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Hemostatic Profile of Holstein Heifers Depending on Age

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Abstract

We have studied the age-related changes in indicators of platelet and plasma-coagulation hemostasis in heifers in the plant-based nutrition phase. Holstein heifers were studied within the research. Blood was taken from animals aged 3, 6, 9, 12 and 15 months. Also, the hemostatic profile was determined in them. It was found that 9 months is a critical age in the dynamics of PLT, PCT, MPV, PDW, and P-LCR. It affects the direction of their changes. The indicator of extrinsic (prothrombin activity, prothrombin ratio, INR) and intrinsic (APTT) coagulation pathways balance the biological significance of each other. The age-related dynamics of fibrin formation indicators (prothrombin time, fibrinogen) and the anticoagulant system (antithrombin III) is intended to maintain the fluid blood condition. According to the principal component analysis, a total variability of hemostatic blood composition is associated with the age of heifers defining 73.18% of the explained variance of parameters. The obtained data may be used as a standardized one in evaluating the hemostasis condition in the body of Holstein heifers as well as in performing therapeutic interventions with drugs affecting blood coagulation.

Disciplinary: Hematology, Animal Science, Biology.

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1 Introduction

The hemostatic system is a protective mechanism enabling to stop the bleeding in case of vascular damage [1] as well as regulating the viscoelastic properties of blood. It has got the

following main structural and functional components: blood cells (platelets), vessel walls, and plasma enzyme systems.

Platelets are specific small anucleate cells [2] produced by bone marrow megalokaryocytes [3]. They are eliminated in the reticuloendothelial system by macrophages [4]. Cells in a subpopulation of platelets differ not only in size and density but also in function. For example, adhesive and aggregating function, as well as vasotrophic one of platelets, decreases as their life span in the bloodstream increases; the concentration and transport function are associated with cell density [5]. According to [1], platelets are not only mediators of hemostasis but also of immunity. They can bind to bacteria and produce immunomodulating chemokines [6]. Granules are of key importance in terms of physiological functions implementation of platelets. Thus, α-granules contain various proteins, chemokines, cytokines, and growth factors ensuring normal cell function [7]. Dense granules contain molecules of ADP, serotonin, glutamate, histamine, and calcium, which are essential for platelet activation [8]. Lysosomes contain proteolytic enzymes [9] and enzymes hydrolyzing glycoproteins, glycolipids, and glycosaminoglycans [10].

Platelets are involved in the formation of the "first wave" of hemostasis (accumulation of cells at the place of vascular disruption) due to the ability to adhere, activation and aggregation, as well as in the "second wave" (blood clotting). This is due to the ability of activated cells to enhance the formation of thrombin. It initiates the conversion of fibrinogen into fibrin [3-4]. These cells are the main subjects in stopping bleeding when the vascular wall is damaged.

The patterns of functioning of the hemostatic system have been studied for a long time, but most of these researches have been performed in humane medicine. Only recently some papers have appeared concerning the following: in animals, unlike humans, the homeostatic balance of platelet functions (primary hemostasis) and the coagulation system (secondary hemostasis) has specific features [11]. In turn, exposure to a large number of endo- and exogenous factors cause hemostatic derangements. For example, it was found that camel platelets have a lower aggregation than human cells. Meanwhile, the breed of camels affects the quantitative expression of this parameter [12]. According to [13], calves, goats, and pigs have considerably higher blood clotting parameters in comparison with humans. It is dog plasma that is characterized by lower values of prothrombin time, activated partial thromboplastin and thrombin time, and considerably higher concentrations of fibrinogen and factor VIII coagulation protein than human one [14]. The clotting time in sheep and humans is about the same [15]. Pigs have a fibrinolytic pathway similar to humans [15]. Nevertheless, the influence of the anti-aggregation ability of the vascular wall on the processes of hemostasis increases in them with age [16]. The differences in the rate of thrombin and plasmin formation in various animal and human species are up to 10 times, which is due to substantial alterations in the regulation of hemostasis [17]. In the body of cattle, the condition of the hemostatic system depends on the breed, health status, gestation phase, lactation phase, number of births, and productivity [18-20]. Moreover, environmental conditions of cultivation also

affect. For example, in calves of Holstein breed in the conditions of the Kursk magnetic anomaly, the aggregation activity of platelets reduces with their normal content in the blood [21].

