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Original Contribution

STUDY ON OXIDATIVE STRESS BIOMARKERS IN WORKERS OF THE COLLIERY INDUSTRY

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ABSTRACT

The aim of the present research was to evaluate the effect of the surroundings on the oxidative status of workers in the mining industry. By means of two different analytical techniques have been investigated selected biomarkers of oxidative stress. By spectrophotometric methods have been studied the levels of lipid peroxidation products (MDA), catalase (CAT) activity, superoxide dismutase (SOD) activity in blood samples isolated from 30 vulcanizers exposed to trichlorethylene and 30 welders exposed to iron and manganese aerosols. Both real time biomarkers of oxidative stress such as levels of ascorbate radicals and reactive oxygen species (ROS) were also evaluated by electron paramagnetic resonance (EPR) spectroscopy. The control group consisted of 20 healthy volunteers at age close to that of the vulcanizers and welders was also studied.

By the present research were demonstrated increased levels of the studied oxidative stress biomarkers in workers from colliery "Troyanovo-1" and "Troianovo-north" to "Collieries Maritsa Iztok" EAD compared with healthy volunteers. It was supposed that increased production of reactive oxygen species might due to harmful surroundings under which the workers operate.

Key words: Trichloroethylene, vulcanisation, biomarkers, oxidative stress

INTRODUCTION

One of the main occupations in the colliery industry are machine operator curing of rubber products and welder. In the performance of their duties welders are exposed to the toxic effects of iron and manganese aerosols and vulcanizer to the toxic effects of trichloroethylene (TCE).

By inhalation of TCE is absorbed approximately 37 - 64% trichloroethylene by the lungs (1, 2) TCE is well soluble in lipids and therefore accumulates in organs containing high levels of adipose tissue, such as, the lungs, liver, kidneys and central nervous system (3). The metabolism of TCE occurs largely in the liver followed by excretion primarily in urine or unchanged in expired air (4). Based on the epidemiologic and experimental data, it is considered that TCE is potential human health hazard for noncancer toxicity to the central nervous system, kidney, liver, immune system, male reproductive system, and the developing fetus (5).

Different epidemiological studies indicate neurobehavioural, reproductive, and respiratory effects in workers exposed to manganese (6). Neurobehavioural effects most often expressed as the disturbances in the control of movements of the hand. Various studies have shown neurochemistry of manganese toxicity affected the levels of dopamine as an initial increase in dopamine followed by a longer-term decrease in humans, monkeys and rodents by exposure to manganese (7). Some authors have suggested that manganese neurotoxicity due to excessive manganese in the oxidation of dopamine, resulting in free radicals and cytotoxicity (8).

Purpose of the present study was to evaluate the oxidative status of workers in the colliery industry working under hazardous working conditions by assessing of biomarkers as lipid peroxidation products, catalase (CAT) activity,

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superoxide dismutase (SOD) activity. The levels of "real time" biomarkers of oxidative stress such as ascorbate radicals ($Asc^{\cdot}$) and reactive oxygen species (ROS) products were also studied by electron paramagnetic resonance (EPR) spectroscopy.

MATERIALS AND METHODS

PATIENTS AND BIOLOGICAL MATERIAL

Studies were conducted in blood samples obtained from 60 workers, 30 vulcanizers exposed to trichlorethylene and 30 welders exposed to iron and manganese aerosols from colliery "Troyanovo-1" and "Troianovo-north" to "Collieries Maritsa Iztok" EAD. The workers were divided in three groups. The 1st consisted of 15 vulcanizers without changes in sera levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), while the 2nd group was vulcanizers with deviations from the reference values of serum activity of AST and ALT. As it is known AST and ALT are markers of liver function and are part of the annual prophylactic medical examinations which are carried out to workers in colliery "Troyanovo-1" and "Troianovo-north" to " Collieries Maritsa Iztok" EAD. The 3rd group was composed of 30 welders. The control group consisted of 20 healthy volunteers at age close to that of the rest three groups.

Informed consent was obtained from all participants in the study according to the ethical guidelines of the Helsinki Declaration.

