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Phenotypical analysis of Chloramphenicol toxicity in *Drosophila*

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Abstract

Background: Chloramphenicol is a broad-spectrum antibiotic with serious side effects, including aplastic anemia and gray baby syndrome. While Drosophila melanogaster is a cost-effective and genetically relevant model for toxicological studies, its response to chloramphenicol remains unexamined. This study explores the toxic effects of chloramphenicol in Drosophila to provide insights into its broader biological impact.

Methods: This study aims to analyze the toxicity of chloramphenicol in terms of developmental toxicity, locomotor activity, morphology and gene expression status (sod1, sod2, tom40, and indy) in Drosophila melanogaster. The study was conducted on Oregon-R strain D. melanogaster larvae fed with chloramphenicol at concentrations of 625; 1,875; 3,125; and 4,375 ppm.

Results: Developmental toxicity assay revealed that chloramphenicol exposure significantly delayed developmental progression, as evidenced by a prolonged transition from larval to pupal stages at concentrations of 3,125 and 4,375 ppm. However, no significant alterations were observed in locomotor activity or morphological characteristics. Moreover, chloramphenicol exposure in D. melanogaster appeared to exert toxic effects by significantly altering the expression of sod1, sod2, and tom40, while having no detectable impact on indy gene expression.

Conclusion: High concentrations of chloramphenicol significantly impair larval development in D. melanogaster and alter gene expression profiles. However, adult flies exhibit no observable morphological or locomotor abnormalities compared to controls. These findings highlight that Drosophila larvae are susceptible to chloramphenicol toxicity, making it an ideal phase for assessing the detrimental effects of chloramphenicol.

Keywords: Chloramphenicol; toxicity; fruit fly; phenotype; sod1; sod2; tom40; indy

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Introduction

Chloramphenicol is a broad-spectrum antibiotic that was first isolated from *Streptomyces venezuelae* in 1947 in Venezuela and has been used clinically since 1949 (Abdollahi & Mostafalou, 2014). Chloramphenicol was initially used for the treatment of typhoid fever, but its use began to decline due to it being considered an old drug and the emergence of *Salmonella enterica* serovar *Typhi* resistant to chloramphenicol. However, because of its low cost and broad-spectrum coverage, chloramphenicol is still included in the World Health Organization's List of Essential Medicines (Gude, 2020). Chloramphenicol is bacteriostatic but can also be bactericidal at high concentrations or when used against highly susceptible microorganisms. Chloramphenicol inhibits protein synthesis and is used to treat surface eye infections such as conjunctivitis and otitis externa, cholera, and typhoid fever. (Oong & Tadi, 2023)

In the United States, chloramphenicol initially

phased out of common use due to concerns over its potential hematopoietic toxicity, which can lead to aplastic anemia. However, its continued application as an antibiotic persists, as the therapeutic benefits are deemed to outweigh the associated risks (Eric, 2007). In addition to aplastic anemia, gray baby syndrome is a well-documented and severe adverse effect of chloramphenicol toxicity in neonates (Syed et al., 2021). In a study involving 64 neonates treated with chloramphenicol, 10 exhibited clinical signs associated with toxicity (Grayson et al., 2017). Moreover, chloramphenicol is a known contributor to aplastic anemia, accounting for 44% of drug-induced cases and 22% of all reported cases between 1950 and 1965 (Maluf et al., 2009). Its incidence among treated patients is estimated at 1 in 20,000 (Syed et al., 2021).

Previous studies on chloramphenicol toxicity have primarily relied on animal models such as rats, rabbits, and monkeys. However, these investigations have mainly focused on evaluating the teratogenic and acute effects of chloramphenicol, without conducting a comprehensive analysis of its broader toxicological impacts (Czeizel et al., 2000). Additionally, these animal models present several challenges, including high costs, ethical concerns, slow reproduction rates, and long lifespans. To address these limitations, we propose the use of the invertebrate model organism *Drosophila melanogaster* (fruit fly). The fruit fly is a promising alternative due to its cost-effectiveness, ethical advantages, and significant genetic similarity to mammals (Pandey & Nichols, 2011; Pratama et al., 2024). The short lifespan and rapid reproduction of *Drosophila* allow for large-scale toxicity studies to be conducted more efficiently and rapidly than in mammalian models (Cho et al., 2024; Rand et al., 2014). Although chloramphenicol has demonstrated antibacterial properties in infected *D. melanogaster* (Mudjahid et al., 2022; Sultan et al., 2001), no studies have yet explored the mechanisms and toxic effects of chloramphenicol in this model organism.

