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Effects of two commonly used anticoagulants on haematology and erythrocyte morphology of rainbow trout (*Oncorhynchus mykiss*)

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Abstract
The present literature gives ambiguous information regarding the use of anticoagulants for fish blood collection. Many workers recommend the use of heparin as the preferred anticoagulant for routine haematology, however the salts of EDTA are also considered appropriate by some workers. The present study was, therefore, carried out with an aim to evaluate the effects of two commonly used anticoagulants i.e., lithium-heparin and di-sodium EDTA with a concentration of 20 I.U/ml and 1.0 mg/ml on the haematological parameters and erythrocyte morphology of coldwater fish *Oncorhynchus mykiss*. Haematological analysis including blood cell counts and haematocrit was done within one hour of the sampling. Samples collected with Na_2EDTA showed increased haematocrit values. Haemoglobin concentration and red blood cell counts were lower in Na_2EDTA collected samples as compared to heparinized samples, while WBC counts did not differ significantly ($P>0.05$). Mean corpuscular volume (MCV) was increased, while a significant decrease ($P<0.05$) was observed in mean corpuscular haemoglobin concentration (MCHC) in Na_2EDTA treated samples. Giemsa stained blood smears showed RBC swelling, anisocytosis, anisonucleosis and hemolyzed erythrocytes in Na_2EDTA treated sample, while no significant change ($P>0.05$) was observed in Li-heparin treated samples. The present study suggests the use of Li-heparin as a preferred anticoagulant for routine haematological analysis of fish blood.

Keywords: *Oncorhynchus mykiss*, Lithium–heparin, Di-sodium EDTA, Haematological analysis, Erythrocyte morphology.

1. Introduction
Fish haematology has assumed greater significance due to increasing emphasis on fish culture and greater awareness of the pollution of natural fresh water resources. One of the important tools for the development of a successful aquaculture system involves the study of physiological and haematological characteristics of cultured fish, especially in regard to the use in diagnosis and prognosis of morbid conditions, including the deleterious effects of infection [1] Haematological parameters have become attractive and important tool in monitoring environmental quality, water pollution, physiological status and the health condition of aquatic organisms [2, 3, 4, 5]. Fish haematology has revealed that interpretation of blood parameters is quite difficult, since variations in the blood are caused by internal and external factors. Though, many automatized clinical tools have been successfully employed to evaluate mammalian blood, but such tools have not been developed yet for fish haematology.
Haematological results are often influenced by a number of factors including anticoagulants used, method of analysis, the storage temperature, and the time lapse between when sample was taken and when they were analyzed [6]. Fish blood samples almost always require an anticoagulant treatment due to the presence of a large number of thrombocytes which cause the blood to clot quickly as compared to other vertebrates. Anticoagulants are additives that inhibit the clotting of blood and thereby ensuring that the concentration of the substance to be measured is changed as little as possible before the analytical process [7]. Haematology provides useful information as long as the addition of an anticoagulant causes no alteration, which could complicate the interpretation of the resulting data [8]. It is important to obtain a reliable blood sample, which represents the true status of the animal, ensuring the quality of the data and the correct interpretation of results [9].

Different salts of heparin and EDTA are the most commonly used anticoagulants in fish haematology [10]. Available literature shows that heparin is the most preferred anticoagulant [9, 10, 11, 12], while some authors consider EDTA salts as appropriate for fish blood analyses [13, 14, 15]. In fact, EDTA is the preferred choice for blood cell counts due to its general availability, ease of preparation, widespread use and relatively low cost [16]. The mode of action of EDTA is by binding calcium ions [17], 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 [18]. Heparin binds to and accelerates the activity of antithrombin III, which inhibits the action of thrombin and other proteases necessary for coagulation [17, 19]. Blood cells of various animals show different reactions to various anticoagulants [20]. Keeping in consideration the varying results of haematological parameters being reported on both the anticoagulants in different fish species, the present study was conducted with an aim to determine and compare how these two commonly used anticoagulants may affect the results of routine haematology and erythrocyte morphology of rainbow trout.

2. Material and methods

2.1 Experimental fish

Juvenile rainbow trout in apparent good health were procured from State Government Fishery Department Fish Farm, Dachigam J & K India. 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 14.6 ± 1.6 ^{0}C, D.O 6.8 mg L^{-1} ± 1 and pH 7.1-7.4 with 12:12 h photoperiod, fitted with a continuous water flow-through system. The fish were acclimated for a fortnight, during which they were fed to satiation a fish meal based formulated diet in two servings per day at 08:00 and 17:00 hours.

