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7-chloro-4-(phenylselanyl) quinoline prevents dopamine depletion in a *Drosophila melanogaster* model of Parkinson's-like disease

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ABSTRACT

Neurodegeneration in Parkinson's disease appears to be caused by multiple factors, including oxidative damage and an increase in acetylcholinesterase expression that can culminate in loss of dopaminergic neurons. A selenium-containing quinoline derivative, 7-chloro-4-(phenylselanyl) quinoline (4-PSQ), shows important pharmacological actions mainly attributed to its antioxidant and anticholinesterase properties. Thus, this study investigated the neuroprotective effect of 4-PSQ in a model of Parkinson's-like disease induced by rotenone (ROT) in *Drosophila melanogaster* and verified whether these effects are related to selenium levels. Adult flies were divided into: [1] control, [2] 4-PSQ (25 μM), [3] ROT (500 μM), and [4] 4-PSQ (25 μM) + ROT (500 μM) groups and exposed to a diet containing ROT and/or 4-PSQ for 7 days, according to their respective groups. Survival, behavioral, and *ex vivo* analyses were performed. Dopamine levels, reactive species levels (RS), lipid peroxidation (LPO), superoxide dismutase (SOD) and catalase (CAT) activity, and proteic thiol (PSH) and non-proteic thiol (NPSH) content in the head region were analyzed, while acetylcholinesterase (AChE) activity and selenium levels in the head and body regions were analyzed. 4-PSQ was able to reverse the ROT-induced deficits in flies, reestablish dopamine and selenium levels, reverse cholinergic deficits, improve motor function, and ameliorate mortality. Furthermore, 4-PSQ also reduced RS levels and LPO, and restored the activities of the antioxidant enzymes, SOD and CAT. Interestingly, a positive relationship between dopamine and selenium levels could be seen. Our results demonstrate the neuroprotective effect of 4-PSQ, and we suggest that the compound may act *via* different mechanisms, such as improving antioxidant defenses and consequently reducing oxidative damages, as well as having an anticholinesterase action, which together can prevent dopamine depletion, as these actions were correlated with the presence of selenium in the 4-PSQ molecule.

Abbreviations: 4-PSQ, 7-chloro-4-(phenylselanyl) quinoline; ROT, rotenone; PD, Parkinson's disease; GC/MS, gas chromatography with mass spectrometry; CRM, certified reference material; ERM, European Reference Materials; ICP-MS, inductively coupled plasma mass spectrometry; RSD, relative standard deviation; LOD, limit of detection; HPLC, high performance liquid chromatography; AChE, acetylcholinesterase; AcSCh, acetylthiocholine; RS, reactive species; LPO, lipid peroxidation; TBARS, thiobarbituric acid reactive substance; SOD, superoxide dismutase; CAT, catalase; PSH, proteic thiol; NPSH, non-proteic thiol; BBB, blood–brain barrier

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1. Introduction

Parkinson's disease (PD) is a neurodegenerative disturbance that presents with progressive and selective loss of nigrostriatal dopaminergic neurons, resulting in a deficiency of dopamine (DA) in the striatum [1,2]. The decreased viability of dopaminergic neurons leads to the onset of symptoms such as rigidity, bradykinesia, resting tremor, and postural instability [1,3,4]. Studies suggest possible mechanisms for neurodegeneration in PD, such as mitochondrial impairment, abnormal protein aggregation, apoptosis and oxidative stress, factors that may culminate in neuronal injury [1,3,5]. Recently, a possible relationship between an increase in acetylcholinesterase expression in the process of cellular apoptosis in a neurotoxin model of PD has been highlighted [6].

Therefore, molecules capable of preventing oxidative stress and inhibiting the acetylcholinesterase enzyme could protect dopaminergic neurons, suggesting a potential novel treatment for PD. In this sense, organic selenium compounds stand out as a new prospect among synthetic substances with therapeutic potential due to their pharmacological activities [7–10]. In addition, studies point important actions of quinoline compounds in conjunction with selenium organics [11–13]. Recently, 7-chloro-4-(phenylselanyl) quinoline (4-PSQ) has been highlighted due to its pharmacological actions, characterized as a multitarget drug, mainly due to its antioxidant [11–16], anti-inflammatory [12,17], and anticholinesterase actions [14,18], which led us to consider its use as a potential drug to be tested in a model of PD induced by the neurotoxin, rotenone (ROT).

Drosophila melanogaster, like vertebrates, have dopaminergic neurons of the central nervous system that are involved in locomotor control [3,19,20]. Additionally, fly models have shown strong PD related phenotypes, including mitochondrial dysfunction, reactive oxygen species production, loss of dopaminergic neurons and reduced locomotion [19,20].

Based on this, we aimed to evaluate the neuroprotective effects of 4-PSQ, in a model of Parkinson's-like disease induced by rotenone in *Drosophila melanogaster*.

2. Materials and methods

2.1. Chemicals

4-PSQ (Fig. 1) was prepared and characterized according to the method previously described by Savegnago et al. [11]. Analysis of the ^{1}H NMR and ^{13}C NMR spectra showed analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of the 4-PSQ (99.9%) was determined using gas chromatography combined with mass spectrometry (GC/MS). ROT was purchased from Sigma-Aldrich (St. Louis, MO, USA) and other reagents were of analytical grade from our laboratory in the Campus of UNIPAMPA.

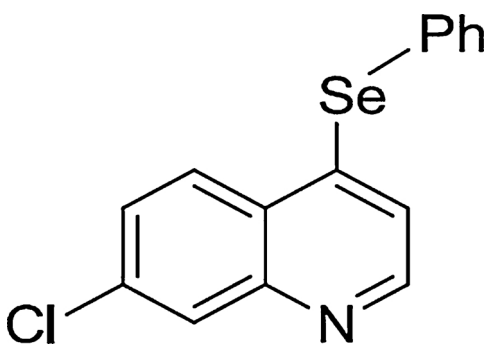

Fig. 1. Chemical structure of 7-chloro-4-(phenylselanyl) quinoline (4-PSQ).

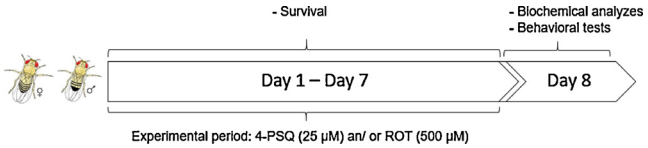

Fig. 2. Schedule of the experimental protocol.

2.2. Drosophila melanogaster stock and culture

The experiments were carried out using *Drosophila melanogaster* (Harwich strain) obtained from the National Species Center (Bowling Green, Ohio, USA). The flies were reared and maintained for about 4 days in an incubator (BOD) at a controlled temperature environment of 25 ± 1 °C and 60–70% humidity with a 12-hour light/12-hour dark cycle. The flies were kept in glass bottles with 10 mL of standard food comprising corn flour (76.59%), wheat germ (8.51%), sugar (7.23%), powdered milk (7.23%), salt (0.43%), an antifungal agent (Nipagin), and a pinch of dry yeast.

3. Experimental protocol

3.1. ROT exposure and 4-PSQ treatment

The experiment protocol (Fig. 2) was performed with flies of both sexes, aged 1–4 days, divided into four groups consisting of 50 flies each: [1] control group, [2] 4-PSQ (25 µM) group, [3] ROT (500 µM) group, and [4] 4-PSQ (25 µM) + ROT (500 µM) group. Flies were concomitantly exposed to a diet containing ROT and/or 4-PSQ for 7 days, according to their respective groups.

ROT (500 µM) was dissolved in ethanol at a concentration similar to that used by Hosamani and Muralidhara [21], Hosamani et al. [22], and Araujo et al. [23] in their published studies with *Drosophila melanogaster*. 4-PSQ (25 µM) was dissolved in ethanol, and this concentration was chosen based on a pilot experiment where flies were exposed to 4-PSQ at various concentrations (12,5 µM, 25 µM, 50 µM, and 100 µM) to determine the effect of 4-PSQ alone on the survival of flies during the experimental period of 7 days. A concentration of 25 µM was considered satisfactory when assessing mortality and the negative geotaxis behavioral test in flies, because it was effective and did not cause toxicity to the flies.

