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ROLE OF XANTHINE DEHYDROGENASE AND AGING ON THE INNATE IMMUNE RESPONSE OF *DROSOPHILA*

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ABSTRACT

It has been proposed that uric acid is an important scavenger of deleterious oxygen species and peroxynitrite in biological systems. The cellular sources responsible for the generation of damage-causing reactive oxygen species (ROS) are widespread. Xanthine dehydrogenase (XDH) / oxidase (XOD) catalyzes the oxidation of xanthine to uric acid. The rosy *(ry)* gene encodes XDH/XOD in *Drosophila melanogaster.* XDH codes for uric acid which is a ROS scavenger. XOD however is an enzyme system implicated in ROS production. In this study, we investigated the roles of XDH in the fly's immune defense response to infection and in the aging process. We first compared ROS generation and nitric oxide (NO) level in the whole body and the gut of XDH mutant with those of wild type. Our results suggested that XDH has a protective effect with respect to both ROS and NO generations, particularly in the gut. We also examined the effect of a XDH deletion mutant on the relative sensitivity of the organism against bacterial infection, on the immune inducibility of antimicrobial peptides and on the effect of aging in the defensive response to infection. Our results strongly suggest that XDH plays an important role in the innate immune response and that the age-associated deterioration of the innate immune response might be, at least in part, associated with the loss of XDH activity in the aging process.

INTRODUCTION

The xanthine oxidoreductase (XD: xanthine dehydrogenase + oxidase (XDH/XOD)) system is a major cellular source of superoxide radical production in disease processes such as ischemia-reperfusion, radiation injury, and other inflammatory conditions (1,2). XDH catalyzes the oxidation of xanthine to uric acid. Uric acid has been shown to have important anti-oxidative properties that guard against oxidative damage in lipids, proteins, and nucleic acids (3). In *Drosophila*, urate-null mutants of the *ry* gene, which encodes XDH/XOD in that organism, are hypersensitive to paraquat, ionizing radiation, and hyperoxia suggesting that this gene product plays a major role in the organism's defenses against oxidative stress (4).

The oxidative stress theory of aging proposes that aging occurs as a consequence of the deleterious effects of oxygen free radicals produced during the course of cellular metabolism (5). The major cellular sources responsible for the generation of damage causing reactive oxygen species (ROS), such as $O_2^{\bullet}$, H_2O_2, and $^{\bullet}OH$ are found throughout the cell : in the mitochondria or peroxisomes, as well as being generated by various enzymes such as cyclooxygenase (COX), NADPH dehydrogenase, and NADPH oxidase ROS (6). The XOD enzyme system is also implicated in ROS production by activated phagocytic cells, as examplified by ischaemia-reperfusion injuries of the myocardium and small intestine (7,8). In addition to ROS, reactive nitrogen species (RNS) such as $NO^{\bullet}$, $NO^{\bullet}_2$, HNO_2 and $ONOO^-$, have been shown to produce massive amounts of free radicals in response to challenges and to also lead to pro-inflammatory processes (9).

Recently, the 'Inflammatory Hypothesis on Aging' was suggested with the hope of providing a new perspective into the molecular mechanisms that bridge aging and age-related disease at the molecular levels (6). This theory suggests that inflammation releases pro-inflammatory mediators which indirectly lead to an increased ROS production. The theory also suggests that the aging process result in the induction of these same pro-inflammatory mediators, thus accounting in part for the observed age-related increase in ROS levels and decrease in functional status (10). In addition, the well documented age-associated decline in the systemic immune response of vertebrates contributes to both a decreased resistance to pathogens and a decreased ability to control the inflammatory processes associated with infection, leading to an increased morbidity and mortality (11,12).

Although substantial attention has been given to the status of XOD as a major source of ROS in vertebrates, the role that XDH/XOD may play in relation to the aging process has received little attention. Studies in rat suggesting a role of XDH/XOD in the aging process were recently reported (6,13). XDH/XOD expression at

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both the enzyme activity and gene expression levels are known to be upregulated under conditions associated with the inflammatory response suggesting that these gene products may play a role in the aging of the immune system as well (14).

Using *Drosophila* as a model system, the present study was designed to investigate (1), the protective roles of XDH in the organism's defense response to infection and (2), the effect of the aging process on its innate immune response.

RESULTS

Longevity of XDH mutant

To investigate the role of XDH in the aging process using *Drosophila melanogaster* as a model system, we examined the longevity of XDH mutant and wild type strain. XDH mutant flies showed a shortened lifespan as compared to the wild type flies, as shown in Figure 1.

