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Cigarette Smoke and Oxidative Stress Indices in Male Active Smokers

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ABSTRACT | Increased generation of reactive oxygen species and peroxidation of biomolecules associated with cigarette smoking have been implicated in multiple organ dysfunctions among smokers. This study assessed the oxidative stress indices, including nitric oxide (NO), reduced glutathione (GSH), ferritin, malondialdehyde (MDA), total antioxidant capacity (TAC), and total plasma peroxide (TPP), and oxidative stress index (OSI), as well as cotinine levels in relation to duration of smoking in male active smokers in Calabar, Nigeria. Ninety consenting male subjects aged 18–60 years comprising 50 smokers and 40 nonsmokers were studied. Anthropometric indices, blood pressure, and socio-demographic information were obtained using standard methods. Oxidative stress indices; GSH, ferritin, NO, MDA, TAC, and TPP were estimated by colorimetric methods and cotinine by ELISA method. Data were analyzed using ANOVA, LSD post-hoc and Pearson's correlation at $p < 0.05$. The results showed that the systolic blood pressure, TPP, OSI, NO, MDA, ferritin, and cotinine levels were significantly higher in smokers compared to nonsmokers. Increasing duration of smoking was associated with increased MDA and decreased GSH and NO levels, while increasing number of cigarette sticks smoked per day was associated with decreased MDA levels. Cotinine correlated positively with ferritin ($r = 0.387$, $p = 0.005$) and TPP ($r = 0.377$, $p = 0.007$) only in smokers. In conclusion, cigarette smoking results in enhanced NO, ferritin, and lipid peroxidation, with concomitant depletion of GSH which may lead to oxidative stress and smoking-related illness in cigarette smokers studied.

KEYWORDS | Antioxidants; Cigarette smoke; Cotinine; Ferritin; Free radicals; Lipid peroxidation; Oxidative stress

ABBREVIATIONS | BMI, body mass index; CAT, catalase; GPx, glutathione peroxidase; GSH, reduced glutathione; MDA, malondialdehyde; NO, nitric oxide; OSI, oxidative stress index; ROS, reactive oxygen species; SOD, superoxide dismutase; TAC, total antioxidant capacity; TBARS, thiobarbituric acid reactive substances; TPP, total plasma peroxide; WC, waist circumference; WHR, waist-to-hip ratio

CONTENTS

1. INTRODUCTION

Cigarette smoking still remains an enormous public health problem and is now the world's single leading cause of several preventable diseases and premature deaths. Smoking has been described as the only risk factor shared by four major non-communicable diseases; cardiovascular disease, diabetes, cancer and chronic respiratory diseases [1]. Adverse effects of cigarette smoking have been linked to the diverse effects of the complex mixture of chemical constituents of cigarette smoke on biological systems [2] Cigarette smoke constitutes about 5,000 compounds and 10^{17} free radicals per puff, many of which are able to induce the generation of reactive oxygen or nitrogen species (ROS/RNS) [3] such as superoxide, hydrogen peroxide (H_2O_2), hydroxyl radicals, and peroxyl radicals [2]. These reactive species in turn are capable of initiating and promoting oxidative damage [2] by inactivating endogenous antioxidants enzymes like superoxide dismutase (SOD), catalase (CAT), and non-enzymatic antioxidants as reduced glutathione (GSH) and ascorbate, leading to the oxidant/antioxidant imbalance and hence oxidative and nitrosative stress [4]. Oxidative damage to macromolecules such as lipids, proteins, and DNA has been implicated as the major pathologic mechanism of all smoking-related diseases. Several studies have reported in vivo and in vitro depletion of antioxidants as a result of cigarette smoking [5]. Some have reported an increase, decrease, or no effect of cigarette smoke on the levels of some indices of oxidative stress. These inconsistencies and disparities may be attributed to the different contrasting pathways and mechanisms by which the different constituents of cigarette smoke exert their various effects on biological systems [6–8].

Although the relationship between cigarette smoking and oxidative stress indices has been established, variability in genetics, environmental, dietary, lifestyle, and individual peculiarities may have diverse effects on studies across different populations of smokers. The relative or absolute contributions of smoking to perturbations in levels of some biomarkers of oxidative stress in active male smokers in Calabar are still uncertain and are therefore assessed in this study.

