



Full Length Research Paper

Preliminary analysis of oxidative stress parameters in Lister Hooded rats after an acute oral treatment with 2,5-dimethoxyamphetamine

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The correlation between the toxicity of some common amphetamines and oxidative stress is well documented. In this study 2,5-dimethoxyamphetamine (2,5-DMA), a widely diffused designer drug with hallucinogenic properties, was investigated for its capacity to induce oxidative stress in rats after an acute oral treatment. The assessed parameters were: reduced glutathione (GSH) as a cellular antioxidant, thiobarbituric acid-reactive substances (TBARS) as biomarkers of lipid peroxidation and the trolox equivalent antioxidant capacity (TEAC) in plasma, liver, testes and kidneys. GSH levels in whole blood and in the organs mentioned above were determined using a mass spectrometer, while all the other parameters were spectrophotometrically assayed. A significant decrease of GSH in liver, an increase of TBARS in plasma, liver and testes, and a simultaneous decrease in the total antioxidant capacity of the plasma were observed. These findings suggest that 2,5-DMA induces an imbalance between oxidants and antioxidants leading to an oxidative stress status.

Keywords: 2,5-DMA, GSH, lipid peroxidation, rats, TEAC

INTRODUCTION

Amphetamines are broadly diffused recreational drugs which are used mainly for their agreeable stimulating effects (Nieddu et al., 2005). In general, the so-called designer drugs are a class of substances of abuse constituted by molecules with pharmacological effects similar to those of controlled substances, but with differences in structure which allow them to evade the provisions of drug laws (Carrollet al., 2012). In addition to legal issues, these drugs are often dangerous because

they have unknown safety profiles, and combined with high potential of abuse, they can generate devastating health consequences (Nieddu et al., 2014). Abuse of illicit drugs leads to toxic effects in different organs, depending on the administration pathway. Several lines of evidence support a role for oxidative stress in the toxicity induced by many drugs of abuse in different organs such as the brain, heart, liver or kidneys (Cunha-Oliveira et al., 2013). The oxidative stress status is defined as a persistent imbalance between antioxidant and pro-oxidant processes, in favor of the latter. The result of this phenomenon is the excessive production of free radicals as reactive oxygen species (ROS) (Sies 1991). At low physiological concentrations, ROS may function as

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signaling molecules; they play an important role in the immunological response and participate in the regulation of various cell activities (e.g., mitosis). However, at high concentrations, ROS damage cellular components, including proteins (enzymes, receptors), lipids and DNA, and they may consequently lead to apoptosis and cell death (Halliwell, 2011; Halliwell, 2006; Halliwell and Lee, 2010). The involvement of ROS in methamphetamine toxicity is supported by several studies which reported decreased glutathione (GSH) levels, reduced levels and activities of antioxidant enzymes, and increased lipid peroxidation and protein carbonylation, all of which are hallmarks of oxidative stress (Huang et al., 2013). Lipid peroxidation is a normally-occurring, complex consequence of oxidative stress and it is defined as the oxidative degeneration of lipids by free radical chain reaction. Membrane phospholipids are plentiful throughout biological systems and are thus easily accessible to all reactive species. In particular, ROS are more damaging in tissues like brain, which has a high density of phospholipid membranes (Dzwart et al., 1999). Polyunsaturated fatty acids and other lipids are oxidized to form an array of bioactive molecules including conjugated dienes, lipid hydroperoxides and malondialdehyde, which is one of several low-molecular-weight end-products of lipid peroxidation that reacts with thiobarbituric acid (Jobes, 2008). The oxidative stress status, caused by exposure to drugs of abuse, may derive from direct or indirect effects, and may occur after drug exposure or during withdrawal from the drug. In this latter case, too, an increase in the levels of oxidants compared to antioxidant defense systems leads to oxidation of proteins, phospholipids or DNA, leading to cell dysfunction. In literature, several scientific articles report the oxidative stress status induced by the administration of methylenedioxymethamphetamine (MDMA), methamphetamine and amphetamine in the nervous system and in various organs and tissues (Cunha-Oliveira et al., 2013; Huang et al., 2013; Cerratani et al., 2011; Ninkovic et al., 2008). In this study 2,5-dimethoxyamphetamine (2,5-DMA) was selected as a model toxicant causing oxidative stress. 2,5-DMA is an amphetamine designer drug with two methoxyl groups in positions 2 and 5 of the phenyl-ring and a primary amine moiety separated from the phenyl ring by two carbon atoms. It is widely diffused in the illicit drug market even though it is listed as a controlled substance in many countries, including Italy (Italian Official Gazette N 266/2007). Several studies report 2,5-DMA quantification in biological matrices (Nieddu et al., 2005; Kanai et al., 2008), but no studies regarding its capacity to induce oxidative stress are currently available. The initial hypothesis was that, being an amphetamine analogue, its toxicity could also be associated with its ability to cause an oxidative stress status. In the present work we compared the levels of oxidative stress indices between healthy controls and rats treated with a single oral dose

