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Original Article

Teratogenicity and hepatotoxicity of rifampicin exposure in Zebrafish

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Abstract: Antibiotics belong to a large group of pharmaceutical substances that tend to kill or prevent the growth of bacteria like rifampicin and other antibiotics. The overuse of rifampicin has resulted in the accumulation in the natural environment and has potential health hazard that involves carcinogenicity, mutagenicity, reproductive toxicity, and neurotoxicity. This study focuses on the toxic effects of rifampicin on zebrafish embryos and follows OECD 236 guidelines. The embryos are treated with 1.25, 2.5, 5, and 10 mg/L of rifampicin for a range of 4-96 hours post-fertilization. Exposed zebrafish embryos showed a variety of deformities in somites, spines, tails, hearts, and yolk sacs. Heart rate was decreasing with increasing centration of rifampicin. For adults, we exposed 5, 10, and 20 mg/L of rifampicin. The hepatotoxicity was assessed by expression of SOD, GH1, and TNF-α gene and observed a spike in the expressed at a low dose (5 mg/L) by 2.13-fold, 15.6-fold, and 3.53-fold, respectively. MDA levels were 0.32 and 0.39 nM at 10 and 20 mg/L of rifampicin, respectively. Therefore, zebrafish provide new insights into the toxicological effects of pharmaceuticals, and we found teratogenicity and rifampicin-induced hepatotoxicity in adult zebrafish.

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Introduction

Antibiotics are compounds that can inhibit microorganisms' metabolic activity and growth (Stokes et al., 2019). They can be natural, synthetic, or semi-synthetic and are frequently utilized as growth promoters in addition to treating or preventing infectious diseases (JiJie et al., 2021). As a result of their widespread usage, poor absorption, and high water solubility, it is only a matter of time before they accumulate in soil and water bodies and pose a serious threat to the environment and human health (Cai et al., 2019; Alengebawy et al., 2019). The majority of antibiotics have a half-life of a few hours to several days, but the residuals are continuously released into aquatic environments (Kulik et al., 2023). As a result, they are regarded as a 'pseudo-persistent' organic pollutant (Carvalho et al., 2016).

Rifampicin is a semi-synthetic antibiotic used to treat infections caused by methicillin-resistant *Staphylococcus aureus* and Mycobacterium (Peek et al., 2020). It is one of the most effective drugs for treating tuberculosis (Abbasi et al., 2018). Once ingested, rifampicin is only partially digested before being eliminated in the urine or feces (Cai et al., 2019). It has recently been found in wastewater from sewage treatment plants and the present treatment systems cannot remove the pollutant completely (Khan et al., 2022). As a result, the deposits are likely to cause endocrine disorders and chronic toxicity to aquatic life and humans.

Zebrafish (*Danio rerio*) are an excellent model organism in terms of size, cost, ease of care, and various features of their growth that are utilized in toxicity studies (Chahardehi et al., 2020). All major systems mature within 72 hours post fertilization (hpf) in zebrafish embryos, which are transparent at early developmental stages and are able to develop ex vivo (Bauer et al., 2021). The fish embryo toxicity test (FET) enables researchers to evaluate unexpected toxicity at early developmental stages and has grown in popularity (Bailey et al., 2013). Zebrafish has been considered an ideal option for acute toxicity studies due to its small size (2-4 cm), short life cycle, and high fertility and, hence, recognized as an excellent

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substitute for animal models (Vasamsetti et al., 2020).

The toxic effects of rifampicin on zebrafish have not been studied well. Therefore, the current study was designed to assess the teratogenic potential of rifampicin on zebrafish embryos. The study followed Organization for Economic Cooperation and Development (OECD) guideline 236 to obtain a dose-related response of zebrafish embryos to rifampicin. Furthermore, we assessed the toxicity of rifampicin in zebrafish liver tissues, with the specific aim of studying the gene expression of superoxide dismutase (SOD), growth hormone 1 (GH1), and tumor necrosis factor-alpha (TNF-α) and their role in hepatotoxicity. In addition, malondialdehyde (MDA) levels were determined in the liver to study the potential oxidative effects of rifampicin on zebrafish.

