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Research Article

Protective Effect of Vitamin C against Behavioral, Histological Changes and Mortality Rate Induced by Paraquat in *Drosophila melanogaster*

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Abstract

Paraquat (PQ) is known to cause fatal intoxications to humans and animals mainly by the generation of reactive species. However, none is known on the transgenerational toxicity of PQ and the effect of the supplementation of the standard antioxidant vitamin C (VIT C) to counteract PQ-induced toxicity in *Drosophila melanogaster*. Here, we investigated the possible protective effect of VIT C on behavioral, mortality rate, and histological changes in different generations of *D. melanogaster* following PQ exposure. Flies of both genders (P generation), of 1-3 days of age, were exposed separately at different concentrations of PQ (0.5, 2.5, 5 mg/g), and VIT C (25, 100, 200 mM) for 7 days (first exposure). The second generation of flies (F_1) was exposed to the most toxic PQ concentration (2.5 mg/g) in the presence of vitamin C (25, 100, 200 mM) again for a week (second exposure), and the locomotor performance of flies resulting from this exposure was also evaluated (F2 generation). The number of death flies was recorded daily and their locomotor performance was evaluated by negative geotaxis assay. The histological analysis of the brain of flies was also performed. The results demonstrated that PQ significantly decreased the lifespan and altered the locomotor activity of flies. PQ (2.5 mg/g) promoted neurodegeneration evidenced by the appearance of vacuolated areas in brain of flies. However, treatment with VIT C decreased alterations in mortality rate and locomotor activity, as well as preventing neurotoxicity in the brain of PQ-exposed flies. Taken together, our result highlight VIT C as a potential therapeutic agent against PQ toxicity.

Keywords: Antioxidants; Paraquat (PQ); Vitamin C (VIT C); Flies; Toxicity.

Introduction

Herbicides have been used worldwide over the years to delay growth of weeds and eliminate phytopathologicalpragues that cause damage to agricultural plantations [1,2]. However, their use is known to have environmental impacts and cause health related problems [3]. Of particular pathological importance, herbicides are recognized as possible teratogenic, mutagenic and carcinogenic agents, as well as endocrine disruptors; and their long-term exposure can promote acute to chronic intoxication [4,5], leading to neurodegenerative diseases [6], and reproductive disturbances [7].

Paraquat (PQ) (1,1'dimethyl-4-4'-bipyridynium dichloride) is among the most widely used herbicides worldwide, and its action is increased when combined with the herbicide Diquat [8,9]. In spite of its wide use in agriculture, numerous studies have demonstrated that PQ cancause fatal intoxications for both humans and animals [10-19].

Substantial evidence from the literature indicate that PQ toxicity is mainly attributed to the increased production of superoxide anion, through oxidative stress induction mechanisms, generating more toxic reactive species, such as hydroxyl radicals (OH^-) and hydrogen peroxide (H_2O_2), which have been associated with the depletion of endogenous antioxidant defense systems [2,19-22]. The accumulation of reactive species and the subsequent depletion of reducing agents create an environment of intense oxidative stress that can, consequently cause damage to the lipids, proteins, mitochondria and DNA, thus leading to the alteration of cellular functions [23-26].

Natural antioxidants have been reported to attenuate oxidative stress by eliminating free radicals and increasing endogenous antioxidant defences [27-31]. One of such compound is vitamin C (ascorbic acid), an important non-enzimatic antioxidant that acts as a potent reducing agent [32-36].

Over the past decades, the fruit fly, *Drosophila melanogaster* has considerable attracted the attention of scientists as an alternative model due to its 75% genetic similarity with mammals. In addition, it has been used to increase our understanding on the neuropathological and toxicological processes involved in the etiology of several diseases, such as Parkinson, Alzheimer, Cancer and chronic diseases [37-41]. Furthermore, *Drosophila* has a relatively fast reproductive cycle, small body size and easy maintenance in laboratory conditions [42].

Although vitamin C (VIT C) is well-known for its antioxidant activity, little is known about the effect of VIT C supplementation on the transgenerational transmission of *Drosophila melanogaster* after exposure to PQ. Therefore, this study aimed to investigate the possible protective effect of this vitamin C on behavioural, mortality rate,

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and histological changes in different generations of *D. melanogaster* following PQ exposure.

