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Protective effect of grape seed and skin extract against diabetes-induced oxidative stress and renal dysfunction in virgin and pregnant rat

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ABSTRACT

The present work deal with the effect of alloxan-induced diabetes on kidney oxidative stress and dysfunction of virgin and pregnant rat as well as the protection that may be afforded by high dosage GSSE (4 g/kg) treatment.

Diabetes affected negatively several kidney function parameters as creatinemia, uremia, uricemia and proteinuria without affecting kidney index. Diabetes also induced an oxidative stress characterized by increased lipid and protein oxidation, a drop in antioxidant enzyme defenses as catalase, superoxide-dismutase, glutathione-peroxidase, an alteration in transition metals as free iron, copper, selenium and associated enzymes and an increase in calpain and acetyl-cholinesterase activities. Tremendously, GSSE treatment protected efficiently against all the deleterious effects of diabetes-induced kidney dysfunction in both virgin and pregnant animals.

High dosage GSSE is a safe and potent anti-oxidant that may be further tested in clinical trials for the long-term preservation of kidney function especially in multiple pregnancies.

1. Introduction

Normal pregnancy is an enhanced oxidative stress status characterized by high energy demand and increased O_2 utilization as well as dynamic changes in whole organism. Diabetes occurring during the course of gestation is a serious complication whose prevalence has strikingly increased in multiethnic populations during the past 20 years [1]. Diabetes is associated with a high level of oxidative stress resulting from an imbalance between reactive oxygen species (ROS) production and the radical scavenging capacity of both enzymatic and non-enzymatic antioxidant systems [2]. Although diabetes is potentially multi-organ threatening, the kidney and the heart appeared as the most obviously altered organs.

Grape seed and skin extract (GSSE) is a complex polyphenolics mixture from grapes containing flavonoïds, non flavonoïds, proanthocyanidins and stilbenes as resveratrol [3]. GSSE is widely used as a nutritional supplement owing to its antioxidant [4], anti-inflammatory [5], health promoting and multi-organ protective properties as in the case of heart, kidney and brain failure (Nassiri-Asl and Hosseinzadeh [6]).

The present work aimed at analyzing the impact of experimentally-induced diabetes on the oxidative stress status into the kidney of virgin and pregnant rat, as well as the protection afforded by high dosage GSSE.

2. Materials and methods

2.1. Reagents and diets

Alloxan and the calpain substrate *N*-succinyl-Leu-Tyr-Amido 4-methyl coumarin were purchased from Sigma-Aldrich Chemical Company St. Louis, USA. Rodent chow was obtained from BADR, Bizerte, Tunisia.

2.2. GSSE preparation

Grape seed and skin extract (GSSE) was obtained from a grape cultivar (Carignan) of *Vitis vinifera* from northern Tunisia. Seeds

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were separated from skin and grounded separately. Powder mixture containing grape seed (50%) and skin (50%) was dissolved in 5% ethanol (v/v). After vigorous stirring and centrifugation at 10000*g* for 15 min at 4 °C, supernatant containing soluble polyphenols was daily administered to animals at high dosage of 4 g/kg. Total phenolic content was determined by the Folin-Ciocalteau colorimetric method [7], flavonoids and condensed tannins according to Dewanto et al. [8] and Sun et al. [9] respectively (Table 1). GSSE composition was established by LC–MS/MS analysis (Table 2).

2.3. Animals and treatment

Adult virgin or pregnant female rats weighing 180–220 g were used in agreement with the NIH guidelines for the use and care of animals [10] and housed in animal facility under controlled temperature with a 12-h light/12-h dark schedule with free access to food and water. Female rats were caged with sexually mature male rats. Vaginal smear was collected to check the presence of sperm and day zero of pregnancy was determined by sperm-positive vaginal smear. Once pregnant, females were divided into eight groups of six animals each:

Group I: virgin (V)
Group II: V + GSSE
Group III: V + Allox
Group IV: V + Allox + GSSE
Group V: pregnant (P)
Group VI: P + GSSE
Group VII: P + Allox
Group VIII: P + Allox + GSSE

Group I, III, V and VII were daily treated with vehicle (5% ethanol) by intra-peritoneal route and group II, IV, VI and VIII received GSSE at 4 g/kg bw.

Diabetes was induced on day 1 of the study using alloxan (150 mg/kg) in virgin (group III and IV) and pregnant (group VII and VIII) animals and every 5 days, blood glucose was monitored using one-touch glucometer. At the end of the treatment period (at gestational day 21), rats were killed by decapitation under deep anesthesia with urethane (40 mg/mL), their blood collected into heparinized tubes and processed for plasma analyses of diabetes, oxidative stress and kidney function parameters. Kidney was carefully dissected, weighed, homogenized and used for biochemical determination of oxidative stress status, transition metals and associated enzyme activities.

