Brazilian Dental Journal (2016) 27(1): 56-59
http://dx.doi.org/10.1590/0103-6440201600385
ISSN 0103-6440

In Situ and *In Vitro* Effects of Two Bleaching Treatments on Human Enamel Hardness

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The aim of this study was to evaluate *in vitro* and *in situ* the effects of two bleaching treatments on human enamel surface microhardness. Sixty enamel slabs from recently extracted thirty molars were used. The specimens were polished with sandpapers under water-cooling. The enamel samples were randomly divided in four groups, treated with 10% hydrogen peroxide (HP) or Whitening Strips (WS) containing 10% hydrogen peroxide and using two conditions: *in vitro* or *in situ* model. For *in situ* condition, six volunteers wore an intra-oral appliance containing enamel slabs, while for *in vitro* condition the specimens were kept in deionized water after the bleaching protocols. The bleaching treatments were applied one-hour daily for 14 days. Similar amounts of bleaching agents were used in both conditions. Before and after bleaching treatments, microhardness was measured. Statistical analysis (ANOVA and Tukey test) showed that in the *in situ* condition there was no statistically significant microhardness reduction in the bleached enamel ($p>0.05$). Significant decrease in hardness was observed for enamel slabs bleached with both treatments in the *in vitro* condition ($p<0.05$). Regarding the bleaching agents, *in situ* results showed no difference between HP and WS, while *in vitro* WS produced the lowest hardness value. It could be concluded that there was no deleterious effect on enamel produced by any of the bleaching protocols used in the *in situ* model. The reduction of hardness was only observed *in vitro*.

Key Words: tooth bleaching, hardness, hydrogen peroxide, *in situ*, *in vitro*.

Introduction

There is a high demand in dental offices nowadays for treatments that improve the aesthetic appearance. Face aesthetics harmony has been associated with perfect smiles and satisfaction with dental appearance. In this context, tooth color plays an important role in the perceptions of aesthetics and satisfaction with dental appearance (1,2). Tooth bleaching has become a popular treatment, with a large variability of commercial presentations that may be applied in the dental office, at-home under the supervision of the dentist or using over-the-counter (OTC) products without professional supervision (3). At-home bleaching treatment is the preferred treatment protocol for vital teeth according to the Brazilian dentists, using low concentration gels with mouthguards, while OTC products are used/recommended by 5% of these dentists (4).

Vital tooth bleaching is a non-invasive treatment that can be used to improve tooth color. Depending on the used kind of product and application, the color improvement may be clinically relevant and even maintained for long periods (up to two years) (1). Usually the bleaching treatments are well accepted by patients (5) and could have a positive impact in the individuals' oral health quality of life (2). While there is some sound scientific evidence regarding the effectiveness from at-home and in office treatments, the over-the-counter products, despite their increased use, still present little evidence of their bleaching effect, except for hydrogen-containing bleaching strips (3,6).

One of the potential adverse effect related to vital bleaching is hypersensitivity (6), which depends upon the used bleaching concentration. Usually, sensitivity is mild to moderate but transient, and could be treated with remineralizing agents or interrupting the treatment (7). Sensitivity may have a negative impact on patients (2). The sensitivity observed during the bleaching treatment has been related to the removal of minerals, resulting in increased porosity on the enamel surface and subsurface (8). The decrease in enamel hardness has been used frequently as an indicator of the mineral loss following bleaching procedures (9). Most *in vitro* studies demonstrated a significant reduction in the enamel hardness (10,11). However, few studies simulated the conditions observed in the oral cavity, as in such conditions the decrease in hardness could hardly be seen, due to the remineralizing effect of human saliva (9).

Therefore, the aim of this study was to evaluate the microhardness of human enamel using *in vitro* and *in situ* models and two different bleaching protocols (at

home and OTC products). The tested hypothesis was that when bleaching protocols were performed under *in situ* conditions in the oral cavity, the harmful effect of mineral loss would not be observed, despite the bleaching protocol.

Material and Methods

Ethical Aspects

The Ethics Research Committee of the University of Cruz Alta, Cruz Alta, RS, Brazil approved this clinical trial (protocol number 462.123). Based on pre-established criteria, six volunteers agreed to participate in the *in situ* part and signed an informed consent form. Human teeth were obtained from the tooth bank and consent terms for donations were also obtained.

Preparation of Enamel Specimens

Thirty third molars freshly extracted for orthodontic reasons were used in this study. . All teeth were examined under magnification (40×) to detect defects on the surface. The crowns of the selected tooth were cut at the CE-junction and the pulp was removed. The crowns were sectioned longitudinally from the middle third of buccal and lingual surfaces (5 mm x 5 mm x 2 mm) (9).

Sixty enamel slabs were obtained. The samples were embedded in 1% chloramine and stored at 5 °C until their use. Before the hardness measures, all specimens' surfaces were prepared. The specimens were included in a metallic matrix and the enamel surface polished for 40 s under water-cooling with sandpapers of decreasing grits (400, 600 and 1200) to obtain flat standardized enamel surfaces.

