Relationship between oxidative stress biomarkers and micronuclei scoring as a biomarker for DNA damage in Egyptian nicotine-dependent smokers: A cross-sectional study

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Background/aim
About one-fifth of cancer deaths are caused by tobacco smoking. Nicotine-related DNA damage is thought to be the result of oxidative stress, whereas DNA damage can be represented in the form of binucleated micronuclei (BMNi). The aim of this study is to determine the relationship between oxidative stress biomarkers and micronuclei scoring in nicotine-dependent smokers.

Subjects and methods
The present study enrolled 112 Egyptian male participants, of which 45 nicotine-dependent smokers matched with 67 nonsmokers. All participants have been clinically evaluated and investigated for oxidative stress biomarkers, cytogenetic analysis of BMNi, and urinary cotinine creatinine ratio concentration.

Results
The smokers showed significantly higher mean values of the serum malondialdehayde concentration ($P<0.05$), and lower mean values of both reduced glutathione concentration and the serum total antioxidant capacity ($P<0.05$) in comparison to the nonsmokers. Moreover, the smokers showed significantly higher mean values of binucleated micronuclei score ($P<0.05$) than the nonsmokers. The linear regression model predicted that each increase in the serum malondialdehyde by 1 nmol/ml could increase the BMNi score by 2.7. On the other side, it is predicted that each decrease in the age of starting smoking by a year could be associated with a decrease in the plasma level of reduced glutathione by 0.5 mg/dl.

Conclusion
Serum level of MDA is a reliable tool to predict the binucleated micronuclei score in smokers. It is suggested that smoking at earlier ages worsens the natural antioxidant defense system. Smoking cessation and the use of supplementary antioxidants by smokers may regress the oxidative stress-induced DNA-damage.

Keywords:
glutathione, malondialdehayde, micronuclei, oxidative stress, smoking

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Introduction

Tobacco smoking is the most common risk factor for the incidence of different types of cancer worldwide including lung cancer. It is estimated that nine of every 10 cases of lung cancer are caused by tobacco use [1] and one of every six deaths is caused by cancer. Further, more than 80% of smokers live in low and middle countries [2].

Cigarette smoke is composed of over 4,500 components in its gaseous and particulate phases. These include direct carcinogens (methylcholanthrene, benzopyrenes, and acrolein), toxins (carbon monoxide, nicotine, ammonia, acetone, and hydroquinone), reactive solids with chemically catalytic surfaces, and oxidants (superoxide and nitrogen oxides) [3]. Moreover, cigarette smoke contains 10^{17} oxidant molecules per puff [4].

Under normal physiological conditions, there is a balance between oxidants and endogenous antioxidants in the body, such as reduced Glutathione (GSH) and Alpha Lipoic Acid (ALA). Oxidative stress (OxS) refers to a disturbance caused by an imbalance between the generation of free radicals and the antioxidant system, which causes damage to biomolecules and cells leading to many chronic degenerative diseases, cardiovascular diseases, cancer, chronic obstructive pulmonary disease, and much other pathology [5]. Smoking may increase oxidative stress not only through the production of reactive oxygen radicals in smoke but also through a weakening of the antioxidant defense systems [6].

Malondialdehyde ($CH_2(CHO)_2$, is an organic compound and is considered a marker for oxidative stress and cell membrane damage caused by reactive oxygen species (ROS) or free radicals such as $O_2^{\bullet-}$ (superoxide anion radical), $^{\bullet}OH$ (hydroxyl radical) and H_2O_2 (hydrogen peroxide). Micronuclei (BMNi) are considered a reliable biomarker for genotoxic exposure and genomic instability in mammalian cells [7]. Nicotine consumption by cigarette smokers has significant genotoxic effects in human cells, including the upper aerodigestive tract as well as peripheral lymphocytes [8]. Nicotine-induced DNA damage is assumed to be a consequence of oxidative stress; whereas DNA damage can be represented in the form of micronuclei (MNi) [9].

The main aim of this study is to explore the relationship between oxidative stress biomarkers and the micronuclei in nicotine-dependent smokers. Identifying this correlation could help in clinical practice as a preventive measure to minimize smoking-related health hazards.

