Chapter 9

Pancreatic Islet Beta-Cell Apoptosis in Experimental Diabetes Mellitus

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Additional information is available at the end of the chapter

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1. Introduction

For screening and detailed studying of antidiabetic medications, various genetic and non-genetic experimental models of diabetes mellitus were used (Islam S., Loots D.T. 2009). And though they are not absolutely equivalent to etiopathogenetic mechanisms of human pathological conditions, each of them represents itself as an integral tool for research into genetic, endocrine, metabolic, morphological changes of this disease (Sarvilina I.V., Maclakov Y.S. 2008).

The most commonly used diabetic experimental models are non-genetic models that use hydrophilic β-cell glucose analogues, such as alloxan, streptozotocin, chlorozotocin, cyproheptadine, etc. The common mechanism of action of these substance includes degradation of pancreatic islet β-cells by means of: 1) generation of oxygen free radicals that destroy the integrity of a cell, 2) alkylation of DNA and subsequent activation of poly-ADP-ribose-synthetase - reduction of NAD to β-cell, and 3) inhibition of active transport of calcium and calmodulin-activated protein kinase (Rees D.A., Alcolado J.C. 2005). In this type of experimental models of diabetes mellitus streptozotocin (an N-nitrosourea derivative of glucosamine) is most commonly used (McNeill J.H. 1999). Depending on cytotoxin dosage used in the experiment (45-70 mg/kg) and route of administration (i.p., i.v.), it is possible to model and simulate different states of carbohydrate metabolism based on a specific clinical type of diabetes mellitus (DM mixed (type 1-2) latent or «hidden» diabetes) (Srinivasan K. et al. 2007). Although diabetes mellitus usually has obvious clinical symptoms such as hyperglycemia, glucosuria, polyuria, polydipsia, severe weight loss, it is difficult to measure the contribution of each of the links to the pathogenesis of diabetes and to assess the extent of pancreatic islet β-cell damage and death. The toxic effect of alloxan and streptozotocin on cells in pancreatic islets manifests itself not only by necrosis but also by apoptosis of pancreatic islet β-cells (Daisy Mythili M., et al. 2004). The study of apoptotic mechanisms

will enable us to identify specific targets for purposeful creation and development of anti-diabetic medications.

2. Materials and methods

The experiments were performed on 180 adult male Wistar rats weighing from 280.0 to 300.0 g (Table 1). The animals were kept under standard vivarium conditions at pharmacology department of Volgograd Medical University, and were provided with a nutritionally balanced diet that the laboratory animals consume consistently. A pilot study was approved by the Central Regional Independent Ethics Committee (protocol № 1-06; № 43-2006; № 70-2008; № 89-2009), and was performed in accordance with GLP when conducting preclinical studies in Russia. All animal experimentation was carried out in compliance with the International Guidelines of the European Convention for the Protection of Vertebrate Animals used in experimental studies (1997).

Group	Day of experiment	Amount of animals
Intact control	3, 7, 14, 28	40
Alloxan-induced diabetes	3, 7, 14, 28	40
Streptozotocin-induced diabetes	7, 28	20
Immune-dependent diabetes	3, 7, 14, 28	40
Streptozotocin-nicotinamide-induced diabetes	3, 7, 14, 28	40

Table 1. Group distribution of experimental animals

Plasma glucose, insulin and C-peptide concentrations were measured in experimental laboratory animals. The blood glucose was determined in samples obtained from the tail vein of rats by enzymatic method, using "Glucose FKD" assay kits (Russia) on SF-46 spectrophotometer at λ=450 nm in 10 mm cuvettes. Animals with blood glucose levels >15 mmol/L were enrolled in the experiment (Akbarzadeh A. et al. 2007). Plasma insulin and C-peptide concentrations were determined on an automated enzyme immunoassay «SUNRISE» analyzer (TECAN, Austria), using DRG Insulin ElisaKit and DRG C-peptide ElisaKit.

Alloxan-induced diabetes simulation model was developed by means of intraperitoneal administration of alloxan at a dose of 120 mg/kg. Tissue samples of animals were collected and submitted for routine histopathological investigation at 3, 7, 14 and 28 days of the experiment.

Experimental streptozotocin-induced diabetes simulation model was developed using streptozotocin (Sigma) (45 mg/kg, intravenously once a day) (Baranov V.G. 1983). Tissue samples were collected and submitted for routine histopathological investigation at 7 and 28 days of the experiment.

Experimental immune-dependent diabetes simulation model was developed by giving a subcutaneous injection of 0.2 ml of complete Freund's adjuvant (CFA) (Grand Island Biological Company, USA). Subsequently, daily intravenous injections of streptozotocin (Sigma, USA) (20 mg/kg) were given to the animals for 5 days, resulting in the development of insulin-dependent diabetes (Ziegler B. 1990). Tissue samples were collected and submitted for routine histopathological investigation at 3, 7, 14 and 28 days of the experiment.

Streptozotocin-nicotinamide-induced diabetes model was developed by giving an injection of streptozotocin (Sigma, USA) (intraperitoneally - 65 mg/kg citrate buffer, pH = 4.5) with a preliminary (15 minutes prior to the procedure) administration of nicotinamide (intraperitoneally - 230 mg/kg prepared in 0.9 % solution of sodium chloride) (Islam S., et al. 2009). Tissue samples were collected and submitted for routine histopathological investigation at 3, 7, 14 and 28 days of the experiment.

Pancreatic tissue was divided into three segments including intestinal, gastric and splenic parts, and then they were fixed in 10% solution of neutral buffered formalin (pH 7.4) for 24 hours. 5-6-mm thick slices were obtained on rotary microtomes and were mounted on slides. Tissue sections were stained with hematoxylin and eosin using standard histological stain techniques (Korzhevsky D.E. 2005).

For detection of α-and β- endocrine cells in the islets of Langerhans the primary antibodies against insulin and glucagon were used (Table 2). To study apoptosis, the primary antibodies to proteins, such as caspase 3, TRAIL (TNF-related apoptosis-inducing ligand), MDM2, Bcl 2, p53, Bax, NF-kB, as well as eNOS were used.

№	Antibody	Clone	Manufacturer
1	Insulin	Polyclonal	DakoCytomation, Denmark
		Ab-6 (INS04 + INS05)	LabVision, UK
		2D11-H5	Novocastra, UK
2	Glucagon	Polyclonal	Novocastra, UK
3	Caspase 3	JHM62	Novocastra, UK
		Rb-1197-P0	NeoMarkers, USA
4	TRAIL	27B12	Novocastra, UK
5	MDM2	1B10	Novocastra, UK
6	Bcl 2	sc-7382	Santa Cruz Biotechnology, USA
		124	Dako Cytomation, Denmark
7	p53	Polyclonal	Dako Cytomation, Denmark
		Ab-1 (Pab 240) MS-104-P0	NeoMarkers, USA
8	Bax	Polyclonal	BD Biosciences Pharmingen, USA
9	NOS-3	RN5	Novocastra, UK
10	NF-kB	Polyclonal	Diagnostic BioSystems, USA

Table 2. IHC Primary Antibodies

Immunohistochemistry was performed according to manufacturers' protocols using ABC (Novocastra, UK), «UltraVision» (Lab Vision, UK) and «EnVision» (Dako Cytomation, Denmark) antibody detection systems and a chromogen, diaminobenzidine under the protocol on high temperature antigen unmasking technique using «*Pascal*» mini autoclave (Dako Cytomation, Denmark) (Kumar G.L. et al. 2009). The reliability of the obtained results was defined using both positive and negative control antigens, as well as negative control antibodies.

Immunohistochemical reaction was based on visual evaluation, taking into account the intensity of color, or on determination of the specific amount of positively stained cells (Allred DC, et al. 1998).