Initial Measurements

Knoop microhardness test was used. Before bleaching treatments, the hardness of the samples was obtained using a micro hardness-testing machine (Buehler, Model 1600, Lake Buff, IL, USA). Three indentations on each specimen were made using a 50 g load for 10 s. The indentations were performed at a 100 µm distance between them to avoid any interference between indentations.

The enamel slabs were then randomly assigned to four different groups (n=15) considering the type of condition (*in situ* or *in vitro* condition) and the used bleaching agent (10% hydrogen peroxide gel – HP, or 10% hydrogen peroxide in strips – WS). Composition and details of the bleaching agents used in the study are in Table 1.

Table 1. Characteristics of the tested bleaching agents

Group	Bleaching agent	Chemical characteristics	Manufacturer	Application technique
1	White Class	10% H_2O_2 Calcium, KNO_3 NaF,	FGM	60 min/day
2	White Strips	10% Hydrogen peroxide	Oral B	60 min/day

In Situ Conditions

Six undergraduate dental students volunteered for the *in situ* experiment. First, full-arch maxillary impressions and stone cast models based in the impressions were obtained. Six intra-oral acrylic appliances were prepared and thirty enamel slabs were randomly allocated and included in these appliances as follows: three appliances with 4 specimens and three appliances with 6 specimens each.

The volunteers were instructed to remove the appliances from the oral cavity once a day to perform the bleaching. The enamel slabs were covered with 0.05 mL of 10% hydrogen peroxide (HP) and with a piece of 5 mm x 5 mm of whitening strips (WS) for one hour. After this period, the bleaching gel or the strip was removed from enamel surface and the volunteers placed the appliances again in their oral cavities, for more 23 h to simulate clinical conditions. The bleaching protocols were conducted for 14 days.

In Vitro Conditions

The enamel slabs received the application of 0.05 mL from either 10% HP gel or WS, which remained in place for one hour. After removal of the bleaching agent, the enamel slabs were placed individually in containers with deionized water for the next 23 h. The bleaching protocols were performed for 14 days.

Final Measurements

Twenty-four hours after bleaching conclusion in the tested conditions, the enamel slabs were removed from the appliances or the containers, included and fixed in previously manufactured acrylic resin matrices. The bleached enamel surfaces were submitted to Knoop microhardness test using the same protocol as for the initial measurements.

Statistical Analysis

Statistical analysis was performed using the Sigma Stat 3.5 statistical software package (Informer Technologies, Inc., San Jose, CA, USA). Equality of variance ($p>0.05$) was first performed to see if the values showed a normal distribution. Mean values were obtained for each specimen considering all the three measurements. Three factors were under investigation: study method (*in vitro* and *in situ*), time (before and after bleaching) and type of bleaching product (10% HP or WS). Analysis of variance (ANOVA) for repeated measures was used. An additional Tukey's post-hoc test was carried out to identify differences among the groups. All analyses were

carried out with a confidence level of 5% ($p<0.05$).

Results

Knoop hardness values exhibited a normal distribution in the study. Table 2 shows the results from hardness measurements considering the different tested conditions. In the *in situ* condition, no statistically significant difference in enamel hardness could be observed for both bleaching protocols. Significant decrease in enamel hardness was only observed for the specimens treated with HP or WS in the *in vitro* condition ($p<0.05$). Comparing the two bleaching protocols (HP or WS), only in the *in vitro* condition, a lower harness was observed for WS compared with the enamel treated with HP. The results from the *in situ* model showed no statistical difference between the bleaching protocols.

Discussion

The hypothesis tested in the present study was accepted. Despite the bleaching protocol (10% HP gel or 10% HP whitening strips), no significant difference in the enamel hardness was observed, different from the *in vitro* condition, which showed a significant decrease of hardness for both bleaching agents.

Previous reports have demonstrated that the use of hydrogen peroxide or carbamide peroxide over enamel surface produced a decrease in hardness when applied *in vitro* (12,13). In this *in vitro* condition, bleaching agents may significantly alter the enamel surface and the composition could also be modified, due to the removal of some components such as calcium and phosphate, and in consequence, the hardness could be reduced (13,14). On the other hand, when peroxide agents are applied in conditions resembling the oral cavity like in the *in situ* methods, usually the decrease in hardness is reduced or avoided due to the human saliva action, and even with the mineral loss occurred the remineralizing effect of saliva (9,15). A recent study (16) showed microhardness decreasing immediately after bleaching; however, after 7 days post- bleaching, microhardness values were the same regardless the type of treatments. These findings reinforce the importance of the remineralizing potential of saliva (16). Saliva protects from demineralization by the presence of the salivary pellicle, providing calcium and phosphorous, its buffer capacity able to neutralize the environment and due to its clearance potential (17). *In situ* studies showed that the salivary pellicle formed over enamel could protect the enamel surface within a period of 3 min from citric acid and reduce de effects of erosive acids for 2 h (18,19). Thus, it was speculated that the presence of the salivary pellicle could be responsible for the lack of changes in enamel microhardness(15), demonstrating that the loss of mineral tissue observed *in vitro* following bleaching treatments is not prone to happen in clinical situations. Other *in situ* studies corroborate these findings, as enamel hardness was not affected by acidic challenge in the presence of saliva (15,20).

