



Correction of Biochemical Parameters in Diabetic Rats by Macrophage Activity Modulation

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Abstract

Type 1 and type 2 diabetes mellitus (T1DM and T2DM) are characterized with destructive processes in organs due to hyperglycemia, protein glycation, oxidative stress, gluconeogenesis and lack of anabolic effects of insulin. The violations of various organs are manifested by a change in the biochemical parameters of the blood. Since macrophages are capable of regulating both autoimmune reactions and the process of regeneration of damaged tissues, the use of a macrophage modulator sodium 3-aminophthalhydrazide (SA) could be a promising method for correction the organ damage. The aim of the work is to reveal the possibility of SA to correct plasma parameters of the organ damage in diabetic rats. Fifty male Wistar rats weighing 240-250 g were used in accordance with the Directive 2010/63/EU. Alloxan (300 mg/kg) was administered intraperitoneally to simulate T1DM; nicotinamide (110 mg/kg) and streptozotocin (65 mg/kg) were administered to simulate T2DM. Diabetic rats received 20 intramuscular injections of SA (2 mg/kg). Biochemical, ELISA, histological and immunohistochemical methods were used. The treating diabetic rats by SA was accompanied by a partial correction of diabetes-specific parameters (glucose, insulin) in T1DM and T2DM groups, correction of organ damage parameters (aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase activity; total protein, urea, creatinine level) in T2DM group, in contrast to T1DM, in which was not ameliorated the parameters of liver damage (ALT and AST/ALT). The reason for the correction of biochemical parameters is the increased division of beta cells and insulin production as a result of the action of the immunomodulator 3-aminophthalhydrazide.

Keywords: Type 1 diabetes mellitus; Type 2 diabetes mellitus; Biochemical parameters; Organ violations; Immunomodulator

Introduction

Diabetes mellitus is a widespread disease characterized by progressive course, chronic complications and organ damage which makes it socially significant [1]. The triggering factor of disorders known in the pathogenesis of diabetes is an impaired glucose homeostasis. The reason for the disruption of glucose homeostasis in type 1 diabetes mellitus (T1DM) is the destruction of a significant number of β -cells in pancreatic islets and insulin deficiency, while in type 2 diabetes mellitus (T2DM) there is insulin resistance and a

partial loss of β -cells [2]. In both cases disruption of glucose homeostasis results in damaging consequences to many vital organs such as kidney, heart, organs of the immune system, eyes as well as the nervous system [3]. There are several mechanisms for the destruction of organs and tissues in diabetes.

Hyperglycemia compensates not only by the removal of excess glucose in the urine, but also by glycation of proteins in various organs and tissues, which leads to microangiopathy, retinopathy, and neuropathy [4]. Glycated proteins become unrecognizable for the immune system and become the target of an autoimmune attack [5]. The walls of the blood capillaries, altered as a result of glycation, become poorly permeable to energy substrates, oxygen and end metabolic products [6]. Deficiency of oxygen, glucose, and other energy substrates reduces the resistance of organs and tissues to destruction. The inaccessibility of glucose for insulin-dependent cells due to a lack of insulin in T1DM or a decrease in the number of insulin receptors in T2DM, as well as due to microangiopathy, leads to an increase in the level of counter regulatory hormones such as glucagon, cortisol, epinephrine and growth hormone, especially in T1DM [7]. The catabolic effect of the counter regulating hormones also contributes to the destruction of organs [8].

These dramatic events in diabetes are complemented by oxidative stress [9]. According to some authors, the cause of oxidative stress is an excessive amount of glucose in the blood [10]. Other reasons of oxidative stress in diabetes are hypoxia [11] and elevated levels of free fatty acids [12]. Hypoxia helps to release reactive oxygen species and free fatty acids are substrates for free radical oxidation. Peroxidation undergoes not only free fatty acids in the blood, but also phospholipids in the membranes of various cells and contributes to cell death.

