

Sardine protein diet increases plasma glucagon-like peptide-1 levels and prevents tissue oxidative stress in rats fed a high-fructose diet

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Abstract. The current study investigated whether sardine protein mitigates the adverse effects of fructose on plasma glucagon-like peptide-1 (GLP-1) and oxidative stress in rats. Rats were fed casein (C) or sardine protein (S) with or without high-fructose (HF) for 2 months. Plasma glucose, insulin, GLP-1, lipid and protein oxidation and antioxidant enzymes were assayed. HF rats developed obesity, hyperglycemia, hyperinsulinemia, insulin resistance and oxidative stress despite reduced energy and food intakes. High plasma creatinine and uric acid levels, in addition to albuminuria were observed in the HF groups. The S-HF diet reduced plasma glucose, insulin, creatinine, uric acid and homeostasis model assessment-insulin resistance index levels, however increased GLP-1 levels compared with the C-HF diet. Hydroperoxides were reduced in the liver, kidney, heart and muscle of S-HF fed rats compared with C-HF fed rats. A reduction in liver, kidney and heart carbonyls was observed in S-HF fed rats compared with C-HF fed rats. Reduced levels of nitric oxide (NO) were detected in the liver, kidney and heart of the S-HF fed rats compared with C-HF fed rats. The S diet compared with the C diet reduced levels of liver hydroperoxides, heart carbonyls and kidney NO. The S-HF diet compared with the C-HF diet increased the levels of liver and kidney superoxide dismutase, liver and muscle catalase, liver, heart and muscle glutathione peroxidase and liver ascorbic acid. The S diet prevented and reversed insulin resistance and oxidative stress, and may have benefits in patients with metabolic syndrome.

Introduction

Metabolic syndrome (MS) is considered to be a clustering of metabolic alterations conferring a high risk of developing type-2 diabetes (T2D) and cardiovascular disease (CVD) (1,2). The prevalence of MS has markedly increased worldwide due to modern lifestyles and an increased consumption of high-sugar diets, in particular fructose (3). Previous data suggests that fructose consumption in humans results in increased visceral adiposity, lipid dysregulation and reduced insulin sensitivity, all of which have been associated with increased risk for CVD and T2D (4). Fructose has become an important causative factor in the development of MS (4,5). The fructose-fed rat is therefore used as an animal model for insulin resistance, and is considered to mirror MS observed in humans (6). Animal studies have demonstrated that rats fed a high-fructose (HF) diet exhibit hepatic oxidative damage and an altered lipid metabolism due to hepatic stress as a result of the burden on the fructose metabolism (7).

Previous studies have focused upon types of seafood that may be beneficial in preventing MS and possibly reducing the risk of various diseases (8,9). The reduced incidence of CVD among populations consuming fish-rich diets has been attributed to a greater proportion of omega-3 polyunsaturated fatty acids in fish oil (10-12). There is evidence that the type of protein in the diet may serve an important role in the secretion of insulin by the pancreas (13) and in the regulation of hepatic lipogenesis mediated by sterol regulatory element binding protein-1 (14). In previous animal studies regarding the health effects of fish protein, fish proteins have been demonstrated to prevent insulin resistance in high-fat fed obese rats (15,16), and diminish the development of high blood pressure and hypercholesterolemia (17,18). Furthermore, in insulin-resistant males and females the consumption of a cod protein diet for 4 weeks improved insulin sensitivity compared with a diet of lean beef, pork, veal, eggs, milk and milk products (19).

The objective of the current study was to investigate the specific effect of the type of dietary protein on insulin resistance, plasma glucagon-like peptide-1 (GLP-1), in addition to oxidative stress in the tissues of HF-fed rats.

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Materials and methods

Preparation of sardine protein. Fish protein was isolated from sardine fillets obtained from a fishery (Oran, Algeria). The heads, internal organs and bones of the sardines were removed. The proteins of the muscle tissue were then solubilized in 10 volumes of water with NaOH (Sigma-Aldrich, St. Louis, MO, USA) added to obtain pH 10.5 according to the method outlined by Undeland *et al* (20). The mixture was centrifuged (18,000 x g, 20 min, 4°C), causing the light oil fraction to rise to the top of the suspension, then the muscle proteins were precipitated and collected. The crude protein content was determined by the Kjeldahl method (21). The crude fat content was measured by the Soxhlet method (22). The moisture content was calculated as the loss in weight following drying at 105°C for 24 h. The ash amount was analyzed by direct ignition at 550°C for 24 h. The amino acid composition of the sardine protein, as determined through analysis by a commercial service (Institute of Chemistry, Center for Technical and Scientific Research in Physical-Chemical Analysis, Algiers, Algeria), is presented in Table I.

