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INFLUENCE OF GLUTATHIONE REDUCTASE ON DIQUAT NEUROTOXCITY ASSESSED BY OXIDATIVE/NITROSATIVE STRESS IN THE CORTEX OF INTRASTRIATALLY TREATED RATS

DJUKIC MIRJANA*, JOVANOVIC MARINA**, NINKOVIC MILICA**, STEVANOVIC IVANA**, DJURDJEVIC D** and VASIC UNA***

**University of Belgrade, Faculty of Pharmacy, Belgrade, Serbia*
***Institute for Medical Research, Military Medical Academy, Belgrade, Serbia*
****University of Belgrade, Gynecology and Obstetrics clinic, Belgrade, Srbija*

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In this study we examined if the response of the cortex against diquat (DQ), intrastriatally (i.s.) applied to Wistar rats, was mediated by oxidative/nitrosative stress (OS/NS). In particular, we were focused on the glutathione (GSH) antioxidative role, thus we applied i.s. glutathione reductase (GR) in the pre-treatment of DQ administration. Superoxide anion radical ($O_2^{\bullet -}$), nitrate (NO_3^-), malondialdehyde (MDA) and superoxide dismutase (SOD), were measured in ipsi- and contra- lateral sides of the cortex, at 30 minutes, 24 hours and 7 days post treatment.

The redox balance was not significantly changed in the cortex of sham operated and intact groups. Also, no differences were observed between the ipsi- and contra- lateral side of the cortex. Lethargy and mortality (30-40%) of the animals in the DQ group within 24 hrs, coincided with rapidly developed lipid peroxidation supported by OS/NS upon i.s. DQ administration. Strong redox potential of DQ probably resulted in a huge deprivation of molecular oxygen. The pre-treatment with GR acted neuro-protectively, based on animal survival and absence of lethargy, although, lipid peroxidation was not developed in the GR+DQ group, OS was documented by a high concentration of $O_2^{\bullet -}$ (within 24 hrs), descending and eventually inhibiting SOD activity (at 7 days).

Key words: diquat, free radicals, glutathione reductase, lipid peroxidation, neurotoxicity, nitrosative stress, oxidative stress

INTRODUCTION

Diquat (DQ) (1,1'-ethylene-2,2'-bipyridylium), has been used extensively since the mid twenties as a non-selective contact herbicide, aquatic weed control agent, seed desiccant and sugarcane flowering suppressant agent. Occupational exposure to DQ does not pose a health risk if the recommendations for use are followed. Accidental cases are usually due to ingestion of decanted DQ. Diquat poisoning by suicidal or accidental ingestion is much less common compared to

its dipiridylium analogue, paraquat (PQ). Systemic exposure to DQ leads to gastrointestinal, liver and kidney toxicity in humans (and monkeys) and causes clinical syndromes such as: severe diarrhoea associated with disorders of water metabolism, general depression and lethargy as the most commonly seen central nervous system (CNS) effects, although some authors reported tremors and convulsions (INCHEM, 1984; Pasi, 1978). Pathophysiological mechanisms of changes in the CNS has not been clarified, although it is known that DQ passes the blood-brain barrier (WSSA Herbicide Handbook Committee, 1989; Kušić *et al*., 1974).

Diquat toxicity is mediated by free radical production, similarly to PQ. In the presence of molecular oxygen (O_2) production of superoxide anion radical ($O_2^{\bullet -}$) occurs during DQ redox-cycling metabolism (Wolfgang *et al*., 1991; Fuke *et al*., 1993). Generation of $O_2^{\bullet -}$ – may lead to many potentially cytotoxic effects, including the membrane-damaging process of lipid peroxidation (LPO). During redox metabolism, DQ^{2+} (commercial product: a salt of a dication) undergoes the one-electron reduction (in the presence of electron donors: cytochrome P450 reductase) to form the radical ($DQ^{\bullet +}$). In aerobic conditions, $DQ^{\bullet +}$ reacts with molecular O_2 to produce superoxide anion radical ($O_2^{\bullet -}$) and DQ^{2+}, initiating a free radicals chain reaction, which is essential for the DQ cytotoxic effect (Fussella *et al*., 2011).

Nitrates (NO_3^-) are the major final metabolic product of RNS and thus can be used as a marker of NO_x production (Moncada *et al*., 1991; Curcic Jovanovic *et al*., 2007).

Glutathione (GSH) acts as a neuromodulator, neurotransmitter and neurohormone in the CNS and is the crucial endogenous antioxidant. During OS, depletion of GSH occurs (Ninkovic *et al*., 2003; Ninkovic *et al*., 2008). It could be at the expense of its oxidation to glutathione disulfide (GSSG) (GSH is a donor of reducing equivalents for the glutathione peroxidase (GPx) catalyzed reduction of lipid hydro peroxides) and conjugation with thiol, nitroso and metal ions (Dringer, 2000; Wu, 2004; Meister, 1988; Ballatori *et al*., 2009; Douglas, 1987; Leaver, and George,1998; Mieyal *et al*., 2008; Smith, 2005; Kehrer and Lund, 1994). Recycling of GSSG back to GSH by glutathione reductase (GR) is particularly important to maintain GSH concentrations at levels necessary to achieve its antioxidative role. Led by the fact that GR does not pass the blood-brain barrier, we decided to administer GR intrastriataly (*i.s.*). In this toxicological-experimental mechanistic study we examined if the pre-treatment with GR, applied *i.s.*, would achieve a neuroprotective role against toxic effects of DQ administered *i.s.* A positive outcome would have confirmed that the oxidative/nitrosative damage of vulnerable brain regions (the cortex in this particular study) was a consequence of reduced antioxidant defence, primarily due to GSH oxidation to GSSG, in DQ neurotoxicity.

