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Selenium deficiency induced an inflammatory response by the HSP60 - TLR2-MAPKs signalling pathway in the liver of carp

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ABSTRACT

Selenium (Se) is one of the essential trace elements for immune regulation and antioxidant systems in fish growth. The dietary Se plays an important role in immune regulation and inflammation by regulating HSPs and TLRs in liver of many animals. The liver is an important digestive organ in carp. Liver damage can seriously affect the growth and survival of carp. This study was conducted to determine whether Se regulated liver inflammation by affecting HSPs-TLR2 signalling and the potential mechanisms of action in common carp. The gene was analysed by qPCR. The proteins of inflammatory factors were detected by ELISA. The others proteins were analysed by Western blot. The results indicated the Se concentrations in blood and liver tissues were significantly influenced by dietary Se. The Se deficiency increased the expression of HSP60 and TLR2 and the secretion of the proinflammatory factor TNF-α, IL-1β and IL-6, induced a low secretion of the anti-inflammatory TGF-β, but the Se supplements could transform these events. Further research showed that with the dose-dependently decrease of Se, the HSP60 expressions were increased, and the MAPKs pathway were significantly activated by the phosphorylation of p38, JNK and ERK in liver tissue and cell. The results provide evidence that Se deficiency induced and exacerbated inflammatory injury to the liver through the HSP60 and TLR2-MAPKs signalling pathways in carp.

1. Introduction

Selenium (Se) is one of the essential trace elements in organisms, including vertebrates, molluscs and microorganisms [1]. Se was considered a toxic substance at the time of its discovery. In 1957, it was determined that Se was a beneficial microelement of the organism that inhibits the third factor of feeding liver necrosis in rats [2]. With the discovery of GPx as the earliest known bioactive form of Se, its important biological functions became widely valued. Numerous studies have confirmed that Se plays an important role in many aspects of health, such as muscle metabolism [3], chemoprevention [4], redox reaction [5], immune function [6] and inflammation [7]. Furthermore, Se is an essential nutrient for growth, immune regulation and antioxidant systems in fish [8–10]. The biology of selenium has been closely associated with dietary Se intake in vertebrates. Dietary Se plays an important role in immunoregulation and inflammation [11,12]. The necessity and requirements of dietary Se have been estimated in multiple species of fish [13]. Though selenosis causes multiple injuries in fish, the effect of a dietary selenium deficiency in fish could not be ignored. (see Table 1).

It has been reported that Se deficiency was closely related to the occurrence of many diseases and impairments to physiological function in various organisms under many conditions [14–16]. Se deficiency inhibited the growth of organs and decreased immune function, which led to many inflammatory diseases [17,18]. Most studies of dietary Se focused on quantity requirements or related diseases. However, the effects of Se deficiency on inflammation are still unclear, especially in fish. A previous study found that low intake of Se has been implicated in the increased incidence of gastrointestinal tract inflammation [19]. Se deficiency facilitates inflammation through the regulation of HSPs and Toll-like receptor 2 (TLR2), which is a pattern recognition receptor (PRR). The HSPs are expressed in all kinds of fish [20]. TLRs are imperative for the initiation of the immune response, both in fish [21] and others vertebrate [22].

Compared to higher animals, though there is no fine differentiation of the acinar structure in the liver, and it is a key organ for storing glycogen and fat [23]. Daily feeding was found to be a major cause of the regulation of the physiological function of the liver in fish [24]. High levels of carbohydrates in the diets of fish induced liver injuries through excessive accumulation of glycogen [25]. The lack of Se could cause immune deficiency, inhibit growth and induce oxidative stress in the livers of fish [26]. However, little is known about the effect of Se on

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Table 1
Primers used for quantitative real-time PCR.

Name	Primer sequence	Product size (bp)
HSP60	Sense: 5′- ATAAGGAAAGCAGCACGAA-3′ Anti-sense: 5′- AAGGCAAACTGGACAGAAAA-3′	199
TLR2	Sense: 5′- CTATCAAGTGCTCCTCAAA-3′ Anti-sense: 5′- CCTCACCCATGTAGTATGT-3′	198
p38	Sense: 5′- GTTGATGTTCAGGCTGTG-3′ Anti-sense:5′- GACGGTAGACATTCTGGTAG-3′	209
ERK	Sense: 5′- AAAATCAAAGAAGCCTACTCCC-3′ Anti-sense: 5′- TTCCCAACTCCTGCGACA-3′	214
JNK	Sense: 5′- CATTCGTTGTTGCCTTGC-3′ Anti-sense:5′- TGTAGTGGGCGTCCTGTG-3′	225
TNF-α	Sense: 5′- ATAACCAGATCGTGATCCCACA -3′ Anti-sense: 5′- TTCGCCCTTCCGACCTCA -3′	195
IL-1β	Sense: 5′- GCCAGACCTGTAGCCCTAG -3′ Anti-sense: 5′- TGTCCGTGCTGATGAACC -3′	229
IL-6	Sense: 5′- ACAGTTTGTGGAGGAGTT -3′ Anti-sense:5′- GGAGTAGGGTTGATTGAG -3′	209
TGF-β	Sense: 5′- ACGAAGCGAGGAGGAGTA -3′ Anti-sense: 5′- TTCTGGAGACGGAAGACG -3′	192
β-actin	Sense: 5′- CCATCGTCCACCGCAAAT -3′ Anti-sense:5′- CGCCTCCTTCATCGTTCC-3′	207

