ORIGINAL ARTICLE

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An in vitro evaluation of the effect of intracoronal bleaching in teeth discolored by triple-antibiotic paste

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Purpose: The purpose of the study is to evaluate the effect of intracoronal bleaching agents (IBA) on discolored teeth with triple-antibiotic paste (TAP).

Methods: Twenty-six extracted maxillary incisors were discolored for 3 weeks with TAP, containing metronidazole, ciprofloxacin, and doxycycline. Following CIE L*a*b* system, colors were measured at baseline and at 1, 2, and 3 weeks. Specimens, bleached intracoronally, were randomly divided into two groups (n = 13): 35% hydrogen peroxide (HP) and 37% carbamide peroxide (CP). Color changes (ΔE) were based on the spectrophotometric measurements at baseline, 3rd, 6th, and 9th days and analyzed using analysis of variance, Bonferroni, and Student t-tests ($p < 0.05$).

Results: The discoloration increased from baseline to week 3 with significant difference ($p < 0.05$). The highest ΔE value was observed at week 3 (16.54 ± 5.90). In HP, there was no significant difference on the 6th day compared to the 3rd day ($p > 0.05$), but a significant difference on the 9th day ($p < 0.05$). In CP, ΔE was not statistically different among evaluated days. On day 9, HP had a significantly higher ΔE than CP ($p = 0.032$).

Conclusion: TAP caused clinically unacceptable ΔE, increasing over time (ΔE ≥ 3.3). IBA was found effective on discolored teeth. 35% Hydrogen Peroxide was more effective than Carbamide Peroxide on the 9th days.

Keywords: Anti-bacterial agent, bleaching agents, carbamide peroxide, hydrogen peroxide, tooth discoloration.

Introduction

Single-tooth discoloration can be annoying for patients and present an esthetic challenge for the clinicians (1). The restoration of single discolored teeth has been changing from full coverage restorations, to single tooth restorations with veneers, which are more minimally invasive (2,3). However, in such cases, clinical problems such as marginal adaptation, marginal staining, esthetic failure, debonding, and fracture are inevitable (4). The walking bleach technique has been the most widely used since its introduction in 1961 to simplify and shorten the chair time and facilitate patient compliance (5). The success rate will vary depending on the etiology and severity of the discoloration. The post-eruptive intrinsic local discoloration is often caused by pulpal remnants or excessive root

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canal materials left in the pulp chamber (6,7). Removing all tissues and performing proper intracoronal bleaching are generally a successful outcome for that cases (8). The other cause is pulp degeneration caused by trauma, resulting in hemolysis of red blood cells and subsequent release of hemoglobin. Iron and hydrogen sulfide reactions produce an intense pigment *iron sulfide* that severely affects the tooth's color (9). The other intrinsic factor is intracanal medicaments (10). In these cases, one of the problematic discolorations is caused by the use of triple-antibiotic paste (TAP) in Regenerative Endodontic Treatment (RET) (11,12).

RET is a biological treatment aimed at replacing affected or necrotic pulp tissue with a pulp-dentin complex to maintain the durability and functionality of the tooth (13). With this treatment, root development is continued, root length is improved, dentin wall thickness is increased, and the closure of the apex is achieved (14). Due to the thickness of root canal walls and to protect dentin tissue during mechanical preparation, chemical disinfection should be focused (13,15).

The latest statement of the American Association of Endodontists does not recommend any instrumentation. Thus, irrigation, and intracanal medication have become essential for disinfection and biofilm disruption (13). Calcium hydroxide is prevalent for its antibacterial properties, but may not be effective against Enterococcus faecalis and Candida albicans, which are resistant to pH changes (16). To overcome the limitations of calcium hydroxide, triple antibiotic paste has been proposed as an intracanal medicament. It is a mixture of Ciprofloxacin, Metronidazole, and Minocycline at a concentration of 1:1:1:1 with distilled water (17). In regenerative studies, it is common practice to use TAP between sessions, but coronal discoloration has been reported in many cases (11,12). Despite this one major drawback of this approach, the AAE Clinical Considerations for a Regenerative Procedure Revised (4/1/2018) guideline still recommends the use of a low concentration of TAP (1–5 mg/mL) (18).

