EFFECT OF THE TRIMETAZIDINE DERIVATIVE S-15176 DIFUMARATE SALT ON THE LEUKOCYTE FORMULA AND CYTOCHEMICAL INDICES OF THE PERIPHERAL BLOOD NEUTROPHILS OF C57BL/6 MICE IN EXPERIMENTAL TYPE II DIABETES MELLITUS

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Abstract. Type 2 diabetes mellitus (T2D) is one of the most common endocrine diseases in the world. It is characterized by dysfunction of pancreatic β-cells, insulin resistance, and hyperglycemia. Despite numerous studies on the pathogenesis and risk factors of diabetes, there is still no consensus on how best to diagnose, classify, and treat the disease. Accumulating evidence suggests that antidiabetic drugs can course unwanted side effects. S-15176 difumarate salt, a novel derivative of trimetazidine, inhibits the rate-limiting enzyme of fatty acid β-oxidation carnitine palmitoyltransferase I and has anti-ischemic properties, which makes it useful as an antidiabetic agent. Here, the effect of chronic treatment with S-15176 difumarate salt on the leukocyte formula and cytochemical indices of the peripheral blood neutrophils (myeloperoxidase activity, free phospholipids, and lysosomal cationic proteins) of C57Bl/6 mice in control and experimental T2D was studied. It was found that in the control group, S-15176 difumarate salt decreased the number of neutrophils containing lysosomal cationic proteins and the cytochemical coefficient of the cells. The treatment of diabetic mice with S-15176 had no significant effect on the activity of myeloperoxidase and free phospholipids in neutrophils. The results obtained suggest that the use of S-15176 difumarate salt as an antidiabetic drug does not induce changes in peripheral blood markers associated with immune-related adverse effects.

Keywords: type 2 diabetes, S-15176, leukocyte formula, myeloperoxidase, lysosomal cationic proteins.

List of Abbreviations
T2D – type 2 diabetes
MCC – mean cytochemical coefficient
STZ – streptozotocin
DMSO – dimethylsulfoxide
LCP – lysosomal cationic proteins

Introduction

For many years there has been no consensus on the best way to determine, diagnose and classify T2D, although research to identify risk factors for diabetes has been progressing for a long time. It's well-known that not all population groups are equally susceptible to this disease (Fletcher *et al*., 2002), i. e. in high-income countries, the rate of undiagnosed diabetes patients is as high as 30% (Henning, 2018).

Diabetes is a general term for heterogeneous metabolic disorders with chronic hyperglycemia being the most common symptom. Impaired insulin secretion and/or malfunction of its action is one of the causes of this disease (Kerner & Brückel, 2014). Type 2 diabetes is one of the most common metabolic disorders caused by a combination of two main factors: insufficient insulin secretion by pancreatic β-cells and the inability of insulin-susceptible tissues to react to insulin (Galicia-Garcia *et al*., 2020).

In connection with the above, the purpose of the current study is to assess some parameters of peripheral blood leukocytes in mice with induced type 2 diabetes and during its correction with the novel anti-ischemic drug S-15176 difumarate salt.

Methods

The work was done in the Laboratory of Mitochondrial Transport of Institute of Theoretical

and Experimental Biophysics Russian Academy of Sciences and at the Department of Biochemistry, Cell Biology and Microbiology Mari State University.

The object of the study was blood leukocytes of C57Bl/6 mice with induced type 2 diabetes.

The total number of mice was 21. Only male mice aged 4 to 5 weeks and weighing 14-16 g were involved in the study.

During the research, all mice were divided into four groups:

1. Control mice (C) (n = 5).
2. Control mice treated with the drug S-15176 (C + S-15176) (n = 4).
3. Mice with induced type 2 diabetes (T2D) (n = 6).
4. Mice with induced type 2 diabetes and treated with the drug S-15176 (T2D + S-15176) (n = 6).

To induce type 2 diabetes, the mice were fed a high-fat diet for four weeks. This was followed by daily administration of a low dose of streptozotocin (STZ) intraperitoneally (35 mg/kg body weight, freshly made before the injection) dissolved in ice-cold 0.1M citrate buffer (pH 4.5) for five consecutive days. On day 33, the diet was cancelled, and then mice were kept on a normal balanced diet for four weeks.

