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Tooth discolouration analysis induced by two Calcium Silicate-based Materials: *in vitro* study

Author: Ana Rita Bernardes Nascimento

Advisor: Paulo Palma, DMD, MSc

Co-advisor: João Carlos Ramos, DMD, PhD

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Tooth discolouration analysis induced by two Calcium Silicate-based Materials: *in vitro* study

Rita Nascimento*, Paulo Palma**, João Carlos Ramos***

* 5th year student of Integrated Master in Dentistry, Faculty of Medicine, University of Coimbra

** Assistant Lecturer and PhD student of Health Sciences, Faculty of Medicine, University of Coimbra

*** Assistant Professor of Integrated Master in Dentistry, Faculty of Medicine, of University of Coimbra

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Faculty of Medicine of the University of Coimbra
The Dentistry Department of Coimbra University Hospitals
Av. Bissaya Barreto, Blocos de Celas
3000-075 Coimbra
Portugal

Author's e-mail: ritznasc@gmail.com

Abstract

Aim: to evaluate the over time chromatic alterations/discolouration in tooth crowns induced by two Calcium silicate based-materials (CSMs) - White mineral trioxide aggregate (WMTA- ProRoot® MTA) and Biodentine™ - used to fill pulp chambers.

Materials and Methods: Twenty-eight premolar human teeth were sectioned 2 mm below cemento-enamel junction (CEJ). Pulp tissues were extirpated through the cervical cut, and occlusal standardized cavities were prepared. The specimens were split according to a stratified random sampling by value into four groups: Group 1- Negative control (filled with dry sterile cotton pellets) (n=4); Group 2- Positive control (filled with blood moistened sterile cotton pellets) (n=4); Group 3- WMTA (ProRoot® MTA) (n=10); Group 4- Biodentine™ (n=10). Colour measurements were recorded with Colorimeter (PR®-650 SpectraScan® Colorimeter, Topanga Canyon Place Chatsworth, CA) at 3 time points: T0, baseline; T1, immediately after material placement, and T2, 6 weeks after. The measurements were taken under standardized conditions: dark room and a white cardboard box illuminated with a LED light of 5500k and 6 watts. Data were transformed into values of the CIE L*a*b* colour system, and the corresponding colour differences (ΔE) values were calculated. Between the measurements the specimens were stored in tap water, in the dark, in a 100% humidity environment at 37°C, with normal atmospheric gas levels. The results were analysed using repeated ANOVA measurements, unilateral t-Student test considering a cut point of 2.3, mixed ANOVA procedures, Kruskal-Wallis test, Mann-Whitney test, and one-way ANOVA with Tukey post-hoc tests. The significance level was set at $\alpha<0.05$.

Results: There were not found statistically significant differences in colour alteration (ΔE) between the two CMSs (group 3-WMTA and 4-Biodentine™), for all time intervals. Relative to L* parameter, it is possible to observe statistically significant differences, between WMTA and Biodentine™.

Conclusion: Concerning global colour variation (ΔE), there was no significant difference between the two CMSs (WMTA and Biodentine™), for all time intervals, over time. However, relative to L* parameter, it was possible to observe statistically significant differences, between WMTA and Biodentine™, being the L* variation for WMTA higher, indicating a greater tooth discolouration in terms of value.

Keywords: Tooth discolouration, tooth staining, white MTA, Biodentine, spectophometry, CIE colour system .

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1. Introduction

The aesthetic appearance of teeth is an issue of primary concern both to patients and professionals(1). One of the most common clinical situations that impairs the aesthetic smile appearance is an anterior tooth submitted to endodontic treatment, that often undergoes discolouration (2-4), and significantly affects the quality of life of patients(5). Progressive discolouration of endodontically treated teeth was primarily related to the ingression of materials into the dentinal tubules(3,4). However, visible crown discoloration may be caused by the presence of material remnants in the pulp chamber, which gets darker over time, thus transferring the colouration through the hard tissues (6).

Considering the increasing demands for aesthetics, biomaterials used in clinical endodontics should be chromatically stable, present optical properties similar to dental structures and exhibit no staining effects on hard dental tissues over time(7). Thus, the choice of a specific material should not rely solely on their biological and functional aspects, but also on aesthetic considerations (2,8). Material-induced tooth discolouration may be prevented to some extent by avoiding substances with a high risk of staining(2).

In the clinical setting, professionals have to deal regularly with exposed dental pulps. For those cases and under specific circumstances a direct pulp capping can be performed and elected as the treatment of choice(9). This treatment aims at the maintenance of pulp vitality and involves the placement of a biocompatible agent on pulp tissue that has been exposed. Two major objectives are the sealing of the pulp pathways against bacterial penetration while encouraging cell differentiation to wall off the exposure site by initiating a dentine bridge, and the maintenance of a healthy pulp tissue(10). In some cases all of the coronal pulp must be removed, and subsequently a pulpotomy has to be performed adjacent to the root pulp, particularly in cases of large pulp exposure or when clear superficial pulp infection can be noticed(11). Wide ranges of materials have been suggested for dealing with pulp exposure(10). Some studies have considered that calcium hydroxide should be considered the gold standard for pulp wounds treatment(12-14). This compound was found to be able to stimulate dentin bridge formation leading to pulp healing, and thus providing high success rates for clinical procedures(15). However, some of the limitations reported with calcium hydroxide include the presence of tunnels in the dentine barrier, extensive dentine formation obliterating the pulp chamber, high solubility in oral fluids, lack of adhesion and degradation after acid etching. Therefore, this material may fail to provide an effective long-term barrier against bacterial

penetration(16). Due to those limitations, a variety of new materials have recently been proposed as candidates for direct pulp capping.

Calcium silicate–based materials (CSMs) such as mineral trioxide aggregate (MTA)(17,18) and Biodentine™(19), have antibacterial properties and excellent biocompatibility, sealing ability, and regenerative capabilities(17,18). These significant inherent advantages make them versatile materials that can be used in several biological treatment options(20).

Biologically, MTA is regarded nowadays as the material of choice in vital pulp therapy cases (pulp capping, partial pulpotomy, pulpotomy), or to seal pathways of communication between the root canal system and the external root surface (perforations, apexification or retrograde filling)(18,21,22). Besides the good biocompatibility, MTA has been recognized as a bioactive material that is, conductive, hard tissue and inductive hard tissue (22,23). However, despite such good characteristics, the main drawbacks of MTA include: discolouration potential, presence of toxic elements in the material composition, demanding handling characteristics, long setting time, high material cost, absence of a known solvent for this material, and the difficulty of its removal after curing(23). The original colour of MTA was grey (grey MTA or GMTA), and it had a serious potential of tooth discolouration. Because of this disadvantage, white MTA (WMTA) was introduced for application in aesthetically sensitive areas (22,23). Nevertheless, studies have reported tooth discoloration after using both kinds of MTA (24,25). Also, some other clinical studies reported tooth discoloration when WMTA was applied in pulpotomies, in apical barriers or coronal biological barriers in cases of pulp revascularization (26,27). Conversely, a recent but single case report, indicated that marginal gingival discolouration induced by GMTA was improved when replaced by WMTA(28).

Biodentine™ (Septodont, Saint Maur des Fossés, France) is the newest calcium silicate-based material that exhibits physical and chemical properties similar to those described for certain Portland cement derivatives(29). This material, with dentin-like mechanical properties, can be used as a dentin substitute on crowns and roots. Compared to MTA, Biodentine™ handles easily, and has a short setting time of 12 minutes(30). On the biological level, it is perfectly biocompatible(19) and capable of inducing reactionary dentin apposition by stimulating odontoblast activity(31) and reparative dentin, by induction of cell differentiation(32). Unlike other Portland cement-based products, it is sufficiently stable so that it can be used both for pulp protection and temporary fillings(33). However, there are no available data on the colour stability of Biodentine™(20).

The aim of this study is to evaluate the specific degree of chromatic alterations/discolouration in tooth crowns induced by WMTA (ProRoot® MTA, Dentsply Tulsa Dental, Johnson City, TN) and Biodentine™ (Septodont, Saint Maur des Fossés, France), over time, as determined by the Comission International de l'Eclairage's (CIE) L*, a* and b* system, and the corresponding colour difference values (ΔE). The null hypothesis (Ho) is that there are no statistically significant differences in colour variation between the two CSMs (WMTA and Biodentine™), over time.

