BIO Web of Conferences **95**, 01048 (2024) https://doi.org/10.1051/bioconf/20249501048
CIBTA-III-2024

Discussion of the biochemical mechanisms of copper deficiency in Karakul sheep bred in the Hungry Steppe and its prevention

Mars Safin[1], *Davletbay* Ibragimov[1*], and *Feruza* Ibragimova[2]

[1]Samarkand State University of Veterinary Medicine, Livestock and Biotechnologies, Samarkand, Uzbekistan
[2]Samarkand State University, Samarkand, Uzbekistan

Abstract. Based on the literature data and the results of our own research, the article provides a detailed discussion of the mechanisms of occurrence, development and deepening of copper metabolism in Karakul sheep grazing in the biogeochemical province of the Hungry Steppe of the Republic of Uzbekistan. Studies have established correlations 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, between the activity of copper-containing enzymes in the blood and liver. Based on the use of these correlations, a convenient test has been developed, using which it is possible to establish the supply of the organism of Karakul sheep with copper, bred in the conditions of the Hungry Steppe. As a result of testing this test technique, a flock of sheep of 465 heads was examined and 76 (16.34%) heads with copper deficiency were identified, of which 8 (1.72%) had clear signs of endemic ataxia. Subsequently, these animals were fed with copper sulfate in the form of a salt mixture mixed with table salt, and their complete recovery was achieved.

1 Introduction

Karakul breeding is one of the main leading branches of animal husbandry in the Republic of Uzbekistan. Its further development is connected with the improvement of production technology, the development of scientific foundations for the full feeding of Karakul sheep, the maximum use of the biological potential of their productivity based on the achievements of modem science, and the prevention of losses caused by various diseases. Endemic diseases caused by copper deficiency, accompanied by the death of some sheep and a significant decrease in their productivity, cause great damage to astrakhan breeding. The purpose 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.

* Corresponding author: shernazarov.1987@gmail.ru

2 Materials and methods

Research material. The material was blood and liver samples of Karakul sheep from Nurabad district of Samarkand region (physiological norm) and Zaamin district of Jizzakh region (Hunger-steppe biogeochemical province of copper deficiency). Whole blood, blood serum, erythrocytes, whole liver and subcellular liver organelles were subjected to the study. In the above biomaterials, the content of copper, zinc and the activity of copper-containing enzymes ceruloplasmin (CP), superoxide dismutase (SOD) and the content of metallothionein were determined. Fractionation of liver cytosol proteins was also carried out.

Research methods. The liver was homogenized according to the conventional method in a homogenizer with a Teflon pestle in a solution of 0.25 M sucrose containing 0.005 M $MgCO_3$ (tissue-solution ratio 1:9). Subcellular organelles were obtained by differential centrifugation: nuclei with cell fragments, large granules, microsomes, and cytosol (2). The content of copper in the blood, liver and the distribution of this metal in the subcellular organelles of hepatocytes were studied.

Determination of the content of copper and zinc was carried out after the biomaterials were ashed in porcelain cups at a temperature of 400-450°C, HNO_3 concentrate was added (three times) and subjected to evaporation Further the samples were dissolved in a 0.1 N HC1 solution, turning them into a mineralizate, which was subsequently used for atomic absorption analysis. Atomic absorption analysis was carried out on a Saturn instrument; an pendant fluorescent LSP-1 lamp was used as a light source in the case of copper, and a high-frequency spectral VSB- 2 lamp powered by an electrodeless lamp power supply PPBL-3 instrument was used for zinc. The analytical lines for copper were 324.7 nm and for zinc 213.8 nm.

Superoxide dismutase activity was determined spectrophotometrically (17), based on the ability of proteins to inhibit the reaction of reduction in an alkaline medium of nitrosine tetrazolium by a superoxide radical to formazan.

The activity of ceruloplasmin was determined with paraphenylenediamine (19) at pH 6.0. The activity of cytochrome oxidase was determined by spectrophotometric assessment of the intensity of oxidation of the reduced cytochrome with cytochrome oxidase contained in the test solution (2).

The determination of the activity of superoxide dismutase was carried out on the basis of measuring the drop in optical density at 600 nm of 2,6- dichlorophenolindophenol, which is reduced during the oxidation of succinate (2.23).