liver inflammation. Therefore, the present study was conducted to determine whether selenium regulates liver inflammation by affecting HSPs-TLR2 signalling and the potential mechanisms of action in common carp.

2. Materials and methods

2.1. Animals

Sixty juvenile common carp (body weight 90.53 ± 4.53 g, length 12.13 ± 0.93 cm) were purchased from a freshwater fish farm. The carp were randomly distributed in three laboratory tanks (2.0 × 1.0 × 0.8 m). Photoperiod was 12-h light/12-h dark. The carp diet was made with corn oil and corn starch, which were obtained from a Se-deficiency region of Jilin Province in China and was shown by analysis to contain 0.013 mg of Se/kg. Organic Se (selenomethionine) was used as the Se source and was supplemented at 0, 0.7, and 1.5 mg Se/kg-diet. The diet Se concentrations were measured as 0.015, 0.80, and 1.49 mg Se/kg-diet. The water in the tanks was kept at 21 ± 0.5 °C, pH 7.2 ± 0.2, and dissolved oxygen was maintained above 8 mgL^{-1}. One-third of the water was replaced daily in the tank. All experimental procedures were conducted with the approval of the Institutional Animal Care and Use Committee of Hubei University.

2.2. Experimental groups

The fish were divided into three groups (20 fish per tank) as follows: 1) Dietary Selenium Deficiency (DG): the fish were fed with the diet with Se concentrations at 0.015 mg Se/kg-diet; 2) Dietary Selenium Normal (NG): the fish were fed with the diet with Se concentrations at 0.80 mg Se/kg-diet; and 3) Dietary Selenium Supplements (SG): the fish were fed with the diet with Se concentrations at 1.49 mg Se/kg-diet. After 60 days of feeding, the fish were anaesthetized with tricaine methane sulphonate buffered with 0.4 g/L $NaHCO_3$. The blood and liver were harvested for measuring the Se concentrations. Another portion of the liver tissues was used for further analyses.

2.3. Cell isolation and culture

Liver cells were prepared. The liver tissues were aseptically harvested and minced into paste. Using RPMI-1640 medium, the cells were washed twice. Erythrocyte removal was conducted in sterile 60-mm petri dishes, and the single-cell suspension was then collected and filtered through a 200-mesh cell sieve. The cells were collected and resuspended in RPMI-1640 containing 10% FCS, incubated for 4 h at 37 °C, cleared of fibroblasts, and cultured in RPMI-1640 containing 10% FCS at 37 °C with 5% CO_2. The media was changed to serum-free media after 24 h. Liver cells were incubated with selenomethionine (0, 1, and 10 μM) for 24 h at 37 °C and 5% CO_2 in six-well plates. The cytotoxic effects of Se to the cells were assessed by an MTT assay.

2.4. ELISA assays of inflammation

The liver tissues were homogenized with PBS on ice and centrifuged at 2000 g for 40 min at 4 °C, and then the supernatants were collected. The liver cell culture supernatants were collected. All the supernatants were assayed for TNF-α, IL-1β, IL-6, and TGF-β levels using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (BioLegend, Inc, Camino Santa Fe, Suite E, San Diego, CA, USA).

2.5. Quantitative real-time polymerase chain reaction (qPCR)

The liver tissues were homogenized with PBS on ice. The liver cells were collected. The total RNA was isolated from the tissues and cells using TRIzol according to the manufacturer's instructions (Invitrogen, China). The RNA concentration and purity were determined spectrophotometrically at 260/280 nm and then reverse transcribed into cDNA. For qPCR, specific primers were used for the detection of TNF-α, IL-1β, IL-6, TGF-β, p38, JNK, ERK, TLR2, HSP60 and β-actin. The qPCR was performed in a 40-μl reaction mixture containing 28 μl of 2 × SYBR Green I PCR Master Mix (TaKaRa, China), 2 μl of diluted cDNA, 1 μl of each primer (10 μM), 1.6 μl of 50 × ROX reference Dye II, and 8.4 μl of PCR-grade water on an ABI PRISM 7500 Detection System (Applied Biosystems, USA). β-actin was used as control. The results were expressed as $2^{-\Delta\Delta Ct}$.

