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ORIGINAL PAPER

Genetic repression of the antioxidant enzymes reduces the lifespan in *Drosophila melanogaster*

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Abstract

Aging is a biological process associated with gradual loss of function caused by cellular and molecular damages ultimately leading to mortality. Free radicals are implicated in oxidative damage which affects the longevity of organisms. Natural cellular defenses involving antioxidant enzymes delay or prevent oxidative damage and, therefore, influence the aging process and longevity has been shown in many species including *Drosophila*. We and others have shown that oxidative resistance is an important mechanism in the aging process in *Drosophila*. Therefore, we hypothesized that repressing endogenous antioxidant defenses shortens longevity in *Drosophila*. To study the influence of natural defense mechanisms against oxidative stress in aging, we have investigated the effect of genetic repression of the antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT), on longevity in *Drosophila* using transgenic RNAi flies and in vivo inhibition of the enzymes with chemical inhibitors. RNAi lines of *Drosophila* viz., *UAS-sod1-IR* and *UAS-cat-IR*, are driven ubiquitously using *Act5C-Gal4* and *Tubulin-Gal4* to achieve the suppression of SOD1 and CAT activities, respectively. We show that genetic repression of SOD1 and CAT by RNAi in transgenic flies led to drastically reduced longevity (SOD1, 77%; CAT, 83%), presenting the evidence for the role of endogenous antioxidant defenses in lifespan extension in *Drosophila*. Further, our study shows that the enzyme inhibitors, diethyldithiocarbamate and 3-amino-1,2,4-triazole, although lower the enzyme activities in vivo in flies, but did not affect longevity, which could be attributed to the factors such as bioavailability and metabolism of the inhibitors and adaptive mechanisms involving de novo synthesis of the enzymes. Our study of genetic repression using transgenic RNAi provides experimental evidence that extended longevity is associated with endogenous antioxidant defenses and aging is correlated with oxidative stress resistance.

Keywords RNAi · Longevity · Chemical inhibitors

Introduction

Aging, a complex biological process, involves the accumulation of cellular damage, loss of protective defenses leading to a decline in redox homeostasis, and increased susceptibility to stressors (Lushchak 2021). Comorbidities that accompany aging have oxidative stress as a major influencing factor (Ames et al. 1993). Disruption of redox homeostasis, as a consequence of imbalance in steady-state reactive oxygen species (ROS) levels maintained by endogenous and exogenous antioxidants, is one of the contributing factors in aging. Oxidative stress results in dysfunctional cellular metabolism and its regulation (Lushchak 2014). Oxidative stress resistance, the ability of an organism to contain oxidative damage, plays a major role in longevity (Kuether and Arking 1999; Niveditha et al. 2017; Deepashree et al. 2019).

Several theories have been put forth to elucidate the relationship between oxidative stress and aging. From the earlier free radical theory of aging (Harman 1956) to the later free radical theory of frailty (Vina 2019), researchers have proposed the mitochondrial theory (Miquel et al. 1980), oxidative stress theory (Beckman and Ames 1998), redox theory of aging (Jones 2015), to cite a few. Recently, the polymerase γ focused theory of mitochondrial aging links the build-up of polymerase-induced mtDNA

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mutations and aging (Ziada et al. 2020). Owing to the contradictory findings from studies in *Caenorhabditis elegans* and mice, the role of oxidative stress in aging is debatable and demands a significant modification to the theory to understand the relationship between oxidative stress and aging (Gems and Doonan 2009). Although oxidative damage is a major contributor to aging, synergistic effects of changes in oxidative stress in multiple cellular components are required to have a significant overall impact on aging (Ikeno and Flores 2020). Recently, Clement and Luo (2020) proposed to modify the free radical theory of aging from "organisms age because cells accumulate ROS-dependent damage over time" to "organisms age because cells accumulate oxidants'-dependent damage and oxidants'-dependent senescent characteristics over time."

However, there are examples for a clear association between oxidative stress, antioxidant defenses, and aging as shown in crickets, *Gryllodes sigillatus* (Archer et al. 2013). Antioxidants quench or inhibit the reactions caused by free radicals that ultimately prevent or delay cellular damage (Warraich et al. 2020). Although studies have shown that antioxidants can increase the average lifespan by neutralizing the deleterious effects of ROS, the relationship between antioxidant enzyme levels and lifespan is not straightforward (Santos-Sanchez et al. 2019). The antioxidants terminate oxidative chain reactions by neutralizing free radicals, making free radicals unavailable for further chain reactions that produce damaging molecular species (Warraich et al. 2020). The endogenous enzymatic antioxidant system, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), and glutamyl transpeptidase (GT), forms the main defense system against ROS in vivo. Although the antioxidant enzymes can neutralize the ROS levels, decreased activity of an enzyme with age or a genetic defect affects the efficiency of free radical detoxification (Xiong 2010). Even though studies suggest the beneficial effects of antioxidants on health, large clinical studies have not found any benefits and reported that excess antioxidant supplements have negative effects (Aversa et al. 2016).

The fruit fly, *Drosophila melanogaster,* has served as a favorite model organism for research on aging (Lints and Soliman 1988). One of the successful approaches using *Drosophila* has been to employ artificial selection to create extended longevity phenotypes to investigate the mechanisms underlying aging and senescence (Rose 1984; Partridge and Fowler 1992; Arking et al. 1996; Arking et al. 2000; Buck and Arking 2002; Arking et al. 2002; Wit et al. 2013, Deepashree et al. 2017). Extended longevity strains are valuable as models to study the role of oxidative stress and antioxidant defenses in longevity (Kuether and Arking 1999; Deepashree et al. 2019).

The possible role of SOD in relation to oxidative stress and longevity has been extensively studied in *D. melanogaster* (Orr and Sohal 1993; Phillips et al. 1995; Yang et al. 2013). SOD is shown to play an important role in attenuating oxidative damage in vivo, thereby delaying the onset of age-related neurodegenerative diseases and aging (Warner 1994). Earlier studies have reported that overexpression of SOD could extend the lifespan of several organisms (Guarente and Kenyon 2000). SOD null mutant in *Drosophila* is short-lived and hypersensitive to paraquat (Phillips et al. 1989). CAT and peroxidase are other forms of antioxidant defenses against ROS. CAT overexpression was found to have a significant effect on H_2O_2 resistance in the fly (Sun and Tower 1999).