The most common materials used for non-vital tooth bleaching are sodium perborate, carbamide peroxide (CP), and hydrogen peroxide (HP) (19). HP and sodium perborate release oxygen-free radicals, whereas CP dissociates into HP and urea (20). The exact bleaching mechanism is based on the oxidation reaction. Highly reactive oxygen (O) and perhydroxyl (HO_2) radicals can be produced by HP (H_2O_2). Oxygen-free radicals are produced more in acidic environments, while highly oxidizing perhydroxyl radicals are produced more in basic environments (21). These reactive radicals penetrate the organic matrix and the color of the dentin can be altered. However, using a higher percentage of H_2O_2 may increase the risk of cervical root resorption. It may occur as an inflammatory reaction by the leakage of H_2O_2 through the dentinal tubules into the periodontal ligament or a foreign body reaction by the protein denaturation or activation of osteoclast by a decrease in pH by H_2O_2 (22). Therefore, the placement of the barrier, selection, and application of bleaching material is of utmost importance (21,22). For this reason, aggressive methods and powerful substances should be avoided. Using a higher percentage of H_2O_2 may increase the risk of invasive resorption (22). Therefore, CP presents a safer alternative bleaching agent. 10% CP releases 3.5% HP, while 35% CP releases 10% HP (23).

In the literature, several methods have been described for the bleaching of teeth discolored by TAP. Intracoronal bleaching was performed with 35% HP (24), 37% CP (25), 35% HP, and 37% CP with +Nd-YAG laser irradiation (26), SP and distilled water (24,27), and SP application with heat and ultrasonic instrument (28). However, there is no consensus on the ideal bleaching agent, and the number of bleaching studies on TAP-discolored teeth is limited. Therefore, this in vitro study aimed to evaluate the effect of intracoronal bleaching agents (IBA) on teeth discolored by TAP, addressing a much-debated question of whether a lower concentration of bleaching agent could achieve clinically acceptable esthetic results. The null hypothesis were that the effect of TAP on coronal discoloration would not be time-dependent and that there would be no significant differences between the tested bleaching agents.

Materials and Methods

The manuscript of this laboratory study has been written according to Preferred Reporting Items for Laboratory Studies in Endodontology (PRILE) 2021 guidelines (29) (Table 1).

The study protocol was conducted by the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Tooth Selection

Twenty-six extracted, caries-free human maxillary central and lateral incisors without any defects, pathological resorption, calcification, or existing restoration were used in this study. None of the teeth had received previous endodontic therapy. The teeth were collected after obtaining the patient's informed consent under a protocol approved by the Research Ethics Committee of Istanbul Okan University School of Medicine and Research Institute (Approval No: 154, April 27, 2022). The periodontal remnants of the extracted teeth were cleaned. The teeth were

Table 1. Preferred reporting items for laboratory studies in endodontology 2021 guidelines. (A/Y) and most-cited

