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AGE PECULIARITIES OF MORPHOFUNCTIONAL CHANGES OF THE LIVER AT EARLY STAGES OF DIABETES MELLITUS DEVELOPMENT WITH THE USE OF CLUSTER ANALYSIS

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In all age groups of animals, we observed regular changes in the morphometric parameters of hepatocytes of different clusters: there was a decrease in the area of hepatocytes, which is associated with marked processes of glycogenolysis that are confirmed by histological and ultrastructural studies. On the14 th and 28 th days of diabetes mellitus, as compared to the control group, the number of hepatocytes in clusters varies: in rats aged 24 months in C1 and C3 it decreases by 8–7 % and 6-10 %, whereas in C2 it increases by 14–17 %. In rats aged 12 months, as compared with the control group of animals, on the14th day, the number of hepatocytes in C1 decreased by 3.64 %, while in C2 it increases by 5.37 %, while on the 28th day in C2 it decreases by 3.69 %, while in C3 it increases by 4.81 % (in all cases $p<0.05$). Increase in the activity of aminotransferases in both plasma and liver homogenates, but the De Ritis ratio does not statistically differ from the control indicators, showing the development of compensatory processes in the liver and preserving its complete functional capacity.

Key words: liver, hepatocyte, streptozotocin-induced diabetes mellitus.

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ВІКОВІ ОСОЛИВОСТІ МОРФО-ФУНКЦІОНАЛЬНИХ ЗМІН ПЕЧІНКИ У РАННІ ТЕРМІНИ РОЗВИТКУ ЦУКРОВОГО ДІАБЕТУ З ВИКОРИСТАННЯМ КЛАСТЕРНОГО АНАЛІЗУ

У тварин різних вікових груп при цукровому діабеті простежуються закономірні зміни морфометричних параметрів гепатоцитів різних кластерів: зменшення площі гепатоцитів, пов'язане з вираженими процесами глікогенолізу, що підтверджується даними гістологічних та ультраструктурних досліджень. На 14 і 28 доби цукрового діабету, порівняно з контролем, кількість гепатоцитів у кластерах змінюється: у 24-міс. тварин у С1 і С3 зменшується на 8–7 % та 6–10 %, натомість у С2 зростає на 14–17 %. У 12-міс. щурів, порівняно з контролем, на 14 добу кількість гепатоцитів у С1 зменшується на 3,64 %, а в С2 збільшується на 5,37 %; на 28 добу – у С2 зменшується на 3,69 %, а в С3 зростає на 4,81 % (у всіх випадках $p<0,05$). Спостерігається достовірне підвищення активності амінотрасфераз як у плазмі крові, так і гомогенатах печінки, проте коефіцієнт де Рітіса статистично значуще не відрізняється від контрольних показників, що вказує на розвиток компенсаторних процесів у печінці та збереження повною мірою її функціональної здатності.

Ключові слова: печінка, гепатоцит, стрептозотоциновий цукровий діабет.

The study is a fragment of the research project "Age features of pathomorphogenesis in some organs of neuroendocrine, cardiovascular, digestive and respiratory systems in diabetes mellitus", state registration No. 0116U003598.

Various pathological processes that occur in the damaged liver occupy one of the important places in gastroenterology [10]. These changes can be caused by a wide range of factors, one of which is diabetes mellitus (DM) [11]. The number of patients with DM is increasing exponentially every year, and this encourages scientists to improve existing and seek new methods of treatment and diagnosis of this pathology. In Ukraine, an annual increase in the number of patients with DM is registered by an average of 5-7%, among children under 17 years of age this figure is 3–4 % and 6 % – among children under 6 years [2, 4]. However, the real number of patients in Ukraine, as shown by the results of epidemiological studies, is 2-2.5 times higher due to undiagnosed cases of the disease [15]. The medical and social problem of diabetes is that it is one of the most common endocrine diseases in the world, and its complications (angio-, neuropathy) lead to disability, incapacitation and mortality of patients [2, 7]. One of these complications is diabetic hepatopathy, which, according to various authors, is diagnosed in 33–88 % of patients [13]. The subclinical course of this disorder leads to the fact that specific signs of hepatobiliary system disease (bitterness in the mouth, heaviness in the right hypochondrium, subicterіcity of mucous membranes, dyspeptic phenomena) are diagnosed in only 18–33 % of patients, however, the real figure reaches 24–88 % of patients with DM [6, 13]. It should be noted that in the available Ukrainian- and Russian-language scientific literature there are no works that would study the structure of the liver in experimental diabetes in the postnatal period of ontogenesis. The number of studies published in English-language sources are insufficient and often contradictory [5]. Cluster analysis has not been used in any scientific work on morphometric analysis of the liver.

