Modulatory Effect of Dietary Copper Nanoparticles and Vitamin C Supplementations on Growth Performance, Hematological and Immune Parameters, Oxidative Status, Histology, and Disease Resistance Against Yersinia Ruckeri in Rainbow Trout (Oncorhynchus Mykiss)

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Abstract

The present study aimed to evaluate the effects of dietary copper nanoparticles (Cu-NPs) and vitamin C (VC) supplementations on rainbow trout (*Oncorhynchus mykiss*) juveniles. Six trial diets were supplemented with Cu-NPs and VC including 0/0 (T1, control diet), 0/250 (T2), 0/500 (T3), 2/250 (T4), 2/500 (T5), and 2/0 (T6) mg Cu-NPs/VC per kg diet. After the feeding trial for 60 days, the fish were challenged with *Yersinia ruckeri* and the survival rate was calculated for 15 days. Based on the data analysis, weight gain (WG), feed conversion ratio (FCR), specific growth rate (SGR), protein efficiency ratio (PER), lysozyme, alternative complement activity (ACH50), catalase (CAT), glutathione peroxidase (GPX), hematocrit (Hct) and mean corpuscular volume (MCV) were significantly ($p < 0.05$) affected by the Cu-NPs factor. Meanwhile, VC was a significant factor for hemoglobin (Hb) and superoxide dismutase (SOD) ($p < 0.05$). The results showed that the Cu-NPs and/or VC-supplemented diets improved WG, FCR, SGR, PER, lysozyme, ACH50, SOD, CAT, GPX, Hb, Hct, and MCV when compared with the control group ($p < 0.05$). The expressions of TNF-α, IL-1ß, IL-10, SOD, CAT, and GPX genes were significantly ($p < 0.05$) decreased in the fish fed on T3, T4, and T5 diets versus the control. In addition, the dietary Cu-NPs and VC supplementations significantly enhanced resistance against pathogens and led to the control of infection in rainbow trout. In conclusion, Cu-NPs and VC administered as feed additive at 2/250–500 mg/kg elevated the growth performance, antioxidant capacity, and health of rainbow trout.

1. Introduction

A key factor of aquaculture viability in all rearing systems is optimal nutrition. Mineral supplements, such as copper, are used to improve growth and food metabolism, strengthen the immune system and antioxidant capacity, and regulate ion exchange and osmotic balance (El Basuini et al. 2016; Mohseni et al. 2014; Lin et al. 2008).

Copper is an essential trace element that is involved in various physiological and biological processes in fish. It plays an essential role in the structure of liver enzymes, melanin and skin pigments, bone and connective tissue formation, myelin maintenance in the nervous system, and hemoglobin synthesis (Havar and Hardy 2002; El Basuini et al. 2016). Many studies have investigated the effective role of dietary copper concentrations and types on various fish species including *Penaeus vannamei* (Davis et al. 1993), *P. monodon* (Lee and Shiau 2002), *Oreochromis niloticus* (El Shaiu et al. 2003), *Haliotis discus hannai* (Wang et al. 2009), *Ctenopharyngodon idella* (Tang et al. 2013), *Huso huso* (Mohseni et al. 2014) and *Pagrus major* (El Basuini et al. 2016).

Nanoparticle forms of essential elements have been shown to be more effective and beneficial than their traditional forms in biological systems through promoting bioavailability, thereby facilitating uptake and utilization (El Basuini et al. 2016; Gharaei et al. 2020a). Nanometer dimensions, large active surface, multiple active centers, and greater catalytic efficiency improve bioavailability and some functional aspects of these metals (Izquier et al. 2016; Rather et al. 2011). Copper nanoparticles (Cu-NPs) are a new form of the copper source that has widely been used in dietary supplementation in aquatic nutrition (El Basuini et al. 2016; Wang et al. 2015).

Vitamin C (VC) or ascorbic acid is a vital water-soluble micronutrient that is essential for physiological functions in animals, including aquatic animals (Fraealossi et al. 2001; Grosso et al. 2013). Vitamin C cannot be synthesized from D-glucose due to the lack of L-gluconolactone oxidase and must be obtained from exogenous sources (Wahli et al. 2003; Chahardeh Baladehi et al. 2017). VC, as a cofactor of many enzymes, plays a role in synthesizing collagen, tyrosine, cartilage, and endothelium of vessels, iron metabolism, and hematology, improving growth and reproduction, strengthening the antioxidant and immune system, and improving survival rates in aquatic animal (Combs 2008; Dawood and Koshio 2016). Some previous research has shown that a high level of VC can play a vigorous inhibitor role in contrast with Cu absorption and/or enzymatic depend on Cu lead to iron utilization decreasing and results iron toxicity (Pekiner and Nebiogliu 1994). Additionally, it is established that dietary VC can decrease the risk of Cu toxicity and Cu supplementation reduces the risk of hypervitaminosis (Watts 1989). In a review paper, Dawood and Kushio (2016) state that VC requirements in aquatic animal species depend on species, size, and feeding behavior.

Although extensive research has addressed the effect of VC supplementation, there is limited knowledge on the interaction of VC and Cu-NPs in fish. Therefore, the present study aimed to assess the effect of dietary VC and/or Cu-NP supplements on growth performance, hematological indices, antioxidant status, histological parameters, immune response, expression of some important genes like TNF-α, IL-1b, and IL-10 in kidney and disease resistance against *Y. ruckeri* in juvenile rainbow trout as one of the most important aquaculture species in the Pacific Ocean in Asia and North America.

2. Materials And Methods

2.1Experimental diets

The basal diet formulation is presented in Table 1. The results of the chemical analysis of the trial diets are shown in Table 2. Cu-NPs (Sigma-Aldrich, 99% purity, NPs size <75 µm) was used as the Cu source, and Stay-C (L-ascorbyl-2-mono phosphate-Ca/Na, Cayman Co., 95% purity) as the VC source. Six trial diets were prepared including two levels of Cu-NPs (0 and 2 mg kg^{-1} dry feed) (El Bausini et al. 2016) and three levels of VC (0, 250, and 500 mg kg^{-1} dry feed) (Dawood et al. 2016) (the control diet without Cu-NPs and/or VC (T1), T2, T3, T4, T5, and T6). They were supplemented to the basal diet according to a 2Í3 factorial design. In preparing the trial diets, ingredients were mixed in a blender for 15 min. The Cu source was mixed with the lipid sources for 15 min and then added to the other ingredients. In the next step, the premixed ingredients were mixed with water and then passed through a meat grinder to prepare pellets with 2 mm diameter, which were dried on nylon screens at 45ºC and kept in two-layer plastics at -20ºC until they were consumed. The actual concentration of proximate composition of Cu and VC was measured in each diet (Table 2).

