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Prospects for the use of exenatide in the correction of experimental diabetic cardiomyopathies

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Abstract
The purpose of this study is to shine a light on the structural changes of atrial cardiomyocytes of rats with streptozotocin-induced diabetes mellitus (DM) and the course of its treatment. DM was modeled by a single intraperitoneal administration of streptozotocin (diluted in 0.1 M of citrate buffer with the pH of 4.5 at a dose of 6 mg per 100 grams of body mass). Antidiabetic therapy was performed with exenatide starting from the 14th day of the experiment. It is established that daily exenatide injections lead to a good glycemic control only at the initial stage of the experiment. However, its use as a monotherapy is not justified for correction of diabetes due to poor glycemic control and levels of glycosylated hemoglobin during later terms of the experiment. Using exenatide in experiment, we did not observe the repair of the structure of atrial cardiomyocytes in the late stages of experimental streptozotocin-induced DM.

Keywords: Diabetes mellitus, myocardium, rats, exenatide

Introduction
DM remains one of the most pressing problems of modern medicine [1]. According to official statistics nowadays in Ukraine about 1.2 million patients with DM are registered. According to data from the World Health Organization and the International Diabetic Federation, the number of patients with diabetes in the world among the adult population aged 20-79 in 2015 is 415 million, and by 2040, the predicted number of patients is 642 million, which will cover 10% of the Earth's population [2].

Patients with type 1 DM usually have a 3-5 times higher risk in developing cardiovascular diseases and death than the general population. Obesity, dyslipidemia, insulin resistance, hyperglycemia, and DM are powerful independent risk factors for heart events [3].

For several decades, scientists around the world have been investigating the endocrine function of the heart [4, 5].

Many researchers have been studying the level of atrial natriuretic peptide (ANP) in hypertension, heart failure, pulmonary hypertension, and DM. Some researchers identified the relationship between the degree of granulation of secretory atrial cardiomyocytes and the level of ANP in the blood of patients with DM [6].

Research results point to the importance of studying the secretory function of the heart. In particular, Wang TJ *et al*. showed elevated levels of ANP in patients with heart failure, atrial fibrillation, and myocardial infarction. Even in the absence of symptoms in patients with heart failure, scientists associate elevated levels of natriuretic peptides with high risk of cardiovascular events in the future [7].

ANP is a hormone that plays an important role in regulating the volume of circulating blood and vascular tone. The level of the hormone in blood plasma increases with heart failure. One of the complications of DM is diabetic cardiomyopathy, which in turn contributes to the development of heart failure.

Despite the significantly higher risk of developing cardiovascular disease in patients with DM, modern methods of treatment have proven their effectiveness in reducing the cardiovascular morbidity and mortality [8].

To reduce cardiovascular disease and mortality in patients with type 2 DM, it is necessary, above all, to achieve glycemic control. Under conditions of insulin deficiency and chronic hyperglycemia, lipid peroxidation (LP) is intensified. Excessive production of LP products has a damaging effect on the cell surface, destabilizes membranes and promotes cellular degradation [9].

Exenatide is a hypoglycemic agent and is used for treatment of type 2 DM. Exenatide is the first drug belonging to an entirely new class of drugs for the treatment of DM type 2 – incretin mimetics [10].

Data from clinical studies indicate that exenatide therapy provides a significant improvement in glycemic control in patients with type 2 DM. Exenatide is a potent stimulant of incretin (glucagon-like peptide-1), which increases glucose-dependent insulin secretion and has other hypoglycemic effects of incretines (suppression of inadequately increased glucagon secretion and improving the function of B-cells).

Data from many clinical studies indicate that exenatide therapy provides a significant improvement in glycemic control in patients with type 2 DM.

According to the updated recommendations of the American Diabetes Association (ADA), it is emphasized that patients with type 2 DM and with atherosclerotic cardiovascular disease who have not achieved glycemic goals through lifestyle modification and metformin should consider the use of a drug that reduces the risk of developing serious cardiovascular events and cardiovascular death [11].

