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Synergistic Effects of Amino Acid Combination in Streptozotocin-Induced Diabetic Rats: Amelioration of Hyperglycemia, Hematological Aberrations, and Pancreatic Damage

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Abstract

Background: This study aimed to investigate the therapeutic potential of an amino acid combination (AAC) comprising gamma-aminobutyric acid, β-alanine, glutamine, and ethanolamine-O-sulfate for synergistic effects in type 1 diabetes mellitus (T1DM).
Methods and Results: The experiments were conducted on albino male rats, divided into three groups: control rats, streptozotocin (STZ)-induced diabetic rats, and diabetic rats treated with AAC. Fasting blood glucose levels and hematological parameters were monitored. Morphological analysis of blood smears and histopathological examination of the pancreas were performed using histological (Hematoxylin and Eosin, Giemsa staining) methods. AAC treatment significantly reduced fasting blood glucose levels and improved hematological parameters, including red and white blood cell counts, which were initially decreased due to STZ-induced diabetes. AAC treatment mitigated the erythrocyte abnormalities observed in diabetic rats. Histopathological examination of the pancreas revealed that AAC partially restored the structural integrity of its tissue, resulting in a slight increase in the size and number of shrunken islets of Langerhans.
Conclusions: AAC exerts a synergistic effect, improving glycemic control, ameliorating hematological aberrations, and promoting partial regeneration of pancreatic islets in T1DM rats. This study highlights the potential of AAC as a therapeutic agent for managing T1DM and its associated complications.(**International Journal of Biomedicine. 2025;15(3):583-589.**)

Keywords: glycemic control • blood cell count • blood cell morphology • pancreas

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Abbreviations

AAC, amino acid combination; **Ala**, β-alanine; **DM**, diabetes mellitus; **DC**, differential count; **EOS**, ethanolamine-O-sulfate; **FBGL**, fasting blood glucose level; **GABA**, gamma-aminobutyric acid; **Gln**, glutamine; **RBCs**, red blood cells, **STZ**, streptozotocin; **T1DM**, type 1 diabetes mellitus; **T2DM**, type 2 diabetes mellitus; **WBCs**, white blood cells.

Introduction

Diabetes mellitus (DM) is a common chronic metabolic disorder characterized by a deficiency of insulin, resulting in hyperglycemia of a defined degree, with or without glucosuria, and hyperlipidemia. In addition to the metabolic dysregulation itself, this leads to numerous long-term secondary complications, including stroke,[1] neuropathy,[2] nephropathy,[3] retinopathy,[4] ulcers,[5] and gangrene in the extremities,[6] as well as an increased risk of cardiovascular disease leading to mortality.[7] It is known that DM weakens the body's antioxidant defenses,[8] which, together with hyperglycemia

and lipid exchange, causes a decrease in transparency of the phospholipid membrane of peripheral tissue cells and, consequently, damage to β-cells of the islets of Langerhans in the pancreas.

It is known that, due to chronic decompression of carbohydrate metabolism in patients with DM, a complex of metabolic disorders develops, pathologically affecting the morpho-functional state of peripheral blood cells. Therefore, nonenzymatic glycosylation of hemoglobin and the erythrocyte membrane proteins leads to the appearance of abnormal cell forms in the peripheral blood. Enhanced concentration of free radicals and undirected proteolysis products in the patient's organism with DM destabilizes erythrocyte cell membranes. Ultimately, the severity of metabolic stress in diabetes, complicated by angiopathy, leads to a decrease in the number of erythrocytes circulating in the blood, an increase in platelet activity, and the development of cell-depressive immunodeficiency.[9]

There are two types of DM. Type 1 diabetes mellitus (T1DM) is characterized by autoimmune destruction of insulin-producing β cells in the pancreas by CD4+ and CD8+ T cells and macrophages infiltrating the islets.[10] Type 2 diabetes mellitus (T2DM) is a non-insulin-dependent disease caused by a metabolic disorder that develops as a result of the tissue cells' decreased sensitivity to insulin. The prevalence of DM tends to increase. The rising incidence of diabetes underscores the urgent need for effective therapy with multifaceted mechanisms of lowering blood glucose levels and correcting compromised physiological oxidative mechanisms.[11]

Physicians currently have two main strategies for managing patients with T1DM: the first involves preventing the onset of autoimmunity, while the second focuses on reversing the effects of existing autoimmunity and promoting β-cell regeneration. The detection of β-cells in individuals with long-standing T1DM, despite ongoing autoimmune activity, suggests that there may be ongoing β-cell formation.[12] Substantial evidence indicates that an increase in β-cell mass during normal growth and following injury is driven by the division of existing β-cells and the formation of new β-cells from ductal or pancreatic progenitor cells.[13] This insight has opened avenues for developing innovative techniques to enhance β-cell regeneration.

