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Effect of sub-chronic exposure to selenium and *Allium mongolicum Regel* flavonoids on *Channa argus*: Bioaccumulation, oxidative stress, immune responses and immune-related signaling molecules

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ABSTRACT

Selenium (Se) is a micronutrient that becomes toxic when present at higher concentrations in fish tissues. *Allium mongolicum Regel* flavonoids (AMRF) have been documented to possess antioxidant, immunoenhancement and anti-inflammation properties. The aim of this study was to investigate the protective effects and potential mechanisms of dietary supplementation of AMRF and Se exposure on oxidative stress, immune responses and immune-related genes expression in *Channa argus*. A total of 480 *C. argus* were randomly divided into eight groups housed in twenty-four 200 L glass aquarium (3 tanks per group, 20 fish per tank). The fish were exposed for 56 days to waterborne Se at 0, 50, 100 and 200 μg/L and/or dietary AMRF at 40 mg/kg. The result indicated that AMRF exerted significant protective effects by preventing alterations in the levels of bioaccumulation, malondialdehyde, lysozyme, complement C3 and immunoglobulin M. AMRF also assists in the elevation of catalase and glutathione peroxidase in the liver and spleen while regulating the expression of immune-related genes including NF-κB p65, IκB-α, TNF-α, IL-1β, IL-8, HSP70, HSP90, and glucocorticoid receptor after 56 days of Se exposure. Our results suggest that administration of AMRF (40 mg/kg) has the potential to combat Se toxicity in *C. argus*.

1. Introduction

Numerous trace elements including Se, Cd, Mn, Co, Pb, Hg and Sn with unknown biological role represent one of the most widespread and serious form of environmental contamination [1]. Selenium (Se) is an essential trace-element widely distributed in natural environment [2]. Se is also an essential micronutrient for lipid metabolism or maintaining the cell reduction-oxidation balance in animals and has the capacity to exert toxic effects at excessive levels [2,3]. However, the margin between baseline nutritional requirements and toxic levels of Se is very narrow [4]. Generally, Se levels are in the range of 1–10 μg/L in natural water though levels can exceed above 50–1000 μg/L in industrial and agricultural wastewater [3]. In addition, industrial manufacturing, coal mining, petrochemical, agricultural production and aquatic drainwater can release high levels of selenium [5].

As is well known, trace element from their living environment can be accumulated in fish tissues [6]. Se can also accumulate in fish upon occurrence of high levels of waterborne Se [7]. Se accumulation can cause negative effects, such as inflammation, immune response and oxidative stress [7]. Increases in oxidative stress can induce overproduction of reactive oxygen species (ROS) in many organisms and cause damage to animals and humans [1]. Aerobic organisms contain an active antioxidant defense system that harbors a variety of defense enzymes that prevent or limit tissue damage by counteracting ROS production [2]. Endogenous antioxidant enzymes, such as catalase (CAT) and glutathione peroxidase (GPx) play an important role in scavenging harmful reactive oxygen derivatives [8], which are used to evaluate the impact of Se exposure in fish [7]. In addition, excess selenium levels affects immune function in conjunction with oxidative stress [9]. Apart from oxidative stress and immune responses, Se can also induce overexpression of inflammatory cytokines which can elicit harmful reactions in the body [7]. Therefore, the studies of potential

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antagonists against Se-induced immune response and oxidative stress are very urgent and important.

Naturally occurring products, i.e. medicinal plants and their extracts, are widely used in the research into the antagonistic effects of environmental pollutants on environmental pollutants due to their safety and effectiveness. *Allium mongolicum Regel* (AMR) belongs to the member of *Liliaceous*, and *allium* families [10]. As a characteristic wild vegetable, AMR has high nutritional value, unique flavor, rich in protein, fat, minerals, flavonoids, polysaccharides and other components [2]. Flavonoids, possessing antioxidant, immunoenhancement and anti-inflammatory properties, are the primary active ingredients in AMR [11]. Many recent studies have suggested that diets rich in flavonoids can reduce the toxicity of toxic pollutants, probably by their roles in the prevention of oxidative damage [12]. Our previous study has suggested that *Allium mongolicum Regel* flavonoids (AMRF) have several biological functions, including promoting growth, immune, antioxidant status and disease resistance in *Channa argus* [2]. Up to now, some plant flavonoids have been used as an immunopotentiator for humans and animals, including flavonoids from orange peel [13], cocoa flavonoids [14] and Korean thistle *Cirsium maackii* flavonoids [15]. They all can stimulate antioxidant defense mechanisms, anti-inflammatory defense mechanisms and provoke the immune response defense mechanisms.

Channa argus is an economically important species of freshwater fish cultured in China [16]. However, there are limited toxicological studies investigating waterborne Se exposure in *C. argus*. To the best of our knowledge, no reports have been undertaken to investigated the effects of AMRF supplementation in relation to Se toxicity. Thus, the present study was conducted to investigate the protective effects of dietary supplementation AMRF and Se exposure on the Se accumulation, oxidative stress, immune responses and immune-related genes expression in *C. argus*.

2. Materials and methods

2.1. Flavonoids preparation

Fresh *A. mongolicum Regel* were purchased from a commercial pasture (Tongliao, China). The AMRF were extracted as described in our previous study [2]. After extraction, AMRF fraction were lyophilized and stored at −40 °C until used.

