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The efficacy of *Aesculus hippocastanum* seeds on diabetic nephropathy in a streptozotocin-induced diabetic rat model

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ABSTRACT

Cytokines, such as transforming growth factor (TGF)-ß1, and increased oxidative stress are considered to be responsible for the development of diabetic nephropathy. We hypothesized that *Aesculus hippocastanum* (AH) seeds may have preventive effects on oxidative stress and TGF-β-related diabetic nephropathy in streptozotocin (STZ)-induced diabetic nephropathy in rats. Twenty-one male Sprague-Dawley albino rats were divided into three groups (n = 7). Except for the control group, they all had diabetic nephropathy induced by an intraperitoneal injection of STZ. While the diabetes group did not receive any medication, the diabetes + AH group was given the medication for 4 weeks. After the experiment, analyses were performed to evaluate the glomerular area, severity of sclerosis, and fibronectin immunoexpression, as well as levels of malondialdehyde (MDA), TGF-β, blood urea nitrogen (BUN), blood glucose, creatinine, and proteinuria. It was found that glomerular area, severity of sclerosis, fibronectin immunoexpression, and levels of MDA, TGF-β, BUN, creatinine, and proteinuria were decreased in the diabetes + AH group. It is known that diabetic nephropathy is induced, to a large extent, by hyperglycemia. In the present study, AH extract ameliorated diabetic nephropathy without decrease in blood glucose levels. In the study, AH seeds showed beneficial effects on the functional properties of the kidney and microscopic improvements in diabetic nephropathy.

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1. Introduction

Diabetic nephropathy (DN) is a common, severe complication of diabetes, developing in 20–40% of diabetic patients [1]. DN is characterized by diffuse glomerulosclerosis and nephrotic syndrome, which are caused by microangiopathy of capillaries in the kidney glomeruli [2].

There are several mechanisms suggested for the pathophysiology of DN. Glomerular hyperfiltration and hyperperfusion represent one mechanism related with high blood glucose levels [3]. Glomerular glucose hyperfiltration causes NaCl loss and results in decreased NaCl presentation to the macula densa. Activated macula densa–mediated feedback mechanisms increase the levels of angiotensin II, which in turn causes glomerular fibrosis and sclerosis with the stimulation of transforming growth factor (TGF)-ß1 (a hypertrophic, prosclerotic cytokine) [4,5]. Another postulated mechanism for DN is the nonenzymatic glycosylation of tissue proteins resulting from chronic hyperglycemia. The glomerular basal membrane is adversely affected by the formation and deposition of advanced glycation end-products [6]. In addition, these advanced products can alter signal transduction, which can be involved in the pathogenesis of DN via alteration of signaling molecules, such as cytokines, hormones, and free radicals [1].

Another postulated mechanism is chronic hyperglycemia–induced protein kinase C (PKC) activation. This has been associated with alterations in blood flow in renal and glomerular vessels, extracellular matrix expansion, basement membrane thickening, hyperfiltration, enhanced angiogenesis, and excessive apoptosis [7]. PKC activation leads to increased activity of mitogen-activated protein kinase (MAPK) [8]. MAPK activity, PKC activity, and TGF-β1 activation, which is commonly related with oxidative stress and reactive oxygen species (ROS), cause hyperfiltration and glomerular basement membrane thickening [9].

Recent studies have tried to define the pathogenesis of DN via oxidative stress mechanisms. Even before symptoms of diabetes become clinically apparent, hyperglycemia specifically induces oxidative stress [10]. The first event that results in kidney damage is the increased production of reactive oxygen species into the

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mitochondria due to the oversupply of NADH and $FADH_2$ in the oxidative phosphorylation chain [11]. This is the result of an oversupply of the Krebs' cycle with pyruvate, which is in turn the result of an increased glucose influx into the cell. The above sequence of events takes place in renal endothelial cells, which are unable to downregulate the glucose transporter-1 despite the hyperglycemic environment [12]. From this event originates a cascade of other detrimental events, such as PKC activation, production of advanced glycation end-products, TGF-β upregulation, and cytosolic NAPDPH consumption. In previous studies, products of lipoxidation in the mesangial matrix was detected via histological analysis of human kidney biopsy specimens [13,14].

The seeds of the *Aesculus hippocastanum* (AH) plant (horse chestnut) are used as a drug to treat venous disorders, such as varicose veins, phlebitis, leg ulcers, and hemorrhoids. AH is an astringent and anti-inflammatory plant that helps to decrease the symptoms of certain illnesses, such as arthritis, strains, and tendonitis [15,16]. Recent studies have shown that AH seeds can decrease the oxidative stress and anti-inflammation effects resulting from decreased levels of cytokines like tumor necrosis factor (TNF)-α and interleukin (IL)-1β in different tissues [17–19].

In this study, we hypothesized that AH seeds may have preventive effects on oxidative stress and TGF-β-related DN in streptozotocin (STZ)-induced DN in rats.

