

# Deficient and excess dietary selenium levels affect growth performance, blood cells apoptosis and liver HSP70 expression in juvenile yellow catfish *Pelteobagrus fulvidraco*

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**Abstract** We investigated the effects of deficient and excess dietary selenium (Se) on growth, blood cells apoptosis and liver heat shock protein 70 (HSP70) expression in juvenile yellow catfish (*Pelteobagrus fulvidraco*). After 8 weeks, yellow catfish (initial weight:  $2.12 \pm 0.01$  g) fed isonitrogenous and isolipid diets containing  $<0.05$  (deficient dietary Se) or 6.5 (excess dietary Se) mg Se/kg displayed a significantly lower weight gain ratio (WGR) than those fed a diet containing 0.23 (normal dietary Se) mg Se/kg. As dietary Se levels increased, liver Se concentration, glutathione peroxidase activity and the hepatosomatic index increased significantly. Plasma glucose concentration was highest in the normal treatment compared with the excess dietary Se treatment. Both deficient and excess dietary Se lead to increased reactive oxygen species (ROS) production and apoptosis ratio

in blood cells, whereas only excess dietary Se increased their cytoplasmic free- $\text{Ca}^{2+}$  (CF- $\text{Ca}^{2+}$ ) concentration. Excess dietary Se also resulted in the highest level of HSP70 expression, thereby possibly providing a protective mechanism against oxidative stress. These results indicate that both deficient and excess dietary Se restrained the growth of juvenile yellow catfish and caused oxidative stress. The overproduction of ROS may act as a signal molecule mediate apoptosis when dietary Se deficiency. Both ROS and CF- $\text{Ca}^{2+}$  were recorded when dietary Se excess, suggesting that  $\text{Ca}^{2+}$  may be activated by Se and play a major role during Se-induced oxidative stress and cell apoptosis.

**Keywords** Selenium (Se) · Growth · Apoptosis · Heat shock protein 70 · Yellow catfish

## Introduction

Se is an essential trace element required by fish to maintain a normal growth and optimal immune system. Se plays an important role in the biosynthesis of the enzyme glutathione peroxidase (GPx, EC 1.11.1.9) (Rotruck et al. 1973) and in the activity of deiodinases (Kohrle et al. 2005). The GPx family of enzymes functions as part of the body's antioxidant system, protects cell membranes against oxidative damage by catalysing reactions, which are necessary for the conversion of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and

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fatty acid hydroperoxides into water and fatty acid alcohol by using reduced glutathione, and also plays an important role in cellular signalling by mediating H<sub>2</sub>O<sub>2</sub> concentrations (Brigelius-Flohe 1999). The deiodinases are necessary for the activation of thyroid hormone T<sub>4</sub> to biologically active T<sub>3</sub> (Beckett et al. 1993; Kohrle et al. 2005) and control the ratio and levels of thyroid hormones, which are critical for regulating growth, metabolism (Kohrle et al. 2005) and metamorphosis in fish (Power et al. 2001; Witt et al. 2009).

An adequate intake of Se has many potential health benefits for aquatic animals, including improved growth, survival and immune response (Hilton et al. 1980; Lin and Shiao 2005; Wang et al. 2012). On the other hand, a Se deficiency is particularly damaging to cellular and mitochondrial membranes (Combs and Combs 1986) and results in reduced weight gain and oxidative stress of fingerling channel catfish (*Ictalurus punctatus*) (Gatlin and Wilson 1984) and Atlantic salmon (*Salmo salar*) (Bell et al. 1987). However, there is a narrow range between the beneficial and toxic levels of Se (NRC 1980; Kieliszek and Blazejak 2013). In rainbow trout (*Oncorhynchus mykiss*), the level for chronic Se toxicity (3 mg/kg) is only tenfold higher than the required levels of Se (0.3 mg/kg) (Hilton et al. 1980). Se in toxic level reduces the survival, length and weight of aquatic animals and causes oxidative stress (Hunn et al. 1987; Misra and Niyogi 2009; Lee et al. 2010; Wang et al. 2012; Le and Fotedar 2014).

Apoptosis or programmed cell death is a major mechanism for eliminating critically damaged cells and is followed by compensatory cell regeneration to maintain tissue structure and function (Elmore 2007). Apoptosis has been suggested as a sensitive and early indicator of chronic chemical stress and as an immune regulatory mechanism is conserved in fish demonstrating its importance in maintaining immunological homeostasis (Weyts et al. 1997). Heavy metals such as cadmium, copper, arsenic, silver and zinc are known inducers of apoptosis (Faverney et al. 2001; Gao et al. 2013; Kim et al. 2014). Although Se-induced apoptosis has been described in *poeciliopsis lucida* and rainbow trout (Misra and Niyogi 2009; Misra et al. 2012; Selvaraj et al. 2013), there is little information on Se-induced apoptosis in aquatic animals. Oxidative stress has been linked to apoptosis in various systems. For example, an overproduction of

ROS, NO and CF-Ca<sup>2+</sup> can induce apoptosis (Faverney et al. 2001; Reynaud et al. 2004; Xian et al. 2010, 2013). Heat shock proteins (HSPs) play a key role in either preventing or repairing cellular damage to maintain protein integrity in the presence of stress such as heat or chemicals (Iwama et al. 1998). HSP family member HSP70 protects cells from the deleterious effects of ROS (Favatiere et al. 1997; Polla 1998) and cytotoxicity (Salminen et al. 1996). HSP70 expression is induced by stress and is therefore commonly used as an indicator of cellular stress in animals (Sherman and Goldberg 2001). Recent studies have shown that HSP70 also protects cells from apoptosis at multiple points in the apoptotic process and provides a sensitive molecular biomarker for monitoring aquatic heavy metal pollution (Mallouk et al. 1999; Feng et al. 2003; Kim et al. 2014).

