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Protective effect of dietary vitamin E on immunological and biochemical induction through silver nanoparticles (AgNPs) inclusion in diet and silver salt ($AgNO_3$) exposure on Zebrafish (*Danio rerio*)

Seyed Aliakbar Hedayati[a], Hamed Ghafari Farsani[b], Saeid Shahbazi Naserabad[c], Seyed Hossein Hoseinifar[a], Hien Van Doan[d,e,*]

[a] Department of Fisheries, Faculty of Fisheries and Environmental Sciences, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran
[b] Young Researchers and Elite Club, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran
[c] Young Researchers and Elite Club, Yasooj Branch, Islamic Azad University, Yasooj, Iran
[d] Department of Animal and Aquatic Sciences, Faculty of Agriculture, Chiang Mai University, Chiang Mai 50200, Thailand
[e] Science and Technology Research Institute, Chiang Mai University, 239 Huay keaw Rd., Suthep, Muang, Chiang Mai 50200, Thailand

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ABSTRACT

The present study evaluated silver nanoparticle (AgNPs) toxicity using biomarkers of oxidative and metabolic stress, immunological impairment and cellular damage in zebrafish (*Danio rerio*), as well as the optimal dose of vitamin E neutralizing undesirable effects. Fish were fed for ten days and eight study groups were investigated: controls, AgNPs exposure alone (1.5 mg L^{-1}) and combined with three different vitamin E doses (1.5 mg L^{-1} of AgNPs + vitamin E 100, 200 or 400 mg kg^{-1} of food), also one positive control group exposed to $AgNO_3$ alone or combined with the same vitamin E doses. *D. rerio* exposed to AgNPs alone or combined with the lower vitamin E dose showed overall worse results in comparison with the control groups and the groups combining nanoparticles and 200 or 400 mg kg^{-1} of food of vitamin E-supplemented diet. AgNPs caused cell impairment by increasing LDH activity and cortisol levels, generated oxidative stress by inhibiting SOD and CAT activity and immunosuppression by inhibiting ACH50 and lysozyme activity. The groups exposed to Ag salt showed the same response-pattern found for the NPs groups, reinforcing that Ag toxicity of AgNPs is mediated by Ag^{+}. In conclusion, although AgNPs are toxic to *Danio rerio*, vitamin E supplementation at 200 or 400 mg kg^{-1} can act protectively against its toxic effects.

1. Introduction

Silver nanoparticles (AgNPs) are among the most used nanoproducts, due to their antibacterial properties (Aschberger et al., 2011; Yue et al., 2015). AgNPs applications range from cosmetics, textiles and disinfection products to food supplements (Navarro et al., 2008; Yue et al., 2015). Studies have reported considerable leaching of silver from AgNPs products (Stevenson et al., 2013). This leads to the releasing of both the nanoparticles and dissolved silver into the aquatic environment, and raises concern about the adverse effects that AgNPs may exert on aquatic organisms (Navarro et al., 2008; Stevenson et al., 2013).

AgNPs toxic effects reflect, in part, the toxicity of the metal itself (Griffitt et al., 2008a,b; Powers et al., 2011) Since silver (Ag) is highly toxic at low ionic concentrations, toxicological studies involving this element have been, increasingly, receiving greater attention (Choi et al., 2010). In this regard, previous studies have demonstrated that exposure to nanosilver may be related to the generation of oxidative stress (Kim et al., 2007), damage to cell membranes and DNA (Pal et al., 2007) or to the interaction of Ag^{+} ions with proteins and enzymes (Yamanaka et al., 2005; Navarro et al., 2008).

It has been proven that AgNPs are toxic to a variety of aquatic organisms (Asharani et al., 2008; Griffitt et al., 2008a,b; Farkas et al., 2011; Asghari et al., 2012; Lapresta-Fernández et al., 2012). More specifically in Zebrafish, AgNPs were proven to be neurotoxic (Powers et al., 2011), embryotoxic (Asharani et al., 2008; Massarsky et al., 2013), cardiotoxic (Asharani et al., 2008) and oxidative stress promoters, as well as the cause for alterations in global gene expression profiles (Griffitt et al., 2013; Massarsky et al., 2013). Although numerous studies have demonstrated the toxic effects of AgNPs, there is

* Corresponding author at: Department of Animal and Aquatic Sciences, Faculty of Agriculture, Chiang Mai University, Chiang Mai 50200, Thailand.
E-mail address: hien.d@cmu.ac.th (H. Van Doan).

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still a lack of understanding on how and what could protect organisms from the deleterious effects of these compounds.

