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Mechanisms underlying taurine protection against copper sulfate-induced toxicity and inflammation in zebrafish

Chenlin Yu[a,b,*], **Tiantian Chen**[a]

[a] Collaborative Innovation Center for Biomedicine, Shanghai University of Medicine and Health Sciences, Shanghai 201318 China

[b] School of Pharmacy, Shanghai University of Medicine and Health Sciences, Shanghai 201318 China

[*]Corresponding author, e-mail: ycl0408@163.com

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ABSTRACT: This study investigates the protective effects of taurine (TAU) against copper sulfate ($CuSO_4$)-induced toxicity and inflammation in zebrafish. Zebrafish larvae were exposed to various concentrations of TAU and $CuSO_4$, followed by assessments of morphological changes, survival rates, oxidative stress, inflammatory responses, and programmed cell death. Results demonstrate that TAU significantly reduced $CuSO_4$-induced mortality and morphological abnormalities, with a mortality rate of 13.3% in the TAU-treated group compared to 83.3% in the $CuSO_4$-only group. Additionally, TAU treatment down-regulated the expression of oxidative stress and inflammation-related genes, including *hsp70* and *cox-2*, and decreased levels of pro-inflammatory mediator genes such as *nf-κb, tnf-α, il-1β*, and *il-6*. Furthermore, TAU effectively reduced the apoptosis rate in zebrafish larvae by inhibiting the pro-apoptotic gene *bax*. These findings highlight the potential of TAU as a therapeutic agent for protecting aquatic organisms from heavy metal toxicity via its antioxidant and anti-inflammatory mechanisms.

KEYWORDS: taurine, copper sulfate, toxicity, antioxidants, zebrafish

INTRODUCTION

In recent years, the increasing prevalence of heavy metal contamination in aquatic environments presents a significant threat to both ecological and human health. Copper ranks among the heavy metals frequently detected in water ecosystems [1]. While copper serves as a micronutrient within certain concentration ranges, an imbalance in its intracellular levels can prove toxic to various cell types [2] and is particularly harmful to aquatic organisms and humans [3]. Excessive exposure to $CuSO_4$ induces oxidative stress, resulting in systemic toxicity and inflammation. In humans, absorbed $CuSO_4$ binds to metallothionein within gastrointestinal mucosal cells, facilitating its excretion via bile or storage in the liver. Chronic exposure has been associated with adverse effects, including renal failure, hepatic cirrhosis, and alterations in lipid metabolism. Notably, $CuSO_4$-induced oxidative stress accelerates inflammation, worsening chronic conditions such as neurodegenerative diseases, diabetes, and cardiovascular disorders [4]. The production of reactive oxygen species (ROS) is central to these toxic effects, as metal-induced oxidative stress activates inflammatory pathways. An overproduction of ROS enhances the inflammatory response by up-regulating the expression of pro-inflammatory genes [5]. Inflammation and oxidative stress exert reciprocal influences [6]; consequently, exposure to toxic copper concentrations in humans or aquatic species triggers an inflammatory response [7].

Taurine (2-aminoethanesulfonic acid, TAU), a sulfur-containing amino acid, predominantly exists in a free state within interstitial and intracellular fluids, acting as a regulator by maintaining osmotic pressure in cells [8,9]. As a potent modulator of the immune system and pro-inflammatory responses, TAU has demonstrated capabilities in tissue repair, enhancement of antioxidant capacity, immune improvement, and alleviation of metal toxicity [10]. However, the effectiveness of TAU in mitigating $CuSO_4$-induced toxicity and inflammation remains unproven.

Zebrafish exhibit numerous advantages, including small size, rapid growth, relatively short life cycle, ease of reproduction, and early body transparency. These characteristics enable efficient genetic screening and real-time visualization of embryos [11]. Additionally, the immune system of zebrafish is highly conserved, with immune cell types and morphology closely resembling those of humans [12]. Under stress, fish experience an increase in the production of oxygen free radicals in tissues or cells [13], resulting in elevated levels of ROS that lead to oxidative damage [14]. Zebrafish demonstrate a robust innate immune response to infection and tissue injury, rendering them prone to inflammatory reactions, thereby establishing them as an excellent model for studying inflammation and wound healing.