2. Materials and methods

2.1. Animals

Approval for all procedures in the experiment was received from the Ege University Animal Research Ethics Committee. In the study, we used 21 male Sprague Dawley rats. The rats weighed 200–220 g. The animals were fed with standard rat food pellets (Abalim®, Abalioglu Food Inc., Denizli, Turkey) ad libitum and kept in cages with standard laboratory conditions. Animals with blood glucose levels higher than 120 mg/ml were not included in the experiment.

2.2. Suspension of A. hippocastanum seeds

In the study, Venotrex® tablets (Abdi Ibrahim, Istanbul, Turkey) were used for preparing AH seed suspensions. The tablets are made from AH seeds, and each contains 263.2 mg of dry extract, adjusted to 50 mg with triterpene glycosides, calculated as anhydrous aescin which is a potentially main active compound. The extraction agent is 50% ethanol. The tablets were crushed and diluted with tap water, and a suspension was prepared.

2.3. Experimental protocol

Seven of 21 rats were separated as the control group (n = 7), and no drug was administered to them. Meanwhile, 60 mg/kg of STZ (Sigma-Aldrich, MO, USA) in physiological saline containing 0.2 M Na-citrate to adjust the pH level to 4.0 was administered to 14 of the 21 rats intraperitoneally (i.p.), as described previously by Gajdosîk et al. [20]. One dose of STZ induces type 1 diabetes [21]. Through this model, persistent disease was initiated; this was characterized by severe hyperglycemia with major clinical signs of diabetes mellitus, such as DN [20,22]. After 24 h, blood glucose levels were evaluated using a glucometer (Boehringer-Mannheim; Indianapolis, IN, USA). Rats were fasted for 8 h, and blood glucose levels higher than 250 mg/dl were accepted as showing that the rats had diabetes. The 14 rats with diabetes were randomly divided into the following two groups: the diabetes group and the diabetes + AH group. The diabetes group (n = 7) was given no medication. Meanwhile, in the diabetes + AH group (n = 7), a suspension of AH seeds in 20 ml/kg/day of tap water to supply 50 mg/kg/day of aescin was administered to by oral gavage for 4 weeks. The rats in the control and diabetes groups only received 20 ml/kg/day of tap water provided by oral gavage.

At the end of the 4 weeks, the rats were anesthetized by an i.p. injection of 80 mg/kg of ketamine (Alfamine, Alfasan, Woerden, Netherlands) and 7 mg/kg of xylazine (Alfazyne, Alfasan, Woerden, Netherlands) mixture. Blood samples for biochemical analysis were taken by cardiac puncture. For histopathological examination, nephrectomy was carried out, and for protein analysis, urine samples were obtained using a stick.

2.4. Histopathological examination of renal tissue

Kidneys were incubated with 200 ml of 4% formaldehyde in 0.1 M phosphate-buffered saline (PBS), and 5 μm sections from all paraffin-embedded, formalin-fixed renal tissues were stained with hematoxylin and eosin (H&E). The sections were assessed with a light microscope (Olympus BX51, Olympus Co., Tokyo, Japan) and photographed at high resolution with a digital camera (Olympus C-5050, Olympus Co., Tokyo, Japan). Each section was evaluated using Image-Pro Express 1.4.5 software (Media Cybernetics, Bethesda, MD, USA). The averages of 50 glomeruli from each animal were used for analysis. The glomerulosclerosis degree, mesangial matrix expansion, and glomerular hypertrophy in the sections were examined. The degree of glomerulosclerosis was assessed using semi-quantitative scoring, as described by Raji et al. [23]. The sclerosis degree of each glomerulus was scored from 0 to 4+, as follows: 0, no lesion; 1+, sclerosis of <25% of the glomerulus; and 2+, 3+, and 4+, sclerosis of 25–50%, >50–75%, and >75% of the glomerulus, respectively.

2.5. Immunoexpression of fibronectin

To eliminate endogenous peroxidase activity, sections were incubated with 10% hydrogen peroxide for 30 min, and 1/100 rabbit fibronectin immunoglobulin G (Bioss, Inc., Woburn, MA, USA) was used as the primary antibody. Following incubation with 10% goat serum for 1 h at room temperature, sections were incubated with the antibodies for 24 h at 4 °C. A Histostain-Plus Bulk Kit (Bioss, Inc.) for primary antibodies was used for antibody detection, and 3,3′ diaminobenzidine was used for visualization. After washing with PBS, sections were evaluated and photographed. A minimum of 50 glomeruli and tubular cells per field in 10 fields of tissue sections were investigated at 100× optical zoom. Brown cytoplasmic immunostaining was considered positive for immunoexpression and scored systemically.

2.6. Biochemical assays

2.6.1. Measurement of plasma cytokine levels

Rat blood samples were centrifuged at 3000 rpm for 10 min at room temperature and stored at −20 °C until assay. Plasma cytokine levels were measured by enzyme-linked immunosorbent assay (ELISA) using a commercially available TGF-β kit. The plasma samples were tested in duplicate according to the manufacturer's instructions [24].

2.6.2. Evaluation of malondialdehyde (MDA)

Lipid peroxidation was evaluated in plasma samples by measuring MDA, which was used as an oxidative stress marker [25]. MDA was assayed by measuring the thiobarbituric acid reactive substances (TBARS) [26]. To measure TBARS, 100 μL

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