Insulin resistance induced by a high-fructose diet potentiates thioacetamide hepatotoxicity

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ABSTRACT

Introduction: Insulin resistance (IR) is recognised as an aetiopathogenic factor for a variety of liver diseases. This study investigated the susceptibility of the liver to the toxic actions of thioacetamide (TA) in a rat model of IR, induced by feeding the rats a high-fructose diet (60 g/100 g) for 30 days.

Methods: Hepatic function and damage were assessed at 0 hour and at 6, 12, 24 and 36 hours after a sublethal dose of TA (300 mg/kg intraperitoneally) was administered.

Results: After 30 days of fructose feeding, the rats showed IR, a decline in their liver antioxidant status and a rise in lipid peroxidation. Liver dysfunction in fructose-fed rats was evident from a rise in transaminase and total bilirubin, a decrease in the albumin/globulin ratio in plasma, a decrease in nitrite and arginase, and an increase in protein carbonyl and nitrosothiol content in the liver. Increased staining for the 3-nitrotyrosine antibody was observed in the fructose-fed rat livers as compared to the controls. TA (300 mg/kg) caused 80 percent mortality in fructose-fed rats within 48 hours, while no death occurred among the controls.

Conclusion: Fructose-fed rats suffered from liver dysfunction and damage. TA caused liver injury in both control and fructose-fed rats. Time-based studies showed that progressive liver injury occurred only in rats that were fructose-fed from 6, 12 and 24 hours after TA administration, with a peak at 36 hours. In control diet-fed rats, the extent of damage was maximal at 24 hours, and declined at 36 hours. Thus, the toxic effects of TA are potentiated due to compromised liver function in the setting of IR.

Keywords: CYP2E1, fructose, insulin resistance, mortality, thioacetamide

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INTRODUCTION

Insulin resistance (IR) represents a collection of disorders that include dyslipidaemia, obesity, impaired glucose tolerance and hypertension, and predisposes one to type 2 diabetes mellitus. IR is also associated with non-alcoholic fatty liver disease, including the more severe form, non-alcoholic steatohepatitis (NASH).[1] IR and post-prandial hyperinsulinaemia are considered as risk factors for the development of hepatic cell carcinoma and reduced long-term survival in patients with liver cirrhosis.[2] Hepatosteatosis has recently been included as one of the features of insulin resistance syndrome.[3]

A diet high in fructose (> 60/100 g) induces IR in animals, and rats that are fed a high dose of fructose are considered in forming a nutritional model for IR.[4] Fructose consumption has been shown to induce dyslipidaemia, low grade hepatic inflammation and the activation of stress-sensitive pathways in the liver. There is evidence for lipotoxicity in the livers of fructose-fed animals.[5]

Thioacetamide (TA) is a potent centrilobular hepatotoxicant that was used as a fungicide several decades ago. Its toxic effects have been attributed to the metabolic product that results from its bioactivation. TA undergoes a two-step bioactivation that is mediated by the microsomal cytochrome P450 isozyme (CYP2E1) to thioacetamide sulphoxide, and further, to a reactive metabolite, thioacetamide-S, S-dioxide.[6] Reactive intermediates in this pathway covalently bind to hepatic macromolecules and cause liver injury and centrilobular necrosis.[7] Hepatotoxins are widely used in animal models to induce acute liver damage.[8] There are no studies in insulin-resistant rodent models with regard to hepatic sensitivity to TA-mediated injury. Based on the above, the present study was designed to demonstrate liver impairment in fructose-fed insulin-resistant rats and to investigate whether IR increases the susceptibility of the liver to the acute hepatotoxic effects of TA.

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Table I. Levels of plasma glucose and insulin and homeostatic model assessment values of the experimental rats at the end of 30 days.

Parameter	CON	FRU
Glucose (mg/dl)	76.8 ± 4.5	152.8 ± 7.2[a]
Insulin (μU/ml)	45.0 ± 3.4	80.0 ± 6.2[a]
HOMA	8.5 ± 0.5	30.1 ± 2.1[a]

NB Values are mean ± standard deviation of four animals from each group. [a]Significant when compared to control rats at $p < 0.05$ (ANOVA followed by Duncan's multiple range test).
CON: control; FRU: fructose; HOMA: homeostatic model assessment.

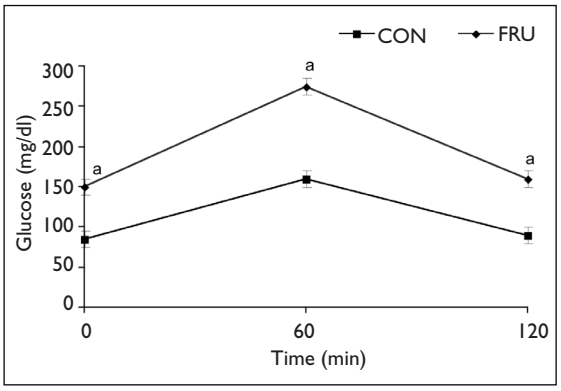


Fig. 1 Plasma glucose concentration levels in response to the oral glucose challenge in experimental rats. Values are mean ± standard deviation; n = 4. CON: control group; FRU: fructose-fed group; a: significant at $p < 0.05$ compared to CON; ANOVA followed by Duncan's multiple range test.