of 2,5-DMA (20 mg/Kg) in order to study the effects of this drug on induction of oxidative stress. Amphetamine dose and treatment regimen were chosen according to previously published data concerning structurally related amphetamines (Cerratani et al., 2011; Ninkovic et al., 2008; Berankova et al., 2007; Beita et al., 2000). The ability of 2,5-DMA to induce oxidative stress was investigated by assessing GSH levels as a cellular antioxidant, thiobarbituric acid-reactive substances (TBARS) as biomarkers of lipid peroxidation and the trolox equivalent antioxidant capacity (TEAC) in the blood, liver, kidneys, and testes. Furthermore, possible liver damage was investigated by assaying hepatic transaminase levels and total bilirubin in plasma; in fact it has been reported that plasma levels of alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin were significantly increased in rabbits following administration of drugs of abuse (Al-Motarreb et al., 2002; Al-Habori et al., 2002).

MATERIALS AND METHODS

Materials

The 2,5-dimethoxyamphetamine (2,5-DMA) was purchased from Lipomed (Arllesheim, Switzerland). Deionized and distilled water was purified through a Milli Q water system (Millipore, Billerica, MA, USA). Other reagents and solvents used were of the highest commercial quality and were obtained from Sigma-Aldrich (Milano, Italy).

Animals

Male Lister Hooded rats, weighing 280–300g, were obtained from Harlan Laboratories (Indianapolis, IN, USA). They were allowed a one-week acclimatisation period in the laboratory's animal facility before the beginning of the experiment. Rats were housed at constant room temperature (24 ± 1 °C) and humidity ($60\pm 5\%$) with a 12-h light–dark cycle, and had free access to standard rat chow (Mucedola, Milan, Italy) and tap water. All procedures complied with the standard stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, MD, USA) and were approved by the local Animal Care and Use Committee.

Animals treatment with 2,5-DMA

Animals were randomly selected and kept in separate cages before the study. Rats were divided into two groups (five rats per group): the control group and the treated group. Control animals received a single oral

dose of water. Rats belonging to the treated group received a single oral dose of 2,5-DMA (freshly dissolved in water at appropriate concentration) at 20 mg/Kg body weight.

Sample preparation

Three hours after the treatment, control and treated rats were sacrificed after being anesthetized with an excess of anesthetic. Before being sacrificed, blood was directly collected from the heart into EDTA tubes. A precipitating solution containing N-ethylmaleimide (NEM) a derivatizing agent to prevent the excessive oxidation of GSH was prepared to a final concentration of 20 mM NEM, 2% sulfosalicylic acid and 2 mM EDTA in 15% methanol as described by Moore et al. (2013) 100 μ l of blood was mixed with 400 μ l of the precipitating solution, vortexed and allowed to incubate at room temperature for 45 min. Following derivatization, samples were centrifuged (14000 g, 5 min, +4°C) and the obtained supernatant, containing the complex GSH-NEM, was transferred into polypropylene tubes and stored at -80°C until analysis. The remaining blood was centrifuged (500 g, 10 min, +4°C) and plasma was collected and stored at -80°C until use for the TBARS, TEAC and transaminases assay. Liver, kidneys and testes tissues were collected from each animal, weighed and transferred into tubes containing polyphosphate buffer (PBS) to wash them of excess blood. The clean tissues were transferred into tubes containing 800 μ l of PBS with 0.1% Triton-X 100 per 100 mg of tissue. Homogenization was carried out using an electric homogenizer at room temperature. Homogenates were centrifuged (1500 g, 10 min, +4°C) and supernatants were collected and stored at -80°C until analysis. One hundred microlitres of supernatant was treated without being frozen and was added to 400 μ l of the precipitating solution (above described), vortexed and allowed to incubate at room temperature for 45 min. After derivatization, samples were centrifuged (14000 g, 5 min, +4°C) and the obtained supernatant, containing the complex GSH-NEM, was transferred into polypropylene tubes and stored at -80°C until analysis.

