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Laura Machado Martins Quagliatto

Efeitos dos meios de armazenamento na composição química da dentina humana

Effects of storage media on the chemical composition of human dentin

Dissertação apresentada à Faculdade de Odontologia da Universidade Federal de Uberlândia, como requisito parcial para obtenção do título de Mestre em Clínica Odontológica Integrada

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ATA

Ata da defesa de DISSERTAÇÃO DE MESTRADO junto ao Programa de Pós-graduação em Odontologia da Faculdade de Odontologia da Universidade Federal de Uberlândia.

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Projeto de Pesquisa de vinculação: Propriedades Físicas e Biológicas dos Materiais Odontológicos e das Estruturas Dentais.

As **quatorze horas e trinta minutos** do dia **vinte de fevereiro de 2019** no Anfiteatro Bloco 4L Anexo A, sala 23 Campus Umuarama da Universidade Federal de Uberlândia, reuniu-se a Banca Examinadora, designada pelo Colegiado do Programa de Pós-graduação em janeiro de 2019, assim composta: Professores Doutores: Carlos José Soares (UFU); Fabiola Galbiatti de Carvalho Carlo (UFJF); e o orientador(a) do(a) candidato(a): **Veridiana Resende Novais Simamoto.**

Iniciando os trabalhos o(a) presidente da mesa **Dra. Veridiana Resende Novais Simamoto** apresentou a Comissão Examinadora e o candidato(a), agradeceu a presença do público, e concedeu ao Discente a palavra para a exposição do seu trabalho. A duração da apresentação do Discente e o tempo de argüição e resposta foram conforme as normas do Programa.

A seguir o senhor(a) presidente concedeu a palavra, pela ordem sucessivamente, aos (às) examinadore (as), que passaram a argüir o(a) candidato(a). Finalizada a argüição, que se desenvolveu dentro dos termos regimentais, a Banca, em sessão secreta, atribuiu os conceitos finais.

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Nada mais havendo a tratar foram encerrados os trabalhos às 16 horas e 45minutos. Foi lavrada a presente ata que após lida e achada conforme foi assinada eletronicamente pela Banca Examinadora.

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“ Apesar dos nossos defeitos, precisamos enxergar que somos pérolas únicas no teatro da vida e entender que não existem pessoas de sucesso ou pessoas fracassadas. O que existe são pessoas que lutam pelos seus sonhos ou desistem deles. ”

Augusto Cury

SUMÁRIO

LISTA DE ABREVIATURAS E SIGLAS

FTIR - Espectroscopia Infravermelha Transformada de Fourier

EDS - Espectroscopia de Energia Dispersiva de Raios-X

DI – Água destilada

DE – Água deionizada

U – Água ultrapura

S – Soro fisiológico

AS – Saliva artificial

F – Dentes congelados

Ec – Condutividade elétrica

HA – Hidroxiapatita

CHAp – Hidroxiapatita carbonatada

RESUMO

RESUMO

O objetivo do foi avaliar o efeito de diferentes meios de armazenamento de dentes para estudos *in vitro* na composição química da dentina. 70 terceiros molares hígidos foram selecionados e divididos em 7 grupos de acordo com o tipo de armazenamento (n=10) - Grupo controle: amostras preparadas e analisadas em menos de 24 horas da extração dentária, não sendo armazenadas em nenhum meio; Grupo DI: amostras armazenadas em água destilada; Grupo DE: amostras armazenadas em água deionizada; Grupo U: amostras armazenadas em água ultrapura; Grupo S: amostras armazenadas em soro fisiológico; Grupo AS: amostras armazenadas em saliva artificial; Grupo F: amostras congeladas a – 20º C. Os dentes foram cortados e após 30 dias de armazenamento as amostras foram avaliadas utilizando Espectroscopia Infravermelha Transformada de Fourier (FTIR), por meio das razões: fosfato/amida I; carbonato/fosfato; e amida I/amida III. Em seguida, as mesmas amostras foram submetidas à análise de composição atômica através da Espectroscopia de Energia Dispersiva de Raios-X (EDS) que mensurou os níveis de C, Na, Mg, P, Ca e a razão Ca/P. Os dados foram avaliados por meio da Análise de Variância - ANOVA One-Way seguido pelo Teste de Comparação Múltipla de Dunnett ($p < 0,05$). A condutividade elétrica e o pH dos meios foram avaliados antes e após o uso como armazenamento. O FTIR mostrou diferença entre o grupo controle e o grupo água ultrapura ($p < 0,001$) para a relação fosfato/amida I, bem como para a saliva artificial ($p = 0,033$). Para a relação carbonato/fosfato, houve diferença do grupo controle para a saliva artificial ($p = 0,012$) e para a água desionizada ($p = 0,002$). Para a relação amida I/amida III, houve diferença significativa do grupo controle para a água destilada ($p < 0,001$), solução salina ($p < 0,001$) e água desionizada ($p = 0,009$). Ao avaliar a relação Ca/P, a análise estatística mostrou que as amostras da água deionizada, saliva artificial e água ultrapura foram diferentes do grupo controle ($p < 0,001$). Observou-se um aumento da condutividade elétrica para todos os meios após o armazenamento das amostras, sendo mais evidente na água ultrapura. Concluiu-se que os meios de armazenamento podem interagir com a composição química da dentina. As características na dentina armazenada em água ultrapura apresentou a maior diferença em relação a detina do grupo controle; enquanto que a dentina congelada apresentou os resultados mais semelhantes.

Palavras-chave: Dentina, FTIR, Propriedades químicas

Abstract

ABSTRACT

The purpose of this study was to evaluate the effect of different tooth storage media for in vitro studies on the chemical composition of dentin. Sixty healthy third molars were selected and divided into 7 groups according to the type of storage (n = 10). Control group: samples prepared and analyzed in less than 24 hours of dental extraction, and were not stored in any medium; Group DI: samples stored in distilled water; Group DE: samples stored in deionized water; Group U: samples stored in ultrapure water; Group S: samples stored in physiological saline; Group AS: samples stored in artificial saliva; Group F: samples frozen at -20° C. The teeth were cut and after 30 days of storage the samples were evaluated using Fourier Transform Infrared Spectroscopy (FTIR), using the following ratios: phosphate / amide I; carbonate / phosphate; and amide I / amide III. Then, the same samples were submitted to atomic composition analysis using X-ray Dispersive Energy Spectroscopy (EDX), which measured the levels of C, Na, Mg, P, Ca and the Ca / P ratio. Data were evaluated using the Analysis of Variance - One-Way ANOVA followed by Dunnett's Multiple Comparison Test ($p < 0.05$). The electrical conductivity and pH of the media were evaluated before and after use as storage. The FTIR showed a difference between the control group and the ultrapure water group ($p < 0.001$) for the phosphate / amide I ratio, as well as for artificial saliva ($p = 0.033$). For the carbonate / phosphate ratio, there was difference of the control group for the artificial saliva ($p = 0.012$) and for the deionized water ($p = 0.002$). For the amide I / amide III ratio, there was a significant difference of the control group for distilled water ($p < 0.001$), saline ($p < 0.001$) and deionized water ($p = 0.009$). When evaluating the Ca / P ratio, the statistical analysis showed that the samples of deionized water, artificial saliva and ultrapure water were different from the control group ($p < 0.001$). An increase in electrical conductivity was observed for all media after storage of the samples, being more evident in ultrapure water. It was concluded that the storage media can interact with the chemical composition of dentin. The characteristics of the dentin stored in ultrapure water presented the greatest difference in relation to the detina of the control group; while frozen dentin presented the most similar results.

