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Original Contribution

Paraquat induces behavioral changes and cortical and striatal mitochondrial dysfunction

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ABSTRACT

Paraquat is a highly toxic quaternary nitrogen herbicide capable of increasing superoxide anion production. The aim of this research was to evaluate various behavioral changes and study cortical, hippocampal, and striatal mitochondrial function in an experimental model of paraquat toxicity in rats. Paraquat (10 mg/kg ip) was administered weekly for a month. Anxiety-like behavior was evidenced in the paraquat-treated group as shown by a diminished time spent in, and fewer entries into, the open arms of an elevated-plus maze. Also, paraquat treatment induced a deficit in the sense of smell. In biochemical assays, NADH-cytochrome *c* reductase activity was significantly inhibited by 25 and 34% in cortical and striatal submitochondrial membranes, respectively. Striatal cytochrome oxidase activity was decreased by 24% after paraquat treatment. Also, cortical and striatal mitochondria showed 55 and 74% increased State 4 respiratory rates, respectively. Paraquat treatment decreased striatal State 3 oxygen consumption by 33%. Respiratory controls were markedly decreased in cortical and striatal mitochondria, indicating mitochondrial dysfunction after paraquat treatment, together with mitochondrial depolarization and increased hydrogen peroxide production rates. We demonstrate that paraquat induced alterations in nonmotor symptoms and cortical and striatal mitochondrial dysfunction.

Paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride) is a highly toxic quaternary nitrogen herbicide. Because of its low cost, rapid action, and environmental characteristics it is widely used in agricultural and other settings. Recently, it has come under investigation as a potential environmental neurotoxin associated with increased risk for neurodegenerative disease developing after chronic exposure. In addition, there is an epidemiological correlation between the incidence of sporadic Parkinson disease and environmental exposure to paraquat [1]. Various in vivo studies have shown that paraquat administered repeatedly from 5 days to 6 weeks in mice or rats produces weak or moderate decreases in the number of dopaminergic neurons of the substantia nigra, striatal levels of dopamine and its metabolites, expression and activity of tyrosine hydroxylase, and immunoreactivity of the dopamine transporter [2–4]. These results suggest that, at least within the nigrostriatal system, chronic poisoning with paraquat may trigger in rodents mechanisms similar to those operating in Parkinson disease. In humans, motor impairments cannot be detected until approximately 70–80% of striatal dopamine has already been lost; however, other nonmotor symptoms are evident before the onset of motor disturbances. These include hyposmia/anosmia, gastrointestinal disturbances, sleep abnormalities, autonomic dysfunction, anxiety, depression, and, at later stages, impaired cognition [5,6].

Paraquat toxicity is related to its ability to redox cycle, accepting an electron from an appropriate donor with subsequent reduction in oxygen to produce superoxide anion while also regenerating the parent compound [7].

Castello and colleagues reported that mitochondria are the major source of paraquat-induced reactive oxygen species (ROS) production [8]. Also, Fukushima et al. showed that paraquat may generate ROS by accepting electrons from purified Complex I of the mitochondrial respiratory chain [9] with the subsequent inhibition of this mitochondrial complex; thus, the interaction of paraquat with mitochondria remains an important aspect of its toxicity.

Many neurodegenerative diseases have been associated with mitochondrial dysfunction [10,11]. Defects in mitochondrial electron transfer activities have been detected in Alzheimer, Parkinson, and Huntington disease [12–14]. In particular, several lines of evidence implicate Parkinson disease as a free radical disease involving mitochondrial dysfunction leading to energy failure [15]. Previous in vitro studies from our laboratory showed that direct effects of paraquat on brain cortex mitochondria are associated with increased

Abbreviations: $DiOC_6$, 3,3″′-dihexyloxacarbocyanine iodide; EDTA, ethylenediaminetetraacetic acid; EPM, elevated-plus maze; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; HRP, horseradish peroxidase; MAO, monoamine oxidase; ROS, reactive oxygen species; SOD, superoxide dismutase.

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superoxide production and depolarization of these organelles, showing a clear mitochondrial dysfunction [16].

The aim of this study was to evaluate various behavioral changes in an experimental model of paraquat toxicity, together with the study of cortical, hippocampal, and striatal mitochondrial function. Evaluation of mitochondrial function was achieved by various parameters, such as electron transfer activity, mitochondrial oxygen consumption, mitochondrial membrane potential, hydrogen peroxide (H_2O_2) production rates, and monoamine oxidase (MAO) activity.

Materials and methods

Animals

A total of 24 from two cohorts of Sprague–Dawley female rats (all acquired from the School of Pharmacy and Biochemistry, University of Buenos Aires), weighing 200–250 g at the beginning of experiments (ca. 2.5 months of age), were kept in a soundproof room under a 12/12 h light/dark cycle (lights on at 7:00 AM) and controlled temperature (22 ± 2 °C). Food and water were available ad libitum. All efforts were made to minimize the number of animals used and their pain and discomfort, according to the principles and directives of the European Communities Council Directives (86/609/EEC) and according to the NIH *Guide for the Care and Use of Laboratory Animals.* The procedures also received approval from the local ethics committee. Paraquat was dissolved in saline and administered at a dose of 10 mg/kg ip. Rats treated with paraquat ($n = 12$) received one injection weekly for 4 weeks. This dose of paraquat, which does not induce any systemic toxicity, was chosen in accordance with earlier work [2,17]. Control animals ($n = 12$) were treated with saline ip once a week. Behavioral tests were performed weekly during the experiment starting in the middle of the morning (10:00 AM). Biochemical assays were carried out 2 days after the end of treatment (Fig. 1).

Behavioral tests

Body weight gain was monitored throughout the treatment of both control and paraquat-treated animals. Anxiety-like behavior and nonsocial olfactory acuity were evaluated 3 days after each injection during the whole experiment (Fig. 1).

