The effect of vitamin C on urinary level of an oxidative stress biomarker during at-home tooth bleaching process: a randomized double blinded clinical trial

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Summary

***Introduction*: Oxidizing agents which are used as tooth whitening agents can induce an oxidative stress, a situation that initiates some systemic diseases. This clinical study aims to evaluate the effect of ascorbic acid (vitamin c) on urinary level of an oxidative stress biomarker during the at-home tooth whitening period.**
***Material and methods*: Thirty healthy patients who requested for the tooth whitening were involved in this trial. Specified bleaching trays were fabricated for both arches after making an impression. Each participant was given two syringes containing 15% carbamide peroxide gel and instructed to apply it for 6 h per night for 14 consecutive nights. Patients were divided into two equal groups. In the experimental group, the patients applied two tablets containing 500 mg of vitamin C every night along with the tooth whitening period. Urine samples were obtained in the morning before the study was started and were repeated in 5, 10, 15th days and five days after the expiration of the bleaching period. TBARS test was employed to evaluate the urinary level of malondialdehyde (MDA) as an oxidative stress biomarker. Data were analyzed by means of independent sample t-test and repeated measurement analysis.**
***Results*: Twenty-nine subjects completed the study. The MDA level increased during the bleaching period in both groups; however, the difference was not significant (P>0.05). In addition, the ascorbic acid application could not present a significant difference in the MDA level (P= 0.34).**
***Conclusion*: The application of 15% carbamaide peroxide did not significantly elevate the oxidative stress biomarker in human urine (IRCTID: IRCT2012113011618N1).**

Key words: ascorbic acid, at-home tooth bleaching, oxidant and antioxidant, oxidative stress, vitamin C.

Introduction

Discolored anterior teeth are perceived as a great esthetic problem by most patients. Tooth whitening or bleaching is a very conservative method which employs oxidizing agents to achieve a lighter and more desirable tooth color. During the course of bleaching, long-chain pigmented molecules oxidize and split into smaller, lighter ones with the release of carbon, water and oxygen (1).
The bleaching of vital teeth can be accomplished by either in-office or at-home technique of which the second method is performed by the patients themselves. The at-home bleaching method has created an easy performing, safe and low cost bleaching technique that is available to all socioeconomic classes of the patients. During this procedure, carbamide peroxide, a combination of hydrogen peroxide as an effective tooth whitening agent and urea, are commonly applied in a custom-fit tray for a few minutes to several hours each day, with regards to peroxide concentration and manufacturer recommendations. Although strong evidences supported the effectiveness of this method, nevertheless, it has lower clinical control compared to in-office one (2).
A number of concerns over systemic side effects of the bleaching agents have been raised because the

patient may have swallowed these oxidizing compounds which inevitably come in contact with their teeth and soft tissues for a long time especially during night. Genotoxicity, cytotoxicity, and carcinogenicity of the tooth whitening materials have been reported by many previous animal studies (3-9). They indicated that hydrogen peroxide might act as a promoter and ingestion of carbamide peroxide in a dose-dependent manner, induced acute mucosa ulcerations in rats' stomach. Notwithstanding, multiple exposures to hydrogen peroxide might reduced food consumption, weight gain, and result to changes in blood chemistry (5). The presence of adenoma and duodenum carcinoma alongside hyperplasia was reported following the ingestion of 0.1 and 0.4% (w/v) of hydrogen peroxide solution for 8 weeks in rats (5-7). Timblin et al. (10) presented an increase over-expression of proto-oncogen *c-jun* protein in human tracheal epithelial cells. However, the International Agency for Research on Cancer (IARC) has concluded that there is no clear evidence in animal and human experiments for the carcinogenicity of hydrogen peroxide (11).

Certainly, oxidizing agents such as tooth bleaching compounds can disturb oxidant-antioxidant body balance in favor of oxidants and can induce a potentially harmful challenge known as "oxidative stress", which has a potential in damaging cell structures, alters their functions and contributes in several pathological conditions and common diseases (12,13). One of the most important outcomes from the oxidant damage is lipid peroxidation (13). Lipid peroxides are unstable and decompose to form a group of reactive carbonyl compounds like malondialdehide (MDA) (14), which can be quantified with thiobarbituric acid-reactive substances test (TBARS) (15). A recent study conducted by Akbari et al. (16) regarding the systemic side effects of the bleaching agents on human health showed that these agents have the potential to disturb the body balance and induce the oxidative stress. They revealed that the serum concentrations of the MDA, total antioxidant capacity (TAC), and pro-oxidant-antioxidant balance (PAB) were increased significantly after the tooth bleaching period.

