# Oxidative stress induced by chlorpromazine in patients treated and acutely poisoned with this drug

## Oksidativni stres izazvan hlorpromazinom kod bolesnika lečenih i akutno otrovanih ovim lekom

Bratislav Dejanović\*, Vesna Vuković-Dejanović[†], Ivana Stevanović[‡§], Ivana Stojanović[||], Gordana Mandić Gajić[§¶], Sanda Dilber\*\*

\*Military Medical Center "Karaburma", Belgrade, Serbia; [†]Institute for Rehabilitation, Belgrade, Serbia; [‡]Institute for Medical Research, [¶]Clinic of Psychiatry, Military Medical Academy, Belgrade, Serbia; [||]Institute for Biochemistry, Faculty of Medicine, University of Niš, Niš, Serbia; \*\*Department of Organic Chemistry, Faculty of Pharmacy, University of Belgrade, Belgrade, Serbia; [§]Faculty of Medicine of the Military Medical Academy, University of Defence, Belgrade, Serbia

**Abstract**

**Background/Aim.** Although chlorpromazine (CPZ) is an antipsychotic drug widely used in clinical practice for a long time, its mechanism of action has not been entirely defined. An extremely difficult managing of patients acutely poisoned with CPZ is additional reason for detailed studying its toxicity mechanisms. In this clinical study, we investigated whether the oxidative stress (OS) mediates CPZ toxic effects in the exposed patients. **Methods.** The patients were organized into 3 groups: the T-group – hospitalized patients receiving therapeutic doses of 75–150 mg CPZ/day; the overdosed group, divided into two subgroups: the group M and the group S – mildly (CPZ serum concentration: $0.21 \pm 0.05$ mg/L) and severely (CPZ serum concentration: $2.66 \pm 0.25$ mg/L) poisoned patients, respectively, and the group C (control group of healthy volunteers). Oxidative stress parameters [total antioxidative status (TAS) and malondialdehyde (MDA) in plasma] and superoxide dismutase (SOD) activity in erythrocytes were measured spectrophotometrically, and CPZ concentrations in serum were monitored chromatographically. One set of measurements was performed in the group C and T, whereas two sets of measurements (after 24 hours and 48 hours) were done in the poisoned patients, groups M and S. **Results.** A decrease of TAS and increase of SOD activity were obtained in both subgroups of the poisoned patients, compared to the controls and the group receiving therapeutic doses of CPZ. A significant increase of MDA was achieved in severely poisoned patients, compared to all other groups. **Conclusion.** Changed oxidative stress parameters in patients poisoned with chlorpromazine indicate involvement of oxidative stress in the toxicity mechanism(s) of chlorpromazine.

**Key words:**
chlorpromazine; poisoning; oxidative stress; malondialdehyde; superoxide dismutase.

**Apstrakt**

**Uvod/Cilj.** Iako hlorpromazin (CPZ) pripada grupi antipsihotika i široko se primenjuje u kliničkoj praksi već duže vreme, njegov mehanizam dejstva još uvek nije potpuno definisan. Izuzetno teško stanje bolesnika nakon akutnog trovanja CPZ dodatni je razlog za detaljno proučavanje mehanizama toksičnosti leka. U ovoj kliničkoj studiji, ispitivali smo da li je oksidativni stres (OS) uključen u toksične efekte kod bolesnika nakon akutnog trovanja CPZ. **Metode.** Bolesnici su bili podeljeni u tri grupe: T-grupa – hospitalizovani bolesnici koji su dobijali terapijsku dozu leka (75–150 mg CPZ dnevno); grupa akutno otrovanih osoba, podeljena u dve podgrupe: grupa M (blago trovanje: koncentracija CPZ u serumu: $0.21 \pm 0.05$ mg/L) i grupa S (teško trovanje: koncentracija CPZ u serumu: $2.66 \pm 0.25$ mg/L) i grupa C (kontrolna grupa zdravih dobrovoljaca). Parametri OS [ukupni antioksidativni status (TAS) i malondialdehid (MDA) u plazmi] i aktivnost superoksid dizmutaze (SOD) u hemolizatu eritrocita određivani su spektrofotometrijski, dok je koncentracija CPZ u serumu praćena hromatografski. Svi parametri određivani su jednokratno u grupi C i T, dok su u grupi M i S merenja izvršena u dva termina, posle 24 h i 48 h nakon trovanja. **Rezultati.** Smanjenje TAS i povećanje aktivnosti SOD dobijeno je u obe grupe bolesnika nakon trovanja, u poređenju sa kontrolnom grupom i grupom bolesnika koja je bila na terapijskim dozama CPZ. Značajno povećanje MDA dobijeno je u grupi bolesnika sa teškim trovanjem, u poređenju sa svim drugim grupama. **Zaključak.** Promene parametara oksidativnog stresa kod bolesnika nakon trovanja hlorpromazinona pokazuju uključenost oksidativnog stresa u mehanizme toksičnosti ovog leka.

