

Original Article

Different dietary contribution to hepatic inflammatory and lipogenic factor mRNA expression

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Abstract: Aim: We studied the effect of diet on hepatic toll like receptor 4 signaling pathways, insulin resistance, and fatty acid oxidation in a rat model. Methods: Animals were fed a normal, high-fructose, high-fat, or a high-fructose and fat diet for 16 weeks and weight, energy production, hepatic pathology, biochemistry, insulin resistance, toll like receptor 4 mRNA expression and fatty acid oxidation were measured. Result: Rats developed nonalcoholic hepatitis and gained weight after a high-fructose and fat and a high-fat diet compared with the high-fructose and normal diet. Signaling pathways activated by toll like receptor 4-the MyD88-TIRAP and the TRIF-TRAM pathways-were activated with all diets except the normal diet. Insulin and insulin receptor substrate mRNA were decreased in the liver and testicular adipose tissue. Peroxisome proliferator-activated receptor- α decreased in testicular adipose tissue in all but normally fed rats and sterol regulatory element-binding protein 1 and fatty acid synthase increased all but normal diets. Conclusion: High fat with and without high-fructose caused liver steatosis, induced insulin resistance, and promoted activation of inflammatory signals and fatty acid oxidation.

Keywords: High-fat diet, high-fructose and fat diet, non-alcoholic fatty liver disease

Introduction

Obesity is a global epidemic and associated diseases such as non-alcoholic fatty liver disease (NAFLD) are the most common manifestations of metabolic derangement [1]. NAFLD can range from simple steatosis to non-alcoholic steatohepatitis (NASH) to liver cirrhosis and hepatocellular carcinoma [2]. Diet contributes greatly to NAFLD, specifically high-fructose and high-fat intake which have been linked to hepatic steatosis and inflammation. Fructose can dysregulate metabolism and this is associated with NAFLD, exacerbating lipogenesis, dyslipidemia, visceral adiposity, and insulin resistance, as well as possibly altering the intestinal microbiome [3-6]. As much as ten weeks of high-fructose intake can change liver function markers (but as few as two weeks did not), hepatic triglycerides (TG), gamma-glutamyltransferase, and uric acid, but 6 months of reduced fructose improved these markers and insulin sensitivity in NAFLD patients [7-9]. Also 4 weeks of fructose restriction for children with NAFLD improved adipose insulin sensitivity and LDL oxidation [10]. High-fat diets are used to

establish NASH in a rat model by increasing fatty acid intake causing hepatic lipid accumulation and ROS. Free fatty acid products and LPS can induce release of downstream inflammatory factors which occur in high-fat-diet induced NAFLD [11]. High fat intake can induce the expression of TLR4 and activate downstream pathways. A high-fructose/high-fat diet is typical for most individuals, and our rat model of dietary induced NAFLD indicated that TLR4 participated in the induction of NASH. Therefore, we investigated liver pathology, serum biochemistry, TLR4 signaling pathways, insulin sensitivity-related transcription factors and fatty acid oxidation for a normal (N), high-fructose (HF), high-fat (HFAT) and a high-fructose/high-fat diet (HFHF).

Materials and methods

Animals and treatments

Forty Male Sprague-Dawley rats (140-160 g) were purchased from Shanghai Experimental Animal Center of Chinese Academy of Sciences (Shanghai, China) and were housed in a speci-

Table 1. Composition of experimental diets

	Control	HFD
Protein, g/100 g	22. 1	19. 4
Carbohydrate, g/100 g	52	45. 8
Fat, g/100 g	5. 28	16. 6
Cholesterol, g/100 g	0	2
Protein, kcal/g	0. 88	0. 77
Carbohydrate, kcal/g	2. 08	1. 83
Fat, kcal/g	0. 48	1. 49
Total kcal/g	3. 44	4. 09

