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Original article

Protective Effects of Agmatine against Chlorpromazine-Induced Toxicity in the Liver of Wistar Rats

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SUMMARY

The metabolic pathways of chlorpromazine (CPZ) toxicity were tracked by assessing oxidative/nitrosative stress markers. The main objective of the study was to test the hypothesis that agmatine (AGM) prevents oxidative/nitrosative stress in the liver of Wistar rats 15 days after administration of CPZ. All tested substances were administered intraperitoneally (i.p.) for 15 consecutive days. The rats were divided into four groups: the control group (C, 0.9 % saline solution), the CPZ group (CPZ, 38.7 mg/kg b.w.), the CPZ+AGM group (AGM, 75 mg/kg b.w. immediately after CPZ, 38.7 mg/kg b.w. i.p.) and the AGM group (AGM, 75 mg/kg b.w.).

Rats were decapitated 15 days after the appropriate treatment. In the CPZ group, CPZ concentration was significantly increased compared to C values ($p<0.01$), while AGM treatment induced the significant decrease in CPZ concentration in the CPZ+AGM group ($p<0.05$) and the AGM group ($p<0.01$). CPZ application to healthy rats did not lead to any changes of lipid peroxidation in the liver compared to the C group, but AGM treatment decreased that parameter compared to the CPZ group ($p<0.05$). In CPZ liver homogenates, nitrite and nitrate concentrations were increased compared to controls ($p<0.001$), and AGM treatment diminished that parameter in the CPZ group ($p<0.05$), as well as in the AGM group ($p<0.001$). In CPZ animals, glutathione level and catalase activity were decreased in comparison with C values ($p<0.01$ respectively), but AGM treatment increased the activity of catalase in comparison with CPZ animals ($p<0.05$ respectively). Western blot analysis supported biochemical findings in all groups. Our results showed that treatment with AGM significantly supressed the oxidative/nitrosative stress parameters and restored antioxidant defense in rat liver.

Key words: **agmatine, antioxidant defense, chlorpromazine, liver, oxidative stress**

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INTRODUCTION

Oxidative and nitrosative stress are involved in the pathophysiology of various neurological disorders. Chronic treatment with neuroleptics increases the production of free radicals and the development of oxidative stress (OS) (1). Typical antipsychotics lead to increased OS by altering the levels of antioxidant enzymes, and cause oxidative damage, particularly lipid peroxidation (LPO) in the brain (2).

Chlorpromazine (CPZ) is a typical antipsychotic that may cause distressing side effects involving the extrapyramidal tract (3). The mechanism of CPZ-induced liver injury has been proposed, but has not been fully clarified, since many factors were found to be implicated in its adverse effects on the liver. CPZ leads to a dose-related impairment in bile secretion and changing hepatocyte and canalicular membrane fluidity, which consequently affects the functional integrity of these sites. It has been shown that oral administration of CPZ for two weeks causes infiltration of inflammatory cells and leads to focal necrosis (4).

The mechanism responsible for CPZ-induced injury includes damage initiated by the activation of Kupffer cells, which release proinflammatory cytokines and stimulate the migration and accumulation of neutrophils and monocytes in the liver. Activated inflammatory cells amplificate primary injury induced by CPZ (5).

It is known that neuroleptics increase free radical production and development of OS (1). Tiobarbituric acid reactive supstances (TBARS) are markers of LPO and were significantly increased after CPZ poisoning (6)

The induction of LPO may be a major factor in oxidative- and nitrosative-mediated liver damage (7).

Nitric oxide (NO) is the product of a five-electron oxidation of the amino acid L-arginine. It can produce hydroxyl radicals (OH•) as well as nitrogen dioxide radical (8). Nitric oxide is produced by the action of the isoenzymes of NO synthases (NOS). It may react with thiol groups in amino acids and proteins and form relatively stable nitroso-thiols (9). Also, NO can be coupled with superoxide anion radical ($O_2^{\bullet -}$) to produce peroxynitrite ($ONOO^-$), a harmful compound to cellular structures, which has been linked to several interactions that may contribute to cellular damage, including LPO (10).

Under physiological conditions, the potential for free radical-mediated damage is kept in check by the antioxidant defense system, which is composed of enzymatic and non-enzymatic components (2). Our previous study showed that CPZ increased the production of free radicals and affected the antioxidant enzyme activity in rat liver (11). 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 (12).