Despite accumulating evidence indicating that copper stress disrupts antioxidant defenses, induces inflammation and apoptosis, and causes developmental toxicity, the specific protective efficacy of TAU against copper-induced toxicity, along with its comprehensive underlying mechanisms, remains largely unexplored in aquatic animals. The zebrafish (*Danio rerio*) larva represents an ideal model for mechanistic investigations in

aquaculture research due to its small size, optical transparency, genetic tractability, and highly conserved vertebrate physiology and immune system. Consequently, this study aims to systematically investigate the effects of TAU treatment on the development of zebrafish larvae exposed to $CuSO_4$, while further exploring the associated molecular mechanisms involving oxidative stress, NF-κB signaling, and apoptosis regulation. This approach seeks to address the existing knowledge gap regarding the anti-inflammatory and cytoprotective role of TAU against copper-induced toxicity and to provide a theoretical basis for its potential application in managing the health of aquatic organisms.

MATERIALS AND METHODS

Zebrafish strains and culture

Wild-type AB zebrafish (*D. rerio*) were obtained from Nanjing Ezerinka Biotechnology Co., Ltd., Nanjing, China. The zebrafish were fed three times daily, and males and females were placed in breeding tanks the day prior to mating. Mating and spawning occurred within 1 h after the lights were turned on in the morning. Fertilized eggs were selected under a microscope and placed in E3 medium at 28 ± 0.5 ℃, with medium replacement occurring after 24 h. Healthy embryos and larvae at 2 and 72 h post-fertilization (hpf) were utilized for subsequent experiments.

Main reagents

Copper sulfate pentahydrate ($CuSO_4 \cdot 5\,H_2O$) was procured from Shanghai Yuanye Bio-Technology Co., Ltd., Shanghai, China. TAU and dimethyl sulfoxide (DMSO) were sourced from Aladdin, Shanghai, China, while 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and methyl cellulose were obtained from Merck Co., Ltd., Shanghai, China. The E3 medium composition included NaCl (29.4 g/100 ml), KCl (1.27 g/100 ml), $CaCl_2 \cdot 2\,H_2O$ (4.85 g/100 ml), and $MgSO_4 \cdot 7\,H_2O$ (8.13 g/100 ml).

Experimental design

Safety test for TAU

DMSO at 0.1% served as a co-solvent for dissolving TAU in E3 medium, resulting in solutions at concentrations of 100, 200, 300, and 400 mg/l. An experimental evaluation was conducted to determine the safe concentration of TAU, wherein zebrafish larvae at 2 hpf were exposed to E3 medium with various concentrations of TAU for 96 h. The lethal and teratogenic effects of TAU on zebrafish were recorded at 24-h, 48-h, 72-h, and 96-h intervals.

Inflammation induced by $CuSO_4$ in zebrafish

At 72 hpf, well-developed zebrafish larvae were exposed to $CuSO_4$ (20 μM) for 48 h. Morphological assessments were performed using an inverted microscope, focusing on phenotypic characteristics such as surface hyperemia, yolk sac edema, and spinal curvature. A preliminary evaluation of the success of the inflammatory model construction was conducted based on the analysis of these indicators.

Effect of TAU on $CuSO_4$-induced inflammation in zebrafish model

Subsequently, zebrafish at 72 hpf were treated with $CuSO_4$ (20 μM) in combination with TAU at concentrations of 200, 300, 325, and 350 mg/l for 48 h. Morphological changes in zebrafish were examined using an inverted microscope, while survival counts, malformation counts, and heart rates were recorded, allowing for the calculation of mortality and malformation rates. These experiments aimed to evaluate the protective effects of TAU against $CuSO_4$-induced inflammation in a zebrafish model. The calculation formulae were as follows:

