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Zhurakivska O.Ya.,
Doctor of Medical Sciences, Professor of the Department of Human Anatomy of Ivano-Frankivsk National Medical University, Ministry of Health of Ukraine, Halyts`ka str. 2, Ivano-Frankivsk, Ukraine, 76000, e-mail: zhurakivska.o.ya@gmail.com
Koshkin O.Ye.
Teaching assistant of the Department of Pediatric dentistry of Ivano-Frankivsk National Medical University, Ministry of Health of Ukraine, Ivano-Frankivsk, Ukraine, e-mail: koshkinoleg@ukr.net

AGE PECULIARITIES OF MORPHOFUNCTIONAL CHANGES OF TEMPORAL MUSCLE IN EXPERIMENTAL DIABETES MELLITUS

Abstract. *The changes in the structural components of the temporal muscle of 2-month-old white male rats in streptozotocin-induced diabetes mellitus (SDM) were investigated. It is established, that on the 14th day of SDM in the temporal muscle, stress-reactive changes in response to hyperglycemia are observed, which are manifested by spasm of arterioles, a decrease of the muscle fiber area by 1.4 times due to a decreased volume density of glycogen in 2.5 times. In the long term course of SDM (56 days) in temporal muscle compensatory-atrophic changes are noted. On the background of hyperglycemia and high levels of glycosylated hemoglobin, the development of diabetic microangiopathy was observed in vessels of hemomicrocirculatory bloodstream, which led to destructive changes in muscle fibers and nervous system of the temporal muscle. We found a decrease in muscle fiber area in 1.7 times and neuromuscular terminations in 3.2 times (in all cases p <0.05). However, it is worth noting the compensatory processes in the temporal muscle, which were manifested by the restoration of ultrastructure of myofibrils, increase of the volume density of glycogen and the formation of young mitochondria in sarcoplasm of muscle fibers. Appearance of young endotheliocytes in the vessels of hemomicrocirculatory bloodstream, that had electron-dense cytoplasm with a large number of micropinocytotic vesicles.*

Key words: *temporal muscle, muscle fiber, neuromuscular ending, streptozotocin-induced diabetes mellitus.*

Introduction. Over the past decade, there has been an increase in the incidence of endocrine diseases, in the structure of which the first place in the world belongs to diabetes mellitus (DM) [2, 15]. In various countries, the number of patients diagnosed with this disease ranges from 4-7% of the general population and tends to increase [2, 4]. Diabetes mellitus is the key medical and social problem of the public health care system in all countries of the world, which is associated with continuous increase of disease incidence, severe complications and high mortality rates, especially among the working-age population [16, 20, 21]. One of the DM complications is diabetic myopathy, which occurs in 88% of cases [5] and is often combined with other complications of diabetes mellitus such as: macro- and microangiopathy, cardiovascular diseases, nephropathy, retinopathy, neuropathy [4, 7, 8, 12, 18]. Diabetic myopathy is manifested by sharp pain and swelling. Usually the pathological process involves the muscles of the lower extremities, particularly the thighs – 80% of cases and the lower leg in 20% [6, 19]. Palpable, painful mass is present in 34-44 % of patients, and fever occurs in about 10% of cases [5].

Taking the above into account, the **aim** of our investigation was to study the dynamics of histo-ultrastructural changes of temporal muscle of 2-month-old rats in the progression of streptozotocin-induced diabetes mellitus development.

Material and methods. The test material involved the temporal muscles of twenty 2-month-old white outbred male rats, which were divided into two groups: intact and experimental. Diabetes in experimental group animals was induced by a single intraperitoneal injection of streptozotocin (dissolved in 0.1M citrate buffer solution with pH 4.5) at a dose of 7mg per 100g of body mass. Glucose levels of experimental group animals were determined daily by taking blood

samples from the tail vein using test strips on an "Accu-Chec" (Germany) glucometer. Animals with blood glucose level higher than 13 mmol/l were selected for the study and material was collected during the 14th and 56th days of the experiment. The methods of investigation included: histological methods (hematoxylin and eosin staining, Masson's trichrome staining), histochemical (Shabadash's method of glycogen detection, Bilshovsky-Gross method of impregnation), biochemical and electron-microscopic ones.

All the procedures with test animals during the experiment were carried out 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.