4-PSQ and ROT were placed into the flies' feed at a final concentration of 25 µM and 500 µM, respectively. The diet treatment consisted of 1% w/v yeast beer, 2% w/v sucrose, 1% w/v milk powder, 1% agar w/v, and 0.08% w/v nipagin. The control group additionally received the same vehicle as that of the ROT and 4-PSQ groups, and the final concentration of vehicle (ethanol) in medium was 0.5%.

4. *In vivo* assays

4.1. Survival rate

The flies were observed daily and survival was assessed by counting the number of live flies per day until the end of the experimental period of 7 days. For the evaluation of survival data, 500 flies were included in each group, with the total number of flies representing the sum of ten independent experiments (50 flies/each replicated treatment).

4.2. Negative geotaxis

Negative geotaxis is a widely used behavioral test to evaluate locomotor performance. In this study, the control and treated flies performed the negative geotaxis test after 7 days of treatment [23,24]. Briefly, 15 flies from each group were separately immobilized and anesthetized using ice and placed individually in a 1.5-cm diameter glass test tube. After a 10-minute recovery period, the test was begun.

Regarding the analysis, we measured the time spent by each fly to reach a height of 8 cm measured from the bottom of the test tube, in a maximum evaluation time of 120 s. The test was repeated five times for each fly and data analyzed according to the averaged time. Three independent experiments were performed, using a total of 45 flies per group.

4.3. Open field test

To evaluate the behavior and exploratory activities of the flies, an open field test of the control and treated groups was performed as per Hirth (2010) [25]. In this test 15 flies from each group were kept in a petri dish divided into squares measuring one centimeter each. They were kept there for a 10-minute recovery period before beginning the test. The exploratory activity and movement of flies were evaluated by counting the number of squares crossed/explored by each fly for a 60-second period. Furthermore, the open field trial was evaluated using the velocity (cm/s) maintained during this exploratory activity. For this analysis, we performed four independent experiments, with a total of 60 flies per group.

5. *Ex vivo* assays

5.1. Homogenized preparation

For biochemical analysis, the flies were first euthanized on ice. The head and body regions were then carefully separated and individually homogenized in ice-cold HEPES buffer (20 mM, pH 7.0), 1:10 (flies/volume μL), for 2 min immediately after centrifugation of the samples according to the desired analysis. The supernatant from the samples was then removed and used for the biochemical assays. The present study examined only the head region of the flies in all *ex vivo* assays, except for determination of selenium concentration and acetylcholinesterase activity, which were analyzed in the head and body regions. All biochemical determinations were performed in duplicate. For each behavioral or biochemical analysis, at least three independent experiments were performed.

5.2. Determination of Selenium concentration

Head and body masses of flies were measured using an analytical balance (AUY220, Shimadzu, Philippines), with a maximum load of 220 g and a resolution of 0.0001 g. In the digestion process, the head (around 4 mg) or body (around 25 mg) of flies were transferred to microtubes (2 mL), 250 μL of HNO_3 were added and were heated in a water bath (Thermomix BM - B, Braun Biotech International, Germany) at ± 95 °C for 1 h. After cooling, the digests were diluted with water up to 2 mL. Nitric acid (Merck, Germany) used in this study was purified using a sub-boiling system (Duopur, Milestone, Italy), and ultrapure water (18 MΩ cm) was obtained from a purification system (Mega Up, Megapurity, South Korea). Six independent experiments for each group of head and body were performed.

The determination of Se in the head and body was performed using an inductively coupled plasma mass spectrometer (NexION 300X, Perkin-Elmer, Canada), equipped with a concentric nebulizer (Meinhard Associates, USA), cyclonic spray chamber (Glass Expansion Inc., Australia), and quartz torch with a quartz injector tube (2 mm i.d.). Instrumental performance was optimized following previous work published in the literature [26,27]. The Se-82 isotope was measured based on reported studies in the literature [26,27]. External calibration was performed using reference solutions of Se (0.01–10 μg L^{-1}), which were prepared by sequential dilution of a stock solution (1000 mg L^{-1}, Merck) in 5% HNO_3. The same monoelemental stock standard solution was used in recovery tests to evaluate the accuracy of the determination step. High-purity argon (99.998%, White Martins, Brazil) was used for plasma generation, nebulization, and as an auxiliary gas in the determination step. Results were expressed considering the flies weight (ng of Se per gram of head or body weight).

In order to evaluate the accuracy of the analytical method, the digestion of a certified reference material (CRM) of fish muscle (BB422) from European Reference Materials (ERM) was performed under the same conditions as for the fly samples and Se was determined by inductively coupled plasma mass spectrometry (ICP-MS). The obtained concentration of Se (1.20 ± 0.08 μg g^{-1}) for ERM was in agreement ($P < 0.05$) with the reference value for Se (1.33 ± 0.13 μg g^{-1}). The relative standard deviation (RSD) was lower than 7%. Thus, the analytical method used in this work presented suitable accuracy and precision for Se determination. The limit of detection (LOD) was calculated from the mean of the blank values plus three times the standard deviation obtained for ten replicates of blank. The sample mass, the final volume of digests and, when necessary, the dilution factor were taken into account. Limits of detection for the determination of Se in the head and body were 124 ng g^{-1} and 21 ng g^{-1}, respectively; different values were obtained in part because of different sample masses used in the sample preparation method (median mass for the head was 4 mg and for the body was 25 mg).

5.3. Determination of DA levels

DA levels were determined using high performance liquid chromatography (HPLC) according to the procedure proposed by Dalpiaz et al. [28]. In the preparation of the sample, the heads of 20 flies per treatment group were homogenized in 100 μL of sodium phosphate buffer (0.1 M, pH 7.4) with 1 mM EDTA, and then centrifuged at 17857 rpm for 10 min at 4 °C, forming a supernatant that was evaluated using HPLC. Three to five independent experiments (20 flies per group) were performed.

5.4. Determination of acetylcholinesterase (AChE) activity

The AChE activity in the head and body regions was determined through a homogenized sample of a total of 20 flies from each group in 200 μL of 20 mM HEPES buffer (pH 7.0) and 20 bodies in 800 μL of 20 mM HEPES buffer (pH 7.0) and centrifuged at 1000 rpm for 5 min at 4 °C, according to Ellmann et al. [29]. The reaction was prepared with 0.25 M KPi buffer (pH 8.0) and 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB 5 mM), and this was added to the supernatant sample solution (50 μL) and to acetylthiocholine (AcSCh) (7.25 mM). The reaction was then monitored for 2 min at 412 nm. The AChE activity is expressed as μmol AcSCh/h/mg protein. The total of 20 flies per group represents the sum of three and five independent experiments performed for head and body samples, respectively.

5.5. Determination of reactive species levels

The level of reactive species (RS) was determined using a spectrofluorometric method, according to Pérez-Severiano et al. [30]. Therefore, 10 flies per group were euthanized on ice, and the heads were homogenized in 500 μL 10 mM Tris buffer (pH 7). Afterwards, the head homogenate was centrifuged at 3570 rpm for 5 min at 4 °C and the supernatant was removed, and used for assay quantification of 2′,7′-dichloroflurescein diacetate (DCF-DA) oxidation. The fluorescence emission of DCF resulting from DCF-DA oxidation was monitored after 1 h at an excitation wavelength of 488 nm and an emission wavelength of 520 nm using a fluorescence spectrometer. The results were obtained and are expressed as a percentage of the control DCF formation in arbitrary units (AU). The mean of three to four independent experiments was used (30–40 flies for each group).

5.6. Determination of thiobarbituric acid reactive substances

Lipid peroxidation (LPO) and products in the head region were

obtained using thiobarbituric acid reactive substance (TBARS) according to the protocol proposed by Ohkawa et al. [31] with minor modifications. Briefly, the heads of 12 flies from each treatment group were homogenized in 500 µL 0.1 M phosphate buffer (pH 7.0) and centrifuged at 1000 rpm for 5 min (4 °C). After centrifugation, the supernatant was incubated in 0.45 M acetic acid/HCl buffer (pH 3.4), 0.28% thiobarbituric acid, and 1.2% SDS at 95 °C for 60 min. The absorbance at 532 nm was then measured. The results of TBARS were normalized based on the protein concentration of the respective fly head regions and are expressed as nmol malondialdehyde (MDA)/mg protein. For this analysis, we performed four to five independent experiments (a total of 36 to 60 flies for each group, respectively).