Previous studies that investigated the role of antioxidant enzymes in lifespan extension, focused primarily on overexpressing SOD and CAT (Orr and Sohal 1993; Phillips et al. 1995; Sun and Tower 1999). Antioxidant enzyme activity was reported to be reduced in response to the higher concentration of oxidants (Lushchak 2014). Therefore, inhibiting antioxidant enzymes seemed an alternate approach to understand their role in aging. In this study, the role of antioxidant defenses in longevity was investigated by repressing the expression or inhibiting the activities of SOD and CAT. This was achieved via two approaches: (1) using specific chemical inhibitors and (2) genetic repression by using transgenic RNA-mediated interference (RNAi) flies. Diethyldithiocarbamate (DDTC) and 3-amino-1,2,4-triazole (ATZ) are the well-known inhibitors of SOD and CAT, respectively. The ease of feeding inhibitors with food media in flies makes it a convenient model for studying the effect of in vivo inhibition of antioxidant enzymes on longevity. DDTC, a thiol-containing compound, is a potent metal ion-chelating agent, which inhibits the enzyme activity of CuZnSOD (by copper chelation) and causes reduced scavenging of O_2^- (Arnelle et al. 1997). ATZ inhibits CAT activity by binding irreversibly to the active site of the protein apoenzyme (Margoliash et al. 1959; Ruiz-Ojeda et al. 2016).

The chemical inhibition of the enzymes was carried out in flies with extended longevity phenotype (ELP) of *D. melanogaster* isolated and characterized in our laboratory through artificial selection (Deepashree et al. 2017). The ELP flies exhibit life-history traits such as uncompromised fecundity (no tradeoff between extended lifespan with reproduction; fecundity remained same as progenitor line), resistance against cold, and enhanced antioxidant defenses (Deepashree et al. 2017). Earlier we have shown the influence of the mitochondrial genome and oxidative stress resistance in extended longevity in this phenotype by demonstrating the role of maternal effect in the offsprings of ELP (Deepashree et al. 2018, 2019). As our ELP is derived from an inbred population of *D. melanogaster* (over 50 years), it is expected to have the least genetic heterogeneity and thus

makes it a suitable model to understand the mechanisms underlying extended longevity, including the role of antioxidant defenses in longevity (Deepashree et al. 2019). In addition to the chemical inhibitors, we have employed RNAi flies to genetically repress gene expression of the antioxidant enzymes to further support our hypothesis that antioxidant defenses play a role in extended longevity. RNAi methodology has been applied in other animal models including *C. elegans* and *Drosophila* (Kirby et al. 2002). In this study, *UAS-sod1-IR* and *UAS-cat-IR* were driven ubiquitously using *Act5C-Gal4* (*Act-Gal4*) and *Tubulin-Gal4* (*Tub-Gal4*) driver lines to achieve the suppression of SOD1 and CAT enzyme activities, respectively. Our study is the first, to our knowledge, to demonstrate the role of antioxidant defenses in longevity using an ELP of *D. melanogaster* by inhibiting endogenous antioxidant enzymes. Furthermore, this study shows that RNAi genetically repressing the antioxidant defenses in flies that affect longevity. Therefore, using the two approaches, we provide experimental evidence to support the role of antioxidant defenses in longevity.

Materials and methods

Materials

Pyrogallol, diethyldithiocarbamate (DDTC), and 3-amino-1,2,4-triazole (ATZ) were procured from Sigma Chemicals Co., St. Louis, USA. All the other chemicals used were of analytical grade.

Fly stocks

Vienna RNAi Drosophila stocks, *UAS-sod1-IR* (Transformant ID 31552) and *UAS-cat-IR* (Transformant ID 6283), were obtained from Drosophila stock center, National Center for Biological Sciences (NCBS), Bengaluru, India. *Tub-Gal4* and *Act-Gal4* were obtained from the Department of Molecular Reproduction, Development and Genetics, Indian Institute of Science, Bengaluru, India. Stocks were built up in the vivarium, at 22 ± 1 °C under constant light–dark cycle with 70%–80% humidity, via serial transfer of flies into fresh culture bottles with wheat-cream–agar media (40 mL in 200 mL volume milk bottles) every 3–4 days to maintain uniformity in density and food availability. Adult flies eclosed from cultures set up at a density of about 100 eggs/bottle and raised under uniform conditions of temperature, humidity, and quantity of food were employed for the present study.

ELP flies used in this were isolated based on selection for longer lifespan from the base population of *D. melanogaster* flies (Oregon K strain) maintained (for > 100 generations) in the Drosophila Stock Center, University of Mysore, Mysuru (Deepashree et al. 2012, 2017). The selection regime involved isolation of longer lifespan flies among normal lifespan flies using postponed senescence as a variable (Rose 1984). Selection for extended longevity was achieved by isolating inbred flies which lived longer than the average lifespan of base stock (~ 60 days) and referred to as the normal lifespan (NLS) line described earlier (Deepashree et al. 2017). Initially, fifty iso-female lines derived from the base (NLS) stock were set up. Flies which lived for more than 60 days (average lifespan of NLS line) were isolated and progenies of such population (surviving older females were able to reproduce and most of them were fertile) were again monitored for longevity. The progenies collected from the next generation were progressively postponed to 70–80 days. The postponement was increased further (80–90, 90–100 days, etc.) in the next generations and this was carried out for 10 generations in vivarium condition. The selected population with a longer lifespan was pooled separately from their parent and sib lines. Several long-lived isolines were isolated from NLS stock and then several such long-lived isolines were pooled to create a long-lived population. This longer living line (lifespan > 100 days) is referred to as the long lifespan (LLS) line. Currently, the LLS population has completed more than 100 generations in our laboratory.

Experimental plan

Genetic repression using RNAi transgenic flies

UAS-sod1-IR, *UAS-cat-IR*, *Tub-Gal4*, and *Act-Gal4* flies were used. The driver lines, *Tub-Gal4* and *Act-Gal4*, were used to achieve ubiquitous expression throughout the body. *UAS-sod1-IR* flies were driven using *Act-Gal4* to achieve RNAi post-transcriptional gene silencing of the *sod1* gene. Similarly, *cat* gene silencing was achieved by driving *UAS-cat-IR* flies with *Tub-Gal4*.

Chemical inhibition in ELP flies

Both NLS and LLS flies were treated with DDTC (1 mM) and ATZ (1.5 mM) through the food medium. Male and female flies were maintained in separate media vials supplemented with inhibitors. For standardizing the concentration, flies were fed with DDTC and ATZ (concentrations ranging from 100 µM to 10 mM) separately as well as assayed for enzyme activities after treatment. The concentration with the highest percentage inhibition in enzyme activity was used for the lifespan assay.

Survivability

For the first part of the experiment, RNAi lines were driven and the lifespan of parents (UAS and Gal4 lines), as well as

F_1 progenies, were assessed. Effects of *sod1* gene silencing on longevity were investigated by assessing the lifespan of male and female *UAS-sod1-IR* and *Act-Gal4* flies (parents) and progenies obtained (*Act > sod1*). The lifespan of both male and female *UAS-cat-IR* and *Tub-Gal4* (parents) and F_1 progenies (*Tub > cat*) were assessed for *cat* gene silencing. Adult lifespan was assessed by setting up 20 vials per line containing 10 males and 10 females separately (10 vials per sex). Flies were transferred to fresh food vials every alternate day for a month and 4–5 days depending on the condition of the media thereafter. Mortality was recorded daily; and flies dying, if any, were not replaced during the course of the assay, which was continued until the death of all flies.

