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Effect of rosmarinic acid on insulin sensitivity, glyoxalase system and oxidative events in liver of fructose-fed mice.

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Abstract

The study investigates the effects of rosmarinic acid (RA) on insulin sensitivity, protein glycation and oxidative events in fructose-fed mice, a model of insulin resistance (IR). Experiments were performed in four groups of animals administered either fructose diet or starch diet with and without RA administration. Insulin sensitivity indices were computed at the end of the treatment period. Redox homeostasis in liver was determined by assaying lipid peroxidative markers and antioxidants in the liver. Glyoxalase system and protein damage were assessed by assaying aldehydes, glyoxalase I and II, protein carbonyls, total thiols and nitrosothiols. Protein glycation was studied by measuring glycated hemoglobin, fructosamine and advanced glycation end products. Mitochondrial function was assessed by assaying succinate dehydrogenase and calcium ATPase. Fructose administration caused glycation of proteins, changes in metabolic parameters, inactivation of the glyoxalase system and depletion of antioxidants. Oxidative stress and reduced mitochondrial function were observed. Administration of RA to fructose-fed mice mitigated the above alterations. The data suggest that metabolic and redox disturbances in this dietary model of IR could be mitigated by RA. The antioxidant action of RA could be one of the contributing mechanisms for the improvement of insulin sensitivity.

Key words: *Antioxidants, fructose, glycation, glyoxalase, insulin resistance, rosmarinic acid.*

Introduction

Chronic changes in carbohydrate composition of the diet have an impact on intrahepatic milieu specifically by inducing adaptative changes in hepatic glucose metabolism that characterize obesity and type 2 diabetes. For instance, rats administered a high fructose diet develop impaired glucose tolerance and mild obesity secondary to a defect in insulin action[1]. Exposure of the liver to a high fructose load increases hepatic gluconeogenesis, reduces the ability of insulin to suppress hepatic glucose production, and stimulates lipogenesis and triglyceride accumulation. [2]

Studies have emphasized that fructose feeding also facilitates oxidative and nitrosative damage in the liver.[3, 4] Enhanced reactive oxygen species (ROS) production, a defect in nitric oxide (NO•) production and oxygen radical mediated NO• inactivation have been documented in rats fed a high fructose diet.[5,6] We have recently demonstrated the increased accumulation of nitrotyrosylated proteins in fructose-fed rat.[7]

The glyoxalase system consists of 2 enzymes, glyoxalase I and glyoxalase II. Glyoxalase I catalyses the formation of S-D-lactoylglutathione by the reaction between methylglyoxal (MG) and glutathione (GSH) while glyoxalase II catalyses the hydrolysis of S-D-lactoylglutathione to D-lactic acid and GSH. Glyoxal may be formed as degradation products of autoxidation, of glucose or from glucose adducts to proteins.[8] Glyoxal is more reactive than glucose and can react non-enzymatically with proteins forming crosslinks and adducts, the degradation of which can be associated with oxidative stress. Thus, the glyoxalase system converts the levels of toxic α-oxoaldehydes to nontoxic R-2-hydroxy acids. The physiological substrate of the glyoxalase system, MG, could be formed non-enzymatically from dihydroxyacetone phosphate and glyceraldehyde–3 phosphate. When fructose is consumed as the sole source of carbohydrate, there is increased flux through the glycolytic pathway and increased formation of intermediates of glycolysis and an increased production of oxoaldehydes could be expected upon fructose feeding.

Plant phenolics are multifunctional antioxidants and they might act at one or more steps in the oxidative stress cascade.[9] Rosmarinic acid (α-O- caffeoyl –3, 4 dihydroxy phenyllactic acid, RA) is a diphenolic derivative of caffeic acid, found as a secondary metabolite in many species of herbs and spices such as rosemary, salvia, sweet basil and mint that belong to the families of *Boraginaceae* or *Laminaceae.* These plants are widely used as culinary herbs, especially in Mediterranean dishes and have long been used in traditional medicine in Southern Europe, Japan and India for the treatment of numerous maladies including diabetes.[10] RA has been shown to be a potent inhibitor of superoxide (O_2 •-) and nitric oxide synthases (NOS) and an effective

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protector against peroxinitrite-mediated tissue damage.[11] Though we know much about the antioxidant activity of RA, there is a lack of information on the effect of RA in combating oxidative stress in the insulin resistance state. In view of all the above, the present study was carried out to investigate the effect of RA on whole body insulin sensitivity, protein glycation, glyoxalase system and oxidative events in liver of mice fed a high fructose diet. In addition, the effect of RA on the utilization of glucose in diaphragm *in vitro* was studied in the presence and absence of insulin and reported.

Materials and methods

Chemicals

RA, MG, glutathione reductase, nitrate reductase and S-D-lactoylglutathione were purchased from the Sigma Chemical Company, St. Louis, MO, USA. All other chemicals used in this study were from Sisco Research Laboratories (P) Ltd, Mumbai, India. Deionized water was used in all analytical procedures.

Treatment and maintenance of animals

Adult (3 week old) Swiss albino male mice of body weight 25-30 g were obtained from the Central Animal House, Rajah Muthiah Medical College, Annamalai University. Animals were housed in polypropylene cages under controlled conditions on a 12h light/12h dark cycle. Animals received a standard pellet diet (Karnataka State Agro corporation Ltd. Agro Feeds division, Bangalore, India) and water, *ad libitum* for a period of one week. Animal handling and experimental procedures were approved and cleared by the Institutional Ethics Committee of Animal Care (IAEC), Rajah Muthiah Medical College, Annamalai University.

