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Original Research Article

Selenium nanoparticles in aquaculture: Unique advantages in the production of Se-enriched grass carp (*Ctenopharyngodon idella*)

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ABSTRACT

The production of selenium-enriched fish can contribute to alleviating selenium deficiency in human diets. However, it is still unclear which selenium source, as an additive, can efficiently and cost-effectively produce high-quality selenium-enriched fish. This study evaluated the effects of selenium nanoparticles (SeNP), selenite, and selenomethionine (SeMet) on the growth, antioxidant capacity, selenium content, selenium speciation, and meat quality of grass carp. Ten diets were prepared, including a basal diet (BD) and three concentrations (0.1, 0.3, and 0.9 mg/kg) of SeNP, selenite, and SeMet. A total of 600 fish (250.79 ± 1.57 g) were randomly assigned to 30 tanks (3 tanks/group). Fish were fed the experimental diet three times daily for 60 d. In this study, SeNP most significantly promoted the growth and antioxidant capacity of grass carp, with 0.3 mg/kg SeNP identified as the optimal additive concentration. Additionally, SeNP demonstrated equally excellent bioavailability as SeMet and significantly increased the content of SeMet in grass carp (*Ctenopharyngodon idella*) muscle. Furthermore, compared to SeMet and selenite, dietary SeNP could more significantly enhance the content of selenocysteine ($SeCys_2$) and methylselenocysteine (MeSeCys) in grass carp muscle tissue. In addition, we have demonstrated that $SeCys_2$ and MeSeCys promote apoptosis of cancer cells (HeLa) through the mitochondrial apoptotic pathway (involving Bax and Bcl-2). Furthermore, as an additive, 0.3 mg/kg SeNP significantly improved the flesh quality of grass carp by reducing crude fat and heavy metal content, as well as increasing the levels of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and the ratio of n-3/n-6 polyunsaturated fatty acid (PUFA). In summary, SeNP is the most suitable additive for producing selenium-enriched fish.

1. Introduction

Selenium (Se) is an essential micronutrient that plays a vital role in animal and human health. It is a crucial element in the production of 25 selenoproteins, which play various roles in redox balance, immunity, reproduction, and the metabolism of thyroid hormones (Ferreira et al., 2021; Kieliszek and Błażejak, 2016). In China, over 70% of the population is affected by Se deficiency, putting more than 70 million people at risk of health problems related to this deficiency (Gao et al., 2011). The global issue of Se deficiency in diets has been acknowledged, with an estimated 0.5 to 1 billion individuals worldwide having inadequate Se intake levels (Winkel et al., 2012), and addressing this issue is an urgent priority. Meat is the primary source of Se in the human diet (Wang et al., 2018). Fish offers a much healthier food source compared to terrestrial meat products (including processed meats, red meat, and poultry) in the global fight against malnutrition and obesity (Tacon et al., 2020). In 2018, global grass carp (*Ctenopharyngodon*

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idella) production exceeded 5,700 thousand tons, making it the most widely produced fish in the world (Li et al., 2023). Therefore, the cultivation of grass carp enriched with Se may be a secure and efficient solution to tackle the issue of Se deficiency in human diets. However, determining the appropriate Se source and concentration as an additive remains a significant challenge in the production of Se-enriched fish.

Se exists in various forms and oxidation states in the environment, including selenite and selenate (Se^{4+}, Se^{6+}), solid-state Se (selenium nanoparticles), and biogenic Se forms such as selenomethionine and selenocysteine (Wadhwani et al., 2016). The concentration and chemical form of Se not only impact its metabolic pathway and bioavailability in organisms but also affect their physiological functions (Weekley and Harris, 2013). Generally, organic forms such as selenomethionine (SeMet), selenocysteine ($SeCys_2$), and methylselenocysteine (MeSeCys) are considered to have greater bioavailability than inorganic forms (Maseko et al., 2013). Selenium nanoparticles (SeNP), the novel form of Se in the form of nanoparticles, have gained significant attention for their bioavailability (Wu et al., 2012). Analyzing the Se speciation of Se-enriched products is crucial in accurately evaluating their applications, as the biological functions and metabolism of Se are highly dependent on its chemical form. This makes Se speciation analysis significant in determining the effectiveness and safety of these products (Ip et al., 2000). Appropriate Se form additives in feed not only enhance the production of beneficial Se forms in fish meat but also maintain or even enhance its nutritional value. Nutritional value indicators, including protein, lipids, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), as well as safety indices such as heavy metals, can be used to evaluate the flesh quality of fish (Li et al., 2023).

Therefore, the aim of this study is: (1) to compare the growth, antioxidant capacity, and deposition efficiency of different dietary Se forms (selenite, SeMet, and SeNP) in grass carp, as well as their impact on the Se species in the muscle; (2) to study the impact of these Se sources on the flesh quality of grass carp (muscle composition, heavy metals, and fatty acids); (3) to evaluate the health benefits (the effect on cancer cells) of Se-enriched grass carp after fed with different Se sources.

2. Materials and methods

2.1. Animal ethics statement

All animal experiments were conducted following the ARRIVE guidelines and approved by the Animal Care and Use Committee of Northwest A&F University (NWAFU-DKXC-20210606).

2.2. Reagents and standards

Protease K, trypsin, protease type XIV, and 100 mM Tris were purchased from Beijing Solarbio Science & Technology Co., Ltd (Beijing, China). Citric acid and sodium 1-hexanesulfonate from Aladdin Chemical (Shanghai, China) were purchased to detect Se speciation separation. The SeMet, MeSeCys, selenate, and selenite used in this study were purchased from Sigma–Aldrich (St Louis, Mo, USA). Se stock standard solutions (1 μg/mL) were purchased from SPEX CertiPrep (Metuchen, USA). The Beijing Institute of Chemical Reagents (Beijing, China) provided the high-grade pure nitric acid (HNO_3) and methanol used in this study.

2.3. Biosynthesis and observation of SeNP

The biogenic SeNP used in this study was produced using *Bacillus subtilis* following our previous study (Zhu et al., 2023). In brief, 8 mM Na_2SeO_3 and *B. subtilis* were added to lysogeny broth liquid medium and incubated for 24 h at 37 °C in a rotary shaker. Subsequently, the biogenic SeNP was extracted for scanning electron microscopy after ultrasonication.

Scanning electron microscopy (SEM, Hitachi S-4800, Tokyo, Japan) was utilized to observe the SeNP following the method described by Cremonini et al. (2016). Initially, the samples were fixed for 24 h at 4 °C with 2.5% glutaraldehyde and then treated with isoamyl acetate before undergoing critical-point drying. The dehydrated samples were placed on a silicon slice using a series of increasing ethanol concentrations (30%, 50%, 70%, 80%, and 100%). The morphology of SeNP was observed using SEM. Subsequently, an XL30 ESEM (FEI, Hillsboro, OR, USA) with an EDAX microanalytical system was used to analyze the elemental composition of the SeNP. To determine the size distribution of SeNP, the PCCS technique (NANOPHOX 1 to 10,000 nm, Sympatec GmbH, Germany) was utilized in this study.

2.4. Experimental diets

To meet the nutritional requirements of grass carp (Zheng et al., 2018), ten diets were prepared, including a basal diet (BD) and three concentrations (0.1, 0.3, and 0.9 mg/kg) of SeNP, selenite, and SeMet. Sigma–Aldrich provided sodium selenite (purity 99%) for fish feeding, while Chuanqi Medical Co. Ltd (Nanjing, China) supplied SeMet (purity 98%). The ingredients and different Se sources were mixed, extruded, and dried by forced draft for 24 h at room temperature and then stored at −20 °C until use. The formulation and composition of the basal diet are shown in Table S1.

2.5. Experimental design and sample collection

Grass carp in good health were obtained from a fishing location in Zhashui City, Shanxi Province, China. A total of 600 grass carp with an initial body weight (IBW) of 250.79 ± 1.57 were randomly allocated into thirty experimental net cages, each containing 20 fish, and each treatment group had three replicate cages (with approximately 400 L of water). Flowing water was provided to the ponds during the 60-d feeding period. A microporous oxygenation device was installed in each cage to maintain a continuous supply of dissolved oxygen at a minimum level of 7 mg/L. During the feeding period, the temperature varied between 20 and 31 °C, and the average values for pH and dissolved oxygen were 8.2 ± 0.6 and 7.8 ± 0.3 mg/L, respectively. Daily checks were conducted on water parameters to ensure a stable water environment. Before the experiment, all fish were acclimated for two weeks in mesh cages with three feedings per day (08:30, 12:30, and 16:30). In addition, the feeding frequency was kept the same throughout the experiment, and the fish were fed with the experimental diets three times daily to apparent satiation. Unconsumed feed was removed, dried for 24 h, and weighed to calculate feed efficiency.

After the rearing experiment was completed, all fish were euthanized with 0.5 g/L MS-222 (Sigma, St. Louis, MO, USA) to obtain the final body weight (FBW) of fish in each tank. To collect blood samples, the syringe was used to obtain blood from the caudal vein of four randomly selected fish per cage. The samples were allowed to clot at 4 °C for 6 h, and then they were centrifuged (825 × *g*, 4 °C, 15 min) to obtain serum, which was stored at −80 °C for subsequent antioxidant capacity analysis. After drawing blood samples, the fillets on the left side, between the dorsal fin and the head above the lateral line, were rapidly excised with a sharp scalpel, snap-frozen in liquid nitrogen, and stored at −80 °C. These samples were then used for subsequent analysis of total Se and heavy metals. Next, fillets were collected from 9 fish (3 fish per cage) randomly selected from each treatment for Se species

analysis using the same method and from the same position on the fish. Muscle composition and fatty acid analysis were performed on 4 fish randomly selected from each cage, respectively. Similarly, the muscle samples were taken from the left dorsal fin to the lateral line of the head.