In the second part, feeding flies containing the inhibitors were employed to inhibit antioxidant enzymes in vivo. Freshly eclosed adult flies (both NLS and LLS) from vials set up at a density of about 20 eggs per vial were collected. Separate assays were carried out to examine the lifespan of male and female flies of both lines. Adult lifespan was assessed by setting up 20 vials per line containing 10 males and 10 females separately (10 vials per sex). Flies were transferred to fresh food vials containing DDTC (1 mM) and ATZ (1.5 mM), separately, every alternate day for a month and 4–5 days depending on the condition of the media thereafter. Mortality was recorded daily; any flies dying were not replaced during the course of the assay, which was continued until the death of all flies.

Antioxidant enzyme activities

The whole-body homogenate was prepared using 0.1 M sodium phosphate buffer (pH 7.4), centrifuged at 5000 rpm for 10 min at 4 °C and the supernatant was used for biochemical assays. All the assays were carried out both in 1-week-old NLS and LLS lines as well as in transgenic lines employing Beckman Coulter spectrophotometer (Beckman Counter Inc., CA, USA). For every assay (depending on the amount of protein required), 50 flies of four replicates were employed (repeated several times for consistent values) for each line and sex group.

SOD activity was assayed by monitoring the inhibition of pyrogallol auto-oxidation (Marklund and Marklund 1974) in a total volume of 3 mL reaction mixture containing fly homogenate and 0.1 M Tris–HCl buffer (pH 8.2). The assay mixture was transferred to a 3-mL cuvette and the reaction was started by adding 0.5 mL 2 mM pyrogallol. The rate of increase in absorbance was monitored for 2 min at 420 nm. The increase in the absorbance at 420 nm after the addition of pyrogallol was inhibited by the presence of SOD. One unit of SOD is described as the amount of enzyme required to cause 50% inhibition of pyrogallol autoxidation per 3 mL of assay mixture. Results have been expressed as units of enzyme activity per mg protein of tissue homogenate.

CAT activity was measured following the method of Aebi (1984). One milliliter reaction mixture contained 0.1 M sodium phosphate buffer (pH 7), the enzyme sample (fly homogenate), and substrate (H_2O_2). The decrease in H_2O_2 level was monitored by observing the rate of decrease in absorbance for 2 min at 240 nm and expressed as mmoles of H_2O_2 decomposed/min/mg protein.

Protein estimation was carried out using Lowry's method with bovine serum albumin as standard (Lowry et al. 1951).

Statistical analysis

The SPSS software (version 14.0) was used for statistical analysis. The survivorship curves were analyzed using Kaplan–Meier survival plots. Log-rank test (Mantel–cox) was used to calculate the significance between control and treated NLS and LLS flies (DDTC and ATZ-treated); *UAS-sod1-IR, Act-Gal4* and *Act > sod1*; and *UAS-cat-IR, Tub-Gal4*, and *Tub > cat*. The cumulative survival of flies was plotted against the time (days). The time of death of each fly is considered for the event until all the fly dies. The treatment (DDTC and ATZ) and the knockdown effect were considered as explanatory variables. To analyze the fixed and random effects of parameters on survival, a linear mixed-effects model with a restricted maximum likelihood criterion was carried out. The treatment (DDTC and ATZ) factor was considered as a fixed effect, whereas, line (NLS and LLS) and sex (male and female) were considered as random effects. Data for biochemical investigations were subjected to factorial analysis of variance with sex and lines as fixed factors, and individual values as dependent factors. Values are represented as mean ± standard error of the mean. Multiple comparisons were carried out by Duncan's post-hoc test.

Results

Genetic repression using RNAi transgenic flies

Survivability/lifespan analysis

Log-rank (Mantel–Cox) test (χ^2) and *P* values revealed that the lifespan differed significantly between parent (*UAS-sod1-IR* and *Act-Gal4*) and *Act > sod1* flies (Table 1). The Kaplan–Meier survivorship curves of groups (*UAS-sod1-IR*, *Act-Gal4*, *Act > sod1* ♂ and ♀) show that there was a significant difference in lifespan (Fig. 1a). The mean lifespan in both the sexes in *Act > sod1* (male, 36.66 ± 1.36; female, 40.33 ± 1.81 days) varied significantly shorter from the other lines. Variations in mean lifespan between groups (*UAS-sod1-IR*, *Act-Gal4*, *Act > sod1* ♂ and ♀) are shown in Table 1.

Table 1 Log-rank test and *P* values obtained for the Kaplan–Meier survivorship curve of *Actin5C-gal4* and *Tubulin-Gal4* driving the expression of *UAS-sod1-IR* and *UAS-cat-IR*

Groups	Mean lifespan (days)	Log-rank (Mantel–Cox) test (χ^2) value	*P* value (*df* = 3)
SOD1			
UAS-sod1-IR	52.62 ± 2.21		
Actin5C-gal4	83.69 ± 2.29		
Actin-Gal4 > UAS-sod1-IR ♂	36.66 ± 1.36	252.92	0.000
Actin-Gal4 > UAS-sod1-IR ♀	40.33 ± 1.81	228.63	0.000
CAT			
UAS-cat-IR	82.87 ± 2.06		
Tubulin-Gal4	55.50 ± 2.77		
Tubulin-Gal4 > UAS-cat-IR ♂	10.43 ± 0.79	319.92	0.000
Tubulin-Gal4 > UAS-cat-IR ♀	14.50 ± 1.30	246.61	0.000

