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Original Article

Effect of dietary nanosilver on gut proteases and general performance in Zebrafish (*Danio rerio*)

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Abstract: The present study was conducted to evaluate the effect of dietary inclusion of silver nanoparticle on general performance and digestive proteases in zebra fish. Four experimental diets were designed with a concentration of 5, 20 and 40 parts per billion (ppb) nanoparticles in the diet (named S5, S20, and S40, respectively) and control group without any nanoparticles (S0). There was no significant difference was observed in FI and FCR between the dietary treatments. At the end of the experiment, S20 exhibited highest WG and SGR followed by S40 compared with other treatments while, there was no significant difference observed between S0 and S5. Similar trends were also observed in total protease enzyme activity. To evaluate the protease enzyme patterns on gut extract, substrate SDS-PAGE was performed and the inhibition of zymogram was studied. The results showed that there was no difference in banding patterns between S0 and S5 with EDTA treated samples whereas two extra bands of molecular weight (MW) 67 and 37 appeared in S20 and S40, were inhibited by EDTA indicating the presence of metalloprotease in those dietary regimes. There were no differences in the banding patterns of PMSF treated samples suggesting that the total serine protease remains unaffected by the dietary regimes. To conclude, we found 20 ppb inclusion of silver nanoparticle in fish diet improves general performance and induces metalloprotease activity in fish. Further detailed study is required before establishing dietary inclusion of silver nanoparticle for industrial purposes.

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Introduction

Over the years, technological applications in aquaculture have been associated with intensification of the applied systems for increased production with economic profitability. Besides high density culture systems, efforts are also being made to achieve high-growth performances and early weaning by shortening productive cycles. Especially in a situation of juvenile stocking, the animals are often subjected to focus all their physiological resources for high performances thereby making them more sensitive to infection due to different pathological and environmental factors. The uses of antibiotics as a growth promoter in basal diets are in practice, particularly in case of monogastric animals (Cromwell, 1991). However, continuous use of antibiotics leads to its retention in animal tissues, which may provoke antibiotic resistance both in animals and consumers thereby raising questions for food security. In this scenario, dietary inclusion of trace elements and nanoparticles is an ideal option, where levels of inclusion must be enough to satisfy metabolic needs and most importantly must be within the permissible limits of tissue retention and environmental hazards.

Nanoparticles received considerable attention in the recent years because of their ability to deliver a wide range of molecules to the body and for a sustained period of time. So far, several nanoparticle-based therapeutic and diagnostic agents have been

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developed (Chakraborty et al., 2013). Historically, silver compounds are in practice for controlling microbial proliferation. The modern-day applications of silver compounds are mostly for medical (antimicrobial agent in wound dressing, catheters, etc.) and as domestic appliances. Silver nitrate is unstable and can be toxic to tissues (Atiyeh et al., 2008) whereas nanosilver is a submicronic and colloidal form of metallic silver (1-100 nm size) which has presently got wide commercial attention (Rai et al., 2009) due to its pronounced impact than bulk silver metal as antimicrobials (Mohanty et al., 2012; Sarkar, 2010). It is more stable to hydrochloric acid in the gut and absorbed at a much lower extent by cells. Therefore, it is minimally toxic and exhibits a higher antimicrobial effect (Choi et al., 2008). One of the studies shows silver nanoparticles is more effective against Gram-positive and Gram-negative pathogens as compared with acid-fast bacteria. Furthermore, silver nanoparticles are not cytotoxic to macrophages at the bactericidal concentration and can augment intracellular killing potential of macrophages (Mohanty et al., 2012).

Despite its potential effect on digestive microbial biodiversity and function, roles of nanosilver in relation with growth, immunological status, digestive enzyme activity, metalloproteinase regulation and intestinal structure of farmed animals are already published (Wright et al., 2002). Silver nanoparticle can cross the cell membranes and penetrate into the tissues to perform biological activities. However, this depends upon pathological and immunological status of the subject, size, structure and purity of the particle, dose and the method of delivery. Some authors suggest that silver promotes an increase of zinc and copper concentration over epithelial tissue, thus indirectly stimulating its positive effects for metabolism (Lansdown, 2002).

