Original Article
Platelet mitochondrial dysfunction of DM rats and DM patients

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Abstract: It is known that cardiovascular complications plays important roles in the development of diabetes mellitus (DM) and platelet dysfunction is one of the key reasons which led to microangiopathy. This study was designed to investigate the mitochondria function changes of platelet in DM rats and DM patients. The results showed that the platelets viability, platelet adenosine triphosphate (ATP) content and platelet mitochondrial ATP content of DM rats were lower than that of normal rats; when incubated in vitro for 24 h, platelet number and mitochondrial membrane potential (MMP) of DM rats were lower than that of normal rats, reactive oxygen species (ROS) was higher than that of normal rats. For DM patients, their platelet number and ROS were higher and MMP was lower than those of normal people; when incubated in vitro for 24 h, platelet viability of DM patients was lower than that of normal people. Platelet ultra-microstructures of DM rats and DM patients were abnormal. These results suggested that platelet mitochondrial function of both DM rats and DM patients was impaired when compared to normal rats and normal people, respectively. Platelets may be applied as a biomarker to observe the mitochondrial changes during DM.

Keywords: Platelet, mitochondria, oxidative stress, diabetic

Introduction

Because of its high morbidity and poor curative effect, diabetes mellitus (DM) seriously affected the normal life and work of nearly 9.7 percent Chinese people [1]. Hyperglycaemia is the main symptom of DM, and it causes various of physiological and biochemical alterations, such as metabolic disorder [2], deoxyribonucleic acid (DNA) damage [3], cell apoptosis [4] and platelet hyperactive [5]. So the complications of DM affect almost every human organ. Microangiopathy is unquestionably the most common and the worst of all the complications. Microangiopathy caused blindness, renal failure and nerve damage, and diabetes-accelerated atherosclerosis leads to increased risk of myocardial infarction, stroke and limb amputation [2, 6].

Brownlee [6] summarized previous researches and concluded that mitochondrial damage was the unifying hypothesis linking the four mechanisms about how hyperglycaemia causes diabetic complications: increased polyol pathway flux; increased advanced glycation end-product (AGE) formation; activation of protein kinase C (PKC) isoforms and increased hexosamine pathway flux. Hyperglycaemia made more glucose to be oxidized in the tricarboxylic acid cycle (TAC) and excessive electron donors nicotinamide adenine dinucleotide hydrogen (NADH) and reduced flavin adenine dinucleotide (FADH2) were pushed into the electron transport chain thus leading to an increase in ROS generation [5, 6]. Mitochondrial function is impaired during the production of ROS. ROS can cause damage to respiratory capacity, mitochondrial DNA (mt DNA) [7] and expression of respiratory chain complexes [8, 9].

The early diagnosis of DM is rather important for prognosis of DM patients. Now, the blood glucose level is the common indicator for diagnosis of DM [10]. But the insidious onset of DM before symptoms develop limits the early detection which leads to poor prognosis [11]. Therefore, the convenient and effective way to solve these problems is necessary [12]. To get platelet from experimental animal and human is rela-

tively easy. Platelet has no nucleus but contains mitochondria, so it can be used for research on mitochondrion [13, 14]. Platelet changes may reflect cardiovascular complication conditions of DM patients [13]. Therefore, platelet may be used to observe what happened to the mitochondria during the whole course of DM. But present references about mitochondrial function of DM platelet are rare.

In this experiment, platelets of DM rats and DM patients were used in order to know how DM affected mitochondrial function and to clarify the mitochondrial mechanism of DM complications.

Methods

Reagents

JC-1 (5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolcarbocyanineiodide), MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), and Streptozocin (STZ) were bought from Sigma, USA. 2', 7'-dichlorofluorescindiacetate (DCFH-DA) was bought from Biotium, USA. Mitochondria isolation kit was bought from Thermo Scientific, USA. JC-1 was stocked as 1 mg/mL dimethyl sulfoxide (DMSO) solution. MTT was stocked as 5 mg/mL DMSO solution. DCFH-DA was stocked as 10 mM DMSO solution. They were stored at -20°C before use [15]. STZ was dissolved in 0.1 M citrate buffer pH 4.2-4.5 when used [16]. ATP Assay Kit and BCA Protein Assay Kit were bought from Beyotime, China.

Platelet preparation

Platelet rich plasma (PRP) was prepared as previously described [15]. briefly, after rats were anesthetized with chloral hydrate (10%, 350 mg/kg Body weight, BW), blood was obtained via puncture of the right ventricle with a syringe containing ethylene diaminetetraacetic acid (EDTA) (5 mmol/L). Human blood was obtained by puncture of the antecubital vein with an anticoagulant tube. Then blood was centrifuged at 150× g for 20 min immediately, and the supernatant was platelet rich plasma (PRP). PRP was centrifuged at 800× g for 10 min to obtain platelet. Platelet was re-suspended according to the procedure of grouping.