The type of bleaching agent and the concentration of these agents may also affect the bleaching efficacy and the potential adverse effect (2). Hydrogen peroxide is a stronger bleaching agent than carbamide peroxide. In the present study, the same concentration of hydrogen peroxide was used, but in different application methods: HP gel and whitening strips. Whitening strips are an OTC method easy to use and less expensive than at-home or in-office bleaching methods, presenting some efficacy in tooth color recovery (21,22) and these are some of the reasons for their growing popularity (4). Few studies have investigated enamel microhardness after the use of hydrogen peroxide strips and the results demonstrated no deleterious effect on the enamel microhardness (23,24). In the present study, when applied *in situ*, there was no difference in enamel hardness between the enamel slabs bleached with 10% HP gel or 10% HP WS. In the *in vitro* condition, a higher decrease in hardness was observed for WS group compared to HP gel group. A previous study has shown that enamel microhardness after the application of 10% carbamide peroxide gel and 6.5% hydrogen peroxide strips was significantly reduced *in vitro* (24).

These results may be explained by calcium in the 10% HP gel (White Class). The manufacturer suggests that calcium is intended to minimize the enamel demineralization process. Studies have reported that adding calcium into the bleaching agents prevents changes in enamel hardness and morphology without reducing bleaching efficacy, *in vitro* (25).

There are some limitations to be considered. The difference between products is one of the points to be highlighted. The 10% HP (White Class-FGM), besides hydrogen peroxide, has also potassium nitrate, sodium fluoride and calcium in its composition. The white strips do

Table 2. Means (SD) of micro hardness (KHN) pre and post bleaching for different conditions (*in situ* and *in vitro*) and different products (hydrogen peroxide - HP and Whitening Strips - WS)

Conditions	Pre-bleaching HP	Post-bleaching HP	Pre-bleaching WS	Post-bleaching WS
	Mean (KHN)±SD	Mean (KHN)±SD	Mean (KHN)±SD	Mean (KHN)±SD
In Situ	244.17±37.88 Aa	247.05±31.11 Aa	240.37±36.18 Aa	244.17±37.88 Aa
In Vitro	238.97±51.85 Aa	213.41±34.24 Bb	217.55±32.63 Aa	187.78±34.28 Bc

Different superscript letters in the same column and subscript letters in the same row indicate statistically significant difference ($p<0.05$).

not present calcium and this could influence the results. Another fact is that samples were stored in deionised water in the *in vitro* essay. However, the study of Parreira et al, found no different microhardness results using saliva or distilled water *in vitro* (12).

This study highlights the importance of study design in the evaluation of adverse effects following bleaching treatments. It seems that the results pointing for adverse effects on enamel surface after bleaching observed by *in vitro* studies should be considered with caution, since the *in situ* models have demonstrated that they could not occur in the clinical condition.

Within the limitations of this study, it could be concluded that there is no deleterious effect of bleaching protocols using 10% HP or WS when using a model that simulates the oral cavity and the reduction of hardness could only be observed *in vitro*.

Resumo

O objetivo deste estudo foi avaliar *in vitro* e *in situ* os efeitos de dois tratamentos clareadores sobre a microdureza do esmalte dental humano. Sessenta blocos de esmalte obtidos de trinta molares recentemente extraídos foram utilizados. Os espécimes foram polidos com lixas sob refrigeração com água. As amostras de esmalte foram divididas aleatoriamente em quatro grupos e tratadas com 10% de peróxido de hidrogênio (HP) ou fitas de clareamento (WS) contendo 10% de peróxido de hidrogênio, testadas em duas condições experimentais: *in vitro* ou *in situ*. Para o ensaio in situ, seis voluntários foram selecionados e usaram um aparelho intra-oral contendo blocos de esmalte, enquanto que para a condição in vitro, os espécimes foram mantidos em água deionizada após os protocolos de clareamento. Os tratamentos clareadores foram aplicadas durante uma hora/dia durante 14 dias. Quantidades semelhantes de agentes de clareamento foram usadas nas duas condições. Antes e após os tratamentos de clareamento, a análise da microdureza foi realizada. A análise estatística (ANOVA e teste de Tukey) mostrou que, na condição in situ não houve redução estatisticamente significante na microdureza do esmalte clareado ($p>0,05$). Diminuição significativa na dureza foi observada nos blocos de esmalte clareados, em ambos os tratamentos, na condição in vitro ($p <0,05$). Em relação aos agentes de clareamento, os resultados in situ não mostraram nenhuma diferença entre a HP e WS, enquanto que in vitro, WS produziu o menor valor de dureza. Conclui-se que não houve nenhum efeito deletério na dureza do esmalte, em nenhum dos protocolos de clareamento ao usar um modelo in situ. A diminuição da dureza foi observada somente na condição *in vitro*.

Acknowledgements

The authors are grateful to the TWAS/CNPQ (process 83903402087/190268/2010-7) for the full-time Postgraduate Fellowship provided to one of the co-authors.

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Received July 2, 2015
Accepted December 22, 2015