2.2. Diet preparation

The experimental diets were the same as those used in our previous study [7]. Commercial diet of *Channa argus* (Dry matter: crude protein 48.1%, crude lipid 11.3%, ash 12.3% carbohydrate 20.1% gross energy, 19.3 kJ/g and protein:energy ratio, 26.0 mg/kJ, Alpha Feed Co., Ltd Shenzhen, China) was used as experimental diet. The AMRF were sprayed into the basal diet slowly with 40 mg/kg [2]. The diet was uniformly mixed in a micromixer at room temperature, and dried under aseptic conditions, pelleted and stored at −4 °C until used.

2.3. Experimental fish and living conditions

C. argus, with an average weight of 60.55 ± 0.55 g, were obtained from a commercial aquatic fry farm (Huzhou, China). Fish were transferred to 200 L glass aquaria with aerated filtered dechlorinated water and acclimated to the laboratory conditions for 14 days. Experimental condition in the tanks were as follows temperature: 26 ± 2 °C; pH: 7.1 ± 0.1; ammonia: less than 0.5 mg/L; nitrites: less than 0.05 mg/L and dissolved oxygen: 6.21 ± 0.41 mg/L and light cycle (13 h light/11 h dark photoperiod). All fish were fed basal diet twice daily at 1–2% of body weight during acclimatization.

2.4. Feed and experimental design

After acclimation, 480 *C. argus* were randomly divided into eight groups housed in twenty-four 200 L glass aquarium (3 tanks per group, 20 fish per tank). Each group was exposed to waterborne Se and/or dietary AMRF. Treatments were as follows: Se groups (basal diet) and AMRF groups (basal supplemented with AMRF 40 mg/kg). The groups were divided as follows: S0A0 (control group, basal diet), S0A1 (AMRF group, AMRF 40 mg/kg), S1A1 (Se 50 μg/L, AMRF 40 mg/kg), S1A0 (Se 50 μg/L, basal diet), S2A1 (Se 100 μg/L, AMRF 40 mg/kg), S2A0 (Se 100 μg/L, basal diet), S3A1 (Se 200 μg/L, AMRF 40 mg/kg) and S3A0 (Se 200 μg/L, basal diet). The fish were fed three times (06:00, 12:00 and 18:00) a day for 56 days at rate of 3–4% of body weight. The Se concentrations that were used were approximately equivalent or higher than those present in the environment [7], which would be safe for *C. argus*. Sodium selenite (Sigma, St. Louis, MO, USA) solution was dissolved in the respective glass aquarium. Tank bottom debris was removed by siphon daily and half of the water in each tank was changed every two days and then Se was added upon the experiment concentration.

2.5. Tissue sampling

Fish were fasted 24 h before collecting samples. At the end of each period (at 28 and 56 days), fish were anesthetized using 300 mg/L of Methane-Sulfonate-222 (MS-222). The blood from five fish randomly sampled from each tank was collected by caudal venipuncture, centrifuged at 1000 g for 10 min to obtain serum, stored at −20 °C for further analysis. Then, fish liver, kidney, spleen, intestine, gill, and muscle were quickly removed. All tissue samples were flash-frozen in liquid nitrogen and stored at −80 °C until analyzed.

2.6. Detection of Se accumulation

Six tissues (liver, kidney, spleen, intestine, gill, and muscle) and water samples from each group were sampled on days 28 and 56 to observe Se concentration. All tissues were dried in an oven at 120 °C before analysis and the dried samples were cold-digested with 20 mL of concentrated nitric acid (65% HNO_3). The digested samples were harvested by centrifugation at 14,000 × *g* for 20 min at 4 °C and the clear liquid used for Se determination in an atomic absorption spectrometer AA-6300 (Shimadzu, Japan). Water sample of 10 mL, collected from glass aquarium of different groups, were analyzed for the level of Se in the same way as tissue sample. The actual waterborne Se concentration is showed in Table 1.

Table 1
Nominal and actual Se concentration in water (μg/L).

Groups	Nominal concentration	Actual Se concentration in the experiment days	
		28 days	56 days
S0A1	0	0.03 ± 0.02[a]	0.07 ± 0.05[a]
S0A0	0	0.07 ± 0.03[a]	0.08 ± 0.04[a]
S1A1	50	52.94 ± 0.17[b]	51.42 ± 0.36[b]
S1A0	50	50.29 ± 0.23[b]	53.15 ± 0.29[b]
S2A1	100	106.14 ± 0.46[c]	104.67 ± 0.57[c]
S2A0	100	100.87 ± 0.41[c]	102.14 ± 0.94[c]
S3A1	200	204.63 ± 0.32[d]	206.71 ± 0.54[d]
S3A0	200	202.55 ± 0.63[d]	205.28 ± 0.29[d]

Note: The Data are presented as mean ± S.D. (n = 6). Values in rows with same superscript does not differ significantly ($P > 0.05$). S0A0: control group; S0A1: AMRF 40 mg/kg group; S1A1: Se 50 μg/L, AMRF 40 mg/kg group; S1A0: Se 50 μg/L, basal diet group; S2A1: Se 100 μg/L, AMRF 40 mg/kg group; S2A0: Se 100 μg/L, basal diet group; S3A1: Se 200 μg/L, AMRF 40 mg/kg group; S3A0: Se 200 μg/L, basal diet group .

2.7. Antioxidant parameters

The liver and spleen were used to analyze the antioxidant parameters. Catalase (CAT), glutathione peroxidase (GPx) activity and malondialdehyde (MDA) content were measured according to the method described by Zhou et al. (2015) [17] and Jiang et al. (2017) [18].

