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ORIGINAL ARTICLE

Tooth discoloration induced by calcium silicate–based cements: effect of dentin surface pre-treatments in regenerative endodontics

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Abstract

This in vitro study evaluated whether dentin bonding agent application or Nd: YAG laser irradiation can reduce tooth discoloration induced by different calcium silicate–based cements (CSCs) under blood-contaminated conditions simulating regenerative endodontic procedures. Ninety extracted human maxillary incisors were standardized and randomly assigned to nine groups ($n = 10$) according to pre-treatment strategy (no treatment, universal adhesive, or Nd: YAG laser) and CSC type (ProRoot MTA, Biodentine, or TheraCal PT). Following blood application and clot formation, Spongostan and the assigned CSC were placed in the coronal third of the canal. Specimens were restored with resin composite and stored at 37 °C and 100% humidity. Color measurements were performed spectrophotometrically at baseline and after 7, 30, 90, and 180 days. Color changes were calculated using ΔE_{00} and whiteness index difference (ΔWID) values. Data were analyzed using three-way repeated-measures ANOVA and Sidak post hoc tests ($\alpha = 0.05$). All groups exhibited time-dependent discoloration. In ProRoot MTA groups, both dentin bonding agent and Nd: YAG laser pre-treatments significantly reduced discoloration compared with untreated controls ($p < 0.05$), with no significant difference between the two approaches ($p > 0.05$). Biodentine and TheraCal PT demonstrated significantly greater color stability than ProRoot MTA ($p < 0.05$). Within the limitations of this study, neither dentin bonding agent nor Nd: YAG laser pre-treatment completely prevented CSC-related discoloration. ProRoot MTA showed the greatest discoloration potential, even after preventive dentin pre-treatment, and therefore may not be the preferred material for anterior regenerative endodontic cases in which esthetics are critical. Biodentine and TheraCal PT demonstrated better color stability and may be more suitable alternatives for esthetically demanding regions, while dentin bonding or Nd: YAG laser pre-treatment appears to provide only limited, material-dependent benefit.

Keywords Regenerative endodontics · Calcium silicate–based cements · Tooth discoloration · Dentin bonding agent · Nd:YAG laser

Introduction

Regenerative endodontic procedures (REPs) represent an established biologically based treatment approach for immature permanent teeth with necrotic pulps and incomplete root development [1, 2]. Grounded in tissue engineering principles, REPs aim to restore the pulp–dentin complex by promoting biological healing and continued root maturation [3]. Beyond the resolution of clinical symptoms and apical pathology, these procedures contribute to dentinal wall thickening and apical closure, thereby supporting improved structural integrity of affected teeth [4, 5]. The clinical protocol typically includes minimal canal instrumentation, chemical disinfection, intracanal medication, and the induction of a blood clot or scaffold to facilitate cell

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migration and tissue regeneration. Following scaffold formation, a biocompatible coronal barrier—frequently a calcium silicate–based cement (CSC)—is placed to protect the regenerative environment and support tissue healing [3, 6].

Despite their biological advantages, tooth discoloration has been consistently reported as an undesirable outcome of regenerative endodontic treatments, particularly in anterior teeth where esthetic demands are high [3]. Discoloration may arise at multiple stages of the REP protocol, including irrigant use, blood clot induction, and the placement of CSC materials [7]. Blood components such as hemoglobin, hematin, and erythrocytes may penetrate dentinal tubules and alter the optical properties of the tooth structure, resulting in visible color changes [7, 8]. In addition, the inherent porosity of CSCs used as coronal barriers may facilitate the entrapment of blood-derived products, which may further contribute to discoloration [9, 10].

Mineral trioxide aggregate (MTA) has long been widely used in regenerative endodontics due to its favorable biocompatibility and bioactivity; however, its potential to cause tooth discoloration has been well documented [11]. This effect has primarily been associated with the presence of bismuth oxide as a radiopacifying agent and its interaction with dentin, irrigants, and blood components [12]. To overcome these limitations, alternative CSCs with modified formulations have been introduced. Biodentine (Septodont, Saint-Maur-des-Fosses, France), which incorporates zirconium oxide instead of bismuth oxide, has demonstrated comparable biological properties along with improved handling characteristics and enhanced color stability relative to MTA [12]. More recently, TheraCal PT (Bisco Inc, Schaumburg, IL), a dual-cure resin-modified calcium silicate–based cement, has been developed, offering rapid setting, calcium ion release, and the possibility of same-visit definitive restoration [13, 14]. However, data regarding its discoloration potential in regenerative scenarios remain limited.

To reduce discoloration associated with CSC placement, dentin pre-treatment strategies aimed at sealing dentinal tubules have been proposed. The application of dentin bonding agents (DBAs) prior to CSC placement has been shown to limit the penetration of blood-derived pigments by creating a physical barrier within the tubules [15]. As an alternative approach, Nd: YAG laser irradiation has been suggested for dentinal tubule occlusion. Through a process of surface melting and re-solidification, Nd: YAG laser treatment can narrow or close dentinal tubules without inducing cracks or surface defects, suggesting a potential adjunctive approach for discoloration control [15, 16].

Although numerous studies have investigated tooth discoloration associated with REP, most have focused on comparisons between CSC material [7, 10, 17, 18] while preventive strategies have received comparatively limited attention [8, 15, 19, 20]. Moreover, no previous study has comprehensively assessed the combined influence of material type, dentin pre-treatment strategy, and time within a single factorial design under blood-contaminated conditions that simulate regenerative endodontic procedures. In particular, data regarding the discoloration behavior of recently introduced materials such as TheraCal PT in such conditions are still scarce. Therefore, the present in vitro study was designed to address this gap by evaluating the combined effects of Nd: YAG laser irradiation and dentin bonding agent application on discoloration induced by different calcium silicate–based cements over time under simulated regenerative conditions. The null hypotheses were that (i) dentin pre-treatment method, (ii) calcium silicate–based cement type, and (iii) evaluation time would not significantly affect tooth discoloration associated with REP.

Materials and methods

The study protocol was approved by the institutional ethics committee (Approval no: 2023-05/03). A sample size calculation was performed using G*Power 3.1 software (Heinrich Heine University, Düsseldorf, Germany), based on data from a previous study [18]. The analysis determined that a minimum of 10 samples per group would be required to achieve 95% power with a significance level (α) of 0.05, assuming a correlation of 0.5 among repeated measures and an effect size of 0.25.