Material and Methods

Animals

Flies of both gender, aged 1 to 3 days old, were used in this study. They were obtained from the Laboratory of Biology and Toxicology (BIOTOX) of the Regional University of Cariri-URCA. The flies were kept in glass flasks containing culture medium constituted of 1 kg of corn meal, 50 g of ground soybeans, 50g of powdered milk, 50g of sugar and 10 g of salt. In addition, they were mainainedin a light/dark cycle of 12h: 12h, under constant temperature and humidity (25 ± 15°C; 60% respectively), as described by Abolaji et al. [42] and adapted by Nunes and collaborators [40].

Treatment schedule

Exposure of parent generation (P) flies to PQ and VIT C: To test the effect of different concentrations of PQ and VIT C, the flies, regardless of gender, were exposed for 7 days (1 week) to the diet supplemented with PQ in increasing concentrations of 0.5, 2.5 and 5.0 mg/g diet, or VIT C in increasing concentrations (25, 100 and 200 mM), and the count of the number of dead flies (mortality rate) was recorded daily at the same time.

For this first experiment, the flies were divided into seven groups (30 flies for each group in triplicate), six of which received the following treatment: Group I - PQ 0.5 mg/g; Group II - PQ 2.5 mg/g; Group III - PQ 5.0 mg/g; Group IV - VIT C 25 mM; Group V - VIT C 100 mM; Group VI - VIT C 200 mM. The flies in the control group received neither VIT C nor PQ.

At the end of the 7-days period, the surviving flies from the P generation underwent locomotor activity test (negative geotaxis assay) and later discarded. However, the resulting pupae from the P generation were allowed to hatch to constitute the F_1 generation (2nd generation). Of note, the locomotor activity of flies of the F_1 generation was also performed for comparative purpose.

PQ exposure and treatment with VIT C in F_1 generation flies: Based on the preliminar result obtained from PQ exposure to P generation about the mortality rate, the concentration of 2.5 mg/g of diet was selected to investigate the possible protective effect of vitamin C against the transgenerational contamination caused by PQ exposure. For this purpose, 30 flies (per group, in triplicate) of both gender of F_1 generation were exposed to diet supplemented with 2.5 mg PQ/g of diet with or without (PQ alone) different concentrations of VIT C (25, 100 and 200 mM). The control diet did not received VIT C or PQ.

For the second experiment, the flies were divided into five groups, four of which received treatment: Group I - PQ (2.5 mg/g); Group II - PQ (2.5 mg/g) + VIT C (25 mM); Group III - PQ (2.5 mg/g) + VIT C (100 mM); and Group IV - PQ (2.5 mg/g) + VIT C (200 mM). The daily count of the number of dead flies from the F_1 generation was performed during the second 7-day exposure periodand later submitted to negative geotaxis essay. Then, the flasks were emptied, leaving the pupae of this second exposure to hatchin order to form the F_2 generation of flies (3rd generation). The F_2 generation of flies were then submitted to negative geotaxis assay after completing one week for comparative purposes.

Nagative geotaxis assay (evaluation of locomotor activity): The locomotor performance of the P, F_1 and F_2 generations flies was investigated by the negative geotaxis assay, which is based on the evaluation of the locomotor activity of the flies. Briefly, the flies were anesthetized on ice, and 10 flies of each flask were placed into vertically oriented glass tubes and allowed to recover for 10 min. The flies were then shaken down to the bottom of the tubes and the number of flies that climbed the 5 cm distance in 6 seconds, as well as those that remained below the marked line in the tube (5 cm), were recorded for analysis. The assay was repeated three times with 3 min interval between the assays, according to experimental procedures previously described by Kiss et al. [43] and adapted by Nunes et al. [40].

Histological analysis of the brain of *Drosophila melanogaster*

The F_1 generation flies were selected to perform the histological analysis in order to know if the increasing concentrations of VIT C (25, 100 and 200 mM) prevented the PQ neurotoxicity in the most toxic concentration (2.5 mg/g). For this, some samples were immediately immersed in 10% buffered formaldehyde and fixed for 24 h, then washed in a buffer solution, dehydrated in alcohol in an increasing series of concentrations (50-100%) and included in glycol methacrylate (historesin) (Historesin Leica®).

