Effect of pH of bleaching agent on tooth bleaching action *in vitro*

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This study investigated the effect of pH of bleaching agent, photo-irradiation time or application times on bleaching action using hematoporphyrin-stained papers (HSPs) and artificially stained bovine-teeth (BT). 23% H2O2 with pH 5.5, 6.0, 7.0, 8.0 and 9.0 were applied on the specimens. HSP was photo-irradiated for 1, 3 and 5 min. BT were photo-irradiated for 10 min and the bleaching was repeated ten times (n=10). CIE L*a*b* of the specimens were measured before and after the procedure. Data were analyzed by repeated-measures ANOVA followed by multiple comparisons with Bonferroni correction. For the HSP, longer irradiation time and higher pH yielded significantly higher color difference (ΔE). As for BT, increasing application times and higher pH resulted in higher ΔE. It was concluded that the pH of the bleaching agent significantly improved the bleaching effect with increased photo-irradiation time for HSP and with an increase of repeated application times for BT.

Keywords: Tooth bleaching, pH, Photo-irradiation, Hematoporphyrin, Bovine teeth

INTRODUCTION

Tooth bleaching is one of the most conservative and cost-effective treatments to enhance the esthetics of discolored teeth. The light-absorbing and light-reflecting nature of an object is altered by this treatment and increases its perception of whiteness. Three types of bleaching techniques are currently used for vital teeth: in-office bleaching with the bleaching agent applied by a dental professional in the office; at-home bleaching with dentist’s prescription for application by the patient at home; and over-the-counter (OTC) bleaching products such as whitening strips, gels, rinses11. Generally, bleaching agents used for in-office and at-home bleaching contain hydrogen peroxide or carbamide peroxide as an active ingredient. The majority of in-office bleaching products contain high concentration (25%–40%) of H2O2 to produce a higher amount of free-radicals2. The bleaching effect is affected by various factors, such as the concentration of H2O2,3,4, application-period, number of bleaching times5, temperature6, catalyst7, and pH8.

Bleaching agents with different pH are available in the market ranging from pH 3.7 to pH 11.1. Among them, the in-office bleaching products have a pH between 3.67 and 6.539. Their pH is mostly acidic to increase the shelf life10. At present, manufacturers have paid their attention to the acidity of bleaching gels, because low-pH can produce various harmful effects on the structure and properties of the tooth, such as changes in chemical composition, surface morphology, reduction in hardness and fracture resistance11-15. Conversely, previous studies have shown that alkaline bleaching agent improved the bleaching efficacy10,16 while minimizing the detrimental modifications on enamel surface16. Despite the advantages, high-pH of bleaching products raise concerns about the safety of the soft tissues as they cause mild irritation to severe ulcerations. According to OECD (Organization of Economic Co-operation and Development) guidelines, there is a correlation between the pH of the substance and degree of dermal irritation. The pH extremes as <2.0 and >11.5 may have strong local effects as corrosion of skin or mucosa17. Thus, when the pH of the bleaching agent lies between these two values, it can be assumed as safe without corrosion or irritation effect. However, adverse effects might be also caused by the oxidation of H2O2. Some bleaching agents contain H2O2 and the pH-conditioner separately as two-bottles or two-syringes to maintain the stability of H2O2 before usage and induce the immediate alkalization upon the opening of the products18.

Determining the pH, maximum bleaching efficacy and safety are the important factors should take into consideration when developing a new bleaching material. Nevertheless, few articles were published regarding the effects of pH values on tooth bleaching8,10. Particularly, in these studies, different pH-conditioners were used to prepare the bleaching agent with different pH values. As an example, HCl and NaOH solutions were added in H2O2 to adjust different pH of bleaching groups. This methodology might not be ideal because it was revealed that various pH-conditioners could eventually affect the bleaching actions in different ways18. For the comparison of the bleaching effect of the materials with different pH, the usage of the same ingredients is necessary. Therefore, the present study aimed to evaluate the effect of pH of tooth bleaching agent and photo-irradiation time or application times on bleaching action using hematoporphyrin-stained paper (HSP) and
bovine-teeth (BT) with different pH of bleaching agents prepared by the same components. The null hypotheses were that: (1) the pH of the bleaching agent and photo-irradiation time did not affect the bleaching efficacy in HSP; (2) the pH and application times did not affect the bleaching efficacy in BT.

