Detection of Hydroxyl and Perhydroxyl Radical Generation from Bleaching Agents with Nuclear Magnetic Resonance Spectroscopy

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Objective: *Children/adolescent's orodental structures are different in anatomy and physiology from that of adults, therefore require special attention for bleaching with oxidative materials. Hydroxyl radical (*$OH^{\cdot}$*) generation from bleaching agents has been considered directly related to both its clinical efficacy and hazardous effect on orodental structures. Nonetheless bleaching agents, indirectly releasing hydrogen peroxide (*H_2O_2*), are considered safer yet clinically efficient. Apart from* $OH^{\cdot}$*, perhydroxyl radicals (*$HO_2^{\cdot}$*) too, were detected in bleaching chemistry but not yet in dentistry. Therefore, the study aims to detect the* $OH^{\cdot}$ *and* $HO_2^{\cdot}$ *from bleaching agents with their relative integral value (RIV) using* ^{31}P *nuclear magnetic resonance (*$^{31}PNMR$*) spectroscope.* ***Study design:*** *Radicals were generated with UV light in 30%* H_2O_2*, 35% carbamide peroxide (CP), sodium perborate tetrahydrate (SPT) and; neutral and alkaline 30%* H_2O_2*. Radicals were spin-trapped with DIPPMPO in NMR tubes for each test agents as a function of time (0, 1, 2, 3min) at their original pH. Peaks were detected for* $OH^{\cdot}$ *and* $HO_2^{\cdot}$ *on NMR spectrograph. RIV were read and compared for individual radicals detected.* ***Results:*** *Only* $OH^{\cdot}$ *were detected from acidic and neutral bleaching agent (30% acidic and neutral* H_2O_2*, 35%CP); both* $HO_2^{\cdot}$ *and* $OH^{\cdot}$ *from 30% alkaline* H_2O_2*; while only* $HO_2^{\cdot}$ *from more alkaline SPT. RIV for* $OH^{\cdot}$ *was maximum at 1min irradiation of acidic 30%*H_2O_2 *and 35%CP and minimum at 1min irradiation of neutral 30%*H_2O_2*. RIV for* $HO_2^{\cdot}$ *was maximum at 0min irradiation of alkaline 30%*H_2O_2 *and minimum at 2min irradiation of SPT.* ***Conclusion:*** *The bleaching agents having pH- neutral and acidic were always associated with* $OH^{\cdot}$*; weak alkaline with both* $OH^{\cdot}$ *and* $HO_2^{\cdot}$*; and strong alkaline with* $HO_2^{\cdot}$ *only. It is recommended to check the pH of the bleaching agents and if found acidic, should be made alkaline to minimize oxidative damage to enamel itself and then to pulp/periodontal tissues.*

Keywords: *Bleaching, Hydrogen peroxide, Hydroxyl radicals, Perhydroxyl radicals, Reactive oxygen species*

Abbreviations*:* **H_2O_2***: hydrogen peroxide;* **CP***: carbamide peroxide;* **SP:** *sodium perborate;* **SPT***: sodium perborate tetrahydrate;* **ROS***: reactive oxygen species;* **$^{31}PNMR$***:* ^{31}P *nuclear magnetic resonance spectroscope;* **RIV***: relative integral value;* **$OH^{\cdot}$***: hydroxyl radical;* **$HO_2^{\cdot}$***: perhydroxyl radical;* **$O_2^{\cdot}$***: super oxide radical;* **DIPPMPO***: 5-(Diisopropoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide;* **DEPMPO***: 5-diethoxyphosphoryl-5-methyl-1-pyrroline-n-oxide;* **DMPO***: 5,5-dimethyl-1-pyrroline-N-oxide;* **D_2O***: heavy water;* **EDTA***: ethylene diamine tetra acetic acid.*

INTRODUCTION

Increased interest in bleaching in pediatric dentistry[1-3] has drawn pedodontists' attention towards the chemistry of commercially available oxidative bleaching agents to be safely applied in children. As described by Sharma *et al.*[1], primary and permanent teeth including their supporting structures are quite different physiologically and anatomically. Nonetheless what is subtoxic or physiologic in permanent dentition may prove toxic in deciduous dentition.[1] In view of supporting studies and case reports, a joint statement was published by many societies in Britain[4] recommending minimal invasive initial procedures for esthetic dentistry. Recently American Academy of Pediatric Dentistry[5] recommended minimal effective concentrations of H_2O_2 or CP for extra-coronal and SP with water for intra-coronal bleaching procedures. Therefore bleaching material need to be chosen smartly especially in children.

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Many studies[1-9] has investigated the hazardous/therapeutic effects of various bleaching material, but the basic mechanism that which chemical property of a particular material makes it hazardous or biofriendly still need to be probed out. Rotstein *et al.*[6] demonstrated radicular penetration H_2O_2 during intra canal bleaching with H_2O_2. Later Sharma *et al.*[7] found significantly lesser radicular penetration of H_2O_2 with alkaline sodium SPT compared to H_2O_2 alone or mixed with SPT. Dahlstorm *et al.*[8] for the first time detected generation of $OH^{\cdot}$ from H_2O_2 and suggested its direct correlation with both hazardous and bleaching efficacy. Farmer *et al.*[9] detected reduced generation of $OH^{\cdot}$ when a reducing agent i.e. thiourea was applied prior to intra-coronal H_2O_2. Similarly for extra-coronal bleaching, CP and SP have been proved better compared to pure H_2O_2 products.[3, 10-12] CP chemically contains H_2O_2 and urea, the contents very similar to the thiourea+H_2O_2 used by Farmer *et al.*[9] Urea in both conditions get decomposed to carbon dioxide and ammonia.[13] It was thought that high pH of ammonia facilitates the bleaching process as less activation energy is required for free radical formation from parent H_2O_2 molecule, resulting in improved yield compared to acidic medium.[14] Nonetheless pH was not mentioned. It would be interesting to investigate whether ammonia in CP actually results in alkaline medium as it is a weak alkali and carbon dioxide is a weak acid. In presence of both type of ions, resultant alkalinity or acidity will be dependent on relative concentration of particular ion and even then it will be weak acidic or alkaline.