Keywords: Chemical properties; Dentin; FTIR

Introdução e Referencial teórico

INTRODUÇÃO E REFERENCIAL TEÓRICO

Na Odontologia, a busca por situações laboratoriais que simulem as condições do meio bucal representa um importante fator para o correto desenvolvimento das pesquisas. Um número importante de testes in vitro utilizam dentes humanos extraídos como unidade amostral, o que parece ser a opção mais adequada. No entanto, esses experimentos precisam estar atentos à manutenção das características das estruturas dentárias para que se possa alcançar resultados mais confiáveis e fidedignos.

Sabe-se que a composição química da estrutura dental influencia diretamente nas suas propriedades e desempenho clínico (Marshall et al., 1997). Sendo assim, o conhecimento da composição dos substratos dentários e as variáveis que podem interferir nesse arranjo é primordial para a execução e entendimento de trabalhos *in vitro*.

A dentina madura de dentes vertebrados é um tecido mineralizado, duro, elástico e avascular que sustenta o esmalte e envolve a câmara pulpar central. É composto de aproximadamente 70% de mineral, 20% de matriz orgânica e 10% de água em peso e 45%, 33% e 22% em volume, respectivamente (Nanci A., 2003). A fase orgânica contém cerca de 90% de proteínas fibrosas (principalmente colágeno do tipo I), e o restante é composto por lipídios e proteínas não-colagênicas da matriz (Xu & Wang, 2012). Já a fase mineral consiste principalmente em fosfato de cálcio na forma de cristais de hidroxilapatita (HA) ($Ca_{10}(PO_4)_6(OH)_2$) e o teor de cálcio e fósforo varia de 34 a 39% e 16 a 18% em peso, respectivamente (Kang et al., 2004).

Os átomos de cálcio e fósforo formam um arranjo hexagonal no plano perpendicular ao eixo cristalino (eixo c, figura 1.1). Colunas constituídas pelo empilhamento de triângulos equiláteros de íons óxidos (O^{2-}) e de íons cálcio (Ca^{2+}) estão ligados entre si por íons fosfato (Elliot, 1994). Os átomos de oxigênio dos íons hidroxila estão situados a 0.9 Å abaixo do plano formado pelos triângulos de cálcio e a ligação O-H forma um ângulo de aproximadamente 30° com a direção C (figura 1.2). Dos quatro átomos de oxigênio que constituem os grupos fosfatos, dois estão situados em planos perpendiculares à direção C e os outros dois são paralelos a esta direção (Kay et al., 1964).

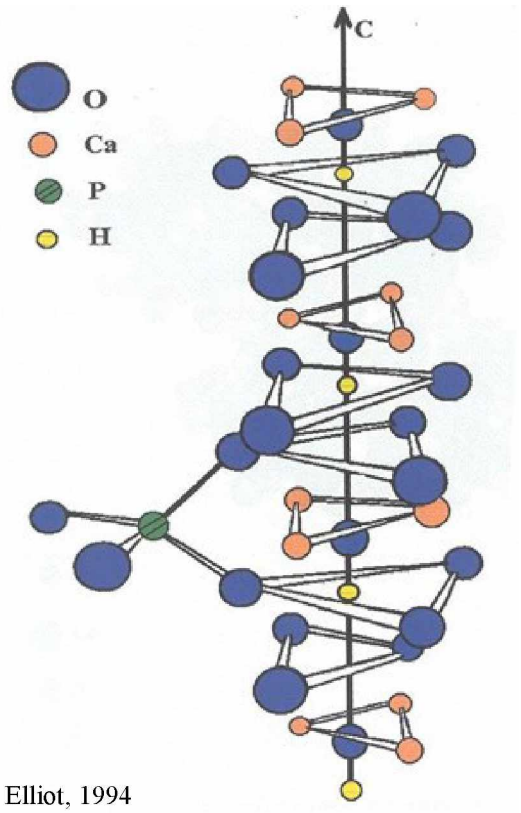

Fig. 1.1 Estrutura da Hidroxiapatita ao lonfo do eico C

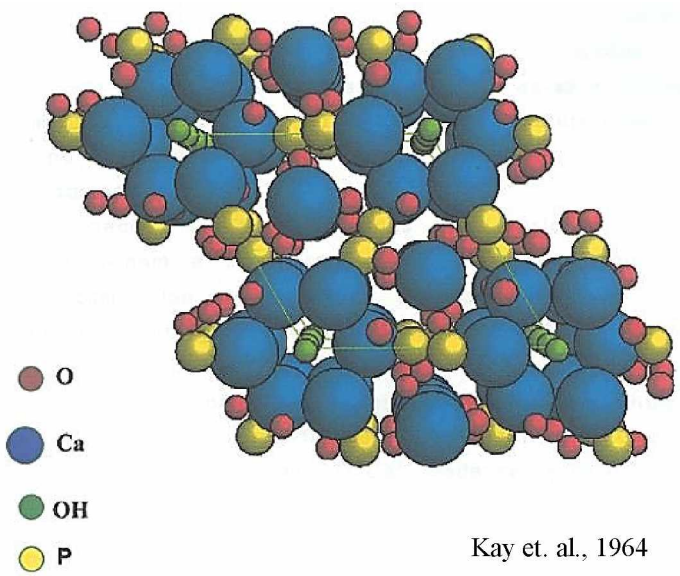

Fig. 1.2 Esrtutura da Hidroxiapatita – célula unitária

Os cristais de hidroxiapatita na dentina são dispostos de maneira orientada, com seu longo eixo paralelo ao eixo da fibrila da proteína predominante da matriz, o colágeno. Estão na forma de placas achatadas com dimensões aproximadas de 60 a 70 nm de comprimento, 20 a 30 nm de largura e 3 a 4 nm de espessura. A orientação, o tamanho e o local de formação desses cristais são regulados por componentes da matriz extracelular (Teruel et. al., 2015).

