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Pharmacological Role of Deoxycholic Acid in the Regulation of Aging in *Drosophila melanogaster*

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Abstract: Aging is characterized by gradually weakening normal cell functions, leading to a progressive decline in biological and physical abilities. Such phenotypes have been linked to mitochondrial dysfunction. Deoxycholic acid (DA) is a secondary bile acid that can activate signal transduction pathways that regulate the transcription of genes related to mitochondrial damage. This study was conducted to determine the *in vivo* anti-aging activity of DA using the model organism *Drosophila melanogaster*. To achieve this, phenotypical assessments were performed on the lifespan and locomotor of D. melanogaster and molecular analysis on the expression of mitochondrial-related genes. The results showed that DA was relatively safe to consume and could extend the lifespan of *D. melanogaster*. Furthermore, subsequent molecular analysis on endogenous antioxidants and mitochondrial-related human-homolog genes in *D. melanogaster* revealed that the administration of DA induced the expression of *srl* and *tom40*, but not *sod1*, *sod2*, *cat*, *pepck*, and *indy*. Taken together, our results suggest the prospective role of DA in regulating aging in Drosophila, which might be translationally relevant to humans.

Keywords: bile acid; fruit fly; longevity; RTqPCR; *in vivo*.

1. Introduction

Deoxycholic acid (DA) is a secondary bile acid produced endogenously in the human intestine [1] and functions to break down and aid the absorption of dietary fat by the gastrointestinal tract [1, 2]. Subcutaneous injection of DA has been reported to be able to stimulate the emulsification of dietary fat, promoting the lysis of adipocytes [3]. Synthetic DA has been approved by the U.S. Food and Drug Administration (U.S. FDA), indicating the reduction of submental fat [4, 5]. Interestingly, previous results revealed that bile acids could activate signal transduction pathways that regulate the transcription of target genes for inflammation, mitochondrial damage, and oxidative stress [6-8], which are considered some of the hallmarks of aging. This occurs through direct activation of G protein-coupled via direct ligand-mediated activation of nuclear receptors such as the farnesoid X receptor (FXR) [7, 9]. Owing to these recent advancements in the pharmacological functions and targets of bile acids,

we seek to understand whether DA can interfere with aging-related events at the molecular and phenotypical levels.

Aging is mostly characterized by a gradual decrease in normal cell function, leading to a progressive decline in biological, physical, and psychological abilities [10, 11]. Aging is a complex, multifactorial, and heterogeneous process caused by various interactions between genes and the environment. Other factors that promote aging are the accumulation of damage to DNA, mitochondria, and other cell parts [12, 13]. Indeed, accumulated evidence has shown that mitochondrial damage and oxidative stress are important factors that contribute to the aging process [13]. Hence, research on aging concentrates on mitochondria physiology and biochemistry as promising targets to decelerate the aging process [13, 14]. However, it remains unclear whether DA possesses anti-aging activity and how the effect, if it does exist, can be stimulated in a proper manner.

To assess the anti-aging activity of drug candidates, including DA, one may need to conduct *in vitro* and *in vivo* pre-clinical experiments before conducting clinical research [15]. In aging research, the fruit fly model organism *Drosophila melanogaster* can be used to examine the anti-aging effects of prospective drug candidates [16, 17]. This insect has several advantages, including cost-effectiveness, fast growth, and easy maintenance in the laboratory [18-20]. Drosophila shares 75% of genetic homology with humans and has a relatively short lifespan (2-3 months), which is ideal for aging-related research [16, 21].

Based on currently available data and in light of the existence of a proper model organism, we investigated the prospective anti-aging activity of DA in the *in vivo* setting. Here we report that DA can prolong the lifespan of *D. melanogaster* without affecting the fly locomotor. These phenotypes were observed under *srl* and *tom40*-mediated regulation of aging in Drosophila, which might be translationally relevant to humans

2. Materials and Methods

2.1. Sample preparation.

Deoxycholic acid was purchased from Sigma and prepared by dissolving it in 70% ethanol. Subsequently, DA solution was diluted to achieve a series of concentrations (250 μM, 50 μM, 10 μM and 2 μM).

2.2. Fly stock.

The wild-type line of *Drosophila melanogaster* (Oregon R) was used in this study. Male flies aged 4-7 days were used in all experiments. These flies were placed in a culture vial containing standard cornmeal-agar food and maintained in standard conditions (25°C, 12 hours light and 12 hours dark cycle).