This study aims to investigate the toxic effects of chloramphenicol on *D. melanogaster* by analyzing developmental toxicity, locomotor activity, morphological changes, and gene expression profiles of key genes related to oxidative stress and metabolism (*sod1*, *sod2*, *tom40*, and *indy*). By utilizing *D. melanogaster*, we seek to provide a deeper understanding of the mechanisms underlying chloramphenicol toxicity in a cost-effective and ethically advantageous system. This approach not only addresses the limitations associated with traditional animal models but also offers new insights into the broader toxicological impacts of chloramphenicol, particularly during the larval stage, which has been identified as a critical phase for assessing its detrimental effects.

Method

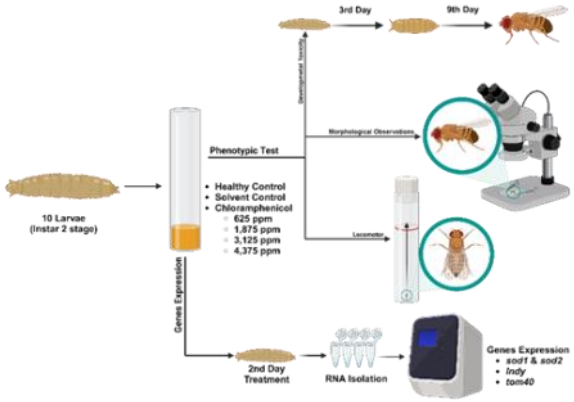

Figure 1. Experimental design for phenotypic analysis in chloramphenicol-treated *D. melanogaster*.

Drosophila Larvae Stock. This study utilized second-instar larvae of *D. melanogaster* genotype Oregon R. The larvae were maintained as stock in culture vials containing standard fly food and were kept at 25°C.

Preparation of Fly Food Supplemented with Chloramphenicol. The fly food was prepared with varying concentrations of chloramphenicol. A stock solution of chloramphenicol was created at 50,000 ppm by dissolving 500 mg of Chloramphenicol (Sigma-Aldrich®) in 10 mL of 70% ethanol (Onemed®). This stock solution was subsequently incorporated into the feed, the composition of which is detailed in Table 1.

Table 1. Composition of Treatment

Composition in each vial	HC	SC	CHL (ppm)			
			625	1,875	3,125	4,375
Corn meal[1] (mg)	375	375	375	375	375	375
Yeast[2] (mg)	125	125	125	125	125	125
Agar[3] (mg)	45	45	45	45	45	45
Glucose[4] (mg)	225	225	225	225	225	225
Propionic acid[5] (µl)	20	20	20	20	20	20
MP[6] (µl)	22.5	22.5	22.5	22.5	22.5	22.5
CHL 50.000 ppm (µl)	-	-	62.5	187.5	312.5	437.5
70% ethanol[7] (µl)	-	437.5	-	-	-	-
Water (ml)	Ad 5	Ad 5	Ad 5	Ad 5	Ad 5	Ad 5

Elken Healthy Food, Nawasagena Pangan Kreatif, Indonesia; [2] Brewer's yeast, Health Paradise Sdn, Malaysia; [3] Swallow®, PT Dunia Bintang Walet, Indonesia; [4] Gulaku®, PT Sweet Indolampung, Indonesia; [5] Merck, Germany; [6] Techno Pharmchem, India; [7] onemed® PT. Jayamas Medica Industri Tbk, Indonesia. (Note: HC= healthy control; SC= solvent control; MP= Methylparaben; CHL= Chloramphenicol)

Experimental Design. The study involved six experimental groups: a healthy control, a

