High intake of fructose, a simple monosaccharide, provokes glucocorticoid dysregulation in mice

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Abstract

The consumption of modern cafeteria diet rich in refined sugars is one factor responsible for the present epidemic of insulin resistance and type 2 diabetes. The present study examines the short and long term effects of feeding high quantities of fructose, a simple sugar on glucocorticoid (GC) action in mice. Male Swiss albino mice were provided a diet containing 60% fructose as the single source of carbohydrate for 30 or 60 days. We analyzed the plasma insulin and glucose levels, plasma and hepatic corticosterone (CORT) levels, the mRNA level of G6P translocase (G6PT) and hexose-6- phosphate dehydrogenase (H6PDH), the protein and mRNA level of microsomal 11-β-hydroxysteroid dehydrogenase type 1 (11βHSD1) and the compartmentalization of glucocorticoid receptor (GR) in the cytoplasm and nucleus of liver tissue. Mice subjected to long-term fructose feeding (60 days) showed elevated hepatic GC level, 11βHSD1 enzyme activity and GR nuclear translocation paralleled with increased plasma insulin and glucose levels. Short-term fructose feeding (30 days) in mice did not cause any significant difference between the tissue CORT levels and the 11βHSD1 and GR protein levels when compared to control animals. These findings suggest that long-term high fructose feeding increases intra-hepatic GC action which may have a mediatory role in diet-induced insulin resistance and associated metabolic changes.

Index terms: Insulin resistance, type 2 diabetes, fructose, 11-β-hydroxysteroid dehydrogenase type 1, glucocorticoid receptor.

1. INTRODUCTION

Fructose, a monosaccharide, is an imperative dietary source of carbohydrates which, naturally occurs in the free form in fruits and honey and in the combined form as half of the disaccharide sucrose [1]. Presently, fructose has become the primary component of modern human diet owing to the indiscriminate use of fructose syrup as a sweetener in carbonated beverages, canned fruits, jams, dairy products and baked goods [2, 3]. The absorption and metabolism of fructose differs from that of glucose [4]. Fructose is readily assimilated and rapidly metabolized in the liver by fructokinase enzyme to fructose-1-phosphate, which is subsequently cleaved by aldolase to form trioses that are precursors of triacylglycerols (TG). This pathway bypasses the phosphofructokinase step, the key regulatory step of glycolysis, leading to unregulated production of trioses culminating into lipogenesis. A high flux of fructose to the liver disturbs the normal hepatic carbohydrate metabolism resulting into two major consequences (i) perturbation of glucose metabolism and glucose uptake pathways, and (ii) a significantly enhanced rate of de novo lipogenesis and TG synthesis driven by the high flux of precursors, glycerol and acyl portions of TG molecules [5, 6]. Fructose-induced metabolic disturbances appear to be responsible for the rising incidence of insulin resistance, type 2 diabetes (T2D), non-alcoholic fatty liver disease, obesity and cardiovascular diseases [7].

GCs regulate the energy homeostasis and body fat repartition through maintaining the whole body glucose homeostasis and lipid metabolism [8]. GC mediates its action through glucocorticoid receptor (GR), which upon activation translocates and binds to the glucocorticoid response elements (GREs) within the nucleus [9]. Elevated intracellular GC levels, as seen in Cushing's syndrome contributes to the development of abnormal fat distribution and hepatic lipid accumulation, obesity, insulin resistance and other metabolic disorders [10]. Excess GC has been reported in insulin-resistant patients associated with hyperglycemia, dyslipidemia and fatty liver and similarly, mice with increased 11-β-hydroxysteroid dehydrogenase type 1 (11βHSD1) expression in mesenteric adipose tissue suffer from impaired glucose tolerance, hyperphagia, and elevated blood lipids [11, 12]. These studies suggest that enhanced GC action may be directly related to the pathophysiology of insulin resistance and associated metabolic disorders.