2.8. Serum immunity assays

Serum lysozyme activity, complement C3 and immunoglobulin M (IgM) concentration were measured by using the method published by Li et al. (2019) [2].

2.9. Reverse-transcriptase real-time PCR

The genes (NF-κB p65, IκB-α, TNF-α, IL-1β, IL-8, HSP70, HSP90, and Glucocorticoid receptor (GR)) expression were analyzed in liver samples and spleen at 56 days after the start of feed experiment. Total RNA was extracted using a TRIzol kit (Takara, Dalian, China). The quality and concentration of RNA were analyzed by using NanoDrop 2000 spectrophotometry (Thermo scientific, USA). Complementary DNA (cDNA) was synthesized using a reverse transcriptase synthesis kit (Takara, Dalian, China). Real-time PCR was used to determine gene expression levels. The primer sequences are indicated in Table 2. PCR reaction mixtures included SYBR qPCR Mix (10 μL), forward and reverse primer (10 mM, 1 μL), cDNA (1 μL), DEPC-treated water (7 μL). The reaction conditions were as follows 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, anneal for 30 s and 72 °C for 30 s. The levels of gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method and normalized using β-actin expression.

2.10. Statistical analysis

The results are presented as mean ± S.D. The data were analyzed with one-way analysis of variance (ANOVA) to determine the significant differences. Tukey's multiple range test was used to compare the mean values ($P < 0.05$) to indicate significant differences. Analysis were conducted using SPSS statistics 19.0 software (IBM, USA).

2.11. Compliance with ethics requirements

All the experimental animals used in the study were performed in accordance with the NIH Guide for the Care and approved by the Ethics Committee of Jilin Agricultural University with ID no. 20121008.

3. Results

3.1. Se accumulation

Se accumulation levels in kidney, liver, spleen, intestine, gill, and muscle of *C. argus* during the 28 and 56 days period of waterborne exposure Se and/or dietary AMRF is shown in Fig. 1.

After 28 and 56 days, a significant increase in Se accumulation ($P < 0.05$) were observed in kidney, liver, spleen, intestine, gill, and muscle with the increase of waterborne Se levels from 0 to 200 μg/L. The same Se dose plus AMRF groups were reduced significantly ($P < 0.05$) in the Se accumulation levels compared with the same Se dose groups in all studied tissues at 28 and 56 days.

3.2. Antioxidant status

Liver and spleen antioxidant parameters (MDA, CAT and GPx) of *C. argus* during the 28 and 56 days period of exposure to waterborne Se and/or dietary AMRF are summarized in Fig. 2.

The results showed that the levels of MDA in liver and spleen significantly increased ($P < 0.05$) with the increased Se doses. However, when fish were supplemented with AMRF the increase in MDA content was attenuated. In the liver and spleen, compared with the same Se dose groups, treatment with AMRF were significantly decreased ($P < 0.05$) the MDA content. The differences between co-treatment with AMRF and Se groups (50 and 100 μg/L Se group in liver, 50 μg/L in spleen Se group) and control group were not significant ($P > 0.05$) at 28 and 56 days (Fig. 2A and B).

Compared with the control group, no notable CAT activity change was observed in the liver and spleen after Se exposure, excluding a significantly increase at exposure to 100 mg/L Se at 28 days. Compared with the same Se dose groups, treatment with AMRF significantly increased ($P < 0.05$) CAT activity in the liver and spleen of *C. argus* at 28 and 56 days (Fig. 2C and D).

Compared with the control groups, the activities of GPx in liver was notably increased ($P < 0.05$) after Se exposure at 28 and 56 days and in spleen over 100 μg/L of Se exposure at 28 days. Compared with the same Se dose groups, treatment with AMRF significantly increased ($P < 0.05$) GPx activity in the liver and spleen of *C. argus* at 28 and 56 days (Fig. 2E and F).

Table 2
Primer sequences and optimal annealing temperatures (OAT, °C) for polymerase chain reactions.

Name	Sequence (5′-3′)	OTA	GenBank ID/Reference	Efficiency %
GR-F	GGGAAAGACCAGGACTCATA	60	Li et al. (2019)[a]	98
GR-R	TTCTTGGTTTTCCGTGCTTC			
HSP90–F	TGTATGTCAGGAGGGTGTTT	55	Li et al. (2019)[a]	99
HSP90-R	TAGATTGATTTCTGGTTTTC			
HSP70–F	ATTTTGAATGTGTCTGCGGT	56	Li et al. (2019)[a]	98
HSP70-R	ACTTGCTGATGATGGGGTTA			
IL-1-F	GTTTACCTGAACATGTCGGCTTACG	59	Li et al. (2019)[a]	97
IL-1-R	AGGGTGCTGATGTTCAGCCCA			
IL-8-F	GAGTCTGAGCAGCCTGGGAGT	61	Li et al. (2019)[a]	99
IL-8-R	CTGTTCGCCGGTTTTCAGTG			
TNF-α-F	ACAATACCACCCCAGGTCCCA	61	Li et al. (2019)[a]	101
TNF-α-R	ACGCAGCATCCTCTCATCCAT			
NF-κB p65-F	CAGCCAAAACCAAGAGGGAT	62	Li et al. (2019)[a]	99
NF-κB p65-R	TCGGCTTCGTAGTAGCCATG			
IκBα-F	AAAATGTTACCGTGCCAGGAC	60	Li et al. (2019)[a]	98
IκBα-R	ATGTATCACCGTCGTCAGTC			
β-actin-F	CACTGTGCCCATCTACGAG	57	Li et al. (2019)[a]	96
β-actin-R	CCATCTCCTGCTCGAAGTC			

[a] M. Li, X. Zhu, J. Tian, M. Liu, G. Wang, Dietary flavonoids from *Allium mongolicum Regel* promotes growth, improves immune, antioxidant status, immune-related signaling molecules and disease resistance in juvenile northern snakehead fish (*Channa argus*), Aquaculture 501 (2019) 473–481.

