ORIGINAL ARTICLES AAEM

Ann Agric Environ Med 2007, 14, 247-252

THE ROLE OF NITRIC OXIDE IN PARAQUAT-INDUCED OXIDATIVE STRESS IN RAT STRIATUM

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Djukic M, Curcic Jovanovic M, Ninkovic M, Vasiljevic I, Jovanovic M: The role of nitric oxide in paraquat-induced oxidative stress in rat striatum. *Ann Agric Environ Med* 2007, **14**, 247-252.

Abstract: The role of nitric oxide (NO) in paraquat (PQ)-induced neurotoxicity is still not fully understood. In this study we used N^G-nitro-L-arginine methyl ester (L-NAME), a non-selective nitric oxide synthase (NOS) inhibitor, in order to examine the effects of NO, reactive oxygen species (ROS) generation and lipid peroxidation (LPO) development during PQ-mediated neurotoxicity. Oxidative stress development in the striatum of Wistar rats intrastriatally (i.s.) poisoned with PQ (and in some cases pre-treated with L-NAME) was investigated by measuring superoxide anion ($O_2^{\bullet-}$), malondialdehyde (MDA) and nitrate (NO_3^-), 30 min, 24 hours and 7 days after treatment. L-NAME pre-treatment provided the possibility to distinguish the role of ROS from reactive nitrogen species (RNS) in oxidative stress development induced by PQ. Our results confirm the involvement of NO in PQ-mediated neurotoxicity and reduced LPO by L-NAME pre-treatment implying that the latter has a protective role.

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Key words: paraquat, oxidative stress, nitric oxide, L-NAME, Wistar rats, brain.

INTRODUCTION

Occupational or accidental exposure to high doses of pesticides can result in life-threatening poisoning [2]. Paraquat (1,1'-dimethyl-4,4'-bipyridinium) (PQ) is a fast-acting non-selective contact herbicide, and is extremely toxic to humans and animals by all routes of exposure [6].

As a redox-cycling compound, PQ exerts its toxicity through increased production of free radicals [28, 31, 33]. Target organs affected during PQ poisoning are the lung and the kidney. The neurotoxic effects of PQ are relatively unknown [3].

When in the divalent cationic form, PQ^{2+} (which is extremely electrophylic), it undergoes one electron reduction to a mono-cationic radical ($PQ^{\bullet+}$) by the cytosolic enzymes NADPH oxidase and nitric oxide (NO) synthase (NOS), and via the mitochondrial complex I [3, 6, 10]. The limiting factor for this reaction is the available amount of NADPH (the major source of electrons) [28, 31]. $PQ^{\bullet+}$ can react with molecular oxygen to form the superoxide anion radical ($O_2^{\bullet-}$) which contributes to oxidative cell damage [33].

There is substantial evidence indicating elevated oxidative stress during PQ poisoning including increased lipid peroxidation (LPO), diminished energy metabolism and decreased cytochrome oxidase activity [21]. Dopaminergic neurons may be preferentially targeted by PQ because of their significant vulnerability to reactive oxygen species (ROS)-mediated oxidative injury [3]. Compared to other neuronal cells, dopaminergic cells are much more sensitive to oxidative injury [6, 9, 18]. The cell bodies of dopaminergic neurons, located within the substantia nigra (SN), send projections that terminate and release dopamine in the striatum (two essential brain regions for maintaining normal motor function). The loss of SN neurons results in

Received: 6 December 2006
Accepted: 12 October 2007

reduced synthesis and release of dopamine from striatal nerve terminals [14].

In addition, stimulation of the N-methyl-*d*-aspartate (NMDA) form of glutamate receptors followed by Ca^{2+} cell influx and NMDA receptor-dependent production of NO by NOS seems to play a role in PQ neurotoxicity [6, 21, 26, 27]. PQ stimulates glutamate efflux initiating excitotoxicity mediated by reactive nitrogen species (RNS) [6, 26, 27].