Taking into account the fact that high fructose content is an important factor responsible for adverse outcomes of the diet, we chose to examine the short (30 days) and long term (60 days) effects of high fructose consumption on the GC hormone action in the liver tissue. We hypothesized that diet

containing 60 % fructose can cause enhanced prereceptor GC action, increased GC levels and GR activation in the liver tissue which may play a role in the reduction of insulin sensitivity. Therefore, we analyzed the effects of high fructose consumption on plasma and liver GC level, 11βHSD1 and GR mRNA and protein levels and also, the blood insulin and glucose levels at different time points of diet manipulation, i.e., at the end of 30 days and 60 days.

2. MATERIALS AND METHODS

Chemicals and assay kits

Fine chemicals and solvents of analytical grade used in this study were obtained either from HIMEDIA Laboratories Pvt., Ltd., Mumbai, India or Sisco Research Laboratories Pvt. Ltd., Mumbai, India.

Animal maintenance

Adult male *Mus musculus* albino mice of Swiss strain (25–30 g) were obtained and maintained in the Central Animal House, Rajah Muthiah Medical College and Hospital (RMMC and H). The animals were housed individually in polypropylene cages for two different experimental periods of 30 days and 60 days and the animal room was maintained under hygienic conditions at a temperature ranging from 22 – 24 °C with 12 h light/12 h dark cycle and constant humidity. The study was approved by the Institutional Animal Ethical Committee (IAEC), RMMC and H, Annamalai Nagar (Reg. No. 160/1999/CPCSEA/977) and, all the animal procedures carried out in this study were according to the guidelines of the IAEC.

Diet

The composition of fructose diet and starch diet were identical except that the corn starch was replaced with an equal quantity of fructose and, were freshly prepared in the laboratory. A metabolizable energy of 3.650 Kcal/g was gained from the diet, of which 65.75 % was obtained from starch in the control diet and from fructose in the fructose diet. The diet composition and the duration of the study are based on our earlier study [13].

Animal groups

The animals were randomly divided into four experimental groups (n=6) as follows: Group 1: Mice fed control diet for 30 days (CON_{30}), Group 2: Mice fed control diet for 60 days (CON_{60}), Group 3: Mice fed high fructose diet for 30 days ($HFrD_{30}$), and Group 4: Mice fed high fructose diet for 60 days ($HFrD_{60}$).

Sample collection

At the end of 30th or 60th day of the experimental period, the mice were subjected to overnight (12 h) fasting and then sacrificed by cervical dislocation. The blood samples were collected from individual mice in tubes containing the anticoagulant heparin. After centrifugation (1000 × g for 20 min) the plasma was separated and was stored at -20 °C for the future analyses. Liver tissue was immediately dissected out after decapitation, washed in ice-cold saline to remove the adhering blood, dried, weighed accurately, frozen and stored at -80 °C until use. The subcellular fractions of cytosolic, nuclear and microsomal were separated from the liver tissue homogenates by the procedure adopted from Spencer *et al*. (2000) and Park *et al*. (2004) [14, 15].

Biochemical assays

Plasma glucose and insulin levels were estimated using reagent kit and by the solid phase enzyme linked immunosorbent assay (ELISA), respectively. Plasma and tissue corticosterone (CORT) level were determined by the fluorometric procedure of Silber *et al.* (1958) [16].

Quantitative real time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the liver tissue using TRIzol Reagent (Genei, Bangalore, India) and its concentration and purity were evaluated using the BioPhotometer Plus (Eppendorf, Hamburg, Germany) at an absorbance of 260 and 280 nm. Reverse transcription of total RNA was performed using M-MuLV-reverse transcriptase (50 U/µl) (Thermo scientific, Pittsburgh,USA) and OligodT primers (0.2 µg/mL) (Genei, Bangalore, India) and the quantification of cDNA was done and stored at -80 °C until use. After quantification, PCR amplification of target genes was performed in a mixture (total volume 20 µl) constituting 100 µg cDNA, 1 µl each of 50 pM reverse and forward primers (Sigma-Aldrich, St Louis, MO, USA) and 10 µl SYBR green master mix (Kapabiosystems, Boston, USA). The thermocycling conditions implemented in the present study were as follows: initial denaturation 95 °C for 3 min, denaturation 95 °C for 1-3 s, annealing 60 ± 3 °C for 30 s and extension 60 °C for 30 s for 40 cycles. The reactions were run in quadruplicate for each sample and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene

served as the internal control. Using the formula $2^{-\Delta\Delta Ct}$, the Ct values obtained for each target gene [G6P translocase (G6PT), hexose-6- phosphate dehydrogenase (H6PDH), 11βHSD1, GR] (Table 1) were normalized with that of GAPDH gene. After normalization of each gene with GAPDH, the calculated relative quantity was expressed in bar graphs as fold change with respect to control.

