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Original Article

Pharmacological Effect of Caffeine on *Drosophila melanogaster*: A Proof-of-Concept *in vivo* Study for Nootropic Investigation

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Abstract

A comprehensive investigation into drug candidates with nootropic activity using a proper and high throughput yet economical model organism is an important issue to consider. This proof-of-concept study was carried out to determine whether *Drosophila melanogaster* can be used as an *in vivo* screening platform to assess the nootropic activity of certain candidates for the treatment of neurodegenerative diseases. To test this, caffeine was used as a nootropic compound and a Drosophila mutant line lacking PGRP-LB with hyperactivation of NF-κB leading to early death with neurodegenerative phenotype was used as a model organism. Caffeine was orally administered via food to the PGRP-LB mutant of *D. melanogaster* at different concentrations (0.4 mM, 0.08 mM, 0.016 mM) prior to phenotypical observations of the survival and locomotor activity, as well as gene expression analysis, to assess the expression level of *sod1, sod2,* and *cat* genes. The results pointed out that the lifespan of *D. melanogaster* treated with 0.016 mM caffeine was dramatically increased; nonetheless, no changes were observed in the locomotor activity. Phenotypical analysis using a T-maze vial test demonstrated a good cognitive improvement in response to caffeine administration. Molecular analysis revealed that caffeine at a concentration of 0,016 mM induced the expression of the endogenous antioxidant genes *sod1* and *cat*, but not *sod2*, signifying that the increased lifespan may be associated with a marked improvement in cytoplasmic antioxidant function. In general, the findings of the present study are in line with those previously observed in the mammalian model organism. Therefore, it can be concluded that *D. melanogaster* can be used as a model organism in preliminary investigation and screening of nootropic candidates prior to further testing in its mammalian counterparts.

Keywords: Nootropic, Caffeine, Neurodegenerative disorders, Fruit fly, Cognitive function

1. Introduction

Aging as a process in life characterized by growth (1) can affect the nervous system and other organs, leading to the loss of tissue function over time and accumulation of cellular damage, increased oxidative stress, and changes in body metabolism (2). Aging can be influenced by many factors, including genetics, environment, metabolism, and reproduction (3, 4). It has been estimated that 25% of the world's population will be over the age of 60 by 2030 (5, 6). This demographic status exerts dramatic impacts on various aspects, such as psychological, social, political, and

economic (7, 8). Aging can affect the brain, for instance, in cognitive disorders, such as decreased memory function, learning, motor coordination, as well as attention disorders which can be associated with nervous system degeneration (2).

In this regard, it is crucial to discover and develop drug compounds, such as nootropics, that can overcome neurodegenerative disorders. The discovery of new chemical compounds that can improve cognitive function or even return the cognitive abilities of older people to their youth time is an exciting prospect (9). One successful attempt to increase cognitive function is the consumption of foods and drinks that contain caffeine (10) which is a psychoactive substance widely used around the world. In the United States, about 85% of adults consume caffeine, either in beverages, such as coffee, or food (10, 11). In the studies conducted on mammals, caffeine has been shown to have a neuroprotective effect as an adenosine receptor ligand by the activation or inhibition of adenosine receptors subtypes A1 and A2A, reducing the amount of amyloid-β in the brain and increasing motor activity (12). The epidemiological evidence suggested that coffee consumption can reduce cognitive decline and dementia, as well as memory loss in humans (13).

The pre-clinical stage of nootropic drug discovery is currently carried out using mammals, such as mice, before proceeding with clinical trials (14, 15). This process is crucial to ensure the pharmacological effect, safety, and doses required to yield the effect. Nonetheless, this process takes time and requires sufficient, if not high, budget allocation. Moreover, the use of mammalian animals in the pre-clinical test is quite challenging due to a series of strict rules and the requirement of ethical clearance (16, 17). Due to these limitations, the use of alternative *in vivo* models similar to humans is urgently required. To tackle this, several model organisms, including the fruit fly, *Drosophila melanogaster*, were introduced.

