

SERUM ANTIOXIDANT PARAMETERS IN PATIENTS POISONED BY DIFFERENT XENOBIOTICS

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Abstract: There is a great diversity of the acute drugs overdose cases in clinical toxicology. Clinical situation is complicated by the coexistence of factors predisposing to the development of adverse drug reactions (chronic use of drugs, polypharmacy, alcohol or drugs dependence, nutritional disorders) and by the presence of chronic organ damage, especially the liver and the kidney. The aim of this study was to evaluate whether there are sensitive plasma markers belonging to the antioxidant system in patients exposed to various xenobiotics. We measured the activity of antioxidant parameters: catalase (CAT), glutathione peroxidase (GPX3), glutathione (GSH), sulfhydryl groups (-SH), carbonyl groups (=CO) and free radicals (2,2-diphenyl-1-picrylhydrazyl, DPPH, assay) in serum of 49 patients with acute intoxication caused by carbamazepine (CBZ, n = 9), mixed drug intoxication (MDI) (n = 9), alcohol withdrawal syndrome (AWS, n = 9), acetaminophen (APAP, n = 7), tricyclic antidepressants (TCAs) (n = 5), valproic acids (VA, n = 4), narcotics (N, n = 3), and three others (benzodiazepines, BZD, n = 2; barbiturates, n = 1). The results were compared with the parameters of not intoxicated patients (n = 39). All patients had lower catalase activity in comparison to the control group (41.9 ± 16.5 vs. 196.0 ± 82.2 U/mg protein, $p = 0.000$), while the increase of GSH level was particularly apparent only in patients with AWS (391.3 ± 257.9 $\mu\text{mol/mg}$ protein) compared to the control group (171.4 ± 88.4 $\mu\text{mol/mg}$ protein, $p = 0.034$) and to patients intoxicated with carbamazepine (152.8 ± 102.5 $\mu\text{mol/mg}$ protein, $p = 0.027$). Some differences, but without statistical significance, were also observed in GPX3 activity between different groups of poisoned patients.

Keywords: antioxidants defense, xenobiotics, enzyme activities

Most acute poisonings are due to single drug overdose but more and more often in clinical practice we observe multidrug poisonings, especially in patients with alcohol and drug addiction or with long pharmacological treatment of psychiatric diseases. Clinical situation is complicated by complex interaction between toxic potential of the drug and coexistence of risk factors predisposing to the development of drug toxicity and adverse drug reactions. Metabolism of xenobiotics takes place largely in the

liver, which accounts for the organ's susceptibility to metabolism-dependent, drug-induced injury. Drug metabolites can be electrophilic active chemicals or free radicals that promote oxidative stress.

The metabolism of xenobiotics detoxification runs in two phases: phase I spans the reactions mediated by cytochrome P450, and phase II comprises the conjugation of xenobiotics' derivatives with organic and inorganic acids as well as with glycine and methionine. Biological sense of those reactions

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is primarily to increase the water solubility of xenobiotics or their metabolites and to increase their elimination from the body in this way (1, 2). Detoxification processes do not always cause toxicity reduction of xenobiotics, sometimes they can also increase toxicity, leading to the generation of reactive oxygen species (ROS), reactive nitrogen species (RNS) and/or alteration in antioxidant and the scavenging enzymes system. Both ROS and RNS can damage cellular organelles causing lipid peroxidation, and protein and DNA oxidation (2). Whether ROS and RNS activity has a causal or propagating role in human diseases associated with oxidative stress, remains an unresolved question. Moreover, even if the alteration of one or more biomarkers of oxidative stress has been reported to occur virtually in all diseases, data on the oxidant/antioxidant balance in the course of poisoning are still extremely scarce (3).

We assume that severely poisoned patients could suffer from oxidative stress caused by ROS and RNS. Sources of ROS/RNS during poisoning include e.g.: production of the active metabolites including lipid hydroperoxides, singlet oxygen and hydrogen peroxide (1); adducts formations of the active metabolites with cellular proteins, lipids (2); the release of cytokines from immune cells, with subsequent activation of inflammatory cascades and an increase of the expression of adhesion molecules (3); severe hypotension with ischemia/reperfusion-induced tissue damage (4-6). Inflammation and tissue injury during poisoning result in an accumulation of granulocytes in organs and lead to greater generation of ROS, which further perpetuates or increases the inflammatory response and tissue injury (7). Because of deep disturbance of consciousness, respiratory failure and lung aspiration complications, poisoned patients frequently require the use of mechanical ventilation with high levels of oxygen for adequate brain and other vital organs oxygenation. Prolonged exposure to hyperoxia may also damage pulmonary epithelial cells with ROS stimulation. Taking into account ROS/RNS induction, poisoned patients should have reduced plasma and intracellular levels of antioxidants and free electron scavengers or cofactors, and decreased activity of the enzymatic system which is involved in ROS detoxification (8).

