ประสิทธิภาพของเครื่องวิเคราะห์การดูดกลืนแสงชนิดไมโครเพลต ในการตรวจหาไฮโดรเจนเพอร์ออกไซด์จากสารฟอกสีฟัน

The Efficacy of a Microplate Spectrophotometer for the Detection of Hydrogen Peroxide from Bleaching Agents

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บทคัดย่อ

ไฮโดรเจนเพอร์ออกไซด์จากสารฟอกสีฟันสามารถซึมผ่านเนื้อฟันเข้าสู่โพรงเนื้อเยื่อในได้อย่างง่ายดาย รวมทั้งยังก่อให้เกิดอันตรายต่อโพรงเนื้อเยื่อในได้ งานวิจัยนี้จัดทำขึ้นโดยมีวัตถุประสงค์เพื่อตรวจหาปริมาณไฮโดรเจนเพอร์ออกไซด์ที่ซึมผ่านแผ่นฟันวัวเข้าสู่โพรงเนื้อเยื่อในจำลอง ภายหลังจากการฟอกสีฟันที่ระยะเวลา 1 ชั่วโมง ด้วยสารฟอกสี 3 ชนิด ขั้นตอนการวิจัยจะใช้ฟันวัวที่เตรียมไว้หนา 3.2 มม. ประกอบเข้ากับอุปกรณ์โพรงเนื้อเยื่อในจำลองภายในบรรจุสารละลายแอซีเทตบัฟเฟอร์ไว้เต็ม ชิ้นงานจะถูกแบ่งออกเป็น 3 กลุ่มตามชนิดของสารฟอก

Abstract

Hydrogen peroxide from bleaching agents can easily penetrate through the tooth structure into the pulpal cavity and may cause damage to pulpal cells. The objective of this study was to evaluate the amount of hydrogen peroxide penetration through the bovine tooth disc into the artificial pulp chamber after bleaching with three different bleaching agents for 1 hour. Bovine tooth discs with a thickness of 3.2 mm were prepared and placed into a modified artificial pulp chamber

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สีฟันที่ใช้ คือ กลุ่มที่ 1 ฟอกสีฟันด้วย Opalescence™ Boost ความเข้มข้นร้อยละ 40 (ไฮโดรเจนเพอร์ออกไซด์) กลุ่มที่ 2 ฟอกสีฟันด้วย Opalescence™ PF ความเข้มข้นร้อยละ 20 (คาร์บาไมด์เพอร์ออกไซด์) และกลุ่มที่ 3 ฟอกสีฟันด้วย Opalescence™ PF ความเข้มข้นร้อยละ 10 (คาร์บาไมด์เพอร์ออกไซด์) ภายหลังจากการฟอกสีฟันเป็นระยะเวลา 1 ชั่วโมง จึงนำสารละลายในโพรงเนื้อเยื่อในจำลองมาตรวจวัดหาปริมาณไฮโดรเจนเพอร์ออกไซด์ผ่านกระบวนการวิเคราะห์การดูดกลืนแสง โดยใช้เครื่องวิเคราะห์การดูดกลืนแสง 2 ชนิด คือ เครื่องวิเคราะห์การดูดกลืนแสงชนิดที่ใช้คิวเวตต์เป็นภาชนะใส่สารตรวจวัด และเครื่องวิเคราะห์การดูดกลืนแสงชนิดไมโครเพลต ผลการวิจัยพบว่า กลุ่มที่ใช้สารฟอกสีฟันที่ระดับความเข้มข้นที่สูงกว่า จะสามารถตรวจพบปริมาณไฮโดรเจนเพอร์ออกไซด์ที่มากกว่า โดยปริมาณไฮโดรเจนเพอร์ออกไซด์ที่มีค่าสูงสุดพบในกลุ่มที่ 1 ไฮโดรเจนเพอร์ออกไซด์ ความเข้มข้นร้อยละ 40 พบปริมาณ 0.0185 ไมโครกรัม/มล. เมื่อใช้เครื่องวิเคราะห์การดูดกลืนแสงชนิดที่ใช้คิวเวตต์เป็นภาชนะ และพบปริมาณ 0.1080 ไมโครกรัม/มล. เมื่อใช้เครื่องวิเคราะห์การดูดกลืนแสงชนิดไมโครเพลต จากผลที่ได้จึงสามารถสรุปได้ว่า เครื่องวิเคราะห์การดูดกลืนแสงชนิดไมโครเพลตมีประสิทธิภาพในการตรวจวัดปริมาณไฮโดรเจนเพอร์ออกไซด์สูงกว่าชนิดที่ใช้คิวเวตต์เป็นภาชนะ

