EXPERIMENTAL WORKS

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GLUCOSE DEPRIVATION-INDUCED GLYCOGEN DEGRADATION AND VIABILITY ARE ALTERED IN PERIPHERAL BLOOD MONONUCLEAR CELLS OF TYPE 2 DIABETES PATIENTS

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The glycogen pathway plays an important role in glucose homeostasis. Impairment of the glycogen pathway has been linked to diabetes mellitus. The aim of the study is to compare the levels of glucose deprivation-induced glycogen degradation and cell viability in peripheral blood mononuclear cells from type 2 diabetes mellitus patients and healthy controls. This was a case-control study comprising 45 T2DM patients and 45 healthy controls. PBMCs were prepared from peripheral blood by density gradient centrifugation. Glycogen levels were measured by the periodic acid-schiff (PAS) staining method. Glycogen degradation was measured as percent change in PAS-stained cells before and after glucose deprivation. PBMC viability was measured by trypan-blue assay. The levels of glucose deprivation-induced glycogen degradation were 55.4% (IQR: 50.6–61.3) in the T2DM group and 70.5% (IQR: 63.9–72.2) in the healthy control group. The difference between the two groups was statistically significant (P = 0.001). The levels of glucose deprivation-induced cell viability were 70.9% (IQR: 66.3–77.1) in the T2DM group and 87.8% (IQR: 83.7–90.7) in the healthy control group. The difference between the two groups was statistically significant (P = 0.001). Together these results indicate that the glucose deprivation-induced glycogen degradation and viability are reduced in PBMCs of T2DM patients.

K e y w o r d s: type 2 diabetes mellitus, glycogen pathway, glucose deprivation, peripheral blood mononuclear cells, cell viability.

The glycogen pathway plays an important role in intracellular glucose homeostasis. When the cellular glucose levels surpass the cellular energy requirement, the excess glucose is stored as glycogen. The stored glycogen is then degraded into glucose when the cellular glucose level is lower. Large stores of glycogen are found in hepatocytes and skeletal muscle cells. Glycogen stores have also been observed in red blood cells, astrocytic glial cells, cardiomyocytes, renal tubular cells, Schwann cells, adipocytes [1-6].

The glycogen pathway is extensively studied in liver and skeletal muscle cells [7-9]. However, there is limited information on its role in other cells such as the peripheral blood mononuclear cells (PBMCs). There is growing interest in the role of PBMCs in diabetes as these cells are chronically exposed to abnormally high levels of glucose [10]. Furthermore,

altered metabolic, cellular, and immune functions have been observed in the PBMCs of diabetic patients [10-12].

Glycogen becomes the main source of glucose during hypoglycemia. Diabetic patients experience several bouts of hypoglycemia due to intensive anti-hyperglycemic therapy [13]. The mismatch between the dosage of anti-glycemic medications and physiological needs is influenced by lifestyle factors such as fasting, dieting, strenuous physical activity, and alcohol consumption [14, 15]. In addition, defects in glucose counter regulatory mechanisms have also been linked to hypoglycemia [16]. Glycogen degradation is one such regulatory mechanism that is reported to be defective in the liver of diabetic patients [17]. Whether glycogen degradation is also impaired in the PBMCs of diabetic patients is not known.

Glycogen stored in PBMCs does not play a major role in regulating plasma glucose levels. However, it may influence PBMC viability during hypoglycemia. Studies have shown that impairment of glycogen degradation under glucose-deprived conditions induces cell death [18]. The aim of this study was to evaluate the status of glycogen degradation in the PBMCs of type 2 diabetes patients (T2DM) and its impact on cell viability.

