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The Effect of Gliflozin Therapy on TNF-α Secretion by Cultured Monocytes in Patients with Diabetes

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Abstract: Currently it's well established that chronic inflammation is a key pathogenetic mechanism in the development of Type 2 diabetes. The study aims to explore the inflammatory response of monocytes in diabetic patients compared to healthy subjects, focusing on TNF-α and to investigate the impact of SGLT2 inhibitors on monocyte inflammation. The study included 20 patients with newly diagnosed type 2 diabetes and 20 control subjects aged 50-79 years old, all participants provided written informed consent upon the inclusion. The secretion of pro-inflammatory cytokine TNF-α in both non-stimulated and LPS-stimulated conditions by blood-derived monocytes at baseline and after 3 months of dapagliflozin therapy 10 mg daily was assessed. Monocytes were isolated using Ficoll gradient centrifugation and CD14+ cell magnetic separation. TNF-α concentration was measured using ELISA. Diabetic patients showed a significant increase in TNF-α secretion by cultured monocytes (both non-stimulated and LPS-stimulated) compared to control subjects. After a 3-month dapagliflozin therapy, there was a significant reduction in TNF-α secretion. The study concludes that monocytes in diabetic individuals exhibit pro-inflammatory activation. Additionally, the antidiabetic therapy using SGLT2 inhibitors showed efficacy in reducing the pro-inflammatory status of monocytes. This suggests that SGLT2 inhibitors could be beneficial not only for controlling glucose levels but also for preventing diabetes-associated diseases by addressing inflammation.

Keywords: Diabetes Mellitus, Monocytes, Inflammatory Cytokines, Pro-Inflammatory Activation, Immune Tolerance

Introduction

Chronic inflammation has become firmly established as a pivotal pathogenetic mechanism in the progression of Type 2 diabetes (Nedosugova *et al*., 2022). Present-day therapeutic advancements in antidiabetic medications, particularly Sodium Glucose co-Transporter 2 (SGLT2) inhibitors, have demonstrated remarkable efficacy in diabetes treatment. These agents offer multifaceted benefits, prominently their anti-inflammatory properties. Consequently, they are extensively employed in managing and preventing inflammatory complications linked to diabetes, such as atherosclerotic cardiovascular disease and chronic kidney disease (Scisciola *et al*., 2022; Solomon *et al*., 2023). The compelling anti-cytokine effect of SGLT2 inhibitors has been substantiated through animal model studies (Theofilis *et al*., 2022). Several investigations have confirmed the expression of SGLT-2 in monocytes (Semo *et al*., 2023). Therefore, exploring the impact of SGLT-2 inhibitors on cytokine secretion by circulating monocytes is essential to assess their anti-cytokine efficacy in diabetic patients. Circulating monocytes are recognized as primary contributors throughout the continuum of chronic inflammation, being principal producers of inflammatory mediators, including cytokines (Kapellos *et al*., 2019).

Among these cytokines, Tumor Necrosis Factor-alpha (TNF-α) assumes a critical role in the genesis and progression of chronic inflammation in Type 2 diabetes. It stands as a key cytokine implicated in the pathogenesis of insulin resistance, a precursor to diabetes progression (Akash *et al*., 2018). Currently, exploring the inflammatory response of monocytes within the context of trained immunity emerges as an imperative focus in contemporary scientific inquiry. This exploration holds promise as a potential therapeutic target for devising immunomodulatory strategies in the clinical management of chronic inflammatory diseases, including diabetes (Keating *et al*., 2020; Funes *et al*., 2022). The primary objective of this study is to investigate the inflammatory response of circulating monocytes in diabetic patients compared to healthy subjects, particularly in terms of TNF-α. Additionally, the study aims to evaluate the inflammatory activation of monocytes under the influence of SGLT2 inhibitors.