คำสำคัญ: การฟอกสีฟัน ไฮโดรเจนเพอร์ออกไซด์ ไมโครเพลต เครื่องวิเคราะห์การดูดกลืนแสง

filled with acetate buffer solution. The specimens were divided into 3 groups and bleached with one of following bleaching agents: group-1, 40% Opalescence™ Boost (hydrogen peroxide); group-2, 20% Opalescence™ PF (carbamide peroxide); and group-3, 10% Opalescence™ PF (carbamide peroxide). The amount of peroxide that penetrated into the artificial pulp chamber was evaluated using either a standard spectrophotometer with cuvette reader or microplate spectrophotometer after 1 hour of bleaching. An increase in the amount of hydrogen peroxide penetration corresponded to an increase in the concentration of bleaching products. The highest amount of hydrogen peroxide penetration was found in the group bleached with 40% hydrogen peroxide (0.0185 µg/ml for standard spectrophotometer, 0.1080 µg/ml for microplate spectrophotometer). The microplate spectrophotometer exhibited greater efficacy for detecting the amount of hydrogen peroxide compared with the standard spectrophotometer.

Keywords: bleaching, hydrogen peroxide, microplate, spectrophotometer

Introduction

Tooth bleaching is commonly used as the patient's first introduction to esthetic dentistry. There are many ways to whiten teeth from minimally invasive treatments, such as tooth bleaching, micro-abrasion and macro-abrasion, to aggressive treatments, such as direct veneer, indirect veneer and prosthetic restoration.[1]

Tooth sensitivity is one of the major adverse effects of bleaching treatment caused by reversible pulpitis. Unquestionably, hydrogen peroxide can easily penetrate through the tooth structure into the pulpal cavity[2] and damage pulpal cells.[4] The severity of pulpal injury is directly related to the amount of hydrogen peroxide penetration. From a previous study, the amount of hydrogen peroxide of 40-50 µg/ml have been suggested to be toxic to

pulpal cells and might cause pulpitis.[3]

Numerous methods are available to quantify the amount of hydrogen peroxide. Peroxidase enzyme catalyzer with spectrophotometry was the standard method to quantify the amount of hydrogen peroxide since 1900s up until now. Peroxidase enzyme catalyzes the transfer of electrons from hydrogen peroxide to a colorimetric indicator. Then, the color change based on the amount of hydrogen peroxide is measured using the spectrophotometer. The absorbance of 596-nm light by the sample is compared with a reference curve generated by standard hydrogen peroxide solutions.[5,6] This method is commonly used to quantitate the amount of hydrogen peroxide in bleaching agents.[7] A previous study in 2015 reported that there were 15 out of 21 experimental studies, from 1991-2014, using peroxidase enzyme catalyzer with spectrophotometry for detection of hydrogen peroxide penetration.[7] Currently, a spectrophotometer with a microplate reader has been introduced for microvolume detection of hydrogen peroxide.[8] Nonetheless, high performance liquid chromatography (HPLC) has been introduced as a very effective technique for detection, identification, and quantification of substances in mixture including hydrogen peroxide. This HPLC was commonly used in pharmaceutical industry and medical purposes.[9] There are a limitation of dental researches using this method for quantifying the amount of hydrogen peroxide penetration.[9,10] A previous study in 2015 introduced the HPLC technique for detection of hydrogen peroxide releasing from dental bleaching products and hair products.[10] This method was also relative to the oxidative reaction of triphenylphosphine and hydrogen peroxide resulted in a formation of triphenylphosphine oxide which was quantified by HPLC/UV method. Even if this method provided a high accuracy and specificity, the quality of chemical reagents using in this method should be equal to or higher than the HPLC grade which is meaning of the expensive cost. Moreover, the experimental data may not be compared with the previous studies which generally used peroxidase enzyme catalyzer with spectrophotometry.[10] Consequently, peroxidase enzyme catalyzer analytical technique with microplate spectrophotometer was used for detection of hydrogen peroxide in the attempt to compare the current results with the previous results of detection by a commonly used spectrophotometer with a cuvette reader.

