

DOES THE SUBACUTE (4-WEEK) EXPOSURE TO FORMALDEHYDE INHALATION LEAD TO OXIDANT/ANTIOXIDANT IMBALANCE IN RAT LIVER?

Sadık Söğüt¹, Ahmet Songur², Oğuz Aslan Özen², Hüseyin Özyurt³, Mustafa Sarsılmaz⁴

¹Mustafa Kemal University, Faculty of Medicine, Department of Biochemistry, ²Afyon Kocatepe University, Faculty of Medicine, Department of Anatomy, ³Gaziosmanpaşa University, Faculty of Medicine, Department of Biochemistry, ⁴Fırat University, Faculty of Medicine, Department of Anatomy

The aim of this experimental study is to evaluate oxidant/antioxidant status and the end products of lipid peroxidation in the liver of rats exposed to subacute formaldehyde (FA) inhalation (4-weeks). Thirty male Wistar albino rats divided into 3 separate groups randomly (10 for each group). Rats were exposed to atmospheres containing 0, 10 and 20 ppm FA continuously (8 hours per day, 5 days per week). Reduced glutathione (GSH), malondialdehyde (MDA) and nitric oxide (NO) levels, as well as xanthine oxidase (XO), and myeloperoxidase (MPO) activities were measured in rat liver homogenates. The results showed a remarkable reduction of GSH content (10 and 20 ppm FA) and XO activity (20 ppm FA) in liver tissues from rats exposed to for 4-weeks ($p < 0.005$, $p < 0.0001$ and $p < 0.0001$, respectively). MDA and NO levels did not change in both two groups (10 ppm and 20 ppm). These findings suggest that the antioxidant system of liver tissue is moderately impaired by excessive FA exposure. The GSH has an important influence especially on subacute FA liver toxicity. GSH-related cellular defensive mechanisms may be depressed and susceptibility to oxidative damage may high in rats exposed to FA subacutely. **Key words:** Formaldehyde, liver, lipid peroxidation, glutathione, oxidant/antioxidant enzymes

INTRODUCTION

Formaldehyde (FA) has been used extensively to protect cadavers from decaying for decades in Anatomy laboratories of Medical Faculties in Turkey (1). It is obviously known that it has some adverse effects on human body. It has been also shown that FA may reveal certain pathologies: When it is ingested orally, plasma protein and albumin levels decreased, hyperkeratosis in the forestomach, and focal gastritis in the glandular stomach appeared (2). All types of gene mutations and chromosomal rearrangements were reported (3) and the damaged chromosome cells were 43% as compared to 1,7% of control value (4). Formaldehyde was found to induce rhinitis, degeneration, frank necrosis, hyperplasia and squamous metaplasia of the ciliated

and non-ciliated nasal respiratory epithelium (5). Feron et al. reported that when rats exposed to FA by short-term inhalation nasal respiratory epithelium severely damaged by FA vapour often does not regenerate and in some cases tumors develop (6).

It is still obscure whether FA can induce toxic effects in distant organs such as liver, kidney, testes, etc. Glutathione-dependent formaldehyde dehydrogenase (GSH-FDH) has an universal role in biological FA oxidation to formic acid (7) (Figure 1). We hypothesized that when FA was ingested and/or inhaled excessively, GSH-FDH will detoxify this compound more than normal metabolic situation and GSH will run out in the liver which is the main metabolic organ in the human body. GSH (L- γ -glutamyl-L-cysteinylglycine) has antioxidant properties and is abundantly present in liver tissue. GSH is involved in the reactions by various enzymes and it protects against reactive oxygen species (ROS) and toxic compounds such as FA. Xanthine oxidase (XO) is one of the major in vivo superoxide generator

Correspondence: Sadık Söğüt
Mustafa Kemal University, Medical Faculty, Department
of Biochemistry, 31100 Antakya/Hatay
Tel: 0 326 214 16 49 (ext: 117)
Fax: 0 326 214 49 77
E-mail: sadiksogut2@hotmail.com

and activated to utilize accumulated purines in the ischemic part of the organ in some circumstances such as ischemia/reperfusion. It is very important to know whether XO activity of the liver tissue is increased or decreased, because oxygen radicals and antioxidants play an important role in the detoxifying process of FA.