METHODS

The chemicals, including TA (99% pure) and solvents used in the present study, were of analytical grade and were purchased from the Sisco Research Laboratories Pvt Ltd, Mumbai, India. The kits that were used for glucose and insulin assays were obtained from Agappe Diagnostics Pvt Ltd, Kerala, India and Boehringer Mahnheim, Germany, respectively. Anti-3-nitrotyrosine (3-NT) antibody was obtained as a generous gift from Prof Dr Luke I Skweda, University of Oklahoma Health Sciences Centre, USA.

Adult male Wistar rats whose body weight ranged from 150–160 g were obtained from the Central Animal House, Rajah Muthiah Medical College and Hospital, Annamalai University, India. They were housed in an environmentally controlled room that was maintained at a temperature of 22°C ± 2°C and humidity 55% ± 5%, with a 12-hour light/dark cycle. The animals received a standard pellet diet (Karnataka State Agro Corporation, Bangalore, India) and tap water *ad libitum*. They were cared for according to the principles and guidelines of the Institutional Ethical Committee of Animal Care, Rajah Muthiah Medical College and Hospital, Annamalai University, and all treatment procedures were approved by the Committee.

After the acclimatisation period of one week, the animals were randomly divided into two groups (n = 20 in each group). 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. On Day 29, the body weight of each animal was recorded. Blood samples collected from the control (n = 4) and fructose-fed rats (n = 4) were used for analysis of glucose and insulin. Blood glucose and plasma insulin were assayed. An oral glucose tolerance test (OGTT) was performed,[9] and the OGTT curve was drawn using the glucose levels observed at various time points (0, 60 and 120 minutes). The homeostatic model assessment (HOMA) value as a measure of IR was calculated using the following formula: fasting insulin (μU/L) × fasting glucose (mmol/L)/22.5.[10] On the following day, the control and fructose-fed rats were administered TA (300 mg/kg in saline, intraperitoneally). The animals were then sacrificed by decapitation at different time intervals (6, 12, 24 and 36 hours, n = 4 in each group) after the administration of TA. A set of control and fructose-fed animals (n = 4 in each group) were sacrificed without TA administration (0 hour) in order to study the basal hepatocellular damage in fructose-fed rats. Blood samples and the livers were collected from four animals in each group for each time point. Plasma was separated by centrifugation and used for analysis. The liver tissues were washed in ice-cold saline, and a homogenate was prepared using 0.1M Tris-HCl buffer, pH 7.4 and used for biochemical assays.

Liver damage was evaluated by measuring the cellular enzymes in plasma. The aminotransferase, gamma glutamyl transferase (GGT), lactate dehydrogenase (LDH), total bilirubin, and the albumin/globulin (A/G) ratio were assayed using the standard analytical procedure.[11] Lipid peroxidation was determined by measuring the thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxides. The extent of protein damage was assessed by protein carbonyl assay in the plasma and liver. Nitrite and nitrosothiol as well as the activities of glyoxalases I and II were measured. The procedures have been outlined in another study.[12] The measurement of glutathione peroxidase (GPx), vitamin C, vitamin E and reduced glutathione by assay methods have also been outlined in a previous study.[13]

Table II. Levels of hepatic marker enzymes, total bilirubin, total protein and albumin/globulin ratio in the plasma of experimental rats.

Parameter	Group	Time (hr) after TA administration				
		0	6	12	24	36
AST (IU/L)	CON	77.1 ± 4.6	86.2 ± 6.6	133.5 ± 6.9	161.5 ± 10.5	95.6 ± 5.6†
	FRU	165.1 ± 7.0*	179.2 ± 6.0*	235.9 ± 9.1*	278.1 ± 10.9*	294.3 ± 17.2*
ALT (IU/L)	CON	72.2 ± 3.5	87.2 ± 6.4	141.0 ± 8.0	187.8 ± 18.1	100.2 ± 6.3†
	FRU	140.4 ± 8.2*	179.9 ± 9.4*	210.6 ± 9.5*	262.8 ± 10.4*	287.9 ± 12.9*
GGT (IU/L)	CON	2.4 ± 0.2	2.6 ± 0.2	4.1 ± 0.4	5.96 ± 0.4	3.1 ± 0.4†
	FRU	5.2 ± 0.2*	6.5 ± 0.5*	8.4 ± 0.4*	10.6 ± 0.4*	13.5 ± 1.02*
LDH (IU/L)	CON	318.3 ± 17.9	330.6 ± 16.2	350.1 ± 19.4	428.4 ± 25.6	339.1 ± 15.5†
	FRU	424.5 ± 18.2*	452.7 ± 22.6*	475.2 ± 23.7*	494.1 ± 24.8*	520.2 ± 30.4*
Total bilirubin (mg/dl)	CON	0.5 ± 0.04	0.7 ± 0.04	1.3 ± 0.2	1.6 ± 0.2	0.8 ± 0.08†
	FRU	1.02 ± 0.06*	1.2 ± 0.1*	2.07 ± 0.2*	2.9 ± 0.2*	3.6 ± 0.2*
Total protein (g/dl)	CON	7.8 ± 0.5	7.3 ± 0.4	6.9 ± 0.3	6.2 ± 0.3	6.4 ± 0.2†
	FRU	7.5 ± 0.4*	7.03 ± 0.4*	6.7 ± 0.4*	5.9 ± 0.3*	4.3 ± 0.2*
A/G ratio	CON	1.6 ± 0.08	1.5 ± 0. 2	1.3 ± 0.05	1.0 ± 0.06	1.4 ± 0.1†
	FRU	0.8 ± 0.03*	0.7 ± 0.06*	0.5 ± 0.04*	0.4 ± 0.03*	0.3 ± 0.03*

