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A fruit fly-based approach to unraveling enteropathy-causing pharmaceuticals

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Abstract

Enteropathy is a gastrointestinal disorder characterized by inflammation in the small intestine and one of the causes of enteropathy is the side effects of certain drugs, such as non-steroidal anti-inflammatory drugs (NSAIDs). The mechanism of NSAIDs, such as indomethacin, could inhibit prostaglandin synthesis, leading to a decrease in mucus production and small intestine integrity. To test the effects of a drug, it is necessary to undergo preclinical testing using animal models. Commonly used animal models such as mice and rats have several drawbacks including high cost, ethical issues, and long lifespan. Therefore, alternatives such as using invertebrate animals like *Drosophila melanogaster* as a more economical in vivo platform with genetic similarity to mammals and devoid of ethical concerns are needed. The aim of this study was to evaluate *Drosophila melanogaster* as an in vivo model organism in testing the side effects of pharmaceuticals that cause enteropathy. In this study, flies aged 3–5 days were starved and then placed into treatment vials comprising untreated control and indomethacin-treated (3.75 mM, 7.5 mM, and 15 mM). Survival analysis was conducted during the treatment period, followed by a Smurf assay test after seven days of treatment. Subsequently, the expression of pro-inflammatory cytokine-related genes (*drs* and *totA*), mitochondria stability-related genes (*tom40*), and endogenous antioxidant-related genes (*sod1, sod2,* and *cat*) was performed using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Our data indicated that indomethacin did not impact lifespan or cause intestinal damage. However, we observed increased expression of pro-inflammatory cytokine-related genes, including *drs*, and a twofold increase in *totA* gene expression. Furthermore, there was a significant upregulation of mitochondrial stability gene *tom40*, endogenous antioxidant genes *sod1* and *cat*, and a threefold increase in *sod2* at 15 mM indomethacin. Although no phenotypical changes in gut integrity were detected, the increased expression of pro-inflammatory cytokine genes suggests the occurrence of inflammation in the indomethacin-treated flies.

Keywords: Intestine, enteropathy, NSAID, fruit fly, Smurf assay

Introduction

Enteropathy is a disorder occurring in the small intestine that exhibits numerous pathological characteristics such as villous atrophy, crypt hyperplasia, increased permeability, inflammatory

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cell infiltration, and malabsorption that could affect growth and disrupt nutrient intake [1]. Enteropathy was not considered a serious medical condition at first. However, the impact of enteropathy on stunting children's growth in developing nations has raised significant concerns regarding its implications for public health in these countries [2]. A previous study has shown a high prevalence of enteropathy among malnourished children living in unhygienic conditions, posing a major concern across developing nations [3]. Additionally, impaired neurocognitive development and reduced vaccine effectiveness have been associated with enteropathy [4].

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the medications that can cause enteropathy. This is supported by previous studies stating that one of the main manifestations of NSAID side effects on the gastrointestinal tract is enteropathy. This is also reinforced by the development of capsule endoscopy (VEC) and double-balloon enteroscopy (DBE) technologies for diagnosing damage that occurs in the intestines, especially the small intestine [5,6]. In NSAID-induced enteropathy, mucus production is reduced and mucosal thinning occurs, leading to an increase in intestinal permeability [6]. A reduction in the mucus layer alongside heightened permeability will amplify the mucosal contact with aggressive luminal elements like bile, enzymes, and bacteria, thus intensifying the immune response [7].

Evaluating a drug's effects requires a series of steps, involving preclinical testing with experimental animals. While mice and rats are commonly employed in such studies, this model presents drawbacks due to its high costs, ethical complexities, slow reproductive rates, and longer life expectancies [8]. Hence, there is a demand for alternative experimental animals that are more economical and devoid of ethical issues, while still bearing similarities to mammals. The fruit fly (*Drosophila melanogaster*) has been a proven model organism in genetic studies due to its resemblance to mammals [9]. *D. melanogaster* has a complex and dynamic intestinal system that resembles mammalian tissue, physiology, and anatomy of the intestine, and it produces gastrointestinal hormones [10]. Besides genetic similarity and the structure of its intestinal tract mirroring that of mammals, *D. melanogaster* is also easier to maintain, cost-effective, and not constrained by ethical issues [11]. It has also been tested as a platform to assess the effectiveness of drug candidates. Our previous studies have demonstrated its utility in evaluating curcumin for treating leaky gut [12], dexamethasone and aspirin for treating inflammation [13], vitamin D as an immunomodulator [14], the antibacterial potential of the green algae *Ulva reticulata* [15,16], *Hibiscus sabdariffa* [17,18], and *Vitex trifolia* [19], as well as the effectiveness of caffeine as a nootropic candidate [20]. The aim of this study was to explore the utilization of *D. melanogaster* as an in vivo platform to assess the potential side effects of a drug that could lead to enteropathy.

