UNIVERSIDADE DE SÃO PAULO
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In-office bleaching using violet LED with and without gel (6% H_2O_2): evaluation of pH levels and enamel microhardness

Clareamento em consultório usando LED violeta com e sem gel clareador (6% H_2O_2): avaliação do pH e da microdureza do esmalte

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“A única forma de chegar ao impossível é acreditar que é possível”.

Lewis Carroll

ABSTRACT

In-office bleaching using violet LED with and without gel (6% H_2O_2): evaluation of pH levels and enamel microhardness

To reduce bleaching side effects and enamel surface alterations, recent protocols using violet LED light (LEDv), alone or associated with low concentrations of hydrogen peroxide (HP) have gained interest. Objective: this *in vitro* study evaluated the effect of three different in-office bleaching techniques on bovine enamel microhardness and the pH variation of peroxide agents during the protocols. Methodology: For Knoop microhardness (KNH) analyses, enamel fragments were divided into 3 groups (n=10): LEDv –hybrid violet LED/Laser light (Whitening Lase Premium, DMC) (10x2' LEDv + 10x30" light off, 2 cycles); HP6%LEDv – 6% HP gel (Nano White Flex, DMC) + LEDv/Laser (Whitening Lase Premium, DMC) (15x1' LEDv + 15x1' light off); HP35% - 35% HP gel (Nano White Flex, DMC) (1x45'). For pH measurements, all bovine teeth were divided into 2 groups (n=10): HP35% and HP6%LEDv. KHN was measured at baseline (T_0), 24h after bleaching (T_1) and after 7 days in artificial saliva (T_7). Initial and final bleaching gels pHs were obtained utilizing a pH meter. KHN was evaluated by the Wald-type permutation statistic, aligned rank transformation statistical test, Wilcoxon and Mann-Whitney tests; pH levels were evaluated by the Welch-James and Wilcoxon tests ($p<0.05$). Results: HP35% and HP6%LEDv presented a decrease in KHN from T_0 to T_1 ($p=0.0039$; $p=0.001$, respectively), with no difference among them ($p>0.05$); baselines values were recovered at T_7 ($p=0.313$ HP35%; $p=0.557$ HP6%LEDv). For LEDv, no significant difference was found between KHN at T_1 and T_0 ($p=0.5286$); at T_7 KHN increased in comparison to T_0 ($p=0.029$). HP6%LEDv and HP35% presented a reduction of pH values ($p=0.0029$; $p=0.0284$, respectively); HP6%LEDv showed greater reduction ($p=0.0004$). Conclusions: Bleaching with LEDv alone was the only treatment that didn't reduce enamel microhardness. HP6%LEDv led to a decrease in KHN values similar to the high concentrated gel. After seven days in artificial saliva, initial KHN was recovered. Although the pH of both gels decreased during the treatment, it remained above the critical value.

Key Words: Bleaching Agents. Hardness. Hydrogen-Ion Concentration. Light Source. Tooth Bleaching

RESUMO

O clareamento de consultório usando apenas luz LED violeta (LEDv) ou sua associação com géis de peróxido de hidrogênio (PH) de baixa concentração tem ganhado interesse, uma vez que efeitos colaterais e alterações na superfície do esmalte podem ser evitados. Objetivo: esse estudo *in vitro* avaliou o efeito de três diferentes protocolos de clareamento de consultório na microdureza do esmalte bovino e a variação do pH dos agentes clareadores durante o tratamento. Metodologia: para a análise de microdureza Knoop (KHN), fragmentos de esmalte foram divididos em 3 grupos (n=10): LEDv - clareamento com luz LED violeta/Laser (Whitening Lase Premium, DMC) (10x2' LEDv + 10x30" descanso, 2 ciclos); HP6%LEDv – clareamento com gel de peróxido de hidrogênio 6% (NanoWhite Flex, DMC) + LEDv/Laser (Whitening Lase Premiu, DMC) (15x1' LEDv + 15x1' descanso); HP35% - clareamento com gel de peróxido de hidrogênio 35% (Nanowhite Flex, DMC) (1x45'). Para as medições de pH, dentes bovinos inteiros foram divididos em 2 grupos (n=10): HP35% e HP6%LEDv. KHN foi medida antes do clareamento (T_0); 24 horas após o clareamento (T_1) e após 7 dias em saliva artificial (T_7). O pH inicial e final dos géis clareadores foi obtido usando um pHmetro. A microdureza foi avaliada pela estatística de Wald usando permutações no cálculo do valor de p e um método de transformação de ranks alinhados para ANOVA fatorial não paramétrica. Comparações múltiplas foram realizadas pelos testes de Wilcoxon e Mann-Whitney; pH foi avaliado pela estatística de Welch-James e as comparações múltiplas pelo teste de Wilcoxon ($p<0,05$). Resultados: HP35% e HP6%LEDv apresentaram diminuição da microdureza do esmalte de T_0 para T_1 ($p=0,0039$; $p=0,001$, respectivamente), sem diferença entre os grupos ($p>0,05$); os valores iniciais foram recuperados em T_7 ($p=0,313$ HP35%; $p=0,557$ HP6%LEDv). Para LEDv, não foi encontrada diferença na microdureza em T_1 e T_0 ($p=0,5286$); em T_7 KHN aumentou em comparação à T_0 ($p=0,029$). HP6%LEDv e HP35% apresentaram redução nos valores de pH ($p=0,0029$; $p=0,0284$, respectivamente); HP6%LEDv apresentou maior redução ($p=0,0004$). Conclusões: clareamento usando apenas LEDv foi o único tratamento que não reduziu a microdureza do esmalte. HP6%LEDv reduziu os valores de KHN de forma semelhante ao gel de alta concentração. Após 7 dias em saliva artificial, a microdureza inicial foi recuperada. Embora o pH de ambos os géis clareadores tenha diminuído

durante o tratamento, ele permaneceu acima do crítico para desmineralização do esmalte.