Photographs of histological sections saved in tif format were used for morphometric studies. Morphometry was performed using ImageJ software version 1.47t. It became possible to determine the area of muscle fibers and their nuclei, as well as the area of neuromuscular endings. The profile area of arterioles, capillaries, venules, their walls and lumen were measured. Vohenvort index (VI) was measured in arterioles and capillaries using the corresponding formula[13]. Computerized data processing was performed using the STATISTICA package (StatSoft, Inc. (2010), STATISTICA (data analysis software system), version 10.

Results of investigation and their discussion. On the 14th and 56th days of streptozotocin-induced DM development the levels of glucose and glycosylated hemoglobin increased to 14.29 ± 1.33 mmol / l (control 3.85 ± 0.76 mmol / l, p = 0.0159) and 15.42 ± 1 , 33 mmol / l (control 3.26 ± 0.84 mmol / l, p = 0.0036), 6.46 ± 0.14% (control 1.98 ± 0.32%, p = 0.0023) and 9.74 ± 0.17% (control 2.14 ± 0.65%, p = 0.0023), which indicates the development of decompensated diabetes mellitus.

On the 14th day of streptozotocin-induced DM development, we discovered a significant decrease in the area of muscle fibers to 365.43 ± 46.95 μm^2 (control – 513.42 ± 20.23 μm^2, p = 0.0001), whereas the area of their nuclei did not change significantly and made up 12.49 ± 2.35 μm^2 (control – 13.69 ± 3.04 μm^2, p = 0.2447). In our opinion, such changes are associated with the decrease of glycogen inclusions in muscle fibers, which is confirmed by its unequal distribution: in some muscle fibers it is found only in the periphery, while in others it is absent (Fig. 1a). Statistical analysis showed a significant 2.5-fold decrease in the volume density of glycogen granules as compared to the control indices (p = 0.0017). Histological specimens show arteriolar spasm whereupon the lumen of some of them assumes a slit-like shape (Fig. 1b). Inner elastic membrane resembles a dark helix and is not visualized along the entire perimeter of the vessel. However, the statistical studies show a significant decrease in the lumen of the arterioles due to a decrease in the area of their lumen (Table 1), whereas the area of the wall does not significantly change, leading to the increase of VI to 424.65±43.54% (control – 358.44±58.09%, (p=0.0001) and points to the decrease in their blood flow capacity. We did not manage to detect significant morphometric changes on the part of metabolic and capacitance vessels (Table 1), but venous plethora was observed on histological preparations.

At this time of the experiment, the number of capillaries per 0.1 mm^2 was not significantly different from the control values and made up 96.3 ± 4.01 (control – 99.2 ± 2.49, p = 0.0696).

The study of preparations impregnated by the Bilshovsky-Gross method revealed thickening which formed along the myelinated nerve fibers (MNF) (Fig. 1c). The area of neuromuscular junctions was not significantly different from the control values and made up 340.90 ± 63.88 μm^2 (control – 337.11 ± 35.71 μm^2, p = 0.2885).

At the ultrastructural level, mitochondria underwent the most pronounced changes in the muscle fibers of the temporal muscle on the 14th day of the SDM development. Their matrix is lucid, cristae become disorganized, somewhere destroyed. Separate muscle fibers have segmental contractures. An increase in the number of micropinocytotic vesicules in the cytoplasm of endothelial cells is observed in the links of hemo-

Table

Morphometric changes of the hemomicrocirculatory bed in experimental diabetes mellitus

HMCB Vessels	Group of Animals	Vessel Area	Lumen Area	Wall Area
14th day				
arterioles	SDM	221.99±29.11*	46.92±11.81*	176.75±19.01
	control	280.68±49.50	61.57±10.14	219.10±42.76
capillaries	SDM	17.77±2.79	7.53±1.01	10.23±2.71
	control	15.42±3.30	7.32±1.65	8.09±2.16
venules	SDM	231.95±29.15	106.78±10.56	125.17±20.51
	control	230.14±35.29	102.75±10.65	127.40±27.56
56th day				
arterioles	SDM	327.16±19.05#	32.75±7.10*	198.41±14.51*,#
	control	315.80±28.63	72.85±15.86	242.95±22.16
capillaries	SDM	16.15±5.44	3.85±1.43*,#	12.30±5.41*,#
	control	15.65±3.21	7.69±1.57	7.96±2.24
venules	SDM	275.45±33.85#	105.19±33.40*	170.25±35.69*,#
	control	308.25±40.60	149.27±24.82	158.97±28.93

Notes:

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

microcirculatory bed, indicating an increase in transendothelial transport. In the axoplasm of neuromuscular synapses, the mitochondrial matrix is lucid and the number of synaptic vesicules is increased by 12.8% as compared to the control indices (p = 0.6547).