The purpose of the study was to establish the peculiarities of morphofunctional changes in the liver in experimental streptozotocin induced diabetes using cluster analysis.

Material and methods. The study involved 42 mature male Wistar rats aged 12 (180-220g) and 24 (320–330 g) months, which were divided into two groups. Group I included 30 animals (in 15 rats of different age groups) whose diabetes was induced by a single intraperitoneal injection of streptozotocin (dissolved in 0.1M citrate buffer solution with pH 4.5) at the rate of: 12 months – 6mg/100g of body weight, 24 months – 5mg/100g of body weight. Group II (control) included 12 animals (6 rats of different ages), which in an equivalent dose were injected 0.1 M citrate buffer with a pH of 4.5 intraperitoneally. The blood glucose levels of animals were measured daily in the morning on an empty stomach with an "Accu-Chek Active" glucometer ("Roche Diagnostics GmbH", Germany). The study material was collected on the 14th and 28th days of the ESID. Under ether anesthesia, decapitation was carried out with simultaneous collection of blood in a test tube for biochemical analysis. The blood level of HbA1c was established in a regulated laboratory "Diameb". Studies of ALT and AST activity in blood serum (μmol/h*ml) and liver homogenates (μmol/h*g) were performed in the regulated laboratory of the Center of Bioelementology of Ivano-Frankivsk National Medical University by means of Reitman-Frankel method, using a set of (Filisit-Diagnostics, Ukraine) reagents.

All the experiments on test rats were carried out in compliance with all the ethical requirements and in agreement with the regulations of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Strasbourg, 1986), Council Directive 86/609/EEC (1986), the Law of Ukraine "On protection of animals from ill-treatment" of December 15, 2009 and orders of the Ministry of Health of Ukraine No. 690 dated September 23, 2009, No. 616 dated August 3, 2012 (expert consensus document of the Commission on the ethical issues of the state higher educational establishment "Ivano-Frankivsk national medical university", minute No 104/18 from October 25, 2018).

Hematoxylin and eosin staining, Masson's trichrome staining, Shabadash's method and Sudan III staining techniques were used for histological and histochemical examination of the liver. For electron microscopic examination, samples of liver tissue were fixed in 2 % solution of osmium tetroxide, and the study was performed and contrasted by means of conventional procedure. The study of the material was performed on an electron microscope PEM-125 K at an accelerating voltage of 75 kV, with subsequent photography.

The index of the liver relative mass was calculated using the following formula: Mr=(Ma/BMa)x100, where Mr stands for the relative mass of the liver, Ma – absolute mass of the liver, BMa – body mass of the animal [1].

Photomicrographs of histological, histochemical and semifine sections saved in TIFF file format were used for morphometric studies. Morphometry was performed using "ImageJ" software. It became possible to identify the following data: indices of mononuclear hepatocytes, which were located within one liver acinus (profile area of hepatocytes, profile areas of their nuclei, nucleo-cytoplasmic ratio (NCR)) and to determine their affinity to different zones of the liver acinus (central, intermediate and peripheral).

We have used cluster analysis (Ward's hierarchical clustering method and K-means method), and chi-square test for the analysis of contingency tables [8]. Computerized data processing was performed using the STATISTICA package (StatSoft, Inc. (2010), STATISTICA (data analysis software system), version 10. www.statsoft.com.) and Excel. Statistical changes were considered significant when the level of statistical significance was $p<0.05$.