Collective finding of various studies are inclining toward the fact that alkalinity/acidity of bleaching agent may be anyhow related to the biofriendly/hazardous nature of it. Apart from $OH^{\cdot}$, many other oxidative radicals too are formed in the chemistry of H_2O_2 bleaching. These radicals along with parent molecule i.e. H_2O_2 are collectively known as reactive oxygen species (ROS). The possibility of $HO_2^{\cdot}$ among ROS has been discussed previously in dental literature.[13] Different ROS individually have different reactivity and life span, directly related to their oxidative potential. Oxidation potential of $OH^{\cdot}$ is more than $HO_2^{\cdot}$ or H_2O_2 (1.78v, 2.80v and 1.70v for H2O2, $OH^{\cdot}$ and $HO_2^{\cdot}$ respectively).[15]

Sharma *et al.*[7] assumed that perhaps generation of different oxidative radicals were dependent on pH of bleaching agent i.e. more $OH^{\cdot}$ in acidic and more $HO_2^{\cdot}$ in alkaline medium. Smith and Argyropoulos[16] found greater concentration of $HO_2^{\cdot}$ than $OH^{\cdot}$ in neutral and alkaline H_2O_2 solution compared to acidic H_2O_2. They concluded $HO_2^{\cdot}$ radical should be the key species, participating in H_2O_2 chemistry, most likely playing more significant role in alkaline peroxide chemistry than originally thought.

Nonetheless till now among many ROS, only $OH^{\cdot}$ generation has been detected[8-9], leaving a lacuna in the knowledge of bleaching chemistry in dentistry. This knowledge may prove important to solve the dilemma of role of different ROS during bleaching from various commercial bleaching materials and allowing a pedodontist/ endodontist to modify the bleaching chemistry to make it more physiologic for orodental tissues.

Therefore, this study intends to detect $OH^{\cdot}$ and $HO_2^{\cdot}$ during bleaching reaction from various commonly used oxidative bleaching agents- 30% H_2O_2, 35%CP and SPT.

MATERIALS AND METHOD

Prior permission for the study was obtained from institutional ethical research committee. The whole experiment was performed in the chemistry lab of Indian Institute of Technology (IIT), Indore, India. The spin trapping reagent DIPPMPO was purchased from Enzo Life Sciences, Inc., New York, USA and stored at -20°C in deep freeze refrigerator. CP (100 %) in solid form was provided by Omatek Laboratories Pvt. Ltd, Indore, India. Other bleaching agents, 30%w/vH_2O_2 (Thermo fisher scientific India Pvt. Ltd, Mumbai, India) and SPT Pure (LOBA Chemie PVT. Ltd, Mumbai, India) were obtained from our own department.

Heavy water (D_2O, Sigma-Aldrich, St. Louis, Missouri, USA) (3.2 ml) was taken in a vial and then purged of air with argon gas for 30 min before using it as a solvent for test agents.

To make 35% CP solution, .35 mg of 100% CP in solid form was dissolved in 1ml of distilled water. This was then standardized and used for sample preparation.

Standardization of amount of bleaching agents

With the help of micropipette (with digital readings) 30%H_2O_2 and prepared 35%CP solution were dropped on cotton piece on labial surface of the extracted central incisor till it was saturated with solution. The three drops of 20µL (60µL) amount of each solution was thus standardized for the experiment. SPT (20mg, in powder form) was mixed with distilled water to make paste like consistency (2g:1ml)[5, 6] and 20mg of this paste was standardized, for found to be sufficient to cover the labial surface of central incisor.

Trapping of free radicals as a function of time from experimental bleaching agents at their original pH

Standardized amount of each sample was taken and pH was measured using electronic pH meter (**Thermo Fisher Scientific,** Navi Mumbai, India) (table-1-3). In each NMR tube 200µL D_2O, 60µL of 30% H2O2/35% carbamide peroxide or 20mg of SPT paste, 65mM DIPPMPO, 10µL of 0.01M EDTA (Titriplex III GR, Merck Specialties Pvt. Ltd, Mumbai, India)) and 50**µL buffer solution were added together for NMR analysis.** The quantification and strength of all reagents were according to Smith and Argyropoulos.[16]

Testing solutions of bleaching agents were added with EDTA in order to remove trace metal ions to stop fenton reaction which tends to shift the bleaching chemistry towards abundant production of $OH^{\cdot}$.[7, 9, 16, 17] Furthermore, to exclude the possibility of superoxide radical ($HO_2^{\cdot}/O_2^{\cdot}$) the bleaching agent solutions were purged of air with argon, for these radicals seem to exist in equilibrium with aqueous H_2O_2 when in contact with either air or oxygen.[16]

