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Synergistic effects of dietary nano selenium and vitamin C on growth, feeding, and physiological parameters of mahseer fish (*Tor putitora*)

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ABSTRACT

The current study was conducted to determine the synergistic effects of dietary nano selenium (Nano Se) and vitamin C on growth, feeding, and physiological parameters of juvenile mahseer, *Tor putitora*. L-ascorbyl-2-polyphosphate (APP) was used as a source of vitamin C. Four semi-purified experimental diets were prepared. A basal diet kept without the supplementation of any micronutrient and the other three diets were formulated such that three different levels of APP (100, 200, and 300 mg kg^{-1}) were used in combination with a pre-determined dose of Nano Se (0.68 mg kg^{-1}). The results showed that both the micronutrients positively synergized the effects of each other. APP at the rate of 300 mg kg^{-1} showed strong interaction with Nano Se. The APP_{300} + Nano $Se_{0.68}$ mg kg^{-1} diet supplemented diet significantly decreased ($P < 0.05$) the feed conversion ratio (FCR) while significantly increased ($P < 0.05$) the weight gain percentage (WG%), feed conversion efficiency (FCE%), specific growth rate (SGR), and serum growth hormone (GH) concentration. Similarly, the physiological parameters such as red blood cells count (RBCs), hemoglobin level (Hb), hematocrit value (Hct), and serum lysozyme activity were also significantly increased in group of fish fed diet supplemented with APP_{100} mg kg^{-1} in combination with Nano $Se_{0.68}$ mg kg^{-1} as compared to the control group. The present results clearly indicated the beneficent synergistic effects of Nano Se and APP in mahseer fish. Moreover, the current finding also supported our hypothesis that Nano Se and APP potentiate positively the effect of each other when both the micronutrients are supplemented together in the same fish feed.

1. Introduction

Minerals and vitamins are two important micronutrients which are required for the maintenance of normal body functions. A proper amount of these micronutrients is required for the normal catalytic processes within the enzymatic system which consists of a variety of enzyme activities linked with the metabolic, endocrine and immune systems (Tomkins, 2002; Keen et al., 2004). The deficiency of these micronutrients causes many metabolic disorders and diseases/infections through their negative influence on the physiological system in fish and other animals (Percival, 1995; Watanabe et al., 1997; Ekiz et al., 2005; Lin and Shiau, 2005). However, their surplus amounts also cause severe toxicity (Watanabe et al., 1997; Hamilton, 2004).

Among micronutrients, selenium and vitamin C both are strong antioxidants and are required for the proper growth and physiological health of animals. Selenium when available in sufficient amount plays an important role in the maintenance of normal body functions of fish and other aquatic species; however, its deficiency weakens the normal physiological health of the body (Ewan, 1976; Raza, 2012; Jamil, 2013; Khan et al., 2016). Similarly, vitamin C is another important micronutrient which is also required in proper amount for the improvement of fish growth and physiological health. This vitamin should be supplemented in fish feed regularly because most of the teleost fish are unable to synthesize ascorbic acid and thus they require proper supplementation of vitamin C in their diet (Al-Amoudi et al., 1992; Dabrowski, 2001; Shalata and Neumann, 2001; Ai et al., 2004; Lin and Shiau, 2005; Khan et al., 2015).

As both micronutrients act as biological antioxidants and help in the protection of cellular membranes from oxidative damage and improve the growth and physiological health of fish and other animals (Ong and Packer, 1992; Wang et al., 1994; Shalata and

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Neumann, 2001; Gaber, 2009; Zuberi et al., 2011; Khan et al., 2015, 2016). Thus due to their ability for interaction, the determination of the synergistic effects among these two important dietary micronutrients may be very useful especially for fish being living in an aquatic environment. By the getting both micronutrients together will enable it to better grow and survive in the current threatening situation of the aquatic environment as currently most water bodies of the world is facing pollution related problems.