Animals and diets. A total of 24 male Wistar rats obtained from Iffa-Credo (I'Arbresle, France) weighing 190-200 g at the beginning of the experiment were used for the current study. The rats were kept in a laboratory animal house with a 12 h light:dark cycle (07:00-19:00). Throughout the experiment, the temperature of the animal room was maintained at 24°C and humidity at 60%. Rats were assigned to four equal-weight groups and fed the following diets for 2 months: Group 1 (C-HF), diet containing 20% casein (C) and 64% fructose (Prolabo, Paris, France); group 2 (S-HF), 20% sardine protein and 64% fructose; group 3 (C), 20% casein; group 4 (S), 20% sardine protein. The compositions of the experimental diets are presented in Table II. Diets were isoenergetic (16.28 MJ/kg) and contained identical amounts of lipids, vitamins (UAR 200; UAR, Villemoisson-sur-Orge, France), minerals (UAR 205 B; UAR) and fiber. Food and water were provided *ad libitum*. The body weights of the animals were recorded every week and food intake was measured daily. The food efficiency ratio was calculated as follows: Weight gain (g) / food intake (g), where weight gain was calculated as the difference between the final weight and the initial weight of each rat during the 60-day experiment and food intake was determined as the difference between the rest amount of food and the amount of food administered for each rat during the 60-day experiment. The glycemia was measured weekly using a handheld glucometer (Accu-Chek Aviva; Roche Diagnostics, Basel, Switzerland) with blood obtained from the caudal vein. The general guidelines for the Care and Use of Laboratory Animals as set forth by the Council of European Communities were followed (23).

Blood, urine and tissue samples. At the end of the experimental period, the rats were sacrificed by anesthesia using intraperitoneal injection of sodium pentobarbital (60 mg/kg body weight; Abbott Laboratories, North Chicago, IL, USA) following overnight starvation. Blood samples were collected from the abdominal aorta in citric acid tubes, and the plasma was separated by centrifugation (3,000 x g, 15 min, 4°C) and stored at -70°C until required for chemical analysis.

Table I. Amino acid composition of dietary proteins (g/100 g protein).

Amino acids	Casein	Sardine protein
Alanine	2.9	6.8
Arginine	3.5	5.5
Cystine (eine)	0.4	1.2
Glycine	1.7	4.3
Histidine	2.9	2.0
Methionine	2.8	2.9
Leucine	8.9	9.2
Serine	4.9	3.4
Tyrosine	5.3	4.2
Valine	6.4	4.4
Isoleucine	5.2	4.2
Lysine	7.6	9.4
Phenylalanine	4.8	3.6
Glutamic acid	20.2	17.2
Aspartic acid	6.7	10.4
Tryptophan	1.2	1.2
Proline	10.6	4.1
Threonine	3.9	4.3
Lysine/arginine	2.17	1.70

Liver, kidney, heart and gastrocnemius muscle tissues were harvested, washed with ice-cold 150 mmol/l NaCl (Sigma-Aldrich), weighed and immediately frozen at -70°C until required for analysis. Urine samples were collected on day 59 of the experiment in the four groups.

Analytical procedures. Plasma glucose was analyzed by the method previously described by Bergmeyer *et al* (24). Insulin in plasma was measured by radioimmunoassay according to Leclercq-Meyer *et al* (25). These measurements were used to calculate the homeostasis model assessment (HOMA) index (mmol/l/22.5): [The product of the plasma insulin concentration (mmol/l) x plasma D-glucose concentration (mmol/l)]. GLP-1 level was measured in plasma using a GLP-1 (Active) ELISA kit (BioVendor Research and Diagnostic Products, Karasek, Czech Republic). Plasma fructose levels were determined enzymatically using a BioSentec Glucose/Fructose/Sucrose kit (BioSentec, Toulouse, France). Protein concentrations were measured according to the method of Lowry *et al* (26) using bovine serum albumin (Sigma-Aldrich) as a standard. Plasma and urine creatinine, uric acid and albumin levels were determined using an enzymatic Spinreact Colorimetric Kinetic Jaffé kit (cat. no. 1001110), a Uricase-POD Spinreact Enzymatic Colorimetric kit (cat. no. 1001011), and a Bromocresol Green Spinreact Colorimetric kit (cat. no. 1001020; Spinreact, Girona, Spain).

Tissue analysis. The level of lipid peroxidation in the liver, kidney, heart and muscle tissues was studied by measuring thiobarbituric acid reactive substances (TBARS) in tissue homogenates using the method of Quintanilha *et al* (27). For TBARS measurement, the tissue homogenates were deprot-

Table II. Composition of the experimental diets.

