Universidade Estadual de Campinas

Faculdade de Odontologia de Piracicaba

MARÍA DEL CARMEN CHOQUE YAYA

EFEITO DA ENZIMA CATALASE E PEROXIDASE NA COR E RESISTÊNCIA DE UNIÃO DO ESMALTE E DENTINA APÓS CLAREAMENTO.

EFFECT OF CATALASE AND PEROXIDASE ENZYME ON COLOR AND BOND STRENGTH OF BLEACHED ENAMEL AND DENTIN

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Dissertação apresentada à Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas como parte dos requisitos exigidos para obtenção do Título de Mestra em Clínica Odontológica, na Área de Dentística.

Dissertation presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Master in Clinical Dentistry in Restorative Dentistry Area.

Orientadora: Profa. Dra. Débora Alves Nunes Leite Lima

Este exemplar corresponde à versão final da dissertação defendida pela aluna María del Carmen Choque Yaya e orientada pela Profa. Dra. Débora Alves Nunes Leite Lima.

Piracicaba
2019

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca da Faculdade de Odontologia de Piracicaba
Marilene Girello - CRB 8/6159

C456e

Choque Yaya, María del Carmen, 1989-
Efeito da enzima catalase y peroxidase na cor e resistência de união do esmalte e dentina após clareamento / María del Carmen Choque Yaya. – Piracicaba, SP : [s.n.], 2019.

Orientador: Débora Alves Nunes Leite Lima.
Dissertação (mestrado) – Universidade Estadual de Campinas, Faculdade de Odontologia de Piracicaba.

1. Dentes - Clareamento. 2. Antioxidantes. 3. Peroxidase. 4. Esmalte dentário. 5. Dentina. I. Lima, Débora Alves Nunes Leite, 1978-. II. Universidade Estadual de Campinas. Faculdade de Odontologia de Piracicaba. III. Título.

Informações para Biblioteca Digital

Título em outro idioma: Effect of catalase and peroxidase enzyme and color on bond strength of bleached enamel and dentin
Palavras-chave em inglês:
Teeth - Bleaching
Antioxidants
Peroxidase
Dental enamel
Dentin
Área de concentração: Dentística
Titulação: Mestra em Clínica Odontológica
Banca examinadora:
Débora Alves Nunes Leite Lima [Orientador]
Lúcia Trazzi Prieto Aureliano
Waldemir Francisco Vieira Júnior
Data de defesa: 09-10-2019
Programa de Pós-Graduação: Clínica Odontológica

Identificação e informações acadêmicas do(a) aluno(a)
- ORCID do autor: https://orcid.org/0000-0003-0046-6495
- Currículo Lattes do autor: http://lattes.cnpq.br/7668502311192742

UNIVERSIDADE ESTADUAL DE CAMPINAS
Faculdade de Odontologia de Piracicaba

A Comissão Julgadora dos trabalhos de Defesa de Dissertação de Mestrado, em sessão pública realizada em 09 de Outubro de 2019, considerou a candidata MARÍA DEL CARMEN CHOQUE YAYA aprovada.

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A Ata da defesa, assinada pelos membros da Comissão Examinadora, consta no SIGA/Sistema de Fluxo de Dissertação/Tese e na Secretaria do Programa da Unidade.

Dedicado à María Emília e o Víctor

Agradecimentos

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Código de Financiamento 001.

Agradeço a Deus por ser meu sustento nessa caminhada.

À Universidade Estadual de Campinas – UNICAMP, na pessoa do seu Magnífico Reitor Prof. Dr. Marcelo Knobel.

À Faculdade de Odontologia de Piracicaba – FOP, na pessoa de seu Diretor Prof. Dr. Francisco Haiter Neto e seu Diretor Associado o Prof. Dr. Flávio Henrique Baggio Aguiar.

À Profª. Drª. Karina Gonzales Silvério Ruiz, Coordenadora Geral do Programa de Pós-Graduação da FOP – UNICAMP.

Ao Prof. Dr. Valentim Adelino Ricardo Barão, Coordenador do Curso de Pós-Graduação em Clínica Odontológica da FOP – UNICAMP.

Agradeço a minha orientadora, Profa. Dra. Débora Alves Nunes Leite Lima, pela sua ajuda constante, pela ênfase que coloca nas coisas que realiza, que foi possível a realização do meu trabalho.

Aos professores da banca de defesa, Profa. Dra. Lúcia Prieto, Prof. Dr. Waldemir Vieira, Prof. Dr. Henrique Vieira e Prof. Dr. Luis Roberto Martins, por ser um exemplo para mim, por sua dedicação, perseverança e carisma com certeza levo um grande aprendizado deles tanto na parte profissional quanto no pessoal.

Ao Prof. Dr. Juliano Lemos Bicas e aos professores da banca de qualificação, Prof. Dr. Américo Correr, Prof. Dr. Pedro Ricomini e Profa. Dra. Maria Aguiar Jordão por demonstrar dedicação e amor no que fazem, que não só é refletido nos seus trabalhos publicados também na qualidade de pessoas, a humildade que mostra a sua grandeza.

Aos amigos desta caminhada, agradeço na pessoa do Rodrigo Lins por ser a luz que me direcionou nos momentos que precisei, pela sua dedicação no que gosta de fazer, pelo sorriso que nunca faltou que me deram forças para continuar, a Joyce Figueiredo pela sua amizade, pela companhia nas incontáveis noites de laboratório, a Mariana Flor por me brindar sua amizade e conselhos durante a pós-graduação, a Danielle Ferreira e Janaina Damasceno pela

sua disposição para me ajudar na finalização do meu trabalho, a Maria Cibelle Pauli pela sua ajuda na realização deste projeto.

Aos meus amigos do Peru, do El Salvador que no Brasil me deram apoio constante, pelos conselhos e pelas risadas brindadas que com certeza fizeram mais leve esta caminhada.

Aos meus colegas da turma de Mestrado da Dentística, meus sinceros agradecimentos a cada um de vocês que contribuíram para que eu estivesse mais perto dos meus objetivos.

Aos professores da Área de Dentística agradeço pela formação que recebi que contribuiu com minha evolução; levo grandes aprendizados para minha vida.

A minha família que é a minha base, obrigada pelo seu apoio incondicional, pela confiança que colocaram em mim, pelo seu exemplo que me levaram a conquistar meus sonhos, amo vocês.

Ao Erick Alpaca Zevallos por acreditar em mim, por me brindar seu apoio nos momentos importantes da minha vida.

Obrigada a todos que contribuíram para que este momento pudesse acontecer.

“Trata a um ser humano como ele é e ele permanecerá sendo aquilo; tratá-lo como pode chegar a ser e vai se tornar em aquilo que foi chamado a ser”.

Goethe.

Resumo

Este estudo *in vitro* avaliou o efeito da catalase e peroxidase na cor e na resistência de união do esmalte e da dentina após o clareamento quando restaurada imediatamente. Foram utilizados cento e noventa e dois blocos dentais (esmalte/dentina) bovinos e saliva artificial como meio de armazenamento. Foram divididos noventa e seis blocos para avaliar o esmalte (E), aleatoriamente em seis grupos (n=16): GCE Grupo controle, sem clareamento, PHE Clareamento com Peróxido de Hidrogênio (PH) 35%, PHC1E PH 35% + catalase 1000 U/mL, PHC10E PH 35% + catalase 10000 U/mL, PHP1E PH 35% + peroxidase 1000 U/mL, PHP10E PH 35% + peroxidase 10000 U/mL. Para avaliar a dentina (D) foram divididos da mesma forma, noventa e seis blocos divididos aleatoriamente em seis grupos (n=16): GCD, PHD, PHC1D, PHC10D, PHP1D, PHP10D. As enzimas foram obtidas nas concentrações de 1000 U/ml e 10000 U/ml para serem aplicadas sobre a superfície do esmalte logo após o clareamento com algodão saturado por 10 minutos. O clareamento foi realizado em esmalte com o uso do PH 35% em duas sessões, com intervalo de 7 dias. A análise da cor foi realizada em três tempos T0 (antes do clareamento), T1 (depois do clareamento) e T2 (depois da enzima) com um espectrofotômetro de refletância. Sendo considerado como resultado os valores de ΔL^*, Δa^*, Δb^* (CIELab) e ΔE_{00} (CIEDE 2000) entre os diferentes tempos $\Delta 1$(T1–T0), $\Delta 2$(T2-T0) e $\Delta 3$(T2-T1). Para a avaliação da resistência de união foi realizada a confecção dos pilares de resina composta flow nos grupos de esmalte e nos de dentina. Todos os grupos foram submetido ao teste de microcisalhamento, operando a célula de carga 5-N e velocidade de 0,5 mm / Min utilizando um fio ortodôntico com análise do padrão de fratura que foi avaliado em lupa estereoscópica (Leica Microsystems). O padrão de fraturas foi classificado em adesivas, coesivas e mistas. Os dados da análise de cor foram analisados pelos testes kruskal-Wallis e Friedman e Dunn, os dados de resistência de união por ANOVA one way e teste de Tukey, com nível de significância de 5%. Na análise dos dados de resistência de união houve uma diferença estatística ($p<0,05$) entre o grupo PHE quando comparado com o grupo GCE, observando uma resistência de união menor após clareamento. Os grupos com o uso das enzimas catalase e peroxidase após o clareamento para adesão imediata tanto no esmalte quanto na dentina obtiveram um aumento na resistência de união aproximando-se ao grupo controle, porem sem diferença estatística. Não foi encontrada diferença entre as concentrações utilizadas (1 000U/ml e 10 000U/ml). O padrão de fratura mais predominante foi a fratura tipo adesiva tanto no esmalte quanto na dentina. Os grupos PHC1, PHC10, PHP1, PHP10, tanto no esmalte quanto na dentina não apresentaram variação de cor estatisticamente significativa ($p> 0,05$). Conclui-se que o uso das enzimas catalase e peroxidase não influenciou na resistência de união ao esmalte e à dentina após o clareamento. A aplicação das enzimas catalase e peroxidase não influenciou na cor do substrato dental, mantendo a efetividade clareadora.

Palavras chaves: Clareamento dental. Antioxidantes. Peroxidase. Esmalte. Dentina.

Abstract

This *in vitro* study evaluated the effect of catalase and peroxidase on color and bond strength to enamel and dentin after bleaching when restored immediately. One hundred and ninety - two bovine dental blocks (enamel/dentin) and artificial saliva as storage medium were used. Ninety-six blocks were randomly divided to evaluate enamel (E), in six groups (n = 16): CGE Control group, no bleaching, HPE Bleaching with Hydrogen Peroxide (HP) 35%, HPC1E HP 35%+catalase 1000U/mL, HPC10E HP 35%+catalase 10000 U/mL, HPP1E HP35%+peroxidase 1000U/mL, HPP10E HP 35%+peroxidase 10000 U/mL. To evaluate dentin (D) were randomly divided equally, into six groups (n = 16): CGD, HPD, HPC1D, HPC10D, HPP1D, HPP10D. Enzymes were obtained at concentrations of 1000U/mL and 10000U/mL to be applied on enamel surface after bleaching with saturated cotton for 10 minutes. The bleaching procedure was performed in enamel with use of HP 35% in two sessions, with interval of 7 days. Color analysis was performed at three times T0 (before bleaching), T1 (after bleaching) and T2 (after enzyme) with a reflectance spectrophotometer. Considering as a result the values of ΔL *, Δa *, Δb * (CIELab) and ΔE_{00} (CIEDE 2000) between the different times Δ1 (T1 – T0), Δ2 (T2-T0) and Δ3 (T2-T1). For the evaluation of the bond strength, the composite resin flow pillars were made in the enamel and dentin groups. All groups were submitted to the micro-shear test, operating the load cell 5-N and velocity of 0.5 mm/min using an orthodontic wire with analysis of the fracture pattern that was evaluated in a stereoscopic magnifying glass (Leica Microsystems). The fracture pattern was classified as adhesive, cohesive and mixed. The color analysis data were analyzed by kruskal-Wallis and Friedman and Dunn tests, the bond strength data by one way ANOVA and Tukey's test, with a significance level of 5%. In analysis of bond strength data there was a statistical difference ($p < 0.05$) between HPE group when compared to CGE group, observing a lower bond strength after bleaching. The groups with the use of catalase and peroxidase enzymes after bleaching for immediate adhesion in both enamel and dentin obtained an increase in bond strength approaching the CG, but without statistical difference. No difference was found between the concentrations used (1 000U / mL and 10 000U / mL). The most predominant fracture pattern was the adhesive type fracture in both enamel and dentin. The groups HPC1, HPC10, HPP1, HPP10, in both enamel and dentin showed no statistically significant color variation ($p > 0.05$). It was concluded that the use of catalase and peroxidase enzymes did not influence the bond strength to enamel and dentin after bleaching. The application of catalase and peroxidase enzymes did not influence the color of the dental substrate, maintaining the bleaching effectiveness.

Keywords: Dental bleaching. Antioxidants. Peroxidase. Enamel. Dentin.

SUMÁRIO

1 INTRODUÇÃO

O clareamento dental representa atualmente uma alternativa conservadora e efetiva de tratamento para a maioria dos dentes com alteração de cor. Na presença de restaurações com resina ou prótese de dentes anteriores, o paciente deverá estar ciente que esses componentes apresentaram uma cor diferente dos dentes naturais que foram clareados, uma vez que o agente clareador não altera a cor das restaurações existentes (Gouveia et al, 2019) e a sua substituição após o clareamento torna-se necessária. O inconveniente da substituição imediata após o clareamento dental se deve a que o clareamento diminui a resistência de união (RU) do compósito ao esmalte e a dentina quando a restauração é realizada imediatamente após o término do tratamento clareador (Lago et al., 2013; Lai et al., 2002).