Grouping

Experimental animals: A total of 50 Sprague Dawley (SD) rats, half female and half male

(3-month old, 250~300 g body weight, BW) were provided by the animal facility of Sun Yat-Sen University. They were housed under standard conditions, 25°C room temperature, 12 h light/dark cycle, access to standard diet and water freely. Immediately before experiments, they were randomly divided into two groups: DM group and normal group. The DM model was established according to Kamboj [16]: following an overnight fasting, rats were randomized to receive either 55 mg/kg BW of STZ diluted in 0.1 M citrate buffer pH 4.2-4.5 (DM group) or citrate buffer only (normal group) through intraperitoneal injection. Seventy-two hours after injection, rats plasma glucose level was measured and the rats with plasma glucose values >15 mM were assigned as DM and recruited to this experiment. Four weeks after that, platelets were prepared and mitochondrial function was tested immediately and after incubated for 24 h: platelet of DM rats in PBS containing 30 mmol/L glucose and platelet of normal rats in PBS containing 5 mmol/L glucose at 37°C.

Clinical subjects: DM patients were diagnosed according to the following criteria: 1) glycolated hemoglobin ≥6.5%; 2) fasting blood-glucose ≥7.0 mmol/l; 3) oral glucose tolerance test ≥11.1 mmol/l. Ten type 2 DM patients (6 males and 4 females, mean age 65.20±10.72 years, range 55-76 years, average body weight 70.0±10.2 kg, mean fasting blood-glucose level 10.9±3.3 mmol/L) diagnosed for the first time, were recruited from the patients hospitalized in the Department of Endocrinology of the Third Affiliated Hospital of Sun Yat-Sen University on March 2013. Ten sex- and age-matched healthy subjects (6 males and 4 females, mean age 66.79±7.58 years, range 55-76 years, average body weight 64.9±10.1 kg, mean fasting blood-glucose level 4.5±0.6 mmol/L) were also recruited. Platelets were prepared and mitochondrial function was tested immediately and after incubated still for 24 h: platelet of DM patients in PBS containing 10 mmol/L glucose and platelet of normal people in PBS containing 5 mmol/L glucose at 37°C.

Platelet counting

Platelet counting was done by blood counting chamber. The blood counting chamber was divided into 9 big squares and there are 25 medium squares in the central big square. Platelets numbers (N) in the medium squares

of the four corners and the middle of the big square was recorded. 10 μL PRP diluted 10 times by platelet-free plasma was added to the blood counting chamber covered by a coverslip. Platelet number/L=5N×10^8.

Platelet viability

Platelet viability was determined by MTT according to Shi et al [16]. 15 μL platelet, 135 μL PBS-glucose, and 10 μL MTT were added to each well of 96-well plate. After incubated at 37°C for 4 h, 100 μL DMSO was added to each well and the plate was shaken for 10 min. With PBS and DMSO as the blank, the absorbance was read with Microplate Reader (SUNRISE, TECAN Switzerland) at a wavelength of 490 nm.

Platelet mitochondrial membrane potential

Platelet MMP was determined as previously described [16]. Platelet was stained with JC-1 solution for 20 min in the dark at room temperature (about 25°C). Platelet MMP was measured by flow cytometry (BD FACS Calibur USA). Green and red fluorescence was measured on the FL1 (PMT 488 nm BP) and FL2 (PMT 536 nm BP) channels, respectively. The ratio of red/green fluorescence intensity was analyzed by WinMDI 2.9 software (the Scripps Research Institute, USA).

Platelet ROS

Platelet ROS was measured by fluorescence dye DCFH-DA [17]. DCFH-DA was added into platelet at the final concentration of 10 μM and then incubated for 30 min at 37°C in the dark. Then platelet green fluorescence was measured by flow cytometry on the FL1 (PMT 488 nm BP) (BD FACS Calibur USA). The platelet ratio showed green fluorescence was analyzed by WinMDI 2.9 software (the Scripps Research Institute, USA).

Platelet ATP content

ATP content was measured by the ATP Assay Kit, followed a firefly luciferase method. Platelet ATP was obtained as the following: 1mL platelet suspension was centrifuged at 800× g for 10 min at 4°C and 400 μL lysis buffer was added to the pellet. After centrifuged at 12000× g for 10 min at 4°C, the supernatant was placed into a new tube.