Ingredient	C (g/kg diet)	S (g/kg diet)	C-HF (g/kg diet)	S-HF (g/kg diet)
C	200	-	200	-
S	-	200	-	200
Fructose	-	-	640	640
Corn starch	590	590	-	-
Sucrose	50	50	-	-
Sunflower oil	50	50	50	50
Cellulose	50	50	50	50
Vitamin (UAR 200)	20	20	20	20
Mineral (UAR 205 B)	40	40	40	40

Diets were isoenergetic (16.28 MJ/kg of diet) and given in powdered form. The 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. The salt mixture provides the following amounts (mg/kg diet): CaHPO_4 , 17200; KCl, 4,000; NaCl, 400; MgO, 420; MgSO_4 , 2,000; Fe_2O_3 , 120; $\text{Fe}_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$, 200; trace elements, 400; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 98; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 20; ZnSO_4 , 80; $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$, 0.16; KI, 0.32. C, casein; S, sardine protein; HF, high-fructose.

einized with 10% trichloroacetic acid (TCA) (Sigma-Aldrich) and the precipitate was treated with thiobarbituric acid (Sigma-Aldrich) at 90°C for 1 h. The pink color formed gave a measure of the TBARS. The concentration was expressed as $\mu\text{mol/g}$ tissue. Additionally, liver, kidney, heart and muscle hydroperoxides were assayed using the method described by Eymard & Genot (28). The color developed was read at 560 nm using a Beckman Coulter DU 640 spectrophotometer (Beckman Coulter, Inc., Cridersville, OH, USA). The concentration was expressed as $\mu\text{mol/g}$ tissue. The level of protein carbonyl was measured by the method of Levine *et al* (29). The tissue was homogenized in 10 mM HEPES buffer (Sigma-Aldrich) containing 137 mM NaCl, 4.0 mM potassium chloride, 1.0 mM potassium dihydrogen phosphate and 0.6 mM magnesium sulfate (Sigma-Aldrich). The homogenate was centrifuged at 40,000 \times g for 20 min at 25°C. The supernatant was mixed with dinitrophenyl hydrazine in 2M hydrochloric acid and allowed to stand at room temperature for 1 h. The protein-hydrazone derivative was precipitated with TCA and the precipitate was washed three times with ethanol-ethylacetate (1:1) (Sigma-Aldrich). The color in the supernatant was read at 390 nm using a Beckman Coulter DU 640 spectrophotometer. The concentrations were expressed as nmol/g tissue. Tissue nitric oxide (NO) was assessed using the Griess reagent (sulfanilamide and N-naphthyl ethylenediamine) (30). Tissue homogenates were clarified by zinc sulfate solution (Sigma-Aldrich), NO_3 (Sigma-Aldrich) was then reduced to NO_2 by cadmium (Sigma-Aldrich) overnight at 20°C under agitation. 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. Sodium nitrite (Sigma-Aldrich) was used for the standard curve. The data were expressed as $\mu\text{mol/g}$ tissue.

Antioxidant enzyme activity. Liver, kidney, heart and muscle tissue homogenates 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), were used for 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) determinations. Tissue SOD activity was determined using a SOD and GSH-Px Reagent Assay Cayman Chemical kit (Cayman Chemical 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. The SOD activity was measured by the degree of inhibition of the reaction, using a spectrophotometer. The results were expressed as U/mg of protein. CAT activity was determined according to the method described by Aebi (31) and the results were expressed as nmol/mg of protein. Tissue GPH-Px activity was measured using an enzymatic method with a kit from Cayman Chemical Company. The data were expressed in nmol/min/mg of protein.

Liver α -tocopherol and ascorbic acid levels. Liver α -tocopherol levels were determined by the method as described by Baker *et al* (32). The level of α -tocopherol was estimated by the reduction of ferric ions to ferrous ions by α -tocopherol and the formation of a red-colored complex with 2,2-dipyridyl was measured at 520 nm using a Beckman Coulter DU 640 spectrophotometer. Ascorbic acid concentrations were measured using a LiChrospher 100 RP18 high performance liquid chromatography instrument (EMD Millipore, Billerica, MA, USA).

Statistical analysis. Values are presented as the mean \pm standard deviation of six rats per group. Statistical analysis of the data was conducted with Statistica software, version 6 (Dell Software, Aliso Viejo, CA, USA). Data were analyzed using two-way analysis of variance with the type of protein and fructose as independent variables. When the interaction was significant, Fisher's protected least significant difference test

Table III. Growth parameters of experimental and control rats.

Variables	Diets					Analysis of variance (P-values)			
	C	S	C-HF	S-HF	Prot	Fru	Prot x Fru		
Energy intake (kJ/day)	392.34±36.52	320.62±20.16 ^a	294.26±3.05 ^b	269.95±16.40 ^{ab}	P<0.05	P<0.05	P<0.05		
Body weight gain (g/8 weeks)	144.90±10.16	119.97±16.49 ^a	160.12±9.17 ^b	133.00±9.45 ^{ab}	P<0.05	P<0.05	P<0.05		
Food efficiency	0.10±0.005	0.10±0.009	0.14±0.008 ^b	0.14±0.01 ^b	NS	P<0.05	NS		
Tissue absolute weight (g)									
Liver	9.67±1.23	7.60±0.73 ^a	10.73±0.62 ^b	9.89±0.49 ^{ab}	P<0.05	P<0.05	P<0.05		
Kidney	1.82±0.24	1.58±0.20	2.22±0.21 ^b	2.26±0.07 ^b	NS	P<0.05	NS		
Heart	0.76±0.04	0.70±0.05	0.92±0.10 ^b	0.88±0.16 ^b	NS	P<0.05	NS		
Muscle	1.50±0.06	1.63±0.13	1.78±0.15 ^b	1.55±0.08 ^a	NS	P<0.05	P<0.05		

Values are presented as the mean ± standard deviation. ^aP<0.05, sardine protein vs. casein, ^bP<0.05, fructose supplementation vs. no supplementation. C, casein; S, sardine protein; HF, high-fructose; Prot, protein; Fru, high-fructose; NS, not significant.

