Mestrado Integrado em Medicina Dentária

Faculdade de Medicina da Universidade de Coimbra

Teeth discoloration after regenerative endodontic procedures with calcium silicate cements

An ex vivo study

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An *ex vivo* study

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Abstract

Introduction: The aim of the present *ex vivo* study was to assess and compare coronal discoloration induced by four endodontic biomaterials used in regenerative endodontic procedures – TotalFill® BC RRM™ putty (FKG), PulpGuard (Coltene), Biodentine™ (Septodont) and ProRoot® MTA (Dentisply).

Methods: Fifty-four teeth were sectioned perpendicular to its long axis, 2 mm apical to the cementoenamel junction and prepared from the apical aspect. After the preparation of apical access to the pulp chamber, specimens were randomly divided into 10 groups: group 1, negative control (*n=3*); group 2, positive control (*n=3*); group 3, TotalFill® BC RRM™ putty + saline (*n=6*); group 4, TotalFill® BC RRM™ putty + blood (*n=6*); group 5, PulpGuard + saline (*n=6*); group 6, PulpGuard + blood (*n=6*); group 7, Biodentine™ + saline (*n=6*); group 8, Biodentine™ + blood (*n=6*); group 9, ProRoot® MTA + saline (*n=6*); group 10, ProRoot® MTA + blood (*n=6*). After filling, the teeth were sealed with SDR Flow+ Bulk Fill Flowable A2 (Dentisply Caulk, Milford, USA). Color was assessed at baseline (before material placement), immediately after material filing, after 72 hours of storage, after 7 days of storage and after 4 weeks (1 month) of storage, using the Comission International de l'Eclairage L*a*b* system. Color changes, ΔE, was compared amoung the different groups and over time.

Results: There are significant differences regarding biomaterial's color variation (ΔE), considering material, treatment or both. If solely material or treatment is considered regardless time, there are no statistical significant differences. Concerning the obtained results for T_{1M}, Biodentine is the material with better color stability, followed by PulpGuard and TotallFill BC, and MTA is the material with lowest color stability.

Conclusions: After a 1-month period of evaluation, blood exposure might not be a critical factor in biomaterials color variation. Biodentine shows the greatest color stability and MTA presented the lowest, consequently leading to an increase of tooth discoloration over time. The selection of the biomaterial should consider material's discoloration potential.

Keywords: Biodentine, mineral trioxide aggregate, PulpGuard, regenerative endodontic procedures, tooth discoloration, TotalFill BC RRM putty, vital pulp therapy.

Resumo

Introdução: O objetivo do presente estudo *ex vivo* foi avaliar e comparar a descoloração coronal induzida por quatro biomateriais endodônticos utilizados em procedimentos endodônticos regenerativos - TotalFill® BC RRM™ putty (FKG), PulpGuard (Coltene), Biodentine™ (Septodont) and ProRoot® MTA (Dentisply).

Métodos: Cinquenta e quatro dentes foram seccionados perpendicularmente ao seu longo eixo, 2 mm apicalmente à junção amelocementária e preparados a partir da sua porção apical. Após a preparação da câmara pulpar, as amostras foram divididas em 10 grupos: grupo 1, controlo negativo (*n=3*); grupo 2, controlo positivo (*n=3*); grupo 3, TotalFill® BC RRM™ putty + soro (*n=6*); grupo 4, TotalFill® BC RRM™ putty + sangue (*n=6*); grupo 5, PulpGuard + soro (*n=6*); grupo 6, PulpGuard + sangue (*n=6*); grupo 7, Biodentine™ + soro (*n=6*); grupo 8, Biodentine™ + sangue (*n=6*); grupo 9, ProRoot® MTA + soro (*n=6*); grupo 10, ProRoot® MTA + sangue (*n=6*). Após preenchimento com o material, os dentes foram restaurados com SDR Flow+ Bulk Fill Flowable A2 (Dentisply Caulk, Milford, USA). A cor foi avaliada no momento inicial (antes da colocação do material), imediatamente após colocação, após 72 horas, após 7 dias e após 1 mês, utilizado o Sistema L*a*b* da Comission International de l'Eclairage. A variação de cor, ΔE, foi comparada entre os diferentes grupos e ao longo do tempo.

Resultados: Foram encontradas diferenças estatisticamente significativas relativamente à variação de cor dos biomateriais (ΔE), tendo em conta o material, o tratamento ou ambos. Se apenas o material ou o tratamento forem considerados, independentemente do tempo, não se encontram diferenças estatísticas significativas. Relativamente ao tempo de avaliação T_{1M}, o Biodentine é o material que apresente melhor estabilidade de cor, seguido pelo PulpGuard e pelo TotalFill BC, apresentando o MTA a pior estabilidade de cor.

Conclusão: Após um período de avaliação de 1 mês, a contaminação do material por sangue pode não ser um fator crítico para a descoloração dos biomateriais. O Biodentine é o material que apresenta maior estabilidade de cor, e o MTA o que apresenta menor, conduzindo por esse motivo a um aumento da descoloração dentária ao longo do tempo. Assim, a seleção do biomaterial deve ter em consideração o potencial de descoloração dos materiais.

Palavras-chave: Biodentine, mineral trioxide aggregate, PulpGuard, regenerative endodontic procedures, tooth discoloration, TotalFill BC RRM putty, vital pulp therapy.

