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Neuroprotective action of aspirin on Paraquat intoxication in on *Drosophila melanogaster*

Ação neuroprotetora da aspirina em *Drosophila melanogaster* intoxicadas por Paraquat

Acción neuroprotectora de la aspirina en la intoxicación por Paraquat en *Drosophila melanogaster*

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Abstract
Acetylsalicylic acid or aspirin is the most widely used drug globally for its anti-inflammatory characteristics, although little is known about its actions on the central nervous system (CNS). We investigated aspirin's potential neuroprotective effects against paraquat-induced neurotoxicity (PQ) in the present study. Adult male wild-type flies were exposed to a diet containing PQ (3 mM) and/or aspirin (1 μM; 5 μM; 10 μM). Flies fed with PQ reduced locomotion and increased mortality. PQ-induced neurotoxicity has also been associated with a marked decrease in acetylcholinesterase (AChE) activity and lipid peroxidation. Co-exposure to aspirin (5 μM) increased survival, improved motor performance, increased AChE activity, and decreased lipid peroxidation. Our results suggest aspirin's neuroprotective effects, probably due to its lysosomal action and antioxidant characteristics. Thus, we demonstrate that the *Drosophila melanogaster* model can elucidate basic aspirin mechanisms to assist the evaluations carried out in higher animals to minimize the neurodegenerative effects caused by diseases such as Parkinson's and Alzheimer's.
Keywords: *Drosophila melanogaster*; Aspirin; Neurodegeneration.

Resumo
O ácido acetilsalicílico ou aspirina é o medicamento mais utilizado no mundo por suas características anti-inflamatórias, embora pouco se saiba sobre suas ações no sistema nervoso central (SNC). No presente estudo, investigamos os potenciais efeitos neuroprotetores da aspirina contra a neurotoxicidade induzida pelo paraquat (PQ). Moscas adultas machos do tipo selvagem foram expostas a dieta contendo PQ (3 mM) e/ ou aspirina (1 μM; 5 μM;10 μM). As moscas alimentadas com PQ reduziram a locomoção e aumentaram a mortalidade. A neurotoxicidade induzida pelo PQ também foi associada a uma diminuição acentuada na atividade da acetilcolinesterase (AChE) e peroxidação lipídica. A co-exposição à aspirina (5 μM) aumentou a sobrevida, melhorou o desempenho motor, aumentou a atividade da atividade da AChE e diminuiu a peroxidação lipídica. Nossos resultados sugerem efeitos neuroprotetores da aspirina, provavelmente devido à sua ação lisossomal e características antioxidantes. Assim, demostramos que a utilização do modelo *Drosophila melanogaster* possa elucidar mecanismos básicos da aspirina de maneira à auxiliar as avaliações realizadas em animais superiores que visam minimizar os efeitos neurodegenerativos causadas por doenças como Parkinson e Alzheimer.
Palavras-chave: *Drosophila melanogaster*; Aspirina; Neurodegeneração.

Resumen
El ácido acetilsalicílico o aspirina es el fármaco más utilizado en el mundo por sus características antiinflamatorias, aunque se sabe poco sobre sus acciones sobre el sistema nervioso central (SNC). En el presente estudio, investigamos

los posibles efectos neuroprotectores de la aspirina contra la neurotoxicidad inducida por paraquat (PQ). Las moscas machos adultos de tipo salvaje se expusieron a una dieta que contenía PQ (3 mM) y / o aspirina (1 μM; 5 μM; 10 μM). Las moscas alimentadas con PQ redujeron la locomoción y aumentaron la mortalidad. La neurotoxicidad inducida por PQ también se ha asociado con una marcada disminución de la actividad de la acetilcolinesterasa (AChE) y la peroxidación de lípidos. La coexposición a la aspirina (5 μM) aumentó la supervivencia, mejoró el rendimiento motor, aumentó la actividad de la AChE y disminuyó la peroxidación de lípidos. Nuestros resultados sugieren efectos neuroprotectores de la aspirina, probablemente debido a su acción lisosomal y características antioxidantes. Así, demostramos que el uso del modelo de *Drosophila melanogaster* puede dilucidar los mecanismos básicos de la aspirina para ayudar a las evaluaciones realizadas en animales superiores que tienen como objetivo minimizar los efectos neurodegenerativos provocados por enfermedades como el Parkinson y el Alzheimer.
Palabras clave: *Drosophila melanogaster*; Aspirina; Neurodegeneración.

