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Effects of agmatine on chlorpromazine toxicity in the liver of Wistar rats: the possible role of oxidant/antioxidant imbalance

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Abstract: Chlorpromazine (CPZ) is a member of a widely used class of antipsychotic agents. The metabolic pathways of CPZ toxicity were examined by monitoring oxidative/nitrosative stress markers. The aim of the study was to investigate the hypothesis that agmatine (AGM) prevents oxidative stress in the liver of Wistar rats 48 h after administration of CPZ. All tested compounds were administered intraperitoneally (i.p.) in one single dose. The animals were divided into control (C, 0.9% saline solution), CPZ (CPZ, 38.7 mg/kg b.w.), CPZ+AGM (AGM, 75 mg/kg b.w. immediately after CPZ, 38.7 mg/kg b.w. i.p.), and AGM (AGM, 75 mg/kg b.w.) groups. Rats were sacrificed by decapitation 48 h after treatment. The CPZ and CPZ+AGM treatments significantly increased thiobarbituric acid reactive substances (TBARS), the nitrite and nitrate (NO_2+NO_3) concentration, and superoxide anion ($O_2^{\bullet-}$) production in rat liver homogenates compared with C values. CPZ injection decreased the capacity of the antioxidant defense system: superoxide dismutase (SOD) activity, catalase (CAT) activity, total glutathione (GSH) content, glutathione peroxidase (GPx) activity, and glutathione reductase (GR) activity compared with the values of the C group. However, treatment with AGM increased antioxidant capacity in the rat liver; it increased the CAT activity, GSH concentration, GPx activity, and GR activity compared with the values of the CPZ rats. Immunohistochemical staining of ED1 in rats showed an increase in the number of positive cells 48 h after acute CPZ administration compared with the C group. Our results showed that AGM has no protective effects on parameters of oxidative and/or nitrosative stress in the liver but that it absolutely protective effects on the antioxidant defense system and restores the antioxidant capacity in liver tissue after administration of CPZ.
Key words: agmatine, chlorpromazine, liver, macrophages, oxidative stress

Introduction

Neuroleptics are used extensively in the treatment of schizophrenia and other affective disorders. The molecular mechanisms by which neuroleptics increase reactive oxygen (ROS) and reactive nitrogen species are unknown. Neuroleptics act by blocking dopamine receptors [10], which in turn lead to increased production of hydrogen peroxide (H_2O_2), resulting in oxidative stress (OS) [50]. Dopamine is primarily metabolized through oxidation by monoamine oxidase and creates H_2O_2. Hydrogen peroxide can further react with iron or copper

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ions to produce the hydroxyl radical, which is the most toxic of free radicals. Increased dopamine turnover by neuroleptics could lead to excessive production of these potentially damaging free radicals [17]. Oxygen free radicals are also reported to diminish dopamine transporter function, further increasing the extracellular dopamine levels [19].

Chlorpromazine (CPZ) is a typical antipsychotic that may cause distressing side effects involving the extrapyramidal tract [39]. The role of OS in acute CPZ intoxication is not completely understood, but it is known that treatment with neuroleptics increases free radical production and OS [4]. Thiobarbituric acid reactive substances (TBARS) are markers of lipid peroxidation (LPO) and are significantly increased after CPZ poisoning [26]. Induction of LPO is involved in oxidative and nitrosative stress-mediated liver damage [51].

Our previous study showed that CPZ increases the production of free radicals and affects the antioxidant enzyme activity in the rat liver [12]. It is known that OS in the liver is a consequence of increased production of free radicals and decreased capacity of antioxidant defense systems in hepatocytes [41]. Kupffer cells and neutrophils that infiltrate the liver represent one of the sources of ROS. The main sources of ROS within hepatocytes are mitochondrial functional abnormalities, cytochrome P450 2E1, nicotinamide adenine dinucleotide phosphate oxidase, cyclooxygenase and lipoxygenase pathways, and iron overload [28]. Furthermore, decreased antioxidant capacity of hepatocytes contributes to ROS-induced liver injury. This is confirmed by the positive correlation of superoxide dismutase (SOD), catalase (CAT), and reduced glutathione (GSH) level with the degree of liver impairment [25]. Oxygen free radicals cause LPO followed by oxidative modification of proteins and DNA in hepatocytes. Products of LPO cause mitochondrial damage that inhibits the electron transport chain in mitochondria, leading to a further increase of ROS production. Also, ROS activate hepatic stellate cells, which results in deposition of extracellular matrix, contributing to development of liver fibrosis and cirrhosis. It is known that ROS induce synthesis of the inducible isoform of nitric oxide synthase (iNOS), which leads to an increase in the NO level. The liver is one of the major organs that experience severe alterations due to an increase in the steady concentration of both ROS and reactive nitrogen species [48]. ROS indeed act on the fatty acid side chains of lipids present in different cellular membranes, in particular, mitochondrial membranes that are exposed directly and frequently to superoxide anion ($O_2^{\bullet-}$) generated upon cellular respiration. The oxidative damage of mitochondrial macromolecules such as mtDNA, proteins, and lipids, along with the protein synthesis machinery in liver cells, induces mitochondrial dysfunction. These molecular processes trigger a further increase in the steady concentration of ROS, resulting in energy depletion leading to cell death, which eventually damages the organ and its function [55].

