



Original Article

The effects of dietary organic selenium on growth, body composition and hematological parameters of common Carp (*Cyprinus carpio*) reared in recirculating aquaculture system

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Abstract



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A feeding trial was conducted to examine the effects of different levels of dietary organic selenium (Se) on the growth, body composition, and hematological indices of common carp. Se element was supplemented to the basal diet at the rates of 0.0, 0.5, 1 and 2 mgkg⁻¹. Fish were randomly stocked at a rate of 25 fish into 500L tanks in the Recirculating Aquaculture System (RAS) system for 10 weeks. Weight gain, final body weight, and specific growth rate were significantly higher ($p < 0.05$) in fish fed 0.5 mgkg⁻¹ followed by 1 and 2 mgkg⁻¹ treatment diets fish. The lowest growth rate values were detected in fish-fed basal diets. Feed consumption of fish fed Se was not significantly different ($p > 0.05$), also the survival rate was similar among the treatment groups. Whole-body composition showed that fish fed 1 mgkg⁻¹ had significantly higher ($p < 0.05$) body moisture and protein as dry weight basis. The highest fiber content was in fish fed 2 mgkg⁻¹, but ash was significantly higher in fish fed only 0.5 mgkg⁻¹ and fat remained unaffected. Whole-body Se concentrations were significantly ($p < 0.05$) increased with increasing Se levels in test diets. The red blood cells and hemoglobin were significantly higher ($p < 0.05$) in fish fed only 0.5mgkg⁻¹ and the basal diet. The white blood cells increased in fish fed the basal and 2 mgkg⁻¹ diet, but the lymphocyte percentage was increased ($p < 0.05$) in fish fed basal and 0.5 mgkg⁻¹. The study concludes that dietary Se has an overall positive effect on common carp. Growth performance, protein content in fish body, selenium deposition and some hematological parameters were observed in fish fed dietary organic selenium specifically in fish fed 0.5 and 1 mgkg⁻¹. Therefore, inclusion of Se in the diet from 0.5 to 1 mgkg⁻¹ would be an optimal level for common carp feed.

Keywords: Body composition, *Cyprinus carpio*, Growth, Haematological indices, Selenium

1. Introduction

Selenium (Se) is a trace mineral that has recently gained significant interest in aquaculture nutrition and it is a crucial element needed for fish growth [1–5]. It is a part of the enzyme glutathione peroxidase and hence is important in biological systems. It converts to selenoproteins which are responsible for various cellular biological functions [6, 7]. Therefore, it is necessary for aquaculture feed for typical growth and physiological processes [1]. Dietary deficiency of Se impairs growth rate and increases mortality [6, 8]. The crucial biological role of Se is related to its presence in the structure of enzymes and proteins. Se element is a naturally occurring mineral in the environment and is most easily metabolized in organic form. It exists in water in trace amounts, primarily in the form of selenates and selenites [9]. Various factors influence the bioavailability of Se. Its chemical form is considered one of the main influential factors [10]. It is most effectively absorbed in the presence of other elements such as vitamins E, D, and A [6]. Moreover, proteins, fats, and heavy metals influence the bioavailability of Se. Inversely, excessive concentra-

tion of Se more than requirements is toxic as it accumulates in fish tissue [11, 12]. Various chemical forms of Se are used in fish diets. Organic Se is widely used in aquaculture as it is more bioavailable and biologically active than inorganic forms [13].

As a result, various researches have reported the influences of dietary Se on various fish species using different forms and concentrations. The inclusion rate of Se depends on fish species and culture conditions. Numerous studies about the effects of Se have been carried out on several species, such as grouper (*Epinephelus malabaricus*) [14], channel catfish (*Ictalurus punctatus*) [15], cobia (*Rachycentron canadum*) [16], Nile tilapia (*Oreochromis niloticus*) [16], in cyprinid species such as crucian carp (*Carassius Carassius*) [8, 18], gibel carp (*Carassius auratus gibelio*) [8, 13], silver carp (*Hypophthalmichthys molitrix*) [19], and few studies on common carp [20–23].