Palavras chave: Clareamento Dental. Clareadores. Concentração de Íons de Hidrogênio. Dureza. Fontes de luz

LIST OF FIGURES

Figure 1 - Box-plot graphic of difference between pH values 41

LIST OF TABLES

Table 1 -	Materials for the study	39
Table 2 -	Equipment for the study	39
Table 3 -	Bleaching protocols	39
Table 4 –	Descriptive statistics for microhardness differences. Different upper case letters means statistically significant difference between groups; ** ($p<0.05$)……	40

LIST OF ABBREVIATIONS AND ACRONYMS

HP	Hydrogen peroxide
LEDv	Violet LED
KHN	Knoop microhardness
AS	Artificial saliva
DW	Distilled water
WTSP	Wald-type permutation statistic
ARA	Aligned rank transformation statistical test for ANOVA
TiO_N	Titanium dioxide doped with nitrogen

SUMMARY

1 **INTRODUCTION** 19

2 **ARTICLE** 25

3 **ARTICLE ILLUSTRATIONS** 39

4 **DISCUSSION** 45

5 **FINAL CONSIDERATIONS** 53

REFERENCES 57

APPENDIXES 65

1 INTRODUCTION

1 **INTRODUCTION**

The demand for aesthetic procedures in Dentistry is increasing since white and well-aligned teeth play an important role for a better overall face appearance. In this context, tooth bleaching is one of the most popular, effective and conservative treatment[1-7] and can be performed in-office or at home. In-office bleaching has some advantages as no need of patient cooperation, full control of the whole procedure and allows the patient to obtain visible results even after only one appointment[1,6,8-15].

Hydrogen peroxide (HP) is the most commonly used bleaching agent and is manufactured in concentrations ranging from 3% to 40%[1,6,12,15-21]. At home bleaching procedures must use low concentrated HP agents. For in-office dental bleaching, low or high concentrated products can be applied, depending on the patient's age, tooth sensitivity and the time required for whitening effect. The peroxide mechanism to promote bleaching is not fully understood[15,19,22]. The most accepted theory speculates that hydrogen peroxide releases free radicals, which diffuse through tooth enamel and dentin oxidizing and reducing complex chromophore molecules that are responsible for dental pigmentation. As consequence, pigments are disrupted into smaller molecules that reflects less light[1,2,7,18].

There are several protocols available for in-office bleaching: applying only peroxide bleaching gels; bleaching using only violet LED light or bleaching using HP gel associated with a light source[7,23,24]. The technique that uses only the bleaching gel is the most widely used because of the satisfactory results and no need of buying de light source equipment, which results in less treatment cost[18]. In this modality, the professional usually applies high concentrated peroxide bleaching gel for a time ranging from 30 to 60 minutes[1,16,23,25,26]. Although high-concentrations of hydrogen peroxide in the whitening gel results in a faster bleaching outcome[1,2,4,5,9,14,23,27], the risk for postoperative sensitivity increases with concentration, once dental sensitivity is related to the diffusion of free radicals through enamel and dentin[2,4,6,8,23,26,28-31]. Also, the contact time between the bleaching gel and the dental surface can influence on tooth sensibility[2,4,25], and shorter bleaching times are recommended. In this context, the association of HP gels with hybrid LED light can be performed to reduce the gel application time[4].

In addition, higher peroxide concentrations have been related to greater enamel surface alterations and cells damage[20,26,32-35]. Mondelli, et al.[34] (2015) and Grazioli, et al.[20] (2018) studied the effect of different bleaching gels concentrations on the enamel surface and reported that the low concentrated HP bleaching gel did not lead to a decrease in enamel microhardness after bleaching, while higher concentrations significantly reduced KHN values. Similarly, Lewinstein, et al.[33] (2004) and Klaric, et al.[32] (2015) evaluated enamel microhardness after bleaching with gels with different concentrations and acidity and demonstrated that the lower the pH, the greater the microhardness loss, as well as high concentrated gels led to a greater microhardness reduction. In addition, bleaching gel with high concentration of HP (35%) was reported to cause alterations on cell morphology, and it was direct proportional to the contact time of the product with dental structure, and alterations were found even with an extremely low application time (5min)[26]. For these reasons, new products and technologies have been developed for in-office dental bleaching. Recent protocols using low concentrated peroxide bleaching gels or even bleaching without peroxide agents have gained interest[4,5,20,24,30,36-38].

According to the Guide of the European Community[38], only bleaching products containing concentrations of >0.1 to 6% of HP present or released are considered safe to the patients. Keeping this context in mind, in order to maintain the whitening effectiveness of high concentrated bleaching gels and increase the safety of bleaching treatment, manufactures proposed the incorporation of nanoparticles of titanium dioxide doped with nitrogen (TiO_N) photo-catalyst into lower concentrations of HP (3.5–6%)[16,29]. This new generation of low concentrated HP gels containing TiO_N has been analyzed in recent studies[3,5,29,38]. Researchers[3,5,38] demonstrated the effectiveness of 6% HP with nitrogen-doped titanium dioxide light activated bleaching agent in clinical studies, with no difference in subjective color evaluations between 6% and 35% HP gels. The catalytic activity occurs when the TiO_N nanoparticles are exposed to wavelengths < 535 nm[38]; enhancing the generation of reactive oxygen species and improving the efficacy of bleaching gel[3,5]. The incorporation of catalystic nanoparticules allows 6%HP to still be effective with a reduced risk of sensitivity[38,40].

Recently launched in the market, a violet LED light system for in office bleaching, presenting wavelength of approximately 405nm-410nm can be used with or without peroxide agents[37,41]. When associated with HP bleaching gels, the light is absorbed

and is partially converted into heat, increasing the kinetic energy of the molecules of bleaching gel and its decomposition into free radicals radicals[11-13,30,33,42]. The aim of this association is to provide faster clinical procedures and more comfort for both patient and professional[2,6,12,17,24,42].

The promising technique of bleaching using violet LED light alone has been performed more recently in order to avoid any side effect caused by bleaching gels in the enamel surface, although more evidences are required to recommend this bleaching protocol[23,37,40]. The mechanism of action of violet light is due to its short wavelength and high frequency interacting with pigment molecules. This is possible because its wavelength coincides with the absorption peak of chromophore molecules, breaking them into smaller molecules[4,24,30]. Studies[2,37] reported the effectiveness of whitening with violet LED light alone and absence of post-treatment hypersensitivity[24].

