

# Pancreatic $\beta$ -cell protective effect of novel GABA derivatives in rats with type 2 diabetes

Ivan N. Tyurenkov<sup>1</sup>, Dmitry A. Bakulin<sup>1</sup>, Yulia I. Velikorodnaya<sup>1,2</sup>, Alexander V. Borisov<sup>1</sup>, Elizaveta E. Abrosimova<sup>1</sup>, Alexey V. Smirnov<sup>1,2</sup>, Grigoryi L. Snigur<sup>1,2</sup>, Svyatoslav S. Surin<sup>1,2</sup>, Daria A. Kavalerova<sup>1</sup>, Olga S. Vasilyeva<sup>3</sup>

*1 Volgograd State Medical University, 1 Pavshikh Bortsov Sq., Volgograd 400131 Russia*

*2 Volgograd Medical Scientific Center, 1 Pavshikh Bortsov Sq., Volgograd 400131 Russia*

*3 Herzen State Pedagogical University of Russia, 48 Moyka River Embankment, Saint Petersburg 191186 Russia*

Corresponding author: Dmitry A. Bakulin ([mbfdoc@gmail.com](mailto:mbfdoc@gmail.com))

Academic editor: Oleg Gudyrev ♦ Received 22 April 2023 ♦ Accepted 08 July 2023 ♦ Published 30 September 2023

**Citation:** Tyurenkov IN, Bakulin DA, Velikorodnaya YuI, Borisov AV, Abrosimova EE, Smirnov AV, Snigur GL, Surin SS, Kavalerova DA, Vasilyeva OS (2023) Pancreatic  $\beta$ -cell protective effect of novel GABA derivatives in rats with type 2 diabetes. Research Results in Pharmacology 9(3): 59–70. <https://doi.org/10.18413/rrpharmacology.9.10042>

## Abstract

**Introduction:** Gamma-aminobutyric acid (GABA) and GABAergic compounds emerged as potential therapeutic agents for diabetes mellitus and its complications. GABA acts as an inhibitory neurotransmitter in the central nervous system and as an extracellular signaling molecule in pancreatic islets, exerting beneficial effects on insulin secretion, glucagon production, apoptosis, beta-cell survival, and regeneration. **Aim:** This study aimed to compare the efficacy of GABA and GABAergic compounds as pancreatic  $\beta$ -cell protective agents in aged rats (18 months) with prolonged hyperglycemia induced by streptozotocin-nicotinamide injection.

**Materials and Methods:** Male outbred albino rats aged 12 months were intraperitoneally injected with streptozotocin (65 mg/kg) and nicotinamide (230 mg/kg). Over the next 6 months, the level of glycemia was monitored every 4 weeks. Further, rats with postprandial glycemia levels between 11 and 18 mmol/L were selected. The experimental groups were treated with GABA and GABAergic compounds (compositions 2 and 3) for 1 month, while the control group received saline. An oral glucose tolerance test (OGTT) was performed after treatment. Blood and samples of the pancreatic tissue (splenic part) were collected for enzyme-linked immunosorbent assay (GLP-1, TNF- $\alpha$  serum level and NF- $\kappa$ B, Nrf2, Klotho tissue homogenate level), immunohistochemistry (islet NF- $\kappa$ B, Nrf2 and Klotho protein expression) and immunofluorescence assays (islet insulin and glucagon expression).

**Results:** The research findings demonstrate significant hypoglycemic effects of the studied GABA derivatives in aged rats with prolonged hyperglycemia. These GABA derivatives effects were accompanied with increased GLP-1 production and improved pancreatic  $\beta$ -cell function and mass. Furthermore, elevated levels of Klotho protein and Nrf2 transcription factor, along with the suppression of NF- $\kappa$ B transcription factor after treatment, may play a crucial role in the  $\beta$ -cell protective effects of these GABA derivatives.

**Conclusion:** Novel GABA derivatives exhibit significant pancreatic  $\beta$ -cell protective effects that may be mediated by enhanced GLP-1, Klotho protein, and Nrf2 transcription factor, and suppressed NF- $\kappa$ B transcription factor. These results highlight the potential of GABA derivatives as promising therapeutic agents for managing diabetes mellitus and its associated complications.

[illegible]diabetes mellitus, GLP-1, insulin, Klotho protein, NF- $\kappa$ B, Nrf2, streptozotocin

Gamma-aminobutyric acid (GABA) and GABAergic compounds are considered promising agents that meet these conditions (Soltani et al. 2011; Prud'homme et al. 2017, 2022; Al-Kuraishy et al. 2021; Antoni et al. 2022). GABA acts as a primary inhibitory neurotransmitter in the central nervous system and as an extracellular signaling molecule in pancreatic islets, where it stimulates insulin secretion, reduces glucagon production, suppresses apoptosis and beta-cell death, and enhances their regeneration. These effects of GABA are mediated through the activation of the Klotho protein (Prud'homme et al. 2017, 2022), SIRT-1, nuclear transcription factor Nrf-2, and indirectly through PI3K/Akt, which are the key mechanisms in regulating the expression of antioxidant response genes. It has been established that GABA inhibits the pro-inflammatory transcription factor NF- $\kappa$ B (Prud'homme et al. 2017, 2022; Typiak and

and Piwkowska 2021), which activates autoreactive T-cells, and their hyperactivity in monocytes and dendritic cells stimulates the production of IL-1 $\beta$ , IL-6, TNF- $\alpha$ , iNOS, and other pro-inflammatory cytokines. This ultimately leads to inflammation activation, oxidative stress, nitrosative stress, damage to beta cells, endothelial cells, neurons, and others, contributing to the progression of diabetes mellitus and the development of its complications. Therefore, the activation of the GABA system in pancreatic beta cells suppresses inflammation, oxidative stress, and nitrosative stress. This holds significant importance in the treatment of diabetes mellitus and its complications and in the development of new and safe therapeutic strategies.

Studies on the pancreatic  $\beta$ -cell protective effects of GABA *in vivo* have often been conducted using the streptozotocin (STZ) model of diabetes induction in young rats, followed by the administration of the test substances for 2–4 weeks (Indrowati et al. 2017; Liu et al. 2017). In clinical conditions, diabetes mellitus more commonly develops in older age, where various organs and tissues, including the carbohydrate metabolism regulatory system, have already accumulated damage. The balance between cell cycle activators and inhibitors shifts in favor of the latter, leading to accelerated apoptosis and death of beta cells, impaired regeneration and functional state, and reduced efficiency of the carbohydrate metabolism regulatory system (Dai et al. 2017; Arrojo e Drigo 2021). Therefore, in this study, we investigated the  $\beta$ -cell protective effects in old rats with prolonged hyperglycemia (24 weeks).

Previously, substances with polyvalent action and high therapeutic potential were identified when studying the neuro-, cardio-, and angioprotective effects of GABA derivatives and 2-pyrrolidone. Compositions or salts with biologically active acids (such as succinic, citric, malic, L-arginine, etc.) were created based on these substances, which showed greater activity compared to the individual components when used separately. This study presents the results of investigating the  $\beta$ -cell protective effects of GABA and compositions numbered 2 and 3 (Fig. 1).

Literature data indicate the effectiveness of GABA as a  $\beta$ -cell protective agent, but to achieve a therapeutic effect in humans, it is necessary to take GABA in very high doses, no less than 3–4.5 g per day. GABA is commonly used as a dietary supplement at a dose of 2 g, three times a day. Meanwhile, various GABA derivatives can have a direct or indirect impact on the GABAergic system of pancreatic islets at much lower concentrations, thereby increasing the effectiveness of anti-diabetic

therapy, including the survival and regeneration of beta cells, stimulation of insulin synthesis, and prevention of diabetes complications (Al-Kuraishy et al. 2021).

This study aimed to compare the efficacy of GABA and GABAergic compounds as pancreatic  $\beta$ -cell protective agents in aged rats (18 months) with prolonged hyperglycemia induced by streptozotocin-nicotinamide injection.

## Materials and Methods

### Ethics statement

All experiments were performed in accordance with the legislation of the Russian Federation and the technical standards of the Eurasian Economic Union for good laboratory practice (GOST R 53434–2009, GOST R 51000.4–2011) and Directive 2010/63/EU of the European Parliament and the Council of the European Union. The study protocol was reviewed and approved by the Regional Independent Ethics Committee of Volgograd region, registration number: IRB 00005839 IORG 0004900 (OHRP), Minutes No. 2022/116 dated March 04, 2022.