It is known that β -cells can recover from ductal, acinus, and islet non-insulin producing cells that have the ability to reprogram [13,14]. According to the latest data, insulin producing cells can form not only in the pancreas, but also in the liver, myocardium and other organs [15]. To one degree or another, the cells of all organs are capable of regeneration, but in diabetes, the rate of regeneration is less than the rate of cell death. The lack of anabolic effects of insulin in type 1 diabetes and in the late stage of type 2 diabetes is also a significant factor preventing cell regeneration in various organs [16].

Thus, the key pathological processes that cause the destruction of various organs in diabetes are hyperglycemia, protein glycation, autoimmune reactions, gluconeogenesis, lack of anabolic effects of insulin and oxidative stress. Therefore, a possible anti diabetic drug has to affect several pathogenetic mechanisms.

Sodium 3-aminophthalhydrazide (SA) is able to change the phenotype of macrophage-monocytes from pro-inflammatory M1 to anti-inflammatory M2, and also has an anti oxidative property [17]. Since it is macrophages that are capable of regulating both autoimmune reactions and the process of regeneration of damaged tissues, the use of a macrophage modulator could be a promising method for correction the cause and consequences of diabetes, such as organ damage [18]. Previously, we found acceleration of regeneration by SA in several experimental models, including myocardial infarction [19] and diabetes mellitus [20]. The violations of the metabolic pathways and damage of various organs are manifested by a change in the biochemical parameters of the blood [21].

The aim of the work is to reveal the possibility of SA to correct plasma parameters of the organ damage in diabetic rats.

Material and Methods

Male Wistar rats 12 weeks old showing no symptoms of any disease were purchased from the Institute of Immunology and Physiology, Russian Academy of Science (RAS), Ekaterinburg, Russia. All animal experimental procedures were performed in accordance with the principles formulated at the Directive 2010/63/EU and were approved by the Institute of Animal Care and Use Committee at the Institute of Immunology and Physiology, RAS. All animals were kept under equal conditions (12 hour light/12 hour dark), were housed 5 animals in a cage and were fed according to the customary schedule with free access to water. The experiment was carried out on 50 rats weighing 240-250 g. The animals were divided into 5 groups of 10 rats per group: 1- control group (intact rats), 2-T1DM, 3-T1DM+SA, 4-T2DM, 5-T2DM+SA. Alloxan monohydrate (Sigma, USA) aqueous solution at a dose 300 mg/kg body weight was administered to the rats intraperitoneally after 16-hour fasting to simulate T1DM [20]. T2DM was simulated after 16-hour fasting by intraperitoneal injection of streptozotocin (Sigma, USA) in 0.1 M citrate buffer pH 4.5 at a dose of 65 mg/kg with a previous intraperitoneal injection of an aqueous solution of nicotinamide at a dose of 110 mg/kg [22,23]. Fasting blood glucose was determined on day 30th after diabetes modeling to verify the models of T1DM and T2DM, then aqueous solutions of the SA were administered to the diabetic rats in groups T1DM+SA and T2DM+SA intramuscularly (2 mg/kg per day, in total 20 injections for 30 days). Animals of all groups were removed from the experiment on the 60th day after the start of the experiment by intramuscular injection of sodium pentobarbital 40 mg/kg. Blood samples were collected *via* cardiac puncture. Heparin has been added as an anticoagulant. Plasma was separated by centrifugation at 3000 rpm for 10 minutes for biochemical and ELISA analysis. Media laparotomy was carried out to collect pancreas for morphometry.

Biochemical parameters in the blood were determined by spectrophotometric methods using readymade reagent kits. The activity of aspartate aminotransferase (AST, E.C.2.6.1.1), alanine aminotransferase (ALT, E.C.2.6.1.2), alkaline phosphatase (ALP, E.C. 3.1.3.1), α -amylase (3.2.1.1) and the concentration of glucose, urea, creatinine and protein were determined in plasma by kits ("Vital Diagnostics", Russian Federation). The content of glycated hemoglobin (HbA1c) in whole blood was determined by the method of affinity gel chromatography using the GLIKOGEMOTEST kit ("ELTA", Russian

Federation). Optical density was measured by a Beckman DU-800 spectrophotometer (US). Plasma insulin level was determined by ELISA method using Rat/Mouse Insulin ELISA kit ("Millipore", US) and LAZURITE AUTOMATED ELISA SYSTEM ("Dynex technologies", US).

