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Original Article

Troxerutin, a Bioflavonoid, Improves Oxidative Stress in Blood of Streptozotocin-Induced Type-1 Diabetic Rats

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Abstract

Type 1 diabetes is a chronic disease characterized by the body's inability to produce insulin due to destruction of the beta cells. There is increasing evidence that reactive oxygen species (ROS) play a major role in the development of diabetic complications. The purpose of this study is to investigate the effects of troxerutin administration on oxidative stress markers in blood of STZ-induced diabetic rats. Male Wistar rats were divided into 4 groups as: control (con), control-troxerutin (CON-TRX), diabetes (Dia), diabetic-troxerutin (DIA-TRX). Type 1 diabetes was induced by injection of streptozotocin (STZ) (i.p, 55mg/kg) and lasted for 10 weeks. Animals received oral administration of troxerutin (150 mg/kg) for 4 weeks. At the end of study, malondialdehyde (MDA, the main product of lipid peroxidation), activity of antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT) were measured spectrophotometrically. Induction of diabetes with STZ resulted in increased MDA levels and decreased blood antioxidant capacity as compared with those of controls ($P<0.05$). Pre-treatment of diabetic rats with troxerutin significantly decreased the levels of MDA ($P<0.01$) and increased the activity of antioxidant enzymes SOD, GPX, and CAT compared to untreated-diabetic groups. Troxerutin had no significant influence on non-diabetic rats. These findings showed that troxerutin may prevent oxidative complications of diabetic circumstances by elevating antioxidant enzymes activities and reducing lipid peroxidation.

Keywords: diabetes, troxerutin, oxidative stress, antioxidant, malondialdehyde , superoxide dismutase, glutathione peroxidase, catalase

1. Introduction

Diabetes mellitus is major causes of morbidity and mortality worldwide. It has been accounted that the number of adults affected by diabetes in the world will enhance from 135 million in 1995 to 300 million in the year 2025 [1, 2]. Hyperglycemia is the most main risk factor for diabetic

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complications, resulting from uncontrolled glucose regulation [3]. Numerous studies demonstrated that hyperglycemia-induced production of free radicals and generation of oxidative stress, contributes to the expansion and development of diabetes and related contributions [4]. Hyperglycemia can progress an enhancement in oxidative stress markers such as membrane lipid peroxidation. The level of lipid peroxidation in cells was directly relative to the glucose amount [5]. The formation in the lipid peroxidation was avoidable after the control of glycemia in streptozotocin-treated diabetic rats [6]. Oxidative stress is defined in overall as addition formation and/or insufficient removal of extremely reactive molecules such as reactive oxygen species (ROS)[7]. ROS highly reactive and can injury protein, RNA, and DNA structure.

However, they are mostly reduced by internal antioxidant defense mechanisms in order to reserve a stable intracellular milieu[8]. Oxidative stress occurs when the amount of ROS surpasses the levels of antioxidants. Reactive species can be removed by a number of antioxidant mechanisms. Superoxide dismutase (SOD), a plentiful free radical scavenger, converts •O2- to H2O2, which is then detoxified to water either by catalase or by glutathione peroxidase. SOD delivers the body's important defense against the highly damaging free radicals and is thereby a main protective mechanism against damage induced by oxidative stress [9]. Thus, in the diabetic cases, the increase of oxidative stress is most possibly a result of abnormal metabolism of glycemia. Therefore, considerable attention has focused on the application of protective agents in various diseases which can decrease these free radicals and thereby improve the oxidative damage to biomolecules. Numerous studies have reported that active dietary ingredients, such as phytochemicals, have promising cytoprotective effects in many pathological conditions. These phytochemicals comprise phenolic compounds, alkaloids, lectins, terpenoids, isoprenoids, and quinones [10]. Thus, it became clear that improving oxidative stress through treatment with antioxidants might be a beneficial strategy for decreasing diabetic complications.