2. MATERIALS AND METHODS

2.1. Selection of Subjects

The subjects of this study were apparently healthy regular male cigarette smokers that have not been diagnosed of any smoking-related illness and non-smokers aged between 18–60 years. The smokers were recruited in drinking and smoking joints and motor parks within Calabar metropolis. The non-smokers were recruited in residential areas in the same environment. Informed consent was sought and obtained from all subjects before recruitment into the study. This study was carried out in accordance with the Ethical Principles for Medical Research Involving Human Subjects as outlined in the Helsinki Declaration in 1975 and subsequent revisions.

A total number of 50 male cigarette smokers (26 moderate smokers and 24 light smokers) were recruited into the study. Smokers of different brands of cigarette were recruited but these brands were not

taken into consideration. In this study, smokers were classified based on smoking pack-years as either heavy smokers (> 30 pack-years), moderate smokers (8–30 pack-years) or light smokers (< 8 pack-years), where pack-year is the number of packs of cigarette smoked per day × number of smoking years or number of pack-years = (number of cigarettes smoked per day/20) × number of years smoked (1 pack has 20 cigarettes) [9]. The non-cigarette smokers (control) were 40 in number. They were those who have never smoked before and do not like the smell of cigarette smoke.

Anthropometric indices such as height and weight were obtained and used in calculating the body mass index (BMI). Socio-demographic data were collected by an interviewer-administered structured questionnaire aiming to determine age, educational levels, socioeconomic status, and social habits (such as smoking, years of smoking, number of packs of cigarette smoked per day, consumption of alcoholic beverages and drug addictions). Information on general health and history of past disease(s) were collected according to the British Medical Research Council questionnaire (BMRC, 1960). Individuals with a history of chronic organ or systemic illness and long-term medication were excluded from the study.

2.2. Sample Collection

Five milliliters of venous whole blood sample were collected from all subjects of the study into plain anticoagulant free sample containers, allowed to clot and retract and then centrifuged at 500 *g* for 10 min at room temperature. Serum samples were collected and stored at –20°C for laboratory estimation of cotinine, nitric oxide (NO), GSH, ferritin, malondialdehyde (MDA), total antioxidant capacity (TAC), total plasma peroxide (TPP), and calculation of oxidative stress index (OSI).

2.3. Laboratory Methods

2.3.1. Determination of TAC

A standard solution of Fe-EDTA complex reacts with H_2O_2 by a Fenton type reaction, leading to the formation of hydroxyl radicals. These ROS degrade benzoate resulting in the release of TBARS (thiobarbituric acid reactive substances). Antioxidants from the added sample cause suppression of the production of TBARS. This reaction is measured spectrophotometrically at 532 nm, and the inhibition of color development is defined as the TAC of the sample [10].

2.3.2. Estimation of TPP

TPP was determined using the reaction of ferrous-butylated hydroxytoluene-xylenol orange complex (FOX-2 reagent) with serum peroxides which yields a colored complex that was measured spectrophotometrically at 560 nm, according to the FOX-2 method. The FOX-2 test system is based on the oxidation of ferrous ions to ferric ions by various types of peroxides present in the serum samples, to produce a colored ferric-xylenol orange complex whose absorbance can be measured [11].

2.3.3. Calculation of OSI

The ratio of TPP to TAC was calculated as the oxidative stress index, an indicator of the degree of oxidative stress: OSI (%) = [TPP (μM H_2O_2) × 100] ÷ [TAC μM].

2.3.4. Estimation of NO

The Griess test was used for detecting total levels of nitrite or nitrous acid in the samples. The NO-containing compounds in the serum combines with alpha-naphthylamine to produce pink azo dye whose absorbance was measured at a wavelength of 540 nm. Total nitrite and nitrate levels were represented as total nitric oxide metabolites (NOx) and measurement of NOx is considered a direct marker of in vivo NO production [12].

2.3.5. Estimation of GSH

Estimation of GSH was carried out following the modified standard Ellman's method. The reagent, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent) reacts with GSH to form the chromophore, 5-thionitrobenzoic acid (TNB) and GS-TNB which is measured spectrophotometrically at 412 nm [13].