Materials and Methods

Chemicals: Rifampicin capsules I.P. (R-Cin 300, Lupin Ltd.) were purchased at a local market, Kattigenahalli, Yelahanka, Bangalore, India.

Zebrafish maintenance and embryo collection: The fish were housed in a glass aquarium (20 L capacity), supplied with RO water and aeration. The photoperiod and temperature conditions were 14 h of light /10 h of darkness and 25±1°C, respectively. The zebrafish were fed dried blood worms and micro pellets twice a day. Mass spawning in a male-to-female ratio of 1:2 was used to harvest embryos. The fish were placed in a breeding tank containing RO water and were allowed to breed overnight. The fertilized eggs were collected the next day and thoroughly washed with RO water to remove debris that had adhered to its surface.

Exposure protocols for embryos and adult zebrafish: Tests were carried out in accordance with OECD guideline 236. The fertilized embryos were placed in a petri plate containing RO water (control) and various rifampicin doses (1.25, 2.5, 5, and 10 mg). The plates were inoculated with 30 eggs per plate, and they contained 25 ml of RO water with test solutions. The experiments were replicated three times. The morphological abnormalities were observed using a compound microscope at 24, 48, 72, and 96 hpf. Abnormal somites were observed at 24 hpf; abnormal eye pigmentation was assessed at 48 hpf; pericardial edema and yolk sac edema were evaluated at 72 hpf; and spine curvature at 96 hpf.

Adult zebrafish of both sexes were acclimated in an aerated glass aquarium containing RO water. Zebrafish were removed from the aquarium and placed in circular tanks containing 2L RO water, and each fish weighed 0.3-0.4 g. For treatment, rifampicin solutions of various concentrations (5, 10, and 20 mg/L) were sonicated for 30s and added to the circular tanks. After 24 hours, an equal amount of water was removed and replaced by fresh RO water containing various rifampicin concentrations. The fish were fed dried blood worms and micro pellets twice a day. After 96 hours of treatment, the fish were quickly euthanized in melting ice. The liver tissues were separated and used for the analysis immediately.

Gene expression studies by real-time PCR: The liver sample from each experimental group of zebrafish exposed to different concentrations (5, 10, and 20 mg/L) of rifampicin was collected separately for total RNA extraction using TRIzol reagent according to the manufacturer's instructions. One microgram of DNase-treated (RQ1 DNase I, Promega) total RNA and an oligo (dT)- adaptor primer were used to synthesize cDNA using MMLV reverse transcriptase (NEB). The cDNA was quantified using the Nano Drop 2000C spectrophotometer (Thermo Scientific) at 260 nm. The mRNA level of immune-related genes was determined using SYBR green quantitative real-time PCR in the ABI PRISM 7300 Sequence Detection System (ABI). The amplification was performed in a 10 mL reaction volume containing 5 mL of 2X SYBR Green Master Mix (ABI), 1 mL of diluted cDNA, 0.5 mL of each primer, and 3.5 mL of DEPC-treated water. DEPC-treated water was used as the negative control to replace the cDNA template. The thermal profile for the SYBR green real-time PCR was 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 72°C for 1 min. The expression of immune-related genes was normalized to the expression of the β-actin gene for each sample. The details of the primer sets used in this assay are shown in Table 1.

Table 1. List of primer used for the real-time PCR analysis.