Control mice were kept on a low-fat diet and drinking water. Starting from day 40 mice from group 2 and 4 were treated with S-15176 (1.5 mg per 1 kg body weight per day) intraperitoneally for 20 days (Belosludtseva *et al*., 2020). The drug was dissolved in dimethylsulfoxide (DMSO). Glucose concentration was determined using One Touch Select Plus (LifeScan Inc, Switzerland).

Blood was collected in an Eppendorfs after sacrificing mice by decapitation; after that smears were prepared according to generally accepted methods. To calculate leukocyte formula smears were stained according to Romanovsky. To determine myeloperoxidase activity and free phospholipids content standard assays «Diachim-Cyto-Stain» (Saint-Petersburg, Russia) were used. Determination of lysosomal cationic proteins in the cytoplasm of leukocytes was made with bromophenol blue (Shubich, 1974).

Assessment of cytochemical reactions was made with semi-quantitative method proposed by Kaplow (Hayhou & Kvaglino, 1993). For all reactions 100 neutrophils were counted under an immersion magnification microscope and for each cell degree of color intensity was determined. The absence of color in the cytoplasm was taken as a zero degree; the presence of single colored grains in the cytoplasm was taken as the first degree; if the test substance filled almost the entire cell those reactions were classified as the second degree; reactions in which intensely colored grains filled the entire cytoplasm completely (often covering the nucleus as well) were taken as the third degree.

The proportion of positively reacting cells was determined by subtracting from 100 observed cells the number of cells in which there were no reactions.

For an objective assessment of the true picture of cellular metabolism the mean cytochemical coefficient (MCC) was calculated with the following formula:

$$MCC = (0a + 1b + 2c + 3d) / 100,$$

where the numbers designate the intensity of the color while the letters designate cell count (%) with positive reaction.

All protocols are approved by the Ethics Committee of the Institute of Theoretical and Experimental Biophysics Russian Academy of Sciences (protocol № 13/2020 from 17.02.2020).

Statistical analysis was carried out using R software package (version 4.1.0) and the following sub-packages: ggplot2 (version 3.3.3), FSA (version 0.8.32). To perform multiple pairwise comparisons Dunn's Kruskal-Wallis test with Benjamini-Hochberg adjustments was used because of small sample sizes and non-normal distribution (preliminary checked with Shapiro-Wilk test for normality).

Results

To begin with, the success of diabetes induction was established. The blood glucose level in mice of all control groups ranges from 9 to 10 mmol/L (Fig. 1) and it is significantly lower than in the corresponding groups of diabetic animals (p = 0.0126 and p = 0.0149) and those