Substituições iônicas na estrutura apatítica freqüentemente ocorrem onde um íon é substituído por outro do mesmo sinal, mas de carga diferente (Elliot, 1994). Substituições acopladas de íons carbonato, por exemplo, são de extrema importância porque a natureza mineral da dentina consiste principalmente de hidroxiapatita carbonatada (CHAp), contendo 4-6% em peso de carbonato (Xu & Wang, 2012; Sonju e Ruyter, 1997). Sob diferentes condições, o íon carbonato pode ocupar dois locais diferentes na estrutura da hidroxiapatita: pode substituir os grupos hidroxila (OH), originando hidroxiapatita carbonatada tipo A, e pode substituir o sítio fosfato (PO_4^3), formando hidroxiapatita carbonatada tipo B, tais ocupações ocorrem numa razão de 10:1 (LeGeros, 199). Essa substituição iônica pode afetar a solubilidade da dentina (Rohanizadeh et al., 1999), ou seja, os grupos carbonatos não alteram a cristalinidade da hidroxiapatita em si, mas podem acelerar os processos de dissolução da estrutura, o que é verificado nas cáries dentárias e nos processos de reabsorção óssea.

Além do carbonato, a estrutura hidroxiapatita permite que vários outros íons sejam incorporados em centros catiônicos (Ca^{2+}) e aniônicos (OH, PO_4^3) com grande facilidade (Sonju e Ruyter, 1997). Sódio (Na^+), potássio (K^+) e magnésio (Mg^{2+}), por exemplo, podem substituir o sítio de cálcio, fluoreto (F^-) e cloreto (Cl^-), por sua vez, podem ser encontrados na posição hidroxila dando origem à fluorapatita e cloroapatita, respectivamente. Essas substituições determinam diferentes comportamentos físico-químicos e biológicos da estrutura (Kay et al., 2004), pois podem alterar a cristalinidade, os parâmetros de rede, as dimensões dos cristais, a textuta superficial, a estabilidade e a solubilidade da estrutura da hidroxiapatita.

Alguns fatores determinantes podem interferir nessas substituições e no arranjo da composição química da dentina, entre eles destacam-se o método de armazenamento realizado, o tempo e o tipo de substância utilizada para estocagem de dentes utilizados

em estudos experimentias (Aquilino et al., 1987; Sultana et al., 2006; Santana et al., 2008). Sempre que se trabalha com tecidos dentários é importante que estes sejam submetidos a métodos de desinfecção e sejam armazenados em meios adequados que possam manter, o máximo possível, a condição normal desses substratos (Silva et al., 2006; Humel et al., 2007; Goodis et al., 1993). Uma grande variedade de meios tem sido usada nos estudos experimentais, tais como a água destilada e água deionizada (com ou sem agentes anti-bacterianos acrescidos), álcool, azida sódica, cloramina T, formol, glutaraldeído, hipoclorito de sódio, saliva artificial, soro fisiológico e timol (Goodis et al., 1991; Straw et al., 1996; Secilmis et al., 2013). A constituição dessas substâncias pode interagir com os compostos químicos da dentina, alterando suas características e propriedades intrínsecas; no entanto, os seus efeitos na estrutura dentária ainda não são claramente conhecidos e interpretados.

A execução de estudos a cerca dessa compreensão e esclarecimentos torna-se indispensável, uma vez que não há na literatura uma padronização dos meios utilizados na conservação dos dentes após extração (Humel et al., 2007; Goodis et al., 1993; Straw et al., 1996; Sultana et al., 2006).

Objetivos

1. OBJETIVO

O objetivo desse trabalho foi avaliar o efeito de diferentes soluções de armazenamento de dentes nas propriedades químicas da dentina, buscando uma padronização para os estudos que envolvam mesmas variáveis. Compreender melhor como as condições de armazenamento afetam a estrutura dentária, possibilitará embasamento e segurança na escolha do meio de estocagem ideal para os diferentes estudos científicos.

Capítulo

1. CAPÍTULO 1

Artigo 1

Effects of storage media on the chemical composition of human dentin

***Artigo a ser enviado para o periódico Archives of Oral Biology**

ABSTRACT

Objective: To evaluate the effect of different storage media on the chemical composition of human dentin.

Materials and Methods: The chemical composition was evaluated by comparing control group with the experimental groups, stored for 30 days and randomly divided according to the storage media (n=10): distilled water, deionized water, ultrapure water, saline solution, artificial saliva, freezing. FTIR evaluated the ratios phosphate/amide I; carbonate/phosphate; and amide I/amide III. EDX measured C, Na, Mg, P, Ca and Ca/P levels. All data were tested for significant differences using one-way ANOVA followed by the Dunnett Multiple Comparison Test ($p < 0.05$). The electrical conductivity and pH of the media were evaluated before and after be used as storage.

Results: FTIR showed difference between the control group and the ultrapure water group ($p < 0.001$) for the phosphate/amide I ratio, as well as for artificial saliva ($p=0.033$). For the carbonate/phosphate ratio, there was difference between the control group with the artificial saliva ($p = 0.012$) and deionized water ($p = 0.002$). For the Amide I/Amide III ratio, there was difference between the control group with distilled water ($p < 0.001$), saline solution ($p < 0.001$) and with deionized water ($p=0.009$). When evaluating the Ca/P ratio, statistical analysis showed that the deionized water, artificial saliva and ultrapure water were different from the control group ($p < 0.001$). An increase in electrical conductivity was observed for all media after storage of the samples, being more evident in ultrapure water.

Conclusion: The characteristics observed in the dentine stored in ultrapure water presented the greatest difference in relation to the dentin of the control group (which would be ideal); while frozen dentin presented the most similar results.

Keywords: Chemical properties; Human dentin; Storage media; Fourier transform infrared spectroscopy

INTRODUCTION

For a better result and greater clinical relevance, the scientific studies in the area of dentistry need to construct laboratory situations that simulate, to the maximum, the conditions of the buccal environment. In this sense, the care with the methods of storage of the extracted human teeth is extremely important for the maintenance of its properties (Humel et al., 2007; Strawn et al., 1996; Sultana et al., 2006).

In the literature, there is no standardized and recommended substance for dental preservation and disinfection after extraction. Thus, a wide variety of storage media has been used in laboratory studies: distilled water and deionized water (with or without the addition of antibacterial agents) alcohol, sodium azide, chloramine T, formaldehyde, glutaraldehyde, sodium hypochlorite, artificial saliva, saline and thymol (Silva et al., 2006, Aquilino et al., 1987). In this way, it is possible that similar studies, with the same factor under study, present different results due to variables such as the type of storage and the medium used for tooth storage (Santana et al., 2008).

The chemical composition of dental tissues is one of the great properties that can be influenced by the storage medium. Their study becomes clinically relevant, since systemic alterations and therapies can alter the chemical nature and performance of dental structures (Cunha et al., 2017, Velo et al., 2018, Abbassy et al., 2015; Gutowska et al., 2011). Therefore, it is indispensable that the researcher is attentive to the storage method of choice, choosing the medium that influences as little as possible the characteristics of the sample and substrate evaluated.