T. putitora (Hamilton) belongs to the family Cyprinidae, distributed along the Himalaya belt in Pakistan, Bhutan, and Bangladesh, India, and is an important game and highly valued food fish (Shrestha, 1990; Nautiyal, 1994). Its common name is golden mahseer and it is a column feeder and omnivorous at the adult stage while planktivorous at juvenile stage (Dubey, 1985; Negi, 1994; Shrestha, 1997). Mahseer populations have been declining in most of their natural habitats due to the impacts caused by the industrialization, urbanization, and agricultural developments which are causing ecological alterations and physical changes in the natural environment in lakes and rivers of mid hills (Das and Joshi, 1994; Shrestha, 1994; Joshi, 1988). Another problem is that growth of mahseer in captivity is slower than the growth of fish reared in the wild (Shrestha, 1997). Thus there is a great need to pay attention to the improvement of captive environment for the better and faster growth of this important fish. Some preliminary research studies on the diet development and the use of diets of different nature for feeding mahseer showed beneficent results (Bista et al., 1998). But the existing studies especially about the micronutrients nutrition are not sufficient.

Most of the earlier research work regarding dietary vitamin C and selenium is related to the dietary requirements of these micronutrients separately for various fish species while insufficient literature is available on their combined use in fish feed. In our previous experiments conducted at our laboratory, have already been documented the individual better levels of both of these micronutrients for *T. putitora* (Raza, 2012; Jamil, 2013; Khan et al., 2015, 2016). Thus after determining the individual requirements of these micronutrients and keeping in view their response in the previous studies we made a hypothesis. The hypothesis was that being strong antioxidants, their combined supplementation will synergize the effects of each other and will show better synergistic effects on the growth and physiology of fish as compared to their individual supplementation. The present study was therefore planned to determine the synergistic effects of nano form selenium (Nano Se) and vitamin C (APP) on growth, feeding, and physiological parameters in juvenile mahseer, *T. putitora*.

2. Materials and methods

2.1. Nano Se synthesis

Nano Se was prepared through the precipitation method as described by Jamil (2013) and Khan et al. (2016) for *T. putitora* species and characterized by the X-ray diffraction technique. The calculated average size of the Nano Se was 90 nm.

2.2. Animals and diets

The healthy and uniform sized fish were bought from a fish farm and were transferred to the laboratory. First the fish were acclimatized to the laboratory conditions for about two weeks while feeding it with a control diet. After acclimation, 10 fish (regardless of their sex) of average body weight (2.30 ± 0.04 g) were stocked per tank. The control diet was formulated as shown in Table 1. Four semi-purified and *iso*-nitrogenous diets consisted of one control diet and three tested diets. The control diet was prepared without the supplementation of either test micronutrient while the other three diets were prepared such that three different APP levels (100, 200, and 300 mg APP kg^{-1} diet) were mixed with a predetermined dose of Nano Se (0.68 mg Nano Se mg kg^{-1} diet), respectively. All the ingredients were mixed completely in a mixer. Then the control diet (without the mixing of the test micronutrients) and each of the other three diets (each diet contained proper amounts of APP in combination with the predetermined dose of Nano Se) were extruded into pellets. After extrusion, the feed pellets were packed into plastic bags and kept at −20 °C until use.

Table 1
Formulation of basal diet for *T. putitora*.

Ingredients	Amount (gram/kilogram)
Soybean meal	130
Wheat bran	10
Fish meal	250
Wheat flour	10
Gluten 60%	500
Sunflower meal	50
Rice bran	10
[a]Premix	10
Di-calcium phosphate (DCP)	10
Canola oil	20
Total sum	1,000.00 g kg^{-1}

[a] Premix contained vitamins (except vitamin C), amino acids, and minerals (except selenium).

2.3. Experimental design

The 60 day feeding experiment was conducted in a flow-through system. The trial was conducted in a triplicate form and totals 12 fiber tanks were used, with 90 liters of water capacity per tank. Each tank was randomly assigned to one of the three replicates of each of the four dietary treatments. The conditions of the experiment were semi-static and freshwater was used throughout the trial.