Troxerutin is a derived of the natural bioflavonoid rutin, hydroxylethylated (–CH2–CH2–OH) at the 3', 4' and 7th locations on the rutin skeleton (Figure1). Troxerutin is a yellow powder that dissolves in water and its solubility increases with increasing temperature [11]. It has been confirmed that troxerutin have antioxidative, anti-inflammatory, antihyperlipidemic, and nephroprotective effects, and it is revealed as an effective agent in the treatment of

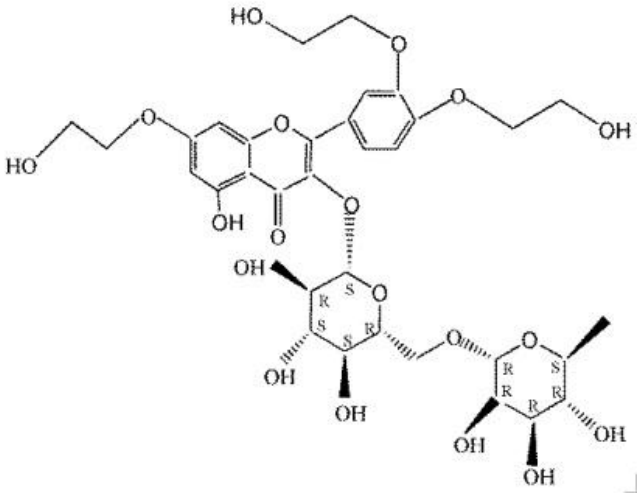

Figure 1. The chemical structure of troxerutin.

cardiovascular diseases [12-14]. Recent studies have shown that troxerutin administration effectively reduce blood glucose level, advanced glycation end products and reactive oxygen species levels in high cholesterol and fructose diet-fed mice [15, 16]. Troxerutin by improving the expression of insulin signaling molecules and increasing glucose uptake in cells play an important role in management of diabetes mellitus [17]. The antioxidative activity of troxerutin in type 1 diabetes has not been investigated so far. Therefore, this study was designed to evaluate the antioxidative effect of troxerutin in blood of streptozotocin-induced type 1 diabetic rats.

2. Materials and Methods

2.1. Animals

Male wistar rats (200-250g) were used in this experimental study. The animals were housed at a constant room temperature (24˚C), a relative humidity for 50%, and a 12 h dark/light cycles that had access to food and water *ad libitum*. This study was approved by the Animal Ethics Committee in accordance with the instruction for the care and use of laboratory animals prepared by Tabriz University of Medical Sciences.

2.2. Animal Grouping

Thirty two rats were randomly divided into four groups as following (n=8/each):

1- Control (CON): vehicle treated healthy rats
2- Control-Troxerutin (CON-TRX): healthy rats were treated with troxerutin (150 mg/kg, orally) once a day for 30 days.
3- Diabetic (DIA): vehicle treated diabetic
4- rats
5- Diabetic-Troxerutin (DIA-TRX): diabetic rats were treated with troxerutin (150 mg/kg, orally) once a day for 30 days.

2.3. Development of Type1 Diabetic Rats

Diabetes was induced by intraperitoneal injection of streptozotocin (STZ; 55mg/kg/body weight; from Sigma, Germany) dissolved in 0.2 ml of 0.1 M citrate buffer, pH 4.5. Control rats were injected with the vehicle (citrate buffer) alone. Three days after STZ injection, development of diabetes was confirmed from tail vein blood glucose sampling. Rats with blood glucose levels of 300 mg/dl and higher were considered as type 1 diabetic rat. Four weeks after diabetes induction, troxerutin (150 mg/kg; from Sigma, Germany) was administrated once a day orally through the gavage tube for 4 weeks [15, 17, 18].

2.4. Blood Collection and Sample Processing

At the end of experiment, the rats were sacrificed under anesthesia with ketamine/xylazine (60/10 mg/kg, i.p.). Blood samples were collected from the inferior vena cava and were centrifuged at 1400g at 4 C for 10 min. The supernatants were used for the biochemical evaluation of serum.