2. Materials and methods

2.1 Tooth preparation

Twenty-eight extracted premolar human teeth were used. All tooth crowns were clinically and radiographically examined to be free of caries, restorations and pathologic discolorations. The roots were sectioned 2 mm below the cemento-enamel junction (CEJ). The coronal surfaces of each tooth were meticulously cleaned with scalers and polished with pumice and water, removing calculus and extrinsic stains. Pulp tissues were extirpated, via the cervical cut with Hedstroem files. No attempt was performed to remove the smear layer. A single step self-etching dental adhesive (Xeno® III, Dentsply De Trey, Konstanz, Germany) was placed at the base of each tooth crown, and cured for 10 seconds (Bluephase® G2, Ivoclar-Vivadent, Schaan, Liechtenstein). Each tooth was then inserted in a standardized putty model that was partially filled with a fluid composite resin (Sinergy ®D6 Flow, Coltène Whaledent, Altstatten, Switzerland) and polymerized (Bluephase® G2, Ivoclar - Vivadent, Schaan, Liechteistein) for 40 seconds, in order to achieve a stable basis for each tooth. Standardized occlusal cavities were prepared under copious water coolant with the same cone bur (980.130, Komet, U.S.A.), until the pulp chamber was reached. The samples were stored in tap water, refrigerated at 3ºC, until initiating the experimental protocol.

2.2 Experimental and control groups

The specimens were split into a stratified random sampling by value, into 4 groups according the material used to fill the standardized cavities: 2 experimental groups (n=10) and 2 control groups (n=4) (**Table I)**.

Table I. Experimental and control groups

Group	Filling material	Manufacturer	Composition	Lot and exp. date
1 (negative control)	Dry sterile cotton pellets	---	---	---
2 (positive control)	Blood moistened sterile cotton pellets	---	---	---
3	ProRoot ® MTA	Dentsply Tulsa Dental, Johnson City, TN	75 % Portland cement (Pc), 20 % bismuth oxide (Bi_2O_3), and 5 % calcium sulfate dehydrate	12001879 2015/04
4	Biodentine ™	Septodont, Saint Maur dés Fosses, France	**Powder:**Tri-calcium silicate (C3S), Di-calcium Silicate (C2S), Calcium Carbonate and Oxide, Iron Oxide, Zirconium Oxide. **Liquid:** Calcium Chloride, Hydrosoluble polymer.	B05574 2014/04

--- No applicable

Colour measurements were recorded at 3 time points: T0: baseline (after preparation of the cavities, but before placement of the materials); T1: immediately after filling material placement and provisional restoration; and T2: after 6 weeks upon retrieval.

After T0, for the experimental groups (3 and 4), WMTA (ProRoot MTA, Dentsply Tulsa Dental, Johnson City, TN) and Biodentine™ (Septodont, Saint Maur dés Fosses, France) were prepared according to the manufacturer's recommendations. With a cylinder amalgam carrier it was possible to fill the cavities with a standardized volume of material (a cylinder with 2mm diameter and 5mm height). Then, the materials were compressed into the pulp chamber with a compacter. In the control groups (1 and 2), dry sterile cotton pellets with and without human blood (collected by pricking the finger of a volunteer), were placed into the cavities, also with the aid of a compacter. **Figure 1** represents a random sample of each of the 4 groups, with the different materials inserted, before the application of the provisional restoration.

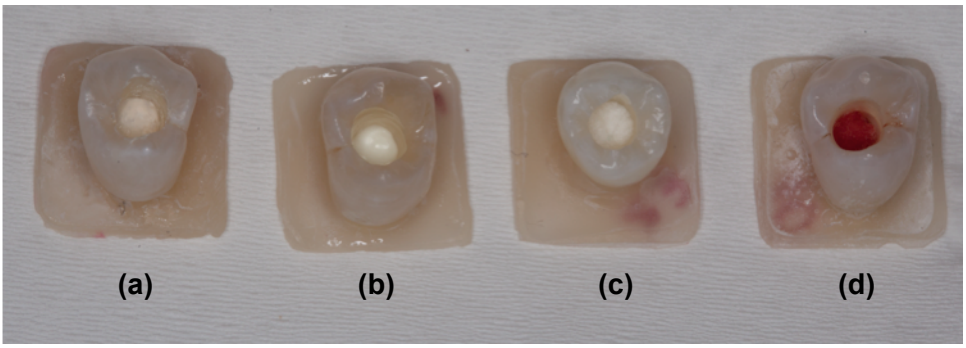

Figure 1. Random sample of each of the 4 groups, with the different materials inserted, before the application of the provisional restoration. **(a)** Group 3: WMTA; **(b)** Group 4: Biodentine™; **(c)** Group 1: Negative control; **(d)** Group 2: Positive control.

After de base filling material, the cavities were restored with a restorative glass ionomer cement (Ketac™ Fil Plus Aplicap™, 3M ESPE, AG, Germany) colour A1, which was prepared as indicated by the manufacturer's recommendations, and T1 measurements were performed. After the measurements in T0 and T1, the specimens were stored in the dark, in a 100% humidity environment at 37°C with normal atmospheric gas levels, until 6 weeks upon retrieval, moment of the third measurement (T2).

2.3 Colorimeter measurements

To evaluate and compare the specific degree of chromatic alterations/discolouration in tooth crowns, induced by the materials, a Colorimeter (PR®-650 SpectraScan® Colorimeter, Topanga Canyon Place Chatsworth, CA) was used by a single operator (**Figure 2)**.

Figure 2. Colorimeter PR®-650 SpectraScan® (Topanga Canyon Place Chatsworth, CA) used in the experiment. Retrieved from: http://www.hometheater.com/content/ht-measurements-explained-rgb-triangle-death-video-measurements-page-2 .

The measurements were performed in a dark room, under standardized conditions of distance and direction, in a custom-built measuring station. This custom-built measuring station was composed by a sconce with a LED light of 5500k and 6 watts (simulating natural day light), and a white cardboard around the light zone (for greater light reflection during the measurements). **Figure 3** represents a scheme of this custom-built measuring station.

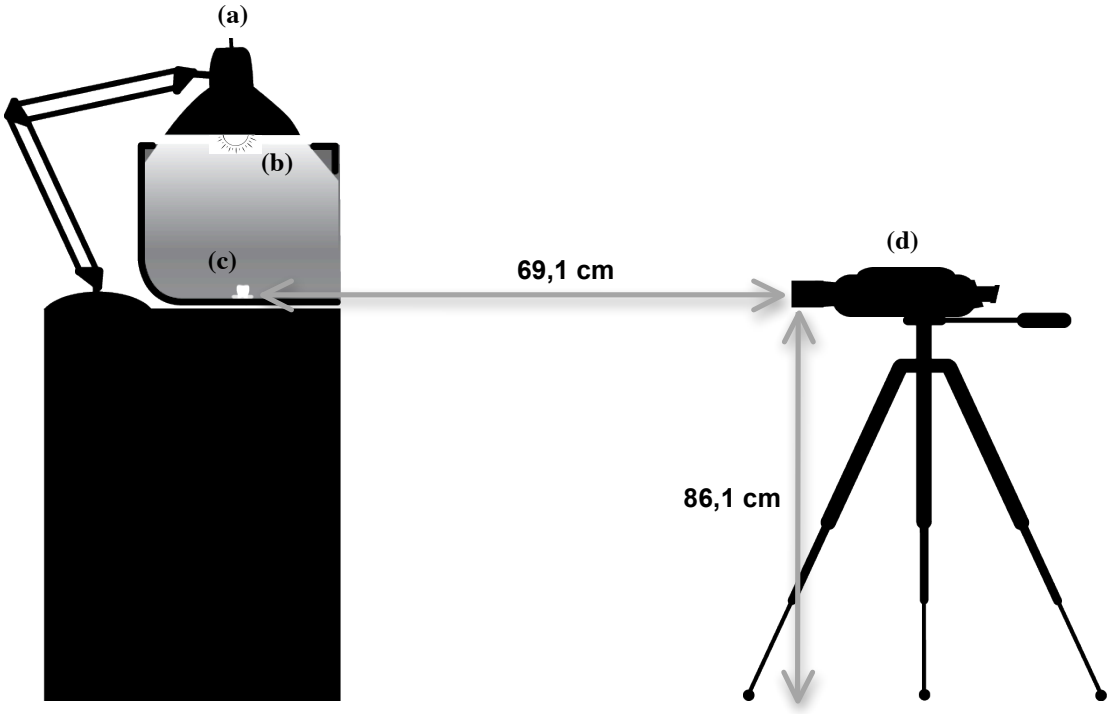

Figure 3. Custom-built measuring station scheme, showing the standardized measures: **(a)** Sconce; **(b)** LED light; **(c)** Sample; **(d)** Colorimeter

The Colorimeter objective presents a black sphere corresponding to the area where colour measurement was performed calibrating the sphere localization up to the tooth CEJ and between tooth mesio-distal distance, as represented in **Figure 4 (b)**.

