

Original Article

Changes in the Expression of Regulatory MicroRNAs – miR-21 and miR-155 – in Gut-Associated Lymphoid Tissue Cells of Rats with Streptozotocin-Induced Diabetes and After the Administration of a Non-Selective TNF- α Blocker

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Abstract

Introduction: The development of type 1 diabetes can be triggered by genetic predisposition as well as changes occurring in the gut-associated lymphoid tissue. This study aimed to investigate the transcriptional activity of the miR-21 and miR-155 genes in gut-associated lymphoid tissue cells of rats with streptozotocin-induced diabetes, both untreated and treated with pentoxifylline, a non-specific blocker of TNF- α . **Material and Methods:** Experimental diabetes mellitus was induced by single intraperitoneal administration of streptozotocin at a dose of 50 mg/kg body weight. Pentoxifylline was administered orally at a dose of 9 mg/kg body weight for 2 or 4 weeks from the first day of streptozotocin-induced diabetes. The expression of miR-21 and miR155 genes was studied using real-time quantitative polymerase chain reaction. **Results:** Streptozotocin-induced diabetes led to the transcriptional induction of the miR-21 and miR155 genes. Pentoxifylline administration to the experimental animals led to the 3-fold downward trend of miR-21 gene expression on day 28 of the experiment. **Conclusions:** The expression of miR-21 and miR155 genes in immune cells may be used as markers of several autoimmune pathologies progression such as type 1 diabetes due to their effect on the balance of pro- and anti-inflammatory factors.

Keywords: mRNA, diabetes mellitus, pentoxifylline.

Introduction

Diabetes mellitus (DM) is a multifactorial metabolic disorder, characterized by chronic hyperglycemia leading to significant physiological, biochemical, and histological changes in the affected organisms [1-4]. The development of type 1 diabetes (T1D) can be triggered by genetic predisposition as well as changes occurring in the gut-associated lymphoid tissue (GALT) combined with an imbalance in the composition of the intestinal microbiome. These changes are associated with the development of chronic inflammation as a result of the activation of both the innate and adaptive

parts of the immune response [5, 6].

Our previous studies found that the development of streptozotocin-induced diabetes (STZ-induced diabetes) in rats is accompanied by changes in the level of gene expression of the entero-insular axis [7] and cytoarchitectonics of TLR2+ and TLR4+ lymphocytes in GALT [8]. Transcriptional induction of autoimmune tolerance regulator genes [9], differentiation of Th1, Th17, Treg-cells [10] and proinflammatory cytokines [11] play an essential role in the progression of both DM and a range of inflammatory diseases.

At the same time, regulatory microRNAs-miR-21 and miR-155 play an important role in maintaining the



pro- and anti-inflammatory signaling balance and its violations in autoimmune pathologies [12]. miR-21 is one of the most highly expressed members of the small non-coding microRNA family in many mammalian cell types [13]. Recent studies have confirmed a key role of miR-21 in the development of inflammation and the negative regulation of the proinflammatory response. Thus, miR-21 is considered to be one of the key mediators of inflammation in macrophages, and its inhibition in leukocytes modulates the inflammatory response. In essence, miR-21 induction can be seen as a “molecular rheostat” regulating the inflammatory switch. Emerging studies indicate that miR-21 promotes inflammation and plays essential roles during the pathogenesis of autoimmune diseases, including T1D, psoriasis, multiple sclerosis (MS), rheumatoid arthritis (RA), and systemic lupus erythematosus (SLE) [14].

miR-155 is one of the first identified and, to date, the most studied miRNA, and has been linked to various cellular processes such as modulation of immune responses and oncogenesis. Previous studies have identified miR-155 as a crucial positive regulator of Th1 immune response in autoimmune diseases [15]. Different pathogen-associated molecular patterns (PAMPs), alarmins, proinflammatory cytokines (TNF α , IL-1 β , INF γ) are inducers of miR-155 expression, whose generation is intensified in infections and injuries [16]. On the other hand, anti-inflammatory cytokines (IL-10, TGF- β), resolvins, glucocorticoids, and negative post-transcriptional regulators effectively reduce the intensity of miR-155 expression [17].