MATERIALS AND METHODS

Animals

The experimental animals were treated according to Guidelines for Animal Study, No. 282-12/2002 (Ethics Committee of the Military Medical Academy, Belgrade, Serbia and Montenegro). The experiments were performed on adult male *Wistar* rats weighing approximately 220 g, randomly divided into two control groups (the intact group n=8, and the sham-operated, n=24) and three experimental groups (n=24, each) which were further subdivided into three subgroups (n=8) according to the time of sacrificing. The rats were housed in cages under standardized housing conditions (ambient temperature of 23±2°C, relative humidity of 55±3% and a light/dark cycle of 13/11 hours) and had free access to standard laboratory pellet food and tap water. All the experiments were performed after a 7 days period of adaptation to laboratory conditions, and were carried out between 9 a.m. and 1 p.m.

Experimental design

Rats were anaesthetized by sodium pentobarbital (45 mg/kg per body weight) given intraperitoneally. In this study we used yeast GR, according to: a) literature evidence of its homology with GR sequences of rats and humans, substrate specificity, kinetic characteristics and substrate affinity; and b) our positive previous experience in different animal models (unpublished data) (Cardoso *et al*., 2008; Prado *et al*., 2004; Ondaraza and Abney, 1997; Collinson and Dawes, 1995). Testing substances were administrated as single doses*, i.s.* in final volumes of 10 μL, which is insufficient to burden nerve tissue. For this purposes we used a Hamilton syringe, which was accurately coordinated by using a stereotaxic instrument for small laboratory animals (coordinates: 8.4 mm behind the bregma, 2.6 mm left from the midline suture and 4.8 mm ventral from dura) (König and Klippel, 1963). The experiment was accomplished with the following five experimental groups, which received different testing substances: the intact group (not treated, n=8), the sham-operated rats (10 μL of saline), n=24; the GR group (GR, 15.63 U/10 μL), n=24; the DQ group (2.5 μg DQ /10 μL, *i.e.* 0.01 μM/10 μL) n=24; and the GR+DQ group (GR, 15.63 U/5 μL, immediately before DQ administration, 0.01 μM/5 μL), n=24. The animals were sacrificed by decapitation at 30 mins, 24 hrs and 7 days after the treatments. Biochemical parameters of OS were measured in the ipsi- and contra- lateral side of the ortex. To exclude the possibility that mechanical injury caused OS in the VBRs, we compared OS parameters between the sham-operated and the intact groups.

Reagents

All chemicals were of analytical grade. The following compounds were used in this study: Diquat - Galokson® (200 g/L) (Galenika - Zemun, Serbia); Sodium pentobarbital – Vetanarcol® (0.162 g/mL) (Werfft - Chemie, Vienna, Austria); 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; 2500 U/1.6 mL (9.2 mg prot/mL – biuret) 170 U/mg protein (Note: 1 unit

reduces 1 μmol GSSG/min, pH 7.6 at 25°C); saline solution (0.9% w/v) (Hospital Pharmacy Military Medical Academy, Belgrade, Serbia); glutathione, glutathione disulfide and NADPH (Boehringer Corp. - London, UK); $NaNO_3$ (Mallinckrodt Chemical Works - St. Louis, MO, USA); ethylenediaminetetraacetic acid – EDTA, epinephrine (Sigma Aldrich - Sr. Louis, USA); sodium phosphate – Na_2HPO_4, potassium dihydrogen phosphate – KH_2PO_4, acetonitrile, trichloroacetic acid, thiobarbituric acid, sulfosalicylic acid, sodium gluconate, methanol, nitro blue tetrazolium (Merck - Darmstadt, Germany); sodium tetraborate and boric acid (Zorka - Sabac, Serbia); carbonate buffer (50 mM, pH 10.2), (Serva, Feinbiochemica - Heidelberg, New York). Deionised water was prepared by the Millipore milli-Q water purification system (Waters - Millipore, Milford, MA, USA).

Tissue preparation

Homogenates of the cortex were prepared from individual rats as described earlier (Gurd *et al*., 1974). The ipsi- and contra- lateral side of the cortex were removed from the brain tissue and kept on ice during the whole procedure. Slices of the cortex were transferred separately into cold buffered sucrose (0.25 mol/L sucrose, 0.1 mmol/L EDTA in sodium-potassium phosphate buffer, pH 7). Aliquots (1 mL) were placed into a glass tube homogeniser (Tehnica Zelezniki Manufacturing, Slovenia). Homogenization was performed twice with a teflon pestle at 800 rpm (1,000 × g) for 15 min at 4°C. The supernatant was centrifuged at 2,500 × g for 30 min at 4°C. The resulting precipitate was suspended in 1.5 mL of deionised water. Solubilisation of subcellular membranes in hypotonic solution was performed by constant mixing for 1 h using a Pasteur pipette. Thereafter, homogenates were centrifuged at 2,000 × g for 15 min at 4°C and the resulting supernatant was used for analysis. Total protein concentration was estimated with bovine serum albumin as a standard (Lowry *et al*., 1951).

