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The Effect of Smoking on Biomarkers of (Anti) oxidant Status

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Abstract

The influence of smoking on the levels of several biomarkers of oxidative stress, antioxidant status and redox status have been investigated in 48 healthy men with a mean age of 25 y. The biomarkers of oxidative stress are the reactive oxygen metabolytes (ROM) and the total oxidant status (TOS). The biomarkers for the antioxidant activity are the biological antioxidant potential (BAP), the ferric reducing ability of plasma (FRAP), the total antioxidant status (TAS), the assay for the defense against the oxidation by hypochlorous acid (OXY) and uric acid (UA). The total thiol levels (TTL) have been measured as a biomarker for the redox status.

The average concentration of ROM was 14% higher in smokers compared with non-smokers ($p<0.05$), whereas TOS was 4.9% higher (not statistically significant).The average concentrations of BAP, FRAP and TAS decreased with smoking with 3.6 ($p<0.025$), 3.6 (non-significant) and 6.1% ($p<0.025$), respectively. Alsouric acid, the main antioxidant in serum and a major contributor to the antioxidant status in serum, decreased by 10.6 % ($p<0.025$) in smokers. The biomarker of the redox status (TTL) was not influenced by smoking.

From these results, it is concluded that in epidemiological studies the effect of smoking should be taken in account when using oxidative stress and antioxidant biomarkers.

Keywords: Biomarkers; Oxidative stress; Antioxidant status; Redox status; Smoking

Introduction

Biomarker studies of oxidative stress and antioxidant status are frequently used to determine the risk of developing chronic diseases, such as cancer, cardiovascular diseases or cognitive decline. The influence of lifestyle factors can have a substantial effect on the biomarker concentrations and therefore these factors, including smoking, should be taken into account in biomarker-based studies [1].

During smoking, free radicals are formed that can activate inflammatory cells which generate high levels of reactive oxygen metabolites [2,3]. Therefore smokers are subjected to an increased oxidative stress situation, which can result in an imbalance between oxidants and antioxidants [4]. As a result, biomarkers of oxidative stress, antioxidant and redox status will be influenced by smoking behavior [5,6].

In the present study the influence of smoking on biomarkers of both oxidative stress, redox and antioxidant status was investigated in a healthy male population.

Materials and Methods

The 48 human volunteers (all men) were selected from 50 healthy candidates for military service. According to a smoking questionnaire, 23 volunteers were smokers and 25 volunteers were non-smokers. The mean age of the smokers was 25.5 y and of the non-smokers 24.9 y. The smokers smoked 10-20 cigarettes/day for more than 5 years, whereas the non-smokers did not smoke for at least 5 years. Two volunteers did not have a clear statement about the smoking status. Fasting blood samples were drawn from the antecubital vein. Blood samples were processedby centrifugation and aliquots of serum were stored at -70°C until analysis.

The study was performed and approved under ethical guidance of Dr. Dusan Stojanovik as documented by the Command of the Military Medical Center, Ministry of Defense of the Republic of Macedonia, and described in Document N no. 04-7/18 from the Army Mail 2990/80, Skopje, Republic of Macedonia.

The ROM assay

The Reactive Oxygen Metabolites (ROM) were measured by the kit (dROMs) from Diacron (Grosseto, Italy). The method measures the concentration of hydroperoxides which are present in the serum sample. The method is based on the principle that, in an acidic solution (pH=4.8), iron is released from transferrin and is available to catalyze the reaction of hydroperoxides to alkoxyl and peroxyl radicals, which further react with chromogen N,N-diethyl-p-phenylenediamine. Upon oxidation, the chromogen is transformed in red colored cation which is measured at 505 nm. The results of the test are expressed in CARR U (Carratelli Units). Each CARR U corresponds to 0.08 mg H_2O_2/100 mL sample. The reagents from the kit of Diacron were adjusted for use on the autoanalyzer LX20-Pro from Beckman-Coulter (Woerden, the Netherlands).

The TOS assay

The Total Oxidant Status (TOS) was measured by the kit from RelAssay Diagnostics (Gaziantep, Turkey). The method is based on the principle that oxidants which are present in the sample can oxidize the ferrous ions to ferric ions [7]. Then, in an acidic medium, the ferric ions form a colored complex with a chromogen. The intensity of the color was measured at 530 nm. The assay was calibrated with H_2O_2, and the results of the assay are expressed in μmol H_2O_2 Eq/L. The assay was performed in microtiter plates.

The BAP assay

For measurement of the Biological Antioxidant Potential (BAP) the kit from Diacron (Grosseto, Italy) was used. The assay is based on the decrease in absorption when Fe^{3+} ions that bind to a thiocyanate-derived substrate, are reduced to Fe^{2+}. The absorbance was measuredat 505 nm, and the amount of reduced ferric ions can be calculated. The results of the assay are expressed as μ Eq ferric ions reducing antioxidants per L of sample. The kit from Diacron was adjusted for the use on the auto-analyzer LX20-Pro from Beckman-Coulter (Woerden, the Netherlands).

