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Minocycline Protection against Paraquat Toxicity in *Drosophila melanogaster*

E. Bonilla[1,3], S. Medina-Leendertz[1*], M. Mora[1], JR. Vielma[1], L. Atencio-Bracho[2], Y. Bravo[1], M. Paz[1], A. Leal-Yépez[1], JL. Arcaya[3]

[1]Laboratorio de Neurobiología, Centro de Investigaciones Biomédicas, Instituto Venezolano de Investigaciones Científicas (IVIC), Estado Zulia, Venezuela
[2]Laboratorio de Genética y Biología Molecular, Departamento de Biología, Facultad Experimental de Ciencias, Universidad del Zulia, Maracaibo, Estado Zulia, Venezuela
[3]Facultad de Medicina, Instituto de Investigaciones Clínicas "Dr. Américo Negrette", Universidad del Zulia, Maracaibo, Estado Zulia, Venezuela
*sml24567@gmail.com

Abstract

We determined the effect of long-term administration (12 days) of minocycline (Mino) on the oxidative stress induced by paraquat (PQ) in *D. melanogaster*. After intoxication with PQ (40 mM) for 36 hours, the intoxicated group was subdivided in three groups: PQ (no further treatment after PQ intoxication), PQ-Mino (treated with Mino [0.05 mM] for 12 days after intoxication) and PQ-Control (exposed to PQ and maintained in standard medium for 12 days after intoxication). Two additional groups, not treated with PQ, were added: Control and Mino. After 36 hours intoxication the concentrations of H2O2, the activities of superoxide dismutase (SOD), Catalase (CAT), glutathione peroxidase (GSH-Px) and nitric oxide synthase (NOS) in the PQ group were increased, but the levels of nitrite and glutathione (GSH) were reduced with respect to the Control. In the group PQ also were observed an increase in the concentrations of MDA, protein carbonyl and the 8-hydroxydeoxyguanosine (8-OHdG) compared with Control. The post-treatment by 12 days with minocycline (PQ-Mino) mitigated oxidative damage induced by PQ as evidenced by the reduction in the concentration of the oxidative stress markers and by decrease damage to the lipid membrane (MDA), protein (protein carbonyl), DNA (8-OHdG) and in an increase of the extension of the life cycle of the flies with respect to PQ-Control.

Keywords: *Drosophila melanogaster*, minocycline, oxidative stress, paraquat.

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Introduction

Paraquat (1,1-dimethyl-4, 4'-bipyridinium dichloride) (PQ), a quaternary ammonium compound with a similar structure to MPP+, the active metabolite of MPTP (phenyl-1-methyl-4-1,2,3,6-tetrahydropyridine) is highly toxic. This compound has the ability to induce neurotoxic effects which resemble the characteristics of Parkinson's disease in rodents, in nonhuman primates and in humans. [1] Paraquat toxicity starts with the generation of the superoxide radical followed by other reactive oxygen species (ROS). [2] Acute paraquat intoxication can increase the levels of nitric oxide (NO) and the expression of inducible nitric oxide synthase (iNOS), resulting in the formation of peroxynitrite. [3] Additionally, NOS acts as a diaphorase that increases the production of the superoxide anion. [4]It is known, that free radicals play an important role in many diseases and pathological conditions such as: cancer, diabetes, multiple sclerosis etc. [5]

Drosophila melanogaster has been used as a suitable organism to model the damage caused by pesticides as PQ and rotenone, on dopaminergic neurons. [6] Reactive oxygen species are continuously produced in aerobic organisms as part of the physiological and metabolic processes. These ROS endogenously produced and oxygen

* *Author for correspondence*

free radicals have important biological functions, but because of their reactive nature, they generate oxidative damage of membrane lipids, proteins and DNA. [5, 7]