Agmatine (AGM) is an amine that is formed by decarboxylation of L-arginine by the enzyme arginine decarboxylase and hydrolyzed by the enzyme agmatinase to putrescine [46]. Recent research has demonstrated a variety of physiological and pharmacological effects of exogenous AGM [38, 47, 56]. The liver plays a crucial physiological role in the maintenance of AGM homeostasis in the organism [6, 33]. Studies have shown that AGM can reduce LPS-induced acute hepatic injury in mice via suppression of NF-κB translocation and reduction of the synthesis and release of cytokines [33]. Hepatoprotective effects of AGM have also been shown in mice with fulminant hepatic failure, and this may have been related to its ability to suppress OS, NO synthesis, and TNF-α production [16]. Therefore, AGM may serve as a novel therapeutic strategy for hepatic inflammatory diseases. On the other hand, biochemical analysis revealed that CPZ treatment significantly induced LPO and decreased GSH levels, as well as the antioxidant defense enzymes SOD and CAT, in experimental rats [39].

Based on this background, the aim of our study was to investigate the role of ROS or reactive nitrogen species and the efficiency of antioxidant protection in the rat liver in acute CPZ intoxication after AGM treatment.

Materials and Methods

Reagents

All chemicals used in this study were of analytical grade and from the following sources. DTNB, NaH_2PO_4, ammonium molybdate, ammonium acetate, NADPH, and NADH were purchased from Merck (Darmstadt, Germany). $Na_2HPO_4 \times 2H_2O$, TCA, nitroblue-tetrazolium, gelatin, Na_2CO_3, $NaHCO_3$, epinephrine, EDTA, NAD^+, methanol, and GSSG (oxidized form) were purchased from Serva, Feinbiochemica GmbH & Co., Hei-

delberg, Germany. TBA was purchased from ICN Biomedicals Aurora, OH, USA, and acetonitrile was purchased from J. T. Baker, Deventer, Netherlands. Glutathione reductase (EC 1.6.4.2), Type III, from yeast [9001-48-3], Sigma-Aldrich Corporation (St. Louis, MO, USA) − highly refined suspension in 3.6 M $(NH_4)_2SO_4$, at pH 7.0; 2,500 U/1.6 ml (9.2 mg protein/ml − biuret) 170 U/mg proteins (note: 1 unit reduces 1 μmol GSSG/min, pH 7.6 at 25°C). Sodium nitrate ($NaNO_3$) was purchased from Mallinckrodt Chemical Works, St. Louis, MO, USA. Agmatine and an analytical standard for CPZ were purchased from Sigma-Aldrich Corporation, St. Louis, MO, USA (catalog number C8138), as were sulfanilic acid and N-(1-naphthyl) ethylendiamine dihydrochloride. Chlorpromazine (Largactil) was purchased from Galenika, Serbia. Saline solution (0.9% w/v) was purchased from a hospital pharmacy (Military Medical Academy, Belgrade, Serbia). All drugs solutions were prepared on the day of the experiment.

Animals

The experimental animals were treated according to the Guidelines for Animal Study (No. 282–12/2002, Ethics Committee of the Military Medical Academy, Belgrade, Serbia and Montenegro). Male adult Wistar rats, 2 months old, with a body mass of 200 ± 50 g were used for experiments. The rats were housed in cages under standardized housing conditions (ambient temperature of 23 ± 2°C, relative humidity of 55 ± 3%, and a light/dark cycle of 13/11 h) and had free access to standard laboratory pellet food and tap water. All the experiments were performed after 7 days of adaptation to laboratory conditions and were carried out between 9 a.m. and 1 p.m.

Experimental procedure

The experiment was accomplished with the following (four) experimental groups, which received different test substances: the sham control group (C group, 0.9% saline solution), n=10; the CPZ group (CPZ-HCl 38.7 mg/kg b.w.), n=10; the CPZ+AGM group (AGM, 75 mg/kg b.w. i.p., immediately after CPZ-HCl administration, 38.7 mg/kg b.w.), n=10; and the AGM group (AGM, 75 mg/kg b.w. i.p.), n=10.

The dose of CPZ (37.8 mg/kg b.w.) was selected on the basis of tests performed by other authors, and it represents 2/3 of the median lethal dose (LD_{50}) for rats [42]. For the dose of AGM (75 mg/kg b.w.), we determined the dose based on our previous studies on other models, which showed that a dose of 75 mg/kg b.w. was not toxic, as judged by no change in body weight or food intake in rats, and did not result in any other visible morphological changes [52].

