Daily Subacute Paraquat Exposure Decreases Muscle Function and Substantia Nigra Dopamine Level

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Summary

The use of the herbicide paraquat (1,1'-dimethyl-4,4'-bipyridylium dichloride; PQ) which is widely used in agriculture is known to cause dopaminergic neurotoxicity. However, the mechanisms underlying this effect are not fully understood. This present study investigated the behavioral manifestations, motor coordination, and dopaminergic neurodegeneration following exposure to PQ. Male rats were injected with PQ (10 mg/kg i.p.) daily for three weeks. Rotarod systems were used for measuring locomotor activity and motor coordination. The effects of PQ on dorsiflexor, electrophysiologically-induced muscle contraction were studied. Dopamine concentrations in the ventral mesencephalon were measured by high performance liquid chromatography and the number of dopaminergic neurons in substantia nigra pars compacta was estimated by tyrosine hydroxylase immunohistochemistry. PQ induced difficulty in movement and significant reduction in motor activity and twitch tension at the *dorsiflexor* skeletal muscle. The number of tyrosine hydroxylase positive neurons was significantly less in the substantia nigra pars compacta and nigral dopamine level was significantly reduced in PQ treated animals (20.4±3.4 pg/mg) when compared with control animals (55.0±2.4 pg/mg wet tissue). Daily treatment of PQ for three weeks induces selective dopaminergic neuronal loss in the substantia nigra and significant behavioral and peripheral motor deficit effects.

Key words

Paraquat • Dopamine • Skeletal muscle • Locomotor activity

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Introduction

Paraquat (PQ, 1,1′-dimethyl-4,4′-bipyridinium dichloride) is a widely used herbicide in agriculture whose chemical structure is very similar to the active form of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a neurotoxin that leads to parkinsonism in animals (Bloem *et al.* 1990) and humans (Langston *et al.* 1983). This suggests an association between the use of this pesticide in agriculture and incidence of Parkinson's disease. Indeed, intraperitoneal (i.p.) administration of PQ in rodents causes loss of DA neurons in the substantia nigra (SN) as a key feature in parkinsonism with associated decrease in locomotor activity (Brooks *et al.* 1999, McCormack *et al.* 2002).

Epidemiologic studies indicate that exposure to PQ in human through agricultural usage can be a risk factor in the incidence of neurodegeneration in both animal and human models (Dinis-Oliveira *et al.* 2006, McCormack *et al.* 2005, Thiruchelvam *et al.* 2000a,b, 2003, Djukic *et al.* 2007). A strong correlation has been reported between exposure to PQ and Parkinson's disease (PD) incidence (Berry *et al.* 2010) and confirmed by clinical studies conducted in Canada, Taiwan, and the United States (Liou *et al.* 1997). This correlation is supported by animal studies showing that PQ produces toxicity on dopaminergic neurons of the rat and mouse brain (McCormack *et al.* 2002, McCormack *et al.* 2005). However, it is unclear how PQ triggers toxicity in dopaminergic neurons. PQ exposure results in mitochondrial dysfunction and microglial activation leading to increased generation of reactive oxygen

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species (ROS), which in turn damages dopaminergic neurons. PQ also decreases the binding of dopamine (DA) to dopamine transporter (DAT) and inhibits DA uptake, thereby disturbing DA homeostasis (Ossowska *et al.* 2005). Paraquat mediates nigral dopaminergic neuronal apoptotic machinery through sequential phosphorylation of c-Jun N-terminal kinase and c-Jun and the activation of caspase-3 which induces sequential neuronal death (Peng *et al.* 2004).

PQ is one of the most widely used herbicides in the world. It is rapidly acting and nonselective herbicide. Occupational and professional exposure to PQ may occur by inhalation or dermal route and is considered as an etiological factor of PD (Moretto and Colosio 2011). Suicidal poisonings are related to ingestion of PQ (Gawarammana and Buckley 2011). Ingestion of concentrated solutions (12-20 %) of PQ leads to early death due to organs failure. Patients with acute PQ poisoning show damage to the lungs, liver, and kidneys, and some also show symptoms of central nervous system toxicity (Di Monte 2003). There is no specific treatment for PQ poisoning, thus management of poisonings is to relieve symptoms and complications (Gawarammana and Buckley 2011).

