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Mitochondrial Dysfunction Associated with Low Expression of Bcl-2: an Inflammatory Mediator in Diabetic Patients with Atherosclerosis

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Objective: To investigate mitochondrial dysfunction and pattern of expression of B-cell lymphoma 2 (Bcl-2) and correlate these with cardiovascular diseases (CVD) in type 2 diabetes mellitus (T2DM).

Background: Diabetes mellitus (DM) raises the risk of CVD, which contributes significantly to the high mortality rates associated with the disease. The role of mitochondrial dysfunction and apoptosis in the development of CVD has been suggested, however the mechanisms involved are unknown.

Methods: Type 2 diabetic mellitus patients with atherosclerosis (T2DM + ATHER, n=40), T2DM patients (n=40) and a control group of age-matched non-diabetic individuals (n=35) were enrolled in this study. Blood samples were collected and analysed for the presence of anti-apoptotic-related factors, Bcl-2 in Peripheral mononuclear cells by qPCR as well as mitochondrial dysfunction by immunofluorescence analysis. Serum TNF-α and antioxidant enzymes (SOD) were measured by ELISA.

Result: Bcl-2 expression was significantly decreased in patients with T2DM + ATHER and T2DM ($P < 0.05$) compared to the control group. MitoSpy Red staining revealed nuclear condensation and fragmentation in T2DM + ATHER and T2DM. There was a significant increase in pro-inflammatory cytokine TNF-α while the levels of SOD were significantly decreased in the T2DM + ATHER and T2DM patient group ($P<0.05$) compared to the control group.

Conclusion: These results suggest that mitochondrial dysfunction was associated with oxidative stress and inflammation in patients with T2DM +ATHER.

Key words: Atherosclerosis; mitochondrial dysfunction; apoptosis; oxidative stress

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Diabetes mellitus is major risk factor for the development of cardiovascular diseases (CVD) principally atherosclerosis. Previous evidence suggests that myocardial metabolism is altered in diabetes, which likely contributes to the development of cardiac dysfunction (1). Mitochondrial dysfunction and increased reactive oxygen species (ROS) generation are significantly related to diabetic heart damage. Mitochondria are regarded as the house of cellular energy and the center of metabolism in the heart. Recently, studies reported that any damage in the mitochondria can impair cellular function and has been linked with pathogenesis of diabetic cardiomyopathy (2).

Different mechanisms have been described to control mitochondrial quality, including mitochondrial fission and fusion, mitophagy and biogenesis. Therefore, a failure of these mechanisms indicates mitochondrial damage, which has been observed in the hearts of diabetic patients (3). Oxidative stress-mediated myocardial injury is a consequence of an imbalance between free radical generation and elimination. Overproduction of ROS and impairment of mitochondrial dynamics result in mitochondrial dysfunction which may cause the development of several cardiac diseases (4). Moreover, oxidative stress stimulates the expression of several pro-inflammatory cytokines like Interleukin (IL)-1 and tumour necrosis factor (TNF)-α, which is in turn associated with mitochondrial dysfunction (5, 6).

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Apoptosis in human hearts is associated with pathological conditions including acute myocardial infarction (7, 8), complete heart block (9) atherosclerosis (10), restenosis (11) and end-stage hypertrophic cardiomyopathy (12). Apoptosis is programmed cell death, a complex process that is triggered by mitochondrial and endoplasmic reticulum–stress responses and oxidative stress. Apoptotic signalling can occur via two pathways, extrinsic or intrinsic (the mitochondrial-dependent pathway) (13). Different mediators are involved in these pathways, which are classified as apoptotic or antiapoptotic factors. The B-cell lymphoma 2 (Bcl-2) is a family of proteins consisting of anti- and proapoptotic members, and the Bcl-2 protooncogene inhibits apoptosis (14, 15).

This study aimed to investigate a possible relationship between mitochondrial dysfunction and the expression of Bcl-2 to investigate possible biochemical mechanisms in diabetic patients with cardiovascular disease.