NB Values are mean ± standard deviation of four animals from each group. *Significant when compared to CON-treated rats at respective time periods. †Non-significant when compared to CON-treated rats at 0 hours at the level of $p < 0.05$ (ANOVA followed by Duncan's multiple range test).
TA: thioacetamide; CON: control group; FRU: fructose group; AST: aspartate transaminase; ALT: alanine transaminase; GGT: gamma glutamyl transferase; LDH: lactate dehydrogenase; A/G: albumin/globulin

Table III. Levels of TBARS, LHP and the activities of GPx in the plasma and liver of experimental animals.

Parameter	Group	Time (hr) after TA administration				
		0	6	12	24	36
Plasma TBARS (μmol/dl)	CON	2.4 ± 0.1	2.3 ± 0.1	2.7 ± 0.2	3.1 ± 0.2	2.4 ± 0.2[b]
	FRU	3.4 ± 0.2[a]	3.6 ± 0.2[a]	3.8 ± 0.3[a]	4.3 ± 0.3[a]	4.6 ± 0.3
LHP (mmol/dl)	CON	0.8 ± 0.05	0.9 ± 0.07	0.9 ± 0.06	1.1 ± 0.09	1.0 ± 0.08[b]
	FRU	1.3 ± 0.09[a]	1.5 ± 0.10[a]	1.6 ± 0.1[a]	1.6 ± 0.1[a]	1.8 ± 0.1
Liver TBARS (nmol/mg)	CON	1.5 ± 0.08	1.6 ± 0.1	1.7 ± 0.1	2.7 ± 0.2	1.6 ± 0.1[b]
	FRU	2.9 ± 0.2[a]	2.9 ± 0.2[a]	3.1 ± 0.2[a]	3.3 ± 0.2[a]	3.8 ± 0.3
LHP (nmol/mg protein)	CON	1.2 ± 0.07	1.1 ± 0.04	1.5 ± 0.1	1.8 ± 0.1	1.4 ± 0.1[b]
	FRU	2.1 ± 0.1[a]	2.2 ± 0.2[a]	2.4 ± 0.2[a]	2.6 ± 0.2[a]	3.4 ± 0.2
GPx (μmol of GSH utilised/min/mg protein)	CON	8.0 ± 0.5	8.2 ± 0.4	7.5 ± 0.4	6.0 ± 0.3	9.8 ± 0.5
	FRU	5.0 ± 0.3	5.1 ± 0.4	4.8 ± 0.3	3.2 ± 0.1	4.5 ± 0.3
GR (μmol of NADPH oxidised/hr/mg protein)	CON	36 ± 2.1	34 ± 2.1	28 ± 1.5	21 ± 2.0	33 ± 2.5
	FRU	19 ± 1.2	18 ± 1.2	15 ± 1.3	17 ± 1.2	13 ± 1.1

NB Values are mean ± standard deviation of four animals from each group. [a]Significant when compared to CON-treated rats at respective time periods. [b]Non-significant when compared to CON-treated rats at 0 hours at the level of $p < 0.05$ (ANOVA followed by Duncan's multiple range test).
TA: thioacetamide; CON: control group; FRU: fructose group; GPx: glutathione peroxidase; GR: glutathione reductase; NADPH: nicotinamide adenine dinucleotide phosphate; TBARS: thiobarbituric acid reactive substances; LHP: lipid hydroperoxides; GSH: reduced glutathione.

Arginase was measured in the liver homogenate.[14] For the lethality experiments, another set of controls (n = 10) and the fructose-fed (n = 10) animals were administered TA (300 mg/kg, intraperitoneally) and observed every 12 hours for 72 hours. The survival or mortality rate was recorded.

The liver tissue was washed in ice-cold normal saline, cut into small pieces and fixed immediately in 10% phosphate-buffered formalin. The liver sections (4–5 μm in thickness) were dehydrated in graded alcohol, embedded in paraffin and stained with haematoxylin and eosin (H&E) for histological examination. For the

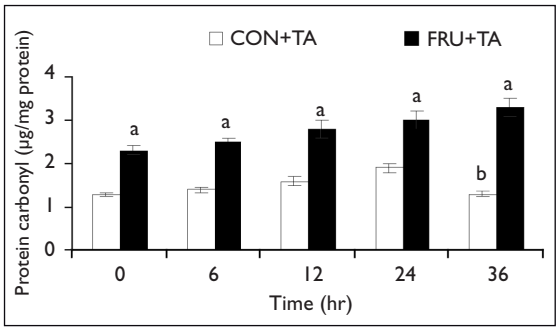


Fig. 2 Concentration levels of protein carbonyl in the liver of the experimental rats. Values are mean ± standard deviation (n = 4 in each group). a: significant as compared to CON + TA treated rats at respective time periods; b: non-significant as compared to CON + TA treated rats at 0 hours at $p < 0.05$ (ANOVA followed by Duncan's multiple range test). CON + TA: control + thioacetamide group; FRU + TA: fructose + thioacetamide group.

