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Original Article

Effect of disulfiram on ketamine-induced cardiotoxicity in rats

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Abstract: It is known that ketamine increases the production of catecholamines, causing oxidative damage to the heart. Suppression of the production of catecholamines by disulfiram, a drug with antioxidant properties, indicates that disulfiram may decrease ketamine-induced cardiotoxicity. The objective of the present study was to investigate the effect of disulfiram on ketamine-induced cardiotoxicity in rats. Disulfiram was administered by oral gavage in doses of 25 mg/kg to rats in the DK-25 group and 50 mg/kg to rats in the DK-50 group. Distilled water was applied in the ketamine control (KC) and healthy (HG) rat groups. At one hour after drug administration and subsequently at ten-minute intervals, a 60 mg/kg dose of ketamine was intraperitoneally injected in the rats in all groups other than HG, and anesthesia was maintained for three hours. Disulfiram prevented both increase in the levels of parameters indicating oxidative and myocardial damage and decrease of antioxidant levels in the heart tissue with ketamine in a dose-dependent manner. Disulfiram better prevented occurrence of cardiotoxicity with ketamine in the 50 mg/kg dose than in the 25 mg/kg dose. It is concluded that disulfiram may usefully be applied in clinical practice in the prevention of cardiotoxicity as observed during anesthesia with ketamine.

Keywords: Ketamine, disulfiram, cardiotoxicity, oxidant, antioxidant, rat

Introduction

Ketamine is known as a phencyclidine-derived intravenous anesthetic agent, which is applied in the initiation of general anesthesia and short-time surgical procedures [1]. Anesthesia-creating doses of ketamine cause serious side effects [2], which are said to arise from sympathomimetic activity [3] that results from the excessive production of catecholamines such as adrenaline and noradrenaline. Increased sympathomimetic activity increases the heart's need for oxygen [4], and excessive production of catecholamines causes oxidative damage to the heart [5]. The cardiotoxicity of catecholamines results from their strong positive chronotropic and inotropic effects, increasing pre- and post-charge of the heart and reducing coronary perfusion pressure in diastole [6, 7]. Although the pathogenesis of myocardial damage stimulated by catecholamines is multifactorial, it has been suggested that oxidative stress plays an important role [8, 9]. Previous studies have shown that ketamine increases levels of endogenous adrenaline, noradrenaline and dopamine in a dose-dependent manner [10]. This knowledge from the literature indicates that ketamine creates a cardiotoxic effect by increasing the amount of endogenous catecholamines. This in turn shows that suppression of the excessive production of endogenous catecholamines and antioxidant therapy may be beneficial in reducing the cardiotoxic effect of ketamine. Disulfiram, which was tested in this study against ketamine cardiotoxicity, is a sulfur compound used in the treatment of chronic alcoholism [11]. Disulfiram blocks formation of noradrenaline from dopamine in the adrenal glands by inhibiting the dopamine β-hydroxylase enzyme [12, 13]. As adrenaline is synthesized from noradrenaline in the adrenal medulla [14], suppression of noradrenaline production will also supress the production of adrenaline. Again, disulfiram has

been shown to prevent increase in malondialdehyde (MDA), which is an oxidant parameter applied to ischemia reperfusion in ovarian tissue, as well as decrease in total glutathione (tGSH), which is an antioxidant parameter [15]. Suppression of production of catecholamines by disulfiram (a drug with antioxidant properties) indicates that disulfiram might reduce ketamine-induced cardiotoxicity. As we could find no previous study in the literature concerning prevention of the cardiotoxic effect of ketamine using disulfiram, the objective of this study was to biochemically investigate the effect of disulfiram on ketamine-induced cardiotoxicity in rats.

Material and methods

Animals

Experimental animals were obtained from the Recep Tayyip Erdoğan University, Medical Experimental Research and Application Center. A total of 32 male Albino Wistar rats, weighing between 220 g and 235 g, were randomly selected for use in the experiment. The animals were housed and fed in the pharmacology laboratory at normal room temperature (22°C) for one week before the experiment to ensure adaptation to their environment. The protocols and procedures were approved by the local Animal Experimentation Ethics Committee (Date: 12.12.2014; Meeting No.: 2014/78).