2.2 Treatments and sample collection

Juvenile rainbow trout were taken from a reproduction center (Shahid Motahari, Yasouj, Iran), transported alive to the laboratory, and acclimated to the experimental conditions for two weeks prior to the beginning of the feeding trial. The fish (initial average weight 15.1±1.05 g) were randomly distributed into six groups with triplicate tanks within each group (18 tanks (200 L of each) and 12 fish per tank). During 60 days, the fish were fed with various test diets three times a day at 08:00, 13:00, and 18:00 based on 3% of the body weight. The quality of water during the experimental period including, temperature, pH, and dissolved oxygen were recorded to be 16 ± 2ºC, 7.1 ± 0.3, and 6.4 ± 0.2 mg l^{-1}, respectively. At the end of the trial, the fish fasted, and their growth performance, weight gain (WG%), specific growth rate (SGR%), feed conversion ratio (FCR), survival rate (%), and protein efficiency ratio (PER) were measured by the following equations (Jafarinejad et al. 2018):WG % = (final body weight (g) −initial body weight (g)) × 100SGR % = (Ln final body weight (g)−Ln initial body weight) × 100 / experimental daysFCR = dry feed fed / wet weight gainPER %=[(final body weight (g)- initial body weight (g)) / dry protein intake (g)] ×100Survival rate % = (final number of fish/ initial number of fish) × 100

2.3 Hematological and biochemical measurement

Blood was collected from the caudal vein of three fishes from each tank by a non-heparinized syringe (3 ml) for hematological analysis. Afterward, partial whole blood was introduced into heparinized microtubes and used to measure red blood cell (RBC), white blood cell (WBC), hematocrit (Hct), hemoglobin (Hb), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and mean corpuscular volume (MCV) by the procedures described in Gharaei et al. (2020b) and Adel and Khara (2016). For biochemical analysis, blood sera were separated by centrifuging at 3000 rpm for 10 min (Gharaei et al., 2010). Catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX), and malondialdehyde (MDA) (n=6 from each group) in serum samples were measured with a commercial chemical calorimetric enzyme assay kit (ZellBio GmbH, Germany) by the procedures described in Gharaei et al. (2020b). The lysozyme activity in serum samples was assayed using the method of Ellis (1990) and Gharaei et al. (2020b). Alternative complement activity (ACH50) in serum samples was assessed using the protocol presented by Yano (1992).

2.4 Measurement of gene expression

To compare mRNA expression levels, the posterior parts of the intestinal tissues from each treatment group (N = 5) were randomly collected at the end of the trial, frozen, and kept at -80°C until use. Total RNA extraction was carried out in the intestinal samples by using the Takapou Zist Kit (Tehran, Iran) following the manufacturer's instructions. RNA integrity was verified by ethidium bromide staining of the 28S and 18S ribosomal RNA bands (as a marker) on 1.2% agarose gel. To remove DNA contaminants, the extracted RNA was treated with RNA-free DNase (Takara, Japan), and the reverse was transcribed to cDNA by a superscript cDNA synthesis kit (AccuPawer® CycleScript RT PreMix, Germany) following the manufacturer's instructions. The mRNA expression levels of CAT, SOD, GPX, TNF-α, IL-1β, and IL-10 genes in the intestinal of the rainbow trout were evaluated by fluorescent real-time quantitative PCR. The specific

primers for CAT, SOD, GPX, TNF-α, IL-1β, IL-10, and β-actin (housekeeping gene) were designed according to the cDNA sequences of rainbow trout in GenBank (Nootash et al. 2013; Hosseini et al. 2020) and thermocycling conditions as indicated in Table 3. All primers were synthesized by TakapouZist Co., Ltd. and amplified fragments length of 70–295 bp. Real-time quantitative PCR was conducted in a quantitative thermal cycler (Mastercycler® eprealplex, Eppendorf, Germany). Three replicates were performed for each sample. The threshold cycle (CT) was determined manually for each run. The PCR efficiency of each set of primers was determined using serial 10-fold dilutions of cDNA, and resulting plots of CT vs. the logarithmic cDNA dilution, using the efficiency equation (E):

$E = 10^{(-1/slope)}$

Gene expression data were analyzed using the $2^{-\Delta\Delta CT}$ method after it was verified that the primers were amplified with an efficiency of 97-99% (Gharaei et al. 2011). The data for all treatment groups were compared to the control group.

2.5 Bacterial challenge test

At the end of the experiment period, the survivals of fish in each treated group were challenged by the *Yersinia ruckeri* (BCCM/LMG3279) strain. This test was carried out as described by (Gharaei et al. 2020b). The mortality percentage of fish in each group was measured after a 15-day resistance test period and the relative percentage of survival (RPS) was determined by the following formula: RPS=100-[(test mortality/control mortality) Í100]

2.6 Histopathological analysis

On the last day of the trial, six randomly selected fish of each treated group were sampled. After anesthetized with 200 mg l^{-1} MS222 (Faggio et al. 2014), gill, liver, kidney, and intestine tissue samples with the size of 1×1×0.5 cm were preserved in 10% neutral buffered formalin for fixation. Afterward, the tissue preparation with the routine protocol (dehydration in ethanol, clearing in xylene, impregnation, and embedding in melted paraffin, sectioning at 5 µm with a rotary microtome, and staining with hematoxylin-eosin standard staining method) was performed for the histopathological investigation by light microscopy (Mohammadi et al. 2020).

2.7 Statistical analysis

Normality and homogeneity of the data were tested before performed parametric tests. The data (means ± SE) from each group were subjected to one-way analysis of variance (ANOVA), and Tukey's post hoc test was used to rank the groups in SPSS (version 18) if significant differences ($p < 0.05$) were detected. The effects of dietary Cu-NPs and VC levels, as well as their interactions, were tested by two-way ANOVA.

3. Results

Fluctuations of growth performance including WG, FCR, SGR, PER, and survival rate of juvenile common carp treated with different levels of dietary Cu-NPs or/and VC supplementation for 60 days are shown in Table 4. According to the experiments, WG, FCR, SGR, and PER were significantly affected only by Cu-NPs supplementation. In addition, the WG, SGR, and PER values were significantly higher ($p < 0.05$) in all treatment groups than in the control group (T1). The mortality rate in all treatment groups did not show any significant differences ($p < 0.05$). In addition, the FCR value was significantly ($p < 0.05$) decreased in all treatment groups compared with the control group (T1). Similarly, based on two-way ANOVA analysis, neither VC supplementation nor its interaction with Cu-NPs was effective on the FCR parameter and the other growth parameters (Table 4). The survival rate, which varied from 97.22% to 100%, was not significantly different among the groups ($p > 0.05$).

As shown in Table 5, the lysozyme activity and ACH50 values were not significantly affected by the VC supplementation and the interaction of Cu-NPs Í VC. On the other hand, only Cu-NPs was the significant factor for the lysozyme activity and ACH50 levels of the fish fed on the trial diets when compared with the control group (T1). However, the fish fed on the T4 and T5 diets showed significantly higher ($p < 0.05$) lysozyme activity value than those fed on the T1 diet. But, the ACH50 value in all treatment groups was significantly ($p < 0.05$) more pronounced than that in the control group (T1). The highest level of ACH50 was recorded in the fish fed on the T5 diet (Table 5).