A RU é comprometida devido ao fato de que o peróxido de hidrogênio (PH) deixa uma camada de oxigênio residual livre, responsável pela quebra das moléculas cromóforas maiores em moléculas menores e menos pigmentadas (Lago et al., 2013; Lai et al., 2002) o que interfere também com a polimerização do sistema adesivo inibindo a infiltração do adesivo na superfície condicionada (Lai et al., 2002; Dishman et al., 1994). A adesão é feita através da formação de microestruturas denominadas “tags”, ou prolongamentos resinosos, porém, quando a superfície é clareada os “tags” apresentam-se fragmentados, pobremente definidos e em menor profundidade do que na superfície não clareada (Sundfeld et al., 2005). O PH apresenta um alto poder de penetração em esmalte e dentina devido a seu baixo peso molecular (Vidhya et al., 2011). Por este motivo o tempo recomendado para procedimentos adesivos varia entre 7 e 21 dias após o clareamento dental a fim de reverter a diminuição da resistência de união (Monaghan e Lautenschlager, 1992; Cavalli et al., 2001; Basting et al., 2004), mas uma espera tão longa pode resultar na insatisfação ou desconforto para um paciente na procura de estética imediata.

Com o intuito de reverter a RU afetada e diminuir o tempo para procedimentos adesivos, antioxidantes têm sido estudados para esse fim após clareamento dental. Como os antioxidantes enzimáticos, tais como a peroxidase (Elorza et al., 2012), catalase e a superóxido dismutase (Villareal e Einer, 2004; Feiz et al., 2017) (Sigma-Aldrich, St Louis, MO, USA), estas enzimas aumentaram os valores de RU, só a peroxidase e catalase obtiveram valores próximos aos do grupo controle. Como desvantagens do uso destas enzimas podemos citar o custo elevado, tempo demorado e dificuldade de transporte devido a importação. Dentre os antioxidantes não enzimáticos temos o ascorbato de sódio a 10% (Turkun e kaya, 2004; Whang

e Shin, 2015), que restabelece a RU diminuída, mas favorece o acúmulo de Streptococcus mutans (Khamyerdi et al., 2016) e pigmenta a superfície clareada (da Silva, 2006).

Já entre os antioxidantes herbais ou oriundos de plantas medicinais temos: a solução de chá verde (Lambert e Elias, 2010; Berger et al, 2013), a salvia 10%, o extrato de semente de uva 5% (Khamyerdi et al., 2016), a casca de pinho 5% (Subramonian et al., 2015), e a casca da romã 5% (Kumar et al., 2016), que possuem proantocianidina responsável pelo poder antioxidante, muito mais potente que os antioxidantes não enzimáticos, como as vitaminas C, E e betacaroteno (Leigh, 2003) mas afirma-se que devido a seu alto peso molecular tenha menor penetração na estrutura dentária (Kumar et al., 2016). Entretanto estes estudos foram analisados apenas em esmalte.

Apesar da variedade de substâncias antioxidantes para restaurar ou diminuir os efeitos adversos dos agentes clareadores sobre a RU no esmalte e na dentina após o clareamento dental, ainda não se comercializa um produto antioxidante de aplicação clínica. A falta de padronização da literatura e a presença das desvantagens apresentadas apontam à necessidade de mais estudos sobre antioxidantes que possuam uma utilização efetiva para o sucesso clínico imediato e a longo prazo. Então, a busca por um protocolo adequado que justifique a indicação de um antioxidante em casos de necessidade imediata do tratamento restaurador se faz necessária.

Desse modo estudamos algumas enzimas também conhecidas como biocatalisadores, essas enzimas podem ser extraídas de tecidos animais (catalase, pancreatina, tripsina, pepsina e renina) e fontes vegetais (peroxidase, papaína, bromelina, ficina, malte) (Bon et al., 2008).

A atividade de uma enzima é expressa em unidades (U), definida como a quantidade de enzima necessária para catalisar a transformação de 1 μmol de substrato por min. sob condições ótimas (Motamed et al., 2009). De acordo com o poder e especificidade por catalisar a decomposição do PH e a biocompatibilidade utilizamos as enzimas catalase e peroxidase para tal fim.

A enzima catalase faz parte do sistema de defesa do corpo humano, geralmente em células com aumento do estresse oxidativo, como citosol, mitocôndrias e peroxissomas (Scriver et al., 2009), atuando na decomposição do PH em água e oxigênio (Schonbaum e Chance, 1976). A temperatura ótima varia de 25°C a 30°C e a um pH ótimo de 6 a 7.5 (Alpteki, 2008).

As peroxidases são um grupo de enzimas que catalisam a oxidação de um substrato usando PH como molécula aceptora, formando moléculas de água. As peroxidases são uma das enzimas de maior estabilidade térmica presentes em frutas e vegetais (Berbicz e Clemente, 2001; Maciel et al., 2006), e que pode ser facilmente extraída (Everse, 2013). A sua temperatura ótima varia de 35°C a 37°C e a um pH ótimo de 6 a 7 (Roling et al., 2000).

Sabendo as propriedades das enzimas observamos que não há na literatura evidências científicas que utilizem a peroxidase obtida do nabo para reverter os valores de RU pós-clareamento. Dessa forma, o objetivo desse estudo *in vitro* foi avaliar o efeito do uso das enzimas catalase e peroxidase na resistência de união ao esmalte e à dentina clareada e avaliar o efeito das enzimas sobre a cor.

2 ARTIGO: EFFECT OF CATALASE AND PEROXIDASE ENZYME ON COLOR AND BOND STRENGTH OF BLEACHED ENAMEL AND DENTIN

Artigo submetido ao periódico Journal of Dentistry (Anexo 1)

ABSTRACT

OBJECTIVE: Evaluate the effect of catalase and peroxidase on color and micro-shear bond strength (SBS) to enamel and dentin after bleaching when restored immediately.

METHODS: Bovine dental blocks were randomly assigned to evaluate enamel (E) and dentin (D), in six groups each one (n=16): CG control group, HP Hydrogen peroxide (HP) 35%, HPC1 HP35% + catalase 1000U/mL, HPC10 HP35% + catalase 10000U/mL, HPP1 HP35% + peroxidase 1000U/mL, HPP10 HP35% + peroxidase 10000U/mL. The storage medium was artificial saliva. Bleaching procedure was performed in enamel, in two sessions, with interval of 7 days. To evaluate the color we used CIEDE 2000 color difference in three times, T0 (before bleaching), T1 (after bleaching) and T2 (after the enzyme) were analyzed. We used ΔL*, Δa*, Δb* and ΔE between the different times Δ1 (T1-T0), Δ2 (T2-T0) and Δ3 (T2-T1). The composite resin abutments were made in enamel and dentin for SBS evaluation, subjected to micro-shear test, analysis of the fracture pattern that was evaluated on a stereoscopic magnifying lens. Posteriorly statistical analysis was performed with significance level of 5%.

RESULTS: In both, a statistically significant difference between the HPE group when compared to the GCE group, showing lower adhesive resistance after bleaching. The groups HPC1, HPC10, HPP1, HPP10 of E and D obtained an increase in bond strength approaching the CG, however without presenting a statistically significant difference, for the color variation there was no significant statistical difference in the dental substrate ($p > 0.05$).

CONCLUSIONS: Catalase and peroxidase enzymes did not influence the enamel and dentin bond strength after bleaching and did not influence the color result obtained on the dental substrate.

Keywords: Dental Bleaching, Antioxidants, Peroxidase, Enamel, Dentin.

INTRODUCTION

Dental bleaching currently represents a conservative and effective treatment for most teeth with color changes. In presence of anterior teeth restorations, the bleaching agent does not change the color (1) and its substitution after bleaching may become necessary. The disadvantage of immediate substitution after bleaching is that hydrogen peroxide (HP) decreases the bond strength (SBS) of the composite to enamel and dentin when restoration is performed immediately after bleaching (2,3).

SBS is compromised due to the fact that HP leaves a free residual oxygen layer responsible for breaking larger pigmented molecules into smaller and less pigmented molecules (2,3). This residual oxygen interferes with adhesive system polymerization, reducing its infiltration (3,4). Adhesion is achieved by the formation of "resin tags", when the dental surface is bleached the tags are fragmented, poorly defined and with lower infiltration than on unbleached dental surface (5). HP has a high penetration power in enamel and dentine due to its low molecular weight (6).

Therefore, the recommended time for adhesive procedures ranges from 7 - 21 days after tooth bleaching to eliminate free residual oxygen (1, 7). However, such a long time may result in dissatisfaction or discomfort of a patient searching for immediate aesthetics.

Antioxidants have been studied to reverse decrease in SBS after tooth bleaching, such as peroxidase, catalase and superoxide dismutase (8,9), 10% sodium ascorbate (10), green tea solution (11), 10% sage, 5% pineapple extract (12), pine bark 5% (13), pomegranate peel 5% (14). Nonetheless, they presented some disadvantages such as high cost, shipping issues (8,9), favoring accumulation of *Streptococcus mutans* (12), bleached surface pigmentation (15), low penetration into dental structure due to high molecular weight (16).

Lack of standardization on literature and some disadvantages of the actual antioxidants used, further studies are required in order to find antioxidants that reestablish SBS values of resinous materials to bleached substrates that allow an effective use for immediate and long-term clinical success. Therefore, the search for an adequate protocol that justifies the use of an antioxidant in cases of immediate need for restorative treatment is necessary.

According to power and specificity for catalyzing HP decomposition and biocompatibility we used catalase and peroxidase for this purpose. These enzymes are oxirredutase hydrogen peroxide, present catalytic function of decomposition of HP in water and oxygen, substances innocuous for the organism (17).

Enzymes can be extracted from animal tissues and plant sources (18). The activity of an enzyme is expressed in units (U), defined as amount of enzyme needed to catalyze the

transformation of 1 μmol of substrate per min under optimal conditions (19), as the catalase enzyme, it is part of defense system of the human body, usually in cells with increased oxidative stress, such as cytosol, mitochondria and peroxisomes (20). The optimum temperature ranges from 25 ° C to 30 ° C and an optimum pH of 6 to 7.5 (21), and the peroxidases enzymes are one of enzymes with higher thermal stability present in fruits and vegetables (22); it can be easily extracted (23). The optimum temperature ranges from 35 ° C to 37 ° C, at an optimum pH of 6 to 7 (22,24).

Due to its biocompatibility, low cost, ease of obtaining and thermal stability, it is widely used in the textile, pharmaceutical and food industries. In order to reverse SBS values after bleaching, we looked for peroxidase and observed that there is no scientific evidence in the literature that used it enzyme obtained from the turnip, thus we have the following null hypotheses: (1) The use of catalase and peroxidase enzymes does not influence bond strength in enamel and dentin bleaching. (2) The use of catalase and peroxidase enzymes does not influence in color.

MATERIALS & METHODS

Experimental Design

Experimental units:	192 fragments of teeth obtained from bovine incisors.	
Factors under study	Treatment protocol in 6 levels	**CG Control group** HP 35% HP 35% + Catalase 1 000 U/mL HP 35% + Catalase 10 000 U/mL HP 35% + Peroxidase 1 000 U/mL HP 35% + Peroxidase 10 000 U/mL
	Time in 3 levels	Before bleaching After Bleaching After Enzyme
Response variable:	Micro-shear test Fracture pattern Color evaluation by reflectance spectroscopy (CIEDE 2000)	

Specimen Preparation

Extracted bovine incisors were used. The crowns were separated from the roots by using a double sided diamond disc (KG Sorensen, Barueri, SP, Brazil), which was mounted on an electric micromotor (NSK, Joinville, SC, Brazil) operating under constant irrigation. The inclusion criteria are incisive bovine healthy and exclusion criteria are presence of dentin

exposition, presence of pigmentation, cracks, fractures and caries for selected bovine incisors, these were stored in 0.1% thymol (Proderma, Piracicaba, São Paulo, Brazil) solution at 4°C until required.

Dental blocks (5mm x 5mm) were obtained from the bucal surface, using a diamond cutting disc (4" × 012 × ½, Buehler, Illinois, USA) coupled to a metallographic cutter (Isomet 1000; Buehler, Lake Buff, IL, USA). Enamel and dentin thickness were standardized (1mm of enamel and 1.75mm of dentin). The dentin surface was flattened and the enamel surface were polished using a rotary polisher (AROTEC, Cotia, SP, Brazi) equipped with silicon carbide paper discs 600, 1200 and 2000 SiC (Norton, São Paulo, SP, Brazil). At the end each stage, all specimens were immersed in ultrasonic tank (Marconi, Piracicaba, SP, Brazil) with distilled water for 10 min to remove both residual particles and the smear layer. Next the blocks were polished with felt discs (TOP, RAM and SUPRA AROTEC Cotia, SP, Brazil) associated with diamond paste (3цm, ¼ цm AROTEC Cotia, SP, Brazil) for one minute each one and rinse distilled water to get rid of debris layers, in between each stage. Also, each specimen was marked with a diamond bur #1012 (KG Sorensen, Barueri, SP, Brazil) on one side to standardize the sample position in the spectrophotometer. The dental blocks were stored in distilled water and refrigerated at 4°C, until required.