Reduced Glutathione

Glutathione (GSH) was measured using a liquid chromatographic system with triple quadrupole mass spectrometry detection (LC-MS/MS). GSH was quantified in whole blood, liver, testes and kidneys using thiosalicylic acid (TSA) as an internal standard (IS). IS was selected because, like GSH, it possesses an -SH group prone to derivatization by NEM, forming the TSA-NEM adduct (Camera et al., 2001). For the construction of the calibration curve, both thiosalicylic acid and GSH standards were treated with the same precipitating solution used to prepare the samples, in order to obtain TSA-NEM and GSH-NEM. LC-MS/MS analyses were

performed on a Varian 310-MS triple quadrupole mass spectrometer (Varian, Palo Alto, CA, USA). Derivatized samples were conveniently mixed with the IS solution (using dilution factors for blood, liver, testes and kidney of 500, 100, 10, 0, respectively), vortexed briefly and injected into the LC system using a 5 μ l loop. Chromatographic separation was achieved on a LUNA C₁₈ column (5 μ m, 100 \times 2.1 mm Phenomenex, Bologna, Italy) fitted with a Phenomenex C18 security guard cartridge (4 \times 2.0 mm ID). A linear gradient with 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) was performed as follows: 1 min at 5% B; in 0.5 min solvent B was increased from 5 to 97%, and in another 1.5 min it was decreased to 95%. Then, in 0.5 min solvent B was decreased from 95% to 5% and remained constant for 2.5 min. The total runtime was 6 min. All chromatographic solvents were degassed online with a vacuum degasser from Varian. The MS/MS spectrometer was fitted with an electrospray interface (ESI) operating in positive mode. The ESI source conditions were: capillary voltage, 5000 V; drying gas temperature, 200 °C; nebulizer gas pressure, 50 psi (both nebulizer and drying gas were high-purity nitrogen); electron multiplier voltage, 1795 V. For operation in MS/MS mode, the collision gas used was argon with a pressure of 2mTorr in the collision cell. For the optimization of detection conditions, direct infusion of standard solutions (250 ng/ml) was made using a T connection with mobile phase at a flow rate of 400 μ l/min. For both the GSH-NEM complex and its internal standard, two transitions were used; the most abundant was used for the quantitation and the other was used to confirm the analyte identity. The transitions related to free GSH were also monitored in order to verify the complete derivatization of GSH with NEM, and none was detected. Individual parameters for GSH-NEM, IS and GSH are listed in table 1 below. The values of GSH in the samples were calculated using a standard curve built in 15% methanol (range from 10 to 10000 ng/ml) and expressed as μ moles of GSH per gram wet weight of tissue.

Lipid peroxidation

Thiobarbituric acid-reactive substances (TBARS) were evaluated by spectrophotometric detection according to the TBARS assay described by Spanier and Taylor (1991), with some modifications. 100 μ l of sample were thawed and added to 100 μ l of 33% glacial acetic acid, 75 μ l of 10% sodium dodecyl sulphate (SDS), 100 μ l of 50 mM Tris-HCl pH 7.4 and 250 μ l of 0.75% thiobarbituric acid (TBA). The mixture was then incubated for 1 h at 100°C and immediately cooled on ice. After 10 min, 200 μ l of 33% acetic acid were added and samples were centrifuged for 20 min at 7000 g. The supernatant absorbance was then read with a Thermo Elecrom Corporation Genesys 10UV spectrophotometer at 535 nm. The values of TBARS in the samples were calculated

Table 1. Liquid chromatography–tandem mass spectrometry parameters

Analyte	RT (min)	Capillary (V)	MRM transitions (<i>m/z</i>)	CE (eV)
GSH	2.3	50	308.3→ 179.1	-10
			308.3→ 161.9	-14
GSH-NEM	3.1	32	433.4→ 304.0	-11.5
			433.4→ 286.9	-16.5
IS	3.4	30	280.4→ 262	-7.5
			280.4→ 163.3	-20

RT retention time, *MRM* multiple reaction monitoring, *CE* collision energy

using a standard curve and expressed as nmoles of TBARS formed per gram wet weight of tissue.

