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RESEARCH ARTICLE

Curcumin-mediated gene expression changes in *Drosophila melanogaster*

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Abstract

Background: Curcumin has been suggested to be useful in the treatment of numerous diseases, including autoinflammation, that has been implicated in some pathological conditions. Experimentally, autoinflammatory phenotypes were observed in the short-lived Peptidoglycan recognition protein LB (PGRP-LB) mutant of *Drosophila melanogaster*. **Objective:** This study aimed to determine the effect of curcumin on the expression of ageing-related genes in the autoinflammatory model of *D. melanogaster*. **Method**: This study was performed using five test groups including untreated control, solvent control, and three groups given a series of curcumin concentrations: 10 µM, 50 µM, and 250 µM, separately. Survival assay and gene expression studies were carried out on these test groups. **Result**: The results revealed that the lifespan of the curcumin-treated groups was significantly improved in comparison to the control groups. Such phenotype was accompanied by the increased expression of *srl* and *hsp22* genes in most, if not all, of the curcumin-treated groups and elevated expression of *tom40*, *pepck,* and *cat* genes was specifically detectable only in groups treated with 250 µM curcumin. On the contrary, the expression of *indy* was significantly reduced upon the administration of curcumin at all given concentrations. **Conclusion**: Based on these results, it can be inferred that supplementation of curcumin can improve the lifespan of the PGRP-LB mutant flies and this might be related to the changes in the expression of ageing-related genes.

Introduction

Autoinflammatory disorders are medical conditions related to the dysfunction of the innate immune system, commonly characterised by an improper activation of innate immune responses (Betrains *et al*., 2021). Autoinflammatory phenotypes have been implicated in several non-communicable diseases, including neurodegenerative diseases and metabolic syndromes (Kastner *et al.,* 2010; Ciccarelli *et al*., 2014). Autoinflammatory condition is evolutionarily conserved (Asri *et al*., 2019) and can affect neuronal integrity (Nainu *et al*., 2019), which eventually will have tremendous effects on the process of ageing and the lifespan of a species (Kastner *et al*., 2010). Ageing is defined as a progressive process, a general imbalance of function resulting from susceptibility to environmental changes and an increased risk of disease and death. This process takes place normally and varies depending on the condition of each individual's body (López-Otín *et al*., 2013; McIntyre *et al*., 2021). Excessive reactive oxygen species (ROS) production during mitochondrial damage may trigger the oxidation of membrane lipids and proteins as well as the mutations of mitochondrial DNA, which in turn may provoke cell damage and lead to ageing (López-Armada *et al*., 2013; Riley & Tait, 2020). Mitochondria are involved in many major cellular processes and therefore require continuous control to maintain their health (Jadiya & Tomar, 2020). Three types of mechanisms are available to ensure the integrity of mitochondrial proteins; reactive oxygen species that

are broken down by antioxidant enzymes (i.e. *cat*), clearance of damaged mitochondria by mitophagy (i.e. *tom40*) and protein folding/degradation by chaperone molecules and proteases (i.e. *hsp22*) (Cedikova *et al*., 2016; Jadiya & Tomar, 2020).

The transcription factor NF-κB that regulates inflammation, oxidative stress, and the expression level of cytokines has also been associated with caloric restriction (Jung *et al*., 2009; Zhang *et al*., 2016). Caloric restriction is thought to suppress NF-κB activation and immune response (Taniguchi & Karin, 2018). The gene that plays a role in the calorie restriction process is the SLC13A2 gene which homologues to the "*I'm not dead yet* (*indy*) gene and *spargel* (*srl*) gene" in *Drosophila melanogaster*. Decreased expression of *indy* can reduce the amount of Adenosine triphosphate/ Adenosine triphosphate (ATP/ADP) that can trigger mitochondrial biogenesis through increased levels of *srl* and prevent an increase in the inflammatory responses (Olivo-Marston *et al*., 2014; Rogers & Rogina, 2015; Huang *et al*., 2019). In addition, there is also an efficient use of ATP when caloric restriction occurs and triggers gluconeogenesis in response to a lack of glucose intake by an enzyme called phosphoenolpyruvate carboxykinase (pepck) (Onken *et al.*, 2020).

