https://doi.org/10.46389/rjd-2020-1046
www.rjdnmd.org

Rom J Diabetes Nutr Metab Dis
2020; volume 27, issue 4, pages 309-315

Original Research

Mitochondrial dysfunction of mesenchymal stem cells isolated from blood with type 2 diabetic patients

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Received: 17 July 2020 / Accepted: 28 October 2020

Abstract

The Aim of the Study: is to focus on mitochondrial dysfunction, in the context of NASH, mitochondrial function in stem cells is likely to be impaired. **Materials and Methods:** Mesenchymal stem cell separated of peripheral blood from diabetes 2 type (DT2) patients was collected in the context of a clinical protocol authorized by the local Ethics Committee of Ukraine Association of Biobank (Ukraine), with a license from the Ministry of Health of Ukraine 04/10/2018 №1813 and 27/03/2019 №1231 by the national competent authority for biobank cord blood, cell and, tissue therapy. The study population (n = 96) was represented by diabetic patients from SI «ZIGUS NAMSU» in Kharkiv, Ukraine, and healthy volunteers. Patients were divided into two groups: group I consisted of patients with diabetes 2 type (DT2), group II - patients with DT2 complicated course of NASH (DT2 + NASH). The control group consisted of 25 conditionally healthy persons (men and women) of the same age. **Conclusion:** In the modern scientific space, various directions have been proposed in the diagnosis of metabolic syndrome and the treatment of D2T.

Introduction

Metabolic syndrome and finally diabetes 2 type (DT2) as a result of progressive obesity, insulin resistance, abnormal cholesterol or triglyceride levels are newfound problems in the current endocrinology. As reported by the International Diabetes Federation, in the entire world 382 million of adults (8.3%) are living with diabetes; the number is estimated to increase to 592 million in the next 20 years. More than 260 million people will be afflicted globally by 2022 [1–2].

Two main and interacting components determine the normal level of glucose in the blood, this is the reaction to insulin of skeletal muscle and liver, production of insulin by beta cells of the pancreas, under the influence of glucose. Therefore, two levels of defect have been identified, insulin resistance and progression to hyperglycemia. Modern data integrating these two hypotheses have a common, new direction inherent in the development of T2DM – mitochondrial dysfunction. [3–4].

Hepatic dysfunction in the form of nonalcoholic fatty liver disease (NAFLD) is commonly

observed in patients with T2DM [5–8]. Whether NAFLD is a cause or consequence of the diabetic pathology remains a topic of contention; however, the alterations in hepatic energy substrate metabolism and mitochondrial function in T2DM patients with NAFLD are well characterized. Decreased insulin sensitivity of the liver accompanied by increased hepatic fat storage are two such major metabolic changes found in the diabetic patients [9–11]. Mitochondria-intrinsic perturbations in obese, insulin-resistant patients with nonalcoholic steatohepatitis (NASH) include lower maximal respiration, increased mitochondrial uncoupling, and increased proton leak [12]. These findings are further strengthened by the observation of decreased ATP content and turnover in the T2DM liver [13–15].

Currently, significant progress has been made in the implementation of the anti-beta strategy, both at the clinical level and at the level of the national health system. The study of the metabolic syndrome and the search for an effective treatment for diabetes enable physicians to more deeply implement preclinical diagnostic correlates that allow the prevention of diabetes development at the primary level. Most effective in treatment includes e.g., glucagon-like peptide (GLP-1) mimetic, dipeptidyl-peptidase-4 (DPP-4) inhibitors, sodium glucose transporter-2 (SGLT2) inhibitors, but also surgical gastric correction, diet-related therapy, such as calorie restriction and finally mesenchymal stem cells application [5–8]. The other half of the 20th century has become a new stage in diabetes patients, so Friedenstein, and the knowledge about MSC is gaining biomedical significance in the natural sciences of biosystems. Unique cytophysiological properties of this stem cell population have led to developing a concept, in which their clinical application is consequently implemented [16–19].