The determination of acid phosphatase was carried out based on the measurement of paranitrophenol, which is formed during the enzymatic hydrolysis of paranitrophenyl phosphate and has a characteristic absorption maximum at 410 mn according to Anders-Schepinsky in the Karlikov modification (4) in an alkaline medium.

Gelfiltration of the liver cytosol of Karakul sheep was carried out on a K-25 column (45x2 cm) on Sephadex G-100, and for the elution of copper- and zinc- containing proteins and enzymes, a Tris buffer with pH 7.0 was used in a gradient of 0.25 M NaCl.

The results obtained were subjected to statistical processing, the arithmetic mean, standard deviation, mean arithmetic error, and reliability index were calculated. We also carried out a correlation analysis of the relationship of the studied indicators, determined the correlation coefficients and derived the regression

equation. Those indicators were taken into account in which the difference between the compared groups was significant when the probability of the obtained difference was less than 0.05 (that is, $P < 0.05$, $P < 0.01$ and $P < 0.001$).

3 Results and Discussion

The role of copper in the feeding of karakul sheep is revealed in the works of Risch (6), Makhmudov (5), Daminov (3), Abdullaev (1), where the presence of biogeochemical provinces, the balance of the pasture diet for copper, physiological parameters and features of the distribution of metal between organs and tissues in the norm and in violation of its metabolism. To a lesser extent, the biochemical mechanisms of the occurrence, 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 deficiency in Karakul sheep on the territory of Uzbekistan urgently requires such studies.

This paper presents materials related to the use of literature data and the results of our own research, and on this basis, we discuss the biochemical mechanisms of the occurrence, development and deepening of copper deficiency in Karakul sheep grazing in natural pastures of the biogeochemical province of the Hungry Steppe. In Karakul sheep in this zone, copper deficiency first appears, which is caused by a moderate copper deficiency due to the high content of its antagonists (sulfates and molybdenum) in the pasture diet, subsequently turning into a pronounced form. According to the severity of the clinical manifestation, they are distinguished as "moderate" and "pronounced" forms of copper deficiency. Initially, Karakul sheep grazing in pastures with a high content of molybdenum and sulfates in soils, plants and drinking water show a moderate deficiency of copper, which is accompanied by a decrease in their productivity without specific clinical signs of copper deficiency and a decrease in the level of copper in the liver by 2-3 times, compared with the norm (5,7,8). Subsequently, a pronounced form of copper deficiency 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, anemia in newborn lambs, endemic hepatitis (1,5,7), in which depigmentation of the coat occurs, deterioration of the hair structure and curl quality (1,5). Along with a shaip decrease in the level of copper in the liver, there is also a significant decrease in its amount in other organs and tissues. So, in muscle and nervous tissues, the copper content decreases by 3 times, in the blood and endocrine glands by 2 times, in parenchymal organs - by 20-30% compared with the physiological norm. An analysis of the biochemical parameters of copper deficiency in Karakul sheep showed that this inhibited the activity of hepatic sulfide oxidase, there was a decrease in the concentration of ceruloplasmin, inhibition of the activity of monoamine oxidase and cholinesterase in blood serum, cytochrome oxidase, succinate dehydrogenase in the liver and brain tissues (1.5,6,7,8, 9.10,13,15), as well as an increase in the activity of serum aminotransferases, aldolase and alkaline phosphatase (1,5,7,811,12,13,14).

In view of the fact that the liver is the main organ of metabolic transformations of copper (23) in the body of animals, researchers, in order to reveal the biochemical mechanisms of copper metabolism in physiological and pathological conditions of the body, pay special attention to the study of the subcellular localization of copper, copper-containing proteins and enzymes in the hepatocyte. When studying the liver subcellular fractions isolated by us using their marker enzymes, it turned out that in sheep the distribution of cell organelles over subcellular fractions differs from the picture observed in humans and rats. These differences, for example, were found when determining the activity of the marker enzyme of lysosomes - acid phosphatase (Table 1).

Table 1. Acid phosphatase activity in subcellular liver fractions of healthy Karakul sheep.