The present study was directed to potentially benefit the influence of agmatine (AGM) on oxidative stress development during CPZ toxicity. The liver plays a crucial physiological role in the maintenance of AGM homeostasis (13, 14). Studies have shown that AGM may serve as a novel therapeutic strategy for hepatic inflammatory diseases (15, 16). Contrary, biochemical analysis in experimental rats revealed that CPZ treatment significantly induced LPO and decreased glutathione (GSH) levels, as well as antioxidant defense enzymes superoxide dismutase (SOD) and catalase (CAT) (3). Based on these findings, the main objective of our research was to investigate the role of reactive oxygen (ROS) and nitrogen (RNS) species, as well as the efficiency of antioxidant protection in rat liver in subacute CPZ intoxication after AGM treatment.

MATERIAL AND METHODS

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 body mass 200 ± 50 g, were used for the experiment. Groups of two or three rats per cage (Erath, FRG), were housed in an air conditioned room at the temperature of 23 ± 2 ºC with 55 ± 10 % humidity and with lights on 12 h/day (07.00-19.00 h). The animals were given a commercial rat diet and tap water *ad libitum*.

Experimental procedure

The experiment was accomplished with the following (four) experimental groups, which received different testing substances: the control group (C, 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 animals were sacrificed by decapitation 15 days after the treatments. For the same purpose, the liver were excised and stored at -20 °C.

Determination of CPZ concentration

The concentration of CPZ was determined in the liver using a high performance liquid chromatography-tandem mass spectrometry (HPLC MS / MS) (17).

In one gram of liver tissue, 4 mL of acidic acetonitrile was added and the sample was homogenizated on Ultra Turax, then centrifuged for 10 minutes at 3500 rpm. After centrifugation the supernatant was decanted into clean tube and 6 mL 10 % NaCl solution were added to the supernatant. The purification was performed on C-18 columns, which were conditioned by passing of 5 mL of methanol, folowed by 5 mL of water. After the sample extract was loaded onto conditioned SPE colums and passed trough, SPE columns were washed with 1 mL 0.01 mol H_2SO_4. CPZ was eluted from SPE colums with 2x3 mL mixture of acidic acetonitrile and methanol (50:50), eluated then evaporated under the stream of nitrogen and the residue was dissolved in 1mL of a mixture of acidic acetonitrile and methanol (50:50).

The method was performed on HPLC MS/MS Waters Acquity with TQD detector. The chromatographic conditions for HPLC MS/MS were as follows: guard column and a reversed phase column C-18; 2.1 x 100 mm; 3.5 μm; temperature 35 °C, mobile phase A- 0.1 % HCOOH in water : B-methanol. Gradient: 0 min - 5 min 95 % A, 5 min - 6 min 30 % A, 6 min – 7 min 0 % A, 7 min-13 min 95 % A, a mobile phase flow rate was 0.4 mL/min. The mass detector in the positive ESI mode: protonated molecular ion: m/z 319.3 → 86. 319,3 → 245.9 for CPZ. The voltage on the capillaries was 3.5 kV. Cone voltage 35 V.

The stock standard solution of 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

The 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 by a homogenizer with a Teflon pestle at 800 rpm for 15 minutes at 4 °C. The supernatant was centrifuged at 2500 g for 30 minutes at 4 °C. The resulting precipitate was suspended in 1.5 mL of deionised water. Homogenates were centrifuged at 2000 g for 15 minutes at 4 °C and the resulting supernatant was used for analysis (18). Total protein concentration was estimated with bovine serum albumin as a standard (19).

Lipid peroxidation in forebrain cortex was measured as tiobarbituric acid reactive substances production (TBARS), as described by Girotti et al. (20). Data were expressed as nmol per mg of proteins.

After deproteinization, the production of NO was evaluated by measuring nitrite and nitrate concentrations (NO_2+NO_3). Nitrates were previously transformed into nitrites by cadmium reduction (21). Nitrites were assayed directly spectrophotometrically 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.

Total glutathione (GSH+1/2GSSG, in GSH equivalents) content was determined with 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 (22). The results were expressed as nmol 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 the tissue (23). Kinetic analysis was performed at 405 nm. Units of CAT activity is defined as the number micromol H_2O_2 reduced per minute (μmol H_2O_2/min). Data were expressed as U CAT per mg of proteins.