Introduction

Tooth discoloration is a major aesthetic concern reported in multiple studies as an undesirable result of regenerative endodontic procedures (REPs). (1–10) Regenerative endodontics is defined as “biologically based procedures designed to replace damaged tooth structures, including dentine and root structures, as well as cells of the pulp-dentine complex”. (11) Therefore, regenerative endodontic procedures aim to regenerate the pulp-dentine complex damaged due to infection, trauma or development anomalies, in immature permanent teeth with pulp necrosis. (1) The association between color variation and the use of different endodontic materials in REPs, namely calcium silicate-based cements (CSCs) (12) such as mineral trioxide aggregate (MTA) and Biodentine™ (Septodont, Saint-Maur-des-Fossés, France) (5,6), is described. Thus, the selection of the biomaterial must consider functional, biological and aesthetic aspects, including its discoloration potential. (2,4,12,13)

MTA was developed as a filling material, presenting a wide span of clinical applications including REPs, pulp capping procedures, apexification and apexogenesis, as well as root resorptions, furcation defects and perforation repairs. (5,7,8,14–17) Although this bioceramic shows excellent biocompatibility and bioactivity, the original formulation (gray MTA) was associated with coronal discoloration. (5,8,9,13–15,17–20) This well-known drawback triggered the development of white MTA, by modifying the originally marketed composition. However, the latter formulation still results in dental staining. (7,8,9,11–16,18,22,23) Considering MTA main disadvantages, including its discoloration potential, several biomaterials were introduced, such as Biodentine, TotalFill® BC RRM™ putty (FKG, La-Chaux-de-Fonds, Switzerland) (5,23) and PulpGuard (Coltene/Whaledent, Altstätten, Switzerland). (24)

Nowadays, multiple hypothesis have been outlined regarding the underlying mechanisms of color alteration following REPs, with the presence of bismuth oxide within the material composition as radiopacifier (13–16,18,21,22,25) and blood contamination (2,6,10,13,15,16,21,22,26) being suggested as key-factors to trigger and exacerbate discoloration, respectively.

Current literature states that Biodentine presents superior color stability over MTA, which might be associated with the faster setting time of the formerly mentioned biomaterial. Therefore, bearing this potential explanation in mind, this study aims to analyse the discoloration potential of two more recently introduced calcium silicate-based cements - TotalFill BC and Pulp Guard – which were selected because of their shorter setting time (approximately 2 hours and 3 minutes, respectively) compared to

MTA. Moreover, the present study belongs to an ongoing research line that aims to investigate and compare the discoloration potential of several available bioactive cements used in regenerative therapy. Since the last study of the mentioned research project was conducted in acrylic teeth and evaluated the role played by blood on color stability of both Biodentine and MTA, (6) the present study intends to confirm the possible interaction between different biomaterials and blood, when in contact with human teeth dental structure.

Thus, the aim of the present *ex vivo* study was to assess and compare coronal discoloration induced by four endodontic biomaterials used in vital pulp therapy – ProRoot® MTA (Denstisply Tulsa Dental, Johnson City,TN, USA), Biodentine, TotalFill BC and PulpGuard – in the presence of blood. The null hypothesis states that there are no statistically significant differences between the experimental groups.

Materials and Methods

Specimen preparation

Fifty-four (54) premolars extracted for orthodontic purposes or periodontal reasons were included in the present study. The number of samples included in the present study was based on a previous sample size calculation, performed in G*Power 3.1 software. Only teeth clinically and radiographically free of caries, cracks, restorations and pathologic or extrinsic discolorations were selected for the experimental procedures. External surfaces of each tooth were visually inspected and cleaned with ultrasonic scaler and periodontal scalers, and polished with pumice and water in order to remove any organic material, calculus or extrinsic staining.

Root sectioning perpendicular to the long axis was performed 2 mm apical to the cementoenamel junction in all teeth.

The access cavity was performed in all specimens through retropreparation using a cylindrical diamond bur (2-mm diameter, Drendel+Zweiling, DIAMANT GmbH, Schürendreder Weg 27, 32689 Kalletal, Germany) in high speed turbine under copious irrigation. Cavities centered on the pulp chamber with 4 mm depth and 2 mm diameter were obtained, always ensuring a peripheral minimum of 1 mm of enamel and 1 mm of dentin. The access cavities were then irrigated with sodium hypochlorite (2,5% NaOCl) to remove pulp tissue remnants, followed by a 17% EDTA (CanalPro™ EDTA, Coltene/Whaledent, Altstätten, Switzerland) irrigation to eliminate the smear-layer and expose tubular dentin and a final rinse with saline solution (0,9% NaCl).

Specimens were stored in saline solution until experimental procedures were performed.

Blood collection

Blood (6 mL) sample was collected from one participant by venepuncture after informed consent, according to the approval of the Ethical Committee of IRB of the Faculty of Medicine – University of Coimbra (notification CE-001/2013). The blood collection tubes were sterile and interior coated with spray-dried tripotassium ethylenediaminetetraacetic acid (K3EDTA) to prevent clotting, thus allowing both hematology analysis and handling during sample preparation. Hematologic testing included a baseline hematocrit (percentage volume of erythrocytes) determination - hematocrit details in table 1. Blood samples were sealed and stored at 4ºC until used.

Table 1. Hematocrit, erythrocytes and hemoglobin values.

	Results	Reference values
Hematocrit	37.2 %	36.0 – 46.0 %
Erythrocytes	4.22 $x10^{12}$/L	3.80 – 4.80 $x10^{12}$/L
Hemoglobin	11.9 g/dL	12.0 – 16.0 g/dL

Experimental setup

The samples were randomly divided in 10 groups by the stratified random sampling method: one negative control group (*n*=3), one positive control group (*n*=3), and 8 experimental groups (*n*=48), as seen in figure 1.