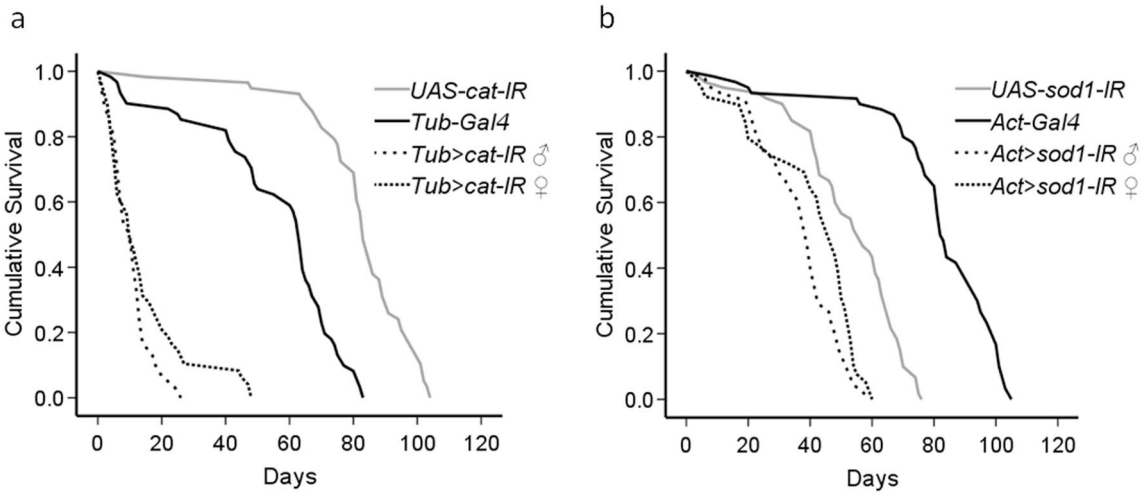

Fig. 1 Knockdown of SOD1 and CAT leads to a marked reduction in lifespan of flies. Kaplan–Meier survivorship curves of *Act5C-gal4* driving the expression of *UAS-sod1-IR* and *Tubulin-Gal4* driving the expression of *UAS-cat-IR* flies. Cumulative survival of *UAS-sod1-IR, Act5C-gal4*, *Act-Gal4 > UAS-sod1-IR* **a** and *UAS-cat-IR, Tubulin-Gal4*, *Tubulin-Gal4 > UAS-cat-IR* **b** are plotted against time (days). *CAT* catalase, *SOD* superoxide dismutase

Log-rank (Mantel–Cox) test (χ^2) and *P* values revealed that the lifespan differed significantly between parent (*UAS-cat-IR* and *Tub-Gal4*) and *Tub > cat* flies (Table 1). The Kaplan–Meier survivorship curves of groups (*UAS-cat-IR*, *Tub-Gal4*, *Tub > cat* ♂ and ♀) show that lifespan is markedly affected by knockdown of catalase ubiquitously (Fig. 1b). The lifespan of both male (10.43 ± 1.12 days) and female *Tub > cat* flies (14.50 ± 1.85 days) varied significantly from their parents (*UAS-cat-IR* and *Tub-Gal4*). *Tub > cat* flies showed a drastic decrease of 84.61% in lifespan when compared to the mean lifespan of their parents. Variations in mean lifespan between groups (*UAS-cat-IR*, *Tub-Gal4*, *Tub > cat* ♂ and ♀) have been compiled in Table 1.

Antioxidant enzyme activities

SOD activity was higher in *UAS-sod1-IR* flies (0.61 ± 0.04 Units/mg protein) when compared with the *Act-Gal4* line (0.36 ± 0.06 Units/mg protein). A drastic decline in SOD activity was observed in *Act > sod1* flies when compared to *UAS-sod1-IR* and *Act-Gal4* flies (Fig. 2a). There was no significant sex difference in SOD activity in *Act > sod1* flies. The percentage inhibition of CuZnSOD (SOD1) activity by RNA interference in the *Act > sod1* flies is shown in Table 2. One-way ANOVA showed that there is a significant difference in SOD activity between groups. A significant difference in SOD activity was observed between *UAS-sod1-IR*, *Act-Gal4* and *Act > sod1* flies ($F = 37.107$, $P = 0.002$).

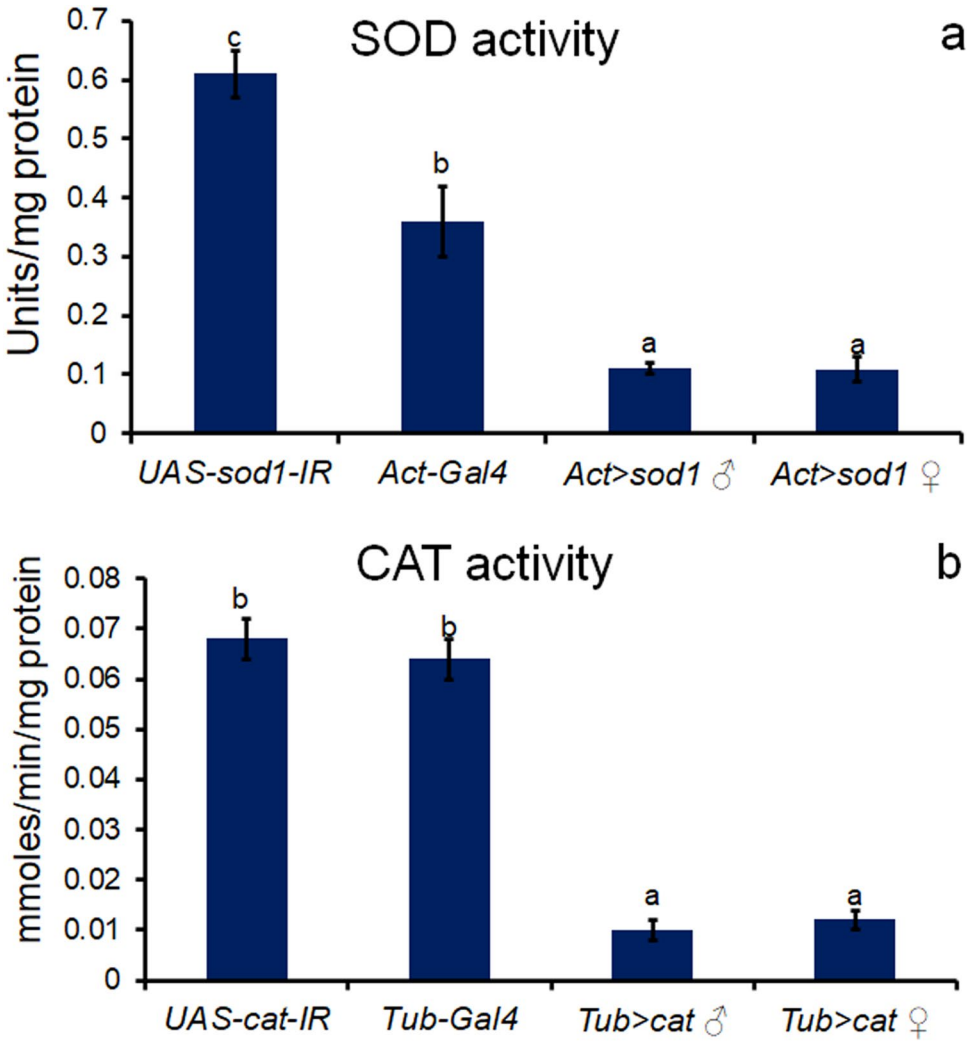

Fig. 2 Ubiquitous knockdown of SOD1 and CAT caused remarkable suppression of enzyme activity. **a** SOD activity in *UAS-sod1-IR, Act5C-gal4*, and *Act-Gal4 > UAS-sod1-IR* male and female flies. **b** CAT activity in *UAS-cat-IR, Tubulin-Gal4*, and *Tubulin-Gal4 >* UAS-*cat-IR* male and female flies. Each value represents the mean ± SE. Values denoted with different alphabets differ significantly ($P < 0.05$). *CAT* catalase, *SOD* superoxide dismutase