N^G-nitro-L-arginine methyl ester (L-NAME), a competitive non-specific inhibitor of NOS, has been found to restrain the development of oxidative and nitrosative stress in neuronal cells by reducing NO synthesis [29]. Nitrate (NO_3^-) is the major final metabolic product of NO and other RNS [8, 25, 33].

In order to reveal the role of NO in PQ-mediated neurotoxicity, we tested a hypothesis that L-NAME pre-treatment could possibly distinguish the effects of ROS on oxidative stress from those of RNS. Furthermore, we sought to determine whether L-NAME exerted a protective effect.

EXPERIMENTAL

Animals. Adult male Wistar rats weighing approximately 220 g were used for the experiment. The rats 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 with a of light/dark cycle of 13/11 hours. Treatment and care of animals was humane according to the Guidelines for Animal Study number 282-12/2002. The whole study was approved by the Ethics Committee of the Military Medical Academy, Belgrade, Serbia.

Experimental design. After adaptation, the rats were randomly assigned to 5 groups. One group of 8 was used as the control (untreated animals) and sacrificed at time zero. The rats in the other groups were intraperitoneally anesthetised with sodium pentobarbital (45 mg/kg body weight) before intrastriatal (i.s.) administration of the following: control group (n = 8) treated with 10 μL of 0.9% NaCl solution; PQ group (n = 24) – animals were poisoned with PQ with one single dose (2.5 μg/10 μL); L-NAME + PQ group (n = 24) – animals were pre-treated with L-NAME with one single dose (10 μg/10 μL) 30 minutes before PQ administration and L-NAME group (n = 24) – animals were treated with L-NAME with one single dose (10 μg/10 μL). The rats in all the groups were sacrificed by decapitation at 30 min, 24 hours and 7 days (8 animals at each time point), heads were immediately frozen in liquid nitrogen and stored frozen at -70ºC until analysis. The administration of PQ, L-NAME and NaCl required the use of 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 4.8mm ventral from the dura) [24].

Reagents. All chemicals were of analytical grade or better. Paraquat - Galokson® (200 g/L) was purchased from Galenika (Zemun, Serbia); Sodium pentobarbital – Vetanarcol® (0.162 g/mL) was purchased from Werfft-Chemie (Vienna, Austria); NaCl solution (0.9% w/v) was provided by the Hospital Pharmacy (Military Medical Academy, Belgrade). L-NAME was purchased from Sigma (Munich, Germany) and $NaNO_3$ from Mallinckrodt Chemical Works (St. Louis, MO, USA). Sodium gluconate, EDTA, Na_2HPO_4, KH_2PO_4, glycerol, methanol and acetonitrile were purchased from Merck (Darmstadt, Germany). Sodium tetraborate and boric acid were purchased from Zorka (Sabac, Serbia). Deionised water was prepared by the Millipore milli-Q water purification system (Waters-Millipore, Milford, MA, USA).

The tissue preparation. Homogenates of rat striatum were prepared according to Guard *et al.* method [12]. In brief, the brain was removed before extraction of the striatum, ipsi- and contra- lateral side from incompletely defrosted brain tissue at all times kept on ice. Slices of striatum were transferred separately into cold buffered sucrose (0.25 mol/l sucrose containing 0.1 mmol/l EDTA in potassium-sodium phosphate buffer, pH=7). An aliquot (1 ml) was placed into a glass tube homogeniser (Tehnica Zelezniki Manufacturing, Slovenia) and homogenisation 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 recovered and dissolved in 1.5 ml of deionised water. Solubilisation of subcellular membranes in hypotonic solution was performed by constant mixing using a pasteur pipette for 1 h. Thereafter, homogenates were centrifuged at 2,000 × g for 15 min at 4°C and the resulting supernatant was stored at –70°C until analysis [12].

The concentration of total protein in the striatum homogenates was estimated according to the method of Lowry *et al.* (1951) with bovine serum albumin as a standard [19].