Chemicals

For laboratory experimentation, sodium thiopental was obtained from IE-Ulagay (Turkey) and ketamine from Pfizer (Turkey); disulfiram was purchased from Sigma Co., (Germany).

Study groups

The animals were divided into four groups: disulfiram-25 + ketamine (DK-25), disulfiram-50 + ketamine (DK-50), ketamine control (KC) and healthy (HG) groups.

Experimental procedure

Rats in the DK-25 group were administered 25 mg/kg disulfiram, and rats in the DK-50 group received 50 mg/kg disulfiram by oral gavage. At one hour following drug administration, ketamine of 60 mg/kg dose was intraperitoneally (IP) injected in all the rat groups other than HG, and anesthesia was maintained for three hours, repeating the dose every ten minutes. After this time, all the animals were sacrified by decapitation, and oxidant/antioxidant parameters were measured in the heart tissue. Creatine Kinase (CK), Creatine Kinase MB (CK-MB) and Troponin I (TP I) values were measured in the blood samples collected from tail veins before the rats were sacrified.

Biochemical experimental procedure

Preparation of samples: In order to determine MPO in the heart tissue, 0.5% HDTMAB (hexadecyl trimethyl ammonium bromide) containing potassium phosphate buffer at pH = 6 for determination of MDA 1.15% potassium chloride solution were completed to 2 ml in phosphate buffer at pH = 7.5 for the other measurements and homogenized on ice cold. The solution was then centrifuged at 10,000 rpm for 15 min at + 4°C, and the supernatant was used as the sample for analysis.

Malondialdehyde (MDA) analysis: MDA measurements were based on the method used by Ohkawa et al., [16] involving spectrophotometrical measurement of absorbance of the pink-colored complex formed by thiobarbituric acid (TBA) and MDA at high temperature (95°C) at a wavelength of 532 nm. Homogenates were centrifuged at 5000 g for 20 minutes, and these supernatants were used to determine the amount of MDA; 250 µl homogenate, 100 µl 8% sodium dodecyl sulfate (SDS), 750 µl 20% acetic acid, 750 µl 0.08% TBA and 150 µl purified water were pipetted into capped test tubes and vortexed. The mixture was left for incubation at 100°C for 60 minutes before 2.5 mL n-butanol was added to it, and spectrophotometric measurement was conducted. The amounts of red color formed were read at 532 nm using cuvettes of 3 mL and, taking acount of dilution coefficients, MDA amounts in the samples were determined using the standard chart, created through the previously prepared MDA stock solution.

Determination of myeloperoxidase (MPO) activity: To determine MPO in homogenates of the heart tissue, 0.5% HDTMAB (0.5% hexadecyl trimethyl ammonium bromide) containing potassium phosphate buffer at pH = 6 was prepared. The solution was then centrifuged at 10,000 rpm for 15 min at + 4°C, and the supernatant was used as the sample for analysis. In determining the activity of the MPO

enzyme, an oxidation reaction with MPO-mediated H2O2 involving 4-aminoantipyrine/phenol was used as substrate [17].

NO analysis: Tissue nitric oxide (NO) levels were measured as total nitrite + nitrate levels with the use of the Griess reagent as previously described [18]. The Griess reagent consists of sulfanilamide and *N*-(1-napthyl)-ethylenediamine. The method is based on a two-step process. The first step is the conversion of nitrate into nitrite using a nitrate reductase; the second step is the addition of the Griess reagent, which converts nitrite into a deep purple azo compound. Photometric measurement of absorbance was conducted at 540 nm because this azo chromophore accurately determines nitrite concentration. NO levels were expressed as μmol/g protein.