Hepatocyte fractions	Acid phosphatase activity		
	IU/g	IU	%
Liver total activity	352.2+5.6	352.2±5.6	100
Nuclei with fragments	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

As can be seen from Table 1, from the hepatic total activity of acid phosphatase, the share of nuclei with fragments accounts for 36.7±0.3%, and for large granules - 52.9±0.2%, and for microsomes + cytosol - 10.4± 0.2%. These results allow us to consider that sheep hepatocytes contain large lysosomes under physiological conditions, the sedimentation rate of which is similar to that of mitochondria, and for some of the lysosomes it approaches the nuclear sedimentation rate. The heterogeneity of the fraction of hepatocytes precipitated at 12000g for 20 minutes is a specific feature of the liver of ruminants. In humans and rats, almost “pure” mitochondria are sedimented under these conditions. The presence of copper-enriched lysosomes in the mitochondrial fraction in rats occurs only when they are loaded with copper (22) and in humans - in Wilson's disease (18,21). As an additional control, we isolated subcellular fractions of the liver of three healthy people who died in a car accident. Subcellular fractions were isolated by differential centrifugation and the activity of the marker enzyme lysosomal acid phosphatase and the copper content were determined in them. The results of the analyzes showed that neither in the mitochondrial nor in the nuclear fraction with fragments of human hepatocytes, acid phosphatase activity is normally detected. Analysis of copper in subcellular fractions of the human liver showed that it is close to the literature data (18,21), and testified to the satisfactory level of the analytical part of our studies.

To determine the proportion of copper-enriched 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 acid phosphatase, a marker enzyme of lysosomes, were determined. It was found that the nuclear fraction with cell fragments contained 36.6±3.1%, large granules 49.9±1.3% and microsomes with cytosol 13.9±0.5% of the total liver cell copper (7). As for the activity of acid phosphatase, they were (Figure 1), respectively, in the above order 36.7±0.3%, 52.9±0.2% and 10.4+0.2% of the total hepatocyte activity (7).

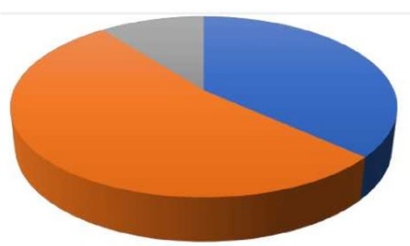
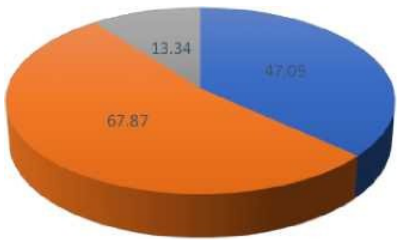


Fig. 1. Acid phosphatase activity in subcellular fractions of Karakul sheep liver (in IU).

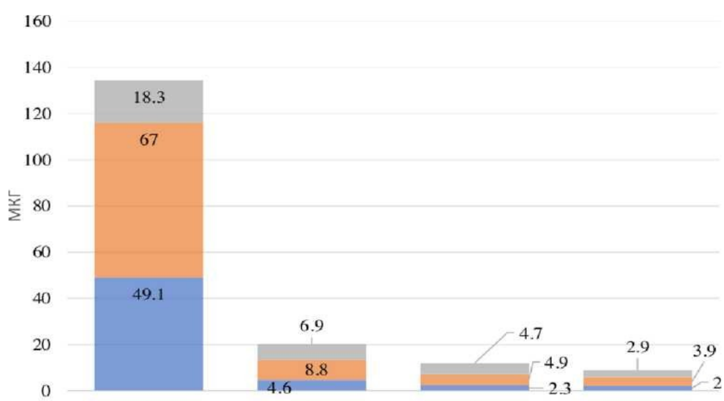
Thus, it should be noted that the distribution of copper in subcellular fractions of the liver of sheep differs from those in humans and rats, in which the highest concentration of copper is found in the cytosol, then in descending order - in mitochonria, microsomes and nuclei with fragments, and the difference in activity of acid phosphatase in subcellular fractions of hepatocytes is not detected. We mm—nm_iiiii—_i_nn_ carried out analyzes to determine the content of copper in the subcellular fractions of the liver cells in healthy Karakul sheep and these results are consistent with the data of other authors, which, with a copper content in the liver of 102.0 pg/g (14), 78.18 pg/g(10) and 128.3pg/g (7), respectively, 37.7%, 41.9% and 36.7% are found in the nuclear fraction: in the fraction of large granules 51.4%; 44.7%; 52.9% and in the fraction of microsomes with cytosol 10.9%, 13.4%; and 10.4% copper of hepatocytes (Figure 2).



