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Original Article

Perturbations in Redox Status, Biochemical Indices, and Expression of *XBP1s* and *NOX4* in the Liver of *Channa Punctatus* Following Exposure to Mancozeb

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Abstract

Background and objectives: Due to the increased demand for food for the growing population, pesticides are widely used to control diseases and boost productivity. This study was designed to evaluate the toxic effects of the fungicide, Mancozeb (MZ), in the liver of the fish strain *Channa punctatus*.

Methods: Fifty-four healthy *C. punctatus* fish (24 ± 4.0 g, 11.0 ± 2.0 cm) were divided into three groups (n = 18 per group): control, T1 (20% of 96 h-LC_{50} – 2.068 mg/L) and T2 (40% of 96 h-LC_{50} – 4.136 mg/L). Reactive oxygen species, redox imbalance, and liver biomarkers were measured after 20, 40, and 60 d of MZ exposure. Transcriptional profiling of *XBP1s* and *NOX4* genes was performed after 60 d.

Results: There were significant ($p < 0.05$) increases in reactive oxygen species induction, oxidative stress biomarkers (lactate dehydrogenase enzyme activity, glutathione peroxidase, superoxide dismutase and catalase), and liver biomarkers (alanine transaminase, aspartate transaminase, alkaline phosphatase, and total bilirubin) after 20, 40, and 60 d of MZ exposure. However, there were significant ($p < 0.05$) decreases in superoxide dismutase and catalase after 40 d. There was a significant ($p < 0.05$) upregulation in *XBP1s* (5.1-fold) and *NOX4* (3.3-fold) gene expression in the T2 group after 60 d. These results collectively evinces the inflammatory response triggered by MZ. It may serve as early bio-indicators of endoplasmic reticulum stress and in prevention and treatment of liver diseases.

Conclusions: The present study established that MZ is an oxidative stress inducer that may lead to liver diseases like liver steatohepatitis, non-alcoholic fatty liver disease, and non-alcoholic liver steatohepatitis. Further studies are required to elucidate the different mechanisms and signaling pathways that can minimize liver injury.

Keywords: Mancozeb; Liver injury; Oxidative stress; *XBP1s*; *NOX4*.

Abbreviations: ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; C, control group; CAT, catalase; cDNA, complementary DNA; CTCF, corrected total cell fluorescence; DCF, 2′,7′-dichlorofluorescein; DCF-DA, dichlorodihydrofluorescein diacetate; ETU, ethylene thiourea; GPx, glutathione peroxidase; H_2DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; H_2O_2, hydrogen peroxide; LDH, lactate dehydrogenase; MZ, Mancozeb; NADH, nicotinamide adenine dinucleotide; NALS, non-alcoholic liver steatohepatitis; *NOX4*, NADPH Oxidase 4; OS, oxidative stress; PCR, polymerase chain reaction; ROS, reactive oxygen species; SOD, superoxide dismutase; T, treatment group; TB, total bilirubin; *XBP1s*, spliced X-box binding protein.

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Introduction

The fungicide Mancozeb (MZ) has wide applications in agricultural and non-agricultural sectors. MZ is a chelate of manganese and zinc cations along with ethylene bis-dithiocarbamates.[1] It is widely used in controlling fungal diseases in crops. The Fungicide Resistance Action Committee has placed this fungicide in category M, a multi-site action fungicide.[2] MZ is effective against a wide range of fungal types and is thus used for multiple agricultural purposes.[3] The broad acceptance of MZ over other commercially available pesticides is not astonishing due to its low acute toxicity and broad spectrum action. The half-life of MZ is only 1 to 2 days, so it does not remain in the soil for an extended period. MZ is also photolabile and has low solubility in water. In addition to its agricultural activities, MZ has also been used as a vulcanizer and accelerator in the rubber industry, a slimicide in water coolant systems, and a metal scavenger in sewage treatment plants.[4] With its common use, it is important to understand if MZ has any toxicity.

The metabolites or breakdown products of MZ, including ethylene thiourea (ETU), ethylene bisisothiocyanate sulfide, and ethylene bisisothiocyanate, are formed when MZ is exposed to sunlight. These metabolites have a high water solubility and are not easily degradable. ETU is the primary metabolite of MZ and with a half-life of 1 to 2 weeks. ETU can harm the soil as well as aquatic flora and fauna. A previous study reported that decomposition of MZ resulted in high concentrations of manganese in the brains of the fish, *Cyprinus carpio*.[5] High amounts of manganese and lower levels of ETU were detected in the soil used for banana production in tropical Mexico; while these ratios of manganese and ETU were reversed in the sub-surface and surface waters.[6] Due to its breakdown into ETU, MZ is classified as a probable carcinogen B2 by the United States Environmental Protection Agency.[7] MZ is a teratogen, neurotoxin, disruptor of redox equilibrium, developmental and reproductive inhibitor, and carcinogen.[8] MZ can enter water bodies through surface run-off and agricultural waste disposal, thus contaminating these water bodies and killing or harming non-target organisms. Moreover, MZ is capable of instigating morphological abnormalities such as body axis distortion, DNA damage, cell death, and changes in behavioral patterns during zebrafish development.[9]