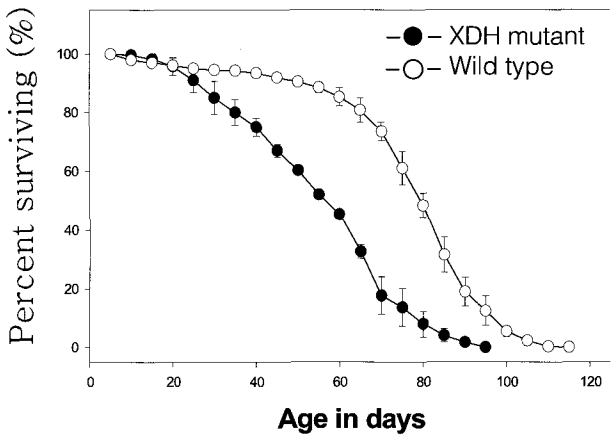

Figure 1: Survival curves of wild type (*Oregon R*) and XDH mutant strain (ry^{506}) used in this study. About 50 males were in each vial containing a standard medium, the flies were transferred to fresh vials every 3~4 days at which time the numbers of dead flies were recorded. About 400 males were scored for each lifespan experiments.

Mean life time (± standard error) in XDH mutant strain and wild type were 56.53±0.95 and 77.93±1.06, respectively. A Kaplan-Meier survival analysis (log-rank test = 260.43, df=1, P<0.0001) showed that these longevity differences are significant.

Effect of XDH on ROS generation and NO levels

In order to determine whether the shortened life span of the XDH mutant flies was associated with excessive oxidative stress, we measured total ROS and NO generations in XDH mutant and wild type flies. ROS generations in whole body of flies were measured using the dichlorofluoroscein (DCF) method. NO levels in the whole body of adult were assayed by Griess spectrometric measurements. Figure 2a shows that the ROS generation in the whole body of XDH mutants was slightly higher than in wild type, being significantly higher in males but not in females. XDH is known to have a very high activity in the intestine (15,16), which has one of the most rapid cell turnover rates of any tissue in the body. We therefore examined ROS generation in the guts of XDH mutant and wild type strain. Figure 2b shows that significantly increased ROS generation in the gut of XDH mutants relative to controls was detected in both sexes.

Figure 2a:

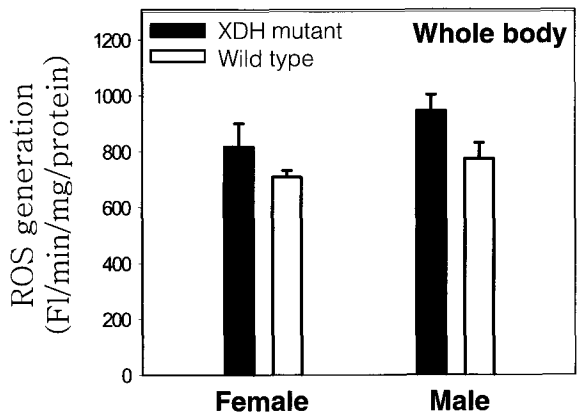

Figure 2b:

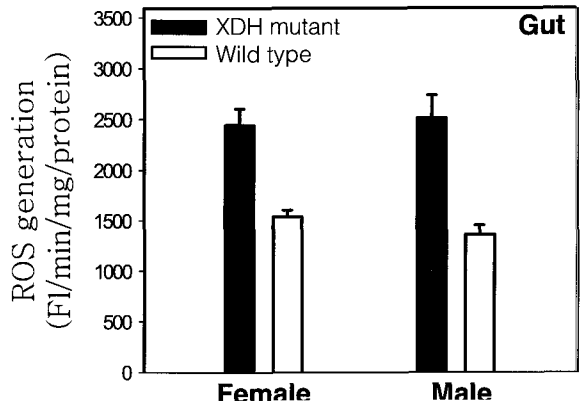

Figure 2: Comparisons of ROS generation in XDH mutant and wild type strain. Whole body of 5 adults (3 days old, a) and dissected guts of 10 adults (3 days old, b) were used by DCFDA assay. Changes of fluorescence intensity were measured with excitation and emission wavelength of 486 nm and 530 nm, respectively for 1 hr. Each values is the mean±standard deviation of 8 assays were performed. A statistical significance between wild type and XDH mutant strain is marked as *P<0.05 and **P<0.01.

