Effects of Pumpkin (*Cucurbita pepo* L.) Seed Protein on Blood Pressure, Plasma Lipids, Leptin, Adiponectin, and Oxidative Stress in Rats with Fructose-Induced Metabolic Syndrome

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ABSTRACT: This study evaluates the potential effects of pumpkin seeds protein on blood pressure (BP), plasma adiponectin, leptin levels, and oxidative stress in rats with fructose-induced metabolic syndrome. Twenty four male Wistar albino rats were divided into four groups and fed a 20% casein diet, 20% casein diet supplemented with pumpkin protein, 20% casein diet with 64% D-fructose, or 20% casein diet with pumpkin protein and 64% D-fructose for 8 weeks. Continuous fructose feeding induced an increase in plasma insulin/glucose ratio, BP, insulin and glucose, aspartate aminotransferase, alanine aminotransferase (ALT), alkaline phosphatase (ALP), creatinine, urea, and uric acid levels, and a decrease in the liver and muscle glycogen stores. In addition, elevated levels of total cholesterol (TC), triglycerides (TG), and leptin and lowered adiponectin levels were observed in rats fed a fructose-enriched diet. These groups also exhibited lower plasma levels of ascorbic acid and glutathione, higher thiobarbituric acid-reactive substances, hydroperoxide, carbonyl, and nitric oxide in both the liver and kidneys than rats fed the control diet. Interestingly, pumpkin seed protein treatment significantly counteracted alterations induced by fructose improving glucose, insulin, BP, TG, TC, ALT, and ALP levels, increasing liver and muscle glycogen stores, adiponectin level, and adiponectin/leptin ratio, and reducing plasma leptin levels. In addition, rats fed pumpkin protein with a high-fructose diet improved oxidative stress in the liver and kidneys. In conclusion, proteins from *Cucurbita pepo* L. seeds effectively improve metabolic parameters and protect against oxidative stress induced by a high-fructose diet.

Keywords: adiponectin, leptin, metabolic syndrome, oxidative stress, pumpkin seed protein

INTRODUCTION

Metabolic syndrome (MS) represents a group of metabolic alterations characterized by an increased risk of developing cardiovascular diseases, obesity, hypertension, diabetes, and hypercholesterolemia that affect public health worldwide (Spahis et al., 2017; Watt et al., 2019). These alterations frequently cluster together, contributing to cardiovascular morbidity and mortality (Stepanova et al., 2010). The prevalence of MS is increasing to epidemic proportions, and its prevalence varied from 8% to 43% in men and 7% to 56% in women worldwide (Cameron et al., 2004). The etiology of MS is multi-factorial and involves environmental and genetic factors (Jellinger et al., 2012).

The main dietary component that predisposes to the development of MS is the sugar in sweetened drinks. The common sweeteners are sucrose (50% saccharose and 50% fructose) and high-fructose corn syrup (HFCS, up to 55% fructose). It was postulated that a high-fructose (HF) diet is the main cause of MS development (Aydin et al., 2014). In addition, data from studies agree that excess fructose intake has detrimental effects on multiple cardiometabolic risk factors (Lim et al., 2019; Santos et

al., 2019).

Fructose, commonly added as HFCS or sucrose, in processed food is believed to result in less satiety than other sugars, thus increasing caloric intake, which is mainly through sweetened beverages (Aydin et al., 2014). Adipose tissue hormones have an essential influence in the prognosis of MS; in particular, adiponectin and leptin contribute to the development and progression of diseases associated with obesity, including hypertension, atherosclerosis, and type 2 diabetes (Lancha et al., 2012).

It is well established that dietary changes play a key role in the development of MS. Natural products are chemically diverse and has improved the pharmaceutical industry over the centuries (Sharifi-Rad et al., 2018a; Sharifi-Rad et al., 2018b). Plants and herbs have been applied in both the prevention and treatment of human disorders since ancient times (Prakash Mishra et al., 2018). One of these plants are pumpkins, which are used in the treatment of many metabolic disorders due to its components and phytochemicals beneficial for health (Yadav et al., 2010). Pumpkins (*Cucurbita*) belong to the Cucurbitaceae family, which is comprised of many species consumed as food and is mostly found in China, Pakistan, India, Yugoslavia, Argentina, the Mexican regions, America, and Brazil (Jia et al., 2003; Andrade-Cetto and Heinrich, 2005). Three main types are cultivated: *Cucurbita pepo*, *Cucurbita maxima,* and *Cucurbita moschata* (Lee et al., 2003). The health benefits of pumpkin seeds are attributed to their macro- and micronutrient composition; proteins, triterpenes, lignans, phytosterols, polyunsaturated fatty acids, antioxidative phenolic compounds, carotenoids, tocopherol, and minerals (Kim et al., 2012). To our knowledge, there are no investigations on the effects of protein from *C. pepo* L. on MS. Hence, the aim of this study was to evaluate the MS-associated abnormalities in rats fed a HF diet and explore whether co-treatment with pumpkin seed protein could counteract these metabolic alterations.

MATERIALS AND METHODS

Plant material and preparation of protein
Pumpkin seeds were obtained from the local market of Nedroma in Western Algeria. The seeds were then thoroughly cleaned with water and dried in the shade. After drying, the seeds were skinned and powdered. Then, the grounded pumpkin seed floor was homogenized with 10 mmol/L of sodium sulfite solution, and the pH was increased to 10 with 1 mmol/L of NaOH. The homogenate was continuously stirred and incubated overnight at 4°C, then centrifuged at 3,000 g for 20 min at 4°C. The supernatant was removed, brought to pH 4.5 with 5 mmol/L of H_2SO_4, and continuously stirred and incubated overnight at 4°C. After centrifugation, the pellet was dried at

37°C. The chemical compositions of pumpkin protein powder (g/100 g) show that the main nutritional components were proteins (80 g), lipids (8.92 g), ashes (1.33 g), moisture (3.36 g), and fibers (0.04 g). It also had high amounts of arginine, aspartate, and glutamic acid but was deficient in lysine, tryptophan, and methionine (Table 1).