Elevated-plus maze test

Anxiety-like behavior was evaluated by the elevated-plus maze (EPM) test. The apparatus (made of Plexiglas) comprised two open arms (30×70 cm) alternating at right angles with two closed arms ($30 \times 70 \times 30$ cm), delimiting a central area, the surface of which was 49 cm^2. The whole maze was elevated 60 cm above the floor. Previous to the start of the test, and to habituate the animals to the apparatus, they were individually placed in a rectangular Plexiglas arena (40×40 cm) for 5 min. After that, the rats were placed in the central area of the maze, facing one of the closed arms, and were allowed to explore it for 5 min as previously described [18]. The animal's behavior was videotaped and the percentage of entries into and the time spent in each arm over the total exploration, in both open and closed arms, were calculated following a four-paw criterion: entry into the arm of the EPM was defined as the animal placing all four paws in that particular part of the maze. It is well known that rodents show natural aversion to open surfaces. Behavioral and physiological manifestations of fear (i.e., freezing, defecation, etc.) were seen when they were exposed to an open environment. Based on this fact, the elevated-plus maze test estimates the anxiety-like behavior of the animal by the reduction in the number of open-arm entries (%TSO) and the time spent in them (%FEO) [18]. The test time was recorded using a Sony Handycam video camera. The maze's arms were equally illuminated so that the animals did not perceive lighting differences. Each arm was cleaned with 10% EtOH every time between trials.

Nonsocial olfactory acuity test

To measure nonsocial olfactory acuity, methods were modified from previous ones used by another author [19]. Glass plates with 25 µl of either a novel scent (lemon, peppermint, or vanilla) or water were presented sequentially to the animals. Time spent sniffing each glass plate was recorded for a 3-min session. The obtained results represented the proportion of time sniffing the novel odor. The test time was recorded, again using a Sony Handycam video camera.

Isolation of mitochondria

Brains were quickly removed. Dissection was performed as described by Madison and Edson with some modifications [20]. Immediately after dissection, the cerebral cortex, hippocampus, and striatum from three control and three treated rats were pooled for each experimental point. Tissues were weighed and homogenized (1/5 w/v) in an ice-cold medium consisting of 0.23 M mannitol, 0.07 M sucrose, 10 mM Tris–HCl, and 1 mM EDTA, pH 7.4. Homogenates were centrifuged at 700*g* for 10 min to discard nuclei and cell debris and the supernatant obtained was centrifuged at 8000*g* for 10 min. The resulting pellet containing mitochondria was washed and resuspended in the same buffer at a protein concentration of 20–25 mg/ml [21]. The entire procedure was carried out at 0–2 °C. Submitochondrial membranes were obtained by twice freezing and thawing the mitochondrial preparation and were homogenized by passage through a tuberculin syringe with a needle [22]. The preparation obtained consisted in a fraction of outer and inner membranes, which do not present restriction to substrate access.

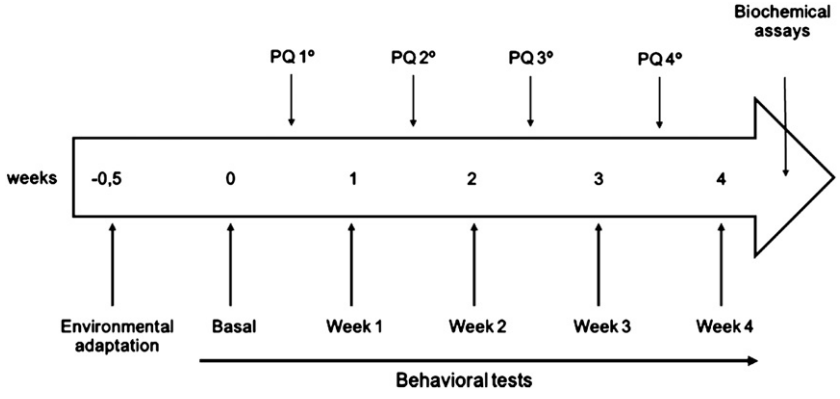

Fig. 1. Time line and experiments. Rats received intraperitoneal treatment with paraquat at a dose of 10 mg/kg or an equivalent of normal saline once a week for a total of four injections and behavioral tests were tested every week for 4 weeks. Two days after the last injection of paraquat, the rats were sacrificed and mitochondria from various brain areas were isolated for biochemical assays.

For mitochondrial membrane potential determinations, brain areas were homogenized in MSH buffer (0.23 M mannitol, 0.07 M sucrose, 5 mM Hepes, pH 7.4) supplemented with 1 mM EDTA and the final mitochondrial pellet was resuspended in MSH buffer without EDTA supplementation.

Protein content was assayed using the Folin phenol reagent and bovine serum albumin as standard [23].

Mitochondrial function and enzyme activities

Mitochondrial electron transfer activities

NADH-cytochrome *c* reductase (Complex I–III) activity was measured in submitochondrial membranes by following spectrophotometrically the reduction of cytochrome *c* at 30 °C at 550 nm ($\varepsilon = 19.6\ mM^{-1}\ cm^{-1}$) in a reaction medium containing 100 mM phosphate buffer (pH 7.4), 0.2 mM NADH, 25 μM cytochrome *c*, and 0.5 mM KCN. Enzyme activity was expressed in nmol cytochrome *c* reduced/min · mg protein. Succinate-cytochrome *c* reductase (Complex II–III) activity was similarly determined and expressed, except that 20 mM succinate was substituted for the NADH.

Cytochrome oxidase activity (Complex IV) was assayed spectrophotometrically at 550 nm by following the rate of oxidation of reduced cytochrome *c*[24]. Cytochrome *c* was reduced with dithionite, which was removed afterward by eluting through a Sephadex-G25 column with potassium phosphate buffer (10 mM), pH 7.4. The reaction was initiated by the addition of 50 μM reduced cytochrome *c* to submitochondrial membranes (0.5 mg/ml) and the rate of reduced cytochrome *c* oxidation was determined as a pseudo-first-order reaction constant (k') (expressed as k'/mg protein).