Immunoblotting

The protein samples were extracted and quantified through the method of Lowry *et al.* (1951) [17]. The proteins were separated by 6 % and 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970) [18]. The resolved proteins were transferred onto a polyvinylidene difluoride membrane (0.45 µm) using transfer buffer (25 mM Tris, 192 mM glycine and 20 % methanol) at 40 V for one and half hours. After this, to reduce the non-specific protein binding, the membranes were subjected to blocking using Tris buffered saline – Tween 20 (TBST) containing 3% bovine serum albumin (pH 7.4) for 2 h. Following blocking, the membrane were incubated with primary antibodies specific to 11βHSD1 (Cayman Chemicals, 1:50) and GR (Cell Signaling, 1:1000) followed by secondary antibody (rabbit monoclonal). The bands were normalized through stripping and re-probing the membranes with β-actin antibody. Using enhanced chemiluminescence (ECL) kit (Thermo Scientific Super Signal West Pico Chemiluminescent Substrate, Rockford, USA) the immunoactive proteins were visualize and the protein band densities were measured using Image J software (National Institute of Health Bethesda, MD, USA).

Statistical Analyses

The obtained results were expressed as means ± SD for 6 mice from each group for biochemical analysis and 4 mice from each group for immunoblotting and for qPCR. Significance of differences among the groups were evaluated using One–way ANOVA followed by Tukey's test ($P<0.05$). All the statistical analyses were performed using GraphPad Prism 5 for Windows (Version 5.01; GraphPad Software, San Diego, CA, USA).

3. RESULTS

Plasma glucose, insulin and insulin sensitivity indices

Elevated plasma glucose and insulin levels, higher HOMA and lower QUICKI values were observed in $HFrD_{30}$ and $HFrD_{60}$ groups in comparison with their respective control groups and the changes were statistically significant (Table 2). However, animals fed with HFrD for 60 days showed higher values of glucose (by 24 %) and insulin (by 33 %) when compared with $HFrD_{30}$ group.

Corticosterone levels in plasma and liver tissue

Corticosterone level in liver tissue was higher in $HFrD_{60}$ group (by 75 %) as compared to CON_{60} group (Table 3). The hepatic corticosterone level of $HFrD_{30}$ group animals showed no significant difference when compared to CON_{30} group. HFrD feeding for 60 days in mice increased the hepatic corticosterone level by 68 % as compared to $HFrD_{30}$ group. The plasma corticosterone levels showed no significant difference among the groups.

Western blotting analysis of 11ϐHSD1 and GR proteins in liver tissue

The protein abundance of 11βHSD1 and GR proteins in liver tissue was given in Figure 1A. Fructose consumption for 30 days showed lesser difference in the protein levels of microsomal 11βHSD1, while no significant difference was observed in the protein levels of cytosolic and nuclear GR when compared to CON_{30} group. The continued administration of HFrD for 60 days in mice showed a significant increase in the microsomal 11βHSD1 protein level (by 37 %) as compared to control mice. The protein level of microsomal 11βHSD1 was significantly increased by 46 % in mice fed with HFrD for 60 days as compared to $HFrD_{30}$ group. In liver, the cytosolic GR expression was decreased by 41 % and the nuclear expression of GR was increased by 30 % in $HFrD_{60}$ group when compared to mice fed with HFrD for 30 days. No significant differences were observed between CON_{30} and CON_{60} groups.

G6PT, H6PDH, 11ϐHSD1 and GR mRNA levels

The mRNA abundance of G6PT, H6PDH and 11βHSD1 in liver of $HFrD_{60}$ group was significantly increased by 71 %, 76 % and 80 % as compared to $HFrD_{30}$ group (Figure 2). In $HFrD_{30}$ group the mRNA levels of G6PT, H6PDH and 11βHSD1 showed no significant difference as compared to control animals. The relative mRNA expression of GR was unaltered in all the four groups.

