



Effects of Sodium-Heparin and Dipotassium EDTA on the Haematological Parameters and Blood Cell Morphology of Freshwater Fish *Schizothorax labiatus* (McClelland, 1842)

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Abstract: The present study was conducted with an aim of evaluating the effects of sodium-heparin (20 I.U/ml) and di-potassium EDTA (1.0mg/ml) on the haematological parameters and blood cell morphology of freshwater fish *Schizothorax labiatus*. For blood sampling, fifteen fishes of uniform weight (250 ± 18.5 g) and length (25 ± 2.1 cm) were selected. All the haematological analysis were carried out immediately after sampling. An increase in the haematocrit level was observed in the samples collected with K_2 EDTA. Red blood cell counts were lower in K_2 EDTA collected samples as compared to heparinized samples. The WBC counts and haemoglobin concentrations did not differ significantly ($p > 0.05$). Mean corpuscular haemoglobin (MCH) and mean corpuscular volume (MCV) increased in K_2 EDTA sample, while a significant decrease ($p < 0.05$) was observed in mean corpuscular haemoglobin concentration (MCHC). Cell morphometric analysis showed an increase in the RBC area in samples treated with K_2 EDTA as compared to heparinized sample. There was however, no significant difference ($p > 0.05$) observed in WBC and thrombocyte area of K_2 EDTA and heparinized sample. The present study suggests the use of Na-heparin as a preferred anticoagulant for routine haematological analysis of fish blood.

Keywords: Sodium–heparin, Di-potassium EDTA, Haematological analysis, Cell morphology.

Introduction

In recent time haematological and immunological study of fish has assumed greater significance due to the increasing emphasis on fish culture and greater awareness of the pollution of natural fresh water resources (Blaxhall, 1972). Haematological and biochemical parameters are used as health indicators to detect the structural and functional status of fish under stress condition (Pimpao *et al.*, 2007; Suvetha *et al.*, 2010). The haematological characteristics have also been used as diagnostic tool to evaluating physiological changes including fish health (Satheeshkumar *et al.*, 2010.). Many automatic clinical tools have been developed for mammalian haematological analysis, but due to the unavailability of these automatic tools for fish, manual methods are employed.

The interpretation of blood parameters is quite difficult, since variations in the blood are caused by internal and external factors including anticoagulants used, method of analysis, the storage temperature, and the time lapse between sample analyses. Fish blood contains a large number of thrombocytes due to which it clots quickly, and samples almost always require anticoagulant treatment. Anticoagulants are additives that inhibit the clotting of blood and thereby ensure that the concentration of the substance to be measured is changed as little as possible before the analytical process (Guder, 2001). Blood cells of various animals show different reactions to various anticoagulants (Witeska and Wargocka, 2011). In fish haematology the most commonly used anticoagulants are different salts of heparin and EDTA (Walencik and Witeska, 2007). EDTA acts by

binding calcium ions, which are essential in the coagulation cascade and for cell-to-cell interaction. Due to this property, the EDTA salt is used as a blood anticoagulant because it chelates calcium ions which promote blood clotting (Jain, 1993). Heparin binds to and accelerates the activity of antithrombin III, which inhibits the action of thrombin and other proteases necessary for coagulation (Harr *et al.*, 2005).

Many workers have used EDTA salts as most appropriate for fish blood analyses (Blaxhall and Daisley, 1973; Sala-Rabanal Jalis 2003), while others recommend heparin as the preferred anticoagulant (Walencik and Witeska, 2007; Ishikawa *et al.*, 2010; Clark *et al.*, 2011). Haematology provides useful information as long as the addition of an anticoagulant causes no alteration. The aim of the present study was to study the effects of sodium-heparin and di-potassium-EDTA on the haematological parameters and blood cell morphology of *Schizothorax labiatus* so as to suggest the better anticoagulant for routine fish blood analysis.

Materials and Methods

Experimental Fish

Adult specimens of *Schizothorax labiatus* (McClelland, 1842) weighing 250 ± 18.5 g and average length 25 ± 2.1 cm were obtained from a local fish farm. The fish were transported in polythene bags filled with water and oxygen and brought to wet laboratory at the Department of Zoology, University of Kashmir. To rule out any possible microbial infection, the fish were given a prophylactic dip in KMnO_4 (5 mg L^{-1}) and stocked in indoor circular fish tank (water volume = 600 L) at $16.6 \pm 1.4^\circ \text{C}$, D.O $6.6 \text{ mgL} \pm 1$ and pH 7.1-7.5 with 12:12 h photoperiod.