The aim of this study is to evaluate antioxidant status and lipid peroxidation in the liver of rats exposed to FA by inhalation subacutely (4-weeks). For this purpose, GSH as an index of non-enzymatic antioxidant status of the liver tissue; malondialdehyde (MDA), as an index of *in vivo* lipid peroxidation; xanthine oxidase, a superoxide radical ($O_2^{\cdot-}$) generator; nitric oxide ($\cdot NO$), a free radical; and myeloperoxidase (MPO), as an index of leukocyte sequestration to the liver tissue were studied in liver homogenates.

MATERIALS AND METHODS

Chemicals and instruments

Xanthine oxidase, xanthine, 5,5'-Dithiobis (2-nitrobenzoic acid), glycine, N-naphthyl ethylenediamine, sulphanilamide, thiobarbituric acid, 1,1,3,3-tetramethoxypropane were purchased from Sigma Chemical Co (St Louis, MO, USA). $CuCl_2$, bovine serum albumin, H_2O_2 , EDTA, Na_2CO_3 , $(NH_4)_2SO_4$, chloroform, thiobarbituric acid, trichloroacetic acid, sodium nitrite, ethanol, NaCl, KH_2PO_4 , $NaHPO_4 \cdot 2H_2O$ from Merck (Germany). Cadmium granule was purchased from Fluka (Germany). LKB Biochrom Ultraspec Plus uv/visible spectrophotometer (Cambridge, UK) was used to measure XO, NO, GSH, MPO, and protein measurements. Fluorescence Spectrophotometer (Hitachi Model F-4010, Japan) was used to measure tissue MDA levels.

The production of FA gas

The formaldehyde gas was generated from paraformaldehyde (Merck KGaA, 64271 Darmstadt, Germany) by thermal depolymerization according to a method described by Chang (8). Formaldehyde level of the test atmosphere was monitored at regular intervals by a digital formaldehyde-meter (Environmental Sensors Co. Boca Raton FL 33431 USA).