Platelet mitochondria were isolated using mitochondria isolation kit according to the manufacturer's protocol. Briefly, platelet was centrifuged at 800× g for 10 min and the supernatant was removed. Then platelet was homogenized on ice with a hand-held Dounce homogenizer in 800 μL ice-cold mitochondria isolation medium (10 mM HEPES, pH 7.5, containing 200 mM mannitol, 70 mM sucrose, 1mM EGTA and 1% fatty acid-free BSA) The homogenate was centrifuged at 700× g for 5 min. The supernatant was kept on ice, and subsequently transferred carefully into a new tube and centrifuged at 12000× g for 15 min. The resulting pellets were re-suspended in 500 μL of mitochondria isolation medium and subjected to a second centrifugation at 12000× g for 5 min. The final pellets were re-suspended in mitochondrial lysis buffer (approximately 40 mL per 100 mg tissue) and were kept on ice. All centrifugation steps were performed at 4°C.

100 μL test buffer and 100 μL supernatant were added into 96-well white plate and assayed by a luminometer (Infinite F500, TECAN Switzerland). The standard curve of ATP concentration was determined by a known amount (0.01 μM-10 μM). Finally ATP concentration was adjusted by protein content measured by BCA Assay Kit.

Platelet ultra-microstructure

PRP was centrifuged at 800× g for 10 min, the supernatant was removed and the pellet was re-suspended with PBS and centrifuged again. 2.5% glutaraldehyde and 1% osmic acid was added to the pellet. All the above steps were performed at 4°C. The platelet was then embedded with Epon-812, cut into ultrathin sections, stained with both lead nitrate and uranium acetate and observed using an electron microscope (EMS) (Tecnai Spirit, USA).

Statistical analyses

All statistical analyses were performed by SPSS19.0 software (Statistical Package for the Social Science). Kolmogorov-Smirnov test and Levene's test of Equal Variances were applied for normal distribution and variance homogeneity respectively. The data were analyzed with independent-sample t test, values are presented as mean ± SD and α was set at 0.05.
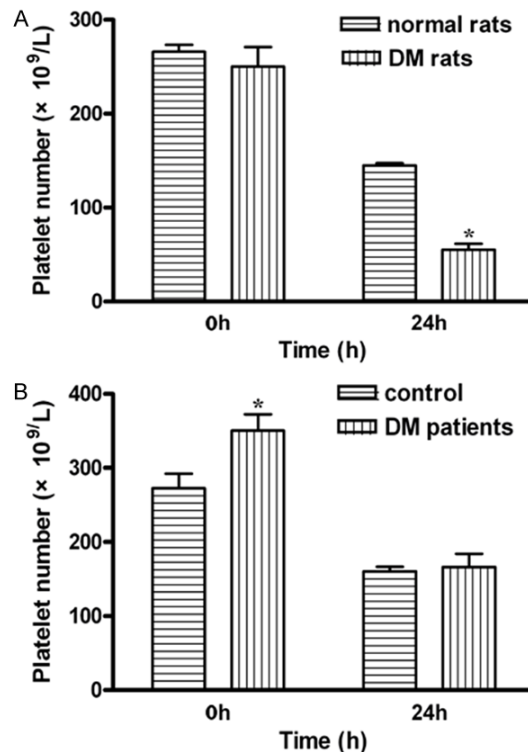
Figure 1. Platelet number. A. After incubation for 24 h, platelet number of DM rats was significantly less than that of normal rats, (F=5.276, *P*=0.000). B. Platelet numbers of DM patients were higher (*F*=6.720, *P*=0.041) than that of normal people. Values are presented as mean ± SD. *Compared with normal people, *P*<0.05; #compared with normal rat, *P*<0.01.
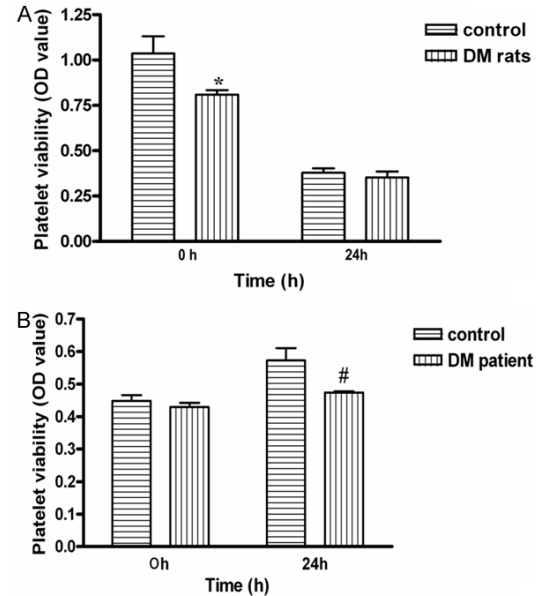

Figure 2. Platelet viability. A. There was a detectable difference between platelet viabilities of normal rats and DM rats, (F=14.137, P=0.046). B. Incubated for 24 h can make the platelet viability of DM patients lower than that of the normal people (F=1.038, P=0.043), values are presented as mean ± SD. *Compared with platelet of normal rat, P<0.05; #compared with platelet of normal people, P<0.05.