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solvent control, and four groups exposed to chloramphenicol at feed concentrations of 625; 1,875; 3,125; and 4,375 ppm. Each group was replicated five times, with ten second-instar *D. melanogaster* larvae placed in each vial. The larvae were allowed to consume their respective diets for two days before RNA isolation. Three phenotypic tests were conducted: developmental toxicity, locomotor assessment, and morphological observation. The developmental toxicity test evaluated the number of larvae that pupated by the third day and the number of pupae that emerged as adult flies by the ninth day. The locomotor test involved placing adult flies from each exposed group into empty vials (Biologix®) marked with a 5 cm line; the number of flies that successfully crossed the line within 10 seconds was recorded. Morphological observations were performed on the eighth day by visually inspecting the eyes, wings, thorax, and legs of adult flies across all six groups. Additionally, molecular analysis was conducted by assessing the expression levels of the *sod1*, *sod2*, *tom40*, and *indy* genes (**Figure 1**).

Gene Expression Analysis. RNA samples were extracted from ten live *D. melanogaster* larvae per treatment group using the PureLink™ RNA Mini Kit (Invitrogen™). Gene expression levels were analyzed via reverse transcriptase quantitative PCR (RT-qPCR). RT-qPCR was performed using specific primer sets to amplify *sod1*, *sod2*, *tom40*, and *indy*, with a total reaction volume of 10 µL per PCR tube. The ribosomal protein gene (*rp49*) served as an internal control. The PCR amplification was conducted for 40 cycles consisted of the following steps: (1) initial hold at 37°C for 15 minutes, (2) second hold at 95°C for 10 minutes, (3) denaturation step at 95°C for 10 seconds, (4) annealing step at 60°C for 30 seconds, and (5) extension step at 72°C for 30 seconds.

Table 2. Sequences of primer sets used in this study (Asfa et al., 2023; Cassar et al., 2015)

Genes	Primer Sequences	
	Forward	Reverse
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'
tom40	5'– TGC ACG TGT GCT ACT ACC AG – 3'	5'– ATT CCG CCT CTG AAG ACC AG – 3'
indy	5'– CTG CCC AAC TCT GTC CTC TTA CTG – 3'	5'– CAG GAT CAG GTA CAG AGG ATG GAT – 3'
rp49	5'– GAC GCT TCA AGG GAC AGT ATC TG – 3'	5'– AAA CGC GGT TCT GCA TGA G – 3'

Data Processing and Analysis. The gene expression data were analyzed using Qiagen software, followed by statistical evaluation using one-way ANOVA with Dunnett's post hoc test. For the developmental toxicity test, data processing to generate the developmental delay curve was conducted using both one-way ANOVA and two-way ANOVA in GraphPad Prism® 9.

Result and Discussion

The phenotypic characteristics of the model organism *D. melanogaster* serve as key indicators for assessing the effects of a substance by examining its morphology and behavior. These characteristics include lifespan extension or reduction, alterations in developmental stages, and behavioral changes (Panchal & Tiwari, 2017; Pandey & Nichols, 2011). Various phenotypic assays are commonly employed in our lab to study fly development and evaluate the impact of compounds on *D. melanogaster*, including developmental toxicity tests, locomotor assessments, and morphological observations (Khaerani et al., 2024; Rumata et al., 2023).

We first assessed the developmental toxicity of chloramphenicol in *Drosophila* larvae. The results showed no significant difference in larval development between the healthy control and solvent control groups (**Figure 2A–2B**), indicating that the solvent used in the study did not interfere with larval progression to the pupal and adult stages. Additionally, larvae exposed to chloramphenicol at 625 and 1,875 ppm exhibited no significant developmental differences compared to the solvent control. However, treatment with chloramphenicol at 3,125 and 4,375 ppm significantly reduced the number of larvae developing into pupae compared to the solvent control (**Figure 2A**). Despite this, the transition from pupae to adult flies remained unaffected at all chloramphenicol concentrations (**Figure 2B**).

