Effects of Hydrogen Peroxide Concentration and Activation Time on Hydroxyl Radical Generation and the Bleaching Effect

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Running title: Hydoxyl Radical Generated during Bleaching

Abstract

Purpose: Tooth bleaching is widely performed in clinical practice. According to Goldstein, the mechanism behind tooth bleaching is the decomposition of a carbon double bond (chromatic) molecule in a discolored tooth to an achromatic substance via hydroxyl radicals generated from hydrogen peroxide. In this study, we sought to clarify the mechanism by investigating the efficacy of bleaching and time taken to activate the hydroxyl radicals generated from various concentrations of hydrogen peroxide.

Methods: Hydrogen peroxide solutions containing 3%, 10%, 20%, and 30% hydrogen peroxide were mixed with a spin trapping agent. The hydroxyl radicals generated were measured by electron spin resonance. Activation was achieved by light irradiation at a

wavelength of 440 nm for 0.5, 1, 2, 3, 5, and 10 min. Filter papers dyed with β-carotene were immersed in 3%, 10%, 20%, and 30% hydrogen peroxide solutions and irradiated for 0.5, 2, and 10 min. The color difference, ΔE*ab, was calculated from the CIELab values at baseline and after bleaching.

Results: The generation of hydroxyl radicals increased at higher concentrations of hydrogen peroxide and longer irradiation times. The ΔE*ab values also showed the same tendency as the results showing the amounts of hydroxyl radicals generated. That is, the ΔE*ab value increased as the concentrations and activation times increased.

Conclusions: The concentrations of hydrogen peroxide and the activation times were important for the generation of hydroxyl peroxide radicals. However, based on the amounts

of hydroxyl radicals and the ΔE*ab values, other factors such as the catalysts, temperature rise, and the physical properties of the bleaching agent appear to be related to the efficacy. To pursue effective and safe tooth bleaching materials, other factors in addition to the concentration of hydrogen peroxide and the application time should be considered.

Key words

tooth bleaching, hydrogen peroxide, hydroxyl radicals, β-carotene, color difference

Introduction

Tooth whitening is performed to improve the aesthetics of the teeth and has become a popular treatment. Among a variety of bleaching methods, in-office bleaching involves the application of a bleaching agent containing hydrogen peroxide onto the surface of the tooth enamel and a visible light is used to activate the bleaching agent. In-office bleaching is an effective and safe method because it is performed by a dentist in the dental office.

In 2015, we investigated the relationship between temperature rise and the bleaching effect using irradiation at different wavelengths and light intensities.[1)] In 2016, we investigated the amounts of radicals generated during bleaching using luciferin.[2)] In 2017,

we examined the influence of pH on the generation of hydroxyl radicals via ESR equipment.[3)]

As shown in Fig. 1, the bleaching mechanism can be attributed to the radicals generated by the activation of hydrogen peroxide and the decomposition of a chromatic molecule to an achromatic molecule, according to Goldstein and colleagues.[4)]

Hydrogen peroxide decomposes into hydroxyl and hydroperoxyl radicals under visible light, ultraviolet light, heat, and catalysts in neutral to alkaline conditions. Hydroxyl radicals and hydroperoxyl radicals cleave double bonds, which are chromatic molecular groups, but hydroxyl radicals are more effective and have a shorter lifespan.

The decomposition of hydrogen peroxide, the generation of the radical, and its action have been previously reported.[5–7)]

Electron spin resonance (ESR) method can be used to observe the absorption of electromagnetic waves by electron spins. If unpaired electrons exist in the substance investigated, the direction of the spin is usually oriented in random directions and the magnetic moments are canceled out. When unpaired electrons are placed in a magnetic field, they take two energy states: a stable state, which moves in the same direction as the magnetic field and an unstable state, which moves in the opposite direction. It is thought that the amounts of free radicals generated in a substance depend on the amount of

ionization by radiation; therefore, the absorbed dose can be evaluated from the integrated value of the ESR spectrum.

Fig. 2 shows the spin trapping agent 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) and a typical hydroxyl radical waveform. DMPO is used as a spin trapping agent to measure the amount of hydroxyl radicals generated. The lifetime of the hydroxyl radical is very short and cannot be measured by regular ESR. DMPO is thus used to prolong the lifetime of the hydroxyl radical and make it measurable by ESR at room temperature. The intensity of the DMPO-OH signal was measured as hydroxyl radical signal intensity ratio with respect to the intensity of Mn marker used as an internal standard. The horizontal axis represents the magnetic field strength (mT), and the vertical axis shows the relative amounts of radicals

detected. Mn markers are observed at both ends of the characteristic 1:2:2:1 waveform.

The relative signal intensity (RSI) is the radical signal value X divided by the Mn marker signal value. Because the radicals have extremely short lifetimes,[8)] spin trapping agents are usually used for detection with ESR.

The bleaching effect of the radicals was determined by calculating the color difference (ΔE*ab) before and after dyed filter paper was bleached. The null hypothesizes to be tested were to investigate whether increasing the concentration of hydrogen peroxide and prolonging the irradiation (activation) time increased the amount of hydroxyl radical generation and increased the color difference ΔE*ab of the dyed filter paper.

Materials and Methods

1) Preparation of various concentration of HP solutions

Fig. 3 illustrates the preparation of the 3%, 10%, 20%, and 30% hydrogen peroxide (HP) solutions. We used 30%HP (Wako Pure Chemical Industries Ltd., Tokyo, Japan) as received, and diluted this concentration with distilled water to prepare the lower concentrations. The HP solutions (130 μL of each concentration) were added to 20 μL of DMPO (Chemical Dojin Co., Ltd., Tokyo, slightly acidic) and 50 μL of phosphate buffer (pH 7.41). DMPO was used for the spin trapping method of ESR. A pH meter (PH-6011, CUSTOM Corp., Tokyo, Japan) was used to check the pH of each solution. The pH levels

of the 3%HP and 10%HP solutions were 6.4, and those of 20%HP and 30%HP were 6.2.

All procedures were performed at 18 ± 2 °C.

2) Procedures for hydroxyl radical measurement by ESR

Fig. 4 shows the procedures used to measure the hydroxyl radicals. Each of the solutions was suctioned into the ESR capillary tube. Irradiation with a halogen light with a wavelength of 440 nm (ES-USH500, Ushio, Tokyo, Japan) was used to activate the HP solution for 0.5, 1, 2, 3, 5, and 10 min.[9–12)] The distance between the light unit tip and the capillary tube was approximately 30 mm.