Tooth selection

A total of 90 extracted human permanent maxillary incisor teeth were included in this study. All teeth were extracted for periodontal reasons and had fully developed root apices. Prior to the experimental procedures, each tooth was examined under a dental operating microscope (DOM; OMS2380, Zumax, Suzhou, China) to detect and exclude specimens with any visible fractures, cracks, caries, or structural defects. Standardized periapical radiographs were obtained from both bucco-lingual and mesio-distal angles to confirm the presence of a single root and single canal morphology (Vertucci Type I). Only teeth with a root canal curvature less than 10° were selected.

To minimize structural variability, teeth extracted from patients aged between 40 and 60 years were selected to achieve greater consistency in dentinal tubule characteristics. This age range was preferred to standardize dentinal tubule diameter and distribution, thereby reducing variability in dentin permeability and its potential influence on discoloration outcomes. Specimens presenting with caries, old restorations, previous root canal treatments, anatomical anomalies, internal or external resorptions, post-core restorations, pulp canal calcifications, or pre-existing discoloration were excluded. Until the start of the experiment, all selected teeth were stored in distilled water at room temperature to prevent dehydration.

Sample preparation and grouping

The apical portion of each tooth was sectioned to obtain a standardized length of 10 mm from the buccal cemento-enamel junction (CEJ). Endodontic access cavities were prepared, and the buccal wall thickness was standardized to 3 ± 0.5 mm. Root canals were enlarged using Gates-Glidden drills (#3–#5; Mani Inc., Japan) along the entire canal length. Root ends were sealed with resin composite (Filtek Z350, 3 M ESPE, USA). The sample preparation was performed by a single operator (P.Ş).

The REP protocol recommended by the American Association of Endodontists (AAE) was strictly followed [21]. The root canals were irrigated with 20 mL of 2.5% sodium hypochlorite (NaOCl; Cerkamed, Poland) and 20 mL of 17% etilen-diamine-tetra-aseticacid (EDTA; Cerkamed, Poland) using 30G side-vented needle (PD Irriflex, Vevey, Switzerland) placing 1 mm short from the apex. The canals were then dried with sterile paper points (VDW, Germany). Then calcium hydroxide paste (Opacal, PD, Vevey, Switzerland) was placed into the root canals. A sterile cotton pellet was placed to pulp chamber, and the access cavities were temporarily closed (Cavit; 3 M ESPE, St. Paul, USA) All procedures were performed under DOM by an experienced endodontist (B.A). Samples were stored in 37 °C and %100 humidity for 2 weeks.

For the second appointment scenario, the temporary restoration and medicament were removed, and the canals were irrigated with 20 mL of 17% EDTA and dried. A sterile Teflon tape was placed at the canal orifice. Specimens were randomly allocated into nine experimental groups ($n = 10$) based on dentin surface pre-treatment strategy and calcium silicate–based cement (CSC) type (Table 1).

Three dentin pre-treatment protocols were used:

- Control group: No pre-treatment (bonding agent or laser) was applied to the internal dentin walls of the pulp chamber.
- Bonding group: A universal adhesive with a self-etch approach (OptiBond Universal, Kerr Corporation, USA) was applied to the dentin walls by scrubbing for 20 s, followed by 5 s of gentle air drying, and then light curing for 10 s.
- Laser group: The dentin surfaces of the pulp chamber were irradiated using an Nd: YAG laser (Fotona, Ljubljana, Slovenia) at 1 W power, 10 Hz frequency, and 50 µs pulse duration, using a 300 µm fiber optic tip without water cooling. The laser tip was positioned 1 mm from the surface, and the irradiation was applied with back-and-forth sweeping motions in both occluso-apical and mesio-distal directions for 60 s [19]. The Nd: YAG laser parameters used in this study were selected based on previous studies demonstrating effective dentinal tubule occlusion through melting and resolidification mechanisms, while avoiding thermal damage to dentin and pulpal tissue [16].

All procedures were performed by the same operator to minimize variability (T.O).

Blood clot formation and CSC material placement

Following the application of dentinal tubule sealing strategies, Fresh human blood (0.2 mL) was obtained from a healthy volunteer (TÖ) and injected into the canal to a level approximately 4 mm below the CEJ using an insulin syringe. Specimens were left undisturbed for 15 min to allow clot formation. A hemostatic sponge (Spongostan; Cutanplast, Italy) was placed over the clot.

Depending on the group assignment, one of the three CSCs - ProRoot MTA (Dentsply Maillefer, Switzerland),

Table 1 Experimental Design, Surface Pre-treatment Strategies, and Composition of Calcium Silicate–Based Cements

Material	Main composition	Radiopacifer	Setting time	Dentin Pre-treatment strategy	*n*
ProRoot MTA	Tricalcium silicate, dicalcium silicate, calcium aluminate, calcium sulfate	Bismuth oxide	Approx. 4 h	No pre-treatment (control)	10
				Bonding	10
				Laser irradiation	10
Biodentin	Tricalcium silicate, iron oxide, calcium carbonate, calcium oxide	Zirconium oxide	Approx. 12 min	No pre-treatment (control)	10
				Bonding	10
				Laser irradiation	10
TheraCal PT	Resin-modified calcium silicate matrix with Portland cement particles	Barium zirconate	Dual-cure / maximum setting time 5 min	No pre-treatment (control)	10
				Bonding	10
				Laser irradiation	10

Biodentine or TheraCal PT - was prepared in accordance with the manufacturer's instructions. The selected material was applied to the coronal third of the canal using a micro apical placement system (MAP; Produits Dentaires SA, Switzerland) to a uniform thickness of 3 mm, positioned 1 mm below the CEJ. To confirm the accuracy of material placement, periapical radiographs were taken. The access cavities were temporarily sealed, and specimens were stored at 37 °C in 100% humidity for 24 h to allow complete setting of the material. After this period, the coronal cavities were permanently restored using resin composite (Filtek Z350, 3 M ESPE, USA) (Fig. 1). All samples were maintained under controlled temperature and humidity conditions throughout the experimental period. All CSC placement procedures were by an experience endodontist (B.A).