In the body of farm animals, the hemostatic system is closely associated with somatic characteristics [22], affecting the rate of their growth and development as well as the formation of productive qualities. Nevertheless, in most species, including cattle, there are scattered data concerning the parameters of the hemostatic system. They do not permit the identification of specific standards even by animal species. According to the fact that the regularities of the formation of hemostasis in the body of young cattle have not been identified, the purpose of our paper was to evaluate the age-related changes in the parameters of platelet and plasma-coagulation hemostasis in Holstein heifers in the plant-based nutrition phase.

2 Materials and Methods

Ethical Statement. The study was confirmed by the Bioethics Committee of the South Ural State Agrarian University. It was done in compliance with the principles of humane treatment of animals.

Animals. The research was performed in 2020-2021 under the conditions of Belagash LLP being a part of the Agrofirma TNK LLP group of companies (Republic of Kazakhstan). The company specializes in grain production and dairy farming. In 2011, the company imported young cows and heifers of the Holstein-Friesian breed from the USA. The company is engaged in breeding these animals to the present. The technology of young-stock breeding provides for their maintenance up to 60 days of age in the unit for newborns. For feeding, an imitation of powdered milk is used being drunk 3 times a day. Then the calves are separated by gender: the bulls are transferred to the fattening unit; the heifers to the farming unit. In the unit for heifers, there are sections for the nursery (from 2 to 7 months) and sections for coupling (from 7 months). The way of keeping is a cubicle system. The enterprise has its own feed processing building and mainly uses a feed of its own production. The feeding is twofold. In the nursery sections, heifers are fed with concentrated feed mixture, hay, and vitamin granules; from the age of 4 months, succulent feeds are added. Animal feeding regimes correspond to the standards of the All-Russian Institute of Animal Husbandry [23].

Sample Collection and Analysis. To carry out the research, an experimental group (n=10) was formed from 3-month-old heifers under the principle of approximate analogues. The blood was taken in the morning (before feeding) in animals aged 3, 6, 9, 12, and 15 months. Prior to blood sampling, the heifers of the experimental group were subjected to a clinical examination.

Blood from animals was obtained by vacuum method from the tail vein using a vacuum-containing system. To do this, the tail of the animal was fixed with a hand in the middle third area and slowly raised up. The injection site (in the area of 2-5 tail vertebrae) was disinfected with alcohol; the needle was inserted at an angle of 90 ° until it stops at a depth of 5-10 mm. Blood samples were collected in vacuum tubes (VACUETTE) designed for the study of the hemostatic

system (blue lid) and hematologic studies (purple lid). The test tube was carefully turned over 4 times after taking the blood to mix with the stabilizer.

The blood in the insulated shipping container was delivered to the Laboratory of I. V. Smolin LLP (the city of Kostanay) on the first day after its sampling. The thrombocytogram was part of a hematologic study performed on Sysmex, XS-500I hematology analyzer (Japan). It included platelet count (PLT), plateletcrit (PCT), mean platelet volume (MPV), platelet distribution width (PDW), and large platelet ratio (P-LCR).

ACL TOP 500 automatic coagulation analyzer (USA) was used to identify the parameters of plasma coagulation hemostasis. It included the determination of activated partial thromboplastin time (APTT), international normalized ratio (INR), prothrombin activity, prothrombin ratio, thrombin time, fibrinogen, and antithrombin III concentrations.