$$\text{Mortality rate (\%)} = (\text{deaths/total samples}) \times 100\%$$

$$\text{Abnormality (\%)} = (\text{deformities/total samples}) \times 100\%$$

ROS analysis

The ROS levels in zebrafish larvae were assessed using the ROS probe, 2′-7′-dichlorodihydrofluorescein diacetate (DCFH-DA), which reacts with ROS to produce fluorescent dichlorofluorescein (DCF). After treatment, zebrafish larvae (30 per group) were stained with a 15 μmol/l DCFH-DA solution in the dark for 1 h. Following anesthesia, microscopy images were captured using a fluorescence microscope, and fluorescence intensity was quantified to determine ROS levels.

Real-time quantitative PCR (qRT-PCR)

qRT-PCR was conducted to measure the mRNA expression of inflammation-related genes using ReverTra Ace™ qPCR RT Master Mix with gDNA Remover (TOYOBO, Shanghai, China). The $2^{-\Delta\Delta Ct}$ method facilitated the comparative analysis of gene expression, with β-actin serving as the internal control. The primers utilized in this study were obtained from Qingke, Shanghai, China and are listed in Table 1.

Statistical analysis

Data analysis and visualization were performed using GraphPad Prism software. One-way ANOVA was applied for multiple group comparisons, and results were expressed as the mean ± standard deviation. Statistical significance was set at $p < 0.05$.

RESULTS

Determination of the dose of TAU

Zebrafish at 2 hpf were placed in six-well plates with 10 fish per well and exposed to various concentrations of TAU. The results indicated that zebrafish treated with 100, 200, 300, and 400 mg/l TAU for 96 h exhibited deformities at 400 mg/l, resulting in a 0% survival rate (Fig. 1). A concentration range of 0–350 mg/l TAU

Table 1 Sequences of the primers of target genes for qPCR.

Gene	Forward (5′−3′)	Reverse (5′−3′)
β-actin	TCCGGTATGTGCAAAGCCGG	CCACATCTGCTGGAAGGTGG
il-1β	ATGGCGAACGTCATCCAAGAG	TTCAAGTCGCTGCTTCCGGCT
tnf-α	GGGCAATCAACAAGATGGAAG	GCAGCTGATGTGCAAAGACAC
il-6	TGCTACACTGGCTACACTCTT	CACATCCTGAACTTCGTCTCC
cox-2	TGGGATTTGACATCGTTA	ATTTCACATCTGGCACCT
nf-κb	ACAAGACGCAAGGAGCCCAG	AACTGTCTCTTGCACAAAGGGCTCA
hsp70	GCTGACGAGGAGGAGGAGAA	GAGGAGGAGGAGGAGGAGGA
bcl-2	GAGGATGACTTCTCTCGGCG	CAGGCTGGAAGGAGAAGATG
bax	GTTTCATCCAGGATCGAGCAG	CATCTTCTTCCAGATGGTGA