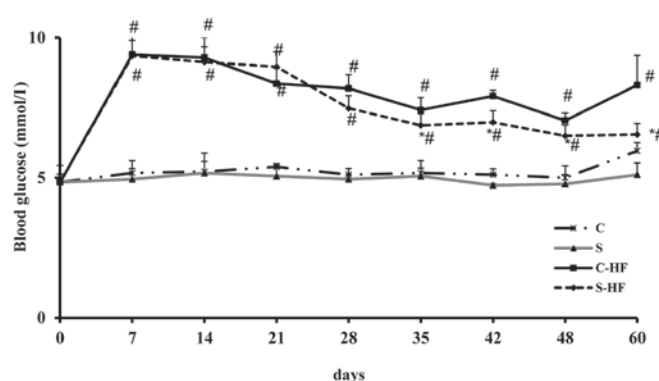


Figure 1. Time course of blood glucose of experimental and control rats. Values are presented as the mean ± standard deviation. ^aP<0.05, sardine protein vs. casein, ^bP<0.05, fructose supplementation vs. no supplementation. C, casein; S, sardine protein; HF, high-fructose.

was performed. P<0.05 was considered to indicate a statistically significant difference.

Results

Energy intake and tissues weights. Following 8 weeks of feeding, the HF-fed rats were significantly heavier than the control rats, despite a low cumulative energy intake over the 8 weeks of study (P<0.05; Table III). Consequently, food efficiency was significantly higher (P<0.05) in HF-fed animals as compared with control animals. Consistent with this, the weights of the liver, kidney and heart were greater in rats on the HF diets compared with control rats. The constituents (g/100 g) of the protein obtained by these operations were 93 g protein, 0.9 g lipids, 2.5 g ash and 3.6 g moisture. Rats on sardine protein diets gained less weight and had reduced food and energy intakes than those fed casein diets. The liver weight was significantly reduced with S and S-HF diets, while heart, kidney and skeletal muscle weights were not significantly different among HF rats with any of the protein sources.

Time course of blood glucose. The results of the time course of glycemia assessed in the rats is presented in Fig. 1. Rats receiving HF diets exhibited significantly higher blood glucose levels at 7, 14, 21, 28, 35, 42, 48 and 60 days compared with control diets. In S-HF rats, the glycemia was significantly reduced at 35, 42, 48 and 60 days of the experiment compared with C-HF rats. In addition, the values recorded in the S group were significantly reduced at days 48 and 60 compared with the C group. However, the results of blood glucose were closely comparable between the two protein groups on days 7, 14, 21 and 28 of the experimental period.

Metabolic alterations. Following 8 weeks on the HF diet, it was observed that plasma glucose was significantly increased as demonstrated by the data presented in Table IV. Plasma insulin levels were increased only in S-HF fed rats as compared with S fed rats. The HOMA-insulin resistance (HOMA-IR) index was observed to be higher in the HF groups compared with the control groups. Likewise, plasma GLP-1 levels were significantly reduced in the HF group compared with control rats. In addition, rats on HF diets had higher plasma

Table IV. Metabolic and hormonal data of experimental and control rats.

Variables	Diets				Analysis of variance (P-values)		
	C	S	C-HF	S-HF	Prot	Fru	Prot x Fru
Plasma D-Glucose (mmol/l)	5.96±0.30	5.11±0.42	8.31±1.06 ^a	6.54±0.47 ^{ab}	NS	P<0.05	P<0.05
Plasma insulin (μ U/ml)	53.59±2.62	32.98±8.33 ^a	66.65±5.97	43.22±12.25 ^{ab}	P<0.05	P<0.05	P<0.05
HOMA-IR	14.88±7.51	7.74±2.31 ^a	21.94±5.43 ^b	12.70±4.46 ^{ab}	P<0.05	P<0.05	P<0.05
Plasma fructose (mmol/l)	0.80±0.12	0.49±0.09 ^a	1.00±0.17 ^b	0.83±0.14 ^{ab}	P<0.05	P<0.05	P<0.05
Plasma GLP-1 (pg/ml)	13.64±2.44	14.03±3.30 ^a	8.26±2.54 ^b	10.63±2.48 ^{ab}	P<0.05	P<0.05	P<0.05
Plasma creatinine (mg/dl)	0.44±0.05	0.33±0.07 ^a	0.68±0.18 ^b	0.43±0.06 ^{ab}	P<0.05	P<0.05	P<0.05
Plasma uric acid (mg/dl)	3.72±1.10	2.94±0.75 ^a	9.24±1.94 ^b	7.21±2.19 ^{ab}	P<0.05	P<0.05	P<0.05
Urinary albumin (g/dl)	109.22±15.18	78.09±16.24 ^a	178.60±14.21 ^b	149.43±5.60 ^{ab}	P<0.05	P<0.05	P<0.05
Urinary creatinine (mg/dl)	12.34±1.43	10.23±2.13	21.17±0.63 ^b	14.04±2.52 ^{ab}	NS	P<0.05	P<0.05
Urinary uric acid (mg/dl)	23.02±2.80	16.45±1.33 ^a	33.03±5.71 ^b	29.10±5.37 ^b	P<0.05	P<0.05	NS