Yellow catfish is a freshwater aquatic species in China that is widely cultured in the provinces of Liaoning, Hubei, Sichuan and Zhejiang. Yellow catfish is highly popular in China and a promising market exists in Japan, South Korea and East and South Asia. Recently, with the development of intensive aquaculture farming, questions have been raised regarding the effect of heavy metals in the food or environment on farmed fish. However, the influence of dietary Se on this fish has not yet been investigated. Therefore, the main objectives of this study are to determine the effects of deficient and excess dietary Se on yellow catfish by determining (1) growth performance, main plasma biochemical indices and GPx activity, (2) oxidative stress as indicated by blood cells ROS production, CF-Ca<sup>2+</sup> concentration, NO production and apoptosis and (3) HSP70 expression in the liver.

## Materials and methods

### Experimental diets

The formula and approximate composition of the basal diet are given in Table 1. It was formulated with purified ingredients to provide 39.96 % crude protein from casein and 9.93 % crude lipid from fish oil and lecithin oil (4:1). The normal dietary Se concentration was according to our study of yellow catfish dietary Se requirements (0.23 mg/kg dry weight. Based on the WGR, the dietary Se requirement of juvenile yellow

**Table 1** Ingredients and proximate composition of the basal diet

Ingredients	Percents in diet (%, dry weight)
Casein <sup>a</sup>	46
Corn starch <sup>a</sup>	26.5
Fish oil <sup>a</sup>	8
Lecithin oil <sup>a</sup>	2
Vitamin premix <sup>b</sup>	0.5
Mineral mixture <sup>c</sup>	1.5
Choline chloride <sup>a</sup>	0.5
L-ascorbyl-2-phosphate <sup>a</sup>	0.1
Sodium chloride <sup>a</sup>	0.4
Ca(H <sub>2</sub> PO <sub>4</sub> ) <sub>2</sub> <sup>a</sup>	2
Betaine <sup>a</sup>	0.5
Carboxymethylcellulose <sup>a</sup>	3
Microcrystalline cellulose <sup>a</sup>	9
<i>Proximate composition</i>	
Crude protein	39.96
Crude lipid	9.93
Ash	5.37
Moisture	6.42

<sup>a</sup> Obtained from Guangzhou Fishtech Fisheries Science & Technology Co., LTD, Institute of Animal Science, Guangdong Academy of Agricultural Sciences (Guangzhou, China)

<sup>b</sup> Provided by the Institute of Animal Science, Guangdong Academy of Agricultural Sciences (Guangzhou, China). Vitamin premix (/kg diet): vitamin A, 2000 IU; vitamin D<sub>3</sub>, 700 IU; vitamin E, 10 mg; vitamin K<sub>3</sub>, 2.5 mg; thiamine, 2.5 mg; riboflavin, 5 mg; pyridoxine, 3 mg; vitaminB<sub>12</sub>, 0.01 mg; niacin, 17.5 mg; D-calcium pantothenate, 10 mg; folic acid, 0.8 mg; biotin, 0.045 mg; inositol, 25 mg

<sup>c</sup> Provided by the Institute of Animal Science, Guangdong Academy of Agricultural Sciences (Guangzhou, China). Mineral premix (/kg diet): MgSO<sub>4</sub>·H<sub>2</sub>O, 280 mg; CuSO<sub>4</sub>·5H<sub>2</sub>O, 6 mg; FeSO<sub>4</sub>·H<sub>2</sub>O, 30 mg; MnSO<sub>4</sub>·H<sub>2</sub>O, 4.9 mg; FeSO<sub>4</sub>·H<sub>2</sub>O, 30 mg; ZnSO<sub>4</sub>·H<sub>2</sub>O, 86.95 mg; feed grade calcium iodate (5 %), 15 mg; feed grade cobalt sulphate (5 %), 3 mg

catfish was estimated to be 0.20 mg/kg, using nonlinear regression analysis (unpublished). We chose the excess dietary Se concentration within the 4.6–6.5 mg Se/kg range, which is known to cause adverse effects in fish regardless of whether they are cold or warmwater species (Hamilton et al. 1990; Hamilton 2003). The experimental diets were supplemented with Se as Na<sub>2</sub>SeO<sub>3</sub> (Analytical Reagent, Tianjin Guangfu Fine Chemical Research Institute, China). The resulting dietary Se concentrations were <0.05

(deficient dietary Se), 0.23 (normal dietary Se), 6.5 (excess dietary Se) mg Se/kg as determined by flame photometry after wet decomposition according to the Association of Official Analytical Chemists (AOAC) (1995). The diets were prepared by mixing the ingredients thoroughly with water prior to pressure pelleting with a 5 mm die. Pellets were dried in an oven at 50 °C for 24 h. The diets were stored at –20 °C until used.

#### Feeding trials and sample collection

The 8-week feeding trial was conducted in an indoor flow-through system. During the experiment, water temperature ranged from 26 to 32 °C, dissolved oxygen was maintained above 6 mg/L, ammonia-N was below 0.2 mg/L, pH ranged from 7.0 to 7.5, and half the water in the system was exchanged daily. A 12-h dark/12-h light photoperiod was maintained during the entire experiment. Juvenile yellow catfish used in the study were obtained from a private hatchery. Before starting the feeding trial, fish were acclimated to the experimental conditions and fed a commercial diet (crude protein, 42.24 %; lipid, 7.06 %; ash, 10.06 %; moisture, 9.42 % and 0.46 mg Se/kg) for 2 weeks. Two hundred and twenty-five fish initially weighing  $2.12 \pm 0.01$  g were randomly distributed into 9 tanks (350 L capacity and 300 L water), with 25 fish per tank. Experimental diets were randomly assigned to three tanks per diet. Fish were fed to satiation twice daily (9:00 am and 4:00 pm) for 8 weeks. Daily feeding rate was about 4 % of total body weight. Indeed, few feed remained during the experiment, and we adjusted daily ration according to prior feeding responses. If diet remained, it would be collected by siphoning and then be dried and weighed. The actual amount of feed consumption was calculated for FCR calculation.