Measurements

Parameters of OS/NS (superoxide anion radicals, nitrates, malondialdehyde, superoxide dismutase) were measured in both sides (ispilateral and contralateral) of the cortex, after the treatments at 30 min, 24 hrs and 7 days.

Superoxide anion radical ($O_2^{\bullet -}$)

Content of $O_2^{\bullet -}$ was quantified by the method based on the reduction of nitrobluetetrazolium – NBT to monoformazan by $O_2^{\bullet -}$. The yellow colour of the reduced product was measured spectrophotometrically at 550 nm (Auclair and Voisin, 1985) The results were expressed as μmol reduced NBT per mg of protein.

Nitrates (NO_3^-)

Deproteination of brain homogenates was performed using acetonitrile (sample: acetonitrile, 2:1, v/v), then centrifuged and the supernatant was filtered (0.45 μm) before chromatographic analysis (ion-exchange HPLC) (Curcic Jovanovic *et al*., 2007). A mobile phase composed of borate buffer/gluconate concentrate, methanol, acetonitrile and deionized water in a ratio 2:12:12:74

(v/v/v/v) (pH 8.5) was used for isocratic elution at a flow rate of 1.3 mL/min at room temperature. Spectroscopic detection was performed at a single wavelength of 214 nm. For NO_3^- determination, 50 μL of filtrate was injected into the HPLC system. The results were expressed as nmol NO_3^- per mg of protein.

Malondialdehyde (MDA)

Malondialdehyde (MDA) was determined by the spectrophotometric method of Ohkawa and co-workers (Baydasë *et al*., 2001). MDA, a secondary product of LPO, gives a red colored pigment after incubation with thiobarbituric acid-TBA reagent (15% trichloroacetic acid and 0.375% TBA, water solution), at 95°C at pH 3.5. Absorbance was measured at 532 nm. The results were expressed as pmol MDA per mg of protein.

Superoxide dismutase (SOD)

Activity of SOD was measured spectrophotometrically, as the inhibition of spontaneous autooxidation of epinephrine at 480 nm. The kinetics of the sample enzyme activity was followed in a carbonate buffer (50 mM, pH 10.2, containing 0.1 mM EDTA), after the addition of 10 mM epinephrine (Sun and Zigman, 1978). The results were expressed as U SOD per mg of protein.

Statistical analysis

Data analysis was performed using Statistica software version 7.0 (Stat Soft, Inc.). Parameters of OS were presented graphically for the ispilateral and in tabular form for the contralateral side of the VBRs. Data are shown as mean ± standard deviation. Parameters of OS measured at different time points within each group were compared by the independent Student's t-test. OS parameters for the same time point, but between the groups, were compared using ANOVA with the Tukey's post hoc test. Differences were considered statistically significant for $p<0.05$(*), $p<0.01$(**) and $p<0.001$(***).

RESULTS

Differences between the control (the intact group) and the sham-operated groups were not significant. Results of the biochemical analysis of oxidative/nitrosative stress (OS/NS) parameters in ipsilateral and contralateral side of the cortex were presented graphically (Graphs 1 – 4).

Mortality (30% - 40%) within 2-3 hours after awakening from anesthesia was observed only in the DQ group (0.01 μM of DQ were *i.s.* administered). Lethargy was the only neurological symptom observed in that group.

Only significant values of the OS parameters are presented in this section. All changes of measured biochemical parameters of OS/NS occurred almost equally between ipsi- and contra- lateral cortex, accordingly, the interpretation of results related to bilateral cortex of treated rats.

Superoxide anion radical ($O_2^{\bullet-}$)

In the DQ group, $O_2^{\bullet-}$ content was initially (at 30th min) significantly increased ($p<0.05$), afterwards the descending course resulted in a significantly reduced level ($p<0.05$), at 7 days, compared to the controls. In the GR+DQ group, significantly elevated $O_2^{\bullet-}$ content was measured at 30 min and 24 hrs ($p<0.05$ and $p<0.01$, respectively). Statistical difference of $O_2^{\bullet-}$ content was obtained between the pre-treated and poisoned groups, at 24 hrs (GR+DQ >DQ; $p<0.05$) (Graph 1).