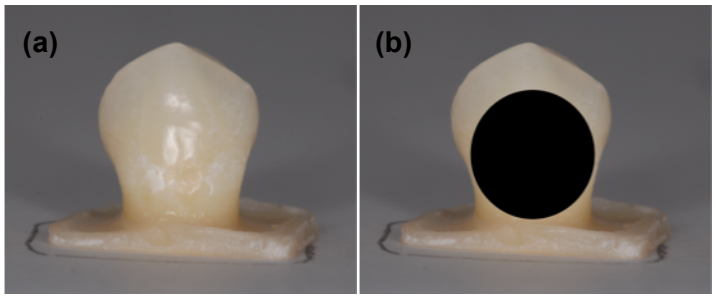

Figure 4. **(a)** Open sight visualization of a sample; **(b)** Sample looked thought colorimeter, revealing the black sphere localized up to the CEJ tooth mesio-distal distance.

2.4 Tooth shade assessment

Instrumental measurements, including dental colorimeters and spectrophotometers, commonly utilize the International Commission on Illumination (CIE, from the french *Comission International de l'Eclairage's*) L*a*b* system. CIE is an organization recognized by ISO as an international standardization body on the subject of light, vision, and colour(34). In 1931, CIE has defined mathematically a colour space (CIE XYZ), which defines a colour by three coordinates. In 1976, a new perceptual colour space L*a*b* (CIE LAB) was introduced, and aimed at finding greater uniformity of colour perception relative to XYZ space. The total colour difference between two objects can be expressed numerically by their Euclidean distance, in ΔE values. Therefore, the difference between two colours, (L^*_1, a^*_1, b^*_1) e (L^*_2, a^*_2, b^*_2) is given by:

$$\Delta E=[(L^*_1 - L^*_2)^2 +(a^*_1 - a^*_2)^2 + (b^*_1 - b^*_2)^2]^{1/2}$$

In this study, the colour measurements were taken directly in CIE 1931 *xyY* colour space (x,y coordinates and luminance Y, cd/m^2). It is important to refer that perceptible threshold of chromatic alterations for the human eye is defined by $\Delta E \geq 2.3$, and ΔE is defined by the expression $\Delta E = [(\Delta L^*)^2 +(\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$, where ΔL is the change in luminance [from 0 (black) to 100 (white)], Δa^* is the change in the red (positive a*) to green (negative a*) parameter, and Δb^* is the change in the yellow (positive b*) to blue (negative b*) parameter. Therefore, it was necessary to transform the initial colour coordinates into the colour space L*a*b*. To determine these coordinates L*, a* e b*, the coordinates X, Y e Z have to be calculated first:

$$X = \frac{x}{y} Y$$

$$Y = Y$$

$$Z = \frac{1 - x - y}{y} Y$$

With these values, it was possible to calculate L*a*b* coordinates:

$$L^* = 116\, f\left(\frac{Y}{Y_0}\right) - 16$$

$$a^* = 500\left[f\left(\frac{X}{X_0}\right) - f\left(\frac{Y}{Y_0}\right)\right]$$

$$b^* = 200\left[f\left(\frac{Y}{Y_0}\right) - f\left(\frac{Z}{Z_0}\right)\right]$$

- X_0,Y_0, Z_0 : white point reference (in this case, the initial background measure).
- Function f: $f(x) = \begin{cases} x^{\frac{1}{3}} & se \quad x > \left(\frac{6}{29}\right)^3 \\ \frac{1}{3}\left(\frac{29}{6}\right)^2 x + \frac{4}{29} & se \quad x \leq \left(\frac{6}{29}\right)^3 \end{cases}$

2.5 Statistical analysis

Statistical analysis was performed using IBM® SPSS® Statistics, version 20.

Evaluation of variation of chromatic coordinates L* a* b* over time (T0, T1, T2) for each group (1,2,3 and 4), was carried out with repeated ANOVA measurements.

In order to analyse colour changes for the 4 groups over time, three different colour intervals (ΔE) were calculated: ΔE (T0-T1), ΔE (T1-T2) and ΔE (T0-T2), using the expression $\Delta E = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$. For these time intervals, in each group, a unilateral t-Student test was realized considering the cut point value of 2.3 as the minimum threshold for perceptible changes in colour difference.

Mixed ANOVA procedures were used to verify coordinate L* evolution, considering time (T0, T1 and T2) as the within subjects effects and considering groups (1,2,3 and 4) as between subjects effects.

Kruskal-Wallis test was realized in order to discover if there were statistically significant differences between the groups (1,2,3 and 4), for all experimental moments, regarding L* variation parameter. With Mann-Whitney test it was possible to analyse which group pairs had statistically significant differences in coordinate L*.

Colour differences (ΔE) between T0 and T1, T1 and T2, T0 and T2, were calculated and compared for the 4 groups, using a paired sample test. One-way ANOVA and Tukey post-hoc tests were applied to verify the significant statistical differences between the groups (1,2,3 and 4), regarding colour variation.

The significance level was set at $\alpha < 0.05$.

3. Results

In **Table II**, the descriptive statistics obtained for chromatic coordinates are represented, in the different groups measured over time (T0, T1 and T2). The luminance Y (Cd/m^2), the chromatic coordinates x y are the direct measurements that were performed, whereas L*, a* e b* were calculated according to the formulae previously presented.

Table II. Chromatic coordinates for the 4 groups in T0, T1 and T2.

		Group 1		Group 2		Group 3		Group 4	
		Average	*std*	Average	*std*	Average	*std*	Average	*std*
T0	**Y**	975	58	974	55	954	63	970	41
	x	.365	.007	.365	.006	.367	.006	.364	.004
	y	.378	.006	.378	.005	.379	.005	.377	.003
	L*	90	2	90	2	89	2	90	1
	a*	.585	.634	.484	.261	.792	.716	.619	.400
	b*	7.012	3.694	6.798	3.163	7.524	2.872	6.209	1.914
T1	**Y**	957	64	923	17	944	45	943	47
	x	.366	.006	.363	.003	.366	.005	.365	.004
	y	.379	.005	.374	.003	.379	.004	.378	.004
	L*	89	2	88	1	89	2	89	2
	a*	.495	.637	1.356	.350	.590	.687	.787	.385
	b*	7.516	3,039	4.962	1,571	7.344	2.456	6.904	2,205
T2	**Y**	959	93	811	32	882	53	941	47
	x	.372	.007	.368	.003	.371	.004	.371	.003
	y	.384	.006	.378	.002	.384	.003	.383	.003
	L*	89	3	84	1	87	2	89	2
	a*	.759	.722	1.925	.496	.952	.912	1.018	.454
	b*	10.659	3.613	7.085	1.065	10.099	1.983	9.972	1.720

Coordinate L*

Figure 5 shows the graphics relative to coordinate L* variation, over time (T0, T1,T2) for each group (1,2,3 and 4). For group 1 (negative control), no statistically significant differences were observed over time, $F(2,6)=0.897$, $p=0.456$. It also can be observed that the L* values for this group, decreased from T0-T1 and suffered a small increase from T1-T2. On the other hand, for groups 2 (positive control), 3

(WMTA) and 4 (Biodentine™), statistically significant differences were observed over time: $F(2,6)=35.604$, $p<0.001$; $F(2,18)=34.839$, $p<0.001$; $F(2,16)=10.065$, $p<0.001$, respectively. In these groups, a decrease in L* values was observed with increasing time. Alterations were greater for group 2 (positive control) $F(2,6)=35.604$, $p<0.001$, as represented in the corresponding graphic, and the decrease was constant over time. Comparing group 3 $F(2,18)=34.839$, $p<0.001$, with group 4 $F(2,16)=10.065$, $p<0.001$, group 3 had a greater decrease in L* values than group 4. Analysing the graphics, group 4 decreased from T0-T1, whereas in group 3, a decrease in the same values was only observed from T1-T2.