Similarly, PQ is toxic to animals and can cause damage to lungs and brain, among other tissues. Conspicuously, PQ has been reported to increase the risk for sporadic PD *via* its toxicity to DA neurons in the substantia nigra (Thiruchelvam *et al.* 2003, 2005). PQ toxicity is caused by oxidative stress due to redox cycling, a process in which it accepts an electron from an appropriate donor and subsequently reduces O_2 to produce superoxide anion radical ($O_{2-\cdot}$) (Drechsel and Patel 2008). These observations emphasize the need to systematically monitor exposures and apply experimental approaches to understand and anticipate mechanisms that could result in PQ-induced neurotoxicity.

The present study aimed to explore the behavioral manifestations of PQ induced toxicity on the locomotor activity and motor coordination. We aimed also to investigate the skeletal muscle electrophysiological modifications and to determine the level of DA in substantia nigra following PQ treatment.

Materials and Methods

This project was reviewed and approved by the Institutional Review Board of the United Arab Emirates University, and experiments were performed in accordance with protocols approved by the Institutional Animal Care and Research Advisory Committee.

Animals and treatment

All the experiments were performed on Male Wistar rats (body weight, 210±15 g). Animals were housed in groups of 4 in polypropylene cages with a controlled light and dark cycle of 12 hours each at 24-26 °C. Food and water were available *ad libitum*. PQ (Sigma, St. Louis, MO), dissolved in normal saline, was injected at 10 mg/kg i.p. daily for 3 consecutive weeks to induce neurotoxicity. Equal number of rats served as control and received normal saline in similar way. At the end of three weeks of treatment and after 30 min of last treatment with PQ, the individual groups of rats were subjected to following tests.

Behavioral studies

Behavioral studies included measuring motor coordination in rats. Rotarod systems (Harvard apparatus, MA, USA) were used for measuring locomotor activity and motor coordination. The animals were placed on a rod with 3-cm diameter, which is rotating at a constant speed (20 RPM). The total time during which the animals remain by coordinating and balancing on the rod and continuously moving forward to show the forward gait to avoid falling off the rod was compared between control and PQ treated group of animals (n=10, in each group). The latency to fall represented a measure of motor activity and motor coordination of the animals. Prior to the actual test, all the animals were trained on rotarod twice a day for one week.

Electrophysiological recording

The electrophysiological recordings were performed by exposing *dorsiflexor* muscle with its motor nerve *in-situ* and stimulating electrically to observe the changes in twitch tension. At the time of experiments, rats (n=8, in each group) were anesthetized with urethane 25 % solution (2.5 mg/g bodyweight, i.p.) and the flexor digitorum superficialis muscle was dissected out. Care was taken not to damage the muscle. The *dorsiflexor* muscle was chosen because it encloses predominantly fast twitch fibers and its location makes *in-situ* physiological recording possible. Muscle contraction ability was investigated by measuring isometric twitch tensions. Isometric twitch tensions (evoked either directly by stimulation of the muscle or indirectly by stimulation of motor nerve) were measured after the tendinous

insertion of electrodes and was attached to a force displacement transducer (Model FT-03C, Grass Technologies, RI, USA). The output was differentially amplified and displayed on a chart recorder for analysis. Twitch responses to supramaximal stimuli delivered to the *dorsiflexor* nerve at 1 Hz were recorded in *dorsiflexor* muscle. Direct muscle stimulation was accomplished by placing two wide platinum wires underneath the muscle. Twitches were evoked either directly or indirectly using a stimulator (Model S44, Grass Technologies, RI, USA) delivering 5 VDC square wave pulses of 0.5 ms duration. The muscle was lengthened until a maximum twitch response was elicited. This was achieved usually when the muscle was stretched by 1.1 times its resting length. Normal Krebs solution was used to irrigate the exposed muscle and nerve.

