

Levels of Selective Oxidative Stress Markers and Antioxidant Enzymes in the Blood of Hubble-Bubble Smokers

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Abstract

Background: Hubble-bubble smoking is a common smoking practice spreading fast among young adults, even in western countries. People think it is less toxic than cigarette smoking.

Aim: The aim of this study was to investigate the levels of oxidative stress markers and antioxidants in the blood of hubble-bubble (HB) smokers compared to controls as a sign of toxicity since disease process is caused by abnormal antioxidant capacity.

Methods: Blood samples were collected by authorized personnel from recruited healthy volunteers (28 habitual HB smokers and 18 nonsmokers). The levels of oxidative stress markers (malondialdehyde (MDA), and protein carbonyl), antioxidants superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and lipids were measured.

Results: The protein carbonyl level in the HB smokers' group (2.58 mmol/gm of protein) was significantly higher than in the control group (2.58 ±0.8 vs. 2.04 ±0.8 mmol/gm of protein), while the CAT level in the HB smokers' group was significantly lower than the control group (22545.4±504 vs 24772.0±344.6 mU/ml). Also, a clear difference in GPx level was found between study groups (1091.6±122 vs. 3144.9± 409mU/ml). However, no significant differences were observed between the two groups regarding the SOD and MDA levels or the concentration of the lipid.

Conclusions: HB smoking is associated with increased levels of carbonyl proteins, decreased levels of catalase, and glutathione peroxidase in the plasma, which may contribute to several adverse health effects associated with HB smoking.

Keywords: Hubble-bubble, antioxidants, smoking, tobacco

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Introduction

Hubble-bubble (HB) smoking, also known as nargileh, argileh, shisha, hookah, goza, oriental pipe, and waterpipe, is a 400-year-old method of tobacco smoking. A small amount

(15–25g) of tobacco is usually placed in a bowl, covered with fenestrated aluminum foil, and topped with a piece of burning charcoal to keep the tobacco burning. With each puff, the HB smoker draws air through the burning tobacco; the smoke passes into a water container via a stem and then enters a hose before finally reaching the mouth [1]. The origin of this method is thought to be India or China [2–4]. In

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most Arab countries, HB smoking has long been common among elderly men living in poor areas, and it was practiced only in cafes [2]. This trend, however, has changed dramatically during the past few years. There was a surge in the use of HB smoking especially in some Arab countries, e.g., Lebanon, Jordan, Egypt, and Syria, as well as in Europe and the United States [2]. HB smoking has become common among men and women of all age groups from all social classes [2, 5]. Several factors have contributed to its resurgence, including the common misbelief that HB smoking is less threatening than cigarette smoking since the smoke initially passes through water that removes harmful constituents; a lack of regulation and law in most countries; the influence of media and false advertising; socio-cultural attitudes; and, personal and interpersonal influences which have made it more socially acceptable than smoking cigarettes, especially among women and young adults [6]. Moreover, the introduction of honeyed tobacco (mu'assel: 30% tobacco, 70% honey), with one or more fruit flavors providing mild smooth aromatic smoke [3, 7], has also added to the increase in HB smoking.

Although HB is mainly prevalent in the Middle East, Arabian Gulf, Turkey, India, Pakistan, Bangladesh, and some regions of China, its use is increasing globally, especially among adolescents and young adults. It has been claimed that one hundred million people worldwide smoke HB daily [2–4]. Several studies have shown an alarming prevalence of HB smoking among school students, especially in the United States, Arabian Gulf, and Lebanon [7–8], as well as among university students, especially in the Arabian Gulf, the United Kingdom, the United States, and some Middle East countries [7, 9–12].

The response has been at both the international and national levels. In 2005, the WHO issued an advisory note stating that HB smoking poses serious health problems to both active and passive smokers and it called for better regulations to reduce the spread of HB smoking, such as prohibiting its use in public places and addressing the misleading labeling of HB tobacco products [13]. In 2007, the American Lung Association called for more research on the health effects of waterpipe use and labeled HB smoking an ‘emerging deadly trend’ that is spreading among young adults (18-to-24-year olds), who seem to be attracted to its sweeter and smoother smoke [12, 14].

