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ANIMAL SCIENCE

Blood cell alterations in *Colossoma macropomum* juveniles caused by silver nanoparticles

JULIANA O. MENESES, FERNANDA DOS SANTOS CUNHA, JOEL ARTUR R. DIAS, NATALINO DA COSTA SOUSA, MÁRCIA VALÉRIA S. DO COUTO, ANA FLÁVIA S. DA CUNHA, PETERSON EMMANUEL G. PAIXÃO, HIGO A. ABE, VICTOR RUAN S. NASCIMENTO, JULIANA C. CARDOSO, LUIZ P. DA COSTA & RODRIGO Y. FUJIMOTO

Abstract: This study evaluated the median lethal concentration of silver nanoparticles and their effects in fish tambaqui *Colossoma macropomum*. Therefore, an acute toxicity assay was carried out in completely randomized design evaluating six different concentrations of silver nanoparticles on blood parameters of tambaqui. The silver nanoparticles were produced by chemical reduction with polyvinyl alcohol (AgNP-PVA). The lethal concentration 50% (LC50) was estimated using probit regression. The blood was collected, analyzed and the data were submitted to T-test (dying x surviving fish) and Tukey test (surviving fish). An increase in glucose, hematocrit, total plasma protein, hemoglobin, erythrocytes, leukocytes, monocytes, and neutrophils as well as reduced MCV (mean corpuscular volume) in dying fish compared to surviving fish were observed. Survived fish exposed to 187.5 µg/L showed an increase in hematocrit, MCV, and MCH and a reduction in erythrocytes, total numbers of leukocyte, thrombocyte, lymphocyte, and neutrophil. The fish exposed to concentrations below 125 µg/L, had returned the blood parameter to baselines compared to control. The estimated LC50 was 165.09 µg/L and was classified as highly toxic for the fish tambaqui. In higher concentrations, it causes an acute respiratory toxicity, but in concentrations below 125 µg/L, the fish can adapt to the stressing agent.

Key words: Aquaculture, biotechnology, hematology, nanotechnology.

INTRODUCTION

The fish tambaqui *Colossoma macropomum* stand out as the second most reared and commercialized fish in Brazil. However, attached to this crescent captivity production, there is a crescent concerning about fish diseases (fungi, bacteria and viruses) due to intensification of production (Marques et al. 2020, Hilsdorf et al. 2022).

The use of antibiotics and xenobiotics, such as malachite green, methylene blue, or copper sulfate, are frequently used by fish farmers to control diseases in aquaculture. However, they do not use them properly, sometimes by inadequate concentration or time of exposure, resulting the selection of resistant pathogens and carcinogenic or toxic effects to the aquatic organisms (Huang et al. 2015, Lafferty et al. 2015, Luis et al. 2019). Malachite green could be cited in this context, since its misused leads to a quickly intoxication causing uncoordinated moves and provoking physiological alteration of the liver (Sudova et al. 2007)

Nowadays, different studies are looking for eco-friendly alternatives to control fish diseases

like fungal or bacterial infection. Particularly, the use of nanotechnology in aquaculture can provide specific alternative to conventional treatments. Its potential is mainly related to the nano size (<100nm) and smart delivery of substances being more efficient for medicinal procedures (Cao et al. 2015, Rather et al. 2016, Sharma & Langer 2014, Khosravi-Katuli et al. 2017, Vijayakumar et al. 2017, Luis et al. 2019). Among the nanotechnology products, the metallic nanoparticles, including silver, gold and copper are highlighted due their antimicrobial potential against bacteria, fungi, viruses, and parasites (Márquez et al. 2018, Yunus et al. 2019, Romo-Quiñonez et al. 2020, Shaalan et al. 2020). However, few studies have been conducted about the toxicity of metallic nanoparticles for aquatic organisms (mainly tropical species) (Ramachandran et al. 2018, Botha et al. 2015).

The acute toxicity assay are important tools to evaluate the new therapeutic products to aquaculture ensuring safe use, adequate handling, and mitigation of side effects of disease control (Sonone et al. 2020). Allied to acute toxicity assay, hematological studies have been conducted to better understanding of any toxic effect on organism (Shaluei et al. 2013, Couto et al. 2018, Yunus et al. 2019).