MATERIALS AND METHODS

Subjects: 40 patients with type 2 diabetes mellitus and atherosclerosis (T2DM + ATHER, N = 40), 40 individuals with Type 2 diabetes mellitus (n=40), and 35 individuals (n=35) with no clinically diagnosed atherosclerosis and no diabetes mellitus (the control group), were included in this study. Outpatient cardiology and vascular surgery practices were used to recruit patients with clinical atherosclerotic cardiovascular disease. The criterion for diabetes mellitus is fasting blood glucose levels >126 mg/dL, while coronary artery disease is defined as angiography or a documented history of myocardial infarction. All subjects signed a written informed consent form, and all study methods were approved by the Ethical Committee of the Diwaniyah Teaching Hospital and the University of Al- Qadisiyah (Al-Diwaniyah, Iraq).

Methods

All the groups underwent a full clinical history, examination, and CVD study. Venous blood samples (5 mL) were obtained from the patients and the control group and divided into parts: One component was centrifuged (Kokusan centrifuge, Germany) at 3000 RPM for 10 minutes to extract plasma, which was then stored in closed plastic tubes at -20 °C until analysis. Glucose was measured with a commercial kit, while TNF-α and superoxide dismutase (SOD) levels in the plasma were measured by ELISA (Elabscience, China). Peripheral blood mononuclear cells were isolated from the second part of the blood by differential centrifugation.

For the isolation of lymphocytes and monocytes, venous blood was collected into density gradient solution tubes and spun at 3000 rpm for 30 minutes at room temperature. Cell layers were removed, pelleted, and kept at 80 °C until qPCR measurements of Bcl-2 expression were performed. Total RNAminiprep kit (Favorgen biotech, Taiwan) was used, cDNA was synthesized and qPCR for Bcl2 was used for analysis. The resulting cDNA was mixed with specific primers (forward primer: TGGATGACTTGAGTACCTGAAC, reverse primer: ACAGCCAGGAGAAATCAAAC), and Bright Green master mix (Abm, Canada). For the housekeeping gene, a-actin was used.

Mitochondrial dysfunction was measured by fluorescence microscope using MitoSpy staining. For analysis of mitochondrial dysfunction, peripheral monocytes were stained with MitoSpy Red that localized to the mitochondria based on its membrane potential (BioLegend, UK).

Statistical Analysis

Data are expressed as mean ± SEM. Statistical analysis was carried out using SPSS, the significant differences between groups were determined by using one way ANOVAs. The probability of ($P < 0.05$) was considered significant.

RESULTS

Clinical and Biochemical Characteristics of Study Groups:

The patients' characteristics and clinical parameters are shown in Table 1. In terms of age, gender, and BMI, no significant differences were detected between the patient groups and the control group ($p > 0.05$). Compared with the T2DM patients and the control, fasting blood sugar (FBS), systolic blood pressure (SBP), triglycerides (TG), total cholesterol (TC) and low density lipoprotein cholesterol (LDL-c) levels were higher in the T2DM + ATHER group ($P< 0.05$), which also had lower high density cholesterol (HDL-c) levels. These results indicate that atherosclerosis was initiated and developed by risk factors such as obesity, dyslipidaemia and hypertension.

Table 1. Patients' characteristics and clinical parameters

Parameter	T2DM + ATHER Mean ±SEM	T2DM Mean ±SEM	Control Mean ±SEM	P-value
Age (year)	55.7± 3.2	54.23± 4.5	54.4±2.2	*P* >0.05
BMI	25.2 ± 3.8	24± 3.1	23.5± 4.2	*P* >0.05
Systolic BP(mmHg)	145.02±16.03 *	138.02±14.15 *	126.55±13.22	*P*< 0.05
Diastolic BP(mmHg)	82.3±5.6	81.3±2.4	80.1±3.2	P>0.05
Total cholesterol mg/dl)	234.63 ± 44.23*	195.03 ± 55.48*	112.16 ± 23.31	*P*< 0.01
Triglycerides (mg/dl)	278.3 ±81.31*	204.9 ± 64.25*	115.5 ±21.22	*P*< 0.01
HDL-c (mg/dl)	40.9±1.2*	42.5±1.7*	48.6±2.2	*P*< 0.05
LDL-c (mg/dl)	154.46±6.3*	145.95±11.03*	120.12±13.2	*P*< 0.01
FBS (mg/dl)	265.3 ±61.40*	213.12±71.15*	94.56 ± 9.28	*P*< 0.01