Experimental animals
Male Wistar albino rats (n=24) weighing 190 to 200 g at the start of the study were obtained from the Animal Research Center, Pasteur Institute Algiers, Algeria. All animals were housed at a constant temperature of 23°C and relative humidity of 60% with a 12-h light/dark cycle. The rats were randomly divided into 4 separate groups of six rats each. The first group (C) was fed a diet with 20% casein. The second group (P) was fed a diet with 20% casein and an oral dose of pumpkin seed protein. The third group (C-HF) was fed a diet with 20% casein supplemented with 64% fructose (Prolabo, Paris, France, 67.19% of total energy). The fourth group (P-HF) was fed a diet with 20% casein plus 64% fructose and an oral dose of pumpkin seed protein for 8 weeks. Pumpkin protein was administered daily by oral gavage at a dose of 1 g/kg. The animals were weighed once a week, and food and water intakes were measured daily.

The animal experimental protocols were approved by the Institutional Animal Ethics Committee of University of Oran1-Ahmed Ben Bella (registration no. 13/355/2015, approval no. DZ-TN371/13) and carried out according to

Table 1. Composition of the experimental diets[1] (unit: g/kg)

Ingredient	C	P	C-HF	P-HF
Casein	200	—	200	—
Fructose	—	—	640	640
Corn starch	590	590	—	—
Sucrose	50	50	—	—
Sunflower oil	50	50	50	50
Cellulose	50	50	50	50
Vitamin[2]	20	20	20	20
Mineral[3]	40	40	40	40

[1]Diets were isoenergetic (16.28 MJ/kg of diet) and given in powdered form.
[2]UAR 200 (UAR, Villemoisson-sur-Orge, France). Vitamin mixture provides the following amounts (mg/kg diet): retinol, 12; cholecalciferol, 0.125; thiamine, 40; riboflavin, 30; pantothenic acid, 140; pyridoxine, 20; inositol, 300; cyanocobalamin, 0.1; ascorbic acid, 1.600; dl-α-tocopherol, 340; menadione, 80; nicotinic acid, 200; *para*-aminobenzoic acid, 100; folic acid, 10; biotin, 0.6.
[3]UAR 205 B (UAR, Villemoisson-sur-Orge, France). The salt mixture provides the following amounts (mg/kg diet): $CaHPO_4$, 17,200; KCl, 4,000; NaCl, 400; MgO, 420; $MgSO_4$, 2,000; Fe_2O_3, 120; $Fe_2SO_4 \cdot 7H_2O$, 200; trace elements, 400; $MnSO_4 \cdot H_2O$, 98; $CuSO_4 \cdot 5H_2O$, 20; $ZnSO_4$, 80; $CoSO_4 \cdot 7H_2O$, 0.16; KI, 0.32.
C, control with 20% casein diet; P, 20% casein supplemented with an oral dose of pumpkin seed protein; C-HF, 20% casein supplemented with 64% fructose; P-HF, 20% casein plus 64% fructose and an oral dose of pumpkin seed protein.

the guidelines for the care and use of laboratory animals recommended by the Council of the European Union (1986).

Systolic blood pressure

On day 54 of the experimental period, the systolic blood pressure (BP) of prewarmed and conscious rats was measured using the tail-cuff method with sphygmomanometer technique (PowerLab/400, logiciel Chart for Windows, ADInstruments Inc., Dunedin, New Zealand). The instrument used was a CODA rat tail system BP (Kent Scientific Corp., Torrington, CT, USA).

Blood and tissues collection

After 8 weeks of feeding, the overnight-fasted rats were anesthetized with an intraperitoneal injection of pentobarbital (60 mg/kg) (Sigma-Aldrich Co., St. Louis, MO, USA), and blood samples were withdrawn from the abdominal aorta and collected into tubes containing Na^2-ethylenediaminetetraacetic acid (0.1%) (Sigma-Aldrich Co.). The plasma was obtained by low-speed centrifugation at 3,000 g for 20 min at 4°C and stored in multiple aliquots at -70°C until analysis. The liver, kidney, and muscle specimens were quickly excised, rinsed with a cold saline solution, weighed, and immediately frozen at -70°C until analysis.

Chemical analysis

Plasma analysis: The fasting plasma glucose (glucose assay kit, BioSystems, Barcelona, Spain) and insulin (rat insulin ELISA kit, SpiBio, Montigny Le Bretonneux, France) levels were measured on the eighth week of the experiment. Plasma levels of albumin, triglycerides (TG), total cholesterol (TC), calcium, iron, creatinine, uric acid, urea, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) were determined using commercially available standard assay kits (Spinreact, Girona, Spain). The AST/ALT ratio was then calculated. The ascorbic acid level in plasma was determined using the method of Jagota and Dani (1982).

The total glutathione level was determined according to the method of Anderson (1985).

Measurement of hepatic and renal glycogen levels: The mixture of liver and kidney tissues and a 3-fold volume of alkali solution were placed in boiling water for 20 min. After centrifugation at 2,000 g for 10 min, the extract was used to determine glycogen content (Sadasivan and Manikam, 1996).

Plasma leptin and adiponectin levels: Plasma levels of adiponectin were measured using a commercial kit (BioVision, Inc., Milpitas, CA, USA). Leptin levels were assessed using commercial enzyme immune assay kit. The results were expressed as nanogram of adiponectin and leptin per milliliter of plasma.