Vitamin E comprises fat-soluble compounds with antioxidant activity. It is found naturally within food and is available as a dietary supplement (Traber and Atkinson, 2007). Since it is a peroxyl radical scavenger, it is important in preserving cellular membrane integrity and, thus, maintaining cellular bioactivity (Traber and Atkinson, 2007). Vitamin E is also important in maintaining and modulation immune functions in fish (Cuesta et al., 2001; Pearce et al., 2003; Puangkaew et al., 2004)

In ecotoxicology, physiological responses measured with the aid of several biomarkers, such as immunological response, serum metabolites and enzymatic activities, providing valuable insights and correlation on adverse effects of xenobiotics in exposed organisms (Lewis et al., 1999; Rao, 2006; Kavitha and Rao, 2007). Since data on the effects of AgNPs on serum metabolite and immunological parameters in fish and the possible protective effects of vitamin E are scarce, the main aim of this study, was to determine what is the optimal dose of vitamin E to counteract the damage caused through AgNPs exposure on a well-known vertebrate model for toxicity assessment, the Zebrafish (*Danio rerio*) using a multi-biomarker approach (Rubinstein, 2003; Parng, 2005).

2. Material and methods

2.1. Fish and experimental conditions

One thousand and eighty Zebrafish (*Danio rerio*) (Zebrafish, Cypriniformes: Cyprinidae), weighing 1.93 ± 0.2 g, were obtained from Gorgan, Iran. Fish were acclimatized for one week prior to the experiment and fed three times a day with a commercial food (ALLER, DENMARK Co. – with no additives). A 12:12 light/dark photoperiod and water temperature (26.0 ± 1.0 °C) was maintained constant. The following water physico-chemical parameters, namely pH (8.0 ± 8.4), dissolved oxygen levels (6.0 mg L^{-1}) and ammonia, nitrite, and nitrate (< 0.1 mg L^{-1}), were monitored daily. Sub-lethal dose of 1.5 mg L^{-1} AgNPs was chosen based on previous studies (Griffitt et al., 2008a,b; Bowman et al., 2012; Hedayati et al., 2015). Half of the LC_{50} concentration found from present experiments (Appendix 1), 0.15 mg L^{-1} of $AgNO_3$, was chosen for the positive control with the Ag salt.

The experiments were carried out in triplicate and consisted of eight test groups: 0 + 0 - fish fed the control diet; 0 + 1.5 - fish fed the control diet + 1.5 mg L^{-1} of AgNPs; 100 + 0 - fish fed the control diet + vitamin E (100 mg kg^{-1} of food); 100 + 1.5 - fish fed the control diet + vitamin E (100 mg kg^{-1} of food) + 1.5 mg L^{-1} of AgNPs; 200 + 0 - fish fed the control diet + vitamin E (200 mg kg^{-1} of food); 200 + 1.5 - fish fed the control diet + vitamin E (200 mg kg^{-1} of food) + 1.5 mg L^{-1} of AgNPs; 400 + 0 - fish fed the control diet + vitamin E (400 mg kg^{-1} of food); 400 + 1.5 - fish fed the control diet + vitamin E (400 mg kg^{-1} of food) + 1.5 mg L^{-1} of AgNPs; and one positive control group where fish were exposed to 0.15 mg L^{-1} waterborne $AgNO_3$: 0 + $AgNO_3$ - fish fed the control diet + 0.15 mg L^{-1} of $AgNO_3$; 100 + $AgNO_3$ - fish fed the control diet + vitamin E (100 mg kg^{-1} of food) + 0.15 mg L^{-1} of $AgNO_3$; 200 + $AgNO_3$ - fish fed the control diet + vitamin E (200 mg kg^{-1} of food) + 0.15 mg L^{-1} of $AgNO_3$; 400 + $AgNO_3$ - fish fed the control diet + vitamin E (400 mg kg^{-1} of food) + 0.15 mg L^{-1} of $AgNO_3$. Fish were randomly distributed into 30 L tanks. Fish were fed three times, at 8:00 am and 14:00 pm and 20:00 pm, for 10 days. Sampling occurred after 10 days exposure.

2.2. Food preparation, AgNPs suspension and $AgNo_3$ stock solution

The basic diet common to all groups contained 51.1% crude protein, 13.6% total lipid, 10.2% ash and 3.5% fiber. AgNPs at 1.5 mg L^{-1} were added to the food isolated or combined with three doses of vitamin-E (100, 200 or 400 mg kg^{-1} of food). The positive control groups also received vitamin-E supplementation on the food with the same doses of the AgNPs groups. AgNPs nanocolloids were prepared according to the manufacturer's (Nanosany Company, Iran) instructions (Antimicrobial Product 2 brand, 4000 ppm nanosilver concentration, mean particle size of 20 nm). $AgNO_3$ was purchased from Sigma-Aldrich (≥99.99%) and a stock solution of 1000 mg L^{-1} used deionized water and was prepared fresh before the exposure. All-rac-α-tocopherol, a synthetic-source of vitamin E, was purchased from Merck (Germany). The oil coating method using canola oil was employed to add vitamin E to the fish diet (Treves-Brown, 2000; Ghafari Farsani et al., 2017).