Ashouri *et al.* [21] reported the improved growth and antioxidant status of common carp using 1 mg of nano-Se, while Saffari *et al.* found that nano-Se was more efficient than both organic and inorganic forms [20]. Gaber repor-

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ted an improved growth and survival rate in common carp by selenite [23]. Similar to the present study, Ani *et al.* found some positive effects of Se on common carp performance using 0.03 mgkg⁻¹ organic Se [22].

The ideal dietary Se requirement of common carp, a fish with significant economic importance and widespread distribution globally, is the subject of few research. The present study aimed to investigate the effect of different inclusion rates of organic Se in feed on growth performance, body composition, and blood hematological indices of common carp juveniles.

2. Materials and Methods

2.1. Experimental design

Common carp juveniles were achieved from a local fish hatchery, in Erbil, Iraq. Before the experiment, fish were acclimated and fed a control diet. The experiment was conducted in the RAS system at the Agricultural Research Center, Erbil, Iraq. The system volume was 18m³, which consisted of polyethylene tanks, drum filter, protein skimmer and biological filter. Furthermore, an ozone generator and UV sterilizer were included in the system. A randomized complete block design was applied for treatment allocation, and each 12:500L polyethylene tank was stocked with 25 fish. The system was maintained and cleaned during the trial.

2.2. Test Diets and experimental duration

Fish were fed the basal diet during the acclimation period for two weeks. Then, three test diets were prepared and used afterwards. basal diet was formulated according to the required ingredients and its nutritional value was balanced for growing common carp in RAS culture. Three test diets were supplemented with Sel-plex (1000) obtained from Alltech Company as a basis for organic Se. The three test diets were prepared containing 0.5, 1, and

2 mgkg⁻¹ Se (Table 1). Fish were fed two times a day at a specific amount of 3% of the test diets for 10 weeks.

2.3. Water quality

The physical and chemical parameters of the water were maintained within the optimal range for common carp culture in the RAS system. Dissolved oxygen (DO), pH, and Temperature were checked every day. Ammonia (NH₃), Nitrite (No₂) and Nitrate (No₃) were measured once every 5 days. Throughout the experiment, temperature was maintained at 22.45±0.90 °C (Mean±SD); DO 8.15±0.64 mgL⁻¹; PH 8.34±0.06; NH₃ 0.22±0.01 mgL⁻¹; No₂ 1.66±0.69 mgL⁻¹ and No₃ 2.5±1.21 mgL⁻¹.

2.4. Growth performance

Fish were weighed every week to measure growth parameters. Samples were collected for various measurements at the end of the experiment. At the end of week 10,(end of experiment), all fish were weighed to assess growth measurements. Weight gain (WG), specific growth rate (SGR), survival rate, feed conversion efficiency (FCE), protein efficiency ratio (PER), and feed conversion ratio (FCR) were calculated and measured according to the following equations;

$$WG = \text{Final Weight (g)} - \text{Initial weight(g)}$$

$$SGR = \frac{(\ln \text{ final weight} - \ln \text{ initial weight})g \times 100}{\text{rearing days (days)}}$$

$$FCR = \frac{\text{Total feed given (g)}}{\text{Total weight gain (g)}}$$

$$PER = \frac{\text{Total wet weight gain(g)}}{\text{Dry weight of protein in diet (g)}}$$

Table 1. Formulation and proximate composition (% dry matter) of experimental diets containing different concentrations of organic selenium (Se).