2.4. Biochemical analyses

Plasma insulin was measured using the ultrasensitive rat insulin ELISA Kit (Alpco Diagnostics). Briefly samples were added to a 96-well microplate pre-coated with a monoclonal antibody specific for rat insulin. After incubation for one hour at 37 °C, the wells were washed and blotted dry. Then a chromogenic substrate 3,3′,5,5′-Tetra-Methyl-Benzidine (TMB) was added and the microplate further incubated under shaking. The reaction was terminated after addition of a stop solution and the optical density measured with a microplate reader (Biotek ELX 800) at 450 nm.

Table 1
Phenolics levels in Carignan GSSE.

Phenolics	Seed	Skin
Total phenolics (mg/g extract)	67.00 ± 1.48	51.00 ± 3.76
Total flavonoïds (mg/g extract)	16.00 ± 0.90	14.00 ± 1.32
Non flavonoïds (mg/g extract)	51.00 ± 0.36	37.00 ± 4.83
Condensed tannins (mg/g extract)	1.22 ± 0.13	3.43 ± 0.16
Total anthocyanins (μg/g extract)	0.997 ± 0.013	0.962 ± 0.011

Table 2
LC–MS/MS data of some phenolic compounds found in Carignan GSSE.

Compounds	Relative abundance (%)	
	Seed	Skin
Catechin	2.27	0.36
Epicatechin	2.85	0.37
Procyanidin dimmer	0.47	ND
Procyanidin trimer	ND	ND
Quercetin	0.64	0.47
Resveratrol	0.14	ND
Rutin	1.51	0.5
Vanillin	10.67	7.75
Gallic acid	50.3	32.77
P-coumaric acid	ND	0.38
Rosmarinic acid	ND	0.75
2,5-dihydroxybenzoïc acid	30.58	51.96
Caffeic acid	ND	2.8
Chlorogenic acid	ND	0.34
Ferulic acid	0.55	1.46

Note: ND, not detected.

Plasma prolactin was determined using the demeditec prolactin rat ELISA kit (Demeditec Diagnostics GmbH, Germany) according to Beach et al. [11].

Lipids were extracted according to Folch et al. [12]. and triglyceride and total cholesterol determined using commercially available kits from Biomaghreb (Tunisia). Lipase activity was measured according to Humbert et al. [13] using *p*-nitrophenol dodecanoate as substrate. The reaction mixture containing 5 mmol *p*-nitrophenol dodecanoate, 50 mmol Tris–HCl buffer pH 8.5 and 50 μL of sample was incubated at 37 °C for 1 h, and the reaction stopped with 60 mmol EDTA. After centrifugation at 10000*g* for 5 min, absorbance was measured at 412 nm.

Kidney lipoperoxidation was evaluated by malondialdehyde (MDA) [14]. Homogenate was mixed with butylatedhydroxy-toluene/trichloroacetic acid (BHT/TCA) solution containing 1% BHT dissolved in 20% and centrifuged at 4000*g* for 15 min at 4 °C. Then, we added to supernatant 0.6 N HCl and 120 mmol L^{-1} thiobarbituric acid (TBA) in a 26 mmol L^{-1} Tris buffer. The mixture was heated at 80 °C for 10 min. After cooling, absorbance was measured at 532 nm. MDA concentration was calculated using the absorbance coefficient of the MDA-TBA complex 1.56×10^5 cm^{-1} M^{-1}.

Oxidative damage to proteins was measured by quantifying protein carbonylation according to Levine et al. [15]. Proteins were precipitated with 20% TCA and after centrifugation at 11000*g* for 3 min and 4 °C (Beckman, Allegra 64R), pellet was dissolved in 10 mM dinitrophenylhydrazine (DNPH) containing phosphate buffer. After 3 washings with ethanol-ethylacetate (1:1), pellet was dissolved in 20 mM potassium phosphate buffer pH 2.3 containing 6 M guanidine chloride and absorbance measured at 366 nm using the molar extinction coefficient of 22000 M^{-1} cm^{-1}

Total protein was determined according to Ohnishi and Barr [16]. Non-protein thiols (NPSH) were determined spectrophotometrically according to Ellman [17]. Kidney homogenates were also used for endogenous antioxidant enzyme activities as glutathione peroxidase, catalase and superoxide dismutase.

Glutathione peroxidase activity (GPx; E.C.1.11.1.9.) [18], was determined by following the oxidation of NADPH in the presence of glutathione reductase catalyzing the reduction of GSSG formed by the peroxidase. Absorbance was measured at 340 nm. Catalase activity (CAT; E.C.1.11.1.6.) was measured according to the method of Aebi [19]. Briefly, the reaction mixture contained 50 mM phosphate buffer (pH 7.0) and 200 mM H_2O_2, and it was recorded spectrophotometrically at 240 nm. CAT was defined in μmol of H_2O_2 degraded per minute per milligram of protein. Superoxide dismutase activity (SOD; E.C.1.15.1.1.) [20] was assayed by the