Therefore, the objective of this study was to evaluate the lethal concentration 50% ($LC_{50\text{-}96h}$) of silver nanoparticles stabilized with polyvinyl alcohol (AgNP-PVA), and their effects on hematological parameters and survival of juvenile tambaqui (*Colossoma macropomum*).

MATERIALS AND METHODS

Characterization of nanoparticle

The AgNP-PVA used in the toxicity test was produced by Meneses et al. (2021). They had a spheroidal form (5.54 ± 2.27 nm) (transmission electron microscopy) with plasmon band in the range of 396–407 nm, zeta potential of -19.6 ± 0.9 mV (less than -30 mV), and the stock solution concentration of 2500 mg/L of silver nanoparticle.

Acclimatation

Juvenile tambaqui *Colossoma macropomum* (3-4cm and 4-6g) were acclimated for 10 days according to Ibama (1987) in 2 tanks (500 L) at stocking density of 0.4 fish/L. The tanks had mechanic and biological filters, forced aeration, and a heater adjusted to 30 °C. In this period, the fish were fed once a day *ad libtum* with a commercial extruded feed for omnivorous fish (32% protein). During the acclimation, the water parameters of temperature (°C – equipment YSI® model 55-12FT), dissolved oxygen (mg/L- YSI® 55-12FT), pH (AKROM® modelo KR20), and electrical conductivity (µS/cm- YSI® 30-10FT) were determined daily. Total ammonia concentration (mg/L- HANNA® HI93715) was measured at the beginning and end of the experiment.

Acute toxicity test

Prior to the acute toxicity assay, screening and sensitivity tests were conducted according to Claudiano et al. (2012) and Florêncio et al. (2014), respectively. The ethical committee of Tiradentes University approved the present study (CEUA/030318R).

The toxicity test was conducted in a static system without water exchange. Water quality parameters, including temperature (27.66 ± 0.52 °C), dissolved oxygen (5.40 ± 0.84 mg/L), pH (6.56 ± 0.24), electrical conductivity (147.83 ± 16.49 µS/cm), and toxic ammonia (0.004 ± 0.001 mg/L), were monitored and remained adequate for this fish species following the recommendation of Brandão et al. (2004).

The acute toxicity test was performed in a completely randomized design with one control and five concentrations of AgNP-PVA (62.5, 125,

187.5, 250, and 312.5 µg/L) in triplicate. Each experimental unit contained five fish (mean biomass of 33.8 ± 0.70 g). The exposure time was 96 hours, and the animals were fasted during the experiment. The mortality was evaluated, and blood parameters were determined.

Blood parameters

Hematological analysis was conducted on the dying fish (minimal swimming, loss of response to stimuli, and minimum opercular beat frequency) throughout the trial time, as well as surviving fish at the end of the experiment (96 h).

The fish were anesthetized (60 mg/L eugenol) and the blood collected by caudal vein puncture with sterilize syringes containing 10% EDTA. The blood smears were stained with *Newprov*® *Panotic* to determine thrombocytes, erythrocytes, and total leukocytes (Tavares-Dias & Moraes 2004, Ranzani-Paiva et al. 2013). The total number of erythrocytes (cell × 10^6/µL) (Neubauer chamber) (Garcia-Navarro 2005), hematocrit percentage (microhematocrit method) (Goldenfarb et al. 1971), hemoglobin concentration (g/dL, LAB-TP 6000 PLUS®), glucose concentration (mg/dL, Accu Chek® Active), total protein plasma levels, and hematimetric indexes (mean corpuscular volume – MCV; mean Corpuscular hemoglobin – MCH; and mean corpuscular hemoglobin concentration – MCHC) were determined according to Vallada (1999).

Statistical analysis

The median lethal concentration ($LC_{50:96h}$) was estimated using the probit regression model. To evaluate the acute toxicity effect of AgNP, a comparison of blood data between dying and surviving fish (96 h according to Islam et al. [2017]) was carried out through the T-test for independent samples (unilateral p=0.05). However, the treatment 250 µg/L was not included in this analysis due the low number (n=1) of surviving fish.