Reagents

All chemicals used in this study were of analytical grade. DTNB, NaH_2PO_4, ammonium molybdate, NADPH and NADH were purchased from Merck (Darmstadt, Germany). Na_2HPO_4 x $2H_2O$, TCA, methanol and GSSG (oxidized form) were purchased from Serva, Feinbiochemica GmbH & Co Heidelberg, New York. TBA was purchased from

ICN Biomedicals Inc., Ohio, and acetonitrile was purchased from Backer J.T., Deventer, Netherlands. Glutathione reductase (EC 1.6.4.2), Type III, from yeast [9001-48-3], Sigma Chemical Co (St Luis, MO, USA) – highly refined suspension in 3.6 M $(NH_4)_2SO_4$, at pH 7.0; 2500U/1.6 mL (9.2 mg prot/mL – biuret) 170 U/mg proteins (Note: 1 unit reduces 1 μmol GSSG/min, pH 7.6 at 25 ºC). Sodium nitrate ($NaNO_3$) was purchased from Mallinckrodt Chemical Works – St. Louis, MO, USA. Analytical standard for CPZ was purchased from Sigma-Aldrich Corporation, St. Louis, MO, USA; catalog number C8138, as well as sulphanilic acid and N-(1-naphthyl)ethylendiamine dihydrochloride. Saline solution (0.9 % w/v) was purchased by the Hospital Pharmacy (Military Medical Academy, Belgrade, Serbia). All solutions were made on the day when the experiments were done.

Western blot analysis

After decapitation, livers were dissected and pooled from three animals. The selected tissue was homogenized with a hand-held pestle in sodium dodecyl sulfate (SDS) sample buffer (10 ml/mg), which contained a cocktail of proteinase and phosphatase inhibitors (24). The electrophoresis samples were heated at 100 °C for 5 minutes and loaded onto 10 % SDS-polyacrylamide gels with standard Laemmli solutions. The proteins were electroblotted onto a polyvinylidene difluoride membrane, which were placed in a blocking solution (Tris-buffered saline with 0.02 % Tween TBS-T and 5 % non-fat dry milk) for 1 hour, and incubated overnight under gentle agitation with primary antibody mouse anti-ED1 (1:7000 Abcam, Cambridge, UK) and mouse anti-β-tubulin (1:1000; Sigma, St Louis, MO, USA). Bound primary antibodies were detected with a horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoreactive bands were visualized on X-ray films using chemiluminescence. Optical densities of immunoreactive bands from 4 independent blots were calculated in Image Quant program. The densities of ED1 and β-tubulin immunoreactive bands were quantified with background subtraction. Squares of identical sizes were drawn around each band to measure density, and background near that band was subtracted. For each blot, optical densities were normalized against β-tubulin levels.

Statistical analysis

One Way ANOVA and Kolmogorov-Smirnov test were used (Software GraphPad Prism, version 5.01) for statistical data analysis. The data are presented as mean ± SEM. The statistical significance of differences was determined by $p < 0.05$.

RESULTS

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

CPZ concentration in the rat liver

The concentration of CPZ was significantly increased in the liver of CPZ-treated animals compared to the controls (Table 1). The treatment with AGM alone or applied together with CPZ decreased CPZ concentration in the liver, compared to the CPZ group.

Table 1. ***CPZ concentration (ppm) in the rat liver 15 days after appropriate treatment***

	CPZ concentration
C	1.30 ± 0.60
CPZ	4.70 ± 1.79**
CPZ+AGM	2.40 ± 1.11#
AGM	1.35 ± 0.91##

Concentrations of parameters of oxidative/nitrosative status in the rat liver

The concentration of TBARS in the CPZ+AGM group was significantly decreased 15 days after the treatment in the liver, compared to CPZ-induced TBARS increase (compared to the control values) in the CPZ group of animals (Table 2).

Table 2. Concentrations of parameters of oxidative/nitrosative stress – TBARS (nmol/mg proteins), NO_2+NO_3 (nmol/mg proteins) and antioxidative defense – GSH (nmol/mg proteins), CAT (U/mg proteins) in the liver of Wistar rats

	TBARS (nmol/mg proteins)	NO_2+NO_3 (nmol/mg proteins)	GSH (nmol/mg proteins)	CAT (U/mg proteins)
C	7.34 ± 1.68	12.00 ± 1.15	14.50 ± 1.98	84.10 ± 4.21
CPZ	8.27 ± 1.41	20.78 ± 2.60***	9.73 ± 1.12**	70.90 ± 6.88**
CPZ+AGM	6.33 ± 0.45$^{\#}$	16.83 ± 2.19$^{***,\#}$	8.35 ± 1.00$^{***,\#}$	79.68 ± 5.62$^{\#}$
AGM	9.37 ± 0.94	12.35 ± 2.02$^{\#\#\#}$	18.18 ± 2.68$^{*,\#\#\#}$	84.83 ± 11.16$^{\#}$

The administration of CPZ resulted in NO_2+NO_3 concentrations increase 15 days after the tretmant in the liver, compared to controls (Table 2). In the CPZ+AGM and AGM group of animals, NO_2+NO_3 concentrations were decreased in the liver, compared to CPZ-treated group.

