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Quercetin Ameliorates Endothelial Dysfunction and Prevents Elevation of Asymmetrical Dimethylarginine in Experimental Diabetes

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ABSTRACT

Antioxidants that derived from food have a powerful potential effects on several diseases associated with oxidative stress. The goal of our work was to evaluate the role of quercetin on endothelial dysfunction and asymmetrical dimethylarginine (ADMA) levels in experimental diabetes, rats were divided into four groups including control, quercetin, diabetic and treated groups. Diabetic rats were treated with quercetin; blood glucose and plasma insulin were estimated. Liver superoxide dismutase (SOD), reduced glutathione (GSH) and plasma nitric oxide (NO) were measured. ADMA was estimated by reversed phase HPLC. Our results revealed an increase in insulin resistance and ADMA concomitant with a decrease in plasma nitric oxide, liver superoxide dismutase and reduced glutathione activities while treatment with quercetin significantly ameliorated these parameters in treated group compared to diabetic group. Also a negative correlation between ADMA and NO was found accompanied with a positive correlation between ADMA and insulin resistance. The important role of quercetin in attenuating endothelial dysfunction and elevated ADMA levels provides strong evidence that it may be a promising antidiabetic and protective natural product against cardiovascular complications related to diabetes.

Keywords: Quercetin**,** Endothelial dysfunction, Nitric oxide, HPLC, ADMA, Diabetes mellitus.

INTRODUCTION

Diabetes mellitus is a complicated disease that associated with hyperglycemia, declined insulin production and deteriorated insulin action leading to the disability of glucose molecules to transport from the blood to the tissues, resulting in rising of both blood sugar and urinary glucose excretion [1]. This process is distinguished by impaired production of NO, the antiplatelet adhesion and vasodilator factor and also reduction of NO bioavailability [2].

NO inhibits platelet accumulation, cellular adhesion, leucocytes immigration and the proliferation of vascular smooth muscle [3, 4]. The main and the very important action of nitric oxide is the providing of vascular homeostasis. When nitric oxide decreases, endothelial homeostasis is impaired and endothelial dysfunction starts [5].

ADMA is considered as a key that participates in endothelial dysfunction. It is produced by endothelial cells and presents in plasma in amounts that are enough to prohibit NO production [6, 7]. Elevation of ADMA levels is associated with hypertension, hypercholesterolemia and cardiovascular diseases.

Streptozotocin (STZ) induced-experimental diabetes, mainly by releasing of oxygen free radicals and enhancing pancreatic necrosis [8, 9].

There is a mounting confirmation evincing the role of free radicals in many diseases including hypertension and diabetes mellitus. The imbalance between free radicals and antioxidants ability to scavenge them results in a state of

oxidative stress [10]. Superoxide anion inactivates NO and limits its availability for smooth muscle relaxation [11]. Therefore, antioxidant treatment is a very important approach to regulate ROS and has a beneficial therapeutic effect on NO activation.

Food derived antioxidants such as flavonoids and vitamins have been used to prevent many chronic diseases. Flavonoids are natural polyphenolic substances widely distributed in the plant kingdom [12] and they have important pharmacological actions versus many diseases including allergy, inflammation, hypertension and diabetes [13]. Quercetin is so far the most plenteous flavonoid in our diet, including berries, apples, onion, red wine, tea, seeds and nuts [12, 14].

Thus, it is from interest to evaluate the potential effect of querectin in preventing diabetes-associated endothelial dysfunction and how far it is able to attenuate elevation of ADMA and prevent nitric oxide reduction in experimental diabetes.

MATERIALS AND METHODS

Materials

Streptozotocin (STZ), ADMA (HPLC standard), O-Phthaldialdehyde (OPA), tetrahydrofuran (THF) and mercaptoethanol were purchased from Sigma Aldrich Medical Company St. Louis USA.

Experimental animals

Male albino rats weighting 180-200 g were obtained from the animal house of National Research Centre, Giza, Egypt. Animals were housed in individual suspended stainless steel cages in a controlled environment (22-25°c) and 12 hour light, 12 hour dark. The animals had free access to water and standard rodent chow diet. All animals received human care in compliance with guidelines of the Ethical Committee of National Research Centre, Egypt and followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals.