The animals were sacrificed by decapitation 48 h after the treatments. After sacrifice, livers were excised and stored at −20°C.

Determination of CPZ concentration

The concentration of CPZ was determined in the liver using high-performance liquid chromatography-tandem mass spectrometry (HPLC MS/MS) [27].

Four milliliters of acidic acetonitrile was added to 1 g of liver tissue, and the sample was homogenized on an ULTRA-TURRAX and then centrifugted for 10 min at 3,500 rpm. After centrifugation, the supernatant was decanted into a clean tube, and 6 ml 10% NaCl solution was then added to the supernatant. Purification was performed on C-18 columns, which were conditioned by passing with 5 ml of methanol followed by 5 ml of water. After the sample extract was loaded onto conditioned SPE columns and passed through, the SPE columns were washed with 1 ml 0.01 mol H_2SO_4. CPZ was eluted from SPE columns with a 2 × 3 ml mixture of acidic acetonitrile and methanol (50:50), the eluate was then evaporated under a stream of nitrogen, and the residue was dissolved in 1ml of a mixture of acidic acetonitrile and methanol (50:50).

HPLC MS/MS was performed on a Waters ACQUITY HPLC MS/MS system equipped with a TQD detector. The chromatographic conditions for HPLC MS/MS were as follows: guard column and reversed phase C-18 column (2.1 × 100 mm; 3.5 μm); temperature, 35°C; mobile phase A, 0.1% HCOOH in water; mobile phase B, methanol; mobile phase A gradient for 0 min to 5 min, 95%, 5 min to 6 min, 30%, 6 min to 7 min, 0%, and 7 min to 13 min, 95%; and a mobile phase flow rate of 0.4 ml/min. The mass detector was set in positive ESI mode (protonated molecular ion: m/z 86 to 319.3 and 245.9 to 319.3 for CPZ), the voltage on the capillaries was 3.5 kV, and the cone voltage was 35 V.

The stock standard solution for CPZ was prepared in methanol (concentration 0.897 mg/ml), and standard working solutions were prepared by diluting the stock standard solution in mobile phase.

Measurement of oxidative/nitrosative status parameters

Liver tissue was dissected on ice, and slices of the liver tissue were transferred separately into cold buffered sucrose (0.25 mol/l sucrose, 0.1 mmol/l EDTA in 50 mM sodium-potassium phosphate buffer, pH 7.2). Homogenization of the tissue in the sucrose medium was performed with a homogenizer (Tehnica Zelezniki Manufacturing, Slovenia) with a Teflon pestle at 800 rpm (1,000 g) for 15 min at 4°C. The supernatant was centrifuged at 2,500 g for 30 min at 4°C. The resulting precipitate was suspended in 1.5 ml of deionized water. Solubilization of subcellular membranes in hypotonic solution was performed by constant mixing for 1 h using a Pasteur pipette. Thereafter, homogenates were centrifuged at 2,000 g for 15 min at 4°C, and the resulting supernatant was used for analysis. Total protein concentration was estimated with bovine serum albumin as a standard.

Lipid peroxidation in the liver was measured as TBARS production, as described by Girotti *et al.* [21]. Data were expressed as nmol per mg of proteins.

After deproteinization, the production of NO was evaluated by measuring NO_2+NO_3 concentrations. Nitrates were initially transformed into nitrites by cadmium reduction [40]. Nitrites were assayed directly by spectrophotometry at 492 nm using the colorimetric method of Griess (Griess reagent: 1.5% sulfanilamide in 1 mol HCl plus 0.15% N-(1-naphthyl) ethylendiamine dihydrochloride in distilled water). The results were expressed as nmol per mg of proteins.

Superoxide anion content was determined through the reduction of nitroblue-tetrazolium (NBT) in an alkaline nitrogen-saturated medium. Kinetic analysis was performed at 550 nm [3]. The results were expressed as nmol reduced NBT per min per mg of proteins.

SOD (EC 1.15.1.1) activity was measured spectrophotometrically as the inhibition of spontaneous autoxidation of epinephrine at 480 nm. The kinetics of sample enzyme activity were followed in a carbonate buffer (50 mmol, pH 10.2), containing 0.1 mmol EDTA, after the addition of 10 mmol epinephrine [54]. Data were expressed as U SOD per mg of proteins.

Catalase activity was determined by spectrophotometric method. Ammonium molybdate forms a yellow complex with H_2O_2 and is suitable for measuring both serum and CAT activity in tissue [22]. Kinetic analysis was performed at 405 nm. Units of CAT activity were defined as the number of micromols of H_2O_2 reduced per min (μmol H_2O_2/min). Data were expressed as U CAT per mg of proteins.

Total glutathione (GSH+1/2GSSG, in GSH equivalents) content was determined by DTNB-GSSG reductase recycling assay. The rate of formation of 5-thio-2-nitrobenzoic acid (TNB), which is proportional to the total GSH concentration, was followed spectrophotometrically at 412 nm [1]. The results were expressed as nmol per mg of proteins.