immunohistochemical localisation of nitrated proteins, serial sections were deparaffinised, rehydrated and incubated with the 3-NT polyclonal antibody at 4°C overnight according to the manufacturer's instructions. Sections were incubated overnight, with a 1:700 dilution of anti-3-NT antibody. Detection was done using a supersensitive polymer-horseradish peroxidase immunohistochemistry detection system (Biogenex Laboratories, San Ramon, CA, USA). After washing, the slides were incubated with diaminobenzidene substrate solution for 5 minutes. Sections were counterstained with haematoxylin and observed under light microscopy.

The values were stated as mean ± standard deviation. The differences between the groups were analysed using ANOVA, followed by Duncan's multiple range test. The level of statistical significance was set at $p < 0.05$.

RESULTS

An increase in the body weight of the animals was observed at the end of the experimental period. Fructose-fed animals (FRU) gained more weight than control animals (CON). The final body weight was: CON 180.14 ± 12.0 g and FRU 203.0 ± 13.1 g. The levels of plasma glucose and insulin as well as the values of HOMA in the control and fructose-fed rats are shown in Table I. The plasma glucose and insulin levels were significantly higher in the fructose-fed rats as compared to those in the control rats. IR in fructose-fed rats was indicated by higher HOMA values as compared to that in the control rats. Fig. 1 shows the results of the OGTT in the experimental animals. Significant elevations ($p < 0.05$) were observed in the glucose level at all time points in fructose-fed rats as compared to that in the control rats.

Table II shows the activities of aspartate transaminase, alanine transaminase and GGT, the levels of total bilirubin and the A/G ratio. Compared to the controls, fructose-fed rats registered a significant increase in enzyme activity at 0 hour, thus indicating liver dysfunction. Plasma enzyme activity increased in the control rats after TA administration up to 24 hours. After 36 hours, there was a decline in its activity. On the other hand, fructose-fed rats showed a time-dependent increase in enzyme activity from 6 hours onward, and this peaked at 36 hours. TA administration led to increased plasma bilirubin in control rats at 6 hours, further elevation at 24 hours and finally a decline at 36 hours to a near-control value. In fructose-fed rats, plasma bilirubin increased at 6 hours and peaked at 36 hours (3–4 mg/dl), which indicated liver dysfunction and injury. The plasma A/G ratio was reduced in both control and fructose-fed rats upon TA administration. The values were near-normal in control rats after 36 hours, while in fructose-fed rats, there was a decline in values at 36 hours.

Table III shows the levels of oxidative stress indices such as TBARS and lipid hydroperoxides. The levels were higher in fructose-fed rats than in control rats under basal conditions (0 hour). Upon TA administration, the values peaked at 24 hours in control rats and at 36 hours in fructose-fed diabetic rats. Here again, in control rats, the levels declined at 36 hours, while in fructose-fed rats, a further increase was observed after 36 hours. Fig. 2 shows the levels of protein carbonyl in the livers. The values were higher in fructose-fed rats than in control diet-fed rats at basal conditions and at all time points after TA administration. The levels declined in control rats at 36 hours while in fructose-fed rats, a further increase was observed after 36 hours.

The activities of the enzymatic antioxidants, such as GPx and glutathione reductase in the liver, are presented in Table III, while the levels of non-enzymatic antioxidants, such as vitamins C and E, and reduced glutathione in the plasma and liver of experimental animals, are shown in Table IV. A significant reduction was observed in the enzymic and non-enzymic antioxidant levels after TA administration in both the CON and FRU groups. Improvements in the antioxidants levels were seen in the control animals that were fed TA (CON + TA group) after 36 hours, while fructose-fed rats showed a progressive decline in the antioxidant status.

The nitrite levels decreased while the nitrosothiol levels increased in control and fructose-fed rats upon the administration of TA (Table V). These alterations were time-dependent. The changes progressed for 24 hours for control rats, and after 36 hours, the values were almost normal. On the other hand, in fructose-fed rats, the

Table IV. Levels of nitrite and nitrosothiol in the plasma and liver of the experimental rats.

Parameter	Group	Time (hr) after TA administration				
		0	6	12	24	36
Plasma						
Nitrite (μmol/L)	CON	15.1 ± 0.7	14.7 ± 0.3	12.9 ± 0.5	9.8 ± 0.4	13.5 ± 0.7†
	FRU	9.1 ± 0.5*	8.6 ± 0.4*	7.9 ± 0.7*	7.4 ± 0.5*	6.5 ± 0.4*
Nitrosothiol (nmol/ml)	CON	7.3 ± 0.4	7.9 ± 0.4	12.2 ± 0.6	15.6 ± 0.5	10.9 ± 0.6†
	FRU	15.8 ± 0.8*	16.3 ± 0.8*	18.8 ± 0.6*	22.9 ± 0.8*	29.4 ± 1.5*
Liver						
Nitrite (μmol/mg protein)	CON	16.2 ± 0.5	15.3 ± 0.8	13.2 ± 0.5	11.2 ± 0.4	15.4 ± 0.8†
	FRU	11.1 ± 0.6*	10.7 ± 0.6*	9.5 ± 0.6*	8.03 ± 0.5*	6.82 ± 0.3*
Nitrosothiol (μmol/mg protein)	CON	35.0 ± 2.3	41.1 ± 2.9	50.5 ± 3.8	66.3 ± 6.02	45.6 ± 2.3†
	FRU	62.1 ± 2.9*	65.0 ± 5.5*	74.0 ± 4.2*	95.2 ± 3.5*	117.2 ± 6.7*