Results of the study and their discussion. At early stages of experimental streptozotocin induced diabetes (ESID) development (14-28 days) the levels of glucose and HbA1c in blood of animals from different groups has considerably increased: in 12-month-old animals these indices have made up14.35±0.51 mmol/l (control – 4.64±0.39 mmol/l, p=0.0036) and 15.99±0.48 mmol/l (control – 4.63±0.39 mmol/l, p=0.0001), 7.03±0.47 % (control – 2.34±0.19 %, p=0.0325) and 8.33±0.32 % (control – 2.33±0.1 %, p=0.0236); and in 24-month-old animals they have increased to 14.06±0.41 mmol/l (control – 5.62±0.66 mmol/l, p=0.0012) and 15.74±0.49 mmol/l (control – 5.43±0.6 mmol/l, p=0.0001), 7.21±0.19 % (control – 2.41±0.37 %, p=0.0056) and 8.18±0.41 % (control – 2.34±0.33 %, p=0.0236). On the 14th day of ESID development, the mass of the liver in 12- and 24-month-old rats does not considerably change, as compared with the control indices, but the mass of the animals decreases, which leads to an increase in the relative mass of the liver (table 1). On the 28th day, the relative mass of the liver continues to increase against the background of a decrease in the mass of the liver and body mass of the test animals (table 1).

On the 28th day of ESID development in 24-month-old animals we observed a significant number of binuclear hepatocytes (Fig. 1a) and an increase in lipid droplets in individual hepatocytes of different zones (fig. 1c), there is also a leukocytic infiltration of the portal triads of hepatic lobules (fig. 1d). A significant decrease in the number of glycogen granules in the hepatocytes of 12- and 24-month-old animals

(fig. 1a, b) leads to the lucidity of cytoplasm. It should be noted that most of the glycogen is lost by the hepatocytes of the central and peripheral zones, while the hepatocytes of the intermediate zone still contain glycogen. Dilated great veins, sinusoidal capillaries, perisinusoidal spaces are observed.

Table 1

Massometric parameters of 12-month-old rats at different stages of streptozotocin induced diabetes mellitus

Period of the experiment	Group of animals	Mass of the body (g)	Mass of the liver (g)	Relative mass of the liver (%)
12-month-old rats				
14th day	ESID	213±2.74*	8.98±0.21	4.22±0.13*
	control	225±5.01	8.91±0.35	3.96±0.16
28th day	ESID	208±2.74*#	10.04±0.17*#	4.83±0.11*#
	control	244.33±8.14	9.67±0.1	3.96±0.15
24-month-old rats				
14th day	ESID	297.80±15.63*	11.97±0.38	4.02±0.11*
	control	333.67±3.21	10.29±0.37	3.08±0.08
28th day	ESID	289±4.18*	14.26±0.5*#	4.94±0.23*#
	control	340.67±1.15	10.49±0.19	3.08±0.06

Notes: 1) the probability indices as compared to the control * – $p<0.05$; 2) the probability indices as compared to the previous term of experiment # – $p<0.05$.

In the early stages of ESID development we have observed a decrease in the number of glycogen granules in hepatocytes in animals of different ages at the electron microscopic level. This may be confirmed by the "desolation" of hepatocytes, or the so-called "glycogen spaces" (fig. 2 a, c), which are observed in the perinuclear space, where no intracellular organelles are visualized.

