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Disodium EDTA used as anticoagulant causes hemolysis in common carp blood

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Abstract: Disodium EDTA used as anticoagulant for common carp blood caused a significant increase and high variability in hematocrit readings comparing to the heparinized samples. Na_2EDTA induced erythrocyte swelling, causing cell membrane disruption (hemolysis). The nuclei released from destroyed cells changed shape from oval to round, and underwent gradual swelling and vacuolation, followed by karyolysis. The degree of observed changes was similar at all Na_2EDTA concentrations (0.1, 0.5, and 1.0 mg/mL); on the other hand, percentage of abnormal and destroyed cells showed considerable differences among blood sampled from various carp individuals. The obtained results revealed that Na_2EDTA caused gradual damage to erythrocytes, and thus should not be applied as anticoagulant for blood analysis of common carp.

Key words: Fish, erythrocytes, hematocrit, heparin

Introduction

Blood for diagnostic analyses is usually treated with anticoagulants to prevent clotting. Heparin is a natural endogenous anticoagulant agent acting both in blood vessels in vivo, and ex vivo when added to a blood sample. Heparin inhibits conversion of prothrombin into active thrombin, and thus prevents conversion of fibrinogen into fibrin. The mechanism of EDTA anticoagulant action is based on inhibition of thrombocyte aggregation and various reactions of hemostatic cascade due to chelation of free Ca^{2+} ions.

Blood cells of various animals show different reactions to various anticoagulants. In human and other mammal hematology EDTA (ethylenediaminetetraacetic acid) salts (sodium or potassium) are most commonly applied, and occasionally also heparin, citric, or oxalic acid salts are used (1,2). In avian hematology both heparin and EDTA salts are considered equally appropriate (3), while for reptile blood analyses heparin is recommended (3,4); however, Harr et al. (5) did not observe any differences in hematologic values in samples from pythons collected on heparin or EDTA.

Fish blood clots extremely quickly, and samples almost always require anticoagulant treatment. In fish hematology both anticoagulants are applied. Some authors consider EDTA salts the most appropriate for fish blood analyses (6,7), while others recommend heparin (8-10). However, the results of various studies showed different effects of both anticoagulants on

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hematological parameters, even in the same species. According to Hattingh (11), heparin concentrations ranging from 0.03 to 3 mg/mL reduced hematocrit values of *Cyprinus carpio* blood, while 1 mg/mL of Na_2EDTA was the most appropriate anticoagulant, and did not alter hematocrit values for 2 days from sampling. In contrast, the results of our previous study (12) revealed that Na_2EDTA at concentrations 0.1-1.0 mg/mL increased sensitivity of common carp erythrocytes to hemolysis, and made hematocrit readings impossible. Similarly, Korcock et al. (13) reported an increase in hematocrit of *Salmo gairdneri* blood treated with 5 mg/mL of EDTA, comparing to the heparinized samples. Tavares-Dias and Silva Sandrim (14) obtained higher hematocrit and hemoglobin levels in heparin-treated blood samples of *Colossoma macropomum*, compared to the samples with EDTA, and concluded that the latter was a more appropriate anticoagulant. Therefore, the issue of the effects of various anticoagulants on fish red blood cells and hematological parameters still needs to be clarified.

The aim of present study was a detailed quantitative comparison of hematocrit values, susceptibility to hemolysis, and erythrocyte morphology in the blood of common carp treated with sodium heparin and Na_2EDTA.

Materials and methods

Blood of 10 healthy 18-month-old common carp (*Cyprinus carpio* L.) of body mass 80-100 g was used in the experiment. The fish were obtained from the rearing pond of the Inland Fisheries Institute in Żabieniec, and kept for 12 months under laboratory conditions in a flow-through aerated tank, at 20-22 °C, and fed once a day carp starter Aller Aqua Classic 4 mm.

Blood (about 500 μL from each of 10 fish) was collected by heart puncture, using chilled needles, into chilled plastic Eppendorf tubes containing no anticoagulant. Then blood of each fish was immediately transferred to 4 tubes containing dried anticoagulants: sodium heparin or Na_2EDTA, to obtain final concentrations of anticoagulants in blood: for heparin 50 IU/mL, and for Na_2EDTA 0.1, 0.5, and 1.0 mg/mL of blood, similar to the method applied in human hematology (1). Blood was stored for 2 h at 4 °C (standard blood storage procedure), and gently mixed every 15 min. After this time, hematocrit (Ht) was measured in microhematocrit capillaries, after centrifugation for 5 min at 12,000 rpm.