Photo images were captured with an «AxioScope» microscope (Carl Zeiss, Germany) and a «PowerShot» digital camera (Canon, Japan). Morphometric analysis was performed using "VideoTestMorfo-4" software (Russia). In the course of the study we determined the relationship between the total α-and β- endocrine cell area and the total area of the islet (S,%), between the volume fraction (ML, %) of islets and exocrine glands, as well as the area of β-cell nuclei (S, μm^2). We also measured apoptotic index (AI), i.e. the relative amount of β-endocrine cells with apoptotic structural and immunohistochemical changes.

The research results were processed using basic statistical analysis techniques as well as "Video TestMorfo-4», Excel Microsoft Office (Microsoft, USA) and STATISTICA 6.0 software (StatSoft Inc., USA). The analysis of the parameters for normally-distributed values was performed using Student's t test. Nonparametric statistics was calculated using Mann-Whitney test. To compare qualitative variables, the chi-square test and the Fisher exact test were used. Differences were considered significant if error probability was $p < 0.05$.

3. Results

All animals in the intact control group showed stable plasma biochemical parameters which did not exceed the physiological norms during an observation period (glucose - 4,02 ± 0,1 mmol/L (Fig. 1), insulin - 1,65 ± 0,04 mU/ml., C-peptide - 4,65 ± 0,16 ng/ml.

Morphological study of the intact control group showed that the exocrine part of the pancreas has an alveolar-tubular structure which exhibited a division into lobules separated by interlobular connective tissue. Exocrinotcytes tended to be highly differentiated and they formed acini. Pancreatic islets in all parts of the pancreas were round or slightly oval in shape. They were single or were arranged in clusters around the intralobular excretory ducts. The total volume fraction of islet tissue in the gastric and splenic segments was almost twice as much as the volume fraction of islets of the intestinal segment (Table 3). The total area of nuclear β-endocrine cells in all segments of the pancreas had no statistically significant differences during all periods of observation. Insulin-positive cells were predominantly found in the central part of the pancreatic islets; however, glucagon-positive cells were more frequently encountered at the periphery. In this case there was a statistically significant increase ($p < 0.05$) in β-cell area of the splenic segment compared with the intestinal and gastric

segments of the pancreas (Fig. 2A). The maximum α-endocrine cell area was determined in the intestinal and gastric segments of the pancreas; however, this area was statistically significant lower ($p < 0.05$) in the splenic segment (Table 3). There was a statistically significant ($p \leq 0.05$) direct relationship between the total volume fraction of pancreatic islets and the area of β-endocrine cells depending on their location. The amount of islets and β-endocrine cells tended to increase in the following segments of the pancreas: intestinal →gastric → splenic.

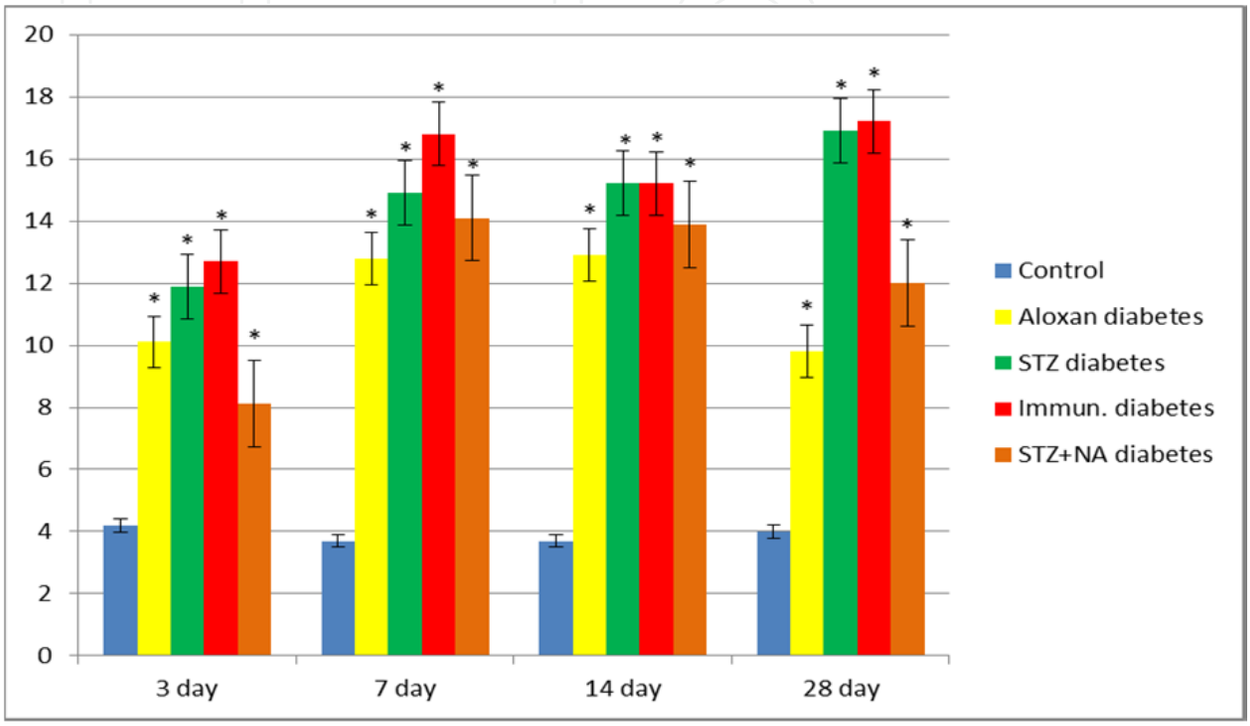


Figure 1. Blood glucose dynamics (mmol/L) in rats with different models of experimental diabetes. * - Significant changes compared with the intact control group.

Indicator	Day of experiment	Segment of the pancreas		
		Intestinal	Gastric	Splenic
Volume fraction of islets,%	3	6,8±2,5	12,6±6,0	13,8±7,3
	7	6,7±2,2	12,0±4,5	12,9±2,1
	14	6,9±3,1	12,1±5,2	14,0±2,9
	28	6,6±4,2	12,8±4,5	14,3±5,2
Total area of β-cells,%	3	51,3±9,2	64,6±9,1	**78,2±6,1****
	7	53,8±7,6	63,4±7,0	**75,4±8,7****
	14	52,1±5,5	64,5±8,7	**79,4±4,6****
	28	54,3±5,4	66,2±3,6	**77,3±4,3****
Total area of α-cells,%	3	37,4±5,2	31,2±3,5	**22,2±3,4****
	7	39,3±4,3	30,4±4,0	**21,3±3,0****
	14	38,5±3,5	32,4±3,2	**19,7±2,3****
	28	37,1±1,0	30,2±1,2	**17,9±1,3****

Indicator	Day of experiment	Segment of the pancreas		
		Intestinal	Gastric	Splenic
Total area of β-cell nuclei, μm²	3	24,2±1,2	24,4±1,0	23,9±0,2
	7	24,3±1,3	23,8±1,2	24,7±1,0
	14	24,3±1,2	24,8±1,3	24,6±1,3
	28	24,4±1,0	24,3±1,2	24,7±2,0

* - Significant difference in the intestinal segment of the pancreas ($p < 0.05$),
** - Significant difference in the gastric segment of the pancreas ($p < 0.05$).

Table 3. Morphometric indicators of pancreatic islets in the intact control group (M ± m).

Bax protein expression was negative in all islet cells. A weak positive cytoplasmic staining of islet cells in the central part of the islet for Bcl-2 protein was reported. In the nuclei of individual β-endocrine cells ambiguous or weak expression of p53 protein was determined. Most of β-islet cells had a positive nuclear staining for MDM2 protein. In some β-endocrine cells cytoplasmic expression of caspase 3 and TRAIL was poorly defined or ambiguous (Table 4) (Fig.3A, 4A). Apoptotic index was low (Table 5).

Group	Day of experiment	Primary antibodies							
		p53	Caspase-3	TRAIL	Bax	Bcl-2	MDM2	NOS-3	NF-kB
Intensity of expression									
Intact control	3	-	+	+	-	+	++	-	++
	7	-	+	+	-	+	++	-	++
	14	-	+	+	-	+	++	-	++
	28	-	+	+	-	+	++	-	++
Amount of cells									
Intact control	3	-	+	+	-	+	+++	-	++
	7	-	+	+	-	+	+++	-	++
	14	-	+	+	-	+	+++	-	++
	28	-	+	+	-	+	+++	-	++

Table 4. Immunohistochemical characteristics of β-cells

Alloxan-induced diabetes

In alloxan-induced diabetes simulation model a significant increase in blood glucose of rats compared with the intact control was reported (Fig. 1).