Ingredients		Ingredient quantities in different dietary Se level			
		0	0.5	1	2
Fish meal 65% anchovy	(%)	28	28	28	28
Soybean 46% (expel)	(%)	32	32	32	32
Corn	(%)	16.6	16.6	16.6	16.6
Wheat bran	(%)	8	8	8	8
Wheat (10CP)	(%)	12	12	12	12
Oil	(%)	3	3	3	3
Vitamin premix free Se ^a	(%)	0.4	0.4	0.4	0.4
Sel-plex ^b	mg kg ⁻¹	0	0.5	1	2
Proximate composition of test diets					
Protein	(%)	35.80	35.79	35.50	36.30
Ash	(%)	8.72	8.87	9.10	9.1
Fiber	(%)	3.33	3.71	3.81	3.28
Moisture	(%)	6.29	6.20	6.15	6.12
Lipid	(%)	7.77	7.45	7.43	7.56
Energy	kcal/kg	3061	3037	3039	3051

a: Vitamin premix contains the followings (mgkg⁻¹): vitamin B1, 20; vitamin B2, 20; vitamin B5, 25; vitamin B6, 10; vitamin B12, 3; vitamin A, 90; vitamin K3, 10 vitamin D3, 20; Iron, 12000; Copper, 4000; Zinc, 10000; Manganese, 12000.

b: Sel-plex[®] 1000 From Alltech Company, Branch of Turkey.

$$FCE = \frac{\text{Total weight gain(g)}}{\text{Total feed given (g)}} \times 100$$

$$\text{Survival rate\%} = \frac{\text{No. of initial fish stocked}}{\text{No. of fish harvested}} \times 100$$

2.5. Diets and whole-body composition

Samples were randomly selected and weighed before being sacrificed with an overdose of MS-222 (Tricaine methanesulfonate). They were dried in an oven for 24 hours at 105°C and were ground and analyzed for proximate composition. The deposition of organic Se in fish samples and test diets was also examined. Moisture, ash, protein and lipid were analyzed using standard proximate composition: moisture measured by oven-dry samples at 105°C to a consistent weight and ash by combustion for 24 hours at 550 °C. Kjeldahl method was used to determine protein and lipid was determined by ether extraction using the Soxhlet method. Se concentration in fish samples and diets was determined using the Atomic Absorption Spectroscopy (AAS) method which is described by Tinggi (1999), Ministry of Science and Technology, Baghdad [24].

2.6. Hematological analysis

Fish samples were randomly selected and anesthetized with 100 mgL⁻¹ of MS-222. Blood was collected from the caudal vein and placed in 5mg EDTA tubes. Hematological analysis of red blood cells (RBC), hematocrit (HCT), hemoglobin (HGB), platelet count (PLT), mean platelet volume (MPV), mean corpuscular hemoglobin concentration (MCHC) and mean corpuscular hemoglobin (MCH) were measured using hematology Analyzer (MCL 3800, China). Other hematological parameters were measured, such as monocyte percentage (MON%), lymphocyte

percentage (LYM%), white blood cells (WBC), and granulocyte percentage (GRA%) using a hematology analyzer as stated above.

2.7. Statistical analysis

The data was performed for homogeneity and normality of variance using the shapiro-wilk test. The data was analyzed through a one-way analysis of variance (ANOVA). Multiple comparisons and significances were tested at ($p < 0.05$), and for further significances, a post-hoc test followed by Tukey's and Duncan's test were applied. Descriptive statistics were presented as mean±SD.

3. Results

In the current study, the inclusion of 0.5 mgkg⁻¹ of dietary Se significantly improved ($p < 0.05$) FBW, WG and SGR (Table 2). The FBW in the fish-fed basal diet was 57.06 g and 54.41g in treatment three with the highest Se level. The lowest FBW was noticed in the fish-fed basal diet. Moreover, WG in the treatment 0.5mgkg⁻¹ was 30.10g and significantly higher ($p < 0.05$) than other treatments followed by treatment three.

In addition, no significant differences ($p > 0.05$) were observed in feed utilization parameters among the different treatment groups (Table 3). FCR, PER, TFI and FCE were similar in all treatment groups. Furthermore, no significant differences ($p > 0.05$) were noticed in survival rates among feeding treatments. basal diet-fed fish had a survival rate of 92% compared to 90% in the highest Se-fed fish.