The study focused on mitochondrial dysfunction was performed to unveil novel metabolism-related clues that may shed light on pathophysiology of T2D.

Materials and methods

The study population (n = 96) was represented by diabetic patients from SI «ZIGUS NAMSU» in Kharkiv, Ukraine, and healthy volunteers. All participants received and signed the informed consent, and the Ethical Committee approved the study. Mesenchymal stem cell separated of peripheral blood from T2DM patients was collected in the context of a clinical protocol authorized by the local Ethics Committee of Ukraine Association of Biobank (Ukraine), with a license from the Ministry of Health of Ukraine 04/10/2018 №1813 and 27/03/2019 №1231 by the national competent authority for biobank cord blood, cell, and tissue therapy.

The mean age of patients in the study was 61±6,3 years. Gender data: 56 (58,3%) – male, 40 (41,7%) – female. All studied patients had T2DM in anamnesis and met the World Health Organization diabetes and glucose intolerance criteria such as fasting plasma glucose(FPG) ≥126 mg/dL (7.0 mmol/L) or 2-hour oral glucose tolerance test (OGTT) plasma glucose ≥200 mg/dL (11.1 mmol/L), as well as polydipsia and polyuria in complex of symptoms.

Inclusion criteria: ability to read, understand, fill in and sign written informed consent; mentally healthy and ability to carry out the procedures of the study protocol; clinical history of type 2 diabetes mellitus (T2DM) according to the Expert Committee on the Diagnosis and classification of diabetes mellitus; the start of diseases of T2DM disease at ≥70 years of age; T2DM duration ≥5 and ≤15 years at the time of consent signature; level of C-peptide 0.28–2.1 ng/mL; HbA1c ≥ 7.1; patients must have been treated with SMT for minimum of five months prior to randomization. The injection insulin dose and metformin doses should be stable over the four months prior to randomization total insulin daily dose (TDD) at time of randomization should not exceed 1.0 units/day/kg; HbA1c≥7.1 and ≤9.0%.

Depending on the persistence of signs of hepatic dysfunction patients were divided in two groups: group I – patients with DT2, group II – patients with DT2 and NASH (DT2+NASH). The control group consisted of 25 healthy male and female comparable with age.

Exclusion criteria: insulin requirements of >1.0 units/day/kg; HbA1c >9.1%. (at the time of consent signature); C-reactive protein >2.85; arterial hypertension: SBP >160 mmHg or DBP >100 mmHg; evidence of renal dysfunction, serum

https://doi.org/10.46389/rjd-2020-1046

creatinine >1.6 mg/dl; proteinuria >290 mg/day; evidence of acute coronary syndrome in past 8 months and/or cardiovascular disease on physical exam.

For female exclusion criteria – pregnancy and/or presently breast-feeding, or unwillingness to use effective contraceptive measures for the duration of the study.

The exclusion criteria in men and women are- active infection including hepatitis C and B, HIV, or Tuberculosis. A history of coagulopathy and/or Factor V deficiency by INR>1.3, PTT>38, PT>13, or medical condition requiring long-term anticoagulant therapy.

Cell culture

All patients gave their written informed consent. MSCs were isolated from peripheral blood (PB) from method magnetic-separated in used automatic system AutoMACS, seeding 50,000 mononucleotide cells/cm^2 in RPMI (1x) +GlutaMAX medium (Gibco Life Technologies, Canada) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific), in CELLdisc™ a range of cell culture surfaces from 1,000 cm^2 up to 1 square meter.

The cultures were incubated at 37°C, 20% O_2, 5% CO_2 with used automatic system FibraStage (New Brunswick Scientific, USA). Medium changes were performed twice a week. Two weeks after initial seeding, primary MSC colonies were detached with a 10 min incubation at 37°C with Trypsine-EDTA 0.05% (Gibco Life Technologies, Canada) and replated at 4200 cells/cm2 in the same medium. Passage 6 MSCs were used for all experiments.