Animals and procedure of FA exposure

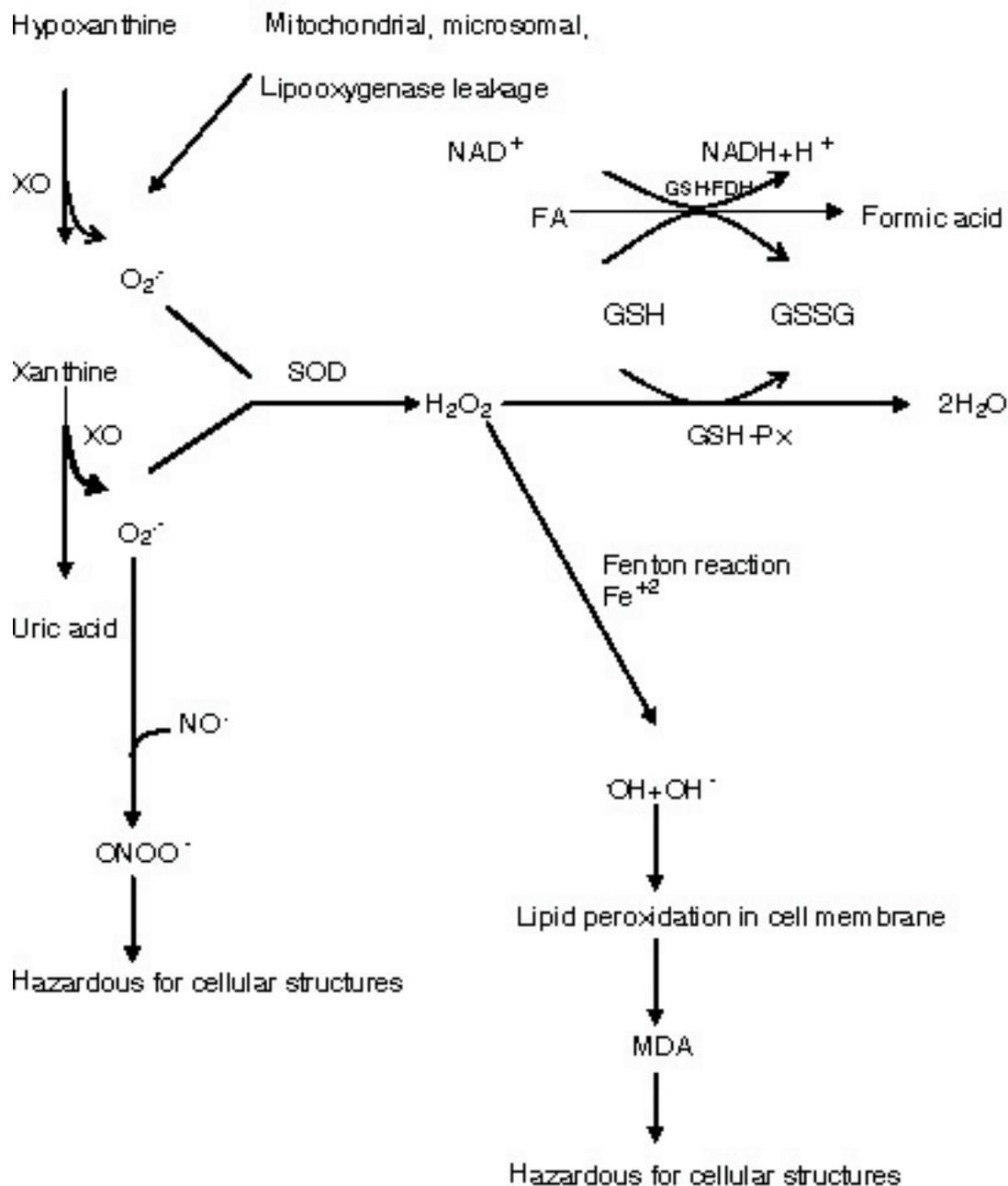
The experimental protocol was approved by the Ethical Committee of Firat University on Human and Animal Experimentation. Additionally, Principles of laboratory animal care (NIH publication no. 85-23, revised 1985) were followed. Three groups of adult male albino Wistar rats consisting of each 10 rats (weighing 250 ± 5 gm) were obtained from the Biomedical Research Unit of the Institute of Health Sciences, Firat University, Elazığ. After an acclimatization period of 1 week, the rats were assigned by computer randomization to 3 different groups and then the last two groups were exposed (whole body exposure) to atmospheres containing 10 and 20 ppm formaldehyde continuously (8 h a day), 5 days per week, during 4 weeks. The exposure was performed in horizontally placed glass chambers. Animals were housed (5 rats per cage) in a chamber ventilated with 10 lt/min, maintained at 25 ± 2 °C, with a relative humidity of 45-55% and a 12-h light/dark cycle during the observation periods. Neither food nor drinking water was present in the inhalation chambers during the exposures. During the non-exposure periods the animals were provided with bottled tap water and the Institute's stock diet for rats *ad libitum*. The rats checked daily and body weights were recorded weekly. At the end of the 4-week exposure period, the rats were killed by decapitation under ether anesthesia, autopsied and examined grossly for pathological changes. The tissues was stored at $-70^\circ C$ until biochemical analyses.

The preparation of liver tissues for biochemical analyses

After liver tissues were obtained for biochemical analysis, the specimens were washed out from contaminating blood with ice-cold buffered saline. They were weighed and homogenized in Tris-HCl buffer (pH 7.4) for three minutes at 16000 rpm with a homogenizer (Ultra Turrax Type T-25-B; IKA Laborotechnic, Staufen, Germany). The homogenates were then centrifuged for 1 hour at 4 °C at 5000 x g. All the measurements were made at this homogenate.

MDA level determination

Tissue MDA levels were determined by the method described by Wasowicz (9). Briefly, MDA was reacted with thiobarbituric acid by incubating for 1 hour at $95-100$ °C.