Percentage of hemolysis was evaluated using a hemolysis test: 20 μL of blood from each of 10 fish was added to 2 cm^3 of physiological NaCl solution (0.6%), and incubated for 30 min at room temperature. After this time the samples were centrifuged for 5 min at 1000 rpm, supernatant was carefully transferred into new tubes, and extinction was read spectrophotometrically at 540 nm wavelength. Percentage of hemolysis was calculated according to the formula:

hemolysis [%] = (A × 100%) / B, where

A – extinction of sample with physiological solution,

B – extinction of sample with distilled water (100% hemolysis).

Blood smears were also made from samples of 8 fish treated with each anticoagulant, and stained with May-Grunwald and Giemsa solutions. A detailed erythrocyte morphology analysis was done: 300 cells were viewed in each smear, and number of normal, abnormal, and hemolyzed erythrocytes was noted. Percentage of erythrocytes showing various anomalies was calculated. Photographs of anomalies were taken using a Nikon Coolpix digital camera connected to a Nikon-Eclipse E 600 microscope, and computer image analysis system CoolView. The significance of differences was evaluated using the non-parametric Mann-Whitney U test, at $P \leq 0.05$.

Results

Hematocrit values in all Na_2EDTA-treated blood samples were significantly higher and much more variable compared to those in the heparin (H) samples (Table) and similar at all anticoagulant concentrations. In samples with Na_2EDTA percentage of hemolyzed cells (obtained using both methods: spectrophotometric, and direct counting in smears) was also significantly higher than in heparinized blood. The results obtained with the 2 methods were similar except for higher hemolysis level in H

Table. Hematocrit values, hemolysis measured spectrophotometrically (*), and erythrocyte morphological pattern in common carp blood sampled using 50 IU/mL of heparin (H) or 0.1, 0.5, and 1.0 mg/mL of Na_2EDTA (different letter denominators indicate significant differences, Mann-Whitney U test, at $P \leq 0.05$, for hematocrit and hemolysis* n = 10, erythrocyte morphology n = 8).

Parameter	H	Na_2EDTA 0.1	Na_2EDTA 0.5	Na_2EDTA 1.0
Hematocrit [%]	24.1±4.0 [a]	43.4±14.6 [b]	40.5±13.5 [b]	39.1±10.9 [b]
Hemolysis* [%]	9.1±8.0 [a]	45.7±33.4 [b]	55.4±34.7 [b]	48.9±32.8 [b]
Hemolyzed erythrocytes [%]	2.0±4.0 [a]	49.0±31.0 [b]	48.0±35.0 [b]	53.0±38.0 [b]
Swollen erythrocytes [%]	2.7 ± 5.1 [a]	22.0 ± 20.0 [b]	25.0 ± 23.0 [b]	28.0 ± 28.0 [b]
Bare nucleus vacuolated [%]	0.0 [a]	27.0 ± 23.0 [b]	28.0 ± 28.0 [b]	30.0 ± 25.0 [b]
Bare nucleus condensed [%]	0.0 [a]	12.0 ± 15.0 [b]	8.8 ± 17.0 [b]	9.8 ± 16.0 [b]
Bare nucleus oval [%]	0.0 [a]	2.8 ± 3.2 [b]	5.3 ± 6.0 [b]	4.7 ± 6.0 [a]
Bare nucleus shrunk [%]	0.0 [a]	0.5 ± 1.4 [a]	0.3 ± 0.7 [a]	0.8 ± 1.1 [a]
Karyolysis [%]	2.2 ± 3.9 [a]	5.5 ± 5.0 [b]	3.8 ± 3.2 [a]	7.6 ± 9.6 [b]

samples measured spectrophotometrically compared to the smears.

Analysis of blood smears revealed that in H samples most erythrocytes were intact (Figure 1A), and sporadically observed hemolysis was always complete, accompanied by karyolysis (Figure 1B), while in all Na_2EDTA samples destruction of erythrocyte membrane and release of cytoplasm with hemoglobin preceded nucleus destruction, and numerous bare nuclei were observed (Table), usually showing an abnormal round shape (Figure 1C). Most of them were considerably swollen, significantly larger compared to normal nuclei of the intact erythrocytes (Table), and showed a vacuolated, foamy structure. Some of the bare nuclei showed a lower degree of destruction, were smaller than the vacuolated ones (but also significantly larger comparing to the intact ones), and were more condensed. Very few bare nuclei remained oval, and some were shrunken, chestnut-like, with projections. Among non-hemolyzed erythrocytes, in all Na_2EDTA samples most cells showed anomalies, mostly swelling (Table, Figure 1D), and very few remained intact.

