide have less toxic effects on pulp cells (14). A study by Soares et al. (2015) was conducted on evaluating the oxidative stress caused by toxic concentrations of hydrogen peroxide on dental pulp cells. The comparison of the cells treated with hydrogen peroxide and the control group showed that both the concentrations mentioned are toxic to pulp cells and cause severe oxidative stress and a significant reduction in cell survival (15). In a study by Bentti et al. (2004), they examined the impact of buccal surface on the permeation of hydrogen peroxide to dental pulp. The results showed that there was a significant difference in permeation of hydrogen peroxide with and without resin composite compared to buccal surface ($p=0.001$). They concluded that hydrogen peroxide 35% had a greater permeation than hydrogen peroxide 10% (16).

Knowing possible damages caused by teeth whitening and bleaching agents can be a good way to prevent these damages, as well as development of an appropriate treatment for the treatment and prevention of chemical damages to the dental stem cells. Therefore, the awareness about the process of these injuries and how to prevent them can significantly reduce health care costs in the health department. Therefore, the purpose of this study is to evaluate the effects of hydrogen peroxide and carbamide peroxide as teeth whitening agents on dental pulp-derived stem cells.

Materials and methods

The type of the present study is experimental, laboratory and basic. The cell population studied is human dental pulp stem cells (hDPSCs). The pH of the solution measurement was adjusted using pH meter at 4.7 value. To conduct this study, 20 healthy impacted wisdom teeth from patients aged 18-45 years due to orthodontic reasons or incomplete growth were used. After extracting the teeth and collecting the samples in 50 ml Falcon tubes containing PBS and 2% pene/strp, they were transferred to the laboratory environment and kept at -4 ° C. In order to isolate the pulp stem cells from CEJ site, the teeth were transversely broken with a surgical cutter, and the dental pulp was extracted from the pulp chamber using narrow sterile forceps. They were divided into 10-20 mm pieces by a sterile forceps, and they were exposed to the collagenase enzyme type IV (Invitrogen) for an hour at 37 ° C and 5% CO2. Then this complex incubated for 2 hours at 37 ˚C and 5% CO2 inside the incubator. During this period, a moderate pipette of tissue parts was performed repeatedly to help its digestion. After 2 hours, in order to digest the enzyme by sampler, 2 times the digestion of the enzyme, the MEM α medium containing 15% fetal bovine serum, 100 u/ml penicillin, 2.5 g/mlμ amphotericin and 100 u/ml streptomycin was added to Falcon and centrifuged for 10 minutes at 1800 rpm. After completion of the centrifugation, the supernatant was removed, and by tapping the end of the Falcon tube than contains the cell mass, cells were isolated. Cells were mixed in the MEMα culture medium containing 15% fetal bovine serum (FBS), 100 u/ml penicillin, 2.5 g/mlμ amphotericin and 100 u/ml streptomycin, Gutamix 1% and Nonessential amino acid 1%, and were transferred to 6 cm Petri dishes with sampler in the amount of cc2, and incubated at 37° C and 5% CO2.

Two days after culture, human dental pulp stem cells were examined under an inverted microscope for the level of growth, the discoloration of medium and microbial contamination.

In order to compare the growth rate of cells from culturing, the time for doubling of cells during the culture period (first to fourth passages) was measured. For this purpose, the following formula was used: in this relation, Population Doubling Time (PDT) is the number of doubling the cell population, N0 is the number of cells at the beginning of culture, N is the number of cells at the end of culture, and CT is the culture time.

$$PDT = CT/\log \frac{N}{N0 * 3.31}$$

In order to treat the cells, they were cultured with H2O2 cells in a 96-well plate with a density of 3500 cell/cm^2 and allowed for 24 hours to adhere to the 96-well plate surface. Then, the previous medium was discarded and the cells were treated with an enriched αMEM medium containing H2O2 concentrations of 50, 100, 180, 250 and 300 μm, and they were kept at the incubator for 24 hours at 37 ° C with humidity and 5% CO2.

