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Comparative assessment of individual RONS in serum of smokers compared with non-smokers and their correlation with the lipid profile and antioxidant status

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Abstract

Objective: Cigarette smoking generates free radicals, such as reactive oxygen and nitrogen species (RONS) that contribute to many diseases. The aim of this study was to compare the levels of individual RONS in serum from smokers and non-smokers, and to examine their impact on lipid profiles and the endogenous antioxidant status, which is represented by vitamins C and E.

Methods: Ninety-four healthy Egyptian volunteers (48 smokers and 46 non-smokers) were enrolled. Blood samples were collected and analysed for common haematological tests, lipid profiles, and serum antioxidants. Six reactive oxygen species and three reactive nitrogen species were measured.

Results: A significant increase in radical levels was observed, as well as significant increases in haemoglobin (Hb), haematocrit, platelet count, total cholesterol, triglycerides, low-density lipoprotein cholesterol, and very low-density lipoprotein cholesterol in smokers compared with non-smokers. In contrast, high-density lipoprotein cholesterol was significantly reduced in smokers compared with non-smokers. A moderate negative correlation was found between serum levels of vitamins C and E and $O_2^{-\bullet}$, $HO^{\bullet}$, H_2O_2, $NO^{\bullet}$, and $ONO^{\bullet}$, reflecting a negative impact of elevated RONS levels on the endogenous antioxidant status.

Conclusion: These results may increase our understanding of the pathological role of smoking in several diseases.

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Introduction

Cigarette smoking is a major risk factor for the development and progression of several diseases via diverse underlying mechanisms.[1–3] Among these mechanisms, the free radical-induced oxidative effect has been suggested to play a significant role in the pathogenesis of cardiovascular diseases and cancer.[4,5] It has been argued that the increased production of reactive species associated with smoking may exceed the capacity of the endogenous antioxidant defence system, resulting in oxidative damage and lipid peroxidation.[6–9] Although cigarette smoke is a rich source of free radical and non-radical oxidants, direct exposure to cigarette smoke represents only a portion of the total oxidative stress that is found in living tissues. Additionally, smoking contributes to further endogenous oxidant formation that magnifies the inflammatory immune responses.[10,11]

Reactive oxygen and nitrogen species (RONS) are currently the most widely studied free radical oxidants in living organisms. The most important reactive oxygen species (ROS) generated by cigarette smoking are the superoxide anion ($O_2^{-\bullet}$), the hydroxyl radical ($HO^{\bullet}$), the singlet oxygen radical ($^1O_2$), hypochlorous acid (HClO), hydrogen peroxide (H_2O_2), and the peroxyl radical ($ROO^{\bullet}$).[5,7,8,12,13] These ROS species significantly affect the haematological and oxidative stress biomarkers in both active and passive smokers.[14] The most important reactive nitrogen species (RNS) found in cigarette smoke are nitrogen dioxide ($ONO^{\bullet}$), nitric oxide ($NO^{\bullet}$), and peroxynitrite ($ONOO^-$).[4] Although endogenous nitric oxide is involved in the endothelial vasodilatory mechanism, its availability is decreased by the effects of oxygen radicals. Additionally, this reaction produces peroxynitrite, which, in turn, enhances oxidative stress.[4,8]

In normal physiology, there is a dynamic equilibrium between ROS activity and antioxidant defence capacity.[15] When this equilibrium shifts in favour of ROS, either by a reduction in antioxidant defences or an increase in ROS production, the oxidative stress increases, leading to potential cellular damage.[16]

Because RONS have a short lifetime, it has been difficult to measure their levels, especially in biologic fluids.[17] However, the introduction of specific fluorescence-based probes has made this type of measurement possible.[17,18] Thus, we measured individual RONS in serum from rheumatoid arthritis patients in a recent study.[19]

To date, most of the studies involving smokers have been concerned with assessing the total oxidative stress without focusing on the individual RONS and their expected pathological roles.[14,16] Therefore, the aim of this study was to comparatively measure the levels of individual RONS in serum from smokers and non-smokers, and to highlight their impact on the lipid profile and the endogenous antioxidant status, which is represented by vitamins C and E. These levels will be correlated with the individual RONS levels to assess the oxidant–antioxidant balance. Our results are expected to shed light on reactive species

that play a central role in smoking-induced diseases.

Materials & methods

Study design

This study was conducted between August 2016 and January 2017, and it was approved by the Research Ethics Committee of the Faculty of Pharmacy in Assiut University, Assiut, Egypt (no. 1451/2017). A detailed questionnaire on demography information and smoking status was distributed to visitors of outpatient clinics at Assiut University who attended the clinics for a routine check-up. All patients with infectious diseases and chronic diseases, and those who were on long-term medications were excluded. Additionally, persons who were subjected to any of the following within the 3 months before the start of the study were excluded: antibiotics, steroids, thiazide diuretics, nonsteroidal anti-inflammatory drugs, immunomodulatory drugs, drugs that affect lipid profiles, hospitalisation, surgery, radiotherapy, and previous history of direct smoking (for current non-smokers). Ninety-four healthy volunteers were enrolled and divided into two groups based on their smoking habit. The smokers group included 48 subjects (all male), and the non-smokers group included 46 subjects (32 males and 14 females). Informed consent was obtained from each participant.