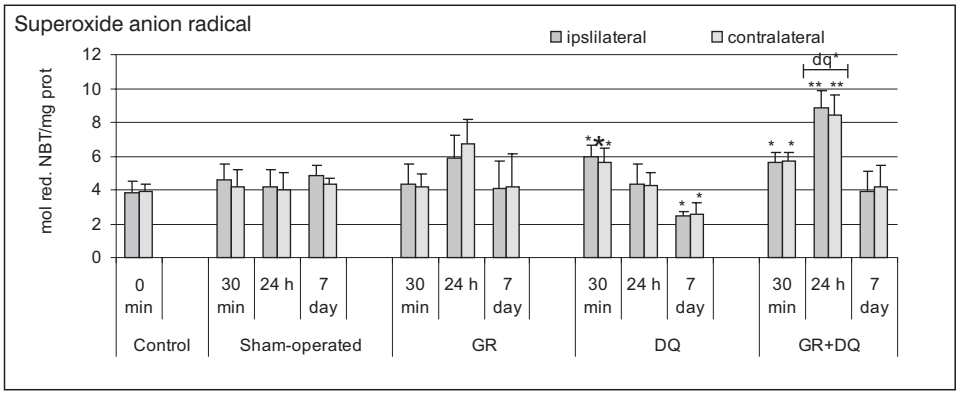

Figure 1. Content of superoxide anion radical ($O_2^{\bullet-}$) in bilateral cortex of rats after single intrastriatal administration of diquat (DQ group), glutathione reductase (GR group) and glutathione reductase in the pretreatment of diquat administration (GR+DQ group).
Content of $O_2^{\bullet-}$ is expressed as: μmol red. NBT/mg proteins. *See the experimental conditions presented in the subsection: Experimental design. Values are means ± SD (n=8 rats per each time point: 30 min, 24 hrs, 7 days). One-way ANOVA followed by post-hoc Tukey test were used for statistical analysis; $p<0.05$ was considered as significant for the same time point. Statistically significant differences are marked as follows: $p<0.05$(*), $p<0.01$(**) and $p<0.001$(***) – compared to the control group; and: $p<0.05$ (dq*), $p<0.01$ (dq**) and $p<0.001$ (dq***) – compared to group DQ.

Nitrates (NO_3^-)

In the DQ group, nitrates were significantly elevated during the whole experiment ($p<0.05$), compared to the controls. In the GR+DQ group, NO_3^- content significantly increased only in the contralateral cortex ($p<0.05$) at 30 min, compared to the controls. Statistical difference of NO_3^-content was obtained between the pre-treated and poisoned groups, at 24 hrs (GR+DQ<DQ; $p<0.05$) (Graph 2).

Malondialdehyde (MDA)

In the DQ group, MDA levels were significantly elevated in the ipsilateral cortex at 30 min ($p<0.05$) and bilaterally at 24 hrs ($p<0.05$). No significant differences for MDA were observed in the GR+DQ group, compared to the controls. Statistical difference of MDA levels was obtained between the pre-treated and poisoned groups, within 24 hrs (GR+DQ<DQ; $p<0.05$) (Graph 3).

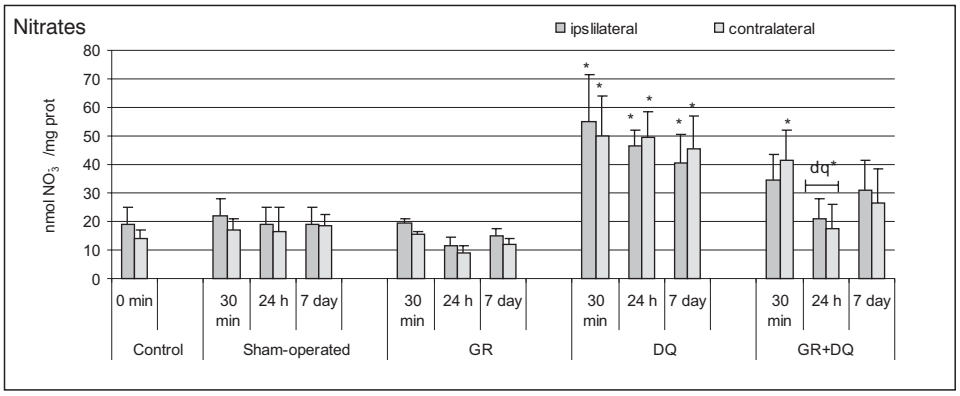

Figure 2. Content of nitrates (NO_3^-) in bilateral cortex of rats after single intrastriatal administration diquat (DQ group), glutathione reductase (GR group) and glutathione reductase in the pretreatment of diquat administration (GR+DQ group).
Nitrates are expressed as: nmol NO_3^-/mg proteins. *See the experimental conditions presented in the subsection: Experimental design. Values are means ± SD (n=8 rats per each time point: 30 min, 24 hrs, 7 days). One-way ANOVA followed by post-hoc Tukey test were used for statistical analysis; $p<0.05$ was considered as significant for the same time point. Statistically significant differences are marked as follows: $p<0.05$(*), $p<0.01$(**) and $p<0.001$(***) – compared to the control group; and: $p<0.05$ (dq*), $p<0.01$ (dq**) and $p<0.001$ (dq***) – compared to group DQ.