METHODS

Induction of diabetes

STZ was dissolved in 50 mM sodium citrate (pH 4.5) solution containing 150 mM NaCl. The solution containing (6.0 mg/100g body weight) was subcutaneously administrated in rats; fasting blood sugar was estimated after 3 days to confirm the development of diabetes mellitus [15].

Experimental design

Forty male albino rats were used in this study and divided into four groups (ten rats in each group) as follows:
Group I (control group): healthy rats received a vehicle.
Group II (quercetin group): healthy rats received intra peritoneal (i.p) injection of querectin (15 mg/kg body weight/day) for 8 weeks.
Group III (diabetic group): diabetic rats received a vehicle.
Group IV (treated group): diabetic rats received i.p. injection of querectin (15 mg/kg body weight/day) for 8 weeks [16].

After the experimental period; blood was withdrawn from the retro-orbital venous plexus of the eye using capillary tubes and collected in a- tubes contain sodium fluoride for blood glucose estimation and b- tubes contain anticoagulant for other biochemical analysis.

Liver was removed quickly and placed in iced normal saline, perfused with normal saline solution to remove blood cells, blotted on filter paper and frozen at -80°C. The frozen tissues were cut into small pieces and homogenized in 5 ml cold buffer (0.5 g of Na_2HPO_4 and 0.7 g of NaH_2PO_4 per 500 ml deionized water (pH 7.4) per gram tissue, then centrifuged at 4000 rpm for 15 min at 4°C , the supernatant was kept for oxidant/antioxidant parameters estimation [17].

Fasting blood sugar was estimated by colorimetric method using commercial kit purchased from Vitro Scient, Egypt, based on the method described previously [18]. Oxidant/antioxidant parameters were determined by commercial kits; liver malondialdehyde (MDA) [19], superoxide dismutase (SOD) [20] and plasma nitric oxide (NO) [21] were estimated by colorimetric methods according to the methods described previously. Reduced glutathione (GSH) was determined according to the method of Beautler et al. [22]. All kits were purchased from BioMed. Diagnostics.

Plasma insulin level was estimated by ELISA according to Yalow and Bauman [23] using BioSoure INS-EASIA Kit. Insulin resistance was calculated from the equation:

Insulin resistance = fasting glucose (mg dl–1) X fasting insulin (μIU ml–1)/405 [24].

Determination of plasma asymmetric dimethylarginine by HPLC
Plasma ADMA was determined by HPLC method modified from the method described previously [25].

Sample preparation
25 mg 5-sulfosalicylic acid (5-SSA) were added to 1 mL plasma, mix well and left for10 min. in an ice-bath; the precipitated protein was removed by centrifugation at 4000 r.p.m. using cooling centrifuge (Laborzentrifugen, 2K15, Sigma, Germany) for 10 min., the supernatant was filtered through hydrophilic PVDF 0.45 μm filter; then mix 50 μL from the filtered solution with 500 μL of OPA solution and left for 3 min. before injecting onto HPLC.

HPLC condition
Ten μl of sample-OPA were injected onto HPLC; separation was achieved on reversed phase column (150 X 4.6 mm C18). The mobile phase consisted of sodium acetate buffer, methanol and THF (A, 82:17:1; B, 22:77:1, % v, respectively) and eluted in a flow rate 1.0 ml/min by gradient method (table 1). Column temperature adjusted at 37°C and fluorescence detector was set at 338 and 425 nm (excitation and emission) respectively. A linear standard curve was constructed by plotting peak areas versus the corresponding concentrations. The concentration in samples was obtained from the standard curve.

Table (1): Gradient method for ADMA analysis

Time (min)	Mobile phase A (% v)	Mobile phase B (% v)
0.0	100	0
5	90	10
15	60	40
25	25	75
28	10	90
32	100	0

Statistical analysis
Results were expressed as mean ± standard error. Data were analyzed by independent sample t test (SPSS) version 15 followed by (LSD) test to compare significance between groups. Difference was considered significant when P value <0.05.