The experimental diets were fed to the triplicate groups of fish thrice a day (6:00a.m.; 12:00p.m.; 5:00p.m.) until apparent satiation. The water quality parameters were maintained at the proper levels as reported for the under study species by Khan et al. (2015). During the experimental period, the average water pH ranged from7 to 8 and temperature ranged from 21 to 23 °C. The ammonia level measured was lower than 0.20 mg L^{-1}, while the dissolve oxygen content (DO_2) ranged from 6 to 6.5 mg L^{-1}.

2.4. Samples collection

At the end of the 60 day experiment, fish were fasted for 24 h prior to sampling to allow the evacuation of food in the gastrointestinal tract. On the following day, fish were sampled as follows; One by one all fish were removed from each tank and euthanized in a tricaine methanesulfonate solution (MS 222, 0.5 g L^{-1}; Argent Chemical Laboratories, Redmount, WA, USA) respectively. Each euthanized fish was blotted dry on the paper towels and weighed. After weighting, all fish per tank were subjected to the blood collection process and the blood was collected by the tail ablation method (Khan et al., 2015, 2016). The remaining blood was centrifuged at 3500 rpm for 15 min. The serum was separated and stored at −20 °C for the analysis of the serum growth hormone concentration and serum lysozyme activity.

2.5. Growth and feeding parameters

The weight gain percentage (WG%), feed conversion ratio (FCR), feed conversion efficiency (FCE%), and specific growth rate (SGR%), was calculated according to the method followed by Khan et al. (2015).

Table 2
Synergistic effects of the diets supplemented with different levels of APP in combination with Nano Se on the growth and feeding parameters of juvenile *T. putitora*.

Parameters/Diets	WG%	SGR (%)	FCE (%)	FCR
Basal diet	64.6 ± 0.52^{g}	0.40 ± 0.02^{g}	28.06 ± 0.13^{g}	3.56 ± 0.06^{a}
APP_{100} + Nano $Se_{0.68}$	98.76 ± 1.76^{d}	0.59 ± 0.09^{d}	42.43 ± 0.36^{d}	2.35 ± 0.01^{d}
APP_{200} + Nano $Se_{0.68}$	104.46 ± 0.70^{c}	0.62 ± 0.01^{c}	45.24 ± 0.11^{c}	2.21 ± 0.04^{e}
APP_{300} + Nano $Se_{0.68}$	125.41 ± 0.74^{a}	0.72 ± 0.03^{a}	50.93 ± 0.08^{a}	1.96 ± 0.04^{g}

Data are represented as Mean ± SE (n = 3). Means followed by a different letter within a column are significantly different (P < 0.05) (ANOVA followed by Tukey's LSD test).

2.6. Serum growth hormone assay

For the analysis of serum growth hormone, the method described by Khan et al. (2015, 2016) was followed. Serum GH concentration in all group units was estimated by using a Micro ELISA HGH kit (Amgenix MicroLISATM, USA).

2.7. Physiological parameters

About 1 ml blood was collected in VACUETTE EDTA K3 tubes for the analysis of hematological parameters while the rest of blood was centrifuged and was used for the analysis of serum lysozyme activity. The blood parameters such as, red blood cell (RBC) count ($106\ \mu L^{-1}$), hemoglobin (Hb) level ($g\ dL^{-1}$), and hematocrit (Hct%) were determined by using the hematology analyzer (Sysmax KX-21 Japan).

An important innate immunity parameter, the lysozyme activity was determined according to the method followed by Khan et al. (2016). The absorbance was observed with the help of spectrophotometer at 450 nm after 1 min intervals for 10 min and the lysozyme activity ($\mu g\ mL^{-1}$) was calculated by using hen egg white lysozyme (Sigma-Aldrich) as a standard.

2.8. Statistical analysis

Obtained results were analyzed through one-way analysis of variance (ANOVA) by using the Statisix 8.1 analytical software. When any significant difference was detected, a Tukey's LSD (least significant difference) test was used to compare the means. Treatment differences were measured with the significance level at (P < 0.05).

