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Speculation On The Biochemical Mechanisms And Preventive Actions Of Copper Deficiency In Karakul Sheep In A Famine

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Annotation. Based on literature data and the results of our own research, this article provides a detailed analysis of copper metabolism in sheep. At the same time, the issues of the emergence, development, and deepening of copper deficiency in sheep grazed in the conditions of the biogeochemical province of the Hungry Steppe are revealed. Studies have established correlation relationships between the copper content in the liver and blood, between the copper content in the blood and the activity of copper-containing blood enzymes, between the copper content in the blood and the activity of copper-containing liver enzymes, and between the activity of copper-containing blood and liver enzymes. Based on the use of these correlation relationships, a convenient test has been developed, with which it is possible to establish the availability of copper for Karakul sheep bred in the conditions of the Hungry Steppe. Using this method of testing a flock consisting of 465 sheep was examined and 76 (16.34%) copper deficient animals were identified, of which 8 (1.72%) had obvious signs of endemic ataxia. Subsequently, the examined animals were fed copper sulfate in the form of a salt mixture mixed with sodium chloride and complete improvement in the hralth of the sheep herd was achieved.

Keywords: copper metabolism, subcellular organelles of the liver, physiological norm, moderate and pronounced copper deficiency, copper content, metallothionein, correlation relationship, regression equation, feeding with copper sulfate.

1. INTRODUCTION

Karakul breeding is one of the main leading branches of animal husbandry in the Republic of Uzbekistan. Its further development is associated with the improvement in production technology, the development of the scientific basis for the full feeding of Karakul sheep, the maximum use of the biological potential of their productivity based on the achievements of modern science, and the prevention of losses caused by various diseases. Endemic diseases caused by copper insufficiency, accompanied by the death of part of the sheep head and a significant decrease in their productivity, cause great damage to Karakul breeding. The aim of this work was to study the pathological mechanisms underlying the development of copper deficiency as a theoretical basis for the development of its diagnosis, treatment and prevention in Karakul sheep.

2. MATERIAL AND METHODOLOGY.

1.1. Study material. The material was blood and liver samples of Karakul sheep from the Nurabad district of the Samarkand region (physiological norm) and the Zaamin district of the Jizzakh region (the Hungry Steppe biogeochemical province whit copper failure). The study examined whole blood, blood serum, red blood cells, whole liver and subcellular organelles of the liver. In the above biomaterials, the content of copper and zinc and the activity of the copper-containing enzymes ceruloplasmin (CP) superoxide dismutase (SOD) and the content of metallothionein were determined. Fractionation of liver cytosolic proteins was also carried out.

1.2. Research Methods. Liver homogenization was carried out according to the generally accepted method in a homogenizer with a Teflon pestle in a solution of 0.25 M sucrose containing 0.005 M $MgCl_2$ (tissue-solution ratio 1: 9). Subcellular organelles were obtained by differential centrifugation: nuclei with cell debris, large granules, microsomes and cytosol [2]. The copper content in the blood and liver well as the distribution of this metal in the subcellular organelles of hepatocytes was studied.

The content of copper and zinc was determined after the ashing of biomaterials in porcelain dishes at a temperature of 400-450°C, Conc entrated. HNO_3 was added and evaporated (three times), and the samples were subsequently dissolved in a 0.1 N HCl solution, converting them into a mineralizate, which was later used for atomic absorption analysis. Atomic absorption analysis was performed on a Saturn instrument, and the LSP-1 lamp was used as a light source for copper, and for the VSB-2 zinc powered by the PPBL-3 device. The analytical wavelengths for copper were 324.7 nm, and those for zinc were 213.8 nm.

Superoxide dismutase activity was determined spectrophotometrically (19) based on the ability of proteins to inhibit the reduction of nitroblue tetrozolium by a superoxide radical in formazan in an alkaline medium. The activity of ceruloplasmin was determined by paraphenylenediamine [21] at a pH of 6.0. The activity of cytochrome oxidase was determined by spectrophotometric evaluation of the oxidation intensity of reduced cytochrome with cytochrome oxidase contained in the test solution [2, 17]. The activity of superoxide dismutase was determined on the basis of measuring the optical density drop at 600 nm of 2,6-dichlorophenolindophenol, which is reduced by succinate oxidation [2]. Determination of acid phosphatase was carried out on the basis of measurements of p-nitrophenol formed during the enzymatic hydrolysis of p-nitrophenyl phosphate and possessing a characteristic absorption maximum in an alkaline medium at 410 nm according to the methods of Anders-Schepinsky modified by Karlikov [4]. The obtained results were subjected to statistical processing, and the arithmetic mean value, standard deviation, arithmetic mean error, and reliability index were calculated [5,7,8]. A correlation analysis of the relationship between the studied indicators was also carried out, the correlation coefficients were determined, and the regression equation was derived. We took into account those indicators in which the difference between the compared groups was significant when the probability of the obtained difference was less than 0.05 (i.e., $P <0.05$, $P <0.01$, and $P <0.001$).