The rat pancreases were fixed in 10% formalin for 24 hours, processed for customary histological evaluation, and embedded into paraffin blocks using an automatic processor (Leica EG 1160). Tissue sections of 3-4 mm thick were made using a microtome and stained with Hematoxylin and Eosin (HE) dye. Tissues were examined under light microscope (Leica DM 2500), and image analysis was done using Video Test-Morphology 5 software Immunohistochemical (IHC).

Immunohistochemical procedure was performed using the Avidin-Biotin Peroxidase Complex (ABC) method. For the detection of Ki67-positive or insulin-positive cells, 4 μ m of paraffin sections of pancreas was deparaffinized, rehydrated, and incubated in antigen retrieval buffer (0.01 M citrate buffer, pH 6.0) using a microwave oven for 15 mins. Pancreatic tissues were incubated overnight at 4°C with anti-insulin antibodies (Clone E11D7, Millipore) diluted at 1:200 or anti-Ki67 antibodies (Leica, Biosystems) diluted at 1:25. After the Di-Amino-Benzidine (DAB) nickel reaction, the sections were counterstained using hematoxylin. Morphometric studies of the islets of the pancreas (PI) in each experimental group included: calculation of the total number of PI per 1 mm² of pancreatic parenchyma, PI diameter (μ m), total number of cells in PI, number of β -cells in PI and number of Ki67+ β -cells in PI.

Statistical analysis was performed using OriginPro 9.0 software (Original Lab Corporation, US). Data are presented as mean \pm standard error of the mean. The statistical significance of the differences in the data obtained was estimated using the nonparametric Mann-Whitney criterion (U).

Results

Glucose in blood plasma increased almost 4 times in rats of T1DM group and 2.4 times in rats of T2DM group compared to the indicator of control group (Table 1). The content of glycated hemoglobin also increased with T1DM and with T2DM by 1.9 times relative to the norm. The plasma insulin level in rats of type 1 diabetes decreased significantly, almost 2.3 times and more moderately in type 2, 1.3 times relative to the control group. The difference in glucose content in rats T1DM and T2DM was significant at $p < 0.05$.

	1	2	3	4	5
Parameter	Control	T1DM	T1DM+SA	T2DM	T2DM+SA
Glucose mmol/L	5.1 \pm 0.2	20.1 \pm 1.3 [*]	9.5 \pm 1.6 ^{* 2}	12.3 \pm 0.2 ^{* 2}	7.7 \pm 0.4 ^{* 4}
HbA1c%	4.3 \pm 0.3	8.2 \pm 0.5 [*]	4.8 \pm 1.2 ²	8.0 \pm 0.5 [*]	4.8 \pm 0.6 ⁴
Insulin μ IU/L	55.6 \pm 0.9	24.7 \pm 2.2 [*]	44.8 \pm 2.2 ^{* 2}	41.9 \pm 0.9 ^{* 2}	44.6 \pm 0.5 ^{* 4}

Table 1: Parameters characterizing the development of diabetes mellitus. ^{*}: Differences with control group are significant at $p < 0.05$; ², ⁴: Differences with groups 2 or 4 are significant at $p < 0.05$.

After injections of SA in diabetic rats, the glucose content decreased 2.1 times in the T1DM+SA group and 1.6 times in the T2DM+SA group compared to that in the T1DM and T2DM groups. However, the

glucose level in rats treated with SA remained higher than that of the control group (Table 1). The action of SA was accompanied by an increase in the amount of insulin in both groups of T1DM+SA and

T2DM+SA, but not a normalization of the indicator. At the same time, in both groups of diabetic rats treated with SA, the amount of glycated hemoglobin decreased to the control level.

During the study of parameters characterizing the state of various organs (Table 2), we found an increase in the activity of AST and ALT

in the blood plasma of rats of the T1DM and T2DM rats compared with the control group tests, while there were no significant differences between the activities of the corresponding aminotransferases in these groups. In both groups of diabetic rats, the ratio of AST/ALT activities was reduced compared to that in the control group.