Values are presented as the mean \pm standard deviation. *P<0.05, sardine protein vs. casein; ^{ab}P<0.05, fructose supplementation vs. no supplementation. C, casein; S, sardine protein; HF, high-fructose; Prot, protein; Fru, high-fructose; HOMA-IR, homeostasis model assessment insulin resistance index; GLP-1, glucagon-like peptide-1; NS, not significant.

fructose levels than those fed control diets. Plasma and urine creatinine and uric acid, and urinary albumin concentration were significantly higher (P<0.05) in rats fed the HF diet than in those on the C diet. In the rats fed the HF diet, it was observed that the S-HF diet, compared with the C-HF diet, attenuated the rise in plasma glucose (21%), insulin (35%), HOMA-IR (42%) and fructose (17%), and increased the GLP-1 (29%) level. Consumption of the S diet significantly reduced plasma insulin, fructose and HOMA-IR compared with the C diet. Rats fed the S-HF diet exhibited reduced plasma levels of creatinine (37%) and uric acid (22%), and urine levels of creatinine (34%) and albumin concentration (16%) compared with rats fed the C-HF diet. In addition, the S diet reduced plasma levels of creatinine (25%) and uric acid (21%), and urinary levels of uric acid (29%) and albumin concentration (29%) compared with the C diet.

Tissues lipid and protein oxidation and nitric oxide levels. Table V presents the status of oxidative stress parameters in the tissues of experimental and control rats. Addition of fructose to the protein diets resulted in oxidative stress that was demonstrated by the increase in TBARS in the heart and liver tissues compared with control diets. In the kidneys, the concentration of TBARS was significantly increased in rats fed the C-HF diet compared with rats fed the C-diet. Higher hydroperoxide and protein carbonyl levels, however reduced NO contents in all tissues were observed in all HF-fed rats compared with control rats. Additionally, following the treatment with sardine protein (S-HF group), hydroperoxide concentrations in the liver (31%), heart (16%), kidney (11%) and muscle (19%) tissues were significantly reduced compared with the C-HF group. In addition, the S group rats presented with low hydroperoxide (25%) levels in the liver tissue. Protein carbonyl and NO contents were significantly reduced in the liver, kidney and heart tissues of S-HF fed rats compared with C-HF fed rats. Feeding rats the S diet led to reduced carbonyls in the heart and reduced NO concentrations in the kidney and heart compared with the C diet.

Antioxidant enzymatic activities in the tissues. Following 8 weeks of feeding, the diets supplemented with high dietary fructose induced a significant reduction in the activity of SOD, CAT and GSH-Px in all tissues in comparison with the control diets (Fig. 2) (33). Feeding rats the S diet resulted in an increase in liver SOD activity (21%) and in the CAT activity of the liver (13%) and muscle (28%) compared with the C diet. The administration of the S-HF diet to rats increased SOD activity in liver (31%) and kidney (14%) tissues compared with the C-HF diet. Furthermore, liver and muscle CAT activity was increased in S-HF fed rats by 28 and 48%, respectively, when compared with the C-HF fed rats. The activity of GSH-Px, which serves a role in peroxide removal, was significantly higher in the liver (31%), heart (51%) and muscle (24%) homogenates of S-HF fed rats than C-HF fed rats.

Liver ascorbic acid and α -tocopherol levels. A reduction in liver ascorbic acid and α -tocopherol levels was observed in the HF fed rats compared with the control diets (Table VI). Administration of sardine protein to rats with or without fruc-

Table V. Oxidative stress markers and nitric oxide levels in tissues of experimental and control rats.