In the literature, data tackling HB health hazards is less prevalent compared to that on cigarette smoking. This might be due to the fact that HB smoking is mostly a non-western habit, and many HB smokers are also cigarette co-smokers [2]. Nevertheless, the limited existing scientific data regarding HB smoking suggest pathological consequences for the most part similar to those associated with cigarette smoking, as well as an additional risk factor of infection related to the method of HB smoking [2, 15–16]. It has been shown that HB smoking has acute and chronic effects on cardiac and pulmonary functions [15, 16]. Smoking HB produces a comparable amount of nicotine to cigarette smoking but more carbon monoxide and tar. The carbon monoxide and heavy metals concentrations in HB smoke are higher than in cigarette smoke, and so these subjects inhale more nicotine and heavy metals because of the mode of smoking; this is influenced by the frequency of puffing and the length of the smoking session [16].

To maintain cell survival, cellular antioxidants detoxify reactive oxygen species (ROS) with antioxidant enzymes (AOE) (e.g.,

superoxide dismutase [SOD], catalase, and glutathione peroxidase [GPx]) and a group of low molecular weight compounds including glutathione and nutritionally derived antioxidants such as vitamins and flavonoids [17]. An imbalance in the production of ROS and the endogenous antioxidant capacity leads to a state of 'oxidative stress' that damages DNA, proteins, and lipids by oxidation, impairing cellular function and contributing to the pathogenesis of several diseases such as diabetes mellitus, pulmonary and cardiovascular diseases, cancer, neurodegenerative disorders, and aging [17–18]. Cigarette smoke causes the direct delivery of large amounts of oxidants and free radicals and induces the production of endogenous ROS by inflammatory cells. It contains numerous oxidants, pro-oxidants, carbonyl compounds and free radicals [19].

With each puff of a tobacco cigarette, a huge number of oxidants in both the tar and gas phases can be inhaled [19]. Cigarette smokers often have lower blood levels of antioxidants compared to nonsmokers; however, it is still unclear whether this results from decreased dietary intake of food rich in antioxidants or the depletion of circulating antioxidants through chronic exposure to smoke, or both. Erythrocytes in particular are at a high risk of oxidation due to their function as an oxygen carrier and their lack of repair mechanisms for damaged parts. They depend on antioxidant defensive components, which consist mainly of AOE [20].

Several studies have compared the level of ROS and antioxidant enzymes between smokers and non-smokers. The increased prevalence of HB smoking has led to an increase in research examining its association with chronic conditions and end-organ damage.

It is crucial to address the underlying mechanisms of disease caused by HB smoking, such as oxidative stress [2]. However, there are only a few studies on the role of oxidative stress in HB smoking. In a study performed on healthy Saudi males, it was found that the serum concentration of total antioxidant capacity of HB smokers was significantly lower than that of non-smokers [4].

Since the main constituent of mu'assel is tobacco, it is expected, therefore, that smoking HB should have the same effect regarding AOE and the oxidative markers in the human blood. In order to clarify the above assumption, we undertook the present study to investigate the chronic effect of HB smoking on the level of the three AOE—SOD, CAT, and GPx—and the oxidative markers MDA and CP. Obviously, this study aims to correlate HB smoking with possible health hazards caused by nicotine and its metabolites.

Materials and Methods

All chemicals were purchased from reputable sources; thiobarbituric acid (TBA) came from Riedel-de Haën, HCL from Biosar, 2,4-dinitrophenylhydrazine (DNPH) from Alfa Aesar, guanidium hydrochloride molecular biology grade from Promega, and ethanol-ethyl acetate from GFC Chemicals. SOD, CAT, and GPx activity were measured spectrophotometrically using a microplate reader (Synergy HTX, multi-mode order), from Biotech. The software program used was Gen5, version 2.07.