Mitochondrial respiration

Oxygen uptake was determined in intact isolated mitochondria (0.5–1 mg/ml) with a two-channel respirometer for high-resolution respirometry (Oroboros Oxygraph, Paar KG, Graz, Austria). Mitochondrial respiratory rates were measured in a reaction medium containing 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris–HCl (pH 7.4), 1 mM EDTA, 4 mM $MgCl_2$, 5 mM phosphate, and 0.2% bovine serum albumin at 30 °C. Malate (6 mM) and glutamate (6 mM) were used as substrates for Complex I to measure State 4 respiration and 1 mM ADP was added to measure State 3 respiration [25]. Oxygen uptake was expressed in ng-atom O/min.mg protein. The respiratory control ratio (State 3 respiration/State 4 respiration) was determined to evaluate if the isolation procedure or paraquat treatment affected mitochondrial physiology [26].

Hydrogen peroxide production

Hydrogen peroxide generation was determined in intact isolated mitochondria (0.1–0.3 mg/ml) by the scopoletin–HRP method, following the decrease in fluorescence intensity at 365–450 nm (λ_{ex}–λ_{em}) at 37 °C [27]. The reaction medium consisted of 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris–HCl (pH 7.4), 0.8 μM HRP, 1 μM scopoletin, 7 mM succinate, 6 mM glutamate, and 0.3 μM SOD. Hydrogen peroxide is a product of superoxide anion dismutation. However, part of this superoxide anion is transported to cytosol before its conversion to H_2O_2. Addition of SOD to the reaction medium converts the superoxide anion transported to the cytosol into H_2O_2. This method allows one to determine the H_2O_2 that diffuses from the mitochondria into the Tris buffer. Calibration was made using H_2O_2 (0.05–0.35 μM) as a standard to express the fluorescence changes as nmol H_2O_2/min · mg protein.

Mitochondrial membrane potential

Intact isolated mitochondria were loaded with the potentiometric cationic probe $DiOC_6$ at 30 nM for 20 min at 37 °C in MSH buffer supplemented with 5 mM malate, 5 mM glutamate, and 1 mM phosphate. The procedure was carried out in a dark room. Mitochondria were acquired by a FACScan flow cytometer equipped with a 488-nm argon laser and a 615-nm red diode laser [28]. Mitochondrial fluorescence with no probe and after 0.5 μM FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) treatment was measured as negative and positive controls, respectively. To quantify the resulting changes in membrane potential, a common marker indicating the relative fluorescence intensity of the mitochondrial population analyzed was used.

Monoamine oxidase activity

MAO activity was measured in submitochondrial membranes (0.5 mg/ml) by following spectrophotometrically the oxidation of 100 μM kynuramine in a reaction medium containing 50 mM phosphate buffer, pH 7.4, at 30 °C. Kinetics studies were followed at 360 nm ($\varepsilon = 4.28\ mM^{-1}\ cm^{-1}$) [29].

Drugs and chemicals

Paraquat, kynuramine, ADP, EDTA, glutamic acid, malic acid, mannitol, scopoletin, HRP, succinate, sucrose, SOD, cytochrome *c*, Trizma base, FCCP, and antimycin were purchased from Sigma Chemical (St. Louis, MO, USA). Other reagents were of analytical grade.

Statistical analysis

Results are presented as means ± SEM. Before each analysis, test variables were checked for normality, so all data were evaluated by the Kolmogorov–Smirnov test to follow a posterior parametric or nonparametric statistical analysis. Biochemical results were compared using the unpaired independent Student's *t* test to analyze the significance of differences between two groups. Behavioral results were performed using repeated-measures two-factor ANOVA and unpaired independent samples Student's *t* test. SPSS (version 13.0) statistical software was used and a difference was considered statistically significant when $p<0.05$.

Results

Behavioral tests

Paraquat-treated rats display alterations in body weight gain

The animals' body weight (monitored weekly) is shown in Fig. 2. Paraquat-treated and control rats gained body weight on average ($p<0.001$, in-group differences for all the evaluated time points). However, the body weight gain of the paraquat-treated group was lower than that of controls in the last week ($p<0.01$, paraquat vs control group at the fourth week). It is important to remark that the

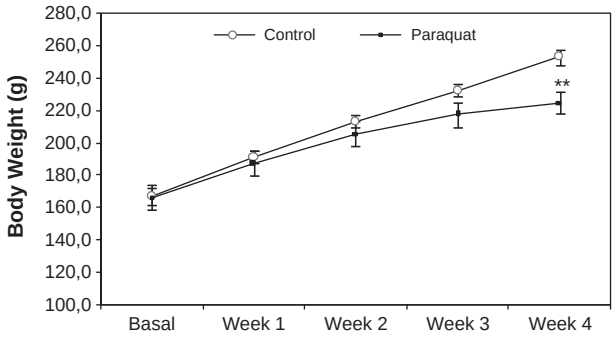

Fig. 2. Effect of paraquat treatment on body weight gain. The animals' body weight was monitored weekly, as described under Materials and methods. Results represent the body weight ± SEM for 12 animals per group. Unpaired independent samples Student's *t* test was used (**$p<0.01$ compared with control value at the fourth week).

rats survived the treatment well and their physical appearance did not change during the experiment.

Paraquat-treated rats display an anxiety-like phenotype

Anxiety-like behavior was measured in control and paraquat-treated rats using the elevated-plus maze test. As shown in Fig. 3A, animals treated with paraquat spent significantly less time in the open arms (%TSO) compared to controls ($p<0.001$, control vs paraquat treated rats from the first to the fourth week). There were in-group differences for paraquat treatment at the first and third week ($p<0.05$, in-group paraquat treatment, comparisons between basal and first and third weeks). Another parameter, evaluated by the maze, was the frequency of entrance into open arms (%FEO) (Fig. 3B). The control group displayed similar %FEO throughout the evaluation. Also, animals treated with paraquat entered fewer times into the open arms from the first week of paraquat treatment ($p<0.05$, in-group paraquat treatment, basal vs first and second weeks; $p<0.01$, basal vs third and fourth weeks). However, control group frequencies were higher than those for the paraquat-treated group in all the cases. Significance values between control and paraquat-treated animals for %FEO are explained in detail in the Fig. 3B legend.