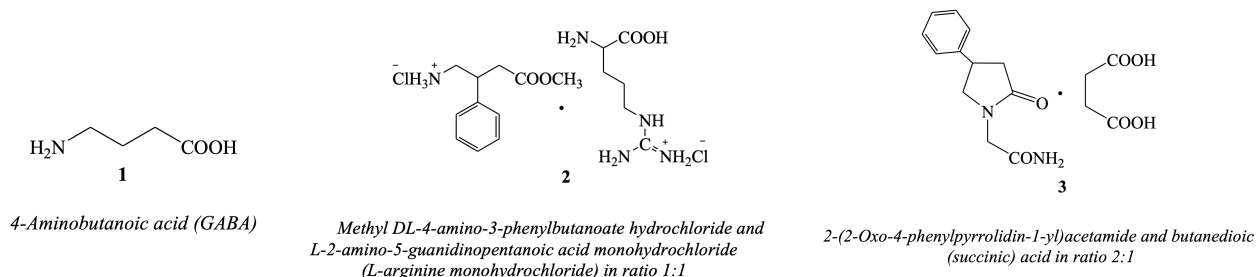
### Experimental animals

The study used 12-month outbred albino male rats (body weight 350 g), purchased from Stolbovaya Nursery for Laboratory Animals (Moscow oblast, Russian Federation). The age of the rats at the beginning of the experiment was 12 months, and at the time of treatment and testing – 18–19 months, which corresponded to the age of old rats. The rats were housed in standard vivarium facilities: 20–25°C and 40–60% humidity in a standard 12/12-h light–dark cycle with food and tap water *ad libitum*.

### Test compounds

Compositions 2 and 3 were selected from a series of GABA derivatives and 2-pyrrolidone for inclusion in the presented study funded by the Russian Science Foundation (Grant No. 21-15-00192) starting from April 19, 2021.

Composition 2 (a composition of linear GABA derivative with L-arginine) possesses pronounced endothelioprotective effects by activating endothelial nitric oxide synthase (eNOS) and increasing NO production by the endothelium. This suggests its potential for further investigation as a means for preventing and treating diabetic angiopathies. Considering the important



**Figure 1.** Test compounds. **Note:** Reference drug: 4-Aminobutanoic acid (GABA); Composition 2: Methyl DL-4-amino-3-phenylbutanoate hydrochloride and L-2-amino-5-guanidinopentanoic acid monohydrochloride (L-arginine monohydrochloride) in ratio 1:1; Composition 3: 2-(2-Oxo-4-phenylpyrrolidin-1-yl)acetamide and butanedioic (succinic) acid in ratio 2:1.

role of nitric oxide in regulating pancreatic function, a substance that stimulates nitric oxide production may also have  $\beta$ -cell protective properties.

Composition 3 (composition of 2-pyrrolidone derivative with succinic acid) exhibits nootropic, neuro-, cardio-, and endothelioprotective effects, as well as antidepressant and anxiolytic actions. Both compositions were selected in preliminary screening studies to investigate their  $\beta$ -cell protective effects in the alloxan-induced diabetic model, using tests of rats survival, preservation of beta-cell mass (indirectly measured by the ability to stimulate insulin production), and a glucose tolerance test with measurements of glycemia, glucagon-like peptide-1 (GLP-1), and insulin levels. Based on the obtained results (unpublished data), these substances showed more pronounced  $\beta$ -cell protective and hypoglycemic effects compared to GABA and other structural analogs. GABA was chosen as a reference substance to compare the  $\beta$ -cell protective properties, which are well-documented in the literature.

### Experimental design

The study design is shown in Figure 2.

The rats were induced with streptozotocin-nicotinamide-induced diabetes by administering streptozotocin (intraperitoneally, 65 mg/kg) following pretreatment (15 minutes prior) with nicotinamide (intraperitoneally, 230 mg/kg in 0.9% sodium chloride solution). After 3 days, rats with postprandial (2 hours after oral glucose administration at a dose of 4 g/kg) glycemia levels  $> 11$  mmol/L and  $< 18$  mmol/L were selected for the study. Every 4 weeks, the level of glycemia was measured. The duration of DM was 6 months. During this period, prolonged diabetes mellitus caused the death of 12% of the diabetic rats.

After 6 months of observation the following STZ administration, an oral glucose tolerance test (OGTT) was performed, and based on the measured blood glucose levels, the rats were randomly divided into four groups with comparable mean blood glucose levels: the 1<sup>st</sup> group received saline (0.9% NaCl) orally (negative control group with diabetes), and the 2<sup>nd</sup>, 3<sup>rd</sup>, and 4<sup>th</sup> groups received the respective investigated substances (GABA,

composition 2 and 3) orally at doses of 1000, 20, and 50 mg/kg for 30 days. Intact rats of the same age without diabetes, strain were used as positive controls.

After the course of oral administration of the investigated substances, an oral glucose tolerance test (OGTT) was performed by administering glucose at a dose of 4 g/kg; and blood glucose levels were measured at 30, 60, and 120 minutes with subsequent calculation of the area under the curve (AUC<sub>0-120</sub>). Blood samples were also collected from the rats to determine the levels of GLP-1 and TNF- $\alpha$ .

Subsequently, after euthanasia (decapitation under 450 mg/kg chloral hydrate anesthesia), samples of the pancreatic tissue (splenic part) were collected for enzyme-linked immunosorbent assay, immunohistochemistry and immunofluorescence assays.

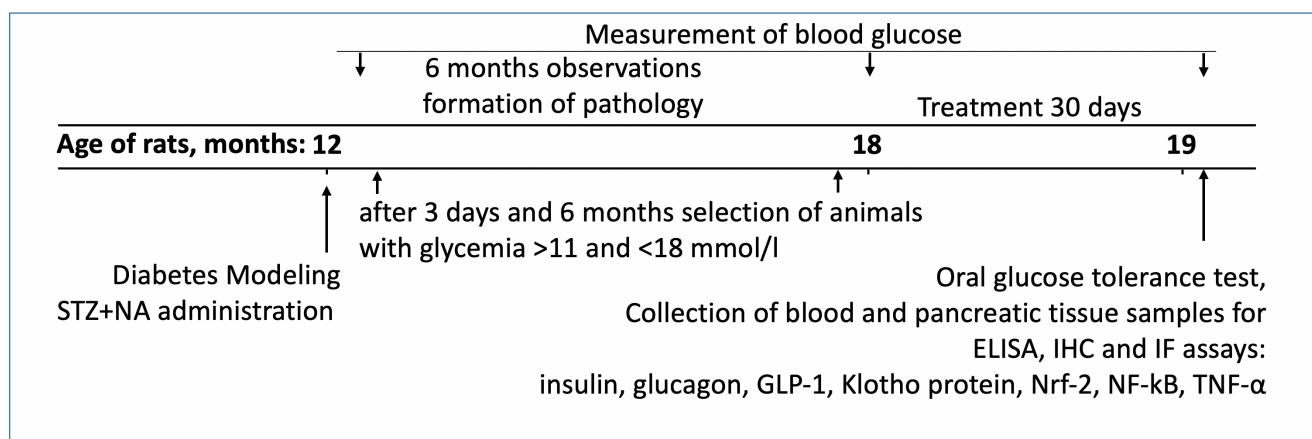
The Contour TS glucometer and corresponding test strips (Bayer, Germany) were used to measure blood glucose levels. Blood samples were collected by puncturing the sublingual vein.

### Blood serum obtaining

Blood samples from control and experimental rats were collected into tubes without anticoagulant. The blood samples were allowed to clot at room temperature for approximately 60 minutes. Subsequently, the samples were centrifuged at 1000g for 20 minutes to separate the clot from the serum. The serum was carefully collected and stored at -80°C until further analysis.