A number of authors have proven that exenatide significantly increases the 1st and the 2nd phases of insulin secretion in hyperglycemia, contributes to the reduction patients' body mass, reduces the level of triglycerides, low density lipoproteins, as well as diastolic pressure and increases the level of high-density lipoprotein in patients with 2nd type DM [12, 13]. However, there is no information about the influence of this drug on endocrine apparatus of the heart.

The purpose of our study was to establish on an electron microscopic level the peculiarities of the morphological rearrangement of cardiomyocytes and the endocrine system of the heart in experimental DM and its correction with exenatide while taking into account that exenatide prevents the development of cardiovascular complications in DM and the urgency of the problem of correction of diabetes.

Materials and Methods

Pieces of atria and auricles of the heart of 10 white male Wistar rats (weighing 180-220 g) were used as a material for the study. Animals were divided into 3 groups: 1 - intact rats (3 animals), 2 - 4 animals with streptozotocine-induced DM, 3 - 9 animals with streptozotocin-induced DM receiving antidiabetic therapy. Experimental diabetes mellitus (EDM) in animals of 2nd and 3rd groups was induced by a single intraperitoneal administration of streptozotocin (dissolved in 0.1 M citrate buffer solution with the pH of 4.5) at a dose of 6 mg per 100 grams of body mass. Animals of the 3rd group, began receiving the antidiabetic therapy from the 14th day of diabetes development: 3rd group (3 animals) was receiving exenatide ("Baetta", Eli Lilli, USA) at a dose of 0.04 μg/100g of body mass/day subcutaneously in the morning 30 minutes before feeding. Euthanasia of animals was carried out under thiopental anesthesia by means of decapitation followed by blood collection in a test tube for biochemical studies. The development of EDM was controlled by the daily determination of blood glucose, which was measured from a drop of blood of the caudal vein using test strips at the "Accu Shec" glucometer (Germany). Material was taken on the 28th and 56th day of the experiment. Histological and electron microscopic research methods were used. The volume density (Vi = Pi / Pt x100%, where Vi – stands for volume density, Pi – the number of points within the studied object, Pt – total number of points of the test-system) of secretory granules (SG), the area of cardiomyocytes and their nuclei, nucleocytoplasmic index (NCI) (the ratio of nuclear profile area to cytoplasmic area) were determined.

Morphometry was performed on the specimens in NIH USA "Image J" software as manual operation taking into account magnifications. Computer data processing was carried out using the statistical package Stat. Soft. Inc; Tulsa, OK, USA; Statistica 6.

Results of the investigation

On the 28th day of EDM blood glucose level reached 15,42±1,34 mmol/l (control - 5,12±1,0 mmol/l, $p<0,001$), level of HbA_{1c} – (9,01±0,26)%, (control – 2,36±0,98%, $p<0,001$). At the histological examination of atrial cardiomyocytes, there were contractions of some myofibrils, moderate stromal edema, deformation of individual muscle fibers. Separate muscle fibers were fragmented. The nuclei of cardiomyocytes had irregular shape.

In secretory atrial cardiomyocytes using electron microscopy the enlightenment of sarcoplasm, edema of myofibrils and myofilaments were detected. Sarcolemma was loose and in some places it formed invaginations inside the cell. Sarcoplasm of secretory atrial cardiomyocytes had a moderate electron-optical density. Throughout the sarcoplasm, especially in the subsarcolemmal space, we have found mitochondria with an enlightened matrix and collapse of the crests. Locally we observed lysis of myofibrils, edema of nuclei, grouping of heterochromatin into the breasts and its condensation along the inner surface of the nuclear shell. The perinuclear space was extended, the cariolemma formed finger-like protuberances. Near the nucleus a hypertrophied Golgi complex was located. Granular endoplasmic net cisterns were expanded with uneven contours. Ribosomes were freely located in the cytoplasm, sometimes forming polysomes.

The volume density of SG in the right and left auricles was significantly increased, compared with the control group. In the right auricle, we observed a significant increase in volume density of young and diffusing SG. Young SG are characterized with matrix of high electron density, which is surrounded by membrane with a light rim underneath it. The contours of diffusing SG are blurred, the outer membrane is absent. In the left auricle, the volume density of the 3rd type of SG was significantly increased (table 1).

