

The effects of gene therapy with PEI-pDNA complex containing human preproinsulin gene on structural and ultrastructural characteristics of several organs in mice of different age at experimental diabetes mellitus



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ABSTRACT

Diabetes mellitus (DM) is one of the leading age-related diseases. Its prevalence, according to WHO, will increase steadily, therefore the study of the age-related features of its morphogenesis and search for new approaches to its treatment, taking into account the age, remains one of the topical issue of modern medicine and biology.

The purpose of the research was to study the age-related features of the effect of gene therapy with PEI-pDNA complex containing the human preproinsulin gene on the morpho-functional characteristics of organs mostly affected by diabetic dysfunction at streptozotocin-induced DM in mice of different ages.

MATERIALS AND METHODS. *DM was induced in 3- and 20-month old mice by the administration of streptozotocin (40 mg/kg on 0.1 M citrate buffer, once a day, for 5 days). Four weeks after the development of persistent hyperglycemia, the polyethylenimine (PEI)-plasmid DNA complex, containing the human preproinsulin gene, was injected into the liver. On the 30th day after the plasmid administration, morpho-functional features of the pancreas, myocardium, liver and kidney of young and old mice were studied using histological, electron microscopic, histochemical and immunohistochemical techniques.*

RESULTS. *In the young animals, the use of the PEI-pDNA complex with the human preproinsulin gene had a certain healing effect on the structure and ultrastructure of the studied organs, reduced the dystrophic and destructive alterations in the cells of their parenchyma and capillaries, reduced the intensity of apoptosis, and stimulated the development of compensatory-adaptive hyperplastic processes. In the old animals, there was a slight positive effect of the gene therapy or no effects. Often, some of the structural and ultrastructural parameters of several organs worsened; the high intensity of apoptosis and the development of complications (insulinitis) persisted.*

CONCLUSIONS. *The effect of the PEI-pDNA complex containing the human preproinsulin gene on the morpho-functional characteristics of internal organs in mice at DM has different efficacy at different ages: high in the young animals and minor effect, lack of it, or additional deterioration in the old ones, which manifested itself in various tissues in different degree.*

KEY WORDS: *diabetes mellitus; gene therapy; preproinsulin; apoptosis; aging*

Diabetes mellitus (DM) is one of the most common multifactorial age-dependent endocrine diseases, accompanied by numerous complications. WHO epidemiologists previously predicted that by 2025 the number of patients with DM will exceed 400 million, 80-90 % of which will be patients with type 2 diabetes. By 2010, the number of diseased at the age of 20-79 years had to be 6.4 %, covering 285 million, and by 2030 – should have increased to 7.7 %, covering 439 million [1]. But today experts predict that by 2030 the number of patients with diabetes in the world will amount to not even 336 million and not 440 million, as expected, but 552 million, acquiring the scale of the «non-infectious» epidemic of the 21st century [2, 3]. According to the International Diabetes Federation (IDF), as of 2015, there are 415 million patients on the planet, and according to forecasts for 2040 the number of people with DM will increase to 642 million [3].

Recently, throughout the world, and in Ukraine in particular, there has been an increase in the number of people over 60, that is, there is an obvious aging of the population. Significant prevalence of diabetes is particularly characteristic of this age category, which makes this pathology one of the main objects of gerontological research [4, 5].

Serious complications at DM, affected practically all human body systems, significantly increase the risk of developing micro- and macrovascular diseases and the mortality rate, primarily from cardiovascular disease [6-10]. Of particular interest are endothelial injuries due to the special role of endothelial dysfunction in the pathogenesis of diabetes [8, 11-13]. Also of great importance in the study of diabetes pathophysiology are the mechanisms of cell death associated with the pathogenesis of this disease and the development of its complications [11, 14]. All this determines the need to investigate morpho-functional changes in the cellular, non-cellular components and capillaries of various organs that are most subjected to diabetic dysfunction, in the modeling of DM and its correction, taking into account the features of these processes at different ages.

In a large number of studies devoted to the search for methods of DM correction, there are researches in the field of gene therapy. The staff of the Institute of Molecular Biology and Genetics of NASU under the guidance of Academician V. A. Kordium designed the eukaryotic expression vector of the human preproinsulin gene for subsequent delivery to non-endocrine mammalian cells in order to develop experimental gene therapy for type 1 diabetes [15]. The sequence of the human preproinsulin gene is highly homologous to the sequence of the preproinsulin gene of experimental animals, and has high internal homology due to multiple short repeats [15].

The purpose of the research – to study the structural, ultrastructural, histochemical and morphometric features, as well as the intensity of apoptosis, in the pancreas, myocardium, liver and kidney in mice of different ages with streptozotocin (STZ)-induced diabetes mellitus and its correction using gene therapy with PEI-pDNA complex containing the human preproinsulin gene.

MATERIALS AND METHODS

The experiments were carried out according to the existing bioethical norms of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986), as well as article 26 of the Law of Ukraine on the Protection of Animals against Cruelty (No. 3447-IV, 21.02.2006).

The studies were carried out on C57BL/6j mice of two age groups (the 3-month and 18-20-month olds), which were divided into three groups: I – control; II – mice with induced DM; III – mice with diabetes and gene therapy (DM+GT) using the PEI-pDNA complex, containing the human preproinsulin gene. Diabetes mellitus was modeled by intraperitoneal injection of streptozotocin (*Sigma*, USA) at a dose of 40 mg/kg per 0.2 ml of 0.1 M citrate buffer (pH = 5) for 5 days. As a control group, intact animals of the same age were used, which were intraperitoneally injected with 0.2 ml of citrate buffer.