Minocycline (Mino) is an antibiotic that belongs to the family of tetracyclines. In addition to its antibiotic properties, some authors have shown that it has antioxidant properties, either directly by its ability to capture free radicals or by its direct action on the enzyme complexes forming free radicals or indirectly by blocking the activation of microglia. [8,9]Minocycline is protective against acute injury to the central nervous system and neurodegenerative disease models, including mouse model of Parkinson's disease, Amyotrophic Lateral Sclerosis, and Huntington´s disease. [8]In mitochondria, Mino prevents the collapse of the membrane potential and the release of apoptotic factors. [8] Huang *et al.,* (2012) demonstrated that the mechanism of minocycline in preventing PQ-induced apoptosis might be mediated by attenuating endoplasmic reticulum (ER) stress and mitochondrial dysfunction, which respectively result in caspase 12 activation and the release of H_2O_2, HtrA2/Omi and Smac/Diablo. [10]

In the study we determined the effect of long-term administration of Mino on oxidative stress markers (H_2O_2, GSH, NO, SOD, CAT, GSH-Px NOS) and to determine the damage to the membrane lipids, proteins and DNA induced by acute PQ intoxication in *D. melanogaster*.

Materials and Methods

Experimental animals stock

Male flies of *D. melanogaster* (wild-type, Oregon R strain) were used. Flies were maintain]ed in 12h/12h light/dark cycle at 25°C and fed standard corn meal prepared as described previously. [11]

Paraquat-acute intoxication and antioxidant post-treatment

Two days old flies (300 per group) were transferred to vials containing only filter paper soaked with 40 mM paraquat (SIGMA) in 5% sucrose solution. After 36 hours intoxication, flies were divided in three groups: PQ (**no further treatment after PQ intoxication,** the flies were sacrificed after 36 hours of the PQ intoxication with their respective control), PQ-Mino (treated with Mino [0.05 mM; SIGMA] for 12 days after intoxication) and PQ-Control (36 hours of PQ intoxication and maintained in standard corn meal for 12 days after intoxication). Two additional groups without pre-intoxication with PQ were used: Control (maintained in standard corn meal) and Mino (maintained in standard corn meal and treated with minocycline for 12 days). The flies were transferred daily to fresh medium during 12 days. Three replicates of each treatment were carried out.

Hydrogen peroxide determination

A homogenate of whole body of *D. melanogaster* was prepared with 50 flies in 500 µL PBS, using a glass-glass homogenizer. The homogenate was centrifuged at 10,000 g for 5 min. at 4°C. Ninety µL of the supernatant were removed to determine H_2O_2. The OXISELEC™ hydrogen peroxide Assay Kit (CELL BIOLABS, INC) was used. The absorbance was read at 540 nm. The results were expressed in nmol H_2O_2/mg protein. Assays were performed in triplicate.

Determination of nitric oxide (•NO)

The QuantiChrom™ (D2NO-100) kit was used for determining the concentration of nitrites (NO_2^-) present in the samples; it is designed to measure the production of •NO, followed by the reduction of nitrate to nitrite using an improved Griess method. Whole body homogenates of *D. melanogaster* were prepared with 50 flies in 500 µL of PBS, using a glass-glass homogenizer. The homogenates were centrifuged at 10,000 g for 5 min at 4 °C. The supernatants were used for the determination of NO_2, following the protocol described in the kit. Absorbance was read at 540 nm. Results were expressed in µM of nitrite/mg protein and assays were performed in triplicate.

Determination of total protein

In all cases where the determination of the protein concentration was needed, was used the Bicinchoninic Acid Protein Assay Kit BCA1 (SIGMA). The absorbance was measured at 562 nm (SYNERGY HT, BIOTECK). Assays were performed in triplicates. The results were expressed in mg protein/mL.

Activity of cytosolic superoxide dismutase (SOD, EC 1.15.1.1)

Whole body homogenates were prepared from *D. melanogaster* with 10 flies in 400 µL of ice - cold lyses

buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 0.1 mM EDTA, 0.5% Triton X-100), using a glass - glass homogenizer. The homogenates were centrifuged at 12,000 g for 10 min at 4°C. The supernatant was removed to determine SOD activity. The OXISELEC™ superoxide dismutase activity assay kit (CELL BIOLABS, INC) was used to measure the enzyme activity. Seventy µL of the supernatant were removed and the activity was determined following the kit instruction. The absorbance was read at 490 nm. The results were expressed in Units/mg protein. Assays were performed in triplicate.