2.5. Lipid Peroxidation Measurement Malondialdehyde

Lipid peroxides are unstable and decompose to produce a series of compounds including reactive carbonyl compounds. Polyunsaturated fatty acid peroxides generate malondialdehyde (MDA) and its measurement has been used as indicator of lipid peroxidation, which is examined by measuring thiobarbituric acid reactive substances (TBARS) in homogenates. Briefly, the samples (250 µL) were mixed with 1 mL 10% trichloroacetic acid (TCA) and 1 mL of 0.67% thiobarbituric acid. Then, the samples were heated in a boiling water bath for 15 minutes and n-butylalchohol (2:1 v: v) was added to the solution. After centrifugation (5 minutes, 3500 g) at room temperature, TBARS was approved from the absorbance at 535 nm, using a spectrophotometer (Pharmacia Biotech; England) and the values obtained were expressed as nmol per 100 mg tissue protein [19, 20]. The intra- and inter-assay coefficient of variations on measurements done on the same sample was less than 5% and 7%, respectively.

2.6. Superoxide Dismutase Measurement

Superoxide dismutase (SOD) activity was determined using a RANSOD kit (RandoxCrumlin, UK). SOD activity was measured at 505 nm by a spectrophotometer. In this method, xanthine and xanthine oxidase were used to generate superoxide radicals able to react with 2-[4-iodophenyl]-3-[4-nitrophenol]-5-phenyl tetrazolium chloride (ITN) to form a red formazan dye. Concentrations of substrates were 0.05 mmol/L for xanthine and 0.025 mmol/L for ITN. Superoxide dismutase activity was assessed by the degree of inhibition of this reaction. After calculating the percent of inhibition using related formula, SOD activity was calculated by comparing with the standard curve and was

expressed as U/mg protein [21, 22]. The inter-sample intra-assay variation and coefficient of variation were less than 8%.

2.7. Glutathione Peroxidase Measurement

Glutathione peroxidase (GPX) activity was determined using a RANSEL kit (Randox Crumlin, UK). GPX catalyzes the oxidation of glutathione (at a concentration of 4 mmol/L) by cumene hydroperoxide. In the presence of glutathione reductase (at a concentration ≥ 0.5 units/L) and 0.28 mmol/L of NADPH, oxidized glutathione is immediately converted to the reduced form with concomitant oxidation of NADPH to NAD+. The decrease in absorbance at 340 nm (37°C) was measured using a spectrophotometer, and then GPX concentration was calculated and expressed as U/mg protein. The coefficient of variation for intra-assay and inter-assay were 7%, and 8%, respectively [23].

2.8. Catalase Measurement

CAT activity was assayed by the method of Aebi(24). Briefly, to a quartz cuvette, 0.65 ml of the phosphate buffer (50 mmol/l; pH 7.0) and 50 µl sample were added, and the reaction was started by addition of 0.3 ml of 30 mM hydrogen peroxide (H2O2). The decomposition of H2O2 was monitored at 240 nm at 25 °C CAT activity was calculated as nM H2O2 consumed/min/mg of tissue protein.

2.9. Statistical Analysis

All values were expressed as means ± SEM. The between-group parameters were analyzed using *one-way ANOVA* followed by *Tukey* post-hoc test. Differences were considered statistically significant when $P<0.05$.

3. Results and Discussion

3.1. Results

3.1.1. Effect of Troxerutinon Lipid Peroxidation

As shown in Figure 2, the level of lipid peroxides increased significantly in DIA group in comparison with CON group($p<0.001$). After 4 weeks of treatment of diabetic rats with troxerutin, the level of MDA decreased in plasma of diabetic rats as compared with DIA group ($p<0.01$).