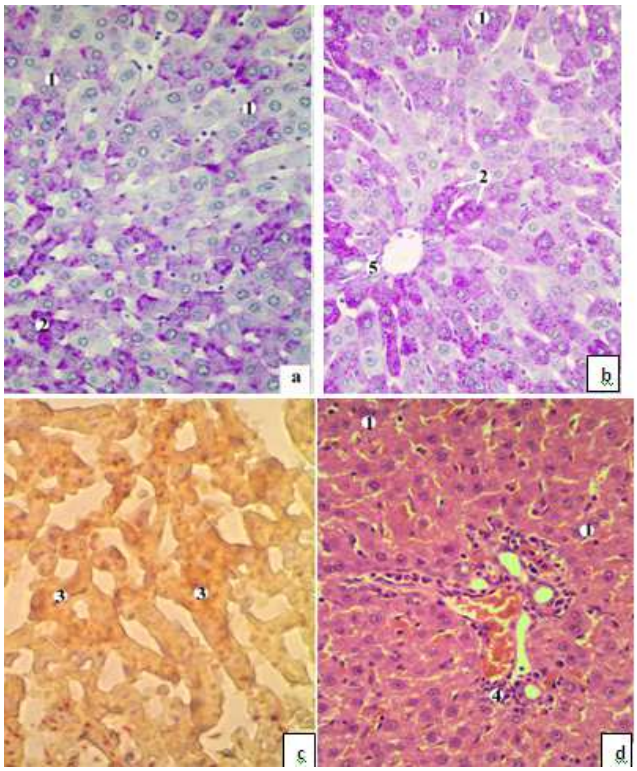

Fig. 1. Increase in the number of binuclear hepatocytes (a) and lipid droplets (c) in them, lympho-plasmacytic infiltration of the portal tracts (d) in 24-months-old rats and reduction of glycogen inclusions in the peripheral zone of the hepatic lobe (b) of 12-month-old rats on the 28th day of ESID development. Staining: Shabadash's staining (a, b), Sudan III (c), Masson's trichrome staining (d). Microphotographs. Magnification: a-d) x400. Marking: 1 – binuclear hepatocytes, 2 – glycogen granules, 3 – lipid droplets, 4 – lymphocytic infiltration of the portal tracts, 5 – triad of the liver.

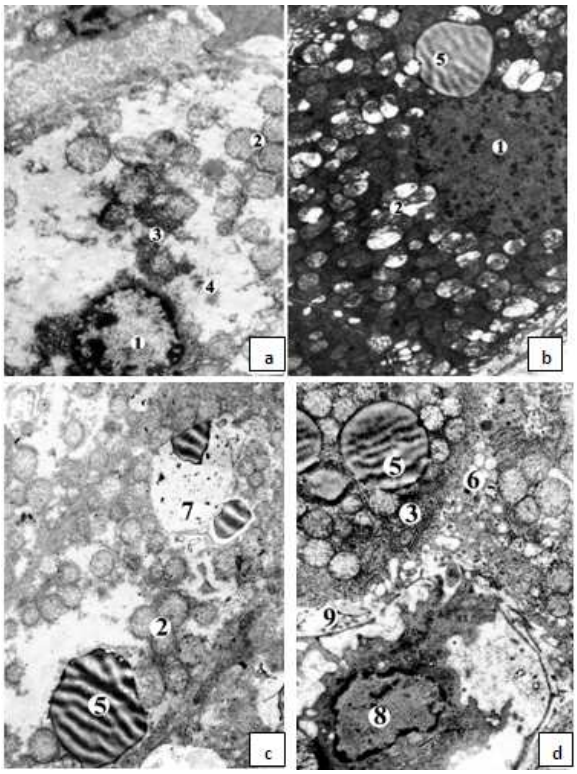

Fig. 2. The presence of empty cytoplasmic fields without glycogen in light (a, c) and destructive changes in the mitochondria of dark (b) hepatocytes in 12-months-old rats on the 28th day of ESID development. Electronic microphotographs. Magnification: a) x6400, b) x4800. Marking: 1 – nucleus of hepatocyte, 2 – mitochondria, 3 – RER, 4 – glycogen-free spaces, 5 – lipid droplets, 6 – SER, 7 – vacuole, 8 – nucleus of the stellate macrophagocyte, 9 – Disse's space.

The nuclei of hepatocytes are of spherical shape, chromatin is condensed in the form of lumps along the inner surface of the nuclear envelope. The latter has irregular contours due to invagination.