The variations in the activity of antioxidative enzymes (SOD, CAT, and GPX) are presented in Table 6. The two-way ANOVA test revealed that VC supplementation and Cu-NPs Í VC were the significant factors ($p < 0.05$) for the SOD value, meanwhile the CAT and GPX levels were significantly ($p < 0.05$) affected only by Cu-NPs supplementation. According to the tests, the significant difference ($p < 0.05$) in the mean values of SOD gain among all treatment groups when compared with the control group (T1). In addition, the CAT and GPX contents were significantly increased in the fish fed on the T4 and T5 diets when compared with the control group (T1). Furthermore, the MDA value did not vary significantly ($p > 0.05$) with dietary treatments at the end of the trial.

After 60 days of treatment, there were significant differences in the hematological parameters as shown in Table 7. The Hb value was not significantly ($p < 0.05$) affected by Cu-NPs supplementation and the interaction of Cu-NPs Í VC, but the Hct and MCV values were significantly ($p < 0.05$) influenced only by the VC supplementation factor.

Cu-NPs had no effects on Hb, Hct, and MCV when the diet was not supplemented with VC. However, in the diets containing 250 and 500 mg VC and 2 mg Cu-NPs supplementation, significant increases ($p > 0.05$) were observed in blood Hb, Hct, and MCV. Meanwhile, the RBC, WBC, and MCHC values did not significantly ($p > 0.05$) vary with dietary treatments at the end of the trial.

The relative mRNA expressions of TNF-α, IL-1ß, IL-10, SOD, CAT, and GPX genes in the intestinal after 60 days are shown in Table 8. The analysis of variance demonstrated that the interactive effects of dietary

Cu-NPs and VC were significant on all target genes in the intestinal tissue. In the intestinal tissue of the fish fed on the T3, T4, and T5 diets, the expression of TNF-α, IL-1ß, IL-10, SOD, CAT, and GPX was significantly decreased versus the control group (Table 8).

Histopathological evaluation of the gills, kidneys, liver, and spleen revealed no damage in different tissues of fish treated with Cu-NPs and VCs (Figures 1-4).

The resistance of juvenile rainbow trout to the pathogen (*Y. ruckeri*) after treatment with different diets is depicted in Figure 5. The trend of survival percentage indicated that Cu-NPs/CV supplementations significantly enhanced ($p < 0.05$) the fish resistance against *Y. ruckeri* when compared with the control. In addition, 15 days post-challenge with this bacterium, highest relative percentage of survival was recorded in fish fed on the T5 diet (75%) and followed by those fed on the T4 diet (70%).

4. Discussion

Recent studies have demonstrated that minerals and vitamins such as Cu and VC are required for normal cell functioning, improvement of growth performance, and physiological and immunological process of aquatic species (El Basuini et al. 2016; Dawood and Koshio 2016; Chen et al. 2015; Ai et al. 2006; Watanabe et al. 1997). Also, it has been reported that nanominerals such as Cu nanoparticles have novel features such as high surface activity, larger specific surface area, high catalytic efficiency, high surface active centers, and stronger adsorption capacity, making them more capable of crossing biological barriers so that they are rapidly absorbed by cells and exhibit higher bioavailability than mineral salts (Gharaei et al. 2020a; Izquierdo et al. 2016; Rather et al. 2011).

The present study showed that growth indices including WG, FCR, SGR, and PER were significantly affected by Cu-NPs and VC supplementations, which is in parallel with previous studies according to which dietary Cu and CV supplementations improved growth indices, immune response, and antioxidant status in several species of fish (El Basuini et al. 2016, Mohseni et al. 2014; Tang et al. 2013; Faramarzi 2012; Sabatini et al. 2009; Wang et al. 2009). The growth-promoting effects of Cu-NPs may be explained by the fact that optimal dietary copper induces growth by improving metabolism, activities of the brush border, and preventing lipid peroxidation and protein oxidation in the hepatopancreas and intestine (Tang et al. 2013). Although it has been demonstrated that growth performance is significantly increased in grass carp (*Ctenopharyngodon idella*), beluga (*Huso huso*), red sea bream (*Pagrus major*), and freshwater prawn (*Macrobrachium rosenbergii*) under the effect of dietary Cu supplementation (Tang et al. 2013; Mohseni et al. 2014; El Basuini et al. 2016; Muralisankar et al. 2015). However, unlike this study, Gatlin and Wilson (1986) and Lorentzen et al. (1998) reported no significant effect of Cu supplementation on WG and feed efficiency of channel catfish (*Ictalurus punctatus*) and Atlantic salmon (*Salmo salar*), respectively. Therefore, the severity of the response to copper supplementation and its different impact on growth performance may be attributed to species, age, Cu dosage, and Cu chemical forms.

Crustaceans and fish species are limitedly capable of VC synthesis, so adding it to the diet of farmed species is crucial for improving growth performance and maintaining normal physiological functions

(Dawood and Kushio 2016; Chen et al. 2015). Improved growth performance through the nutrition of VC often appears as a result of the increased feed efficiency of the diet as proven by the previous studies on red seabream (*P. major*) (Dawood et al. 2016), Caspianrouch (*Rutilus rutilus caspicus*) (Roosta et al. 2014), common carp (*C. carpio*) (Liu et al. 2011), cobia (*Rachycentron canadum*) (Zhou et al. 2012), *Oreochromis spilurus* (Al-Armoudi et al. 1992), and Korean rockfish (*Sebastes schegelii*) (Kim and Kang 2015). In the present study, the growth performance reached a significantly higher level in the fish fed on the T5 diet (2 mg kg^{-1} Cu-NPs mixed with 500 mg kg^{-1} VC) compared with the control group. Adel and Khara (2016) presented that the highest WG and SGR and lowest FCR in rainbow trout fingerlings were observed in those fed on 250 mg kg^{-1} VC. Yousefı et al. (2013) evidenced that the growth performance of *Barbus sharpeyi* was improved by VC supplementation. Similarly, Faramarzi (2012) indicated that the growth performance of common carp was increased in the fish fed on dietary VC supplementation at a rate of 800–2000 mg kg^{-1} diet. Unlike this study, it was reported that dietary VC supplementation did not influence the growth performance in large yellow croaker (*Pseudosciaena crocea*) juveniles (Ai et al. 2006). However, our results in this study revealed that Cu-NPs combined with VC have a synergism effect on the growth indices and the physiological status of rainbow trout.

Lysozyme and ACH50 widely participate as humoral components in the innate defense system, so they are important for fish protection against diseases (Kaya et al. 2016). The antibacterial activity of the complement system, reported in various fish, has been suggested as one of the most important mechanisms of bacterial killing and clearing in fish, which can be activated by various immune stimuli (Srivastava & Pandey 2015). One of the triggers of complement activation is cytokines, some of which are effective in regulating proteins involved in iron metabolism, such as ceruloplasmin (Di Bella et al. 2017). In our study, lysozyme activity enzyme and ACH50 value were enhanced significantly ($p < 0.05$) in fish fed on the Cu-NPs and/or CV supplemented diet. This incremental fluctuation may be due to the immune suppressive effects of Cu-NPs (Kaya et al. 2016). This result coincides with the investigation of El Basuini et al. (2016) and Mohseni et al. (2014) who reported increasing lysozyme level in *P. major* and *H. huso* fed on dietary Cu-NPs and inorganic copper supplementations, respectively. In addition, Gharaei et al. (2020a) indicated that the lysozyme and ACH50 values were increased in *H. huso* fed on the dietary Chitosan-Zn NPs supplemented diet. Unfortunately, there is a lack of knowledge on the effect of nanoparticles on the ACH50 level in fish. However, it has been demonstrated that VC is a strong inducer of the immune system, especially non-specific immunity as it results in enhanced lysozyme activity level reported in various fish species including *P. major* (El Basuini et al. 2016); *O. mykiss* (Adel and Khara 2016); *Pseudosciaena crocea* (Ai et al. 2004, 2006); *Takifugu rubripes* (Eo and Lee 2008); *Scophthalmus maximus* (Lin and Shiau 2005), *Pangasianodon gigas* (Pimpimol et al. 2012). Qinghui et al. (2004) observed increased fish lysozyme and ACH50 values when the dietary VC supplementation was enhanced up to 489.0 mg kg^{-1}. Similarly, Chen et al. (2003) demonstrated that the ACH50 level in golden shiner (*Notemigonus crysoleucas*) was increased under the effects of dietary VC supplementation.