The fruit fly, *D. melanogaster*, has 75% genetic similarity to humans and is equipped with comparable signal transduction pathways and homologous protein functions in the nervous system (17, 18). In addition, *D. melanogaster* provides several other advantages, such as cost-effectiveness, speedy growth, and easy maintenance (17). Therefore, once it is experimentally proven that a similar nootropic effect can be observed in *D. melanogaster* as in the mammalian models, the exploration of potential nootropic drug compounds/supplements from natural ingredients can be carried out in the future. This can be parallel with the strategic plan of the world's Sustainable Development Goals to fulfill the discovery, creation, and development of innovative science and technology in the health sector, especially in the field of infectious and non-infectious diseases (including degenerative diseases). In light of the aforementioned issues, the present study aimed to confirm whether *D. melanogaster* can be used as a model organism for the assessment of nootropic drug candidates in *in vivo* pre-clinical settings.

2. Materials and Methods

2.1. Sample Preparation

Caffeine (Soho Nootropics, U.S.) was dissolved using distilled water to obtain a 2% caffeine in water prior to further dilution into 0.4% and 0.08%. All caffeine concentrations were subsequently added to the fly food in a ratio of 1:4.

2.2. Fly Stocks and Maintenance

In this study, we used fruit fly (*D. melanogaster*) as a model organism. Male and female flies aged 2-4 days old with genotype Oregon-R (wildtype) and PGRP-LB$^{\Delta}$ (mutant lacking functional PGRP-LB, used as an autoinflammatory model) were used in all experiments. All fly lines were obtained from the Host Defense and Responses Laboratory (Kanazawa University, Japan) and steadily maintained using regular fly food in plastic Drosophila vials.

2.3. Survival Assay

Survival assay was carried out according to a previously established protocol (19) with some modifications to assess the effect of caffeine on the

lifespan of the PGRP-LB$^{\Delta}$ (PGRP-LB mutant). In brief, 40 *D. melanogaster* were assigned to four groups: Group I was the untreated control (without caffeine) and Group II-IV were caffeine-treated groups. In Group II-IV, fruit flies were maintained in caffeine-containing food at concentrations of 0.4 mM (Group II), 0.08 mM (Group III), and 0.016 mM (Group IV). The lifespan of *D. melanogaster* was observed up to 45-50 days.

2.4. Locomotor Assay

The locomotor assay was carried out using the negative geotaxis method according to previously published protocol (20), with slight modifications. In a nutshell, all live *D. melanogaster* from each group tested in this experiment were placed separately into empty marked test vials equipped with a clear finish line. The test vials were gently tapped to ensure all flies were at the bottom of the vial and subsequently observed for 15 sec. All the *D. melanogaster* that was able to cross the finish line at the marked vial was counted.

2.5. Cognitive Improvement Test

Cognitive testing of *D. melanogaster* was performed using a previously established T-maze protocol (21), with slight modifications based on Ali, Escala (20). Briefly, the T-maze is set up in a clean condition and free from fly corpse. Two groups of fruit flies that were maintained in the presence or absence of 0.016 mM caffeine were used in this experiment. All fruit flies were subjected to a 6-hour fasting procedure and subsequently placed in the T-maze elevator (connecting the starting chamber and test chamber) prior to transferring to the starting chamber. A dark ambience was assigned to the left compartment (A), and a light ambience was assigned to the right compartment (B). Grape syrup, used as a reward in this experiment, was paired with compartment B (with light), while nothing was paired with compartment A (without light). During the initiation stage, all *D. melanogaster* in the starting chamber were allowed to explore the T-maze space for two min.

