Assessment of coronary microleakage marker capacity of three dyes

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ABSTRACT

Objective: The aim of this study was to assess the marker capacity of 2% methylene blue, 2% Rhodamine B and 5% nickel sulfate. **Methods:** After biomechanical canal preparation in 84 single-root pre-molar teeth extracted from human beings, the access cavities were sealed with Coltosol® and the specimens were made impermeable, except for 1 mm adjacent to the temporary sealing. The samples were immersed in the staining solutions and kept in an oven at 37 °C for 3 and 7 days, and were submitted to thermal cycling. During this period, 300 cycles (5 °C and 55 °C) of 30 seconds each were performed in a specific appliance with a digital programmer for temperature, time and number of cycles. Longitudinal sections of the specimens were obtained and observed under stereomicroscopic lens. **Results:** The statistical results by the Analysis of Variance showed a significant difference ($p<0.05$) among the groups and between the two time intervals assessed. **Conclusion:** There was greater leakage in the 7 day interval in all groups, and the Rhodamine B dye exhibited the higher mean leakage depth values in the two time intervals, followed by methylene blue and nickel sulfate.

Keywords: Dyes. Microleakage.

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Introduction

The efficacy of coronary access, disinfection, modeling, and hermetic and three-dimensional filling of the entire root canal system, to provide the return of the tooth to its physiological function in the stomatognathic system as soon as possible, determines the success of endodontic therapy. Thus, in order to achieve success, it is imperative for the root canal to be adequately sealed. It is known that in the course of time, the success of endodontic treatment is safeguarded by this sealing that preserves the root canal from possible recurrent contaminations.

It has been difficult to find a temporary restorative material that resists the more accentuated imbalances, such as those that occur in the oral cavity as a result of the ingestion of liquid or solid foods with different temperatures. Marginal microleakage basically depends on two factors: Material/enamel-dentin interface and physical-chemical properties of the temporary sealing materials.[1] Thus, the solubility, capacity to disintegrate and dimensional instability (contraction and expansion) of the material prejudice the maintenance of coronary sealing, contributing to the increased passage of microorganisms, toxins and chemical substances from the oral cavity to inside the dentin tubules.

To identify marginal microleakage in temporary sealing materials, different methods can be used, among them the following are mentioned: dyes,[2,3,4] radioisotopes,[5,6] microorganisms,[7,8] fluid filtration,[9] the identification of ions and histochemical[10,11] and electrochemical processes.[12,13]

In vitro studies using dyes to assess microleakage have been conducted because they are easy to use and allow safe interpretation of results. Various marker substances have been used, and the following were mentioned: methylene blue, fluorescein, basic fuchsin, silver nitrate, Rhodamine B, nickel sulfate and Indian ink. A huge discrepancy has been observed with respect to the concentrations of these dyes and also in relation to the immersion time of specimens in them.

Methylene blue is an organic acid dye in the phenothiazine class, with molecular formula $C_{16}H_{18}N_3SCl$, widely used as a marker solution. This dye is highly soluble in water and easily penetrates the tooth structures, without undergoing adsorption by the mineral matrix, in addition to having low molecular weight, similar to the size of the nutrient molecules of microorganisms.[14]

Rhodamine B is a basic intense organic dye, with molecular formula $C_{28}H_{31}ClN_2$, soluble in water at ambient temperature, also solvent in alcohols and common organic solvents, in addition to being highly stable. It presents with red coloring, and when it reacts with Sb, Hg, Au and Bi chlorides, it turns purple. When this substance is diluted, it is capable of producing fluorescence.[15,16] Due to this property, Rhodamine B has also been used as marker to assess marginal coronal microleakage in temporary sealing materials.

Nickel sulfate is an inorganic compound with molecular formula $NiSO_4$, and is obtained by dissolution of the metal, oxide or nickel carbonate in a diluted sulfuric acid solution.[15] The metal nickel in minimally alkaline solutions with ammonia or in acid solutions buffered with sodium acetate, form a precipitated red reagent, almost insoluble in water, called nickel-dimethylglyoxime.[16] The nickel is revealed by means of an alcohol solution of dimethylglyoxime. The reaction of the nickel with dimethylglyoxime produces the formation of the Ni-dimethylglyoxime complex that presents a red color. The identification limit is 0.16 μg of nickel.

The chemical method to assess the dimensional stability of temporary sealing materials involves nickel ion leakage through the access cavity walls in the direction of the root canal.[1] The contact of the nickel ions with the revealing solution (dimethylglyoxime) impregnated in the cotton ball and paper cone lead to the formation of a red coloring substance, the nickel-dimethylglyoxime complex ($C_8H_{14}N_4NiO_4$). The presence of this substance inside the root canal allows the occurrence of microleakage to be detected.