The weight gain (WG), specific growth rate (SGR), weight gain rate (WGR), and feed conversion ratio (FCR) were calculated based on the following equations:

FI = feed intake (total amount of the dry feed consumed);

WG (g) = final body weight (g) − initial body weight (g);

SGR (%/d) = [ln final weight (g) − ln initial weight (g)]/number of days × 100;

WGR (%) = 100 × [final body weight (g) − initial body weight (g)]/initial body weight (g);

FCR = feed intake/(final weight of fish − initial weight of fish).

2.6. Analyses of serum antioxidant capacity

Nanjing Jiancheng Bioengineering Institute (Nanjing, China) provided assay kits to measure the serum total antioxidant capacity (T-AOC), malonaldehyde (MDA) content, and hydrogen peroxide (H_2O_2), which were used following the manufacturer's instructions.

2.7. Analyses of the total Se content and Se species

To detect the Se content in fish muscle and BD, the method in a previous study was used in this study (Zhang et al., 2020). Briefly, the dried muscle samples or BD, weighing 0.2 g each, were placed in 50 mL digestion tubes to which 8 mL of HNO3 was added. The mixture was allowed to stand overnight at room temperature and subsequently heated using a microwave digestion system (CEM-MARSX, CEM Corporation, Matthews, NC, USA) with the following program: 5 min at 120 °C, 10 min at 150 °C, and 20 min at 190 °C. Subsequently, the digestion tubes were placed into an electric evaporation block until the liquid in the tubes evaporated to approximately 1 mL. The solution was then transferred to a 25-mL volumetric flask, and the digestion tubes were rinsed 3 times with ultrapure water and poured into the volumetric flask. Finally, the solution was diluted with ultrapure water to the mark on the flask and then prepared for machine detection.

To analyze the Se species of fish muscle and SeNP, a method with some modifications (Zhang et al., 2020) was used in this study. Briefly, the fish muscle was freeze-dried at −60 °C, followed by grinding into powder with liquid nitrogen. Next, 0.2 g of the powder was accurately weighed and added to a 50-mL centrifuge tube containing 20 mL of 100 mM Tris−HCl buffer (pH 7.4) and 0.2 g of trypsin. The mixture was thoroughly mixed and then sonicated for 1 h, followed by shaking at 37 °C for 1 h. Protease type XIV (0.2 g) was added, and the mixture was shaken at 40 °C for 8 h, followed by the addition of 0.2 g proteinase K and shaking at 50 °C for 14 h. After centrifugation at 10,000 × *g* for 10 min, the supernatant was filtered through a 0.22-μm filter (Millipore, Bedford, MA, USA), followed by further processing using a 15-mL 10.0 kDa ultra centrifugal filter (Millipore, Bedford, MA, USA). The obtained sample was evaporated to 2 mL at 43 °C using a rotary vacuum evaporator (rE-52A, Shanghai Yarong Biochemical Instrument Factory, Shanghai, China). Finally, the sample was analyzed using Agilent 7900 ICP–MS. The same process was also used to analyze the Se composition of the SeNP surface.

2.8. Analyses of proximate compositions, heavy metals and fatty acid composition of grass carp muscle

The proximate composition of BD and muscle samples was analyzed using standard procedures outlined by AOAC (1995). In short, the sample was placed in a 105 °C oven until complete evaporation of moisture and then kept at a constant weight. The water content of the sample was measured thereafter. Furthermore, the crude protein content was determined by the Kjeldahl method (nitrogen × 6.25), while the crude fat content was determined by the Soxhlet extraction method. The crude ash content of the samples was measured using a muffle furnace. The methods described by Bosch et al. (2016) were used to determine the concentration of copper, cadmium (Cd), and arsenic (As) in the muscle. To analyze meat samples, a small amount of homogenized meat (approximately 0.3 g) was mixed with two acids (2 mL HCl and 8 mL HNO_3) in a microwave digester for 20 min at 160 °C. The solution was then diluted to 10 mL with ultrapure water and analyzed using an Agilent 7900 ICP–MS machine. In addition, the fatty acid composition of muscle was detected according to the methods recommended by Gou et al. (2020). First, the sample lipids were extracted using chloroform-methanol in a 2:1 (vol/vol) ratio. Second, the lipid fraction was dissolved by adding 1 mL of normal hexane, followed by a 1 h methyl esterification process with 1 mL of 0.4 M potassium hydroxide methanol. Following the addition of 2 mL double-distilled water, the resulting mixture was divided into two layers. Finally, after separating the upper layer, it was subjected to GC analysis, and a single FAME was quantified by comparing it to a recognized standard (47015-U, Sigma−Aldrich, Inc.).

2.9. Cell lines and cell culture

Human cervical adenocarcinoma HeLa, human embryonic kidney (HEK293T) cells were obtained from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in DMEM, high glucose, GlutaMAX (Thermo Fisher Scientific, Waltham, USA) with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. All cells were cultured in uncoated plastic flasks (Corning, New York, USA) at 37 °C in a humidified atmosphere, with 5% CO_2.

2.10. Assessment of the cell viability

Cell viability was measured to compare the in vitro cytotoxicity of $SeCys_2$ and MeSeCys on HeLa and HEK293T cells. HeLa and HEK293T cells were seeded in 96-well plates at a density of 1×10^4 cells/well. After cell adhesion, when the cell density reached 70%, the original culture medium was removed, and the serum-free medium (similar to the above medium but without serum) containing the appropriate concentrations of $SeCys_2$ and MeSeCys was added. After 36 h, the culture medium was discarded and the wells were washed with PBS. Subsequently, 100 μL of CCK-8 solution was added to each well and incubated for 2 h. The absorbance was then measured at a wavelength of 490 nm using a microplate reader. The cell viability of the treated groups was calculated by comparing their absorbance values to that of the control group. Each treatment was replicated six times.

2.11. Assessment of the cell nuclear morphology

The 4′,6-Diamidino-2-phenylindole (DAPI) staining of cell nuclei was performed to examine the nuclear morphology of HEK293T and HeLa cells after treatment with 100 μM $SeCys_2$ and 400 μM MeSeCys for 36 h. Following treatment, the cells were washed twice with phosphate buffer saline (PBS) and then fixed with 4%

paraformaldehyde. Subsequently, permeabilization was achieved using 5% Triton X-100. Finally, the cells were observed and imaged using a Nikon fluorescent microscope. Each treatment was replicated four times.

2.12. Annexin V-FITC/PI staining

Briefly, HEK293T and HeLa cells were subcultured in 6-well plates and cultured until they reached 70% confluence. The original culture medium was then removed, and the cells were incubated with 100 μM $SeCys_2$ and 400 μM MeSeCys diluted in serum-free medium for 36 h. Subsequently, the cells were washed twice with PBS and resuspended in a binding buffer. Then, 10 μL of propidium iodide (PI) and 5 μL of Annexin V-FITC were added to the cell suspension, followed by incubation on ice in the dark for 15 min. Finally, the apoptotic ratio of the samples was analyzed by flow cytometry, and the results were processed by FlowJo software version 7.6. Each treatment was replicated three times.

2.13. Western blot

After lysing HeLa and HEK293T cells using RIPA lysis buffer following the manufacturer's instructions, the protein concentration was determined using a BCA assay kit. Subsequently, proteins