Table 2. Primers used

Primer	Forward (5'-3')	Reverse (5'-3')
TLR4	GCCGGAAGTTATTGTGGTG	CCACTCGAGGTAGGTGTTT
Myd88	AGGCTGCTAGAGTTGCTAGCCTTG	GGTTGCGTATGATTTCTGGCAGTC
TRIF	TTCTGGTCACTGCAGGGAT	ATGGATAACCCAGGGCCTT
TNF- α	ATCCGAGATGTGGAAGTGGC	CGATCACCCCGAAGTTCAGT
NF- κ B	GGCAGCACTCCTTATCAA	GGTGTCGTCCTCCATCGTAG
IRF3	TAAGGGAGATCGGCTGGCTG	TGATGGAGAGGTCCC CAAGG
PPAR- α	ACTATGGAGTCCACGCATGTGA	TTGTCGTACGCCAGCTTTAGC
FAS	TCTGAGGGTTTGGAGTTG	ATTTGGTGTGCTGGTTC
Insulin	AAACAGCACCTTTGTGGTCT	GGGTCCACTTCACGAC
IRS1	ACGCTCCAGTGAGGATTTAAGCA	GGTCTGGTTGTGAATCGTGAA
β -actin	AACCCTAAGGCCAACCGTGAAG	TCATGAGGTAGTCTGTCAGGT

fied and opportunistic pathogen-free (SOPF) barrier facility. All procedures were approved by the Institutional Animal Care and Use Committee of XinHua hospital affiliated to Shanghai Jiao Tong University School of Medicine. All rats were given humane care in a temperature-controlled environment with a 12:12-h light-dark cycle. Rats were randomized to one of four treatment groups and for 16 weeks, animals (n=10/group) had free access to plain tap water and standard chow (normal diet, N), or to water enriched with 20% (w/v) fructose and standard chow (HF), a high-fat diet (HFAT, 88% standard diet, 10% fat and 2% cholesterol) or water enriched with 20% (w/v) high-fructose/high-fat diet (HFHF). Diet calories appear in **Table 1**. After the experimental period, rats were anesthetized with pentobarbital sodium (30 mg/kg, ip) and blood was collected from the portal vein. Liver and testicular adipose tissues were removed, snap-frozen or fixed in 4% neutral-buffered formalin for analysis.

Histopathological evaluation

Rat liver specimens were formalin fixed, paraffin-embedded, cut into 4- μ m sections and hematoxylin and eosin (H&E) staining was used to

derive pathological scores by two pathologists blinded to the study groups. Histological scores were based on NAFLD activity scores (NAS). Census was achieved with a third hepatopathologist if necessary.

RNA isolation and real-time RT-PCR (fluorescent quantitative PCR)

Total RNA was extracted from liver and testicular adipose tissue samples with Trizol reagent (Invitrogen, Carlsbad, CA) and complementary DNA was synthesized according to the manufacturer's instructions (Takara, Shiga, Japan). Real-time quantitative PCR (SYBR Green) assays were performed using SYBR Premix Ex TaqTM (Takara, Shiga, Japan) in an Applied Biosystems 7500 sequence detector (Roche, Indianapolis, IN). The comparative CT method was used to measure target, normalized to an endogenous reference (β -actin) and relative to a calibrator ($2^{-\Delta\Delta Ct}$). Primers used are summarized in **Table 2**.

Statistical analysis

Results are reported as means \pm SEM. ANOVA with Bonferroni's *post hoc* test to confirm statistical significance among treatment groups ($P < 0.05$ considered statistically significant).

Results

Dietary effect on caloric intake and body weight

As shown in **Figure 1**, dietary intake affects body weight over 16 weeks and normal diets appear to cause less weight gain than HFAT or HFHF diets ($P < 0.05$). No significant difference existed between HFAT and HFHF ($P > 0.05$). Caloric intake in the HFAT or HFHF diet is significantly higher than normal and HF diet group ($P < 0.05$).

Dietary effect on liver histology and serum biochemistry

Figures 2, 3 and **Table 3** show that control animals did not change over the experimental peri-