Total glutathione (tGSH) analysis: The amount of GSH in the total homogenate was measured according to the method of Sedlak and Lindsay, with some modifications [19]. The sample was weighed and homogenized in 2 mL of 50 mmol/L Tris-HCl buffer containing 20 mmol/L EDTA and 0.2 mmol/L sucrose at pH = 7.5. The homogenate was immediately precipitated with 0.1 mL of 25% trichloroacetic acid; the precipitate was removed after centrifugation at 4200 rpm for 40 min at 4°C, and the supernatant was used to determine GSH level. A total of 1500 μL of measurement buffer (200 mmol/L Tris-HCl buffer containing 0.2 mmol/L EDTA at pH = 7.5), 500 μL supernatant, 100 μL DTNB (10 mmol/L) and 7900 μL methanol were added to a tube and then vortexed and incubated for 30 min at 37°C. Used as a chromogen, 5,5-Dithiobis (2-nitrobenzoic acid) (DTNB) formed a yellow-colored complex with sulfhydryl groups. Absorbance was measured at 412 nm, using a spectrophotometer (Beckman DU 500, USA). The standard curve was obtained by using reduced glutathione.

Glutathione reductase (GSHRd) analysis: GR activity was determined spectrophotometrically by measuring the rate of NADPH oxidation at 340 nm, according to Carlberg and Mannervik's method [20]. After tissue homogenization, supernatant was used for GR measurement. After the addition of NADPH and GSSG, the chronometer was switched on, and absorbance was measured for 5 min at 30-minute intervals at 340 nm, using spectrophotometric methods.

Glutathione s-transferase (GST) activity: GST activity was determined by Habig and Jakoby [21]. Briefly, the enzyme's activity was assayed spectrophotometrically at 340 nm in a 4 mL cuvette containing 0.1 M PBS (pH = 6.5), 30 mM GSH, 30 mM 1-chloro-2,6-dinitrobenzene, and tissue homogenate.

Superoxide dismutase (SOD) analysis: Measurements were performed according to the method described by Sun et al. [22]. SOD forms when xanthine is converted into uric acid by xanthine oxidase. If nitro blue tetrazolium (NBT) is added to this reaction, SOD reacts with NBT, producing a purple-colored formazan dye. The sample was weighed and homogenized in 2 mL of 20 mmol/L phosphate buffer containing 10 mmol/L EDTA at pH = 7.8. The sample was centrifuged at 6000 rpm for 10 minutes, and the brilliant supernatant was then used as an assay sample. The measurement mixture, containing 2450 μL measurement mixture (0.3 mmol/L xanthine, 0.6 mmol/L EDTA, 150 μmol/L NBT, 0.4 mol/L Na_2CO_3, 1 g/l bovine serum albumin), 500 μL supernatant and 50 μL xanthine oxidase (167 U/l), was vortexed and then incubated for 10 minutes. At the end of the reaction, formazan was produced. The absorbance of the purple-colored formazan was measured at 560 nm. As more of the enzyme exists, the least O2- radical that reacts with NBT occurs.

Determination of creatine kinase (CK): Creatine kinase in the plasma collected from the animals was photometrically measured using the Roche/Hitachi cobas c 701 system. Using the prepared test reagents, all steps of the assay were performed in line with the procedure. UV testing was applied according to the following reactions:

$$\text{Creatine phosphate} + \text{ADP} \xrightarrow{\text{CK}} \text{Kreatine} + \text{ATP}$$

$$\text{ATP} + \text{D-glucose} \xrightarrow{\text{HK}} \text{ADP} + \text{G6P}$$

$$\text{G6P} + \text{NADP}^{+} \xrightarrow{\text{G6PDH}} \text{D-6- phosphogluconate} + \text{NADPH}^{+}$$

Equimolar amounts of NADPH and ATP form at the same rate. The rate of formation of NADPH, which is photometrically measured at 340 nm, is directly proportional to CK activity.