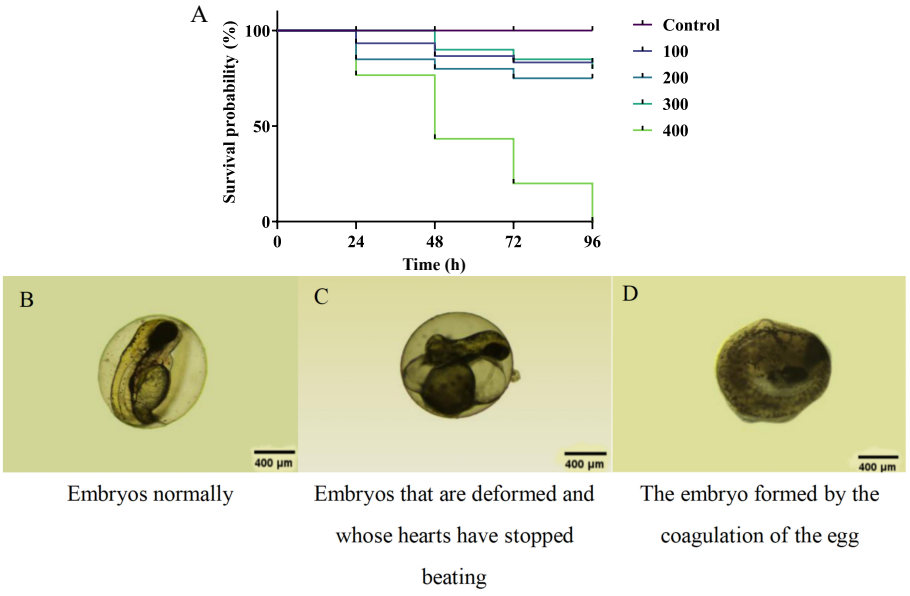

Fig. 1 Results of intervention concentration determination of TAU. (A) Survival curves of zebrafish at different TAU doses within 96 h. (B) Representative image of a normally developed embryo. (C) Representative image of a deformed embryo with cardiac arrest. (D) Representative image of egg coagulation and death.

was determined to be safe for zebrafish administration, leading to the selection of 200, 300, 325, and 350 mg/l for further experiments.

Protective activity of TAU against $CuSO_4$ toxicity in zebrafish larvae

In this study, exposure to 20 μM $CuSO_4$ resulted in significant mortality among zebrafish larvae (Fig. 2). However, treatment with TAU at a dose of 350 mg/l effectively mitigated $CuSO_4$ toxicity, enhancing survival rates. Mortality among larvae treated solely with $CuSO_4$ reached 83.3%, while the co-administration with TAU at 325 mg/l reduced the mortality rate to 13.3%. At the higher TAU concentration of 350 mg/l, no dead larvae were observed after 24 h. Moreover, visual assessments revealed that treatment with $CuSO_4$ alone caused notable toxicity in larvae, characterized by trunk curvature and impaired motility. In contrast, larvae subjected to combined treatments of $CuSO_4$ and TAU exhibited a reduction in morphological abnormalities. At the highest dose of TAU (350 mg/l), zebrafish larvae showed protection against $CuSO_4$ toxicity, with no observable morphological deviations (Fig. 3). These findings demonstrate that TAU exerts a protective effect against $CuSO_4$ toxicity in a dose-dependent manner. To elucidate the protective mechanisms of TAU, further evaluation of its antioxidant and anti-inflammatory activities was conducted.

TAU alleviates $CuSO_4$-induced oxidative stress in inflamed zebrafish embryos

ROS in zebrafish were detected using the fluorescent probe DCFH-DA. Treatment with TAU effectively inhibited ROS formation (Fig. 3), with fluorescence intensity significantly reduced compared to the $CuSO_4$ group. Concentrations of TAU at 200, 300, 325, and 350 mg/l significantly decreased ROS fluorescence intensity compared to the control. To further investigate the underlying mechanisms of its antioxidative action, the effects of TAU on the expression of two