The characterization and standardization of MSCs are as plastic-adherent and spindle-shape, expression of antigen markers (CD73+, CD90+, CD105+, and CD45–, CD34–, CD14–, CD79–) on their surface and differentiation potential [19].

Live cell imaging

For identification of mitochondrial localization, cells were loaded with 200nMMitotracker Green FM in RPMI medium (no phenol red) in HBSS (Sigma-Aldrich). The images were obtained using a Zeiss LSM 400, a compact confocal scanning microscope equipped with 40X oil immersion objective.

The 488 nm was used to excite PKH26 Red Fluorescent Cell Linker (Kit for General Cell Membrane Labeling). Sigma's PKH26 dye is a red fluorescent cell labeling dye used for both in vitro/in vivo live cell imaging which was measured between 505 and 530 nm. All data presented were obtained from at least five coverslips and two to three different cell preparations.

For measurements of mitochondrial membrane potential ($\Delta\Psi m$), cells plated on 22 mm glass coverslips were loaded for 30 min at room temperature with 25 nMtetramethylrhodaminemethylester (TMRM; Invitrogen) in a HEPES buffered saline solution (HBSS) composed of 156 mM NaCl, three mMKCl, two mM MgSO4, 1.25 mM KH2PO4, two mM CaCl2, 10 mM glucose and 10 mM HEPES; pH adjusted to 7.35 with NaOH. The dye remained present in the media at the time of recording. The TMRM is used in the redistribution mode to assess $\Delta\Psi m$, and therefore a reduction in TMRM fluorescence represents mitochondrial depolarization.

For measurement of mitochondrial ROS production, cells were pre-incubated with Red DND-99 (L7528) and 75 nMMitoTracker® Green FM® for 10 min at room temperature measurements were produced using 580 nm excitation and emission above 600 nm.

Measurement of NADH/FAD redox index

The NADH auto-fluorescence was measured using an epifluorescence inverted microscope with a 20X fluorite objective. Excitation light at a wavelength of 350 nm was provided by a Xenon arc lamp, the beam passing through a monochromator (Life Technologies (ThermoFisher) EVOS XL Inverted Imaging Digital Microscope). Emitted fluorescence light was reflected through a 455 nm long-pass filter to a All EVOS® fluorescence imaging systems and the Countess® II FL Automated Cell Counter (Thermo Scientific EVOS Light Cube, DAPI) and digitized. Imaging data were collected

and analyzed using software from EVOS®. FAD auto-fluorescence was monitored using a Zeiss 710 VIS CLSM equipped with a META detection system and a 40x oil immersion objective. Excitation was using the 454 nm Argon laser line and fluorescence was measured from 505 to 550 nm. Illumination intensity was kept to a minimum (at 0.1–0.2% of laser output) to avoid phototoxicity and the pinhole set to give an optical slice of ~2 µm. FAD and NADH redox indexes and mitochondrial pools were estimated according the method described in Bartolome et al [8].

ATP assay method in MSCs

ATP was measured by luciferin-luciferase technique [29, 30] in which the amount of light generated by the reaction of ATP with recombinant luciferase is dependent on the ATP concentration. Sensitivity was augmented by addition of the D-luciferin to the luciferase. A 50 µl sample of MSCs, lysed with TCA 10% (tricoloroaceticacid) and neutralized with KOH 1 M and diluted with hepes buffer 100 mM pH 7.8 (1:64), injected into a cuvette containing 10 µlluciferin (sigma), 10 µl Mgso4, 10 µlluciferase (1 mg/ml). The peak light efflux from cuvette to which either known ATP standards or samples are added was determined using a luminometer (Sirius tube Luminometer, Berthold Detection System, Germany), a ATP standard curve was obtained on the day of each experiment.

