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Impact of Ethanol Exposure on Survival and the Expression of Endogenous Antioxidants in *Drosophila melanogaster*

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Abstract

Background Ethanol, a widely consumed psychoactive substance, disrupts cellular and molecular processes, leading to a range of adverse physiological effects. Prolonged exposure to ethanol has been associated with severe neurological disorders, underscoring the importance of investigating its toxicological impact. This study aims to evaluate the effects of ethanol exposure on the phenotypical characteristics of Drosophila melanogaster, with a particular focus on survival and the expression of endogenous antioxidant-related genes.

Methods The study utilized an ethanol exposure assay on male w1118 flies, assessing survival and the expression of the sod1, sod2, and cat genes through reverse transcriptase quantitative PCR (RT-qPCR).

Results Ethanol exposure negatively affected survival in a concentration-dependent manner, with lower survival rates observed in Drosophila groups exposed to higher ethanol concentrations. Additionally, molecular analysis highlighted the significant role of the endogenous antioxidant gene cat in promoting survival in Drosophila under ethanol exposure.

Conclusion The findings of this study indicate that ethanol exposure is lethal to D. melanogaster in a concentration-dependent manner, with catalase playing a key role in mitigating its toxic effects and enhancing fly survival.

Keywords: Ethanol; fruit flies; toxicity; antioxidants; catalase

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Introduction

Ethanol, a widely used psychoactive agent, has profound effects on various biological systems (Almeida-Gonzalez et al., 2022). Its disruption of cellular and molecular processes can lead to adverse physiological outcomes, especially at high concentrations. Ethanol's toxicological impact is well-documented, including hepatotoxicity (Boby et al., 2021), neurotoxicity (Fernandes et al., 2017), and damage to other organ systems (Obad et al., 2018). Prolonged ethanol exposure is particularly linked to severe neurological disorders and behavioral dysfunctions, highlighting its significance as a critical area of research to better understand its broad effects on living organisms.

Model organisms are essential in scientific research, especially in the fields of toxicology and molecular biology. Mammalian models have been extensively used due to their relevance in understanding complex biological processes within simplified systems. Despite evolutionary differences from humans, many molecular pathways and physiological mechanisms—such as those involved in stress responses, detoxification, and the metabolism of toxic compounds—are highly conserved across species. However, the traditional reliance on rodent models, though

valuable, is often constrained by high costs and labor-intensive demands. This has led to a growing shift towards the use of simpler, more cost-effective model organisms (Doke & Dhawale, 2015).

Drosophila melanogaster, commonly referred to as the fruit fly, is one of the most extensively utilized and powerful simple model organisms in pharmacological and toxicological research (Huang & Lee, 2023; Rand et al., 2023; Rumata et al., 2023). This model, with its short life cycle, ease of handling, and genetic versatility, are increasingly favored for preliminary toxicity studies (Rand et al., 2023). *Drosophila* exhibits a significant degree of genetic homology with humans, including genes involved in stress responses signaling pathways (Hardiyanti et al., 2024; Zou et al., 2000), which are relevant to ethanol exposure. Its well-characterized genome and the availability of genetic tools make it an ideal candidate for investigating both the behavioral and molecular effects of ethanol (Rand et al., 2023). Furthermore, its short lifespan and rapid reproductive cycle enable the study of ethanol-toxicity in a relatively short time frame (Moraes & Montagne, 2021; Rand et al., 2023).

This study aims to investigate the impact of ethanol exposure on the phenotypic traits of *D. melanogaster*. By assessing parameters such as survival rates and the expression of genes associated with oxidative stress, we seek to enhance our understanding of ethanol's effects on metazoans. The findings of this research are expected to provide valuable insights into the toxicity of volatile compounds and highlight the utility of *D. melanogaster* as an efficient, cost-effective, and scalable model for laboratory-based toxicological research.

Methods

Drosophila Stock. This study utilized male *w1118 D. melanogaster* (stock provided by the Laboratory of Host Defense and Responses at Kanazawa University), aged 5–7 days. The flies were bred and maintained in culture vials containing standard fly food and were kept at 25°C during the experiment.

Ethanol Preparation. A series of ethanol concentrations was prepared from a 96% ethanol stock solution, which was diluted with water to achieve final concentrations of 85%, 65%, 45%, 25%, and 5% ethanol.

Ethanol Exposure and Survival Assay. The vials and stopper plugs were arranged as shown in **Figure 1**. A total of 10 *Drosophila* were placed into each vial and sealed with a plug. For each ethanol concentration, 1000 µL of ethanol was applied to the top of the plug, which was then covered with an additional plug. Survival was monitored over a 60-minute period by counting the number of flies that remained alive. During the observation period, each vial was tapped three times at 10-minute intervals. (Sandhu et al., 2015).