The FRAP assay

The measurement of the Ferric Reducing Ability of Plasma (FRAP) was done by the assay based on the method of Benzie and Strain [8], slightly modified. The method is based on the principle of reduction of ferric-tripyridyltriazine complex to ferrous form, upon which an intense blue color develops, and the change of absorbance is measured at 593 nm (kinetic method). We have measured the ferric reducing ability of plasma in a microplate format, by the end-point approach. The absorbance was measured on a Chem Well analyzer (Palm City, FL, USA) at 600 nm, against reagent blank. Standards of 500, 1000 and 2000 μmol/L FeSO4were used for calibration of the assay. The results of the test are expressed as μmol/L $FeSO_4$.

The TAS assay

The Total Antioxidant Status (TAS) was measured by the test kit from RelAssay Diagnostics (Gaziantep, Turkey). The method is based on the reduction of colored ABTS radical by antioxidants that are present in the sample. The absorbance is measured at 660 nm. The kit from RelAssay was adjusted for the use on the auto-analyzer LX20-Pro from Beckman-Coulter (Woerden, the Netherlands).

The TTL assay

The Total Thiols Levels (TTL) were measured using also the reagent kit from RelAssay Diagnostics (Gaziantep, Turkey). The method is based on the reaction of plasma thiols with 5,5'-dithiobis-(2-nitrobenzoic acid), resulting in an increase in absorption at 412 nm. The results of the test are expressed in μmol/L. The kit from RelAssay was adjusted for the use on the auto-analyzer LX20-Pro from Beckman-Coulter (Woerden, the Netherlands).

The OXY assay

The OXY-Adsorbent test from Diacron (Grosseto, Italy) measures the ability to oppose against oxidation, induced by hypochlorous acid. Unreacted HClO radicals further react with the chromogen solution of N,N-diethyl-p-phenylenediamine and forms a colored complex, which is measured at 505 nm. The results of the test are expressed as μmol HClO/mL. The kit from Diacron was adjusted for the use on the auto-analyzer LX20-Pro from Beckman-Coulter (Woerden, the Netherlands).

The uric acid assay

Uric acid (UA) was measured by the enzymatic method using uricase and was performed on an auto-analyzer LX20-Pro from Beckman-Coulter (Woerden, the Netherlands) with a dedicated kit for uric acid.

Statistics

The results of all measurements are expressed as mean ± standard deviation. The distribution of data was assessed with Kolmogorov-Smirnov test, using Statistica-7 software.

Because all data sets were normally distributed, further analysis was performed with Student's t-test, two-sample equal variance or two-sample unequal variance, as appropriate, using Microsoft Excel. The differences between groups were considered as statistically significant if $p<0.05$.

The coefficients of correlation between the assays were calculated with Microsoft Excel. The statistical significance of the coefficients of correlation was assessed according to the number of subjects within the group, using a common statistical table [9]. The correlation coefficient was considered as statistically significant when $p<0.05$.

Results

In this human volunteers study two assays for oxidative stress, ROM and TOS were used. Both biomarkers show a higher level in the serum of smokers of 13.8 and 4.9 % for ROM and TOS, respectively, as shown in Figure 1. The increase of ROM is statistically significant ($p<0.05$). The large variability of the TOS assay prohibits a statistical significance.

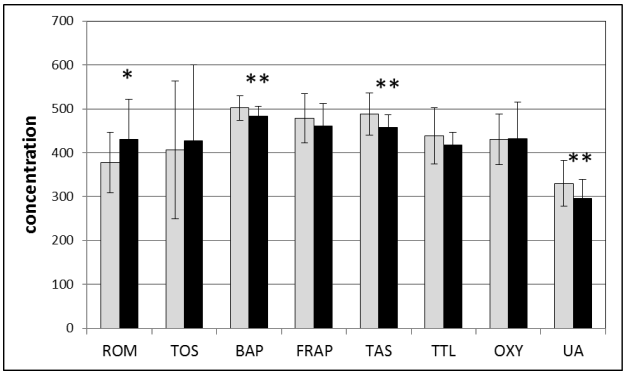

Figure 1: The effect of smoking on several biomarkers in human serum of non-smokers (grey bars) and smokers (black bars): ROM (CARR U), TOS (μmol H_2O_2Eq/L), BAP (μEq/L), FRAP (μmol/L), TAS (mmol/L), TTL (μmol/L), OXY (μmol HClO/mL) and UA (μmol/L). The concentrations of TOS and TAS were multiplied by a factor 200 and 300, respectively, and the concentration of BAP and FRAP were divided by a factor 5 and 3, respectively, to fit in the figure. Statistics: * $p<0.05$ and ** $p<0.025$.

Three of the biomarkers of the antioxidant status (BAP, FRAP and TAS) show lower levels in smokers but by a small percentage only, ranging between 3.6 to 6.1 % (Table 1). The decreases of BAP and TAS were statistically significant with p-values of 0.016 and 0.012, respectively.

In the OXY test the same concentrations were observed between the smokers and non-smokers.

The total thiol assay reflects the redox status. In smokers, this biomarker was 4.8 % lower compared with non-smokers, but this decrease was not statistically significant.

