Available online at www.ijpcr.com
International Journal of Pharmaceutical and Clinical Research 2016; 8(6): 578-582

ISSN- 0975 1556

Research Article

Cigarette Smoking Provoked Proinflammatory Cytokines and Oxidative Stress in Healthy Smokers

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Available Online: 8th June, 2016

ABSTRACT
Cigarette smoking is a major risk factor in the development of various lung diseases. The mechanisms of these diseases include the inappropriate production of inflammatory cytokines and generation various reactive oxygen species (ROS) and reactive nitrogen species (RNS) that can be inactivate endogenous antioxidants mechanisms. This study aimed to evaluate the effect of cigarette smoking on proinflammatory cytokines and antioxidant enzyme activities related to oxidative stress in apparently healthy smokers. The study included 45 apparently healthy smokers divided into mild, moderate and heavy smokers according the smoking index and 40 healthy non smoker males served as control group Inflammatory markers, interleukin-6 (IL-6), tissue necrosis factor-α (TNF-α), antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) as well as total homocysteine (tHcy), malondialdehyde (MDA), protein carbonyl content (PCC), were measured in plasma of all participants. The results revealed significant difference between smokers as compared with nonsmokers in levels of tHcy, MDA, PCC, IL-6, TNF-α, Vit C, GSH, CAT and SOD and significant difference among mild, moderate and heavy smokers. These findings insist that smoking leads to increase free radical load and relatively low antioxidant status which results in an imbalance between oxidant/antioxidant status, reinforcing recommendations concerning the benefits of smoking cessation.

Keywords: Cigarette smoking, smoking index, oxidative stress, inflammation.

INTRODUCTION

Cigarette smoking is considered one of the most important risk factors for the development of coronary and pulmonary diseases as well as increased risk of cancer[1]. Many epidemiological studies have attempted to explain the mechanisms that associated with cigarette smoking; a basic hypothesis is that free radicals may be the most critical factors triggering antioxidant depletion, lipid peroxidation, and protein modification. Cigarette smoke constitutes about 5000 compounds and 10^{17} free radicals per puff, many of which are able to induce reactive oxygen or nitrogen species that can be inactivate endogenous antioxidants enzymes like superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH) as well as ascorbate, the redox form of vitamin C which considered an important physiological antioxidant, so the oxidant/antioxidant balance of smokers becomes disturbed[2]. The excess superoxide that forms in response to exposure to cigarette smoke may be one risk factors for dangerous hazards of smoking, it generates as a result of oxidative stress to the potential thiol self-oxidation of highly reactive sulfhydryl (-SH) group of homocysteine[3]. Products arising from lipid peroxidation as malondialdehyde (MDA) can in turn react with cigarette smoke constituents, creating additional toxic products[4]. Carbonyl groups may be introduced into proteins by reactions with malondialdehyde. Introduction of carbonyl groups into amino acid residues is the most general byproduct of oxidative damage of proteins, among a wide variety of protein oxidations, and quantification, carbonyl content is generally used to estimate total protein oxidation. Thus, exposure to cigarette smoking, leads to an increase in the level of oxidized protein[5]. Several studies have reported increase some cytokines especially interleukin-6 (IL-6) and tissue necrosis factors-α (TNF-α) as results of increased oxidative damage in apparently healthy individuals[6]. The toxic products resulting from both the direct and secondary reactions with cigarette smoke are thought to activate inflammatory immune responses, which may themselves be altered by cigarette smoke constituents and play an influential role in smoking-related oxidative tissue damage[7]. The aim of this study was designed to evaluate the oxidant/antioxidant status [total homocysteine (tHcy), MDA, protein carbonyl content (PCC), and antioxidants; (GSH), vitamin C, CAT and SOD] and inflammatory markers [(IL-6) and tissue necrotic factors-α (TNF-α)] in plasma of healthy smokers and nonsmokers Egyptian males.

SUBJECTS AND METHODS

Selection of Subjects

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Table 1: Clinical characteristics of the study groups.