2.3.6. Estimation of MDA

MDA formed from the breakdown of polyunsaturated fatty acid serves as a convenient index for deter-

mining the extent of the peroxidation products that react with thiobarbituric acid to give a red species absorbing at 532 nm [14].

2.4. Statistical Analysis

Data analysis was done using the statistical package for social sciences (SPSS version 20.0, IBM, USA). Analysis of variance (ANOVA) was used to test significance of variations within and among group means and Fisher's least significant difference (LSD) post-hoc test was used for comparison of multiple group means. Pearson's correlation was used to determine associations between variables. A probability value $p < 0.05$ was considered statistically significant.

3. RESULTS

Table 1 shows the mean age, BMI, waist circumference (WC), waist-to-hip ratio (WHR), blood pressure, cotinine, ferritin, TPP, TAC, OSI, GSH, NO, and MDA in smokers and nonsmokers. The systolic blood pressure, TPP, OSI, NO, MDA, ferritin, and cotinine levels were significantly higher in smokers compared to nonsmokers ($p < 0.05$). No significant differences were observed in the age, BMI, WC, WHR, diastolic blood pressure, TAC, and GSH levels of both groups ($p > 0.05$).

The effect of duration of smoking and number of cigarettes sticks smoked per day on cotinine, ferritin, TPP, TAC, OSI, GSH, NO, and MDA in smokers were shown in **Table 2**. Duration of smoking and number of cigarette sticks smoked per day did not seem to have any effect on the levels of all the indices studied ($p > 0.05$).

Table 3 shows comparison of effect of duration of smoking and number of cigarettes sticks smoked per day on GSH, NO, and MDA in smokers using LSD post-hoc. The GSH levels of those who have been smoking for < 5 years were significantly higher than those who have been smoking for > 10 years ($p < 0.05$), the NO of those who has been smoking < 5 years were significantly higher than those who have been smoking for 5–10 years, while the MDA levels for those who have been smoking for 5–10 years were significantly lower than those who have been smoking for > 10 years ($p < 0.05$). The MDA levels of those who smoke more than 10 sticks of cigarettes per day were significantly lower than those who smoke less than 10 cigarettes per day.

Figure 1 shows the correlation plot of cotinine against ferritin in smokers. A significant positive correlation was observed between ferritin and cotinine levels of smokers studied ($r = 0.387$, $p = 0.005$).

Figure 2 shows the correlation plot of TPP against cotinine levels in smokers. A significant positive correlation was observed between cotinine and TPP levels of smokers studied ($r = 0.377$, $p = 0.007$).

4. DISCUSSION

Smoking-related diseases have been linked to the various components of cigarette smoke and their specific yet to be determined effects on vital organs and tissues. Adverse health effects of cigarette smoking have been attributed to smoking-induced generation of ROS and oxidative stress and their deleterious effects on biomolecules such as lipids, membrane proteins, and nucleic acids. The levels of some biomarkers of oxidative stress in relation to duration of smoking and number of cigarettes smoked were estimated in active male smokers.

In this study, the systolic blood pressures of smokers were significantly higher than non-smokers. Cigarette smoking has been associated with an acute and marked increase in blood pressure and heart rate [15]. Increased blood pressure in smokers has been related to the toxic effects of nicotine (a major toxic component of cigarette smoke) and carbon monoxide generated by cigarette smoking [16]. Nicotine has been shown to stimulate sympathetic nerves over activity, which increases myocardial oxygen consumption through a rise in blood pressure, heart rate, and myocardial contractility often leading to endothelial dysfunction [17]. Carbon monoxide has been shown to exert a direct toxic effect by causing structural lesions and changes to the arterial vasculature resulting in elevation in the blood pressure. Smoking-mediated elevation in blood pressure may involve an initial vasoconstriction mechanism mediated by nicotine which causes acute but transient increase in systolic blood pressure. This phase is followed by a decrease in blood pressure as a consequence of depressant effects of chronic nicotine intake. Simultaneously, carbon monoxide acts directly on the arterial wall causing structurally irreversible alterations. Structur-