The NO levels in both sexes of the XDH mutant was also higher than in the wild type strain, as shown in Figure 3. These results suggest that the normally high activity of XDH in the gut of the wild type animal may have a protective effect on both ROS and NO generations, both in the gut itself and in the body as a whole.

Role of XDH in the defensive response to infection

In order to investigate the role of the XDH gene in defensive response to infection, we first examined ROS generation in individuals after bacterial challenge in wild type and XDH mutant adults. ROS generation in the XDH mutant animals increased after infection, as shown

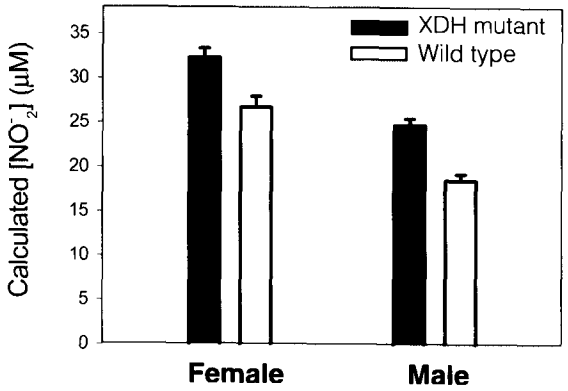

Figure 3: Comparisons of NO levels in XDH mutant and wild type strain. 10 adult females or males (3 days old) per group were used in each experiment. NO concentration was measured by a microplate assay based on the Griess reagent. The absorbance at 540 nm was measured and NO_2^- was quantified $NaNO_2$. Each value is the mean±standard deviation of 6 independent experiments. A statistical significance between wild type and XDH mutant strain is marked as **P<0.01.

in Figure 4, while there was no change in wild type flies during these same times. These results suggest that XDH plays an important role in modulating the normal defense response to infection.

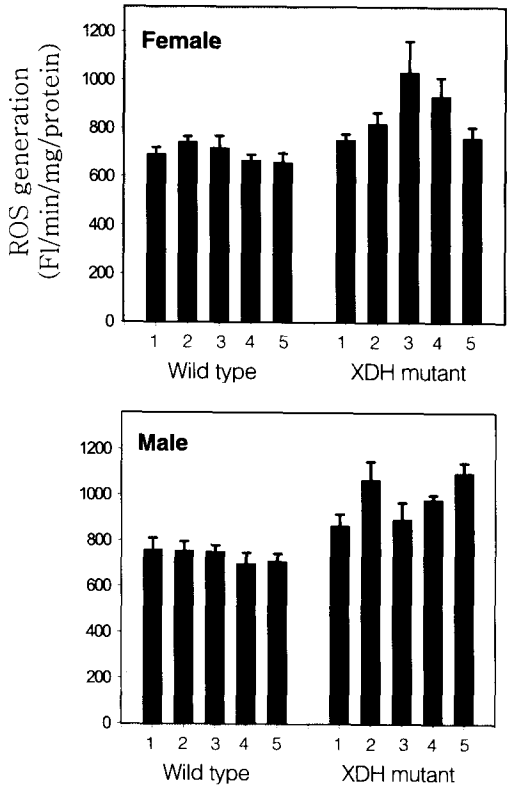

Figure 4: ROS generation of wild type and XDH mutant after bacterial infection. Values of ROS level in wild type and XDH mutant strain were measured at 30 min (lane 2), 1 hr (lane 3), 2 hr (lane 4), and 4 hr (lane 5) after bacterial infection. Lane 1 is the level of the noninfected. Averaged values of ROS generation were obtained from 7 experiments. A statistical significance between the noninfected control and the infected groups at different time is marked as *P<0.05 and **P<0.01.

To investigate the role of XDH activity in the innate immunity, we examined the sensitivity of XDH mutants to bacterial challenge. Known quantities of *E. coli* containing an Amp^{r}plasmid were introduced into adults by pricking. The defensive abilities of the wild type, the XDH deletion mutant, and the Relish mutant strain were assayed by comparing the relative levels of bacterial growth under defined conditions. Mutation in Relish have been shown to prevent normal induction of antibacterial peptide genes in response to infection (17,18,19) and so we used this mutant as a positive control. Figure 5 shows that at 24 hr after bacterial infection, the number of colony forming units (cfu) in XDH mutant, was 40 to 376 times that in wild type strain. The bacterial growth of the *Relish^{E20}* mutant was similar to that of XDH mutant, suggesting that the absence of XDH also impairs the animal's infection defense system.