Total antioxidant activity

Trolox equivalent antioxidant capacity (TEAC) was measured using the method described by Re et al. (1999) and modified by Lewinska et al (2007). Briefly, a fresh solution was prepared by dissolving 19.5 mg of 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) and 3.3 mg of potassium persulphate in 7 ml of phosphate buffer (0.1 mol/L, pH 7.4). This solution was stored in the dark for 12 h to allow the reaction to be completed. It was then diluted (1:80) in phosphate buffer (0.1 mol/L, pH 7.4) and mixed thoroughly to give an absorbance between 0.9 and 1.0 AU at 734 nm. Samples (diluted 1:50 in phosphate buffer) were added to diluted ABTS solution and after 3 min the absorbance of the mixture was measured at 734 nm twice in a spectrophotometer (Thermo Ecom Corporation Genesys 10 UV, Madison, Wisconsin, USA). The extent of ABTS bleaching (decrease in absorbance, corrected for a small decrease in absorbance of ABTS solution alone) is proportional to the activity of antioxidants in a given sample. The antioxidant capacity was expressed as TEAC, that is the concentration of trolox needed to produce the same effect as the sample studied. Calculations were made on the basis of a standard curve obtained for a trolox solution (5–20 µM trolox in a total volume of 550 ml). The values of TEAC in the samples were expressed as mmol per gram of proteins in plasma and as µmoles of TEAC formed per gram wet weight of tissue. The quantification of total protein content in plasma was performed using the Lowry method (Lowry et al., 1951).

Transaminases and Bilirubin

Alanine Aminotransferase ALT/GPT, Aspartate Aminotransferase AST/GOT and total Bilirubin were assayed using spectrophotometric kits from Biosystem

(Barcelona, Spain).

Statistical analysis

Data were reported as mean ± standard deviation. Differences between groups were determined by using the Student's *t*-test. A probability (*P*) value less than 0.05 was considered significant. Statistical analysis was performed by SPSS 8.0 software (SPSS, Inc., Chicago, Illinois, USA).

RESULTS

Reduced Glutathione

The treatment with a single oral dose of 2,5-DMA (20 mg/Kg) caused a significant decrease of GSH levels in liver when compared with controls (Figure 1). The assay was also performed in whole blood, kidneys and testes, and results are shown in table 2 below. The values of GSH were expressed as µmoles of GSH formed per gram wet weight of tissue and as mM in blood. Chromatograms of GSH-NEM and TSA-NEM in a standard solution and in the liver of a treated and a control rat are shown in figures 2 and 3, respectively.

Lipid Peroxidation

Lipid peroxidation assay was performed to determine the TBARS level in plasma and in tissue homogenates of liver, kidneys and testes of control and treated rats. The results are presented in table 3 below. Three hours after treatment with a single oral dose of 2,5-DMA (20 mg/Kg), the TBARS levels in plasma (Figure 4), liver (Figure 5) and testes (Figure 6) were significantly increased when compared with the control group. The analysis in kidneys did not reveal any significant difference between the two groups of rats studied. The values of TBARS were expressed as nmoles of TBARS formed per gram wet weight of tissue and as µM in plasma.