Marking: 1 - glycogen granules, 2 – spasm of arteriol, 3 - normal arteriol, 4 - arterio-venular anastomos, 5 – adhesion of erythrocyte, 6 - terminal axon branches, 7 - nuclei of terminal neurolemocytes, 8 – swelling of endomysium, 9 - reduction of axon spouting in neuromuscular endings.

On the 56th day of SDM course we have observed a significant increase in the area of muscle fibers, up to 444.09 ± 58.85 μm^2 as compared to the previous experimental period (p <0.0001), though they remaine below the control parameters (p <0.0001). The area of nuclei increased to 15.79 ± 2.67 μm^2 as compared to the

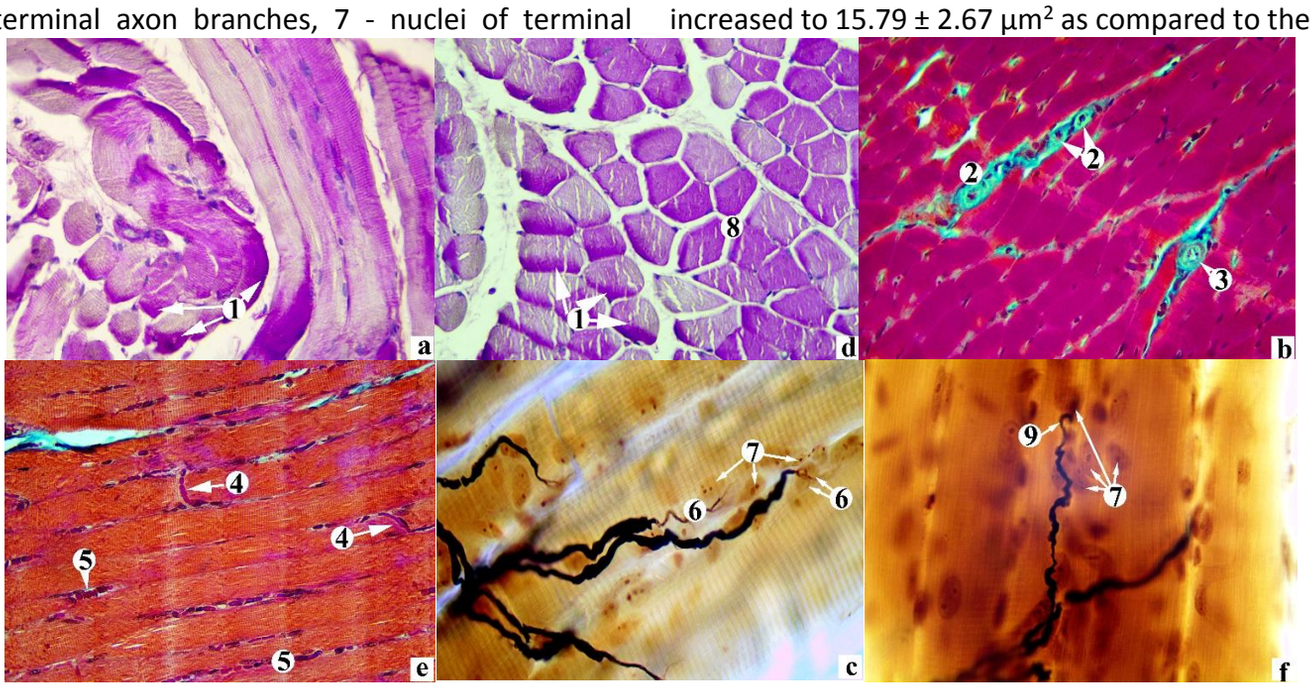

Fig. 1. Histostructural changes of temporal muscle of 2-month-old rats on the 14th day (a-c) and 56th day (d-f) of streptozotocin diabetes mellitus. Coloring: Shabadash (a, d), Masson trichrome (b, e), Bielschowski-Gross impregnation (c, f). Microphotographs. Magnification: a, b, d, e) x400; c, f) x1000.

