Action of chlorhexidine and sodium hypochlorite over dentin microhardness

Daniel **DALLA-LANA**[1]
Priscila Daiane Schneider **HANAUER**[2]

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ABSTRACT

Objective: Evaluate the microhardness of bovine dentin tissue after exposure to endodontic irrigating solutions. **Methods:** Bovine dentin samples were randomly divided into 6 groups (n = 10) and submitted to the following treatments: saline (60 min); 2.5% NaOCl (sodium hypochlorite) (60 min); 2% gel CHX (chlorhexidine) (60 min); 17% EDTA (Ethylenediamine tetraacetic acid) (1 min); 2.5% NaOCl + 17% EDTA (60 + 1 min) and 2% gel CHX + 17% EDTA (60 + 1 min). Knoop microhardness was used for the measurements. Data were evaluated by ANOVA test followed by Duncans Method at 5% significance level. **Results:** The 2.5% NaOCl solution, followed or not by 17% EDTA, significative reduced the dentin microhardness ($p < 0.05$). Exposures to 2% gel chlorexidine, followed or not by 17% EDTA, did not result in alterations at dentin tissue microhardness ($p > 0.05$). **Conclusion:** 2.5% NaOCl significatively reduces the microhardness of bovine dentin after 60 minutes of exposure.

Keywords: Chlorhexidine. Dentin. Hardness. Root canal irrigants. Sodium hypochlorite.

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[1] Universidade de Passo Fundo, Programa de Pós-Graduação em Odontologia (Passo Fundo/RS, Brazil).

[2] Faculdade de Especialização na Área de Saúde do Rio Grande do Sul, Especialização em Endodontia (Passo Fundo/RS, Brazil).

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Contact address: Daniel Dalla-Lana
E-mail: danieldlana@yahoo.com.br - danieldallalana@gmail.com

Introduction

The two major challenges of endodontic therapy are represented by the disinfection process of the root canal, as well as the prevention of recontamination with adequated sealing of the canal system.[1] To achieve these requirements endodontists need to use root canal irrigants as assistents during the prepare stage.

A high percentage of walls of the root canal that are not touched by endodontic instruments is demonstrated in many studies. These results demonstrate and reaffirm the importance of the irrigating solutions in order to achieve an adequate disinfection.[2]

An ideal irrigant, in addition to antimicrobial capacity and biocompatibility, should have minimal effects on the physical properties of dentin tissue, since changes in dentin microhardness may predispose the dental element to fracture. The same is true for the decrease in flexural strength, where smaller loads will already be sufficient to lead to failure of the dentin tissue.[3]

Some studies demonstrate that endodontic procedures are capable of promoting a reduction of only 5% in dental stiffness, which would not be able to cause the weakening of this structure.[4]

Irrigating substances are used as the main agent for removal of debris left by instrumentation, also dissolve tissues and lubricate the canal during biomechanical preparation.[5] The most widely used solution in endodontics is NaOCl due to its potencial to dissolve necrotic tissues and antimicrobial effectiveness.[6] The association of a solution to NaOCl, such as EDTA, with chelating capacity, is also necessary for the removal of the smear layer.[7] For some schools, chlorhexidine has been used as an irrigant solution because of its antimicrobial property and low toxicity.[8]

The effect of irrigating solutions on dentin ultrastructure is still unclear. The acknowledgment about the kind of solution, concentrations, application time, and sequence of use is important to achieve the right sanitization without jeopardizing the dentin ultrastructure quality.[9] And, consider the effect of the irrigant on the dentin organic and inorganic matrices is an importante aspect to be studied.[10]

Chlorhexidine has been widely used as an irrigant or intracanal medication, in some studies it has been shown to be more efficient than 5.25% sodium hypochlorite against *Enterococcus faecalis*[11] and presents substantivity between 72 hours to 4 weeks.[12,13] However, organized bacterial growth in biofilm is very difficult to be eliminated, and chlorhexidine 2% is not capable of breaking it. Thus, sodium hypochlorite is the only irrigant solution capable of causing its disruption, which also applies to the dissolution capacity of organic tissue, which has been the main disadvantage of chlorhexidine.[11]

Some authors suggest the use of sodium hypochlorite in order to eliminate the organic tissue, then the use of 17% EDTA to remove the smear layer and in the sequence the use of chlorhexidine in order to increase the disinfection power due to its substantivity,[11,14] however, the chemical interaction between sodium hypochlorite and chlorhexidine may lead to color changes in the dental element[13] and the precipitate formed by this interaction interferes with the sealing of the sealing material.

