

Effects of smokeless dipping tobacco (*Naswar*) consumption on antioxidant enzymes and lipid profile in its users

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Abstract: Dipping tobacco, traditionally referred to as moist snuff, is a type of finely ground, moistened smokeless tobacco product. *Naswar* is stuffed in the floor of the mouth under the lower lip, or inside the cheek, for extended periods of time. Tobacco use causes dyslipidemia and also induces oxidative stress, leading to alteration in levels of antioxidant enzymes. Dyslipidemia and oxidative stress in turn play a vital role in the development of cardiovascular disease (CVD). Studies conducted on smokeless tobacco products reveal contradictory findings regarding its effects on lipid profile and antioxidant enzymes. As use of *Naswar* is quite common in Pakistan, the current study aimed to evaluate levels of the antioxidant enzymes viz glutathione per oxidase (GPx) and super oxide dismutase (SOD), alongside lipid profile parameters such as total cholesterol, triglycerides, High density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C) to assess the risk of adverse cardiovascular events in *Naswar* users. 90 Healthy males aged 16-43 years, who consumed *Naswar* daily, were selected for the study, alongside 68 age-matched non-tobacco users as controls. Both GPx and SOD levels as well as serum HDL-C were significantly reduced ($P < 0.01$) in *Naswar* users, whereas serum total cholesterol, LDL-C, triglycerides and LDL-C/HDL-C ratio were significantly increased ($P < 0.01$) in *Naswar* consumers compared to controls. Our findings indicate deleterious effects of *Naswar* usage on health by causing altered lipid profile and antioxidant enzymes thereby placing its consumers at an increased risk of cardiovascular disease.

Keywords: Smokeless dipping tobacco; *Naswar*; glutathione per oxidase; super oxide dismutase; lipid profile.

INTRODUCTION

Naswar is a dipping type of smokeless tobacco product (STP) commonly used in Pakistan, Afghanistan, Iran and South Africa and is made from tobacco belonging to *Nicotina rustica* species. STP is used without combustion and this eliminates the danger of direct exposure of toxic combustion compounds to the lung and other tissues of the user and the people around. Use of STP may result in other health hazards, local or systemic according to the content of various toxic products, including nicotine and tobacco-specific nitrosamines (Thomsen, 2008). The tobacco leaves are sun-dried, finely ground and then mixed with calcium oxide and wood ash. Flavorings such as cardamom, menthol and coloring agents are also added. Packed in small polythene bags, the size of tea bags, a pinch of *Naswar* (about 1g) is consumed by placing it in the mouth cavity, usually between the oral mucosa and gingival cavity or sometimes under the tongue. After about half an hour it is then spat out (Zakiullah *et al.*, 2011).

Naswar usage has been linked with oral cavity and esophageal cancers (Saleem *et al.*, 2013). It has also been reported to contain an average nicotine content of 14.667mg/g (Zakiullah *et al.*, 2011) which is the principal addictive substance in tobacco. Nicotine has sympathomimetic effects and thus causes the release of

catecholamines resulting in increased heart rate and cardiac contractility, vasoconstriction and a transient rise in blood pressure. Nicotine can also cause insulin resistance and endothelial dysfunction. All of these effects of nicotine on cardiovascular system could potentially lead to the development of atherosclerosis which is well documented in cigarette smokers (Benowitz, 2010) who reportedly suffer from an atherogenic lipid profile, particularly decreased High Density Lipoprotein Cholesterol (HDL-C), resulting in an enhanced risk of cardiovascular disease (CVD) (He *et al.*, 2013). Several studies indicate that smokers tend to have increased levels of oxidized Low Density Lipoprotein-Cholesterol (LDL-C), which are incorporated in foam cells (via macrophages) leading to the formation of an atherosclerotic plaque (Benowitz and Gourlay, 1997).

Atherosclerosis is associated with free radical damage and the oxidative modification of LDL that in turn involves lipid per oxidation (Adak and Nazri, 2007). Thus, lipid per oxidation products may in turn disrupt lipid and protein metabolism, causing changes in the cell membrane and alteration in enzyme function (Pioruńska-stolzmann *et al.*, 1999). Smokeless tobacco generates highly reactive electrophiles due to the presence of nitrosamines, potentially resulting in the formation of free radicals and development of oxidative stress as compared to pure nicotine (Yildiz *et al.*, 1999). The generation of free radicals leads to DNA damage, increased lipid per oxidation and alterations in the levels of antioxidant

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enzymes such as glutathione per oxidase (GPx), super oxide dismutase (SOD) and catalase (CAT) (Naga Sirisha and Manohar, 2013). These antioxidant enzymes comprise the first line of defense against any free radical damage (Shrestha *et al.*, 2012). Glutathione with its redox enzymes, functions by detoxifying free radicals via GPx. SOD converts super oxide radicals into hydrogen peroxide (H₂O₂) which in turn is converted to water by GPx and catalase (Pasupathi *et al.*, 2009).