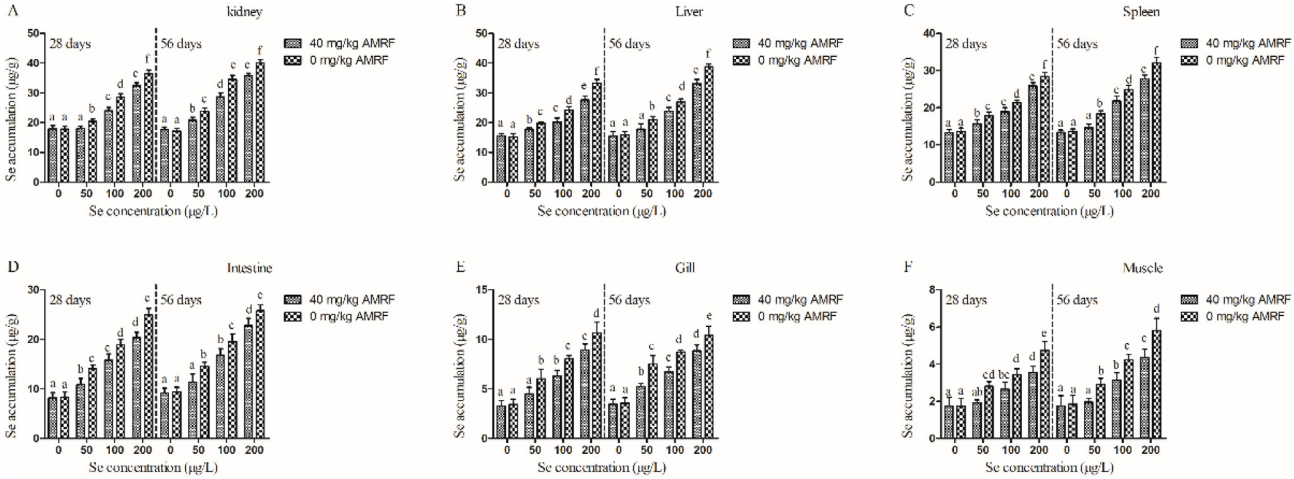

Fig. 1. Se accumulation (Kidney (A), Liver (B), Spleen (C), Intesine (D), Gill (E) and Muscle (F)) of *Channa argus* (n = 6) after exposure to different dose of Se (0 μg/L, 50 μg/L, 100 μg/L and 200 μg/L) and/or dietary AMRF 40 mg/kg at 28 and 56 days. Data are expressed as the mean ± S.D. Bar with different letters are significantly ($P < 0.05$) different by Tukey test on the same sampling interval.

3.3. Serum immunity assays

Serum LA, IgM and C3 of the *C. argus* after 28 and 56 days exposed to waterborne Se and/or dietary AMRF are presented in Fig. 3.

As shown in Fig. 3, Se exposure resulted in an elevation in serum LA, IgM and C3 levels. However, with dietary AMRF the increase in LA, IgM and C3 levels were attenuated. Compared with the same Se dose groups, dietary AMRF exhibited an decrease in LA, IgM and C3 levels ($P < 0.05$). In addition, the differences between co-treatment with AMRF and Se groups and control group were not significant ($P > 0.05$) at 28 and 56 days (except in 0 μg/L Se groups).

3.4. Real-time PCR analysis

As shown in Fig. 4, TNF-α, IL-1β and IL-8 gene expression were significantly affected in liver and spleen of *C. argus* after 56 days. Compared with control group, the expression of TNF-α, IL-1β and IL-8 were significantly increased ($P < 0.05$) in liver and spleen of *C. argus* after 56 days with increasing Se exposure levels. However, with dietary AMRF the elevation in TNF-α, IL-1β and IL-8 levels were attenuated. Compared with the same Se dose groups, dietary AMRF exhibited an decrease in TNF-α, IL-1β and IL-8 levels ($P < 0.05$) (except in 0 μg/L Se groups).

As shown in Fig. 5, NF-κB p65, IκB-α and GR gene expression were significantly affected in liver and spleen of *C. argus* after 56 days. Compared with control group, the expression of NF-κB p65 was significantly increased ($P < 0.05$) in liver and spleen of *C. argus* after 56 days with increasing Se exposure levels. Compared with the same Se dose groups, dietary AMRF significantly decreased ($P < 0.05$) the expression of NF-κB p65 in liver and spleen. In contrast, compared with the same Se dose groups, dietary AMRF significantly increased ($P < 0.05$) the expression of IκB-α and GR (except in 0 μg/L Se groups).