Biochemical analysis

Fasting venous blood samples were collected from the study subjects under aseptic conditions. A sample of the fresh whole blood from each subject was analysed using an automatic haematology analyser Celltac G (Nihon Kohden Co., Tokyo, Japan) to determine haemoglobin (Hb), packed cell volume (PCV, haematocrit), red blood cells (RBCs), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), total white blood cells (WBC), neutrophils, eosinophils, lymphocytes, monocytes, and platelets counts.

Serum samples were obtained from the blood samples by centrifugation at $2000 \times g$ for 10 minutes at 4°C. The plasma was analysed using a Cobas c 311 analyzer (Roche Diagnostics GmbH, Mannheim, Germany) to determine the total cholesterol, triglyceride, and high-density, low-density, and very low-density lipoprotein cholesterol (HDL-C, LDL-C, and VLDL-C, respectively) levels. Serum vitamin C and E levels were analysed using reversed-phase high performance liquid chromatography (HPLC) methods.[20,21]

Serum RONS levels were measured using specific fluorescence-based reagents. The superoxide anion level was determined by measuring the level of fluorogenic ethidium (E^+), which forms in an oxidation reaction of superoxide anion with hydroethidine (HE) (Sigma-Aldrich, Seelze, Germany). Potassium superoxide (Sigma-Aldrich) was used as a reference standard for the superoxide anion.[22] The Singlet Oxygen Sensor Green (SOSG) reagent (Molecular Probes, Eugene, Oregon, USA) was used for the specific fluorescence determination of singlet oxygen using 5,10,15,20-tetrakis(N-methyl-4-pyridyl)-21H,23H-porphine (TMPyP), a photosensitising agent, to produce singlet oxygen as a reference standard for calibration purposes.[23] The selective monitoring of hydroxyl radical was performed through fluorescence determination, which was achieved by coumarin-3-carboxylic acid (3-CCA) (Sigma-Aldrich), where the hydroxyl radical was generated through Fenton's reaction of a mixture containing hydrogen peroxide, ferrous ammonium sulfate, and phosphate buffer (pH 7.4).[24] Fluorescence detection using Amplex® Red Hydrogen Peroxide/Peroxidase kit (Molecular Probes) was used for the specific

detection of the hydrogen peroxide level.[25] The hypochlorite anion was selectively determined using 2-[6-(4′-amino) phenoxy-3H-xanthen-3-on-9-yl] benzoic acid (APF) reagent (Sigma-Aldrich), followed by fluorescence detection using sodium hypochlorite (Sigma-Aldrich) as a standard.[26] The lipid peroxidation sensor, 4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4adiaza-s-indacene-3-undecanoic acid (BODIPY581/591 C11) (Molecular Probes) was used for the fluorescence detection of the peroxyl radical using tert-butyl hydroperoxide (Sigma-Aldrich) to generate peroxyl radicals for calibration.[27] To measure nitric oxide radical, 4,5-diaminofluorescein (DAF-2) (Sigma-Aldrich) was used as a specific fluorescence reagent, and spermine nonoate (Sigma-Aldrich) was used as the nitric oxide radical donor.[28] A specific fluorescence kit of 2,3-diaminonaphthalene (DAN) (Sigma-Aldrich) was used to detect nitrite.[29] Finally, peroxynitrite was reduced by nitrate reductase (Sigma-Aldrich) to nitrite, and was detected using DAN. Sodium nitrate and sodium nitrite (Sigma-Aldrich) were used as calibration reference standards for the nitrate and nitrite radicals, respectively.

Statistical analysis

Statistical Package for the Social Sciences (SPSS), version 20 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. RONS levels were expressed as the mean and standard error of the mean (SEM). One-way analysis of variance (ANOVA) was used to compare means, with Tukey's test used for post-hoc analysis. Differences were considered to be highly significant, significant, or nonsignificant for $P \leq 0.001$, $P \leq 0.05$, or $P > 0.05$, respectively. Pearson's correlation coefficient (r) was used to determine correlations between variables. The correlation strength was strictly defined using Evans' method, and was considered to be very strong, strong, moderate, weak, or very weak if $(0.8 \leq r \leq 1.0)$, $(0.6 \leq r < 0.8)$, $(0.4 \leq r < 0.6)$, $(0.2 \leq r < 0.4)$, or $(0.0 \leq r < 0.2)$, respectively.[30]

Results

Participant features and biochemical analyses

As shown in Table 1, there were no significant differences in age, height, weight, body mass index (BMI), and systolic and diastolic blood pressures (BP) between the smokers and non-smokers groups.

Table 2 shows the results of the haematology tests, lipid profiling, and endogenous serum antioxidant levels between the smokers and non-smokers groups. For the

Table 1. Selected features of smoker and non-smoker healthy participants.

Feature	Smokers group* (n = 48)	Non-smokers group* (n = 46)	*P* Value
Age (years)	32.6 ± 9.4	30.9 ± 10.5	NS
Weight (kg)	63.8 ± 12.7	61.4 ± 11.8	NS
Height (cm)	165.7 ± 12.0	163.3 ± 12.7	NS
BMI (kg/m^2)	23.2 ± 4.4	22.9 ± 4.1	NS
Number of cigarettes smoked/day	16.7 ± 6.3	–	–
Smoking duration (years)	8.5 ± 4.8	–	–
Systolic BP (mmHg)	116.9 ± 8.5	119.1 ± 7.7	NS
Diastolic BP (mmHg)	77.4 ± 6.9	79.2 ± 5.3	NS

*Mean ± SEM.