Further, 2 µm cuts were done in microtome (Leica®) model RM 2245 equipped with glass razor. The histological sectionswere distended in distilled water and placed on laminas, dried in a buffer at 60°C for 1 min and submitted to toluidine blue staining technique. The laminas in triplicate per animal were analyzed under a light microscope Leica®DM500 and photographed by Leica EC3 locked to the microscope and to Leica® Application Suite (LAS) EZ for histological analysis, according to procedures previously described by Kretzschmar et al. [44], Kosmidis et al. [45] and Dutta et al. [46].

Statistical analysis

Two-way ANOVA followed by Benferroni test was performed using statistical software GraphPad Prism version 6.0, to find out significant variations among different groups of different generations. The results were expressed as mean ± SEM and considered statistically significant when $p < 0.05$.

Results

As depicted in (Figure 1), the exposure of generation P flies to PQ at concentrations of 2.5 and 5 mg/g of diet caused a significant increase in the mortality rate when compared to the control ($p < 0.05$), with the higher mortality at concentration of 2.5 mg/g (Figure 1A). However, 0.5 mg/g of PQ (group I) did not cause any effect on the survival of flies when compared with the control ($p > 0.05$), and the other groups exposed to the PQ (groups II and III) (Figure 1A and 1B). A similar effect was observed with the highest concentration of VIT C (200 mM) used (group VI) when compared to the control ($p > 0.05$). Throughout the 7-day exposure period, it is possible to notice that this concentration of VIT C exerted a greater protective activity in the survival rate of generation P flies compared to the other groups exposed to the VIT C (groups IV and V) (Figure 1A). The cumulative number of generation P dead flies, obtained at the end of the period

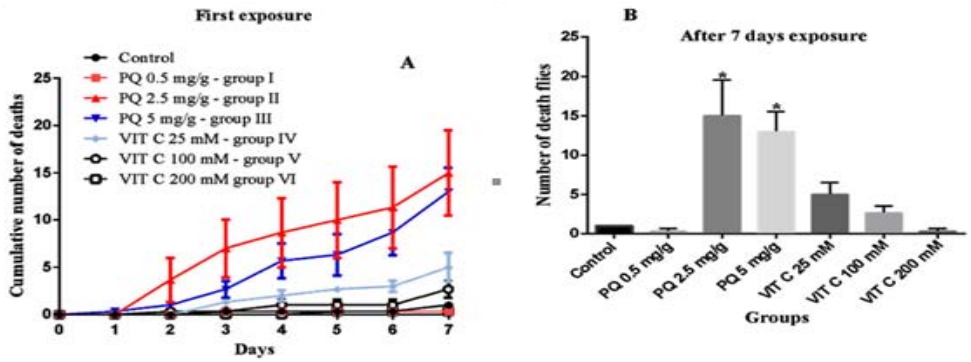

Figure 1: Mortality rate of flies of the parental generation (P) after exposure to PQ and VIT C alone. (A) Indicates the mortality rate over the 7-day exposure time and (B), the mortality at the end of the exposure period (cumulative number of deaths). The results are the average of n = 3 independent experiments. * Indicates significant difference in relation to the control ($p < 0.05$).

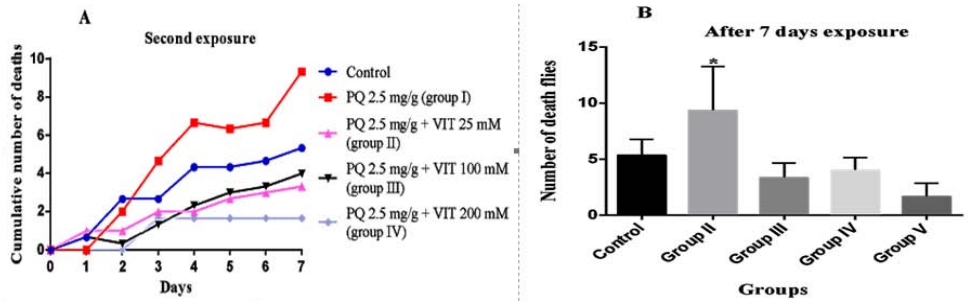

Figure 2: Mortality rate of F1 generation flies exposed to a diet containing PQ (2.5 mg/g) alone or in the presence of increasing concentrations of VIT C. (A) Cumulative number of deaths over time of exposure and (B), mortality on day 7. The results are the average of n = 3 independent experiments. * Indicates significant difference with the control ($p < 0.05$).

of exposure to PQ and VIT C separately, shows that there was a slight reduction in the mortality rate with the increase in the concentration of VIT C when compared to the control ($p > 0.05$; Figure 1B).