Results

Platelet number

In animals, platelet number of normal rats was (265.9±16.1) and (144.8±53.2)×10⁹/L immediately after sacrifice and 24 h incubation; platelet numbers of DM rats were (250.2±50.3) and (55.1±15.2)×10⁹/L at same time points. After 24 h incubation, platelet number of DM rats was significantly less than control, (F= 5.276, *P*=0.000) (**Figure 1A**). For human, platelet numbers of DM patients were higher (*F*=6.720, *P*=0.041) than that of normal people (**Figure 1B**) immediately after blood collection. Values are presented as mean ± SD.

Platelet viability

As is shown in **Figure 2**, there was a detectable difference between platelet viabilities of nor-

mal rats and DM rats at 37°C, (1.03±0.21) and (0.81±0.06), (F=14.137, P=0.046). Longer incubation (24 h) did not show any difference. The OD values of platelet of DM patient were (0.42±0.03) and (0.44±0.08), while those of the normal people were (0.45±0.04) and (0.57±0.08) immediately after blood collection and 24 h incubation, respectively. Incubated for 24 h made the platelet viability of DM patient lower than that of the normal people (F=1.038, P=0.043), values are presented as mean ± SD.

Platelet reactive oxygen species (ROS)

Platelet ROS of normal rats at 37°C was (3.130±0.382)% and (4.173±0.989)%; the ROS of DM rats was (6.861±5.066)% and (10.764± 4.066)%. Platelet ROS of DM rats was higher than that of normal rats after 24 h incubation (F=4.805, P=0.004) (**Figure 3A**). Platelet ROS of normal people was (34.545±3.617)% and (88.342±2.433)%; platelet ROS of DM patients was (84.402±6.382)% and (94.068±1.278)% (**Figure 3C**). Compared to normal people, platelets of DM patient generated more ROS (F=
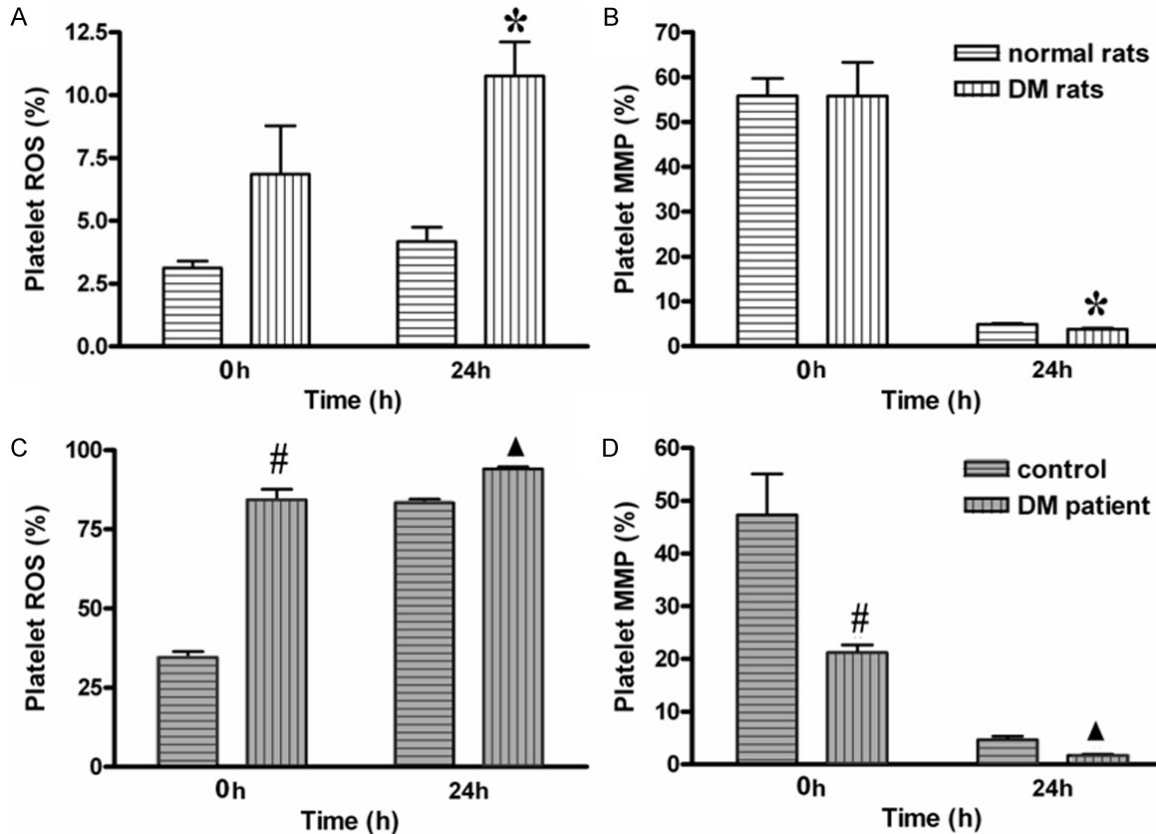
Figure 3. Platelet ROS and MMP. A. 24 h incubation made platelet ROS of DM rats lower than that of normal rats (F=4.805, P=0.004). B. 24 h incubation made platelet MMP of DM rats lower than that of normal rats (F=0.284, P=0.005); C. Compared to normal people, platelet of DM patients generated more ROS (F=4.987, P=0.000; F=7.879, P=0.001). D. The platelet MMP of DM patients was lower than that of normal people (F=5.478, P=0.019; F=3.131, P=0.003). Values are presented as mean ± SD. *Compared with platelet of normal rats at 24 h, P<0.05; #compared with platelet of normal people at 2 h, P<0.05; ▲compared with platelet of normal people at 24 h, P<0.05.