There is very limited information on dietary application of silver nanoparticles in fish feed. Therefore, this is a timely approach to evaluate the encouraging effects of dietary nanoparticles within nontoxic levels of administration. Aim of this study was to verify the effects of the nanosilver in the feed for zebra fish juveniles. The main purpose was to evaluate the general digestive performances during the fast grow-out phase of the fish to provide basic information for use of nanosilver for nutritional research.

In an ecotoxicological assessment study, 75% survival rate was observed for zebra fish even at dietary inclusion of 0.25 ppm (parts per million) nanosilver for 15 days (Perello, 2013). Even if there are no reports by now; however, there might be a risk of bio-magnification in such concentration and further research is required to verify. In this scenario, dietary concentrations in ppb (part per billion) can be considered as nontoxic level and may retain beneficial effects of nanoparticles. In one of the pathogenecity tests conducted at our laboratory, we observed that the zone of inhibition against Gram-negative *Aeromonas hydrophila* were evident from 5 ppb nanosilver concentration (unpublished data). For the present study, we have considered 5 ppb to 40 ppb of dietary inclusions of nanosilver in the diet.

Zebra fish (*Danio rerio*) is selected for our current study as it not only mimics other aquaculture varieties, but also a preclinical model for many biological studies (Hsu et al., 2007; Chakaraborty et al., 2009, 2011; Chakraborty and Agoramoorthy 2010). In addition, genetics and molecular basis of zebra fish have been extensively mapped (Hill et al., 2005). Hence, any experimental trends, including nutritional exposition exhibited by this fish can be applied to other aquaculture species as well as the vertebrate regime. This research provides basic information about digestive performances of dietary nanosilver as a trace element in the diet.

Materials and Methods

The experiment was conducted at the fish rearing facility of KIIT School of Biotechnology, Bhubaneswar, during autumn 2012. Zebra fish juveniles (40 days after hatching, length 10.5 ± 1.6 mm) were obtained from local fish supplier, M/sAquatech, Bhubaneswar. Fish were kept in 500 L FRP (Fiberglass Reinforced Plastic) tank for two

Table 1. Water quality parameters during experimental period.

Water quality parameters	0 days	15 days				30 days			
		S0	S5	S20	S40	S0	S5	S20	S40
pH	7.2 ± 0.3	7.3±0.03	7.2±0.01	7.3±0.06	7.1±0.02	7.1±0.3	7.1±0.4	7.1±0.4	7.2±0.5
Ammonia (ppm)	0.19 ± 0.04	0.14±0.03	0.20±0.13	0.25±0.03	0.31±0.04	0.22±0.02	0.26±0.03	0.28±0.07	0.23±0.03
D.O. (ppm)	5.8±0.2	6.4±0.6	6.5±0.34	6.8±0.27	6.6±0.19	5.8±0.22	6.3±0.37	6.6±0.29	6.4±0.5
CO_2 (ppm)	0.15±0.02	0.14±0.04	0.12±0.03	0.09±0.02	0.22±0.03	0.13±0.03	0.10±0.04	0.12±0.01	0.08±0.04
Nitrate (ppm)	0.06±0.01	0.07±0.01	0.14±0.04	0.20±0.02	0.16±0.03	0.09±0.01	0.13±0.04	0.11±0.03	0.16±0.03
Alkalinity (ppm)	60±2.2	73±5	65±6	81±7	72±6	91±6.84	72±6.84	80±6.84	79±6.84

Table 2. Ingredients and crude nutrient composition of the experimental diets.