**Ključne reči:**
hlorpromazin; trovanje; stres, oksidativni; malondialdehid; peroksid dismutaza.

**Correspondence to:** Bratislav Dejanović, Military Medical Center "Karaburma", Severni bulevar 1, 11 000 Belgrade, Serbia. Phone: +381 11 3609 372, Fax: +381 11 266 2722. E–mail: bracadejanovic@yahoo.com

## Introduction

Chlorpromazine (CPZ) is an antipsychotic drug widely used in clinical practice for a long time, but its mechanism of action is not yet fully defined. The drug is dopamine D2 receptor antagonist, but also it has an affinity for other receptors as well [1]. Also, by its binding to calmodulin ($Ca^{2+}$- binding messenger protein) it may influence on a variety of $Ca^{2+}$-calmodulin dependent enzymes [2]. It is known that an excessive stimulation of $Ca^{2+}$-activated processes, such as those catalyzed by nonlysosomal proteases, endonucleases and phospholipases, may be the predominant cytotoxic event for a variety of toxic insults [3].

It is also known that CPZ has been shown to lower peroxidative damage *in vivo* and to protect cells from lethal injury presumably by acting as an antioxidant [4, 5]. However, potent prooxidative effect of CPZ was documented by the generation of reactive oxygen species (ROS) within 15 minutes of CPZ treatment in differentiated human hepatoma HepaRG cells [6]. Energy disruption by CPZ was blamed for exacerbation of its toxicity.

Recent studies showed that chronic administration of CPZ reduces the activity of manganese superoxide dismutase (MnSOD) and copper/zinc SOD (CuZnSOD) (after 21 days), and catalase and glutathione (GSH) (after 180 days) and increases lipid peroxidation (LPO) based on the concentration of malonylaldehyde (MDA) determination [7, 8].

Oxidative stress (OS) is implicated in the pathophysiology of many diseases including neurological ones [8]. Reactive oxygen species, such as hydrogen peroxide ($H_2O_2$), superoxide radicals ($O_2^{\bullet-}$), hydroxyl radicals (OH•) are known to damage various cellular components, including membrane lipids, proteins, DNA and thereby contribute to cellular dysfunction [9]. The products of LPO (carbonyl compounds) readily react with amino acids residues, such as cysteine or lysine, and disturb protein function. Under normal conditions, antioxidant enzymes (like SOD, glutathione peroxidase, catalase, etc.) provide adequate protection against free radicals (FRs) harmful effects on all kind of biomolecules, unlike in OS when extrem FRs production occurs, and endogenous antioxidative system is insufficient to prevent oxidative injury of cells. There is evidence that the mitochondrial pathology and OS (reduced activities of antioxidant enzymes in plasma, red blood cells, and cerebrospinal fluid in patients) may be the most critical concerns in the pathophysiology and outcome of schizophrenia [10]. Such changes in oxidative defense mechanisms in patients with schizophrenia may be exacerbated by the treatment with antipsychotics having pro-oxidant properties [11].

Having in mind these contradictory findings, the aim of this study was to test the hypothesis that CPZ provokes OS development in dose-dependent manner. Therapeutic (T), mild (M) and severe (S) toxic doses of CPZ were tested in exposed patients by assessing total antioxidative status (TAS) and MDA concentration in plasma and SOD activity in erythrocytes.