previous observation period (p = 0.0098), but did not differ significantly from the control parameters (control – 14.52 ± 4.27 μm^2, p = 0.4093). Histological preparations show swelling of endo- and perimysium (Fig. 1d), defibration and focal lysis of individual muscle fibers and their histolymphocytic infiltration, which may indicate the development of aseptic myositis. As compared to the previous experimental period, muscle fibers are moderately and equally filled with glycogen granules (Fig. 1d), indicating partial compensation of metabolic changes in the test muscle.

Multiple nummular erythrocytic sludges, aggregation of erythrocytes and thrombocytes to the luminal endothelial surface were discovered in the hemomicrocirculatory bed. The wall area of all microvessels (Table 1) increases, which, when narrowing the lumen of arterioles and capillaries, leads to an increase in their VI to 1057.50 ± 72.09% (control – 347.69 ± 76.87%, p = 0.0001) and 377.49 ± 51.16% (control – 105.75 ± 28.94%, p = 0.0001), indicating significant decrease in their blood flow capacity. At the same time the area of venules significantly increases (Table. 1). Such restructuring of microvessels points to the opening of arterio-venular anastomoses (Fig. 1e), which has a compensatory-protective value, since it provides shunting blood into the venous bed. Such changes in angioarchitectonics of the temporal muscle lead to a decrease in the number of hemocapillaries per 0.1 mm^2 to 58.8 ± 8.38 as compared to the previous experiment period and control period (in all cases p<0.05).

Histological preparations impregnated by the Bilshovsky-Gross method show increased number and size of varices of the preterminal parts of MNF, decrease in axon sprouting (Fig. 1f), which is confirmed by the morphometric findings, namely: the area of neuromuscular connections decreases to 186.32 ± 34.26μm^2 (control – 511.35 ± 66.24 μm^2, p= 0.00005). The heterogeneity of the muscle fiber structure was observed on the ultrastructural level. In some cases, we observed karyorrhexis, lysis of individual myofibrils, expansion of the structural components of the sarcoplasmic reticulum, destruction of mitochondrial cristae with subsequent vacuolation, presence of myelin-like inclusions in mitochondria (Fig. 2 a). While in other muscle fibers, we observed intracellular regenerative processes, which were manifested by: the preserved ultrastructure of myofibrils, the presence of glycogen granules in sarcoplasm, the formation of young mitochondria with electron-dense matrix and fuzzily differentiated cristae.

Electron microscopic examination revealed stratification of the lamella of the MNF myelin sheath, widening of the periaxonal space (Fig. 2c). Axoplasm was of low electron density in terminals of axons which form neuromuscular synapses. Here we have found mitochondria with lucid matrix, small number of neurofilaments, microtubulesa and synaptic vesicles. Post-synaptic folds are disorganized, sometimes shortened and destroyed in neuromuscular synapses (Fig. 2d).

In the vessels of the hemomicrocirculatory bed, the most pronounced changes were observed in endothelial cells. Their cytoplasm was electron-dense with a large number of micropinocytotic vesicles (Fig. 2 e-f). The lumenal surface of the plasmolemma forms long and short fingerlike protrusions in the lumen of the microcirculation vessels, with subsequent formation of microclasmatosis, which, along with hemoreologic disorders, impairs blood supply to the muscle.

Basal membrane of the capillaries undergoes marked changes. It loses its three-layer structure, considerably thickens due to its proliferation in the form of separate plates (Fig. 2 f), which is one of the markers of diabetic microangiopathy.

Marking: 1 - destructively altered mitochondria, 2 - young mitochondria, 3 - glycogen granules, 4 - myofibril lysis, 5 – stratification of myelin sheath, 6 - disorganization and destruction of synaptic folds in neuromuscular synapses, 7 - finger-shaped protrusions of the lumenal surface of the plasmolemma of endothelial cells, 8 - thickening of the inner elastic membrane, 9 - multilayered basal membrane.