To investigate the effect of CP on the dental pulp stem cells, the fourth passage cells with a density of 500 cell/well were cultured in each well of the 96-well plate and incubated for 24 hours. After completion of incubation, cells with

concentrations of 0.9, 0.95, 0.71, 0.47 and 0.24 mg/ml were treated with CP, and kept in the incubator for 24 hours at 37 ° C with humidity and 5% CO2.

Multi Table Tournament (MTT) technique or (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyletrazolium Bromide) was used to evaluate the cellular proliferation.

After completion of incubation for 24 hours, the supernatant medium of the cells was removed and the cells were kept in a new medium containing 10% FBS for 3 days to return to the primary state (recovery time). Then, 180 µl of a medium without FBS and 20 µl of MTT solution at 5 mg/mL concentration were added to each well, and plate surrounding was covered with aluminum foil. After incubation for 4 hours at 37 ° C, the supernatant medium of cells was slowly removed and 200 µl of dimethyl sulfoxide was added to dissolve the formed crystals, then for uniformity, the plate was placed on a shaker machine for 10 minutes at 100 rpm, and the light absorbance of the plate was read by a Plate Reader microtiter at 570 and 490 wavelengths.

In order to reduce test errors, DMSO was added into several wells without cells, OD (optical density) was read along with other wells and finally removed from the wells. The IC50 of cells were calculated using the Grap Pad Prism software. To evaluate the results of MTT assay, one-way ANOVA test and Tukey HSD test were used. The results of other tests were analyzed by Student's t-test using SPSS ver.16 software. The techniques were repeated three times.

Results

In this study, an enzyme digestion technique was used. Figure 1A shows the location of the tooth fracture. The findings from cell culture under a microscope showed that colonies derived from pulp tissue are similar to fibroblast-like cells and they are needle-shaped and long (Figure B2). In Figure C, D 2, the cores of dental pulp stem cells stained with acridine orange and ethidium bromide are depicted.