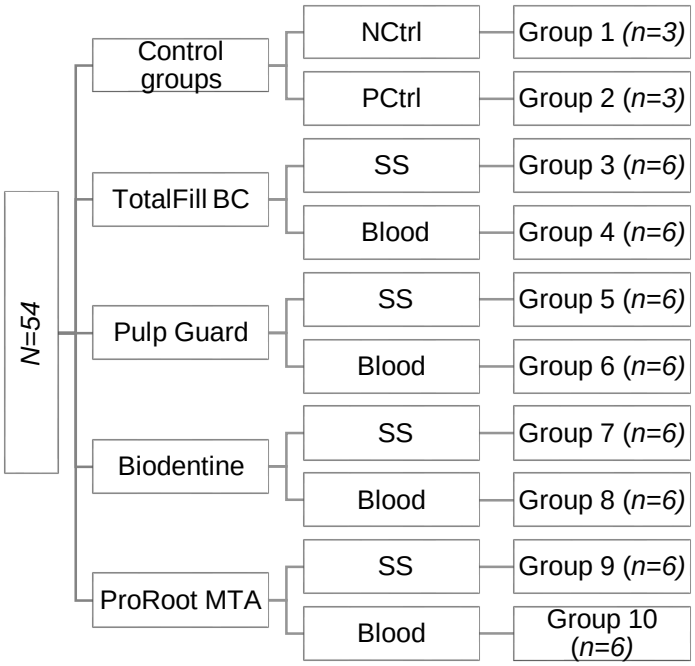


Figure 1. Description of the experimental groups (NCtrl: negative control; PCtrl: positive control; ss: saline solution)

After preoperative color measurement of each specimen, cavities in **group 1** and **group 2** were filled with a sterile cotton pellet moistened with saline (negative control) and blood (positive control), respectively. The access cavities of the remaining experimental groups were filled with different combinations of biomaterial (TotalFil BC, PulpGuard, Biodentine, ProRoot MTA) and liquid solution (blood or saline solution) as follows:

Group 3: TotalFill BC + saline solution;

Group 4: TotalFill BC + blood;

Group 5: PulpGuard + saline solution;

Group 6: PulpGuard + blood;

Group 7: Biodentine + saline solution;

Group 8: Biodentine + blood;

Group 9: ProRoot MTA + saline solution;

Group 10: ProRoot MTA + blood.

Cavities were firstly filled with either blood (groups 4, 6, 8 and 10) or saline solution (groups 3, 5, 7 and 9), using a syringe and needle with lateral exit (Kendall, MonojectTM, New York, USA). Subsequently, biomaterials were prepared according to the manufacturers' instructions (table 2) and inserted into the cavities directly over the liquid solutions.

All cavities were then sealed with a resin-based flowable composite - SDR Flow+ Bulk Fill Flowable A2 (Dentsply Caulk, Milford, USA) - light-cured for 20 s with a polywave LED curing light source (Bluephase® Style, Ivoclar Vivadent AG, Schaan, Liechtenstein).

Tooth filling and photographic record were performed in the laboratory at 23,5ºC in a 52% humidity environment.

Immediate postoperative color measurements were recorded and specimens were stored in a dark environment, in an incubator (Gallenkamp, London, UK) at 37ºC and 100% humidity until subsequent color measurement evaluation periods.

Photographic record

Photographic register was performed with a Canon EOS 5DsR camera using a Canon EF 100mm f/2.8L Macro IS USM Lens and a Canon Macro Twin Lite MT-24EX with emiters positioned at a 45º angle and cross polarization filters (polar_eyes, Emulation Group).

The following settings were used: F22 aperture, ISO 100, 1/125 shutter speed, Flash at Manual ½ power and custom white balance (with a 18% grey card – eLAB – Emulation Group).

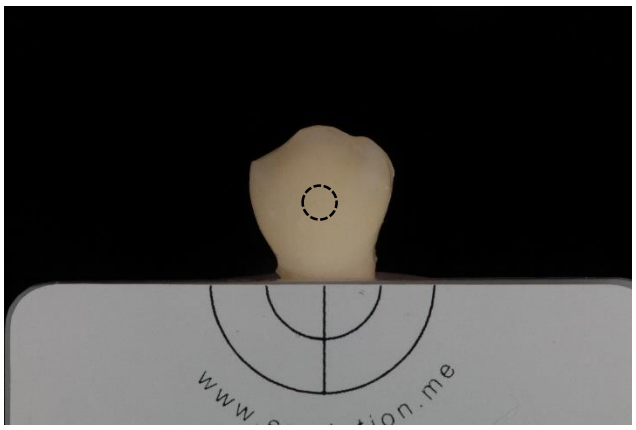
The pictures were saved in RAW file format.

The photographs were taken from de buccal wall/side of the teeth.

In order to assure standard positioning of the samples, a silicone device (Virtual Refill Putty Fast Set, Ivoclar Vivadent AG, Schaan, Liechtenstein) was prepared to hold the specimens and the grey card scale was used as a reference to properly center and place the samples, as seen in figure 2.

In post-production, before taking color measurements, all photographic registers were calibrated using the White Balance Selector and the Exposure slider of Adobe Photoshop Lightroom (Adobe Systems, CA, USA).

Figure 2. Positioning device for photographic record.



Tooth Color Measurement

Shade analysis was performed at the five following evaluation periods:

$\mathbf{T_0}$: baseline (after cavity preparation, before biomaterial placement);

$\mathbf{T_{PO}}$: immediately after biomaterial placement and provisional restoration;

$\mathbf{T_{72H}}$: after 72 hours of storage;

$\mathbf{T_{7D}}$: after 7 days of storage;

$\mathbf{T_{1M}}$: after 1 month of storage.

Color assessment was performed by a single operator using ImageJ (National Institutes of Health, NIH) software, considering a central circular area, regarding the height and width of the tooth crown, with the total area of 256x256px.

Table 2. Materials' compositions, manufacturers, preparation procedures, lot numbers and expiration dates.

