Original Article

# Suppression of Hepatic Oxidative Events and Regulation of Enos Expression in the Liver by Chrysin in High Fructose-fed Rats

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## Abstract

Rats fed high dietary fructose are documented to form an acquired model of insulin resistance leads to diabetes mellitus. The present work investigates the involvement of kinins in the effects of chrysin in high fructose-fed rats' leads to hypertensive conditions. Previous studies show that chrysin promotes insulin sensitivity in fructose-fed rats. The effects of chrysin on blood pressure, plasma glucose, insulin, and the insulin sensitivity index were determined. Male Wistar rats of body weight 160–180 g were fed either diet containing starch (60% carbohydrate) or fructose (60% fructose diet). From the 16th day of feeding, rats in each dietary group were divided into two, and treated or not with chrysin (25 mg/kg b.w/day). The increases in systolic blood pressure, hyperglycemia, and hyperinsulinemia were controlled by chrysin administration in fructose-fed rats. Fructose-fed rats had higher blood pressure and elevated plasma levels of glucose and insulin. After 45 days, oxidative and nitrosative damage and endothelial nitric oxide synthase (eNOS) expression and hepatocyte apoptosis were determined. To evaluate whether nitric oxide (NO) plays a role in chrysin action, insulin sensitivity indices, fasting plasma glucose and insulin were assessed in response to co-administration of High Fructose Fed Diet (HFFD) rats. Fructose feeding caused oxidative damage to proteins and lipids and resulted in reduced antioxidant status, eNOS expression and nitrite level. Treatment with chrysin altered all these parameters to levels not significantly different from control. Treatment with chrysin improved insulin sensitivity. However, reduced oxidative events with simultaneous increase in NO bioavailability may be involved in the insulin-sensitizing and cytoprotective effects of chrysin in fructose-fed rats.

Keywords: Blood pressure, chrysin, endothelial nitric oxide synthase, high fructose fed rats, nitric oxide

## Introduction

Rats fed high dosage of fructose in diet (60g/100g diet) form a useful model of the multi-metabolic syndrome or syndrome X, a clinical condition which involves a cluster of abnormalities such as insulin resistance, hyperinsulinemia, glucose intolerance, dyslipidemia and hypertension. The insulin resistance in fructose-fed rats is associated with the defects in insulin signaling pathways.[1] The sites of fructose-induced insulin resistance are documented to be the liver, skeletal muscle and adipose tissue.[2]

Nitric oxide (NO), a free radical, is produced in cells through nitric oxide synthase (NOS) reaction that oxidizes the terminal guanidine nitrogen of L-arginine and converts it to L-citrulline in the presence of oxygen and cofactors.[3] Effects of NO can be both protective and toxic to cells, depending on the nature of the NO-derived species, the surrounding intracellular milieu, and the cellular context. NO has potent vasodilatory,[4] anti-inflammatory[5] and anti proliferative effects.[6] As a beneficial metabolite, NO reacts with lipid peroxides and functions as an antioxidant.[7] However, at high concentrations, NO reacts with O2°- and forms a highly reactive species called peroxynitrite (ONOO ) that has deleterious consequences in cells.

Evidence suggests the existence of a link between insulin resistance (IR) and NO action. For example, among the three NOS isoforms, endothelial NOS (eNOS) regulates blood flow to insulin-sensitive tissues and its activity is impaired in insulin-resistant

individuals.[8] Mice with targeted disruption in eNOS genes develop IR.[9] Inhibition of NOS impairs microvascular recruitment and blunts muscle glucose uptake in response to insulin,[10] suggesting that insulin-mediated glucose disposal is NO dependent. The vasodilatory effect of insulin has also been reported to be dependent on eNOS-mediated NO production. Low physiologic concentrations of NO may play a role in the stimulation of glucose utilization by insulin.[11]