On the 56th day of EDM, the animals in group 2, had their glucose and HbA_{1c} levels increase to 18.21±0.22 mmol/l (control 5.31±0.23 mmol/l, $p<0.001$) and 9,31±0.25% (control 2.32±0.09%). At the ultrastructural level, along with the manifestations of diabetic microangiopathy, we have been observing a deepening of myocardial degenerative processes, and changes in atrial cardiomyocytes. Furthermore, we detected the disappearance of glycogen grains, the appearance of droplets of fat, swelling of nuclei of cardiomyocytes. Contour of cariolemma was uneven, with finger-shaped bulging. On the surface of the cisterns of the granular endoplasmic net, the amount of ribosomes was reduced. Sarcoplasm of typical cardiomyocytes was lightened, contained small vacuoles. The electron microscopic density of the mitochondrial matrix was reduced, their crests were disintegrated and partially destroyed (Figure 1a). Some cardiomyocytes were smaller in size, wrinkled, contained hypochromic nuclei.

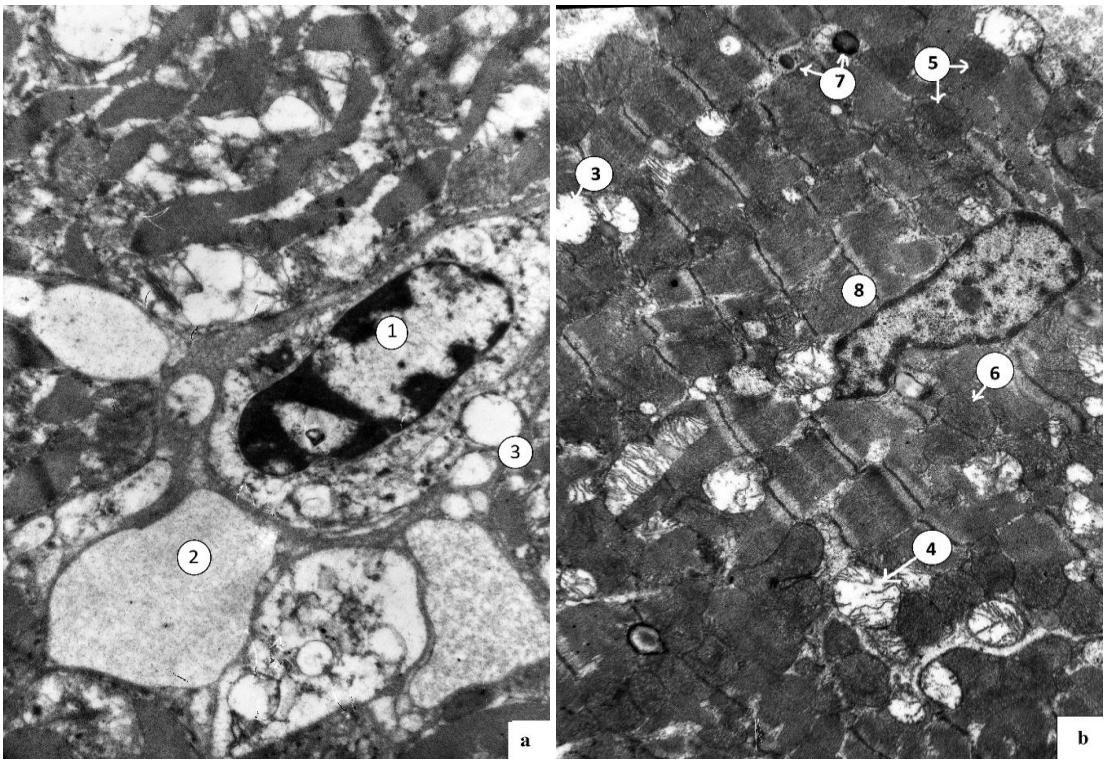
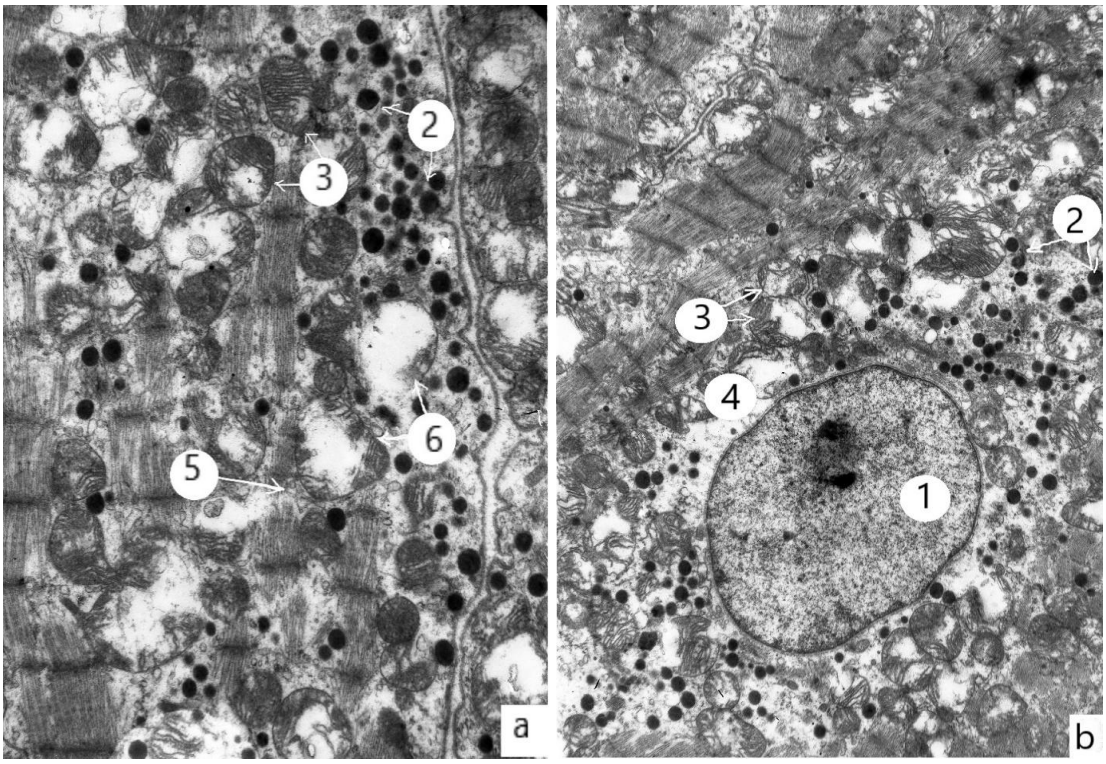