LABORATORY ANALYSIS

Determination of the lipid peroxidation products

The levels of MDA reactive products in the blood samples were determined by the tiobarbituric acid (TBA) method according to Plaser and Cushman 1966, with some modification by Gadjeva et al., (9). Results were expressed as micromols per mg protein MDA (μM/mgPr).

Determination of catalase activity

CAT activity in the erythrocyte lysates was assessed by the method described by Beers and Sizer (10). Briefly, hydrogen peroxide (30 mM) was used as a substrate and the decrease in its concentration at 22°C in phosphate buffer (50mM, pH7.0) was followed spectrophotometrically at 240 nm. One unit of CAT activity is defined as enzyme amount that degraded 1 μM H_2O_2 per minute. Results were expressed as international units per mg of total protein (IU/mgPr).

Determination of Superoxide dismutase activity

SOD activity was measured in the erythrocyte lysates as described by Sun et al. with minor modifications (11). The xanthine/xanthine oxidase system was used to produce the superoxide anion. This anion reduces nitroblue tetrazolium (NBT) to formazan, which is detected at 560 nm. SOD of the sample removes the superoxide anion and inhibits the reduction. The level of this reduction is used as a measure of SOD activity (12). The concentrations of xanthine, xanthine oxidase, and nitroblue tetrazolium in the assay were respectively 50 μM, 10 U/ml, and 0.125 mM. One unit of enzymatic activity is defined as an amount of that causes 50% inhibition of NBT reduction to formazin. The results were expressed as international units per mg of total protein (IU/mgPr).

Electron paramagnetic resonance (EPR) studies

All EPR measurements were performed at room temperature (18-23°C) and relative humidity 40 % on a X-band EMX^{micro}, spectrometer Bruker, Germany, equipped with standard Resonator. Experiments were carried out in triplicate. Spectral processing was performed using Bruker WIN-EPR and Simphonia software.

EPR *ex vivo* evaluation the levels of ascorbate radicals and ROS products in the studied blood sera

At *in vivo* conditions endogenic ascorbic acid can be oxidized by ROS to a stable ascorbate radical (13) and the last can be detected only by direct EPR spectroscopy which is the only method does not interfere with the biochemical processes (14). The levels of ascorbate radicals were studied according to Bailey, 2004 with some modification. Briefly, to 100μL serum was added 900μL DMSO, and after centrifugation at 4000 rpm for 10 min at 4ºC the supernatant was collected and immediately transferred into a quartz tubes and placed in EPR cavity (15).The level of ascorbate radicals was calculated as the height of the first peak of EPR spectrum and result was expressed in arbitrary units. EPR settings were as follows: 3505.00 G center field, 20.00 mW microwave power, 1.00 G modulation amplitude, 15 G sweep width, a receiver gain $1x10^5$, 40.96 ms time constant, 60.42s sweep time, 10 scans per sample.

EPR *ex vivo* evaluation the levels of ROS products in the studied blood sera

To investigate in real time formation of reactive oxygen species (ROS) in the sera of the workers and controls was used EPR spectroscopy combined with *ex vivo* PBN spin trapping. The spin trap PBN, upon reaction with unstable radicals forms a relatively stable spin adduct that can be subsequently detected by EPR spectroscopy. The levels of ROS were determined according Shi et al., 2005 with modifications by Zheleva et al., 2011 (16). Briefly, to 100μL serum was added 900μL 50 mM PBN dissolved in DMSO and after centrifugation at 4000 rpm for 10 min at 4ºC EPR spectrum of the supernatant was recorded. The levels of ROS products were calculated as double integrated plots of EPR spectra and results were expressed in arbitrary units. EPR setting were as follows: Center field – 3503.73 G, Microwave power – 20.00 mW, Modulation amplitude - 5G, Sweep width – 50 G, Receiver gain -1 x 10^5, Time constant – 81.92 ms, Sweep time – 125.95 sec. Scans per sample -5 scans.

Statistical analysis

Statistical analysis was performed with Statistica 7, StaSoft, Inc. And the results were expressed as means ± S.E. Statistical analysis was performed with Student's t-test. $P < 0.05$ was considered statistically significant.