Generation of radicals with the UV irradiation and NMR analysis was done according to Smith and Argyropoulos.[17] All the NMR tubes for each samples were irradiated with UV light in a UV light chamber (short wavelength 360 nm, Jyoti Scientific Laboratories, Gwalior, India)) for 0, 1, 2 and 3 min respectively for 30% H_2O_2, 35% CP and SPT, while for 0 and 1min for neutral and alkaline 30% H_2O_2. All these samples were analysed using ^{31}P NMR spectroscopy (Model *AVANCE III 400 Ascend Bruker BioSpin International AG*, Switzerland) operating at 400MHz. Scanning for each sample was completed in 1min 31 sec for 32 scans at pulse of 25µsec. Orthophosphoric acid (85%) was used as external standard with chemical shift set at zero. Chemical shifts of radicals to be detected were reported relative to external standard. The area under the peak at particular chemical shift for detected radicals represented relative integral value (RIV, suggestive of relative molar concentration) was also recorded.

Trapping of free radicals as a function of pH and time from neutral and alkaline H_2O_2 (30% w/v)

In order to cross check whether the generation of different types of radicals is actually dependent on pH of bleaching agent, main molecule i.e. H_2O_2 was tested in the same manner as described above after adjusting its pH to neutral and alkaline with buffering capsules.

Buffering capsules (Merck Specialties Pvt. Ltd, Mumbai, India) of neutral or alkaline pH were added to 100ml of distilled water and pH was confirmed on pH meter. Neutral (pH 6.94) or alkaline buffer solution (pH 8.89) 50µL at a time was added to two NMR tube. The tubes were irradiated with UV light in the same manner as above for 0 and 1 min and then analyzed using ^{31}PNMR.

RESULTS

DIPPMPO-radical adducts were detected in NMR spectra. Sample of original NMR spectrum obtained from a particular bleaching agent at a time is shown in figure-1f. The NMR spectra for each bleaching agent irradiated for different time was compounded with the help of inbuilt software of computer attached with ^{31}PNMR and shown in figure-1a-e. Both $OH^{\cdot}$ and $HO_2^{\cdot}$ radicals were detected using ^{31}PNMR. Detection of $OH^{\cdot}$ and $HO_2^{\cdot}$ radicals in acidic, neutral and alkaline 30% H_2O_2, 35%CP, SPT is summarized in table-1. Chemical shifts and RIVs for detected $OH^{\cdot}$ and $HO_2^{\cdot}$ are summarised in table 1&2, while comparison among them in figure-2.

Acidic 30%*H_2O_2*: No $HO_2^{\cdot}$ adducts were detected (figure-1a, table-1). The RIV suggested that more amount of $OH^{\cdot}$ adducts were formed at 1min then gradually decreased till 3-minute irradiation time (figure-1a&2, table-2).

35% CP: No $HO_2^{\cdot}$ adducts were detected. The RIV suggested that initially $OH^{\cdot}$ adducts, making solutions were increased up to 2min then decreased at 3min irradiation time. (figure-1b&2, table-2)

SPT: $HO_2^{\cdot}$ adducts were detected but $OH^{\cdot}$ adducts were not detected. The RIV for $HO_2^{\cdot}$ adducts suggested that it was high at 0min, gradually reduced up to 2min then increased at 3 min to maximum concentration approximately same as initial concentration. (figure-1c&2, table-2)

Neutral 30% H2O2: Only $OH^{\cdot}$ were detected. The peaks of some unidentified radical adduct too were detected at 1.64 ppm on NMR spectroscopy graph for both irradiation times at 0 and 1 min respectively. RIV for $OH^{\cdot}$ suggested that amount of OH adduct was reduced at 1 min irradiation time. RIVs of hydroxyl and unidentified radical adducts suggests that relatively more amount of unidentified radical adduct was present in neutral H_2O_2 bleaching agent. (figure-1d&2, table-2)

Alkaline 30% H2O2: Both $OH^{\cdot}$ and $HO_2^{\cdot}$ were detected (figure-1d, table-1). The RIV for $OH^{\cdot}$ was (table-2, figure-2). The RIV for $HO_2^{\cdot}$ suggested that it was reduced at 1min. RIVs of $OH^{\cdot}$ and $HO_2^{\cdot}$ were suggestive of that relatively more amount of $HO_2^{\cdot}$ compared to $OH^{\cdot}$ were formed in alkaline H_2O_2 bleaching agent and both radicals were reduced at 1min. (figure-1e&2, table-2)

Table-3 shows that acidic pH of bleaching agents was always associated with $OH^{\cdot}$ while the alkaline one with $HO_2^{\cdot}$. When solution was strongly alkaline as in SPT, only $HO_2^{\cdot}$ were detected.

Figure 1: NMR spectra of tested bleaching agents.