After acclimatization for a period of one week, the animals were divided into four groups consisting of six mice each and were maintained as follows:

Group 1 (6 mice): CON control animals received the control diet containing starch and tap water.

Group 2 (6 mice): FRU- fructose-fed animals received the fructose - rich diet and water.

Group 3 (6 mice): FRU+RA-these animals received the fructose diet and were administered RA (100mg / kg/ day) orally.

Group 4 (6 mice): CON+RA- received the control diet and were administrated RA (100mg/kg/day), orally.

Animals were maintained in the respective groups for 60 days. Food and water were provided *ad libitum* to the animals. Food intake, body weight and fluid intake were determined at regular intervals. The diet composition is given in Table -1. On the 59th day of experimental period, the mice were fasted overnight. Blood samples were collected by sinoocular puncture. Samples were again collected at 120 minutes after administration of glucose (2g /kg). The blood glucose concentration was quantitated using a kit from Agappe Diagnostics Pvt Ltd, Kerala, India. Plasma insulin was estimated using an ELISA kit (Accubind, Monobind Chemicals Ltd., CA, USA). Insulin resistance/sensitivity was assessed by computing indices namely, insulin sensitivity index ($ISI_{0,120}$)[12], homeostatic model assessment (HOMA)[13] and quantitative insulin check index (QUICKI)[14]. The formulae used are given below:

$$ISI_{0,120} = \frac{MCR}{\text{Log } MSI}$$

$$MCR = \frac{m}{MPG}$$

Where, MCR is Metabolic Clearance Rate,

MPG = mean plasma glucose, the mean of 0 and 120 min glucose values

MSI = Mean serum insulin (mU/L) calculated as the mean of the 0 and 120 min insulin values.

m= (75000 mg+ (0 min glucose – 120 min glucose x 0.19 x BW/ 120 min)

HOMA = Insulin (µU/L) x Glucose (mmol /L)/22.5

$$QUICKI = \frac{1}{\log(\text{Glucose mg / dL}) + \log(\text{Insulin µU / mL})}$$

On the 60th day of experimental period, the animals were put on overnight fast and sacrificed by cervical decapitation the next day under deep anesthesia with ketamine hydrochloride (35 mg/Kg). Blood was collected in tubes containing ethylene diaminetetraacetic acid (EDTA). The body was cut opened and liver tissue was excised, washed in ice cold saline and homogenate was prepared in cold 0.1 M phosphate buffer, pH 7.4 or HEPES buffer. Plasma was separated by centrifugation at 1500g for 10 minutes. Assays were done in whole blood, plasma, liver homogenate and liver mitochondria.

Biochemical Assays

Assay of glycated Hb (Hb A_1C), fructosamine and advanced glycated end products (AGE)

Glycated Hb was estimated by the method of Rao and Pattabiraman[15] and expressed as percentage of total Hb. Fructosamine was analyzed by the method Johnson et al.[16] In brief, solution mixture contained 0.1 mL plasma and 1mL of 0.25mM nitroblue tetrazolium in 0.1M sodium carbonate. The amadori product obtained from plasma protein glycation reduces NBT to a tetrazindyl radical NBT^+ that produces a dye with absorption maximum at 530nm.

Deoxymorpholino fructose was used as the standard. Plasma AGEs were assayed by the method of Yanagisawa et al.[17]

Assay of GSH and GSSG

GSH and GSSG were estimated in blood and in liver mitochondria by the method of Teitz.[18] GSH was determined in protein free supernatants by the yellow colour developed upon adding disodium hydrogen phosphate and 2-dithonitro benzoic acid (DTNB). Total GSH+GSSG was determined after the addition of N-ethyl maleimide to the sample and by the standard recycling method using glutathione reductase after 30 minutes. The absorbance was read at 412 nm after the addition of disodium hydrogen phosphate and DTNB. The GSSG concentration was calculated by subtracting GSH from total GSH+GSSG.

Assay of nitrite and nitrosothiols in liver

Total nitrite concentration, as an index of tissue NO level was determined by the method of Rock et al [19] using Griess reagent. Nitrate was reduced to nitrite by the addition of nitrate reductase. The samples were treated with, 70% sulphosalicylic acid. The sulphanilamide–diazonium salt was then reacted with N- (1-naphthyl) ethylenediamine (0.3%) to produce a chromophore, the color of which was read at 540nm. For nitrosothiol (RSNO) estimation, 500µL of 0.2% mercuric chloride and 1% sulphanilamide were added to 0.5mL of liver homogenate. This was then reacted with 500µl of 0.3% N- (1-naphthyl) ethylenediamine. Addition of mercuric chloride releases NO from RSNO to form nitrite that reacts with Griess reagent. After 10 minutes the colour was measured at 540 nm. S-nitrosoglutathione (GSNO) standard was prepared by mixing GSH and nitrite at concentrations of 100mM. Values are expressed as mmol of GSNO/mg protein.[20] Background absorbance of nitrite in tubes containing all solutions except mercuric chloride was subtracted from RSNO signal.