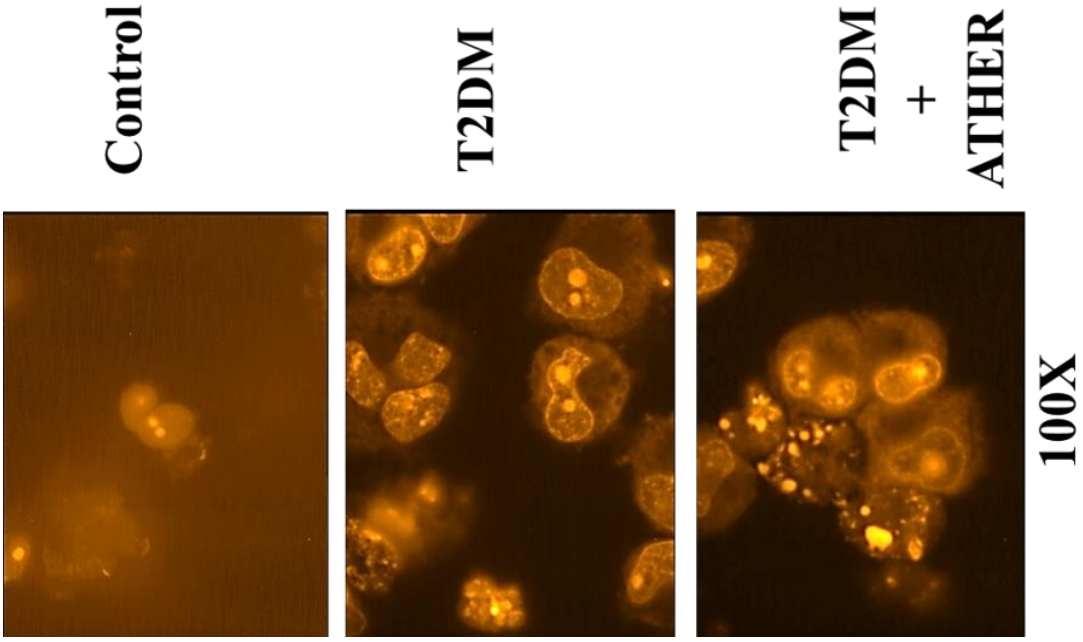

Figure 1. Morphological changes associated with mitochondrial dysfunction in T2DM + ATHER, T2DM, and control groups

Mitochondrial DNA Damage

MitoSpy Red staining indicated nuclear condensation and fragmentation associated with apoptosis in dispersed cells from the T2DM + ATHER and T2DM groups compared to the control group, with lower mitochondrial masses and apoptotic bodies (Fig .1).

Mitochondrial DNA Damage with Inflammatory Cytokines

TNF-α serum levels were significantly increased in the patient groups compared to the control group (*P* value < 0.05), as shown in Fig. 2. This increase was related to high levels of mitochondrial dysfunction.

As a marker of oxidative stress, serum SOD activity was significantly decreased in the T2DM + ATHER and T2DM groups compared to the control ($P < 0.05$, Fig. 3). qPCR data analysis showed that the expression of anti-apoptotic Bcl-2 was significantly decreased in the patient groups compared to the control group ($p<0.05$, Fig. 4).

