Na_2EDTA anticoagulant impaired blood samples from the teleost *Piaractus mesopotamicus*[1]

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ABSTRACT.- Farias T.H.V., Pereira N.L., Pádua S.B., Alves L.O., Sakabe R., Belo M.A.A. & Pilarski F. 2016. **Na_2EDTA anticoagulant impaired blood samples from the teleost *Piaractus mesopotamicus*.** *Pesquisa Veterinária Brasileira 36(5):431-435.* Centro de Aquicultura da Universidade Estadual Paulista, Rodovia Paulo Donato Castellane s/n, Jaboticabal, SP 14884-900, Brazil. E-mail: lapoa_caunesp@hotmail.com

The present study aimed to evaluate the effects of Na heparin and Na_2EDTA on blood of *Piaractus mesopotamicus* (360.7±42.4g, 26.4±1.0cm). Twenty fishes were sampled in two experiment trials, ten for erythrocyte fragility analysis and ten for hematologic and plasma biochemical study. The blood collected by venous-caudal puncture was fractioned and stored in anticoagulants solution: Na_2EDTA 10%, Na_2EDTA 3%, Na heparin 5000 IU and Na heparin 100 IU. Plasmatic levels of calcium presented in the Na_2EDTA stored samples were about 80% lower than both heparin groups. Blood samples of *P. mesopotamicus* stored with Na_2EDTA demonstrated increase in the hematocrit and MCV, and decrease in MCHC. The dose-response effect was observed in this study. The results are reinforced by the higher levels of plasmatic protein and hemolysis presented in the Na_2EDTA 10% stored blood, confirming the deleterious effect of this anticoagulant treatment on the quality of blood samples. Na_2EDTA is not indicated to store *P. mesopotamicus* blood samples, but sodium heparin at 100 IU is the most recommended anticoagulant, since this treatment presented the lower rate of alterations in the stored blood.

INDEX TERMS: Fish hematology, blood clotting, EDTA, Heparin, *Piaractus mesopotamicus.*

RESUMO.- [**Anticoagulante Na_2EDTA danifica amostras de sangue do teleósteo *Piaractus mesopotamicus*.**] O presente estudo teve por objetivo avaliar os efeitos da heparina sódica e Na_2EDTA no sangue *de Piaractus mesopotamicus* (360,7±42,4g, 26,4±1,0cm). Foram amostrados vinte peixes em dois ensaios experimentais, sendo dez peixes utilizados para análise da fragilidade dos eritrócitos e dez peixes para análise dos parâmetros hematológicos e estudo bioquímico do plasma. O sangue coletado por punção veno-caudal foi aliquotado e armazenado em diferentes soluções anticoagulantes: Na_2EDTA 10%, Na_2EDTA 3%, heparina sódica 5.000 UI e heparina sódica 100 UI. Níveis plasmáticos de cálcio apresentados nas amostras armazenados em Na_2EDTA diminuíram cerca de 80% em relação aos dois grupos armazenados com heparina. Amostras de sangue de pacus armazenados com Na_2EDTA demonstraram aumento do hematócrito e VCM, e diminuição na CHCM. O efeito dose-resposta foi observado neste estudo. Estes resultados são reforçados pelos níveis mais elevados de proteína plasmática e hemólise apresentado no sangue armazenado com Na_2EDTA 10%, o que confirma o efeito deletério desse tratamento anticoagulante na qualidade de amostras de sangue. Na_2EDTA não é indicada para armazenar amostras de sangue de *P. mesopotamicus*, e heparina sódica a 100 UI é o anticoagulante mais recomendado, uma vez que este tratamento apresentou a menor taxa de alterações no sangue armazenado.

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TERMOS DE INDEXAÇÃO: Hematologia de peixes, coagulação sanguínea**,** EDTA, Heparina, pacus, *Piaractus mesopotamicus.*

INTRODUCTION

Although blood parameters are routinely used to determine the health status of fish and to monitor their physiological conditions, almost the whole methodology is adapted from other animals such as mammals, resulting in some certain difficulties since the blood sampling until the analysis processes. Also, it is know that fish blood parameters can be influenced by several factors, such as seasonal variations, genetics, age, gender, stock density, stress and water quality (Ekanen et al. 2012, Belo et al. 2013, Castro et al. 2014a), what could explain the lack of information on the reference values of health and ill fish.

