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Antioxidant Activity by DPPH Assay of Potential Solutions to be Applied on Bleached Teeth

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The aim of this study was to assess, using the DPPH assay, the antioxidant activity of several substances that could be proposed to immediately revert the problems caused by bleaching procedures. The percentage of antioxidant activity (AA%) of 10% ascorbic acid solution (AAcidS), 10% ascorbic acid gel (AAcidG), 10% sodium ascorbate solution (SodAsS), 10% sodium ascorbate gel (SodAsG), 10% sodium bicarbonate (Bicarb), Neutralize® (NE), Desensibilize® (DES), catalase C-40 at 10 mg/mL (CAT), 10% alcohol solution of alpha-tocopherol (VitE), Listerine® (LIS), 0.12% chlorhexidine (CHX), *Croton Lechleri* (CL), 10 % aqueous solution of *Uncaria Tomentosa* (UT), artificial saliva (ArtS) and 0.05% sodium fluoride (NaF) was assessed in triplicate by 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) free radical assay. All substances exhibited antioxidant activity, except for CL. AAcidS, AAcidG and VitE exhibited the highest AA% ($p<0.05$). On the contrary, CHX, NE, LIS and NaF showed the lowest AA% ($p<0.05$). In conclusion, AAcidS, AAcidG, SodAsS, SodAsG and VitE presented the highest antioxidant activity among substances tested in this study. The DPPH assay provides an easy and rapid way to evaluate potential antioxidants.

Key Words*:* antioxidant, dental adhesive, free radical.

INTRODUCTION

Hydrogen or carbamide peroxides commonly used for tooth bleaching have been associated with low bond strength values of adhesive restorations placed immediately after bleaching (1). The action mechanism of bleaching agents is based on a complex oxidation reaction,which releases oxygen free radicals that penetrate through the porosities of the enamel prism to the dentin, possibly due to the low molecular weight (about 30 g/mol) of these substances (2). These residual oxidant substances, like oxygen and other free radicals, are reported to interfere with the adhesion of restorative materials and inhibit their adequate polymerization (1).

To overcome this problem, several antioxidant agents have been proposed, such as sodium ascorbate, ascorbic acid, butylhydroxianisole, catalase, ethanol, acetone, glutathione peroxidise, alpha-tocopherol and sodium bicarbonate (3-6).

Components of mouthrinses, such as chlorhexidine, essential oils and sodium fluoride, have antioxidant activity and have been recommended for prevention of caries and periodontal diseases (7). In the same manner, commercially available products, like catalase at 1.25% (Neutralize®, FGM Dental Products, Joinville, SC, Brazil) and 5% potassium nitrate and 2% sodium fluoride (Dessensibilize KF® 2%; FGM Dental Products), which contain antioxidant compounds, could also be used to increase the bond strength to bleached teeth.

Due to antioxidant activity of naturally occurring

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substances in higher plants, attention has increased on the protective activity of these natural antioxidants against chronic disorders caused by oxidative process. *Croton Lechleri* (CL) and *Uncaria tomentosa* (UT) are plants from the Amazon River basin widely used for inflammatory disorders and with exhibited antioxidant and radical scavenging activity (8,9).

Although most *in vitro* studies have used artificial saliva (ArtS) as a storage medium for bleached teeth prior to bonding procedures (3-6), they have not evaluated or discussed the possible antioxidant effect of this storage medium on immediately increase bond strength values of bleached teeth. However, to the best of our knowledge, the antioxidant activity of these products (commercial products, natural plants and saliva) has not yet been evaluated. Another important point is that, there is lack of standardization regarding the methods used for measure the potential antioxidants of the substances (8), usually indicated in the literature (1-6).

DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical method is an antioxidant assay based on electron-transfer that produces a violet solution in ethanol (10). This free radical, stable at room temperature, is reduced in the presence of an antioxidant molecule, giving rise to colorless ethanol solution. The use of the DPPH assay provides an easy and rapid way to evaluate antioxidants by spectrophotometry (10), so it can be useful to assess various products at a time.

Due to the lack of evidence about which solution can be more effective as an antioxidant or even if there are other solutions with equal or more capacity to eliminate free radicals from dental surfaces after bleaching procedures, the purpose of this study was to evaluate the antioxidant activity of several agents proposed for reversion of problems caused by bleaching procedures using the DPPH free radical assay.