Determination of catalase (CAT, EC 1.11.1.6)

A homogenate of whole body of *D. melanogaster* was prepared with 10 flies in 500 µL ice-cold PBS with 1mM EDTA per gram of tissue, using a glass-glass grinder. The homogenate was centrifuged at 10,000 g for 15 min at 4 °C. The supernatant was removed to determine the catalase activity using the OXISELEC™ Catalase activity Assay Kit (CELL BIOLABS, INC). Twenty µL of the supernatant were removed and the activity of catalase was determined following the protocol described in the kit. The absorbance was read at 520 nm. Assays were performed in triplicate. The results were expressed in Units/mg protein.

Determination of glutathione peroxidase (GSH-Px, EC 1.11.1.9)

The homogenates of whole body of *D. melanogaster* were prepared with 70 flies in 500 µL of PBS using a glass-glass homogenizer. The homogenates were centrifuged at 10,000 g for 10 min at 4°C. The supernatants were used to determine the glutathione peroxidase activity, using the EnzyChrom™ (EGPX-100) kit; 360 µL of distilled water were placed in a tube for NADPH, for the positive control, 500 µL of assay buffer was added to the respective tube. 6 mM NADPH was prepared by mixing 12 µL of calibrator with 188 µL of distilled water; 10 µL of the samples and the positive control of GPX were transferred to the wells, including a control containing 10 µL of buffer. To each well 84 µL of assay buffer, 2 µL glutathione, 3 µL of 35 mM NADPH, and 8 µL GR enzyme were added. Of this working reagent, 90 µL were added to each sample and control. Five µL of diluted cumene hydroperoxide were added and mixed with vortex for 30 seconds. This solution was diluted 1:10 in water to obtain a 1X substrate solution. For all samples and controls, 100 µL of the diluted solution of cumene hydroperoxide 1X were added. The plate was covered, stirring gently to mix the contents and the absorbance were read at 340 nm at time 0 (T0) and after 4 minutes (T4). The results of GPX activity were expressed in Units/L. Assays were performed in triplicate.

Determination of glutathione

The EnzyChrom™ assay kit GSH/GSSG was used (EGTT-100). To measure the concentration of glutathione 40 flies were homogenized in 200 µL of cold buffer [50 mM phosphate (pH 7.0), 1 mM EDTA and 20 µL capturer]. The homogenates were centrifuged at 10,000 g × 5 min at 4°C and the supernatants were transferred to clean tubes for the deproteinization. For the determination of reduced glutathione (GSH) 40 flies were homogenized in 1 mL of cold buffer (50 mM phosphate pH 7.0 and 1 mM EDTA). The samples were centrifuged for 15 minutes (10,000 g at 4°C); the supernatants were transferred to clean tubes to carry out the deproteinization using a solution of 5% metaphosphoric acid (RX MPA). A standard curve for GSH was prepared following the manufacturer's instructions and the absorbance was read at 412 nm at 0 min and 10 min. Assays were performed in triplicate. The results were expressed in µM of GSH/mg protein.

Determination of the activity of nitric oxide synthase (NOS, EC 1.14.13.39)

Nitric oxide synthase was determined using the EnzyChrom™ kit (ENOS-100) (BioAssay SYSTEMS). The assay consists of two steps: in the first step, the NOS enzyme reacts with the substrate to form •NO that was measured indirectly by following the reduction of nitrate to nitrite using the improved Griess method. Seventy flies per treatment group were homogenized in 1X PBS (pH 7.4) and centrifuged for 15 minutes (10,000 g at 4°C). The supernatants were used to determine the NOS activity according to the kit's instructions. The results were reported in Units/L. Assays were performed in triplicate.