Materials and Methods

Design of the Study

The study included patients aged 50-79 years with newly diagnosed Type 2 diabetes who had not previously received treatment with hypoglycemic preparations and a control group of participants without diabetes, matched by age and gender. Exclusion criteria were cancer diseases, alcoholism, uncontrolled arterial hypertension, chronic heart failure class III-IV NYHA, decompensated renal or hepatic insufficiency, a history of HIV infection, syphilis, viral hepatitis, or tuberculosis. All study participants underwent a clinical and laboratory examination that included the following parameters: Body Mass Index (BMI), family history of diabetes, arterial blood pressure, biochemical indicators of diabetes glucose and glycated Hemoglobin (HbA1c) blood levels, and parameters of lipids profile total cholesterol, triglycerides, High-Density Lipoproteins (HDL), Low Density Lipoproteins (LDL). Blood samples were taken from each study participant. Then, a culture of circulating monocytes/macrophages was isolated to evaluate the immune response of cultured cells to inflammatory stimulation by measuring TNF-α secretion. Diabetic patients were prescribed therapy with dapagliflozin 10 mg/day, after a 3-month treatment period they underwent the second examination. The pro-inflammatory status of monocytes of the studied groups of patients was investigated before the beginning of dapagliflozin therapy and after the 3-month treatment period.

Primary Culture of Circulating Monocytes Macrophages

Circulating monocytes were separated from 30 mL of whole blood by isolating the mononuclear leukocyte fraction at Ficoll gradient centrifugation. Next, CD14+ cells were obtained using LS Columns and paramagnetic nanoparticles CD14+ MicroBeads (Miltenyi Biotec Inc., USA). The isolated monocytes were planted in three wells of a plate and cultivated in X-VIVO serum-free medium at 37°C. Pro-inflammatory stimulation with Lipopolysaccharide (LPS), Escherichia coli serotype O111:B4 (Sigma-Aldrich Co., USA) at a concentration of 1 µg/mL was performed in wells 1 and 2 on the first day. In previous studies the different concentrations of LPS were used for inflammatory stimulation of cultured cells from 100-5000 ng/mg (Edgar *et al*., 2021; Alvarado-Vázquez *et al*., 2018), so in this study, the concentration of 1000 ng/mL was used based on pilot experiments those demonstrated no significant difference in cytokine secretion upon LPS stimulation at a concentration of 10, 100 and 1000 ng/mL. LPS stimulation was not performed in well 3, which was used to assess the basal (non-stimulated) secretion of cytokines. Culture fluid samples were collected after 24 h of incubation after the first LPS-stimulation (LPS-stimulated secretion) for measurements of TNF-α concentrations, then the monocytes were allowed to rest for five days and were restimulated (re-stimulated secretion) on the sixth day of cultivation in well 2. Well, 1 was used to assess the secretion of cytokines without re-stimulation in the second stage of the experiment. On the 7^{th} day of incubation, the viability and number of cells were assessed. After the collection of medium samples, cells attached to the well surface were detached with trypsin and counted (Freshney, 2015). The number of cells on the 7^{th} day of the experiment averaged 400,000 cells, which was 80% of the initially planted cells (500,000 cells in each well). Storage of culture fluid samples was carried out in a freezer at a temperature of -70°C. The concentration of cytokine (pg/mL) in cell culture supernatant samples was determined by the ELISA method (Human TNF-alpha/TNFSF1A DuoSet ELISA, R and D Systems Inc., USA). The sensitivity of the ELISA kit is 2 pg/mL. Pro-inflammatory activation of monocytes was calculated as the ratio of LPS-stimulated and non-stimulated secretion of inflammatory cytokines after 24 h of incubation with and without LPS. The tolerance of immune response was calculated by assessing the re-stimulated secretion of inflammatory cytokines in 24 h after the second LPS stimulation.

