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Silymarin ameliorates expression of urotensin II (U-II) and its receptor (UTR) and attenuates toxic oxidative stress in the heart of rats with type 2 diabetes

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ABSTRACT

Type 2 diabetes mellitus (T2DM) is associated with an increased risk of cardiovascular disease (CVD). Urotensin II ((U-II)) and its receptor (UTR) are involved in the progression of CVD through enhancement in the production of reactive oxygen species (ROS). Since silymarin (SMN) is a natural agent with anti-diabetic effects, this study aimed to investigate the antioxidant potency of SMN on the expression of (U-II)/UTR system and oxidative stress status in the heart of type 2 diabetic rats. Thirty-six male Wistar rats were randomly divided into six groups (n = 6). Control and diabetic groups treated with or without SMN (60 and 120 mg/kg/day) for 2 months. Fasting blood sugar (FBS), insulin, lipid profile, creatine kinase-MB ((CK-MB)), lactate dehydrogenase (LDH) and markers of oxidative stress were measured by spectrophotometric methods while (U-II) and UTR gene expression was determined by qPCR method. SMN significantly reduced the FBS level, increased the concentration of insulin and improved HOMA-IR. SMN prevented diabetes-induced weight loss, and attenuated the increased levels of total oxidative status (TOS), malondialdehyde (MDA), and nitric oxide (NO). Diabetes-induced reduction of total thiol molecules content (TTM) was normalized to the normal level in SMN treated rats. SMN significantly modulated serum lipid profile, reduced the expression of (U-II) and UTR in the heart, and improved histopathological changes in the heart tissues. Therefore, the current study indicated that SMN ameliorated unpleasant diabetic characteristics via down-regulation of (U-II) and UTR gene expression and modulation of oxidative stress in the heart tissue of type 2 diabetic rats.

1. Introduction

Diabetes mellitus (DM) is one of the fastest growing health problems in the world. The latest estimates by the International Diabetes Federation project that 592 million worldwide will have DM by 2035 [1,2]. Many investigators have indicated that development of cardiovascular disease (CVD) has frequently been observed in diabetic patients, and it is currently one the major causes of morbidity in these patients [3,4]. Therefore, it is believed that the association of hyperglycemia with various cardiac complications is the leading cause of morbidity in the diabetic population [5].

Urotensin II (U-II) is a cyclic undecapeptide with activity on the cardiovascular and nervous system [6]. U-II receptor (UTR), a member of the G protein-coupled receptors (GPCRs) family also named as GPR14, is highly expressed in the cardiovascular system. In human, the expression of U-II and UTR is upregulated in cardiovascular diseases, including heart failure, hypertension, and coronary artery disease [7] as well as type 2 diabetes [8,9]. There is a positive correlation between the extent of congenital heart failure (CHF) and plasma U-II concentrations [10,11] with 2.1-fold enhancement compared with controls [12]. Overexpression of the U-II in atherosclerotic lesions where infiltration of macrophage occurs [13], may accelerate the atherosclerosis through acting synergistically with mildly oxidized LDL (ox-LDL) level and activate proliferation of vascular smooth muscle cell (VSMC) [14]. Also the formation of foam cells in atherosclerosis is affected by an upregulation of U-II which in turn induces expression of acyl-coenzyme A: cholesterol acyltransferase-1 (ACAT-1) in macrophages [15,16]. Besides its important role in the CVD, U-II/UTR system also plays a significant role in diabetes and its complications. U-II stimulates translocation of NADPH oxidase subunits and enhances the production of

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reactive oxygen species (ROS) thus, ROS are somehow involved in the U-II induced heart disease [17]. Oxidative stress due to the increased generation of ROS from physiologic cellular reactions, inflammatory reactions and cell injury plays a key role in the pathogenesis and complications of diabetes mellitus [18]. However, hyperglycemia can also induce oxidative toxic stress through various mechanisms and decline antioxidant capacity [19]. Unfortunately, most hypoglycemic drugs such as glibenclamide, gliclazide, and tolbutamide, have limited efficacy and show a variety of adverse effects: including weight gain, hypoglycemia, bone loss, and increased risk of cardiovascular events [20,21]. Medicinal plants are important sources of active natural products differing in their biological properties and applications in the management of various human diseases [22]. Silymarin (SMN) is a flavonoid mixture extracted from *Silybum marianum (milk thistle)* that grows in various parts of the world. SMN contains flavolignans such as silybin, silycristin, silydianin and isosilybin [23]. SMN functions as a free radical scavenger and it has been hypothesized that the protective effect of SMN on tissue damage may be attributed to the increase in the activity of antioxidant enzymes and inhibition of lipid peroxidation [24]. Moreover, an antidiabetic and cardioprotective activity property of silymarin have also been reported [25].

Because of the key role of U-II and its receptor and oxidative stress in cardiovascular system diseases, the aim of the present study is to investigate the anti-oxidant effects of SMN in the heart tissues of type-2 diabetic rats and on the gene expression levels of U-II and UTR.

2. Material and methods

2.1. Chemicals and drugs

Livergol tablets containing silymarin was obtained from Goldaru (Goldaru Co., Iran). Each coated tablet contained dried extract of Sylibum marianum equivalent to 70 mg Silymarin (it should be noted that the flavonolignans including silybin, silychristin, silydianin, and isosilybin which may be extracted from *Silybum marianum* plant are collectively known as Silymarin). Streptozotocin (STZ) and Nicotinamide (NIC) were purchased from the Sigma Company (Sigma Chemical Co., USA). Insulin-specific ELISA kit was obtained from Mercodia (Mercodia Diagnostics, Sweden) whereas ZellBio (ZellBio GmbH, Germany) supplied colorimetric assay kit for determination of NO. All the other chemicals used were of analytical grade.