Discussion

High hematocrit readings in Na_2EDTA samples observed in the present study were related to erythrocyte swelling. This, according to Smit et al. (15), might have been caused by an increase in pCO_2 and acidification due to treatment with acidic EDTA salt. Swelling of fish erythrocytes in EDTA-treated samples was also observed by Blaxhall (16) and Korcock et al. (13) in fish, and by Olsen et al. (17) in mammal blood. Increased hematocrit values and osmotic fragility in samples of avian blood with EDTA were also noted by Mafuvadze and Erlwanger (18). In contrast, Morris et al. (19) reported a decrease in hematocrit values in mammal blood samples with EDTA compared to heparinized blood.

Gradual destruction of erythrocytes was observed in Na_2EDTA samples, irrespectively of the anticoagulant concentration. Sodium EDTA caused cell swelling, followed by disintegration of the outer membranes of erythrocytes, which resulted in release of the nucleus. Bare nuclei underwent further destruction: swelled, finally nuclear membrane broke, and complete karyolysis took place. In heparin

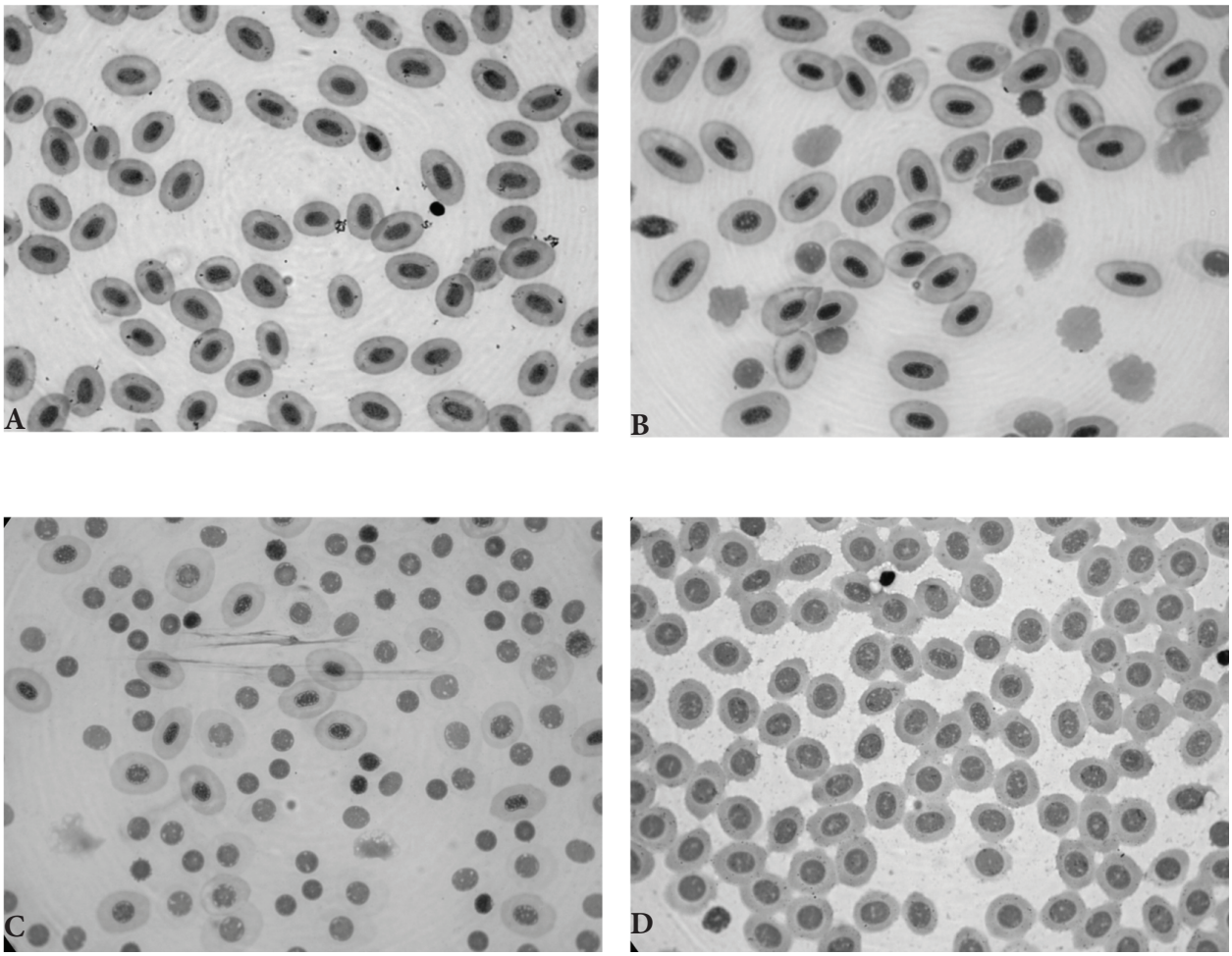

Figure 1. Alterations in erythrocyte morphology induced by Na_2EDTA in common carp blood: A – normal erythrocytes in heparinized sample, B – hemolyzed erythrocytes in heparinized sample, C – bare nuclei in Na_2EDTA sample, D – swollen erythrocytes in Na_2EDTA sample.

samples very few anomalies were observed, and they probably were not caused by the anticoagulant. No data were found in the literature concerning the sequence of morphological changes in erythrocytes during anticoagulant-induced hemolysis.

Hemolysis of fish erythrocytes in blood treated with EDTA was observed by Van Vliet et al. (9) in tilapia, and by Walencik and Witeska (12) in common carp. Increased osmotic fragility of EDTA-treated erythrocytes was also reported in human blood (20,21). According to Sarkar et al. (22), osmotic fragility of erythrocytes of various animals differ, and may be affected by various factors, e.g. the applied anticoagulant. Hemolysis indicates adverse effect of Na_2EDTA on erythrocyte membrane structure, permeability, and stability, which was probably related to decalcination induced by the chelating action of anticoagulant (6,11). According to Hattingh and Smith (3), also reptilian erythrocytes undergo hemolysis in decalcinated solutions. Orlov et al. (23) reported hemolysis in carp but not in human erythrocytes treated with EDTA. However, no hemolysis was observed when intracellular Ca^{2+} chelator (BAPTA) was added. The authors concluded that extracellular calcium is necessary for integrity of external membranes of nucleated cells. According to Lagunes et al. (24), decalcination of extracellular environment increases cell membrane permeability to Na^+ ions, and thus may induce cell swelling due to the increase in water uptake.