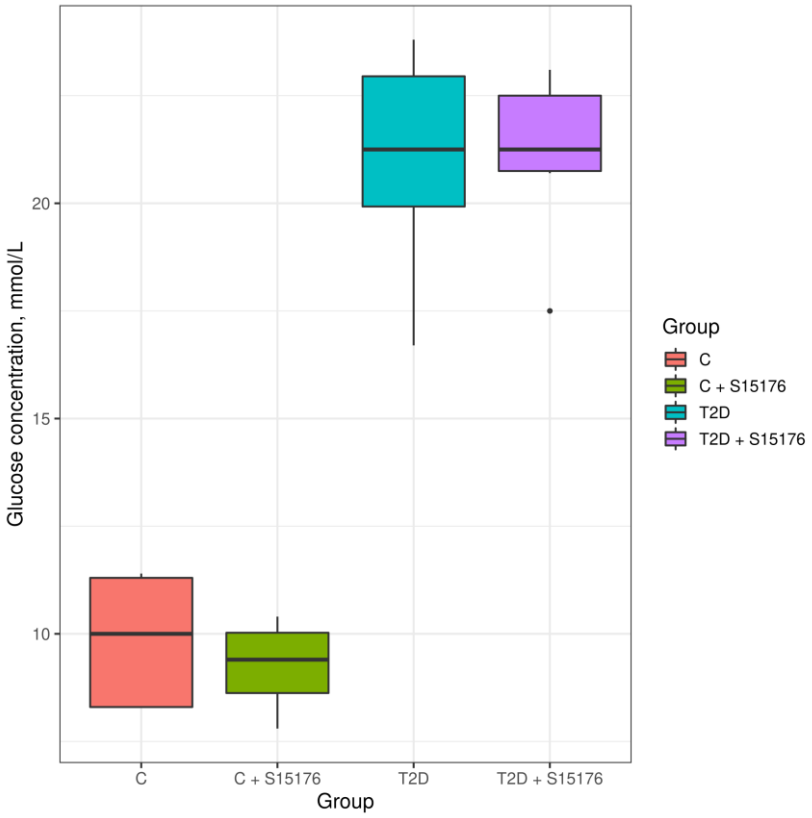

Fig. 1. Glucose concentration in blood (mmol/L)

Table 1

Dunn (1964) Kruskal-Wallis multiple comparison of glucose concentration in blood between mice groups under study

Comparison	*p*-value
C – C + S15176	0.8947
C – T2D	0.0126
C + S15176 – T2D	0.0149
C – T2D + S15176	0.0168
C + S15176 – T2D + S15176	0.0297
T2D – T2D + S15176	1.0000

treated for diabetes ($p = 0.0168$ and $p = 0.0297$). When the drug was administered to control and diabetic mice, the glucose level did not change (Table 1).

At the next stage, the leukocyte formula was calculated. It was found that the proportions of stab neutrophils in all studied groups of mice did not differ (Fig. 2A). An exception is the blood leukoformula of diabetic mice treated with the drug, where a decrease in the proportion of these cells was noted ($p = 0.0489$).

When determining the proportion of segmented neutrophils, it was found that the administration of S-15176 to control animals leads to a significant increase in the proportion of these cells relative to that of both groups of