Subjects and methods

Subjects and study design

This is descriptive cross-sectional study has been conducted in the National Research Center, Egypt as part from the project entitled "Nicotine Dependence as environmental health problem, the efficacy of different approaches for its management" During the eleventh research plan under number 11010179. It included 112 eligible participants; of which 45 eligible male nicotine-dependent smokers, and 67 age- sex-matched nonsmokers as a control group.

Inclusion and exclusion criteria

All participants were cancer free and apparently healthy. Participants who had any compromising disease such as diabetes, under dietary supplementations one year before the blood sampling, exposed to DNA-cytotoxic drugs or ionizing radiation one year before the sampling or occupationally exposed to chemicals, such as organic solvents, were excluded from this study.

Ethical consideration

The present study was conducted with the Code of Ethics of the World Medical Association, according to the principles expressed in the Declaration of Helsinki. This study has been approved by the local Ethics Committee of the National Research Centre, Cairo, Egypt with approval number 16-402/2016-2019. A written informed consent was provided by each participant prior to their inclusion in the study.

Methods

All the participants were interviewed to fulfill a pretested questionnaire form and to be assessed clinically. Detailed personal, smoking history, Alcohol consumption, and special habits, occupational, medical, and family histories of cancers were covered in the questionnaire.

The body mass index (BMI) was calculated as a person's weight in kilograms divided by the square of height in meters [10]. The nicotine dependence status of the smokers was assessed using heaviness of smoking index (HSI), score $\geq$4 was considered a highly nicotine-dependent smoker as described by Lim *et al.* [11].

Biochemical assays

Assay of the oxidative stress biomarkers

Serum levels of Malondialdehyde (MDA) and total antioxidant capacity (TAC) were assayed calorimetrically as described in Satoh [12], Koracevic *et al.* [13], and Metwally *et al.* [14] respectively. Reduced glutathione (GSH) has been assayed in the heparinized blood using Colorimetric Method as described in Beutler *et al.* [15].

Assay of the nicotine metabolite

Urinary Cotinine Concentration was assayed using Enzyme Linked Immuno-sorbent assay(ELISA), a colorimetric method. A commercial cotinine Elisa kits from Sigma-Aldrish Co. LLC., USA, was used [16], and the concentration was presented as pg/ml. Creatinine concentration in the urine was assayed using calorimetric, Alkaline picrate method (Jaffe) as described by Bartles [17], using Calorimetric assay kit (Cayman, USA). The concentration was expressed as mg/dl. Cotinine Creatinine Ratio (CCR) then was calculated and expressed as Pg/mg. Cr.as recommended by Benowitz [18].

Cytogenetic analysis

A blood sample (3–3.5 ml) was withdrawn from each participant into a sterile heparin-coated vacutainer. Culture tubes were prepared as follows: each 100 ml bottle of RPMI1640 was added to 25 ml fetal bovine serum, 4 ml L-glutamine, 1.5 ml penicillin/streptomycin solution, and 5 ml of phytohemagglutinine. They were mixed well and distributed in sterile falcon's flat-tipped 5 ml tubes, and then 0.4 ml blood was added for each culture tube.

Table 1 General characteristics of nicotine-dependent smokers and nonsmokers

Characteristics	Nicotine-dependent Smokers (*n*=45)	Nonsmokers (*n*=67)	*P value*
Age (years)	40.7±9.9	37.5±9.9	0.200
Body Mass index (kg/m^2)	29.1±6.2	27.5±7	0.400
Number of cigarette/day	25.4±12.4	——-	
Duration of smoking (years)	20.4±9.6	——-	
Initial age of starting smoking(years)	15.1±3.6	——-	
Heaviness of smoking index Score(HSI)	4.1±1.7	——-	
Cotinine/Creatinine Ratio (CCR) (pg/mg cr.)	25.7±8.7	10.1±6.5	<0.001*

*Significant at $P<0.05$, using Student's *t*-test.