Methods

Materials

The drug utilized in this study was indomethacin (CAS: 53-86-1, purity 98.0%), obtained from Xi'an Natural Biotechnology Co., Ltd. (Xi'an, China). Indomethacin is one NSAID that has the potential to cause damage to the intestines, characterized by severe inflammation, hyperemia, edema, and infiltration of inflammatory cells, particularly neutrophils [21].

Fly stock

D. melanogaster wild type (*Oregon R, OR*) was obtained from the Host Defense and Response Laboratory at Kanazawa University, Japan, and has been consistently maintained at the Pharmacology and Toxicology Laboratory in the Faculty of Pharmacy, Universitas Hasanuddin, Indonesia. The flies were cultured on a diet comprising sugar, agar, cornmeal, yeast, propionic acid, and methylparaben dissolved in distilled water. Cultivation conditions involved maintaining the flies at 25°C with alternating light-dark cycles, each lasting 12 hours.

Study design and setting

In this study, adult flies aged 3–5 days were starved in empty vials for two hours, and a total of ten flies were introduced into vials containing filter paper soaked in 5% glucose solution (untreated control) and vials containing filter paper soaked in 5% sucrose solution and indomethacin with different doses (3.75 mM, 7.5 mM, and 15 mM). The flies then were

transferred into treatment vials and their lifespan was observed for seven days. After that, a Smurf assay was conducted to identify disruptions in the intestinal integrity of the fruit flies by transferring them in to food mixed with Brilliant blue dye and left for a day. Flies experiencing gut damage were marked by changes in the color of their bodies after consuming the food [22].

Following the Smurf assay testing, the surviving flies, after seven days of treatment, were then subjected to gene expression analysis utilizing the reverse transcription-quantitative polymerase chain reaction (RT-qPCR) method. The genes encompassed pro-inflammatory cytokine-related genes (*drs* and *totA*), a mitochondria stability-related gene (*tom40*), and endogenous antioxidant-related genes (*sod1, sod2,* and *cat*). The experimental design is depicted in **Figure 1**.