Gene	Accession No.	Sequences
BETA ACTIN	AF025305	Forward: GTTGGTATGGGACAGAAAGA Reverse: GGCGTAACCCTCGTAGAT
SUPEROXIDE DISMUTASE (SOD)	BC165134	Forward: ATGGTGAACAAGGCCGTTTG Reverse: CTATCGGTTGGCCCACCATG
GROWTH HORMONE (GH1)	BC116501	Forward: CATCAGCGTGCTCATCAA Reverse: CGCAACCCTCAGGTAAGT
TUMOR NECROSIS FACTOR-ALPHA (TNF-A)	AY28371	Forward: TGACCCTTCAGGACAATC Reverse: CAACATCTTCAACGCACA

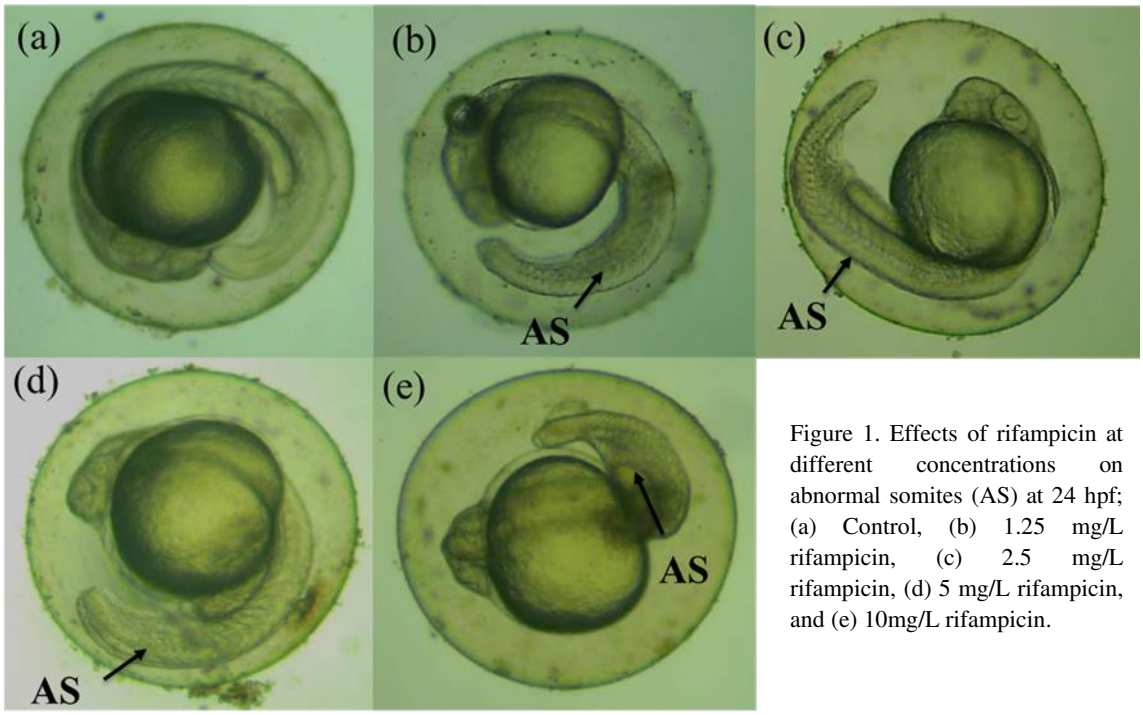

Figure 1. Effects of rifampicin at different concentrations on abnormal somites (AS) at 24 hpf; (a) Control, (b) 1.25 mg/L rifampicin, (c) 2.5 mg/L rifampicin, (d) 5 mg/L rifampicin, and (e) 10mg/L rifampicin.

Malondialdehyde (MDA) levels: After 96 hours of rifampicin exposure, the treated adult zebrafish were euthanized, and their liver samples were harvested in 1 ml of MDA reagent (TAE+TBE buffer). MDA level was calculated as per the protocol by Dhindsa et al. (1981). The liver samples were crushed and homogenized using an MDA reagent. The homogenate was incubated in a water bath at 95°C for 30 minutes. After incubation, the samples were centrifuged at 10,000 rpm for 15 mins. The supernatant was collected, and MDA levels were measured using the UV/vis spectrophotometer.

Statistical analysis: One-way analysis of variance was applied using GraphPad Prism 5.0. Dunnett multiple tests were used for one-way ANOVA at a 95 % confidence level to find a significant difference between control and treatment. A minimum of three replicates were used, and each data point is presented as the average of three biological replicates with standard deviation.