6. Antioxidant enzymes activities

6.1. Determination of superoxide dismutase activity

The superoxide dismutase (SOD) activity in the head of flies was assayed by evaluating the inhibition of quercetin auto-oxidation, according to the procedure of Kostyuk and Potapovich [32], and modifications as made by Franco et al. [33]. The heads of 20 flies in each group were homogenized and centrifuged (at 14000 rpm for 30 min at 4 °C). SOD was then measured. The reaction mixture contained sodium phosphate buffer (0.025 M/EDTA 0,1 mM, pH 10), *N,N,N,N*-tetramethylethylenediamine (TEMED), and 10 µL of the head tissue sample. The reaction was started by adding 0.15% quercetin dissolved in dimethyl formamide and monitored for 2 min at 406 nm. Results represent the mean of three independent experiments with a total of 20 flies in each group. The enzymatic activity is expressed as U/mg protein (one unit is defined as the amount of enzyme required to inhibit the rate of autooxidation of quercitin by 50% at 26 °C).

6.2. Determination of catalase activity

The catalase (CAT) activity in the fly head was spectrophotometrically determined according to Aebi [34] with modifications as per Paula et al. [24]. A total of 20 fly heads per group were homogenized and centrifuged (at 14000 rpm for 30 min at 4 °C). The reaction mixture contained phosphate buffer (0.25 M/EDTA 2.5 mM, pH 7.0), hydrogen peroxide (10 mM), Triton X-100 (0.012%), and 30 µL head sample; the reactions were evaluated at 240 nm for 2 min. Enzyme activity is expressed as U/mg protein (1 U decomposes 1 µmol H_2O_2/min at pH 7 and 25 °C) and was determined from three to four independent experiments (20 flies per group).

6.3. Determination of thiol content

The determination of protein (PSH) and non-protein (NPSH) thiol content was estimated based on spectrophotometry, as per Ellmann et al. [35]. To determine thiol content, 10 head of flies per group were homogenized, and later added to 0.5 M perchloric acid and centrifuged (at 10,000 rpm for 5 min at 4 °C). For evaluation of non-protein thiol, the supernatant was used, and the pellet reserved for later analyses of non-protein thiol. Next, DTNB 5 mM was added; the mixture was then kept for 15 min in the dark, and read at 412 nm using a spectrophotometer. For evaluation of non-protein thiol measures, the pellet was resuspended in Tris/HCl 0.5 M with pH 8.0, after the supernatant had been removed. Next, 5 mM DTNB was added and the mixture was kept for 15 min (temperature and light suitable) and read at 412 nm using a spectrophotometer. Results represent the mean of three independent experiments (10 flies in each group) and are shown as a percentage of the control group.

6.4. Determination of protein concentration

The protein concentration in the head and body regions was evaluated using the method of Bradford (1976) [36] with standard bovine serum albumin.

6.5. Statistical analysis

Differences in lifespan were analyzed by comparing the survival curves by log-rank (Mantel-Cox) test. The results of other analyses were performed by one-way analysis of variance (ANOVA), followed by Newman-Keuls test or two-way ANOVA (4-PSQ × ROT) followed by Bonferroni *post hoc* test where appropriate. Pearson's correlation coefficient was used to examine the correlation between the analyzed parameters. GraphPad Prism, version 6 (San Diego, CA, USA) was used for all statistical analyses and their respective figures (2–8). The results of descriptive statistical data are presented as mean(s) ± standard error (SE) of the mean. Probability values less than 0.05 ($p < 0.05$) were considered statistically significant.

7. Results

7.1. Effect of 4-PSQ on the survival rate of Drosophila melanogaster

The exposure of adult flies, for an experimental period of 7 days, to ROT decreased the survival rates compared with those in the control group. The survival rates of the 4-PSQ and 4-PSQ + ROT groups were similar, and all groups had greater survival when compared to the ROT-alone group, evidencing the effectiveness of 4-PSQ in preventing ROT-induced mortality ($p < 0.05$; Fig. 3).

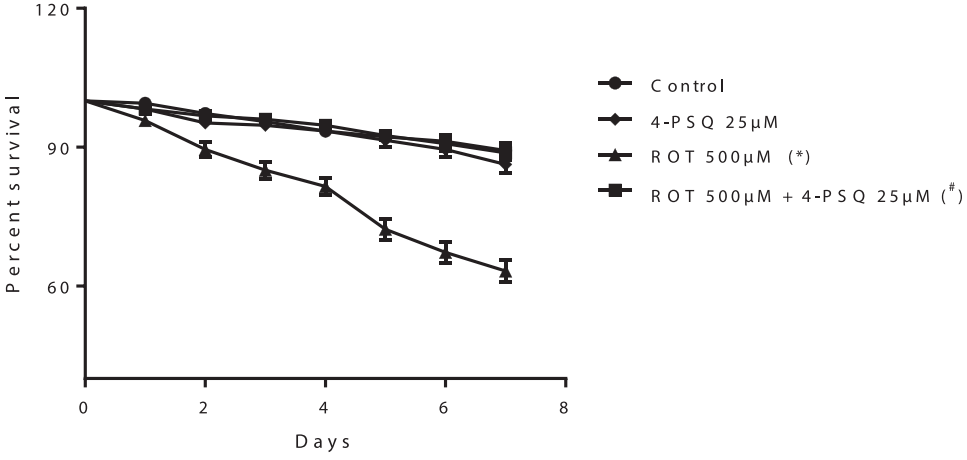

Fig. 3. Effect of 7-chloro-4-(phenylselanyl) quinoline (4-PSQ) on the survival rate of flies exposed to rotenone (ROT). Data were collected every 24 h for each group over 7 days. The total number of flies (500 per group) represents the sum of ten independent experiments. Lifespan measurement was determined by comparing the survival curves from Mantel-Cox log-rank tests and multiple comparisons were corrected using the Bonferroni test. * Significant difference from the control group; # significant difference between ROT and ROT + 4-PSQ ($p < 0.05$).