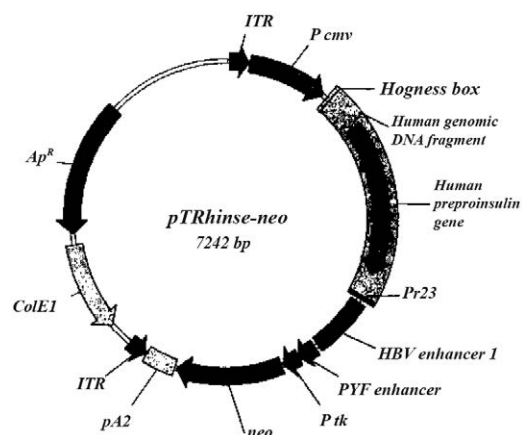


Fig. 1. Scheme of plasmid construction containing human preproinsulin gene [15].

The development of hyperglycemia was controlled using a glucometer (*Accu-Chek Active*, Germany). Blood sampling was performed by retroorbital venous sinus puncture on an empty stomach. The animals were withdrawn from the experiment 5 weeks after the development of persistent hyperglycemia. Further involvement in the experiment was impossible due to poor physical condition and high mortality of the old animals. The obtained tissue made it possible to evaluate the entire complex of reactive changes in the studied organs at the peak of the pathological process without any theoretically possible self-healing or application of experimental correction of the disease.

Some animals with STZ-induced DM of both age groups underwent gene therapy. Four weeks after the development of persistent hyperglycemia, a solution containing a plasmid vector for the delivery of the human preproinsulin gene, which was obtained in the department of regulatory mechanisms of the cell of the Institute of Molecular Biology and Genetics of the National Academy of Sciences of Ukraine, was injected into their liver [15]. Plasmid (**Fig. 1**) constructed with the human preproinsulin gene, precipitated with polyethylenimine (PEI), 25kD, was prepared *ex tempore* 10 minutes before administration. The volume of the precipitated pDNA was 90 µl, the content of plasmid pDNA in the preparation was 10 µg per animal, pDNA/PEI weight ratio was 1:2. The procedure for injection of the drug is described in detail in our previous paper [16].

On the 30th day after plasmid administration, the animals were euthanized. We used 12 mice in control group (7 young and 5 old), 16 mice with STZ-induced DM (10 young and 6 old), and 12 mice with induced DM that received the plasmid with the human preproinsulin gene (6 young and 6 old).

The tissue of the pancreas, left ventricle of myocardium, liver and kidney was studied using standard histological as well as electron microscopy techniques. The pancreas was fixed in Bouin's fixative, histological sections 5 µm thick, obtained on a rotational microtome HM 325 (*Microm*, USA), stained with hematoxylin and eosin, as well as sections of all other organs. The sections of pancreas also were stained with aldehyde-fuchsin by Gomori for detection of secretory granules in β-cells of pancreatic islets. The obtained histological specimens were studied and photographed at x40, x100, x200 and x400 magnification using a BX51 microscope and DP-Soft 3.2 software (*Olympus*, Japan). In all studied groups of experimental animals (control, DM, DM+GT in two age groups) the following morphometric parameters were estimated: the number of pancreatic islets per 1 mm² of the sections, the specific volume of endocrine tissue in the total volume of pancreas; specific volume of β-cells in the total volume of pancreatic islets; the number of insulinocytes with dystrophic changes and the number of lymphocytes in pancreatic islets. Ultrathin sections of the myocardium, liver and kidney, made with an LKB-III ultramicrotome

(Bromma, Sweden), were analyzed under an electron microscope TEM-125C (SEMI, Ukraine).

Apoptosis was detected using an TUNEL assay with ApopTag® Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon, USA), as detailed in our previous article [16]. The apoptotic index (AI) was calculated as the number of TUNEL-positive cells per 1 mm² of the section area.

Statistical analysis was carried out using parametric and nonparametric statistics with Excel (Microsoft, USA) and Statistica 6.0 software (Stat Soft Inc., USA). Wilcoxon-Mann-Whitney U-test was used for statistical hypothesis testing. The differences are significant at α = 0.05. The median (Me [Q 25; Q 75]), the minimum and maximum values (min-max) were used in the table.

RESULTS AND DISCUSSION

In the modeling of diabetes in the animals of both age groups, the development of circulatory disorders, mainly the capillaries, as well as the dystrophic and alterative changes in the parenchyma of the studied organs, often acquired features of severe destructive damage leading to their necrosis and apoptosis. TUNEL assay at DM detected increased apoptotic cell death (mostly endotheliocytes of the blood capillaries of various organs, pancreatic beta cells, hepatocytes and epithelial cells of the proximal tubules of the kidneys). In the old animals, these changes were much more pronounced than in the young animals, often becoming irreversible. In all studied organs, the mean AI values during DM modeling were also significantly higher in the old animals (Table 1). Especially demonstrative, these differences were found in the pancreatic islets (the median of AI in the young mice was 9.83 TUNEL⁺ cells/mm² and in the old mice – 20.27 TUNEL⁺ cells/mm²) and even more – in the myocardium (0.14 TUNEL⁺ cells/mm² – in the young ones and 26.49 TUNEL⁺ cells/mm² – in the old mice).

The application of gene therapy in the young animals with diabetes has a certain healing effect on the structure and ultrastructure of the pancreas, myocardium, liver and kidney, reducing the severity of dystrophic and destructive alterations and stimulating the development of compensatory hyperplasia at the cellular and subcellular levels. In addition, in the majority of the studied cells, there was a significant decrease in the intensity of apoptosis, which indicates a pronounced protective effect of the PEI-pDNA complex in the young animals.

The application of gene therapy in old animals did not have a pronounced healing effect on the structure and ultrastructure of the studied organs. There were deep alterative changes of their cellular components and capillaries. In some cases, there was a development of pathologic processes (insulinitis in the pancreas). At the same time, the signs of apoptosis (sharply increased at DM, were much manifested than in the young animals) decreased insignificantly (in hepatocytes, cardiomyocytes), practically did not change (in epithelial cells of the proximal tubules of the kidneys), or even increased (in insulinocytes of pancreatic islets).