4. DISCUSSION

Compelling evidences confirm that high fructose intake can have deleterious metabolic effects such as dyslipidemia and impaired insulin sensitivity, but the effect of fructose on GC dysregulation remains unclear. The present work investigated the adverse effects of short term (30 days) and long term (60 days) fructose feeding on the prereceptor activation of GCs that mediate the development of insulin resistance and obesity-related metabolic disorders.

The major findings of the present study show that high fructose feeding impairs the insulin sensitivity and the adverse effects of fructose on GC metabolism are to a greater extent in mice fed 60 % dietary fructose for 60 days than in those fed for 30 days. The long term (60 days) consumption of high fructose diet enhanced the G6PT-H6PDH-11βHSD1 triad activity followed by elevated GC levels and GR activation.

The long term exposure (60 days) to fructose-rich diet did not affect the plasma GC level, while the hepatic GC level was significantly elevated. Elevation in hepatic GC level was in parallel with increased mRNA levels of G6PT, H6PDH and 11βHSD1 genes and coincided with the increased 11βHSD1 protein level. 11βHSD1 catalyzes the reversible interconversion of GC by using nicotinamide adenine dinucleotide phosphate ($NADP^+$) or NADPH as a cofactor generated by H6PDH and alongside, the substrate supply for H6PDH activity is maintained by G6PT. This observation is in accordance with results from previous studies demonstrating that fructose consumption up-regulates the G6PT-H6PDH-11βHSD1 triad activity in both human and animal models [19-21]. Bursac *et al.* (2013) and Vasiljević *et al.* (2013) reported that consumption of 10 % fructose solution for 9 weeks increased the 11βHSD1 activity and thus elevated GC levels and enhanced GC action in the adipose tissue of Wistar rats [22, 23]. Similarly, Wang *et al*. observed that the increased hepatic G6PT and H6PDH expression and enhanced 11βHSD1activity and local GC action contributed to the development of type 2 diabetes in *db/db* mice [24]. Thus, the elevation of both mRNA and protein level of 11βHSD1 noticed in our study in the liver of fructose-fed mice was predictable. It may be concluded that simultaneous elevation in G6PT, H6PDH and 11βHSD1 mRNA levels is very likely responsible for the increased hepatic GC concentration observed herein. Meanwhile, the short term controlled fructose feeding had no significant effect on the activity of G6PT-H6PDH-11βHSD1 triad system and hepatic GC levels.

Next, we sought to examine the activation of GR by characterizing the intracellular distribution of GR in liver. We found that fructose feeding for 60 days activated GR through enhancing the translocation of GR to the nucleus from the cytoplasm. Furthermore, the GR mRNA was unchanged in

the liver of mice after 60 days exposure to high fructose diet. This suggests that down-regulation of GR is not at the transcriptional level but could be a post translational modifications like phosphorylation. Our findings suggest that long term (60 days) exposure to high fructose diet up-regulated G6PT-H6PDH-11βHSD1 activity, elevated hepatic GC level and increased nuclear translocation and activation of GR resulting in enhanced GC action which may contribute to the development of insulin resistance, hyperglycemia and hyperlipidemia.

Controlled diet manipulation studies state that diets high in fructose induce features of metabolic syndrome like hyperinsulinemia, hypertriglyceridemia, hypertension, and obesity in rodent models [25-27]. In the present study, fructose feeding for 30 days and 60 days impaired the insulin sensitivity which was indicated through increased plasma glucose and insulin concentrations, and by higher value of HOMA-IR and lower value of QUICKI.

Overall the results suggest that consumption of fructose diet for 30 days can increase glucose and insulin levels without any change in GC levels whereas fructose feeding for 60 days results in enhanced hepatic GC action through upregulating prereceptor GC signaling and activating GR thereby drastically increasing the plasma glucose and insulin levels. Thus, excess consumption of fast foods containing high amounts of fructose must be avoided since chronic exposure of fructose has an influence on GC action which may hasten the development of metabolic disorders.