MATERIALS AND METHODS

Preparation of the bleaching material

Table 1 lists the materials and preparation formula to create different pH of bleaching agents. The bleaching agents were prepared by mixing with 30% H₂O₂, sodium carbonate (Na₂CO₃) carboxymethyl cellulose sodium (CMCS) and distilled water according to a pre-calculated formula. The bleaching agents of different pH 5.5, 6.0, 7.0, 8.0 and 9.0 with 23% H₂O₂ were prepared by adjusting the Na₂CO₃ amount. The final concentration of the bleaching material was decided according to a previous study. The pH-conditioner was added to the bleaching agent just before using it, because the alkaline pH can accelerate the degradation of H₂O₂. The pH values of the bleaching gel were measured using a portable pH meter (LaquaTwin compact pH meter, Horiba, Kyoto, Japan).

Preparation of HSP specimens

HSP was used in the first experiment. The 0.24 g weight of hematoporphyrin powder (Wako pure chemical, Tokyo, Japan) was dissolved in 300 mL of ethanol and a 0.1 wt% hematoporphyrin solution was prepared. Photo printing paper (Canon, Tokyo, Japan) was immersed in a dark room. Subsequently, the specimens were cut in the solution for 5 min and allowed to naturally dry. 0.1 wt% hematoporphyrin solution was prepared. Photo printing paper (Canon, Tokyo, Japan) was immersed in a dark room. Subsequently, the specimens were cut in the solution for 5 min and allowed to naturally dry. The specimens were embedded in a cylindrical acrylic (Sankyorikagaku, Saitama, Japan) under running water until reaching approximately 1-mm of remaining enamel. The thickness of the remaining enamel was checked by a digital vernier caliper (Mitutoyo, Kanagawa, Japan). Bovine incisors were used for the second experiment. Soft tissue remnants attached to the teeth were cleaned gently by a scalpel. Labial enamel surfaces were ground with 280–800 grit silicon carbide papers (Sankyorikagaku, Saitama, Japan) under running water until reaching approximately 1-mm of remaining enamel. The thickness of the remaining enamel was checked by a digital vernier caliper (Mitutoyo, Kanagawa, Japan). Two specimens with a size of approximately 5×5 mm were obtained from the flatten labial surface of each tooth by the rotary diamond saw (Mini Lab-cutter mc-110, Maruto instrument, Tokyo, Japan). Dentin surface was covered with dental wax (Utility wax, GC) and the specimens were embedded in a cylindrical acrylic tube with 10-mm in height and 10-mm in internal diameter using dental self-curing acrylic resin (Unifast III, GC). At the setting of resin, enamel surface was exposed to the outside and the pulpal surface of dentin covered with wax was exposed in the tube (Fig. 1).

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Manufacturer</th>
<th>pH 5.5</th>
<th>pH 6.0</th>
<th>pH 7.0</th>
<th>pH 8.0</th>
<th>pH 9.0</th>
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<tbody>
<tr>
<td>H₂O₂ (µL)</td>
<td>Wako pure chemical, Tokyo, Japan</td>
<td>1,000</td>
<td>1,000</td>
<td>1,000</td>
<td>1,000</td>
<td>1,000</td>
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<tr>
<td>Active ingredient</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>DW (µL) Carrier</td>
<td>Wako pure chemical</td>
<td>500</td>
<td>500</td>
<td>500</td>
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<td>500</td>
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<tr>
<td>CMCS (mg) Thickener</td>
<td>Wako pure chemical</td>
<td>0.35</td>
<td>0.35</td>
<td>0.35</td>
<td>0.35</td>
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<tr>
<td>Na₂CO₃ (mg) pH conditioner</td>
<td>Wako pure chemical</td>
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<td>0.002</td>
<td>0.015</td>
<td>0.33</td>
<td>1.6</td>
</tr>
</tbody>
</table>


Bleaching procedure and color-measurement of HSP specimens

The specimens were randomly allocated into five main experimental groups according to the pH values of the bleaching agents (pH 5.5, 6.0, 7.0, 8.0 and 9.0). Each group was subdivided into three groups (n=10) according to the photo-irradiation time (1, 3 and 5 min). The 2-mm-thick bleaching agent was applied evenly on the specimens and photo-irradiation was performed for 1, 3 or 5 min by an arch type violet-LED light unit for tooth bleaching (Cosmo Blue, GC, Tokyo, Japan). Irradiation time was determined by the result of the pilot study. Subsequently, the bleaching agent was wiped off using a piece of damp gauze. CIE L*a*b* values were measured by the colorimeter and photographs were taken. Color difference (ΔE) of HSP before and after the bleaching was calculated according to the following equation:

\[
\Delta E = \sqrt{((\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2)}
\]