As shown in Fig. 6, HSP70 and HSP90 gene expression were significantly affected in liver and spleen of *C. argus* after 56 days. Compared with control group, the expression of HSP70 and HSP90 were significantly increased ($P < 0.05$) in liver and spleen of *C. argus* after 56 days with increasing Se exposure levels. Compared with the same Se dose groups, dietary AMRF significantly decreased ($P < 0.05$) the expression of HSP70 and HSP90 in liver and spleen of *C. argus* after 56 days.

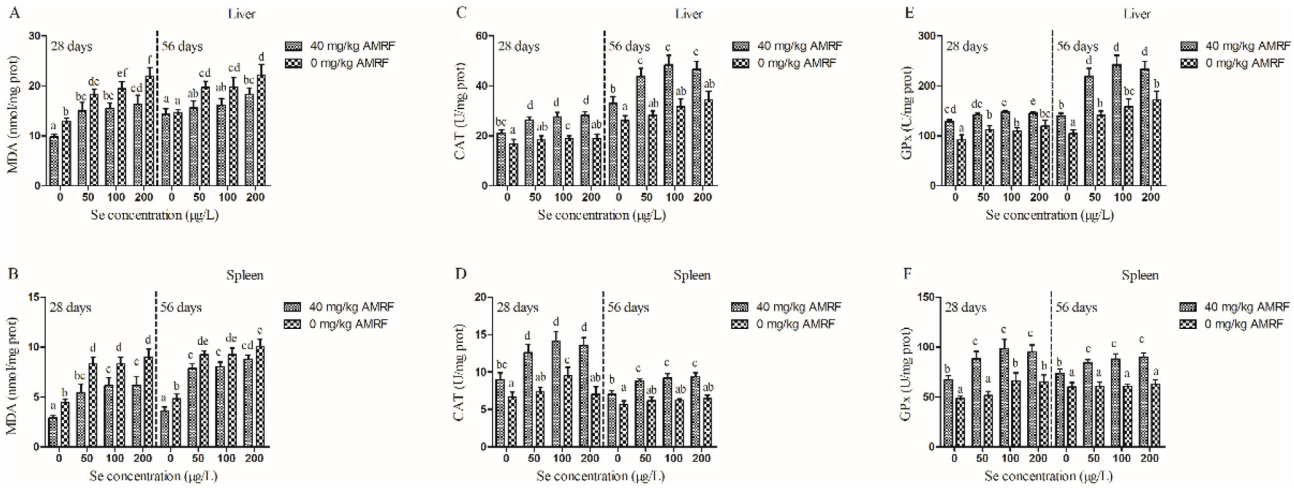

Fig. 2. Oxidative stress parameters in the liver and spleen (MDA (A, B), CAT activity (C, D) and GPx activity (E, F)) of *Channa argus* (n = 6) after exposure to different dose of Se (0 μg/L, 50 μg/L, 100 μg/L and 200 μg/L) and/or dietary AMRF 40 mg/kg at 28 and 56 days. Data are expressed as the mean ± S.D. Bar with different letters are significantly ($P < 0.05$) different by Tukey test on the same sampling interval.

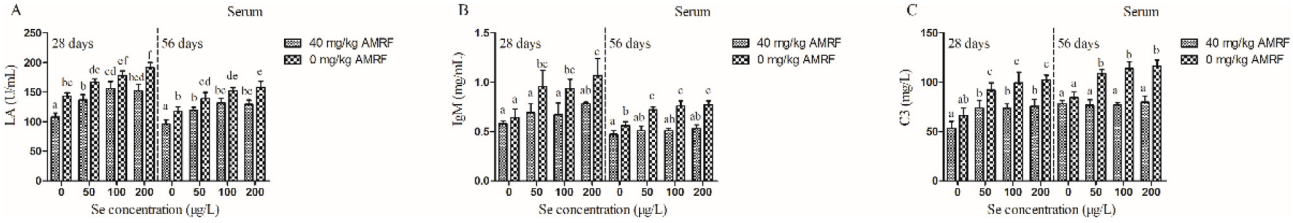

Fig. 3. Serum lysozyme activity (LA) (A), IgM (B) and complement C3 (C) of *Channa argus* (n = 6) after exposure to different dose of Se (0 μg/L, 50 μg/L, 100 μg/L and 200 μg/L) and/or dietary AMRF 40 mg/kg at 28 and 56 days. Data are expressed as the mean ± S.D. Bar with different letters are significantly ($P < 0.05$) different by Tukey test on the same sampling interval.

4. Discussion

4.1. Se accumulation

Due to bioaccumulation, trace elements in aquatic animals are higher than the surrounding water [7]. *Allium mongolicum Regel* flavonoids, one of the most effective possibly component of *Allium mongolicum Regel*, are plant chemicals with oestrogen activity [2]. Muqier et al. (2017) [10] and Zhou et al. (2015) [17] have suggested that flavonoids and isoflavones could promote growth, growth-related hormones, antioxidant and immune responses. In the present study, Se accumulation in the kidney and liver of *C. argus* significantly elevated following exposure to waterborne Se. However, dietary supplemented with AMRF substantially decreased Se accumulation in the kidney and liver following exposure to waterborne Se. The kidney and liver are considered as target organ for toxic substances accumulation, since they play a major role in eliminating and redistributing toxic substances after uptake through the gills [19]. Kim and Kang (2014) [6] demonstrated that the kidney and liver are the major organs that accumulate Se in *Pagrus major*, and are also important tissues of the detoxification mechanism. Our previous study also have found that the bioaccumulation pattern of exposure to waterborne Se in different tissues was: kidney > liver > spleen > intestine > gill > muscle [7]. In the present study, Se accumulation in the spleen, gill and intestine of *C. argus* substantially enhanced but lower than the kidney and liver. Similar to the liver and kidney, dietary supplementation with AMRF significantly decreased Se accumulation in the spleen, intestine, gill and muscle of *C. argus* following exposure to waterborne Se. Karaytug et al. (2007) [20] and Yin et al. (2018) [1] demonstrated that spleen, intestine and gill are the major metabolically active tissues, so it is easy to accumulate toxic substances. Compared with the same Se dose groups, dietary AMRF groups significantly reduced ($P < 0.05$) Se accumulation levels in all studied tissues at 28 and 56 days. To date, no information is available about how plant flavonoids or other extracts attenuate Se accumulation in aquatic organisms. The underlying mechanism need further investigations.