Two culture tubes were set up for each partici. To detect micronuclei, Cytokinesis-block micronucleus assay (CBMN) in peripheral blood lymphocytes was done. After initiation of culture at 37°C by 44 h (h), cytochalasin-B (Cytochalasin-B from Drechslera dematioidea, Sigma –Aldrich (now Merck)) was added for blocking of cytokinesis. Subsequently, scoring is selectively directed to binucleated cells (BN), as these cells can present MNi [19], then the culture was completed for another 24-28 h before harvest and Giemsa staining. Five hundred BN were studied and multiplied by two for each case for scoring of MNi. BN cells with MNi were photographed using computer-supported image analyzer (computer-assisted camera system; Applied Imaging, San Jose, CA, USA). Scoring of MNi in BN cells prevents confusing effects caused by altered cell division kinetics [20].

Statistical analysis

The collected data have been computerized and statistically analyzed using IBM –SPSS version 20.0 software (Statistical Package for Social Science). Quantitative data were presented as mean values ± standard deviation (SD) and compared Student's *t* test for two groups. The correlation between individual variables was tested using Pearson correlation coefficient (*r*). Linear Regression analysis has been used to predict the changes in one variable based on the change of the other. *P values* ≤ 0.05 were considered statistically significant.

Results

A total of 112 male participants were recruited in the study, including 45 nicotine-dependent smokers matched by age and sex with 67 nonsmokers (controls). No significant difference was observed between the smokers and nonsmokers in their body mass index (BMI). A statistically significant higher cotinine creatinine ratio was detected among the smokers group in comparison to the nonsmokers.

Table 2 The oxidative stress biomarkers and micronuclei scoring in smokers and nonsmokers

Biomarker	Nicotine-dependent Smokers (*n*=45)	Nonsmokers (*n*=67)	*P value*
Malondialdehayde (nmol/ml)	1.8±0.7	1.4±0.2	0.001*
Reduced Glutathione GSH (mg/dl)	1.7±0.4	2.1±0.3	<0.001*
Total antioxidant Capacity (mmol/l)	2.7±0.9	3.3±0.9	0.020*
BMNi score	5.6±4.4	1.1±2.1	<0.001*

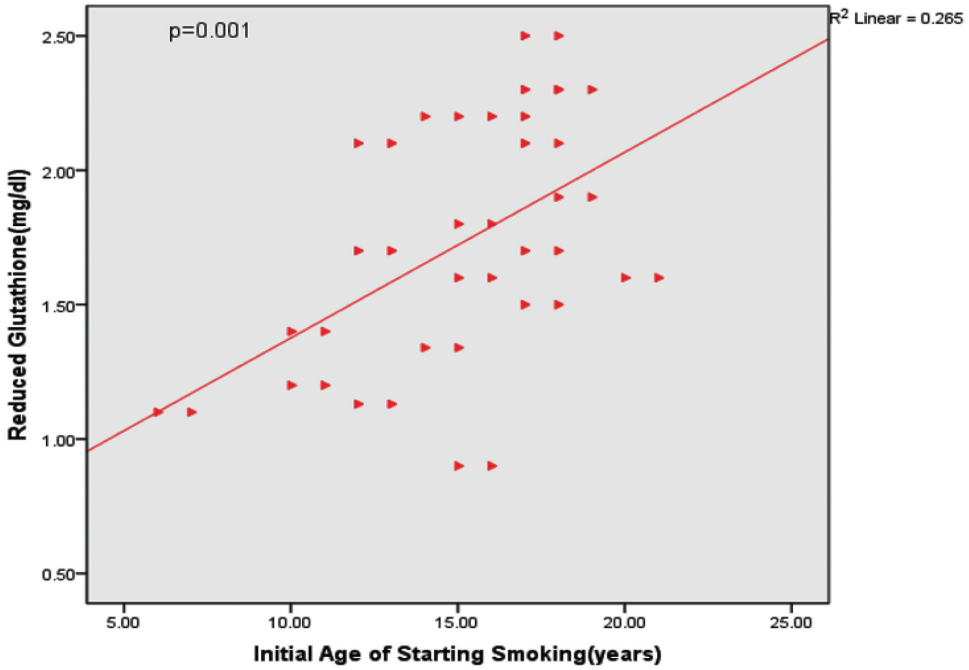
BMNi, Binucleated Micronuclei in T-Lymphocytes. *Significant at $P<0.05$, using Student's *t*-test.

Table 1 summarizes the general characteristics of the smokers and nonsmokers group.