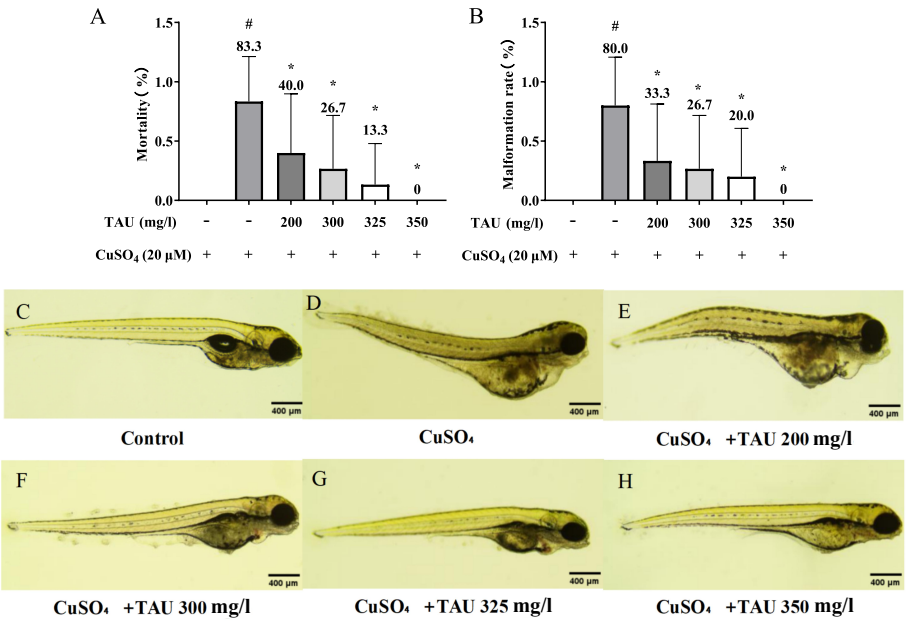

Fig. 2 Effects of TAU on the zebrafish inflammation model. (A) Mortality of zebrafish treated with $CuSO_4$ and different doses of TAU. (B) Malformation rate of zebrafish treated with $CuSO_4$ and different doses of TAU. (C–H) Representative images of zebrafish treated with $CuSO_4$ and different doses of TAU. (C) Control, (D) $CuSO_4$, (E) $CuSO_4$ + TAU 200 mg/l, (F) $CuSO_4$ + TAU 300 mg/l, (G) $CuSO_4$ + TAU 325 mg/l, (H) $CuSO_4$ + TAU 350 mg/l. $CuSO_4$ used was 20 μM. $^{*}p < 0.05$ compared to the control group; $^{\#}p < 0.05$ compared to the $CuSO_4$-treated group.