Several studies show conflicting results related to the effect of smokeless tobacco use on lipid profile. In a study conducted on 2840 adult smokeless tobacco users, 2.5 times risk of hypercholesterolemia was reported when compared to non-users (Tucker, 1989). On the other hand there are studies conducted mainly on Swedish snuff that have shown no difference in the levels of total cholesterol, HDL-C, LDL-C, triglycerides and other lipid fractions between snuff users and nontobacco users (Siegel *et al.*, 1992; Wallenfeldt *et al.*, 2001).

Therefore, this study is designed to evaluate lipid profile parameters and levels of antioxidant enzymes in *Naswar* consumers in order to ascertain the risk of development of cardiovascular disease in relation to *Naswar* consumption.

MATERIAL AND METHODS

Place of the study

The present study was conducted at the Department of Biochemistry, University of Karachi from January 2014 till October 2014. Ethical approval was obtained from the institutional ethical committee at the University of Karachi.

Subjects

Healthy male subjects aged 16-43 years were recruited for this study, following written, informed consent. Subjects were divided into a control group comprising 68 healthy subjects with no history of tobacco use in any form, with age matched test group comprising 90 exclusive *Naswar* users who were consuming it for duration of 1-25 years. Subjects with history of tobacco usage other than *Naswar* were excluded from the study.

Additionally, subjects suffering from diabetes mellitus, obesity, cardiovascular, pulmonary or renal disorders or taking any antioxidant vitamin supplements were also excluded from the study.

Methods

3 ml blood was drawn following overnight 12 hrs fast. Blood was centrifuged at 3000 rpm and serum isolated and frozen at -20°C until lipid profile parameters analysis. Whole blood was also collected in lithium heparin tubes for estimation of GPx and SOD.

Sample preparation

After centrifuging whole blood for 10 minutes at 3000 rpm plasma was aspirated. Erythrocytes were then washed with 0.9% sodium chloride solution followed by centrifugation for 10 minutes at 3000 rpm. This was repeated four times. The erythrocyte pellet was re-suspended in cold redistilled water and incubated for 15 minutes at 4°C. To keep the % inhibition between 30% and 60%, 25 fold dilution of the resultant lysate with 0.01M Phosphate Buffer (pH 7.0) was carried out.

Estimation of super oxide dismutase

SOD was assayed using the Ransod kit (Randox Laboratories, Crumlin and Antrim, UK). Its basic principle refers to the method established by McCord and Fridovich (McCord and Fridovich, 1969). Manufacturer's instructions were followed and readings were taken using Rx Monza, semi-automated analyzer.

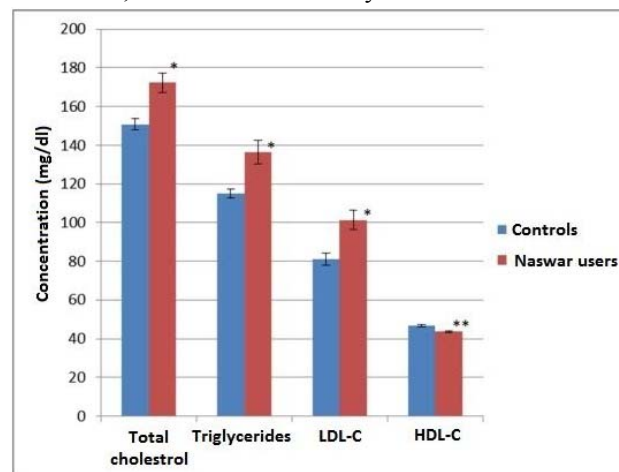


Fig. 1: Effect of *Naswar* usage on lipid profile. Experimental details are given in material and methods section. Values are given as mean± SEM. Statistical analysis was performed using student's t-test. The significance of the difference is indicated by *P<0.01 and **p<0.001 from controls. LDL-C =Low Density Lipoprotein-Cholesterol, HDL-C =High Density Lipoprotein-Cholesterol.

Estimation of glutathione per oxidase

GPx assay was performed using Ransel GPx kit that employs the method based on that of Paglia and Valentine (1967). Reduced Glutathione (GSH) is oxidized by cumenehydro peroxide in the presence of GPx. The oxidized glutathione (GSSG) is then reduced by NADPH in the presence of glutathione reductase. The decrease in absorbance at 340nm was measured. Readings were taken on Rx Monza, semi-automated analyzer.

Estimation of lipid profile parameters

Serum total cholesterol, triglycerides and HDL-C were measured using Randox kits. Friedewald formula was used to calculate serum LDL-C.

$$\text{LDL-C} = \text{Total cholesterol} - \text{Triglycerides} - \text{HDL-C} \quad 5$$

STATISTICAL ANALYSIS

The data was analyzed by statistical programme (SPSS) using student's independent samples *t*-test. $P < 0.05$ was taken as statistically significant.