In addition, UA was determined because it is the most important contributor to the total antioxidant status. UA decreased with 10.6 % in smokers with a statistically significant p-value of 0.017 (Table 1).

Biomarker	% Change	P-value
ROM	13.8	0.030*
TOS	4.92	0.671
BAP	-3.62	0.016**
FRAP	-3.56	0.282
TAS	-6.13	0.012**
TTL	-4.78	0.141
OXY	0.70	0.890
UA	-10.61	0.017**
*p<0.05; **p<0.025.		

Table 1: The mean percentage of change in sera of smoking human volunteers compared with non-smoking volunteers.

Non-smokers (N=25)	BAP	FRAP	ROM	TAS	TOS	TTL	UA	OXY
BAP	1.00							
FRAP	0.17	1.00						
ROM	-0.18	0.09	1.00					
TAS	0.45*	0.84**	0.01	1.00				
TOS	-0.36	0.18	0.10	-0.12	1.00			
TTL	0.47*	-0.15	-0.03	0.17	-0.21	1.00		
URIC	0.30	0.86**	-0.01	0.94**	-0.08	-0.01	1.00	
OXY	0.13	0.36	-0.03	0.48*	-0.22	-0.01	0.56**	1.00
Smokers (N=23)	BAP	FRAP	ROM	TAS	TOS	TTL	UA	OXY
BAP	1.00							
FRAP	-0.19	1.00						
ROM	0.18	0.13	1.00					
TAS	0.02	0.80**	-0.14	1.00				
TOS	0.04	-0.05	0.12	-0.23	1.00			
TTL	0.50*	0.20	-0.29	0.30	0.19	1.00		
URIC	-0.15	0.89**	0.07	0.89**	-0.22	0.17	1.00	
OXY	-0.26	-0.05	0.00	-0.19	0.14	-0.06	-0.08	1.00
*p<0.05; **p<0.01.								

Table 2: Coefficients of correlation between the assays as performed on serum samples of non-smokers and smokers. For non-smokers the threshold value for a statistical significant correlation of $p<0.05$ is $r=0.396$, and for a statistical significance of $p<0.01$ this value is 0.505. For smokers these threshold values are $r=0.413$ and $r=0.526$, respectively.

The correlations between the various biomarkers of oxidant and antioxidant status are different for smokers and non-smokers. In Table 2 the correlations are shown between all biomarker assays presented in this study. The statistically significant correlations between BAP and TAS, as well as between OXY and TAS and between OXY and UA within the group of non-smokers are not present in the smokers group.

Discussion

In biomarker studies a number of modifying factors have to be taken into account such as storage conditions [10], the influence of post-prandial effects [11], circadian rhythm [12,13], body mass index [14] and the smoking behavior. In the present study the influence of smoking on number biomarkers of oxidative stress was investigated in healthy young men. As biomarkers of oxidative stress, ROM and TOS were measured, for the antioxidant activity BAP, FRAP, TAS, OXY and UA. TTL was measured as a biomarker for the redox status. Since epidemiological studies usually require a high throughput of samples, we have focused on biomarkers that can be measured on an auto-analyzer. Some of these biomarkers, such as the ROM and TTL in serum, have proven their value recently in large epidemiological studies [15-18]. Although the biomarkers of total antioxidant status are prone to some criticism recently [19], we have included a number of these biomarkers also to investigate their mutual correlation with and without the influence of smoking.

It was found that the oxidative stress biomarkers ROM and TOS showed an increase of 13.8 and 4.9 % in smokers vs non-smokers. Two other studies found also a small, but statistically significant increase in ROM [15,16]. In these studies the absolute increase of ROM as a consequence of smoking was not given because ROM was part of an integrated parameter of oxidative stress, the oxidative index. On the other handc Kotani et al. [20] and Keretetse et al. [21] did not find a relation of ROM with smoking behavior. For the TOS assay no data have been reported in literature.

The levels of the three antioxidant biomarkers (BAP, FRAP and TAS) decreased with smoking but to a smaller percentage compared to the increase of ROM. Also uric acid decreased by 10.6 % in smokers. Since uric acid is the main determinant of the antioxidant assays in serum or plasma [22], it could be expected that the assays for the antioxidant status also would measure lower values. In a recent paper [23], we reported the correlation coefficients of uric acid with BAP, FRAP, TAS and OXY. FRAP and TAS showed the highest correlation coefficient of 0.869 and 0.922, respectively. BAP and OXY showed

much lower correlation coefficients of 0.302 and 0.272, respectively. The 10.6 % decrease of UA in smokers, however, is not reflected in the decrease of FRAP (3.6 %) and TAS assay (6.1%). The loss of some correlation of TAS and OXY with some other biomarkers of antioxidant status as a result of smoking, remained to be explained.

Our study indicates once more that smokers have elevated concentration of oxidative stress biomarkers and also a compromised antioxidant status. Therefore, in biomarker-driven studies correction for smoking should be taken in account as a modifying factor on biomarkers of oxidative stress and antioxidant status. The redox status seems to be less sensitive to smoking.

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Declaration of interest

The authors stated that there are no conflicts of interest regarding the publication of this study.

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