ESR spectra were measured with a JES-X320 spectrometer (JEOL, Tokyo, Japan). The ESR settings were as follows: measurement time, 20 s; microwave power, 3 mW; magnetic field, 325 ± 5 mT; sweep time, 20 s; and modulation, 0.03 s.

3) ΔE*ab value obtaind from between dyed and bleached specimens

Fig. 5 shows the procedures used to obtain the ΔE*ab values of the bleached dyed paper.

Preparation of the dyed paper: Among the colored polyenes, β-carotene was chosen as the dye because it has a double bond (chromic) with a simple structure and a small molecular weight; however, it does not cause tooth discoloration. To prepare the 0.01% dye solution, we dissolved β-carotene (Lucarotin 10CWD, BASF SE, Ludwigshafen, Germany) with distilled water. The solution was pale brown. Filter papers (JIS P3801,

ADVANTEC, Tokyo, Japan) 15 x 15 mm were dipped into the dye solution for 10 min and dried. A total of 96 dyed specimen papers were divided among the four HP concentration groups and then grouped by three irradiation times. Each group contained 8 specimens. As shown in Fig. 3, HP solutions at concentrations of 3%, 10%, 20%, and 30% without DMPO were prepared. Each dyed paper was measured with a colorimeter (ShadeEye NCC, Shofu, Kyoto, Japan) to obtain the baseline colorimetric measurements as CIELab values.

Bleaching procedure: 24 dyed papers were dipped into 3%HP solution and irradiated for 0.5 min, 2 min, and 10 min at 440 nm from a distance of approximately 60 mm. Similarly, 24 dyed paper groups were dipped in 10%HP, 20%HP, and 30%HP and sub-divided into

0.5 min, 2 min, and 10 min irradiation groups. After bleaching, the specimens were remeasured via ShadeEye NCC.

The color difference ΔE*ab values were calculated from the baseline L*0 a*0b*0 values and L*1 a*1b* 1 values after light irradiation.

The formula for calculating the color difference ΔE*ab was:

$$\Delta E^{*}ab = \sqrt{(L^{*}1 - L^{*}0)^{2} + (a^{*}1 - a^{*}0)^{2} + (b^{*}1 - b^{*}0)^{2}}$$

where L*0, a*0, and b*0 represent the baseline CIELab values, and L*1, a*1, and b*1 the CIELab values after bleaching.

The mean ΔE*ab values and standard deviations (SDs) were calculated and statistically analyzed with the Scheffe exact test ($p<0.05$).

Results

1) Waveform of hydroxyl radicals from 3%HP at each irradiation time

Fig. 6 shows the waveforms of the hydroxyl radicals from 3%HP at each irradiation time.

The irradiation times were 0, 0.5, 1, 2, 3, 5, and 10 min. The transverse axis represents the

mT (magnetic field) and the vertical axis shows the hydroxyl RSI. Each waveform shows

that the typical signal of a hydroxyl radical (DMPO-OH), as the transverse direction of

waveform, had peaks of the Mn marker, signal peaks of 1:2:2:1, and another Mn marker.

Large RSI values indicate larger numbers of hydroxyl radicals. As the irradiation time

increased, the amount of hydroxyl radicals increased.

2) Hydroxyl radical generation at each irradiation time for each concentration group

Fig. 7 shows the hydroxyl radicals produced at the irradiation times for each concentration. The generation of hydroxyl radicals was greater at longer irradiation times. At a low concentration, that is, 3%HP, the increase was negligible. However, at concentrations of 10%, 20%, and 30%, HP increased the rate of generation, especially at 20% and 30%. Irradiation lasting 0.5 and 1 min barely increased the amounts of radicals in 3%HP, while a slight increase was found in 10%HP, 20%HP, and 30%HP. At irradiation times of 2, 3, 5, and 10 min, a concentration of 3%HP slightly increased the rate, while 10%HP and 20%HP increased the rate according to the concentration. A comparison of 20%HP and

30%HP showed that the increment was not proportional to the concentration, despite a

1.5-fold difference in concentration.

3)Color difference ΔE*ab value at irradiation time and each HP concentration group

Fig. 8 shows the color difference ΔE*ab value at each irradiation time and HP

concentration. The transverse axis indicates the irradiation time (min), while the vertical

axis indicates the color difference ΔE*ab. A T-mark indicates the standard deviation.

Asterisks indicate a significant difference by the Scheffe exact test ($p<0.05$). Fig. 8 is

similar to the graph showing the amounts of radicals generated (Fig. 7). The ΔE*ab value

increased as the HP concentration and irradiation time increased.

Discussion

Various conditions necessary for tooth bleaching with hydrogen peroxide have been reported.[13–22] Many researchers have described the bleaching effects of the materials, while some have described tooth sensitivity caused by the components penetrating the dentin at certain hydrogen peroxide concentrations and activation times.[13, 14] Some papers have reported that the microstructure of the enamel breaks down when high concentrations of hydrogen peroxide are applied.[15–18] Furthermore, even for in-office bleaching, a low concentration of hydrogen peroxide is preferable; thus, the concentration of hydrogen peroxide should be low.

Kawamoto et al. reported the relationship between hydrogen peroxide and laser or light irradiation and the production of hydroxyl radicals.[19] Factors considered to be important include the hydrogen peroxide concentration, pH, activation method, operation time, conditions of the light activation method (i.e., wavelength, intensity, and use of a high-energy laser), presence of a catalyst and photocatalyst, and thermal action.

The rate of chemical reactions is dependent on temperature, and thus temperature is also an important factor for the reactions in tooth bleaching.[1, 2] However, in-office bleaching is performed in the oral cavity, and the upper limit should be 40 to 50 °C.[20, 21]

We used various concentrations of hydrogen peroxide and activation times and measured the hydroxyl radicals by the ESR method. More hydroxyl radicals evolved at higher

concentrations and longer activation times, as reported previously in many studies. The detection of radicals by the ESR method involves many challenges that must be overcome, including the conditions needed to detect specific radicals, properties of the samples, conditions for generating the radicals, and measurement times. In particular, the concentration and amount of the spin trapping agent used (DMPO) are important for obtaining RSI measurements under consistent conditions. Specimens evaluated by the ESR method must be in a liquid state because they need to be suctioned into a capillary tube. We used a liquid sample with hydrogen peroxide, DMPO, and phosphate buffer.[8, 20, 23)] Commercially available bleaching agents have paste-like or gel-like properties, so it is difficult to measure the radicals generated by them with the ESR method. In another

simple method, radicals can be detected by chemical luminescence using luciferin.