3. Results

3.1. Growth and feeding parameters

No mortality was observed. The synergistic effect of dietary Nano Se along with different levels of APP on the growth and feeding parameters of *T. putitora* is shown in Table 2. The addition of dietary Nano Se in combination with APP into the fish feed showed significant effect on the growth and feeding parameters of *T. putitora*. The dietary Nano Se and APP supplementation

synergized the effect of each other and significantly higher (P < 0.05) weight gain percentage (WG%), feed conversion efficiency (FCE%), specific growth rate (SGR%), while significantly lower (P < 0.05) feed conversion ratio (FCR) was observed in the group of fish fed APP_{300} + Nano $Se_{0.68}$ $mg\ kg^{-1}$.

3.2. Serum growth hormone level

Serum growth hormone results are given in Table 3. The lowest GH level was observed in group of fish reared on the basal diet. However, the supplementation of dietary Nano Se along with APP significantly increased the serum growth hormone concentration

Table 3
Synergistic effects of the diets supplemented with different levels of APP in combination with Nano Se on the serum growth hormone level in juvenile *T. putitora*.

Parameters/diets	Serum growth hormone ($ng\ mL^{-1}$)
Basal diet	0.09 ± 0.06^{a}
APP_{100} + Nano $Se_{0.68}$	0.100 ± 0.08^{b}
APP_{200} + Nano $Se_{0.68}$	0.115 ± 0.06^{c}
APP_{300} + Nano $Se_{0.68}$	0.312 ± 0.09^{d}

Data are represented as Mean ± SE (n = 3). Means are followed by a different letter within a column are significantly different (P < 0.05) (ANOVA followed by Tukey's LSD test).

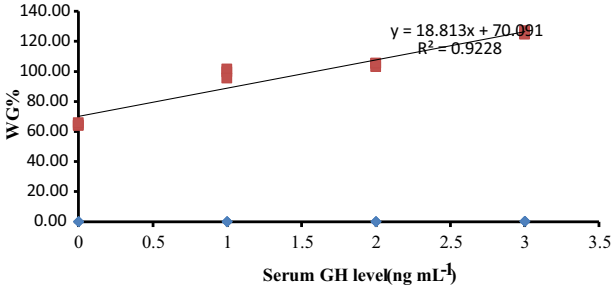

Fig. 1. Positive co-relation between the WG% and serum growth hormone level.

Table 4
Synergistic effects of the diets supplemented with different levels of APP in combination with Nano Se on the hematology of juvenile *T. putitora*.

Parameters/ diets	RBC count (106 μL)	Hb level ($g\ dL^{-1}$)	Htc (%)
Basal diet	2.26 ± 0.08^{a}	7.3 ± 0.20^{a}	32.92 ± 0.01^{a}
APP_{100} + Nano $_{Se0.68}$	2.64 ± 0.13^{b}	$8 \pm 0.05c^{b}$	33.96 ± 0.03^{b}
APP_{200} + Nano $Se_{0.68}$	2.98 ± 0.03^{c}	8.4 ± 0.07^{c}	34.53 ± 0.08^{c}
APP_{300} + Nano $Se_{0.68}$	3.2 ± 0.05^{d}	9.16 ± 0.14^{d}	36.4 ± 0.30^{d}

Data are represented as Mean ± SE (n = 3). Means are followed by a different letter within a column are significantly different (P < 0.05) (ANOVA followed by Tukey's LSD test).

in *T. putitora*. The supplementation of APP at the rate of 300 $mg\ kg^{-1}$ in combination with Nano Se at the rate of 0.68 $mg\ kg^{-1}$ showed strong synergistic effects and significantly increased (P < 0.05) the serum growth hormone concentration as compared to the diets contained lower doses of APP in combination with Nano Se as well the control diet.

Moreover, a positive linear co-relation between the weight gain percentage (WG%) and the serum growth hormone level of *T. putitora* which is shown in Fig. 1.

3.3. Physiological parameters

The effects of dietary Nano Se in combination with different levels of APP on the hematological parameters like red blood cells (RBCs) count, hemoglobin (Hb) level, and hematocrit (Hct%) are

shown in Table 4. The dietary Se Nano supplementation in combination with APP showed positive synergistic effects on the hematology of *T. putitora*. Thus, the hematological parameters like RBCs count, Hb level, and Hct%, were significantly increased (P < 0.05) in the group of fish fed diet supplemented with dietary APP_{300} $mg\ kg^{-1}$ in combination with the dietary Nano $Se_{0.68}$ $mg\ kg^{-1}$ as compared to the control group.