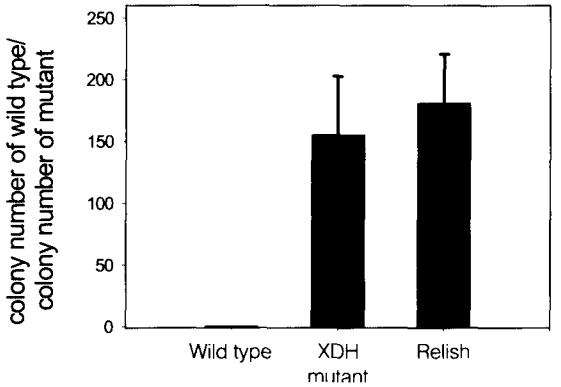

Figure 5: Sensitivity of XDH mutant against bacterial infection. Bacterial growth in wild type, XDH mutant and Relish mutant adults (2 days old, female) at 24 hr after *E. coli* infection were examined. Sensitivities of XDH mutant and Relish mutant were indicated as the ratio of the number of cfu in the mutant relative to the number of cfu in the wild type control. Averaged values obtained from 7 independent experiments are shown as error bar graph. Relish, a member of Rel family, known to participate in the response to bacterial challenge (18), is used as a positive control. A statistical significance between wild type and mutant strains is marked as *P<0.05 and **P<0.01.

Figure 6: The RT-PCR analysis of expression of various kðB-dependent antimicrobial peptide genes in *Drosophila* wild type and XDH mutant flies. At 6 hr after infection, total RNA from dissected guts was extracted with RNAzol™ reagent and first cDNA was synthesized by using First cDNA synthesis kit. The 25 mðl PCR mixture contained cDNA derived from 100 ng of total RNA, 1.25 units of *Thermus aquaticus* DNA polymerase, 200 mðM of each dNTP, 200 nM of each primer, 1.5 mM $MgCl_2$.

We also examined whether the infection induced synthesis of antimicrobial peptide genes is affected in the gut of XDH mutant flies following microbial challenge. We performed semi-quantitative RT-PCR analysis using specific primers for each gene. Figure 6 shows that, in the absence of XDH activity, immune inducibility of *drosocin* and *metchnikowin* was severely impaired whereas that of *drosomycin* and *diptericin B* was only slightly affected. Interestingly, mutant flies constitutively expressed *defensin* gene, but had no noticeable effect on the immune inducibility of *diptericin A*. These results show that XDH mediated signaling pathway is required for some, but not all, of the innate immune genes, suggesting the existence of differential ROS mediated regulatory mechanism in the induction of various antimicrobial peptide genes.

Effect of aging on the defense response to infection

In order to determine the effect of aging on the defense response to infection, we examined the ability of young, middle aged and old flies of the wild type and XDH strains to resist bacterial infection. As shown in Figure 7, the number of colony forming bacteria found after bacterial challenge in wild type flies increased in an age-associated pattern, suggesting that the effectiveness of the innate immune response of *Drosophila* normally decreases with age. There was no age-related change in the XDH mutant flies. Note, however, that the XDH mutant animals have ~40 fold higher levels of cfu relative to wild type. Taken together, these results demonstrate that there exists an aging associated deterioration of the innate immune response in normal animals, that this immune response is partly dependent on XDH activity, and that the loss of this immune response might be partly due to the loss of XDH activity in the aging process.

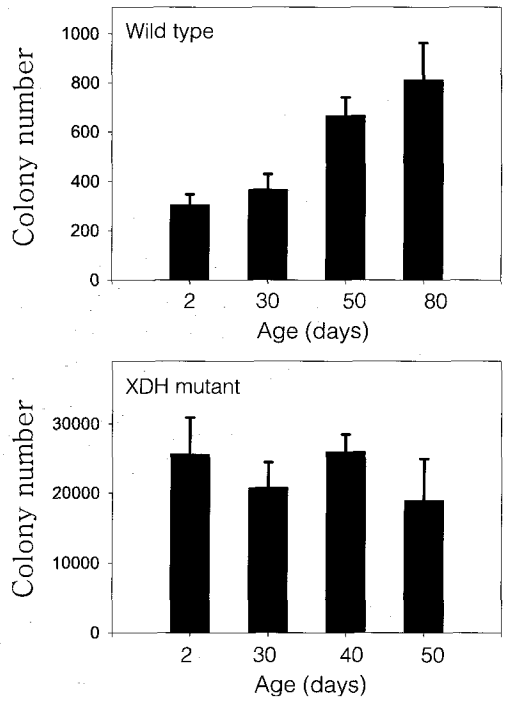

Figure 7: Effect of age on bacterial growth after *E. coli* infection in wild type and XDH mutant flies. 8 females per group at 24 hr after *E. coli* infection were homogenized in LB media and spread on LB plates containing Ampicillin. Averaged values were obtained from 5 independent experiments and are shown as the mean±standard deviation. In wild type, the number of colonies increased significantly in an age-related manner, but XDH mutant showed no significant change in colony numbers. Note the large difference in colony numbers between wild type and mutant animals. A statistical significance between young aged control and old aged groups is marked as *P<0.05 and **P<0.01.