RESULTS

On **Figure 1** are shown MDA levels measured in the blood samples of the workers and controls enroled in this study. As can be seen, in the 1st group, MDA levels were significantly higher than those of the control group (4.104 ± 0.121 μM vs. 3.56 ± 0.07μM, $P < 0.005$). The levels of MDA in the 2nd group were also significantly higher comparing to those of control group (4.83 ± 0.19μM vs. 3.56 ± 0.07μM, $P < 0.0002$). Only in the 3rd group, the levels of MDA were significantly decreased compared to those of the control group (3.15 ± 0.05 μM vs. 3.56 ± 0.07μM, $P < 0.002$).

On **Figure 2** are presented the levels of erythrocyte CAT activity measured in the four groups. As is seen, statistical significantly higher levels of CAT activity were found for the 1st and 2nd group comparing to those of the control group (21839.23 ± 2512.064 IU/gHb vs. 4518.03 ± 658.455 IU/gHb, $P < 0.0001$) and (29159.33 ± 2931.695 vs. 4518.03 ± 658.455 IU/gHb, $P < 0.00005$), respectively. In the 3rd group, the erythrocyte CAT activity was significantly decreased in comparison with the control group (1772.79 ± 173.539 IU/gHb vs. 4518.03 ± 658.455 IU/gHb, $P < 0.01$).

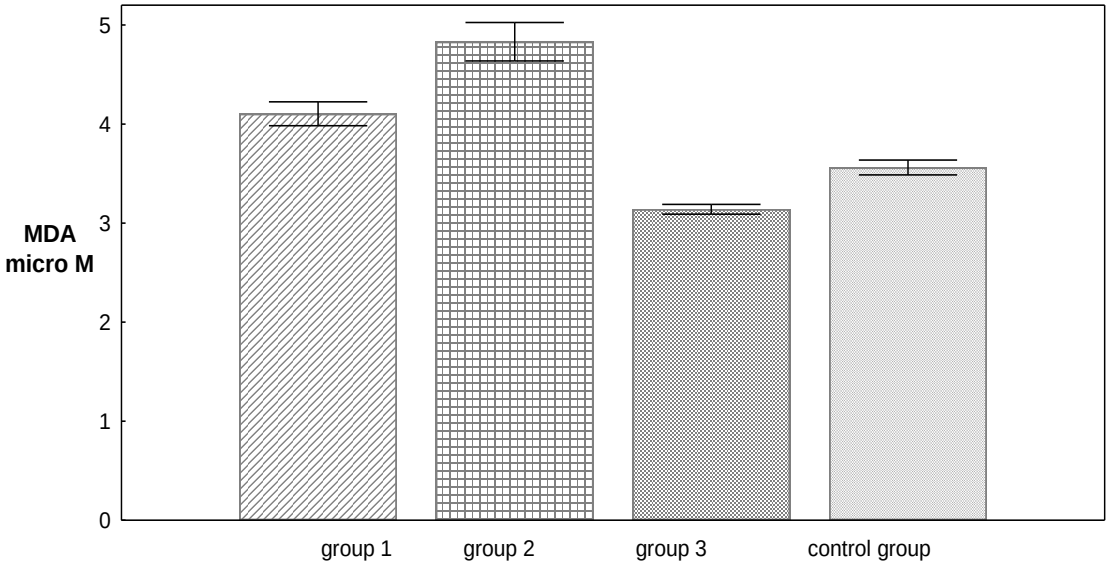

Figure 1. Levels of MDA reactive products in blood samples of the workers and healthy controls. Results are expressed as μM MDA products per mg protein.