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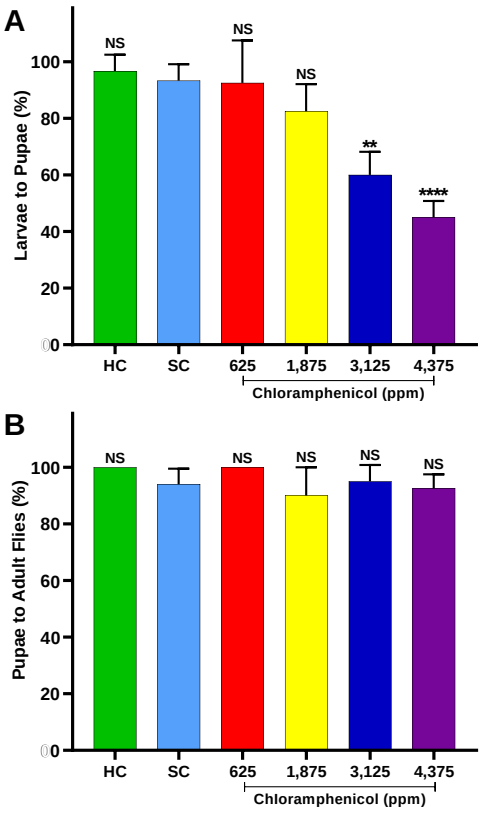

Figure 2. Development of *Drosophila melanogaster.* (A) larval transition to pupae on day 3 and (B) pupal transition into adult flies on day 9 post-treatment. HC, healthy control; SC, solvent control; NS, non-significant; **, $p < 0.01$; ****, $p < 0.0001$.

The observed developmental toxicity of chloramphenicol in *D. melanogaster* suggests a dose-dependent effect on larval progression. While lower concentrations (625 and 1,875 ppm) did not significantly impact larval development, higher concentrations (3,125 and 4,375 ppm) notably reduced the number of larvae transitioning into pupae. This finding aligns with previous studies indicating that high-dose antibiotic exposure can disrupt developmental processes, potentially by interfering with mitochondrial function, protein synthesis, or hormonal regulation essential for metamorphosis (Suárez-Rivero et al., 2021). However, the absence of a significant effect on pupal-to-adult transition suggests that chloramphenicol primarily affects early-stage development rather than later metamorphic processes.

The lack of developmental impairment at the adult stage, despite significant larval toxicity at higher doses, may be attributed to compensatory mechanisms that mitigate the adverse effects of chloramphenicol during later developmental stages. One possible explanation is the differential sensitivity of larval and pupal tissues to chloramphenicol exposure, where early-stage development is more vulnerable due to higher metabolic demands and active cellular proliferation (Merkey et al., 2011). Additionally, the results indicate that the solvent used in this study did not interfere with developmental outcomes, reinforcing the reliability of the observed chloramphenicol effects. Further investigations into the molecular mechanisms underlying this selective developmental toxicity, particularly its impact on mitochondrial activity and stress response pathways, could provide deeper insights into the specific mode of action of chloramphenicol in *D. melanogaster*.

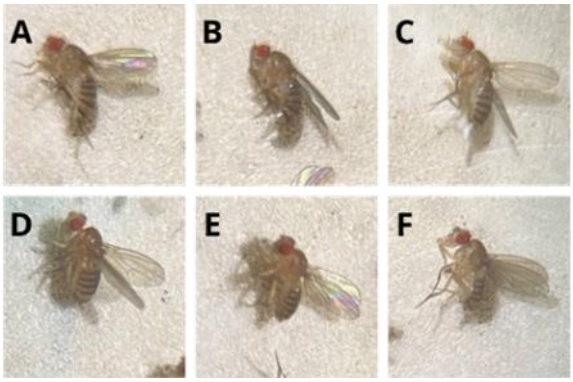

Figure 3. Morphology of adult *Drosophila melanogaster* from each test group. (A) healthy control; (B) solvent control; chloramphenicol at concentration of (C) 625 ppm; (D) 1,875 ppm; (E) 3,125 ppm; (F) 4,375 ppm.

We next observed the morphology of adult flies developed in each treatment groups to confirm whether the absence of developmental impact to pupal-adult flies' phase did not results in morphological problems. As displayed in **Figure 3** no distinguishable differences were observed among the treatment groups. All flies exhibited similar characteristics in terms of body color, size, and the shape of their eyes, wings, and legs compared to the healthy and solvent control groups. Previous research has indicated that antibiotics, including chloramphenicol, can inhibit organismal development, as demonstrated in studies involving various model organisms such as chicken embryos, zebrafish, and rabbits (EFSA, 2014). Similarly, (Slykerman et al., 2023), reported that children exposed to antibiotics before three months of age were 2.24 times more likely to experience growth delays compared to those exposed after twelve months of age. The pupal stage in *D. melanogaster* represents a critical period of metamorphosis, during which extensive tissue remodeling occurs between the larval and adult stages (Merkey et al., 2011). Previous study has shown that during this stage, significant cellular changes take place, including the destruction or restructuring of certain tissues and organs in insects such as *D. melanogaster* (Rolff et al., 2019).