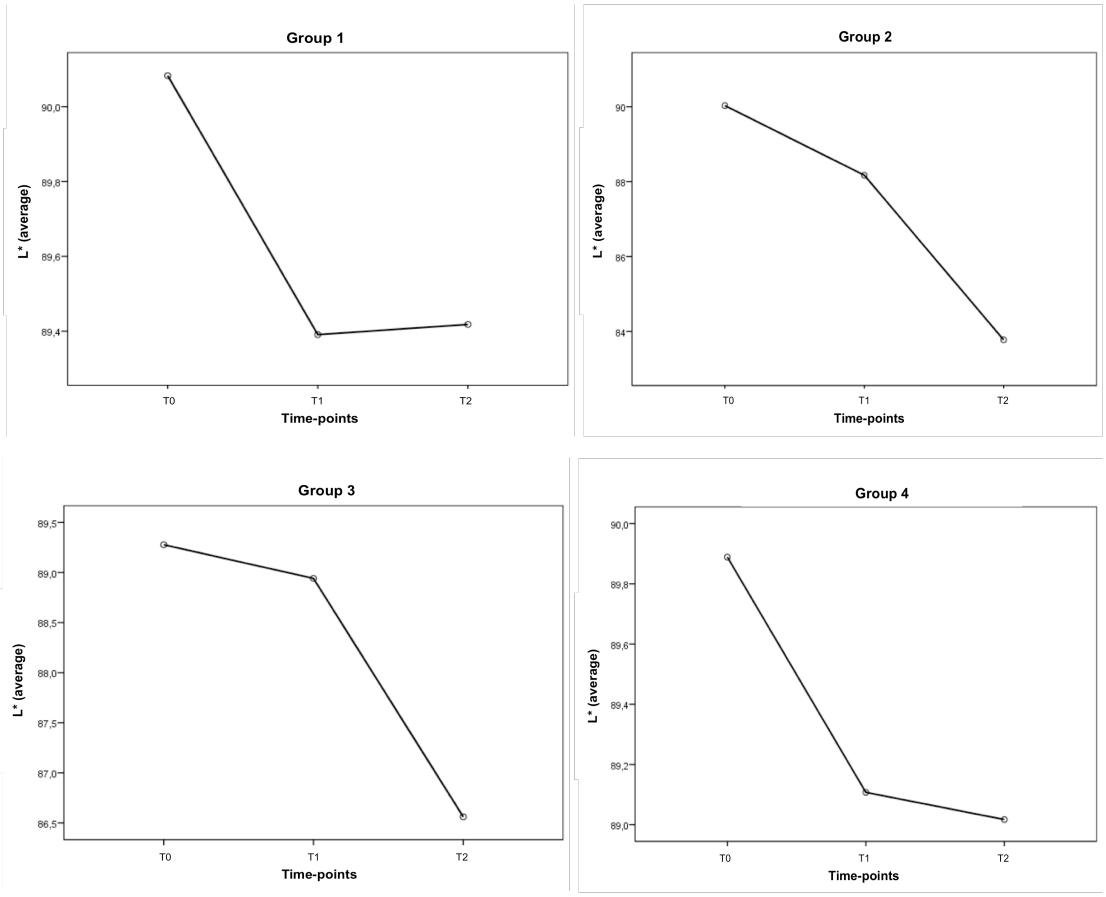

Figure 5. Coordinate L* variation, over time (T0, T1,T2), for each one of the groups.

Figure 6 represents coordinate L* evolution over time (T0, T1, T2), for each group. Mixed ANOVA procedures revealed statistically significant differences, over time, in the four groups, $F(6,46)=16.766$, $p<0.001$ for the all experimental study. However, main effects for the group were not statistically significant, $F(3,23)=1.618$, $p=0.213$. Time was a more significant factor contributing to the discolouration than the type of material applied in each group, $F(1,23)=154.490$, $p<0.001$.

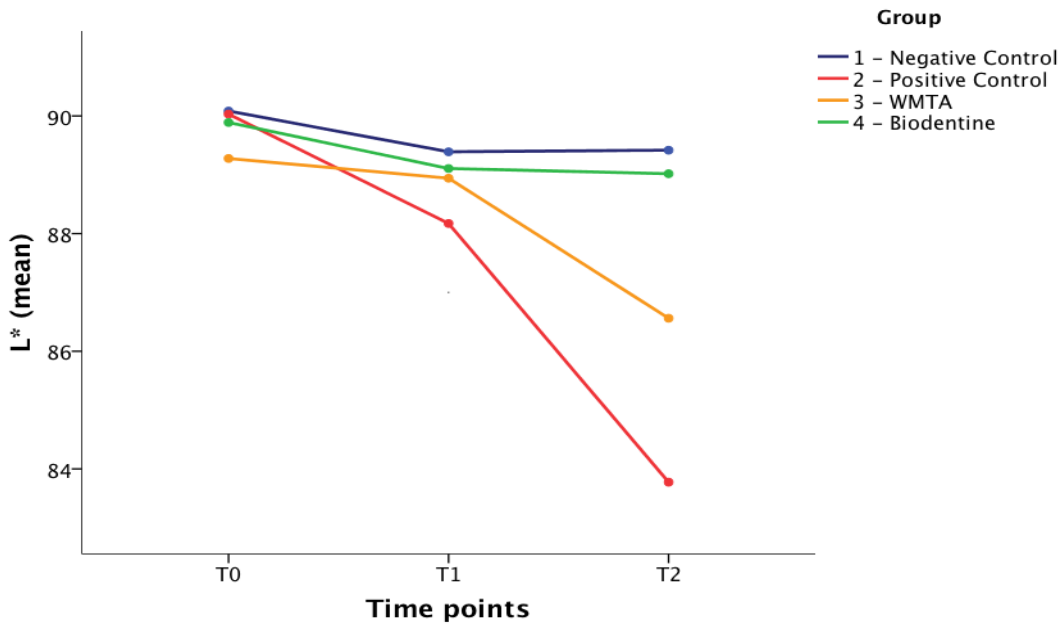

Figure 6. Overlapped L* coordinates evolution of over time (T0, T1, T2), of the four groups.

The coordinate L* variation was also verified, between time intervals (T1-T0) and (T2-T1), for each group. In **Table III** the different coordinate L* variation averages are represented.

Table III. Coordinate L* variation averages, between time intervals (T1-T0) and (T2-T1), for each group.

Group	**L*** in time intervals	Average	***std***
1	**L* (T1-T0)**	- 0.69	0.19
	L* (T2-T1)	0.03	0.66
2	**L* (T1-T0)**	- 1.86	0.79
	L* (T2-T1)	- 4.40	0.60
3	**L* (T1-T0)**	- 0.34	0.40
	L* (T2-T1)	- 2.38	0.40
4	**L* (T1-T0)**	- 0.78	0.24
	L* (T2-T1)	- 0.09	0.20

Regarding this L* variation, Kruskal-Wallis test, no statistically significant differences were shown, $\chi^2(3)=2.915$; p =0.405 between the groups (1,2,3 and 4), for the time interval T0-T1. However, for the time interval T1-T2, statistically significant differences are present between the groups $\chi^2(3)=19.865$; $p<0.001$. Analysing T1-T2,

statistically significant differences were found for all group pairs, except between groups 1 and group 4. **Table IV.** represents the results of application of Mann-Whitney test, between the group pairs.

Table IV. Results of Mann-Whitney test, between the groups, showing statistically significant differences for all group pairs except between groups 1 and 4.

Groups	2	3	4
1	**U**=0.00 **Z**=-2.309 **p=0.021***	**U**=5.00 **Z**=-2.121 **p=0.034***	**U**=14.00 **Z**=-0.849 **p**=0.396
2		**U**=4.00 **Z**=-2.263 **p=0.024***	**U**=0.00 **Z**=-2.828 **p=0.005***
3			**U**=0.00 **Z**=-3.780 **p<0.001***

In the box-plot graph (**Figure 7)** representing the coordinate L* variation, it is possible to observe the differences previously detected and referred.

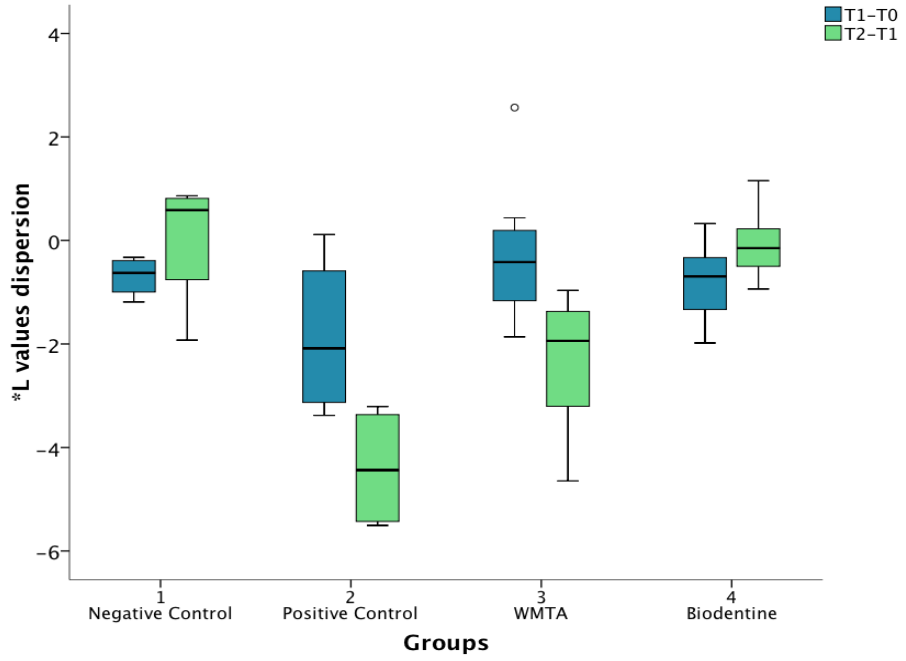

Figure 7. Box-plot representing Coordinate L* values dispersion in the three time points.