After the 8-week feeding trial, the fish were fasted for 24 h, then individually weighed and counted. Six randomly selected fish per tank were anesthetized in diluted MS-222 (ethyl 3-aminobenzoate methanesulfonate, Tricaine; Suzhou SciYoung BioMedicine Technology Co., Ltd) (60 mg/L) and used to collect blood and liver samples as follows. Blood was drawn from the caudal vein of the individual fish, collected in a syringe and transferred into a tube containing sodium EDTA as an anticoagulant, and centrifuged immediately at 3500×g for 10 min at 4 °C. The plasma was

preserved at  $-78\text{ }^{\circ}\text{C}$  for analysis. After collecting blood, the livers were removed and weighed. Part of each liver was stored in a plastic tube at  $-78\text{ }^{\circ}\text{C}$  for Se analysis. The other part of each liver was homogenized for 1 min with an ultrasonic cell crusher (Scientz Co., China) in ten times its volume of ice-cold 0.9 % physiological saline (0.9 % NaCl) and subsequently centrifuged at 4000 rpm for 10 min at  $4\text{ }^{\circ}\text{C}$ . The supernatant was transferred to a separate tube and stored at  $-78\text{ }^{\circ}\text{C}$  for further analysis.

A further three randomly selected fish from each tank were also anesthetized in diluted MS-222 and used for collection of blood cells for the apoptosis assay and whole livers for RNA isolation as follows. Blood was extracted from each fish by a 25-gage needle and 2.5 mL syringe containing an equal volume of ice-cold anticoagulant solution (AS, heparin sodium 10 g/L, sodium chloride 6.5 g/L, calcium chloride 0.12 g/L, sodium dihydrogen phosphate 0.01 g/L, sodium bicarbonate 0.2 g/L, potassium chloride 0.14 g/L). Each diluted blood cells solution was transferred into a separate microcentrifuge tube held on ice, further diluted with AS to obtain a final concentration of about  $1 \times 10^6$  cells/mL and then used for flow cytometric analysis. The three livers were excised, pooled, immediately frozen in liquid nitrogen and stored at  $-78\text{ }^{\circ}\text{C}$  for RNA isolation.

#### Hepatic parameters and plasma biochemical indices

Plasma biochemical indices were determined by a fully automatic biochemical analyser (HITACHI 7170A). GPx activity and malondialdehyde (MDA) content were determined using commercial kits (NJBI Clinical Reagent, Nanjing, China) following the manufacturer's instructions. The Se concentrations in liver were analysed by flame photometry after wet decomposition according to the AOAC (1995). The protein concentration in liver supernatant was measured spectrophotometrically according to Bradford (1976) using bovine serum albumin as a standard.

#### Growth performance parameters

Growth performance parameters were calculated as follows: weight gain ratio (WGR, %) =  $100 \times (\text{final body weight} - \text{initial body weight})/\text{initial body weight}$ ; feed conversion ratio (FCR) = feed consumption (total

feed casting – total feed residue) (g)/body weight gain (g); survival ratio (%) =  $100 \times (\text{final amount of fish})/(\text{initial amount of fish})$ ; hepatosomatic index (HSI) =  $100 \times (\text{liver weight (g)}/\text{body weight (g)})$ .

#### Flow cytometry

Flow cytometry was performed with a FACSCalibur equipped with a single argon ion laser with filtered emission at 488 nm. Photomultiplier bandpass filters for fluorescence were 530/30 nm (green fluorescence, FL1) and 582/42 nm (yellow/orange fluorescence, FL2). For each subsample, 10,000 events were counted. Cell Quest<sup>®</sup> software (Becton–Dickinson Immunocytometry Systems, San Jose, CA, USA) was used to create logical markers and mean fluorescence intensity analyses of fluorescence data.

#### Reactive oxygen species (ROS) production

ROS levels were measured using the cell-permeant probe 2',7'-dichlorofluorescein diacetate (DCFH-DA, Sigma) as described previously (Xian et al. 2011). Once inside the cell, DCFH-DA is deacetylated by cytosolic enzymes to the nonfluorescent polar molecule dichlorofluorescein (DCFH), which is trapped inside the cell and oxidized by ROS to the highly fluorescent 2',7'-dichlorofluorescein (DCF,  $\lambda$  excitation = 498,  $\lambda$  emission = 522). DCFH-DA solutions were prepared by dissolving DCFH-DA in dimethyl sulphoxide (DMSO) to 1 mM and stored at  $-20\text{ }^{\circ}\text{C}$ . A volume of 200  $\mu\text{L}$  blood cells suspension was incubated with 10  $\mu\text{M}$  DCFH-DA for 30 min at room temperature in the dark after which the DCF fluorescence was measured at 530/30 nm (FL1 filter) by flow cytometry. ROS production was expressed as mean fluorescence of DCF in arbitrary units (A.U.) (Xian et al. 2013).

#### NO production

Intracellular NO production was measured using the fluorescent probe 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA, Sigma). DAF-FM DA solutions were prepared by dissolving DAF-FM DA in dimethyl sulphoxide (DMSO) to 1 mM and stored at  $-20\text{ }^{\circ}\text{C}$ . A volume of 200  $\mu\text{L}$  blood cells suspension was incubated with 10  $\mu\text{M}$  DAF-FM DA

for 60 min at room temperature in the dark after which the DAF-FM fluorescence was measured at 530/30 nm (FL1 filter) by flow cytometry. NO production was expressed as mean fluorescence of DAF-FM in arbitrary units (A.U.) (Xian et al. 2013).

#### Cytoplasmic free-Ca<sup>2+</sup> (CF-Ca<sup>2+</sup>) concentration

The concentration of CF-Ca<sup>2+</sup> was determined using the cell-permeant probe fluo-3/acetoxymethyl ester (fluo-3/AM, Sigma) as described previously (Xian et al. 2010). Fluo-3/AM solutions were prepared by dissolving fluo-3/AM in dimethyl sulphoxide (DMSO) to 1 mM and stored at -20 °C. A volume of 200 µL blood cells suspension was incubated with 10 µM fluo-3/AM for 30 min at room temperature in the dark after which the fluo-3 fluorescence of cells was measured at 530/30 nm (FL1 filter) by flow cytometry. Esterase activity was expressed as the mean fluorescence of fluo-3 in arbitrary units (A.U.) (Xian et al. 2013).