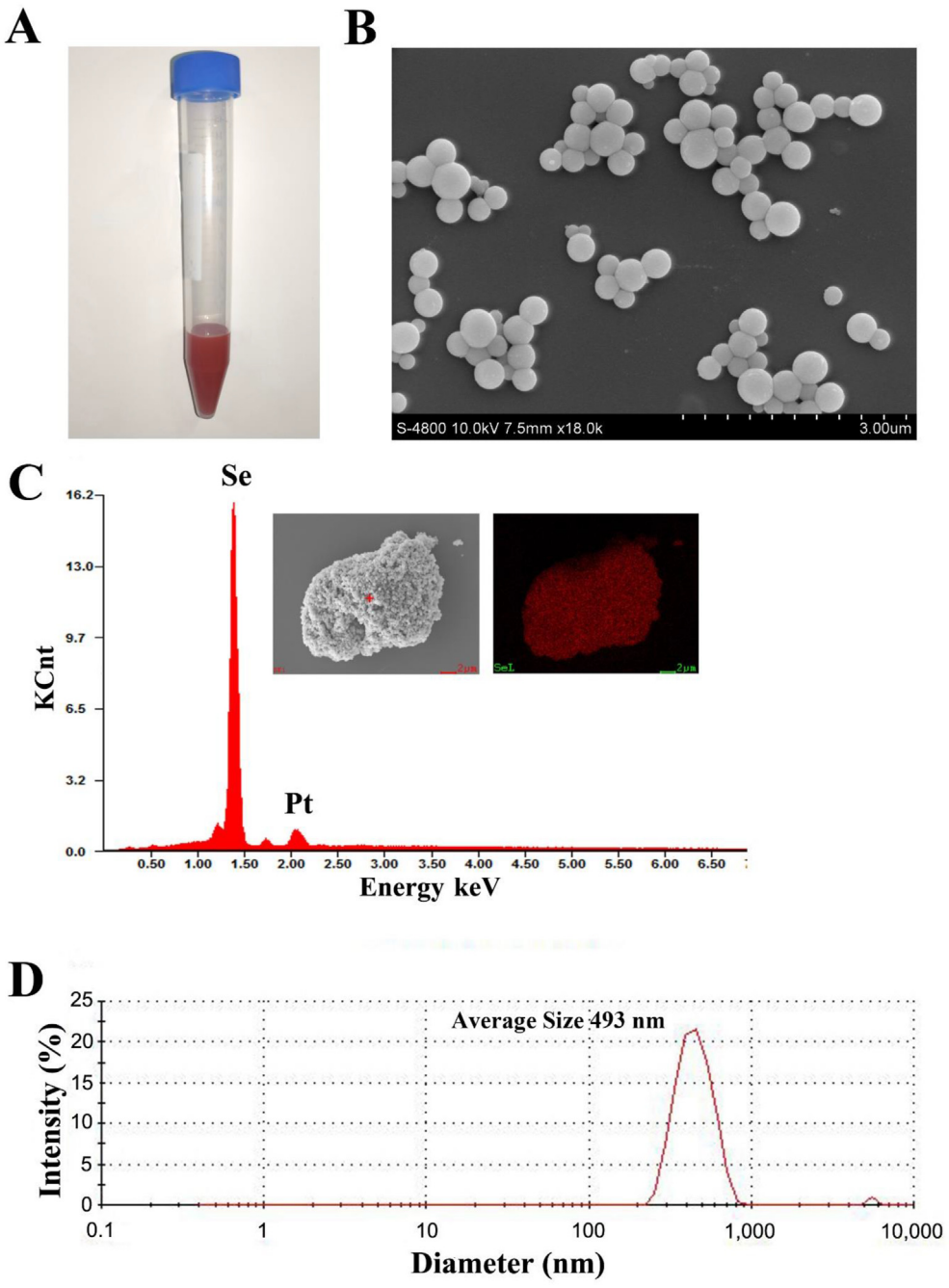

Fig. 1. Scanning electron microscopy analysis of SeNP. (A) SeNP were extracted from *B. subtilis* which enriched SeNP. (B) The ultrastructure of SeNP was observed using scanning electron microscopy. (C) SeNP was further determined by an EDAX microanalytical system (FEI, Hillsboro, OR, USA). In Fig. 1C, the image on the left shows the ultrastructure of SeNP, and the red image on the right shows the location of SeNP. (D) The average particle size of SeNP was detected by the PCCS technique. SeNP = selenium nanoparticles; PCCS = photon cross correlation spectroscopy; *B. subtilis* = *Bacillus subtilis*; KCnt = 1,000 counts; Se = selenium; Pt = platinum.

of various sizes were separated by 12% SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane (Merck Millipore, USA). The PVDF membrane was incubated in 5% nonfat dry milk, diluted in Tris-buffered saline containing 0.1% Tween 20, for 2 h at 25 °C. Following this, the membrane was incubated overnight at 4 °C with the corresponding primary antibodies. Subsequently, the membrane was further incubated at 4 °C for 2 h with the appropriate secondary antibodies. The protein bands were detected using a chemiluminescence reagent (Amersham, GE Healthcare Life-sciences) for visualization. Finally, the bands were captured using a gel imager (GenoSens 2100, Clinx). The protein bands were semi-quantified using ImageJ software. Each treatment was replicated three times.

2.14. Statistical analysis

Statistical analysis was conducted using SPSS 22.0 software (SPSS, Inc., Chicago, IL, USA). Normality and homogeneity of variance for all data were assessed using one-way ANOVA. Duncan's post-hoc test was employed for analyzing the experimental data. All values are presented as the mean ± SD and are derived from a minimum of 3 independent repetitions, and significance was considered at $P < 0.05$.

3. Results

3.1. Characterization of SeNP

XL30 ESEM equipped with an EDAX microanalytical system was used to visualize the morphology and elemental composition of the SeNP extracted from *B. subtilis* (Fig. 1A to D). The results of this study revealed that these nanoparticles exhibited a granular morphology, and their particle sizes displayed significant nonuniformity (Fig. 1B). The elemental analysis of the extracted nanoparticles revealed that Se was the primary constituent, with the additional detection of Pt elements (Fig. 1C). The presence of Pt was attributed to the platinum coating used during the scanning electron microscopy imaging process. The average particle size of SeNP was measured to be 493 nm (Fig. 1D). In addition, we observed the presence of selenite, selenite, SeMet, $SeCys_2$, and MeSeCys on the surface of SeNP. Notably, the content of $SeCys_2$ was the highest, followed by MeSeCys (Fig. S1). Additionally, we also found an unknown peak at approximately 5.6 min.

3.2. The impact of various Se forms on the growth performance of grass carp

Table 1 shows that in the SeNP group, feeding at 0.1, 0.3, and 0.9 mg/kg SeNP significantly increased FBW ($P < 0.01$; $P < 0.01$; $P < 0.01$, respectively), WG ($P = 0.01$; $P < 0.01$; $P < 0.01$, respectively), WGR ($P = 0.03$; $P < 0.01$; $P < 0.01$, respectively), and SGR ($P = 0.02$; $P < 0.01$; $P < 0.01$, respectively) of grass carp compared to the control group. Regarding FCR, only the SeNP-0.3 and SeNP-0.9 groups significantly reduced FCR ($P < 0.01$; $P = 0.02$, respectively) of grass carp. In the selenite group, only the selenite-0.3 group exhibited a significant increase in FBW ($P = 0.02$) and WG ($P = 0.04$) compared to the control group. In the SeMet group, feeding at 0.1, 0.3, and 0.9 mg/kg SeMet significantly increased FBW ($P = 0.01$; $P < 0.01$; $P < 0.01$, respectively), WG ($P = 0.01$; $P < 0.01$; $P < 0.01$, respectively), WGR ($P = 0.02$; $P < 0.01$; $P < 0.01$, respectively), and SGR ($P = 0.02$; $P < 0.01$; $P < 0.01$, respectively) compared to the control group. Regarding FCR, only the SeMet-0.3 group exhibited a significant reduction in FCR ($P = 0.02$). In summary, among the different Se sources, the group fed with 0.3 mg/kg demonstrated the best growth performance, with SeNP having the most prominent effect ($P < 0.01$).

3.3. The impact of various Se treatments on the bioavailability and Se speciation in grass carp muscle

Table S2 illustrates the satisfactory linear responses for both total Se and Se species. The mean recoveries of the ICP–MS method used to determine the total Se content in muscle ranged from 96.4% to 108.9%. The known Se concentration of GBW10045a is 0.06 ± 0.01 mg/kg, and our measured value is 0.061 ± 0.01 mg/kg, demonstrating the accuracy of our measurement. After treating grass carp with different concentrations of selenite, the Se content in the muscle increased from 0.221 ± 0.008 mg/kg in the selenite-0.1 group to 0.395 ± 0.016 mg/kg in the selenite-0.9 group, representing an increase of 19% to 114% compared to the control group. For SeNP, the Se content in muscle increased from 0.282 ± 0.008 mg/kg in the SeNP-0.1 group to 0.629 ± 0.020 mg/kg in the SeNP-0.9 group, representing an increase of 52% to 240% compared to the control group. For SeMet, the Se content in muscle increased from 0.260 ± 0.049 mg/kg in the SeMet-0.1 group to 0.592 ± 0.063 mg/kg in the SeMet-0.9 group, representing an increase of 40.5% to 220% compared to the control group (Fig. 2A). Compared to selenite, feeding 0.1 to 0.9 mg/kg SeMet and SeNP

Table 1
Growth performance and biological parameters of grass carp fed the experimental diets.[1]