PRILE 2021 Checklist of items to be included when reporting laboratory studies in Endodontology*			
Section/ Topic	**Item Number**	**Checklist Items**	**Reported on page number**
Title	1a	The Title must identify the study as being laboratory-based, e.g. "laboratory investigation" or "in vitro," or "ex vivo" or another appropriate term	1
	1b	The area/field of interest must be provided (briefly) in the Title	1
Keywords	2a	At least two keywords related to the subject and content of the	1
Abstract		investigation must be provided	
Abstract	3a	The rationale/justification of what the investigation contributes to the literature and/or addresses a gap in knowledge must be provided	1
	3b	The aim/objectives of the investigation must be provided	1
	3c	The body of the Abstract must describe the materials and methods used in the investigation and include information on data management and statistical analysis	1
	3d	The body of the Abstract must describe the most significant scientific results for all experimental and control groups	1
	3e	The main conclusion(s) of the study must be provided	1
Introduction	4a	A background summary of the scientific investigation with relevant information must be provided	1-2
	4b	The aim(s), purpose(s) or hypothesis(es) of an investigation must be provided ensuring they align with the methods and results	2
Materials and Methods	5a	A clear ethics statement and the ethical approval granted by an ethics board, such as an Institutional Review Board or Institutional Animal Care and Use Committee, must be described	2
	5b	When harvesting cells and tissues for research, all the legal, ethical, and welfare rights of human subjects and animal donors must be respected and applicable procedures described	2
	5c	The use of reference samples must be included, as well as negative and positive control samples, and the adequacy of the sample size justified	4
	5d	Sufficient information about the methods/materials/supplies/samples/ specimens/instruments used in the study must be provided to enable it to be replicated	2,4-5
	5e	The use of categories must be defined, reliable and be described in detail	4-5
	5f	The numbers of replicated identical samples must be described within ach test group. The number of times each test was repeated must be described	4-5
	5g	The details of all the sterilization, disinfection, and handling conditions must be provided, if relevant	4-5
	5h	The process of randomization and allocation concealment, including who generated the random allocation sequence, who decided on which specimens to be included and who assigned specimens to the intervention must be provided(if applicable)	4
	5i	The process of blinding the operator who is conducting the experiment (if applicable) and the examiners when assessing the results must be provided	-
	5j	Information on data management and analysis including the statistical tests and software used must be provided	5
Results	6a	The estimated effect size and its precision for all the objective (primary and secondary) for each group including controls must be provided	5-6
	6b	Information on the loss of samples during experimentation and the reasons must be provided, if relevant	-
	6c	All the statistical results, including all comparisons between groups must be provided	5-6

Discussion	7a	The relevant literature and status of the hypothesis must be described	6-7
	7b	The true significance of the investigation must be described	6-7
	7c	The strength(s) of the study must be described	6-7
	7d	The limitations of the study must be described	7
	7e	The implications for future research must be described	7
Conclusion(s)	8a	The rationale for the conclusion(s) must be provided	7
	8b	Explicit conclusion(s) must be provided, i.e. the main "take-away" lessons	7
Funding and support	9a	Sources of funding and other support (such as supply of drugs, equipment) as well as the role of funders must be acknowledged and described	7
Conflicts of interest	10a	An explicit statement on conflicts of interest must be provided	7
Quality of images	11a	Details of the relevant equipment, software and settings used to acquire the image(s) must be described in the text or legend	-
	11b	If an image(s) is included in the manuscript, the reason why the image(s) was acquired and why it is included must be provided in the text	-
	11c	The circumstances (conditions) under which the image(s) were viewed and evaluated must be provided in the text	-
	11d	The resolution and any magnification of the image(s) or any modifications/ enhancements (e.g. brightness, image smoothing, staining etc.) that were carried out must be described in the text or legend	--
	11e	An interpretation of the findings (meaning and implications) from the image (s) must be provided in the text	
	11f	The legend associated with each image must describe clearly what the subject is and what specific feature(s) it illustrates	-
	11g	Markers/labels must be used to identify the key information in the image(s) and defined in the legend	-
	11h	If relevant, the legend of each image must include an explanation whether it is pre-experiment, intra-experiment or post-experiment and, if relevant, how images over time were standardised	-

*From: Nagendrababu V, Murray PE, Ordinola-Zapata R, Peters OA, Rôças IN, Siqueira JF Jr, Priya E, Jayaraman J, Pulikkotil SJ, Camilleri J, Boutsioukis C, Rossi-Fedele G, Dummer PMH (2021) PRILE 2021 guidelines for reporting laboratory studies in Endodontology: a consensus-based development. International Endodontic Journal May 3. doi: 10.1111/iej.13542. https://onlinelibrary.wiley.com/doi/abs/10.1111/iej.13542
For further details visit: http://pride-endodonticguidelines.org/prile

kept in 0.5% chloramine T (Merck, Darmstadt, Germany) for 1 week, and then, the teeth were stored at 4°C in distilled water and used within 1 month.