Material	Composition	Manufacturer	Preparation procedure	Lot Number	Expiration Date
Biodentine™	Powder: tricalcium silicate, dicalcium silicate, calcium carbonate and oxide, iron oxide, zirconium oxide Liquid: calcium chloride, hydrosoluble polymer	Septodont, Saint-Maur-des-Fossés, France	1. Pour 5 drops of liquid into the capsule 2. Place the capsule on a mixing device 3. Mix for 30 seconds	B21190	11/2019
ProRoot® MTA	Tricalcium silicate, bismuth oxide, dicalcium silicate, tricalcium aluminate, calcium sulfate dehydrate or gypsum	Denstisply Tulsa Dental, Johnson City,TN, USA	Mix powder/liquid ratio 1:3	177918	08/2020
PulpGuard	Silicates, polydimethylsiloxane, silicon oils, platinum catalyst, zinc oxide, zirconium dioxide, bioactive glass, pigment	Coltene/Whaledent, Altstätten, Switzerland	Ready to apply using auto-mixing tips	2018120-P3-RR	08/2019
TotalFill® BC RRM™ putty	Tricalcium silicate, tantalum oxide, zirconium oxide	FKG, La-Chaux-de-Fonds, Switzerland	No mixing is required 1. Remove the desired amount of material and place it on a clean glass slab. 2. Place the material into the root canal with a sterile plastic instrument and compress it.	1702BPP	11/2019
SDR™ Bulk fill flowable composite	Barium-alumino-fluoro-borosilicate glass, strontium-alumino-fluoro-silicate glass,modified urethanedimethacrylateresin, EBPADMA,TEGMA, CQ, photoaccelerator,BHT,UV stabilizer,titanium dioxide,iron oxide pigments,fluorescing agent	Dentisply DeTreyGmbH,Konstanz, Germany	1. Dispense SDR™ material 2. Light-cure for at least 20 seconds	1803000656	02/2021

Measurements were performed using the color space defined by the Comission International de l'Eclairage (CIE) L*a*b* system, with L* values corresponding to the lightness or luminance (ranging from 0 [black] to 100 [white]), a* values matching the red-green axis (red – positive a*; green – negative a*) and the values of b* exhibiting the yellow-blue axis (yellow – positive b*; blue – negative b*).

ΔE describes the color variation between baseline and each of the subsequent color measurement periods of evaluation (T_{P0}, T_{72H}, T_{7D}, T_{1M}), determined by using the following formula:

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

Perceptible chromatic alterations for the human eye were defined at the threshold value of $\Delta E \geq 3.3$. (27)

Statistical analysis

Statistical analysis was performed with IBM SPSS Statistics version 24 software and the significance level was set at α=0.05. The description of the results within each one of the materials (TotalFill BC, PulpGuard, Biodentine and ProRoot MTA) and each of the treatment options (saline solution or blood) was performed using the mean and standard deviation values. Evaluation of ΔE values over time considering both biomaterial and treatment (blood or saline solution) was performed using a repeated ANOVA measures with two independent factors. Analysis of the results of the last period of evaluation were made using a two-way ANOVA. Additionally, description of the results of the control groups was performed to allow group comparisons.

Results

There are statistically significant differences in the assessed variables ($F(3, 92.128) = 48.729$; **$p<0.001$**) regarding the color variation (ΔE) over time. Statistical differences are also found considering the factor material ($F(9, 92.128) = 11.673$; **$p<0.001$**), the factor treatment ($F(3, 92.128) = 5.127$; **$p=0.002$**) or both material and treatment ($F(9, 92.128) = 3.643$; **$p=0.008$**). (Tables 4-6)

On the other hand, if solely the material is considered regardless the timing of evaluation, no statistical significant differences are found ($F(3, 40) = 3.468$; $p=0.076$), which also applies to treatment ($F(1, 40) = 2.084$; $p=0.157$). These findings mainly result from the dispersion observed in the different biomaterials.

Concerning the obtained results for T_{1M}, statistically significant differences are observed regarding the material (F(3, 40) = 3.400; **p=0.027**), whereas no differences were reported regarding the treatment (F(1, 40) = 1.665; p=0.204). (Figures 3 and 4)

Table 3. Mean and standard deviation values of each of the L*a*b* coordinates of each experimental group for all periods of evaluation in the presence of **saline solution**. ΔE values express color variation from baseline (T_0) to each evaluation point (T_{PO}, T_{72H}, T_{7D}, T_{1M}).

Saline solution		**BC (*n*=6)**		**PG (*n*=6)**		**BIO (*n*=6)**		**MTA (*n*=6)**	
		$\bar{x}$	sd	$\bar{x}$	$(sd$	$\bar{x}$	sd	$\bar{x}$	sd
T_0	L*	60.13	5.43	59.21	3.44	55.11	5.43	59.24	5.63
	a*	0.79	2.71	1.41	3.07	2.79	2.97	1.97	2.41
	b*	13.04	6.81	15.11	6.47	16.44	4.67	15.57	6.54
T_{P0}	L*	61.87	4.72	60.75	3.61	56.31	4.63	60.43	5.56
	a*	0.95	3.08	1.48	2.75	3.18	2.79	2.05	2.46
	b*	14.18	6.81	15.37	5.06	16.61	4.15	14.69	5.99
	ΔE 1	2.33	0.87	2.50	0.45	1.90	0.71	1.79	0.84
T_{72H}	L*	61.24	5.16	60.74	3.60	55.55	4.36	55.62	5.24
	a*	1.15	2.91	1.22	2.90	3.73	2.59	1.10	1.62
	b*	14.78	6.44	15.74	6.27	17.10	4.26	12.00	5.27
	ΔE 2	2.41	0.73	1.74	0.84	1.78	1.01	5.49	1.07
T_{7D}	L*	60.79	4.88	56.82	3.56	52.46	5.02	52.29	5.11
	a*	1.24	3.02	1.19	2.73	3.32	2.73	0.83	1.47
	b*	14.56	6.57	14.57	5.97	16.20	3.77	11.06	4.89
	ΔE 3	2.21	0.50	2.58	1.53	2.89	0.70	8.63	1.17
T_{1M}	L*	60.69	5.68	59.96	3.30	54.78	3.97	54.10	5.72
	a*	0.97	3.21	1.24	2.89	3.71	2.54	0.31	1.68
	b*	15.64	5.81	15.86	6.40	16.47	3.90	11.57	4.44
	ΔE 4	2.98	1.59	2.04	1.03	2.55	1.16	7.22	1.94

BC: TotalFill BC; BIO: Biodentine; MTA: ProRoot MTA; PG: PulpGuard; sd: standard deviation

Table 4. Mean and standard deviation values of each of the L*a*b* coordinates of each experimental group for all periods of evaluation, in the presence of **blood**. ΔE values express color variation from baseline (T_0) to each evaluation point (T_{PO}, T_{72H}, T_{7D}, T_{1M}).