2.2. Animals

Thirty-six male Wistar rats weighing 220 ± 10 gr were purchased from The Animal Care Center (Hamadan University of Medical Sciences). Animals were acclimatized at 22 ± 2 °C, 12-h light/dark cycle, and relative humidity of 60 ± 5% for one week with ad libitum access to the standard rat chow diet and tap water. The research was approved by the Medical Ethics Committee of Hamadan University of Medical Sciences (IR.UMSHA.REC.1395.412) and experiments were conducted in accordance with the internationally accepted principles for laboratory animal use and care as found in the European Community guidelines (EEC Directive of 1986; 86/609/EEC).

2.3. Work design

The animals were divided into six (n = 6) following groups: Normal control (C); normal control groups receiving 60 or 120 mg/kg of SMN (C + SMN 60 and C + SMN 120); diabetic control (D); and diabetic rats receiving either 60 or 120 mg/kg of SMN (D + SMN 60 and D + SMN 120). Treatments were administered once per day by oral gavage for 2 months. To induce type 2 diabetes in overnight fasted rats, nicotinamide (NIC) was first injected (120 mg/kg; i.p) and 15 minutes later, rats were received Streptozotocin (60 mg/kg, dissolved in 0.1 M of citrate buffer pH 4.5) intraperitoneally. The development of T2DM in rats was confirmed by determination of fasting blood sugar (FBS) 3 days after STZ-NIC injection. The animals with a fasting blood sugar level of above 150 mg/dl were considered as diabetic [26].

At the end of the experiment and after an overnight fasting, rats were anesthetized with ketamine (50 mg/kg), blood samples were collected, and serum samples were separated and stored at −20 °C for biochemical analysis. In addition, the heart tissues were liberated from surrounding tissues, dissected and cleaned with ice-cold normal saline. A small part of heart tissues were fixed at 10% neutral buffer formalin for histopathological evaluation while the remaining's were transferred into cryotubes, kept in liquid nitrogen for 1 h, and finally were stored at −70 °C until further analysis.

2.4. Biochemical assays

FBS was measured with a glucometer (Accuchek; Roche, Germany). The serum insulin level was measured by ELISA kit (Mercodia, Uppsala, Sweden) whereas homeostatic model assessment for insulin resistance (HOMA-IR) was calculated as: HOMA-IR = Fasting insulin (μU/ml) × FBS (mg/dl) /405 [27]. Serum total cholesterol, triglycerides and HDL-C were measured according to the rutin procedure using commercially available kits (Pars Azmun, Iran). Serum LDL-C level was calculated from the Friedewald formula (LDL-C = TC - [HDL-C + TG/5]) [28] while serum VLDL-C concentration was calculated according to the Nobert formula (VLDL-C = TG/5) [29]. Atherogenic Index was calculated as a ratio of TG/HDL-C and the activities of serum CK-MB and LDH were determined using Pars Azmun kits (Pars Azmun Co., Iran).

2.5. Oxidative stress assay

MDA as marker of lipid peroxidation was measured in the plasma and heart tissue using thiobarbituric acid method based on the reaction of MDA with TBA. Total antioxidant capacity (TAC) was determined using ferric reducing antioxidant power assay (FRAP) [30] while total oxidant status (TOS) was measured by the oxidation of ferrous with xylenol orange [31] and the oxidative stress index (OSI) was calculated as TOS/TAC [32]. Total thiol molecules (TTM) were determined using Ellmans reagent (DTNB; 5,5-dithio-bis-[2-nitrobenzoic acid]) according to the Hu method [33] and nitric oxide (NO) content was assayed calorimetrically (ZellBio GmbH, Ulm-Germany), according to the manufacturer's instructions.

2.6. Total RNA isolation and real-time quantitative PCR

Total RNA was extracted manually from heart tissues using the RNX-Plus (Sinaclon, Tehran, Iran), according to the manufacturer's protocol. Expression of U-II and UTR genes were measured by Real time qPCR using SYBR premix Ex TaqTM II (TaKaRa Biotechnology, Japan) on a Roche Light Cycler 96 System (Roche Life Science Deutschland GmbH, Germany). cDNA was synthesized by reverse transcription of total RNA (500 ng) using the Prime Script RT reagent kit (TaKaRa Biotechnology, Japan). The gene specific primers used in the present study were as: forward: 5′-CCCGCGAGTACAACCTTCT-3′ and reverse: 5′-CGTCATCCATGGCGAACT-3′ for housekeeping gene β-Actin, forward: 5′-GTCGTCATGGACAGGGTG-3′ and reverse: 5′ GAGGGTTTATT TCTCATAGTGG-3′ for U-II, and forward: 5′-GCACGCCAGCATCTT CAC-3′ and reverse: 5′-TGGTCCCAAAGAGCAACG-3′ for UTR gene. Relative gene expressions (fold changes) were calculated as $2^{-\Delta\Delta CT}$ [34].

2.7. Histopathological examination of heart tissues

For histopathological evaluations, a small piece of heart tissue was fixed with 10% formalin and embedded in paraffin. Serial sections (thickness of 5 μm) were processed and stained with hematoxylin and