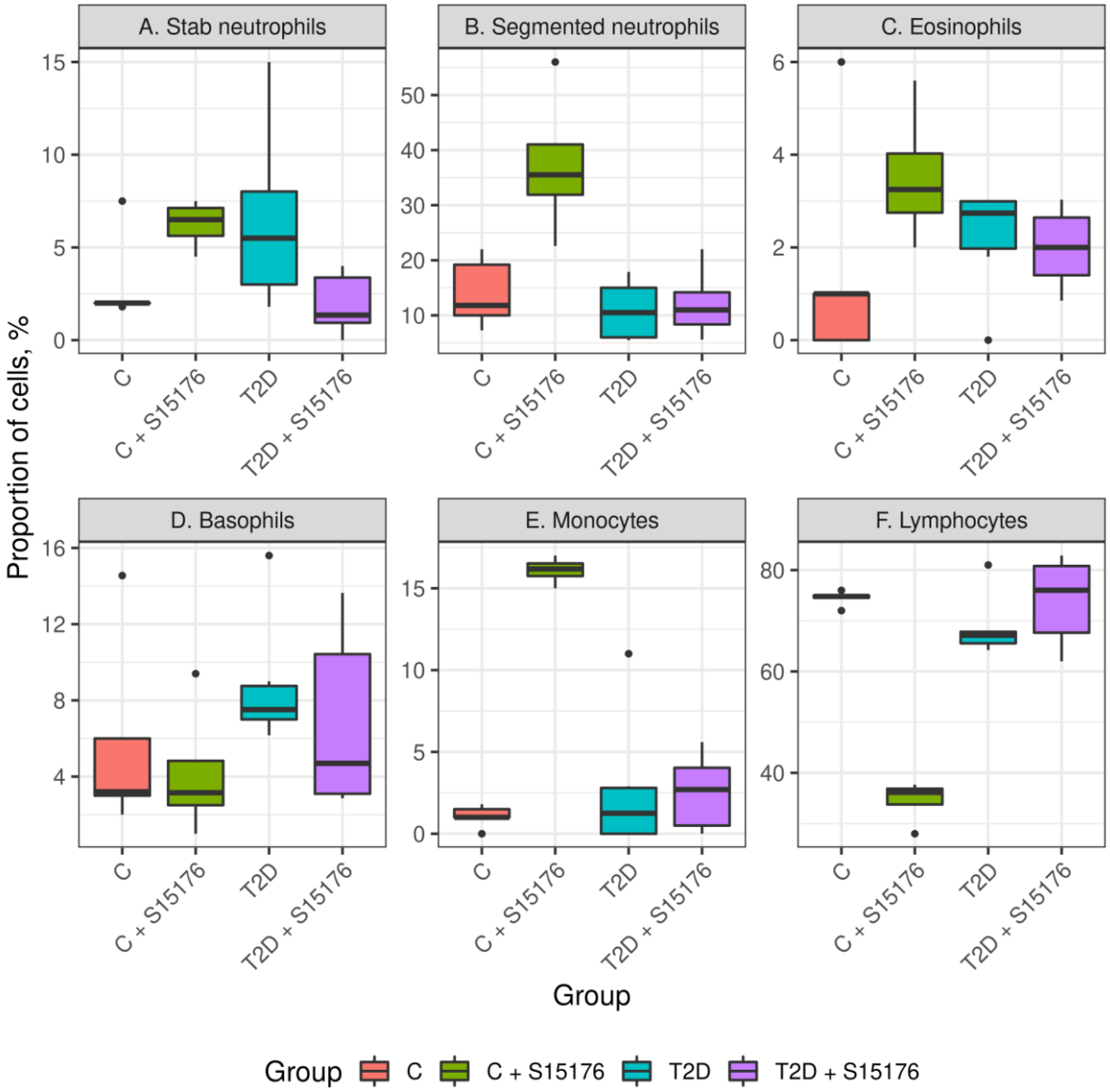

Fig. 2. Proportion of cells in total leukocyte formula (%)

diabetic mice ($p = 0.0163$ and $p = 0.0278$). The drug had no effect on the proportion of these cells in treated mice (Fig. 2B).

The content of eosinophils and basophils is the same in all four studied groups of mice (Fig. 2C, 2D). The effect of the drug on the proportion of these cells has not been established.

When determining monocytes proportion in the leukoformula, it was found that with the introduction of the drug S-15176, an increase in the proportion of these cells in the control group was observed up to 16%, which significantly differs from the indicator of all other groups ($p = 0.0212$; $p = 0.0198$; $p = 0.0460$) (Fig. 2E).

In terms of the lymphocyte content, the leukoformula of the control group of healthy mice treated with the drug differs in the lowest content of these cells (decrease down to 33%) from the control group of healthy mice ($p = 0.0137$), as well as from diabetic mice treated with the drug ($p = 0.0153$) (Fig. 2F).

In terms of the proportion of neutrophils containing myeloperoxidase, as well as its average cytochemical coefficient, no significant