On the other hand, NO overproduction through induction of inducible NOS (iNOS), another isoform of NOS, in inflammatory conditions appears to inhibit insulin's metabolic actions. The expression of iNOS is induced during obesity[12] and diabetes[11] due to an increase in free fatty acids (FFA)[13] and glucose levels.[14] Increased NO production has been shown to down regulate insulin signaling by reducing the activation of PI3-kinase and PKB/Akt.[15]

Plant polyphenolic compounds the flavonoids consist of number of classes, as flavanols, flavones and flavans. A naturally occurring flavones, Chrysin (5, 7-dihydroxy flavones structure shown in Fig. 1) contained in flowers blue passion flower (Passiflora caerulea), Indian trumpet flower, as well as in edible of mushrooms,[16] honey and propolis.[17] At the same time it possess antioxidant capacity, anti-inflammatory activity, anti-allergic, anti-cancer, ant estrogenic, anxiolytic,[18] antihypertensive properties.[19] Chrysin having tyrosinase inhibitory activity, moderate aromatase inhibitory activity, and another important role are inhibits estradiol-induced DNA synthesis. C-iso-prenylated hydrophobic derivatives of chrysin are potential P-glycoprotein modulators in tumour cells.[20] The present study aimed to evaluate the effect of chrysin on NO, eNOS and iNOS proteins in the High Fructose Fed Diet (HFFD) rats against the control and unsupplemented groups.

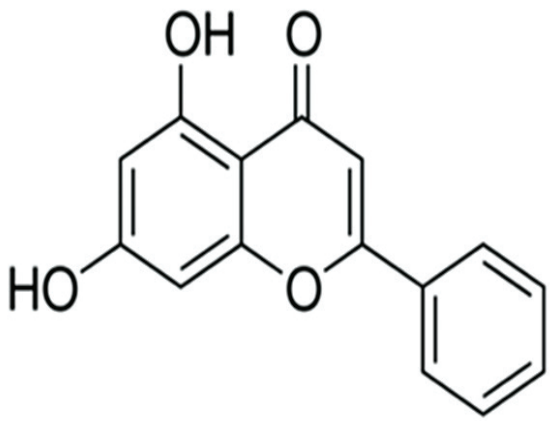


Figure 1: The different subgroups of flavones

## Materials and Methods

### Animals

Healthy male adult albino rats (Wistar strain) 6-7 weeks old, weighing 160-180g was procured from "Sri Venkateswara Enterprises", Bangalore, India. They were housed in a clean sterile polypropylene cages with proper aeration and lighting (12 ± 1 hr day / night rhythm) throughout the experimental period. During the course of the experiments, the temperature was maintained between 27°C ± 2°C. The animals were fed with commercially available pelleted rat feed (Gold-Mohur, M/S Hindustan Lever Ltd, Mumbai, India) during the acclimatization period and water ad libitum. The usage and handling of experimental rats was done according to the rules and regulations given by the Institutional Ethics Committee.

After one week of acclimatization the animals were divided into two batches. One batch was provided with a control diet containing starch as the source of carbohydrate and the other was fed a fructose-enriched diet for 45 days. They were fed either a control diet, containing 60% corn starch, 20% casein, 0.7% methionine, 5% groundnut oil, 10.6% wheat bran, 3.5% salt mixture and 0.2% vitamin mixture, or a high-fructose diet, which had the same composition as the control diet, except that corn starch was replaced with an equal amount of fructose. The total experimental duration was 45 days. Supplementation of chrysin (25mg kg-1 body weight) was given orally for the last 15 days of the experimental period. This dose selected based on our previous studies 19. The rats were divided into four groups and consisting of six rats each.

### Experimental Design

Group I : Normal control rats.

Group II : Control rats treated with chrysin (25 mg kg-1 body weight) twice daily for a period of last 15 days of the experimental period.

Group III : High Fructose fed rats (>60% fructose for 45 days).

Group IV : High Fructose fed rats treated with chrysin (25 mg kg-1 body weight) twice daily for last 15 days of the experimental period.