Aside body's defense, exogenous antioxidants as therapeutic adjuncts may well improve the inherent human antioxidant capacity and overcome the oxidative damages. Considering the effectiveness of antioxidant vitamins like vitamin C on the oxidative stress induced diseases such as Alzheimer disease (17) and atherosclerosis (13) and its effective role in inhibiting lipid peroxidation (15), it may be necessary to equip the human body with a variety of external origin antioxidants in order to counter balance harmful effects of the tooth whitening oxidants (12).

To the best of our knowledge, no study has been carried out on humans regarding the effect of ascorbic acid on tooth bleaching induced oxidative stress. This clinical trial was carried out to investigate whether the oxidative stress following at-home bleaching could be reduced by shifting pro-oxidant-antioxidant balance in favor of antioxidants via the use of vitamin C supplements. The null hypothesis was that, the ascorbic acid did not affect the urinary level of the MDA as a marker of oxidative stress.

Material and methods

Study population

Participants in this randomized double-blind clinical trial were selected from dental students with undesirable tooth discoloration complaint in Mashhad Dental School (the second biggest city in Iran). Thirty (19 females and 11 males) healthy volunteers signed a detailed informed consent and this investigation was approved by the Ethical Board of Mashhad University of Medical Sciences (Approval number: 901092).

The subjects with any of the following criteria were excluded from this assay:

1. Participants who had previous anterior restorations, tooth decay, exposed root surfaces, broken teeth, enamel erosions or poor oral hygiene that needs further treatments.
2. Participants who experienced the tooth whitening procedures or they used antioxidant drugs (vitamin C and vitamin E supplements) in the past six months.
3. Patients who are suffering from systemic or enzymatic disorders that disturb oxidant-antioxidant balance and smokers due to synergic oxidant with the bleaching agents.
4. Women who are pregnant or lactating mothers.
5. Volunteers with temporomandibular disorders or having para-function habits like broxism and clenching contraindicated for the at-home tooth bleaching method.

Bleaching procedure

To produce an accurate negative mold, alginate impressions (Bayer, Leverkusen, Germany) were obtained from both arches for each participant. Thereafter, casts were poured with dental stone powder (Tara, Kheyzaran, Isfahan, Iran) and were trimmed to a horseshoe shape with no palatal or tongue sections, without damaging tooth surfaces and gingival margins. To incorporate reservoir spaces, several layers of nail polish were applied on labial tooth surfaces of stone casts. The bleaching trays were fabricated with 0.035 inch vacuum formed sheets utilizing a vacuum tray-forming machine (Ultravac; Ultradent Products Inc., South Jordan, USA). Subsequently, the trays were trimmed to form scalloped borders on 2 mm far away from the gingival margins. Two 3-ml syringes of 15% carbamide peroxide gel (Opalescence, Ultradent Products Inc., South Jordan, USA) were given to each participant. The patients were instructed to place adequate amount of the bleaching agent into the tray to cover the facial surfaces of the teeth which are visible during laughing and speaking. To obtain maximum benefits of the product and patient compliance, participants were asked to wear bleaching trays for at least 6 h per night (according to

the manufacturer's instruction) for 2 weeks. After the loaded tray is seated, the patients were instructed to gently remove any excess of the bleaching material with a tissue or a brush. They were cautioned to discontinue the use of bleaching agents in the situations of tooth hypersensitivity or gingival problems and alert research team immediately.
In the end, the volunteers were randomly assigned in equal numbers to one of the two treatment groups. The randomization process was carried out by a third person who was not involved in the research protocol. In the experimental group, patients were instructed to chew two tablets containing 500 mg of vitamin C (Sunkist, Sunkist Growers, USA) before using the bleaching tray every night along with the tooth whitening procedure. Participants were reminded by a research operator through telephone to take their daily doses of the ascorbic acid. In the control group, no drug was prescribed. The whole procedure was clarified by an expert operator for each participant to increase adherence to the protocol. Neither the operator nor the statistician knew the group allocation, both were blinded to the protocol.

Urine sample
One milliliter of participants' morning urine was taken on days 0, 5, 10, 15 and 20 of the study. The first sample was collected in the morning before the initiation of bleaching procedure and was continued every five days until the final sample which was gathered five days after the expiration of the whitening period. Regarding sequencing sample collection, the samples were refrigerated at -80°C with no special treatment, according to commercial kit recommendation until initiating the assay.