MATERIAL AND METHODS

Fifteen substances were tested: 1) Aqueous solution of 10% ascorbic acid (AAcidS; Sigma Chemical Co., St. Louis, MO, USA; batch#: 91H0179); 2) Carbopol gel of 10% ascorbic acid (AAcidG); 3) Aqueous solution of 10% sodium ascorbate (SodAsS; Sigma Chemical Co.; batch#: A4034); 4) Natrosol gel of 10% sodium ascorbate (SodAsG); 5) Aqueous solution of 10% sodium bicarbonate (Bicarb; Lase Peroxide Sensy II; São Carlos, SP, Brazil; batch#: 413/09); 6) 1.25% catalase solution (NE; Neutralize®; FGM Dental Products, batch#: 190608); 7) 5% potassium nitrate and 2% sodium fluoride gel (DES; Desensibilize®; FGM Dental Products; batch# 250209); 8) Aqueous solution of C-40 catalase (10 mg/mL, 16,000 units *per* milligram of protein) (CAT; Sigma Chemical Co; batch# 037K7022); 9) 10% alpha-tocopherol in ethanol (VitE; Fleming Pharmacy, Ponta Grossa, PR, Brazil); 10) Listerine® (LIS; Johnson & Johnson Industrial Ltda., São José dos Campos, SP, Brazil; batch# 1359B06); 11) 0.12% chlorhexidine digluconate (CHX; Fleming Pharmacy); 12) Viscous latex *in natura* of *Croton Lechleri* (CL; World Natural, Guayaquil, Equator; batch# y3459); 13) 10% *Uncaria Tomentosa* aqueous solution (UT; Fleming Pharmacy); 14) ArtS (1 g sodium carboxymethylcellulose, 4.3 g xylitol, 0.1 g potassium chloride, 0.1 g sodium chloride, 0.02 mg sodium fluoride, 5 mg magnesium chloride, 5 mg calcium chloride, 40 mg potassium phosphate, 1 mg potassium thiocyanate and 100 g distilled deionized water) (Fleming Pharmacy); 15) 0.05% sodium fluoride (NaF; Fleming Pharmacy).

The percentage of antioxidant activity (AA%) of each substance was assessed by DPPH free radical assay. The measurement of the DPPH radical scavenging activity was performed according to methodology described by Brand-Williams et al. (11). The samples were reacted with the stable DPPH radical in an ethanol solution. The reaction mixture consisted of adding 0.5 mL of sample, 3 mL of absolute ethanol and 0.3 mL of DPPH radical solution 0.5 mM in ethanol. When DPPH reacts with an antioxidant compound, which can donate hydrogen, it is reduced. The changes in color (from deep violet to light yellow) were read [Absorbance (Abs)] at 517 nm after 100 min of reaction using a UV-VIS spectrophotometer (DU 800; Beckman Coulter, Fullerton, CA, USA). The mixture of ethanol (3.3 mL) and sample (0.5 mL) serve as blank. The control solution was prepared by mixing ethanol (3.5 mL) and DPPH radical solution (0.3 mL). The scavenging activity percentage (AA%) was determined according to Mensor et al. (12):

$$AA\% = 100 - \left[\frac{(Abs_{sample} - Abs_{blank})x100}{Abs_{control}} \right]$$

Statistical Analysis

The experiment was done in triplicate for each substance. The results were expressed as percentage decrease with respect to control values and compared

by one-way ANOVA and Tukey's test. A difference was considered statistically significant if $p \leq 0.05$.

RESULTS

The results are presented in Table 1. All substances exhibited antioxidant activity, except for CL. The substances can be divided in three main groups , as depicted in Table 1. The first group was composed by five substances with higher values of antioxidant activity. In this group, the highest values were observed for AAcidS (95.7%) followed by AcidG, VitE and SodAsG ($p>0.05$). Bicarb (74.1%) differed significantly only from AAcidS ($p<0.05$). The second group was composed by four substances with intermediary antioxidant activity - SodAsS (51.8%), CAT, ArtS and UT (34.2%) ($p>0.05$). Both ArtS and UT were similar to several of the third group. However, the values of antioxidant activity were always higher for these substances. In the third group, only six substances with lower less antioxidant activity were included: DES (23%), CHX, NE, LIS, NaF and CL. According to Table 1, significant different was found there is only between DES and NaF (9.7%) ($p<0.05$). The DPPH free radical assay can be considered reliable and reproducible because in all products the coefficient of variation is lower (Table 1).

Table 1. Means, standard deviations and coefficients of variation of antioxidant activity in percentage (AA%) of the tested substances.

Treatment agent	Mean	Standard deviation	Coefficient of variation (%)	(*)
1st group				
AAcidS	95.65	0.56	0.58	a
AAcidG	95.33	1.07	1.12	a,b
VitE	92.81	1.95	2.10	a,b
SodAsG	76.04	14.59	19.2	a,b
Bicarb	74.09	10.53	14.2	b
2nd group				
SodAsS	51.79	14.2	27.4	c
CAT	48.05	7.17	14.9	c
ArtS	36.44	5.91	16.2	c,d
UT	34.19	4.20	12.3	c,d
DES	23.02	2.57	11.2	d
3rd group				
CHX	18.50	1.90	10.3	d,e
NE	18.12	4.61	25.4	d,e
LIS	17.38	4.20	24.2	d,e
NaF	9.72	1.44	14.8	e
CL	0.00	0.00	0.00	(**)

(*) Different letters indicate statistically significance among the groups ($p<0.05$). (**) CL did not show any activity.