Table 2 Percent inhibition of SOD and CAT activity in *Actin-Gal4 > UAS-sod1-IR* and *Tubulin-Gal4 > UAS-cat-IR* flies as well as NLS and LLS flies treated with DDTC and ATZ

Groups	% inhibition of SOD1 activity after RNAi and DDTC treatment	% inhibition of CAT activity after RNAi and ATZ treatment
Actin-Gal4 > UAS-sod1-IR		
Male	77.11	NA
Female	77.52	NA
Tubulin-Gal4 > UAS-cat-IR		
Male	NA	84.84
Female	NA	81.81
NLS		
Male	72.12	74.13
Female	73.03	75.15
LLS		
Male	69.68	72.46
Female	70.28	73.25

ATZ 3-amino-1,2,4-triazole, *CAT* catalase, *DDTC* diethyldithiocarbamate, *LLS* long lifespan, *NLS* normal lifespan, *RNAi* RNA interference, *SOD* superoxide dismutase

A marked difference in CAT activity between parent (*UAS-cat-IR* and *Tub-Gal4*) and *Tub > cat* flies was observed (Fig. 2b). The CAT activity decreased drastically in the *Tub > cat* flies, irrespective of sex. Table 2 shows the percent inhibition of CAT activity by RNAi in the *Tub > cat* flies. One-way ANOVA of the data showed that there is a significant difference in CAT activity between groups. A significant difference in CAT activity was observed between *UAS-cat-IR*, *Tub-Gal4* and *Tub > cat* flies ($F = 82.207$, $P = 0.000$).

Chemical inhibition of the enzymes in ELP

Survivability/lifespan analysis

Log-rank (Mantel–Cox) test (χ^2) and *P* values revealed that there was no significant difference in lifespan between treated and control groups, except for LLS females (Table 3). The Kaplan–Meier survivorship curves of groups (sex and line) showed that there is no difference in lifespan between control and DDTC-treated flies (Fig. 3a–d). LLS flies showed a relatively shortened lifespan after DDTC treatment compared with NLS flies. Variations in mean lifespan between groups (sex, line, and treatment) are given in Table 3. The results on survivability show that there is no effect of DDTC treatment on the lifespan of flies. The effect of DDTC treatment on lifespan was analyzed by adjusting sex and line as random effects. Results revealed a significant effect ($P = 0.044$) of treatment on survival (Suppl Table 1).

Log-rank (Mantel–Cox) test (χ^2) and *P* values revealed that significant differences in lifespan between treated and control groups were observed only in LLS flies, but not in NLS flies (Table 3). The Kaplan–Meier survivorship curves of groups (sex and line) showed that there is no difference in lifespan between control and ATZ-treated flies (Fig. 4a–d). LLS flies showed a relatively shortened lifespan after ATZ treatment compared with NLS flies. Variations in mean lifespan between groups (sex, line, and treatment) are given in Table 3. The results on survivability show that there is no effect of ATZ treatment on the lifespan of flies. The effect of ATZ treatment on lifespan was analyzed by adjusting sex and line as random effects. Results revealed a significant effect ($P = 0.006$) of treatment on survival (Suppl Table 1).

Table 3 Log-rank test and *P* values obtained for the Kaplan–Meier survivorship curve between NLS and LLS flies treated with DDTC and ATZ

Groups ($df = 1$)	Mean lifespan (days)	Log-rank (Mantel–Cox) test (χ^2) value	*P* value
NLS			
Male			
Control	61.75 ± 1.79	0.47	0.49
DDTC treated	61.15 ± 2.56		
Female			
Control	65.21 ± 2.67	0.14	0.71
DDTC treated	63.75 ± 3.77		
LLS			
Male			
Control	79.20 ± 4.76	2.75	0.09
DDTC treated	69.78 ± 4.93		
Female			
Control	96.33 ± 3.75	4.69	**0.03**
DDTC treated	89.47 ± 2.10		
NLS			
Male			
Control	60.95 ± 1.81	0.002	0.97
ATG treated	59.80 ± 3.19		
Female			
Control	68.84 ± 1.23	0.30	0.58
ATG treated	71.17 ± 0.56		
LLS			
Male			
Control	83.60 ± 5.39	11.34	**0.001**
ATG treated	70.35 ± 3.85		
Female			
Control	98.04 ± 4.01	6.51	**0.01**
ATG treated	79.38 ± 6.70		

Bold *P* value represents significant difference in lifespan between control and treated flies

ATZ 3-amino-1,2,4-triazole, *DDTC* diethyldithiocarbamate, *NLS* normal lifespan, *LLS* long lifespan

Antioxidant enzyme activities

SOD activity was higher in LLS flies (male, 0.497 ± 0.01; female, 0.414 ± 0.02 Units/mg protein), irrespective of sex, when compared to NLS flies (male, 0.287 ± 0.01; female, 0.254 ± 0.03 Units/mg protein). Following 24 h treatment with DDTC, SOD activity decreased drastically in all the groups (sex and line). Figure 5a illustrates a significant decrease in the SOD activity in the treated group when compared to the control group. The percent inhibition of CuZnSOD activity by DDTC treatment in all the groups (sex and line) is shown in Table 2. One-way ANOVA showed that there is no significant difference between treated groups (sex and line). A significant difference was observed between lines ($F = 63.929$, $P = 0.000$); however, a significant difference between sexes was observed only in the LLS line (Table 4).

CAT activity was higher in LLS flies (male, 0.098 ± 0.004; female, 0.086 ± 0.005 mmoles/min/mg protein), irrespective of sex, when compared to NLS flies (male, 0.058 ± 0.004; female, 0.069 ± 0.002 mmoles/min/mg protein). Following 24 h treatment with ATZ, CAT activity decreased drastically in all the groups (sex and line). Figure 5b illustrates a significant decrease in CAT activity in the treated group