## Methods

### Participants

The following groups of participants were organized in this cross-sectional study: the group C – the control group of healthy individuals, nonsmokers, on no medication, excluding personal or family histories of psychiatric/neurological illness; the group T – the hospitalized patients on therapeutic doses of 75–150 mg CPZ/day divided in three single daily doses, for minimum 60 days; and two groups of poisoned patients, subdivided into two categories according to the poisoning severity score (PSS) and measured serum concentration of CPZ: the group M – mildly poisoned patients (PSS: 0–1; CPZ in serum: 0.21 ± 0.05 mg/L) and the group S – severely poisoned patients (PSS: 2–3; CPZ in serum: 2.66 ± 0.25 mg/L). Patients with PSS 4 were not included in this study. The study was conducted in the Military Medical Academy in Belgrade. It was approved by the Ethics Committee of the Military Medical Academy, Belgrade, Serbia.

### Reagents

All chemicals used in this study were of analytical grade. Also, all drugs solutions were prepared on the day of the experiment.

### Sample preparation

Considering the circadian rhythm, blood samples were taken between 08:00 and 09:00 a. m. just before the breakfast.

Immediately after the blood test, tubes with lithium-heparin was put on ice to preserve the activity of SOD in erythrocytes. Blood with EDTA was immediately analyzed and it was determined from the concentration of hemoglobin, which is expressed through the activity of the SOD isoenzymes.

### The chromatographic quantification of serum CPZ

Samples were prepared as follows: 5 g of previously homogenized and thawed sample was transferred to the polypropylene centrifuge tubes, and 20 mL of 0.1 mM sulfuric acid in acetonitrile was added. After homogenization on ultra-turrax, samples were sonicated for 5 minutes and centrifuged at 2,400 g for 10 minutes. Supernatant was decanted into the clean centrifuge tubes and the extracts were purified. Columns were conditioned with 5 mL of methanol and 5 mL of water, followed by sample loading and washing with 0.01 M sulfuric acid. CPZ was eluted with 6 mL of 0.1 mM sulfuric acid in acetonitrile and methanol (50 : 50 v/v). Eluates were evaporated at 40°C in the gentle stream of nitrogen. Dry residue was dissolved in mobile phase and 20 uL was injected into the high pressure liquid chromatography (HPLC) system.

Concentrations of CPZ were made by diluting stock and working standard solutions to achieve calibration concentrations expected to meet the therapeutic levels in serum of patients. Stock solution was prepared by dissolving the standard in methanol to the concentration of 0.1 mg/mL. Working solution was also prepared in methanol by diluting stock solution to the concentration of 1 ug/mL. Concentrations of calibration solutions (25 ng/mL, 50 ng/mL, 75 ng/mL and 100 ng/mL) were prepared by dissolving working solution in acetonitrile. The mobile phase was a mixture of 0.03 M sodium acetate-acetonitrile (67 : 33 v/v). Flow rate was set to

1 mL/min isocratic elution through the HPLC column. Detection was achieved by measuring ultra-violet absorption at 250 nm.

Five-point calibration (including zero) was performed at the beginning of each batch of samples, followed by blank and fortified samples [12].

Oxidative stress parameters (TAS, SOD, MDA) were determined spectrophotometrically. One set of measurements was performed in the group C and T, whereas two sets of measurements (after 24 hours and 48 hours) were done in the poisoned patients (groups M and S).

### TAS determination

Total antioxidant status was performed by commercial "Randox" test on automatic biochemical analyzer AXON Technicon. The principle of the reaction is based on *in vitro* reduction of blue 2,2-azino-di-(3-etilbenztiazonil sulfonate) (ABTS) cation radical (ABTS•+) into its colorless molecular form, ABTS, by endogenous antioxidants. The intesity of blue color diminishes with the increase of TAS. This TAS assay is often referred to as the Trolox equivalent antioxidant capacity (TEAC) assay and is expressed as mmol/L [13].

### SOD activity

Superoxide dismutase activity (including all three SOD isoenzymes) was determined by commercial "Randox" test on an automatic biochemical analyzer AXON Technicon. The dismutation of $O_2^{\bullet-}$ into $H_2O_2$ is catalyzed by SOD. The principle of the method is based on the reaction of xanthine and xantine oxidase that provides $O_2^{\bullet-}$ which reacts with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T) forming red-colored formazan. The higher intensity of red color means the less activity of SOD. The results were expressed as U of SOD *per* mg Hb.