Assay of aldehydes, glyoxalase I and II activity

The concentration of aldehydes in liver was measured by a fluorescence method.[21] Aliquots of 1ml of homogenate were extracted with 6 ml of chloroform-methanol (2:1) and vortexed. The extract was mixed with 6 ml of water and centrifuged at 3000g for 5 minutes. To 2ml of the chloroform layer, 0.2 ml of methanol was added and the fluorescence intensity of the solution was measured at an excitation wavelength of 360 nm and an emission wavelength of 430 nm, using a Perkin-Elmer 512 double beam fluorescent spectrophotometer. Quinine sulphate (0.1µg/ml) in 0.1 M in sulphuric acid (H_2SO_4) was used as the standard. The concentration of aldehyde conjugates are given as µmol of quinine sulphate (QS) equivalent / g tissue. Glyoxalase I was assayed by measuring the rate of formation of S-D-lactoylglutathione. The assay mixture contained 7.9mM MG, 1mM GSH, and 14.6mM magnesium sulfate, and 182mM imidazole HCl, pH 7.0. After 5 minutes, 0.1 ml of sample (50 µg protein) was added and increase in absorption at 240 nm was measured and the activity was calculated using the co-efficient 2.86/mM/cm.[22] The enzyme activity is calculated as µmole/g / min of the product formed. One unit of the enzyme is defined as the amount of enzyme catalyzing the formation of 1µmol of S-D-lactoylglutathione/min/mg protein under the assay conditions. Glyoxalase II was assayed by measuring formation of GSH from S-D-lactoylglutathione.[23] The reaction was started by the addition 0.5 ml of 1.5mM S-D-lactoylglutathione to 0.1ml of sample and GSH formation was measured after 15 min by reaction with 0.75 mM DTNB. The activity was expressed as mmol GSH formed/min/mg protein.

Isolation of liver mitochondria and assay of enzymes

Liver mitochondria was isolated by differential centrifugation according to Johnson and Lordy.[24] About 500 mg of tissue was homogenized in 2ml of cold 0.25M sucrose, using glass homogenizer and made up to 5ml, cell debris was removed by filtration through cheese cloth. The pellet obtained when spun at 16,300 ×g for 20 minutes was used as the mitochondrial fraction. Isolated mitochondria were resuspended in 3ml of 0.1M phosphate buffer, pH 7.4. The mitochondrial purity was assessed by assaying the specific activity of succinate dehydrogenase. Protein determination in cell homogenate and mitochondria were carried out by the method Lowry et al.[25]

Determination of mitochondrial thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxide (LHP) levels

For TBARS measurement, the mitochondrial preparation was deproteinized with 10% trichloroacetic acid (TCA) and the precipitate was treated with thiobarbituric acid (TBA) at 90°c for 1hour. The pink color formed gave a measure of TBARS. 1, 1’ 3, 3’ -tetra methoxy propane was used as the standard and the concentration was expressed as µmol/mg protein.[26] LHP content was measured in methanol-extracted mitochondrial homogenate. To 0.2ml of aliquot of sample, 1.8ml of the reagent, which contained 90ml of methanol, 10ml of 250mM sulphuric acid, 88mg of butylated hydroxytoluene, 7.6mg of xylenol orange and 9.8mg of ferrous ammonium sulphate, was added. The colour developed was read at 560nm.[27]

Assay of protein carbonyls and total thiols

The levels of protein carbonyl (PC) groups and total thiols (TSH) were measured by the methods of Levine et al[28] and Sedlak and Lindsay[29] respectively. For PC, the protein-hydrazone derivative formed by addition of 2, 4 dinitro phenyl hydrazine to sample was precipitated with 20% TCA. The precipitate was washed thrice with ethanol-ethyl acetate (1:1) mixture and centrifuged again to repellet the precipitate. Guanidine-HCl was added to dissolve the precipitate and the absorbance was read at 320nm. For TSH, liver homogenate was treated with DTNB and made up to 10 ml with absolute methanol. The mixture was centrifuged at 3000g for 15 minutes. The absorbance of the clear supernatant was read at 412nm with GSH as the standard.

Measurement of antioxidants

Enzymatic antioxidants superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione S-transferase (GST) were assayed in liver. Non

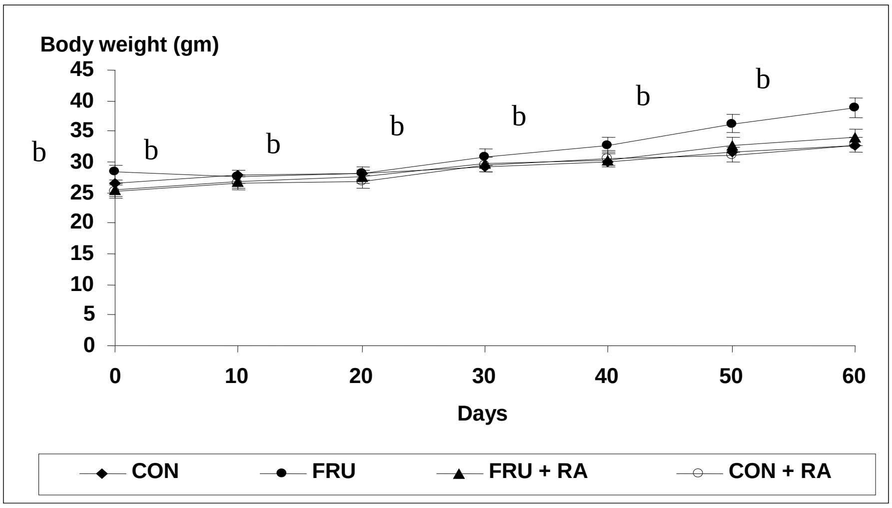

Figure 1: Effect of fructose and RA on body weight. The body weight of FRU group significantly increased ($p < 0.05$) as compared to CON. Body weight of RA treated fructose-fed mice increased but did not differ from body weight of the CON group ($p < 0.05$, $n = 6$). [b]Significant as compared to CON ($p < 0.05$, $n = 6$). (CON-control, FRU-fructose, FRU+RA-fructose + rosmarinic acid, CON + RA-control + rosmarinic acid). Values are mean ± SD of six experiments.