2.3. Sampling

All fish were anaesthetized with clove powder (200 mg kg^{-1}, 20 min) and killed after 10 days of continuous feeding. Blood was collected without any anticoagulant from the caudal vessels of all fish using 1 mL sterile syringes and allowed to clot at room temperature for 2 h. The samples were then centrifuged at 3000 rpm for 15 min at 4 °C. The serum was pooled ($n = 5$) and stored at −80 °C for immunological and enzymatic parameter analyses (Ghafari Farsani et al., 2017).

2.4. Immunological, oxidative stress and metabolic stress biomarkers

Serum lysozyme activity was measured by the addition of 50 μL of *D. rerio* serum to 2 mL of a *Micrococcus lysodeikticus* suspension (0.2 mg mL^{-1}) in a 0.05 M sodium phosphate buffer (pH 6.2). The reaction was carried out at room temperature and absorbance's were determined spectrophotometrically at 450 nm after 0.25 min and 5 min. One unit of lysozyme activity (U mL^{-1}) was defined as the amount of enzyme causing an absorbance decrease of 0.001 min^{-1}. The method was calibrated using a standard curve with lysozyme from hen egg whites (Sigma, USA) in PBS based on the turbid metric method (Parry et al., 1965). Alternative complement pathway activity (ACH50) was determined based on the methods described by Yano (1992) and Sunyer and Tort (1995) and assessed with the modifications described by Yeh et al. (2008). The volume of supernatant complement leading to 50% hemolysis (ACH50) was determined, and the number of ACH50 U mL^{-1} was calculated.

Superoxide dismutase (SOD) activity was determined using a commercial kit (Pars Azmoon Co., Tehran, Iran) and catalase (CAT) activity was determined according to Aebi (1984) by the decrease in absorbance at 240 nm using 50 mM H_2O_2 as substrate.

Serum enzyme activity comprised lactate dehydrogenase (LDH), alkaline phosphatase (ALP), alanine aminotransferase (ALT), acid phosphatase (ACP) and aspartate aminotransferase (AST), estimated using commercial kits (Pars Azmoon Co, Tehran, Iran) and analyzed according to Peyghan and Takamy (2002).

Glucose, cortisol, total protein, albumin and globulin were determined using commercial kits (Pars Azmoon Co, Tehran, Iran). Cortisol and glucose assays were carried out as described by Shaluei et al. (2012). Total protein content was determined based on the method reported by Bradford (1976) using bovine serum albumin as standard. Serum albumin concentrations were determined in acidic pH with Bromocresol Green reagent. Serum globulin concentrations were calculated by subtracting the albumin values from the total serum protein concentrations (Bayunova et al., 2002).

2.5. Statistical procedures

Data was statistically analyzed and graphs were generated using the GraphPad Prism v5 software (GraphPad Software, Inc.). Comparisons among groups were performed using a one-way analysis of variance (ANOVA) followed by the Bonferroni's or Dunnett's Multiple Comparison Test. Tukey's test was applied to test inter-grouping homogeneity. Statistical significance was set at $p < 0.05$. All data were expressed as means ± standard error of the means.