The nicotine-dependent smokers showed significantly higher mean values of the serum Malondialdehayde (MDA) concentration, and lower mean values of both reduced glutathione (GSH) concentration and the serum total antioxidant Capacity(TAC) in comparison to the nonsmokers. Moreover, the smokers showed significantly higher mean values of binucleated micronuclei score than the nonsmokers (Table 2). The normal Binucleated Lymphocyte cells are demonstrated in (Fig. 2a, b) and the Micronucleated Binucleated Lymphocyte cells are demonstrated in (Fig. 3a, b).

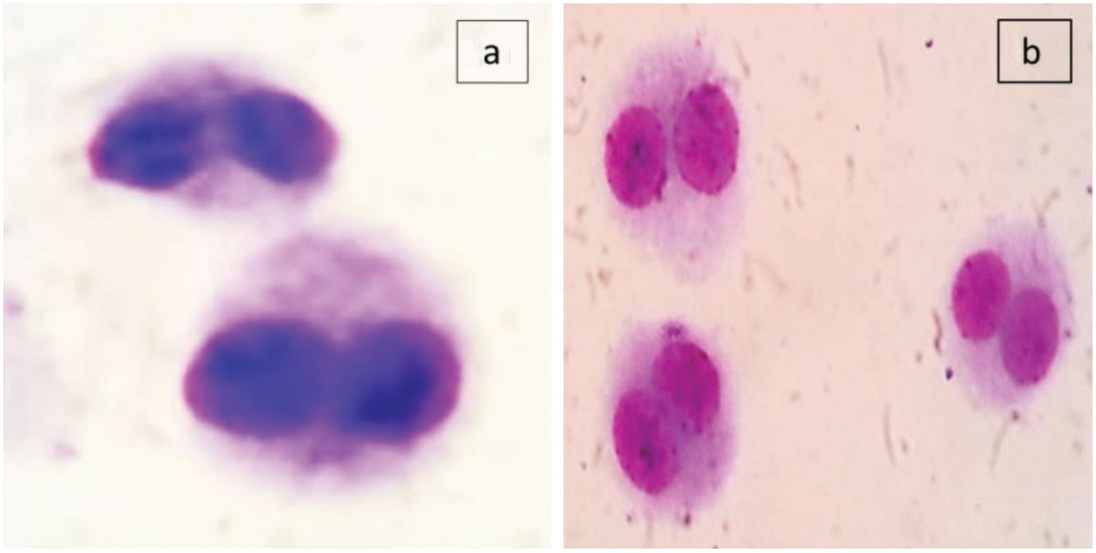
A significant direct linear correlation could be detected between the binucleated micronuclei score (BMNi) and the serum malondialdehayde ($r=0.4$, $P<0.0001$). On the other side, no significant correlation could be detected neither with the Reduced Glutathione nor with the total antioxidant capacity concentrations ($P>0.05$). The linear regression model predicted that each increase in the serum malondialdehayde by 1 nmo/ml could increase in the BMNi score by 2.7 ($P<0.0001$). Furthermore, a significant direct linear correlation could be detected between BMNi score and

Figure 1



Pearson correlation between the serum level of reduced glutathione and the initial age of starting smoking.