Interestingly, even though MZ is widely used as a fungicide globally and has known toxic effects on many organisms, it had not been considered a toxic substance either by the United States Environmental Protection Agency or the Agency for Toxic Substances and Disease Registry until recently. Although pesticides have been shown to be effective in controlling food inflation and increasing crop yield, the agents can harm non-target organisms, too. This has drawn the attention of environmentalists and scientists. Certain pesticides have been banned or discontinued due to bioaccumulation in tissues or their lethality to non-target organisms. The exhaustive use of these chemicals exerted ill effects on the environment affecting terrestrial and aquatic life, disrupting the ecological balance, and thus are considered a potential threat to the ecosystem. In this study, we focused on the toxic effects of MZ specifically in the liver of *C. punctatus*. Since the liver plays a pivotal role in detoxification, biotransformation, and removal of xenobiotics, we chose to investigate MZ-induced liver toxicity. MZ is a known hepatotoxic agent and has the potential to cause liver diseases such as liver steatosis, non-alcoholic fatty liver disease, and non-alcoholic liver steatohepatitis (NALS).[8,10–12] It has been reported that MZ can accumulate in human tissues, too, especially among farmers who use MZ in their fields.[13] Moreover, there have been reports of MZ accumulation in food sources, such as in vegetable crops.[14,15]

We chose to study MZ toxicity in *C. punctatus* because this fish is widely distributed and consumed in the South-Asian region. It is a bottom feeder, air-breathing fish, with yearlong availability, and is sensitive to changes in its environment.[16] The government of India also promotes the commercial production of murrel fishes (https://dst.gov.in/sites/default/files/Murrel.PDF). We assessed the effects of sublethal concentrations of MZ in the liver of *C. punctatus* by measuring biomarkers of oxidative stress [superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and lactate dehydrogenase (LDH) activity], liver injury [alkaline phosphatase (ALP), aspartate transaminase (AST), alanine transaminase (ALT), and total bilirubin (TB), along with reactive oxygen species (ROS) induction], and transcriptional profiling [spliced X-box binding protein (*XBP1s*) and NADPH Oxidase 4 (*NOX4*)].

Materials and methods

Test chemical

The test chemical, Mancozeb- 75% (Wettable powder) with the trade name Lumineb was bought from a local dealer at Daliganj, Lucknow, India. The fungicide Lumineb was manufactured and traded by Monsoon Agrochemicals Pvt. Ltd. at Azad Nagar, New Delhi- 110033 with a batch number (M0152/031). All other chemicals used in the experimental study were of analytical grade.

Animal model and acclimatization

C. punctatus (24 ± 4.0 g, 11.0 ± 2.0 cm) were hand-netted from the outskirts of Lucknow (longitude 26.87° and latitude 80.89°), India, and transported to the lab in wide-mouthed plastic tubs. They were given a prophylactic treatment of 0.05% $KMnO_4$ solution to cure skin fungal disease, if any. After the treatment, they were washed in bulk with running tap water and transferred to 1000 L aquaria prefilled with 15 d aged tap water for acclimatization. The aquaria water was checked for necessary water parameters: total dissolved solids (184.56 ± 3.8 mg/L), hardness (186.8 ± 3.50 $CaCO_3$ mg/L), dissolved oxygen (6.9 ± 0.4 mg/L), temperature (T) (25.5 ± 2.0°C), and pH (7.2 ± 0.2).[17] During the acclimatization period, the fish were fed twice a day at 8:00 am and 6:00 pm with food pellets manufactured by Perfect Companion Group Ltd., Thailand, at a rate of 2% of the fish weight.[18] Feeding was stopped a day before the start of the toxicological study.[19]

Experimental setup

Calculation of 96 h-LC_{50} of Mancozeb

The median lethal concentration until 96 h (96 h-LC_{50}) for MZ was calculated by uniform bioassays. To find the major toxicity range, six fish were placed in each aquarium with six different concentrations of MZ: 40.0, 35.0, 30.0, 25.0, 20.0, and 15.0 mg/L for 96 h. There was 100% mortality in all the aforementioned concentrations, and the toxicity range was predicted to be below 15 mg/L. To determine the definitive concentration, six fish were released in each glass aquarium that had MZ concentrations below 15.0 mg/L (13.5, 12.0, 10.5, 9.0, 7.5, 6.0, 4.5, 3.0, and 1.5 mg/L). Mortality in every aquarium was recorded at a regular interval of 24 h. Based on the mortality, the 96 h-LC_{50} was determined using the 'Trimmed Spearman-Karber' method to be 10.34 mg/L.[20]

Experimental layout

After acclimatization, 54 healthy fish were assigned to three groups: the first group was designated as the control (C), the other two were designated as the treatment groups: T1 as 20% of 96 h-LC_{50} (2.068 mg/L) and T2 as 40% of 96 h-LC_{50} (4.136 mg/L) of MZ. The study was conducted in triplicate (6 fish per group × three experiments; a total of 18 fish per group). The water in the treatment groups was completely replaced twice a week to remove excretory waste. Upon completion of the exposure period, two fish were selected in an unbiased manner from each group and anesthetized with MS222 (0.3 g/L).[21] Blood was isolated through heart puncture using a 1 mL sterile hypodermic syringe manufactured by Nipro (lot no. 20L01K88). Blood was collected in ethylene diamine tetra acetic acid coated vials and kept at −20°C for further biochemical examination and ROS estimation. Livers were harvested from the fish to examine fluctuations in oxidative stress parameters such as SOD, CAT, GPx, and LDH activity, as well as transcriptional profiling of two oxidative stress-related genes (*XBP1s* and *NOX4*).