2.3. Survival assay.

A survival assay was performed to examine the effect of DA on the lifespan of *Oregon R* line *D. melanogaster* (Figure 1). Flies were divided into five groups, each consisting of 15 male flies. One group fed normal fly food (without DA) was designated as the untreated control, whereas four groups were treated with food-containing DA with concentrations of 250 μM, 50 μM, 10 μM, and 2 μM, respectively. All groups were monitored daily for their survival rates.

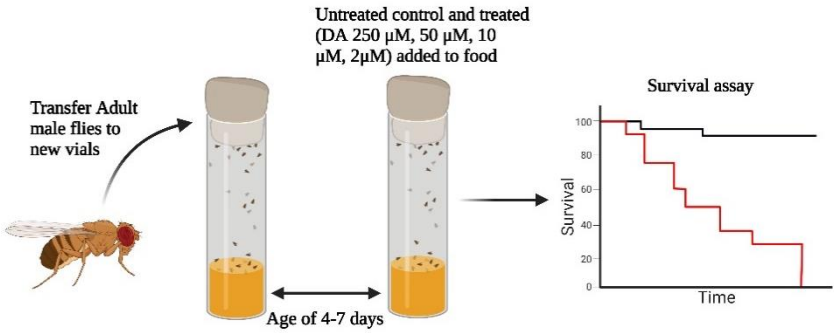

Figure 1. Experimental design. Four groups of adult flies were treated with fly food containing DA at different concentrations (250 µM, 50 µM, 10 µM and 2µM). One group maintained in the absence of any treatments was used as the untreated control. DA, deoxycholic acid.

2.4. Locomotor assay.

The locomotor assay was performed on all fly groups using the negative geotaxis method, as previously described [22, 23], with slight modifications. Flies were placed in a marked empty vial designated as a locomotor testing vial. The test vial is placed in an upright position in front of the climbing wall. In this test, the test vial (containing the flies to be tested) is tapped down to ensure that all the flies stay at the bottom of the vial. After that, events in the testing vial for up to 15 seconds were observed and recorded. The number of flies that rise to cross the marked finish line is calculated. The experiment was repeated three times.

2.5. Gene expression assay.

Total RNA isolation was performed using live flies on all fly groups post-treatment. From each group, five live flies were subjected to RNA isolation procedure using Pure Link™ RNA Mini Kit (Invitrogen™, Thermo Fisher Scientific Inc.). The amount of total RNA in each sample was measured using a spectrophotometer (BioDrop, Biochrom, Ltd.), and the Reverse Transcriptase Quantitative PCR (RT-qPCR) technique was subsequently used to examine the expression of target genes. The expression of *sod1*, *sod2*, *cat*, *srl*, *pepck*, *tom40*, and *indy* in all treatment groups were examined separately 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. The Rotor-Gene Q thermal cycler (Qiagen, Germany) was utilized for the RT-qPCR experiment, and the level of the ribosomal protein rp49 was used as the internal control. The running profile of RT-qPCR was as follows: 37°C for 15 minutes, 95°C for 10 minutes, followed by 40 cycles of amplification (95°C for 10 seconds, 60°C for 30 seconds, and 72°C for 30 seconds were used for each cycle). Verifying the expected amplified product was done based on the standard melting curve profiles from 60°C to 95°C. All generated 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 1.

Table 1. Primers used in the RT-qPCR assay.

Genes	Forward primer	Reverse primer
sod1	5' – AGG TCA ACA TCA CCG ACT CC – 3'	5' – GTT GAC TTG CTC AGC TCG TG – 3'
sod2	5' – TGG CCA CAT CAA CCA CAC – 3'	5'– TTC CAC TGC GAC TCG ATG – 3'
cat	5' – TTC CTG GAT GAG ATG TCG CAC T – 3'	5' – TTC TGG GTG TGA ATG AAG CTG G – 3'
srl	5' – CTC TTG GAG TCC GAG ATC CGC AA – 3'	5' – GGG ACC GCG AGC TGA TGG TT – 3'
Pepck	5' – CCG CCG AGA ACC TTA TTG TG – 3'	5' – AGA ATC AAC ATG TGC TCG GC – 3'
tom40	5' – TGC ACG TGT GCT ACT ACC AG – 3'	5' – ATT CCG CCT CTG AGA CCAG – 3'
indy	5' – CTG CCC AAC TCT GTC CTC TTA CT – 3'	5' – CAG GAT CAG GTA CAG AGG ATG GAT – 3'