Diet and hepatic inflammatory and lipogenic factor

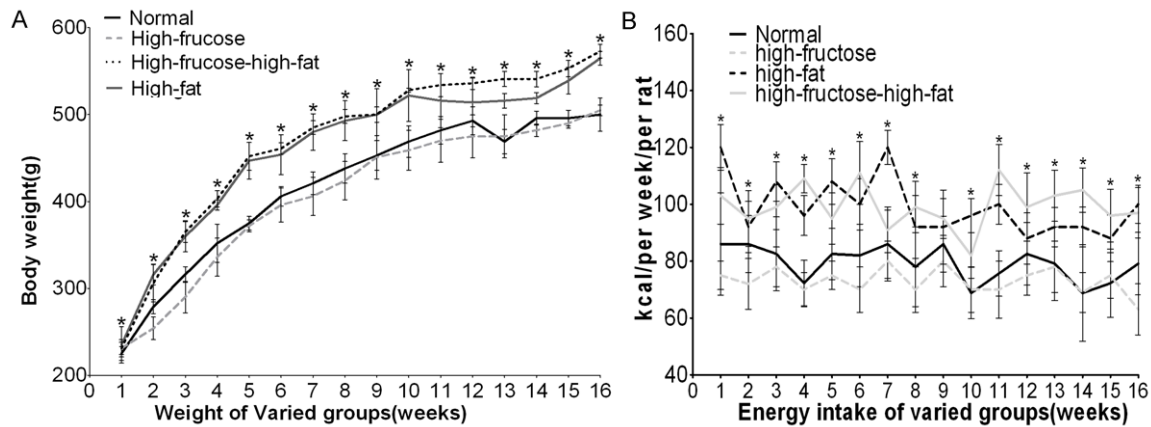


Figure 1. Effect of diet on weight gain and energy intake in the rat after 16 weeks. Data are means \pm SEM (n=10) N: Normal; HF: 20% fructose diet; HFAT: high-fat diet; HFHF: high-fructose-high-fat (* P <0.05 HFAT and HFHF versus N and NHF).

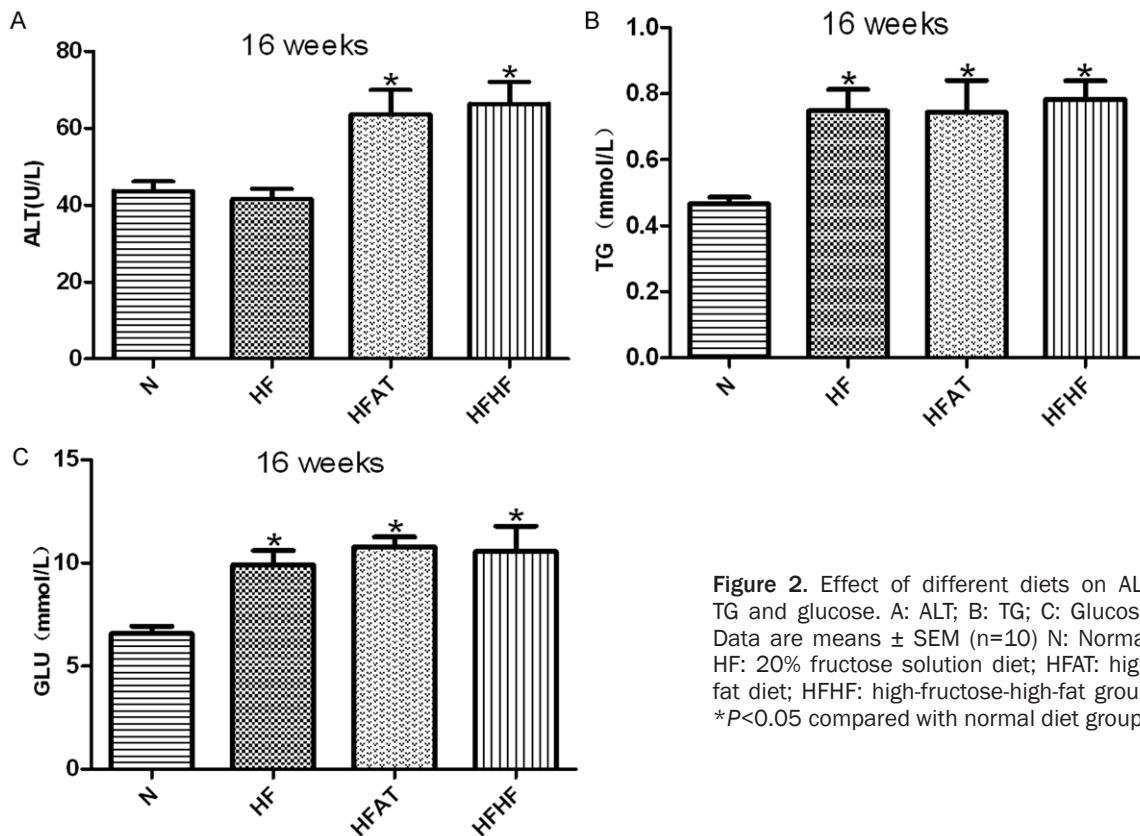


Figure 2. Effect of different diets on ALT, TG and glucose. A: ALT; B: TG; C: Glucose. Data are means \pm SEM (n=10) N: Normal; HF: 20% fructose solution diet; HFAT: high-fat diet; HFHF: high-fructose-high-fat group; * P <0.05 compared with normal diet group.