Table 1. Primer sequences for polymerase chain reaction

Target genes	Primer sequences	Primer length	Accession no.
β-actin	F: 5′-GTG CCC ATC TAC GAG GGT TA-3′	20	AF057040.1
	R: 5′-AAG GAA GGA AGG CTG GAA GA-3′	20	
NOX4	F: 5′-AGA TAT TCT GGT ACA CGC AC-3′	20	XM_005173419.4
	R: 5′-GAA ACT ATG GCA ACA GGA GA-3′	20	
XBP1s	F: 5′-TGT TGC GAG ACA AGA CGA-3′	18	KX364065.1
	R: 5′-CCT GCA CCT GCT GCG GAC T-3′	19	

NOX4, NADPH Oxidase 4; *XBP1s*, spliced X-box binding protein.

Estimation of generated ROS

The collected blood was incubated for 30 min with 20 µM non-fluorescent 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) dye (Sigma Aldrich, USA). The slides were prepared and dried in the dark. When H_2DCF-DA is exposed to the presence of oxygen radicals like H_2O_2, it is oxidized to a green fluorescent 2′,7′-dichlorofluorescein (DCF) dye. Fluorescence was observed using a fluorescence microscope (Nikon Corporation K 12432). The excitation and emission wavelengths were 485 and 528 nm, respectively. Fluorescent intensities are represented as fold changes with respect to the control for the different groups and were predicted using Image J software.[22] The ROS generated was represented as corrected total cell fluorescence (CTCF) by applying the formula:

$$\text{CTCF} = \text{Integrated density} - (\text{Area of selected cell} \times \text{Mean fluorescence of the background})$$

Analysis of SOD and CAT activity

Estimation of SOD and CAT activity was determined in the liver tissue homogenates using the modified methods of Kakkar *et al.* and Aebi *et al.,* respectively.[23,24] For SOD activity, 200 µL of tissue homogenate was mixed with 1.2 mL of sodium pyrophosphate buffer, 100 µL of PMS, and 300 µL of NBT. The enzymatic reaction was initiated by adding 200 µL of NADH for 5 min. To complete this reaction, glacial acetic acid was added. For CAT activity, 1 mL of sodium phosphate buffer was mixed with 50 µL of the tissue homogenate. The enzymatic reaction was initiated by adding 500 µL of H_2O_2. The absorbance of SOD and CAT was estimated using a UV-VIS spectrophotometer (Shimadzu, UV-1800 pharma spec) at 560 nm and 240 nm, respectively. The activity of SOD and CAT enzymes was calculated and expressed as µm/min/mg protein. The extinction coefficient (ε) for CAT is 0.041/µm/cm.

Analysis of GPx activity

GPx activity was measured using the modified method of Flohé and Günzler.[25] A reaction mixture of 1–0.3 mL of tissue homogenate and phosphate buffer (0.1 m, pH 7.4), 0.2 mL of GSH (2 mm), and 0.1 mL each of sodium azide (10 mm) and H_2O_2 (1 mm) was prepared and incubated at 37°C for 15 min. After 15 min, 0.5 mL of 10% TCA was added to terminate the reaction, followed by centrifugation at 3,000 rpm for 5 min. Next, 0.1 mL of the supernatant was mixed well with 0.2 mL of phosphate buffer (0.1 m, pH 7.4) and 0.7 mL of DTNB (4 mg/mL). Absorbance at 420 nm was recorded using the Shimadzu UV/Vis 1800 pharma spec spectrophotometer.[26]

Analysis of LDH enzyme activity

LDH activity (L-lactate nicotinamide adenine dinucleotide1 oxidoreductase; EC – 1.1.1.27) was measured using the methods described by Phukan *et al*. and Wróblewski and Ladue.[27,28] The supernatant (100 µL or 0.1 mL) obtained after centrifugation of the liver tissue homogenate at 9,000 rpm for 30 min at 4°C was mixed with 2.4 mL of 0.1 M phosphate buffer (pH 7.5) and 0.1 mL of DPNH (α-Nicotinamide adenine dinucleotide, reduced disodium salt) or α-nicotinamide adenine dinucleotide (NADH) solution, which was prepared by adding 2 mg NADH in 1 mL of phosphate buffer. After 20 min, 0.1 mL of 0.02 M sodium pyruvate (2.5 mg/mL of distilled water) was added to start the reaction. After 1 min, the absorbance was recorded at 340 nm every 30 s for 3 min. The enzymatic activity was expressed as units mg. protein^{-1} min^{-1} at 37°C. One unit of enzyme activity was defined as the change in optical density of 0.001 per min or Δ0.001 OD/min.

