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ORIGINAL ARTICLE

# Intrastriatal pre-treatment with L-NAME protects rats from diquat neurotoxcity

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## Abstract

**Introduction:** Contact herbicide diquat (DQ), redox cycling compound, mediates its systemic toxicity throughout the enlarged production of free radicals. Target organs are liver and kidney in humans. To-date, the mechanism of DQ-induced neurotoxicity has not been rationalized.

**Objective:** The objectives of the study were to examine the ability of DQ to induce oxidative stress (OS) and/or nitrosative stress (NS) upon intrastriatal (*i.s.*) administration and to investigate the role of nitric oxide ($NO_x$) using NG-nitro-L-arginine methyl ester (L-NAME), a non-selective inhibitor of nitric oxide synthase (NOS) in the pretreatment of DQ *i.s.* administration.

**Material and Methods:** The experiment was conducted on Wistar rats, randomly divided in experimental groups, receiving different treatments *i.s.* applied. Parameters of OS/NS such as: superoxide anion radical ($O_2^{\bullet-}$), superoxide dismutase (SOD), malondialdehyde (MDA) and nitrates ($NO_3^-$) were measured in the cortex (bilaterally), at 30$^{th}$ min, 24 hours and 7 days after the treatments.

**Results:** Lethargy and high mortality rate were observed only in the DQ group (within 24 hours and 2-3 hours, respectively) after awakening from anesthesia. Markedly increased production of $NO_x$ and $O_2^{\bullet-}$ along with elevated lipid peroxidation altogether contributed to DQ neurotoxicity. The most importantly, the L-NAME *i.s.* pretreatment protected treated animals from dying and diminished OS/NS response against DQ-induced neurotoxicity.

**Conclusion:** The *i.s.* pretreatment with L-NAME resulted in neuroprotection against DQ neurotoxity, based on animal survival and reduced LPO in the cortex.

## Abbreviations

DQ – diquat; *i.s.* – intrastriatally; *i.p.* – intraperitoneally; L-NAME – NG-nitro-L-arginine methyl ester; LPO – lipid peroxidation; $O_2$ – molecular oxygen; MDA – malondialdehyde; NADPH – nicotinamide adenine dinucleotide phosphate; NMDA – N-methyl-*d*-aspartate; $NO_x$ – nitrogen oxide; $NO_3^-$ – nitrate; NS – nitrosative stress; $O_2^{\bullet-}$ – superoxide anion radical; OS – oxidative stress; PQ – paraquat; SOD – superoxide dismutase; RNS – reactive nitrogen species; ROS – reactive oxygen species; VBRs – vulnerable brain regions

## Key words

diquat, oxidative stress, nitric oxide, L-NAME, *Wistar* rats, brain

## INTRODUCTION

Diquat (DQ), 1,1'-ethylene-2,2'-bipyridylium (Fig. 1), is a contact herbicide, aquatic weed control agent, seed desiccant and sugarcane flowering suppressant agent. Occupational exposure to DQ, mainly of agriculture workers, does not pose a health risk if properly handled/used. Suicidal or accidental ingestion of decanted DQ is much less common than poisoning with the more widely-used herbicide paraquat (PQ), its bipyridylium analogue.

As a redox cycling compound, DQ exerts its toxicity through free radical chain reactions. During its metabolism, the formation of superoxide anion radical ($O_2^{\bullet-}$) occurs in the presence of molecular oxygen ($O_2$), similar to PQ [1, 2, 3]. The limiting factor for this reaction is nicotinamide adenine dinucleotide phosphate (NADPH), the major source of reducing equivalents in cells [4, 5]. Free radical mediated systemic toxicity of DQ poisoning is addressed to its nephro- and hepato-toxicity in humans [6, 7, 8]. The mechanisms of DQ neurotoxicity are still unknown. Reactive oxygen species (ROS), as well as, reactive nitrogen species (RNS) have been implicated in pathophysiology of many neurological disorders [9]. Vulnerable brain regions (VBRs) (cortex, striatum and hippocampus) are susceptible to oxidative/nitrosative injury because of their specific anatomy and functionality in the central nervous system [10]. In the presented study, the cortex was used to examine DQ neurotixic effects on ROS/RNS production.