RESULTS

Naswar users mean age was 27.71 ± 0.84 and controls subject mean age was 25.74 ± 0.68 . Figs. 1 and 2 show that serum triglycerides, total cholesterol, LDL-C levels as well as the LDL-C to HDL-C ratio were significantly elevated ($P < 0.01$) in *Naswar* consumers compared to the control group. However, serum HDL-C cholesterol was significantly decreased ($P < 0.001$) in *Naswar* users compared to the control group. Additionally, levels of glutathione per oxidase and super oxide dismutase were significantly decreased ($P < 0.01$) in *Naswar* users compared to the control group, indicating that *Naswar* users are in a state of oxidative stress (figs. 3 and 4).

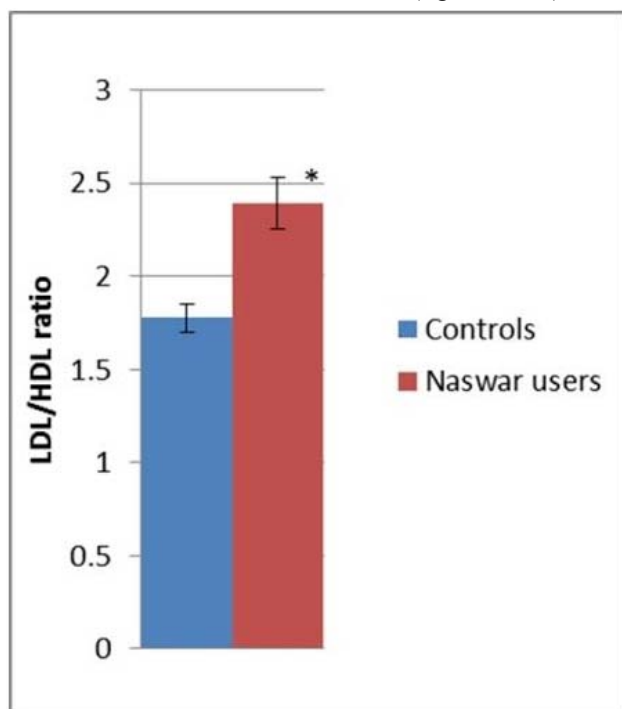


Fig. 2: LDL / HDL ratio in controls versus *Naswar* users. Experimental details are given in material and methods section. Values are given as mean \pm SEM. Statistical analysis was performed using student's *t*-test. The significance of the difference is indicated by $*P < 0.001$ from controls. LDL-C=Low Density Lipoprotein Cholesterol, HDL-C=High Density Lipoprotein Cholesterol.

DISCUSSION

In the present study we determined the levels of the antioxidant enzymes GPx and SOD, as well as the lipid profile to ascertain the risk of CVD in *Naswar* users.

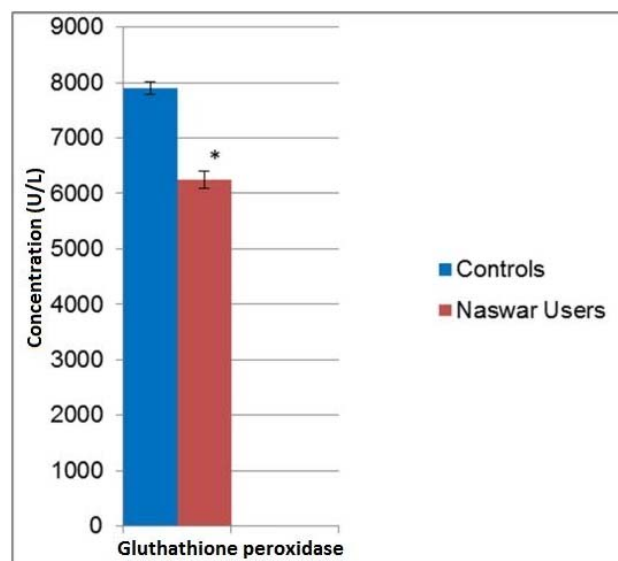


Fig. 3: Effect of *Naswar* usage on glutathione per oxidase. Experimental details are given in material and methods section. Statistical analysis was performed using student's *t*-test Values are given as mean \pm SEM for controls ($n=48$) versus *Naswar* users ($n=72$). The significance of the difference is indicated by $*P < 0.001$ from controls.