Thiobarbituric Acid Reactive Substances (TBARS) Assay
To evaluate the effect of ascorbic acid on the urinary level of the MDA, as a product of lipid peroxidation as well as an index of the oxidative stress, TBARS test (Cayman Chemical, Ann Arbor, MI, USA, Item Number 10009055) was used based on an established method (18). The pink colored MDA-TBA adduct was formed by the reaction of MDA and thiobarbituric acid (TBA) under high temperature (90-100°C) and acidic conditions. The absorbance of the MDA-TBA complex was read at 532 nm and the concentration of the MDA in samples was calculated using a standard curve.

Statistical analysis

The normality of data distribution was examined by Kolmogorov Smirnov test. The difference of the MDA values between the five measurements was carried out using repeated measurements and Independent sample test. SPSS version 11.5 software (SPSS, Chicago, IL, USA) was used for statistical analysis while level of statistical significance was set at 0.05.

Results

Twenty-nine participants (19 females and 10 males) completed the two-week study period. One of the participants was excluded from the evaluation as a result of low cooperation, and no adherence to the study protocol. None of the participants who completed the study reported any signs of tooth hypersensitivity or gingival irritation.
Table 1 presents the mean, standard deviation and minimum and maximum value of the MDA in both control and intervention groups between five sampling intervals.
The Kolmogorov Smirnow test demonstrated that the MDA concentration was normally distributed in all measurements (P>0.05).
According to repeated measurement analysis, the urinary concentration of the MDA in both groups gradually increased during the study period that suddenly subsided at the final sample session (Fig. 1), however, no significant difference presented between these five sample sessions in each of the control and experimental groups (P value= 0.7 and 0.66, respectively). Furthermore, this analysis showed no significant difference in the MDA concentration during study period between two groups (P value= 0.86).
Independent sample t-test showed no significant difference between two groups on the baseline with other sampling sessions (P value= 0.79, 0.80, 0.81 and 0.34, respectively).
The data regarding the comparison of the urinary concentration of the MDA between the first and other sampling intervals are presented in Table 2.

Discussion

The findings of the current research could not reveal a significant increase in the MDA concentration in urine samples during the tooth bleaching procedure (P=0.7). Literally, this study has been designed to protect tooth bleaching volunteers from the systemic side effects of the whitening agents by vitamin C supplementation, based on a recent study, which was carried out by the same investigators who indicated that the serum concentrations of the MDA, total antioxidant capacity (TAC), and prooxidant-antioxidant balance (PAB) were increased significantly after the tooth bleaching period (16). Although this study could not reveal similar results, monitoring of the MDA concentration from onset to end of the study period showed the gradual elevation of the MDA that suddenly decreased in the final sample session which was taken five days after the expiration of the whitening period. Thus, it cannot entirely rule out the probably tooth bleaching induced oxidative damage. To clarify different outcomes between these two studies, the Authors need to emphasize on some points. Initially, the difference between these two experiments may be attributed to the kind of samples that were taken for the oxidative stress assay. The evaluation

Table 1. The mean, standard deviation, minimum and maximum value of the MDA in both groups between five sampling intervals.

Sampling intervals	Study groups	Mean±SD	Min	Max
First	Control	0.072±0.011	0.059	0.104
	Intervention	0.074±0.008	0.063	O.092
	Total	0.073±0.009	0.059	0.104
Second	Control	0.074±0.013	0.059	0.109
	Intervention	0.074±0.013	0.059	0.112
	Total	0.074±0.013	0.059	0.112
Third	Control	0.076±0.017	0.059	0.114
	Intervention	0.076±0.011	0.057	0.102
	Total	0.076±0.015	0.057	0.114
Fourth	Control	0.080±0.017	0.051	0.106
	Intervention	0.080±0.017	0.063	0.111
	Total	0.080±0.017	0.051	0.111
Fifth	Control	0.077±0.011	0.065	0.112
	Intervention	0.073±0.012	0.058	0.101
	Total	0.075±0.011	0.058	0.112