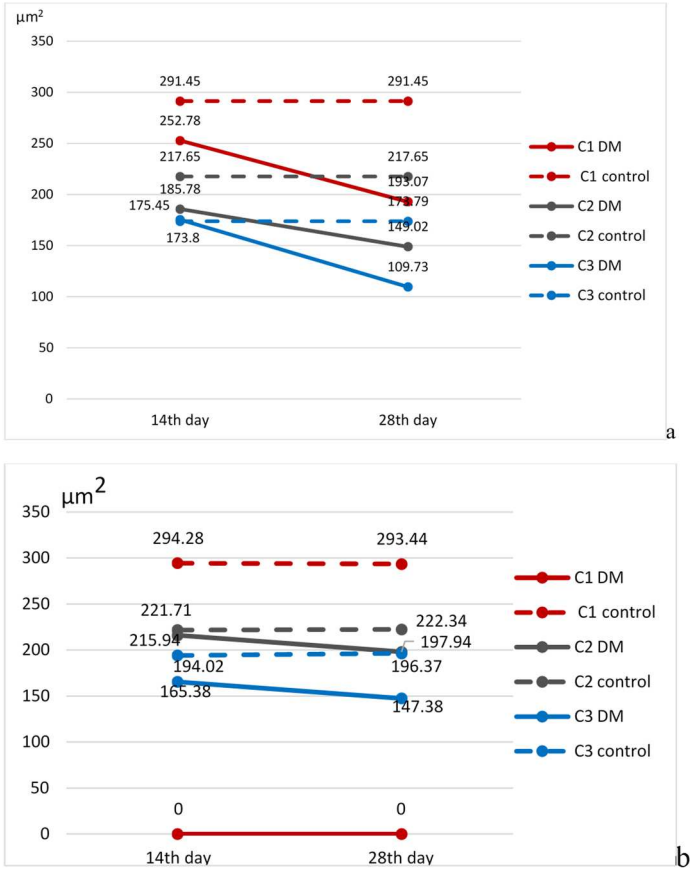

Fig. 3. Dynamics of changes in the area of hepatocytes in 12-months-old (a), and 24-month-old (b) rats in different clusters in the early stages of ESID development.

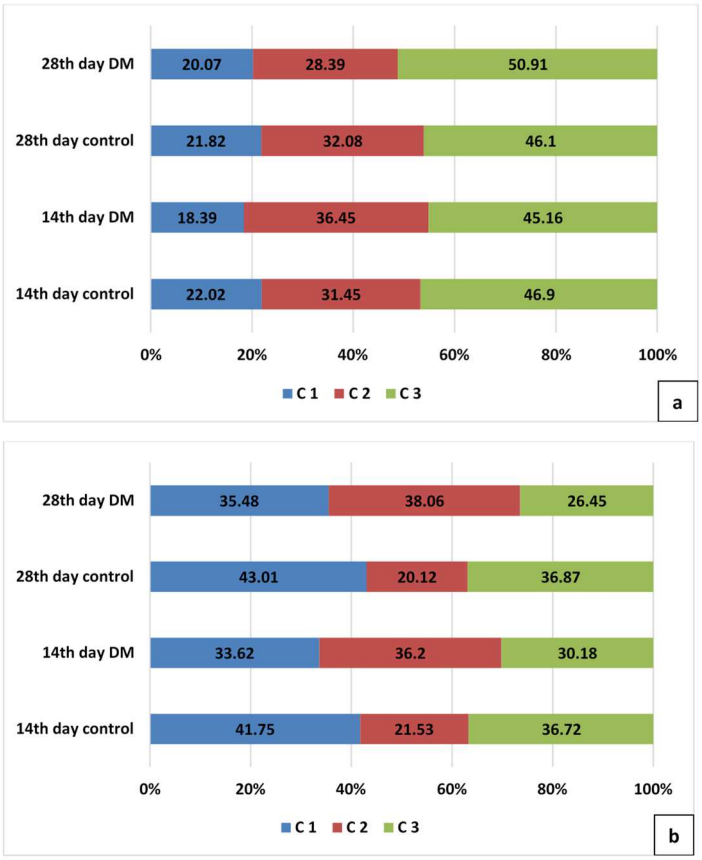

Fig. 4. Quantitative ratio of hepatocytes of different clusters in the hepatic lobule of 12- (a) and 24-month-old (b) rats in the early stages of ESID development.