3. RESULTS AND DISCUSSION.

The role of copper in the feeding of Karakul sheep was disclosed in the works of Rish [9], Makhmudov [6], Daminov [3] and Abdullaev [1], where the presence of biogeochemical provinces, the balance of the pasture ration for copper, physiological parameters and features of the distribution of metal between organs and tissues being normal or in violation of its metabolism were established. To a lesser extent, the biochemical mechanisms of the onset, development, and deepening of diseases associated with copper deficiency have been deciphered, and preventive measures have not been developed to prevent this disease. At the same time, the widespread occurrence of copper insufficiency in Karakul sheep in the territory of Uzbekistan urgently requires such studies.

In this paper, materials are presented concerning a wider generalization of the literature data and the results of our own research, and based on this, we will discuss the biochemical mechanisms of the emergence, development, and deepening of copper deficiency in Karakul sheep grazed in natural pastures of the biogeochemical province of the Hungry Steppe. First, in Karakul sheep in this zone, copper deficiency occurs, which is caused by a moderate copper deficiency due to the high content of its antagonists (sulfates and molybdenum) in the pasture diet, which later turns into a pronounced form. According to the severity of the clinical manifestations, they are distinguished as “moderate” or “severe” forms of copper failure. Initially, Karakul sheep grazing in pastures with a high content of molybdenum and sulfates in soils, plants and drinking water exhibit a moderate copper deficiency accompanied by a decrease in their productivity without the manifestation of specific clinical signs of copper failure and a decrease in copper levels in the liver by 2–3 times compared with the norm [9,10,15]. Subsequently, pronounced hypocuprosis develops, in which the amount of copper in the liver decreases to 2-5 mg/kg of fresh tissue, which is 20-40 times lower than the physiological norm. As a result, young animals develop endemic ataxia, anaemia in new-born lambs, and endemic hepatitis [1,6,10,11, ,13,16], which causes depigmentation of the coat and deterioration of the hair structure and curl quality [1,6]. Along with a sharp decrease in the level of copper in the liver, there is also a significant decrease in its amount in other organs and tissues. Therefore, in muscle and nervous tissues, the copper content decreases by 3 times, in the blood and endocrine glands by 2 times, and in parenchymal organs - by 20-30% compared with the physiological norm. Analysis of biochemical parameters of Karakul sheep with copper deficiency showed that hepatic sulfoxidase activity is inhibited; the ceruloplasmin concentration is reduced; serum monoamine oxidase, serum cholinesterase, cytochrome oxidase, and succinate dehydrogenase in the liver and brain tissues are inhibited [1,6,9,10,12,14,15,25]; and serum aminotransferase, aldolase and alkaline phosphatase activities are increased [1,9,18,23].

Because the liver is the main organ of the metabolic transformations of copper [25] in animals, researchers should uncover the biochemical mechanisms of copper metabolism in physiological and pathological conditions of the body and pay special attention to the subcellular localization of copper, copper-containing proteins and enzymes in hepatocytes. When studying the subcellular fractions that we isolated using marker enzymes, it turned out that in sheep the distribution of cellular organelles by subcellular fractions differs from the pattern observed in humans and rats. These differences, for example, were found when determining the activity of the marker enzyme of lysosomes, namely, acid phosphatase (Table 1).