Genes	Forward primer	Reverse primer
rp49	5' – GAC GCT TCA AGG GAC AGT ATC TG – 3'	5' – AAA CGC GGT TCT GCA TGA G – 3'

2.6. Data processing.

The survival data were visualized as a Kaplan-Meier graph and statistically analyzed using Log Rank. In contrast, locomotor and gene expression data were processed and analyzed statistically using the One-Way ANOVA approach followed by post hoc analysis and visualized as a bar graph. Data are presented as mean ± SD for all statistical analyses, and p-values less than 0.05 are considered significant. All data were processed and visualized using GraphPad Prism® 9.

3. Results and Discussion

3.1. Low concentration of deoxycholic acid treatment increased the lifespan of D. melanogaster.

In this research, we investigated the pharmacological activity of DA in *D. melanogaster* at both phenotypical and molecular levels. Drosophila is a model organism that has contributed to many innovative and important discoveries, including in identifying critical components of signaling pathways that are conserved among metazoan species, including humans [24, 25]. At the phenotypical level, we examined the effect of DA on the survival and locomotor of flies. Meanwhile, at the molecular level, we examined the possible changes in the expression of three antioxidant genes (*sod1*, *sod2*, and *cat*) and four aging-related genes (*srl*, *pepck*, *tom40*, and *indy*).

In general, the pharmacological effect of certain chemical entities shall not impair the Drosophila lifespan [26]. Therefore, survival assay shall serve as a simple and direct experimental endpoint to investigate the harmful effect of potential drug candidates and their beneficial effect on the prevention of aging. In this study, we first carried out a survival assay to determine whether DA can negatively affect the lifespan of *D. melanogaster*. The assay was carried out on males of *D. melanogaster* using four different concentrations of DA: 250 µM, 50 µM, 10 µM, and 2 µM. Flies aged 4-7 days were given DA orally (incorporated into the fly food) and observed for almost 60 days. As shown in Figure 2, treatment of *D. melanogaster* to DA for about 60 days resulted in significant changes in fly survival rate. These results indicate that administering various concentrations of DA can increase fly survival. It is crucial to note that a low concentration of DA was able to improve the flies lifespan better than its counterparts treated with higher concentrations of DA.

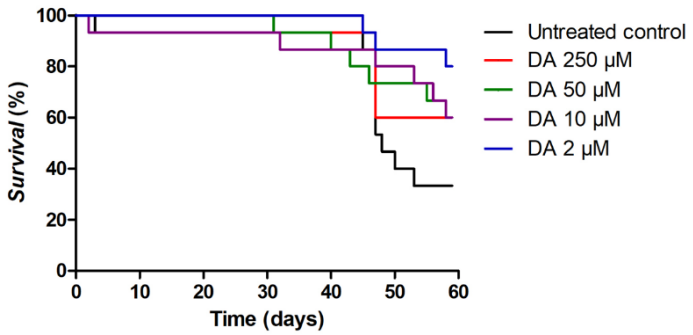

Figure 2. Significant changes in the survival of *D. melanogaster* (Oregon R) after treatment with DA at different concentrations. Adult male flies aged 4-7 days were divided into five groups, and four assigned groups

were treated with DA at different concentrations. One group maintained in the absence of treatments was used as the untreated control. DA, deoxycholic acid.

3.2. Insignificant changes in the locomotor of D. melanogaster upon deoxycholic acid treatment.

Based on the fly survival result (Figure 2), it is apparent that fruit fly *D. melanogaster* did not experience any negative effect on its lifespan after DA treatment, suggesting that DA is safe to consume by flies at the given concentrations. To gain more knowledge on the prospective effect of DA on the phenotypical traits of *D. melanogaster*, we examined its effect on the fly locomotor. Locomotor activity has been suggested to be correlated with longevity [27], and impaired locomotor is sometimes observable upon introduction of certain exogenous substances/drugs [28]. Therefore, carrying out a locomotor assay may provide additional insight into the negative impact of DA on the phenotypical trait of metazoan species. As shown in Figure 3, the treatment of flies with DA at all concentrations resulted in insignificant changes in the locomotor activity during the experiment, compared to the untreated control, suggesting that DA did not impair the locomotor activity of *D. melanogaster*.