2.6. Western blot analyses

The total protein of the liver tissues and cells were extracted according to the manufacturer's recommended protocol (Invitrogen, Beijing, China). The protein concentrations were determined using the BCA Protein Assay Kit. The expressions of p38, JNK, ERK, TLR2, HSP60 and β-actin, and the phosphorylation of p38, JNK, and ERK were analysed by Western blot. Samples with equal amounts of protein (50 μg) were fractionated on 12% SDS polyacrylamide gels and then transferred to polyvinylidene difluoride membranes. After blocking with 5% skim milk in 0.1% Tween 20 in TBS for 2 h, the samples were incubated with primary antibodies of p38, JNK, ERK, TLR2 (Cell Signalling Technology, Inc.), HSP60 and β-actinin (Abcam plc. Inc.) diluent buffer (5% (w/v) BSA) overnight at 4 °C at a 1:500 dilution. Then, incubations with 1:3000 dilutions (v/v) of the secondary antibodies were conducted for 2 h. The results were observed with an Enhanced Chemiluminescence Detection System. β-actin was used as a loading control.

2.7. Statistical analyses

The cytokine assays and protein levels were statistically analysed with SPSS statistical software for Windows (version 13; SPSS Inc., Chicago, IL, USA). The data were assessed using the Tukey-Kramer method for multiple comparisons. All values are expressed as the means ± SD. The significance was determined with one-way analysis of variance at a significance level of $p < 0.05$.

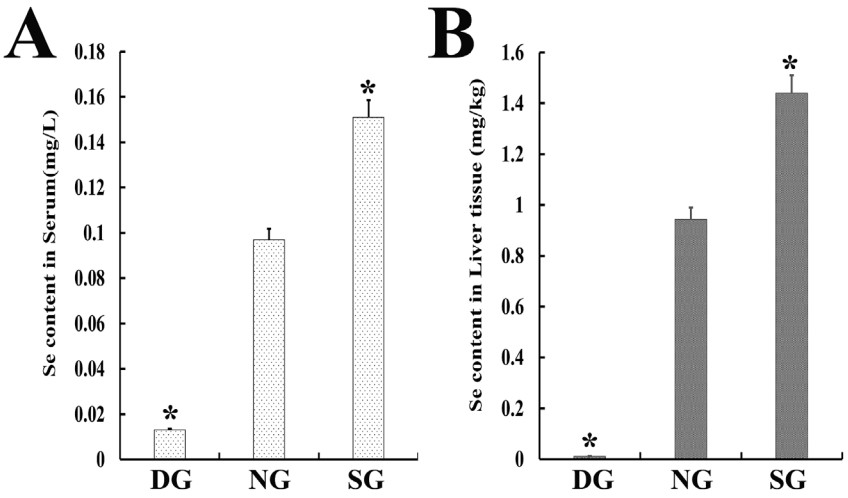


Fig. 1. Blood and liver selenium concentrations with different diets. (A) Se content of the serum. (B) Se content in liver tissues. DG, the fish fed a dietary Se deficiency at 0.015 mg Se/kg-diet. NG, the fish fed normal dietary Se at 0.80 mg Se/kg-diet. SG, the fish fed dietary Se supplements at 1.49 mg Se/kg-diet. Data are presented as the means ± SD. (n = 10 per group). *$p < 0.01$ indicates a significant difference from the NG fish.