In addition to the tendency to reduce hydrogen peroxide bleaching concentration, new generations of bleaching gels have been introduced with neutral/alkaline pH in order to minimize enamel surface alterations[8,10,18,28,43]. Also, recent studies[43,44] showed that bleaching gels with basic pH presents greater bleaching effect. Although alkaline peroxide agents have been developed, studies report that during bleaching procedure, there is a trend toward a decrease in bleaching gels pH from the initial times to the end[42,45,46]. Therefore, it is recommended replenishing the bleaching gel during the treatment. To provide the best cost-benefit to the dentist and more comfort to the patient, manufactures enhanced the neutral/alkaline bleaching gels formulations for maintaining the basic pH of the gel during the whole bleaching procedure[8,47]. Therefore, those new bleaching agents can be applied only one time in enamel surface, because its pH remains stable.

Although studies in the literature evaluated the pH of several concentrations of bleaching gels, the authors of the present study did not find any study regarding the pH of in office bleaching gels with hydrogen peroxide concentration lower than 10%. Also, studies are still required to investigate the stability of bleaching gels pH and the possibility of only one application of the product. Bleaching protocols using low concentrated bleaching agents and bleaching with violet LED light alone are available for the dentists with the objective of reducing deleterious effects in enamel. However, few studies evaluated the safety of those recent bleaching protocols, as regarding the microhardness. Therefore, it needs investigation.

Thus, the aim of this *in vitro* study was to evaluate the effect of different bleaching protocols on bovine enamel microhardness. In addition, the pH variation of the different bleaching agents during the gel application time was determined. The null hypotheses were (1) there is no difference between the bleaching protocols regarding enamel microhardness and, (2) the pH of the bleaching agents does not change during bleaching procedure.

2 ARTICLE

2 **ARTICLE**

The article presented in this Dissertation was written according to the Journal of Applied Oral Science instructions and guidelines for article submission

Evaluation of pH levels and the effect of recent violet LED light in office bleaching protocol alone or associated with 6% H_2O_2 on enamel microhardness

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ABSTRACT

To reduce bleaching side effects and enamel surface alterations, recent protocols using violet LED light (LEDv), alone or associated with low concentrations of hydrogen peroxide (HP) have gained interest. Objective: this *in vitro* study evaluated the effect of three different in-office bleaching techniques on bovine enamel microhardness and the pH variation of peroxide agents during the protocols. Methodology: For Knoop microhardness (KNH) analyses, enamel fragments were divided into 3 groups (n=10): LEDv –hybrid violet LED/Laser light (10x2' LEDv + 10x30" light off, 2 cycles); HP6%LEDv –6% HP gel + LEDv/Laser (15x1' LEDv + 15x1' light off); HP35% - 35% HP (1x45'). For pH measurements, all bovine teeth were divided into 2 groups (n=10): HP35% and HP6%LEDv. KHN was measured at baseline (T_0), 24h after bleaching (T_1) and after 7 days in artificial saliva (T_7). Initial and final bleaching gels pHs were obtained utilizing a pH meter. KHN was evaluated by the Wald-type permutation statistic, aligned rank transformation statistical test, Wilcoxon and Mann-Whitney tests; pH levels were evaluated by the Welch-James and Wilcoxon tests ($p<0.05$). Results: HP35% and HP6%LEDv presented a decrease in KHN from T_0 to T_1 ($p=0.0039$; $p=0.001$, respectively), with no difference among them ($p>0.05$); baselines values were recovered at T_7 ($p=0.313$ HP35%; $p=0.557$ HP6%LEDv). For LEDv, no significant difference was found between KHN at T_1 and T_0 ($p=0.5286$); at T_7 KHN increased in comparison to T_0 ($p=0.029$). HP6%LEDv and HP35% presented a reduction of pH values ($p=0.0029$; $p=0.0284$, respectively); HP6%LEDv showed greater reduction ($p=0.0004$). Conclusions: Bleaching with LEDv alone was the only treatment that didn't reduce enamel microhardness. HP6%LEDv led to a decrease in KHN values similar to the high concentrated gel. After seven days in artificial saliva, initial KHN was recovered. Although the pH of both gels decreased during the treatment, it remained above the critical value.

Key Words: Tooth Bleaching. Light Source. Bleaching Agents. Hardness. Hydrogen-Ion Concentration

INTRODUCTION

In-office bleaching is a popular, effective and conservative treatment[1-4]. Hydrogen peroxide (HP) is the most commonly used bleaching agent and is manufactured in concentrations ranging from 3% to 40%[4-7]. High-concentrated peroxide bleaching gel is widely used because satisfactory results can be achieved in only one appointment[2,3,4,8,9]. However, the risk for postoperative sensitivity increases with the concentration[2,4,6,10-13] and at higher peroxide concentrations, there is a great risk of enamel surface alterations and cell damage[7,11,14-17]. For

these reasons, new products and technologies have been developed for in-office dental bleaching using novel protocols with low-concentrated peroxide bleaching gels or even bleaching without peroxide agents [2,3,7,13,18-21].

Accordingly, a new generation of low-concentrated HP gels containing nanoparticles of titanium dioxide doped with nitrogen (TiO_N) photo-catalyst has been proposed. These nanoparticles are added to maintain the whitening effectiveness of the low-concentrated HP (3.5–6%) bleaching gels similar to high-concentrated gels, while increasing the safety of the treatmet[5,12]. The catalytic activity occurs when the TiO_N nanoparticles are exposed to wavelengths < 535 nm[22]; enhancing the generation of reactive oxygen species and improving the efficacy of the bleaching gel[1,3]. Another type of technology, the hybrid violet LED light system (LEDv) for in-office bleaching was recently launched in the market, which presents wavelengths of approximately 405nm-410nm and can be used with or without peroxide agents[20,22].

This promising technique of bleaching using LEDv alone has been performed more recently to avoid any side effect caused by bleaching gels in the enamel surface, although more evidences are required to recommend it in a clinical scenario [2,13,20]. The mechanism of action of violet-light is due to its short wavelength and high frequency interacting with the pigment molecules. This is possible because its wavelength coincides with the absorption peak of the chromophore molecules, breaking them into smaller molecules[2,13,18].

Some studies have also reported that bleaching side effects and enamel surface alterations are influenced by the acidity of HP gels, as lower bleaching agent's pH lead to greater alterations on the dental structure[15,16,22-26]. In this context, new generations of bleaching gels have been introduced with neutral/alkaline pH; however, during the bleaching procedure, there is a trend toward a decrease in the bleaching gels' pH from the initial times to the end[24,26,27] and the recommendation is replenishing the gel during the treatment. To provide the best cost-benefit to the dentist and more comfort to the patient, manufacturers enhanced the neutral/alkaline bleaching gels formulations for maintaining the basic pH of the gel during the entire bleaching procedure[10,28]. Therefore, these new bleaching agents can be applied only once on the enamel surface because its pH remains stable.