BMI, body mass index; BP, blood pressure; NS, non-significant; SEM, standard error of the mean.

Table 2. Comparison of haematology tests, lipid profile and endogenous serum antioxidants between smokers and non-smokers groups.

Parameter	Smokers group* (n = 48)	Non-smokers group* (n = 46)	*P* Value
Haematological parameters			
Hb (g/dL)	14.4 ± 1.2	11.3 ± 0.9	≤0.01 (S)
PCV (%)	44.5 ± 1.4	38.9 ± 1.1	≤0.01 (S)
RBCs ($10^6/\mu L$)	4.9 ± 0.3	4.6 ± 0.2	>0.05 (NS)
MCV (fL)	85.7 ± 2.6	83.4 ± 2.4	>0.05 (NS)
MCH (pg)	31.2 ± 0.9	27.9 ± 1.2	≤0.05 (S)
MCHC (g/dL)	32.8 ± 1.4	29.4 ± 1.0	≤0.01 (S)
Total WBCs ($10^3/\mu L$)	7.4 ± 0.4	6.7 ± 0.2	>0.05 (NS)
Neutrophils (%)	62.5 ± 2.3	62.0 ± 3.1	>0.05 (NS)
Eosinophils (%)	5.1 ± 0.05	5.7 ± 0.06	>0.05 (NS)
Lymphocytes (%)	28.5 ± 1.7	28.1 ± 1.3	>0.05 (NS)
Monocytes (%)	3.9 ± 0.04	4.2 ± 0.05	>0.05 (NS)
Platelets ($10^3/\mu L$)	265.2 ± 15.7	215.9 ± 14.3	≤0.01 (S)
Lipid profile			
Total Cholesterol (mg/dL)	197.7 ± 25.2	155.2 ± 18.5	≤0.001 (HS)
HDL-Cholesterol (mg/dL)	35.6 ± 4.6	42.5 ± 5.1	≤0.001 (HS)
LDL-Cholesterol (mg/dL)	128.7 ± 8.8	88.4 ± 7.6	≤0.001 (HS)
VLDL-Cholesterol (mg/dL)	33.4 ± 3.7	24.3 ± 2.7	≤0.001 (HS)
Triglycerides (mg/dL)	166.8 ± 15.3	121.7 ± 13.1	≤0.001 (HS)
Serum antioxidants			
Vitamin C (mg/dL)	0.75 ± 0.15	1.19 ± 0.22	≤0.001 (HS)
Vitamin E (mg/dL)	1.08 ± 0.33	1.98 ± 0.41	≤0.001 (HS)

*Mean ± SEM.

Hb, haemoglobin; HS, highly significant; HDL, high density lipoprotein; LDL, low density lipoprotein; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; MCV, mean corpuscular volume; NS, nonsignificant; PCV, packed cell volume (haematocrit); RBCs, red blood cells; S, significant; SEM, standard error of the mean; VLDL, very low-density lipoprotein; WBCs, white blood cells.

haematology tests, there were no significant differences in the RBC count, MCV, and total and differential WBC count. However, Hb, PCV, MCH, MCHC, and platelet count were significantly higher ($P \leq 0.05$) in the smokers group. The lipid profile for smokers showed a highly significant increase ($P \leq 0.001$) in the levels of total cholesterol, LDL-C, VLDL-C, and triglycerides, but a highly significant decrease in HDL compared with the non-smokers. There was a highly significant decrease in serum endogenous antioxidants (vitamin C and vitamin E) in the smokers group compared with the non-smokers group.

Individual serum RONS levels in smokers and non-smokers groups

Serum levels of individual ROS and RNS in the study subjects are presented in Table 3. Except for singlet oxygen, which was found to be significantly higher in the smokers group ($P \leq 0.05$), all other radical levels were highly significantly increased in the smokers group compared with the non-smokers group ($P \leq 0.001$). The mean

Table 3. Serum levels of reactive oxygen and nitrogen species in smokers and non-smokers.

Reactive species levels	Smokers group* (n=48)	Non-smokers group* (n=46)	*P* Value
Superoxide anion (nM)	259.6 ± 63.1	145.3 ± 52.7	≤0.001 (HS)
Hydroxyl radical (nM)	275.3 ± 57.2	149.5 ± 38.1	≤0.001 (HS)
Singlet oxygen (nM)	116.0 ± 47.9	92.1 ± 12.7	≤0.05 (S)
Hydrogen peroxide (nM)	891.4 ± 153.7	235.1 ± 64.8	≤0.001 (HS)
Hypochlorite radical (nM)	107.2 ± 18.6	53.4 ± 9.5	≤0.001 (HS)
Peroxyl radical (nM)	138.1 ± 21.5	76.2 ± 11.9	≤0.001 (HS)
Nitric oxide (μM)	25.5 ± 8.3	13.1 ± 6.6	≤0.001 (HS)
Nitrogen dioxide (μM)	3.36 ± 1.0	1.8 ± 0.8	≤0.001 (HS)
Peroxynitrite (μM)	4.72 ± 1.25	2.54 ± 0.7	≤0.001 (HS)

*Mean ± SEM.
HS, highly significant; S, significant; SEM, standard error of the mean.

serum levels of the quantified RONS in the smokers and non-smokers groups are shown in Figure 1.

