DOI: 10.46909/journalalse-2021-023
Available online: https://jurnalalse.com
Print: ISSN 2784 – 0379
Online: ISSN 2784 – 0360

Original Article
Journal of Applied Life Sciences and Environment
Vol. LIV, Issue 3 (187) / 2021: 264-272

THE IMPACT OF TWO ANTICOAGULANTS ON ERYTHROCYTES MORPHOLOGY IN DIFFERENT VERTEBRATE SPECIES

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Received: Nov. 26, 2021. Revised: Jan. 10, 2022. Accepted: Jan. 14, 2022. Published online: Jan. 27, 2022

ABSTRACT. In this study, we aimed to highlight the influence of anticoagulants on erythrocyte morphometry in different vertebrate species. Anticoagulants are a category of substances that inhibit blood clotting through various mechanisms. Due to this property, they are used to collect blood samples for a wide range of laboratory tests. The literature mentions that the use of anticoagulants produces morphological changes of erythrocytes, thus influencing results. Blood samples were collected from three warm-blooded vertebrate species (horse, rabbit, and chicken) and one lower vertebrate species with nucleated erythrocytes (fish) in vacutainers with Heparin and EDTA (ethylenediaminetetraacetic acid), in a normal concentration and a double concentration. At the time of harvesting, control smears were performed. In order to be able to compare the effects produced by anticoagulants on the morphology of erythrocytes, they were evaluated morphometrically at intervals of 3, 6, and 24 hrs. after harvest. The following features were evaluated using the Toup View software: length, width, surface and perimeter of erythrocytes for species with anucleated erythrocytes. The same characteristics were evaluated in the nucleus for species with nucleated erythrocytes. The data obtained were processed with statistical programs to highlight changes in erythrocyte morphology produced by anticoagulants.

Keywords: blood cells; length-width ratio; cell surface; shape; vacutainer.

INTRODUCTION

Blood sampling is useful in the diagnosis of clinical diseases and also in the routine management of animal welfare (Harikrishnan *et al.,* 2018). Research in the field of haematology and blood biochemistry in animals often involves collecting samples in anticoagulant media to maintain the liquid state of blood for a longer period (Barrelet and Ricketts, 2002). Anticoagulant use, according to some studies, frequently results in erroneous results, particularly in terms of erythrocyte indices, due to the morphological alterations that

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anticoagulants cause at the cellular level (Walencik and Witeska, 2007).

Usually, anticoagulants intervene and stop a certain stage of the coagulation process, but some anticoagulants stop two or more stages depending on their nature, which is why we have different types of anticoagulants depending on the investigations to be performed in hematology (Mireşan *et al.,* 2003).

Anticoagulants are substances that inhibit the clotting process of blood or plasma, both *in vivo* and *in vitro.*

Blood samples can be drawn into tubes with a variety of preservatives, anticoagulants, and other chemicals, and then stored at room temperature, refrigerated, or frozen (World Health Organization, 2002). The goal is to keep the sample, as well as the drug or its metabolites of interest, in a stable state from the moment of collection through analysis (Kulkarni *et al.,* 2016).

Sodium citrate, EDTA or heparin or even a gel whose density is between that of the blood cells and blood plasma are examples of additions.

Furthermore, some tubes contain additives that can maintain some blood components or chemicals, such as glucose (Kulkarni *et al.,* 2016).

Erythrocytes are the most abundant elements in the composition of blood (Nemkov *et al.,* 2018). Their number is expressed in units of $106/mm^3$ and shows variations depending on the species ($5\times106/mm^3$ in humans, $5.5\times106/mm^3$ in cattle, $8\times106/mm^3$ in horses, $4\times106/mm^3$ in chickens, $14\times106/mm^3$ in sheep, and $7\times106/mm^3$ in pigs) (Mireşan *et al.,* 2003). There are small variations in these values, given the age, gestational status, sex of the individual, and altitude, and may be differences between different populations of the same species. Deviations from normal values occur only in pathological cases (Mohri and Rezapoor, 2009; Ahyayauch *et al.,* 2013). Erythrocytes transport and exchange oxygen and carbon dioxide between the respiratory system and other tissues.

Endothelial cells are activated to synthesize nitric oxide (NO).

Because the oxygenation of erythrocytes in the pulmonary capillaries favours the discharge of carbon dioxide. This induces the contraction and release of ATP and the activation of the synthesis capacity of nitric oxide by endothelial cells and its complexation with haemoglobin.