3.1.2. Effect of Troxerutinon Glutathione Peroxidase

One-way ANOVA showed that the glutathione peroxidase levels were significantly lower in non-treatment diabetic rats than in control rats ($p<0.001$) and troxerutin-treated control rats ($p<0.001$). A comparison between the diabetic rats treated with troxerutin and non treated diabetic group exhibited significantly difference among these groups. Data showed that troxerutin could improve glutathione peroxidase activity in diabetic rats ($p<0.05$) (Figure 3).

3.1.3. Effect of Troxerutin on Superoxide Dismutase

Results demonstrated that induction of diabetes reduced superoxide dismutase activity in diabetic group as compared with treated

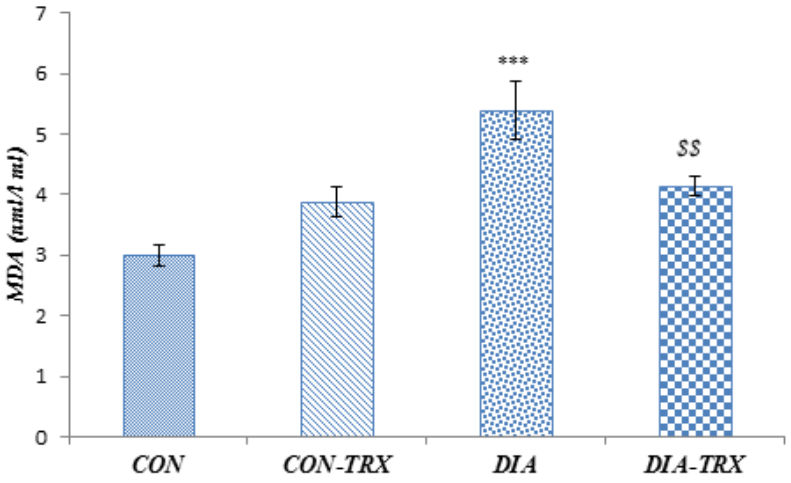

Figure 2. The effect of troxerutin on MDA level in plasma of diabetic and non-diabetic rats. Data are shown as mean ± S.E.M. ***p<0.001 indicated significantly change compared with control (CON) group and $^{\$\$}$p<0.01 indicated significantly change compared with DIA group.

(p<0.001) and non-treated (p<0.001) controls. As in Figure 4, troxerutin administration significantly increased superoxide dismutase levels in the blood of diabetic rats as compared to non treated diabetic group (p<0.05).

3.1.4. Effect of Troxerutin on Catalase

Figure 5 shows that induction of type 1 diabetes resulted in a significant decrease in catalase activity in the blood of diabetic rats compared with CON group (p<0.001). Furthermore, troxerutin administration significantly increased the levels of catalase in DIA-TRX group as compared with untreated diabetic group (p<0.001).

3.2. Discussion

The oxidative stress and reduced antioxidant capacity as one of the main manifestations of diabetes mellitus were apparent in blood of diabetic rats in our study. However, administration of troxerutin in type 1 diabetic rats restored significantly the blood levels of antioxidant enzymes and plasma levels of lipid peroxidation.

Type 1 diabetes is accompanied with the hyperglycemia-induced raised levels of reactive oxygen species (ROS) [1, 25]. Blocking ROS generation has been shown to prevent hyperglycemia-related damages [26].These reactive species can be removed by a number of endogenous enzymatic and non-enzymatic antioxidant mechanisms. However, there are evidences that the antioxidant defense is finished in diabetic patients, proposing a disturbed capacity of scavenging of harmful free radicals [27]. Therefore, increased production of free radicals and impaired antioxidant defense capabilities in diabetic conditions designate a central contribution of ROS in the onset, progression, and pathological consequences