Turnip Peroxidase Extraction and Peroxidase Quantitative Determination

The test done was UV-vis. We use Guiacol 50mM, H_2O_2 20mM, 5% ethanol and 0.1M phosphate buffer pH 7.0. Pipette 0.623 mL of Guiacol, 0.195 mL of H_2O_2, 5 mL of ethanol and complete up to 100mL with phosphate buffer. Pipette 3 mL of this "substrate" into a cuvette of the spectrophotometer (DU 800, Beckman Coulter, CA, USA). The machine was reset with the "substrate" and add 0.5 mL of the soluble peroxidase extract (10 g turnips in 100 mL of buffer phosphate) (19) mixed rapidly the formed solution by inverting tube and placed in the spectrophotometer, this value was initial absorbance, measured again after 1 minute with diluted enzyme sample (0.9 mL of phosphate buffer + 0.1 mL of sample) for the value to be between 0.1 and 0.9. The enzyme activity was calculated by defining a unit of activity as increase of 0.001 in absorbance at 420 nm per minute of reaction per g. of sample (25).

The results of analysis are in table 1. From analysis in triplicate we used the best value obtained within the range of 0.1 and 0.9 absorbance; with this we obtained the concentration of enzyme with the following formula.

1U = 0.001 Absorbance / minute

Enzymatic Activity (t 0 min) = 0

Enzymatic Activity (t 10 min) = 0.52

EA = Amount of product formed / minute of reaction

EA = 1 500 U / 0.5 mL of the soluble peroxidase extract

EA = 3 000 U/mL

3 000 U --------- 1 mL

X --------- 100 mL

X = 300 000 U / 10 g de Nabo

X = 30 000 U/ g de Nabo

The enzyme was maintained stable for 10 minutes at 30 times dilution, being 1 000 U / mL; we also analyzed a higher concentration, being 10 000 U/mL.

Obtaining of Peroxidase Enzyme

The soluble peroxidase extract was obtained for each experimental part on the day of use. Slices of turnip were chopped; 60 g of turnip were weighed. The material was homogenized with 60 mL of 100 mM phosphate buffer solution, pH 7, for one and half minute. The suspension obtained was filtered on gauze and cotton, and the filtrate was centrifuged at 10 000 rpm for 20 min at 4 ° C. The supernatant was collected and stored at -18 ° C. (24).

Catalase Quantitative Determination

Catalase enzyme from bovine liver (Sigma-Aldrich, St Louis, MO, USA) was antioxidants used.

Enzymatic Activity = 2 000 U/mg (26)

5 mg/mL = 10 000 U/mL

The catalase and peroxidase were diluted in 100 mM phosphate buffer solution, pH 7, to obtain the concentrations tested (1 000 U/mL and 10 000 U/mL) for enamel and dentin groups.

Pre – bleaching preparation

The enamel and dentin groups were randomly divided. All specimens were storage in saliva artificial (Proderma, Piracicaba, São Paulo, Brazil) within labeled eppendorf 24 hours before bleaching procedure.

Bleaching Procedure

After 24 hours storage in artificial saliva, the specimens were bleached with 35% hydrogen peroxide (Whiteness HP, FGM, Joinville, SC, Brazil) and had their dentine facets

enclosed in double-sided tape (3M ESPE, St, Paul, MN, USA). Gel was applied to enamel according to the manufacturer´s instructions, three applications for 15min each, totalizing 45min of gel application. The bleaching gel was removed using flexible plastic cotton-tipped rods between each application and in the end of the whole process all specimens were thoroughly washed with water distilled. The bleaching procedure was repeated after 7 days. The specimens were stored in artificial saliva (27) in the incubator at 37°C between of sessions. All groups had daily saliva exchange; the control group remained in saliva during the procedure.

Antioxidant Application

Immediately after the second bleaching session, the specimens were washed with distilled water for 30 sec and dried with absorbent paper. In corresponding groups (G3E-G6E and G3D-G6D), catalase and peroxidase solutions were applied by covering bovine enamel with saturated cotton for 10 min (28). After antioxidant treatment, enamel surfaces were rinsed thoroughly with a water distilled for 30 s and air-dried.

After bleaching, all specimens of both enamel and dentin were included in polyester resin leaving surfaces of enamel exposed. The dentin groups following their inclusion were flattened using 600grift SiC paper under a water spray to create a standard smear layer, just before each adhesive procedure to simulate to the clinical treatment method, in which the smear layer was created by rotary instruments (29) until to the mark made with diamond bur #1012, made in enamel-dentin junction, leaving the dentin surface exposed, standardizing depth.

Color measurements

A spectrophotometer (Konica Minolta CM-700d-Konica Minolta Investment Ltd. Sensing Business Division, Shanghai, China) was used to measure the color of tooth in three times. At the baseline, when the specimens were without bleaching (initial/T0), after second sessions (after bleaching/T1) and after of catalase and peroxidase applications (after enzyme/T2).

The spectrophotometer was used according to the manufacturer´s instructions, was previously calibrated. Specimens were laced in a teflon device (specimen holder) inside a light cabin (GTI Mini Matcher MM1e, GTI Graphic Technology Inc., Newburgh, NY, USA) with a daylight lamp, ambient light was standardized during the measurement process. The color of the specimens were measured and quantified in terms of the CIEDE 2000 color difference for ΔE_{00} and ΔE (CIELab) color space system for ΔL^*, Δa^*, Δb^*. L^*, a^*, b^*, coordinate values established by members of the commission internationale de l´Eclariage (CIE) Technical Committee, in which the L^* axis represents the degree of lightness within a sample and ranges

from 0 (black) to 100 (white). The a* coordinates represents degree of green/red color. The b* coordinates represents degree of blue/yellow color. The measurement of color change in the different times was made by calculating the variation of L* (ΔL1=L*after bleaching – L*initial, ΔL2=L*after antioxidant – L*initial, ΔL3=L*after antioxidant – L*after bleaching), a* (Δa1=a*after bleaching – a*initial, Δa2=a*after antioxidant – a* initial, Δa3=a*after antioxidant – a*after bleaching), and b* (Δb1=b* after bleaching – b*initial, Δb2=b*after antioxidant – b*initial, Δb3=b*after antioxidant – b*after bleaching). The overall color variation was analyzed using the CIEDE 2000 system (ΔE00) according to the following equation: and the total color change (ΔE_{00}) was calculated according to the following formula (30):

$$\Delta E' = \left[\left(\frac{\Delta L'}{KLSL}\right)^2 + \left(\frac{\Delta C'}{KCSC}\right)^2 + \left(\frac{\Delta H'}{KHSH}\right)^2 + RT\left(\frac{\Delta C'}{KCSC}\right)\left(\frac{\Delta H'}{KHSH}\right)\right]^{1/2}$$

And through CIELab expressed according to the formula below:

$$\Delta E = [(L_1 - L_0)^2 + (a_1 - a_0)^2 + (b_1 - b_0)^2]^{1/2}.$$

Adhesive Procedures

After the antioxidant application treatments, specimen's surfaces were delimiting the area where the bonding was made, using adhesive tape with two holes of 1.1mm (31). Adhesive procedure was applied according to manufacturer instructions. The conditioning of the enamel and dentin surface was performed with 37% phosphoric acid (Condac, Joinville, SC, Brazil) for 30 and 15 seconds respectively, rinsed for 15 seconds and dried enamel surfaces for 10 seconds and dentin surface dried with absorbent paper. Two consecutive layers of adhesive (Single Bond 2, 3M ESPE, St, Paul, MN, USA) were applied by a microbrush actively for 15 seconds with interval of air- dried for 5 seconds. Before adhesive polymerization, cylindrical matrices made of perforated noodles (32) with diameter of 1.1mm and 1.5mm height (Furadinho 6, Pastificio Santa Amália, São Paulo SP, Brazil) were positioned on to the adhesive holes of the bounding tape. Adhesive was light-cured (VALO Cordless, Ultradent, South Jordan, UT, USA – 1200 mW/cm^2) for 10 seconds. To build up resins abutments, a flowable resin composite (Filtek Z 350, 3M ESPE, St, Paul, MN, USA) was filled on the matrices and light curing for 40s. After 2 hours storage in distilled water the matrices were removed along with adhesive tape.

Microshear Test

After 24 hours storage in distilled water at 37°C, the specimens were locked in a microshear device couple to a universal testing machine (4411 Instron, Norwood, MA, USA) operating the 5-N load cell and 0.5 mm / min velocity using an orthodontic wire (Morelli Ortodontia, Sorocaba, Brazil). A shear load was applied to the base of the composite cylinder. The values found in kilograms-force (Kgf) were converted into Megapascals (MPa) by dividing the force (Kgf) by the adhesive interface (cm^2).

Fracture pattern

After fracture the specimens were observed at a magnification of 50X using a stereomicroscope (Leica Microsystems, Wetzlar, Germany). The fracture pattern was classified as adhesive (failure at the enamel/composite interface), cohesive (failure in the enamel or composite) and mixed (combination of adhesive and cohesive failures).

Statistical analysis

The L value was analyzed by mixed models for time-repeated measurements. For data of value "a" and "b" were analyzed by the nonparametric tests of Kruskal-Wallis and Friedman. Color variations (deltas) were analyzed by Kruskal-Wallis and Dunn tests. Analysis of variance one-way (ANOVA) and Tukey's tests were used for analyzed bond strength data. Fracture pattern was analyzed used chi-square test. These analyses were performed in the SAS and R programs, with a significance level of 5%.

RESULTS

Peroxidase Quantitative Determination

The absorbance of the solution, in triplicate, was read in the spectrophotometer. By applying a calibration curve and the Lambert-Beer Law, the enzyme concentration was calculated from the absorbance (33), as can be seen in table 1.

Table 1 - Mean (standard deviation) of enzyme activity as a function of concentration and time

Time	Concentration		
	10x	30x	100x
1 minute	1.13 (0.11) Ac	0.28 (0.06) Bb	0.05 (0.02) Ca
5 minutes	2.09 (0.10) Ab	0.46 (0.04) Ba	0.09 (0.02) Ca
10 minutes	2.65 (0.14) Aa	0.52 (0.06) Ba	0.08 (0.02) Ca

Means followed by distinct letters (upper case in horizontal and lower case in vertical) differ from each other ($p \leq 0.05$). p (concentration) <0.0001; p (time) <0.0001; p (concentration x time) <0.0001.

Power Test: It is possesses

The power of the test was calculated in the Gpower program. The parameters used in calculate were: significance level of 0.05, mean effect size (f = 0.38), according to Cohen (1988) and 6 treatments. The sample size of 16 experimental units per group provided the power of the test of at least 0.80 in the performed analyzes (34).

Shear Bond Strength

One-way ANOVA and Tukey´s tests showed that for enamel the bond strength was significantly higher in the non-bleaching group than in the group with HP 35% ($p < 0.05$). The other groups presented intermediate resistance, not differing from the groups no treatment and PH 35% ($p > 0.05$). For dentin there was no significant difference between groups ($p > 0.05$). These results can be observed in Table 2.

Table 2 - Mean (standard deviation) of the bond strength (Mpa), in the micro-shear test, as a function of the group

Group	**E**namel	**D**entin
Control Group	29.77 (5.39) a	29.99 (7.56) a
HP 35%	23.15 (5.76) b	24.85 (8.17) a
HP35% + C 1000 U/mL	28.53 (5.28) ab	27.44 (8.96) a
HP35% + C 10000 U/mL	28.62 (5.07) ab	28.51 (8.01) a
HP35% + P 1000 U/ mL	27.72 (5.52) ab	27.67 (6.33) a
HP35% + P 10000 U/ mL	28.87 (4.53) ab	29.43 (8.62) a
p-value	0,0238	0,5294

Means followed by distinct vertical letters differ from each other ($p \leq 0.05$).

Fracture pattern

Adhesive fracture was the dominant fracture pattern in all groups, for both, enamel and dentin, as can be seen in table 3. The group CGE and CGD had 12.5% of abutment with a cohesive type fracture. HPC1D and HPC10D groups had 21.9% and 25.0% of cohesive fracture, respectively, ($p < 0.05$).

Table 3 - Fracture pattern

Group	**E**namel			**D**entin		
	Adhesive	Cohesive	Mixed	Adhesive	Cohesive	Mixed
Control Group	19 (59.4)	4 (12.5%)	9 (28.1)	18 (56.2)	4 (12.5%)	10 (31.2)
HP 35%	28 (93.3)	0 (0.0%)	2 (6.7)	25 (78.1)	0 (0.0%)	7 (21.9)
HP35% + C 1000 U/mL	23 (71.9)	0 (0.0%)	9 (28.1)	20 (62.5)	7 (21.9%)	5 (15.6)
HP35% + C 10000 U/mL	27 (84.4)	1 (3.1%)	4 (12.5)	18 (56.2)	8 (25.0%)	6 (18.8)
HP35% + P 1000 U/ mL	23 (71.9)	2 (6.2%)	7 (21.9)	18 (56.2)	2 (6.2%)	12 (37.5)
HP35% + P 10000 U/ mL	28 (87.5)	2 (6.2%)	2 (6.2)	15 (46.9)	6 (18.8%)	11 (34.4)
p-value	0,0293			0,0488		

Color measurements

Tables 4 to 7 respectively show color variation between after bleaching and initial (Δ1); after antioxidants and initial (Δ2); and after antioxidants and after bleaching (Δ3), of ΔE, Δa, Δb, ΔL.

It is observed in Table 4 that, in the three times intervals, there was only significant difference of ΔE_{00} (CIEDE2000) between the unbleached group and the other groups ($p < 0.05$). There was no significant difference between the groups receiving 35% PH regarding ΔE_{00} (CIEDE2000).