Characteristics	Nonsmokers	Smokers	Smokers		
			mild	moderate	heavy
Number	40	45	13	17	15
Age (years)	33 ± 3	35 ± 4	30 ± 5	33 ± 4	36 ± 4
Systolic blood pressure (mmHg)	122 ± 7	141 ± 6^{A}	129 ± 5	132 ± 6	149 ± 5^{A,B,C}
Diastolic blood pressure (mmHg)	73 ± 5	93 ± 4^{A}	77 ± 4	79 ± 5	95 ± 4^{A,B,C}
Body mass index (kg/m^2)	26.8 ± 2.4	21.8 ± 2.3^{A}	24.0 ± 2.1	23.1 ± 1.4	20.2 ± 1.2^{A,B,C}
Smoking status	-	-	12	17	25^{B,C}
Smoking index (pack- year)	-	-	< 200	200-400	> 400^{A,B,C}

A = *$p<0.05$versus nonsmokers group; B = *$p<0.05$versus mild smokers group; C = *$p<0.05$versus moderate smokers group

Table 2: lipid profile in nonsmokers and smokers.

Parameters	Nonsmokers	Smokers	Smokers		
			mild	Moderate	heavy
TG (mg/dl)	81.3 ± 9.4	181.3 ± 11.6^{A}	152.8 ± 12.2	187.7 ± 11.1	228.2 ± 16.3^{A,B,C}
T-cholesterol (mg/dl)	159.5 ± 13.5	268.7 ± 19.8^{A}	220.5 ± 18.4	270 ± 21.7	321.7 ± 24.8^{A,B,C}
HDL-C (mg/dl)	53.7 ± 4.7	41.8 ± 6.6^{A}	45.2 ± 3.5	43.6 ± 2.6	37.7 ± 2.5^{A,B,C}
LDL-C (mg/dl)	84.4 ± 7.2	164.8 ± 9.4^{A}	121.7 ± 10.8	153.7 ± 11.4	189.8 ± 12.5^{A,B,C}

Plasma tHcy, MDA and PCC levels in healthy nonsmokers and smokers.

Parameters	Nonsmokers	Smokers	Smokers		
			mild	Moderate	Heavy
tHcy (µmol/L)	8.24 ± 1.2	17.2 ± 2.3^{A}	12.4 ± 1.7	14.7 ± 2.2	18.3 ± 2.5^{A,B,C}
MDA (nmol/mg protein)	1.16 ± 0.08	3.91 ± 0.17^{A}	0.32 ± 0.04	2.37 ± 0.12	3.89 ± 0.18^{A,B,C}
PCC (nmol/mg protein)	0.23 ± 0.01	0.52 ± 0.03^{A}	0.31 ± 0.07	0.39 ± 0.05	0.54 ± 0.04^{A,B,C}
Parameters	Nonsmokers	Smokers	Smokers		
			mild	Moderate	heavy
IL-6 (ng/mL)	0.71 ± 0.02	1.65 ± 0.05^{A}	0.83 ± 0.03	1.14 ± 0.07	1.72 ± 0.06^{A,B,C}
TNF-α (ng/mL)	1.04 ± 0.01	2.13 ± 0.08^{A}	1.15 ± 0.04	1.73 ± 0.06	2.21 ± 0.07^{A,B,C}

Forty five smoker males aged 20 to 40 years were randomly selected from out patient's clinic, National Research Centre Giza, Egypt and 40 males served as controls. The smokers were classified into 3 groups according to duration and number of cigarette of smoking into mild, moderate and heavy smokers. Through clinical examination was performed, cases had diabetes mellitus or any other metabolic disorders as well as cases taking drugs were excluded. Anthropometric measurements including weight (kg) and height (cm); for each participants were performed, body mass index (BMI); as weight (kg) divided by height (m)2; was calculated, following the recommendations of the International Biological program[8]. Blood pressure was measured with a standard mercury sphygmomanometer. A written informed consent was taken from all the participants prior to enrolment into the study. The protocol of this study was approved by the "Ethical Committee" of the "National Research Centre".

Blood samples

Venous blood samples were withdrawn from all participants after 14 hours overnight fasting in heparinized tubes. The samples were centrifuged at 3000 rpm for 10 minutes; plasma was separated and kept at -80ºC until estimation of the different biochemical parameters.

Biochemical assays

Lipid profile

Total cholesterol[9], triglycerides[10], HDL-cholesterol[11] were measured using commercially available kits provided by Biocon Diagnostic, Germany. LDL-cholesterol was calculated according to equation developed by Friedewald, 1972[12]. LDL- C= total cholesterol- (HDL-C + triglyceride/5)

IL-6 and TNF-α

They were measured by a commercially available enzyme-linked immunosorbent assay (ELISA) (R&D Systems, UK) according to the manufacturer's specifications.