Damage to membrane lipids (lipid peroxidation determination)

Mitochondria from whole body of *D. melanogaster* were prepared by differential centrifugation, following the protocol described by Fernandez-Vizarra *et al.*, (2009) and

modified by Mora *et al.* (2014). [12-13] All reagents were from SIGMA- ALDRICH, USA. Briefly, with glass-glass grinder, a homogenate was prepared with one hundred and fifty flies in 990 µL ice-cold Tris-sucrose buffer (0.32 M sucrose, 1 mM EDTA and 10 mM Tris-HCl at pH 7.4) containing 1% butylated hydroxytoluene (BHT). The homogenate was centrifuged 15000 g for 2 minutes at 4 °C (IEC CENTRA MP4R INTERNATIONAL EQUIPMENT COMPANY). The supernatant was removed carefully and the pellet, which contains the mitochondria, was centrifuged 15000 g for 2 minutes at 4 °C three times in ice-cold Tris-sucrose buffer.

Determination of malondialdehyde

The mitochondrial fraction was re-suspended in phosphate-buffered saline (PBS), following the protocol described in the OXISELEC™ TBARS assays kit (CELL BIOLABS, INC). The absorbance was read at 532 nm (SYNERGY HT, BIOTECK). The results were expressed in nmoles MDA/mg protein. Assays were performed in triplicate.

Proteins damage (determination of carbonyl protein)

The protein damage was determined following the ELISA kit for the determination of protein carbonyl: OxiSelect™ (STA-310) (CELL BIOLABS, INC.). This assay is based on the derivatization of the carbonyl groups with dinitrophenylhydrazine (DNPH), followed by immunedetection with an anti-DNP antibody. Seventy flies per group were homogenized in PBS (pH 7.4) and centrifuged at 10,000 g at 4°C; the supernatants were used for testing. The protein concentrations of all samples were adjusted to 10 µg/ mL/well. The standard curve was prepared using bovine serum albumin (BSA) at a concentration of 10 µg/mL per well in PBS. One hundred microliters of samples and BSA standard (oxidized and reduced) were added and incubated at 37°C for 2 hours. After the respective washes, 100 µL of the DNPH working solution were added and the samples were incubated for 45 min at room temperature in the dark. One hundred µL of the diluted antibody (anti-DNP) were added to all wells that were incubated at room temperature for 1 hour with shaking. Absorbance was read at 450 nm immediately. Assays were performed in triplicate. The results were expressed in nmol/mg.

Oxidative DNA damage

For DNA purification seventy animals from the different treatments were homogenized in 1 mL digestion buffer (100 mM NaCl, 10 mM Tris-Cl, pH 8.0; 25 mM EDTA, pH 8.0; 0.5% SDS, 0.1 mg/mL proteinase K) and incubated at 50 °C for 16 hours, then centrifuged at 13,200 g at 4°C for 5 minutes (IEC Central MP4R INTERNATIONAL EQUIPMENT COMPANY). The supernatants were transferred to sterile tubes for RNase digestion. The samples were incubated for 1 hour at 37°C, and then an equal volume of phenol/chloroform/isoamyl alcohol was added and centrifuged at 13,200 g for 10 minutes. Next, the upper aqueous phase was transferred to sterile tubes and 1/2 volume of 7.5 M ammonium acetate and 3 volumes of 100% of ethanol were added to precipitate the DNA. The tubes were incubated for 12 hours at -20°C. The samples were centrifuged at 13,200 g for 15 min to recover DNA, which was re-suspended and washed with 70% ethanol and centrifuged at 13,200 g for 2 min; the supernatants were decanted and the tubes were dried on absorbent paper. The DNA was re-suspended in TE buffer and stored at -20°C until used. Samples of the obtained DNA were separated and observed by agarose (0.7%) gel electrophoresis with ethidium bromide [0.5 mg/mL].

Measurement of DNA damage (8-hydroxy-d-guanosine)

Oxidative damage to DNA was determined using the competitive ELISA kit OxiSelect™ from Cell Biolabs, Inc. (STA-320) for the quantification of 8-OHdG. Standards of 8-OHdG and unknown samples were initially added to a plate containing the conjugate preadsorbed 8-OHdG/ ABS. After an incubation time, a monoclonal antibody (anti-8-OHdG) was placed. Subsequently a conjugated secondary antibody (HRP) was added. The content of 8-OHdG in the unknown samples was determined by comparison with a predetermined standard curve.