Assessment of GPx activity was performed using a commercial kit [45]. GPx catalyzes the oxidation of reduced GSH by cumene hydroperoxide. In the presence of reduced glutathione reductase (GR) and nicotinamide adenine dinucleotide phosphate reduced form (NADPH), oxidized reduced GSH is immediately converted to the reduced form with concomitant oxidation of NADPH-$NADP^+$. The decrease in absorbance at 340 nm was measured [45]. Units of enzyme activity of GPx were defined as the number of micromols of NADPH oxidized per min (μmol NADPH/min). Data were expressed as U GPx per mg of proteins.

The method for determining the activity of GR was based on the ability of GR to catalyze the reduction of GSSG to GSH by oxidation of the coenzyme NADPH to $NADP^+$ [20]. In the reaction, we used 100 mmol NAD^+ as the standard. The units for enzyme activity were defined as the number of micromols of NADPH oxidized per min (μmol NADPH). The results were expressed as U GR per mg of proteins.

Immunohistochemistry

For immunohistochemical analyses, livers (four per group) were quickly removed and fixed in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS; pH 7.4) for 12 h at 4°C. For cryoprotection, liver tissue was transferred into graded sucrose (10–30% in 0.1 M PBS, pH 7.4). The livers were frozen in 2-methyl butane and kept at −80°C until sectioning on a cryotome. Liver sections (25 μm thick) were collected serially, mounted on Superfrost glass slides, dried for 2 h at room temperature, and stored at −20°C until staining. Slides were incubated with appropriate dilution of mouse monoclonal ED1 antibody 1:100 (Abcam, Cambridge, UK), raised against rat lysosomal membrane antigens of activated macrophages for 60 min [29]. Afterwards, slides were incubated with the peroxidase-labeled polymer (Dako-Cytomation) conjugated to goat anti-mouse immunoglobulins for 30 min. The immunoreaction products were visualized with 3′3-diaminobenzidine (DAB, Dako,

Glostrup, Denmark) according to the manufacturer's instructions. After dehydration and clearing, sections were mounted with mounting medium DPX (Sigma-Aldrich, St. Louis, MO, USA) and examined under a Zeiss Axio Vert microscope (Zeiss, Gottingen, Germany).

Quantitative analysis

Tissue sections were observed using Zeiss Axio Vert microscope (Zeiss, Gottingen, Germany). Three sections of three animals per group were used to statistical determination. Quantitative analysis of immunolabeled cells was made using Image J software. For each sections we captured 4 photomicrographs which were marked as regions of interest (ROI). Immunohistochemical data were expressed as mean value of cell number ± standard deviation (SD).

Statistical analysis

After verifying a normal distribution in all groups, using the Kolmogorov-Smirnov test, the data were presented as mean ± SD values. The data were analyzed statistically by ANOVA followed by Tukey's test. A linear regression analysis was performed using the GraphPad Prism statistical software to determine the relation between the obtained values of parameters. The SD statistical significance of differences was determined by $P<0.05$. Also, immunohistochemical data from ED1 were analyzed statistically by one-way ANOVA using Tukey's test. Three sections of three animals per group were used for statistical analysis. Immunohistochemical data were expressed as mean cell number ± SD values. Statistical significance was defined as $P<0.05$.

Results

The results of our study revealed that CPZ treatment induced different changes in parameters of OS and antioxidant capacity in liver samples of experimental animals.

CPZ concentration in the rat liver

The concentration of CPZ was increased in the CPZ group of animals compared with the C and AGM groups (Table 1). However, administration of CPZ with AGM led to a reduction in drug concentration compared with the CPZ group.

Table 1. CPZ concentration (ppm) in the rat liver 48 h after treatment

Groups	CPZ concentration
C	–
CPZ	1.08 ± 0.39*
CPZ+AGM	0.05 ± 0.01†
AGM	–

The data are expressed as mean ± SD values. Statistical significance: *$P<0.05$ (compared with the control group; one-way ANOVA and Tukey's test); †$P<0.05$ (compared with the CPZ group; one-way ANOVA and Tukey's test).

Concentrations of parameters of oxidative status in the rat liver

In the CPZ group, the TBARS concentration was significantly increased in the liver ($P<0.05$) at 48 h compared with the C group. Also, in both the CPZ+AGM and AGM groups, the TBARS concentrations were significantly elevated in the liver at 48 h of treatment compared with the C group (Fig. 1A).

Administration of CPZ significantly increased the NO_2+NO_3 concentration in the liver 48 h after treatment ($P<0.001$) compared to the concentration in the C group (Fig. 1B). Also, both the CPZ+AGM ($P<0.001$) and AGM ($P<0.05$) groups, the NO_2+NO_3 concentration was significantly elevated in the liver in the same time period compared with that in the C group. In the AGM group, the NO_2+NO_3 concentration decreased ($P<0.001$) in the liver compared with that in the CPZ group.