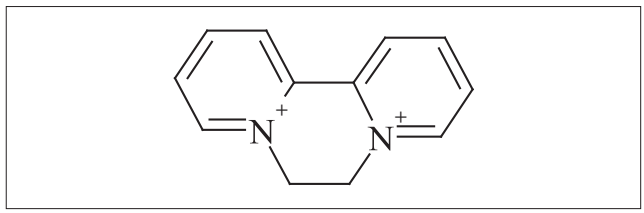

**Figure 1.** Diquat

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Peroxynitrite anion ($ONOO^-$) is recognized as a very harmful RNS that reacts with all classes of bio-molecules (including hydroxylation of aromatic amino acids, nitration of tyrosine and oxidation of thiols and lipids), contributing to oxidative cell damage [11, 12]. Among others, $ONOO^-$ is the source of the most powerful ROS, hydroxyl radical ($OH^-$), which is one of the most potent ROS to induce cell damage. It has been suggested that $ONOO^-$ formation is a primary pathway of $NO_x$ metabolism, based on the high rate of reaction between $NO^-$ and $O_2^{\bullet-}$. Accordingly, suppression/ inhibition of $NO_x$ synthesis by NG-nitro-L-arginine methyl ester (L-NAME) might act protectively [13]. L-NAME, a competitive non-specific inhibitor of nitrogen oxide synthase (NOS), has been found to restrain the development of nitrosative stress (NS) in neuronal cells by reducing nitrogen oxide ($NO_x$) synthesis [13]. Also, the application of L-NAME makes it possible to discriminate outcomes of brain response to direct harmful exposure to DQ between ROS and reactive nitrogen species (RN). Nitrates ($NO_3^-$) are the major final metabolic product of RNS and thus can be used as a marker of $NO_x$ production [1, 14, 15].

In order to reveal the role of $NO_x$ in overall oxidative/ nitrosative stress (OS/NS) response to DQ neurotoxicity, L-NAME was applied intrastriatally (*i.s.*) in the pre-treatment of DQ *i.s.* administration. Based on the examined parameters of OS/NS from the bilateral cortex of the treated animals and their survival, it was possible to evaluate whether the *i.s.* pre-treatment with L-NAME could achieve a protective effect against DQ neurotoxicity.

## MATERIAL AND METHOD

**Animals.** Adult male Wistar rats (~ 220g) were housed in separate cages with free access to food and water. For adaptation purposes, the rats were kept for 7 days at room temperature prior to the experiment in a light/dark cycle during 13/11 hours. Treatment and care of animals were provided ethically according to the Guidelines for Animal Study, No. 322-10/2010. The entire study was approved by the Ethics Committee of the Military Medical Academy in Belgrade, Republic of Serbia.

**Experimental design.** After adaptation, the rats were randomly assigned to 5 groups, as follows: 1) control-intact group (untreated animals, n=8); 2) sham operated group (n = 24) treated with 10 µL of saline ( 0.9% NaCl); 3) DQ group (n = 24) – animals poisoned with one single dose of DQ (2.5 µg/10 µL); 4) L-NAME + DQ group (n = 24) – animals pre-treated with one single dose of L-NAME (10 µg/10 µL) 30 min. before DQ *i.s.* administration; 5) L-NAME group (n = 24) – animals treated with one single dose of L-NAME (10 µg/10 µL). Rats were intraperitoneally (*i.p.*) anesthetized with sodium pentobarbital (45 mg/kg body weight) before *i.s.* application of one single dose of the tested compounds.

The rats were sacrificed by decapitation at 30 min, 24 hours and 7 days after the treatments (8 animals at each time point) and the heads were immediately frozen in liquid nitrogen and stored at -70 °C until analysis. The tested substances were administered by a Hamilton syringe, 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 the dura) [16].

**Reagents.** All chemicals were of analytical grade. The following compounds were used in this study: Diquat – Reglone® (200 g/L) (Zeneca – Agrochemicals); 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 3.6M $(NH_4)_2SO_4$, at pH 7.0; 2500 U/1.6 mL (9.2 mg prot/mL – biuret) 170 U/mg proteins. (Note: 1 unit reduces 1µmol 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); sodium gluconate, ethylenediaminetetraacetic acid – EDTA (Sigma, St. Louis, MO, USA); sodium phosphate -$Na_2HPO_4$, potassium dihydrogen phosphate – $KH_2PO_4$, glycerol, methanol, acetonitrile, trichloroacetic acid, thiobarbituric acid (Merck, Darmstadt, Germany); sodium tetraborate and boric acid (Zorka, Sabac, Serbia); carbonate buffer (50 mM, pH 10.2), (Serva, Feinbiochemica, Heidelberg/New York). Deionized water was prepared by the Millipore milli-Q water purification system (Waters-Millipore, Milford, MA, USA).