The whole-body composition was measured on a dry weight basis. The results represented significant differences ($p < 0.05$) in protein, fiber and ash content of fish fed 1, 2 and 0.5 mgkg⁻¹ respectively. Body protein content was significantly higher ($p < 0.05$) in fish fed 1 mgkg⁻¹ which was 56.46% compared with fish fed a basal diet of 48.00% (Table 4). Fish fed 2 mgkg⁻¹ had significantly ($p < 0.05$)

Table 2. Growth rate parameters of common carp fed different levels of dietary organic.

Parameters	Dietary Selenium mg kg ⁻¹			
	0	0.5	1	2
IBW(g)	26.72 ± 0.38	26.96 ± 0.26	26.69 ± 0.42	26.84 ± 0.36
FBW(g)	50.92 ± 0.61 ^c	57.06 ± 2.85 ^a	53.09 ± 0.97 ^{bc}	54.41 ± 0.90 ^{ab}
WG(g)	24.20 ± 0.26 ^c	30.10 ± 2.83 ^a	26.40 ± 0.97 ^{bc}	27.57 ± 1.17 ^{ab}
SGR (%)	0.92 ± 0.006 ^c	1.07 ± 0.07 ^a	0.98 ± 0.03 ^{bc}	1.00 ± 0.03 ^{ab}
TWG	503.33±5.50	578.04±40.91	517.81±40.87	562.06±23.30
Survival rate %	92.0±0.00	88.0±6.92	89.33±4.61	90.0±2.30

Values are means ± SD ($n = 3$). Values with different superscript letters are significantly different ($p < 0.05$).

Abbreviations: IBW, initial body weight; FBW, final body weight; WG, weight gain; SGR, specific growth rate; TWG; total weight gain.

Table 3. Feed utilization parameters of common carp fed different levels of dietary Se.

Parameters	Dietary Selenium mg kg ⁻¹			
	0	0.5	1	2
FCR	3.36±0.08 ^a	3.09±0.25 ^a	3.33±0.31 ^a	3.14±0.14 ^a
TFI/g	1696.28±55.57.08 ^a	1783.84±21.29 ^a	1718.16±44.32 ^a	1764.36±43.36 ^a
FCE (%)	29.68 ± 0.72 ^a	32.41 ± 2.51 ^a	30.16 ± 2.75 ^a	31.86± 1.40 ^a
PER (%)	0.82 ± 0.02 ^a	0.90 ± 0.07 ^a	0.84 ± 0.07 ^a	0.87 ± 0.03 ^a

Abbreviations: FCR; Food conversion ratio, TFI; Total feed intake, FCE; Feed conversion efficiency, PER, protein efficiency ratio; PI; production Index.

Values are means ± SD ($n = 3$). Values with different superscript letters are significantly different ($p < 0.05$).

the highest fiber content of 1.66% followed by 0.97% in fish only that fed a basal diet. Ash content of fish fed only 0.5 mgkg⁻¹ was 9.43 % which was significantly higher (p<0.05) than the other treatment groups, but digestible energy was better in the basal diet. Whole-body retention and deposition of Se showed that fish fed 2 mgkg⁻¹ Se had significantly (p<0.05) more Se (0.97 mgkg⁻¹) (Table 4) compared to other fish.

In the present study, fish-fed basal and 0.5 mgkg⁻¹ diet exhibited a significant increase (p<0.05) in both the blood RBC and HGB. Blood RBC and HGB in fish fed basal diet were 29.67 gL⁻¹ and 85.21 gL⁻¹ followed by fish fed 0.5mgkg⁻¹ diet which was 29.18 10¹²L⁻¹ and 86.96 gL⁻¹ respectively (Table 5). The percentages of blood WBC and LYM were affected (p<0.05) with dietary Se inclusion (Figure 1). Fish-fed diet 2 mgkg⁻¹ showed the highest level of blood WBC followed by the fish-fed basal diet, but the percentage of blood LYM was significantly higher in the fish-fed basal diet followed by fish-fed only 0.5 mgkg⁻¹. However, the percentages of blood MON and GRA remained unaffected in all feeding treatments (Figure 1).