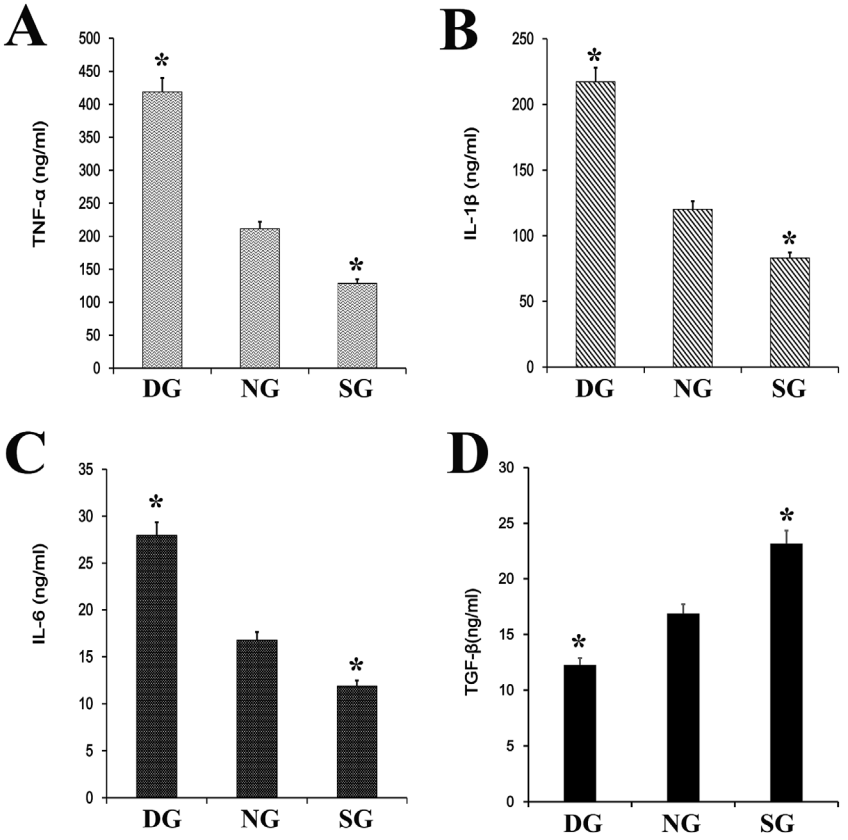


Fig. 2. The productions of inflammatory factors in liver tissue. (A) The TNF-α protein expression levels in liver tissues. (B) The IL-1β protein expression levels in liver tissues. (C) The IL-6 protein expression levels in liver tissues. (D) The TGF-β protein expression levels in liver tissues. DG, the fish fed a dietary Se deficiency at 0.015 mg Se/kg-diet. NG, the fish fed normal dietary Se at 0.80 mg Se/kg-diet. SG, the fish fed dietary Se supplements at 1.49 mg Se/kg-diet. Data are presented as the means ± SD. (n = 10 per group). *$p < 0.01$ indicates a significant difference from the NG fish.

3. Results

3.1. Selenium concentrations in the blood and liver

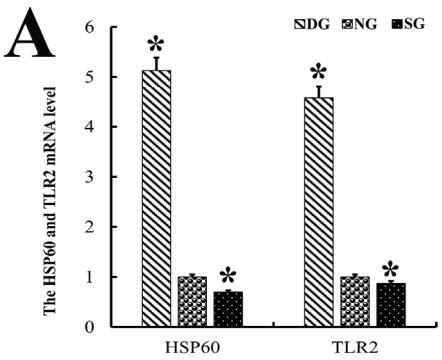
The selenium concentrations in the blood and various tissues were significantly influenced by the dietary selenium. The results of blood and liver selenium concentrations are shown in Fig. 1. The blood selenium concentration was positively correlated with the content of selenium in feed. In the DG, the blood selenium concentration was significantly reduced. As the diet selenium concentrations increased, the blood selenium concentration was significantly increased in the NG and SG (Fig. 1A). The liver selenium content had a similar trend, and compared to the NG fish, the selenium concentration was significantly reduced in the DG and significantly augmented in the SG fish (Fig. 1B).

3.2. The production of inflammatory factors in livers

The liver tissues were homogenized and the supernatants were collected. The proinflammatory and anti-inflammatory factors were detected. The results were shown in Fig. 2. The Se deficiency induced an increase in TNF-α, IL-1β, and IL-6 in the DG fish. This growth was significantly reduced in the NG fish. In the SG fish, the effect demonstrated further relief from inflammation. TGF-β serves as an anti-inflammatory factor in fish. A similar trend was found in that Se deficiency could inhibit the TGF-β expression, and the selenium supplements facilitated TGF-β expression in the NG and SG fish.

3.3. Effect of dietary Se on HSP60 and TLR2 in the liver

Slightly different from mammals, TLR2 is a major inflammatory



B

The Correlation analysis of HSP60 with inflammation factor in tissues

	TNF-α	IL-1β	IL-6	TGF-β
HSP60	0.99502	0.98592	0.98491	0.98614
TLR2	0.98794	0.99901	0.99696	0.99594

a the value of Correlation coefficient (r), r > 0.95 significant positive correlation.

