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ORIGINAL ARTICLE

Development and Reversal of Discolouration in Roots Filled with Ledermix or Doxymix Pastes Stored in the Dark for Three Months then Daylight for Twenty Four Months

Basil ATHANASSIADIS, Laurence James WALSH, Paul Vincent ABBOTT

ABSTRACT

Objective: To explore the long-term effects on discolouration by demeclocycline HCl (Ledermix, LED) or doxycycline hyclate (Doxymix, DOX) pastes placed in extracted human teeth over a 27-month period under different storage conditions.

Methods: The canals in 38 teeth were prepared carefully, to minimize exposure to contamination from irrigants, then either LED (Lederle Pharmaceuticals, Germany) or DOX (Ozdent, Australia) were placed. Samples were stored in the dark for 3 months followed by daylight for 24 months. The storage conditions varied as follows: Group 1: Open access, dry storage (OD); Group 2: Closed access, dry storage (CD); Group 3: Open access, wet storage (OW); Group 4: Closed access, wet storage (n=4 for each material). Additional teeth were used as controls: Polyethylene glycol only in a closed canal; and saline only irrigation with LED paste in a closed canal. Standardised digital photographs were taken over 27 months and evaluated for changes in luminosity.

Results: Darkening of tooth structure occurred in all LED groups and in the two DOX groups that were stored wet, during exposure to light, with a faster rate with LED. The most rapid staining occurred with LED in moist conditions with an open access cavity. The least staining occurred with DOX in samples stored dry. With prolonged exposure to light, a reversal in staining occurred with DOX at 3 months and LED at 9 months.

Conclusion: Staining of tooth structure is influenced by the choice of medicament, and by exposure to moisture and air. Light has a bimodal effect, first driving staining, but later reversing it. This can be explained by different wavelengths of light causing photodegradation and photo-oxidation of tetracyclines and their complexes with tooth mineral.

Keywords: Access cavity, Doxymix, Ledermix, light, staining, water

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From the Private Practice Brisbane (B.A. ✉ basildent@bigpond.com), Queensland, Australia; The University of Queensland (L.J.W.), School of Dentistry, Herston, Australia; UWA Dental School (P.V.A.), The University of Western Australia, Perth, Australia

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HIGHLIGHTS

- Discolouration of tooth structure from tetracycline endodontic pastes is greater for demeclocycline hydrochloride than for doxycycline hyclate.
- Exposure to air and moisture are key factors driving the process of discolouration.
- Exposure to light over a prolonged period of time leads to a reversal in staining.

INTRODUCTION

The discolouration of tooth roots during treatment with tetracycline-containing medicaments such as Ledermix paste (LED, Lederle Pharmaceuticals, Wolfratshausen, Germany) was reported in two laboratory studies conducted nearly 20 years ago (1, 2). These studies have provided the platform on which later studies have been undertaken using similar methodology. The conditions that were used in such studies are now not considered to represent the clinical situation under which endodontic treatment occurs (3). It is now recognised that there are three key factors - light, moisture (water) and oxidation - that cause the progressive colour changes responsible for discolouration of tooth roots when tetracycline-containing medicaments are used (3). LED remains a commonly used material in endodontics in some global markets, and hence understanding the factors which worsen or attenuate discolouration is important for clinical practice (3).To date there have been no long-term laboratory studies that track discolouration over the long term and control all the variables that could influence the outcomes.

While studies indicate light accelerates the discolouration process for tetracyclines, blue light was shown to be the specific wavelength of sunlight involved in interacting with the tetracycline molecule and it led to the formation of organic molecules which absorb light (4). While visible blue light can cause photo-degradation of tetracyclines to form a red quinone complex, visible green light can reverse this. This principle is the basis for using intense green light as a treatment for reversing tetracycline discolouration (5, 6). Aqueous solutions of the red quinone complex have been shown to change from a red-purple colour to a light yellow or clear solution following exposure to intense light (7, 8). Extracted primary teeth that were stained because of the systemic use of tetracycline antibiotics showed a reduction in staining over a period of 14 months exposure to daylight (9). Intense visible green light (532-535 nm wavelength) from a KTP laser or an array of 75 green LEDs for 30 minutes reversed the red-purple staining of human dentine caused by the tetracycline, demeclocycline (10).

Previous reports have suggested that the discolouration of teeth caused by tetracycline appears to be permanent (7) but a review of the literature shows that exposure to sunlight (which contains visible blue and visible green light) could have bi-modal effects on staining from tetracyclines that are particularly light sensitive, such as demeclocycline. Two studies using extracted rat teeth which were stained with systemic tetracyclines reported darkening on initial exposure to daylight with lightening occurring after 3 weeks of continuous daylight exposure (11). When a more intense ultra-violet light was used, it took only 24 hours for lightening to occur, with no subsequent discolouration when further exposed to incandescent light for the following two months. These findings suggest that the bleaching effect may be irreversible (8).

As a result of the inherent sensitivity of demeclocycline to visible light, there has been interest in replacing this active ingredient of LED with a tetracycline that is less susceptible to oxidative degradation, and one that possesses better antimicrobial properties, such as doxycycline. Even at a sub-antimicrobial dose, doxycycline has useful anti-inflammatory properties (12) which may make it useful for managing both apical periodontitis and inflammatory resorption of teeth. It is highly effective against E. faecalis, a pathogen found in infected root canal systems, when used both as an irrigant (13) and as a medicament (14). These benefits distinguish it from clindamycin, which lacks anti-inflammatory and anti-resorptive properties (3). Clindamycin also lacks effectiveness against E. faecalis because of intrinsic resistance (15, 16), and it is not substantive since it does not bind to dentine. Furthermore, clindamycin has a much shorter half-life in tissue (4 hrs) than either demeclocycline (half-life 10-12 hrs) or doxycycline (half-life 18-24 hrs) (17). Doxycycline hyclate is one of the major active ingredients in Doxymix paste (DOX), a new medicament being manufactured by Ozdent Pty Ltd (Sydney, Australia).

The aim of this study was to explore the long-term responses of roots to medicament pastes containing either demeclocycline or doxycycline, when stored in the dark for three months followed by exposure to daylight for 24 months, with the roots stored in either wet or dry conditions. In a parallel experiment on the bench, the influence of diffuse light on the two pastes was assessed in microtiter plates over 18 months in room air or with oxygen depletion.