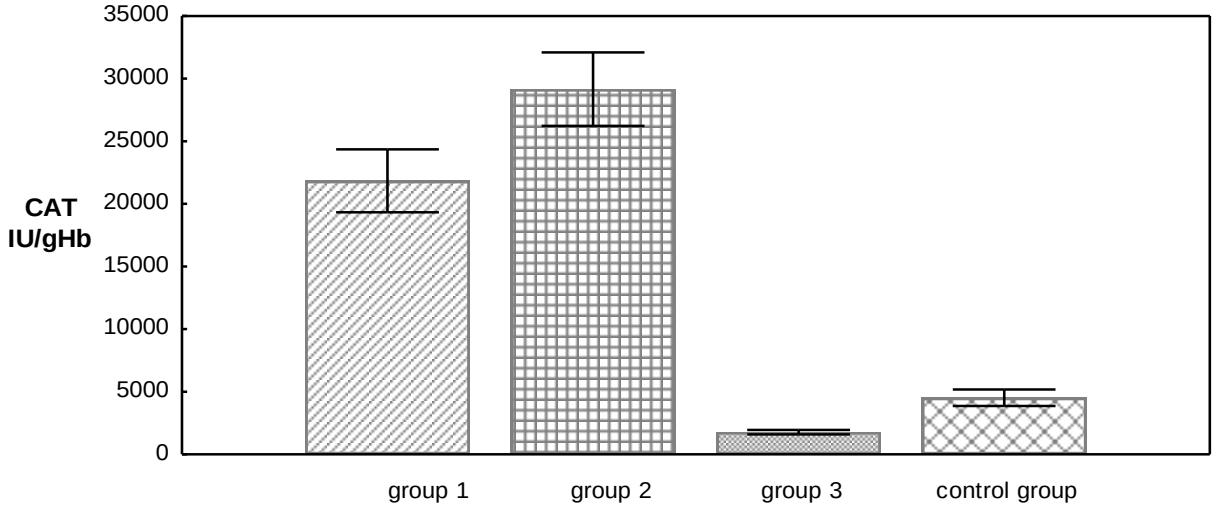

Figure 2. Levels of CAT activity in the erythrocyte lysates expressed as IU/gHb.

The erythrocyte SOD activity in all groups of workers was significantly higher compared with the control group. The levels of erythrocyte SOD activity in the 1st and 2nd group were significantly higher, comparing with the controls, respectively (369.408 ± 58.563 IU/gHb vs. 111.263 ± 10.899 IU/gHb, $P < 0.01$) and (677.527 ± 65.740 IU/gHb vs. 111.263 ± 10.899 IU/gHb, $P < 0.00001$). There was a significant increase in erythrocyte SOD activity of the 3rd group comparing to that of the control group (290.750 ± 28.353 IU/gHb vs. 111.263 ± 10.899 IU/gHb, $P < 0.0001$).

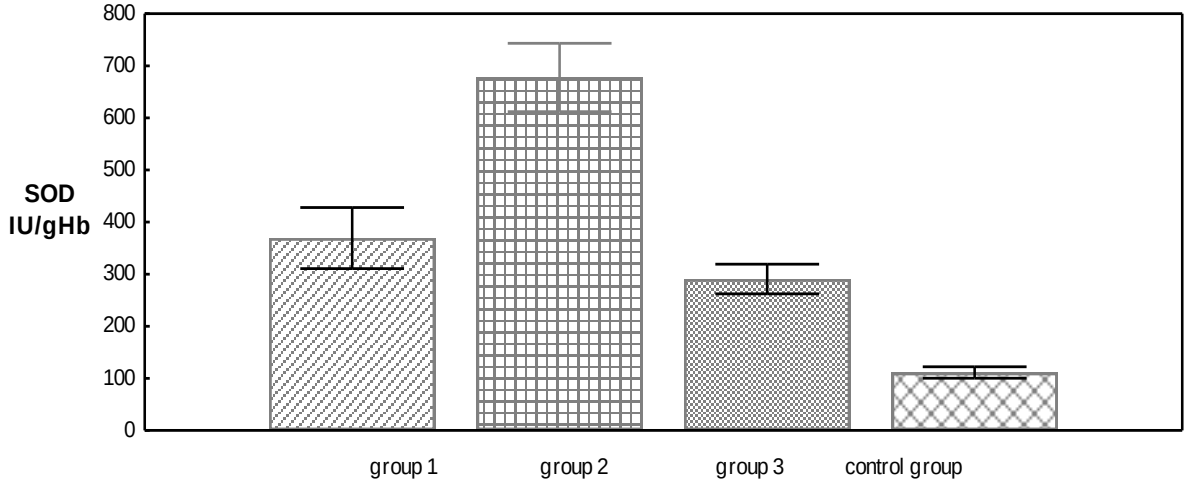

Figure 3. Levels of SOD activity in the erythrocyte lysates expressed as IU/gHb.