Materials and Methods

Study design and subjects. A case-control study was designed by including T2DM patients (n = 45) and healthy individuals (n = 45). The present study was approved by the institutional ethics committee, (Ref.no: SDUMC/KLR/IEC/30/2019-20 dated 06-June-2019). T2DM patients were recruited from the Department of General Medicine, R. L. Jalappa Hospital and Research Centre, attached to Sri Devaraj Urs Medical College, India. Informed consent was obtained in writing before starting the study. We followed the criteria of the Indian Council of Medical Research for diagnosing T2DM [19]. The inclusion criteria for the selection of T2DM were: (i) patients of both genders (ii) between the ages 30–80 years (iii) fasting blood glucose ≥ 126 mg/dl. (iv) HbA1c > 6.5% in the last test performed in the 12 months before the study. (v) The exclusion criteria for the patient selection were (i) micro-vascular complications and (ii) chronic co-morbidity (hypertension, diabetic foot ulcers, rheumatoid arthritis, and cardiovascular complications). The inclusion criteria for the selection of control subjects were: (i) healthy individuals of both gender and age, between the ages 30–80 years (ii) no known history of any chronic disease (iii) HbA1c < 6.5% in the last test performed in the 12 months before the study.

PBMC isolation. The PBMC was isolated from the whole blood using Ficoll-histopaque (Merck, Darmstadt, Germany) [20]. Briefly, 1 ml of anticoagulated blood was layered on 1 ml of Ficoll-histopaque and centrifuged at 3000 rpm for 30 min without break. The PBMCs layer was collected in a 15 ml falcon tube and washed twice with 10 ml of 1 X phosphate-buffered saline (PBS). The number of PBMCs was counted using a hemocytometer and used for in vitro culture.

PBMC culture and glucose treatment. PBMCs were grown in two steps. The culture medium contained glucose in the first step but not in the second step. For the first step, the cultures were prepared by adding approximately 1×10^5 PBMCs to 5 ml of glucose-deficient medium which comprised of glucose-free Roswell Park Memorial Institute medium (Gibco, New York, USA) supplemented with 1% antibiotics, 20% heat-inactivated Fetal Bovine Serum (FBS), and 300 µl (30 µg/ml) of phytohaemagglutinin (PHA). The cultures were treated with 10 mmol/l glucose and incubated for 48 h in a 5% CO_2 incubator at 37°C. After 48 hs of incubation, PBMCs were pelleted down by centrifugation at 2000 rpm for 10 min and washed with 1X PBS. The washed pellet was suspended in 50 µl 1X PBS and divided into two parts. One part was used for the determination of baseline glycogen content and cell viability. The remaining suspension was used for setting up the culture for the second step. The culture medium in the second step was as before but did not contain 10 mmol/l glucose supplementation. The cultures were incubated for 24 h and pelleted down by centrifugation at 2000 rpm for 10 min and washed with 1X PBS. The pellet was used to measure glucose-deprivation induced glycogen degradation, glucose deprivation-induced cell viability in PBMCs from T2DM patients, and healthy control [21].

Determination of glycogen degradation. Glycogen levels in the cultured PBMCs were determined by the periodic acid-schiff (PAS) staining method [21]. Briefly, slides were washed with 1X PBS and treated with fixative (37% formaldehyde and 99% Ethanol). PBMCs were then placed on the slides for 1 min. Then, slides were treated with 1% periodic acid for 5 min and Schiff's reagent (SRL Biolabs, Maharashtra, India) for 15 min followed by counterstain with haematoxylin for 30-45 sec. Approximate-

ly 80-85% of PBMCs were scored. The percentage of PAS-stained cells (glycogen positive cells) was used as the measure for glycogen level. The amount of glycogen degradation was determined as below:

Glycogen degradation (%) = [Glycogen levels before glucose deprivation (%)] – [Glycogen levels after glucose deprivation (%)]

Cell viability assay. The viability of the cultured PBMCs was determined by the trypan-blue assay [22]. Briefly, a 1:1 ratio of cell suspension and 0.4% trypan blue (Gibco, New York, USA) was mixed and loaded into a hemocytometer. Stained cells were scored as non-viable and unstained cells were scored as viable. The magnitude of cell viability after induction of glucose deprivation was determined as below:

Viability(%) = [Viable cells before glucose deprivation (%)] – [Viable cells after glucose deprivation (%)]

Statistical analysis. SPSS Statistics was used for performing statistical analysis (IBM SPSS Statistics for Windows, version 24.0 (IBM Corp., Armonk, N.Y., USA) [23]. The data were checked for normal distribution by Shapiro–Wilk test using Q–Q plots and normality plots. The mean and standard deviation were calculated for data showing normal distribution. The median and interquartile range were calculated for data not following a normal distribution. Parametric tests were used for analyzing data following a normal distribution. Whereas, non-parametric tests were used for data not showing normal distribution. The difference was considered to be statistically significant if the p-value was less than 0.05.