Emerging evidence suggests that amino acids may play an important role in preventing diabetes and its associated complications. Gamma-aminobutyric acid (GABA) has been studied for its potential therapeutic effects in both T1DM and T2DM. GABA is known for its role in the central nervous system. Studies have shown that GABA can inhibit the autoimmune response, thereby preserving insulin production and potentially reducing the onset of diabetes.[14,15] Noteworthy, the recent publications in the field of experimental diabetes give evidence about the protective and regenerative effects of GABA on pancreatic beta cells and demonstrate its potential in reversing diabetes in diabetic mouse models.[16]

Another amino acid, β-alanine (Ala), a dietary supplement primarily known for its role in muscle performance, has not been extensively studied concerning T1DM. However, there is limited evidence suggesting that Ala could influence metabolic pathways that are relevant to diabetes. Its role in synthesizing carnosine is not directly related to T1DM but could indirectly affect metabolic health, a significant aspect of managing diabetes. The benefits of β-alanine administration in combination with increased physical activity in patients with T2DM led to a reduction in glycemia and a simultaneous improvement in physical capacity.[17]

It has been shown that glutamine (Gln) significantly affects glucose metabolism and insulin sensitivity, including nitrogen transport, immune function, and gut health. Research has demonstrated that Gln can improve insulin sensitivity and reduce hyperglycemia in T1DM and T2DM models.[18,19] Its ability to reduce oxidative stress and support immune function is also beneficial in managing DM, particularly in reducing complications associated with the disease.

It is known that ethanolamine-O-sulfate (EOS) increases GABA levels by inhibiting GABA transaminase. While EOS is not widely studied in the context of diabetes, the resulting increase in GABA levels could have protective effects similar to those observed with direct GABA supplementation. This could contribute to β-cell preservation and reduced autoimmune activity in T1DM, though specific studies on EOS in diabetes are limited. This suggests potential therapeutic uses for EOS in managing diabetes, particularly through the modulation of GABAergic pathways.[20] Studies have shown that administering both EOS and Gln produced a protective effect on the amino acids in the brain and the pancreas of rats with experimental STZ diabetes.[21] We also revealed the effect of the combined administration of the above-mentioned 3 neuroactive amino acid mixture in T1DM.[22] In particular, to create an antidiabetic preparation, Prof. Kamalyan compiled a mixture that included GABA, Gln, Ala, and EOS. The study showed the promising therapeutic effects of this mixture on the function of the pancreas in alloxan-induced diabetic rats [23]

Our study explored how combining investigating compounds (GABA, Ala, Gln, and EOS) might impact T1DM. We have attempted to use them in combination for their probable synergistic effect, which can enhance therapeutic efficacy by acting through multiple mechanisms. We hope the amino acid combination (AAC) is a synergistic pharmacological agent that acts simultaneously and dynamically.

This study aimed to investigate the therapeutic potential of AAC comprising gamma-aminobutyric acid, β-alanine, glutamine, and ethanolamine-O-sulfate for synergistic effects in T1DM.

Materials and Methods

Animals

The experiments were conducted on 18 albino male rats weighing 180-240 g. Animals were maintained at a controlled temperature of 23 ± 4 °C with a humidity of 55 ± 5% and a 12-hr dark-light cycle. The rats were fed a standard rat chow and tap water *ad libitum*. Animal care, regular monitoring for general health conditions and weight, and all-animal study experiments were performed based on the IACUC policies and animal care standards, regulations adopted by the Armenian Ethical Committee of the Institute of Biochemistry

named after H. Buniatian, National Academy of Science of the Republic of Armenia (IRB0001621 - IORG0009782).

Induction of Experimental Diabetes

Streptozotocin (STZ) is a cytotoxic alkylating agent that induces swift and irreversible necrosis in pancreatic β-cell islets.[24] It is primarily used to induce T1DM in experimental models, especially rodents.

Rats were induced to be diabetic by a single intraperitoneal injection of 60 mg/kg STZ (Sigma-Aldrich Co., USA) dissolved in 50 mM sodium citrate buffer, pH 4.5 (2.0 ml/kg body weight).[25] The induction of DM was confirmed after STZ treatment by estimating elevated fasting blood glucose level (FBGL). Only those rats with FBGL ≥ 13 mmol/L were included in the study.