Among the alterations, several can be due to post--sampling proceedings, in especial the misuse of anticoagulants. The blood coagulation is one of the main causes of the quality deterioration of a hematological analysis, also being responsible for alterations in the immunological and biochemical parameters (Pádua et al. 2012). The most used anticoagulants for fish are the sodium heparin and the sodium ethylendiamintetracetic acid (NA_2EDTA). The heparin acts accelerating the action of antithrombin III, inhibiting the action of thrombin and, consequently, the continuity of the coagulation cascade. On the other hand, the EDTA is able to chelate ions, including calcium (Ca2+), important in several steps of the blood clotting (Harr et al. 2005). However, according to Ishikawa et al. (2010), both anticoagulants can promote changes on the blood parameters in teleost fish.

The pacu (*Piaractus mesopotamicus*), an emergent species in the world aquaculture and considered a good experimental model, is a native teleost fish of the Parana--Paraguay Basin, and is of importance in the South America for human consumption, angling and aquaculture (Belo et al. 2005, 2012). The pacu has proven to be a good bioindicator of water quality, and in accordance with Castro et al. (2014b) this species has been used in ecotoxicity studies for registration of chemicals in Brazil, and no information about the effects caused by the use of anticoagulants on the hematological and biochemical parameters has been related in the literature. Hence, the present study aimed to evaluate the effects of different concentrations of Na_2EDTA and sodium heparin on the osmotic fragility of erythrocytes and some routinely blood analysis in order to determine which anticoagulant is more indicated to this species.

MATERIALS AND METHODS

Fish and experimental conditions. In the present study were used 20 specimens of *Piaractus mesopotamicus* juveniles (360.7g±42.0 g) obtained from a commercial fish farm (Sertãozinho, São Paulo, Brazil). The animals were maintained in 500 L tanks for one month in order to acclimate to the experimental conditions, with continuous aeration and water change, under constant water quality parameters (temperature 26.0±2.0°C, pH 7.9±0.1, oxygen 6.7±0.69mg L^{-1}, ammonium 0.1±0.1mg L^{-1}). The animals were fed a commercial feed (32% of crude protein), twice a day, until the apparent satiation.

Compliance with Ethical Standards. The experimental protocol was approved by Ethics Committee on the Use of Animals from São Paulo State University, process nº22.518/10.

Blood sampling and anticoagulant test assay. Ten fish were captured using a net and mechanically immobilized in a wet piece of fabric. The sampling of the blood of each fish was realized through veno-caudal puncture, using sterile syringes (3mL) and hypodermic needles (25x7mm), containing no anticoagulants, as recommended by Ishikawa et al. (2010). Trying to not promote any hemolitic effects besides the possibly caused by the anticoagulants, we decided to use no anesthetic chemicals (Korcock et al. 1988). The blood collected, circa of 0.5mL, was quickly placed in four polyethylene tubes (1.5mL): The first and second contained 15µL of NA_2EDTA, at a concentration of 3% (0.3mg mL^{-1} of blood) and 10% (1mg mL^{-1} of blood), respectively. At last, the third and fourth tubes contained 15 µL of sodium heparin at concentration of 5.000 UI (75 UI mL^{-1} of blood) and 100 UI (1.5 UI mL^{-1} of blood), obtained from the dilution of the heparin 5.000 UI in physiological solution (0.65% of NaCl, 1:50) (Ishikawa et al. 2010).

Total blood NBT assay. The reactive oxygen species (ROS) production of the blood leukocytes was evaluated using nitroblue tetrazolium (NBT) reduction following the method of Biller--Takahashi et al. (2013). Briefly, 100 µL of blood mixed with the anticoagulant was added to 100 µL of phosphate-buffered-saline (pH 8.4) containing NBT (Sigma, USA). After 30 minutes of incubation in the dark, 50 µL of this solution was added to 1mL of N,N-dimethylformamide (Sigma, USA), shaken, and centrifuged at 700 g for 10 minutes. The optical density of the supernatant was measured in a conventional spectrophotometer, at a wave length of 540nm.

Plasmatic protein and ionic analysis. The remaining blood from the hematological analysis was centrifuged at 300g during 10 minutes and the plasma obtained was used to the determination of the plasmatic protein and plasmatic chloride (Labtest®, Lagoa Santa, MG, Brazil). In order to determine the plasmatic concentration of calcium, sodium and potassium was used a selective ion analyzer (Iselab® Drake, São José do Rio Preto/SP, Brazil).

