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Zinc alleviates arsenism in common carp: Varied change profiles of cytokines and tight junction proteins among two intestinal segments

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ABSTRACT

This study was designed to evaluate the effects of zinc on inflammation and tight junction (TJ) in different intestinal regions of common carp under sub-chronic arsenic insult. Fish were exposed to zinc (0, 1 mg/L) and arsenic trioxide (0, 2.83 mg/L) in individual or combination for a month. Inflammatory infiltration and TJ structure changes were displayed by H&E staining and transmission electron microscope. To further explore these changes, biochemical indicator (SOD), gene or protein expressions of inflammatory responses (NF-κB, IL-1β, IL-6 and IL-8) and TJ proteins (Occludin, Claudins and ZOs) were determined. In the anterior intestine, arsenic decreased activity of SOD, mRNA levels of Occludin, Claudins and ZOs, increased mRNA levels of ILs. However, unlike the anterior intestine, arsenic has an upregulation effects of Occludin and Claudin-4 in the mid intestine. These anomalies induced by arsenic, except IL-8, were completely or partially recovered by zinc co-administration. Furthermore, transcription factor (NF-κB) nuclear translocation paralleled with its downstream genes in both intestinal regions. In conclusion, our results unambiguously suggested that under arsenic stress, zinc can partly relieve intestinal inflammation and disruption of tight junction segment-dependently.

1. Introduction

Aquatic biota are major repositories for arsenic (As), which originated from both natural and anthropogenic sources [1,2]. Arsenical toxicity rests with numerous interacting factors especially its chemical speciation [1], therefore it is not synonymous with assessment of associated risks by quantifying total As in samples. Under normoxic water environment, most of the absorbed As (V) is metabolized into its reduced form As (III), which is more toxic to freshwater organisms [3].

In various models including mammalian species [4], poultry [5,6] or fish species [7], disruption of cellular redox homeostasis has been suggested as the primary mechanisms of arsenical toxicity, which is mainly associated with the production of reactive oxygen species (ROS) and depletion of antioxidant enzymes. In addition, this burst of oxygen clusters can lead to the activation of a redox sensitive nuclear transcription factor, nuclear factor kappa-B (NF-κB) and its cascaded inflammatory cytokines [8]. A depletion of cellular superoxide dismutase (SOD) activity and the consistently raised secretion of proinflammatory cytokines Interleukin (IL-1β, IL-6) and Tumor Necrosis Factor- alpha (TNF-α) was reported in a model of As-exposed rats [9]. In conclusion, these past studies suggest that As toxicity was triggered through provoking oxidative stress and NF-κB cascaded cellular inflammatory events.

The penetration of pathogenic and commensal bacteria from the lumen are the primary predisposing factors for autoimmune disease, inflammatory bowel diseases (IBD) and metal poisoning events [10,11]. Apical tight junction (TJ) proteins serve as a pivotal section in the maintenance of the epithelial barrier function and control of paracellular permeability. To date, a variety of protein components of TJ, such as Occludin, Claudin and Zonula occludens (ZO)-1, have been identified [12], which scarcity unambiguously participates in inflammation-induced disruption in the intestinal mucosa [13]. Under pathophysiologic conditions, inflammatory cytokines and the disruption of TJ networks have been investigated on aggravating some gastrointestinal diseases, including IBD [14,15]. The activation of NF-κB signal induces Occludin and ZO-1 breakdown and their relocation [16–18]. However, studies in local inflammation and the barrier function of gut epithelium induced by As in fish is still scanty.

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Biotoxicity of As is confounded by its interactions with other elements or compounds. For example, excessive copper was shown to aggravate myopathy in chickens residing by As-contaminated diets [19]. By contrary, simultaneous supplementation of few antioxidants like vitamins, N-acetyl cysteine, and some micronutrients like Se along with chelating agents have been found to antagonize As toxicity more effectively [20–22]. As a constitutive roles of more than 200 enzymes, zinc (Zn) functions in a variety of physiological processes, including gene expression, protein synthesis, cell division, and growth [23]. Specially, Zn is responsible for activating Cu–Zn SOD to fight against oxidative stress. Flourishing evidences suggest that Zn supplementation could attenuate heterotoxin-induced organ toxicity [24,25], and reduce the absorption of the metals from the gastrointestinal tract [26,27]. When Zn was administered prior to As, it also contributes to a decrease in As toxicity by reducing As organic residues in mice [28]. The mechanism of such interactions are warranted for more in-depth research.

The present study has been designed to evaluate the effects of Zn on the enterotoxicity of As on the morphology and development of reflexes in inflammation and TJ in the foregut and midgut of common carp. Furthermore, it may open new prospects for Zn to act as a nutritional and protective supplement for farmers who suffer As poisoning.