Statistical Analysis. The statistical analysis was done using the Statistica 6.0 software. It included normality testing of the distribution of values in the sample using the Shapiro-Wilk test, calculation of the mean value (X) and its standard error (Sx). To evaluate the total variability of hemostasis indicators in the body of heifers, the principal components method was used [24]. The similarity was defined using the Spearman correlation coefficient; the number of main components was determined by the Cattell's scree test [25]. The statistical significance of the links was taken equal to $P \leqslant 0.05$. The PAST package [26] was used to perform calculations.

3 Results

One of the crucial parts of the hematologic study is platelets and their indices. They make it possible to control the condition of the hemostatic system in the animal body. Its indicators describe the functions of platelets, allowing to identification of primary bleeding disorders [2]. Thus, it is essential to know the reference limits of the platelet parameter in the body of animals in a physiological state.

For example, the number of platelets in the blood of Holstein heifers in the raising phase steadily increased, exceeding the level of 3 months of age by 1.50 times (Table 1). On average, during the raising phase, it amounted to 356.78±6.74 109/l. The thrombocrit value, i.e. the volume fraction of cells in the blood [2], was not equal to the number of platelets. The age-related variability of thrombocrit, as well as platelet indices, had the following direction. Their value increased from 3 months to 9 months of age, at which it reached a maximum, and then declined, reaching a minimum at the age of coupling time (Table 1). Meanwhile, the average value of the parameter for the entire phase of heifer breeding was equal to 0.23±0.02%.

Platelets circulating in the blood have a certain size, which can be indirectly judged by the size of mean platelet volume (Table 1). For example, in the blood of 3-6 months old heifers, the MPV had the lowest value and amounted to 6.20± 0.03 and 6.50± 0.05 fl. By 9 months of age, cell volume increased to 7.70±0.13 fl and then decreased to 7.20±0.03 fl by the end of the heifer raising phase. The average value for the studied phase of postnatal ontogenesis of animals was 7.03±0.05 fl.

Such parameters as PDW and P-LCR were characterized by similar age-related variability; their average values during the raising were 7.79±0.05 and 5.84±0.15%, respectively.

Table 1: Platelets and their indices in Holstein heifers (n=10)

Indicator	Age of heifers, months					Average for raising phase
	3	6	9	12	15	
PLT, 10^9 /l	294.30±4.31	305.30±2.20*	351.30±9.17*	391.30±12.81*	441.70±5.22*	356.78±6.74
PCT, %	0.18±0.01	0.21±0.01	0.29±0.01	0.25±0.02*	0.22±0.04*	0.23±0.02
MPV, fl	6.20±0.03	6.50±0.05	7.70±0.13*	7.57±0.05*	7.20±0.03*	7.03±0.05
PDW, %	6.73±0.04	6.80±0.03	9.60±0.03*	8.50±0.05*	7.33±0.04*	7.79±0.05
P-LCR, %	3.52±0.04	3.76±0.04	7.70±0.03*	7.47±0.48*	6.76±0.16	5.84±0.15

Note: * - P≤0.05 in relation to the age of 3 months

Therefore, the number of platelets in the blood of heifers during the raising phase increased with age. Platelet and thrombocrit indices changed in the form of an ascending parabola with a maximum at 9 months of age. It is this age that can be attributed to the "critical" one in the formation of functional activity of thrombopoiesis in the body of growing animals. It is reasonable to assume that the revealed dynamics of platelets and their characteristics provide the required level of liquid properties of blood, determining the perfusion of internal organs, the intensity of metabolism, and the rate of growth and development of heifers.