A (20), B (18) and C (7,13). H - Nuclei with fragments IZZI - Large granules;ED - Microsomes + cytosol.

Fig. 2. The content of copper in subcellular fractions of the liver of healthy sheep in micrograms.

Thus, based on the above literature data and the results of our own research, it can be noted that the liver of an adult human normally contains 6-12 mg/kg, and the liver of a rat 2-3 mg/kg of this element. In sheep, such low concentrations are found only in conditions of severe copper deficiency, accompanied by endemic ataxia. With moderate copper deficiency, not associated with pathology, sheep liver usually contains more than 8 mg/kg of copper oer fresh tissue. In this regard, it was of particular interest to study the distribution of copper in the subcellular fractions of the liver of sheep, in which the content of this metal in the liver approached its level in the liver of humans and rats. Sheep livers were fractionated containing an average of 128.8 (normal), 20.0 (moderate copper deficiency), 12.0 (severe deficiency) and 8.8.0 (endemic ataxia) mg/kg of copper per fresh tissue. The conducted studies (Figure 2) showed that with moderate copper deficiency, the decrease in the copper content in the liver was 6.65 times, in the nuclei with cell debris 10.7 times, in large granules 7.61 times, in the microsomal fraction with cytosol 2.65 times, times (P <0.01). With severe copper deficiency, these indicators were below the physiological norm,respectively, by 11.2; 21.3; 13.7 and 3.89 times, and with endemic ataxia - 15.3; 24.6; 17.2 and 6.31 times (P<0.0l).



□ - Nucleus with cellular fragments; E - Large granules; H - Microsomes + cytosol.

Fig. 3. Changes in the level of copper in the liver and in its subcellular fractions of karakul sheep under various states of copper metabolism: 1- Physiological norm; 2 - Moderate copper deficiency; 3 - Severe copper deficiency; 4 - Enzootic ataxia.

In order to elucidate the mechanisms of copper deficiency in sheep, we carried out the fractionation of liver cytosol proteins in healthy, copper-deficient sheep and the identification of copper- and zinc-containing proteins.

Chromatographic fractionation of sheep liver cytosol reveals I, II, III, IY and Y protein fractions, of which II, IY and Y, regardless of the state of copper metabolism, are copper-containing with a molecular weight of about 150,000, 30,000 and 10,000 Da, respectively, identified by us as ceruloplasmin , superoxide dismutase and metallothionein. As for zinc, this trace element is found in all five protein fractions. In healthy sheep, the ceruloplasmin and superoxide dismutase protein fractions contain 25% and 22% of cytosol copper, respectively, and the metallothionein fraction, 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 physiological conditions, the metallothionein fraction contains 1.35 pg of copper and about 1.3 pg of zinc, which indicates an equal content of copper and zinc thionein in it. Copper deficiency is accompanied by an increase in the content of zinc both in the cytosol (2.4 times) and in all protein fractions (I and Y - 4 times, III, IY - 2 times and II 1.6 times). It was also noted that with severe copper deficiency, a shift in the absorption maximum of the metalthioneion fraction of the cytosol from 255 nm to 215 nm, which proves the presence of the zinc-thiolate chromophore group of zinc-thionein in the fraction. In addition, the copper content and the activity of copper-containing enzymes in blood serum (CP), blood erythrocytes (SOD) and liver cytosol (CP and SOD) were determined in these experimental animals, which showed that with severe copper deficiency, there is a significant decrease in the content of copper and activity of copper-containing enzymes

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

Copper exchange status	**Object of study**	**Copper content in //g/ml or/./g/g**	**CPU activity in mg%**	**SOD activity in mg%**
Physiological norm	Red blood cells	0.92±0.1	-	11.6±1.08
	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	Red blood cells	0.44+0.06	-	6.38±0.9
	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±l .0	-	-

As can be seen from Table 2, a decrease in the copper content in sheep with copper deficiency is accompanied by a decrease in this element in erythrocytes by 52.2%, in blood serum by 47.0%, in the liver by 86.6%. in its cytosol by 83.1% and a decrease in CP activity in blood serum by 46.8%, in the cytosol of the liver by 63.8%, SOD in erythrocytes by 45.0% and in the cytosol of the liver by 50.0%, indicating close correlation between these indicators. In order to identify the degree of correlation, we carried out a correlation analysis, using the indicators given above, which made it possible to calculate the correlation coefficients and establish the presence of reliability between the compared indicators (Table 3).