Coordinate a*

Figure 8 shows the graphics relative to coordinate a* variation, over time (T0, T1,T2), for each group. Statistically significant differences were not observed for group 1 (negative control) over time, $F(2,6)=3.694$, $p=0.090$. For group 2 (positive control), group 3 (WMTA) and group 4 (Biodentine™), statistically significant differences are observed: $F(2,6)=42.250$, $p<0.001$; $F(1.269,18)=6.125$, $p=0.025$ considering Greenhouse-Geiser effects; $F(2,16)=10.586$, $p=0.001$, respectively. The overall Δa^* values were greater for group 2, $F(2,6)=42.250$, $p<0.001$, as represented in the corresponding graphic. The increase in a* values for this group was constant over time. A similar situation was also observed for group 4. Relative to group 3, the a* values decreased from T0-T1, whereas from T1-T2 the a* values increased, which was equally observed for group 1. Comparing group 3 $F(1.269,18)=6.125$, $p=0.025$ (considering Greenhouse-Geiser effects), and group 4, $F(2,16)=10.586$, $p=0.001$, the variation in a* values was greater in group 4, and this variation was much more constant over time than for group 3.

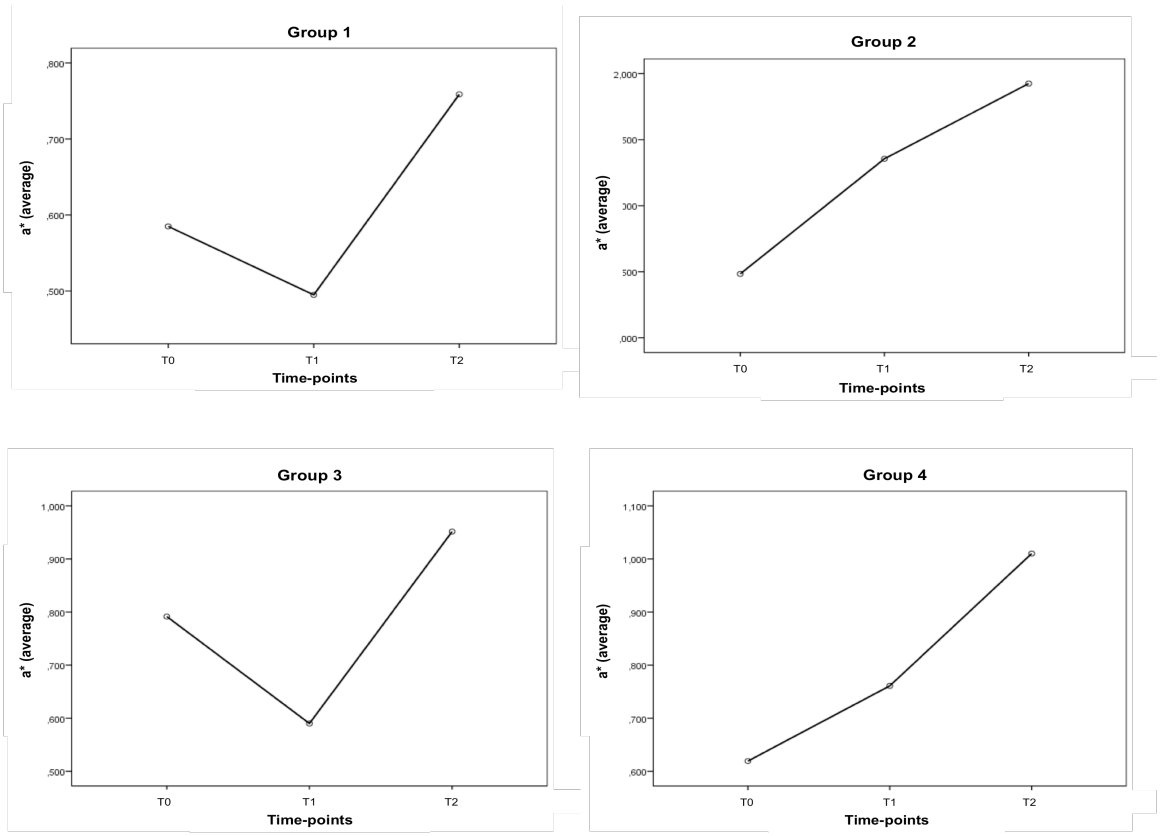

Figure 8. Coordinate a* variation, over time (T0, T1,T2), for group each one of the groups (1,2,3 and 4).

Coordinate b*

Figure 9 shows the graphics relative to coordinate b* variation, over time (T0, T1 and T2), for each group. For group 2 (positive control), no statistically significant differences were observed over time, $F(2,6)=2.576$, $p=0.156$. Noting the corresponding graphic there is a decrease from T0-T1 and an increase from T1-T2, despite not being significant. For group 1 (negative control), group 3 (WMTA) and group 4 (Biodentine™), statistically significant differences were observed over time: $F(2,6)=59.846$, $p<0.001$; $F(2,18)=16.264$, $p<0.001$; $F(2,16)=82.608$, $p<0.001$, respectively. Analysing the graphics, it's possible to observe that for groups 1 and 4, values increased over time, however, in group 3 values first suffered a little decrease from T0-T1, and a subsequent increase from T1-T2. The overall Δb^* values were greater for group 4 $F(2,16)=82.608$; $p<0.001$, and then for group 1 $F(2,6)=59.846$, $p<0.001$.

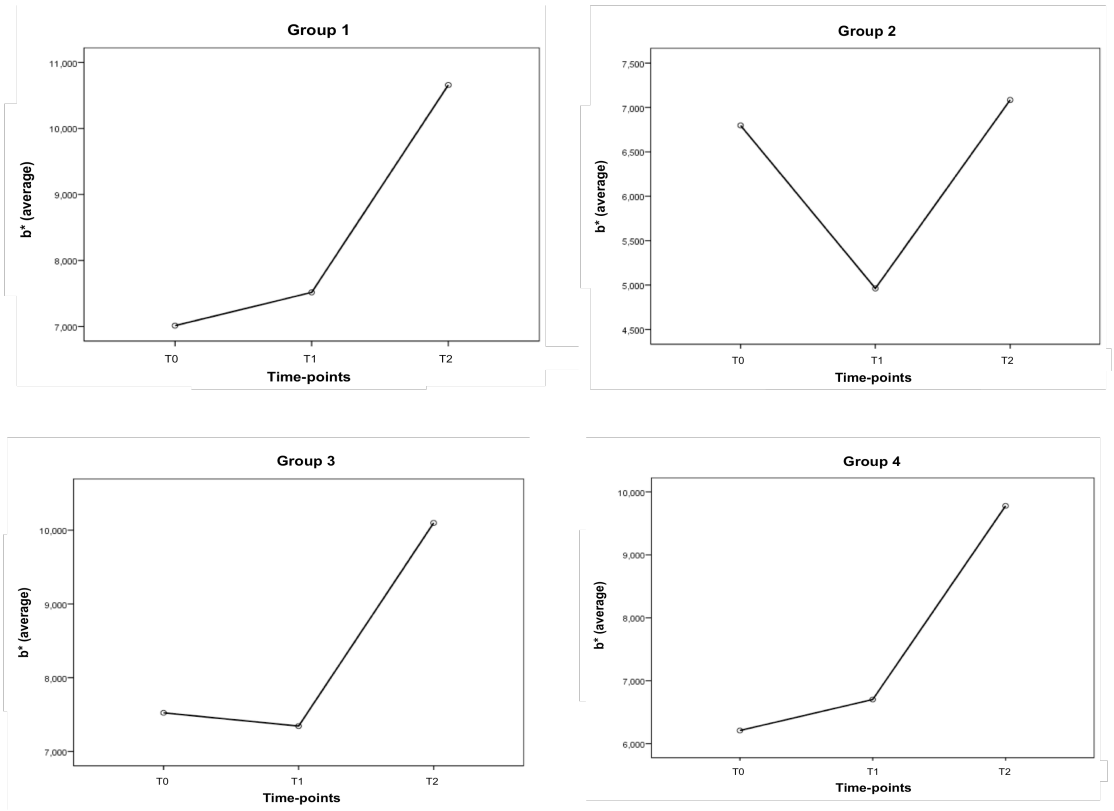

Figure 9. Coordinate b* variation, over time (T0, T1,T2), for group each one of the groups.