The analysis of morpho-functional changes in the organs at DM modeling and the search for methods of its correction is impossible without taking into account the level of glucose in the blood serum in animals of all experimental groups, which we carried out earlier [17]. The obtained results of morphological studies were well correlated with these biochemical markers. According to the obtained data, at STZ-induced diabetes in both age groups, the glucose level increased more than threefold (and in the young animals it was slightly higher than in the old ones), which corresponded to the development of persistent hyperglycemia in them. With the application of the PEI-pDNA complex containing the human preproinsulin gene, the concentration of glucose in the blood decreased in the young animals almost threefold, approaching the control level, and in the old ones – approximately twice, remaining quite increased [17].

These main points can be illustrated by the example of organs that are considered the most open to injury at DM. However, the morpho-functional changes in the pancreas are the triggering mechanism of all the studied phenomena, associated with the possible development of diabetic complications. At the modeling of STZ-induced DM, which is more corresponding to type 1 diabetes, the evaluation of morpho-functional changes in the pancreas is critically important under experimental conditions. Therefore, in the context of the purpose of this paper, it is necessary to cite the data of our previous studies [17].

Histological study of pancreas. When studying the pancreas, we noted that the morphometric parameters (the number of pancreatic islets per area and the specific volume of pancreatic islets in the total volume of the pancreatic tissue) in the old animals were significantly lower than in the young animals, and the level of apoptosis was significantly higher due to age-related changes in this gland. At the same time, the indicator of functional activity of pancreatic islets (the number of insulinocytes with A-F-positive secretory granules in the cytoplasm) differed little in

Table 1. Apoptotic index in different organs of young and old mice in groups DM and DM+GT compared to control group (number of TUNEL⁺ cells per 1 mm² of section).

	GROUPS	YOUNG MICE			OLD MICE		
		min-max	Me	Q ₂₅ -Q ₇₅	min-max	Me	Q ₂₅ -Q ₇₅
Pancreas	Control	0.00-0.02	0.01	0.005-0.01	2.0-24.52	3.75	3.25-8.37
	DM	3.12-17.67	9.83*	6.19-14.03	6.11-39.11	20.27*#	12.60-30.27
	DM+GT	0.20-2.37	0.63*	0.25-1.66	18.81-31.24	27.06*#	24.92-30.77
Liver	Control	0.32-1.87	0.86	0.47-1.48	0.55-5.93	2.46	1.82-4.02
	DM	3.16-13.96	11.62*	6.42-13.76	7.81-14.60	10.15*	8.14-13.21
	DM+GT	0.94-9.92	6.10*#	4.12-9.44	2.25-12.67	8.96*#†	4.42-12.00
Myocardium	Control	0.00-0.07	0.03	0.02-0.06	6.32-28.43	10.18#	7.88-13.12
	DM	0.12-0.18	0.14*	0.13-0.16	18.80-41.99	26.49*#	22.63-31.30
	DM+GT	0.04-0.16	0.08*	0.04-0.10	8.02-24.72	14.15*#	11.30-18.93
Kidney	Control	0.09-3.09	2.10	1.31-2.73	1.41-7.54	4.07#	3.40-5.73
	DM	4.31-10.92	7.95*	7.86-8.99	7.48-14.44	9.37*#	8.95-13.28
	DM+GT	4.34-9.21	5.72*	5.41-6.56	6.45-12.04	7.58*#	6.93-9.45

Notes: * – significant differences at α = 0,05 compared to control group, # – compared to young mice, † – compared to group DM.

the young and old control animals, although its spread was significantly larger in the old, which reflects the heterogeneity of the morphological features characteristic of aging (Fig. 2).

While inducing DM, the quantitative parameters of pancreatic islets decreased in both age groups, and the level of functional activity of the insulocytes decreased particularly sharply in the old mice, reflecting the deeper destructive dystrophic and atrophic processes in the pancreatic islets at DM in old age. The level of apoptosis increased significantly, especially in the old mice, which was correlated to much deeper structural damage to the pancreas, which we noted in this age group.

When the plasmid complex was administered to the young animals with DM the amount of the insulocytes with A-F-positive granules in the cytoplasm increased (fig. 2). The level of apoptosis in the pancreas, as determined by the TUNEL assay, was significantly lower than in the animals that did not receive treatment, which was an objective criterion for the effectiveness of gene therapy at this age. A different pattern was found in the old mice. The number of functionally active insulocytes increased insignificantly (fig. 2). If the introduction of the plasmid complex in the young animals with DM led to a significant decrease in the apoptosis index approaching the control values (median of AI 9.83 TUNEL⁺ cells/mm² in DM group; 0.63 TUNEL⁺ cells/mm² – in group DM+GT), then in the old ones – there was not only decrease in this index, but even its growth was noted (20.27 TUNEL⁺ cells/mm² – at DM; 27.06 TUNEL⁺ cells/mm² – at DM+GT) (Table 1). In addition, apoptosis affected not only insulocytes in pancreatic islets, but also ductal epitheliocytes, capillary endotheliocytes, exocrine cells, and lymphocytes infiltrated the islets (Fig. 3).

Therefore, if the morpho-functional changes in the pancreas of the young and old animals were predominantly similar at DM modeling, differing mainly in the intensity, then in the correction of DM by gene therapy, they turned out to be different in a number of indices. In the young animals, there was a certain normalization of the studied features in varying degrees, and in the old animals, a significant impairment of them, which led to an increase in alternative changes, the development of pathological processes, and an increase of apoptosis, whose level was higher than at DM.