NO_3^- determination. Firstly, deproteinisation of brain homogenates was performed using acetonitrile (sample: acetonitrile, 2:1, v/v). After centrifugation, the supernatant was filtered through a membrane (0.45 μm) prior to chromatographic analysis (ion-exchange HPLC, as described by Curcic Jovanovic *et al.* [5]. In brief, a mobile phase [pH = 8.5 composed of borate buffer/gluconate concentrate, methanol, acetonitrile and de-ionised water in a ratio 2:12:12:74 (v/v/v/v)] 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.

MDA determination. MDA (an indicator of the level of LPO) was determined by following the method described by Villacara *et al.* 1989 [32]. The method is based on the

reaction between thiobarbituric acid (TBA) and MDA which produces a yellow colour that absorbs at 533 nm.

$O_2^{\bullet-}$ determination. Its presence was quantitated by following a 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. The results were expressed as nmol of reduced NBT/mg protein [1].

Statistical analysis. Data are expressed as means ± SD. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the Fishers LSD multiple range test when appropriate. Values of $p<0.05$ were considered significant. Spearman's correlation analysis was performed for the relationships between all the measured parameters. All statistical calculations were performed using Statistica version 5.0.

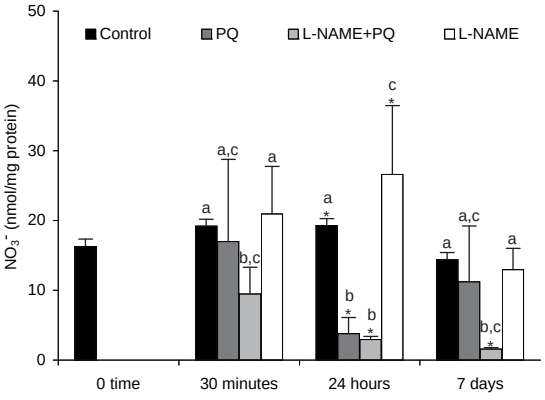

Figure 1. NO_3^- content in ipsilateral striatum of rats after single intrastriatal administration of PQ, L-NAME and L-NAME + PQ.
Values are means ± SD (n = 8). Means not sharing the same letter are significantly different ($p<0.05$, for the same time point). *Statistically different from control group (zero time); p-values were obtained by one-way ANOVA followed by Fisher's test.

RESULTS

The results of the measured parameters are presented graphically for the ipsilateral and in tabular form for the contralateral side of the striatum.

NO_3^-. A significant decline in the concentration of NO_3^- was observed after 24 hours in both the ipsilateral and contralateral side of striatum isolated from rats poisoned with PQ. In the L-NAME + PQ group, NO_3^- in the striatum was lower than in controls and gradually decreased with time until an almost negligible level was present after 7 days (Fig. 1, Tab. 1).

$O_2^{\bullet-}$ radical. A significant reduction in the level of $O_2^{\bullet-}$ radical was observed after 24 hours of PQ poisoning. In the L-NAME+PQ group a significantly elevated concentration

Table 1. Concentrations of measured parameters in contralateral striatum of Wistar rats.