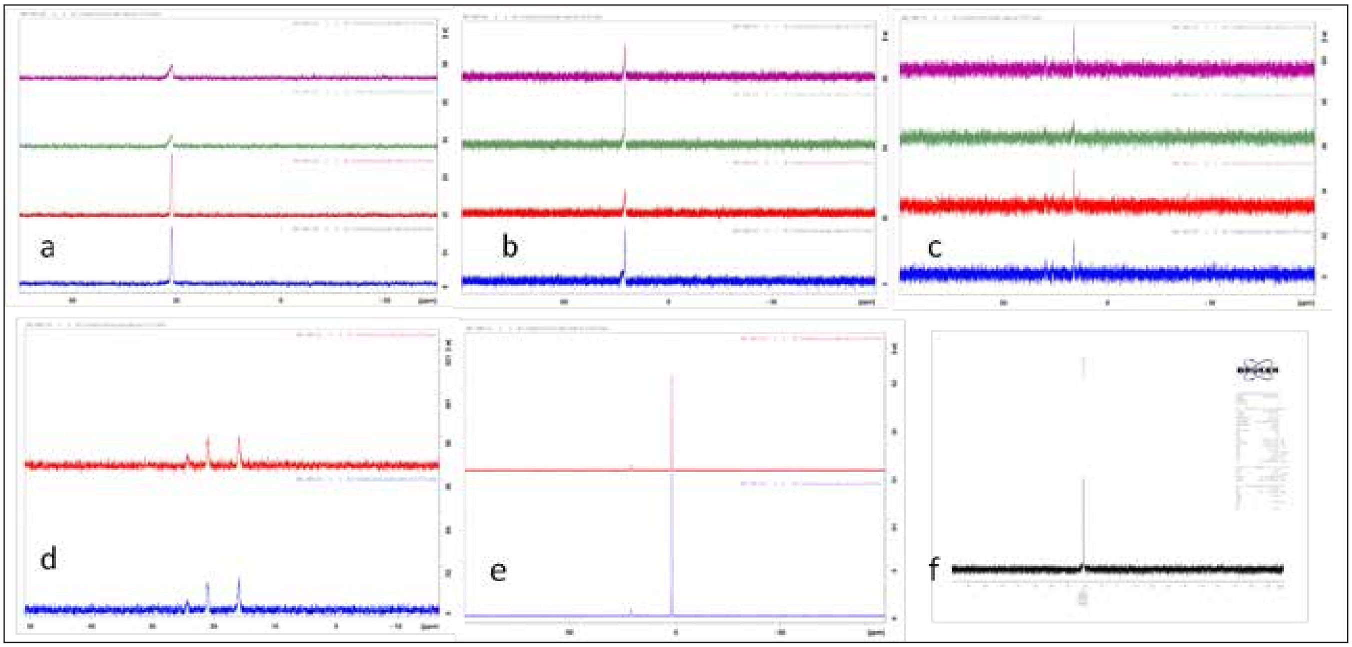

Table-1: Chemical shifts of radicals generated from various bleaching agents detected at NMR spectra

Bleaching Agents	pH	Irradiation time	DIPPMPO / OH$^{\cdot}$		DIPPMPO / OOH$^{\cdot}$		Chemical shift of DIPPMPO
			Chemical shift	Mean	Chemical shift	Mean	
30% Hydrogen Peroxide	Acidic (2.45)	0 sec	21	20.99± 0.02	-	-	17.9
		1 min	21				
		2 min	20.96				
		3 min	20.99				
	Neutral (6.94)	0 sec	20.94	20.93± 0.02	-	-	17.9
		1 min	20.91				
	Basic (8.89)	0 sec	20.95	20.96± 0.01	15.84	15.86 ±0.03	17.9
		1 min	20.97		15.88		
35% Carbamide Peroxide	Acidic (5.31)	0 sec	21.06	21.06± 0.02	-	-	17.9
		1 min	21.05		-		
		2 min	21.05		-		
		3 min	21.09		-		
Sodium Perborate Tetrahydrate	Alkaline (9.90)	0 sec	-	-	16.04	16.10 ±0.08	17.9
		1 min	-		16.08		
		2 min	-		16.22		
		3 min	-		16.07		

Bleaching Agents	pH	Irradiation time	DIPPMPO / OH$^{\cdot}$		DIPPMPO / OOH$^{\cdot}$		Chemical shift of DIPPMPO
			Chemical shift	Mean	Chemical shift	Mean	
30% Hydrogen Peroxide	Acidic (2.45)	0 sec	21	20.99± 0.02	-	-	17.9
		1 min	21				
		2 min	20.96				
		3 min	20.99				
	Neutral (6.94)	0 sec	20.94	20.93± 0.02	-	-	17.9
		1 min	20.91				
	Alkaline (8.89)	0 sec	20.95	20.96± 0.01	15.84	15.86 ±0.03	17.9
		1 min	20.97		15.88		
35% Carbamide Peroxide	Acidic (5.31)	0 sec	21.06	21.06± 0.02	-	-	17.9
		1 min	21.05		-		
		2 min	21.05		-		
		3 min	21.09		-		
Sodium Perborate Tetrahydrate	Alkaline (9.90)	0 sec	-	-	16.04	16.10 ±0.08	17.9
		1 min	-		16.08		
		2 min	-		16.22		
		3 min	-		16.07		

Table 2: Relative integral values of radicals generated from various bleaching agents, detected at NMR spectra

Bleaching agents	pH	Irradiation time	DIPPMPO / OH·	DIPPMPO / OOH·
		Relative integral value	Relative integral value	
30% Hydrogen Peroxide Neutral (6.94) Basic (8.89)	Acidic	0 sec	93.94	
	(2.45) 1 min	100		-
	2 min	93.60		
	3 min	84.43		
		0 sec	29.14	-
	1 min	26.82		
		0 sec	33.23	40.23
	1 min	31.07	37.82	
35% Carbamide Peroxide	Acidic	0 sec	79.05	-
	(5.31) 1 min	87.79	-	
	2 min	100	-	
	3 min	74.11	-	
Sodium Perborate Tetrahydrate	Basic	0 sec	-	39.30
	(9.90) 1 min	-	27	
	2 min	-	10.30	
	3 min	-	40.08	