Experimental Design

In the experiment, 18 normal healthy rats were used. The animals were weighed and divided into three groups consisting of 6 rats: Group 1 (Control): normal healthy rats; Group 2 (STZ): STZ-induced diabetic rats; and Group 3 (STZ+AAC): STZ-induced diabetic rats treated with AAC.

The diabetes model was induced in 12 animals (Groups 2 and 3) by i/p injection of STZ at a dose of 60 mg/kg. The animals of the control group were injected with an appropriate amount of citrate buffer instead of STZ. The level of peripheral blood glucose (mmol/L) was measured daily in the morning after overnight fasting by taking a drop of blood from the proximal ventral tail vein and using a glucometer (Accu-Chek Active, Roche, Germany). Rats with blood glucose concentrations over 13 mmol/L were considered to have T1DM and were used for further experiments. The control group was selected among intact animals, which had glucose concentrations in the range of 5.0–5.8 mmol/L.

Treatment of Diabetic Rats with AAC

Pharmacological evaluation of the antidiabetic activity of the combination of GABA, Ala, Gln, and EOS was carried out using the STZ-induced diabetes model in rats.

Five days after the injection of STZ (receiving well-established diabetes) to improve the animals' health, Group 3 rats were weighed and given i/p injections of AAC solution (100 mg GABA, 50 mg Glu, 100 mg Ala, 300 mg EOS/2.0 ml/kg) daily for 10 days. An appropriate amount of normal saline was injected into Group 1 and Group 2 animals for all consecutive days. Blood glucose was determined each day during the treatment period.

Histopathological Study

At the end of the treatment, the animals were fasted overnight, anesthetized by i/p injection of 40-50 mg/kg Nembutal, and subjected to instant euthanasia, after which the pancreas of all animal groups and the blood samples were collected to perform morphological investigations.

The blood samples were collected into EDTA-containing vacutainer tubes and used immediately to determine hematological values—white blood cell count (WBCs), red blood cell count (RBCs), and differential count (DC), including lymphocytes, monocytes, basophils, neutrophils, and eosinophils—using an automated hematology analyzer Uritmedical BH-40.

Standard methods were used to prepare blood smears using Giemsa histological staining.[26] Pancreas of all animal groups were isolated; samples were fixed in 10% buffered formalin and embedded in paraffin; 5-10 μm sections were prepared and stained with Hematoxylin-Eosin (H&E).[27] Blood smears and the pancreatic tissue specimens were examined/photographed with a light microscope (Optica B-1000FL-HBO; Camera- C-P20).

Statistical Analysis

Data were statistically analyzed using SigmaStat software. All values are presented as mean ± standard error of the mean (SEM). The statistically reliable comparison was performed by using the Student's t-test. Statistical significance was considered at $P<0.05$.

Results

After a single i/p injection of STZ at a dose of 60 mg/kg, stable hyperglycemia was observed after 5 days. A nearly 7-fold increase in the fasting blood glucose level in experimental rats was observed compared to the control. The animals exhibited the following symptoms: increased water consumption (more than 120 mL), frequent urination, sudden weight loss, hair loss, and depression. In Group 3 animals, the daily treatment of AAC reduced the FBGL by 80%, reaching the norm on Day 3 ($P<0.001$), persisting for several days, and then gradually increased in the following days, to 14% vs. untreated STZ rats (Fig. 1).

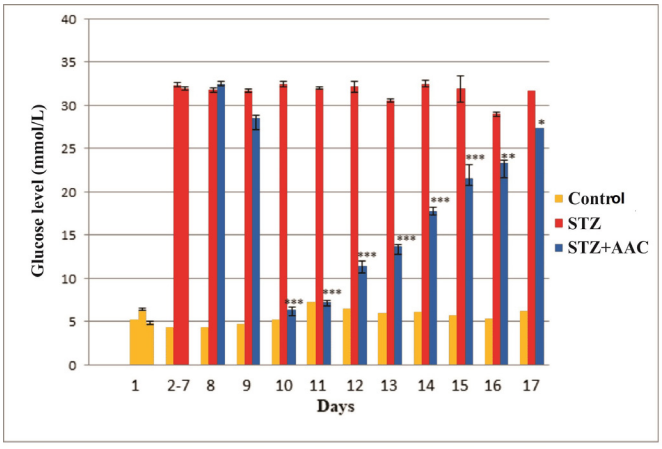

Fig.1. *Results of daily FBGL measurements in the male albino rats of the study groups. Data are shown as mean ± SEM. *P<0.05, ** P<0.01, and *** P<0.001 compared to the STZ group.*