Longevity

For the longevity experiments, four groups of two days old flies (300 flies per group) were used: PQ-Mino, PQ-Control, Control and Mino. Flies were counted and transferred to fresh medium daily for their entire life cycle. Three replicates of each experiment were done.

Statistical analysis

Data were expressed as means ± SEM and the significance was determined by Student's t-test and one-way ANOVA, and differences among groups were evaluated using the test of Tukey for multiple comparisons. Differences were considered significant when $p<0.05$.

Results

As shown in Table 1 the intoxication with PQ for 36 hours increased the concentration of the H_20_2 and the activities of the enzymes SOD, CAT, GSH-Px and NOS with respect to control ($p<0.01$). These increments continued in the PQ-Control group after 12 days post intoxication with PQ. In contrast, the concentration of glutathione (GSH) and nitrite were significantly decreased by the intoxication with PQ ($p<0.01$). Moreover, an increase in the concentration of oxidative damage markers to lipid membranes (mitochondrial MDA), proteins (protein carbonyl), DNA (8-OHdG) when compared with control group were observed.

Table 1. Oxidative stress markers in adult male *D. melanogaster* exposed to PQ (40 mM) for 36 hours.

	Control	PQ
ROS ([1]H_2O_2)	0.810 ± 0.080	4.130 ± 0.277 ***
RNS ([2]Nitrite)	16.535 ± 0.486	10.413 ± 0.558***
Enzymatic activity		
[3]SOD	198.150 ± 1.363	433.270 ± 2.702***
[4]Cat	0.121 ± 0.009	0.242 ± 0.003***
[5]GSH-Px	6.700 ± 0.232	21.040 ± 0.567***
[6]NOS	0.007170 ± 0.000013	0.022400 ± 0.000083***
Endogenous antioxidant		
[7]GSH	14.684 ± 0.440	6.723 ± 0.315***
Oxidative Damage		
Lipid peroxidation [8]Mitochondrial MDA	6.970 ± 0.261	15.930 ± 0.554***
Proteins damage [9]Carbonyl Protein	6.280 ± 0.260	10.001 ± 0.287***
DNA damage [10]8-OHdG	1.360 ± 0.102	5.470 ± 0.154***

Data was analyzed by Student's t-test and PQ was compared to control group, ***$p<0.01$. [1]nmol H_2O_2/mg protein; [2]µM of nitrite/ mg protein; [3]Units/mg protein; [4]Units/mg protein; [5]Units/L; [6]Units/L; [7]µM of GSH/mg protein; [8]nmol MDA/mg protein; [9]nmol/mg; [10]ng / mL of 8-OHdG.

Table 2. Effect of minocycline on oxidative stress markers in *D. melanogaster* without and with pre-intoxication with PQ (40mM) for 36 hours and then treated with Mino (0.05 mM) for 12 days. Without PQ pre-intoxication With PQ pre-intoxication

	Control	Mino	PQ-Control	PQ-Mino
ROS ([1]H_2O_2)	1.095 ± 0.042	0.525 ± 0.023•	4.283 ± 0.176++	1.457 ± 0.025∞∞
RNS ([2]Nitrite)	13.136 ± 0.744	10.609 ± 0.567••	8.897 ± 0.371++	10.686 ± 0.319▪▪
Enzymatic activity				
[3]SOD	192.57 ± 0.613	231.00 ± 0.747••	675.285 ± 1.599++	401.700 ± 1.449▪▪∞∞
[4]Cat	0.139 ± 0.006	0.192 ± 0.023	0.333 ± 0.008++	0.212 ± 0.006▪∞∞
[5]GSH-Px	6.28 ± 0.261	16.280 ± 0.367••	27.42 ± 0.802++	25.45 ± 0.635▪▪∞
[6]NOS	0.0061 ± 0.00077	0.0033 ± 0.00038••	0.0068 ± 0.00078	0.0011 ± ∞∞ 0.00019▪▪
Endogenous antioxidant				
[7]GSH	16.212 ± 0.583	12.778 ± 0.434••	6.469 ± 0.461++	16.020 ± 0.396∞∞
Oxidative Damage				
Lipid peroxidation [8]MitochondrialMDA	6.805 ± 0.143	5.609 ± 0.120••	8.775 ± 0.155++	5.742 ± 0.135▪∞∞
Proteins damage [9]Carbonyl Protein	6.440 ± 0.298	6.320 ± 0.213	8.310 ± 0.232++	7.700 ± 0.237▪
DNA damage [10]8-OHdG	2.370 ± 0.060	1.170 ± 0.072••	3.830 ± 0.145++	2.660 ± 0.129∞∞