Fig 1: Submicroscopic changes of cardiomyocytes of the left atrium on the 56th day of EDM (a) and it's correction with exenatide (b). Magnification: a) 6400, b) 4800. 1-nucleus of apoptotic changed cardiomyocyte, 2- hydroponic dystrophy of cardiomyocyte, 3- vacuated dystrophy of cardiomyocyte, 4- destructively changed mitochondria, 5- young mitochondria, 6- mature mitochondria, 7- lysosomes, 8- sarcomere.

At this time of the experiment, we detected the phenomena of inflammatory infiltration of the myocardium. The dystrophic changes in myocardium were characterized by the presence of atrophied cells with the phenomena of karyopyknosis, the nuclei of many cells were deformed. There was an excessive growth of the connective tissue between the cardiomyocytes.

We detected the alteration of secretory atrial cardiomyocytes: reduction of volume density of SG compared with the previous term of study, yet noticeable progressive increase in volume density of diffusing SG (table 1). Most of the granules were subsarcollemal, among them mature and diffusing granules were predominant (Figure 2a). In some secretory atrial cardiomyocytes, we found hyperplasia of the structural components of the Golgi apparatus, granular endoplasmic reticulum.



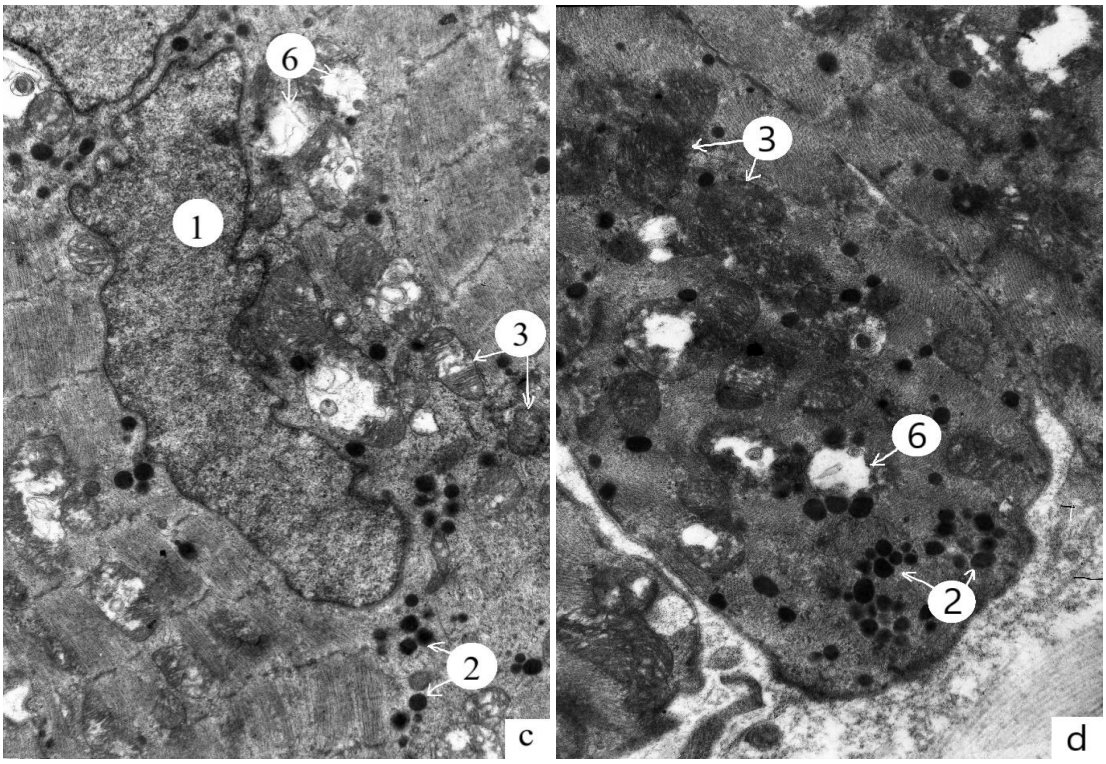


Fig 2: Ultrastructural rearrangement of secretory atrial cardiomyocytes in EDM (a, b) and its correction with exenatide (c, d). Magnification: a) 6400, b) 4800, c) 6400, d) 8000. 1-nucleus, 2-secretory granules, 3-mitochondria, 4- paranuclear edema, 5- lysis of myofibrils, 6- vacuoles.

On the 28th day of the experiment, the 3rd group of animals that were treated with exenatide for 14 days, had their blood glucose levels decreased significantly to 9.23±1.02 mmol/l (p<0.01) compared with with the 2nd group of animals, but such indicators are significantly higher than the intact group of animals (p<0.05). In animals of group 3, the level of HbA_{1c} decreased to 6.23±1.31% (p<0.05), however, it remained higher than the intact group of animals (p<0.001).

After 14 days of treatment in 2nd and 3rd groups of animals there was no significant difference between morphometric parameters of the left and right atrial cardiomyocytes (in each case p<0,05, see table 2), though there was a significant increase of nuclei of cardiomyocytes of the left atria. Between 1st and 2nd groups of animals there was no significant difference between morphometric parameters of atrial cardiomyocytes as well (table 2). So, according to morphometric data, monotherapy with exenatide contributes to the restoration of quantitative parameters of atrial cardiomyocytes, which was confirmed with histological and ultrastructural examination.