Item[2]	IBW, g/fish	FBW, g/fish	WG, g/fish	FI, g/fish	WGR, %	SGR, %/day	FCR
BD	251.47 ± 1.69	443.51 ± 4.50^{e}	192.04 ± 4.97^{f}	292.2 ± 12.82	76.37 ± 2.25^{e}	0.95 ± 0.02^{e}	1.52 ± 0.04^{ab}
SeNP-0.1	250.68 ± 1.36	456.42 ± 2.66^{cd}	205.74 ± 2.41^{bcde}	300.77 ± 4.29	82.07 ± 1.10^{bcd}	1.00 ± 0.01^{bcd}	1.46 ± 0.03^{abcd}
SeNP-0.3	250.99 ± 1.39	481.30 ± 8.60^{a}	230.31 ± 8.38^{a}	306.09 ± 10.12	91.76 ± 3.34^{a}	1.08 ± 0.03^{a}	1.33 ± 0.03^{e}
SeNP-0.9	251.14 ± 2.65	468.50 ± 7.50^{b}	217.36 ± 10.11^{b}	306.36 ± 5.50	86.59 ± 4.94^{b}	1.04 ± 0.04^{b}	1.41 ± 0.08^{de}
Selenite-0.1	251.23 ± 2.10	450.32 ± 3.08^{de}	199.09 ± 5.18^{ef}	297.79 ± 13.3	79.26 ± 2.73^{de}	0.97 ± 0.03^{de}	1.50 ± 0.06^{abcd}
Selenite-0.3	251.95 ± 1.71	454.61 ± 4.51^{cd}	202.66 ± 6.13^{cde}	306.05 ± 6.30	80.45 ± 2.96^{cde}	0.98 ± 0.03^{cde}	1.51 ± 0.03^{abc}
Selenite-0.9	250.02 ± 1.65	450.72 ± 1.03^{de}	200.70 ± 2.40^{def}	308.43 ± 4.16	80.28 ± 1.46^{cde}	0.98 ± 0.01^{cde}	1.54 ± 0.03^{a}
SeMet-0.1	250.29 ± 1.00	456.24 ± 3.34^{cd}	205.95 ± 2.35^{bcde}	311.13 ± 4.46	82.28 ± 0.62^{bcd}	1.00 ± 0.01^{bcd}	1.51 ± 0.03^{abc}
SeMet-0.3	249.62 ± 1.55	463.18 ± 5.79^{bc}	213.55 ± 7.15^{bc}	303.46 ± 10.91	85.56 ± 3.35^{bc}	1.03 ± 0.03^{bc}	1.42 ± 0.06^{cd}
SeMet-0.9	250.51 ± 1.40	462.75 ± 6.01^{bc}	212.24 ± 7.35^{bcd}	303.10 ± 6.73	84.73 ± 3.40^{bcd}	1.02 ± 0.03^{bc}	1.43 ± 0.06^{bcd}
P-value	0.83	<0.01	<0.01	0.32	<0.01	<0.01	<0.01

BD = basal diet; SeNP = selenium nanoparticles; SeMet = selenomethionine; IBW = initial body weight; FBW = final body weight; WG = weight gain; FI = feed intake (total amount of the dry feed consumed); WGR = weight gain rate; SGR = specific growth rate; FCR = feed conversion ratio.

[1] Values are mean ± SD ($n = 3$). Values within a column that do not share a common superscript are significantly different ($P < 0.05$).

[2] BD is the basal diet; SeNP-0.1, 0.3 and 0.9 are the basal diet supplemented with selenium nanoparticles at 0.1, 0.3 and 0.9 mg/kg, respectively; Selenite-0.1, 0.3 and 0.9 are the basal diet supplemented with selenite at 0.1, 0.3 and 0.9 mg/kg, respectively; SeMet-0.1, 0.3 and 0.9 are the basal diet supplement with selenomethionine at 0.1, 0.3 and 0.9 mg/kg.

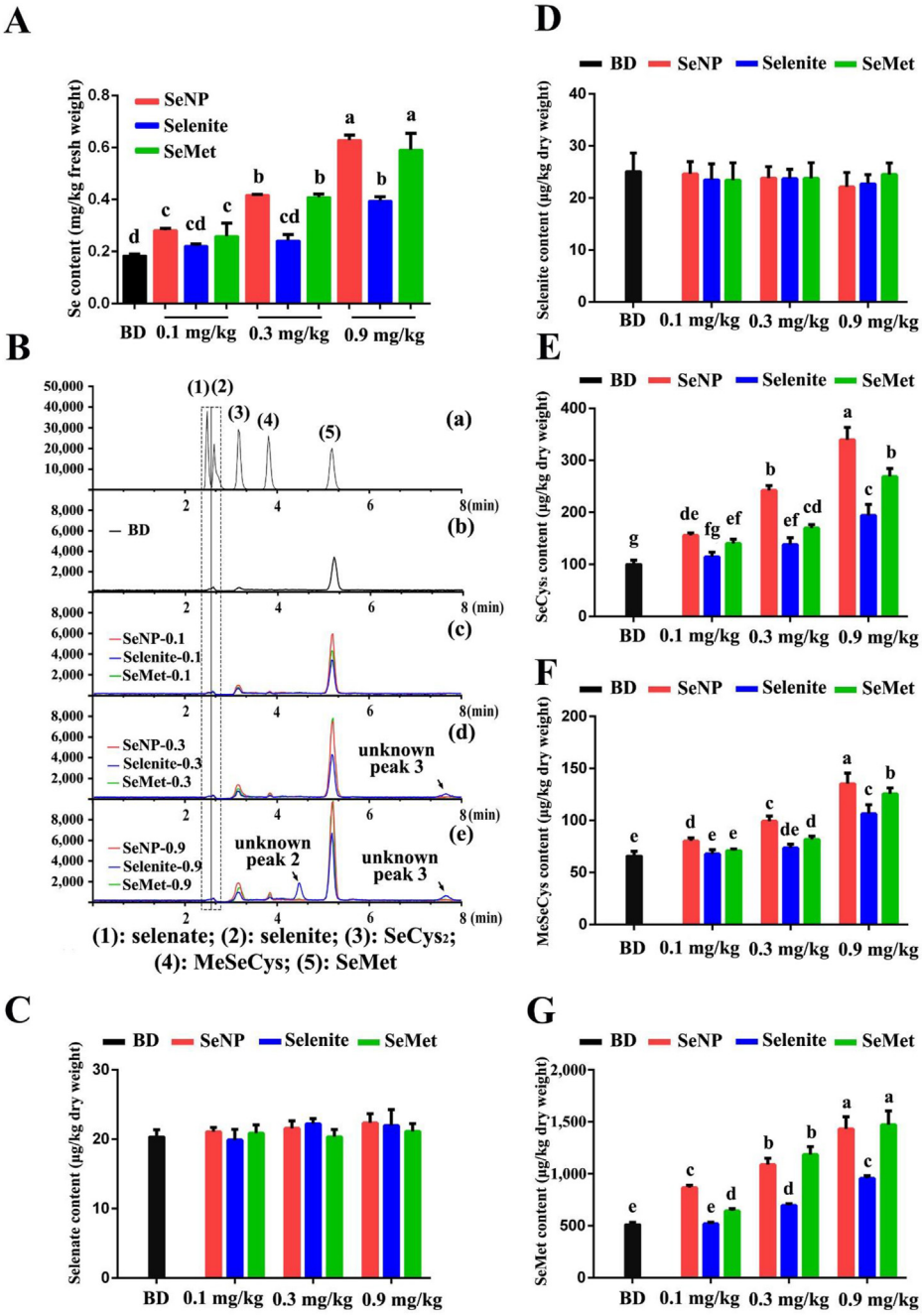

Fig. 2. Assessment of the bioavailability and speciation of selenium in grass carp muscle under varying selenium treatments. (A) Deposition efficiency of different selenium sources at varying concentrations in grass carp muscle ($n = 3$). (B) Assessment of speciation of selenium in grass carp muscle under varying selenium treatments. (a) Chromatograms for five standard selenocompounds (1-5), (b) basal diet (BD) treatment group, (c) 0.1 mg/kg SeNP, selenite and SeMet treatment, (d) 0.3 mg/kg SeNP, selenite and SeMet treatment groups, (e) 0.9 mg/kg SeNP, selenite and SeMet treatment. (C-G) Selenate, selenite, $SeCys_2$, MeSeCys, and SeMet contents in grass carp muscle ($n = 3$). SeNP = selenium nanoparticles; SeMet = selenomethionine; $SeCys_2$ = selenocysteine; MeSeCys = methylselenocysteine. Bars without a common superscript indicate statistically significant differences ($P < 0.05$). Data expressed as means ± SD.

resulted in higher Se content in the muscle of grass carp. The deposition of Se in the muscles of grass carp was significantly influenced by the dietary Se source, with the order of enrichment efficiency being SeNP ≈ SeMet > selenite.

Fig. 2B displays the concentration of each Se species in the muscle of grass carp under various Se source treatments. To assess the efficiency of our enzyme extraction method, we conducted a preliminary experiment to determine the enzyme extraction efficiency (0.3 mg/kg) (Table S3). The suitability of the enzyme hydrolysis method was demonstrated by the mean enzyme extraction efficiency, ranging from 89.09% to 90.18%. Both selenite and selenate were detected in grass carp muscle, but no significant changes were observed in their concentrations under different concentrations and sources of Se treatments (Fig. 2B to D) ($P = 0.92$; $P = 0.77$, respectively). However, compared to BD groups, there was a significant increase in the content of $SeCys_2$ when supplemented

with dietary SeNP at 0.1, 0.3, and 0.9 mg/kg ($P < 0.01$; $P < 0.01$; $P < 0.01$, respectively) and SeMet at 0.1, 0.3 and 0.9 mg/kg ($P < 0.01$; $P < 0.01$; $P < 0.01$, respectively). Additionally, significant increases in $SeCys_2$ were observed only when the feeding concentration of selenite exceeded 0.3 mg/kg. SeNP exhibited the most pronounced promoting effect, and it also showed a dose-dependent response (Fig. 2B and E). Similarly, our results also demonstrated a dose-dependent increase in MeSeCys in the muscle of grass carp when supplemented with diets containing 0.1 to 0.9 mg/kg Se. SeNP exhibited the most significant promoting effect (Fig. 2B and F). In addition, significant differences in SeMet levels also were observed in the muscle of grass carp after exposure to various Se sources and supplementation levels (Fig. 2B and G). Compared to selenite, both SeNP and SeMet significantly increased the SeMet content in the muscle of grass carp after various Se treatments at 0.1 mg/kg ($P < 0.01$), with SeNP showing the most substantial effect. Under 0.3 and 0.9 mg/kg Se treatments, SeNP, selenite, and SeMet significantly increased the SeMet content in the grass carp muscle compared to the BD group ($P < 0.01$), with no significant difference between SeNP and SeMet. Interestingly, our results revealed two unknown peaks (unknown peaks 2 and 3) in the groups fed 0.3 and 0.9 mg/kg selenite.