Biochemical studies – estimation of dopamine

Animals were humanely decapitated and tissue samples from ventral mesencephalon were removed and placed on ice. The samples were weighed and transferred to pre-cooled lightproof Eppendorf tubes. Two milliliters of 0.1 M perchloric acid containing 0.1 M citric acid and 0.001 M ethylenediaminetetraacetic acid was added to each tube. The tubes were submerged in ice pellets and the tissues were homogenized using a motorized homogenizer (Ultra Turrax T25basic, IKA, Werke) for 2 min. The homogenized tissue was centrifuged in a refrigerated centrifuge (2 °C) at 5400xg for 10 min. The supernatant was aspirated into Eppendorf tubes and filtered through 0.45-µm mesh microfilter before being injected into the HPLC system.

The HPLC system consisted of a dual plunger pump (Waters® 616, Waters® Corporation, MA, USA), controller (Waters® 6000 S), autosampler (Waters® 717S), and a pulsed electrochemical detector (Decade II, Antec Leyden®, The Netherlands). The glassy carbon electrode was equipped with the applied potential of 800 mV and current of 10 nA, and with Empower® 2.0, the operating software, from Waters®. The standard catecholamine mobile phase from Chromsystems® (Mobile Phase Catecholamine Part No: 5001) was used at the flow rate of 0.8 ml/min and run overnight to stabilize the baseline. Calibration solutions (Chromsystems®, Plasma calibration standards) containing 386 pg/ml of DA were injected repeatedly to establish the integrity of the column, and the stability of the mobile phase and the reproducibility of the retention time. Peak areas of each component were recorded and averaged to calculate the final concentration of DA in the tissue sample.

Tyrosine hydroxylase immunohistochemistry in substantia nigra pars compacta (SNc)

The rats (n=6 in each group) were humanely killed with an over dose of urethane 25 % solution (2.5 g/kg bodyweight, i.p.) and perfused with 10 % formalin in phosphate buffer, transcardially. The brains were removed, postfixed in the same fixative solution overnight and then imbedded in paraffin. Coronal paraffin sections (10 µm) of the midbrain were cut and collected serially and processed for detection of tyrosine hydroxylase (TH) using the avidin-biotin-complex (ABC) method. Briefly, after microwave treatment for antigen retrieval, the sections were incubated with TH-antibody raised in rabbit (Millipore, diluted 1:10,000) overnight. After rinsing in phosphate buffered saline (PBS), the sections were incubated in biotinylated goat anti-rabbit IgG (Jackson, 1:500) for 1 h then in extravidin-peroxidase conjugate (Sigma, 1:1000) for another hour. To visualize any TH immunoreactivity the sections were incubated for 5 min in a solution containing 25 mg diaminobenzidine dissolved in 50 ml of 0.1 M phosphate buffer (PB, pH 7.4) with 7.5 µl hydrogen peroxide (30 %) and 1 ml nickel chloride (3.5 %) added to intensify the reaction. Finally, the sections were rinsed with water, dehydrated in graded alcohol, cleared in xylene and mounted with. All antibodies were diluted in PBS containing 0.3 % Triton.

Representative digital images were captured using a Zeiss AxioCamHRc digital camera with AxioVision 3.0 software (Carl Zeiss, Germany). The survival of DA producing neurons was determined by the number of TH positive (TH^+) neurons in the SNc. Six sections from each animal (n=6) separated by at least 30 µm from the middle of nigra 5.2-5.8 mm behind bregma (Paxinos and Watson 2007) were counted. Only TH-positive profiles displaying obvious nuclei which were identified by the lack of cytoplasmic staining in the middle of the neurons were included in this analysis. The labels of immunohistochemical images were coded and these images were carefully and blindly (without the knowledge of animal treatment) assessed by an expert histopathologist and reported about the changes observed in the tissue sections.