Whereas in-office bleaching protocols using low-concentrated bleaching and bleaching with LEDv alone are available for the dentists with the objective of reducing any deleterious effects on the enamel, few studies evaluated the safety of these recent bleaching protocols regarding the microhardness. Studies are still required to investigate the pH of the bleaching gels and its stability and the possibility of not replenishing the product. Thus, the aim of the present *in vitro* study was to evaluate the effect of different bleaching protocols (using violet LED light alone; bleaching with 6% HP gel containing TiO_2N nanoparticles associated

with violet LED light; 35% HP gel) on bovine enamel microhardness. In addition, the pH variation of the different bleaching agents during the gel application time was determined.

MATERIAL AND METHODS

This in vitro study evaluated two response variables: enamel microhardness and bleaching gels pH. The present study has two study factors: (1) bleaching protocol at three levels [35% hydrogen peroxide gel (HP35%); 6% hydrogen peroxide bleaching + violet LED/Laser light (HP6%LEDv); violet LED/Laser light (LEDv)]; (2) time at two levels for microhardness variable [immediately after treatment and after one week in artificial saliva], and two levels for the pH variable [initial and final].

Fifty bovine maxillary central incisors were used for the study, thirty as enamel fragments and twenty as the entire bovine tooth. After extraction, they were stored in physiologic saline containing 0.1% thymol and kept in a refrigerator at approximately 4°C until the specimen preparation. The materials and equipment used in the present study are described in Tables 1 and 2, respectively.

Microhardness test

For the microhardness analyses, enamel fragments were obtained from thirty bovine teeth. Thirty flattened and polished enamel fragments (4 × 4 × 2 mm) were obtained and microhardness measurements were taken before initial exposure to the bleaching protocols (baseline; T_0), immediately post-treatment (24h after bleaching; T_1) and 7 days after immersion in artificial saliva (T_7). The Knoop microhardness (KHN) measurements were recorded using the MicroMet 6040 microhardness tester (Buehler LTD, Lake Bluff, IL, USA) with a load of 25g applied for 5 seconds[17,32]. Three indentations, 100µm apart, were made in the center of the enamel fragments. The measurements were performed on each specimen at each evaluation time.

The microhardness differences between T_0 and T_1 and between T_0 and T_7 were used for statistical analyses. Positive values indicate that the microhardness increased and negative values indicate that the final microhardness was lower than the baseline value.

Bleaching procedures

After the initial microhardness measurements, a stratified randomization was made to divide the specimens into 3 groups (n=10)[17]: HP35% (control); HP6%LEDv and LEDv, to receive the different bleaching protocols described in Table 3.

At the end of the bleaching protocols that applied peroxide gels on the enamel surface (HP35% and HP6%LEDv), the excess of the gel was removed with gauze and distilled water (DW). Next, the specimens were immersed in an ultrasonic bath with DW for 2 min (Merce,

Campinas, SP, Brazil). The specimens were then polished for 20s with a wet felt disc impregnated for polishing (DMC Equipamentos Ltda., São Carlos, SP, Brazil), followed by the ultrasonic bath for 2min. In sequence, a desensitizer (Nano White, DMC Equipamentos Ltda., São Carlos, SP, Brazil) was applied for 4min on the enamel surface and the specimens were then submitted to another 2 min ultrasonic bath.

After the bleaching procedures, the specimens were kept in DW for 24 h. Next, the immediate KHN measurements were taken. After the second measurement, the specimens were stored in artificial saliva (AS) at 37°C, for 7 days. The AS was specifically formulated for the re-mineralization of the dental hard tissues and contained: 1.5 mM $Ca(NO_3)$ $2.4H_2O$, 0.9 mM Na_2HPO_4, $2H_2O$, 0.15 M KCl, 0.02 M TRIS and 0.05 ppm F (pH 7.0)[29]. The solution was changed daily. After this period, the last microhardness test was performed on the specimens.

Bleaching gels pH measurements

For the bleaching gels pH analyses, twenty entire bovine teeth were used. Prophylaxis with pumice and water using a Robinson brush (KG Sorensen Ind. E Com. LTD, São Paulo, SP, Brazil) at low speed was done to clean the teeth. After the preparation, all teeth were stored in DW until being used for the experiment in order to avoid dehydration. The specimens were randomly divided into 2 groups (n=10): HP35% and HP6%LEDv, to receive the different bleaching protocols described in Table 3. Since the LEDv group did not have peroxide gel applied on the enamel surface, the pH measurements were not performed in this group. During the bleaching protocol, the teeth roots were maintained in DW to avoid dehydration.

To analyze the pH levels of the bleaching gels, a portable pH meter with a digital display was used (Model pH100, pHTEK, Curitiba, PR, Brazil). Prior to the beginning of each group of readings, the pH meter was calibrated with two standard solutions (pH 4.0 and 7.0). The pH of the bleaching gels was measured at the initial and final time of the different bleaching protocols. The gel was taken to the electrode with a microbrush (KG Sorensen Ind. E Com. LTD, São Paulo, SP, Brazil) and after each reading, the electrode was cleaned with DW.

Statistical analysis

All of the analyses were performed in software R, version 4.0.0, and a 5% significance level ($p<0.05$) was used in all of the analyses. After normality and homoscedasticity evaluation by Q-Q Plots and Residual versus Predicted plots, differences in microhardness were analyzed by means of a Wald-type permutation statistic test (WTPS). As a complementary analysis, the Aligned Rank Transform (ARA) was conducted to analyze the effects of each group and moment in the microhardness. Both the ARA and Mann-Whitney tests compared group behaviors in microhardness, while both the ARA and Wilcoxon signed-rank tests compared differences in the moments of observation.

For pH levels analysis, the Levene's test was used. Shapiro-Wilk checked the normality of pH data per group. Since the null hypothesis of normality was rejected, the Welch-James test was used to compare the pH of the groups. The comparison between the moments was carried out by the Wilcoxon signed-rank test.