XO: xanthine oxidase, $O_2^{\cdot-}$: superoxide, NO: nitric oxide, SOD: superoxide dismutase, CAT: catalase, H_2O_2 : hydrogen peroxide, GSH: reduced glutathione (L- γ -glutamyl-L-sisteinylglycine), GSH-Px: glutathione peroxidase, GSSG: oxidized glutathione, FA: formaldehyde, GSH-FDH: glutathione-dependent formaldehyde dehydrogenase, $\cdot OH$: hydroxyl radical (the most potent free oxygen radical), MDA: malondialdehyde, $ONOO^{\cdot}$: peroxynitrite

Figure 1. Schematic representation of oxidant/antioxidant mechanisms of liver tissue
 This figure adapted from our previous publication (Mol Psychiatr 2001;6:66-73)

Following the reaction, fluorescence intensity was measured in the n-butanol phase with fluorescence spectrophotometer adjusting wavelengths excitation at 525nm and emission at 547nm, by comparing with a standard solution of 1,1,3,3 tetramethoxypropane. Results were expressed in terms of $\mu\text{mol/g}$ wet tissue.

XO activity determination

XO (EC 1.2.3.2) activity was measured spectrophotometrically by the formation of uric acid from xanthine through the increase in absorbance at 293 nm, according to Prajda and Weber's method (10). Tissue homogenate was incubated (50 μL) for 30 min at 37 °C in 2.85 mL of medium containing phosphate

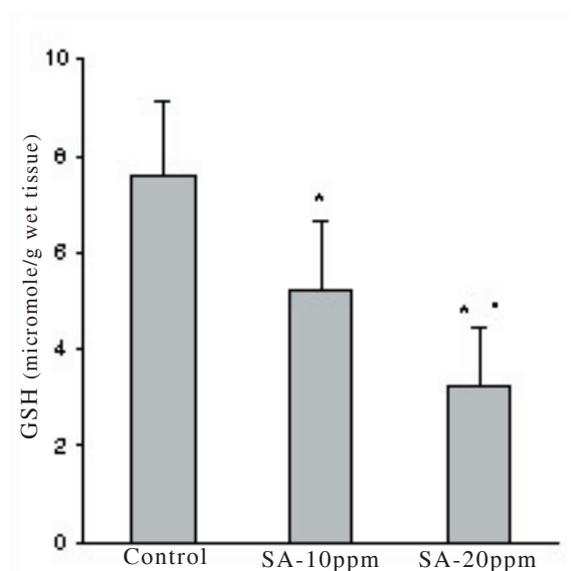


Figure 2. Glutathione (GSH) concentrations in liver tissue homogenate of rats exposed to FA in different concentrations. Values are expressed as means \pm S.D.: *significantly lower than control rats; # significantly lower than 10 ppm group.

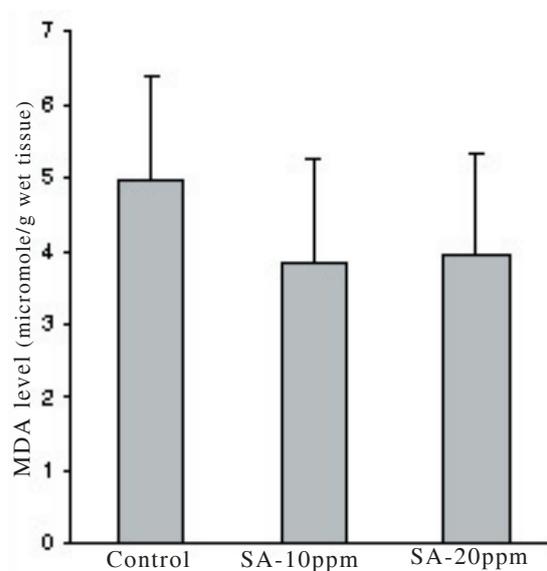


Figure 3. Malondialdehyde (MDA) levels in liver tissue homogenate of rats exposed to FA in different concentrations.

buffer (pH 7.5, 50 mM) and xanthine (0.067 mmol final concentration in each tubes).