The purpose of this study was to evaluate the amount of hydrogen peroxide penetration through the tooth disc into the artificial pulp chamber after bleaching with three different bleaching agents for 1 hour as measured with either standard spectrophotometer or microplate spectrophotometer.

Materials and methods

The project was approved by Mahidol University, Institute Animal Care and Use Committee. (No.U1-06386-2560) Forty-five bovine incisors from adult bovine slaughtered for consumption in a legitimate and pass standard slaughterhouse regulated by Department of Livestock Development, Ministry of Agriculture and Cooperatives, were used to prepare experimental specimens. The enamel surface was analyzed using a stereomicroscopy (Stereo Microscope Model Eclipse E400 POL, Nikon, Tokyo, Japan). The specimens with cracks or flaws in the enamel were excluded.

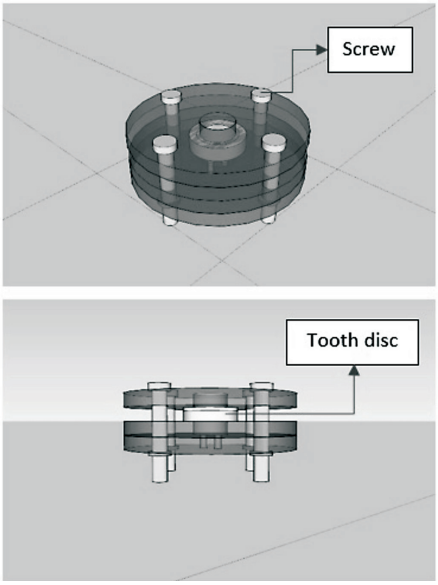
The selected bovine incisors were cleaned with periodontal curettes and polished with a mixture of pumice and water. Then, the specimens were cut at the middle third area of the buccal surface into a cylindrical shape (6 mm in diameter) using rough

fissure diamond burs under copious water.

The cylindrical specimens were ground on a dentine surface perpendicular to the long axis by manual rotation using 600-grit silicon carbide paper (Buehler, Buehler Ltd., Lake Bluff, IL, USA) until the specimens reached a thickness of 3.2 mm (the entire thickness of enamel and dentine from natural bovine teeth) as measured with a digital Vernier caliper (Mitutoyo, Tokyo, Japan). Then, 17% ethylenediaminetetraacetate solution (Endo Clean, M Dent, Bangkok, Thailand) or EDTA was applied for 1 minute to remove the smear layer formed during preparation. All tooth discs were rinsed and stored in distilled water for less than 24 hours before starting the experiment.

These specimens were divided into 3 groups according to the concentration and composition of bleaching agents as follows: group-1 (40HP) was 40% Opalescence Boost (Hydrogen peroxide, Ultradent, South Jordan, USA), group-2 (20CP) was 20% Opalescence PF (Carbamide peroxide, Ultradent, South Jordan, USA) containing potassium nitrate and fluoride, and group-3 (10CP) was 10% Opalescence PF (Carbamide peroxide, Ultradent, South Jordan, USA) containing potassium nitrate and fluoride.

The specimens were put into a device that was modified from an artificial pulp chamber developed at the Laboratory of Experimental Pathology and Biomaterials, Araraquara School of Dentistry, UNESP.[11] Each device included 3 acrylic discs. Two discs with an opening of 5 mm allowing the placement of a specimen between these 2 discs in the proper position were prepared. The remaining disc with 2 perforations to allow the circulation of solution in and out to quantify peroxide penetration covered the bottom of lower disc. Between the acrylic discs, a thin silicone disc was inserted to prevent excess penetration of the bleaching agent. All compartments were tightly fixed with 4 screws. A diagram of the modified artificial pulp chamber model is presented in Figure 1.