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TABLES

Table 1. List of primers used for qRT-PCR

S.No.	Gene	Forward 5'<-sequence->3'	Reverse 5'<-sequence->3'	Product length
1.	G6PT	CAAAGGAGGAGAGCACCCTA	CCCCAGTCTGTACAGCAAGTC	109
2.	H6PDH	GCCAGTTTCTATGAGGAGTATGG	TGCTGATGTTGAGAGGCAGT	103
3.	11βHSD1	TTCTAAACCACATCACTCAGACCT	CATGACCACGTAGCTGAGGA	101
4.	GR	AGGAATTCAGCAAGCCACTG	CAGGGTAGGGGTAAGCTGTG	101
Internal control				
4.	GAPDH	AGAACATCATCCCTGCATCC	ACACATTGGGGGTAGGAACA	123

Table 2. Plasma glucose and insulin levels and insulin sensitivity indices

Parameters	CON_{30}	CON_{60}	$HFrD_{30}$	$HFrD_{60}$
Glucose (mg/dL)	75.2 ± 3.5^{a}	75.6 ± 4.2^{a}	91.6 ± 6.1^{b}	123.6 ± 7.7^{c}
Insulin (μU/mL)	20.5 ± 1.3^{a}	21.1 ± 0.9^{a}	27.7 ± 1.7^{b}	34.3 ± 2.4^{c}
HOMA	0.63 ± 0.03^{a}	0.70 ± 0.04^{a}	1.04 ± 0.04^{b}	1.74 ± 0.07^{c}
QUICKI	0.31 ± 0.1^{a}	0.30 ± 0.1^{a}	0.29 ± 0.2^{b}	0.27 ± 0.1^{c}

CON 30 – Control diet for 30 days; CON 60 – Control diet for 60 days; HFrD30 - High fructose diet for 30 days; HFrD60 - High fructose diet for 60 days.

Values are means ± SD of 6 mice from each group. Values bearing different alphabets in their superscript differ significantly from each other. One-way ANOVA ($P<0.05$) followed by Tukey's test.

HOMA = Insulin (μU/L) x Glucose (mg/dL)/2430

QUICKI = 1/[log (glucose mg/dL) + log (insulin mU/L)]

Table 3. Plasma and liver corticosterone levels

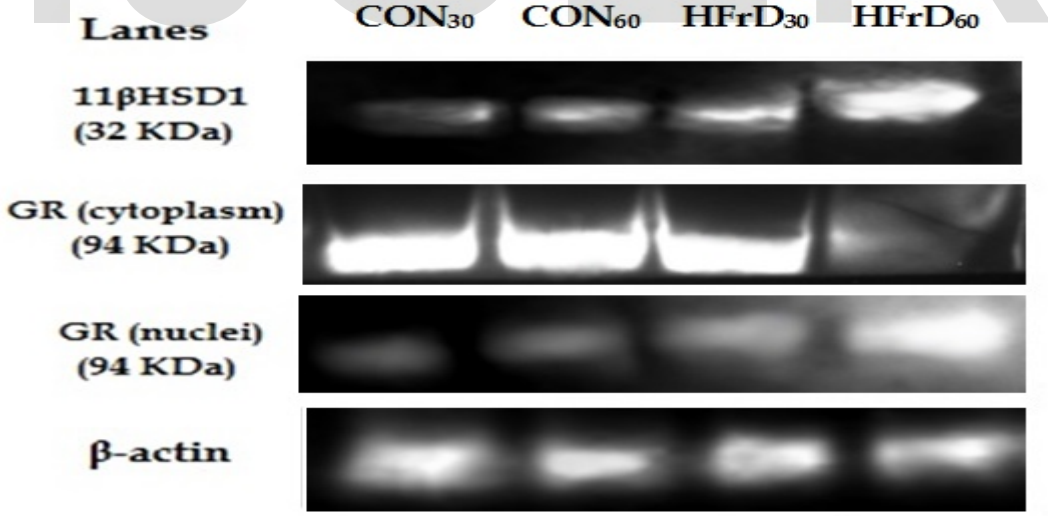
Parameters	CON_{30}	CON_{60}	$HFrD_{30}$	$HFrD_{60}$
Plasma corticosterone (ng/mL)	181.5±43.41	187.1±49.34	183.6±43.67	180.9±42.11
Liver corticosterone (ng/mg)	2.35±0.13 [a]	2.41±0.11 [a]	2.80±0.14 [a]	4.22±0.17 [b]