Blood Sampling and Anticoagulant Treatment

During blood sampling, fishes were netted carefully imparting minimum stress and placed

in a fish trough. Using a chilled needle fitted to a 3 ml syringe, blood was drawn from the caudal vein. Blood smears were made on the spot from anticoagulant free blood, which were used as control to be compared against the blood smears prepared from anticoagulant treated blood. Blood sample was equally transferred to sterilized blood collection tubes, containing the respective anticoagulant i.e., Na-Heparin (20 I.U/ml) and K_2EDTA (1.0mg/ml) which were prepared by dissolving the dry salts of Na-Heparin and K_2EDTA (Loba Chemie, India) in phosphate buffer saline (PBS, pH 6.8). Blood vials were kept in an ice-bath until further analysis. All the haematological parameters were analyzed within 2 hours after sample collection.

Total Erythrocyte Count (RBC) and Total Leucocyte Count (WBC)

RBC and WBC counts were done simultaneously in a Neubauer haemocytometer with Natt Herrick's (1952) diluent (1:200). The blood cell counting was done under a microscope.

Haemoglobin (Hb)

Drabkin's cyanomethemoglobin method (1946) was used to estimate the haemoglobin content of the blood. 20 μl of blood was mixed with 5 ml of Drabkin's reagent (Loba Chemie, India), left to stand for 15 minutes and then absorbance measurement was done at 540 nm. Absorbance of test sample was plotted against that of haemoglobin standard (Ranbaxy India) to get the haemoglobin concentration. Haemoglobin test samples were centrifuged, prior to reading the absorbance, to remove dispersed nuclear material.

Haematocrit (Hct)

Blood samples (50 μl) were taken in micro haematocrit capillaries and centrifuged in a micro centrifuge (REMI RM-12C BL, India) spun in at 12,000 rpm for 5 min to obtain haematocrit value which were reported as percentage. Blood indices including mean cor-

puscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated from RBC, Hct and Hb according to the following formulae: $MCV = (PCV \times 10) / RBC$, $MCH = (Hb \times 10) / RBC$ and $MCHC = (Hb \times 100) / Hct$ (Dacie and Lewis, 1991).

Blood Cell Morphology

Using grease free glass slides, two blood smears were prepared for each blood sample. After air drying, the smears were fixed in 100% methanol for 5-8 min and stained with May-Grunwald-Giemsa stain. Using a compound microscope (Magnus MLX-Tr, India) blood smears were examined at 1000x magnification. Digital microphotographs were obtained with a digital DSLR camera (Cannon EOS 70D) attached to microscope and blood cell morphometric analysis was done with 'ImageJ' image analysis software (National Institute of Health) calibrated to a stage micrometer.

Statistical Analysis

All statistical analyses were carried out with SPSS 11.5 (SPSS Inc., Chicago, IL, USA). Haematological parameters were summarized as mean \pm standard deviation (SD) and differences between Na-heparin and K_2 EDTA anticoagulated blood samples were statistically analyzed using the Student's t-test. Results were considered significant at $p < 0.05$.

Results and Discussion

Haematological Parameters

Table 1 shows the result of the blood samples treated with Na-Heparin (20 I.U/ml of blood) and K_2 EDTA (1.0 mg/ml of blood). Haematocrit values in all K_2 EDTA treated samples were significantly higher ($p < 0.05$) and much more variable compared to those of heparin collected samples. The total RBC counts between blood samples obtained

with K_2 EDTA and those obtained with Na-Heparin showed Significant ($p < 0.05$) differences. Mean corpuscular volume (MCV) was increased in K_2 EDTA sample, while a significant decrease ($p < 0.05$) was found in mean corpuscular haemoglobin concentration (MCHC). Haemoglobin concentration, total WBC counts and leucocyte morphology did not differ significantly ($p > 0.05$) among the samples treated with Na-heparin and K_2 EDTA.

Cell Morphometric Analysis

Characterization and identification of blood cells was done by light microscopy. Blood smear analysis revealed considerable erythrocyte swelling leading to significant enlargement in all the K_2 EDTA treated samples (Fig. 1d) as compared to the untreated blood samples (Fig. 1a and 1b), while in heparinized samples, most of the erythrocytes were normal and intact (Fig. 1c). In Na_2 EDTA treated samples, bare nuclei released by haemolyzed erythrocytes were also observed (Fig. 1d). Leukocytes in K_2 EDTA and Na-Heparin anticoagulated blood showed no morphological changes.