Bleaching agents	pH		Irradiation time Relative integral value	DIPPMPO / OH·	DIPPMPO / OOH·
				Relative integral value	
30% Hydrogen Peroxide	Acidic (2.45)		0 sec	93.94	
		1 min	100		-
		2 min	93.60		
		3 min	84.43		
	Neutral (6.94)		0 sec	29.14	-
		1 min	26.82		
	Basic (8.89)		0 sec	33.23	40.23
		1 min	31.07	37.82	
35% Carbamide Peroxide	Acidic (5.31)		0 sec	79.05	-
		1 min	87.79	-	
		2 min	100	-	
		3 min	74.11	-	
Sodium Perborate Tetrahydrate	Basic (9.90)		0 sec	-	39.30
		1 min	-	27	
		2 min	-	10.30	
		3 min	-	40.08	

Table 3: Detected radicals as a function of pH of various bleaching agents

Bleaching agents	pH	Detected radicals	
30% Hydrogen peroxide (acidic)	2.45	OH·	
30% Hydrogen peroxide (alkaline)	8.89	OH·	OOH·
30% Hydrogen peroxide (neutral)	6.94	OH·	
35% carbamide peroxide (acidic)	5.31	OH·	
Sodium perborate tetrahydrate (alkaline)	9.90		OOH·

Figure 2: Pictorial presentation of Relative integral values of tested bleaching agents.

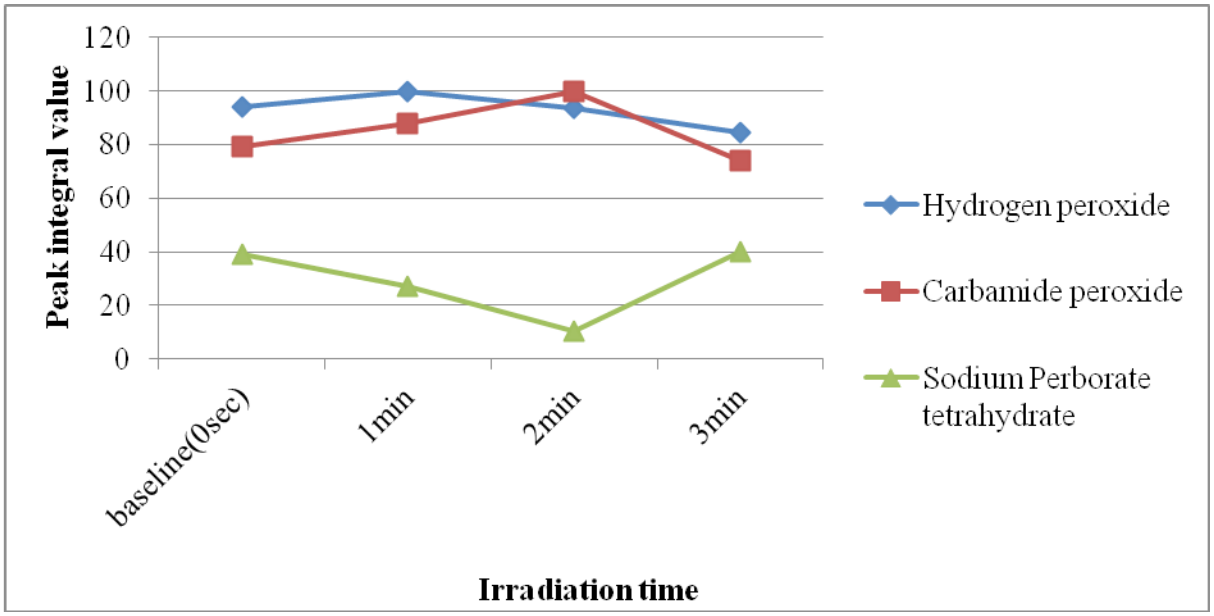

Figure 3: a. Mechanism of spin trapping of hydroxyl and perhydroxyl radicals using DIPPMPO. b. Conversion of hydroxyl to perhydroxyl radicals in alkaline medium. c. Base catalyzed decomposition of peroxide to form perhydroxyl and superoxide radicals.

a.

$$\mathrm{HOOH} \xrightarrow{\text{UV light}} 2\mathrm{OH}^{\cdot} \quad (1)$$

$$\mathrm{DIPPMPO} + \mathrm{OH.} \longrightarrow \mathrm{DIPPMPO\text{-}OH.}\ \text{(radical adduct)} \quad (2)$$

$$\mathrm{OH.} + \mathrm{H2O2} \longrightarrow \mathrm{H2O} + \mathrm{HOO.} \quad (3)$$

$$\mathrm{DIPPMPO} + \mathrm{HOO.} \longrightarrow \mathrm{DIPPMPO\text{-}OOH.}\ \text{(radical adduct)} \quad (4)$$

b.

$$\mathrm{H2O2} + \mathrm{OH}^{-} \longleftrightarrow \mathrm{HOO}^{-} + \mathrm{H_2O} \quad (1)$$

$$\mathrm{H2O2} + \mathrm{HOO}^{-} \longleftrightarrow \mathrm{OH}^{\cdot} + \mathrm{O_2}^{\cdot -} + \mathrm{H_2O} \quad (2)$$

$$\mathrm{HOO}^{-} + \mathrm{OH}^{\cdot} \longleftrightarrow \mathrm{O2}^{\cdot -} + \mathrm{H_2O} + \mathrm{H_2O} \quad (3)$$

c.