Results

Demographic and clinical characteristics. The demographic and clinical details of the study participants are summarized in Table. In both the groups, 47% were females and 53% were males. The mean age of the study participants was 54 ± 7.3 years in the T2DM patients and 53 ± 7.3 healthy controls.

Glucose deprivation-induced glycogen degradation. The glycogen levels were measured in PBMCs at two-time intervals. The first measurement was carried out before glucose deprivation (i.e., in glucose proficient condition) and the second measurement was carried out in the glucose-deprived conditions. The results are graphically represented in Fig. 1. The glycogen levels did not show normal distribution. Therefore, median and interquartile ranges (IQR) were calculated for both the study groups. Before the induction of glucose deprivation, the median glycogen levels were 81% in the T2DM group and 83% in the healthy control group. There was no statistically significant difference between the two groups (P = 0.551; Mann-Whitney U test; 2-tailed).

Table. Demographic and clinical profile of study participants

Parameter	T2DM patients (n = 45)	Healthy controls (n = 45)
Age (Years)	54 ± 7.5	53 ± 7.3
Gender (Male/Female)	24/21	22/23
Blood Pressure (mmHg) (Diastolic/Systolic)	86 ± 6.5/147 ± 17.5	79 ± 8.5/120 ± 10.8
Anti-diabetic medications:		
• Metformin	42.7%	Not applicable
• Glimepiride	24.2%	
• Insulin	33.1%	
The average duration of disease	2.4 ± 0.8	-
Fasting blood glucose (mg/dl)	143 ± 16.1	108 ± 10.1
Random blood glucose (mg/dl)	175 ± 19.2	129 ± 8.3
Postprandial blood glucose (mg/dl)	164 ± 24.7	124 ± 15.3
Hemoglobin A1c (%)	7.6 ± 0.8	5.8 ± 0.4
Low Density Lipoprotein (mg/dl)	173 ± 16.0	123 ± 14.3
High Density Lipoprotein (mg/dl)	80 ± 9.5	43 ± 9.0