Gene Expression Analysis. RNA was isolated from five flies from each treatment group and transferred into Treff tubes. Extraction was performed using the Total RNA Isolation System (Promega), following the manufacturer's protocol. The expression levels of *sod1*, *sod2*, and *cat* genes were quantitatively assessed using the reverse transcriptase quantitative PCR (RT-qPCR) method. RT-qPCR analyses were conducted using forward and reverse primers for *sod1*, *sod2*, and *cat* (**Table 1**) in a 10 µL reaction volume, employing the GoTaq® 1-Step RT-qPCR System (Promega) according to the manufacturer's instructions. RNA levels of the host ribosomal protein *rp49* (used as an internal control) were assessed using a separate set of *rp49* primers (**Table 1**). The RT-qPCR was performed with a Rotor-Gene Q thermal cycler (Qiagen, Germany) under the following conditions: (1) RT activation at 37°C for 15 minutes, (2) RT inactivation at 95°C for 10 minutes, (3) Denaturation at 95°C for 10 seconds, (4) Annealing at 60°C for 30 seconds, and (5) Extension at 72°C for 10 seconds, conducted for 40 cycles.

Data Processing and Analysis. The data obtained from the survival tests were analyzed using the Log-Rank test. Gene expression data were analyzed using One-Way ANOVA with post hoc Tukey's test, utilizing GraphPad Prism® 9.

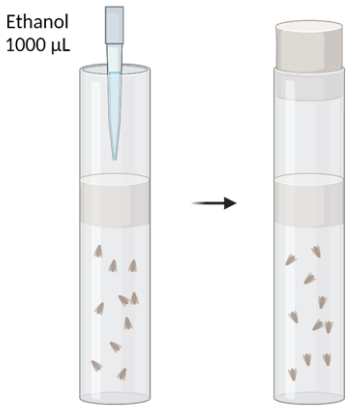

Figure 1. Schematic Representation of the Experimental Design for Ethanol Exposure in *Drosophila*.

Result and Discussion

This study aimed to evaluate the effects of ethanol exposure on *Drosophila* using a straightforward method (Sandhu et al., 2015). Phenotypic analysis indicated that ethanol exposure negatively impacted survival in a concentration-dependent manner. Higher ethanol concentrations resulted in increased acute mortality rates in *D. melanogaster* (**Figure 2**), similar to what we have observed previously (Rosa et al., 2021).

Earlier studies have demonstrated that ethanol is metabolized in *Drosophila* via the enzyme

alcohol dehydrogenase (Nunez et al., 2023). This metabolic pathway generates free radicals, leading to the induction of oxidative stress (Das & Vasudevan, 2007), which may contribute to the acute toxicity observed in Drosophila.

Table 1. Primer sequences used in RT-qPCR

Genes	Primer Sequences	
	Forward	Reverse
sod1	5'–CTCTTGGAGTCCGAGATCCGCAA–3'	5'-GGGACCGCGAGCTGATGGTT–3'
sod2	5'–CCGCCGAGAACCTTATTGTG–3'	5'–AGAATCAACATGTGCTCGGC–3'
cat	5'–TTCCTGGATGAGATGTCGCACT–3'	5'–TTCTGGGTGTGAATGAAGCTGG–3'
rp49	5'–GACGCTTCAAGGGACAGTATCTG–3'	5'–AAACGCGGTTCTGCATGAG–3'

Under normal conditions, oxidative stress is managed by endogenous antioxidant systems (Jomova et al., 2024). Key components of this defense mechanism include Superoxide Dismutases (SODs) and Catalase (Cat), which play crucial roles in the dismutation of superoxide radicals ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) (Jomova et al., 2024). To investigate the mechanisms underlying ethanol exposure acute toxicity in *Drosophila*, we conducted molecular assays to examine the expression levels of endogenous antioxidants *sod1*, *sod2*, and *cat* using the RT-qPCR method.

According to **Figure 3**, molecular analysis revealed that ethanol exposure did not significantly alter the expression of the endogenous antioxidant genes *sod1* and *sod2* (**Figures 3A and 3B**). However, it significantly affected the *cat* gene (**Figure 3C**), with expression levels of *cat* decreasing as ethanol concentration increased. Similar findings have been reported in mice, where high ethanol concentrations inhibit catalase activity (Das & Vasudevan, 2005).