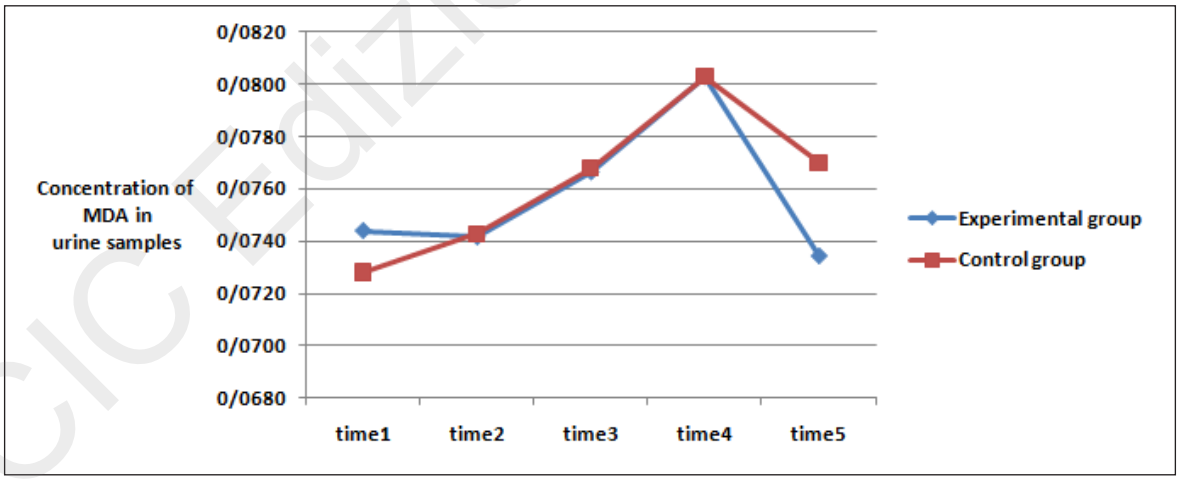

Figure 1. Repeated measurement analysis of the groups.

of oxidative stress biomarkers in human serum samples is more popular than that of urine. On the other hand, ethical consideration did not permit researchers to take five blood samples for the duration of 20 days (one blood sample in each five days). Moreover, it was determined that urinary level of the MDA is significantly lower than that of blood (19, 20) and can hardly be traced in situations with minute quantities in human serum. In fact, the current research was designed to monitor oxidant status during the bleaching period and after its expiration, as a multiple time series method in response to the limitation of the previous study with no evaluation of oxidant changes after the bleaching period, so as to determine the time

Table 2. The comparison of the urinary concentration of MDA between first with other sampling intervals.

Comparisons	Study groups	Number	Mean ± SD	*P-value*
First compared with second interval	Intervention	14	0.0002±0.013	0.793
	Control	15	-0.0015±0.020	
First compared with third interval	Intervention	14	-0.0022±0.013	0.809
	Control	15	-0.0040±0.022	
First compared with fourth interval	Intervention	14	-0.0059±0.017	0.819
	Control	15	-0.0075±0.019	
First compared with fifth interval	Intervention	14	0.0010±0.013	0.343
	Control	15	-0.042±0.015	

needed for the human body to completely recover from the oxidative stress. Also in a previous study (16), 9% hydrogen peroxide was used as the whitening agent, but in this study, relying on long, successful clinical application, the 15% carbamide peroxide was utilized which released about 5% hydrogen peroxide. Finally, the participants of this study were selected from dental students who strictly adhered to the described bleaching protocol. Furthermore, the whole procedure was completely described for each patient by an expert clinician and the patients try to follow the treatment instructions immediately. The well educated participants used the proper amount of the bleaching agents and removed excess on tray borders to minimize leakage, gingival irritation and swallowing. This was based on previous obtained facts which emphasized that the at-home bleaching procedure should be carried out with high ethical standards and under full professional supervision, in order to decrease potential systemic side effects (5).

There is concern regarding the possible adverse effects of the at-home bleaching agents by swallowing a minute quantity and absorption through the gastrointestinal tract or local absorption through the gingival, especially when they are applied with no dental supervision (21). The probably systemic side effects of hydrogen peroxide are dependent on the amount and the concentration of its compounds ingested. According to the study of Dahl et al. (5), accidental ingestion of 35% hydrogen peroxide has resulted in fatal or near-fatal poisonings. Nevertheless, it is not a major concern as regards carbamide peroxide which yields lower concentration of hydrogen peroxide (2). In addition, Cherry et al. (3) indicated acute toxicological effects of ingested 35% carbamide peroxide in female rats. However, they reported that commercial products containing 10 or 15% carbamide peroxide showed milder symptoms than 35% carbamide peroxide concentration.