Results

Abnormal somite and eye development: The exposure to rifampicin during the zebrafish embryonic development caused morphological malformations of organs. One of the developmental defects observed was altered axial curvature resulting from defects observed in the myotomes of the somites. Zebrafish embryos in control (RO water) showed the development of normal somites. However, the embryos treated with the increasing doses of rifampicin, i.e., 1.25, 2.5, 5, and 10 mg of rifampicin, showed abnormal somites (Fig. 1). We found the change in the phenotypes of the somites caused due to

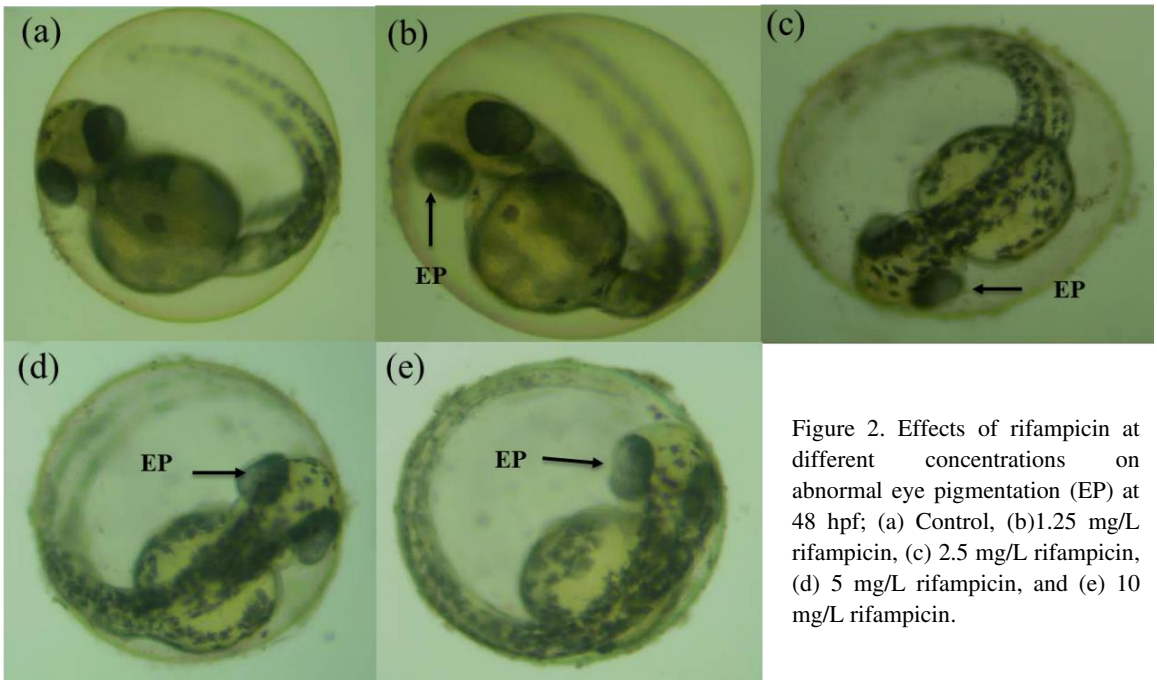

Figure 2. Effects of rifampicin at different concentrations on abnormal eye pigmentation (EP) at 48 hpf; (a) Control, (b)1.25 mg/L rifampicin, (c) 2.5 mg/L rifampicin, (d) 5 mg/L rifampicin, and (e) 10 mg/L rifampicin.

rifampicin-induced toxicity in zebrafish somitogenesis. Zebrafish embryos in control (RO water) showed the development of normal eyes. However, the embryos treated with increasing doses of rifampicin, i.e., 1.25, 2.5, 5, and 10 mg of rifampicin, showed abnormal eye pigmentation (Fig. 2).