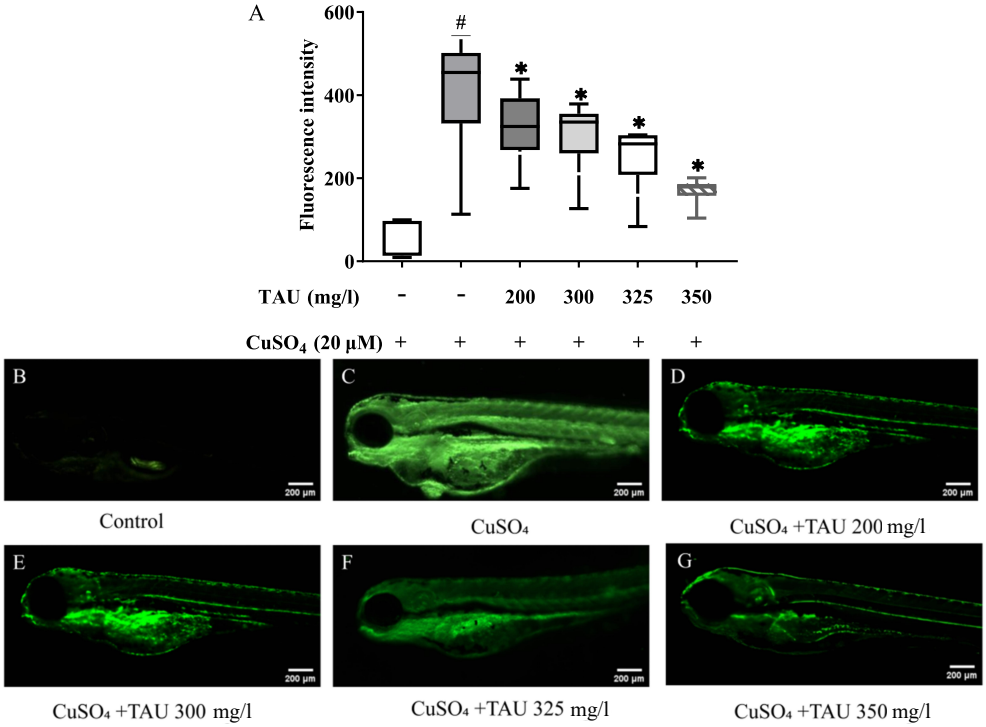

Fig. 3 The impact of TAU on the generation of ROS in zebrafish larvae. (A) Fluorescence intensity of ROS in zebrafish. (B–G) Fluorescence intensity images of zebrafish larvae after treatment with 20 μM $CuSO_4$ and different doses of TAU. (B) Control, (C) $CuSO_4$, (D) $CuSO_4$ + TAU 200 mg/l, (E) $CuSO_4$ + TAU 300 mg/l, (F) $CuSO_4$ + TAU 325 mg/l, (G) $CuSO_4$ + TAU 350 mg/l. Compared to the control group, $^{*}p < 0.05$. Compared to the $CuSO_4$-treated group, $^{\#}p < 0.05$.

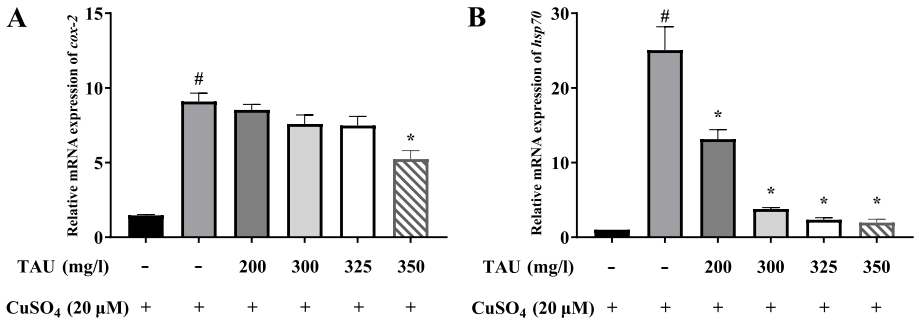

Fig. 4 Effects of TAU on antioxidant capacity-related mRNA expression in zebrafish larvae under waterborne copper exposure. (A) Expression of *cox-2* gene relative to β-actin. (B) Expression of *hsp70* gene relative to β-actin. $^{*}p < 0.05$ compared to the control group; $^{\#}p < 0.05$ compared to the $CuSO_4$-treated group.

antioxidant-associated genes, cyclooxygenase-2 (*cox-2*) and heat shock protein 70 (*hsp70*), were evaluated. In the presence of $CuSO_4$, the expression of *hsp70* was significantly increased compared to the control group (Fig. 4). However, following 48 h of TAU treatment, *hsp70* expression was significantly downregulated relative to the $CuSO_4$ group, with significant reductions observed in the 200, 300, 325, and 350 mg/l TAU groups (Fig. 4). Furthermore, exposure to copper sulfate resulted in a marked increase in *cox-2* expression compared to the control group, which was significantly mitigated by TAU at 350 mg/l.

TAU protects zebrafish embryos from $CuSO_4$-induced inflammation

The mRNA expression levels of nuclear factor-κB (*nf-κb*), tumor necrosis factor-α (*tnf-α*), interleukin-1β (*il-1β*), and interleukin-6 (*il-6*) were measured via RT-PCR. Compared to the control group, these expression levels were significantly elevated in the $CuSO_4$ group ($p < 0.05$), while the 325 and 350 mg/l TAU groups exhibited notable decreases in expression compared to the $CuSO_4$ group ($p < 0.05$) (Fig. 5).

TAU inhibits $CuSO_4$-induced cell apoptosis in zebrafish

Moreover, the gene expression levels of key apoptosis-related factors, Bcl-2-associated X (*bax*) and B-cell lymphoma-2 (*bcl-2*), were also evaluated using RT-PCR. The expression ratio of *bax*, which signifies cellular apoptosis, was significantly upregulated in the $CuSO_4$ group, but this elevation was markedly inhibited by pretreatment with TAU at 300, 325, and 350 mg/l (Fig. 6). Additionally, *bcl-2* expression was substantially reduced in the $CuSO_4$ group compared to the control group ($p < 0.05$), while upregulation was observed in the 325 and 350 mg/l TAU groups ($p < 0.05$) (Fig. 6). These data suggest that TAU at 325 and 350 mg/l exhibits a significant protective effect against $CuSO_4$-induced apoptosis in zebrafish.