รูปที่ 1 *ส่วนประกอบของโพรงเนื้อเยื่อในจำลอง*

Figure 1 *The diagram of all compartments of the modified artificial pulp chamber*

Bleaching was performed using 3 bleaching agents according to the manufacturer's recommendations. In total, 30 mg of bleaching agent was applied to each sample and remained in contact for 1 hour (hr).

To quantify the peroxide penetration through tooth disc, the artificial pulp chambers were filled with 1 ml of acetate buffer solution (2 mol/l, pH=4.5) by mixing 0.1 M acetic acid and 0.1 M sodium acetate (tri-hydrate). The dentine side remained in contact with the acetate buffer solution during all bleaching procedures.

After the bleaching processes, 100 µl of acetate buffer solution inside the chamber were removed and mixed with 2750 µl of distilled water, 100 µl of leucocrystal violet (0.5 mg/ml dissolved in 0.5%

HCl (Sigma Chemical, St Louis, MO, USA)), and 50 µl of peroxidase type II from Horseradish (1 mg/ml (Sigma Chemical, St Louis, MO, USA)).

This procedure was based on the chemical reaction of hydrogen peroxide with leucocrystal violet, which was catalyzed by peroxidase enzyme.[6] The color of the mixed solution varied in intensity due to the quantity of penetrated peroxide. As a consequence of this variation, it was possible to indirectly calculate the amount of penetrated peroxide in the solution using a spectrophotometry method.

Spectrophotometry was used to read the amount of hydrogen peroxide penetration using 2 techniques of spectrophotometer. Readings will be taken with a standard spectrophotometer with cuvette reader (Genesys 10S Vis, Thermo Fisher Scientific, Waltham, MA, USA) and hybrid multimode microplate spectrophotometer (Epoch, BioTek, Winooski, VT, USA) at 596-nm wavelength. The calibration curve was used to calculate the quantity of hydrogen peroxide contained in each solution. The measurement was repeated thrice for each solution for all different application times. The calibration curve was generated by dividing the known-concentration of hydrogen peroxide by its absorbance measured from each spectrophotometer.

The means and standard deviation of hydrogen peroxide concentration (µg/ml) were calculated. Data were organized, and the distribution and homogeneity of variance were analyzed using Komogorov-Smirnov test and Levene's test. Then, one-way ANOVA at a 95% significance level and Duncan's post hoc test were used for analysis. Thus, the independent t-test was used for further analysis of differences between the testing method, and the correlation between testing methods was also analyzed within each material at a 95% significance level.

Results

The results from standard spectrophotometer analysis were in Table 1. Analysis with one-way ANOVA at a 95% significance level showed a significant effect of the bleaching type on the amount of hydrogen peroxide penetration (p=0.002). The data were also analyzed using Duncan's test for multiple comparison. The highest amount of hydrogen peroxide penetration was found in the group bleached with 40HP (0.0185), and this level was significantly higher compared with other groups. In contrast, the lowest amount was observed in the group bleached with 10CP (0.0026), but the difference was not significant (p=0.818) compared with the group bleached with 20CP (0.0037).

ตารางที่ 1 *ปริมาณไฮโดรเจนเพอร์ออกไซด์ที่ซึมผ่าน (ไมโครกรัม/มล.) ภายหลังจากฟอกสีฟันเป็นระยะเวลา 1 ชั่วโมง โดยใช้เครื่องวิเคราะห์การดูดกลืนแสงชนิดที่ใช้คิวเวตต์เป็นภาชนะ*

Table 1 *The amount of hydrogen peroxide penetration (µg/ml) after 1-hr application as determined by the standard spectrophotometer with cuvette reader*

Spectrophotometer	**Bleaching type**		
	40HP	20CP	10CP
H_2O_2 (µg/ml)	0.0185± 0.0177[a]	0.0037± 0.0098[b]	0.0026± 0.0099[b]

The data (mean ± standard deviation) with the same superscripts are not statistically different (p>0.05).

The results from microplate spectrophotometer were in Table 2. Analysis with one-way ANOVA at a 95% significance level also revealed a significant effect of bleaching types on the amount of hydrogen peroxide penetration (p=0.000). Further analysis using Duncan's test for multiple comparison revealed that the highest amount of hydrogen peroxide penetration was detected in the group bleached with 40HP (0.1080). This level was significantly higher compared with other groups. The lowest amount of hydrogen peroxide penetration was observed in the group bleached with 10CP (0.0107).

