Aquaculture 531 (2021) 735965

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Co-administration of zinc for treating and preventing arsenism in common carp *Cyprinus carpio*: An alternative to avoid physiological and cellular damages

Yu Wang[a,1], Hongjing Zhao[a,1], Menghao Guo[a], Mengyao Mu[a], Hui Zong[b], Mingwei Xing[a,*]

[a] College of Wildlife and Protected Area, Northeast Forestry University, Harbin 150040, Heilongjiang, PR China
[b] Guangdong Polytechnic of Science and Trade, Guangzhou 510000, PR China

ARTICLE INFO

Keywords:
Cyprinus carpio
Arsenite
Oxidative stress
Inflammatory
Endoplasmic reticulum stress

ABSTRACT

Arsenic (As) contamination in water bodies has adverse effects on aquatic organisms. Zinc (Zn), as an important enzyme cofactor and efficient reactive oxygen species scavenger, plays an indispensable role in cellular homeostasis and biological functions. However, there has been less investigation on As−Zn interaction in common carp *(Cyprinus carpio)*. We hypothesize that Zn is a candidate for the treatment and prevention of arsenism. In general, feed efficiency, growth performance and organ indices did not significantly differ between the As or/and Zn group and the control groups ($p > 0.05$). However, arsenite significantly altered the intestinal morphological features and suppressed intestinal tight junction proteins (claudins, occluding, and zonula occludens) as indicated by mRNA and protein levels. Moreover, oxidative stress (malondialdehyde and organic 8-hydroxy-2-deoxyguanosine), inflammatory (nuclear factor kappa-B and pro-inflammatory cytokines) and detoxification parameters (metallothionein) in intestine and liver increased following arsenite exposure ($p < 0.05$), concomitant with innate immunity suppression (C4 and IgM; $p < 0.01$). These anomalies led to abnormal lipid metabolism (SREBP-1c pathway; $p < 0.01$) and endoplasmic reticulum stress (PERK-eIF2α, IRE1α and ATF6; $p < 0.01$). Overall, As impaired general physiological and cellular functions, and compromised the immune system of carp. Zn^{2+} restored these alterations, as evidenced by the reduction in inflammation and endoplasmic reticulum stress via the activation of the antioxidant system. Thus, Zn supplementation can be used to treat and prevent arsenism; it is a useful alternative to prevent physiological and cellular damage in the inland culture of carp.

1. Introduction

Arsenic (As) is the most toxic metals (https://www.atsdr.cdc.gov/spl/). Owning to various natural and anthropogenic processes, As is widely found in aquatic environments (Sharma and Sohn, 2009). High concentrations of inorganic As (≥ 1000 μg/L) are routinely detected in areas with high As-containing groundwater, mine waste, or mill tailings (Chen et al., 2018; Mensah et al., 2020). Recently, As contamination in animal-derived foods, especially sea foods, has led to high incidence of arsenicosis in many countries (Fendorf et al., 2010; Ma et al., 2018). Thus, As pollution may be harmful to aquatic organisms and might be transferred to higher levels along the food chain, including human beings, causing severe health problems (Rahman et al., 2012; Wang et al., 2020a). Acute As exposure in zebrafish (*Danio rerio*) is lethal and could cause abnormalities at individual and cellular levels, including abnormal motor activity, anxiety, and brain extracellular nucleotide hydrolysis (Baldissarelli et al., 2012). Following chronic exposure, As is sublethal and might induce further continuous and extensive events, including oxidative stress and metabolism disorders in common carp (*Cyprinus carpio*) (Ventura-Lima et al., 2009; Kumari et al., 2016); body malformation in zebrafish (*D. rerio*) (Ma et al., 2015); immunosuppression in European sea bass (*Dicentrarchus labrax*) and common carp (*C. carpio*) (Cordero et al., 2018; Wang et al., 2020b); and DNA damage in goldfish (*Carassius auratus Linnaeus*) (Kumar et al., 2014). However, the potential effects of chronic exposure to As in vivo have not been thoroughly evaluated.

Intracellular Zn^{2+} is regulated by different Zn transferrins (including Zn^{2+} importers (ZIPs), Zn^{2+} transporters (ZnTs) and metallothioneins (MTs) (Kambe et al., 2015; Li et al., 2019), and is essential for maintaining cell growth and metabolism (Medeiros et al.,

* Corresponding author.
E-mail address: xingmingwei@nefu.edu.cn (M. Xing).
[1] Yu Wang and Hongjing Zhao contributed equally to this article.

https://doi.org/10.1016/j.aquaculture.2020.735965
Received 12 May 2020; Received in revised form 21 September 2020; Accepted 23 September 2020
Available online 29 September 2020
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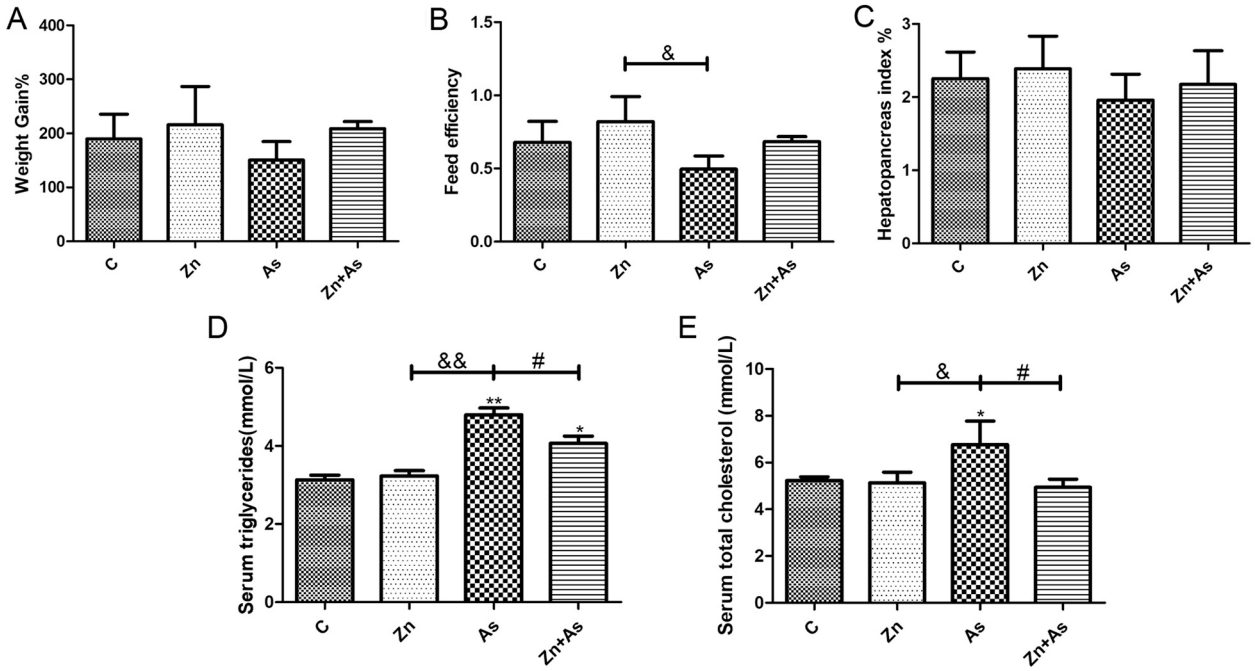

Fig. 1. (A) weight gain, (B) feed efficiency, (C) hepatosomatic index, (D) serum triglyceride (TG), (E) serum total cholesterol (TCHO). The * indicates significant differences between control group and experience group ($p^{*} < 0.05$, $p^{**} < 0.01$), the $^{\&}$ indicates significant differences between zinc group and arsenic group ($p^{\&} < 0.05$, $p^{\&\&} < 0.01$), the $^{\#}$ indicates significant differences between arsenic group and zinc + arsenic group ($p^{\#} < 0.05$, $p^{\#\#} < 0.01$).