	1	2	3	4	5
Parameter	Control	T1DM	T1DM+SA	T2DM	T2DM+SA
AST $\mu\text{mol}/\text{min}\cdot\text{L}$	16.5 \pm 1.0	25.8 \pm 1.0*	18.1 \pm 2.7 ²	20.3 \pm 0.7*	16.6 \pm 1.1 ⁴
ALT $\mu\text{mol}/\text{min}\cdot\text{L}$	12.9 \pm 0.9	26.2 \pm 3.2*	25.0 \pm 1.8 *	20.8 \pm 1.1*	11.4 \pm 0.6 ⁴
AST/ALT	1.29 \pm 0.06	0.98 \pm 0.09*	0.78 \pm 0.04 *	0.98 \pm 0.04*	1.46 \pm 0.06 ⁴
ALP $\mu\text{mol}/\text{min}\cdot\text{L}$	60.4 \pm 2.3	56.3 \pm 6.3	59.2 \pm 6.8	78.5 \pm 10.3	61.1 \pm 4.1
α -Amylase $\text{mg}/\text{s}\cdot\text{L}$	29.8 \pm 2.9	31.7 \pm 1.8	37.1 \pm 2.6	28.3 \pm 4.1	27.1 \pm 1.3
Urea mmol/L	5.1 \pm 0.3	11.6 \pm 0.8*	5.6 \pm 0.8 ²	10.3 \pm 1.5*	7.0 \pm 2.4 ⁴
Creatinine $\mu\text{mol}/\text{L}$	64.1 \pm 2.5	114.7 \pm 2.4*	58.6 \pm 0.8 ²	53.3 \pm 5.9 ²	54.9 \pm 4.3
Total protein g/L	72.0 \pm 2.7	57.4 \pm 2.6*	65.1 \pm 3.0	60.8 \pm 4.3	69.7 \pm 4.4

Table 2: Parameters characterizing the state of various organs. *: Differences with control group are significant at $p < 0.05$; 2,4: Differences with groups 2 or 4 are significant at $p < 0.05$.

A decrease in the activity of both aminotransferases and an increase of their ratio followed the SA injections in the T2DM+SA group, whereas in the T1DM+SA group only AST activity was normalized, and ALT activity and AST/ALT coefficient remained at the level of untreated animals. There were no significant changes in ALP activity in the groups of untreated and the treated SA diabetic rats. The value of α -amylase activity in the blood plasma of rats of all the studied groups did not have significant differences (Table 2).

The urea content in the blood plasma of rats of T1DM and T2DM groups was almost twice as high as the control level without significant differences between these groups. In the T1DM group, creatinine concentration also doubled relative to the control one. After SA injections, the urea concentration in the blood plasma of both diabetic rat groups decreased, and the creatinine concentration also normalized in the T1DM group (Table 2).

The amount of total protein in blood plasma decreased only in animals with type 1 diabetes, but not in type 2 diabetes. Normalization of total protein in blood plasma followed by injections of SA.

In the study of morphometric indicators in the group of rats T2DM, no significant changes were found in the number of pancreatic islets, their diameter and the total number of cells in the pancreatic islet relative to the indicators in the control group (Table 3), at the same time, the number of β -cells in the islet of the island significantly decreased compared to the control rat parameter.

In group T1DM, the total number of Pancreatic Islets (PI) decreased significantly compared to the control and remained at the same low level after injection of SA, in contrast to the group of T2DM, where the number of PI did not decrease after modeling diabetes and exceeded the level of control after injection of SA. In group T1DM, the diameter of pancreatic islets increased relative to the control index. In type 1 diabetic rats treated with SA, the diameter of PI normalized. In the groups T2DM and T1DM+SA, the diameter of PI did not differ significantly from that in the control group (Table 3).

We did not reveal significant changes of the total cell number in pancreatic islets of diabetic rats and diabetic rats treated with SA compared with control rats. At the same time, the number of β -cells in the islets of T1DM group dramatically decreased (almost 10 times) and in the T2DM group this indicator decreased almost three times. The administration of SA was accompanied by a partial restoration of the β -cell number in the T1DM+SA and T2DM+SA groups, since this indicator increased in both groups, but did not reach the control level.