### MDA concentrations

The LPO was determined by MDA quantification, using thiobarbituric acid reactive substances (TBAR) method. Namely, TBAR (15% trichloroacetic acid + 0.375% TBA + 0.25% mol HCl) reacts with MDA, originating from polysaturated fatty acid peroxidation. Formed MDA was measured spectrophotometrically at 533 nm. The results were expressed as mmol/L.

### Statistical analysis

Types of data distribution within the groups were analyzed by Kolmogorov-Smirnov test. All data were analyzed statistically by one way ANOVA using Dunnett`s C-test. The statistical program GraphPad Prism was used. Statistical significance was defined at $p < 0.05$. The data was presented as mean ± SD.

### Results

The demographic characteristics of subjects in the study are given in Table 1.

Generally, there were more females than males in all the studied groups, but with no differences in age among them.

The serum CPZ concentrations in the group T were within the therapeutic range (data not shown). The serum CPZ concentrations in the patients from the group S (2.66 ± 0.25 mg/L) were much higher than in the patients from the group M (0.21 ± 0.05 mg/L).

After 24 hours of poisoning, TAS values were lower in both groups (Figure 1) than in the controls ($p < 0.05$), as well as in the patients from the group T ($p < 0.01$). On the contrary, the SOD activities were higher in both M and S group (Figure 2), than in the controls ($p < 0.05$), at the same time (24 hours), as well as in the patients from the group T ($p < 0.05$). Both T and M group had the same MDA concentrations (Figure 3) as the control group, but we registered the significant MDA increase in the patients from the group S ($p < 0.001$). A significant negative correlation (Figure 4) between the serum CPZ concentration and TAS level in plasma of the patients from the group S (r = -0.54, $p = 0.0161$) was obtained after 24 hours of the poisoning.

After 48 hours of poisonings the results were similar for all the examined parameters: significantly decreased TAS level ($p < 0.05$) and increased SOD activity (M patients, $p < 0.05$ and the patients from the group S, $p < 0.01$) in the groups M and S, compared to the controls, as well as in the group T (Figures 1, 2). Also, in the patients from the group S, we registered a significant MDA increase ($p < 0.001$) compared to all the other groups (Figure 3).

### Discussion

Our results showed that CPZ induces OS development in the dose-dependent manner in exposed humans, based on

**Table 1**

**Characteristics of healthy volunteers and patients exposed to chlorpromazine (CPZ) enroled in the study**

| Study groups | Participants (n) | Females (n) | Average age ($\bar{x} \pm SD$) | Males (n) | Average age ($\bar{x} \pm SD$) |
|---|---|---|---|---|---|
| Control | 30 | 10 | 43.5 ± 10.91 | 20 | 41.8 ± 17.72 |
| T | 39 | 14 | 39.7 ± 13.23 | 25 | 31.9 ± 10.70 |
| M | 13 | 7 | 46.7 ± 11.33 | 6 | 35.7 ± 8.83 |
| S | 19 | 10 | 38.9 ± 9.45 | 9 | 32.1 ± 5.55 |

**The control group of healthy individuals, nonsmokers, on no medication, with no personal or family histories of psychiatric/neurological illness; the group T – hospitalized patients on therapeutic doses of CPZ; the group M – patients with mild CPZ poisoning; the group S – patients with severe CPZ poisoning.**
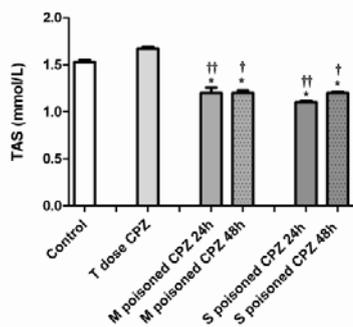
**Fig. 1 – Total antioxidant status (TAS) level (mmol/L) in the control group and in the groups of patients with therapeutic doses (T-dose) of chlorpromazine (CPZ), as well as in the groups of CPZ mild (M) and severe (S) poisoned patients. Bars in the graph represent the mean ± SD. Labels of statistical significance: * – compared to the control group; † – compared to the group T. A statistical significance was considered at: *·†$p < 0.05$, ††$p < 0.01$, One Way ANOVA, Dunnett`s C-test.**
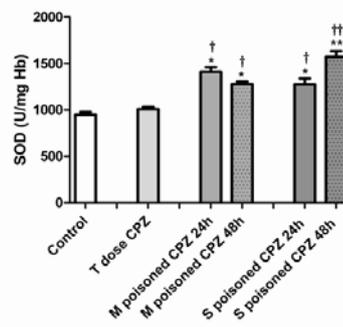