However, luciferin detects all radical species and is not specific for the detection of only

hydroxyl radicals. [2, 24, 25)]

In this experiment, the results of hydroxyl radical detection by ESR and the color change

results were in good agreement. Specifically, more hydroxyl radicals were generated and

bleaching effect was stronger when the hydrogen peroxide concentration and activation

time were increased. Therefore the null hypothesis was adopted. When increasing the

concentration of hydrogen peroxide and prolonging the irradiation (activation) time

increased the amount of hydroxyl radical generation and increased the color difference

ΔE*ab of the dyed filter paper. However, there was a difference in the amount of increase.

Hydroxyl radicals have an extremely short lifetime and are continuously generated, cleave double bonds instantaneously, and disappear. ESR measures hydroxyl radicals at the moment they are generated. The bleaching of the dyed paper can be considered to represent the accumulation of the double bonds cleaved. In other words, the ESR measurement is instantaneous and the color difference measurement is cumulative.

Tooth bleaching can also be done at home. In this method, a 10% carbamide peroxide gel is placed in direct contact with the surface of the teeth in a mouth tray for 2 h each day for several weeks. Carbamide peroxide decomposes into urea and hydrogen peroxide to provide a bleaching action. The relationship between the lifetime of the hydroxyl radicals, effects on the teeth, and contact time is based on empirical evidence.

The bleaching agents and bleaching samples are indispensable for obtaining objective measurements of the bleaching effect. Clinically, discolored teeth are the targets, [26–29] but experimentally, extracted teeth or specimens dyed with some pigment are used.[30] Brown egg shells have also been used for a simple screening test.[1, 31] In this experiment, the bleaching effect was determined by bleaching the dyed filter papers were stained with β-carotene.

Pure β-carotene is soluble in chloroform but not in water. In this study, we used Lucarotin (BASF SE), which contains 10% β-carotene, 50–70% glucose, and 15–30% gelatin. Lucarotin is water soluble and powdery. β-carotene is not a substance that causes discoloration of teeth. Therefore, Lucarotin had impurities that influenced the result. When

dyed, the specimens turned brown and when bleached, they became lighter (L*) and changed to a pale brown color to the naked eye. Color difference values were calculated from CIELab values before and after bleaching under each condition and the bleaching effect was examined. These results were in good agreement with those of the radical generation experiment. That is, higher concentrations of hydrogen peroxide and longer activation times resulted in a greater bleaching effect. However, although the amounts of radicals generated and the bleaching effects were similar, the correlation was not strong and it was thought that other factors influenced the results.

Tooth bleaching agents must be safe and whiten teeth effectively to the desired color within a short time. The results of this experiment showed that higher concentrations and

longer activation times are effective, but this is not useful for the design of potential bleaching agents. Although the upper limits of the concentration of hydrogen peroxide and activation time could be inferred from this experiment, other factors that could not be investigated here must be considered.

Conclusions

1) The concentrations of hydrogen peroxide played important roles in the generation of hydroxyl peroxide radicals.

2) The activation time was also important for the generation of hydroxyl peroxide radicals.

3) Although similar results were obtained for radical generation and color difference change as the concentration of hydrogen peroxide was higher and the activation time was longer, it was suggested the ESR measurement of instantaneous occurrence of hydroxyl radicals generation, measurement of color difference was to represent the total amount of double bonds degradation.

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過酸化水素濃度と活性化時間がヒドロキシラジカル発生と漂白におよぼす影響

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ランニングタイトル： 漂白時のヒドロキシラジカル発生量

抄録

目的：歯の漂白は臨床診療で広く使用されている。歯の漂白メカニズムは、「過酸化水素から発生するヒドロキシルラジカルが、有色二重結合分子を分解し無色分子にとする」とされている。本研究の目的は、この漂白メカニズムを明らかにするため、過酸化水素の濃度および活性化時間を変化させ、ヒドロキシルラジカル発生量と漂白効果を検討することである。

材料と方法：濃度 3,10,20 と 30%の過酸化水素水とスピントラッピング剤(5,5-dimethyl-1-pyrroline-N-oxide (DMPO))と混合し ESR (Electro Spin Resonance) 法によりヒドロキシラジカル発生量を検討した。過酸化水素の活性化は、波長 450nm のハロゲン光源を使用し 0.5,1,2,3,5,10 分の照射時間を行った。

漂白効果の検討は、β-カロテンで染色した濾紙を 3,10,20,30%過酸化水素水に浸漬し、0.5,2,10 分間照射し、漂白前後の CIELab 値から色差 ΔE*ab を算出した。色差値が大きいほど漂白効果が高いと判定した。

結果：ヒドロキシルラジカルの生成は、過酸化水素の濃度が高く照射時間が長いと増加した。ΔE 値はヒドロキシラジカル発生量と同様の傾向を示した。すなわち、ΔE* ab は、高い濃度、長い活性化時間であるほど増加した。

結論： 過酸化水素の濃度および活性化時間は、過酸化水素ラジカルの生成において重要な役割を果たした。触媒の存在、温度および漂白剤の物理的特性を含む要因に加え、ヒドロキシルラジカル量と色差値 ΔE* ab 値は、歯の漂白効果と関連していた。効果的かつ安