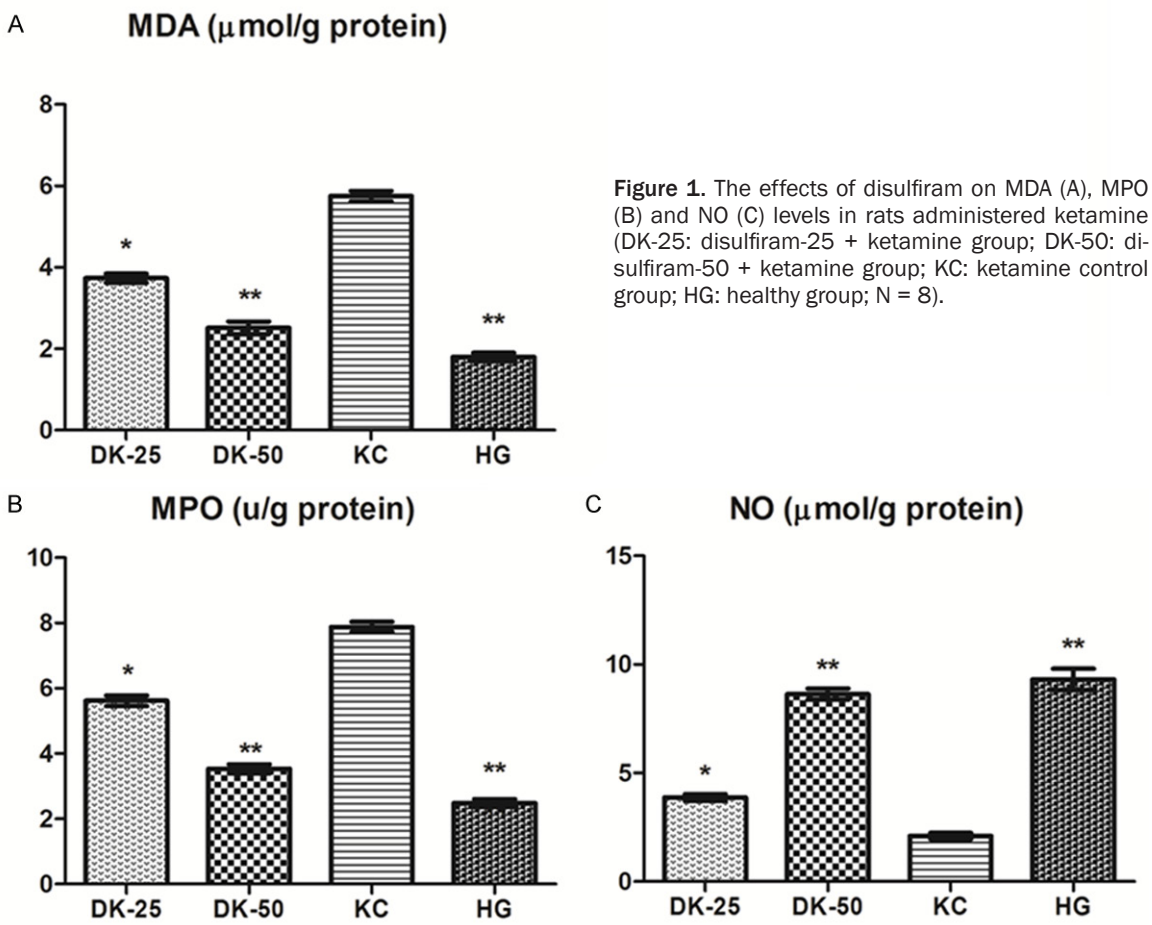

Figure 1. The effects of disulfiram on MDA (A), MPO (B) and NO (C) levels in rats administered ketamine (DK-25: disulfiram-25 + ketamine group; DK-50: disulfiram-50 + ketamine group; KC: ketamine control group; HG: healthy group; N = 8).

Determination of creatine kinase MB (CK-MB): Determination of creatine kinase MB in the plasma collected from the animals was photometrically measured in the Roche/Hitachi cobas c 701 system. Using the prepared test reagents, all steps of the assay were performed with immunological UV testing in line with the procedure. The CK-MB isoenzyme consists of two subunits, CK-M and CK-B, both of which have an active place. The catalytic activity of CK-M subunits in the sample was inhibited up to 99.6% with the help of CK-M-specific antibodies, without influencing CK-B subunits. The remaining activity of CK-B, corresponding to half of CK-MB activity, was determined using the total CK method.