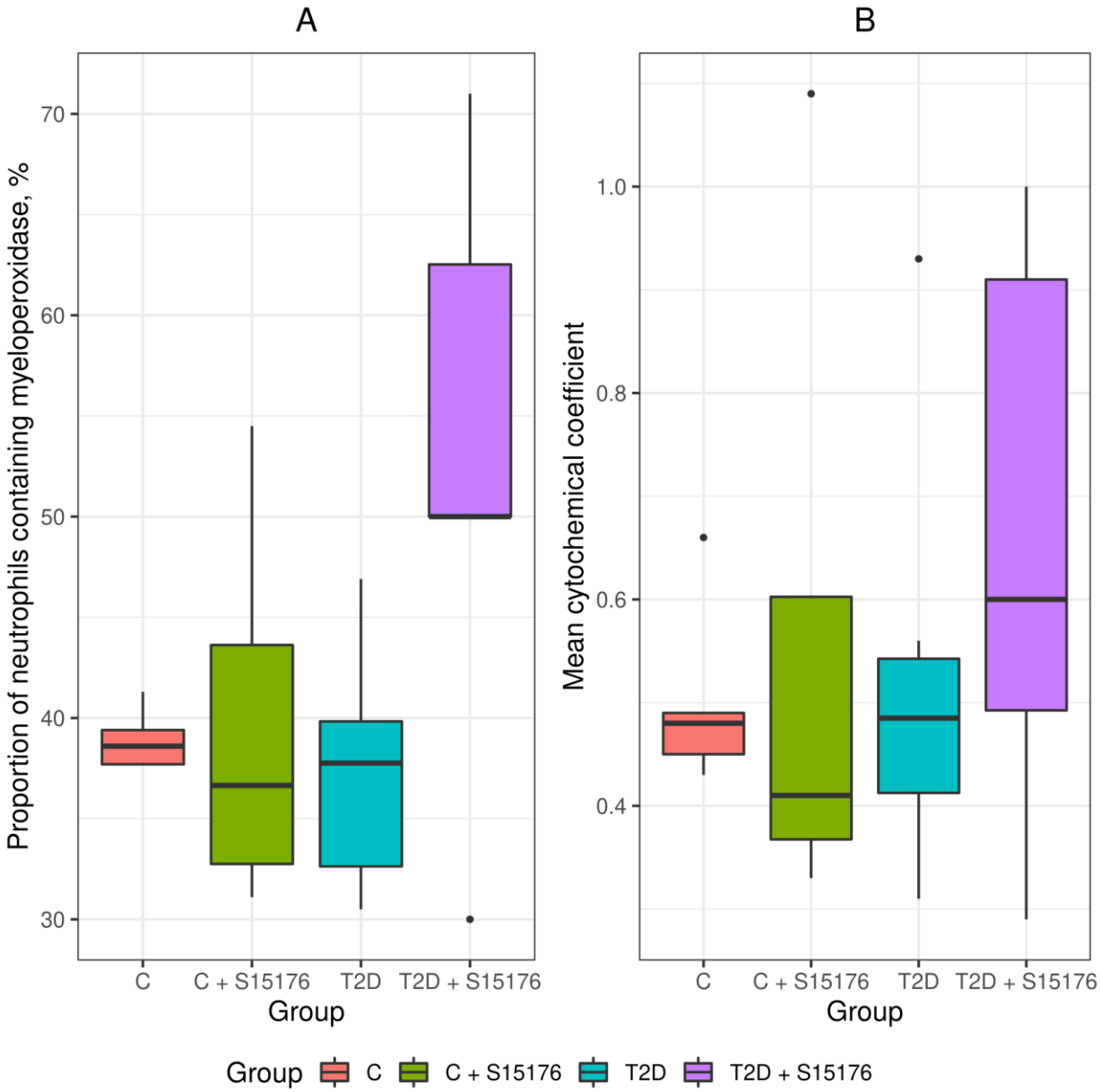

Fig. 3. Proportion of neutrophils containing myeloperoxidase (%) and its mean cytochemical coefficient

differences were found between the groups of mice under study. (Fig. 3A, 3B).

The diabetic group of mice treated with the drug significantly differs in the greater proportion of neutrophils containing phospholipids from the control group of healthy mice, as well as from the control group that received the drug (Table 2; Fig. 4A). In addition, in the control group that received the drug S-15176, the value of the indicator was significantly lower than in the diabetic group of mice that did not receive therapy. In terms of the average cytochemical coefficient, significant differences were observed only between the control group of healthy mice and the groups of diabetic mice, in which the value of the coefficient was significantly higher (Table 3; Fig. 4B).

This drug affects the content and activity of lysosomal cationic proteins. Significant differences both in the proportion of neutrophils containing lysosomal cationic proteins and in the corresponding MCC were found only between