At the 2nd week of treatment in the 3rd group of animals at the ultrastructure level, there were intracellular regenerative processes characterized by hyperplasia of the granulosa endoplasmic net, the appearance of young mitochondria in cytoplasm, whose crests were clearly differentiated. Along with this we also encountered mitochondria with destructive changes. Cardiomyocytes were visualized in all animals of group 3, which didn't differ from those in intact animals by ultrastructural organization. We detected cardiomyocytes with a large amount of SG with an electron-bearing matrix. Volume density of SG in secretory atrial cardiomyocytes decreased in comparison with animals of the 2nd group, but remained significantly higher than the intact group of animals (table 1).

Table 1: Volume density of SG in different parts of atria in EDM and its correction with exenatide

Section of myocardium	Types of SG	1st group (intact rats)	28th day		56th day	
			2nd group	3rd group	2nd group	3rd group
Right auricle	1st	1,79±0,09	3,74±0,12*	2,16±0,14	2,68±0,12*	2,81±0,18
	2nd	3,56±0,18	4,98±0,17*	4,12±0,12	3,36±1,12	4,18±0,12
	3rd	3,09±0,19	5,46±0,21*	3,88±1,16	5,98±0,18*	4,44±0,28
	All SG	8,44±0,15	14,18±0,16	10,16±0,47	12,02±0.47*	11,43±0,19
Left auricle	1st	0,94±0,08	1,02±0,08	0,98±0,06	0,6±0,08	1,21±0,06
	2nd	1,25±0,09	1,23±0,11	1,36±0,09	1,42±0,06	1,51±0,09
	3rd	1,88±0,12	3,25±1,14*	2,16±0,11	2,96±0,09*	2,71±0,19
	All SG	4,07±0,09	5,5±0,44*	4,5±0,09	4,98±0,08*	5,43±1,81

Note: 1)*p<0,05 – a significant difference between 1st and 2nd group of animals; 2) #p<0,05 – a significant difference between 2nd and 3rd groups of animals; 3) ^p<0,05 – a significant difference between 1st and 3rd groups of animals.

Table 2: Morphometric characteristic of cardiomyocytes in experimental DM and its correction

Section of myo-cardium	Indicator	1st group (intact rats)	28th day		56th day	
			2nd group	3rd group	2nd group	3rd group
Right atrium	Area of cells (μm 2)	166,85±17,77	156,78±10,06*	164,44±4,94	145,42±10,15*	149,59±7,97*,#,^
	Area of nucleus (μm 2)	11,98±1,23	11,19±0,96*	11,72±0,75	11,17±0,88*	13,41±1,20*,#,^
	NCI	0,08±0,006	0,08±0,006	0,08±0,005	0,08±0,007*	0,1±0,009*,#,^
Left atrium	Area of cells (μm 2)	166,29±10,85	160,74±12,06*	165,32±6,30	144,77±11,32*	151,19±6,44*,#,^
	Area of nucleus (μm 2)	12,03±1,27	11,17±0,89*	11,86±1,09*,#	11,26±0,79*	10,23±0,70*,#,^
	NCI	0,07±0,008	0,08±0,008*	0,08±0,006	0,08±0,007*	0,07±0,005*,#,^

Note: 1)*p<0, 05 – a significant difference between 1st and 2nd group of animals; 2) #p<0, 05 – a significant difference between 2nd and 3rd groups of animals; 3) ^p<0, 05 – a significant difference between 1st and 3rd groups of animals.

After 42 days from the beginning of the EDM correction in animals of group 3, glucose and HbA_{1c} levels in the blood were significantly lower than in the 2nd group of animals and accordingly amounted to 10.12±0.83 mmol/l (p<0.05) and 6,84±0.69% mmol/l (p<0.05). Such indications are significantly lower than those with streptozotocin DM which didn't get treatment (p<0.01), did not differ significantly from the previous experiment period (in each case p>0,05), and significantly higher than intact group (p<0.01).

After 42 days of treatment on the 56th day of EDM the area of atrial cardiomyocytes was significantly smaller in comparison with intact animals, though - significantly bigger in comparison with 2nd group of animals (see table 2). The area of cardiomyocytes' nuclei in the right atrium has significantly increased in comparison with 1st and 2nd animal groups, in return, in the left atrium the area of nuclei has significantly decreased in comparison with 1st and 2nd groups. Such morphometric changes of cardiomyocytes in the 3rd group have led to a significant increase of NCI in the right atrium, in comparison with 1st and 2nd groups of animals. However, in the left atrium this indicator has significantly decreased.