The effect of the PEI-pDNA complex containing the human preproinsulin gene at experimental DM promotes not only the recovery of the morpho-functional characteristics of the pancreas in young animals, but also the structures and ultrastructure of the heart, liver, and kid-

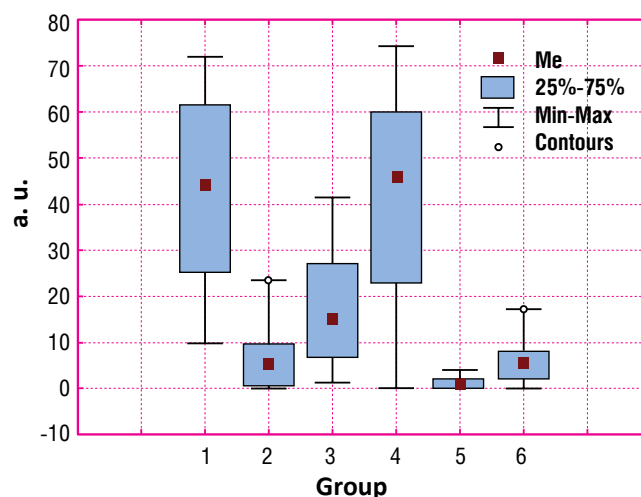


Fig. 2. Diagrams of specific volume of insulocytes containing A-F-positive secretory granules in pancreas of young (1, 2, 3) and old (4, 5, 6) mice in groups DM (2, 5) and DM+GT (3, 6) compared to control group (1, 4).

neys. The severity of destructive changes and edema in the myocardium and liver parenchyma decreases. The effect of the plasmid prevents damage to the glomeruli in kidney, although it does not repair the damage to the renal tubule in the form of granular dystrophy and edema of the epithelium cytoplasm. In old animals under similar experimental conditions, various changes occur in all studied organs, which indicate a pronounced age-related effect of the gene therapy.

The histological study of the myocardium. The histological examination of the myocardium in mice with experimental DM of both age groups showed impaired blood circulation (plethora of arteries of a small and medium diameter, foci of perivascular edema followed by extension to the connective tissue stroma, which is simultaneously infiltrated by leukocytes, lymphocytes and macrophages; focal edema and hyalinosis of arterial wall; aggregation and adhesion to the surface of the vascular endothelium of erythrocytes and platelets, which indicates an abnormality

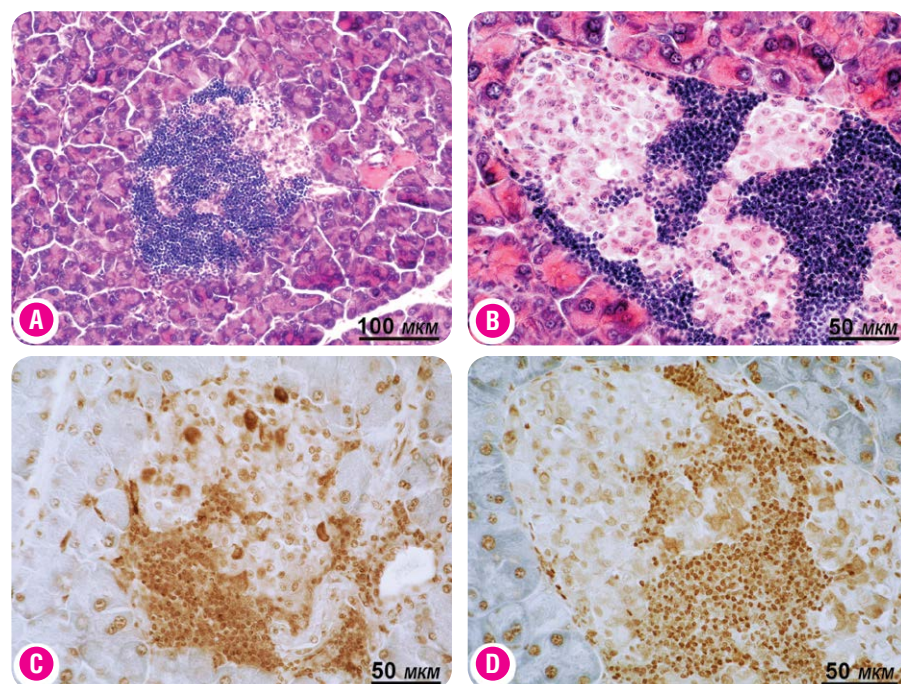


Fig. 3. Microphotographs of pancreatic sections of old mice in group DM+GT. Structural changes in pancreatic islets: A, B – infiltration of pancreatic islets by lymphocytes with the signs of insulitis, hematoxylin and eosin staining; C, D – apoptosis of insulocytes and lymphocytes, infiltrated pancreatic islets, TUNEL assay.

of blood rheology) and dystrophic changes in cardiomyocytes, as well as the development of their contractile damages and focal fibrosis. In the old animals with DM, the dystrophic changes in the myocardium were much more pronounced than in the young mice.

Under the impact of the PEI-pDNA complex against the background of a certain normalization of the myocardial structure, focal perivascular edema and fibrosis persisted; microcirculatory disorders in intramural vessels, foci of pericellular edema of cardiomyocytes, as well as their focal dystrophy in the form of edema, swelling and myocytolysis, distortion and hyperrelaxation of the sarcomeres (Fig. 4 A, B). This type of cardiomyocytes dystrophy is a consequence, mainly, of their ischemic injury. In the older animals of the DM+GT group, these processes were more pronounced. There was an increase in dystrophic changes in cardiomyocytes and capillaries, as well as an increase in lymphocytic infiltration of interstitium.

The ultrastructural analysis of myocardium of mice with DM revealed focal dystrophic changes of cardiomyocytes in the form of severe edema of sarcoplasm, disorders of the sarcotubular system and deep damage to the contractile apparatus of cells (lysis of myofibrils, their disintegration, homogenization), and ultrastructure of capillaries (necrotic or apoptotic changes of endotheliocytes, widening of the pericapillary space, increase in the number of pericapillary fibrous structures, Fig. 4 C).

The application of gene therapy at DM in young animals led to a certain normalization of the myocardial ultrastructure (Fig. 4 D). Edema of cardiomyocytes and pericapillary spaces significantly decreased. The structure of the contractile apparatus improved, striation of myofibrils was clearly expressed in most cells, with clearly visible Z lines, indicating a reduction in the contractile dysfunction that is typical at DM. At the same time, there were no significant changes in apoptotic activity either in the modeling of DM or in its gene therapy correction. In addition, there were a number of changes in the myocardium of the young animals that had a compensatory type – most evident hypertrophy of cardiomyocytes nuclei (in some cases they were several times larger than their usual sizes), hypertrophy and hyperplasia of the nucleoli, mitochondrial hyperplasia. Nucleolar hypertrophy was also observed in the nuclei of endothelial cells of capillaries, as well as fibroblasts.