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Table 1 The activity of acid phosphatase in subcellular fractions of the liver of healthy Karakul sheep

Fraction of Hepatocyte	Acid Phosphatase Activity		
	IU/g	***IU***	***%***
Liver total activity	*352,2±5,6*	*352,2±5,6*	*100*
A nucleus with cell debris	*425,5±2,9*	*128,8±2,2*	*36,7±0,3*
Large granules	*715,8±3,8*	*185,6±2,4*	*52,9±0,2*
Microsomes + cytosol	*80,7±1,3*	*36,7±1,3*	*10,4±0,2*

As seen from Table 1, from the total hepatic activity of acid phosphatase, nuclei with fragments accounted for 36.7 ± 0.3%, large granules accountrd 52.9 ± 0.2%, and microsomes + cytosol accounted for 10.4 ± 0 , 2%. These results suggest that sheep hepatocytes contain large lysosomes under physiologically normal conditions, the sedimentation rate of which is similar to that of mitochondria, and for some lysosomes it approaches the sedimentation rate of nuclei. The heterogeneity of the hepatocyte fraction deposited at 12,000 g for 20 minutes is a specific feature of the liver of ruminants. In humans and rats, under these conditions, almost “pure” mitochondria are found in the sediment[20,23]. The presence of copper enriched lysosomes in the mitochondrial fraction in rats occurs only when they are loaded with copper [24], and in humans, with Wilson’s disease [23]. As an additional control, we carried out the isolation of subcellular fractions of the liver of three healthy people who died in a car accident. Subcellular fractions were isolated by differential centrifugation. Fractions and the activity of the marker enzyme lysosomal acid phosphatase and the copper content were determined. The results of the analyses showed that in the mitochondrial and nuclear fractions with fragments of human hepatocytes, acid phosphatase activity was not normally found. Analysis of copper in the subcellular fractions of the human liver showed that it is close to published data [23], and indicated a satisfactory level of the analytical part of our studies.

To determine the proportion of copper-rich lysosomes in the subcellular fractions of the liver of healthy Karakul sheep, after their isolation by differential centrifugation, the copper content and the activity of the acid phosphatase-marker enzyme of lysosomes were determined. It was found that the nuclear fraction with cell debris contained 36.6 ± 3.1%, large granules 49.9 ± 1.3% and microsomes with cytosol 13.9 ± 0.5% of the total copper of the liver cell [18]. The activity of acid phosphatase, amounted to 36.7 ± 0.3%, 52.9 ± 0.2% and 10.4 ± 0.2% of the total hepatocytic activity (Fig. 1), respectively [18].

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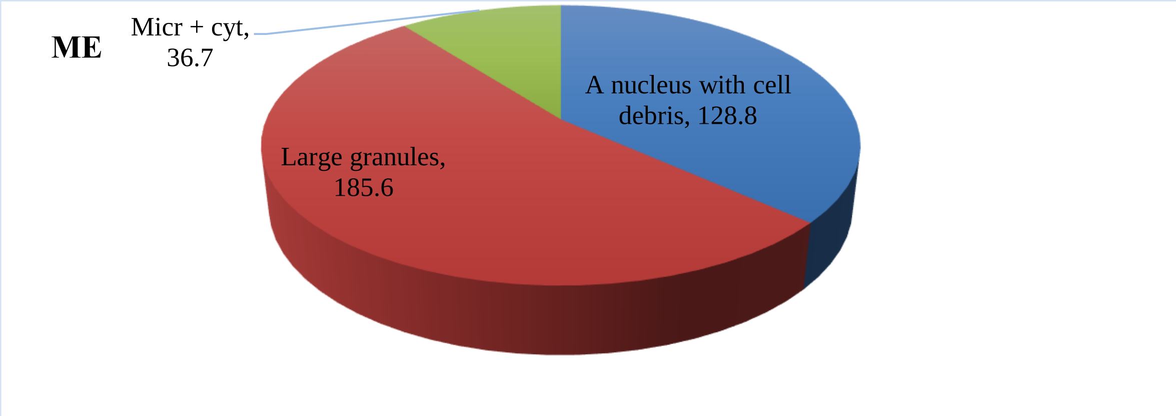

Fig. 1. The activity of acid phosphatase in subcellular fractions of the liver of Karakul sheep (in IU)

Thus, it should be noted that the distribution of copper in the subcellular fractions of the liver of sheep differs from those in humans and rats in which the highest concentration of copper is found in the cytosol; then, in decreasing order, in mitochondria, microsomes and nuclei with fragments, and the difference in acid phosphatase activity in subcellular fractions of hepatocytes is not detected. We carried out analyses to determine the copper content in the subcellular fractions of liver cells in healthy Karakul sheep and these results are consistent with data from other authors in which the copper contents in the liver were 102.0 µg/g [22], 78.18 µg/g [18] and 128.3 µg/g [10,14]; 37.7%, 41.9% and 36.7% was found in the nuclear fraction, respectively; in the fraction with large granules 51.4%, 44.7%, 52.9%; and in the fraction of microsomes with cytosol 10.9%, 13.4%, and 10.4% of hepatocyte copper (Fig. 2).