Time	Parameter	Groups			
		Control	PQ	L-NAME+PQ	L-NAME
30 min	NO_3^-	$16.14 \pm 6.73^{a, b}$	$19.72 \pm 9.45^{d, b}$	$9.68 \pm 2.82^{a, c}$	28.46 ± 13.13^{d}
	$O_2^{\bullet-}$	2.44 ± 0.54^{a}	3.86 ± 0.87^{a}	$5.44 \pm 1.44^{b*}$	3.66 ± 0.83^{a}
	MDA	59.00 ± 20.66^{a}	$103.40 \pm 25.59^{b*}$	$96.71 \pm 19.11^{b*}$	$108.98 \pm 9.72^{b*}$
24 h	NO_3^-	17.92 ± 5.77^{a}	$3.50 \pm 2.22^{b*}$	$5.08 \pm 2.04^{b*}$	40.80 ± 20.91^{c}
	$O_2^{\bullet-}$	2.91 ± 0.91^{c}	$1.06 \pm 0.45^{a*}$	$5.25 \pm 1.39^{b*}$	2.81 ± 0.77^{c}
	MDA	$68.02 \pm 14.80^{a, d}$	55.73 ± 13.04^{a}	$76.99 \pm 17.40^{b, d*}$	$110.82 \pm 23.9^{c*}$
7 days	NO_3^-	14.29 ± 3.66^{a}	12.60 ± 4.71^{a}	$3.09 \pm 1.59^{a*}$	16.91 ± 5.21^{a}
	$O_2^{\bullet-}$	3.54 ± 0.83^{a}	4.24 ± 1.75^{a}	3.20 ± 1.65^{a}	3.64 ± 0.64^{a}
	MDA	$63.76 \pm 8.94^{a, c}$	$95.17 \pm 12.62^{b*}$	$86.58 \pm 23.53^{b, c*}$	$74.05 \pm 19.11^{b, c}$

Control group (n = 8) – treated with 10 µL of 0.9% NaCl solution; PQ group (n = 24) – poisoned with one single dose of PQ (2.5 µg/10 µL); L-NAME + PQ group (n = 24) – pre-treated with L-NAME with one single dose (10 µg/10 µL) 30 minutes before PQ administration and L-NAME group (n = 24) – treated with L-NAME with one single dose (10 µg/10 µL). NO_3^- are expressed as nmol/mg protein; $O2^{\bullet-}$ as mmol red. NBT/mg protein and MDA as pmol/mg protein. Values are means ± SD (n = 8). Means not sharing the same letter are significantly different ($p < 0.05$, for the same time point). *Statistically different from control group (zero time); p-values were obtained by one-way ANOVA followed by Fisher's test.

Table 2. Spearman's correlation coefficients between NO_3^- and $O_2^{\bullet-}$, and NO_3^- and MDA for each time point within the experiment.

	r_s-values							
	ipsilateral striatum				contralateral striatum			
	0	30 min	24 h	7 d	0	30 min	24 h	7 d
NO_3^- and $O_2^{\bullet-}$	-0.26	-0.08	+0.88	-0.10	+0.44	-0.70	+0.40	-0.80
NO_3^- and MDA	-0.25	-0.31	+0.65	+0.10	-0.61	-0.60	-0.80	+0.40

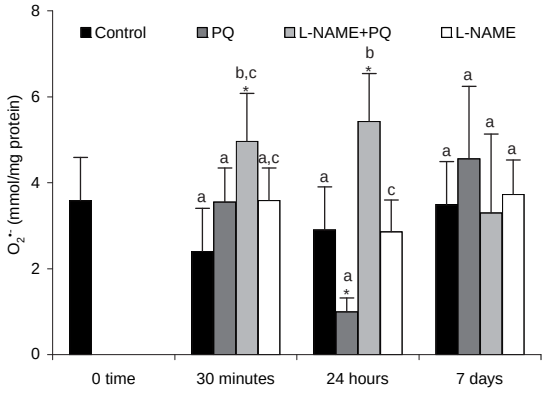

Figure 2. $O_2^{\bullet-}$ concentration in ipsilateral striatum of rats after single intrastriatal administration of PQ, L-NAME and L-NAME + PQ.
Values are means ± SD (n = 8). Means not sharing the same letter are significantly different ($p<0.05$, for the same time point). *Statistically different from control group (zero time); p-values were obtained by one-way ANOVA followed by Fisher's test.