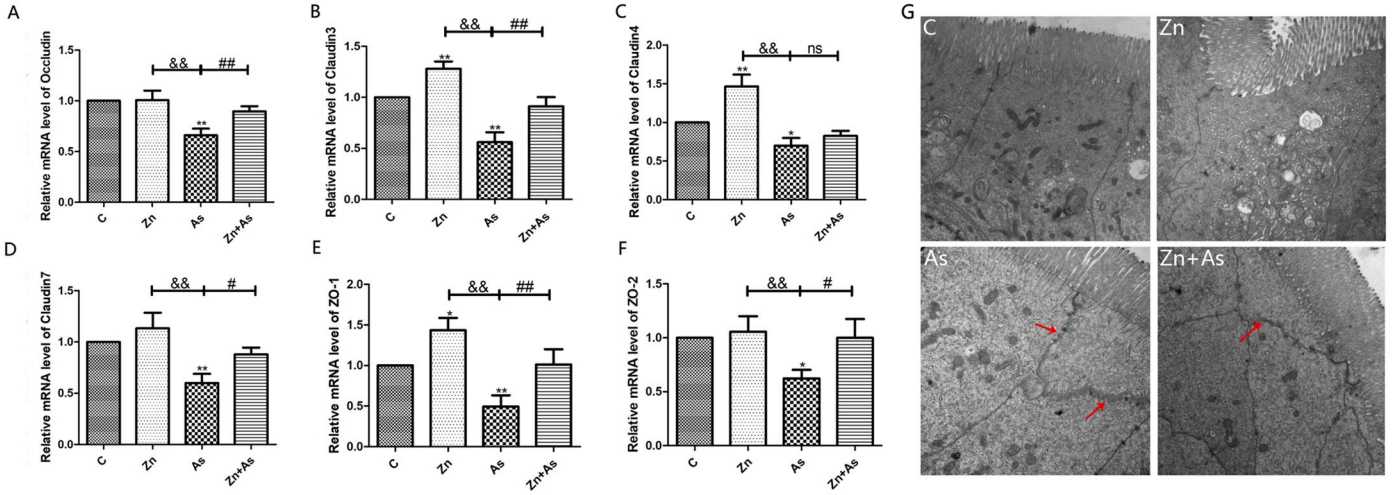

Fig. 2. Tight junction proteins mRNA levels and transmission electron microscope (TEM) observation of intestinal. (A) Occludin (B) Claudin-3 (C) Claudin-4 (D) claudin-7 (E) ZO-1 (F) ZO-2 (G) control group, zinc group, arsenic group, zinc + arsenic group TEM, red arrows: tight junctions. The * indicates significant differences between control group and experience group ($p^{*} < 0.05$, $p^{**} < 0.01$), the $^{\&}$ indicates significant differences between zinc group and arsenic group ($p^{\&} < 0.05$, $p^{\&\&} < 0.01$), the $^{\#}$ indicates significant differences between arsenic group and zinc + arsenic group ($p^{\#} < 0.05$, $p^{\#\#} < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2020). Supplementation of antioxidants such as ascorbic acid and vitamin E (Ramanathan et al., 2002; Kannan and Flora, 2004); *n*-acetylcysteine (Flora, 1999); and micronutrients such as selenium (Jamwal et al., 2019) is effective in treating arsenism. From the perspective of improving neurotoxicity and reproductive toxicity caused by As, Zn supplementation is comparable to α-tocopherol (Kumar and Reddy, 2018) and ascorbic acid (Altoe et al., 2017), respectively. Considering nutrition, diets containing Zn at 117.1 mg/kg increased weight gain, reduced feed intake, and improved antioxidant activity in the hepatopancreas of juvenile Pacific white shrimp (*Litopenaeus vannamei*) (Shi et al., 2020). Song et al. (2017) also confirmed the positive effects of Zn supplementation in enhancing growth performance, physical barrier function, and intestinal immunity of juvenile grass carp (*Ctenopharyngodon idella*). Moreover, Zn supplementation has been reported to mitigate cellular metabolic stress in *Pangasianodon hypophthalmus* exposed to lead (Pb) and high temperature (Kumar et al., 2020). More recently, the enhanced production of reactive oxygen species (ROS) has been reported to contribute to cell injury associated with As exposure (Hu et al., 2020). A pioneering study reported arsenite tolerance in mice treated with Zn (Kreppel et al., 1994), which might be attributed to the restoration of the pro-oxidant/antioxidant balance (Rahman et al., 2019). Zn^{2+} can be used as a cost-effective, easily available, and practical candidate for the treatment and prevention of arsenism in common carp. We hypothesized that As-induced, systemic, pathological changes are ROS-dependent and driven by oxidative damage, which might be alleviated by Zn supplementation.