Variables	Diets				Analysis of variance (P-values)		
	C	S	C-HF	S-HF	Prot	Fru	Prot x Fru
Liver							
TBARS ($\mu\text{mol/g}$)	0.88 \pm 0.03	0.81 \pm 0.09	0.92 \pm 0.08 ^b	0.91 \pm 0.09 ^b	NS	P<0.05	NS
LHP ($\mu\text{mol/g}$)	1.78 \pm 0.25	1.34 \pm 0.06 ^a	2.24 \pm 0.03 ^b	1.96 \pm 0.02 ^{ab}	P<0.05	P<0.05	P<0.05
Carbonyls (nmol/g)	78.02 \pm 6.01	67.30 \pm 1.35	237.92 \pm 7.80 ^b	200.43 \pm 11.84 ^{ab}	NS	P<0.05	P<0.05
NO ($\mu\text{mol/g}$)	606.00 \pm 73.99	452.75 \pm 92.47	358.14 \pm 29.87 ^b	295.81 \pm 34.04 ^{ab}	NS	P<0.05	P<0.05
Kidney							
TBARS ($\mu\text{mol/g}$)	0.57 \pm 0.05	0.60 \pm 0.09	0.75 \pm 0.04 ^b	0.69 \pm 0.07	NS	P<0.05	NS
LHP ($\mu\text{mol/g}$)	1.63 \pm 0.11	1.46 \pm 0.08	1.78 \pm 0.39 ^b	1.59 \pm 0.12 ^{ab}	NS	P<0.05	P<0.05
Carbonyls (nmol/g)	56.00 \pm 28.21	40.50 \pm 15.15	117.56 \pm 21.74 ^b	94.71 \pm 28.55 ^{ab}	NS	P<0.05	P<0.05
NO ($\mu\text{mol/g}$)	260.49 \pm 8.41	225.39 \pm 49.80 ^a	116.27 \pm 5.18 ^b	90.21 \pm 10.60 ^{ab}	P<0.05	P<0.05	P<0.05
Heart							
TBARS ($\mu\text{mol/g}$)	0.33 \pm 0.02	0.32 \pm 0.02	0.63 \pm 0.02 ^b	0.51 \pm 0.01 ^b	NS	P<0.05	NS
LHP ($\mu\text{mol/g}$)	1.40 \pm 0.22	1.37 \pm 0.32	2.19 \pm 0.45 ^b	1.84 \pm 0.25 ^{ab}	NS	P<0.05	P<0.05
Carbonyls (nmol/g)	88.84 \pm 14.51	67.91 \pm 16.82 ^a	148.90 \pm 29.67 ^b	126.69 \pm 12.96 ^{ab}	P<0.05	P<0.05	P<0.05
NO ($\mu\text{mol/g}$)	192.95 \pm 15.14	131.62 \pm 12.66 ^a	81.57 \pm 8.87 ^b	74.90 \pm 11.62 ^{ab}	P<0.05	P<0.05	P<0.05
Muscle							
TBARS ($\mu\text{mol/g}$)	0.18 \pm 0.04	0.18 \pm 0.02	0.22 \pm 0.01	0.21 \pm 0.03	NS	NS	NS
LHP ($\mu\text{mol/g}$)	0.33 \pm 0.02	0.32 \pm 0.02	0.63 \pm 0.02 ^b	0.51 \pm 0.01 ^{ab}	NS	P<0.05	P<0.05
Carbonyls (nmol/g)	127.21 \pm 6.28	123.58 \pm 5.66	187.04 \pm 6.55 ^b	166.23 \pm 15.01 ^b	NS	P<0.05	NS
NO ($\mu\text{mol/g}$)	123.96 \pm 4.80	116.14 \pm 4.87	85.59 \pm 9.72 ^b	75.55 \pm 12.05 ^b	NS	P<0.05	NS

Values are presented as the mean \pm standard deviation of six rats per group. ^aP<0.05, sardine protein vs. casein, ^bP<0.05, fructose supplementation vs. no supplementation. C, casein; S, sardine protein; HF, high-fructose; Prot, protein; Fru, high-fructose; TBARS, thiobarbituric acid reactive substances; LHP, lipid hydroperoxides; NO, nitric oxide; NS, not significant.

Table VI. Liver ascorbic acid and α -tocopherol levels of experimental and control rats.

Variables	Diets				Analysis of variance (P-values)		
	C	S	C-HF	S-HF	Prot	Fru	Prot x Fru
Ascorbic acid (mg/g)	1.09 \pm 0.05	1.2 \pm 0.08	0.67 \pm 0.07 ^b	0.81 \pm 0.08 ^{ab}	NS	P<0.05	P<0.05
α -tocopherol (mg/g)	0.63 \pm 0.010	0.70 \pm 0.03 ^a	0.40 \pm 0.04 ^b	0.45 \pm 0.06 ^b	P<0.05	P<0.05	NS

Values are presented as the mean \pm standard deviation of six rats per group. ^aP<0.05, sardine protein vs. casein, ^bP<0.05, fructose supplementation vs. no supplementation. C, casein; S, sardine protein; HF, high-fructose; Prot, protein; Fru, high-fructose; NS, not significant.