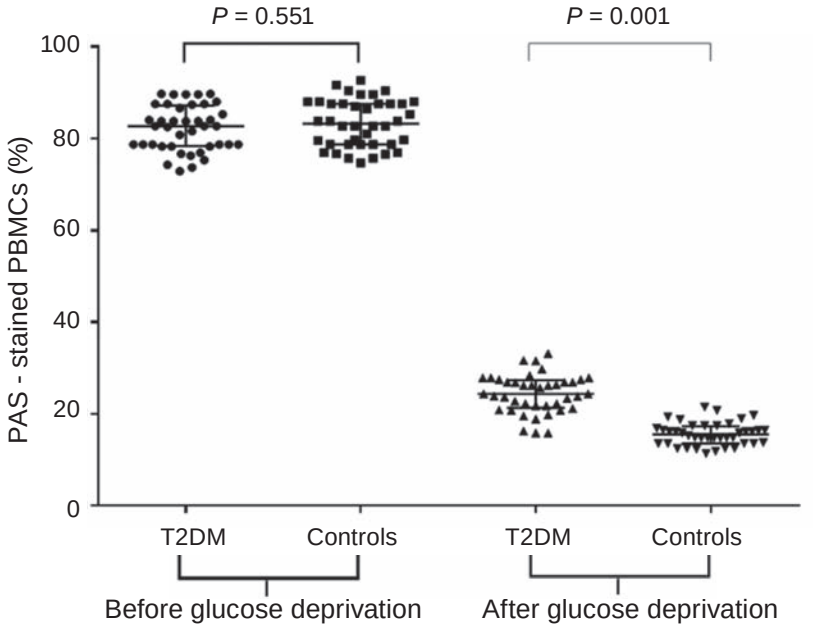

Fig. 1. Effect of glucose deprivation on glycogen levels in PBMCs

This indicates that the baseline levels of glycogen in the two groups were comparable before the induction of glucose deprivation. After inducing glucose deprivation, the median glycogen levels were 27.5% (IQR: 26.2–28.4) in the T2DM group and 14.8% (IQR: 14.8–16.1) in the healthy control group. There was a statistically significant difference in the levels between the two groups (P = 0.001; Mann-Whitney U test; 2-tailed). The levels of glycogen degradation in the two groups are graphically represented in Fig. 2. The median levels of glycogen degradation were 55.4% (IQR: 50.6–61.3) in the T2DM group and 70.5% (IQR: 63.9–72.2) in the healthy control group. The levels of glycogen degradation were 0.8 times lower in the T2DM patients compared to the healthy control group. The difference between the two groups was found to be statistically significant (P = 0.001; Mann-Whitney U test; 2-tailed).

Comparison of glucose deprivation-induced cell viability. In addition to glycogen levels, cell viability was also measured in the PBMCs. Likewise, the measurements were carried out before and after the induction of glucose deprivation. The results are graphically represented in Fig. 3. The percentage of viable cells was used as the measure of cell viability. Cell viability levels did not show normal distribution. Therefore, median and IQR were calculated for both groups. Before the induction of glucose deprivation, the median cell viability levels were 81% in the T2DM group and 83% in the healthy control group. There was no statistically significant difference between the two groups (P = 0.958; Mann-Whitney U test; 2-tailed). This indicates that the baseline levels of cell viability in the two groups were comparable before the induction of glucose deprivation. After inducing glucose deprivation, the median cell viability levels were 71.1% (IQR = 67.1–77.0) in the T2DM group and 87.1% (IQR = 84.4–91.3) in the healthy control group. The difference in the cell viability levels of the two groups was statistically significant (P = 0.001; Mann-Whitney U test; 2-tailed). The percentage of cell viability in the two groups was graphically represented in Fig. 4. The percentage of cell viability in the T2DM group was 70.9% (IQR: 66.3–77.1) and 87.8% (IQR: 83.7–90.7) in the healthy control group. The percentage of cell viability was 0.8 times lower in the T2DM group compared to those from healthy controls. The difference in the levels of the two groups was statistically significant (P = 0.001; Mann-Whitney U test; 2-tailed).

Correlation between glucose deprivation-induced glycogen degradation and cell viability. Correlation between glycogen degradation levels and cell viability was determined after combining the data from both the study groups. The study parameters did not show normal distribution therefore Spearman's rank-order correlation test was used. The graphical representation of the results is given in Fig. 5. The two parameters showed statistically significant correlation (P = 0.001; r = -0.44; Spear-

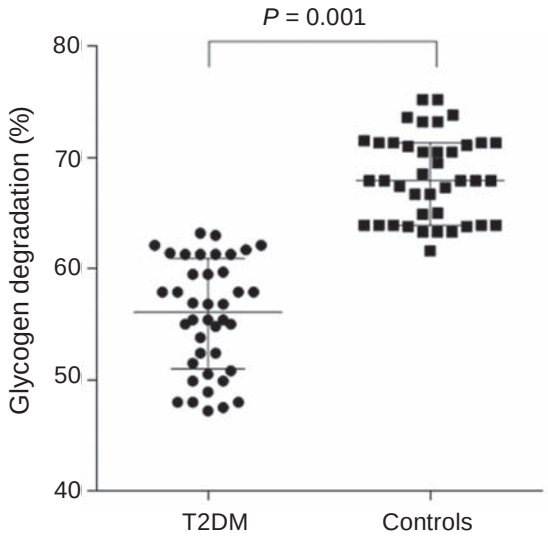

Fig. 2. Glycogen degradation in PBMCs from T2DM and healthy controls

man's rank-order correlation test). The correlation was negative i.e., reciprocal in the relationship and the magnitude of correlation was moderate. Furthermore, the correlation analysis was also carried out for each study group separately. The correlation was negative in both T2DM (r = -0.22; P = 0.03; Spearman's rank-order correlation test) and control (r = -0.24; P = 0.02; Spearman's rank-order correlation test) groups. However, the magnitude of correlation was weak or negligible in both the groups.