Because the development of T1D, like other chronic, immunologically-mediated diseases, is closely linked to the overproduction of proinflammatory cytokines, an important way of its correction is to reduce the activation of proinflammatory signaling. One of these critical systemic cytokines is tumor necrosis factor α (TNF α) [18], and its primary source is GALT cells [19].

Thus, this study aimed to investigate the transcriptional activity of the miR-21 and miR-155 genes in GALT cells of rats with streptozotocin-induced diabetes, both untreated and treated with pentoxifylline as a non-specific blocker of TNF- α .

Material and Methods

The experimental animals, white Wistar mature male rats (n=80) obtained from the nursery of

Veterinary Medicine Association Ltd. “Biomodelservis” (Kyiv) were housed under standard conditions, with proper diet and water ad libitum at the animal facility of Zaporizhzhia State Medical University. Animal treatment and all experimental procedures were performed in compliance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes. The study was approved by the Ethical Committee of Zaporizhzhia State Medical University.

Experimental study design comprised five groups: nondiabetic untreated animals - control (group 1; n=16); animals with experimental diabetes mellitus (EDM), 14 days after streptozotocin (STZ) administration (group 2; n=16); animals with EDM, 28 days after STZ administration (group 3; n=16); animals with EDM, 14 days after STZ administration, and treated with pentoxifylline (PTX) (group 4; n=16); animals with EDM, 28 days after STZ administration, and treated with PTX (group 5; n=16).

EDM was induced by a single intraperitoneal administration of STZ (Sigma Chemical, USA) at a dose of 50 mg/kg body weight. Immediately prior to the administration, STZ was dissolved in 0.1 M citrate buffer (pH 4.5). The period from STZ administration to termination of the experiment was interpreted as the duration of EDM. The control group received a corresponding amount of citrate buffer.

Blood glucose concentration was determined using the glucose oxidase method with a BIONIME Rightest TM GM 110 glucometer (Switzerland) 12 hours and then on days 1, 2, 3, 5, 7, 10, 14, and 28 after STZ administration.

Blood samples were taken from the tail vein. Animals with a fasting glucose level of > 8.0 mmol/l were selected for the study. Glucose concentration was determined after 6 hours of starvation on the third day after STZ administration.

PTX was administered orally at a dose of 9 mg/kg body weight for 2 or 4 weeks from the first day of EDM induction. We used PTX, a methylxanthine derivative, and a non-selective phosphodiesterase inhibitor because it has been reported that it might also influence the function of immune cells and the production of cytokines. In particular, PTX was shown to inhibit efficiently TNF- α transcription in various in vitro and in vivo systems [20].

Real-time reverse transcription-polymerase chain reaction (RT-PCR) was used to analyze the expression of genes. Tissue samples (ileum with isolated

lymphoid follicles) embedded in paraffin were cut with a microtome (slice thickness of 15 μ m) and placed in Eppendorf tubes (Eppendorf AG, Germany). The tissue samples were dewaxed by incubation in xylene twice for 5 minutes, then in 100% ethanol twice for 5 minutes. Isolation of total RNA from rat tissues was performed using the Trizol RNA Prep 100 Kit (IZOGEN, RF) according to the manufacturer's protocol.