od but the HFAT diet caused induced serious hepatocellular steatosis, lobular inflammation and ballooning. NAFLD activity scores (NAS) for HFAT were elevated and 90% of the animals developed NASH. A HFHF diet caused higher NAFLD scores and the same proportion of animals developed NASH. ALTs were greater in the HFAT- and HFHF-fed animals as well as TG and

glucose (P <0.05). No significant difference existed between HFAT and HFHF (P >0.05) rats, but serum TG and glucose were greater than those in the HFHF group.

Dietary effect on hepatic inflammation

TLR4 expression, myeloid differentiation primary response gene 88 (MyD88), TIR-domain-

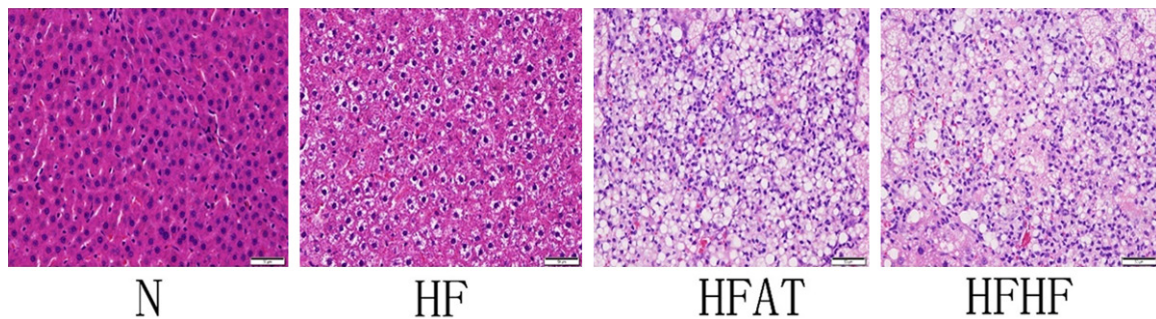


Figure 3. Representative photomicrographs (200x) of H&E stained liver sections.

Table 3. NAS for rat hepatic histology

	Steatosis (0-3)				lobular inflammation (0-3)				Ballooning (0-2)			NAFLD activity score (mean \pm SEM)	NASH (%)
	0	1	2	3	0	1	2	3	0	1	2		
Control	0	0	0	0	2	8	0	0	10	0	0	0.8 \pm 0.42	0
HF	0	6	4	0	7	3	0	0	10	0	0	1.70 \pm 0.82	0
HFAT	0	2	6	2	0	3	3	4	2	6	2	5.20 \pm 0.78*	80%
HFHF	0	3	5	2	0	1	5	4	1	5	4	5.40 \pm 0.84*	90%

containing adaptor inducing interferon- β (TRIF) mRNA, TNF- α , nuclear factor-kappa B (NF- κ B) and interferon regulatory factor 3 (IRF3) were measured (**Figure 4**). TLR4 mRNA was greater with HF, HFAT, and HFHF diets after 16 weeks ($P < 0.05$). Myd88 mRNA was significantly higher after a HFHF diet and a HFAT diet ($P < 0.05$). TRIF and NF- κ B were greater after a HF, HFHF, and HFAT diet as well ($P < 0.05$) (**Figure 4**). TNF- α was greater for all diets compared to control chow as was IRF3 ($P < 0.05$).

Dietary effect on PPAR- α , FAS, SREBP1, IRS1 and insulin mRNA in liver and adipose tissue

To measure dietary effects on lipogenesis and fatty acid oxidation genes in adipose tissue, mRNA PPAR- α , FAS, and SREBP1 were measured (**Figure 5**). Expression of PPAR- α was significantly lower in adipose tissue of rats fed a HFAT and HFHF diet compared to N and HF diets after 16 weeks ($P < 0.05$). In contrast, SREBP 1 was significantly higher in adipose tissue after a HFAT and HFHF diet, and a HFHF diet decreased insulin and IRS1 mRNA in testicular adipose tissue compared with N and HF diets ($P < 0.05$), and all intervention groups had less hepatic insulin and IRS1 mRNA compared with N diet (**Figures 6, 7**).