Figure 2

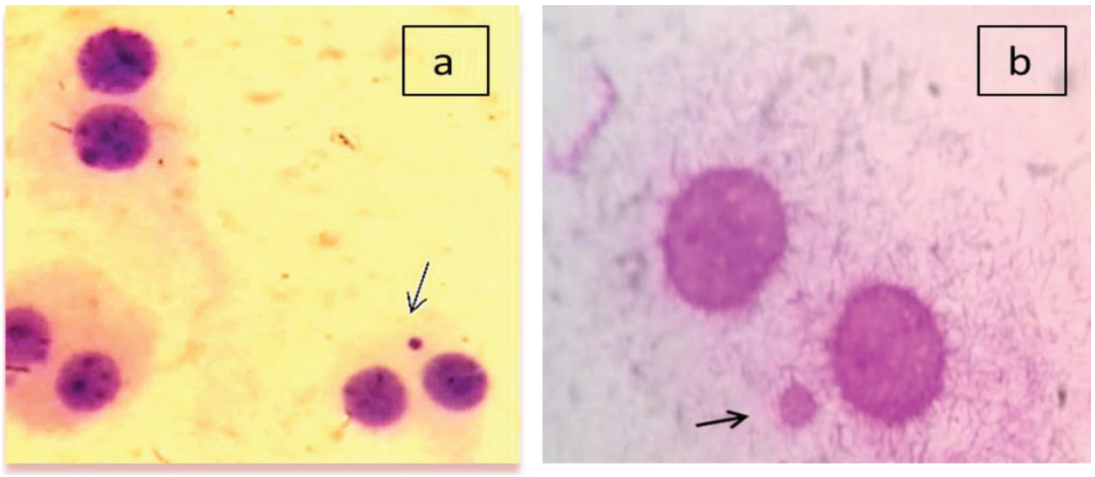


Photomicrograph showed normal Binucleated T- Lymphocyte cells using computer-assisted image analysis system in the peripheral blood showed the absence of micronuclei (MNi), Original magnification X100 (2a) and (2b).

the heaviness of the smoking index score (HSI) (Table 3).

An inverse linear correlation could be detected between the smoker's age and their GSH concentrations(r=0.2, *P*=0.02). After controlling the effect of age as a confounding factor, a significant direct linear correlation could be detected between the age of starting smoking for the first time and the reduced glutathione concentrations (mg/dl) as illustrated in (Fig. 1). It is predicted that each decrease in the age of starting smoking by a year, could be associated with a decrease in the plasma level of reduced glutathione (GSH) by 0.5 mg/dl. However, no significant

Figure 3



Micronucleated Binucleated T-Lymphocyte cells in the peripheral blood, an arrow pointed to the micronucleus (3a and 3b). It has been photographed by the author using computer-assisted image analysis system (Photomicrograph), we can note the specific criteria of micronucleus (MNi). It is smaller in size than the main nucleus, round or oval in shape, and not connected to the main nucleus. Original magnification X100 (3a) and (3b).

Table 3 Linear regression model predicts the relationship between the smoking, oxidative stress biomarkers and micronuclei score as a marker for DNA damage

	Unstandardized coefficients (95%CI)		Standardized coefficients		
Oxidative stress marker	Beta	SE	B	t value	*P value*
Malondialdehayde (nmol/ml)	2.7	0.6	0.4	4.5	<0.001*
Reduced Glutathione GSH(mg/dl)	–1.1	0.7	–0.2	–1.5	0.110
Total antioxidant Capacity (mmol/l)	–0.3	0.3	–0.09	–0.9	0.374
Heaviness of smoking index Score(HSI)	1.2	0.5	0.5	2.5	0.020*

* Significant at $P<0.05$.

Table 4 Linear regression model predicts the relationship between the age of starting smoking and the oxidative stress biomarkers

	Unstandardized coefficients (95%CI)		Standardized coefficients		
Oxidative stress marker	Beta	SE	B	t value	*P value*
Malondialdehayde (nmol/ml)	–0.06	0.6	–0.01	–0.09	0.926
Reduced Glutathione GSH(mg/dl)	0.5	0.2	0.4	2.7	0.001*
Total antioxidant Capacity (mmol/l)	0.8	0.5	0.2	1.5	0.158

* Significant at $P<0.05$.

correlation could be detected either with the serum Malondialdehayde (nmol/ml) or with the serum total antioxidant Capacity (mmol/l), as shown Table 4.

Discussion

Cigarette smoking increases the likelihood to get lung cancer or die due to lung cancer 15-30 times than nonsmokers [21]. However, the mechanism of this carcinogenicity is still under the study. It has been reported that the reactive oxygen species (ROS) which are present in large amount in cigarette smoke, causes ROS-induced oxidative DNA damage that plays a major role in the development of lung cancer [22] In our recent previous study, we proved an association between smoking as an environmental pollutant and the binucleated micronuclei (BMNi) in the peripheral blood T-lymphocytes, as an early marker of DNA damage [23]. In the present study, we studied the relationship between the oxidative stress

biomarkers and the binucleated micronuclei (BMNi) in the peripheral blood T-lymphocytes.