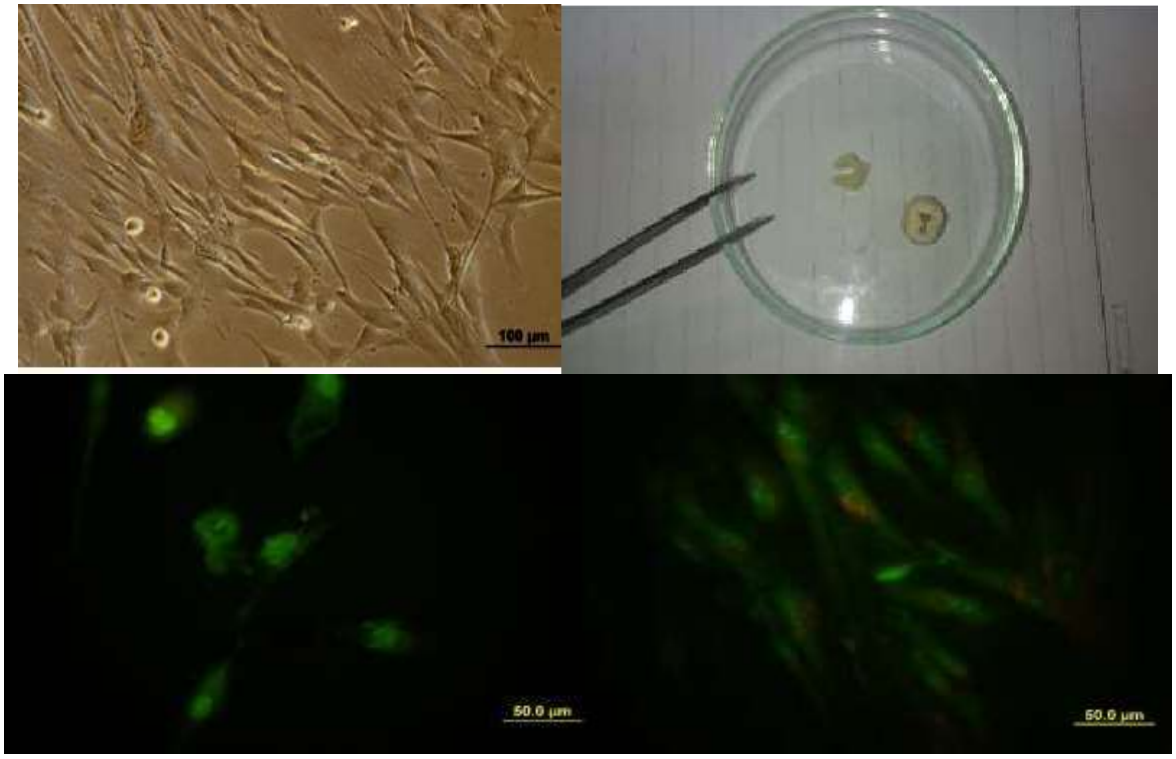


Figure 1. *Human dental pulp stem cells. Figure A shows how to fracture teeth for extracting stem cells. Figure B shows the human dental pulp stem cells that are similar to fibroblastic cells. Figure C shows the staining of cells with AO/EB.*

The time needed for proliferation and doubling of the cells was about 22 hours. MTT technique was used to evaluate the effect of hydrogen peroxide on cells. The results showed that viability of cells at concentrations of 50 and 100 µm was statistically significant, so that the growth of the cells was almost similar to the control