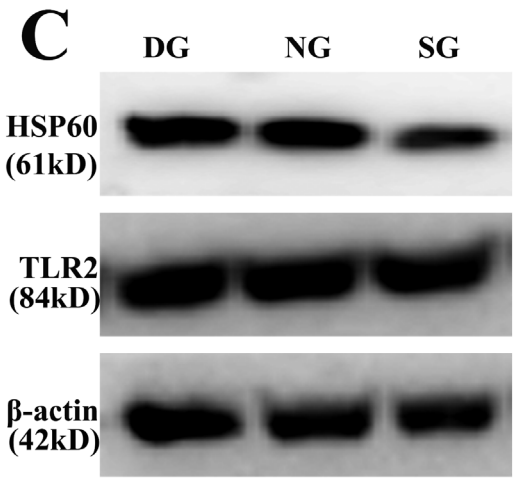


Fig. 3. The expression levels of HSP60 and TLR2. (A) The mRNA levels of HSP60 and TLR2 in liver tissues. (B) The correlation analysis with inflammatory factors in liver tissue. (C) The protein expression levels of HSP60 and TLR2 in liver tissues. β-actin was used as a control. DG, the fish fed a dietary Se deficiency at 0.015 mg Se/kg-diet. NG, the fish fed normal dietary Se at 0.80 mg Se/kg-diet. SG, the fish fed dietary Se supplements at 1.49 mg Se/kg-diet. Data are presented as the means ± SD. (n = 10 per group). *p < 0.01 indicates a significant difference from the NG fish.

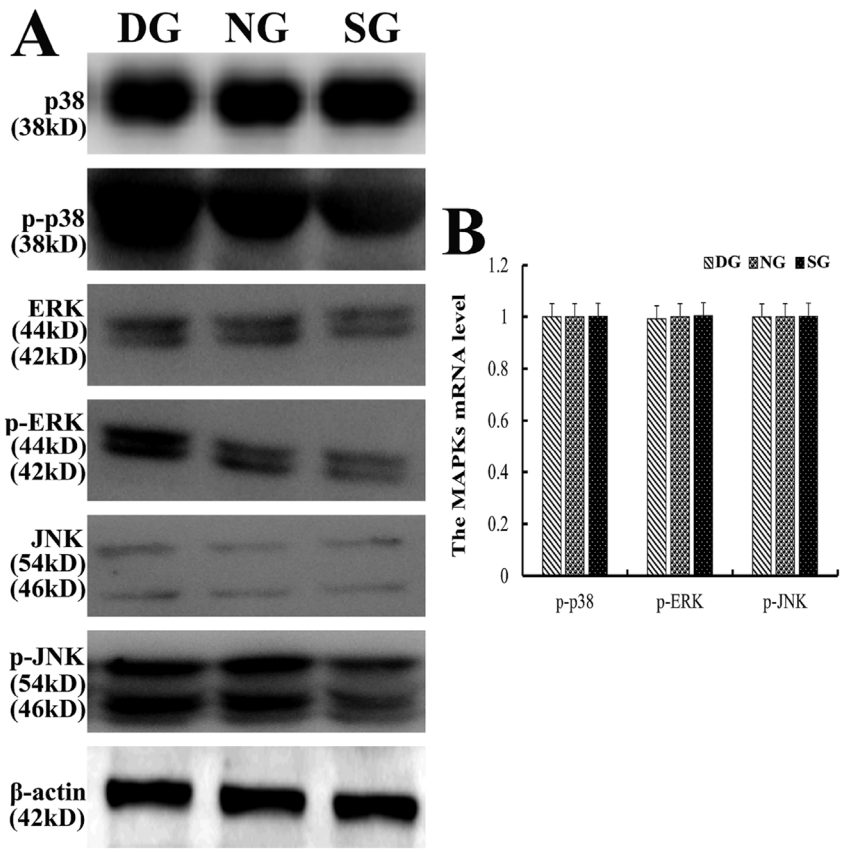


Fig. 4. The MAPKs pathway activation in liver tissues. (A) The MAPK protein and MAPK phosphorylation levels in tissues. (B) The mRNA levels of p38, ERK and JNK in liver tissues. β-actin was used as a control. DG, the fish fed a dietary Se deficiency at 0.015 mg Se/kg-diet. NG, the fish fed normal dietary Se at 0.80 mg Se/kg-diet. SG, the fish fed dietary Se supplements at 1.49 mg Se/kg-diet. Data are presented as the means ± SD. (n = 10 per group). *p < 0.01 indicates a significant difference from the NG fish.

factor in carp. Additionally, HSP60 is an inducer associated with TLR2. The results showed that dietary Se could significantly influence the expression of HSP60 (Fig. 3). Compared to the NG fish, the HSP60 was significantly increased in the DG fish, but there was low expression in the SG fish. Consistent with this change, the expression of TLR2 increased in the DG fish, and then the correlation analysis with the inflammatory factor was conducted. The results show the changes in HSP60 and TLR2 expression had a significant correlation with TNF-α, IL-1β, IL-6, and TGF-β expression (Fig. 3B).