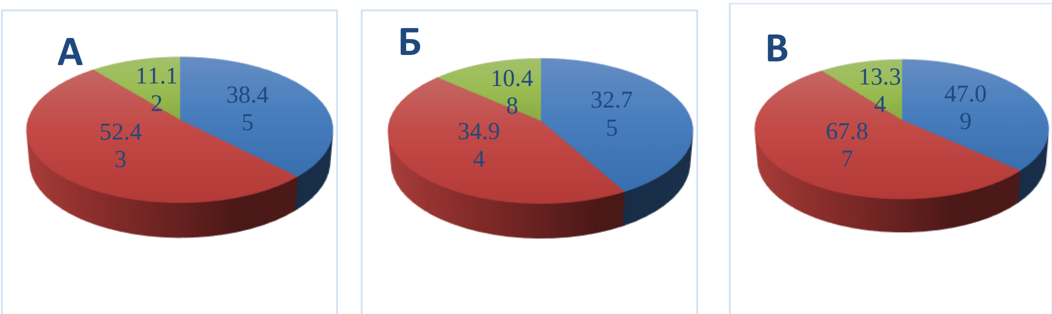

Fig. 1. The copper content in the subcellular fractions of the liver of healthy sheep in mcg. A (22), B (18) and C (10). ● A nucleus with cell debris; ● Large granules; ● Microsomes + cytosol

Thus, on the basis of the above literature data and the results of our own research, it can be noted that the adult liver contains 6-12 mg/kg copper normally, and the rat liver contains 2-3 mg/kg of this element. In sheep, such low concentrations were found only in conditions of severe copper deficiency, accompanied by endemic ataxia. With a moderate deficiency of non-pathological copper, the sheep liver usually contains more than 8 mg/kg of copper in fresh tissue. In this regard, it was of certain interest to study the distribution of copper in the subcellular fractions of sheep hepatocytes, in which the content of this metal in the liver was close to its level in the liver of humans and rats. Sheep liver fractionation was performed, containing on average of 128,8 (normal), 20,0 (moderate copper deficiency), 12,0 (severe insufficiency) and 8,80 (endemic ataxia) mg/kg of copper i fresh tissue. Studies (Fig. 2) showed that with moderate copper deficiency, the decrease in copper content in the liver was 6.65 times, in nuclei with cell debris 10,7 times, in large granules 7,61 times, and in the microsomal fraction with cytosol 2,65 times (P <0.01). With severe copper failure, these indicators were below the physiological normal level by 11,2, 21,3, 13,7 and 3,89 times respectively, and with endemic ataxia by 15,3, 24,6, 17,2 and 6,31 times (P <0.01).