Curcumin is a diarylheptanoid and a medically promising phenolic pigment accountable for the yellow color of turmeric (Hewlings & Kalman, 2017). Previous *in vivo* studies have reported that curcumin can increase fruit flies' survival, in the absence of infection (Evangelakou *et al*., 2019), delineating the possible pharmacological effect of curcumin on lifespan improvement and ageing prevention. Such phenotype has been attributed to its antioxidant properties. This compound manages to prevent the increment of ROS and neutralises nitric oxide (NO) (Chongtham & Agrawal, 2016). Recent *in vivo* study, furthermore, demonstrated that curcumin can downregulate the expression of the NF-κB-mediated pro-inflammatory gene in *D. melanogaster* (Nainu *et al*., 2022), implying that curcumin may reduce inflammation by its role as an immunomodulator. However, despite the knowledge that curcumin can prolong fruit flies survival and possess a pharmacological role to overcome inflammation, the mechanisms to achieve such an effect remain unclear.

Based on such notion, the authors carried out an experimental *in vivo* study to determine the effect of curcumin on the survival of the autoinflammatory fly model. Later, the expression of human-homologue ageing-related genes that have been reported to play roles in the ageing process of fruit fly *D. melanogaster was examined*. Results obtained in this study may provide hints on the mechanism of action of curcumin to treat autoinflammatory-related ageing in the metazoan species.

Methods

Fly stocks

In this study, the authors used two-four days old adult Peptidoglycan recognition protein LB (*PGRP-LB$^{\Delta}$) D. melanogaste*r. This is a mutant line lacking the expression of *PGRP-LB*, that has been shown to experience overactivation of the NF-κB immune deficiency (Imd) pathway in *D. melanogaster* (Asfa *et al.*, 2022), upon the introduction of a proper ligand. Flies were constantly maintained in the fly culture vials under standard laboratory conditions (25°C, 12 hours light and 12 hours dark cycles, and normal cornmeal-based fly food).

Fly food and compound preparation

The preparation of a standard fly food requires corn flour, sugar, yeast, agar, methylparaben, propionic acid, and aqua dest. Curcumin (Sigma Aldrich, Cas No. 458-37-7; C1386-10G) was prepared as a 50 mM stock solution using 96% ethanol (Merck) as the solvent and subsequently diluted using the same solvent to obtain concentrations of 10 mM and 2 mM. Each curcumin solution was added to the fly food separately to obtain final concentrations of 10 µM, 50 µM, and 250 µM.

Survival assay

A survival assay was carried out to examine the effect of curcumin on the lifespan of the *PGRP-LB$^{\Delta}$* mutant line of *D. melanogaste*r. Each group consisted of ten male flies. In this study, six groups were used. One group was given normal fly food (without curcumin) and thus served as the untreated control group. The other four groups were designated as the solvent control group (flies were given 96% ethanol-containing fly food) and the curcumin-treated groups (flies were given fly food containing curcumin at concentrations of 10, 50, and 250 µM, respectively). All groups were daily monitored for their survival rates. Observations were made for up to 30 days and every three days, food was changed until the end of the observation.

Gene expression analysis

Total Ribonucleic acid (RNA) isolation was performed on all fly groups. From each group, five live flies were subjected to an RNA isolation procedure using a Pure Link RNA Mini kit (Invitrogen, Thermo Fisher Scientific Inc.). The concentration of RNA in all samples was

determined using a nano spectrophotometer (BioDrop, Biochrom, Ltd.) and processed using the Reverse Transcriptase Quantitative PCR (RT-qPCR) method. The expression of *tom40, cat, hsp22, indy, srl,* and *pepck* genes were separately examined in all treatment groups by RT-qPCR, in a reaction volume of 10 µl each, using the SuperScript III Platinum SYBR Green One-Step RT-qPCR kit with ROX (Invitrogen, Thermo Fisher Scientific Inc.) according to the manufacturer's protocols, using the Rotor-Gene Q thermal cycler (Qiagen, Germany). The expected amplified product is verified based on the standard melting curve analysis. As an internal control in the RT-qPCR assay, the level of ribosomal protein rp49 was examined using the rp49 primer set. The following RT-qPCR running profile was used: 37°C for 15 minutes, 95°C for 10 minutes, 40 repeated cycles of amplification in which each cycle was performed at 95°C for 10 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, and a final run of melting curve analysis spanning from 60°C to 95°C. All PCR data were then processed using Q-Gene and subjected to gene expression analysis. A list of primers used in the RT-qPCR is provided in Table I.