Analyses of different hydrogen peroxide penetration levels as determined by different evaluating methods are demonstrated in Figure 2. Statistically significant differences were found between the two spectrophotometers with different reading methods (p<0.05) for each bleaching agent. The use of a standard spectrophotometer with cuvette reader yielded a reduced amount of detected hydrogen peroxide compared with microplate spectrophotometer.

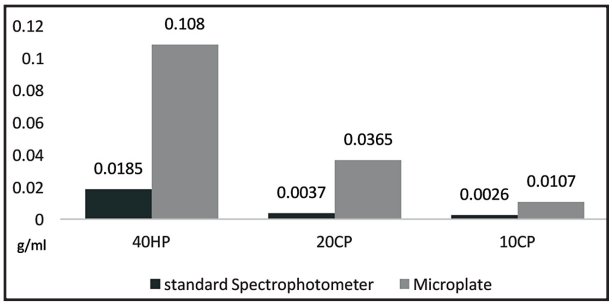
The correlation between results from the two spectrophotometers with different reading methods on the amount of hydrogen peroxide (µg/ml) was further analyzed by Pearson correlation at the 95% significance level as demonstrated in Table 3. High correlation was found with R = 0.95 for the 40HP group, R = 0.96 for the 20CP group and R = 0.99 for the 10CP group.

ตารางที่ 2 *ปริมาณไฮโดรเจนเพอร์ออกไซด์ที่ซึมผ่าน (ไมโครกรัม/มล.) ภายหลังจากฟอกสีฟันเป็นระยะเวลา 1 ชั่วโมง โดยใช้เครื่องวิเคราะห์การดูดกลืนแสงชนิดไมโครเพลต*

Table 2 *The amount of hydrogen peroxide penetration (µg/ml) after 1-hr application based on the microplate spectrophotometer*

Microplate	Bleaching type		
	40HP	20CP	10CP
H_2O_2 (µg/ml)	0.1080± 0.0164[a]	0.0365± 0.0105[b]	0.0107± 0.0243[c]

The data (mean ± standard deviation) with the same superscripts are not statistically different (p>0.05).



รูปที่ 2 *ปริมาณไฮโดรเจนเพอร์ออกไซด์ที่ซึมผ่าน (ไมโคร-กรัม/มล.) ภายหลังจากฟอกสีฟันเป็นระยะเวลา 1 ชั่วโมง โดยใช้เครื่องวิเคราะห์การดูดกลืนแสงชนิดที่ใช้คิวเวตต์เป็นภาชนะ และชนิดไมโครเพลต*

Figure 2 *The amount of hydrogen peroxide penetration (µg/ml) after 1-hr application as determined by standard spectrophotometer and microplate spectrophotometer*

ตารางที่ 3 *ความสัมพันธ์ที่ได้จากผลการตรวจวัดไฮโดรเจนเพอร์ออกไซด์ (ไมโครกรัม/มล.) ระหว่างเครื่องวัดการดูดกลืนแสงชนิดที่ใช้คิวเวตต์เป็นภาชนะ และชนิดไมโครเพลต*

Table 3 *The correlation between results from the two spectrophotometers with different reading methods on the amount of hydrogen peroxide (µg/ml)*

Correlation	Bleaching type		
	40HP	20CP	10CP
R	0.95	0.96	0.99

Discussion

The small molecule H_2O_2 is released from bleaching agents, can penetrate rapidly through the tooth structure, can reach the dentin tissue shortly after the application of a bleaching agent to the enamel, and then can enter the pulp.[12] H_2O_2 and products derived from decomposition of bleaching agents react with chromophores, breaking them down into smaller compounds and resulting in brighter teeth.[7,13,14] Unfortunately, this oxidative reaction is not only limited to chromophores but also may cause a number of undesirable side effects.[15,16] Thus, studies evaluating the penetration of H_2O_2 into the pulp chamber are required to establish a safe and effective bleaching therapy.[17-19]

The methodology used for quantifying the trans-enamel and trans-dentinal penetration of H_2O_2 in this study was modified from a previous report by Goncalves and co-worker[20], which demonstrated significant sensitivity for detecting small amounts of H_2O_2. In our study, the contact between the dentin surface and the acetate buffer solution allowed for stabilization of H_2O_2 penetration into the artificial pulp chamber, and this procedure was employed for further investigation of H_2O_2 diffusion through the dental tissues as measured by either the standard spectrophotometer with cuvette reader and the microplate spectrophotometer.