When used as intracanal medication, the authors demonstrate that chlorhexidine is more effective than calcium hydroxide in eliminating *Enterococcus faecalis* and that if mixed, its antimicrobial effectiveness can be increased. Regarding bond strength, chlorhexidine presents inhibitory action of metalloproteinases, which would cause degradation of the unprotected collagen of the hybrid layer leading to failures in the adhesion of restorations. Clinically used at indicated concentrations, the biocompatibility of chlorhexidine is acceptable, and in rare cases may cause allergic reactions.[11]

Future studies should be performed to verify if chlorhexidine 2% as an irrigant and intracanal medication in the presence of blood, plasma and other fluids is as effective as NaOCl, until then it can not be considered superior to sodium hypochlorite,[14] once it has the indication of being an alternative as an irrigat in relation to antimicrobial activity, but it is important to point out it does not have the capacity to dissolve organic tissue[12,13]

In relation to the mechanical properties of dentinal tissue, both solutions significantly reduce microhardness, not presenting statistical differences between them when the measurements are carried out at 500 and 1000 micrometers of the surface of the root canal.[15]

Reduction of the microhardness of the canal surface is a desirable feature. The use of chelating agents during the biomechanical preparation of the root canals

promotes the removal of the smear layer, increases the penetration of the irrigating solutions inside the dentinal tubules, improving the disinfection capacity and reduces the dentin microhardness facilitating the action of the endodontic instruments within the root canal.[16]

The use of NaOCl alone, or followed by ultrasonification, or followed by the use of EDTA, promotes changes in dentin ultrastructure, resulting in surface collagen displacement and thinning of the fibrils, as well as extensive erosion of peritubular and intratubular dentin.[9]

When used NaOCl 5% followed by irrigation with EDTA 17% (2 minutes), without the use of NaOCl after EDTA solution, erosion in the dental tissue is minimal. However, a final irrigation with NaOCl 5% (1 minute) after the use of EDTA 17% (2 minutes) should be avoided or done with great caution to avoid chemical weakening of the root.[17]

Based on information in the literature about the interactions of endodontic substances with dentin tissue, the aim of this study was to evaluate the influence of the root canal irrigants and auxiliary chemical substances, used during the biomechanical preparation of the root canal system, on the microhardness of dentinal tissue of bovine teeth, considering the hypothesis that the solutions negatively influence the mechanical properties of dentinal tissue.

Material and methods

Twenty bovine teeth extracted from carcasses of animals slaughtered at the Henrich slaughterhouse in the city of Passo Fundo, RS, were used, which were washed and stored in a sterilized distilled water bottle, renewed every 5 days. It was selected teeth with only one root canal.

The coronary portions were removed with diamond disc (KG-SORENSE®, Cotia-SP, Brazil) and the root length was standardized at 20 mm. The root canal prepare was performed with LA Axxess drill (SybronEndoOrange, SP, Brazil.), in the sequence #1, #2 and #3, at the working lenght. The working length was determined with manual #15 K-file (Dentsply-Maillefer), reducing one millimeter after the file was visualized in the apical foramen.

The irrigating substance used for the preparation of the samples was the saline solution. The irrigating substance was carried into the root canal by a 21 mm long cannula, NavTip® (UltradentProducts Inc, South Jordam, USA), and a 5ml syringe. After the use of each drill, irrigation was performed with a volume of 5 ml of the solution and the patency with a k # 15 file.

After preparation, the samples were sectioned into 5 mm lengths (Fig 1) and randomly divided into 6 groups (n = 10). Each root resulted in 3 experimental units. The cervical portion of the slice was marked and polished with felt discs and aluminum oxide paste. The specimens were washed with distilled water to remove residues and then stored in individual vials (per group) containing sterile distilled water. Afterwards, the samples were immersed for 60 minutes in the solutions, according to the classification of the groups (Table 1), except for the EDTA solution, where the 1-minute period was used. After the immersion period, the samples were stored in sterile distilled water until the microhardness test was performed.