1. Introduction

Neurodegenerative diseases are a heterogeneous group of disorders characterized by progressive degeneration of the CNS structure and function or peripheral nervous system. The most common neurodegenerative diseases include Alzheimer's disease and Parkinson's disease (Erkkinen et al., 2018). Together, these diseases affect more than 50 million people worldwide (Livingston et al., 2020).

In Alzheimer's disease (AD), the known pathogenesis is based on the deposition of ß-amyloid plaques and neurofibrils in the brain parenchyma in specific parts such as the cerebral cortex and hippocampus (Sereniki & Vital, 2008). Thus, synaptic connection interruption resulting in cerebral atrophy, causing the symptoms most observed in AD patients, such as cognitive skills deficit and memory loss (Iqbal et al., 2010).

Parkinson's disease, on the other hand, is characterized by motor imbalances with the loss of dopaminergic neurons in the substantia nigra in the brain and the appearance of Lewy bodies, cytoplasmic inclusions formed by alpha-synuclein (Baltazar et al., 2014).

These two diseases do not yet have treatments that inhibit the neurodegenerative process. However, several other therapies have been used to reduce the effects caused by these diseases. Studies linked most of these diseases to environmental factors, such as the paraquat herbicide (PQ) (Baltazar et al., 2014; Niveditha et al., 2017; Yan et al., 2016).

Paraquat is a highly toxic herbicide that induces reactive oxygen species (ROS) production, leading to oxidative stress. Also, it alters mitochondrial metabolism (Soares et al., 2017). The CNS has a high need for O_2 and large amounts of polyunsaturated fatty acids in its composition. Therefore, it becomes more susceptible to free radicals' prejudicial action, which might lead to the oxidation of proteins, DNA, mRNA, and lipid peroxidation—outing the effect of neurotoxicity and neurodegeneration present (Sereniki & Vital, 2008). This compound, in numerous studies, is used as a drug capable of mimicking effects related to neurodegenerative processes in several animals, including rats and mice (Bus et al., 1976; Wang et al., 2017) and nerve cells of *Drosophila melanogaster* (de Oliveira Souza et al., 2019; Niveditha et al., 2017; Soares et al., 2017; Souza et al., 2017).

Here, we test the effect of acetylsalicylic acid, commonly known as aspirin, in association with PQ in *Drosophila*. Aspirin is one of the most used pharmaceutical products in medical practice. Aspirin is one of the most used pharmaceutical products in medical practice. Besides its extensive use as an analgesic and antipyretic, aspirin is also beneficial to atherosclerosis, cardiovascular disease, and various cancer types (Aubin et al., 1998).

Previous studies have explored the neuroprotective effect of aspirin under different disease conditions. It has been shown to have protective effects in models for Parkinson's disease (Aubin et al., 1998). Moreover, epidemiological studies have shown that high doses of aspirin have a lower prevalence of AD and better cognitive function maintenance (Kern et al., 2012; Shi et al., 2020), such as induce lysosomal biogenesis (Ghosh et al., 2007; Melo, 2019) in mouse cell model by activating PPARα, and this mechanism would allow cleaning in the accumulation of amyloid-beta plaques in AD (Chandra et

al., 2018). However, although the epidemiological results point to aspirin's potential role in preventing and treating neurodegenerative aspects, its therapeutic potential and the underlying molecular mechanism need further investigation. We investigated aspirin's neuroprotective effect in the *Drosophila melanogaster* model in this context.

2. Methodology

2.1 Fly strains

The male used in this work were from the w^{1118} strain obtained from Bloomington Stock Center (BDSC # 3605), Indiana, USA. We kept the flies in the Entomology Laboratory culture, UNOESTE - Campus II- Bloco Q, Presidente Prudente on a standard diet and were reared under 25 ± 1 °C and 12:12 h light/dark cycle. Males and females were kept in bottles containing 25 mL of traditional medium (Diet) and synchronized so that Fl could be collected after they emerge from the pupal. We used males between 0 - 6 hours old after the pupil left for all experiments.