Ingredients (g/kg)	S0	S5	S20	S40
Wheat flour	36	36	36	36
Fishmeal	35	35	35	35
Corn gluten	15	15	15	15
Mustard oil	8	8	8	8
Cod liver oil[a]	2	2	2	2
Vitamin premix[b]	2	2	2	2
Mineral premix[c]	2	2	2	2
5 ml nanosilver solution adjusted to µg/kg	0	5	20	40
Nutritional Analysis*				
Crude Protein	41.2	40.3	40.1	40.1
Total lipid	8.4	7.8	8.2	8.6
Ash	14.5	14.2	14.2	14.4

weeks acclimatization before transferring to experimental tanks fitted with flow through fresh water exchange. After acclimatization, fish were randomly stocked at a density of 100 juveniles in 100 L polyfibre aquaria. Three replicates were maintained for each feeding regime with 12-hour light: 12-hour dark photoperiod. The fish were reared in static water tanks with 100% water exchange on every third day. Throughout the experiment, the rearing water was constantly supplied with oxygen by air stone diffusers to maintain the dissolved oxygen above 6 mg/L. Major water quality parameters were monitored throughout the experiment, and all ethical protocol were followed during the entire experiment (Table 1).

Organically coated silver nanoparticle was procured from Sigma Aldrich, Kolkata, India. The size and shape of the silver nanoparticle (90 nM) were confirmed in Scanning Electron Microscopy (JEM 2100, JEOL, Japan) operating at 300 kV (Fig. 1). The nanoparticle was suspended in ultrapure water (Milli Q) and its concentration was adjusted to 5, 20 and 40 µg/kg in three volumes of 5 ml. An artificial fishmeal based diet (40% protein) was formulated from wheat flour, mustard oil cake, vitamin mixes as per previous studies of Rathore et al. (2005). The detailed composition of diets is presented in Table 2. The pre-weighed dry ingredients were carefully blended using a laboratory food mixer. The mixtures were the primed with water to yield a suitable mash. Moist diets were made into 500 µM pellet size and dried at 40°C in a fan assisted drying cabinet. This diet was treated as control (S0) as three experimental sets were formulated by adding 5 ml of silver nanoparticle in three different proportions of 5 ppb (S5), 20 ppb (S20) and 40 ppb (S40), respectively. The fish were feed 4% of body weight twice a day until visible apparent satiation. Leftover feed in the tank were removed by siphoning, and their dry weight was recorded. The difference between initial

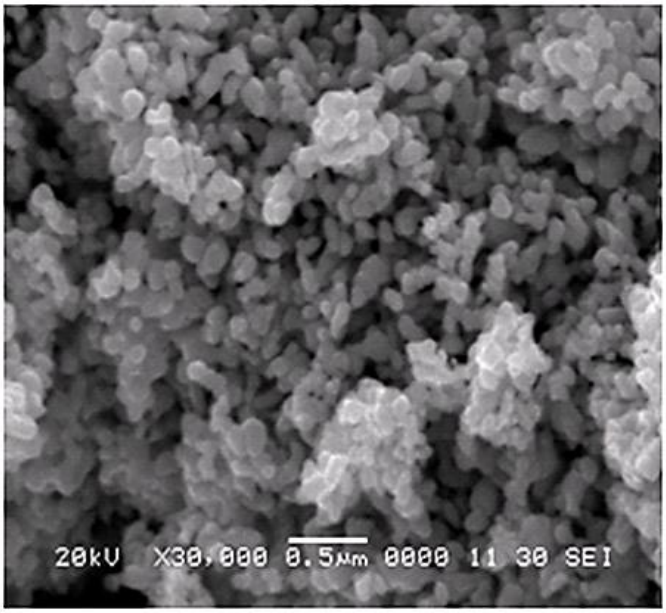

Figure 1. SEM micrograph of silver nanoparticle used in the study.

administered diet and the unfed diet were used to calculate the feed intake. Ten fish were collected randomly from each tank at weekly intervals to estimate the food requirement of the growing juvenile.