4. Discussion

Se element is a crucial micronutrient for fish feed [1, 12]. Different dietary supplementation levels of organic Se in common carp feed were examined. The effects were observed on FBW, WG and SGR. Fish fed only 0.5 mgkg⁻¹ had significantly better FBW, WG, and SGR. The growth rate of common carp decreased with increasing Se in the diet by more than 1 mgkg⁻¹. Changes in growth rate could be related to the level of selenium in the diet, which

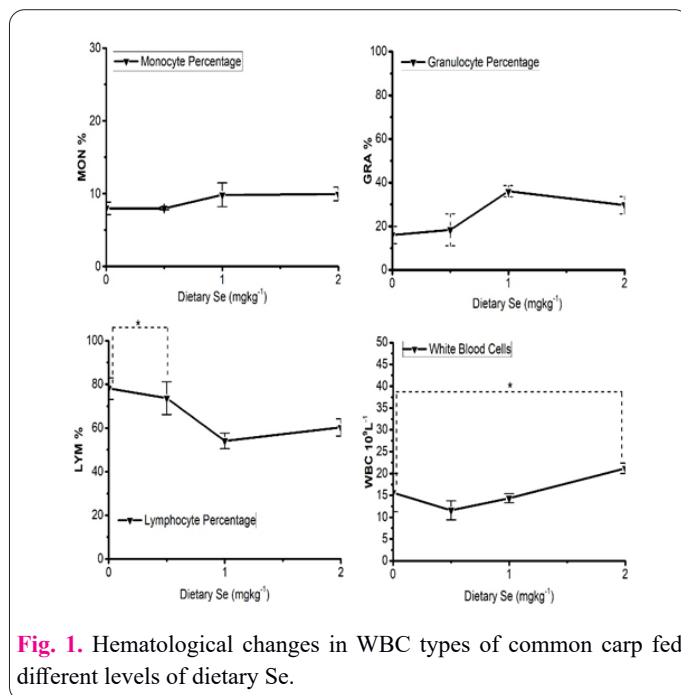


Fig. 1. Hematological changes in WBC types of common carp fed different levels of dietary Se.

might make high selenium in the diet more than optimal requirements for common carp which eventually reduces growth. The overall positive effects were noticed in fish fed 0.5 mgkg⁻¹. Similarly, significant effects were reported in common carp by Saffari *et al.* [20] and Ashouri *et al.* [21]. Saffari *et al.* [20] used different sources of Se, such as nano-Se, organic Se, and inorganic Se. They reported that using 0.7 mgkg⁻¹ of nano-Se significantly increased final

Table 4. Whole- Body Proximate Composition (as dry weight basis) of common carp fed different levels of dietary Se.

Parameters	Dietary Selenium mg kg ⁻¹			
	0	0.5	1	2
Moisture	1.49 ± 0.12c	1.75 ± 0.10bc	2.56 ± 0.35a	2.12 ± 0.24b
Protein	48.00 ± 1.43b	51.68 ± 3.58ab	56.46 ± 3.22a	52.94 ± 1.90ab
Fat	30.58 ± 2.30a	28.48 ± 2.89a	26.94 ± 2.05a	26.88 ± 1.57a
Fiber	0.97 ± 0.03b	0.64 ± 0.24c	1.20 ± 0.13ab	1.66 ± 0.42a
Ash	7.62 ± 0.61c	9.43 ± 0.51a	8.88 ± 0.23ab	8.49 ± 0.25b
Energy	4675 ± 129.46a	4510 ± 165.78ab	4404 ± 94.20b	4414 ± 78.03b
Se*	0.62±0.02c	0.64±0.03c	0.79±0.04b	0.97±0.03a

Values are means ± SD (n = 3). Values with different superscript letters are significantly different (p < 0.05).