Correlations between individual RONS and serology markers in smokers

As shown in Table 4, the correlations between individual ROS serum levels and lipid profiles can be summarised as follows: moderate to strong positive correlation between total cholesterol and $O_2^{-\bullet}$, $HO^{\bullet}$, H_2O_2, and $ClO^{\bullet}$; a moderate positive correlation between triglycerides and $O_2^{-\bullet}$, $HO^{\bullet}$, and H_2O_2; a moderate positive correlation between both LDL and VLDL and $HO^{\bullet}$; a moderate negative correlation between HDL and both $O_2^{-\bullet}$and $HO^{\bullet}$; and very weak to weak positive correlations for the rest of the ROS and lipid profiles.

For RNS, the correlations were as follows: a moderate positive correlation between total cholesterol and $NO^{\bullet}$, $ONO^{\bullet}$, and $ONOO^{-}$; a weak positive correlation between triglycerides, LDL, VLDL, $NO^{\bullet}$, $ONO^{\bullet}$, and $ONOO^{-}$; and a weak negative correlation between HDL and $NO^{\bullet}$, $ONO^{\bullet}$, and $ONOO^{-}$.

Generally, positive correlations were found between total cholesterol, triglycerides, LDL, VLDL, and all RONS. The strength of the correlations were in the following descending order: $HO^{\bullet}$, H_2O_2, $O_2^{-\bullet}$, $ClO^{\bullet}$, $ROO^{\bullet}$, $ONO^{\bullet}$, $ONOO^{-}$, $NO^{\bullet}$, and $^1O_2$, whereas the only negative correlation was between HDL and all assessed reactive species. Figure 2 summarises the correlations between individual RONS and the lipid profiles for the smokers group.

For the endogenous antioxidant status (Table 4), we found that smokers had moderate to strong negative correlations between both vitamins C and E and the oxygen radicals $O_2^{-\bullet}$, $HO^{\bullet}$, and H_2O_2, as well as weak negative correlations with $^1O_2$ and $ClO^{\bullet}$. For RNS, moderate negative correlations were found in smokers between vitamin C and $NO^{\bullet}$ and $ONO^{\bullet}$, while a weak negative correlation was found with $ONOO^{-}$. Finally, a moderate negative correlation was found between vitamin E and $NO^{\bullet}$, as well as a weak negative correlation between vitamin E and $ONO^{\bullet}$ and $ONOO^{-}$.

Discussion

Chronic exposure to cigarette smoke affects a wide range of immunological and haematological parameters in humans.[31,32] The main objective of the current study was to measure the serum levels of individual RONS in smokers and non-smokers to

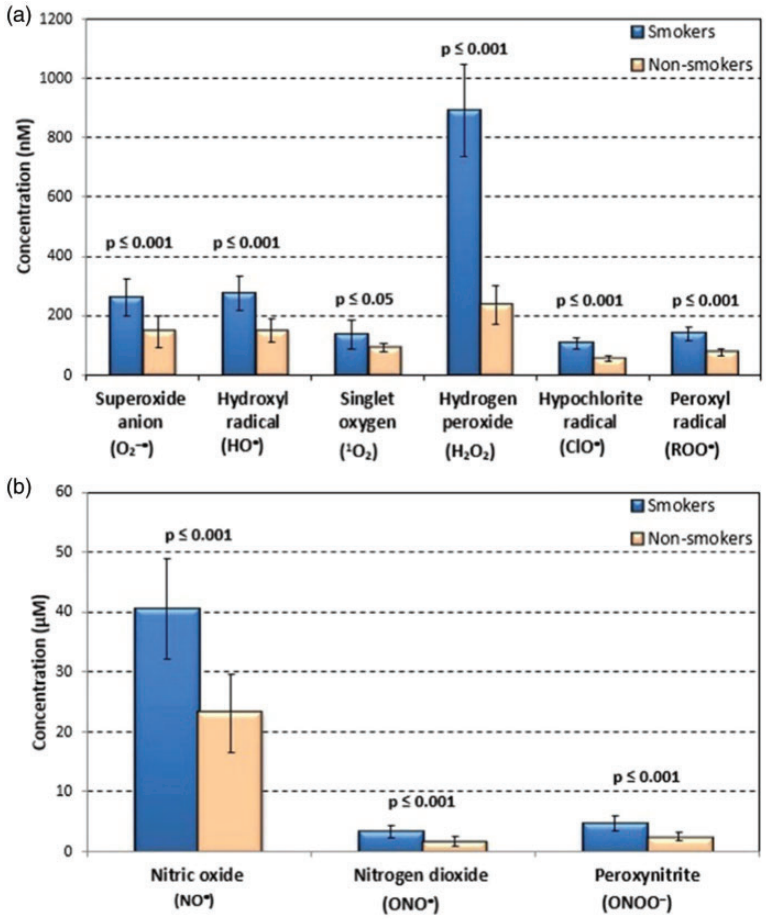

Figure 1. Mean serum levels of (a) reactive oxygen species and (b) reactive nitrogen species in smokers and non-smokers groups.