Selection of subjects

A total of 46 healthy Jordanian individuals ranging in age from 19–43 years were included in this study. Eighteen were non-smokers (aged 22 ± 5.6) and 28 were HB smokers (aged 21.9 ± 3.5). An individual was considered a smoker

if they had smoked tobacco (mu'assel) using only a water-pipe twice a day for at least a year. An individual was considered a non-smoker if they had never smoked tobacco in any form. Subjects with a history of cardiovascular, endocrine, or gastrointestinal disorders or those who smoked cigarettes, cigars, pipes, or any other form of tobacco, in combination with HB, were excluded. All included subjects were not taking any medications or nutritional supplements at the time of the study, had no history of recent acute illness, no clinical evidence suggestive of cardiopulmonary disease or any chronic respiratory disease, and no history of drug or alcohol dependence.

All participants completed a questionnaire to provide data concerning their name, address, age, occupation, education, smoking habits, mu'assel brand, medical history (with an emphasis on the respiratory system), socioeconomic status, education, income, and lifestyle (i.e., food, alcohol, coffee, and vitamin consumption, and exercise level), etc. The study was approved by the institutional review board of the University of Jordan and the University of Jordan Hospital.

All participants were informed about the purpose and protocol of the study and asked to give informed consent. The study was conducted in full accordance with the tenets of the Declaration of Helsinki and conformed to the STROBE (Strengthening the Reporting of Observational Studies in Epidemiology) statement for case-control studies.

Blood Collection

After 12 hours of fasting, a venous blood sample was collected by authorized personnel in an EDTA and plain blood collection tubes under aseptic conditions. EDTA tube samples were centrifuged at 1,000g for ten minutes at

4°C and then plasma was analyzed to determine lipid peroxidation (MDA) and protein carbonyl (CP) levels, to verify if there were any lipid metabolism abnormalities that might affect lipid peroxidation results. Blood serum from plain tubes was used for lipid profile analysis. RBCs were lysed and used for the determination of the AOE levels: SOD, CAT, and GPx.

Malondialdehyde assay

Malondialdehyde (MDA) was measured in plasma using the method described by Nwanjo et al. [21]. In brief, 0.1 ml of plasma was treated with 2 ml of (1:1:1 ratio) TBA-TCA-HCL reagent (TBA 0.37%: 0.25 N HCL: 15% TCA) and placed in a boiling water bath (100°C) for 15 min, cooled and centrifuged at 4,600 rpm for an hour, and then the clear supernatant was measured at 535 nm against reference blank.

Protein carbonyl assay

Protein determination was performed according to a modification of the Biuret method. Protein carbonyl was measured in plasma using the 2,4-dinitrophenylhydrazine (DNPH) spectrophotometric assay method described by Levine et al. [22] and modified by Hernández-Marco et al. [23]. We used 20 µl of plasma and 0.4 ml of 10 mM DNPH. The contents were thoroughly mixed and incubated in a dark room for one hour, and the tubes were shaken every 15 minutes. The plasma was deproteinized with 0.5 ml of 100% trichloroacetic acid, incubated on ice for 15 minutes, and then centrifuged at 12,000 rpm for 10 minutes. The pellets were washed with ethanol/ethyl acetoacetate (1:1 v/v) three times, and solubilized with 1 ml of guanidine 6 M. It was then incubated for 30 minutes at 37°C and monitored spectrophotometrically at 373 nm, with a coefficient of extinction 22 mM⁻¹ cm⁻¹. Results are expressed as nmol/mg protein.

Superoxide dismutase (SOD) assay

SOD activity was assayed using Abcam kits (Cat#: ab65354, UK) according to the manufacturer's instructions. In the SOD assay protocol, superoxide anions are produced by the action of xanthine oxidase. SOD catalyzes the dismutation of the superoxide anion into hydrogen peroxide and O₂, and then superoxide anions act on WST-1 to produce a water-soluble formazan dye detectable by the increase in absorbance at 450 nm. The greater the activity of SOD in the sample, the less formazan dye is produced. SOD activity results were expressed as a % of the inhibition rate.

Catalase (CAT) assay

CAT activity was assayed using Abcam kits (Cat#: ab83464, UK) according to the manufacturer's instructions. In the catalase activity assay protocol, the catalase present in the sample reacts with hydrogen peroxide (H₂O₂) to produce water and oxygen. The unconverted H₂O₂ reacts with a probe to produce a product measurable by colorimetric analysis at optical density (OD) 570 nm. Therefore, the catalase activity present in the sample is inversely proportional to the signal obtained.