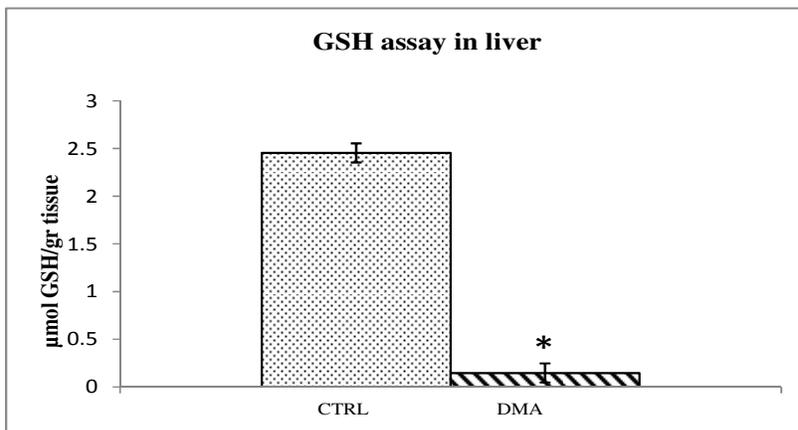


Figure 1. Acute effect of 2,5-DMA on GSH content in liver. Data are represented as mean±SD; n=5 rats.* P<0.05

Table 2. Acute effect of 2,5-DMA (20 mg/Kg) on GSH levels in organs (μmol GSH per gram wet tissue) and blood (mM) of rats examined

Matrices	Control group	Treated group
Blood	7.62 ± 1.25	6.2 ± 0.89
Liver	2.45 ± 0.04	0.145 ± 0.07 *
Kidneys	< LOD	< LOD
Testes	0.07 ± 0.04	0.06 ± 0.02

LOD limit of detection

Each value represents the mean ± SD; n=5 rats

*P<0.05 when compared with control group

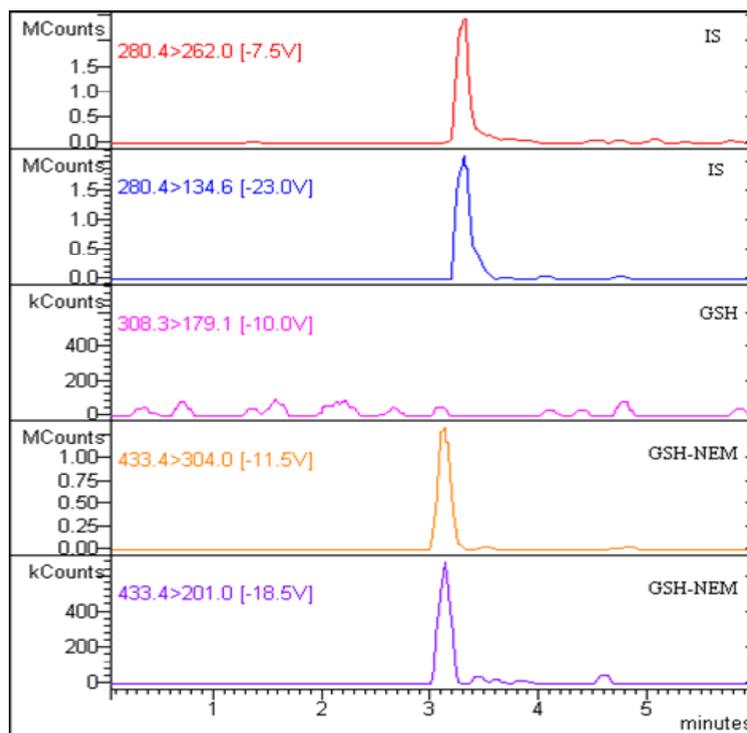


Figure 2. Chromatogram of a standard solution of GSH-NEM (150 ng/mL) and IS (500 ng/mL)