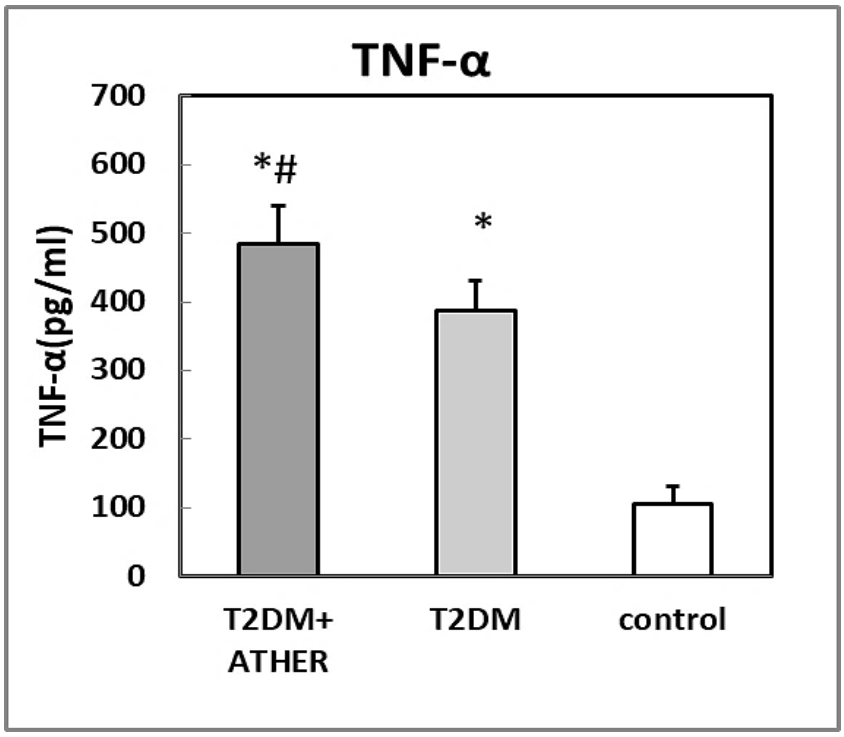

Figure 2. TNF-levels in plasma from T2DM + ATHER, T2DM, and control patients. *indicates significant differences relative to the control group, # indicates significant differences between patient groups (P 0.05).

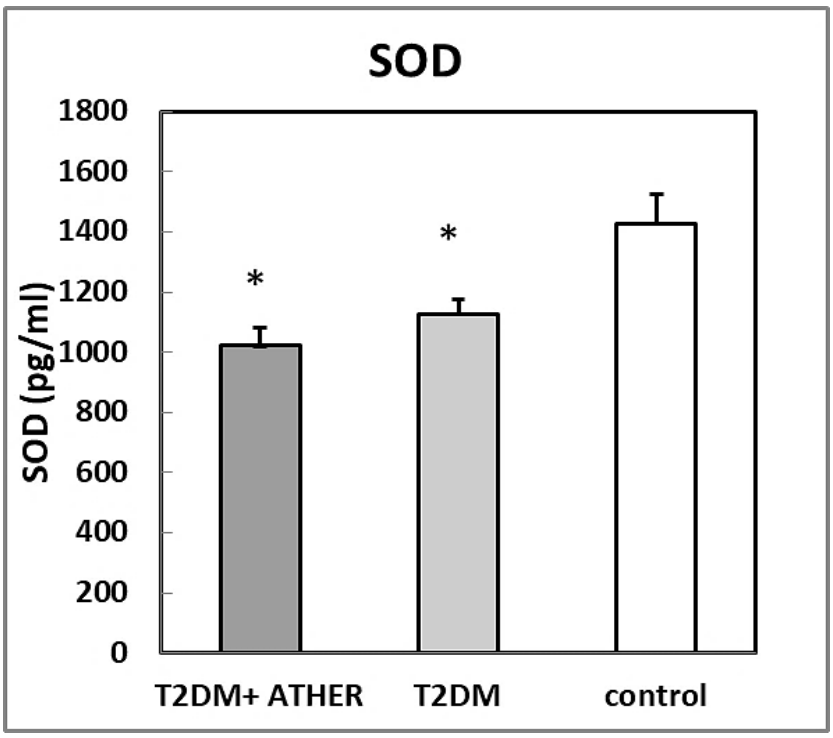

Figure 3. SOD levels in plasma patients with T2DM + ATHER, T2DM, and control groups. Data are expressed as mean ± SEM, *indicates statistically significant differences relative to the control group ($P \leq 0.05$).

DISCUSSION

The present study characterized mitochondrial dysfunction and its association with inflammation and apoptosis from diabetic patients with and without CVD. The levels of TG, TC and LDL-c were significantly increased in T2DM + ATHR, while HDL-c levels were relatively lower compared to the control group (Table 1). Consistent with these results, previous studies have recorded that dyslipidaemia was higher in T2DM patients compared to healthy subjects. The mechanism explaining dyslipidaemia in the pathogenesis of T2DM is β-cell dysfunction and increased fatty acid influx secondary to insulin resistance (15). The major findings of this study were that there was an increase in mitochondrial dysfunction in subjects with a clinical diagnosis of atherosclerosis, and that oxygen and nutrients should be supplied to the tissues. As a result, the human heart must constantly pump blood, resulting in a loss of energy, even when at rest (16). Because the mitochondria provide most of this energy and are necessary for proper heart function, cardiovascular illnesses are linked to mitochondrial malfunction (17).