**The tissue preparation.** Cortex homogenates were prepared from individual rats as described earlier [17]. The ipsi- and contra-lateral side of the cortex were removed from brain tissue and kept on ice during the whole procedure. Slices of the VBRs 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 homogenizer (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 deionized 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 used for analysis. Total protein concentration was estimated with the Lowry method, using bovine serum albumin as a standard [18].

**Measurements.** Parameters of OS/NS (superoxide anion radicals, superoxide dismutase, malondialdehyde and nitrates) were measured in bilateral cortex, at 30 min, 24 hours and 7 days after the treatments.

**Superoxide anion radical ($O_2^{\bullet-}$).** Content of $O_2^{\bullet-}$ was quantified by the method based on the equimolar reduction of nitrobluetetrazolium to monoformazan by $O_2^{\bullet-}$. The yellow colour of the reduced product was measured spectrophotometrically at 550 nm [19].

The results were expressed as µmol reduced NBT/ mg proteins which corresponds to µmol $O_2^{\bullet-}$/ mg proteins.

**Nitrate ($NO_3^-$).** Deproteinisation of brain homogenates was performed using acetonitrile (sample: acetonitrile, 2:1, v/v), then centrifuged and the supernatant filtered (0.45 µm) before the chromatographic analysis (ion-exchange HPLC). 50 µL of filtrate was injected into the HPLC system for $NO_3^-$ analysis [15]. A mobile phase consisting of borate buffer/ gluconate concentrate, methanol, acetonitrile and deionized

water in a ratio of 2:12:12:74 (v/v/v/v) (pH 8.5) was used for isocraticelution at a flow rate of 1.3 ml/min, at room temperature. Spectroscopic detection was performed at a single wavelength of 214 nm. The results were expressed as nmol $NO_3^-$/mg proteins.

**Malondialdehyde (MDA).** Malondialdehyde (MDA), the degradation product of lipid peroxidation, was determined spectrophotometrically (method of Ohkawa et al.) [20]. MDA gives a red coloured 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/mg proteins.

**Superoxide dismutase (SOD).** The activity of SOD was measured spectrophotometrically, as the inhibition of spontaneous autooxidation of epinephrine at 480nm. Kinetics of sample enzyme activity followed in a carbonate buffer (50 mM, pH 10.2, containing 0.1 mM EDTA), after the addition of 10 mM epinephrine [21]. The results were expressed as U SOD/mg proteins.

**Statistical analysis.** Data are expressed as average ± SD. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the Tukey *post-hoc* test. Differences were considered significant at $p<0.05$. Spearman's correlation analysis was used to establish the correlations between measured parameters. All statistical calculations were performed using programme Statistica version 7.0.

## RESULTS

Results for the ipsilateral cortex are presented graphically (Figures 2-5), whilst results for bilateral cortex are sumerrized in Table 1. Differences between the intact and sham-operated groups were not found to be significant (data not shown); therefore, the sham-operated group was used as the control group for further comparisons. A similar trend of changes (without statistical differences) of examined parameters were obtained for contra- and ipsi- lateral-cortex, for the same time points and the equal treatments, except for the DQ group at 24$^{th}$ hours for $NO_3^-$ (Tab. 1). The mortality rate of rats was observed only in the DQ group (30-40 %) within 2-3 h after awakening from the anesthesia. Lethargy was the only neurological symptom observed in the DQ group. In the L-NAME and the L-NAME+DQ groups, all the rats survived. Attention has been focused on the significant results of the OS/NS parameters in this section.

**Superoxide anion radical ($O_2^{\cdot-}$).** Opposite to the significantly low amount of $O_2^{\cdot-}$ in the DQ group, a markedly high concentration of $O_2^{\cdot-}$ was achieved in the L-NAME+DQ group in the bilateral cortex, 7 days after the treatments.

**Nitrate ($NO_3^-$).** The $NO_3^-$ level was significantly higher in the ipsilateral cortex of the DQ group, contrary to the L-NAME group (significantly lower), compared to the sham-operated group, during whole experiment. Within 24 h, in the L-NAME+DQ group, the $NO_3^-$ content was found to be significantly reduced compared, to the sham-operated group. At 24 h, the content of $NO_3^-$ was statistically significant different between contra- and ipsi- lateral cortex in the DQ group.