MATERIALS AND METHODS

LED and DOX pastes in microtiter plates over 18 months

This arm was included in the study to assess the changes in the materials themselves over time, independent of contact with teeth. LED and DOX pastes were placed into the 200 µL wells of microtiter plates. The pastes were placed into wells that were separated by one blank well, to avoid contamination of the materials with one another. The plates were placed on a shelf and kept for eighteen months at room temperature (ranging from 24°C- 30°C) with a humidity range of 40%-55%. They were exposed to diffuse daylight but not direct sunlight. Plates were organised into 2 groups. One group was exposed to the normal atmosphere. The other "no oxygen group" were stored in a sealed clear glass jar in an atmosphere from which all the oxygen had been consumed by burning a small candle at the base of the jar. The plates were photographed immediately after placement (day 0) and at several time points during storage (2 days, 14 days, and then at 3, 6, and 18 months), so that changes in colour could be tracked over time. In the no-oxygen group, after completing each session of photography, the plates were returned to the glass jar, and the candle was lit once again and the jar sealed to re-establish an oxygen-free atmosphere. Standardised photographs were taken using a digital camera (as outlined below) and combined to form a montage (Fig. 1).

Teeth

A total of 38 extracted human permanent teeth from patients aged 40-70 years were used. The teeth were extracted for clinical reasons unrelated to this study such as extensive decay or periodontal disease and were analysed randomly. The teeth were collected with informed consent from patients attending a private general dental practice. The study was conducted in accordance with the Declaration of Helsinki.

All soft tissue remnants were removed from the teeth by mechanical debridement before the crowns were sectioned from the roots with a high-speed diamond bur under water spray. Teeth with multiple roots were sectioned to provide roots with single root canals (e.g. palatal roots of maxillary molars and distal roots of mandibular molars). The longest and widest roots were chosen, which included distal roots of lower molars and palatal roots of upper molars. The roots were checked for the absence of fractures or root surface caries. The root canals were also checked for patency. All roots were kept in distilled water containing 0.1% thymol until root canal preparation was carried out.

The length of each root was measured from the access form to the apex, excluding the temporary restoration, using digital vernier callipers to the nearest 0.01 mm (model Dig LCD0, Harman, Salt Lake City, UT, USA), with 3 repeat measurements being made. Three repeat measurements were done for each sample in the eight experimental groups (four LED and four DOX) and the average used.

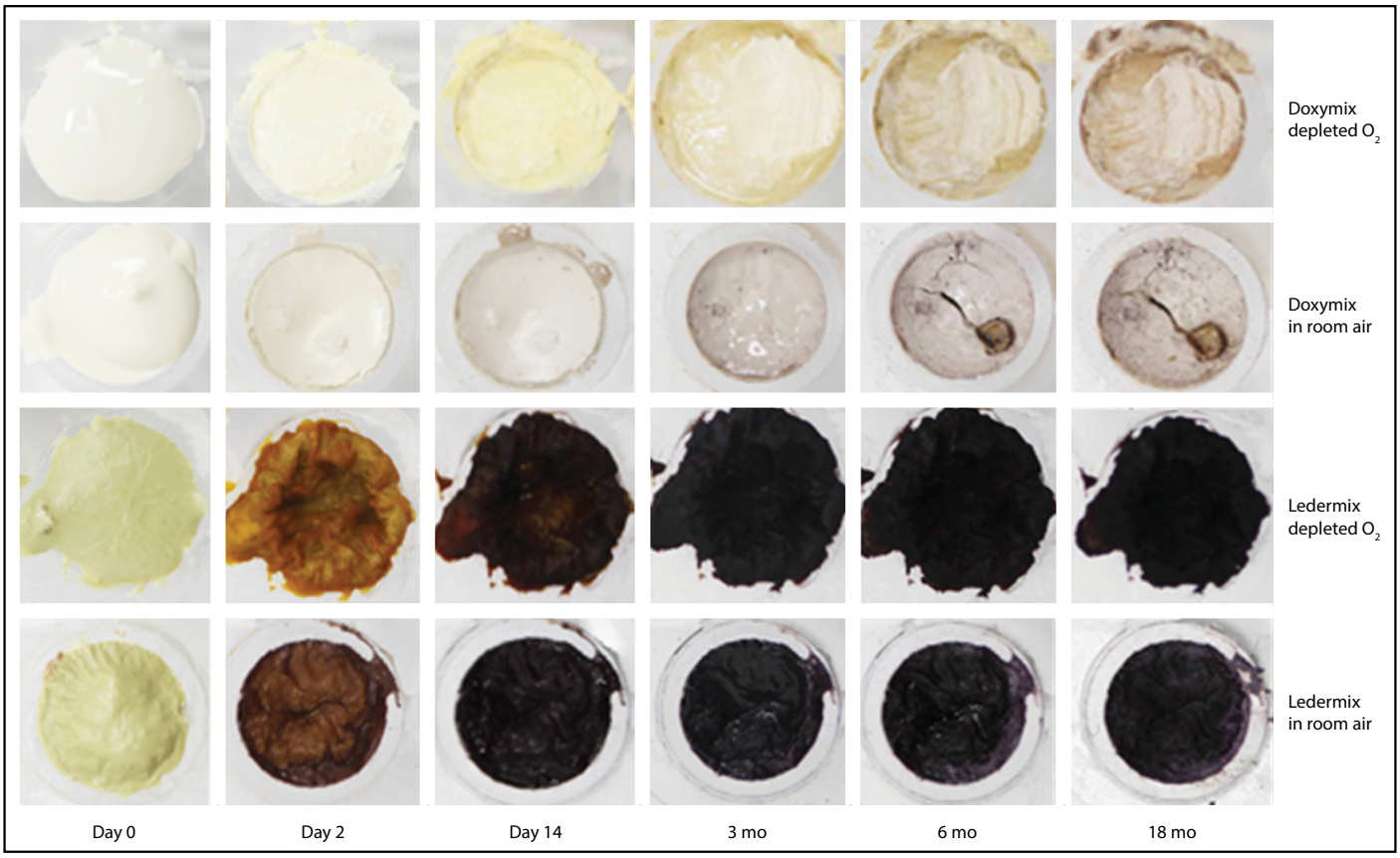

Figure 1. A montage of images of representative wells showing across the row, the same sample over 18 months. All images have been standardized for luminosity. Samples are designated as either Ledermix (LED) and or Doxymix (DOXY). The samples were exposed to ambient light, either in normal room air or sealed in a glass with depleted oxygen.

Root canal treatment

Root canal preparation involved first using a size 10 K file (Dentsply Sirona, Ballaigues, Switzerland) with a lubricant (Glyde, Dentsply Maillefer, Johnson City, Tennessee, USA), followed by SX and S1 files (Protaper Gold S files, Dentsply Maillefer, Ballaigues, Switzerland) to open the orifices, in combination with irrigation using 2% sodium hypochlorite (NaOCl) (DentaLife, Melbourne, Australia). Then, rotary nickel-titanium files (EndoWave, J. Morita Europe GmbH, Dietzenbach, Germany) were used with 2% NaOCl (10 mL) irrigation until an apical size 35 with 0.04 taper was obtained. To complete the root canal preparation, 8 mL of 17% EDTA (DentaLife, Ringwood, Australia) was used. Finally, in order to minimize any residues of NaOCl or EDTA in the samples, the roots were soaked in distilled water for two weeks in sealed containers that were placed in daylight.