In addition to the acute effect, a possible adaptive effect on fish was evaluated by comparing the hematological data of surviving fish in the treatments. The data were submitted to the premises of Shapiro Wilk normality and Levene homoscedasticity tests followed by analysis of variance (ANOVA) and Tukey post-test ($p<0.05$) for the means comparison with the aid of BioEstat 5.3 and Past software (Zar 2009).

RESULTS

In the acute toxicity test, an increase in the number of dead fish was observed with the increased AgNP-PVA concentration and exposure time (Table I). The median lethal concentration ($LC_{50:96h}$) was 165.09 µg/L, with lower and upper limits of 141.04 µg/L and 189.14 µg/L, respectively. The fish exposed to the two higher concentrations (250 and 312.5 µg/L) presented irregular breathing and sudden circular movements at the water's surface, during the first minutes of exposure. After a period of 30 to 60 min, they remained at the bottom of the tank, exhibited increased opercular frequencies, lost equilibrium, and eventually died.

Hematological analysis

The moribund fish (62 to 187.5 µg/L at 96 h) exhibited increased blood glucose, hematocrit, total plasma protein, hemoglobin, erythrocytes, leukocytes, monocytes, neutrophil and MCV values comparing to surviving fish (Figure 1 and 2).

The surviving fish (62.5, 125, and 187.5 µg/L) after 96 h of nanoparticle exposure displayed no significant difference of blood glucose, hemoglobin concentration, total protein plasma level, MCHC, and monocytes ($p>0.05$, Figures 3 and 4). However, hematocrit, erythrocyte, MCV,

Table I. Accumulated mortality of tambaquis exposed to acute toxicity test with AgNP-PVA over time (n=15 for each concentration tested).

AgNP-PVA Concentrations	Accumulated mortality (%)			
	24h	48h	72h	96h
Control	0	0	0	0
62.5 **µg/L**	0	0	0	0
125 **µg/L**	6.66	26.66	26.66	33.33
187.5 **µg/L**	46.66	53.33	53.33	60.00
250 **µg/L**	86.66	93.33	93.33	93.33
312.5 **µg/L**	100	100	100	100

and MCH values showed significant differences among the treatments ($p<0.05$). At concentration of 187.5 µg/L, the fish presented increased hematocrit, MCV, and MCH and reduced erythrocytes. At this concentration, a reduction in the total leukocyte number, thrombocyte, lymphocyte, and neutrophil also was observed (Figures 3 and 4). In concentrations below 125 µg/L, the blood parameter of surviving fish returned to baselines compared to control, except to erythrocytes, MCV, and neutrophils.

DISCUSSION

Nanomaterials have been used due to their numerous applications in many areas, including medicine, textile industry, food, agriculture, and the gas and oil industry (Islam et al. 2017, Prasad 2017, Singh et al. 2017, Bajpai et al. 2018, El- Sayed & Kamela 2020). However, due to this increased use, concern has grown about the dangers that these nanoparticles posed to the environment. Silver nanoparticles (AgNP) are among the most used. They have been evaluated to control pathogens and improve the water quality in aquaculture (Dasgupta et al. 2017, Ismail et al. 2017). However, to ensure their safe use, toxicological studies should be carried out to elucidate their toxic effect to living organisms with the objective of predicting or controlling their nanotoxicity (Abramenko et al. 2018).

In the present study, the median lethal concentration of silver nanoparticles was 165.09 µg L^{-1}, which was classified as highly toxic for tambaqui *Colossoma macropomum* according to Zucker (1985). The LC_{50} of present work was higher than the $LC_{50:48h}$ of 84 µg/L AgNP- PVP (Polyvinylpyrrolidone, 30–40 nm particle size) for zebrafish (*Danio rerio* -0.42 ± 0.04 g) (Bilberg et al. 2012). For 18 g of *Cyprinus carpio*, the LC_{50} of silver nanoparticles (Nanosil®) (LC_{50}= 73.8 mg/L) was higher than present study (Hedayati et al. 2012a). This variability of acute toxicity depends on many variables including size, age, and health condition of the organisms tested (Hedayati et al. 2012b), as well as the stabilizing agent used in nanoparticle production (Bilberg et al. 2012). The data indicate a relationship between the sensitivity of organism and their weight.