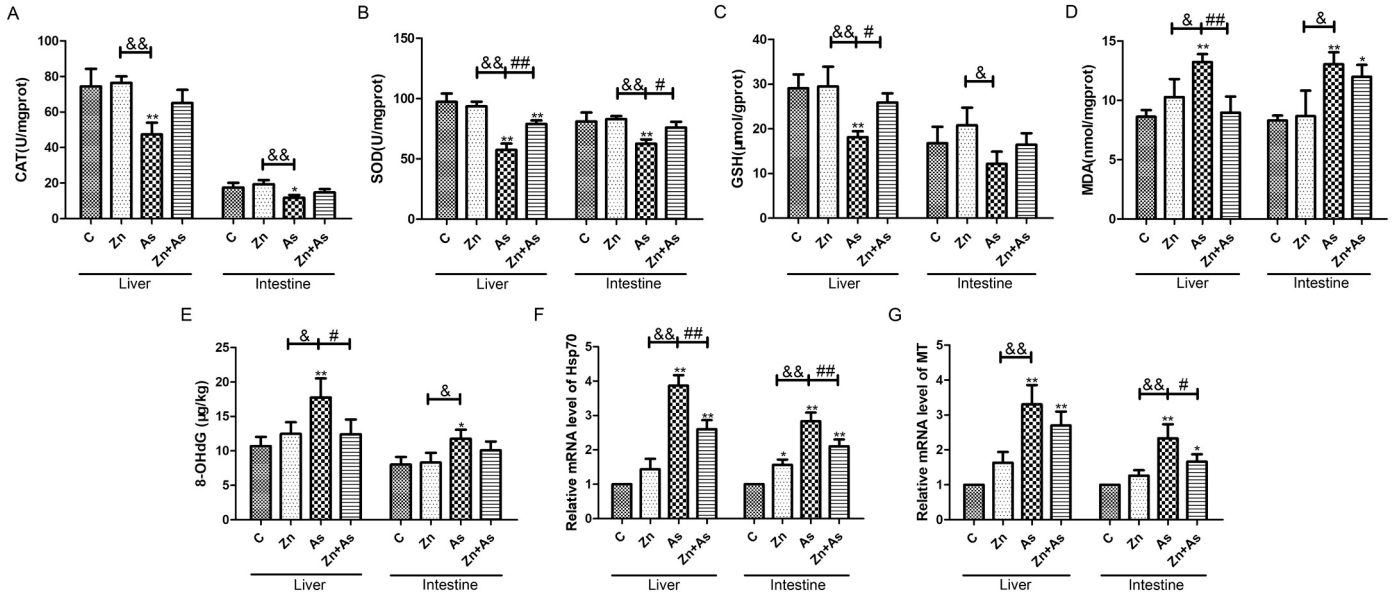

Fig. 3. Oxidative stress responses of liver and intestinal. (A) activities of CAT, (B) activities of SOD, (C) content of GSH, (D) content of MDA, (E) content of 8-OHdG, (F) mRNA level of HSP70, (G) mRNA level of MT. The * indicates significant differences between control group and experience group ($p^{*} < 0.05$, $p^{**} < 0.01$), the $^{\&}$ indicates significant differences between zinc group and arsenic group ($p^{\&} < 0.05$, $p^{\&\&} < 0.01$), the $^{\#}$ indicates significant differences between arsenic group and zinc + arsenic group ($p^{\#} < 0.05$, $p^{\#\#} < 0.01$).

In addition to participating in biomolecule synthesis and xenobiotic detoxification, the endoplasmic reticulum (ER) also plays a role in metabolic processes (Fu et al., 2012). Certain xenobiotics such as Cu^{2+} (Song et al., 2016) and Pb^{2+} (Zhu et al., 2013), induce ER stress in teleosts, leading to unfolded protein response (UPR) in the ER lumen. In yellow catfish (*Pelteobagrus fulvidraco*), ER stress and liver lipid metabolic abnormality were found to co-occur with other toxicological responses (Song et al., 2016). Mechanistically, hepatic lipid homeostasis is regulated by numerous ER membrane-bound transcriptional factors and nuclear receptors, such as sterol regulatory element-binding protein 1C (SREBP-1c), liver X receptor alpha (LXRa), and peroxisome proliferator-activated receptors (PPARγ) (Wang et al., 2019b; Lee et al., 2008). However, the exact mechanism underlying arsenite-induced ER stress and the role of Zn in fish, particularly considering the regulation of these transcriptional factors, remains poorly understood.

In this study, common carps were subjected to environmentally relevant concentrations of As for 30 days. In addition, a protective concentration of Zn was added (Malekpouri et al., 2011; Zhao et al., 2019b). The chronic systemic effects of As on global growth parameters and intestinal and hepatic damages were investigated. More importantly, the potential detoxification effect of Zn exposure on As-treated fish was assessed in the aquatic environment. We anticipate that this strategy could be extensively applied to assess and prevent the health risks associated with environmental pollutants in fish and humans.

2. Materials and methods

2.1. Animals and samples

$ZnCl_2$ (CAS No. 7646-85-7) and arsenic trioxide (As_2O_3) (CAS No. 1327-53-3) were purchased from Sigma-Aldrich (St. Louis, MO, USA). In total, 120 common carps (mean length, 16.65 ± 1.35 cm; mean weight, 110.50 ± 11.39 g) were purchased from Harbin Fishing Ground. Compressed air was supplied via air stones from air pumps, and pH of 7.0–8.0, water temperature of 27.0 ± 1.5 °C, total ammonia nitrogen concentration of < 0.05 mg/L, and dissolved oxygen level of > 6.0 mg/L were maintained. The natural light cycle of 12-h day: night was maintained. The fish were randomly divided into four groups with 30 fish in each group, 3 tanks per group and 10 fish per tank: control group (no As and Zn supplementation), As group (2.83 mg/L) (Zhao et al., 2019b), Zn group (1 mg/L) (Malekpouri et al., 2011) and As group (2.83 mg/L) + Zn (1.0 mg/L). After acclimatizing the fishes for 14 days, they underwent 30 days of semi-static exposure. All fishes were manually fed a commercial basal diet (ingredient and nutritional composition is shown in Table. S1) at a rate of 2% of the body weight, three times a day (08:00, 12:00, and 17:00). The experimental procedures were in accordance with the Guide for Use and Care of Laboratory Animals (European Communities Council Directive 86/609/EEC), and animal maintenance was approved by the local ethics committee at NEFU (approval no. UT-31; June 20, 2014).

2.2. Determination of physical characteristics

After 30 days of exposure followed by 1 day of fasting, all surviving fish were weighed to determine weight gain. During the experiment, deaths were recorded to determine the survival rate. Feed efficiency was calculated as the total wet weight (g)/the total feed (g). The liver index was calculated according to the following formula: (liver weight (g)/body weight (g) × 100%.

2.3. Biochemical assays

After inducing anesthesia with 20 mg/L of 3-aminobenzoic acid ethyl ester methanesulfonate (MS-222; Aladdin, Shanghai China), fish were sacrificed for biochemical assays. The serum, hindgut and liver were. Serum complement 3 (C3, 450 nm), C4 (450 nm), immunoglobulin M (IgM, 450 nm) levels and organic 8-hydroxy-2-deoxyguanosine (8-OHdG, 450 nm) levels were spectrophotometrically detected using ELISA kit (Jiangsu Kete Biological Technology, MEIMIAN, China) with a microplate reader (Thermo Scientific, USA). Briefly, the standard sample was first diluted. Then, blank was placed in the control well and was adjusted to zero with double-distilled water, without sample and enzyme reagent, the other steps were the same. Subsequently, 50 μL of standard sample was accurately added to the enzyme label coated plate. Then 40 μL of sample diluent was added into the sample well to be tested and then 10 μL sample to be tested.