Treatment with medicaments

The roots were blotted dry using absorbent paper towels, and the canals were dried using paper points until no visible moisture remained. A medicament paste (either LED or DOX) was then placed into the root canal using a spiral root filler (size 2) (Dentsply Sirona, Maillefer, Ballaigues, Switzerland) attached to a low-speed handpiece. Uniform filling was achieved by continuing to spiral the paste in the canal until the paste was visible at the apex and the access cavity. Roots were randomly assigned to one of the two medicament groups. To prevent contamination, fresh gloves were used when working with each individual root, and the external surfaces of the roots were wiped repeatedly with gauze soaked in 70% clear isopropyl alcohol (DentaLife, Melbourne, Australia) to remove any remnants of the medicaments. Four groups of four roots each were filled with either LED or DOX paste.

The coronal orifices of the canals were either left open or were closed with a temporary filling material. In the samples where the coronal orifices were to be closed, the coronal ends of the canals were first cleared of excess medicament by repeatedly using cotton wool pellets moistened with 70% isopropyl alcohol until no remnants of the medicament were seen. The coronal openings were then filled immediately following placement of the medicament with a 1 mm layer of Cavit W (3M ESPE, St Paul, MN, USA) followed by a 2 mm layer of resin modified glass ionomer cement (Riva, SDI, Melbourne, Australia) which was also extended across the cut coronal face of the root. No cotton wool was used beneath the restorations.

The samples were then immediately placed into individual clear, labelled 50 mL containers which were sealed with a lid and stored in an incubator at 37-40 °C for three months with no light. All groups were kept together at the same temperature in the incubator for the first three months. The humidity within the incubator during the three months of roots storage

varied between 35-45%, as measured using a dual thermometer and hygrometer unit (model 46618, Reptile One, Sydney, Australia). The conditions under which the prepared samples were stored varied as follows - Group 1: Open canal, dry storage (OD); Group 2: Closed canal, dry storage (CD); Group 3: Open canal, wet storage (OW); Group 4: Closed canal, wet storage (CW). The prefix letters L or D were added to the group designation to distinguish whether LED or DOX had been used. Experimental groups contained 4 samples per group, with roots allocated randomly to experimental groups.

For dry storage, there was no distilled water in the storage jars. For wet storage, the roots were placed lengthwise on gauze squares at the base of the jar. The gauze was loaded with 3.0 mL of distilled water initially, and then additional 1.0 mL volumes of distilled water added every two months for the duration of the experiment. This ensured that a small pool of water remained underneath the roots, so that with a closed lid the relative humidity within the containers remained in the range of 90-100% during wet storage was sealed with the lid to trap the moisture. The roots were not covered with or submerged under distilled water to prevent the paste from washing out.

Control groups

Two control groups were included. In one control group (n=3), the root canals were filled with polyethylene glycol (PEG) 400/4000 only (mixture of PEG 400 and PEG 4000 in a ratio of 10:1), as this is the base vehicle used in both medicaments. In order to assess the influence of NaOCl, the other control group (n=4) had the root canals prepared using saline as the only irrigation solution. These roots were filled with LED and exposed to light only. In both instances, as the roots had already been soaked for 2 weeks in distilled water prior to adding the PEG or LED, no additional rinsing with distilled water was done prior to placing these agents, instead the canals were dried using paper points and then the material placed. The coronal ends of the canals in both control groups were filled with Cavit and glass ionomer cement, and they were then stored in clear plastic containers with moistened gauze as described above. One operator performed all the experiments using pastes in roots and in microtiter plates.

Digital photography

Digital photographs of the samples were taken at day 0 and then after 1 and 3 months. After the three-month incubation period, the samples were removed from the incubator and then exposed to ambient light for a further 24 months. They were maintained in their original clear plastic containers on a shelf in a glass-panelled room at a room temperature of 24-30°C and with relative humidity from 40-55%. Although they were exposed to diffuse and indirect sunlight, there was no direct sunlight on the samples at any point. Samples were photographed after 1, 3, 6, 9, 12, 16 and 24 months. The photographs were taken under standard conditions - that is, the same distance from the camera to the samples (25 cm) and the same background (white paper) with a CMYK colour calibration standard included in each image. A digital camera (Mark II EOS 5D, Canon, Tokyo, Japan) was fitted with a 105 mm macro lens and a ring flash (Macro Ring Lite MR-14EX). The same settings were used for all exposures.

Image analysis

Using the background and the CMYK internal colour reference included in each image, all images were standardized using the luminosity values obtained with the histogram tool in Adobe Photoshop Elements ™ version 15.0 software (Adobe Systems Incorporated, San Jose, CA, USA). All images were corrected so that the luminosity value for the background was 231. Luminosity measures the brightness of a photographed image with no units of measurement. When the image becomes darker the luminosity value drops. If the luminosity values increase to be above the values obtained when the roots were first filled, then there has been lightening of the roots and the number is expressed as a negative. The percentage changes in luminosity from baseline were calculated.

Statistical analysis

Statistical analyses of changes over time for the same root were done by comparing the raw data at Day 0 to data from each time point, as listed in Table 1, using Instat for Windows version 3 software (GraphPad, San Diego, CA, USA). Repeated measures analysis of variance with Tukey post-hoc tests were undertaken to compare treatment groups, while changes over time in each group were analysed using a paired t test (two-tailed), with a threshold of $P<0.05$. The significance of changes was indicated as $P<0.05$ (*), $P<0.01$ (**), and $P<0.001$ (***). Since there were both darkened and lightened roots, the letters d (darkened) or l (lightened) were used with the asterisks to indicate whether the root darkened or lightened.

Delta-E values for aged samples compared with the time zero baseline for the relevant material were calculated from RGB values using an online calculator (colormine.org). Values for Delta-E range from 0 to 100. Delta-E values were used because they are a measure of change in the visual perception of two given colours, so are relevant to understanding whether the changes that occur will be discernible to the human eye. As a 'distance' between two colours, a value of 1.0 is the smallest colour difference a highly trained human eye can see. The average human eye cannot detect any colour differences with a Delta-E value of 3 or less, and Delta-E values between 3 and 6 are usually considered acceptable for colour or shade matching. Thus, for this study, values of 3.3 and above were considered to represent meaningful change. The statistical analysis of luminosity data and calculations for delta E were done by an operator who was independent from the clinician who performed the experiments.