RESULTS

Microhardness

When the microhardness was analyzed, the data showed evidence of significant effects of group (p=0.007 for WTSP; p=0.013 for ARA) and moment (p=0.001 for WTSP; p=0.0003 for ARA). There was no evidence to reject the null hypothesis of no interaction effect between the group and moment (p=0.236 for WTSP; p=0.340 for ARA). Regarding comparison between groups, the data reported significant differences between the LEDv and HP35% (p=0.0165 for ARA; p=0.002 for the Mann-Whitney test) and conflicting results between the LEDv and HP6%LEDv (p=0.0559 for ARA; p=0.033 for the Mann-Whitney test). A boundary value of p was observed for the comparison between the LEDv and HP6%LEDv, and, if a level of 10% of significance is under consideration, the data would reject the null hypothesis of equality of microhardness for the two groups. The data did not show evidence to reject the null hypotheses that the reduction observed for the median of microhardness observed for HP35% was the same as the HP6%LEDv (p=0.7709 for ARA; p=0.399 for the Mann-Whitney test).

Table 4 shows the descriptive statistics for microhardness difference between the baseline KHN values and KHN at T_1 and T_7, for the three bleaching protocols. Data showed that the microhardness difference values between T_1 and T_0 were greater than between T_7 and T_0 (p=0.0002 for ARA; p=0.0009 for the Wilcoxon Sign Test). There was no statically significant difference between microhardness median at T_1 and T_0 for the LEDv group (p=0.5286; Wilcoxon Sign Test). For the HP35% and HP6%LEDv groups, there was a significant reduction in microhardness median between T_1 and T_0 (p=0.0039 for the HP35%; p=0,001 for the HP6%LEDv; Wilcoxon Sign Test). The microhardness median at T_7 increased when compared to T_0 for LEDv (p=0.029; Mann Whitney test). For the HP35% and HP6%LEDv groups, there was no significant difference between microhardness medians at T_7 and T_0 (p=0.313 for HP35%; p=0.557 for the HP6%LEDv; Mann-Whitney test), indicating a microhardness recovery after 7 days in artificial saliva.

pH measurements

The decrease in pH values from initial to the end of bleaching procedure (ΔpH) was concentrated between -0.25 and 0 for the HP35% group, and for the HP6%LEDv group, this value was between -0.5 and -0.75 (Figure 1). The initial pH mean was 7.66 for HP35% and 9.28 for HP6%LEDv; the final pH mean was 7.53 and 8.68, respectively.

The Levene's test did not reject the null hypotheses of equality of variance between groups (p=0.192). The Shapiro-Wilk test rejected the null hypothesis of normality of pH data for the HP35% group (p=0.0070), but this hypothesis was not rejected for the HP6%LEDv group (p=0.6954). The Wilcoxon sign-rank test showed a reduction of the pH median for both HP6%LEDv and HP35% groups from the initial moment to the end of the bleaching protocols (p=0.0029 and p=0.0284, respectively). Data suggests that the reduction was greater for the group HP6%LEDv (p=0.0004; Welch-James test).

DISCUSSION

In the present study, a 6% HP bleaching gel containing TiO_N was used associated with a hybrid violet LED light. Manufacturer's instructions were followed for the three protocols employed in the present study, which include polishing and desensitizer application in specimens that received the bleaching gels (HP35% and HP6%LEDv) and the absence of these stages for the LEDv group. As indicated in the instruction manual, the bleaching gel was applied once on the enamel surface without replenishing.

Products with pH values above the critical level for enamel dissolution (pH 5.5) are incapable of causing enamel demineralization[10,15,23]. Therefore, studies[14,30,31] support the protocol of not replenishing the gel if the pH of the product is maintained at safe levels. In the present study, both the 35% and 6% HP bleaching gels presented a decrease in pH values (p=0.0029 and p=0.0284, respectively) from the initial to the end of treatment (ΔpH mean = 0.13 for HP35% group and 0.6 for the HP6%LEDv group). This result is in agreement with previous studies[26,27]; however, even with this reduction, the pH values were above the critical level for enamel dissolution for both bleaching gels during the whole procedure, allowing its unique application on the enamel surface.

Regarding enamel microhardness, there was a decrease in KNH values from T_0 to T_1 for the HP35% (p=0.0039) and HP6%LEDv groups (p=0.001), with no significant difference among them (p>0.05); but there wasn't a significant microhardness loss for the LEDv group (p=0.5286). The present study results agree with studies[8,32,33] that reported similar behaviors regarding enamel surface alteration for different bleaching gels concentrations. In contrast, other studies[7,15-17] observed that higher HP concentrations led to greater surface alterations. Desensitizer containing sodium fluoride and potassium nitrate was applied after the bleaching procedure in specimens of the HP35% and HP6%LEDv groups in order to follow the manufacture's instructions. The application of this product may have masked the reduction in microhardness provided by the bleaching agent[9,16,34], resulting in no difference among agents.

In the present study, a significant reduction of KHN immediately after bleaching occurred for the HP35% (p=0.0039) and HP6%LEDv groups (p=0.0039), even using neutral/alkaline gels. Our results agree with previous studies[7,15,25,34,35] that also used neutral/alkaline bleaching

gels and presented reduction in enamel microhardness. Magalhães, et al.[25] (2012) and Crastechini, et al.[34] (2018) speculated that this KHN loss promoted by neutral gels could be related to the demineralization caused by the low concentrations of calcium and phosphate ions and high concentrations of sodium and chloride ions in the bleaching gels, which can cause undersaturation with respect to the hydroxyapatite and can explain the decrease in enamel microhardness in the present study. Recent studies[6,36] found a reaction of HP with enamel proteins. Since changes in the organic content can affect enamel integrity[25,34], another explanation for the loss of microhardness reported by the present study can be related to the oxidative reaction of the HP with the organic matrix.

In the LEDv group, there was no significant microhardness loss after bleaching (p=0.5286). Bleaching without peroxide agents is a very recent topic and studies about it have focused on the efficacy of this protocol[2,18,20]. The results of the present study agree with the few studies that evaluated enamel surface alterations after bleaching with violet LED[19,37]. This novel technique using violet LED light alone has been reported to promote color alterations[2,18,20,37]. Since no surface alteration was observed for this treatment, it seems to be a promising protocol, although more studies should be done to recommend it.