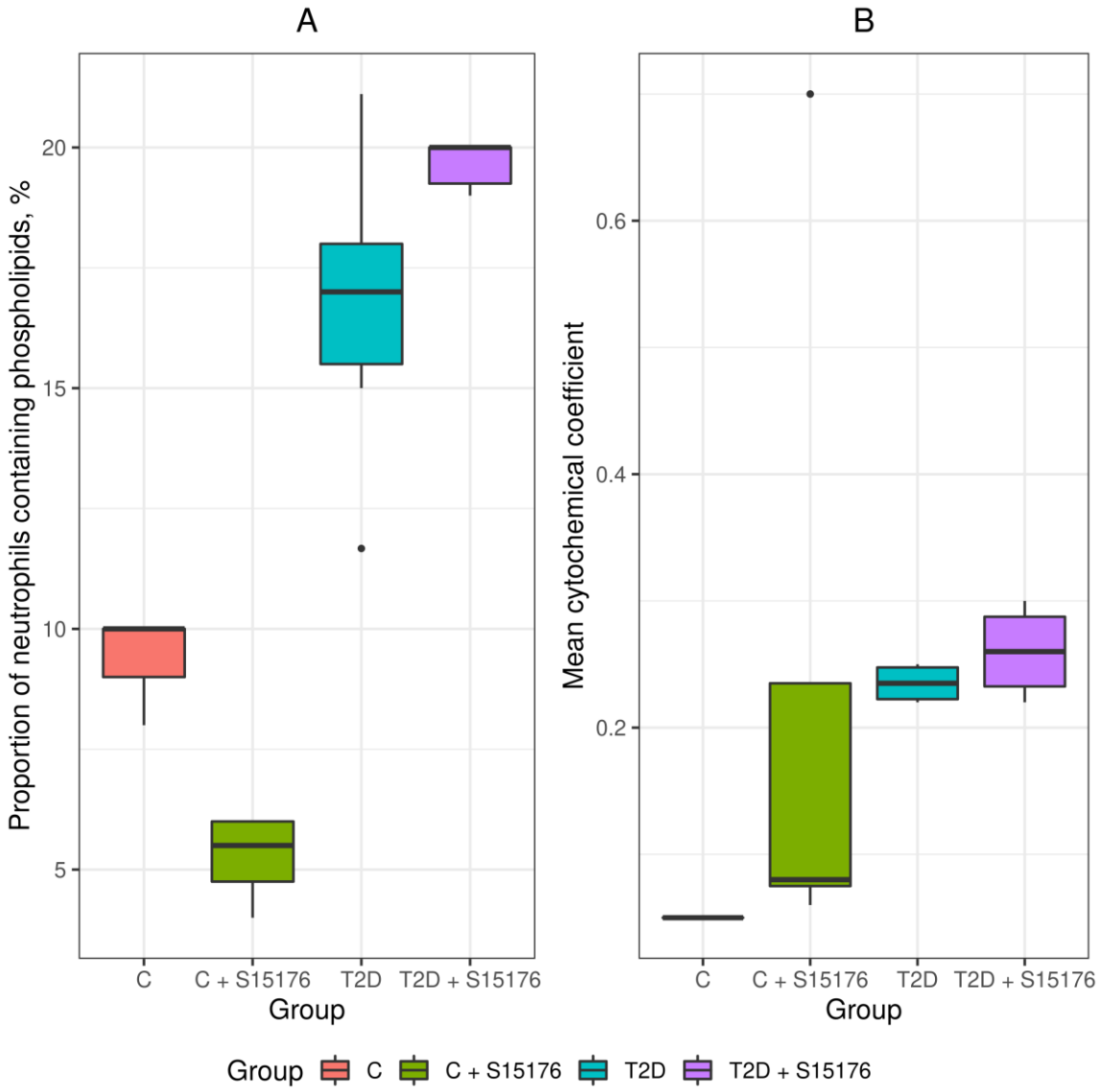

Fig. 4. Proportion of neutrophils containing free phospholipids (%) and its mean cytochemical coefficient

Table 2

Dunn (1964) Kruskal-Wallis multiple comparison of proportion of neutrophils containing free phospholipids between mice groups under study

Comparison	*p*-value
C – C + S15176	0.2770
C – T2D	0.1229
C + S15176 – T2D	0.0115
C – T2D + S15176	0.0149
C + S15176 – T2D + S15176	0.0010
T2D – T2D + S15176	0.3138

Table 3

Dunn (1964) Kruskal-Wallis multiple comparison of mean cytochemical coefficient of neutrophils containing free phospholipids between mice groups under study

Comparison	*p*-value
C – C + S15176	0.1380
C – T2D	0.0165
C + S15176 – T2D	0.5703
C – T2D + S15176	0.0040
C + S15176 – T2D + S15176	0.2894
T2D – T2D + S15176	0.5110

Table 4

Dunn (1964) Kruskal-Wallis multiple comparison of proportion of neutrophils containing lysosomal cationic proteins between mice groups under study

Comparison	*p*-value
C – C + S15176	0.4781
C – T2D	0.0622
C + S15176 – T2D	0.0067
C – T2D + S15176	0.4674
C + S15176 – T2D + S15176	0.8190
T2D – T2D + S15176	0.0093

Table 5

Dunn (1964) Kruskal-Wallis multiple comparison of mean cytochemical coefficient of neutrophils containing lysosomal cationic proteins between mice groups under study