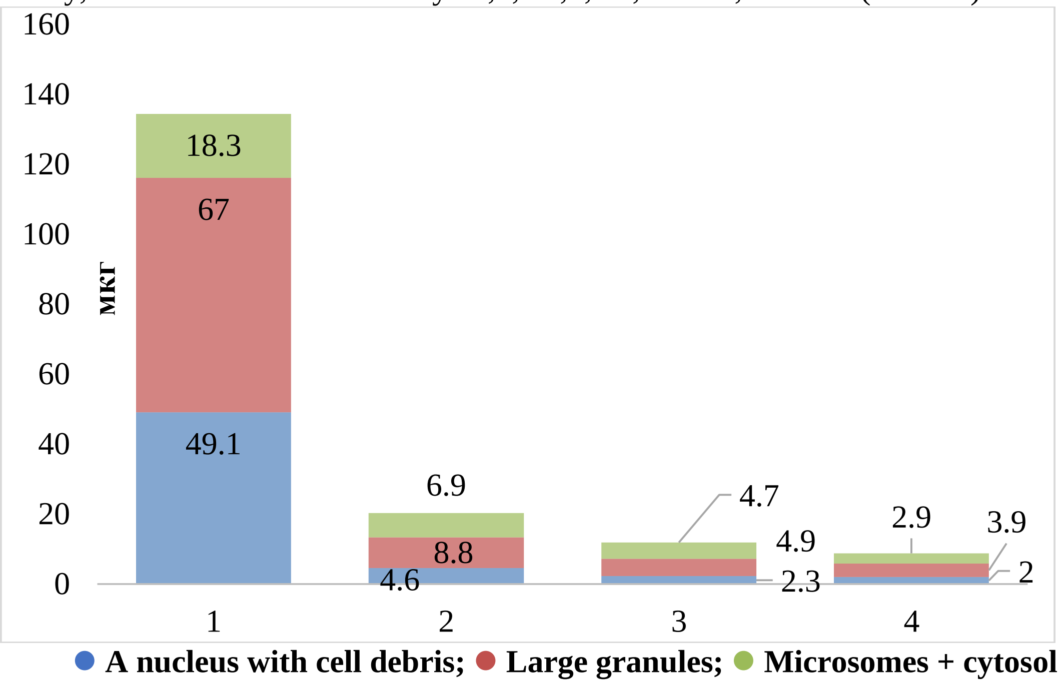

Fig. 2. Changes in the level of copper in the liver and in its subcellular fractions in Karakul sheep. Various conditions of copper exchange: 1-Physiological norm; 2-Moderate copper failure; 3 - Severe copper failure; 4- Enzootic ataxia

To elucidate the mechanisms of the occurrence of copper deficiency in sheep, we carried out fractionation of the liver cytosol proteins of healthy and copper-deficient sheep and determined their copper content and the activity of copper-containing enzymes in the serum (CP), red blood cells (SOD), and liver cytosol (CP and SOD).

Chromatographic fractionation of the liver liver cytosol reveals I, II, III, IY, and Y protein fractions, of which II, IY, and Y, regardless of the state of copper exchange, are copper-containing with molecular weights of approximately 150,000, 30,000, and 10,000. Respectively. We subsequently identified ceruloplasmin, superoxide dismutase and metallothionein. For zinc, this trace element was found in all five protein fractions. In healthy sheep, ceruloplasmin and superoxide dismutase protein fractions contained 25% and 22% copper cytosol, respectively, and metallothionein contained 53%. Copper deficiency causes a decrease in the copper content in these protein fractions by 1,3, 2,0 and 3,0 times, respectively. Under physiologically normal conditions, 1,35 μg of copper and approximately 1,3 μg of zinc are present in the metallothionein fraction, which indicates equal contents of copper and zinc thionine. Copper deficiency was accompanied by an increase in the zinc content both in the cytosol (2,4 times) and in all protein fractions (I and Y 4 times, III and IV 2 times and II 1.6 times). It was also noted that with severe copper failure, the shift in the absorption maximum of the metallothionein fraction of the cytosol is from 255 nm to 215 nm, which proves the presence of the zinc thiolate chromophore group of zinc thionine in the fraction.

With severe copper deficiency, there was a significant decrease in the copper content and the activity of copper-containing enzymes both in the liver and its cytosol and in the blood of sheep (Table 2).

Table 2. The copper content and activity of copper-containing enzymes in the blood and liver of sheep

Copper exchange state	Object of study	Copper content in µg/m or µg/g	CP activity in mg%	SOD activity in mg%
Physiological norm	Erythrocytes	0,92±0,1	-	11,6±1,08
	Blood serum	1,0±0,01	18,44±1,4	-
	Liver cytosol	14,2±0,8	78,5±2,7	0,86±0,07
	Liver	89,0±6,6	-	-
Severe copper deficiency	Erythrocytes	0,44±0,06		6,38±0,9
	Blood serum	0,53±0,03	9,8±1,59	
	Liver cytosol	2,4±0,2	28,42±0,75	0,43±0,08
	Liver	12,0±1,0	-	-

As shown in Table 2, a decrease in the copper content in sheep with copper failure was accompanied by a decrease in this element in red blood cells by 52.2%, blood serum by 47.0%, and the liver by 86.6%. In the liver cytosol. The decrease was 83.1% with a decrease in the activity of CP in serum by 46.8%. In the cytosol of the liver, there was a decrease of 63.8%, SOD in red blood cells decreased by 45.0% and in the cytosol of the liver, the decrease was 50.0%, indicating the close correlations of these indicators among themselves.

To identify the degree of correlation, we carried out a correlation analysis using the above indicators which allowed us to calculate the correlation coefficients and establish the presence of reliability between the compared indicators (Table 3).