The flies that managed to stay in compartment B during two min of testing were counted and compared to their counterparts in compartment A. This experiment was repeated three times. At the data collection stage, all flies were subjected to a similar process: flies were given two choices of compartment, compartment A without grape syrup and compartment B with the grape syrup. The test was carried out without light conditions in both chambers. This step was performed in a dark condition to eliminate the phototaxis potential of lighting to Drosophila and ensure that flies use their olfactory abilities to locate food containing compartment. After two min, light was turned on and flies in compartment B were counted. Upon the completion of this test, flies were returned to the original vial.

2.6. Gene Expression Analysis

The isolation of total RNA was carried out using flies from each group. In brief, five live flies from each group were transferred to Treff tubes before being crushed using a micropestle. The *D. melanogaster* RNA was extracted using Wizard SV Total RNA Isolation System (Promega). Total RNA was quantitatively measured using a nano spectrophotometer (BioDrop, U.S.).The level expression of *sod1*, *sod2*, and *cat* genes was assessed by quantitative reverse transcriptase PCR (RT-qPCR) method using three different sets of primers: *sod1* primer set (*sod1* forward primer: 5'–AGGTCAACATCACCGACTCC–3' and *sod1* reverse primer: 5'–GTTGACTTGCTCAGCTCGTG–3'), *sod2* primer set (*sod2* forward primer: 5'–TGGCCACATCAACCACAC–3' and *sod2* reverse primer: 5'– TTCCACTGCGACTCGATG –3') and *cat* primer set (*cat* forward primer: 5'–TTCCTGGATGAGATGTCGCACT–3' and *cat* reverse primer: 5'–TTCTGGGTGTGAATGAAGCTGG–3').

Each RT-qPCR reaction was performed in a 20 µl reaction volume using the GoTaq® 1-Step RT-qPCR

System (Promega) according to the manufacturer's instructions. The expected product verification is validated using a post-amplification melt curve profile. As an internal control in the RT-qPCR assay, *rp49* ribosomal protein levels were examined using a set of *rp49* primers (*rp49* forward primer: 5'–AGATCGTGAAGAAGCGCACCAAG–3' and *rp49* reverse primer: 5'–CACCAGGAACTTCTTGAATCCGG–3'). Rotor-Gene Q thermal cycler (Qiagen, Germany) was used with the following profiles: 37°C for 15 min, 95°C for 10 min, and set at 95°C for 10 sec, 60°C for 30 sec, and 72°C for 30 sec for 40 repeated cycles, followed by the analysis of the melt curve from 60°C to 95°C. The obtained data were analyzed using the relative quantification method.

2.7. Data Processing and Analysis

All data obtained in the survival assay were analyzed using the Kaplan-Meier curve, coupled with Log-Rank statistical analysis. Locomotor, cognitive, and gene expression analyses of data were processed using the One-way ANOVA method. All statistical analyses were performed using GraphPad Prism® 9. Data were presented as mean±S.D, and a p-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Caffeine Increases the Lifespan of PGRP-LB$^{\Delta}$ *D. melanogaster*

One of the phenotypic parameters related to aging is lifespan (22, 23); therefore, survival assay is a simple approach to evaluate the effect of a drug on lifespan. The present study compared the lifespan of two *D. melanogaster*: Oregon-R (wildtype) and PGRP-LB$^{\Delta}$ (PGRP-LB mutant). As illustrated in figure 1, the lifespan scores of Oregon-R and the PGRP-LB$^{\Delta}$ were around 63-64 and 35-36 days, respectively. This is probably due to the knockout of the PGRP-LB gene and has been reported elsewhere (24, 25) upon the stimulation of a proper ligand. As an amidase, PGRP-LB plays an important role in the negative regulation of the Imd pathway (NF-κB-homologue in *D. melanogaster*) by regulating the immune response against Gram-negative bacterial infections. The loss of PGRP-LB disrupts such function and impairs the physiological condition of flies, including the induction of premature neurodegenerative conditions and aging (25). Consequently, we decided to use PGRP-LB$^{\Delta}$ flies in the next experiments.