An elevation in haematocrit values and a decrease in haemoglobin concentration and RBC counts were observed in K_2 EDTA treated samples. K_2 EDTA also induced RBC swelling and erythrocyte haemolysis, while no significant change of haematological parameters was observed in Na-heparin treated samples. Similar observations were reported by Walencik and Witeska (2007) and Ishikawa *et al.* (2010). EDTA salt can cause acidification and an increase in pCO_2 (Smit *et al.*, 1977), due to which haematocrit levels might have elevated. This phenomenon was observed by Korcock *et al.* (1988), Walencik and Witeska (2007) and Witeska and Wargocka (2011). Hattingh (1975) compared the effects of heparin and EDTA on haematocrit of five species of fish and found that EDTA had a tendency to increase haematocrit in fish, and in some species induced

Table 1 Hematological parameters of *Schizothorax labiatus* (McClelland, 1842) (n=15) treated with anticoagulant sodium-heparin (20 I.U/ml of blood) and di-potassium-EDTA (1.0mg/ml of blood)*

Anticoagulant used	Hct (%)	Hb (gdl ⁻¹)	RBC (x10 ⁶ /mm)	WBC (x10 ³ /mm)	MCV (fl)	MCH (pg)	MCHC (gdl ⁻¹)
Na-Heparin	34.32± 2.01 ^a	8.19 ± 0.43 ^a	1.42 ± 0.07 ^a	12.18 ± 3.20 ^a	241.1±8.96 ^a	57.59 ± 1.16 ^a	23.91 ± 1.31 ^a
K ₂ EDTA	39.20 ± 1.95 ^b	6.90 ± 0.70 ^a	1.22 ± 0.06 ^b	11.45 ± 2.71 ^a	321.4 ± 8.38 ^b	56.45 ± 2.77 ^a	17.57 ± 1.01 ^b

Hct, Haematocrit; Hb, Haemoglobin concentration; RBC, Red blood cell count; WBC, White blood cell count; MCV, Mean corpuscular volume; MCH, Mean corpuscular haemoglobin; MCHC, Mean corpuscular haemoglobin concentration;

Values are mean ± SD. Means with different superscripts are significantly different ($p < 0.05$)