2. Materials and methods

2.1. Experimental designs

One hundred and twenty uniform-sized juvenile common carp (16.65 ± 1.35 cm, 110.5 ± 11.39 g) were procured from the fish farm located near Harbin and randomly maintained in 12 laboratory tanks (100 × 60 × 45 cm). Water samples were randomly collected from each aquarium to determine water-quality parameters twice a day. Water hardness (6.75 mg/L as $CaCO_3$, 1.65 mg/L as sodium and 4.23 mg/L as chloride) was determined by the ethylene diamine tetra-acetic acid titrimetric method. Measurements of dissolved oxygen (6 mg/L) were done by Winkler method and water pH (pH = 6.9) were measured using a pH-meter. The mean temperature for tested water qualities was 17.5 °C. The fish were maintained under a 12 h light and 12 h dark photoperiod, and fed with commercial dry food pellets twice a day. According to 10% 96h-LC_{50}, the dosages of Zn^{2+} (1 mg/L) [29] and As_2O_3 (2.83 mg/L) [30] were used. After a 2-week-acclimation in charcoal-filtered aerated tap water, common carp were arbitrarily divided into 4 groups (30/group): control group, Zn-group, As-group and As + Zn group. During the treatment period, sub-lethal effects like the level of activity, swimming ability and color changes visually were monitored. Animal maintenance and experimental procedures were in accordance with the Guide for Use and Care of Laboratory Animals (European Communities Council Directive 86/609/EEC) and approved by local ethics committee at Northeast Forestry University (approval no. UT-31; 20 June 2014) [31].

After a 24 h starvation before sampling, fish were anaesthetized with tricaine methanesulfonate (MS-222) solution. Then they were weighed and dissected on ice. Anterior and mid intestines were obtained and kept at −80 °C for the subsequent analysis.

2.2. SOD activity assay

Anterior and mid intestines were homogenized in 10 vol (w v^{-1}) of ice-cold physiological saline and centrifuged at 6000 × g for 20 min at 4 °C. The supernatant was conserved and used to determine the SOD activity. Using wst-1 assay, SOD activity was examined according to the manufacturer's instruction (NJJCBIO, Nanjing, China).

2.3. Histological observation and electron microscopy

The anterior intestine and mid intestinal samples (5 mm) were rapidly collected. Hematoxylin and eosin (H&E) staining was employed in morphological score [32]. The mean villus height and width were measured, the number of inflammatory cells and the goblet cells (number per villus) were counted.

The specimens were subjected to ultrathin sectioning and the staining process is described before [33]. Under JEM-1200ES electron microscope and Image pro-plus6.0, we assessed the space between the tight junctions.

Table 1
A list of primers in qPCR analysis of mRNA expression of the target genes.

Genes	GenBank accession	Primer sequence
IL-1β	XM_019080073.1	GAGTGAACTGCACCAAACAAC
		GTCGGCACTGTCAGAGTAAAT
IL-6	AY102632.1	GGACGACAGAGCTCTTAGAAAC
		GGCCAGAGACTGTTGATACTG
IL-8	KU881637.1	CTGTCGCTGCATTGAAACTG
		TTCAGGGTGGCAATGATCTC
occludin	XM_019113097.1	GGAGAGACTGCCAACGATTT
		TTCGGCACACAGTTCCTTATAG
claudin-3	XM_019094930.1	CGGGACTTCTACAACCCAATAC
		ACAGCAATGCTCCTCCAATTA
claudin-4	XM_019075830.1	GCTGGGTCAGGCCATTTA
		CTGTACCCTCCTCTCTCTATGT
claudin-7	JQ767155.1	GATGATCGTTGGGATCCTTCTC
		TGGCAATGCGGGACTTT
claudin-11	XM_019104625.1	GTGCTGGGAGGACTTCTTATAC
		GAGAGAATCCGAACGACATCAG
claudin-15	XR_002016662.1	GGGCTTTCTGAGCTGGATAAT
		ACATCCACAGGTTCTCGTAAAG
ZO-1	XM_019089195.1	CGAAATGACACGGGCTATGA
		AGCTTGAGTACGAGGGAGAA
ZO-2	KM112095.1	TACAGCGGGACTCTAAAATGG
		TCACACGGTCGTTCTCAAAG
β-actin	NM_181601.5	CCTTCCAGCAGATGTGGATTAG
		TGAAGTGGTAACAGTCCGTTTAG

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

Total RNA were extracted using a Total RNA Kit (Invitrogen, the U.S.A). After passing the quality inspection, the cDNA was synthesized using Hiscript QRT SuperMix (Taraka, Japan). The relative mRNA levels of cytokines- and TJ-related genes (Table 1) were performed on a LightCycler® 96 (Roche, Switzerland) and determined with the FastStart Universal SYBR Green Master reagents (Roche, Switzerland). The mRNA expression levels were calculated using the $2^{-\Delta\Delta CT}$ method as previously described [5].