Sample Size Calculation

PASS 11 software (NCSS, LLC, Kaysville, Utah) was used to calculate the sample size. In the Power analysis, with d (effect size): 1.170, the minimum sample size for Power: 0.80 and α: 0.05 was defined as n = 13 for each group.

Experimental Procedure

Following Traditional Endodontic Access Cavity preparation, the root canals were irrigated using 10 mL 5.25% NaOCl (Wizard, Rehber Kimya, Türkiye), 10 mL 17% EDTA (Wizard, Rehber Kimya, Türkiye), and 10 mL distilled water with 30 gauge irrigation needle (Genject A. Ş., Türkiye). The TAP was prepared by mixing equal powder of metronidazole (Flagly, Eczacibaşi), ciprofloxacin (Ciprо, Biofarma), and doxycycline (Tetradoks, Actavis) with distilled water in a ratio of 1:1:1:3. After drying the root canals using paper points, TAP was placed into the root canals using a lentulo (Dentsply Maillefer, Ballaigues, Switzerland) and remained below the cementoenamel junction (CEJ). A small piece of the cotton pellet was placed on the CEJ and the access cavities were covered with a temporary filling material (Cavit G, 3M ESPE, Germany). Teeth were stored in distilled water within Eppendorf tubes and incubated for 3 weeks at 37°C in 100% humidity (JSGI-100T, Gongju, Kore). Color measurements were made as described in the next section. The data were recorded at baseline, 1 week (1w), 2 weeks (2w), and 3 weeks (3w) after TAP was applied, respectively.

After 3w, TAP paste was removed from the root canals using copious, gentle irrigation of 20 mL of 17% EDTA with a 30 gauge endodontic needle (Genject A.Ş., Türkiye) (18). Then, the root canals were dried with paper points

Table 2. Assessment of color changes of TAP staining

Time	ΔE Mean±SD	[1]p
1w	9.84±5.424[A]	
2w	13.20±5.80[B]	0.000*
3w	16.54±5.90[C]	
1w–2w[2]p	0.000*	
1w–3w[2]p	0.000*	
2w–3w[2]p	0.000*	

[1]Repeated measures analysis of variance; [2]Bonferroni test. *p<0.05. Different letters indicate statistically significant difference.

(Diadent Group International Inc., Chongju, Korea). As a barrier, glass ionomer cement (Ketac Easy Molar, 3M ESPE, St Paul, USA) was applied in 3–4 mm.

Color Measurement

In this study, a composite frame was prepared to standardize the color measurements. A 6-mm diameter in a circle shape of bulk-fill composite resin (Filtek Bulkfill, 3M ESPE, St Paul, USA) was applied 2-mm coronal to CEJ on the fascial surfaces of the teeth. This way, the spectrophotometer's probe tip (Easyshade, VITA Zahnfabrick, Bad Säckingen, Germany) could be placed in the exact location.

Before measuring, the spectrophotometer was calibrated. On the gray background, the measurements were repeated 3 times for each sample, and average values of color parameters were determined. The L*, a*, and b* values were recorded according to the Commission Internationale de l'Eclairage CIELAB coordinates. The color difference (ΔE) was calculated using the formula:

$$\Delta E = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$$

Intracoronal Bleaching Steps

The specimens were randomly divided into two groups (n = 13) according to IBA:

- Group HP - 35% HP (Opalescence Endo, Ultradent)
- Group CP - 37% CP (Whiteness Super Endo, FGM).

The bleaching agents were placed in the pulp chamber of the cavity and a cotton pellet was placed over the gel. A temporary filling material (Cavit G, 3M ESPE, Germany) was placed over the access cavity until the next session, which took place 3 days later. This procedure was repeated in each bleaching session for up to 9 days. During this process, specimens were immersed in an Eppendorf tube that contained distilled water and kept in an incubator at 37ºC and 100% humidity.

The color measurements of bleached teeth were determined on the 3rd, 6th, and 9th days. CIE L*a*b* values were recorded and color changes were computed according to the formula. In the present study, ΔE ≤ 3.3 was interpreted as a clinically acceptable difference.