TABLE 1. Age, BMI, WC, WHR, blood pressure, cotinine, ferritin, TPP, TAC, OSI, GSH, NO, and MDA in smokers and nonsmokers

Parameter	Nonsmokers (n = 40)	Smokers (n = 50)	p Value
Age (years)	33.75 ± 6.92	35.56 ± 10.87	0.340
BMI (kg/m^2)	24.10 ± 3.22	24.32 ± 2.86	0.736
WC (cm)	84.20 ± 10.93	80.44 ± 9.18	0.086
WHR	0.89 ± 0.09	0.86 ± .11	0.341
S.BP (mm Hg)	122.75 ± 7.50	130.36 ± 15.75	0.004
D.BP (mm Hg)	80.75 ± 9.97	77.64 ± 11.35	0.171
Cotinine (ng/ml)	0.83 ± 1.12	77.87 ± 51.55	< 0.001
Ferritin (µg/L)	40.51 ± 9.18	100.50 ± 43.67	< 0.001
TPP (µM H_2O_2)	82.47 ± 30.96	137.22 ± 9.71	< 0.001
TAC (µM)	869.30 ± 163.10	840.10 ± 199.81	0.458
OSI (%)	11.16 ± 7.58	17.46 ± 11.66	0.003
GSH (µM)	13.87 ± 3.36	12.66 ± 3.15	0.084
NO (µM)	20.25 ± 3.46	29.10 ± 14.68	< 0.001
MDA(µM)	11.70 ± 6.97	32.38 ± 20.70	< 0.001

Note: BMI, body mass index; D.BP, diastolic blood pressure; GSH, reduced glutathione; MDA, malondialdehyde; NO, nitric oxide. OSI, oxidative stress; S.BP, systolic blood pressure; TAC, total antioxidant capacity; TPP, total plasma peroxides; WC, waist circumference; WHR, waist-to-hip ratio.

TABLE 2. Effect of duration of smoking and number of cigarettes sticks smoked per day on cotinine, ferritin, TPP, TAC, OSI, GSH, NO, and MDA in smokers

Parameter	Group			F ratio	p Value
Duration	*< 5 years (n = 9)*	*5–10 yrs (n =13)*	*> 10 yrs (n = 28)*		
Cotinine (ng/ml)	85.57 ± 56.87	80.23 ± 50.03	83.38 ± 53.50	0.029	0.972
Ferritin (µg/L)	106.94 ± 50.16	114.45 ± 44.51	88.32 ± 43.82	1.681	0.197
TPP (µM H_2O_2)	93.55 ± 35.37	96.69 ± 41.58	90.00 ± 38.22	0.138	0.871
TAC(µM)	935.11 ± 90.96	879.54 ± 98.37	842.50 ± 194.8	1.177	0.317
OSI (%)	9.95 ± 3.30	10.99 ± 4.53	11.47 ± 6.25	0.270	0.765
GSH (µM)	16.44 ± 4.09	13.92 ± 2.87	13.36 ± 3.07	3.145	0.052
NO (µM	36.11 ± 17.48	21.92 ± 4.86	30.28 ± 15.88	2.835	0.069
MDA(µM)	28.88 ± 14.8	23.77 ± 8.85	38.41 ± 24.53	2.578	0.087
Cig. Sticks/Day	*< 5 sticks (n = 33)*	*5–10 sticks (n = 10)*	*> 10 sticks (n =7)*		
Cotinine (ng/ml)	75.13 ± 51.28	87.82 ± 55.32	88.94 ± 52.38	0.357	0.702
Ferritin (µg/L)	99.81 ± 38.35	111.56 ± 56.87	92.80 ± 53.15	0.411	0.665
TPP (µM H_2O_2)	95.69 ± 39.83	92.30 ± 34.45	85.57 ± 36.60	0.207	0.813
TAC (µM)	876.84 ± 175.4	872.10 ± 111.25	858.28 ± 162.2	0.038	0.963
OSI (%)	11.73 ± 6.42	10.44 ± 3.18	10.19 ± 4.62	0.336	0.716
GSH (µM)	14.2727 ± 3.89	14.70 ± 2.1108	14.00 ± 2.30	0.094	0.910
NO (µM)	31.00 ± 16.62	23.60 ± 6.04	27.57 ± 12.93	1.011	0.372
MDA (µM)	34.33 ± 21.06	36.50 ± 20.68	16.03 ± 13.13	2.665	0.080

Note: Cig., cigarette; GSH, reduced glutathione; MDA, malondialdehyde; NO, nitric oxide; OSI, oxidative stress; TAC, total antioxidant capacity; TPP, total plasma peroxides.