2.5. Western blotting

The preparation of intestinal tissue homogenates and western blot analysis of routine protein expression were carried out using previous procedures [34]. On the other hand, cytoplasmic and nuclear proteins were obtained by Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime). BCA Assay Kit (Beyotime) was used to determine protein concentration. After protein being separated on SDS-PAGE and transferred to nitrocellulose filter membranes, the membranes were blocked and then blotted with primary antibodies, including NF-κB, iκB-α, Occludin, Claudin3, ZO-1 (Wanleibio, China) and p-iκB-α, Histone H3 (Abcom, UK), β-actin (Proteintech, China). Specific bands were detected with ECL Assay Kit (Bipec Biopharma) and quantified by Image J [35].

2.6. Statistical analysis

Principal Component Analysis (PCA) and Pearson's correlation coefficients (PCC) were performed in SPSS (version 20.0). After passing a normal distribution and equal variance testing, all the data were analyzed by one-way ANOVA and presented as the mean ± SD. A

Table 2
SOD activity and histological scores.

Groups	SOD		Intestinal villus				Number per villus		Number per mm^2		Intestinal tight junction	
	U/mgprot		Length (μm)		Width (μm)		Goblet cells (× 10)		Inflammatory cells ($\times 10^3$)		Space (nm)	
	Anterior	Mid	Anterior	Mid	Anterior	Mid	Anterior	Mid	Anterior	Mid	Anterior	Mid
Control	39 ± 6.4	36 ± 5.8	417 ± 30	506 ± 37d	35 ± 5.9	48 ± 7c	3.9 ± 0.8	4.1 ± 0.8	1.4 ± 0.3	2.3 ± 0.3d	4.0 ± 0.5	3.8 ± 0.6
Zn	49 ± 5.5b	41 ± 2.6d	372 ± 52	440 ± 47ac	40 ± 3.5	49 ± 4.5	3.9 ± 0.6	4.4 ± 0.4	1.1 ± 0.1	2.1 ± 0.2d	3.8 ± 0.4	3.3 ± 0.6
As	21 ± 4.1b	26 ± 3.7bc	220 ± 32b	232 ± 51b	62 ± 14b	62 ± 5.1a	5.5 ± 0.5b	5.1 ± 0.5b	8.1 ± 0.5b	6.2 ± 0.4bd	38 ± 5.9b	31 ± 4.5bd
AsZn	33 ± 3.4a	30 ± 4.3a	395 ± 51	460 ± 44c	47 ± 16a	39 ± 4.3	4.0 ± 0.5	3.7 ± 0.4	2.2 ± 0.3b	2.7 ± 0.6ac	5.1 ± 1.0	4.9 ± 1.1

Values are means ± SD (n = 6), $P^a < 0.05$, $P^b < 0.01$ *vs* control group; $P^c < 0.05$, $P^d < 0.01$ *vs* the same treated group in anterior intestine.

significant difference was considered at $P < 0.05$.

3. Result

3.1. Antioxidase indicator

The effects of As stress and Zn as antioxidase indicator was shown in Table 2. In both the anterior and mid intestines, Zn alone had some strengthening effect on SOD activity compared with the control, Zn with As stress recovered SOD activity compared with As stress alone, in which group SOD activity displays significant decrement ($P < 0.01$).

3.2. Histological changes and scores

In the intestines of control and Zn-treated group, few inflammatory cells infiltration indicates a physiological inflammation (Table 2, Fig. 1A and E). While in the As group, as shown in Table 2, Fig. 1C and G, As induced a large number of inflammatory cells infiltration into the mucosa, and then shedding and swelling of intestinal villi, and massive goblet cells compared to control group ($P < 0.05$). In addition, the intestinal villi appeared to be shortened and widened significantly in the As group ($P < 0.05$) (Table 2, Fig. 1C and G). In the co-administration group, reduced inflammatory infiltration and intact intestinal villi were observed compared with the As (Table 2, Fig. 1D and H) in anterior and mid intestine. These results indicated that Zn exerts certain anti-inflammatory effects when intestine was insulted by As.

3.3. TJ ultrastructure

Under the TEM, TJ structure appears as a black compact electron band that starts at the top of the epithelium and extends to the base layer. As shown in Table 2 and Fig. 2C and G, the As group in both anterior and mid intestine displayed irregular widening of the intercellular space ($P < 0.05$), which was narrower than the As group in co-administration group ($P < 0.05$) (Table 2, Fig. 2D and H).

3.4. Levels of inflammatory indicators

The gene transcriptional changes of inflammatory indicators were shown in Fig. 3. In the both intestines, As alone increased mRNA levels of ILs ($P < 0.05$) compared with that of control. These anomalies induced by As, except IL-8, were completely or partially recovered by Zn co-administration ($P < 0.05$) (Fig. 3C).