Osmotic fragility of erythrocytes and coagulation efficacy assay. To the determination of the osmotic fragility of the erythrocytes were used the ten remaining animals in the tank, and blood sampling procedure followed the same as described above. Dilutions were made in serial from the stock solution (PBS, pH 7.4, 10.5%), in the following concentrations: 0.65, 0.54, 0.43, 0.32, 0.21 e 0.10% of NaCl-PO_4, as described by Parpart et al. (1947). The blood was diluted in 1:100 concentration in each one of the NaCl-PO_4 solutions, stored at room temperature for 30 min and homogenized each 10 min. After this, the solution was centrifuged at 2000 rpm for 5 min and the supernatant hemoglobin was determined according to Collier (1944). The rest of the blood was maintained at 6°C for 10h and, after this, visually evaluated in order to classify the occurrence of coagulation and/or hemolysis (Hattingh & Smith 1976).

Hematology. After maintained at room temperature (25°C) during 15 minutes, the blood was used to determination of the hematocrit following the technic of the microhematocrit, hemoglobin by the method of the cyanomethahemoglobin, and red blood cell count (RBC) in hemocytometer after dilution of the blood in citrate formaldehyde (1:200) solution. The hematimetric indexes were calculated according to the equations of Wintrobe. Differential leukocyte count was performed on blood smears stained with May-Grünwald-Giensa-Wright counting 200 cells. For the quantification of total leukocytes and thrombocytes were counted the number of erythrocytes, leukocytes and thrombocytes in 10 fields for each blood smear. The values for total leukocytes and

thrombocytes were determined by the equation: Total number of leukocytes or thrombocytes (µL) = [(number of leukocytes or thrombocytes counted in the smear) X (erythrocyte global count per µL)]/ Number of erythrocytes counted in the blood smear (Belo et al. 2014).

Statistical analysis. To the statistical analysis of the data was performed a variance analysis (ANOVA) and, when significant ($p<0.05$), the means were compared using the Tukey's test. The data was transformed when necessary and all the statistical procedure was realized in R Software.

RESULTS

In the osmotic fragility of erythrocytes assay, the cells of the heparinized blood and containing NA_2EDTA 3% presented a higher resistance to the hypotonic solution when compared to the NA_2EDTA 10% group, which showed significant increase ($p<0.05$) in the cell lysis between 0.35 to 0.65% NaCl-PO_4 solution. These analyze showed dose-response effect (Figure 1). Blood containing NA_2EDTA 10% showed higher scores ($p<0.0001$) of hemolysis after 10 hours at 6° C (Table 1).

Blood biochemical parameters presented significant differences according to the anticoagulant used (Table 1). The values of plasmatic protein were higher in the samples containing NA_2EDTA 10% (p=0.,0088) when compared to NA_2EDTA 3% and Heparin 100 IU, but showed no differences when compared to the Heparin 5000 IU group.

The samples containing Heparin 100 IU showed significant increase ($p<0.0001$) in the plasmatic chloride values when compared to NA_2EDTA 3 and 10%, and blood containing heparin 5000 IU presented no differences from any of the groups (Table 1). On the other hand, blood containing

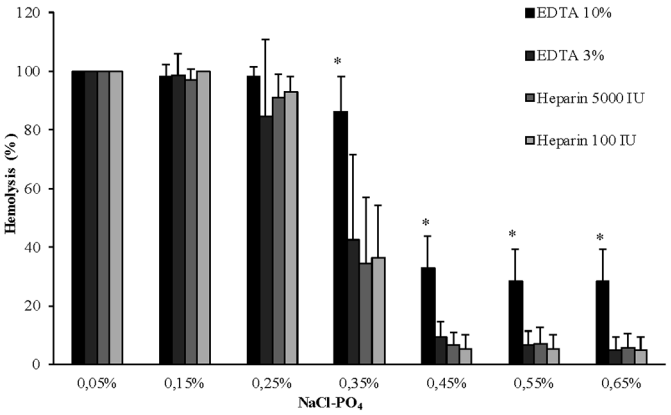

Fig.1. Osmotic fragility of erythrocytes of *Piaractus mesopotamicus* blood stored in different anticoagulants ($p<0.05$).