Tooth color measurements

Tooth color was measured using a spectrophotometer (VITA Easyshade Advance 4.0; VITA Zahnfabrik, Germany). Measurements were obtained from the central buccal surface against a standardized white background (CIE calibrated tile). For each specimen, three consecutive readings were recorded and averaged. The device was calibrated before each measurement session according to the manufacturer's instructions. The same calibrated device was used throughout the study, and all measurements were carried out by a single trained operator who was blind to all experimental procedures (YSU). To ensure uniform baseline distribution, specimens were allocated equally according to tooth shade (A2, A3, B2) across groups [19, 22].

Color evaluations were performed according to the CIE L*a*b* color space system. In this system, the L* coordinate indicates lightness (ranging from 0 = black to 100 = white), a* represents the green (–) to red (+) spectrum (typically −70 to + 70), and b* corresponds to the blue (–) to yellow (+) spectrum (–80 to + 100). After baseline color assessment (T0), the teeth were stored in artificial saliva and kept in an incubator at 37 °C to simulate oral environmental conditions and the aging process. The artificial saliva was refreshed weekly. Subsequent color measurements were taken at T1 (7 days), T2 (30 days), T3 (90 days), and T4 (180 days) to evaluate time-dependent discoloration compared to baseline L*a*b* values.

All color changes were quantified using the CIEDE2000 (ΔE_{00}) color difference formula [23, 24], and the CIELAB-based whiteness index (WID) [22, 25]:

$$\Delta E_{00} = \left[\left(\frac{\Delta L'}{K_L S_L} \right)^2 + \left(\frac{\Delta C'}{K_C S_C} \right)^2 + \left(\frac{\Delta H'}{K_H S_H} \right)^2 + R_T \left(\frac{\Delta C'}{K_C S_C} \right) \left(\frac{\Delta H'}{K_H S_H} \right) \right]^{1/2}$$

$$\mathrm{WID} = 0.511\,L^* - 2.324a^* - 1.100b^*$$

Color differences were calculated using the CIEDE2000 (ΔE00) formula, which provides improved perceptual uniformity compared with the traditional CIELAB color difference formula.

In the WID equation, the coefficients 0.511 (P), 2.324 (Q), and 1.100 (R) are weighting factors assigned to the CIELAB coordinates. These coefficients were established through psychophysical visual assessments linking perceived whiteness to color coordinates approaching reference white (L* = 100, a* = 0, b* = 0). Accordingly, higher

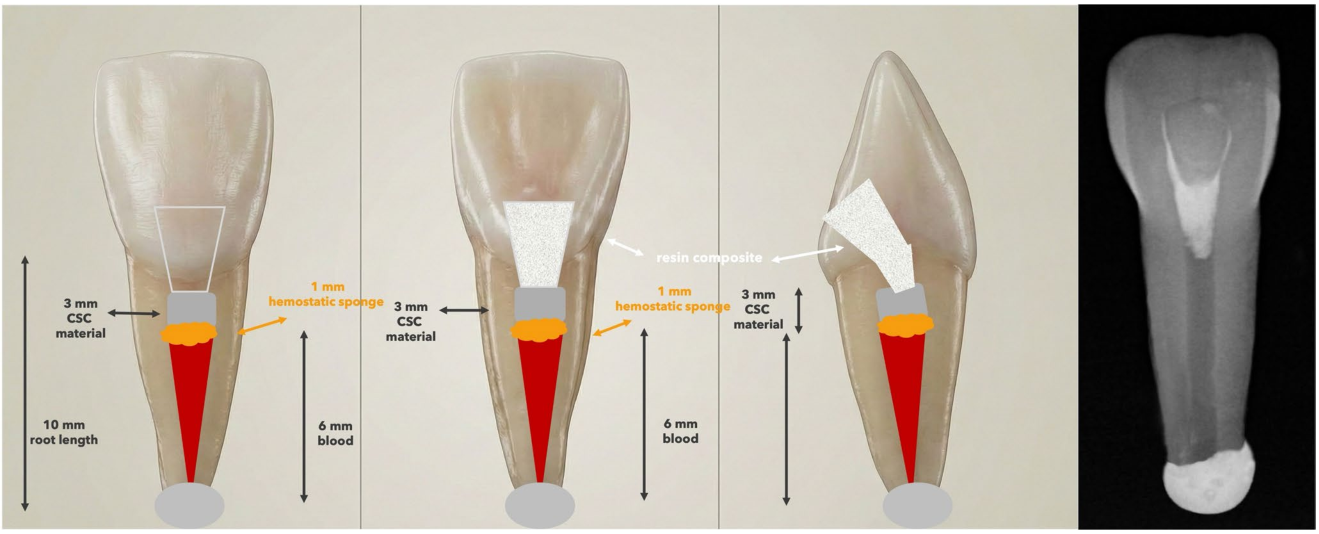

Fig. 1 Schematic representation of the standardized experimental model used to simulate regenerative endodontic procedures, illustrating 6-mm blood clot formation and placement of a 3-mm-thick calcium silicate–based cement (CSC) extending to 1 mm below the cemento-enamel junction (CEJ), followed by coronal resin composite restoration. A representative periapical radiograph confirms the position and thickness of the CSC

lightness (L* values closer to 100) increases the WID score, whereas chromatic deviations are reflected by negative weighting of a* and b* values, such that greater departures from zero result in lower WID values [22, 25]. For statistical analysis, changes in whiteness over time were expressed as the difference between baseline and each subsequent measurement interval (ΔWID).

Statistical analysis

Descriptive statistics for each variable were calculated and presented as "Mean ± Standard Error of Mean (SEM)". Data were subjected to three-factor mixed-design ANOVA (analysis of variance) using General Linear Model procedure for repeated measurements. The model included "Material", "Group" and "Time" as the main effects and their two-way and three-way interaction terms. In cases where Mauchley's test revealed that the assumption of sphericity violated, the Greenhouse-Geisser adjustment was applied. Simple effect analysis with Sidak adjustment was used to break down any significant interaction effect terms as post hoc analysis. A probability value of less than 0.05 was considered significant, unless otherwise noted. SPSS 21 was used for statistical analysis.