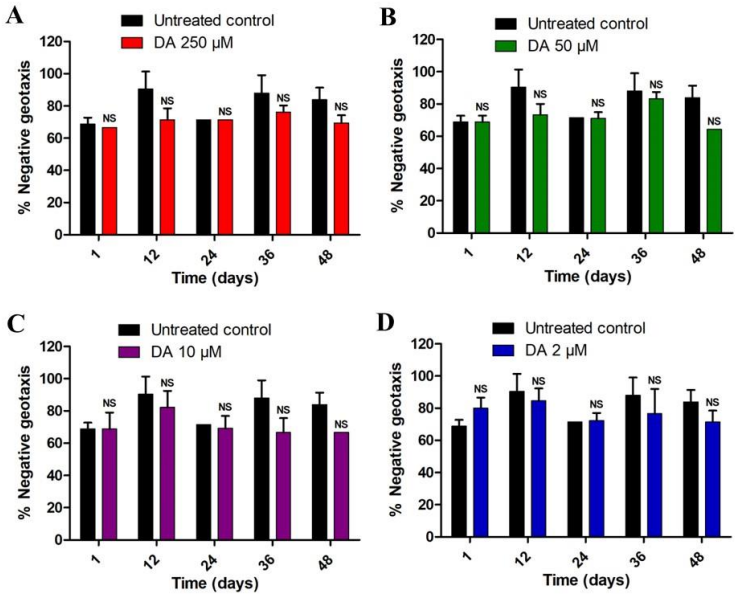

Figure 3. Insignificant changes in the locomotor of *D. melanogaster* after treatment with DA at different concentrations: 250 µM (A), 50 µM (B), 10 µM (C), and 2 µM (D). Adult male flies at the age of 4-7 days were divided into five groups, and the four assigned groups were then treated with DA at different concentrations. One group maintained in the absence of any treatments was used as the untreated control. DA, deoxycholic acid; NS, not significant.

3.3. Specific modulation of aging-related genes but not on endogenous antioxidant genes upon deoxycholic acid treatment.

Drosophila melanogaster has been suggested as a versatile model organism in nutrition and aging research [15, 16]. It has been reported that aging is associated with oxidative stress and mitochondrial damage [29, 30]. There are several genes associated with aging, four of which are the genes encoding spargel (*srl*), phosphoenolpyruvate carboxykinase (*pepck*), translocase outer membrane 40 (*tom40*), and I'm not dead yet (*indy*). The *srl* gene in *D. melanogaster* is homologous to PPAR-γ coactivator 1-α (PGC-1α) in humans [31]. PGC-1α

and its homologs have been identified as major regulators of mammalian mitochondrial biogenesis. [26].

In *D. melanogaster*, increased expression of the *srl* gene will increase mitochondrial activity both during development and in the adult stage [27]. The protein that functions as the main regulator of mitochondrial biogenesis, both in mammals and in Drosophila, is spargel [26]. Based on the data, it is apparent that DA could significantly increase the expression of the *srl* (Figure 4A) and *tom40* (Figure 4C) genes but not the other two genes (*pepck* and *indy*) at a lower concentration compared to the untreated control. These results suggest that DA-treated flies might experience increased energy production via stimulation of the mitochondrial. Such phenotype shall be advantageous for Drosophila activities in the movement and reproduction. Furthermore, increased expression of the *srl* and *tom40* genes also has been implicated in the increased Drosophila lifespan [28].