Statistical Analysis

The software SPSS Statistics v. 27.0 (SPSS Inc., USA) was used for statistical analysis. Shapiro-Wilk's W test was used to test the type of distribution. Quantitative data are presented as a mean value and standard deviation. Mann-Whitney U-test was used for between-group comparisons of the data in diabetic and control groups, Wilcoxon signed-rank test was used for within-group comparisons to analyze the dynamics of clinical data and inflammatory response of monocytes of diabetic patients after the treatment period. Significance was defined at the 0.05 level of confidence.

Results

Totally 40 participants 20 patients with newly diagnosed diabetes at baseline and 20 control subjects were included in the study. The study was performed in accordance with the principles of the Declaration of Helsinki of 1975 (revised version of 2013) and approved by the local ethics committee of the Sechenov First Moscow State Medical University (protocol #04-21, February 18, 2021. Informed consent was taken from all study participants upon enrollment. The clinical and laboratory characteristics of study participants are presented in Table 1.

Control and group with diabetes had no difference in basic clinical characteristics such as age, gender, BMI, and family history of Type 2 diabetes mellitus. As for laboratory characteristics, the blood levels of glucose and glycated hemoglobin were significantly higher in patients with diabetes as well as disorder of lipid profile, in particular, a significant increase of triglycerides and LDL and reduced blood level of HDL were observed in the group with diabetes. After 3 months of dapagliflozin therapy blood glucose level in patients with diabetes has decreased to 6.3 (0.4) mmol/L, p<0.001, HbA1c has decreased to 6.5 (0.4) %, p = 0.003. Non-significant beneficial changes in lipids profile were also observed under dapagliflozin therapy.

The results of TNF-α secretion measurements in cell culture supernatant are presented in Table 2.

The significant increase of non-stimulated and LPS-stimulated secretion of TNF-α in primary culture of monocytes in patients with diabetes in comparison with control subjects was demonstrated after 24 h of incubation. After 3-months treatment period the secretion of TNF- decreased significantly at all points. The pro-inflammatory activation of cultured monocytes was significantly higher in the control group, 33.4 (25.9) versus 19.9 (14.7) in the group with diabetes, p = 0.038. After 3 months of dapagliflozin therapy, the ratio of LPS-stimulated and basal secretion of TNF-α increased to 26.4 (15.2), but the dynamics weren't statistically significant, p = 0.064. Figure 1 presents the ratio of LPS-stimulated and non-stimulated secretion of TNF-α in the control and group with diabetes at baseline and after the treatment period.

Secretion of TNF-α was significantly higher in a group with diabetes on the 7th day after 24 h re-stimulation with and without LPS. However, the secretion of TNF-α didn't increase in response to the repeat LPS stimulation in comparison with cells without re-stimulation in control as well as in groups with diabetes.

Table 1: Characteristics of study participants

Characteristics	Control group	Group with diabetes	Significance, p
Age, years	57 (4)	59 (6)	0.118
Gender, m/f	6/14	9/11	0.246
BMI, kg/m^2	28.0 (3.200)	30.5 (5.000)	0.069
Family history of diabetes, %	31.0	30.0	0.859
Blood glucose, mmol/L	4.9 (0.400)	8.2 (1.300)	<0.001*
HbA1c, %	5.9 (0.200)	7.1 (0.600)	<0.001*
Total cholesterol, mg/dL	222.4 (26.40)	218.6 (51.20)	0.813
TG, mg/dL	111.6 (42.80)	223.1 (122.0)	0.009*
HDL, mg/dL	133.6 (143.3)	45.7 (13.10)	0.011*
LDL, mg/dL	89.6 (46.80)	144.6 (48.20)	0.004*

Data presented as mean value (standard deviation); BMI, body mass index; HbA1c, glycated hemoglobin; HDL, high-density lipoproteins; TG, triglycerides; LDL, low-density lipoproteins)