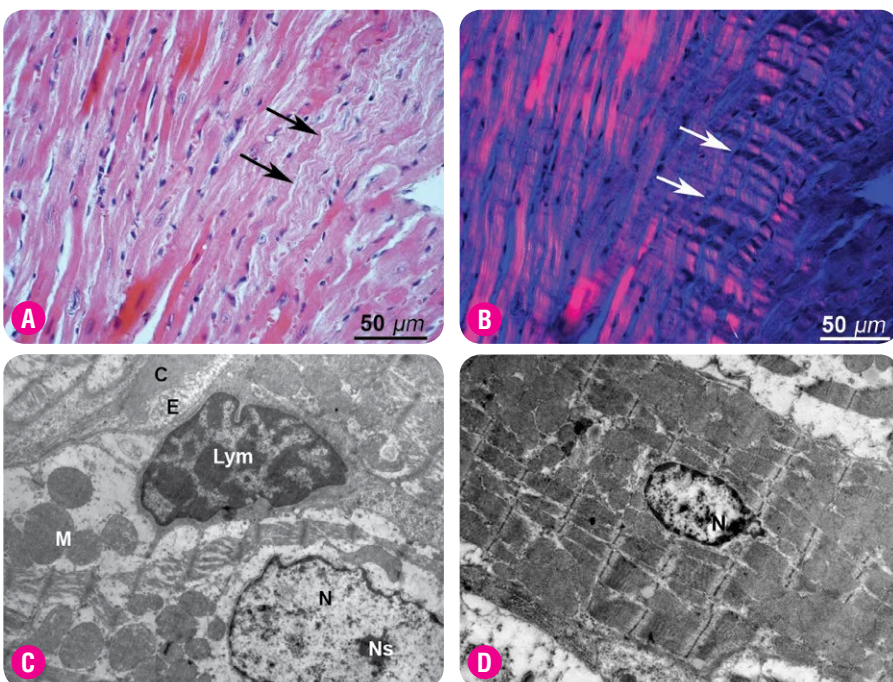
In the old animals of the DM+GT group, against a background of some improvement in the myocardial ultrastructure, dystrophic changes in the cells and capillaries were persisted in the form of lysis of myofibrils and

subsarcolemmal edema, increase of lymphocytic infiltration of the interstitium, edema of connective tissue stroma, damage to endothelial cells of the capillaries, while compensatory adaptive processes were extremely poorly expressed.

At the same time, significant age differences were noted in the study of apoptosis in the myocardium. While there were no changes in apoptotic activity in young animals either in the modeling of DM or in its correction using gene therapy, in old animals there was a sharp increase in the apoptosis of cardiomyocytes. In the young animals, median of AI were 0.03 TUNEL+ cells/mm² in control group, 0.14 TUNEL+ cells/mm² – in DM group and 0.08 TUNEL+ cells/mm² – in DM+GT group, but, in the old ones, these indicators increased several times (10,18 TUNEL+ cells/mm² – control group, 26,49 TUNEL+ cells/mm² – DM group, 14,15 TUNEL+ cells/mm² – group DM+GT, Table 1). Consequently, when modeling DM in old animals, this indicator increased almost threefold in comparison with the control, while in DM+GT group it decreased almost twofold. Therefore, despite severe structural and ultrastructural damage to the myocardium of the old animals, detected after gene therapy, apoptotic cell death decreased.

Dystrophic changes in cardiomyocytes, their apoptosis, as well as myocardial fibrosis and damage to capillaries at DM are considered to be key pathogenetic factors in the development of diabetic cardiomyopathy [6, 7, 9]. Since these changes are much more pronounced in the old animals (both in DM and in DM+GT groups), these age differences can be considered as a morphological manifestation of more severe DM complications in the cardiovascular system during aging.

The histological study of the kidney. The study of the kidney of animals of both age groups with STZ-induced DM noted the development of diffuse mesangial sclerosis due to thickening of the basal membrane of the glomerular capillaries as a result of the deposition of hyaline-like masses. A distinctive feature of glomerulosclerosis, which developed at the DM modeling, was a diffuse type of the lesions of the renal glomeruli, mainly juxtamedullary nephrons, as well as tubulointerstitial nephritis. It was characterized by the development of moderate focal edema of the interstitial connective tissue in the renal medulla, the expansion of the proximal tubule lumen, the albuminous degeneration of their tubular epithelium, and the presence of the hyaline cylinders in the moderately enlarged lumens of the renal distal tubules, indicative of the development of proteinuria. In the old mice, the nature of the pathological changes developing in the kidneys at STZ-induced DM was similar, but differed



◀ Fig. 4. Microphotographs of myocardial sections of young mice in group DM (C) and DM+GT (A, B, D). Histological (A, B) and ultrastructural (C, D) changes: A, B – distortion and hyperrelaxation of the sarcomeres (arrows), hematoxylin and eosin staining; C – cardiomyocytes with sarcoplasmic edema and myofibril destruction, necrosis of endothelial cell; D – normalization of the cardiomyocytes ultrastructure, moderate subsarcolemmal edema in surrounding cells. Light (A), polarized (B) and electron (C, D) microscopy.

Notes: Lym – lymphocyte, M – mitochondrion, C – capillary vessel, E – endothelial cell, N – nucleus, Ns – nucleolus.

in the intensity and prevalence due to the development of age-related sclerosis in the kidneys. Particularly evident changes affected basal membranes and capillary endothelial cells.