4.2. Antioxidant response

Trace metals accumulation in the aquatic animals could induce oxidative stress [21]. The presence of toxic substances in the organs have been shown to promote the synthesis of ROS [1]. Trace element-induced ROS production is usually associated with cell damage caused by lipid alterations. MDA, a product of lipid peroxidation, is a reliable index of oxidative stress and cellular injures [22]. Therefore, the inhibition of MDA can be a potential intervention approach for oxidative stress. Our previous study demonstrated Se exposure could elevate MDA content both in spleen and liver of *C. argus* [7]. The present study further confirms oxidative stress effects of Se exposure in *C. argus*. Administration of soybean isoflavones has been reported to reduce hepatic MDA concentrations in *Trachinotus ovatus* [17]. In the present study, AMRF treatments were found to attenuate the increase of MDA concentrations in the liver and spleen of *C. argus* following exposure to waterborne Se.

Antioxidant defenses are complex and effective sensors that connect contaminant and metabolic changes in fish. The changes of endogenous antioxidant enzymes activity after exposure to trace elements reflect the oxidative stress effect of fish [9]. CAT, endogenous antioxidative molecules, play a major role in counteracting ROS and oxygen toxicity [23]. GPx is a selenium-containing enzyme that catalyzes the reduction

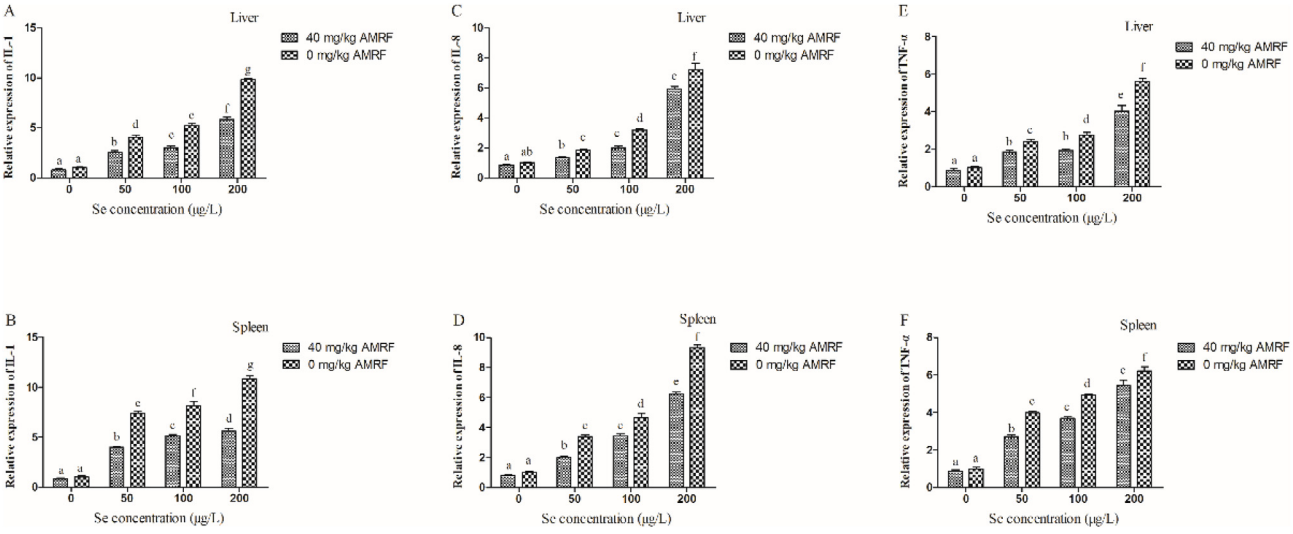

Fig. 4. Relative gene expression (TNF-α, IL-1β and IL-8) in the liver and spleen of *Channa argus* (n = 6) after exposure to different dose of Se (0 μg/L, 50 μg/L, 100 μg/L and 200 μg/L) and/or dietary AMRF 40 mg/kg at 56 days. Data are expressed as the mean ± S.D. Bar with different letters are significantly ($P < 0.05$) different by Tukey test on the same sampling interval.