RESULTS

Exposure to diffuse light caused colour changes in the medicament pastes stored in microtiter plates. The influence of light was greater on LED, which contains demeclocycline, than on DOX, which contains doxycycline. LED changed from a lime green colour to black after just 14 days, whilst DOX changed from white to a light grey colour. Storage in an oxygen-depleted atmosphere slowed the rate of change in both groups, with the DOX remaining white for up to three months, after which a slight grey colour change occurred. With LED, the paste became black by 14 days but at a slower rate in the no oxygen group (Fig. 1).

TABLE 1. Changes in luminosity values over time

Time (months)	Ledermix Paste				Doxymix Paste				Control 1	Control 2
	LOW	LCW	LCD	LOD	DOW	DCW	DCD	DOD	PEG Saline	LCW
Dark 1 mo	7.35*d	4.23*d	1.04 NS	7.33*d	2.17 NS	3.54***d	-4.27 NS	-1.17 NS	2.52*d	
Dark 3 mo	11.71**d	10.88*d	4.48*d	11.73**d	3.28**d	4.67**d	-2.87 NS	-0.82 NS	0.29 NS	
Daylight										
1 mo	42.78***d	21.8*d	12.28*d	20.81*d	15.66**d	9.5*d	0.7 NS	8.06 NS	NA	16.11**d
3 mo	41.14**d	24.13*d	10.72*d	19.46*d	9.99*d	11.67*d	-3.88 NS	6.77 NS	0.06 NS	21.11!**d
6 mo	40.21**d	26.78*d	10.11*d	22.14*d	5.54*d	11.67*d	-5.31 NS	4.35 NS	-4.25 NS	8.9**
9 mo	17.31*d	32.44**d	7.36 NS	20.05*d	0.27 NS	7.62*d	-7.03 NS	2.07 NS	-0.8 NS	9.08 NS
12 mo	25.33*d	28.83**d	3.35 NS	19.59 NS	2.45 NS	4.31 NS	-8.72*l	-0.58 NS	-0.88 NS	15.76***d
16 mo	13.35*d	24.09*d	2.32 NS	16.71 NS	4.11*d	9.22*d	-11.23*l	-3.56 NS	-1.65 NS	17.66**d
24 mo	9.66*d	29.77*d	1.86 NS	15.07 NS	-3.51 NS	-1.81 NS	-13.30*l	-7.14 NS	-5.45 NS	17.32**d

Data show percentage changes in luminosity values of the roots for three months in the dark followed by 24 months in light. Maximum discolouration time points for each group are highlighted with a grey colour in the table cells. A negative value indicates the root has lightened whilst a positive value indicates darkening of roots. The significance of changes from baseline is shown as follows: *: P<0.05; **: P<0.01; ***: P<0.001. The letter d (darkened) or l (lightened) follows the asterisk to indicate whether the roots darkened or lightened. Control 1 contained the polyethylene glycol vehicle 400/4000 (PEG) in a ratio of 10:1 used in both LED and DOX, while Control 2 was Ledermix in closed wet storage conditions (replicate a root canal with closed access and sitting in moisture) but with canals irrigated with saline rather than with NaOCl during preparation. OW: Open canal, wet storage; CW: Closed canal, wet storage; CD: Closed canal, dry storage; OD: Open canal, dry storage; NA: not available; NS: Not significant; The prefix letters L or D were added to the group designation to distinguish between LED and DOX

For the tooth samples, composite panels of sequential images showing the discolouration effect from the two different tetracycline-containing pastes and the effect of dry versus wet storage are shown in Figures 2 and 3 for LED and DOX, respectively. The results for the eight groups and two controls are outlined in Table 1.

Measurements of sample length showed comparable root length for roots across the four LED and four DOX groups, with in average length in millimetres as follows: LCW: 12.86, LCD: 13.76, LOW: 14.00, LOD: 14.61; DCW: 15.16, DCD: 13.49, DOW: 14.06, and DOD: 13.43. Differences between sample groups were not statistically significant (P=0.689).

Control groups

The first control group with roots filled with PEG 400/4000 paste showed minimal discolouration when stored in the dark (maximum of 2.5% after one month) but on exposure to daylight, there was lightening of the roots via photo-bleaching. This occurred from the three months interval, and they continued to lighten for up to 24 months. There was no discolouration noted on the roots as a result of using NaOCl/EDTA irrigation followed by placement of a PEG 400/4000 base. However, the roots in the second control group which had only saline for irrigation during the canal instrumentation procedure and LED paste in the canals showed only half the discolouration compared to the corresponding group – that is, LCW with NaOCl/EDTA irrigation. This latter group reached its maximum discolouration at nine months (32.4%) and ended up with slightly less darkening of 29.8% at the end of the study. Control group two with saline irrigation only and LED reached its maximum discolouration at three months (21.1%) and lightened to 17.3% by the end of the study.

Storage in the dark for (0-3 months)

For the period where the roots were stored in the dark, three of the four LED groups darkened between 10.9% - 11.7%, with one group (LCD) darkening only by 4.5%. In the two DOX groups stored dry (DCD, DOD), the roots lightened in colour. The two DOX groups stored wet (DCW, DOW) had minimal discolouration (3.3-4.7% darker). The wet storage groups darkened the most, with the LED paste (LOW, LCW) roots darkening 2.5 times more than the DOX paste (DOD, DCD) roots when stored in the dark.

Storage in the light (3-24 months)

During the first six months of exposure to light, darkening occurred in all four LED groups (10.1-42.8%) and in three of the DOX groups (5.5-15.7%). The most pronounced darkening occurred when the root canals were left open and the roots were stored wet (DOW), while the least darkening occurred when the root canals were closed and the roots were stored dry (DCD).

The peak level of discolouration was reached at different time points, with four groups peaking after one month (LED: LOW, LCD; DOX: DOW, DOD). The DCW group peaked at three months, the LOD group at six months, and the LCW group at nine months. After 24 months, all DOX groups were photobleached to be lighter than when first assessed, while all the roots from the LED groups were darker than when they were first assessed (Table 1).

The first six months following exposure to light was when most of the colour changes occurred, and Table 2 highlights the differences between wet vs dry and open vs closed amongst the groups. The relative extent of discolouration for LED wet versus dry storage was 2:1, and open versus closed was 1.7:1, while for DOX, wet versus dry storage was 6:1, and open versus closed storage was 2:1. Comparisons between the two medicaments showed that LED stains anywhere between three to nine times more than DOX when comparing the two pastes between the wet, dry, open and closed groups.