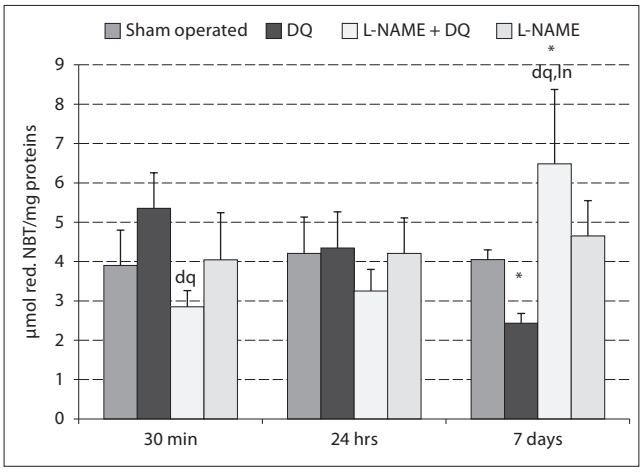


**Figure 2.** Superoxide anion radical

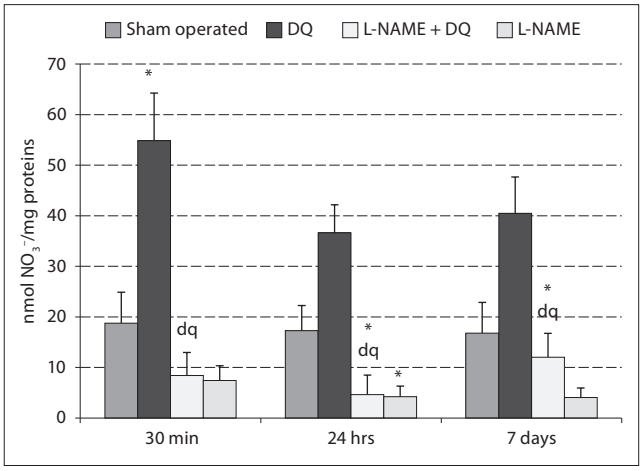


**Figure 3.** Nitrates

**Superoxide dismutase activity (SOD).** Statistically reduced SOD activity was measured in the bilateral cortex in the L-NAME+DQ group, at 24 h and after 7 days; and in the L-NAME group, during the whole experiment, compared to the control group (sham- operated).

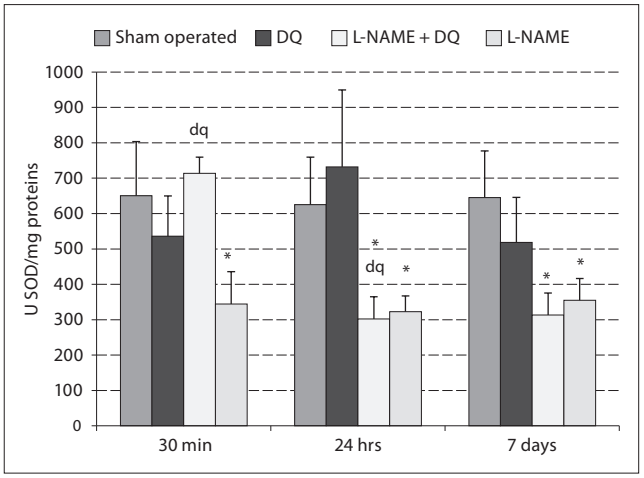


**Figure 4.** SOD

**Table 1.** Parameters of oxidative/nitrosative stress [superoxide anion radical ($O_2^{\bullet-}$), superoxide dismutase (SOD), malondialdehyde (MDA) and nitrate ($NO3^-$)] in the ipsi- and contra- lateral cortex, 30 min, 24 hours and 7 days after the treatments (DQ, L-NAME and L-NAME + DQ)