2.2 Anticoagulants Used

Anticoagulant solutions were prepared by dissolving dry salts of lithium-heparin (20 I.U/25 µL) and disodium-EDTA (1.0 mg/25 µL) in phosphate buffer saline (PBS, pH 6.8).

2.3 Blood Sampling

To minimize the stress, fishes were carefully netted and positioned ventral side up (with gill region submerged) in a V-shaped, partially water-filled and padded sampling trough. Blood was collected from the caudal vein using a chilled needle with a 3 ml syringe without any anticoagulant, with the whole sampling event taking no more than 3 minutes. Blood smears from the well mixed blood were made on the spot and rest of the blood sample was equally transferred to blood collecting vials (0.5 ml in each tube) containing the respective anticoagulants (10 I.U Li-Heparin per 0.5 ml and 0.5 mg Na_2EDTA per 0.5 ml). All the blood samples were kept in an ice-bath until further analysis.

2.4 Haematological analysis and erythrocyte morphology

All the haematological parameters were analyzed within 2 hours after the sampling was done. Total erythrocyte and leucocyte counts were done with a Neubauer haemocytometer (Marienfeld-Superior, Lauda-Königshofen, Germany) by using Natt Herrick's [21] diluent with a ratio of 1:200. Haemoglobin content of blood was estimated spectrophotometrically by the method of Drabkin [22]. Heparinized as well as EDTA treated blood (50µl) was 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. Blood indices including mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated according to Dacie and Lewis [23].

Uniformly spread blood smears were prepared for each blood sample and left to air dry. Dried smears were fixed in absolute methanol for 5-8 min and Giemsa stained. Examination of smears was conducted under a compound microscope (Magnus MLX-Tr, India) at 1000x magnification and digital microphotographs were obtained.

2.5. Statistical Analysis

Data shown represents the mean ± standard deviation (SD) and differences between haematological parameters of Li-heparin and Na_2EDTA anticoagulated blood samples were statistically analyzed using the Student's *t*-test. Results were considered significant at $P<0.05$. Statistical analyses were carried out using SPSS version 10.5.

3. Results

Comparison of haematological parameters of the blood samples treated with Li-heparin and Na_2EDTA has been presented in table 1.

Table 1: Haematological parameters of rainbow trout, *Oncorhynchus mykiss* (n=20) treated with anticoagulant lithium-heparin (20 I.U/ml of blood) and di-sodium-EDTA (1.0 mg/ml of blood)*

Anticoagulant	HCT (%)	Hb (gdl^{-1})	RBC ($x10^6/mm^3$)	WBC ($x10^3/mm^3$)	MCV (fl)	MCH (pg)	MCHC (gdl^{-1})
Li-Heparin	35.38^a±2.09	9.26^a ±0.55	1.21^a±0.05	12.45^a± 2.71	292.39^a±8.69	76.53^a±2.07	26.20^a±1.42
Na_2EDTA	40.26^b±2.02	7.83^b ±0.60	1.01^b± 0.06	13.18^a± 3.20	396.26^b±9.62	76.99^a±1.74	19.43^b±0.58

(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 ($P<0.05$)

A significant ($P<0.05$) increase in the haematocrit values was observed in all the Na_2EDTA treated samples compared to those of Li-heparin treated samples. Significant ($P<0.05$) differences were also found in haemoglobin concentration and total RBC counts between blood samples obtained with Na_2EDTA and those obtained with Li-heparin. Mean corpuscular volume (MCV) was increased, while a significant decrease ($P<0.05$) was observed in mean corpuscular

haemoglobin concentration (MCHC) in Na_2EDTA treated sample. Total WBC counts and leucocyte morphology did not differ significantly ($P>0.05$) in the samples treated with Li-heparin and Na_2-EDTA.

Blood cells were identified and characterized by light microscopy. Analysis of blood smears revealed that in heparinized samples, most of the erythrocytes were normal and intact (Fig. 1b), while in all Na_2EDTA treated samples, erythrocytes showed considerable swelling leading to cell enlargement (Fig. 1c). Bare nuclei released by haemolyzed erythrocytes were also observed in Na_2EDTA treated samples (Fig. 1d). No morphological changes were found in the leukocytes of Na_2EDTA and Li-heparin treated blood samples.

4. Discussion

In the present study the values of some haematological parameters showed significant differences among the blood samples collected with Na_2EDTA and Li-heparin. Na_2EDTA caused an elevation in haematocrit values and lowered the haemoglobin concentration and RBC counts. It also induced RBC swelling, anisocytosis (unequal cell size), anisonucleosis (unequal nucleus size), and caused haemolysis of erythrocytes, while no significant change was observed in Li-heparin treated samples.