Discussion

The purpose of this study was to check whether glucose deprivation-induced glycogen degradation and cell viability are altered in the PBMCs of T2DM patients. The main finding of this study is: (i) glucose deprivation-induced glycogen degradation was reduced in the PBMCs from T2DM patients, (ii) glucose deprivation-induced cell viability of PBMCs was reduced in T2DM patients, and (iii) glucose deprivation-induced glycogen degradation and cell viability of PBMCs are reciprocally related. These results indicate that PBMCs from T2DM patients have a reduced capacity to mobilize glucose via glycogen degradation when exogenous supply is limited. Furthermore, these results indicate that the reduced capacity for glycogen degradation may be linked to the reduced viability of PBMCs in T2DM patients. To the best of our knowledge, this is the first study to examine the role of glycogen catabolism in PBMCs derived from T2DM patients under conditions of glucose limitation.

Studies have shown that glycogen metabolism is abnormal in T2DM. Glycogen metabolisms in the liver and skeletal muscles are comparatively lower in T2DM patients than in healthy volunteers [7, 24]. The lowered levels appear to be linked to impairment of glycogen synthesis. Levels of glycogen synthase are considerably reduced in the skeletal muscle of T2DM patients [25]. Defects in

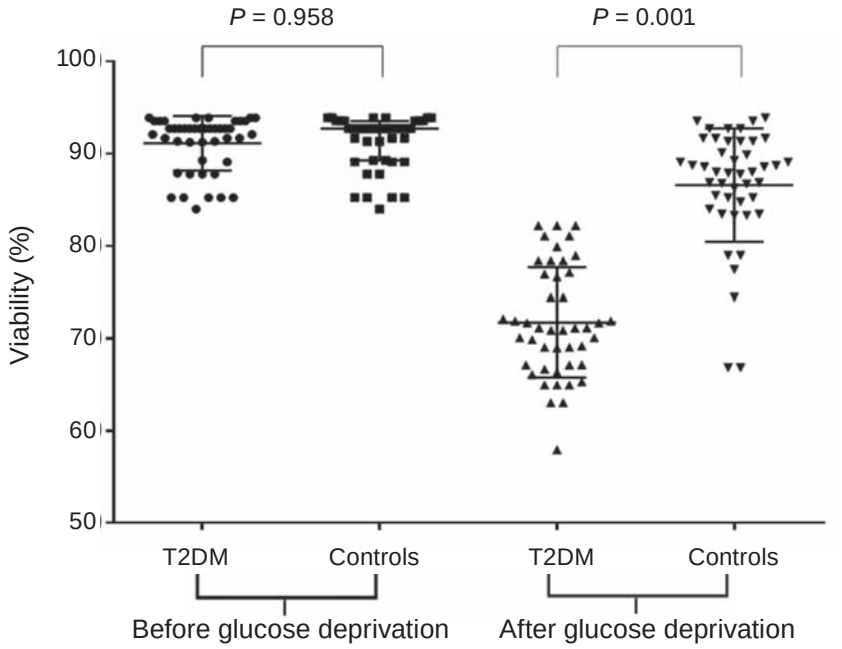

Fig. 3. Effect of glucose deprivation on PBMC viability

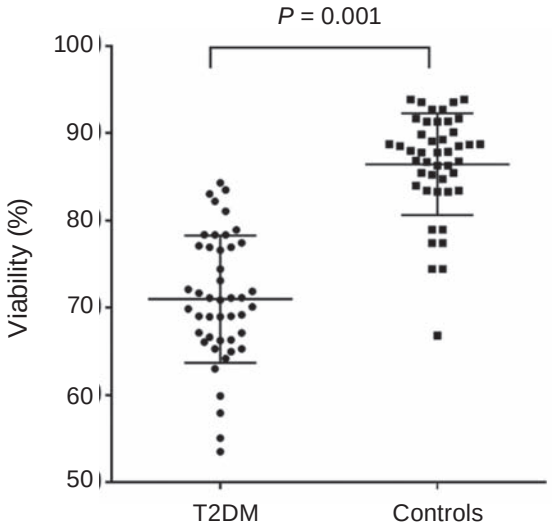

Fig. 4. Glucose deprivation-induced viability of PBMCs from T2DM and healthy controls