Unfortunately, in each of the previous cases that reported toxic, fatal or near fatal effects of hydrogen peroxide, the amount of hydrogen peroxide swallowed was unknown. Considering the dosage and application mode, concerns with potential systemic health risk with the tooth bleaching agents have largely diminished (22). It was reported that the frequency of genetic mutation induced by 10% carbamide peroxide is relatively similar to a physiological saline control (23). Sometimes other ingredients in the bleaching gels such as carbopol or glycerin may be responsible for the poisoning rather than the bleaching agent itself (4). In spite of this, salivary peroxidase as a body enzymatic equipment rapidly decomposes large amount of hydrogen peroxide in the oral cavity (22). It has been determined that the oral cavity is capable of decomposing more than 29 mg of hydrogen peroxide per minute, whereas for a night guard bleaching of both arches using 10% carbamide peroxide, the total hydrogen peroxide exposure dose was estimated to be approximately 3.5 mg (24).

On the other hand, the *in vitro* studies that have frequently emphasized the carcinogenicity, mutagenicity and teratogenicity of H_2O_2 (6-9) could not be reproduced *in vivo* due to inappropriate design, conduct and assessment of the results (24). The overall data on bleaching studies accumulated over the last 20 years also confirmed the results of the present study, which concluded that the use of low concentrations of hydrogen peroxide is still safe (25-28).

In the current study, due to the ability of well-designed trays to make precise contact with the teeth with no gingival exposure, no participant dropout was reported due to gingival irritation or tooth hypersensitivity. Indeed, the gingival irritation which has been reported by the at-home bleaching method that used low concentration of carbamide peroxide in custom made trays is more likely attributed to an ill-fitting tray rather than the bleaching agent itself (29).

Although several *in vitro* studies have reported promising findings about the use of different antioxidant agents for treatment and/or prevention of the oxidative damages caused by hydrogen peroxide (30-32), the present study investigators indicated that the vitamin C supplementation did not significantly reduce the urinary level of the MDA as a marker of oxidative stress following the tooth bleaching procedure with 15% carbamide peroxide (P=0.66), thus the null hypothesis could not reject. Literally, obtained results

from the *in vitro* studies cannot necessarily be extrapolated to the clinical situation. It seems that the amount of antioxidant delivered to cultured cells in the *in vitro* studies was much higher than the level of antioxidant reached in the extracellular fluid after oral administration of 500 mg of ascorbic acid (30-32).
In the clinical setting, the achieved outcomes on protective antioxidant role of vitamin C administration are conflicting. In spite of the study of Harats et al. (33) who determined the effective role of vitamin C against oxidative stress in smokers, two other studies in which the diets of smokers were supplemented with the vitamin C could not significantly reduce plasma TBARS levels (34, 35). These findings are in line with the results of Padayatty et al. (13) who reported that although diet rich in fruits and vegetables is associated with lower risk of cardiovascular disease and cancer, it is not clear if vitamin C contributes to these benefits. In addition, they concluded that the vitamin C treatment in humans could not change oxidation biomarkers or clinical outcomes which completely confirmed the outcome of this study. In the field of vitamin C supplementations, the unique study of Nyyssönen et al. (35) is of particular interest presented a significant increase in plasma TBARS after regular and slow release of vitamin C and the Authors could not find any logical etiology for this phenomenon.
The present study concluded that the night guard tooth bleaching with 15% carbamide peroxide will not induce oxidative stress and systemic side effects if the procedure fully described for each patient is regularly reviewed and monitored. The American Dental Association encourages all patients who are interested in tooth bleaching to completely follow dental professional advices (22). However, due to the importance of the carcinogenicity and relatively limited data available on the topic for tooth bleaching, questions and debates over the systemic risks of bleaching may arise periodically. Thus, further clinical research is encouraged to clarify the controversy and concerns on systemic side effects of hydrogen peroxide as a tooth whitening agent.

Conclusions

Within the limitations of the current study, the Authors could not reveal that the tooth bleaching agents promoted oxidative stress with lipid peroxidation monitoring on MDA biomarker in human urine samples. But the Authors cannot entirely rule out the benefits of the ascorbic acid on tooth bleaching induced oxidative stress.

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Conflict of interest

The Authors of this manuscript clarify that they have no proprietary, financial, or other personal interest of any sort which has been used in this study.

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