### Chemicals

Fructose, bovine serum albumin, glucose-6-phosphate, γ-glutamyl paranitroaniline, nicotinamide adenine dinucleotide ($NAD^+$, NADH), nicotinamide adenine dinucleotide phosphate ($NADP^+$, NADPH), reduced glutathione, oxidized glutathione, adenosine triphosphate (ATP), adenosine monophosphate (AMP) and 1,2,4-aminonapthol sulphonic acid were obtained from Sigma Chemical Company, ST. Louis, MO, USA.

All other chemicals and reagents used were of analytical grade with highest purity. They were obtained from Glaxo Laboratories, Mumbai, SD Fine Chemicals, Mumbai and Sisco Research Laboratories, Pvt. Ltd., India.

## Collection of Samples

At the end of experimental period, the rats were fasted overnight and killed by cervical decapitation under mild ether anesthesia. Blood was collected in heparinised tubes to separate the plasma. Liver tissue are immediately dissected out, washed in ice-cold saline to remove the adhering blood, dried, weighed accurately, frozen and stored at 80°C until use.

## Biochemical analysis

Blood pressure (BP) measurement was carried out in six animals from each group by the direct catheterization method.[21] Blood glucose was estimated by the method of Sasaki et al.,.[22] Plasma insulin was assayed by radioimmunoassay using a double-antibody technique.[23]

Plasma levels of advanced oxidation protein products (AOPP)[24] and the total antioxidant potential, ferric reducing ability of plasma (FRAP)[25], and the intracellular levels of ROS were measured.[26]

## Analysis of eNOS and iNOS proteins in liver

Proteins were extracted from 200 mg frozen liver samples. The sample was homogenized at 4° C in 2 mL of buffer containing 50 mM Tris–HCl pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM sodium chloride, 1 mM sodium vandate, and 10 mM of protease inhibitor cocktail and centrifuged (1000g for 10 min at 4°C). The supernatant obtained was again centrifuged (10,000g for 20 min at 4°C) and the pellet obtained was resuspended in homogenization buffer. After measuring protein concentration, samples containing 50 lg of protein were denatured and separated by 10% sodium dodecyl sulphate – poly acrylamide gel electrophoresis (SDS–PAGE). Proteins were then transferred onto a nitrocellulose membrane (Sigma–Aldrich, St. Louis, USA) using a transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol) at 80 mA for 1 h and 30 min. Nonspecific binding on membrane was blocked using buffer (5% skimmed milk powder in 0.5 M Tris-buffered saline containing 0.1% Tween-20 (TBS-T) at room temperature for 2-4 h and the membrane was probed overnight with gentle agitation at 4°C with either anti- eNOS (rabbit polyclonal; 1:700 dilution) or anti - iNOS (rabbit polyclonal; 1:200 dilution), antibodies diluted in TBS-T containing 3% bovine serum albumin (BSA). After washing with TBS-T for 10 min, the membranes were incubated for 2 h at 4°C with horse radish peroxidase (HRP)-conjugated anti-rabbit secondary antibody. For immuno-detection, the membrane was washed thrice with TBS-T buffer and incubated with enhanced chemi luminescence solution (ECL) as per manufacturer's specifications (Pierce ECL kit, USA) and exposed to a X-ray film. The protein bands were quantified by densitometry software (AlphaEase Fm software, Alphainnotech Co, Johanesburg, South Africa). Protein levels were normalized against β-actin. The data for each group is expressed as fold change relative to the control group considered as 1.0.

## Statistical analysis

All the grouped data were evaluated statistically, and the significance of changes caused by the treatment was determined using one-way analysis of variance followed by Duncan's Multiple Range Test by using SPSS version 20 for Windows. Results are presented as means 6 SD values of 6 rats from each group. The statistical significance was set at $P < 0.05$.