The number of mitochondria is reduced, their cristae are disorganized and shortened, sometimes undergoing lysis, the matrix is lucid, the coat of mitochondria, in particular the outer one, is fluffy, sometimes with signs of lysis. Reduction and shortening of hepatocyte microvilli both around the sinusoidal pole and turned towards the bile capillary can be observed. Changes in the hepatocytes of the central zone, in particular cluster 3 (C3) are also aggravated. We have noticed the presence of hepatocytes of higher electron density with a large number of destructively altered mitochondria, reduction of Golgi complex, rough (granular) endoplasmic reticulum (RER) and smooth (SER) endoplasmic reticulum, enlarged lipid droplets (Fig. 2b). Along with hepatocytes with vacuolar dystrophy, hepatocytes are visualized, the structure of which does not differ from the control group of rats (fig. 2d).

In the early stages of ESID development regular changes in the morphometric parameters of hepatocytes of different clusters are observed in all age groups of animals (fig. 3), namely: a decrease in the area of hepatocytes and their nuclei which is associated with marked glycogenolysis, as confirmed by histological and ultrastructural studies.

Another common pattern of changes in different clusters in the early stages of ESID development is a decrease in the area of nuclei in animals of different age groups. Changes in the quantitative indices of hepatocytes lead to a redistribution of the percentage of different clusters of hepatocytes. In 12-month-old rats, as compared with controle indices, on the 14th day of experiment the number of hepatocytes in C1 decreased by 3.64 %, and in C2 increases by 5.37 %; on day 28 – the indices observed in C2 decrease by 3.69 %, and in C3 increase by 4.81 % (in all cases $p<0.05$) (fig. 4). The number of hepatocytes in C1 and C3 decreases by 8-7% and 6–10 % in 24-month-old rats on the 14^{th}-28^{th} days, while in C2 their number increases by 14–17% (fig. 4).

In the early stages of ESDM (14-28^{th} days) in 12-month-old rats there was a significant increase in ALT activity by 2.53 and 3.53 times, AST – by 2.38 and 3.73

times, compared with the control group, in the serum (Table 2). However, De Ritis ratio is statistically insignificantly different from the control values, which indicates the development of compensatory processes in the liver in experimental diabetes and the full preservation of its functional capacity (table 2). In liver homogenates, ALT activity on the 14th day increases by 1.28 times, on the 28th day – by 1.54 times, and ACT activity increases by 1.39 times and 1.37 times, respectively (table 2).

In 24- month-old rats on the 14-28th days of ESDM development there is an increase in the activity of enzymes of cytolysis of hepatocytes in the serum (ALT – by 2.54 and 3.64 times, AST – by 2.36 and 3.84 times) compared with the control group (table 2). On the 28th day of the experiment, in both blood serum and liver homogenate of 24-month-old rats, the activity of aminotransferases continues to increase. As for De Ritis ratio, it is less than the control values on the 14th day of the experiment, and on the 28th day it increases and does not differ significantly from the control (table 2).

It should be noted that the most pronounced decrease in the area of hepatocytes is observed in C1 and C3. Such changes can be explained by the fact that, according to various authors [3], hepatocytes of the peripheral zone are the first to respond to metabolic changes in the blood, so in response to hyperglycemia and hypoinsulinemia the processes of gluconeogenesis are activated, leading to the decrease in their area (as confirmed by our studies). Therefore, C3 hepatocytes are glycogen depots and their area starts decreasing on the 28th day of the experiment, when C1 hepatocytes do not contain glycogen to ensure the processes of gluconeogenesis.