At day 3 of the experiment a well pronounced interstitial edema associated with hyperemia of blood vessels and capillaries in the acinar tissue of pancreatic islets was determined histologically. Compared with the intact control group, the total volume fraction of the pancreatic islets was not significantly reduced. However, there was a slight decrease in the area of β-cells in all segments of the pancreas (Table 5). The cells with moderate dystrophic

and destructive changes were revealed in the central part of the islets of Langerhans. Immunohistochemical reaction in the central part of the pancreatic islets revealed insulin-positive cells with marked expression of insulin. A slight increase in the size of β-cell nuclei was reported. Compared with the intact control group, the total area of islet cells in all segments of the pancreas was slightly reduced. The total glucagon-positive cell area did not change as compared with the control group (Table 5).

At day 7 the islets were swollen, hyperemic and collapsed. There was no statistically significant reduction in the total volume fraction of islets in all segments of the pancreas. In the central part of the islets we observed moderate necrobiotic changes associated with a significant decrease ($p < 0.05$) in the total β-cell area predominantly in the gastric and splenic segments when compared with the intact control group (Table 5). There was a statistically significant increase ($p < 0.05$) in the area of α-endocrine cells in the gastric and splenic segments of the pancreas. β-cell necrosis was accompanied by moderate leukocyte infiltration of the stroma and acinar cells with only few leukocytes in the islets. The area of nuclear β-islet cells in all segments of the pancreas increased by 4.5 μm^2; however, no statistically significant changes were identified when compared with the intact control group.

Edema significantly reduced by day 14; however, blood vessels of the acinar tissue and capillaries of the islets of Langerhans were still slightly hyperemic. There were focal sclerotic changes in some islets. Compared with the control group, there were not any statistically significant changes in the islet volume fraction in all segments of the pancreas. Insulin-positive cells tended to be located in a random pattern; however, glucagon-positive cells were predominantly found at the periphery. The total β-endocrine cell area in the gastric and splenic segments, as compared with the intact control group, was significantly decreased ($p < 0.05$), while the total α-cell area was increased ($p < 0.05$) (Table 5) (Fig.2B). There was moderate hypertrophy of β-cell nuclei in all segments of the pancreas.

At 28 day edema, hyperemia of blood vessels and inflammatory infiltration were replaced by focal sclerotic changes of the acinar tissue and pancreatic islets. As before, a slight decrease in the islet volume fraction in all segments of the pancreas was reported. Also, in all segments of the pancreas the total area of β-cells was slightly increased, compared with day 14 of the experiment, but was significantly lower ($p < 0.05$) as compared with the animals in the intact control group. The volume fraction of α-endocrine cells slightly decreased but was statistically greater ($p < 0.05$) than in the intact control group (Table 5). The average area of nuclei was slightly decreased as compared with the intact control group and with day 14 of the experiment.

Indicator	**Day of experiment**		**Segment of the pancreas**		
			Intestinal	**Gastric**	**Splenic**
Volume fraction of islets,%	3	intact	6,8±2,5	12,6±6,0	13,8±7,3
		diabetes	6,7±2,1	11,8±5,4	13,9±10,2
	7	intact	6,7±2,2	12,0±4,5	12,9±2,1
		diabetes	4,5±3,1	11,2±3,2	11,1±5,2

Indicator	Day of experiment		Segment of the pancreas		
			Intestinal	Gastric	Splenic
	14	intact	6,9±3,1	12,1±5,2	14,0±2,9
		diabetes	4,2±1,4	8,4±1,6	9,3±2,1
	28	intact	6,8±2,1	11,9±3,7	13,7±3,2
		diabetes	3,1±1,1	7,6±2,3	9,5±2,4
Total area of β-cells, %	3	intact	51,3±9,2	64,6±9,1	78,2±6,1
		diabetes	50,4±8,5	65,9±8,2	69,8±4,9
	7	intact	53,8±7,6	63,4±7,0	75,4±7,7
		diabetes	42,2±2,1	**45,5±1,4***	**58,1±1,4***
	14	intact	52,1±5,5	64,5±8,7	79,4±4,6
		diabetes	40,4±2,4	**39,7±2,2***	**57,4±2,0***
	28	intact	51,1±7,5	65,2±6,0	77,4±4,4
		diabetes	**32,3±1,3***	**40,2±1,4***	**49,7±2,1***
Total area fraction of α-cells,%	3	intact	37,4±5,2	31,2±3,5	22,2±3,4
		diabetes	36,5±4,8	30,8±2,5	25,7±2,4
	7	intact	39,3±4,3	30,4±4,0	21,3±3,0
		diabetes	41,1±4,1	**49,3±2,1***	**35,4±2,2***
	14	intact	38,5±3,5	32,4±3,2	19,7±2,3
		diabetes	32,2±1,5	**47,4±2,0***	**32,2±2,1***
	28	intact	30,0±4,4	31,4±3,9	18,9±3,2
		diabetes	**30,1±2,1***	**42,3±3,1***	**30,1±4,1***
Total area of β-cell nuclei, μm²	3	intact	24,2±1,2	24,4±1,0	23,9±0,2
		diabetes	27,8±5,9	27,5±5,5	26,6±4,6
	7	intact	24,3±1,3	23,8±1,2	24,7±1,0
		diabetes	29,5±4,3	28,4±3,2	29,6±4,3
	14	intact	24,3±1,2	24,8±1,3	24,6±1,3
		diabetes	30,3±3,2	29,6±2,3	30,4±3,4
	28	intact	23,9±1,2	24,2±1,2	24,6±1,2
		diabetes	28,5±5,1	27,4±3,4	28,5±4,0

* - Significant difference compared with the intact control group ($p < 0.05$).

Table 5. Morphometric indicators of pancreatic islets in rats with alloxan-induced diabetes (M ± m)

Immunohistochemical study which, was performed at day 3 and day 28, showed negative staining of β-endocrine cells for p53, TRAIL, endothelial NO-synthase and Bcl-2 proteins (Fig. 3B) in all segments of the pancreas. At day 3 we observed mild cytoplasmic staining for anti-caspase-3 and anti-Bax-antibody in some endocrine cells. Compared with the intact control group, apoptotic index was significantly increased ($p < 0.05$) during all observation periods. The expression of NF-kB and MDM2 proteins tended to decrease. At 7-14 day moderate cytoplasmic staining for caspase-3 and Bax proteins accompanied by decreased

amount of cells with NF-kB-and MDM2-positive stained nuclei was reported. At day 7 there was a significant ($p < 0.05$) increase in the amount of apoptotic β-cells, compared with day 3 of the experiment, which was followed by a significant ($p < 0.05$) decrease by day 14 (Table 12). At day 28 the amount of cells and the intensity of expression of apoptotic markers significantly decreased and anti-apoptotic proteins were expressed in large quantities in the endocrine cells of pancreatic islets. Apoptotic index was significantly decreased as compared with day 7; however, it was still statistically more significant than in the intact control group (Table 6).