### Preparation of homogenates

The isolated pancreatic tissue samples from control and experimental rats were fragmented and washed in chilled phosphate-buffered saline to remove blood. The tissues were then stored at -80°C until further analysis. Immediately before the analysis, the tissue samples were homogenized in lysis buffer (1 mL of lysis buffer per 50 mg of tissue sample) using a glass homogenizer on ice. The resulting suspension was sonicated using an ultrasonic disperser until it became clear. The solutions were then centrifuged for 5 minutes at 10000g, and the resulting supernatant was used for ELISA.



**Figure 2.** Study Design. *Note:* STZ-NA – streptozotocin-nicotinamide diabetes model; ELISA – enzyme-linked immunosorbent assay; IHC – immunohistochemistry assay; IF – immunofluorescence assay; GLP-1 – glucagon-like peptide-1; NRF2 – nuclear factor erythroid 2-related factor 2; NF-kB – nuclear factor kappa B; TNF- $\alpha$  – tumor necrosis factor alpha.



### Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assays (ELISA) were performed using commercially available kits (Cloud-Clone Corp., USA) following the manufacturer's instructions. Concentrations of glucagon-like peptide-1 (GLP-1) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) were determined in the serum. The homogenates of pancreatic tissue were used to measure the levels of transcription factors NF- $\kappa$ B and Nrf2, as well as Klotho protein. Optical density was measured using a microplate automatic analyzer, SPECTROstar Nano (BMGLabtech, Germany), at a wavelength of 450 nm.

### Tissue processing for immunohistochemical staining

Pancreatic tissue samples were fixed in 10% neutral buffered formalin for 1 day at 22-24°C for immunohistochemical (IHC) and immunofluorescent (IF) staining. The samples were dehydrated through a series of ascending-strength alcohol baths, cleared in chloroform using the Cytadel 2000 tissue processor (Shandon, UK), and embedded in Histomix paraffin medium (BioVitrum, Russia). Paraffin blocks were cut into 5- $\mu$ m-thick sections using the HM340E rotary microtome (MICROM, Germany), and the sections were mounted on poly-L-lysine-coated slides (Menzel, Germany).

### Immunohistochemical staining

For IHC analysis, after deparaffinization and rehydration, the paraffin sections were incubated in 3% hydrogen peroxide for 20 minutes to block endogenous peroxidase activity. The immunohistochemical reactions were performed using a peroxidase-polymer visualization system according to the manufacturer's instructions (Lab Vision, USA). Antigen retrieval was achieved by boiling the sections at 100°C in 0.01 M citrate buffer (pH 6.0) for 20 minutes. The sections were then incubated overnight at 4°C in a humid chamber with primary antibodies against NF- $\kappa$ B p65 (Cloud-Clone, USA), Nrf2 (1:100, Affinity Bioscience), and Klotho protein (1:100, Affinity Bioscience). Peroxidase was visualized using the 3,3'-diaminobenzidine substrate kit. The sections were counterstained with Mayer's hematoxylin. Negative controls were prepared without incubation with primary antibodies, while following all other steps of the protocol.

The slides were examined and photographed using an AxioScope.A1 microscope (ZEISS, Germany) equipped with an AxioCam MRc5 digital camera. The acquired images were processed using the ZENpro 2012 software (ZEISS, Germany).

### Immunofluorescent staining

To assess the functional state of the pancreatic islets in experimental rats, immunofluorescent staining was performed for insulin and glucagon. Immunofluorescent staining was carried out after deparaffinization using a standard protocol with antigen retrieval in "Dewax and HIER Buffer L" (Thermo Scientific, USA) at a temperature of 98°C for 30 minutes. The sections were incubated with primary antibodies against insulin (1:200, Abcam, USA) and glucagon (1:200, Abcam, USA) for 12 hours in a humid chamber. Alexa Fluor 647 (1:500, Abcam, USA) and Alexa Fluor 488 (1:500, Abcam, USA)

were used as secondary antibodies. Nuclei were counterstained with DAPI (Thermo Scientific, USA) at room temperature for 15 minutes. The sections were mounted in a non-fluorescent mounting medium under coverslips. The obtained results were analyzed using an Axio Imager.A2 fluorescence microscope (ZEISS, Germany) at 200x magnification with Ikaros software (Meta Systems, Germany).

### Morphometry

In the morphometric analysis, the specific amounts of insulin or glucagon in the pancreatic islets were evaluated using the following formulas (1 and 2):

$$\text{Insulin Index (II)} = \frac{SI \times 100}{(SI+Sg)}, \quad (1)$$

where II is the insulin index (%), SI is the area of immunopositive material (insulin) in  $\mu\text{m}^2$ , Sg is the area of immunopositive material (glucagon) in  $\mu\text{m}^2$ .

$$\text{Glucagon Index (GI)} = \frac{Sg \times 100}{(SI+Sg)}, \quad (2)$$

where GI is the glucagon index (%), SI is the area of immunopositive material (insulin) in  $\mu\text{m}^2$ , Sg is the area of immunopositive material (glucagon) in  $\mu\text{m}^2$ .

The expression levels of insulin and glucagon were assessed by calculating the area of immunopositive material in the pancreatic islets from at least 30 random fields of view.

### Statistical analysis

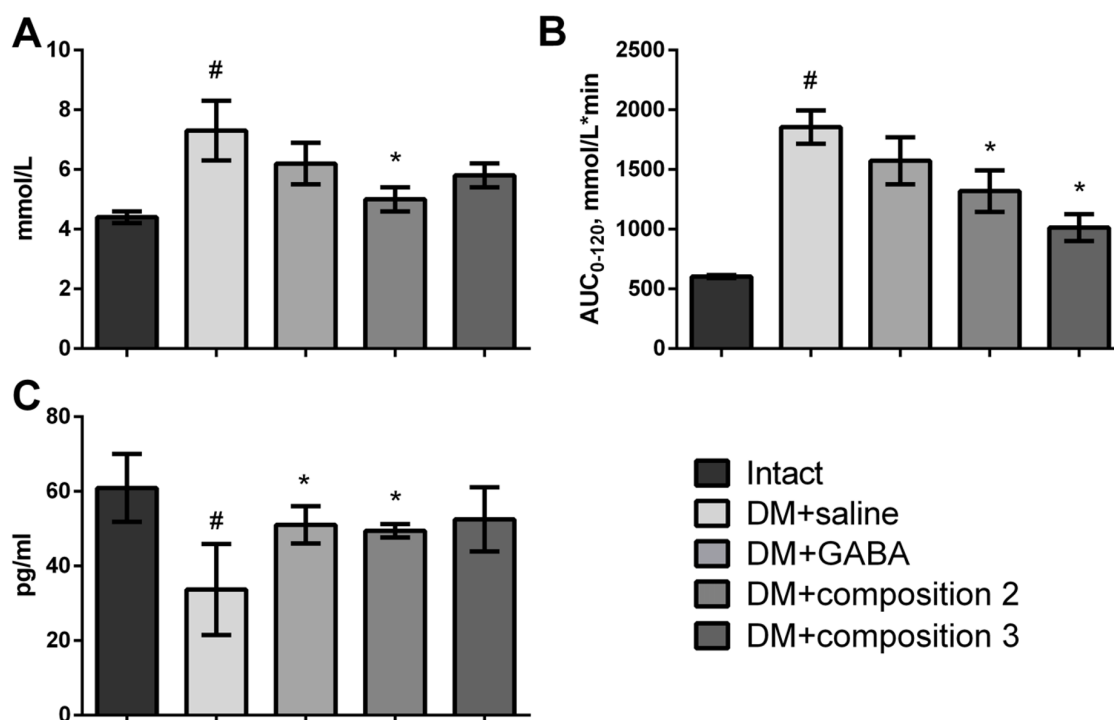
Statistical analysis was performed with Microsoft Office Excel 2016 (Microsoft, USA) and Prism 6 (GraphPad Software Inc., USA). The intergroup differences were assessed using the Kruskal-Wallis rank analysis and the Dunn post-hoc test. All data presented as the mean and standard error of the mean (SEM). The differences were considered statistically significant at  $p < 0.05$ .

## Results

### Effects of the investigated compositions on blood glucose level, glucose utilization and serum GLP-1 level

Rats with DM without treatment showed significantly higher blood glucose levels after a 4-hour fasting compared to the intact group (by 66%), and the serum GLP-1 level was 45% lower. Moreover, the glucose utilization rate during the oral glucose tolerance test differed significantly: the area under the glucose-time curve (AUC<sub>0-120</sub>) in the untreated DM group was three times larger than in the intact group (Fig. 3).