TABLE 3. Comparison of effect of duration of smoking and number of cigarettes sticks smoked per day on GSH, NO and MDA in smokers using LSD post-hoc

Parameter	Groups		Mean difference	p Value
Duration	*< 5 yrs (n = 9)*	*> 10 yrs (n = 28)*		
GSH (µM)	16.44 ± 4.09	13.36 ±3.07	3.09 ± 1.23	0.016
	< 5yrs (n = 9)	*5–10yrs (n = 13)*		
NO (µM)	36.11 ± 17.48	21.921 ± 4.85	14.18 ± 6.17	0.026
	5–10yrs (n = 13)	*>10yrs (n=28)*		
MDA (µM)	23.77 ± 8.85	38.41 ± 24.53	–14.64 ± 6.74	0.035
Cig. Stick/Day	*< 5 sticks (n = 33)*	*> 10 sticks (n = 7)*		
MDA (µM)	34.33 ± 21.06	16.03 ± 13.13	18.29 ± 8.38	0.034
	5–10 sticks (n = 10)	*> 10 sticks (n = 7)*		
MDA(µM)	36.50 ± 20.68	16.03 ± 13.13	20.46 ± 9.93	0.045

Note: Cig., cigarette; GSH, reduced glutathione; MDA, malondialdehyde; NO, nitric oxide.

al alterations tend to affect the blood pressure which becomes irreversibly elevated. Chronic cigarette smoking has been shown to induce arterial stiffness which may persist for a decade after smoking cessation [16]. Cigarette smoke-induced increase in blood pressure has also been related to the physiological activities of NO. NO has been associated with the regulation of blood pressure and regional blood flow. Rees et al. [18] found that the pharmacological blockage of NO synthesis induced a dose-dependent, long-lasting increase in mean systemic arterial blood pressure. NO bioavailability has been shown to be significantly decreased by cigarette smoking [18].

Higher levels of TPP, MDA, and OSI were observed in smokers compared to nonsmokers. Both in vivo and in vitro studies have demonstrated that chemical constituents in cigarette smoke have the potential to generate ROS and induce oxidative stress by increasing the pro-oxidant burden and/or decreasing antioxidant protection. Cigarette smoke-derived ROS cause oxidative damage to cellular components and activate numerous signal pathways that modulate cellular responses and may ultimately lead to pathological changes in cell function [5, 19, 20]. Nicotine has been shown to disrupt the mitochondrial respiratory chain leading to increased generation of superoxide anion and hydrogen peroxide [21]. This induced increase in pro-oxidants can result in lipid peroxidation, induction of DNA strand break, inactivation of certain proteins and rupture of membranes, disruption of cellular functions and integrity that are associated with numerous adverse health effects [22]. MDA and TPP are products of lipid peroxidation hence higher levels seen in smokers is commensurate with increased ROS generation associated with smoking. However, MDA levels of smokers who have been smoking for more than 10 years and those who smoke more than 10 sticks of cigarette per day were significantly lower than those who have smoked for less than 10 years and who smoke less than 10 sticks of cigarette per day. This may be attributed to the fact chronicity may be associated with adaptation mechanisms. Chronic smoking may induce increased activity of antioxidant system to neutralize the increased ROS associated with smoking in other to restore redox equilibrium between pro-oxidants and antioxidants.