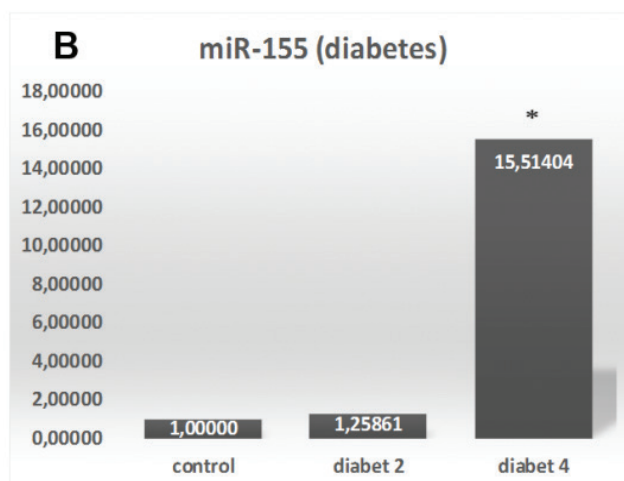
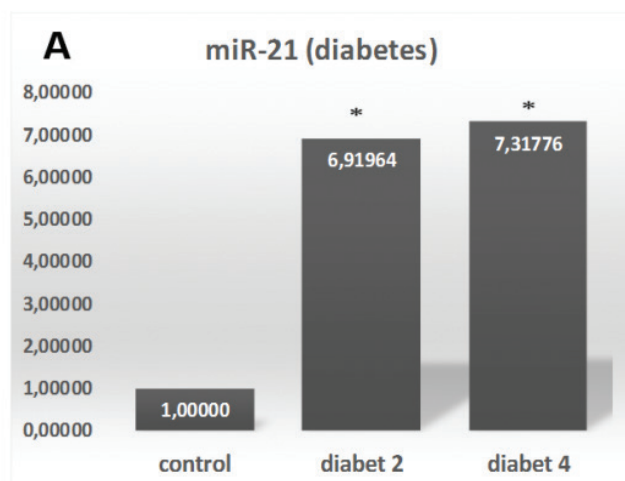
The concentration and quality of isolated total RNA were determined on a Libra S32PC spectrophotometer (Biochrom Ltd., England). For the subsequent reverse transcription procedure, RNA samples were selected with the following parameters: ratio A260/A280 within the range of 1.8-2.2. Reverse transcription (cDNA synthesis) was performed using the TaqMan[®] MicroRNA Reverse Transcription Kit (Life Technologies, USA), specific miR-21 and miR-155 loop primers (rno-mir-21 Stem-loop Sequence: UGUACCACCUUGUCGGGUAGCUUAUCA-GACUGAUGUUGACUGUUGAAUCUCAUGGCAA-CAGCAGUCGAUGGGCUGUCUGACAUUUUGGUAUC; rno-mir-155 Stem-loop Sequence: CUGUUAAGC-UAAUUGUGAUAGGGGUUUUGGCCUCUGACUGA-CUCCUACCUGUUAGCAUUAACAG), small nuclear U6 RNAs (endogenous control) and 10 ng of RNA as an internal control. Real-time quantitative PCR was conducted using TaqMan[®] MicroRNA Assays (Life Technologies, USA): U6 small nuclear RNA (ID 001973) and rno-miR-21-5p (Assay ID 000397, Mature miRNA Sequence: UAGCUUAUCAGACUGAUGUUGA, miRBase Accession Number MIMAT0000790, Chromosome Location: Chr. 10 - 73902210 - 73902301 [-] on Build Rnor_6.0) and rno-miR-155-5p (Assay ID 002571, Mature miRNA Sequence: UUAUUGC-UAAUUGUGAUAGGGGU, miRBase Accession Number MIMAT0030409, Chromosome Location: Chr. 11

- 24176603 - 24176667 [+] on Build Rnor_6.0). The PCR temperature cycles were as follows: initial denaturation for 10 minutes at 95°C and 40 cycles for 15 seconds at 95°C and 60 seconds at 60°C on a CFX 96 thermocycler (Bio-Rad, USA).

PCR data analysis was performed using the CFX Manager[™] software (Bio-Rad, USA). All amplification reactions on individual samples were performed in three replications. All experimental data were processed using Microsoft Excel 2019 (Microsoft Corp., USA) and STATISTICA 13 (TIBCO Software Inc., 2018). For all indices, we calculated the sample mean (M), its variance, and standard error (m). Significance of the differences between the sets of experimental and control groups data was determined using Student's t-test (t), probability of sample means distribution (p), and confidence interval. Statistical significance was set at 0.05.

Results

It was established that relative normalized expression of the miR-21 gene in ileum cells of rats with STZ-induced diabetes significantly increased by 6.9 times on day 14 of the experiment and by 7.3 times on day 28 of the experiment compared to the control group (Figure 1A). PTX administration to the experimental animals led to a downward trend of expression of the miR-21 gene on day 14 of the experiment; however, these changes were not statistically significant (Figure 1C). On day 28 of the STZ-induced diabetes development in rats treated with PTX, a significant decrease in the expression of the miR-21 gene by 3.0 times compared to rats with STZ-induced diabetes was established (Figure 1E).