From a clinical point of view, it is important to search for biomarkers of ROS induction and, on the other hand, biomarkers indicating the consumption of the antioxidant system. The usefulness of an ideal biomarker of oxidative damage lies in its ability to provide a valid and early indication of disease

and/or its progression. We can use a number of assays which may be used to evaluate enzymatic and non-enzymatic defensive mechanisms which reduce harmful effects of ROS/RNS. Those antioxidative mechanisms may be considered as measures of biological response and adaptation. But selective measurement of the antioxidant capacity does not provide relevant information on the overall antioxidant status. Moreover, total antioxidant capacity is manifested not only by concentration/activity of individual antioxidants but also by their synergistic action.

From a variety of oxidative/antioxidative parameters we selected for our study the following parameters: sulfhydryl groups (-SH), carbonyl groups (=CO), catalase (CAT), plasma glutathione peroxidase (GPX3), glutathione (GSH), free radicals (2,2-diphenyl-1-picrylhydrazyl, DPPH assay) in the blood serum. The major role of sulfhydryl groups lies also in stabilizing the three-dimensional structure of proteins, thereby preserving their functional properties. Sulfhydryls play an important role in biochemistry as disulfide bonds connect amino acids together for functional purpose in secondary, tertiary or quaternary protein structures. Sulfhydryl groups can be easily oxidized, and thiolates act as potent nucleophiles. Lipoxidation (LPO) and glycation processes lead to the formation of low molecular mass reactive carbonyl species (RCO). Carbonyl compounds, like most other intermediates and by-products of metabolism, are electrophilic and thus, highly reactive when they meet different cellular nucleophilic groups (sulfhydryls, imidazoles and hydroxyls) as well as oxygen atoms of macromolecules such as proteins, nucleic acids and aminophospholipids, resulting in their nonenzymatic and irreversible modification, and formation of a variety of adducts and crosslinks collectively named ALEs (advanced lipoxidation end products) and AGEs (advanced glycation end products) (9-11). As the consequences of these effects, the disorders of the cellular metabolism and organelles may be observed. It was proposed to describe this process as "carbonyl stress" (12, 13). Therefore, at molecular level, carbonyl groups indicate chemical modification of biomolecules and the extent of damage of the cellular structure and function.

The human antioxidant system is involved in the protection against ROS. It comprises enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT) and nonenzymatic species, i.a., reduced glutathion, uric acid, creatinine. Other parameters such as sulfhydryl (-SH) or carbonyl (=CO) groups are indicators of damage caused by ROS. The first line of defense is formed

by cytosolic and mitochondrial SODs which catalyze the dismutation of superoxide anion radicals to hydrogen peroxide. Hydrogen peroxide is then removed by GPX or by CAT, found in the cytosol and mitochondria of most tissues.

Catalase activity becomes important at higher concentrations of hydrogen peroxide, at which the enzyme decomposes most of this compound to water and oxygen (14). Decreased CAT or GPX activity may lead to overproduction of superoxide and hydrogen peroxide which are substrates of the Fenton reaction leading to production of the most reactive oxygen radical – the hydroxyl radical with oxidative damage of the cellular DNA, proteins and lipids.

Glutathione is the most abundant sulfhydryl-containing substance in cells. It can be oxidized to form a glutathione radical (GS^{\bullet}). Although GS^{\bullet} is a pro-oxidant radical, it can combine with another GS^{\bullet} to yield glutathione disulfide (GSSG) which is then reduced to GSH by the NADPH-dependent glutathione reductase. GSH reacts with a variety of xenobiotic electrophilic compounds in the catalytic reaction of glutathione-S-transferase. GSH effectively scavenges ROS like a lipid peroxy radical, peroxynitrite and H_2O_2 directly and indirectly through enzymatic reactions. GSH can conjugate with NO, resulting in the formation of a S-nitroso-glutathione adduct which is cleaved by the thioredoxin system to release GSH and NO. GSH interacts with sulfhydryl containing proteins which play important roles in the regulation of cellular redox homeostasis (15).