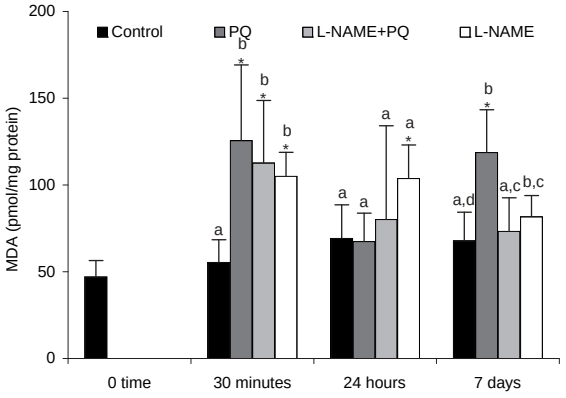

Figure 3. MDA in ipsilateral striatum of rats after single intrastriatal administration of PQ, L-NAME and L-NAME + PQ.
Values are means ± SD (n = 8). Means not sharing the same letter are significantly different ($p<0.05$, for the same time point). *Statistically different from control group (zero time); p-values were obtained by one-way ANOVA followed by Fisher's test.

of $O_2^{\bullet-}$ was found within the first 24 hours. The difference between the concentrations of $O_2^{\bullet-}$ obtained in PQ and L-NAME+PQ groups was significant at the 24 hours time point (Fig. 2, Tab. 1).

LPO. After PQ administration MDA levels were significantly elevated at 30 min and 7 days. However, in the L-NAME + PQ group the MDA level was elevated only at 30 min. In the latter group, the MDA values declined and after 7 days were close to control values (Fig. 3, Tab. 1).

Correlation between NO_3^- and $O_2^{\bullet-}$, and NO_3^- and MDA. At all time points the NO_3^- concentration was positively correlated with that of $O_2^{\bullet-}$ concentration in brain striatum after PQ intoxication, with observed statistical significance (r_s=+0.44, p=0.0098) (Fig. 4).

At all time points the NO_3^- concentration was positively correlated with that of MDA with observed statistical significance (r_s=+0.52, p=0.0016) (Fig. 5, Tab. 2).

DISCUSSION

Our results confirm the neurotoxic effects of PQ, based on observed increases in both $O_2^{\bullet-}$ production and LPO. LPO results in a progressive loss of membrane fluidity, reduces membrane potential and increases the permeability to ions such as Ca^{2+} [21]. Decreased levels of NO_3^- found after PQ poisoning suggest NO depletion via a PQ redox-cycling metabolism, probably via reaction with $O_2^{\bullet-}$, whereby the harmful peroxynitrite ($ONOO^{\bullet-}$) anion is generated. Pretreatment with L-NAME provided the possibility to distinguish the effects of ROS from RNS with respect to kinetic and spatial propagation of oxidative lipid injury induced by PQ. Almost identical results were found on both sides of the brain area. The consequences of NOS inhibition could be attributed to the down-regulation of NADPH oxidase [22, 23], decreased production of $ONOO^{\bullet-}$ and reduced excitotoxicity of NMDA receptors, known in the literature as a feature of PQ-mediated neurotoxicity. Thus, our study

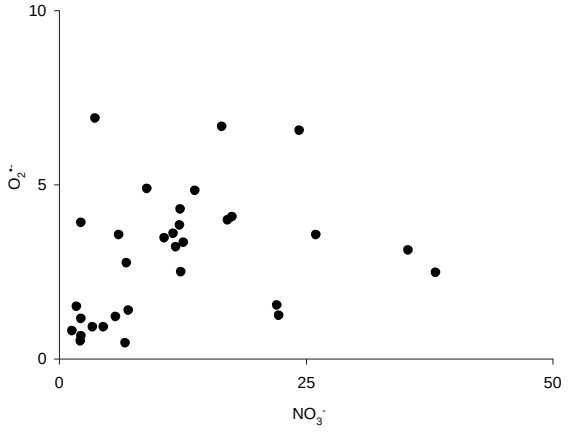

Figure 4. Correlation between NO_3^- and $O_2^{\bullet-}$ concentrations in striatum of rats poisoned with PQ at all time points.