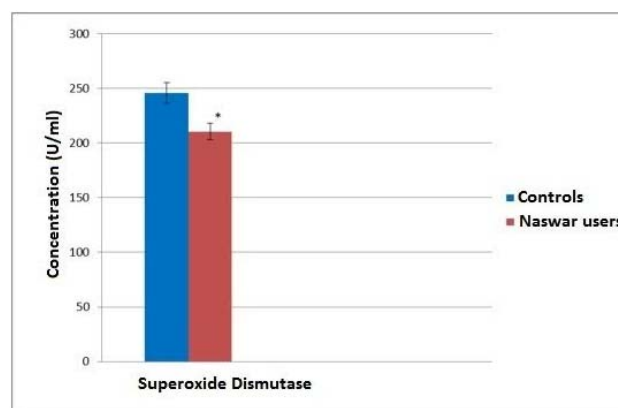


Fig. 4: Effect of *Naswar* usage on super oxide dismutase. Experimental details are given in material and methods section. Statistical analysis was performed using student's *t*-test Values are given as mean \pm SEM for controls ($n=34$) versus *Naswar* users ($n=50$). The significance of the difference is indicated by $*P < 0.01$ from controls.

We found decreased levels of super oxide dismutase and glutathione per oxidase in *Naswar* consumers compared to controls. It reportedly contains toxic levels of arsenic, lead, cadmium, and nitrates, which could potentially be converted to nitrites. The presence of nitrates, nitrites, a high nicotine content and an average alkaline pH of 8.5, all potentially favor the formation of tobacco specific nitrosamines (Zakiullah *et al.*, 2011). TSNA's are group of carcinogens (Stepanov *et al.*, 2008) that contribute to the occurrence of oral cavity, esophageal and lung cancers in tobacco users (Hecht and Hoffmann, 1988) and also lead

to oxidative stress in the human body, prolonged exposure to which could lead to antioxidant depletion (Kilinc *et al.*, 2004). GPx and SOD are antioxidant enzymes that protect the cell against oxidative damages. Our observations indicating decreased SOD and GPx levels indicate that *Naswar* usage may result in oxidative stress, leading to consumption of antioxidants and thereby placing *Naswar* consumers at an enhanced risk of oral cavity and gastrointestinal tract cancers as several studies have confirmed that tobacco leaves contain high As and Cd concentrations (Zakiullah *et al.* 2011; Borgerding *et al.*, 2012; Khilifi *et al.*, 2014) atherosclerosis and numerous other systemic disorders. No study thus far has examined the effect of *Naswar* usage on antioxidant enzymes. However, a Turkish study on Maras powder (a smokeless tobacco product) reported an increased state of oxidative stress via raised levels of malondialdehyde and reduction in the levels of SOD and Catalase its users (Kilinc *et al.*, 2004). Another study conducted on tobacco chewers has also reported oxidative stress and decreased concentrations of antioxidant enzymes in its users (Samal *et al.*, 2006).

The present study also shows increased cholesterol, triglycerides and LDL-C concentrations as well as an increased LDL-C/ HDL-C ratio, but a decreased level of HDL-C in the serum of *Naswar* users compared to controls. These findings may be attributed to the sympathomimetic effects of nicotine. Nicotine stimulates the sympathetic nervous system, thereby causing circulatory catecholamine release raising levels of free fatty acids which in turn stimulate the secretion of very low density lipoprotein cholesterol (VLDL-C) from the liver and therefore of triglycerides. Free fatty acids have also been found to stimulate synthesis and secretion of cholesterol by the liver (Benowitz and Gourlay, 1997). Indeed, an increased LDL-C/HDL-C ratio is closely linked to the development of CVD (Adak 2010) Triglyceride-rich lipoproteins are most important for the progression of early atherosclerotic plaques, while cholesterol-rich lipoproteins predominantly affect the late atherosclerotic process (Pioruńska-stolzmans *et al.*, 1999) To the best of our knowledge this is the first study assessing the impact of *Naswar* on lipid profile in its users. However, there are studies on other smokeless tobacco products such as gutka (Purushottama Dass *et al.*, 2013) and Maras powder (Bozkus *et al.*, 2014) in which increased levels of total cholesterol, LDL-C and triglycerides and decreased levels of HDL-C have been reported in smokeless tobacco users as compared to non-users.

Although, the levels of total cholesterol, triglycerides, HDL-C and LDL-C in *Naswar* users are not in the range to cause dyslipidemia but are significantly increased as compared to controls, in addition decreases in levels of antioxidant enzymes could increase the risk for development of CVD. Furthermore studies conducted in

the western population which are mostly on Swedish snuff have reported a mixed picture with one showing no risk (Wennberg *et al.*, 2007) and the other an increased risk for adverse cardiovascular events such as myocardial infarction, stroke and ischemic heart disease, in snuff users compared to non-users (Hergens *et al.*, 2007).

CONCLUSION

It can therefore be concluded that in a developing country such as Pakistan, that witnesses a high consumption of *Naswar*, alteration in the lipid profile and antioxidant enzymes, comprise risk factors for the development of cardiovascular disease, could signify a large public health problem.

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