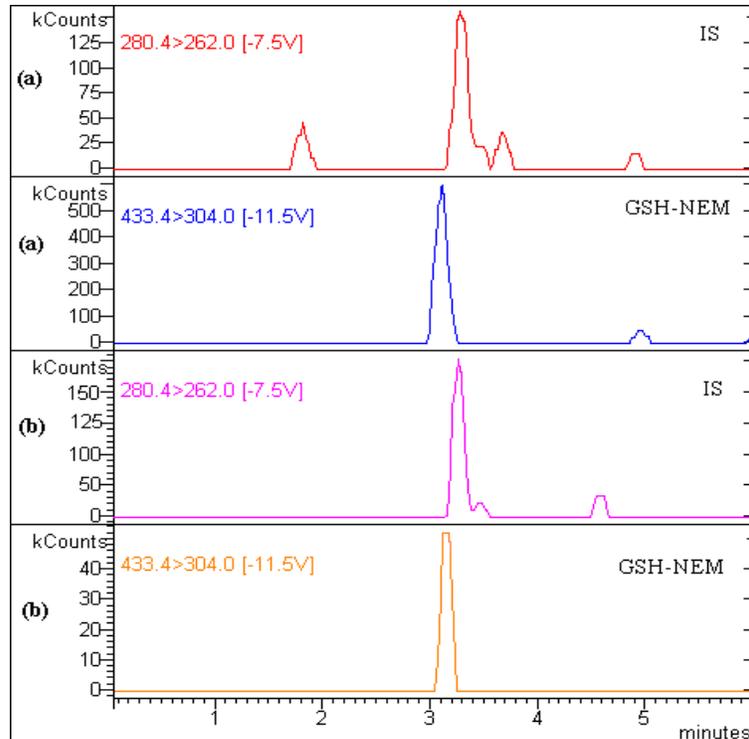


Figure 3. Chromatogram of GSH-NEM and IS in liver of a control (a) and a treated rat (b)

Table 3. Acute effect of 2,5-DMA on TBARS levels in plasma (μM), and organs (nmol TBARS per gram wet tissue) of rats examined

Matrices	Control group	Treated group
Plasma	18.48 \pm 1.48	26.16 \pm 2.85*
Liver	220 \pm 35	276 \pm 3.6*
Kidneys	125.86 \pm 27.26	116.19 \pm 8.08
Testes	59,46 \pm 11,3	104,7 \pm 10,9**

Each value represents the mean \pm SD; n=5 rats
 **P<0.01; *P<0.05 when compared with control group

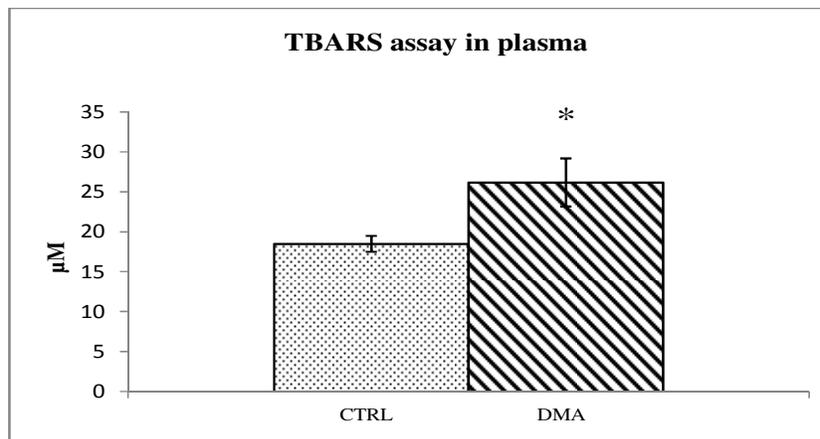


Figure 4. Acute effect of 2,5-DMA on TBARS content in plasma. Data are represented as mean \pm SD; n=5 rats.* P<0.05

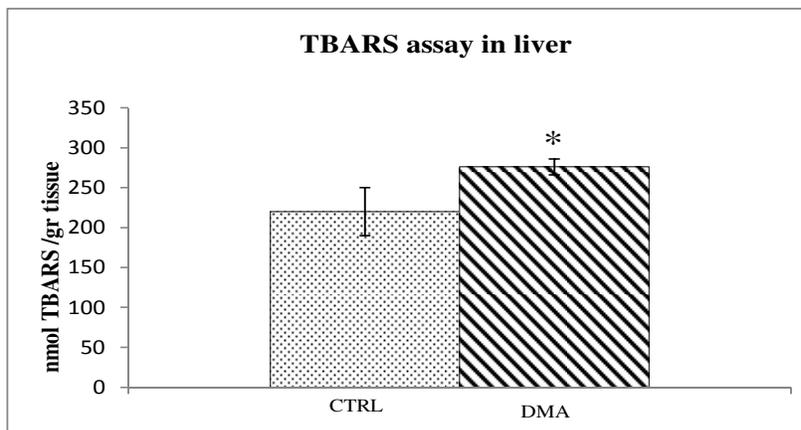


Figure 5. Acute effect of 2,5-DMA on TBARS content in liver. Data are represented as mean±SD; n=5 rats. * P<0.05