Glutathione peroxidase assay (GPx)

GPx activity was determined using Abcam kits (Cat#: ab102530, UK) according to the manufacturer's instructions. In the glutathione peroxidase assay protocol, glutathione peroxidase (GPx) oxidizes GSH to produce GSSG as part of the reaction in which it reduces cumene hydroperoxide. Glutathione reductase (GR) then reduces the GSSG to produce GSH, and in the same reaction consumes NADPH. The decrease of NADPH (measured at OD 340

nm) is proportional to GPx activity.

Statistical Analysis

Data were statistically analyzed using the unpaired Student's t-test and differences were considered significant whenever the *p-value* was <0.05. Data in the table are expressed as mean ± standard deviation. In the figure, data are expressed as percentage ± SEM vs. controls.

Results

The study group is composed of 28 (19 males and nine females) volunteers who had smoked HB at least twice weekly for one year. The mean age of the smokers was 21.9 ± 3.5 years (range 18–34 years). Recruited subjects reported smoking for about 1–14 years (average = 4.3 years). The control group had 18 (8 males and 10 females) healthy volunteers who had never smoked tobacco of any form. The mean age of the control group was 22 ± 5.6 years (range 19–43 years).

No statistical differences were noted regarding gender.

As seen in Figure 1 and Table 1, the plasma level of protein oxidative product (carbonyl proteins) in the smokers' group was significantly higher than in the control group (125 ± 6 vs. 100 ± 9%). However, no significant difference was observed in the lipid oxidative product MDA between the two groups. The anti-oxidative enzymes (CAT and GPX) in HB smokers were significantly lower than in the control group (91% ± 1 and 34% ± 2 lower than their control counterparts, respectively, Fig. 1 and Table 1). No significant differences were observed between the study groups regarding the SOD enzyme as well as cholesterol, HDL, LDL, and triglyceride levels.

Table 1: Biomarker comparison of oxidative stress and antioxidants in HB smokers v. controls

	Hubble-bubble smokers n = 28	Control group n = 18	Student's <i>t</i> -test <i>p</i> value
Carbonyl Proteins	2.58 ± 0.8 nanomole/gm of protein	2.04 ± 0.8 nanomole/gm of protein	< 0.05
MDA	3.04 ± 0.5	2.99 ± 0.6	> 0.05
Catalase	22545.4±504 mU/ml	24772.0±344.6 mU/ml	< 0.01
GPx	1091.6±122 mU/ml	3144.9± 409mU/ml	< 0.001
SOD (inhibition rate %)	92.7 ± 16.6	87.7 ± 20.7	> 0.05
Cholesterol	175.51 ± 44.63	164.60 ± 33.72	> 0.05
HDL	51.97 ± 11.91	51.99 ± 14.29	> 0.05
LDL	106.54 ± 37.64	101.40 ± 31.30	> 0.05
Triglyceride	82.55 ± 36.91	83.13 ± 34.60	> 0.05

GPx: glutathione peroxidase; MDA: malonyl-dialdehyde; SOD: superoxide dismutase; HDL: high density lipoproteins; LDL: low density lipoproteins. Data shown in table expressed as mean ± SD for HB smokers and non-smokers. The last column shows the *p* value for statistical analysis between the two groups. Data expressed as mean ± standard deviation.