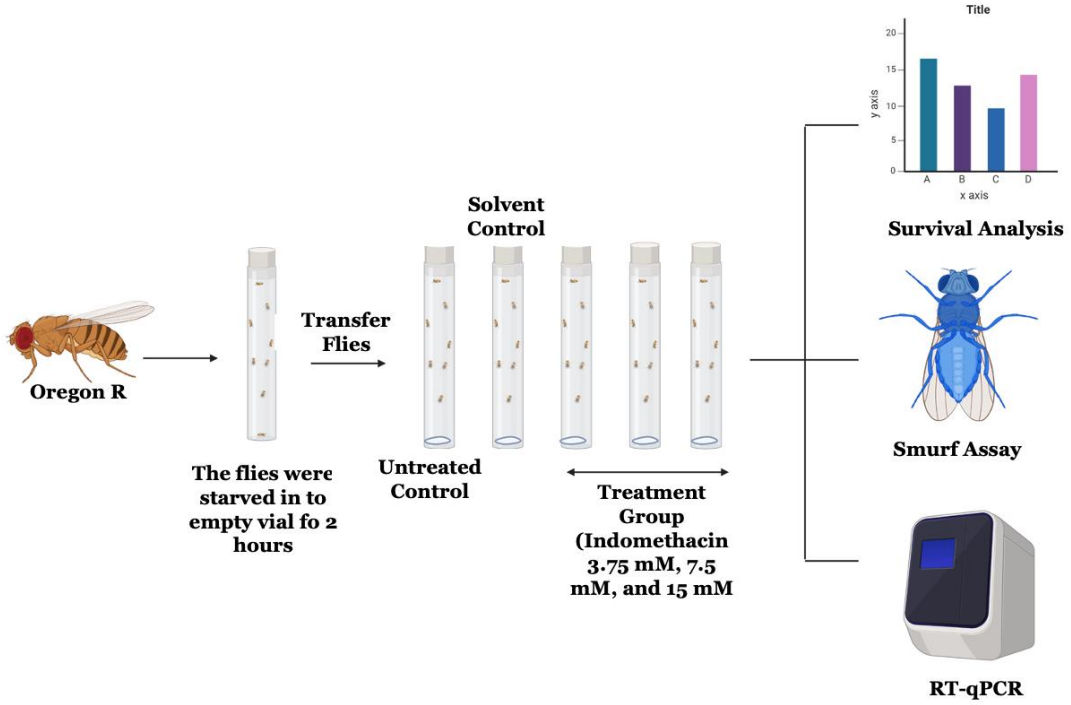

Figure 1. Study design to examine the enteropathy effect of indomethacin in *Drosophila melanogaster*.

Enteropathy induction

For enteropathy induction, the indomethacin stock solution was prepared by diluting it in 96% ethanol. Subsequently, working solutions were formulated at concentrations of 3.75 mM, 7.5 mM, and 15 mM. Filter paper containing a 5% glucose solution was prepared and then treated with the respective working solutions according to the treatment groups, and carefully placed into vials. The flies that were starved for two hours were transferred to treatment vials and observed for seven days. The filter paper was replaced daily. Seven days later, the Smurf assay was performed to visually assess the intestinal damage.

Survival analysis

Analyses of survival rates were utilized to measure the lifespan of *D. melanogaster*, serving as a parameter to elucidate the dynamics of the fly's life cycle. This analytical approach enables the identification of factors influencing the extension or reduction of the fly's lifespan [23]. The experimental design involved three experimental groups administered indomethacin as an inducer at different doses and a control group without indomethacin. The experiment was conducted by observing the number of flies alive during the administration of indomethacin treatment; the number of surviving flies was recorded daily.

Intestinal barrier integrity analysis (Smurf assay)

To assess the intestinal integrity in *D. melanogaster*, the Smurf assay was conducted. This assay, as previously described [24], involves the use of a non-absorbent Brilliant blue FDC dye (PT. Harapan Indo Warna Lestari, Tangerang, Indonesia) in standard food. In brief, adult flies that

were treated with indomethacin for seven days were transferred to food supplemented with 2.5% Brilliant blue FDC dye for 24 hours. The occurrence of the Smurf phenotype was determined by observing changes in the color of the flies' bodies. If the entire body of a fly turned blue after consuming colored food, it was classified as Smurf, whereas flies that did not show any color change on their bodies after consuming the food or only exhibited partial changes were classified as non-Smurf.

Gene expression analysis

To evaluate the effect of indomethacin administration on gene expression in *D. melanogaster*, expression analysis of genes related to pro-inflammatory cytokines (*drs* and *totA*), mitochondrial stability (*tom40*), and endogenous antioxidant (*sod1*, *sod2*, and *cat*) were determined. Drosomycin (encoded by *drs*) is an antimicrobial peptide (AMP) regulated by the NF-κB pathway, and Turandot A (encoded by *totA*) is regulated by the JAK-STAT pathway. These two proteins are expressed in response to inflammation. Translocase of other membrane 40 (encoded by *tom40*) correlates with the number of cell deaths, and superoxide dismutase 1 and 2 (encoded by *sod1* and *sod2*) and catalase (encoded by *cat*) are responsible for neutralizing reactive oxygen species (ROS) as a consequence of inflammation. Five *D. melanogaster* adults that had been treated with indomethacin for seven days were collected for RNA isolation using the Pure Link RNA Mini Kit following the manufacturer's guidelines (Invitrogen, Thermo Fisher Scientific Inc., Massachusetts, US). The expression levels of targeted genes were assessed utilizing the RT-qPCR method. The RT-qPCR assay was conducted in a 10 µL reaction volume employing the SuperScript III Platinum SYBR Green One-Step RT-qPCR with ROX following the manufacturer's instruction (Invitrogen, Thermo Fisher Scientific Inc., Massachusetts, US). RT-qPCR runs with specific primers for the target genes (**Table 1**) were performed in a 10 µL reaction volume, comprising one cycle at 37°C for 15 min, followed by 95°C for 10 min, and subsequently 40 cycles of 95°C for 10 sec, 60°C for 30 sec, and 72°C for 30 sec. A standard melt curve analysis was conducted in each RT-qPCR run to validate the specific amplification of the expected product. The host's ribosomal protein gene, *rp49*, was employed as an internal control.