NF-κB pathway was demonstrated to be activated indicated by the level of p-IκB-α and NF-κB nuclear translocation. In control groups of two intestinal regions, NF-κB resided in the cytoplasm as an inactive form and absent from the nucleus. As stress resulted in an increased p-IκB-α ($P < 0.01$), accompanied by a shift of NF-κB towards the nuclear area in the cytoplasm (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4357005/figure/f4/ Fig. 4). However, treatment with Zn suppressed the phosphorylation of IκB-α and attenuated NF-κB nuclear translocation ($P < 0.05$) compared with As stress group.

3.5. Levels of TJ indicators

The gene transcriptional and translational changes of TJ proteins were shown in Figs. 5 and 6. In the anterior intestine, As decreased the mRNA levels of Occludin, Claudins and ZOs compared with the control (Fig. 5A–D). In the mid intestine, As stress alone decreased Claudin3/7/11/15 and ZOs compared with the control accompanying by increased Occludin and Claudin4. As stress with Zn recovered these abnormal expressions to the control completely or partially (Figs. 5 and 6). It is interesting to note that Zn alone had a benefit in both intestinal segments indicated by increased TJ proteins in comparison with the control.

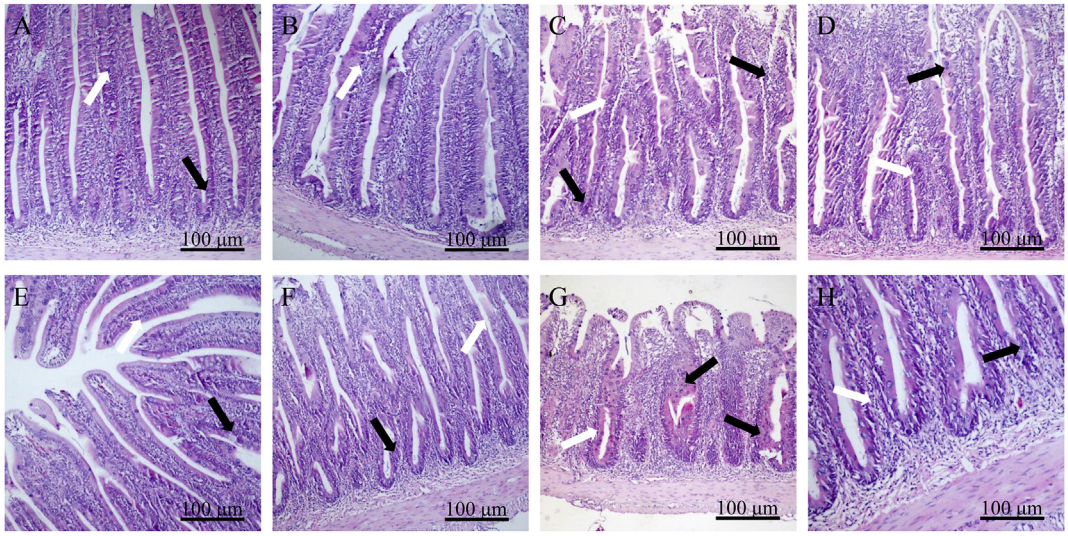

Fig. 1. Histological changes of the intestines. A–D: Anterior intestine, E–H: Mid intestine, A&E: control group, B&F: Zn group, C&G: As group. D&H: Zn + As group. In each panel, white arrows indicate goblet cells; black arrows indicate inflammatory cells.

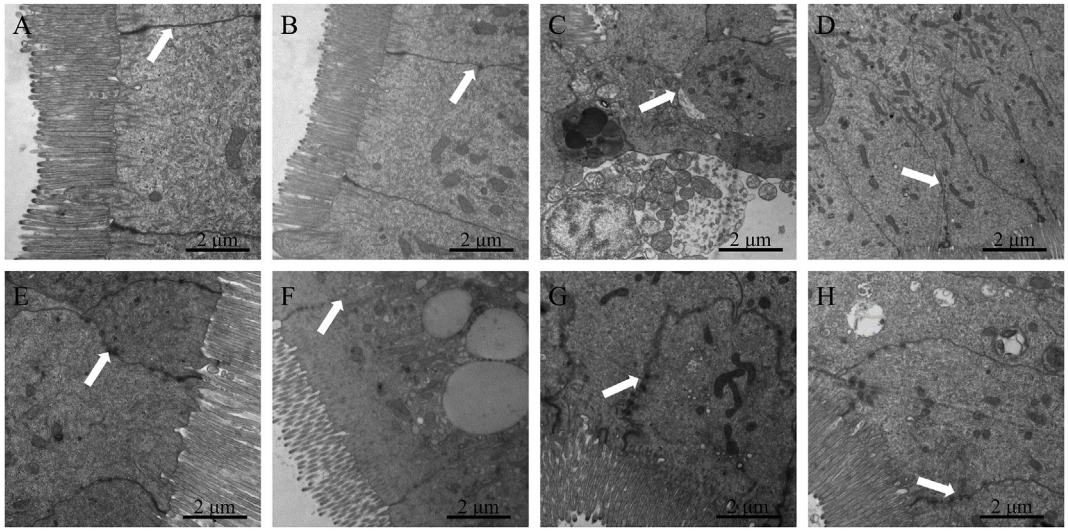

Fig. 2. TJ structural changes of the intestines. A–D: Anterior intestine, E–H: Mid intestine, A&E: control group, B&F: Zn group, C&G: As group. D&H: Zn + As group. In each panel, white arrows indicate tight junctions.