Oxidative stress in hepatic and renal tissues

The hepatic and renal tissue homogenates were prepared on ice at a ratio of 1 g wet tissue to 9 mL of 150 mmol/L KCl using an ultraturrax homogenizer. Free radical damage was determined by measuring thiobarbituric acid-reactive substances (TBARS) using malondialdehyde as a standard, as described by Quintanilha et al. (1982). Tissue lipid hydroperoxide (LHP) levels were evaluated according to the method of Eymard and Genot (2003). Tissue carbonyl concentrations were determined using the assay described by Levine et al. (1990). Nitric oxide (NO) was assessed using Griess reagent (sulfanilamide and N-naphthyl ethylene diamine) (Cortas and Wakid, 1990). Tissue homogenates were clarified by zinc sulfate solution (Sigma-Aldrich Co.), and NO_3 (Sigma-Aldrich Co.) was then reduced to NO_2 by cadmium (Sigma-Aldrich Co.) overnight at 20°C under continuous stirring. Samples were added to the Griess reagent and incubated for 20 min at room temperature. The absorbance of these solutions was measured at 540 nm using a Beckman Coulter DU 640 spectrophotometer (Beckman Coulter, Brea, CA, USA). Sodium nitrite (Sigma-Aldrich Co.) was used for the standard curve.

Antioxidant enzyme activities in hepatic and renal tissues

The hepatic and renal tissue homogenates were prepared on ice at a ratio of 1 g wet tissue to 9 mL 150 mmol/L KCl using a POLYTRON® PT 2100 homogenizer (Kinematica AG, Lucerne, Switzerland) and used to determine the activities of superoxide dismutase (SOD; EC 1.15.1. 1), glutathione peroxidase (GSH-Px; EC 1.11.1.9), and catalase (CAT; EC 1.11.1.6). Tissue protein concentrations were determined using bovine serum albumin as described by Lowry et al. (1951). Tissue SOD activity was measured by the nicotinamide adenine dinucleotide oxidation procedure (Cayman Company, Ann Arbor, MI, USA). Briefly, the method uses xanthine and xanthine oxidase to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride to form a formazan dye. SOD activity was then measured by the degree of inhibition of the reaction using a spectrophotometer. The results were expressed as U/g of protein. CAT activity was determined according to the method described by Aebi (1974), and the results were expressed as U/mg of protein. The hepatic and renal GPH-Px activities were measured using an enzymatic method using a kit from Cayman Chemical Company (Ann Arbor, MI, USA), and the data were expressed in nmol/min/mL.

Statistical analysis

Data are expressed as means±standard deviation or standard error of the mean for continuous variables and percentages for categorical variables. Statistical analyses were

performed using the IBM SPSS Statistics 20 (IBM Corp., Armonk, NY, USA). Data were tested using two-way analysis of variance (ANOVA) with the type of protein and fructose content as independent variables. The Student's *t*-test was performed to analyze differences between two groups. Differences were considered significant at $P<0.05$.

RESULTS

Body weight, daily energy intake, gross weight gain efficiency ratio, and abdominal circumference of rats on pumpkin seed protein treatment

Table 2 illustrates the final body weight, average daily energy intake, and abdominal circumference of the rats. The data indicates that all groups exhibited a comparable final body weight as reflected by a similar gross weight gain efficiency ratio, which represents the amount of weight gain in grams per calorie consumed.

In addition, the abdominal circumference and metabolic body weight were unaffected by the diets. The daily energy intake was lower in the C-HF group compared with that in the C group; the values remained unchanged when comparing the P vs. C, P-HF vs. P, and P-HF vs. C-HF groups. Treatment of rats with pumpkin seed protein in the presence or absence of fructose did not affect these values compared to those of the untreated groups.

Absolute and relative liver and kidney weights

The absolute and relative weights of the liver and kidney are shown in Table 3. After 8 weeks of treatment, the absolute liver weight of the C-HF and P-HF groups increased by 55% and 39%, respectively, compared to that of the controls. The relative liver weight, expressed as g/100 g body weight, in the C-HF and P-HF groups increased by 35% and 22%, respectively, compared to that of the controls. Compared to the C group, the absolute and relative kidney weights of the C-HF group significantly decreased by approximately 10% and 13%, respectively, while those of the P-HF group did not experience significant changes in these parameters. Pumpkin seed protein administration decreased the absolute liver weights of the P and P-HF groups by approximately 22% and 31%, respectively, compared to those of the C and C-HF groups. In addition, a notable increase in the absolute (137%)

Table 2. Food intake, body weight, and metabolic body weight of experimental and control rats

Variable	Diet				P-value		
	C	P	C-HF	P-HF	P	F	P+F
Initial body weight (g)	197.66±5.75	209.83±9.08	209.66±9.95	209.66±4.83	NS	NS	NS
Final body weight (g)	321.16±22.83	302.50±13.33	326.00±19.72	327.00±13.02	NS	NS	NS
Energy intake (kcal/d)	72.12±1.04	73.04±0.19	68.11±1.51[#]	72.49±2.80	NS	<0.05	NS
Gross weight gain efficiency ratio (g/kcal)	0.028±0.002	0.027±0.004	0.028±0.004[#]	0.021±0.002*[#]	NS	<0.05	<0.05
Water intake (mL/d)	23.04±0.12	23.69±0.39	25.90±0.14[#]	24.78±0.69	NS	<0.05	NS
Metabolic body weight (BW[0.75])	75.83±4.04	72.52±2.41	76.69±3.49	76.88±2.30	NS	NS	NS
Abdominal circumference (cm)	18.91±0.49	18.85±0.98	18.91±0.66	19.55±0.71	NS	NS	NS

Values are mean±SEM of 6 rats per group.
*P vs. C, *P-HF vs. C-HF, [#]C-HF vs. C, and [#]P-HF vs. P at $P<0.05$.
C, control with 20% casein diet; P, 20% casein supplemented with an oral dose of pumpkin seed protein; C-HF, 20% casein supplemented with 64% fructose; P-HF, 20% casein plus 64% fructose and an oral dose of pumpkin seed protein; F, fructose; NS, not significant.