Statistically Analysis

All statistical analyses were performed using IBM SPSS Statistics 22 (IBM SPSS, Turkey). The parameters were assessed by Kolmogorov–Smirnov and Shapiro–Wilk tests, and it was found that the parameters were normally distributed. Student t-test was used for comparisons of parameters between two groups. Repeated measures analysis of variance was used for intragroup comparisons of the parameters and the Bonferroni test was used for post hoc comparisons. Significance was analyzed at $p < 0.05$ level.

Results

There is a statistically significant difference regarding the color changes (ΔE) among the evaluated periods of the 1st (1w), 2nd (2w), and 3rd weeks (3w) ($p = 0.000$). The most significant color change was observed in the 3w ($p < 0.05$) (Table 2).

Table 3. Evaluation of color changes of bleached teeth

Time	Group HP ΔE Mean±SD	Group CP ΔE Mean±SD	[1]p
3rd day	12.24±6.21[Aa]	9.81±4.26[Aa]	0.256
6th day	15.75±5.52[Aa]	11.11±6.56[Aa]	0.063
9th day	19.28±6.02[Ba]	14.14±5.46[Ab]	0.032*
[2]p	0.002*	0.087	
3rd–6th[3]p	0.170	1.000	
3rd–9th[3]p	0.003*	0.109	
6th–9th[3]p	0.005*	0.158	

[1]Student t test; [2]Repeated measures analysis of variance; [3]Bonferroni test. *p<0.05. Different capital letters in the column indicate differences between times. Different lowercase letters in rows indicate differences between groups.

In the Group HP, there was no significant difference on the 6th day when compared to the 3rd day ($p > 0.05$), but there was a significant difference on the 9th day ($p < 0.05$) (Table 3).

In Group CP, the color change was not statistically significant differed among the evaluated days ($p = 0.087$) (Table 3).

The comparison of the bleaching agents showed no statistical difference between the 3rd and 6th day ($p > 0.05$). However, on day 9, the Group HP had a significantly higher mean ΔE than the Group CP ($p = 0.032$) (Table 3).

Discussion

Teeth discoloration, which can be due to various causes, significantly contributes to esthetic failure. Particularly, a single discolored tooth creates an esthetic paradox (30). Bleaching is an excellent opportunity to treat these teeth conservatively without needing veneers or full crowns (21). However, the etiology of the case is just as crucial as the bleaching technique and materials affecting the result. In studies, it has been reported that trauma- or necrosis-induced discoloration has a better prognosis when compared to intracanal medicaments or metallic stains (7). One of these intracanal medicaments is TAP, a mixture of three antibiotics (25). TAP has antimicrobial properties against Enterococcus faecalis and a potently influences on "regeneration and revascularization" to maintain vitality (13,31). However, tooth discoloration has been associated with TAP (13,32).

In regenerative endodontic treatment, to minimize discoloration, changing the antibiotic paste's content and using a dentin bonding agent have been recommended (19). However, our understanding of the effect of currently used bleaching materials on these discolored teeth needs to be improved. This is particularly important when patients consult the clinic with esthetic concerns about anterior tooth discoloration. While some research has been carried out on intracoronal bleaching with different agents (27), studies have yet to attempt to compare the bleaching effect of CP and HP agents on TAP discolored teeth. Hence, the present study was conducted to evaluate and compare the bleaching effect of CP and HP on TAP discolored teeth.

HP, sodium perborate, and CP are effective agents commonly used in intracoronal bleaching (6,7). However, since sodium perborate is not legal in Europe, it is not preferred anymore. While providing an effective whitening, there is a risk of weakening the mechanical properties of the dental hard tissues (33), penetration of the agent into the dentin tubules (34), and external cervical root resorption (22). The safe usage of these agents has gained in importance for the clinician.

With bleaching procedures, satisfactory whitening results can be visible after 2–4 sessions, depending on the degree of discoloration. Patients should be informed about "over-bleaching" and assess the color daily to avoid this (7). Therefore, in this study, bleaching agents were applied in three sessions and renewed every 3 days to mimic clinical conditions (9).