In the kidneys of the young mice with DM that underwent gene therapy, morphological changes indicating its pronounced positive effect were revealed. There was a decrease in structural damage not only to the renal glomeruli, but also to the renal tubule. At the same time, the PEI-pDNA complex did not completely eliminate the STZ-induced damage to the tubules in the form of granular dystrophy and edema of the epithelium cytoplasm, mostly of the proximal tubules. There was a complex of dystrophic alteration in the kidneys of the old mice with DM which underwent gene therapy, unlike in the young ones (Fig. 5).

There were often found glomeruli with focal sclerosis in the cortex and medulla of the kidneys. A coat-sleeve-like lymphocytic infiltration around the intratubular arteries were identified. In this case, the walls of such arteries were thickened due to the edema of all layers and, sometimes, plasma infiltration and hyalinosis. Also, in the old mice of DM+GT group, unlike in the young mice, there were often noted foci of lymphohistiocytic infiltration and sclerosis in the connective tissue stroma.

The immunohistochemical and morphometric studies as well as electron microscopic studies showed a sharp increase in the intensity of the apoptosis in the kidneys of mice at STZ-induced DM and a marked decrease at gene therapy with the PEI-pDNA complex in the young ones, which indicates a pronounced protective effect of this treatment. At the same time, the protective effect of the PEI-pDNA complex directly depends on the age of the experimental animals that determines the features and type of the age-related pathology. In the young animals with DM, the administration of the plasmid complex led to a significant decrease in the apoptosis index (median of AI was 7.95 TUNEL⁺ cells/mm² in DM group, 5.72 TUNEL⁺ cells/mm² – in group DM+GT). In the old ones, this decrease was less (9.37 TUNEL⁺ cells/mm² in DM group, 7.58 TUNEL⁺ cells/mm² – in group DM+GT, Table 1).

The histological study of the liver. Histological and ultrastructural studies of the liver of the mice with STZ-induced DM indicate a disorders of blood circulation, as well as marked dystrophic changes in hepatocytes due to impaired intracellular fluid circulation (intracellular edema, fatty, hydropic, balloon dystrophy) and protein metabolism disorders (hyaline dystrophy). In the end, this led either to necrosis of hepatocytes, or to their death through apoptosis. Numerous studies of histological chang-

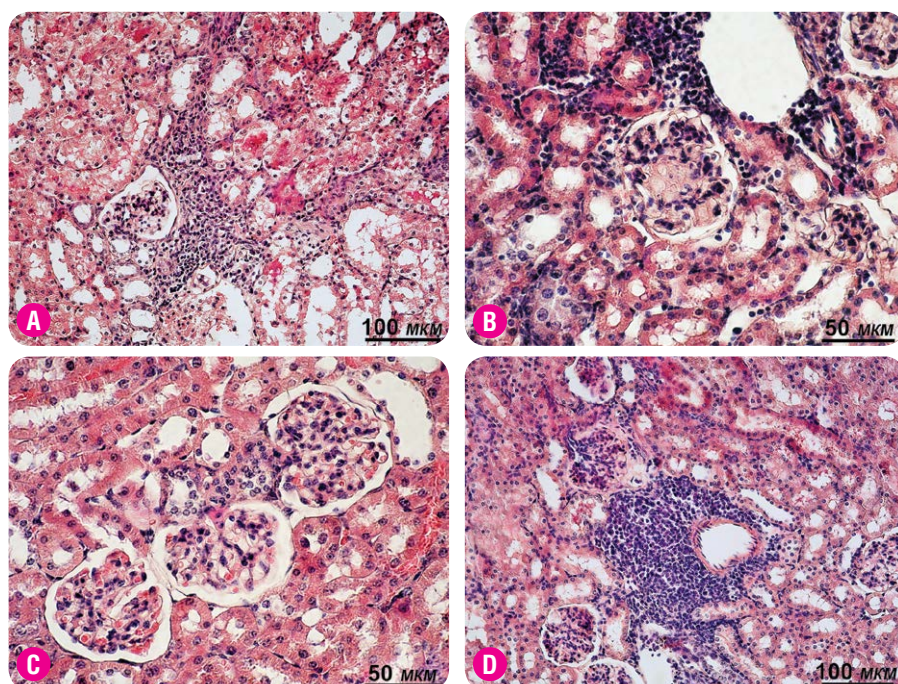
es in the liver at the modeling of DM by introduction of streptozotocin or alloxan indicate the development of steatosis, up to steatohepatitis and fibrosis, which is also observed in people with diabetes [18].

Structural and ultrastructural changes in the liver of the old mice with STZ-induced diabetes were more pronounced than in the young mice (Fig. 6, 7). They are characterized by disorder of the liver lobular structure, marked damage of the blood vessels wall, injury of the endothelium and tissue reaction to its damage in the form of foci of leukocyte infiltration directly under the endothelium. Also there were pronounced dystrophic and necrotic changes in hepatocytes, as well as their apoptosis; development of foci of lymphocytes and macrophages infiltration at the site of hepatocyte death, followed by their transformation into lymphocyte-macrophage and/or macrophage granulomas. Steatosis was much more developed than in the young animals with DM. Perivascular fibrosis was predominantly focal.

Destructive changes in mitochondria of hepatocytes in the form of focal clearing of the matrix, destruction of cristae, formation of myelin bodies and damage of mitochondrial membranes were observed. The disorders of mitochondrial functions in liver at DM was noted by many researchers, both in humans and when modeling this pathology in animals [8, 19-20]. Reduction in the level of oxygen consumption, oxidative phosphorylation and synthesis of ATP in mitochondria at DM is a reflection and consequence of damage to their ultrastructure. It can be assumed that functional violations (as well as structural ones) of the energy producing system of liver cells at DM manifest themselves more in older age periods.

In the young animals exposed to the PEI-pDNA complex, complete preservation of the liver structure of the, minor dystrophic changes in hepatocytes in the form of edema and vacuolization of their cytoplasm were noted. At the same time, there were regenerative changes aimed at maintaining tissue homeostasis, which was violated by streptozotocin. Such rearrangements were manifested by an increase in the number of binuclear hepatocytes, hyperplasia of the granular endoplasmic reticulum, and an increase in the mitotic activity of hepatocytes.