Table 1. Primers used in the RT-qPCR assay

Genes	Forward primer	Reverse primer
drs	5'–TTGTTCGCCCTCTTCGCTGCTCT–3'	5'–GCATCCTTCGCACCAGCACTTAC–3'
totA	5'–GAATAGCCCATGCATAGAGGAC–3'	5'–CCAAAATGAATTCTTCAACTGCT–3'
tom40	5'–TGCACGTGTGCTACTACCAG–3'	5'–ATTCCGCCTCTGAAGACCAG–3'
sod1	5'–AGGTCAACATCACCGACTCC–3'	5'–GTTGACTTGCTCAGCTCGTG–3'
cat	5'–TTCCTGGATGAGATGTCGCACT–3'	5'–TTCTGGGTGTGAATGAAGGTGG–3'
sod2	5'–TGGCCACATCAACCACAC–3'	5'–TTCCACTGCGACTCGATG–3'
rp49	5'–GACGCTTCAAGGGACAGTATCTG–3'	5'–AAACGCGGTTCTGCATGAG–3'

Statistical analysis

All datasets obtained from both the survival and gene expression were processed utilizing GraphPad Prism 9 (GraphPad Software, Boston, US). The survival assay data were visually represented through a bar graph and subjected to statistical analysis employing the Log Rank method. The estimated mRNA levels for all treatment groups were illustrated in bar graphs and statistically analyzed using one-way ANOVA, followed by Dunnett analysis. The presentation of data included mean values accompanied by standard deviation (mean±SD), with statistical significance considered at $p<0.05$.

Results

Indomethacin administration in *Drosophila* did not induce an enteropathy-like phenotype

Indomethacin functions by inhibiting prostaglandin production, a mediator of pain. However, this inhibition can lead to reduced mucus production, potentially causing intestinal hyperpermeability and thinning of tight junctions, which may trigger an inflammatory response. To investigate whether such events occur in indomethacin-treated *D. melanogaster*, we employed a Smurf assay. However, our results indicated that the prolonged administration of indomethacin

did not induce enteropathy in *D. melanogaster*. This is evidenced by the absence of widespread coloration in the flies' bodies, as illustrated in **Figure 2**. This observation suggested that indomethacin may not induce an enteropathy-like phenotype in *D. melanogaster*.

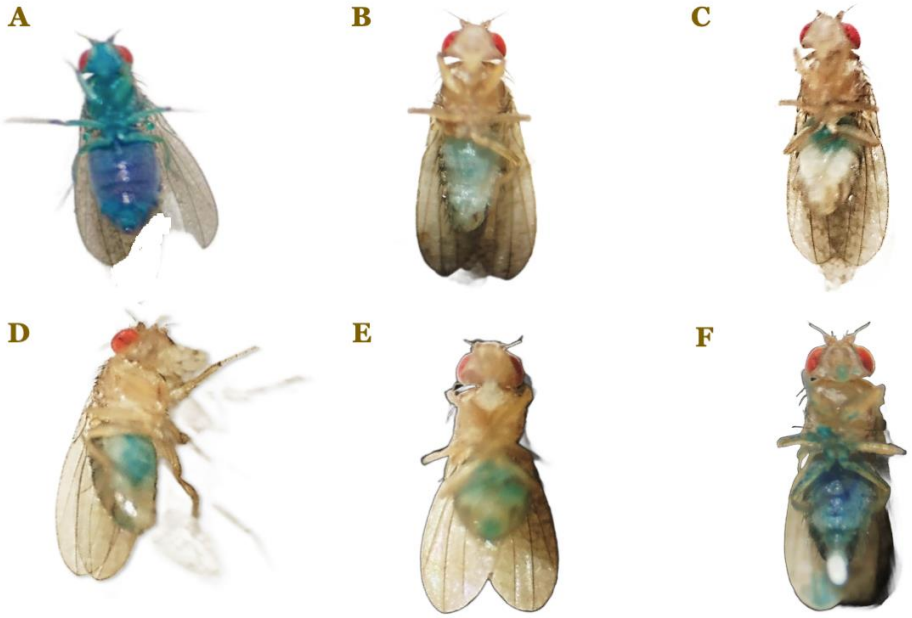

Figure 2. Exposure to indomethacin did not induce enteropathy-like phenotype in *Drosophila melanogaster*. Flies experiencing enteropathy were characterized by a change in body color referred to as 'Smurf' (A) [12]. Flies with a non-Smurf phenotype can be seen in the untreated control (B), solvent control (C), indomethacin 3.75 mM (D), indomethacin 7.5 mM (E), and indomethacin 15 mM (F) groups.