Determination of troponin I (TP I): Troponin I levels in the plasma collected from the animals was measured using the ELFA (Enzyme-Linked Fluorescent Assay) technique in the VIDAS Troponin I Ultra kit. Using the prepared test reagents in the kit, all steps of the assay were automatically performed in the VIDAS device. The sample was transferred into the well containing anti-cardiac troponin I antibodies, which were marked with alkaline phosphatase (conjugate). The sample-conjugate mixture was transferred and left in the solid phase receptacle, providing binding to troponin I and conjugate, which were bound to the inner wall of the antigen solid phase binder. Unbound content was removed by irrigation. Conjugate enzyme catalyzes hydrolysis of 4-methyl umbellipheryl phosphate substrate to 4-methyl umbelliferone, which is a product with a fluorescence measured at 450 nm. Density of fluorescence is proportional to the antigen concentration in the sample.

Statistical analysis

All data were subjected to one-way analysis of variance using Statistical Package for Social Sciences (SPSS) 18.0 (Armonk, NY, USA) software. Differences among groups were obtained using the least significant difference option, and significance was declared at P less-than- or

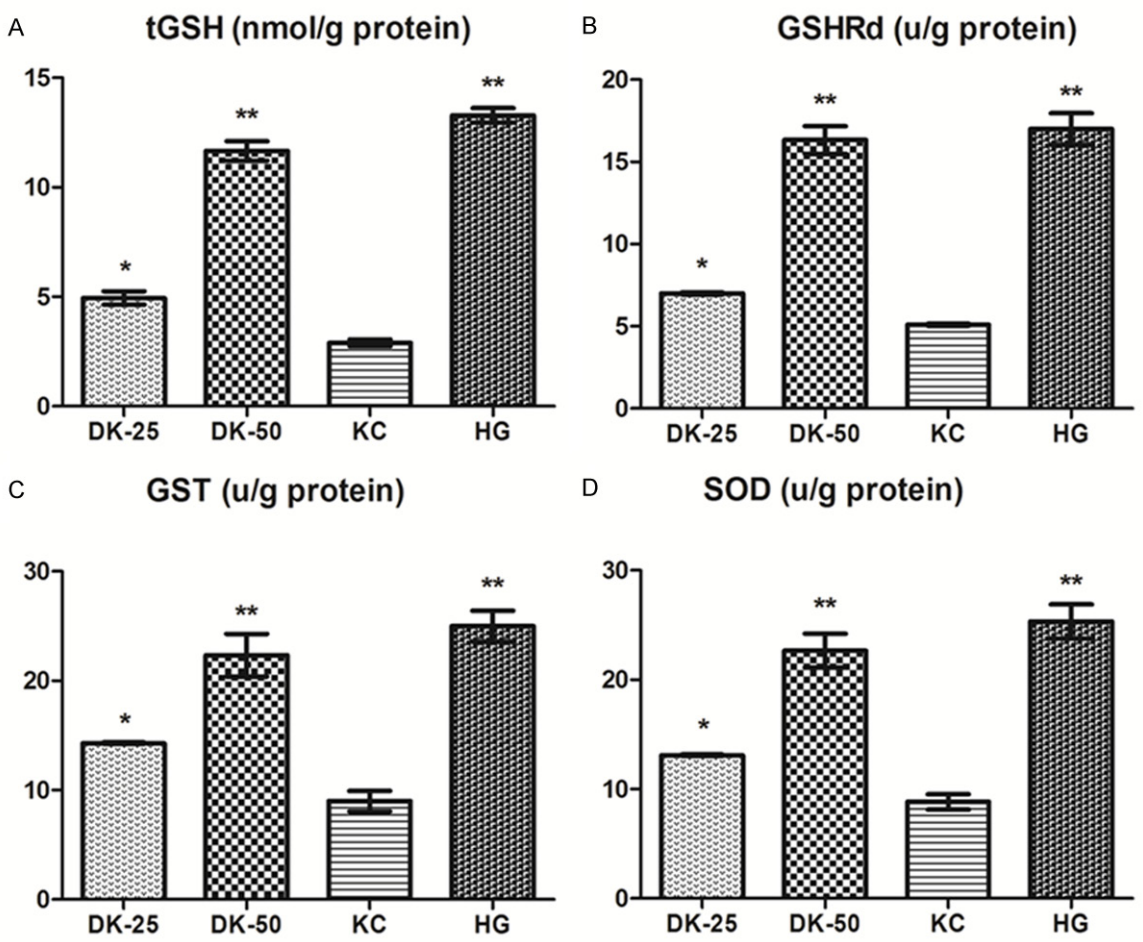

Figure 2. The effects of disulfiram on tGSH (A), GSHRd (B), GST (C) and SOD (D) levels in rats administered ketamine (DK-25: disulfiram-25 + ketamine group; DK-50: disulfiram-50 + ketamine group; KC: ketamine control group; HG: healthy group; N = 8).

equals slant $P < 0.05$. The results are expressed as mean ± SEM.