Electron microscopy revealed less intensity of destructive changes in hepatocytes and capillaries. At the same time, structural signs of high functional activity were found in hepatocytes, manifested by hypertrophy of the cytoplasm, nuclei and nucleoli, an increase in convolution of the nuclear membrane, hyperplasia of mitochondria, mainly by condensation,



◀ Fig. 5. Microphotographs of kidney sections of old mice in group DM+GT: A – infiltration of stroma by lymphocytes, stromal sclerosis; B – focal glomerular sclerosis; C – focal enlargement and plethora of glomerular capillaries; D – perivascular foci of lymphoid infiltration, glomerular sclerosis. Hematoxylin and eosin staining, light microscopy.

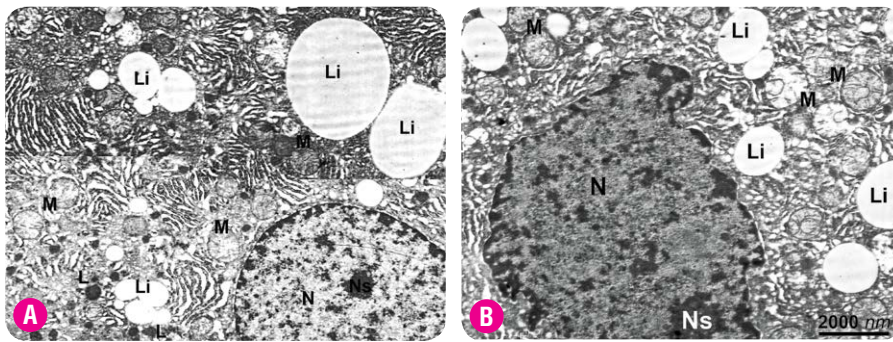
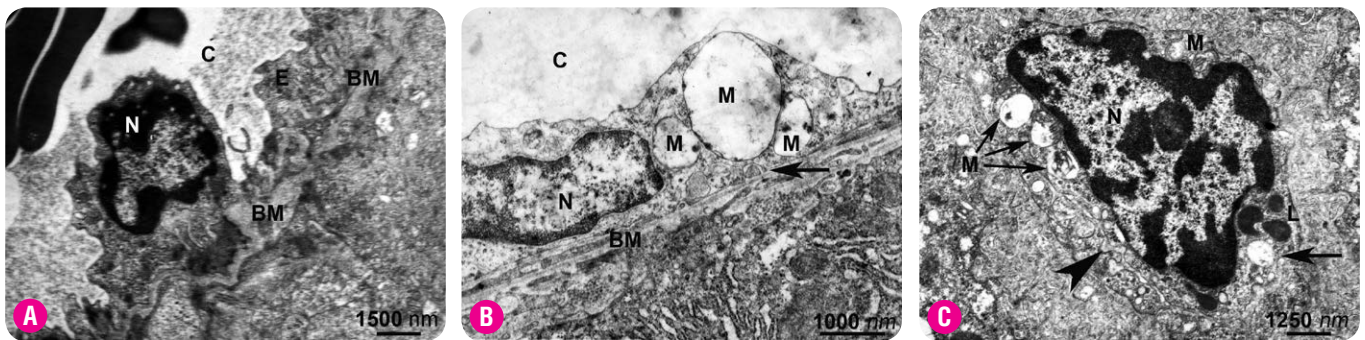


Fig. 7. Merged microphotographs of liver sections of old mice in group DM (A) and DM+GT (B, C). Ultrastructural changes in capillaries: A – heterochromatization of the nucleus, focal changes in the thickness of the basal membrane; B – edema and swelling of mitochondria with destruction of cristae, an increase in the number and polymorphism of microvesicles in the basal cytoplasm with the formation of multivesicular bodies, focal expansion and compaction of basal membrane; C – a closed capillary with a narrowed lumen (▶), heterochromatization of the nucleus and destruction of mitochondria with the formation of vesicles (↑) and myelin bodies (→). Electron microscopy.

Notes: BM – basal membrane, M – mitochondrion, L – lysosome, C – capillary vessel, E – endothelial cell, N – nucleus.

Fig. 6. Merged microphotographs of liver sections of old mice in group DM+GT. Ultrastructural changes in hepatocytes: A – cytoplasm compaction due to the arrangement of the organelles, expansion of granular endoplasmic reticulum, clearing of the mitochondrial matrix, disorganization and partial destruction of the cristae, accumulation of large lipid inclusions and lysosomes in the cytoplasm, compaction of nucleoli in the nucleus; B – hypertrophy of the nucleus, convolution of nuclear membrane and compaction of the karyoplasm with chromatin hypercondensation, an increase in the number of dystrophic organelles and lipid inclusions in the cytoplasm. Electron microscopy.

Notes: Li – lipid inclusion, L – lysosome, M – mitochondrion, N – nucleus, Ns – nucleolus.



and «young» organelles closely connected with numerous granular endoplasmic reticulum. In addition, the positive effect of the PEI-pDNA complex at STZ-induced DM on the liver structure of the young animals was characterized by a marked decrease in the frequency of monocellular necrosis and hepatocytes apoptosis.

In the liver of the old mice with STZ-induced DM exposed to the PEI-pDNA complex, the development of dystrophic changes of varying intensity were at the ultrastructural level (Fig. 6, 7). There was a significant condensation of the hepatocytes cytoplasm, with a marked decrease in the number of glycogen granules. A sharp increase in the number of lipid inclusions was noted, which indicates the development of fatty degeneration, expansion of the granular endoplasmic reticulum, and a reduction in the number of ribosomes bound to endoplasmic reticulum. Attention was drawn to the pronounced destruction of mitochondria with disorientation, and often degradation of the cristae and clearing of their matrix.