Triglyceride (TG, 510 nm) and total cholesterol (TCHO, 500 nm)

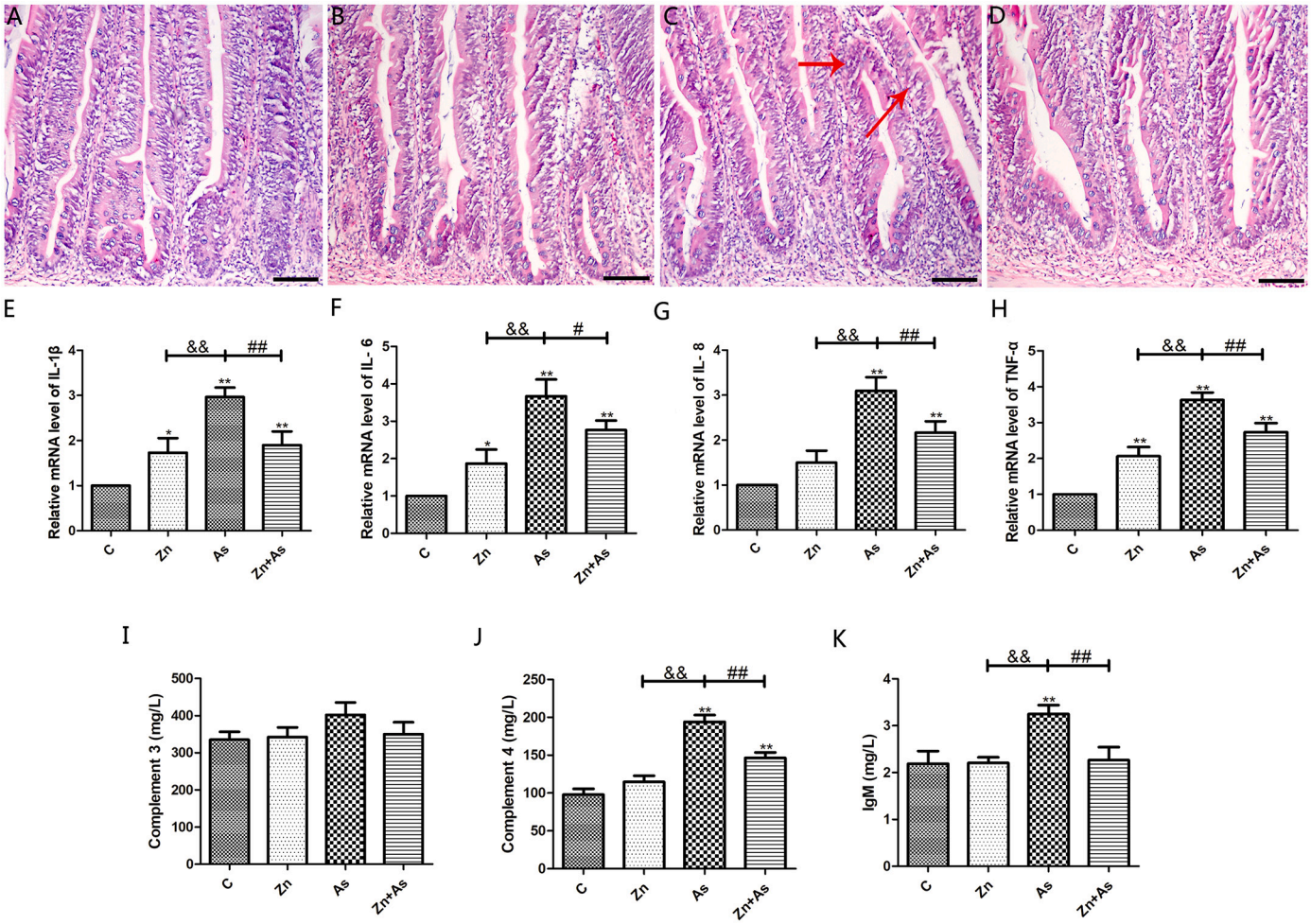

Fig. 4. Intestinal histopathological structure and inflammatory indicators mRNA levels. (A) control group, (B) zinc group, (C) arsenic group, (D) zinc + arsenic group. Red arrows: villous injury. (*E*-H) IL-1β, IL-6, IL-8, TNF-α, (I) levels of Complement 3, (J) levels of Complement 4, (K) levels of IgM. The * indicates significant differences between control group and experience group ($p^* < 0.05$, $p^{**} < 0.01$), the $^{\&}$ indicates significant differences between zinc group and arsenic group ($p^{\&} < 0.05$, $p^{\&\&} < 0.01$), the $^{\#}$ indicates significant differences between arsenic group and zinc + arsenic group ($p^{\#} < 0.05$, $p^{\#\#} < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

levels of serum were spectrophotometrically determined using commercial assay kits (NJJCBIO, China) with a microplate reader (Thermo Scientific, USA). Briefly, for blank, 2.5 μL double-distilled water +250 μL working solution was used; for standard, 2.5 μL calibrator +250 μL working solution was used; and for sample assessment, 2.5 μL sample + 250 μL working solution was used.

Malondialdehyde (MDA) and glutathione (GSH) levels and superoxide dismutase (SOD) and catalase (CAT) activities of liver and intestine were spectrophotometrically determined using commercial assay kits (NJJCBIO, China) with a microplate reader (Thermo Scientific, USA). Specifically, for MDA level determination, blank solution (absolute ethanol + working solution 1), standard solution (MDA standard + working solution 1), sample solution (sample to be tested + working solution 1), and control solution (sample to be tested + working solution 2) were made. Double-distilled water was used for calibration, and then, the absorbance of each solution was measured at 532 nm and at an optical path of 0.5 cm. For determining GSH level, blank (100 μL reagent 1), standard (100 μL GSH standard), and sample (100 μL sample supernatant) solutions were made, according to manufacturer instructions. At 405 nm, the absorbance of each pore was measured. For determining SOD activity, the control well (20 μL distilled water +20 μL enzyme working solution +200 μL substrate solution), control blank well (20 μL distilled water +20 μL enzyme diluent +200 μL substrate solution), sample well (20 μL sample to be tested +20 μL enzyme working solution +200 μL substrate working solution), and blank sample well (20 μL sample to be tested +20 μL enzyme diluent +200 μL substrate solution) were formulated. The absorbance of each well was measured at 450 nm. For determining CAT activity, the reference and sample tubes were set according to manufacturer instructions. Double-distilled water was used to calibrate the kit to zero, and then, the absorbance value of each solution was measured at 405 nm with an optical path of 0.5 cm.

2.4. Histomorphology and electron microscopy observations

The hindgut and liver tissues (1.0 mm^3) were stained with H&E and oil red O, and were then subjected to histological observation according to the method reported by Barbieri et al. (2016) and Zhao et al. (2017) using a light microscope (Eclipse Ci-L, Japan). For electron microscopy, liver and intestinal tissues were extracted and immediately sectioned. Staining was conducted according to the method reported by Zhao et al. (2017). Microphotographs were obtained using JEM-1200ES, Japan.