## Results

Table 1 show significant increased the levels of AOPP, intracellular ROS production, as well as decreased FRAP level were observed in HFFD rats as compared to control rats. Supplementation of chrysin in HFFD rats (group IV) the levels fructosa-

| Parameters | Control | Control+25 mg chysin | HFFD rats | HFFD rats+25 mg chrysin |
|---|---|---|---|---|
| Intra cellular ROS generation in liver (mean fluorescence intensity) | 353.72±3.04 [a] | 348.36±3.73[a] | 598.43±5.50[b] | 359.28±3.84[c] |
| AOPP (µmol/L) | 89.9 ± 6.28 [a] | 88.64 ± 6.04 [a] | 142 ± 11.8 [b] | 94.42 ± 6.36 [c] |
| FRAP plasma (µmol/L) | 926.5 ± 38.27 [a] | 925.3 ± 34.8 [a] | 595.4 ± 40.4 [b] | 922 ± 37.4 [c] |

Each value is mean ± S.D. for six rats in each group.
Values not sharing a common superscripts (a, b and c) differ significantly at $P < 0.05$ (DMRT).

Table 1: ROS, AOPP, and FRAP in plasma and heart tissue of experimental animals

mine, glycated hemoglobin, AOPP, intracellular ROS production and FRAP are reverse to near normal as compared to group III. But there is no significant difference between group I and II.

## Effects of chrysin on BP levels

Table 2 gives the BP values at the end of the 15th, 30th and 45th days of the experimental period. The SBP of fructose-fed rats was significantly higher than that of the controls. BP values were significantly lower in fructose-fed animals treated with chrysin as compared with fructose-fed rats. There was no significant alteration in BP values in control rats treated with chrysin as compared with control rats.

## Effects of chrysin on plasma glucose, insulin levels and Blood pressure measurement

Table 2 shows the levels of plasma glucose, insulin and blood pressure measurement of control and experimental animals. A significant increase in plasma glucose, insulin and blood pressure measurement were observed in fructose-fed rats. The levels were not significantly altered in control rats treated with chrysin as compared with those of control rats, whereas administration of chrysin to the fructose-fed rats controlled the hyperglycemia.

| Parameters | Control | Control+25 mg chysin | HFFD rats | HFFD rats+25 mg chrysin |
|---|---|---|---|---|
| Blood pressure (mmHg) 15th day | 94.53±3.38 [a] | 95.50±3.28 [a] | 152.26±5.07[b] | 99.34±3.49 [c] |
| 30th day | 95.6±5.37 [a] | 97.4±5.07[a] | 164.60±5.61[b] | 100.45±5.63 [c] |
| 45th day | 98.5±5.92 [a] | 98.83±5.54[a] | 178.84±5.72[b] | 104.65±6.35 [c] |
| Glucose (mg/dL) 15th day | 86.99±3.42[a] | 87.04±3.42[a] | 108.85±4.61[b] | 88.09±3.54[c] |
| 30th day | 88.82±5.83 [a] | 87.52±5.53[a] | 119.08±5.66[b] | 92.39±5.04[c] |
| 45th day | 88.67±5.27 [a] | 86.02±5.36[a] | 130.46±5.72[b] | 93.28±5.8[c] |
| Insulin (µU/mL) 15th day | 52.10±3.07[a] | 51.86±3. 13[a] | 87.49±4.10[b] | 54.38±3.34[c] |
| 30th day | 53.51±3.25[a] | 52.67±3.54[a] | 94.64±5.37 [b] | 56.34±3.22[c] |
| 45th day | 52.67±3.37[a] | 51.86±3.48[a] | 99.58±5.69[b] | 54.93±3. 18[c] |
| Angiotensin-converting enzyme (mU/mL) | 47.17±0.45[a] | 45.24±0.99[a] | 73.75±0.54[b] | 49.83±0.38[a] |

Table 2: Blood pressure and levels of glucose, insulin, and angiotensin-converting enzyme activity in plasma of control and experimental animals

Each value is mean ± S.D. for six rats in each group.
Values not sharing a common superscripts (a, b and c) differ significantly at P < 0.05 (DMRT).