\(\Delta L\): the difference of L* values between baseline and after bleaching
\(\Delta a\): the difference of a* values between baseline and after bleaching
\(\Delta b\): the difference of b* values between baseline and after bleaching

Preparation of BT specimens and artificial staining

Bovine incisors were used for the second experiment. Soft tissue remnants attached to the teeth were cleaned gently by a scalpel. Labial enamel surfaces were ground with 280–800 grit silicon carbide papers (Sankyorikagaku, Saitama, Japan) under running water until reaching approximately 1-mm of remaining enamel. The thickness of the remaining enamel was checked by a digital vernier caliper (Mitutoyo, Kanagawa, Japan). Two specimens with a size of approximately 5×5 mm were obtained from the flatten labial surface of each tooth by the rotary diamond saw (Mini Lab-cutter mc-110, Maruto instrument, Tokyo, Japan). Dentin surface was covered with dental wax (Utility wax, GC) and the specimens were embedded in a cylindrical acrylic tube with 10-mm in height and 10-mm in internal diameter using dental self-curing acrylic resin (Unifast III, GC). At the setting of resin, enamel surface was exposed to the outside and the pulpal surface of dentin covered with wax was exposed in the tube (Fig. 1).
After polymerization of embedding resin, the wax was removed and the dentin surface was irrigated with 5% sodium hypochlorite solution (Wako pure chemical) for 1 min to remove organic tissue remnants. Dentin surfaces were etched with 40% phosphoric acid (K-etchant gel, Kuraray Noritake Dental, Tokyo, Japan) for 10 s to open the dentinal tubules to enhance the stain uptake into the dentin. Then the enamel surfaces of specimens were polished with 1000-grit and 1200-grit silicon carbide paper to obtain smooth and flat enamel surfaces. Finally, the specimens were cleaned by an ultrasonic cleaner for 3 min.

Artificial staining of the specimens was done with black tea extract. Tea solution was prepared by immersion of two tea bags (Lipton yellow label tea bags, Unilever Japan, Tokyo, Japan) in 50 mL of fresh boiling water for 5 min. The specimens were immersed in the extract and stored in an incubator for 7 days at 37°C. The solution was stirred once a day to avoid precipitation of the solution and changed on the fourth day of the experiment.

After removal from the solution, the specimens were rinsed under tap water and air-dried. The color of the enamel surface was measured with the colorimeter as mentioned above. CIE $L^*$ $a^*$ $b^*$ values were recorded as the baseline data. Specimen selection was based on $L^*$ value and only the specimens with the $L^*$ value between 45–50 were selected for the experiment to minimize the variation among the specimens. Photograph of each experimental surface was taken by the same digital camera used in the HSP experiment. The total specimen number was 50 and randomly assigned to five experimental groups ($n=10$).

**Bleaching procedure and color-measurement of BT**

The same five bleaching agents with different pH were prepared and used as in the HSP experiment. The 2-mm-thick bleaching agent was applied on the enamel surface and photo-irradiation was done for 10 min by the same violet-LED bleaching light unit (Cosmo blue). After light exposure, the bleaching agent was removed with damp cotton, washed thoroughly under tap water and dried gently. Color-measurement was performed and photographs were taken. Bleaching procedure and color-measurement were repeated ten times. The $\Delta E$ before and after each bleaching time was calculated from obtained $L^*$, $a^*$ and $b^*$ values as same as HSP experiment.

**Statistical analysis**

Kolmogorov-Smirnov test and Shapiro-Wilk test were performed to check the normal distribution of $\Delta L$, $\Delta a$, $\Delta b$ and $\Delta E$ data of HSP and BT. The data of HSP and BT were statistically analyzed using repeated-measures ANOVA followed by multiple comparisons with Bonferroni correction ($\alpha=0.05$) with the factors of pH of bleaching agent and photo-irradiation time for HSP and pH of bleaching agent and number of application times for BT (SPSS statistics Ver 23, IBM, Armonk, NY, USA). Mauchly’s test indicated that the assumption of sphericity had been violated, therefore Greenhouse–Geisser estimates was used to report the significance.