Spectrophotometers are the most widely used instruments for color matching. They offer the advantage of monitoring and measuring color changes by minimizing subjective errors (35). In this study, the effect of different bleaching agents on teeth colored with TAP was evaluated using a spectrophotometer. To eliminate any inconsistency during the measurements, a composite frame was applied on the buccal surface of the teeth to ensure that measurements were always taken from the exact location.

The current study showed that when used in a mixture, TAP resulted in a non-acceptable coronal discoloration even after 1 week ($\Delta E > 3.3$) and this effect increased with time. The highest level of discoloration occurred on the 21st day, so the first null hypothesis is rejected. This finding agrees with the results obtained by other researchers (28,36,37).

Although the discoloration mechanism of TAP has not been fully explained, it is believed that minocycline in its content causes discoloration by chelation with calcium ions (12,25). To avoid tooth discoloration, it has been reported that minocycline, a tetracycline derivative, was replaced with doxycycline (38). In a previous study, TAP with minocycline ($\Delta E = 32.42 \pm 6.09$) showed a significantly higher ΔE compared to TAP with doxycycline ($\Delta E = 11.64$) in the 3 weeks, but both values presented unacceptable ΔE (26). This study used a mixture of ciprofloxacin, metronidazole, and doxycycline was used as a TAP paste. Similarly, in our study, the coronal discoloration increased with time, and values were found to be unacceptable in the 3 weeks ($\Delta E = 16.54 \pm 5.90$). This finding suggested that doxycycline, a tetracycline antibiotic, was the cause of the discoloration.

In accordance with the results obtained in our study in which human teeth were used, Parikh et al. (37) reported the highest discoloration on day 21 ($\Delta E = 20.32$) in bovine teeth. Since the number and diameter of dentin tubules in the crown of bovine teeth are similar to those of human teeth (39), human and bovine teeth have been used in studies investigating tooth discoloration.

In the present study, the whitening effect increased daily, but a significant difference was only observed between

the second and third sessions in the HP-treated samples. When the bleaching agents were compared, a significant difference was observed only on the day 9th. Therefore, the second null hypothesis was rejected, which states that no significant differences would exist between the tested bleaching agents. This result is attributed to the bleaching effect increasing as the application time and concentration of HP increase. Several *in vitro* studies have evaluated the intracoronal bleaching of discolored teeth with TAP (24,26-28). In one of the studies using 35% HP gel (24), the authors did not notice significant differences ($p = 0.175$) in the color change, between the second (8 days) and the third (12 days) sessions. The other study reported the highest bleaching effect on the 12th day using 35% HP gel (26). However, these studies are difficult to compare directly with the present study because of differences in the number and frequency of bleaching sessions. In our study, the second bleaching session was performed on the 6th, and the third session on the 9th day.

In this study, CP showed a gradual bleaching effect during the tested period. This finding may be correlated to the content of *carbopol and glycerine* in the composition, which provides a long-term effect of this agent by delaying the release of HP (40). The most surprising aspect of the data in this study is that 35% HP gel produced a similar bleaching effect as 35% CP to the 6th-day measurement. As 35% CP releases 10% HP, it would be expected that 35% HP gel would have a more noticeable impact. When the studies with similar findings are reviewed, it is noteworthy that Lim *et al.* (41) evaluated the color change using human visual inspection in artificially blood-staining teeth. In a systematic review and meta-analysis study, it was reported that CP (35%, 37%), HP (35%), and the combination of sodium perborate with HP (3%, 30%) did not significantly differ from each other in endodontically treated discolored teeth (2).

The main limitation of this study was that it was performed *in vitro* conditions. The combination of blood with residual antibiotic pastes in the canal may have contributed to staining in clinical conditions. In addition, as the prognosis of bleaching is time-dependent, further research should be carried out to investigate the long-term success under both *in vivo* and *in vitro* conditions.

Conclusion

This study has demonstrated that TAP caused clinically unacceptable discoloration of teeth, which increased with time. CP and HP have a whitening effect on discolored teeth. However, the HP agent had the highest effect on the 9th day.

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