3.4. The impact of various Se forms on the antioxidant ability of grass carp

The antioxidant capacity of grass carp after feeding different Se sources was detected in this study (Table 2). When 0.1 mg/kg of each of the three Se sources was added to the diet, there was no significant effect on T-AOC in the serum of grass carp ($P = 0.34$). However, all three Se sources at a concentration of 0.3 mg/kg significantly increased the T-AOC in grass carp serum ($P < 0.01$). Furthermore, 0.9 mg/kg of SeNP also significantly increased the T-AOC in grass carp serum ($P < 0.01$), while selenite and SeMet showed no significant impact on T-AOC ($P = 0.72$; $P = 0.06$, respectively). Regarding MDA, the addition of 0.1 to 0.9 mg/kg of SeNP ($P < 0.01$) and SeMet ($P < 0.01$) in the feed significantly decreased the levels of MDA. Similarly, only when the selenite addition was at a concentration of 0.3 mg/kg, a significant decrease in MDA levels was observed ($P < 0.01$). For H_2O_2, the inclusion of SeNP in the feed at concentrations of 0.1 to 0.9 mg/kg significantly reduced H_2O_2 levels ($P < 0.01$). Similarly, the inclusion of SeMet at levels of 0.3 and 0.9 mg/kg in the diet led to significant decreases in H_2O_2 levels ($P < 0.05$). However, when the dietary concentration of SeMet was 0.1 mg/kg, there was no significant difference in serum H_2O_2 levels compared to the control group ($P = 0.06$). In contrast, only the inclusion of selenite at a concentration of 0.3 mg/kg resulted in a significant reduction in H_2O_2 levels ($P = 0.02$). In summary, among the three Se sources, the SeNP group exhibited the strongest antioxidant capacity at the 0.3 mg/kg concentration. These results suggest that the addition of 0.3 mg/kg Se is the most suitable concentration for promoting the growth and antioxidant capacity of grass carp. Compared to selenite and SeMet, SeNP demonstrated the strongest antioxidant and growth-promoting effects at the 0.3 mg/kg concentration.

Table 2
The serum antioxidant capacity of grass carp fed the experimental diets.[1]

Item[2]	T-AOC, U/mL	MDA, μM	H_2O_2, mM
BD	4.09 ± 0.31^{de}	7.84 ± 0.47^{a}	0.15 ± 0.00^{a}
SeNP-0.1	4.31 ± 0.25^{cde}	4.18 ± 0.48^{bc}	0.14 ± 0.01^{cd}
SeNP-0.3	6.24 ± 0.05^{a}	2.74 ± 0.24^{c}	0.11 ± 0.00^{f}
SeNP-0.9	5.45 ± 0.09^{b}	3.04 ± 0.20^{c}	0.13 ± 0.00^{e}
Selenite-0.1	3.84 ± 0.32^{e}	6.87 ± 0.67^{a}	0.15 ± 0.01^{ab}
Selenite-0.3	4.84 ± 0.62^{bc}	4.69 ± 0.49^{b}	0.14 ± 0.00^{bc}
Selenite-0.9	3.99 ± 0.46^{e}	7.40 ± 1.48^{a}	0.15 ± 0.01^{ab}
SeMet-0.1	4.26 ± 0.39^{cde}	4.22 ± 1.43^{bc}	0.14 ± 0.00^{abc}
SeMet-0.3	5.37 ± 0.14^{b}	2.74 ± 0.32^{c}	0.13 ± 0.00^{de}
SeMet-0.9	4.67 ± 0.14^{cd}	4.93 ± 0.12^{b}	0.14 ± 0.00^{bc}
P-value	<0.01	<0.01	<0.01

BD = basal diet; T-AOC = total antioxidant capacity; MDA = malonaldehyde; H_2O_2 = superoxide dismutase.

[1] Values are mean ± SD ($n = 3$). Values within a column that do not share a common superscript are significantly different ($P < 0.05$).

[2] BD is the basal diet; SeNP-0.1, 0.3 and 0.9 are the basal diet supplemented with selenium nanoparticles at 0.1, 0.3 and 0.9 mg/kg, respectively; Selenite-0.1, 0.3 and 0.9 are the basal diet supplemented with selenite at 0.1, 0.3 and 0.9 mg/kg, respectively; SeMet-0.1, 0.3 and 0.9 are the basal diet supplement with selenomethionine at 0.1, 0.3 and 0.9 mg/kg, respectively.

3.5. The impact of various Se forms on heavy metals, proximate compositions, and fatty acids of grass carp muscle

The good linear responses of the heavy metals are shown in Table S4. As depicted in Fig. 3A, supplementing grass carp with three forms of Se (SeNP, selenite, SeMet) at a dosage of 0.3 mg/kg significantly reduced the plumbum (Pb) content in grass carp muscle ($P < 0.01$), with SeNP and SeMet exhibiting the most significant reductions (approximately 82% to 90%). Furthermore, the findings revealed that SeNP notably decreased the As content in grass carp muscle (by approximately 18%; $P < 0.01$), while the SeMet and selenite groups exhibited no significant alterations (Fig. 3B) ($P = 0.27$; $P = 0.49$, respectively). Feeding grass carp with SeNP, selenite, and SeMet resulted in 94%, 70%, and 83% reductions in Cd content in grass carp muscle, respectively (Fig. 3C). Additionally, the three forms of Se resulted in a decrease of approximately 40% to 49% of zinc (Zn) content in the muscle tissue (Fig. 3D). However, feeding the three forms of Se did not significantly reduce the content of strontium (Sr) and mercury (Hg) in the muscle tissue (Fig. 3E and F). The observed differences in the effects of feeding the three Se sources on As content may be attributed to the unique metabolic mechanism of SeNP in grass carp, whereas the differences in Pb and Cd content may be attributed to their different metabolic mechanisms or bioavailability.

For the proximate composition of grass carp muscle (Fig. 3G to J), the addition of the three Se sources in the diet did not affect the content of crude protein ($P = 0.57$), crude ash ($P = 0.49$), and moisture ($P = 0.44$) in the muscle tissue. However, all three forms of Se, including SeNP, selenite, and SeMet were able to significantly reduce the crude lipid content in grass carp muscle ($P < 0.01$; $P = 0.03$; $P < 0.03$, respectively), with SeNP showing the most significant effect (reducing it by approximately 33%).

The results presented in Table 3 demonstrate that compared with the control group, the fatty acid content in grass carp muscle significantly differed after feeding on the three forms of Se sources. For saturated fatty acid (SFA), feeding three Se sources significantly increased the contents of C16:0 and C18:0, resulting in increased ΣSFA contents ($P = 0.02$; $P < 0.01$; $P = 0.02$, respectively). Notably, sodium selenite had the most significant promoting effect. However, it's worth noting that SeNP significantly reduced the content of C14:0 ($P = 0.03$), which is contrary to the effect of selenite ($P = 0.01$). For monounsaturated fatty acid (MUFA), all three Se sources significantly reduced the content of C18:1n-9, resulting in a significant decrease in total MUFA content ($P < 0.01$), with selenite playing the most significant impact. Feeding three Se sources had no significant effect on Σn-6 polyunsaturated fatty acid (PUFA) ($P = 0.91$) but increased the content of Σn-3 PUFA ($P < 0.01$) (mainly C20:5n-3 (EPA) and C22:6n-3 (DHA)), which led to an increase in the Σn-3/Σn-6 PUFA value, and the highest Σn-3/Σn-6 PUFA ratio was observed in the SeNP group.