Table 4. Median (minimum value; maximum value) of variance in color, ΔE 2000 (CIEDE) of enamel, as a function of group.

Group	Time		
	ΔE1	**ΔE2**	**ΔE3**
Control Group	0,28 (0,02; 1,81) b	0,28 (0,02; 1,95) b	0,22 (0,11; 0,46) b
HP 35%	5,37 (3,63; 8,19) a	5,49 (3,92; 9,03) a	0,72 (0,14; 2,62) a
HP35% + C 1000 U/mL	5,32 (4,27; 10,64) a	5,48 (4,40; 9,89) a	0,68 (0,15; 4,51) a
HP35% + C 10000 U/mL	5,30 (3,22; 8,38) a	5,55 (3,52; 9,08) a	0,61 (0,05; 2,40) a
HP35% + P 1000 U/ mL	5,09 (3,11; 7,73) a	4,88 (3,41; 8,29) a	0,93 (0,17; 2,55) a
HP35% + P 10000 U/ mL	5,73 (3,60; 8,01) a	4,39 (3,92; 8,31) a	0,70 (0,28; 2,45) a
p-valor	<0,0001	<0,0001	<0,0001

Medians followed by distinct vertical letters differ from each other ($p \leq 0.05$).

(ΔE1)After bleaching - initial; (ΔE2)After enzyme - initial; (ΔE3)After enzyme - after bleaching.

Table 5 - Median values (Minimum / Maximum) of ΔL as a function group.

Group	Time		
	ΔL1	**ΔL2**	**ΔL3**
Control Group	-0,12 (-0,46; 0,45) b	-0,11 (-0,50; 0,45) b	-0,06 (-0,39; 0,57) b
HP 35%	5,72 (3,27; 7,88) a	5,96 (3,27; 9,38) a	0,34 (-0,18; 1,50) ab
HP35% + C 1000 U/mL	5,74 (3,50; 8,16) a	6,10 (3,45; 9,43) a	0,13 (-0,23; 2,14) ab
HP35% + C 10000 U/mL	5,91 (2,77; 8,61) a	5,97 (2,28; 8,52) a	0,27 (-3,66; 1,44) ab
HP35% + P 1000 U/ mL	5,09 (3,05; 8,37) a	5,11 (4,01; 9,32) a	0,66 (-1,29; 1,72) a
HP35% + P 10000 U/ mL	5,94 (3,73; 7,46) a	6,12 (3,82; 7,76) a	0,14 (-0,90; 1,24) ab
p-value	<0,0001	<0,0001	0,0225

Medians followed by different letters in the columns indicate statistical differences ($p \leq 0,05$).

(ΔL1)After bleaching - initial; (ΔL2)After enzyme - initial; (ΔL3)After enzyme - after bleaching.

Table 6 - Median values (Minimum / Maximum) of Δa as a function group.

Group	Time		
	Δa1	**Δa2**	**Δa3**
Control Group	-0,02 (-0,22; 0,20) a	-0,07 (-0,32; 0,16) a	-0,06 (-0,15; 0,04) a
HP 35%	0,56 (-2,53; 2,70) a	0,13 (-0,95; 2,71) a	-0,20 (-1,33; 1,59) ab
HP35% + C 1000 U/mL	0,66 (-1,07; 3,76) a	0,22 (-1,15; 2,02) a	-0,29 (-3,33; 0,21) ab
HP35% + C 10000 U/mL	-0,22 (-1,40; 2,39) a	-0,21 (-1,50; 1,43) a	-0,09 (-1,70; 0,43) ab
HP35% + P 1000 U/ mL	0,29 (-1,12; 2,81) a	-0,29 (-1,28; 0,94) a	-0,28 (-1,87; 0,00) b
HP35% + P 10000 U/ mL	-0,25 (-1,51; 3,50) a	-0,34 (-1,53; 1,63) a	-0,29 (-1,87; 0,30) ab
p-value	0,1808	0,0421	0,0225

Medians followed by different letters in the columns indicate statistical differences (p≤0,05).

(Δa1)After bleaching - initial; (Δa2)After enzyme - initial; (Δa3)After enzyme - after bleaching.

Table 7 - Median values (Minimum / Maximum) of Δb as a function group.

Group	Time		
	Δb1	**Δb2**	**Δb3**
Control Group	-0,02 (-0,46; 2,70) a	-0,06 (-0,81; 2,92) a	-0,08 (-0,40; 0,74) a
HP 35%	-4,93 (-9,15; -2,23) b	-5,58 (-9,90; -3,09) b	-0,55 (-1,17; 0,43) b
HP35% + C 1000 U/mL	-5,96 (-11,02; -2,48) b	-6,56 (-11,96; -3,14) b	-0,52 (-0,94; -0,04) b
HP35% + C 10000 U/mL	-4,83 (-11,67; -3,36) b	-5,21 (-12,01; -3,47) b	-0,32 (-1,29; 0,30) ab
HP35% + P 1000 U/ mL	-4,63 (-7,21; -2,20) b	-5,36 (-9,02; -2,65) b	-0,44 (-1,81; -0,10) b
HP35% + P 10000 U/ mL	-5,27 (-8,99; -3,90) b	-5,72 (-9,21; -4,33) b	-0,46 (-0,76; 0,06) b
p-value	<0,0001	<0,0001	0,0014

Medians followed by different letters in the columns indicate statistical differences (p≤0,05).

(Δb1)After bleaching - initial; (Δb2)After enzyme - initial; (Δb3)After enzyme - after bleaching.

In the three times intervals, there was only significant difference of ΔE 2000 (CIEDE) (measure of the total color variance) between the unbleached group and the other groups ($p < 0.05$). There was no significant difference between the groups receiving 35% PH regarding ΔE2000 (CIEDE).

ΔL (measure of lightness variation) of control group, the values were lower than other groups, whereas the values of Δb (measure of yellowing) of control group were higher than other groups, not differing between them. In the analysis of Δa (measure of greenish ou rednish) of all the groups, they did not differ between them.

DISCUSSION

After statistical analysis, the null hypotheses were accepted, since treatment protocols presented did not have a statistically significant influence on bond strength in enamel and dentin bleaching

The observed decrease of SBS in the HPE and HPD groups is probably attributed to the presence of residual oxygen left by hydrogen peroxide. Responsible for tooth bleaching, and also interferes with polymerization by inhibiting the infiltration of adhesive on conditioned surface (4). This finding is in according to Sundfeld et al (2005), which showed, in the bleached group, formation of smaller, thinner, less frequent, poorly defined and non-uniform “tags”, different from that shown in control group. Adhesion at 7, 14 and 21 days after bleaching, the tags were long, well defined, distributed and contiguous, which shows a good penetration of adhesive material in enamel (5).

The exact depth of this layer of oxygen-rich enamel is not known. However, it should be greater than 5 to 10 μm; otherwise, acid conditioning procedure would have removed it (4). According to Mattos et al. (2003), the low molecular weight of HP allows it a high power of penetration (35).

HP solution at 35% (m/v) shows 35% H_2O_2 and 65% H_2O by mass. The HP decomposes into molecular oxygen and heat; in dilute solutions, heat is easily absorbed by water (36).

Then, we could say that free radicals present in this case are derivatives of the molecular oxygen metabolism, but the term "free radical" is not the ideal term, since some of them do not present unpaired electrons in their last layer, so it is better to use the term reactive oxygen species (ROS) (37).

The O_2 undergoes tetravalent reduction, with acceptance of four electrons, resulting in H_2O formation. During this process the ROS are formed, such as superoxide radicals (O_2^-), being unstable, with a half-life of milliseconds and, therefore, hardly crosses membranes. However, it has a longer half-life than hydroxyl (OH), the OH that are the most reactive species known in biological systems, and have a very short half-life of approximately 10^{-9}s, the hydroperoxyl (HO_2) is present in small proportions and hydrogen peroxide (H_2O_2) which has a long half-life and is able to cross lipid layers and react with transition metals and some hemoproteins (21). Normally, complete reduction of O_2 occurs in the mitochondria and the ROS reactivity is neutralized with entry of four electrons.

As we know ROS are slowly leached by saliva, taking from 7 to 21 days for this, so we can say that oxidant derived from HP must be chemically stable. This excludes the superoxide radical, hydroxyl, for example, since both are short half-life species. Among the possibilities, H_2O_2 itself is a better candidate, since it brings together these properties (31).

For this reason, we used catalase and peroxidase enzymes because they are specific for H_2O_2, catalyzing the reduction of H_2O_2 in O_2 and water, as well as their biocompatibility and thermal stability up to 40°C (21) and 55°C (19) great from 6-7.

In addition, these enzymes are released endogenously as reported by Karaarslan et al. (2018), due to the oxidative stress generated by bleaching that activates the pulp cell defense system, promoting an enzymatic degradation of HP to avoid excessive damage to dental tissues (38).

Catalase enzyme has been used in lyophilized form, a lyophilization method, to keep enzymes stable for a long time, including years, but the process generates high operating and capital costs. Lyophilization is usually reserved for high-value proteins (39), which makes obtaining the enzyme expensive in addition to importation costs. According to Alptekin et al. (2008) that used this enzyme in soluble form, reducing costs, can be stored at 5 ° C for up to 11 days, after the enzyme completely loses its activity. This author also reports studies that described that from day 18 the enzyme lost 50% of its activity and at 4 ° C the enzyme lost all activity in 20 days, but their use in soluble mode is stable only for some days of 11-20 days (21), probably making it unfeasible for production.

According to Chagas et al. (2014) the enzymatic activity of turnip peroxidase, storage it at 3° C and at -20° C, was maintained at an average of 80% in 90 days. After 170 days of storage, at -20° C the enzyme lost 33.4% of enzymatic activity, at 3° C showed loss of 52.4%, which can be attributed to fungi growth, which occurred after 80 days of storage (40). The groups HPP1E, HPP10E and HPP1D, HPP10D presented values close to the CGE, although not being statistically significant but due to the possibility of making its production enable its stability of up to 90 days with easy storage in refrigerator or freezer it is suggested to do more studies with different concentrations.

Meanwhile for our study we used lyophilized catalase that was stored at -20° C, according to manufacturer, and used at room temperature. The peroxidase was centrifuged at 4 ° C to separate the supernatant from the precipitate and obtain a cell free enzyme used at room temperature without storing them to ensure their maximum enzymatic activity at optimal temperature and pH. The lack of purification of enzyme was a limitation of our study, being able to obtain better results with this procedure. The application of two enzymes in this case was done by placing cotton saturated with the enzymes on the surface of the enamel (23) to approximate the clinical reality.

As for bleaching procedure, the HP was applied three times of 15 minutes each application, due to rapid degradation that presents the bleaching agent and instability of pH, making it acidic, pH of 5, after time recommended by manufacturer and supported by literature (41). According to Motamed et al. the enzyme turnip peroxidase can maintain its stable activity in acidic environment in a pH range of 2.6 to 5.0 for 15 h. (19). However, in our study we used the pH of 7 for use of both enzymes.

Enzymes catalase and peroxidase have a much higher molecular weight than hydrogen peroxide, but they are still smaller than average diameter of enamel structures, facilitating its penetration. Electron microscopy studies showed a mean diameter of the enamel prisms of ~ 4-7 μm. (42) and the hydroxyapatite crystals range from 15 to 50 nm, having occasional pores between them of ~ 20-30 nm (43). Catalase and peroxidase have a molecular weight of 60KDa and ~ 44KDa, respectively (26,44), with a diameter of approximately ~3-5nm (45), which could indicate that enzymes are able to penetrate into the dentin, as evidenced in G3D-G6D groups with values close to the control group with no statistical difference between them.

Another factor that may have influenced these values may be the removal of enamel that was made to release dentin, because the mechanical removal would generate a chemical imbalance of internal and external environments, allowing the HP to exit through molecular diffusion of medium of higher concentration to that of lower concentration. In order to expose the dentin we used the 600 water sandpaper to leave a layer of standardized smear layer (29), as well as the depth of wear, with the limitation of the marking made on the lateral surface of the samples at enamel-dentin junction.

The fracture pattern of the abutments was evaluated to determine the weakest part of the bond in different groups. As expected in groups that were restored immediately revealed high percentages of adhesive fracture. The groups where the antioxidant enzymes were applied presented mixed and cohesive failure. Indicating that application of antioxidants increases bond strength of the composite to enamel and dentin after bleaching (46).

For color evaluation we performed two bleaching sessions with a 7 day interval simulating the technique of bleaching in office where we can evidence a significant color change to be able to test influence of antioxidants on color.

In color analysis, the null hypothesis was accepted, since use of catalase and peroxidase enzymes did not influence the color in enamel after bleaching.

The CIEDE 2000 color-difference presents a reliable and objective tool for determination of tooth color. The ΔL * (difference in the white and black scale) was considered positive as increase of brightness and Δb * (difference in yellow and blue) negative as reduction of yellowish as indicative of a bleaching benefit.

Studies have shown that ΔE (total color variation) values of approximately 3.3 are clinically relevant (47), also found that values from 0-2 were imperceptible, from 2-3 only perceptible, from 3-8 moderately perceptible, and values above 8 were markedly perceptible (48).

In our study, ΔE analysis showed moderate to markedly perceptible values, evidencing that 35% HP bleaching was effective in all bleached enamel groups. Color variation after application of antioxidants - after bleaching was 0.78 - 1.16, being imperceptible according to Guler et al. (2005) (48).