Although there was a microhardness loss in groups bleached with peroxide agents, this loss seems to be clinically negligible. The microhardness median after 7 days in AS (artificial saliva) was not statistically different from the initial microhardness for the HP35% and HP6%LEDv groups (p=0.313 and p=0.557, respectively). This finding is in accordance with the results already found in the literature[15,17,19,35]. Studies reported that human saliva and AS are effective agents to recuperate enamel microhardness and can overcome the detrimental effectives promoted by the bleaching procedure[15,17,37]. Therefore, the closer to oral cavity conditions the samples are submitted to, less microhardness loss is expected. For the LEDv group, there was an increase in microhardness from T_0 to T_7 (p=0.029), which confirms the remineralizing potential of the solution used in the present study.

Based on the current results, bleaching with 35%HP; 6%HP with TiO_N + LEDv or bleaching with LEDv alone can be considered safe regarding enamel microhardness alterations if the manufacturer's instructions are followed. Bleaching with LEDv alone can be considered the safest of the three protocols since no surface alterations was observed. The effect of HP35% and HP6%LEDv on the enamel microhardness was similar. Further *in vivo* studies are needed to compare these protocols regarding tooth sensibility and efficacy to indicate one or the other. It is also worth emphasizing the importance of the saliva to recuperate the enamel microhardness after bleaching. The absence of a control group with only artificial saliva can be a limitation of the present study; however, all experimented groups were maintained in the same standard conditions since the specimens preparations. In addition, the flattening and polishing of the enamel surface required for the microhardness test removes the

aprismatic enamel that is more mineralized, which can result in overestimated results compared to a clinical situation.

CONCLUSIONS

Under the limitations of the present *in vitro* study, it can be concluded that:

- LEDv didn't decrease the enamel microhardness. After 7 days in artificial saliva, the KHN values increased compared to the baseline.
- HP6%LEDv and HP35% decreased enamel microhardness immediately after bleaching, with no significant difference among them. After 7 days in artificial saliva, the KHN values were similar to baseline for both groups.
- The pH of both the HP35% and HP6%LEDv groups decreased from the initial to the end of treatment, but remained above the critical value for enamel dissolution. HP6%LEDv presented a greater decrease in pH values.

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Table 1 - Materials for the study

Table 2 - Equipment for the study

Table 3 – Bleaching protocols

Table 4 – Descriptive statistics for microhardness differences. Different upper case letters means statistically significant difference between groups; ** ($p<0.05$)

Figure 1 – Box-plot graphic of difference between pH values

3 ARTICLE ILLUSTRATIONS

Table 1 – Materials for the study

Material	Characteristics	Manufacturer
Bleaching Kit: Nano White Flex 6% and 35%	1- 6% Hydrogen Peroxide gel nitrogen-doped titanium dioxide (TiO2) nanoparticles 2- 35% Hydrogen Peroxide gel nitrogen-doped titanium dioxide (TiO2) nanoparticles 3- Desensitizer: purified water, essence, thickener, preservative, potassium nitrate and sodium fluoride 4- Impregnated felt discs for polishing	DMC Equipamentos LTDA., São Carlos, SP. Brazil

Table 2 – Equipment for the study

Equipment	Manufacturer
1- LED/Laser Hybrid Light: **Whitening Lase Premium** 6 blue LEDs light (450nm ± 10nm); 6 violet LEDs light (405 nm ± 5 nm); infrared laser light (808nm ± 10nm); red laser light (660nm ± 10nm)	DMC Equipamentos LTDA., São Carlos, SP, Brazil
2- Microhardness tester: **MicroMet 6040**	Buehler LTD, Lake Bluff, IL, EUA
3- pH meter: **pH 100**	pHTEK, Curitiba, PR, Brazil

Table 3 - Bleaching protocol

(continues)

Group	Characteristics	Bleaching protocol
HP35%	Bleaching with 35% Nano White Flex (DMC Equipamentos LTDA., São Carlos, SP. Brazil)	1 session with one bleaching gel application Total bleaching time = 45 min After the bleaching procedure, the specimens of this group were polished and the desensitizer was applied

Table 3 - Bleaching protocol

(conclusion)

Group	Characteristics	Bleaching protocol
HP6%LEDv	Bleaching with 6% Nano White Flex associated with violet LED/Laser hybrid light - Whitening Lase Premium	1 session with one bleaching gel application After bleaching gel application, it was light activated for 1min followed by 1min interval. Performed were 15 light activations and 15 intervals (15x1' LEDv + 15x1' interval) Total bleaching time = 30 min After the bleaching procedure, the specimens of this group were polished and the desensitizer was applied
LEDv	Bleaching with violet LED/Laser hybrid light - Whitening Lase Premium (DMC Equipamentos LTDA., São Carlos, SP. Brazil)	1 session with 2 consecutive light irradiation cycles The LEDv was activated for 2min followed by 30s of interval. Performed were10 light activations and 10 intervals in each cycle of 25min. Between the cycles, 5 min of rest was done 1 cycle (25 min) = 10x2' LEDv on + 10x30" LEDv off Total bleaching time = 50 min This bleaching protocol did not require polishing and desensitizer application

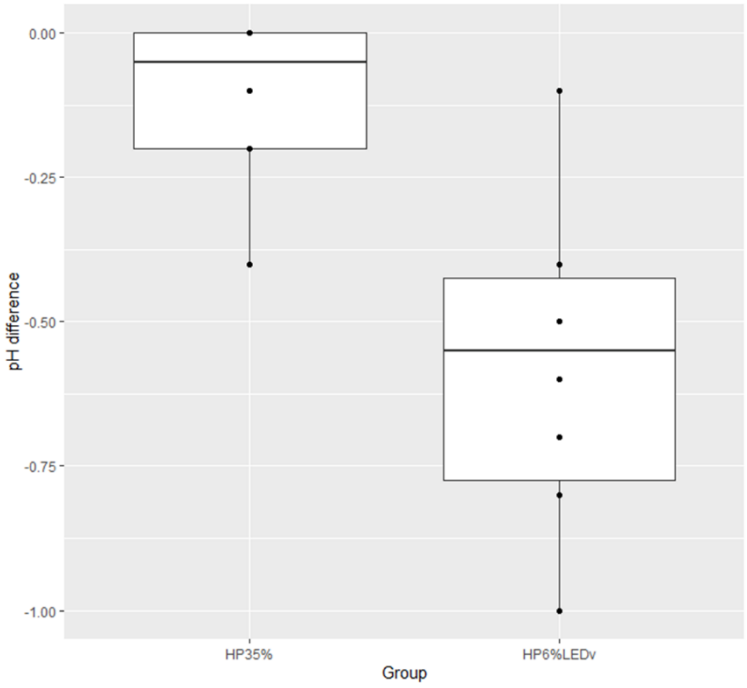
Table 4 – Descriptive statistics for microhardness differences. Different upper case letters means statistically significant difference between groups; ** ($p<0.05$)

* The microhardness differences between T_0 and T_1 and between T_0 and T_7 were used for statistical analyses. Positive values indicate that the microhardness increased and negative values indicate that the final microhardness was lower than the baseline value.