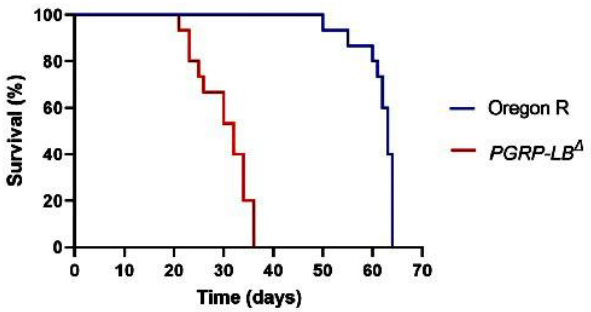

Figure 1. Comparison of lifespan of *D. melanogaster* Oregon R (wildtype) and PGRP-LB$^{\Delta}$ (mutant). Mutant line lacking PGRP-LB experienced early death phenotype, compared to Oregon flies.

Following that, we used PGRP-LB$^{\Delta}$ flies in the evaluation of the nootropic effect of caffeine. In the current study, caffeine was used as an example of nootropic compounds. As displayed in figure 2, higher concentrations of caffeine (0.4 mM and 0.08 mM) yielded a negative effect on the survival of flies, while caffeine at a lower concentration (0.016 mM) was safe and can promote longer survivorship of the PGRP-LB$^{\Delta}$ flies, in comparison to untreated control flies. This result indicates that caffeine acts in a concentration-dependent manner in *D. melanogaster*.

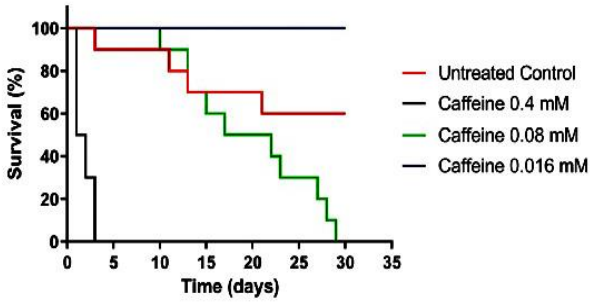

Figure 2. Survival of PGRP-LB mutant in the presence and absence of caffeine at different concentrations. Adult fruit flies aged 2-4 days were assigned to different groups and subjected to intended treatments. The flies which received no additional treatment were designated as untreated control.

3.2. Caffeine Did Not Affect the Locomotor of PGRP-LB$^{\Delta}$ *D. melanogaster*

One of the observable phenotypic parameters of aging is the status of locomotor activity which denotes the ability of a subject to move from one location to another (20). In response to the consumption of certain pharmaceutical preparations, locomotor activity can be enhanced, declined, or remain steady. Subsequently, we assessed the effect of caffeine on locomotor activity of PGRP-LB$^{\Delta}$ *D. melanogaster*. It seems that routine consumption of 0.016 mM caffeine did not affect the locomotor activity of PGRP-LB$^{\Delta}$ flies, at least until 10 days of caffeine treatment (Figure 3). This result suggested that caffeine can increase the lifespan of flies without affecting their movement.

3.3. Caffeine Improves the Cognitive Function of PGRP-LB$^{\Delta}$ *D. melanogaster*

Increased lifespan in PGRP-LB$^{\Delta}$ flies provides an indication of physiological improvement. Nevertheless, the association between this improvement and the ability of flies to recognize and memorize certain objects has remained unclear; consequently, we carried out a cognitive T-maze test to assess this issue. As depicted in figure 4, PGRP-LB$^{\Delta}$ flies that consumed 0.016 mM caffeine had a better ability to locate food compartments, compared to their untreated counterparts, signifying that caffeine consumption could improve the cognitive function of flies, at least in nutrition seeking-related activities.