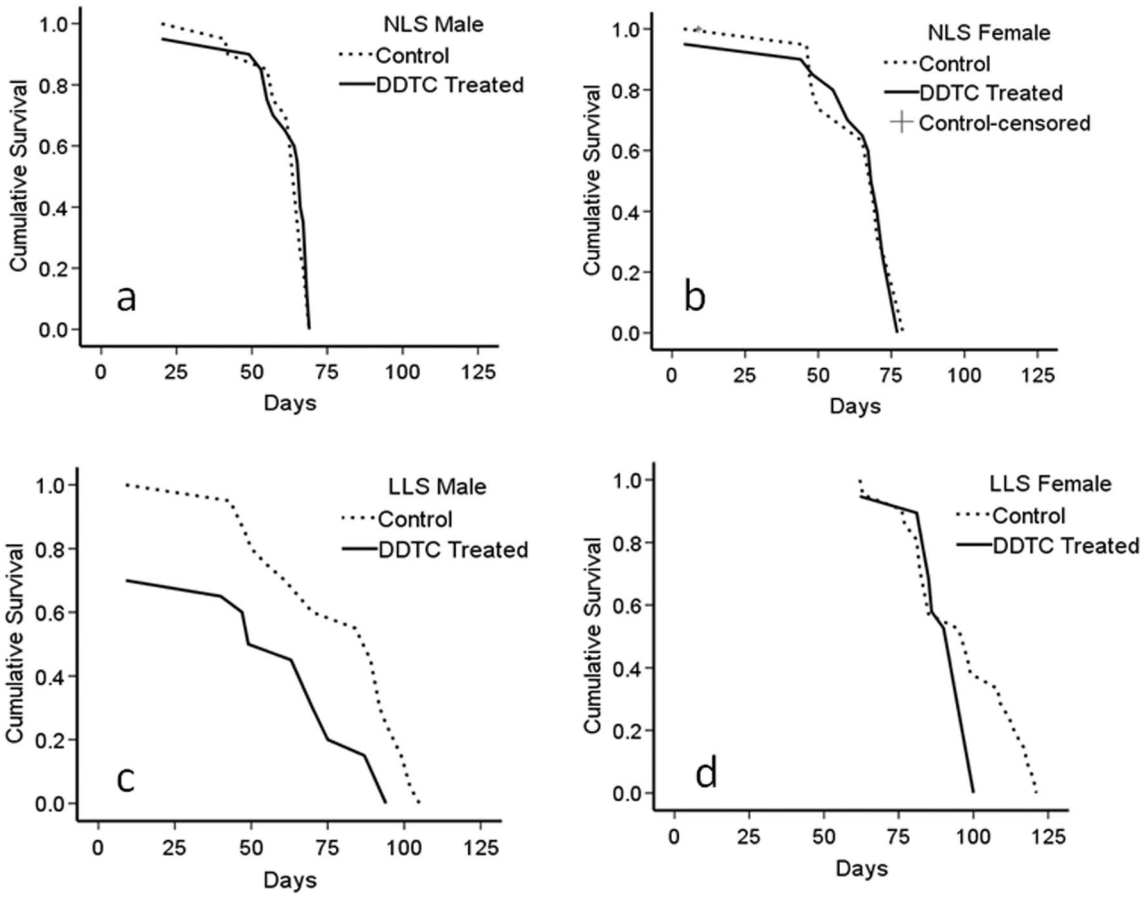

Fig. 3 Effect of DDTC on lifespan was not significant. Kaplan–Meier survivorship curves of normal (NLS) and long lifespan (LLS) lines of *D. melanogaster* treated with DDTC (SOD activity inhibitor). Cumulative survival is plotted against age (days) in males and females of NLS **a**, **b** and LLS **c**, **d** flies. Censored data (+sign on the curves) represents missing value due to accidental death. DDTC, diethyldithiocarbamate

when compared to the control group. The percent inhibition of catalase activity by ATZ treatment in all the groups (sex and line) is shown in Table 2. One-way ANOVA showed that there is no significant difference between treated groups (sex and line). A significant difference was observed between lines ($F = 53.392$, $P = 0.000$); however, a significant difference between sexes was observed only in the LLS line (Table 4).

Discussion

The endogenous antioxidant system comprises the enzymes (SOD, CAT, GPx, and thioredoxin), hydrophilic antioxidants (urate, ascorbate, glutathione, and flavonoids), and lipophilic radical antioxidants (tocopherol, carotenoid, and ubiquinol) (He et al. 2017). SOD1, SOD2, and catalase are important enzymes in the detoxification of ROS and H_2O_2 in cells that constitute redox balance implicated in aging (Phillips et al. 2000; He et al. 2017). Though SOD overexpression increases lifespan, the role of other factors in redox homeostasis is not clear (reviewed by Gems and Doonan 2009). Higher activities of SOD, CAT, and glutathione-s-transferase have been observed in the long-living strain of *Drosophila* (Dudas and Arking 1995). Flies with RNAi-mediated silencing of *SOD2* have been shown to have reduced lifespan (Kirby et al. 2002). However, altered expression of SOD and catalase genes in *C. elegans* and mice failed to affect the lifespan (reviewed by Gems and Doonan 2009). Le Bourg (2001), in his review, has opined that antioxidant enzymes are poorly connected to the normal aging process, but they allow the organism to cope with stressful conditions. Overexpression of SOD alleles was shown to be associated with postponed aging in laboratory *Drosophila* stocks (Tyler et al. 1993). However, an earlier study reported that there was no relation between mean lifespan and higher activity of catalase in *D. melanogaster* (Durusoy et al. 1995).

RNAi provides a powerful genetic tool for gene function analysis (Alic et al. 2012). In our study, the GAL4/UAS binary regulatory system was employed to express