Table 3. Absolute and relative liver and kidney weights of experimental and control rats

Variable	Diet				P-value		
	C	P	C-HF	P-HF	P	F	P+F
Liver							
Absolute (g)	11.06±0.87	8.61±0.55*	17.18±1.94[#]	11.94±0.75*[#]	<0.05	<0.05	<0.05
Relative (g/100 g body weight)	3.44±0.24	2.85±0.23*	5.30±0.86[#]	3.65±0.24*[#]	<0.05	<0.05	<0.05
Kidney							
Absolute (g)	1.93±0.15	1.85±0.13	1.73±0.19[#]	2.04±0.16*[#]	NS	<0.05	<0.05
Relative (g/100 g body weight)	0.61±0.06	0.60±0.06	0.53±0.07[#]	0.62±0.03*[#]	NS	<0.05	<0.05

Values are mean±SEM of 6 rats per group.
*P vs. C, *P-HF vs. C-HF, [#]C-HF vs. C, and [#]P-HF vs. P at $P<0.05$.
C, control with 20% casein diet; P, 20% casein supplemented with an oral dose of pumpkin seed protein; C-HF, 20% casein supplemented with 64% fructose; P-HF, 20% casein plus 64% fructose and an oral dose of pumpkin seed protein; F, fructose; NS, not significant.

Table 4. Plasma metabolic and hormonal changes in experimental and control rats

Variable	Diet				P-value		
	C	P	C-HF	P-HF	P	F	P+F
Glucose (mmol/L)	6.10±0.09	5.69±0.08*	7.66±0.23[#]	7.05±0.11*[#]	<0.05	<0.05	<0.05
Insulin (pmol/L)	153.11±5.67	135.31±2.53*	324.25±6.56[#]	302.72±6.68*[#]	<0.05	<0.05	<0.05
Insulin/glucose	25.10±0.01	23.70±0.03	42.33±0.03[#]	42.94±0.01[#]	NS	<0.05	NS
Blood pressure (mmHg)	118.48±1.49	118.37±0.69	129.05±0.97[#]	127.66±0.53*[#]	NS	<0.05	<0.05
TG (mmol/L)	1.34±0.07	0.94±0.09*	4.00±0.37[#]	2.95±0.17*[#]	<0.05	<0.05	<0.05
TC (mmol/L)	2.83±0.61	2.01±0.31*	6.48±1.11[#]	5.18±0.43*[#]	<0.05	<0.05	<0.05
Albumin (g/L)	39.32±0.48	38.81±0.41	42.36±0.73[#]	41.22±0.67*[#]	NS	<0.05	<0.05
Calcium (mg/dL)	8.58±0.49	8.58±0.40	6.68±0.55[#]	7.91±0.23*[#]	NS	<0.05	<0.05
Iron (mmol/L)	12.39±0.04	12.39±0.12	8.91±0.10[#]	10.88±0.38*[#]	NS	<0.05	<0.05
Ascorbic acid (μmol/L)	111.51±6.43	111.72±4.14	91.26±5.06[#]	94.67±4.06[#]	NS	<0.05	NS
Glutathione (μmol/L)	965.87±10.97	967.24±7.33	633.84±13.76[#]	654.86±28.39[#]	NS	<0.05	NS

Values are mean±SEM of 6 rats per group.
*P vs. C, *P-HF vs. C-HF, [#]C-HF vs. C, and [#]P-HF vs. P at $P<0.05$.
C, control with 20% casein diet; P, 20% casein supplemented with an oral dose of pumpkin seed protein; C-HF, 20% casein supplemented with 64% fructose; P-HF, 20% casein plus 64% fructose and an oral dose of pumpkin seed protein; F, fructose; TG, triglycerides; TC, total cholesterol; NS, not significant.

and relative (17%) kidney weights was observed in the P-HF group compared to the C-HF group. The relative liver weight was 31% and 17% lower in rats fed pumpkin seed protein with or without fructose, respectively, than in those fed casein diets. No significant difference was observed in rats without MS.

BP and plasma metabolic data
As shown in Table 4, an HF diet caused several metabolic abnormalities in rats. In particular, it increased levels of plasma glucose, insulin, TG, TC, and albumin as well as the insulin/glucose ratio. Furthermore, these animal models not only developed hypertension but also had decreased levels of plasma ascorbic acid, glutathione, calcium, iron, and albumin compared to those of the controls. Interestingly, pumpkin seed protein treatment reversed these changes and resulted in a significant decrease ($P<0.05$) in levels of glucose, insulin, BP, TG, TC, and albumin and increase in levels of calcium and iron; levels of ascorbic acid and glutathione remained unchanged compared to those fed a C-HF diet. In addition, significantly low plasma glucose, insulin, TG, and TC levels were observed in normal rats fed pumpkin seed protein compared to those fed a C diet ($P<0.05$).

Liver and kidney functions
The liver and kidney functions are described in Table 5. Compared to control rats, those with diets with fructose had significantly increased levels of ALT, AST, and ALP and increased AST/ALT ratio (all $P<0.05$). Furthermore, the plasma levels of creatinine, uric acid, and urea were significantly higher in fructose-fed rats compared to those of control rats ($P<0.05$).

Treatment of fructose-fed rats with pumpkin seed protein (P-HF) resulted in lower ALT (29%), ALP (6%), uric acid (12%), and urea (13%) levels and AST/ALT ratio (30%) compared with rats fed the C-HF diet. The plasma urea levels were approximately 10% lower in the P group than in the C group.