3.4. Effect of dietary Se on the MAPKs pathway in liver tissue

The MAPKs pathway, which is downstream of TLR2, had an important role in the regulation of inflammatory factors. The mRNA expression of p38, JNK and ERK in tissues was assessed using qPCR analysis (Fig. 4B). Compared to the NG fish, the mRNA expression levels of p38, JNK and ERK were no different than in the DG and SG fish. Then, the expression of p38, JNK and ERK proteins in liver tissues was assessed using Western blot analysis (Fig. 4A). Further analysis revealed that compared to the NG, the DG showed significantly increased levels of phosphorylated p38, JNK and ERK in the liver tissues. However,

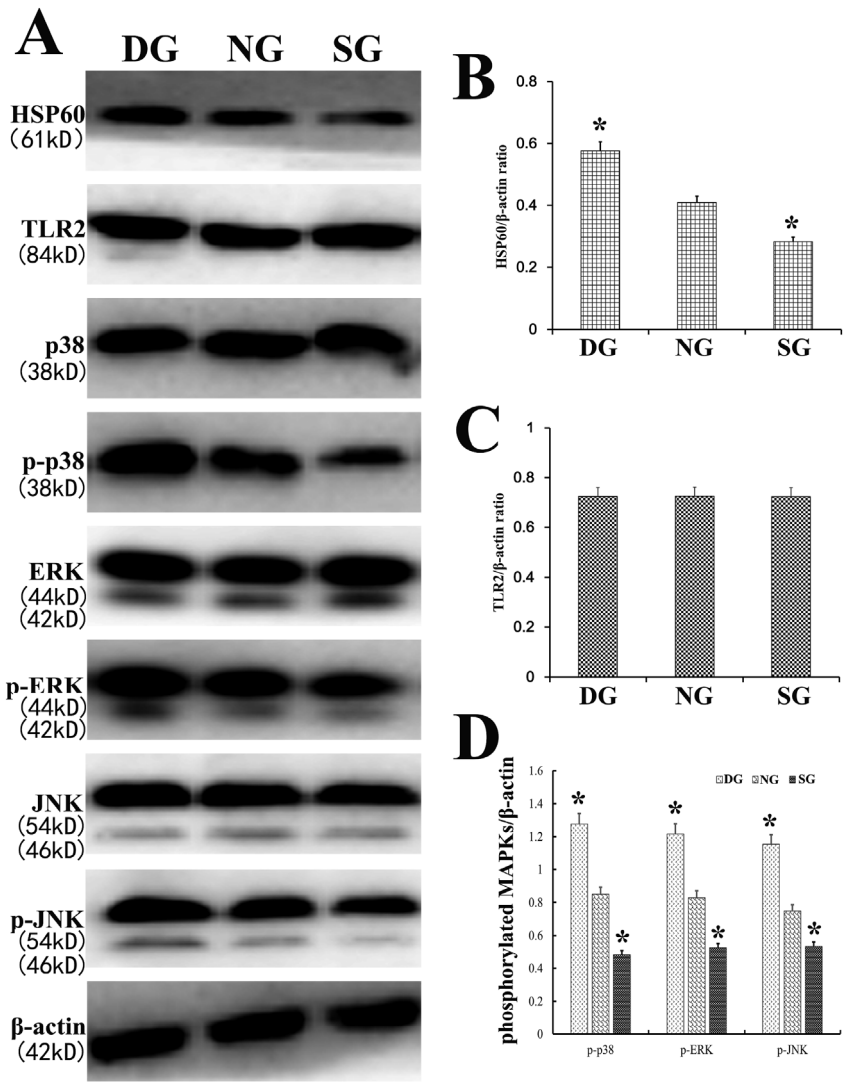


Fig. 5. Effect of Se on the HSP60-TLR2-MAPKs pathway activation in liver cells. (A) The protein expression and phosphorylation levels in cells. (B) The expression of HSP60 in cells. (C) The expression of TLR2 in cells. (D) The phosphorylation levels of MAPKs in cells. β-actin was used as a control. DG, incubated with selenomethionine 0 μM. NG, incubated with selenomethionine 1.0 μM. SG, incubated with selenomethionine 10 μM. Data are presented as the means ± SD. (n = 10 per group). *p < 0.01 indicates a significant difference from the NG cells.

these levels were significantly reduced in the SG fish (Fig. 4A). As the dietary selenium dose was reduced, the phosphorylation of p38, JNK and ERK noticeably increased.

3.5. Effect of Se on the HSP60-TLR2-MAPKs pathway in liver cells

To confirm the effect of Se on the proteins, liver cells were isolated and cultured with different Se contents. First, the cytotoxic effects of the selenomethionine (0, 1, and 10 μM) to cells were assessed by an MTT assay (Fig. 5). No cytotoxic effect was observed, and the cell vitality was no different. Then the expressions of HSP60, TLR2, p38, JNK and ERK proteins in the liver cells were assessed using Western blot analysis (Fig. 5). The results demonstrated that as the selenium dose was reduced, the HSP60 expression increased, and the phosphorylation of p38, JNK and ERK noticeably increased in liver cells.