2.2 Determination Paraquat and aspirin

The choice of the PQ concentration used in this study was based on the flies' survival curves' profile exposed to this herbicide. Males of *Drosophila melanogaster* up to 6 hours of age were divided into the following groups: (1) Control; (2) 1 mM PQ; (3) 2 mM PQ; (4) 3 mM PQ; (5) 4 mM PQ. PQ was added to the treatment medium containing 2% w/v agar, 1% sucrose, 1% powdered milk, and 0.8% nipagin. The flies were exposed to treatments for 5 days (25 ± 1 ° C with 60% relative humidity.) The 1 μM; 5 μM; 10 μM concentration of aspirin were added to food based on works already described (Song et al., 2017). Thus, the flies were co-exposed to PQ (3 mM) and three aspirin concentrations (1 μM; 5 μM; 10 μM) for 5 days.

2.3 Longevity assays

For this purpose, we placed sixty flies of each group into separate vials in groups of 20 flies. Flies were transferred to vials containing fresh food every 1 day, and the number of dead flies was registered daily. The total number of flies represents the sum of three independent experiments (20 flies/treatment replica) (Linford et al., 2013).

2.4 Negative geotaxis assay

The flies' locomotor functions were determined using the negative geotaxis test (Benzer, 1967; Hurley & Staveley, 2021). The flies were classified under brief ice anesthesia and placed in a vertical glass column (length 20 cm, diameter 1.5 cm/ 10 flies). After recovery from cold exposure (approximately 30 min), gently beat the flies on the column's bottom. Flies that reached the top of the column (14 cm) within 7 s were counted. The assay was repeated six times, and the data were expressed as the average of six attempts to replicate. Also, included 30 flies per group for the test, and the total number of flies represents the sum of three independent experiments (10 flies per group).

2.5 Quantitative Real-Time Polymerase Chain Reaction

Head RNA was extracted from flies using TRIZOL® (Invitrogen) according to the manufacturer's specifications. We used 500 ng of total RNA extracted and the High-capacity cDNA Reverse Transcription Kit (AppliedBiosystems) to obtain the cDNA. Quantitative real-time PCR experiments were performed on StepOne Plus (PE Applied Biosystems) using the GoTaq® qPCR Master Mix (Promega). Sequence information of the primers listed in Table 1. We used the ΔΔCt method (Livak & Schmittgen, 2001) to compare expression levels.

Table 1. List of primer sequences used for RT-PCR analysis in this study.

		Sequence	Reference
Superoxide Dismutase (SOD1)		5'-GAACTACTTTGCTGAGGTGG 3'-GGATCTGCAAGTAG-TTCGGT	(Haddadi et al., 2014)
Catalase		5'-TCAACATCACCGACTCCAAG 3'-CAGC-GTTGCCCGTTGACTT	
Rpl32		5'-AGGGTATCGACAACAGAGTG 3'-GAACTTCTTGAATCCGGTGG	

Source: Authors.

2.6 Behavioral analysis

To track the locomotor behavior of *D. melanogaster*, we introduce 10 males in a 12.7 cm diameter chamber with sloping walls and record their movements for 15 min and then analyze them in the Ctrax software (Version 0.5.18. Mac; available at http://ctrax.sourceforge.net). This software is free and uses the analysis of videos to determine the speed and behavior of flies in a given space (Branson et al., 2009).

2.7 Homogenate preparation

At the end of the treatment period, we used 10 heads per replicate for the acetylcholinesterase (AChE) activity assays. In the lipid peroxidation assay, 50 heads were used per replicate. Heads were then homogenized in ice-cold 50 mM phosphate buffer, pH 7.4. The homogenate was centrifuged at 3000 x g for 10 min at 4 °C, and AChE activity was determined in the supernatant. Lipid peroxidation was determined in the homogenate.