Results

Evaluation of color change (ΔE_{00})

Three-way repeated-measures ANOVA demonstrated that material type, discoloration-prevention method, and time each had a significant main effect on ΔE_{00} values ($p < 0.05$) (Table 2), (Fig. 2). A significant interaction between material type and discoloration-prevention method was also observed ($p < 0.05$), indicating that the effectiveness of dentin bonding agent or Nd: YAG laser pre-treatment depended on the CSCs used. No significant interactions involving time were detected ($p > 0.05$), suggesting that although discoloration increased over time, the relative differences among materials and pre-treatment strategies remained consistent throughout the experimental period.

Across all groups, ΔE_{00} values increased significantly over time, with the highest discoloration recorded at 6 months compared with the 1- and 3-month time points ($p < 0.05$). For all materials and pre-treatment groups, mean ΔE_{00} values exceeded the perceptibility (0.8) and acceptability (1.8) thresholds at all evaluation periods, indicating clinically perceptible discoloration.

Among the tested materials, ProRoot MTA demonstrated the highest discoloration potential overall. In the absence of dentin pre-treatment, ProRoot MTA exhibited significantly

Table 2 Mean ± SEM ΔE00 values for each material and discoloration prevention method at all evaluation periods

		ΔE 1 (D0-W1)	ΔE 2 (D0-M1)	ΔE 3 (D0-M3)	ΔE 4 (D0-M6)	P-value(s)						
Material	**Discoloration prevention methods**	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Time (T)	Material (M)	Group (G)	M*G	T*M	T*G	T*M*G
Proroot MTA	Control	$9.62 \pm 1.98^{\text{ab, A,x}}$	$9.81 \pm 0.98^{\text{b, A,x}}$	$9.64 \pm 0.97^{\text{b, A,x}}$	$11.71 \pm 0.57^{\text{a, A,x}}$	0,009	0,02	0,043	0,019	0,746	0,967	0,989
	Bonding	$7.17 \pm 0.33^{\text{ab, B,x}}$	$6.63 \pm 0.35^{\text{b, B,x}}$	$6.85 \pm 0.39^{\text{b, B,x}}$	$8.36 \pm 0.72^{\text{a, B,x}}$							
	Laser irradiation	$7.29 \pm 0.84^{\text{ab, B,x}}$	$7.2 \pm 0.41^{\text{b, B,x}}$	$6.86 \pm 0.61^{\text{b, B,x}}$	$8.07 \pm 0.95^{\text{a, B,x}}$							
Biodentin	Control	$7.57 \pm 0.46^{\text{ab, A,y}}$	$6.79 \pm 0.5^{\text{b, A, y}}$	$7.12 \pm 0.8^{\text{b, A,y}}$	$7.72 \pm 1.04^{\text{a, A,y}}$							
	Bonding	$7.13 \pm 0.63^{\text{ab, A,x}}$	$6.58 \pm 0.57^{\text{b, A,x}}$	$7.43 \pm 0.65^{\text{b, A,x}}$	$7.8 \pm 0.77^{\text{a, A,x}}$							
	Laser irradiation	$6.9 \pm 0.88^{\text{ab, A,x}}$	$6.04 \pm 0.23^{\text{b, A,x}}$	$6.8 \pm 0.53^{\text{b, A,x}}$	$7.36 \pm 0.91^{\text{a, A,x}}$							
Therecal PT	Control	$7.04 \pm 1.01^{\text{ab, A,y}}$	$6.23 \pm 0.71^{\text{b, A,y}}$	$7.01 \pm 0.84^{\text{b, A,y}}$	$7.88 \pm 1.12^{\text{a, A,y}}$							
	Bonding	$7.5 \pm 0.88^{\text{ab, A,x}}$	$7.36 \pm 0.94^{\text{b, A,x}}$	$6.56 \pm 1.26^{\text{b, A,x}}$	$8.24 \pm 1.16^{\text{a, A,x}}$							
	Laser irradiation	$7.68 \pm 0.58^{\text{ab, A,x}}$	$7.28 \pm 0.93^{\text{b, A,x}}$	$5.75 \pm 0.59^{\text{b, A,x}}$	$7.82 \pm 1.4^{\text{a, A,x}}$							

a,b: Different lowercase letters within the same row indicate statistically significant differences over time for each material and discoloration prevention method ($p < 0.05$)

A,B: Different uppercase letters within the same column indicate statistically significant differences among discoloration prevention methods for each material separately ($p < 0.05$)

x,y: Different lowercase letters (x,y) within the same column indicate statistically significant differences among materials for each discoloration prevention method separately ($p < 0.05$)