Table 2. Indicators of plasma coagulation hemostasis in Holstein heifers (n=10)

Indicator	Age of heifers, months					Average for raising phase
	3	6	9	12	15	
Quick prothrombin ratio	2.31± 0.03	2.13± 0.01*	1.80± 0.08*	1.95± 0.07*	2.48± 0.16	2.13± 0.07
Prothrombin activity, %	32.63± 0.41	35.93± 0.30*	45.70± 2.24	41.10± 1.95*	31.70± 2.39	37.41± 1.46
INR, IU	2.40± 0.03	2.22± 0.02*	1.86± 0.08*	2.02± 0.08*	2.60± 0.18	2.22± 0.08
APTT, sec	67.50± 4.47	58.70± 1.34	56.70± 1.39*	57.17± 2.92*	59.10± 4.04	59.83± 2.90
Thrombin time, sec	22.50± 0.59	19.07± 0.36*	18.70± 0.37*	18.53± 0.65*	18.07± 0.79*	19.37± 0.55
Fibrinogen, mg/l	2013.00± 46.50	1457.00± 30.81*	1414.00± 38.76*	1403.00± 43.54*	1377.00± 32.29*	1532.80± 38.38
Antithrombin III, mg/l	0.33± 0.05	0.37± 0.04	0.37± 0.04	0.35± 0.02*	0.33± 0.05	0.35± 0.04

Note: * - P≤0.05 in relation to the age of 3 months

The components of plasma coagulation homeostasis are responsible for direct blood clotting, which follows an extrinsic and intrinsic pathway, differing only in the mechanisms of activation of clotting factors.

The condition of the extrinsic blood coagulation pathway was judged by the status of the prothrombin complex. In the studies we characterized it by the amount of prothrombin activity, the value of the international normalized ratio (INR) and the prothrombin ratio. The age variability of the values of these parameters has revealed a critical point. It is 9 months old heifers. From this phase, the indicators changed the direction of alterations (Table 2). Therefore, the activity of prothrombin, starting from the age of 3 months, increased and reached the maximum value in the

blood of 9-month-old heifers (45.70 ±2.24%), which corresponded to the minimum level of prothrombin ratio (1.80±0.08) and INR (1.86±0.08 IU). Then the prothrombin activity began to decrease and had the lowest value in the blood of 15-month-old animals (31.70 ±2.39%) with the highest values of prothrombin ratio (2.48±0.16) and INR (2.60±0.18 IU).

Activated partial thromboplastin time (APTT) was defined to characterize the intrinsic coagulation pathway. Its age dynamics were opposite to the prothrombin activity. From the age of 3 months, the APTT value decreased, reaching a minimum in the blood of 9-month-old heifers (56.70±1.39 s), and then increased by the end of the raising phase to 59.10±4.04 sec (Table 2).

Therefore, the key parameters of the extrinsic and internal coagulation pathways were in reciprocal relationships, balancing the biological effects of each other.

The final stage of blood clotting - fibrin formation was evaluated by the value of thrombin time and fibrinogen concentration. The value of these parameters in the blood of heifers during the phase of their raising systematically declined by 19.69 and 31.59%, respectively (Table 2).

The antithrombin III concentrations gave an idea of the anticoagulant system condition in the body of heifers. The level of the parameter did not depend on age. It fluctuated in the range of 0.33-0.37 mg/l, averaging 0.35±0.04 mg/l during the phase of growing heifers in plant-based feeding. Thus, we have evaluated the condition of plasma-coagulation homeostasis in the body of heifers using basic coagulation tests (thrombin, prothrombin, and APTT). This made it possible to establish the fluctuations of blood clotting factors aimed at maintaining its fluid state, ensuring the performance of its physiological functions, as well as the ability to thrombosis (if necessary).

At the next stage, we attempted to describe the complete variability of the parameters characterizing the hemostatic system in the body of Holstein heifers during the plant-based raising phase using the principal component meth-od [24]. To identify statistically significant factors, the Cattell's scree test was used [25]. According to the graph, two factors were significant: the main component 1 (MC1) and the main component 2 (MC2), and the rest fell into the “factorial scree”. Loads of hemostasis parameters on MC 1 and MC 2 are given in Table 3.