$O_2^{\bullet-}$ production was significantly increased in the liver of both the CPZ ($P<0.001$) and CPZ+AGM ($P<0.001$) groups compared with the C group (Fig. 1C). The concentration of $O_2^{\bullet-}$ was significantly decreased in the liver of the AGM ($P<0.001$) group compared with the CPZ group 48 h after treatment.

Administration of CPZ led to a decrease in SOD activity ($P<0.001$) compared with the C group 48 h after treatment (Fig. 1D). Treatment with AGM together with CPZ decreased the activity of SOD in the liver compared with the levels of activity in the C group ($P<0.001$) and CPZ group ($P<0.05$). However, AGM alone increased SOD activity in the liver ($P<0.001$) compared with that in the CPZ group.

In the CPZ ($P<0.001$) and CPZ+AGM ($P<0.05$) groups, CAT activity was significantly decreased in the liver compared with that in the C group (Fig. 1E). Administration of AGM, alone ($P<0.001$) or with CPZ ($P<0.01$), resulted in an ancrease in CAT activity in the

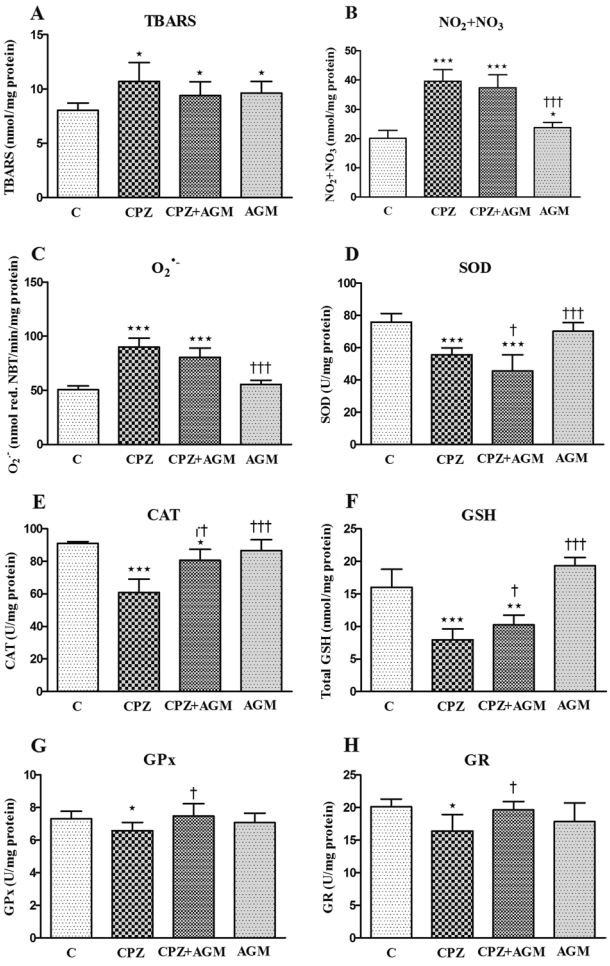

Fig. 1. Oxidative stress in the rat liver 48 h after treatment: A) TBARS concentration (nmol/mg proteins), B) NO_2+NO_3 concentration (nmol/mg proteins), C) $O_2^{\bullet-}$ production (nmol reduced NBT/min/mg proteins), D) SOD activity (U/mg proteins), E) CAT activity (U/mg proteins), F) GSH content (nmol/mg proteins), G) GPx activity (U/mg proteins), H) GR activity (U/mg proteins). Bars in the graphs represent mean ± SD values for 7 animals in each group. Statistical significance: **P*<0.05; ** *P*<0.01; *** *P*<0.001 (compared with the control group; one-way ANOVA and Tukey's test). †*P*<0.05; ††*P*<0.01; †††*P*<0.001 (compared with the CPZ group; one-way ANOVA and Tukey's test).