Comparison	*p*-value
C – C + S15176	0.2536
C – T2D	0.1600
C + S15176 – T2D	0.0128
C – T2D + S15176	0.4278
C + S15176 – T2D + S15176	0.5726
T2D – T2D + S15176	0.0152

the diabetic group of mice and the groups receiving the drug (Tables 4, 5). A significant decrease in the proportion of cells with these proteins was revealed in diabetic mice treated with the indicated drug (approximately from 42% to 25%, p = 0.0093) (Fig. 5A). A similar pattern was observed for the dynamics of MCC of cationic proteins (p = 0.0152; Fig. 5B). In the control mice treated with this drug, only a tendency towards a decrease in the indicated cytochemical parameters was noted (Tables 4, 5).

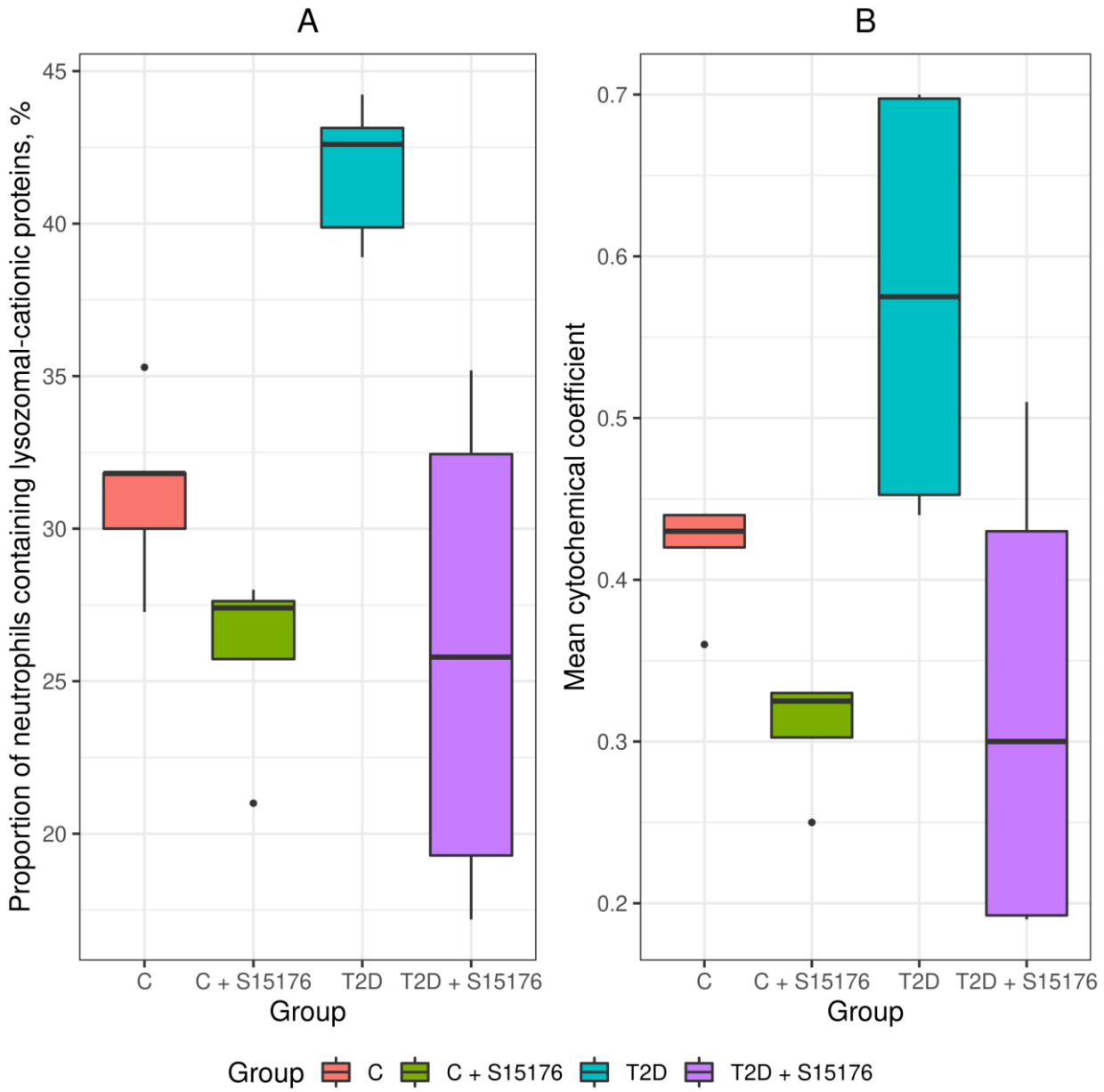

Fig. 5. Proportion of neutrophils containing lysosomal cationic proteins (%) and its mean cytochemical coefficient

Discussion

In the course of our experiment, type 2 diabetes was modeled in C57Bl/6 mice. The blood glucose level in mice of both diabetic groups is significantly higher (approximately 2 times) than in the control groups. Studies have shown that S-15176 has no effect on lowering blood glucose levels in both control and diabetic mice.

The administration of S-15176 to control mice causes a changes in the leukocyte formula (an increase in neutrophils and monocytes is noted), in which the lymphoid profile of the blood of the mice is reduced. The observed rearrangements of the leukoformula are possibly aimed at increasing the level of natural resistance by increasing the proportion of cells with pronounced anti-inflammatory effects. However, in diabetic mice treated with this drug, such rearrangements were not observed. Although it is known that neutrophilic leuko-

cytes are involved in the pathogenesis of diabetes, and an increase in their number may be observed (Zach, 2016).

We did not find any effect of S-15176 on the cytochemical parameters of myeloperoxidase. It is possible that this enzyme is not at all associated with the pathogenesis of diabetes, although, as it seems to us, its cytochemical parameters in diabetic mice could differ from those of other groups of mice. At the same time, it is known that the activity of neutrophils, which is largely due to the activity of this particular enzyme, depends on the duration of diabetes and the content of glucose in the blood (Tarabrina *et al*., 2017).

The cytochemical assessment of the lipid content revealed a tendency for their increase when the drug was administered to diabetic mice. Lipids in leukocytes function as a building material. They are part of specific neutrophilic granules and take part in the construction of other cellular organelles. The obtained shifts in the values of phospholipids in diabetic mice can be regarded as a compensatory response in this pathology.