#### Apoptotic cell ratio

Apoptotic blood cells were quantified using the annexin V-FITC/PI apoptosis detection kit (Invitrogen) following the manufacturer's instructions. Briefly, blood cells were washed with AS and resuspended at about  $3 \times 10^6$  cells/mL in  $1 \times$  annexin V binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl<sub>2</sub>). Two hundred microlitres of blood cells sample was stained with 2.5 µL of annexin V-FITC and 5 µL of 50 µg/mL PI working solution for 15 min in the dark. Then, 50 µL  $1 \times$  annexin V binding buffer was added to each tube, and the cells were immediately analysed by flow cytometry. Results were expressed as annexin V-FITC/PI in a dot plot. Cells stained negative with both probes represent live cells (quadrant 1). Cells stained positive with annexin V-FITC and negative with PI are early apoptotic cells (quadrant 2). Cells stained positive with both annexin V-FITC and PI are in the end stage of apoptosis, undergoing necrosis, or already dead (quadrant 3). The apoptotic cell ratio was expressed as the percentage of cells in quadrants 2 and 3 (Fig. 1).

#### RNA extraction and reverse transcription

Total RNA was extracted using the RNAiso reagent (Takara, Japan), according to the manufacturer's

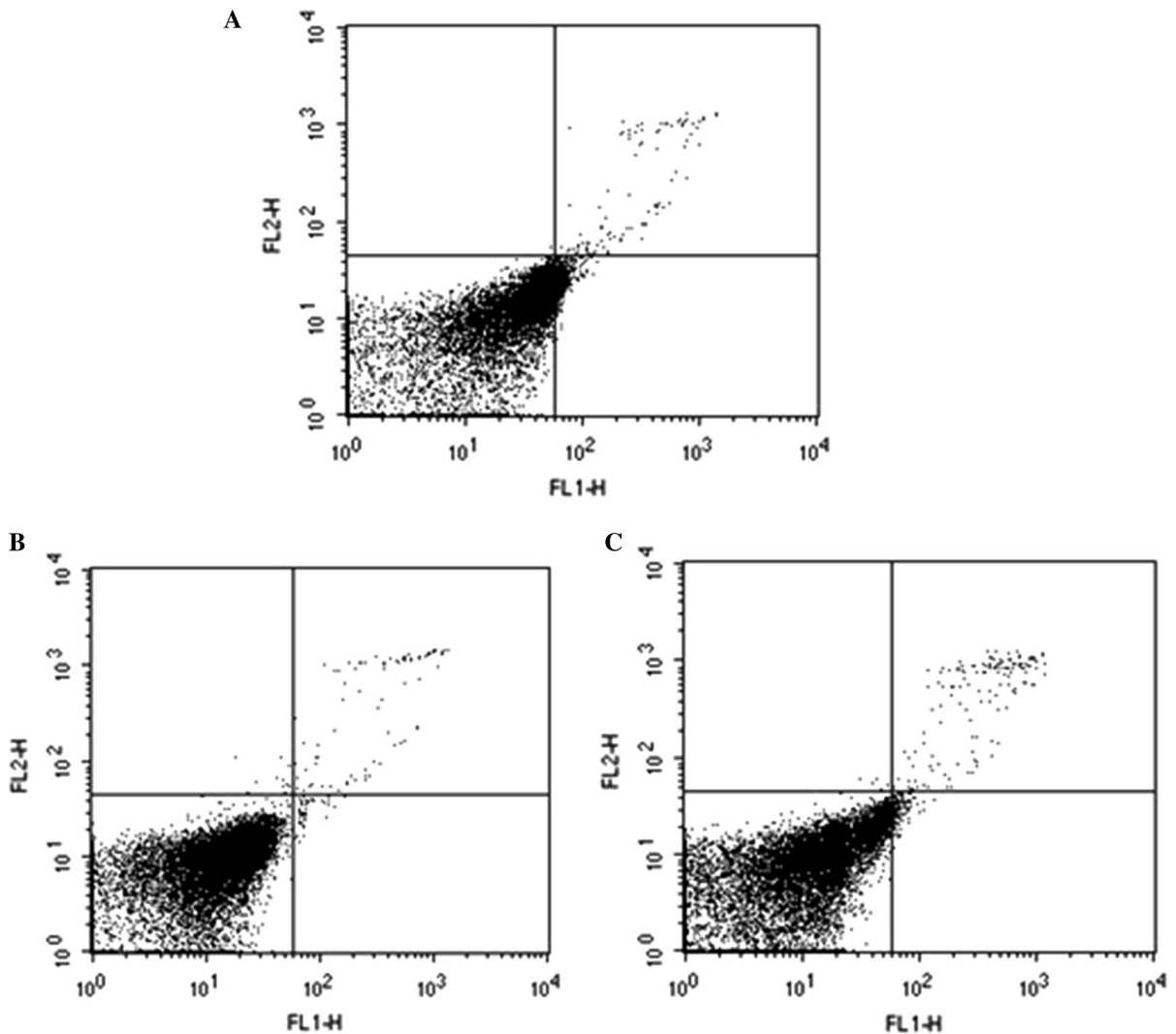
instructions. RNA quality was checked by measuring the absorbance at 260 and 280 nm using spectrophotometry (Thermo Scientific NanoDrop ND2000), and its integrity was confirmed by 1 % agarose gel electrophoresis. First-strand cDNA was transcribed from hepatopancreatic poly(A) RNA by incubating 13 µL RNA-Primer mix (1 µg total RNA, 10 µM random primers, 2.4 µM oligo-d (T)18 primers) at 65 °C for 10 min then adding 22 µL RTase mix (200 U of M-MLV RTase, 25 U of RNase inhibitor (Takara, Dalian, China),  $1 \times$  RT buffer, 1 mM of each dNTP) and incubating at 37 °C for 60 min. The cDNA mix was diluted to 1:4 and stored at -78 °C for subsequent real-time quantitative PCR (RT-PCR).