Group	Day of experiment	Primary antibodies							
		p53	Caspase-3	TRAIL	Bax	Bcl-2	MDM2	NOS-3	NF-kB
Intensity of expression									
Intact control	3	-	+	+	-	+	++	-	++
	7	-	+	+	-	+	++	-	++
	14	-	+	+	-	+	++	-	++
	28	-	+	+	-	+	++	-	++
Diabetes	3	-	+	-	+	-	++	-	++
	7	-	++	-	++	-	+	-	+
	14	-	++	-	++	-	+	-	+
	28	-	+	-	+	-	++	-	++
Amount of cells									
Intact control	3	-	+	+	-	+	+++	-	++
	7	-	+	+	-	+	+++	-	++
	14	-	+	+	-	+	+++	-	++
	28	-	+	+	-	+	+++	-	++
Diabetes	3	-	+	-	+	-	+	-	+
	7	-	++	-	++	-	++	-	+
	14	-	++	-	++	-	++	-	+
	28	-	+	-	+	-	++	-	+

Table 6. Immunohistochemical characteristics of β-endocrine cells in alloxan-induced diabetes

Within the period from day 7 to day 14 we observed cytoplasmic staining for caspase-3 and Bax proteins accompanied by decreased amount of cells with NF-kB-and MDM2-positive stained nuclei (Fig. 4B). At day 7 there was a significant ($p < 0.05$) increase in the amount of apoptotic β-cells compared with day 3 of the experiment, which was followed by a significant ($p < 0.05$) decrease by day 14 (Table 12).

Thus, in alloxan-induced diabetes we observe a decrease in the total β-cell area from day 7 to day 28 of observation which is accompanied by a simultaneous increase in the total α-cell area. These changes occur in all segments of the pancreas; however, they are predominantly obvious and are statistically significant in the gastric and splenic segments. Along with

pronounced necrotic changes in the cells of the insular apparatus of the pancreas, there is also an increase in apoptogenic activity of β-endocrine cells due to the damage to mitochondrial membranes (increased expression of Bax and inhibition of Bcl-2 proteins), which might be caused by impaired intracellular calcium homeostasis and activation of the mitochondrial apoptotic pathway with subsequent activation of caspase cascade without the involvement of p53 protein (Table 12).

Streptozotocin-induced diabetes

In streptozotocin-induced diabetes a statistically significant elevation of plasma glucose levels, as compared with those in the intact control animals, was reported (Fig. 1).

Histological investigation which was performed at day 7 showed pronounced hyperemia of blood vessels associated with lymphocytic infiltration and marked edema of the interlobular connective tissue. There were not any statistically significant differences in the total volume fraction of islets either with regard to the intact control group, or to different segments of the pancreas. We revealed marked necrotic changes in endocrine cells both in the central part and at the periphery of the islets (Fig. 2C). We also stated a statistically significant decrease ($p < 0.05$) in the area of β-cells in all segments of the pancreas. However, a significant increase ($p < 0.05$) in the volume fraction of α-cells exclusively in the gastric and splenic segments of the pancreas was reported. There was marked hypertrophy of β-islet cell nuclei ($p < 0.05$) predominantly in the splenic segment of the pancreas (Table 7).

By day 28 moderate hyperemia of blood capillaries of pancreatic islets and accumulation of lymphocytes at some sites were observed. Focal necrotic changes were reported in some islets, whereas other islets underwent sclerotic changes. No significant changes in the islet volume fraction were observed. The total β-islet cell area was still significantly lower ($p < 0.05$) compared with the intact control group during the same period of observation. The total volume fraction of α-endocrine cells was significantly increased ($p < 0.05$) exclusively in the splenic segment of the pancreas. The nuclei of β-cells were hypertrophic and hypertrophy was statistically significant ($p < 0.05$) in the cells of the splenic segment of the pancreas (Table 7).

Indicator	Day of experiment		Segment of the pancreas		
			Intestinal	Gastric	Splenic
Volume fraction of islets,%	7	intact	6,7±2,2	12,0±4,5	12,9±2,1
		diabetes	4,7±3,1	10,2±6,7	11,8±1,5
	28	intact	6,8±2,1	11,9±3,7	13,7±3,2
		diabetes	3,2±2,1	8,2±3,1	9,7±2,3
Total area of β-cells,%	7	intact	53,8±7,6	63,4±7,0	75,4±8,7
		diabetes	**24,3±2,1***	**39,2±3,2***	**40,1±2,0***
	28	intact	51,1±7,5	65,2±6,0	77,4±4,4
		diabetes	**29,8±5,5***	**43,2±5,4***	**47,7±8,2***
Total area of α-cells,%	7	intact	39,3±4,3	30,4±4,0	21,3±3,0
		diabetes	44,2±4,5	**42,3±2,2***	**29,8±2,1***

Indicator	Day of experiment		Segment of the pancreas		
			Intestinal	Gastric	Splenic
	28	intact	40,0±4,4	31,4±3,9	18,9±3,2
		diabetes	40,9±4,8	42,7±4,8	**30,3±4,7***
Total area of β-cell nuclei, μm²	7	intact	24,3±1,3	23,8±1,2	24,7±1,0
		diabetes	23,8±3,1	25,7±2,5	**29,5±1,8***
	28	intact	23,9±1,2	24,2±1,2	24,6±1,2
		diabetes	25,3±1,2	25,9±1,0	**29,3±1,1***

* - Significant difference compared with the intact control group ($p < 0.05$).

Table 7. Morphometric indicators of pancreatic islets in rats with streptozotocin-induced diabetes (M ± m)

Immunohistochemical reaction, which was performed at day 7, revealed that most endocrine cells exhibited marked expression of proapoptotic proteins, such as caspase-3, TRAIL and Bax (Fig. 3C, 4C). Activation of apoptosis was accompanied by inhibited expression of anti-apoptotic Bcl-2, MDM2, nuclear factor NF-kB proteins (Table 8). There was a statistically significant increase in the amount of cells with morphological features of apoptosis when compared with the intact control group (Table 8).

By day 28 there was a decrease in the amount of β-cells as well as in the intensity of expression of caspase 3 in the surviving cells (Table 12). We did not observe any significant changes in expression of either mitochondrial proapoptotic Bax protein or membrane TRAIL (Table 8). Compared with the intact control group, there was a statistically significant increase in apoptotic index. With regard to day 7 of observation, a statistically significant decrease in apoptotic index was stated (Table 8). Moderate expression of endothelial NO-synthase in the capillaries of pancreatic islets was found during all observation periods (Table 8).

Characteristic	Day of experiment	Primary antibodies							
		p53	Каспаза-3	TRAIL	Bax	Bcl-2	MDM2	NOS-3	NF-kB
Intensity of expression									
Intact control	7	-	+	+	-	+	++	-	++
	28	-	+	+	-	+	++	-	++
Diabetes	7	-	+++	+	++	-	+	+	+
	28	-	++	+	++	-	+	+	+
Amount of cells									
Intact control	7	-	+	+	-	+	+++	-	++
	28	-	+	+	-	+	+++	-	++
Diabetes	7	-	++	+	+	-	+	+	+
	28	-	+	+	+	-	++	+	++

Table 8. Immunohistochemical characteristics of β-endocrine cells in streptozotocin-induced diabetes

Thus, the development of streptozotocin-induced diabetes is associated not only with hyperglycemia, but also with marked necrotic changes in endocrine cells of pancreatic islets, decreased β-islet cell area accompanied by hyperplasia of their nuclei, increased α-endocrine cell area, as well as increased apoptotic index (Table 12). High expression levels of endothelial NO-synthase in the capillaries of pancreatic islets are indicative of endothelial dysfunction.

Immune-dependent diabetes

Persistent hyperglycemia and a reduction in plasma insulin by 67.8% were reported in immune-dependent diabetes (Fig. 1), as compared with the intact control group.

Microscopic examination, which was performed at day 7, found that the total volume fraction of the islets decreased when compared with the intact control group. The islets had an irregular shape due to marked necrotic changes in the endocrine cells, hyperemia of the capillaries, as well as mild lymphocytic infiltration. There were single insulin-positive cells or they were arranged in small clusters in the central part of the islets around the capillaries. We observed a significant decrease ($p < 0.05$) in the total β-cell area in all segments of the pancreas as compared with the intact controls (Table 9). The total α-endocrine cell area was significantly increased exclusively in the splenic segment of the pancreas.

At day 14 some symptoms of insulitis, i.e. inflammation of insular cells, persisted. Compared with the intact control group, the area covered by β-endocrine cells in all segments of the pancreas tended to become smaller ($p < 0.05$) due to marked necrotic changes in β-endocrine cells (Fig. 2D). However, we observed moderate hypertrophy of the nuclei of β-islet cells as well as an increase in the area covered by α-endocrine cells in the splenic segment of the pancreas (Table 9). Some pancreatic islets were reported to undergo sclerotic changes.