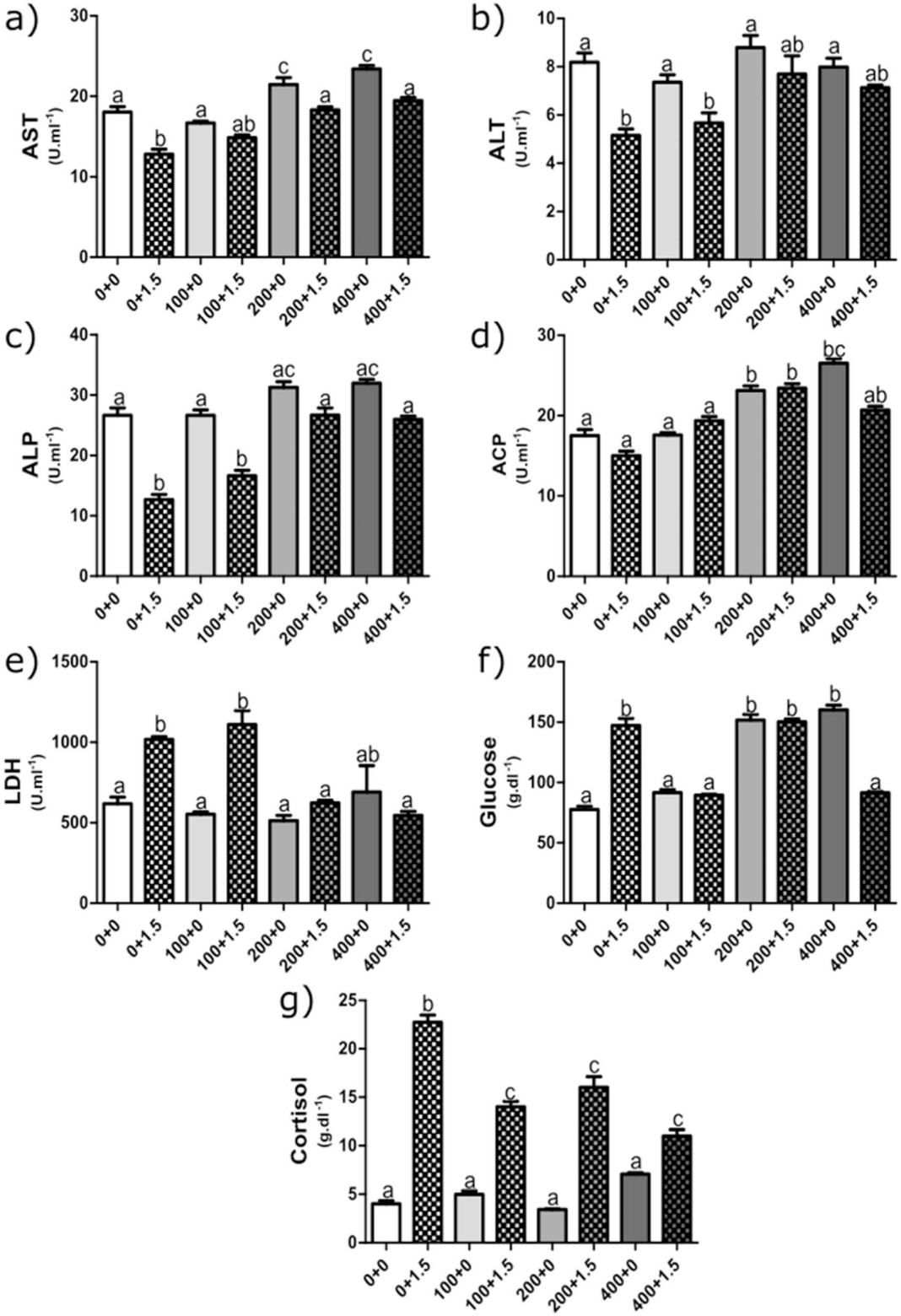

Fig. 1. Cellular damage biochemical biomarkers in *D. rerio* serum. a) AST activity (U mL^{-1}); b) ALT activity (U mL^{-1}); c) ALP activity (U mL^{-1}); d) ACP activity (U mL^{-1}); e) LDH levels (U mL^{-1}); f) glucose levels (g dL^{-1}); g) cortisol levels (g dL^{-1}). 0 + 0, 0 + 1.5, 100 + 0, 100 + 1.5, 200 + 0, 200 + 1.5, 400 + 0 and 400 + 1.5 refer to the eight test groups. Data are expressed as means ± standard error of the means. Different letters indicate significant differences between groups ($p < 0.05$).

3. Results

Serum enzymes AST, ALT and ALP (Fig. 1a, b and c) exhibited lower activities in the 1.5 + 0 and 1.5 + 100 groups compared to the control. The groups treated with NPs and 200 or 400 of vitamin E or vitamin E alone showed no differences in activity when compared to the group that did not ingest the vitamin E supplementation or the AgNPs. Strong to moderate positive (0.7–0.9) correlations between ALT, AST and ALP

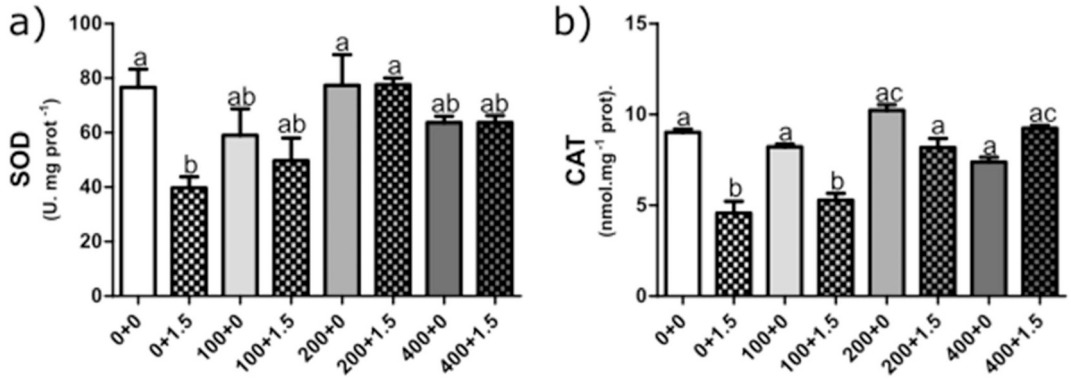

Fig. 2. Oxidative stress biochemical biomarkers in *D. rerio* serum. a) SOD activity (U. mg prot^{-1}); b) CAT activity (nmol·mg^{-1} prot). 0 + 0, 0 + 1.5, 100 + 0, 100 + 1.5, 200 + 0, 200 + 1.5, 400 + 0 and 400 + 1.5 refer to the eight test groups. Data are expressed as means ± standard error of the means. Different letters indicate significant differences between groups ($p < 0.05$).