Discussion. During early stages of SDM course (14th day), we have observed a decrease in MF area by 28.8%, which, in our opinion, was associated with the decrease in their glycogen content. Such changes are associated with the increase of contra-insular hormones, particularly cortisol, in blood in response to hyperglycemia,

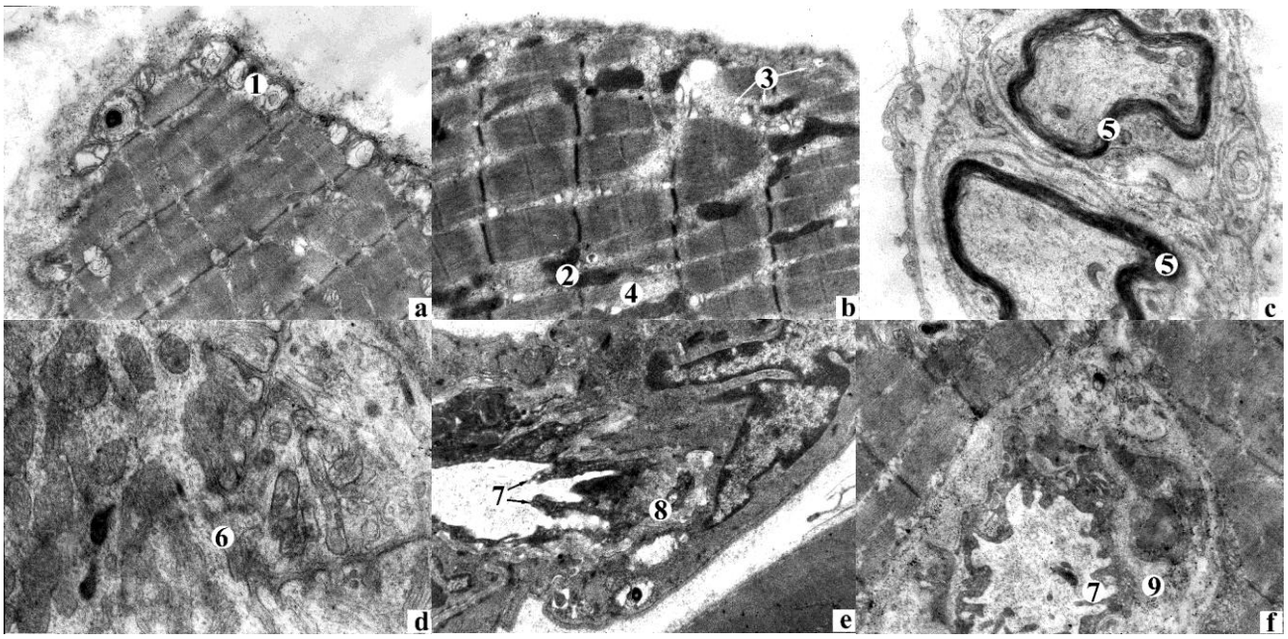

Fig. 2. Ultrastructural changes of muscle fibers (a, b), myelin nerve fibers (c), neuromuscular synapses (d), arterioles (e), capillaries (f) on the 56th day of the course of SDM. Magnification: a, e, f) x8000; b, c) x9600, d) x16000.

[15, 16], as they promote gluconeogenesis in insulin deficiency. It is worth noting that the hyperproduction of contra-insular hormones in DM is one of the pathogenic links in the development of diabetic angiopathies [10, 11, 18]. At the same time, some authors note high levels of cortisol in the early stages of SDM development [15], which, according to our research, leads to the spasm of afferent vessels and is accompanied by a significant decrease in their lumen by 1.8 times, resulting in the increase in VI by 1.7 times and indicates a decrease in the blood flow capacity of these microvessels. Disturbances in the microcirculatory system lead to hypoxic damage to the muscle fibers, which, according to our research and the studies of other authors, manifest themselves as segmental contractions of myofibrils and destructive mitochondrial changes [20, 21].