4.987, P=0.000; F=7.879, P=0.001), values are presented as mean ± SD.

Platelet mitochondrial membrane potential

Representative flow cytometry dot plots of platelet showing JC-1 staining at 37°C were shown in **Figure 4**. Platelet MMP of normal rats at 37°C was (55.825±8.65)% and (4.325 ±0.350)%; for DM rats, the MMP was (55.725±16.925)% and (3.875±0.400)%. 24 h incubation made platelet MMP of DM rats lower than that of normal rats (F=0.284, P=0.005) (**Figure 3B**).

Platelet MMP of DM patients and normal people after incubation were shown in **Figure 3D**. The MMP of DM patients were (47.3±19.0)% and (4.6±1.5)%, while those of the normal people were (21.7±4.3)% and (1.7±0.5)%, respectively. The platelet MMP of DM patients was lower than that of normal people (F=5.478,

P=0.019; F=3.131, P=0.003), values are presented as mean ± SD.

Platelet and platelet mitochondria ATP content

As we can see from **Figure 5A**, platelet ATP content of DM rats was (8.805±1.617) nmol/mgprot, significantly lower than that of normal rats, (16.965±3.871) nmol/mgprot (P=0.006).

Platelet mitochondria ATP content of normal rats was (3.124±0.064) and (1.465±0.010) nmol/mgprot; for DM rats, platelet mitochondria ATP content was (1.806±0.591) and (0.995±0.161) nmol/mgprot. There were significant differences between the two groups (F=0.000, P=0.001; F=2.176, P=0.044) (**Figure 5B**), values are presented as mean ± SD.