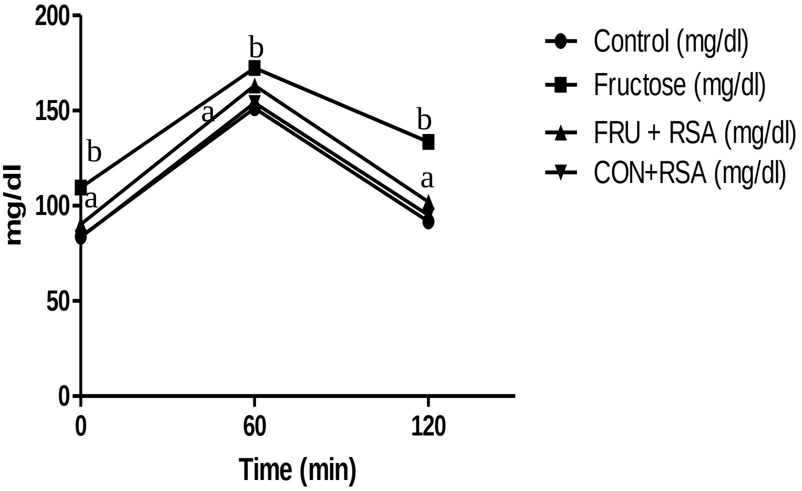

Figure 2: Plasma glucose concentrations in response to oral glucose load in control and experimental animals (means ± S.D, n=6). (CON-control, FRU-fructose, FRU+RA-fructose + rosmarinic acid, CON + RA-control + rosmarinic acid). Values are mean ± SD of six experiments. [b] - when compared with CON, $P < 0.05$; [a] - when compared with FRU, $P < 0.05$.

enzymatic antioxidants vitamin E and ascorbic acid were assayed in plasma and liver. The procedures for the above assays are given elsewhere.[30]

Uptake of glucose by rat diaphragm

Diaphragm was removed from normal control mice and glucose utilization in diaphragm was analyzed by the method of Haugaard and Haugaard.[31] Glucose utilization under basal conditions was determined in normal diaphragm and in the presence or absence of additives. The incubation mixture contained the following: 0.04 M sodium phosphate (pH 7.2), 0.005 M potassium chloride, 0.004 M magnesium chloride, 0.006 M glucose, 0.08 M sodium chloride with and without RA and/or insulin. The amount of glucose utilized was determined by measuring glucose levels in the medium after the incubation period (0min, 30min, 60min and 120min).

Statistical analysis

Values are expressed as means ± SD. Data within the groups are analyzed using one-way analysis of variance followed by Duncan's multiple range test. A value of $p<0.05$ was considered statistically significant

Results

Body weight changes

Figure1 illustrates the body weight changes observed in the animals during the course of the experimental period. As

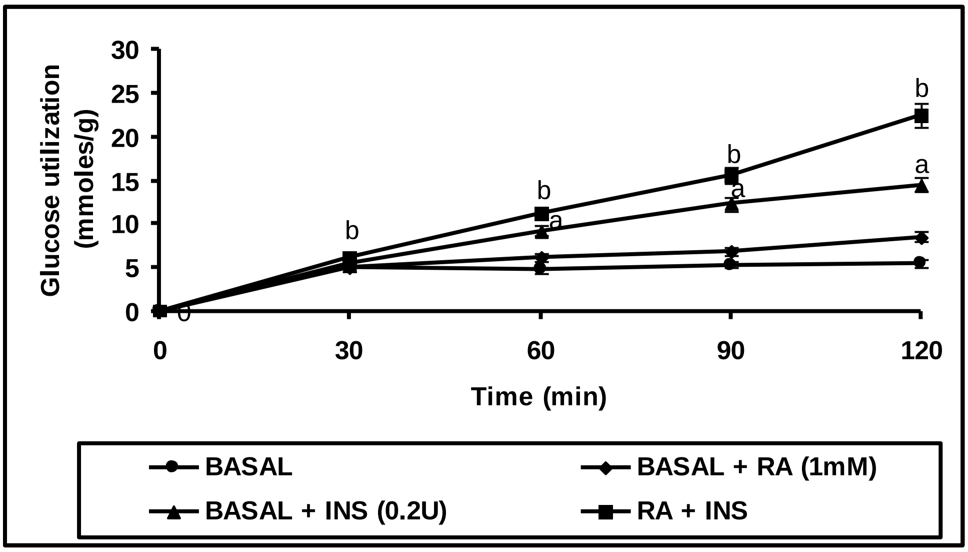

Figure 3: Effect RA on glucose utilization by the diaphragm in the presence and absence of insulin/RA with time. Both RA (1mM) and insulin (0.2U/mL) individually increased glucose utilization. When present together the effect was synergistic. (CON-control, RA-rosmarinic acid, INS-insulin). Values are mean ± SD of six experiments. (a-significant at $P<0.05$ when compared with CON; b-significant at $P<0.05$ when compared with CON).

shown in the figure the mean body weights of the animals progressively increased during the experimental period [final body weight (g) CON-32.6±3.21, FRU-38.8±3.7, FRU+RA-34.0±2.9, CON+RA-32.8±2.7]. However, mice fed fructose diet alone (FRU) became 19% heavier at the end of the experimental period. The mean final body weights were not significantly different from one another for the other groups.