As mentioned earlier (see Materials and Methods section), the flies originating from P generation (i.e., F_1 generation) were exposed to food supplemented with PQ (2.5 mg/g of diet) in the presence or absence (PQ alone) of different concentrations of VIT C (25, 100 and 200 mM). Figure 2A shows that PQ (2.5 mg/g) caused a significant increase in the number of dead flies in time-dependent manner when compared to the control ($p < 0.05$). But, when PQ (2.5 mg/g) was co-exposed with different concentrations of VIT C (groups II to IV), the mortality rate was significantly reduced below control, when compared with PQ-alone exposed flies (Figure 2A and 2B). The highest protective effect of vitamin C against the intoxication induced by herbicide PQ was observed at concentration of 200 mM (Figure 2A and 2B). This result may indicate the potential of vitamin C to counteract PQ toxicity in *Drosophila*.

The locomotor performance of the P and F_1 generations flies was investigated by the negative geotaxis test. Two-way ANOVA multiple comparisons followed by Tukey´s post test revealed no significant difference when comparing the locomotor activity of P generation flies *vs*. F_1 generation flies, all of them exposed to PQ and VIT C and at all the concentrations tested. However, there was a significant decrease in the locomotor activity of P and F_1 generations of flies exposed to PQ at concentrations of 5 mg/g and 2.5 mg/g of diet, respectively, when compared to the control of both generations ($p < 0.05$; Figure 3).

To test the potential protective effect of VIT C under the changes in locomotor activity induced by PQ at the concentration that most exerted toxic activity (second exposure), the F_1 and F_2 generations of flies were used. As depicted in Figure 4, there was a slight decrease in the locomotor activity of flies of both generations exposed to PQ (2.5 mg/g of diet) in comparison to control of the F_1 and F_2 generations ($p < 0.05$). As expected, this effect was attenuated by co-treatment with different concentrations of VIT C, although it has not been statistically different compared to the flies exposed to PQ alone from F_1 or F_2 generations ($p > 0.05$). Two-way ANOVA revealed no significant difference between groups from both generations ($p > 0.05$).

As previously mentioned, histological analysis of the brain of F_1 generation flies was carried out in order to find out of VIT C was able to prevent neurodegeneration caused by PQ at the most toxic concentration (2.5 mg/g). In the histological analysis of the brain samples of the flies of the control group (Figure 5A), the frontal section of the optical areas of the retina (Re), optic chiasm, lamina (La), medulla (Me) and lobe (Lo) showed normal cellular characteristics, whereas the group exposed to PQ at concentration of 2.5 mg/g (Figure 5B) shows degenerative changes that appear as vascular lesions in the various regions of the brain. However, the groups exposed simultaneously to PQ and VIT C in increasing concentrations of 25, 100 and 200 mM (images C, D, E, respectively), showed histological

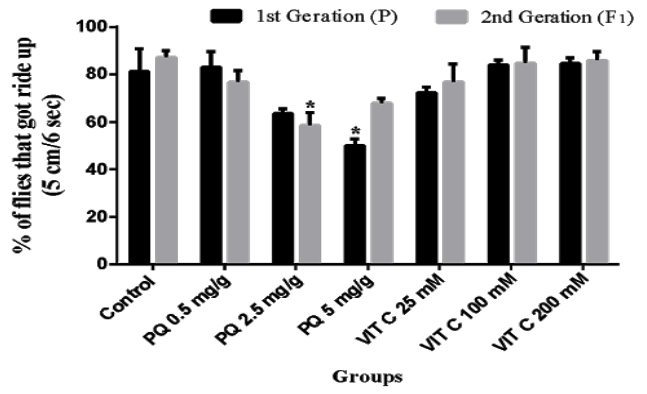

Figure 3: Percentage of flies of generation P (1st generation) and F1 (2nd generation) exposed to PQ or VIT C that rose during the negative geotaxis test, performed after the first exposure. * Indicates significant difference in relation to the control.