In the experimental groups receiving GABA derivatives (compounds 2 and 3), there was an improvement in glucose metabolism (fasting blood glucose level and glucose utilization rate). Additionally, in the groups receiving GABA and its derivatives, the serum GLP-1 level was approximately 50% higher than in the control group, indicating the ability of the studied compounds to enhance incretin production (Fig. 3).

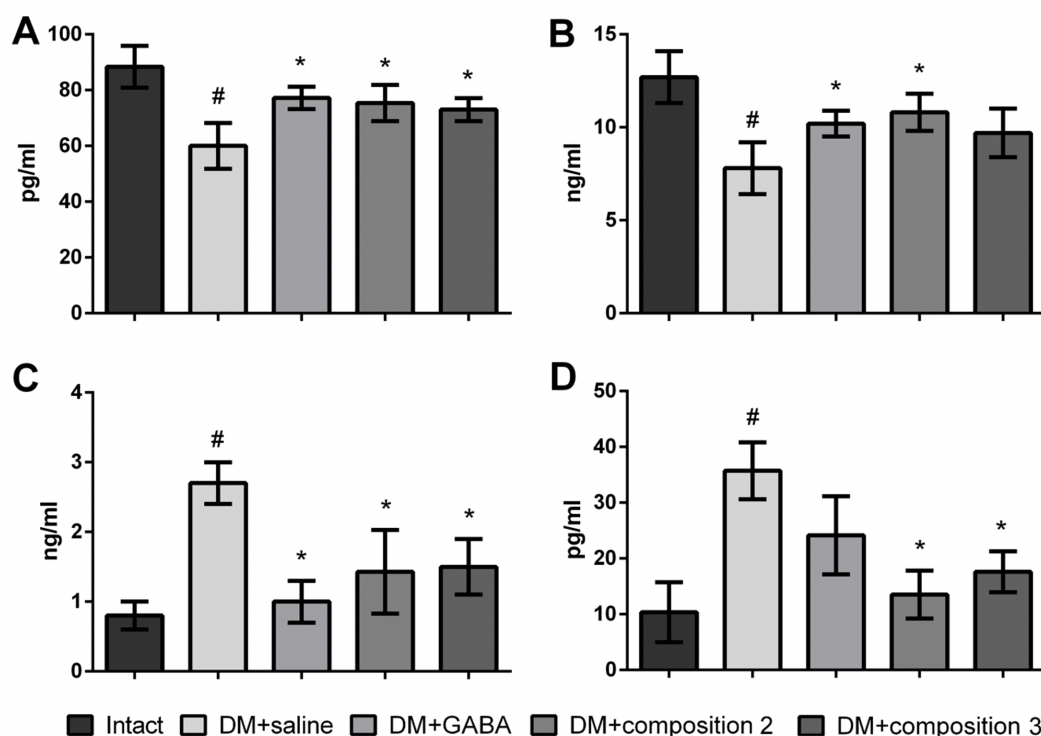


**Figure 3.** Fasting blood glucose level (A), area under the curve "glucose level-time" (B) and serum GLP-1 level (C). *Note:* # – p<0.05 compared to Intact group; \* – p<0.05 compared to DM+saline group (Kruskal-Wallis rank analysis and the Dunn post-test); data shown as the mean ± SEM.

#### Effects of the investigated compositions on Klotho protein levels in pancreatic tissues

In pancreatic tissue homogenates, it was noted that in rats with experimental diabetes without treatment, the level of

Klotho protein was 30% lower compared to intact rats, while in rats treated with GABA and compositions 2 and 3, the level of Klotho protein was higher by 24%, 21%, and 17%, respectively (Fig. 4A).

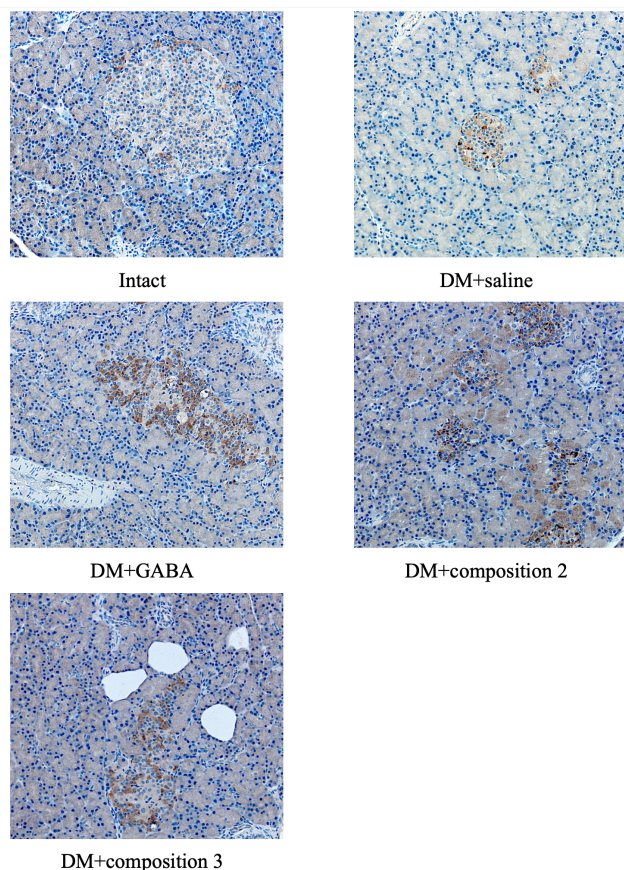


**Figure 4.** The content of protein Klotho (A), Nrf2 (B) and Nf-kB (C) in pancreatic tissue homogenates; serum TNFα level (D). *Note:* # – p<0.05 compared to Intact group; \* – p<0.05 compared to DM+saline group (Kruskal-Wallis rank analysis and the Dunn post-test); data shown as the mean ± SEM.

Immunohistochemical analysis showed that in the pancreatic islets of intact control rats, Klotho protein was localized in the cytoplasm of cells located at the periphery of the Langerhans islets (Fig. 5).

In rats with diabetes without treatment, most of the islet cells contained Klotho protein as delicate homogeneous granules, and only a few cells had large dark granules (Fig. 5).

In the pancreatic islets of rats treated with GABA, a moderate increase in Klotho protein expression was observed. After the administration of compositions 2 and 3, most of the endocrine islet cells in the pancreas showed a weakly positive reaction to Klotho protein. In a small portion of the cells, the immunopositive material was detected as large dense granules (Fig. 5). Additionally, a weakly positive reaction to Klotho protein was also observed in the cytoplasm of exocrine cells surrounding the islets (Fig. 5).



**Figure 5.** Immunolocalization of Klotho protein in the pancreatic tissues of experimental rats. Antibodies against Klotho protein. Peroxidase-antiperoxidase method. Nuclei stained with Mayer's hematoxylin. Magnification 200.

#### Effects of the investigated compositions on the level of transcription factor Nrf2 in pancreatic tissues

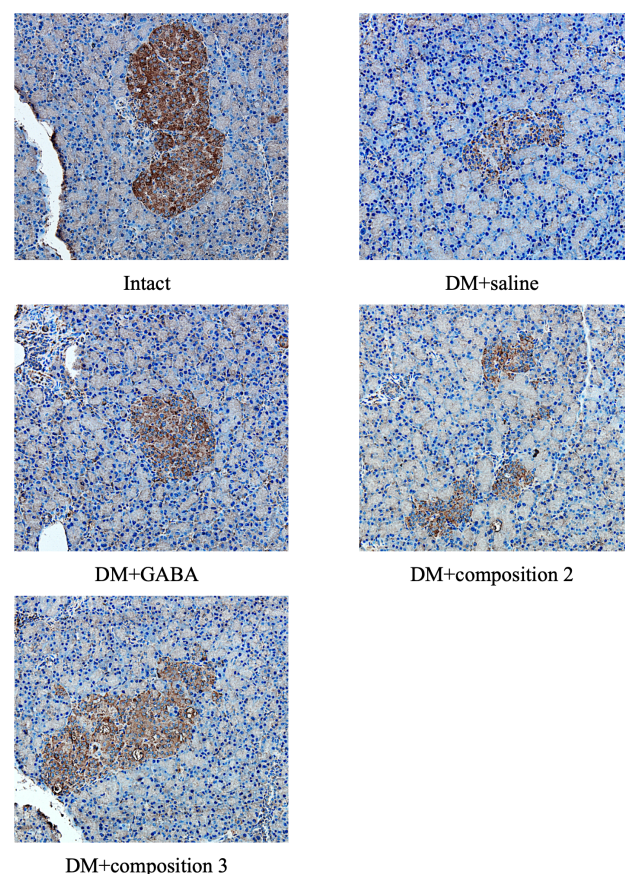
In pancreatic tissue homogenates, the level of transcription factor Nrf2 was significantly lower compared to intact animals (Fig. 4B). In rats treated with GABA and composition 2, the content of Nrf2 in pancreatic tissue homogenates was 31% and 38% higher than in the DM+saline control group.