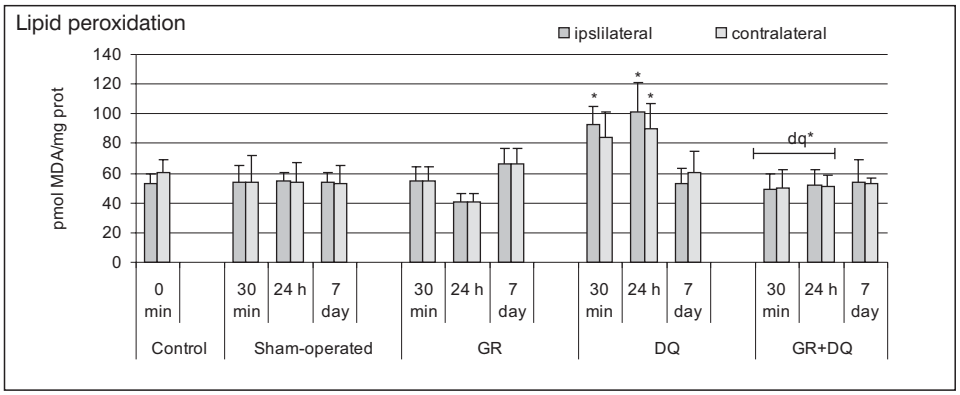

Figure 3. Lipid peroxidation (malondialdehyde, MDA) in the bilateral cortex of rats after a single intrastriatal administration of diquat (DQ group), glutathione reductase (GR group) and glutathione reductase in the pretreatment of diquat administration (GR+DQ group).
Lipid peroxidation is expressed as: pmol MDA/mg proteins. *See the experimental conditions presented in the subsection: Experimental design. Values are means ± SD (n=8 rats per each time point: 30 min, 24 hrs, 7 days). One-way ANOVA followed by post-hoc Tukey test were used for statistical analysis; $p<0.05$ was considered as significant for the same time point. Statistically significant differences are marked as follows: $p<0.05$(*), $p<0.01$(**) and $p<0.001$(***) – compared to the control group; and: $p<0.05$ (dq*), $p<0.01$ (dq**) and $p<0.001$ (dq***) – compared to group DQ.

Superoxide dismutase activity (SOD)

In the DQ group, no significant differences for SOD activity were observed. In the GR+DQ group, SOD activity was significantly decreased compared to the controls, since the first measurement at 30 min, 24 hrs ($p<0.01$), until the 7th day (total inhibition) ($p<0.001$). Statistical differences for SOD activity were obtained between the pre-treated and poisoned groups, for ipsilateral cortex at 30 min and 24 hrs, and bilaterally at 7 days (GR+DQ<DQ; $p<0.05$, within 24 hrs; GR+DQ<<DQ; $p<0.01$, 7th days) (Graph 4).

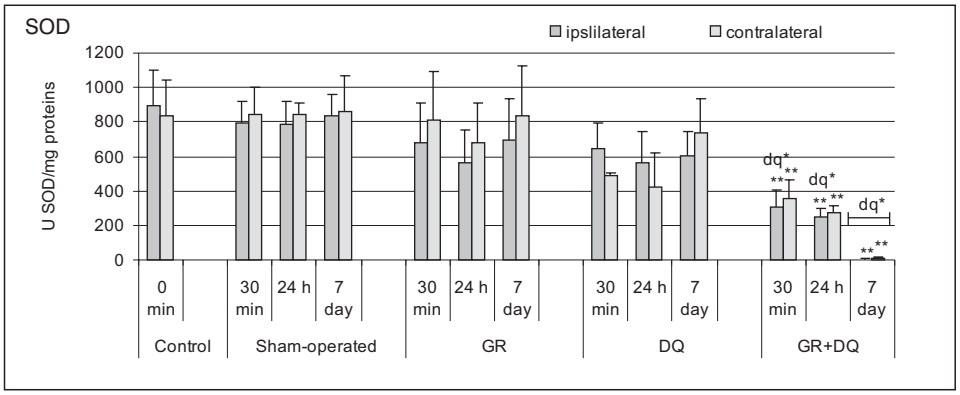

Figure 4. Activity of superoxide dismutase (SOD) in the bilateral cortex of rats after a single intrastriatal administration of diquat (DQ group), glutathione reductase (GR group) and glutathione reductase in the pretreatment of diquat administration (GR+DQ group).
Activity of SOD is expressed as: U SOD /mg proteins. *See the experimental conditions presented in the subsection: Experimental design. Values are means ± SD (n=8 rats per each time point: 30 min, 24 hrs, 7 days). One-way ANOVA followed by post-hoc Tukey test were used for statistical analysis; $p<0.05$ was considered as significant for the same time point. Statistically significant differences are marked as follows: $p<0.05$(*), $p<0.01$(**) and $p<0.001$(***) – compared to the control group; and: $p<0.05$ (dq*), $p<0.01$ (dq**) and $p<0.001$ (dq***) – compared to group DQ.

DISCUSSION

In this study we showed for the first time, that GR applied *i.s.* in the pre-treatment of DQ neurotoxicity achieved a neuroprotective effect, based on the animal survival, absence of lethargy and lack of lipid peroxidation. Our study confirmed that antioxidative effect of GSH is one of the crucial contributors against oxidative/nitrosative injury induced by DQ.