Data were analyzed by one-way ANOVA test and differences among experimental groups were tested for significance using the test of Tukey for multiple comparisons. [1]nmol H_2O_2/mg protein; [2]µM of nitrite/ mg protein; [3]Units/mg protein; [4]Units/mg protein; [5]Units/L; [6]Units/L; [7]µM of GSH/mg protein; [8]nmol MDA/mg protein; [9]nmol/mg; [10]ng / mL of 8-OHdG. Control compared with Mino •$p<0.05$; ••$p<0.01$; Control compared with PQ-Control +$p<0.05$; ++$p<0.01$;Control compared with PQ-Mino ▪ $p<0.05$ ▪▪$p<0.01$; PQ-Control compared with PQ-Mino ∞∞$p<0.01$.

In the PQ-Control group a significant increase in the concentration of H_20_2 and enzymatic activities of SOD, CAT and GSH-Px were detected with respect to control group ($p<0.01$). On the other hand, the concentrations of GSH and nitrites were decreased with respect to control ($p<0.01$). An increase in the concentration of mitochondrial MDA, 8-OHdG ($p<0.01$) and protein carbonyl ($p<0.05$) in the PQ-Control with respect to control were observed (Table 2).

After 12 days of treatment with Mino and post-intoxication with PQ (PQ-Mino), a significant decrease of the concentration of H_20_2 and of the enzymatic activities of SOD, CAT, NOS ($p<0.01$) and GSH-Px ($p<0.05$) were observed with respect to PQ-control. The concentration of glutathione (GSH) was significantly increased when compared with PQ-control group ($p<0.01$). Also, a significant decrease in the concentration of the oxidative damage markers to lipid membrane (mitochondrial MDA) and DNA (8-OHdG) were observed with respect to PQ-control ($p<0.01$) (Table 2).

In the PQ-Mino group an increase of the enzymatic activities of SOD, GSH-Px ($p<0.01$) and Cat ($p<0.05$) were detected with respect to control group. In contrast, the activity of NOS were decreased when compared with control ($p<0.01$). An increase in the concentration of protein carbonyl ($p<0.05$), and a decrease in the concentration of nitrite ($p<0.01$) and in mitochondrial MDA were observed with respect to control group ($p<0.05$) (Table 2).

At 12 days of treatment with Mino without pre-intoxication (Mino), a significantly decreased concentration of H_20_2 ($p<0.05$), nitrite, GSH and NOS, andan increase in the enzymatic activities of SOD and GHS-Px were observed with respect to control ($p<0.01$). In the same group, a decrease in the concentration of mitochondrial MDA and the 8-OhdG were detected when compared with Control ($p<0.01$). The concentration of protein carbonyl showed no significant difference when compared with Control.

In the group of flies treated with Mino the longevity was significantly increased when compared to control (111 ± 0.5 versus 78 ± 0.7 days, $p<0.01$). In the group PQ-Mino a significant increase was also observed with respect to the PQ-Control group (94 ± 0.8 and 45.7 ± 1.2 days, respectively). A significant difference on the 50% survival rate in the PQ-Mino group (40 ± 0.96 days) with respect to PQ-Control (12 ± 1.1 days) was detected ($p<0.01$).