When counting Ki67 positive cells among islet β -cells, a significant decrease was found in the T1DM group, while in the T2DM group the number of these cells increased significantly compared to the control and to the T1DM group (Table 3). In the type 1 diabetic rats treated with SA the number of Ki67+cells normalized, and in the T2DM+SA group, there were more cells than in the control animals and untreated rats of the T2DM group.

	1	2	3	4	5
Parameter	Control	T1DM	T1DM+SA	T2DM	T2DM+SA
Number of PI/ mm^2	1.77 \pm 0.15	1.38 \pm 0.23*	1.24 \pm 0.22*	1.61 \pm 0.27	2.51 \pm 0.30 ⁴
The diameter of PI, μm	80.2 \pm 6.8	120.2 \pm 12.5*	76.3 \pm 5.4 ²	93.7 \pm 5.6	76.9 \pm 6.4

Number of cells in the PI/mm ²	9874 ± 954	9929 ± 412	10740 ± 499	8390 ± 579	99015 ± 502
Number of β-cells in the PI/mm ²	5898 ± 215	589 ± 30*	1844 ± 141 ²	1766 ± 440 ²	3663 ± 735 ⁴
Number of Ki67+β-cells in the PI/mm ²	30.5 ± 4.8	8.8 ± 3.9*	31.1 ± 7.0 ²	112.8 ± 11.1 ²	236.8 ± 15.6 ⁴

Table 3: Morphometric parameters of rat pancreas. PI: Pancreatic Islet; *: Differences with control group are significant at p<0.05; ^{2,4}: Differences with groups 2 or 4 are significant at p<0.05.

Discussion

A significant deviation of glucose, HbA1c and insulin from control parameters in T1DM group is consistent with the type 1 diabetes mellitus, and moderate increase in glucose and a slight decrease in insulin in T2DM group are consistent with the type 2 diabetes mellitus model [2]. The change in the insulin content in the blood plasma of rats T2DM corresponds to the stage of the disease, characterized by a partial loss of β-cells [24].

Glycated hemoglobin is a widely used test for the evaluation not only glycemic control, but the development of chronic diabetes complications [25]. HbA1c is a more reliable indicator than glucose, as it assesses the state of hyperglycemia for at least one month. Despite the difference in glucose content, the level of glycated hemoglobin in both groups of diabetic rats is similar; therefore, a similar degree of development of chronic complications in these groups of animals can be assumed. A change in the characterizing diabetes mellitus tests towards the normal levels and partial correction of hyperglycemia during the modulation of macrophage activity by means of SA confirmed the previously obtained data [19,20].

An increase in the activity of AST and ALT in groups of diabetic rats indicates the manifestation of a cytolytic syndrome [26]. Since aminotransferases localize in many organs and tissues, the increased activity of these enzymes in plasma does not allow determining accurately the place of cytolysis. Damage to the liver or myocardium is usually judged by the AST/ALT coefficient. A decrease in this coefficient in our experiment in rats of the T1DM and T2DM groups indicated a predominant damage to the hepatocytes. The cause of hepatocyte cytolysis can be both the toxicity of alloxan and streptozotocin, as well as the manifestation of chronic glucose toxicity due to glycation of hepatocyte proteins and autoimmune reactions to these proteins [5,10]. More pronounced hyperglycemia in the T1DM group compared to the same in the T2DM group and the lack of correction of ALT and AST/ALT in the T1DM group made the assumption of the influence of chronic glucose toxicity more credible.

ALP isoenzymes are found in osteoblasts of bone tissue and in the liver [27]. The activity of the ALP bone isoenzyme increases in blood plasma when osteoblasts are activated, for example, in a growing body or when osteoporosis is compensated, but in the second case, the activity of acid phosphatase, a specific osteoclast enzyme, also increases [28]. The hepatic isoenzyme ALP localizes on the hepatocyte membrane facing the bile capillary, and it normally secretes into bile, as it participates in digestion, eliminating phosphoric acid from organic substrates. When the hepatocyte membrane is damaged, ALP passes into sinusoids, and its activity in the blood increases. The values of ALP activity obtained in our experiment in rats with type 1 and 2 diabetes, not differing from the control one, indicated the absence of cholestatic syndrome in these groups.