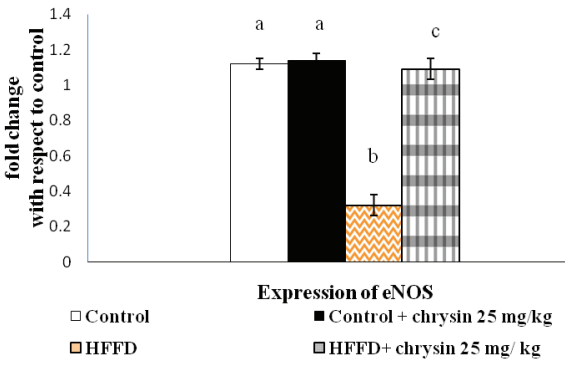


Figure 2: Effect of chrysin in expression of eNOS level in liver of experimental animals

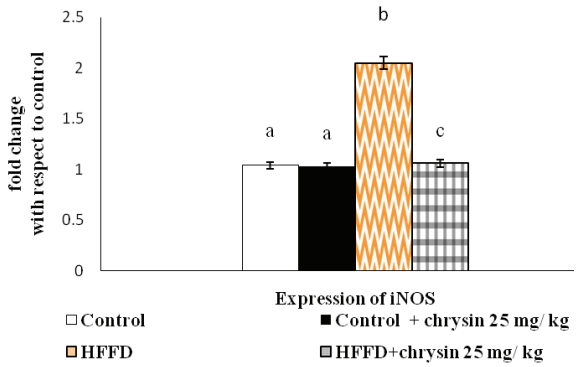


Figure 3: Effect of chrysin in expression of iNOS level in liver of experimental animals

Columns are mean ± SD of six rats from each group.
Columns not sharing a common superscripts (a, b and c) differ significantly at P < 0.05 (DMRT).

## Chrysin increases the expression of eNOS and decreases the expression of iNOS

Liver of experimental animals showed decreases expression of eNOS and increases expression of iNOS in HFFD rats as compared to control rats (Fig. 2,-3 respectively). Treatment with chrysin resulted in higher expression of eNOS and lower expression of iNOS when compared to chrysin-unsupplemented HFFD rats.

There are no changes between group I and II. Chrysin (25 mg/kg of B.W) is effective dose for all parameters significant effect in HFFD rats as compared to control rats. Chrysin in normal rats didn't show any significant.

## Discussion

In this study, we used the HFFD-rats model to investigate the disease protective effect of chrysin Fructose feeding induced a significant increase Reactive oxygen species (ROS), and Advanced oxidation protein products (AOPP). Excess ROS can cause cardiac dysfunction and failure via cellular damage by causing lipid peroxidation, protein oxidation, and nitration. ROS generation due to fructose consumption can be attributed to unregulated glycolytic pathway, depletion of ATP due to excess fructose metabolism, increased lipid levels and an increased flux through the Krebs cycle.[27] Consistent with previous reports,[28] fructose feeding elevated intracellular ROS and AOPP levels. AOPP indicates the extent of oxidative damage to circulating proteins especially albumin and is measured by reaction between plasma protein and chlorinated oxidants.[29] The total antioxidant power of plasma is assayed as a function of ferric reducing ability of plasma and is contributed by the non enzymic antioxidants of plasma.[26] ISRN Decline in the FRAP value is responsible for the elevated intracellular levels of ROS and plasma AOPP levels in treatment chrysin with HFFD rats. Chrysin being an antioxidant significantly reduced oxidative damage to proteins, thereby decreasing AOPP levels in circulation. Several studies have noted that chrysin is a potent antioxidant, and it has been shown to scavenge oxygen-derived free radicals in vitro at various concentrations. The total antioxidant capacity of animals measured by FRAP assay signifies its antioxidant power and cytoprotective effects.