3.6. Bioinformatics analysis

The results of PCA were used to evaluate the relationship between the status of the inflammatory indicators and the TJ proteins under the four treatments. Two principal components clearly split inflammatory indicators and the TJ proteins into two groups along the first PCA axis, PC1 (Fig. 7A and B). In this axis, a positive correlation was detected for IL-1β, IL-6 and IL-8 in anterior intestine. While in mid intestine, that

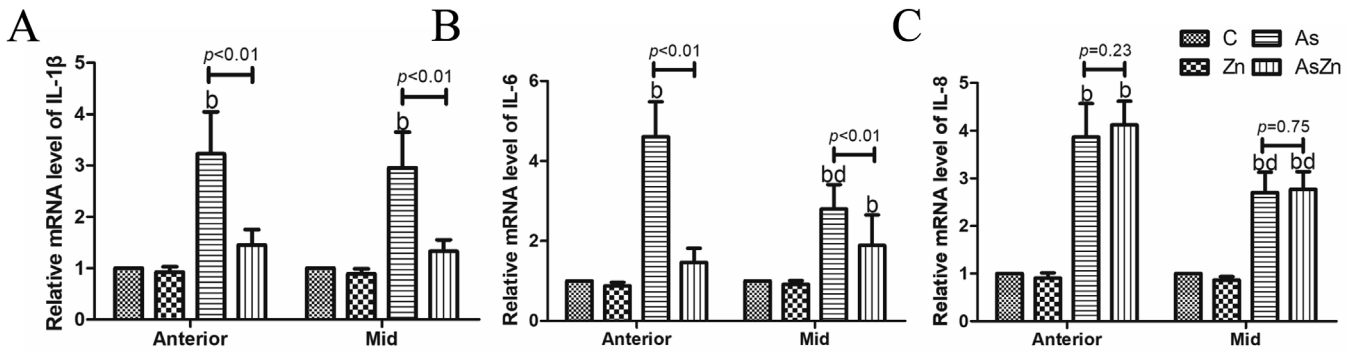

Fig. 3. Relative expression of inflammatory indicators. Values are given as mean ± SD from six fish in each group. $P^a < 0.05$, $P^b < 0.01$ vs control group; $Pc < 0.05$, $P^d < 0.01$ vs the anterior intestine; $^*P < 0.05$, $^{**}P < 0.01$; ns, no significant.

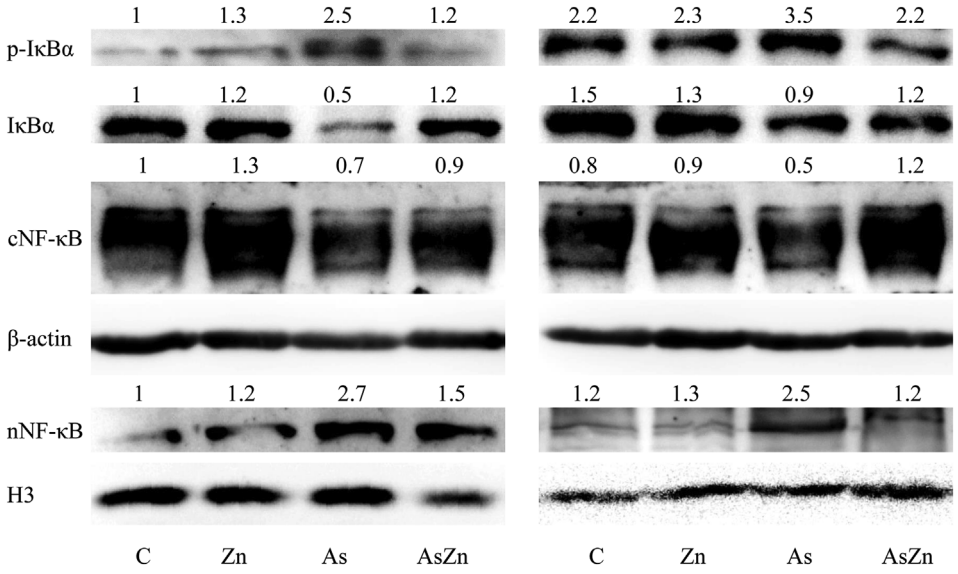

Fig. 4. The activation of NF-κB pathway. Western blot analysis of the protein levels of IκB-α, p-IκB-α, cytoplasmic NF-κB, β-actin, nuclear NF-κB and Histone 3 in the anterior intestine (the left) and the mid intestine (the right) overexposed to $ZnCl_2$ and/or As_2O_3. The density of bands were quantified by Image J and normalized to control group of the anterior intestine.

relationship occurred in IL-1β, IL-6, IL-8, Claudin4 and Occludin.