Effect of indomethacin exposure on the lifespan

Our initial examination revealed no significant impact of indomethacin on the gastrointestinal integrity of *D. melanogaster*. Despite the absence of apparent gut damage in the indomethacin-treated flies, we were intrigued to investigate whether indomethacin treatment could influence the lifespan of *Drosophila*. To address this, we systematically evaluated the effect of indomethacin on the lifespan of *D. melanogaster*, conducting daily observations to ascertain the number of surviving flies throughout the treatment period. Our findings indicated that there was no discernible difference in survival among all indomethacin-treated groups compared to the control group, as illustrated in **Figure 3**. This data further suggested that indomethacin may not induce any apparent toxicities in *D. melanogaster*, at least within the initial seven days of treatment, at dose ranges between 3.75 and 15 mM.

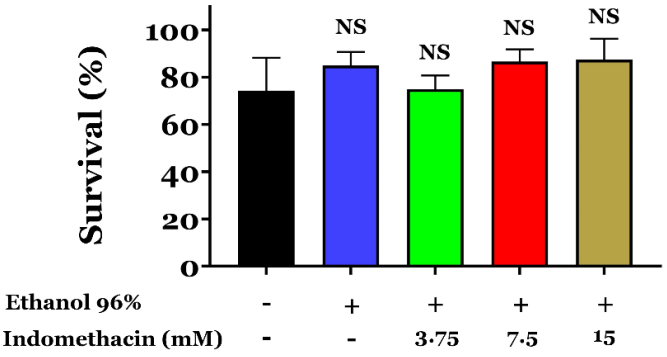

Figure 3. Effect of indomethacin administration on the lifespan of adult *Drosophila melanogaster*. Administration of indomethacin to *D. melanogaster* did not result in a significant (survival rate 85%) decrease in the lifespan of *D. melanogaster* compared to the control group. NS: not significant.

Indomethacin exposure resulted in the upregulation of NF-κB and JAK/STAT-related genes

Although there was no decrease in lifespan and no visible signs of gut damage in the *D. melanogaster* phenotype after indomethacin administration, this could potentially influence the genotype of *D. melanogaster*, particularly genes associated with the NF-kB and JAK-STAT pathways, which are pathways linked to inflammatory conditions. To verify this, RT-qPCR analysis was conducted to evaluate the effect of indomethacin administration on the expression of *drs* and *totA*, which are genes expressed when the NF-kB and JAK-STAT pathways are activated. Our results indicated that the administration of 15 mM indomethacin resulted in an increase in the expression of *drs* and *totA* genes compared to the untreated group (**Figure 4**).