Table 5. Liver and kidney parameters in experimental and control rats

Variable	Diet				P-value		
	C	P	C-HF	P-HF	P	F	P+F
AST (U/L)	17.50±1.51	17.80±1.30	38.16±1.72[#]	35.00±1.41[#]	NS	<0.05	NS
ALT (U/L)	22.66±1.50	20.80±1.92	41.50±1.04[#]	29.50±1.51*[#]	NS	<0.05	<0.05
AST/ALT	0.77±0.06	0.85±0.08*	0.91±0.03[#]	1.18±0.05*[#]	<0.05	<0.05	<0.05
ALP (U/L)	193.00±6.78	184.80±6.30	292.16±6.70[#]	274.83±4.99*[#]	NS	<0.05	<0.05
Creatinine (mg/dL)	0.49±0.08	0.48±0.06	0.79±0.19[#]	0.78±0.09[#]	NS	<0.05	NS
Uric acid (mg/dL)	3.35±0.36	3.11±0.15	8.47±0.78[#]	7.49±0.52*[#]	NS	<0.05	<0.05
Urea (mg/dL)	3.81±0.34	3.43±0.23*	8.67±0.74[#]	7.50±0.38*[#]	<0.05	<0.05	<0.05

Values are mean±SEM of 6 rats per group.
*P vs. C, *P-HF vs. C-HF, [#]C-HF vs. C, and [#]P-HF vs. P at $P<0.05$.
C, control with 20% casein diet; P, 20% casein supplemented with an oral dose of pumpkin seed protein; C-HF, 20% casein supplemented with 64% fructose; P-HF, 20% casein plus 64% fructose and an oral dose of pumpkin seed protein; F, fructose; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; NS, not significant.

Table 6. Liver and muscle glycogen levels in experimental and control rats (unit: μg glucose/mg tissue)

Variable	Diet				P-value		
	C	P	C-HF	P-HF	P	F	P+F
Liver	61.65±2.54	62.57±1.70	43.49±6.07[#]	52.44±4.43[*#]	NS	<0.05	<0.05
Muscle	8.91±0.52	9.37±0.31	6.34±0.38[#]	8.02±0.24[*#]	NS	<0.05	<0.05

Values are mean±SEM of 6 rats per group.
*P vs. C, *P-HF vs. C-HF, #C-HF vs. C, and #P-HF vs. P at P<0.05.
C, control with 20% casein diet; P, 20% casein supplemented with an oral dose of pumpkin seed protein; C-HF, 20% casein supplemented with 64% fructose; P-HF, 20% casein plus 64% fructose and an oral dose of pumpkin seed protein; F, fructose; NS, not significant.

Table 7. Plasma leptin and adiponectin levels and adiponectin/leptin ratio in experimental and control rats

Variable	Diet				P-value		
	C	P	C-HF	P-HF	P	F	P+F
Leptin (ng/mL)	6.93±0.13	6.74±0.15	13.28±0.17[#]	12.52±0.38[*#]	NS	<0.05	<0.05
Adiponectin	454.24±13.71	458.57±4.90	233.37±6.22[#]	252.05±8.22[*#]	NS	<0.05	<0.05
Adiponectin/leptin	65.91±2.06	67.98±1.49[*]	17.61±0.35[#]	20.12±0.57[*#]	<0.05	<0.05	<0.05

Values are mean±SEM of 6 rats per group.
*P vs. C, *P-HF vs. C-HF, #C-HF vs. C, and #P-HF vs. P at P<0.05.
C, control with 20% casein diet; P, 20% casein supplemented with an oral dose of pumpkin seed protein; C-HF, 20% casein supplemented with 64% fructose; P-HF, 20% casein plus 64% fructose and an oral dose of pumpkin seed protein; F, fructose; NS, not significant.

The liver and muscle glycogen levels

Table 6 shows the glycogen levels in the muscles and liver. Compared with the control groups, the HF groups had significantly elevated glycogen stores in both the liver and muscles (P<0.05). Treatment of HF rats with pumpkin seed protein resulted in a significant increase in glycogen stored in the liver (21%) and muscles (26%) compared to those in the C-HF group (P<0.05).

Plasma leptin and adiponectin levels

Table 7 illustrates the plasma values of leptin and adiponectin. The mean leptin levels were approximately 2 and 1.85 times higher in HF rats than in control rats. The plasma concentrations of adiponectin were 1.95 and 1.82 times lower in HF rats than in controls. Furthermore, rats fed the HF diet showed a significantly lower adiponectin/leptin ratio than normal rats (P<0.05). Compared to those fed with the C-HF diet, rats fed with the pumpkin protein diet had approximately 1.06-fold lower leptin levels and approximately 1.08-fold higher plasma adiponectin levels. Furthermore, the adiponectin/leptin ratio increased by nearly 14% in rats fed with pumpkin protein than in those fed the C-HF diet.

Oxidative stress in the liver and kidneys

Levels of TBARS, LHP, carbonyl, and NO in the liver and kidneys are shown in Fig. 1. Our data showed that continuous consumption of a HF diet resulted in oxidative stress in rats, which was evidenced by a significant increase in the levels of TBARS, carbonyls, and LHP and a decrease in NO in the hepatic and renal homogenates.

Administration of oral pumpkin protein to the fructose-fed groups significantly improved hepatic levels of TBARS and LHP and significantly increased the bioavailability of NO in both the liver and kidneys (P<0.05). We also observed a significant decrease in the levels of TBARS and carbonyls in the kidneys of normal rats fed pumpkin protein compared to those fed with casein (P<0.05).