Oral glucose tolerance test

Figure 2 gives the results of the oral glucose tolerance test in the experimental animal. The mean fasting glucose level was higher in the fructose- fed mice as compared to control mice. Significant elevations were observed in the glucose level at 60 and 120 min after the oral glucose load in the fructose fed mice. On the other hand, fasting glucose concentration was normal and significantly lower in fructose-fed mice treated with RA than the untreated fructose- fed mice. The response to oral glucose load was normal in RA treated CON and FRU mice. Area under curve, $\text{AUC}_{\text{glucose}}$ (mg/ml/min) for the experimental animals were CON=143.43±12.36; FRU=176.34 ± 14.65; FRU + RA=155.66 ± 10.32; CON + RA=146.02 ± 9.65; and $\text{AUC}_{\text{insulin}}$ (μU/mL/min) were CON=3702 ± 270.1; FRU=5603 ± 370.3; FRU + RA=4488 ± 225.3; CON + RA=3759 ± 186.6. Both $\text{AUC}_{\text{glucose}}$ and $\text{AUC}_{\text{insulin}}$ were significantly higher in fructose-fed mice as compared to that of control mice. RA supplementation to fructose-fed mice significantly reduced both $\text{AUC}_{\text{glucose}}$ and $\text{AUC}_{\text{insulin}}$ values.

Table 1: Composition of diet (g/ 100g)

Ingredients	Control diet	High-fructose diet
Corn starch	60	-
Fructose	-	60
Casein (fat free)	20	20
Methionine	0.7	0.7
Groundnut oil	5	5
Wheat bran	10.6	10.6
Salt mixture ♣	3.5	3.5
Vitamin mixture *	0.2	0.2

♣ The composition of mineral mix (g/kg) $MgSO_4$. $7H_2O$-30.5; NaCl -65.2; KCl - 105.7; KH_2PO_4-200.2; $MgCO_3$ - 3.65; Mg $(OH)_2$. $3H_2O$ - 38.8; $FeC_6H_5O_7.5H_2O$ - 40.0; $CaCO_3$-512.4; KI-0.8; NaF-09.$CuSO_4.5H_2O$-1.4; $MnSO_4$-0.4, and $CONH_3$-0.05.

*One kilogram of vitamin mix contained thiamine mononitrate, 3g; riboflavin, 3g; Pyridoxine HCl, 3.5g; nicotinamide, 15g;d-calcium pantothenate, 8g; folic acid, 1g; d- biotin, 0.1g; cyanocobalamin, 5 mg; Vitamin A acetate, 0.6g; α-tocopherol acetate, 25g, and choline chloride, 10g.

Glucose utilization in rat diaphragm

Glucose disposal by diaphragm was followed with time and the results are reported in Fig 3. RA significantly increased glucose utilization as compared to untreated mixture. For the first one hour glucose disposal was slow, and it excreted a stimulatory effect during the subsequent period of incubation. Thus RA was capable of maintaining the ability of the tissue to metabolize glucose. Insulin, however immediately increased glucose utilization. In the presence of both RA and insulin, the utilization of glucose was greater than when they were present alone.

Glucose, insulin and protein glycation

There was a significant elevation in the levels of glucose, insulin, fructosamine and glycated hemoglobin at the 60th day of fructose feeding. Co-treatment with RA reduced the levels of glucose, insulin, fructosamine and glycated hemoglobin to near normal values. The values did not differ significantly between CON and CON+RA (Table 2). Increased AGE - linked fluorescence was observed in plasma of fructose – fed rats. Treatment with RA reduced the levels of AGEs.

Table 2: Levels of glucose, insulin and fructosamine in plasma and glycated Hb in blood at end of the experimental period.

Parameters	Control	Fructose	Fructose+ RA	Control + RA
Glucose (mM)	4.65 ± 0.30	6.08 ± 0.45^a	5.01± 0.35^b	4.60 ± 0.35
Insulin (μU/ml)	18.6 ± 1.04	30.2 ± 2.5^a	19.8 ± 1.4^b	18.9 ± 1.5
Fructosamine (mmol/L)	0.76 ± 0.06	1.30 ± 1.0^a	0.84 ± 0.05^b	0.73 ± 0.07
Glycated Hb(% total Hb)	1.14 ± 0.09	2.3 ± 0.16^a	1.28 ± 0.09^b	1.16 ± 0.09
AGE (AU)	35.5 ± 2.3	65.2 ± 4.8^a	43.4 ± 3.2^b	34.1 ± 2.7

Values are means ± SD of 6 animals from each group. CON-control mice; FRU-fructose-fed mice; FRU+RA-Fructose-fed mice treated with RA; CON+RA-control mice treated with RA. AU – Arbitrary units,^{a} Significant as compared to CON (p<.05; ANOVA followed by DMRT), ^{b} Significant as compared to FRU (p<.05; ANOVA followed by DMRT)

Table 3: Measures of insulin resistance/insulin sensitivity indices.