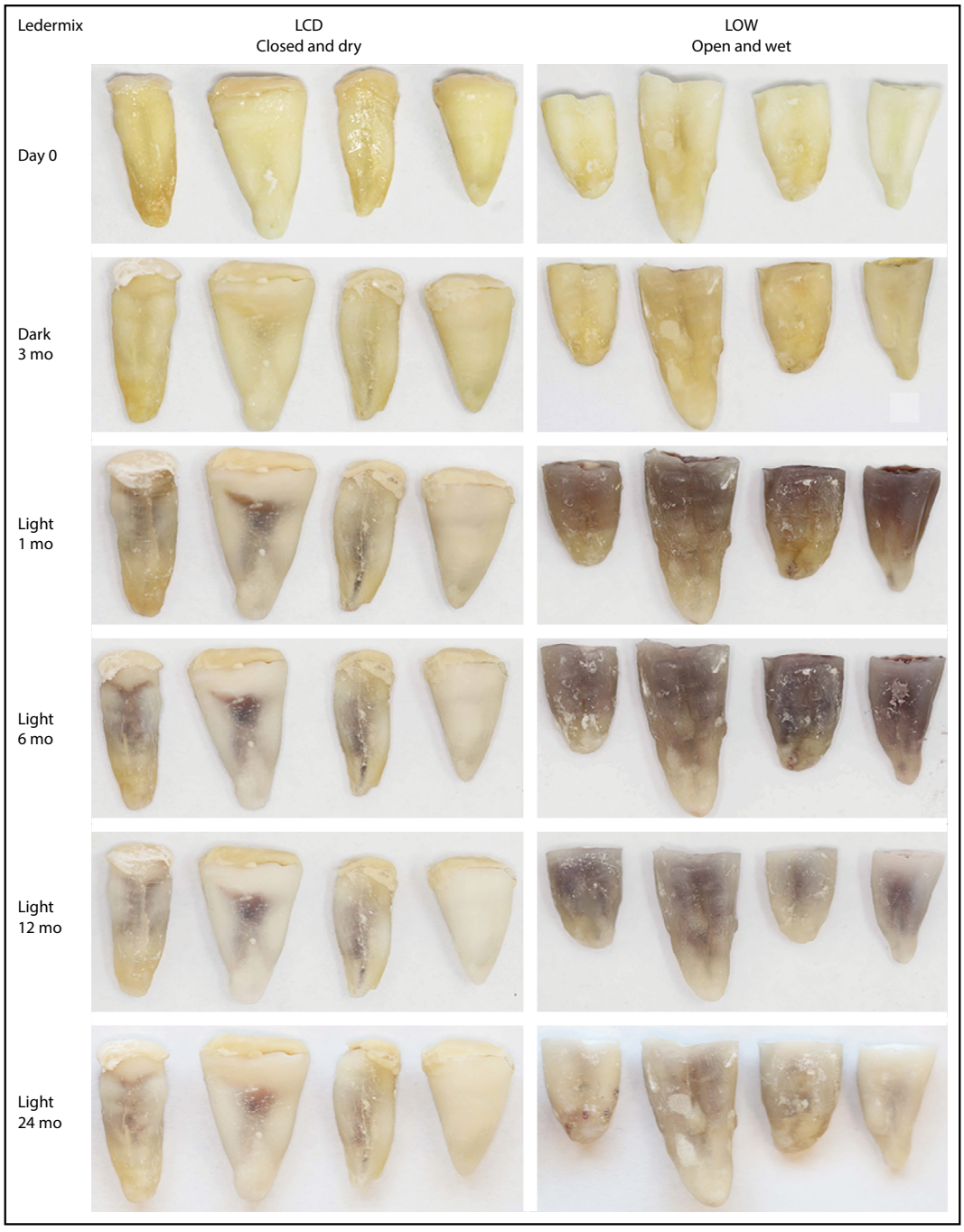

Figure 2. Samples treated with DOX, arranged in the same manner as Figure 1, with closed and dry samples (left), or open and wet samples (right) followed over 27 months. Note how the extent of discolouration caused by exposure to light for 1 month is much less for DOX than for LED (in Fig. 1). Both groups once again show progressive reductions in staining over 6 to 24 months of light exposure

CD: Closed canal, dry storage; OW: Open canal, wet storage; DOX: Doxymix, LED: Ledermix

Figure 4 summarizes the data trends from Table 1. The baseline value refers to the colour present at the start of the experiment. After 24 months exposure to daylight, two of the four LED groups (LOD, LOW) had partial lightening after reaching their peak discolouration, and one group (LCD) darkened and then recovered to its baseline colour. Group LCW (with NaOCl/EDTA irrigation) and control group 2 (LED after saline irrigation) darkened but did not recover the colour compared to baseline. Only one LED group recovered to baseline (LCD). After 24 months of daylight, two DOX groups (DOW, DCW) recovered to baseline, one showed no change (DOD) - as did control group 1 - and one lightened to be lighter than baseline (DCD). This highlights the difference in staining with DOX, which had no staining in three of the four groups (DOD, DOW, DCW), while one group (DCD) lightened. For LED, three of the four groups darkened (LCW, LOW, LOD) but did not lighten back to their baseline values, and one group (LCD) showed no changes at the final observation period. In control group 1 with PEG 400/4000 as the medicament after NaOCl and EDTA were used for irrigation, the roots did not discolour.

