Impact of various reagents during blood collection from poultry to analyze morpho-biochemical indicators

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Abstract. The work provides experimental data on the impact of anticoagulants (K3 - EDTA, 3.8% sodium citrate and lithium heparin) and coagulation activator on morpho-biochemical indicators in the blood samples of broiler chickens of cross-selection of Agricultural Center "Smena" at the age of 35 days. The studies were carried out with the use of semi-automatic flow analyzer Sinnowa BS-3000P (SINNOWA Medical Science & Technology Co., Ltd, China) and veterinary automatic blood analyzer DF-50 by Dymind Biotech (PRC) with the use of original reagents. The results showed that the most optimal reagent for determination of morphological indicators of the poultry blood was EDTA. The average white blood count (WBC) is lower in blood samples with citrate and heparin in contrast to EDTA by 30.5% and 24.1%, respectively. The average red blood count (RBC) is lower in tubes with sodium citrate (by 37.9 %) and lithium heparin (by 13.8 %) in contrast to K3-EDTA. When the sodium citrate anticoagulant is used, the blood biochemistry results for all positions of the experiment, excluding calcium, are lower than in heparin, and its closest values are lower than in coagulation activator, excluding trypsin activity. That's why, the most optimal reagents for biochemistry, including for blood enzyme tests, should be considered the tubes with heparin or coagulation activator.

1 Introduction

The morphological and biochemical blood indicators are important for determining the physical status and health of the live-stock animals and poultry, including for mycotoxicosis diagnostics. Recently, the classical research methods have been replaced with automatic blood and biochemical analyzers with a set of reagents, i.e. there has been the significant upgrading of the instrumental base of scientific laboratories that has led to a high accuracy of research results requiring the determination of new reference values, as

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well as to increase in the speed of analysis. The scientific literature has accumulated wide and contradictory material on blood morphology and biochemistry in poultry [1], this is often related to the use of various reagents for blood plasma and serum production.

The tubes of different manufacturers that are currently used for blood sampling contain necessary concentrations of anticoagulants (sodium citrate, ethylenediaminotetraacetic acid (EDTA), lithium heparin and others). The existing anticoagulants ensure the preservation of the components without significant changes in the course of sample analysis and are characterized by the properties that inhibit the blood components coagulation process. This prevents blood coagulation due to the binding of calcium ions (EDTA, sodium citrate), inhibition of thrombin activity (heparin, hirudin) [2,3]. There is enough information in the literature on the effect of various types of anticoagulants on hematological indicators in human or animals, but there is limited information on the use of tubes for studying blood samples of poultry. In this regard, in order to obtain more correct analysis results, it is important to choose the correct anticoagulant for blood collection. Therefore, the purpose of this work was to compare the impact of anticoagulants (K3 - EDTA, 3.8% sodium citrate and lithium heparin) on morpho-biochemical indicators in the blood samples of broiler chickens of cross-selection of Agricultural Center "Smena" at the age of 35 days.

2 Materials and research methods

The blood collected from broiler chickens of cross-selection of Agricultural Center "Smena" at the age of 35 days in the morning hours, in fasting state, was tested during the experiment. The produced plasma was tested on the semi-automatic flow analyzer Sinnowa BS-3000P (SINNOWA Medical Science & Technology Co., Ltd, China) with the use of biochemical sets (DIAKON-VET, Russia) by determining total protein, alkaline phosphatase, glucose, cholesterol, calcium, phosphorus. The trypsin activity in blood plasma was tested by using the benzoyl-DL-arginine nitroanilide (BAPNA) as a substrate on the semi-automatic biochemical analyzer BS3000P (KHP) by means of kinetic method [4]. The hematological tests were performed on the veterinary automatic blood analyzer DF-50 by Dymind Biotech (PRC) using the original reagents. The tests were performed with simultaneous use of different tubes: with K3 EDTA, lithium heparin, sodium citrate and coagulation activator. The samples were centrifuged at 3000 rpm for 5 minutes to separate plasma from the formed elements.