全な歯の漂白剤を開発するには、過酸化水素の濃度および活性化時間のみならず、他の要因も考慮する必要があると考えられた。

キーワード：歯の漂白、過酸化水素、ヒドロキシラジカル、β-カロテン、色差

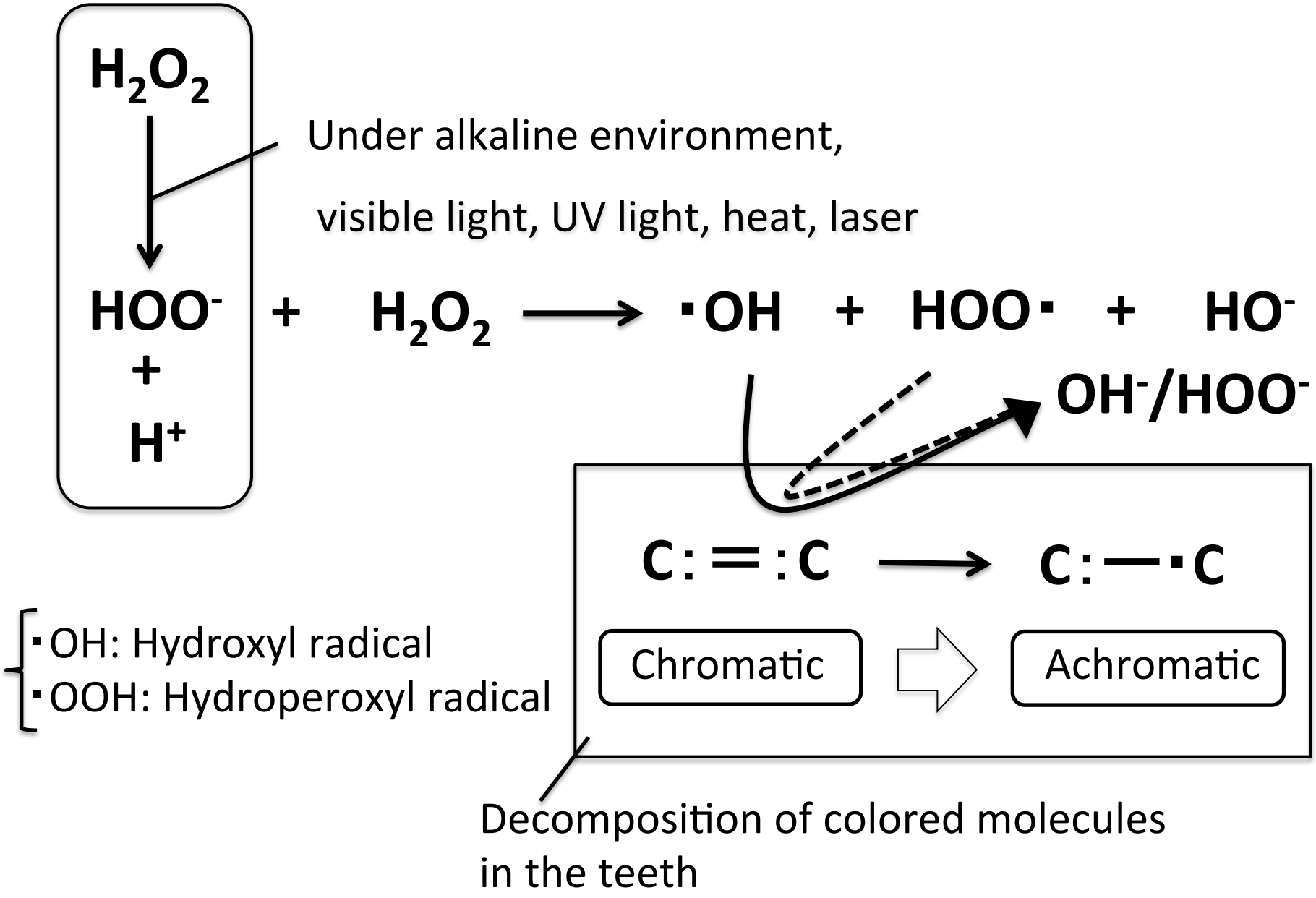

Fig. 1. Decomposition of HP and cutting of double bond