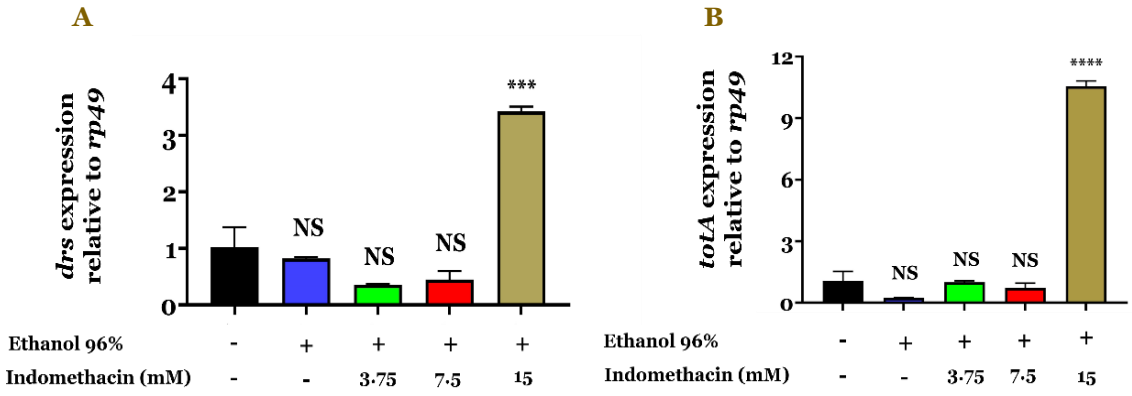

Figure 4. Expression levels of *drs* and *totA* upon the administration of indomethacin. Upregulated expression of *drs* (A) and *totA* (B) was observed only in *Drosophila melanogaster* groups treated with indomethacin 15 mM. NS: not significant; *** statistically significant at $p<0.001$; **** statistically significant at $p<0.0001$.

Indomethacin exposure led to an overexpression of translocase of outer membrane 40 (*tom40*) gene

The increase in gene expression levels associated with the NF-kB and JAK-STAT pathways due to a high dose of indomethacin administration was likely caused by inflammation and cellular stress responses. This condition may be triggered by oxidative stress, which can result in cellular damage. To verify this, we conducted an analysis of the expression of *tom40*, which is associated with mitochondrial stability. The expression analysis data indicated that there was an increase in the expression level of the *tom40* gene upon administration of 15 mM indomethacin compared to the untreated group (**Figure 5**).

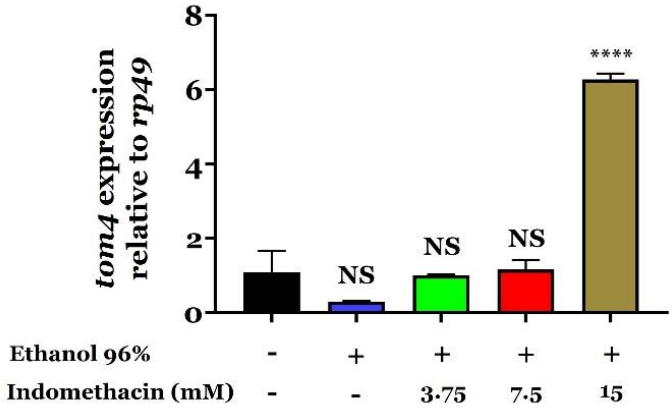

Figure 5. Overexpression of translocase of the outer membrane 40 (*tom40*) gene was observed in *Drosophila melanogaster* groups treated with higher concentrations of indomethacin. NS: not significant; **** statistically significant at $p<0.0001$.

Indomethacin exposure resulted in elevated expression of endogenous antioxidant genes

In the previous findings, we observed changes in the transcriptional activity of the *tom40* gene following indomethacin administration suggesting that cellular damage response occurred. Furthermore, we measured the expression levels of endogenous antioxidant-related genes to further investigate whether the oxidative stress effects occurring within the cells are also mediated by the presence of antioxidants within the cells, specifically superoxide dismutase 1 and 2 (*sod1* and *sod2*) and catalase (*cat*). The observation results revealed an augmentation in the expression levels of *sod1* (**Figure 6A**), *sod2* (**Figure 6B**), and *cat* (**Figure 6C**) in the indomethacin-treated group, particularly at the concentration of 15 mM.