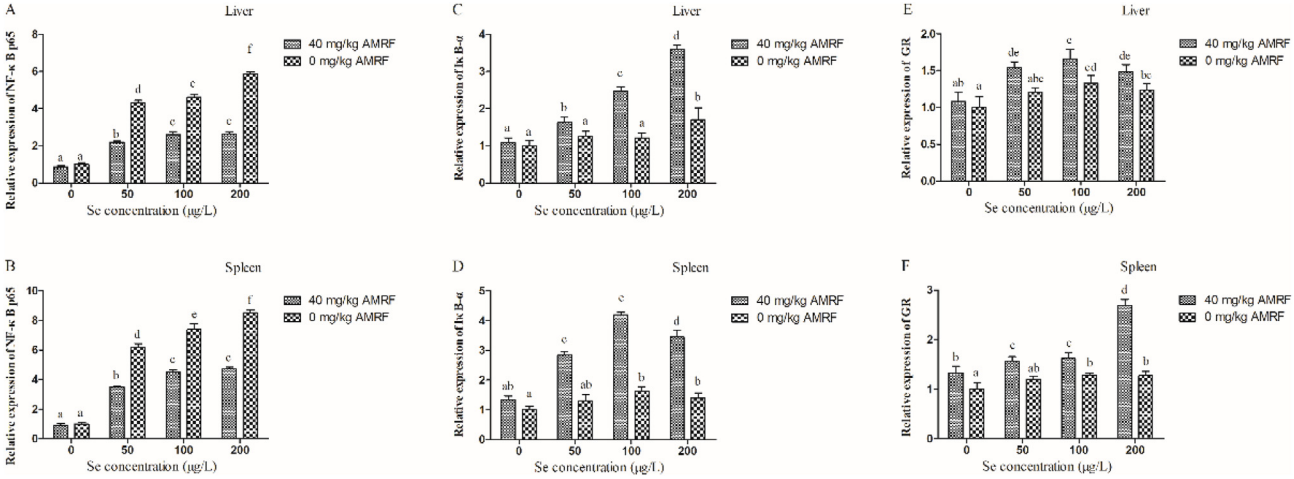

Fig. 5. Relative gene expression (NF-κB p65, IκB-α and GR) in the liver and spleen of *Channa argus* (n = 6) after exposure to different dose of Se (0 μg/L, 50 μg/L, 100 μg/L and 200 μg/L) and/or dietary AMRF 40 mg/kg at 56 days. Data are expressed as the mean ± S.D. Bar with different letters are significantly ($P < 0.05$) different by Tukey test on the same sampling interval.

of both H_2O_2, protein carbonyl and MDA [24,25]. As important antioxidative enzymes for the antioxidant defense system against oxidative stress, CAT and GPx are widely distributed in human and fish cells. Consequently, the promotion of CAT and GPx activity can be a potential intervention approach for oxidative stress. In the present study and our previous study showed that CAT and GPx both in spleen and liver of *C. argus* were not significantly enhanced by increasing Se exposure concentration, may result in a failure to protect the tissues against reactive oxygen metabolites or MDA. The findings of the present study demonstrated that AMRF treatments significantly increased the levels of CAT and GPx both in spleen and liver of *C. argus* following exposure to waterborne Se, which was a pattern opposite to that of the MDA content. The elevation of CAT and GPx activity in spleen and liver of *C. argus* is beneficial for reducing lipidic superoxide damage caused by Se exposure.

4.3. Serum immune responses

The oxidative stress and trace metals accumulation can induce immune disorders in animal cells and particularly in fish [1]. Immune indicators are reliable parameters for monitoring the potential impact of environmental hazards on fish health. Lysozyme, complements and IgM, important components of the immune system, widely distributed in the mucus, serum and ova of fish that not only eliminate bacteria or pathogens but also activates phagocytes or promote inflammatory responses [2,3,26]. Se has been reported to increase the levels of lysozyme and IgM in the serum of fish [7]. In addition, higher lysozyme and IgM levels are also observed in disease fish [27]. A previous study has also shown that trace metals accumulation have negative impacts on the immune system of fish [7]. In the present study, the levels of C3, IgM and lysozyme were significantly increased in serum of *C. argus*