Parameters	Control	Fructose	Fructose+ RA	Control + RA
HOMA	3.84±0.38	8.2±0.79^a	4.42±0.43^b	3.86±0.28
ISI$_{0,120}$	106.06±0.16	69.4±0.09^a	94.9±0.08^b	106.1±0.005
QUICKI	0.313±0.004	0.284±0.005^a	0.307±0.005^b	0.314±0.003

Values are means ± SD of 6 animals from each group; CON-control mice; FRU-fructose-fed mice; FRU+RA-Fructose-fed mice treated with RA; CON+RA-control mice treated with RA;^{a}Significant as compared to CON (p<.05; ANOVA followed by DMRT);^{b}Significant as compared to FRU (p<.05; ANOVA followed by DMRT);HOMA-homeostatic model assessmentISI$_{0,120}$-insulin sensitivity index;QUICKI-quantitative insulin check index; Computations were done as given under materials and methods.

Table 4: Levels of lipid peroxidation indices, protein carbonyl and aldehydes in liver mitochondria and cytosol of experimental animals.

Parameters	Control	Fructose	Fructose+ RA	Control + RA
TBARS (nmol/mg mit. protein)	0.90 ± 0.05	2.11± 0.13^a	0.96 ± 0.04^b	0.87 ± 0.07
LHP(μmol/mg tissue)	0.91± 0.07	1.89 ± 0.12^a	1.01 ± 0.04^b	0.91 ± 0.07
Proteincarbonyl (μmol/mg protein)	1.24 ± 0.09	2.21 ± 0.18^a	1.37 ± 0.10^b	1.22 ± 0.09
Aldehyde (μmole of Q.S eq /g tissue)	0.85 ± 0.05	1.02 ± 0.09^a	0.92 ± 0.04^b	0.84 ± 0.05

Values are means ± SD of 6 animals from each group.;CON-control mice; FRU-fructose-fed mice; FRU+RA-Fructose-fed mice treated with RA; CON+RA-control mice treated with RA, Q.S eq- Quinine sulphate equivalent.;^{a} Significant as compared to CON (p<.05; ANOVA followed by DMRT); ^{b} Significant as compared to FRU (p<.05; ANOVA followed by DMRT)

Table 5: Concentration of reduced glutathione (GSH), oxidized glutathione (GSSG), vitamin E and vitamin C in plasma of experimental animals

Parameters	Control	Fructose	Fructose+ RA	Control + RA
GSH^A	341.5 ± 28.3	205.4 ± 17.6^a	315.8 ±25.8^b	342.5 ±27.5
GSSG^A	5.67 ± 0.36	2.86 ± 0.19^a	5.20 ± 0.46^b	5.60 ± 0.51
GSSG/GSH	0.017 ± 0.001	0.013 ± 0.001^a	0.016 ± 0.001^b	0.016 ± 0.001
Vitamin E^B	1.13 ± 0.07	0.30 ± 0.03^a	1.03 ± 0.06^b	1.12 ± 0.10
Vitamin C^B	1.15± 0.07	0.31± 0.01^a	1.09 ± 0.04^b	1.16 ± 0.07

Values are means ± SD of 6 animals from each group.; CON-control mice; FRU-fructose-fed mice; FRU+RA- fructose fed rats treated with RA; CON+RA-control mice treated with RA; A-μmol/L; B – mg/dL; ^{a}Significant as compared to CON (P<.05; ANOVA followed by DMRT); ^{b}Significant as compared to FRU (P<.05; ANOVA followed by DMRT)

Insulin sensitivity indices

HOMA, ISI and QUICKI values were significantly altered in FRU when compared to CON and the levels were close to normal, in RA treated HFD-fed mice (Table 3).

Oxidative stress markers

Table 4 gives the status of oxidative stress markers such as LHP, TBARS, PC and aldehydes in mitochondria. FRU group showed significantly higher levels of these parameters as compared to CON. In FRU+RA, the levels of these substances were significantly lowered (P<0.05) as compared to FRU.

Non-enzymatic antioxidants

Levels of non-enzymatic antioxidants in plasma of experimental animals are given in the Table 5. The levels were significantly lower in FRU than in CON. In FRU+RA the activities of non-enzymatic antioxidant levels were significantly higher as compared to untreated FRU. CON+RA showed increased antioxidant levels in these mice which however were not significant as compared to control.

Table 6: Activities of enzymatic antioxidants in liver of experimental animals.

Parameters	Control	Fructose	Fructose+ RA	Control + RA
SOD[A]	5.34 ± 0.43	3.76 ± 0.21[a]	4.93 ± 0.43[b]	5.33 ± 0.42
CAT [B]	50.8 ± 4.06	26.4 ± 2.1[a]	47.3 ± 3.3[b]	51.9 ± 4.5
GPx[C]	7.0 ± 0.44	5.13 ± 0.49[a]	6.57 ± 0.43[b]	7.1 ± 0.58
GST [D]	3.74 ± 0.18	1.88 ± 0.14[a]	3.54 ± 0.26[b]	3.76 ± 0.23

Values are means ± SD of 6 animals from each group.;CON-control mice; FRU-fructose-fed mice; FRU+RA-Fructose-fed mice treated with RA; CON+RA-control mice treated with RA.;[a] Significant as compared to CON (p< .05; ANOVA followed by DMRT);[b] Significant as compared to FRU (p< .05; ANOVA followed by DMRT);A- U/mg protein; B- µmoles of H_2O_2 consumed /min/mg protein; C- µg of GSH consumed /min/mg protein;D- µmoles of 1-chloro 2, 4-dinitrobenzene - GSH conjugate formed/min/mg protein.