Table I: List of RT-qPCR primers used in this study

Gene	Forward primer sequence	Reverse primer sequence
cat	5'-TTCCTGGATGAGATGTCGCAC T-3'	5'-TTCTGGGTGTGAATGAAGCTGG-3'
tom40	5'-TGCACGTGTGCTACTACCAG-3'	5'-ATTCCGCCTCTGAAGACCAG-3'
hsp22	5'-CCAGATCTTCAATGTGTCTCTCTGCG-3'	5'–TTCCTGGATGAGATGTCGCACT– 3'
indy	5'-CTGCCCAACTCTGTCCTCTTACTG-3'	5'-CAGGATCAGGTACAGAGGATGGAT-3'
srl	5'-CTCTTGGAGTCCGAGATCCGCAA-3'	5'-GGGACCGCGAG CTGATGGTT-3'
pepck	5'-CCGCCGAGAA CCTTATTGTG-3'	5'-AGAATCAACATGT GCTCGGC-3'

Statistical analysis

All results were obtained from at least three independent replicates and further processed using GraphPad Prism 8. The survival data were visualised as a Kaplan–Meier graph and statistically analysed using the Log Rank approach. The statistical analysis of gene expression data was carried out using one-way ANOVA followed by Tukey HSD post hoc analysis and visualised as a bar graph. Data were presented as mean ± S.D for all statistical analyses, and *p*-values of less than 0.05 were considered statistically significant.

Results

Curcumin improved the lifespan of PGRP-LB$^{\Delta}$ D. melanogaster

A survival assay was carried out to determine the survival of *PGRP-LB$^{\Delta}$ D. melanogaster* after treatment with curcumin. Previously, the authors showed that the *PGRP-LB$^{\Delta}$* has a shorter lifespan than the Oregon R (wildtype) flies (Nainu *et al*., 2022). This is probably due to the loss of the PGRP-LB protein that plays an important function in the homeostatic regulation of the NF-kB pathway, Relish, in *D. melanogaste*r. In the absence of this PGRP-LB protein, overexpression of immune-related genes, under the Imd (NF-κB) pathway was observed (Kounatidis *et al*., 2017; Asfa *et al*., 2022; Nainu *et al*., 2022). Based on the observation of the survival assay, the *PGRP-LB$^{\Delta}$* flies in the untreated control group succumbed up to 100% on day 30, while on the same day, there were still live *PGRP-LB$^{\Delta}$* in the curcumin-treated groups at all concentrations. Adult PGRP-LB$^{\Delta}$ aged two-four days were divided into five different groups and subjected to the intended treatments. Flies that received no additional treatment and 96% ethanol were assigned as untreated control and solvent control, respectively (Figure 1).

Curcumin supplementation upregulated the expression of antioxidant-related genes

The supplementation of curcumin at concentrations of 10 and 50 µM did not have a significant effect on the expression level of the *cat* and *tom40* genes. However, a significant increase in the expression of those genes was observed on flies treated with 250 µM curcumin compared to the solvent control group (Figure 2a and 2b, respectively). Meanwhile, the expression of *hsp22* was upregulated in a concentration-dependent manner in two of the three curcumin-treated groups, the ones treated with 50 and 250 µM (Figure 2c). Adult *PGRP-LB$^{\Delta}$* aged two-four days were divided into four different groups and subjected to the intended treatments. A group of flies that received 96% ethanol was assigned as solvent control.

Curcumin supplementation upregulates the expression of srl and pepck but downregulates the expression of Indy

Next, the gene expression analysis on three ageing-related genes: *srl, pepck*, and *indy* was carried out. The expression of *srl* was enhanced once the *PGRP-LB*$^{\Delta}$ flies were incubated in the food-containing curcumin at 50 and 250 µM (Figure 3a). Likewise, the expression of *pepck* was upregulated, but only in the 250 µM curcumin-treated group (Figure 3b). On the contrary, curcumin supplementation at all given concentrations reduced the expression of *indy*, in comparison to the ones expressed in the flies of the solvent control group (Figure 3c). Adult *PGRP-LB*$^{\Delta}$ aged two–four days were divided into four different groups and subjected to the intended treatments. A Group of flies that received 96% ethanol was assigned as solvent control.