The microhardness test was performed in the metallography laboratory in the School of Mechanical Engineering of the University of Passo Fundo, using the microdriometer device (HMV-2 Shimadzu, Tokyo, Japan) with the knoop indenter, applying a load of 25 g for the period of 15 seconds. The samples were fixed in a miniwalrus, one at a time, and three indentations, 200 μm far each other, were performed at a depth of 500 μm of dentin, counted from the wall of the root canal, towards the cementum tissue, in the cervical portion of the slice (Fig 1). The mean value of the 3 indentations was the microhardness value used for the statistical analysis.

For the statistical analysis the data were transformed into log10 and evaluated by the ANOVA test followed by the post hoc of Duncan's Method, at a significance level of 5%.

Results

The results showed that the use of 2.5% sodium hypochlorite solution, with or without prior exposure to 17% EDTA, reached the lowest values of dentin microhardness ($p < 0.05$). Although no statistically significant difference was found between the groups that used the hypochlorite solution and chlorhexidine ($p > 0.05$), the samples exposed to chlorhexidine gel 2%, with or without prior exposure to 17% EDTA, did not present significant reductions in microhardness values of the dentin tissue ($p > 0.05$). The use of the 17% EDTA solution for 1 minute did not promote changes in dentin microhardness (Table 2).

Table 1. Classification of groups.

Group	Substance	Period (min.)
1	Saline	60
2	2.5% sodium hypochlorite	60
3	2% chlorhexidine	60
4	17% EDTA	1
5	17% EDTA + 2.5% sodium hypochlorite	1 + 60
6	17% EDTA + 2% chlorhexidine	1 + 60