Blood		BC (*n*=6)		PG (*n*=6)		BIO (*n*=6)		MTA (*n*=6)	
		$\bar{x}$	sd	$\bar{x}$	sd	$\bar{x}$	sd	$\bar{x}$	sd
T_0	L*	59.07	7.76	58.59	6.93	60.64	4.16	58.41	5.07
	a*	0.52	2.53	1.99	2.19	0.81	2.35	11.93	25.69
	b*	11.02	4.32	15.86	6.82	12.78	5.46	13.04	7.79
T_{P0}	L*	59.37	6.96	58.63	4.85	60.50	3.97	58.60	4.32
	a*	2.75	3.17	3.94	1.99	0.89	2.18	1.45	1.57
	b*	10.33	4.51	14.32	7.08	12.18	4.80	13.46	4.33
	ΔE 1	3.12	2.13	3.29	1.22	1.59	1.38	12.74	25.47
T_{72H}	L*	58.65	5.90	54.98	5.94	60.92	4.09	54.52	4.06
	a*	1.68	2.99	4.78	3.71	1.39	2.49	-0.02	2.05
	b*	11.37	3.51	15.80	4.85	13.96	4.52	11.23	4.02
	ΔE 2	2.49	0.91	5.55	2.90	2.07	0.97	15.75	25.40
T_{7D}	L*	55.77	5.47	51.01	5.64	57.26	3.91	51.27	3.99
	a*	1.54	2.88	3.97	3.36	0.96	1.94	-0.38	2.02
	b*	10.17	3.30	14.52	4.56	12.44	3.77	9.74	3.81
	ΔE 3	4.60	1.75	8.41	2.97	3.83	1.20	18.67	24.07
T_{1M}	L*	58.93	5.82	54.44	5.41	60.13	3.75	53.06	3.58
	a*	1.21	2.96	3.30	3.16	1.22	2.60	-0.67	1.93
	b*	11.00	2.65	15.22	4.65	13.58	4.11	10.22	2.86
	ΔE 4	3.23	1.48	5.13	3.03	2.06	1.27	17.68	24.83

BC: TotalFill BC; BIO: Biodentine; MTA: ProRoot MTA PG: PulpGuard; sd: standard deviation

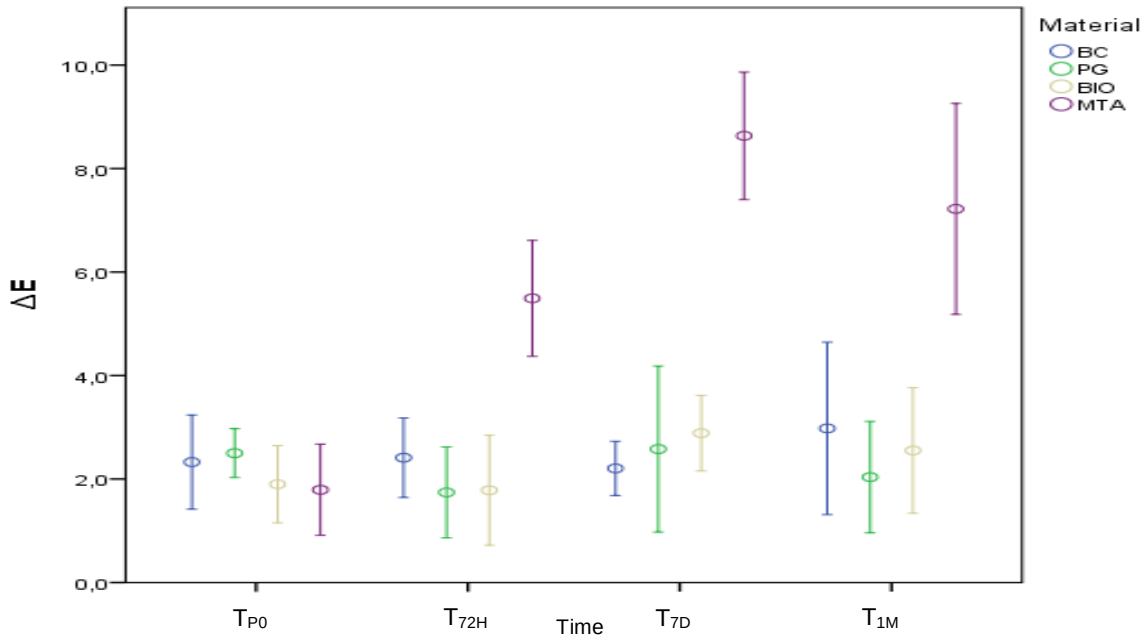


Figure 3. ΔE variation of the groups with **saline solution** over time.