Statistical analysis

The calculated mean of data derived from each experiment was presented as bar graphs and the standard

error of mean (S.E.M) was plotted as error bar (mean±S.E.M). The results from the control and PQ treated groups were compared using unpaired Student's t-test in the software Graphpad Prism version 4. For all the experiments, the sample size was 6-10 and the significance level of 0.05 (95 % confidence) was considered as a cut off.

Results

The weight of the rats were assessed and recorded during and after the treatment of PQ. No significant changes were observed in the weight of PQ treated group (204.0±2.7 g, n=10 initially and 247.2±2.4 g, n=10 after three weeks) when compared to the saline treated control group (204.9±3.4 g, n=10 initially and 257.8±6.3 g, n=10, after three weeks) (Fig. 1). No significant effect was found in feeding, drinking and ability of the animals to maintain normal growth and full health.

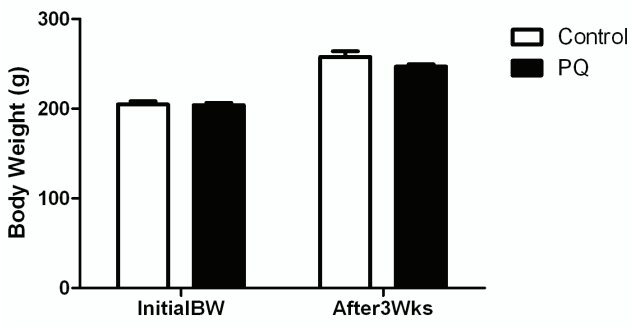

Fig. 1. Effects of paraquat on body weight initially at the start of the treatment and after 3 weeks treatment and compared to control to the paraquat treatment. Data in bar graph are mean±S.E.M, n=10.

Behavioral studies

Behavioral manifestations were measured following exposure to PQ. These included measuring locomotor activity and motor coordination in rats. PQ treatment modified locomotor activity and motor coordination as investigated by performance on the rotarod instrument. PQ treated animals lost their coordination and the normal motor control and showed weakness when compared to the control group to walk continuously or walk forward to avoid falling off the rotarod. Statistical analysis showed that animals that received PQ stayed for much ($P<0.05$) shorter period (124±33 s, n=10) on the rotarod compared to control group (380±99 s, n=10) (Fig. 2).

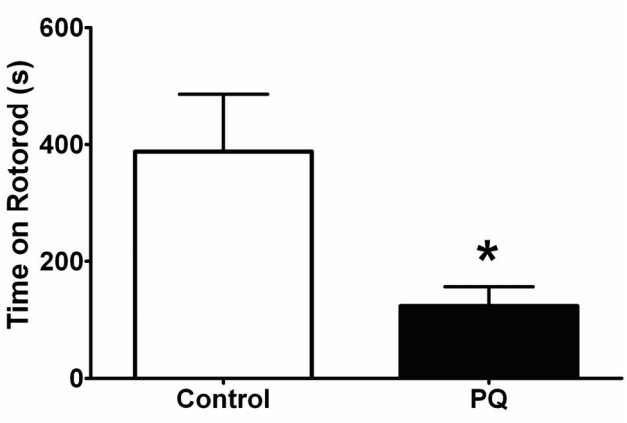

Fig. 2. Effects of paraquat on latency to fall from rotarod compared to control. Data in bar graph are mean±S.E.M, n=10, * $P<0.05$.

Electrophysiological studies

Muscle contraction ability was investigated by measuring isometric twitch tensions (evoked either directly by stimulation of the muscle or indirectly by stimulation of motor nerve). Twitch tensions (n=10) were recorded after the tendinous insertions were attached to a force displacement transducer. Isometric force of contraction in response to indirect supramaximal nerve and direct muscle stimulation were reduced in PQ treated animals (Table 1). PQ treatment significantly ($P<0.05$) reduced force and tension of muscle contraction. Muscles from PQ treated rats generated a significantly smaller force of contraction upon both direct and indirect stimulation. However, PQ treatment had no effect on contractile speed, rise time or half time of decay (Table 1).