Parametric correlation coefficients were shown in Fig. 7C which was separated by the anterior and mid intestines. In the anterior intestine (the beneath), positive correlations were observed inside inflammatory indicators and the TJ proteins. However, in the mid intestine (the upper), the positive correlations observed in Claudin4 and Occludin became the contrary.

4. Discussion

The present study described the adverse effects of the arsenite sub-chronic intake within the intestinal tract as well as the protective role of Zn supplementation after such contamination. Co-administration of Zn during As exposure exerted significant protection to the altered intestinal tissue injury, oxidative stress (SOD), inflammation, and tight junction.

As the first line of enzymatic antioxidant defense, SOD activity has been shown strong correlation with the environmental conditions [36]. A reasonable explanation on the derogative SOD activity in the As group could rely on the burst of ROS, which compromise cellular viability in an irreversible manner [37,38]. Zn supplementation to As-insulted fish significantly recovered the SOD activity (Table 2). This might be due to the antioxidative property of Zn, a SOD constitutive element. What's more, morphology was also remarkably affected by As. In this study, infiltrated inflammatory cells, shedding and swelling intestinal villi, and massive goblet cells were observed in the intestine of common carp insulted by As. On the contrary, Zn supplementation protected the intestines from the oxidative damage caused by As, as also suggested by Altoé et al. [39] while working with rats.

One of the histopathological damages in intestine of common carp in this study is swelling and increasing number of neutrophil infiltration, indicating a result of IBD against the severe pathological changes. Inflammatory cytokine release plays a cascaded role in IBD. NF-κB, an important regulatory factor of inflammation, initiates the expression of inflammatory indicators including TNF-α, IL-1β, IL-6 and IL-8 [40]. The increased protein level of p-IκBα and accompanying NF-κB nuclear translocation indicate transcription initiation of inflammatory cascades. The above-mentioned inflammatory mediators were found increased in As-insulted intestinal tract, suggesting the occurrence of inflammation, which was then ameliorated when Zn was co-administrated. The present study indicated that Zn treatment not only improved the symptoms of As-treated fishes, including the amelioration of histological injury,

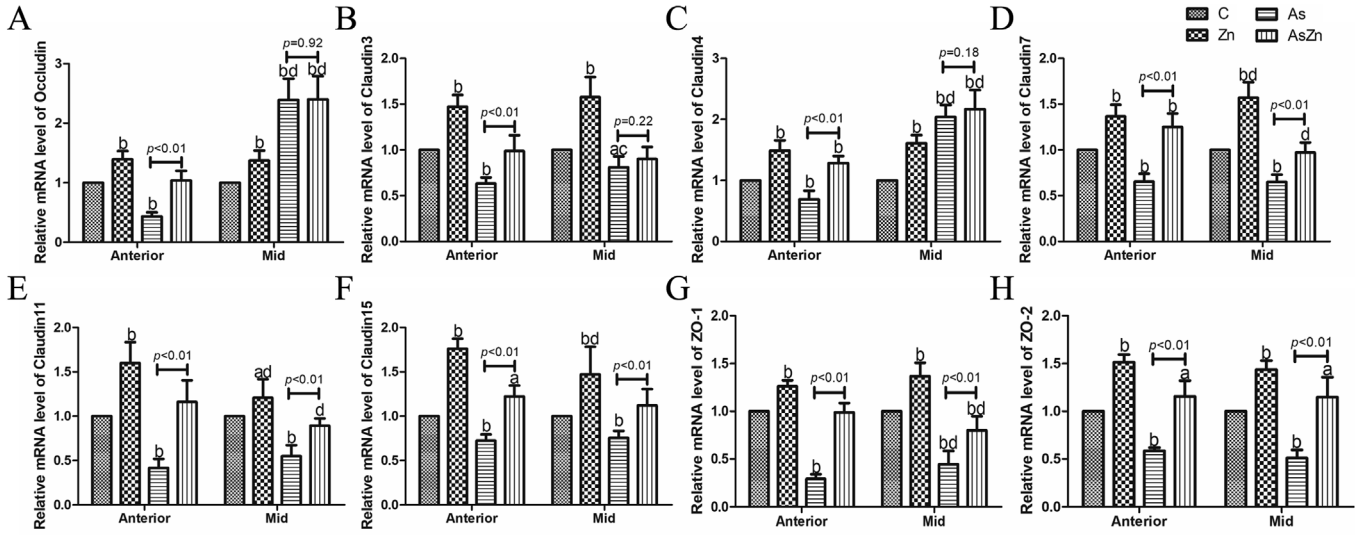

Fig. 5. Relative expression of tight junction indicators. Values are given as mean ± SD from six fish in each group. $P^a < 0.05$, $P^b < 0.01$ vs control group; $Pc < 0.05$, $P^d < 0.01$ vs the anterior intestine; $^*P < 0.05$, $^{**}P < 0.01$; ns, no significant.