3.4. Caffeine Enhances the Expression of *sod1* and *cat* Genes

Aging has been suggested to be associated with enhanced activity of reactive oxygen species (ROS) (2, 4). To prevent ROS-mediated aging, cells require endogenous antioxidants. Several endogenous antioxidants that have been reported to play a vital role in the neutralization of ROS are superoxide dismutases (*sod*s), such as *sod1* and *sod2*, as well as *catalase* (*cat*) (26, 27). To determine whether the improvement of PGRP-LB$^{\Delta}$ lifespan by caffeine was achieved through the role of enhanced endogenous antioxidant activity, we assessed the expression levels of *sod1*, *sod2*, and *cat* in PGRP-LB$^{\Delta}$ flies upon treatment with 0.016 mM caffeine. As a result, it was found that caffeine at a concentration of 0.016 mM could increase the expression of *sod1* and *cat* genes, but not *sod2* (Figure 5). Such overexpression profiles of endogenous antioxidants have been reported to be important in the reduction of ROS levels (28, 29).

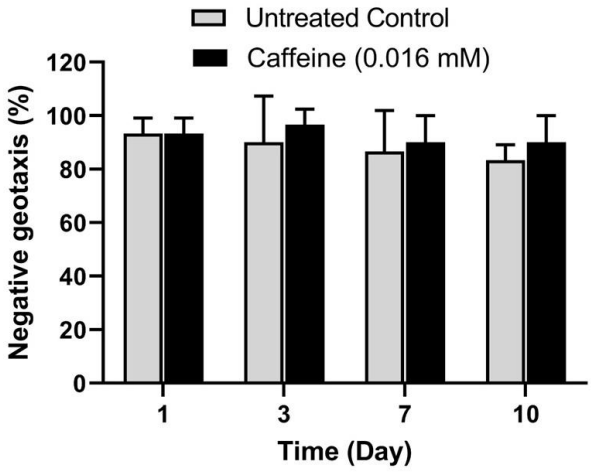

Figure 3. Locomotor of PGRP-LB mutant in the presence and absence of caffeine at 0.016 mM. Adult fruit flies aged 2-4 days were divided into different groups and subjected to intended treatments. The flies which received no additional treatment were designated as untreated control.

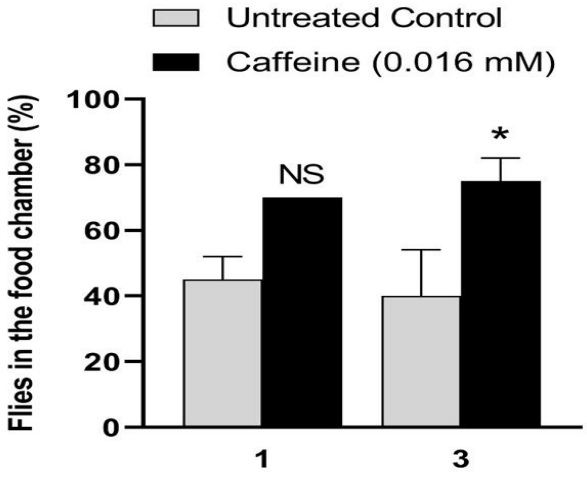

Figure 4. Cognitive improvement in the PGRP-LB mutant in the presence of caffeine at 0.016 mM. Adult fruit flies aged 2-4 days were divided into different groups and subjected to intended treatments. The flies which received no additional treatment were designated as untreated control (***P*<0.01).