Table V represents the averages ($\bar{x}$), and corresponding standard deviations (*std*), for colour difference intervals (ΔE) between T0-T1, T1-T2, T0-T2, in all groups.

Table V. Colour differences (ΔE) between T0-T1, T1-T1, T0-T1, for all groups.

ΔE	Group 1		Group 2		Group 3		Group 4	
	Average	*std*	Average	*std*	Average	*std*	Average	*std*
T0-T1	1.145	.365	3.413	.786	1.820	1.527	1.252	.803
T1-T2	3.3310	0.909	4.9988	1.5944	3.7976	1.301	3.1597	0.9935
T0-T2	3.923	.629	6.802	1.816	4.095	1.041	3.728	.651

Figure 10 represents the graphics relative to the 3 colour differences (ΔE) for each group: ΔE (T0-T1), ΔE (T1-T2), ΔE (T0-T2).

Relative to ΔE (T0-T1), perceptible threshold of chromatic alterations for the human eye (ΔE≥2.3), were not observed for group 1 (negative control), $t(3)= -6.326$, $p=0.996$, group 3 (WMTA), $t(9)=-0.994$, $p=0.827$, and for group 4 (Biodentine™), $t(8)=-3.917$, $p=0.998$. However, for group 2 (positive control), colour alteration is statistically superior to ΔE=2.3, $t(3)=2.834$, $p=0.033$, being also the only group in T0-T1 with statistically significant colour alteration. Between T0-T1, analysing the correspondent graphic, the group which had a higher degree of colour alteration (ΔE) in descending order, was: group 2, group 3, group 4, and group 1, respectively.

Regarding ΔE (T1-T2), perceptible threshold of chromatic alterations for the human eye (ΔE≥2.3), is not observed for group 1, $t(3)=2.267$, $p=0.054$. However, colour difference is statistically superior to ΔE=2.3 for: group 2, $t(3)=3,385$, $p=0.0215$; group 3, $t(3)=3.561$, $p=0.003$; and group 4, $t(3)=2.737$, $p=0.0115$.. Between T1-T2, analysing the corresponding graphic, the group which had a higher degree of colour alteration (ΔE) in descending order, was: group 2, group 3, group 1 and group 4, respectively.

Relative to ΔE (T0-T2), perceptible threshold of chromatic alterations for the human eye (ΔE≥2.3) is observed for all the groups: group 1, $t(3)= 5.163$ $p=0.007$; group 2, $t(3)= 4.960$, $p=0.008$; group 3, $t(9)= 5.455$, $p<0.001$; group 4, $t(8)=6.583$, $p<0.001$. These colour differences are statistically significant for all the 4 groups. Between T0-T2, analysing the corresponding graphic, the group which had a higher degree of colour alteration (ΔE) in descending order, was: group 2, group 3, group 1, and group 4.

For the three time intervals, group 2 achieved the ΔE minimum value of 2.3, being perceptible threshold to chromatic alterations for the human eye. Group 3 and

group 4, were just perceptibility threshold to chromatic alterations, in ΔE (T1-T2) and ΔE (T0-2). In these two time intervals, group 3 had a greater colour change than group 4.

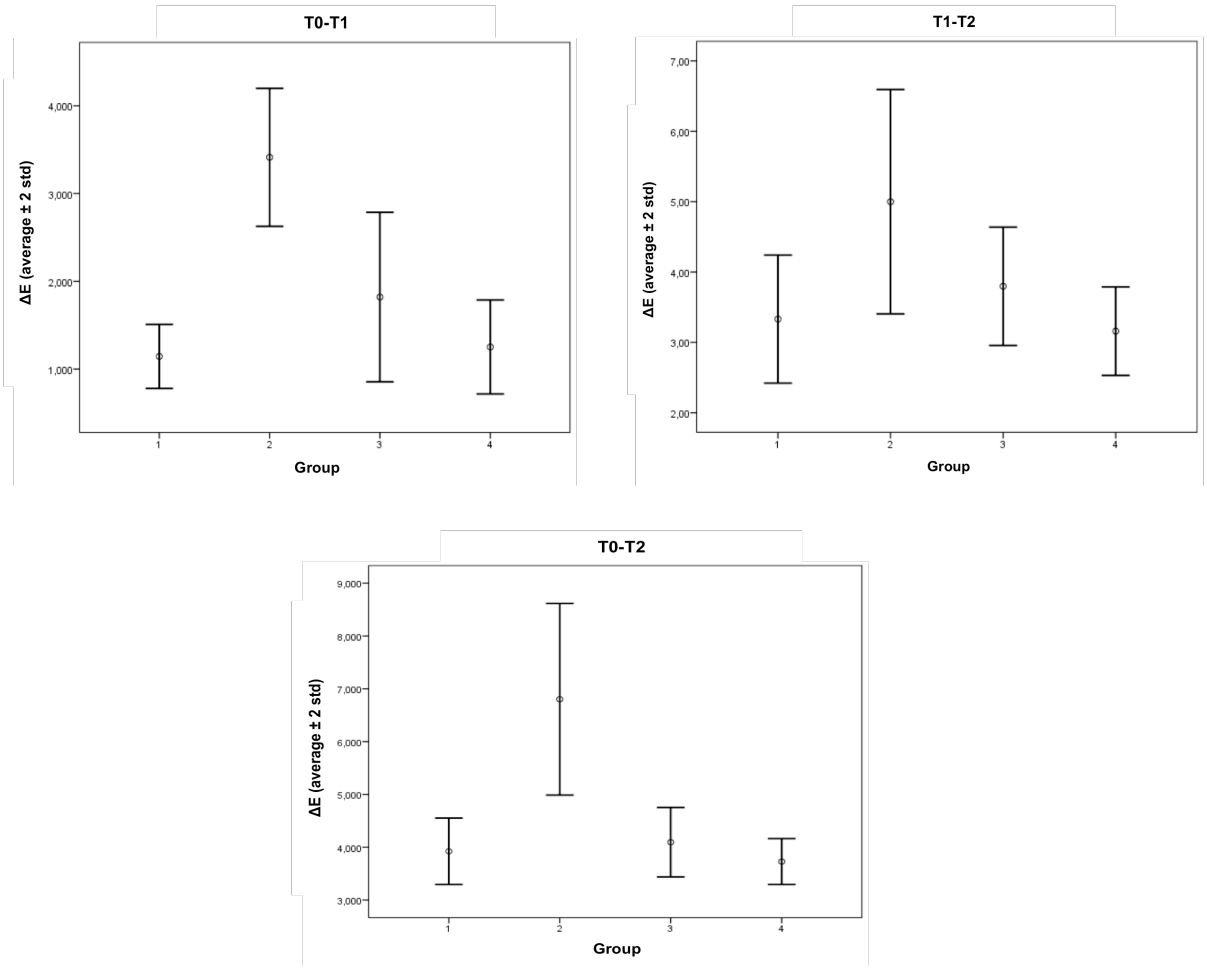

Figure 10. Graphics relative to the 3 colour differences (ΔE) for each group: ΔE (T0-T1), ΔE(T1-T2), ΔE (T0-T2). Error bars represent mean ± 2 std.

In **Figure 11** is shown the results of colour differences (ΔE), relative to T0-T1 and T1-T2, between groups.