Influence on heartbeat and pericardial edema: The heart rate measurement in zebrafish has been used to indicate cardiac function (Gut et al., 2017). Reduction in the heart rate is an indicator of cardiac toxicity that was observed in response to the drug rifampicin. Zebrafish embryos in control (RO water) showed normal heart rates. However, the embryos treated with the increasing doses of rifampicin showed a comparative decrease in the heart rate (Fig. 3). The results revealed that the abnormal heart rate is dose- and time-dependent. Pericardial edema has the potential to interfere with normal developmental landmarks, leading to abnormalities such as cardiac looping defects, bradycardia, and kidney malfunctions. The yolk sac environment is highly lipophilic and has low water permeability. However, the toxic effect of rifampicin has impaired the osmotic gradient's maintenance, resulting in excessive water uptake into the embryo or edema (Karilyn et al., 2018). Zebrafish embryos in control (RO water)

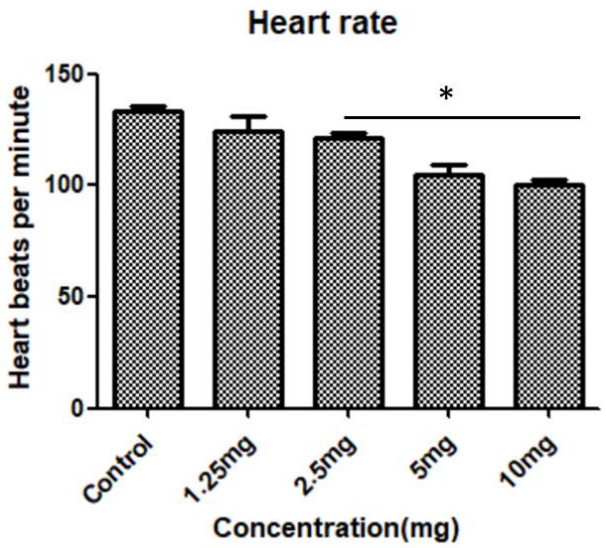

Figure 3. Average heartbeats per minute at designated concentrations of rifampicin. The heart beats were counted at 48hpf. * Represent significant at *P<0.05*. Concentration (mg/L).

showed no edema formation. However, the embryos treated with increasing doses of rifampicin showed the formation of pericardial edema and yolk sac edema with an increase in the concentration (Fig. 4). The results reveal that the formation of pericardial edema and yolk sac edema are dose- and time-dependent.

Abnormal embryo and spine curvature: Exposure to rifampicin during zebrafish embryonic development has led to abnormal spine curvature at 96 hpf. Zebrafish embryos in control (RO water), 1.25