Assessment of biochemical parameters

Increases in liver biomarker enzymes, such as ALT, AST, and ALP, is indicative of liver ailments. The activity of ALT, AST, and ALP was thus estimated using the method of Trivedi *et al*.[29,30] TB levels were recorded using the modified method of Perry *et al*.[31] The activity of ALT, AST, and ALP enzymes are represented in IU/L of serum.[32] TB was calculated using the method adopted by Bharti and Rasool.[32]

Transcriptomic analysis of genes related to oxidative stress like XBP1s and NOX4 by qRT- polymerase chain reaction (PCR)

Part of the liver tissues harvested from the fish were stored in TRIzol reagent (Invitrogen, USA) and the tissue homogenate was prepared for RNA isolation. To purify the RNA, the sample was mixed with the deoxyribonuclease enzyme. RNA integrity was measured using a Nanodrop (Thermo Scientific, USA; 2000/2000c) at 260 nm. The primers were designed and procured from Integrated DNA Technologies (Table 1). The Revert Aid H Minus Synthesis kit (K1632; Thermo Scientific, USA) was used to prepare complementary DNA (cDNA). After cDNA synthesis, a reaction mixture comprised of an SYBR Green qPCR Master mix (2K0251; Thermo Scientific, USA) along with the forward and reverse primers, cDNA, and nuclease free water was prepared and loaded for amplification of DNA in CFX96™ (C1000 Thermal Cycler, BioRad, USA). DNA amplification was done in three simple steps: denaturation, annealing, and polymerization. The amplified PCR products were captured on 1% agarose gels containing an intercalating agent and ethidium bromide (BioRad, USA). Images of the amplified DNA were acquired using the ChemiDoc system (BioRad, USA) and band intensities were determined using Quantity One software (BioRad, USA). The methods of Livak and Schmittgen, Iheagwam et al., and Gupta et al. were followed to calculate the expression of the target genes.[33–35]

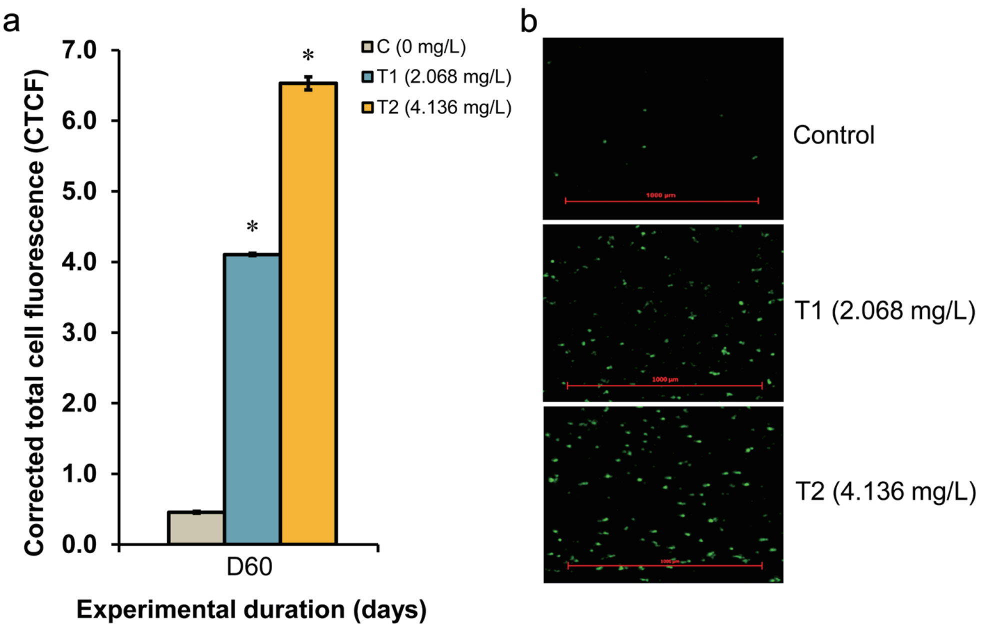

Fig. 1. ROS induction in the erythrocytes of control and treated groups (T1 and T2). (a) The corrected total cell fluorescence (CTCF) values in fish exposed to T1 (2.068 mg/L) and T2 (4.136 mg/L) after 60 days. The stated values are mean ± standard error; n = 3 fish were taken from each group; *$p < 0.05$. (b) Image of ROS captured using a non-fluorescent DCF-DA dye that oxidizes to green fluorescent DCF dye. C, control group; DCF, 2′,7′-dichlorofluorescein; DCF-DA, dichlorodihydrofluorescein diacetate; ROS, reactive oxygen species; T, treatment group.

Statistical analyses

Data were analyzed using a one-way analysis of variance and Tukey's post hoc test with a level of significance set at $p < 0.05$. The Statistical Package for Social Sciences software (v. 20.0) was used to analyze the data. Image J software (v. 1.50, USA) was used for ROS estimation. Graphs were prepared using GraphPad Prism 9.0. Transcriptional gene expression was determined using the software Quality One (v. 4.6.2.70, BioRad).