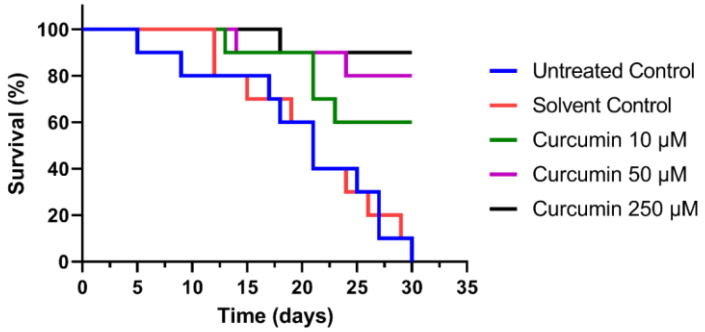
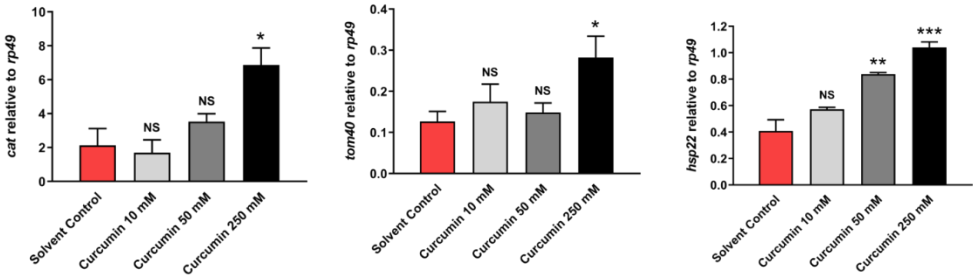
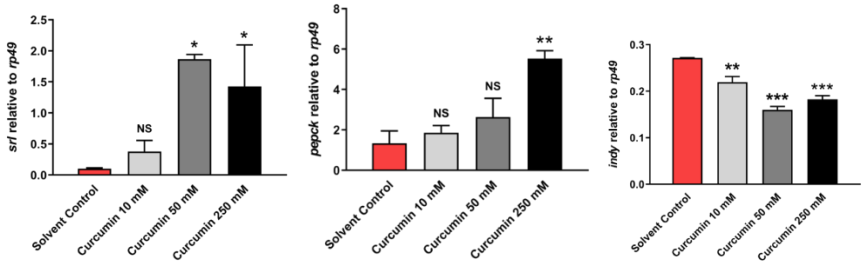


Figure 1: Survival of *PGRP-LB*$^{\Delta}$ flies in the presence and absence of curcumin supplementation



NS, Non Significant; *, $p < 0,05$; **, $p < 0.01$; ***, $p < 0.001$

Figure 2: Enhanced expression of a) *cat* b) *tom40* c) *hsp22,* after curcumin supplementation at high concentration



NS, Non Significant; *, $p < 0,05$; **, $p < 0.01$; ***, $p < 0.001$

Figure 3: Improved expression of a) *srl* b) *pepck* c) *indy* after curcumin supplementation.

Discussion

Autoinflammatory phenotypes can be observed in an experimental model organism, including the *PGRP-LB$^{\Delta}$ D. melanogaste*r. Previously, the authors' group showed that the *PGRP-LB$^{\Delta}$* flies have shorter lifespans than their Oregon R wildtype counterparts (Asfa *et al*., 2022; Nainu *et al*., 2022). This is probably due to the loss of the PGRP-LB protein which functions as a negative regulator of the Imd pathway in *D. melanogaste*r (Orlans *et al*., 2021). In the absence of PGRP-LB protein, an autoinflammatory condition similar to that in humans may occur, leading to the decline of flies' survival.

From this study, it can be seen that the lifespan of *PGRP-LB$^{\Delta}$* flies was improved in the presence of curcumin (Figure 1), suggesting that curcumin can help to prolong the lifespan of *PGRP-LB$^{\Delta}$* flies. Since *PGRP-LB$^{\Delta}$* flies have been reported to experience overexpression of immune-related genes (possibly, delineates the overactivation of Imd-mediated immune pathway), the authors sought to examine whether that condition might alter the expression of ageing-related genes which eventually leads to the autoinflammatory-related short-lived phenotype observed in this study and other previous studies.

Several studies have revealed the association between high levels of ROS and mitochondrial dysfunction, especially during ageing (Cui *et al*., 2012) and in certain pathological disorders (Guo *et al*., 2013; Mikhed *et al*., 2015; Bhatti *et al*., 2017; Tirichen *et al.,* 2021). Mitochondrial damage can increase the production of mitochondrial reactive oxygen species (ROS) which eventually leads to the oxidization of membrane lipids, proteins, and disruption of mitochondrial DNA integrity, all of which can trigger oxidative stress and autoinflammatory condition (Guo *et al.,* 2013). Indeed, a close association of oxidative stress (due to the elevated production of ROS by mitochondria) with the occurrence of dysfunctional tissue phenotypes has been widely reported (Guo *et al*., 2013; Mikhed *et al*., 2015; Bhatti *et al*., 2017; Mc Auley *et al*., 2017).