Paraquat-treated rats display progressive olfactory discrimination deficits

Rats were tested for olfactory acuity using the scents of lemon, vanilla, and peppermint beginning at the basal point and during the whole treatment (four weekly points) as shown in Fig. 4. When given the choice between a novel odor (vanilla, lemon, or peppermint) and water, control groups showed a preferential exploration of the novel scent, approximately 70%, during the treatment ($p<0.001$, significant difference between water and novel odor). This result was also observed for rats and mice by another author who worked with Parkinson animal models [18]. In general, there were no in-group differences for controls. Rats treated with paraquat lost the ability to discriminate between vanilla and water from the second week of paraquat treatment (Figs. 4A and B). The observed differences referred to in-group differences, which indicate that animals treated with paraquat spent significantly less time sniffing vanilla from the second to the fourth week than at the basal and first points. The same happened when the rats were given the choice between peppermint and water (Figs. 4C and D). Together with this, the rats spent more time sniffing water than peppermint at the second and third week. The exact same behavioral pattern was observed when given the choice between lemon and water (Figs. 4E and F). Significance values between control and paraquat-treated animals for vanilla, peppermint, and lemon are explained in detail in the Fig. 4 legend.

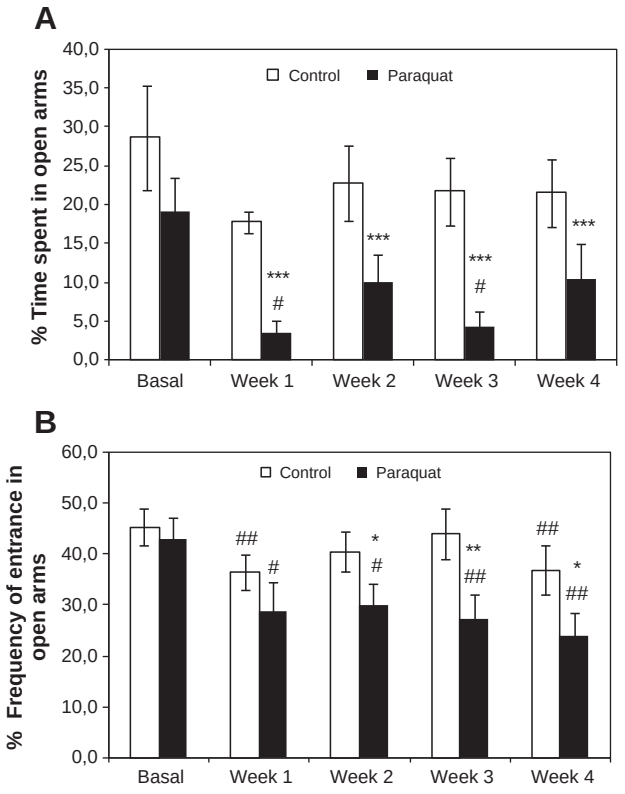

Fig. 3. Effect of paraquat treatment on anxiety-like behavior. Anxiety-like behavior was measured using the elevated-plus maze test, as described under Materials and methods. (A) %TSO. Bars represent the means of proportion of time (%) spent in open arms ± SEM. Repeated-measures two-factor ANOVA was used (#$p<0.05$) for in-group difference compared with basal point. Unpaired independent samples Student's *t* test was used (***$p<0.001$) for intergroup differences. (B) %FEO. Bars represent the means of proportion of entrance into open arms ± SEM. Repeated-measures two-factor ANOVA was used (#$p<0.05$, ##$p<0.01$) for in-group difference compared with basal point. Unpaired independent samples Student's *t* test was used (*$p<0.05$, **$p<0.01$) for intergroup differences.

Mitochondrial function and enzyme activities

Paraquat treatment modifies cortical and striatal mitochondrial electron transfer activities

Mitochondrial respiratory complex activities were measured in submitochondrial membranes from various brain areas of control or paraquat-treated rats. Table 1 shows that NADH-cytochrome *c* reductase activity was significantly inhibited by 25 and 34% in cortical and striatal submitochondrial membranes, respectively, from paraquat-treated animals ($p<0.05$, compared with control values), but no changes were observed in succinate-cytochrome *c* reductase activity in these areas. A decreased NADH-cytochrome *c* reductase (Complex I–III) activity with an unaffected succinate-cytochrome *c* reductase (Complex II–III) activity corresponds to a decreased Complex I (NADH-ubiquinone reductase) activity. Therefore, paraquat treatment did not affect Complex III activity. No significant changes were observed in hippocampal mitochondrial Complex I, II, and III activities.

Cytochrome oxidase (Complex IV) activity was decreased by 24% in striatal submitochondrial membranes from paraquat-treated rats ($p<0.05$, compared with control animals); however, this effect was not observed in cortical and hippocampal submitochondrial membranes after paraquat treatment.

Paraquat treatment impairs cortical and striatal mitochondrial respiration

The malate–glutamate-dependent oxygen consumption was measured in State 4 (at rest or controlled respiration) and in State 3 (active respiration, the maximal physiological rate of O_2 uptake and ATP synthesis) [30]. The respiratory control ratio (the most sensitive indicator of mitochondrial oxidative phosphorylation coupling) was calculated as the relationship between State 3 and State 4 respiration rate.

Table 2 shows oxygen consumption rates of cortical, hippocampal, and striatal intact mitochondria isolated from control and paraquat-treated animals. Cortical and striatal mitochondria from paraquat-treated rats showed 55 and 74% increased State 4 respiratory rates, respectively ($p<0.05$ vs control). Also, paraquat treatment decreased striatal oxygen consumption by 33% in State 3 ($p<0.05$, compared with State 3 control value). Respiratory controls of cortical and striatal mitochondria from paraquat-treated rats were 1.75 ± 0.08 and 1.56 ± 0.07, respectively ($p<0.05$, compared with control values), indicating the presence of dysfunctional mitochondria after in vivo treatment with paraquat. No significant changes were observed in State 4 and State 3 respiratory rates in hippocampal mitochondria from paraquat group.