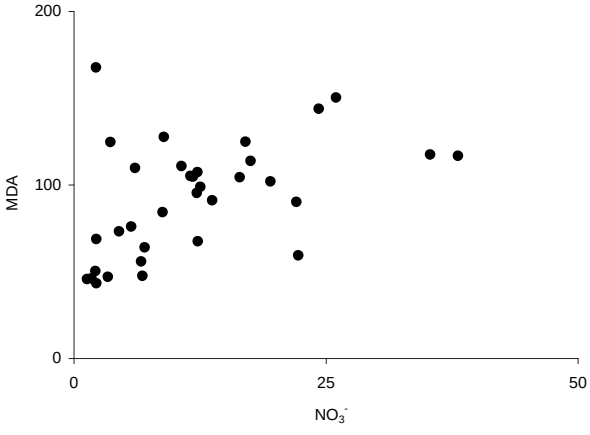

Figure 5. Correlation between NO_3^- and MDA concentration in striatum of rats poisoned with PQ at all time points.

confirms NO involvement in PQ-mediated neurotoxicity involving harmful effects of RNS on LPO, as well as the protective effect offered by L-NAME in pre-treated rats.

L-NAME's protective effect on PQ intoxication with respect to NO synthesis was clear from our results (Fig. 1). Immediately after treatment, the NO_3^- level was reduced two-fold compared to controls.

A most likely explanation is that endogenous NO, already present in rat striatum prior to NOS inhibition by L-NAME, promptly reacted with $O_2^{\bullet-}$ to form $ONOO^{\bullet-}$. In addition, lower NO_3^- concentrations in PQ-treated rats compared to controls point to the involvement of NO in PQ redox-cycling metabolism. Depletion of NO could be explained by $ONOO^{\bullet-}$ formation [8]. Shimizu *et al.* found elevated extracellular NO levels after PQ perfusion and almost negligible levels after 24 hours of PQ exposure, which is in accordance with our results. Perhaps reduced synthesis of ATP in mitochondria and energy depletion are explanations for such results [26, 27]. The anticipated inhibition of NOS in rats treated only with L-NAME was not observed. Importantly, the highest inhibition of NOS was obtained in rats exposed to L-NAME + PQ, which implies synergistic or potentiating effects of these substances.

According to Muzaffar and co-workers, some NO donors can inhibit NADPH oxidase [22, 23]. We speculate that inhibition of NO synthesis by L-NAME forced PQ redox cycling with a consequent elevation of ROS production. Considerably higher LPO in PQ-treated rats immediately after administration and after 7 days emphasises the more harmful effects of RNS, compared to that observed in the L-NAME + PQ-treated rats, in which the effects of ROS were dominant. If ROS are less harmful to lipids than $ONOO^{\bullet-}$ we envisage that this could be the reason why MDA values were similar to controls in the L-NAME + PQ group after 24 hours and 7 days. These findings support our hypothesis regarding the protective effect of L-NAME.

Significantly elevated $O_2^{\bullet-}$ levels in the striatum of rats in the L-NAME + PQ group (within the first 24 hours compared to the PQ group) could be explained by elevated $O_2^{\bullet-}$ production (induced by PQ), inhibited NO synthesis and insufficient capacity of superoxide dismutase (SOD) [4]. A recent study from Edabi and Sharma demonstrated that $ONOO^{\bullet-}$ inhibited manganese SOD (Mn-SOD) contributing to elevated $O_2^{\bullet-}$ [6, 7]. $ONOO^{\bullet-}$, a more aggressive oxidant than $O_2^{\bullet-}$, triggers LPO, which is in accordance with our observed results [33]. Therefore, increased MDA in rats poisoned with PQ might be ascribed to the deleterious effects of $ONOO^-$ rather than $O_2^{\bullet-}$ [8, 16, 17, 33]. The dual role of NO, both anti-oxidative and pro-oxidative, is currently a subject of scientific contention [13]. Despite this, we believe that both could contribute to LPO. Its pro-oxidative role could be attributed to $ONOO^{\bullet-}$ [8, 16, 17, 33]. In contrast, its anti-oxidative role could be explained by the termination of LPO by the formation of nitrated lipid adducts, including $RONO/RNO_2$ and $ROONO/RONO_2$, or via the induction of various anti-oxidant defence enzymes [8, 25]. Positive correlation between NO_3^- and MDA and between NO_3^- and $O_2^{\bullet-}$ at all time points provides evidence that favours the involvement of NO in PQ-mediated toxicity. It has been suggested that $ONOO^{\bullet-}$ formation is a primary pathway of NO metabolism which is related to the high rate of reaction between NO and $O_2^{\bullet-}$ [16, 17].