DPPH is a stable free radical and it is widely used to test the ability of antioxidants to act as free radical scavengers or hydrogen donors, and to evaluate total antioxidant activity of serum. DPPH has an odd electron which becomes paired with hydrogen from another free radical scavenging native antioxidant to form the reduced DPPH-H. The resulting decolorization of the serum specimen is stoichiometric with respect to the number of electrons captured. The DPPH method is rapid and inexpensive, and it is not specific to any particular antioxidant component but applies to the overall antioxidant capacity of the sample.

The markers used in our study were chosen because of the simplicity of their measurement and because they appear to yield useful information on antioxidative system status. We measured several markers because the most accurate and clinically relevant approach to evaluating oxidative damage is to measure many different types of damage from different biomolecules. The aim of the study was to

investigate the influence of different xenobiotics on antioxidant parameters in serum of patients either exposed to various xenobiotics or with alcohol addiction, and to evaluate whether there are sensitive plasma markers belonging to the response of the antioxidant system in such patients.

EXPERIMENTAL

Patients

The patients (aged 17-80, mean 41 ± 15 years) included in the study group were hospitalized because of acute intoxications by carbamazepine ($n = 9$), mixed drug intoxication (MDI) ($n = 9$), alcohol withdrawal syndrome (AWS, $n = 9$), acetaminophen (APAP, $n = 7$), tricyclic antidepressants (TCAs) ($n = 5$), valproic acids (VA, $n = 4$), narcotics (N, $n = 3$) and three others (benzodiazepines, BZD, $n = 2$; barbiturates, $n = 1$). Serum and urine from patients was frozen after collection at $-80^{\circ}C$ until analysis.

The studies were conducted with approval of Bioethical Commission of Jagiellonian University (KBET/108/B/2011 30. 06. 2011).

All patients were symptomatic and routine biochemical analysis of the blood samples (total bilirubin, alanine aminotransferase, aspartate aminotransferase, γ -glutamyl transpeptidase, international normalized ratio, urea, creatinine, creatine phosphokinase) was conducted for each patient to diagnose the organ damage (i.e., liver, kidney, muscles). Acute poisoning was diagnosed based on history, clinical symptoms and confirmation of the presence in serum or urine of at least one of the following xenobiotics: carbamazepine, ethanol, acetaminophen, tricyclic antidepressants, valproic acids, benzodiazepines, phenobarbital, salicylates, phenothiazines, amlodipine, baclofen, dextromethorphan, metoprolol, mianserine, propafenone or zolpidem. Additionally, alcohol dependence was diagnosed based on psychological tests. The control group was recruited among healthy volunteers aged 15-76, mean 43 ± 20 ($n = 39$).

Methods

Antioxidant parameters: catalase was measured by the Aebi method (16), -SH groups by the Hu method (17), =CO groups by the Levine method (18) and glutathione peroxidase by the Paglia and Valentine method (19). Percentage of inhibition (%) of free radicals was measured using the DPPH test (20).

Statistical approach

Mean values and standard deviations were calculated for all antioxidant parameters. The differ-

Table 1. Antioxidant parameters (arithmetic mean \pm SD) in various groups of patients and in controls.