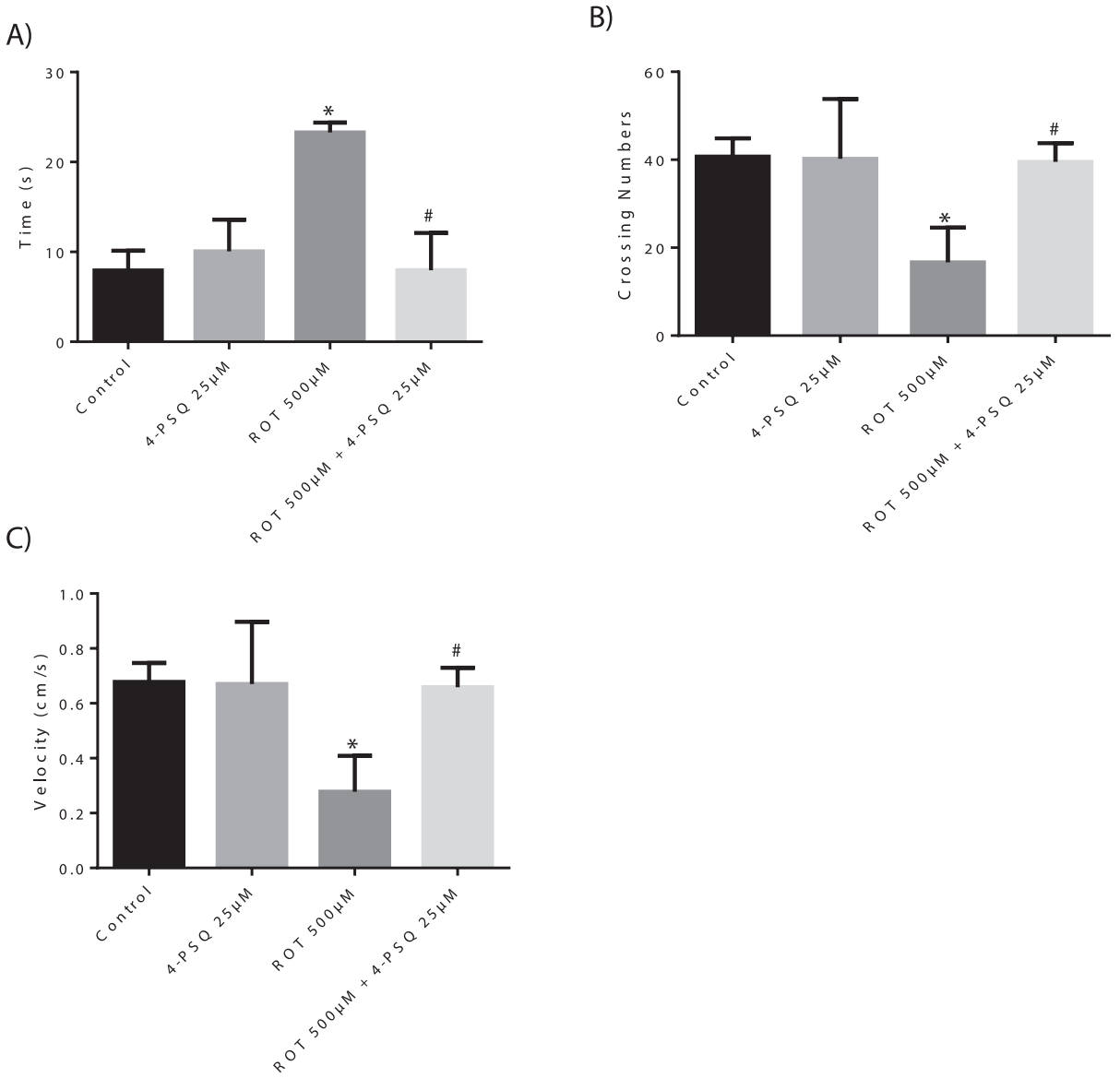

Fig. 4. Effect of 7-chloro-4-(phenylselanyl) quinoline (4-PSQ) on geotaxis response (climbing) and exploratory activities of flies exposed to rotenone (ROT) over 7 days. (A) Negative geotaxis assay; (B) open-field, and (C) velocity (cm/s) on open field. Fifteen flies per group were included for the negative geotaxis and open-field tests (total of 45 flies). These values represent the sum of three or four independent experiments. Values are mean ± SE, and significance determined by two-way analysis of variance (ANOVA) followed by Newman-Keuls test. * Significant difference from the control group; # significant difference between ROT and ROT + 4-PSQ ($p < 0.05$).

7.2. Locomotor performance

Figs. 3A and 2 B shows the consequences of treatments on locomotor behavior and exploratory activities. The two-way ANOVA of the locomotor behavior of the flies showed a 4PSQ × ROT interaction ($F_{(1,8)} = 25.68$, $p = 0.0010$; two-way ANOVA). *Post hoc* comparisons showed that the exposure to ROT compromised the locomotor behavior of the flies, verified by the decrease (approximately 194%) in the climbing rate compared with that in the control group ($p < 0.05$). Treatment with 4-PSQ prevented significantly the negative effects on climbing performance induced by ROT (Fig. 4A).

Fig. 3B and C shows the effect of treatment on exploratory activity. The two-away ANOVA revealed a 4-PSQ × ROT interaction ($F_{(1,12)} = 7.723$, $p = 0.0167$; two-way ANOVA; Fig. 4B and C). In the open field test, there was a reduction in the exploratory activity (approximately 59%) and velocity of exploration (approximately 59%) of flies in the ROT group relative to the control group ($p < 0.05$), characterizing a locomotor injury. 4-PSQ was effective in preventing the locomotor deficit and increasing the velocity of exploration of the flies.

7.3. Selenium levels

The two-way ANOVA revealed a significant interaction between 4-PSQ and ROT in the selenium levels in the head of flies [$F_{(1,2)} = 72.12$; $p < 0.0001$; Fig. 5A]. In relation to the body, the two-way ANOVA demonstrated a significant main effect of 4-PSQ [$F_{(1,2)} = 169,3$; $p < 0,0001$; Fig. 5B] and ROT [$F_{(1,2)} = 58,30$; $p < 0.0001$; Fig. 4B] on the levels of selenium. *Post hoc* analysis showed that flies exposed to ROT had reduced levels of selenium in the head (approximately 80%) and body (approximately 88%) compared to those in the control group. Additionally, flies treated only with 4-PSQ had higher selenium levels in the head and body regions (383% and 270%, respectively) than the control group. There was an increase in selenium levels in the head (763%) and body (1353%) of flies in the group treated with 4-PSQ + ROT when compared with the levels in the ROT group ($p < 0.05$), evidencing the compound's ability to restore selenium

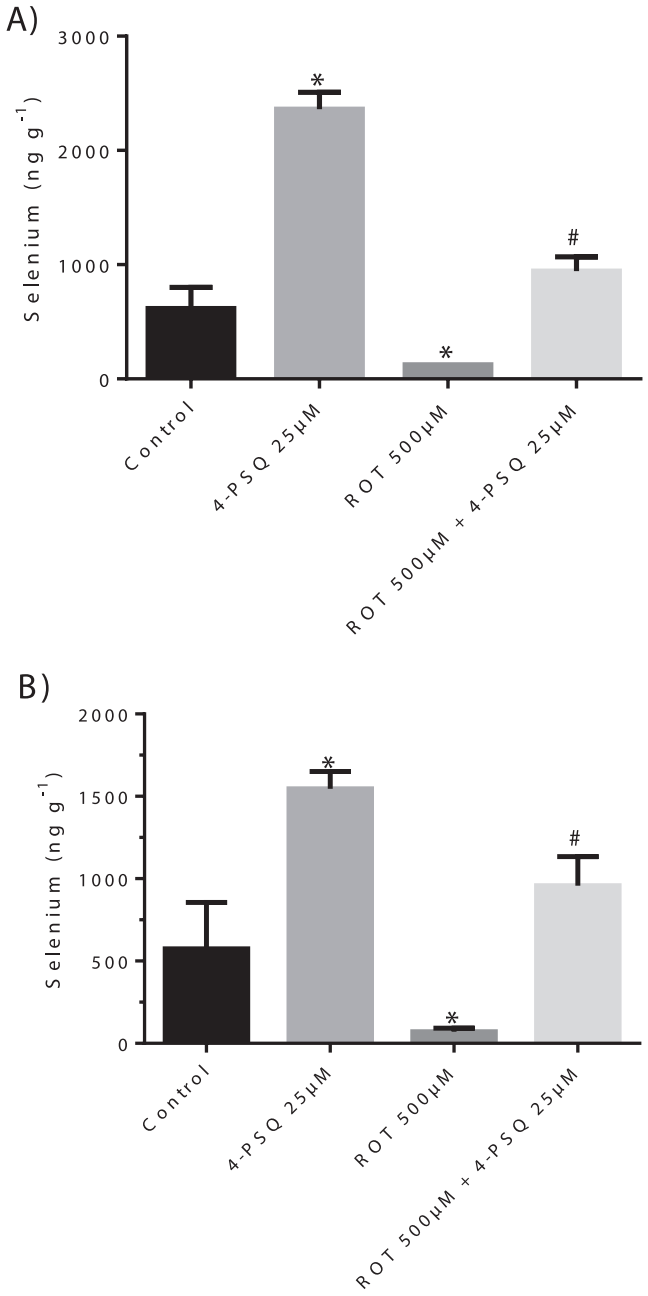

Fig. 5. Effect of 7-chloro-4-(phenylselanyl) quinoline (4-PSQ) on rotenone (ROT)-induced alterations in selenium levels in the head (4A) and body (4B) of adult *Drosophila melanogaster*. Values are mean ± SE. Significance determined by two-way analysis of variance (ANOVA) followed by Newman-Keuls *post hoc* test. * Significant difference from the control group; # significant difference between ROT and ROT + 4-PSQ ($p < 0.05$).