In the process of deoxygenation, the content of nitric oxide in the peripheral tissues decreases and at the same time the content of carbon dioxide increases. This causes erythrocyte swelling and vasodilation (Premont *et al.,* 2021). The diameter of the erythrocyte is measured in μm and has the following values depending on the species: 8.3 μm in humans, 7-8 μm in rabbits, 3.5 μm in goats, 5-6 μm in equine, and 7.5 μm in chicken (Mireşan *et al.,* 2003).

All mammals' erythrocytes are anucleated, and the majority of them are in the shape of biconcave discs termed discocytes and have a life span of approximately 120 days (Barrelet and Ricketts, 2002). Erythrocytes in fish are nucleated ellipsoidal cells with varying volumes and have a life span from 13 to 500 days. Environmental conditions and especially, water temperature and its dissolved oxygen content, seasonal changes and fish activity affect the number of erythrocytes (Witeska, 2013; Cocan *et al.,* 2018; Uiuiu *et al.,* 2021).

Therefore, our research aimed to highlight the influence of anticoagulants on erythrocyte morphology in different vertebrate species: two species with anucleated erythrocytes (horse and rabbit) and two species with nucleated erythrocytes (chickens and fish).

MATERIALS AND METHODS

Blood samples were collected from different vertebrate species: horse (*Equus caballus*, Linnaeus 1758) - adult male, rabbit (*Lepus europaeus*, Pallas 1778) - adult male, black bullhead (*Ameiurus melas*, Rafinesque 1820) - adult female, and domestic chicken (*Gallus gallus domesticus*, Linnaeus 1758) - adult female (one specimen/species). Collection methods were as follows: in horses from the jugular vein, in rabbits from the ear veins, in fish by puncturing the caudal vein, and in chickens from the ulnar veins.

After extraction, the blood samples were transferred to vacutainers where the anticoagulant is also located. For this experiment, the anticoagulants LiHep (Lithium heparin) and K3 EDTA (ethylenediaminetetraacetic acid) were used. The vacutainers were filled with blood as follows: normal anticoagulant concentration (up to the black marker found on the label of the vacutainer) and double anticoagulant concentration (up to half of the vacutainer). During blood harvesting procedures, there are situations where the operator is unable to harvest the recommended blood volume specified by the producer on the vacutainer due to age, size, and physiological status of the animal. Investigations on the effect of double anticoagulant concentration were performed to avoid situations where, at the time of blood sampling, the amount of blood indicated by the vacutainer manufacturer is not observed. The control smears were made on the spot and on a blade, a drop of blood was inserted, which was spread with the help of a blunt blade then stained with a Diakit Panoptic quick staining kit with the three constituents: Reag-Fix Panoptic (fixator), Reag-Red Panoptic (dye with eosinophilic action), and Reag-Blue Panoptic (dye with basophilic action) to highlight the figurative elements and their fixation. From the anticoagulant vacutainers, smears were made at intervals of 3, 6, and 24 hrs. The used method was identical to that of the control samples. The effect of anticoagulants on erythrocyte morphology was evaluated at 3, 6, and 24 hrs. after blood collection. Using the ToupView software, the following characteristics were evaluated: cell surface and length-width ratio. Cell surface and cell length-width ratio may show the deformation of the blood cells (RBC) over time under the action of different concentrations and different anticoagulants, which could result in the biased interpretation of the haematological panel.

The smears were examined with a Nikon Eclipse 50i microscope with a 60×objective. The microscope is equipped with a Nikon DS-Fi1 camera, and with the help of NIS-Elements Viewer 3.0, software images were taken with the following settings: fast focus 1280×960, quality capture 2560×1920, auto exposure, AECompensation +1.0EV, and contrast antireflex.

For each smear, 10 images were taken and 10 erythrocytes were measured from each image. A total of 15600 measurements were performed in total. The collected data were processed in Microsoft Excel and GraphPad Prism 8 software. ANOVA one-way test and Dunnett's multiple comparisons test were computed to determine the differences between the control group data and each experimental treatment.

RESULTS AND DISCUSSION

Based on the conducted experiments, it can be seen that the erythrocyte surface in horses has similar values when collected on EDTA in the

recommended (normal) dose and when analysed after 24 hrs. from sampling. A similar situation is encountered when analysing the surface of erythrocytes in the blood collected on a double concentration of EDTA analysed 6 hrs. after sampling. The other treatments show significant differences compared to the values of the control group (*Table 1*).