The antioxidant defense system is highly correlated with the health and safety of fish, and its major enzymes (SOD, CAT, GPX, and MDA) decompose reactive oxygen species (ROS) into a less reactive form (Sheikh Asadi et al. 2018; Dekani et al. 2018). The role of SOD is to stimulate the oxidation and reduction

of superoxide anions to hydrogen peroxides, which are then used as a substrate by the CAT and GPX enzymes (Saffari et al. 2016). As shown in Table 5, maximum activities of the SOD, CAT, and GPX enzyme are observed in the fish fed on the T5 (2 mg kg^{-1} Cu-NPs mixed with 500 mg kg^{-1} VC) diet compared with the control group. Previous studies have shown that Cu-containing diet, Cu/Zn-SOD enzyme activities in hepatocyte of rainbow trout (*Oncorhynchus mykiss*) (Osredkar and Sustar, 2011; Trenzado et al. 2009) and grass carp (*Ctenopharyngodon idella*) (Tang et al. 2013) and GPX increases in the plasma of goldfish (*Carassius auratus gibelio*) (Shao et al. 2010). In fact, copper is positively associated with the antioxidant defense system (Fang et al. 2013) and the effect of dietary copper on stopping oxidative damage may be related to the reaction with ROS such as anion superoxides and hydroxyl radicals (Tang et al. 2013). On the other hand, ceruloplasmin is a Cu-containing protein whose activity increases with appropriate levels of dietary Cu (Shaiu and Ning 2003). This Cu-containing protein is capable of stopping superoxide radical production (Valko et al. 2007) and hydroxyl radical formation (Zhang et al. 2013). One of the major antioxidant additives in the fish diet and food industry is VC, which alleviates oxidative stress (Dawood et al. 2016; Geo et al. 2013). VC plays an important role in scavenging free radicals (ROS and reactive nitrogen species) by acting as an early electron donor and reducing the agent (Dawood et al. 2016). Many previous studies have stated that dietary VC supplementation increases the SOD, CAT, and GPX activities in yellow catfish (*Pelteobarus fulvidaco*) (Liang et al. 2015), Siberian sturgeon (*Acipenser baerii*) (Xie et al. 2006), and black carp (*Mylopharyngodon piceus*) (Hu et al. 2013). Therefore, the results suggested that dietary Cu-NPs + VC supplementation are probably able to increase the antioxidant level and they have a synergistic interactive effect on inducing the antioxidant system. Our results showed no significant variations in the MDA value in all treatments. On the contrary, Jankowski et al. (2020) reported a reduction of MDA concentration under the effects of various forms of Cu (mineral and nanoparticle) in turkeys.

Hematological assessments can provide an indication of the physiological status of fish (Behera et al. 2013). In the present study, the Hb, Hct, and MCV values were increased more significantly in the fish fed on the T5 diet (2 mg kg^{-1} Cu-NPs mixed with 500 mg kg^{-1} VC) than the control fish, suggesting the positive effect of Cu-NPs + VC on physiological responses. The lower values of these hematological parameters in the control group indicate the necessity of adding Cu and VC to the diet to improve blood counts. The measured levels of blood variables in the normal range are for trout health, which confirms the effects of non-toxic Cu-NPs used under the present experimental conditions. It was confirmed that increased Hb indicates a stress response or increased hematopoiesis (Clauss et al. 2008). While the fish fed on Cu-NPs/VC were healthier than the control group, which was determined by the level of antioxidant, safety, and survival rates in the bacterial stress test. Thus, an increase in RBC, Hct, and Hb associated with hematopoiesis increased or decreased hemolysis (Hosseini et al. 2018). This may be due to the role of Cu as a combination of many enzymes and glycoproteins that aid in the synthesis of hemoglobin (Nordberg et al. 2015; Dawood et al. 2020). Ceruloplasmin (a liver-derived protein) is required to release iron and transfer it from cells and tissues to plasma. There are several copper molecules in the structure of this protein, and its synthesis in the liver requires the presence of copper. In fact, copper deficiency impairs the ability of iron absorption or release from tissues for hemoglobin synthesis (Haver and Hardy 2008). The same results were recorded by Adel and Khara (2016) and Zhou et al. (2012) for pirarucu (*Arapaima gas*) and cobia (*R. canadum*), respectively.

To investigate the effects of Cu-NPs and VC on inflammatory and antioxidant responses, we measured the expression of several gene biomarkers including three pro-inflammatory cytokines (TNF-α, IL-10, and IL-1ß) and three antioxidant systems (CAT, SOD, and GPX). The results of the present study showed that the expression levels of TNF-α, IL-10, and IL-1ß genes were decreased in the intestine of the fish in the T3, T4, and T5 treatment groups. TNF-α (tumor necrosis factor) is known as a multifunctional cytokine that plays a key role in cell-mediated inflammatory immunity responses (Lykouras et al. 2008; Mocellin et al. 2015). IL-1ß acts as a mediator of the inflammatory response and helps reduce inflammatory pain sensitivity in various cellular activities, including cell proliferation and apoptosis by inducing cyclooxygenase-2 (PTGS2 /COK2) in the central nervous system. TNF-α and IL-1ß are considered important indicators of phagocytic activity and they are the first cytokines produced in the early stages of inflammation in fish (Skadberg et al. 2015). IL-10 is known as a cytokine synthesis inhibitory factor that minimizes damage to target cells by suppressing the transcription of pro-inflammatory cytokine (Shafiei-Jahani et al. 2020). The significant reduction in the expression of pro-inflammatory cytokine genes in fish fed on the diets containing Cu-NPs/VC supplements can be interpreted as their significantly down-regulated synergistic effect on the immune response, which was also dose-dependent. IL-10 has also been reported to be capable of degrading pro-inflammatory cytokine mRNA, reducing TNF-α receptor expression, and regulating macrophage-derived TNF-α and IL-1 secretion (Opal et al. 1998; Opal and DePalo 2000). Suska et al. (2003) reported that cellular Cu sites induce the secretion of TNF-α and IL-1ß by inflammatory cells *ex vivo* and *in vivo*. On the other hand, TNF-α has been shown to increase phagocytosis of neutrophils under apoptosis. Thus, Cu-NPs may reduce the production of pro-inflammatory cytokine because Cu ion is closely related to RNA and DNA. Antioxidant vitamins, including VC, can increase immune function by increasing the proliferation of lymphocytes and macrophages (Jang et al. 2014). Changes in gene expression reported in this study suggest a low inflammatory potential of the Cu-NPs / VCs tested. In this regard and consistent with our results, Yun et al. (2012) and Jang et al. (2014) reported that dietary supplemental VC significantly reduced TNF-α, IL-1ß, and IL-6 mRNA levels in mice and broiler chick, respectively.