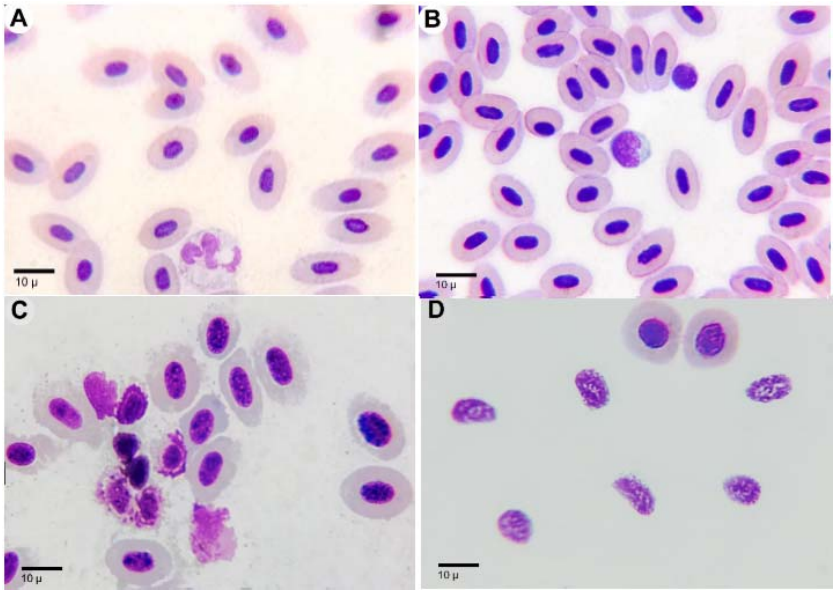

Fig 1: Light micrographs of Giemsa stained blood film of rainbow trout (*Oncorhynchus mykiss*). (A) No anticoagulant added, showing normal and intact erythrocytes (B) Li-heparin treated, showing normal and intact erythrocytes (C) Na_2EDTA treated, showing hemolysis of erythrocytes and anisocytosis (D) Na_2EDTA treated, showing swollen erythrocytes and bare erythrocyte nuclei.

Similar observations were reported by Walencik and Witeska [10] and Ishikawa *et al*. [12]. EDTA salt can cause acidification and an increase in pCO_2 [24], which might have led to the elevation in haematocrit levels. Similar phenomenon was observed by Blaxhall [25], Hattingh [26], Korcock *et al*. [27], Walencik and Witeska [10] and Witeska and Wargocka [20]. Hattingh [26] 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 haemolysis, while heparin produced very little change in erythrocyte volume and haematocrit values. When blood of *Blennius pholis* was incubated for 20 min with 10 mg/mL of EDTA, a distortion of erythrocyte shape followed by lysis was observed [28]. EDTA sequesters Ca^{2+} ions responsible for the activation of Na^+ and K^+ ions in the cell membrane allowing the free entry of water into the cell, promoting-swelling and its consequent lysis [18].

In the present study, Na_2EDTA treated samples showed a decrease in RBC count, which could be due to the haemolytic action of EDTA. EDTA treatment causing erythrocyte haemolysis have been reported in fish [27, 10, 29] and other vertebrates [30, 31] leading to low RBC counts and decreased haemoglobin concentration. EDTA increases the osmotic fragility of erythrocytes, making them susceptible to lysis. Increased osmotic fragility in the blood samples treated with EDTA was observed by Walencik and Witeska [10] in common carp and Mafuvadze and Erlwanger [32] in ostritches, 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 [20], 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 [13, 26].

Heparin is considered a more suitable anticoagulant as it is known to cause little alteration in corpuscular size and its varying concentrations have little effect on the haematocrit values [33, 34]. In the present study, no significant effects of Li-heparin were observed on the haematological parameters and cell morphology which was also observed by Walencik and Witeska [10] in *Cyprinus carpio* and Ishikawa *et al*. [12] in hybrid surubim catfish.

5. Conclusions

The results of the present study indicate that Li-heparin should be used as the preferable anticoagulant for blood sampling in *O. mykiss* as it imparts minimum changes to haematological parameters, while Na_2EDTA cannot be applied as an anticoagulant for haematological analysis, since it induces erythrocyte haemolysis and membrane distortion affecting red blood cell parameter readings. Further studies are recommended to investigate the effect of EDTA and heparin on the plasma biochemical profile of rainbow trout.

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