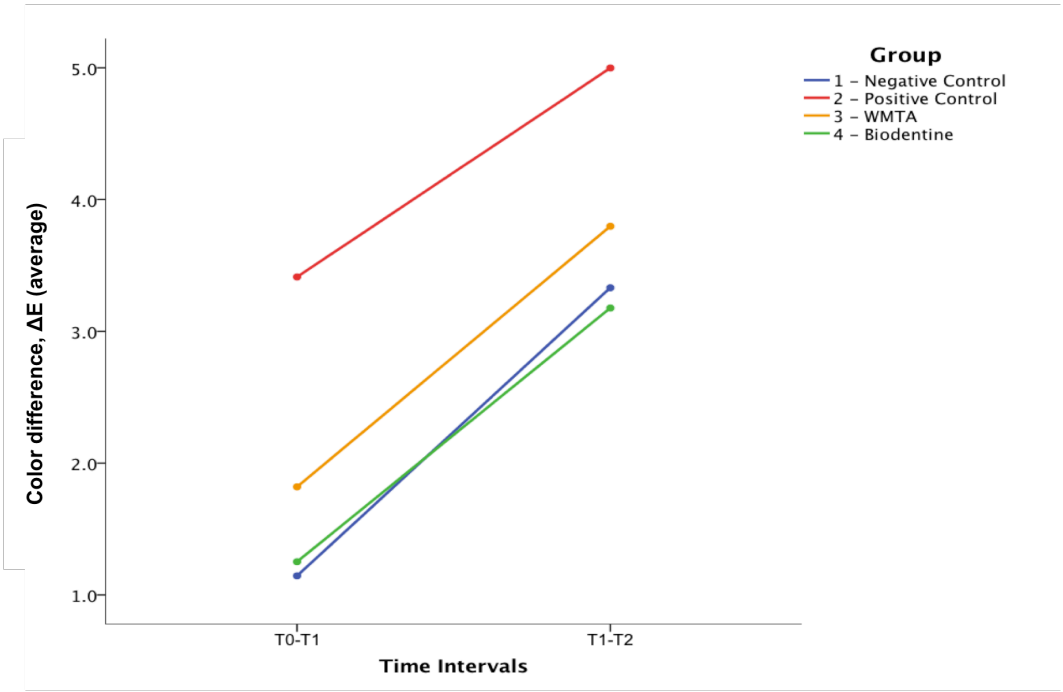

Figure 11. ΔE (T0-T1) and ΔE (T1-T2), over time, between groups.

Figure 12 shown the results of colour differences (ΔE), relative to T0-T1 and T0-T2, between groups.

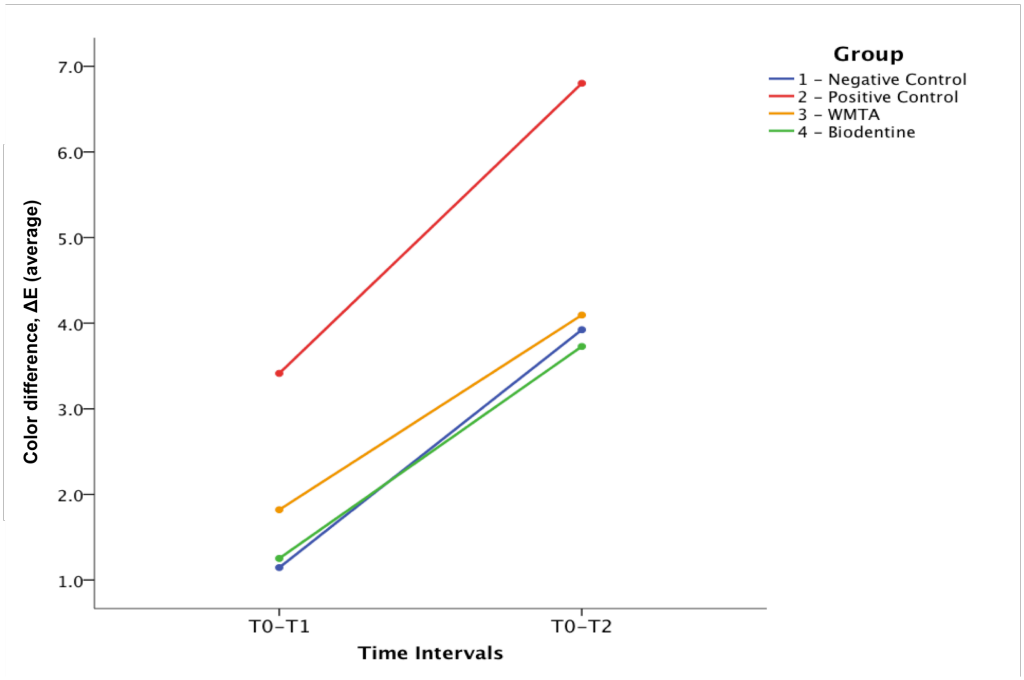

Figure 12. ΔE (T0-T1) and ΔE (T0-T2), over time, between groups.

It is possible to observe that:

- between T0-T1, there are statistically significant differences in at least two groups, $F(3,23)=3.998$, $p=0.020$. Using the pos-hoc tests, we can observe that the differences detected are significant between group 2 (positive control) and group 1 (negative control) ($p=0.039$), and between group 2 (positive control) and group 4 (Biodentine™) ($p=0.018$).
- from T1 to T2, there are no statistically significant differences between the groups, $F(3,24)=2.357$, $p=0.097$.
- between T0-T2, there are statistically significant differences $F(3,23)=9.156$, $p<0.001$. Using pos-hoc tests, we can see that the differences detected are significant between group 2 (positive control) and group 1 (negative control) ($p=0.003$), between group 2 (positive control) and group 3 (WMTA) ($p=0.001$), and between group 2 (positive control) and group 4 (Biodentine™) ($p<0.001$).

Analysing **Figures 11** and **12**, it is possible to see that group 2, had the greatest colour alteration over time (T0-T1 and T0-T2). On other hand, there were not found statistically significant differences in colour alteration (ΔE), between the two CMSs (group 3 and 4), for all time intervals.

According to data relative to coordinate L* variation over time between groups, the null hypothesis should be rejected.

4. Discussion

In recent years, various clinical colour-measuring devices have become available(35). These devices, are efficacious to quantify/analyse the natural tooth colour(36,37), and allow a more uniform and accurate communication between technicians and dentists(36). Some of this technology can be used in colour research, as it is done in the present study.

Overall, and despite de short term period of evaluation, the results of this study show that there is a strong tendency for crowns to "darken" with time which is greater for some materials than others, and is driven by intrinsic changes in the material, as well as changes caused in the adjacent dentine.

With regard to the alterations in L* parameter, the standard values vary, as indicated, from 0 (black) to 100 (white). In this study a significant decrease was measured in all experimental groups with increasing time, except in group 1 (negative control) $F(2,6)=0.897$, $p=0.456$. However, besides not being significant, it is possible to observe a small increase in L* values from T1-T2. Similarly, this finding was also present in a study by Felman *et al.*(2013)(38), also in the negative control group with sterile cotton pellets, but moistened with saline solution. Before the insertion of the sterile cotton pellets, the authors used a final rinse of sodium hypochlorite, for canal irrigation. Despite the factors responsible for the lightening of the negative control teeth being uncertain, they supposed that this canal irrigation could have induced a mild bleaching effect, which would have affected all teeth in the study and thus being a constant for all the experimental groups in their study. On the other hand, removing the smear layer with EDTA and NaOCl could have promoted the infiltration of the materials through the dentin tubules, which caused the discolouration associated to the experimental groups and positive control whereas inducing bleaching of the negative control. In the present study, no sodium hypochlorite was used, or other canal irrigation besides tap water for hydration. So we assume that this slight lightening in group 1 may be explained by the reduction of dentin volume which is perceptible as diminution of the saturation of the sample. In terms of visual perception, the descriptive analysis of L* values in this study, indicates a darkening effect in tooth crowns, which has been highlighted already in Ioannidis *et al.*(2013)(25). The alterations observed for this parameter in the WMTA (group 3) $F(2,18)=34.839$, $p<0.001$, were more intense compared with the Biodentine™(group 4) $F(2,16)=10.065$, $p<0.001$, in the overall experimental time (T0-T2). However, Biodentine™ had a more intense decrease in L* values than WMTA from T0-T1, whereas in WMTA a greater decrease, in the same values, was observed from T1-T2. This indicates that when Biodentine™ was placed in the teeth

cavities (before T1), the luminance suffered immediately a decrease, darkening the crowns; from T1-T2, this decrease was less marked. Relative to WMTA, L* parameter decreased contrary to Biodentine™. After the WMTA placement in teeth cavities (before T1), the luminance decreased in a gradual way, compared with the sharper decrease analysed from T1-T2. It would be interesting to continue the analysis of luminance variation of the crowns treated with both the CMSs for longer periods (more than 6 weeks), to verify if L* values for WMTA will continue decreasing in a marked way, whereas for Biodentine™ that decrease would be much more gradual or if there is a stabilization in *L values. One possible contributing factor for this could be the MTA setting time which takes significantly longer (mean of 165±5 min)(22) than for Biodentine™ (12 min)(30). Therefore between T0-T1, MTA was setting, while Biodentine™ had already done so. The resulting products of the setting reaction could be responsible for the immediate decrease in tooth discolouration seen first for Biodentine™. This material's own intrinsic colour and opacity should also be considered in this topic of discussion. Regarding L* variation, there are no statistically significant differences, $\chi^2(3)=2.915$; p=0.405 between the groups for the time interval T0-T1. However, for the time interval T1-T2, statistically significant differences are present between all the groups $\chi^2(3)=19.865$; p<0.001, except between group 1 and group 4. The L* parameter between T1-T2 was statistically higher for WMTA than Biodentine™ on the global variation [WMTA - L*(T1-T0):-0.34, L*(T2-T1):- 2.38; Biodentine ™- L*(T1-T0):- 0.78; L*(T2-T1):- 0.09].