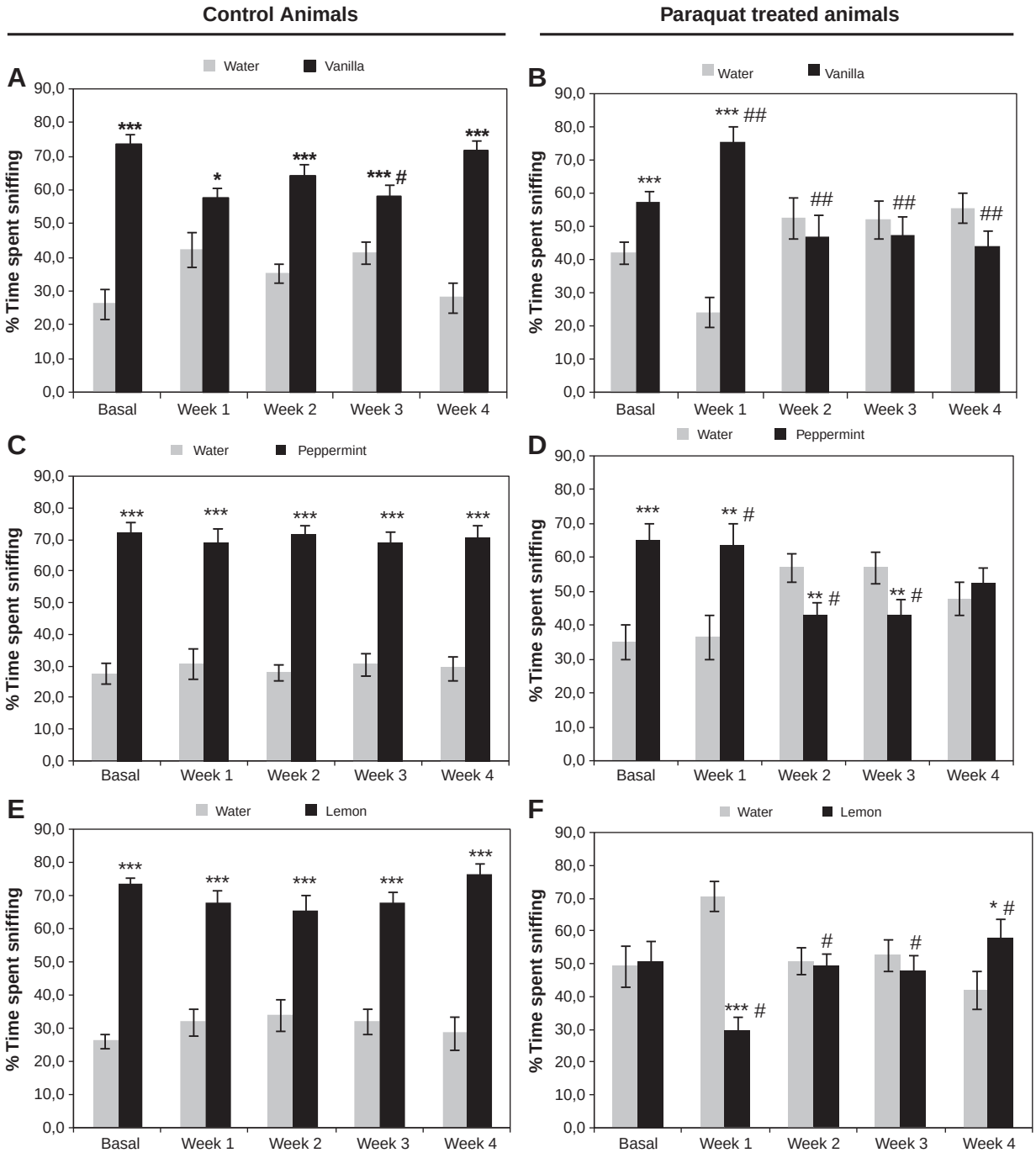

Fig. 4. Effect of paraquat treatment on olfactory discrimination. Rats were tested for olfactory acuity using various scents, as described under Materials and methods. Bars represent the time spent investigating each scent ± SEM for 12 animals per group. (A) Control animals—vanilla. Unpaired independent samples Student's *t* test was used (*$p<0.05$, ***$p<0.001$) for intergroup differences compared with basal point. Repeated-measures two-factor ANOVA was used (#$p<0.01$) for in-group difference compared with basal point. (B) Paraquat treated animals—vanilla. Unpaired independent samples Student's *t* test was used (***$p<0.001$) for intergroup differences compared with basal point. Repeated-measures two-factor ANOVA was used (##$p<0.01$) for in-group difference compared with basal point. (C) Control animals—peppermint. Unpaired independent samples Student's *t* test was used (***$p<0.001$) for intergroup differences compared with basal point. (D) Paraquat-treated animals—peppermint. Unpaired independent samples Student's *t* test was used (**$p<0.01$, ***$p<0.001$) for intergroup differences compared with basal point. Repeated-measures two-factor ANOVA was used (#$p<0.05$) for in-group difference compared with basal point. (E) Control animals—lemon. Unpaired independent samples Student's *t* test was used (***$p<0.001$) for intergroup differences compared with basal point. (F) Paraquat-treated animals–lemon. Unpaired independent samples Student's *t* test was used (*$p<0.05$, ***$p<0.001$) for intergroup differences compared with basal point. Repeated-measures two-factor ANOVA was used (#$p<0.05$) for in-group difference compared with basal point.

Paraquat treatment induces cortical and striatal mitochondrial depolarization

Mitochondrial potential represents a parameter of mitochondrial function. Intact mitochondria isolated from various brain areas of control and paraquat-treated animals were used to determine membrane potential using flow cytometry and $DiOC_6$ as a fluorescent probe. The mitochondrial population and the autofluorescence level of unloaded samples are shown for all tissues: cerebral cortex (Fig. 5A), hippocampus (Fig. 5B), and striatum (Fig. 5C). As shown by the histograms, paraquat treatment induced an important decrease in FL-1 $DiOC_6$ fluorescence in cortical and striatal mitochondria compared with control mitochondria (Figs. 5A and C), indicating mitochondrial depolarization. However, paraquat did not disturb hippocampal mitochondrial polarization (Fig. 5B). As expected, an

Table 1
Effects of paraquat treatment on mitochondrial electron transfer activity from various brain areas.

