**Original Article**

# Metformin improves liver antioxidant potential in rats fed a high-fructose diet

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Increased lipid peroxidation plays a role in the pathology associated with fructose feeding. The present study reports the effects of metformin on the liver lipid peroxidation and antioxidant defence system of rats fed a high-fructose diet. The experimental animals were divided into two batches of 12 animals each. The control batch received a control diet containing 60% starch; the second batch was given a high-fructose diet containing 60% fructose as the sole source of carbohydrate. At the end of second week these were each subdivided into two groups. One was given metformin (50 mg/kg body weight/day in water) by intragastric intubation and the other group was left untreated. The rats were continued on the same dietary regimen for the next two weeks. After the experimental period of four weeks, liver lipid peroxidation and antioxidant status were quantified. Enhanced thiobarbituric acid-reactive substance reactivity and lipid hydroperoxides were observed in high-fructose-fed rats. However, the activities of enzymic antioxidants were lower in this group. Administration of metformin attenuated the rise in lipid peroxidation and improved the antioxidant potential in high-fructose-fed rats. Metformin did not have any effect on the antioxidant status of control rats. Attenuation of lipid peroxidation by metformin could be related to its insulin sensitising action.

**Key words: antioxidants, high-fructose diet, insulin, lipid peroxidation, metformin.**

## Introduction

Rats fed a high-fructose diet (>60% of total calories) provide a useful animal model of insulin resistance.[1–3] The sites of fructose-induced insulin resistance are documented to be the liver,[2] skeletal muscle[4] and adipose tissue.[5] The rats also develop a cluster of abnormalities, which include hypertension, hypertriglyceridaemia and glucose intolerance in addition to hyperinsulinaemia.[6]

Recently, the relationship between oxidative stress and insulin action has attracted many researchers. It has been suggested that oxidative stress can impair insulin action.[7,8] In a previous report we have demonstrated increased erythrocyte lipid peroxidation in high fructose-fed rats.[9] Further, supplementation of α-tocopherol, an antioxidant, improved antioxidant potential and insulin action in high-fructose-fed rats.[10]

Metformin, an insulin sensitiser, has become the established treatment for type 2 diabetes mellitus. Proposed mechanisms for the antihyperglycaemic effect include enhanced insulin-stimulated glucose uptake from the blood into the tissues, decreased glucose production in the liver by suppression of hepatic gluconeogenesis and decreased intestinal absorption of glucose.[11]

Based on these observations we initiated a study to see whether improved insulin action by metformin could improve antioxidant potential in a state of insulin resistance. We now report the effects of fructose feeding and metformin cotreatment on the liver antioxidant system in rats.

## Materials and methods

### *Animals and experimental diet*

Male adult Wistar rats weighing 170–190 g were obtained from the Central Animal House, Rajah Muthiah Medical College, Annamalai University, Annamalai Nagar, India. They were housed in the animal room under controlled conditions on a 12 h light/dark cycle. They all received a standard pellet diet (Karnataka State Agro Corporation, Agro Feeds Division, Bangalore, India) and water ad libitum. The animals used in the present study were cared for according to the principles and guidelines of the Ethical Committee of Animal Care of Annamalai University, which is in accordance with the Indian National Law on animal care and use.

After one week of acclimatisation 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 two weeks. The composition of the diets is given in Table 1. At the beginning of the third week, rats in each set were subdivided randomly into two groups and one group in each was administered with metformin (50 mg/kg body weight/day)

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by gastric intubation for a period of two weeks. The following experimental groups, consisting of six rats each, were maintained as follows:

***Group 1.*** Control animals (CON) received the control diet and tap water ad libitum for 4 weeks.

***Group 2.*** Fructose-fed animals (FRU) received the fructose-enriched diet and tap water ad libitum for 4 weeks.

***Group 3.*** Fructose-fed animals received the fructose diet and tap water ad libitum for 4 weeks. Metformin was given during the third and fourth weeks (FRU-MET).