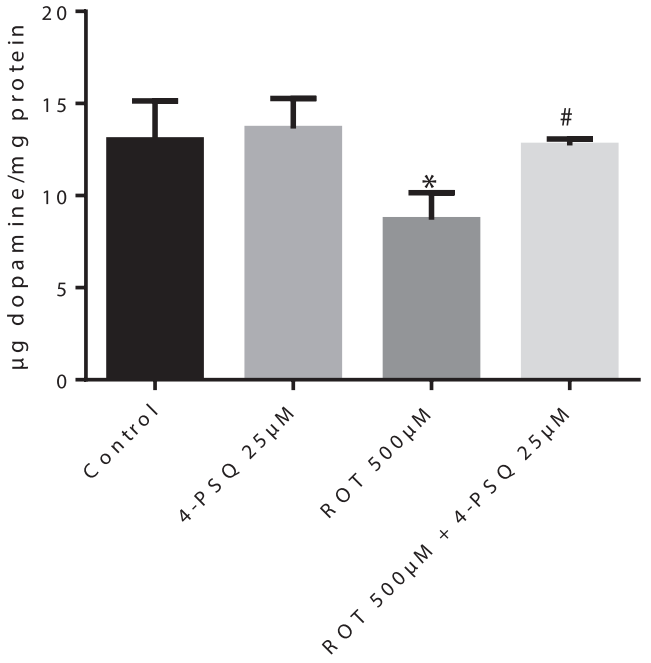

Fig. 6. Effect of 7-day treatment with 7-chloro-4-(phenylselanyl) quinoline (4-PSQ) on rotenone (ROT)-induced alterations in dopamine levels in the homogenized head region of adult *Drosophila melanogaster*. Values are mean ± SE (n = 20 flies per replicate, three to five replicates used). Significance determined by two-way analysis of variance (ANOVA) followed by Newman-Keuls *post hoc* test. * Significant difference from the control group; # significant difference between ROT and ROT + 4-PSQ ($p < 0.05$).

Fig. 7. Effects of treatment with 7-chloro-4-(phenylselanyl) quinoline (4-PSQ) on correlation between selenium levels and dopamine (DA) levels (B) in the head of flies. Pearson's correlation analysis was considered significant difference when $p < 0.05$.

levels in the head and body of flies.

7.4. DA levels

Fig. 6 illustrates the effects of treatments when analyzing the DA levels in the head of flies. Two-way ANOVA revealed an interaction between 4-PSQ and ROT [$F_{(1,13)} = 4.857$; $p = 0.0462$; Fig. 5]. The *post hoc* test showed that flies exposed to ROT had reduced levels of DA (approximately 33%) in the head compared to that of the control group. However, the data from the group treated with 4-PSQ and ROT simultaneously demonstrated that the 4-PSQ protected against the effect of ROT, since it increased DA levels in the head of flies ($p < 0.05$).

In the Pearson's correlation analysis, we observed a significant positive correlation between DA and selenium levels in the head of flies (r = 0.8342, $p = 0.0007$, n = 12; Fig. 7).

7.5. AChE activity

The two-way ANOVA revealed a significant interaction between 4-PSQ and ROT in the head [$F_{(1,8)} = 15.12$; $p = 0.0046$] and body [$F_{(1,16)} = 33.99$; $p < 0.0001$]. *Post hoc* analysis showed that the flies exposed to ROT had a significant increase in AChE activity in the head (approximately 60%) and body (approximately 51%), while additional treatment with 4-PSQ prevented against an increase in AChE activity in

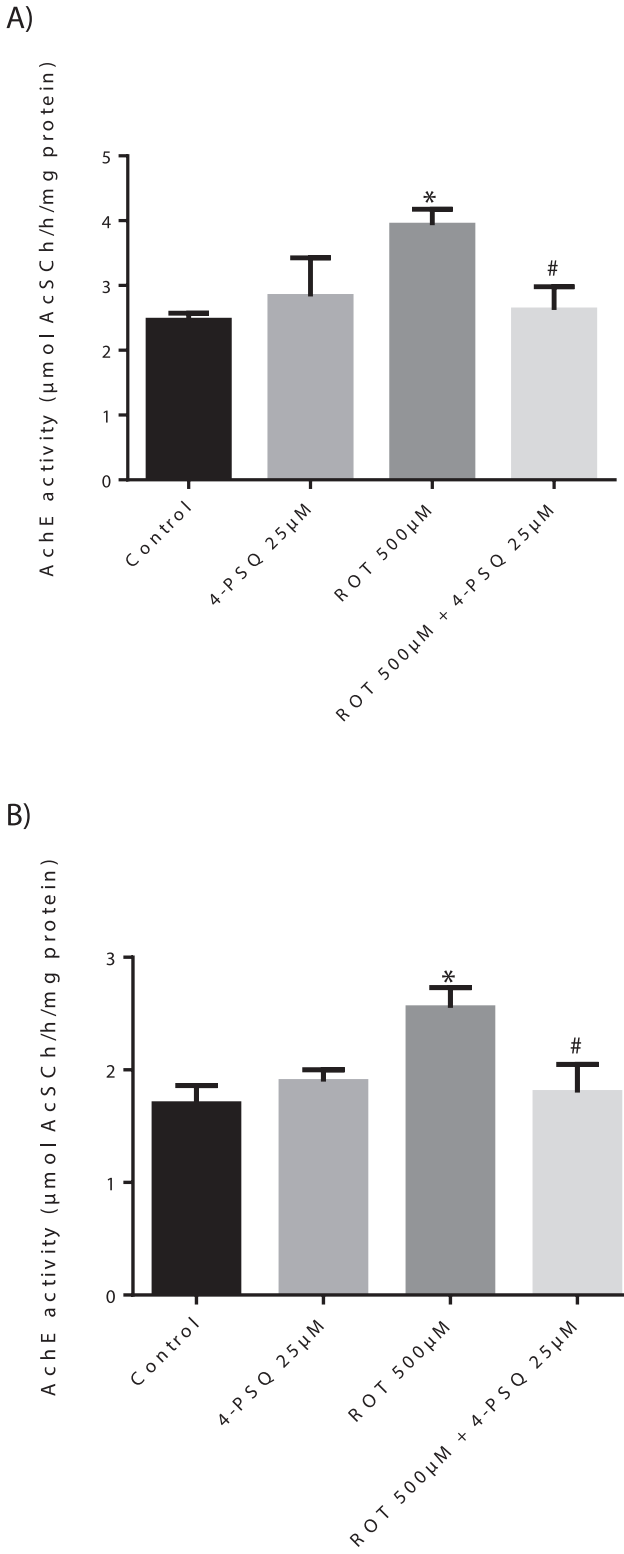

Fig. 8. Effect of 7-chloro-4-(phenylselanyl) quinoline (4-PSQ) on rotenone (ROT)-induced alterations in AChE activity (µmol/min/mg protein) in the head (A) and body (B) regions of adult *Drosophila melanogaster*. Values are mean ± SE (n = 20 flies per replicate, three and five replicates used, respectively). Significance determined by two-way analysis of variance (ANOVA) followed by Newman-Keuls test. * Significant difference from the control group; # significant difference between ROT and ROT + 4-PSQ ($p < 0.05$).