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

Statistical indicators	Blood copper		Liver copper		Blood copper	SOD of erythro cytes	CP blood serum
	CCP blood serum	eSOD of erythro cytes	C CPof the liver cytosol	SOD of the liver cytosol	Liver copper	OD of the liver cytosol	CP of the liver cytosol
Physiologica				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	00.93	00.84	00.95	0.94	0.81	0.90	0.94
P	<0.001	<0.01	<0.001	<0.001	<0.01	<0.001	<0.001

As can be seen from Table 3, there are significant relationships between the copper content and the activity of the blood serum CP and SOD of erythrocytes, between the copper content in the liver and the activity of the CP and SOD of the liver cytosol, between the copper content in the blood and the copper content in the liver, between the activity of SOD erythrocytes and SOD activity of the liver cytosol, between the activity of the blood serum CP and the activity of the CP of the liver cytosol. The presence of correlation relationships between these indicators allowed

Table 4. Regression equation between copper content and activity of blood and liver enzymes in sheep.

Indicators	Regression Equation
Blood copper / Serum CP	Y=23.9X1.36
Blood copper / SOD of erythrocytes	Y= 16.3.X-1.87
Liver copper / Liver CP	Y=25.83+0.55X
Liver copper / Liver SOD	Y=0.343+0.006 X
Blood copper / Liver Copper	Y=79.5X - 6.8
Blood cirrhosis / Liver cirrhosis	Y=4.53X-10.45
SOD of blood erythrocytes / SOD of liver	Y=0.82+12.67 X

The derived regression equations prove a reliable relationship between the given indicators and at the same time make it possible to use them as a convenient test for determining the body's supply of Karakul sheep with copper. So, for this, by determining the activity of the CP and SOD in the blood of sheep and combining these indicators with the regression equation, it is possible to establish the level of supply of their body with copper. In the biogeochemical province of the Hungry Steppe, which is unfavorable in terms of copper metabolism, the establishment of a copper deficiency in sheep by this method before the appearance of clinical signs of the disease will allow timely implementation of health measures. But at the same time, the main attention should be paid to 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% (14). In this regard, a reliable conclusion of the copper metabolism of animals can be made on the analysis of both enzymes and on a sufficient number of animals to exclude variants caused by genetic factors. The proposed diagnostic test to identify the state of copper metabolism by determining the activity of the

CP and SOD was tested in the farm of the Zaaminsky district. During testing, 465 heads of sheep were examined and 76 (16.34%) heads with copper deficiency were identified, of which 8 (1.72%) had clear signs of endemic ataxia. According to our recommendation, all the unfavorable livestock was fed (free access) with a salt mixture containing sodium chloride and copper sulfate (at the rate of 0.2 kg $CuSO_4$ per 100 kg NaCl) and as a result, complete recovery of the animals was achieved.

4 Conclusion

- Unlike other animal species, for example, rats, in ruminants, including Karakul sheep, the main place of copper concentration in the hepatocyte is lysosomes, which are present in the fraction of large granules (about 50%) and in nuclei with cell debris (about 1/3). Cytosol and microsomal fraction contain, respectively, 10-17% and 4.0-9.5% of hepatocyte copper.
- Copper deficiency in sheep is manifested by a decrease in the concentration of copper and the activity of copper-containing enzymes (CP and SOD) in the blood serum, hepatocyte and in its cytosol, while the content of copper II, IY and Y-protein fractions of the cytosol decreases (by 1.3, 2.0 and 3.0 times, respectively), and the zinc content in all five protein fractions of the cytosol increases (4 times in I and Y, 2 times in III, IY, and 1.6 times in II,respectively).
- In case of copper insufficiency, a correlative relationship has been established for a decrease in the indicators of copper content, the activity of copper- containing enzymes in the blood, liver and its cytosol. Determining the activity of the CP and SOD of the blood and using the regression equation, it is possible to establish the degree of provision of the body of sheep with copper, and on this basis, a diagnostic test was developed and proposed to establish the copper exchange of the sheep population.
- The proposed diagnostic test to identify the state of copper metabolism was tested in a farm in the Zaaminsky district. During testing, 465 heads of sheep were examined and 76 (16.34%) heads with copper deficiency were identified, of which 8 (1.72%) heads had clear signs of endemic ataxia, and as a result of feeding with a salt mixture, their recovery was achieved.