In the CPZ group, total GSH content was significantly decreased in the liver compared to the controls (Table 2). Total GSH content significantly decreased in the CPZ+AGM group compared to CPZ group in the liver 15 days after the treatment. Contrary, total GSH concentration increased in AGM group compared to both control and CPZ group in the liver (Table 2).

In the CPZ group, after 15 days, CAT activity was significantly lower in the liver, compared to the control (Table 2). However, CAT activity significantly increased in both CPZ+AGM and AGM groups in the liver, compared to CPZ-treated animals 15 days after the treatment.

Western blot analysis

To assess the pattern of ED1 protein expression following CPZ-induced liver injury in rats and after AGM treatment, livers were isolated 15 days post-injury. Immunoblot analysis showed that ED1 was present as a single band with a molecular mass of about 37 kDa (Figure 1). There was a significant increase in ED1 expression in physiological control compared to all other groups (CPZ, CPZ+AGM, AGM).

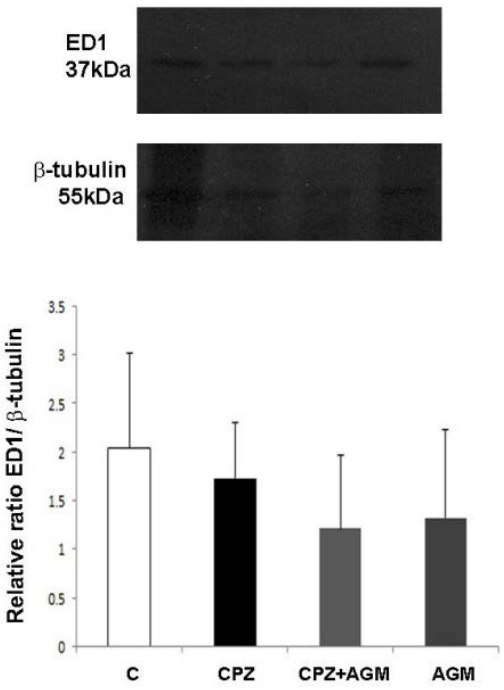

Figure 1. ***Quantitative immunoblot detection of ED1 protein levels in the liver isolated from control, CPZ, CPZ+AGM and AGM group 15 days after the treatment. Bars represent mean ED1 protein abundance (± SEM) from three independent determinations expressed relative to β-tubulin. Significance level is shown in the graph (*p < 0.05 vs. control), which is accompanied by a representative immunoblot.***

DISCUSSION

Presented results showed that OS played an important role in subacute CPZ-induced liver injury in rats, which can disturb the balance between ROS/RNS production and antioxidant defense in the liver.

In the rat liver, there was a greater increase in CPZ concentration compared to the brain. It confirms previous findings of the largest amount of deposited CPZ in the liver with the most important role for the manifestation of its harmful effects (25, 26). Subacute application of CPZ during 15 days induced the increase of concentration of the drug in rat liver compared with the control values (Table 1). However, the administration of CPZ+AGM significantly decreased CPZ concentration in the liver, indicating that the presence of AGM reduced the deposition of CPZ.

Our research showed no significant change in the concentration of TBARS in the liver after CPZ administration (but a slight increase), while the combined treatment CPZ+AGM after 15 days significantly reduced the concentration of TBARS compared to the controls (Table 2). The liver has the greatest sensitivity to changes caused by CPZ and the reduction of TBARS by AGM may be the result of some induced mechanism of hepatoprotection (27).

It has been reported that low concentrations of AGM (10–100 mM) are able to amplify an OS pathway, which is triggered by the reaction products of AGM oxidation (28). However, if AGM is present at higher concentrations (e.g. 1–2 mM), it does not affect mitochondrial respiration and is ineffective in inducing OS (29).

Increased NO_2+NO_3 concentrations accompanied by a reduced total GSH content in the liver tissue homogenates 15 days after CPZ administration indicate that nitrosative stress associated with antioxidative defense system damage is present in this kind of liver damage (Table 2). The treatment with AGM leads to decreased NO_2+NO_3 concentrations compared to control group, which could be explained by protective effect of AGM on the mechanism of secondary inflammation (30).

The liver is an important source of GSH to the other peripheral tissues, so the intensive metabolism of xenobiotics in this organ could lead to GSH reduction and decline in GSH concentration in other peripheral tissues (31, 32). Glutathione neutralizes ROS within cells directly or through cycle glutathione peroxidase/GSH (33). Significant reductions in total GSH concentration 15 days after subacute CPZ administration in the liver compared to the controls is consistent with the results of the research groups from other laboratories (3, 4).