#### Real-time quantitative PCR

The liver tissue expression of HSP70 and  $\beta$ -actin was analysed by RT-PCR using primers based on the published HSP70 (FJ376083.1) and  $\beta$ -actin (EU161066.1) mRNA sequences as follows:  $\beta$ -actin forward primer, 5'-GGCAATGAGAGGTTTCAGG TG-3' and reverse primer, 5'-TCTCATGGATGCCG-CAGGA-3'; HSP70 forward primer, 5'-TGGAG-GAGGGTCTTCTGGAC-3' and reverse primer, 5'-CACCAAAGAAAACAAACGGACTG -3'. The RT-PCR amplifications were carried out in a total reaction volume of 20 µL, containing 10 µL SYBR Green 2  $\times$  Supermix (Applied Biosystems, USA), 2 µL of the 1:4 diluted cDNA (synthesized as described above), 2 µL each of 3 µM forward and reverse primers and 4 µL ddH<sub>2</sub>O, in the CFX Connect Real-Time PCR Detection System. The PCR program consisted of 95 °C for 12 min, followed by 40 cycles of 95 °C for 10 s, 58 °C for 10 s and 72 °C for 10 s. Sequences were verified by melting curve analysis, and relative gene expression levels were calculated using  $2^{-\Delta\Delta ct}$  method. All samples were run in triplicate, and each assay was repeated three times.

#### Statistical analysis

All data are expressed as mean  $\pm$  standard error and were subjected to one-way analysis of variance (ANOVA) after being checked for any violations of the ANOVA model. When overall differences were significant at less than 0.05, the Fisher's least significant difference (LSD) test was used to compare the means. All statistical analyses were performed using



**Fig. 1** Flow cytometric analysis of blood cells stained with annexin V-FITC (Ann V-FITC) and PI from juvenile yellow catfish fed deficient (**a**), normal (**b**) or excess (**c**) Se diets. Unchanged cells (Ann V-FIT-/PI-) are located in quadrant 1

(*bottom left*), early apoptotic cells (Ann V-FITC+/PI-) in quadrant 2 (*bottom right*), late apoptotic cells and necrotic cells (Ann V-FITC+/PI+) in quadrant 3 (*top right*)

the SPSS 11.5 program (SPSS Inc, Chicago, IL, USA). The level of significant difference was set at  $P < 0.05$ .

## Results

### Growth performance and biochemical indices

Fish fed deficient or excess dietary Se had a lower WGR and higher FCR compared to those fed normal

dietary Se ( $P < 0.05$ ). However, the survival ratio was not affected by changes in dietary Se ( $P > 0.05$ ) (Table 2), whereas changes in dietary Se also did not significantly affect plasma concentrations of albumin (ALB), globin (GLB), triglyceride (TG), cholesterol (CHO), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) ( $P > 0.05$ ), but the concentration of glucose (GLU) was significantly higher in the normal treatment compared with the excess dietary Se treatment ( $P < 0.05$ ) (Table 3).

**Table 2** Growth performances of juvenile yellow catfish fed deficient, normal or excess Se diets for 8 weeks (mean  $\pm$  S.E.,  $n = 3$ )

Treatments	WGR (%)	FCR	Survival ratio (%)
Deficient dietary Se (<0.05 mg/kg)	485.0 $\pm$ 21.9 <sup>b</sup>	1.02 $\pm$ 0.04 <sup>a</sup>	100
Normal dietary Se (0.23 mg/kg)	638.5 $\pm$ 48.5 <sup>a</sup>	0.81 $\pm$ 0.06 <sup>b</sup>	100
Excess dietary Se (6.5 mg/kg)	564.2 $\pm$ 38.6 <sup>ab</sup>	0.89 $\pm$ 0.02 <sup>b</sup>	100

Values in each column without a common superscript are significantly different ( $P < 0.05$ )

### Se concentrations, hepatosomatic index, GPx activity and MDA content

As dietary Se levels increased, liver Se concentrations and the HSI increased significantly ( $P < 0.05$ ) (Table 4) and displayed a dose–response relationship. GPx activity also increased with increasing dietary Se levels ( $P < 0.05$ ), but MDA content was not significantly affected ( $P > 0.05$ ) (Table 3).

### Blood cells and HSP70 expression

Fish fed either deficient or excess dietary Se had significantly increased blood cells apoptotic ratios (Fig. 1; Table 5) and ROS induction ( $P < 0.05$ ) (Table 5). Only the excess dietary Se group displayed a significant increase in CF-Ca<sup>2+</sup> concentration ( $P < 0.05$ ) (Table 5) and up-regulated HSP70 expression ( $P < 0.05$ ) (Fig. 2). All groups displayed a relatively constant NO production (Table 5).

### Discussion

In this study, the WGR of juvenile yellow catfish fed deficient or excess Se diets (485.0  $\pm$  21.9 and 564.2  $\pm$  38.6 %, respectively) was reduced compared to those fed a normal Se diet (638.5  $\pm$  48.5 %). Similar result has been reported that reduced growth is one of the first symptoms observed in Atlantic salmon (Bell et al. 1987) fed deficient Se diets. It has been confirmed that Se is a structural component of deiodinase, which is responsible for the metabolism of thyroid hormones. For the deficiency of Se, the activity of this enzyme may be suboptimal and exert an adverse influence by reducing insulin sensitivity of target tissues (Thomas and Pfeiffer 2012; Wiernsperger and Rapin 2010), then affected carbohydrate and lipid homoeostasis and prevented the storage of metabolic fuels after food intake (Steinbrenner 2013), which together reduce overall growth. Growth and development might also be reduced due to the toxic effects of high Se (Kim and Kang 2014), which

**Table 3** Main plasma biochemical indices of juvenile yellow catfish fed deficient, normal or excess Se diets for 8 weeks (mean  $\pm$  S.E.,  $n = 3$ )