The results of the immunohistochemical (IHC) analysis revealed that Nrf2 was localized in the cytoplasm of islet

cells as coarse granular deposits in intact rat pancreas (Fig. 6).

In rats with induced diabetes, a significant reduction in immunopositive material was observed in the cytoplasm of cells forming Langerhans islets (Fig. 6), and the reaction to Nrf2 appeared as weakly expressed homogeneous deposits with only a few cells showing large granules.

In rats with diabetes receiving GABA, compositions 2 and 3, the level of Nrf2 immunopositive analyte in the islet cells of pancreatic tissues was higher compared to the control group.



**Figure 6.** Immunolocalization of Nrf2 in the pancreatic tissues of experimental rats. Antibodies against Nrf2. Peroxidase-antiperoxidase method. Nuclei stained with Mayer's hematoxylin. Magnification 200.

#### Effects of the investigated compositions on the level of transcription factor NF- $\kappa$ B in pancreatic tissues

The study of NF- $\kappa$ B concentration in pancreatic homogenates showed that this nuclear transcription factor was 3.4 times higher in the pancreatic tissues of rats with induced diabetes compared to intact rats (Fig. 4C). Rats receiving the investigated compositions for 30 days had a statistically significant but slightly higher level of NF- $\kappa$ B compared to the control group and intact rats.

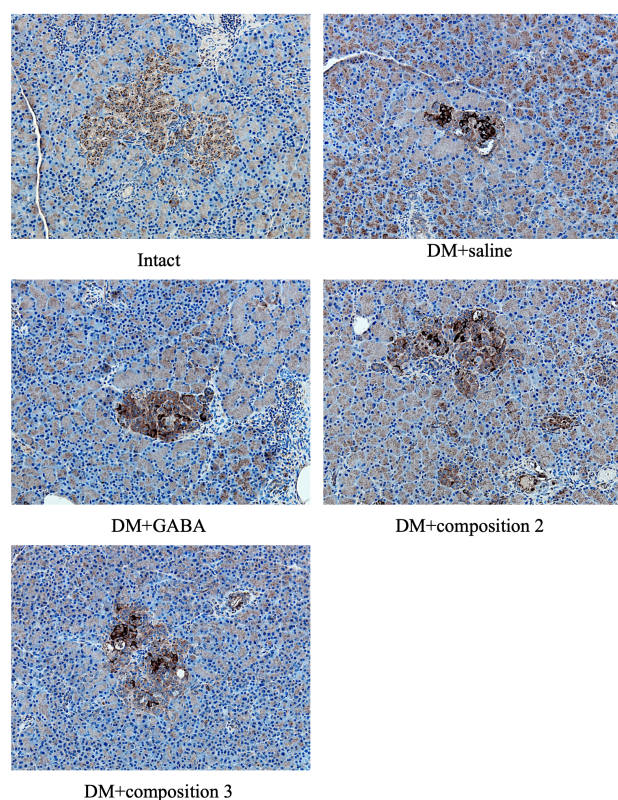
It could be presumed that the increase in NF- $\kappa$ B levels would lead to the activation of pro-inflammatory factors. To clarify the anti-inflammatory effects of the investigated compounds, we measured the level of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), as a downstream component of the signaling pathway initiated by NF- $\kappa$ B activation.



In the control group of rats with diabetes, the TNF level was 2.8 times higher than in intact rats (Fig. 4D). Rats receiving GABA, compositions 2 and 3 for 30 days showed significantly lower levels of TNF- $\alpha$  in the pancreatic tissues compared to the control group.

It should be noted that in the pancreatic tissues of intact rats, NF- $\kappa$ B was predominantly detected by IHC in the cytoplasm of endocrine islet cells (Fig. 7). The zymogen zone of the pancreatic acini also showed moderate reactivity to NF- $\kappa$ B. In the pancreatic tissues of rats with induced diabetes, significant activation of NF- $\kappa$ B was observed in residual islet cells, where the immunopositive material completely filled the cytoplasm. Furthermore, in a significant portion of cells, the examined factor was present in the nuclear zone (Fig. 7).

In the experimental groups that received treatment after 6 months of hyperglycemia, a low density of immunopositive material was recorded in islet cells. Only in isolated cells, high material density was observed. In acinar cells, the content of NF- $\kappa$ B was significantly lower than in other structures of the pancreas (Fig. 7).



**Figure 7.** Immunolocalization of NF- $\kappa$ B in the pancreatic tissues of experimental rats. Antibodies against NF- $\kappa$ B. Peroxidase-antiperoxidase method. Nuclei stained with Mayer's hematoxylin. Magnification 200.

### Effects of the investigated compositions on the functional state of the pancreas

According to the results of immunofluorescent staining, the group with untreated diabetes mellitus showed a statistically significant decrease in the specific amount of immunopositive insulin material compared to the median area of immunopositive material in the intact group:  $10.86 \mu\text{m}^2$  [4.809; 18.20] versus  $81.61 \mu\text{m}^2$  [76.32; 90.56] (Fig. 8).

The administration of GABA only slightly increased the content of immunopositive insulin material compared to the control group of animals:  $18.07 \mu\text{m}^2$  [10.70; 23.44] versus  $10.86 \mu\text{m}^2$  [4.809; 18.20]. However, the administration of composition 2 positively affected the preservation of the functional ability of  $\beta$ -cells to produce insulin, resulting in significantly higher levels of immunopositive insulin material compared to the group with induced diabetes:  $53.34 \mu\text{m}^2$  [30.76; 74.37] versus  $10.86 \mu\text{m}^2$  [4.809; 18.20] (Fig. 8).

The median value of immunopositive glucagon material in the intact group of animals was  $18.39 \mu\text{m}^2$  [9.438; 23.68]. In animals with diabetes, the level of immunopositive material was more than 4 times higher:  $89.14 \mu\text{m}^2$  [81.8; 95.2].

In animals with diabetes receiving GABA and composition 3 for 30 days, the specific amount of immunopositive material was slightly lower than in the control group of animals. However, in animals receiving composition 2, the level of immunopositive glucagon material was almost twice lower than in the control group,  $46.66 \mu\text{m}^2$  [25.63; 69.24] versus  $89.14 \mu\text{m}^2$  [81.80; 95.12] in the untreated group (Fig. 8).

Thus, the greatest preservation of the area of immunopositive material (insulin) was observed in the experimental group of animals with diabetes mellitus after the course administration of composition 2.