activities were found. LDH activity (Fig. 1e) was higher in the groups treated with AgNPs alone and AgNPs combined with the lowest vitamin E dose when compared to all other groups.

Glucose levels (Fig. 1f) were higher in the 1.5 + 0, 200 + 0, 200 + 1.5 and 400 + 0 groups when compared to the control and 100 + 0, 100 + 1.5 and 400 + 1.5 groups. Cortisol levels (Fig. 1g) were higher in all the groups treated with AgNPs when compared to the control or the groups exposed to NPs + vitamin E. However, the group exposed only to the AgNPs displayed higher cortisol levels compared to the groups that combined NPs exposure and vitamin E. Cortisol showed strong to moderate negative (0.8–0.7) correlations with ALP, ALT and AST activity and a moderate negative correlation with ACH50 activity.

SOD and CAT activities (Fig. 2a and b) were lower in the groups exposed to AgNPs alone and AgNPs combined to the lowest vitamin E dose compared to all groups and the control. Serum biochemical parameters of total protein and albumin presented lower levels in the 0 + 1.5 and 100 + 1.5 groups compared to all other analyzed experimental groups. However, the groups treated with 200 mg and 400 mg of vitamin E supplementation displayed higher levels of both total protein and albumin (Fig. 3a and b). The group supplemented with the highest dose of vitamin E presented higher globulin levels than all the other groups in the present study (Fig. 3c). Humoral innate immune parameters, ACH50 and lysozyme activity, presented lower activities in the groups exposed to the NPs alone or combined with the lowest dose of vitamin E supplementation (Fig. 3d and e).

Ag^{+} measured concentration in water on groups exposed to the Ag salt was 0.097 ± 0.002 mg L^{-1}. Overall, the groups exposed to AgNO3 alone or supplemented with vitamin E showed the same trend observed for the NPs exposed groups (Table 1).

4. Discussion

AST, ALT, ALP, ACP and LDH enzymes are predominant in the liver (Kori-Siakpere et al., 2012). When damage occurs to hepatic tissues, these enzymes are released into the bloodstream leading to increased activities in plasma (Velisek et al., 2009; Shahsavani et al., 2010; Kumari et al., 2011; Atli et al., 2015). However, the results of the study indicate decreased AST, ALT, ALP and ACP activity in groups exposed to AgNPs alone or associated with the lower vitamin E dose. Similar results have been reported for rats exposed to AgNPs (Sulaiman et al., 2015). It is postulated that this decrease can be caused by the inactivation of thiol (–SH) groups and amino transaminases by the AgNPs, consequently causing the malfunctioning of several proteins and reactions (Abbas et al., 2011; Adeyemi and Whiteley, 2013). Fish exposed to pesticides have also exhibited lower activities of these enzymes (Sastry and Sharma, 1980; Luskova et al., 2002; Khoshbavar-Rostami et al., 2004).

On the other hand, LDH activity was higher in the same groups where ALT, AST, ALP and ACP activities were found to be inhibited, suggesting impairment of liver function (Kang et al., 2012; Wu and Zhou, 2013; Ghafari Farsani et al., 2017; Shadegan and Banaee, 2018). In addition, as LDH is responsible for the interconversion of pyruvate to lactate in glycolysis, changes in the energy production and cellular metabolism pathways during AgNPs exposure was possible (Osman et al., 2007; Vieira et al., 2008; Domingues et al., 2010). Accordingly, increased circulating cortisol in all groups exposed to the AgNPs was observed, establishing a link between the changes detected in LDH and stress responses in exposed zebrafish. Cortisol is an established biomarker of stress-intensity (Barton, 2002; Shaluei et al., 2012; Akbary et al., 2016) and plays a role in increasing energy availability through gluconeogenesis (Saravanan et al., 2011), thereby confirming the disruption of energy production by the cellular metabolism. Several studies have reported increased plasma cortisol after exposure to metals, nanomaterials and, more importantly, AgNPs (Laflamme et al., 2000; Brauner and Wood, 2002; Katuli et al., 2014; Ghafari Farsani et al., 2017).