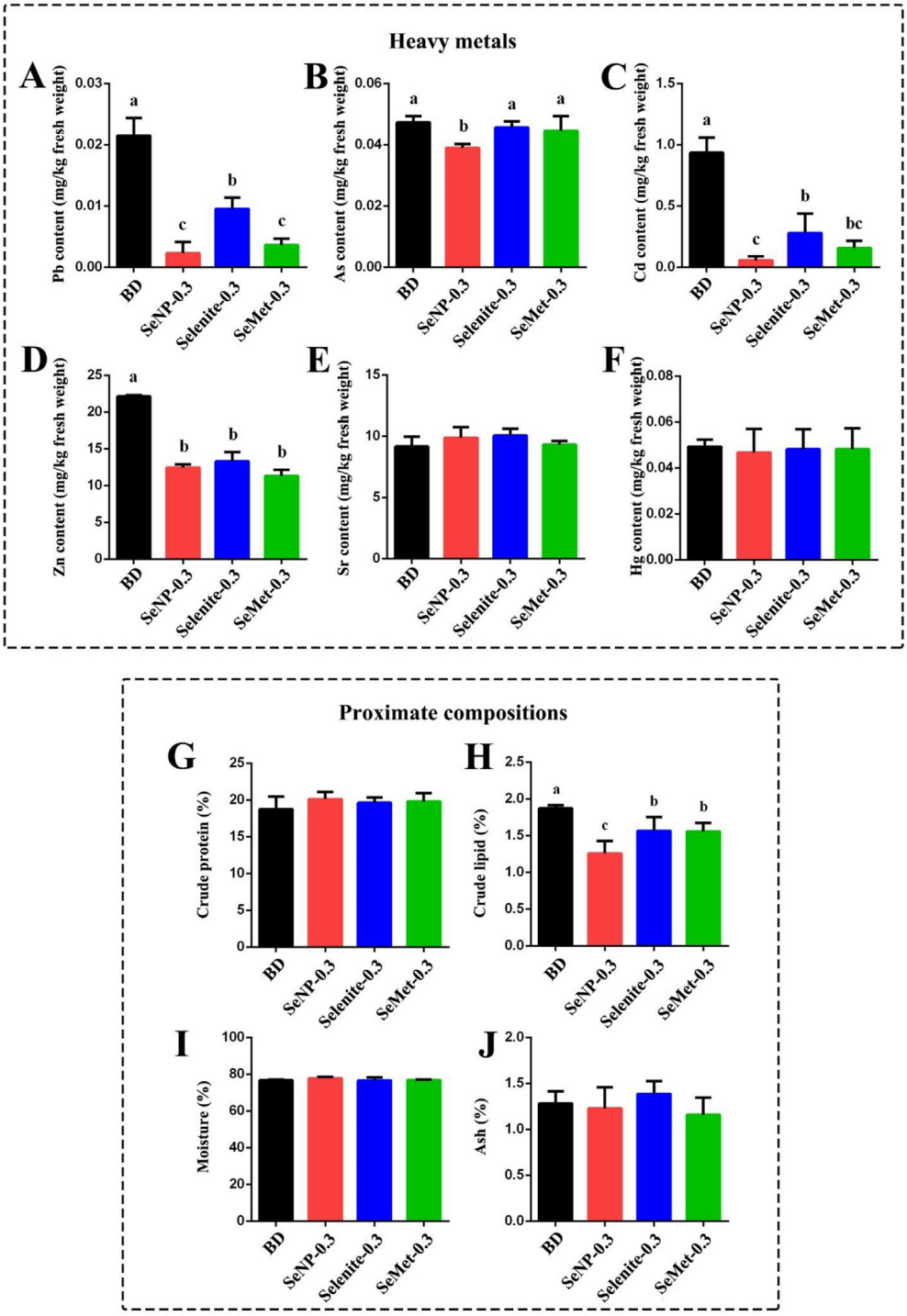

Fig. 3. Heavy metal element content and proximate composition analysis of grass carp muscle ($n = 3$). (A to F) Pb, As, Cd, Zn, Sr, and Hg contents in grass carp muscle. (G to J) The proportions of crude protein, crude lipid, moisture, and ash in grass carp muscle. BD = basal diet; SeNP = selenium nanoparticles; SeMet = selenomethionine; Pb = plumbum, As = arsenic, Cd = cadmium, Zn = zinc, Sr = strontium, Hg = mercury. Bars without a common superscript indicate statistically significant differences ($P < 0.05$). Data expressed as means ± SD.

3.6. The health benefits (the effect on cancer cells) of $SeCys_2$ and MeSeCys

HEK293T and HeLa cells were used to assess the function of $SeCys_2$ and MeSeCys. The results obtained from CCK-8 assays revealed that $SeCys_2$, at concentrations ≥100 μM, significantly inhibited the viability of HeLa cells. Specifically, at a concentration of 100 μM, $SeCys_2$ markedly suppressed the viability of HeLa cells ($P < 0.01$) while having no effect on HEK293T cells ($P = 0.35$). Concerning MeSeCys, a concentration of 400 μM exhibited no toxicity against HEK293T cells but significantly inhibited the viability of HeLa cells (Fig. 4A and B).

In addition, we observed similar differences through DAPI staining (Fig. 4C). Specifically, 100 μM $SeCys_2$ had no significant effect on the nuclear morphology of HEK293T cells, whereas it induced noticeable abnormalities in the nuclei of HeLa cells.

Table 3
Fatty acid composition of grass carp muscle (% total fatty acids).[1]

Item	Groups[2]				*P*-value
	BD	SeNP-0.3	Selenite-0.3	SeMet-0.3	
C14:0	2.69 ± 0.03^{b}	1.78 ± 0.09^{c}	3.77 ± 0.81^{a}	2.77 ± 0.15^{b}	<0.01
C16:0	20.87 ± 0.77^{c}	23.42 ± 0.96^{b}	25.26 ± 0.70^{a}	22.21 ± 0.86^{bc}	<0.01
C18:0	7.71 ± 0.00^{c}	9.69 ± 0.15^{b}	13.16 ± 1.25^{a}	10.09 ± 0.92^{b}	<0.01
ΣSFA[3]	31.27 ± 0.78^{c}	34.90 ± 0.87^{b}	42.18 ± 2.55^{a}	35.06 ± 1.31^{b}	<0.01
C16:1n-7	9.32 ± 1.09^{c}	9.44 ± 0.58^{bc}	10.76 ± 0.65^{ab}	11.35 ± 0.27^{a}	0.02
C18:1n-9	34.31 ± 0.43^{a}	28.37 ± 0.65^{b}	21.67 ± 1.00^{c}	27.11 ± 0.41^{b}	<0.01
ΣMUFA[4]	43.62 ± 1.06^{a}	37.81 ± 1.22^{b}	32.43 ± 0.79^{c}	38.47 ± 0.14^{b}	<0.01
C18:2n-6	15.81 ± 1.37	14.13 ± 0.57	12.53 ± 2.37	13.99 ± 1.23	0.15
C18:3n-6	0.22 ± 0.01^{b}	0.22 ± 0.01^{b}	0.19 ± 0.02^{c}	0.32 ± 0.01^{a}	<0.01
C20:4n-6	1.54 ± 0.01^{c}	2.5 ± 0.16^{b}	4.02 ± 0.10^{a}	2.69 ± 0.23^{b}	<0.01
Σn-6 PUFA[5]	17.56 ± 1.37	16.85 ± 0.41	16.74 ± 2.44	17.00 ± 1.01	0.90
C18:3n-3	3.08 ± 0.37^{a}	1.62 ± 0.24^{b}	2.40 ± 0.09^{a}	2.69 ± 0.64^{a}	0.01
C20:5n-3 (EPA)	1.08 ± 0.09^{d}	2.29 ± 0.02^{a}	1.26 ± 0.04^{c}	1.71 ± 0.16^{b}	<0.01
C22:5n-3	0.52 ± 0.06	0.53 ± 0.06	0.56 ± 0.02	0.53 ± 0.10	0.857
C22:6n-3 (DHA)	2.86 ± 0.15^{c}	6.02 ± 0.05^{a}	4.43 ± 0.12^{b}	4.55 ± 0.53^{b}	<0.01
Σn-3 PUFA[6]	7.55 ± 0.36^{d}	10.45 ± 0.23^{a}	8.65 ± 0.09^{c}	9.47 ± 0.22^{b}	<0.01
Σn-3/ΣN-6 PUFA	0.43 ± 0.02^{d}	0.62 ± 0.02^{a}	0.52 ± 0.07^{b}	0.56 ± 0.02^{ab}	<0.01
Total	100.00	100.00	100.00	100.00	

BD = basal diet; SeNP = selenium nanoparticles; SeMet = selenomethionine; EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid.
[1] Values are mean ± SD ($n = 3$). Values within a row that do not share a common superscript are significantly different ($P < 0.05$).
[2] BD is the basal diet; SeNP-0.1, 0.3 and 0.9 are the basal diet supplemented with selenium nanoparticles at 0.1, 0.3 and 0.9 mg/kg, respectively; Selenite-0.1, 0.3 and 0.9 are the basal diet supplemented with selenite at 0.1, 0.3 and 0.9 mg/kg, respectively; SeMet-0.1, 0.3 and 0.9 are the basal diet supplement with selenomethionine at 0.1, 0.3 and 0.9 mg/kg, respectively.
[3] ΣSFA (saturated fatty acid) = C14:0 + C16:0 + C18:0.
[4] ΣMUFA (monounsaturated fatty acid) = C16:1n-7 + C18:1n-9.
[5] Σn-6 PUFA (polyunsaturated fatty acid) = C18:2n-6 + C18:3n-6 + C20:4n-6.
[6] Σn-3 PUFA (polyunsaturated fatty acid) = C18:3n-3 + C20:5n-3 + C22:5n-3 + C22:6n-3.

Similarly, 400 μM MeSeCys induced significant nuclear abnormalities in HeLa cells while having no significant impact on the nuclear morphology of HEK293T cells. The yellow arrows in Fig. 4C denote aberrant nuclear morphology, indicative of apoptotic cells.

To further investigate the differential effects of $SeCys_2$ and MeSeCys on apoptosis in HEK293T and HeLa cells, flow cytometry was utilized to assess the apoptotic status of the cells. The results demonstrated that 100 μM $SeCys_2$ significantly increased the proportion of early and late apoptosis in HeLa cells ($P < 0.01$), while showing no significant effect on HEK293T cells ($P = 0.75$). Similar results were observed in the 400 μM MeSeCys treatment group (Fig. 5A to C).

Furthermore, the Western blot results also indicated that both 100 μM $SeCys_2$ and 400 μM MeSeCys elevated the levels of Bax protein and decreased the levels of Bcl-2 protein in HeLa cells ($P < 0.01$), resulting in an increase in the Bax/Bcl-2 ratio. However, no significant effect was observed on HEK293T cells (Fig. 5D to F).