Group of patients	Parameters						
	-SH (mmol/mg)	=CO (nmol/mg)	CAT (U/mg)	GPX3 (U/mg protein)	GSH (mmol/mg)	Percentage of inhibition (%)	
CBZ	0.0111 \pm 0.0016	1.040 \pm 0.327	44.5 \pm 16.7 ^a	26.5 \pm 2.0	152.8 \pm 102.5 ^d	4.21 \pm 2.91	
MDI	0.0111 \pm 0.0015	1.519 \pm 0.300	38.0 \pm 17.4 ^a	27.8 \pm 2.0	244.5 \pm 220.4	3.09 \pm 1.80	
AWS	0.0132 \pm 0.0016	1.292 \pm 0.191	45.5 \pm 22.0 ^a	28.3 \pm 1.3	391.3 \pm 257.9 ^e	5.03 \pm 2.29	
APAP	0.0111 \pm 0.0024	1.169 \pm 0.338	39.2 \pm 16.9 ^a	27.9 \pm 1.9	333.5 \pm 224.4	4.36 \pm 2.83	
TCAAs	0.0143 \pm 0.0014	1.008 \pm 0.299	44.2 \pm 16.8 ^a	28.5 \pm 1.8	292.0 \pm 168.6	4.92 \pm 3.15	
VA	0.0109 \pm 0.0008	1.175 \pm 0.201	47.3 \pm 10.6 ^a	27.9 \pm 2.1	167.1 \pm 98.7	5.30 \pm 3.45	
N	0.0120 \pm 0.0049	1.905 \pm 1.035	39.1 \pm 11.9 ^a	31.6 \pm 6.0	233.7 \pm 34.6	4.82 \pm 1.03	
Others	0.0107 \pm 0.0033	1.316 \pm 0.218	33.5 \pm 12.9 ^a	25.0 \pm 5.5	176.7 \pm 132.5	4.79 \pm 2.56	
Control	0.0114 \pm 0.0024	1.333 \pm 0.567	196.0 \pm 82.2 ^b	28.7 \pm 1.8	171.4 \pm 88.4 ^a	5.90 \pm 6.35	

Meaning of abbreviations: CBZ - carbamazepine, MDI - mixed drug intoxication, AWS - alcohol withdrawal syndrome, APAP - acetaminophen, TCAAs - tricyclic antidepressants, VA - valproic acids, N - narcotics, -SH - sulfhydryl groups, =CO - carbonyl groups, CAT - catalase, GPX3 - plasma glutathione peroxidase, GSH - glutathione; the results in columns with different letters in superscript differ with given statistical significance: a - b: $p < 0.0001$; c - d: $p < 0.05$

ences between groups were checked using the ANOVA test with the Tukey *post hoc* test. The homogeneity of variances were tested by means of the Levene's test. In case of non-homogenous variances, the Welch test was applied to compare groups with different diagnosis. Spearman's correlation coefficients were calculated for all pairs of parameters, separately in groups of patients and controls. The Fisher two-tailed test was used to assess the significance of the difference between two Spearman correlation coefficients.

A probability level of $p < 0.05$ was considered to be statistically significant. Principal Component Analysis (PCA) was used in order to reveal possible differences or similarities between the patients and the controls in multi-dimensional space established by the whole set of investigated parameters. Before using this method, the variables had been standardized. Statistical calculations were carried out using the commercially available packages: SPSS Statistics 22 (IBM) and Statistica PL v.10 (StatSoft).

RESULTS

The descriptive statistics and a comparison of antioxidant parameters in the study and control groups were detailed in Table 1. All patients with diagnosed acute xenobiotics poisoning and the patients with alcohol withdrawal syndrome had statistically significant lower catalase activity in comparison to the control group (41.9 ± 16.5 vs. 196.0 ± 82.2 U/mg, $p = 0.000$). The patients with AWS had higher GSH levels (391.3 ± 257.9 μ mol/mg) compared to the control group (171.4 ± 88.4 μ mol/mg, $p = 0.034$) or to the patients intoxicated with carbamazepine (152.8 ± 102.5 μ mol/mg, $p = 0.027$).

We did not find any other differences between the studied groups and controls. The first three principal components (PC) of the PCA model, with respective eigenvalues 1.49, 1.22 and 1.09, explained 63.1% of total variance of the original parameters. The first PC was loaded mainly by GSH and GPX3 (positively, with corresponding weights: 0.778, 0.747) and % inhibit (negatively, with weight = -0.438), while the second (i.e., vertical) PC was loaded predominantly by CAT (weight = 0.615) and =CO (weight = 0.591). Figure 1 shows patients and controls scores

in the space established by the first two principal components of the PCA model. Spearman rank correlation coefficients, calculated separately for the patients and the controls, and the significance of the difference between them are shown in Table 2.

DISCUSSION

The literature referring to the ROS studies is enormous. The studies are performed using available analytical methods and there are no “gold standard” assays of free radical activity (15, 21). Several approaches such as determination of endogenous antioxidant levels, measurement of the products of oxidized macromolecules and direct detection of free radicals are most often used. To assess endogenous antioxidant capacity, we examined the concentrations of antioxidants like GSH

and the activity of antioxidant enzymes like CAT and GPX3 in plasma.