| *Parameters of oxidative/nitrosative stress* | Cortex | Time | Experimental Groups (n = 8 rats / time point) | | | |
|---|---|---|---|---|---|---|
| | | | Sham operated | DQ | L-NAME+DQ | L-NAME |
| ***Superoxide anion radical*** µmol red. NBT/mg proteins | **ipsilateral** | **30 min** | **3.90 ± 0.90** | **5.35 ± 0.69** | **2.85 ± 0.41 $^{dq}$** | **4.04 ± 1.20** |
| | | **24 hrs** | **4.21 ± 0.92** | **4.34 ± 1.18** | **3.25 ± 0.55** | **4.21 ± 0.90** |
| | | **7 days** | **4.05 ± 0.25** | **2.43 ± 0.27 $^{*}$** | **6.48 ± 1.89 $^{*,dq,ln}$** | **4.65 ± 0.90** |
| | *contralateral* | *30 min* | *4.02 ± 0.54* | *5.06 ± 1.19* | *2.79 ± 0.26 $^{dq}$* | *3.22 ± 1.29* |
| | | *24 hrs* | *3.91 ± 0.91* | *4.84 ± 0.79* | *3.03 ± 1.26 $^{dq}$* | *3.41 ± 1.14* |
| | | *7 day* | *3.54 ± 0.83* | *2.52 ± 0.70* | *6.23 ± 1.46 $^{*,dq}$* | *3.54 ± 1.20* |
| ***Superoxide dismutase*** U/mg proteins | **ipsilateral** | **30 min** | **650.45 ± 152.99** | **536.22 ± 113.38** | **713.95 ± 45.32 $^{dq}$** | **344.27 ± 91.80 $^{*}$** |
| | | **24 hrs** | **625.06 ± 134.50** | **732.21 ± 217.28** | **302.00 ± 62.91 $^{*,dq}$** | **322.43 ± 44.90 $^{*}$** |
| | | **7 day** | **645.00 ± 132.20** | **518.62 ± 127.22** | **313.32 ± 61.87 $^{*}$** | **355.04 ± 61.30 $^{*}$** |
| | *contralateral* | *30 min* | *660.88 ± 202.99* | *553.55 ± 133.55 $^{*}$* | *618.66 ± 201.24 $^{*,ln}$* | *319.61 ± 67.94* |
| | | *24 hrs* | *655.06 ± 184.5* | *631.15 ± 170.48 $^{*}$* | *309.87 ± 75.34 $^{dq}$* | *308.59 ± 36.81* |
| | | *7 day* | *645.00 ± 198.2* | *527.97 ± 450.76* | *388.45 ± 151.81* | *318.40 ± 43.71* |
| ***Lipid peroxidation*** pmol MDA/mg proteins | **ipsilateral** | **30 min** | **54.02 ± 14.80** | **92.75 ± 12.72 $^{*}$** | **97.31 ± 11.67 $^{*,ln}$** | **58.05 ± 10.80** |
| | | **24 hrs** | **56.00 ± 10.66** | **100.80 ± 14.85** | **55.92 ± 4.92 $^{*,dq}$** | **56.54 ± 10.70** |
| | | **7 day** | **55.76 ± 8.94** | **53.05 ± 10.20** | **58.11 ± 8.79** | **59.84 ± 14.80** |
| | *contralateral* | *30 min* | *59.00 ± 12.66* | *94.07 ± 27.02* | *88.22 ± 12.88 $^{ln}$* | *50.05 ± 15.81 $^{*}$* |
| | | *24 hrs* | *68.02 ± 14.80* | *99.92 ± 26.95* | *56.65 ± 11.31 $^{*,dq}$* | *48.54 ± 17.7 $^{*}$* |
| | | *7 day* | *63.76 ± 8.94* | *50.33 ± 24.85* | *60.58 ± 13.42 $^{*}$* | *49.84 ± 11.81 $^{*}$* |
| ***Nitrates*** nmol/mg proteins | **ipsilateral** | **30 min** | **18.78 ± 6.09** | **54.85 ± 9.42 $^{*}$** | **8.42 ± 4.56 $^{dq}$** | **7.42 ± 2.90** |
| | | **24 hrs** | **17.30 ± 4.97** | **36.61 ± 5.57** | **4.62 ± 3.88 $^{*,dq}$** | **4.22 ± 2.10 $^{*}$** |
| | | **7 day** | **16.78 ± 6.09** | **40.50 ± 7.19** | **12.04 ± 4.72 $^{*,dq}$** | **4.04 ± 1.90** |
| | *contralateral* | *30 min* | *16.04 ± 2.73* | *50.09 ± 13.83 $^{*}$* | *12.11 ± 4.67 $^{dq}$* | *12.54 ± 6.53* |
| | | *24 hrs* | *15.92 ± 3.77* | *59.69 ± 13.91 $^{*,ipsi}$* | *4.61 ± 2.76 $^{dq}$* | *9.03 ± 1.62* |
| | | *7 day* | *14.29 ± 3.66* | *45.46 ± 18.39 $^{*}$* | *4.24 ± 2.11 $^{dq}$* | *4.15 ± 0.94* |

* – compared to control group; dq – compared to DQ group; ln - compared to L-NAME group, ipsi – compared to ipsi lateral side of the cortex.