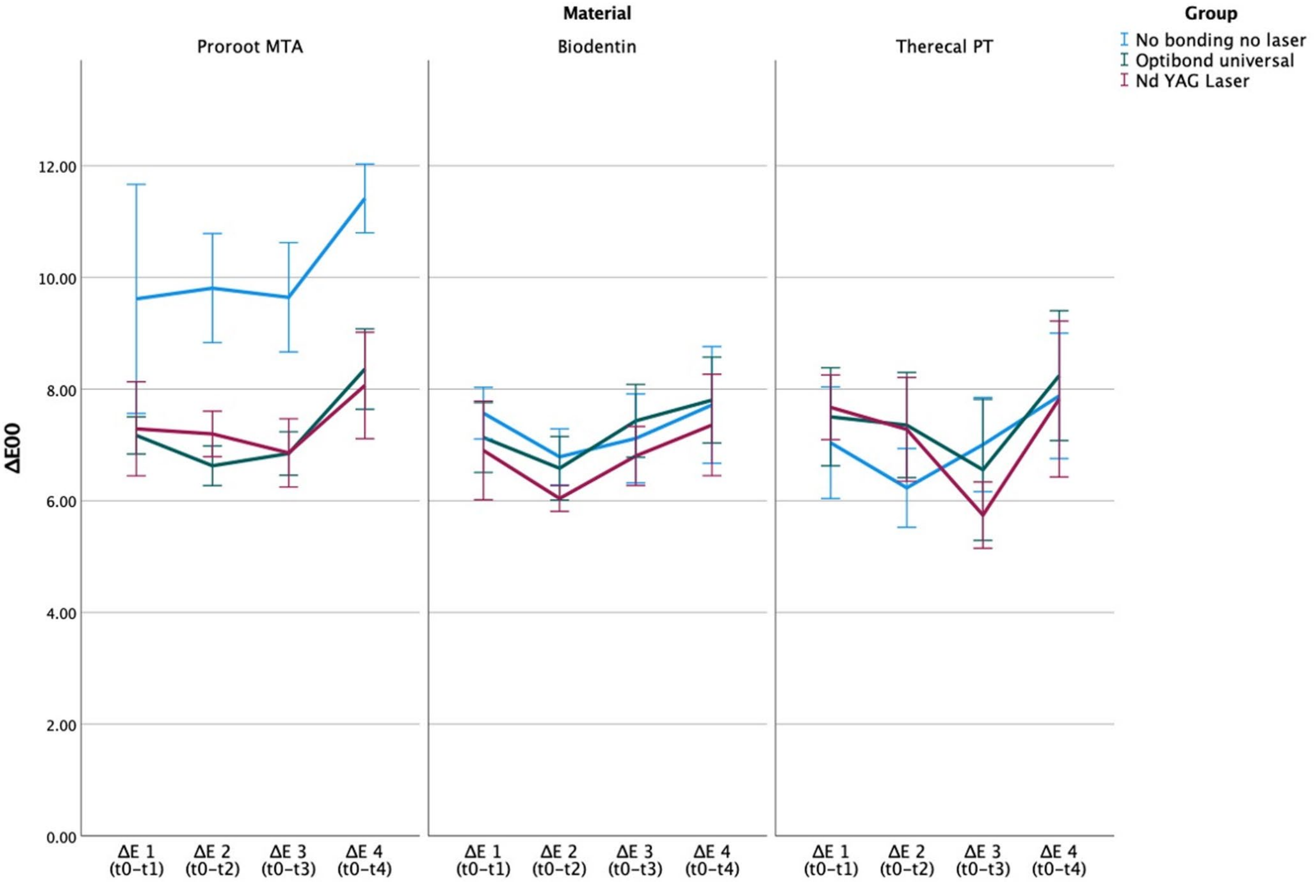

Fig. 2 Changes in ΔE00 values over time for each calcium silicate–based material and discoloration prevention method. All recorded ΔE00 values exceeded the perceptibility (0.8) and acceptability (1.77) thresholds. Error bars represent SEM

higher ΔE_{00} values than Biodentine and TheraCal PT at all time points ($p<0.05$). In contrast, Biodentine and TheraCal PT showed significantly greater color stability.

The effectiveness of discoloration-prevention strategies was material-dependent. In ProRoot MTA groups, both dentin bonding agent and Nd: YAG laser pre-treatment significantly reduced discoloration compared with untreated controls at all time points ($p<0.05$). However, neither approach completely prevented color change, and no significant difference was observed between the two preventive methods ($p>0.05$). Conversely, for Biodentine and TheraCal PT, dentin pre-treatment methods did not significantly influence ΔE_{00} values at any evaluation period ($p>0.05$). Clinically, these findings suggest that intrinsic material composition may play a more decisive role in discoloration behavior than dentin surface pre-treatment alone.

Evaluation of whiteness index (ΔWID)

Three-way repeated measures ANOVA revealed a significant main effect of material on ΔWID values ($p<0.05$), whereas the main effects of time and discoloration-prevention method were not significant ($p>0.05$). However, significant time × material, time × discoloration-prevention method, and time × material × discoloration-prevention method interaction effects were detected ($p<0.05$) (Table 3) (Fig. 3).

Time-dependent changes in ΔWID values were material and method-specific. Significant temporal changes were observed in ProRoot MTA (control), Biodentine (bonding), and TheraCal PT (bonding and Nd: YAG laser) groups ($p<0.05$), whereas no significant changes were detected in the remaining combinations ($p>0.05$).

Material-related differences in ΔWID values became more evident at later evaluation periods, particularly within bonding groups. ProRoot MTA generally exhibited higher ΔWID changes than Biodentine and TheraCal PT, reflecting a greater tendency toward clinically unfavorable color alteration.

Table 3 Mean ± SEM WID values for each material and discoloration prevention method at all evaluation periods

Material	**Discoloration prevention methods**	WID 1 (D0-W1) Mean ± SEM	WID 2 (D0-M1) Mean ± SEM	WID 3 (D0-M3) Mean ± SEM	WID 4 (D0-M6) Mean ± SEM	P-value(s) Time (T)	Material (M)	Group (G)	M*G	T*M	T*G	T*M*G
Proroot MTA	Control	$10.04 \pm 5.66^{b, B,y}$	$21.43 \pm 4.39^{a, A, x}$	$23.32 \pm 3.23^{a, A,x}$	$20.26 \pm 4.01^{a, A,x}$	0,336	0,046	0,346	0,061	0,001	0,014	0,018
	Bonding	$24.25 \pm 1.81^{a, A,x}$	$23.44 \pm 1.88^{a, A,x}$	$23.86 \pm 1.68^{a, A,x}$	$25.58 \pm 2.37^{a, A,x}$							
	Laser irradiation	$18.4 \pm 3.23^{a, B,x}$	$20.87 \pm 1.87^{a, A,x}$	$21.66 \pm 1.96^{a, A,x}$	$23.31 \pm 2.18^{a, A,x}$							
Biodentin	Control	$27.96 \pm 2.32^{a, A,x}$	$24.27 \pm 2.98^{a, A,x}$	$24.65 \pm 3.04^{a, A,x}$	$25.34 \pm 2.57^{a, A,x}$							
	Bonding	$19.61 \pm 2.02^{a, B,x}$	$16.92 \pm 1.82^{b, B,y}$	$16.85 \pm 1.91^{b, A,y}$	$17.63 \pm 2.41^{ab, B,y}$							
	Laser irradiation	$14.33 \pm 1.33^{a, B,x}$	$15.37 \pm 2.12^{a, B,x}$	$18.53 \pm 2.35^{a, A,x}$	$14.59 \pm 3.92^{a, B,x}$							
Therecal PT	Control	$15.5 \pm 4.47^{a, A,xy}$	$16.19 \pm 2.73^{a, A,x}$	$19.4 \pm 7.09^{a, A,x}$	$21.4 \pm 3.24^{a, A,x}$							
	Bonding	$17.6 \pm 2.03^{a, A,x}$	$12.32 \pm 2.09^{b, A,y}$	$13.59 \pm 1.69^{b, A,y}$	$9.74 \pm 1.51^{b, C,z}$							
	Laser irradiation	$20.19 \pm 3.59^{a, A,x}$	$14.43 \pm 3.81^{b, A,x}$	$16.04 \pm 3.79^{b, A,x}$	$15.74 \pm 2.57^{b, B,x}$							

a, b: Different lowercase letters within the same row indicate statistically significant differences in ΔWID values over time for each material and discoloration prevention method ($p<0.05$).