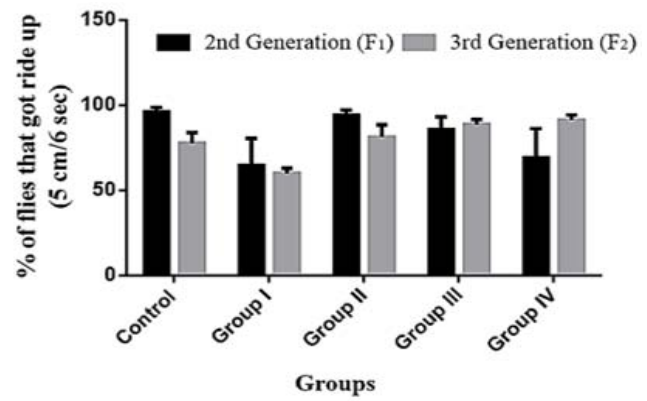

Figure 4: Percentage of F_1 (2nd generation) and F_2 (3rd generation) flies exposed to PQ alone or in combination with different concentrations of VIT C that rose during the negative geotaxis test, performed after the second exposure. Group I - PQ (2.5 mg/g); Group II - PQ (2.5 mg/g) + VIT C (25 mM); Group III - PQ (2.5 mg/g) + VIT C (100 mM); Group IV - PQ (2.5 mg/g) + VIT C (200 mM).

characteristics similar to those of the control, indicating preserved brain areas. Therefore, VIT C at the concentrations tested here was able to prevent the harmful effects of PQ in the brain of the flies (Figures 5C, D and E).

Discussion

In this study, we used fruit fly *Drosophila melanogaster* as a model species to evaluate the effects of VITC (ascorbic acid) on PQ-induced toxicity. *D. melanogaster* has been widely used in biology since the early 20th century, which has played important roles in genetics, embryonic development, disease-related signal transduction, and as a human disease model [26,47,48]. Moreover, *D. melanogaster* had stood out for being an excellent model for gerontological and toxicological research, where adult flies appear to show many of the manifestations of cellular senescence observed in mammals [20,33,38,49,50].

Oxidative stress is a condition characterized by the shift in the balance of the antioxidant defense mechanisms of the organism [51], that happen due to production of Reactive Oxygen Species (ROS) known to cause damage to lipids, proteins, RNA, and DNA [24,52], resulting consequently, in cell death if not properly managed [38]. According to Ayala et al. [30], different studies consider oxidative stress as the main mechanism of PQ-induced toxicity, suggesting that a therapy with the use of antioxidant substances such as melatonin [53], serotonin, [54], lipoic acid [20], vitamin E, vitamin A, and vitamin C [55], taurine, caffeine [51,56], 4-hydroxyisophthalic acid (DHA-I), quercetin, and nicotinamide [57,58], can be a viable alternative to counteract PQ-induced reactive species generation. This is at least in part, because antioxidants are exogenous or endogenous molecules that mitigate any form of oxidative stress, by directly eliminating reactive species and increasing endogenous antioxidant defences [11,26,27,59,60].

In fact, researches have shown that treatment with antioxidants is associated with a low incidence of degenerative diseases, including cancer, Alzheimer's, Parkinson's and coronary heart disease [29,61-63]. Nevertheless, it is important to take into account that antioxidant substances can also promote adverse effects on the body depending on the dosage and the frequency of use [34]. Of note, the complete removal of reactive species by supplementation with antioxidants can disrupt cell signalling pathways and increase the risk of chronic disease [64,65], since reactive species play crucial roles in maintaining normal cell function at relatively low concentrations [52,66,67].

In the current study, VIT C was used to test its potential protective effect against PQ-induced toxicity in *Drosophila*. Vitamin C is a water-soluble substance that functions as an antioxidant in the aqueous phase [56], and an essential micronutrient that is associated with several biochemical and biological functions [31,68]. In addition, VIT C has long been considered a potent antioxidant because it is able to eliminate free radicals associated with oxidative stress and, consequently, interrupt the underlying oxidative reactions [36,41,69-71], via the modulation of antioxidant enzymes activity, such as Superoxide Dismutase (SOD), catalase, and Glutathione Peroxidase (GPx), possibiliting even the use of this vitamin in anticancer treatments [62,63,72,73].