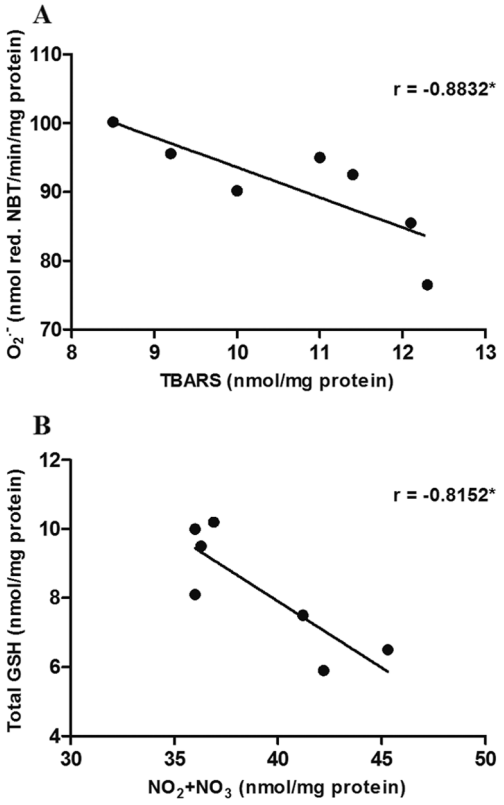

Fig. 2. The correlation of A) TBARS concentration (nmol/mg proteins) and $O_2^{\bullet-}$ level (nmol reduced NBT/min/mg proteins) and B) NO_2+NO_3 concentration (nmol/mg proteins) and total GSH content (nmol/mg proteins) in the liver of CPZ-treated animals. As the TBARS concentration increases, the $O_2^{\bullet-}$ level decreases in parallel (r=−0.8832); at the same time, as the NO_2+NO_3 concentration increases, the total GSH concentration decreases (r=−0.8152) in the liver of rats (Pearson's correlation). There is a negative linear relationship between these variables (*P*<0.05, respectively).

liver 48 h after tretmant compared with that in the CPZ group.

In the CPZ group (*P*<0.001) and CPZ+AGM group (*P*<0.01), total GSH content was significantly decreased in the liver compared with that in the C group (Fig. 1F). Total GSH content increased in the liver in both the CPZ+AGM (*P*<0.05) and AGM (*P*<0.001) groups compared with the CPZ group 48 h after treatment.

Administration of CPZ resulted in a decrease in GPx activity (*P*<0.05) in the liver compared with the level of activity in the C group (Fig. 1G). In the CPZ+AGM group, GPx activity increased (*P*<0.05) compared with that in the CPZ group.

In the CPZ group, GR activity was significantly lower (*P*<0.05) in the liver at 48 h compared with that in the C group (Fig. 1H). However, GR activity significantly increased (*P*<0.05) in the CPZ+AGM group compared with the CPZ group 48 h after treatment.

Our results suggest a significant negative correlation between the TBARS concentration and $O_2^{\bullet-}$ production (r=−0.8832, *P*<0.05) (Fig. 2A) as well as between the NO_2+NO_3 concentration and total GSH content (r=−0.8152, *P*<0.05) (Fig. 2B) in liver 48 h after CPZ administration.

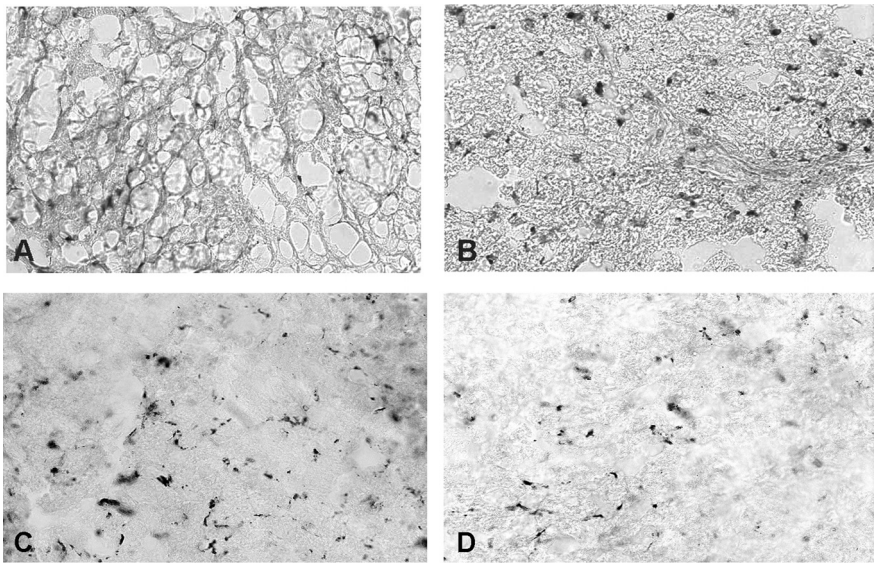

Fig. 3. Representative photomicrographs of ED1 staining by immunohistochemistry of liver sections in the C group (A), CPZ group (B), CPZ+AGM group (C), and AGM group (D). The number of tested rats was 3 for each group. Increased expression of ED1 was noted in liver tissue sections of Wistar rats in the CPZ group compared with sections obtained from the other investigated group (C, CPZ+AGM, AGM). Original magnification, × 400.

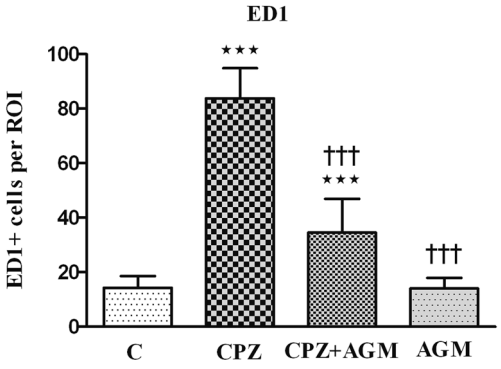

Fig. 4. Quantitative analysis of ED1-positive cells per region of interest (ROI) in the rat liver 48 h after treatment. Bars in the graph represent mean cell number ± SD values. Statistical significance: ***P<0.001 (compared with the control group; one-way ANOVA and Tukey's test). †††P<0.001 (compared with the CPZ group; one-way ANOVA and Tukey's test).