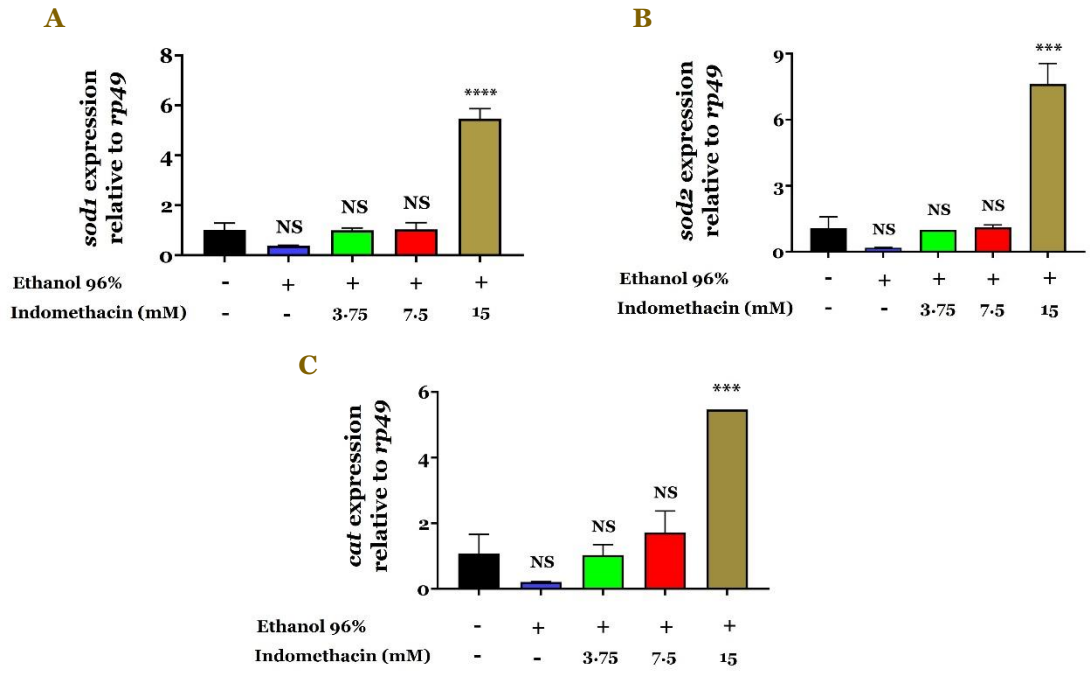

Figure 6. Elevated expression levels of endogenous antioxidant genes *sod1* (A), *sod2* (B), and c*at* (C) upon administration of 15 mM indomethacin. NS: not significant; *** statistically significant at $p<0.001$; **** statistically significant at $p< 0.0001$.

Discussion

This study sought to investigate whether continuous exposure to indomethacin could result in enteropathy in *D. melanogaster*. A previous report indicated that indomethacin induces inflammation in the gastrointestinal tract of rats [25]. Inflammation, a physiological response to external or internal stimuli, can lead to oxidative stress at the cellular level. These stress conditions can drive pathological progression, causing additional tissue damage and perpetuating chronic inflammatory responses. To evaluate this hypothesis, we employed a Smurf assay, a technique previously utilized for identifying intestinal damage in *D. melanogaster* by detecting colored food leakage from their intestinal tract [24]. Our data indicated that indomethacin may not induce enteropathy in *D. melanogaster*. The lack of an enteropathy-related phenotype in *D. melanogaster* could potentially be attributed to the inadequate potency of the indomethacin concentration, which may not have inflicted significant damage to the intestine of *D. melanogaster*, thereby preventing the manifestation of the smurf phenotype in this model organism. Alternatively, indomethacin may not induce an enteropathy phenotype in *Drosophila* due to its lack of efficacy in this species.

Although exposure to indomethacin did not manifest observable changes in the phenotype of *D. melanogaster*, it does not preclude the possibility of influencing gene expression in the organism. This likelihood arises from the intricate genotype-phenotype relationship, where the genotype establishes the genetic potential of an organism, and the phenotype represents the physically manifested genetic information influenced by various factors such as the environment.

Consequently, alterations in the genotype, such as gene mutations, may occur even if their phenotypic effects are not immediately evident [26].

In our study, we postulated that changes in the expression of pro-inflammatory genes, cell stress-related genes, and endogenous antioxidant genes may occur at a molecular level following indomethacin treatment, despite the lack of visible phenotypic changes. To assess this hypothesis, we carried out a parallel experiment in which *Drosophila* was treated with varying concentrations of indomethacin. The surviving flies, after seven days of treatment, were then subjected to RNA isolation, followed by RT-qPCR analysis. Our data indicated an increase in the expression of *drs* (**Figure 4A**), *totA* (**Figure 4B**), *tom40* (**Figure 5**), *sod1* (**Figure 6A**), *sod2* (**Figure 6B**), and *cat* (**Figure 6C**) during administration of 15 mM indomethacin.

The expression of AMPs, including *drs*, is known to be regulated by the NF-κB pathway [27]. Activation of the Toll receptor in *D. melanogaster* leads to the formation of a signal complex involving homotypic. The Toll-IL-1-receptor (TIR) is a component of Toll-like receptors (TLRs) located in the cytoplasm, which are responsible for immune and inflammatory responses [28,29] and interactions with adaptor proteins like Tube, MyD88, and kinase Pelle [30]. Subsequently, this activation triggers NF-κB, Dif, and Dorsal to translocate into the cell nucleus, initiating the release of AMPs and cytokines as part of the inflammatory response [31,32]. In light of our findings, there was a noticeable increase in the expression of the *drs* gene following the administration of 15 mM indomethacin. This result suggests a potential occurrence of inflammation. However, it is noteworthy that despite this molecular indication, observable phenotypic changes indicative of intestinal damage have not manifested yet.