Heparin in double concentration, regardless of storage time (3, 6, 24 hrs.), did not lead to changes in the length-width ratio of horse erythrocytes. A similar situation was obtained with the double dose of EDTA after 24 hrs. of storage.

Similar values to those of the control group of rabbit erythrocyte surface were observed for the following treatments: 3 hrs. after sample, blood obtained on EDTA in normal concentration was analysed and 6 hrs. later, blood collected on EDTA in double concentration was analysed, and blood collected on heparin in normal concentration, for both 3 and 24 hrs. after sampling (*Table 2*).

Table 1 - Dunnett's multiple comparisons test: RBC surface and RBC length-width ratio in horses

Control vs.	Mean Difference	95.00% CI of diff.	Significant?	Summary	Adjusted P-value
Horse (cell surface - μm^2)					
EDTA-24h-Normal	-0.3988	-0.9388 to 0.1413	No	ns	0.272
EDTA-6h-Double	0.04657	-0.4935 to 0.5866	No	ns	0.9997
EDTA-3h-Normal	1.307	0.7673 to 1.847	Yes	****	< 0.0001
EDTA-6h-Normal	0.9765	0.4364 to 1.516	Yes	****	< 0.0001
EDTA-3h-Double	2.31	1.770 to 2.850	Yes	****	< 0.0001
Heparin-3h-Normal	1.187	0.6466 to 1.727	Yes	****	< 0.0001
Heparin-6h-Normal	2.996	2.456 to 3.536	Yes	****	< 0.0001
Heparin-24h-Normal	1.986	1.446 to 2.526	Yes	****	< 0.0001
Heparin-24h-Double	1.149	0.6087 to 1.689	Yes	****	< 0.0001
EDTA-24h-Double	0.866	0.3260 to 1.406	Yes	***	0.0001
Heparin-3h-Double	0.8243	0.2842 to 1.364	Yes	***	0.0003
Heparin-6h-Double	0.6169	0.07685 to 1.157	Yes	*	0.0155
Horse (cell length-width ratio)					
EDTA-24h-Double	0.007671	-0.02379 to 0.03913	No	ns	0.9958
Heparin-3h-Double	-0.0157	-0.04716 to 0.01576	No	ns	0.7364
Heparin-6h-Double	-0.01027	-0.04173 to 0.02119	No	ns	0.9758
Heparin-24h-Double	-0.03089	-0.06235 to 0.0005767	No	ns	0.0574
EDTA-3h-Normal	-0.2366	-0.2680 to -0.2051	Yes	****	< 0.0001
EDTA-6h-Normal	-0.1431	-0.1746 to -0.1116	Yes	****	< 0.0001
EDTA-24h-Normal	-0.1545	-0.1859 to -0.1230	Yes	****	< 0.0001
EDTA-3h-Double	-0.09063	-0.1221 to -0.05917	Yes	****	< 0.0001
Heparin-6h-Normal	-0.06586	-0.09733 to -0.03440	Yes	****	< 0.0001
Heparin-24h-Normal	-0.04309	-0.07455 to -0.01163	Yes	**	0.0017
EDTA-6h-Double	-0.03591	-0.06737 to -0.004449	Yes	*	0.0156
Heparin-3h-Normal	-0.03456	-0.06602 to -0.003099	Yes	*	0.0226

Legend: ns - P-value ≥ 0.05 (not significant); * - P-value from 0.01 to 0.05 (significant); ** - P-value from 0.001 to 0.01 (very significant); *** - P-value from 0.0001 to 0.001 (extremely significant); **** - P-value < 0.0001 (extremely significant)

Regarding the length-width ratio of rabbit erythrocytes, similar values with those of the control group were observed for the treatment on EDTA in double concentration, analysed 24 hrs. after sampling. The same situation was observed in the case of heparin treatments in normal concentration after 3 and 24 hrs., and in the case of double heparin concentration in 24 hrs.