Index	Se levels (mg Se/kg)		
	Deficient dietary Se (<0.05 mg/kg)	Normal dietary Se (0.23 mg/kg)	Excess dietary Se (6.5 mg/kg)
ALB (g/L)	9.07 $\pm$ 2.32 <sup>a</sup>	10.8 $\pm$ 4.9 <sup>a</sup>	6.97 $\pm$ 0.15 <sup>a</sup>
GLB (g/L)	27.1 $\pm$ 5.6 <sup>a</sup>	16.4 $\pm$ 4.8 <sup>a</sup>	20.6 $\pm$ 0.3 <sup>a</sup>
TG (mmol/L)	3.13 $\pm$ 0.30 <sup>a</sup>	2.34 $\pm$ 0.26 <sup>a</sup>	3.09 $\pm$ 0.29 <sup>a</sup>
CHO (mmol/L)	3.16 $\pm$ 0.25 <sup>a</sup>	2.62 $\pm$ 0.22 <sup>a</sup>	2.82 $\pm$ 0.14 <sup>a</sup>
GLU (mmol/L)	5.54 $\pm$ 0.55 <sup>ab</sup>	6.35 $\pm$ 0.49 <sup>a</sup>	4.33 $\pm$ 0.27 <sup>b</sup>
ALT(U/L)	25.7 $\pm$ 12.2 <sup>a</sup>	16.0 $\pm$ 2.7 <sup>a</sup>	16.3 $\pm$ 0.9 <sup>a</sup>
AST(U/L)	599.3 $\pm$ 12.6 <sup>a</sup>	459.7 $\pm$ 31.8 <sup>a</sup>	629.3 $\pm$ 87.3 <sup>a</sup>
LDH	2676.7 $\pm$ 1187.2 <sup>a</sup>	1213.7 $\pm$ 74.8 <sup>a</sup>	1577.0 $\pm$ 117.5 <sup>a</sup>

Values in each column without a common superscript are significantly different ( $P < 0.05$ )

ALB albumin, GLB globin, TG triglyceride, CHO cholesterol, GLU glucose, ALT alanine aminotransferase, AST aspartate aminotransferase, LDH lactate dehydrogenase

**Table 4** Se concentration, HIS, GPx activity and MDA content in liver of juvenile yellow catfish fed deficient, normal or excess Se diets for 8 weeks (mean  $\pm$  S.E.,  $n = 3$ )

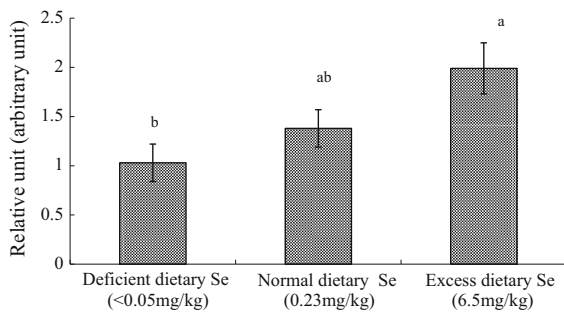
Treatments	Se ( $\mu\text{g/g}$ dry liver weight)	HIS	GPx	MDA
Deficient dietary Se (<0.05 mg/kg)	$0.13 \pm 0.01^b$	$1.65 \pm 0.02^b$	$28.2 \pm 0.2^c$	$1.01 \pm 0.10^a$
Normal dietary Se (0.23 mg/kg)	$0.51 \pm 0.08^b$	$1.70 \pm 0.04^b$	$35.8 \pm 1.5^b$	$0.91 \pm 0.12^a$
Excess dietary Se (6.5 mg/kg)	$3.20 \pm 0.55^a$	$1.96 \pm 0.03^a$	$69.7 \pm 6.8^a$	$0.97 \pm 0.07^a$

Values in each column without a common superscript are significantly different ( $P < 0.05$ )

**Table 5** ROS production, NO production, CF-Ca<sup>2+</sup> concentration and apoptotic ratio in blood cells of juvenile yellow catfish fed deficient, normal or excess Se diets for 8 weeks (mean  $\pm$  S.E.,  $n = 6-9$ )

Treatments	ROS	NO	CF-Ca <sup>2+</sup>	Apoptotic ratio
Deficient dietary Se (<0.05 mg/kg)	$53.5 \pm 4.1^a$	$45.0 \pm 2.0^a$	$55.6 \pm 2.6^b$	$2.44 \pm 0.27^a$
Normal dietary Se (0.23 mg/kg)	$31.2 \pm 2.7^b$	$43.8 \pm 2.0^a$	$61.0 \pm 4.1^b$	$1.57 \pm 0.08^b$
Excess dietary Se (6.5 mg/kg)	$60.7 \pm 3.6^a$	$49.4 \pm 3.3^a$	$74.8 \pm 4.5^a$	$2.69 \pm 0.20^a$

Values in each column without a common superscript are significantly different ( $P < 0.05$ )

**Fig. 2** Expression of HSP70 in liver from juvenile yellow catfish fed deficient, normal or excess Se diets for 8 weeks.  $\beta$ -Actin was used as the loading control. The bar diagram shows the relative HSP70 expression level in liver. Results are expressed as mean  $\pm$  SE ( $n = 3$ ). Bars denoted with different letters indicate a significant mean difference in expression between treatments ( $P < 0.05$ )

have been reported in the studies of rainbow trout (Hilton et al. 1980), channel catfish (Gatlin and Wilson 1984), juvenile grouper (*Epinephelus malabaricus*) (Lin and Shiau 2005), juvenile yellowtail kingfish (*Seriola lalandi*) (Le and Fotedar 2014) and red seabream (*Pagrus major*) (Kim and Kang 2014). The oxidative stress of Se toxicity caused glycogen depletion of hepatocytes, additional energy cost and then depressed growth rates (Riu et al. 2014). These results indicated that for optimal growth, animals require an optimized Se supplementation. It is of particular interest that fish fed deficient Se diet had a