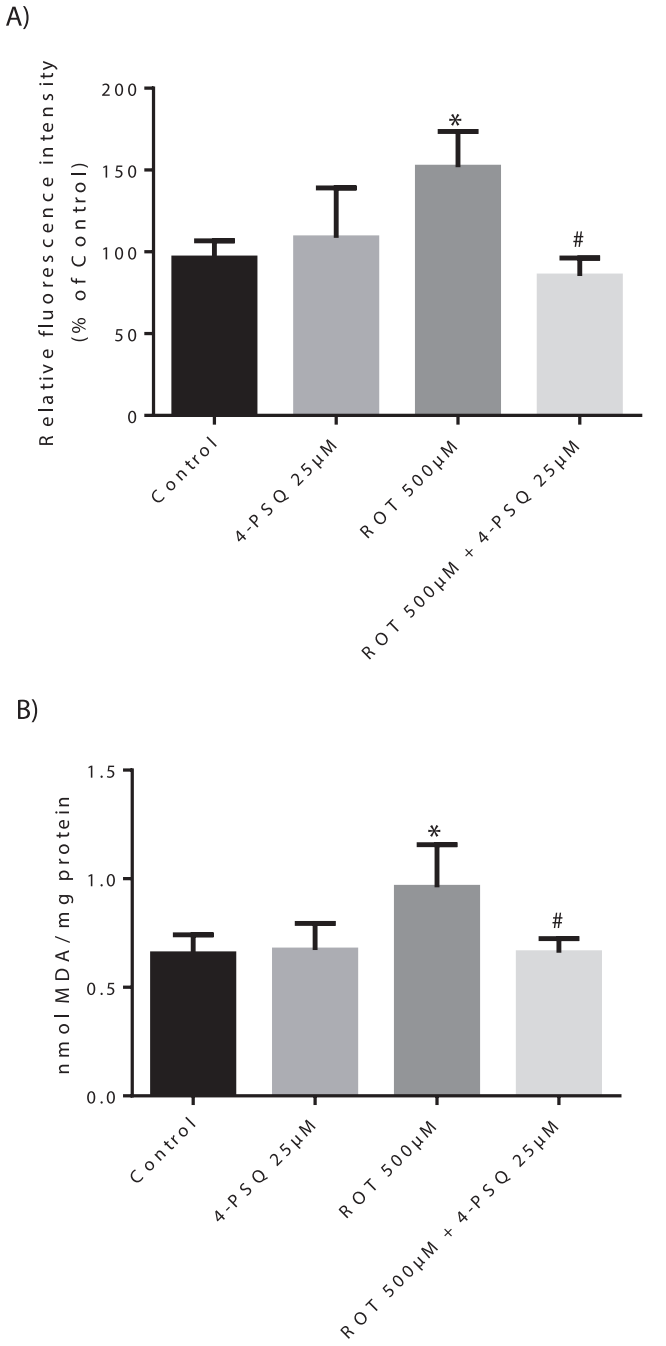

Fig. 9. Effect of 7-day treatment with 7-chloro-4-(phenylselanyl) quinoline (4-PSQ) for 7 days on rotenone (ROT)-induced alterations in markers of endogenous oxidative stress in the head region of adult *Drosophila melanogaster*. (A) Reactive species levels (RS) and (B) levels of lipid peroxidation (LPO) in the head region of adult *Drosophila melanogaster*. Values are mean ± SE (n = 10 and 12 flies, respectively, per replicate, three to five replicates used). Significance determined by two-way analysis of variance (ANOVA) followed by Newman-Keuls test. * Significant difference from the control group; # significant difference between ROT and ROT + 4-PSQ ($p < 0.05$).

the head and body of flies (Fig. 8A and B, $p < 0.05$).

7.6. Biomarkers of oxidative stress

Fig. 9A shows the RS levels. The two-way ANOVA showed a significant interaction between 4-PSQ and ROT [$F_{(1,9)} = 13.17$; $p = 0.0055$] in the RS levels. *Post hoc* analysis revealed that flies exposed to ROT had significantly increase in the production of RS (about

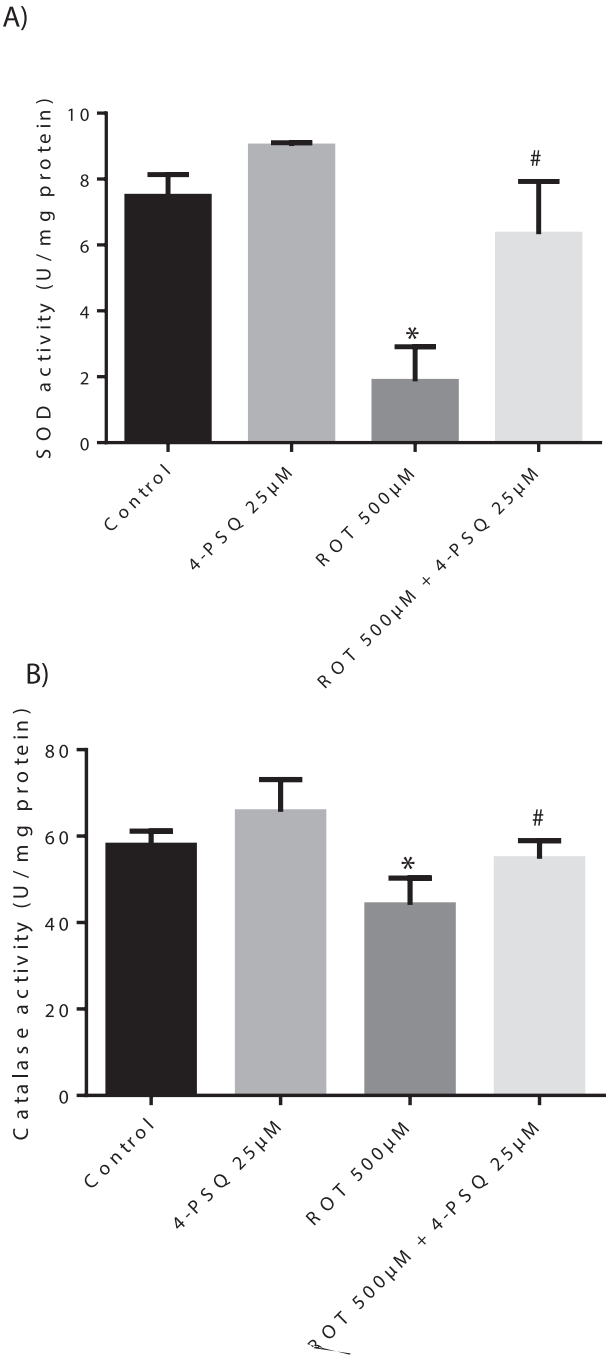

Fig. 10. Effect of 7-day treatment with 7-chloro-4-(phenylselanyl) quinoline (4-PSQ) for 7 days on alterations induced by rotenone (ROT) on the activities of antioxidant enzymes in the homogenized head regions of adult *Drosophila melanogaster*. (A) Superoxide dismutase (SOD) and (B) catalase (CAT). Values are mean ± SE (n = 20 flies per replicate, three to four replicates used). Significance determined by two-way analysis of variance (ANOVA) followed by Newman-Keuls test. * Significant difference from the control group; # significant difference between ROT and ROT + 4-PSQ ($p < 0.05$).

58%) when compared with the control group ($p < 0.05$). Additionally, there was a significant decrease in the production of RS in the group exposed to ROT and treated with 4-PSQ when compared with the untreated group.

Fig. 9B evidenced the effects the treatment LPO and products in the head region. In the two-away analysis it was possible to verify an interaction between 4-PSQ and ROT [$F_{(1,14)} = 6.560$, $p = 0,0226$; Fig. 8B] in the LPO. We observed increased in LPO (about 48%) in flies exposed to ROT, while the treatment with 4-PSQ was effective in reducing LPO ($p < 0.05$).

7.7. Antioxidant defenses

The Fig. 10A and B shows the results of enzymatic activity of SOD and CAT. Two-way ANOVA revealed a significant interaction between 4-PSQ and ROT for SOD activity [4-PSQ × ROT interaction $F_{(1,8)} = 6.365$, $p = 0.0356$], but no significant interaction for CAT activity [4-PSQ × ROT interaction $F_{(1,9)} = 0.2221$, $p = 0.6486$]. Post-hoc analysis revealed inhibition of SOD (about 75%) and CAT (about 24%) activities in the ROT compared to the control group. Furthermore, 4-PSQ was successful in preventing the inhibition of SOD and CAT activities (Fig. 9A and B; $p < 0.05$).

7.8. Thiol determination

Two-way ANOVA revealed no significant interaction between 4-PSQ and ROT for PSH [4-PSQ × ROT interaction $F_{(1,8)} = 0.1962$, $p = 0.6695$] or NPSH [4-PSQ × ROT interaction $F_{(1,8)} = 0.4131$, $p = 0.5384$]. Similarly, in the *post hoc* analysis, there were no statistical differences between groups with regard to the PSH and NPSH in the head regions (data not shown).