The small size of the nanoparticles is the reason for their toxicity to fish. The high area:volume ratio of the nanoparticles allows interaction with living cells, releasing silver ions into the cell, causing intracellular damages (Wijnhoven et al. 2009, Bianchini & Wood 2003). Erratic swimming, quick breathing, and constant agitation of fish were observed when exposed to nanoparticles. This cell–nanoparticle interaction

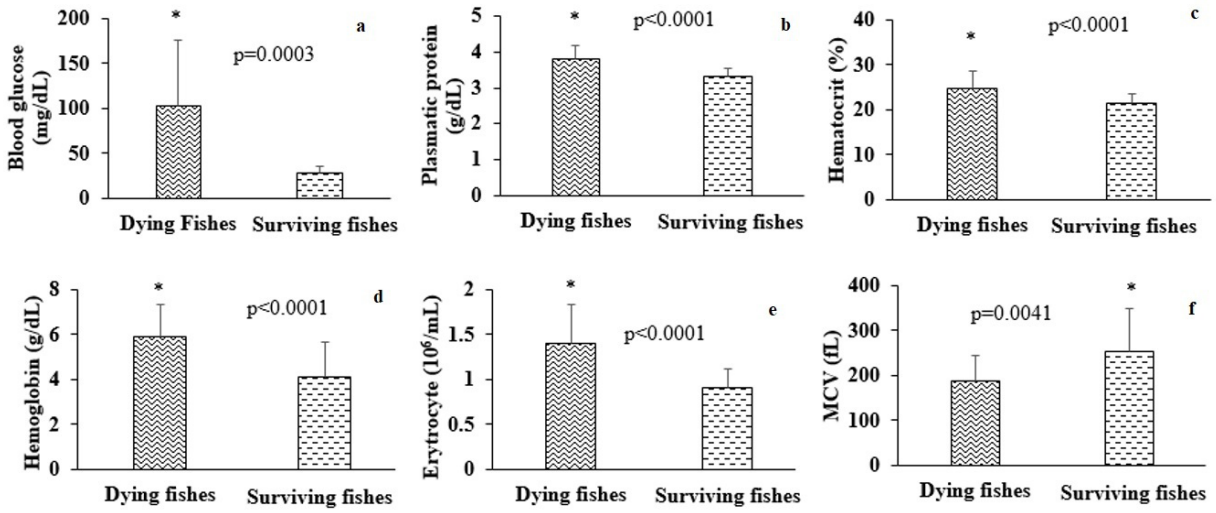

Figure 1. Red blood cells and biochemical parameters between dying and surviving fish tambaqui after acute toxicity test using silver nanoparticle-polyvinyl alcohol (AgNP-PVA). a. Blood glucose; b. Plasmatic protein; c. Hematocrit; d. Hemoglobin; e. Erytrocyte; f. MCV. (*): statistical difference for independent samples by t-test (p=0.05).

have caused an immediate respiratory toxicity in the gills, with subsequent loss of equilibrium through the time, and consequently death 24-48 hours at the highest concentration. This respiratory toxicity has been reported in the scientific literature for zebrafish (*Danio rerio*) (Bilberg et al. 2012) and catfish (*Clarias batrachus*) (Pandit & Sinha 2018).

The acute respiratory toxicity indicated by the fish behavior and cited by Bilberg et al. (2012) was corroborated by hematological response of tambaquis. Dying fish presented changes in erythrogram with increased number of erythrocytes, hemoglobin, and hematocrit and reduced mean corpuscular volume (MVC). These changes are associated with increased demand for oxygen to compensate the impaired gas exchange, which can be attributed to the accumulation of nanoparticles in the cells and to the histological damage in gills, including lamellar fusion and aneurysm (Rajkumar et al. 2016).