Results

96 h-LC_{50} of MZ for C. punctatus

The 96 h-LC_{50} of MZ was 10.34 mg/L, and the 95% upper and lower confidence limits were 12.12 mg/L and 8.81 mg/L, respectively.

Estimation of ROS levels

The ROS levels in the blood harvested from the *C. punctatus* exposed to MZ in groups T1 and T2 as CTCF were significantly higher after 60 d compared to the control ($p < 0.05$). The fold changes after 60 d were 7.98- and 13.28-fold for T1 and T2, respectively. Fluorescence images provided further evidence of ROS generation (Fig. 1).

Analysis of oxidative stress biomarkers

The oxidative stress biomarkers (SOD and CAT) followed the same trend as ROS generation, with increased levels after 20 and 60 d of MZ exposure; however, there were decreases in these levels at 40 days for both enzymes. After 20 and 60 d of MZ exposure, the fold change percentages in SOD were 33.8% and 65.5% in T1 and 70.2% and 84.4% in T2, respectively. Similarly, the fold change percentages for CAT were 29.9% and 61.2% in T1 and 80.0% and 104.3% in T2, respectively. After 40 d, the fold change percentages for SOD and CAT decreased to 7.5% and 3.3% in T1 and 30.9% and 14.25% in T2, respectively. The activity of both GPx and LDH continuously increased at 20, 40, and 60 d after MZ exposure. The recorded fold changes in GPx activity were 0.21-, 1.76-, and 7.46-fold for T1 and 0.63-, 4.53-, and 13.37-fold for T2, respectively. The fold change percentages for LDH activity were 42.15%, 59.07%, and 90.69% for T1 and 79.04%, 105%, and 133.13% for T2, respectively. The fold change increases and decreases in all of the aforementioned oxidative parameters were significantly different compared to the control group ($p < 0.05$) (Fig. 2).

Analysis of liver biomarker enzymes

The fold changes in prominent liver biomarker enzymes (ALT, AST, ALP, and TB) were significantly upregulated in T1 and T2 after 20, 40, and 60 d of MZ exposure compared to the control ($p < 0.05$) (Fig. 3). Specifically, the fold changes for ALT were 0.42-, 0.82-, and 1.47-fold in T1 and 1.6-, 2.6-, and 3.5-fold in T2, respectively. The fold changes for AST and ALP were 0.2-, 0.6-, and 1.1-fold 1 and 0.52-, 0.83-, and 1.79-fold in T1, and 1.6-, 2.6-, and 3.4-fold and 1.7-, 2.5-, and 4.1-fold in T2, respectively. The TB values were also significantly increased after 20, 40, and 60 d of MZ exposure ($p < 0.05$), with fold changes of 1.05-, 5.7-, and 8.5-fold in T1 and 0.7-, 1.8-, and 2.9–fold in T2, respectively.

Expression of genes related to oxidative stress and endoplasmic reticulum stress

XBP1s and *NOX4* are expressed in response to oxidative stress. *XBP1s* expression was significantly upregulated in both exposure groups ($p < 0.05$), with fold activities of 2.4 and 5.1 in T1 and T2, respectively. Whereas, *NOX4* was significantly upregulated in T2 ($p < 0.05$), with a 3.3-fold increase, but there was no significant change in expression in T1 (0.6-fold, $p > 0.05$) (Fig. 4).