References

1. D.V. Abdullaev, Copper in the body of Karakul sheep and its relationship with zinc and vitamin C under normal conditions and copper deficiency, Author's abstract. Diss. Ph.D. biol. Sci., Samarkand, 27 (1967)
2. V.S. Asatiani, Enzyme analysis methods (Nauka, Moscow, 1969)
3. R.A. Daminov, M.A. Rish, The role of copper metabolism disorders in the pathogenesis of icterohemoglobinuria in Karakul sheep. In the book: Microelements in agriculture and medicine, Ulan-Ude, 520-524 (1968)
4. D.V. Karlikov, Phosphatases in pig blood serum, their heritability and relationship with economically useful traits, Author's abstract. Diss. Ph.D. biol. Sci. Dudrovitsy, 17 (1968)
5. M. M. Makhmudov, The influence of the microelement copper on the productivity of Karakul sheep in the zone of copper deficiency. Author's abstract. Diss. Cand. Biol. Sciences-Tashkent, 22 (1964)
6. M.A. Rish, Biogeochemical provinces of Western Uzbekistan. Author's abstract. Diss. Doctor of Biological Sciences, Moscow, 40 (1964)

7. M.G. Safin, Copper in the hepatocytes of Karakul sheep is normal and with disorders of its metabolism. Abstract of thesis, Candidate of Biological Sciences – Borovsk, Kaluga region, 18 (1985)
8. G. Safin, Effects of grazing conditions on copper metabolism in sheep. SamDU. Bulletin of scientific research. Scientific and theoretical services magazine, Samarkand, **3**, 89-93 (2003)
9. M.G. Safin, Study of ecological, biochemical and diagnostic aspects of hypocurosis in Karakul sheep, Tr. Peasant State University named after Cyril and mythology, Luga Leningrad region (2003)
10. F.A. Ruziev, N.M. Sadikova, M.G. Safin, N.E. Yoldashev, Study of ecophysiological and biochemical aspects of copper metabolism in coracle sheep. Collection of materials of the fourth international scientific conference "Monitoring of the spread and prevention of dangerous diseases of animals and poultry", Samarkand, 233-240 (2011)
11. M.G. Safin, “Copper in the hepatocytes of Karakul sheep is normal and with disorders of its metabolism Samarkand, Monograph, 140 (2023)
12. L.Ya. Simonov, Superoxide dismutase of Karakul sheep and its inheritance. Abstract of thesis. Candidate of Biological Sciences Yerevan, 23 (1980)
13. B. Bazarov, Z. Rajamuradov, M. Safin, A. Rajabov, D.K. Hayitov, M. Kuziev, SH. Aminjonov, M. Ismayilova, J. Kudratov, M. Khujabekov, D. Khaydarov, The productivity, chemical composition and nutritional value of pastures dominated by Artemisia diffusa and Cousinia resinosa in arid lands of southwestern Uzbekistan. B IOD I V E R S I T A S ISSN: 1412-033X, Volume 24, Number 7, July 2023 E-ISSN: 2085-4722, 3916-3923 DOI: 10.13057/biodiv/d24073
14. S.R. Gooneratne, J.G. Mc Howell, J. Gawthorne, Intracellular distribution or cjpptr in the liver of normal and cjpper loaded sheep, **27**, 30-37 (1979)
15. M. Nishikimi, N.A. Rao, K. Vagi, The occurence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxigen, Biochem. Biophys. Res. Commun., **46**, **2**, 849-854 (1972)
16. H. Porter, Tissue copper proteins in Wilson’s disease. Intracellular distribution and chromatographic fractionation, Arch.of Neurol., **11**, 341-349 (1964)