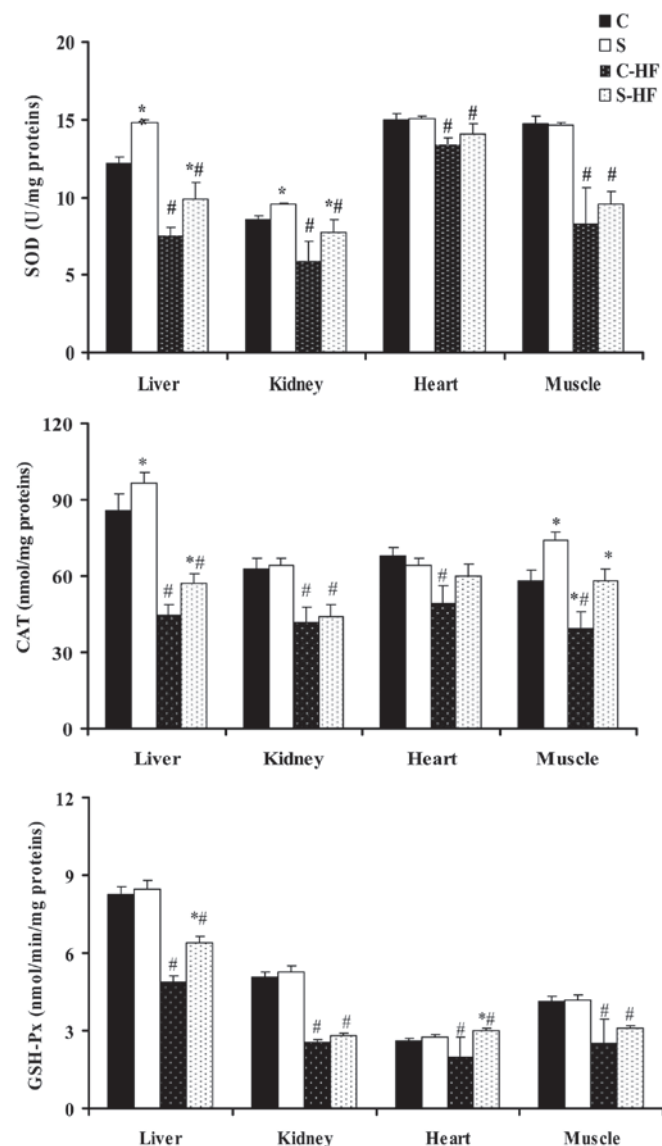


Figure 2. Activity of antioxidant enzymes in tissues of experimental and control rats. Values are presented as the mean \pm standard deviation of six rats per group. *P<0.05, sardine protein vs. casein, **P<0.05, fructose supplementation vs. no supplementation. SOD, superoxide dismutase; CAT, catalase; GSH-Px, glutathione peroxidase; C, casein; S, sardine protein; HF, high-fructose.

tose significantly increased levels of liver ascorbic acid relative to casein-fed rats. In addition, the levels of liver α -tocopherol were higher in the S group than in the C group.

Discussion

An HF diet favors the development of several metabolic alterations in the rat (3,34-36). The results from the current study indicate that the weight gain and liver, kidney, heart and muscle weights in the HF group were significantly greater than those of control groups, despite reduced energy intake, suggesting a low energy expenditure. These results are in line with a previous study (37). Intake of sardine protein suppressed HF-diet-mediated body, liver and muscle weight gain compared with casein, which is attributable in part to the satietogenic effects of sardine protein. According to Borzoei *et al* (38), fish protein may have a stronger satiating effect compared with beef and chicken protein. Alternatively, the reduced levels of essential amino acids including isoleucine, phenylalanine, tyrosine, valine and histidine, in sardine protein (Table I), may have been responsible for this low rate of growth. These results are consistent with those obtained in salmon protein-fed insulin resistant rats (39) and in spontaneously hypertensive rats fed with fish protein compared with those fed with casein (17).

In addition, these data imply that the reduction in weight gain, liver weight and muscle weight as a result of the sardine protein diet may be responsible for improving insulin sensitivity in HF fed rats. Rats assigned to an HF diet developed hyperglycemia, hyperinsulinemia and insulin resistance. Furthermore, this animal model exhibited significant elevations in blood glucose levels at all time points, compared with the control rats. The beneficial effect of sardine protein consumption in the HF group was demonstrated by a reduction in glycemia at all time points, a reduction in plasma D-glucose and insulin concentrations following overnight starvation, and a reduced HOMA index and plasma fructose. These observations are in agreement with previous studies on fish protein (15,40). It is widely recognized that certain bioactive agents (hormones) produced by the gastrointestinal system are able to modulate the secretory activities of the islets of Langerhans (41,42). The current study demonstrated that the GLP-1 response in the HF group was significantly reduced compared with the controls. Treatment with sardine protein attenuated the reduction in GLP-1 levels resulting from the HF diet, coinciding with reduced plasma glucose, insulin concentrations and reduced weight gain. Consequently, previous studies have identified GLP-1 to be highly effective in reducing blood glucose levels in patients with T2D (43-45), and GLP-1 has been reported to serve an important role in normalizing fasting hyperglycemia (46). Furthermore, GLP-1 acts as an

incretin to reduce blood glucose, via stimulation of insulin secretion from islet β cells, and in addition is able to inhibit gastric emptying and acid secretion, reducing food ingestion and glucagon secretion (47). Alternatively, this protein-dependent difference in insulin resistance may be attributed to the difference in the amino acid composition. Different amino acids appear to affect insulin secretion in different ways, with the branched-chain amino acids leucine, isoleucine and valine observed to increase insulin secretion more than other amino acids (48). In the sardine protein used in the present study and in cod and soy proteins, these amino acids are present in reduced amounts compared with casein (49). Increased levels of glycine, alanine and glutamic acid in sardine proteins may have a positive effect on glycemia, due to the fact that reduced glycemia in patients with T2D supplemented with alanine, glycine, aspartic and glutamine acids was observed by Natarajan Sulochana *et al* (50).