Examination of the total number of RBCs, WBCs, DC of leukocytes of all groups of animals was performed to determine the percentage of each type of white blood cell present in the blood. Diabetic rats showed a significant decrease in the total number of RBCs and WBCs than the normal control

group rats. Differential count showed that neutrophils and lymphocytes levels were significantly decreased. An increased number of monocytes was observed in diabetic rats (Table 1). Changes in DC eosinophils and basophils were within normal limits. After 10 days of treatment by AAC, these indicators gradually return to normal. Blood cell counts appeared to recover from the STZ-induced decrease observed in the treatment group, reaching baseline levels like those observed in the control group. These findings correlate with the positive effect of AAC on blood glucose levels.

Table 1.

Total number of RBCs, WBCs, and DC of leukocytes of the studied rat groups.

Parameter	Control (n=6)	STZ (n=6)	STZ+AAC (n=6)	RR*
RBC ($\times10^{12}$/L)	8.445±0.164	6.395±0.086	8.868±0.111	7.8-10.2
WBC ($\times10^{9}$/L)	10.731±0.107	7.370±0.070	11.267±0.117	9.7-12.9
Neutrophils, %	21.083±0.49	13.067±0.088	16.817±0.111	13-36
Lymphocytes, %	75.000±0.577	58.250±0.167	66.167±0.358	61-86
Monocytes, %	1.233±0.084	6.017±0.060	3.95±0.070	1-4
Eosinophyls, %	0.730±0.042	0.853±0.015	0.812±0.023	0-2
Basophils, %	0.455±0.0226	0.168±0.012	0.352±0.005	0.3-0.5

**RR, reference range. Intergroup comparison of all animal groups showed significant P-values (P<0.05).*

Blood cell morphology is a key tool in laboratory hematology. Deviations from the norm in size, shape, color, distribution, or presence of inclusion bodies suggest possible pathological processes. Data from the morpho-functional study demonstrated that the leukocytes, platelets, and erythrocytes of the control rats' blood were normal (Fig. 2A). Erythrocytes were discoid (biconcave), about 7–8 μm in diameter, which corresponds to the size of the nucleus of a small lymphocyte and with a central area of pallor, which occupies a third of the diameter of erythrocytes and well hemoglobinized on the outer two-thirds of the diameter of the cells without any inclusions.

Of particular interest was the study of the blood smears of the diabetic untreated rats, which demonstrated morphological aberrations, shape abnormalities (poikilocytes), rouleaux formations or agglutinations, partial destruction (hemolysis) of the erythrocytes under the influence of STZ (Fig. 2B,C). We observed codocytes, also known as target cells (with a dark central hemoglobinized area, surrounded by a white ring, an area of relative pallor, followed by a dark outer, peripheral second ring containing a band of hemoglobin;[28] echinocytes or burr cells (reversible condition, referred to a form of red blood cell that have an abnormal cell membrane characterized by many small, evenly spaced thorny projections);[29] spherocytes (smaller than normal red blood cells with a lack central pallor, occurring in the setting of immune-mediated hemolysis or red cell membrane defect resulting from plasma membrane protein deficiency).[30] We also found disruption of the leukocytes' membrane integrity and morphological abnormalities (Fig. 2C,D), such as neutrophils with hypersegmented nuclei.

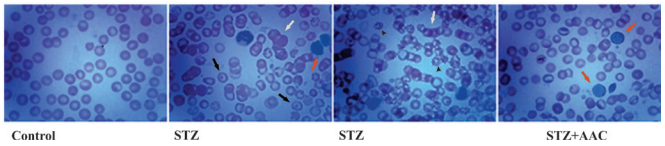

Fig. 2. *Blood cell morphology of the studied rat groups. Codocytes or target cells (black arrows), rouleaux formations or agglutinations (white arrows), leucocytes with impaired membrane integrity and morphological abnormalities (red arrows), echinocytes or burr cells (black arrowhead). Staining with Giemsa. Magnification: 1000x.*

The observation of the blood smears of the AAC-treated group showed that RBCs were generally morphologically normal compared to the blood of the diabetic untreated rats. An increased number of WBCs, such as small and large lymphocytes, was also detected.

