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Morphological Aspects of Diabetic Gastroparesis

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Abstract.

The research work highlights issues relating to studying morphological signs of diabetic gastroparesis. On the forty-second day of the development of experimental streptozotocin-induced diabetes mellitus neuronal hydropic degeneration confirmed by the results of morphometry and ultrastructural investigations was observed in the intermuscular plexus of the rats' stomach. Pycnomorphous cells were found. Axonal degeneration defining the neurogenic nature of the damage to unmyelinated nerve fibers was also present. The processes of apoptosis were initiated in the interstitial cells of Cajal leading to their death. Such changes occurred on the background of the development of diabetic microangiopathy which caused pronounced destructive changes in the smooth myocytes resulting in the violation of their contractility due to circulatory and hemic hypoxias. Thus, experimental diabetes mellitus in rats changes motor-evacuation function of the stomach by destructive changes in the myogenic and neurogenic factors regulating it.

Keywords: stomach; intermuscular plexus; interstitial cells of Cajal; myocytes; streptozotocin-induced diabetes mellitus

Problem statement and analysis of the recent research

Diabetes mellitus (DM) has a great socioeconomic impact which is determined by both the costs for medical care and social welfare support for patients (due to incapacitation, disability, and premature death). Diabetic gastroparesis (DG) is one of the complications of DM which involves a variety of neuromuscular dysfunctions of the stomach including abnormalities of gastric contractility (reduced motor-evacuation function of the stomach resulting from damage to the sympathetic and parasympathetic divisions of the autonomic nervous system) and abnormal gastric myoelectrical activity in patients with DM [3,4]. Nowadays, considerable attention is paid to the investigation of the functional activity of the stomach in case of DM both experimentally and practically. It has been caused by deterioration in quality of life in patients and the occurrence of complications. Several pathogenetic mechanisms of the development of diabetic gastropathy have been described in today's scientific literature. They include: the theory of autonomic neuropathy (changes in the expression of nitric oxide synthase (NOS) in neuronal cells of the gastrointestinal tract) [2, 5, 6], the concept of "toxicity of postprandial hyperglycemia" (hyperglycemia slows down gastric emptying) [7], reduced levels of acyl ghrelin and elevated levels of des-acyl ghrelin slowing down the peristalsis [10], increased concentrations of Helicobacter pylori [4], and psycho-emotional stress [4]. The autonomic nervous system and interstitial cells of Cajal (they generate slow waves with a frequency of 3-4 cycles per minute and are involved in stomach peristalsis) are known to be directly involved in the regulation of gastrointestinal motility [11, 12]. However, their morphological changes in case of DM have been insufficiently described in the scientific literature.

Therefore, **the objective** of our research was to determine morphological and functional changes in the nervous apparatus of the stomach and its smooth myocytes during the early stages of experimental DM.

Materials and Methods

15 male Wistar rats at the age of 12 months were examined. They were divided into 2 groups: the control group (5 animals) and the experimental group (10 animals). In the experimental group diabetes mellitus was induced by an

intraperitoneal injection of streptozotocin (6 mg/100 g body weight), in the control group 0.1 M citrate buffer, pH 4.5, was injected (6 mg/100 g body weight). The development of DM was evaluated measuring blood glucose level which was determined collecting a drop of blood from tail vein with the help of a blood glucose meter ("Accu-Chec", Germany) and test strips. Glycated hemoglobin (HbA_{1C}) levels were measured using an extremely sensitive ion exchange liquid chromatography method (laboratory "Diameb").

On the 14th and 42nd day of the experiment the material for the investigation was taken. Histological (the Nissl staining) and electron-microscopic study (small pieces of material (the stomach) were fixed in a 2% solution of osmium tetroxide using standard contrasting technique) were used. The material was investigated at a magnification of 1,200 to 12,000 times by means of electron microscope PEM-125K at an acceleration voltage of 75 kV. Histological specimens and semi-thin sections stained with a 2% solution of methylene blue were examined using light microscope 300 MS (THR) with a connected Digital camera for microscope DCM 900. Morphometry was performed using image processing program NIH USA ImageJ in a manual mode considering magnifications. Structural changes at each stage of the study were analyzed in 50 fields of view. The surface area of the profile of neurons and their nuclei, nucleocytoplasmic index (NCI), and nuclear shape factor (NSF) were determined.