Table 2

Activity of aminotransferases in blood serum and liver homogenates in streptozotocin induced diabetes mellitus

Period of the experiment		ALT (μmol / (h*ml)), blood serum	ALT (μmol / (h*g)), homogenate	AST (μmol / (h*ml)), blood serum	ACT (μmol / (h*g)), homogenate	De Ritis Ratio
12-month-old rats						
14th day	ESID	0.99± 0.13*	26.31±2.02*	1.12 ±0.14*	29.51±2.22*	1.13±0.04
	Control	0.39±0.02	20.43±1.13	0.47 ±0.02	21.11±8.01	1.21±0.04
28th day	ESID	1.45 ±0.05*#	31.72±9.01*#	1.83±0.11*#	29.09±1.83*	1.26±0.05#
	Control	0.41±0.02	20.51±0.82	0.49±0.02	21.22±7.04	1.2±0.01
24-month-old rats						
14th day	ESID	0.7± 0.13*	18.53±2.03*	0.78±0.14*	19.52±2.21*	1.11±0.04*
	Control	0.28±0.02	14.11±1.11	0.33±0.02	15.03±7.89	1.18±0.03
28th day	ESID	1.02±0.05*#	22.19±9.01*#	1.27±0.11*#	20.41±1.82*	1.25±0.05*#
	Control	0.28±0.02	13.52±0.87	0.33±0.02	15.04±7.06	1.18±0.03

Notes: 1) * – the probability indices as compared to the control, $p<0.05$; 2) # – the probability indices as compared to the previous term of experiment $p<0.05$.

Another common pattern of changes in different clusters in the early stages of ESID development is a decrease in the area of nuclei in animals of different age groups. Such quantitative changes are accompanied by a qualitative restructuring of hepatocytes, namely: a decrease in the electron-optical density of the karyoplasm, an increase in the number of destructive changes in mitochondria with lysis of their cristae, expansion and destruction of RER cisternae. The abovementioned changes indicate a decrease in the energy supply of hepatocytes and inhibition of their metabolic activity due to the decrease in insulin and glucagon levels [9]. According to these authors, both hormones stimulate RNA and protein synthesis in hepatocytes. Therefore, the decrease in the area of the nuclei and RER reduction are the signs of inhibition of the protein-synthesizing function of hepatocytes. However, we have observed a significant increase in the activity of aminotransferases in 12-months-old animals in the early stages of ESID development as compared with the control group, but the De Ritis Ratio does not differ significantly from the control indices, indicating the development of compensatory processes in the liver and preservation of its functional capacity. We did not observe a decrease in the nuclei of hepatocytes of different clusters in 24-months-old rats in the early stages of the experiment, so we believe that the functional activity of hepatocytes in different areas is preserved, as in 12-months-old animals, which is confirmed by biochemical studies. In particular, the De Ritis Ratio on the 14th day of the experiment was significantly lower than the control indices, and by the 28th day it increases and does not differ significantly from the control indices. Such qualitative and quantitative

restructuring of the liver in 24-months-old rats in the early stages of ESID development may be considered as compensatory.

Changes in the quantitative indices of hepatocytes lead to a redistribution of the percentage of different clusters of hepatocytes. In 12-month-old rats, as compared with controle indices, on the 14th day of experiment the number of hepatocytes in C1 decreased, and in C2 increases; on day 28 – the indices observed in C2 decrease by and in C3 increase. We think that, the increase of C3 cells is a compensatory reaction, because the cells of this cluster predominate in the central zone, their main function is detoxification [3], and they are the main pool for young hepatocytes formation [12, 14].

The number of hepatocytes in C1 and C3 decreases in 24-month-old rats on the 14th-28th days, while in C2 their number increases. This redistribution of clusters may indicate low liver regeneration potential of 24-month-old rats and reduced detoxification function [9].

Conclusions

1. In the early stages of ESID (14-28 days) development the levels of glucose and HbA1c in the blood of different groups of animals increases significantly, in 12-months-old animals by 4.6–3.4 times and 3-3.6 times, respectively, and in 24-months-old animals – 2.5-2.9 and 3–5 times (in all cases $p<0.05$), which points to the development of experimental diabetes mellitus of moderate severity.