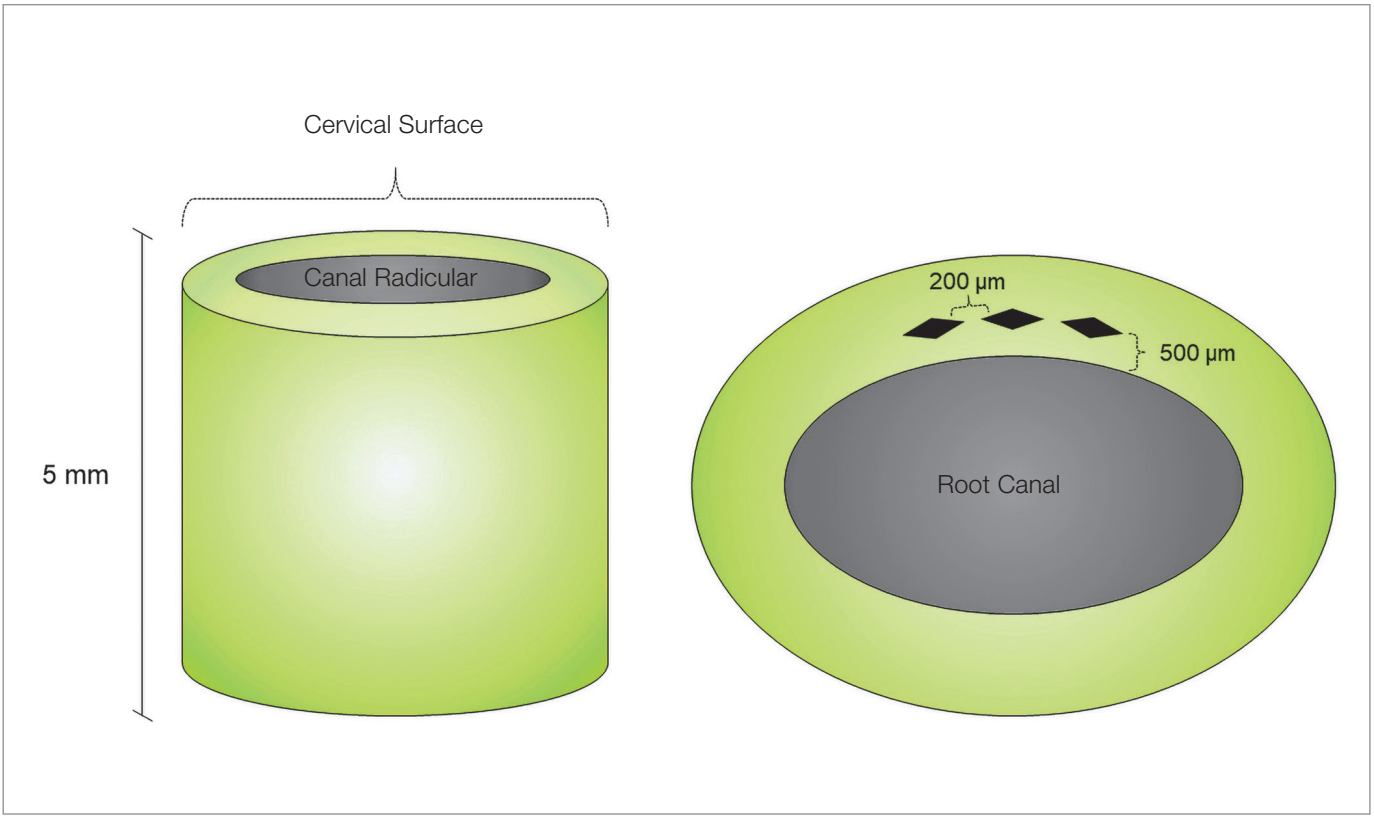

Figure 1. Schematic drawing of sample preparation and indentation sites.

Discussion

The study was conducted using samples of bovine teeth as they can be easily obtained and the dentin of these teeth is similar to the human dentin, with respect to the structure, composition and quantity of dentinal tubules.[18,19] In addition they have demonstrated a standardized morphological structure, since the animals are slaughtered with the same age, assuring similar characteristics and avoiding some variables that are found in human teeth. Finally, they are considered discarded pieces, so the study was not submitted to the ethics committee in scientific research.

Table 2. Mean and standard deviation of treatment groups.

Group	Mean+ / Standard Deviation
1 - Saline	$31,8^{A} \pm 4,5$
2 - 2.5% sodium hypochlorite	$18,1^{B} \pm 6,8$
3 - 2% chlorhexidine	$28,6^{AB} \pm 5,9$
4 - 17% EDTA	$36,2^{A} \pm 11,5$
5 - 17% EDTA + 2.5% sodium hypochlorite	$17,2^{B} \pm 4,4$
6 - 17% EDTA + 2% chlorhexidine	$23,2^{AB} \pm 4,9$

Means followed by different letters in the column are statistically different.

The most commonly used endodontic agents are sodium hypochlorite (NaOCl), chlorhexidine and EDTA. The first two have antimicrobial properties, but sodium hypochlorite has a cytotoxic action when compared to chlorhexidine, which does not have properties of tissue dissolution.[12,14] The current endodontic protocol indicates the use of EDTA after the use of chlorhexidine or hypochlorite as a chelating agent in endodontics, improving the disinfection of the canal system.[11,20] This increase in the disinfection capacity can be related to permeabilization of the dentinal tubules, allowing the access of the bactericidal solutions to the interior of the tubules, which has this depth reached through the activation with ultrasonic devices.[21,22]

The irrigating solutions are used in endodontics to facilitate the action of endodontic instruments and also have the capacity to dissolve organic matter, modify the pH of the medium, control infections and remove Smear layer.[23] Thus, in agreement with other authors,[11] they must present high cleaning power, antimicrobial capacity and biocompatibility.

Several studies look for the best irrigating substance for endodontics, but all irrigants have positive and negative properties, making each solution a complement of each other, since a single type of solution is not able to eliminate all types of debris. Other researchers also warn that the endodontic instruments are not able to prepare the entire area of the root canal walls, making the role of irrigating solutions a very important contribution for de desinfection process.[24,25]

Several concentrations of NaOCl are described in the literature, values of 1, 2.5 and 5.25% are those with tissue dissolving capacity and antimicrobial effectiveness. On the other hand, high concentrations of hypochlorite present greater adverse effects, making it even more cytotoxic and interfering in the properties of the dentin tissue.[26]

Our study used the concentration of 2.5% for sodium hypochlorite solutions as it is recommended in the literature for endodontic therapy.[13] The exposure time was 60 minutes, taking into consideration the clinical aspect of the endodontic therapy, where on average the time of contact with the irrigating solutions and auxiliary chemical substances, after the preparation stages of the operative field, is 60 minutes.