Data and statistical analysis

Statistical tests: unpaired two-tailed Student's tests were performed using Medical Statistica 8.5 and Statistica 8.0 Microsoft Excel software (USA). Differences were considered statistically significant with p-value <0.01. Results are expressed as means ± standard error of the mean (SEM.).

Results

MSCs are taken from 96 diabetic patients who were diagnosed according to American Diabetes Association Guideline 2011 [35]. The baseline characteristic data are summarized in Table 1. In addition, MSCs of 25 healthy human volunteers with equal sex for all was sampled. Average ages for normal control individuals without medication and history of diabetes disease was 37.7 ± 4.9.

Changes in TMRM fluorescence showed a significant decrease in basal ΔΨm in patients with complicated TD2 MSC NASH, so the patients with TD2 MSC showed a significant decrease in mitochondrial membrane potential (p <0.001). Thus, in the TD2+NASH MSC group ΔΨm was reduced to 61.2 ± 3.2% (n = 36; p<0.001), while in the group of patients with diabetes mellitus this indicator was a bit higher – 75.4 ± 3.7% respectively (n = 50; p <0.001). Control group had ΔΨm MSC 85.2 ± 3.1% (n = 25).This data indicates possible mitochondrial impairment in mesenchymal stem cells in patients with TD2+NASH.

Table 1: Content ATP/ADP and mitochondrial membrane potential in MSCs group comparison, as shown in this table the difference between groups are significant (P-value <0.001)

Normal	Normal	Diabetes 2 type	Diabetes 2 type complicated course of NASH
N	25	50	36
BMI	25	37	41, 2
ATP/ADP	2.94 ± 0.11	5.03 ± 0.37	5.03 ± 0.37
HbA1C%	4.86 ± 0.13	4.09 ± 0.08	4.09 ± 0.08
FBS	84.05 ± 2.5	76 ± 0.94	76 ± 0.94
Mitochondrial (ΔΨm)	85.2 ± 3.1%	75.4 ± 3.7%	61.2 ± 3.2%

Note: P value < 0.001; BMI – Body Mass Index [weight/(height)^2] = kg/m^2; FBS – fasting blood sugar.

https://doi.org/10.46389/rjd-2020-1046

The results of study showed the expression of MSCs surface antigens CD with patients TD2 higher with accompanying NASH. So, we found that MSCs showed significantly decreased expression of CD90 by 96% that was also observed in patients TD2 higher with accompanying NASH.

So, in TD2 and NASH group, decreased CD105 surface antigens expression to 4% (n = 36; p<0.001) comparing to 81% (n = 25) of control group respectively that affected the properties stem cell. Based on the results obtained, we can make the assumption that progress of NASH with patients TD2 impairs the mitochondrial function of MSC (Fig. 1).

The total mitochondrial pool of NADH in MSCs with patients DT2 complicated course of NASH was also higher (p = 0.001) than in patients DT2 with level (60.3 ± 4.7%, n = 50) and control group with level (92.0 ± 5.5%, n = 25, (p<0.001)), indicating increased substrate availability for complex I in these cells (Fig. 2).