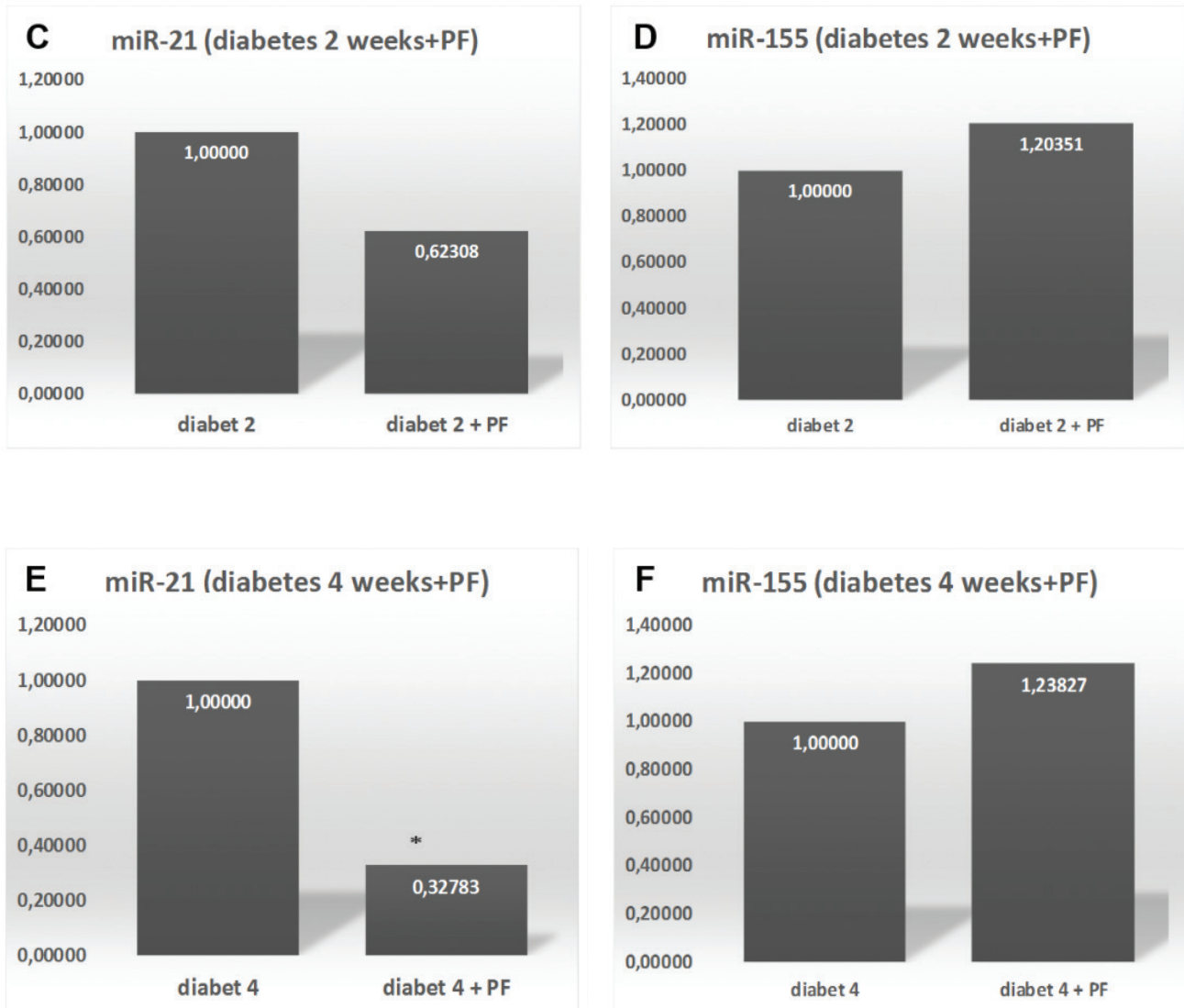


Figure 1 (A-F): Relative normalized number of miR-21 and miR-155 in the rat ileum cells. Normalization using the $\Delta\Delta C_t$ method with U6 as a reference gene. d2, d4: 2nd and 4th week of EDM, respectively; d2 + pentoxifylline, d4 + pentoxifylline: after the administration of pentoxifylline to diabetic animals.