***Group 4.*** Control animals received the control diet and tap water ad libitum for 4 weeks. Metformin was administered during the third and fourth weeks of the experimental period (CON-MET).

### *Biochemical analysis*

After the experimental period the animals were fasted overnight and killed by cervical decapitation. The body was cut open and liver was dissected out into ice-cold saline and then thoroughly rinsed. The tissue was cut into fragments and homogenised to a known volume of buffer using a Potter-Elvejham homogeniser. Aliquots from the homogenate were used for further studies.

### *Lipid peroxidation studies*

The level of lipid peroxidation in liver was studied by measuring the thiobarbituric acid-reactive substances (TBARS) in the liver homogenate by the method of Niehaus and Samuelsson,[12] and lipid hydroperoxides in methanol-extracted liver homogenates by the Fox assay described by Jiang *et al.*[13] For this assay, lipids were extracted from liver by the method of Folch *et al.*[14] The lipid extracts were resuspended in methanol and used for the assay.

**Table 1.** Composition of diets fed to rats for the determination of insulin resistance

| Ingredient (g/100 g) | Control diet | High-fructose diet |
|---|---|---|
| Corn starch | 60 | – |
| Fructose | – | 60 |
| Casein | 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 |

†Composition of the mineral mix (g/kg): $MgSO_4.7H_2O$, 30.5; NaCl, 65.2; KCl, 105.7; $KH_2PO_4$, 200.2; $3MgCO_3.Mg(OH)_2.3H_2O$, 38.8; $FeC_6H_5O_7.5H_2O$, 40.0; $CaCO_3$, 512.4; KI, 0.8; NaF, 0.9; $CuSO_4.5H_2O$, 1.4; $MnSO_4$, 0.4; and $CONH_3$, 0.05. ‡One kilogram of vitamin mix contained: thiamine mononitrate, 3 g; riboflavin, 3 g; pyridoxine HCl, 3.5; nicotinamide, 15 g; d-calcium pantothenate, 8 g; folic acid, 1 g; d-biotin, 0.1 g; cyanocobalamin, 5 mg; vitamin A acetate, 0.6 g; α-tocopherol acetate, 25 g; and choline chloride, 10 g.

### *Enzymic and nonenzymic antioxidants*

Reduced glutathione (GSH) levels were determined by the method of Ellman,[15] ascorbic acid levels by the method of Roe and Kuether,[16] and vitamin E levels by the method of Baker *et al.*[17] Superoxide dismutase (SOD; EC 1.15.1.1) was assayed by the method of Kakkar *et al.*,[18] catalase (CAT; EC 1.11.1.6) by that of Sinha,[19] and glutathione peroxidase (GPx; EC 1.11.1.9) by that of Rotruck *et al.*[20]

Superoxide dismutase was assayed based on inhibition of the formation of NADH-phenazine methosulphate-nitroblue tetrazolium formazon. The enzyme was prepared by the method of Bartosz *et al.*[21] The assay mixture contained 1.2 mL sodium pyrophosphate buffer (0.025 mol/L, pH 8.3), 0.1 mL phenazine metho sulphate (PMS) (186 µmol/L), 0.3 mL nitro blue tetrazolium (NBT) (300 µmol/L), 0.2 mL NADH (780 µmol/L) and an appropriately diluted enzyme preparation. A system devoid of enzyme served as control. One unit was taken as the amount of enzyme that gave 50% inhibition of NBT reduction/mg protein. CAT and GPx activities were assayed by measuring the amount of the substrate consumed (hydrogen peroxide and glutathione, respectively) after carrying out the reaction for a specified period of time.

### *Statistical analysis*

Results were analysed statistically by one-way analysis of variance (ANOVA) followed by Tukey's test. The level of significance was set at $P < 0.05$.

## Results

### *TBARS and Lipid hydroperoxides*

As seen in Table 2, there was a significant ($P < 0.05$) increase in the concentrations of TBARS and lipid hydroperoxides in liver of fructose-fed rats (Group 2) compared with those of control rats (Group 1). The concentrations were significantly lower in fructose-fed rats after treatment with metformin (Group 3). Metformin-treatment to control rats (Group 4) did not significantly alter the peroxidation indices.