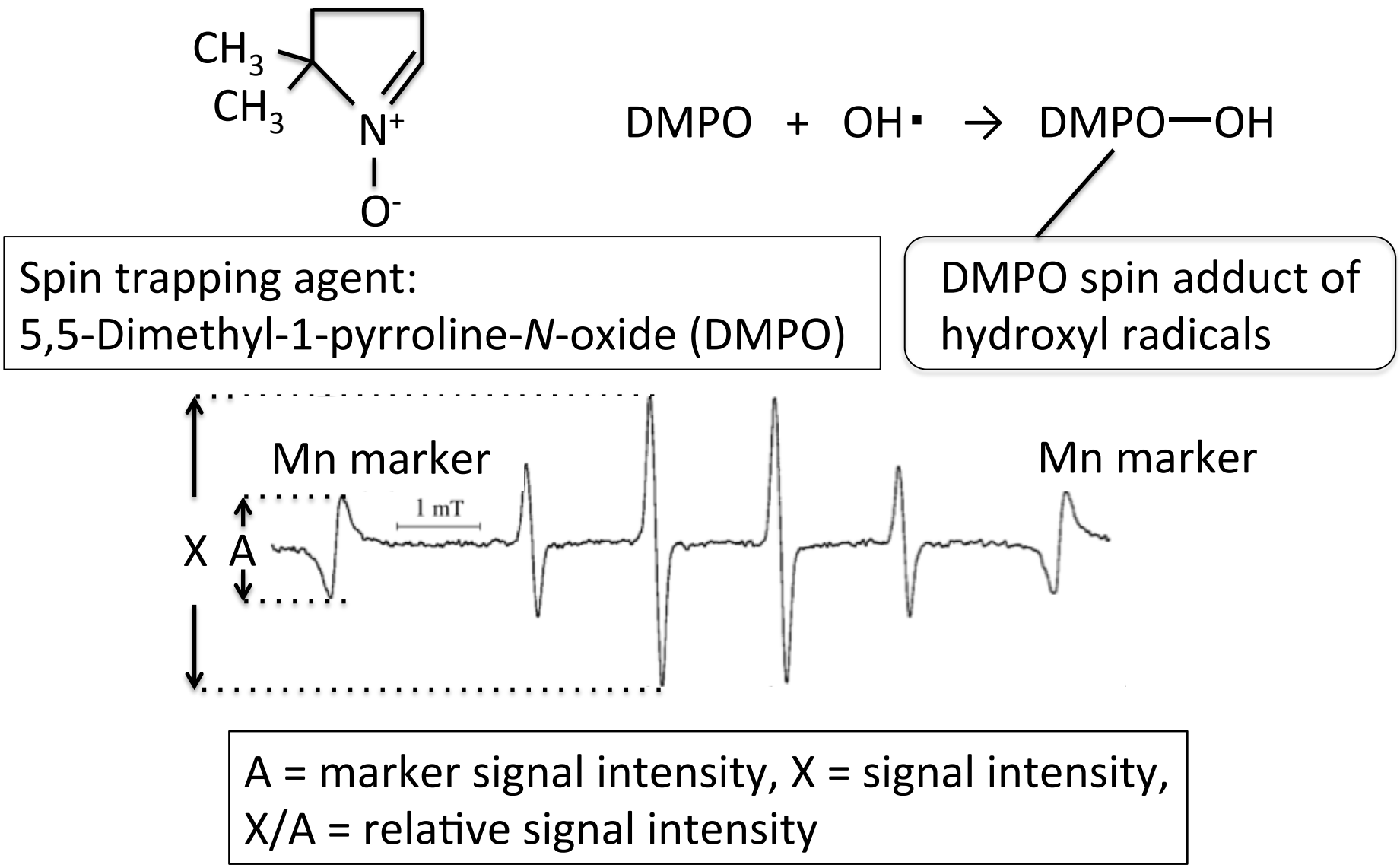

Fig. 2. Spin trapping agent DMPO and a typical hydroxyl radical waveform

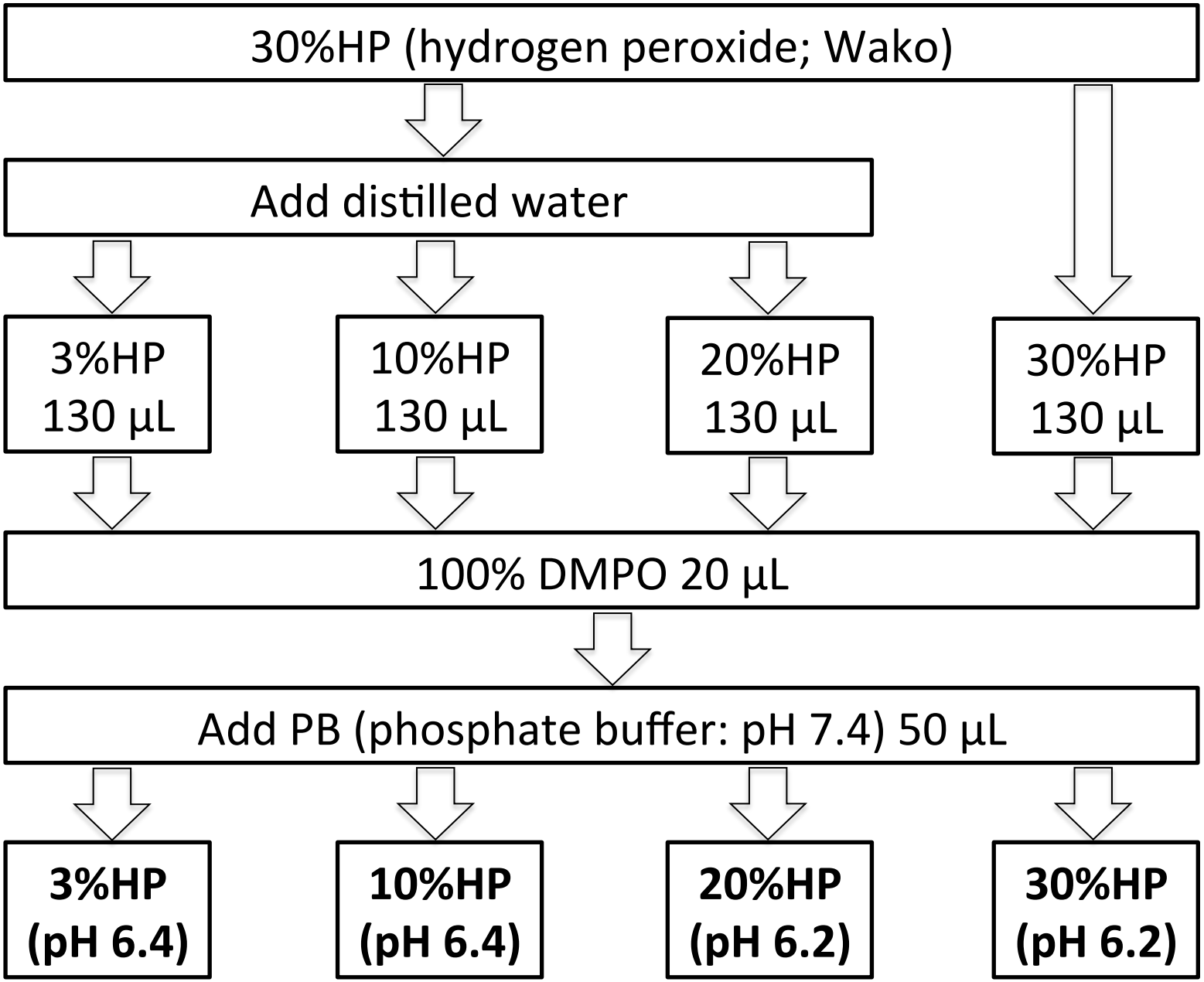