Hemolysis not only affects the values of erythrocyte-related parameters but also may interfere with various plasma constituents. Release of hemoglobin from hemolyzed erythrocytes may result in false high levels of other substances analyzed spectrophotometrically at similar wavelength. On the other hand, decreases in concentrations of some plasma constituents in hemolyzed samples may be related to dilution due to mixing with released intracellular content (25). Morgan et al. (26) reported that hemolysis resulted in an increase in plasma concentrations of iron, potassium, uric acid, total protein, and activities of lactate dehydrogenase (LDH) and alanine aminotransferase (AlAT), and reduction in plasma bilirubin, and activity of alkaline phosphatase (ALP). Similar results were obtained by Garcia Aguilar et al. (27), who reported an increase in plasma levels of magnesium, ferritin, phosphorus, potassium, and triglycerides, accompanied by a reduction in ALP activity. The results obtained by Morris et al. (19) revealed that hemolysis produced false high plasma glucose (by 50%) and fatty acid (by 200%) readings. These data show that hemolysis affects not only the values of red blood parameters, but also other hematological measures. Thus, blood showing any degree of hemolysis is inappropriate for hematological analysis.

The results of the present study indicate that reactions of erythrocytes of various animal species to anticoagulants may be different but the origin of this difference is difficult to explain. The available data indicate that it may result from different buffering capacity of blood (and different degree of acidification by EDTA salts) or from different sensitivity of cell membranes to extracellular decalcination (due to Ca^{2+} chelation by EDTA).

The obtained results indicate that sodium EDTA cannot be applied as anticoagulant for analyses of common carp blood in any concentration since it induces gradual erythrocyte destruction beginning from damage to the outer cell membrane, and then causes damage also to the nuclear membrane. This results in hemolysis, which not only affects red blood parameter readings but also may disturb the results of other blood analyses such as measurements of various plasma constituents.

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