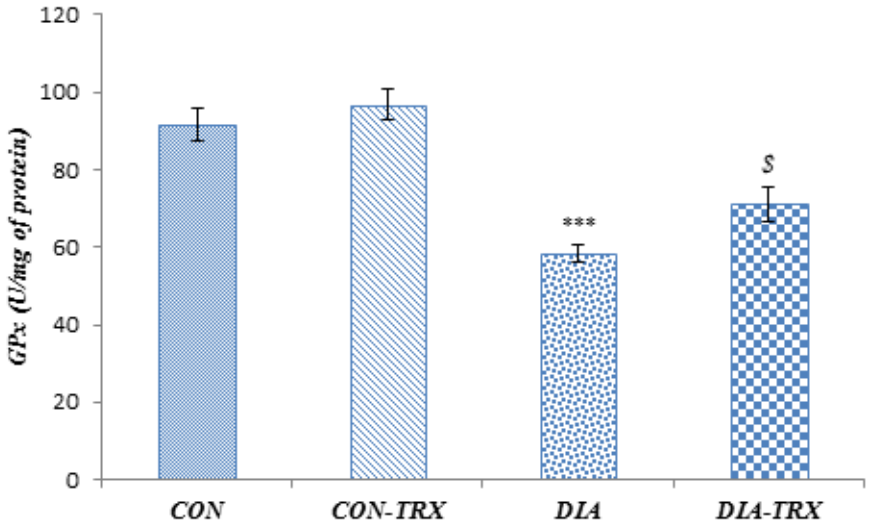

Figure 3. The effect of troxerutinon on glutathione peroxidase level. Data are shown as mean ± S.E.M. for=8 animals, $^{***}p<0.001$ indicated significantly change compared with control group and $^{\$}p<0.05$ indicated significantly change compared with DIA group.

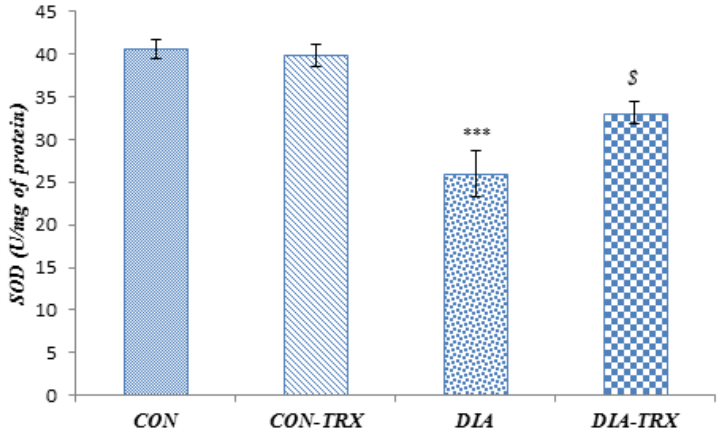

Figure 4. The effect of troxerutin on superoxide dismutase level. Data are shown as mean ± S.E.M. for=8 animals, $^{***}p<0.001$ indicated significantly change compared with control group and $^{\$}p<0.05$ indicated significantly change compared with DIA group.

of this disease [28, 29].As a result, successful efforts to diminish oxidative stress in diabetic models have involved the use of antioxidant supplementation [30].

Troxerutin can be found in tea, coffee, cereal grains, and a variability of fruits and vegetables (12).It has been reported to have a powerful antioxidant effects and prevents injury caused by oxidative stress in different tissues, e.g., liver, kidney, and brain [31-33]. Panat et al (2016) confirm that Troxerutin protects cells against oxidative stress-induced cell death through radical scavenging mechanism [34]. Previous studies by Pingand *et al* (2012), have revealed that that troxerutin

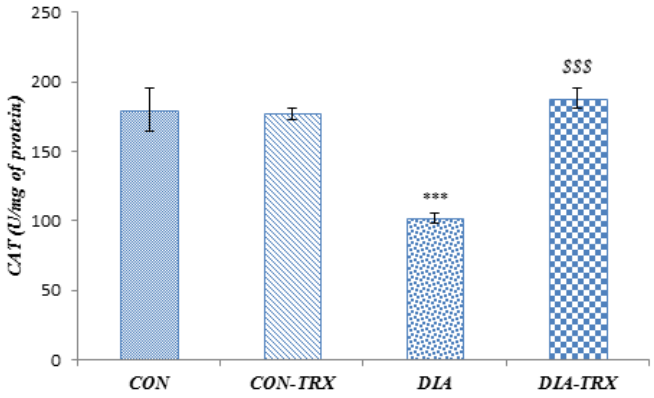

Figure 5. The effect of troxerutin on catalase level. Data are shown as mean ± S.E.M. for=8 animals, ***p<0.001 indicated significantly change compared with control group and $^{\$\$\$}$p<0.001 indicated significantly change compared with DIA group.

protected the cells against γ-irritation-induced cellular injury, including cell death [35].