The luminosity or brightness analysis given by ΔL showed higher positive values in groups with bleaching o and for Δb, the groups with bleaching obtained significantly more negative values, representing reduction of yellowing, when compared with the control group, affirming the effectiveness of bleaching.

The values after antioxidant - baseline are for more positive ΔL* and for Δb* more negative than ΔL1 and Δb1 respectively, not being statistically significant, but this may indicate that the ΔL * and Δb * can be affected by the dehydration. In the clinical practice, a dehydration of the teeth produced by the insulation made to apply the bleaching gel occurs (49), so we could attribute these values obtained to the dehydration produced in the samples.

The results of application of enzymes presented intermediate values for the bond strength, not statistically differing from the groups bleached only with PH 35% and not bleached. Thus, due to this behavior, it is suggested that more studies be performed by testing different concentration of the enzymes.

CONCLUSION

It was concluded that the use of enzymes catalase and peroxidase did not influenced the enamel and dentin bond strength after bleaching. The application of enzymes catalase and peroxidase did not influence the color of dental substrate, maintaining aesthetic result obtained.

ACKNOWLEDGEMENTS

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001.

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3 CONCLUSÃO

Com base nos achados deste estudo foi possível concluir que o uso das enzimas Catalase e Peroxidase não teve influência na alteração de cor e na resistência adesiva ao esmalte e a dentina após o clareamento dental.

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APÊNDICE 1 – METODOLOGIA ILUSTRADA

Confecção das amostras

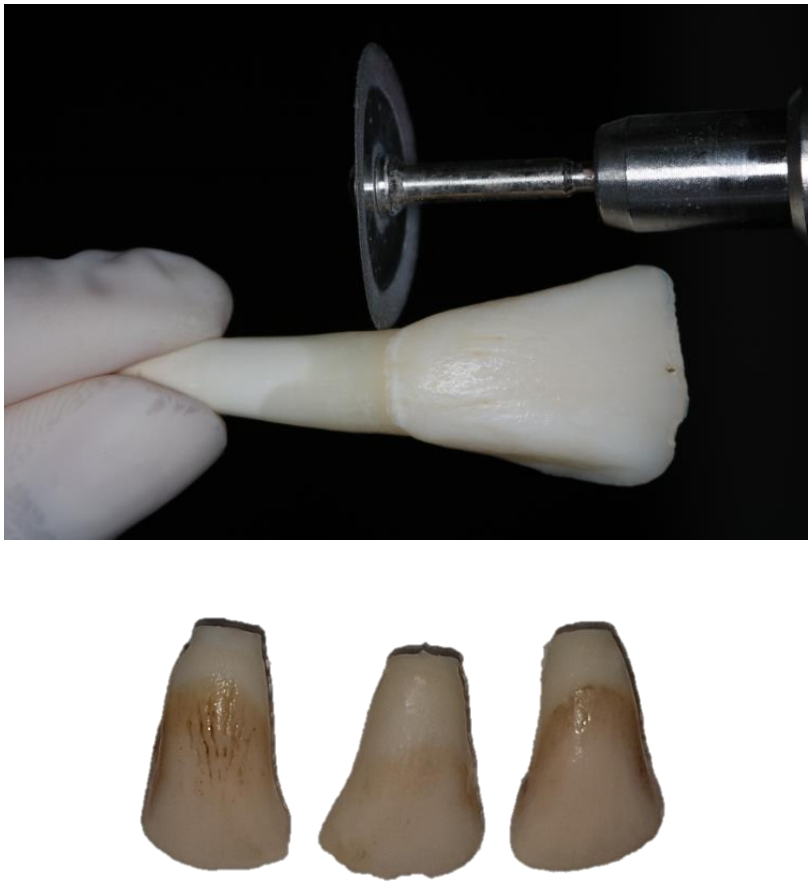


Figura 1 - Incisivo bovino, separação da coroa-raiz com disco diamantado.

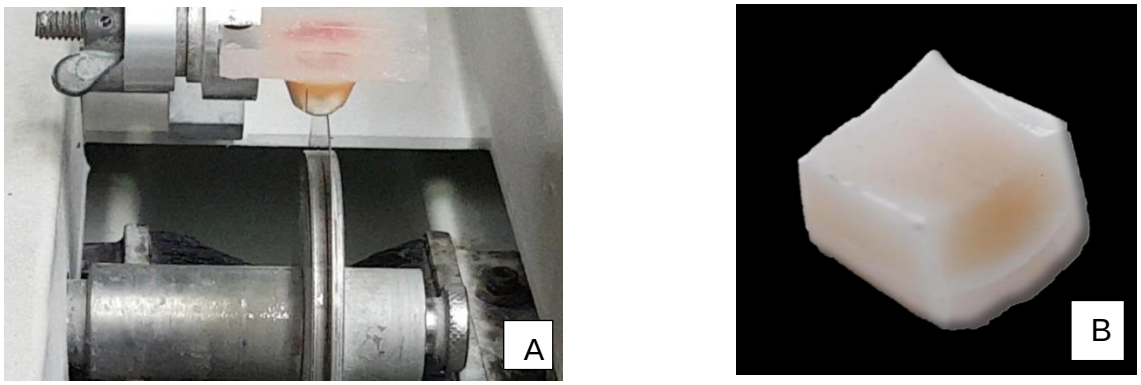
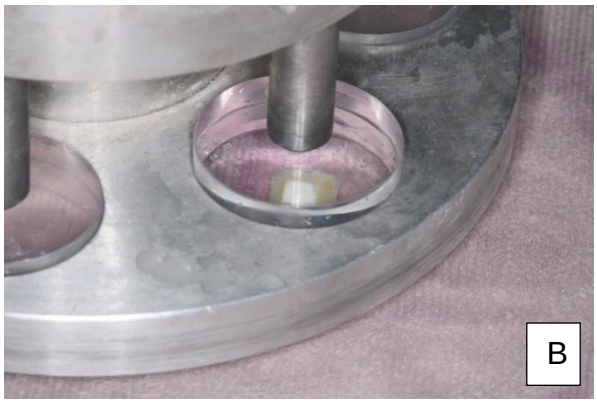


Figura 2 - Coroa posicionada em placa de acrílico na cortadeira metalográfica (A); Bloco dental obtido através do incisivo bovino (B).



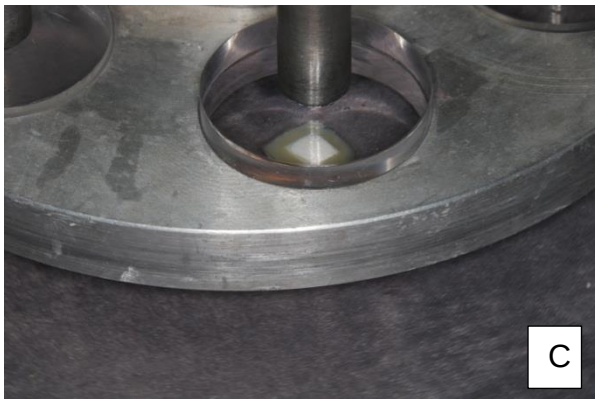


Figura 3 - Polimento das amostras na politriz giratória com lixas de Carbeto de Silício (A); Polimento com discos de feltros e pastas de polimentos (B) (C); Amostras submetidas à limpeza em cuba ultrassônica (D).

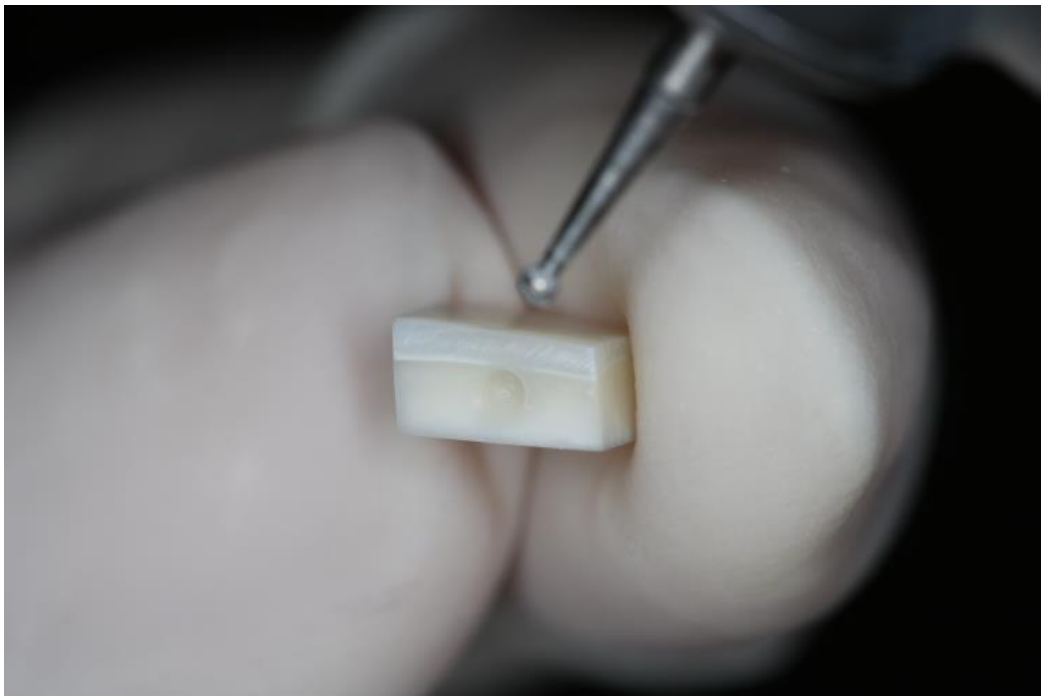


Figura 4 - Marcação das amostras embaixo da junção amelodentinária para padronização na análise de cor e para padronizar a profundidade do desgaste do esmalte para liberar a dentina.

Preparação das enzimas

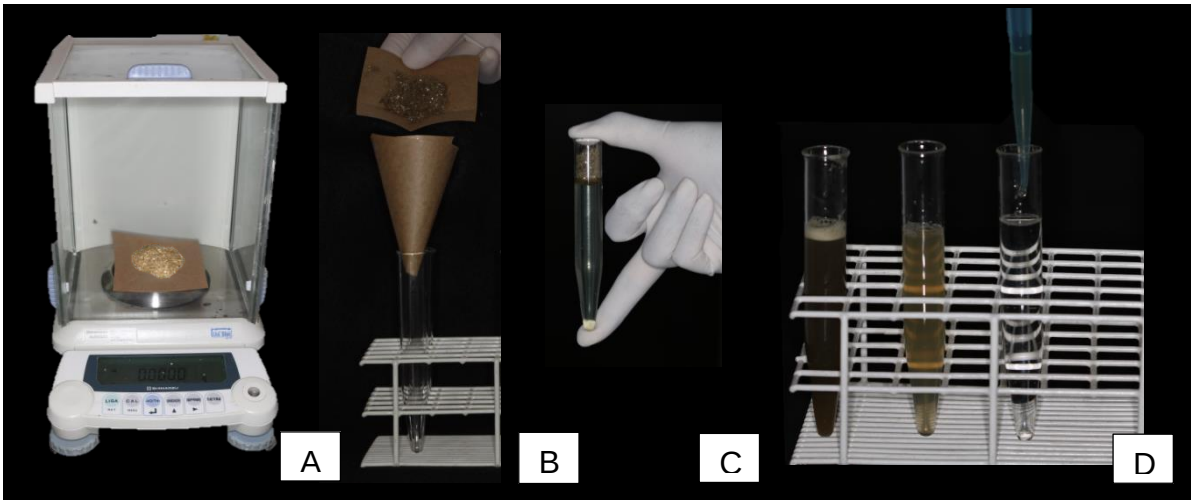


Figura 5 - Preparação da enzima catalase – Pesagem da enzima na balança de precisão (A); Colocação da enzima no tubo de ensaio (B); Mistura da enzima com tampão fosfato por inversão do tubo (C); Diluição da enzima (D).