RESULTS AND DISCUSSION

Endothelial dysfunction is a predictive sign of cardiovascular diseases and atherosclerosis [26]. Quercetin (the present supplemented agent) has both antioxidant and anti-inflammatory properties [27].

Our results showed a significant increase in fasting blood sugar concomitant with a significant reduction in insulin levels in experimental diabetes compared with healthy control. Insulin resistance in this study was calculated and it was significantly increased in diabetic rats compared to control group (table 2).

The elevation in blood glucose and the reduction of plasma insulin level in diabetic group in this study may be resulting from STZ mediated destruction of ß-cells [28], in experimental diabetes, cells become unable to generate more insulin leading to elevated accumulation of blood glucose instead of storage and utilization.

Table 2: Fasting blood glucose and insulin levels in different studied groups

Parameters Groups	Glucose (mg/dl)	Insulin (μIU/ml)	Insulin resistance ($mgdl^{-1}$ μIU ml^{-1})
Control	76.42±1.1	11.4±0.7	2.15 ±0.4
Quercetin	77.23±1.3	11.7±1.3	2.23±0.3
Diabetic	271.72±1.9 ♥	9.0±1.3	6.03 ±0.3 ♥
Treated	175.61±1.7 ♥ #	10.1±1.9	4.38 ±0.1 ♥ #

Results are expressed as mean ± SE, n=10, significant p value < 0.05, ♥ significant difference compared to control group, # significant difference compared to diabetic group.

In addition, STZ-induced diabetes is associated with a production of free radicals and the formation of highly reactive oxygen species that lead to oxidative destruction of all cells' constituents such as DNA, lipids and proteins [5].

Inflammation and also oxidative stress are thought to participate in atherosclerosis (initiation and progression). Our results appear a significantly increase in MDA in diabetic group in comparison to control one; elevation of MDA is resulting from lipids' breaking down in oxidative stress status [29]. Several studies indicated the elevation of lipid peroxidation in diabetes mellitus and its complications [30] and also its association with endothelial dysfunction, the key juvenile in the pathogenesis of cardiovascular disease.

The elevation of MDA level may be considered as a reflection in both enzymatic and nonenzymatic antioxidants of defense systems as was observed in our results, where the elevation of MDA was concomitant with a reduction of both SOD and GSH levels in diabetic group (table 3).

Several studies indicated that experimental diabetes and hyperglycemia are considered important factors that release free radicals and decrease NO as was found in the current study ; this may be related to increase the production of superoxide anions from autoxidation of glucose and advanced glycation end products accumulation due to proteins nonenzymatic cross-linking via oxidative stress that can decrease the NO bioavailability and polyol passageway activation , that rise the use of nicotinamide adenine dinucleotide phosphate and the decline of NO biosynthesis [31]. It was found that, arginase' up regulation is an important factor that inhibits endothelial NO synthase (NOS) - mediated nitric oxide production and that contributes to dysfunction of endothelial cells in diabetes [32, 33].

In the present study ADMA level was increased significantly in diabetic group compared to control group. Several studies indicated that ADMA has been used as an important marker of endothelial dysfunction.

There is a proof that elevation of ADMA level may be a cause or a result of rising insulin resistance [34]. Surprisingly, several investigations indicated that insulin resistance plays an important role in propagation of diabetic complications and retinopathy [35]. However, others indicated that increased ADMA level is an important factor in diabetic complications and retinopathy; these evidences reflect a phenomenon of the association of diabetic complication with both insulin resistance and ADMA elevations [36].

Table 3: Oxidant/antioxidant parameters in different studied groups

Parameters Groups	Liver MDA n mol /g tissue	Liver SOD U/ g tissue	Liver GSH µ mol/g.tissue
Control	34.9 ± 0.45	427.3 ± 1.6	12.10 ±0.5
Quercetin	33.9 ± 0.44	431.2 ± 1.4	12.31±0.2
Diabetic	56.2 ± 0.39 ♥	249 ± 1.5 ♥	8.54 ±0.22 ♥
Treated	39.8 ± 0.59 ♥ #	366 ± 1.7 ♥ #	11.4 ±0.13 #

Results are expressed as mean ±SE, n=10, significant p value < 0.05, ♥ significant difference compared to control group, # significant difference compared to diabetic group.