GSH is rapidly oxidized to GSSG by radicals and other reactive species and then GSSG is exported from cells to the blood; thus, it can provide a valid marker of oxidative stress. To assess ROS-induced protein and lipid oxidation, we determined the production of carbonyl groups and the loss of free sulfhydryl groups, while DPPH was used as a marker of the overall antioxidant capacity.

On the other hand, there is a great diversity of the cases of acute drug overdoses, admitted to the toxicology department. Clinical situation is frequently complicated by the coexistence of risk factors predisposing to the development of adverse drug reactions and drug toxicity. Those involve a complex interaction between toxic potential of the drugs (e.g., reactive metabolites, free radical generation, mitochondrial effects), environmental factors (e.g., con-

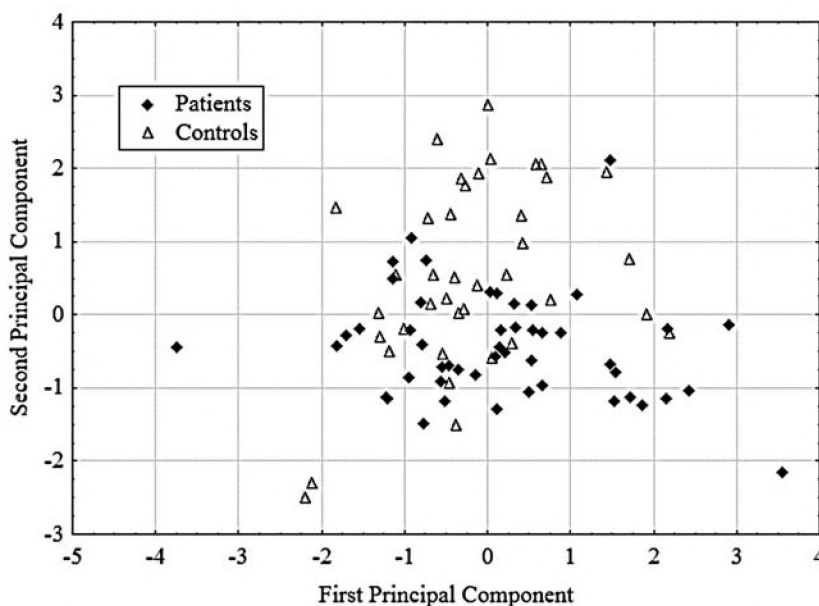


Figure 1. Patients and controls scores in the space established by first two principal components of the PCA model.

Table 2. Spearman rank correlation coefficients for patients and controls ($p < 0.05$), and the significance of the difference between them (NS – not significant).

Pairs of correlated parameters	Patients	Controls	p
CAT and =CO	-0.323	NS	-
CAT and % inhibit.	0.317	-0.388	0.001
=CO and % inhibit.	-0.302	NS	-
GPX3 and GSH	NS	0.500	-

comitant drugs or alcohol use), patient-related factors (e.g., age, gender, underlying diseases, co-medications, nutritional status, activation of the immune system, physical activity) and genetic factors e.g., genetic polymorphisms of genes controlling drug metabolism, detoxification, transport. (1, 2, 22-25). Under physiologic conditions, approximately 1 to 3% of the oxygen consumed by the body is converted into superoxide and other ROS (26). In the course of acute poisoning or chronic drug or ethanol abuse, any person may be at a risk of oxidative stress induced by high rates of oxygen usage (e.g., agitation, respiratory and cardiac failure, hyperthermia). Prolonged exposure to free radicals, even at low concentrations, may result in the damage of biologically important molecules and potentially lead to DNA mutation, tissue injury and disease (27, 28).

Many of the acutely drug poisoned patients and with alcohol addiction chronically use antiepileptic, antidepressant or antipsychotic drugs. It has been reported that long treatment by some antiepileptic drugs (carbamazepine, valproic acids, barbiturates) and tricyclic antidepressant drugs can induce oxidative stress in tissues and organs and lead to their injury. This is reflected by higher lipid peroxidation, protein carbonyl levels, an oxidized glutathione level and inhibition of antioxidant enzymes activities including CAT and GPX (29-32). According to the literature data, CAT activation occurs in a short time (30-60 s.) due to high levels of hydrogen peroxide which arise after acute or chronic exposition to a ROS generating agent (e.g., drug or ethanol overdose). This process may lead to impairment of antioxidant defense manifested in the final fall of CAT activity much below the value observed in the control group, which was also observed in our study.