The morphological changes of platelet mitochondria

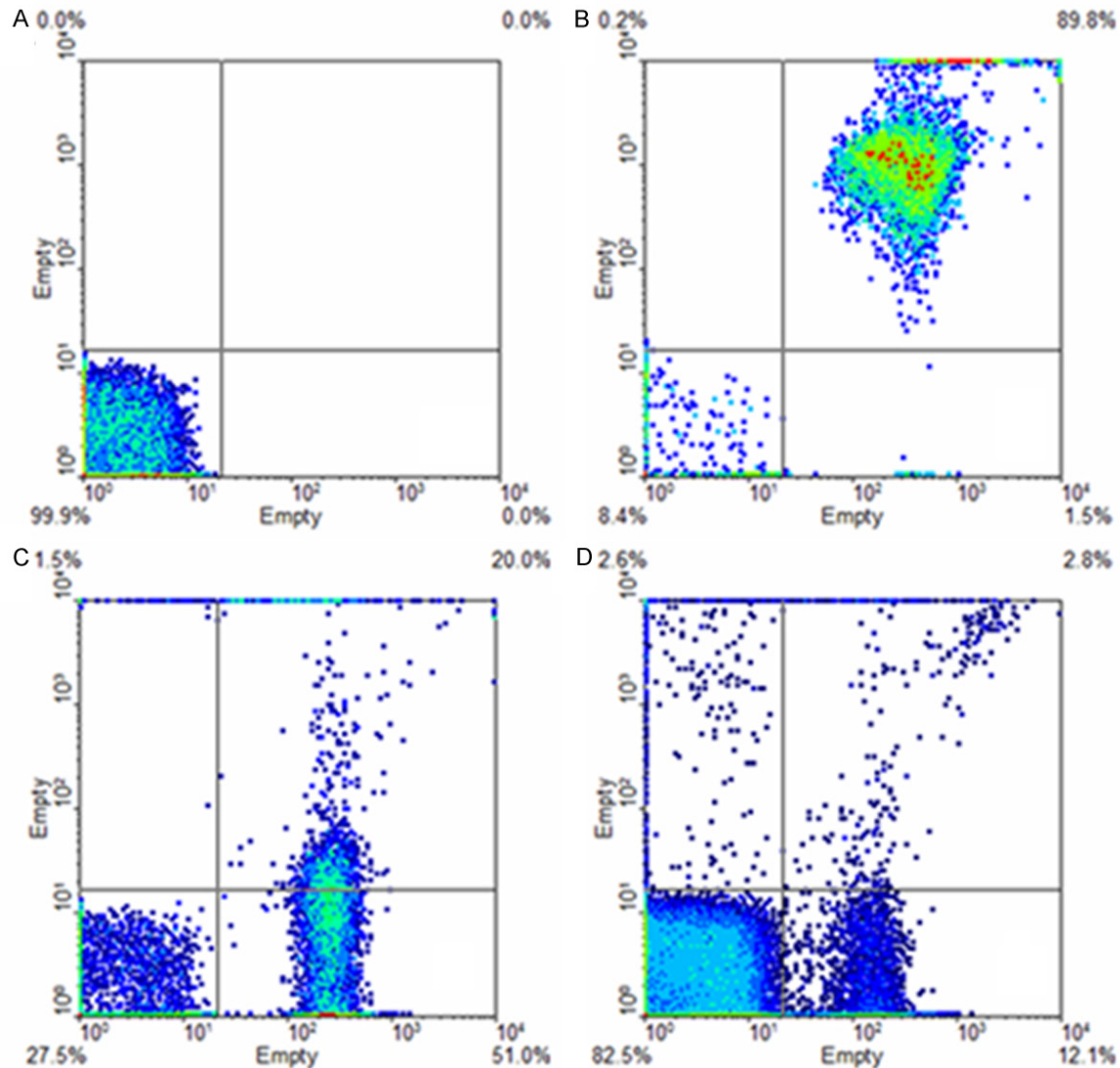No obvious morphological abnormality of mitochondria was observed in normal rat platelet,

Figure 4. Representative flow cytometry dot plots of platelet showing JC-1 staining at 37°C. A. Without staining, approximately no platelet events express red Jaggregates (PMT2, as shown in the upper right gate) and no contains green monomer fluorescence (PMT1, as shown in the lower right gate). B. platelet of normal people, approximately 89.8 percent of the platelet events express red Jaggregates (PMT2, as shown in the upper right gate) and 1.5 percent contains green monomer fluorescence (PMT1, as shown in the lower right gate). C. platelet of DM patients, approximately 20.0 percent of the platelet events express red Jaggregates (PMT2, as shown in the upper right gate) and 50.1 percent contains green monomer fluorescence (PMT1, as shown in the lower right gate). D. platelet of normal people incubated for 24 h, approximately 2.8 percent of the platelet events express red Jaggregates (PMT2, as shown in the upper right gate) and 12.1 percent contains green monomer fluorescence (PMT1, as shown in the lower right gate).

(**Figure 6A**). Outer and inner membranes were clear, and cristae were intact. In platelet of DM rat, abnormal mitochondria with disorganized cristae, α-granules and vacuoles were observed (**Figure 6B**, **6C**).

Mitochondrial ultramicro-structures of platelets of DM patients and normal people were observed. No obvious morphological abnormality of mitochondria was observed in platelets of normal people (**Figure 6D**). Outer and inner

membranes were continuous, and cristae were plentiful. In platelets of DM patents, swelled mitochondria with disorganized cristae, damaged inner membrane, plenty of glycogen, α-granules and vacuoles were detected (**Figure 6E-H**).

Discussion

Mitochondrial dysfunction has been suggested to contribute to both morbidity and develop-
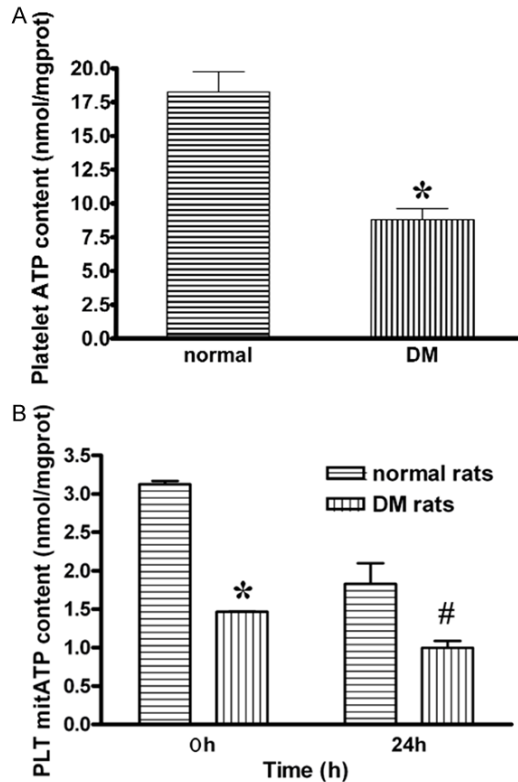
Figure 5. Platelet and platelet mitochondrial ATP content. A. platelet ATP content of DM rats was significantly lower than that of normal people; B. platelet mitochondrial ATP content of the two groups were significant differences (F=0.365, P=0.940; F=2.122, P=0.953). Values are presented as mean ± SD. *Compared with that of normal rats, P<0.05; #compared with that of normal rats at 24 h, P<0.05.