This respiratory shock and hematological alterations also have been reported in toxicity studies after fish exposure to pollutants and heavy metals. Depending on the kind of metal and time of exposure, particularly the hemoglobin could be affected, reducing the oxygen carrying capacity. As commonly observed, the infection by heavy metal can cause an uncoordinated synthesis of blood cells from the hematopoietic system explaining problems like anemia status (blood loss, poor production or cell destruction) (Atamanalp et al. 2011, Ahmed et al. 2020, 2022).

In addition, reduced MCV and increased erythrocytes values (microcytosis) can indicate a high percentage of young cells in the circulation after short exposure to silver, and was observed with other metals, such as aluminum (Alwan et al. 2009). Imani et al. (2015) and Faiz et al. (2015) also reported this reduction in rainbow trout (*Oncorhynchus mykiss*) and carp (*Ctenopharyngodon idella*), respectively, exposed to zinc oxide nanoparticles.

This non-adaptative response is also reflected in leukocytes in dying fish, which presented leukocytosis, monocytosis, and neutrophilia, which are related to a non-adaptation stress phase (Hedayati et al. 2015, Meneses et al. 2020). The increase in these cells

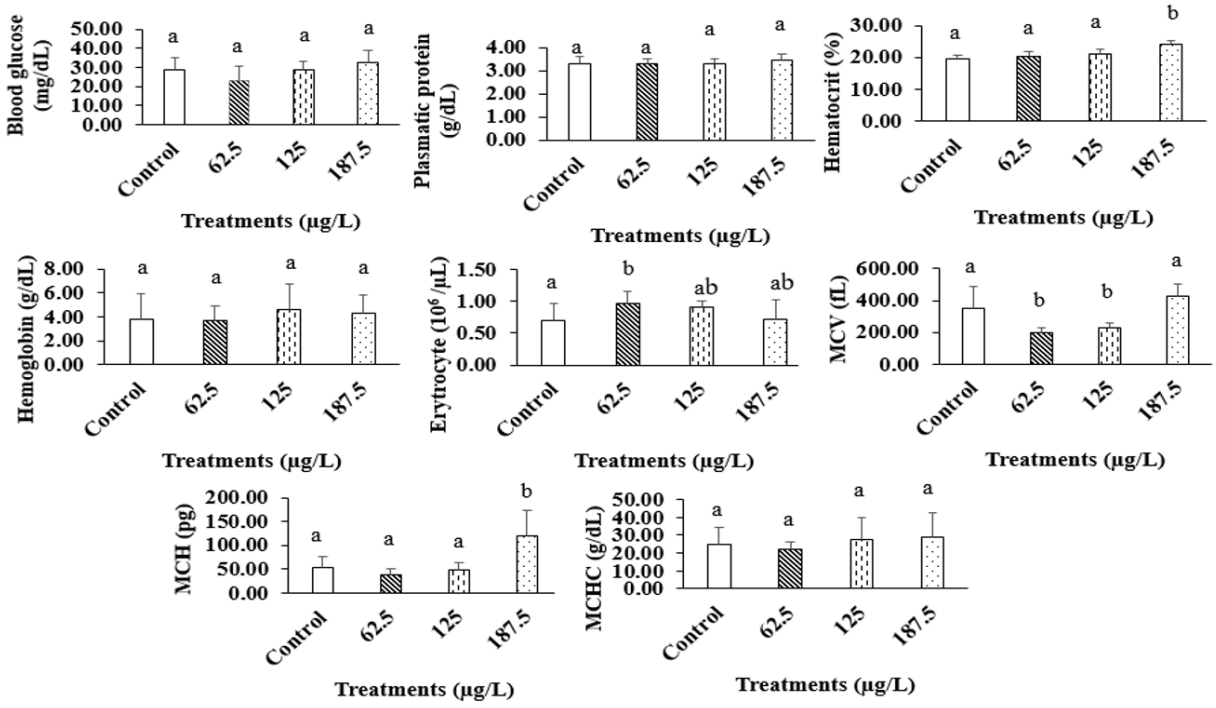

Figure 2. Red blood cells and biochemical parameters of surviving fish tambaqui exposed to different concentrations of silver nanoparticle-polyvinyl alcohol (AgNP-PVA). The different lowercase letters indicate treatment differences by Tukey test at 5% of probability.

in dying fish after short-term exposure of silver nanoparticles is a reaction to the immediate stimulating effect on the immune system, which also adversely affects the innate immune system as reported by Shaluei et al. (2013) and Imani et al. (2015). This, associated with all other erythrogram alterations, demonstrates that fish suffer excessive stress and acute respiratory shock when exposed to high concentrations of AgNP, reaching death in 24 - 48 h.