higher FCR compared to those fed normal Se diet; similar results were also observed in the studies of channel catfish (Wang and Lovell 1997), juvenile grouper (Lin and Shiau 2005) and grass carp (*Cenopharyngodon idellus*) (Su et al. 2007). Su et al. (2007) have reported adding Se to feeding diet enhanced the activities of amylase, protease and lipase enzymes, improved digestive system health and contributed to efficient digestion (Nugroho and Fotedar 2015). Although there was no significant difference of FCR between normal dietary Se treatment and excess dietary Se treatment but the juvenile olive flounder (*Paralichthys olivaceus*) and *Acipenser* fed high Se diets were observed the histopathological lesions of liver or kidney (Lee et al. 2010; Riu et al. 2014), owing to the damage of these important organs, a poorer feed efficiency for a long time might occur (Lee et al. 2010). From these results, we infer that Se plays an important role in maintaining digestive system health and improving feed utilization (Nugroho and Fotedar 2015), which can help fishes obtain the best growth performance with the lowest feed cost. We found that deficient and excess Se diets did not affect mortality, a result similar to studies on other aquatic species, e.g. juvenile abalone (*Haliotis discus hannai* Ino), yellowtail kingfish (*Seriola lalandi*), Siberian sturgeon (*Acipenser baeri*) and white sturgeon (*Acipenser transmontanus*) (Wang et al. 2012; Pacini et al. 2013; Le and Fotedar 2014; Riu et al. 2014). The possible reason may be that the duration of



the feeding trial was not long enough to cause a high mortality of this fish and that the reduced growth is likely a physiological trade-off for achieving a comparatively lower Se-induced mortality (Riu et al. 2014).

Liver have the highest tissue concentrations of Se (Lee et al. 2010). In our study, a dose–response relationship of dietary Se and the liver's Se concentration, its HSI, GPx activity was observed up to 6.5 mg Se/kg diet, liver's Se concentration, its HSI, GPx activity as bio-indicators of dietary Se exposure. In contrast, a decrease in the HSI was observed in the green sturgeon (*Acipenser medirostris*) (Lee et al. 2011; Riu et al. 2014) and juvenile red swamp crayfish (*Procambarus clarkia*) (DÖrr et al. 2013) as dietary Se increased. The authors proposed that a decreased HSI might reflect the depletion in energy reserves under Se-induced stress conditions (DÖrr et al. 2013; Riu et al. 2014). We speculated that the increased HSI in our study as a result of high dietary Se deposition might have caused a physiological stress of juvenile yellow catfish liver. However, there was a lack of further study by histological observation to reflect this pathological stage.

Environmental (e.g. temperature, photoperiod, density and salinity), physiological (e.g. reproductive cycle, age, gender and nutrition) and social (e.g. social hierarchy) factors have been reported to affect blood parameters in fish (Chen et al. 2003; Bowser 1993). Blood chemistry parameters may also be a sensitive method to detect the presence of stressors in the culture environment (Chen et al. 2003). Whereas protein, GLU, LDH and transaminases are sensitive to stress conditions and often used as indicators of stress conditions (Kavitha et al. 2010; Ramesh et al. 2014), TG and CHO are generally affected by the metabolism of carbohydrate, lipid and protein (Andenen et al. 1992) and are used to monitor the course of disease in lipid metabolism disorders (John 2007). In the present study, plasma GLU in juvenile yellow catfish decreased in response to excess Se diets. This significantly decreased parameter usually indicates the fish's anaemic condition, which might have resulted from a large energy consumption caused by the oxidative stress of deficient or excess Se intake. Previous studies have described how fish cope with the increased energy demand created by stress, including Se stress, by elevating their plasma glucose levels to provide various organs/tissue with energy (Barton

2002; Ramesh et al. 2014; Kim and Kang 2014). Whereas the alteration of other involved plasma parameters in this study was not changed significantly, the considerable variation may also be provided the reflection of stress condition (Kim and Kang 2014; Ramesh et al. 2014).

Release of ROS is a critical step in the innate immune response, but if the generation of ROS exceeds a system's ability to neutralize and eliminate them, oxidative stress may occur (Lushchak 2011). Excess ROS directly oxidizes the cell membrane and macromolecules, causing damage to lipids, proteins and DNA, and ultimately leads to cell death (Halliwell and Gutteridge 1989; Kelly et al. 1998). In the present study, deficient dietary Se resulted in a significant increase in ROS and apoptosis in blood cells. Deficiencies of Se are known to cause tissue peroxidation due to a reduced antioxidant capacity resulting from a decreased GPx activity (Bell et al. 1987; Combs and Combs 1986; Behne and Kyriakopoulos 2001). Reddy et al. (1998) have showed that free radical production is greater in exercised rats with a Se deficiency. According to our study, a lack of protective effect by the lower GPx activity in liver of fish fed deficient Se diet may reduce the primary cellular antioxidant defence, released ROS and then induced apoptosis to resist this oxidative stress.

ROS plays a significant role in inducing Se toxicity. Not only can Se itself trigger a generation of ROS, but ROS are also generated intracellularly via the mitochondrial pathway of apoptosis when mitochondrial membranes are damaged (Selvaraj et al. 2013). There are three main ways in which Se results in toxicity: the excess amount of Se can form  $\text{CH}_3\text{Se}-$ , which enters a redox cycle, generates superoxide and oxidative stress, or forms free radicals (Spallholz and Hoffman 2002); selenocysteine as a result of excess Se inhibits the Se methylation metabolism and may cause other Se-related adverse effects (Spallholz and Hoffman 2002); in particular, Se can disrupt proteins via substituting as sulphur in sulphur bonds, resulting in incorrect protein shape, dysfunctional enzymes and impairment of normal cellular biochemistry (Lemly 2002; Spallholz and Hoffman 2002). In the present study, Se toxicity is manifested in ROS overproduction, with apoptosis acting to eliminate the critically damaged cells. Similarly, Selvaraj et al. (2013) have demonstrated that high level of Se exposure induces mitochondrial membrane potential damage, DNA damage and