2.8 Lipid peroxidation (Thiobarbituric acid reactive substances-TBARS)

In the experiment, 100 µL of homogenate was incubated for 1 h at 37 °C. After that, the samples were incubated at 100 °C for 120 min in an acidic medium containing 0.6% TBA (thiobarbituric acid) and 8.1% SDS (sodium do-decyl sulfate) was added. The reaction product was determined at 532 nm, and the results were expressed as % of control after correction by the protein content (Ohkawa et al., 1979).

2.9 Acetylcholinesterase (AChE) activity

For measured the AChE activity, 10 µL of supernatant was mixed with 100 µL of medium containing 0.25 M phosphate buffer (pH 8.0), 5,5′-dithiobis-2-ni-trobenzoic (DTNB) acid [5 M], distilled water (70µL) and 8 mM acetylthiocholine (20 µL), and data were recorded at 412 nm after 2 min and were expressed as nanomoles of substrate hydrolyzed/min/mg protein. AChE activity was expressed relative to the control (Park et al., 2012).

2.10 Protein Assay

Protein was extracted from the treated and controls using phosphate buffer after exposure and estimated by the Bradford method at 595 nm using BSA as the standard (Bradford, 1976). According to the manufacturer's instructions, the samples were diluted 1:10 and added to Bradford's reagent (BioRad®).

2.11 Statistical analysis

The results *in vivo* and *ex vivo* are expressed as SD. All experiments were carried out in triplicate. Multiple

comparisons were performed using one-way ANOVA followed by Bonferroni's multiple comparison test, and the differences were considered significant when p <0.05 (**), 0.01 (***), and 0.001 (****). Statistical analyzes were performed using the GraphPad Prism 9 software.

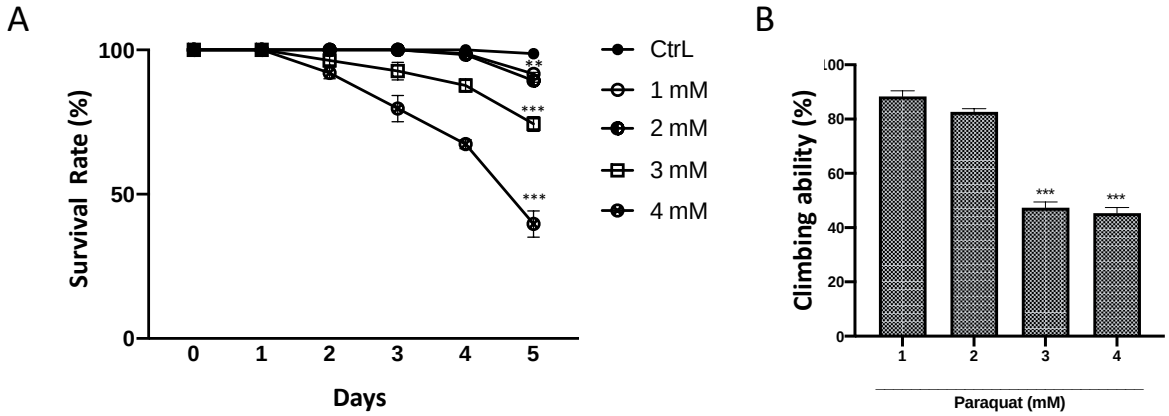
3. Results

3.1 Determination of paraquat and aspirin concentrations.

In Figure 1, we demonstrate the survival test performed on animals exposed to various PQ concentrations. We observed that the PQ concentration of 1 and 2 mM showed almost no difference compared to the control group, except on the last day, where increased the death rate. At the concentration of 3mM there was 25% of dead flies, and in 4 mM there was a severe reduction with more than 50% of dead flies (Figure 1A).

Interestingly, when we submitted the animals that survived the paraquat exposure to the climbing test (Figure 1B), we observed that 3 and 4 mM concentrations had the same climbing rate. However, the concentration of 3 mM had less lethality. So we decided to work with a concentration of 3 mM.