Enzyme activity	Cortex		Hippocampus		Striatum	
	Control	Paraquat	Control	Paraquat	Control	Paraquat
NADH-cytochrome *c* reductase (nmol/min · mg protein)	79 ± 4	59 ± 3*	80 ± 8	79 ± 9	154 ± 8	101 ± 4*
Succinate-cytochrome *c* reductase (nmol/min · mg protein)	5.2 ± 0.4	6.3 ± 0.5	13.5 ± 0.4	12.8 ± 0.8	11 ± 1	13 ± 2
Cytochrome oxidase (k'/mg protein)	266 ± 27	321 ± 42	256 ± 11	238 ± 7	180 ± 13	136 ± 10*

Values represent the means ± SEM of 4–6 individual mitochondria samples, each obtained from a pool of cerebral cortex, hippocampus, or striatum of three rats. Unpaired independent Student's *t* test was used to compare with control value.
*$p<0.05$.

important decrease in $\Delta\Psi_m$ was detected in mitochondria from the three brain areas studied after 0.5 μM FCCP addition (used as a positive control; Figs. 5A, B, and C).

Quantification of $DiOC_6$ fluorescence under the various conditions is presented in Fig. 5D. Paraquat treatment decreased cortical and striatal mitochondrial membrane potential by 25 and 22%, respectively ($p<0.01$, compared with control mitochondria).

Paraquat treatment increases cortical and striatal mitochondrial hydrogen peroxide production

Hydrogen peroxide production rates were determined in intact mitochondria isolated from control and paraquat-treated rats (Fig. 6A). Control H_2O_2 production rates were 0.46 ± 0.01, 0.54 ± 0.03, and 0.63 ± 0.04 nmol/min · mg protein in cortical, hippocampal, and striatal mitochondria, respectively, supplemented with succinate and glutamate. Hydrogen peroxide production rates were significantly increased by 13 and 48% in cortical and striatal mitochondria, respectively, from paraquat-treated animals ($p<0.01$, compared with control values). No significant changes were observed in H_2O_2 production of hippocampal mitochondria from paraquat-treated animals.

Paraquat treatment does not modify monoamine oxidase activity

Activity of MAO was measured in submitochondrial membranes from control and paraquat-treated animals in various brain areas. As shown in Fig. 6B, paraquat treatment was not able to produce changes in MAO activity in any brain area studied.

Discussion

Paraquat induces a decrease in body weight gain and nonmotor symptoms

Although Parkinson disease (PD) has been traditionally considered a neurodegenerative motor disorder, many studies show that there is an increasing recognition of early nonmotor symptoms, suggesting the disease is more multifaceted than commonly thought [18]. Ossowska and colleagues reported that weekly doses of paraquat induced a small decrease in the number of dopaminergic neurons in the substantia nigra [31]. Taking into account that motor symptoms in PD are evidenced with a decrease of 70% in the number of dopaminergic neurons, we focused our study on the evaluation of nonmotor symptoms as predictors of the early stages of Parkinson disease.

Body weight gain was studied throughout the experiment. We found that, although animals gained weight on average, the paraquat group's body weight was significantly lower than that of controls at the fourth week. In this sense, Kang et al. found that body weight significantly decreased in the third week of treatment in an experimental design of twice weekly injection of paraquat for 3 weeks [32]. We consider that the difference between that and our results is explained by the frequency of paraquat injection. As we mentioned before, rats survived the treatment well and their physical appearance and normal activity did not exhibit changes during the experiment. We suggest that the decrease in body weight could be related to an inflammatory stress response to paraquat, which made the rats eat less than control animals, as suggested by other authors [1,33]. Consistent with this, Kang and colleagues associated the loss of body weight with decreased food and water consumption.

Taylor and colleagues demonstrated that severe reduction of vesicular monoamine transporter does trigger anxiety-like behavior that preceded depressive symptoms in mice [18]. Our results show that paraquat treatment induced anxiety-like behavior from the first paraquat injection. This was evidenced by both variables studied: time spent in and frequency of entrance into a maze's open arms. The decrease in frequency of entrance into the open arms was persistent up to the end of the experiment without changes throughout the weekly tests. However, the time spent in the open arms exhibited in-group fluctuations across the treatment. But, when we compared the control with the paraquat-treated group, there was a decrease in both variables week by week.

One of the earliest manifestations of PD is the loss of the sense of smell. In humans, olfaction deficits are presented in the 100% of PD patients and, in the majority of the cases, this change is detected before the onset of motor symptoms [34]. Moreover, it was demonstrated that rodents presented olfactory abnormalities in association with reduced monoamine storage capacity [18]. In this study, we showed that exposure to paraquat during 4 weeks induced

Table 2
Effects of paraquat treatment on mitochondrial oxygen consumption of various brain areas.

Condition	Oxygen consumption (ng-atom O/min.mg protein)					
	Cortex		Hippocampus		Striatum	
	Control	Paraquat	Control	Paraquat	Control	Paraquat
State 4	12.2 ± 0.3	18.9 ± 0.9*	14.3 ± 0.9	17 ± 2	11.7 ± 0.3	20.4 ± 0.5*
State 3	42.2 ± 0.9	39 ± 2	49 ± 7	39 ± 9	48 ± 4	32 ± 2*
Respiratory control	3.46 ± 0.03	2.06 ± 0.08*	3.4 ± 0.2	2.3 ± 0.3	4.10 ± 0.09	1.56 ± 0.07*

Malate and glutamate were used as substrates as described under Materials and methods. Values represent the means ± SEM of four to six individual mitochondria samples, each obtained from a pool of cerebral cortex, hippocampus, or striatum of three rats. Unpaired independent Student's *t* test was used to compare with control value.
*$p<0.05$.