*Whole-body Se concentration determined using Atomic Absorption Spectroscopy (AAS).

Table 5. Hematological parameters of common carp fed different dietary selenium levels.

Parameters	Dietary Selenium mg kg ⁻¹			
	0	0.5	1	2
RBC 10 ¹² L ⁻¹	29.67±1.58a	29.18. ±2.90a	22.33±3.04b	20.01±3.60b
HCT %	358.45±43.45	342.56±62.94	309.03±84.48	232.07±20.75
HGB gL ⁻¹	85.21±9.50a	86.91±15.24a	55.23±19.54b	58.23±5.94b
MPV fl	13.66±0.20a	13.26±0.71a	12.98±1.03a	13.41±0.33a
PLT 10 ⁹ L ⁻¹	7208±790.48a	6782±1086.25a	7081±208.85a	7087±568.26a
MHC pg	2.56±0.38a	3.13±0.80a	2.50±0.25a	3.31±0.96a
MCHC gL ⁻¹	20.66±2.84a	25.33±6.02a	25.16±5.75a	17.16 ±7.37a

Values are means ± SD (n = 3). Values with different superscript letters are significantly different (p < 0.05).

Abbreviations: RBC; Red blood cells, HGB; Hemoglobin, HCT; Hematocrit, PLT; Platelet count, MPV; Mean platelet volume, MCH; Mean corpuscular hemoglobin, MCHC; Mean corpuscular hemoglobin concentration.

body weight compared with other treatment groups, but fish SGR improved in both nano-Se and organic sources, which is in agreement with the results of the present study. Ashouri *et al.* [21] used the same concentration levels of Se but in different from which was nano-Se. They find significant improvement in final body weight and weight gain in fish fed 1 mgkg⁻¹. Enhanced growth of fish-fed Se in various species was reported by Lin and Shiau [14] in grouper, Betancor *et al.* [25] in European sea bass, Han *et al.* [13] in gibel carp, Wang *et al.* [15] in channel catfish, Wang *et al.* [18] in crucian carp, Cotter *et al.* [26] in hybrid striped bass and Küçükbay *et al.* [27] and Rider *et al.* [28] in rainbow trout.

The inclusion of Se in feed has not shown any significant effects on feed utilization parameters of FCR, FCE and PER. This is in agreement with Ashouri *et al.* [21], who found that using 0.7 mgkg⁻¹ Se had no significant effect on the FCR of common carp. The results in the present study were also in agreement with Zhou *et al.* [8], who reported no significant effect of dietary Se on FCR using 0.5 mgkg⁻¹ of organic and nano-Se in crucian carp feed. Similar results were reported in gilthead seabream [30], crucian carp [18], striped bass [26], and largemouth bass [18]. In contrast, Abdul-Tawwab *et al.*, [30] noticed better feed utilization in African catfish fed 0.3 mgkg⁻¹ Se.

No significant results were noticed in fish survival rate among all experimental diets. The result is in agreement with Ashouri *et al.* [21], who've also found that using 0.7 mgkg⁻¹ of Se has no significant effect on common carp survival. Also, using 0.5 mgkg⁻¹ by Zhou *et al.* [8] showed no effect on the survival rate of crucian carp. Similarly, the effects of Se on survival were demonstrated in various studies, including Hardy *et al.* [31] on cutthroat trout, Tashjian *et al.* [32] in sturgeon, Le and Fotedar [33] in yellowtail kingfish, Zhou *et al.* [32] in largemouth bass, and Mechlaoui *et al.* [29] in gilthead seabream. In contrast, Gaber, [23] found a better survival rate in common carp fed 0.24 and 0.32 mgkg⁻¹, but Ani *et al.* [22] observed the influence of 0.03 mgkg⁻¹ Se on the survival rate of common carp fry and juveniles. Liu *et al.* [16] reported a significant enhancement in the survival rate of cobia juveniles fed 1 mgkg⁻¹ Se. Comparison results from the present study with other similar research could be noticed using different levels of selenium in the fish diet. Studies used 0.03 to 0.32 mg kg⁻¹ of Se in the fish diet compared with the present study, whose maximum level of Se in the diet was 2 mgkg⁻¹.