Results

As shown in **Figure 1A**, disulfiram reduced the amount of MDA in the heart tissue, which increased with administration of ketamine, in a dose-dependent manner. In addition, disulfiram reduced the MPO level at doses of 25 and 50 mg/kg, significantly inhibiting the increase in ketamine in heart tissue (**Figure 1B**). The amount of NO in heart tissue was found to be significantly lower in the rat group that received ketamine than in the groups given disulfiram in doses of 25 and 50 mg/kg (**Figure 1C**). As expected, treatment of heart tissue with the 50 mg/kg disulfiram dose was more effective in preventing decrease in the amount of antioxidants by ketamine, including tGSH, GSHRd, GST and SOD, than at 25 mg/kg (**Figure 2A-D**).

CK, CK-MB and TP I analysis results

As seen in **Table 1**, levels of CK and CK-MB were 1100 ± 84 and 390 ± 56 u/l in the heart tissue of rats in the ketamine control group (KC), while these levels were measured as 500 ± 79 and 131 ± 18 u/l in the healthy group (HG), respectively. CK levels were measured as 778 ± 97 u/l for the 25 mg/kg disulfram dose and as 520 ± 50 u/l for the 50 mg/kg dose. CK-MB levels were measured as 239 ± 38 and 151 ± 27 u/l in the groups administered disulfiram doses of 25 and 50 mg/kg, respectively. TP I level was recorded as 1.8 ± 0.18 μg/l in the heart tissue of the rats that received ketamine, while this level was measured as 0.17 ± 0.01 μg/l in the HG group. TP I level was measured as 1.1 ± 0.1 and 0.21 ± 0.01 μg/l in the groups administered 25 and 50 mg/kg doses of disulfiram, respectively.

Table 1. Effect of disulfiram on plasma levels of CK, CK-MB ve TP I in rats administered ketamine

Drugs	Dose (mg/kg)	CK (u/l)	P	CK-MB (u/l)	P	TP I (μg/l)	P
KC	60	1100 ± 84	-	390 ± 56	-	1.8 ± 0.18	-
DK-25	25	778 ± 97	< 0.01	239 ± 38	< 0.01	1.1 ± 0.1	< 0.01
DK-50	50	520 ± 50	< 0.0001	151 ± 27	< 0.0001	0.21 ± 0.01	< 0.0001
HG	-	500 ± 79	< 0.0001	131 ± 18	< 0.0001	0.17 ± 0.01	< 0.0001

Discussion

The effect of disulfiram on cardiotoxicity as developed in rats by administration of ketamine was biochemically investigated. Results of the experiment indicated that levels of MDA and MPO increased and levels of NO, tGSH, GSHRd, GST and SOD decreased in the heart tissue samples of the rats that received ketamine. MDA and MPO are accepted as oxidant and tGSH, GSHRd, GST and SOD as antioxidant parameters [23]. In physiological conditions, oxidant/antioxidant balance is maintained by the predominance of antioxidants; impairment of this balance leads to tissue damage, a condition known as oxidative stress [24]. The existing literature and our results indicate that oxidant/antioxidant balance changes in favor of the oxidants in the heart tissue of the group given ketamine. Impairment of oxidant/antioxidant balance in favor of the oxidants has been reported to develop as a consequence of excessive spending of the antioxidant systems; depending on the spending of antioxidants, elevated free oxygen radicals lead to lipid peroxidation in the cells [24]. MDA, which is the final product of lipid peroxidation, was found to be high in the ketamine group, suggesting that ketamine causes oxidative damage to the heart. It has been shown experimentally in previous studies that ketamine increases endogenous adrenaline and noradrenaline in a dose-dependent manner [10]. Furthermore, excessive production of catecholamines has been reported to cause oxidative damage to the heart [5]. A recent study has found that ketamine significantly increases the amount of MDA in heart tissue even after a single dose, and that long-term use of ketamine further increases the amount of MDA [25].