Leading by Corsanti, Beggeta and co-workers studies on PQ (structural analogue of DQ) neurotoxicity, we applied intrastriatally (*i.s.*) one single dose of DQ 50 mg/kg to examine if OS/NS is included in the overall response of the the vulnerable brain regions (VBRs) against DQ neurotoxicity. Some brain regions, such as: pyramidal neurons of CA1 and CA3 sectors of the hippocampus, small pyramidal neurons and third layer of the cerebral cortex and striatum (*Nucleus*

caudatus and *putamen*) are particularly vulnerable to oxidative injury (Djukic *et al*., 2007), that is why we chose the cortex to explore DQ neurotoxicity pathways. Apparently, the applied dose was sufficient to study the mechanism of DQ neurotoxicity. The experiment lasted 7 days, which is sufficient for the recovery of tissues, if reversible tissue damage occurred (Kang *et al*., 2009; McCormack *et al*., 2005; Widdowson *et al*., 1996).

Based on the obtained results, invasively applied tested compounds (*i.s.* rout of administration) did not aggravate tissue damage. Namely, no significant differences between the results of biochemical OS/NS parameters were observed in the bilateral cortex of the sham-operated and control-intact groups. Instant and spatial propagation of the OS/NS was confirmed by our experiment, based on almost identical responses against the applied treatments in both sides of cortex, over time (Graphs 1- 4) (Cui *et al*., 2004; Halliwell, 2001).

Oxidative stress is linked to nicotinamide adenine dinucleotide phosphate (NADPH) depletion and activation of the hexose monophosphate shunt (HMPS) (Tawara *et al*., 1996). NADPH depletion and energy exhaustion eventually led to cell death. Some redox-cycling compounds completely inhibit the activation of HMPS by inhibiting GR (Schraufstatter *et al*., 1985). There is however no data specifically relating to inhibition of GR with DQ, but if so, we can speculate that the GR pre-treatment could be one rationale more for our experimental approach.

Lethargy and lethality (30-40%) observed within 2-3 hours after awakening from anesthesia, in the group of *Wistar* rats *is.* poisoned with DQ, coincided with LPO measured in bilateral cortex of these animals. Oxidative/nitrosative stress was documented with an initially increased production of $O_2^{\bullet -}$ (at 24th hrs) and markedly elevated NO_3^- in the bilateral cortex during the whole experiment (Graphs 1-4). Nitrates are the final, stable products of reactive nitrogen species (RNS), including various (non)radical forms of nitrogen oxides (NO_x) that indicate their overall metabolism.

Increased concentration of $O_2^{\bullet -}$, measured within 24 hrs of DQ *i.s.* administration was in accordance with DQ redox potential ($E_o' = -349$ mV for DQ) (Miranda *et al*., 2000), Karma *et al.* (2011) showed that DQ was 10 – 40 times more effective at generating ROS than PQ in redox cycling assays with human recombinant NADPH-cytochrome P450 reductase and about 10 times more potent at generating H_2O_2 in the rat liver microsomes (McCormack *et al*., 2005). Enhanced O_2 utilization during redox metabolism of DQ contributes to citotoxicity and leads to anoxia of the affected tissue. It is known that lethargy is caused by anoxia, what is evident in the case of DQ poisonings (Fitsanakis *et al*., 2002). Thus, anoxia is an additional contributing factor to DQ neurotoxicity. Over time the content of $O_2^{\bullet -}$ decreased, possibly due to the reaction with NO_x. We assumed that increased NO_3^- was at the expense of possibly induced NOS expression by DQ (Fukushima *et al*., 1994). Peroxynitrite anion ($ONOO^-$) is the product of spontaneous reaction between $O_2^{\bullet -}$ and acids, nitration of tyrosine and oxidation of thiols and lipids, thus contributing to oxidative cell damage (Muzaffar *et al*., 2005). Additionally, $ONOO^-$ is the source of hydroxyl radicals ($OH^{\bullet}$), which are a potent trigger of LPO, thus inducing cell damage (Paxinos and Watson, 1986).

Enhanced MDA in the bilateral cortex during 24 hrs from the *i.s.* DQ application is in agreement with literature data (Paxinos and Watson, 1986). Our results provided the evidence that RNS are more potent inducers of LPO, compared to ROS for ~ 10^2 times. More specifically, concentrations of NO_3^- in the range of 10-60 nmol/mg protein *vs.* $O_2^{\bullet -}$ in the range of 1-6 μmol/mg protein (ratio: red NBT/ $O_2^{\bullet -}$ is 1/1) resulted in an almost same level of LPO (Graphs 1-3).

Lipid peroxidation results in a progressive loss of membrane fluidity, reduces membrane potential and increases the permeability to ions such as Ca_2^+ (Rubbo and Donnell, 2005). Similarly to PQ, it is possible that DQ might also stimulate glutamate efflux initiating excitotoxicity mediated by RNS (through stimulation of the N-methyl-*d*-aspartate (NMDA) receptors followed by Ca^{2+} cell influx and subsequent induction of NOS) (Ebadi and Sharma, 2003; Rubbo and Donnell, 2005; Shimizu *et al*., 2003; Shimizu *et al*., 2003). Nitric oxide is supposed to be involved in the pathophysiology of neurological disorders due to hypoxia/ anoxia in the brain (in this particular case, anoxia is a consequence of DQ redox metabolism) due to increased release of glutamate and activation of N-methyl-D-aspartate receptors (Fitsanakis *et al*., 2002). Elevated production of NO_x, along with LPO occurrence, coexists in all brain regions tested in anoxia compared to the control, which is in agreement with our results (Fitsanakis *et al*., 2002). L-NAME is able to suppress excitotoxicity (Shimizu *et al*., 2003).