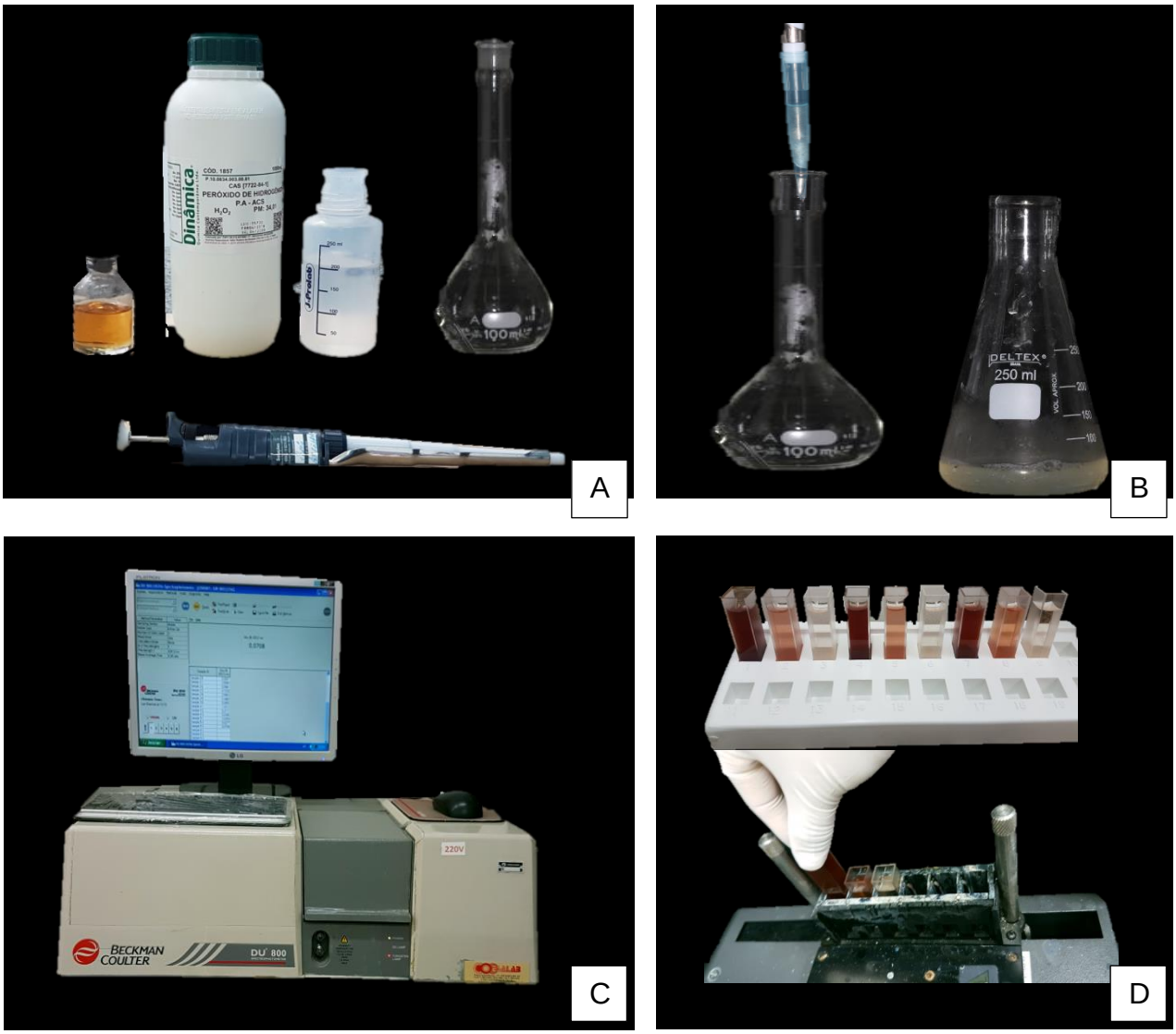


Figura 6 - Materiais para determinação quantitativa da peroxidase, guiacol, H2O2, álcool, balão volumétrico, pipeta (A); Colocação dos materiais no balão volumétrico, e sobrenadante da enzima peroxidase (B); Espectrofotometro (DU 800, Beckman Coulter, CA, USA) (C) para determinação da atividade enzimática. Colocação dos materiais para determinação da atividade enzimática com a peroxidase nas cubetas do espectrofotômetro e colocação delas no espectrofotômetro para leitura (D).

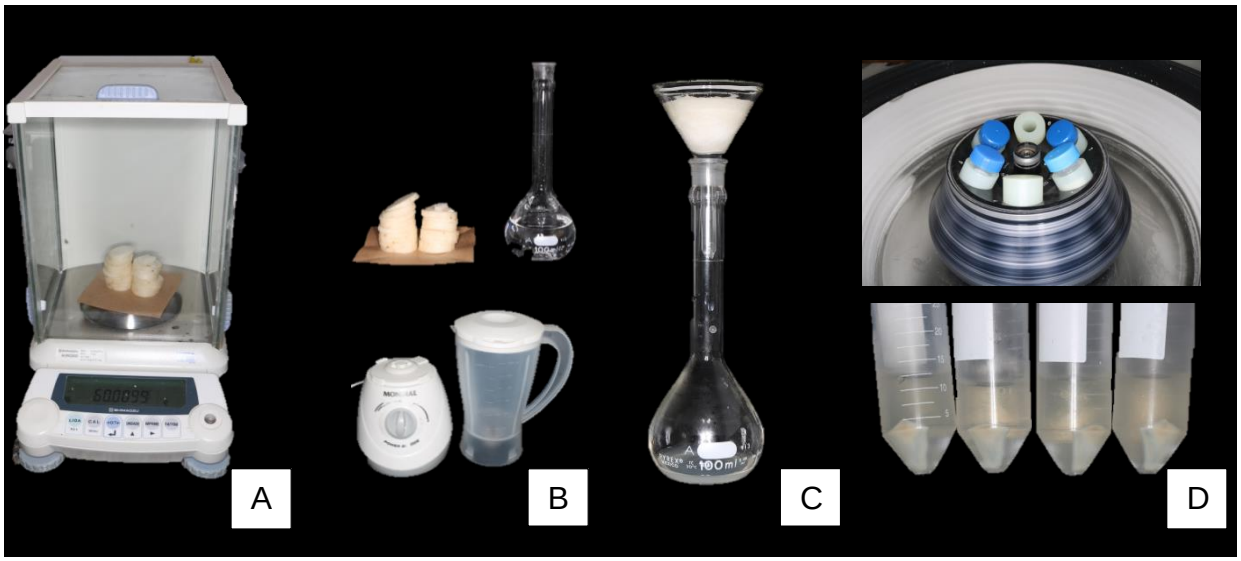


Figura 7 - Preparação da enzima peroxidase – Pesagem da enzima na balança de precisão (A); Homogeneização da enzima no liquificador (B); Filtrado da enzima (C); Centrifugado da enzima para separar o sobrenadante do precipitado (D).

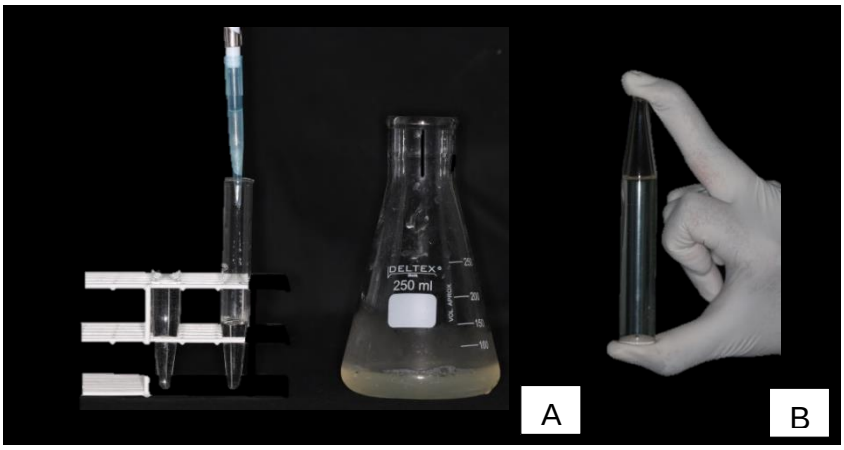


Figura 8 - Diluição da enzima peroxidase com tampão fosfato (A); Mistura da enzima com tampão fosfato por inversão do tubo (B).

Procedimento Clareador

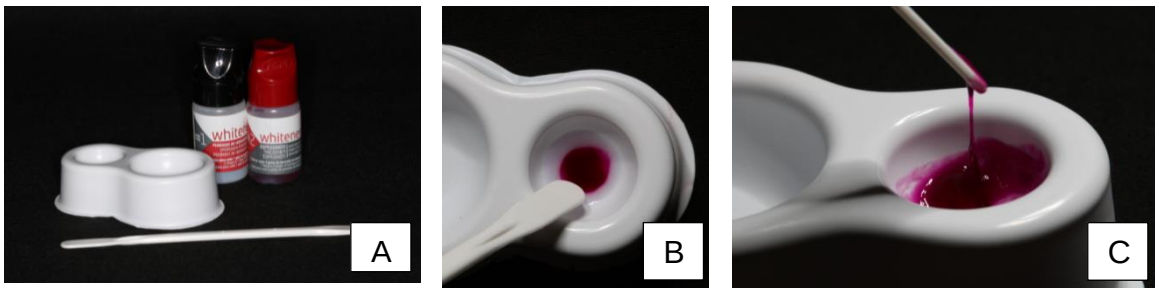


Figura 9 - Gel clareador Whiteness HP (A); Peróxido de hidrogênio e Espessante na proporção 3:1 em recipiente de manipulação (B); Mistura dos componentes (C).

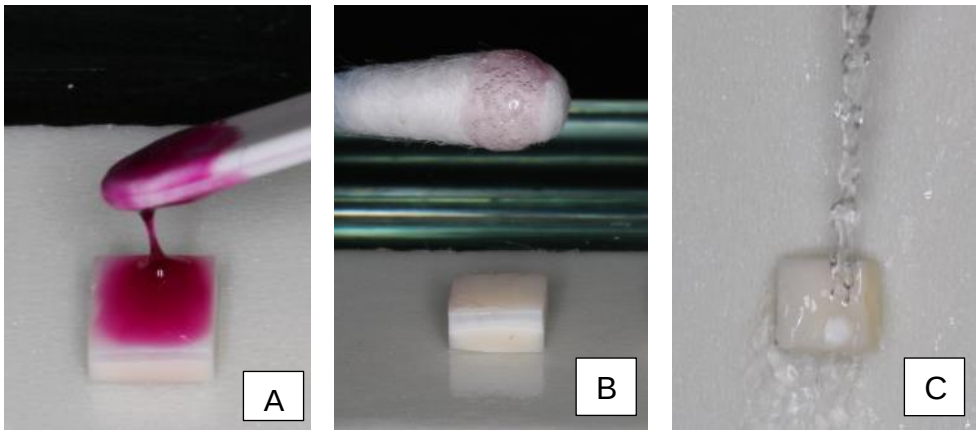


Figura 10 - Aplicação do gel clareador sobre a amostra (A); Remoção do gel clareador com hastes flexíveis com ponta de algodão (B); Remoção completa do gel clareador com irrigação de agua purificada (C).

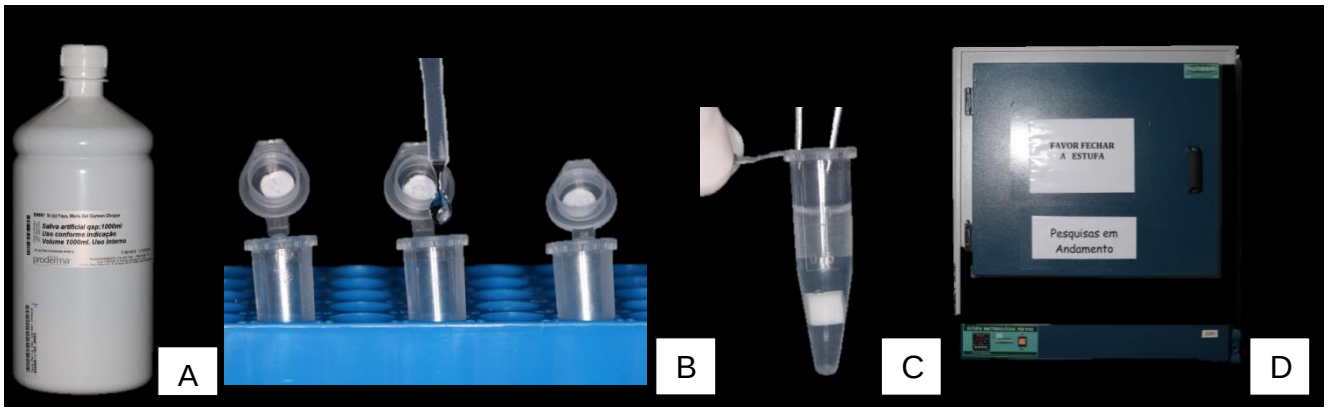


Figura 11 - Saliva artificial (A); Colocação da saliva artificial nos eppendorf (B); Colocação das amostras na saliva artificial durante as sessões de clareamento (C) e armazenados na estufa a 37°C (D).

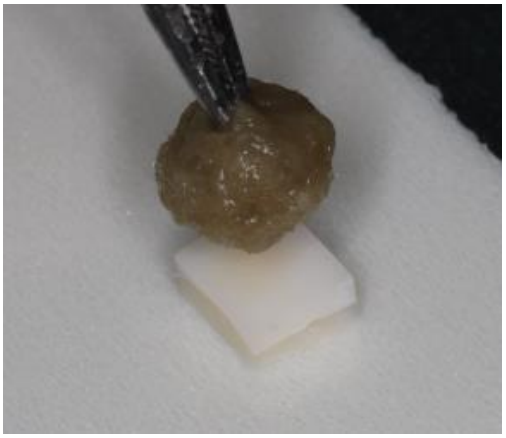


Figura 12 - Aplicação das enzimas nas amostras.