Discussion

Oxidative stress plays a key role on the toxicity induced by the herbicide paraquat. Free radicals generation induced by PQ can cause oxidative damage to proteins, lipids and DNA. We evaluated whether long-term treatment with minocycline after PQ intoxication decreases oxidative stress in *D. melanogaster*.

Exposure to paraquat for 36 hours induced oxidative stress in *Drosophila melanogaster* was evidenced by the increases in the concentration of biomarkers of oxidative stress and oxidative damage observed to lipids, proteins and DNA. Similar results were observed by Hosamani and Muralidhara, (2013) in *D. melanogaster* intoxicated with paraquat for 24 hours. They demonstrated that the acute PQ toxicity is associated to oxidative stress and mitochondrial dysfunction. [14] The mechanisms of PQ toxicity consist of a redox cycling reaction in which the generation of superoxide anions promotes the generation of hydrogen peroxide and hydroxyl radical as well as a depletion of NADPH. [2] Long term administration of minocycline was efficient at increasing the longevity of *D. melanogaster* exposed to 40 mM of PQ. Bonilla *et al*., (2006) demonstrated that the pre-treatment with melatonin, minocycline and lipoic acid and subsequent intoxication with 20 mM PQ, increased the longevity of *D. melanogaster*. [11]

Flies treated with Mino for 12 days, after 36 hours of intoxication with PQ, showed a highly significant increase of lifespan and a decrease of oxidative damage to lipids, proteins and DNA. Mora *et al.,* (2013) compared the effects of ascorbic acid and Mino on the life span, motor activity and lipid peroxidation of *D. melanogaster* and showed that Mino significantly increased the life span when compared to ascorbic acid and the controls. [15] Our results are in agreement with previous reports, which have shown protective effects of Mino on oxidative stress [13, 16].

A significant increase was observed in the concentrations of 8-OHdG and protein carbonyl, and in the activities of GSH-Px and NOS in males of *D. melanogaster* flies at 36 hours after PQ intoxication. A significant decrease in the levels of nitrite and reduced glutathione was detected. These changes persisted 12 days post-intoxication, suggesting that the flies were exposed to a high in vivo oxidative stress and that cellular damage persists after intoxication. However, in flies intoxicated with PQ for 36 hours and post-treated with Mino (PQ-

Mino) for 12 days, a significant decrease in the levels of 8-OHdG when compared to the PQ-control group was detected, which confirms the oxidative injury of the herbicide. Other authors have also shown that exposure of D. melanogaster to PQ at different doses (50, 100, 150, 200 and 500 μM) includes neuroanatomical changes and damage to the integrity of DNA determined by the comet assay under alkaline conditions. [17]

We showed that in the mitochondria of surviving flies it occurs a significant increase in the levels of MDA after 36 hours of intoxication by PQ and this increment continued in the PQ-Control group after 12 days post-intoxication. This finding was indicative of oxidative damage to mitochondrial membranes by PQ. Previous studies have shown that PQ is capable of inducing oxidative stress by producing a significant increase in MDA levels in both the mitochondria and cytosol in *D. melanogaster.* [14, 18] The redox cycling is the main cause of PQ toxicity, and explains the fact that even after 12 days of intoxication with PQ the PQ-Control group showed high levels of MDA.

Long term Mino treatment of flies exposed to PQ for 36h, showed a significant decrease in the concentration of MDA compared with the PQ-control group, showing that Mino mitigated the oxidative damage to membranes caused by PQ. The activity of the antioxidant enzymes SOD and CAT was significantly increased. In addition, an increase in the levels of H_2O_2 in the cytosolic fraction of D. melanogaster males 36 hours after intoxication by PQ and after 12 days post-intoxication was observed. These findings suggest that the oxidative damage still persists 12 days after injury. The increase in the concentration of H_2O_2 was closely related to the increase in CAT activity, given that this enzyme is responsible for conversion of 2 molecules of H_2O_2 to $2O_2$ and H_2O; thus preventing the formation of hydroxyl radicals through a Fenton-type mechanism.