ment of DM [6]. In this study, to observe the changes of platelet mitochondrial function, we measured platelet viability, MMP, ROS and ATP content. The results were that for both DM rats and DM patients, the platelet viability, ATP content and MMP decreased; ROS increased. Our finding suggested that the platelet mitochondrial function was impaired in both DM rats and DM patients.

Mitochondrion processed enzymes needed in TAC and oxidative phosphorylation (OXPHOS), so mitochondrion is usually called energy factory of the cell [6]. ATP content was also used to reflect the ability of mitochondria to generate energy for the normal life of the cell [15]. In this experiment, we found that the platelet ATP content of DM rats was lower than that of normal rats. One of the main reasons maybe the TAC and OXPHOS system cannot work as powerful

as normal. So we measured the platelet mitochondrial ATP content and the results showed that the mitochondrial ATP content was also decreased. Platelet viabilities of both DM rats and DM patients all decreased. The MTT assay reflects the disoxidation ability of succinate dehydrogenase (SDH) in living cells, which is a marker enzyme of mitochondria. Platelet MMP of both DM rats and DM patients decreased. Exposed to high glucose for 7d, MMP of retinal pericytes collapsed [18]. Using Female lepr+/+C57BL/KsJ (db/db) mice as the model, Sourris [19] showed the same results as ours: decreased MMP and ATP content. To clarify why high glucose can interfere in TAC and OXPHOS, we measured the platelet ROS and found that platelets of DM rats and DM patients all showed excessive ROS.

The dysfunction of ETC, especially complex III, can lower the efficiency of the use of oxygen and ROS thus generated. Increased ROS and the damage of respiratory chain complexes could respectively or collaboratively reduce the out shift of protons or increase its backshift to lower ΔpH and MMP. The lowered MMP would open the permeability transition pore (PTP) in the mitochondrial membrane. The opening of PTP would eliminate the residual ΔpH completely, aggravating the reduction of the MMP. Mitochondrial ATP production was mainly depend on electric pressure gradient (mitochondrial membrane potential, MMP) and H+ concentration gradient (pH gradient, ΔpH) formed by proton shift into the mitochondrial intermembrane space accompanied by electron transport [15]. So the reduction of the MMP would inevitably lead to reduced cellular ATP content.