Although the biochemical parameters associated with oxidative stress were not investigated in this study, VIT C demonstrated great protective capacity through prevention of histological changes and reduction of the alterations in the locomotor activity of flies induced by PQ exposure, as well as by increasing the survival rate of the P and F_1 generation flies exposed to the most toxic concentration (2.5 mg/g diet) of the herbicide. Corroborating this fact, Bonilla et al. [20] investigated the effects of VIT C (as well as other natural antioxidant substances) on changes in life expectancy induced by PQ in *D. melanogaster*. In general, VIT C stood out for its great protective capacity, improving the survival rate of the flies, as demonstrated in the experiments of the second exposure (Figure 2).

Of particular interest, Bahadorani et al. [74] evaluated the effects of supplementation of VIT C on the life expectancy of *D. melanogaster* under normoxia and hyperoxia. The authors observed that VIT C extended the survival rate of flies under normoxic conditions, similar to that observed in the first experiment with VIT C alone (see section 3.1). In addition to having exhibited antioxidant activity under hyperoxia, decreasing the oxidative effects of O_2 and

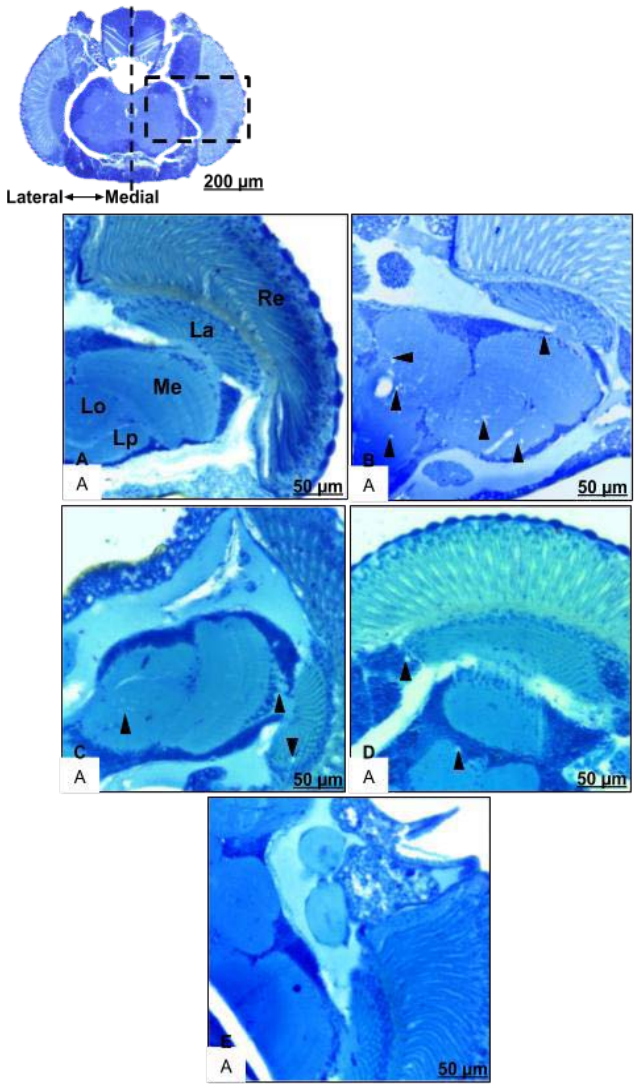

Figure 5: Histological analysis of *Drosophila* brains. A-E, Frontal sections were stained with toluidine blue and examined by bright-field microscopy. (A) Control: the optical areas are the Retina (Re), the optic chiasm (arrow heads), the Lamina (La), Medulla (Me) and Lobula (Lo). (B) Exposure to PQ: histological analysis in the group exposed to PQ shows degenerative changes that appear as vacuolar lesions in the various brain regions (arrows). Groups treated with VIT C (C, D and E) presented preserved brain regions. Scale bar A–E, 50 µm.