Among the methods that determine the chemical composition of dentin, Fourier Transform Infrared Spectroscopy (FTIR) (Severcan et al., 2008; Bachmann et al., 2006) and X-ray diffusion spectroscopy (EDX) (de Morais et al., 2017) are frequently used. The EDX is a method of surface analysis that provides semiquantitative chemical analysis of the sample. It is based on the radiation of samples with high energy electrons and on the observation of X-ray emissions resulting from the core hole via electron de-excitation. The emitted X-rays energies generally differ from element to element, considering that each element has a unique atomic structure (Mitic et al, 2017). FTIR, in turn, provides

structural information of the molecules, identifies chemical bonds by wavelength and intensity of the absorption of infrared light by a specimen (de Miranda et al., 2018).

Knowing the parameters involved in these methods of analysis is important to avoid methodological errors. The way the samples are stored and prepared is a concern, because depending on the medium used new ions and chemical components could be incorporated into the specimens, not only quantifying the dental structure (de Barros da Cunha et al., 2017). Therefore, the purpose of this study was to evaluate the influence of different storage methods on the chemical properties of dentin, by FTIR and EDX analysis. The null hypothesis tested was that different storage methods exhibit similar performance for the chemical properties of dentin.

MATERIALS AND METHODS

Specimen preparation

After approval from the Ethical Committee in Research of the Federal University of Uberlandia (CAAE 49206815.7.0000.5152), seventy non-carious human third molars were collected, cleaned and examined using a stereoscopic microscope (Leica MS5; Leica Microscopy Systems Ltd, Heerbrugg, Switzerland) to check caries and another structural defect absence. The teeth were divided into 7 groups (n = 10) according to the type of storage method: **C**- control group (teeth collected, taken to the cutter and analyzed in less than 24 hours, not being stored in any medium); **DI** - distilled water; **DE** - deionized water; **U** - ultrapure water**; S** - saline solution**; AS** - artificial saliva; and **F** – frozen teeth (specimens frozen at -20 ºC).

After, the teeth were cut using a water-cooled diamond saw (Isomet, 15HC diamond; Buehler Ltd., Lake Bluff, IL, USA) mounted on a precision saw (Isomet 1000; Buehler Ltd., LakeBluff, IL, USA). Three perpendicular cuts along axis of each tooth was done: 1° - at the cementoenamel junction, separating the crown from the root; 2° - 2mm towards the crown; 3° - 4mm in the same direction for removal of the occlusal third. The slice of middle third was used for analysis and had the enamel removed by sectioning to obtain only dentine specimens. Then, all slices were cut longitudinally in mesial-distal

and buccal-lingual directions, resulting in four portions (Figure 1). The specimens were analyzed after 30 days storage. The solution was changed weekly.

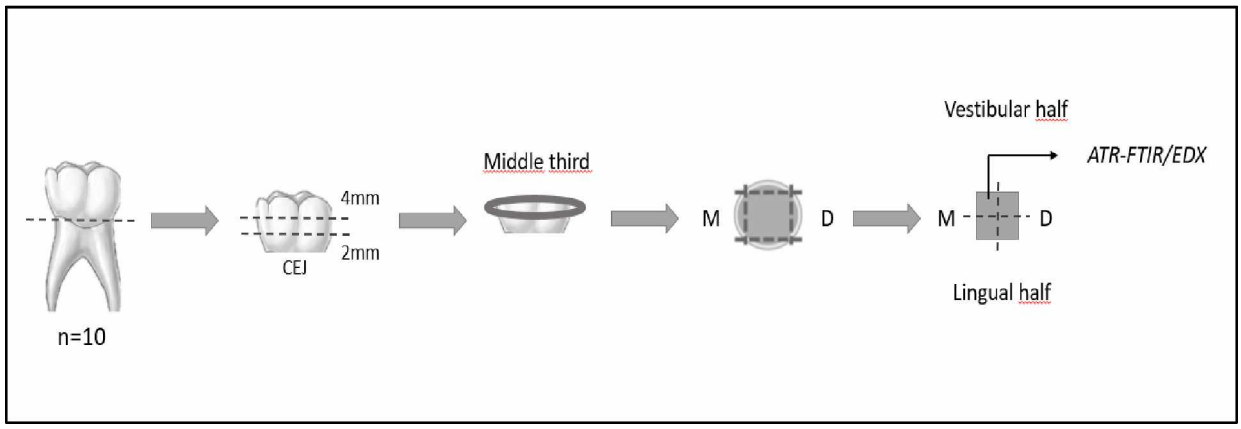

Figure 1. Schematic illustration of the experimental design

Fourier Transform Infrared Spectroscopy (FTIR)

The chemical composition of the specimens was evaluated for each group using attenuated total reflectance/Fourier-transform infrared spectroscopy (ATR/FTIR; Vertex 70 - Bruker, Ettlingen, Germany). Samples were removed from their storage medium and dried with absorbent paper, then each testing surface was carefully positioned against the diamond crystal of the ATR unit, and a constant pressure was applied to facilitate contact. The absorbance spectrum was acquired by scanning the specimens 32 times in the range from 400 to 4000 cm^{-1} at a 4-cm^{-1} resolution and then analyzed by OPUS 6.5 software (Bruker, Ettlingen, Alemanha). After baseline correction and normalization, the integrated areas of specific bands of organic and inorganic dentin content were calculated. The bands evaluated were as follows: amide I (C=O stretching at 1655 cm^{-1}), amide II (out-of-phase combination of N–H bending and C–N stretching at 1544 cm^{-1}), amide III (C–N stretching at 1235 cm^{-1}), carbonate v_2 at 872 cm^{-1} and phosphate v_1, v_3 stretching mode – 960cm^{-1} and 1040 cm^{-1} . Then the following parameters were evaluated: (1) mineral/matrix ratio M:M (expressed by the ratio between phosphate integrated areas and of protein amide I); (2) carbonate/mineral ratio C:M (the ratio of the integrated areas of carbonate to the phosphate) ; (3) amide I/amide III ratio (the ratio of the integrated areas of amide I to the amide III) (Toledano et. al., 2015; Lopes et. al., 2018, de Miranda et. al., 2018).

Energy-Dispersive X-ray Spectroscopy (EDX)

After analysis in the FTIR, the same specimen was sputter-coated (Balzers SDC 050 - Oerlinkon Balzers, Balzers, Liechtensnten) prior to analysis. Semi-quantitative elemental analysis of dentin was performed using an EDX (X-act, Oxford Instruments) spectrometer equipped with a rhodium X-ray tube and a liquid nitrogen (N_2) cooled semiconductor detector, operating at 15 kV. Chemical characterization (wt%) was performed and the concentrations of the following elements were evaluated: C, Na, Mg, P and Ca; as well as the Ca/P ratio of each group was determined.