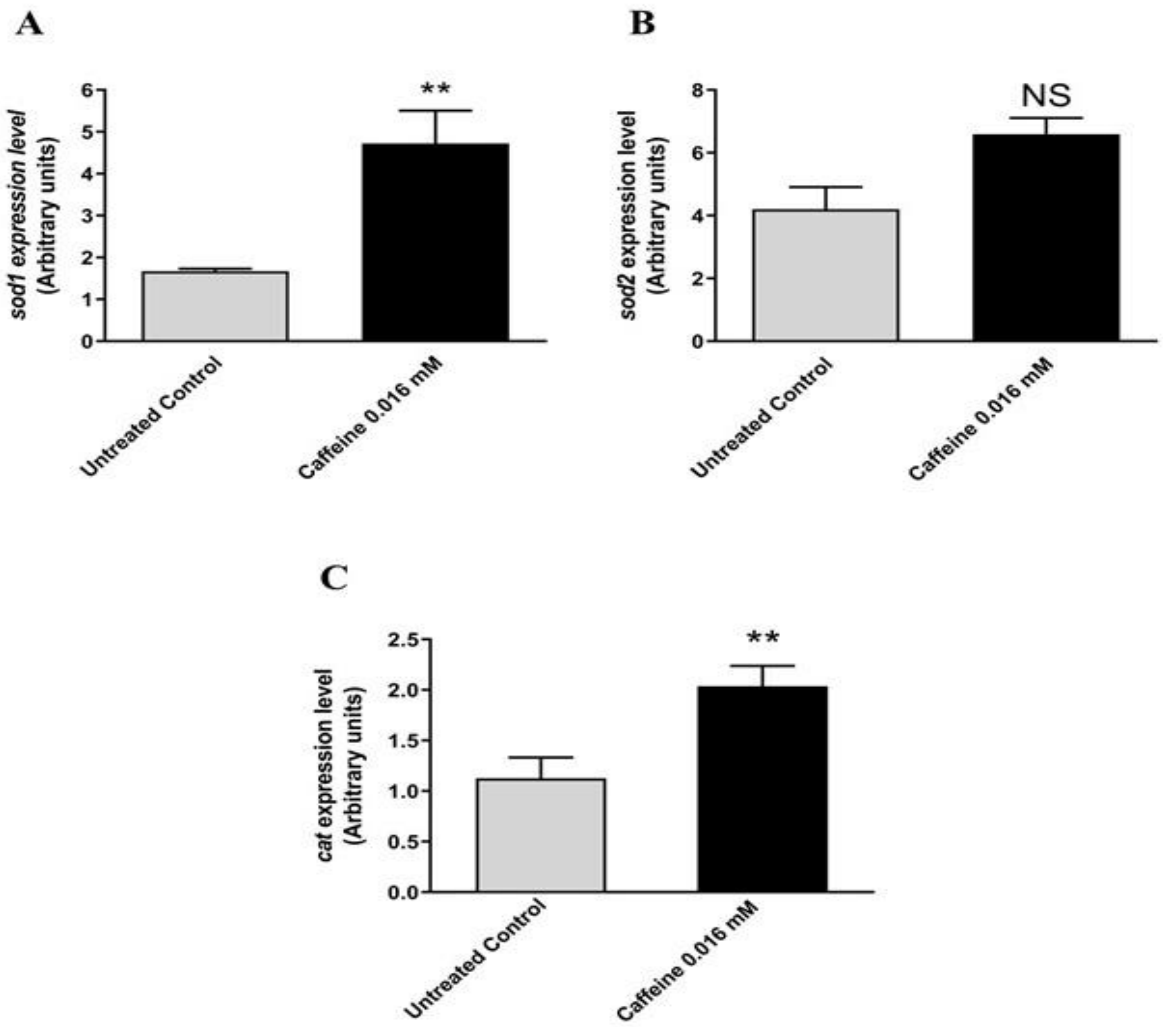

Figure 5. Expression of *sod1* (**A**) *sod2* (**B**) and *cat* (**C**) in the PGRP-LB$^{\Delta}$ in the presence or absence of caffeine at 0.016 mM. Adult fruit flies aged 2-4 days were divided into different groups and subjected to intended treatments. The flies which received no additional treatment were designated as untreated control (NS, Non-Significant; ***P*<0.01).