The current study also demonstrated that excess fructose consumption is closely associated with the increased levels of creatinine and uric acid in plasma and urine, and albuminuria as compared with control diets. In rats fed with the S-HF diet, reduced plasma creatinine (37%) and uric acid (22%) levels, and urine albumin (16%) and creatinine (34%) levels were observed compared with rats fed with the C-HF diet. This suggests that the sardine protein may slow or reverse the progression of established kidney disease and may protect against the development of kidney disease in fructose-induced MS in rats. In addition, the S diet group exhibited reduced plasma creatinine and uric acid levels, and urinary uric acid and albumin levels compared with the C diet group. Similar observations were reported in streptozotocin-induced type 1 diabetes rats fed sardine protein compared with those fed casein (16).

Fructose-induced hyperglycemia is able to increase reactive oxygen species (ROS), resulting in lipid peroxidation and the depletion of the antioxidant defense status in various tissues (51). In the present study, the tissue levels of TBARS, hydroperoxides and carbonyls were increased in HF rats, whilst the activity of antioxidants including SOD, CAT and GSH-Px were reduced in HF rats. These data demonstrate that HF treatment exhibited detrimental effects on antioxidant production and subsequently increased oxidative stress in rats. This accounts for the excessive production of superoxide anions and organic peroxides, and the increased utilization of scavenging free radicals. In addition, fructose itself enhances the formation of ROS *in vitro* (52). These observations are in agreement with previous studies (51,53). Taken together, these alterations reduced the cellular capacity to cope with oxidative stress and are responsible for the increase in biomarkers of oxidative damage. The increase in protein carbonyl content is indicative of oxidative damage in addition to chemical modification. The protein oxidation observations provide additional evidence for carbonyl stress, which arises from oxidative and/or non-oxidative reactions and leads to increased chemical modification of proteins (54). Exposure to the sardine protein diet counteracted the increase in oxidative stress in HF rats and maintained the activity of SOD, CAT and GSH-Px. This implies that fish protein is able to act as a protective agent against potential fructose-induced alterations in rats by attenuating lipid and protein oxidation, and enhancing the

antioxidant capacity of tissues. Similar results were observed in spontaneously hypertensive streptozotocin-induced diabetic rats fed with fish protein compared with those fed casein (55). In the liver, low levels of lipid peroxides were associated with increased activity of SOD, CAT and GSH-Px in S-HF fed rats. This suggests that the livers of S-HF rats exhibit an enhanced protective response to oxidative stress. The possible reduced accumulation of H_2O_2 in the liver as a result of increased activity of CAT in rats fed the S-HF diet is suggested to be explained by the increased glutathione levels stimulating the GSH-Px-mediated reduction of H_2O_2 and organic hydroperoxides. In addition, an increase in the levels of ascorbic acid in the liver of S-HF fed rats may be responsible for the reduced lipid peroxides. The lack of a difference in the CAT activity in the heart and kidney in the current study may imply that these organs are less vulnerable to diet-induced oxidative stress. The reduction in lipid peroxide levels in these organs may have resulted from an increase in SOD activity in the kidney and GSH-Px activity in the heart. These data suggest a reduced susceptibility to oxidative stress in fructose-fed rats. In muscles, however, the reduced lipid peroxidation in rats fed the S-HF diet compared with those fed the C-HF diet may be due to the enhancement of CAT and GSH-Px activities, which may be accounted for by the reduced accumulation of H_2O_2 . The current study demonstrates that the fructose-enriched diets reduced NO levels in tissues compared with control rats, suggesting endothelial dysfunction. These observations may be explained by reduced NO production, inactivation of NO by superoxide radicals, or to increased formation of peroxynitrite (ONOO-) leading to aggravation of cellular injury via membrane damage. This is in agreement with the studies by Sun *et al* (56) and Simão *et al* (57) in patients with MS. NO, which serves a critical role in maintaining normal endothelial function by causing vasodilatation (58), was reduced on treatment with sardine protein. The NO-reducing effect of sardine protein did not result from a reduced relative amount of the NO precursor, arginine, in the amino acid profile of sardine protein. However it may be associated with the reduction in blood pressure, as Ait-Yahia *et al* (17) reported low blood pressure in spontaneously hypertensive rats fed fish protein compared with those fed casein.

In conclusion, chronic fructose consumption leads to detrimental effects, including insulin resistance, metabolic disorder and oxidative stress. Administration of sardine protein was able to prevent and reverse the insulin resistance and oxidative stress induced by the HF diet. Overall, the current study may provide novel insights regarding future human and clinical nutritional approaches aimed at preventing or treating MS.

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