It can be assumed that the reduced CAT activity is due to its increased consumption as a result of an excess of the formed hydrogen peroxide, or the lack of CAT activation due to a decreased CAT's substrate level. Another factor contributing to the reduced serum CAT activity may be the time period between the xenobiotics overdose and the collection of blood samples, as in clinical practice acutely poisoned patients are admitted to the toxicology department usually more than one hour after the drugs overdose.

On the other hand, we did not observe any statistically significant differences between the patients poisoned due to GPX3 activity. GPX3 activity is known also as a marker of oxidative stress vulnerability (33). Intrinsic GPX3 activity is important, because this enzyme, like CAT, converts hydrogen

peroxide to water with simultaneous oxidation of reduced GSH to its oxidized form, GSSG. Our results suggest that hydrogen peroxide transformation involving GPX3 has less significance than CAT in the case of acute poisonings.

We found statistically significant increased GSH levels in the patients with AWS compared to the control group and to the patients intoxicated with carbamazepine. We did not observe significant GSH depletion in any group of patients, even in the case of malnourished patients with alcohol dependence, psychiatric diseases and with recognized alcoholic liver disease with increased serum transaminase activity. This stands in conflict with other data that confirmed the glutathione role as one of the most important endogenous antioxidants in the cell (34-36). A ROS attack may lead to a major depletion of reduced glutathione, and after GSH depletion the toxicity of ethanol is strikingly enhanced (37-39). At the moment, we cannot explain the dissociation between our experimental data and the results of other authors. Future studies of both reduced and oxidized glutathione and glutathionylated proteins (PSSG), and their relative ratios in human plasma may give fundamental information on the intracellular redox status of the whole organism and be useful quantitative systemic biomarkers of oxidative stress and disease risk, and may also provide essential parameters to link environmental influences and progression of changes associated with alcohol dependence (3, 40-43).

In the case of acute ethanol intoxication, main ethanol metabolism is related to alcohol dehydrogenase (ADH). Chronic ethanol consumption causes also induction of the cytochrome P450-dependent formation of reactive metabolites that cause direct toxicity, with lipid and protein peroxidation leading to phospholipids changes in cell membranes, an increased level of protein carbonyls, decreased sulfhydryl groups, and glutathione depletion (34, 35, 44, 45). The third ethanol metabolism pathway connected with CAT activity is less important. In our study, we noted significantly decreased CAT activity in the alcohol addicted group, while no changes in the carbonyls and sulfhydryl groups level were observed.

It is known that acetaminophen initial hepatotoxicity leads to synthesis of active metabolite, N-acetyl-p-benzoquinone imine (NAPQI). NAPQI reacts with hepatic GSH leading to its depletion. Additionally, NAPQI covalently binds to sulfhydryl groups of proteins as acetaminophen-cysteine adducts (46). It is worth underlining that because of

specific antidotal treatment (e.g., with N-acetylcysteine) used to protect the patients against hepatotoxicity, all those patients who received such treatment before admission to the toxicology department were excluded from our study.

According to the acetaminophen hepatotoxicity mechanism, one should suspect a decreased GSH level in the case of severe acute acetaminophen poisoning. However, it is important to note that all patients within the acetaminophen overdose group were so far healthy young people. All of them were admitted to hospital during the first 24 h after the acetaminophen overdose. In this time period, not all the acetaminophen dose was metabolized (acetaminophen was still present in the blood) and this is a possible reason why we did not observe significant GSH depletion.

Some differences in the scatter of patients and controls can be observed in the PCA plot. Namely, almost all patient were localized in a lower cluster (Fig. 1). As the second (vertical) principal component was mainly influenced by CAT (and to a lesser degree by PCO), such a result confirms our finding that CAT can be a particularly sensitive parameter in diagnosing the onset and progression of acute poisoning. Both groups differed also in correlations between some parameters (Table 2) which appeared in one group but not in the other. Most striking is the difference for the correlation between CAT and % inhibition, as it was positive in the patients and negative in the controls. The physiological meaning of such differences should be clarified in future studies.

CONCLUSIONS

The presented results confirm the significantly reduced CAT activity in all patients with acute drugs poisoning and with alcohol addiction. Oxidant/antioxidant imbalances occurring under the influence of acute or chronic exposition to xenobiotics may cause temporary or fixed dysfunction of biological systems.

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Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this article. All authors of the publication have been informed of the intent and approved it.

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