Although there have been many researches to find a sealing material close to the ideal characteristics, little attention has been given to identifying the best marker solution for evaluating coronary marginal microleakage. Furthermore, the pertinent results found in the literature are discrepant due to the use of several dyes and the different immersion times applied to them. The use of staining solutions as leakage markers on materials being tested is an aspect that has been much questioned in recent years. Therefore, there is clearly a need to assess leakage levels revealed by different dyes and compare them, under the same experimental conditions, analyzing their influence on marking leakage in relation to a material used for temporary sealing.

This study assessed coronary microleakage marker capacity of 2% methylene blue, 2% Rhodamine B and 5% nickel sulfate, associated with 1% dimethylglyoxime at intervals of three and seven days, using Coltosol™ cement as temporary sealer.

Material and Methods

It was made a selection of 84 single-rooted human pre-molars with completely formed apexes, extracted by orthodontic indication or periodontal involvement, either healthy or with incipient caries, without detectable cracks after been inspected by a lens with enlargement of four times.

The coronary preparation performed presented proportions of 2.5 mm diameter in the mesiodistal direction and 3.5 mm in the buccolingual direction, carried out with a carbide steel bur #1557, operated in the vertical direction parallel to the long axis of the tooth, coupled to a high speed pen. The measurements were confirmed with a digital caliper. All the internal cavity walls were flatted after removing the roof of the pulp chamber with an Endo-Z bur and an endodontic probe was used to locate the entry to the root canal. After biomechanical preparation of the root canals using oscillatory technique[17] the #25 file was preserved as a memory file.

The teeth were randomly divided into 6 groups of 14 specimens each, and 2 teeth were used as control. The positive control teeth did not have temporary sealing and only the root surface was made impermeable, whereas the negative control was sealed and made completely impermeable. Both were immersed on the methylene blue, Rhodamine B and nickel sulfate dyes (Table 1).

Cotton balls were compacted in the pulp chamber of each specimen, leaving a distance of 4 mm from the cavitary surface angle, measured with a millimetric probe. For the specimens immersed in nickel sulfate solution, #25 absorbent paper cones and cotton balls to be introduced into the pulp chamber were previously treated with an alcohol solution of dimethylglyoxime, respecting the previously determined 4 mm.

Then Coltosol® was inserted in the cavity with the aid of a Hollenback 3S, and was smoothly adapted to the cavity walls by means of a condenser. Its adaptation was observed with a lens with enlargement of four times.

The entire external surface of the teeth was made impermeable, except in the coronary sealing region, respecting the cervical margin of the restoration and about 1 mm around it. The surfaces were covered with two layers of epoxy resin (Araldite™ Hobby) observing an interval of 24 hours for drying between the two applications. When this time had elapsed, the samples were covered with a layer of red colored nail polish (Niasi S/A) to make them completely impermeable, waiting 40 minutes for it to dry.[18] After sealing and respecting the drying times of the materials used to make them impermeable, the teeth were immersed in 6 standardized glass recipients, previously identified in accordance with the group, the dye used and the time interval. The volume of staining solution used in each recipient was standardized at 10 ml, measured by means of a hypodermic syringe, and these substances were prepared by a specific laboratory (Lenza Farmacêutica, Brazil).

The recipients containing the samples immersed in the dyes were placed in a bacteriologic oven where they stayed for a minimum of 8 hours at a temperature of 37 °C and 100% humidity.

The samples were removed from the oven and taken to a duly calibrated and electronically programmed thermal cycling appliance. Two (2) rounds of thermal cycling were conducted at temperatures of 5 °C and 55 °C, totaling 300 cycles of 30 seconds each. Firstly, the uneven numbered groups, that were immersed for 3 days in the staining solution, were thermal cycled and next, the even numbered groups, that remained in the staining solutions for 7 days.

Immediately after the thermal cycling process, the samples were put back into the bacteriologic oven at 37 °C, where they stayed, respecting the immersion time in the dyes, until the time they were sectioned.

After the time intervals had been completed, the teeth were removed from the staining solutions and washed under running water for 5 minutes and dried at ambient temperature for 24 hours to allow the dyes to fix.[18]

Table 1. Division of samples into groups.

GROUPS	DYE USED	TIME INTERVAL
I	2% methylene blue	3 days
II	2% methylene blue	7 days
III	2% Rhodamine B	3 days
IV	2% Rhodamine B	7 days
V	5% nickel sulfate	3 days
VI	5% nickel sulfate	7 days

After drying, longitudinal sectioning of the samples was done in the bucco-lingual direction, using a flexible double-faced diamond disk coupled to a micromotor.