The ferritin levels of smokers were higher than those of nonsmokers studied. Increased ferritin levels have also been demonstrated in current and former smokers. Serum ferritin is an acute-phase reactant and may be increased in the presence of inflammation. Smoking has been associated with oxidative stress and tissue inflammation, thus elevated ferritin levels seen in smokers may therefore be associated with inflammatory reactions as a result of smoking which can rapidly increase the expression of ferritin protein [23]. Serum ferritin reflects total stored iron concentration, and its increase with cigarette smoking suggests an accumulation of the metal in smokers and overabundance relative to metabolic needs. Overabundance of iron catalyzes the formation of ROS [24]. Iron (Fe^{2+}) catalyzes the conversion of hydrogen peroxide to the highly reactive and toxic hydroxyl radical (the Fenton and Haber–Weiss reactions [25]. Cigarette smokers, in addition to having

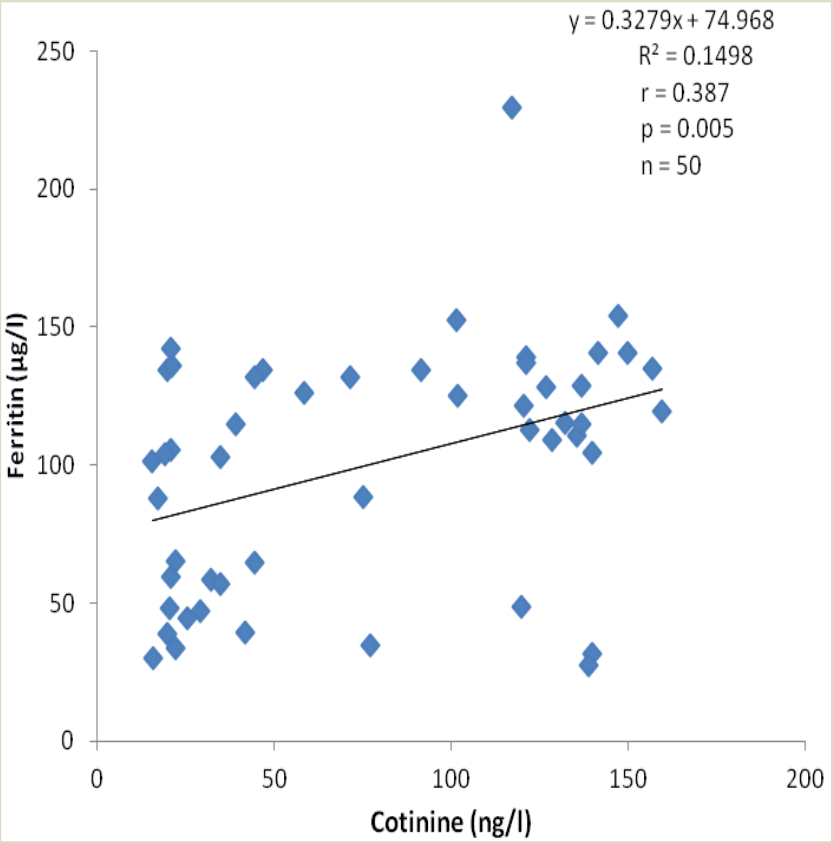

FIGURE 1. Correlation plot of ferritin against cotinine levels in smokers. From the figure, serum cotinine correlated positively with serum ferritin levels in smokers studied. The higher the cotinine levels, the higher the ferritin levels.

increased numbers of alveolar phagocytes that generate high levels of ROS, have also been shown to contain increased amounts of iron in their alveoli thus aggravating ROS generation in smokers [26].

Higher NO levels were seen in smokers compared to nonsmokers studied. This could be attributed to high concentrations of inhaled NO from smoke. NO has been shown to be a chemical component of cigarettes smoke which can directly or indirectly lead to the formation of free radicals and oxidative stress [27]. NO itself at physiological concentrations is relatively unreactive with nonradical molecules. However, NO combines slowly with molecular oxygen in air (over a period of seconds) to form the toxic oxidant and nitrating agent, NO_2. NO may be converted to a number of more reactive derivatives, known collectively as RNS such as NO_2, N_2O_3, N_2O_4, and peroxynitrite. DNA damage and nitration of tyrosine in cells exposed to the gas phase of cigarette smoke have been attributed to the action of RNS [28]. NO_2 reacts rapidly with other smoke constituents to form nitrosocarbon-centered radicals which react instantaneously with molecular oxygen to form peroxyl radicals that react with NO to form alkoxyl radicals and NO_2, thereby creating a continuous cycle [29]. Higher serum NO concentrations have also been reported in smokers [27].