It can be assumed that there was no damage to the extra-island pancreatic tissue in rats of the T1DM and T2DM groups, since the activity of α-amylase in these animal groups remained at the normal level [29].

Urea can accumulate in the blood both with increased production, for example, associated with the deamination of amino acids for gluconeogenesis [30], and as a result of impaired filtration in the kidneys [31]. An increase in not only the concentration of urea, but also creatinine in the blood plasma of type 1 diabetic rats confirms the assumption of impaired filtration in the kidneys of animals, which is one of the signs of diabetic nephropathy [32]. Modulation of macrophage activity ameliorated signs of nephropathy in animals of type 1 diabetes. In the T2DM group, kidney damage was probably much less than in the T1DM group.

The reasons for an insufficient amount of protein in blood plasma can be both a decrease in its synthesis in the liver (most of the blood proteins are synthesized in the liver), and protein loss in case of impaired renal filtration [31,33]. Increased ALT activity and a decrease in the AST/ALT ratio, as well as an increase in the level of urea and creatinine in the blood plasma of T1DM rats, along with hypoproteinemia confirmed destructive changes in both organs.

The revealed in our experiment decrease in the number of islets and number of β-cells in islets in T1DM group occurs as a result of autoimmune reactions and an inflammatory process known as insulinitis [34]. The diameter of the islets in the T1DM group increases as a result of edema, which is one of the manifestations of insulinitis. The process of inflammation in the islets is regulated by macrophages and blood monocytes migrating into the islets. Macrophages and monocytes that take the pro-inflammatory M1 phenotype produce pro-inflammatory cytokines IL-1, IL-6 and TNFα and attract T-lymphocytes. An autoimmune process develops and results in apoptosis of β-cells [17,35]. In type 2 diabetes, insulinitis also occurs. Signs of insulinitis were less pronounced in type T2DM rats than in T1DM rats, and were manifested only in a decrease in the number of β-cells, also less pronounced than in the T1DM group.

Macrophages with the M2 phenotype are capable of producing anti-inflammatory cytokines and growth factors that contribute to the regeneration and survival of not only β-cells, but also cells of various organs [17,18]. Therefore, exposure to macrophages by immunomodulator in order to change their phenotype from pro-inflammatory M1 to anti-inflammatory M2 may be a promising strategy in the treatment of diabetes. Injections of an immunomodulator SA with rats of the T1DM+SA group were followed by an increase in the survival rate of pancreatic islets and β-cells and normalization of island size. In T2DM+SA group modulation of macrophage activity also contributed to the partial regeneration of β-cells.

Ki67 is a well known proliferation marker, which serves to evaluate the regeneration process of various cells, including islet beta cells, when researchers identify physiological and pharmacological factors which might re-initiate β -cell replenishment by replication, neogenesis or transdifferentiation [36]. The restoration of the normal number of Ki67+ β -cells in the islets of diabetic rats treated with SA contributed to an increase in the β -beta cell pool in rats of the T1DM+SA group. A large number of Ki67+ β -cells that exceeded the norm in rats of the T2DM+SA group could be formed as a result of enhanced transdifferentiation of islet cells into insulin-producing ones.

Partial recovery of β -cells after injections of an immunomodulator SA probably contributed to a partial correction of hyperglycemia and parameters characterizing the state of various organs in T1DM and T2DM groups.

Conclusion

- Based on the studied parameters of diabetes mellitus (glucose, insulin) and damage to organs such as the liver, kidneys (AST, ALT, urea, creatinine, total protein), we can conclude that the changes in type 1 diabetes model were more pronounced than with type 2 diabetes.
- The effect of the immunomodulator 3-aminophthalhydrazide was accompanied by a partial correction of diabetes-specific parameters for type 1 and 2 diabetes and a complete correction of organ damage in animals with type 2 diabetes, in contrast to rats with type 1 diabetes, in which was not ameliorated the parameters of liver damage (ALT and AST/ALT).
- The reason for the correction of biochemical parameters is the increased division of beta cells and insulin production as a result of the action of the immunomodulator 3-aminophthalhydrazide.

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