**Malondialdehyde (MDA).** Lipid peroxidation was significantly elevated in the bilateral cortex during 24 h in the DQ group, and at 30 min of the treatment in the L-NAME+DQ group, compared to the sham-operated group.

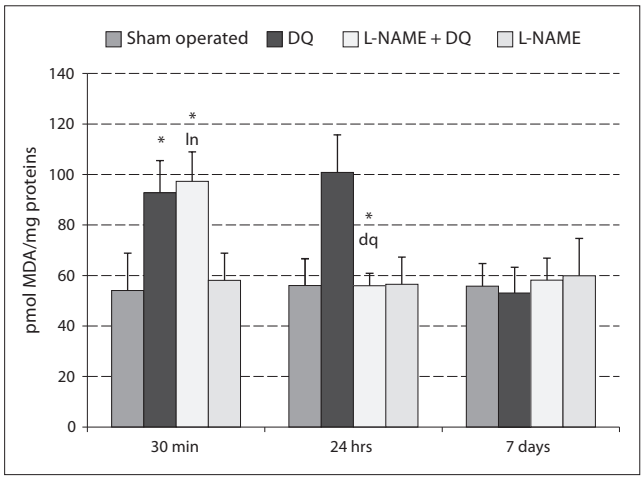


**Figure 5.** Lipid peroxidation

**Table 2.** Spearman's correlation coefficients between parameters of oxidative/nitrosative stress ($O_2^{\bullet-}$. $NO_3^-$. SOD and MDA) for each time point within the experiment

| | $O_2^{\bullet-}$ | MDA | SOD |
|---|---|---|---|
| **MDA** | r = 0.482<br>p = 0.0000* | | |
| **SOD** | r = 0.219<br>p = 0.0084* | r = 0.681<br>p = 0.0000* | |
| **$NO_3^-$** | r = 0. 207<br>p = 0.0128* | r = 0.486<br>p = 0.0000* | r = 0.665<br>p = 0.0000* |

* statistical significance was considered at p<0.05.

**Correlation between $NO_3^-$ and MDA; and $O_2^{\bullet-}$ and MDA.** A positive correlation was achieved between examined parameters of OS/NS at all time points (Tab. 2).

## DISCUSSION

The results of the presented study have shown that *i.s.* administered DQ induced oxidative/nitrosative damage in the bilateral cortex of Wistar rats, assessed by the increased production of $O_2^{\bullet-}$ and $NO_x$, together with elevated LPO (i.e. MDA) in an early phase of the treatment. Spatial and instant propagation of OS/NS induced by DQ was confirmed by almost identical values of measured parameters of OS/NS for both sides of the cortex (Tab. 1). No significant difference between measured parameters of OS/NS was found between the sham-operated and control-intact groups in the cortex, underlining that mechanical injury due to the *i.s.* route of administration of the tested compounds (by Hamilton syringe) itself, did not provoke OS/NS.

The obtained increase of $O_2^{\bullet-}$ in the DQ group is in accordance with the literature, supporting the evidence that DQ redox cycling metabolism leads to enhanced ROS production and $O_2$ consumption. The one-electron reduction of DQ (DQ is in the form of $DQ^{2+}$) by cytochrome P450 reductase generates a bipyridyl cation radical ($DQ^{\bullet+}$); further on, $DQ^{\bullet+}$ reacts with $O_2$ and becomes oxidized back to its parent form ($DQ^{2+}$), while $O_2$ becomes reduced to $O_2^{\bullet-}$ [22]. Compared to PQ, DQ is a more powerful oxidant based on its redox potentials ($E_o$' = -349 mV for DQ and $E_o$' = -446 mV for PQ) [23]. Fussell Karma C. et al., showed that DQ was 10-40 times more effective at generating ROS than PQ in redox cycling (assessment performed with human recombinant NADPH-cytochrome P450 reductase) and about 10 times more potent at generating $H_2O_2$ (using rat liver microsomes)