A, B,C: Different uppercase letters within the same column indicate statistically significant differences among discoloration prevention methods within each material ($p<0.05$).

x, y,z: Different lowercase letters (x, *y,z*) within the same column indicate statistically significant differences among materials within each discoloration prevention method ($p<0.05$).

To enhance the clinical interpretability of the instrumental color measurements, mean CIE L*, a*, and b* values were converted into RGB coordinates to generate data-based visual simulations of specimen color changes. The estimated color appearance of each experimental group over time is presented in Fig. 4 and corresponds with the trends observed in ΔE00 and WID values.

Overall, all tested materials exhibited clinically perceptible discoloration over time, with ProRoot MTA demonstrating the highest color change. Dentin pre-treatment strategies provided only a partial reduction in discoloration in ProRoot MTA, while showing no significant effect for Biodentine and TheraCal PT. Regarding ΔWID, changes in the direction of color shift were primarily dependent on material type and specific material–method combinations rather than time or pre-treatment method alone.

Discussion

Tooth discoloration remains a major esthetic concern in regenerative endodontic procedures, particularly in anterior teeth. According to the literature, approximately 54% of cases undergoing regenerative endodontic procedures (REP) exhibit varying degrees of tooth discoloration, and current whitening protocols have been shown to be insufficient in eliminating this discoloration [26]. Although previous studies have investigated either the discoloration potential of calcium silicate–based cements or the preventive effect of dentin surface pre-treatment strategies separately, no previous study has comprehensively evaluated the combined influence of material type, dentin pre-treatment strategy, and time within a single factorial design under blood-contaminated regenerative conditions, particularly including a recently introduced material such as TheraCal PT. Therefore, the present study evaluated the combined effects of CSC type, dentin pre-treatment strategy, and time on tooth discoloration using both ΔE₀₀ and ΔWID analyses. The findings demonstrated progressive and clinically perceptible discoloration in all groups over time, with ProRoot MTA exhibiting the highest discoloration potential. In contrast, Biodentine and TheraCal PT showed greater color stability, while dentin bonding agent and Nd: YAG laser application provided only partial and material-dependent benefits. Accordingly, the null hypotheses were rejected, as dentin pre-treatment method, CSC type, and evaluation time significantly influenced discoloration behavior.

The present findings demonstrated a significant time-dependent increase in ΔE₀₀ values across all experimental groups, indicating that discoloration progressed throughout the 6-month observation period. This progressive pattern suggests that color alterations associated with CSCs

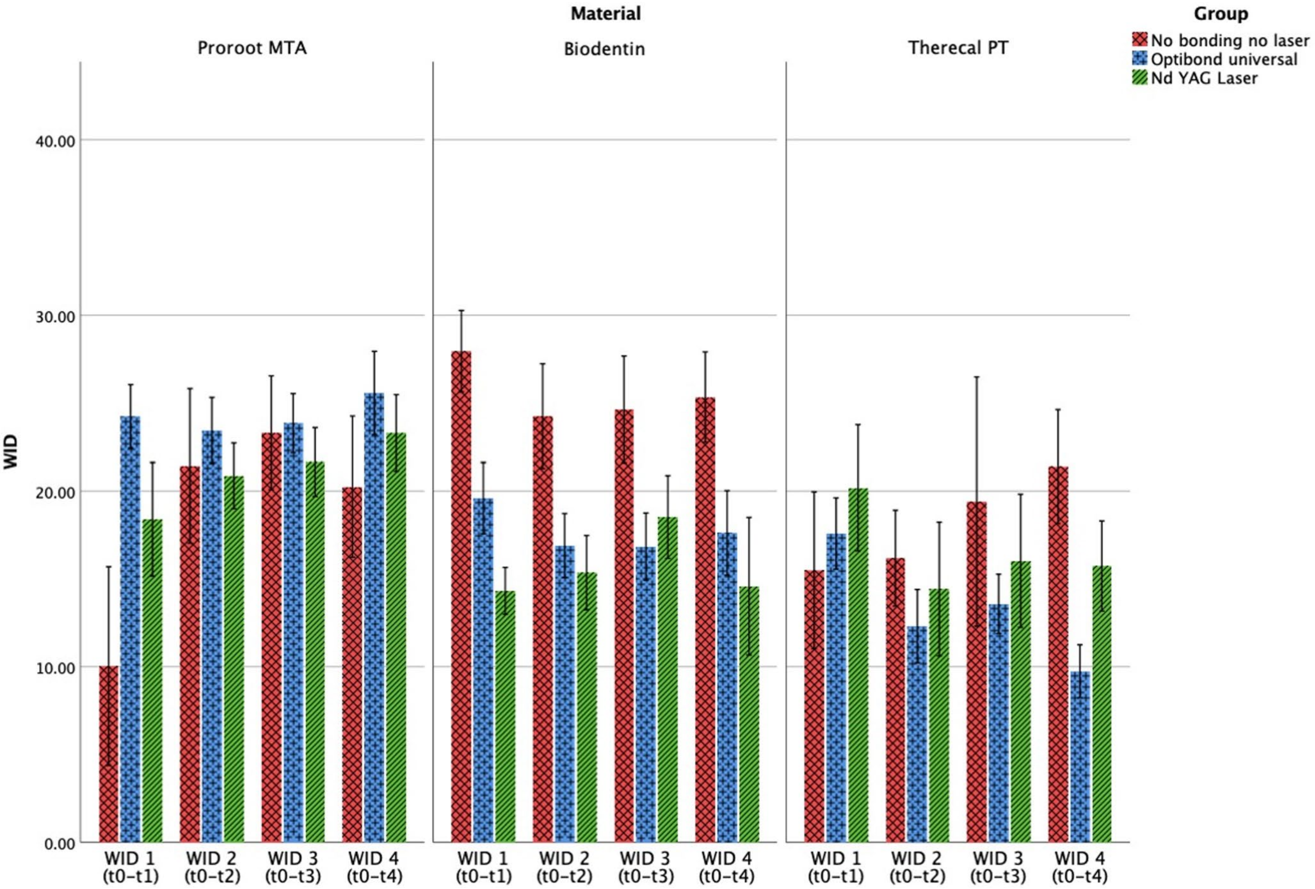

Fig. 3 Changes in whiteness index (WID) values over time for each calcium silicate–based material and discoloration prevention method. Error bars represent SEM