Leitura de cor

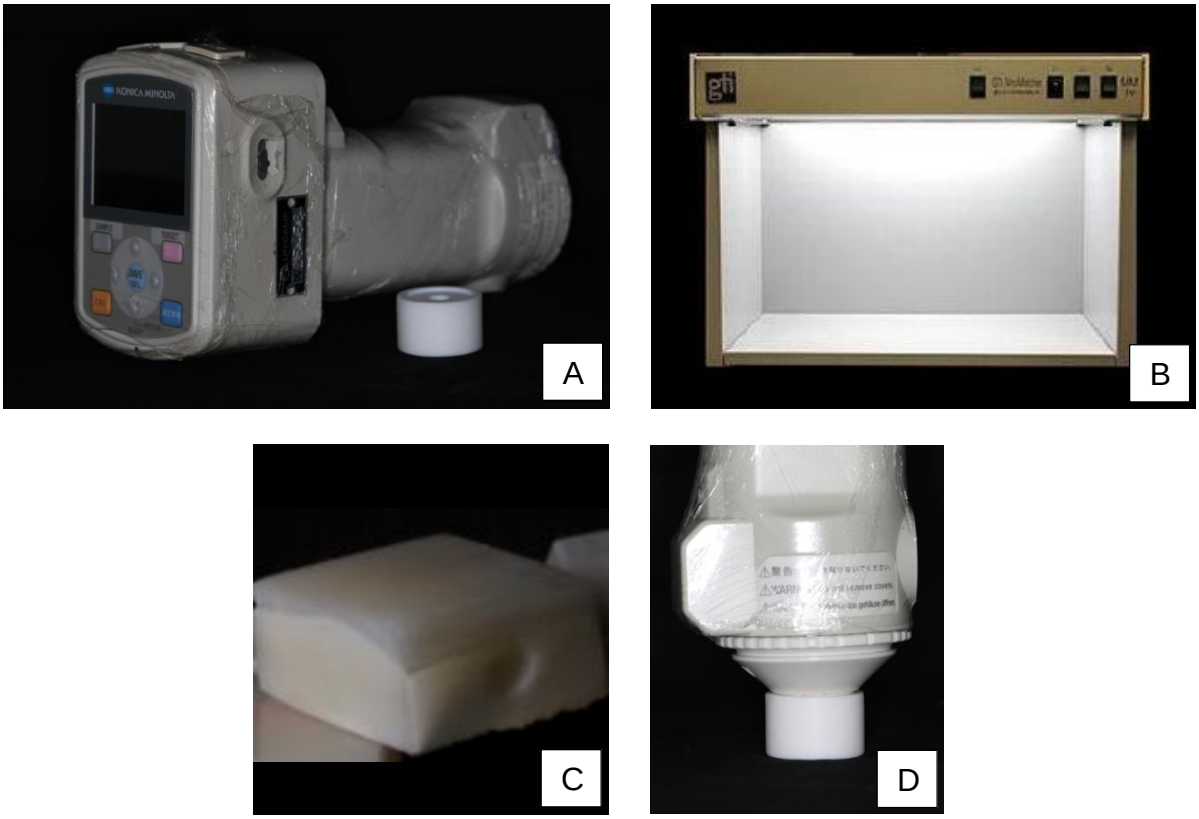


Figura 13 - Espectrofotômetro de refletância Konica Minolta CM-700d (A); Câmara de Luz (B), Amostra marcada na lateral para padronização do posicionamento (C); Posicionamento do espectrofotômetro com relação ao porta amostra para a leitura de cor (D).

Imersão das amostras na resina de poliestireno

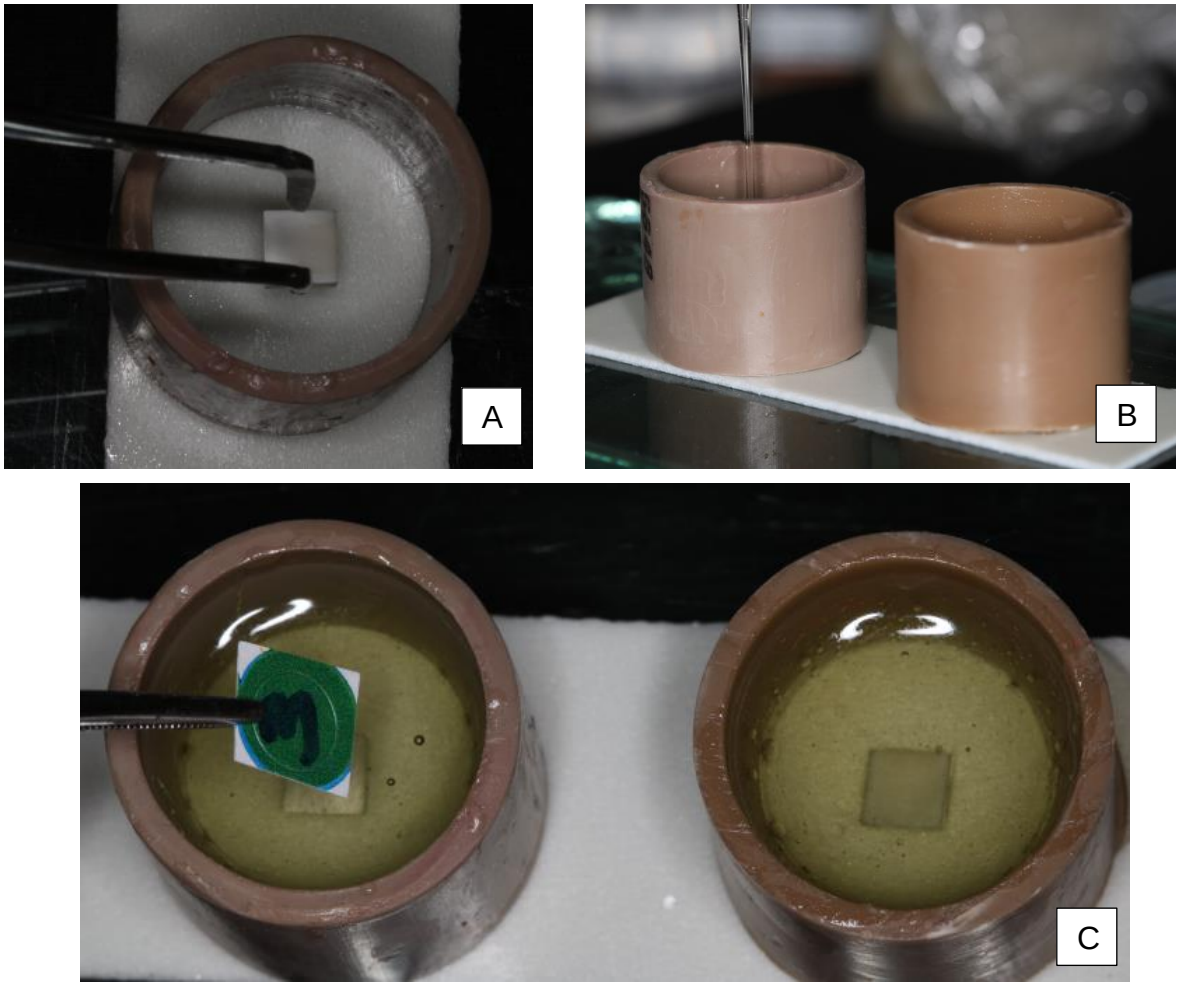


Figura 14 - Colocação da amostra nos canos (A) para imersão na resina de poliestireno (B); Etiquetado das amostras (C).

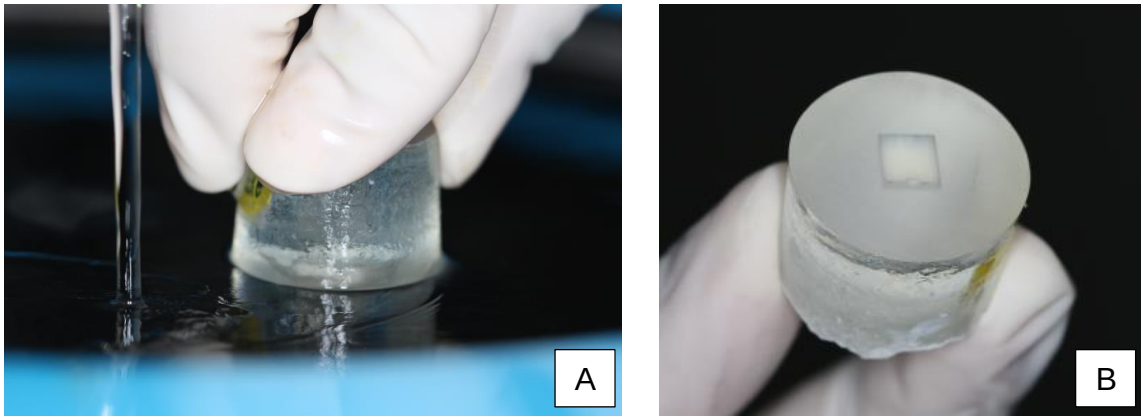


Figura 15 - Desgaste do esmalte para liberação da dentina com lixa de agua #600 (A) para padronização do smear layer até a marcação feita com a broca #1012 (B).

Procedimento Adesivo

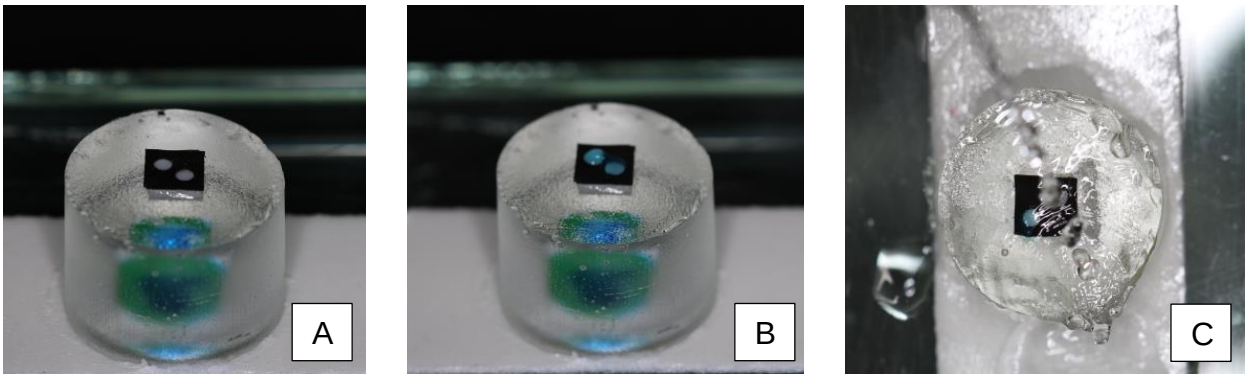


Figura 16 - Colocação da fita delimitadora da área adesiva sobre a estrutura dentaria (A); Aplicação do acido fosfórico a 37% (B); Remoção do acido (C).

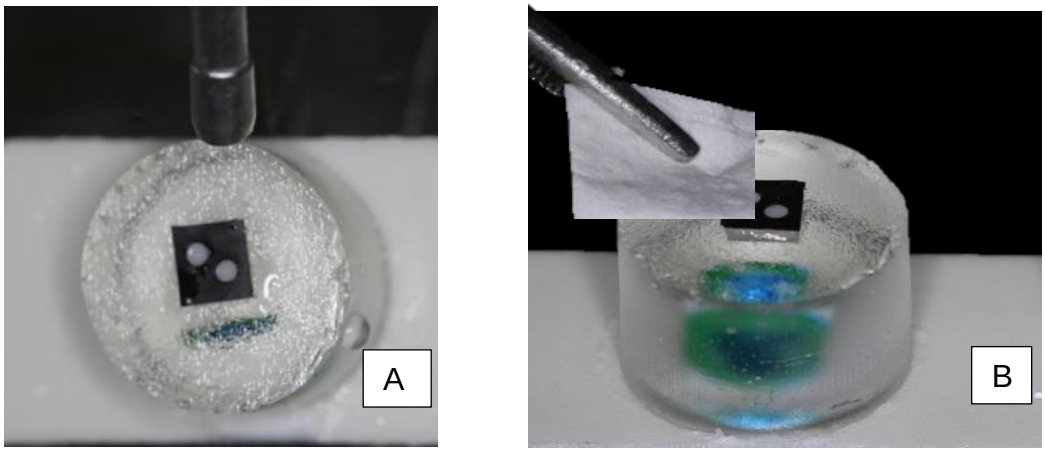


Figura 17 - Secado da superfície de esmalte com aplicação de ar (A) e com papel absorvente a superfície da dentina para manutenção da umidade (B).

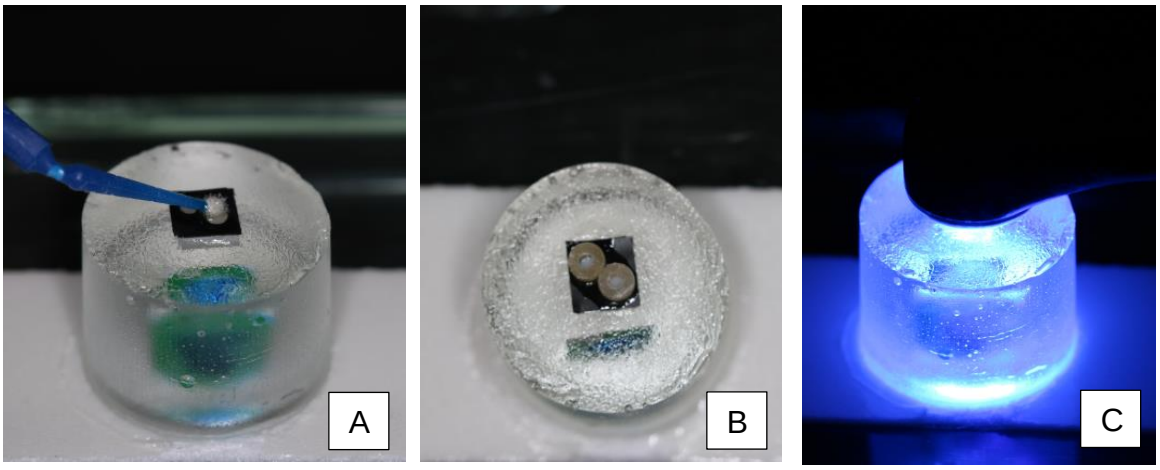


Figura 18 - Aplicação do adesivo (A); Posicionamento da matriz (B) previamente a foto polimerização (C).