According to the findings of the present study, pre-administration of troxerutin in dose of 150 mg/kg daily for four weeks could decrease MDA levels and significantly increased SOD, GPX, and CAT activity in blood of diabetic rats. These findings might be ascribed at least in part to the troxerutin ability to scavenge and avoid free radical generation and improved antioxidant properties.

Antioxidant enzymes can protect cellular compartments and organelles against oxidative damage. The SOD decomposes superoxide radicals (O^{-}_{2}) and generates H_2O_2. H_2O_2 is subsequently decreased to water by CAT in the peroxisomes, or by GPX oxidizing GSH in the cytosol [36, 37]. Many studies have shown that the overproduction of ROS in diabetes would break the balance between ROS generation and antioxidant defenses.

The antioxidant enzymes were maybe exhausted, leading to the reduction of their activity [12, 38, 39]. In the present study, the activities of antioxidant enzymes, including SOD, CAT, and GPX, in blood of diabetic rats were decreased by the injection of STZ and subsequent hyperglycemia. It indicated that hyperglycemia may increase the oxidative stress by the inhibiting the activities of antioxidant enzymes. Interestingly, troxerutin could markedly induce the activities of those antioxidant enzymes in the blood of diabetic rats. This study is the first original article that suggested the protective effect of troxerutin against oxidative stress of blood in STZ-induced type 1 diabetes, by renewing the activity of antioxidant enzymes accompanied with a reduction in oxidative stress. In agreement with these experimental observations, troxerutin diminished nickel-induced oxidative stress in rats [40], improved hepatic lipid homeostasis [41], protected the

mouse brain and kidney from D-galactose injury[12, 42] and suppressed lipid abnormalities in the heart of high-fat-high-fructose diet-fed mice [15].

Furthermore, it is clear that hyperglycemia is the main cause of diabetic complications. Hyperglycemia stimulates cellular ROS production by various major mechanisms including elevated levels of circulating advanced glycation end-products (AGEs), activation of multiple isoforms of PKC, stimulated endoplasmic reticulum stress, increased mitochondrial dysfunction [43, 44]. One of the main causes of hyperglycemia-related injuries is activation of PKC and its downstream target such as NF-κB[45, 46]. It is demonstrated that activation of PKC have been associated with tissue damages in the diabetic models [46]. It can be supposed that troxerutin may inhibit over-activation of PKC or its targets in diabetic condition. In this case, it has been shown that that troxerutin has significant inhibitory effects on NF-κBin renal and hepatic tissues of diabetic and healthy rats [12, 47] and in hippocampus of high cholesterol-fed mice [16, 32, 42].Thus, troxerutin, through above-mentioned mechanisms, is capable to reverse the oxidative potential during STZ-induced type 1 diabetes. Although as limitations of the present study, we could not perform direct measurement of ROS and oxidant anions generation instead of antioxidative enzymes and did not investigate the mechanisms of troxerutin-induced reduction in oxidative stress; however, direct contribution of above-mentioned mechanisms in antioxidative influences of troxerutin warrants further investigation.

4. Conclusion

In conclusion, the present results confirmed that troxerutin administration had protective influences in type 1 diabetes mellitus. Marked reduction in lipid peroxidation (indicated by MDA) and an enhancement in antioxidant enzymes levels following troxerutin administration indicated its antioxidant activity in this model. Therefore, troxerutin could be introduced as a therapeutic agent in oxidative stress-related diseases.

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