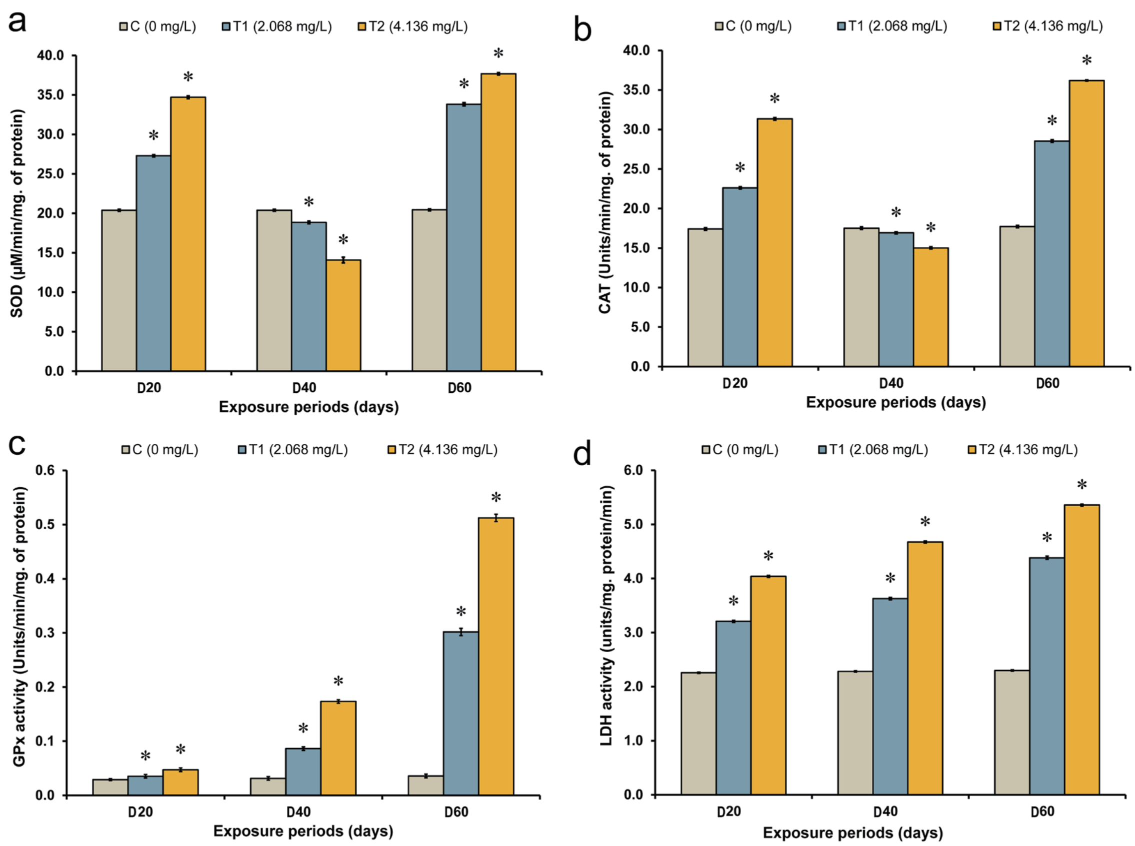

Fig. 2. Oxidative stress in the livers of *C. punctatus* after exposure to different concentrations of MZ. (a) SOD and (b) CAT were significantly decreased in T1 and T2 after 40 d; (c) GPx and (d) LDH activity were significantly increased in T1 and T2 after 20, 40, and 60 d. The stated values are mean ± standard error. * represents significant values $p < 0.05$ of T1 and T2 with respect to C. C, control group; CAT, catalase; GPx, glutathione peroxidase; LDH, lactate dehydrogenase; MZ, Mancozeb; SOD, superoxide dismutase; T, treatment group.

Discussion

There is growing evidence that MZ toxicity can cause excessive oxidative stress which can result in liver injury. This liver toxicity is associated with ROS generation, increased liver injury, oxidative biomarkers, and increased expression of oxidative stress-related genes (*XBP1s* and *NOX4*). Our study provides further evidence of MZ-induced liver toxicity in fish.

Liver cells consist of three major cell subtypes: hepatocytes, Kupffer cells, and hepatic stellate cells. Kupffer cells act as macrophages, and any damage to these cells can lead to NALS, non-alcoholic fatty liver disease, liver steatosis, and liver fibrosis. Liver cell damage can increase oxidative stress,[36] which is defined as a disturbance in the harmony and synchrony of antioxidant enzymes and the pro-oxidants produced by xenobiotics.[37] Anti-oxidant enzymes, such as SOD, CAT, and GPx, form the first formidable defense to counter oxidative stress caused by MZ. In the process of oxidative stress, superoxide radicals are converted to H_2O_2 by SOD. The H_2O_2 formed is neutralized to water and oxygen by CAT.[38] GPx is analogous to CAT as it also removes excessive H_2O_2.[39] In the present study, we observed an increase in SOD and CAT activity after 20 d of MZ exposure, indicating that these enzymes were activated. Surprisingly, after 40 d, the activity of these enzymes suddenly dropped, which was indicative of ROS overproduction. However, after 60 d, the enzyme activity significantly increased, suggesting that the activity of these enzymes was restored. Some studies have reported a decrease in the antioxidant enzymes (SOD and CAT) following oxidative stress.[40–42] Also, there was a uniform and continuously significant increase in the activity of the GPx, which actively attenuated oxidative stress by neutralizing H_2O_2 after every exposure period, particularly after 60 d. Increased LDH activity indicates increased anaerobic metabolism or hypoxic conditions caused by toxicants where pyruvate breaks down to lactate to provide energy.[27] We observed a uniform significant increase in LDH activity in our study after 20, 40, and 60 d, with the highest activity recorded after 60 days, consistent with the onset of hepatic disease and tissue injuries.[43] Our study is consistent with the findings of other studies in fish. Ayanda *et al*. similarly showed that liver injury (ALT, AST, ALP, and TB) and oxidative stress biomarkers (CAT, SOD, GPx, and LDH) were significantly elevated in *C. gariepinus*.[44] Uçar *et al*. also evaluated oxidative stress biomarkers in *Oncorhynchus mykiss*,[45] and Wang *et al*. found that the concentrations of liver injury and oxidative stress biomarkers were significantly elevated in freshwater fish, *Hypophthalmichthys nobilis* when exposed to the herbicide, pendimethalin.[46]

The use of clinical pathology or accurate estimation of serum biochemical parameters can provide vital and practical details in the assessment of liver damage. These methods can also be used to detect the type of liver damage, such as membrane injury, cholestasis, and hepatic function. ALT and AST are classified as liver injury biomarkers whereas ALP and TB are cholestatic enzymes in-