Results from the levels of ascorbate radicals measured in the studied sera are presented on **Figure 4.** In all studied samples was registered EPR doublet signal typical for the ascorbate radical (EPR spectrum is not shown). The level of ascorbate radicals was calculated from the height of first peak of the EPR doublet signal. The levels of ascorbate radicals registered in sera isolated from the workers of the 1st and 3rd group were significantly higher, compared to that of the healthy controls: 3386542 ± 153131.9 arb.units vs. 2315745 ± 82068.8 arb.units, $P < 0.0001$ and 3513047 ± 129523.4 arb.units vs. 2315745 ± 82068.8 arb.units, $P < 0.00001$, respectively. There was also found a significant increase in the levels of ascorbate radicals in the 2nd group compared to the control group (3012881 ± 118757.8 arb.units vs. 2315745 ± 82068.8 arb.units, $P < 0.001$).

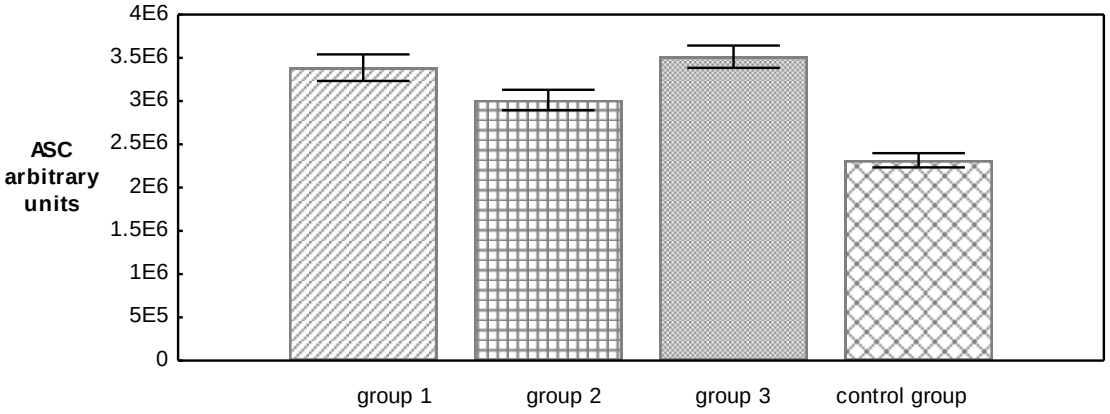

Figure 4. The levels of Ascorbate radicals (expressed in arb. units) registered in blood samples of the studied and control groups

Results from the levels of ROS products measured in the studied sera are presented on **Figure 5.** In all studied samples was registered EPR spectrum of the typical PBN spin adduct consisting of six spectral lines (EPR spectrum is not shown). Based on the values of the calculated hyperfine splitting constants (a^N= 13.88 G and a_β^H= 2.35 G) PBN spin adduct and that the sera were mixed with DMSO solution of PBN in aerobic conditions, the generated free radical species were identified as secondary oxygen centered alkoxy radicals (17). Levels of ROS products were calculated after double integration of the plot of the corresponding EPR spectrum.

As is seen on Figure 5 the levels of ROS products registered in the blood samples isolated from the 1st group of workers were not significantly different from that of the control group (2.527 ± 0.146 arb.units vs. 2.432 ± 0.112 arb.units, $P > 0.05$). There was a significant decrease in the levels of ROS products calculated for the 2nd group compared to the control group (1.910 ± 0.179 arb.units vs. 2.432 ± 0.112 arb.units, $P < 0.05$). Contrary, the levels of ROS products registered in the 3rd group were significantly higher than those of the control group (3.100 ± 0.244 arb.units vs. 2.432 ± 0.112 arb.units, $P < 0.05$).