### *Enzymic antioxidants*

The activities of the enzymes SOD, CAT and GPx in liver were significantly decreased ($P < 0.05$) in fructose-fed rats (Group 1) compared with the control rats (Table 3).

**Table 2.** Levels of thiobarbituric acid-reactive substances and lipid hydroperoxides in liver of control and experimental animals

| Parameters (nmol/mg tissue) | CON | FRU | FRU + MET | CON + MET |
|---|---|---|---|---|
| TBARS | 0.13 ± 0.01 | 0.19 ± 0.01[a,*] | 0.12 ± 0.01[b,*] | 0.14 ± 0.01NS[a] |
| Lipid hydroperoxide | 1.11 ± 0.03 | 1.22 ± 0.04[a,*] | 1.14 ± 0.04[b,*] | 1.06 ± 0.01NS[a] |

Values shown are mean ± SD ($n = 6$). Statistical significance (ANOVA followed by Tukey's test): [a]compared with CON, [b]compared with FRU, *significant at $P < 0.05$. CON, control animals; FRU, fructose-fed animals; NS, not significant; MET, metformin; TBARS, thiobarbituric acid-reactive substance.

Metformin-treated fructose-fed rats (Group 3) showed significantly higher activities of these enzymes compared with those of fructose-fed rats (Group 2). The activities remained unaltered in control rats treated with metformin (Group 4).

***Non-enzymic antioxidants***

The concentrations of the non-enzymic antioxidants (namely, GSH, vitamin C and vitamin E) in liver were significantly decreased ($P < 0.05$) in fructose-fed rats (Group 2) compared with the control rats (Group 1; Table 4). The fructose-fed rats, when treated with metformin (Group 3), showed significant elevation compared with untreated fructose-fed rats (Group 2). In control rats treated with metformin (Group 4), there were no significant alterations.

## Discussion

The development of hyperglycaemia, hypertriglyceridaemia, hyperinsulinaemia and insulin resistance in fructose-fed rats was reported previously by our laboratory.[9,22–25] Co-administration of metformin abolished the effects of fructose and these metabolic alterations were normalised.[25] The present study examines two indices of lipid peroxidation; the TBARS and the lipid hydroperoxides in liver tissue of control and experimental animals. Although the thiobarbituric acid (TBA) test is a very non-specific technique, it can offer an empirical window on the complex process of lipid peroxidation and is used widely as a marker.[26] The TBA test analyses the end products derived from hydroperoxide transformation, metabolism or decomposition. The Fox assay, on the other hand, directly measures the lipid peroxides. The assay is simple and sensitive and has an advantage over the TBA assay in that the confounding factors are eliminated by extraction or precipitation procedures.[13]

Increases in the levels of TBARS and hydroperoxides were observed in the liver of fructose-fed rats. Fructose feeding can induce free radical formation by a number of mechanisms. It causes downregulation of the key enzymes of the hexose monophosphate pathway, namely glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase that generate a reduced environment in the form of NADPH and NADH.[27] Impaired regeneration of NADPH could result in an increased oxidative state of the cell. Further, heightened catabolism of fructose would result in energy depletion in cells, making them more susceptible to peroxidation. In addition to this, hyperglycaemia, hypertriglyceridaemia and hyperinsulinaemia produced by fructose feeding can be related to increased lipid peroxide levels found in these rats.[7,28,29]

The non-enzymic and enzymic antioxidant defence potential in fructose-fed rats was weak and inadequate, which can be attributed to oxidative stress. Reactive oxygen species can themselves reduce the activity of antioxidant enzymes such as CAT and GPx.[30] Cu-Zn SOD activity is reported to play a key role in antioxidant defence mechanisms, particularly during hyperglycaemia and in states of insulin resistance. Cu-Zn SOD is inactivated by the glycation of specific lysine residues.[31]