3.6. Effect of Se on the inflammatory factors in liver cells

Proinflammatory and anti-inflammatory factors were detected with qPCR and ELISA. With the decrease in selenium concentration, noteworthy increases in TNF-α, IL-1β, and IL-6 mRNA were observed in liver cells. The proteins were shown to have same changes according to ELISA. However, TGF-β showed an opposite change in that the mRNA and protein were reduced as the selenium concentration decreased. The expression of these inflammatory factors in liver cells had a similar trend as the trend observed in the tissues. The results provide further to support our conclusion (See Fig. 6).

4. Discussion

The liver is an important digestive organ in carp. A previous study showed the liver was only a fat storage tube in some fish [27]. Liver damage can seriously affect the growth and survival of carp. The microelement Se has a significant role in lipid peroxide transformation [28]. Se was shown to be a protective microelement in the liver of many kinds of animals. A lack of Se could cause immune deficiency, inhibit growth and induce oxidative stress in fish [6,29]. This study involved feeding fish diets of different Se concentrations (0.015, 0.80, and 1.49 mg Se/kg-diet). This study was designed to analyse the effect of Se on the liver in carp.

The oxidative stress could stimulate inflammation in the liver, which exacerbates liver damage [30]. Many reports found that

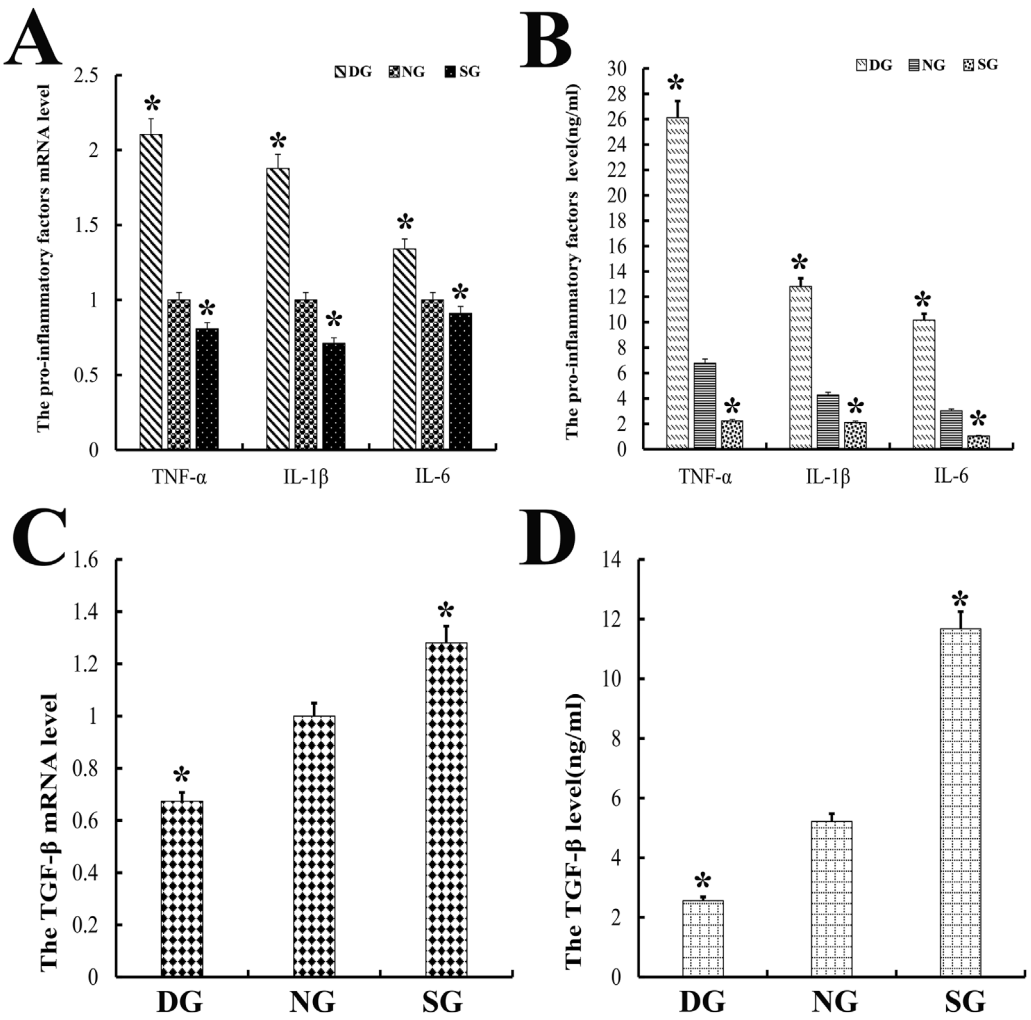


Fig. 6. The expressions of inflammatory factor in liver cell. (A) The TNF-α, IL-1β, and IL-6 mRNA levels in liver cells. (B) The TNF-α, IL-1β, and IL-6 protein expression levels in liver cells. (C) The TGF-β mRNA levels in liver cells. (D) The TGF-β protein expression levels in liver cells. β-actin was used as a control. DG, incubated with selenomethionine 0 μM. NG, incubated with selenomethionine 1.0 μM. SG, incubated with selenomethionine 10 μM. Data are presented as the means ± SD. (n = 10 per group). *p < 0.01 indicates a significant difference from the NG cells.

inflammatory cytokines have a key role in liver damage [31]. Some studies also reported that Se deficiency could induce inflammation in many organs of different animals [12,14,15]. The results of this study show an increase in the proinflammatory factors TNF-α, IL-1β, and IL-6 when Se concentrations were decreased. The proinflammatory cytokines are indicators of inflammation and aggravated tissue damage [32]. TNF-α is defined as an “early” cytokine [33]. IL-1β plays an important role in the regulation of the host immune responses [34]. This factor could damage liver cells and destroy liver structures in fish. According to current knowledge, fish also produce anti-inflammatory cytokines to reduce tissue damage by serving as antagonists against the proinflammatory cytokines. TGF-β was found to be a major factor for inhibiting inflammation in the livers of fish [35]. The results showed that TGF-β increased after Se supplementation, and it decreased under Se-deficient conditions. A previous study indicated that a decrease in TGF-β would exacerbate inflammation damage in liver tissue. Our results showed Se deficiency inhibited TGF-β expression and facilitated the production of TNF-α, IL-1β, and IL-6, which could induce inflammation in carp liver tissue.