Fig. 3. Preparation of 3%HP, 10%HP, 20%HP, and 30%HP solutions

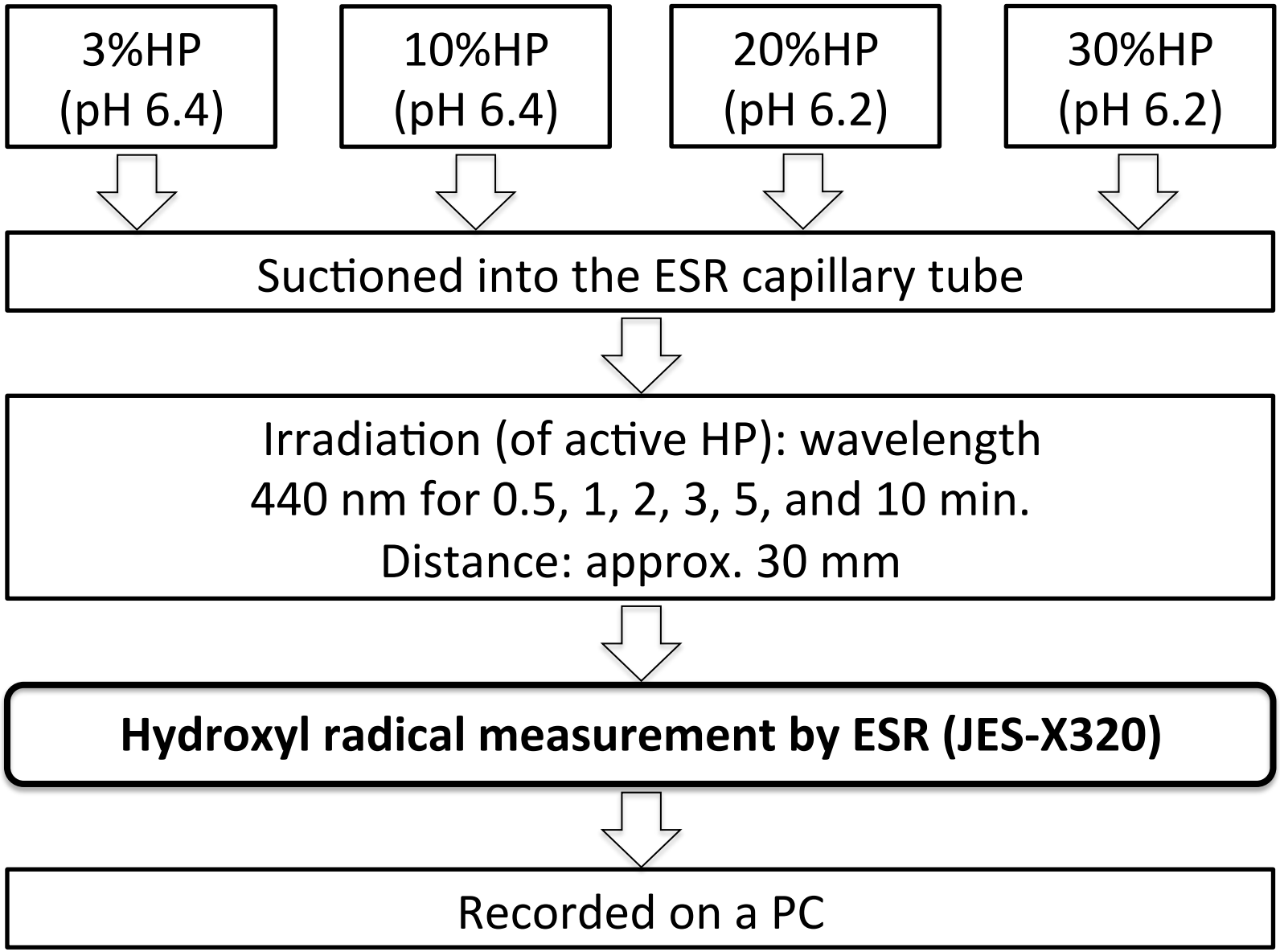

Fig. 4. Procedures for hydroxyl radical measurement by ESR

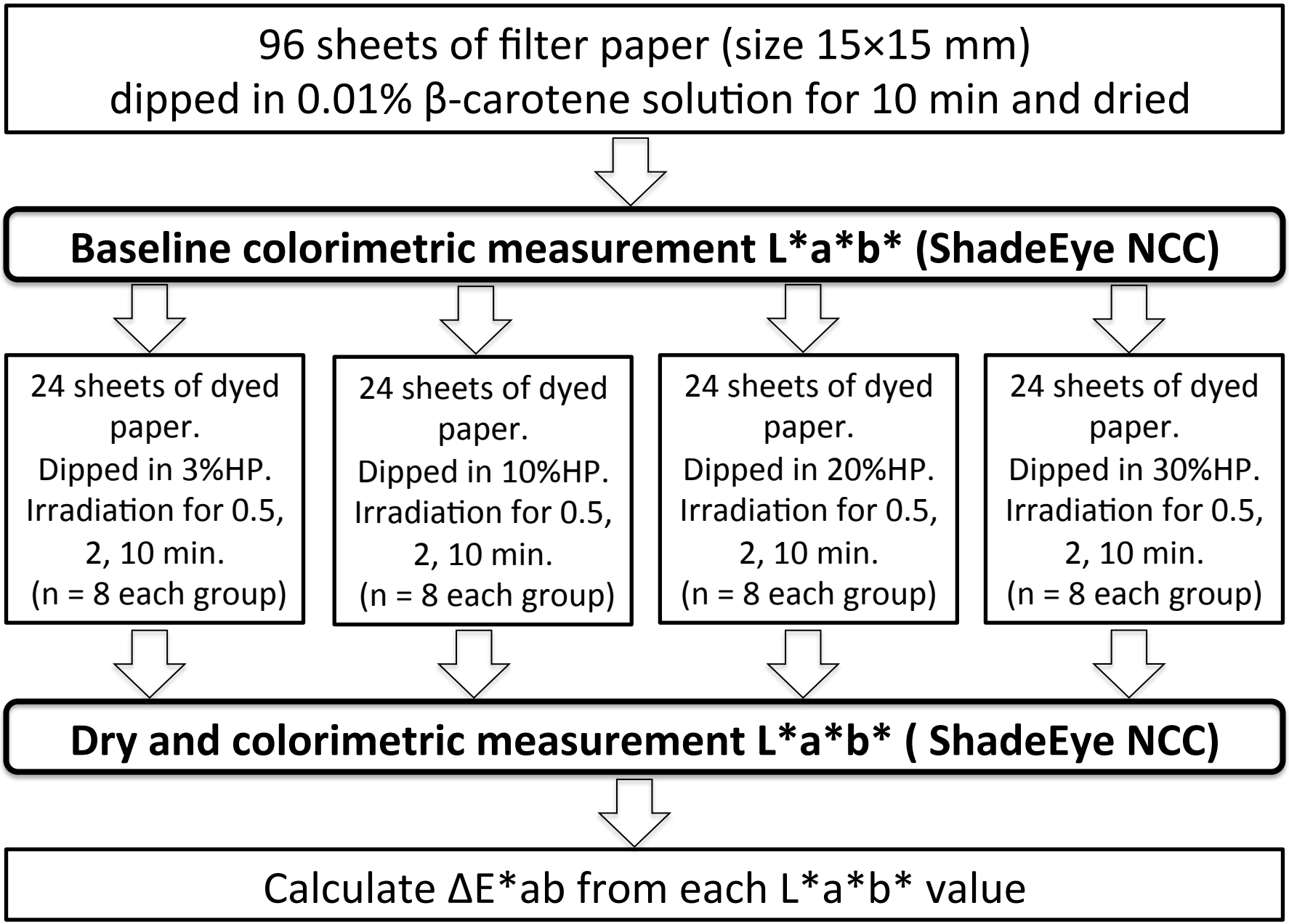