Table 3: Loads of hemostasis parameters on MC 1 and MC 2

Indicator	Main component 1		Main component 2	
	Load	*P*	Load	*P*
PLT, 10^9 /l	**-0.812**	≤0.05	**-0.675**	≤0.05
PCT, %	**-0.749**	≤0.05	**-0.678**	≤0.05
MPV, fl	**-0.763**	≤0.05	**-0.754**	≤0.05
PDW, %	**-0.723**	≤0.05	-0.561	0.087
P-LCR, %	**-0.736**	≤0.05	**0.648**	≤0.05
Quick prothrombin ratio	0.457	0.231	-0.157	0.702
Prothrombin activity, %	**0.912**	≤0.05	0.321	0.468
INR, IU	0.541	0.151	-0.141	0.724
APTT, sec	**0.791**	≤0.05	0.487	0.202
Thrombin time, sec	**0.817**	≤0.05	0.403	0.287
Fibrinogen, mg/l	**0.723**	≤0.05	-0.089	0.867
Antithrombin III, mg/l	0.349	0.412	0.147	0.734
Explained variance	73.18		9.79	

Note: Bold indicates statistically significant effects (P≤ 0.05)

Statistically significant MC1 and MC2 in total explained 82.97% of the variance of hemostasis indicators in the body of Holstein heifers during their raising. Meanwhile, on the main component 1, almost all parameters of cellular and plasma-coagulation hemostasis gave statistically significant loads. This gave us grounds to suppose that it reflects the influence of the age of animals on the formation of hemostatic mechanisms in the body of heifers. It means that age defines the complete variability of hemostatic parameters in the blood by 73.18%. For the main component 2, statistically significant loads were given by the number of platelets and megalothrombocytes, hematocrit and mean platelet volume (Table 3). It is reasonable to suppose that the main component 2 reflected the influence of PLT and their population characteristics on the ability of heifers to coagulate blood if necessary.

Therefore, the principal component method enabled us to establish the following: a complete variability of hemostatic parameters in the blood of heifers depends on their age; the ability to coagulate blood depends on the number of platelets and their functional properties.

4 Discussion

Coagulation is one of the most essential functions of blood. This is a complex physiological process as a result of which a clot forms, preventing blood loss and bleeding. The coagulation system includes the endothelial lining of blood vessels as well as cellular (platelets) and soluble (plasma proteins) blood components [26].

An important component of the hemostatic system is platelets, which recognize vascular damage and trigger coagulation, increasing the formation of a clot at the injured site [27]. The environmental changes affect platelets [4]. Hence, the age-related growth in their number in the bloodstream is the outcome of an increased amount of blood in the body of heifers. That happens to owe to the growth of the body and the compounds of various chemical structures circulating in it.

It is reasonable to suppose that the platelet concentration correlates with the value of thrombocrit, reflecting the volume fraction of cells in the bloodstream [2]. Yet this dependence has not been revealed. For example, in 9 months old animals, the platelet count was 351.30±9.17 109/l with hematocrit 0.29±0.01%; before coupling time, the 15 months old animals showed the platelet count of 441.70±5.22 109/l. It corresponded to the thrombocrit value of 0.22±0.04%. Therefore, the cells, depending on their age, had different sizes and volumes. In accordance with [27], the size (volume) of platelets circulating in the blood varies greatly even in an animal. Earlier it was suggested that the size of platelets is associated with the phase of their life in the bloodstream and varies in the range from young to old cells. However, this idea did not go any further [27].

At present, it has been established that the functional status of the animal body is related to the condition of physiological systems [22]. Thus, not only the number of platelets but also their features reflect the structural development of the body at the appropriate age under the implementation of hereditary information. Therefore, the number of blood plates, their volume fraction in the blood, the average volume of platelets, the variability of the parameter and the

number of megalothrombocytes among themselves are in a balanced condition. Due to it, the blood has optimal liquid properties and can circulate through the system, providing the metabolic needs of cells. According to [27], the variability of platelet parameters is associated with their diverse biological functions. The authors [28] have come to similar conclusions in their studies. They pointed out that in a stable condition of the body, the number of platelets entering and leaving the blood is equal. It means that the production of platelets corresponds to their destruction ("turnover"). The physiological meaning of this comes down to the fact that to keep the number of platelets in the blood while reducing the survival of cells, the rate of their production (turnover) by megakaryocytes accumulates. Platelet homeostasis is primarily controlled by thrombopoietin, synthesized in the liver, bone marrow, and spleen [29].