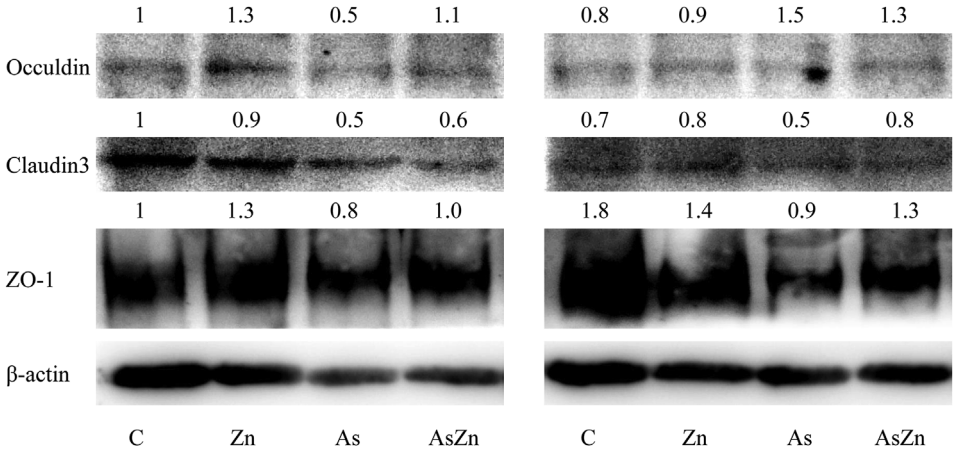

Fig. 6. Protein levels of TJ-related genes. Western blot analysis of the protein levels of Occuldin, Claudin3, ZO-1 and β-actin in the anterior intestine (the left) and the mid intestine (the right) overexposed to $ZnCl_2$ and/or As_2O_3. The density of bands were quantified by Image J and normalized to control group of the anterior intestine.