On the 42nd day of treatment in animals of the 3rd group, the values of the volume density of the granules decreased in comparison with the 2nd group of animals, but were greater than control. There was a decrease in the number of diffusing granules. The cytoplasm of secretory atrial cardiomyocytes contained a large number of vacuoles of various sizes and in the form of cylinders. We encountered mitochondria of the usual structure and with partially destroyed crests. Along with destructively altered cells, we find single cells with pronounced regenerative changes (Fig. 2d).

Discussion

We, like other authors [14], have established that with an increase in the duration of experimental DM, the area of cardiomyocytes decreases. This happens due to several reasons. In the early stages (14 days of EDM), the area of cardiomyocytes decreases due to metabolic changes, in particular the decrease in the amount of glycogen, which was observed by other authors in skeletal muscles [15]. In the long term of the experiment (56 days), we found diabetic microangiopathy of the myocardium, which leads to hypoxia of cardiomyocytes, and as a consequence, to their atrophic changes. Other authors [16, 17] at the EDM point to the development of diabetic myopathy of skeletal muscles, which is characterized by a decrease in the area of muscle fibers and destructive changes in neuromuscular compounds against a background of diabetic microangiopathy.

According to our research, hypoxia and metabolic changes in cardiomyocytes under EDM result in the rearrangement of their endocrine apparatus, which is confirmed by changes in the density of the various types of SG. Taking into account that in EDM the volume density of the 3rd type of SG is significantly increased, we can talk about the predominance of the processes of allocation of ANP from the cell over its synthesis.

The changes we discovered may indicate a development of heart failure and are confirmed by the data of other authors [18], who observed elevated levels of ANP in patients with diabetes mellitus, heart failure or even an increased risk of development of heart failure without clinical signs of the cardiovascular system disease [19, 20]. Choosing a hypoglycemic agent, we were guided by the data of literature sources, in which the cardiovascular effects of incretin mimetics were studied, in particular, exenatide, which was used to treat diabetes.

When choosing a hypoglycemic agent, according to ADA, specific factors need to be taken into account. Comparing agents from different groups, ADA analyzes the efficacy of GPP-1 receptor antagonists, in particular, exenatide. We are talking about high efficiency of the drugs, the absence of cases of hypoglycemia, weight loss, but neutral cardiovascular effects and the impossibility of application (exenatide) with GFR <30 ml / min / 1.73 m2. Exenatide was studied in the EXSCEL study, which involved more than 14,000 patients with type 2 diabetes. The good news was that exenatide does not increase the incidence of cardiovascular complications, including hospitalization due to heart failure, and is not associated with the risk of developing pancreatic cancer and pancreatitis. But, unfortunately, this agonist of GPP-1 has not yet demonstrated the benefits of reducing the risk of adverse cardiovascular events, which was previously shown by liraglutide [21].

In several large clinical studies, scientists compared the effect of insulin and exenatide therapy [22]. In one of these studies, patients with type 2 DM were treated with exenatide instead of insulin, their glycemic control was worse for 38% [23].

Exenatide monotherapy leads to an improvement in the glycemic profile of animals with EDM. However, glucose levels remain higher than in the intact group of animals. The therapy we applied for 14 days has led to a partial restoration of the structural components of cardiomyocytes and secretory atrial cardiomyocytes. However, monotherapy with exenatide for 42 days due to hyperglycemia has led to the development of diabetic microangiopathy and dystrophic changes in cardiomyocytes. Therefore, according to our studies, the use of exenatide for EDM correction is promising only in combination with other antidiabetic drugs.

Conclusions

The use of exenatide in the early stages of the development of an EDM leads to a partial normalization of carbohydrate metabolism. However, the long-term use of exenatide as monotherapy is inadequate, as this drug is not able to support target glucose and glycosylated hemoglobin levels, which significantly increase at the 42nd day of treatment, compared

with the 28th day. This leads to the progression of the destructive processes in cardiomyocytes and secretory atrial cardiomyocytes by type of vacuolar and hydrotopic dystrophy.

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