Fig. 5. ΔE*ab value obtained from dyed and bleached paper

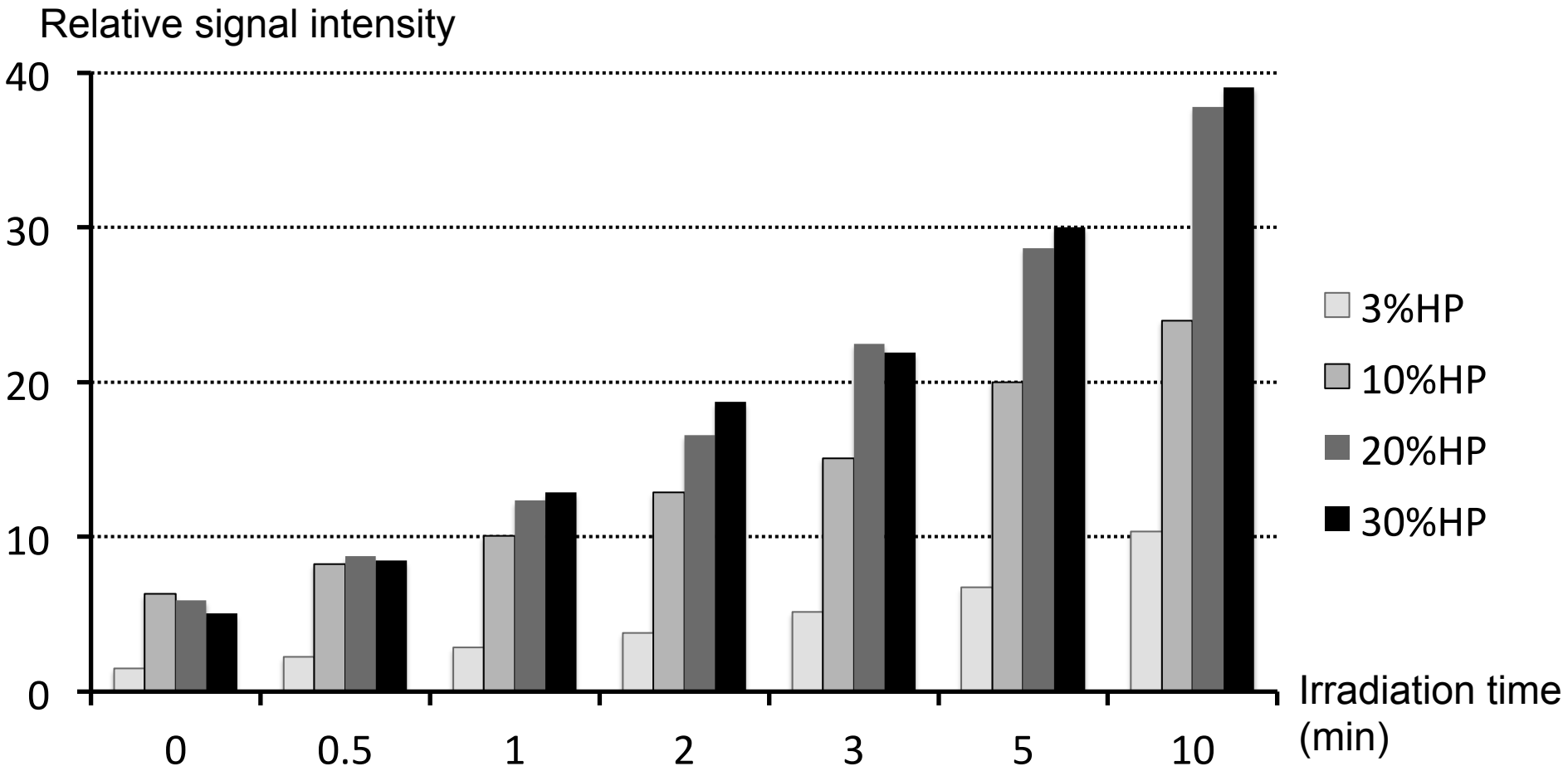

Fig. 7. Hydroxyl radical generation at each irradiation time for each concentration group