increase our understanding of the possible roles of these radicals in the pathology of various diseases. These radicals are either endogenously produced or acquired from exogenous sources. The endogenous sources include immune cell activation, inflammation, excessive exercise, ischaemia, infection, cancer, and rheumatoid arthritis, while the exogenous sources include cigarette smoke, air pollution, alcohol, heavy metals, and certain drugs.[33] Cigarette smoke is a major source of exogenous oxidants, which can initiate redox cycling reactions that generate new free radicals. Moreover, cigarette smoke promotes the activation of neutrophils and macrophages that act as endogenous sources of free radicals.[34] In this study, a highly significant increase in the serum levels of $O_2^{-\bullet}$, $HO^{\bullet}$, H_2O_2, $ClO^{\bullet}$, and $ROO^{\bullet}$ was observed in the smokers group, as well as a significant elevation in $^1O_2$, as shown in Figure 1.

For RNS, a highly significant elevation was observed for $NO^{\bullet}$, $ONO^{\bullet}$, and $ONOO^-$ in the smokers group. Although the $NO^{\bullet}$ radical is much less reactive compared with the other studied serology markers, its effects appear to result from a slow

Table 4. Correlation of serum lipid profile and endogenous antioxidant levels with serum levels of individual reactive oxygen and nitrogen species in the smokers group.

Parameter	$O_2^{-\bullet}$ (r)	$HO^{\bullet}$ (r)	$^1O_2$ (r)	H_2O_2 (r)	$ClO^{\bullet}$ (r)	$ROO^{\bullet}$ (r)	$NO^{\bullet}$ (r)	$ONO^{\bullet}$ (r)	$ONOO^-$ (r)
Lipid profile									
Total Cholesterol	0.54	0.69	0.31	0.57	0.51	0.48	0.43	0.47	0.43
HDL-Cholesterol	−0.44	−0.50	−0.31	−0.34	−0.31	−0.29	−0.30	−0.35	−0.38
LDL-Cholesterol	0.37	0.41	0.28	0.35	0.28	0.24	0.27	0.25	0.27
VLDL-Cholesterol	0.39	0.41	0.15	0.27	0.26	0.30	0.26	0.24	0.21
Triglycerides	0.40	0.54	0.28	0.42	0.39	0.31	0.36	0.38	0.37
Serum antioxidants									
Vitamin C	−0.54	−0.62	−0.24	−0.41	−0.31	−0.23	−0.44	−0.46	−0.39
Vitamin E	−0.51	−0.50	−0.34	−0.42	−0.28	−0.23	−0.40	−0.38	−0.39

$^1O_2$, singlet oxygen; $ClO^{\bullet}$, hypochlorite radical; H_2O_2, hydrogen peroxide; HDL-C, high-density lipoprotein; $HO^{\bullet}$, hydroxyl radical; LDL, low-density lipoprotein; $NO^{\bullet}$, nitric oxide; $O_2^{-\bullet}$, superoxide anion; $ONO^{\bullet}$, nitrogen dioxide; $ONOO^-$, peroxynitrite; r, correlation coefficient; $ROO^{\bullet}$, peroxyl radical; VLDL, very-low-density lipoprotein.

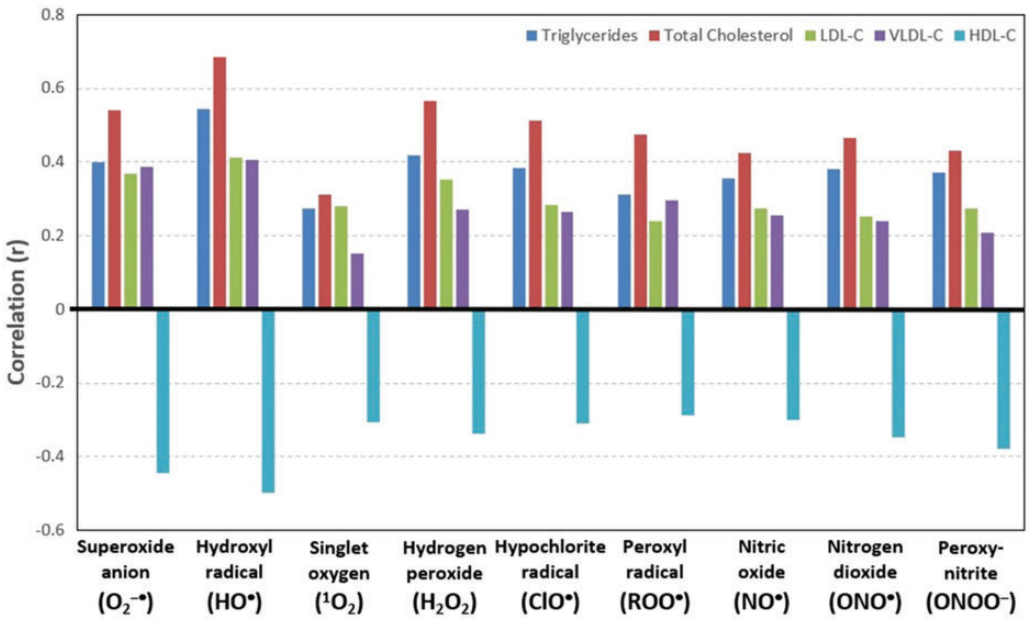

Figure 2. Correlations between individual reactive oxygen and nitrogen species and lipid profiles in serum of the smoker group. HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; VLDL-C, very low-density lipoprotein cholesterol. Correlation strength: very strong, $\pm$ ($0.8 \leq r \leq 1.0$); strong, $\pm$ ($0.6 \leq r < 0.8$); moderate, $\pm$ ($0.4 \leq r < 0.6$); weak, $\pm$ ($0.2 \leq r < 0.4$); very weak, $\pm$ ($0.0 \leq r < 0.2$).