Electronic data processing was performed using statistical software package Stat.Soft.Inc; Tulsa, OK, USA; Statistica 6. Nonparametric methods of the investigations (the Mann-Whitney test, Spearman's rank correlation coefficient (r_s)) were used.

Results and discussion

On the 14th day of the development of experimental DM blood glucose level increased by 13.62 ± 0.72 mmol/l (in the control group it increased by 5.25 ± 0.59 mmol/l, p=0.0001) and HbA_{1c} level increased by 6.84 ± 0.59 % (in the control group it increased by 2.11 ± 0.22 %, p=0.0001). Such biochemical changes indicated the development of a stable type of DM. There was detected a strong direct relationship between glucose and HbA_{1c} levels r_s = 0.78 (p=0.0075).

Surface area of the profile of neuronal nuclei in the intermuscular plexus increased significantly by 34.51 ± 1.32 um^2 (in the control group it increased by 30.14 ± 0.98 um^2, p=0.0001 and no significant changes were observed in surface area of the profile of neurons which constituted 102.06 ± 8.22 um^2 (in the control group it was 99.65 ± 10.50 um^2, p=0.8798) resulting in an increased nucleocytoplasmic index by 0.52 ± 0.05 (in the control group it increased by 0.44 ± 0.06, p=0.0113). NSF was found to be decreased by 0.76 ± 0.01 (in the control group it decreased by 0.84 ± 0.02). At the light-optical level in rats with DM enlarged nuclei were displaced towards the periphery of the perikaryon and cleared. Grains of tigroid were irregularly distributed throughout the neuroplasm of the neuron cell bodies. They formed clusters along the periphery of the perikaryon while around the nuclei chromatolysis was occasionally seen to occur. Capillaries were found to be sanguine. At the ultrastructural level neuronal nuclei contained diffusely scattered granules of euchromatin and separate clusters of electron-dense heterochromatin. The nuclear membrane formed slight invaginations (Fig. 1a), perinuclear space was expanded. Neuronal cytoplasm was found to be of moderate electron-optical density. Dictyosomes and vesicles of the Golgi complex, hypertrophied cisternae of granular endoplasmic reticulum, and 1-2 lysosomes were concentrated close to the nucleus.

There was observed an increase in mitochondrial volume, mitochondrial matrix was cleared and cristae were partially destroyed and disorganized (Fig. 1a). Such morphological and morphometric features of neuronal restructuring indicated their high functional activity [9, 13] that was probably associated with polyphagia and constant intestinal motility for evacuation of food. According to the results of our research in separate unmyelinated nerve fibers axoplasm of low electron optical density was visualized. It contained large mitochondria with cleared matrix and some cristae, slightly decreased numbers of neurotubules (Fig. 1b). The Schwann cell cytoplasm was characterized by an increase in the number and size of breakdown products of myelin and hyperplasia of the endoplasmic reticulum. In the interstitial cells of Cajal autophagosomes (Fig. 1b) and several small vacuoles were found. These cells were on the border of unmyelinated nerve fibers. Reverse dystrophic changes in mitochondria were detected in myocytes. In the hemomicrocirculatory channel, particularly in capillaries, erythrocytic sludges, erythrocytes and platelet adhesion were detected. More pronounced changes were seen in endothelial cells. An increased number of pinocytic vesicles and destructive changes in mitochondria were found in their cytoplasm (Fig. 1c). The nuclei of endothelial and pericapillary cells contained matrix of low electron optical density with marginal heterochromatin (Fig. 1c). Such changes were caused primarily by high levels of HbA_{1c} changing the surface charge of erythrocytes. It leads to true capillary stasis, sludge and agglutination of red blood cells and further results in microthrombosis causing local circulatory and hemic hypoxia and activating cascade of molecular mechanisms of damage to cellular membranes [1, 8, 9].