Transcriptional activity of the miR-155 gene in ileum cells of rats with STZ-induced diabetes significantly increased only on day 28 of the experiment by 15.5 times compared to the control group (Figure 1B). PTX administration to the diabetic animals did not lead to statistically significant changes in the transcriptional activity of the miR-155 gene (Figure 1D, 1F).

Discussion

Recently, the mechanisms of cascading pathogenetic changes taking place during the development of an autoimmune pathology, including T1D, received considerable attention [21]. Immune disorders lead to the development of T1D; simultaneously, hyperglycemia increases the autoimmune response, leading to

a “vicious” circle [9]. Regulatory microRNAs play an important role in maintaining the pro- and anti-inflammatory signaling balance in the development of autoimmune pathologies [12]. The MicroRNA Target Prediction Database (miRDB, <http://mirdb.org>) currently lists 234 predicted targets for rno-miR-155-5p. Additional miR-155 targets have been identified in humans. A bioinformatics analysis performed using TargetScan (www.targetscan.org) to investigate miR-155 functional targets, predicted 552 human mRNAs as potential targets of miR-155.

Another database, miRTarBase (<http://mirtarbase.mbc.nctu.edu.tw/index.php>), lists 898 experimentally validated transcripts directly or indirectly modulated by miR-155. In particular, miR-155 is crucial for the differentiation and function of T and B lymphocytes, as well as myeloid cells [22]. Thus, forced

expression of miR-155 in bone marrow cells induces myeloproliferation and tumorigenesis. The transcription of miR-155 increases in response to lipopolysaccharides (LPS), TNF α , and INF β [23]. A review by Mashima presents the most current information on the role of miR-155 in the immune system [24]. During antigen processing following infection, the lack of miR-155 in dendritic cells (DCs) results in a failure to present antigens to T cells. During T cell activation, miR-155-deficient CD4 $^{+}$ T cells increase Th2 subsets in response to IL-4, while miR-155 overexpression promotes Th1 responses after IFN- γ stimulation [25]. During the effector phase of the immune response, miR-155 plays a key regulator role in pathogen scavenging [26]. Kurowska-Stolarska *et al.* have shown a clear inverse correlation of miR-155 expression level in CD14 $^{+}$ monocytes and CD68 $^{+}$ macrophages in patients with rheumatoid arthritis [27]. A study by Ye *et al.* showed that administration of miR-155 inhibitors to male Sprague-Dawley rats with STZ-induced diabetes significantly reduced the intensity of the inflammatory response and resulted in faster wound healing compared to the intact animals [28]. An experiment on mice with the Dicer gene knockout [29] demonstrated the role of miR-155 as a trigger for DM development (Dicer, a ribonuclease III enzyme is required for processing of mature miRNA forms) [30]. Only 10% of male Dicer-KO mice developed DM pathology 25 days after STZ administration, while 70% of wild-type mice underwent DM induction [29]. However, in knockout miR-155KO mice, the homeostatic ratio of Treg regulatory cells and proinflammatory T lymphocyte populations (Th17) is unbalanced, with a shift towards Th2 differentiation. Thus, miR-155KO mice are resistant to the development of experimental autoimmune encephalomyelitis (EAE) caused by Th17 and Th1 differentiation defects, confirming the role of miR-155 in the induction of inflammation.

Recent studies show that miR-21 also plays a prominent role in the regulation of immune functions [31]. Non-activated T lymphocytes and antigen-presenting cells (APCs) have a low level of miR-21 expression, but after their activation, the level of expression changes dramatically [13]. At the same time, by contributing to the induction and maintenance of inflammation, miR-21 plays an essential role in the pathogenesis of several autoimmune diseases, including T1D, psoriasis, multiple sclerosis, rheumatoid arthritis. The expression of miR-21 is induced by LPS, phorbol 12-myristate 13-acetate (PMA), IL-6 through activation of nuclear factor kappa B (NF- κ B) transcription factors