4. Discussion

4.1. The impact of various dietary Se forms and levels on the growth performance and antioxidant capacity of grass carp

Previous studies have highlighted the potential toxicity of excess selenium in fish (Berntssen et al., 2017; Hardy et al., 2010), which can impact the production of Se-enriched fish. Therefore, the production of Se-enriched grass carp should not only focus on the Se content in the muscle but also pay attention to the health of the grass carp body. Among the three Se sources (SeNP, selenite, SeMet), SeNP exhibited the strongest effect on promoting grass carp growth, while selenite showed the weakest impact on grass carp growth indicators, which is consistent with previous studies on common carp by Saffari et al. (2017). Liu et al. (2021) reported that 0.6 to 0.9 mg/kg SeNP enhanced the growth performance of grass carp, while our results show that 0.3 mg/kg of SeNP is the optimal concentration. This difference may be attributed to the superior bioavailability and reactivity of biogenic SeNP compared to chemically synthesized SeNP (Malyugina et al., 2021).

A previous study demonstrated that compared to sodium selenite and SeMet, dietary supplementation with SeNP could significantly enhance the antioxidant capacity of fish (Saffari et al., 2017), which is consistent with our findings in grass carp. This enhancement could be attributed to SeNP's more effective increase in $SeCys_2$ content, as $SeCys_2$ serves as the active center for antioxidant enzymes in the body (Khanam and Platel, 2016). Moreover, the addition of 0.9 mg/kg selenite did not significantly improve the antioxidant capacity of the grass carp serum, which may be related to the increase of unknown peak 2 [selenourea (SeUr)]. Currently, there is no research indicating the relationship between SeUr and antioxidant capacity, so the specific mechanism still needs to be further explored. In summary, our results suggest that all three Se sources (SeNP, selenite, SeMet) showed better growth and antioxidant performance at an addition level of 0.3 mg/kg compared to the addition levels of 0.1 mg/kg and 0.9 mg/kg. In addition, compared to sodium selenite and SeMet, feeding with SeNP resulted in the strongest antioxidant capacity and growth performance in grass carp.

4.2. The impact of various dietary Se forms on the total Se deposited, Se species in grass carp

Se nutritional supplements have been widely used in fish production (Barbosa et al., 2020; Farzad et al., 2019). In our previous research, we found that there is organic matter on the surface of SeNP (Zhu et al., 2023), which explained that some proteins are attached to the surface of SeNP, and our further research shows that there are six kinds of selenoproteins on the surface. Our study is the first to explore the composition of Se speciation on the surface of biologically produced SeNP.

There is a significant difference in the bioavailability of inorganic Se and organic Se in fish (Le and Fotedar, 2014). We found that the Se concentration in the muscle of grass carp significantly increased

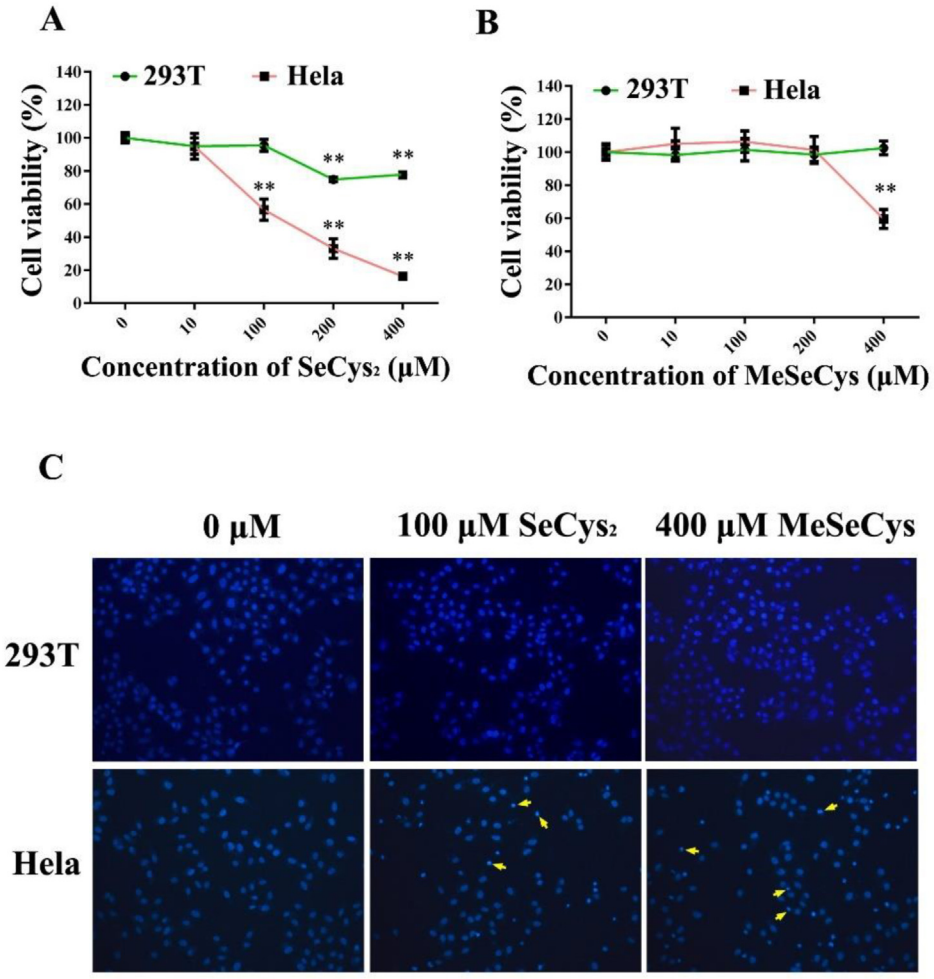

Fig. 4. The effects of $SeCys_2$ and MeSeCys on the viability and nuclear morphology of HEK293T and HeLa cells. (A and B) The effects of different concentrations of $SeCys_2$ and MeSeCys on the viability of HEK293T and HeLa cells ($n = 6$). (C) The effects of 100 μM $SeCys_2$ and 400 μM MeSeCys on the nuclear morphology of HEK293T and HeLa cells ($n = 4$). The yellow arrows represent morphologically abnormal cell nuclei (characterized by nuclear condensation). $SeCys_2$ = selenocysteine; MeSeCys = methylselenocysteine; 293T = human embryonic kidney (HEK293T) cells. All data are presented as means ± SD. *$P < 0.05$, **$P < 0.01$.

after feeding selenite at a concentration of 0.9 mg/kg, while significant increases were observed with SeNP and SeMet at a concentration of 0.1 mg/kg. This suggests that selenite exhibits lower deposition efficiency in grass carp muscle, while SeNP and SeMet demonstrate superior deposition efficiency, which further validates previous research results (Saffari et al., 2017). Therefore, considering only the deposition efficiency, SeNP and SeMet are more suitable as feed additives for producing Se-enriched grass carp.

However, limited previous studies have conducted speciation analysis for Se in fish and only the forms Se (IV), Se (VI), $SeCys_2$, and SeMet were identified in the muscles of Atlantic salmon and trout (Jagtap et al., 2016; Sele et al., 2018). In our study, we identified five forms of Se amino acids in grass carp muscle. In addition, the addition of various Se sources to the feed had no significant impact on selenite and selenate levels in the muscle but significantly influenced SeMet, followed by $SeCys_2$ and MeSeCys levels. These observations may be related to the metabolic pathways of Se in grass carp muscle (Chen et al., 2022). Regarding SeMet in the muscle, SeMet and SeNP showed stronger promoting effects on SeMet accumulation in grass carp muscle, possibly because SeNP and SeMet are more easily absorbed and replace the sulfur atom in methionine in grass carp muscle (Budisa et al., 1995) compared with selenite. Concerning $SeCys_2$ in the muscle, the promotion effect of SeNP and SeMet was significantly higher than that of selenite. Among these, SeNP had the strongest promotion effect. These results may be attributed to the direct conversion of SeMet into $SeCys_2$ or synthesis of $SeCys_2$ via an intermediate metabolite (selenium hydride) while selenite can only be reduced to selenium hydride (depending on the activity of thioredoxin reductase) and then converted into $SeCys_2$ (Kajander et al., 1991; Weekley et al., 2011). The enhancement of $SeCys_2$ by SeNP could be attributed to its easier internalization into cells, which can be metabolized into $SeCys_2$ (Liu et al., 2020). Similarly, for MeSeCys, SeNP exhibited the strongest promotion effect. We speculate that the increase in $SeCys_2$ and MeSeCys in the muscle of grass carp after feeding with SeNP may be related to the organic Se on the surface of SeNP, but the direct effect of SeNP could not be ruled out. The underlying mechanism needs to be further studied. These findings further support our hypothesis that SeNP enhances the antioxidant capacity of grass carp by increasing the levels of $SeCys_2$ and MeSeCys