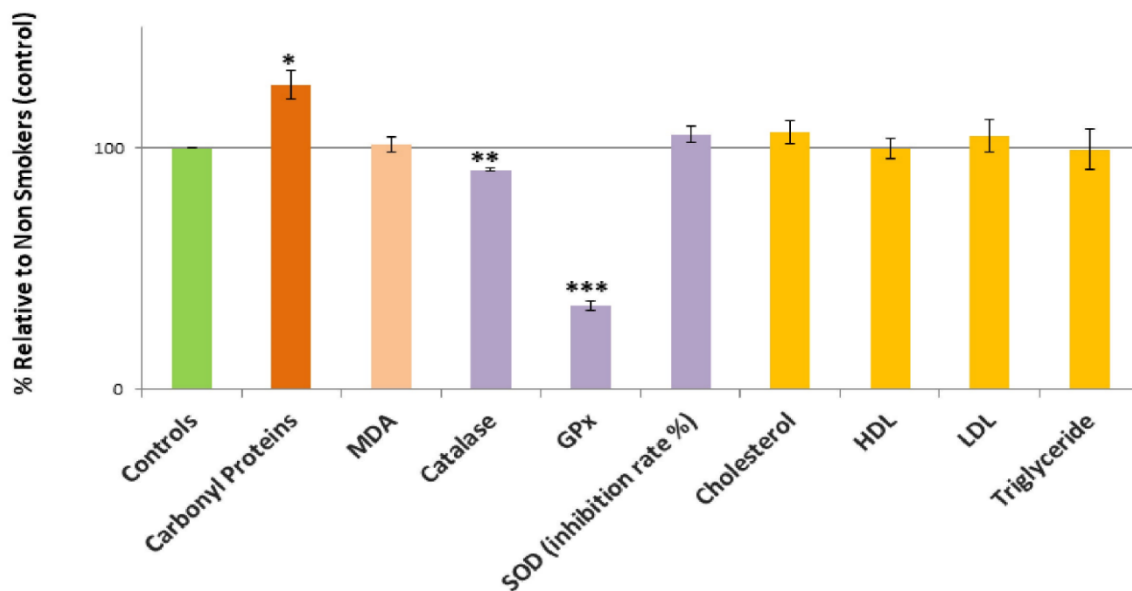


Figure 1: Relative levels of oxidative markers, selective antioxidant enzymes, and lipids in HB smokers (as percentage) vs their levels in non-smokers. *, **, * show the statistical significance when $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. Data expressed as percentage ± SEM vs. controls.**

Discussion

Endogenous and/or exogenous reactive oxygen species perturb cellular functions by causing oxidative modifications of cellular components such as carbohydrates, lipids, proteins and DNA. Age-related development of diseases has been explained by the creation of conditions that lead to oxidative stress, which, in turn, reduces the functional performance of cells as well as their regeneration [24]. In addition, various studies have suggested a strong potential association between HB smoking and the development of pathological conditions that can lead to CV diseases, metabolic disorders, diabetes, lung, oral and bladder cancers, respiratory illnesses, and adverse pregnancy outcomes [25–26]. These can be explained by the inhalation of exogenous ROS during smoking, which results in macromolecular damage by inducing oxidative stress and the development of inflammation and cancers [27]. In this study, we assessed the level of markers for protein carbonylation, lipid peroxidation, and the activity of AOE as potential targets for exogenous reactive species induced by HB smoking.

Measurement of carbonyl proteins (CP) is often used as a biomarker for protein modification in biological samples that may induce oxidative stress. High protein levels in serum and the stability of the compounds are advantages for early detection and application as indicators for oxidative stress [26]. Other studies have reported elevated levels of CP in the plasma and tissues in humans with aging and neurodegenerative diseases [28] and correlated the increase with the development of coronary diseases [29], renal disease [30], and chronic myeloid leukemia [31]. In vitro studies have also shown increased protein carbonylation by exposing bronchial epithelial

and endothelial cells to extracts from cigarette smoke [32].

Our study showed a significant increase in the plasma level of CP among HB smokers. This suggests that carbonyl proteins may be a possible mechanism for HB smoking-induced toxicity and the development of related diseases [33].

MDA is considered the most mutagenic product which yields as a secondary product from lipid peroxidized radicals and can be estimated in biological samples after reaction with thiobarbituric acid (TBA). Some studies showed increased MDA in the plasma of cigarette and HB smokers [17–18]. In animal models, a high correlation was observed after exposure to smoke and increased serum level of MDA [30]. Our analysis showed no significant difference in MDA between HB smokers and non-smokers.

As observed in our study, it is possible that oxidative stress in the serum of HB smokers is more related to protein oxidation rather than lipid peroxidation.