## Discussion

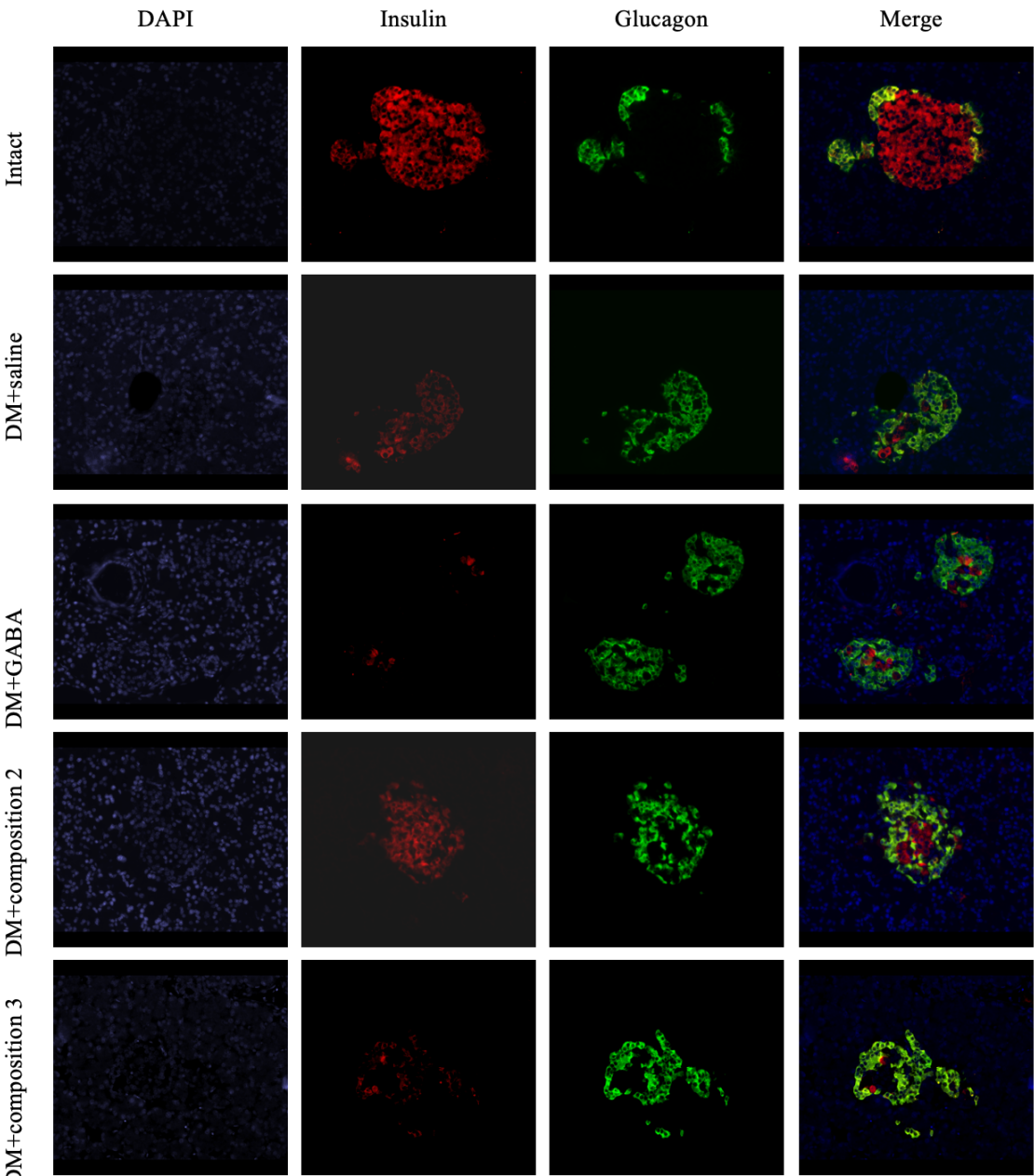
In the present study, we investigated the pancreatic  $\beta$ -cell protective properties of two novel compositions derived from GABA: composition 2 and composition 3, respectively based on GABA and 2-pyrrolidone (a cyclic form of GABA). The results of the study conducted on aged rats (18-19 months) with prolonged hyperglycemia (6 months) demonstrated that GABA exhibited pronounced pancreatic  $\beta$ -cell protective properties, but modified GABA molecules (compositions 2 and 3) eliciting these effects at significantly lower doses.

Soltani et al. (2011) were the first to demonstrate that GABA reduces apoptosis and cell death of pancreatic  $\beta$ -cells and enhances their regeneration in diabetes. When studying the pancreatic  $\beta$ -cell protective properties of GABA on knockout animals based on the Klotho protein, this effect was almost absent, leading to the conclusion that the action of GABA on  $\beta$ -cell function is mediated through the multifunctional Klotho protein.

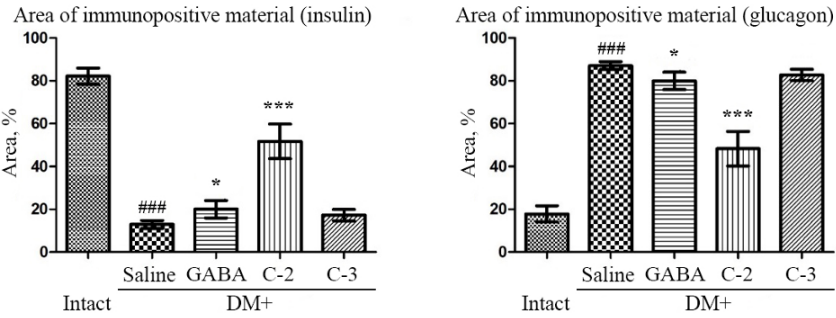
In our study, rats with diabetes exhibited significantly lower content of Klotho protein compared to intact rats. According to some authors, the concentration of Klotho protein can be considered a prognostic factor for the development of chronic complications in diabetes (Prud'homme et al 2017; Zubkiewicz-Kucharska et al. 2021). The decrease in Klotho protein levels in our study may be attributed to prolonged hyperglycemia and aging (Prud'homme et al. 2022; Abraham and Li 2022; Donate-Correa et al. 2023). And it is crucial to note that all the tested compositions in our study increased the level of Klotho protein in pancreatic tissue.

One of the mechanisms of action of GABA and Klotho protein is the suppression of nuclear transcription factor NF- $\kappa$ B activation, which acts as an inflammatory factor by activating various genes responsible for the production of pro-inflammatory mediators and is a key factor in the death of  $\beta$ -cells during *in vivo* islet inflammation.





A



B

**Figure 8.** Localization of insulin and glucagon in the pancreatic islets of rats, immunofluorescent microscopy, magnification  $\times 200$  (A). Amount of immunopositive insulin and glucagon material in pancreatic islets (B). **Note:** ### –  $p < 0.001$  compared to Intact group; \*\*\*\* –  $p < 0.05$ ,  $p < 0.001$  respectively compared to DM+saline group (Kruskal-Wallis rank analysis and the Dunn post-test); data shown as the mean  $\pm$  SEM; C-1 – composition 2; C-2 – composition 3.

It has been proposed that Klotho protein can inhibit NF- $\kappa$ B activity through increased HSP70 levels, which, when located perinuclearly, prevent the translocation of NF- $\kappa$ B into the nucleus. Prud'homme et al. (2014) demonstrated that GABA increases the expression of SIRT-1, which leads to increased NAD<sup>+</sup> levels in  $\beta$ -cells. Activation of SIRT-1 results in the deacetylation of p65, necessary for the activation of the NF- $\kappa$ B pathway, and suppresses its expression and inflammation. These effects are suppressed by both GABA-A and GABA-B receptor antagonists.

According to our experiment, compositions 2 and 3 exhibited the same activating effect on Klotho protein in pancreatic tissue of rats with induced diabetes as GABA, but at much lower doses. At the same time, the modified GABA compounds inhibited TNF- $\alpha$  1.3-1.6 times more strongly compared to pure GABA. However, the inhibitory effect of the original GABA preparations on NF- $\kappa$ B was weaker than that of pure GABA. Such divergent actions of GABA compounds on target molecules can be explained by the complex and mutually exclusive relationship between Klotho protein, TNF- $\alpha$ , and NF- $\kappa$ B. TNF- $\alpha$  promotes phosphorylation of RelA at Ser536, a subunit of NF- $\kappa$ B p65, leading to NF- $\kappa$ B activation and the expression of several pro-inflammatory cytokines, including TNF- $\alpha$  itself. On the other hand, both membrane-bound and soluble Klotho protein inhibit phosphorylation of RelA at Ser536, suppressing NF- $\kappa$ B activation and the TNF- $\alpha$  induced by it, thereby preventing the production of pro-inflammatory molecules. According to our data, the tested compositions exerted pronounced activating effects directly on Klotho protein and inhibitory effects on TNF- $\alpha$ . Suppression of NF- $\kappa$ B expression by GABA compounds is expected to lead to positive effects, particularly with long-term use, as NF- $\kappa$ B also plays a significant role in the development of diabetes mellitus complications.