In a healthy heart, mitochondrial bioenergetic function plays a key role. Defective mitochondrial proteins, oxidative damage, and altered signalling pathways are all associated with heart failure, resulting in inadequate energy production in the myocardium (18). In addition, disease associated with mitochondrial dysfunction affects the heart (19). Atherosclerosis is a chronic inflammatory disorder of the arteries. Cellular responses to inflammation and injury involve TNF-α as a key player. In the cardiovascular system, activation of signal transduction pathways by TNF-α may contribute to vascular dysfunction, the development and progression of atherosclerosis, myocardial infarction and heart failure. The present study investigated the levels of TNF-α in patients with T2DM and atherosclerosis. Increased plasma levels of TNF-α were observed in all patient groups compared to the control (Fig. 2). Several mechanisms have been identified by recent studies in which TNF-α may promote atherogenesis including endothelial adhesion molecule expression (20), activation of macrophages (21), stimulation of smooth muscle cell (SMC) proliferation and migration (22) as well as induction of apoptosis (23). SOD levels were measured in the present study. Low levels of SOD were found in patients with type 2 DM and atherosclerosis, which indicated high oxidative stress (24). Previous studies in patients with CAD indicated that plasma levels of oxidized LDL (25), malondialdehyde (26), and advanced oxidation protein products (27) were significantly higher in those with CAD compared to those without. Enzymatic catalysis and enzyme-mediated ROS generation have been linked to impaired mitochondrial respiration and ATP synthesis, as well as the development of a variety of illnesses, including diabetes and heart failure (28, 29,30, 31).

Oxidative stress has been shown as one of the stimuli for an increased level of mitochondrial fusion (32, 33). Mitochondria fuse when their integrity is impaired, isolating faulty mtDNA gene products from healthy neighbouring mitochondria (34). An accumulation of mutations and damage to mtDNA has been described in patients with CAD (35) and in animal models of myocardial infarction, due to its closeness and vulnerability to mitochondrial ROS (36). In support of these findings, investigations have also shown that cellular stressors such as cardiac ischemia cause greater mitochondrial fission (37).

Altering the calcium handling dynamics of the cell is one way that Bcl-2 could influence mitochondrial function and cell survival. By altering either the mitochondria or the endoplasmic reticulum, Bcl-2 and family members Bax and Bak have been found to modify cellular reactivity to Ca^{2+} (38). Mitochondria use Ca^{2+} released from the endoplasmic reticulum to buffer the cytosolic Ca^{2+} level. In cultured neurons, Bcl-2 can boost the Ca^{2+} buffering capacity of mitochondria from neurons (39) and cardiomyocytes (40), as well as prevent cell death caused by Ca^{2+} (41).

Studies are being conducted to discover whether mitochondrial dysfunction causes atherogenesis or if mitochondrial dysfunctions are reactions to atherosclerosis, as this is currently unknown (42). Recently, it was discovered that overexpression of Bcl-2 reduced cardiomyocyte death in myocardial ischemia models, an inherited type of cardiomyopathy that was significantly improved. Although the significance of Bcl-2 family members in human heart failure is unknown, it is known that both Bcl-2 and Bax are expressed at high levels in failing human hearts (43). Because the Bcl-2 family has the capacity to affect heart conditions such as ischemia, calcium dysregulation, and increased oxidative stress, it is clear that members of the Bcl-2 family are promising therapies for a variety of cardiac illnesses.

CONCLUSIONS

The evidence that mitochondrial damage/dysfunction occurs in both normal aging and atherosclerosis is growing. Mitochondrial failure can lead to an increase in ROS production and calcium dysregulation. Apoptosis and senescence, two important processes in the development of susceptible atherosclerotic plaques, are aided by these consequences. Mitochondrial dysfunction has important metabolic consequences, and its systemic manifestations may contribute to the development of atherosclerosis. Mitochondrial injury and malfunction are thus therapeutic targets for drug development or lifestyle changes.

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