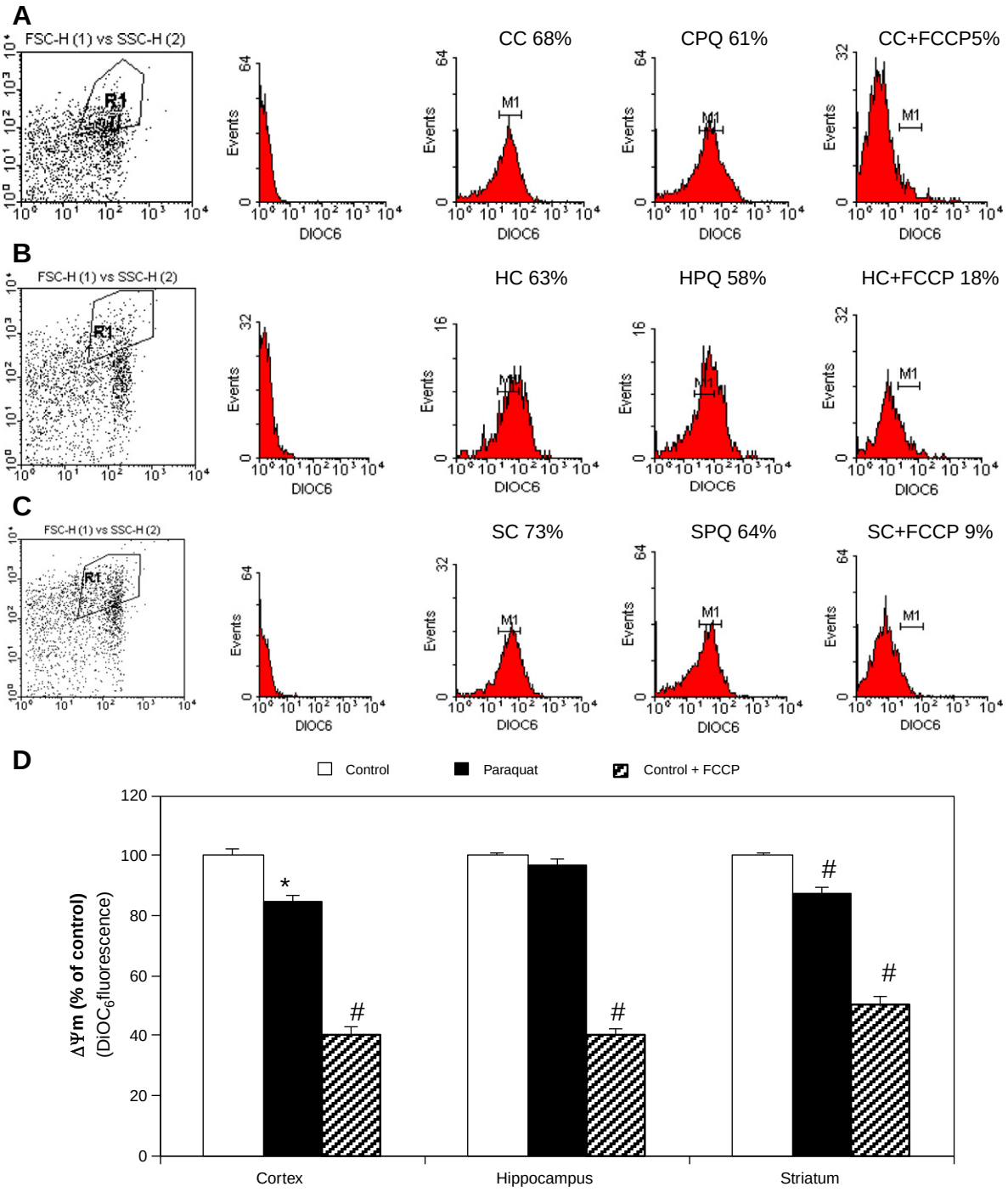

Fig. 5. Evaluation of mitochondrial membrane potential ($\Delta\Psi_m$) through the changes in $DiOC_6$ fluorescence intensity. Histograms of gated mitochondrial events (R1) versus relative fluorescence intensity (FL-1) from control and paraquat-treated animals and FCCP-treated mitochondria are shown. (A) Cerebral cortex, (B) hippocampus, and (C) striatum. Each histogram represents a typical experiment of three. Assessment of $\Delta\Psi_m$ was performed as described under Materials and methods. CC, cerebral cortex control animals; CPQ, cerebral cortex paraquat-treated animals; HC, hippocampus control animals; HPQ, hippocampus paraquat-treated animals; SC, striatum control animals; and SPQ, striatum paraquat-treated animals. (D) Bar graph of $DiOC_6$ fluorescence quantification corresponding to relative values of mitochondrial transmembrane potential, as described. Bars represent the means ± SEM of three experiments. Unpaired independent samples Student's *t* test was used ($^*p<0.01$, compared with control value). Repeated-measures two-factor ANOVA was used ($\#p<0.05$) for in-group difference compared with basal point.

a deficit in the sense of smell. In this context, it is interesting to note that the olfactory deficit observed does not seem to take the course of a stable decline in smell function. For instance, based on our results, animals lost their ability to discriminate novel odors (vanilla, peppermint, or lemon) after the second injection of paraquat and this is conserved to the end of treatment. However, we found an unexpected result when rats were given the choice between lemon and water at the fourth week. Before starting the experiment, the rats did not show a preference for lemon compared with water, but they lost almost all their ability to discriminate lemon after the first injection and they exhibited more time sniffing lemon than water after the fourth injection. This result is quite different from the one we obtained for vanilla and peppermint. We believe that lemon did not have as strong an effect at the basal point as we found for the other odors.