Fish fed 1 mgkg⁻¹ diet had the highest protein content, followed by 2 and 0.5 mgkg⁻¹ diets. Fiber was significantly higher in fish fed 2 mgkg⁻¹. Due to the inverse relationship between fat and protein, fat declined by increasing dietary Se. The improvement of whole-body composition in the present study conflicts with Saffari *et al.* [20] and Ashouri *et al.* [21], both of whom reported that dietary Se nanoparticles did not have any influence on the proximate composition of common carp, which might be due to the diverse forms of Se utilized in these studies. Also, Zhu *et al.* [34] did not report any significant differences in the body composition of gibel carp. Similar effects of Se on fish body composition were reported by Zhu *et al.* [34] and Han *et al.* [13]. Fish fed 1.34 mgkg⁻¹ had significantly higher whole-body Se concentration in gibel carp. Han *et al.* [13] and Zhu *et al.* [34] found a significant increase of Se concentration in fish fed 5 mgkg⁻¹. Fish fed 2 mgkg⁻¹

¹ had more accumulated Se in the whole body. High Se concentration in muscle of fish fed Se was found in crucian [14, 18, 35]. Se accumulates differently in fish muscle than it does in tissue [36]. These variations might be attributed to variations in fish life stages, culture systems, and certain parameters such as temperature or feed quality. Organic Se is more bioavailable since it is delivered intact to target tissues.

Organic and inorganic forms of Se promote overall health status in fish according to numerous studies. Immunity status could be checked by some hematological parameters such as blood serum, blood cells, hematocrit parameters, albumin, Hb, and lysozyme. Increased total count of RBCs, hematocrit and hemoglobin due to the increase of Se levels in fish diet might be attributed to the enhancement of fish health.

In this study, the dietary inclusion of Se had significant influences on some of the fish hematological indices. Abdel-Tawwab *et al.* [30] found a significant increase in RBC, HGB and HCT in catfish fed 0.3 and 0.5 mgkg⁻¹ Se. Sharaf Al-Din [38] in Nile Tilapia and Mushtaq *et al.* [19] in silver carp reported significant effects of dietary Se on HGB, RBC, HCT and WBC. However, values of HCT, MPV, PLT, MHC and MCHC remained unaffected ($p>0.05$) with dietary Se. In a different way, Durigon *et al.* [37] observed that dietary Se increased blood MHC but blood MCHC remained unaffected by Nile tilapia-fed Se, but Mushtaq *et al.* noticed that MCHC was significantly affected by silver carp fed Se [19]. Therefore, the variation in hematological could be due to different fish species, culture conditions and Se forms and level of inclusion in test diets. The present study was carried out in the RAS system, in which water from all dietary treatments was filtered at once, this could minimize the effects of Se and increase interaction among feeding treatments.

5. Conclusion

The effects of different dietary concentrations of organic selenium (Se) on the growth, body composition, and hematological indices of common carp were investigated. The growth, protein content, selenium deposition, and hematological indices of common carp were significantly improved in fish-fed Se. Fish-fed treatment diets containing 0.5 mgkg⁻¹, 1 mgkg⁻¹, and 2 mgkg⁻¹ Se showed a significant weight increase, final body weight, and specific growth rate, whereas fish-fed basal diets showed the lowest growth rate values. For a common carp diet, a Se content of 0.5 to 1 mgkg⁻¹ is ideal. Further studies should be done about the optimal Se requirement in common carp in RAS culture.

Conflict of Interests

The author has no conflicts with any step of the article preparation.

Consent for publications

All authors read and approved the final manuscript for publication.

Ethics approval and consent to participate

No humans or animals were used in the present research.

Informed Consent

The authors declare not to use any patients in this research.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions

All authors conceived and designed the study. All authors read and approved the final manuscript.

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