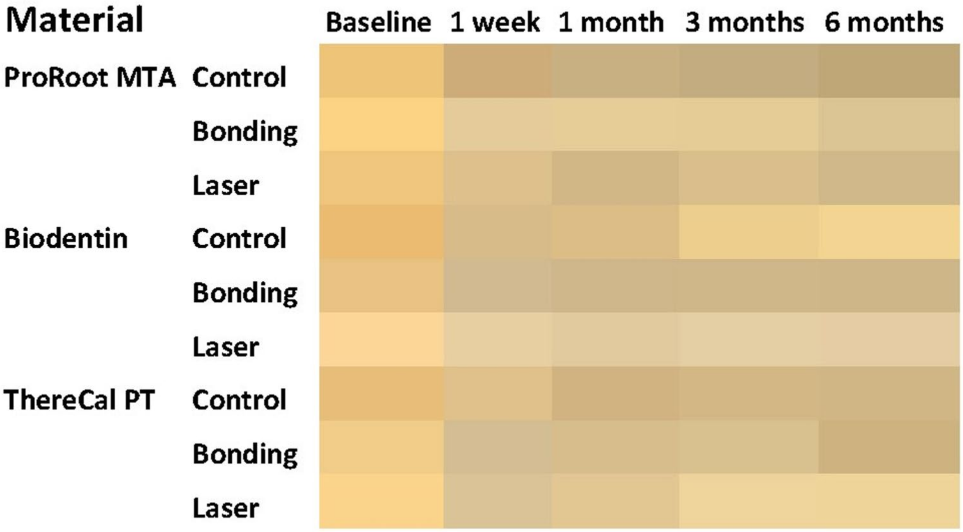

Fig. 4 Data-based visual simulation of specimen color changes derived from mean CIE Lab* values converted to RGB coordinates. The images represent the estimated color appearance of ProRoot MTA, Biodentine, and TheraCal PT under different discoloration prevention protocols at baseline, 1 week, 1 month, 3 months, and 6 months

are not limited to the early post-treatment phase but may continue as material–dentin interactions evolve. In blood-contaminated conditions, hemoglobin degradation products and iron-containing compounds may gradually penetrate dentinal tubules and interact with cement components, contributing to cumulative discoloration over time [18]. The absence of significant time-related interactions in ΔE_{00} further indicates that although the magnitude of discoloration increased, the relative behavior of materials and preventive strategies remained stable during follow-up. These findings underscore the importance of long-term color assessment when evaluating the esthetic performance of CSCs in REP.

In studies evaluating tooth discoloration following regenerative endodontic procedures, follow-up periods have varied considerably, most commonly extending to 3 or 6 months and, in some cases, up to 1 year [8, 19, 20, 22]. In the present study, color measurements were performed at standardized intervals consistent with previously reported protocols, including assessments after restoration and at 1 week, 1 month, 3 months, and 6 months. Previous investigations have shown that, despite the use of various discoloration-prevention strategies, clinically and statistically significant discoloration often becomes more evident at 6 months compared with earlier evaluation periods [8, 19]. Some authors have further demonstrated that color change may continue to increase up to 1 year, with the greatest differences observed at later follow-up intervals [22]. In the current study, although discoloration did not exhibit a strictly linear progression over time, ΔE_{00} values at 6 months were significantly higher than those recorded at 1 and 3 months. This pattern may reflect the complex, polychromatic nature of natural tooth structure, in which subtle optical changes become more apparent over time. Additionally, the gradual release and interaction of chemical constituents from calcium silicate–based cements—particularly MTA-derived materials—may contribute to cumulative discoloration, underscoring the importance of long-term evaluation when assessing esthetic outcomes [27].

Material type significantly influenced discoloration behavior, whereas the effect of dentin pre-treatment strategies appeared comparatively limited. ProRoot MTA demonstrated greater discoloration than Biodentine and TheraCal PT throughout the observation period, emphasizing the dominant role of intrinsic material composition in long-term color stability under blood-contaminated conditions. Consistent with previous studies [12, 17, 28], the higher discoloration potential of ProRoot MTA may be related to the presence of bismuth oxide, which can interact with blood-derived components and undergo oxidation reactions that promote dark pigmentation [29, 30]. In contrast, the improved color stability observed with Biodentine and TheraCal PT may be associated with the use of alternative radiopacifiers such as zirconium oxide and barium zirconate, which are considered less prone to chromogenic reactions [31–33]. In addition, the resin-modified structure and rapid setting behavior of TheraCal PT may further limit blood-derived pigment diffusion during the early setting phase [12, 34]. Similar findings were reported by Karadayı et al., who evaluated zirconium oxide–containing CSCs after root canal obturation and observed that discoloration reached its maximum level at 3 months and remained relatively stable thereafter [35]. In contrast, discoloration in the present study continued to increase up to 6 months. This difference may be attributed to the blood-contaminated regenerative model used in the current study, in which the prolonged interaction between blood-derived pigments and CSC materials may have contributed to ongoing discoloration over time. Collectively, these findings suggest that radiopacifier chemistry and blood interaction play decisive roles in discoloration behavior during regenerative endodontic procedures.

Another factor that may contribute to the discoloration potential of calcium silicate–based cements is their setting time. Previous investigations have suggested that prolonged setting duration may increase blood absorption and subsequent hemolysis, thereby enhancing the interaction between blood-derived pigments and the cement matrix [17, 36]. ProRoot MTA exhibits a relatively long setting time of approximately 4 h [37], whereas Biodentine sets within approximately 12 min [15], and TheraCal PT achieves setting within five minutes under intraoral temperature conditions [14]. The extended setting period of ProRoot MTA may allow greater diffusion and incorporation of hemoglobin degradation products into the material structure before complete hardening occurs. In contrast, the shorter setting times of Biodentine and TheraCal PT may limit the extent of blood infiltration, potentially contributing to their comparatively lower discoloration observed across measurement intervals.