The reaction was stopped by addition of 0.1 mL 100% (w/v) TCA and the mixture was centrifuged at 5000 x g for 15 min. With resultant clear supernatant, absorption at 293 nm was measured against blank. One unit of activity was defined as 1 μ mol of uric acid formed per minute at 37 °C, pH 7.5, and expressed in U/g protein.

NO level determination

Tissue nitrite (NO_2^-) and nitrate (NO_3^-) were estimated as an index of NO production. Therefore, we measured liver tissue concentrations of the stable NO oxidative metabolites (NO_2^- and NO_3^-). Quantitation of nitrate and nitrite was based on the Griess reaction (11). Samples were treated with copperized cadmium in glycine buffer at pH 9.7 (2.5 to 3 g of Cd granules for a 4 mL reaction mixture) to reduce nitrate to nitrite after deproteinization of the samples with Somogyi reagent. Following clean up, the sample was mixed with fresh reagent and the absorbance was measured in a spectrophotometer to give the total nitrite concentration. The concentration of nitrite measured thus represented the total nitrite, i.e. nitrate plus nitrite. A standard curve was established with a set of serial dilutions

(10^{-3} - 10^{-8} mol/L) of sodium nitrite. Linear regression was done using the peak areas from the nitrite standard. The resulting equation was then used to calculate the unknown sample concentrations. Results were expressed as $\mu\text{mol/g}$ prot.

GSH level determination

Reduced glutathione concentration was determined by the titration with 0,1 mmol/L 5,5'-Dithiobis (2-nitrobenzoic acid) in a 0,1 mol/L disodium phosphate buffer solution, pH 8. The formation of the reduced product, thionitrobenzene, was measured spectrophotometrically at 412 nm (12). GSH level was expressed as $\mu\text{mol/g}$ wet tissue.

MPO activity determination

MPO activity was determined using a 4-aminoantipyrine/phenol solution as the substrate for MPO-mediated oxidation by H_2O_2 , and changes in absorbance at 510 nm (A_{510}) were recorded (13). One unit of MPO activity is defined as that which degrades 1 μmol H_2O_2 /min at 25°C. Data are presented as U/g protein.

Statistical analysis

Data were analyzed by using SPSS® for Windows computing program. Nonparametric statistical methods were used to analyze

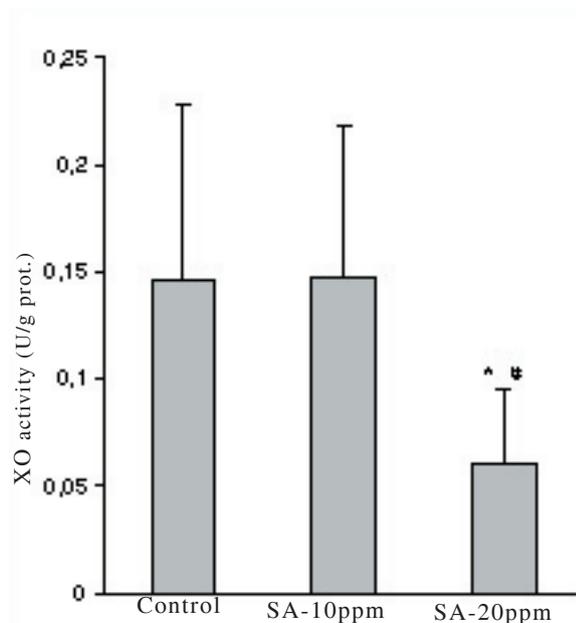


Figure 4. Xanthine oxidase (XO) activities in liver tissue homogenate of rats exposed to FA in different concentrations. Values are expressed as means \pm S.D.: *significantly lower than control rats; #significantly lower than 10 ppm group.

the data. Mann-Whitney U tests were used to examine between group comparisons. Bivariate comparisons were examined using Pearson rank correlation coefficients and values were corrected for ties. Two-tailed significance values were used. Statistical significance was ascribed when p was equal or less than 0.05. The results were presented as means \pm standard deviation.