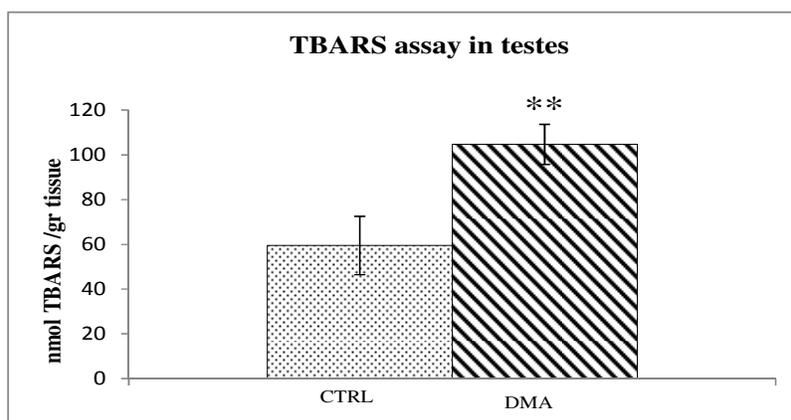


Figure 6. Acute effect of 2,5-DMA on TBARS content in testes. Data are represented as mean±SD; n=5 rats. ** P<0.01

Table 4. Acute effect of 2,5-DMA on TEAC levels in plasma (mmol TEAC per gram of proteins), and organs (μ mol TEAC per gram wet tissue) of rats examined

Matrices	Control group	Treated group
Plasma	0.81 ± 0.10	0.60 ± 0.02*
Liver	227,80 ± 6.47	254,30 ± 15,87
Kidneys	234,64 ± 50,57	259,98 ± 6,71
Testes	213,01 ± 5,70	179,76 ± 6,22

Each value represents the mean ± SD; n=5 rats
*P<0.05 when compared with control group

Total antioxidant activity

TEAC results in plasma and in tissue homogenates of liver, kidneys and testes of control and treated rats are reported in table 4 above. Three hours after treatment with a single oral dose of 2,5-DMA (20 mg/Kg), the TEAC levels in plasma (Figure 7) were significantly decreased

when compared with the control group. The analysis in liver, kidneys and testes did not reveal any significant difference between the two groups of rats studied. The total antioxidant activity was expressed as μ moles of TEAC formed per gram wet weight of tissue and as millimoles per gram of plasmatic proteins.

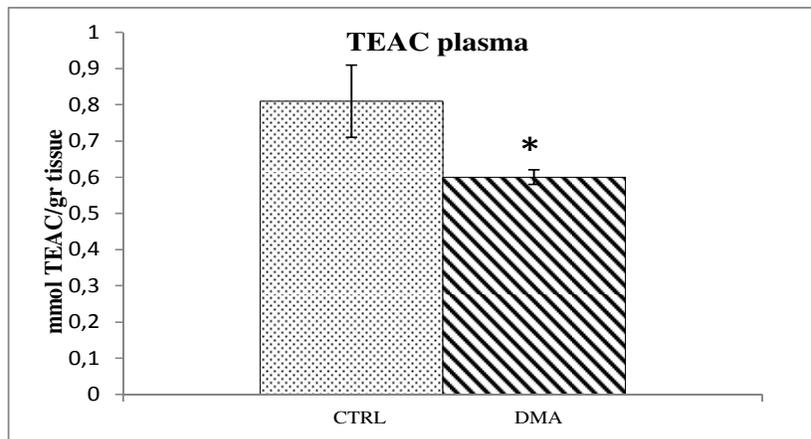


Figure 7. Acute effect of 2,5-DMA on TEAC content in plasma. Data are represented as mean±SD; n=5 rats.* P<0.05

Transaminases

Plasma levels of Alanine Aminotransferase ALT/GPT and Aspartate Aminotransferase AST/GOT in treated rats showed no statistical differences when compared with control rats (30.4 ± 6 U/L versus 30.5 ± 4 U/L for ALT and 62.2 U/L ± 1 versus 61.7 ± 6 U/L for AST).

Bilirubin

Total Bilirubin levels in treated rats showed no statistical differences when compared with control rats (0.2 ± 0.01 mg/dL versus 0.2 ± 0.02 mg/dL).