The amount of hydrogen peroxide penetration represents the residual hydrogen peroxide that remained from the reaction with the colored molecules in the tooth structure and then penetrated into the pulp. Increased penetration was observed with increased concentrations of bleaching agents. This result correlated with previous studies using in-office bleaching agents.[21,22] However, a former study found that the hydrogen peroxide seem to break down to radical ion faster than carbamide peroxide.[23]

The value of the assessment archiving from various methods may be different. In this study, the amount of hydrogen peroxide penetration was analyzed based on 2 techniques of spectrophotometry: microplate spectrophotometer and standard spectrophotometer with cuvette reader. In previous studies, a spectrophotometer with cuvette reader was mainly used, which required a large amount of samples and chemical reagents. A 3-ml total volume of testing sample is required to fill the cuvette for the procedure of analysis.[21,22] Whereas, the microplate spectrophotometer helps to reduce the total volume of analyzed solution to microliters in each well of microplate. However, as mentioned earlier, HPLC is another interesting and effective device for identifying and quantification of hydrogen peroxide. Future study may be carried out the detection of hydrogen peroxide using HPLC technique.

Comparing the amount of hydrogen peroxide penetration in terms of the penetration rate, this study found that 40HP exhibited an increased penetration rate (0.1080 µg/ml/hr) compared with 20CP (0.0365 µg/ml/hr) and 10CP (0.0107 µg/ml/hr). This result indicates that the increased bleaching agent concentration, the increased penetration rate.

The lowest amount of hydrogen peroxide penetration was observed in the 10CP group in both spectrophotometers, which used a home-bleaching agent. However, some studies have discussed hydrogen peroxide toxicity in pulpal cells. From a previous study, 40-50 µg/ml hydrogen peroxide has been suggested to be toxic to pulpal cells and might cause pulpitis.[3] Regarding hydrogen peroxide penetration results, the highest measurement observed was much lower than toxic levels. However, using different measurements or equipment may be obtained in different results. The interpretation for the clinical situation should be concerned that

might be resulted from the sensitivity and reliability of equipment and methodology. Thus, genotoxic and carcinogenic effects of high concentrations of hydrogen peroxide during long-term application have been proposed.[25,26] The human body contains peroxidase and catalase enzymes as a natural defense mechanism, and these enzymes reduce the amount of hydrogen peroxide that passes through the pulp cavity. To avoid the adverse effects that might occur from unsafe use, it is important to consider the concentration used and application time.

Conclusion

Under the conditions of this study, it can be concluded that the amount of hydrogen peroxide penetration was affected by the concentration of the bleaching products. The microplate spectrophotometer exhibits greater efficacy compared with the standard spectrophotometer with cuvette reader, and the microplate spectrophotometer is recommended to quantify relatively low amounts of hydrogen peroxide.