DISCUSSION

Tooth bleaching involves the use of powerful oxidants such as hydrogen peroxide that produce other free radicals and reactive species of oxygen during its kinetics process. These residual oxidant substances are reported to interfere with adhesive system infiltration and inhibit their appropriate polymerization (1). The use of antioxidant agents has thus been proposed for *in vitro* studies to overcome this problem (3-6).

The highest AA% values were observed for vitamins with recognized capacity of radical scavenging like ascorbic acid (vitamin C - AAcidS and AAcidG), its derived sodium salt (sodium ascorbate - SodAsS and SodAsG) and vitamin E (VitE). Several studies have demonstrated the positive effect of sodium ascorbate to reverse the compromised bonding to bleached teeth (6,13). Sodium ascorbate is a sodium salt of ascorbic acid, and they are potent antioxidants capable of quenching reactive free radicals in biological systems (14). Ascorbic acid showed the highest antioxidant activity; independently of its presentation is solution or gel (AAcidS and AAcidG). However, its pH is approximately of 1.8, been inappropriate for clinical use.

On the other hand, sodium ascorbate (SodAsS and SodAsG) has a pH of 7.4, but the AA% was only similar to ascorbic acid when in gel. Although sodium ascorbate did not show significant differences between its forms of preparations (10 or 20% and solution or gel) in terms of reversion bond strength values, some authors only suggested that additives within the hydrogel may reduce the efficacy of the material decreasing diffusability of ascorbate (13). However, comparison of both forms of sodium ascorbate (solution and hydrogel) to overcome the lower bond strength of bleached enamel, revealed that the hydrogel form had better behavior (13).

VitE, a lipid-soluble antioxidant (pH 6.8), has recently been proposed with good results in dentin and enamel (6), showed AA% similar to ascorbic acid.

Previous studies reported that alcohol application on bleached enamel increased bond strength, although the values did not return to the levels of the non-bleached group (15). However, the presence of alcohol in the composition of the VitE formulated for this study may have contributed to the good response in terms of antioxidant activity, since VitE is not miscible in water solutions (6). Future studies are needed to test this hypothesis.

As shown by Buettner (16), VitE and vitamin C are, respectively, water and lipid soluble antioxidant small molecules that cooperate to protect lipids membranes against free radical process in organisms. Although ascorbate has good properties like a free radical scavenger, its shelf life is affected by pH and temperature variations (14). On the other hand, VitE is more oxidizing and stable than ascorbate because of its hydrophobicity.

The majority of studies dealing with *in vitro* bleaching research used ArtS as a storage medium; however, none of them consider its antioxidant activity. There is a consensus that an elapsed time of 1 or 2 weeks following the bleaching treatment is necessary to avoid bonding failures and usually during this elapsed time, the specimens are left in contact with saliva. The ArtS used in the present study showed an AA% of 36.4; however it had no protein components. It is actually a remineralizing solution with an electrolyte composition similar to human saliva but lacking its antioxidant defense molecules, so it could be argued that some of these components have antioxidant activity too. Although human saliva is the first line of defense against free radical-mediated oxidative stress (7) and some of the antioxidants proposed to revert lower bond strength values such as CAT, glutathione peroxidase, AAcid and VitE are also natural constituents of whole saliva, previous *in situ* studies did not consider this property of human saliva (3-6). As demonstrated by Barbosa et al. (17) and Bittencourt et al. (18), the concentration of hydrogen peroxide affects the time elapsed between bleaching and bonding procedure of in situ studies. Further studies assessing the antioxidant activity of human saliva by DPPH must be performed.

As former substances needed to be prepared in laboratorial conditions, commercially available products were also analyzed. The antioxidant activity of mouthrinses for periodontal disease control has previously been confirmed by the TEAC method (7). The antioxidant activity was ascribed to either active principles like methylsalicylate or vehicles like ethanol. However, differences among the antioxidant activity of LIS, NaF and CHX found in a previous study can be attributed to the reaction mechanism of assays used between both experiments (10).

Some products were chosen because they are offered with bleaching kits to be used in case of dentin hypersensitivity or mucosa injury caused by hydrogen peroxide. NE, a commercially available form of catalase, presented almost the half AA% than CAT. Catalase, an H_2O_2 oxidoreductase enzyme, was proposed by Rotstein (19) to eliminate hydrogen peroxide residuals. Then, other authors investigated the neutralizing effect of CAT on the bond strength of bleached teeth, with conflicting results (6,15). In the present study, CAT at 10 mg/mL (pH 7) exhibited half the AA% of ascorbic acid (AAcidS or AAcidG); however, it is difficult to establish comparisons because the manufacturers offer no information about the composition.