By day 28, as before, a statistically significant reduction ($p < 0.05$) in the area covered by β-islet cells in all segments of the pancreas, as compared with the intact control group, was observed. Inflammatory infiltration, swelling and hyperemia of blood vessels tended to reduce, too. However, the amount of islets which developed sclerotic changes increased and β-endocrine cells had moderately hypertrophic nuclei. There was an increase in the area covered by α-endocrine cells in the gastric and splenic segments of the pancreas (Table 9).

Indicator	Day of experiment		Section of the pancreas		
			Intestinal	Gastric	Splenic
Volume fraction of islets,%	7	intact	6,7±2,2	12,0±4,5	12,9±2,1
		diabetes	4,7±3,1	10,2±6,7	11,8±1,5
	14	intact	6,9±3,1	12,1±5,2	14,0±2,9
		diabetes	4,2±1,2	8,9±2,0	9,2±2,2
	28	intact	6,8±2,1	11,9±3,7	13,7±3,2
		diabetes	4,4±1,0	7,4±1,3	8,7±3,4

Indicator	Day of experiment		Section of the pancreas		
			Intestinal	Gastric	Splenic
Total area of β-cells,%	7	intact	53,8±7,6	63,4±7,0	75,4±8,7
		diabetes	**29,8±5,5***	**43,2±5,4***	**47,7±8,2***
	14	intact	52,1±5,5	64,5±8,7	79,4±4,6
		diabetes	**25,4±3,2***	**40,1±3,2***	**42,3±1,2***
	28	intact	51,1±7,5	65,2±6,0	77,4±4,4
		diabetes	**30,2±4,5***	**44,3±2,3***	**40,1±3,2***
Total area of α-cells,%	7	intact	39,3±4,3	30,4±4,0	21,3±3,0
		diabetes	45,2±1,2	40,4±5,1	**56,7±4,6***
	14	intact	38,5±3,5	32,4±3,2	19,7±2,3
		diabetes	44,1±2,1	42,3±4,2	**56,4±3,4***
	28	intact	40,0±4,4	31,4±3,9	18,9±3,2
		diabetes	33,9±3,2	**49,5±4,4***	**50,9±3,2***
Total area of β-cell nuclei, μm²	7	intact	24,3±1,3	23,8±1,2	24,7±1,0
		diabetes	25,8±1,1	25,2±2,3	27,5±2,2
	14	intact	24,3±1,2	24,8±1,3	24,6±1,3
		diabetes	25,6±2,3	24,6±2,3	26,4±2,1
	28	intact	23,9±1,2	24,2±1,2	24,6±1,2
		diabetes	24,9±2,0	25,1±1,2	25,8±2,2

* - Significant difference compared with the intact control group ($p < 0.05$).

Table 9. Morphometric indicators of pancreatic islets in rats with insulin-dependent diabetes (M ± m)

Immunohistochemical study during all observation periods showed absence or doubtful expression of p53, Bax, MDM2 as well as Bcl-2 proteins in certain β-islet cells. Within the period from day 7 to day 14 we observed increased expression of TRAIL proapoptotic protein and caspase 3 protein (compared with the intact control group and at day 14 compared with day 7 of the experiment) (Table 10, 12, Fig. 3D, 4D) in most β-cells accompanied by a slight reduction by day 28. Simultaneously, apoptotic index during all observation periods was significantly increased compared with the intact control group (Table 5). The expression of NO-synthase in the endothelium of the pancreatic islet capillaries during all observation periods enhanced.

Group	Day of experiment, days	Primary antibodies							
		p53	Caspase-3	TRAIL	Bax	Bcl-2	MDM2	NOS-3	NF-kB
Intensity of expression									
Intact control	7	-	+	+	-	+	++	-	++
	14	-	+	+	-	+	++	-	++
	28	-	+	+	-	+	++	-	++

Group	Day of experiment, days	Primary antibodies							
		p53	Caspase-3	TRAIL	Bax	Bcl-2	MDM2	NOS-3	NF-kB
Diabetes	7	-	+++	+++	++	-	+	++	+
	14	-	+++	++	++	-	+	++	+
	28	-	++	+	+	-	++	++	+
Amount of cells									
Intact control	7	-	+	+	-	+	+++	-	++
	14	-	+	+	-	+	+++	-	++
	28	-	+	+	-	+	+++	-	++
Diabetes	7	-	++	+	++	-	++	+	+
	14	-	++	+	++	-	++	+	+
	28	-	++	+	+	-	++	+	+

Table 10. Expression of pro-and β-anti-apoptotic proteins in endocrine cells of insulin-dependent diabetic rats

Thus, it was found that immuno-dependent diabetes is characterized by the following changes: hyperglycemia, reduced area of β-islet cells, inflammatory infiltration of islets, marked β-necrotic changes in endocrine cells, focal sclerosis of the islets of Langerhans.

Streptozotocin-nicotinamide-induced diabetes

The results of the 28-day streptozotocin-nicotinamide-induced diabetes study indicated that plasma C-peptide concentration in the control animals with diabetes increased by 36.8% ($p<0,05$), as compared with intact rats. Blood glucose levels in rats with strep-tozototsin-nicotinamide-induced diabetes during the entire experiment was significantly superior to glycemia in intact animals (Fig. 1).

Histologically, the maximum reduction in the volume fraction of islets was found at day 3 with a tendency to increase by day 28 in all segments of the pancreas.

At day 3 the pancreatic tissue appeared unevenly swollen and hyperemic. There were minor destructive changes of endocrine cells in pancreatic islets. The proportion of β-islet cells in all segments of the pancreas markedly decreased. At the same time no significant changes in the area of β-cells in islets were observed. The islets showed uneven accumulation of insulin-positive cells. Along with the cells which exhibited a marked accumulation of insulin, endocrine cells showing uneven distribution of immunopositive material in the cytoplasm with moderate or weak staining were singled out. Endocrine cells in the splenic segment of the pancreas were characterized by moderate hypertrophy of the nuclei and it was statistically significant, as compared to the intact control (Table 11).

By day 7 swelling hyperemia decreased and a microscopic mosaic image was observed. Both histologically intact and impaired islets of Langerhans were found. In a number of islets we could observe focal necrotic changes of endocrine cells, while others exhibited focal proliferation of connective tissue, and sometimes poorly defined intralobular and periductal

sclerotic changes. Compared with the intact control group, there was a statistically significant reduction in the area of β-cells in the splenic segment. Immunopositive material was unevenly distributed both in the insular cells and within the cytoplasm of an insular cell. Hypertrophy of the nuclei of islet cells was reported in all segments of the pancreas.

At day 14 a mosaic pattern of cell damage was still noted. Both histologically intact islets and islets revealing sclerotic changes were defined. Their amount tended to decrease in all segments of the pancreas. Simultaneously, the total β-cell area decreased in the gastric and splenic segments of the pancreas. Hypertrophy of the nuclei of β-cells persisted in all segments of the pancreas (Fig. 2E).

By day 28 a statistically significant reduction in β-cell area in the gastric segment of the pancreas was reported, as compared with the control group. Hypertrophy of the nuclei of β-cells persisted (Table 11).