2.5. Quantitative real-time PCR (qRT-PCR) analysis

Total RNA from the hindgut and liver tissues were isolated using TRIZOL (Invitrogen, USA). The quantity and quality of total RNA were determined using ULTROSPEC 1100 Pro (Amersham Biosciences), and

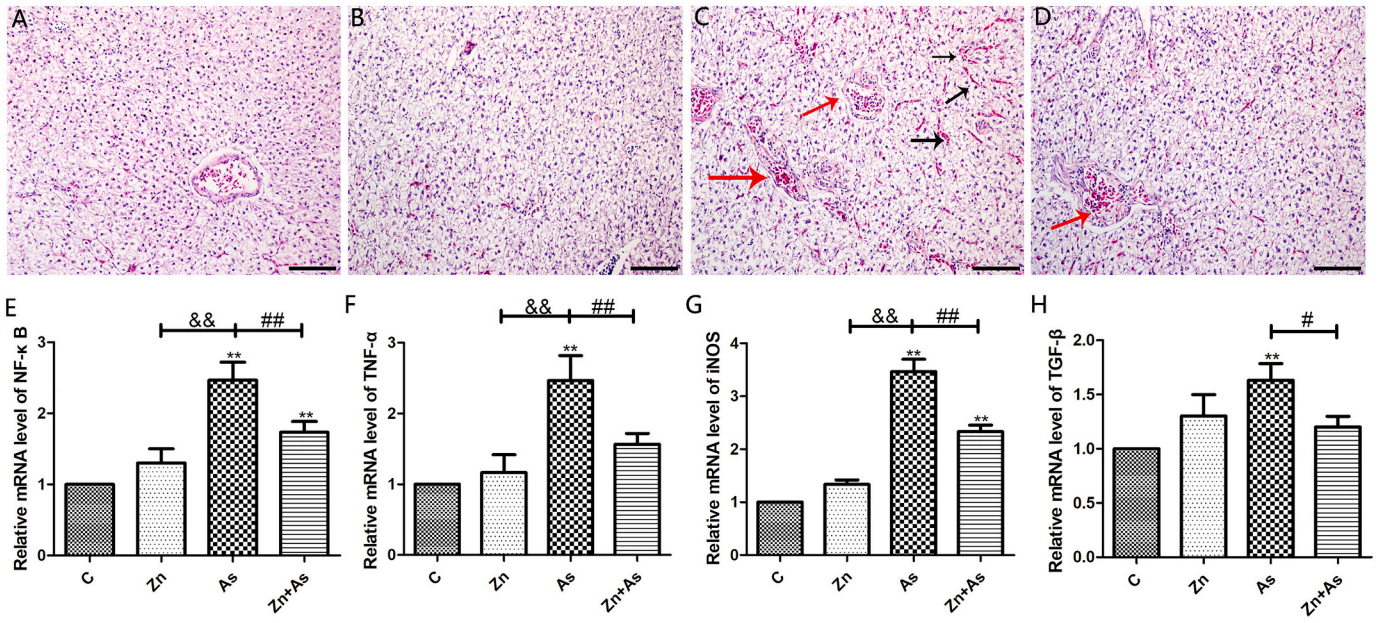

Fig. 5. Liver histopathological structure and inflammatory indicators mRNA levels. (A) control group, (B) zinc group, (C) arsenic group, (D) zinc + arsenic group. Red arrows: bleeding point, black arrows: inflammatory cell infiltrates, (E) NF-κB, (F) TNF-α, (G) iNOS, (H) TGF-β. The * indicates significant differences between control group and experience group ($p^* < 0.05$, $p^{**} < 0.01$), the $^{\&}$ indicates significant differences between zinc group and arsenic group ($p^{\&} < 0.05$, $p^{\&\&} < 0.01$), the $^{\#}$ indicates significant differences between arsenic group and zinc + arsenic group ($p^{\#} < 0.05$, $p^{\#\#} < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

all RNA samples showed good quality with the OD260/280 values ranging from 1.8 to 2.0. RNA for 1 g was reverse transcribed to cDNA using the HiScript II Q Select RT SuperMix for qPCR following the manufacturer's instructions (Vazyme Biotech Co., Ltd). Relative mRNA levels were measured using a LightCycler® 480 with the FastStart Universal SYBR Green Master reagents (Roche, Switzerland). mRNA expression was assessed using the $2^{-\Delta\Delta CT}$ method by normalizing to β-actin, as previously described (Jing et al., 2019). The primer pairs used are shown in Table S2.

2.6. Western blotting

After tissue lysis (Beyotime, China), BCA method was used to determine total protein level (Beijing Solarbio Science & Technology Co., Ltd.). After separation using PAGE gels, total proteins were transferred to nitrocellulose membranes, then detected using glucose-regulated protein 78 (GRP78) (Proteintech, China), phospho-protein kinase-like ER kinase (p-PERK), phosphor-eukaryotic initiation factor-2α (eIF2α) (Bioss, China), PERK, eukaryotic initiation factor-2α (eif2α), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1alpha (IRE1α) (Wanleibio, China). Then, the proteins were normalized using β-actin (Proteintech, China), as previously described (Wang et al., 2018b).

2.7. Data analysis

In this study, fishes were divided into three independent groups with at least three fishes in each group. SPSS (Chicago, IL, USA) was used for data analysis, and GraphPad Prism 5 (version 5.01, Inc., La Jolla) was used for plotting graphs. The data were normally distributed, as assessed by analysis of variance, and were presented as means ± SD (Shapiro–Wilk normality test). Subsequently, all data were tested using Tukey's test. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Growth performance and visceral index of fish following As exposure

No death was recorded during the experiment. Exposure of common carps to As or/and Zn did not retard growth performance, decrease feed efficiency, or lead to atrophied liver compared with no exposure ($p > 0.05$; Fig. 1A–C). Interestingly, a significant decrease of feed efficiency was found in the As group than in the Zn group ($p < 0.05$; Fig. 1B).

3.2. Zn decreased As-induced increased TG and TCHO levels

In the As group, carps showed significant ($p < 0.05$) increases in serum TG and TCHO levels than in the control group. However, these serum indexes were significantly lower in the As + Zn group than in the As group ($p < 0.05$) (Fig. 1D, E).

3.3. Zn restored altered intestinal tight junctions (TJs) in As-exposed carps

From the mRNA profile of the TJ genes, we determined that As significantly suppressed claudin-3/4/7, occludin, and zonula occludens-1/2 (ZO-1/2) levels ($p < 0.05$; Fig. 2A–F). These abnormalities were markedly ameliorated by the co-administration of Zn.

TEM results revealed that, the TJs structure had a black, tight electron band, extending from the top of the epithelium to the base layer. As shown in Fig. 2G, the intercellular space in the intestines of fish in the As group were irregularly widened ($p < 0.05$), which was reduced in the As + Zn group.