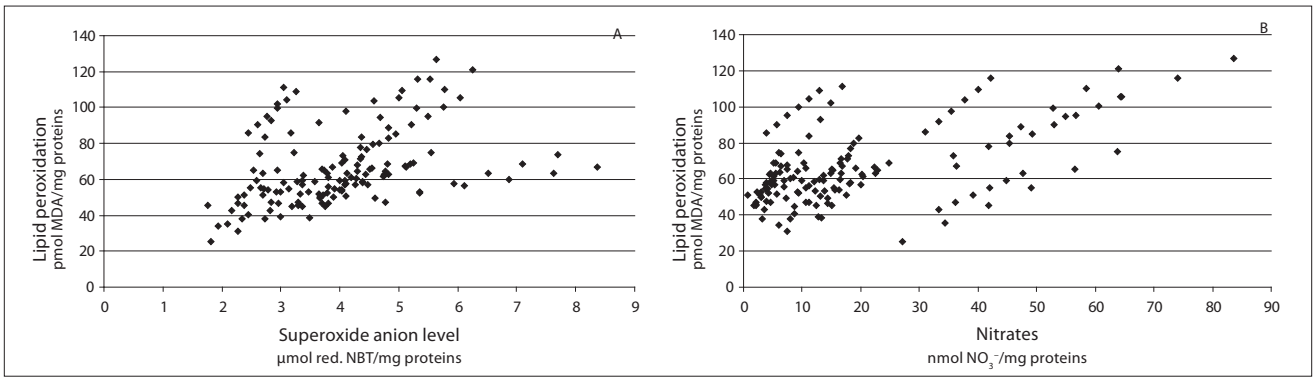


**Figure 6.** Speraman's correlation

[22]. It appears that the redox cycling metabolism of DQ starves tissues of $O_2$ (including brain tissue, based also on own experiment). These data demonstrate that ROS formation during DQ redox cycling can generate OS. Enhanced $O_2$ utilization during redox cycling reduces intracellular $O_2$ and contributes to citotoxicity. Extreme $O_2$ depletion attained by DQ leads to anoxia of an affected tissue. It is known that lethargy (in the presented study it was observed only in the DQ group) is caused by anoxia [9]. The huge consumption of $O_2$ by DQ redox metabolism is an additional contributing factor to DQ neurotoxicity.

The high mortality ratio (30-40%) was observed within 2-3 h after awakening from the anesthesia in the DQ group. Our previous study on PQ neurotoxicty has shown that one single *i.s.* application of PQ (50 mg/kg) resulted in Parkinson's disease like symptoms and was less harmful in terms of mortality, compared to DQ [1, 24]. Thus, oxidative brain injury, documented with markedly elevated production of ROS/RNS and LPO in an initial phase of DQ *i.s.* intoxication (within 24 hours), was probably a consequence of more intense redox turnover of DQ at the time, contributing to lethal a outcome for the treated animals. Lethargy and the high mortality rate in the DQ group emphasizes the susceptibility of cortex to oxidative damage [8].

A marked increase in the production of $NO_3^-$ immediately after *i.s.* DQ application suggests the possibility of NOS expression by DQ [25]. Once NOS are expressed, the formation of large amounts of $NO_x$ may account for the NS-mediated cellular response to the harmful effect of DQ in the bilateral cortex, one of the most vulnerable brain regions for OS [10]. Increased $NO_x$ synthesis indirectly favours the formation of $OH^-$ throughout increased $ONOO^-$ production and its possible homolytic cleavage, thus initiating the process of oxidative cell damage, including LPO and formation of protein adducts, etc. [16, 26]. Enhanced LPO (expressed as MDA content) by DQ *i.s.* injection in the bilateral cortex during the 24 h of the application is in agreement with literature [22, 26]. Depletion of $O_2$ at the expense of an increased production of $O_2^{\bullet-}$ was noticed in the DQ group, especially within the first 24 h. In general, the production of $O_2^{\bullet-}$ dictates the enhanced activity of SOD, thus producing $H_2O_2$, which further triggers chain reactions of free radicals, leading to OS development. Lipid peroxidation results in a progressive loss of membrane fluidity, reduces membrane potential and increases the permeability to ions such as $Ca_2^+$ [27].

Over time, the content of $O_2^{\bullet-}$ decreased in the DQ group possibly due to a lower metabolic rate of DQ, and additionally due to the depletion of $O_2^{\bullet-}$ by $NO_x$ (nitrates were markedly higher in the DQ-group, compared to the others) in favour of the spontaneous reaction of $ONOO^-$ production. Swamy M. et al. showed that elevated production of $NO_x$, coexists with LPO in all brain regions tested in anoxia compared to control, which is in agreement with the results of the presented study [9].