Antioxidant enzyme activities in the liver and kidneys

The antioxidant enzyme activities in the liver and kidneys are presented in Fig. 2. It was observed that consumption of a HF diet in rats decreases the hepatic activities of SOD and CAT and increases that of GSH-Px compared to those fed with control diets. In addition, rats fed the C-HF diet had a lower renal SOD activity than those fed with the C diet. Meanwhile, a higher SOD activity was reported in the P-HF group than in the P group. Although CAT activity was significantly higher in the C-HF group compared to that of the C group (P<0.05), it was lower in the P-HF group compared to the P group. Renal GSH-Px activity was markedly higher (130%) in the P-HF group than in the P group. Treatment with pumpkin protein reduced hepatic activities of SOD and GSH-Px without changing CAT activity in the P-HF and P groups compared to the C-HF and C groups, respectively. In renal tissues, administration of pumpkin protein to fructose-fed rats significantly increased SOD and GSH-Px activities (104%) and reduced CAT activity compared to C-HF rats. In rats without MS, feeding the P diet caused lower renal CAT and GSH-Px activities compared to those fed the C diet.
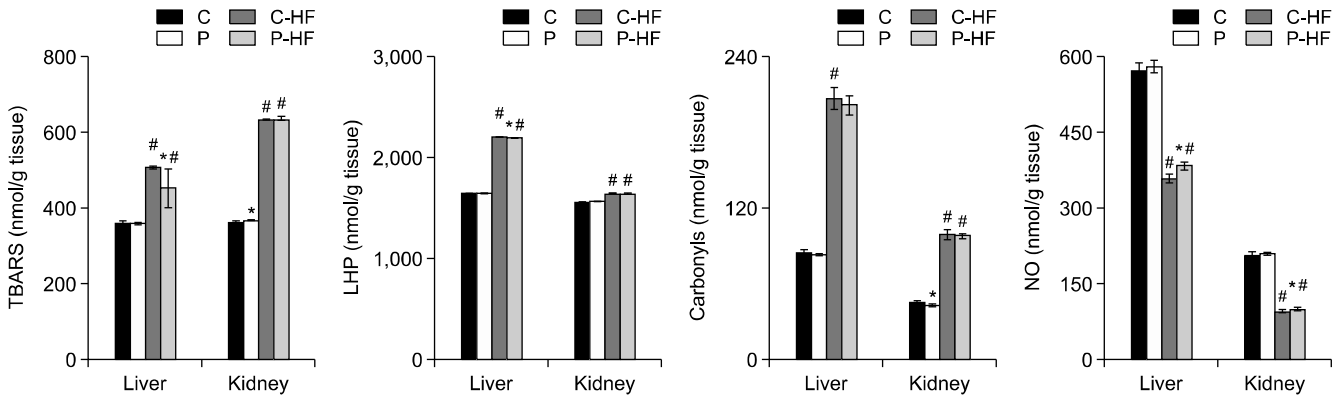
Fig. 1. Lipid and protein oxidation of the liver and kidneys in rats fed with pumpkin protein or casein with or without a high-fructose diet for 8 weeks. Data are presented as mean±standard deviation (6 rats in each group). Pumpkin protein vs. casein with or without fructose (P vs. C and P-HF vs. C-HF at *P<0.05); fructose treatment vs. no fructose treatment (C-HF vs. C and P-HF vs. P at #P<0.05). TBARS, thiobarbituric acid-reactive substances; LHP, lipid hydroperoxide; NO, nitric oxide; C, control with 20% casein diet; P, 20% casein supplemented with an oral dose of pumpkin seed protein; C-HF, 20% casein supplemented with 64% fructose; P-HF, 20% casein plus 64% fructose and an oral dose of pumpkin seed protein.
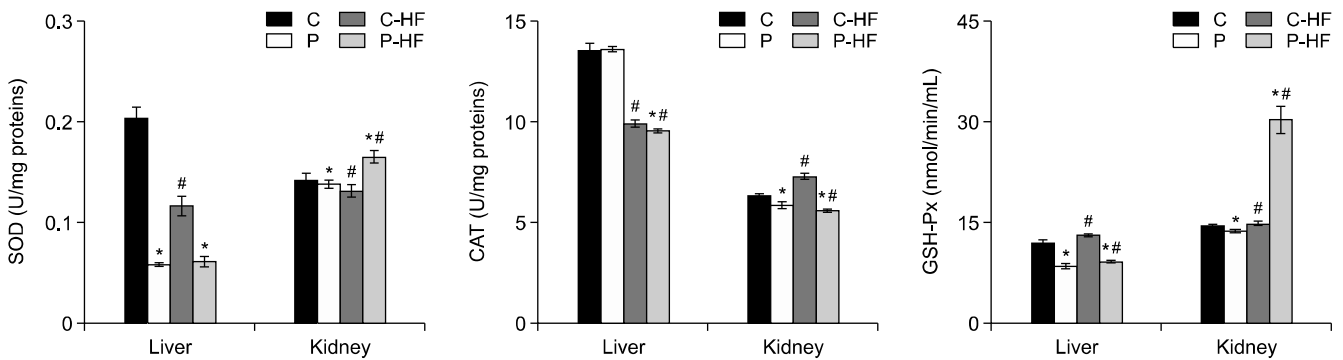

Fig. 2. Antioxidant enzymatic activities of the liver and kidneys in rats fed with pumpkin protein or casein with or without a high-fructose diet for 8 weeks. Data are presented as mean±standard deviation (6 rats in each group). Pumpkin protein with and without fructose vs. casein with or without fructose (P vs. C and P-HF vs. C-HF at *P<0.05); fructose treatment vs. no fructose treatment (C-HF vs. C, P-HF vs. P at #P<0.05). SOD, superoxide dismutase; CAT, catalase; GSH-Px, glutathione peroxidase; C, control with 20% casein diet; P, 20% casein supplemented with an oral dose of pumpkin seed protein; C-HF, 20% casein supplemented with 64% fructose; P-HF, 20% casein plus 64% fructose and an oral dose of pumpkin seed protein.