DISCUSSION

XDH/XOD is a rate-limiting enzyme found in the nucleic acid catabolism of many animal species. Under oxidatively induced conditions, the activation of XOD is considered to be one of the major cellular sources of superoxide production (13). XDH/XOD also catalyzes the oxidation of xanthine to uric acid, which has physiologically important ROS/RNS scavenging properties that help to prevent oxidative damage (3). The higher ROS and NO levels detected in the XDH mutant compared to wild type, detected in the present study, support the idea that XDH probably plays an important defenses role against oxidative damages. The decreased anti-oxidative defenses brought about by the absence of XDH might be related to the shortened lifespan of XDH mutant flies.

Other data implicates ROS and RNS in being intimately involved in extensive inflammatory reactions. In mammals, NO has been shown to be an active molecule among free radical species, in the induction of the vasodilatory response during the inflammatory process (20,21) and large amounts of NO are produced by specific stimuli, such as bacterial lipopolysacchride or inflammatory cytokines (22,23). Bacterial LPS and hypoxia increase XDH/XOD mRNA expression and activity and the regulation of XDH/XOD is associated with injury/inflammation, in mammals (14,24). A rise of ROS generation in XDH mutant after bacterial infection was detected (Figure 4). In the analysis of bacterial growth after *E. coli* infection described above, the XDH mutant flies produced up to 200 fold more clones than wild type (Figure 5). The infection induced levels and patterns of synthesis of the antimicrobial peptide genes were affected in the gut of XDH mutant flies (Figure 6). These results suggest that XDH plays an important role in the defense response against infection and the resulting oxidative stress.

It was recently reported that XDH/XOD is phosphorylated in rat pulmonary microvascular endothelial cells (RPMEC) and that phosphorylation is greatly increased in response to acute hypoxia (25). The phosphorylation is mediated, at least in part, by p38 kinase (25). p38 participates not only in inflammatory responses but also in stress-induced signaling. p38 could exert a negative regulatory influence on the process of NF-κB activation, and eventually control the inflammatory response in mammals (26). The insect immune system appears to be similar to the vertebrate innate immunity, which

includes the activation of macrophages and the induction of acute phase response during infection and injury (27). Therefore, it will be an interesting subject to examine whether the role of XDH in defense response against infection is modulated by the p38 signaling pathway.

The age-associated dysregulation of the immune response contributes to higher incidences of infectious disease in the aged (11,12). In our data, the number of cfu of wild type in analysis of bacterial growth after *E. coli* infection was increased in an age-associated pattern. XDH mutant flies did not show the aging-associate pattern (Figure 7). These results suggest that the age-associated deterioration of the innate immune response might be due, at least in part, to the loss of XDH activity in aging process.

In conclusion, our results showed that XDH plays an important role in the fly's immune defensive response against infection. They further suggest that the age-associated deterioration of the innate immune response might be due, at least in part, to the loss of XDH activity.

MATERIALS AND METHODS

Strains

ry^{506}, a deletion mutant of approximately one-third of the rosy coding region (28), have been known to have no XDH enzyme activity (29). We also examined the XDH enzyme activity in ry^{506} strain used in this study using a fluorimetric assay (30) and confirmed that the ry^{506} strain is in fact XDH-null (data not shown). This line was used for all analyses and is referred to as the XDH mutant strain. We used *Oregon R* as a wild type strain. The ry^{506} mutant was generated in an *Oregon R* strain (31) and thus the two strains share a common genetic background. We did not otherwise make the backgrounds co-isogenic. The *Relish* mutant strain $Relish^{E20}$, which is more sensitive to infection than wild type flies (32), was used as a positive control in experiment of bacterial challenge.

Lifespan

Approximately 50 flies of wild type *(Oregon R)* or XDH mutant strain ry^{506} were maintained in standard vials containing a standard cornmeal agar medium. They were transferred to fresh vials every 3~4 days at which time the numbers of dead flies were recorded.