As general growth performances, the length and weight were measured every week and also for the final sampling at 30 days. Fish were collected at 9 A.M., washed properly through a sieve and were immediately frozen at -20°C. The digestive systems of individual fish were removed using a glass plate maintained at 0°C under a dissecting microscope. Dissected digestive tracts are pooled (100 mg) and washed in PBS (Phosphate buffer saline pH 7.4). Protein lysate was prepared in RIPA buffer at 4°C. The lysate was centrifuged 15 min at 8000 rpm (4°C) and supernatant collected. Total protein was estimated based on Bradford et al. (1976). Total protease activity was evaluated by using 1% azocasein in 50 mM Tris–HCl, pH 7.5 (Garcia-Carreno, 1992). Ten microlitres of enzyme extract was mixed with 0.5 mL of buffer (50 mM Tris–HCl, pH 7.5), 0.5 ml of substrate solution and incubated for 10 min at room temperature. The reaction was stopped by addition of 0.5 ml 20% trichloroacetic acid and then centrifuged at 14000 x g (11480 rpm) for 5 min. The absorbance of the supernatant was recorded at 366 nm. Separation of proteins from extracts was done by 12% SDS-PAGE according to Laemmli (1970). Protein extract (15 µg protein/sample) was loaded into each well and electrophoresis was performed on a mini gel electrophoresis device (G Biosciences, Noida, India). The molecular weights of the proteins were determined by comparison of mobility with known marker proteins, plotting the log10 of Rf values against the molecular weight. The protease composition was studied after separation of proteins by substrate SDS-PAGE (Garcia-Carreno and Haard, 1993). Enzyme preparations containing 5 mU activity were incubated with inhibitors to determine the class of enzymes in the gut extract of zebra fish juveniles. Solutions of serine protease inhibitor PMSF (100 mM) and metalloprotease inhibitor EDTA (20 mM) were prepared and incubated with enzyme preparation 5 mU activity (1:1) at 25°C for 1 hrs prior to loading into the wells. Then they were subjected to substrate SDS-PAGE with sample volume adjusted to 15 µl containing 5 mU activity loaded to each well. After electrophoresis, the gel was immersed in a solution of 3% casein in 50 mM Tris–HCl, (pH 7.5) for 30 min at 5°C to allow the substrate to diffuse into the gel at low enzyme activity followed by incubation at 25°C for 60 min. The gel was then washed, stained and destained. Clear bands (zymograms) were identified as protease activity bands that are compared bands of the enzyme preparation without inhibition. Analysis of physico-chemical parameters of control and experimental sets of aquarium were assayed through standard protocol prescribed by APHA (1992).

Feed intake (FI as % body weight) was the mean feed consumption per fish as a percentage of the fish body weight (calculated to intake per day) for the experimental period. Specific growth rate (SGR) was estimated according to the formula: SGR = 100 x (ln(w2)-ln(w1))/t, where w2 = final fish weight, w1 = initial fish weight and t = duration of the experiment in days. Feed conversion ratio (FCR) was estimated as: FCR = Feed intake/ Growth,

Table 3. General performance of zebrafish fed with different dietary levels of silver nanoparticle.

Treatments	S0	S5	S20	S40	P<
WG (mg)	43.0 ± 3.00	43.3 ± 3.32	55.0 ± 3.06	49.67± 4.98	0.05
FI	1.23 ± 0.01	1.24 ± 0.01	1.27 ± 0.01	1.26 ± 0.04	ns
FCR	0.03 ± 0.002	0.03 ± 0.002	0.02 ± 0.001	0.03 ± 0.003	ns
SGR	0.79 ± 0.04	0.84 ± 0.05	0.98 ± 0.04	0.9 ± 0.07	0.05

WG: Average weight gain, FI: Feed intake, FCR: Feed conversion ratio, SGR: Specific growth rate, ns: Non significant, *: P< 0.05.

Table 4. Banding patterns of protease zymograms from gut extract of zebra fish treated with PMSF and EDTA by substrate SDS-PAGE.