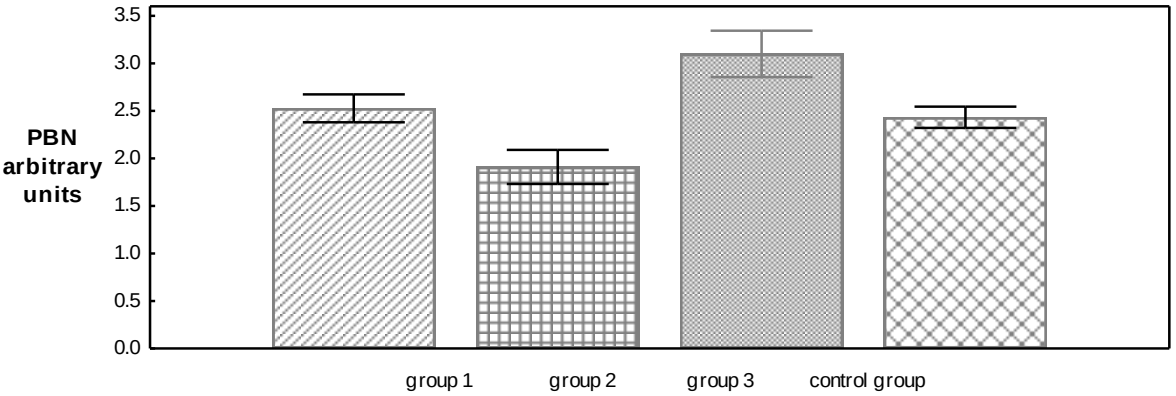

Figure 5. The levels of ROS products (expressed in arb. units) registered in blood samples of the studied and control group

DISCUSSION

The toxicity of TCE is due to its reactive metabolites derived from the reaction of glutathione conjugating with TCE (18). there are a number of reports in support of this statement. Other authors have reported that TCE-induced oxidative stress in the livers of mice is close related to the metabolism of TCE to trichloroacetic acid (19, 20). An imbalance between the production of reactive oxygen species and cellular antioxidants has been demonstrated by another study conducted on mice treated with TCE (21)There are a number of epidemiology studies in workers showing the relationship between TCE exposure and renal cell cancer mortality and incidence, but has not been established the mechanism for induction of malignant disease (22). Our results showed a significant increase in the levels of MDA, CAT, SOD and ascorbate radicals in workers from

group 1 and 2 compared with the control group. It is not surprise that the levels of MDA, SOD and CAT activity determined for the 2nd group were higher than those determined for the 1st group. Workers enrolled in 2nd group have deviations from the reference values of the serum enzyme activity of AST and ALT. This finding means that the level of the oxidative processes in the livers of workers in the 2nd group is higher comparing to that of the 1st group consisted of workers without changes in sera levels of AST and ALT. As it is known AST and ALT are markers of liver function. It should be mentioned, that with an exception for the ROS levels measured in the 2 nd group, the levels of the ascorbate radicals and ROS products in the three groups of workers were higher than those of the healthy controls. This result confirms that oxidative processes are in progress at the time of the study. There exist sufficiently reports which indicate that long-term exposure to TCE may lead to hepatotoxicity, nephrotoxicity and malignancies (23, 24, 25) It is also known that free radical damage have a role in the initiation of carcinogenesis. However, there is no evidence about the relation between reactive oxygen species caused by TCE and initiation of carcinogenesis. Moreover, it is not known whether oxidative stress is a direct consequence of the metabolism of or resulting from TCE-induced hepatotoxicity and nephrotoxicity.

The results of the 3rd group consists of welders exposed to toxic gases showed that the levels of SOD, ascorbate radicals and ROS products were elevated comparing to the controls. It is obvious that a permanent oxidative process, in the workers of the 3rd group is available. On the other hand statistical significant decrease in the levels of MDA measured for the same group means that oxidative damages are overcame by the antioxitant defense of the workers in that group. Insufficient data are available for the oxidative status in workers exposed to manganese. However, there are several epidemiological studies of workers have provided consistent evidence indicating that neurotoxicity is associated with low-level manganese exposure, but it is not exactly elucidated mechanism of the toxicity (7).

CONCLUSION

By the present paper have been demonstrated increased levels of the studied oxidative stress biomarkers in blood samples of workers from colliery "Troyanovo-1" and "Troianovo-north" to "Collieries Maritsa Iztok" EAD, in comparison with those of the healthy volunteers. It was supposed that harmful surroundings under which the workers operate might be the reason for the increased production of reactive oxygen species.

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