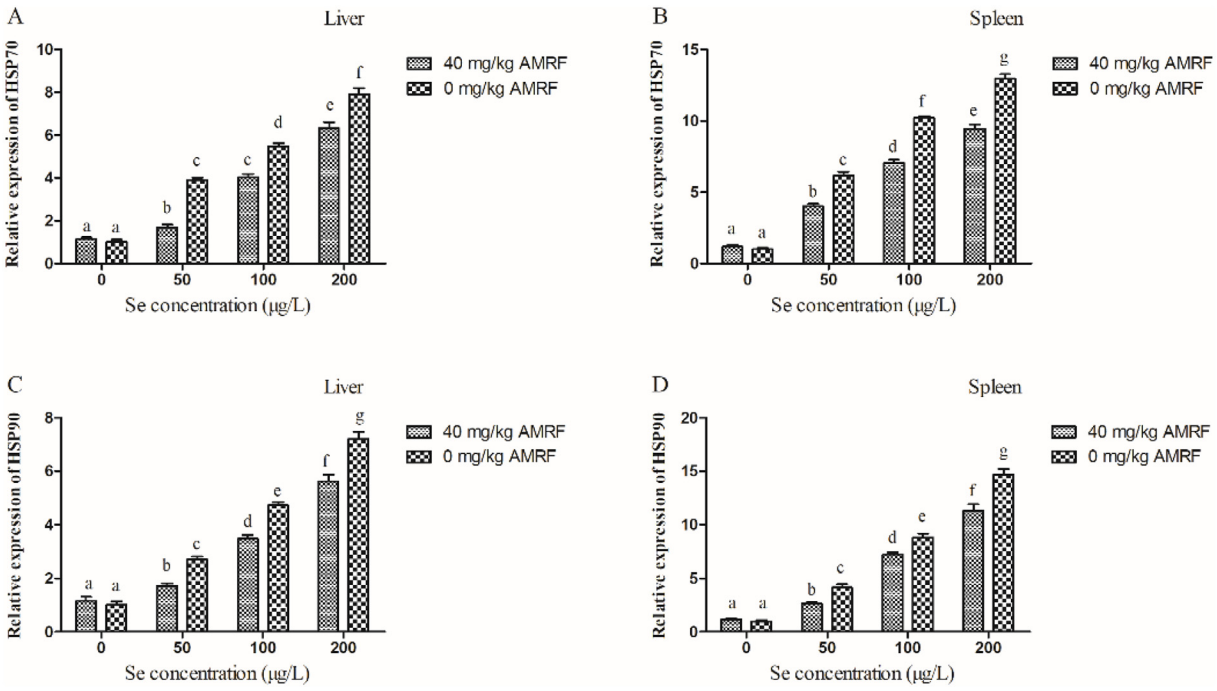

Fig. 6. Relative gene expression (HSP70 and HSP90) in the liver and spleen of *Channa argus* (n = 6) after exposure to different dose of Se (0 μg/L, 50 μg/L, 100 μg/L and 200 μg/L) and/or dietary AMRF 40 mg/kg at 56 days. Data are expressed as the mean ± S.D. Bar with different letters are significantly ($P < 0.05$) different by Tukey test on the same sampling interval.

following exposure to waterborne Se. Our previous study and other reports indicate that diets supplemented with soybean isoflavones or AMRF could increased the levels of C3, IgM and lysozyme in fish [2,17]. However, in this study, AMRF treatments significantly decreased the levels of C3, IgM and lysozyme in serum. The reduction of C3, IgM and lysozyme levels in serum of *C. argus* may benefit to reducing excessive immune response damage caused by Se exposure.

4.4. Immune-related gene expression

Interleukins (ILs) and tumor necrosis (TNFs) are two major cytokines, which originate from lymphocytes, macrophages, monocytes and granulocytes [28]. These molecules play a major role in animal growth, metabolism, antioxidants and immunity. Among these cytokines, IL-1β, IL-8 and TNF-α are potent pro-inflammatory factors that play an important role in the initiation and regulation of inflammatory processes [2]. Previous study have suggested that trace metals including tin and lead can promote the immune response by activating NF-κB protein and upregulating the levels of inflammatory factors [1,28]. Similarly, our previous study [7] and in this study also showed that Se exposure can up-regulate IL-1β and TNF-α gene expression in the liver and spleen of *C. argus*. In this study, AMRF treatments significantly reduced the gene expression of IL-1β and TNF-α in the liver and spleen of *C. argus* after Se exposure.

Previous study showed that plant flavonoids (cocoa and Korean thistle *Cirsium maackii*) [14,15] can reduce inflammatory cytokines levels. Our previous study shows that dietary AMRF can decrease the levels of NF-κB p65 and inflammatory cytokines including TNF-α, IL-1β and IL-8 in *C. argus* [2]. NF-κB is a potent proinflammatory transcription factor, which plays an major role in regulating the gene expression of inflammatory cytokines [29]. Our previous study also found that exposure to waterborne Se elevated the gene expression of inflammatory cytokines that may be attributed to the activation of NF-κB p65 [7]. In response to adverse stimuli including inflammatory cytokines, oxidative stressors, viruses and bacterial products, NF-κB protein was activated by the isolation from its binding protein IκB [30]. In this study, AMRF treatments notably down-regulated the gene expression of NF-κB p65, TNF-α, IL-1β and IL-8 while up-regulated the gene expression of IκB-α after Se exposure. To our knowledge, the current study is the first to suggest AMRF could attenuate Se-induced inflammatory responses in fish.

Glucocorticoid receptors (GR) is a steroid ligand-dependent transcription factors, which can cross talk with NF-κB signaling pathways [31]. Previous study have been suggested that GR can inhibit the gene expression of inflammatory cytokines by interacting with p65 subunit, IκB and IKK [32]. In the present study, we found that dietary AMRF treatments significantly increased the expression of GR in the liver and spleen of *C. argus* after Se exposure, indicating that dietary AMRF could improve anti-inflammatory ability of *C. argus*. Similarly, previous study also reported that administration of glutamine increased the expression of GR in *Jian* carp [33].

Heat shock proteins (HSPs) including HSP70 and HSP90 play an important role in the formation of GR protein complexes [34]. HSP70 and HSP90 are also stress proteins, which can enhance cell tolerance and survival in stress environment [35]. Previous study revealed that the mRNA levels of HSP70 was enhanced following exposure to lead or decreased following probiotic administration [1]. In the present study, dietary AMFR showed a similar trend for the expression of NF-κB p65 and inflammatory cytokines (TNF-α, IL-1β and IL-8) in the liver and spleen of *C. argus*, indicating that dietary AMRF could attenuate Se-induced oxidative damage and inflammation in *C. argus*.

5. Conclusion

In conclusion, the present study was apparently the first report that dietary AMFR can reduce Se-accumulation in organ, decrease oxidative stress, increase immune response and regulate immune-related signaling molecules following Se exposure in *C. argus*. These results indicate that AMFR has the potential to alleviate the negative effects of Se toxicity in aquaculture.

Conflicts of interest

The authors declare that they have no conflict of interest.

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