Indicator	Day of experiment		Segment of the pancreas		
			Intestinal	Gastric	Splenic
Total volume fraction of islets,%	3	intact	6,8±2,5	12,6±6,0	13,8±7,3
		diabetes	3,8±1,0	8,3±1,0	5,4±3,2
	7	intact	6,7±2,2	12,0±4,5	12,9±2,1
		diabetes	4,2±1,2	7,8±1,4	6,7±2,5
	14	intact	6,8±2,1	11,9±3,7	13,7±3,2
		diabetes	5,4±1,3	6,9±1,2	8,9±4,2
	28	intact	6,6±4,2	12,8±4,5	14,3±5,2
		diabetes	5,3±1,1	7,9±1,0	11,3±2,1
Total area of β-cells,%	3	intact	51,3±9,2	64,6±9,1	78,2±6,1
		diabetes	43,4±4,9	52,4±3,4	65,7±3,4
	7	intact	53,8±7,6	63,4±7,0	75,4±7,7
		diabetes	44,6±4,2	53,2±6,1	**57,7±3,3***
	14	intact	51,1±7,5	65,2±6,0	77,4±4,4
		diabetes	37,4±5,6	**47,6±4,3***	**56,2±4,2***
	28	intact	54,3±5,4	66,2±3,6	77,3±4,3
		diabetes	41,2±2,3	**50,2±3,1***	67,8±2,6
Total area of β-cell nuclei, μm²	3	intact	24,2±1,2	24,4±1,0	23,9±0,2
		diabetes	26,4±1,1	26,8±1,0	**26,5±0,9***
	7	intact	24,3±1,3	23,8±1,2	24,7±1,0
		diabetes	**29,8±1,0***	**29,6±1,2***	**30,1±0,6***
	14	intact	23,9±1,2	24,2±1,2	24,6±1,2
		diabetes	**29,1±1,3***	**29,6±1,0***	**30,0±1,0***
	28	intact	24,4±1,0	24,3±1,2	24,7±2,0
		diabetes	**28,7±1,2***	**28,7±1,1***	**29,7±1,2***

* - Significant difference compared with the intact control ($p < 0.05$).

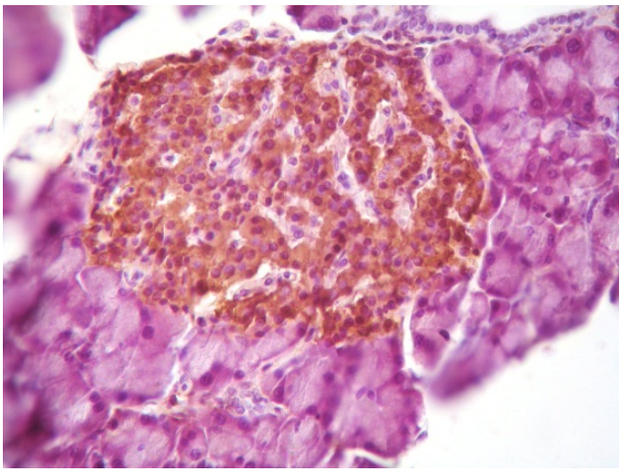
Table 11. Morphometric indicators of pancreatic islets in rats with experimental streptozotocin-nicotinamide-induced diabetes (M ± m)

Day experiment	Intact control	Alloxan-induced diabetes	Streptozotocin-induced diabetes	Immune-dependent diabetes	Streptozotocin-nicotinamide-induced diabetes
3	4,1±0,2	**10,2±1,2***	3,9±0,1	-	5,1±1,8
7	3,9±0,1	**17,4±0,9* ^**	4,0±1,1	**28,6±1,5***	5,4±2,1
14	4,2±1,0	**14,9±1,0* ^**	**32,3±4,2***	**35,2±2,9*#**	4,8±1,9
28	4,0±1,1	**12,5±0,6*** #	**21,3±2,2*** #	**30,3±2,7***	5,0±4,1

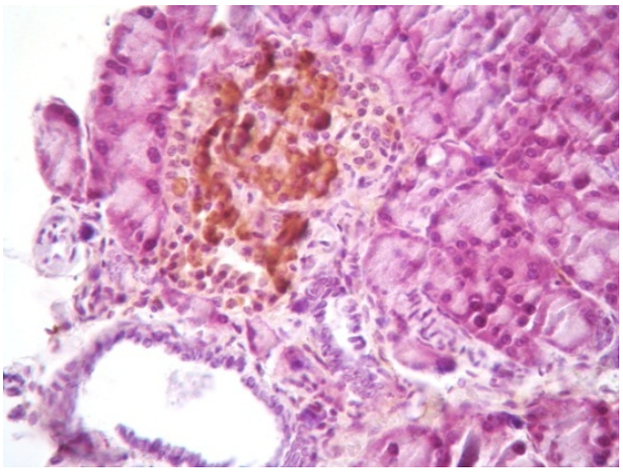
* - Significant difference compared with the intact control ($p < 0.05$) ^ - significant difference compared with day 3 of experiment ($p < 0.05$); # - significant difference compared with day 7 of experiment ($p < 0.05$).

Table 12. Apoptotic index of β-cells (%)

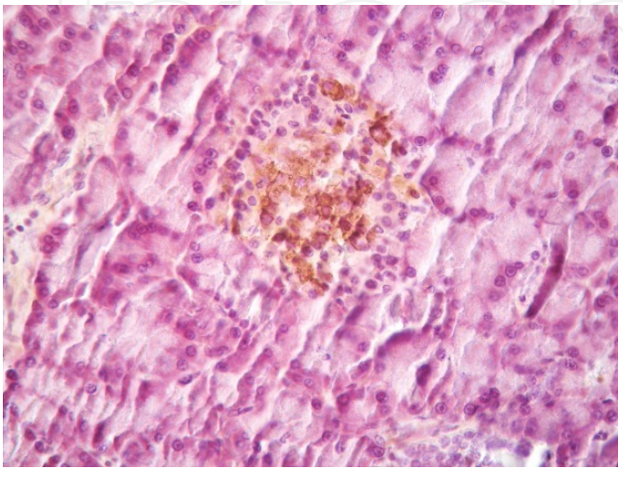
Thus, hyperglycemia as well as reduced plasma C-peptide concentrations are common to experimental streptozotocin-nicotinamide-induced diabetes. Along with biochemical changes, it displays some characteristic morphological changes, including a statistically significant decrease in insular cell area associated with hypertrophy of β-cell nuclei, mosaic islet damage resulting in the destruction of endocrine cells as well as sclerotic changes of pancreatic islets during long-term observation period.