7.9. Effects of 4-PSQ on correlation between DA levels and behavioral and biochemical parameters

In the Pearson's correlation analysis (Table 1), DA levels in the head were significantly negatively correlated with negative geotaxis (r = -0.8336, $p = 0.0008$), AChE activity in the head (r = -0.8118, $p = 0.0013$) and body (r = -0.8731, $p < 0.0001$), RS levels (r = -0.8487, $p = 0.0002$), and LPO (r = -0.8943, $p < 0.0001$). Furthermore, the correlation analysis found a significant positive correlation between DA levels and survival (r = 0.8739, $p = 0.0002$), crossing numbers on the open field test (r = 0.9050, $p < 0.0001$), velocity of open field exploration (r = 0.9051, $p < 0.0001$), and SOD (r = 0.8232, $p = 0.0010$) and CAT (r = 0.9172, $p < 0.0001$) activity. No significant correlation was observed between DA levels and PSH (r = -0.2641, $p = 0.4068$) or NPSH (r = -0.3386, $p = 0.2816$).

8. Discussion

The current study investigated the effects of 4-PSQ in a model of ROT-induced PD in *Drosophila melanogaster*. Our results demonstrate

Table 1
Effects of 7-chloro-4-(phenylselanyl) quinoline (4-PSQ) on correlation between dopamine (DA) levels in the head of flies with behavioral or biochemical parameters.

DA and behavioural and biochemical parameters	*r*	*p*	*n*
DA x Survival	0.8739	0.0002 *	12
DA x Negative geotaxis	- 0.8336	0.0008 *	12
DA x Test Open Field	0.9050	< 0.0001 *	15
DA x Velocity of Open Field	0.9051	< 0.0001 *	15
DA x AchE in head	- 0.8118	0.0013 *	12
DA x AchE in body	- 0.8731	< 0.0001 *	17
DA x RS	- 0.8487	0.0002 *	13
DA x LPO	- 0.8943	< 0.0001 *	17
DA x SOD	0.8232	0.0010 *	12
DA x CAT	0.9172	< 0.0001 *	14
DA x PSH	-0.2641	=0.4068	12
DA x NPSH	-0.3386	=0.2816	12

Acetylcholinesterase (AchE); Reactive species (RS); Lipid peroxidation (LPO); Superoxide dismutase (SOD); Catalase (CAT); Proteic thiol (PSH); Non-proteic thiol (NPSH).

* $p < 0,05$ is considered significant.

the ability of 4-PSQ to prevent the damage caused by this model, and its relation to the levels of selenium in the head of flies. We observed that treatment with 4-PSQ increased selenium levels, as well as prevented oxidative stress, restored antioxidant defenses, and inhibited the action of AChE. These effects can be related to the restored DA levels observed in the head of flies and, consequently the prevention of locomotor impairments and the improvement in the survival of flies exposed to ROT.

Interestingly, the results of the study indicate that treatment with 4-PSQ protects against dopaminergic damage caused by ROT in the head of flies. An increasing number of recent studies have shown that ROT exposure produces similar behavioral and biochemical changes as observed in PD and other neurological diseases [1,37,38]. Regarding the characterization of PD induced by exposure to ROT, our results corroborate with other studies that found DA depletion in *Drosophila melanogaster* [21–23]. The toxicity caused by ROT can lead to signs and symptoms similar to those observed for PD; this is associated with mitochondrial dysfunction due to complex I inhibition, which decreases electron transport chain antioxidant levels, and increases iron levels, and impairs dopaminergic neurons that may be more susceptible to oxidative stress than other neurons in the brain because they contain DA [39]. Surprisingly, an unprecedented and very important finding is that selenium levels are reduced in the group treated with ROT when compared with those in the control group. One hypothesis for this finding would be a compensatory mechanism in which, due to the oxidative stress provoked by ROT, there would be an increase in the demand for selenium in the brain for the production of selenoprotein to combat oxidative insults [40,41]. However, treatment with 4-PSQ was able to reestablish selenium levels in both the head and body of the flies. Consistent with our findings, Khera et al. [42] in a study with placental trophoblast cells verified that cells treated with selenium are more resistant to mitochondrial oxidative stress stimulated by ROT. Ellwanger et al. [8], observed bradykinesia and DNA damage reduction in a paraquat PD model in rats receiving selenium in the form of selenite in drinking water. Another study verified that selenium supplementation reverses the impairment of dopaminergic neurotransmission induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [43]. However, none of these studies measured selenium levels. Therefore, we first verified that selenium levels are decreased in the head of flies in this model of Parkinson's disease.

4-PSQ reduced the depletion of DA caused by ROT, demonstrating its potential to increase levels of DA and its neuroprotective effect, evidenced also by the association between the increase in dopamine levels as the levels of selenium increased in the head of flies. Moreover, evidence indicates the involvement of selenium in dopaminergic protection pathways, possibly due to the high level of reactive oxygen species production during dopamine cycling and autooxidation [44]. It is well documented that 4-PSQ has neuroprotective actions such as those reported by Duarte et al. [14] *in vitro* and Vogt et al. [16] *in vivo*. In this study, the 4-PSQ reduced the depletion of DA, demonstrating the involvement of the dopaminergic system in the neuroprotective effect of this new selenium-containing quinoline compound, and thus may be a possible candidate for the treatment of PD. We believe that 4-PSQ has a mechanism of action similar to that of another organoselenium compound that is well-described in the literature, diphenyl diselenide [$(PhSe)_2$]. Studies conducted by Burger et al. [45], Savegnago et al. [46], and Figueira et al. [47] using $(PhSe)_2$ noted that the pharmacological effects of the compound mainly related to the dopaminergic system of mice. Additionally, a recent study conducted by Sampaio et al. [7] demonstrated that treatment with $(PhSe)_2$ reverses some motor impairments in a 6-hydroxydopamine (6-OHDA) model of PD in rats.

In this study, flies exposed to ROT exhibited increased mortality and locomotor impairment with a phenotype similar to PD. Other studies with ROT also showed high mortality and locomotor deficits in flies exposed to this pesticide, characterizing the model of ROT-induced PD in flies [19,21–23]. In this sense, the reduction in locomotor behavior and deficit in the negative geotaxis and open field tests indicate that the PD model was successfully induced in the flies exposed to ROT. A mitochondrial deficiency caused by ROT can bring about damage to the locomotor system as it contributes to energy deficits of the ambulatory and flight muscles that are rich in mitochondria and have a high energy requirement, affecting locomotor and exploratory behaviors due to inhibition of electron transport chain complex I, caused by this bioenergetic crisis [1,21]. In the present study, we found that flies treated with 4-PSQ exhibited enhanced survival rates against ROT treatment in the co-exposure paradigm showed the ability of 4-PSQ in promoting survival pathways and abrogating locomotor impairments. Ellwanger et. al. [8]. in a study of an experimental model of PD induced by paraquat in rodents pointed that the use of selenium may contribute to the maintenance of locomotor skills. In studies with rodents, the compound 4-PSQ *per se* did not affect the behavior of animals [12,18,48]. Corroborating our findings regarding the neural protection exercised by 4-PSQ, recently its neuroprotective action against anxiety and learning and memory impairment caused by amyloid β-peptide (Aβ) was reported after evaluation using behavioral tests in a murine Alzheimer disease model [18].

Acetylcholine is the primary excitatory neurotransmitter in the central nervous system that plays a pivotal role in the regulation of motor function and locomotion [49,50]. AChE has an important role in the function of cholinergic synapses in the nervous system (central and peripheral), and became an attractive target for the development of new drugs [51]. Evidence suggests a significant role for the degeneration of cholinergic neurons in the pathophysiology of neurodegenerative diseases [52] and the involvement of AChE in the pathogenesis of PD, in which the increased expression of AChE is proposed to induce neuronal cell death [6]. Nonetheless, there are indications that dopaminergic deficits are associated with deterioration of the base ganglion circuit dynamics, resulting in overactivation of the cholinergic system, leading to motor and cognitive impairments [53]. AChE activity was also evaluated in this study, and an increase in AChE activity was found in the head and body of flies exposed to ROT, resulting in changes in locomotor activity with respect to the control group, corroborating the behavioral data and compatible with symptoms of PD. Similar to the present study, Rao et al. [54] verified an increase in AChE activity in a ROT-induced model of PD. In addition, Soares et al. [55] reported that paraquat, another agent similar to ROT and with an herbicidal action, affects the cholinergic system specifically by activating AChE; they suggested that this may be related to the fact that DA depletion caused by paraquat blocks auto-inhibition of acetylcholine release through muscarinic auto-receptors, leading to an excess of acetylcholine in the synaptic cleft and an increase in AChE activity [53]. Several previous reports showed that increased AChE activity is associated with increased neuronal oxidative and nitrosative stress, and alterations in energy metabolism in invertebrates and vertebrates [6].