DISCUSSION

It has been established that fructose overload is the main cause of the development of MS (Pereira et al., 2017). In this study, we aimed to evaluate the therapeutic effects of pumpkin seed protein in reducing the metabolic consequences of fructose-induced MS in rats.

After 8 weeks, the body weight, gross weight gain efficiency ratio, abdominal circumference, and metabolic body weight were virtually identical in all groups, which are consistent with the findings of Shapiro et al. (2008). Fructose has previously been reported to increase hunger and stimulate the appetite (Lowette et al., 2015). Its palatability increased fluid intake and reduced the energy intake in C-HF rats compared to those of controls. Thus, the reduction in calorie intake did not alter the body weight, gross weight gain efficiency ratio, abdominal circumference, and metabolic body weight in C-HF rats. Meanwhile, the P-HF rats did not show changes in these

parameters when compared to those of the controls. We also observed an increase in the liver-to-body weight ratio and absolute liver weight during fructose supplementation, which may possibly be an initial sign of fatty liver (Miatello et al., 2002). Additionally, this hypertrophy was associated to changes in liver function, as shown by the elevated plasma AST, ALT, and ALP levels and increased AST/ALT ratio. These changes, apart from the increased lipogenesis and steatosis (data not published), are suggestive of liver injury. Previous studies have emphasized that a HF diet can lead to liver dysfunction as well as non-alcoholic fatty liver disease, even if these facts are not consistent (Tappy and Lê, 2010; Lozano et al., 2016). Nevertheless, our results corroborate those of Park et al. (2018), who demonstrated cardiac hypertrophy in fructose-fed rats. Nakayama et al. (2010) showed that rats fed a diet with 60% fructose developed renal hypertrophy with tubular cell proliferation and low-grade tubulointerstitial injury, which exacerbated proteinuria, worsened

renal function, and accelerated glomerulosclerosis in a remnant kidney model (Gersch et al., 2007). Furthermore, rats fed a diet with 64% fructose had elevated levels of plasma urea, creatinine, and uric acid after 8 weeks. Increased uric acid production caused by a HF diet may be due to the phosphorylation of fructose to fructose-1-phosphate, resulting in hepatic degradation of ATP to ADP and AMP, endothelial cell dysfunction, and impaired postprandial muscle vasodilatation, which may contribute to insulin resistance (Nakagawa et al., 2006). Administration of oral pumpkin protein ameliorated liver hypertrophy in rats fed a diet with or without fructose, suggesting that it exerts its hepatic anti-hypertrophy effects via a mechanism that involves the reduction of liver toxicity through the improvement not only of the ALT and ALP levels and AST/ALT ratio but also of lipid production and steatosis (data not published). This also suggests that pumpkin proteins help prevent liver diseases and disorders by improving liver function and reducing cell damage. Unfortunately, despite the improvement in the plasma urea and uric acid levels, our results demonstrate that pumpkin protein treatment increased the absolute kidney weight and did not counteract kidney hypertrophy caused by a HF diet.

The results of this investigation showed that when compared to the controls, rats overfed with fructose resulted in elevated plasma glucose and insulin levels, increased insulin/glucose ratio, and decreased liver and muscle glycogen stores, confirming the report of AL-Rasheed et al. (2016). Such changes may be associated with glucose tolerance and insulin resistance when there is an excess fructose supply. These observations could be explained by the fact that fructose does not stimulate insulin secretion from pancreatic β-cells due to the low concentration of the fructose transporter, glucose transporter type 5, in these cells (Elliott et al., 2002). Interestingly, pumpkin protein supplementation counteracted these abnormalities through the amelioration of glucose metabolism by increasing glycogen synthesis and storage in both the liver and muscles in rats induced with MS. Ju and Chang (2001) reported a significant increase in plasma insulin and reduction in blood glucose levels after consumption of both sugar-free pumpkin powder and common pumpkin powder. The possible mechanism by which pumpkin protein leads to hypoglycemia and hypoinsulinemia may be due to the increase in the peripheral utilization of glucose or its release from bound insulin. The study further proposed that the rich protein contents with a wide range of molecular weights (3~60 kDa) in pumpkin seeds improve insulin levels and blood glucose tolerance (Caili et al., 2006).

Moreover, we assessed whether proteins from pumpkin seed lowered the BP in rats fed with a HF diet. The presence of MS in these rats was accompanied by a significant rise in the BP by almost 9% and 8% in the C-HF and P-HF rats, respectively, when compared to those of controls. Insulin resistance and hyperinsulinemia observed in fructose-fed rats may impair endothelial function and thereby contribute to the elevated BP in this model. Overall, our results are consistent with those of other reports (Mostafa-Hedeab et al., 2017; Malakul et al., 2018). Singh et al. (2008) found that the systolic BP of wild-type mice fed a diet with 60% fructose for 12 weeks was 9 mmHg higher than those fed a diet with 60% starch, and that a diet with 60% fructose reduces kidney renin expression by approximately 50% in rats after 2 weeks. Similarly, Pérez-Pérez et al. (2017) found that the ambulatory systolic and diastolic BPs in 74 healthy men increased significantly after they consumed 200 g of fructose daily for 2 weeks. Moreover, increased levels of circulating insulin and fructose metabolites as a result of HF diets can alter endothelial activity, thereby raising the BP. However, it is worth noting that pumpkin protein treatment resulted in a significant decline in the BP of fructose-fed rats as compared to those with fructose-casein. These findings might be explained by the presence of compounds, such as cucurbitin protein, which possesses vasodilatory properties (Chelliah et al., 2018) that are protective against hypertension. Thus, pumpkin seed consumption is highly beneficial for hypertensive patients treated with diuretics that result in a loss of potassium (Arinathan et al., 2003).