NB Values are mean ± standard deviation of four animals from each group. *Significant when compared to CON-treated rats at respective time periods. † Non-significant when compared to CON-treated rats at 0 hours at the level of $p < 0.05$ (ANOVA followed by Duncan's multiple range test).
TA: thioacetamide; CON: control group; FRU: fructose group.

Table V. Levels of lactate dehydrogenase, arginase, cytochrome P450 isozyme (CYP2E1) and the glyoxalase system in the liver of the experimental rats.

Parameter	Group	Time (hr) after TA administration				
		0	6	12	24	36
LDH (IU/mg protein)	CON	15.3 ± 0.9	17.4 ± 1.5	20.6 ± 1.7	33.3 ± 2.5	18.5 ± 1.4†
	FRU	28.4 ± 1.9*	33.5 ± 2.9*	42.5 ± 1.8*	54.6 ± 2.3*	67.0 ± 3.5*
Arginase (μg of urea liberated/min/mg tissue)	CON	1012 ± 50.8	968 ± 49.8	883 ± 52.3	684 ± 36.5	795 ± 51.6†
	FRU	794 ± 48.5*	753 ± 40.1*	666 ± 38.6*	545 ± 30.8*	508 ± 25.4*
CYP2E1 (pmol of p-nitrophenol oxidised/mg protein/min)	CON	0.4 ± 0.03	0.8 ± 0.03	1.1 ± 0.8	1.6 ± 0.1	0.9 ± 0.04†
	FRU	0.9 ± 0.04*	1.5 ± 0.06*	2.05 ± 0.20*	2.6 ± 0.21*	2.9 ± 0.2*
Glyoxalase-I (μmol/min/mg protein)	CON	13.2 ± 0.8	12.3 ± 0.6	11.8 ± 0.6	9.04 ± 0.6	12.8 ± 0.6†
	FRU	8.4 ± 0.6*	7.8 ± 0.4*	6.5 ± 0.4*	6.03 ± 0.5*	5.5 ± 0.3*
Glyoxalase-II (μg of GSH consumed/min/mg protein)	CON	4.8 ± 0.4	4.6 ± 0.3	4.2 ± 0.4	4.06 ± 0.4	4.8 ± 0.2†
	FRU	3.4 ± 0.3*	3.3 ± 0.2*	2.9 ± 0.2*	2.7 ± 0.2*	2.4 ± 0.1*

NB Values are mean ± standard deviation of four animals from each group. *Significant when compared to CON + TA rats at respective time periods. † Non-significant when compared to CON-treated rats at 0 hours at the level of $p < 0.05$ (ANOVA followed by Duncan's multiple range test).
TA: thioacetamide; CON: control group; FRU: fructose group; LDH: lactate dehydrogenase; CYP2E1: cytochrome P450 isozyme.

alterations progressed for 36 hours. Table VI shows the decreased activity of arginase and glyoxalases I and II, and the induction of LDH and CYP2E1 enzymes in the liver after TA administration. These changes progressed for 24 hours for the control rats, after which there was a reversal. In fructose-fed rats, there was no reversal but a tendency toward deterioration.

Fig. 3a–d shows the H&E stained liver sections of the control and fructose-fed rats at 24 and 48 hours after TA administration. At 24 hours, the control rats showed hepatocyte dropout with minimal inflammatory cell infiltration (Fig. 3a), while fructose-treated rats showed macro- and micro-vesicular steatosis and necrosis around the portal triad as well as focal areas of the inflammatory cell infiltrate around the portal triad (Fig. 3b). After 48 hours, the control rat liver architecture appeared normal with mild sinusoidal congestion (Fig. 3c). Steatosis, necrosis and inflammatory cell infiltration were more pronounced after 48 hours, along with fat vacuole, the inflammatory cell infiltrate around the central vein extending up to the portal triad in fructose-fed rats (Fig. 3d).

Fig. 4a–d shows photomicrographs representing the accumulation of 3-NT protein adducts in the livers of

Table VI. Levels of vitamin C, vitamin E and reduced glutathione in the plasma and liver of the experimental animals.