$ONOO^{\bullet-}$ can react with all classes of bio molecules (including the hydroxylation of aromatic amino acids, nitration of tyrosine and oxidation of thiols and lipids) and can significantly contribute to oxidative cell damage [8].

The mitochondrial respiratory chain is the major source of intracellular $O_2^{\bullet-}$ production under physiological conditions (electron "leakage" during the process of O_2 reduction to H_2O). However, PQ metabolism via complex I suppresses $O_2^{\bullet-}$ production through the mitochondrial electron transport chain via NAD(P)H depletion. The significant decrease in $O_2^{\bullet-}$, NO_3^- and MDA observed after 24 hours found in rats poisoned with PQ could therefore be explained by energy insufficient oxidatively damaged neurons.

Such a disorder of mitochondrial function involving mitochondrial depolarisation, Ca^{2+} deregulation and depressed ATP synthesis can lead to apoptotic or necrotic cell death, and may produce early onset and rapid progression of neurological disorders [11]. Dopaminergic neurons are particularly vulnerable to complex I inhibition. In the study by Tawara and co-workers, complex I activity in rat brain decreased with time with a significant effect observed 2 hours after PQ administration, implying that PQ decreases mitochondrial complex I activity within the brain at an early stage after PQ exposure, even before respiratory dysfunction is observed [30].

Edabi and Sharma's studies that focussed on Parkinson's like pathophysiological mechanisms induced by PQ, reported that $ONOO^-$ was an effective inhibitor of enzymes in the mitochondrial respiratory chain which resulted in decreased ATP synthesis due to significantly lower activity of complex I [7, 30].

Behavioural changes induced by PQ emphasise the susceptibility of the nigrostriatal dopaminergic system to oxidative damage [20]. Perhaps pre-treatment with L-NAME prevents development of extrapyramidal behaviour during PQ poisoning.

According to the report by Shimizu and colleagues, L-NAME was able to suppress excitotoxicity [6, 15]. In our present study, we noticed behavioural changes in the group of rats that were administered PQ, characterised by rigour, tremor, diskinesia and rotational behaviour contralaterally from the lesion immediately after recovery from anaesthesia. Rats treated with L-NAME did not exhibit any behavioural changes. Behavioural studies focusing on the impact of xenobiotics on dopamine systems have only recently been documented [6].

Some authors have reported that PQ's toxicity involves glutamate-induced activation of non-NMDA receptors resulting in activation of NMDA receptor-channels. The influx of Ca^{2+} into cells stimulates NOS. Released NO would

diffuse towards dopaminergic terminals and further induce mitochondrial dysfunction by the formation of $ONOO^{\bullet-}$ resulting in continuous and long-lasting dopamine overflow [26, 27]. NO produced by NOS is thought to play an important role in excitotoxicity, probably through the formation of $ONOO^{\bullet-}$.

CONCLUSION

Our study confirms (i) the involvement of NO in PQ-mediated neurotoxicity, (ii) more harmful effects of nitrogen species than oxygen species on oxidative lipid deterioration and (iii) the protective effect of L-NAME if administered in a pre-treatment regime. The absence of behavioural changes, characterised by rigour, tremor, diskinesia and rotational behaviour in the group pre-treated with L-NAME (in addition to the obtained experimental results) implies a protective role of L-NAME during PQ-mediated neurotoxicity.

Acknowledgement

This work was supported by a Grant from the Ministry of Science and Environmental Protection, Republic of Serbia (Project No. 145010). The authors would like to thank Dr. David R. Jones for help in editing the manuscript.

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