other enzymes such as glycogen phosphorylase and glycogen debranching enzyme also appear to play a role in reduced glycogen synthesis in T2DM. As a result, less glucose is available for glycogen synthesis despite hyperglycemia [26]. Insulin resistance also contributes to reduced glycogen synthesis in the skeletal muscle of T2DM patients. Insulin resistance results in insufficient transport of glucose into the cytoplasm [27]. This study adds new dimensions to the glycogen pathway in T2DM. Our observations indicate that glycogen degradation is also disrupted in T2DM.

Glycogen degradation has been shown to affect the immunological functions of leukocytes. Blocking of glycogen degradation in the dendritic cells under low-glucose conditions increases cell death and reduces inflammatory cytokine production [28]. Glycogen metabolism has been linked to anti-oxidant protection in T memory cells. T cells channel glucose 6-phosphate formed due to glycogen degradation into the pentose phosphate pathway for generating NADPH. The resulting cofactor ensures high levels of reduced glutathione [29]. Further studies have shown that the anti-oxidant role of glycogen metabolism is involved in the regulation of macrophage-mediated inflammation [30]. It is now well established that immunity is compromised in T2DM patients [12]. However, the nature of causative factors for the immune impairment is not clear [31]. The results presented herein indicate that abnormal glycogen catabolism in the PBMCs may be one of the contributors in this direction.

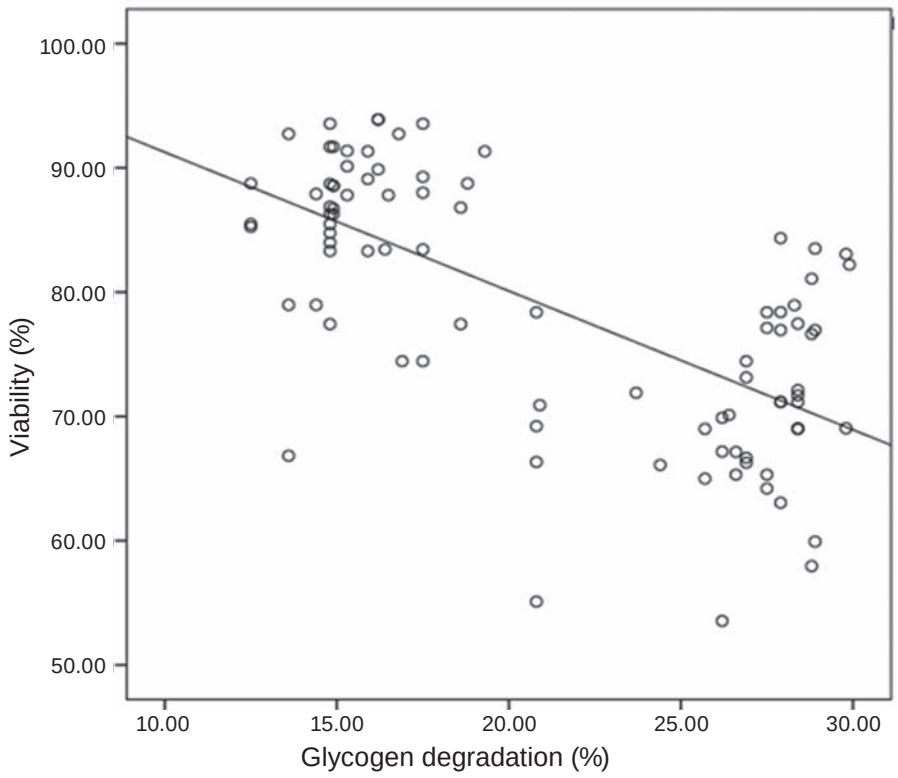

Fig. 5. Correlation between glucose deprivation-induced glycogen degradation and viability in PBMCs