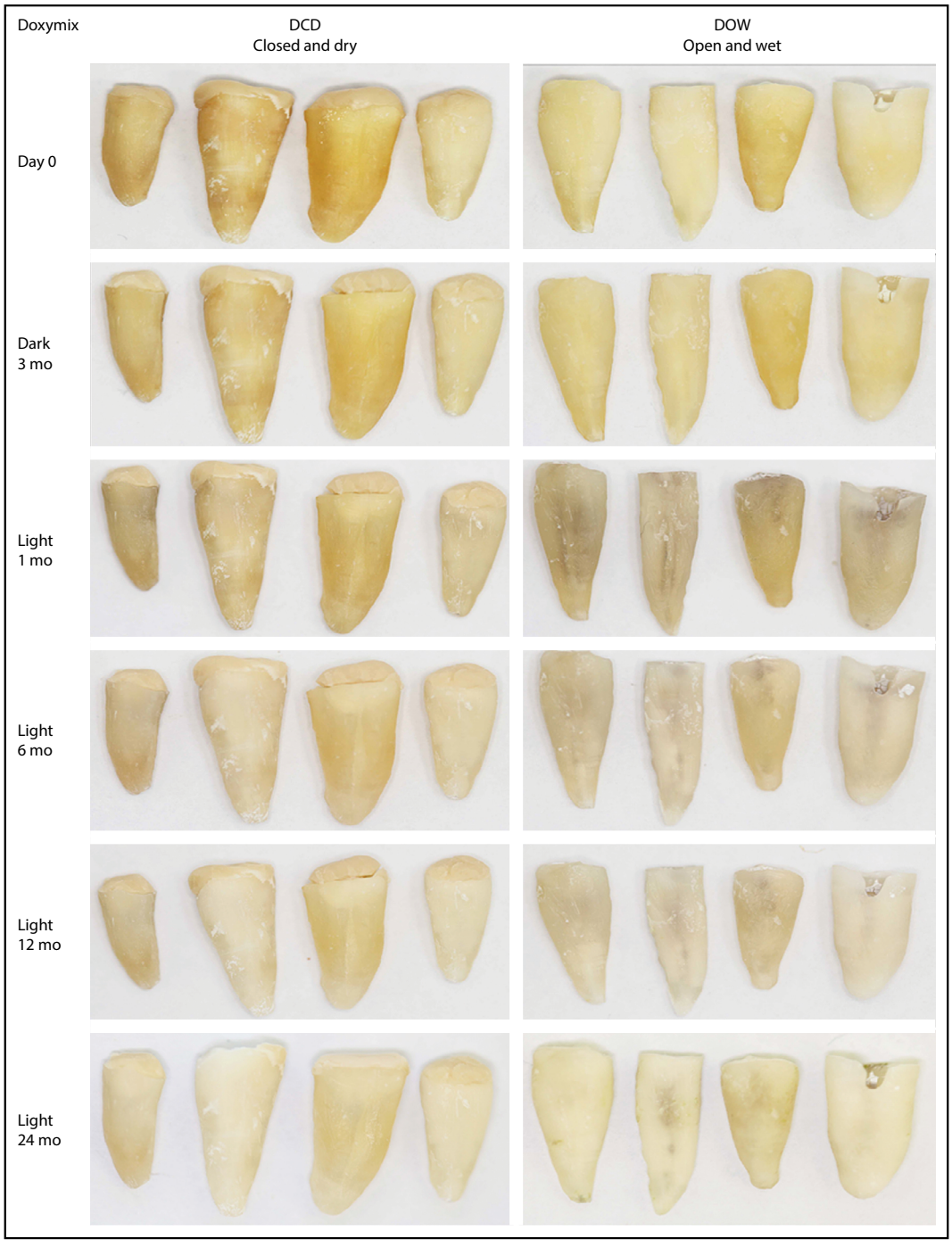

Figure 3. A graphic summarizing the effects of time on the extent of staining and whether lightening, recovery to baseline or recovery below baseline of the roots occurs amongst the four LED and four DOX groups when exposed to no light for three months and daylight for 24 months

CD: Closed canal, dry storage; OW: Open canal, wet storage; DOX: Doxymix, LED: Ledermix

The trends for changes in luminosity were reflected in the data for Delta-E, which compared samples from their baseline throughout the course of the study. These data for medicaments in microtiter plates show a greater extent of colour change for LED versus DOX, and reduced staining when oxygen was depleted, or when samples were kept in the dark (Table 3). The delta E values for roots treated with medicaments show the same trends, with the additional influence of greater staining in wet versus dry storage, and in open versus closed canals (Table 4). The Delta-E data also show the staining reversal effect caused by light. A key example is DOX at 24 months, with Delta-E values less than 3.3, meaning that roots would not be assessed visually as being different from the baseline.

DISCUSSION

In order to illustrate the interactions of variables (choice of tetracycline, exposure to moisture, exposure to the air, exposure to daylight) on the staining of tooth structure, a matrix demonstration experiment was established, allowing each of these factors to be tested separately, with the samples tracked

TABLE 2. Changes in luminosity values in treatment groups with different storage conditions

Dry vs Wet storage				Open vs Closed access			
LED wet	LED dry	DOX wet	DOX dry	LED open	LED closed	DOX open	DOX closed
LCW (24.24%) LOW (41.38%) 32.81%	LCD (11.04%) LOD (20.8%) 15.92%	DCW (10.95%) DOW (10.4%) 10.68%	DCD (-2.83) DOD (6.39%) 1.78%	LOW (41.38%) LOD (20.8%) 31.09%	LCW (24.2%) LCD (11.04%) 17.64%	DOD (6.39%) DOW (10.4%) 8.40%	DCW (10.95%) DCD (-2.83%) 4.06%

Data for experimental groups show the mean percentage change in luminosity, between 1-6 months of light exposure under dry vs wet storage and open vs closed canals (measuring the effect of storage conditions wet, dry, open, closed on colour change for roots with the two different medicaments used). The percentage change listed in the last row is the average of the two values for that column. DOX: Doxymix, LED: Ledermix; CW: Closed canal, wet storage; CD: Closed canal, dry storage; OW: Open canal, wet storage; OD: Open canal, dry storage. The prefix letters L or D were added to the group designation to distinguish between LED and DOX

over an extended period of time (up to 2 years) using standardized digital photography. This experiment was designed to reveal the long-term responses of roots to medicament pastes containing either demeclocycline or doxycycline, under controlled conditions, and free of possible confounding factors prior to the study. In consideration of the literature on contributing factors and confounders (3), other technique improvements were also made, such as no pre-soaking of teeth in NaOCl, closing the access cavities with temporary restorations, not submerging the samples in water during the observation period, using EDTA for the final irrigation step, and soaking the teeth in distilled water for two weeks in sunlight to eliminate traces of irrigants on the outside of specimens.

This is the first study using human teeth in an *in vitro* model to show that the roots with both LED and DOX underwent a reversal in staining when they reached their maximum point of discolouration in daylight conditions within three months and nine months respectively. All roots with DOX became lighter after 24 months compared to their original colour. This lightening in colour beyond their initial measurements was not seen in the roots with LED.

This lightening effect occurs because the red-purple reaction product from demeclocycline and the grey reaction product from doxycycline can be photo-oxidised by visible green light at wavelengths from 520-540 nm (6). Because this type of light is not absorbed by water or hydroxyapatite, it can penetrate deep into the tooth structure to cause photo-bleaching (10). Doxycycline undergoes photo-bleaching better than demeclocycline. From a clinical perspective, replacing demeclocycline with doxycycline is likely to result in less discolouration should it occur as a result of a poorly filled access cavity (3).

Choice of tetracycline

The results of this study demonstrate that the major factors affecting discolouration include light, moisture and oxidation but the first major variable is the choice of the tetracycline antibiotic used. When LED was stored in microtiter plates, the intensity and extent of staining was much greater compared to DOX under the same conditions. Likewise, when LED was placed in the root canals, the intensity and extent of staining of the crowns and roots was much greater compared to DOX under the same conditions. When exposed to light, roots in the two LED groups with wet storage (LCW and LOW) underwent the most intense colour changes with red-purple discolouration. In contrast, the changes in colour with DOX were mild and the affected roots developed a light grey discolouration. Under wet storage conditions, the LED groups (LOW, LCW) darkened three times more than the corresponding DOX groups. In roots with open access cavities, the LED groups darkened to four times the extent of the DOX groups.