The GSH levels in smokers did not differ from those of nonsmokers. Comparable levels of GSH in smokers and nonsmokers may be attributed to adaptive responses that include upregulation of GSH antioxidant defenses in response to cigarette smoking-induced oxidative challenge. The GSH adaptive response consists of a coordinated response between

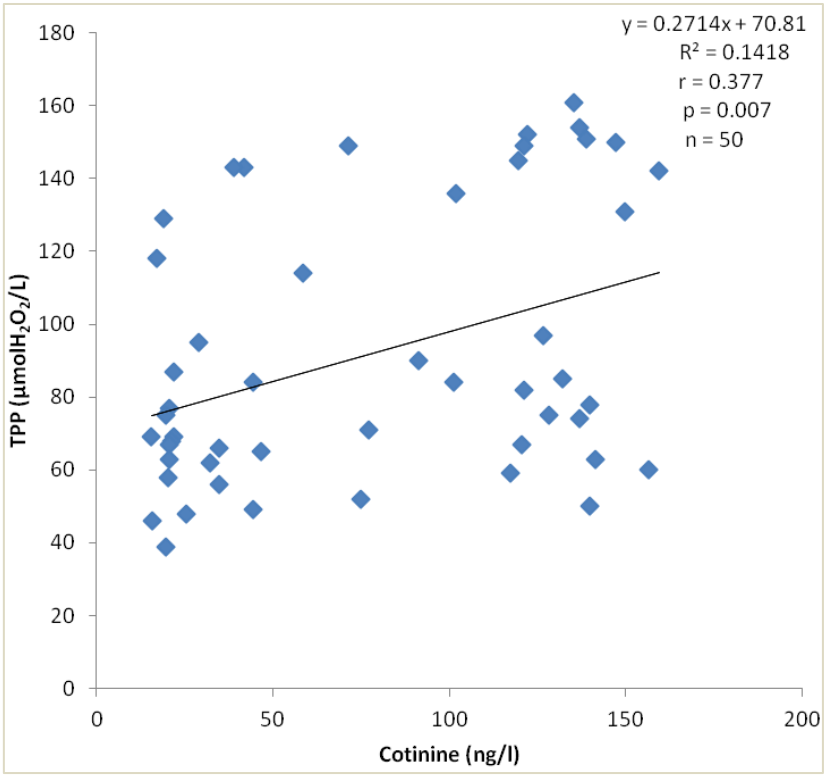

FIGURE 2. Correlation plot of TPP against cotinine levels in smokers. From the figure, serum cotinine correlated positively with the total plasma peroxide levels in smokers studied. The higher the cotinine levels, the higher the levels of lipid peroxidation.

GSH synthesis, utilization, recycling, and transport [30]. However, the GSH levels of those who have been smoking for < 5 years were significantly higher than those who have been smoking for > 10 years. Exposure to cigarette smoke has been reported to cause a decrease in the GSH concentration and in the expression or activity of several antioxidant enzymes such as glutathione peroxidase (GPx), SOD, and CAT [31]. This depletion may directly be associated with elevation in lipid peroxidation which could be attributed to increased ROS generation by cigarette smoking, besides its consumption by the antioxidant enzymes GPx. Acetaldehyde, a major aldehyde from cigarette smoking has been shown to deplete the cells of their GSH by conjugating with it, which further makes the cells more vulnerable to peroxidative damage [2].

Cotinine levels were higher in smokers and correlated positively with ferritin and TPP. Cotinine, a major metabolite of nicotine is currently considered the best indicator of tobacco smoke exposure. It is specific for nicotine, has a longer half-life (15–40 h), and its level is thought to be directly proportional to the quantity of absorbed nicotine, duration, and frequency of exposure. The quantity of nicotine absorbed by smokers is quite variable, being dependent upon its concentration in the smoke, the individual's smoking pattern, and the pH of the smoke [32]. This implies that the greater the exposure to nicotine and oxidants in cigarette smoke, the higher the cotinine value and the resultant lipid peroxidation product (TPP) and iron stores.

5. CONCLUSION

The findings of this study suggest that smoking is associated with increased NO, ferritin, and lipid pe-

roxidation, while increasing duration of smoking with depletion of GSH, which may predispose to oxidative stress and smoking-related complications.

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