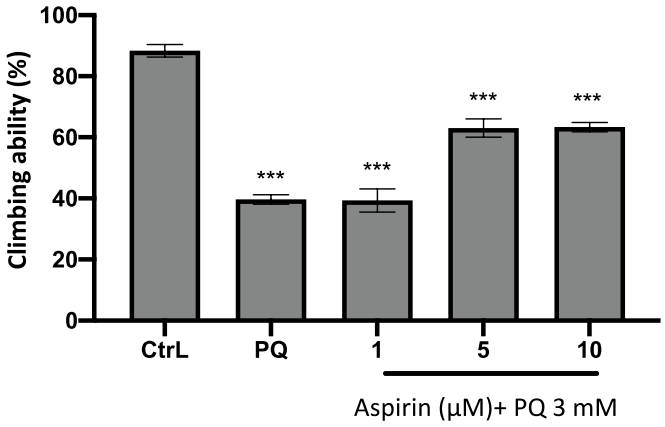
Figure 1. The survival rate of flies exposed to different concentrations of Paraquat.



(A) Data were collected every 24 hours for each group for 5 days. The data are presented as mean ± S.D. The total number of flies (60 per group) represents the sum of three independent experiments. **(B)** Locomotor activity of flies exposed to different concentrations of Paraquat. The total number of flies (30 per group) represents the sum of three independent experiments. Data are presented as mean and SD. One-way ANOVA followed by a multiple comparison test. **p < 0.01 and *** p <0.001 indicates a significant difference concerning the control group. Source: Authors.

In Figure 2, we show aspirin's effect on animals' ability to perform the climbing test when exposed to the PQ. We used concentrations of 1 μM; 5 μM; 10 μM, however, on the concentration of 1 μM was unable to recover values close to the control, demonstrating the inefficiency of this concentration, the importance of 5 and 10 μM of aspirin had similar results. Still, we opted for the use of an intermediate combination (5 μM) of PQ. Additionally, flies exposed to PQ showed deficiencies in locomotor behavior, such as reductions in the rate of climbing, compared to the control group (CtrL). This effect was abolished by co-exposure to aspirin since the flies in this group had a better climbing performance.

Figure 2. Effect of Aspirin on flies subjected to PQ exposure.

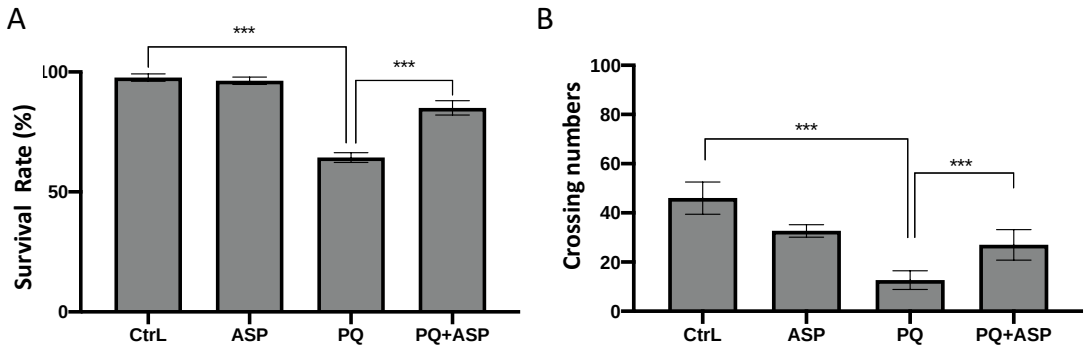


Flies exposed to PQ in the concentration of the 3 µM were to submitted co-treatment with aspirin at concentrations of 5 and 10 µM partially rescued the climbing ability. *** p <0.001 indicates a significant difference between CtrL and treatments. (CtrL- Control; PQ- Paraquat 3 mM; ASP-Aspirin 5 µM). Source: Authors.

3.2 Aspirin improves the survival rate and motility of *D. melanogaster*

Flies exposed to 3 mM PQ showed high mortality compared to control animals after 4 days (Figure 3A). However, PQ and Aspirin's treatments presented a rescue similar to that observed in the control animals. Demonstrating the ability to protect against mortality caused by paraquat. In tracking, flies exposed to PQ showed less exploratory activity than the control group (Figure 3B). This effect was attenuated by co-exposure to aspirin.

Figure 3. Effect of aspirin on (A) animal survival (20 per replicate), (B), and the number of crossed quadrants in the motility test (5 per group) after 5-day exposure.