Relative to a* chromatic parameter, a significant increase was measured in all experimental groups except for group 1 (negative control) F(2,6)=3.694, p=0.090. The alterations observed in the Biodentine™(group 4) F(2,16)=10.586, p=0.001, were more intense compared with WMTA (group 3) F(1.269,18)=6.125, p=0.025, in the overall experimental time (T0-T2). In terms of visual perception, the descriptive analysis of a* values for this study indicates an increase in redness, thus a reduced tendency towards green. However, constant significant increase with time was only observed for groups 2 and 4. Relative to group 3 (WMTA), from T0-T1, a* values decreased, reducing tendency towards red and increasing towards green. From T1-T2, a* values increased, reducing tendency towards green and increasing towards red.

Regarding b* chromatic parameter, a significant increase was measured in all experimental groups except for group 2 (positive control) F(2,6)=2.576, p=0.156. The alterations observed for Biodentine™ F(2,16)=82.608, p<0.001, were more intense compared with WMTA F(2,18)=16.264, p<0.001. There was an increase in

yellowness, and a reduced tendency towards blue. However, relative to group 3, increase was not constant over time despite being significant. Values first suffered a small decrease from T0-T1, while from T1-T2 values increased in yellowness and reduced tendency towards blue.

Positive control (group 2), was associated with clinically perceptible higher tooth colour change ($\Delta E \geq 2.3$), immediately after material placement in tooth cavities (T1) $t(3)=2.834$, $p=0.033$ (**Figure 10: T0-T1**). This finding suggests a rapid and severe pattern of discolouration induced by blood, which is in accordance with the fact that red blood cells are a known tooth staining agent(39).

Relative to group 3 (WMTA), perceptible tooth colour change ($\Delta E \geq 2.3$) occurred between T1 and T2 $t(3)=3.561$, $p=0.003$. This shows that WMTA can cause tooth discolouration in the short/mid-term. In a previous study, WMTA exceeded the perceptibility threshold after 6 months; however, the authors did not report variation in ΔE values, in short and mid-term observation periods(24). Nevertheless, no direct comparisons can be made because of the different experimental methodologies, including the colorimetric method of measurement, the material application method and the sample selection. Another study revealed that WMTA exceeded the perceptibility threshold only 3 months after placement(25) but no extrapolation can be made to the present study due to the reason mentioned above. Besides, in the last study, Ioannidis *et al.*(2013)(25) used a different type of WMTA (Angelus™, Londrina, Brazil). The MTA patent(40) reports the inclusion of 20% bismuth oxide in MTA, which has been verified for ProRoot® MTA(41,42). Camilleri *et al.*(43) reported the inclusion of 10.5% bismuth oxide in MTA Angelus. This could be the explanation for the time of perceptibility changes caused by ProRoot WMTA, which showed significantly darker discolouration earlier in this study, compared to the moment reported by Ioannidis *et al.* (2013) with Angelus MTA. This explanation was also advanced by Vallés et al. (2013)(20). In another study by this author, reporting the colour stability of ProRoot WMTA(44), it was speculated that the formation of metallic bismuth under light irradiation, in an oxygen-free environment, could be the main reason for the darkening of the WMTA samples. It has been reported that Bi_2O_3 undergoes thermal dissociation at high temperature, which yields metallic bismuth and oxygen(45). Increasing the partial pressure of oxygen at high temperature avoids the formation of metallic bismuth, and the sample remains transparent. In the previously referred work(20), Vallés et al. confirmed the same result for the discolouration of WMTA, and, also, that when WMTA was exposed to irradiation with light in an atmosphere that contained oxygen or an oxygen-free environment but without light, it remained stable in colour. In the present study, the teeth cavities were

restored with Ketac™ Fil Plus Aplicap™ (3M ESPE, AG, Germany) which is an auto-cure material not requiring any technique of light irradiation. On the other hand, the samples were not immersed in glycerine gel, but they were stored in a dark oxygen atmosphere, except when the measurements were taken. Thus, it is questionable if a photo-polimerizable composite resin was used to restore the tooth cavities of group 3 (WMTA), in an oxygen-free environment, it would show an earlier and greater discolouration. It' is also important to refer that in the study of Valles *et al.*(2013)(44) the last colour measurement of the samples was done on the 5th day, which is a shorter time compared with the present study.

For the prevention of MTA-induced discolouration, Akbari *et al.*(2012)(24) suggested the application of a double layer of dentine bonding agent in the access cavity, to obstruct penetration of material constituents into dentinal tubules during MTA placement. Even though this approach resulted in significantly less discolouration for WMTA *in vitro*, it is doubtful whether it is applicable in clinical practice. Alternatively, in the presence of discolouration, Belobrov & Parashos (2011)(46) proposed the internal bleaching of pulp chamber after removal of MTA with the aid of magnification.

According to the information supplied in the material safety datasheet, ProRoot MTA consists of 75 % Portland cement, 20 % bismuth oxide (Bi_2O_3), and 5 % calcium sulfate dehydrate(47). Portland Cement (PC) differs from the MTA by the absence of bismuth ions and presence of potassium ions(48). According to Steffen and van Waes (2009)(49), bismuth oxide, which has been added to PC to increase radiopacity, is a possible factor responsible for the discolouration of teeth treated with MTA. Consequently, PC has been suggested as an alternative (50).

Only Vallés *et al.* (2013)(20) analysed the colour stability of Biodentine™ and concluded that this material remained stable over time without discoloration in all experimental groups, regardless of the oxygen environment, light (in an oxygen or an oxygen free-environment), and exposure time. Conversely, in the present study Biodentine™ induced perceptible tooth colour changes ($\Delta E \geq 2.3$), between T1 and T2 $t(3)= 2.737$, $p=0.0115$, which in accordance with our protocol conditions occurred in an environment with oxygen and without light. It should be noticed that in Vallés *et al*. study (2013)(20) colour measurements were only read at 5 days, which is a shorter time compared with the present study. The protocol conditions were also considerably dissimilar, which may have contributed to the difference in results.

For all time intervals, no statistically significant differences in colour alteration were found (ΔE), between the two CMSs (group 3 and 4)**.** .

In the present study, during specimen preparation, the smear layer was not removed, which, according to some researchers (1,6,7), can occlude dentinal tubules, thus preventing the materials from diffusing inside it and inducing discolouration. The fact that group 2 (positive control) immediately showed severe coronal discolouration, means that, despite the presence of smear layer, the blood pigments immediately play an important role in tooth discolouration. The effect of the presence of the smear layer was confirmed by Parsons *et al.* (2001)(51) and Davis *et al.* (6). Davis *et al.*(2002) assessed coronal distribution and colour changes of four commonly used endodontic sealers placed in the pulp chamber after 2 years, without the removal of smear layer(6). As hypothesized, sealer was confined to the chamber with little, if any, penetration of the material into dentin. They refuted the theory that sealer leaks into dentinal tubules over time in the presence of smear layer, causing discolouration. Moreover, they concluded that visible crown discolouration might be caused by the presence of material remnants in the pulp chamber, which gets darker over time, therefore transferring this colouring through the hard tissues. Through this study, we can speculate that if the teeth were split longitudinally, in a buco-lingual direction for evaluation, the discolouration observed in the present study would not be by the infiltration of WMTA and Biodentine™ into dentinal tubules but caused by their own darkening over time. Conversely, it can be questioned that if the smear layer had been removed in the present study, the materials would show an earlier and greater discolouration.

It is important to refer that significant emphasis was given for the preservation of all specimens in a relatively humid condition during the measuring process in order to prevent enamel colour alterations caused by drying that could lead to experimental bias. The dehydration of dental tissues, especially in laboratory and clinical conditions, leads to significant changes in the optical properties, including enhancement of reflectance properties and subsequent lightness increase (52,53).

Despite the standardized experimental set-up, the present model has limitations in fully imitating the clinical situation. Interaction of the endodontic material with salivary components and bacteria may occur if there is leakage at the restoration margins. This may lead to different *in vivo* staining mechanisms.

5. Conclusions

Within the limitations of this *in vitro* study it can be concluded that:

- Concerning global colour variation (ΔE), there was no significant difference between the two CMSs (WMTA and Biodentine™), for all time intervals, over time.
- However, relative to L* parameter, it was possible to observe statistically significant differences, between WMTA and Biodentine™, being the L* variation for WMTA higher, indicating a greater tooth discouloration in terms of value.

Longer evaluation periods, new material combinations, different experimental conditions and *in vivo* studies should be performed in order to clarify the real aetiology and process of tooth discolouration.

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