$$\mathrm{HOO}^{-} + \mathrm{OH}^{\cdot} \longleftrightarrow \mathrm{OH}^{-} + \mathrm{HOO}^{\cdot} \longleftrightarrow \mathrm{O_2}^{\cdot -} + \mathrm{H_2O}$$

DISCUSSION

Since two decades' detection of radicals has become the key point for researchers to make dental bleaching more bio-friendly yet efficient enough clinically. Nonetheless detection of generated different radicals (not ions) is very difficult owing to highly unstable in nature. Spin traps are highly reactive towards free radicals, therefore have been used in the detection of oxygen centered radicals (ROS) mainly $OH^{\cdot}$ and $HO_2^{\cdot}$. Most studies previously used electron paramagnetic resonance (EPR) spectroscopy to detect the radicals. For EPR detection, the spin trapping entails the reaction of nitrones or nitroso spin traps (paramagnetic species) with unstable radical system to form a more stable free radical (radical adduct). Nonetheless, these radical adducts degrade with time, becoming diamagnetic and therefore, EPR undetectable.[19] Argyropoulos *et al*[19] stated that phosphorous containing spin traps not only give rise to radical adducts that have longer half life compared to other spin traps, but also the presence of phosphorous with in radical adducts allowed for the use of ^{31}PNMR spectroscopy to investigate the detailed chemistry of radical reactions in complex H_2O_2 reactions during bleaching. The radical adducts, even after conversion into their final diamagnetic forms, are stable over a long period of time and NMR detectable. Moreover, DIPPMPO, used in our study, has higher partition coefficient (K_p= 2.1) compared to DEPMPO (0.06) and DMPO (0.1).[19] This higher partition coefficient allows DIPPMPO to react readily with radicals having ultra short lives and thus any bias was reduced in this study.

The pH of 30% H_2O_2 was highly acidic (table 1-3). Only $OH^{\cdot}$ were detected from parent bleaching agent at all the times i.e. 0, 1, 2, 3 minutes (table-1, figure-1a). Results of our experiment are in agreement with Smith and Argyropoulos [16] and Sharma *et al.*[7], that there is abundance of $OH^{\cdot}$ radicals when pH of bleaching agents is acidic. Owing to short half life and high reactivy penetrated H_2O_2[6, 7], should generate $OH^{\cdot}$ radicals locally. Detection of $OH^{\cdot}$ in extra-radicular area by Dahlstrom *et al.*[8] and Farmer *et al.*[9], confirm this assumption. The locally generated $OH^{\cdot}$ (oxidative potential, $OH^{\cdot}$=2.80v > H_2O_2=1.78v)[15] react rapidly and nonselectively with nearly all electron rich compounds (enamel as in extra coronal bleaching; or the dentine as in intra coronal bleaching). Unaltered and unreacted H_2O_2, pass through enamel (extra coronal bleaching)[20] or dentine (intra-coronal bleaching)[6-9] to reach to pulpal or to extra-radicular region respectively. In addition to it, the acidity of a bleaching agent makes it more corrosive to inorganic dental structure, which in turn allows more penetration of H_2O_2 molecule.

All these findings suggest that bleaching mechanism of acidic bleaching agents is two edged swords for dental tissues. Generated $OH^{\cdot}$ is cytotoxic[17] and give rise to inflammation in penetrated tissues and in turn is a main cause of so called bleaching related side effects i.e. tooth sensitivity[21-23], gingival irritation[24-25], throat irritation[26], and nausea[26] etc. Even at baseline (0min) presence of $OH^{\cdot}$ were detected from acidic H_2O_2. After irradiation for 1min RIV for $OH^{\cdot}$ was increased. At 2min it almost decreased to original value and at 3min it continued to decrease (table-2, figure-2). Tanaka *et al.*[18] in their ESR study using DMPO as spin trap, stated that under photochemical reactions, the formation of $OH^{\cdot}$ from H_2O_2 increased upto 3min and that the amount of $OH^{\cdot}$ generated changed according to the concentration of H_2O_2 and irradiation time. Contrary to their findings, the RIV for $OH^{\cdot}$ was decreased with time in our experiment. Cai H *et al.*[27] explained that DMPO can make radical adducts with both $OH^{\cdot}$ and $HO_2^{\cdot}$. Therefore, perhaps, the results of Tanaka *et al*[18] were for both $OH^{\cdot}$ as well as $HO_2^{\cdot}$ but as these radicals are difficult to detect as different peaks in ESR, they explained their results on the basis of $OH^{\cdot}$ only and thus in turn differences to our results. This explanation also resolved their query that inspite of adding $OH^{\cdot}$ scavenger in solution, only slight reduction in $OH^{\cdot}$ concentration was found as ESR kept detecting signals of $HO_2^{\cdot}$-DMPO adduct.

CP is a commonly used extra and intra coronal tooth bleaching agent. In our study the pH of 35% carbamide peroxide solution was acidic (table-3). Only $OH^{\cdot}$ were detected from CP at all the times i.e. 0, 1, 2, 3 minutes. Similar to 30% H_2O_2, detection of $HO_2^{\cdot}$ in CP was not found (table-1, figure-1b). RIVs showed that substantial concentration of $OH^{\cdot}$ was there at baseline but was less than 30% H_2O_2. Maximum concentration of $OH^{\cdot}$ was detected at 2min, then after 3min it reached approximately to the value of baseline [table-2, figure-2]. These findings can be correlated both to the pH of parent agent and mode of supply of H_2O_2 molecule. The generation of active H_2O_2 molecule from carbamide peroxide is dependent on its decomposition and therefore are in lesser concentration compared to pure H_2O_2 of same concentration.[28] This also explains why there was a little lag in $OH^{\cdot}$ concentration to reach up to maximum.