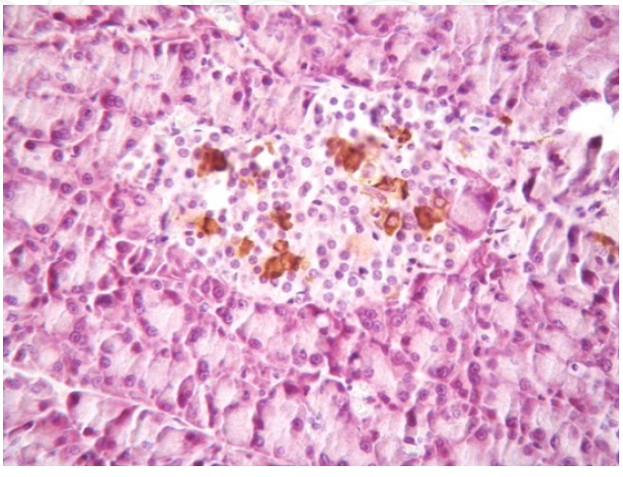
A. Intact control



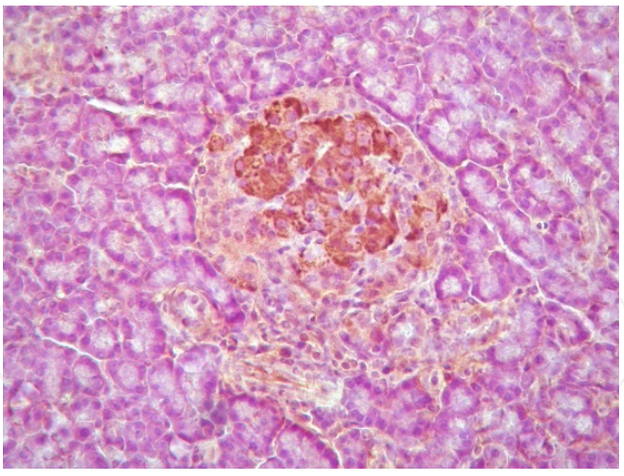
B. Alloxan-induced diabetes



C. Streptozotocin-induced diabetes

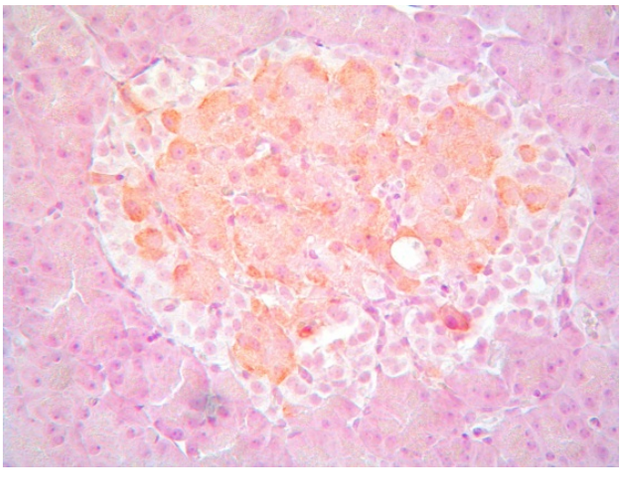


D. Immune-dependent diabetes

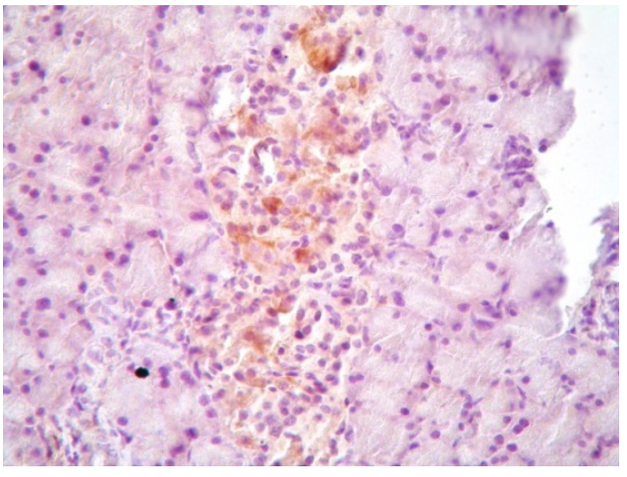


E. Streptozotocin-nicotinamide-induced diabetes

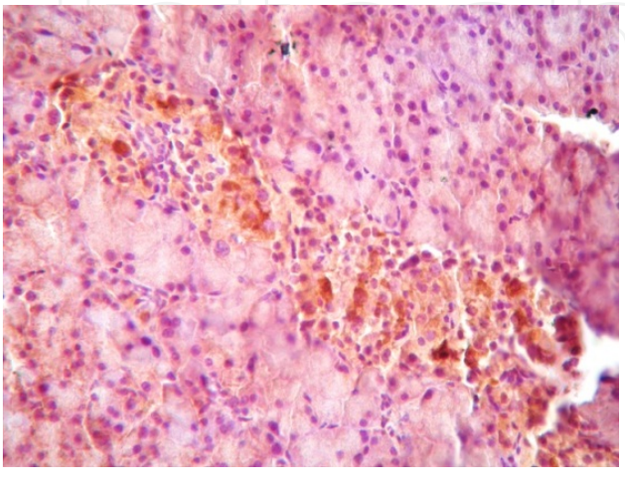
Figure 2. Reduction in the amount of cells under different experimental diabetes conditions, day 14 (Streptozotocin-induced diabetes, day 7), splenic segment of the pancreas. Primary antibody against insulin, DAB staining. Magnification: x400



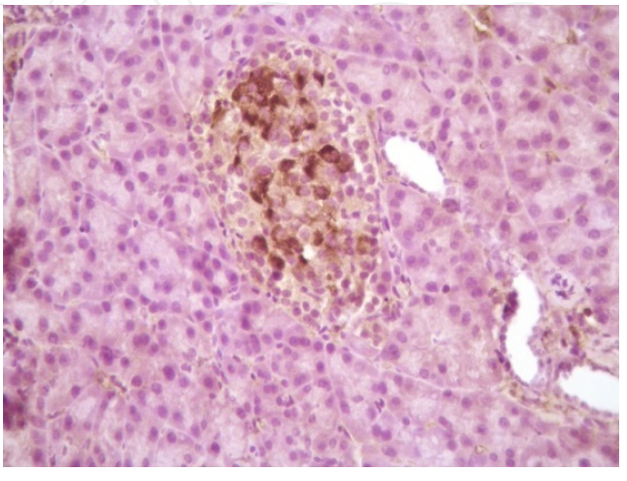
A. Intact control



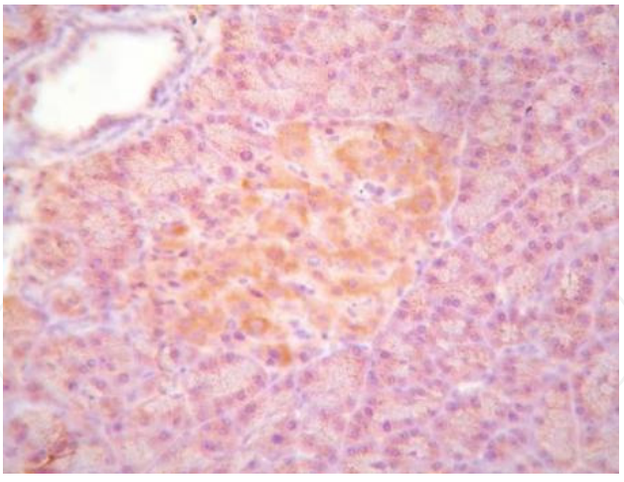
B. Alloxan-induced diabetes



C. Streptozotocin-induced diabetes

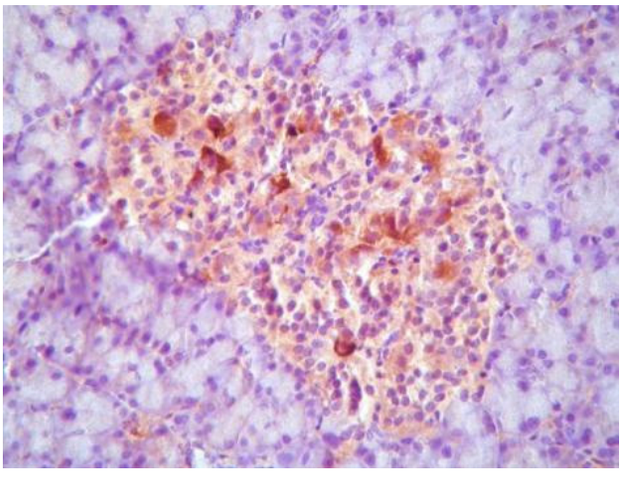


D. Immune-dependent diabetes

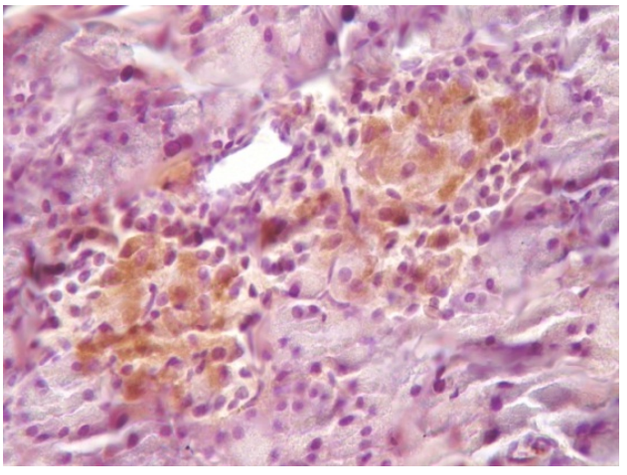


E. Streptozotocin-nicotinamide-induced diabetes

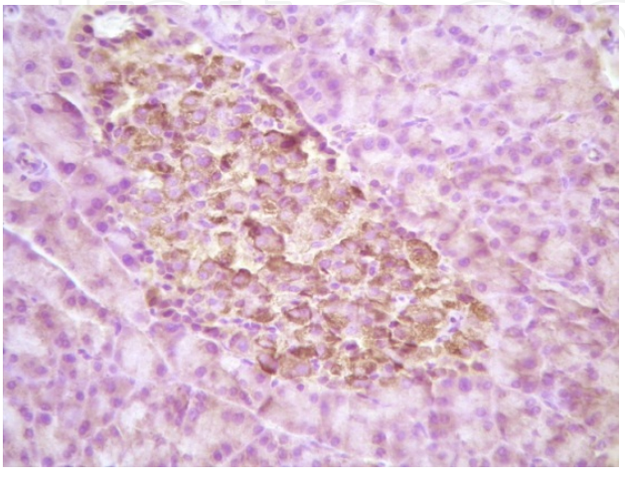
Figure 3. TRAIL expression in pancreatic islet cells under different experimental diabetes conditions, day 14 (Streptozotocin-induced diabetes, day 7), splenic segment of the pancreas. TRAIL primary antibody, DAB staining. Magnification: x400.