AOE can scavenge free radicals and prevent cellular oxidative damage by enzymatic mechanisms. In our study, we examined the main AOE: superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). CAT and GPx levels were significantly lower in the HB smokers' group compared to the non-smokers. However, there was no significant difference between the two groups regarding the level of SOD. Previous work has shown that smoking and its oxidative products did not affect the level of AOE. The same study also reported a decrease mainly in GPx and SOD activities in cigarette smokers [34].

Regarding the effect of HB smoking on the level of AOE, there are limited studies on this subject. Reduced SOD activity has been

reported with acute exposure of experimental animals to water-pipe smoke, while chronic exposure has also reduced CAT and GPx levels [34]. In a study performed on healthy Saudi males, the total antioxidant capacity was significantly lower in HB smokers than in non-smokers [4].

Despite a lack of supporting scientific evidence, people consider HB smoking an innocent habit and, therefore, there has been a global increase in its use. This leads to a false sense of security among HB smokers. Many cigarette smokers hope to benefit when they completely switch to HB, although there is not enough evidence to support the notion that HB smoking is an effective way of quitting cigarette smoking. The literature is full of studies showing that HBS is unsafe even among those who do not use another type of tobacco [6].

Conclusion

Hubble-bubble smoking has been shown in our

study to produce oxidative stress (high serum carbonyl proteins) and an impaired oxidant defense system (low catalase and glutathione peroxidase concentrations), which might predispose such smokers to a variety of diseases similar to those encountered in cigarette smokers. This calls for better regulation and raised awareness about the dangers of this habit.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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قياس مستويات بعض مؤشرات الاجهاد التأكسدي والانزيمات المضادة للأكسدة في الدم عند مدخني الأرجيلة

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الملخص

الهدف: يهدف البحث إلى مقارنة مستويات مؤشرات الإجهاد التأكسدي ومضادات الأكسدة في الدم (البلازما) عند كل من مدخني الأرجيلة وغير المدخنين

المواد والطرائق: تطوع لتطبيق الدراسة 28 شخصاً من مدخني الأرجيلة و 18 متطوعاً من غير المدخنين كمجموعة ضابطة. تم قياس تراكيز مؤشرات الإجهاد التأكسدي الآتية : كاربوتيل البروتين ومالون داي الدهايد (MDA) كما تم قياس الانزيمات المضادة للأكسدة الآتية : سوبر أكسيد ديسميوتيز (SOD) والكاتاليز (CAT) والجلوتاثيون بيروكسيديز (GPx)

النتائج: كان مستوى الكاربونيل بروتين عند مجموعة مدخني الأرجيلة (0.8 ± 2.58 ميلي مول/غم من البروتين) أعلى وبدلالة احصائية مقارنة مع المجموعة الضابطة (0.8 ± 2.04 ميلي مول/غم من البروتين، $P < 0.05$)، ولكن لم يكن هناك فرق واضح بين المجموعتين فيما يتعلق بمستويات الـ MDA. ولوحظ أن مستويات الـ CAT عند مدخني الأرجيلة كانت أقل وبدلالة احصائية مقارنة مع تلك عن المجموعة الضابطة (504 ± 22545.4 mU/ml و 344.6 ± 24772.0 mU/ml على التوالي، $P < 0.01$). كما كانت مستويات GPx عند مدخني الأرجيلة أقل وبدلالة احصائية من تلك عند المجموعة الضابطة (122 ± 1091.6 mU/ml و 409 ± 3144.9 mU/ml على التوالي، $P < 0.001$) ولكن لم يكن هناك فرق واضح بين المجموعتين فيما يتعلق بمستويات SOD

خاتمة: يرتبط تدخين الأرجيلة بزيادة مستويات الكربونيل بروتين وانخفاض مستويات الكاتاليز والجلوتاثيون بيروكسيديز في البلازما، مما قد يساهم في العديد من الآثار الصحية الضارة المرتبطة بتدخين الأرجيلة.

الكلمات الدالة: الأرجيلة، مضادات الأكسدة، التدخين، التبغ.