Cytotoxicity induced by AgNPs is mediated by the generation of reactive oxygen species (ROS) and oxidative stress (Kim et al., 2007; Choi et al., 2010; Lima et al., 2012). The release of the metal ions from the nanoparticles enhances ROS production, leading to oxidative damage, including lipid peroxidation, changes in membrane permeability, protein carbonylation and DNA damage (Xia et al., 2006; Khan et al., 2015; Sayed and Soliman, 2017). Thus, as SOD is the first enzyme to cope with oxygen radicals and CAT facilitates the reduction of H_2O_2 into H_2O (Van der Oost et al., 2003), changes in SOD and CAT activities are expected. In this regard, inhibition of both SOD and CAT activities in the groups exposed to AgNPs alone and AgNPs combined with the lower dose of vitamin E supplementation were observed. Similar results have been reported by Choi et al. (2010), Devi et al. (2015) and Sayed and Soliman (2017). In addition, Atli et al. (2006) have demonstrated that CAT activity is decreased by Ag exposure, although it is stimulated by other metals. SOD and CAT inhibition may lead to the accumulation of oxyradicals and cause oxidative damage, indicating failure of the antioxidant defense mechanism in protecting the organism from damages caused by nanoparticle exposure.

Plasma total protein levels are applied as a sensitive fish health indicator, because they can reflect liver health conditions (John, 2007; Palaniappan and Vijayasundaram, 2009; Katuli et al., 2014). Herein, decreases in serum total protein and albumin levels were observed in AgNPs-treated zebrafish, impairment of liver functions and overall changes in metabolism. These results also suggest changes in protein synthesis, which may be caused due to damage to protein-synthesizing subcellular structures and inhibition of hepatic synthesis of blood proteins (Fontana et al., 1998; Palaniappan and Vijayasundaram, 2009). In addition, AgNPs be responsible for reducing the synthesis of total proteins and albumin in the liver (Gokcimen et al., 2007). Other authors