Parameter	Group	Time (hr) after TA administration				
		0	6	12	24	36
Plasma						
Vitamin E	CON	28.1 ± 2.1	30.9 ± 2.1	23.7 ± 2.1	22.1 ± 1.2	33.4 ± 2.5[b]
	FRU	13.4 ± 1.1[a]	14.6 ± 1.2[a]	9.8 ± 0.5[a]	8.4 ± 0.3[a]	8.6 ± 0.3
Vitamin C	CON	150.8 ± 10.1	144.9 ± 9.2	141.1 ± 11.2	101.2 ± 8.5	143.6 ± 12.5[b]
	FRU	118.3 ± 9.1[a]	95.5 ± 7.1[a]	82.5 ± 6.1[a]	93.1 ± 8.1[a]	91.8 ± 9.2
GSH	CON	29.2 ± 2.1	31.5 ± 2.4	25.2 ± 1.1	24.4 ± 1.1	35.4 ± 2.2[b]
	FRU	11.3 ± 1.2[a]	17.6 ± 1.1[a]	10.8 ± 0.8[a]	9.1 ± 0.4[a]	9.9 ± 0.7
Liver						
Vitamin E	CON	20.4 ± 1.2	19.1 ± 1.2	19.3 ± 1.4	15.1 ± 1.1	19.4 ± 2.1[b]
	FRU	10.8 ± 0.7[a]	12.4 ± 1.1[a]	8.4 ± 0.4[a]	7.08 ± 0.3[a]	9.6 ± 0.6
Vitamin C	CON	53.5 ± 0.4	49.5 ± 3.7	42.9 ± 0.3	20.1 ± 1.4	51.4 ± 4.1
	FRU	31.2 ± 0.3	29.3 ± 1.2	17.9 ± 1.2	11.08 ± 0.4	9.12 ± 0.7
GSH	CON	112.2 ± 9.4	105.7 ± 8.1	95.6 ± 7.5	74.2 ± 4.2	89.48 ± 7.4
	FRU	65.4 ± 3.4	61.8 ± 4.2	51.3 ± 2.9	39.78 ± 2.1	35.18 ± 2.1

NB Values are mean ± standard deviation of four animals from each group. [a]Significant when compared to CON + TA rats at respective time periods. [b]Non-significant when compared to CON rats at 0 hours at the level of $p < 0.05$ (ANOVA followed by Duncan's multiple range test).
GSH: reduced glutathione; TA: thioacetamide; CON: control group; FRU: fructose group

Table VII. Mortality study.

Group	Total no. of rats	No. of dead rats		No. of rats that survived	Mortality (%)
		36 hr	48 hr		
CON + TA	10	-	-	10	0
FRU + TA	10	2	6	2	80

CON + TA: control + thioacetamide group; FRU + TA: fructose + thioacetamide group

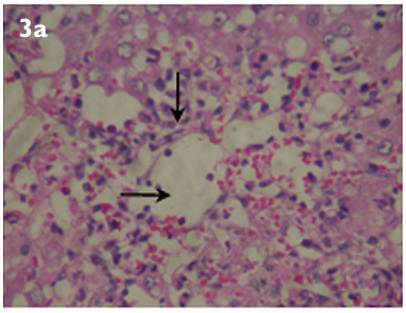
the experimental animals. Negative staining suggests no immunoreactivity for the 3-NT antibody in control rats treated with TA after 24 hours (Fig. 4a). The intensity of 3-NT staining was pronounced in fructose-fed rats treated with TA after 24 hours (Fig. 4b). Immunostaining was negative, even at 48 hours, in control rats treated with TA (Fig. 4c), while staining was more pronounced in fructose-fed rats treated with TA after 48 hours (Fig. 4d).

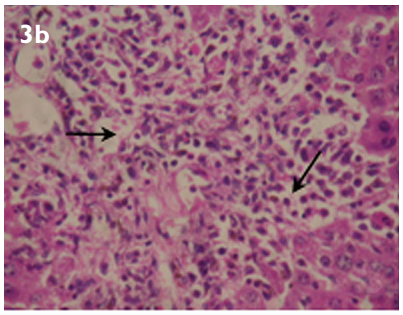
The administration of a normally non-lethal dose of TA (300 mg/kg, intraperitoneally) caused 80% mortality in high fructose-fed rats. Among the ten rats that were fed a fructose diet, eight died between 24 and 48 hours, two after 36 hours and six after 48 hours of TA administration. The remaining two rats were alive up to 72 hours later. The control diet-fed group showed a 100% survival rate for the same dosage (Table VII).

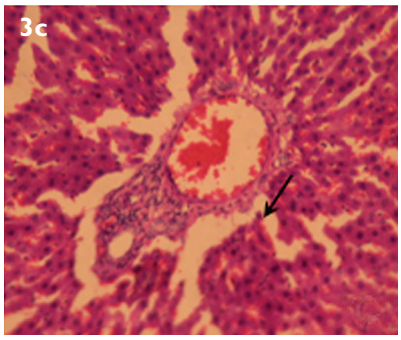
DISCUSSION

Measurements of liver function parameters before TA administration (0 hour) revealed that liver dysfunction occurred due to fructose-feeding, and this potentiated toxin-induced liver injury. Hepatic damage in fructose-fed rats was evident from the increased plasma transaminase, LDH and GGT as well as the decline in hepatic function from the elevated bilirubin level, the decreased A/G ratio and the altered arginase activity. These biochemical changes reflect hepatocellular damage in fructose-fed rats.