Predicted MW of protease bands	Control S0	Band patterns in PMSF treated samples			Band patterns in EDTA treated samples		
		S5	S20	S40	S5	S20	S40
86	✓	✓	✓	✓	✓	✓	✓
73	✓	✓	✓	✓	✓	✓	✓
67			✓	✓		×	×
45	✓	×	×	×	✓	✓	✓
40	✓	✓	✓	✓	✓	✓	✓
37			✓	✓		×	×
34	✓	×	×	×	✓	✓	✓
24	✓	×	×	×	✓	✓	✓
21	✓	×	×	×	✓	✓	✓
18	✓	×	×	×	✓	✓	✓

Molecular weights of bands are calculated by Rf migration plot against medium range protein marker. Presence of band marked (✓) and inhibited bands are marked (×). Absences of bands are left blank.

(where feed intake is the amount of feed consumed by the fish, and growth is the increase of fish biomass during the same period).

Biological and analytical data were subjected to one-way analysis of variance (ANOVA) using Microsoft Excel programme for Windows at a significance level of 0.05.

Results

At the end of the experiment, as compared with that of control, S5 treatment did not show any difference in weight gain whereas, S20 and S40 treatments showed 28% and 14% increase in total weight gain (P<0.05), respectively. Likewise, 24% and 14% increase in SGR were observed on S20 and S40 groups respectively (P<0.05). However, no significant differences observed on FI and FCR between treatments (Table 3). Among all the treatments, S20 exhibited better performance in terms of weight gain and SGR compared with the others.

The specific protease activity was recorded as 0.598 ± 0.07 units-1protein-1 at the beginning of the experiment. By the end of the experiment, the activity increased 5.6 % in S0 (0.632 ± 0.04 units1protein-1) while S5, S20 and S40 showed 6.3% (0.636 ± 0.04 units1protein-1), 21.7% (0.726 ± 0.02 units1protein-1) and 18.8% (0.711 ± 0.03 units1protein-1) enhancement in activity at the end of experimental period (P<0.05) as compared to that of the beginning of the study. At 30 days of rearing, the protease activity showed no significant difference between S0 and S5 whereas that the fish treated with S20 and S40 showed 14.8 and 12.5% enhancement in activity (P<0.05)compared to that of control group (Fig. 2).

Protease activity bands (zymograms) based on substrate SDS-PAGE analysis was compared among control and experimental samples to find out the differences in banding pattern of serine and metalloproteases in the current experiment is presented in Table 4. PMSF treated intestinal extract

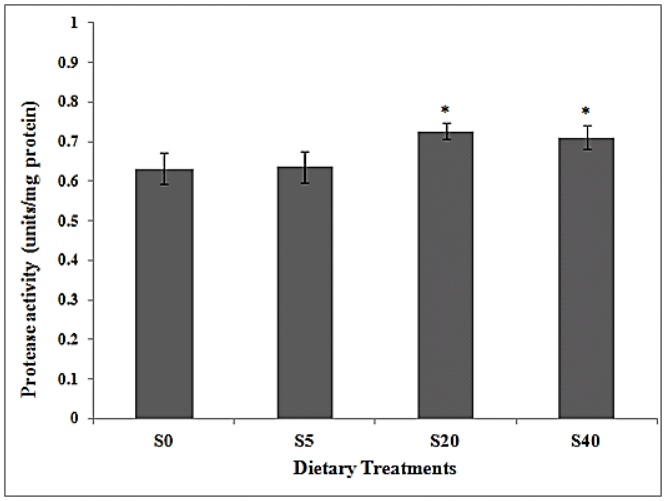

Figure 2. Specific protease activity of zebra fish fed with the different dietary inclusion level of silver nanoparticle at 30 days of rearing. *: significance level $P<0.05$.

did not show any significant differences in banding pattern among the treatments. This implies that the status of serine proteases remained unaffected by silver nanoparticles. Interestingly, in the intestinal extract of S20 and S40, two extra bands of MW about 66 and 37 KDa were observed which were inhibited by EDTA. This indicates the presence of metalloproteases in these dietary regimes. However, these two protease bands were found absent in S0 and S5 and neither any differences in banding pattern among them was observed.