As expected, and described in the literature, sodium hypochlorite solutions interacted with dentin tissue. We can state that the result of this interaction can be interpreted in a negative way, since the reduction of the microhardness of the dentin tissue can make the dental element susceptible to fractures after the restoration stages, or to treatments with intraradicular retainers, mainly in relation to metallic cores where, as a consequence of the difference between the modulus of elasticity of the substrate and the restorative material, root fractures may occur, as described at the literature.[27]

In view of a significant decrease in the values of dentin microhardness after a long period of exposure to 2.5% sodium hypochlorite solution, we can think of reducing the concentration of this solution in order to reduce negative aspects of dentin properties, which can be clarified by new studies, searching for the concentration of NaOCl solution effective in eradicating bacterial contamination without adverse effects on dentin tissue.

The other antibacterial solution tested in this study was chlorhexidine, which has broad spectrum antimicrobial activity, i.e., it binds to hydroxyapatite of the enamel and dentin being released slowly as its concentration decreases in the medium, thus in-

creasing its period of action, called substantivity, which gives antimicrobial effect within 12 weeks after its use in the root canal.[28] Chlorhexidine has biocompatibility and is not irritating to the periapical tissues, being bactericidal in the concentration of 2%.[12] Therefore, it is an excellent alternative for patients allergic to sodium hypochlorite.

The present research used chlorhexidine in the concentration of 2%, as described in the literature,[28] but in the gel form, as indicated in the current endodontic protocols of many Brazilian institutions. The microhardness test results showed no significant changes in dentin tissue after the use of 2% chlorhexidine gel for 60 minutes.

EDTA, a chelating agent, which has the property of sequestering the metal ions of a particular molecular complex, was also one of the solutions tested in this study. The ethylenediaminetetraacetic acid (EDTA) is a specific chelator for the calcium ion and consequently for the dentin, acts on the dentinal tissue promoting a superficial softening, which will facilitate the stage of instrumentation and enlargement of the root canals.[5] Recent studies show that its use for a period of one minute already has enough action on the dentin, once its indication is only that of removal of smear layer and permeabilization of the dentinal tubules.[29]

In this study, the exposure of the dentin to 17% EDTA was 1 minute, according to the protocol of current clinical use suggested in the literature, followed by exposure to some of the tested bactericidal solutions. One of the expected consequences was that the groups treated with sodium hypochlorite after EDTA had a significant reduction of dentin microhardness, characterized by the erosion promoted by NaOCl after the use associated with EDTA.[17] The results did not show significant decreases in microhardness after exposure of the EDTA solution followed by exposure to hypochlorite or chlorhexidine, which was a very satisfactory result, since the function of EDTA is to permeabilize the dentinal tubules, allowing the penetration of bactericidal solutions in the interior of the dentinal tubules, where we know the bacterial contamination, and even biofilm formation, which can make the endodontic infection persistent. Therefore, the use of 17% EDTA for 1 minute seems to be a safe alternative in order to eliminate the smear layer and to permeabilize the dentin tubules, not significantly interfering in the mechanical properties of the dentin.

The use of saline in endodontics is characterized in the function of irrigant, that is, mechanical action for the removal of the debris present inside the root canal system. It is indicated for endodontic preparation protocols that use 2% gel chlorhexidine as auxiliary chemical substance, since, with the pharmaceutical gel form, it is impossible to act as an irrigant. In this research, it was one of the tested solutions, but with the purpose of control, because it is an inert solution on the mechanical properties of the dentin tissue.