(p65 binds to two miR-21 promoter sites with subsequent positive regulation), AP-1, STAT-3 [32]. Most target genes for miR-21 are tumor suppressors that regulate cell proliferation and activation and have anti-apoptotic activity. Inhibition of T-cell apoptosis occurs through the suppression of PDCD4 [33], TIPE2 [34] and FASL [35]. Furthermore, miR-21 regulates T helper cells, stimulating the Th2 subpopulation's differentiation through inhibition of IL-12p35 gene expression in dendritic cells, and Th17 differentiation through SMAD7 suppression [36]. Homing of T lymphocytes into the secondary lymphoid organs also relies on miR-21 through the inhibition of CCR7 expression [37]. Studies show that miR-21 intensifies the transmission of ERK and JNK signals in activated T cells through suppression of Sprouty1, while overexpression of miR-21 stimulates the activity of activator protein 1 (AP-1) and expression of IL-2. This suggests that miR-21 can increase the T-cell response [38]. Murugaiyan *et al.* found that in miR-21 $-/-$ mice, T-cell differentiation into a Th17 subpopulation is diminished, causing their resistance to EAE [36]. miR-21 induces Th17 differentiation by stimulating SMAD-2/3 activation, inhibiting IL-2 expression, and ultimately upregulating the TGF- β signaling pathway. Dong *et al.* found that in peripheral blood mononuclear cells (PBMC) of patients with RA, the expression level of miR-21 was reduced, but this was accompanied by elevated expression of proinflammatory cytokines (IL-17, IL-22, TNF- α) and STAT3 transcription factor [39]. SMAD-7, a negative regulator of TGF- β signaling and direct miR-21 target, also plays a notable role in this pathway. SMAD-7 can both suppress Th17 differentiation by down-regulating the TGF- β signal cascade and boost it by suppressing Treg differentiation. Thus, in patients with RA, the number of FOXP3 $^{+}$ T cells and miR-21 levels were both significantly reduced. This was accompanied by elevated expression and activation of STAT3, as well as a decrease in the levels of the STAT5/pSTAT5 protein and Foxp3 mRNA [39]. However, in another study, although miR-21 levels were elevated in regulatory T cells (Tregs), inhibition of miR-21 in Tregs altered Foxp3 expression [40]. Research on the role of miR-21 in the pathogenesis of autoimmune diseases opens the possibility of using its inhibitors in the treatment of autoimmune diseases. For instance, in a mouse model of autoimmune encephalomyelitis, inhibited expression of miR-21 led to a decrease in the production of proinflammatory cytokines IL-17A, IL-17F, IL-21, and IL-22, producing a positive treatment outcome [34]. Garchow *et al.* found that inhibition of miR-21 transcriptional activity *in vivo*

contributes to the alleviation of autoimmune splenomegaly in a tri-congenic B6.Sle1.Sle2.Sle3 (B6.Sle123) mouse model [41]. In addition, the expression of miR-21 was significantly upregulated in the PBMC of patients with T1D [42]. Furthermore, the use of anti-miR-21 altered the CD4/CD8 T-cell ratio and decreased Fas receptor expression in the lymphocytes.

In summary, although miR-21 and miR-155 are shown to be significantly upregulated or downregulated in several autoimmune diseases, it remains to be determined whether they can be used as biomarkers for the diagnosis and prognosis of autoimmune disease. The results of this investigation demonstrate that STZ-induced diabetes led to the transcriptional induction of the miR-21 and miR155 genes. PTX administration to the experimental animals led to a downward trend of miR-21 gene expression on day 28 of the experiment. MiR-21 and miR-155 are likely among the triggers for the onset and progression of DM, resulting from their effect on the delicate balance of pro- and anti-inflammatory factors, the level of cytokines, modulation of cell differentiation of both adaptive and innate immunity, and homing of immunocompetent cells.

Conclusions

The results of this investigation demonstrate that the expression of regulatory microRNAs (miR-21 and miR155) genes in immune cells may be used as markers of several autoimmune pathologies progression such as type 1 diabetes mellitus due to their effect on the balance of pro- and anti-inflammatory factors, including the level of cytokines, modulation of cell differentiation of both the adaptive and innate immunity, and homing of immunocompetent cells. Pentoxifylline is a potential therapeutic alternative for the treatment of type 1 diabetes mellitus or other autoimmune pathology characterized by excessive production of proinflammatory cytokines.

Conflict of Interest

The authors declare no conflict of interest.

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