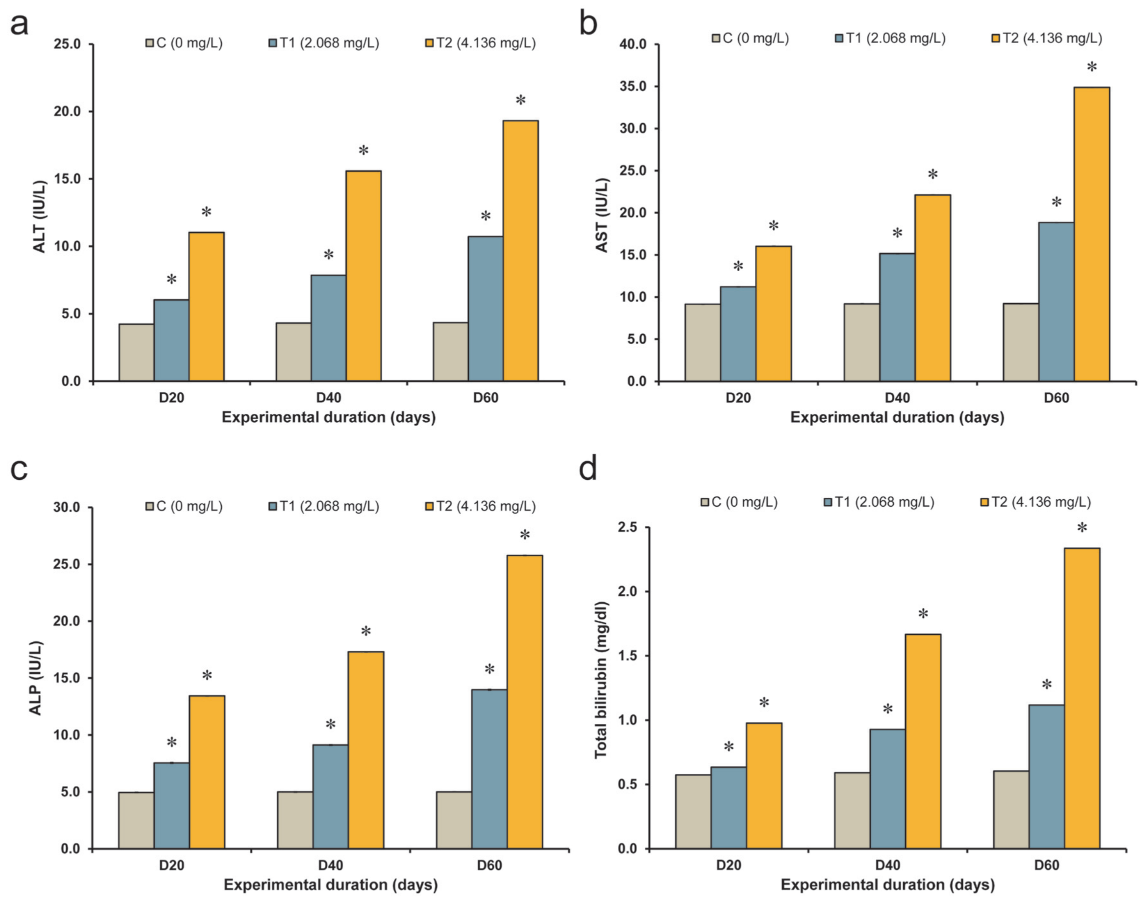

Fig. 3. Liver injury biomarkers. (a) ALT, (b) AST, (c) ALP, and (d) TB in C, T1 (2.068 mg/L), and T2 (4.136 mg/L). There were significant increases in T1 and T2 after 20, 40, and 60 d. The stated values are mean ± standard error. *p < 0.05 of T1 and T2 with respect to C. ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; C, control group; T, treatment group; TB, total bilirubin.

volved with the blockage of bile ducts.[47] Liver biomarker enzymes like AST, ALP, and ALT catalyze transamination reactions and are used in the detection and differential etiologic diagnosis of hepatic disease. Fluctuations in their concentrations can be used as an index of liver injury and tissue health. Changes in these enzymes can be attributed to disruptive hepatocyte membranes.[14] In this study,