elevated production of ROS-induced *Poeciliopsis lucida* hepatoma cell death. The peroxidation of membrane lipids due to ROS insult also increased with increasing selenomethionine (SeMet) exposure in isolated hepatocytes of rainbow trout (Misra et al. 2012), and the peak of activity for generating superoxide from SeMet occurs after early liver development in the embryos of this fish (Palace et al. 2004). To this day, the precise mechanisms of apoptosis induced by Se are not well understood (Philchenkov et al. 2007). However, it is believed that ROS may play a crucial role in Se decreased cell viability and Se-induced apoptosis (Zou et al. 2007). Antioxidant enzymes are considered to be the first line of cellular defence against oxidative damage. GPx as an essential antioxidant enzyme has been reported to protect cell against apoptosis induced by ROS (Fu et al. 2001). It is important to note that though the GPx activity in liver of fish on excess Se diets runs up to  $69.7 \pm 6.8$  U/mg prot, which significantly higher than the  $35.8 \pm 1.5$  U/mg prot for those on normal Se diets, this activity apparently is still inadequate to capture all ROS generated by excess dietary Se intake. This is consistent with the previous observation that the capacity of ROS interception by the enzymatic antioxidant (SOD, GPx and CAT) pathways in trout hepatocytes was inadequate during high SeMet exposure (Misra et al. 2012).

Calcium functions as a second messenger in a variety of cellular processes in different organisms. Agents which increase CF-Ca<sup>2+</sup> have the ability to trigger apoptosis. A former works have shown that Se compounds are able to open Ca<sup>2+</sup> release channels (Wang et al. 2003; Salazar et al. 2008; Misra et al. 2012). A rapid increase in intracellular Ca<sup>2+</sup> released when rainbow trout were exposed to a high dose of SeMet (Misra et al. 2012), while the changes of intracellular Ca<sup>2+</sup> induced by Na<sub>2</sub>SeO<sub>3</sub> also have found in SW480 cells (Wang et al. 2003). Our observations are in agreement with these previous findings. In this experiment, a significant CF-Ca<sup>2+</sup> increase was observed in fish fed excess dietary Se. Interestingly, it is important to note that an increased CF-Ca<sup>2+</sup> concentration was observed only for the excess dietary Se treatment while a strong overproduction of ROS was observed for both deficient and excess dietary Se treatments. Previous studies have shown that there was a close relationship between Ca<sup>2+</sup> and ROS in signal transduction pathways (Wang

et al. 2003). ROS generation has triggered an increase in intracellular Ca<sup>2+</sup> levels, disrupted the Ca<sup>2+</sup> balance (Misra et al. 2012) and then induced apoptosis. However, Wang et al. (2003) have suggested that intracellular ROS is also Ca<sup>2+</sup>-dependent and derived from mitochondria as a result of Ca<sup>2+</sup> changes induced by Na<sub>2</sub>SeO<sub>3</sub>. Apart from the oxidative stress, intracellular Ca<sup>2+</sup> might also be involved in SeMet-induced following exposure to the highest SeMet dose (Dong et al. 2006; Misra et al. 2012). The source of Ca<sup>2+</sup> may derive from either extracellular Ca<sup>2+</sup> or intracellular Ca<sup>2+</sup> stores including endoplasmic reticulum and mitochondria (Bouron 2000). As Se can oxidize a large amount of thiols on cellular membranes and destroy the integrity of cell membranes, extracellular Ca<sup>2+</sup> flows into the cell (Jenkins and Hidiroglou 1971; Misra et al. 2012). Based on these analysis, we suggests that increased CF-Ca<sup>2+</sup> may depend on Se-dependent oxidations and may be from extracellular Ca<sup>2+</sup> influx by oxidation of cellular membrane thiols.

Nitric oxide (NO) functions as a cellular messenger in various physiological and pathological processes. NO contributes to apoptosis via nitrosylation or nitration of key signalling molecules or via the impairment of mitochondrial respiratory chain electron transfer. A previous study in chicken demonstrated Se deficiency induced the higher levels of NO and iNOS, NO played a causative role in the Se deficiency—induced apoptosis of immune cells (Zhang et al. 2013; Zhao et al. 2014). However, NO production was not influenced by the changes in dietary Se in this study, suggesting that NO response against dietary Se may be different between terrestrial and aquatic animals.

Our results showed that excess dietary Se up-regulated HSP70 RNA expression in fish liver. Similarly, studies on abalone (*Haliotis discus hannai*) and intertidal copepod (*Tigriopus japonicus*) have reported HSP70 as a sensitive molecular biomarker, which is highly elevated upon exposure to heavy metals (Wu et al. 2011; Rhee et al. 2013; Kim et al. 2014). It has been shown that upon heavy metal exposure, denatured proteins resulting from the accumulation of toxic ROS induce heat shock protein expression (Dean et al. 1991; Fukuda et al. 1996; Zhang et al. 2009), supporting the notion that increased HSP70 expression may be capable of shifting the response to a large toxic insult from necrosis to apoptosis (Mallouk et al. 1999; Feng et al.

2003) and may be used to protect cells from oxidative stress. However, a negative relationship between the expression of HSP70 and apoptosis in juvenile channel catfish (*Ictalurus punctatus*) ovaries (Weber and Janz 2001) has been reported. This difference in results may be explained by different toxicant concentrations and/or differences between acute and chronic toxicant exposure (Weber and Janz 2001). Recent work by Penglase et al. (2010) has shown that the HSP70 expression of cod (*Gadus morhua* L.) larvae was no different when fed normal rotifers compared to Se-enriched rotifers, because Se-enriched rotifers are less likely to be toxic than sodium selenite. These results suggest that response of HSP70 expression may be connected with the species, Se concentration and exposure duration.

In summary, this study demonstrated that both deficient and excess dietary Se reduce the growth of yellow catfish and cause oxidative stress. ROS overproduction paralleled cell apoptosis could be observed in fish fed diet with deficient or excess Se, CF-Ca<sup>2+</sup> levels increased when dietary Se excess, suggesting that Ca<sup>2+</sup> may play a major role during excess Se-induced oxidative stress and cell apoptosis. NO production was not triggered by dietary Se-induced oxidative stress and therefore took no part in blood cells apoptosis. The HSP70 expression up-regulated upon excess dietary Se intake may serve to resist the stress caused by Se toxicity.

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