It is important to emphasize that after the exposure protocols to the irrigant solutions and auxiliary chemical substances used in this study, the samples were washed with sterile physiological solution in order to remove the residues after the treatments, not allowing the formation of chemical smear layer, and also preventing the formation of the precipitate resulting from the interaction between EDTA and chlorhexidine, like found in another researches.[30]

Our study did not evaluate the microhardness values of the dentin tissue after the ultrasonic activation of the tested solutions, which can also interfere in the mechanical properties of the dentin tissue, since this activation amplifies the effect of the substances, promoting the heating of the same and allowing to reach more regions within the canals and dentinal tubules.

The microhardness of the dentin depends on its mineral concentration, when we measure the hardness values, we can have values altered according to the studied region of the dentinal structure of the bovine teeth. Therefore, in this study, we used as a microhardness value the average of 3 different indentations on the dentin surface in a nearby region.[16] The microhardness test is hardly performed on the surface of the root canal because it is a concave area, with the presence of microscopic peaks and valleys, which prevent a homogeneous defect by the crystal of the indenter, consequently making it impossible to visualize and measure the indentation in the binocular. Thus, the test was performed on the transverse surface along the long dental axis, after polishing and at a depth of 500 µm of the canal surface, as described in the literature.

Conclusion

In this way, from the point of view of microhardness, it is possible to conclude that long exposures to the NaOCl 2.5% solution, preceded or not preceded by exposure to EDTA 17%, should be avoided because can significantly reduce dentin microhardness ($p < 0.05$). The use of 2% gel chlorhexidine can be a good alternative, it had demonstrated to be safe, and did not produce significant changes in the values of dentin microhardness ($p > 0.05$). The use of 17% EDTA over the 1-minute period is enough, because according to the literature the action is effective and acoording to our results, did not produce changes in the microhardness values of the dentin tissue ($p > 0.05$).