group, with the increase in the concentration of hydrogen peroxide, the rate of cell survival decreased. This decrease at high concentrations compared to the control group was statistically significant ($P < 0.05$) (Table 1). As shown in Chart 1, the IC50 of this material was at a concentration of 180 µm.

Table 1. *Evaluating the effect of hydrogen peroxide on dental pulp stem cells by MTT technique*

	Average	SEM
control	100	4.926112
50 Um	67	3.614791
100uM	65	3.371952
180uM	53	4.953891
250uM	34	3.91227
300M	20	2.967899

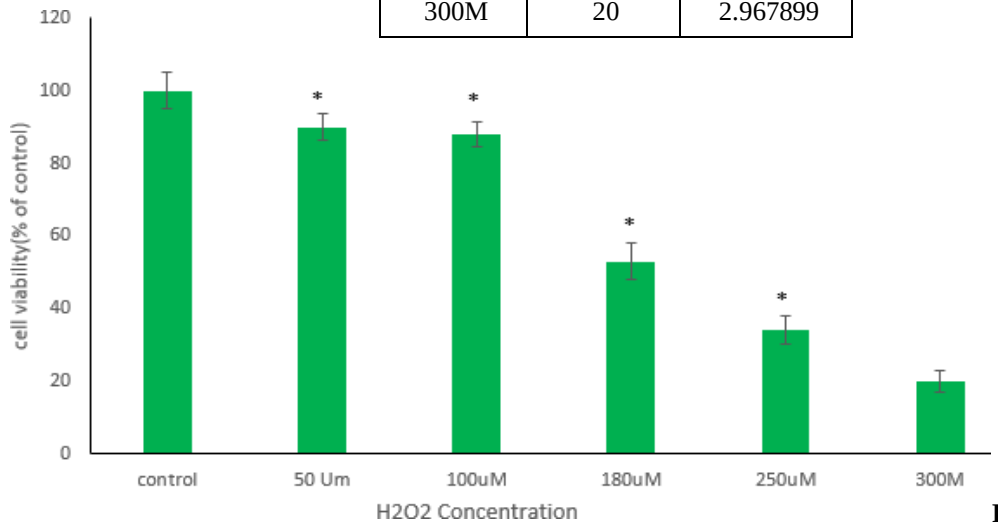
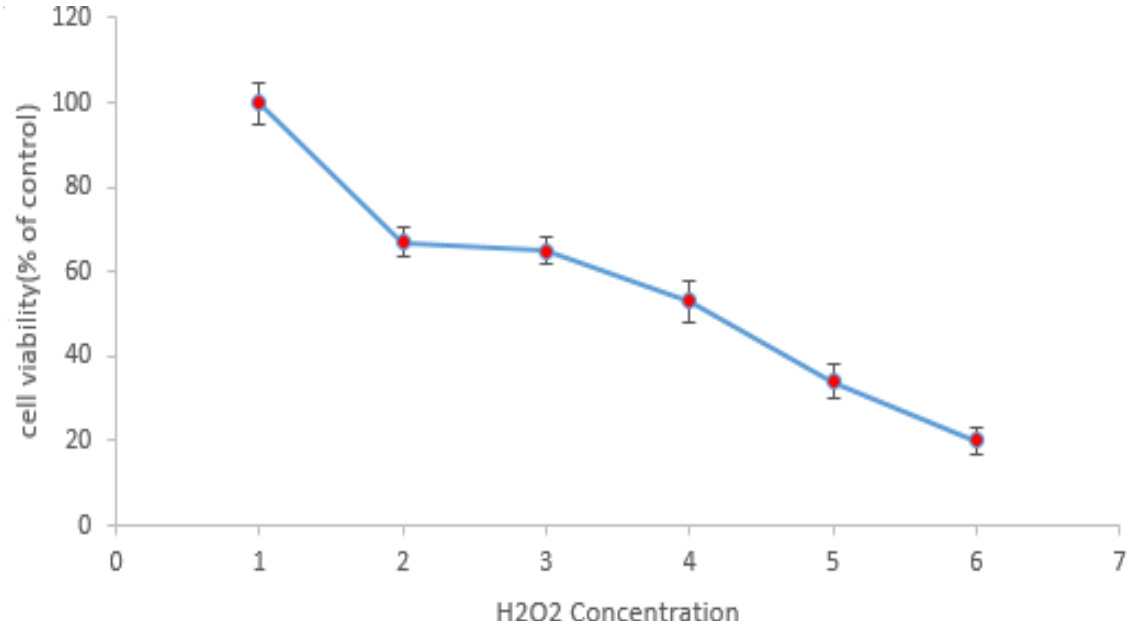