SPT is another commonly used intra-coronal bleaching agent but had been tried for extra coronal bleaching as well.[10-11] In our study, the pH of SPT was alkaline (table 1-3). When it comes in contact with moisture it decomposes to form sodium metaborate and H_2O_2[29] which in turn generates ROS to bleach discolored organic tissues. Contrary to the 30%H_2O_2 and 35%CP, SPT gave peaks other than identified peaks of $OH^{\cdot}$ and no peaks for this radical was detected (table-1, figure-1c). Smith and Argyropoulos[16, 17] detected this peak as of $HO_2^{\cdot}$ in ^{31}PNMR spectra. It can be said that acidic bleaching agents generate $OH^{\cdot}$ while alkaline one the $HO_2^{\cdot}$ and also that the bleaching action in these agents is mainly dependent on $HO_2^{\cdot}$ instead of $OH^{\cdot}$.

Ernst CP *et al* [11] compared the effect of SP mixed with 30% H_2O_2, 10% CP gel, 30% H_2O_2 gel as extra-coronal bleaching agent on topography of enamel and found no significant difference. Their results were very contradictory to other authors, that inspite of low pH (i.e. 2) of 30% H_2O_2 no surface alterations were found but anyhow recommended bleaching gels having pH 6 (Hi Lite and Opalescent). SP (pH 8) in their experiment did not cause any surface alteration of enamel. Except for acidic H_2O_2 all other results of their experiment can be related to our findings that alkaline bleaching agent is safe (in molecular terms- lower or nil concentration of $OH^{\cdot}$ compared to $HO_2^{\cdot}$). Lewinstein *et al.*[10] also compared the effect of extra coronal bleaching agents: 30% H_2O_2 alone or mixed with SP on microhardness of enamel and dentine. They suggested to limit the use of high concentration H_2O_2 and recommended SP as being less damaging to enamel. Many case reports[1] also considered SPT as biofriendly. The pH of SPT combined with water in a powder to liquid ratio of 2g:1 ml was always in an alkaline range from 8.99 to 10.66 as measured by Weiger *et al.*[29] Because generation of H_2O_2 in SPT is dependent on decomposition of its molecule, its pH was found slowly increasing.[28]

RIVs of $HO_2^{\cdot}$ generated by SPT demonstrated that at baseline it was 39.30. It started to drop upto at 1 and 2min. respectively but again raised at 3 min up to baseline [table-2, figure-2]. Some of the

SPT remained in dissociated form in presence of moisture giving rise to more concentration of $HO_2^{\cdot}$ at baseline. The drop and again rise in RIV of $HO_2^{\cdot}$ may be because that H_2O_2 was slowly released from SPT. Our experiment showed that bleaching from SPT is mainly due to $HO_2^{\cdot}$ and that too slowly released.

Detection and relative concentration of reactive oxygen species as a function of pH

According to Abbot and Brown[30], in the absence of metal ions, peroxide decomposition is pH dependent and is a bimolecular reaction that involves the perhydroxyl anion. The increase in superoxide ($O_2^{\cdot}/HO_2^{\cdot}$) radical concentration with pH increase was explained by Argyropoulos DS *et al.*[19] (figure-3b). As one moves to more alkaline pH's more of the H_2O_2 in the system will be in the form of its conjugate base (pKa (H_2O_2) = 11.8), therefore the formation of superoxide radical anion should be more significant. The $OH^{\cdot}$ formed during base catalyzed decomposition of peroxide can subsequently react with perhydroxyl anions to give perhydroxyl and superoxide radicals (figure-3c).[19]

At neutral (pH= 6.94) and acidic (pH= 2.45) H_2O_2, $OH^{\cdot}$ were the only radicals detected. Smith and Argyropoulous[16] found maximum concentration of $OH^{\cdot}$ from H_2O_2 at near neutral pH. Contrary to them we found maximum concentration of these radicals were acidic pH of H_2O_2 (table-2, figure-2). When 30% H_2O_2 was made alkaline (pH= 8.89), hydroxyl and perhydroxyl both radicals were detected (table-1, figure-1). The RIVs of $OH^{\cdot}$ and $HO_2^{\cdot}$ at alkaline pH indicated possible conversion of $OH^{\cdot}$ to $HO_2^{\cdot}$ (figure-3b). RIV of these both radicals seems to be in equilibrium as difference of approximately 7 was maintained between concentration of both radicals at 0 as well as at 1 min. Both radicals were found minimally reduced as a function of time from 0 to 1min. Smith and Argyropoulos[16] found increase in concentration of superoxide with increase in pH which remain in equilibrium with perhydroxyl radical (conjugate base of $O_2^{\cdot}$ in aqueous medium) (figure-3b&c). Our experiment supports their findings.

RIV of $OH^{\cdot}$ at alkaline and neutral pH as compared to acidic H_2O_2 was quite less both at baseline and after 1min (table-2, figure-2). RIV of $OH^{\cdot}$ for alkaline H_2O_2 was little higher as compared to neutral H_2O_2 but very less than acidic H_2O_2. RIV of $HO_2^{\cdot}$ radicals in alkaline H_2O_2 was only little more than it is for $OH^{\cdot}$. This finding was similar to Argyropoulos *et al.*[19] that there started conversion reaction of $OH^{\cdot}$ radical to $HO2^{\cdot}$ at alkaline pH (figure-3, b3&4).