Table 3. Correlation between the content of copper and the activity of copper-containing enzymes in the blood and liver

Statistical indicators	Blood copper	Liver	Blood copper	**SOD** Erythrocytes	**CP** Blood serum

	CP Blood serum	SOD Erythrocytes	CP Liver cytosol	SOD Liver cytosol	Blood Liver	SOD Liver cytosol	CP Liver cytosol
Physiological norm							
r	0,93	0,92	0,95	0 ,82	0,94	0,92	0,86
P<	0,001	0,001	0,001	0,01	0,001	0,001	0,01
Copper deficiency							
r	0,93	0,84	0,95	0 ,94	0,81	0,90	0,94
P<	0,001	0,01	0,001	0,001	0,01	0,001	0,001

As seen from Table 3, there are highly reliable relationships between the copper content and the activity of CP in the blood serum and SOD in red blood cells, between the copper content in the liver and the activity of CP and SOD in the liver cytosol, between the copper content in the blood and the copper content in the liver, between the activity of SOD in red blood cells and the activity of SOD in the liver cytosol, and between the activity of serum CP and the activity of CP in the liver cytosol. The presence of correlation relationships between these indicators allowed us to conduct a regression analysis and derive the regression equations, which are presented in Table 4.

The derived regression equations, proving the presence of a highly reliable relationship between these indicators, make it possible to use them as a convenient test for determining the availability of copper in the body of Karakul sheep. Therefore, by determining the activity of CP and SOD in sheep blood and combining these indicators with the regression equation, one can establish the level of copper supply to their bodies. In dysfunctional copper exchange.

Table 4 The regression equation between the copper content and the activities of enzymes sheep blood and liver

Indicators	Regression equation
Blood copper /CP blood serum	Y=23.9·X--1.36
Blood copper/SOD erythrocytes	Y=16,3·X--1,87
Blood copper/CP liver	Y=25,83+ 0,55·X
Blood copper/SOD liver	Y=0,343+0,006· X
Blood copper/copper liver	Y=79,5·X – 6,8
CP copper/CP liver	Y=4,53·X.--10,45
SOD erythrocytes SOD liver	Y=0,82 +12,67··X

The establishment of a Hungry Steppe biogeochemical province and the establishment of copper deficiency in sheep before the onset of clinical signs of the disease will allow for timely health interventions. However, at the same time, the main focus should be on the fact that the number of animals with a hereditarily low level of these enzymes in the blood in different flocks can be 10-15% [25]. In this regard, a reliable conclusion regarding the copper metabolism of animals can be made by analysing both enzymes and a sufficient number of animals, which allows us to exclude variants caused by genetic factors. The proposed diagnostic test to identify the state of copper metabolism by determining the activity of CP and SOD was tested on a farm in the Zaamin district. During testing, 465 sheep were examined and 76 (16.34%) copper-deficient animals were identified, of which 8 (1.72%) had obvious signs of endemic ataxia. According to our recommendation, all unsuccessful livestock were fed (salt free access) a salt mixture containing sodium chloride and copper sulfate (at a rate of 0.2 kg $CuSO_4$ per 100 kg NaCl), and as a result, animal health was achieved.

4. CONCLUSION

1. Unlike other animal species, for example, rats in ruminants, including Karakul sheep, the main concentration of copper in hepatocytes is in lysosomes present in the fraction of large granules (approximately 50%) and in the nuclei with cell debris (approximately 1/3). The cytosolic and microsomal fractions contained respectively 10-17 and 4.0-9.5% of hepatocyte copper, respectively.
2. Sheep copper deficiency is manifested by a decrease in copper concentration and the activity of copper-containing enzymes (CP and SOD) in blood serum, hepatocytes and their cytosol; copper II, IY and Y-protein fractions of the cytosol (1.3, 2.0 and 3.0 times, respectively), and an increase in the zinc content in all five protein fractions of the cytosol (I and Y-4 times, III and IV-2 times and II-1.6 times).
3. With copper deficiency, the presence of highly correlated bonds has been established to reduce the reliance on indicators of copper content and the activity of copper-containing enzymes in the blood, liver and cytosol. By determining the activity of CP and SOD in the blood and using the regression equation, it is possible to establish the degree of availability of copper to the sheep .
4. In the case of copper failure to reduce reliance on indicators of copper content and the activity of copper-containing enzymes in the blood, liver and cytosol, the presence of highly correlated relationships was established, on the basis of which a regression equation were derived, which can be used as a convenient diagnostic test to determine the degree of supply of copper to Karakul sheep. For this, it is necessary to determine the activity of CP and SOD in the blood and use the regression equations to establish the degree of availability of this element to the sheep.
5. The proposed diagnostic test to identify the state of copper metabolism was tested in a farm in the Zaamin district. During testing, 465 sheep were examined and 76 (16.34%) copper-deficient animals were identified, of which 8 (1.72%) had obvious signs of endemic ataxia, and improvement in the sheep herd was achieved by feeding with salt mixture.

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