Currently, the most preferable anticoagulant for hematological tests is ethylenediaminetetraacetate (EDTA) with the most effective concentration of 1.2 mg/ml of blood which is used for the blood plasma production and can form the soluble high-stable complexes due to formation of chelate compounds with calcium ions. The effectiveness of the analysis for blood plasma separation depends on a number of factors during preparation of the material, i.e. the quality of the analysis will increase when the tube is inverted 5-7 times immediately after blood collection to ensure better mixing of the blood and the anticoagulant and centrifugation at 2000-3000 rpm. During the hematological tests related to blood cells counting, determining the ESR, etc., during the PCR tests (qualitative and quantitative methods), it is advisable to use tubes containing 1.95 mg of EDTA/1 ml of blood. It is advisable to store tubes with collected blood samples for up to 6-10 hours at 4°C, further storage of tubes with blood samples is not recommended for more than 24 hours due to low red cell and white cell counts [5].

The anticoagulant heparin is used to test the electrolyte composition, gas composition, and blood alcohol content. However, heparin is acidic and is not recommended for using in the morphological studies, white cell and platelet counting, polymerase chain reaction (PCR) diagnostics.

The anticoagulant sodium citrate is used to collect venous blood and in testing the coagulation properties of blood. In the process of blood coagulation, the enzyme thrombin converts fibrinogen into fibrin, i.e. activation of initially inactive enzymes occurs due to successive complex reactions with the participation of active enzymes. The ratio of citrate to the amount of blood taken should be 1/9 that is achieved by using tubes with 3.8% or 3.2% sodium citrate solution (0.129 mol/l). When conducting coagulation tests, it is recommended to observe certain rules: immediately after venipuncture, the released tissue thromboplastin can affect the test results, therefore, it is necessary to mix the blood and the anticoagulant, and the amount of blood taken should be at the level as set forth in the requirements [5].

Serum can be produced by blood coagulation and clot retraction (thickening), but external activator (silicon dioxide) is required. In practice, centrifugation of tubes with blood accelerates the clot retraction. To produce the maximum pure serum, the following conditions should be observed: 1. Shake and mix the tube contents; 2 Wait until the blood coagulation process is completed with the tube in vertical position; 3. Perform centrifugation for at least 10 minutes at 3000 rpm.

It is important to note that with subsequent shaking or overturning, the blood serum contamination with clot components can occur, it is allowed to store the blood samples at room temperature for up to 6 hours, and in the cold-storage holding conditions (+ 4°C) - for up to 24 hours. The separated blood serum is used for biochemical and immunological tests in the study of protein composition, enzymes, hormones, tumor markers, HIV infection, hepatitis, etc. [5].

Statistical methods. For statistical processing of the results, the JMP Trial 14.1.0 software was used to calculate the mean value (M) and the standard deviation (±m) and determine the significance of differences by the Student's t-test. Differences were considered statistically significant at $p< 0.05$.

3 Study results and discussion

EDTA is recommended as anticoagulant for hematological tests, since it allows for the best preservation of cellular components and blood cell morphology. We considered this group as control when determining the blood cell morphology and compared the effect of other reagents on morphological indicators of blood (Table 1).

Table 1. Results of morphological blood tests of broiler chickens at the age of 35 days with the use of tubes with different anticoagulants (n=60, M±m).

Hematological indicators	Tubes with anticoagulants		
	K3-EDTA (control)	3.8 % sodium citrate	lithium heparin
WBC, 10^9/l	34.4 ± 0.77	23.9 ± 0.85*	26.1 ± 0.15*
RBC, 10^{12}/l	2.9 ± 0.06	1.8 ± 0.04*	2.5 ± 0.03*
HGB, g/l	146.0 ± 2.56	90.6 ± 1.82*	132.3 ± 2.18*
HCT, %	36.9 ± 0.58	22.2 ± 0.45*	31.7 ± 0.38*

Note: * the differences with the control group are reliable at $p<0.05$

The study results showed the reliable difference between the control and the other tubes. The average white blood count (WBC) is lower in blood samples with citrate and heparin in contrast to EDTA by 30.5% and 24.1%, respectively.