The positive association between $NO_3^-$ (i.e. $NO_x$) and MDA and $O_2^{\bullet-}$ and MDA, observed at all time points and within all treated groups, provide the evidence that both RNS and ROS contribute to DQ-induced lipid peroxidation, emphasizing RNS as a more potent inducer of LPO (Tab. 1,2). Based on a considerably lower range of $NO_3^-$ concentrations (10-60 nmol $NO_3^-$/mg prot) compared to $O_2^{\bullet-}$ (1-6 μmol red. NBT/mg prot, ratio: red NBT/ $O_2^{\bullet-}$ is 1/1) that resulted at almost the same level of LPO, it appears that $NO_x$ is a more potent inducer of LPO for ~ $10^2$ times than $O_2^{\bullet-}$ (Tab. 1, Fig. 2,3).

Similar 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) [7, 27, 28, 29]. 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) because of increased release of glutamate and activation of NMDA receptors [9]. Shimizu et al demonstrated that L-NAME is able to suppress excitotoxicity [29]. Moreover, it has been reported that $ONOO^-$ both activates and inhibits the 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 [30].

Pretreatment with L-NAME provided the possibility to distinguish the effects of ROS from RNS in affected VBRs [1]. The consequences of NOS inhibition by L-NAME could be attributed to the down-regulation of NADPH oxidase [12, 31]. Concentrations of $NO_3^-$ in the L-NAME + DQ and the L-NAME group were considerably lowered (almost 10 times), compared to the DQ group (Tab. 1, Fig. 3). Thus, the presented study supports the notion that RNS are perhaps a crucial contributor to DQ-mediated neurotoxicity. The survival of the animals, together with the absence of lethargy in the group pretreated with L-NAME, is the crucial indicator of its neuroprotective role. Obtained results of biochemical analysis of OS/NS parameters demonstrated that RNS play a more devastating role than ROS in DQ neurotoxicity.

With respect to reduced $NO_x$ synthesis and undeveloped LPO, pre-treatment with L-NAME provides a protective

effect against DQ *i.s.* intoxication (Tab. 1, Fig. 5). The activity of SOD was elevated at 30 min of the treatment in the L-NAME+DQ group. The low amount of $NO_x$ was insufficient to sequestrate produced $O_2^{\bullet-}$; thus, more substrate ($O_2^{\bullet-}$) left to be processed by SOD. Increased LPO observed only within 30 min in the L-NAME+DQ group could be attributed to increased SOD activity, thus significantly producing $H_2O_2$. A positive correlation was found between LPO and SOD activity (Tab. 2). More importantly, no increased production of $ONOO^-$ occurred because NOS inhibition by L-NAME; therefore, no deleterious effect of $ONOO^-$ was accomplished. The obtained results are in accordance with the well-established knowledge that $H_2O_2$ and $ONOO^-$ are also potent reactive species for triggering LPO, partly due to their possibilities to produce $OH^{\bullet}$ [11, 16].

Based on the results in the group treated only with L-NAME, it was shown that L-NAME itself did not induce oxidative lipid injury (Tab. 1, Fig. 5). Additionally, no signs of lethargy or animals dying were noticed, which is in accordance with the study by A. Jelenkovic et al. [32].

The presented study shows for the first time that *i.s.* pre-treatment with L-NAME protects neuronal cells against DQ neurotoxicity.

## CONCLUSION

In conclusion, the results of the presented study confirm following:

1. The *i.s.* route for administering tested compounds does not provoke brain injury, as concluded by comparing the measured parameters of OS/NS response between the sham-operated group and the intact group.
2. DQ neurotoxicity is mediated by OS/NS, evidenced by the increase in production of ROS and RNS (in particular, nitrates, which were statistically higher only in the DQ group) and developed LPO.
3. The difference measured/observed outcomes between the group treated with DQ and the group pretreated with L-NAME indicates that RNS are more potent inducers of LPO. compared to ROS.
4. There is no evidence of L-NAME toxicity at the applied dose.
5. The L-NAME *i.s.* applied prior to DQ *i.s.* administration protects the cortex against DQ neurotoxicity, based on animals survival, absence of lethargy and obtained parameters of biochemical analysis of OS/NS.

### Ethics Committee Approval

The authors have complied with the institutional policies governing the humane and ethical treatment of the experimental subjects, and are willing to share the original data and materials, if so requested. The experimental animals were treated according to Guidelines for Animal Study, No. 322-10/2010 (Ethics Committee of the Military Medical Academy, Belgrade, Republic of Serbia).

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