Table 1. Effect of PQ (10 mg/kg, i.p.) on dorsiflexor muscle contraction of control and PQ treated rats.

Muscle characteristic	Control	PQ treated
Indirect nerve stimulation		
Rise time (ms)	0.49±0.04	0.50±0.06
½ Decay time (ms)	0.44±0.03	0.43±0.04
Twitch tension (g)	8.5±0.3	4.9±0.5*
Direct muscle stimulation		
Rise time (ms)	0.47±0.03	0.52±0.06
½ Decay time (ms)	0.37±0.04	0.48±0.08
Twitch tension (g)	8.1±0.3	3.9±0.7*

Data in the table shown are mean±S.E.M, n=10, * $P<0.05$.

Biochemical studies – estimation of dopamine

PQ treatment reduced the DA level significantly in the ventral mesencephalon when compared to the control group which received saline only ($P<0.01$, n=8). The level of the DA in ventral mesencephalon in PQ treated group was 20.4±3.3 pg/mg while the control group measured 55.1±2.4 pg/mg of wet weight of the tissue (Fig. 3).

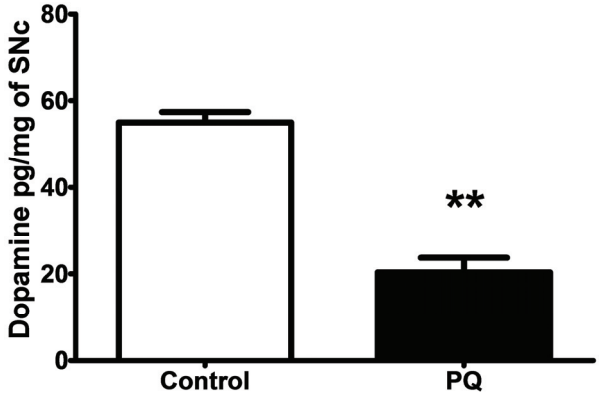

Fig. 3. Effects of paraquat on amount of dopamine estimated by HPLC in substantia nigra compared to control. Data in bar graph are mean±S.E.M, n=8, ** P<0.01.

Immunohistochemical studies – SNc dopamine

In order to determine whether PQ treatment could cause loss of DA neurons, quantification of TH^+ neurons in SNc was carried out. The number of TH^+ neurons (authentic marker of dopaminergic neurons) in the middle of SNc was 45.6±2.4/section and 33.8±3.4/section in control and PQ-treated rats respectively. Statistical analysis showed that chronic PQ exposure significantly reduced about one third of total number of TH^+ neurons in SNc relative to sections from saline treated control rats ($P<0.001$, n=6) (Figs 4 and 5).

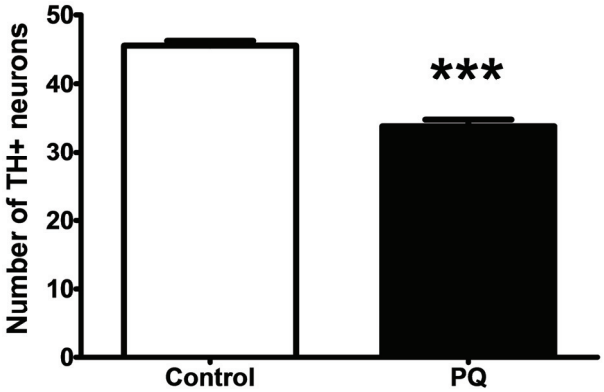

Fig. 4. Effect of paraquat on number of the TH^+ neurons compared to control data in bar graph are mean±S.E.M, n=6, *** P<0.001.