RESULTS

Figure 2 shows the effects of inhaled FA on liver GSH content. GSH levels were gradually decreased in both 10 ppm group (5.23 ± 1.44 $\mu\text{mol/gr}$ wet tissue) and 20 ppm group (3.24 ± 1.21 $\mu\text{mol/gr}$ wet tissue) when compared to control group (7.58 ± 1.54 $\mu\text{mol/gr}$ wet tissue) ($p < 0.005$ and $p < 0.0001$, respectively). FA was found to cause GSH depression in a dose-dependent manner. Figure 3 shows the effects of FA on liver peroxidation in rats subjected to subchronic FA inhalation. There was no statistically significant increase in tissue MDA level in 10 ppm and 20 ppm groups compared to both control group.

The activity of liver oxidant enzyme XO is reported in Figure 4 for the two study groups. Rats exposed to FA resulted in a dose-independent inactivation of XO more

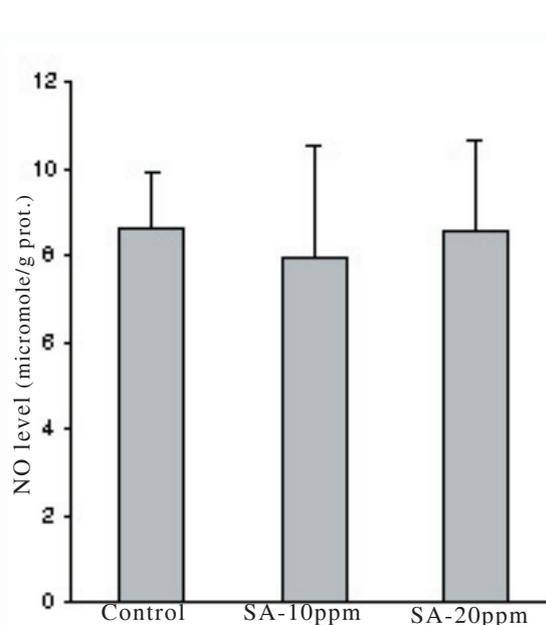


Figure 5. Nitric oxide (NO) concentrations in liver tissue homogenate of rats exposed to FA in different concentrations.

than two-fold. There was a statistically significant decrease in XO activity in 20 ppm group compared to both control and 10 ppm groups. The level of the liver NO is reported in Figure 5. The results show that FA exposure is not associated with the changed NO production in both 10 ppm and 20 ppm exposure.

The activity of liver MPO is presented in Figure 6. There were no activity changes of MPO in 10 ppm and 20 ppm groups compared to control group. There is a statistically significant correlation between NO and MDA in 20 ppm group ($r = 0.676$, $p < 0.035$).

DISCUSSION

Free oxygen radicals are reactive oxygen species and reduction of molecular oxygen produces these radicals. Many of the toxic effects of oxygen can be attributed to these reactive oxygen species. Exposure of membrane lipids of liver cells to oxygen radicals in the presence of iron salts stimulates the process of lipid peroxidation.

Liver GSH plays an important role in protecting membrane lipids of the liver cells from reactive oxygen species (ROS) attack (14); however it is rapidly oxidized when challenged by oxidants released from metabolic events or degrading processes such

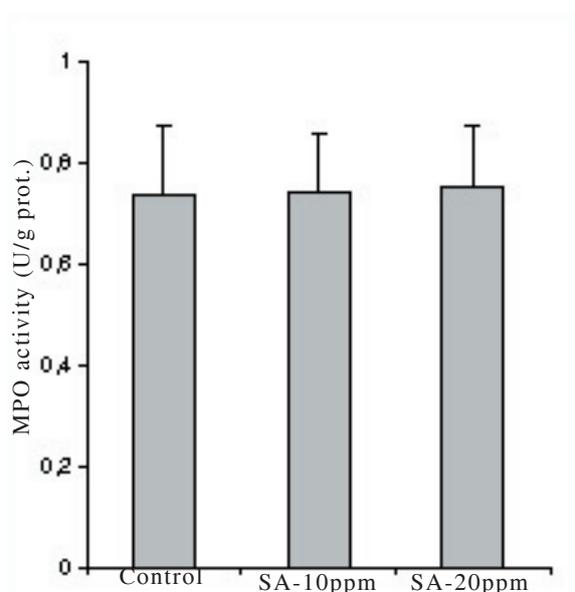


Figure 6. Myeloperoxidase (MPO) activities in liver tissue homogenate of rats exposed to FA in different concentrations. There were no statistically significant differences between the groups.

as GSH-FDH (Figure 1). This rapid depletion may be the reason for the low GSH values in liver tissue.