Total homocycteine (tHcy)

tHcy was estimated by high performance liquid chromatography (HPLC) system, Agilent technologies 1100 series, equipped with a quaternary pump (Quat. pump, G131A model).

Malondialdehyde (MDA) assay

MDA as a marker of lipid peroxidation products was determined by measuring thiobarbituric acid reactive substances (TBARS) content in plasma according to the method of Wong et al. 1987[13]

Protein carbonyl content (PCC) assay

PCC levels were measured according to method described by Levine et al. 1990[14].

Glutathione assay

GSH was determined according to the method of Ellman, 1959[15].

Vitamin C assay

Table 3: Levels of Non-Enzymatic Antioxidants Glutathione and Vitamin C in Plasma from Nonsmokers and Smokers.

Parameters	Nonsmokers	Smokers	Smokers		
			mild	moderate	Heavy
GSH (nmol/mg protein)	33.7 ± 1.21	21.9 ± 2.40 [A]	29.2 ± 2.10	25.6 ± 1.23	20.7 ± 1.10 [A,B,C]
Vitamin C (mg/L)	5.87 ± 0.14	3.11 ± 0.11 [A]	3.41 ± 0.17	2.65 ± 0.13	2.11 ± 0.11[A,B,C]

Table 4: Levels of antioxidants enzymes in nonsmokers and smokers subjects.

Parameters	Nonsmokers	Smokers	Smokers		
			mild	moderate	Heavy
CAT	8.82 ± 0.31	4.62 ± 0.14 [A]	6.31 ± 0.15	5.20 ± 0.13	4.22 ± 0.17[A,B,C]
SOD	35.61 ± 2.26	22.34 ± 1.30 [A]	30.21 ± 1.62	25.72 ± 1.43	21.60 ± 1.11[A,B,C]

Vitamin C was estimated by the method of Denson and Bowers, 1961[16].

Catalase (CAT) activity

CAT activity was determined according to the method described by Aebi 1984[17].

Superoxide dismutase (SOD) activity

SOD activity was determined according to the method of Marklund and Marklund 1974 [18].

Statistical Analysis

Data are expressed as mean ± SE. Statistical significance was determined using student t- test and anova test. A probability value of P less than 0.05 was considered statistically significant.

RESULTS

In the present study no significant differences in weight, height and body mass index were observed between smokers and nonsmokers. Diastolic and systolic blood pressure was significantly higher in smokers than in non-smokers Table (1). Table 2 shows the total cholesterol, TG, and LDL-C levels were significantly increased ($p<0.01$) in smokers compared with nonsmokers, while the HDL-C and does not differ significantly. Plasma tHcy level was significantly increased in healthy smokers compared with nonsmokers. Analysis of the effect of smoking index on tHcy levels revealed a significant difference among mild, moderate and heavy smokers as compared with nonsmokers. Plasma MDA was significantly increased in healthy smokers compared with nonsmokers. Analysis of the effect of smoking index on MDA levels revealed a significant difference among mild, moderate and heavy smokers as compared with nonsmokers. The levels of carbonyl group, as index of protein oxidation in plasma from healthy smokers was significantly higher than those measured in nonsmokers. The levels of carbonyl group in plasma from mild, moderate and heavy smokers were significantly higher than the nonsmokers group. Plasma IL-6 concentration was significantly increased in healthy smokers compared with healthy nonsmokers. Also, plasma TNF-α level was significantly increased in healthy smokers compared with non-smokers. Analysis of the effect of smoking index on IL-6 and TNF-α levels revealed a significant difference among mild, moderate and heavy smokers. Plasma GSH was significantly decreased in healthy smokers compared with healthy nonsmokers. Also, plasma vitamin C was significantly decreased in healthy smokers compared with healthy non-smokers. Analysis of the effect of smoking index on GSH and vitamin C levels revealed a significant difference among mild, moderate and heavy smokers. The activities of plasma enzymatic antioxidants (CAT and SOD) in smokers were significantly lower in smokers than nonsmokers. Analysis of the effect of smoking index on CAT and SOD activities revealed a significant difference among mild, moderate and heavy smokers (Table 4).