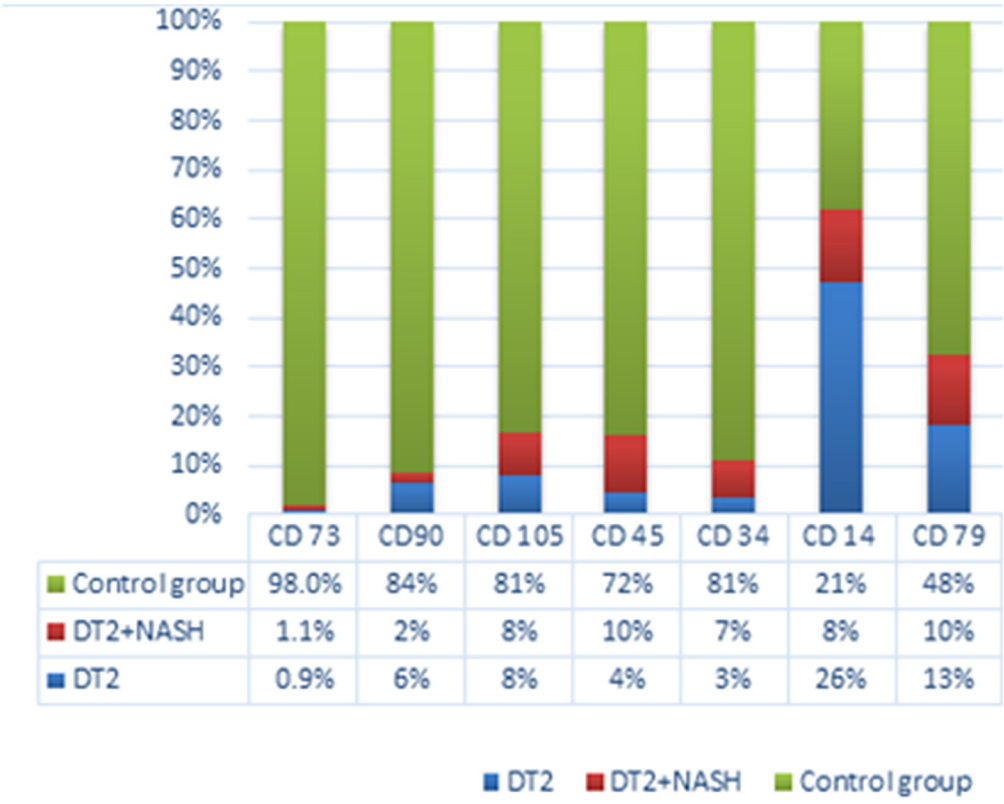
Quantification of the NADH redox index in MSCs from control and patient DT2, as measured with the mitochondria-specific probe established significant differences (p = 0.001) between the MSCs control group and group patients TD2 accompanying NASH, patients of TD2.

Discussion

The results of study showed that the decreased level of CD34 in progenitor stem cells and MSCs is associated with diabetes progression. In the healthy group patients MSCs in undifferentiated state in their positions (pools) remain stable. Despite "trigger" like pro-inflammatory cytokines IL-1 and/or IL-6, INF-α and growth factors VEGEF, TGEF are able to mobilize MSCs and induce their proliferation and homing the decrease in level MSC contributes impairment of all tissue regeneration [12–13].

Interestingly the by results of study MSCs cells in patients with TD2 were characterized by increased expression of CD44, working as an immune cell receptor involved.

Research conducted by Kodama and colleagues in experimental study revealed that CD44



	CD 73	CD90	CD 105	CD 45	CD 34	CD 14	CD 79
Control group	98.0%	84%	81%	72%	81%	21%	48%
DT2+NASH	1.1%	2%	8%	10%	7%	8%	10%
DT2	0.9%	6%	8%	4%	3%	26%	13%

Figure 1: The expression of antigen marker MSCs with patient's DT2 and control group.