ED1 staining

By means of immunohistochemistry, we investigated the expression of ED1 in CPZ-induced liver injury in rats (Fig. 3). We found that ED1-positive cells in the CPZ and CPZ+AGM groups increased by 487% and 142% compared with the C group, respectively (Fig. 4). However, ED1 positivity decreased in the rats treated with CPZ+AGM (59%) and AGM (83%) compared with the CPZ group.

Discussion

Oxidative stress and peroxidation along with subsequent changes in the antioxidant defense system may be responsible for one of the molecular mechanisms of the liver tissue due to CPZ intoxication. Based on our results, it can be concluded that OS plays an important role in acute CPZ-induced liver injury in rats, with CPZ possibly disturbing the balance between reactive oxygen/nitrogen species production and antioxidant protection. Our findings showed that CPZ increases hepatocyte sensitivity to LPO and nitrosative damage. LPO occurs within 48 h after CPZ administration, while CPZ potentiates impairment of the antioxidant defense system in the liver. A decrease in SOD activity simultaneous to a decrease in GSH level contributes to an impaired antioxidant defense system in the liver and makes hepatocytes more sensitive to CPZ-induced damage during stress.

In line with previous findings showing that the largest amount of CPZ is deposited in the liver, where it exerts harmful effects [9, 13], this study shows that CPZ causes an increase in drug concentration after 48 h compared with the concentration in controls but that the use of AGM with CPZ significantly reduces the concentration in the rat liver (Table 1).

The results presented here indicate that acute CPZ

administration leads to liver damage through processes involved in oxidative modification of lipids, which are strengthened by the negative correlation between the concentration of TBARS and $O_2^{\bullet-}$ production 48 h after CPZ administration. Previously, it was shown that CPZ inhibits microsomal NADPH-induced LPO [31], while other authors have shown that the LPO inhibition after CPZ administration is a result of removing free radicals ($OH^{\bullet}$ i $ROO^{\bullet}$) [8]. Combined treatment with CPZ+AGM failed to induce changes in TBARS concentration in comparison with the CPZ group, indicating that the protective effects of AGM are not due to the influence on lipid peroxides in liver tissue [11]. In our study, increased NO_2+NO_3 concentrations were accompanied by a reduced total GSH content in liver tissue homogenates 48 h after CPZ administration compared with those of the control. In this way, it was shown that increasing NO_2+NO_3 concentrations cause antioxidative defense system damage, which was confirmed by the negative correlation with the total GSH content after 48 h. Furthermore, treatment with AGM led to increased NO_2+NO_3 concentrations compared with the concentration in the control group, which can be explained by the mechanism of secondary inflammation [37].

It was previously mentioned that the reaction between NO and $O_2^{\bullet-}$ generated peroxynitrite ($ONOO^-$), which is an extremely strong oxidizing and nitrating agent and can react with all classes of biomolecules. In the liver of rats 48 h after CPZ administration, we found increased $O_2^{\bullet-}$ production compared with the level of the control.

Concurrent with OS, disbalance of antioxidative defense system components occurs. The decrease in SOD activity in the present study can be explained by the severe OS [35]. The observed decrease in SOD activity and increase in $O_2^{\bullet-}$ formation 48 h after CPZ administration indicate the effects of OS on the rat liver. Similar results showing reduced SOD activity after CPZ administration have been reported by other authors [39, 43]. It is believed that these data for reduced enzyme activity after a single CPZ administration can be explained by the LPO formation in the liver tissue, which was confirmed in our experiment in the 48 h period after CPZ administration by comparison with the level of control animals. Inactivation of SOD may also be caused by increased free radical production, which could subsequently cause oxidative modification of proteins of the enzyme; this was not excluded as a possible cause of inhibition of SOD activity after CPZ administration.

Apart from reduced SOD activity, CPZ+AGM administration failed to induce changes in NO_2+NO_3 concentration and $O_2^{\bullet-}$ production in the rat liver compared with the levels in the CPZ group. These results could be explained by $O_2^{\bullet-}$ and NO being expended in the formation of highly toxic $ONOO^-$, which would mean that AGM does not show protective effects on liver tissue in this time period. In addition, NO has a higher affinity for $O_2^{\bullet-}$ with regard to SOD, which may explain the reduced SOD activity due to the lack of a substrate ($O_2^{\bullet-}$) [15].