Table 2: Secretion of inflammatory cytokines

			Group with diabetes	
TNF-α concentration, pg/mL		Control group	Baseline	After 3 months of dapagliflozin therapy
24 h of incubation	Non-stimulated	169.0 (181.00)	319.3 (277.000)	169.0 (181.00)
	LPS-stimulated	3098.5 (1466.5)	4927.6 (3098.46) 0.042*	3467.6 (2135.8) 0.003**
7th day	Without re-stimulation	119.0 (46.700)	163.1 (55.5000) 0.007*	143.4 (58.300) 0.009**
	after the 2nd stimulation	120.4 (58.300)	159.1 (55.8000) 0.032*	138.2 (34.300) 0.003**

Data presented as mean value (standard deviation) LPS, lipopolysaccharide; *, statistics of difference from control participants, p-value; **, statistics of changes after period of treatment, p-value

Discussion

We conducted a comparative analysis of the non-stimulated and LPS-stimulated secretion of TNF-α to study the pro-inflammatory activation of monocytes. We observed a significant increase in TNF-α secretion by monocytes in diabetic patients, aligning with prior findings demonstrating elevated TNF-α levels in newly diagnosed diabetic patients compared to both healthy subjects and individuals with coronary artery disease (Nikiforov *et al*., 2017). Interestingly, while monocytes from healthy individuals exhibited higher TNF-α activation after LPS stimulation compared to the diabetic group, this was potentially attributed to increased basal secretion and reduced responsivity to pro-inflammatory stimulation among diabetic patients. However, this hypothesis necessitates further investigation. Previous studies assessing pro-inflammatory cytokine secretion in macrophage cultures showcased varied results. For instance, one study examining primary human macrophages found differences in IL-6 secretion between control and diabetic groups, while basal TNF-α and MCP-1 secretion remained comparable (Alvarado-Vázquez *et al*., 2018). Conversely, another study on RAW264.7 macrophages under high-glucose conditions demonstrated a substantial increase in LPS-stimulated pro-inflammatory cytokine secretion compared to normal glucose conditions (Suzuki *et al*., 2021).

The concept of trained immunity in innate immune cells has garnered attention in chronic inflammatory diseases like diabetes mellitus (Bekkering *et al*., 2016; Charles-Messance and Sheedy, 2021; Funes *et al*., 2022; Naruse, 2022). Studies suggest that initial pro-inflammatory stimulation might induce a 'trained' state in cells, leading to either tolerance or heightened inflammatory responses upon subsequent stimulations (Bekkering *et al*., 2016; Nikiforov *et al*., 2019). Studies suggest that initial pro-inflammatory stimulation might induce a 'trained' state in cells, leading to either tolerance or heightened inflammatory responses upon subsequent stimulations (Thiem *et al*., 2021). Such triggers, including hyperglycemia, could potentially induce epigenetic changes, fostering a trained macrophage phenotype (Thiem *et al*., 2019; Choudhury *et al*., 2021). Comparison of transcriptomes of human peripheral blood mononuclear cells in basal conditions and after stimulation with LPS + IFN-γ showed the enhanced inflammatory response and IL-6 JAK STAT3 signaling, reactive oxygen and nitrogen species production, IL-α/IL-β signaling and TLR1/2 cascade in cells derived from patients with diabetes versus control subjects that matched the results of the study on diabetic mice (Edgar *et al*., 2021). These data support the hypothesis that trained immunity of monocytes may be an important inflammatory mechanism in the pathogenesis of diabetes mellitus.