Discussion

In a previous study, we successfully used a high-fat diet (88% standard diet, 10% fat and 2% cholesterol) to replicate a NASH model, and this mimicked pathological sequela of NASH and had features of Mets [12]. Here, we used the HFD-diet as well as HFHF and HF diets to study effects on hepatic inflammatory and lipogenic factor mRNA expression. HFAT and HFHF diets offered more energy and caused more weight gain, but HF and N diets were not different. Rats on the HF diet consumed more liquid than controls but ate less of the solid chow. Rats on the HFHF diet ate slightly less than those fed HFAT, but higher caloric content of the diet cancelled the effect of less intake. There were no significant differences between HF and N diets and these data agreed with Tillman's work [13].

Meanwhile, NAS scores and serum ALT were greater in the HFAT and HFHF animals. Fructose can induce slight steatosis and previous studies indicate that liver fat disposition is related to NAFLD which is determined by a balance of lipid synthesis, secretion, and clearance that depend on several metabolic pathways such as fatty acid oxidation, TG esterification, adipose lipolysis and *de novo* lipogenesis as well as and

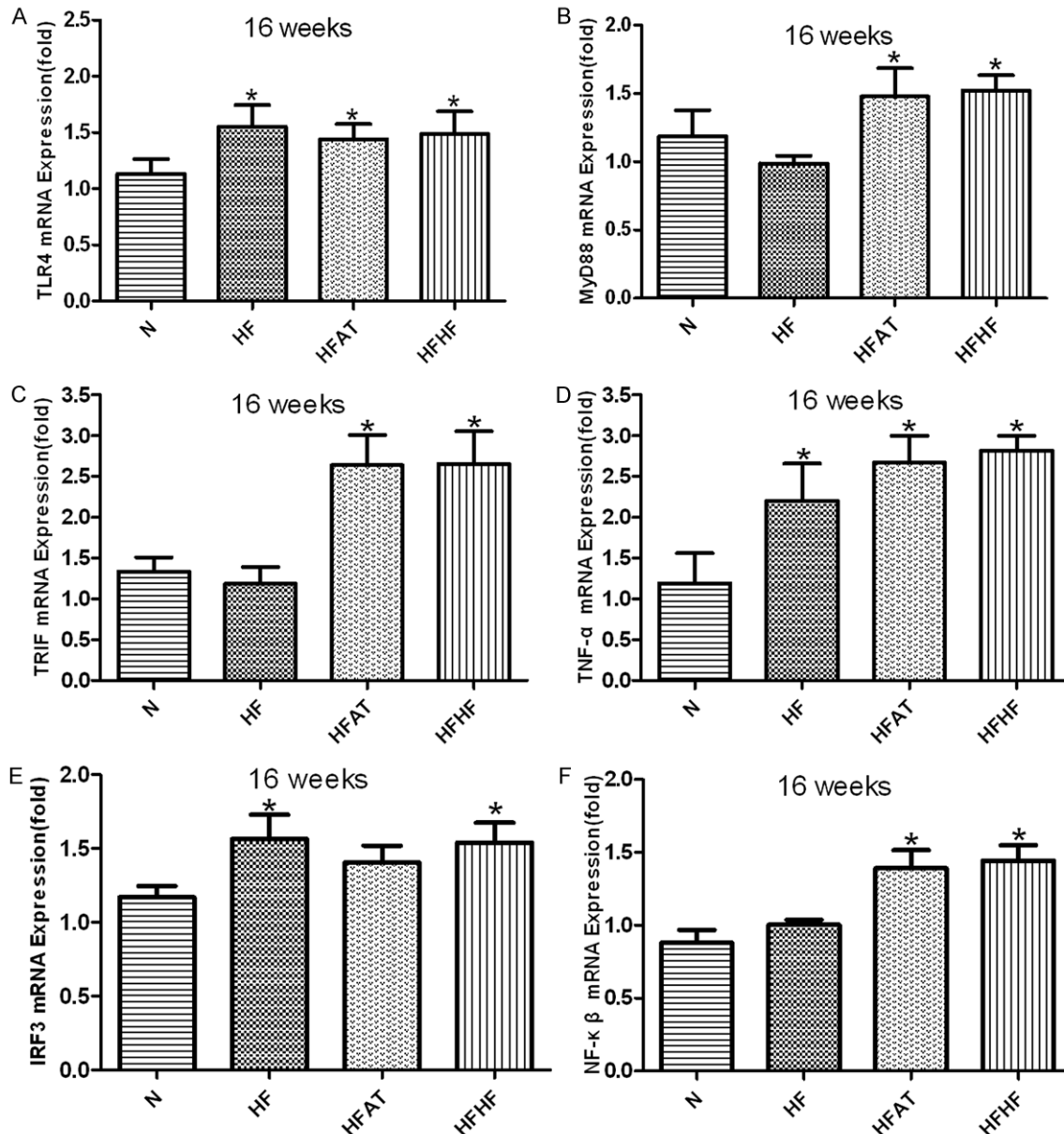