In addition to the release of AMPs, NF-κB activation impacts the release of unpaired 3 (*upd3*), which is the molecule responsible for activating the JAK-STAT pathway in *Drosophila* during septic injury [33], leading to the activation of the JAK/STAT signaling pathway. Activation of the JAK/STAT pathway in adipose tissue stimulates the expression of *totA*. This gene expression, in turn, triggers the secretion of several peptides into the hemolymph. This process enhances resistance to stressors such as bacterial infections, heat shock, and exposure to ultraviolet radiation, thereby bolstering organismal immunity, particularly in animals, against these challenges [34]. The increase in *totA* expression at the 15 mM concentration suggests damage occurring due to the side effects of indomethacin, prompting the upregulation of this gene. However, this damage appears to be relatively minor, as it does not manifest phenotypically. This implies that *totA* expression serves a protective function against oxidative stress or toxins, mitigating significant damage to the body, particularly the intestines of *D. melanogaster*, following exposure to indomethacin.

The *tom40* is a gene involved in the autophagy process, crucial for maintaining mitochondrial function and preventing dysfunction. Dysregulation of mitochondrial autophagy in damaged mitochondria can lead to inflammation-related diseases [35]. Perturbations in mitochondrial permeability and function may lead to elevated levels of reactive oxygen species (ROS) and DNA mutations [36]. Elevated expression of *tom40* has been linked to heightened cell death due to ROS release [37]. Consistent with our findings, we observed an increase in *tom40* gene expression in the group exposed to 15 mM indomethacin (**Figure 5**), suggesting a higher incidence of cell death compared to other groups. Interestingly, despite the occurrence of cell death, no discernible impacts on the fruit fly phenotype were observed. Further analysis, possibly incorporating additional biomarkers, could offer valuable insights into the specific dynamics and implications of cell death in response to indomethacin exposure.

The elevated expression of endogenous antioxidant genes in response to indomethacin treatment was also observed in our study. Our subsequent investigation aimed to assess the expression of antioxidant-related genes, specifically superoxide dismutases (*sod1* and *sod2*) and catalase (*cat*). The observation results revealed an augmentation in the expression levels of *sod1* (**Figure 6A**), *sod2* (**Figure 6B**), and *cat* (**Figure 6C**) in the indomethacin-treated group, particularly at the concentration of 15 mM. This suggests an increase in ROS generation due to the administration of indomethacin at the 15 mM concentration. However, the observed elevation in ROS levels appears to be effectively managed by the upregulation of *sod1*, *sod2*, and *cat* genes. The heightened expression of these antioxidant genes is linked to protection against ROS-induced cellular damage [38].

Notwithstanding our findings, we acknowledge several limitations in our study. The absence of data on the systemic bioavailability of indomethacin is a significant limitation. Additionally, the lack of information regarding the amount of indomethacin administered to *D. melanogaster* to induce enteropathy hinders the comprehensiveness of our findings. These limitations highlight the need for future research focusing on the effects of drugs, particularly indomethacin, in the context of enteropathy induction. Addressing these gaps will undoubtedly contribute to a deeper understanding and broader knowledge base concerning enteropathy.

Conclusion

D. melanogaster, as an invertebrate insect model, holds promise for studying intestinal diseases. However, the administration of indomethacin as an inducer to provoke enteropathy may not be sufficient to cause visible damage in the fly's gut. Despite the absence of observable damage, our findings indicate that the administration of indomethacin at 15 mM can influence gene expression, particularly those associated with inflammation, without visually apparent effects in the gut. Consequently, for subsequent research endeavors, further exposure and increased dosages could be explored to achieve the goal of inducing detectable enteropathy in *D. melanogaster*. This approach may provide valuable insights into the intricate interplay between indomethacin exposure, gene expression alterations, and potential intestinal damage in this model organism.

Ethics approval

Not required.

Competing interests

All the authors declare that there are no conflicts of interest.

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Underlying data

All data underlying the results are available from the corresponding author upon reasonable request.

How to cite

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