Figure 2. *The effect of different concentrations of HP on cell viability of hDPSCs by MTT technique. The results for the control and treated groups are shown as schematics.*



* Significant statistical differences (P <0.05) compared to the control group

The MTT technique was used to evaluate the effect of carbamide peroxide on human dental pulp stem cells. At low concentrations, the reduction of cell growth was not significant, but with increasing the concentrations of carbamide

peroxide, the reduction in cell growth was statistically significant (Table 2). The statistical results are shown in Chart 2. The IC50 of this materal was approximately at a concentration of 1.9, so that the cells had a decrease in growth by about 50%.

Table 2. *Evaluation of the effect of carbamide peroxide on dental pulp stem cells*

	Average	SEM
control	100	0.027682
0.24	95	4.116578
0.47	80	3.08971
0.71	75	7.81798
0.97	65	5.218091
1.9	58	2.55277

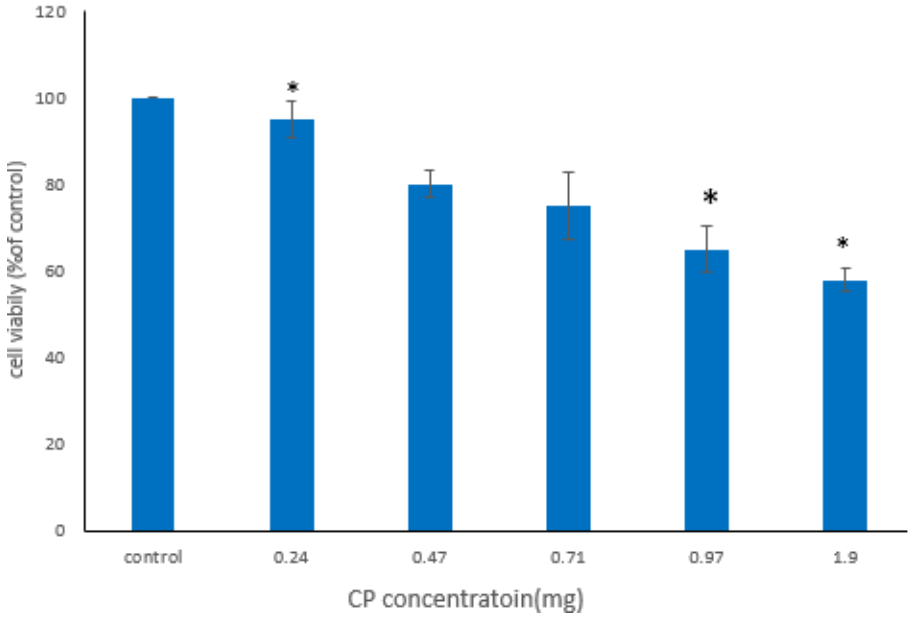
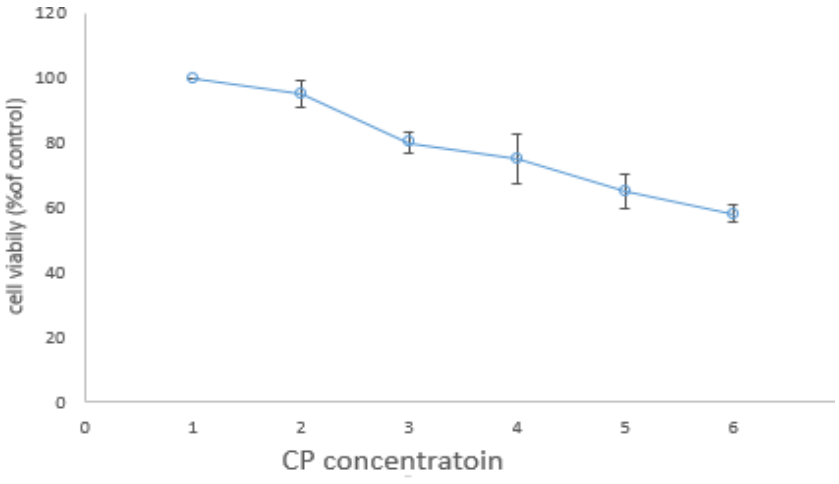


Figure 2. *Effect of different concentrations of CP on the cell viability of hDPSCs by MTT technique. The results for the control and treated groups are shown schematic.*



* Significant statistical differences (P<0.05) compared to the control group

According to the results, it can be said that hydrogen peroxide has a higher toxic effect on the cells than on oxide carbamide. Because, this material has created apoptosis in the cells at much lower concentrations than carbamide peroxide. In addition, the IC50 concentration of hydrogen peroxide is much lower than that of carbamide peroxide, which means that hydrogen peroxide at a much lower concentration than carbamide peroxide can cause 50% cell death, thus it has more toxicity.

Discussion. The topic of beauty of smile and oral and dental health has attracted public attention in recent decades. Studies have shown that the absence of a beautiful smile causes depression (17). One of the most important factors in the beauty is dental discoloration that can disturbed the beauty factor in a person (18). The teeth are discolored due to various causes (systemic or acquired), and this change in color is one of the major issues that has involved dentists to itself. Teeth discoloration is a multifactorial illness that can be caused by lifestyle, disease, injury, and physiological processes and is caused by various factors such as taking some medications, genetic defects, trauma, age and dental caries. up to now, dental bleaching has been reported as the most conservative method of treating teeth discoloration (19-21). Attention to the beauty and appearance of the mouth and teeth has led to the use of a series of materials including whitening materials in the field of dentistry. The use of these materials, though it is easy and simple and increases the quality of dental care, but unreliable and unreasonable use of these materials causes problems. Scientific evidence suggests that these seemingly harmless bleaching agents are oxidizing materials that damage the tissues and dental cells, including stem cells. These materials affect the homeostasis of cells with oxidative phosphorylation mechanisms the production of free radicals and lipid peroxidation, and cause oxidative stress and apoptosis (22).