Figure 4. Effect of diet on hepatic TLR4, MyD88, TRIF, TNF- α , NF- κ β , and IRF3 mRNA expression. A: TLR4 mRNA, B: MyD88 mRNA, C: TRIF mRNA, D: TNF- α mRNA, E: NF- κ β mRNA, F: IRF3 mRNA. Data are means \pm SEM (n=10) N: Normal; HF: 20% fructose diet; HFAT: high-fat diet; HFHF: high-fructose-high-fat (* P <0.05 compared with normal diet).

VLDL synthesis and secretion in hepatic tissue. Particularly, hepatic *de novo* lipogenesis is critical for NAFLD pathogenesis [14]. Synthesis of fatty acids is significantly greater with NAFLD compared to normal individuals [15]. Dietary fatty acids influence hepatic lipogenesis as well [14]. We noted that fatty acids from a HFAT and HFHF diets cause NAFLD and HFAT diets have been historically used to induce animal models of steatosis [16].

NAS scores were higher for HFHF animals compared to HFAT rats but these were not significant. A diet enriched with HFHF can induce liver fibrosis, inflammation, endoplasmic reticular stress and adipogenesis [17]. Thus, we used fructose to induce NASH in an HFHF diet [18] because it can stimulate circulating endotoxin with NAFLD. Interestingly, children with NAFLD who consume HF beverages with a meal had increased postprandial endotoxin in 24 h, and