Amounts of MDA were found to be significantly lower in the group in which disulfiram was tested against ketamine cardiotoxicity than in the group receiving ketamine. We could find no evidence in the literature concerning suppression of MDA in the heart tissue by disulfiram, but disulfiram has been experimentally shown to inhibit increase of MDA in ovarian tissue in a dose-dependent manner [15]. An earlier study, similar to the present one, reported that metyrosine, which inhibits the synthesis of catecholamine, reduces production of oxidant parameters such as MDA and MPO, which increase with ketamine administration [25]. It is known from the literature that, along with MDA, MPO is a strong oxidant resource. MPO is the "neutrophil myeloperoxidase" enzyme that catalyzes the production of hypochlorous acid as a result of the reaction of hydrogen peroxide with chlorine ions. Hypochlorous acid so formed also inactivates α1-antiproteinase, causing tissue damage [26]. Disulfiram also reduces production of MDA and MPO, suggesting that, like metyrosine, disulfiram inhibits the production of catecholamines, protecting the heart against ketamine toxicity. Again, NO level was found to be significantly higher in the heart tissue of the group that received disulfiram than in the group that was given ketamine. It is known that "endothelium-derived relaxing factor" (EDRF) synthesized by vascular endothelium is responsible for the vasodilator response and is equivalent to NO, which has one unpaired electron and is therefore accepted as an oxidant. However, although some by-products that may form as a result of the reaction between NO and the superoxide radical produced in the vascular endothelium are cytotoxic, beneficial effects have been reported on regulation of vascular tonus [27-29]. This evidence from the literature supports our experimental results.

Levels of tGSH, GSHRd ve GST were significantly higher in the disulfiram group than in the ketamine group. There is existing evidence that ketamine decreases tGSH in the heart tissue [25]. High amounts of GSH in the cells indicate cell viability while low amounts show weakening and damage of the immune system in the cells [30]. As the present results indicate, disulfiram prevents decrease of GSHRd, which is important for protection of the amount of tGSH to provide cell viability. GSHRd activity is known to

be higher in healthy tissues than in damaged tissues, and has been shown to be reduced in proportion to the severity of tissue damage [31]. Experimental studies have shown that GST, which is an enzymatic antioxidant, plays a role in cardioprotective activity [32]. Another intracellular enzymatic antioxidant enzyme responsible for cardioprotective activity is SOD [33]. It has been proposed that the cardiotoxic effect of ketamine develops with decrease in levels of SOD [25]. The literature suggests that disulfiram can prevent reduction of SOD activity related to oxidative stress, protecting tissue against oxidative damage [15]. In the present study, levels of CK-MB and TP I, which are markers of myocardial damage, were found to be significantly higher in the ketamine group than in the disulfiram and healthy groups. Levels of CK-MB and TP I in the heart tissue are known to rise in proportion to the severity of oxidative stress [34]. Again, studies have demonstrated that elevated levels of CK-MB and TP I result from disturbances caused by oxidative stress in the myocardial membrane [35, 36]. The levels of TP I have also been used by Porciello et al. to differentiate cardiac and noncardiac causes [37]. Boccara et al. argued that serum levels of heart troponins are biochemical markers that can be detected in the earliest period for diagnosis of acute coronary diseases in humans [38]; even small increases in the troponins have been reported to be crucial indicators of myocardial damage [39].

In conclusion, ketamine is understood to induce oxidative stress in the heart tissue. Disulfiram was seen to prevent oxidative damage to the heart due to ketamine administration in a dose-dependent manner. Disulfiram better prevented decrease of antioxidants in the heart tissue with ketamine in 50 mg/kg doses than in 25 mg/kg doses. These results suggest that disulfiram may be beneficial in clinical practice in the prevention of cardiotoxicity that may develop due to ketamine anesthesia.

Disclosure of conflict of interest

None.

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