DISCUSSION

Copper, an essential trace element, acts as a catalytic cofactor in various enzymes that facilitate energy metabolism and antioxidant reactions [15]. However, excessive environmental inorganic Cu ions can disrupt copper ion homeostasis in zebrafish, leading to the accumulation of ROS and triggering inflammatory responses mediated by oxidative stress damage [16]. Cyclooxygenase-2 (COX-2) and heat shock protein 70 (HSP70) are critical regulators of inflammation and cellular stress responses. COX-2, an inducible enzyme, catalyzes the metabolism of arachidonic acid to produce prostaglandins, which mediate inflammatory responses through upregulated expression during inflammatory stimuli [17]. HSP 70, functioning as a molecular chaperone, exhibits significantly elevated expression under stress conditions and protects cells via its antioxidant properties [18]. In this study, ROS levels significantly increased, and *cox-2* and *hsp70* expressions were upregulated following $CuSO_4$ exposure, while TAU treatment reduced ROS levels and downregulated *cox-2* and *hsp70* expression. These findings indicate that TAU effectively alleviates $CuSO_4$-induced inflammation by modulating inflammation-related protein expression and mitigating oxidative stress.

The activation of inflammatory signaling pathways is pivotal in sustaining and amplifying inflammatory responses [19]. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) serves as a key transcription factor widely implicated in inflammatory responses and immune regulation [20]. Previous studies have demonstrated that NF-κB activation enhances the expression of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, which exacerbate inflammatory responses and may aggravate tissue damage [21]. TNF-α and IL-1β are notable pro-inflammatory factors that promote IL-6 secretion and activation, further intensifying tissue inflammatory damage. In the present study, $CuSO_4$ treatment significantly upregulated NF-κB activity, subsequently increasing the expression

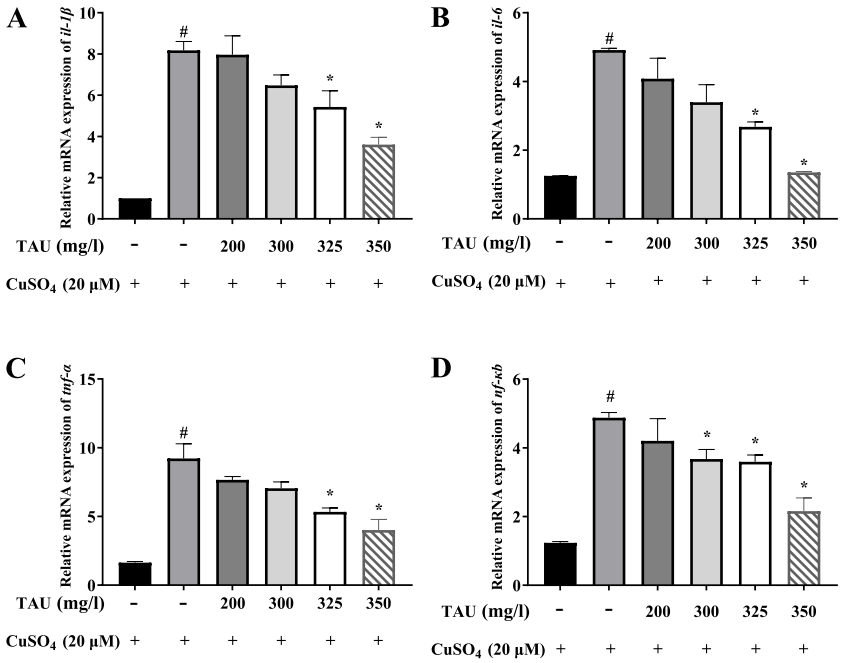

Fig. 5 Effects of TAU on inflammation-related mRNA expression in zebrafish larvae under $CuSO_4$ exposure. (A) Expression of *il-1β* gene (B) Expression of *il-6* gene (C) Expression of *tnf-α* gene (D) Expression of *nf-κb* gene. All expressions of genes are relative to *β*-actin. $^{*}p < 0.05$ compared to the control group; $^{\#}p < 0.05$ compared to the $CuSO_4$-treated group.