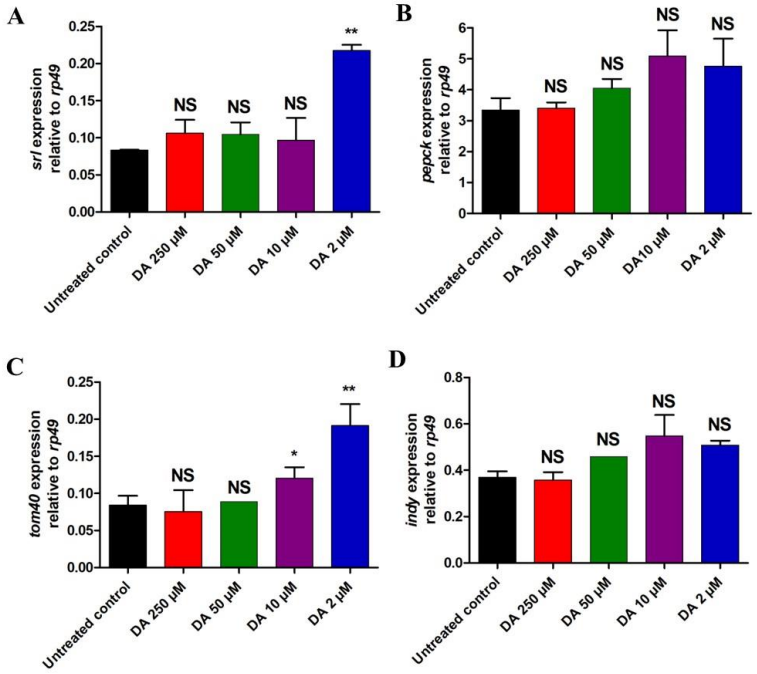

Figure 4. Expression level of *srl* (A), *pepck* (B), *tom40* (C), and *indy* (D) in the Oregon R *D. melanogaster* in the presence or absence of DA. Adult male flies aged 4-7 days were divided into five groups and subjected to intended treatments. Flies that received no additional treatment were designated as the untreated control. RNA was extracted from five live Drosophila in each group, followed by RNA quantification and amplification by RT-qPCR. The level of target genes was compared to rp49 RNA level as an internal control. DA, deoxycholic acid; NS, not significant; * $p < 0.05$; ** $p < 0.01$.

Several studies have revealed the association between the high level of ROS and mitochondrial dysfunction, especially during aging [29, 32-34] and in certain pathological disorders [35-38]. Mitochondrial damage can increase the production of mitochondrial ROS, which eventually leads to the oxidization of membrane lipids and proteins and disruption of mitochondrial DNA integrity, all of which can trigger oxidative stress and autoinflammatory condition [39, 40]. 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 proposed [35, 41-43]. In light of this notion, we examined the expression of three endogenous antioxidant genes in DA-treated flies (*sod1*, *sod2*, and *cat*). Based on Fig. 5, the expression of *sod1*, *sod2*, and *cat* (in the fly groups treated with fly food containing DA at all concentrations) was relatively unchanged, suggesting that these genes may not play a role in

the increased lifespan of the Oregon R flies, different to the ones seen in the previously observed caffeine-treated flies [23].

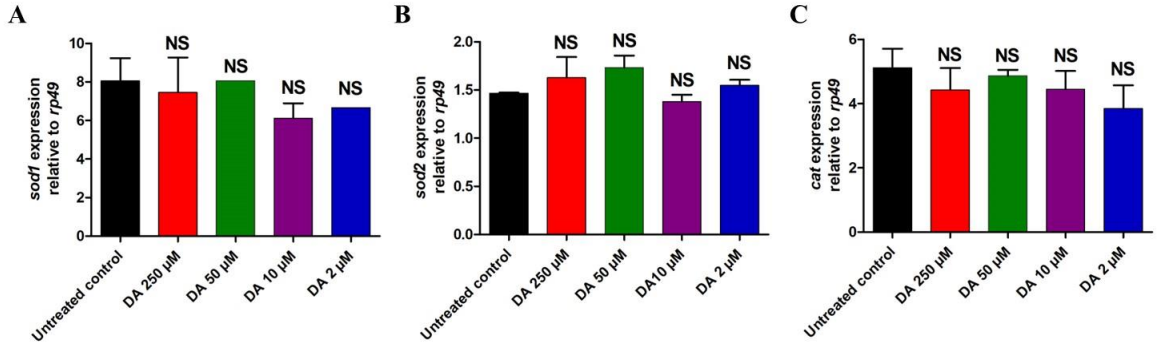

Figure 5. Expression levels of endogenous antioxidant genes *sod1* (A), *sod2* (B), and *cat* (C) in Oregon R *D. melanogaster* in the presence or absence of DA. Adult male flies aged 4-7 days were divided into five groups and subjected to intended treatments. Flies that received no additional treatment were designated as untreated control. RNA was extracted from five live Drosophila in each group, followed by RNA quantification and amplification by RT-qPCR. The level of target genes was compared to rp49 RNA level as an internal control. DA, deoxycholic acid; NS, not significant.

4. Conclusions

In the present experimental study, we demonstrated the *in vivo* anti-aging activity of DA in the *D. melanogaster* model with a relatively insignificant phenotypical trade-off. To the best of our knowledge, this study provides the first evidence of the *in vivo* pharmacological effects of DA, a secondary bile acid with unknown anti-aging function on the metazoan lifespan, using *D. melanogaster* as the model organism.

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Conflicts of Interest

We declare that we have no conflict of interest.

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