Despite the various benefits of copper nanoparticles in aquatic organisms, their toxic effects have also been reported in some cases. In *Epinephelus coioides*, adverse effects on gut, gill, and liver (Wang et al. 2015), *Cyprinus carpio* caused a sharp decrease in alkaline phosphatase and increased T4 and free T4 in blood plasma (Hoseini et al. 2016), *Oncorhynchus mykiss* reduced hematocrit percentage and the amount of potassium and sodium in the blood plasma (Shaw et al. 2012). Nowadays, the interaction between transition metals, e.g., Cu, with VC is well known (Akbıyık et al. 2012) as the rate of VC oxidation stability increases with the fixed concentration of Cu and prevents catalytic oxidation of VC in the presence of a stable Cu complex.

Various studies have shown that dietary VC can produce antioxidants (Biller et al. 2018). VC bonds to ROS in the body and retrieves free radicals through H^+ donation. VC has also been shown to act as a reducing agent, primarily by reducing the transport of metals such as Cu and Fe ions, which react with H_2O_2 to form hydroxyl radicals (Babior 1997).

Despite reports of inducing increased expression of the SOD, CAT, and GPX genes due to the toxicity of Cu-NPs in aquatic organisms (Ramya et al. 2016; Muralisankar et al. 2019; Dawood et al. 2020). The results of this experiment suggested that the Cu-NP level of 2 mg kg^{-1} had no adverse effect on fish and, when applied with VC, had the greatest effect on ROS production. SOD and CAT are related to stress management (Li et al. 2010) and are used as important indicators in the early detection of oxidative pollution. Decreased expression of the SOD, CAT, and GPX genes in fish exposed to Cu-NPs/VC could indicate oxidant eradication. The results of Nile tilapia (*Oreochromis niloticus*) exposed to ZnO-NPs and vitamins C and E (Abdelazim et al. 2018), *O. niloticus* exposed to Ag-NPs (Afifi et al. 2013), and *Carassius auratus* exposed to a mixture of Cu-NPs and ZnO-NPs and cerium oxide-NPs and pure NP (Xia et al. 2013) are in agreement with our findings. It is confirm that VC can destroy the superoxide anion by forming radical semidehydra ascorbate (Abdelazim et al. 2018). The results of our experiment showed significant neutralization in the antioxidant system so that the fish fed on a mixture of Cu-NPs and VC exhibited the lowest expression level of the SOD, CAT, and GPX genes. This ability of VCs to combat possible oxidative damage was caused by exposure to Cu-NPs. Othman et al. (2017) explained that they used VC to prepare ligands for cerium oxide nanoparticles as a tool to facilitate the detection of NP in tissues because they suggested that VC could bind tightly to NPs and showed its performance. It has also been reported that the presence of NPs themselves can increase the VC activity (Astete et al. 2011).

The maximum resistance to *Y. ruckeri* and survival rate in this study were recorded in the fish fed on the T5 diet. Many previous studies have shown that various dietary additives have increased the survival rate and resistance to the pathogen in rainbow trout (Gharaei et al. 2020b; Yilmaz et al. 2018; Aghamirkarimi et al. 2017). The significant enhancement in survival rate in this study may be related to the induction of non-specific immune defenses and antioxidant system by synergistic interaction of Cu-NPs and VC.

In the present study, histopathological alterations were not observed in gill, intestine, liver, and kidney tissues affected by Cu-NPs and Cu-NPs + VC. According to the previous studies, the gill, liver, and kidney are the most sensitive organs to Cu-NP exposure and respond by showing various degrees of necrosis and tissue damage (Ostaszewska et al. 2018). Gills are an important organ with several functions like respiratory osmoregulation and respiratory gas exchange, acid-base balance, and excretion of metabolites. Thus, they are the primary target for a high concentration of Cu-NPs (Ostaszewska et al. 2018).

The intestine is the site of absorption of a huge portion of the nutrients and non-nutrients digested. It has been shown that Cu-NPs are well oxidized to ionic forms in acidic environments and have high adsorption capacity (Pirarat et al. 2011). On the other hand, the height of intestinal villi is an important indicator of the efficiency of digestion and absorption in the gut (Ringoe et al. 2003). Rathore et al. (2019) has reported that dietary VC increases the height of villi in the gut of tilapia (*Oreochromis niloticus*). The combination of Cu-NPs and vitamin C is likely to increase the efficiency of nutrient absorption in the gut by providing a larger surface area as well as an acidic environment of the gut, which strengthens the immune system against the pathogens through the viscous mucin layers that cover the receptors for infectious agents (present on the intestinal mucosa) (Johari et al. 2015).

5. Conclusion

In conclusion, our study identified some changes in growth, blood, biochemical, immune, and histopathology parameters of rainbow trout juveniles under the influence of dietary Cu-NPs and/or CV supplementations. Based on the data obtained, a diet containing 2 mg Cu-NPs combined with 500 mg VC per kg food can improve the growth indices including WG. FCR, SGR, PER, and blood indices including Hb, Hct, MCV, and biochemical and immunological indices including SOD, CAT, GPX, ACH50, and lysozyme. It appears that the synergistic effect of Cu-NPs and VC improves feed utilization, metabolism efficiency, and intestine tissue structure and enhances the antioxidant capacity and immune system in rainbow trout. However, additional studies are required to evaluate the other effects of these supplements on the immune responses and blood parameter variations and why some of the results differ from those of the previous studies. This research indicated that a combination of Cu-NPs and CV could be used in the rainbow diet as a growth promoter as it can improve the physiological conditions and resistance against pathogens.

Declarations

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-Conflicts of interest

The authors declare that they have no competing interests.

-Ethics approval

All procedures were carried out in accordance with the Animal Care and Use Committee guidelines at the Faculty of Sciences of the University of Zabol.

-Consent to participate (include appropriate statements)

The authors agree to collaborate and publish this article.

-Consent for publication (include appropriate statements)

We will transfer the copyright of the article to editorial office for publishing.

-Availability of data and material/ Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

-Code availability

Not applicable

-Authors' contributions (include all authors)

Delavari M.: MSc student; these data are coming from his thesis.

Gharaei A.: Asssociate professor in the Deptartment of Fisheries and the main supervisor for the student. He provided all the scientific suggestions and grants.

Mirdar Harijani J.: He is the second supervisor and provided scientific suggestions.

Davari A.: She was the advisor for the work and provided scientific comments.

Rastiannasab A.: He was the advisor for the scientific group.