Similarly, the serum lysozyme activity was also significantly increased with APP_{300} + Nano $Se_{0.68}$ $mg\ kg^{-1}$ supplementation as compared to the control diet. The serum lysozyme activity results are shown in Table 5.

Table 5
Synergistic effects of the diets supplemented with different levels of APP in combination with Nano Se on the on the serum lysozyme activity of juvenile *T. putitora*.

Parameters/Diets	Serum lysozyme activity (μmol min^{-1} mg^{-1} protein)
Basal diet	0.88 ± 0.08^{a}
APP_{100} + Nano $Se_{0.68}$	2.40 ± 0.06^{b}
APP_{200} + Nano $Se_{0.68}$	3.28 ± 0.07^{c}
APP_{300} + Nano $Se_{0.68}$	4.31 ± 0.06^{d}

Data are represented as Mean ± SE, (n = 3). Means are followed by different letter within the column are significantly different (P < 0.05). (ANOVA followed by Tukey's LSD test).

4. Discussion

Initial studies from our laboratory have already been determined the better dietary levels of both nano selenium (Nano Se) 0.68 mg kg^{-1} (Jamil, 2013; Khan et al., 2016), and vitamin C, l-ascorbyl-2-polyphosphate (APP) 300 mg kg^{-1} diet (Khan et al., 2015) respectively, for *T. putitora*. As some of the earlier research studies showed that synergistic interactions exist between the selenium and other vitamins such as E (Gatlin et al., 1986; Fonseca et al., 2013). However, no information is available about the synergistic effects of dietary vitamin C and selenium in fish. Thus the present study was designed on the basis of the hypothesis that the dietary Nano Se when use in combination with APP in same fish feed, may show strong synergistic interactions. The results obtained revealed that these two important micronutrients when supplied in combination showed strong synergistic interactions and positively potentiated the growth, feeding, and physiological parameters of *T. putitora*.

Earlier research studies showed that adequate amount of dietary selenium is essential for the proper body growth of fish and other animals (Hoffmann and Berry, 2008; Schrauzer and Surai, 2009; Ewan, 1976; Raza, 2012; Khan et al., 2016; Jamil, 2013). In a previous study, it was observed that the diet enriched with vitamin C, E and Se significantly increased the weight gain in tilapia (*O. niloticus*) (Kim and Mahan, 2003). Selenium supplementation also increases the selenium concentration in the muscle tissue of some fish species (Buckley, 2000). Fonseca et al. (2013) observed that the tilapia fillet fed the diet supplemented with 400 mg kg^{-1} vitamin C and vitamin E, respectively and 0.4 mg kg^{-1} selenium oxide showed significantly increased (P < 0.05) weight gain (WG) and feed conversion efficiency (FCE%) as compared to the control diet. The higher growth of tilapia fed the increasing levels of selenium, vitamins C and E can be explained by the facts that both selenium and vitamin C are important micronutrients which are needed for the better growth performance and feeding parameters of fish (NRC, 1993; Wang et al., 2003; Khan et al., 2015, 2016). Their individual supplementation enhances erythrocytes production and prevents high quantity fat accumulation in the liver tissue (Martins et al., 2008; Khan et al., 2015, 2016). The present study also revealed a strong synergistic interaction among dietary Nano Se and APP. Both the micronutrients when supplemented in combination in *T. putitora* feed resulted in significantly increased growth and feeding parameters (weight gain percentage, feed conversion efficiency, feed conversion ratio, specific growth ratio) and significantly decreased the feed conversion ratio.