CON 30 – Control diet for 30 days; CON 60 – Control diet for 60 days; HFrD30 - High fructose diet for 30 days; HFrD60 - High fructose diet for 60 days.
Values are means ± SD of 6 mice from each group. Values bearing different alphabets in their superscript differ significantly from each other. One-way ANOVA (P<0.05) followed by Tukey's test.

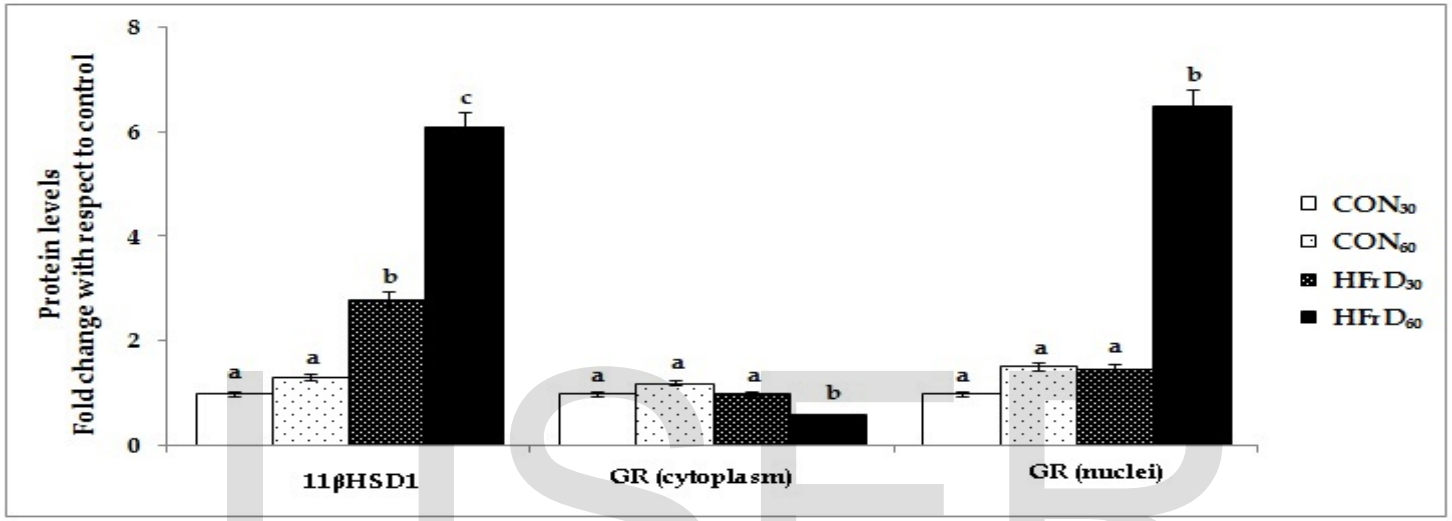
Figures

Fig.1.

A)



B)