All tested compositions, compared to the control, demonstrated the ability to restore GLP-1 production, which is of great importance since it has been established that GLP-1 (and GLP-1 receptor agonists) exhibit significant cytoprotective effects, reducing apoptosis and cell death of  $\beta$ -cells and activating their regeneration. These effects are associated with a significant reduction in oxidative stress markers (superoxide dismutase, glutathione reductase, catalase, glutathione peroxidase, glutathione levels, lipid peroxidation). GLP-1 and GABA synergistically suppress oxidative stress in diabetes through the activation of Nrf2 and downstream cAMP, PI3K, and PKC pathways. Thus, the additional stimulation of Nrf2 through the incretin mechanism, in addition to the action of GABA, may have great significance in the therapy of diabetes. Furthermore, GLP-1, through the activation of glutamate decarboxylase in the pancreas, leads to an increase in GABA concentration in  $\beta$ -cells, and GABA enhances the sensitivity of receptors to GLP-1, resulting in increased insulin synthesis and secretion.

The DM pathogenesis involves opposing nuclear transcription factors Nrf2 and NF- $\kappa$ B. NF- $\kappa$ B increases the expression of pro-inflammatory cytokines and suppresses the activity of antioxidant defense enzymes. On the other hand, Nrf2 protects the structures and functions of the pancreas from damage caused by oxidative and nitrosative stress, achieved through the activation of antioxidant defense enzymes and inhibition

of NF- $\kappa$ B and its associated inflammation (Adoga et al. 2022; Gao et al. 2022).

In turn, all the studied compositions stimulated Nrf2 induction in pancreatic islet cells damaged by streptozotocin, as evidenced by the results of immunofluorescence and immunohistochemistry analysis. The Keap1/Nrf2 signaling pathway is known to be the most important regulator of cellular protection against oxidative stress, potentially playing a vital role in preventing the progression of diabetes. Additionally, it has been shown that Nrf2 prevents the development of diabetes by activating the antioxidant defense system and exerting anti-inflammatory effects, thereby influencing the damage to  $\beta$ -cells (Van der Horst et al. 2022). Induction of Nrf2 in mice has been shown to activate the synthesis of antioxidant enzymes, mitochondrial respiration, and suppress the formation of oxidative DNA adducts and apoptosis of pancreatic  $\beta$ -cells mediated by reactive oxygen species, nitric oxide, and peroxynitrite, thereby contributing to the restoration of pancreatic  $\beta$ -cell mass and insulin secretion.

Considering the key role of Nrf2 in regulating the expression of hundreds of genes in cell protection against oxidative stress, the elevation of its levels in rats with diabetes may underlie the pancreatic  $\beta$ -cell protective effects of GABA derivatives in diabetes. Given the influence of Nrf2 on the activity of the antioxidant system, the production of pro-inflammatory factors, and the maintenance of mitochondrial homeostasis, the activation of the Nrf2 transcription factor can be considered a target for the prevention and treatment of the consequences of prolonged hyperglycemia (Robertson 2023).

The evaluation of the functional activity of islet cells based on immunofluorescence analysis showed that composition 2 exhibited the most effective action, not only promoting the maintenance of insulin-producing cell functions but also suppressing glucagon production. It is known that glucagon secretion in the presence of persistent hyperglycemia is another aspect of the pathophysiology of diabetes, and GABA, by activating GABA-A receptors in  $\alpha$ -cells, inhibits glucagon secretion.

Considering the unidirectional nature of the effects of the studied compositions on factors reflecting the functional state of the pancreas and the regulation systems of carbohydrate homeostasis, it can be concluded that these effects are due to the activation of the GABAergic system. These compositions are promising for further investigation as agents for the prevention and treatment of diabetes and its complications.

## Conclusion

1. The comprehensive study of the  $\beta$ -cell protective potential of new GABA derivatives demonstrates their pronounced hypoglycemic effects in aged rats with prolonged hyperglycemia.
2. The hypoglycemic action of the studied GABA and its derivatives is mediated by increased GLP-1 production and improved functional state and increased mass of pancreatic  $\beta$ -cells.
3. The elevation of Klotho protein levels and the nuclear transcription factor Nrf2, as well as the suppression of nuclear transcription factor NF- $\kappa$ B production, play an important role in the provision of the  $\beta$ -cell protective effects of GABA derivatives.

## Conflict of interest

Authors declare no conflict of interests.

## Financial support

This research is supported by the Russian Science Foundation (project No. 21-15-00192, dated April 19, 2021).

## References

- Abraham CR, Li A (2022) Aging-suppressor Klotho: Prospects in diagnostics and therapeutics. *Ageing Research Reviews* 82: 101766. <https://doi.org/10.1016/j.arr.2022.101766> [PubMed]
- Adoga JO, Channa ML, Nadar A (2022) Type-2 diabetic rat heart: The effect of kolaviron on mTOR-1, P70S60K, PKC- $\alpha$ , NF- $\kappa$ B, SOD-2, NRF-2, eNOS, AKT-1, ACE, and P38 MAPK gene expression profile. *Biomedicine & Pharmacotherapy* 148: 112736. <https://doi.org/10.1016/j.biopha.2022.112736> [PubMed]
- Al-Kuraishy HM, Hussian NR, Al-Naimi MS, Al-Gareeb AI, Al-Mamoori F, Al-Buhadily AK (2021) The potential role of pancreatic  $\gamma$ -aminobutyric acid (GABA) in diabetes mellitus: A critical reappraisal. *International Journal of Preventive Medicine* 12: 19. [https://doi.org/10.4103/ijpvm.IJPVM\\_278\\_19](https://doi.org/10.4103/ijpvm.IJPVM_278_19) [PubMed] [PMC]
- Antoni FA (2022) The case for clinical trials with novel GABAergic drugs in diabetes mellitus and obesity. *Life (Basel, Switzerland)* 12(2): 322. <https://doi.org/10.3390/life12020322> [PubMed] [PMC]
- Arrojo e Drigo R (2021) Probing  $\beta$ -Cell biology in space and time. *Diabetes* 70(10): 2163–2173. <https://doi.org/10.2337/dbi21-0008> [PubMed] [PMC]
- Dai C, Hang Y, Shostak A, Poffenberger G, Hart N, Prasad N, Phillips N, Levy SE, Greiner DL, Shultz LD, Bottino R, Kim SK, Powers AC (2017) Age-dependent human  $\beta$  cell proliferation induced by glucagon-like peptide 1 and calcineurin signaling. *The Journal of Clinical Investigation* 127(10): 3835–3844. <https://doi.org/10.1172/JCI91761> [PubMed] [PMC]
- Dedov II, Shestakova MV, Vikulova OK, Zheleznyakova AV, Isakov MA, Sazonova DV, Mokrysheva NG (2023) Diabetes mellitus in the Russian Federation: Dynamics of epidemiological indicators according to the federal register of diabetes mellitus for the period 2010-2022. *Diabetes Mellitus [Sakharny] Diabet* 26(2): 104–123. <https://doi.org/10.14341/dm13035> [in Russian]
- Donate-Correa J, Martín-Carro B, Cannata-Andía JB, Mora-Fernández C, Navarro-González JF (2023) Klotho, oxidative stress, and mitochondrial damage in kidney disease. *Antioxidants (Basel, Switzerland)* 12(2): 239. <https://doi.org/10.3390/antiox12020239> [PubMed] [PMC]
- Gao W, Guo L, Yang Y, Wang Y, Xia S, Gong H, Zhang BK, Yan M (2022) Dissecting the crosstalk between Nrf2 and NF- $\kappa$ B response pathways in drug-induced toxicity. *Frontiers in Cell and Developmental Biology* 9: 809952. <https://doi.org/10.3389/fcell.2021.809952> [PubMed] [PMC]
- Indrowati M, Pratiwi R, Rumiya, Astuti P (2017) Levels of blood glucose and insulin expression of beta-cells in streptozotocin-induced diabetic rats treated with ethanolic extract of artocarpus altilis leaves and GABA. *Pakistan Journal of Biological Sciences: PJBs* 20(1): 28–35. <https://doi.org/10.3923/pjbs.2017.28.35> [PubMed]
- Liu W, Son DO, Lau HK, Zhou Y, Prud'homme GJ, Jin T, Wang Q (2017) Combined oral administration of GABA and DPP-4 inhibitor prevents beta cell damage and promotes beta cell regeneration in mice. *Frontiers in Pharmacology* 8: 362. <https://doi.org/10.3389/fphar.2017.00362> [PubMed] [PMC]
- Maltese G, Psefteli PM, Rizzo B, Srivastava S, Gnudi L, Mann GE, Siow RC (2017) The anti-ageing hormone klotho induces Nrf2-mediated antioxidant defences in human aortic smooth muscle cells. *Journal of Cellular and Molecular Medicine* 21(3): 621–627. <https://doi.org/10.1111/jcmm.12996> [PubMed] [PMC]
- Prud'homme GJ, Glinka Y, Kurt M, Liu W, Wang Q (2017) The anti-ageing protein Klotho is induced by GABA therapy and exerts protective and stimulatory effects on pancreatic beta cells. *Biochemical and Biophysical Research Communications* 493(4): 1542–1547. <https://doi.org/10.1016/j.bbrc.2017.10.029> [PubMed]
- Prud'homme GJ, Glinka Y, Udovik O, Hasilo C, Paraskevas S, Wang Q (2014) GABA protects pancreatic beta cells against apoptosis by increasing SIRT1 expression and activity. *Biochemical and Biophysical Research Communications* 452(3): 649–654. <https://doi.org/10.1016/j.bbrc.2014.08.135> [PubMed]
- Prud'homme GJ, Kurt M, Wang Q (2022) Pathobiology of the klotho antiaging protein and therapeutic considerations. *Frontiers in Aging* 3: 931331. <https://doi.org/10.3389/fragi.2022.931331> [PubMed] [PMC]
- Robertson RP (2023) Nrf2 and antioxidant response in animal models of type 2 diabetes. *International Journal of Molecular Sciences* 24(4): 3082. <https://doi.org/10.3390/ijms24043082> [PubMed] [PMC]
- Soltani N, Qiu H, Aleksic M, Glinka Y, Zhao F, Liu R, Li Y, Zhang N, Chakrabarti R, Ng T, Jin T, Zhang H, Lu WY, Feng ZP, Prud'homme GJ, Wang Q (2011) GABA exerts protective and regenerative effects on islet beta cells and reverses diabetes. *Proceedings of the National Academy of Sciences of the United States of America* 108(28): 11692–11697. <https://doi.org/10.1073/pnas.1102715108> [PubMed] [PMC]
- Sun H, Saeedi P, Karuranga S, Pinkepank M, Ogurtsova K, Duncan BB, Stein C, Basit A, Chan JCN, Mbanya JC, Pavkov ME, Ramachandran A, Wild SH, James S, Herman WH, Zhang P, Bommer C, Kuo S, Boyko EJ, Magliano DJ (2022) IDF diabetes atlas: Global, regional and country-level diabetes prevalence estimates for 2021 and projections for 2045. *Diabetes Research and Clinical Practice* 183: 109119. <https://doi.org/10.1016/j.diabres.2021.109119> [PubMed]
- Typiak M, Piwkowska A (2021) Antiinflammatory actions of klotho: Implications for therapy of diabetic nephropathy. *International Journal of Molecular Sciences* 22(2): 956. <https://doi.org/10.3390/ijms22020956> [PubMed] [PMC]
- van der Horst D, Carter-Timothe ME, van Grevenynghe J, Laguerre N, Dinkova-Kostova AT, Olgarnier D (2022) Regulation of innate immunity by Nrf2. *Current Opinion in Immunology* 78: 102247. <https://doi.org/10.1016/j.coi.2022.102247> [PubMed]
- Zulkiewicz-Kucharska A, Wikiera B, Noczyńska A (2021) Soluble klotho is decreased in children with type 1 diabetes and correlated with metabolic control. *Frontiers in endocrinology* 12: 709564. <https://doi.org/10.3389/fendo.2021.709564> [PubMed] [PMC]