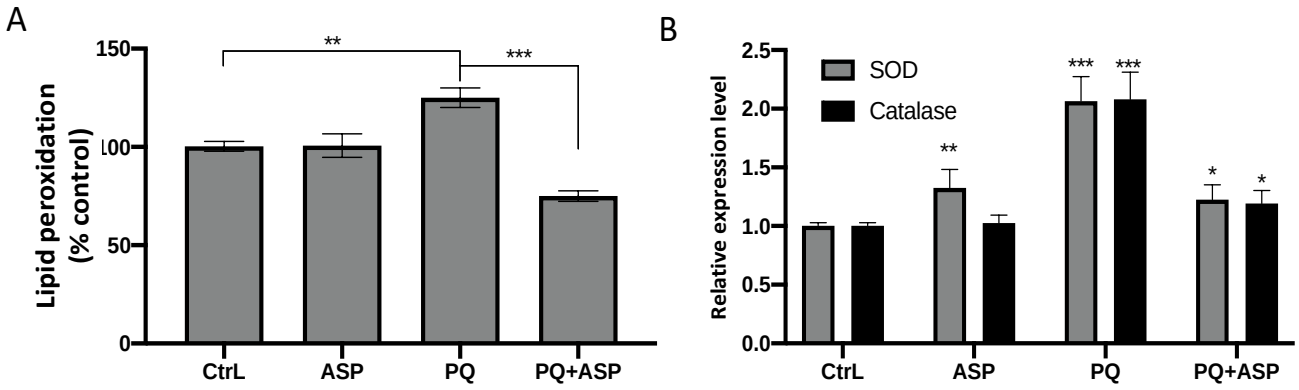
One-way ANOVA followed by multiple comparison *** p <0.001 indicates significance. Abbreviations: CtrL (control), PQ (Paraquat 3 mM), ASP (Aspirin 5 µM). Source: Authors.

3.3 Effect of Aspirin under oxidative markers of flies exposed to Paraquat

In fly heads exposed to Paraquat, there was a significant increase in TBARS (Figure 4A and B), reflecting ROS production induced by PQ. These effects of PQ exposure have been completely normalized with aspirin's addition. Moreover, there were no significant changes in TBARS in flies exposed to aspirin in isolation compared to the control group.

Further, we evaluated the expression of SOD and catalase in the heads of these animals (Figure 4 B), essential enzymes for the control of oxidative stress, whose results showed the increase in transcript levels in animals treated with Paraquat and co-exposed to Aspirin when compared the control group.

Figure 4. Effect of Aspirin on markers related to cell stress.

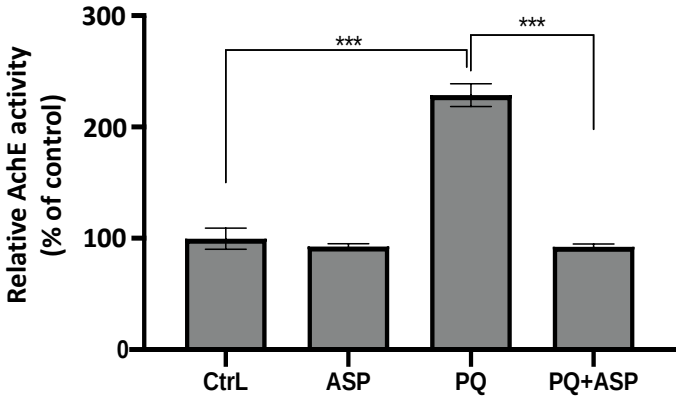


(A) Analysis of lipid peroxidation shows recovery to normal values compared to control and paraquat. (B) RTqPCR shows changes in the SOD and Catalase genes in the brains of animals exposed to Paraquat and rescue in the presence of Aspirin. One-way ANOVA followed by multiple comparison *p < 0.05, **p < 0.01 and *** p <0.001 indicates significance difference in relation to control group. Abbreviations: CtrL (control), PQ (Paraquat 3 mM), ASP (Aspirin 5 μM). Source: Authors.

3.4 Effect of aspirin on acetylcholinesterase (AChE) activity

Levels of AChE exposed to Paraquat are shown to be high. However, co-exposure to aspirin restored AChE activity to the same levels found in control flies (Figure 5).