**Fig. 2 – Superoxide dismutase (SOD) activity (U/mg Hb) in the control group and in the groups of patients with therapeutic doses (T-dose) of chlorpromazine (CPZ) as well as in the groups of CPZ mild (M) and severe (S) poisoned patient. Bars in the graph represent the mean ± SD; Labels of statistical significance: * – compared to the control group; † – compared to T group, a statistical significance was considered at: *·†$p < 0.05$, **·††$p < 0.01$, One-Way ANOVA, Dunnett`s C-test.**
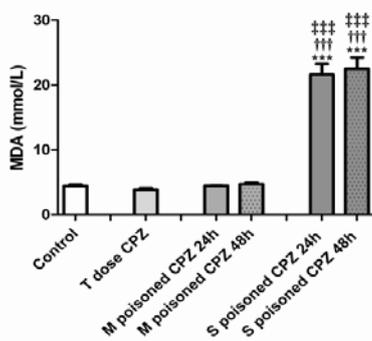


**Fig. 3 – Malondialdehyde (MDA) concentration (mmol/L) in the control group and in the groups of patients with therapeutic doses (T dose) of chlorpromazine (CPZ) as well as in groups with mild (M) and severe (S) CPZ poisoning. Bars in the graph represent the mean ± SD; labels of statistical significance: * – compared to the control group; † – compared to the group T; ‡ – compared to the group M. A statistical significance was considered at: ***·†††·‡‡‡$p < 0.001$, One-Way ANOVA, Dunnett`s C test.**
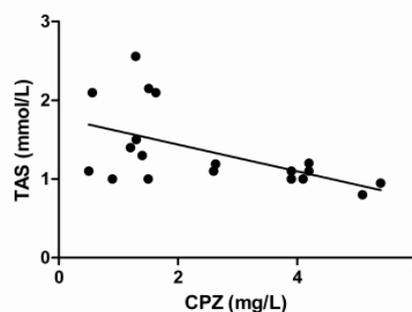


**Fig. 4 – The correlation of the serum concentration of chlorpromazine (CPZ) (mg/L) in the patients with severe poisoning and total antioxidative status (TAS) level (mmol/L) in plasma. In the first 24 hours after poisoning, parallel with the increase of CPZ serum concentration, TAS level in plasma decreases, Pearson`s correlation, r = -0.54. There is a negative linear relationship between these variables ($p < 0.05$).**

the measured OS parameters. Namely, LPO as the major indicator of lipid oxidative injury was highly elevated in the patients poisoned with higher toxic doses of CPZ (PSS: 2–3). Additionally, TAS levels were lower and SOD activities were higher in both the groups, M and S, of the patients than in the controls, as well as in the group T. The obtained results were in an agreement with recent clinical trials that even therapeutic doses of CPZ disrupt antioxidative defense system [14]. In addition to the reduced energy production, the products of anaerobic glucose metabolism lead to the formation of ROS, which affects the deterioration of the course and outcome of poisoning. However, we found that TAS levels in plasma of the patients from the group T did not significantly differ from the control group, indicating that the total antioxidant capacity of the organism in the patients from the group T was not diminished (Figure 1).

The study of Hu and Kulkarni [15] showed that CPZ is oxidized into CPZ cation radical in the presence of $H_2O_2$ and/or other FRs such as $O_2^{\bullet-}$ and hydroxyl radical (HO•). Also, auto-oxidation of dopamine is the potential source of

undergoes, $O_2^{\bullet-}$. However, dopamine is primarily metabolized through oxidation by monoamine oxidase (MAO) to 3,4-dihydroxyphenyl-acetic acid (DOPAC), followed by the formation of $H_2O_2$, which can further react with iron or copper ions to produce OH• (the most toxic of FRs). Thus, the biochemical pathway of dopamine supports the oxidation process in the brain leading to neurological disorders and oxidation of administered neuroleptics such as CPZ, as well.