conversion to $ONO^{\bullet}$ and $ONOO^-$ by oxidation, and it can be present in amounts as high as 500 ppm in cigarette smoke.[35] Cigarette smoke may also affect levels of vascular endothelial nitric oxide, and can also modulate inflammatory reactions through inflammatory cytokines.[36] Additionally, $ONO^{\bullet}$ can react with other

gaseous components of cigarette smoke (such as isoprene) to form alkoxyl radicals. These peroxyl radicals then react with either $NO^{\bullet}$ or $ONOO^{-}$ to form peroxynitrate radicals.[37]

A significant increase in the levels of total cholesterol, LDL-C, VLDL-C, and triglycerides was observed in smokers compared with non-smokers, while a significant decrease in HDL-C levels was seen in smokers compared with non-smokers. These results are similar to previously reported observations.[32,38] Dyslipidaemia is significantly worsened by an increase in smoking duration. This is partly because nicotine in cigarette smoke stimulates the release of adrenaline from the adrenal medulla, which increases serum levels of free fatty acids. These fatty acids, in turn, stimulate the liver to secrete cholesterol, VLDL-C, and triglycerides.[38] Smoking may induce sub-endothelial oedema with lipid accumulation and changes in vascular permeability. Additionally, nicotine decreases vascular activity, worsens endothelium dysfunction, and induces the formation of coronary artery clots.[32] However, LDL-C level is positively correlated with the VLDL-C level and triglycerides, and the HDL-C level is inversely proportional to the LDL-C level.[39,40] Cigarette smoking also induces insulin resistance, which may lead to hyperinsulinaemia, as observed by a marked decrease in lipoprotein lipase and hepatic lipase that transforms VLDL to LDL.[41] Insulin resistance was shown to negatively affect the lipid profile, inducing endothelial dysfunction and oxidative stress and driving the formation of atheroma and the development of cardiovascular diseases.[40] In our study, moderate to strong positive correlations were found between both total cholesterol and triglycerides and $O_2^{-\bullet}$, $HO^{\bullet}$, and H_2O_2, whereas moderate positive correlations were observed between both LDL and VLDL and $HO^{\bullet}$. However, moderate negative correlations were seen between HDL and $O_2^{-\bullet}$and between HDL and $HO^{\bullet}$.

As a rich source of active oxidants, cigarette smoke may cause an imbalance in the endogenous antioxidant defences that are associated with increased production of free ROS.[42] Vitamin C, a strong antioxidant, has a hydrophilic nature and a unique structure that makes it an excellent electron donor for scavenging free RONS.[7] However, vitamin E has a lipophilic nature that enables it to pass through the cell membrane to scavenge free reactive species. The biological activity of vitamin E is almost entirely a result of its antioxidant properties that aid in membrane stabilisation and effectively prevent lipid peroxidation.[43] In this study, a marked decrease in serum levels of antioxidant vitamins C and E in smokers was observed, which resulted in an increased lipid peroxidation rate. Our results also show that a moderate to strong negative correlation exists between vitamin C and E and $O_2^{-\bullet}$, $HO^{\bullet}$, and H_2O_2. Cigarette smoking depletes these serum antioxidants, which are required to scavenge excess free radicals, thereby increasing the rate of lipid peroxidation.[43]

The most significant radicals that affected the serological markers in our study were the hydroxyl and superoxide anion radicals. This result may be explained by the activation of pulmonary alveolar macrophages by cigarette smoke. During this process, $O_2^{-\bullet}$ and H_2O_2 are produced, and these species are converted through an iron-catalysed reaction into $HO^{\bullet}$ radical, leading to biological damage.[12,44]

The significant elevation of Hb concentration and its related markers that occurred in the smokers group in this study was consistent with other reports.[32,38] This elevation is best explained as a compensatory mechanism that increases Hb concentration because of a lack of oxygen-carrying capacity in carboxy-Hb, which results from continuous exposure of the

smoker to carbon monoxide that is generated in the cigarette burning process.[45] Finally, although the effect of smoking on platelet count is controversial, the smokers in our study showed a significant increase in platelet count, which could be attributed to increased platelet aggregation.[46–48]

We acknowledge that there are several limitations to this study. We included subjects from only one geographical region (i.e., Assiut, Egypt), so we cannot generalise the results to other populations. Additionally, we were not able to include female smokers because smoking disclosure for women is socially unacceptable in Egypt.