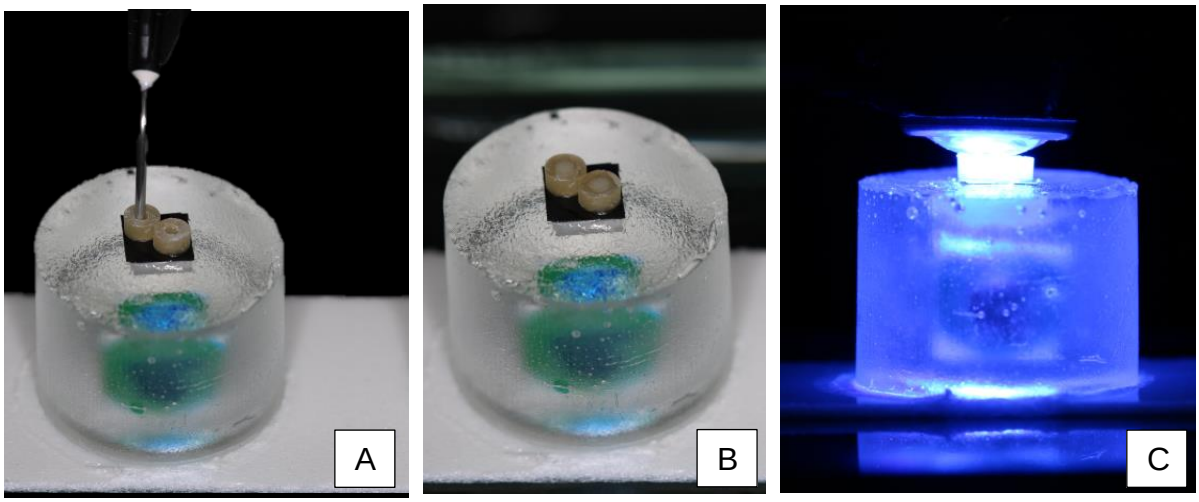


Figura 19 - Aplicação da resina composta tipo flow na matriz (A) (B); Fotopolimerização da resina (C).

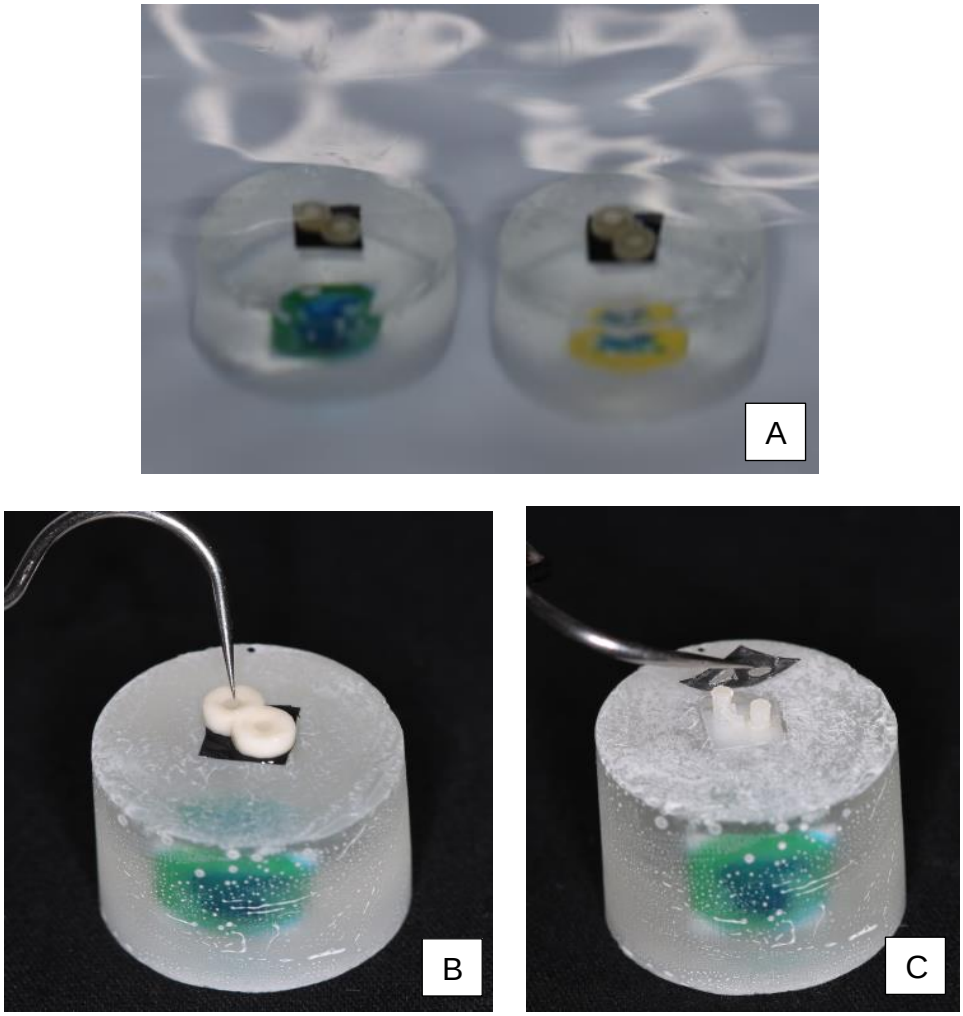


Figura 20 - Imersão em agua para facilitar a remoção da matriz (A); Remoção da matriz (B) e da fita delimitadora com sonda exploradora (C).

Teste de Microcisalhamento

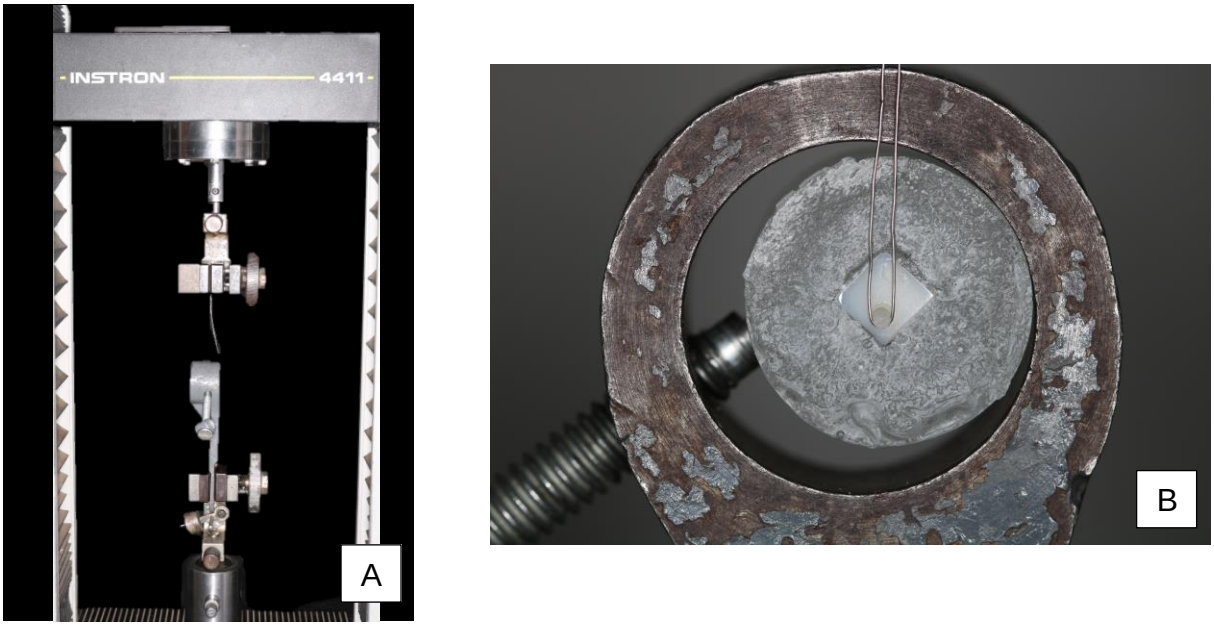


Figura 21 - Instron 4411 (A); Amostra posicionada no dispositivo de Microcisalhamento (B).

Padrão de Fratura

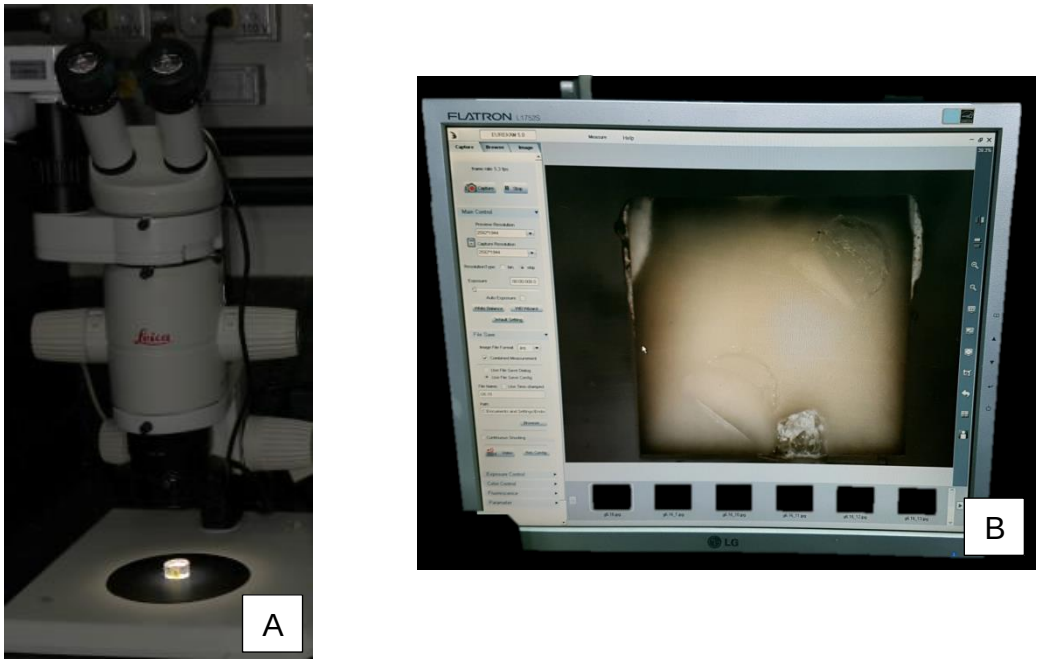
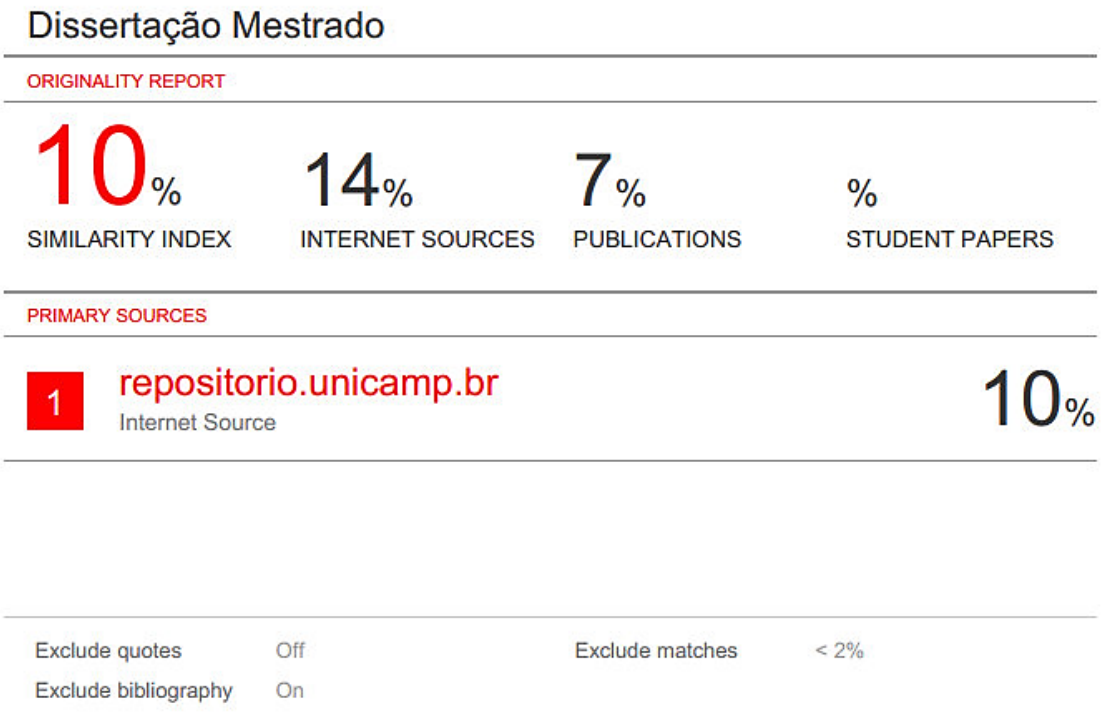


Figura 22 - Amostra posicionada na lupa estereoscópica (Leica Microsystems) (A) para leitura do padrão de fratura (B).

ANEXOS

Anexo 1 - Verificação de originalidade e prevenção de plágio



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Anexo 2 – Documento de submissão do artigo

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You have been listed as a Co-Author of the following submission:

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Corresponding Author: Débora Alves Nunes Leite Lima
Co-Authors: María del Carmen Choque Yaya, MSc; Maria Cibelle Pauli, Doctor Student; Gislaine RICCI Leonardi, Teacher - Doctor; Juliano Lemos Bicas, Teacher - Doctor; Flavio Henrique Baggio Aguiar, Teacher - Doctor;

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