The drug S-15176, when administered to control mice, leads to a tendency to reduce the proportion of neutrophils containing LCP and the value of their MCC. In mice with diabetes, when this drug is administered, a significant decrease in the values of these parameters is observed, which may adversely affect the state of the body. It is well-known that lysosomal cationic proteins are responsible for the nonspecific defense of the body against foreign agents. The main function of LCP is the modulation of enzymatic processes in the cell and a strong bactericidal effect based on the disruption of the structure and function of the membranes of the microbial cell (Pigarevsky, 1978). Deficiency of cationic proteins or their insufficient synthesis leads to a decrease in the functional activity of leukocytes (Kolesnik *et al*., 2019).

When exposed to the drug S-15176, a decrease in the activity of these proteins is observed, which may lead to a decrease in the level of the body's natural resistance. This, in turn, can exacerbate the pathogenesis of diabetes.

The data obtained require further studies to study the effect of this drug on other parameters of leukocytes and blood in general and, probably, can be useful at the stages of preclinical trials of new drugs.

Conclusions

1. Type 2 diabetes (T2D) was modeled in C57Bl/6 mice, which was confirmed by the results of glucose determination. The introduction of the drug S-15176 does not affect the concentration of glucose in the blood.

2. When S-15176 is administered to the control group of mice, the structure of the leukocyte formula changes, which is manifested by an increase in the proportion of segmented neutrophils and monocytes and a decrease in the proportion of lymphocytes. The drug S-15176 does not affect the structure of the leukoformula in diabetic mice.

3. It was established that the proportion of neutrophils containing myeloperoxidase, free phospholipids, lysosomal cationic proteins and the corresponding average cytochemical coefficients are characterized by relatively low values and do not differ in healthy and diabetic mice. An exception is the MCC value of phospholipids, which is higher in neutrophils from diabetic mice. The proportion of cells containing LCP and their MCC decreases in both S-15176-treated control and S-15176-treated diabetic mice.

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