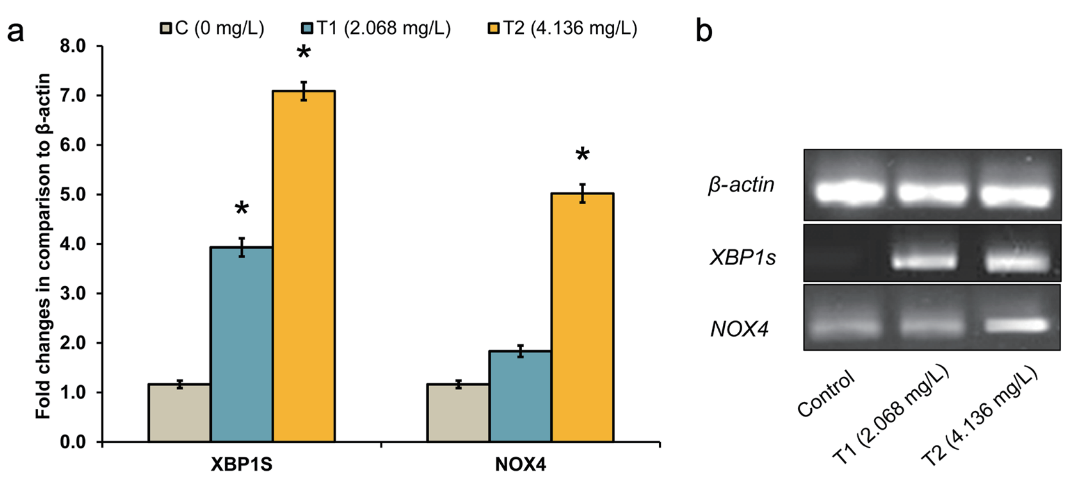

Fig. 4. *XBP1s* and *NOX4* expression after MZ exposure. (a) Relative fold changes compared to *β-actin*. The stated values are mean ± standard error. (b) Band densitometry. *p < 0.05 of T1 (2.068 mg/L) and T2 (4.136 mg/L) with respect to C. C, control group; MZ, Mancozeb; *NOX4*, NADPH Oxidase 4; T, treatment group; *XBP1s*, spliced X-box binding protein.

we observed significant increases in TB after MZ exposure (T1 and T2). This condition is known as hyperbilirubinemia, which is an underlying cause of blocked bile ducts.[32]

As discussed above, *XBP1s* and *NOX4* gene expression are upregulated in response to oxidative stress. *XBP1s* is a member of the bZIP family and is expressed following endoplasmic reticulum stress and aggregation of unfolded proteins, resulting in an unfolded protein response. An experiment conducted by Liu *et al.* demonstrated that downregulation of *XBP1* can cause an increase in oxidative stress due to suppressed CAT activity.[48] The regulation of *XBP1s* can govern the redox balance by modulating the expression of antioxidant enzymes.[49] When ROS increases beyond the tolerable limit, the unfolded protein response is activated. This in turn cleaves *XBP1* into its spliced form (*XBP1s*), which migrates into the nucleus where it can alter various physiological functions. Thus, *XBP1s* play many roles in pathways related to oxidative stress, endoplasmic reticulum stress, disrupted glucose and lipid metabolism, inflammatory responses, and cancer development.[50] In the present study, *XBP1s* were significantly upregulated in both MZ exposure groups. T2 registered a higher fold change consistent with increases in MZ concentration. *NOX4* also has important functions during inflammatory responses and contributes to increased ROS levels, which in turn can trigger many events like hepatic stellate cell activation, liver fibrosis, and apoptosis.[51] *NOX4* can activate *TGF-β* and *TNF-signaling* to initiate apoptotic events, which is an important factor to consider in drug design and disease prognosis. Interestingly, the role of *NOX4* in endoplasmic reticulum stress-induced oxidative stress has also been highlighted.[52] *NOX4* can also stimulate *XBP1s*, which in turn may initiate *RIPK1*-related *NF-κ*B signaling.[53] It was also postulated that pro-inflammatory cytokines in macrophages were produced when toll-like receptors stimulated *XBP1s* via *NOX2* signaling.[53] Furthermore, the role of *NOX4* in apoptosome formation and its ability to influence *caspase*-3 and *Bcl2* expression, as well as cause leakage of cytochrome 'c' from the mitochondria via ROS production, make *NOX4* an interesting focus of future research to treat liver ailments.[54] Activation of *NOX4* in hepatocytes can stimulate quick onset of non-alcoholic associated steatohepatitis and promote apoptosis.[52] In the present study, the expression of *NOX4* was not significantly increased in the T1 group, but there was a significant upregulation of *NOX4* in the T2 group. While some studies have investigated the combined role of *NOX4* and *XBP1s*,[55,56] more in-depth knowledge is required to understand the roles of these two genes in the treatment of liver diseases.

Conclusions

This study showed that MZ is a strong oxidative stress inducer that can trigger *NOX4* and *XBP1s* gene expression in the liver. We also found that ROS levels and their related biomarkers were elevated during liver injury, indicating that MZ toxicity can exert its toxic effects even in sub-lethal concentrations in *C. punctatus* fish. It is well known that ROS overproduction can cause neurodegenerative diseases, cancer, and liver diseases. Future studies will focus on the possible molecular pathways that are affected by MZ toxicity, contributing to a better understanding of disease progression.

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Conflict of interest

The authors declare that there is no conflict of interest.

Author contributions

AAK performed the experiment and wrote the first draft of the manuscript; SD and SS contributed to the finalization of the results; MK corrected the first draft of the manuscript; SPT is responsible for study conception, design of the study, and execution of the experiments.

Ethics statement

All animal studies and animal handling were approved by the Animal Ethics Committee of University of Lucknow (IAEC, Regn. No. 1861/GO/Re/S/16/CPCSEA). All animals received human care in accordance with relevant institutional and national guidelines and regulations.

Data sharing statement

Additional data are available on request.

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