2. Regular changes in the morphometric parameters of hepatocytes of different clusters are observed in all age groups of animals, namely: a decrease in the area of hepatocytes and their nuclei which is associated with marked glycogenolysis, as confirmed by histological and ultrastructural studies. In 12-month-old animals the area of the nuclei of hepatocytes of different clusters decreases (in all cases $p<0.05$), and in 24-months-old rats these indices do not significantly change, as compared with the control indices.

3. A significant increase in the activity of aminotransferases in both, blood plasma and liver homogenates, is observed in animals of different age groups, as compared with the control group, but the De Ritis Ratio does not significantly differ from the control indices, indicating the development of compensatory processes in the liver and preservation of its complete functional capability.

Prospects for further research lie in the fact that the findings of our research may be used for further study of the effects of various antidiabetic agents on liver hepatocytes in the treatment of diabetes mellitus.

References

1. Abdollahi M, Zuki AB, Goh YM. The effects of Momordica charantia on the liver in streptozotocin-induced diabetes in neonatal rats. Afr J Biotechnol. 2010; 9: 5004–12.
2. Chudhary D, Cleveland DA, Agdere L, Graetz R, Etienne D. Does the Use of Diabetes Electronic Medical Record Template Lead to Improvement of Screening for Complications of Tipe 1 Diabetes Mellitus in the Pediatric Population. Pediatrics. 2018; 141(1): 122–8.
3. Colnot S, Peret C. Liver Zonation Molecular Pathology of Liver Diseases. Molecular Pathology Library. 2011; 13: 7–16.
4. Dhurandhar D, Bharihoke V, Kalrah S. A histomorphometric evaluation of effects of sucralose ingestion on liver of albino rats. International Journal of Scientific Researc. 2018; 7(5): 45–6.
5. Garcia- Carrizo F, Priego T, Szostaczuk N, Palou A, Pico C. Sexual dismorphism in the age-induced insulin resistance, liver steatosis, and adipose tissue function in rats. Frontiers in Physiology. 2018; 8(445):1–14.
6. Kumar R. Hepatogenous diabetes: an under estimated problem of liver cirrhosis. Indian J Endocr Metab. 2018; 22: 552–9.
7. Li Y, Zeng KW, Wang XM. Cerebral microangiopathy of diabetes. Zhongguo Zhong Yao Za Zhi. 2017; 42(12): 2247–53.
8. Liao M, Li Yu, Kianifard F, Obi E, Arcona S. Cluster analysis and its application to healthcare claims data: a study of end-stage renal disease patients who initiated hemodialysis. BMC Nephrology. 2016; 17(25): 1–15.
9. Noorafshan A, Esmail-Zadeh B, Bahmanpour S, Poost-Pasand A. Early stereological changes in liver of Sprague-Dawley rats after streptozotocin injection. Indian J Gastroenterol. 2005; 24(3): 104–17.
10. Poisson J, Lemoinne S, Boulanger C, Durand F, Moreau R, Valla D, et al. Liver sinusoidal endothelial cells: Physiology and role in liver diseases. Journal of Hepatology. 2017; 66(1): 212–27.
11. Sherigar JM, Castro JD, Yin YM, Guss D, Mohanty SR. Glycogenic hepatopathy: A narrative review. Word J Hepatol. 2018; 10(2): 172–85.
12. Stanger BZ. Probing hepatocyte heterogeneity. Cell Research. 2015; 25(11):1181–2.
13. Upadhyay R. Diabetic Hepatopathy. Hepatology. The Association of Physicians of India-Medicine Update. 2017; 67: 314–8.
14. Yang J, Mowry LE, Nejak-Bowen KN, Okabe H, Diegel CR, Lang RA, et al. Beta-catenin signaling in murine liver zonation and regeneration: A Wnt-Wnt situation! Hepatology. Author manuscript. 2014 ; 60(3): 964–76. 110.
15. Zelinska NB. Statistics of diabetes mellitus among children in Ukraine (analysis and forecast). Ukrainian Journal of Pediatric Endocrinology. 2015; 3(4): 8–14.

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