In recent years, studies on stem cells has grown dramatically. Stem cells, due to their role in treatment of disease and restoration of tissues, are of great important (17, 23). Stem cells are classified into two main categories, which include embryonic and adult stem cells. These cells are used in applied research in the field of restoration and regenerative medicine. Dental tissue is one of the tissues that is rich in stem cells and is easily accessible. These cells have the ability to differentiate into neurons, fat, odontoblasts, etc. In this regard, it seems that the use of autologous stem cells producing dentin, cement, bone and periodontal ligaments in the future seems likely to be an alternative choice for commonly used treatments and the use in regenerative medicine (22, 24, 25).

Mesenchymal stem cells found in dental tissue include dental pulp stem cells, dental papila and dental follicles, extracted deciduous teeth and periodontal ligaments. These cells can be isolated and purified and grown under special tissue culture conditions and used in the reconstruction of teeth, nerves and bones (26). Among the mechanisms that cause dental injuries, include induction of apoptosis, activation of immune responses and physiological changes in the tooth. One of the routinely used materials in dentistry is whitening materials that have oxidative properties and cause oxidative stress and induce apoptosis (27). Inhibition of signaling pathways in pulp stem cells prevents differentiation and proliferation of stem cells. Therefore, better understanding of pulp stem cell regulation in pathological conditions will certainly help in appropriate therapeutic interventions and repair and reconstruction of tissues. In most bleaching methods, hydrogen peroxide derivatives are used in various concentrations (3-38%) with or without heat, carbamide peroxide (10-30%) or a mixture of sodium peroxide and hydrogen peroxide (28).

In this study, due to the ease of use of dental pulp stem cells, the effect of peroxide hydrogen and carbamide peroxide was investigated on the dental pulp stem cells. The MTT technique was used to evaluate the growth of cells. The results showed that cells, following the treatment with HP and CP at low concentration, indicated a slight decrease in growth, which was statistically significant. So that these two materials at low concentrations did not have a toxic effect on the growth of the cells, however, 50% decreased in the growth was observed at high concentrations. The two materials can change the growth of the cells in a dose-dependent manner. The interesting point is that the toxic effect of hydrogen peroxide on cells is far greater than that of carbamide peroxide on the same cell line. As a result, it can be concluded that carbamide peroxide has less side effects than hydrogen peroxide and its use at a lower dose compared to hydrogen peroxide can be a good method for teeth bleaching. It has also been reported that chemical softening as a result of bleaching may affect the hardness of the restorative materials and the clinical durability of the tooth colored restorations (29). This report has questioned the use of oxidative materials in bleaching.

In line with the current study, a study by Soares et al. (2015) was conducted on the early and late response of dental pulp stem cells to bleaching strategies. This study was carried out using different concentrations of hydrogen peroxide and at different times by MTT assay. The results indicated that the concentration of 35% of this material reduced the cell survival by 93-97% and altered the morphology of the cells. But gels containing 8-10% percent hydrogen peroxide have less toxic effects on pulp cells (14). Also, in line with the current study, a study by Soares et al. (2015) was conducted on the evaluation of oxidative stress caused by toxic concentrations of hydrogen peroxide on dental pulp cells. In this study, dental pulp cells were directly exposed to two concentrations of 0.1 and 0.3 μg/ml of hydrogen peroxide for 30 minutes. Then, the cell survival and oxidative stress were measured by MTT assay. Comparison of the cells treated with hydrogen peroxide with the control group showed that both concentrations were toxic to pulp cells and significantly reduced the survival of cells and cause severe oxidative stress (15). In line with the current study, a study by Hanks et al. (1993) was conducted on

the effects of dental bleaching agents in vitro. This study showed that hydrogen peroxide and carbamide peroxide at high concentrations increase cytotoxicity and permeability of teeth to this materials (24).