Electrical conductivity (Ec) and ph of storage medium

The storages medium were subjected to analysis. Conductivity studies were performed at room temperature on a Tecnopon mCA-150 conductivity meter (version 6.2 software), using a potassium chloride solution (Λ = 146.9 $\mu S\ cm^{-1}$) as standard. The pH values were obtained using a METTLER TOLEDO FiveEasy™ pH meter with a LE438 sensor. The electrical conductivity and pH were evaluated for all media: distilled, deionized, ultrapure water, saline solution and artificial saliva. As a control they were analyzed immediately after the removal of their respective filters and containers of origin. And experimentally, each medium was analyzed after dentin storage. For this, the distobuccal sample of each tooth was used, which would not be tested at any time during the study (Figure 1.1). These dentin samples were submerged in the media corresponding to their experimental group and after 28 days these media were taken for analysis.

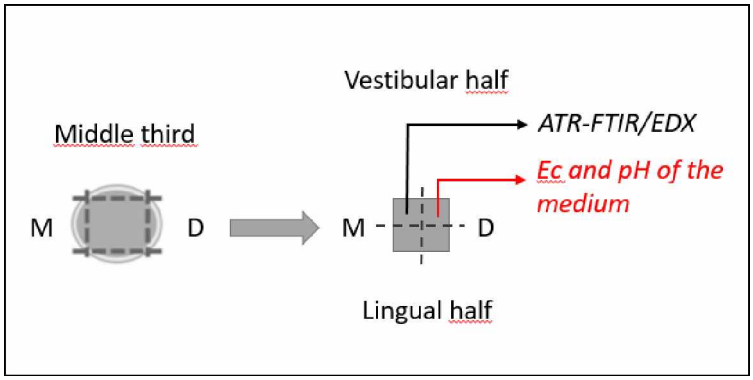

Figure 1.1 Illustration for identification sample stored in the media submitted to Ec and pH analyzes

Statistical analysis

For FTIR and EDX, data were tested for normal distribution (Shapiro-Wilk) and equality of variances (Levene's test). Analysis of variance (one-way ANOVA), considering the factor storage method, followed by Dunnett's test were performed on the chemical composition data of dentin by ATR/FTIR and EDX analysis. Sigma Plot statistical software (version 12.0, Systat Software, Inc., San Jose, CA, USA) was used for analysis and a p value of less than 0.05 was considered to be statistically significant. No statistical analysis was performed for the results of electrical conductivity and pH of the storage media. The purpose of these tests was to verify if there was alteration in the means to relate with the results of the chemical analysis, justifying them more clearly.

RESULTS

ATR/FTIR

The mean of the integrated areas of each band evaluated by ATR/FTIR are shown in Table 1. One-way Anova followed by Dunnett test showed difference between the control group with the group of artificial saliva for amide I ~ 1655 cm^{-1} ($p < 0.001$). For amide II ~1544 cm^{-1}, there was difference with ultrapure water ($p < 0.001$). For amide III ~1235 cm^{-1}, there was a difference between the control group with the following groups: distilled water ($p < 0.001$), artificial saliva ($p < 0.001$), saline solution ($p < 0.001$) and deionized water ($p = 0.018$). The group of ultrapure water and artificial saliva showed differences with the control group for the carbonate ~ 872 cm^{-1} analysis ($p < 0.001$). For the phosphate ~ 960cm^{-1} and 1040 cm^{-1}, only pure water showed a difference ($p < 0.001$) in relation to control group. The mean of the spectra of each group is shown individually in Figure 2a. To facilitate comparisons, such spectra were superimposed on Figure 2B.

Table 1. Mean of the integrated areas of each chemical component evaluated by FTIR

Storage Method	Amide I	Amide II	Amide III	Carbonate	Phosphate
Control	2.16 (0.12)	0.31 (0.08)	0.34 (0.08)	1.80 (0.12)	11.66 (1.74)
Distilled Water	2.03 (0.16)	0.19 (0.04)	0.14 (0.02) *	1.66 (0.21)	10.72 (2.46)
Deionized Water	2.18 (0.3)	0.41 (0.14)	0.28 (0.1) *	1.68 (0.19)	13.39 (0.8)
Ultrapure Water	2.46 (0.44)	0.61 (0.13) *	0.35 (0.05)	1.02 (0.21) *	7.09 (1.59) *
Saline Solution	2.13 (0.31)	0.3 (0.2)	0.2 (0.1) *	1.66 (0.21)	11.47 (2.71)
Artificial Saliva	1.52 (0.29) *	0.23 (0.08)	0.19 (0.05) *	1.27 (0.39) *	10.21 (2.44)
Frozen Teeth	2.06 (0.25)	0.36 (0.07)	0.40 (0.07)	1.78 (0.13)	12.43 (1.38)