This acute stress condition verified by cellular alterations is also demonstrated by increase of blood glucose and total plasma protein in dying fish, which reflects the stressful condition. The fish try to mobilize glucose and plasmatic proteins to supply their energetic demands to maintain their physiological responses (McDonald & Milligan 1997, Martins et al. 2002, Martinez et al. 2004). These responses have also been observed in *Carassius auratus* exposed to silver nanoparticles at a concentration of 0.1 to 0.4 mg/L (Imani et al. 2015).

At lower concentrations over time, the surviving fish showed an adaptive response, returning to normal hematological values compared to the control group. However, the treatment with the concentration of 187.5 µg/L still induced higher values of erythrocytes, hematocrit, and hematimetric indexes, including MCV and MCH, probably associated to erythrocyte swelling due to metal toxicity (Sharma & Langer 2014). Increased MCH values have been observed in carp (*Cyprinus carpio*) (Vali et al. 2020), and increased MCH and MCV values were observed in silvered carp (*Hypophthalmichthys molitrix*) (Shaluei et al. 2013) exposed to AgNP.

In the intermediate treatments (62.5 and 125 µg/L), the erythrocytes and leukocytes returned to normal values (compared to the control), but there was still a reduction in leukocytes in the treatment with 187.5 µg/L. Thus, a loss

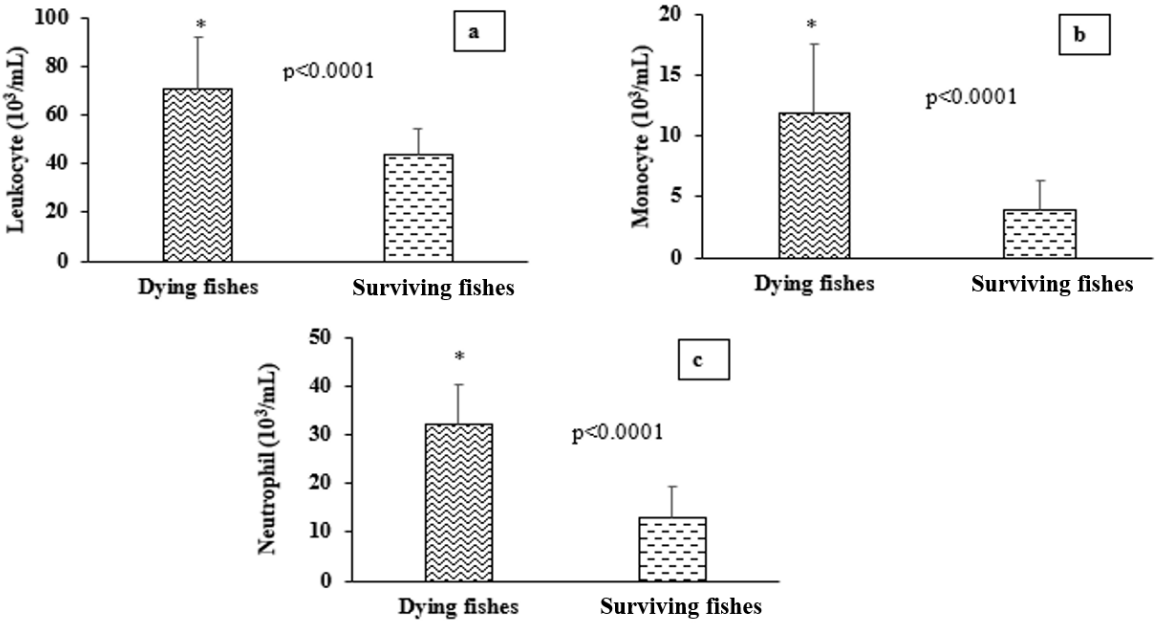

Figure 3. Leukocytes of dying and surviving fish tambaqui after acute toxicity test using silver nanoparticle-polyvinyl alcohol (AgNP-PVA). a. total of leukocytes; b. monocytes; c. neutrophil; (*): statistical difference for independent samples by t-test (p=0.05).

of defense capacity of the organisms was observed in this treatment, which may be due to nanoparticles absorbed by leukocytes as well as immunosuppression and intracellular cytotoxicity induced through the release of Ag^{+} that is highly toxic in cells (Kettler et al. 2016).