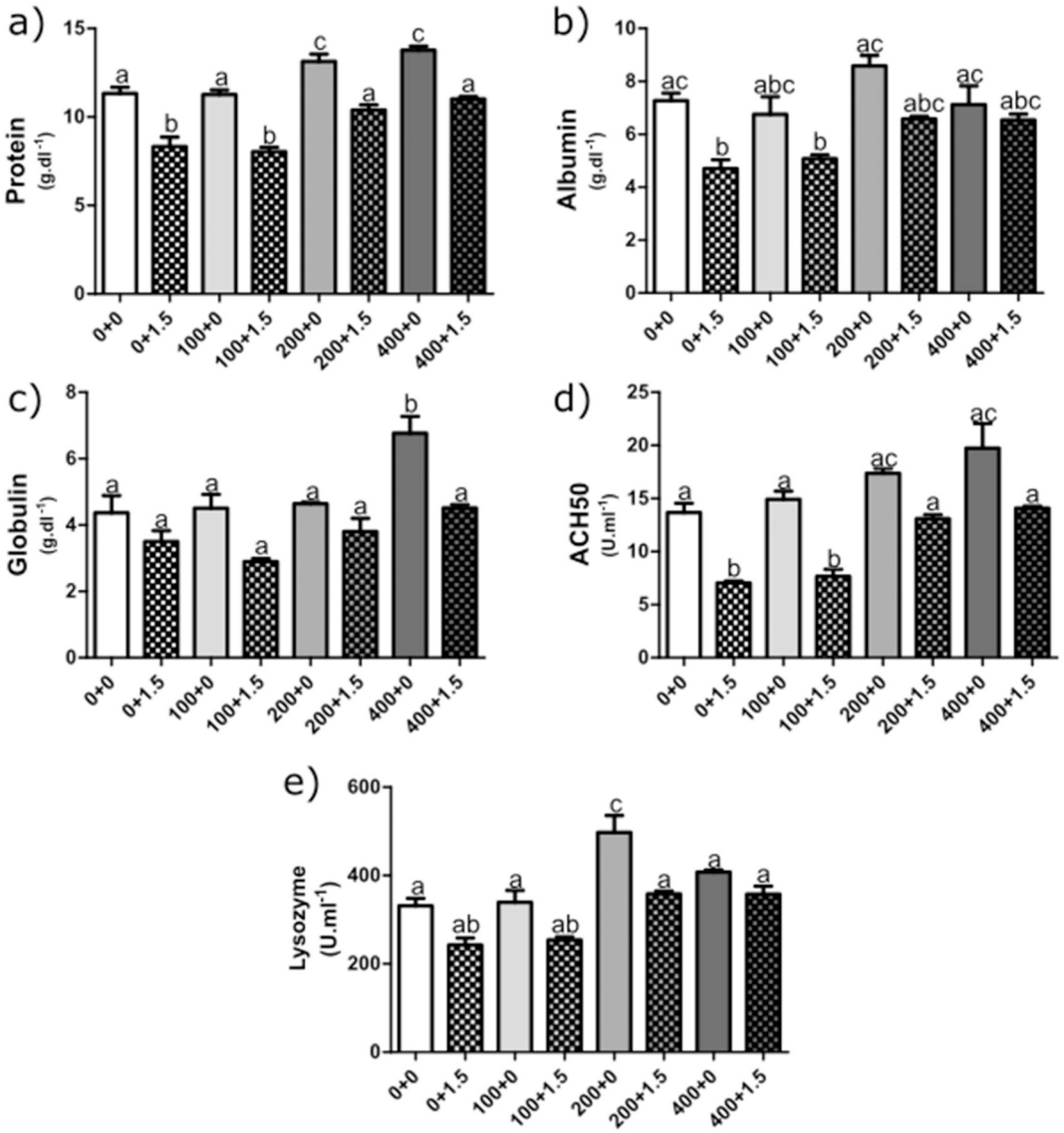

Fig. 3. Biochemical biomarkers of immunological response and metabolism in *D. rerio* serum. a) protein levels (g dL^{-1}); b) albumin levels (g dL^{-1}); c) globulin levels (g dL^{-1}); d) ACH50 activity (U mL^{-1}); e) Lysozyme activity (U mL^{-1}). 0 + 0, 0 + 1.5, 100 + 0, 100 + 1.5, 200 + 0, 200 + 1.5, 400 + 0 and 400 + 1.5 refer to the eight test groups. Data are expressed as mean ± standard error of the mean. Different letters indicate significant differences between groups ($p < 0.05$).

Table 1
Biochemical biomarkers in the serum of *D. rerio* exposed to $AgNO_3$. 0 + 0, 0+ $AgNO_3$, 100+ $AgNO_3$, 200+ $AgNO_3$, 400+ $AgNO_3$ refer to the five test groups. Data are expressed as mean ± standard error of the mean. Asterisks indicate significant differences from the control (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

Positive control ($AgNO_3$) – biochemical biomarkers					
	0 + 0	0 + $AgNO_3$	100 + $AgNO_3$	200 + $AgNO_3$	400 + $AgNO_3$
Glucose (g dL^{-1})	77.67 ± 2.60	150.7 ± 2.33***	96 ± 2.31***	90 ± 2.08*	84 ± 2.31
Cortisol (g dL^{-1})	4 ± 0.31	26.26 ± 1.85***	22.67 ± 1.45***	17.42 ± 1.45**	11.34 ± 0.35
ALT (U mL^{-1})	8.19 ± 0.38	6.09 ± 0.13**	6.45 ± 0.26*	6.96 ± 0.31	7.67 ± 0.45
AST (U mL^{-1})	18.05 ± 0.67	14.20 ± 1.36*	15.11 ± 0.65	18.28 ± 0.66	19.45 ± 1.05
ALP (U mL^{-1})	26.67 ± 1.20	11.73 ± 0.73***	13.50 ± 0.87***	22.29 ± 0.98*	23.62 ± 0.93
ACP (U mL^{-1})	17.5 ± 0.76	15.33 ± 1.21	19.07 ± 0.95	22.51 ± 1.38*	20.73 ± 1.24
LDH (U mL^{-1})	618.3 ± 41.29	1046 ± 29.08***	1028 ± 31.93***	650.7 ± 10.27	579.7 ± 6.69
SOD (U·mg $prot^{-1}$)	76.67 ± 6.64	37 ± 2.31***	54 ± 4.62*	73 ± 2.52	64.33 ± 4.26
CAT (nmol·mg^{-1} prot)	9.02 ± 0.17	4.06 ± 0.70***	5.19 ± 0.54**	7.85 ± 0.59	8.81 ± 0.40
Total Protein (g dL^{-1})	11.33 ± 0.35	7.40 ± 0.37***	8.69 ± 0.28**	10.31 ± 0.35	11.11 ± 0.72
Albumin (g dL^{-1})	7.27 ± 0.29	4.08 ± 0.45**	4.71 ± 0.28**	6.24 ± 0.42	6.35 ± 0.63
Globulin (g dL^{-1})	4.37 ± 0.52	3.42 ± 0.39	3.48 ± 0.31	3.65 ± 0.58	4.12 ± 0.49
Lysozyme (U mL^{-1})	331 ± 17.39	240.3 ± 10.04***	251 ± 9.71**	360.7 ± 6.12	361.8 ± 11.29
Complement - ACH50 (U mL^{-1})	13.67 ± 0.88	6.33 ± 0.25***	7.38 ± 0.26***	13.57 ± 0.75	14.14 ± 0.32

have also reported that total protein and albumin levels are decreased in fish exposed to different pollutants (Vijayan et al., 1997; Velisek et al., 2009).