Table 7: Activities of glyoxalase I and II and level of nitrosothiol and nitrite in liver of experimental animals.

Parameters	Control	Fructose	Fructose+ RA	Control + RA
Glyoxalase I (A)	13.9 ± 1.12	10.9 ± 0.8[a]	13.02 ± 1.11[b]	14.12 ± 1.3
Glyoxalase II (B)	3.51± 0.31	2.20 ± 0.17[a]	3.19 ± 0.24[b]	3.48 ± 0.28
Nitrosothiol (C)	34.1 ± 2.3	58.3 ± 4.8[a]	36.0 ± 2.8[b]	33.1± 3.0
Nitrite (D)	15.91 ± 1.17	6.89 ± 0.57[a]	14.72 ± 1.34[b]	15.46 ± 1.42

Values are means ± SD of 6 animals from each group; CON-control mice; FRU-fructose-fed mice; FRU+RA-Fructose-fed mice treated with RA; CON+RA-control mice treated with RA; [a] Significant as compared to CON (p<.05; ANOVA followed by DMRT);[b] Significant as compared to FRU (p<.05; ANOVA followed by DMRT); A - µmol/g/min; B - µg of GSH consumed/min/mg protein; C - mmol/mg protein; D - µmol/mg protein.

Table 8: Levels of non-enzymatic antioxidants, total thiols, succinate dehydrogenase and Ca2$^+$-ATPase in liver mitochondria of experimental animals.

Parameters	Control	Fructose	Fructose+ RA	Control + RA
GSH (mg/g tissue)	13.31 ± 1.16	5.61 ± 0.52[a]	12.1 ± 1.0[b]	13.41 ± 1.3
GSSG (µg/mg protein)	422.8 ± 39.6	278.55 ± 21.8[a]	391.4 ± 30.4[b]	416.8 ± 35.5
Vit E (µg/mg protein)	1.14 ± 0.09	0.28 ± 0.02[a]	1.05 ± 0.08[b]	1.11± 0.11
Vit C (µg/mg protein)	1.57± 0.13	0.32 ± 0.02[a]	1.43 ± 0.11[b]	1.60 ± 0.14
Total thiols (µg/mg protein)	7.67 ± 0.44	5.66 ± 0.30[a]	7.20 ± 0.71[b]	7.70 ± 0.53
SDH (A)	23.5 ± 1.84	13.6 ± 1.19[a]	22.8 ± 2.0[b]	23.7 ± 1.79
Ca^{2+}- ATPase (B)	0.273 ± 0.023	0.163 ± 0.028[a]	0.248 ± 0.014[b]	0.265 ± 0.022

Values are means ± SD of 6 animals from each group.; CON-control mice; FRU-fructose-fed mice; FRU+RA-Fructose-fed mice treated with RA; CON+RA-control mice treated with RA.; [a] Significant as compared to CON (p<.05; ANOVA followed by DMRT); [b] Significant as compared to FRU (p<.05; ANOVA followed by DMRT); A- µ mol of succinate oxidized / min/ mg protein; B- µ mol of Pi liberated / min /mg protein.

Enzymatic antioxidants

Table 6 shows the activities of enzymatic antioxidants in liver of experimental animals. The activities of SOD, CAT, GPX and GST were significantly decreased by 30%, 45%, 20% and 50% respectively in the fructose-fed animals as compared to control. In FRU+RA, the activities returned back to near normal. The values did not differ significantly between CON and CON+RA.

Glyoxalase I and II

Levels of glyoxalase I and II, nitrosothiol and nitrite in liver of experimental animals are shown in the Table 7. Significantly higher levels of nitrosothiol and lower levels of glyoxalase I and II and nitrite were observed in fructose fed mice. RA administration brought the activities of glyoxalase I and II, and levels of nitrosothiol and nitrite to near normal.

Mitochondrial assays

Concentration of TSH, mitochondrial succinate dehydrogenase and calcium ATPase were significantly lower in fructose-fed mice, as compared to control (Table 8). In CON+RA and FRU+RA groups, it was observed that the levels were similar to CON group.

Discussion

Fructose consumption is associated with the development of insulin resistance in both humans and animals and is well documented in the literature.[2,32] Aberrations in post-receptor events and defects in cellular actions of insulin have been known to occur in fructose-fed animals.[33] The present study observed insulin resistance, hyperglycemia, hyperinsulinemia, increase in protein glycation and an exaggerated response to glucose challenge. Oxidative stress was evidenced by a rise in products of lipid peroxidation and oxidative damage to proteins.