Abouassi *et al* [31] showed changes in the micro morphology and micro hardness of the enamel surface after bleaching with two different concentrations of H_2O_2 (3.6- 10%) and CP (10% and 35%). They found that application of CP and H_2O_2 showed only small quantitative and qualitative differences. Results of our study are in agreement with their findings. Strength of active ingredient in 10 and 35% carbamide peroxide is approximately equal to 3 and 10% H_2O_2. They did not measure the pH of their tested agent. However, as 1) pH of both H_2O_2 and carbamide peroxide were acidic, 2) strength of H_2O_2 i.e. active ingredient too were equal and 3) radical generated too were of same type, results were almost similar in their experiment. Furthermore, a study done by Sun *et al* [32] investigated the effects of acidic and neutral 30% H_2O_2 on enamel in terms of chemical structure, mechanical properties, surface morphology, and tooth color and concluded that neutral 30% H_2O_2 had the same efficiency in tooth bleaching, less deleterious effects on enamel than did the acidic 30% H_2O_2. Results of their study support our findings that acidic and neutral 30% H_2O_2, both generate $OH^{\cdot}$ and concentration of $OH^{\cdot}$ from neutral one were adequate for bleaching but due to difference in pH 30% H_2O_2 had deleterious effect.

Umanah AU *et al* [33] in their study compared the efficacy of 35% carbamide peroxide and SP mixed with water in the bleaching of discolored non vital teeth and found SPT used over a long period as both safe and better bleaching efficacy than 35% carbamide peroxide. Probably $HO_2^{\cdot}$ from SP was responsible for better results. According to Weiger *et al* [29] although tetrahydrate SP presents small amount of active oxygen (10.4%) compared to mono or tryhydrate forms, its setting occurs after 7 days. Probably alkalinity reduces the fast exhaustion of water molecules and allows slow continuous supply of $HO_2^{\cdot}$. Sharma DS *et al.*[1] also recommended SPT + water as biocompatible and user friendly, that it did not cause external cervical root resorption inspite of two-time trauma in bleached permanent tooth. The pH of SPT in our study was alkaline i.e. 9.90, very similar to the previous studies.[11, 29] The biologic compatibility of SPT may be contributed to alkaline nature of it which probably generate $HO_2^{\cdot}$. The results suggested that mainly $HO_2^{\cdot}$ were detected in alkaline bleaching agents i.e. SPT and alkaline 30% H_2O_2 (table-3). Therefore, role of $HO_2^{\cdot}$ should be considered important in alkaline bleaching process. These studies support that slowly released $HO_2^{\cdot}$ to be biofriendly.

The pH of various bleaching agents used in the experiment was measured using electronic pH meter (table-3). It can be clearly seen that acidic pH of bleaching agents was always associated with hydroxyl and the alkaline one with $HO_2^{\cdot}$. When solution is strongly alkaline as in SPT, only $HO_2^{\cdot}$ were detected. This study suggested that in bleaching agents having alkaline pH, $HO_2^{\cdot}$ play a major role in bleaching chemistry.

This is probably the first ^{31}P NMR *in vitro* study, for detection of various radicals generated from various bleaching agents used in dentistry. However further studies need to be carried out to accurately quantify different oxidative radicals generated from bleaching agents.

Our study confirmed that different radicals can be induced by changing its pH and by doing so perhaps deleterious effects of bleaching can be prevented. Litmus paper can be used to check the pH of bleaching agent prior to the procedure. Nonetheless, there is a need of further study to know how to change the pH of bleaching agents from acidic to alkaline.

CONCLUSIONS

On the basis of detected radicals using ^{31}P Nuclear Magnetic Resonance spectroscopy, following conclusions could be drawn:

1. Hydroxyl ($OH^{\cdot}$) as well as perhydroxyl ($HO_2^{\cdot}$) radicals were detected from bleaching agents, generation of which was found to be dependent on pH of bleaching agents.
2. $OH^{\cdot}$ were always detected in bleaching agents with acidic i.e. 30% H_2O_2 and 35% carbamide peroxide.
3. $HO_2^{\cdot}$ were detected in bleaching agents with alkaline pH i.e. alkaline 30% H_2O_2 and SPT.
4. Only $OH^{\cdot}$ were detected, when pH of 30% H_2O_2 was raised to neutral.

5. Relative integral values for $OH^{\cdot}$ for various bleaching agents at different irradiation time was in following order, (1min, acidic H_2O_2) = (2min, CP) > (0min, acidic H_2O_2) > (2min, acidic H_2O_2) > (1min, CP) > (3min, acidic H_2O_2) > (0 sec, CP) > (3min, CP) > (0 sec, alkaline H_2O_2) > (1min, alkaline H_2O_2) > (0min, neutral H_2O_2) > (1min, neutral H_2O_2).
6. Relative integral values for $HO_2^{\cdot}$ for various bleaching agents at different irradiation time was in following order: (0min, alkaline H_2O_2) > (3min, SPT) > (0min, SPT) > (1min, alkaline H_2O_2) > (1min, SPT) > (2min, SP).

On the basis of results of this study and previous case reports, it may be recommended that while using bleaching agents, the pH of the bleaching agents should always be checked and if found to be acidic, the pH should be made alkaline which may help to prevent oxidative damage to enamel itself and then to pulp by altering the bleaching chemistry.

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