Carp submitted to sublethal concentrations of silver nanoparticles showed increased concentration of leukocytes in times soon after exposure; however, in the highest concentration there was a reduction (Vali et al. 2020), which is a similar response to the surviving fish in the present study. Monocytosis and neutrophilia were also observed in the surviving fish, accompanied by an increase in thrombocyte and lymphocyte (62.5 and 125 µg/L). However, a reduction of thrombocyte and lymphocyte were observed in treatment with 187.5 µg/L. Lymphocyte is one of the most important immune response cells, and stressful conditions can cause lymphopenia in fish. Ale et al. (2018) observed lymphopenia together with neutrophilia in curimbatás (*Prochilodus lineatus*) exposed to 25 µg/L of AgNP.

Thrombocytes perform homeostatic buffering or coagulation and are important blood defense cells (Hill & Rowley 1996). Their return to normality in the lower treatment compared to the control group indicates an adaptive response.

In a toxicity study with silver nanoparticles using lower concentrations than the present study (0.02 mg/L), the total leukocytes were also returned to normality after 3 days of exposure (Shalue et al. 2013), indicating an adaptive response. Thus, the tambaqui exposed to lower concentrations (62.5 and 125 µg/L) of silver nanoparticles had an adaptive effect indicated by lower mortalities and hematological parameters that returned to normal level.

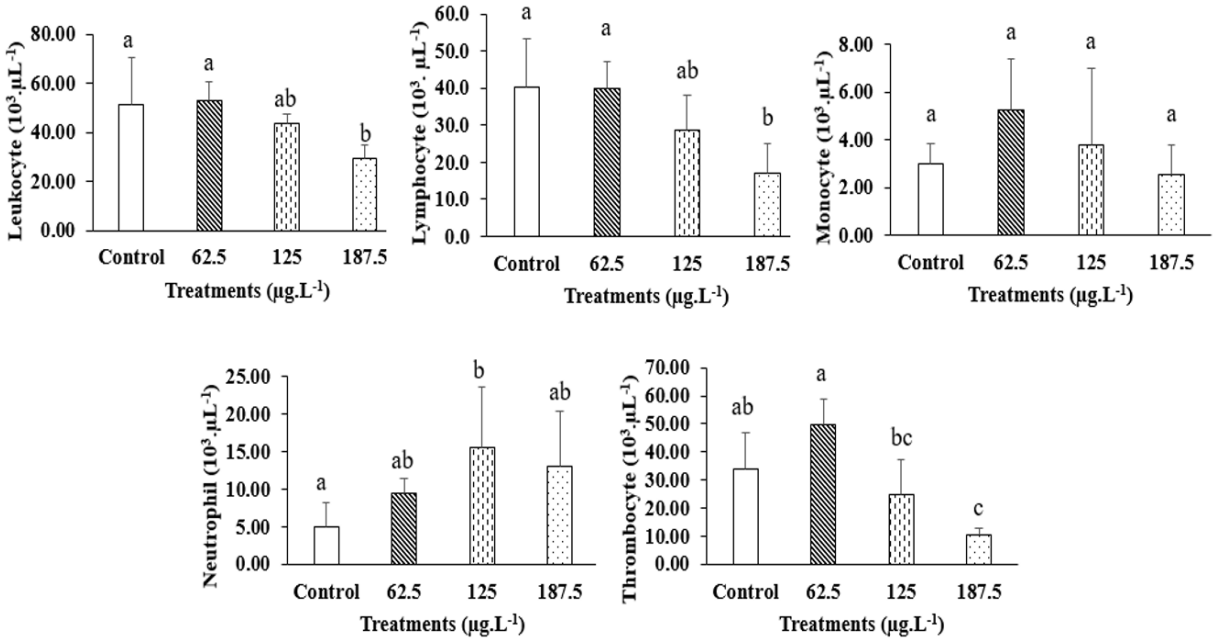

Figure 4. White blood cells and Thrombocytes of surviving fish tambaqui exposed to different silver nanoparticle-polyvinyl alcohol (AgNP-PVA) concentrations. The different lowercase letters indicate treatment differences by Tukey test at 5% of probability.