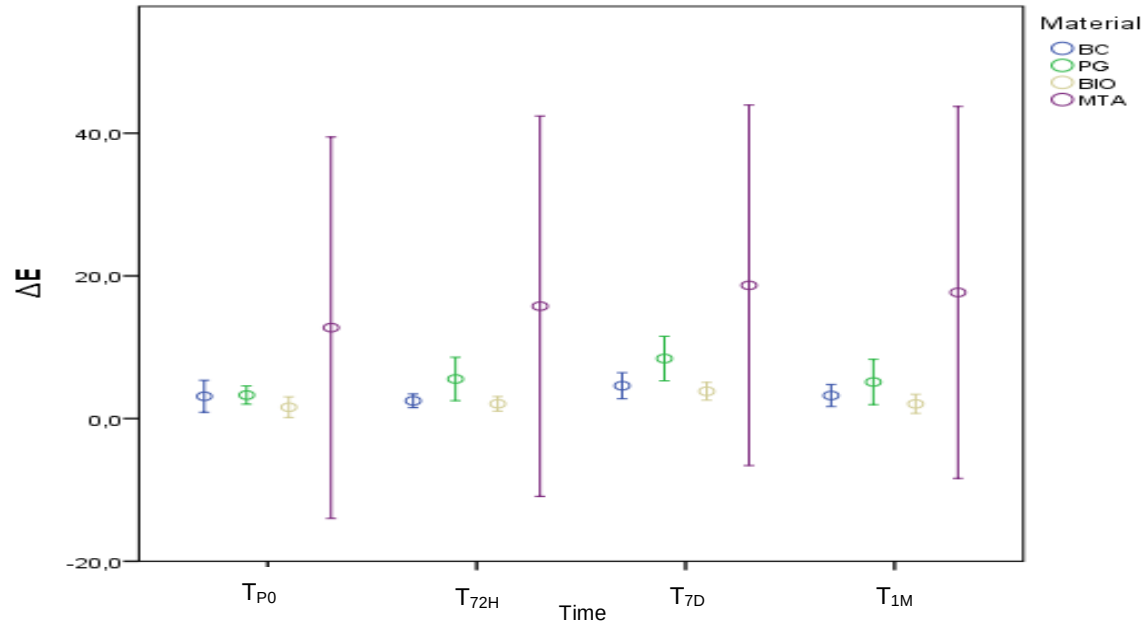


Figure 4. ΔE variation of the groups with **blood** over time.

Table 5. Material's p values after post-hoc tests.

	PG	BIO	MTA
BC	0.999	0.996	0.065
PG	-	0.985	0.087
BIO	-	-	**0.039**

BC: TotalFill BC; BIO: Biodentine; MTA: ProRoot MTA PG: PulpGuard.

Table 6. Mean and standard deviation of ΔE values of the control groups for all periods of evaluation ($\Delta E1$=T_{0}-T_{PO}, $\Delta E2$=T_{0}-T_{72H}, $\Delta E3$=T_{0}-T_{7D}, $\Delta E4$=T_{0}-T_{1M}).

	ΔE1		ΔE2		ΔE3		ΔE4	
	$\bar{x}$	sd	$\bar{x}$	sd	$\bar{x}$	sd	$\bar{x}$	sd
SS	3.82	2.14	2.02	0.73	2.16	0.92	1.73	0.65
Blood	4.41	1.43	9.19	3.53	7.83	2.93	6.30	2.89

sd: standard deviation; ss: saline solution.

Discussion

The aim of the present *ex vivo* study was to evaluate and compare the discoloration potential of four different CSCs used in regenerative endodontic procedures, as well as to assess the role played by blood's contamination on color variation severity when CSCs are applied. Theoretically, recently introduced biomaterials (Biodentine, TotalFill BC and PulpGuard) with a faster setting time when compared to the gold standard MTA might present superior color stability. Moreover, it is crucial to unveil the possible role played by blood when in contact with CSCs since blood exposure is likely to occur in both REPs and vital pulp therapy. An insight on these matters would allow to adequately select the biomaterial to use based on the clinical conditions, ultimately allowing the accomplishment of better esthetic results.

In fact, it has been extensively reported that REPs lead to dental discoloration. This undesirable consequence of regenerative therapy is a major concern for both clinicians and patients, especially when aesthetic area is involved. (1-10) REPs protocol involves the creation of a cervical plug by placing a 3-4mm CSC layer in the coronal portion of the root canal. Endodontic materials should present chromatic stability and optic properties similar to dental

structures and not cause teeth discoloration over time. (13) However, as previously mentioned, the association of CSCs with color variation over time is well-described within literature.

The obtained results show that there are statistically significant differences in ΔE over time (**p<0.001)**, thus the null hypothesis, stating that there are no statistically significant differences between the experimental groups, has been rejected. In fact, statistical differences were found considering the factor material (**p<0.001)**, the factor treatment (**p=0.002)** or interaction of both factors (**p=0.008**), over time.

Several possible mechanisms have been outlined to explain color alteration following CSCs placement, being that the composition of the biomaterial presents as a major contributing factor to CSCs discoloration potential. (22) MTA formula contains bismuth oxide as a radiopacifier (Bi_2O_3), which has been proven to lead to tooth discoloration through its (1) reduction or (2) oxidation when in contact with strong oxidizing agents, such as dentin collagen and sodium hypochlorite, used as an irrigation solution. (22) Both bismuth reduction and oxidation result in dark precipitates formation, consequently leading to tooth staining when bioceramics comprising bismuth oxide are used. In fact, previous studies consistently demonstrate MTA to exhibit the lower color stability when compared to alternative calcium silicate-based cements, (3,4,6,7,9,10,12) hypothesizing that the obtained results could arise from the incorporation of different radiopacifiers such as zirconium oxide and tantalum oxide, instead of bismuth oxide, in the more recently introduced CSCs. (22)

Concerning blood contamination, the overall available scientific evidence shows blood exposure to be a factor that significantly exacerbates CSCs color alteration. (2) This discoloration severity increase by blood might be related with material porosity and presence or absence of smear-layer, which can reduce or increase dentin permeability, respectively. MTA shows a longer setting time (2 hours and 45 minutes) compared with all the remaining three tested materials (Biodentine, 12 minutes; TotalFill BC, 2 hours; PulpGuard, 3 minutes). Therefore, we hypothesized MTA to remain porous for longer which results in increased blood absorption and subsequent hemolysis, with consequently superior discoloration. On the other hand, a recent in vitro study found that the contact with blood after a follow-up period of 6 months does not modify color alterations suffered by the biomaterials. Our findings are in agreement with the results of Palma *et al.,* (6) since after a 1-month period the present study found statistically significant differences regarding the factor material, whereas no differences were reported regarding the treatment (blood/saline solution). It is noteworthy that the study of Palma *et al.* (6) was conducted in an acrylic model, while the present study was performed under ex vivo conditions. However, results from both studies appear to be in accordance, which might corroborate the hypothesis of material being the critical factor for discoloration instead of the conditions of the treatment. According to the present study, regardless the treatment,

after material placement, MTA was the CSC with the highest ΔE at every measurement point, which reflects that MTA presents the lowest color stability of all tested biomaterials. Furthermore, the analysis of MTA ΔE values are mostly perceptible by the human eye ($\Delta E \geq 3.3$), therefore impairing the esthetic outcome of the procedure.