Another study done by Mokhlis et al. (2000) on the clinical assessment of bleaching agents of hydrogen peroxide and carbamide peroxide during daily use has proven that using 20% carbamide peroxide significantly increased whitening compared to 7.5% hydrogen peroxide during the first 14 days of the study (30). Basting et al. (2012) examined the efficacy and sensitivity of teeth to 10% and 20% doses of carbamide peroxide and a dose of 35-38% hydrogen peroxide in bleaching. The results of this study indicated that 43.2% of the participants experienced mild or moderate tooth sensitivity during bleaching treatment. The most common dental sensitivity was found in 71.4% of volunteers that used 20% carbamide peroxide bleaching solution, which may be related to the concentration of peroxide or contact time of this agent with the teeth structure. The dental sensitivity is a side effect that is due to increased permeability of enamel and dentin after easy passage of peroxide into tooth pulp, which is observed in bleaching (33-31), the incidence of this sensitivity was different in the participants in this experiment (34). Another study by Forner et al. (2012) conducted on 20 volunteers for bleaching by a combination of 5% lactoperoxidase and 3% carbamide peroxidase used three times a day for two minutes, showed that that the use of active enzymes of carbamide peroxide is effective at low concentrations and short duration for tooth whitening. Enzymatic bleaching of teeth can increase the efficiency of low concentration of peroxidase and reduce the peroxidase risk on the oral tissue (35).

The findings of some studies suggest that 10% carbamide peroxide causes morphological changes in the enamel surface (36, 37), however, few studies have shown that no significant change can happened (38). Reported side effects indicate that cervical abrasion may be the result of internal bleaching is often observed in teeth treated with thermocatalytic therapy. Dental sensitivity has been observed in 15-78% of patients treated with external bleaching, which is a common side-effect of bleaching (39).

Clinical studies on the effects of hydrogen peroxide suggest that direct contact with hydrogen peroxide causes genotoxic effects in bacteria and culture of epithelial cells, but due to effects of metabolic enzymes, its effect is reduced or completely eliminated. Several studies on cancer, including the hamster species, indicate that hydrogen peroxide (H2O2) is likely to act as a promoter. Tooth whitening products using HP should not be used without gum protection, particularly in patients with damaged soft tissue. 20% HP can cause severe irritation or burns when contacting with skin of eyes (40). High concentrations of carbamide peroxide also cause damage to the enamel (41, 42). Hydrogen peroxide is used as a mouthrinse to eliminate oral ulcers and eliminate anaerobic organisms. An important point in using hydrogen peroxide in bleaching is avoiding high doses and long periods of time (39, 43).

Conclusion. According to the results of this study, it can be definitely stated that the toxic mechanisms of hydrogen peroxide and carbamide peroxide include induction of apoptosis, production of active oxygen species, reduced level of antioxidant enzymes, increased lipid peroxidation and increased oxidative stress, which can easily affect the survival of stem cells and lead to cell death of these cells. Dental stem cells play a vital role in restoring damaged tissues throughout the life, given that they have self-proliferative potential and differentiate into different types of cells. Therefore, disruption and defect in the function of dental stem cells can affect the entire dental tissue of a living being and prepare the condition for destruction of tissue and its aging. Therefore, dental pulp stem cells are considered as an important diagnostic tool to study the toxicity of materials due to the lack of ethical issues and ease-of-use, and with the knowledge that oxidizing materials by what mechanism exert their effects, appropriate therapeutic interventions can be made. With regards to previous studies conducted that showed that increasing the concentration of oxidative materials, the survival rate of the treated cells decreased and we observed the induction of apoptosis and cell death in the treated groups, it seems that in order to reduce the side effects of these materials, it is better to encapsulate the oxidatives in the special nanopolymers, so a small dose of them is consumed and they would have more effective levels by being encapsulated. This may even be economically feasible, which requires more in vitro studies.

Therefore, the results of the current study showed that these two bleaching agents decrease the growth of stems cells in a dose-dependent manner. At low concentrations, these materials can result in resistance to oxidative stress, but at high concentrations, they have toxic effects on cell growth. These compounds can be a good candidate for examination of materials with a double edges, and prior to the use of these materials, appropriate strategies should be applied depending on the type of cell and the effects of bleaching agents.

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