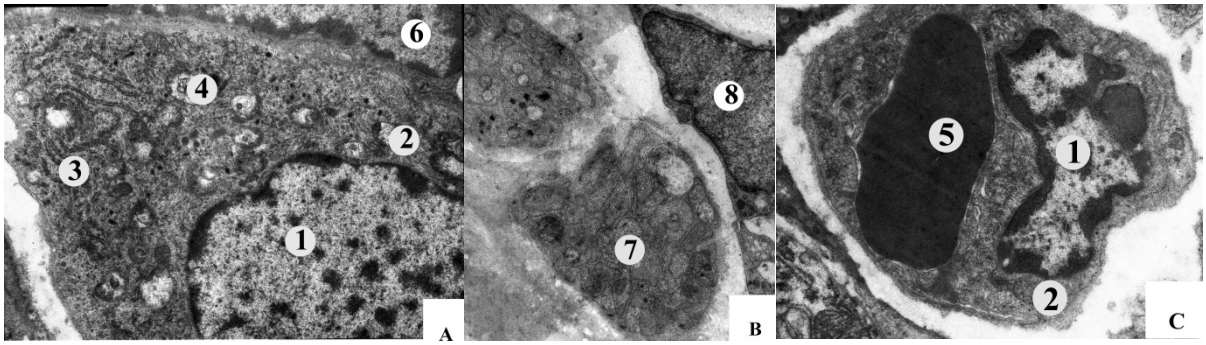

Fig. 1. Ultrastructural restructuring of neuron (a), unmyelinated nerve fibers and interstitial cells of Cajal (b), capillaries (c) on the 14th day of the development of experimental DM. Electronograms. Mag.: a, b) × 8,000, c) × 9,600.
1 - nucleus, 2 - mitochondria, 3 - granular endoplasmic reticulum, 4 - vacuoles, 5 - erythrocyte, 6 - Schwann cell, 7 - unmyelinated nerve fibers, 8 - interstitial nucleus of Cajal

On the 42nd day of the development of experimental DM blood glucose level continued to increase by 16.84 ± 0.77 mmol/l (p=0.0001), HbA_{1c} level increased by 9.72 ± 0.85% (p=0.0001). A strong direct relationship between glucose and HbA_{1c} levels r_s = 0.78 (p=0.0075) was also detected.

Surface area of the profile of neurons in the intermuscular plexus increased significantly by 126.59 ± 5.42 um^2 compared to the control group and the results obtained on the 14th day of the development of experimental DM (p=0.0001, p=0.0002) while no significant changes were observed in surface area of the profile of neuronal nuclei being 33.01 ± 2.25 um^2 (p=0.1859) compared to the results obtained on the 14th day of the development of experimental DM, however, it was significantly higher as compared with the control group (p=0.0041). NSI was found to be decreased by 0.34 ± 0.04 (p=0.0002, p=0.0001). NSF increased by 0.85 ± 0.05 (p=0.0002) compared to the results obtained on the 14th day of the development of experimental DM, however, it was not significantly different from control values (p=0.0041). Such morphometric changes in the neuronal nucleus and body indicated their swelling that was confirmed by the results of light and electron microscopic studies. In particular, on Nissl stained histological specimens karyopyknosis and karyolysis, peripheral chromatolysis and cytoplasmic vacuolation were observed. In other neurons tigroid was finely divided. Chromatolysis was sometimes seen around the nucleus only. On the 42nd day of the development of experimental DM "pycnomorphous" neurons were detected.

At the ultrastructural level in neurons destructive changes were detected including karyopyknosis and karyolysis, expansion and destruction of Golgi apparatus tubules, clearing of the mitochondrial matrix and destruction of the inner mitochondrial membrane, the presence of vacuoles and lipofuscin granules in the neuroplasm (Fig. 2a). In such cells granular endoplasmic reticulum contained several expanded cisternae at the periphery of the perikaryon (Fig. 2a). In addition to destructively changed neurons dark "pycnomorphous" neurons with apoptotic bodies and neurons with preserved ultrastructure were detected. However, the latter were rare. Such morphological changes in neurons are associated, firstly, with the violation of metabolic processes because low insulin levels cause impaired protein synthesis in neurons of the brain and neurofilaments damage [2, 3, 13] resulting in diabetic encephalopathy. Secondly, hyperglycemia causes neuronal apoptosis by tissue acidosis [1, 2, 3, 5]. Thirdly, vacuolar dystrophy of neurons occurs secondary to diabetic microangiopathy. On the 42nd day of the development of experimental DM destructive changes in capillary wall were detected including destruction of membranous structures of cytoplasmic organelles of endothelial cells. The symptoms of microclasmatosis were aggravated (Fig. 2b). In the lumen of the hypothalamic capillaries adhesion and aggregation of platelets and erythrocytic sludges causing disruption of the microcirculation were also detected. The nuclei of endothelial and pericapillary cells were found with cleared nucleoplasm and coarsely granular chromatin gathered in clumps near the inner nuclear membrane (Fig. 2b). The latter formed slight invaginations, as a result, the nuclei became irregular in shape. Detachment of the endothelial cells and exposure of the basal membrane occurred here and there. The latter was thickened due to its hyalinosis.