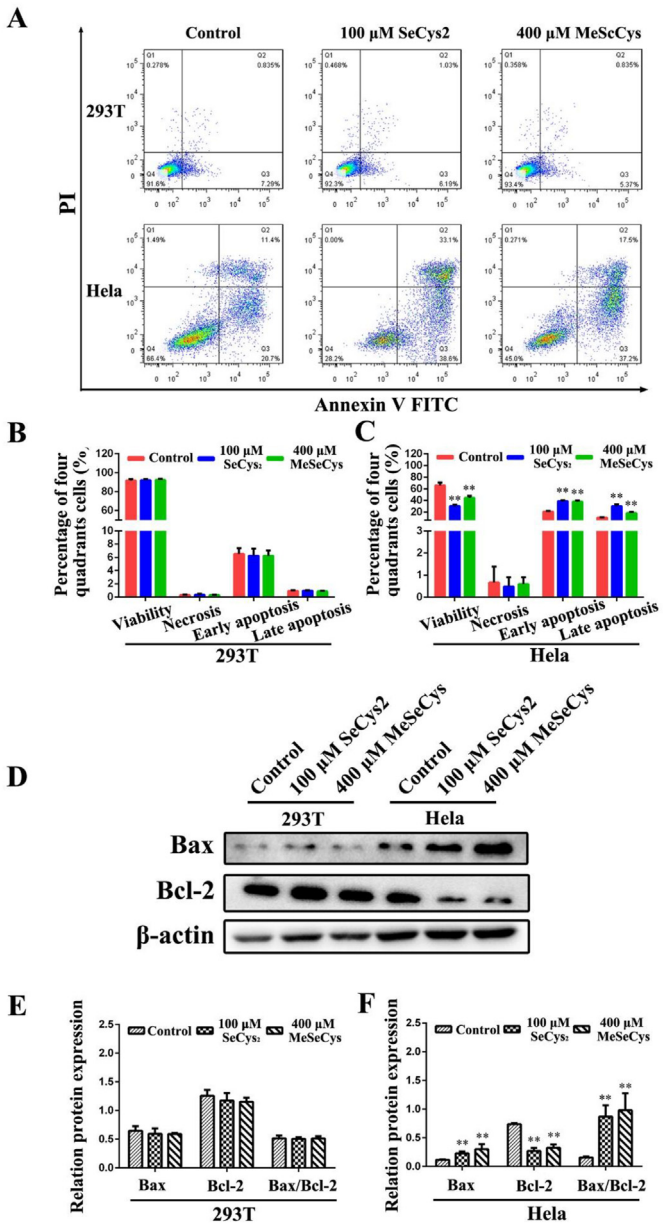

Fig. 5. The effects of $SeCys_2$ and MeSeCys on apoptosis in HEK293T and HeLa cells ($n = 3$). (A to C) Treatment with 100 μM $SeCys_2$ and 400 μM MeSeCys induced apoptosis in HeLa cells, while no significant effect was observed in HEK293T cells. (D) Western blot analysis was performed to detect the effects of 100 μM $SeCys_2$ and 400 μM MeSeCys on the protein expression of Bax and Bcl-2 in HEK293T and HeLa cells ($n = 3$). (E and F) The columns show the statistical analysis of protein abundance in Fig. 5D. $SeCys_2$ = selenocysteine; MeSeCys = methylselenocysteine; HEK293T cell = human embryonic kidney cells. All data are presented as means ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$.

in their bodies, thereby promoting their growth. In addition, we also found two unknown Se species in the selenite-treated groups (0.3 and 0.9 mg/kg). Referring to the research by Zhang et al. (2020), we speculate that unknown peaks 2 and 3 are SeUr and selenoethionine (SeEt), but their metabolic pathways and possible biological functions remain unclear. This is the first study to investigate the effect of different Se sources on the content of Se speciation in fish muscle and the first to identify five Se amino acids in grass carp muscle, providing information for understanding the intricate metabolism of Se in fish.

4.3. Effects of 0.3 mg/kg different dietary Se forms on the proximate composition, heavy metal elements and fatty acids in grass carp muscles

Appropriate Se additives can not only significantly increase the Se content in grass carp but also have no adverse effects on the growth and development of the fish. In fact, they can even promote the fish's growth performance, thereby reducing the production cost of Se-rich fish. According to the results, SeNP and SeMet at concentrations of 0.1 to 0.9 mg/kg demonstrated a significant increase in Se content in the muscle tissue of grass carp. Furthermore, if we consider the daily intake of 100 g of fish meat by individuals (Lin, 2014), the Se-enriched fish produced at various concentrations in our experiments are far from reaching the toxic dose (400 μg/day) for the human body, indicating no potential toxic effects on human health (Fisinin et al., 2009; Food and Nutrition Board USA Institute of Medicine, 2000). At the same time, it fulfills the purpose of supplementing selenium in the human body (Lin, 2014; Wang et al., 2022). It is worth noting that based on the growth and antioxidant indicators of grass carp, a Se supplementation concentration of 0.3 mg/kg is the most suitable. Therefore, we will further investigate the effects of three forms of Se additives at a concentration of 0.3 mg/kg on the quality of grass carp muscle. The content of heavy metal elements, crude composition, and fatty acid composition of muscle can reflect the flesh quality of fish (Li et al., 2023). It has been well established that Se has an antagonistic effect on heavy metals (Zhao et al., 2023). While previous studies have primarily focused on the relationship between heavy metals and sodium selenite (Talas et al., 2008), a comparative analysis of the effects of different Se sources on heavy metal accumulation in fish muscle has not been conducted to date. In this study, the results demonstrated that all three Se sources significantly reduced the levels of Pb, Cd, and Zn in the muscle of grass carp, while there were significant differences in the reduction effects on Pb and Cd, which may be related to the deposition efficiency of different Se sources or the content of Se speciation in the muscle (Dang and Wang, 2011). In contrast, the reduction effect on Zn was independent of the Se source. The positive effect of Zn has been demonstrated by previous studies (Prasad et al., 2004). Therefore, we recommend the combined use of Se and zinc in the production of Se-enriched fish, although the specific dose ratio needs further investigation. In addition, SeNP is the only additive among the three Se sources that can reduce the content of As, further proving that SeNP may be a more suitable Se additive for producing Se-enriched grass carp. Farmed fish are often of low quality due to their comparatively high lipid content (Ergün et al., 2009). Our results revealed that among the three Se sources, SeNP had the most significant effect on reducing crude fat content in grass carp muscle, possibly due to their regulation of lipid metabolism-related genes through the AMPK pathway (Liu et al., 2021).

In addition, fatty acids found in fish muscle are known for their beneficial health effects. The presence of high levels of unsaturated fatty acids, particularly n-3 series polyunsaturated fatty acids such as EPA and DHA, and a balanced Σn3/Σn6 ratio have been shown to play a critical role in reducing the risk of cardiovascular disease (Liu et al., 2019). SeNP significantly increased the content of EPA and DHA as well as the ratio of n-3/n-6 PUFA in grass carp muscle, indicating that using SeNP as an additive for Se-enriched grass carp could improve the health benefits of consuming the muscle of this fish.

4.4. The effect of $SeCys_2$ and MeSeCys on cancer cells

To the best of our knowledge, current research on $SeCys_2$ and MeSeCys primarily focuses on their antioxidant properties

(Marschall et al., 2016), while there is a lack of studies concerning their anticancer effects. Dar et al. (2018) investigated the anticancer activity of steroidal pyrimidines using MCF-7 cells. Additionally, a study has shown that europium SeNP exhibits anticancer activity by reducing the viability and increasing the apoptosis rate of HeLa cells compared to normal cells (HEK293T) (Kim et al., 2016). In this study, two cell lines, HEK293T and HeLa cells, were selected to investigate the effects of $SeCys_2$ and MeSeCys on normal (HEK293T) and cancer cells (HeLa). We observed remarkable pro-apoptosis and anti-proliferation functions of $SeCys_2$ and MeSeCys on HeLa cells in vitro. Bax is a pro-apoptotic mediator belonging to the Bcl-2 family of proteins and is primarily located in the cytosol. However, during apoptosis, Bax relocates to the mitochondria. An increase in Bax levels or a decrease in Bcl-2 levels, leading to an elevated Bax/Bcl-2 ratio, can induce cellular apoptosis. Previous studies have demonstrated that SeNP significantly increases the protein levels of BAX and Bcl-2 in MCF-7 cells (Bhattacharjee et al., 2017). In our study, we found that $SeCys_2$ and MeSeCys also regulate the expression of Bax and Bcl-2 proteins. Therefore, our results suggest that $SeCys_2$ and MeSeCys exert their pro-apoptosis on HeLa cells by modulating the mitochondrial apoptosis pathway through Bax and Bcl-2, which suggested Se-enriched fish meat produced by SeNP as an additive was more beneficial to human health.

5. Conclusion

In summary, our results demonstrate that SeNP can significantly improve the growth parameters and antioxidant capacity of grass carp, and reduce the production cost of Se-enriched fish. SeNP have excellent bioavailability similar to SeMet in grass carp. Compared with selenite and SeMet, the production of Se-enriched fish using SeNP as an additive can provide humans with more $SeCys_2$ and MeSeCys. These two forms of Se promote apoptosis of HeLa by modulating the mitochondrial apoptotic pathway, while they do not harm normal cells (HEK293T). In addition, feeding SeNP also significantly improved the flesh quality of grass carp. Based on these findings, it is reasonable to consider that SeNP could be used as a new additive to produce Se-biofortified aquatic products and ultimately meet the Se requirements of humans.

Author contributions

Chao Zhu: Writing—Reviewing and Editing. **Zifang Wu** and **Qimin Liu:** Data curation, Writing—Original draft preparation. **Xiaolin Wang:** Visualization, Investigation. **Lijuan Zheng:** Software, Validation. **Shuyang He, Fangxia Yang:** Conceptualization, Methodology, Software. **Hong Ji** and **Wuzi Dong:** Supervision.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix supplementary data

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