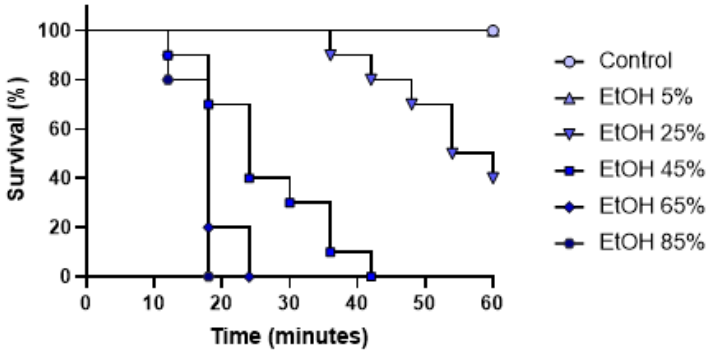

Figure 2. Effect of ethanol exposure on *Drosophila* survival. Ethanol exposure resulted in a concentration-dependent decrease in survival rates of *Drosophila*.

Interestingly, the data shown in **Figure 2** indicate that *Drosophila* exposed to low ethanol concentrations (5%) exhibit a survival rate comparable to the control group. However, molecular assays revealed a significant increase in catalase activity in this group (**Figure 3C**). This finding suggests that catalase, which is typically inactive under normal conditions, may be activated by sustained exposure to low concentrations of ethanol, thereby mitigating ethanol-induced toxicity in *Drosophila*. In contrast, high ethanol concentrations likely cause direct cellular damage, leading to decreased catalase activity and reduced survival.

Overall, the findings of this study indicate that ethanol exposure is lethal to *Drosophila melanogaster* in a concentration-dependent manner. Specifically, higher concentrations of ethanol lead to increased mortality rates among the flies, demonstrating the acute toxicity of ethanol. Molecular analyses reveal that catalase, an important endogenous antioxidant, plays a crucial role in mitigating the toxic effects of ethanol. The expression levels of the *cat* gene, which encodes catalase, decrease with increasing ethanol concentration, suggesting that ethanol may inhibit catalase activity or reduce its expression. This reduction in catalase activity is associated with decreased survival rates of *Drosophila*. Conversely, lower ethanol concentrations seem to activate catalase, potentially providing a protective effect and thereby enhancing fly survival.

A limitation of this study is that we did not conduct a comprehensive genomic analysis of *Drosophila* to identify the full range of potential toxicity mechanisms. Our investigation focused on evaluating the expression of specific genes related to oxidative stress and toxic responses, providing initial insights into ethanol toxicity. However, by not analyzing the entire genome, we may have overlooked complex interactions between various molecular pathways involved in the organism's response to ethanol exposure.

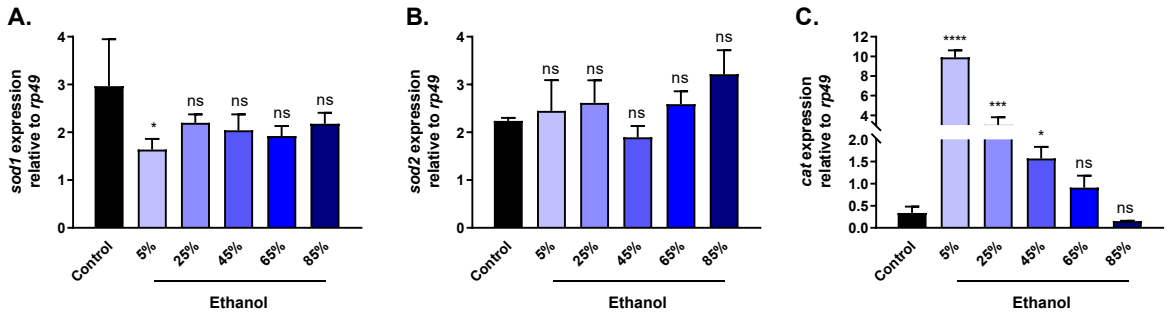

Figure 3. Effect of ethanol exposure on the expression of endogenous antioxidants in *Drosophila*. Ethanol did not significantly alter the expression of the *sod1* (A) and *sod2* (B) genes, but it significantly affected the expression of the *cat* gene (C). *: P≤0.05; ***: P≤0.001; ****: P≤0.0001; ns: P>0.05.

Conclusion

This study highlights the utility of *D. melanogaster* as a promising model for assessing the toxicity of volatile compounds. Our findings demonstrate that inhalation of ethanol decreases Drosophila survival, likely due to its influence on catalase antioxidant activity. This research is expected to contribute important knowledge on the toxicity of volatile compounds, utilizing an efficient, cost-effective, and scalable model for toxicological studies in laboratory settings.

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