In summary, cigarette smoking generates several oxidative and nitrative stressors that may act as serious health hazards. To the best of our knowledge, the current study is the first to monitor individual RONS in smokers. The specific effect of the monitored reactive species on lipid markers, combined with practical insight into serum antioxidant status, were confirmed. Some of the reactive species, such as $HO^{\bullet}$ and $O_2^{-\bullet}$, play predominant roles in cellular damage caused by smoking. Consequently, early intervention to promote smoking cessation may reverse these harmful effects and prevent future health problems. However, further insights into the molecular mechanisms of these reactive species in the pathology of smoking-related diseases will be of a great value.

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Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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References

1. Das SK. Harmful health effects of cigarette smoking. *Mol Cell Biochem* 2003; 253: 159–165.
2. Alberg AJ. Cigarette smoking: health effects and control strategies. *Drugs Today (Barc)* 2008; 44: 895–904.
3. Pirie K, Peto R, Reeves GK, et al. The 21st century hazards of smoking and benefits of stopping: a prospective study of one million women in the UK. *Lancet* 2013; 381: 133–141.
4. Ambrose JA and Barua RS. The pathophysiology of cigarette smoking and cardiovascular disease: an update. *J Am Coll Cardiol* 2004; 43: 1731–1737.
5. Valavanidis A, Vlachogianni T and Fiotakis K. Tobacco Smoke: involvement of reactive oxygen species and stable free radicals in mechanisms of oxidative damage, carcinogenesis and synergistic effects with other respirable particles. *Int J Environ Res Public Health* 2009; 6: 445–462.
6. Kamceva G, Arsova-Sarafinovska Z, Ruskovska T, et al. Cigarette smoking and oxidative stress in patients with coronary artery disease. *Maced J Med Sci* 2016; 4: 636–640.
7. Chávez J, Cano C, Souki A, et al. Effect of cigarette smoking on the oxidant/antioxidant balance in healthy subjects. *Am J Phytomed Clin Ther* 2007; 14: 189–193.
8. Peluffo G, Calcerrada P, Piacenza L, et al. Superoxide-mediated inactivation of nitric oxide and peroxynitrite formation by tobacco smoke in vascular endothelium: studies in cultured cells and smokers. *Am J Physiol* 2009; 296: H1781–H1792.

9. Kasap S, Gönenç A, Şener DE, et al. Serum cardiac markers in patients with acute myocardial infarction: oxidative stress, C-reactive protein and N-Terminal probrain natriuretic peptide. *J Clin Biochem Nutr* 2007; 41: 50–57.
10. Lobo V, Patil A, Phatak A, et al. Free radicals, antioxidants and functional foods: impact on human health. *Pharmacogn Rev* 2010; 4: 118–126.
11. Mu Y, Patters BJ, Midde NM, et al. Tobacco and antiretrovirals modulate transporter, metabolic enzyme, and antioxidant enzyme expression and function in polarized macrophages. *Curr HIV Res* 2018; 16: 354–363.
12. Goel R, Bitzer ZT, Reilly SM, et al. Influence of smoking puff parameters and tobacco varieties on free Radicals yields in cigarette mainstream smoke. *Chem Res Toxicol* 2018; 31: 325–331.
13. Huang MF, Lin WL and Ma YC. A study of reactive oxygen species in mainstream of cigarette. *Indoor Air* 2005; 15: 135–140.
14. Lymperaki E, Makedou K, Iliadis S, et al. Effects of acute cigarette smoking on total blood count and markers of oxidative stress in active and passive smokers. *Hippokratia* 2005; 19: 293–297.
15. Schectman G. Estimating ascorbic acid requirements for cigarette smokers. *Ann NY Acad Sci* 1993; 686: 335–345.
16. Ganapathy V, Manyanga J, Brame L, et al. Electronic cigarette aerosols suppress cellular antioxidant defenses and induce significant oxidative DNA damage. *PLoS One* 2017; 12: e0177780.
17. Gomes A, Fernandes E and Lima JLFC. Fluorescence probes used for detection of reactive oxygen species. *J Biochem Biophys Methods* 2005; 65: 45–80.
18. Chen X, Tian X, Shin I, et al. Fluorescent and luminescent probes for detection of reactive oxygen and nitrogen species. *Chem Soc Rev* 2011; 40: 4783–4804.
19. Khojah HM, Ahmed S, Abdel-Rahman MS, et al. Reactive oxygen and nitrogen species in patients with rheumatoid arthritis as potential biomarkers for disease activity and the role of antioxidants. *Free Radical Biol Med* 2016; 97: 285–291.
20. Craft NE and Park H. Improved HPLC method for vitamin C analysis in serum. *FASEB J* 2010; 24: 537–514.
21. Khan A, Khan MI, Iqbal Z, et al: An optimized and validated RP-HPLC/UV detection method for simultaneous determination of all-trans-retinol (vitamin A) and alpha-tocopherol (vitamin E) in human serum: comparison of different particulate reversed-phase HPLC columns. *J Chromatogr B* 2010; 878: 2339–2347.
22. Benov L, Sztejnberg L and Fridovich I. Critical evaluation of the use of hydroethidine as a measure of superoxide anion radical. *Free Radical Biol Med* 1998; 25: 826–831.
23. Gollmer A, Arnbjerg J, Blaikie FH, et al. Singlet Oxygen Sensor Green(R): photochemical behavior in solution and in a mammalian cell. *Photochem Photobiol* 2011; 87: 671–679.
24. Manevich Y, Held KD and Biaglow JE. Coumarin-3-carboxylic acid as a detector for hydroxyl radicals generated chemically and by gamma radiation. *Radiat Res* 1997; 148: 580–591.
25. Zhou M, Diwu Z, Panchuk-Voloshina N, et al. A stable nonfluorescent derivative of resorufin for the fluorometric determination of trace hydrogen peroxide: applications in detecting the activity of phagocyte NADPH oxidase and other oxidases. *Anal Biochem* 1997; 253:162–168.
26. Setsukinai K, Urano Y, Kakinuma K, et al. Development of novel fluorescence probes that can reliably detect reactive oxygen species and distinguish specific species. *J Biol Chem* 2003; 278: 3170–3175.
27. Drummen GP, van Liebergen LC, Op den Kamp JA, et al: C11-BODIPY(581/591), an oxidation-sensitive fluorescent lipid peroxidation probe: (micro)spectroscopic characterization and validation of methodology. *Free Radic Biol Med* 2002; 33:473–490.
28. Kojima H, Nakatsubo N, Kikuchi K, et al. Detection and imaging of nitric oxide with novel fluorescent indicators: diaminofluoresceins. *Anal Chem* 1998; 70: 2446–2453.
29. Nussler AK, Glanemann M, Schirmeier A, et al. Fluorometric measurement of