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Tables

Table 1

The formulation of the basal diet

Ingredients	g kg^{-1} dry diet
Fish meal	620
Meat powder	60
Soybean meal	20
Wheat flour	80
Canola oil	40
Fish oil	40
Lecithin	30
Vitamin permix[1] (VC free)	30
Mineral premix[2](Cu free)	29.96
-Cellulose + (VC and/or Cu-NPs)α	90.04
[1]Composition of vitamin premix (IU, g or mg/kg): A (3,600,000 IU), D3 (8,000,000 IU), E (14.4 g), K3 (800 mg), B1 (7 g), B2 (2.64 g), Niacin (11.8 g), Calcium pantothenate (3.92 g), B6 (1.17 g), B9 (0.4 g), Biotin (40 mg), Choline chloride (100,000 mg). Aras Bazar Pharmaceutical Co., Mazandaran, Iran. [2]Composition of mineral premix (g/kg): Mn (39.68 g), Fe (20 g), Zn (33.88 g), Co (4 g), I (0.39 g), Se (0.08 g). Aras Bazar Pharmaceutical Co., Mazandaran, Iran.	

Table 2

The chemical analysis of the trial diets

Proximate composition (dry matter basis)						
Trial diets(Cu-NPs/VC mg kg^{-1})	Analyzed copper (mgkg^{-1})	Analyzed VC (mg kg^{-1})	Crud lipid (g kg^{-1})	Ash(g kg^{-1})	Crud protein (g kg^{-1})	Gross energy(kj g^{-1})
T1 (0/0)	1.59	ND	223	101.2	417.5	22.21
T2 (0/250)	1.60	244	226	101.4	413.2	22.33
T3 (0/500)	1.57	491	226.2	102.5	413.3	22.19
T4 (2/250)	3.51	241	230.1	102.3	418.1	22.40
T5 (2/500)	3.48	489	227.8	100.5	415.5	22.31
T6 (2/0)	3.52	ND	222	103.1	410.6	22.37
ND, not detected						

Table 3

The real-time PCR primer sequences and thermocycling condition

Accession no.	Thermocycling condition	Primer sequence (5´— 3´)	Primer	Genes
NM_001160614	95 ◦C 30 s, 35 cycles of 95 ◦C 5 s, 60.4◦C 30 s and 72 ◦C 30 s	GTAGTCGTGGCTCAATGGTAAG	F	SOD
		GCTTTATATTCTGCGGGTCATT	R	
110,490,868	95 ◦C 30 s, 35 cycles of 95 ◦C 5 s, 54.5◦C 30 s and 72 ◦C 30 s	TTGAGGTGACACATGACATCTCT	F	CAT
		ACGGTGGAGAAGCGAATGG	R	
AF281338	95 ◦C 30 s, 35 cycles of 95 ◦C 5 s, 60 ◦C 30 s and 72 ◦C 30 s	AAATTGCCATTCCCCTCCGA	F	GPX
		TCCATCAGGACTGACCAGGA	R	
AJ223954 AJ298294	95 °C 30 s, 35 cycles of 95 °C 5 s, 62 °C 30 s and 72 °C 30 s	ACATTGCCAACCTCATCATCG	F	IL-1β
		TTGAGCAGGTCCTTGTCCTTG	R	
AB118099	95 °C 30 s, 35 cycles of 95 °C 5 s, 60 °C 30 s and 72 °C 30 s	CGACTTTAAATCTCCCATCGAC	F	IL-10
		GCATTGGACGATCTCTTTCTTC	R	
AJ249755.1	95 °C 30 s, 35 cycles of 95 °C 5 s, 60 °C 30 s and 72 °C 30 s	TGGAGGGGTATGCGATGACACCTG	F	TNF-α
		TGAGGCCTTTCTCTCAGCGACAGC	R	
AC006483.3	95 °C 30 s, 35 cycles of 95 °C 5 s, 60 °C 30 s and 72 °C 30 s	TCACCCACACTGTGCCCATCTACGA	F	β-Actin
		CAGCGGAACCGCTCATTGCCAATGG	R	

Table 4

The growth performance and nutrient digestibility of different experimental groups in the rainbow trout for 60 days (Means ± SE, n = 3). Means marked by different letters are significantly different ($p<0.05$).

Parameters					
Trial diets(Cu-NPs/VC mg kg^{-1})	WG%	FCR	SGR%	PER%	SR%
T1 (0/0)	159.24 ± 7.83^{a}	1.88 ± 0.06^{a}	1.58 ± 0.07^{a}	120.52 ± 13.43^{a}	100±0.00
T2 (0/250)	182.04 ± 10.09^{ab}	1.66 ± 0.1^{ab}	1.72 ± 0.05^{ab}	144.61 ± 10.40^{ab}	100±0.00
T3 (0/500)	201.50 ± 9.71^{bc}	1.41 ± 0.03^{bc}	1.83 ± 0.03^{bc}	167.46 ± 6.39^{bc}	100±0.00
T4 (2/250)	247.96 ± 8.53^{d}	1.24 ± 0.03^{d}	2.07 ± 0.08^{d}	191.54 ± 10.86^{c}	100±0.00
T5 (2/500)	236.56 ± 10.32^{cd}	1.26 ± 0.07^{d}	2.01 ± 0.1^{cd}	188.69 ± 15.76^{c}	100±0.00
T6 (2/0)	214.63 ± 11.21^{bcd}	1.35 ± 0.02^{bc}	1.90 ± 0.03^{bcd}	175.95 ± 11.61^{c}	97.22±0.33
Two-way ANOVA (p-value), NS: not significant					
Cu-NPs	$p < 0.05$	$p< 0.05$	$p< 0.05$	$p< 0.05$	NS
VC	NS	NS	NS	NS	NS
Cu-NPs Í VC	NS	NS	NS	NS	NS

Table 5

The changes in immunological parameters in the juvenile rainbow trout at different experimental groups for 60 days (Means ± SE, n = 3). Means marked by different letters are significantly different ($p < 0.05$).

Parameters	Treatment groups (Cu-NPs/VC mg kg^{-1})					
	T1 (0/0)	T2 (0/250)	T3 (0/500)	T4 (2/250)	T5 (2/500)	T6 (2/0)
Lysozyme (U ml^{-1})	50.2 ± 2.1[a]	56.7 ± 2.5[a]	57.1 ± 3.7[a]	85.0 ± 4.5[b]	100.6 ± 5.9[c]	58.4 ± 2.6[a]
ACH50(U ml^{-1})	125.2 ± 1.3[a]	137.9 ± 3.2[b]	140.3 ± 2.3[b]	153.4 ± 4.1[c]	159.1 ± 3.9[c]	134.9 ± 3.8[b]
Two-way ANOVA (p-value), NS: not significant						
Lysozyme		ACH50		MDA		
Cu-NPs	P < 0.05	P < 0.05		NS		
VC	NS	NS		NS		
Cu-NPs Í VC	NS	NS		NS		

Table 6

The changes in antioxidant capacity parameters in the juvenile rainbow trout at different experimental groups for 60 days (Means ± SE, n = 3). Means marked by different letters are significantly different (p < 0.05).