Our present study also revealed that fructose-fed rats had increased plasma TG and TC levels compared to those of controls. Fructose is readily absorbed and rapidly metabolized in the liver, transformed into fatty acids, and released to the systemic circulation in the form of TG, thus increasing not only the TG plasma levels but also the atherogenic risk (Aydin et al., 2014; Pereira et al., 2017). In Syrian golden hamsters fed with a HF diet, the mechanisms underlying fructose-induced dyslipidemia were determined to be caused by the enhanced TG production resulting from the increase in both the very low density lipoprotein particle size and secretion rate by the liver and to the reduction in TG clearance due to lowered lipoprotein lipase activity (Taghibiglou et al., 2000). Parallel to this, insulin resistance observed in fructose-fed rats may be associated with intracellular TG accumulation, which causes lipotoxicity and β-cell damage that leads to the development of diabetes (Ziegler et al., 2001). Liver hypertrophy and elevated plasma lipid and leptin levels observed in fructose-fed rats may cause a reduction in adiponectin production, decreasing the adiponectin/leptin ratio and adiponectin secretion that would result in hepatic lipid accumulation, confirming the metabolic abnormalities caused by fructose. Similar results were observed in Korean adults with type 2 diabetes (Lee et al., 2009) and in MS patients (Scheid and Sweeney, 2014).

A lower adiponectin/leptin ratio may indicate high levels of leptin due to leptin resistance, which is a characteristic of MS. Oral administration of pumpkin protein from *C. pepo* L. to rats for 8 weeks decreased the levels of plasma TC and TG, suggesting that dietary supplementation of pumpkin protein at 1 g/kg/d was sufficient to improve dyslipidemia in fructose-fed rats with MS. These data may be attributed to the enhancement of β-oxidation of fatty acids and suppression of fatty acid synthesis in the liver as well as to the increased adiponectin and leptin levels and adiponectin/leptin ratio, which may collectively result in the improvement of the atherogenic profile and insulin-sensitivity. In addition, high levels of arginine in pumpkin protein and a low lysine/arginine ratio (0.09 in pumpkin protein vs. 2.17 in casein) contribute to reduce lipotoxicity. Thus, protein from *C. pepo* L. could modulate plasma lipid disorders in rats associated with fructose overload. In the study of Rabrenović et al. (2014), pumpkin seeds were found to possess antihyperglycemic, hypolipidemic, antioxidative, and anti-inflammatory effects.

Finally, compared to untreated control rats, we documented an increase in oxidative stress in rats given a diet with 64% fructose, which was evidenced by the markedly elevated TBARS, LHP, and carbonyl levels in both the hepatic and renal tissues. The oxidative damage results from the reduction of plasma ascorbic acid and total glutathione concentrations, defects in the components of the free radical antioxidant enzyme defense system, such as SOD, CAT, and GSH-Px activities, and decrease in plasma adiponectin levels, which may serve as an upstream pathway of increased oxidative stress in MS (Yamauchi and Kadowaki, 2008), leading to increased lipid susceptibility to peroxidation. Rats fed the P-HF diet exhibited higher hepatic and renal SOD and GSH-Px activities but a lower hepatic CAT activity than those fed the P diet. When compared to the C group, the C-HF group had a lower SOD activity in both tissues, lower hepatic CAT and higher hepatic GSH-Px activities, and a higher renal CAT activity. Our findings indicate that there was also a dramatic decrease in the NO bioavailability in the hepatic and renal tissues, probably due to the rise in the plasma uric acid level that inhibits endothelial function as well as the low activity and expression of endothelial nitric oxide synthase (eNOS) (Palanisamy and Venkataraman, 2013; Okamura et al., 2014). Miatello et al. (2002) have shown that eNOS activity was decreased by approximately 30% in mesenteric vessels isolated from fructose-fed rats, while Palanisamy and Venkataraman (2013) have reported that eNOS expression in fructose-fed rats was approximately half of that of control rats. In addition, there is evidence that an overproduction of reactive oxygen species, which is generated by cellular disturbance in glucose or/and lipid metabolism, leads to degradation of NO (Steven et al., 2017). Increased levels of superoxide anions may reduce NO bioavailability. Superoxide radicals cannot be dismuted in the absence of the Cu-Zn SOD enzyme, and the increased levels of radicals can interact with NO to form peroxynitrite radicals, eventually leading to the aggravation of cellular injury via membrane damage (Zhao et al., 2015). In this study, it was found that daily oral administration of 1 g/kg of pumpkin protein to fructose-fed rats counteracted the high hepatic levels of TBARS and LHP despite lower SOD and GSH-Px activities compared to those fed with C-HF. In renal tissues, an increase in GSH-Px and SOD activities, which might protect against superoxide anion elevation, and a decrease in CAT activity were observed despite the unchanged lipid peroxidation. Therefore, it appears that increased NO bioavailability in both tissues after pumpkin protein administration in fructose-fed rats with MS, mainly due to arginine, seems to be responsible for the increased activity of antioxidant enzymes, improved lipid metabolism, and reduced risk of free radical damage.

In conclusion, our data showed that proteins from *C. pepo* L. seeds counteracts the deleterious effects of fructose by improving glycemia, insulinemia, plasma lipid disorders, hepatic function, and BP and ameliorating decreases in adiponectin and leptin levels and adiponectin/leptin ratio. In addition, these proteins possess potent antioxidant effects that prevent oxidative stress and attenuate the risk of free radical abnormalities as well as enhance the antioxidant enzyme defense. These suggest that pumpkin protein is beneficial in the treatment of MS induced by a HF diet.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Investigation, software, formal analysis: AC. Methodology, investigation: FZHC. Statistical analysis: KC. Investigation: EEE. Writing (review, editing, visualization): LP. Conceptualization, resources, funding acquisition, supervision and writing (original draft): DAY.

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