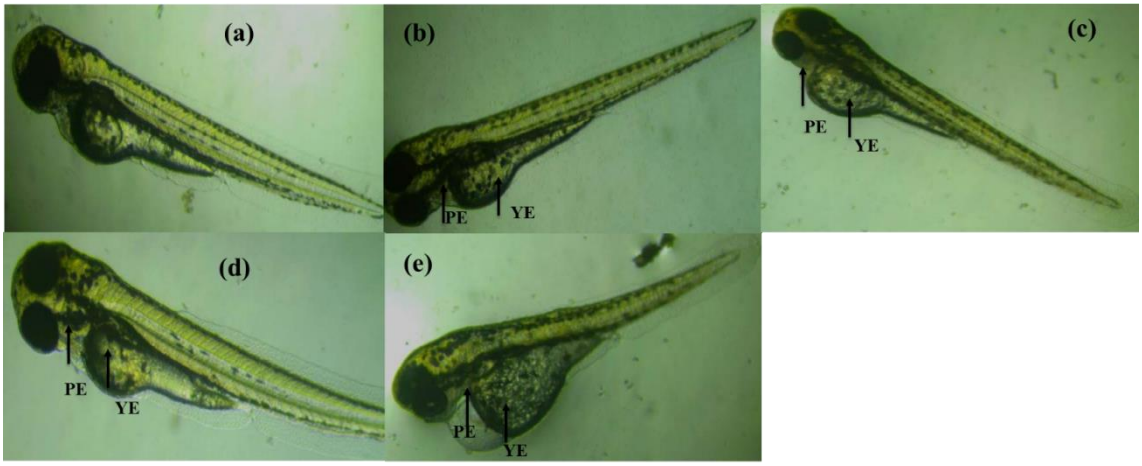

Figure 4. Effects of rifampicin at different concentrations on pericardial edema (PE) and yolk sac edema (YE) at 72 hpf; (a) Control, (b) 1.25 mg/L rifampicin, (c) 2.5 mg/L rifampicin, (d) 5 mg/L rifampicin, and (e) 10 mg/L rifampicin.

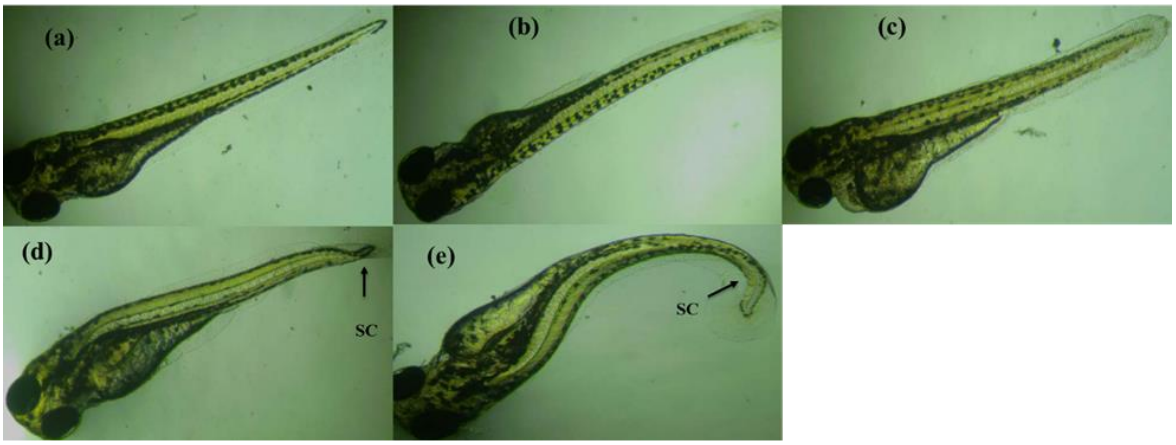

Figure 5. Effects of rifampicin at different concentrations on abnormal spine curvature (SC) at 96 hpf; (a) Control, (b)1.25 mg/L rifampicin, (c) 2.5 mg/L rifampicin, (d) 5 mg/L rifampicin, and (e) 10 mg/L rifampicin.

and 2.5 mg of rifampicin, showed normal spine length and curvature; however, the embryos treated with increasing doses of rifampicin, i.e., 5 and 10 mg of rifampicin, showed abnormal spine curvature (Fig. 5).

Expression of stress and growth-related genes: Superoxide dismutase (SOD), growth hormone 1 (GH1), and tumor necrosis factor-alpha (TNF-α) were selected to study the hepatotoxicity of adult zebrafish exposed to rifampicin (Fig. 6A-C). When exposed to 5 mg/L of rifampicin, the expression of SOD, GH1, and TNF-α were suddenly spiked in the liver and upregulated by 2.13-fold, 15.6-fold, and 3.53-fold, respectively. However, at higher concentrations of rifampicin (10 and 20 mg/L), SOD and TNF-α expression level was significantly downregulated. At 10 mg/L, GH1 expression level was upregulated by 6.35 folds; at 20 mg/L, there was no significant difference (Fig. 6).

Malonaldehyde (MDA) levels in Liver tissue: The quantity of MDA was measured to determine the extent of lipid peroxidation. At 5 mg/L of rifampicin, there was no significant difference from the control. However, treatment with concentrations of 10 and 20 mg/L increased the MDA level by 0.32 and 0.39 nM, respectively (Fig. 7). The hepatic MDA levels in zebrafish compared to controls suggest that rifampicin treatment causes oxidative damage in the liver tissues.