References

1. Ricucci D, Loghin S, Siqueira Jr JF. Exuberant biofilm infection in a lateral canal as the cause of short-term endodontic treatment failure: report of a case. J Endod. 2013;39(5):712-8.
2. Clark-Holke D, Drake D, Walton R, Rivera E, Guthmiller JM. Bacterial penetration through canals of endodontically treated teeth in the presence or absence of the smear layer. J Dent. 2003;31(4):275-81.
3. Machnick TK, Torabinejad M, Munoz CA, Shabahang S. Effect of MTAD on flexural strength and modulus of elasticity of dentin. J Endod. 2003;29(11):747-50.
4. Reeh ES, Messer HH, Douglas WH. Reduction in tooth stiffness as a result of endodontic and restorative procedures. J Endod. 1989;15(11):512-6.
5. Machado SCP. Efeitos provocados pelos irrigantes endodônticos na estrutura do canal radicular [dissertação]. Porto: Universidade Fernando Pessoa; 2015.
6. Torabinejad M, Cho Y, Khademi AA, Bakland LK, Shabahang S. The effect of various concentrations of sodium hypochlorite on the ability of MTAD to remove the smear layer. J Endod. 2003;29(4):233-9.
7. Arslan H, Ayranci LB, Karatas E, Topcuoglu HS, Yavuz MS, Kesim B. Effect of agitation of EDTA with 808-nanometer diode laser on removal of smear layer. J Endod. 2013;39(12):1589-92.
8. Ferraz CC, Gomes BP, Zaia AA, Teixeira FB, Souza-Filho FJ. In vitro assessment of the antimicrobial action and the mechanical ability of chlorhexidine gel as an endodontic irrigant. J Endod. 2001;27(7):452-5.
9. Wagner MH, Rosa RA, Figueiredo JAP, Duarte MAH, Pereira JR, So MVR. Final irrigation protocols may affect intraradicular dentin ultrastructure. Clin Oral Investig. 2017;21(7):2173-82.
10. Oyarzun A, Cordero AM, Whittle M. Immunohistochemical evaluation of the effects of sodium hypochlorite on dentin collagen and glycosaminoglycans. J Endod. 2002;28(3):152-6.
11. Mohammadi Z, Abbott PV. The properties and applications of chlorhexidine in endodontics. Int Endod J. 2009;42(4):288-302.
12. Ercan E, Ozekinci T, Atakul F, Gul K. Antibacterial activity of 2% chlorhexidine gluconate and 5.25% sodium hypochlorite in infected root canal: in vivo study. J Endod. 2004;30(2):84-7.
13. Zehnder M. Root canal irrigants. J Endod. 2006;32(5):389-98.
14. Weber CD, McClanahan SB, Miller GA, Diener-West M, Johnson JD. The effect of passive ultrasonic activation of 2% chlorhexidine or 5.25% sodium hypochlorite irrigant on residual antimicrobial activity in root canals. J Endod. 2003;29(9):562-4.
15. Oliveira LD, Carvalho CA, Nunes W, Valera MC, Camargo CH, Jorge AO. Effects of chlorhexidine and sodium hypochlorite on the microhardness of root canal dentin. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2007;104(4):e125-8.
16. Cruz-Filho AM, Sousa-Neto MD, Savioli RN, Silva RG, Vansan LP, Pécora JD. Effect of chelating solutions on the microhardness of root canal lumen dentin. J Endod. 2011;37(3):358-62.
17. Wang Z, Maezono H, Shen Y, Haapasalo M. Evaluation of root canal dentin erosion after different irrigation methods using energy-dispersive x-ray spectroscopy. J Endod. 2016;42(12):1834-9.
18. Orstavik D, Haapasalo M. Disinfection by endodontic irrigants and dressings of experimentally infected dentinal tubules. Endod Dent Traumatol. 1990;6(4):142-9.
19. Donassollo TAR, Romano AR, Demarco FF, Della-Bona A. Surface microhardness evaluation of enamel and dentin in bovine and human teeth (permanent and deciduos). Rev Odonto Ciênc. 2007;22(58):311-6.
20. Arias-Moliz MT, Ferrer-Luque CM, Espigares-Garcia M, Baca P. Enterococcus faecalis biofilms eradication by root canal irrigants. J Endod. 2009;35(5):711-4.
21. Paiva SSM, Siqueira Jr JF, Rocas IN, Carmo FL, Ferreira DC, Curvelo JAR, et al. Supplementing the antimicrobial effects of chemomechanical debridement with either passive ultrasonic irrigation or a final rinse with chlorhexidine: a clinical study. J Endod. 2012;38(9):1202-6.
22. Beus C, Safavi K, Stratton J, Kaufman B. Comparison of the effect of two endodontic irrigation protocols on the elimination of bacteria from root canal system: a prospective, randomized clinical trial. J Endod. 2012;38(11):1479-83.
23. Ok E, Ertas H, Saygili G, Gok T. Effect of photoactivated disinfection on bond strength of root canal filling. J Endod. 2013;39(11):1428-30.
24. Peters OA. Current challenges and concepts in the preparation of root canal systems: a review. J Endod. 2004;30(8):559-67.
25. Vaudt J, Bitter K, Neumann K, Kielbassa AM. Ex vivo study on root canal instrumentation of two rotary nickel-titanium systems in comparison to stainless steel hand instruments. Int Endod J. 2009;42(1):22-33.
26. Cecchin D, Farina AP, Souza MA, Albarello LL, Schneider AP, Vidal CMP, et al. Evaluation of antimicrobial effectiveness and dentine mechanical properties after use of chemical and natural auxiliary irrigants. J Dent. 2015;43(6):695-702.
27. Barcellos RR, Correia DP, Farina AP, Mesquita MF, Ferraz CC, Cecchin D. Fracture resistance of endodontically treated teeth restored with intra-radicular post: the effects of post system and dentine thickness. J Biomech. 2013;46(15):2572-7.
28. Gomes BP, Vianna ME, Zaia AA, Almeida JF, Souza-Filho FJ, Ferraz CC. Chlorhexidine in endodontics. Braz Dent J. 2013;24(2):89-102.
29. Uzunoglu E, Aktemur S, Uyanik MO, Durmaz V, Nagas E. Effect of ethylenediaminetetraacetic acid on root fracture with respect to concentration at different time exposures. J Endod. 2012;38(8):1110-3.
30. Rasimick BJ, Nekich M, Hladek MM, Musikant BL, Deutsch AS. Interaction between chlorhexidine digluconate and EDTA. J Endod. 2008;34(12):1521-3.