The locomotor test results (**Figure 4**) revealed no significant differences between any of the chloramphenicol-treated groups and the solvent control. This suggests that chloramphenicol exposure does not substantially affect locomotor

function, as movement performance remained consistent across all treatment conditions. The locomotor assay is a widely used approach for evaluating movement disorders in various disease model organisms, including *D. melanogaster*. As behavior is an essential aspect of an organism's life, it can be influenced by genetic background as well as environmental and ecological factors. Furthermore, it has been reported that locomotor performance is closely associated with neuromuscular activity and cognitive functions such as memory (Panchal & Tiwari, 2017).

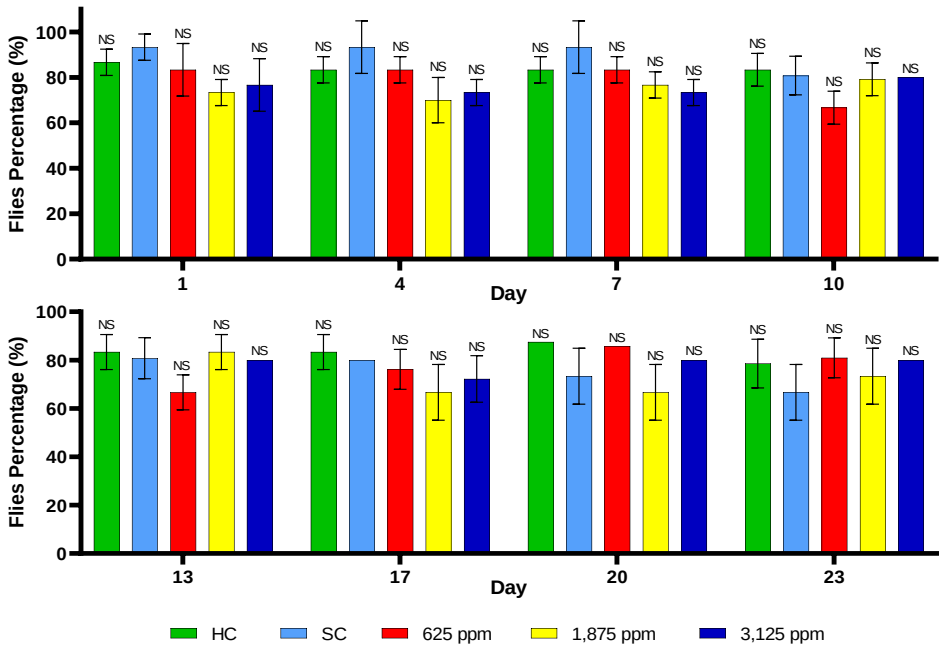

Figure 4. Locomotor activity of *Drosophila melanogaster* in the presence and absence of chloramphenicol. HC, healthy control; SC, solvent control; NS, non-significant

The absence of locomotor impairment following chloramphenicol exposure suggests that, at the tested concentrations, the antibiotic does not interfere with neuromuscular coordination or motor function in *D. melanogaster*. This finding is particularly relevant as locomotor assays are frequently used to assess neurotoxicity and muscle-related dysfunctions in various model organisms (Panchal & Tiwari, 2017). Given that previous studies have reported potential adverse effects of antibiotics on mitochondrial function and neuronal health (Suárez-Rivero et al., 2021), the current results indicate that chloramphenicol does not exert significant neurotoxic effects within the tested dose range. However, while locomotor activity remained unaffected, other behavioral and physiological aspects, such as cognitive function or metabolic stress, may still warrant further investigation. Additional analyses, including neurodegenerative markers and oxidative stress assessments, could provide deeper insights into the broader effects of chloramphenicol exposure in *D. melanogaster*.