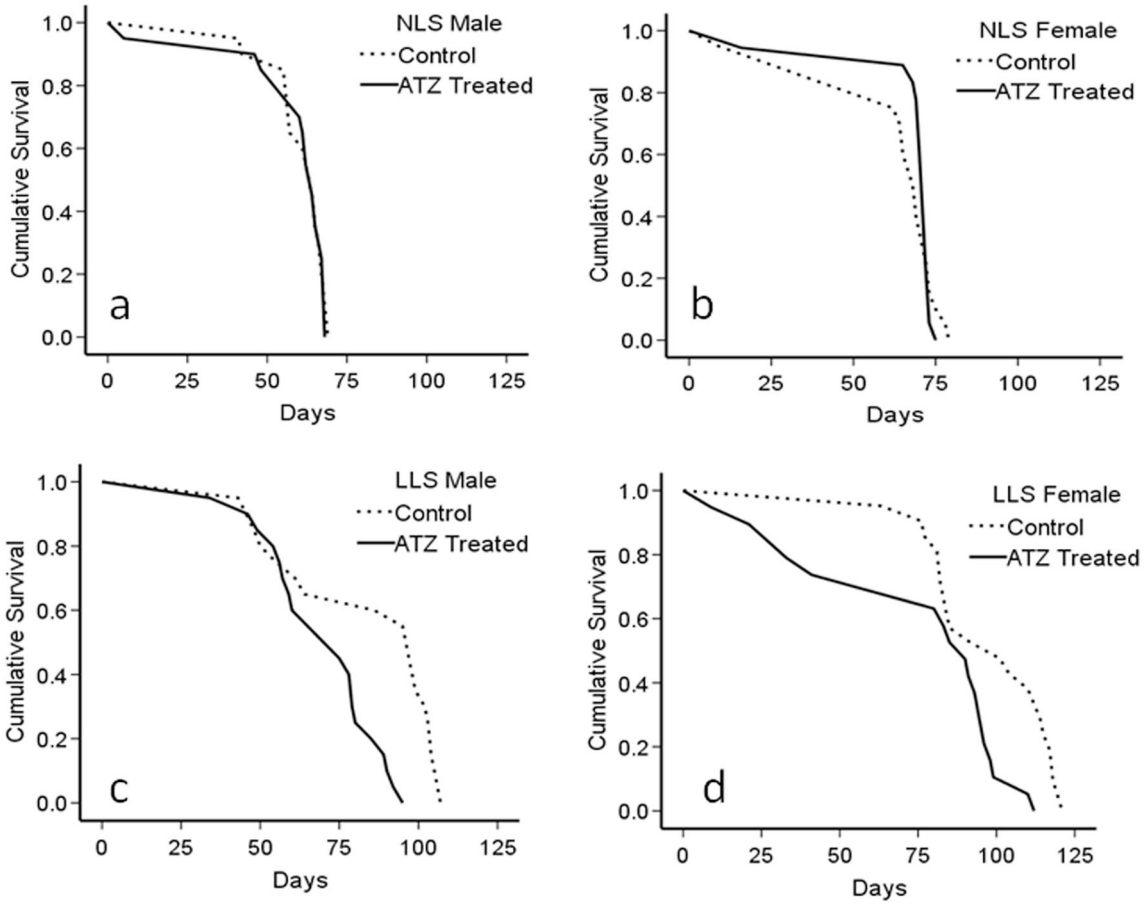

Fig. 4 Effect of ATZ on lifespan was partially significant. Kaplan–Meier survivorship curves of normal (NLS) and long lifespan (LLS) lines of *D. melanogaster* treated with ATZ (catalase activity inhibitor). Cumulative survival is plotted against age (days) in males and females of NLS **a**, **b** and LLS **c**, **d** lines. ATZ, 3-amino-1,2,4-triazole

RNAi using inverted repeat (IR) constructs, by crossing the *UAS-IR-RNAi* lines with the ubiquitously expressing *Tub-Gal4 and Act-Gal4* driver lines. Specific upregulation of the antioxidant defense system genes in flies is accompanied with increased resistance to paraquat-induced oxidative stress involving endogenous ROS (Arking 2001). A study has shown that the conditional transgenic systems to overexpress CuZnSOD in flies extended lifespan up to 48% and, in the case of overexpression of MnSOD, it was extended by 37% (Sun and Tower 1999). In addition, overexpression of catalase led to increased resistance to H_2O_2 without extending lifespan. It has been shown that transgenic strain overexpressing *sod* and *cat* genes was shown to be resistant to paraquat-induced oxidative stress and extended mean longevity, suggesting the influence of the antioxidant defenses against free radicals in aging (Orr and Sohal 1994). Transgenic interventions and genetic experiments have shown that SOD plays an important role in attenuating oxidative damage in vivo thereby delaying aging (Warner 1994).

In general, long-living animal species are reported to have more efficient antioxidant systems showing higher SOD activity than shorter-living species (Junqueira et al. 2004; Tasaki et al. 2017; Negroni et al. 2019). *Sod1*-null mutants of *Drosophila* are reported to show increased susceptibility to oxidative stress by paraquat hypersensitivity, increased DNA damage, infertility as adults, and a dramatically reduced lifespan, surviving no more than three days (Mockett et al. 2003; Blackney et al. 2014). However, overexpression of antioxidant genes did not increase lifespan in mice, and knockdown of MnSOD and GPx1 showed no effect on lifespan (Speakman and Selman 2011). In our study, genetic repression of SOD1 through RNAi led to reduced lifespan (male, 76.8%; female, 77.3%), which is presenting strong evidence for the role of SOD in longevity.

CAT-null mutants of *Drosophila* are reported to survive pre-adult development but have difficulty eclosing into adults (Phillips et al. 2000). CAT-null mutants experience massive mortality over the 2–3 days of adult life,

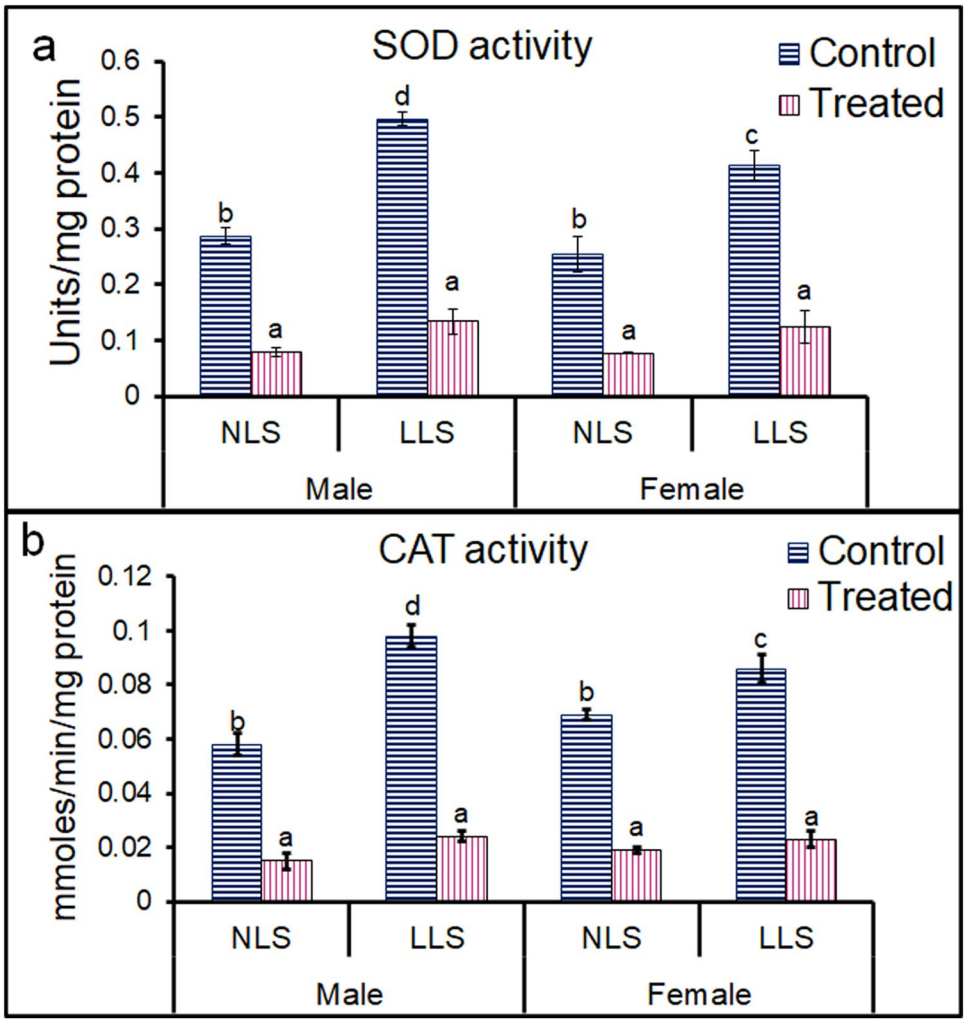

Fig. 5 Drastic reduction in SOD and CAT activities following treatment with inhibitors. **a** Total SOD and **b** CAT activities in normal lifespan (NLS) and long lifespan (LLS) lines of *D. melanogaster* flies treated with diethyldithiocarbamate (DDTC) and 3-amino-1,2,4-triazole (ATZ), respectively. Each value represents the mean ± SE. Values denoted with different alphabets differ significantly ($P < 0.05$). *CAT* catalase, *SOD* superoxide dismutase