Discussion

The present studies have demonstrated that PQ injection (10 mg/kg injected daily i.p. for three weeks) can cause a notable reduction in the amount of DA in SNc and a significant bradykinetic reaction in the rotarod experiment when compared to the control group. The degeneration in TH^+ neurons in PQ treated group was up to 28 % when compared to the control group in the SNc. This significant loss is mainly attributed to the treatment of PQ. Our *in-situ* experiment on twitch response on *dorsiflexor* muscle showed significant decrease in their twitch tensions. However, the rise time and the half decay time of the contraction have not shown a statistically significant change when compared to the control group.

This study also investigated the effects of subacute intraperitoneal injection of PQ. The results showed that PQ produced a decrement in rotarod performance. Animals received daily treatment of PQ for three weeks stayed on the rotarod for much shorter period compared with control. In agreement with previous study (Thiruchelvam *et al.* 2003) in which 10 mg/kg, twice per week for 6 weeks was used, the motor coordination was reduced in the PQ treated rats tested in the similar environment of rotarod, suggesting a strong relationship with the characteristics of neurotoxicity produced by PQ.

The present results demonstrated that the i.p. treatment of PQ, resulted in a significant reduction of muscle contraction when stimulated electrically on *dorsiflexor* muscle with no effect on contractile speed, rise time or half time of decay.

Presynaptically, effects of PQ treatment could result from blocking entry of Ca^{2+} into nerve terminals *via* voltage sensitive channels or it may have an action on the internal Ca^{2+} binding sites and consequently the level of Ca^{2+} sequestering activity inside the nerve terminal (Yoshimura *et al.* 1993, Tauskela *et al.* 2005, Bagatini *et al.* 2011). Postsynaptically, PQ may elicit modifications in the regulation of the myoplasmic transient that could explain altered twitch contractile properties. Indeed, muscle-twitch response after transmitter release from nerve terminals can be changed significantly by changing sarcoplasmic reticulum function with or without alterations in myosin characteristics. However, the unchanged rise and relaxation time of isometric twitch tension probably reflect an intact function of the sarcoplasmic reticulum, as opposed to an alteration in the intrinsic shortening properties of the myofibrils of the muscle. The physiological role of skeletal muscle