Protein levels
Fold change with respect to control
8
6
4
2
0
a
a
b
c
a
a
a
b
a
a
a
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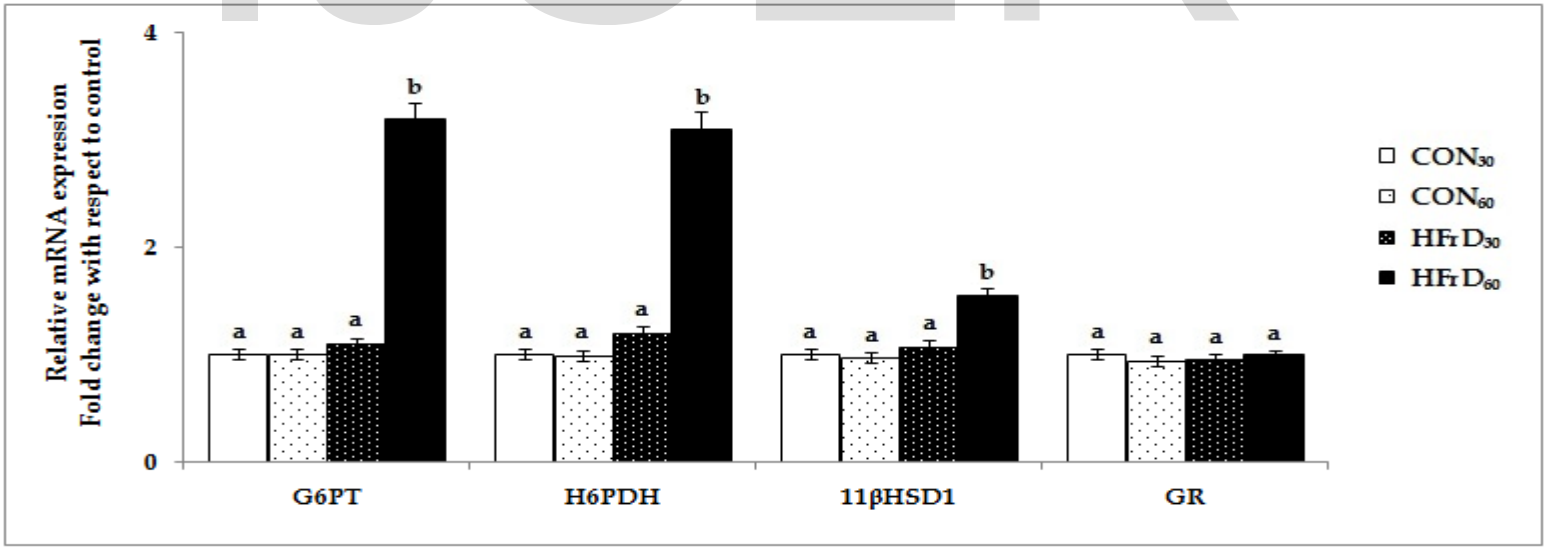
11βHSD1
GR (cytoplasm)
GR (nuclei)
CON30
CON60
HFrD30
HFrD60

Fig. 2.


Relative mRNA expression
Fold change with respect to control
4
2
0
a
a
a
b
a
a
a
b
a
a
a
b
a
a
a
a
G6PT
H6PDH
11βHSD1
GR
CON30
CON60
HFrD30
HFrD60

Legends

Fig. 1. Protein abundance of 11βHSD1 and GR proteins in liver tissue.

(A) Representative immunoblots of 11βHSD1 protein in the microsomal fraction and GR protein in the cytosolic and nuclear fractions from liver. Lane 1- Control diet for 30 days (CON_{30}); Lane 2- Control diet for 60 days (CON_{60}); Lane 3-High fructose diet for 30 days ($HFrD_{30}$); Lane 4 – High fructose diet for 60 days ($HFrD_{60}$). (B) Densitometry data of 11βHSD1 in microsome, GR in cytoplasm and GR in nuclei of liver. Values are means ± SD of 4 mice from each group. Values bearing different alphabets in their superscript differ significantly from each other. One-way ANOVA ($P<0.05$) followed by Tukey's test.

Fig.2. G6PT, H6PDH, 11βHSD1and GR mRNA levels in liver tissue

Figure 2 represents the mRNA expression of G6PT, H6PDH, 11βHSD1 and GR in liver. CON_{30} - Control diet for 30 days; CON_{60} - Control diet for 60 days; $HFrD_{30}$ -High fructose diet for 30 days; $HFrD_{60}$ – High fructose diet for 60 days. The Ct values of the test gene of each group were standardized using Ct value of GAPDH of the same group and the relative quantity was expressed in bar graphs as fold change with respect to control after normalization with GAPDH for each gene. Values are means ± SD of 4 mice from each group. Values bearing different alphabets in their superscript differ significantly from each other. One-way ANOVA ($P<0.05$) followed by Tukey's test.