**RESULTS**

**Experiment using HSP**

The pictures of the typical color change of the specimens in each group during the procedure presented in Fig. 2. All the bleaching groups showed the bleaching effect.
and the degree of bleaching was different among them. Changes of $\Delta L$, $\Delta E$, $\Delta a$, and $\Delta b$ in each experimental group were shown in Figs. 3a, b, c and d respectively. The $\Delta E$ of HSP specimens was significantly influenced by the pH of the bleaching agent and photo-irradiation time ($p<0.001$). The $\Delta E$ gradually increased in all the experimental groups accompanied by the increasing $L^*$ value (the lightness of the specimen) and decreasing $a^*$ (i.e. redness of the specimen has moved towards achromatic color, near-zero point of coordinate) and $b^*$ values (i.e. yellowness of the specimen has moved towards achromatic color, near-zero point of coordinate). The maximum bleaching effect was found in pH 9.0 group with 5 min photo-irradiation time and minimum color change was found in the pH 5.5 group with 1 min photo-irradiation time. According to multiple comparisons with Bonferroni correction, there was a significant difference of $\Delta E$ in between all experimental groups.

**Experiment using BT**

The typical color change of the samples in each experimental group is shown in Fig. 4. Comparable to
the HSP experiment, the color change was clear. In all experimental groups, a bleaching effect was found. Changes of $\Delta L$, $\Delta E$, $\Delta a$, and $\Delta b$ were shown in Figs. 5a, b, c and d respectively. The $\Delta E$ of BT specimens was significantly influenced by the pH of the bleaching agent and number of application times ($p<0.001$). The $\Delta E$ increased in accompany with increased $L^*$ value and decreased $a^*$ and $b^*$ values on each specimen in all the experimental groups. Bonferroni correction showed a significant difference of $\Delta E$ in all experimental groups.

**DISCUSSION**

Bleaching is a whitening process that occurs in a solution or on a surface. Chromophores are organic compounds containing a large number of single and double bonds. Bleaching can be achieved by destroying the double bonds through breaking the conjugated chain, or by oxidation of other chemicals in it. The mechanism of tooth bleaching by H$_2$O$_2$ is similar to the bleaching of wood pulp, cotton, cloth in industry or chromogenic molecules inside the intracrystalline spaces of enamel. Previous studies have investigated the effect of pH in industrial bleaching of cotton fibers or wood pulp using H$_2$O$_2$ with higher pH to accelerate and increase the bleaching efficacy. However, the effect of pH of bleaching agent in tooth deserved further studies.

Numerous methods and substrates have been utilized to evaluate bleaching materials and techniques in vitro, such as human teeth, BT, HSP, and dye solutions. Although the human or BT were used in most studies, the characteristics of each tooth were different from one to another. These characteristics are tooth age, mineralization status, the thickness of enamel or dentin, diameter and number of dentinal tubules, differences in the numbers of organic and inorganic compounds, the extent of penetration of bleaching agent, and initial tooth color. In the present work, the effect of pH on tooth bleaching action was evaluated using HSP and BT specimens. Hematoporphyrin is a blood pigment and HSP paper may be suitable for the evaluation of the bleaching effect because it is very sensitive, easy to prepare and use. It allows the assessment of a larger number of specimens and avoids the complexities resulting from chemical diffusion and optical transmission within the tooth. It is an appropriate evaluation with minimal variation and high reproducibility. BT have been used on bleaching experiments because it was difficult to collect a large number of extracted human incisors. The chemical and physical properties of BT such as composition, heat capacity, hardness, dentinal tubule density and permeability are similar to human teeth.

The black tea was used for staining the teeth in many previous studies concerning tooth bleaching. This agent is widely available and can induce reproducible discoloration in a short time. Since it is difficult to predict the bleaching effect from only the result of HSP, it is necessary to evaluate the bleaching effect using a tooth model. Although the experimental design in this study might be different from the exact clinical situation, the method used with HSP and BT seemed to be suitable and useful as a screening test for bleaching materials to
determine their application methods before usage in the clinical situation\textsuperscript{20}. The accurate evaluation of the color change of HSP and BT was an important step in this experiment. Among the methods for color-measurement, the shade guide is subjective\textsuperscript{20} while the color-measuring device is objective, such as a colorimeter or a spectrophotometer\textsuperscript{34}. Therefore, a colorimeter (NR-II) was used in the present study.