DISCUSSION

The present study compared oxidant/antioxidant status in smokers and nonsmokers. Regarding lipid profile, our results showed that the mean total cholesterol, TG, and LDL-C levels were significantly high among the smokers in comparison to nonsmokers, the rise in blood lipid levels in smokers may be through catecholamine and adenyl cyclase axis induced tissue lipolysis. On the other hand significant low HDL-C levels were observed in nonsmokers, these results are in agreements with results reported by Devaranavadgi et al., 2012[19]. No significant difference was observed in BMI in smokers compared to nonsmokers. The mean systolic and diastolic arterial blood pressure were significantly high in smokers compared to non smokers this may be due to sympathetic nervous over activity[20]. Cigarette smoking induces arterial stiffness which may persist after smoking cessation[21]. The incidence of hypertension is increased among those who smoke 15 or more cigarettes per day[22]. The mean MDA levels as a marker of lipid peroxidation were showed to be increased significantly in smokers as compared to non-smokers, which are in agreement with results reported by Metta[23]. MDA levels showed more significance increase in moderate and heavy smokers, indicating positive correlation between increased oxidative stress and the number of cigarettes smoked per day this is in concordance with Solak et al.,2005[24]. Homocysteine contains a highly reactive sulfhydryl(-SH) group. The sulfhydryl group readily self-oxidizes to form disulfide linkage with other free thiols, along with the generation of superoxide radicals as a byproduct[25]. In the present study tHcy level was significantly increased in smokers compared with nonsmokers. Analysis of the effect of smoking index on tHcy levels revealed a significant difference among mild, moderate and heavy smokers. Elevated plasma homocysteine may induce excessive production of reactive oxygen species, especially superoxide anion formation via increasing the NADPH oxidase activity, thus leading to

greater oxidative stress and decreased antioxidant enzyme activities[26]. Also Homocysteine like sulfhydryl compound can promote the oxidation of LDL, reduce the concentration of HDL cholesterol in plasma by inhibiting the synthesis of apolipoprotein A1 (apo A-1), the main HDL apolipoprotein and increase the serum levels of MDA[27]. Furthermore, our study showed significant increased plasma IL-6 and TNF-α concentrations in smokers compared with nonsmokers. The oxidative stress resulting from elevated serum tHcy can oxidize membrane lipids and proteins and consequently increase the expression of these inflammatory factors. tHcy can be converted to a highly reactive thiolactone which is able to react with proteins forming-NH-CO-adducts, thus affecting body proteins and enzymes. Such an effect may contribute to atherogenesis by enhancing the inflammatory response[28]. The presence of carbonyl groups in proteins has been used as a marker of ROS-mediated protein oxidation. The levels of protein carbonyl content, as index of protein oxidation was significantly high in smokers compared to nonsmokers, also heavy smokers showed significant high levels compared to mild and moderate smokers. These results are in agreement with Kocyigit et al.,2011[29] who reported increased levels of plasma PC in both hand-rolled cigarette smokers and manufactured filter-cigarette smokers as proteins are considered the major targets for reactive oxidants in cells. Regarding antioxidant enzymes our results showed significantly lower CAT, SOD, GSH activity in smokers compared to non smokers and more significance decrease in moderate and heavy smokers, this indicates failure of antioxidant defense mechanisms as scavenging free radicals, detoxifying lipid peroxides[30]. Vitamin C has long been considered one of the most important cellular antioxidant, as it severs as a primary antioxidant by detoxifying exogenous radical species that have entered cells or which have arisen within cells due to excess superoxide generation by mitochondrial metabolism. We found that levels of vitamin C were significantly decreased in smokers compared to non-smokers. Deficiency of vitamin C concentrations was related to a reflex mechanism against the increased oxidative stress. Our results are consistent with Shah et al., 2015[31] who reported significant low levels of vitamin C in smokers compared with non-smokers. The results of present study demonstrate that smoking significantly increases MDA, tHcy, PCC levels and decreases antioxidant enzyme and vitamins C. Moreover significant increase of inflammatory cytokine, IL-6 and TNF-α. These findings insist that smoking leads to increase free radical load this results in an imbalance between oxidant/antioxidant status, reinforcing recommendations concerning the benefits of smoking cessation. These also indicate that the best way to combat this public health problem is to implement comprehensive smoking control measures that include effective smoking prevention strategies.

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