Another application of total serum proteins, along with cortisol levels, is the evaluation of innate immune humoral parameters in fish (Shakoori et al., 2019). In this regard, the decrease of serum proteins and increases in plasma cortisol in AgNPs-exposed fish may be related to a suppression of the innate immune response (Montero et al., 1999; Han et al., 2014). This was corroborated herein, where ACH50 and lysozyme activity were also inhibited in fish exposed to AgNPs alone or combined with the lowest vitamin E dose. Lysozymes and ACH50 display antibacterial and antivirus activities and are important components of the innate immune system (Skouras et al., 2003; Rooijakkers and Van Strijp, 2007). This is in agreement with previous studies and corroborates the fact that AgNPs induce immunosuppression (Gagné et al., 2012; Bruneau et al., 2016). In fact, AgNPs can directly bind to lysozymes and change their structure (Wang et al., 2017).

Biomarkers results, LDH, CAT, SOD, protein levels and ACH50, in the simultaneous treatments combining AgNPs and vitamin E resulted in significant protection against metabolic and oxidative stress and immunosuppression in the groups that underwent 200 mg and 400 mg vitamin E supplementation. The inhibition of ROS formation due to the scavenging property of vitamin E (Al-Jassabi and Khalil, 2006; Ural, 2013; Ghafari Farsani et al., 2017) could have restored SOD and CAT activities. Thus, it is possible that an increase in the activity of these enzymes could also have contributed to the elimination of the ROS-induced production by the AgNPs (Ghafari Farsani et al., 2017).

Still, the toxicity of $AgNO_3$ and AgNPs didn't differ significantly and the vitamin E supplementation had similar positive outcomes in both cases, even if the route of exposure were different. That offset is in accordance with previous studies that already confirmed that acute toxicity of AgNPs to fish is mediated by Ag^+ (Kim et al., 2011; Massarsky et al., 2013; Ribeiro et al., 2014; Groh et al., 2015).

Vitamin E supplementation could, thus, prevent stress-related immunosuppression, by bringing the evaluated immunological parameters back to levels similar to the control group. In addition, vitamin E supplementation has been shown to enhance resistance to diseases and reduce the effects of metabolic stress in humans and fish (Meydani et al., 1990; Montero et al., 1999; Clerton et al., 2001; Puangkaew et al., 2004). However, even though cortisol was decreased, it did not match levels of the control fish, demonstrating that, although overall metabolic stress could be reduced by vitamin E, this compound did not confer total protection against AgNPs exposure.

5. Conclusions

Sublethal doses of 1.5 mg L^{-1} of AgNPs and 0.15 mg L^{-1} of $AgNO_3$ induced oxidative stress responses, metabolic disturbances and triggered immunological responses in zebrafish. However, vitamin E supplementation conferred a protective effect against the adverse effects caused by both AgNPs and $AgNO_3$. The best dose for vitamin E supplementation to counteract Ag exposure adverse effects ranged between 200 and 400 mg kg^{-1} in food. The most adequate biomarkers to assess not only the toxicological effects of the AgNPs or $AgNO_3$, but also the protective effects of vitamin E, were LDH, CAT and ACH50 activities.

Compliance with ethical standards

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Conflict of interest

The authors declare that they have no conflict of interest.

Appendix 1

Table 2
Zebrafish Cumulative mortality (n = 21, each concentration) exposed to acute $AgNO_3$.

$AgNO_3$ lethal concentrations (ppm)	Mortality (No.)				
	Number of samples	24 h	48 h	72 h	96 h
0	21	0	0	0	0
0.1	21	0	0	0	2
0.25	21	0	2	3	10
0.5	21	2	4	6	17
1	21	2	7	13	21
2	21	4	8	16	21

Table 3
Calculation of LC_{50} for Zebrafish exposed to $AgNO_3$.

	Concentration (ppm)			
	24 h	48 h	72 h	96 h
LC_{10}	1.27 ± 0.005	0.40 ± 0.001	0.16 ± 0.001	0.09 ± 0.001
LC_{30}	2.35 ± 0.005	1.40 ± 0.001	0.74 ± 0.001	0.22 ± 0.001
LC_{50}	3.10 ± 0.005	2.09 ± 0.001	1.15 ± 0.001	0.31 ± 0.001
LC_{70}	3.85 ± 0.005	2.77 ± 0.001	1.55 ± 0.001	0.40 ± 0.001
LC_{90}	4.94 ± 0.005	3.77 ± 0.001	2.13 ± 0.001	0.53 ± 0.001
LC_{99}	6.43 ± 0.005	5.14 ± 0.001	2.94 ± 0.001	0.71 ± 0.001

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