DISCUSSION

Oxidative stress is a key player in cytotoxicity induced by amphetamines. Increasing evidence suggests that one effect of MDMA-induced toxicity involves the production of ROS and reactive nitrogen species, which subsequently results in oxidative/nitrosative stress (Fiaschi and Cerratani, 2010). Several scientific papers describe oxidative stress induced by the consumption of MDMA, methamphetamine and amphetamine in the nervous system and in different organs and tissues (Cunha-Oliveira et al., 2013; Huang et al., 2013; Cerratani et al., 2011; Ninkovic et al., 2008). In this work we investigated the possible oxidative stress status induced by acute administration of high doses of 2,5-DMA in rats and this is the first report to investigate this aspect. Induction of oxidative stress was investigated by assessing GSH as a cellular antioxidant, TBARS as biomarkers of lipid peroxidation and the total antioxidant activity, measured as TEAC, in blood, liver, testes and kidneys. A slight decrease in GSH levels, although not statistically significant, was observed in blood. In plasma,

a significant increase in TBARS levels was observed together with a significant decrease in TEAC. Our results are in accordance with several papers in literature where increased lipid peroxidation and decreased antioxidant parameters were observed after d-amphetamine treatment (Simeonova et al., 2010; Govitrapong et al., 2010). Liver is a vulnerable target for amphetamine toxicity, but the mechanism of this hepatotoxicity remains poorly understood. For instance, liver toxicity has been widely recognized for MDMA, which is known to be hepatotoxic to humans. It is well-known that amphetamine abuse is associated with hepatocellular damage through oxidative stress (Vitcheva et al., 2009). As an endogenous antioxidant, GSH is critical for cellular functions and cell survival: it maintains the redox status of the sulfhydryl groups of cellular proteins. GSH plays a crucial protective role against cellular injury, thanks to its oxidant neutralizing, lipid peroxidase and/or tocopheryl radical regenerating activities (Di Mascio et al., 1991). In the liver of rats treated with 2,5-DMA, a significant decrease in GSH levels and a simultaneous increase of TBARS levels were observed. In light of this, our results are in accordance with Carvalho et al. (2002) who reported GSH depletion in liver after an acute intraperitoneal dose of MDMA in mice. This renders the cells more exposed to the effects of reactive compounds, reactive oxygen and reactive nitrogen species, leading to deleterious effects in hepatocytes. Furthermore, although amphetamines toxicity has been often associated with variable clinical as elevation of hepatic enzymes (ALT, AST, alkaline phosphatase) and total bilirubin (Cerratani et al., 2011; Da Silva et al., 2013), in our experimental conditions, no alteration of ALT, AST and bilirubin was observed. This results show that even if an oral treatment with an high dose of 2,5-DMA creates an increased oxidative stress status in liver it seems to not be related to hepatic damage after three hours of treatment.

The influence of amphetamines such as MDMA on the fertility and reproduction of the male rat has been studied by Barenys et al. (2009) which described a high incidence of DNA damage in sperm and testes histopathology after drug assumption. It is well-known that DNA damage in sperm is linked to an increase of oxidative stress (Aitken et al., 2010) and that testes are vulnerable to oxidative stress because of the abundance of highly unsaturated fatty acids and the presence of potential ROS-generating systems (aitken et al., 2008). This is in accordance with our results that demonstrate an increase in levels of oxidative biomarkers after acute 2,5-DMA administration: testes of treated rats showed a significant increase in TBARS levels. Similarly to liver and testes, amphetamines and their metabolites are known to damage the kidney during their excretion process (Song et al., 2010). On the contrary, in this study, the parameters analyzed did not change in kidney, suggesting that this organ is not affected by oxidative stress three hours after oral administration of a high dose of 2,5-DMA. The presented results, showed that, under our experimental conditions, 2,5-DMA induces oxidative stress in plasma, liver and testes, modifying the balance between oxidants and antioxidants.

In conclusion it can be postulated that the enhanced oxidative stress observed, could contribute to the understanding of the mechanisms involved in 2,5 DMA toxicity, an aspect widely studied in the literature concerning other well-known amphetamines.

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