* P value compared to the control with experimental groups

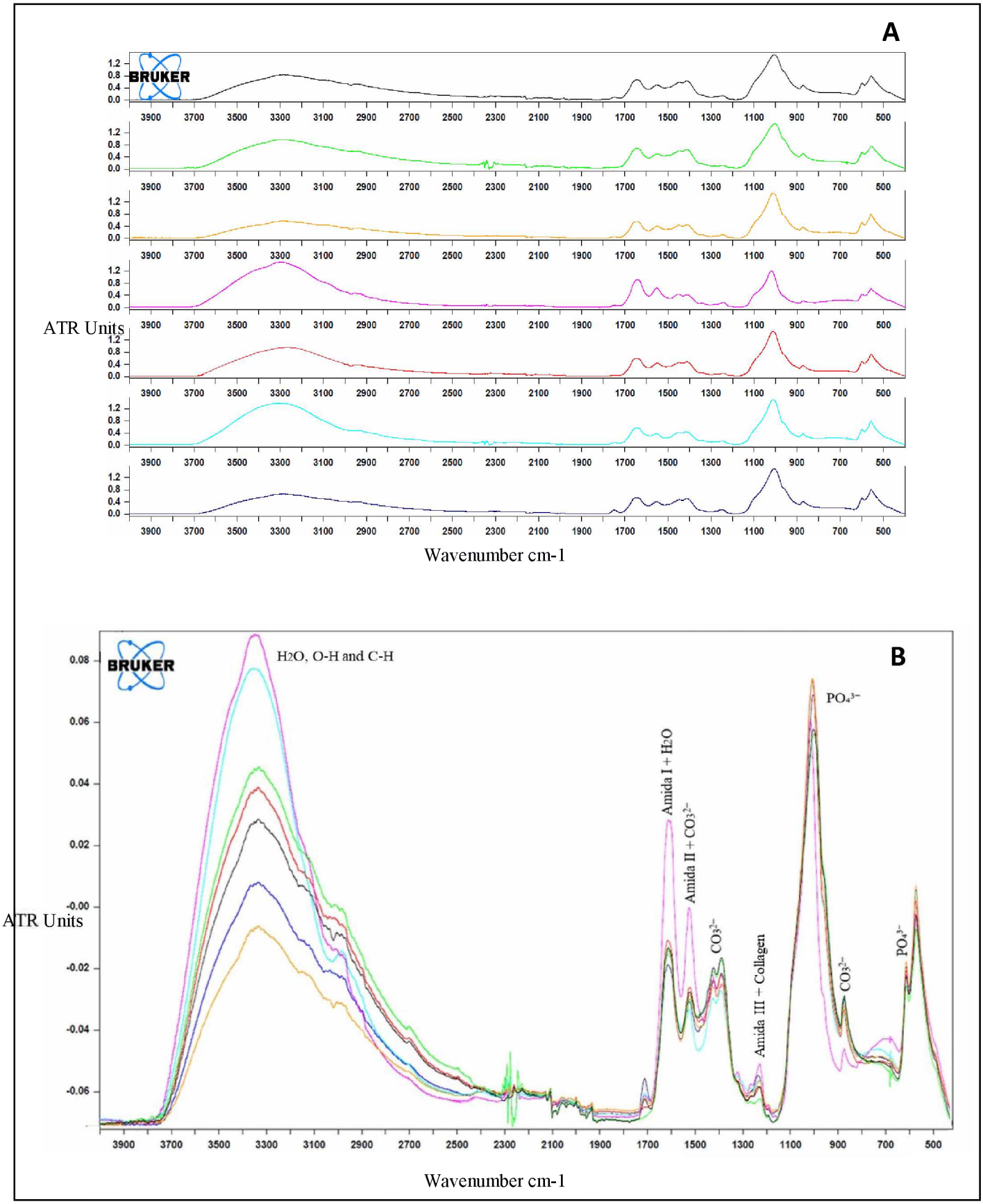

Figure 2. ■ Control group; ■ Distilled water; ■ Deionized water; ■ Ultrapurewater; ■ Saline solution; ■ Saliva artificial; ■ Frozen teeth

Means and standard deviation for ratio of the parameters obtained by ATR/FTIR are shown in Table 2. One-way Anova followed by Dunnett test showed difference between the control group with Ultrapure Water for the ratio phosphate/amide I ($p < 0.001$), as well as for artificial saliva ($p = 0.033$). For the carbonate/phosphate ratio, there was a difference between the control with the artificial saliva group ($p = 0.012$), and between the control and deionized water group ($p = 0.002$). For the Amida I/Amida III ratio, there was a difference between control group with distilled water ($p < 0.001$), saline solution ($p<0.001$) and deionized water ($p=0.009$).

Table 2. Means and standard deviation for the ratios of the parameters evaluated by FTIR

Ratio / Storage Method	Phosphate/ Amide I	Carbonate/ Phosphate	Amide I/ Amide III
Control	5.41 (0.94)	0.16 (0.02)	6.81 (2.13)
Distilled Water	5.29 (1.25)	0.16 (0.03)	14.91 (2.53) *
Deionized Water	6.31 (1.49)	0.13 (0.02) *	9.48 (2,23) *
Ultrapure Water	2.93 (0.66) *	0.15 (0.01)	7.69 (1.75)
Saline Solution	5.36 (0.97)	0.14 (0.02)	12.63 (3.97) *
Artificial Saliva	6.78 (1.62) *	0.12 (0.02) *	7.16 (2.60)
Frozen Teeth	6.18 (1.34)	0.15 (0.02)	5.24 (1.19)

* P value compared to the control with storage methods groups

EDX

Table 3 show the C, Na, Mg, P, Ca and Ca/P values, which expressed in percentages relative to the total amount of all detectable elements in the areas examined of each group after treatment with storage methods. One-way Anova followed by Dunnett test showed difference between control group with ultrapure water for C analysis ($p < 0.001$). For analysis of Na, there was a difference between the control group with artificial saliva ($p < 0.001$), physiological saline ($p < 0.001$) and ultrapure water ($p =$

0.042). Only the group of frozen teeth presented no significant difference for the Mg analysis (p = 0.189), while all other groups presented difference: ultrapure water (p <0.001), artificial saliva (p <0.001), deionized water (p <0.001), distilled water (p <0.001) and saline solution (p <0.001). The results of the P analysis showed a difference between control group with ultrapure water (p <0.001), distilled water (p = 0.006) and artificial saliva (p = 0.006). Only the distilled water presented difference for the Ca analysis (p = 0.041). When evaluating the Ca/ P ratio, statistical analysis showed that the deionized water, artificial saliva and ultrapure water were different from the control group (p <0.001 for all groups).

Table 3. Means and standard deviations of the chemical elements detected by EDX.

Storage Methods	**Chemical Elements of dentin (wt%)**					
	C	*Na*	*Mg*	*P*	*Ca*	*Ca/P*
Control	13.20	0.51	0.73	15.28	27.18	1.78
	(1.94)	(0.09)	(0.08)	(0.95)	(2.65)	(0.08)
Distilled Water	13.12	0.44	0.47	13.67	23.92	1.75
	(1.07)	(0.05)	(0.04) *	(0.57) *	(1.36) *	(0.03)
Deionized water	14.05	0.45	0.45	15.62	29.91	1.91
	(1.56)	(0.04)	(0.07)*	(0.81)	(1.90)	(0.04)*
Ultrapure water	21.26	0.39	0.25	12.86	24.53	1.90
	(3.05)*	(0.07)*	(0.09)*	(1.45)*	(3.54)	(0.07)*
Saline Solution	15.29	1.05	0.54	14.46	24.84	1.72
	(1.46)	(0,18)*	(0.10)*	(0.82)	(2.08)	(0.09)
Artificial Saliva	14.82	1.32	1.03	13.67	26.17	1.91
	(2.45)	(0.27)*	(0.15)*	(1.01)*	(2.19)	(0.04)*
Frozen teeth	12.57	0.64	0.83	14.90	27.18	1.82
	(1.96)	(0.17)	(0.11)	(1.46)	(4.04)	(0.10)

* P value compared to the control with storage methods groups

Ec and pH

Table 4 shows the values of Ec and pH of the storage substances before and after the storage of the dentin samples. A slight increase in pH was observed for all media after dentin storage, as well as for electrical conductivity. Ec variations for the three types of water (distilled, deionized and ultrapure water) were more evident after de storage period.

Table 4. Ec and ph values of storage media before and after storage of samples

Storage Media	**Ec** ***(before)***	**Ec** ***(after)***	**pH** ***(before)***	**pH** ***(after)***
Distilled Water	8.5 µs/cm	80.1 µs/cm	6.5	7.1
Deionized Water	5.5 µs/cm	91.1 µs/cm	6.5	7.2
Ultrapure Water	2.5 µs/cm	158.2 µs/cm	6.5	6.9
Saline Solution	17.4 µs/cm	18.5 µs/cm	6.0	7.1
Artificial Saliva	6.9 µs/cm	7.4 µs/cm	6.9	8.0

DISCUSSION

The null hypothesis was rejected, since the chemical composition of the dentin stored in certain medium was different from the non-stored dentin. This is probably because the chemical composition of these media interacts with some components of the dentin structure (Strawn et al., 1996; Secilmis et al., 2011; Moura et al., 2004).