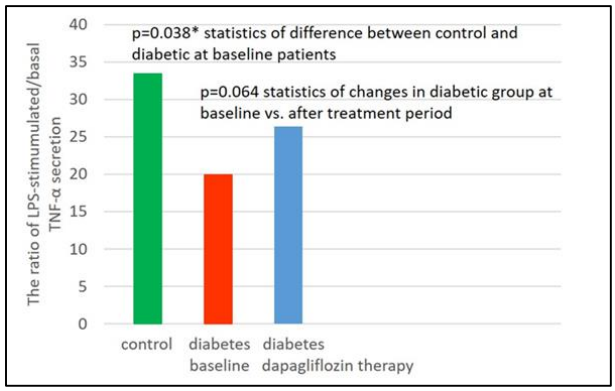

Fig. 1: The pro-inflammatory activation of monocytes in control and group with diabetes

Our investigation revealed an immune response tolerance in cultured human monocytes from both diabetic and control groups upon repeated LPS stimulation. Namely, we didn't observe the response of monocytes/macrophages on repeated stimulation after 5 days of rest in a refreshed medium after the first stimulation with LPS. The secretion of TNF-α by re-stimulated cells was similar to that in cells without re-stimulation. This suggests that trained immunity might develop as the disease progresses, with early diabetes stages primarily characterized by pro-inflammatory monocyte activation. However, further studies across different disease durations and complications are necessary to validate this hypothesis. Moreover, the impact of obesity as a factor triggering pro-inflammatory monocyte activation in diabetes remains significant (Breznik *et al*., 2018). Although our study did not find significant differences in BMI between the control and diabetic groups, assessing the effect of obesity on monocyte inflammation in diabetes warrants further exploration.

After 3 months of antidiabetic therapy with SGLT2 inhibitors (dapagliflozin 10 mg/day), the basal and LPS-stimulated secretion of TNF-α in patients with diabetes reduced significantly and matched the results of TNF-α secretion in non-diabetic participants. At the same time, the ratio of non-stimulated and LPS-stimulated secretion increased significantly in comparison with the baseline value as demonstrated in Fig. 1 due to a decrease in basal secretion and improvement of the immune response of cells. A recent study demonstrated the anti-cytokine effect of the SGLT2 inhibitor canagliflozin, which reduced IL-1β-stimulated secretion of IL-6 and Monocyte Chemoattractant Protein-1 (MCP-1) by cultured human endothelial cells in in vitro model, at the same time the anti-cytokine effect dapagliflozin wasn't observed in that study (Mancini *et al*., 2018). Another SGLT2 inhibitor tofogliflozin demonstrated a significant anti-inflammatory effect, namely, the reduction of MCP-1

gene expression and apoptotic cell death, on cultured human proximal tubular cells exposed to high glucose (Ishibashi *et al*., 2015). The results of the present study confirm the anti-inflammatory efficacy of antidiabetic therapy with SGLT2 inhibitors, which has not been previously studied in terms of the pro-inflammatory response of monocytes, which justifies the pathogenetic therapy with SGLT2 inhibitors not only for hypoglycemic treatment in diabetes mellitus but also for the prevention of diabetes-associated diseases.

Conclusion

The study concludes that monocytes in diabetic individuals exhibit pro-inflammatory activation. Additionally, the antidiabetic therapy using SGLT2 inhibitors showed efficacy in reducing the pro-inflammatory status of monocytes. This suggests that SGLT2 inhibitors could be beneficial not only for controlling glucose levels but also for preventing diabetes-associated diseases by addressing inflammation.

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Author's Contributions

Tatiana Vladimirovna Kirichenko: Conceptualization, written original drafted preparation, written reviewed and edited visualization.

Leyla Azimovna Bochkareva: Investigation resource.

Yuliya Vladimirovna Markina: Formal analysis, data curation, written reviewed, and edited.

Taisiya Vladimirovna Tolstik: Validation, formal analysis and investigation.

Anastasia Ilyinichna Bogatyreva: Validation and investigation.

Alexander Mikhailovich Markin: Methodology, software investigation, and data curation.

Andrey Alexeyevich Tulskiy: Written reviewed and edited.

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Ethics

The study was performed in accordance with the Declaration of Helsinki and approved by the Local Ethics Committee of the Sechenov First Moscow State Medical University on February 18, 2021. Informed consent was obtained from all subjects involved in the study.

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