NA_2EDTA 10% presented the highest ($p<0.0001$) plasmatic sodium concentration, followed by the NA_2EDTA 3% and Heparin 5000 IU, while the lower values of plasma sodium were observed in blood containing Heparin 100 IU.

Samples with Heparin 100 IU showed low levels of plasma potassium when compared blood containing NA_2EDTA 3% (p=0.0198). The NA_2EDTA groups of samples (3 and 10%) presented significant decrease ($p<0.0001$) in the plasmatic calcium when compared to the Heparin groups. No differences were found among the groups of samples containing different anticoagulants in the total blood NBT assay (p=0.6640).

All the anticoagulants investigated in the present study were efficient in the prevention of the blood coagulation of *Piaractus mesopotamicus* for longer than 10 hours

Table 1. Mean values (± SEM)[a] and ANOVA[b] observed in the plasmatic protein, ions, NBT and hemolysis study of *Piaractus mesopotamicus* blood stored with different anticoagulants

Parameters	Na2EDTA		Heparin		*p*
	10%	3%	5000 UI	100 UI	
Plasmatic protein (g/dL)	3.31±0.54A	2.8±1.0B	3. 09±0.24AB	2.92±0.5B	0.0088
Chlorede (meq L-1)	99.43±4.8C	103.1±8.3BC	106.4±3.4AB	109.2±4.4A	<0.0001
Na (meq L-1)	150.2 a±5.96A	139±5.6B	137.5±5.42B	129.2±3.31C	<0.0001
K (meq L-1)	3.44±0.33AB	3.59±0.35A	3.42±0.55AB	2.99±0.45B	0.0198
Ca (meq L-1)	0.200±0.08C	0.226±0.09C	1.032±0.11B	1.216±0.05A	<0.0001
NBT assay (OD)	0. 3402±0.06	0.3615±0.14	0.3347±0.02	0.346±0.04	0.6640
Hemolysis Score (1-5)	2.70±0.25 A	0.42±0.15 B	0.50±0.17 B	0.50±0.17 B	<0.0001

[a] Mean values of 10 samples; SEM (standard error of the mean¬), [b] Means compared in the lines, with at least one letter in common do not differ from each other through Tukey's test (p>0.05).

Table 2. Mean values (± SEM)[a] and ANOVA[b] observed in the hematological study of *Piaractus mesopotamicus* blood stored with different anticoagulants

Parameters	Na2EDTA		Heparin		*p* value
	10%	3%	5000UI	100UI	
Ht (%)	36.9±3.1A	35.9±2.6A	31.9±1.7B	31.5±2.4B	< 0.0001*
Hemoglobin (g.dL-1)	10.2±1.0	10.3±0.8	10.5±0.8	10.2±0.8	0.8430
MCV (fL)	173.5±28.8A	147.5±20.5B	134.1±12.0B	124.2±18.0B	< 0.0001*
MCHC (g dL-1)	27.8±1.0B	28.6±1.3B	33.0±1.3A	32.5 ±2.0A	< 0.0001*
RBC (x 106 µL-1)	2.2±0.4	2.5±0.4	2.4±0.2	2.6±0.4	0.0993
Throm (x 103 µL-1)	71.7±15.5	73.3±11.2	63.6±11.6	68.4±7.3	0,1840
Leuk (x 103 µL-1)	28.9±5.9	36.3±9.0	37.4±5.4	33.7±2.2	0,3060

[a] Mean values of 10 samples; SEM (standard error of the mean¬), [b] Means compared in the lines, with at least one letter in common do not differ from each other through Tukey's test (p>0.05).

(Data not show). At the same time, thrombocyte, leukocyte and erythrocyte counts did not present difference (p>0,05) among treatments (Table 2). Blood samples containing NA_2EDTA (3% and 10%) presented increase in the hematocrit values ($p < 0.0001$) and decrease in the MCHC ($p<0.0001$) when compared to fish blood containing heparin (100 and 5000 IU). The same response profile was observed for MCV analyzes, but only the samples stored with NA_2EDTA 10% presented a significant increase on MCV ($p<0.0001$).

DISCUSSION

Despite the effectiveness of anticoagulants in the control of *Piaractus mesopotamicus* blood clotting process for longer than 10 hours, the results obtained in the present study shows that the hematological and biochemical parameters of *P. mesopotamicus* suffered considerable changes according to the anticoagulant used to the samples preservation.