In the analysis of dentin properties, some variables, such as time and storage method, are important because they can alter the results (Goodis et al., 1993; Tosun et al., 2007; 1996). The lack of standardization of storage methods for in vitro studies may yield unreliable results, since storage media may interfere with dentin characteristics. Based on a review of the literature performed in recent years, it was possible to observe the variability of the storage medium used for FTIR analysis (Table 5).

Table 5. Studies that used FTIR and its storage options

Title	Authors	Year	Material	Storage
Effects of in-office and at-home bleaching on human enamel and dentin: an in vitro application of Fourier Transform Infrared Study	Severcan F et al.	2008	Enamel and Dentin	Deionized water
Infrared Absorption Bands of Enamel and Dentin Tissues from Human and Bovine Teeth	Bachmann L et al.	2003	Enamel and Dentin	Saline solution (9%)
Spectroscopic alterations on enamel and dentin after nanosecond Nd:YAG laser irradiation	Antunes A et al.	2006	Enamel and Dentin	Saline solution (9%)
Tooth Bleaching Increases Dentinal Protease Activity	Sato C et al.	2013	Enamel and Dentin	Frozen at -30ºC
A biofilm cariogenic challenge model for dentin demineralization and dentin bonding analysis	Maske TT et al.	2015	Dentin	Distilled Water
A Fourier Transform Infrared Spectroscopy Analysis of Carious Dentin from Transparent Zone to Normal Zone	Liu Y et al.	2014	Dentin	Phosphate-buffered saline containing 0.002% sodium azide
A Mechanistic study of Plasma Treatment Effects on Demineralized Dentin Surfaces for Improved Adhesive/Dentin Interface Bonding	Dong X et al.	2014	Dentin	Phosphate-buffered saline containing 0.02% sodium azide
Changes in chemical composition and collagen structure of dentine tissue after erbium laser irradiation	Bachmann L et al.	2005	Dentin	Saline solution (9%)
Characterization of Dentin Matrix Biomodified by Galla Chinensis Extract	Deng M et al.	2013	Dentin	Distilled water

Collagen absorption bands in heated and rehydrated dentine	Bachmann L et al.	2005	Dentin	Saline solution (9%)
Compositional analysis of root cementum and dentin after ErYAG laser irradiation compared with CO2 lased and intact roots using Fourier transformed infrared spectroscopy	Sasaki KM et al.	2002	Dentin and cement	Distilled water
Effect of dental tissue conditioners and matrix metalloproteinase inhibitors on type I collagen microstructure analyzed by Fourier transform infrared spectroscopy	Botta SB et al.	2012	Dentin	Distilled Water
Effect of intracanal medicaments used in endodontic regeneration procedures on microhardness and chemical structure of dentin	Yassen GH et al.	2015	Dentin	Thymol 0,1%
Effect of Medicaments Used in Endodontic Regeneration Technique on the Chemical Structure of Human Immature Radicular Dentin: An In Vitro Study	Yassen GH et al.	2013	Dentin	Deionized water
Effect of Nd:YAG Laser Irradiation Pretreatment on the Long-Term Bond Strength of Etch-and-Rinse Adhesive to Dentin	Gan J et al.	2017	Dentin	Saline solution (9%) containing 0.002% sodium azide
Effects of heating on the mechanical and chemical properties of human dentin	Hayashi M et al.	2012	Demineralized dentin	Hanks'balanced saline solution (HBSS)
Molecular weight and galloylation affect grape seed extract constituents' ability to cross-link dentin collagen in clinically relevant time	Liu Y et al.	2015	Demineralized dentin	Saline solution (9%) containing 0.002% sodium azide

Non-thermal atmospheric plasma brush induces HEMA grafting onto dentin collagen	Chen M et al.	2014	Demineralized dentin	Saline solution (9%) containing 0.002% sodium azide
Phase, Compositional, and Morphological Changes of Human Dentin after Nd:YAG Laser Treatment	Lin CP et al.	2001	Dentin	Distilled Water
Proanthocyanidins' efficacy in stabilizing dentin collagen against enzymatic degradation: MALDI-TOF and FTIR analyses	Liu Y et al.	2013	Demineralized dentin	Phosphate-buffered saline containing 0.002% sodium azide
Surface characterization and biological properties of regular dentin, demineralized dentin, and deproteinized dentin	Tabatabaei FS et al.	2016	Dentin	Saline solution

FTIR spectroscopy is the measurement of the wavelength and intensity of the absorption of infrared light by a specimen. Infrared light is energetic enough to excite molecular vibrations at higher energy levels (Cui et al., 2012), such vibrations occur in wave numbers, or varying frequencies in the region of the light spectrum (Lopes, Limirio, Novais & Dechichi, 2018). The number of waves of each IR absorbance peak is determined by the intrinsic physicochemical properties of the corresponding molecule. In particular, the use of FTIR in attenuated total reflection (ATR) mode has attracted a lot of attention due to the small but well controlled depth of penetration and length of the corresponding path of the infrared light in the sample (Kasarian & Chan, 2013; Griffiths & Haseth, 2007). One of the advantages of this method is that it consists of a non-destructive technique that allows the use of the same specimen for different evaluations (Zieba-Palus & Kunicki, 2006). Unlike the FTIR that analyzes the sample at the molecular level, the EDX technique provides a chemical element level analysis and how they are distributed on the sample surface (Roberts-Harry et al., 2000). The emitted X-rays

energies generally differ from element to element, considering that each one has a unique atomic structure (Lopes, Limirio, Novais & Dechichi, 2018). The results of each of these methodologies can generate complementary information about the chemical composition of dentin and its possible alterations.

In the present study, the FTIR analysis showed the presence of the absorption bands corresponding to the collagen region and the inorganic portion in the study groups (Figure 2). The dentin spectrum is composed of mineral phase (phosphate and carbonate of ions) and organic phase (collagen amide I, amide II and amide III) (Kinney et al., 2003). This characteristic spectrum reflects the chemical nature of dentin: a mineralized connective tissue composed of approximately 70% minerals, 20% organic matrix and 10% water by weight (Deng et al., 2013). The mineral content of the dentin consists mainly of hydroxyapatite crystals formed by calcium phosphate ($Ca_{10}(PO_4)_6(OH)_2$) (Nanci A., 2003). Under different conditions, the carbonate ion may occupy two different sites in the hydroxyapatite structure: it may substitute hydroxyl groups OH (carbonated hydroxyapatite type A) and the phosphate site (carbonated hydroxyapatite type B) (Xu & Wang, 2012; Sonju and Ruyter, 1997). This ionic substitution can affect the crystallinity and solubility of dentin (Rohanizadeh et al., 1999; LeGeros, 199).