Macroscopic observations of the longitudinal sections obtained were performed and the linear measurement of dye leakage was taken using a Wild M-8 stereomicroscopic lens under an enlargement of 4 times, with a digital camera coupled to a computerized quantitative analysis system, by means of Image Pro-Plus software calibrated to a 1-mm scale. The images were captured with this program from the most coronal distance on the cotton ball up to the maximum leakage point marked on it, measured in millimeters. The data found were put into tables and submitted to the Analysis of Variance and the *t* test, considering the level of significance of 5% ($p<0.05$), to check the differences among the studied groups.

Results

Table 2 shows that there was significant difference ($p<0.05$) among the three staining solutions used for each one of the evaluated periods.

In the two time intervals, three and seven days, Rhodamine B exhibited a significantly higher leakage depth than methylene blue and nickel sulfate. Furthermore, in relation to the latter two dyes, methylene blue showed greater penetration efficacy compared with nickel sulfate in the two time intervals assessed. On the other hand, no presence of leakage was observed in any of the specimens immersed in the three staining solutions in the negative control in the two time intervals assessed. In the positive control all the samples showed leakage of the dyes.

When the time periods were compared, it was observed a significant difference ($p<0.05$) between the two intervals, in which the leakage depth found in the seven day interval was significantly higher to the one found in the three day interval, in all the analyzed groups (Fig 1).

Table 2. Mean values of the leakage depth measurement (in mm).

GROUP	TIME INTERVALS		
	3 days	7 days	
2% Methylene Blue	1.524	2.597	2.061[b]
2% Rhodamine B	2.760	3.380	3.070[a]
5% Nickel Sulfate	1.191	1.910	1.551[c]
	1.825[b]	2.629[a]	

Means followed by different letters differ among them ($p<0.05$) by the t test.

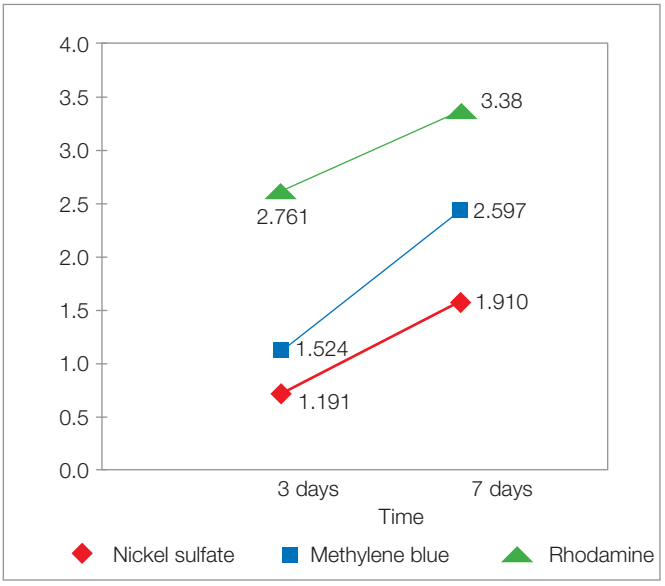

Figure 1. Leakage depth measurement in the two time intervals (in mm).

Discussion

Methylene blue, Rhodamine B and dimethylglyoxime are organic solutions used as indicators for qualitative inorganic determination.[19] The indicators must comply with two conditions: The associated or the ionized forms must present different colors, and the change in coloring must be fast.[20] Any dye with intense color that behaves by presenting a double redox reaction (reduction-oxidation) may be used as an indicator, if its oxidized or reduced forms have different colors.[21] The colors must be sufficiently intense to allow the dye to be used in such small concentrations that its addition to the test mixture insignificantly changes the redox potential. Methylene blue dye is a redox indicator and has an intense blue when oxidized, but it is colorless when reduced. Thus the color stability of organic dyes is an important factor that must be observed in microleakage studies and is related to the hydrogen potential (pH) that should also be assessed. It is known that methylene blue dye presents an acid character and Rhodamine B, a basic one. In contrast, methylene blue has less chemical stability

than the latter, thus it may change color over time or when coming into contact with a basic substance and can be transformed. By changing the pH, methylene blue is converted into leucomethylene (which is its non-visible form) and may change the accuracy of the reading.[22] A staining solution with acid or alkaline characteristics may alter the dentin itself during the period of experimental material setting, thus facilitating the creation of spaces and increasing the possibility of the dye leakage. Thus, an attempt was made in this study to implement a standardization of the pH of staining solutions around 7.0, the same precaution that is recommended in the literature.[23]