Oxidative stress was markedly decreased after 7 days in the DQ group, based on a statistically low concentration of $O_2^{\bullet -}$ and LPO was in the range of control values. Having in mind that SOD activity did not differ from the controls (during the whole experiment), descending concentrations of $O_2^{\bullet -}$ could be on the account of $ONOO^-$ formation. Additionally, since the formation of $O_2^{\bullet -}$ depends on O_2, we assumed that the decline of $O_2^{\bullet -}$ during the experiment in the DQ group could be attributed to enormous depletion of O_2 during DQ red-ox metabolism. Thus, in addition to the reaction of $ONOO^-$ formation, anoxia is equally or even more responsible for $O_2^{\bullet -}$ descending course over time. Antioxidant defence of nervous tissue become exhausted and weakened due to several possibilities: a) deprivation of O_2 and GSH; b) peroxynitrite ($ONOO^-$) formation and consequent nitrosylation of proteins; and c) prooxidative effect of thiyl radical (Djukic *et al*., 2007; Cadens, 1997; Giustarini *et al*., 2004; Ebadi and Sharma, 2003; Lestaevel *et al*., 2003). $ONOO^-$ effectively inhibits enzymes in the mitochondrial respiratory chain followed by reduced adenosine-triphosphate (ATP) synthesis (Djukic *et al.*, 2007; Ebadi and Sharma, 2003; Lestaevel *et al*., 2003). Upon NMDA receptor stimulation, glutamate release correlates with $NO^\bullet$ production. Also, extracellular $NO^\bullet$ movement may provide signalling from activated NMDA receptors into surrounding synaptic terminals of the cerebral cortex (Montague *et al*., 1994).

Moreover, it has been reported that $ONOO^-$ both activates and inhibits COX-1 and COX-2 activities, depending on concentration. Both COX-1 and COX-2 convert arachidonic acid to prostaglandin, resulting in pain and inflammation (Suntres *et al*., 2002). It might be possible that observed inflammatory effect could coexist with OS/NS response to DQ *is.* administration.

In rats pre-treated with GR (the GR+DQ group), the animals did not exhibit signs of lethargy and there was no lethality. Furthermore, LPO did not develop, although, redox imbalance was documented with significantly high $O_2^{\bullet -}$ within 24 hrs, while increased NO_3^- was measured only in contralateral cortex at 30th min and statistically differed from the DQ group, at 24th hrs. Evidently, glutathione recycling affected RNS metabolic turnover in VBRs and resulted in reduced concentration of NO_3^-. It also, might be possible that lower amount of NO_3^- was due to S-nitrosation of glutathione by nitric oxide (Schrammel *et al*., 2003).

Lack of LPO could be attributed to lack of H_2O_2 production due to descending SOD activity, until total inhibition, measured at 7th days. Consequently, dismutation of $O_2^{\bullet -}$ did not adequately occur a significant increas of $O_2^{\bullet -}$ was measured within 24 hrs). It is well known that homolytic cleavage of H_2O_2 results in the production of hidroxyl radical ($HO^{\bullet}$), the most potent reactive oxygen species, that spontaneously initiates LPO. Although, the content of NO_3^- was higher compared to the controls (at 30 mins and 7 days), it was statistically lower than in the DQ group (at 24 hrs content of NO_3^- was in the range of the control values).

Statistically different results for NO_3^- content at 24 hrs between the DQ and the GR+DQ groups and lower values of NO_3^- in the GR+Q group (except increased values in contralateral cortex at 30 mins, nitrates were in the range of the controls) might be explained by the formation of GS-NO mediated by GSH-S-transferase (Douglas, 1987; Leaver and George, 1998; Giustarini *et al*., 2004; Jaffrey *et al*., 2001; Schrammel *et al*., 2003). Berendji *et al*. (1999) reported that NO-donors could decrease intracellular glutathione (GSH) levels in lymphocytes by as much as 75%.

Guided by the fact that GR does not pass across the cell membrane and/or the blood-brain barrier and further, we applied *i.s.* GR, thus subsequent conversion of GSSG into the GSH occurred extracellularlly/interstitially.

The transport of GSH and/or its precursors or breakdown products across the membranes and/or the blood-brain barrier is still a subject of controversy (Wade and Brady, 1981; Ennis *et al*., 1998; Kannan *et al*., 1990; Kannan *et al*., 1992; Zlokovic *et al*., 1994; Favilli *et al*., 1997). It is still not clear whether GSH is transported intact or whether GSH uptake depends on the breakdown of GSH initiated by gGT on the luminal side of the brain capillaries or if the sodium-dependent GSH transporter contributes to the delivery of GSH from the blood into the brain, hence GSH homeostasis of the brain remains to be elucidated (Jain *et al*., 1991; Meister, 1991; Kannan *et al*., 1996; Kannan *et al*., 1999).