The study has been conducted on 45 male nicotine-dependent smokers; they smoke on average 25.4±12.4 cigarettes/day since mean period 20.4±9.6 years. Their heaviness of smoking index score (HSI) ranged from 1 to 6 with mean value 4.1±1.7 indicating moderate to high nicotine-dependent state. The serum levels of the oxidative stress biomarker Malondialdehayde (MDA) were significantly higher in the nicotine-dependent smokers in comparison to their levels in the nonsmokers. In addition, the antioxidant defense enzyme reduced glutathione (GSH) showed significantly lower levels in the smokers than observed in the nonsmokers. Moreover, the total antioxidant capacity (TAC) was significantly lower in the smokers group in comparison to the nonsmokers.

This finding emphasizes the deleterious effect of smoking not only as oxidative stressor, but it could also weaken the natural antioxidant enzyme system which is in agreement with Isik [24].

On the other hand, the presence of the biomarker for DNA damage; binucleated micronuclei (BMNi) in the peripheral blood lymphocyte cells of the smokers, was significantly higher in comparison to the nonsmokers (Table 2). This finding could be explained by the genotoxic effect of tobacco smoking on human cells. Our result was in agreement with Mohammed *et al.* [23], Kleinsasser *et al.* [8], Larramendy *et al.*[25].

Zhang *et al.* [26] reported that, the endogenous formation of malondialdehayde and its reaction with DNA during intracellular oxidative stress forms MDA –DNA adducts which makes it a key biomarker of endogenous DNA damage. It has been reported also by Annamalay [27] that the determination of MDA levels in the blood as well as in tissue homogenates, is one of the important methods to predict the oxidative stress levels.

Interestingly, in this study we detected a significant direct linear correlation between the binucleated micronuclei score (BMNi) and the serum Malondialdehayde (r=0.400, $P<0.001$). The linear regression model predicted that each increase in the serum malondialdehayde by 1 nmol/ml could lead to an increase in the BMNi score by 2.7. On the contrary, no significant correlation could be detected either with the Reduced Glutathione or with the total antioxidant capacity concentrations. This finding may emphasize that the serum Malondialdehayde is not just an oxidative stress biomarker but it is also an indicator for lipid peroxidation and oxidative stress-induced-cell membrane damage. The linear regression model detected also direct linear correlation between the BMNi score and the heaviness of the smoking index of the smokers (Table 3). It has been reported in previous studies that a younger age at initiation of cigarette smoking is associated with an increased risk of lung cancer [28].

Donohue [29] reported higher oxidative stress levels among the older smokers with long-term smoking duration in comparison to the younger ones. In this study, we just detected an inverse linear correlation between the smoker's age and the reduced glutathione GSH (r=-0.200, P=0.02) (nontabulated). However, we could not detect a correlation with the oxidative stress biomarker serum MDA, as well as with the serum total antioxidant capacity (TAC). Moreover, after Adjustment for the smoker's age, a significant direct linear correlation could be detected between the age of smoking onset and the plasma level of reduced glutathione GSH (mg/dl) as illustrated in Fig. 1. It is predicted that each decrease in the age of starting smoking by a year, could be associated by a decrease in the plasma level of reduced glutathione (GSH) by 0.5 mg/dl. On the contrary, no significant correlation could be detected either with the serum Malondialdehayde (nmol/ml) or with the serum total antioxidant Capacity (mmol/l).

Conclusion

Based on our results, cigarette smoking is associated with an elevation of the oxidative stress biomarker MDA; and deteriorates the endogenous antioxidant enzyme activity as indicated by decreased plasma levels of the reduced glutathione and the serum levels of the total antioxidant capacity. The study identifies a direct linear correlation between the oxidative stress biomarker MDA and the score of the DNA-damage biomarker; binucleated micronuclei in the peripheral blood lymphocytes. Interestingly, we found that the serum level of MDA is a reliable tool to predict the binucleated micronuclei score in smokers. It is suggested that smoking at earlier ages worsens the natural antioxidant defense system as indicated by the marked decline in the plasma levels of the antioxidant enzyme; reduced glutathione (GSH) in conjunction with the lower age of smoking onset in the smokers. National smoking cessation programs are a primary preventive measure for tobacco-induced diseases including cancers. We recommend the use

of supplementary antioxidants by smokers to regress or decrease the oxidative stress biomarkers and to prevent progression of oxidative stress-induced DNA-damage and the development of cancer.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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