Maintenance of ample concentrations of antioxidants seems to be necessary for efficient insulin action. For example, expression of the insulin resistance receptor gene requires certain transcription factors whose activity is modulated by GSH.[32] Vitamin E also has a beneficial effect on insulin action, as its supplementation could restore the GSH concentration in fructose-fed rats[10] and improve the physical state of plasma membrane and insulin action in non-insulin dependent diabetes mellitus patients.[33]

Metformin therapy to fructose-fed rats lowered the levels of TBARS and lipid hydroperoxides, indicating a decrease in lipid peroxidation. It is of interest to note that metformin improved red cell antioxidant activities in fructose-fed rats.[34] Furthermore, the imbalance between lipid peroxidation and

**Table 3.** Activities of superoxide dismutase, catalase and glutathione peroxidase in liver of control and experimental animals

| Parameters | CON | FRU | FRU + MET | CON + MET |
|---|---|---|---|---|
| SOD (U/mg protein) | 6.23 ± 0.57 | 4.62 ± 0.48[a,*] | 5.83 ± 0.43[b,*] | 6.69 ± 0.29NS,[a] |
| CAT (μmol of $H_2O_2$/min/mg protein) | 57.36 ± 1.72 | 52.76 ± 2.21[a,*] | 56.94 ± 2.26[b,*] | 59.47 ± 2.57NS[a] |
| GPx (μg/min/mg protein) | 9.20 ± 0.98 | 5.39 ± 0.43[a,*] | 6.92 ± 0.60[b,*] | 8.28 ± 0.20NS[a] |

Values shown are mean ± SD ($n = 6$). Statistical significance (ANOVA followed by Tukey's test): [a]compared with CON, [b]compared with FRU, *significant at $P < 0.05$. CAT, catalase; CON, control animals; FRU, fructose-fed animals; GPx, glutathione peroxidase; NS, not significant; MET, metformin; SOD, superoxide dismutase.

**Table 4.** Levels of non-enzymic antioxidants in liver of control and experimental animals

| Parameters | CON | FRU | FRU + MET | CON + MET |
|---|---|---|---|---|
| GSH (mg/g tissue) | 20.80 ± 1.43 | 18.30 ± 1.93[a,*] | 20.80 ± 1.01[b,*] | 22.2 ± 0.78NS[a] |
| Vitamin C (mg/g tissue) | 0.40 ± 0.04 | 0.25 ± 0.02[a,*] | 0.29 ± 0.02[b,*] | 0.45 ± 0.02NS[a] |
| Vitamin E (mg/g tissue) | 0.33 ± 0.01 | 0.25 ± 0.01[a,*] | 0.31 ± 0.01[b,*] | 0.34 ± 0.01NS[a] |

Values shown are mean ± SD ($n = 6$). Statistical significance (ANOVA followed by Tukey's test): [a]compared with CON, [b]compared with FRU, *significant at $P < 0.05$. CON, control animals; FRU, fructose-fed animals; GSH, reduced glutathione; NS, not significant; MET, metformin.

antioxidant defences in the erythrocytes of diabetic patients was normalised after metformin therapy.[35]

Faure *et al.* suggest that metformin has antioxidant activity in addition to its effect on insulin sensitivity as metformin improved the antioxidant defence in erythrocytes of normal rats. In our study, however, metformin treatment had no effect on the antioxidant parameters in liver of rats fed the control diet containing starch. Thus, the effects of metformin can possibly be attributed to its well-known insulin-sensitising actions (i.e. attenuation of hyperglycaemia, hyperinsulinaemia and hypertriglyeridaemia). Furthermore, the free radical scavenging properties of metformin could not be demonstrated under *in vitro* conditions either at low or high concentrations.[35] Based on this finding, we suggest that stimulation of antioxidant defences and attenuation of lipid peroxidation are attributable to the metabolic control offered by metformin. The beneficial effects of metformin on these parameters could be significant considering the therapeutic potential of the drug.

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