Results of microleakage studies are more reliable since the use of a phosphate ion buffer solution maintains the pH practically constant, even when an acid or a strong base is added. Thus it is important for the marker solution to be buffered and have its pH adjusted to 7.0 or close to it.[20] To evaluate sealer materials, one perceives that in the choice of the dye, little attention has been given to the various molecule sizes and their specific behavior in the face of certain circumstances. The choice is based more on the appearance of the dye.[24] From this aspect, it is important to emphasize that the organic dyes methylene blue and Rhodamine B are molecules that present high molecular weight and little capacity for ionic dissociation, in relation to the nickel sulfate, an inorganic salt. This substance has a low molecular weight compared with the macromolecules of the organic dyes and a high capacity of ionic dissociation when in the form of a solution. Thus, when staining solutions are studied comparatively, their concentrations must be adjusted in order to allow an equal competitiveness of the ions for penetrating the tooth/sealing material interface, when they are kept in the same volume of solution. Thus the relation between the mass and the volume of the staining solution determined for methylene blue and Rhodamine B was 2% and for the nickel sulfate solution, it was 5%.

The penetration depth of staining solutions into the dental structure varies in accordance with the amount of air trapped inside the root canal.[25] In our study, no vacuum was used and dye penetration occurred in a passive manner with a view to similar conditions closer to clinical reality. The elimination of air may induce an overestimate of the extent of microleakage in vivo,[23] although some authors have demonstrated that the use of vacuum gives a more precise assessment of dye penetration through the space existent between the sealing material and the canal walls.[25,26]

In the methodology used in this study, it was opted to use a 4 mm thickness of the sealing material on each one of the specimens. This is in agreement with some studies that proposed a thickness ranging between 3 and 5 mm, sufficient to assure marginal sealing.[2,27]

The option for the Coltosol sealing material, used in this study to assess the dye leakage marker capacity, was due to this cement has properties of expansion during its setting, providing a very dense filling and good marginal sealing.[28,29]

In the present study it was opted to use thermal cycling in an electronically programmed appliance, similarly to another study,[4] because it reproduced extreme temperatures more faithfully, although many authors prefer to simulate these variations by means of manual thermal baths on ice and in an oven[26,30] and by means of a humidifier chamber.[31]

In the literature there is still no standardization of the immersion time of specimens in the dyes.[10,11,26,29,30,32] In this study, periods of three and seven days of immersion time in the dyes were adopted to simulate the usual time between consultations.

The dye penetration depth was measured linearly, using a Wild M-8 stereomicroscope lens at a 4 times enlargement, with a digital camera coupled to a computerized quantitative analysis system, using Image Pro-Plus software. The use of a stereomicroscopic lens was found in other studies[30,33] and these measurements allowed more precise results to be obtained, although literature showed a large number of studies that analyzed marginal microleakage by means of scores.[1,4,32]

The Rhodamine B dye molecule is smaller than that of methylene blue and it is less tensoactive than the latter, as its penetration is greater, which is in agreement with the results of the present study.[22] Other studies also observed that Rhodamine B exhibited the highest intradentinal penetration indexes and allowed an adequate visualization.[34,35] On the other hand, some authors believed that methylene blue favored the reading of marginal microleakage when compared with Rhodamine B and fluorescein.[3]

The specimens immersed in the nickel sulfate staining solution exhibited the lowest penetration indexes among the dyes assessed. No studies making a

comparative assessment of this dye were found, so that the information in this study could be compared

The type of methodology used is an important factor for microleakage study, because the dyes, ions and isotopes present smaller molecular sizes than the bacteria and their by-products. However, if the dye identifies the microleakage, it means that there is a passageway and that the bacterias or its by-products could pass in a shorter or longer period of time. Thus, a tooth submitted to endodontic treatment should receive a well performed definitive restoration as quickly as possible, as defects in marginal adaptation that allow microleakage of saliva may place the entire endodontic treatment at risk.

Conclusion

Within the experimental conditions of this study, and considering the results obtained, it was concluded that:

1. The leakage magnitudes determined by 2% Rhodamine B, when the sealing material Coltosol® was used, were significantly higher than those found for 2% methylene blue and for 5% Nnickel sulfate, indicating the greater coronary microleakage marker capacity of Rhodamine B in relation to the other dyes, both at the interval of three and seven days ($p<0.05$).
2. The 2% methylene blue exhibited greater penetration depth in relation to 5% nickel sulfate in the two time intervals assessed ($p<0.05$).

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