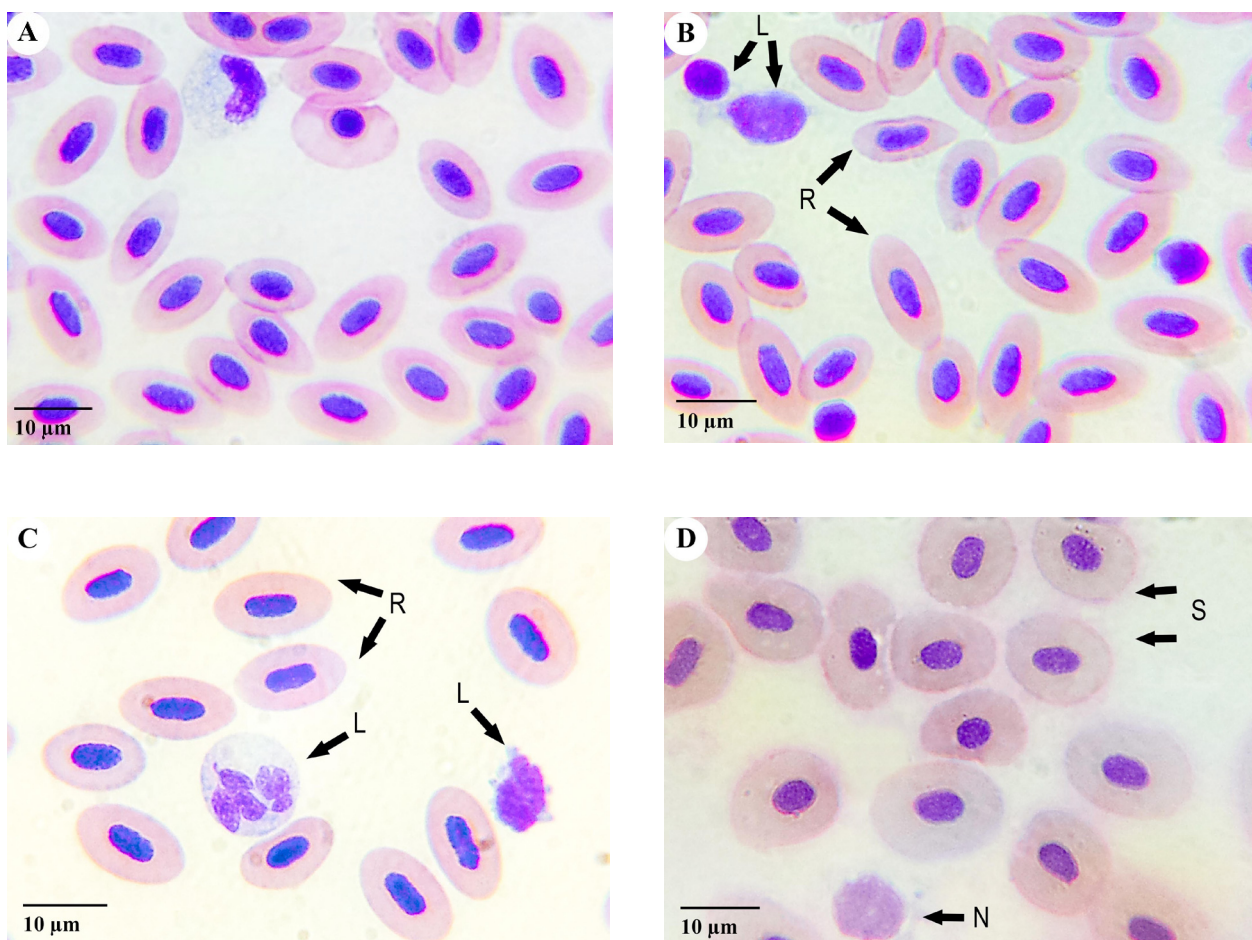


Fig. 1. Light micrographs of May-Grunwald-Giemsa stained blood film of *Schizothorax labiatus* (McClelland, 1842). A and B: Control, showing normal and intact erythrocytes (R); C: Na-heparin treated, showing normal and intact erythrocytes (R) and leucocytes (L); D: K₂EDTA treated, showing erythrocyte swelling (S) and bare erythrocyte nuclei (N).

haemolysis, while heparin produced very little change in erythrocyte volume and haematocrit values. On incubation of blood for 20 min with 10 mg/mL of EDTA, a distortion of erythrocyte shape followed by lysis was observed in *Blennius pholis* (Mainwaring and Rowley, 1985). EDTA sequesters Ca^{2+} ions is responsible for the activation of Na^+ and K^+ ions in the cell membrane which allows the free entry of water into the cell, promoting swelling and its consequent lysis (Jain, 1993). Heparin is known to cause little alteration in corpuscular size and is considered a more suitable anticoagulant, because its varying concentration has little effect on the PCV values (Dubin *et al.*, 1976). In the present study, Na-Heparin caused no significant alteration in RBC morphology, which also corroborate the observation made by Walencik and Witeska (2007), Ishikawa *et al.* (2010) and Witeska and Wargocka (2011) in common carp.

A decrease in RBC count in Na_2EDTA treated samples was observed in the present study, which could be due to the haemolytic action of EDTA. EDTA treatment causing erythrocyte haemolysis have been reported in fish (Walencik and Witeska, 2007; Korcock *et al.*, 1988; Lataretu, 2013) and other vertebrates (Muro *et al.*, 1998; Antwi-Baffour *et al.*, 2013) leading to low RBC counts and decreased haemoglobin concentration. Witeska and Wargocka (2011) observed gradual destruction of erythrocytes in Na_2EDTA samples, where Na_2EDTA caused cell swelling, followed by disintegration of the outer membranes of erythrocytes, which resulted in release of the nucleus. Erythrocytes become more susceptible to lysis as EDTA increases their osmotic fragility. Increased osmotic fragility in the blood samples treated with EDTA was observed by Walencik and Witeska (2007) in *Cyprinus carpio* and Mafuvadze and Erlwanger (2007) in ostriches, which eventually lead to a decrease in the RBC count. A gradual destruction of erythrocytes in Na_2EDTA samples was observed by Witeska and Wargocka (2011), where Na_2EDTA caused cell swelling, followed by disintegration of the outer

membranes of erythrocytes, which resulted in release of the nucleus. Haemolytic action of EDTA can be attributed to the adverse effect of Na_2EDTA on erythrocyte membrane structure, permeability, and stability, which is probably related to decalcination induced by the chelating action of the anticoagulant (Blaxhall, 1972; Hattingh, 1975).

In conclusion Na-heparin is found as the preferable anticoagulant for blood sampling in *Schizothorax labiatus* as it imparts minimum changes to haematological parameters, while K_2EDTA cannot be applied as an anticoagulant for haematological analysis, since it significantly affects the haematological parameters and alters the shape and size of blood cells, affecting red blood cell parameter readings.

Acknowledgements

The authors are grateful to the Head, Department of Zoology, University of Kashmir, Hazratbal, Srinagar, India, for providing the necessary laboratory facilities and also gratefully acknowledge the generous funding from the Department of Science and Technology (DST), Govt of India, New Delhi in the form of DST-FAST Track Young Scientist Project.

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