Nowadays, Biodentine presents a viable alternative biomaterial to MTA. Besides presenting zirconium oxide within its formulation, this bioceramic exhibitis a shorter setting time. Previous studies found greater color stability with Biodentine, (6,12,28) which might be explain by the absence of bismuth oxide within its formulation and because of the faster setting time that possibly limits blood sorption.

More recently, two different biomaterials were introduced (TotalFill BC and PulpGuard), bismuth-free and, as previously referred, with a shorter setting time when compared to MTA, which might be predictive of a favourable result concerning its color stability. To our knowledge, although a favourable cytocompatibility profile of both biomaterials was reported in previous studies (24), there is no published data concerning color variation of both TotalFill BC or PulpGuard. (22,24) According to the results of the present study, TotalFill BC, like Biodentine, do not cause significant color change compared with MTA.

When treated with saline solution MTA presented the highest ΔE in all measurement points, except for T_0, and Biodentine the lowest ΔE in all measurement points, except fot T_{1M}. After 1 month of storage, Pulp Guard presented the lowest ΔE value, followed by Biodentine, TotalFill BC and, finally, MTA. On the other hand, when treated with blood, Biodentine is the material presenting the lowest ΔE, followed by TotalFill BC, PulpGuard and, lastly, MTA, at all measurement points.

To summarize, the choice of the material presents the critical factor for the obtainment of a successful esthetic outcome. Accordingly, to our findings at T_{1M}, Biodentine is the material that shows greater color stability, followed by PulpGuard and TotalFill BC, and MTA is the material with lowest color stability. These considerations are verified either in the presence or absence of blood during regenerative procedures. Furthermore, in addition to showing the lowest discoloration potential, it is noteworthy that previous studies demonstrate that Biodentine might allow the execution of bonding procedures directly over it in an immediate time frame (12 minutes after biomaterial placement), therefore potentially maximizing this procedure's long-term success. (29)

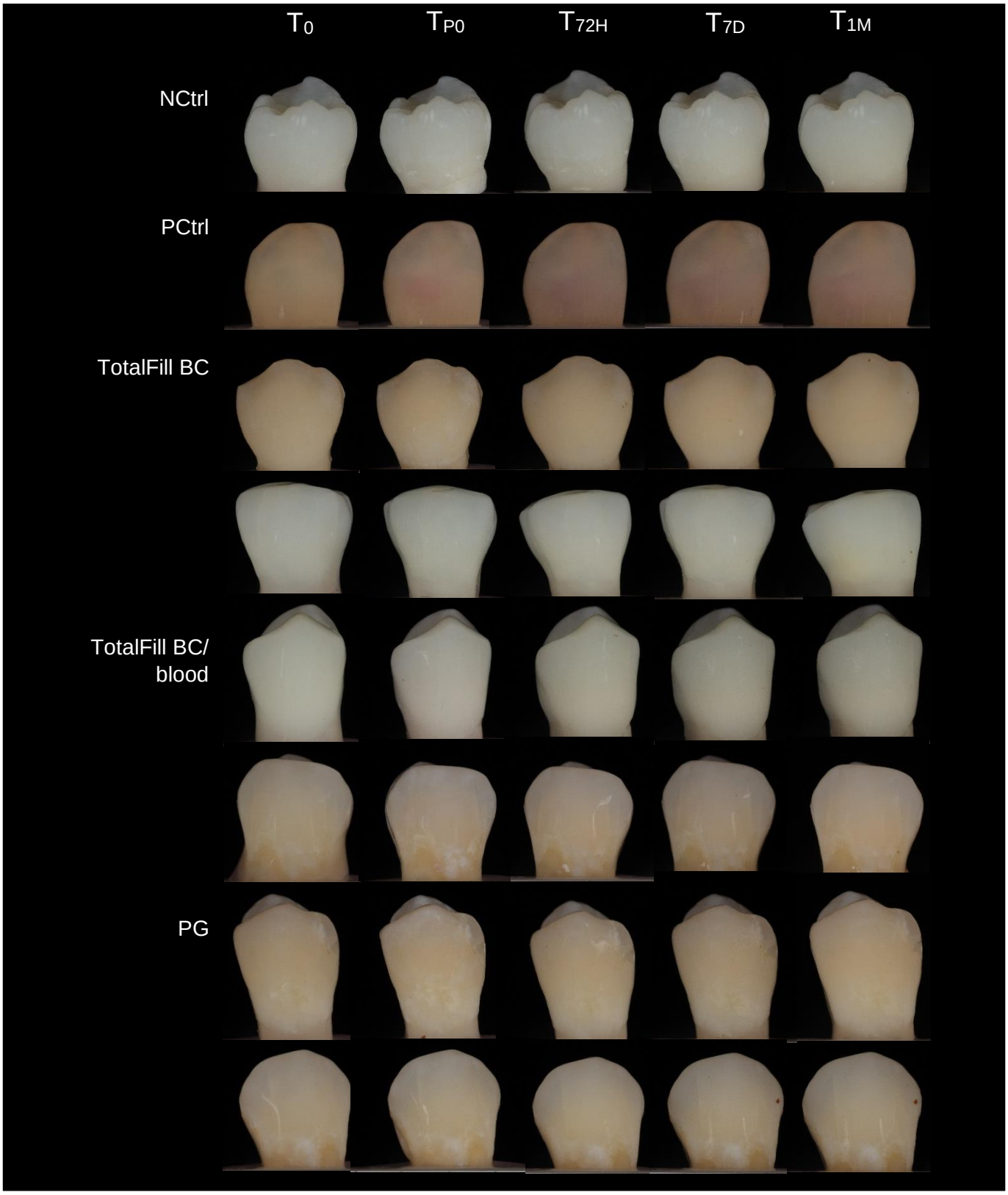
Regarding the limitations of the present study, the absence of positive pulp pressure inherent to an ex vivo model might limit the blood's flow towards the periphery. Therefore, in the future, the role played by blood's exposure should be confirmed in vivo. Moreover, a longer follow-up period is desirable to confirm the obtained results at a long-term perspective.