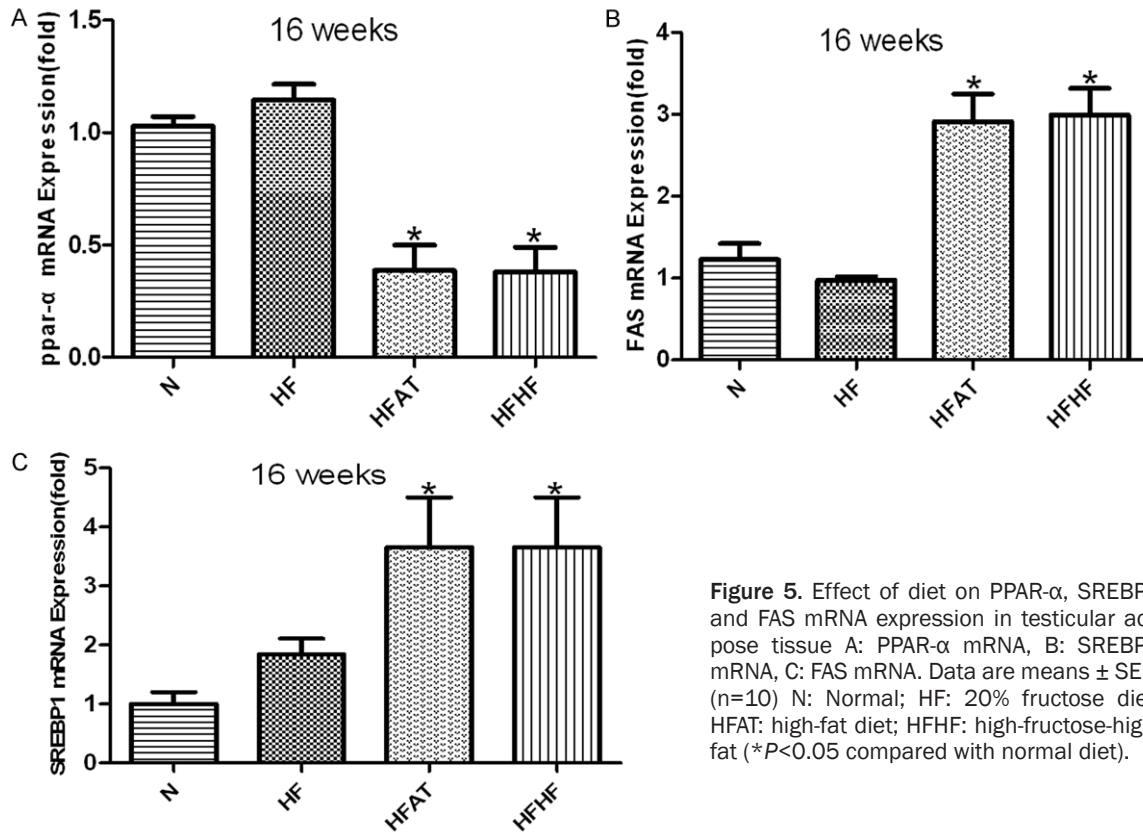


Figure 5. Effect of diet on PPAR- α , SREBP1 and FAS mRNA expression in testicular adipose tissue A: PPAR- α mRNA, B: SREBP1 mRNA, C: FAS mRNA. Data are means \pm SEM (n=10) N: Normal; HF: 20% fructose diet; HFAT: high-fat diet; HFHF: high-fructose-high-fat (* P <0.05 compared with normal diet).

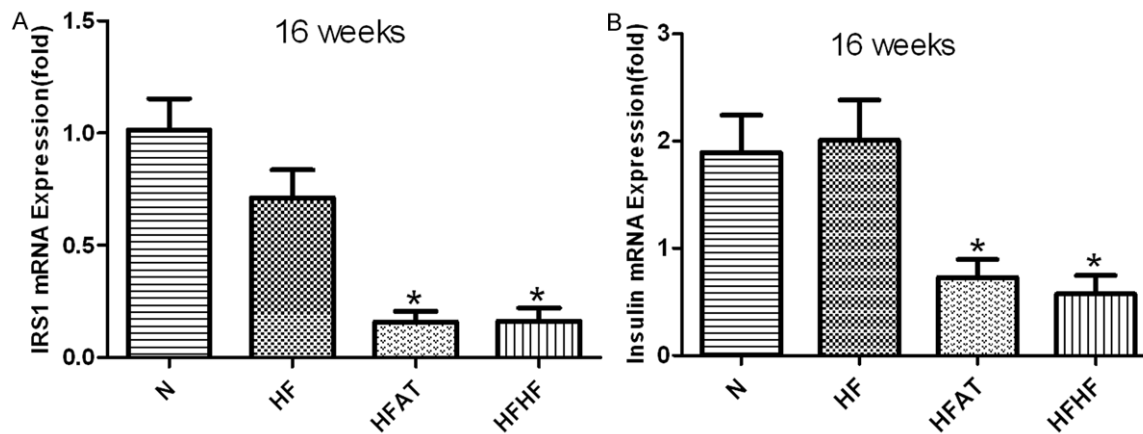


Figure 6. Effect of diets on insulin and IRS1 mRNA expression in testicular adipose tissue A: Insulin mRNA, B: IRS1 mRNA. Data are means \pm SEM (n=10) N: Normal; HF: 20% fructose diet; HFAT: high-fat diet; HFHF: high-fructose-high-fat (* P <0.05 compared with normal diet).

this is associated with NAFLD [19]. Meanwhile, TLR4 contributes to the development of fructose-induced hepatic steatosis [20] although fructose itself cannot induce NASH. Furthermore, fructose also increases enzymes involved in DNL [21].

HFAT dietary induced obesity is associated with insulin resistance and evidence suggests that increased fructose consumption and dyslipidemia, visceral adiposity, and insulin resistance are related [11]. Research suggests that fructose induces hepatic and extrahepatic insulin