Supplementation of quercetin in this study significantly increased liver SOD and GSH concomitant with a reduction of MDA level in treated group (table 3).

It is well documented that quercetin has antioxidant and antihypertensive properties [37], which is mediated by vasodilatory effect [38]. Thus, it has an important role in protecting against cardiovascular diseases.

Quercetin inhibits lipid peroxidation and preserves antioxidant enzymes by two important actions: scavenging of free radicals and promoting the antioxidant defense mechanism. The antioxidant effect of quercetin is due to its ability to diffuse into cell membrane [39] and quench free radicals at different sites in the membrane lipid; the pentahydroxy flavone structure facilitates metal ions chelation through its phenolic structure, therefore scavenging peroxyl and lipid alkoxyl radicals [40].

Moreover, quercetin metabolites inhibit oxidation mediated by peroxynitrite. Similar to free quercetin, they can protect against oxygen free radicals [41].

The reduction of MDA levels was associated with elevation of NO synthase activity, indicating the advantageous effects of quercetin on the endothelial cells are mediated by elevation of NO bioavailability and bioactivity which may be related to the reduction of ADMA synthesis and released by endothelial cells (table 4).

Table 4: Asymmetrical dimethylarginine and nitric oxide levels in different studied groups

Parameters Groups	ADMA µmol/L	NO µmol/L
Control	0.44 ± 0.09	133.5 ±1.2
Quercetin	0.38 ± 0.05	137.3 ±1.3
Diabetic	1.22 ± 0.10 ♥	85.3 ± 1.5 ♥
Treated	0.76 ± 0.11 ♥ #	116.9 ± 1.8 ♥ #

Results are expressed as mean ±SE, n=10, significa nt p value < 0.05, ♥ significant difference compared to control group, # significant difference compared to diabetic group.

In this study, there is a negative correlation between ADMA and NO concomitant with a positive correlation between ADMA and insulin resistance (fig. 1, 2).

Quercetin supplementation increases nitric oxide production, resulting in inducing secondary vasodilation. The increase in blood flow promotes glucose uptake. It was indicated that, nitric oxide enhances glucose transport in skeletal muscles by increasing glucose transporter 4 (GLUT4) levels at the cell surface [5]. As a result, improvement of insulin resistance was observed as was found in the current study.

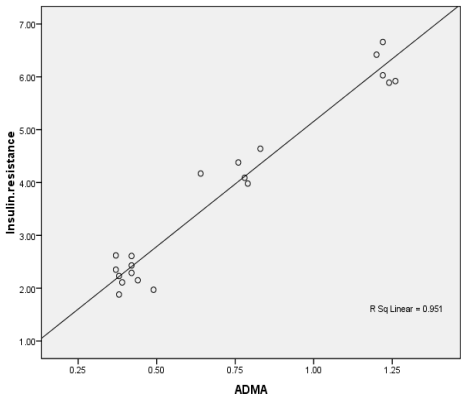

Fig. 1: Correlation between ADMA and insulin resistance

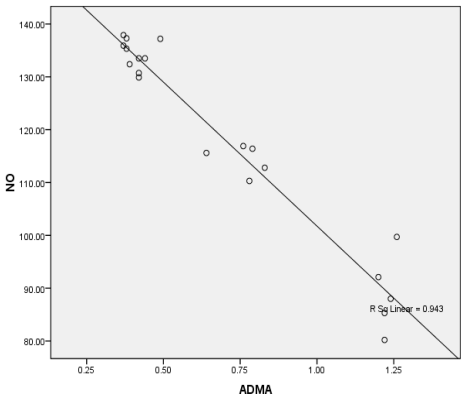

Fig. 2: Correlation between ADMA and nitric oxide

In conclusion, our results appeared that quercetin supplementation is capable of improving endothelial dysfunction, especially that induced by oxidative stress and attenuating atherosclerotic formation. The promising effects of quercetin may be related to its ability to increase nitric oxide bioactivity and bioavailability, also to reduce oxidative stress. Our results support the role of quercetin in attenuation of ADMA elevation in STZ- induced experimental diabetes.

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