As one of the antioxidant enzymes, catalase (encoded by the *cat* gene) has been shown to play a major role in the ageing process (Nandi *et al*., 2019). Indeed, malfunction expression of the *cat* gene can increase the harmful effect of the H_2O_2-mediated oxidative stress (Sullivan-Gunn & Lewandowski, 2013; Pizzino *et al*., 2017; Nandi *et al*., 2019), implicating the beneficial role of catalase in the improvement of the lifespan. Based on Figure 1 and Figure 2a, it can be seen that fly survival was enhanced even if the expression of the *cat* gene was not upregulated (in the fly groups treated with fly food containing 10 and 50 µM of curcumin). Similar to the *cat* expression pattern, the expression of *tom40* also enhanced in the curcumin-treated group but only when flies were given curcumin at a high concentration (250 µM) (Figure 2b). Tom40 (translocase of outer membrane 40), encoded by *tom40*, plays a role in the autophagy process to prevent mitochondrial dysfunction and autophagy becomes important in terms of preventing the generation of free radicals (Liu *et al*., 2018). Based on these results, catalase and Tom40 may only be partially involved in the increased lifespan of the *PGRP-LB$^{\Delta}$* flies. It would be interesting to examine whether other antioxidant genes such as *sod1* and *sod2* also play roles in the increased lifespan of *PGRP-LB$^{\Delta}$* flies, as was seen in the caffeine-treated flies (Asbah *et al*., 2021).

The expression of *hsp22* was upregulated in a concentration-dependent manner in two of the three curcumin-treated groups (Figure 2c) and this is linearly correlated to the lifespan of *PGRP-LB$^{\Delta}$* flies (Figure 1). The *hsp22* gene has been reported to be important in the cellular regulation and has been implicated to play a role in the regulation of mitochondrial metabolism (Tower, 2015). Increased expression of *hsp22* has been shown to reduce oxygen consumption and reduce metabolism, preferentially in the oenocytes (liver-like cells), and leads to increased lifespan (Tower *et al*., 2014).

Failure of mitochondrial biogenesis can result in mitochondrial dysfunction (Dela Cruz & Kang, 2018). PGC-1α (human) and its homologue Spargel (*srl*) (Drosophila) serves as a major regulator of mitochondrial biogenesis, both in mammals as well as in Drosophila (George & Jacobs, 2019). In this study, the authors observed improved expression of the *srl* upon curcumin treatment (in the fly groups treated with fly food containing 50 and 250 µM of curcumin) (Figure 3a), implying that *srl* and its physiological function may also play a role in the enhanced survival of *PGRP-LB$^{\Delta}$* flies. It is important to note that the PGC-1α (and its homologue in Drosophila, Spargel) has been shown to be a promising target in the pharmacological treatment of acute and chronic mitochondrial dysfunction-related neurodegenerative diseases (Simmons *et al*., 2020).

Meanwhile, elevated expression of *pepck* in the presence of curcumin at high concentrations (Figure 3b) implies the possible role of dietary restrictions, which can improve the health and longevity of a species (Onken *et al*., 2020). However, this remains uncertain and shall be the subject of future studies. With the occurrence of dietary restrictions, metazoan species may enter a temporarily low-level glucose state, resulting in the reduction of energy required for cellular activities. In response, the body will produce its own glucose by carrying out the gluconeogenesis stimulated by the enzyme PEPCK which will be used to synthesise

ATP which is useful as an energy source (Yuan *et al*., 2016). Along with that, suppression of *indy* (Figure 3c) plays a role in mitochondrial biogenesis throughout its activation effect of activated protein kinase (AMPK) as a result of a decreased amount of ATP (Dela Cruz & Kang, 2018).

Conclusion

Based on the results obtained in this study, curcumin may prolong the lifespan of *PGRP-LB*$^{\Delta}$ flies, possibly through its pharmacological effect on the expression of antioxidant-related and ageing-related genes, particularly by upregulation of *cat, tom40*, *hsp22*, *srl,* and *pepck* as well as downregulation of *indy.*

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Conflict of Interest

The authors declare no conflict of interest.

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