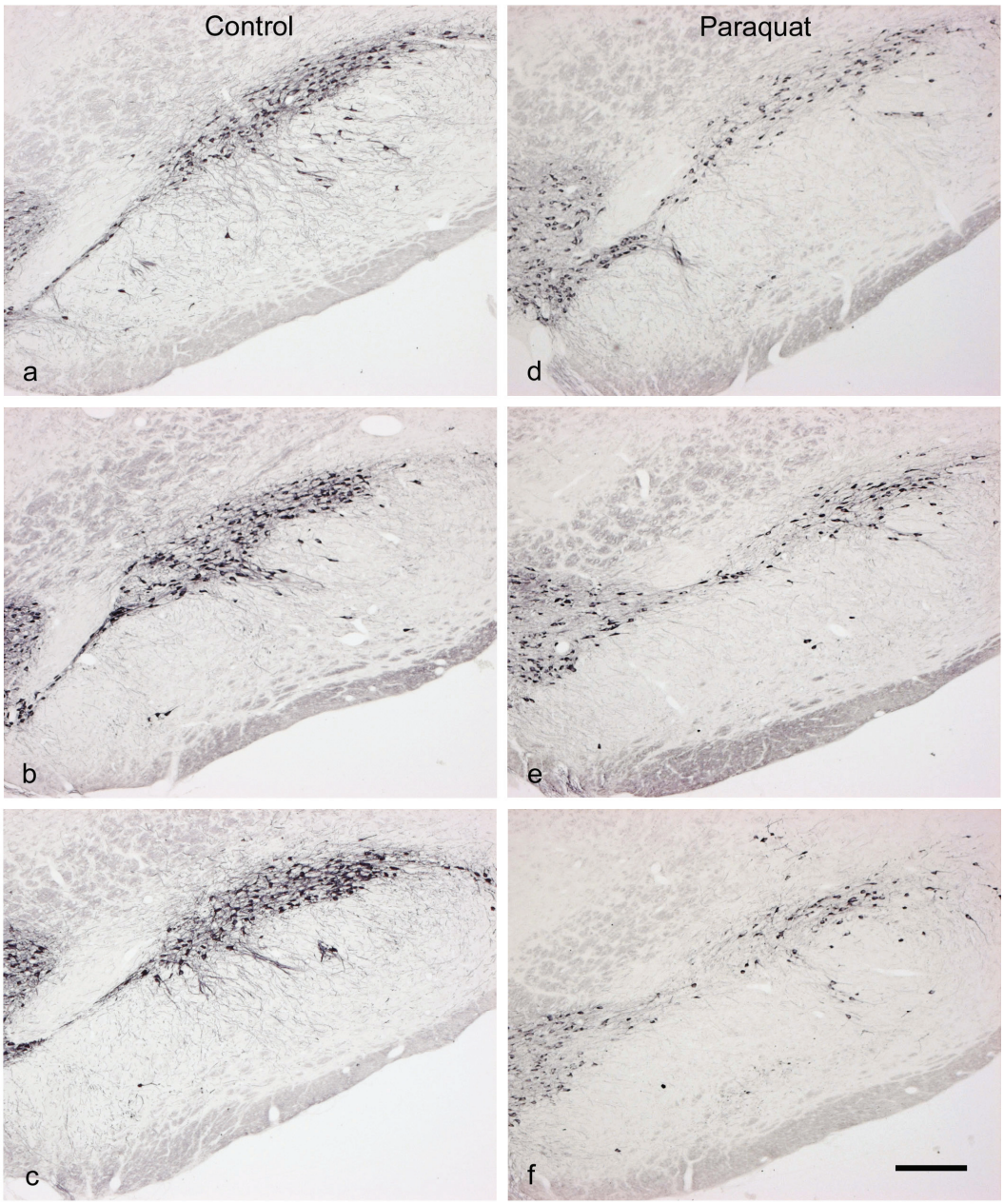

Fig. 5. Images of coronal sections of the midbrain showing dopaminergic neurons in the substantia nigra pars compact (SNc) revealed by TH antibody. Panels (**a**-**c**) are examples represent TH staining from three different control rats (treated with saline) compared with another three different rats that had been treated with paraquat alone (**d**-**f**). Note that paraquat treatment induced a significant loss of TH^+ immunoreactive neurons (d-f), relative to saline (a-c). Scale bar = 250 µm.

sarcoplasmic reticulum is the release and sequestration of Ca^{2+} during the contraction-relaxation cycle, thus regulating the level of contractile apparatus activation. Another possibility is that free radicals are elevated during PQ treatment and may cause muscle membrane damage, with the net results of contractility decrement (Huang *et al.* 2012). Recently, reported data indicated that PQ decreases the mitochondrial membrane potential and increases mitochondrial reactive oxygen species (ROS), supporting mitochondria as the target site of PQ (Huang *et al.* 2012).

A previous study on the neuromuscular function was conducted by directly applying PQ to the diaphragm (an isolated skeletal muscle preparation). The results showed that PQ inhibited acetylcholine contracture blocked the postsynaptic acetylcholine receptors which play a role in the inhibition of the skeletal muscle contracture. In addition, PQ resists the diaphragms from alpha-isometric force of contraction in response to indirect supramaximal nerve and direct muscle stimulation (Lin-

Shiau and Hsu 1994). Taken together with these findings and our results, suggest that the effect of PQ might be both locally mediated through neuromuscular junction and through indirect manifestation from the effect on central dopaminergic system.

The results obtained from histochemical studies depicted that chronic PQ exposure significantly reduced the number of TH^+ neurons in the SNc relative to vehicle-treated rats. Although earlier studies showed no significant effects of PQ treatment on the number of dopaminergic neurons in SNc (Widdowson *et al.* 1996, Thiruchelvam *et al.* 2000a,b), our results are in agreement with those who reported 25-30 % loss of TH^+ neurons (McCormack *et al.* 2002, Mangano *et al.* 2011).