Conclusion. In conclusion, this study provides the first evidence to show that glucose deprivation-induced glycogen degradation is abnormal at the cellular level in T2DM and this appears to result in reduced cell viability. The major limitation of this study is that glycogen synthesis prior to degradation was not measured. Furthermore, the correlation between glycogen degradation and cell viability was weak when analyzed group-wise. Larger sample size would be necessary to confirm this relationship. Future studies should examine this gap so that the pathway can be developed as a target for novel anti-diabetic drugs.

Conflict of interest. Authors have completed the Unified Conflicts of Interest form at http://ukr-biochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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Authors' contributions. All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Praveen Kumar, Prabhakar Kamarthy, and Sharath Balakrishna, The first draft of the manuscript was written by Praveen Kumar and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate. The study was approved by the Institutional Ethics Committee of Sri Devaraj Urs Medical College, India with vide letter number SDUMC/KLR/IEC/30/2019-20 dated 06-June-2019. Informed consent was obtained from the study participants in writing before enrollment in the study.

СПРИЧИНЕНИЙ ДЕПРИВАЦІЄЮ ГЛЮКОЗИ РОЗПАД ГЛІКОГЕНУ В МОНОНУКЛЕАРНИХ КЛІТИНАХ ПЕРИФЕРИЧНОЇ КРОВІ ТА ЇХНЯ ЖИТТЄЗДАТНІСТЬ У ПАЦІЄНТІВ ІЗ ЦУКРОВИМ ДІАБЕТОМ 2 ТИПУ

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Глікогеновий шлях відіграє важливу роль у гомеостазі глюкози. Відомо, що цукровий діабет є наслідком його порушення. Метою дослідження було порівняти рівень розпаду глікогену, спричиненого глюкозною депривацією, та життєздатність мононуклеарних клітин периферичної крові (МКПК) у пацієнтів із цукровим діабетом 2-го типу (ЦД2) та в здорових осіб. Дослідження яке охоплювало 45 пацієнтів із ЦД2 та 45 здорових осіб контрольної групи. Мононуклеарні клітини виділяли з периферичної крові центрифугуванням у градієнті щільності. Депривацію глюкози спричиняли інкубацією клітин у культуральному середовищі, яке не містило 10 мМ добавки глюкози. Рівень глікогену у клітинах вимірювали фарбуванням періодичною кислотою Шиффа (PAS), розпад глікогену виражали як відсоток профарбованих клітин до/після депривації глюкози. Життєздатність клітин вимірювали за допомогою тесту з фарбуванням клітин трипановим синім. Показано, що рівень спричиненого депривацією глюкози роз-

паду глікогену у клітинах становив 55,4% (IQR: 50,6–61,3) у групі ЦД2 та 70,5% (IQR: 63,9–2,2) у контрольній групі, різниця між двома групами була статистично значущою (P = 0,001). Життєздатність клітин після депривації глюкози, становила 70,9% (IQR: 66,3–77,1) у групі ЦД2 та 87,8% (IQR: 83,7–90,7) у здоровій контрольній групі. Різниця між двома групами була статистично значущою (P = 0,001). Разом ці результати вказують на те, що індукована депривацією глюкози деградація глікогену в мононуклеарних клітинах периферичної крові та їхня життєздатність у пацієнтів з ЦД2 є зниженими.

Ключові слова: цукровий діабет 2 типу, глікогеновий шлях, депривація глюкози, мононуклеари периферичної крові, життєздатність клітин.

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