Fructose-fed rat livers exhibited oxidative stress, manifested by increased levels of TBARS, lipid hydroperoxides and protein carbonyl in fructose-fed rats. The development of oxidative stress is known to be an instigator of IR, and we observed an imbalance between the pro- and antioxidant status in fructose-fed rats. Enhanced lipid peroxidation in fructose-fed rats could be associated with the high circulating glucose, which enhances the production of free radicals from glucose auto-oxidation. A decrease in both enzymatic and non-enzymatic antioxidants was observed in fructose-fed rats. GPx is a selenium-containing enzyme, which catalyses the conversion of hydrogen peroxide and various hydroperoxides, using GSH as a reducing agent to form water and corresponding alcohols, respectively.[15] GSH, vitamins C and E are well-known antioxidants, all of which were diminished in the fructose-fed rats.







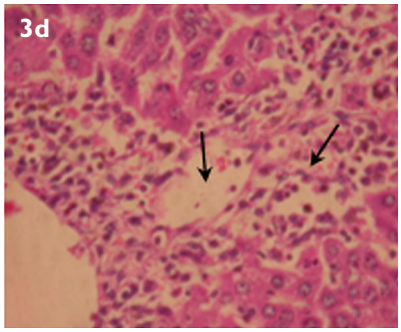
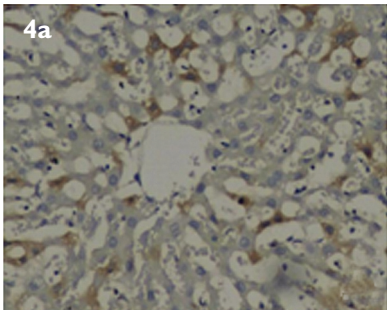
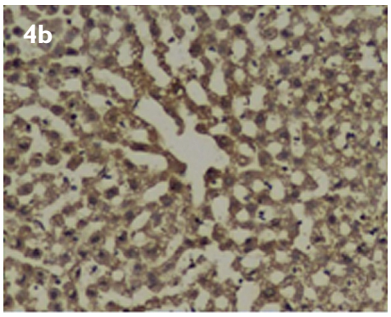
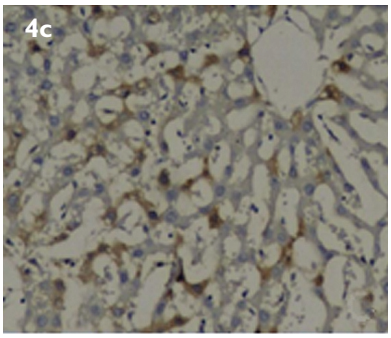


Fig. 3 Photomicrographs show (a) the liver of a control rat treated with thioacetamide (TA) after 24 hours. The arrows show hepatocyte dropout and an inflammatory cell infiltrate; (b) the liver of a fructose (FRU) + TA group rat after 24 hours. The arrows show macro- and micro-vesicular fatty change with cell infiltration around the portal triad; (c) the liver of a control (CON) + TA group rat after 48 hours. The arrow shows the congested sinusoids and normal hepatocytes; and (d) the liver of a FRU + TA group rat after 48 hours. The arrows show fat vacuoles with inflammatory cell infiltration around the central vein (Haematoxylin & eosin, × 20).







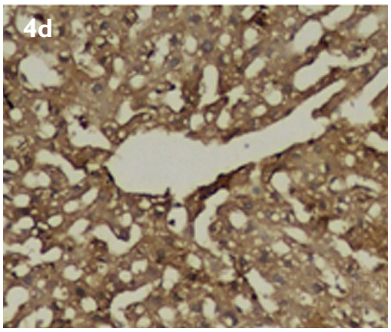


Fig. 4 Photomicrographs for a 3-nitrotyrosine (3-NT) protein adduct show (a) the section of a control rat liver 24 hours after thioacetamide (TA) administration that remains negative for 3-NT antibody; (b) positive staining in a liver section from a fructose-fed rat treated with TA at 24 hours; (c) the liver section of a TA-treated control rat 48 hours after TA administration with no reactivity; and (d) increased immunostaining in fructose-fed rats treated with TA at 48 hours (Haematoxylin, × 20).

An increase in protein carbonyl content is referred to as carbonyl stress and is commonly used as a marker of protein oxidation.[16] Oxidative modifications alter the biological properties of proteins, leading to their fragmentation, increased crosslinking and aggregation, and enzyme dysfunction.[17] Nitrotyrosine protein adducts are formed by reactions between peroxynitrite and tyrosine residues in proteins.[18] The formation of 3-

NT has been used extensively as nitrosative stress and as a biomarker of inflammation.[19] Enzymic and receptor proteins, upon nitration of their nitration residues, undergo irreversible changes that render them non-functional.[20,21] An increase in the protein carbonyl content in fructose-fed rats suggests protein modification by oxidation and nitration. Immunohistochemical detection of 3-NT indicates nitrosative stress.