The largest concentration of CAT is present in the liver. Can and his associates [7] showed that CPZ administration in rats affects the activity of antioxidant enzymes (SOD and CAT) in liver tissue. CPZ leads to structural changes and modifications of membrane permeability of endothelial cells in a dose-dependent manner, which affect hemodynamic resistance vessels *in vivo*. Also, CPZ has prooxidant effects and acts via its metabolites, which are involved in the formation of H_2O_2 by the process of autoxidation [30]. Although it has protective effects against other oxygen radicals ($OH^{\bullet}$ or $O_2^{\bullet-}$), CPZ is not involved in the removal of H_2O_2 [7]. The results of another study on a model of nonalcocholic fatty liver disease also showed that CAT activity may be reduced in the liver [28]. The results of our study show that CPZ led to a decrease in CAT activity in the liver after 48 h compared with the level of activity in the C group. Oxidative stress leads to the activation of a hepatic stellate cells and Kupffer cell proinflammatory response, which is involved in the development of inflammation and fibrosis [32].

One explanation for the reduced antioxidant capacity and reduced CAT activity in the liver of rats after acute and subacute CPZ administration, could be increased CYP2E1 activity in the liver, leading to inactivation of CAT and SOD after CPZ administration [44]. Our results are consistent with these studies, because acute CPZ administration in addition to reduced CAT activity led to decreased SOD activity in the liver of rats compared with the level in the C group, which can be explained by the progressive development of OS. Treatment with CPZ+AGM after 48 h led to an increase in CAT activity as compared with the level of activity in the CPZ group.

The liver is an important source of glutathione for other peripheral tissues [5, 36]. In our experiment, there were significant reductions in total GSH concentration in the liver of rats 48 h after acute CPZ administration with the concentration of the C group. These results are

consistent with research groups from other laboratories, which have shown reduced GSH concentrations, leading to increased OS in the liver [39, 53]. It is generally accepted that acute CPZ administration leads to reduced GSH levels due to its consumption in the GPx reaction when neutralizing free radicals generated by prolonged exposure to this xenobiotic.

Treatment with CPZ+AGM led to an increase in total GSH concentration in the liver after 48 h compared with the concentration of the CPZ group, as was previously shown 24 h after treatment [12]. This increase is probably due to expression of gamma-glutamylcysteine synthetase, which catalyzes the key reaction of GSH biosynthesis [23, 49].

As a result of the increase in OS, the GPx activity of the CPZ group was significantly reduced in the liver 48 h after treatment. Different ROS are responsible for the oxidation of essential sulfhydryl groups (SH) involved in the control of activities of many enzymes [14]. Peroxides can be converted to a less oxidized form H_2O_2. However, the main danger of increased H_2O_2 is reflected in its ability to pass through the cell membrane very quickly; when H_2O_2 enters a cell, it can react with Fe^{2+} and Cu^{2+} ions and form toxic $OH^{\bullet}$, which represents the beginning of the toxic insult. In endothelial cells, peroxides can be converted to hydroxyl ions in the presence of iron. A hydroxyl radical can react with all of the components of the DNA molecule and can be included in the damage of purine and pyrimidine bases [24].

A sufficient amount of reduced GSH maintains GR activity, which indirectly contributes to the antioxidant status [18]. We found a significant decrease of GR in the liver compared with the level of C group, which confirms that the antioxidative defense system was disrupted after acute CPZ administration in rats.

Studies show that GSH together with GPx plays an important role in protecting cells from cytotoxic agents collecting ROS [2]. In addition, it is known that GR participates in the detoxification of xenobiotics, free radicals, and peroxide through the removal of toxic agents with GSH, which ultimately protects cells and organs from damage caused by various toxins [34]. Administration of AGM with CPZ in experimental animals prevents biological oxidative damage. Increased GR activity in the liver of rats 48 h after acute and subacute AGM+CPZ administration may be due to removal of free radicals and reduction of OS. In liver tissue 48 h after acute administration AGM+CPZ caused an increase in enzymatic (CAT, GPx, GR) and nonenzymatic (GSH) components of antioxidant protection compared the levels of the components in the CPZ group, which means that AGM, as a collector of free radicals, preserves the antioxidant status.

Studies also show that administration of CPZ to rats leads to morphological changes, with basic pathological changes in the appearance of pigment granules and intracellular vacuoles within hepatocytes, inflammatory cell infiltrates and degeneration [53]. Our results suggest that ED1-positive cells increased in the CPZ group (487%) compared with the control, whereas treatment with CPZ+AGM reduced the number of positive cells by 59% compared with the number in the CPZ group (Fig. 4).

Administration of AGM partially ameliorated oxidative/nitrosative damage in liver injury after CPZ hepatotoxicity. Administration of AGM does not lead to changes in NO and $O_2^{\bullet-}$, indicating that they are expended in the synthesis of highly toxic $ONOO^-$. These results show that in the 48 h period studied here AGM did not show protective effects on the oxidative and nitrosative stress in the liver tissue, although it did affect the recovery of antioxidant defense in the liver of CPZ-treated rats. There was a trend toward reduction of excessive production of free radicals, but it is obvious that the 48 h period was not sufficient to remove the effects of LPO and nitrosative stress. On the other hand, AGM absolutely has protective effects on the antioxidative defense system.

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