In comparison with the control group, ultrapure water showed less mean of the integrated areas of carbonate (CO_3^{2-}) and phosphate (PO_4^{3-}) peaks (Table 1). This can be explained by the total absence of ions in ultrapure water, which increases the chemical potential for the dissolution of carbonate and phosphate (Lee & Lin, 1997; Habelitz et al., 2002), altering these absorption bands (Figure 2). The electrical conductivity values (Ec), shown in Table 4, can support this discussion. The ultrapure water was the medium that presented lower Ec value before the storage of dentin (2.5 µs / cm). However, it was the medium with the highest value after the storage period (158.2 µs / cm), with a conductivity sixty times greater than the original one. This probably means that the PO_4^{3-} and CO_3^{2-} ions of the dentin were dissolved and incorporated into the ultrapure water.

In terms of variations of the organic matrix, the spectral region of amides I and III is suitable for the study of structural changes in proteins (Wang et al., 2002, Kirchner, 1997). Amide I is the main protein absorption band and is governed mainly by the stretching vibrations of C = O (70-85%) and C - N (10-20%) (Rodrigues et al., 2018).

The reduced means of the amide III integration areas for the distilled water, deionized water, artificial saline and saline groups indicates some structural or compositional molecular differences in the peptide chain (Table 1). This is because amide III is a very complex band, dependent on the details of the force field, the nature of the side chains and the hydrogen bond (Fang & Yang & Wu, 2002). For the saline and artificial saliva, this reduction may be due to the adsorption of ions from these solutions by the dentin substrate and due to the adsorption of water by collagen (Raum et al., 2007). This discussion can be illustrated in Figure 2, an increase of the O-H stretch band (3300 cm^{-1}) and a reduction of the C-N stretch band (1235 cm^{-1}) is observed, which characterizes greater water absorption in the collagen structure.

For the distilled and deionized water groups, this reduction can be justified by the strong dissolution process that occurs in the free ion solutions (Goodis et al., 1991), leading to a breakdown of chemical bonds that alter the structural organization of collagen (Habelitz et al., 2002). The amide bands II remained the same for all groups when compared to the control group, except for ultrapure water; However, changes in amidic peaks I and III are believed to be primarily responsible for structural and molecular changes in the protein (Goodis et al., 1993).

In FTIR spectroscopy, the area under a band is directly proportional to the concentration of the chemical species that gives rise to the specific band. However, it is sometimes preferable to use ratios between reference areas or peak heights, since the comparison parameters resulting from single bands are subject to uncertainties arising from sample to sample variations (Ou-Yang et al., 2001). From the results of the FTIR, it was possible to calculate ratios that illustrate the relationships between organic and inorganic components, bringing more information about the structure of collagen and hydroxyapatite.

The carbonate/phosphate ratio is important because it shows the mineral content of the carbonate in hydroxyapatite (Toledano et al., 2015). Reduction of this ratio was observed in the artificial saliva group due to the decrease in the carbonate area in this group and in the deionized water group (Table 2). However, in comparison with the control group, deionized water did not present significant differences for the carbonate and phosphate bands alone. This leads to the assumption that CO_3^{2-} occupied the hydroxyl

(OH) site, forming type A carbohydrate containing less water (Sonju & Ruyter, 1997), which could be confirmed in the FTIR spectrum (Figure 2). In organic content, the amide I/amide III ratio is important because it portrays the organization of collagen (Toledano et al., 2015). It was observed an increase of this ratio for the groups of distilled water, deionized water and saline solution, due to the reduction of the amide III bands for these groups, already mentioned previously.

The EDX shows that the samples of the distilled, ultrapure and deionized water had lower values of Mg^{2+} due to the absence of ions in these solutions (Tanaskovic-Stankovic et al., 2018). This ion shortage also justifies the reduction in phosphorus levels for ultrapure and distilled water. The solutions containing sodium ion (Na^{+}), saline solution and artificial saliva (for buffer effect), significantly influenced the chemical composition of dentin. The Na^{+} is considered an ion of small size, which may facilitate its entry into the crystalline lattice (Secilmis et al., 2011).

The Ca / P weight ratio determines the rate of mineralization of hydroxyapatite (Velo et al., 2018). The results showed an increase in the Ca / P ratio in the ultrapure water, deionized water and artificial saliva groups compared to the control group. Even with all the changes undergone by these groups when analyzing FTIR, it can be affirmed that there was no dissolution of hydroxyapatite. The pH analysis of the media, set forth in Table 4, confirms this claim. It is known that the rate of dissolution of dentin increases as the pH decreases (West et al., 2001; Vanuspong et al., 2002), and a pH equal to 5.5 already initiates this process of demineralization (Paschalis et al. al., 1996). However, no study medium showed this value. This means that differences in the mineral matrix of dentin stored in ultrapure water and artificial saliva did not lead to demineralization of hydroxyapatite. There was a more characteristic change in the structure and organization of the crystal at a first moment.

The control group sought to preserve the properties of dentin when the tooth is still in the oral cavity. For this, the teeth were collected, prepared and analyzed in less than 24 hours. This situation would be ideal, but in the execution of laboratory work is not feasible, since the collection of samples, the standardization of teeth and the preparation of the same is difficult to be done at the same time. Based on this, and in the results presented in this study, it was observed that the group of frozen teeth was the

closest to the ideal situation. The samples were stored for 30 days at a temperature of – 20 °C and did not present significant differences for any analysis when compared to the control group, showing that it was the only means capable of preserving the chemical characteristics of dentin. After tooth extraction, post-mortem changes can occur that modify the behavior and properties of dentin (Titley et al., 1998) and freezing can prevent this deterioration.

Therefore, for studies that evaluate the chemical composition of dentin, the freezing of samples is the most recommended medium, however more studies need to be done to get a better idea of the chemical interactions between the dentin and its storage medium.

CONCLUSIONS

It can be concluded that different storage solutions can affect the chemical properties of dentin, altering the organic and inorganic compositions. Ultrapure water was considered the worst storage medium for analysis of dentin chemical composition and freezing was considered the best because it preserved the natural properties and characteristics of dentin.

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Anexos

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• Use of parametric tests when non-parametric tests are required

• Inconsistencies between summary statistics and statistical tests such as giving means and standard deviations for data which were analysed with non-parametric tests.

• Multiple comparisons undertaken with multiple t tests or non-parametric equivalents rather than with analysis of variance (ANOVA) or non-parametric equivalents.

• Post hoc tests being used following an ANOVA which has yielded a non-significant result.

• Incomplete names for tests (e.g. stating "Student's t test" without qualifying it by stating "single sample", "paired" or "independent sample")