Measurement of total ROS generation

For determination of total ROS generation, 5 adult flies of wild type and XDH mutant strain were homogenized in homogenizing buffer (50 mM K^+ phosphate buffer, pH 7.4) and centrifuged at 12,000 rpm at 4°C for 10 min. 25 mM DCFDA (2',7'-dichlorofluoroscein diacetate) was added to the extracts and changes in fluorescence intensity were measured on a Fluorescence Microplate Reader (FL500, Bio-Teck Instruments, Winooski, USA) with excitation wavelength of 486 nm and emission wavelength of 530 nm for 1 hr (33,34).

Measurement of NO concentration

NO synthesis was measured by a microplate assay based on the Griess reagent (1% sulfanilamide/ 0.1% N-(1-naphthyl)-ethylenediamide dihydrochloride in 3 N HCl) (35,36). For each assay, 10 adult flies were homogenized and centrifuged at 12,000 rpm at 4°C for 30 min. To measure nitrite, 100 μl extracts and an equal volume of the Griess reagent were incubated at room temperature for 10 min. The absorbance at 540 nm was measured with a microplate spectrophotometer (Molecular Devices, Menlo Park, CA) and NO_2^- was quantified using $NaNO_2$ (10-50 μM) to establish a standard reference for each assay.

Bacterial infection and bacterial survival in infected animals

In bacterial challenge experiments, *E. coli* were introduced into animals by pricking (32,37). *E. coli* containing an Amp^r plasmid were cultured an OD_{600} 1.4 culture in LB. Pricked adult flies (1-2 days old) were incubated at 25°C for 24 hr. Three to five animals at the appropriate time after E. coli infections were homogenized in LB media and spread on LB plates containing Ampicillin (100 μg/ml).

RT-PCR analysis

For the RT-PCR analysis of infection-induced expression of various κB-dependent antimicrobial peptide genes in *Drosophila*, both wild type and XDH mutant flies were infected by pricking with a thin needle previously dipped into a heat-killed mixture of overnight cultures of *E. coli, M. luteus*, and *S. cerevisiae*. At 6 hr after infection, total RNA from dissected guts was extracted with RNAzol™ reagent and first cDNA was synthesized by using First cDNA synthesis kit (Qiagen) according to manufacturer's instruction. The 25 μl PCR mixture contained cDNA derived from 100 ng of total RNA, 1.25 units of *Thermus aquaticus* DNA polymerase, 200 μM of each dNTP, 200 nM of each primer, 1.5 mM $MgCl_2$. The specific primer sequences were as follows: *diptericin A* sense, 5'-ATG CAG TTC ACC ATT GCC GTC-3', and antisense, 5'-TCC AGC TCG GTT CTG AGT TG-3'; *diptericin B* sense, 5'- GCT TAT CCC TAT CCT GAT CTC C-3', antisense, 5'-CCA CCA AGG TGC TGG GCA TAC G-3'; *defensin* sense, 5'-ATG AAG TTC TTC GTT CTC G-3', and antisense, 5'-CAA TTG CGG CAA ACG CAG-3'; *attacin* sense, 5'-TTA ACC TCC AAT CCC GCT GG-3', and antisense, 5'-GCA TCC AGA TTG TGT CTG CC-3'; *drosomycin* sense, 5'-ATG ATG CAG ATC AAG TAC-3', and antisense, 5'-TTA GCA TCC TCC GCA CCA-3'; *metchnikowin* sense 5'- GAT GCA ACT TAA TCT TGG AGC G-3', and antisense, 5'-TTA ATA AAT TGG ACC CGG TCT TGG TTG G-3'; *β-actin* sense, 5'-GAT CAC CAT TGG CAA CGA-3', and antisense, 5'-TCT TGA TCT TGA TGG TCG-3' (38-42).

Statistical analysis

Statistical analysis of survival curves was carried out by using the Kaplan-Meier Survival procedure (Log-rank test). Differences between the assayed values (such as ROS generation, NO levels, and numbers of cfu) of wild type and mutants, noninfected and infected group, and

young aged and old aged group were assessed using the Student's *t*-test.

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ABBREVIATIONS

cfu	colony forming units
COX	cyclooxygenase
DCF	dichlorofluoroscein
DCFDA	2',7'-dichlorofluoroscein diacetate
NO	nitric oxide
RNS	reactive nitrogen species
ROS	reactive oxygen species
ry	rosy
XD	xanthine oxidoreductase
XDH	xanthine dehydrogenase
XOD	xanthine oxidase

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