Commission Internationale de l’Eclairage (CIE) has explained a three-dimensional color space CIE Lab based on three color receptors (red, green, and blue) in the eye. It consists of three axes: $L^*$, $a^*$ and $b^*$. The $L^*$ value represents the lightness of an object. The perfect black has an $L^*$ value of zero and a perfect white has an $L^*$ value of 100. The $a^*$ value represents redness (positive $a^*$) or greeneiness (negative $a^*$). The $b^*$ represents yellowness (positive $b^*$) or blueness (negative $b^*$). Because of the CIE Lab system, the color differences can be presented in units as mentioned below that relate to visual perception and clinical significance. At $0<\Delta E<1$, the difference ($\Delta E$) is not noticed by the observer; $1<\Delta E<2$, the difference is noticed only by the experienced observer; $2<\Delta E<3.5$, the difference is also noticed by the inexperienced observer; $3.5<\Delta E<5$, a clear difference in color is noticed; $5<\Delta E$, observer notices two different colors\textsuperscript{35}.

In this study, pH 5.5 was selected as it is the limitation of pH that can be adjusted without pH-conditioner. Most of the in-office bleaching products had a pH between 3.7 and 7.9\textsuperscript{36}. The pH 6.0 and 7.0 were adopted to previous reports and the pH 8.0 and 9.0 groups were added to evaluate the effect of higher pH. Finally, the pH of each experimental group was pH 5.5, 6.0, 7.0, 8.0 and 9.0.

The usage of the light source in tooth bleaching is controversial. Photo-irradiation was performed by a violet-LED light in this experiment (Cosmo blue) to increase the bleaching efficacy. The peak wavelength of the light unit was 405 nm and its intensity was 55 mW/cm\textsuperscript{2}. Because of short-wavelength and high-vibration frequency it shows less penetrability of dental tissue and greater energy on the surface, resulting in breakage of large pigment molecules with lower heating and pulp protective\textsuperscript{37}.

Both of null hypotheses were rejected, because longer irradiation time and higher pH yielded significantly higher $\Delta E$ in HSP and increasing application times and higher pH resulted in higher $\Delta E$ in BT specimens. The previous studies\textsuperscript{8,10} about the effect of the pH on bleaching action conveyed a higher effect with higher-pH of bleaching agent. One experiment measuring the color of the solution containing chromogens (wine and tobacco solution) reported that the efficacy of $\text{H}_2\text{O}_2$ bleaching was directly proportional to the pH of the solution\textsuperscript{10}. Similar results were obtained in another study\textsuperscript{10}, investigating the chemical activity of $\text{H}_2\text{O}_2$ on chromogens of a tea solution by measuring the absorbance of the solution as a function time. An increase in the speed of the reaction between pH 8.0 and pH 9.0\textsuperscript{10} was found. One \textit{in vitro} study with human teeth showed an increased bleaching efficacy with a bleaching gel with alkaline pH, when compared with acidic gels\textsuperscript{38}.

$\text{H}_2\text{O}_2$ is one of the most active bleaching agents that decomposes into free-radicals, such as hydroxyl, perhydroxyl and superoxide free-radicals. $\text{H}_2\text{O}_2$ bleaching generally proceeds via perhydroxyl free-radical. The formation of perhydroxyl ion is influenced by pH\textsuperscript{35}. Therefore, more free-radical production can be seen in higher pH bleaching agents. As well as in a basic solution, lower activation energy is required for the formation of free-radicals from $\text{H}_2\text{O}_2$, and the reaction rate is higher compared with acidic solutions.

The present study demonstrated that higher pH of the bleaching agent and increased photo-irradiation time could give rise to higher bleaching effect in HSP specimens, as well as higher pH of the bleaching agent and increased number of application times yielded the higher bleaching effect in BT specimens. As the tested pH range was pH 5.5–9.0 all the experimental groups lie within the safe range according to OECD Guidelines and can expect higher bleaching effect with minimum adverse effects. According to this experiment pH 9.0 is the most recommended pH for tooth bleaching, with extended photo-irradiation time and multiple applications. Therefore, it is better to use high-pH bleaching agents within the safety range. However, the result with HSP and BT was difficult to apply directly to \textit{in vivo} treatments. In this \textit{in vitro} study, although we have investigated the effect of pH as an isolated factor, the bleaching effect can be influenced by many factors in the clinical treatments as mentioned above. Therefore, further study with extracted human teeth and subsequently \textit{in vivo} study is necessary to establish a suitable clinical method of using bleaching agents with high-pH to achieve maximum bleaching effect.

**CONCLUSION**

It was concluded that higher pH of the bleaching agent showed higher bleaching effect with an increase of photo-irradiation time for HSP specimens and with an increase of application times for BT specimens.

**CONFLICT OF INTEREST**

The authors do not have any financial interest in the companies whose materials are included in this article.

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