Although the mechanism of action of PQ in destroying DA neurons in Parkinsonism is not fully understood several mechanisms have been proposed. It has been shown that PQ induces dopamine overflow and reduces DA synthesis by N-methyl-D-aspartate (NMDA) receptor activation, associated with Ca^{2+} penetration as a key feature of neurodegeneration. PQ has also been found to decrease the mitochondrial complex I activity of the brain (Tawara *et al.* 1996). Furthermore, it markedly induces α-synuclein up-regulation and aggregation as a consequence of toxicant insult leading to α-synuclein pathology in neurodegenerative disorders (Manning-Bog *et al.* 2002).

This study also investigated the effect of daily PQ exposure (i.p.) for 3 weeks. The reason for that is to try to mimic heavy human exposure scenarios to PQ. While this dosage regimen has affected the muscle function and SN dopamine level, it did not affect the body weight or the general appearance of the animals. Other researchers have also investigated different routes and dosage regimens. Mangano *et al.* (2011) demonstrated that PQ exposure (10 mg/kg, 3 times /wk. for three weeks) could conceivably contribute to motor and non-motor disturbances. McCormack *et al.* (2002) showed that PQ (10 mg/kg once /wk for three weeks) caused selective dopaminergic degeneration without significant decrease in DA level in the striatum. These authors explained their finding by a possible compensatory mechanism by which neurons that survive damage are capable of restoring neurotransmitter tissue levels (McCormack *et al.* 2002). However, Widdowson *et al.* (1996) reported that multiple oral dosing (5 mg/kg/day) for 14 days did not lead to changes in locomotor activity or grip strength or neuropathology or changes in neurochemistry in the nigrostriatal tract. These findings illustrate the importance of the route and dosage regimen in PQ-induced neuromuscular dysfunction.

One of the possibilities of having less TH^+ neurons obtained in PQ treated animals in this study may be due to phenotypic down-regulation of TH^+. However, this is unlikely the case because in two recent studies, in which an unbiased quantification technique was used, the findings indicated that PQ treatment in mice killed TH^+ containing neurons selectively (McCormack *et al.* 2002, Mangano *et al.* 2011).

The results of the current study showed that PQ treatment produce 66 % reduction in the level of DA in ventral mesencephalon measured with HPLC compared with 28 % loss of TH^+ neurons in SNc measured immunocytochemically. Interestingly, similar results have been previously reported which showed that the extent of MPTP-induced DA depletion is usually much greater that extent of neuronal loss in the substantia nigra of mice and monkey (Chan *et al.* 1997, Di Monte *et al.* 2000). This discrepancy might be explained by the usage of different techniques. Immunohistochemical staining is a very efficient technique to provide precise location and accurate estimate of the number of TH^+ neurons in substantia nigra. However, this technique does not provide accurate estimate of the concentration of the neuronal transmitter in nigra. Degenerating neurons, which would very likely produce less amount of TH^+ or DA, will still be stained positively and counted especially when a very sensitive peroxidase method with nickel intensification is employed as the one used in this study.

In these studies, an estimation of DA level resulted in a major decrease in the concentration suggesting a strong correlation to our histopathological reports. Several studies have authenticated the involvement of the DA from the neurons spread through SNc and other regions of mid brain. About sixty percent of reduction in the levels of DA was observed in this current study. Nigrostriatal DA regulates the tone and contraction in skeletal muscles through an influence on postsynaptic D1 and D2 receptors (Korchounov *et al.* 2010, Calabresi *et al.* 2007). To completely estimate the degree and the extent of the progressive neurotoxicity, it will require a long course of study to reproduce the nature of disease which occurs slowly in brain.

Conclusion

In conclusion, the results of the present study have demonstrated that i.p. daily treatment of rats for

3 weeks with PQ induces selective dopaminergic neuronal loss in the substantial nigra and significant behavioral and peripheral motor deficit effects. Additional studies are needed to uncover the mechanisms underlying these effects.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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