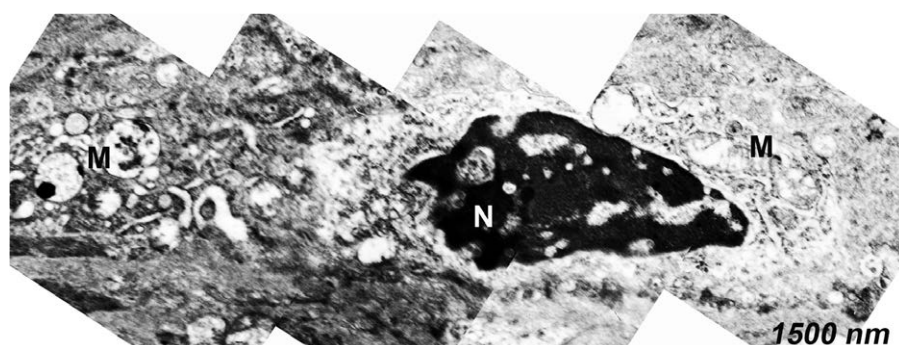
Blood capillaries of the liver at DM after gene therapy also underwent severe ultrastructural changes (mostly in the old mice). In their endothelium, heterochromatization and changes in the shape of the nucleus, destruction of organelles, and formation of numerous vacuoles and vesicles were often detected. The lumen of the capillaries was distinctly narrowed, which led to the formation of closed capillaries. Attention was drawn to the considerable thickening of the basement membrane of the capillaries, its reduplication and development of paravasal fibrosis (Fig. 6).

Stellate macrophages (Kupffer cells) also suffered destructive changes, the heterochromatization of the nucleus and other changes characteristic for the development of apoptosis were often observed at the ultrastructural level (Fig. 8).

The use of TUNEL assay showed a sharp increase in the intensity of apoptosis in the liver of mice with STZ-induced DM (in hepatocytes, capillary endotheliocytes, Kupffer cells, and lymphocytes in perivascular infiltration sites) and a marked decrease in the effects of the PEI-pDNA complex, which indicates its pronounced protective effect. The introduction of the plasmid complex in young animals at diabetes mellitus led to a significant decrease in the apoptosis index (median of AI was 11.62 TUNEL+ cells/mm² in DM group, 6.10 TUNEL+ cells/mm² – in group DM+GT), but in the old animals, this decrease was less pronounced (10,15 TUNEL+ cells/mm² in DM group, 8,96 TUNEL+ cells/mm² – in group DM+GT, Table 1). Thus, the protective effect of gene therapy essentially depends on the age of experimental animals and is determined not only by the intensity of pathological processes at different ages, but also by the range of reparative reactions.

So, as our study has shown, the normalization of the structural and ultrastructural organization of the many organs that are most susceptible to diabetic dysfunction, the basic morphometric parameters of the pancreatic islets and morpho-functional features of β-cells, as well as the apoptosis index in the studied organs (combined with an improvement in glucose concentration in the blood) due to the application of gene therapy by PEI-pDNA complex containing the human preproinsulin gene with indicates the effectiveness of correcting of experimental diabetes in young animals. In addition, the use of gene therapy stimulated the development of compensatory hyperplasia in the myocardium and liver, which is an important indicator of its protective effect in the young animals.

Compared with the young animals, in the old ones, there was a sharp increase in destructive processes in the studied organs at DM modeling,



◀ Fig. 8. Merged microphotographs of liver sections of old mice in group DM+GT: apoptosis of stellate macrophage. Electron microscopy.

Notes: M – mitochondrion, N – nucleus.

which was combined with a dramatic increase in apoptosis. The application of gene therapy in the old animals with DM led to the development of pronounced morphological heterogeneity of tissues, which is also one of the characteristic features of structural manifestations of aging [11]. There is also a slight improvement in the structure and ultrastructure of the cells that make up the basic functional unit of organs and their microvessels, as well as the impairment of many parameters of their structural and ultrastructural organization, the progression of lymphocyte infiltration in the kidney and myocardium, the persistence of high intensity of apoptotic cell death or its amplification in pancreatic islets, the development of complications (acute insulinitis).

The obtained results testify to the significant role of the age factor in the pathomorphism of diabetes and in the development of new approaches for its correction. The age aspect of this problem acquires special significance not only due to a sharp increase in the prevalence of this pathology with age, but also because a number of manifestations of diabetes, in terms of their morphological characteristics, resemble the age changes typical for normal physiological aging [11, 17]. Evidently, the pre-existing

age-related morpho-functional changes in the organs (in which complications occur most often at DM) contribute to the development of deeper diabetic lesions, which is confirmed by the data obtained by us.

One of the possible causes of the observed age differences may be pre-existing age-related changes in the pancreas, which determine the developmental nature of both the pathological process at DM and the reactive changes in response to correction in old age. Despite the existence of contradictory data, the viewpoints about the worsening of β -cells function, the decrease in their proliferative activity and the increase in the level of apoptosis in aging predominate in the literature [21-23]. At the same time, numerous studies performed in animal models, as well as in patients with DM, associated with the study of the mechanisms of injury, death, regeneration and adaptation of β -cells, indicate that they have greater plasticity than previously thought, which allows us to consider their prospective target for finding new treatment of DM [14, 23-24]. Conducting similar studies is appropriate for older age groups, since they can help to clarify the characteristic features of corrective effects at this disease in old age.

CONCLUSION

Our data obtained by histological, electron microscopy, histochemical and immunohistochemical, as well as morphometric studies suggest that the corrective effects of gene therapy with PEI-pDNA complex containing the human preproinsulin gene on the morpho-functional characteristics of organs at streptozotocin-induced diabetes mellitus show different efficacy in different ages.

A high effectiveness of gene therapy in the young animals and a slight effect, its absence, or an additional deterioration in the old ones that manifested itself differently in different tissues, cells and microvessels was noted.

The protective effect of gene therapy directly depends on the age of the experimental animals, which determines not only the features and character of the development of the pathological process, but also the level and range of the reparative reactions. The results of the study indicate the need to take into account the age factor while developing a strategy for diabetes mellitus treatment.

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