We found that 4-PSQ was effective in protecting against the increase in AChE activity caused by ROT in the head and body regions, suggesting its efficacy in protecting cholinergic function at the central and peripheral level. It is an important result that molecules capable of inhibiting the enzyme acetylcholinesterase could protect the dopaminergic neurons arising as a possibility in the treatment of PD, since an increase in acetylcholinesterase expression in the process of cellular apoptosis in neurotoxin-induced models of PD has been observed [6]. Studies using 4-PSQ reported an inhibitory effect on cerebral AChE activity *in vitro* [14,56] and *in vivo*, while also having an effect on the brain structure of mice [18]. Duarte et al. [14] verified this effect only *in vitro;* however, they suggested that due to the fact these selenium-containing quinolone derivatives inhibit AChE activity and improve cognitive enhancement, this compound may be a potential therapeutic agent for the treatment of neurodegenerative disorders. More recently, Pinz et al. [18] in their study of a murine Alzheimer's disease model showed the inhibitory effect of 4-PSQ on AChE. Nevertheless, our data

corroborate those of other studies using other selenium compounds, such as a study that found dietary supplementation with $(PhSe)_2$ decreased AChE activity and improved climbing performance in flies exposed to manganese [57].

Numerous molecular pathways have been proposed for the development of PD, including among them oxidative stress and ROS generation [4]. Indeed, the increased incidence of age-associated diseases, such as PD, reflects the generation of free radicals and associated oxidative stress enhanced in certain regions of the brain during aging [58]. Evidence suggests that ROT-induced neurotoxicity can be attributed to the specific sensitivity of dopaminergic neurons to RS and oxidative damage [37,59]. Moreover, signs of oxidative damage have been detected frequently in dopaminergic neurons from PD patients and animal models, suggesting an implication of oxidative stress in this disease [37,59]. Similarly, in our study, we observed a significant increase in the production of RS and LPO in the ROT treatment group.

Recently, the antioxidant hypothesis of PD has become widely accepted and the potency of various neuroprotective agents in neurotoxin-based models has been demonstrated [2,19,60]. The inhibition of free radicals by antioxidant compounds offers beneficial effects against dopaminergic neurotoxicity. However, many currently available antioxidants cannot readily penetrate the blood–brain barrier (BBB) after systemic administration, pointing to the need to search for novel BBB-compatible antioxidant therapies [43,61]. Thus, an important result here is that 4-PSQ penetrated the BBB after administration since we observed an increase in selenium levels in the head of flies, consequently reversing increases in RS and LPO in the head of flies. These results indicate the potential of 4-PSQ to attenuate oxidative damage while maintaining cellular homeostasis. Following this, we speculate that the protective effect of 4-PSQ in this model of PD can also be attributed to its ability to abrogate oxidative stress. Several different strategies have been proposed to fight oxidative stress in PD, including molecules that can promote endogenous mechanisms to buffer free radicals, such as selenium [60]. Studies of the pesticides ROT [38,62] and Paraquat [8], which have been related to the development of PD, have pointed out that selenium can act as a shield against the oxidative stress caused by such pesticides. Our findings are also consistent with other studies that found potential antioxidant actions of 4-PSQ in *in vitro* and *ex vivo* rodent models against the increase in RS [12,13,16] and LPO [16,56]. 4-PSQ can act on different antioxidant defense lines, protecting against cerebral oxidative damage, suggesting that 4-PSQ acts in a manner similar to other organoselenium compounds [16]. This proposition can be supported by the results of Vogt et al. [16] who found that the phenylselenyl substituent group in the quinoline structure is important for the antioxidant effect of this compound at the brain level. From the results presented in this study, we suggest that 4-PSQ, through its antioxidant action, acts to reduce the production of oxidative stress in neuronal cells.

To better understand the underlying mechanisms of the protective activity of 4-PSQ against ROT-induced oxidative damage, we assessed the activities of antioxidant enzymes (SOD and CAT) in the head of flies. In the present study, there was a significant decrease in SOD and CAT activities in the head regions of ROT-exposed flies. The protective role of SOD and CAT in the cells is related to the elimination of deleterious O_2 and H_2O_2 [50]. Our results evidently point to a compromised antioxidant defense system that can impair cellular function in the head regions of flies exposed to ROT. However, 4-PSQ reversed this inhibition of SOD and CAT activities caused by ROT exposure. Thus, 4-PSQ prevents ROT-associated depletion of the antioxidant defense system through the increased enzyme activity in the treated flies. The marked reduction in RS and increase in SOD and CAT activity after treatment with 4-PSQ revealed its potential antioxidant effect against ROT-induced oxidative stress. Recently, the antioxidant effect of 4-PSQ against cerebral stress was demonstrated, in addition to reducing RS and LPO and increasing CAT and glutathione-S-transferase activities [16]. Furthermore, in a study of the action of 4-PSQ on a model of Alzheimer's disease, the authors point to the multi-target component of this compound, since it is not only an AChE inhibitor in the cerebral cortex and hippocampus, but also reduces LPO levels in the cortex of mice [18]. Thus, our results confirm the antioxidant potential of 4-PSQ, increasing the activity of antioxidant enzymes in the fight against oxidative injury.

The thiol level is an indirect oxidative stress biomarker and thus is indicative of chemical changes in thiol groups of proteins and peptides [50]. In the evaluation of non-enzymatic antioxidant defenses, the main ones are PSH and NPSH, no differences were observed between groups, consistent with the results of other studies of ROT-induced PD [22,23]. However, a study with 4-PSQ that aimed to evaluate the effect of the compound at the brain level in mice showed that 4-PSQ protected completely against the reduction in the levels of PSH and NPSH, evidencing the potential of the compound to act at the level of non-enzymatic protection against oxidative insults [14,16].

Furthermore, we can observe a correlation between the levels of DA and some of the evaluated parameters. Thus, we verified that according to a decrease in DA levels there is an increase in the time spent and velocity in the negative geotaxis test, an increase in AChE activity, and in the oxidative stress parameters (RS and LPO), or *vice versa*, while a decrease in dopamine levels would be correlated to a decrease in fly survival, activity, and exploratory behavior, and activity of antioxidant enzymes (SOD and CAT), or *vice versa*. Thus, a strong correlation can be observed between dopamine levels and fly survival, behavioral parameters, oxidative stress, and antioxidant defenses. These factors demonstrate the adequate establishment of the ROT-induced model of PD in *Drosophila melanogaster*, corroborating other studies in the literature [21–23]. Nonetheless, a positive correlation between DA and selenium levels that is, a relationship between the decrease in DA and selenium levels in the head of flies, was found. These data clearly demonstrate the relationship between AChE activity and oxidative stress on dopaminergic and behavioral changes in this PD model, as well as reinforcing the antioxidant and anticholinesterase action of 4-PSQ associated with its neuroprotective effect in our work, and the relation with selenium levels.

In summary, our results indicate that 4-PSQ can be considered a multi-target molecule, acting in the dopaminergic system through different mechanisms: reducing oxidative damages, improving antioxidant defenses, and exercising an anticholinesterase action, factors which together can protect dopaminergic neurons, preventing dopamine depletion, and consequently reversing the behavioral motor deficits in *Drosophila melanogaster*. Nevertheless, we found that these actions of 4-PSQ are mainly correlated with the presence of selenium in its structure. Therefore, 4-PSQ highlights an important target in the search for compounds with therapeutic potential in the neurodegenerative diseases, such as PD.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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