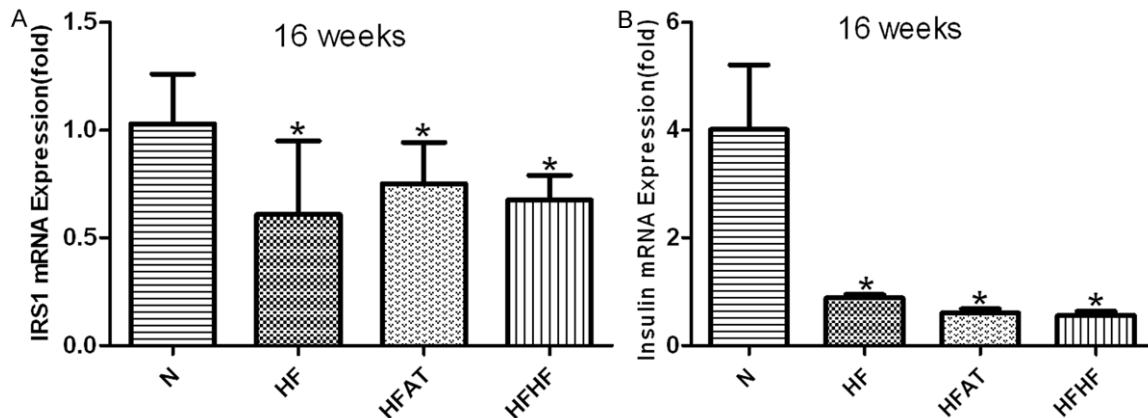


Figure 7. Effects of diet on hepatic insulin and IRS1 mRNA expression A: IRS1 mRNA, B: Insulin mRNA. Data are means \pm SEM (n=10) N: Normal; HF: 20% fructose diet; HFAT: high-fat diet; HFHF: high-fructose-high-fat (* P <0.05 compared with normal diet).

resistance in healthy adults [22]. In this work, hepatic IRS1 and insulin mRNA expression decreased in all non-control diets, but both decreased only in adipose tissue for HFAT and HFHF animals. Fructose did not induce significant insulin resistance in adipose tissue, so we suggest that insulin resistance initially occurred in the liver and then in adipose tissue, and this may be explained by hepatic fructose metabolism. Fructose alone may induce insulin resistance in adipose tissue if it is increased or if prolonged feeding is allowed, but more work is needed to verify this.

Fructose can induce hypertriglyceridemia [23, 24], even short-term high-fructose intake can increase DNL and liver fat [25]. We noted that serum glucose and TG increased significantly for all but control diets and lipogenesis increased as did hyperglycemia both of which are associated with lipid metabolic changes, decreasing oxidation and increasing hepatic TG synthesis.

Because the etiology of NAFLD involves inflammatory factors, we measured TLR4 signaling pathways. TG synthesis due to long-term fructose consumption can improve hepatic fat accumulation and a HF diet can influence intestinal growth and permeability and prompt increased LPS in the portal vein. This increased LPS can activate TLR4 signaling pathways in Kupffer cells, activating expression of NF- κ B and inducing TNF- α which can lead to insulin resistance [26-30].

We observed that TLR4 signaling pathways, MyD88-TIRAP or TRIF-TRAM pathways, were activated in all but control diets. The MyD88-TIRAP pathway leads to rapid activation of NF- κ B and increased TNF- α production. The TRIF-TRAM pathway activates tank-binding kinase 1/inhibitor of kappa B kinase and IRF3, leading to production of type I IFNs and delayed NF- κ B activation. As a key transcription factor for regulating type I IFN, IRF3 is vital for defending exogenous invasion of microbiota by adaptive immunity [31].

Adipose tissue is involved in the formation of NAFLD so we measured lipogenesis and fatty acid oxidation genes, such as PPAR- α , SREBP-1 and its target gene FAS in testicular adipose tissue. PPAR- α decreased after HFAT and HFHF diets only. PPAR- α can upregulate lipid metabolism-related genes and prompt mitochondrial β -oxidation and decrease serum lipids [32]. Defects in PPAR- α can lead to steatohepatitis. Our data agreed with published findings [33] that expression of SREBP-1 increased in all but control diets and FAS, regulated by SREBP1, followed a trend similar to SREBP-1. Increased expression of SREBP-1c can increase *de novo* synthesis of hepatic free fatty acids by insulin stimulation, thus promoting fatty livers [34].

Conclusion

A fructose- and fat-enriched diet can activate hepatic TLR-4 signaling pathways, and insulin resistance transcription factor is increased in

hepatic and adipose tissue. Lipogenesis and fatty acid oxidation genes also change significantly after a HFHF diet. This study will aid in providing a stronger focus on our diets in the future. Eating less high-fat foods and drinking fewer high-fructose beverages, may be the key to preventing NALD and insulin resistance.

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Disclosure of conflict of interest

None.

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