nitrite/nitrate by 2,3-diaminonaphthalene. *Nat Protoc* 2006; 1:2223–2226.
30. Evans JD. *Straightforward statistics for the behavioral sciences*. Pacific Grove: Brooks/Cole Publishing Co., 1996.
31. Saha SP, Bhalla DK, Whayne TF, et al. Cigarette smoke and adverse health effects: an overview of research trends and future needs. *Intl J Angiol* 2007; 16:77–83.
32. Malenica M, Prnjavorac B, Bego T, et al. Effect of cigarette smoking on haematological parameters in healthy population. *Med Arch* 2017; 71: 132–136.
33. Pham-Huy LA, He H and Pham-Huy C. Free radicals, antioxidants in disease and health. *Intl J Biomed Sci* 2008; 4: 89–96.
34. Rahman K. Studies on free radicals, antioxidants, and co-factors. *Clin Interv Aging* 2007; 2: 219–236.
35. Pryor WA and Stone K. Oxidants in cigarette smoke. Radicals, hydrogen peroxide, peroxynitrate, and peroxynitrite. *Ann N Y Acad Sci* 1993; 686: 12–27; Discussion 27–28.
36. Van Keulen HV, Gomes AS, Toffolo MCF, et al. Serum levels of nitric oxide and cytokines in smokers at the beginning and after 4 months of treatment for smoking cessation. *Intl J Cardiol* 2017; 230: 327–331.
37. Pryor WA. Cigarette smoke radicals and the role of free radicals in chemical carcinogenicity. *Environ Health Perspect* 1997; 105: 875–882.
38. Lakshmi A, Lakshmanan A, Kumar G, et al. Effect of intensity of cigarette smoking on haematological and lipid parameters. *J Clin Diag Res* 2014; 8: BC11–BC13.
39. Muscat JE, Harris RE, Haley NJ, et al. Cigarette smoking and plasma cholesterol. *Am Heart J* 1991; 121: 141–147.
40. Hirano T. Pathophysiology of diabetic dyslipidemia. *J Atheroscler Thromb* 2018; 25: 771–782.
41. Fetterman JL, Sammy MJ and Ballinger SW. Mitochondrial toxicity of tobacco smoke and air pollution. *Toxicology* 2017; 391: 18–33.
42. Elmasry SA, Al-Azzawi MA, Ghoneim AH, et al. Role of oxidant–antioxidant imbalance in the pathogenesis of chronic obstructive pulmonary disease. *Egypt J Chest Dis Tuberc* 2015; 64: 813–820.
43. Kharb S and Singh GP. Effect of smoking on lipid profile, lipid peroxidation and antioxidant status in normal subjects and in patients during and after acute myocardial infarction. *Clin Chim Acta* 2000; 302: 213–219.
44. Dikalov S, Itani H, Richmond B, et al. Tobacco smoking induces cardiovascular mitochondrial oxidative stress, promotes endothelial dysfunction, and enhances hypertension. *Am J Physiol Heart Circ Physiol* 2019; 316: H639–H646.
45. Aitchison R and Russell N. Smoking–a major cause of polycythaemia. *J Royal Soc Med* 1988; 81: 89–91.
46. Suwansaksri J, Wiwanitkit V and Soogarun S. Effect of smoking on platelet count and platelet parameters: an observation. *Clin Appl Thromb Hemost* 2004; 10: 287–288.
47. Gumus F, Solak I and Eryilmaz MA. The effects of smoking on neutrophil/lymphocyte, platelet//lymphocyte ratios. *Bratisl Lek Listy* 2018; 119: 116–119.
48. Green MS, Peled I and Najenson T. Gender differences in platelet count and its association with cigarette smoking in a large cohort in Israel. *J Clin Epidemiol* 1992; 45: 77–84.