Moment	Group	Median	Std. Deviation
($T_1 - T_0$)	LEDv	.0000 A	5.29050
	HP35%	-8.5500** B	30.83103
	HP6%LEDv	-11.3200** B	13.49995
	Total	-5.9350	20.05007
($T_7 - T_0$)	LEDv	2.9150** C	5.62424
	HP35%	-2.0650 D	9.31681
	HP6%LEDv	.8150 D	12.95494
	Total	.6800	9.96108

Total	LEDv	.0850	6.09654
	HP35%	-6.9650	24.45357
	HP6%LEDv	-4.3200	14.65244
	Total	-2.0650	16.97338

Figure 1 – Box-plot graphic of difference between pH values



4 DISCUSSION

4 **DISCUSSION**

In the present study, the 6% and 35% HP bleaching gels were applied only one time on the enamel surface. Studies[31,48,49] support a unique application of the gel once the pH of the product is maintained at safe levels. Thus, the acidity of the gel will determine the possibility of not replenishing the peroxide agent during the procedure, once greater acidity have been related to greater changes in tooth structure and also post treatment sensitivity[10,20,33,44,45,47]. Products with pH values above the critical level for enamel dissolution (pH 5.5) are incapable of causing enamel demineralization[8,32,42]. Thus, it is important that the bleaching gel presents high pH and maintain it stable during the whole procedure.

The pH measurements of the bleaching gels used in this study showed that both 35% and 6% HP gels presented a decrease in pH values (p=0.0029 and p=0.0284, respectively) from the initial to the end of treatment (initial/final pH means= 7.66/7.53 for the HP35% group and 9.28/8.68 for the HP6%LEDv group). So, our second null hypothesis that the pH of the bleaching agents do not change during bleaching procedure was rejected. This result is in agreement with some studies[41,45] but does not agree with others[8,46]. Alexandrino, et al.[44] (2014) showed that bleaching gels with the same concentration, the same neutral initial pH value could maintain or decrease the pH during bleaching procedure, depending on the formulation of the product. This can explain the different results obtained in previous studies. Researchers[20,41,45] reported that the higher HP concentrations, the lower the final pH of the gel. This finding was also observed in the present study.

Even though the pH of both bleaching gels decreased during treatment, this value was always above the critical level for enamel dissolution. Therefore, the bleaching protocols performed in this study can be applied only once on the enamel surface and cannot cause enamel demineralization with respect to the pH values of the gels used. Recent studies[31,37,46,49] showed that this technique of one application of neutral/alkaline gels should be performed because of the best cost-benefit for the professional, the reduced risk of accidents related to soft tissues and it is more comfortable the patient. Regarding color change and post-bleaching sensitivity, Almeida, et al.[31] (2015), Vildosola, et al.[37] (2017) and Martins, et al.[46] (2018)

demonstrated equal effectiveness and tooth sensibility for the protocols with or without replenishing the gel.

The possible adverse effects of bleaching on dental structure have been widely studied. To determine small changes in surface that demonstrate the effect of bleaching products on enamel, the microhardness test is considered suitable and is frequently used[34,44,47]. The high concentration and acidity of peroxide bleaching gels have been reported to induce greater enamel surface alterations[20,32-34,44,45]. However, in the present study no significant difference was found between HP35% and HP6%LEDv ($p>0.05$). This result agrees with studies[1,9,50,51] that reported similar behavior regarding enamel surface alteration for different bleaching gels concentrations.

The contradictory results regarding enamel microhardness after bleaching can be related to the use of bleaching gels from different commercial brands, with different formulations and time of applications[9]. Different results can be obtained due to methodological differences regarding the substrate used (human or bovine teeth), microhardness test (Knoop or Vickers) and storage solution (artificial saliva, human saliva, distilled water) chosen for the study[9,33,34,51].

Desensitizer was applied after bleaching procedure in specimens of the HP35% and HP6%LEDv groups in order to follow the manufacture's instructions. The desensitizer available in the bleaching gel kit was used, which contained sodium fluoride and potassium nitrate in its composition. The application of this product may be responsible for the absence of significant differences between HP35% and HP6%LEDv groups, once studies[15,26,33,52] have shown less enamel microhardness decrease or even no decrease after bleaching when fluoride agents were used after bleaching procedures. In this context, the desensitizer gel may have masked the reduction in microhardness provided by the bleaching agent, resulting in no difference among agents. So, it is extremely important to follow the manufacturer's instructions for the best results of the treatment.

In the present study, a significant reduction of KHN immediately after bleaching occurred for the HP35% ($p=0.0039$) and HP6%LEDv groups ($p=0.0039$), even using neutral/alkaline gels. Studies frequently report a decrease in enamel microhardness values immediately after bleaching procedures[13,15,17,20,21,32,34,44]. Klaric, et al.[53] (2013)

and Klaric, et al.[32] (2015) evaluated the microhardness loss promoted by different bleaching gels concentration and acidity, and showed a decrease in microhardness values, even for neutral agents. Our study is in agreement with other studies[20,21,44,47,52] that also used neutral/alkaline bleaching gels and presented reduction in enamel microhardness. In contrast, Borges, et al.[1] (2015) investigated the effect of different HP concentrations on enamel microhardness and showed no differences in the KHN values obtained immediately after bleaching compared to the baseline; however, the specimens of their study were kept in artificial saliva, while in the present study, they were stored in distilled water for rehydration before the immediate measurement.

Magalhães, et al.[47] (2012) and Crastechini, et al.[52] (2018) speculated that the demineralization could be attributed to the low concentrations of calcium and phosphate ions and high concentrations of sodium and chloride ions in bleaching gels, which can cause undersaturation with respect to hydroxyapatite and can explain the decrease in enamel microhardness in the present study.