3.4. Zn decreased oxidative injury and DNA damage in As-exposed carps

Exposure of carps to As significantly decreased the activities of CAT and SOD and the level of GSH in the intestine and liver ($p < 0.01$; Fig. 3A–C). However, these indices increased in the As + Zn group

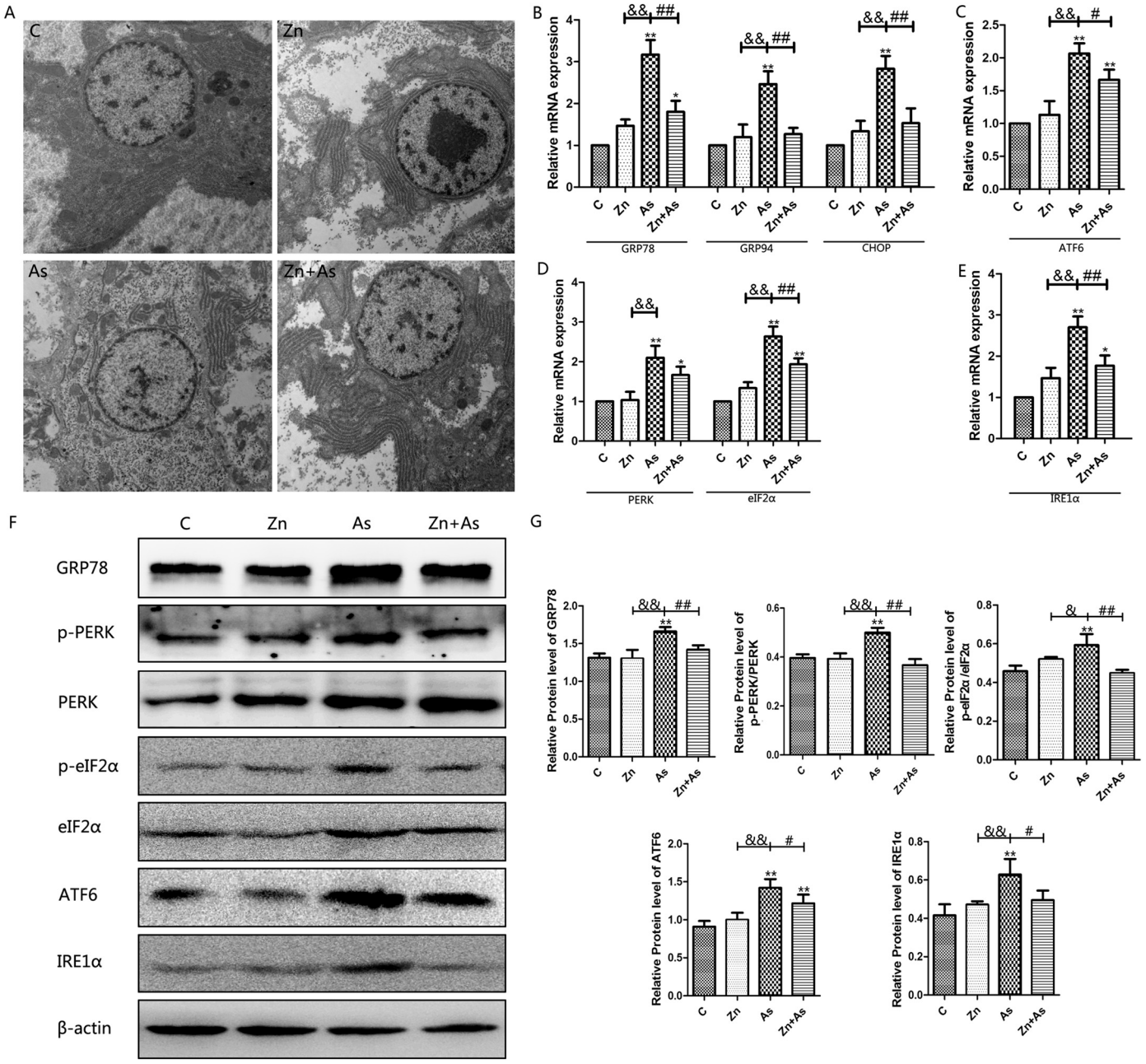

Fig. 6. (A) Liver ultrastructure (TEM, bars 1 μm) of Liver. C group showing the typical appearance of mitochondria with clear cristae and endoplasmic reticulum with flat. Hepatocytes in As groups showing the swelling of endoplasmic reticulum. (B-E) The expression of genes involved in ER stress in carp. (F-G) Protein expression of ER pathway. The * indicates significant differences between control group and experience group ($p^{*} < 0.05$, $p^{**} < 0.01$), the $^{\&}$ indicates significant differences between zinc group and arsenic group ($p^{\&} < 0.05$, $p^{\&\&} < 0.01$), the $^{\#}$ indicates significant differences between arsenic group and zinc + arsenic group ($p^{\#} < 0.05$, $p^{\#\#} < 0.01$).

compared with those in the As group. Zn treatment alleviated the increase in MDA levels and significantly restored oxidative damage in the intestine and liver ($p < 0.01$; Fig. 3D).

Bioaccumulation of heavy metal, such as cadmium, chromium and Pb, is positively correlated with heat shock protein 70 (HSP70) and MTs in fish liver, suggesting that these biomarkers are effective in assessing the quality of aquatic environments contaminated with such metals (Savassi et al., 2020). In line with this, we noted significantly increased expression of HSP70 and MT genes following As exposure in the liver and intestine ($p < 0.01$; Fig. 3F and G), indicating a role of these genes in intracellular defense (Rahman and De Ley, 2017). Subsequently, 8-OHDG, a marker for DNA oxidation, was upregulated in the liver and intestine of As-exposed carps ($p < 0.05$; Fig. 3E). This indicated that oxidative stress-induced by As elicits a detoxification response, causing DNA damage. Simultaneously, detoxification effects were observed in the As + Zn group, as oxidative stress and DNA damage were restored in the liver and intestine.

3.5. Zn normalized innate immunity and inflammation in As-exposed carps

Noticeable damage and fracture of intestinal villi, inflammatory infiltration, cell swelling and vacuolation in the liver were observed (Figs. 4C and 5C). The hind intestine and liver showed intact intestinal villi and reduced inflammatory infiltration in the As + Zn group were