4. Discussion

The PGRP-LB protein is an amidase which plays a major role in the NF-κB homolog Imd (Immune deficiency) pathway in *D. melanogaster* (30). This protein acts in the regulation of the immune response against Gram-negative bacterial infections (31, 32). The absence of PGRP-LB can lead to the hyperactivation of the NF-κB Imd pathway, resulting in a hyperinflammatory response, which can cause aging occurring faster (25). The overexpression of the immune system can induce neurodegenerative events, thereby accelerating aging (33). Neurodegenerative effects can occur in the form of decreased cognitive function, memory loss, and attention disorders (34, 35). At a later stage, neurodegenerative disorders have been linked to an early death phenotype (36, 37). Therefore, PGRP-LB mutant fly (PGRP-LB$^{\Delta}$) can be potentially used as a model organism in the in vivo investigation of drug candidates with nootropic activity to control the aging process.

How caffeine improves the lifespan of PGRP-LB$^{\Delta}$ flies has remained unexplored. As demonstrated in the current study, caffeine might exert its effect on cognitive improvement and lifespan probably via the

enhancement of endogenous antioxidant activity. There have been credible reports of the neuroprotective effect of caffeine on mammalian models via the regulation of Nrf-2 and NF-κB in terms of antioxidant effect to overcome oxidative stress (38, 39). Caffeine has been known as an antioxidant by mechanism as an inhibitor at adenosine receptors A1 and A2, thereby reducing the effects of oxidative stress due to inflammation (39). In agreement with this finding, previous studies using the nematode *C. elegans*, sheep, and pigs pointed to the beneficial effect of caffeine on cognitive function and lifespan (40-42).

One of the causes of neurodegenerative disorders is the excessive production of ROS. A high level of ROS can trigger nerve cell death, and this can be prevented by endogenous antioxidants (43). Endogenous antioxidants, such as superoxide dismutases and catalase, are expressed by humans and *D. melanogaster* (44, 45). In the present study, it was found that caffeine at a 0.016 mM concentration can induce the expression of *sod1* and *cat* in *D. melanogaster*. Such phenotypical features were possibly related to cognitive improvement and enhanced survival of PGRP-LB mutant fly. This is similar to what has been found in the mammalian animal model (46, 47), indicating that the analysis using *D. melanogaster* can provide information on nootropic activity through understanding the mechanisms of inflammatory aging caused by the immune-mediated mechanisms.

Further research is required to elucidate mechanisms involved in the action of caffeine to increase the survival of PGRP-LB$^{\Delta}$ flies. Unveiling the mechanism of aging due to inflammation and how overexpression of antimicrobial peptides in the NF-κB pathway can augment this event can clarify whether the nootropic activity of caffeine and other nootropic agents to alleviate neuroinflammation was achieved through the increased expression of endogenous antioxidants. In general, the results of the present study can provide preliminary information on the effect of caffeine on several phenotypical characteristics related to the neurodegenerative status, such as lifespan, locomotor activity, and cognitive function, as well as the expression of endogenous antioxidant genes.

5. Conclusion

As evidenced by the results of the present study, *D. melanogaster* is a useful in vivo model organism to investigate the effect of caffeine on several phenotypical characteristics related to the neurodegenerative status, such as lifespan, locomotor activity, and cognitive function, as well as the expression of endogenous antioxidant genes. This study further delineated the potential of *D. melanogaster* to screen new drug candidates with a nootropic activity in easier and cheaper ways prior to further tests using the mammalian animal models to examine its safety and efficacy.

Authors' Contribution

Study concept and design: A. A., U. U., A. S. W. P., N.P. and F. N.
Acquisition of data: A. A., U. U., N. R. R. and R. A. R.
Analysis and interpretation of data: A. A., U. U., F. N., T. B. E. and K. D.
Drafting of the manuscript: U. U., N. R. R. and F. N.
Critical revision of the manuscript for important intellectual content: F. N., R. A. R., T. B. E. and K. D.
Statistical analysis: A. A., U. U. and F. N.
Administrative, technical, and material support: A. A., U. U., N. P., A. S. W. P. and F. N.
Study supervision: R. A. R. and F. N.

Ethics

None to be declared.

Conflict of Interest

The authors declare that they have no conflict of interest.

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