Thiyl radical ($RS^{\bullet}$) is produced during GSH metabolic turnover. It is known that $RS^{\bullet}$ enhances LPO. As a strong oxidant ($E^{o\prime}_{RS\bullet/RSH} = +0.9$ V), $RS^{\bullet}$ easily triggers the chain of lipid radical reactions, starting with abstraction of hydrogen (rate of reaction is $\sim 10^7$ $M^{-1}s^{-1}$) from the methylene group of poli-unsaturated fatty acids (PUFA) and/or with addition to double bonds of PUFA (rate of reaction is $\sim 10^8$ $M^{-1}s^{-1}$). Further on, the formed alkyl radical ($R^{\bullet}$) may react with O_2 to form corresponding lipid peroxyl radicals ($ROO^{\bullet}$) (Schafer and Buettner, 2001). Obviously, depleted GSH, thus, consequently reduced antioxidative defence along with massive O_2 deprivation (during extreme redox metabolism of DQ) are

the crucial factors of DQ neurotoxicity (Boonplueang *et al*., 2005). Altogether, discussed mechanistic pathways might coexist in response to the DQ neurotoxic effect.

CONCLUSION

In conclusion, oxidative and nitrosative stress, with an emphasis on LPO, GSH – mediated antioxidative response and oxygen deprivation (causing anoxia) are probably the most important and critical pathophysiological pathways of DQ *i.s.* induced neurotoxicity. Glutathione-based redox regulation and signalling was proved to be the most important antioxidative defence pathway against DQ harmful effects. Cytotoxic effect of DQ could be attributed to oxygen deprivation and GSH depletion. The absence of lethargy and survival in the group pre-treated with GR, altogether indicate a neuroprotective role of *i.s.* administered GR.

The results of the present study confirmed the following: (i) *is.* route of administration of tested compounds does not provoke brain injury, concluding from biochemical analysis of OS/NS parameters and our observation (no significant differences of OS/NS parameters between the intact and the sham–operated group was seen; neither animals were dying, nor any clinical signs of brain tissues' injury was seen in the sham operated group); (ii) no evidence of GR damaging effect was observed; (iii) DQ neurotoxicity is mediated by LPO, increased production of ROS, and RNS, in particular; (iv) depletion of GSH at the expense of its oxidation to GSSG is responsible for reduced antioxidative damage of VBRs after i.s. administration of DQ; (v) the pre-treatment of *i.s.* injected GR accomplished a protective role against harmful *i.s.* DQ poisoning probably due to extracellular/interstitial glutathione recycling.

Our study confirms that extracellularly recycled GSSG by GR, *i.s.* administered, contributes to increased reuptake of GSH (tripeptide: g-L-glutamyl-L-cysteinylglycine) or its precursors or breakdown constituents (structure constituents: amino-acids in single or di-peptide form) into the cells of CNS, thus achieving the protective role.

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Address for correspodence:
Mirjana Djukic, PhD, Associate Professor
Department of Toxicology
Faculty of Pharmacy, University of Belgrade
11221 Belgrade, Serbia
E-mail: mirjana.djukic@pharmacy.bg.ac.rs

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UTICAJ GLUTATION REDUKTAZE NA NEUROTOKSIČNOST DIKVATA: ISPITIVAN JE OKSIDATIVNI/NITROZATIVNI STRES U KORTEKSU INTRASTRIJATALNO TRETIRANIH PACOVA

ĐUKIĆ MIRJANA, JOVANOVIĆ MARINA, NINKOVIĆ MILICA, STEVANOVIĆ IVANA, ĐURĐEVIĆ D i VASIĆ UNA

SADRŽAJ

U ovoj studiji smo ispitali da li je oksidativni/nitrosativni stres (OS/NS), uključen u odgovor korteksa Wistar pacova nakon intrastrijatalne (*i.s.*) izloženosti dikvatu (DK). Posebno smo ispitivali značaj antioksidativne uloge glutationa (GSH), zbog čega smo primenili glutation reduktazu (GR) u predtretmanu davanja DK. Superoksid anjon radikal ($O_2^{\bullet -}$), nitrati (NO_3^-), malondialdehid (MDA) i superoksid dismutaza (SOD), su mereni u obostranom korteksu (ipsi- i kontrastrana), nakon 30 minuta, 24 sati i 7 dana od tretmana.

Redoks balans se nije značajno promenio u korteksu lažno operisanih i netretiranih pacova. Takođe, ne postoji statistički značajna razlika između ipsi- i kontra- strane korteksa. Letargija i mortalitet (30-40%) kod životinja u DK grupi su uočene tokom 24 časa od i.s. trovanja DK, što se poklopilo sa naglim razvojem OS/NS i lipidne peroksidacije. Visok redoks potencijal DK verovatno rezultira opsežnim utroškom molekularnog kiseonika. Zaključeno je da je ostvaren neuroprotektivni učinak predtretmana sa GR, na osnovu preživljavanja životinja i odsustva letargije. Lipidna peroksidacija nije bila razvijena u grupi predtretiranoj sa GR ali je ipak izmerena visoka koncentracija $O_2^{\bullet -}$ (tokom 24 sata) koja zatim opada i na kraju 7. dana u potpunosti inhibira aktivnost SOD.