Recent studies[19,22] have investigated others possible mechanisms of action of HP and the results of some authors can explain the decrease in enamel microhardness of the present study using alkaline bleaching gels. The mechanism of action of hydrogen peroxide to bleach teeth is not fully understood[19,22]. The “chromophore theory” is frequently used to explain the hydrogen peroxide oxidative effects. However, there is no scientific evidence supporting this theory; and the concentration of organic chromophores, if they exist in dental enamel, is extremely low, being under the detection limit of several spectroscopy techniques[19]. Recently, some authors[19,22] found a reaction of HP with enamel proteins. Eimar, et al.[19] (2012) observed that deproteinized enamels were not oxidized, therefore not presenting color change after bleaching. Among the results of their study, Guo, et al.[22] (2018) concluded that aminoacids were responsible for the fluorescence and color properties of HA (hydroxyapatite) and that the bleaching effects of HP might be due to oxidization of the benzene ring in AAAs (amino acids) by free radicals. Therefore, the reduction in the enamel microhardness of the HP35% and HP6%LEDv groups, can be due to the oxidative reaction of HP with the organic matrix, as changes in the organic content can affect enamel integrity[47,52].

In the LEDv group, there was no significant microhardness loss after bleaching ($p=0.5286$). Bleaching without peroxide agents is a very recent topic and studies about

this have focused on the efficacy of this protocol[4,23,24,36]. The results of the present study agree with the few studies that evaluated enamel surface alterations after bleaching with violet LED. Eugenio, et al.[35] (2020) obtained results similar to the present study. They reported no significant difference between the group bleached with violet LED alone and the control group in relation to enamel surface roughness. However, when the light was associated with low concentrated HP gel (7.5%), enamel surface alterations were observed. According to Kury, et al.[39] (2020), the bleaching treatment with violet LED alone showed similar enamel morphology to the untreated group, which shows the safety of its use for dental enamel. This recent bleaching protocol using violet LED light alone has been reported to promote color alterations[4,23,24,36,39]. Since no surface alteration was observed for this protocol, it seems to be a promising technique, although more studies should be done to recommend it.

The present study used two storage solutions. Immediately after bleaching procedures, the specimens were immersed and stored in distilled water for 24 hours for rehydration. After this period, microhardness test was performed and the results showed a significant microhardness loss for HP35% and HP6%LEDv, while LEDv group present no significant differences between baseline values and values obtained at T_1.

After the immediate measurement of microhardness, the specimens were stored in artificial saliva for seven days, with daily changes. This second storage solution was chosen in order to observe the remineralizing potential of the artificial saliva. In our study, after this storage period, the enamel microhardness was reestablished for the HP35% and HP6%LEDv groups, with no significant difference between microhardness median at T_0 and T_7 ($p=0.313$ and $p=0.557$, respectively). Thus, this KHN loss for the groups bleached with peroxide agents seems to be negligible. This finding is in accordance with results already found in the literature[13,32,34,53]. Similarly to the results of this study, Mondelli, et al.[34] (2015) and Parreiras, et al.[13] (2014) showed a decrease in the enamel microhardness when it was obtained immediately after bleaching procedure but, after seven days in artificial saliva, the microhardness was recovered.

Several studies reported that human saliva and artificial saliva are effective agents to recovered enamel microhardness and can overcome the detrimental effectives promoted by the bleaching procedure[13,32,34,53,54]. Therefore, the closer to oral cavity conditions the samples are submitted to, less microhardness loss is expected[54]. For the LEDv group, there was an increase in microhardness from T_0 to T_7 ($p=0.029$), which confirms the remineralizing potential of the solution used in the present study.

Thus, based on the results obtained in the present study, bleaching with 35% HP bleaching gel; 6% HP containing nanoparticles of TiO_N bleaching gel associated with violet LED or bleaching with violet LED light alone can be considered safe regarding enamel microhardness alterations if the manufacturer's instructions are followed. The bleaching technique without peroxide agent, using only violet LED/Laser light was the only protocol that did not cause any significant enamel surface alteration. The HP35% and HP6%LEDv groups presented similar results at the two evaluated times, promoting the same effect in dental enamel in relation to microhardness. The differences between the bleaching gels used in this study were the concentration of HP and the initial and final pH value obtained for each of the agents. Further in vivo studies have to be done to compare the protocols with 35% HP and 6% HP plus light regarding tooth sensibility, pulp cells damage and efficacy of these treatments to indicate one or the other. Due to the results of this study, it is worth emphasize the importance of saliva to recovered the enamel microhardness after bleaching with the protocols used in the experiments of this study.

The results of the present study cannot be directly extrapolated to a clinical situation because it is an *in vitro* study and has some limitations. *In situ* and *in vivo* studies need to be done using these protocols to confirm those findings. In oral cavity, other activities such as abrasion challenges might be presented in conjunction with bleaching procedure. Further studies should present this association. Another limitation of the present study is the absence of saliva and acquired pellicle during bleaching, which could have modified the current results. In addition, the flattening and polishing of the enamel surface required for microhardness test removes the aprismatic enamel that is more mineralized, which can result in overestimated results compared to a clinical situation.

5 FINAL CONSIDERATIONS

5 **FINAL CONSIDERATIONS**

Based on the results of the present study, bleaching with 35% HP; 6% HP with TiO_N + LEDv or bleaching with LEDv alone can be considered safe regarding enamel microhardness alterations if the manufacturer's instructions are followed. In relation to the pH of 6% and 35% HP bleaching gels, it decreased from the initial to the end of both protocols, but remained above the critical value. Therefore, a single application of the two products can be performed safely. Bleaching with LEDv did not cause any surface alterations. Bleaching using low (6%) or high (35%) concentrated HP bleaching gels presented similar effect on enamel microhardness. Further in vivo studies are needed to compare these protocols regarding tooth sensibility and efficacy to indicate one or the other.

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APPENDIXES

APPENDIXES

APÊNCIDE A - DECLARAÇÃO DE USO EXCLUSIVO DE ARTIGO EM DISSERTAÇÃO/TESE

DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN DISSERTATION/THESIS

We hereby declare that we are aware of the article (Evaluation of pH levels and the effect of recent violet LED light in office bleaching protocol alone or associated with 6% HP on enamel microhardness) will be included in (Dissertation/Thesis) of the student (Larissa Luri Almeida Amorim Ikejiri) and may not be used in other works of Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

Bauru, april 7th, 2021.

Larissa Luri Almeida Amorim Ikejiri

Author

Signature

Rafael Francisco Lia Mondelli

Author

Signature