We observed 31 % and 57 % decrease in GSH level of liver tissue in Group I and Group II, respectively. We suggest that this decrease might probably be in response to the extra production of ROS or excessive exposure to FA as stated by some authors (15-17). Glutathione peroxidase (GSH-Px), which is present widely in liver tissue, needs GSH to detoxify hydrogen peroxide (H_2O_2). If the enzymatic antioxidant system can not work properly in liver tissues, there will be an extra free oxygen radical load. In the end of the process, cellular and organelle membranes, which are more sensitive to oxidative attacks than other cellular components, suffer from successive lipid peroxidation and led to be destroyed of cellular structures completely. Although it was not statistically significant, MDA levels were found to be lower in Group I and Group II than those of control animals. We suggest two probable explanations which are important for this contrary results: i) enzymatic and nonenzymatic defense systems against free oxygen radicals, except GSH, might be increased and thus, compensated the effect of GSH and GSH-dependent enzymes such as GSH-Px, glutathione reductase

(GSH-Red) and glutathione-S-transferase (GSH-ST), ii) MDA released by the lipid peroxidation in liver tissues might be metabolized immediately by a mitochondrial MDA-metabolizing enzyme (low specific aldehyde dehydrogenase) and increase in MDA level is not seen.

It was found that the content of GSH was significantly decreased in rat liver after methanol intoxication for 6 hours to 5 days (18-19). Methanol is oxidized by liver alcohol dehydrogenase to FA (20). Formaldehyde in turn is rapidly oxidized by GSH-FDH to formic acid. These findings seem to be concordance with our results. Taken together, one of the reasons of decreased GSH in liver tissue may be this enzymatic degradation process.

It is very difficult to explain why XO activity decreases in liver tissues of rats exposed to FA in Group II. NO is a free radical with "double edged" pathophysiological role in liver tissue, because it can be toxic or protective (21,22). NO is thought to be toxic through its reaction product with O_2^- the peroxynitrite anion ($ONOO^-$), which can further decompose into NO_2 and $\cdot OH$ (23). NO is synthesized by three isoforms of nitric oxide synthases (NOS). Neuronal and endothelial isoforms are regulated by intracellular Ca^{++} . Metabolic reactions that could produce ROS are catalyzed by Ca^{++} -activated enzymes: phospholipase A2 and NOS-1. Inducible NOS activity is independent of Ca^{++} , and the enzyme is expressed in various cells, such as macrophages and neutrophils in response to inflammatory stimuli. In our study, detection of normal NO levels in the study groups I and II suggested that overproduction of free radicals by NOS1 isoform had not been occurred.

In conclusion, GSH improves endurance performance and prevents liver lipid peroxidation during subacute FA inhalation in rats. The GSH content of liver tissue was affected from FA toxicity with dose-dependent manner in our study. Therefore, liver GSH has an important influence on subacute FA toxicity. GSH-related cellular defensive mechanisms may be depressed in liver tissues and therefore susceptibility to oxidative damage may increase in rats exposed to FA subacutely.