Histopathological evaluations were carried out through H&E staining to determine the amount of pancreatic damage in each experimental group of animals.

Morphological investigation of the specimens showed that in the normal healthy rats from the control group, the endocrine part occupied a much smaller area of pancreatic tissue (Fig. 3A). It was represented by dispersed islets of Langerhans - clusters of endocrinocytes penetrated by a dense network of blood capillaries. The islets were predominantly round and oval and were separated from the acini by a thin connective tissue layer. Microscopic examination of pancreatic sections from the untreated diabetic rats revealed degenerative and necrotic changes, as well as shrinkage of the pancreatic islets of Langerhans, decreased islet cell density, marked vacuolization, and a severe reduction in the number of islet cells, which is typical of T1DM (Fig. 3B). However, the pancreatic acinar tissues mostly appeared normal. Treatment with AAC partially restores the morphological structure of pancreatic tissue and contributes to a slight increase in the size of wrinkled islets and the number of endocrinocytes (Fig. 3C). The results of this study show that the use of AAC in diabetes partially induces the regeneration of the islets, thereby restoring the functional activity of the pancreas.

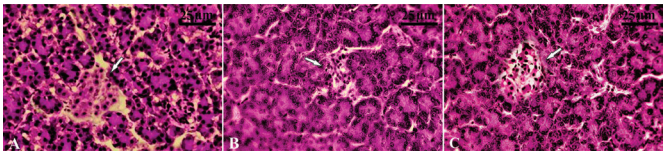

Fig. 3. *Histological image of the pancreas of the studied rat groups. (A) Control group: well-defined acini structures and normal islets of Langerhans, (B) STZ-induced diabetic group: numerous degenerative and necrotic changes in endocrine parts, (C) AAC-treated group: mild improvement of islets of Langerhans area. White arrows show islets of Langerhans. Staining with H&E. Magnification: 400x.*

Discussion

Researchers are actively exploring methods to restore functional β-cell mass as a strategy for diabetes management. These approaches aim to stimulate their replication and regeneration and prevent their damage and death. Potential strategies include promoting the proliferation of residual β-cells, encouraging the differentiation of their progenitors, and inducing the transdifferentiation of non-β-cells into insulin-producing cells, both within and beyond the pancreas.

Persistent hyperglycemia during DM can induce many chemical alterations, which are responsible for most of the clinical complications observed in diabetic patients.

Oxidative stress has been implicated as a primary factor in the progression of diabetes mellitus.[31] Hyperglycemia increases the generation of free radicals by glucose auto-oxidation, damaging vital organs.[32] This necessitates a therapy with multifaceted mechanisms of lowering blood glucose levels and correcting compromised physiological oxidative mechanisms.

As confirmed by our current study, the diabetic group showed an increase in FBGL, suggesting that STZ significantly destroyed the pancreatic β-cells. The combined use of the four investigating compounds included in AAC, based on synergism, has a strongly significant effect on the FBGL. The latter is because GABA, combined with GABA-ergic amino acids and EOS, is involved in carbon and protein metabolism in pancreatic β-cells, partially prevents the destruction of these cells, and, improving its condition, participates in the regulation of hormone secretion and the homeostasis of the islets of Langerhans. However, this effect did not last long, which is explained by the irreversible effects caused by STZ. The use of AAC may cause the release of insulin synthesized by the pancreas. The obtained results correspond with the literature data. It was shown that GABA induced a significant increase in insulin secretion from the pancreas of normal rats. In a diabetic pancreas, GABA evoked an increased insulin secretion, but the increase was statistically insignificant. These findings showed that the number of GABA-like immunoreactive (GABA-LIR) cells is reduced significantly in diabetes. Moreover, GABA is a strong secretagogue of insulin from the pancreas of a normal rat.[33]