The depletion of chrysin and its blood pressure-lowering effects have been reported in human and experimental hypertension19.[19] Chrysin depletion causes impairment of myocardial relaxation and adversely affects heart contractile ability and ion transport.[30] Chrysin lowers arterial pressure by promoting diuresis and vasodilation (our paper). Minute amounts of AII can cause an increase in mean arterial pressure and heart rate, accompanied by increased release of glutamate in spontaneously hypertensive rats (SHR). These changes were partially blocked by using chrysin, an antagonist of glutamate. Co administration of L-NAME caused a rise in BP and attenuated the antihypertensive effect of chrysin in fructose-fed rats. Acute or chronic administration of L-NAME to fructose rats[31] and normal rats can increase BP with renal deterioration. Chrysin increases NO secretion in Tokushima fatty rats, a model of spontaneous type 2 diabetes.

Treatment with chrysin prevented the hyperinsulinemia, hyperglycemia, and insulin resistance induced by high fructose feeding. Insulin resistance and the resultant hyperinsulinemia are documented to be important factors in BP elevation in this model. The potential role of increased insulin sensitivity in response to chrysin on BP reduction can also be suggested. In a previous study, we found that chrysin could improve insulin action and glucose metabolism in fructose-fed rats.[33] chrysin may contribute to increased insulin sensitivity in fructose-fed rats. A role for the kinin system in insulin action has been suggested in rats. Henrikson et al.,[34] reported that kinins could enhance insulin-stimulated glucose transport activity in the skeletal muscle of obese rats. NO can contribute to increased glucose utilization and glucose uptake in muscle and peripheral tissues. Oshida et al.,[35] reported that NO can improve insulin resistance induced by high-fructose feeding. Chrysin is having antihypertensive effects19 is already proved our previous studies as well as reduced the glucose metabolism in high fructose fed rats as compared to group III.

In our study, we have observed decline in NO levels and eNOS expression but increase in iNOS expression and chrysin levels in response to fat overload. ONOO- formation accounts for the decreased availability of NO followed by endothelial dysfunction. In the present study chrysin up regulated the expression of eNOS and NO bioavailability but reduced the levels of nitrosothiols in liver. Many researchers propose that nitrosative stress and iNOS mediated inflammation are involved in the development of IR. For instance, tyrosine nitration of insulin signaling proteins like Insulin receptor-b, IRS-1/-2, and Akt block tyrosine phosphorylation of IRS-1 and downstream signaling possibly through iNOS linked ONOO- formation.[36] Limitation of ONOO- formation may therefore be useful for suppressing nitrosative stress and thereby IR. In this aspect, we suggest that chrysin improves insulin sensitivity by down regulating iNOS expression through a reduction in nitrosative stress.

The expression of iNOS is increased during IR-associated conditions like obesity and diabetes due to excessive production of inducers like glucose, proinflammatory cytokines, FFA and ROS37. iNOS is highly expressed in liver of ob/ob mice which contributes to hyperglycemia and disruption in insulin signaling. Wan et al.,[38] reported that high fat feeding can also cause inflammation through up regulation of iNOS expression. Increased nitrosative stress, particularly protein S-nitrosylation, has been proposed to be involved in the pathogenesis of iNOS-mediated IR.[39] chrysin due to its anti-inflammatory properties might have decreased the expression of iNOS. Total and isoform selective NOS inhibitors are useful tools not only to study the role of NO in the pathophysiological process.

Increased oxidative stress in sugar-induced hypertensive rats may increase ACE activity, which in turn inhibits kininase activity and leads to decreased formation of kinins. The antioxidant action of chrysin may prevent the oxidation of ACE and its activity. Oxidative stress in sugar-induced hypertension is also shown to decrease NOS and NO levels. Again, chrysin may normalize NOS activity and NO levels through its antioxidant action. Reduced ACE activity could contribute to increased accumulation of kinins, eventually activating the kinin B2 receptor and enhancing NO and PG formation. This preliminary study suggests that chrysin may exert its BP-lowering effect and insulin-sensitizing actions and subsequent generation of NO and PGs in high-fructose-fed rats. Further investigations are needed to provide evidence for the implications of chrysin as a candidate in the management of IR, obesity and associated fatty liver disease.

## Acknowledgements

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## Conflict of interest statement

None declared

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