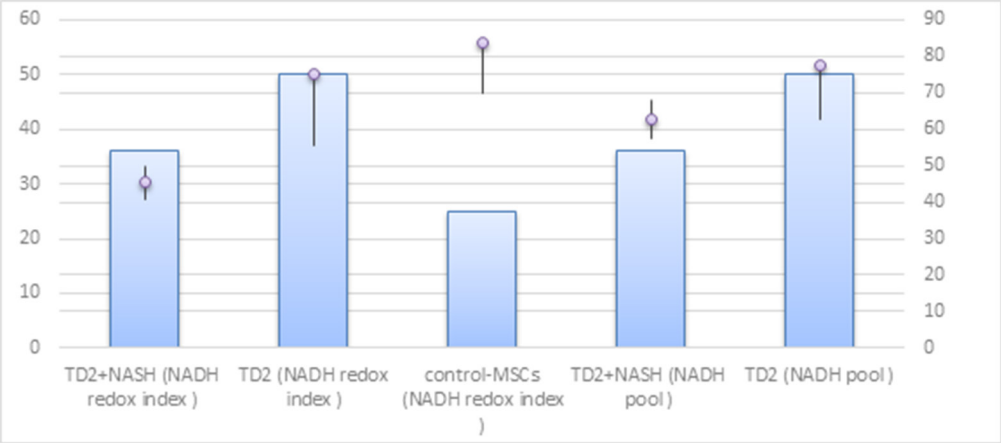


Figure 2: Quantification of the NADH redox index in MSCs from control and patient DT2.

was up regulated in white adipose tissue of obese, diabetic mice.

The same way, CD44 knockout mice fed a high fat diet, did not develop TD2 and/or obesity. The up regulation of CD44 led to migration and infiltration of activated immune cells, increasing the inflammation in adipose tissue; in addition, it was also confirmed that MS adipose tissue was enriched in macrophages secreting IL-1, IL-6 and TNF-alpha. Both obesity and TD2 strongly affect MSC morphology, including actin cytoskeleton organization [14–17].

The NASH affected the expression of patients TD2 MSCs surface antigens, as substantial reduction of CD90, CD105, and CD73 levels was observed. We found that TD2+NASH of MSCs showing significantly decreased expression of CD90 was also observed in patients suffering from TD2. The pro-inflammatory environment of adipose tissue negatively affects MSC stemness.

Stem cell metabolism is a great novel area for research. Changes in mechanisms of energy production in stem cell influence physiology and the course of the disease. Specifically, metabolism as well as changes in energy production has been associated with stem cell self-renewal and differentiation control [10–11].

Living cells are continuously exposed to the harmful effect of exogenous or endogenous reactive oxygen species (ROS). These highly reactive molecules, radicals and non-radicals, have the ability to capture electrons from molecules they come in contact with, including proteins and nucleic acids, leading in consequence to cell damage. Besides, oxidative stress may cause non-specific, post-translational protein modifications, leading to aggregate formation. Being the main source of ROS in cells mitochondrial respiratory complexes I and III are most susceptible to electron leakage, resulting in H_2O_2 formation. In addition, under certain conditions, the electron flux can be intensified, as in increased energetic demand during endurance exercises. On the other hand, mitochondrial function may decrease during aging or degenerative and metabolic diseases.

New evidences indicate the valuable role of mitochondrial dysfunction in the progression of NASH and T2D [18]. The conditions of hyperglycemia and high insulin tolerance lead to imbalances in ROS detoxication inside the cell, resulting in free oxygen radicals-mediated damage in both pathologies. It appears that the accumulation of defective mitochondria contributes to the reduced insulin secretion by β-cells [19-20].

It was shown that MSCs exhibited decreased mitochondrial membrane potential. The ATP/ADP rebalance in these cells was probably activated by excessive amount of ROS and damaged mitochondria, which led to nutrient and ATP deprivation.

Conclusion

The health of patients strongly affects the status of MSCs. Those cells isolated from patients with type 2 diabetes (TD2) or from patients

https://doi.org/10.46389/rjd-2020-1046

with TD2 accompanied by the NASH have the increased incidence of apoptosis, autophagy, accumulation of free radical molecules, and mitochondria deterioration. The mitochondrial membrane potential observed in these cells may be a protective mechanism that provides energy and building blocks to restore cellular homeostasis and control oxidative damage.

Based on presented data, our conclusion is that crucial metabolic aspects of TD2 are indeed recapitulated at the systemic level and perspective therapeutic application of MSC isolated from TD2 patients may be limited due to their dysfunctionality.

Conflict of Interest

The authors declare no conflict of interest.

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