With the course of the experiment (56th day of SDM) we have observed the development of diabetic microangiopathy in temporal muscles, which is principally manifested as hemoreologic disorders, namely: erythrocytic sludge, adhesion of erythrocytes and platelets, microclasmatosis. Most researchers believe that such blood flow changes are associated with high levels of glycosylated hemoglobin, which leads to the change in the shape of erythrocytes (their S-surface charge). Sludge and agglutination of erythrocytes lead to the formation of microthrombi, which result in local circulatory and hemic hypoxia and activate the molecular mechanisms which cause damage to the plasmolemma of endothelial cells [1, 10, 12]. The damage of the latter is also influenced by low levels of vasodilators (nitric oxide and prostacyclin) as DM is associated with the development of ketoacidosis and glycosylation of the N-terminal ends of transmembrane proteins [11]. Sorbitol pathway of glucose metabolism is another factor in the destruction of endothelium in diabetes, as it is associated with the activation of aldose reductase with subsequent sorbitol accumulation in endothelial cells, leading to osmotic edema and destruction of the latter [12, 14]. Hyperglycemia, due to increased activity of glucosyltransferase [1], promotes increased synthesis of glycoproteins in the basal membrane, which, according to our studies, leads to its thickening and proliferation in the form of separate plates and is one of the characteristic signs of diabetic microangiopathy.

Some authors [20] state that in diabetes the function of synaptic apparatus of skeletal muscle is impaired. Autoantibodies to the voltage-gated $Ca2^{+}$ channels at the presynaptic membrane of neuromuscular synapses are considered to be the leading damaging agents. It is a known fact that their effective functioning requires the sufficient amount of energy-related material. However, insulin deficiency in diabetes results in acute

glucose deficiency in neurons and muscle fibers [3], impeding synthesis and isolation of synaptic acetylcholine mediators from axon terminals. Another mechanism of damage to neuromuscular synapses is hypoxia due to the development of diabetic microangiopathy. In this case, the cytoplasm of neurolemocytes is overloaded with a large number of vesicles of different size [20], and the myelin sheath becomes stratified [9], which is clearly observed in our investigations. On the one hand, it may serve as a morphological substrate for the disturbance of oxidative metabolism in SDM, while on the other hand, it explains the hypotrophic changes in the temporal muscle due to impaired energy supply, delayed axonal transport, decreased number of neuromediators and destructive mitochondrial changes [9, 20]. Our studies show that muscle fiber area was 1.7 times smaller than the control indices. However, we should also note the compensatory processes in temporal muscle of immature rats, which were manifested by the following intracellular regenerative phenomena: the restoration of ultrastructure of myofibrils, increase in the number of glycogen granules in sarcoplasm, formation of young mitochondria with electron-dense matrix and obscurely differentiated cristae. Young endotheliocytes with electron-dense cytoplasm and large number of micropinocytotic vesicles were observed in micro-hemo-vessels [17].

Conclusions. Thus, in the early stages of SDM development (14th day), we have observed stress-reactive changes in the temporal muscle in response to hyperglycemia, which were manifested by: the spasm of the arterial link of the hemomicrocirculatory bed, decrease in the area of muscle fibers due to the reduction in volume density of glycogen.

Compensatory-atrophic changes of temporal muscle were observed during the later stages of SDM course (56-70th days). Against the background of hyperglycemia and high levels of glycosylated hemoglobin, the development of diabetic microangiopathy was observed in the hemomicrocirculatory bed resulting in destructive changes to the muscle fibers and nerve apparatus of the temporal muscle.

However, we should also point to the compensatory processes in the temporal muscle of juvenile rats, which were manifested by intracellular regenerative phenomena in muscle fibers and the appearance of young endothelial cells in the micro-hemo-vessels.

Directions for further research consist in comprehensive study of the patterns of changes in muscle fibers, hemomicrocirculatory bed and neuromuscular endings of the temporal muscle with SDM and its correction with various antidiabetic medications.

References:

1. Boychuk TM, Tolstanov DC, Grytsiuk MI, Gozhenko AI. Glycated proteins in diabetes: the phenomenon of formation and pathogenetic effects (review). Actual problems of transport medicine. 2013; 3: 52-59.

2. Chian JL, Kirkman MS, Laffel LM, Peters AL. Type 1 diabetes through the span: a position statement of the American Diabetes Association. Diabetes Care. 2014 Jul;37:2034-54. doi: 10.2337/dc14-1140.

3. Drachman DB. Therapy of Myasthenia Gravis. Myasthenia Gravis and Myasthenic Disorders. 2012: 130–155. Available from: https://doi.org/10.1093/med/9780199738670.003.0006.