The accumulation of ROS in platelet can not only attack ETC, decrease their activity but also impair the mitochondrion DNA repair enzymes [7]. After incubated with high glucose (11 mM) for 12 h, the oxidation rate of glucose and viability of INS-1 cell reduced, the TAC intermediates, such as oxaloacetate, citrate, and α-ketoglutarate decreased; the supplement of oxaloacetate, citrate, and α-ketoglutarate can significantly increased the cell ATP content [20]. The TAC was also impaired in skeletal muscle from DM patients [21]. Incubated with high glucose (30 mM) for 72 h can significantly decrease the activity and expression of mitochondrial complexes I, II, and III [20]. Type 2 DM
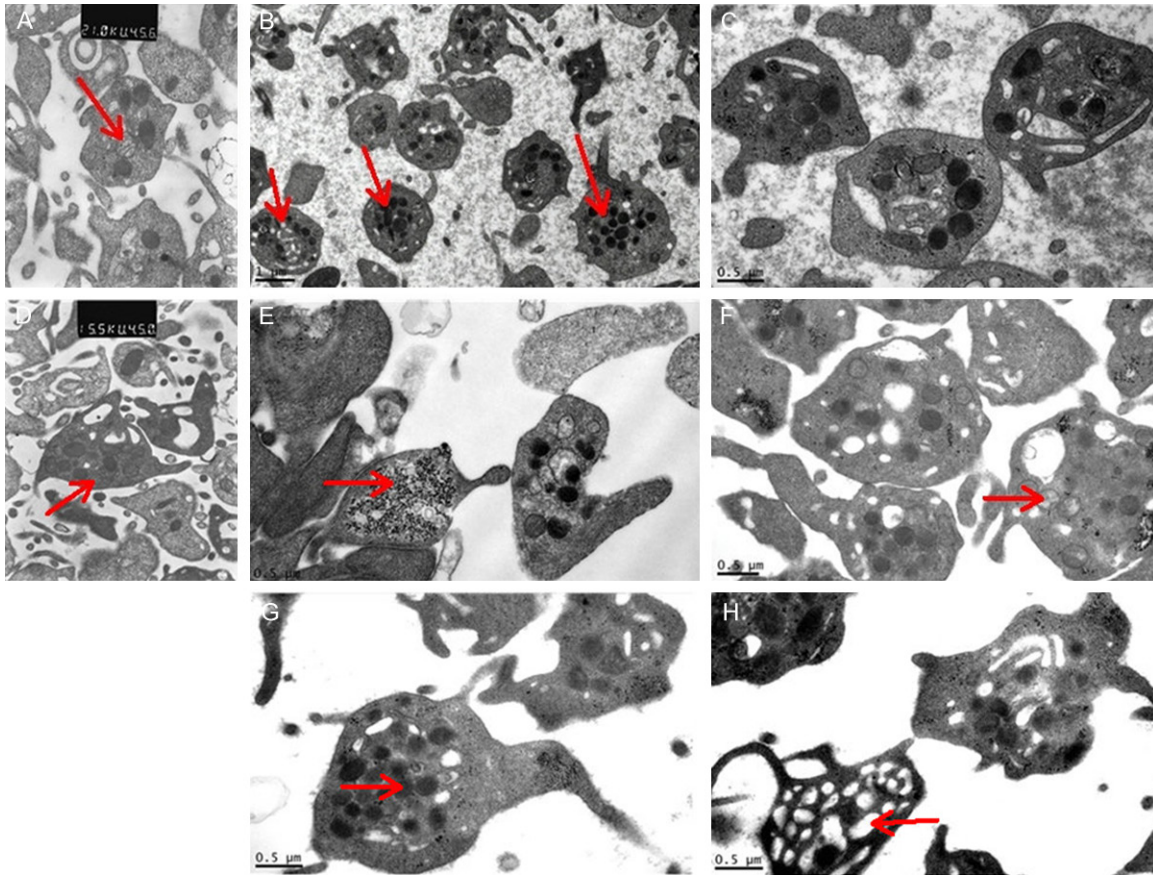
Figure 6. Electron micrograph of platelet of normal rats, DM rats, normal people and DM patients. A. Electron micrograph of platelet of normal rat (bar=0.5 μm). The arrow showed the mitochondrion, the inner and outer membrane and cristae are clear. B. Electron micrograph of platelet of DM rat (bar=0.5 μm). The arrow showed a large number of α-granules and vacuoles gathered in platelet. C. Electron micrograph of platelet of DM rat (bar=0.5 μm). The arrow showed the cristae were discontinuous. D. Electron micrograph of platelet from normal people (bar =1 μm). The arrow showed the mitochondrion, the inner and outer membrane and cristae are clear. E. DM patient platelet (bar=0.5 μm). The arrow showed plenty of glycogen in platelet. F. DM patient platelet (bar=0.5 μm). The arrow showed the disordered mitochondrial, the inner membrane and cristae were discontinuous. G. DM patient platelet (bar =1 μm). The arrow showed a large number of α-granules gathered in platelet. H. DM patient platelet (bar=1 μm). The arrow showed a large number of vacuoles in platelet.

patients showed decreased complex I and IV activity and the expression of the genes involved in energy metabolism, TAC and OXPHOS decreased [22].

In our experiment, ultra-microstructure of platelet mitochondria of DM rats and patients was destroyed: swollen mitochondria with disorganized cristae, damaged inner membrane, plenty of glycogen, α-granules and vacuoles. The detailed reason was well studied [23]. The morphology change may be another evidence to prove the mitochondrion was injured [18].

For DM patients and high risk group, diagnosis early is of great importance because early dis-

covery and prompt therapy can effectively slow down DM progression. Platelets may be used as a biomarker for DM diagnosis due to the following reasons: 1) Platelet extraction is easy and almost invasive, so patient tolerance will be better. 2) Some mitochondrial changes found in other organ can be found in platelet: decreased ATP content and MMP, increased ROS and swollen mitochondria. These all told us that platelet mitochondrial dysfunction can reflect, at least in part, the DM pathology. Mitochondrion lies in the center of progression of DM [6]. So platelet can be used as a peripheral model to observe mitochondrial functional and morphological changes and to clarify the mitochondrial function in DM complications.

Acknowledgements

Disclosure of conflict of interest

None.

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