Figure 5. Neuroprotective effect of Aspirin in flies treated with Paraquat.



Acetylcholinesterase (AChE) activity (10 flies per replica). One-way ANOVA followed by multiple comparisons (*** p <0.001). Abbreviations: CtrL (control), PQ (paraquat 3 mM), ASP (Aspirin 5 μM). Source: Authors.

4. Discussion

Our study suggested a protective role for aspirin by inhibiting the effects caused by PQ when it presented reduced levels of TBARS. Paraquat is commonly used to evaluate oxidative stress; basically, paraquat can accept electrons from various metabolic pathways, including NADPH. After successive modifications, it gives rise to a superoxide anion, a ROS which damages cellular components such as lipids and proteins (Rzezniczak et al., 2011). Although neurotoxicity mechanisms are not very clear, paraquat leads to mitochondrial dysfunction, apoptosis, and autophagy, in addition to inhibiting protein degradation mechanism inducing tauopathies (Baltazar et al., 2014).

For this reason, antioxidants are expected to prevent PQ-induced neurotoxicity. In this way, several drugs and natural extracts are tested for this purpose. Among them are omega 3, turmeric, and propolis (Ayikobua et al., 2018; de Oliveira Souza

et al., 2019; Mishra & Palanivelu, 2008). Our study correlated acetylsalicylic acid, commonly known as aspirin, to a neuroprotective effect on the brain of *D. melanogaster*.

Drosophila melanogaster, better known as the fruit fly, is a model widely used as a genetic model and applied to basic science. Furthermore, it presents several advantages when compared to other models. Among them are: the low maintenance cost, the similarity in metabolic pathways with humans, and the short life cycle (Cheng et al., 2018). Nevertheless, it is widely used to study neurodegeneration, including Alzheimer's and Parkinson's Disease, due to its neuronal similarity and similar responses in inducing these changes, including genetic manipulation and neurotoxic drugs, like paraquat (Park et al., 2012; Rzezniczak et al., 2011). Here we use this model's advantages to study aspirin's possible beneficial effects in animals induced to a neurotoxic process by paraquat.

We demonstrated increased motility and AChE activity (Figure5) on the heads of flies exposed to aspirin and PQ. However, we did not carry out experiments to prove the neurodegenerative process but, yes, speculate based on motility and motor dysfunction tests, supported by previous studies (Riemensperger et al., 2011). We observed an increase in AChE activity in animals exposed to PQ since it affects the cholinergic system. AChE catalyzes acetylcholine hydrolysis, an essential mediator of nerve synapses, and acetylcholine hydrolysis in choline and acetic acid. It is the most important biological component of cholinergic function due to its involvement in membrane integrity and membrane permeability changes that mediate synaptic transmission and conduction (Jahromi et al., 2013).

We assume that low concentrations of aspirin can stimulate lysosomal biogenesis and increase functionality. Lysosomes can act on the degradation of poorly folded proteins in animals' brains exposed to increased stress and neurodegeneration conditions. So, molecular analyses related to lysosomal markers should confirm this hypothesis. In cell models, the aspirin concentrations can increase Aβ astrocytic clearance by increasing the uptake and lysosomal degradation of Aβ by astrocytes (Chandra et al., 2018).

Other studies demonstrate that PQ increases AChE activity, which leads to decreased cholinergic neurotransmission, cell proliferation, increased oxidative stress, neurological dysfunction, and apoptosis (Park et al., 2012). We report a significant increase in AChE levels than the control (Figure5). A decrease in dopamine caused by PQ might be linked to this result as it blocks the autoinhibition of acetylcholine, leading to an increase in acetylcholine in the synaptic cleft seen in other studies (Aosaki et al., 2010).

5. Conclusion

In conclusion, we suggested for the first time that aspirin has neuroprotective activity in the brains of flies subjected to paraquat. We assume that the neuroprotective effect may be correlated with the antioxidant characteristic. However, we understand that more studies should be carried out to verify this drug's actual result. Our group reinforces the importance of in-depth research of mutants of specific neurodegenerations such as Alzheimer's Disease and Parkinson's to describe the molecular mechanisms and whether, under these conditions, aspirin is as promising as in our results.

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