## Author Contributions

- **Ivan N. Tyurenkov**, Doctor Habil of Medical Sciences, Professor, corresponding member of the Russian Academy of Sciences, Head of the Laboratory of Pharmacology of Cardiovascular Drugs, Scientific Center of Innovative Medicines with Pilot Production, Volgograd State Medical University, Volgograd, Russia; e-mail: [fibfuv@mail.ru](mailto:fibfuv@mail.ru); **ORCID ID** <http://orcid.org/0000-0001-7574-3923>. The author was responsible for the idea and planning of the work structure.
- **Dmitry A. Bakulin**, Candidate of Sciences (Medicine), Senior Researcher, Laboratory of Pharmacology of Cardiovascular Drugs, Scientific Center of Innovative Medicines with Pilot Production, Volgograd State Medical

University, Volgograd, Russia; e-mail: [mbfdoc@gmail.com](mailto:mbfdoc@gmail.com); **ORCID ID** <http://orcid.org/0000-0003-4694-3066>. The author was responsible for modeling and control of pathology, design of the final version of the manuscript.

- **Yulia I. Velikorodnaya**, researcher of Laboratory of Pathomorphology, Volgograd Medical Research Center, Volgograd, Russia; e-mail: [Alta-u@mail.ru](mailto:Alta-u@mail.ru); **ORCID ID** <http://orcid.org/0000-0003-2976-6352>. The author was responsible for histochemical staining and assessment of morphological changes, analysis and description of the results.
- **Alexander V. Borisov**, Candidate of Sciences (Medicine), researcher of Laboratory of Pathomorphology, Volgograd Medical Research Center, Volgograd, Russia; email: [borissow1978@rambler.ru](mailto:borissow1978@rambler.ru); **ORCID ID** <http://orcid.org/0000-0003-0202-00082>. The author was responsible for carrying out enzyme immunoassay and describing the results.
- **Elizaveta E. Abrosimova**, graduate student, Department of Pharmacology and Pharmacy, Volgograd State Medical University, Volgograd, Russia; e-mail: [Abrosimova.volgmed@gmail.com](mailto:Abrosimova.volgmed@gmail.com); **ORCID ID** <http://orcid.org/0000-0002-6472-6906>. The author was responsible for carrying out enzyme immunoassay and describing the results.
- **Alexey V. Smirnov**, Doctor Habil. of Medical Sciences, Professor, Head of the Department of Pathological Anatomy, Volgograd State Medical University, Volgograd, Russia; e-mail: [alexeysmirnov.volggmu@gmail.com](mailto:alexeysmirnov.volggmu@gmail.com); **ORCID ID** <https://orcid.org/0000-0001-5351-6105>. The author was responsible for studying the effect of the compositions on the functional state of the pancreas.
- **Grigoriy L. Snigur**, Doctor Habil. of Medical Sciences, Associate Professor, Head of the Department of Biology, Volgograd State Medical University, Volgograd, Russia; e-mail: [sgrigoryl@mail.ru](mailto:sgrigoryl@mail.ru); **ORCID ID** <https://orcid.org/0000-0002-8612-6186>. The author was responsible for studying the effect of the compositions on the functional state of the pancreas and performing immunofluorescence microscopy.
- **Svyatoslav S. Surin**, Senior Lecturer at the Department of Biology, Volgograd State Medical University, Volgograd, Russia; e-mail: [svyatoslavsurin@gmail.com](mailto:svyatoslavsurin@gmail.com); **ORCID ID** <https://orcid.org/0000-0002-7770-5995>. The author was responsible for studying the effect of the compositions on the functional state of the pancreas and performing immunofluorescence microscopy.
- **Daria A. Kavalerova**, Senior Lecturer at the Department of Biology, Volgograd State Medical University, Volgograd, Russia; e-mail: [dareznikova@yandex.ru](mailto:dareznikova@yandex.ru); **ORCID ID** <https://orcid.org/0000-0003-4525-3688>. The author was responsible for studying the effect of the compositions on the functional state of the pancreas and performing immunofluorescence microscopy.
- **Olga S. Vasilyeva**, Candidate of Sciences (Chemistry), Senior Researcher, Laboratory of Nitro Compounds, Herzen Russian State Pedagogical University, St. Petersburg, Russia; e-mail: [ovasja@gmail.com](mailto:ovasja@gmail.com); **ORCID ID** <https://orcid.org/0000-0002-0309-1580>. The author was responsible for the development of the studied compounds.