Our results agree with those reported by Hosamani and Muralidhara (2010, 2013), who obtained a significant increase in the activities of CAT (21-24%) and SOD (25%) 24 hours after exposure to PQ (40 mM) in *D. melanogaster*. [14, 18] Moreover, opposite results were reported in *D. melanogaster* in brain tissue exposed to high concentrations of PQ; the specific activity of SOD decreased as the concentration of PQ became higher. This activity was reduced 67.40% with 200 μM of PQ and 80.76% with 500 μM of PQ. [17]

In our study, a significant increase was found in the levels of protein carbonyl in flies intoxicated with PQ for 36 hours when compared to the Control. But, in the intoxicated animals treated for 12 days with Mino, a significant decrease of protein carbonyl was detected when compared to the PQ-Control flies. Hosamani and Muralidhara (2013) reported increased levels of protein carbonyl in *Drosophila* flies exposed to PQ for 24 hours. [14] Narayanasam *et al*., (2014) determined that the concentration of protein carbonyl was increased in animals intoxicated with PQ as in those in which there was a blockage of the expression of mitochondrial SOD. Oxidative damage to proteins is critical because if sufficient, it can impair the function of receptors, enzymes, transporters and therefore, also harm indirectly other biomolecules. [19]

A significant increase in NOS activity was observed while the levels of nitrite (NO_2^-, indirect indicator levels of •NO) in *D. melanogaster* flies intoxicated with PQ for 36 hours were decreased. Similar results were reported by Ajjuri and O'Donnell (2013) who found an increase in NOS activity in *Drosophila* exposed to PQ (10mM) by 12h although NOS activity levels declined as they approached the 30 hours of exposure. [20] In contrast, they got an increase in nitrite levels at a concentration of 10 mM PQ for 12 hours. We could speculate that this difference is due to the fact that we are using a considerably higher PQ concentration (40 mM) for a longer time (36 hours). Similar results have been observed in experimental models in brain tissue of rats exposed to PQ intoxication. In these studies it has been shown that PQ uses NOS and NADPH as an electron source for generating $O_2^{\bullet-}$ at the expense of the generation of •NO). [21]

After 12 days post-intoxication, in the PQ-Control group NOS activity was similar to those of the control group. Nitrite levels of PQ-control groups at 12 days decreased even below the values of the control group; similar results were reported by Djukie et al., (2007) regarding nitrate content in rat brain tissue at 7 days post exposure to PQ. [21]

Flies intoxicated with PQ for 36 hours and post-treated with Mino (PQ-Mino) for 12 days, showed an decreased in the concentration of nitrite in relation with the Control group. The enzymatic activity of NOS in flies decreased significantly after intoxication with PQ for 36 hours and post-treated with Mino (PQ-Mino) for 12 days with respect to Control. It has been reported that the exposure

of cerebellar granule neurons with •NO in the presence of Mino, can block the neurotoxicity. Mino also protects neurons from the cell death induced by •NO. [22]

A significant increase in the enzymatic activity of GSH-Px was observed. Moreover, there was a decrease in the GSH levels in the cytosolic fractions of *D. melanogaster* flies 36 hours after PQ intoxication and after 12 days post-intoxication, suggesting a redox imbalance in the system.

Conclusion

Paraquat exposure of *Drosophila melanogaster* induced oxidative damage to lipids, proteins and DNA as evidenced by an increase in the in the concentration of MDA, protein carbonyl and 8-OHdG. This effect was mitigated by treatment for 12 days with Mino in all parameters described above. The endogenous enzymatic antioxidant defenses (SOD, CAT and GSH-Px) were activated due to oxidative stress induced by intoxication with PQ for 36h. The activities of these enzymes remained elevated 12 days post-intoxication in animals that were not post-treated with antioxidant. It is possible that Mino could be mitigating the oxidative stress through its direct antioxidant effects or through the chelation of Fe_3^+ to avoid hydroxyl radical formation by the Fenton reaction. Mino seems to be efficient in mitigating the oxidative stress induced by PQ, as evidenced by the significant increase in the survival of the PQ-Mino flies when compared to the PQ-Control group.

Conflict of interest

Authors have no conflict of interest to disclose in this study.

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