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Research Paper

Melatonin and L-carnitin improves endothelial disfunction and oxidative stress in Type 2 diabetic rats

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ABSTRACT

Vascular dysfunction is thought to play a major role in the development of diabetic cardiovascular disease. The roles of endothelial dysfunction, oxidative stress, and dyslipidemia will be considered. Melatonin as well as L-carnitine were shown to possess strong antioxidant properties. Diabetes induced with high fat diet (for 8 weeks) and multipl low doses intraperitoneal injection of STZ (twice, 30 mg/kg/d i.p). The diabetic animals were randomly assigned to one of the experimental groups as follows: Control group (C), high fat diet (HFD), STZ-induced diabetic group (HFD+STZ) , HFD+STZ diabetic group received melatonin (10 mg/kg/d i.p), HFD+STZ diabetic group received L-carnitine (0.6 g/kg/d i.p), and HFD+STZ diabetic group received glibenclamide (5 mg/kg/d, oral). The serum fasting blood glucose, insulin, total cholesterol, HDL- cholesterol, LDL-cholesterol, triglyceride and malondialdehyde (MDA) levels were tested. Acetylcholine induced endothelium-dependent relaxation and sodium nitroprusside induced endothelium-independent relaxation were measured in aortas for estimating endothelial function. Also, glutathione peroxidase (GP*x*), superoxide dismutase (SOD) and nitric oxide (NO) levels activities were determined in rat liver. According to our results melatonin and L-carnitine treatment decreased fasting blood glucose, total cholesterol, and LDL levels. MDA levels significantly decreased with the melatonin treatment whereas SOD levels were not significantly changed between the groups. The results suggest that especially melatonin restores the vascular responses and endothelial dysfunction in diabetes.

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Diabetes Mellitus is a metabolic as well as vascular disease which causes important complications with gradually increasing frequency all over the world. Formation of the free radicals and association between oxidative stress and diabetes complications have recently gained importance in the field old diabetes.

It is well known that the interaction between free oxygen radicals and nitric oxide (NO) cause formation of peroxynitrite and, this cytotoxic oxidant leads to endothelial dysfunction by impairing the function of cellular proteins through the nitration of proteins. Endothelial dysfunction plays a role in especially NO bioavailability of vasodilators, impaired endothelium dependent vasodilation or increased endothelium derived contracting factors, resulting in emergence of diabetes complications [1].

L-Carnitine (3-Hydroxy 4-N-trimethylammonio – butyrate) is an amino acid-like agent involved in the transit of long chain fatty acids which are physically synthesized in the various tissues in living organisms and will be transferred from cytoplasm to the mitochondria matrix, through the inner mitochondrial membrane. In diabetic experimental animals, carnitine levels of pancreas have been found to decrease both in early and advanced stages of diabetes and excretion of carnitine with urine to increase [2]. There are studies suggesting that insulin sensitivity and use of glucose by peripheral tissues increase [3,4]. In the studies conducted with hypertensive and normotensive rats, L-carnitine has been demonstrated to induce endothelium dependent relaxation by increasing the production of nitric oxide [5].

Melatonin is one of the known most potent antioxidants and recent studies indicate that melatonin receptors found in the pancreas islet cells might be protective against harmful outcomes of hyperglycemia [6]. In the other studies, L-carnitine has been

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shown to effectively normalize impaired oxidative condition in diabetic rats induced with STZ [7].

1. Experimental procedures

1.1. Animal model and experimental groups

Thirty five male Wistar rats (250–350 g) had access to laboratory food and water ad libitum. They were housed in cages under standard laboratory conditions (light period between 07:00–19:00 h, 21_2 °C; relative humidity 55%). This study was approved by the Institutional Animal Care Ethics Committee of Ege University, Turkey (2010/137).

The diabetic rat model was developed using a high-fat diet plus multiple low doses of streptozotocin which was similar to that employed in previously studies [8]. The high-fat diet consisted of 22% fat, 48% carbohydrate, and 20% protein with total calorific value 44.3 kJ/kg (Bilgen Lab. Istanbul, Turkey) and control rats were given regular chow consisting of 5% fat, 53% carbohydrate, and 23% protein with total calorific value 25 kJ/kg. Following 4 weeks of dietary intervention, the diabetic group was injected intraperitoneally (i.p.) with low doses of streptozotocin (Sigma, St. Louis, MO, USA, 30 mg/kg, dissolved in 0.1 M sodium citrate buffer, pH 4.4). One week later, blood samples were collected by tail cutting for fasting blood glucose measurements were measured by (Accu-Chek Active-glucometer). Rats with a fasting blood glucose of 7.8 mmol/L were injected with streptozotocin again (30 mg/kg). Control rats were given vehicle citrate buffer (pH 4.4) in a matched volume (0.25 ml/kg) via intraperitoneal injection. 4 weeks after the streptozotocin injection, the fasting blood glucose was measured again, and rats with a fasting blood glucose of $\geq$ 7.8 mmol/L were considered diabetic [9]. The diabetic rats were fed the high-fat diet for another 4 weeks, the control and diabetic rats were then randomly divided into 5 groups: [1] control group (CONN, rats treated with saline in a matched volume), [2] Non treated diabetic group (HFD+STZ), [3] L-carnitine treated diabetic group (HFD+STZ +LC, 0.6 g/kg/d, oral), [4] melatonin treated diabetic group (HFD+STZ +MLT, 10 mg/kg/d, i.p) and [5] glibenclamide treated diabetic group (HFD+STZ+GB, 5 mg/kg/d, i.p.). Melatonin and L-carnitine were dissolved in distillated water and administered intraperitoneally glibenclamide was administrated via oral gavage daily for 2 weeks [10–12]. All the rats were allowed to continue to feed on their respective diets until the end of the study.