Our study showed that in diabetic rats, RBCs and WBCs counts, DC of neutrophils and lymphocytes were found to be significantly decreased, while monocytes, on the contrary, increased. The DC of basophils and neutrophils remained within normal limits. Several studies support this finding. A possible explanation would be cell-specific autoantibodies.[34] Alteration of neutrophil migration may be another reason. The rate of neutrophil migration is lower in T1DM than in T2DM and healthy controls.[35] Moreover, neutropenia can be developed due to neutrophil sequestration in pancreatic tissue or neutrophil infiltration of the islets of Langerhans.[36] Low neutrophil counts are linked with a shortened half-life, increased turnover, and enhanced clearance by macrophages during chronic autoimmune inflammation and islet autoimmunity. Decreased lymphocytes suggest an increase in apoptosis.[37] Additionally, T1DM is characterized by cell-mediated autoimmune destruction of β-cells in the pancreas. This aberrant T-cell activation can destroy immune cells.[38] Activated phagocytosis may be another explanation for affecting neutrophils.[39] Our study revealed an increased monocyte count in the STZ-induced diabetic group. The possible reason may be the severity of diabetic ketoacidosis and evidence of infection. On the other hand, monocytosis may be a leukemoid reaction rather than a systemic inflammatory response. Imbalances in hormones, cytokines, and mediators may also increase monocyte counts.[40]

RBC mass also significantly decreased in diabetic rats compared with non-diabetic controls. Hyperglycemia may have long-term effects that result in the production of ROS, which could lead to irreversible glycation of Hb and RBC membranes.[41] Moreover, elevated levels of chronic hyperglycemia-induced proinflammatory cytokines contribute significantly to insulin resistance and cause anemia. The increase in interleukin-6 in hyperglycemic individuals has an anti-erythropoietic effect that may promote the death of immature RBCs.[42]

In addition to quantitative changes in blood cells, we also found morphological changes in diabetic rats compared to controls. In particular, a high prevalence of changes in erythrocyte morphology was observed. It is known that nonenzymatic glycosylation of proteins in the body induced by diabetic hyperglycemia is another pathway for implementing glucose toxicity.[43] It leads to irreversible structural and functional modifications of the peripheral blood cells, reduces the stability of cells, increases their fragility and deformability (the ability of red blood cells to undergo reversible changes in size and shape), and also changes their aggregation.[44]

Increased lipid peroxides may also lead to RBC membrane damage, hemolysis, and anemia.[45] It has been established that erythrocyte disorders developed as a result of absolute or relative insulin insufficiency significantly impede their functioning. Thus, disorders of erythrocyte morphofunctional state under prolonged hyperglycemia are more pronounced in the development of its decompensation, subsequently leading to quantitative and qualitative disorders of the peripheral link of erythron.

The present study investigated the hematological effects of AAC in STZ-induced diabetic rats. Blood smears from the AAC treatment group showed a nearly normal pattern compared to the blood of diabetic control rats. However, single morphologically changed RBCs and WBCs were still seen.

Our investigations show that after 10 days of treatment by AAC, the blood cell count, DC, and morphological changes gradually return to normal, which is significantly modulated with the regulation of glycemic control. Blood cell counts appeared to recover from the streptozotocin-induced decrease observed in the treated group, reaching baseline levels like those observed in the control group. These findings also correlate with the positive effect of the test substances on blood glucose levels.

The results of H&E staining of pancreatic tissue samples in all groups of rats were evaluated, especially considering the morphology of the islets of Langerhans.

Microscopic examination of the pancreas in healthy rats demonstrated well-defined acini structures with normal islets of Langerhans. In diabetic animals, degenerative and necrotic changes were observed in the endocrine part, such as a decrease in the number and marked destruction and atrophy of the persisted islets of Langerhans, which is why they appear shrunken.[46] Damage to pancreatic β-cells causes the body not to produce insulin, causing hyperglycemia. On the other hand, hyperglycemic conditions can result in the formation of reactive oxygen species (ROS). An increase in ROS that is not balanced with an increase in endogenous antioxidant enzymes, for example, SOD, as a body defense mechanism, can cause oxidative stress and can exacerbate damage to pancreatic β-cells. In the AAC-treated group, the pattern and percentage distribution of the islets of Langerhans were similar to that of diabetic ones. The morphology of the islets of Langerhans, which are slightly enlarged, indicates a moderate increase in the regeneration of the islet β-cells.

Our study shows that in DM, administration of a combined AAC mixture in a relatively short period somewhat regenerates the islets, but cannot significantly slow down pancreatic damage. With long-term use, better results can be expected, which we have planned for the future. It is known that pancreatic Langerhans β-cells are a group of stable cells that can proliferate throughout their life to synthesize insulin again.[47]

In conclusion, the combined action of substances included in AAC in our experiments had a hypoglycemic effect. More importantly, the present results were consistent with the histopathological findings. Interestingly, this study demonstrated significantly improved hematological changes in AAC-treated diabetic animals. This study highlights the potential of AAC as a therapeutic agent for managing T1DM and its associated complications.

Competing Interests

The authors declare that they have no competing interests.

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