Parameters	Treatment groups (Cu-NPs/VC mg kg^{-1})					
	T1 (0/0)	T2 (0/250)	T3 (0/500)	T4 (2/250)	T5 (2/500)	T6 (2/0)
SOD(U ml^{-1})	65.33 ± 2.28^{a}	62.21 ± 3.20^{ab}	53.63 ± 2.91^{b}	45.81 ± 3.62^{c}	42.13 ± 1.97^{c}	59.11 ± 2.81^{ab}
CAT(U ml^{-1})	145.67 ± 12.8^{a}	141.0 ± 9.53^{a}	120.66 ± 8.96^{a}	112.0 ± 11.26^{b}	113.5 ± 7.47^{b}	146.3 ± 6.50^{a}
GPX (U ml^{-1})	680.52 ± 17.3^{a}	692.66 ± 18.1^{a}	601.5 ± 14.0^{ab}	573.30 ± 21.2^{b}	559.4 ± 23.4^{b}	673.71 ± 25.7^{a}
MDA (µmol ml^{-1})	57 ± 6.1	56.4 ± 4.9	58.3 ± 5.03	56.6 ± 4.7	55.6 ± 5.15	58.3 ± 4.2
Two-way ANOVA (p-value), NS: not significant						
SOD			CAT		GPX	
Cu-NPs	NS		P < 0.05		P < 0.05	
VC	P < 0.05		NS		NS	
Cu-NPs Í VC	P < 0.05		NS		NS	

Table 7

The hematological parameters of the rainbow trout juveniles in different experimental groups for 60 days (Means ± SE, n = 3). Means marked by different letters are significantly different (p<0.05)

Parameters							
Trial diets (Cu-NPs/VC mg kg^{-1})	RBC ($í10^6$ mm^{-3})	WBC ($í10^3$ mm^{-3})	Hb (g dl^{-1})	Hct (%)	MCV (fl)	MCH (pg)	MCHC (g dl^{-1})
T1 (0/0)	1.03 ± 0.31	52.3 ± 5.7	5.55 ± 0.2^a	38.6 ± 2.1^{ab}	357.4 ± 5.8^b	53.8 ± 8.1	14.4 ± 0.4
T2 (0/250)	1.1 ± 0.12	51.1 ± 6.8	5.6 ± 0.51^a	40.6 ± 6.6^{ab}	369.1 ± 13.7^b	50.9 ± 7.3	13.8 ± 1.5
T3 (0/500)	0.98 ± 0.15	52.4 ± 5.9	5.6 ± 0.36^a	35.6 ± 4.3^a	363.2± 14.2^b	57.1 ± 9.2	15.7 ± 1.2
T4 (2/250)	1.1 ± 0.28	49.7 ± 4.7	6.2 ± 0.15^b	42.0 ± 1.2^a	381.8 ± 21.1^a	56.4 ± 4.5	14.8 ± 0.8
T5 (2/500)	1.12 ± 0.16	50.3 ± 4.9	6.4 ± 0.4^b	43.6 ± 3.0^a	389.3 ± 25.5^a	57.1 ± 6.8	14.7 ± 1.5
T6 (2/0)	1.11 ± 0.10	49.6 ± 5.3	5.75 ± 0.3^a	41.0 ± 4.5^{ab}	369.4 ± 12.6^b	51.8 ± 6.3	14.0 ± 0.7
Two-way ANOVA (p-value), NS: not significant							
Cu-NPs	NS	NS	NS	$P < 0.05$	$P < 0.05$	NS	NS
VC	NS	NS	$p < 0.05$	NS	NS	NS	NS
Cu-NPs Í VC	NS	NS	$p < 0.05$	NS	NS	NS	NS

Table 8

The relative mRNA levels of the rainbow trout intestine at different experimental groups for 60 days (Means ± SE, n = 5). Means marked by different letters are significantly different ($p < 0.05$).

Trial diets (Cu-NPs/VC mg kg^{-1})						Genes
T6 (2/0)	T5 (2/500)	T4 (2/250)	T3 (0/500)	T2 (0/250)	T1 (0/0)	
$1.67±0.23^{a}$	$0.84±0.06^{c}$	$1.07±0.14^{b}$	$1.15±0.08^{b}$	$1.52±0.12^{a}$	$1.41±0.11^{a}$	TNF-α
$1.81±0.37^{a}$	$1.22±0.20^{c}$	$1.55±0.19^{b}$	$1.63±0.15^{b}$	$1.87±0.29^{a}$	$1.96±0.22^{a}$	IL-1β
$1.97±0.38^{a}$	$1.1±0.09^{b}$	$1.24±0.85^{b}$	$1.72±0.54^{ab}$	$1.92±0.41^{a}$	$2.07±0.23^{a}$	IL-10
$1.41±0.22^{a}$	$0.82±0.16^{b}$	$0.94±0.10^{b}$	$1.19±0.18^{b}$	$1.58±0.11^{a}$	$1.45±0.17^{a}$	SOD
$1.49±0.17^{ab}$	$1.12±0.19^{b}$	$1.31±0.17^{b}$	$1.28±0.07^{b}$	$1.68±0.22^{a}$	$1.91±0.28^{a}$	CAT
$2.05±0.33^{a}$	$1.07±0.21^{b}$	$1.19±0.18^{b}$	$1.75±0.21^{ab}$	$2.15±0.36^{a}$	$2.11±0.72^{a}$	GPX

Figures

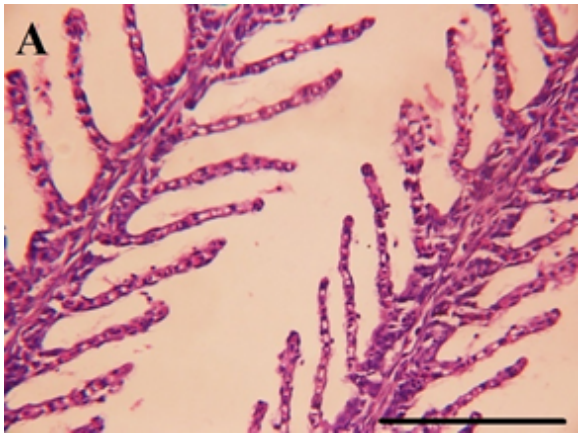

Figure 1

Tissue sections of gills in rainbow trout. (A). Control group (H&E, scale bar= 185 μm).

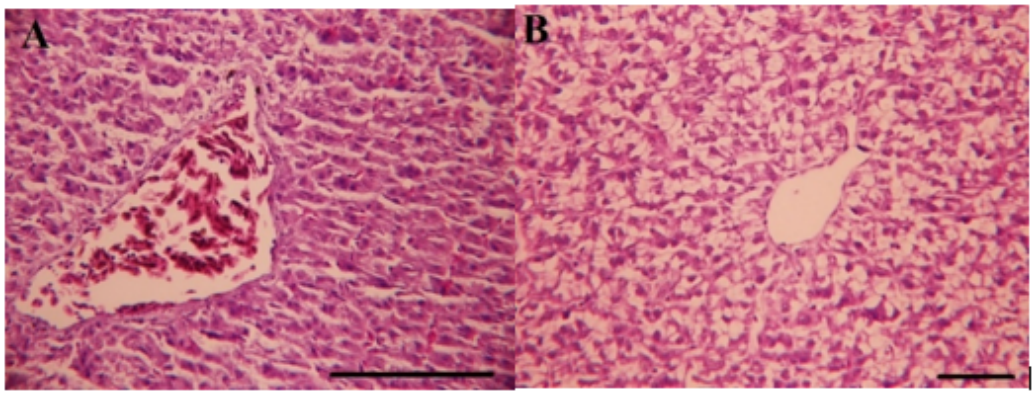

Figure 2

Tissue sections of the liver in rainbow trout. (A): Control group (H&E, scale bar= 150 µm). (B):(H&E, scale bar= 50 µm).