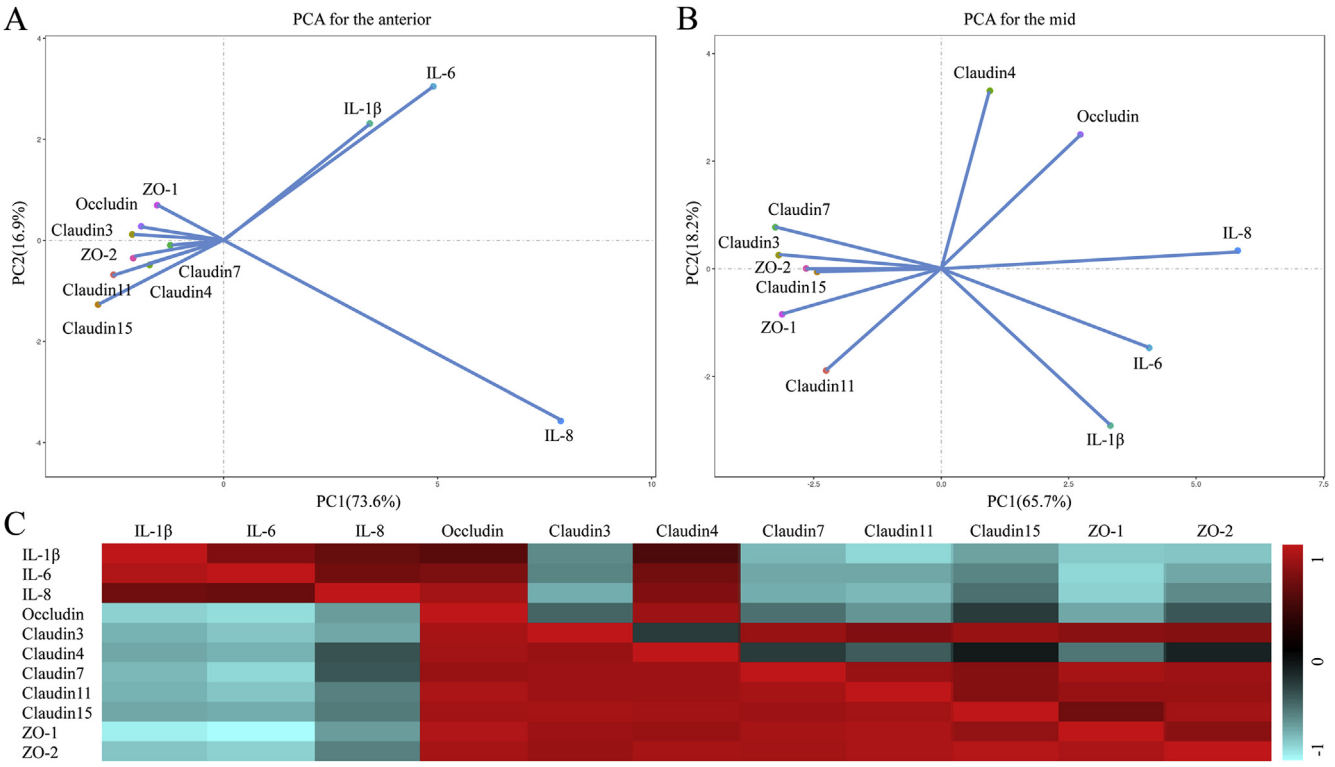

Fig. 7. Bioinformatics analysis. A: Ordination diagram of PCA levels in the anterior intestine (the left) and the mid intestine (the right) overexposed to $ZnCl_2$ and/or As_2O_3. B. Data visualization. The heatmap of Pearson's r correlation coefficient matrix among indicators measured in the anterior intestine (the beneath) and the mid intestine (the upper). Pearson's r correlation coefficient is shown by using the indicated pseudo color scale from −1 (blue) to +1 (red) relative to values for the control group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

but also down-regulated inflammatory indicators including IL-1β and IL-6, without that of IL-8, indicating the potential mechanisms involved in Zn-induced amelioration of As-induced experimental IBD. Our results are consistent with previous studies, which suggested Zn can lead to inhibition of NF-κB dependent inflammatory indicators expression [41,42] by reducing the NF-κB activation and nuclear translocation [43,44]. The discrepancies of unfluctuating IL-8 (Fig. 3C) verus other mitigating indicators (Fig. 3A and B) further suggested that Zn may prevent the deterioration of inflammation independent of IL-8, which was previously demonstrated to lead to intestinal wall injury [45].

Being integral in tight junction formation, occludin localized at tight junctions of both epithelial and endothelial cells [46]. Both claudins and occludins are coupled to cytoskeleton actin filaments with ZO-1 [47], which co-localizes with the cytoplasmic end of occludin at TJs [46,48]. We observed that in both anterior and mid intestine, the barrier was partly damaged by As stress indicated by microstructures (Fig. 2) and TJ proteins (Figs. 5 and 6). These disturbances were alleviated by Zn co-administration although not fully. Increasing evidence suggests that inflammatory indicators act as a pathophysiological regulator affecting the intestinal epithelial TJ permeability [49,50]. Markedly elevated IL-1β and IL-6 levels are accompanied with the increased TJ permeability in the IBD [51,52]. Moreover, the recovery of epithelial barrier function can be promoted by decrease in cytokine production [53]. In this sense, the PCA and PCC carried out in this study demonstrated the existence of a clear negative correlation between the inflammatory indicators and TJ proteins (Fig. 7). Thus, the decrease in inflammatory indicators and increased TJ proteins in As-induced fish treated with Zn may play a role not only in anti-inflammation but also in epithelial barrier function. Previous studies suggested a positive correlation between NF-κB signaling and the reduction of intestinal

permeability [54]. For example, under IL-1β stimulation, Caco-2 cells displayed decreased expression of Occludin protein, which normal junctional localization was distorted, and these anomalies were partially or totally recovered in the presence of an NF-κB inhibitor [52]. These findings indicate the importance of NF-κB activation (by As) in regulating tight junction, and NF-κB (by Zn) inactivation in attenuating their breakdown. In summary, our data revealed that Zn inhibited NF-κB signaling and up-regulated TJ proteins, which may prevent the adversity of As on IBD.

Fish intestine plays a principal role in osmoregulation [55]. However, regional differences in intestinal tract of fish lead to discrepant responses in the oxidative stress, inflammation under metal stresses [56]. For instance, the distal intestine of *Gadus morhua L.* and *Cyprinus carpio L.* have more Ig-positive granulocytes and macrophages than those in the proximal intestine [57,58]. We found that in the mid intestine, the production of Occludin and claudin-4 were stimulated under As stress to protect intestinal barrier function. While different scenario was found in the anterior intestine, where the TJ proteins were decreased by As stress, indicating that mid intestine have a strong ability to resist to As stress. However, Zn exerts superior antioxidative and anti-inflammatory property in anterior intestinal region than in the mid indicated by the activity of SOD and IL-6, Claudin3 and ZO-1 levels. These discrepancies between intestinal segments may be explained by two reasons. The first is that different contact sites of waterborne As exposure lead to an intestinal region-dependent accumulation of As. The second may be ascribed to different absorption efficiencies of antioxidant Zn. Previous studies suggested that compared with the mid intestinal region in fish, the anterior intestinal region was the dominant region for mineral absorption (including Zn) [59,60].

In conclusion, our study demonstrated that the advantageous effects of Zn on the intestinal tracts in As-insulted fish may be attributed to anti-inflammation effect via NF-κB pathway and the maintenance of TJ network. Our results document that as a potential prophylactic agent, Zn would be a step forward in the management of fishery which is suffering As poisoning.

Declaration of competing interest

All authors read and approved the final manuscript. The authors declare that there are no conflict of interest.

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