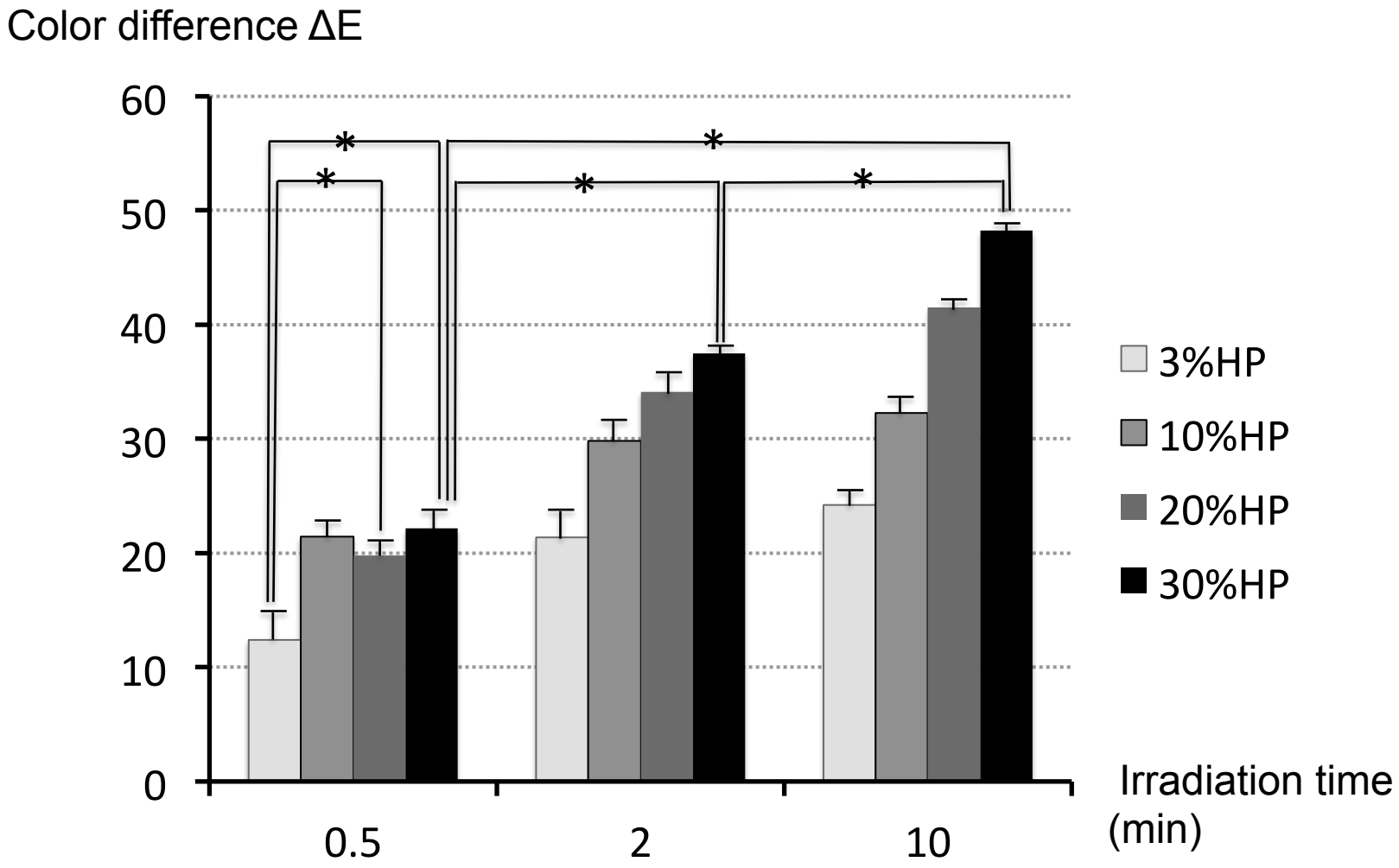

Fig. 8. Color difference ΔE*ab value at irradiation time and each HP concentration group (n = 8)

＊ Significant difference ($p<0.05$, Scheffe)

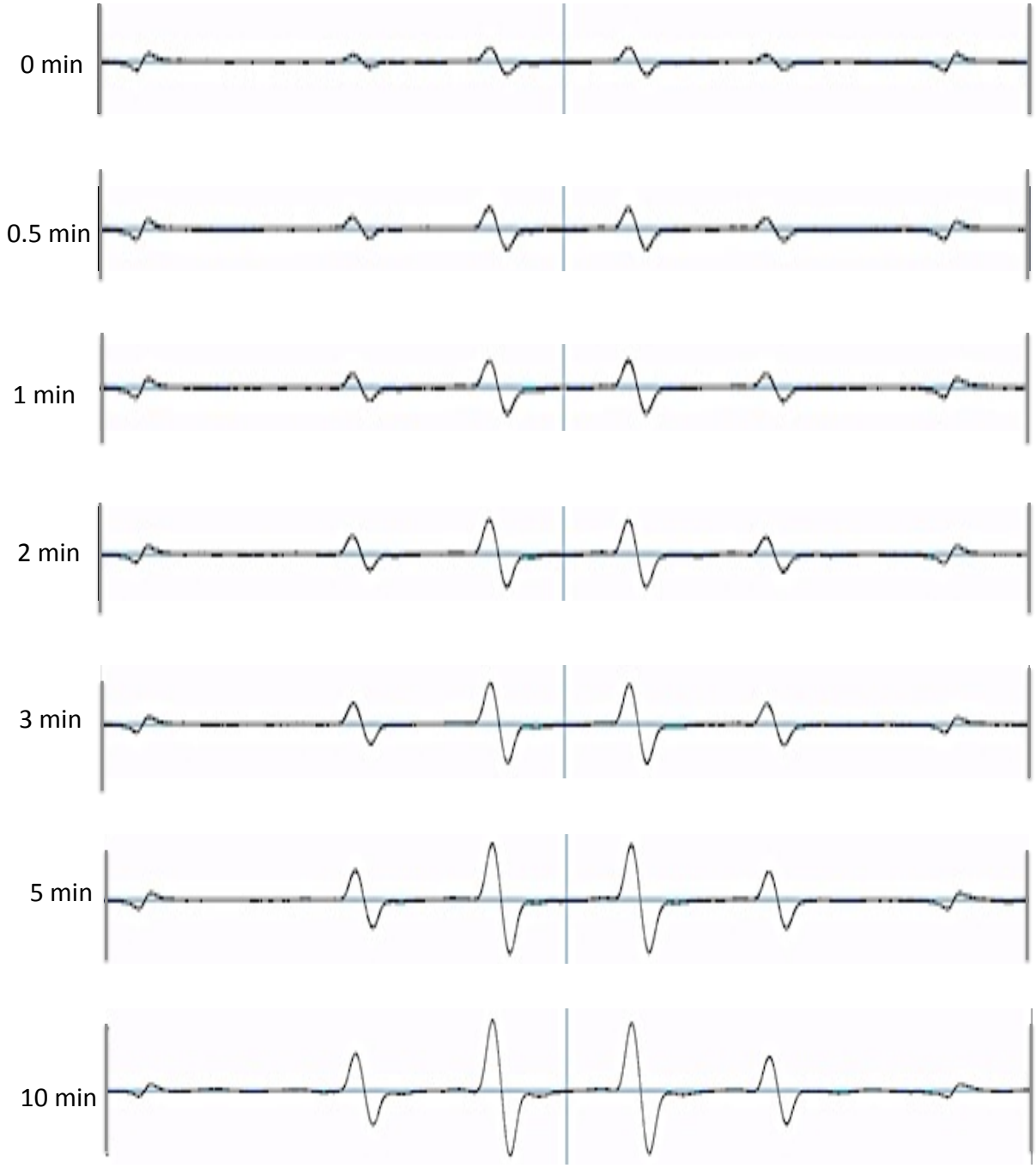

Fig. 6. Waveforms of hydroxyl radicals from 3%HP at each irradiation time