The average red blood count (RBC) is lower in tubes with sodium citrate (by 37.9%) and lithium heparin (by 13.8%) in contrast to K3-EDTA. Hemoglobin (HGB) is also lower in the citrated and heparinized blood samples in contrast to EDTA by 37.9% and 9.4%,

respectively. Hematocrit (HCT) is lower with citrate by 14.7% and with heparin by 5.2% regarding K3-EDTA.

The use of K3-EDTA as anticoagulant caused a significant difference in concentrations of the measured blood parameters. This may be related to the buffer system of the blood. Blood plasma proteins, which have buffer properties, are able to bind both acids and alkalis, and are ampholytes. Hemoglobin - red blood cell protein - hemoglobin (Hb) and oxyhemoglobin (HbO_2) have considerable buffering effect. In the blood capillaries, HbO_2 dissociates into oxygen and Hb. Such dissociation creates favorable conditions for binding of carbonic and other acids due to the alkali reserve. EDTA is the tetrabasic carboxylic acid, soluble in alkali, that facilitates the addition of the Hb molecule to the free residue of this acid. That's why, the tubes with EDTA anticoagulant are better to be used for determining such blood parameters as red blood cells, hemoglobin, hematocrit and erythrocytic indices. In the leucogram indicators (Fig. 1), similar values were obtained when using EDTA and sodium citrate, and they differed significantly in any manner when using the lithium heparin anticoagulant.

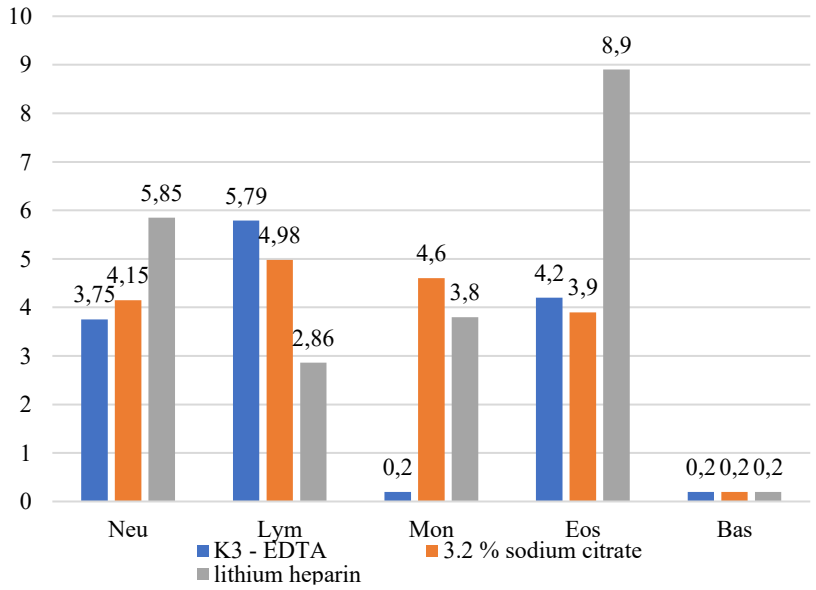

Fig. 1. Leucogram of 35-day old broiler chickens when the tubes with different anticoagulants are used, %.

The Neu and Lym indicators are decreased by 10 times

Figure 1 shows that the percentage of neutrophils (Neu) is the highest in tubes with heparin - 58.5% that is 17% lower than in tubes with citrate, and the lowest in tubes with EDT - 37.5%. The lymphotite content (Lym) is the highest in the samples with EDTA - 57.9%, with citrate it is lower by 8.1%, and the lowest with heparin - 28.6%. The citrated blood shows high monocyte content - 4.6%, the heparinized blood contains 3.8%, EDTA - 0.2%. The eosinophil content (Eos) is the highest with lithium - 8.9%, lower by 4.7% and 5.0% in EDTA and citrate, respectively. There was no difference in the percentage of basophils (Bas) regardless of the type of anticoagulant used.