REFERENCES

- 1- Özen OA, Sarsilmaz M. FA toxicity in inhaled air and preventive measures. *Firat Journal of Medicine* 2000; 9(2):6-12
- 2- Till HP, Woutersen RA, Feron VJ, Clary JJ. Evaluation of the oral toxicity of acetaldehyde and formaldehyde in a 4-week drinking-water study in rats. *Food Chem Toxicol* 1988;26(5):447-52
- 3- Auerbach C, Moutschen-Dahmen M, Moutschen J. Genetic and cytogenetical effects of formaldehyde and related compounds. *Mutat Res* 1977;39(3-4):317-61
- 4- Woodruff RC, Mason JM, Valencia R, Zimmering S. Chemical mutagenesis testing in *Drosophila*. V. Results of 53 coded compounds tested for the National Toxicology Program. *Environ Mutagen* 1985;7(5):677-702
- 5- Cassee FR, Feron VJ. Biochemical and histopathological changes in nasal epithelium of rats after 3-day intermittent exposure to formaldehyde and ozone alone or in combination. *Toxicol Lett* 1994;72(1-3):257-68
- 6- Feron VJ, Bruyntjes JP, Woutersen RA, Immel HR, Appelman LM. Nasal tumors in rats after short-term exposure to a cytotoxic concentration of formaldehyde. *Cancer Lett* 1988;39(1):101-11
- 7- Barber RD, Donohue TJ. Pathways for transcriptional activation of a glutathione-dependent formaldehyde dehydrogenase gene. *J Mol Biol* 1998;280(5):775-84
- 8- Chang JC, Steinhagen WH, Barrow CS. Effect of single or repeated formaldehyde exposure on minute volume of B6C3F1 mice and F-344 rats. *Toxicol Appl Pharmacol* 1981;61(3):451-9
- 9- Wasowicz W, Neve J, Peretz A. Optimized steps in fluorometric determination of thiobarbituric acid-reactive substances in serum: importance of extraction pH and influence of sample preservation and storage. *Clin Chem* 1993;39(12):2522-6
- 10- Prajda N, Weber G. Malign transformation-linked imbalance: decreased XO activity in hepatomas. *FEBS Lett* 1975;59(2):245-9
- 11- Cortas NK and Wakid NW. Determination of inorganic nitrate in serum and urine by a kinetic cadmium-reduction method. *Clin Chem* 1990;36(8):1440-3
- 12- Beutler E, Duron O, Kelly BM. Improved method for the determination of blood glutathione. *J Lab Clin Med* 1963;61(5):882-8
- 13- Wei H, Frenkel K. Relationship of oxidative events and DNA oxidation in SENCAR mice to in vivo promoting activity of phorbol ester-type tumor promoters. *Carcinogenesis* 1993;14:1195-201
- 14- Kobayashi T, Robinson MK, Robinson V et al. Glutathione depletion alters hepatocellular high-energy phosphate metabolism. *J Surg Res* 1993;54(3):189-95
- 15- Ku RH, Billings RE. Relationships between formaldehyde metabolism and toxicity and glutathione concentrations in isolated rat hepatocytes. *Chem Biol Interact* 1984;51(1):25-36
- 16- Farooqui MY, Upreti RK, Ahmed AE, Ensari GA. Influence of intraperitoneally administered formaldehyde on bile production and tissue glutathione levels in rats. *Res Commun Chem Pathol Pharmacol* 1986;53(2):233-6
- 17- Ku RH, Billings R. The role of mitochondrial glutathione and cellular protein sulphhydryls in formaldehyde toxicity in glutathione-depleted rat hepatocytes. *Arch Biochem Biophys* 1986;247(1):183-9
- 18- Skrzydlewska E, Farbiszewski R. Decreased antioxidant defense mechanisms in rat liver after methanol intoxication. *Free Radic Res* 1997;27(4):369-75
- 19- Skrzydlewska E, Farbiszewski R. Glutathione consumption and inactivation of glutathione-related enzymes in liver, erythrocytes and serum of rats after methanol intoxication. *Arch Toxicol* 1997;71(12):741-5
- 20- Porter WH, Moyer TP. Clinical toxicology. In: Tietz fundamentals of clinical chemistry Burtis, CA, Ashwood, ER. [eds] W.B. Saunders Company, Pennsylvania pp 1996:427-56
- 21- Koken T, Inal M. The effect of nitric oxide on ischemia-reperfusion injury in rat liver. *Clin Chim Acta* 1999;288 (1-2):55-62
- 22- Lipton SA, Choi YB, Pan ZH et al. A redox-based mechanism for the neuroprotective and neurodestructive effect of nitric oxide and related nitroso-compounds. *Nature* 1993;364(6438):626-32
- 23- Beckman JS, Crow JP. Pathological implications of nitric oxide, superoxide and peroxynitrite formation. *Biochem Soc Trans* 1993;21(2):330-34.