Morphological assessment is a key component of phenotypic plasticity studies, particularly in *D. melanogaster*, where observable traits can be easily examined (Klingenberg, 2010). This makes the fruit fly an excellent model for studying developmental responses to environmental factors, including drug exposure (Rand, 2010). However, in this study, neither locomotor activity nor morphological traits were significantly altered across the treatment groups. This suggests that chloramphenicol does not adversely affect the external morphology or motor function of *D. melanogaster*, at least within the tested concentration range. These findings indicate that while chloramphenicol may influence developmental progression at higher doses, it does not appear to cause structural deformities or impair locomotor function in adult flies. Further investigations focusing on internal physiological and molecular changes could provide deeper insights into the potential sublethal effects of chloramphenicol exposure.

Finally, we conducted experiments to determine whether the observed developmental changes were associated with alterations in the expression of genes involved in endogenous antioxidant defense and lifespan regulation. Gene expression analysis (**Figure 5**), compared to the solvent control, revealed a significant decrease in *sod1* (**Figure 5A**) and *sod2* (**Figure 5B**) expression at chloramphenicol concentrations of 1,875, 3,125, and 4,375 ppm. Additionally, *tom40* expression (**Figure 5C**) showed a significant reduction at 1,875 ppm but increased at 4,375 ppm, while *indy* expression (**Figure 5D**) remained unchanged across all treatment groups.

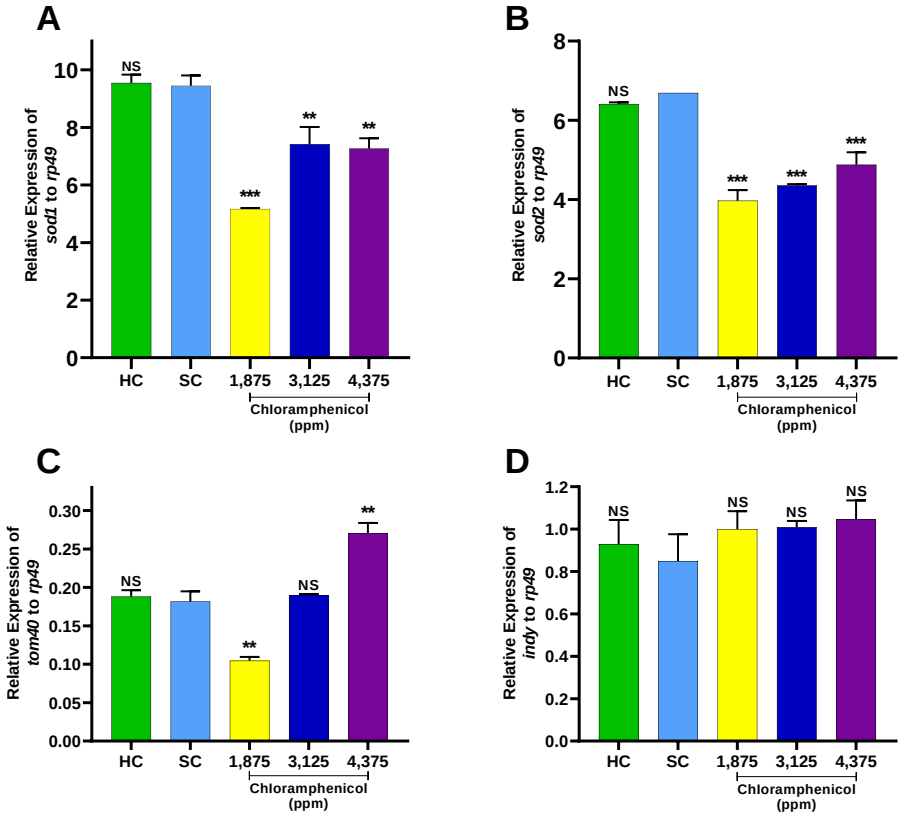

Figure 5. Gene expression analysis of (A) *sod1*, (B) *sod2*, (C) *tom40*, and (D) *indy* in *Drosophila melanogaster* larvae following two days of chloramphenicol exposure. HC, healthy control; SC, solvent control; NS, non-significant; **, $p < 0.01$; ***, $p < 0.001$

To assess the toxic effects of chloramphenicol on *D. melanogaster*, the expression of several genes was analyzed, including the superoxide dismutase genes (*sod1* and *sod2*), translocase of outer membrane 40 (*tom40*), and *I'm not dead yet* (*indy*). The *sod* genes play a crucial role in the initial defense against reactive oxygen species (ROS) by catalyzing the dismutation of superoxide anions (O_2^-) into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2), thereby reducing oxidative damage (Ighodaro & Akinloye, 2018).