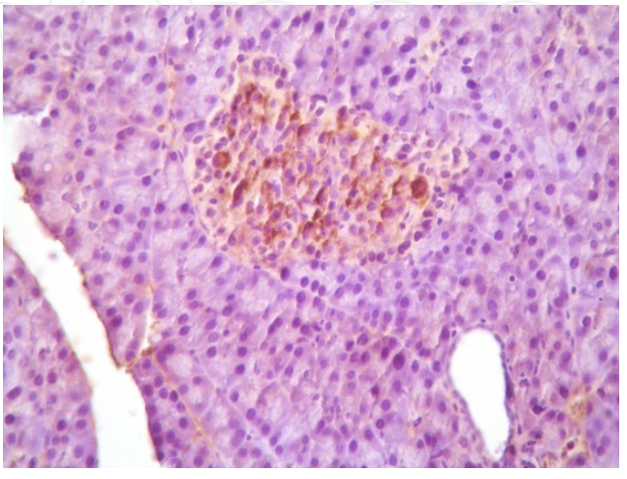
A. Intact control



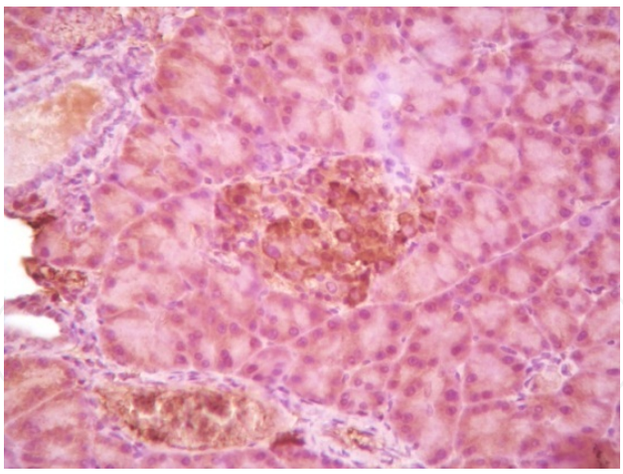
B. Alloxan-induced diabetes



C. Streptozotocin-induced diabetes



D. Immune-dependent diabetes



E. Streptozotocin-nicotinamide-induced diabetes

Figure 4. Apoptotic cells under different experimental diabetes conditions, day 14 (Streptozotocin-induced diabetes, day 7), splenic segment of the pancreas. Caspase 3 primary antibody, DAB staining. Magnification: x400.

4. Discussion

It is known that type I diabetes develops when pancreatic β-cells are damaged due to certain inflammatory, autoimmune and other pathological processes. Selective organ-specific tissue destruction of the insulin-producing pancreatic β-cells is associated with insulin deficiency resulting in impairment of glucose homeostasis. A large body of experimental evidence emphasizes the key role of apoptosis in the pathogenesis of diabetes mellitus (Kim B.M. et al. 2001; Severgina E.S. 2002; Butler A.E. et al. 2003; Bertalli E. et al. 2005; Rees D.A., et al. 2005; Srinivasan K., et al. 2007; Islam S., et al. 2009; Pisarev V.B. et al. 2009). Toxic effects of certain chemicals (e.g. alloxan, streptozotocin, etc.) used to induce diabetes specifically in pancreatic β-cells, manifest themselves by alkylation of DNA and formation of toxic compounds, such as superoxide anion, peroxynitrite, and nitric oxide. Damage to DNA and intracellular structures causes necrosis and activates apoptosis of pancreatic β-cells (Daisy Mythili M., et al. 2004).

Selective toxicity of streptozotocin can be explained by destruction of antiradical protective system and pancreatic β-cell DNA fragmentation. Numerous experiments show that the principal cause of streptozotocin-induced β-cell death is alkylation of DNA. Exposure of cells to streptozotocin results in the formation of toxic compounds, such as superoxide anion, peroxynitrite, and nitric oxide (NO). However, the contribution of NO to the cytotoxic activity remains controversial, because low concentrations of NO in the cells inhibit the inducible forms of NO-synthase, thus reducing DNA fragmentation (Szkudelski T. 2001; Lenzen S. 2008).

Receptor- or mitochondrial-mediated pathway may trigger programmed pancreatic β-cell death (Szkudelski T. 2001). Induction of β-cell apoptosis can occur through a TRAIL-mediated mechanism. DNA damage can activate p53 gene which regulates the expression of

DR5 and/or DR4 death receptors, thus enhancing TRAIL-induced apoptosis (Haupt S., et al. 2003; Kelley R.F., et al. 2005). Our studies provide evidence for activation of TRAIL-induced apoptosis in pancreatic β-cells by administration of streptozotocin on models of streptozotocin-induced and immune-dependent diabetes.

Mitochondrial-mediated apoptotic pathway plays an important role in the development of pancreatic islet damage in diabetes mellitus (Sakurai K., et al. 2001). In alloxan-induced diabetes intracellular calcium homeostasis is typically impaired. *In vitro* and *in vivo* experiments have shown that cytoplasmic calcium concentrations increase in the cells of pancreatic islets. This effect is due to depolarization of cell and mitochondrial membranes of β-endocrine cells which is associated with excessive entry of calcium from the extracellular fluid and intracellular calcium mobilization (Crow M.T., et al. 2004; Jung J.Y., et al. 2004). Thus, considerable gross damage to the intracellular structures by free radicals, oxidation of SH-proteins and impairment of intracellular calcium homeostasis typically result in necrosis and apoptosis, with necrotic processes being more common in alloxan-induced diabetes.

Comparison of morphological data and the dynamics of impairment of β-cell functional activity in experimental models (alloxan-, streptozotocin-, nicotinamide-streptozotocin-induced and immune-dependent diabetes) gives insight into the pathogenesis of experimental diabetes. A high apoptotic index and the prevalence of destructive processes, which enable us to assess not only the extent of pancreatic tissue damage but also to evaluate biochemical parameters, such as plasma glucose, insulin, C-peptide, etc., can be used as markers of severity of experimental diabetes (Snigur G.L., Smirnov A.V. 2010).

Thus, apoptosis is a complex, multi-step, poorly regulated process. Any regulatory impairment can lead to the development of pathological changes in the endocrine apparatus of pancreatic islets including the development of experimental diabetes (Reed J.C., Green D.R. 2011). Each level and element of this system is a potential drug target. The significance of apoptosis determines the need for the refocusing of biomedical research from theoretical biology to clinical medicine. Investigation of apoptotic mechanisms in various pathological conditions enable us to make more accurate diagnoses and prognoses as well as to adjust therapy. The possibility of deliberate control of apoptotic processes is considered to be the basis for developing new medications used to treat many socially important diseases, including the development of anti-diabetic drugs.

Experimental diabetic rats exhibited significant pathohistological changes in the pancreatic islets caused by cytotoxine (streptozotocin+CFA) at all levels of tissue structure. The development of diabetes mellitus was associated with decreased volume fraction of pancreatic islets and β-cells due to inflammation (insulitis) and pronounced destructive changes (e.g. necrosis and apoptosis).

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