Table 4 Results from the factorial analysis of variance for SOD and CAT activity of normal and long lifespan lines of *D. melanogaster* after treatment with diethyldithiocarbamate and 3-amino-1,2,4-triazole

Groups (*df*)	SOD activity		CAT activity	
	F	*P*	*F*	*P*
Sex (1)	4.92	0.057	0.02	0.882
Line (1)	263.9	**0.000**	53.39	**0.000**
Treatment (5)	310.8	**0.000**	554.8	**0.000**
Sex × line (1)	0.95	0.357	8.48	**0.020**
Sex × treatment (1)	3.03	0.120	0.13	0.730
Line × treatment (1)	20.8	**0.002**	20.7	**0.002**
Sex × line × treatment (1)	0.49	0.501	3.20	0.112

Bold *P* value represents significant difference in enzyme activity between line, sex, treatment, sex × line, sex × treatment, line × treatment, and sex × line × treatment

CAT catalase, *SOD* superoxide dismutase

which was noticed in the current study also. In the present study, genetic repression of CAT by RNAi led to drastically reduced lifespan (male, 84.84%; female, 81.8%). Our results provide evidence for the critical role of CAT in longevity. Although SOD1 and CAT activity are negligible in Sod1IR and CatIR in flies, the nature of RNAi silencing makes it unlikely that Sod1IR and CatIR expression completely abolishes SOD1 and CAT synthesis. Thus, the phenotype of a true SOD1 and CAT-null mutant could be more severe than observed here. Drastic reduction in lifespan of flies with repression of SOD1 and CAT activity demonstrates the role of these antioxidant enzymes in the longevity in *Drosophila*. Our study demonstrates that genetic suppression of SOD1 and CAT activity in flies led to drastically reduced lifespan.

Our study is the first, to our knowledge, to examine the role of antioxidant enzymes by inhibiting their activity in vivo by chemical inhibitors on longevity in *Drosophila* flies that were selected for extended longevity. Our results show that both the inhibitors (DDTC and ATZ) inhibit the activities of SOD and CAT in vivo, respectively. An earlier

study has reported the effect of DDTC, an inhibitor of SOD activity, in relation to aging (Sohal et al. 1984; Lushchak et al. 2007). Although the effect of overexpression or lack of SOD activity on lifespan in flies is reported, the effect of inhibition of SOD activity on lifespan is not known. In the present study, the effect of inhibition of CuZnSOD activity on lifespan was assayed by measuring the mean lifespans of age-synchronized cohorts of flies. SOD activity decreased markedly in flies (sex and line) treated with DDTC. However, there was no significant difference in lifespan between control and DDTC-treated flies. This discrepancy or lack of effect on lifespan could be attributed to various reasons, such as the bioavailability, metabolic detoxification of DDTC, and increased synthesis of the enzyme that can overcome the enzyme deficiency in the fly. Moreover, DDTC inhibits only CuZnSOD but not MnSOD activity in vivo. Therefore, the effect on ROS could be partial. In the present study, although DDTC treatment inhibits CuZnSOD activity, it did not affect the longevity of flies.

Catalase is the principal enzymatic mechanism for the removal of H_2O_2 in *Drosophila* since insects lack GPx activity (Orr and Sohal 1992). The compound ATZ binds irreversibly to the active site of the protein apoenzyme resulting in the irreversible inhibition of activity (Margoliash et al. 1959; Ruiz-Ojeda et al. 2016). The effect of ATZ on the activity of the mammalian liver catalase is believed to be due to the accelerated breakdown of catalase moiety rather than the inhibition of the biosynthesis of catalase (Kato 1967). ATZ causes the loss of the activity in the existing catalase molecules without interfering with the de novo enzyme synthesis (Bewley and Laurie-Ahlberg 1984). Lack of CAT activity is reported to decrease viability and lifespan in acatalasemic fly mutants (Durusoy et al. 1995). Although there are studies on the effect of overexpression and lack of CAT activity on lifespan in flies, the effect of inhibition of CAT activity by ATZ on lifespan is not known. In the present study, the effect of inhibition of CAT activity on the lifespan was investigated wherein the in vivo inhibition of CAT activity showed no effect on the lifespan of flies. As in the case of DDTC-treatment, lack of effect on lifespan can be attributed to the bioavailability of ATZ to the fly. Flies were fed with the ATZ for their lifetime in the current study, but ATZ may affect existing catalase molecules without interfering with the de novo enzyme synthesis, which could compensate for the loss of catalase activity. Therefore, although ATZ treatment inhibits CAT activity, it did not affect the lifespan of the flies.

As in vivo inhibition of the enzyme activity did not affect the lifespan in both NLS and LLS flies, which could be attributed to the problem of bioavailability of the inhibitors, MnSOD activity, and GSH levels that were not effective in inducing the oxidative stress. A similar study in multiple independent lines would provide better insight into the role of antioxidant defenses in longevity. Moreover, an effective approach to investigate the role of antioxidant enzymes in aging could be through genetic repression by using RNAi as shown in our study. Our results show a drastic reduction in lifespan of flies with genetic repression of SOD1 and CAT expression, which demonstrates the role of these antioxidant enzymes in longevity in *Drosophila*.

Conclusions

We have examined the role of endogenous antioxidant defenses (SOD and CAT) using two approaches: chemical inhibition and RNAi. Our results show that both the inhibitors (DDTC and ATZ) inhibit the activities of SOD and CAT, in vivo, but, did not reduce the longevity in both NLS and LLS flies, which could be attributed to the factors such as bioavailability, metabolism of the inhibitors and synthesis of the enzymes, and compensatory mechanisms involving MnSOD (SOD2) activity and GSH levels. Genetic repression of SOD and CAT by RNAi in transgenic flies led to a markedly reduced lifespan, which provides strong evidence for the role of antioxidant defenses in aging and longevity. However, further studies are needed to establish the precise involvement of the antioxidant defenses in the overall oxidative stress resistance as a causal mechanism in aging and longevity.

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Data availability All data generated or analyzed during this study are included in this published article (and its supplementary information files).

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