CONCLUSION

The mean lethal concentration (LC_{50}) of silver nanoparticles stabilized with PVA was 165.09 µg/L, which is classified as highly toxic for tambaqui. In higher concentrations, it causes an acute respiratory toxicity but, in concentrations below 125 µg/L the fish presents an adaptation to the stressing agent, returning to normal hematological values.

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JULIANA O. MENESES[1]
https://orcid.org/0000-0002-8680-512X

FERNANDA DOS SANTOS CUNHA[1]
https://orcid.org/0000-0003-2883-7542

JOEL ARTUR R. DIAS[2]
https://orcid.org/0000-0001-9751-0207

NATALINO DA COSTA SOUSA[2]
https://orcid.org/0000-0002-7580-5650

MÁRCIA VALÉRIA S. DO COUTO[2]
https://orcid.org/0000-0001-9235-0996

ANA FLÁVIA S. DA CUNHA[3]
https://orcid.org/0000-0001-9277-3980

PETERSON EMMANUEL G. PAIXÃO[1]
https://orcid.org/0000-0002-8949-4232

HIGO A. ABE[2]
https://orcid.org/0000-0002-5717-9641

VICTOR RUAN S. NASCIMENTO[4]
https://orcid.org/0000-0002-1150-332X

JULIANA C. CARDOSO[1,5]
https://orcid.org/0000-0002-0357-4144

LUIZ P. DA COSTA[6]
https://orcid.org/0000-0002-2632-6485

RODRIGO Y. FUJIMOTO[7]
https://orcid.org /0000-0002-5039-4445

[1]Pós-Graduação em Saúde e Ambiente, Universidade Tiradentes, Avenida Murilo Dantas, 300, 49032-490 Aracaju, SE, Brazil

[2]Pós-Graduação em Ciência Animal, Universidade Federal do Pará, Rua Augusto Côrrea, 1, 66075-110 Belém, PA, Brazil

[3]Pós-Graduação em Biotecnologia, Universidade Tiradentes, Avenida Murilo Dantas, 300, 49032-490 Aracaju, SE, Brazil

[4]Pós-Graduação em Engenharia de Processos, Universidade Tiradentes, Avenida Murilo Dantas, 300, 49032-490 Aracaju, SE, Brazil

[5]Universidade Tiradentes, Instituto de Tecnologia e Pesquisa (ITP), Avenida Murilo Dantas, 300, 49032490, Aracaju, SE, Brazil

[6]Universidade Federal do Amazonas, Instituto de Ciências exatas e Tecnologia (ICET), Avenida General Octavio Jordão Ramos, 1200, 69067-005 Manaus, AM, Brazil

[7]Empresa Brasileira de Pesquisa Agropecuária, Laboratório de Aquicultura, Avenida Beira Mar, 3250, 49025-040 Aracaju, SE, Brazil

Correspondence to: **Rodrigo Yudi Fujimoto**
E-mail: ryfujim@hotmail.com

Author contributions

Juliana Oliveira Meneses (writing the paper, conducting the bioassay, review of literature, analysis of data, review of the paper), Fernanda dos Santos Cunha, Joel Artur Rodrigues and Ana Flávia Santos da Cunha Dias (conducting the bioassay, review of literature), Natalino da Costa Sousa, Márcia Valéria Silva do Couto, Peterson Emmanuel Guimarães Paixão, Higo Andrade Abe and Victor Ruan Silva Nascimento (hematological analysis, review of literature), Juliana Cordeiro Cardoso and Luiz Pereira da Costa (review of the paper, review of the english language, statistical analysis), Rodrigo Yudi Fujimoto (writing the paper, coordinator of the project, review of the data, review of the paper).