1.2. Vasocontractile responses

At the end of treatment, the animals were anesthetized by using an intraperitoneal injection urethane (1000 mg/kg, 20%) after fasting 12 h. Thoracic aorta was removed, placed in cold Krebs-Henseleit solution, cleaned gently of adherent connective tissue and cut into rings (approximately 3 mm length). Blood samples from abdominal aorta and also liver tissue samples were collected, when rats were killed, for the measurement of glucose, insulin, NO, lipid metabolic parameters and some antioxidant enzyme levels.

General parameters of rats (fasting blood glucose and body weight) were measured and recorded at the beginning, before the melatonin and L–carnitine treatment and at the end of experiment.

The aortic rings were suspended under a resting tension 1 g in 20 ml organ chambers containing oxygenated (5% CO_2, 95% O_2) and warmed (37'C) Krebs solution (pH: 7.4) with the following composition (mM): NaCl 112, KCl 5, NaHCO3 25, NaH2PO4 1, MgCl2 0.5, CaCl2 2.5 and glucose 11.5. All preparations were fixed with two stainless steel wires, one was connected to a force displacement transducer (MAY FDT 05, COMMAT Ltd., Ankara, Turkey) for the measurement of isometric contractions and for record on computer using transducer data acquisition system (TDA 94, COMMAT).

After 1 h washing and equilibration period, contractile responses to phenylephrine were taken in rings to determine precontractile tone in vessels. Relaxations induced by acetylcholine (10^{-9}–10^{-5} M) and sodium nitroprusside (10^{-9}–10^{-5} M) were obtained from aortic rings precontracted with EC_{80} concentration of phenylephrine. To determine endothelium-dependent relaxation induced by NO, the contractile responses to N^G-nitro-L-arginine (L-NAME) (10^{-2} M) were recorded in rings precontracted with EC_{50} concentration of phenylephrine.

1.3. Measurement of total cholesterol, LDL, HDL, triglyceride, glucose, NO and insulin

The plasma was prepared with EDTA and separated by centrifugation (10 min, 3000 rpm). Plasma total cholesterol, triglyceride and high-density lipoprotein (HDL) were measured by a commercially available enzyme kit. Plasma LDL was determined using the Friedewald formula: LDL cholesterol=Total cholesterol-HDL-Triglyceride/5 [13]. The concentration of plasma glucose was measured using a glucose kit based on the glucose oxidase method. Insulin was measured by a rat insulin ELISA kit (Millipore, Merck). A nitric oxide fluorometric assay kit (Cayman Chemical, USA) that provides measurement of total nitrate/nitrite concentration was used to assay plasma NO levels.

1.4. MDA assay

The malondialdehyde (MDA) assay was conducted by the thiobarbituric acid method in liver homogenates and plasma. The MDA levels were determined using a molar extinction coefficient of $1.56 \times 105\ M^{-1}\ cm^{-1}$.

1.5. Measurement of tissue antioxidant levels

Liver tissues were washed in 0.9% NaCl solution and approximately 0.5 g tissue samples of liver were homogenized in 0.15 M KCl- 10 mM potassium phosphate buffer, pH:7,4 [tissue to buffer ratio, 1:10 w/v]. Homogenized samples were centrifuged (13000 g, 10 min.) and stored at −80'C until analysis.

Glutathione peroxidase and superoxide dismutase levels in liver tissues were assayed using commercially specific kits (Cayman chemical, USA) and liver catalase activity was determined as described by Aebi [14].

1.6. Statistical analyses

Statistical analyses were performed using SPSS 16.00 for windows software. Data were expressed as arithmetic mean ± standard error (SE) in n number subjects. Difference between the values was considered statistically significant if p value < 0.05. One way variance (Anova) analysis was used in evaluation of the isometric vascular responses and post hoc tukey test was used in comparison of the data having significance. Other non-normal distribution data of the experimental animals were subjected to nonparametric testing. Accordingly, first Kruskal–Wallis test was carried out and in case of difference between the groups Mann–Whitney U test was performed. Variance analysis was made in order to define differences and significances in the repeating measures (fasting blood glucose, body weight etc.).

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