Reactive aldehydes, particularly methyl glyoxal, that are formed during the normal course of metabolism, are scavenged by an efficient detoxification system that comprises two enzymes, glyoxalases I and II.[12] Glyoxalase I catalyses the condensation of GSH with the reactive aldehyde to form hydroxyacylglutathione, while glyoxalase II hydrolyses the product of glyoxalase I to GSH and aldonates.[22,23] Excess methylglyoxal and aldehydes could be formed by the hepatic metabolism of fructose through the glycolytic intermediate. These products can in turn activate the stress response pathways. Modulation of the glyoxalase system occurs during the onset of diabetic complications.[24] Decreased glyoxalase I and II activity represents a harmful situation, as the disposal of methylglyoxal as well as products of lipid peroxidation is less efficient.[25]

A wealth of information on the toxic effects of TA and its mechanism of action is available in the literature.[26-29] The administration of toxic quantities of TA causes fulminant hepatic failure[26] or cirrhosis in experimental animals.[27] TA-induced toxicity includes alterations in biochemical, clinical and histological features that resemble human cirrhosis and morphological features that resemble acute liver damage.[28] Prolonged administration of TA leads to hyperplastic liver nodules, liver cell adenomas, cholangiomas and hepatocarcinomas.[29]

The enhanced formation of reactive oxygen species and lipid peroxidation has been reported in the livers of TA-treated animals.[30] TA has been shown to produce sufficient injury to hepatic parenchyma by causing a large increase in the bilirubin content.[31] A decrease in the level of tissue arginase has shown that a large amount of liver-type arginase leaks immediately into the blood stream from the damaged liver tissue.[32] One report has indicated that TA alters the enzyme activities of the urea cycle.[33]

CYP2E1 plays a key role in the mediation of TA toxicity, and metabolic conversion of TA by CYP2E1 is a prerequisite for the hepatic necrogenic action of TA.[34,35] We observed that the administration of large quantities of fructose induces hepatic CYP2E1 two-fold, thereby substantially enhancing bioactivation-mediated TA-induced liver injury. TA has been shown to induce hepatic damage in a nutritional model of NASH.[36] An increase in the CYP2E1 level in patients with NASH and a rat model of NASH has been previously reported.[37]

The ultimate outcome of TA-induced hepatotoxicity has been documented to be dependent on the extent of tissue repair after injury rather than on bioactivation and/or detoxification mechanisms. Sawant et al have shown that hepatocytes from type 2 diabetic rats that were administered a sub-lethal dose of TA were unable to clear the G1 check point and advance to the S-phase DNA synthesis.[38] The progression of liver injury in fructose-fed rats, even after 36 hours, might be attributed to the inhibition of liver tissue repair, probably due to the failure of cell division and cell cycle arrest in fructose-fed rat livers exposed to TA. This could be responsible for the 80% mortality rate observed in fructose-fed rats that were administered even a non-lethal dose of TA in our study. In control rats, there was a regression of liver injury due to a compensatory liver tissue repair response that resulted in recovery and survival after 36 hours.

A time course study of liver injury provides insight into the extent of toxin-initiated liver injury. In this study, the marker enzymes of liver injury reached a peak at 24 hours after TA administration in control rats, and at 36 hours in fructose-fed rats, thus suggesting the progress of liver cell lysis. Markedly and irreversibly elevating bilirubin levels indicated deteriorating hepatic function, culminating in acute liver failure in the IR rats. At 36 hours after TA administration, the control rats showed remarkable improvement in liver function. Thus, the present study suggests that the administration of a sub-lethal dose of TA caused hepatic injury and 80% mortality in fructose-fed rats at 36 hours, while control rats survived the dosage due to a liver regenerative response.

Changes to the metabolic state of the liver caused by fructose could affect its susceptibility to toxic effects. The availability of cellular adenosine triphosphate (ATP) is a critical requirement for liver regeneration, and administration of compounds that enhance cellular ATP levels promotes the tissue repair process.[39] Although we have not measured the ATP levels, previous studies have shown that the metabolism of fructose causes the depletion of ATP levels.[40] Continued depletion of ATP levels might result in the inhibition of tissue repair, increased progression of liver injury, hepatic failure and mortality in fructose-fed rats. In addition, the formation of advanced glycation end products (AGEs) due to high intracellular glucose might alter liver function, cell proliferation and tissue repair.[38] Various observations have prompted the proposal that the regenerative ability

of the liver could be diminished due to fat accumulation or steatosis.[41] In this context, it is important to note that the increased formation of AGEs in the blood and tissues as well as lipid accumulation in the liver have been reported to take place in fructose-fed rats.[12] In the setting of IR, lipolysis that releases free fatty acids which are taken up by the liver is favoured. Free fatty acids can give rise to metabolites like ceramides and diacylglycerol, which are potentially toxic and cause increased lysosomal fragility, mitochondrial swelling and impairment of cellular membranes.[42] The liver is a major target for the absorbed drugs and xenobiotics because of its unique vascular and metabolic features. Diabetes mellitus potentiates the hepatotoxicity of several compounds such as TA, carbon tetrachloride, bromobenzene, chloroform and acetaminophen in various experimental models.[43]

In summary, we found IR to be associated with a decline in liver function with increased susceptibility to the toxic actions of TA. This could be attributed to fat accumulation, ATP depletion, protein and lipid damage, oxidative stress and increased CYP2E1 activity. Based on our results, it may be reasonable to suggest that potentially hepatotoxic drugs such as hypolipidaemic, antidiabetic (thiazolididiones) and anti-inflammatory drugs (acetaminophen and non-steroidal anti-inflammatory drugs) should be used with caution in patients with IR.

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