The vehicle in both pastes (PEG) when used in the control teeth caused no staining, but rather some lightening occurred which could be due to the presence of peroxides within the polyethylene glycol (18). Thus, the different discolouration patterns seen with the two pastes is likely to be a result of the different chemical structures of the tetracycline components. A similar observation of differences in the extent of discolouration has been made when these two tetracycline antibiotics were administered systemically in both animals (19) and humans (20). With systemic use, demeclocycline caused severe discolouration while doxycycline caused only very mild or no discolouration (20, 21). Doxycycline resulted in the least discolouration.

Exposure to light

The second important variable in tetracycline-induced discolouration is exposure to light. In the present study, the effects of light were due to ambient levels of lighting over prolonged periods of time. This is more relevant clinically than short periods of exposure to direct sunlight or to high intensity lamps, as has been used in previous studies. Exposure to light is a key element in the process of staining from demeclocycline in LED (1, 2). The effect of light was seen in the tooth samples stored in the dark where there was minimal darken-

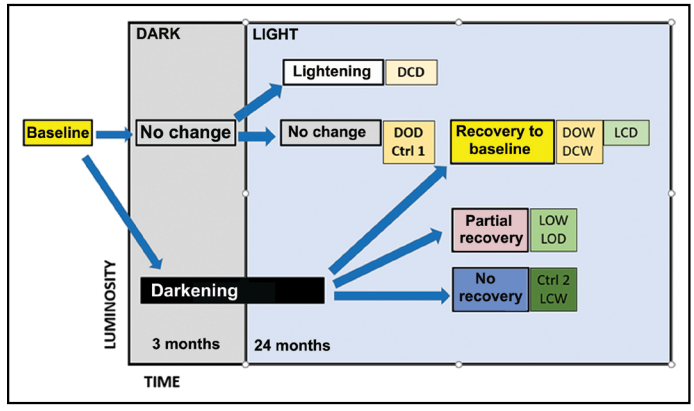

Figure 4. A graphic summarizing the effects of time on the extent of staining and whether lightening, recovery to baseline or recovery below baseline of the roots occurs amongst the experimental groups when exposed to no light for three months and daylight for 24 months.

TABLE 3. Delta-E values for changes in medicament pastes stored in microtiter plates

Time	Stored in Light				Stored in the dark			
	LED		DOX		LED		DOX	
	Air	No O_2	Air	No O_2	Air	No O_2	Air	No O_2
Day 2	57.0	46.6	2.9*	0.2*	6.6	16.7	2.4*	1.3*
Day 14	71.9	71.9	6.2	3.1*	15.3	29.5	0.9*	2.1*
2 months	69.0	72.2	17.1	5.3	23.0	45.9	3.1*	3.7
5 months	70.6	76.4	19.2	14.2	41.2	58.8	7.5	6.5
12 months	69.1	76.0	20.8	19.6	41.5	57.8	11.2	12.1
18 months	69.2	76.4	19.6	15.9	41.8	56.7	8.2	9.6

Data show the Delta-E for aged samples compared with the time zero baseline for the relevant material. Delta-E values were calculated from RGB values using an online calculator (colormine.org). Values shown with an asterisk were less than the threshold of 3.3 used for the study, and so would not be considered a perceptible visual change to an untrained person. DOX: Doxymix, LED: Ledermix

TABLE 4. Delta-E values for changes in the shade of teeth containing medicament pastes

Interval	LED				DOX				Controls			
	LOW	LCW	LCD	LOD	DOW	DCW	DCD	DOD	C1	C2	C3	C4
1 mo Dark	5.5	7.5	3.0*	6.6	1.6*	4.7	4.7	1.3*	7.1	12.1	1.8*	ND
3 mo Dark	8.8	12.3	5.5	10.0	2.4*	5.5	5.6	1.6*	ND	ND	0.2*	ND
1 mo Light	33.3	20.3	11.3	16.8	11.8	9.0	8.0	8.0	19.0	20.5	ND	6.3
3 mo Light	32.0	22.0	10.1	15.7	7.5	10.6	4.9	7.9	15.0	12.0	0.0*	18.9
6 mo Light	31.2	24.0	9.7	17.8	4.1	10.6	4.0*	5.3	14.0	8.0	3.0*	10.3
9 mo Light	13.1	28.2	7.6	16.2	0.2*	7.6	2.8*	3.7	16.2	3.5	0.6*	10.4
12 mo Light	19.3	25.5	4.7	15.8	1.8*	5.2	1.7*	1.8*	11.0	1.5*	0.6*	15.1*
16 mo Light	10.0	22.0	4.0	13.6	3.0*	8.8	0.0*	0.4*	11.8	1.5*	1.2*	16.4
24 mo Light	7.3	26.2	3.6	12.4	2.6*	0.8*	1.3*	2.9*	ND	ND	3.9	16.2

Data show the Delta-E for aged tooth samples compared with the time zero baseline for the relevant material and storage conditions (in columns), at relevant time intervals, in months (mo), across the rows in the table. Delta-E values were calculated from RGB values using an online calculator (colormine.org). Values shown with an asterisk were less than the threshold of 3.3 used for the study, and so would not be considered a perceptible visual change to an untrained person. Controls are as follows: C1: LOD with open lid; C2: LOW with open lid; C3: PEG 400/4000 (in the ratio of 10:1) in roots with a closed lid; and C4: LCW with saline irrigation. LED: Ledermix; DOX: Doxymix; OW: Open canal, wet storage; CW: Closed canal, wet storage; CD: Closed canal, dry storage; OD: Open canal, dry storage; ND: Not done. The prefix letters L or D were added to the group designation to distinguish between LED and DOX

ing of the roots. In contrast, the introduction of light resulted in very rapid changes, most notably darkening of roots filled with LED, rather than those with DOX. The influence of light was also seen in the pastes stored in microtiter plates.

Light has to travel through various structures before it can reach the roots of teeth. These structures include the overlying skin, lips and oral mucosa, the gingiva, bone and periodontal ligament. All colours within the visible light spectrum, other than visible red, are absorbed well within the skin (22, 23). While visible red light can penetrate through hard and soft tissues, light of this wavelength range has minimal effects in terms of tetracycline degradation. Tetracyclines in aqueous solution are most sensitive to visible blue light (450-490 nm), with red light having minimal effects (4).

Scatter events within the tooth structure can attenuate the effects of light. Within enamel, blue light is scattered the most, with only 40% of incident light being transmitted through wet enamel and only 20% through dry enamel (24). Following that, only 10% of transmitted blue light will penetrate through each millimetre of dentine (25). The thickness of cementum ranges between 0.076 mm for teenagers to 0.206 mm for elderly people. Therefore, most of the cementum would have been removed during the preparation of the roots (26). The percentage of light transmission through wet cementum is 65% and 49% through dry cementum which is further attenuated by the dentine (27). Clinically, the blue light component of ambient light would only have an effect on discolouration of anterior teeth if there was a poorly restored access cavity and any LED paste had not been thoroughly cleaned out of the access cavity.