The *tom40* gene encodes a channel-forming subunit of the translocase of the outer mitochondrial membrane (TOM) complex, which facilitates protein import into mitochondria (Humphries et al., 2005). Meanwhile, the *indy* gene encodes a Krebs cycle intermediate transporter, primarily expressed in the intestine and oenocytes of *Drosophila*. This gene is involved in energy metabolism, and its downregulation has been linked to increased longevity and metabolic regulation (Mishra et al., 2021).

Gene expression analysis revealed no significant differences between the healthy control and solvent control groups, confirming that the observed changes were specifically due to chloramphenicol exposure rather than other external factors. A significant reduction in *sod1* expression was observed at chloramphenicol concentrations of 1,875, 3,125, and 4,375 ppm compared to the solvent control (**Figure 5A**). Given that *sod1* is a key enzyme in ROS detoxification, its decreased expression suggests cellular damage, which may impair its production as a response to heightened oxidative stress. A similar phenomenon was reported by (Páez et al., 2008), who found that chloramphenicol exposure significantly increased ROS production and decreased superoxide dismutase (SOD) activity at 32 µg/ml, thereby compromising the cell's first line of defense against ROS.

Similarly, *sod2* expression was significantly reduced at chloramphenicol concentrations of 1,875, 3,125, and 4,375 ppm, with no compensatory increase in expression observed (**Figure 5B**). The differential response between *sod1* and *sod2* may be attributed to their distinct cellular localizations: *sod1* is primarily cytoplasmic, while *sod2* is localized in the mitochondrial matrix (Eleutherio et al., 2021).

The *tom40* gene, a core component of the mitochondrial outer membrane translocase complex, plays an essential role in mitochondrial protein import, biogenesis, and autophagy (Liu et al., 2018). At 1,875 ppm chloramphenicol, a reduction in *tom40* expression was observed (**Figure 5C**), suggesting potential disruptions in mitochondrial protein translocation. Since *tom40* is integral to mitochondrial function, its downregulation may lead to mitochondrial

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biogenesis defects (Pitt & Buchanan, 2021). Interestingly, at 4,375 ppm chloramphenicol, *tom40* expression increased, which may indicate a stress-induced compensatory mechanism. According to (Periasamy et al., 2022), elevated *tom40* levels have been linked to increased cell death, likely due to excessive ROS release and mitochondrial dysfunction. Impairments in mitochondrial permeability and function can elevate ROS levels and lead to DNA mutations (Krittika & Yadav, 2019).

The *indy* gene, which regulates energy homeostasis and metabolism, did not exhibit significant expression changes across treatment groups (**Figure 5D**). This suggests that chloramphenicol does not directly affect the *indy*-mediated metabolic regulatory pathway. Given that reduced *indy* expression has been associated with lifespan extension and metabolic adaptation (Mishra et al., 2021), its stability across treatment groups implies that chloramphenicol-induced toxicity does not influence this specific metabolic mechanism.

Overall, gene expression analysis suggest that chloramphenicol exposure disrupts oxidative stress regulation and mitochondrial function in *Drosophila melanogaster*, as evidenced by significant changes in *sod1, sod2,* and *tom40* expression. However, the *indy* gene remains unaffected, indicating a selective impact of chloramphenicol on cellular pathways involved in ROS defense and mitochondrial homeostasis.

Conclusion

This study reveals that high concentrations of chloramphenicol can hinder the development of *D. melanogaster* larvae and influence the expression of several genes during this stage. However, it is noteworthy that larvae that successfully matured into adult flies displayed no morphological abnormalities or locomotor impairments compared to the control groups. Thus, the larval stage presents the most pronounced toxic effects, making it a suitable model for investigating chemical toxicity mechanisms. The heightened sensitivity of larvae underscores their potential for future toxicological research.

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