Table 2 - Dunnett's multiple comparisons test: RBC surface and RBC length-width ratio in brown hares

Control vs.	Mean Difference	95.00% CI of diff.	Signi-ficant?	Summary	Adjusted P-value
Brown Hare (cell surface - µm^2)					
EDTA-3h-Normal	0.1671	-0.3931 to 0.7273	No	ns	0.9879
EDTA-6h-Double	0.03512	-0.5251 to 0.5953	No	ns	0.9997
Heparin-3h-Normal	-0.5017	-1.062 to 0.05851	No	ns	0.1057
Heparin-24h-Normal	-0.3999	-0.9601 to 0.1603	No	ns	0.3094
EDTA-24h-Normal	-1.143	-1.703 to -0.5826	Yes	****	< 0.0001
EDTA-24h-Double	-1.658	-2.219 to -1.098	Yes	****	< 0.0001
Heparin-6h-Normal	1.081	0.5208 to 1.641	Yes	****	< 0.0001
Heparin-3h-Double	-1.11	-1.670 to -0.5500	Yes	****	< 0.0001
Heparin-6h-Double	-1.166	-1.726 to -0.6056	Yes	****	< 0.0001
EDTA-3h-Double	-0.8908	-1.451 to -0.3306	Yes	***	0.0001
Heparin-24h-Double	-0.8978	-1.458 to -0.3376	Yes	***	0.0001
EDTA-6h-Normal	-0.74	-1.300 to -0.1798	Yes	**	0.0028
Brown Hare (cell length-width ratio)					
EDTA-24h-Double	-0.00312	-0.03108 to 0.02483	No	ns	0.9996
Heparin-3h-Normal	0.008082	-0.01987 to 0.03603	No	ns	0.9896
Heparin-24h-Normal	-0.0175	-0.04545 to 0.01045	No	ns	0.467
Heparin-24h-Double	-0.00782	-0.03578 to 0.02013	No	ns	0.9907
EDTA-3h-Normal	-0.09635	-0.1243 to -0.06840	Yes	****	< 0.0001
EDTA-6h-Normal	-0.1529	-0.1808 to -0.1249	Yes	****	< 0.0001
EDTA-24h-Normal	-0.09613	-0.1241 to -0.06818	Yes	****	< 0.0001
EDTA-3h-Double	-0.07722	-0.1052 to -0.04926	Yes	****	< 0.0001
EDTA-6h-Double	-0.05241	-0.08036 to -0.02446	Yes	****	< 0.0001
Heparin-3h-Double	-0.04061	-0.06856 to -0.01265	Yes	***	0.0007
Heparin-6h-Normal	-0.03689	-0.06484 to -0.008935	Yes	**	0.0028
Heparin-6h-Double	-0.03545	-0.06340 to -0.007494	Yes	**	0.0048

The symbols used have the same meaning as in *Table 1*

As can be seen in *Table 3*, for chicken erythrocytes, similar cell surface values to those of the control group were observed in the following treatments: EDTA in normal concentration at 3 and 24 hrs. after sampling, EDTA in double concentration at 24 hrs. after sampling, and in the case of heparin in normal concentration, 3 hrs. after sampling. Regarding the length-width ratio, all treatments showed extremely significant differences, compared to the control group.

For black bullhead (*Table 4*), the erythrocyte surface had values similar to those of the control group when the blood was collected on heparin in normal concentration and analysed 6 hrs. after sampling and in double heparin concentration, analysed 3 hrs. after

sampling. Regarding the length-width ratio of erythrocytes in black bullhead, we obtained values similar to those of the control group in the following treatments: blood collected on EDTA in double concentration and analysed 24 hrs. after sampling, and for blood collected on heparin in double concentration and analysed 3, 6, and 24 hrs. after sampling.

Table 3 - Dunnett's multiple comparisons test: RBC surface and RBC length-width ratio in chickens