Increased TBARS and LHP levels in liver mitochondria suggest ROS formation and oxidative deterioration of lipids. The TBA test analyzes the end-products derived from hydroperoxide transformation, metabolism or decomposition while LHP measures the rate of initiation of lipid peroxidation and their decomposition to other products. ROS production could be enhanced during fructose feeding by well-described mechanisms like autooxidation and glycation due to hyperglycemia.[34] In addition, hyperinsulinemia, depletion of ATP due to increased catabolism of fructose, increased aldehyde formation and reduced generation of reducing equivalents could be the other contributing mechanisms.[35]

Increase in protein carbonyl and nitrosothiol content and reduction in thiols in fructose-fed rats suggest protein modification by oxidation and nitration. Oxidative damage of mitochondrial proteins can cause disturbances in mitochondrial energy production.[36] In our experiment we observed a decreased activity of complex II enzyme (succinate dehydrogenase) and Ca^{2+}ATPase in fructose-fed mice that might lead to loss of mitochondrial function. Ceriello et al[37] reported that protein modification through increased free radical generation could reduce insulin activity. Low levels of nitrite are observed in our study. Oshida et al[5] observed reduced plasma NO levels and suggested that NO donors can improve insulin sensitivity in fructose-fed rats. Further administration of L-NAME, an inhibitor of NOS can exaggerate the effects of fructose.[5]

GSH is a small tripeptide that exists in the reduced (GSH) and the oxidized (GSSG) forms. Under steady state conditions, cells maintain a resting level of GSSG /GSH known as the redox state. The cycling between GSH and GSSG serves to remove toxic metabolites and regeneration of antioxidants from their radical forms which protects cells from oxidative injury. Thus GSSG/GSH ratio is a commonly used marker for oxidative stress. Fructose treatment significantly lowered the levels of GSH and raised GSSG/GSH ratio indicating excessive oxidation. A reduced tissue GSH level (or increase in oxidized state) has been shown to be associated with diabetes.[38] Since GSH, has direct reaction with oxidants and ROS, depletion in GSH levels is likely to lead to less detoxification of various electrophiles and ROS that are responsible for oxidative damage to proteins and lipids.

Superoxide and H_2O_2 generated during reduction of molecular oxygen or other redox reactions, are catalytically removed by SOD and catalase respectively, to less toxic or non-toxic products. The decrease in the activity of catalase would lead to the accumulation of H_2O_2. GPX is involved in the degradation of H_2O_2 while GST removes toxic hydroperoxides utilizing GSH as their substrates. Reduction in antioxidant enzyme activities could be a consequence of hyperglycemia since these enzyme proteins are shown to be glycated or oxidatively modified upon exposure to glucose.[39]

Modulation of the glyoxalase system has been observed in fructose-fed mice on our study and during the onset of diabetes as well as in the development of clinical diabetic complications.[40] Patients with diabetic complications had a propensity to maintain relatively high levels of plasma glyoxal[41,42] and S-D-lactoylglutathione.[43] Increased formation of aldehydes in tissues has been evidenced and implicated in development of hypertension and secondary complications during prolonged administration of fructose.[44] MG is shown to lower the antioxidant status, leading to oxidative stress.[45]

The chemopreventive/medicinal effects of phenolic antioxidants against oxidative stress-mediated disorders are mostly ascribed to their free radical scavenging action, chelation of redox active metal ions, modulation of gene expression and interaction with the cell signaling pathways.[46] The physiological activities of these phytomedicines are found to be dependent on their amphiphilic characteristics and partial affinity for intracellular membrane systems.[47] Some of them have antidiabetic effects besides antioxidant function.

In this study, we evaluated the effects of RA in a mouse model of insulin resistance. RA has been reported to have potent antioxidant, anti-inflammatory and anticancer activities.[48] Cuvelier et al [49] suggested that the presence of CH=CH-COOH group in RA ensures greater efficiency than the COOH group found in other phenolics and the combination of two acid-phenols in RA leads to an increase in antioxidant efficacy. RA has been suggested to exert antidiabetic action, by its ability to inhibit α-glucosidase activity *in vitro*.[50] The insulin sensitivity effect however has been less well identified and this study is the first of its kind.

Interestingly, we found that the metabolic disturbances in this model could be reversed by treatment with RA. RA was effective in controlling fasting blood glucose and decreasing the hyperglycemia to near fasting levels after the glucose load. RA also increased glucose uptake by the isolated diaphragm. The study also observed that RA reduces protein glycation, oxidative events and the formation of oxidatively modified proteins and lipid peroxidation end products. Normalization of the glyoxalase system can be attributed to reduction in the formation of oxoaldehydes.

Repletion of GSH, the predominant, non-protein sulfhydryl compound by RA, may have an effect on insulin receptor gene activation. Efficient expression of insulin receptor gene requires certain transcription factors that are activated by GSH.[51] Oxidative stress may modulate these transcription factors that are sensitive to changes in the redox state of the cell. Thus, the insulin sensitivity effects of RA may be attributed to the restoration of intracellular redox/metabolic homeostasis and protein glycation. Furthermore, reduction in GSH may affect cell signaling.[52]

High fructose inflicts a metabolic burden on the hepatocyte that selectively increases the stress-sensitive pathways that ultimately reduce the insulin signaling cascade.[53] ROS has been suggested to be one factor that might activate certain stress-sensitive pathways, besides reactive aldehydes and lipid metabolites like ceramides.[54] It is a popular and well-established notion that incidences of IR and type 2 diabetes inversely correlate with tissue antioxidant enzyme activities.[3,55] Such studies imply the possible benefits of supplemental antioxidants in improving insulin sensitivity. We suggest that RA, as an antioxidant, may serve to reduce oxidative stress to promote insulin action in fructose-fed mice. Further studies on the intracellular molecular links between RA action on insulin signaling cascade are obviously necessary.

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