Heat shock proteins (HSPs) are a class of proteins produced during oxidative stress [36]. HSPs can activate TLR signals that induce inflammation in the body. Many studies have found that HSPs induced proinflammatory factor expression [37], but HSP60 has rarely been studied, and the potential mechanisms have also been poorly studied. The present study verified that Se deficiency leads to an increase in HSP60 expression. The HSP60 overexpression was a reason for the inflammation injury [38]. HSPs could induce the TLRs signal. There are some differences between the TLRs signals of fish and mammals [21]. TLR4 was the major signal for the HSPs, but TLR2 was a major activated protein molecule involved in the inflammatory signalling process in carp [39]. TLR2 was examined in further study. The results showed the TLR2 expression was increased with was a dietary selenium deficiency, but there was no effect in the liver cells. The effect on TLR2 was not only in the protein expression but also in the activation of downstream signalling pathways in carp [40]. These results suggest that dietary selenium deficiency induced HSP60 production and TLR2 signalling activation. The results also serve as a reminder that the downstream pathway may be the key pathway in the inflammatory signalling process in carp.

To determine the mechanism behind the inflammatory effects of HSP60, we analysed the expression of the members of the TLR2 downstream pathway. MAPKs are the key signalling molecules in the development of inflammatory responses [41]. Many studies have indicated that the activation of the MAPK pathways increases IL-1β and TNF-α production as well as the inflammatory response [42]. The MAPKs include three principal factors: p38, JNK, and ERK. We measured the expression levels of all three factors both in tissues and cells. The results showed that p38, JNK, and ERK mRNA levels had no change

as HSP60 increased, which was induced by selenium deficiency. The phosphorylation of the p38, JNK, and ERK proteins increased with the HSP60 induced by selenium deficiency. As the dietary selenium increased, the HSP60 was reduced, and the phosphorylation of p38, JNK, and ERK proteins was also inhibited. The MAPKs inhibited phosphorylation, which is a regulatory effect in the inhibition of inflammation [43]. These results showed an increase in HSP60, which was increased by the selenium deficiency, could induce MAPK phosphorylation downstream of TLR2.

In summary, this study showed that dietary selenium was closely related to liver damage in carp. Dietary selenium deficiency induced deficiencies in selenium in the blood and liver, which induced HSP60 overexpression in liver. The TLR2-MAPKs pathway was activated by the increased HSP60, which regulated the TNF-α, IL-1β, IL-6 production. Inflammatory injury to the liver was formed and exacerbated. The results indicate that selenium deficiency induced inflammatory injuries in the liver, and an appropriate increase in dietary selenium helps with growth and disease prevention in carp.

Acknowledgments

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