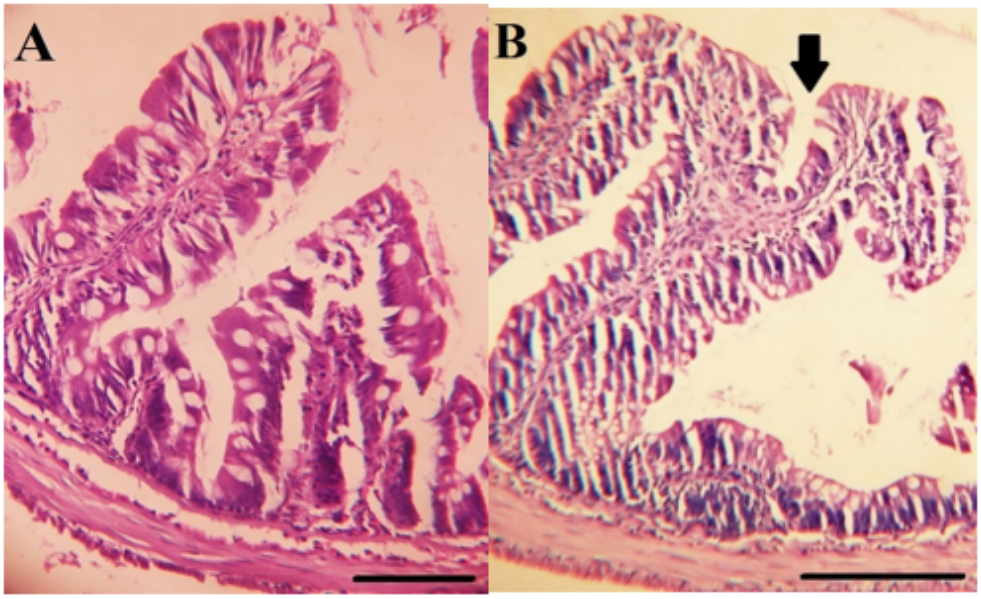

Figure 3

Tissue sections of the intestine in rainbow trout. (A): (H&E, scale bar= 140 µm). (B): (H&E, scale bar= 100 µm).

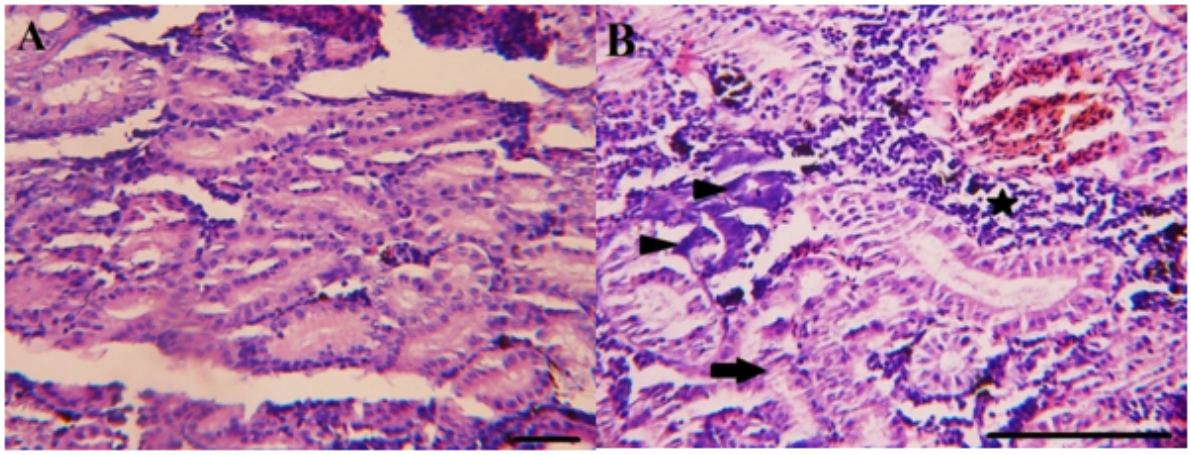

Figure 4

Tissue sections of the kidneys in rainbow trout. (A): (H&E, scale bar= 100 µm). (B): (arrowheads) (H&E, scale bar= 125 µm).

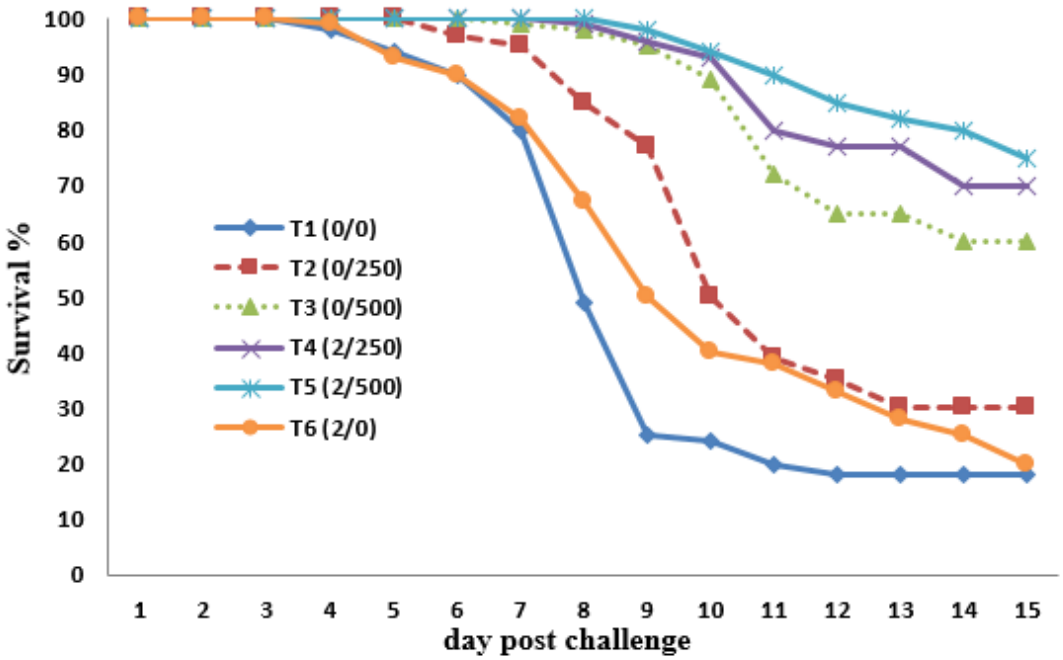

Figure 5

The survival rate of rainbow trout fed on different doses of dietary Cu-NPs and/or VC (Cu-NPs/VC mg kg-1) supplementation during 15-day post-challenge with Yersinia ruckeri.