Control vs.	Mean Difference	95.00% CI of diff.	Signifi-cant?	Summary	Adjusted P-value
Chicken (cell surface - μm^2)					
EDTA-3h-Normal	0.3882	-0.3809 to 1.157	No	ns	0.7243
EDTA-24h-Normal	0.5168	-0.2523 to 1.286	No	ns	0.3799
EDTA-24h-Double	-0.5377	-1.307 to 0.2314	No	ns	0.333
Heparin-3h-Normal	-0.7686	-1.538 to 0.0004981	No	ns	0.0503
EDTA-6h-Double	1.401	0.6321 to 2.170	Yes	****	< 0.0001
Heparin-3h-Double	-5.744	-6.513 to -4.975	Yes	****	< 0.0001
Heparin-24h-Double	-1.662	-2.431 to -0.8930	Yes	****	< 0.0001
Heparin-24h-Normal	-1.126	-1.895 to -0.3566	Yes	***	0.0006
Heparin-6h-Double	-1.14	-1.909 to -0.3712	Yes	***	0.0005
EDTA-6h-Normal	-0.9388	-1.708 to -0.1697	Yes	**	0.0076
EDTA-3h-Double	1.05	0.2814 to 1.820	Yes	**	0.0018
Heparin-6h-Normal	-0.9569	-1.726 to -0.1878	Yes	**	0.006
Chicken (cell length-width ratio)					
EDTA-3h-Normal	0.2393	0.1820 to 0.2965	Yes	****	< 0.0001
EDTA-6h-Normal	0.2627	0.2054 to 0.3200	Yes	****	< 0.0001
EDTA-24h-Normal	0.2812	0.2239 to 0.3385	Yes	****	< 0.0001
EDTA-3h-Double	0.1736	0.1163 to 0.2308	Yes	****	< 0.0001
EDTA-6h-Double	0.136	0.07878 to 0.1933	Yes	****	< 0.0001
EDTA-24h-Double	0.1963	0.1390 to 0.2536	Yes	****	< 0.0001
Heparin-3h-Normal	0.1654	0.1082 to 0.2227	Yes	****	< 0.0001
Heparin-6h-Normal	0.1947	0.1375 to 0.2520	Yes	****	< 0.0001
Heparin-24h-Normal	0.3381	0.2808 to 0.3953	Yes	****	< 0.0001
Heparin-3h-Double	0.4164	0.3591 to 0.4737	Yes	****	< 0.0001
Heparin-6h-Double	0.2171	0.1599 to 0.2744	Yes	****	< 0.0001
Heparin-24h-Double	0.1595	0.1022 to 0.2168	Yes	****	< 0.0001

The symbols used have the same meaning as in *Table 1*

Fig. 1 and *Fig. 2* show the mean values of surfaces and the length-width ratios of erythrocytes for the analysed species (*Fig. 1*) vertical axis shows mean values of erytrocite surfaces and *Fig. 2* shows mean values of length-width ratio of erytrocites). Changes in these parameters can be observed depending on the anticoagulant used, its concentration, and the time of blood storage in the vacutainer. Due to the gradient differences between the internal medium of erythrocytes and anticoagulants, fluctuations of the studied parameters were observed.

Other previous studies (Sanchez-Migallon *et al.,* 2008) analysed the impact of different anticoagulants on haematological values. Our results come in addition to the literature with quantitative data on erythrocyte morphology.

Table 4 - Dunnett's multiple comparisons test:
RBC surface and RBC length-width ratio in black bullheads

Control vs.	Mean Difference	95.00% CI of diff.	Signifi-cant?	Summary	Adjusted P-value
Black bullhead (cell surface - µm^2)					
Heparin-6h-Normal	-0.2708	-1.362 to 0.8200	No	ns	0.9957
Heparin-3h-Double	0.8569	-0.2339 to 1.948	No	ns	0.2095
EDTA-3h-Normal	5.064	3.974 to 6.155	Yes	****	< 0.0001
EDTA-6h-Normal	5.006	3.915 to 6.097	Yes	****	< 0.0001
EDTA-24h-Normal	2.498	1.407 to 3.589	Yes	****	< 0.0001
EDTA-3h-Double	5.014	3.923 to 6.105	Yes	****	< 0.0001
EDTA-6h-Double	4.284	3.193 to 5.374	Yes	****	< 0.0001
EDTA-24h-Double	5.516	4.426 to 6.607	Yes	****	< 0.0001
Heparin-6h-Double	3.016	1.925 to 4.107	Yes	****	< 0.0001
Heparin-24h-Double	1.915	0.8241 to 3.006	Yes	****	< 0.0001
Heparin-3h-Normal	1.689	0.5985 to 2.780	Yes	***	0.0002
Heparin-24h-Normal	-1.725	-2.816 to -0.6344	Yes	***	0.0002
Black bullhead (cell length-width ratio)					
EDTA-24h-Double	-0.03833	-0.1172 to 0.04054	No	ns	0.7629
Heparin-3h-Double	-0.04164	-0.1205 to 0.03723	No	ns	0.675
Heparin-6h-Double	-0.04954	-0.1284 to 0.02933	No	ns	0.4631
Heparin-24h-Double	-0.06006	-0.1389 to 0.01881	No	ns	0.24
EDTA-3h-Normal	-0.2287	-0.3075 to -0.1498	Yes	****	< 0.0001
EDTA-6h-Normal	-0.2272	-0.3061 to -0.1484	Yes	****	< 0.0001
EDTA-24h-Normal	-0.1757	-0.2546 to -0.09686	Yes	****	< 0.0001
Heparin-3h-Normal	-0.3179	-0.3968 to -0.2390	Yes	****	< 0.0001
Heparin-6h-Normal	-0.1875	-0.2664 to -0.1086	Yes	****	< 0.0001
Heparin-24h-Normal	0.1046	0.02575 to 0.1835	Yes	**	0.0026
EDTA-3h-Double	-0.08738	-0.1663 to -0.008513	Yes	*	0.0209
EDTA-6h-Double	-0.08084	-0.1597 to -0.001969	Yes	*	0.0412