Discussion

Growth and metabolic performances are related to homeostatic processes that may reflect the characteristics of nutrients administered in feed. The current study evaluated the effect of dietary nanosilver on general performance and gut proteases in zebrafish. It has been reported that silver nanoparticles applied into the egg (In ovo) can up-regulate the expression of fibroblast growth factor (FGF2) and vascular endothelial growth factor (VEGF) that may stimulate satellite cell proliferation and differentiation (Hotowy et al., 2012). Furthermore, chicken embryos injected with silver nanoparticles affected the expression of genes responsible for muscle development during embryogenesis (Sawoz et al., 2012; Pineda et al., 2012). Growth stimulatory impacts as an additive which ameliorate sanitary profile of animal are inversely proportional to the surrounding environment (Cromwell, 1991). As minute level of microbial load and biotic stresses is prevalent at commercial fish farms, the balancing role of silver nanoparticle is believed to be more apparent, which ultimately tends the fish towards homeostasis and growth. Though there are no reports available on growth promoting role of colloidal silver in fish, it is noted from *in vitro* study that amount of coliforms in pig ileal lysate is reduced significantly after treatment with silver nanoparticles whereas no impact is seen on *Lactobacilli* population when the application doses of silver nanoparticle are enhanced proportionately (Fondevila et al., 2009). These results predicted the fact that colloidal silver in medium dose can inhibit harmful coliforms but do not influence beneficial *Lactobacilli* which was corroborated also through *in vivo* study. Fondevilla et al. (2009) suggested a numerical increase in daily growth of pig at nanosilver enriched diet. In the current study, silver nanoparticle at a dietary dose 20 and 40 ppb showed an indication of improved growth in zebra fish juveniles as compared to other dietary regimes. It may be possible that the nanoparticle exhibiting a probable antimicrobial activity in the digestive system keeps the fish less prone to pathogenic challenges at the gut which in turn provides better digestive activities.

Trace elements like zinc and copper have been shown to induce productive performances in farmed animals and higher vertebrates by reducing post weaning diarrhea, affecting pancreatic and intestinal digestive enzymes activities and maintaining morphology of the intestinal mucosa thereby increasing absorption potential of nutrients (Zhou et al., 1994; Li et al., 2001; Hedemann at al., 2006; Broom et al., 2006). Potentially, silver nanoparticles are also expected to have a similar effect considering the chemical similarity of silver with other metals such as copper. In the present study, the increase in protease activity at 20 ppb and 40 ppb level can be attributed to the possible impact of silver nanoparticles towards digestive performances.

Studies related with the role of silver nanoparticles on metalloproteinases regulation have already been described (Wright et al., 2002; Warriner and Burrell, 2005). The topic use of silver promotes an increase of zinc and copper concentration over epithelial tissue, thus indirectly stimulating its positive effects (Lansdown, 2002). In the present study, the results from enzyme activity suggested that there is increase in number of metalloprotease related enzymes. The result supports the idea of silver nanoparticles inducing the metalloproteases in zebra fish. However, this study is subjected to further detailed characterization of proteases and the involvement of silver nanoparticles.

In conclusion, the present study showed that nanosilver enriched formulated diet can influence the digestive performance in zebra fish. Here, a 20 ppb dietary inclusion of silver trace metal based nanoparticle exhibited as suitable in terms of both protease and metallic enzymatic regulations. This also suggests that 20 ppb dose of silver nanoparticles may induce metalloprotease in zebra fish gut. However, its role as a growth promoter and nutrition is subjected to further studies. This information will contribute for further research on fish physiology and nanoparticles as a feed additive.

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