Catalase can protect living organisms from oxidative damage by the removal of partially reduced oxygen species (34). The highest activity of CAT is present in the liver. One group of authors showed that CPZ administration in rats affected the activity of antioxidant enzymes (SOD and CAT) in liver tissue (35). At a dose-dependent manner CPZ leads to structural changes and modifications of membrane permeability of endothelial cells, which affects hemodynamic resistance vessels *in vivo* (36). Also, CPZ has prooxidant effects and acts through the operation of its metabolites, which are involved in the formation of H_2O_2 by the process of autoxidation (37). In addition to the protective effects against other oxygen radicals ($OH^{\bullet}$ or $O_2^{\bullet-}$), CPZ is not involved in the removal of H_2O_2 (35). Fifteen days of CPZ application led to the decreased CAT activity in the liver compared to the control group (Table 2). This result could direct toward the mechanism of activation of hepatic stellate cells and Kupffer cells' proinflammatory response, which is involved in the development of inflammation and fibrosis (38). In our study, 15 days after subacute CPZ application, there was no difference in positivity of ED1 cells compared to the CPZ+AGM, as well as AGM group of animals on tissue sections of the rat liver. On the basis of the electrophoretic profiles of the ED1 molecules in the liver, we found clear differences in the level of expression of the protein, which were the most pronounced in the control group compared to all other groups 15 days after the treatment (Figure 1).

One explanation for the reduced antioxidant capacity and reduced CAT activity in the liver of rats after subacute CPZ application could be an increased CYP2E1 activity in the liver, which leads to the inactivation of CAT during poisoning (39). The treatment with CPZ+AGM after 15 days leads to an increase in CAT activity as compared to the CPZ-group of animals (Table 2).

The results of this study firstly indicate that treatment with AGM accomplished a protective role against harmful CPZ poisoning. Thus, our findings provide useful information about AGM, which significantly supressed the oxidative and nitrosative stress parameters and restores antioxidant defense in the rat liver.

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Zaštitna uloga agmatina kod toksičnih efekata izazvanih hlorpromazinom u jetri Wistar pacova

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SAŽETAK

Metabolički putevi oštećenja izazvani hlorpromazinom (CPZ) ispitivani su praćenjem markera oksidativnog/nitrozativnog stresa. Cilj studije bio je ispitati hipotezu da li agmatin (AGM) smanjuje oksidativni/nitrozativni stres u jetri Wistar pacova 15 dana posle davanja CPZ. Sve supstance aplikovane su intraperitonealno (i.p.) uzastopno 15 dana. Životinje su podeljene u četiri grupe: kontrolna (C, 0,9 % fiziološki rastvor), CPZ (CPZ, 38,7 mg/kg TM), CPZ+AGM (AGM, 75 mg/kg TM odmah nakon CPZ, 38,7 mg/kg TM i.p.) i AGM (AGM, 75 mg/kg TM).

Pacovi su žrtvovani dekapitacijom 15 dana nakon tretmana. Koncentracija CPZ je u CPZ grupi značajno povećana u poređenju sa kontrolnim vrednostima ($p<0,01$), dok tretman AGM-om dovodi do značajnog smanjenja koncentracije CPZ u CPZ+AGM ($p<0,05$) i AGM grupi ($p<0,01$). Aplikacija CPZ zdravim životinjama ne dovodi do promene koncentracije TBARS u jetri pacova u poređenju sa kontrolom, međutim, tretman AGM-om smanjuje koncentraciju ovog parametra u poređenju sa CPZ grupom ($p<0,05$). U homogenatima jetre CPZ grupe, koncentracija nitrita i nitrata je povećana u poređenju sa kontrolom ($p<0,001$) i tretman AGM-om smanjuje ovaj parametar u CPZ grupi ($p<0,05$), kao i u AGM grupi ($p<0,001$). Kod CPZ pacova smanjena je koncentracija glutationa, kao i aktivnost katalaze u poređenju sa C vrednostima (u svakoj grupi $p<0,01$), dok tretman AGM-om povećava aktivnost katalaze u poređenju sa CPZ životinjama (u svakoj grupi $p<0,05$). Western blot analiza prati biohemijske nalaze u svim grupama. Naši rezultati su pokazali da AGM smanjuje parametre oksidativnog/nitrozativnog stresa i oporavlja antioksidativni kapacitet u jetri pacova.

Ključne reči: **agmatin, antioksidativna odbrana, hlorpromazin, jetra, oksidativni stres**