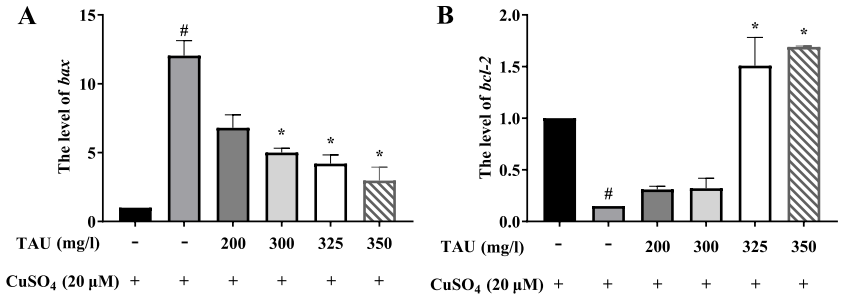

Fig. 6 Effects of TAU on apoptosis in zebrafish larvae under waterborne $CuSO_4$ exposure. (A) Expression of *bax* gene relative to *β*-actin. (B) Expression of *bcl-2* gene relative to *β*-actin. $^{*}p < 0.05$ compared to the control group; $^{\#}p < 0.05$ compared to the $CuSO_4$-treated group.

of pro-inflammatory cytokines (*il-1β*, *il-6*, and *tnf-α*), while TAU alleviated this inflammatory response by inhibiting NF-κB activation and the production of these pro-inflammatory factors. This suggests that TAU mitigates $CuSO_4$-induced inflammation by suppressing NF-κB activation and downregulating the expression of pro-inflammatory cytokines (*il-1β*, *il-6*, and *tnf-α*), thereby protecting tissues from excessive immune responses.

ROS have been implicated in the signaling pathways that lead to NF-κB activation, which is sufficient for inducing inflammatory responses [22]. Additionally, NF-κB is recognized as a key mediator that links apoptosis and inflammation [23]. The current study revealed that transcriptional levels of NF-κB were significantly reduced with TAU administration compared to the $CuSO_4$ group. Furthermore, NF-κB has the capacity to transactivate anti-apoptotic genes, such as *bcl-2*, or promote the production of pro-inflammatory mediators, including TNF-*α* [24]. ROS-induced oxidative stress activates apoptotic pathways by upregulating BAX protein and downregulating BCL-2. BCL-2 family proteins serve as key regulators of apoptosis, with the BAX/BCL-2 ratio determining cell survival or death [25]. In the present study, TAU was shown to inhibit apoptosis by significantly downregulating the mRNA expression levels of *bax* and upregulating *bcl-2*. These findings suggest that TAU may mitigate $CuSO_4$-induced inflammatory responses by modulating the *bax/bcl-2* equilibrium and reducing cell apoptosis. Prior research has established that TAU provides protective benefits by enhancing cellular resilience to

oxidative stress and inflammation through the regulation of apoptosis-related genes [26]. The results of the present study corroborate these earlier findings, further substantiating TAU's potential in reducing both apoptosis and inflammation. The evidence suggests that TAU predominantly suppresses the ROS-induced NF-κB signaling pathway, thereby exerting its anti-apoptotic effects to protect zebrafish from toxicity and inflammation related to oxidative stress.

CONCLUSION

TAU exhibits significant protective effects against $CuSO_4$-induced toxicity in zebrafish larvae, notably decreasing mortality and morphological abnormalities while modulating inflammation and apoptosis pathways. This study highlights TAU's ability to reduce oxidative stress and inflammatory gene expression, indicating its antioxidant properties. These findings emphasize TAU's potential as a therapeutic agent for mitigating heavy metal toxicity and associated inflammation-related conditions. The results not only enhance the understanding of the underlying molecular mechanisms but also pave the way for future research on dietary interventions and pharmacological applications against environmental pollutants.

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