It was discovered that the mean platelet volume (MPV) is an activation marker of large platelets [30]. Meanwhile, large platelets show higher enzymatic and metabolic activity than other cells [31]. Our studies are compatible with this conclusion. In the blood of heifers, the MPV value was proportional to P-LCR one.

Plasma-coagulation homeostasis in conditions of physiological standards is supported by a balance between indicators characterizing the state of the extrinsic and intrinsic coagulation pathway, fibrin formation, and anticoagulation system. The age variability of certain parameters of hemostasis is aimed at maintaining the viscoelastic properties of blood and the ability to clot in case of vascular damage. The balance between the hemostatic system components with pro-and antithrombotic activity is supported by the hemopoietic and liver organs [16, 32]. According to [33], the blood coagulation system development in the body of animals occurs in the first year of life. Yet at birth, they have "excellent" hemostasis.

Additionally, our studies have proven the hypothesis that platelet count is correlated with fibrinogen concentration [4]. This is because the age-related increase in the level of plates in the blood of heifers was followed by a decrease in fibrinogen content. As we know, fibrinogen is a crucial blood clotting protein; its quantity characterizes the rate of conversion of fibrinogen into fibrin, which affects the amount of blood viscosity [26]. Thus, a reduction in thrombin time and fibrinogen concentration is a tool aimed at maintaining blood flow in a growing body. The correlation of thrombin concentration with fibrinogen and its ability to form a fibrin clot was reported in the study [34]. The authors have observed that the amount of thrombin affects the thickness and density of the fibrin clot. In conditions of disordered proportionality between their concentrations, the clots have poor stability [35].

Meanwhile, the amount of thrombin is related to the concentration of activated platelets. Nevertheless, in our studies, the thrombin content, as estimated by thrombin time, reduced with age, and the amount of P-LCR had wave-like dynamics with a peak at the age of 9 months. We consider that the content of circulating P-LCR is not equal to their activity. The same conclusions were drawn in the studies [5], pointing out that up to 30% of circulating platelets have the procoagulant ability.

While analyzing the complete variability of blood indicators reflecting the hemostatic system in the body of Holstein heifers, it was determined that 82.97% of their explained variance is associated with MC1 and MC2. It was found due to by the principal components method. In the main component 1, all indicators of hemostasis, with the exception of the prothrombin ratio, INR and antithrombin III, gave statistically significant loads, which provides an opportunity to link MC1 with the age of heifers. In the main component 2, significant loads were connected with plate-lets and their parameters reflecting the role of cells in maintaining hemostasis.

Therefore, the hemostatic system formation in the body of Holstein heifers depends on the age of the animals and the functional development of physiological systems.

5 Conclusion

This work studies the level of platelets and their parameters in the blood of Holstein heifers during the plant-based raising phase depending on age. The "critical age" in their variability is 9 months, since from this time the direction of the age dynamics of PCT, MPV, PDW, and P-LCR changes.

The indicators of the extrinsic and intrinsic coagulation pathways during heifer raising show reciprocal relationships, balancing the biological significance of each other. The age-related dynamics of fibrin formation indicators (prothrombin time, fibrinogen) and the anticoagulant system (antithrombin III) is intended to maintain the fluid blood condition.

The principal component method demonstrates that the complete variability of the hemostatic composition of blood is related to the age of heifers. It defines 73.18% of the explained variance of parameters.

The obtained data may be used as a standardized one in evaluating the hemostasis condition of Holstein heifers and in performing therapeutic interventions with drugs affecting blood coagulation.

6 Availability of Data and Material

Data can be made available by contacting the corresponding author.

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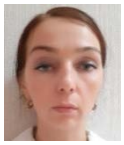

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