Plasmatic levels of calcium presented in the Na_2EDTA stored samples of pacus were about 80 % lower than both heparin groups. The Na_2EDTA acts as a chelating to the anticoagulant factor VI (Ca^{2+}), responsible by the stability and permeability of the cell membrane (Harr et al. 2005), causing an increase of erythrocytes volume. Kaestner (2011) and Bogdanova et al. (2013) described the importance of Ca^{2+} as universal signaling molecule involved in regulating cell cycle, metabolism, structural integrity and volume, and changes in the extracellular levels of Ca^{2+} result in serious dysregulation of hydromineral balance. Since mechanisms of transport are calcium dependent such as Gardos channel recognized in erythrocytes. In this study, increase in sodium levels and decrease in choride was observed in samples stored with Na_2EDTA 10%, confirming this hypothesis. Hemolysis were observed in birds, reptiles (Hattingh & Smith 1976) and carps (Orlov et al. 2005) with poor extracellular levels of calcium, emphasizing the importance of this ion to the preservation of the membrane integrity of nucleated erythrocytes.

Blood samples of pacus stored with Na_2EDTA demonstrated increase in the hematocrit and MCV, and decrease in MCHC. The dose-response effect was observed and samples stored with Na_2EDTA 10% showed more significant results. In samples stored with Na_2EDTA 10% were observed decrease in RBC counts, although statistical difference did not occur among treatments. These results are reinforced by the higher levels of plasmatic protein and hemolysis presented in the Na_2EDTA 10 % stored blood, confirming the deleterious effect of this anticoagulant treatment on the quality of blood samples. In addition, the acidic nature of the EDTA salts can change the pCO_2 and pH of the blood (Witeska & Wargoka 2009) altering the Na^+/K^+ pump function, an important protein that regulates the osmotic gradient in cell (Meyer et al. 1995, Högman et al. 1997, Högman 1998). Also, in heparin 5000 IU samples, the increase of plasmatic protein probably comes from the fact that heparin is a protein, and this group received 50 times more of this anticoagulant then the heparin 100 IU samples.

The evaluation of the osmotic fragility of erythrocytes shows the resistance of these cells in front of osmotic stress simulated by different NaCl concentration solutions, and the degree of hemolysis is measured by the amount hemoglobin liberated by the rupture of the cells (Sarkar et al. 1999). Blood samples of pacus stored with Na_2EDTA 10 % presented increase in the cell lysis between 0.35 to 0.65% NaCl-PO_4 solution. Walencick & Witeska (2007) observed higher fragility in common carp erythrocytes to hipotonic solution since the lesser concentration of Na_2EDTA tests ($0.01mg\ ml^{-1}$) and a positive relation of the osmotic fragility and the increase of the anticoagulant concentrations. Similar results were described in *Blennius pholis* (Mainwaring & Rowley 1985), *Oncorhynchus mykiss* (Korcock et al. 1988), *Oreochromis niloticus* (Ekanen et al. 2012), *Pseudoplatystoma reticulatum* x *P. corruscans* (Ishikawa et al. 2010) and *Colossoma macropomum* (Pádua et al. 2012).

Reactive Oxygen Species (ROS) are substances with the ability to degrade membranes and are extremely aggressive to pathogens during the process of phagocytosis (John et al. 2002), and their production was not influenced by the anticoagulant levels in *P. mesopotamicus* blood, in the same way as observed by Nielsen (1995) in human blood samples stored in heparin, EDTA and oxalate. However, some studies have showed an increase in the ROS production due to the phagocytosis of the cellular debris resulted from the erythrocytes lysis when the blood was added to NA_2EDTA or heparin (Walencick & Witeska 2007, Wan et al. 1992). Although other studies have reported changes in leukocyte and thrombocyte counts in blood samples of *Cyprinus carpio* (Walencik & Witeska 2007) and *Blenius pholis* (Mainwaring & Rowley 1985) stored with EDTA and heparin, blood samples of pacus did not present difference in thrombocyte and leukocyte counts.

CONCLUSIONS

Na_2EDTA is not indicated to store *Piaractus mesopotamicus* blood samples, causing hematological and serum biochemistry alterations and hemolysis, principally when in a high concentration ($1mg\ mL^{-1}$).

On the other hand, sodium heparin at 100 IU is the most recommended anticoagulant, since this treatment presented the lower rate of alterations in the stored blood. Both anticoagulants can be used in total blood NBT assay.

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