Therefore, the experiment results showed that the most optimal reagent for determination of morphological indicators of the poultry blood was EDTA.

The blood biochemistry results of broilers are given in Table 2.

Table 2. Biochemical indicators of blood of broiler chickens at the age of 35 days with the use of different tubes (n=60, M±m).

Biochemical indicators	Lithium heparin (control)	K3 EDTA	3.8% sodium citrate	Coagulation activator
Total protein, g/l	36.3 ± 0.68	39.8 ± 0.56*	30.2 ± 0.80*	38.6 ± 0.58*
Trypsin, U/l	1086.0 ± 36.67	83.2 ± 3.20*	74.1 ± 2.22*	68.2 ± 2.43*
Glucose, mmol/l	11.3 ± 0.49	12.3 ± 0.16	6.9 ± 0.56*	12.3 ± 0.24
Alkaline phosphatase, U/l	3324.0 ± 195.50	5266.0 ± 395.20*	1826 ± 86.7*	3929 ± 358.7
Cholesterol, mmol/l	3.4 ± 0.05	3.2 ± 0.06*	1.8 ± 0.18*	3.3 ± 0.07
Calcium, mmol/l	2.5 ± 0.05	0.6 ± 0.01*	3.2 ± 0.12*	2.9 ± 0.18
Phosphorus, mmol/l	2.6 ± 0.06	2.7 ± 0.05	2.3 ± 0.06*	2.0 ± 0.07*

Note: * the differences are reliable in contrast to the control at $p<0.05$

These tables show that when using the lithium heparin anticoagulant for broiler blood, the total blood protein content differed from EDTA by 9.6% downward, from the coagulation activator - by 6.3%, the control sample exceeded the tube with sodium citrate by 16,8%. The trypsin activity with the lithium heparin anticoagulant exceeded the EDTA samples by 13.0 times, with sodium citrate by 14.6 times, with coagulation activator by 15.9 times. The glucose indicators differ from the lithium heparin specimen when using sodium citrate where the level is 38.9% lower ($p<0.05$). The alkaline phosphatase activity reaches its maximum value when using EDTA that is 58.4% higher than the tube with heparin, and in the tube with sodium citrate, the minimum value is observed which is 45.1% lower than the control. The blood cholesterol content below the control is observed in the tube with EDTA (by 5.9%) and sodium citrate (by 47.1%). The calcium content is significantly less than the control in the tube with EDTA (by 76.0%), and with sodium citrate, on the contrary, it is higher by 28.0%. The phosphorus content is noticeably lower with sodium citrate (by 11.5%) and coagulation activator (by 23.1%). Consequently, when the sodium citrate anticoagulant is used, the blood biochemistry results for all positions of the experiment, excluding calcium, are lower than in heparin, and its closest values are lower than in coagulation activator, excluding trypsin activity.

It is known [5-8] that the mechanism of action of sodium citrate is based on binding of calcium ions in the blood; therefore, it can be assumed that there is a relationship between the calcium ion and the trypsin activity in the blood plasma. A negative correlation was identified in the chicken blood between the trypsin activity and the calcium content [9]. This is consistent with the study results [10,11], which evidence that protease inhibitors are among the most widespread components of plasma protein, that is in significant excess of active proteases, except for cases when activation occurs on surfaces or other pathogens. Chelating agents such as EDTA and citrate do not directly inhibit proteases, but they limit the protease activation in the blood coagulation system by affecting calcium-mediated surface binding and allowing inhibitors to dominate. Our data on the difference in research results when using different blood anticoagulants are consistent with the literature [12].

4 Conclusions

The conducted experimental researches have shown that when taking blood samples, the morpho-biochemical test results differ significantly depending on the type of anticoagulants or coagulation activators. For morphological blood tests of poultry blood, it is recommended to use the tubes with EDTA, and for biochemistry, including for blood enzyme tests, the tubes with heparin or coagulation activator are recommended.

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