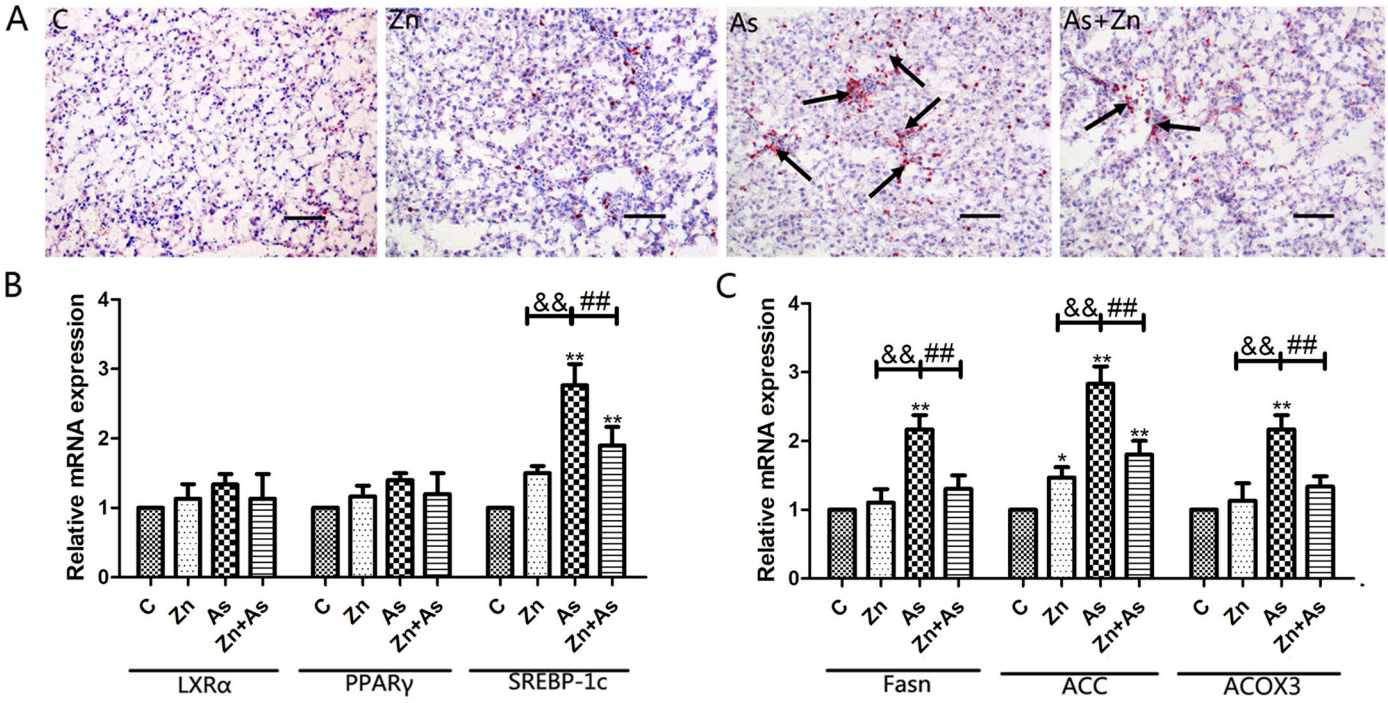

Fig. 7. (A) oil-red O staining, lipid was red-colored and nuclei-blue colored. (B—C) SREBP-1c activation in hepatic lipid metabolism. The * indicates significant differences between control group and experience group ($p^{*} < 0.05$, $p^{**} < 0.01$), the & indicates significant differences between zinc group and arsenic group ($p^{\&} < 0.05$, $p^{\&\&} < 0.01$), the # indicates significant differences between arsenic group and zinc + arsenic group ($p^{\#} < 0.05$, $p^{\#\#} < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

compared with those in the As group (Figs. 4D and 5D, indicating an improvement in inflammation. These results suggest that Zn reduces inflammation induced by As.

To describe changes in immune homeostasis more accurately, specific indicators were assessed in the intestine, blood serum and liver. We found that As exposure induced the upregulation of pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor α (TNF-α) in the intestine ($p < 0.01$; Fig. 4E–H), increased the levels of C3 ($p > 0.05$; Fig. 4I), C4, and IgM in blood serum ($p < 0.01$; Fig. 4J and K); and induced increased nuclear factor kappa-B (NF-κB), TNF-α, inducible nitric oxide synthase (iNOS), and transforming growth factor-β (TGF-β) levels in the liver ($p < 0.01$; Fig. 5E–H). These results indicated that, under As stress, inflammation increased and immune functions reduced in carps. However, compared with As treatment, As + Zn treatment resulted in a significant decrease in IL-1β, IL-6, IL-8, and TNF-α levels in the intestine; a reduction in IgM, C3, and C4 levels in the blood serum; and a decrease in NF-κB, TNF-α, iNOS, and TGF-β levels ($p < 0.05$) in the liver (Figs. 4 and 5).

3.6. Zn decreased hepatic ER stress and UPR in As-exposed carps

Ultrastructural changes revealed that As exposure caused ER swelling and hepatocytes damage (Fig. 6A). We then studied the effect of As—Zn interaction on ER stress and lipid metabolism. Three UPR pathways (PERK–eIF2α, IRE1α, and ATF6) and their downstream genes were examined under As stress (Fig. 6). Results showed that ER stress caused by As treatment increased the transcription of hepatic GRP78/BiP, GRP94, and C/EBP homologous protein (CHOP) ($p < 0.01$; Fig. 6B). However, Zn co-administration significantly downregulated the protein levels of hepatic GRP78, p-PERK, p-eIF2α, ATF-6, and IRE-1α ($p < 0.01$ or $p < 0.05$; Fig. 6F and G). Moreover, the upregulation of the mRNA levels of ATF-6, PERK, eIF2α, and IRE-1α in the liver due to As exposure was were also attenuated by Zn ($p < 0.01$ or $p < 0.05$; Fig. 6C–E).

3.7. Zn decreased hepatic lipid metabolism in as-exposed carps

As shown in Fig. 7, the amount of lipid droplets in hepatocytes increased with As exposure (Fig. 7A). However, the As + Zn group showed decreased number of lipid droplets.

On day 30, the transcriptional levels of SREBP-1c and its targeting enzymes ACC and FAS significantly increased in the As group ($p < 0.01$; Fig. 7B, C), which were markedly attenuated in the As + Zn group ($p < 0.01$; Fig. 7B, C). However, the levels of LXRα and PPARγ remained unchanged, indicating that only SREBP-1c, but not LXRα and PPARγ, was involved in the As-induced abnormality in lipogenesis.

4. Discussion

At the phenotypic level, growth rate is regarded as a critical endpoint to assess chronic toxicity in fish models (Ibrahim et al., 2019). On the contrary, the present study showed no significant change in growth performance, feed efficiency, and liver/body ratio after As stress. However, serum TG and serum TCHO levels showed significant ($p < 0.05$) increases in the As group. These observations can be attributed to two reasons: (1) sub-chronic As exposure at an environmental concentration far from changed phenotype. And (2) metabolic expenditure increased after As exposure for detoxification and homeostasis maintenance. Our results further showed that Zn^{2+} could restore serum index to some extent. Similarly, the protective effect of Zn^{2+} (1 mg/L) on As toxicity (2.83 mg/L) has been reported in the intestinal segments of common carps (Zhao et al., 2019a).

The toxic effects observed in carps following long-term As exposure include reduced antioxidant capacity, altered detoxification mechanisms, and DNA damage (Wang et al., 2018b). In our study, ROS production increased during metabolic processes, eventually increasing oxidative stress, including reduced CAT and SOD activities and GSH level as well as increased MDA level in the intestines and liver of carp, thus exhausting the antioxidant capacity in these organs. In addition, As caused intracellular stress (HSP70) and detoxification (MT) in the liver

and intestines. However, these detoxification mechanisms were not sufficient to eliminate the oxidative damage induced by As exposure, as evidenced by the increased 8-OHDG content. These severe hepatic and intestinal damages increased the perturbations in cellular membrane, and DNA, eventually leading to cell death. However, we provided evidence that Zn had protective roles in As-induced gut and liver injuries by suppressing oxidative stress and detoxification mechanisms. This was confirmed by elevated antioxidant activity and decreased Hsp70 and MT levels in the intestines and livers of As-exposed carps.