the toxic effects of iron (Fe^{2+}). In another study conducted by Suh et al. [56], the authors showed that the administration of VIT C did not affect negatively life expectancy of *D. melanogaster* as highlighted by the previous experiments carried out in this work. Similarly, Bahadorani and Hilliker [74] investigated the effects of VIT C isolated on the lifespan of wild type *Drosophila*. The authors found that at concentrations of 0.02, 0.2 and 2 mM, VIT C had no significant effect on the lifespan of the *Drosophila* compared to the control group. However, when the dose of 20 mM VIT C was included in the culture medium, the lifespan of the flies significantly increased ($p < 0.01$), i.e., reducing the mortality rate. This finding corroborates with the results presented in this study, in which we found that the higher concentration of VIT C (200 mM) used had a greater protective effect when evaluating the mortality rate of the flies.

The protective effects of natural antioxidants such as VIT C were also demonstrated in *D. melanogaster* used as a model in Parkinson's Disease (PD) [32,75,76]. Previous studies have reported that, like VIT C, substances such as quercetin, ellagic acid, nicotinamide and other antioxidants exhibited effective neuroprotective activity by decreasing motor deficits in *Drosophila* used as a model in PD [71,77-79]. In this context, Niveditha et al. [58] demonstrated the potential of natural antioxidants against locomotor impairment induced by PQ in *Drosophila*. Flies exposed to multiple (sub-lethal) dose of PQ showed movement disorder in locomotor activity assay, similar to what was demonstrated by Saur et al. [80] and Khan et al [32], in which the exposure to the herbicide PQ for 24 hours was able to reduce the locomotor activity of the flies, interfering in the process of negative geotaxis. These findings corroborate with what was observed in the locomotor activity tests carried out in this study (see item 3.3). However, as evidenced in the study of Niveditha et al. [58], changes in the locomotor activity of the flies were minimized by treatment with antioxidant, also preventing the underlying neurotoxicity caused by exposure to the herbicide PQ.

The histological analyzes of the brain of F_1 generation flies carried out in this study showed that supplementation with VIT C was able to prevent neurodegenerative progression caused by the deleterious effects of PQ-exposure. Neurodegeneration caused by PQ-induced toxicity was distinguished here as vacuolated areas (Figure 5), as demonstrated through previous experiments performed by Lessing and Bonini [81], Haddadi et al. [82] and Mehdi and Qamar [83]. According to Sunderhaus and Kretzschmar [84], neurodegeneration can be assessed by measuring the size and/or number of vacuoles that have developed, focusing on a specific region of the fly's brain or analysing it whole [85,86]. As depicted in Figure 5, medium-lateral cuts were made to select specific brain regions of the flies in order to show the neurodegeneration caused by the toxicity of PQ (highlighted by the arrows in Figure 5B).

Previous studies showed some changes in the brain of *Drosophila* exposed to sub-chronic dose of PQ [83,58], which corroborates with the results obtained from the histological bright field analysis performed in this study. Interestingly, treatment with vitamin C significantly improved the locomotor performance of flies exposed to PQ, certainly because it markedly prevented the underlying neurotoxicity (Figure 5C, D, E). As pointed by Niveditha and Shivanandappa [87], this fact is noteworthy, since exposure to PQ has been correlated with increased levels of ROS and LPO (lipid peroxidation), as well as the depletion of GSH in the fly's brain, resulting in high levels of oxidative stress and, in the last instance, locomotor impairment. Here, supplementation with VIT C improved locomotor activity of the flies, as well as decreased the mortality rate, and this was associated with the reduction of the vacuolar areas originated by PQ-toxicity in the brain regions. Therefore, the analysis of changes in the normal patterns of locomotor activity of *Drosophila* can provide indications that disturbances in the nervous system are happening concomitantly, which was evidenced through histological analysis as performed in the present study.

In conclusion, PQ (2.5 mg/g of diet) was proved to be toxic to *D. melanogaster*, as evidenced by changes in locomotor activity and

increased mortality rate of the first (P) and second (F_1) generation flies. However, VIT C, besides to prolonging survival rate of flies under normal conditions, prevented PQ-induced changes in locomotor activity of P, F_1 and F_2 flies. In addition, treatment with VIT C prevented neurodegenerative progression in the *Drosophila* F_1 generation brain through reduction of the vacuolar lesions induced by PQ toxicity [4,37,88-91].

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