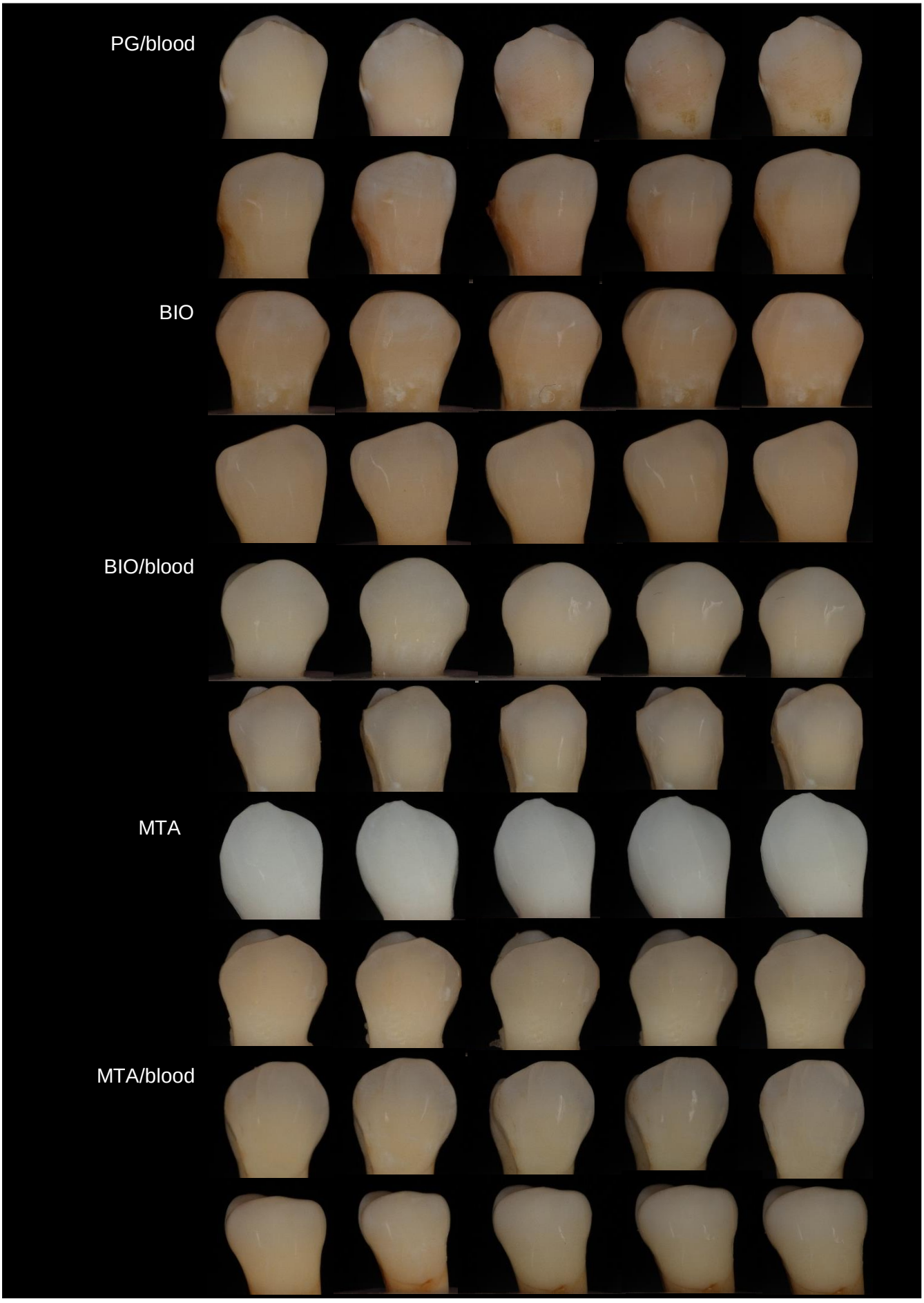
Conclusion

Within the limitations of the present study, our findings suggest that, after a 1-month period of evaluation, blood exposure might not be a critical factor in biomaterials color variation. Biodentine shows the greatest color stability, followed by PulpGuard and TotalFill BC. MTA presented the greatest color variation, consequently leading to an increase of tooth discoloration over time. The selection of the biomaterial should consider materials' discoloration potential.

Appendices

Appendix I. Representative samples of each study group at every measurement point (BC: TotalFill BC; BIO: Biodentine; MTA: ProRoot MTA; NCtrl: negative control; PG: PulpGuard; PCtrl: positive control; T_0: baseline; T_{P0}: immediately after material placement; T_{72H}: 72 hours after material placement; T_{7D}: 7 days after material placement; T_{1M}: 1 month after material placement)




PG/blood
BIO
BIO/blood
MTA
MTA/blood

This work was carried out using the means provided by the laboratory of mechanical tests and preparation of samples of the area of Dental Medicine.

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