The symbols used have the same meaning as in *Table 1*

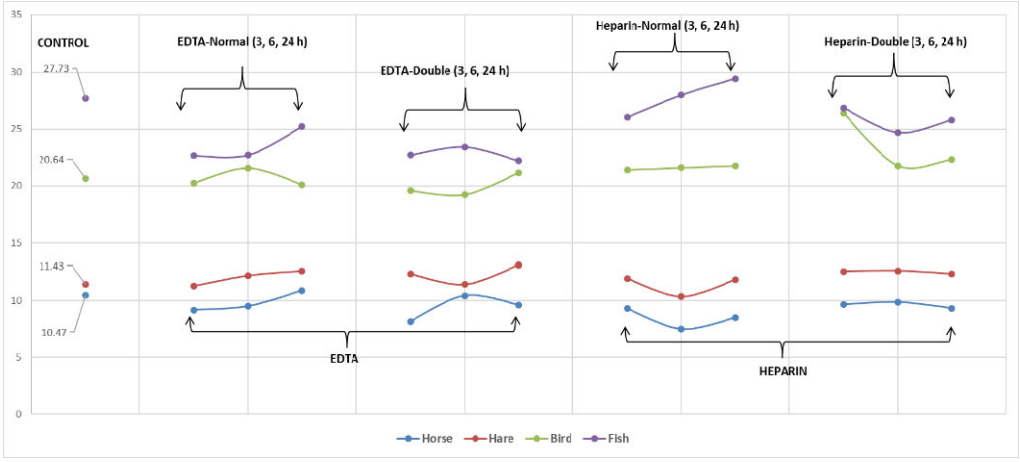

Figure 1 - Mean values distribution of red blood cells surfaces

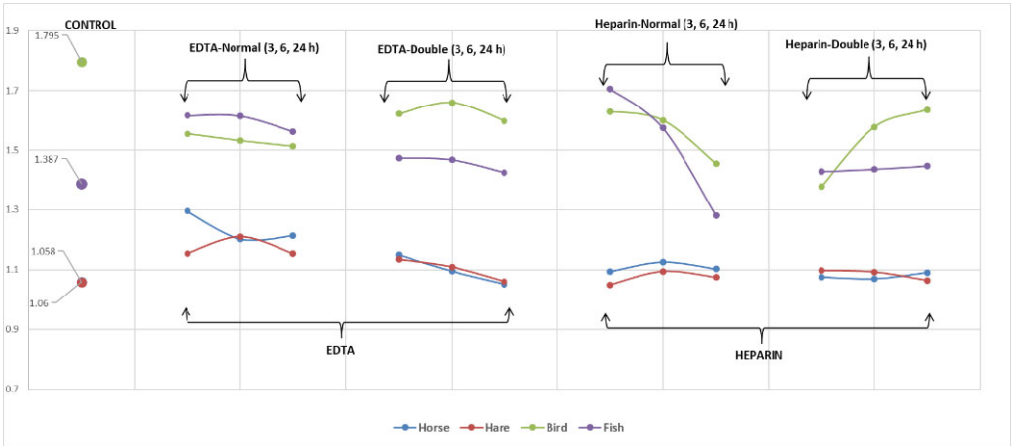

Figure 2 - Mean values distribution of the length-width ratio for red blood cells

CONCLUSIONS

The anticoagulant type, its concentration, and the exposure time of the blood to the anticoagulant are factors that can influence the quality and accuracy of blood analyses. In the tables and figures presented, the recommended concentrations for both types of anticoagulants and the optimal blood analysis time marks can be identified. Failure to observe the recommended concentrations for different types of anticoagulants and the time of exposure of erythrocytes to them may lead to morphological changes. These changes from the increase or decrease in volume can negatively influence certain haematological parameters, in particular haematocrit values. Anticoagulants are isotonic with the internal medium of mammals. In the case of lower vertebrates (birds, fish, reptiles, amphibians), anticoagulants may induce morphological changes in erythrocytes, as the latter have a lower concentration in the internal medium.

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