In the present study, after the peak level of discolouration was reached (between 3-9 months), continued exposure to daylight caused the reversal of staining in roots stained with either LED or DOX. The present study is the first to document this phenomenon occurring in roots under controlled laboratory conditions.

Moisture

The third major variable explored in this study was the presence of moisture since water is required for the hydrolysis of tetracyclines. Moisture will be present clinically when the access cavity restoration is inadequate, or when it breaks down and allows the ingress of saliva and other fluids such as from drinks and food. It is known that when tetracyclines are stored as a powder and kept dry, they are stable and retain their original yellow colour, whereas tetracyclines in aqueous solution undergo rapid degradation (7, 28). Formation of

red-purple quinones in dentine is thirty times slower when there is no water present (7).

In the present study, red-purple staining occurred in the two LED wet groups (LOW, LCW) once they were exposed to light. The roots in the open canals turned a red-purple colour after only 1 month, whilst in roots with closed canals kept in wet storage, the same effect took 6 months to occur. Likewise, moisture accelerated changes in roots with DOX. Hence, it can be concluded that light and moisture are both required for tetracycline discolouration of dentine to occur. This was highlighted in two cases (3) where the combination of light and the breakdown of the access cavity restoration allowed saliva (that is, moisture) to enter the root canal system causing the discolouration of the teeth that had been medicated with LED.

Oxidation

A fourth consideration for discolouration relates to oxidation of tetracyclines. Oxygen is essential for the formation of the red-purple reaction products (29). Past studies of tetracycline antibiotics (including demeclocycline and doxycycline) have demonstrated that oxygen is consumed in this chemical reaction as it occurs in the presence of light, with the solution changing colour from yellow to pink, red or brown, depending on the extent of oxidation (7, 29).

A further factor which could contribute to discolouration is oxidation of tetracyclines by NaOCl. When this irrigant is used prior to medicating the root canal, both hypochlorite ions (OCl-) and hypochlorous acid (HOCl) may be present, depending on the pH of the solution (30). Both are powerful oxidizing agents that can degrade tetracyclines rapidly (31). Adding to this, the free available chlorine from the NaOCl breaks down proteins (such as dentine collagen) into amino acids, giving rise to chloramines which in turn degrade tetracyclines via chlorine substitution reactions) (32). This was clearly demonstrated by the difference between the control group 2 and the LCW groups which showed 1.5 times the staining when NaOCl/EDTA irrigation was used after 24 months of exposure to light. Alternatively, photo-oxidative degradation of tetracyclines can be prevented by rinsing the access cavity and root canal gently with 10% vitamin C (33).

Strengths of the study

The methodology used in the study was designed to control relevant factors that could influence the discolouration process, and to eliminate confounding factors. Improvements in the methodology reduced exposure of the root to oxidants and irrigants. There was no pre-soaking of teeth in oxidant solutions such as hypochlorite and chloramine solutions prior to instrumentation or after instrumentation. In addition, residues of the medicaments within the access cavities were removed. Furthermore, changes to the materials outside of the teeth were also tracked, and the direct effects of environmental oxygen explored by comparing storage in air to storage in an oxygen depleted atmosphere. The inclusion of a PEG vehicle control also allowed the impact of the vehicle on tooth discolouration to be assessed in samples treated exposed to NaOCl and EDTA during root canal treatment.

The period of three months in the dark represents the normal clinical situation when the roots are covered with bone, gingiva and overlying soft tissue. Three months is adequate to cover the majority of cases where medicaments are used even for extended periods such as when treating external inflammatory resorption.

The 24 months period of exposure to light extends what has been done in previous work, which used 6 months (34) or 12 months (35). Light is one of the main causes for tetracycline degradation. Roots can be exposed to light especially in the anterior region because of gingival recession, or an extensive fracture of the coronal tooth structure. The effect of daylight progressed from discolouration (a known outcome with tetracyclines) to then reversal of the discolouration.

The present study tracked both luminosity changes and Delta-E. The latter is important because it allows assessment of whether the colour changes that have occurred in the material in the microtiter plate or in roots containing the material from the baseline would be visually perceptible to the average person. This method has been used in past studies of endodontic discolouration caused by calcium silicate cements (36, 37), with the same value of 3.3 used as a threshold for meaningful colour change in a tooth.

Limitations of the study

While the model used in this study has provided valuable insights into root discolouration by two tetracycline-containing medicaments, it is not an entirely accurate representation of the clinical situation. It does not account for the presence of the soft and hard tissues overlying the radicular and/or coronal tooth structure which could influence the propagation of light. However, roots of extracted teeth have been used in previous studies.

Ideally a life-like model should be explored where teeth can be placed with simulated tissues, or in the jawbones of a human skull and then covering the bone with coloured silicone or other material to simulate the optical properties of bone and gingival tissues. This would also allow the effects of exposure to various light sources to be examined. Such a model could also allow effects of NaOCl and EDTA on other properties of roots, such as compressive strength, to be examined at the same time as assessing discolouration.

Implications for clinical practice

The clinical implications of these findings may be relevant to any endodontic procedures where these tetracycline medicament pastes are used - such as pulpotomy, pulpectomy, regenerative endodontics and replantation. Management of the coronal aspects of the access cavity is the key factor that has a bearing on whether discolouration from tetracycline-containing medicaments occurs, since an open (or inadequately filled) access cavity allows both water and oxygen, the two major drivers of discolouration, to enter the teeth. Such management requires four things: (1) existing restorations should be removed and replaced to provide adequate interim coronal restorations; (2) traces of oxidants in the root canal system could be removed by irrigating the canal with a freshly prepared solution of a suitable antioxidant (e.g. ascorbic acid 10% w/v in water).

If this is not available and EDTA is used as the irrigant, this will both dilute and very slowly inactivate the oxidant actions of NaOCl; (3) the medicament paste must be placed carefully and only within the root canal with any residues of the medicament within the coronal aspect of the access cavity must be removed with cotton pellets soaked in ethanol or EDTA repeatedly, until the cotton wool is clean; and (4) the access cavity should be restored with a two-step temporary restoration (e.g. Cavit overlaid with zinc oxide-eugenol, GIC, or composite resin).

The next stage of treatment (e.g. change of medicament or completion of the root canal filling) should be done in a timely manner to prevent breakdown of the interim and/or temporary restorations which could lead to staining of the tooth. In addition, the replacement of demeclocycline with doxycycline could help reduce the discolouration associated with tetracycline-containing medicaments. If all of these steps are carefully followed, the likelihood of staining when using tetracycline-containing medicaments will be minimised.

Disclosures

Conflict of interest: The authors deny any conflict of interest.

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