Surface pre-treatment significantly influenced discoloration outcomes; however, its effectiveness appeared to be strongly material-dependent. Although dentin bonding agent and Nd: YAG laser application reduced discoloration in ProRoot MTA groups, neither approach completely prevented color change under blood-contaminated conditions. This finding suggests that the protective effect of dentinal tubule sealing may be insufficient against ongoing pigment diffusion over time. In contrast, no significant preventive benefit was observed for Biodentine and TheraCal PT, indicating that when intrinsically more color-stable materials are used, additional surface modification may provide limited clinical advantage. Khoshkhounejad et al. reported that replacing bismuth oxide with zirconium oxide could reduce CSC-related discoloration in the presence of blood [33]. A similar material-dependent discoloration pattern was also reported by Türkoğlu Kayacı et al. [12], who observed lower discoloration for Biodentine and TheraCal PT compared with ProRoot MTA. Zırhlı et al. also compared the discoloration potential of four different CSCs following perforation repair and suggested the use of Biodentine in esthetically sensitive regions because of its lower discoloration tendency [38]. Collectively, these findings support the interpretation that intrinsic material composition may play a more decisive role in long-term discoloration behavior than dentin surface pre-treatment strategies alone. From a clinical perspective, selecting a color-stable CSC may be more effective than relying on dentin surface pre-treatment strategies alone to minimize discoloration in regenerative procedures in esthetic region. Nevertheless, regenerative endodontic procedures are also frequently performed in immature posterior teeth, where esthetic concerns may be less critical and the biological properties of CSC materials may remain the primary consideration during material selection [39, 40].

Beyond the magnitude of overall color change (ΔE_{00}), the analysis of whiteness index differences (ΔWID) provided additional insight into the directionality of discoloration over time. While ΔE_{00} reflects the extent of perceptible color change, ΔWID captures shifts toward lighter or darker appearances, allowing a more clinically nuanced interpretation of esthetic alterations [41]. In the present study, ΔWID values demonstrated a complex interaction pattern among material type, surface pre-treatment, and time, indicating that discoloration was not solely intensity-dependent but also direction-dependent.

In the Biodentine groups, bonding agent application was associated with higher ΔWID values during the early evaluation period, suggesting a transient increase in whiteness shortly after placement. However, this difference was not maintained at later time points, indicating that the initial whitening effect diminished over time. These temporary shifts may be related to early surface modifications, hydration dynamics, or optical scattering changes associated with adhesive infiltration [42] or laser-induced surface alteration [19]. Similarly, in TheraCal PT specimens, both bonding and Nd: YAG laser application resulted in higher early ΔWID values, again reflecting a short-term whitening effect that was not sustained during follow-up. Partovi et al. suggested that this phenomenon may be attributed to the long-term effects of the resin content [43]. As time progressed, however, cumulative pigment diffusion and material–dentin interactions appeared to predominate, resulting in stabilization or reversal of the initial whitening trend. Taken together, these findings suggest that CSC-related discoloration in regenerative settings is a dynamic and multifactorial process, involving both quantitative changes in color magnitude and qualitative shifts in optical directionality.

The present study has several methodological strengths. A key strength was the simultaneous evaluation of material type, dentin pre-treatment strategy, and time within a single factorial design under blood-contaminated conditions simulating regenerative endodontic procedures. The experimental protocol closely replicated the two-visit REP recommended by the American Association of Endodontists, thereby enhancing clinical relevance. In addition, standardized tooth selection, buccal wall thickness, and extended follow-up periods allowed a more reliable assessment of discoloration behavior over time. The combined use of ΔE_{00} and ΔWID analyses provided a comprehensive evaluation of both the magnitude and direction of color change. To the best of our knowledge, this is the first study to evaluate the discoloration behavior of TheraCal PT under blood-contaminated regenerative conditions, providing novel data regarding the esthetic performance of this material.

However, certain limitations should be considered. As an in-vitro investigation, the study cannot fully replicate the complex biological and environmental conditions of the oral cavity, including pulpal response, vascularization, inflammatory processes, saliva, thermal fluctuations, and functional loading. Although blood contamination was simulated, the dynamic biological interactions present in clinical regenerative endodontic procedures may not be entirely reproduced. Only one adhesive protocol and a single laser wavelength and laser parameter setting were tested, which may limit the generalizability of the findings. Furthermore, although discoloration outcomes were quantitatively measured, microscopic evaluation of dentinal tubule occlusion or pigment penetration was not performed; therefore, the underlying mechanisms were not directly verified at the microstructural level. Future studies incorporating microstructural analysis and extended clinical follow-up would help further clarify the interaction between CSC composition, dentin surface modification, and long-term esthetic outcomes.

Conclusion

Within the limitations of this in vitro study, all tested calcium silicate–based cements induced progressive and clinically perceptible discoloration under blood-contaminated regenerative conditions. Although dentin bonding agent application and Nd: YAG laser irradiation partially reduced discoloration associated with ProRoot MTA, neither strategy completely prevented color change, indicating that dentin surface pre-treatment alone may provide limited clinical benefit. Among the tested materials, ProRoot MTA demonstrated the greatest discoloration potential even after preventive procedures, whereas Biodentine and TheraCal PT exhibited superior color stability. Therefore, in esthetically demanding anterior regenerative cases, clinicians should prioritize the selection of intrinsically color-stable materials such as Biodentine or TheraCal PT rather than relying solely on dentin pre-treatment strategies, as ProRoot MTA may not represent the ideal material choice when long-term esthetic outcomes are a primary concern.

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Data availability The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors declare no competing interests.

Ethical approval This study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Ethics Committee of Bahçeşehir University (Protocol No. 2023-05/03; approved on March 1, 2023).

Informed Consent Informed consent was obtained from the individual who voluntarily provided the blood sample used in this study. The participant was a member of the research team, and the procedure was reviewed and approved by the Institutional Ethics Committee to ensure full compliance with ethical standards.
Additionally, extracted teeth were collected from patients who attended the Oral and Maxillofacial Surgery Clinic for routine tooth extraction procedures. Written informed consent was obtained from all patients for the use of their extracted teeth for research purposes. All samples were anonymized prior to inclusion in the study.

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