References

1. Fearon J. Tooth whitening: concepts and controversies. *J Ir Dent Assoc* 2007; 53(3): 132-140.
2. Cooper JS, Bokmeyer TJ, Bowles WH. Penetration of the pulp chamber by carbamide peroxide bleaching agents. *J Endod* 1992; 18(7): 315-317.
3. Guenal I, Sidoti-de Fraisse C, Gaumer S, Mignotte B. Bcl-2 and Hsp27 act at different levels to suppress programmed cell death. *Oncogene* 1997; 15(3): 347-360.
4. Costa CA, Riehl H, Kina JF, Sacono NT, Hebling J. Human pulp responses to in-office tooth bleaching. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2010; 109(4): 59-64.
5. Aussen BS, Kingma Boltjes TY, Wostmann BS. Some fundamental remarks on the polarographic determination of the catalase activity and the H_2O_2 production of bacteria. *Antonie van Leeuwenhoek* 1950; 16(3): 65-78.
6. Mottola HA, Simpson BE, G G. Absorptiometric determination of hydrogen peroxide in submicrogram amounts with leuco crystal violet and peroxidase as catalyst. *Analyt Chem* 1970; 42: 410-411.
7. Kwon SR, Wertz PW. Review of the mechanism of tooth whitening. *J Esthet Restor Dent* 2015; 27(5): 240-257.
8. Lou EK, Damiani F, Heithersay GS. Evaluation of hydroxyl radical diffusion and acidified thiourea as a scavenger during intracoronal bleaching. *J Endod* 2016; 42(7): 1126-1130.
9. Gerber F, Krummen M, Potgeter H, et al. Practical aspects of fast reversed-phase high-performance liquid chromatography using 3 microm particle packed columns and monolithic columns in pharmaceutical development and production working under current good manufacturing practice. *J Chromatogr A* 2004; 1036(2): 127-133.
10. Gimeno P, Bousquet C, Lassu N, et al. High-performance liquid chromatography method for the determination of hydrogen peroxide present or released in teeth bleaching kits and hair cosmetic products. *J Pharm Biomed Anal* 2015; 107: 386-393.
11. Marson FC, Goncalves RS, Silva CO, et al. Penetration of hydrogen peroxide and degradation rate of different bleaching products. *Oper Dent* 2015; 40(1): 72-79.

12. 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. *J Endod* 2007; 33(9): 1074-1077.
13. Nathoo SA. The chemistry and mechanisms of extrinsic and intrinsic discoloration. *J Am Dent Assoc* 1997; 128Suppl: 6S-10S.
14. Joiner A. The bleaching of teeth: a review of the literature. *J Dent* 2006; 34(7): 412-419.
15. Haywood VB. History, safety, and effectiveness of current bleaching techniques and applications of the nightguard vital bleaching technique. *Quintessence Int* 1992; 23(7): 471-488.
16. Dahl JE, Pallesen U. Tooth bleaching--a critical review of the biological aspects. *Crit Rev Oral Biol Med* 2003; 14(4): 292-304.
17. Eimar H, Marelli B, Nazhat SN, et al. The role of enamel crystallography on tooth shade. *J Dent* 2011; 39Suppl3: e3-10.
18. Cavalli V, Rodrigues LK, Paes-Leme AF, et al. Effects of the addition of fluoride and calcium to low-concentrated carbamide peroxide agents on the enamel surface and subsurface. *Photomed Laser Surg* 2011; 29(5): 319-325.
19. Watt BE, Proudfoot AT, Vale JA. Hydrogen peroxide poisoning. *Toxicol Rev* 2004; 23(1): 51-57.
20. Goncalves RS, Costa CA, Soares DG, dos Santos PH, Cintra LT, Briso AL. Effect of different light sources and enamel preconditioning on color change, H_2O_2 penetration, and cytotoxicity in bleached teeth. *Oper Dent* 2016; 41(1): 83-92.
21. Marson FC, Goncalves RS, Silva CO, et al. Penetration of hydrogen peroxide and degradation rate of different bleaching products. *Oper Dent* 2015; 40(1): 72-79.
22. Soares DG, Basso FG, Pontes EC, Garcia Lda F, Hebling J, de Souza Costa CA. Effective tooth-bleaching protocols capable of reducing H_2O_2 diffusion through enamel and dentine. *J Dent* 2014; 42(3): 351-358.
23. Torres CR, Souza CS, Borges AB, Huhtala MF, Caneppele TM. Influence of concentration and activation on hydrogen peroxide diffusion through dental tissues in vitro. *Sci World J* 2013; 2013: 193241.
24. Luque-Martinez I, Reis A, Schroeder M, et al. Comparison of efficacy of tray-delivered carbamide and hydrogen peroxide for at-home bleaching: a systematic review and meta-analysis. *Clin Oral Investig* 2016; 20(7): 1419-1433.
25. Naik S, Tredwin CJ, Scully C. Hydrogen peroxide tooth-whitening (bleaching): review of safety in relation to possible carcinogenesis. *Oral Oncol* 2006; 42(7): 668-674.
26. Tredwin CJ, Naik S, Lewis NJ, Scully C. Hydrogen peroxide tooth-whitening (bleaching) products: review of adverse effects and safety issues. *Br Dent J* 2006; 200(7): 371-376.