DES is a low-viscosity desensitizing gel based on dual desensitizing action for treatment of dentin hypersensitivity produced by tooth bleaching: potassium nitrate acting on nerve desensitization and NaF acting on remineralization of dental surfaces. Tay et al. (20) showed that DES could also be used as pretreatment for dentin hypersensitivity caused by tooth bleaching therapies and that this product did not affect the whitening efficacy. Probably the concentration of NaF and potassium nitrate are not sufficient to counteract the oxidative potential of 35% hydrogen peroxide. NaF alone also showed a lower antioxidant activity.

In the same manner than NE, sodium bicarbonate is sold accompanying in-office bleaching products for being used as a neutralizer when hydrogen peroxide accidentally contacts oral mucosa. Although the antioxidant activity of sodium bicarbonate was higher than both types of catalase (CAT and NE), they had similar behavior in reverting bond strength decrease (3).

Natural antioxidants existing in medicinal plants have been proposed as alternative. A blood-red viscous sap called Dragon's blood with antioxidant properties is released upon making cuts on the bark of CL tree. CL is widely distributed in the upper Amazonian valleys of Ecuador and Peru and marketed as a health product (9). Although PA accounts for up to 90% of dry weight and extracts (H_2O residue extract), fractions (1 and 3) and pure compounds (gallocatechin and epicatechin) of CL have shown to have antioxidant activity by DPPH assay in previous studies (8,21), in the present study

it was impossible to analyze its antioxidant activity because of its blood-red color, as the DPPH assay is a spectrophotometric method. Variations in plant material, extraction method, processing and antioxidant assays employed might affect the concentrations of active compounds that could be reflected in the antioxidant activity.

Previous studies have shown that UT exhibited antioxidant activity even at low concentrations (9,22,23). In the present study, we used the micropulverized form of the decoction bark of UT in aqueous solution at 10%, the same concentration used for the other antioxidants, and one third of the AA% achieved by AAcidS was obtained. This result agrees with those of a previous study (22) in which UT achieved similar results to AAcidS when its concentration was twice or six times the concentration of ascorbic acid. As mentioned by Sandoval et al. (23), the type of UT extract and its concentration are determinant for the DPPH inhibition; the freeze-dried form is a more effective scavenger of DPPH than the micropulverized form of UT.

Despite the higher antioxidant activity of some substances (AAcidS, AAcidG, SodAsS, SodAsG and VitE), it is not clear in the literature which is the minimum antioxidant activity to revert the problems occurred after the bleaching procedure and, unfortunately, the shelf life of these products are shortened and can be affected by storage conditions (temperature, time, light exposure) (24). Further studies are needed to evaluate the neutralizing effect of the some of the tested products to increase the lower bond strength of bleached teeth, and to evaluate the correlation between the antioxidant activity and bond strength values immediately after bleaching procedures.

The DPPH assay provided an easy and rapid way to determine the antioxidant activity of most of the substances tested in this study. It was found that AAcidS, AAcidG, SodAsS, SodAsG and VitE were the substances with higher rates of AA% and are the most promising substances to immediately revert the problems occurring after bleaching procedures.

RESUMO

O objetivo deste estudo foi avaliar, por meio do DPPH, a atividade antioxidante de substâncias que poderiam ser propostas para reverter de imediato os problemas causados pelos procedimentos de clareamento. A porcentagem de atividade antioxidante (AA%) da solução de acido ascórbico 10% (AAcidS), gel de ácido ascórbico a 10% (AAcidG), solução de ascorbato de sódio 10% (SodAsS), gel de ascorbato de sódio 10% (SodAsG), bicarbonato de sódio 10% (Bicarb), Neutralize® (NE), Desensibilize® (DES), catalase C-40 10 mg/mL (CAT), solução alcoólica 10% de alfa-tocoferol (VitE), Listerine® (LIS), clorexidina 0,12% (CHX), CrotonLechleri (CL), solução aquosa 10% de Uncaria Tomentosa (UT), saliva artificial (ArtS) e fluoreto de sódio 0,05% (NaF) foi avaliada em triplicata pelo teste de radicais livres 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH). Todas as substâncias apresentaram atividade antioxidante, exceto a CL. AAcidS, AAcidG e VitE mostraram os maiores valores de AA% ($p<0,05$). Por outro lado, CHX, NE, LIS e NaF mostraram os valores mais baixos de AA% ($p<0,05$). Em conclusão, AAcidS, AAcidG, SodAsS, SodAsG e VitE apresentaram os mais altos valores de atividade antioxidante entre as substâncias testadas. O teste DPPH é um método rápido e fácil para avaliar o potencial antioxidante.

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