Discussions

The study of rifampicin toxicity in aquatic environments is essential due to its widespread use in the pharmaceutical industry. The rifampicin treatment caused developmental defects such as abnormal somites, eye pigmentation, bradycardia, pericardial edema, yolk sac edema, and spine curvature in zebrafish. These results are consistent with previous

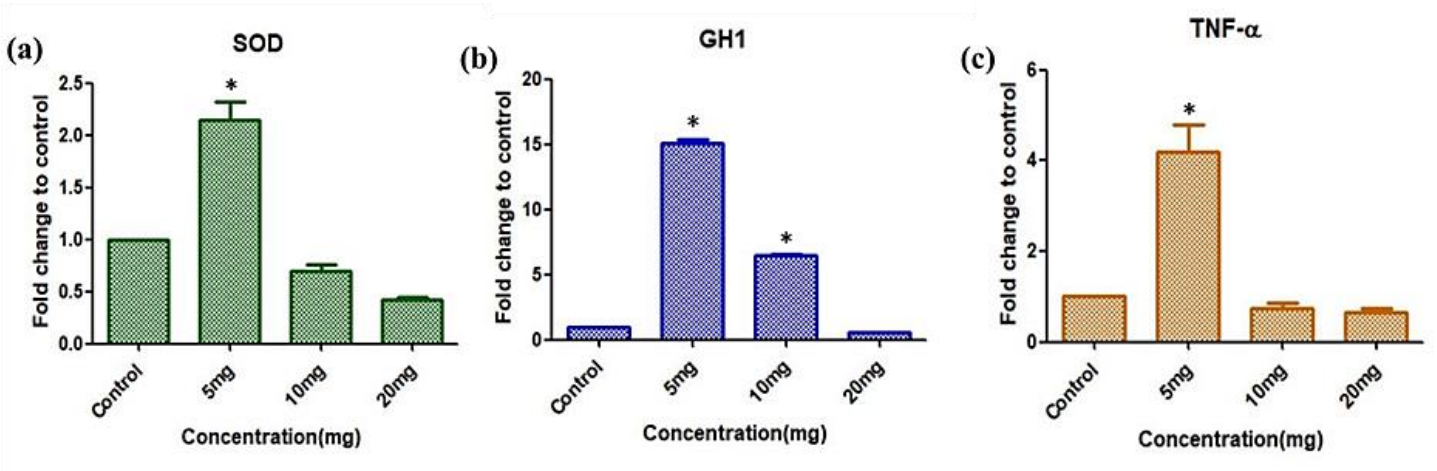

Figure 6. Expression of immune-related genes SOD (A), GH1(B) and TNF-α (C) in the liver tissues of zebrafish treated with rifampicin for 96 h. * Represent significant at $P<0.05$. Concentration (mg/L).

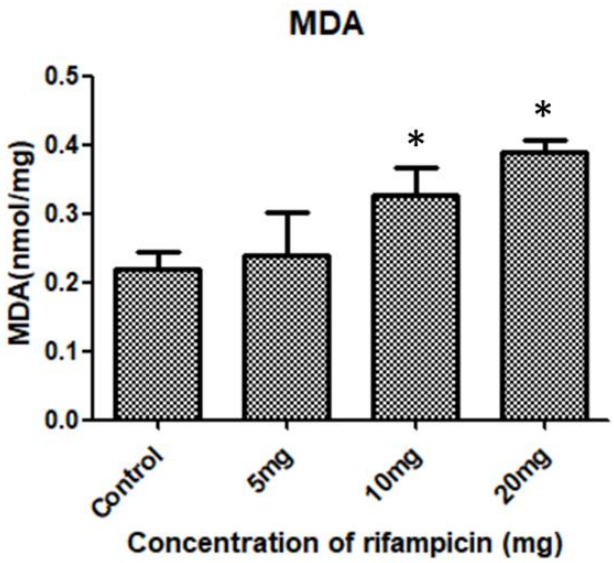

Figure 7. MDA content in the liver tissues of zebrafish treated with rifampicin for 96 h. *Represent significant at P<0.05. Concentration (mg/L).