It has also been shown that different peroxidases and methemoglobin catalyze single electron oxidation of CPZ into biologically active CPZ cation radical. This radical is highly reactive and can interact with a number of endogenous substances and xenobiotics, as well. Neuroleptics block dopamine receptors, which may affect the increased dopamine turnover and, in turn, could eventually lead to the increased production of $H_2O_2$, resulting in OS [9]. It has also been shown that neuroleptics can induce cellular alterations that lead to production of ROS and cell death. In addition, CPZ increases in the level of brain manganese, which in turn may potentiate the damage caused by FRs.

Reacting with GSH, CPZ cation radical leads to the formation of GSH thiol radical cation of CPZ, which is responsible for both pharmacological efficacy and toxic effects of this drug. Inevitably, depleted GSH storage makes antioxidative defense system more fragile, what means that this drug can initiate OS in the brain [16].

A TAS value as the ratio of total antioxidative status in the groups of patients 24 and 48 hours after poisoning with CPZ, was lower than in the control group, as well as in the group T, indicating the consumption of antioxidant potential in the cases of both M and S poisoning (Figure 1). Neuroleptics may also have direct cytotoxic effect *via* the production of toxic metabolites [17].

In this study we obtained a negative linear relationship (Figure 4) between TAS level in plasma and serum CPZ level in the patients from the group S ($p < 0.05$, r = -0.54), confirming the antioxidative defense damage.

Changes in the activities of antioxidative enzymes may provoke an increase in ROS. Increased SOD activity in the poisoned patients from the group M and S and in the group T, indicate that CPZ may induce expression of gene for SOD synthesis (Figure 2). Antipsychotic drugs have been found to induce the expression of immediate early genes such as cfos and cjun, transcription factors, growth factors and peptides [4]. There is evidence that early genes and growth factors can then regulate the expression of antioxidant enzymes which provide a part of neuroprotective mechanisms associated with growth factors [12]. As noted previously, SOD can protect living organisms from oxidative injury by $O_2^{\bullet-}$ sequestration [16]. Increased SOD activities that are registered in both M and in S group are probably induced by the increased production of ROS and CPZ cation radical, consequently.

Membrane fluidity is an important factor that determines inter- and intracellular communication, membrane elasticity and biological transport of proteins and lipids. Guo et al. [18] have proposed that oxidation of membrane lipids leads to the formation of peroxidation degradation products (MDA), which leads to cross-linking reaction of the lipid-lipid and lipid-protein type, thereby rendering the membrane more rigid and less fluid. In patients of the group S, we registered a significant increase in LPO at 24 hours and 48 hours of poisoning (Figure 3). The process of peroxidation is an important indicator of membrane damage, which serves a lot to promote irreversible impairment of essential cellular components and eventually leads to cell death or necrosis. There is considerable evidence that schizophrenic patients have increased levels of LPO. Pall et al. [19] found increased MDA levels in the cerebrospinal fluid of patients taking CPZ.

Increased SOD activity was accompanied with decreased MDA concentration in the group M, emphasizing SOD capability to prevent OS, reducing LPO. This is not the case in the group S, where OS is present in spite of increased SOD activity. These results confirm that the organism was protected from the increased production of FRs in the patients from the group M, while the resulting OS leads to the initiation of the process of LPO in the patients from the group S. In the first 24 hours, after mild CPZ poisoning, the increase of SOD activity is not followed by the MDA increase, indicating a still effective antioxidant potential preventing from LPO. However, FRs production was steadily increased at the same time (24 hours) in the patients from the group S. The increase of SOD in this period is followed by MDA increase, indicating the existence of biological membrane damage by FRs and LPO processes. All of these changes were still present 48 hours of CPZ poisoning in this group.

## Conclusion

Our results showed that chlorpromazine dose-dependent changed of SOD activity and malondialdehyde concentration in all the investigated groups. Oxidative stress, lipid peroxidation and changes of the antioxidant enzyme activity may be responsible for one of the molecular mechanisms of chlorpromazine induced tissue damage. Based on the changes in oxido-reductive status in the patients exposed to chlorpromazine it can be concluded that red-ox toxicity pathway might contribute to the overall chlorpromazine toxic effects in humans. This finding emphases the significance of disturbed oxido-reductive status in patients overdosed by chlorpromazine.

## Acknowledgements

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