Another metabolic toxicity induced by As chronic exposure in carps is innate immune suppression, which is attributed to increased oxidative stress leading to intestinal TJ protein deformation (Fig. 2G). Arsenic exposure may destroy microtubules and F-actin cytoskeleton (Zhao et al., 2019c). By precisely regulating the trans-membrane transport of substances between adjacent cells, TJ proteins play an important role in fish intestinal health. Previous studies have characterized claudin-3/4 as a chief barrier-forming protein, and claudin-7 as a pore-forming protein (Suzuki, 2013; Sanchez et al., 2014). These proteins as well as occluding and ZO-1/2 have a barrier-forming role in the intestine (Duffy et al., 2011; Zhao et al., 2019a). The current study showed that As downregulated the mRNA levels of occludin, claudin 3/4/7, and ZO-1/2 in the hindgut of young common carps, implying a disturbance in the intestinal barrier. However, with Zn supplementation, expression of these proteins was upregulated in the hindgut of the fish via downregulating TJ protein transcription. Interestingly, the altered intestinal permeability following As stress and the recovered integrity following Zn supplementation did not seem to have a direct correlation with our observations of unchanged body weight. Hahn and Baker (1993) also reported that similar levels of Zn supplementation were not effective in improving weight gain despite higher plasma Zn. This would further corroborate the possibility of a luminal effect rather than a systemic one.

Burst of ROS has been reported to induce inflammation (Wang et al., 2018a; Wang et al., 2018c; Zhao et al., 2018). Notably, NF-κB functions as a recruiter and activator of immune cells and then induces transcription of its downstream cytokines such as TNF-α, IL-1β, IL-6 and IL-8 (Chi et al., 2018; Ming et al., 2020). Consistent with these findings, our results showed increased protein or mRNA levels of NF-κB, TNF-α and iNOS in the liver and enhanced mRNA expression of IL-1β, IL-6, IL-8 and TNF-α in the intestine; these results confirmed the pro-inflammatory responses after induced by As. We found that Zn reduced the levels of these pro-inflammatory cytokines and improved intestinal mucosal barrier in carps following As exposure. This might be because Zn supplementation inhibited the synthesis of IL-6, which is produced following TNF-α and IL-1β release in the inflammation cascade, thus impairing TJ structure (Al-Sadi et al., 2009).

A previous study on juvenile turbot (*Scophthalmus maximus L.*) showed that injury to intestinal mucosal integrity leads to the leakage of serum immune parameters, such as immunoglobulin and complement (C), in the lumen (Dai et al., 2020). As humoral defense factors in fish, Cs and immunoglobulins are considered standard inflammatory biomarkers of immunotoxic evaluation in aquatic invertebrates (Whyte, 2007). In the present study, the concentrations of serum IgM, C3, and C4 showed significant increase in carps exposed to As, which was in agreement with previous results (Islam et al., 2012). As expected, these serum immune parameters in carps further suggested the protective effects of Zn on the humoral immune system under As stress.

The above results suggested that, chronic As stress severely impaired the immune system, in general and intestinal barrier as well as caused liver inflammation, as determined using biochemical measurements and pathological observations. However, the mechanism underlying arsenism in unclear at the subcellular level, and Zn might play a momentous role in the treatment and prevention of this condition. Interestingly, ER is an organelle that exhibits sensitive stress responses under toxic effects (Wang et al., 2019a; Xie et al., 2019). Notably, hepatic GRP78 and UPR signaling in tilapia was activated upon exposure to Cu^{2+} (Song et al., 2016). As an adaptive response, UPR activation helps resist the accumulation of misfolded proteins and reduce ER load (Ji, 2008). In the present study, the chaperons and folding sensors in the ER, GRP94 and GRP78/BiP, and three UPR branches, the PERK–eIF2α, IRE1α, and ATF-6 pathways, were significantly displayed upregulated in the As group. This confirmed the induction of ER stress, which was further evidenced by ultrastructural observations. Our experimental results are similar to those of previous metal toxicological studies conducted in mammals, in which cadmium, Pb, nickel, and cobalt induced ER stress (Kitamura and Hiramatsu, 2010; Marta et al., 2008; Wang et al., 2019a).

In vertebrates, lipid metabolism primarily involves lipid uptake, transport, secretion, lipogenesis and lipolysis, and any abnormalities in these processes will lead to disordered metabolism (Karavia et al., 2013; Lu et al., 2013). Arsenic was reported to increase both the cellular lipid droplet (observed using hepatic oil red O staining) and TG content, suggesting a potential cross talk between lipid accumulation and ER stress. These might be attributed to 1) the role of ER as the site of TG synthesis in fat cells (Wolins et al., 2006); 2) SREBP protein activated under ER stress initiates the transcription of its downstream genes, including the SREBP-1c pathways (Gregor and Hotamisligil, 2007); and 3) excessive ER stress increases liver TG accumulation. Similarly, in our study, waterborne As exposure induced ER stress and subsequently activated the SREBP-1c pathway, leading to abnormal lipid deposition in the liver of common carps. Besides SREBP-1c, PPARγ and LXRα are two core transcriptional factors involved in fatty acid synthesis. In the present study, As exposure did not significantly alter the mRNA levels of PPARγ and LXRα, suggesting that SREBP-1c, and not PPARγ or LXRα, increased the level of fatty acid synthetase in carp liver (Dong et al., 2019; Xu et al., 2019).

We then investigated the role of As−Zn interaction in hepatic ER stress-induced alteration in lipid metabolism. Zn has long been recognized for its potential antioxidant properties by inducing MT and attenuating excessive ROS (Aimo et al., 2010). Our results demonstrated the potential of Zn supplementation to reduce oxidative and ER stresses. In our study, Zn inhibited the PERK–eIF2α and IRE1 pathways as well as GRP78/Bip and GRP94 induced by As. Moreover, Zn also significantly attenuated As-induced hepatic lipid accumulation by downregulating the SREBP-1c pathway, which is consistent with the findings of Zhang et al., (2012). Therefore, at the subcellular and molecular levels, the antioxidant properties of Zn alleviate ER stress and abnormal lipid metabolism in the liver.

5. Conclusion

Our results showed that chronic exposure to low environmental concentrations of As severely impaired the immune system, intestinal barrier effect and liver lipid metabolism in common carp. Zn, a strong antioxidant, can relieve systemic arsenism by diminution of inflammatory and ER stresses and improvement of the antioxidant system. Thus, Zn supplementation is a potential alternative for the treatment and prevention of arsenism as it prevents physiological and cellular damage in common carps in inland cultures.

Declaration of Competing Interest

None.

Acknowledgments

This work was supported by the Key Projects of Natural Science Foundation of Heilongjiang Province of China (Grant ZD2020C005); the National Natural Science Foundation of China (Grant No. 31672619); the National Key Research and Development Program of China (Grant No. 2017YFD0501702).

Appendix A. The following are the supplementary data related to this article

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2020.735965.

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