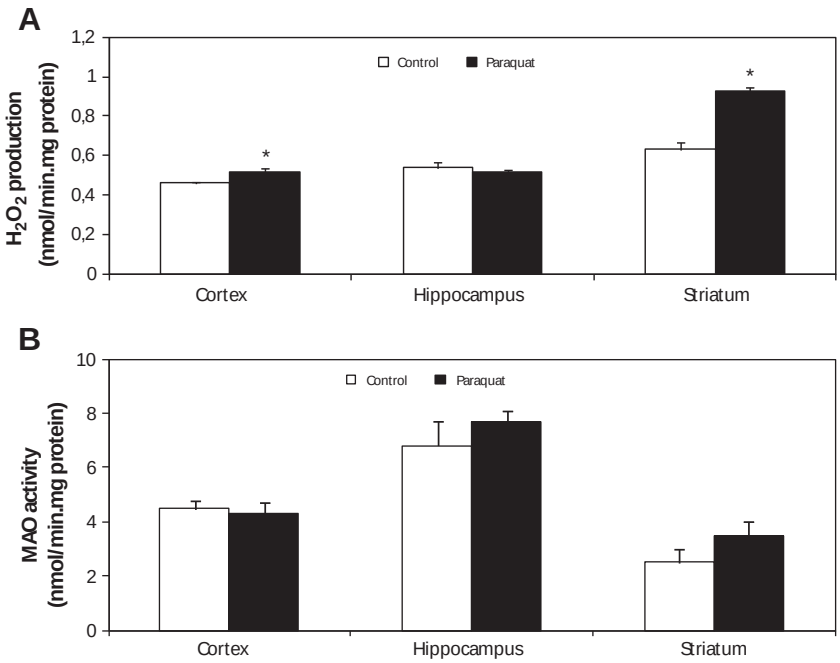

Fig. 6. Effect of paraquat treatment on mitochondrial H_2O_2 production rate and MAO activity from various brain areas. (A) Hydrogen peroxide production. Mitochondria were supplemented with 7 mM succinate and 6 mM glutamate, as described under Materials and methods. Bars represent the means ± SEM of four to six individual mitochondria samples, each obtained from a pool of cerebral cortex, hippocampus, or striatum of three rats. Unpaired independent samples Student's *t* test was used (*$p<0.05$ compared with control value). (B) MAO activity. Submitochondrial membranes were supplemented with 100 μM kynuramine as substrate for the enzyme, as described under Materials and methods. Bars represent the means ± SEM of four to six individual mitochondria samples, each obtained from a pool of cerebral cortex, hippocampus, and striatum of three rats.

Paraquat treatment induces cortical and striatal mitochondrial dysfunction

Paraquat toxicity has been related to mitochondrial superoxide anion production. Enzymes capable of initiating this reaction have been identified in mitochondria, microsomes, plasma membrane, and cytosolic cellular components in various organ systems. It has been described that Complexes I and III of the mitochondrial inner membrane are capable of generating ROS after mitochondria incubation with paraquat [35,36]. We evaluated the effects of in vivo paraquat treatment on respiratory complex activity in three different brain areas. In accordance with Cocheme et al. [35], paraquat treatment inhibited Complex I activity in cortical and striatal mitochondria but no changes were observed in the activity of Complex III of the respiratory chain in any of the brain areas studied. As shown in Table 1, paraquat treatment also inhibited Complex IV activity only in striatal mitochondria.

Disruption of mitochondrial processes was observed in the pathophysiology of several psychiatric and neurological disorders. Results from our laboratory have confirmed that paraquat-induced oxidative stress is mediated by superoxide anion and H_2O_2 production associated with an impairment of mitochondrial respiration, effects prevented by cannabinoid mitochondrial pretreatment [16]. In this study, we evaluated mitochondrial function of cortical, hippocampal, and striatal mitochondria through measurements of oxygen consumption, H_2O_2 production, and mitochondrial membrane potential after in vivo paraquat treatment.

Gray et al. have observed that 100 μM paraquat increases cellular oxygen consumption [37]. This activity was not inhibited by cyanidine or antimycin A, indicating that the actions of paraquat were not dependent on mitochondrial respiration. It is well known that redox cycling reactions consume significant amounts of oxygen, reducing the levels of oxygen available for cellular metabolic processes. Our results show that in vivo paraquat treatment increases State 4 respiration in cortical and striatal mitochondria from paraquat-treated animals. This effect could be a consequence of the increased oxygen utilization by redox cycling of paraquat. Moreover, State 3 oxygen consumption was decreased in striatal mitochondria after paraquat treatment in association with the inhibition of striatal cytochrome oxidase activity.

Hydrogen peroxide is normally formed during basal respiration [38] and also is a product of dopamine metabolism by MAO [39]. Previous reports described that paraquat interacts in vitro with Complex I to generate superoxide anion and H_2O_2[35]. The above results show that in vivo paraquat treatment increased cortical and striatal H_2O_2 production rates. However, no significant changes were observed in MAO activity, suggesting that the observed increase in H_2O_2 is independent of MAO activity. In addition, no significant changes were observed in H_2O_2-catabolizing enzymes (catalase and glutathione peroxidase) measured in mitochondrial fractions from control and paraquat-treated animals (data not shown). Because in our experimental model, 2 days after drug exposure, the paraquat redox cycling was not present anymore, the increased H_2O_2 observed could be due to a permanent alteration in the mitochondrial Complex I induced by paraquat.

Several studies have shown that in vitro paraquat treatment induces the collapse of the mitochondrial membrane potential. This effect was observed in human alveolar epithelial A549 cells [40], human corneal endothelial cells [41], rat adrenal pheochromocytoma PC12 cells [39], and neuroblastoma SH-SY5Y cells [42]. We found that paraquat induced membrane depolarization in cortical and striatal mitochondria from treated animals, indicating mitochondrial dysfunction. The mechanism involved could be related to the effect of paraquat on the permeability transition pore opening by increased superoxide anion production, the increased nitric oxide production [43], or the increased Ca^{2+}-dependent permeability of the inner mitochondrial membrane [44].

It is important to mention that no significant changes were observed after paraquat treatment in any of the mitochondrial functionality parameters evaluated in hippocampal mitochondria, indicating that this area is more resistant to paraquat-induced toxicity in our model of study.

It is postulated that alterations in mitochondrial function and in active oxygen species generation could play a role in the induction of various signaling pathways leading to neuronal death. Dopaminergic neuronal death occurs in animals exposed to paraquat [2,31]. However, the factors or mechanisms by which paraquat kills dopaminergic neurons are poorly understood. Further experiments would be conducted to elucidate the involvement of mitochondria in the molecular mechanism of apoptosis induced by paraquat in our experimental model.

Conclusions

Summing up, this study demonstrates that a weekly dose of paraquat (10 mg/kg ip) for 4 weeks induced alterations in nonmotor symptoms shown by an increase in anxiety-like behavior and a dysfunction in nonsocial olfactory discrimination. Also, it induced cortical and striatal mitochondrial dysfunction as showed by decreased mitochondrial respiratory control, increased H_2O_2 production, and loss of membrane potential. However, it is necessary to carry out further experiments to establish causality between mitochondrial dysfunction and behavioral changes.

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