Like selenium, vitamin C is another essential micronutrient which improves the growth and physiological health of fish. Beside a strong antioxidant, it also accelerates the growth hormone production and thus indirectly increases the body growth in fish (Lovell, 1989; Eipper et al., 1993; James and Manju, 2001; Wang et al., 2003; Kumari and Sahoo, 2005; Ai et al., 2004; Ai et al., 2006; Affonso et al., 2007; Aysun, 2009; Jimenez-Fernandez et al., 2012; Denny-Brown et al., 2012; Zhou et al., 2012; Ullah, 2012; Moein, 2012; Khan et al., 2015; Tran et al., 2006). In the present study, we observed that the dietary APP and Nano Se when used in combination in the same fish feed, strongly synergized the effects of each other. The growth performance and feeding parameters (WG%, SGR%, FCR, FCE%, and serum growth hormone levels) of *T. putitora* were significantly increased by consuming the same feed which was supplemented with the dietary Nano Se (at the rate of 0.68 mg kg^{-1} diet) and APP (at the rate of 300 mg kg^{-1}). These results were more profound than those observed in our previous experiments (Khan et al., 2015, 2016).

The strong antioxidant property of both the selenium (Ong and Packer, 1992; El-Hammady et al., 2007; Molnár et al., 2011; Khan et al., 2016) and vitamin C (Waagbo et al., 1993; Soliman et al., 1994; Chen et al., 2003; Sahoo and Mukherjee, 2003; Nayak et al., 2007; Affonso et al., 2007; NRC, 2011; Pimpimol et al., 2012; Khan et al., 2015) might provide stability and integrity to the blood cells of fish and thus protect them from hemolysis during the normal metabolism and stressful conditions. In the present finding, when both the micronutrients were supplemented together in the same fish feed, it documented that the APP_{300} + Nano $Se_{0.68}$ supplemented diet significantly improved the hematological parameters (RBCs count, Hb level, and Hct%) in juvenile *T. putitora* as compared to the control diet and the diets that contained the low levels of APP in combination with Nano Se. These synergistic effects were significantly better than those observed with the individual supplementation of either vitamin C or selenium in our previous experiments (Khan et al., 2015, 2016).

In fish, the non-specific immune system as compared to the specific immune system is playing a significant role against stress induced by diseases, pollution, and other stressful objects (Anderson, 1992). The supplementation of selenium (Burk et al., 2003; Kumar et al., 2008; Lee et al., 2009; Choi et al., 2013; Khan et al., 2015) and vitamin C (Guha et al., 1993; Fetoui et al., 2008; Korkmaz et al., 2009; Khan, 2014) improves the production of B-lymphocytes. As B-lymphocytes production plays a significant role in the improvement of the lysozyme activity of fish. Thus the improved lysozyme activity then enhances the immunity of fish.

Currently, we supplemented APP and Nao Se in combination in the same diet for observing their synergistic effects. The APP at the rate of 300 mg kg^{-1} showed strong synergistic effects with Nano $Se_{0.68}$ mg kg^{-1} and significantly increased the serum lysozyme activity as compared to the previous feeding trials regarding the micronutrients requirement determination for *T. putitora* (Khan et al., 2015, 2016).

Furthermore, a positive co-relation was observed in the present study among the serum GH concentration and the WG% in *T. putitora* in response to the diets supplemented with dietary Nano Se along with different levels of APP. As in several teleost fish, higher circulating GH levels result in increased feed conversion efficiency and growth rates (Collipp et al., 1984; Won and Borski, 2013; Khan et al., 2015, 2016). The positive co-relation among the serum GH level and the WG% of this study was stronger as compared to the co-relation which was observed in our previous experiments (Khan et al., 2015, 2016).

In conclusion, the results of the present study indicated that dietary vitamin C (at the rate of APP_{300} mg kg^{-1}diet) showed strong synergistic interaction along with Nano $Se_{0.68}$ mg kg^{-1} diet. The APP_{300} + Nano $Se_{0.68}$ mg kg^{-1} supplemented diet significantly increased the growth, feeding, and physiological parameters of *T. putitora*. The results of the present study are significantly better than those obtained in our previous experiments about the vitamin C and Nano Se individual requirements for the same fish species. Thus, this study supported our hypothesis and furthermore, it recommends the supplementation of APP_{300} + Se $Nano_{0.68}$ mg kg^{-1} in

juvenile *T. putitora* feed for better growth, feeding, and physiological parameters.

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