studies showing that antibiotic classes such as aminoglycosides, β-lactum, macrolides, quinolones, sulfonamides, and tetracycline induced developmental abnormalities in zebrafish embryos (JiJie et al., 2021). Being the first functioning organ during embryogenesis, developmental toxicity in zebrafish decreased with increasing concentration (Adrian et al., 2005). As rifampicin exposure caused bradycardia and pericardial edema in zebrafish embryos, it can be considered a cardiotoxic agent that affects both cardiac functioning and development (Zakaria et al., 2018).

It has been found that rifampicin causes several defects in heart development that mainly include a decrease in the heart rate that may be caused by a reduction in the size of the heart and a defect in the positioning of the atrium relative to the ventricle (Basal et al., 2023). The exposure to rifampicin during the zebrafish embryonic development also led to the formation of pericardial edema- a fluid accumulation surrounding the developing heart, which is an abnormal phenotype. Pericardial edema is mainly caused by ionic imbalance, circulatory failure, kidney failure, and permeability defects (Jenna et al., 2023).

Another serious effect of rifampicin on zebrafish embryos was ocular toxicity, which has been proven to cause less retinal pigmentation at 48 hpf (Sato et al., 2023). Anomalies of spinal curvature substantially impact aquatic life because fish with curved spines show swimming difficulties, which may hamper their feeding and predatory habits (Vasamsetti et al., 2022).

SOD, GH1, and TNF- α are important genes to study the toxic effect and immune system (Rashidian et al., 2021). Environmental pollutants have been demonstrated to harm the immune system and impair immune competence in organisms, increasing susceptibility to infectious diseases (Chen et al., 2017). Our results showed that at a dose of 5 mg, immune genes such as SOD, GH-1, and TNF-α were highly elevated in the liver of zebrafish. However, at a concentration of 20 mg, some of these gene expressions were downregulated. These findings suggest that exposing zebrafish to the novel

compound at a concentration of 5 mg may stimulate an immune response, whereas higher concentrations may cause immunosuppression (Zhang et al., 2023).

The study gives crucial insights into the potential immunological effects of new compound exposure, which might be useful for assessing their safety and potential environmental impacts. The increased level of hepatic MDA, a byproduct of cellular lipid peroxidation, suggested rifampicin-induced oxyradicals in the liver tissues (Huang et al., 2016). MDA levels act as one of the best markers of oxidative stress. The degree of membrane lipid oxidative degradation is reflected in MDA levels (Yang et al., 2021). Compared to control, the increase in the MDA levels suggests that rifampicin exposure induces oxidative stress (Xu et al., 2020).

Conclusion

The present study revealed that early-life exposure to rifampicin interferes with zebrafish's proper growth and development. Rifampicin toxicity led to various developmental abnormalities such as abnormal somites, abnormal eye pigmentation, bradycardia, pericardial edema, yolk sac edema, and spine curvature, indicating its teratogenic potential. The toxic effects of rifampicin were dose- and time-dependent and comparable to other antibiotics. The toxic effects were observed at environmentally relevant concentrations; the potential cumulative effects could represent an environmental risk and, thus, should not be neglected. Gene expression of SOD, GH1, and TNF-α was upregulated compared to control at 5 mg. This suggests that 5 mg of rifampicin stimulates an immune response. However, the gene expression was downregulated at 10 and 20 mg of rifampicin, suggesting immunosuppression. Furthermore, rifampicin exposure increased the hepatic MDA levels, leading to the conclusion that rifampicin induces oxidative damage in liver tissues. The data demonstrates that changes in the gene expression of SOD, GH1, and TNF-α and oxidative stress caused due to increased MDA levels are associated with hepatotoxicity.

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