In unmyelinated nerve fibers axoplasm and dendritic matrix were cleared. Varicose thickenings of axons containing separate microtubules, vacuoles and a small number of synaptic vesicles were also found (Fig. 2a). Such changes in nerve fibers indicated axonal dysfunction and delayed anterograde axonal transport in DM [9, 11].

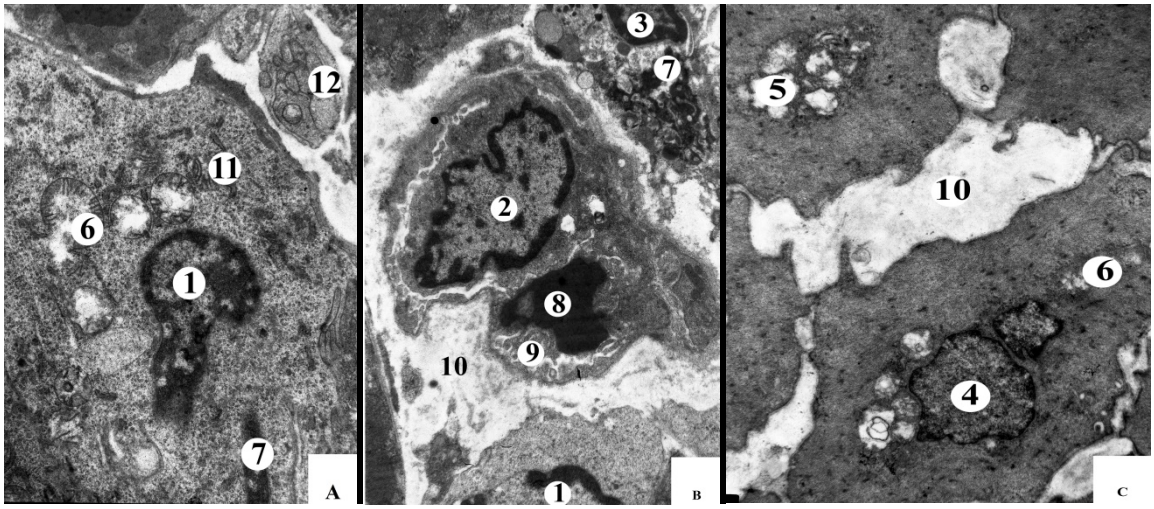

Fig. 2. Ultrastructural restructuring of neuron (a), capillary and interstitial cell of Cajal (b), myocytes (c) on the 42nd day of the development of experimental DM. Electronograms. Mag.: a) × 8,000, b) × 6,400, c) × 4,800.
1 – neuronal nucleus, 2 –endothelial cell nucleus, 3 – interstitial nucleus of Cajal, 4 – myocyte nucleus, 5 - vacuoles, 6 - mitochondria, 7 – apoptotic body, 8 – erythrocyte; 9 – microclasmatosis; 10- connective tissue; 11- granular endoplasmic reticulum

In most interstitial cells of Cajal there was observed karyorrhexis with further nuclear fragmentation and the development of apoptotic bodies (Fig. 2b). Their cytoplasm was of different electron optic density and contained lysosomes, autophagosomes and vacuoles. A reduction in number of interstitial cells of Cajal in DM has also been mentioned by other researchers [4, 5] and according to the results of our study it is caused by their apoptosis.

On the background of circulatory and hemic hypoxia myocytes of the intestinal wall were damaged. Karyorrhexis and karyolysis, cytoplasmic vacuolation, destruction of the inner and outer mitochondrial membranes, disordered arrangement of myofilaments were detected (Fig. 2c). The spaces between myocytes became distended and filled with loose connective tissue. The basal membrane of myocytes was significantly thickened as indicated by other researchers [5, 6, 12] who have recommended to use this morphological sign as a marker of DM.

Conclusions

Diabetic gatroparesis in rats occurs on the 42nd day of the development of experimental streptozotocin-induced DM. It is characterized by destructive changes in neurons of the intermuscular plexus (vacuolar dystrophy, apoptosis), axonal degeneration that defines the neurogenic nature of the damage to unmyelinated nerve fibers, apoptosis of the interstitial cells of Cajal, and vacuolar dystrophy of myocytes.
Such changes occur secondary to microangiopathy.

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