4. Gupta S, Gupta N. The Prevalence of Cardiac Autonomic Neuropathy in Type 2 Diabetes Mellitus. J Med Sci Clin Res. 2017; 5(7): 23-8. Available from: https://doi.org/10.18535/jmscr/v5i7.57

5. Huang BK, Monu JU, Doumanian J. Diabetic myopathy: MRI patterns and current trends. AJR Am J Roentgenol. 2010 Jul;195(1):198-204.

6. Kapur S, Brunet JA, McKendry RJ. Diabetic muscle infarction: case report and review. J Rheumatol. 2004; 31: 190-4

7. Kocdor P, Kaya S, Erdil M, Cureoglu S, Paparella MM, Adams ME. Vascular and Neuroepithelial Histopathology of the Saccule in Humans With Diabetes Mellitus. Otol Neurotol. 2016 Jun; 37(5): 553-7. doi: 10.1097/MAO.0000000000001018.

8. Leung WK, Gao L, Siu PM, Lai CW. Diabetic nephropathy and endothelial dysfunction: Current and future therapies, and emerging of vascular imaging for preclinical renal-kinetic study. Life Sci. 2016; 166: 121-130. doi: 10.1016/j.lfs.2016.10.015.

9. Levitskiy VA, Atamanchuk OV. State of

peripheral neuro-muscular apparatus of tongue in diabetes mellitus of i type. Journal of the Grodno State Medical University. 2014; 2: 70-73.

10. Li Y, Zeng KW, Wang XM. Cerebral microangiopathy of diabetes. Zhongguo Zhong Yao Za Zhi. 2017 Jun; 42(12): 2247-2253. doi:10.19540/j.cnki.cjcmm.2017.0104.

11. Madonna R, Balistreri CR, Geng YJ, De Caterina R. Diabetic microangiopathy: Pathogenetic insights and novel therapeutic approaches. Vascul Pharmacol. 2017; 90: 1-7. doi: 10.1016/j.vph.2017.01.004.

12. Ponto KA, Koenig J, Peto T, Lamparter J, Raum P, Wild PS, et al. Prevalence of diabetic retinopathy in screening-detected diabetes mellitus: results from the Gutenberg Health Study (GHS). Diabetologia. 2016; 59(9): 191-193.

13. Shavrin V.A., Avramenko Yu.N. Comparative morphometry of cerebral microvessels in atherosclerotic and diabetic encephalopathies. Morphologia. 2017;1: 58-61.

14. Stepanova Z, Maksyukov S, Skorobogach M, Tatyanchenko V, Serpionov S, Stepanov D. Prediction of treatment results in patients with postoperative myofascial dysfunction of the masticatory muscle. Medical news of the North Caucasus. 2017; 12(20): 34-42. Available from: https://doi.org/10.14300/mnnc.2017.12051

15. Tkachuk YuL. Morpho-functional changes in pituitary-adrenal system at the early stages of experimental diabetes. World of medicine and biology. 2015; 1: 159-162.

16. Tkachuk YL. Morpho-functional changes of pituitary-adrenal system in the long-term course of experimental diabetes mellitus. Journal of Education, Health and Sport formerly Journal of Health Sciences. 2016; 6(4): 375-384.

17. Tokaruk NS. Characteristics of hemovessels of urinary bladder in rats under diabetes mellitus. Topical problems of modern medicine: Bull. of the Ukr. Med. Dent. Acad. 2016; 16(1): 249-255.

18. Tomita M, Kabeya Y, Okisugi M, Katsuki T, Oikawa Y, Atsumi Y, et al. Diabetic Microangiopathy Is an Independent Predictor of Incident Diabetic Foot Ulcer. J Diabetes Res. 2016; 2016: 5938540. doi: 10.1155/2016/5938540

19. Trujillo-Santos AJ. Diabetic muscle infarction: an underdiagnosed complication of long-standing diabetes. Diabetes Care. 2003 Jan;26(1):211-5.

20. Zhurakivska OYa, Grad AO, Popel SL, Zaiats IT, Krynytska IYa, Marushchak MI et al. Morphological and functional changes of masticatory muscle neuromuscular junctions in the later periods of streptozotocin diabetes mellitus course. World of medicine and biology. 2017; 3: 108-114.

21. Zhurakivska OY, Mykulets TI, Dutchak UM, Klypych YaI et al. Structural Changes of Endocrine System of Myocardium during the Streptozotocin Diabetes Mellitus. World of medicine and biology. 2018; 1: 126-130.