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RESEARCH ARTICLE

Effects of Propylthiouracil-Induced Hypothyroidism on Nonalcoholic Fatty Liver Disease in Rats Fed a High-Fat and High-Cholesterol Diet

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ABSTRACT

Nonalcoholic fatty liver disease (NAFLD) is a common liver disease that manifests as a wide range of pathological conditions ranging from fat accumulation to inflammation, fibrosis and finally cirrhosis. Recently, it suggested that hypothyroidism is closely associated with NAFLD. In addition, 11.7% of hepatocellular carcinoma (HCC) patients were shown to have hypothyroidism. In this study, Wistar rats were fed different high-fat diets (with or without propylthiouracil) and allowed to develop NAFLD. At the end of the experiments, the serum and liver were collected for the analysis of lipid and cholesterol metabolism parameters. Our results showed that the hypothyroid rats had a fleshless body, significantly higher concentrations of total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-c) in the serum and an increased serum alanine transaminase (ALT) level. In addition, the concentration of serum triglycerides and the expression of ApoB100, MTP, SREBP-2 and ABCA1 in the hypothyroid rats showed fluctuating patterns similar to that of liver triglycerides. This finding suggested that propylthiouracil-induced hypothyroidism plays an important role in progressive NAFLD.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the most common liver disease and includes several wide-ranging liver conditions ranging from simple fatty liver to nonalcoholic steatohepatitis (NASH), which is a more serious form of NAFLD that may progress to cirrhosis and hepatocellular carcinoma (HCC) (Fujita *et al*., 2009; Nobili *et al*., 2013). Approximately 10 to 24% of the general population has NAFLD (Musso *et al*., 2003) and approximately 20 to 30% of NAFLD patients may develop histologic signs of fibrosis and necrotizing inflammation, indicative of NASH. Obesity, diabetes, hyperdyslipidemia and insulin resistance are the main features of NAFLD (Marchesini *et al*., 2003), and up to 80% of NAFLD patients are considered obese (Musso *et al*., 2012). Although excessive fat ingestion has recently been linked to obesity and insulin resistance in the general population, the correlation between excessive fat intake and NAFLD in humans is controversial (Hu *et al*., 2001). However, cellular stresses such as oxidative stress, lipid oxidation, direct lipid toxicity and mitochondrial dysfunction induced by drugs and related diseases have been shown to result in NASH (Fujita *et al*., 2009).

Hypothyroidism has been shown to be associated with insulin resistance and dyslipidemia, which are important components of NAFLD (Xu *et al*., 2012). Recently, 11.7% of HCC patients were demonstrated to be affected by hypothyroidism, suggesting a link between hypothyroidism and HCC (Pagadala *et al*., 2012). Propylthiouracil, which has milder teratogenic effects than methimazole, has been considered appropriate for treating maternal hyperthyroidism during the first trimester of pregnancy (Hackmon *et al*., 2012). Although propylthiouracil reduces liver cell damage and increases oxygen delivery to the liver in patients with chronic

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alcohol intake (Carmichael *et al*., 1993), it has the propensity to cause acute liver failure, with 29.4% of patients on this drug dying of fulminant hepatic failure (Aydemir *et al*., 2005; Atawodi *et al*., 2014). Although propylthiouracil has been used to induce hypothyroidism in rats for mechanistic studies (Silva *et al*., 2011), the correlation of hypothyroidism with NAFLD has not been explored extensively. The present paper describes effects of propylthiouracil-induced hypothyroidism on nonalcoholic fatty liver disease in rats fed a high-fat and high-cholesterol diet.

MATERIALS AND METHODS

Animals: Eight-week-old male Wistar rats were purchased from the Center of Experimental Animals of Baiqiuen Medical College of Jilin University (Jilin, China). The animals were housed in plastic cages in a temperature-controlled room at 24±1°C with 50-60% humidity and a 12-h light–dark cycle. The rats were allowed free access to water and food throughout the study. All animal experiments were performed in accordance with the guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Rats adapted to a control diet based on the AIN-93G semi-purified rodent diet (Reeves *et al*., 1993) for 7 days before the experiments were divided into the following groups: (1) the C6 group, which was fed the control diet for 6 weeks; (2) the HF6 group, which was fed a high-fat and high-cholesterol diet for 6 weeks; (3) the HFP2, HFP6 and HFP10 groups, which were fed the HFP diet for 2, 6 and 10 weeks, respectively. Rats in all the groups received water and their diets (Table 1) *ad libitum* during the research period. During the experimental period, the body weight of each rat was measured at baseline and subsequently once every week with a digital scale.

Blood and tissue sample collection: At the end of the experiments, the rats were subjected to 10 to 14 h of fasting, after which they were anesthetized by intramuscular injections of ketamine hydrochloride (100 mg/kg BW) and xylazine hydrochloride (50 mg/kg BW) and euthanized by decapitation. After collecting blood in dry tubes, the serum was stored at -80°C. After exsanguination, the liver was removed and cleaned with saline, and part of the liver was stored at −80°C for further analysis, and part of the tissue was frozen in liquid nitrogen for preparing cryosections for Oil Red O staining. The remaining tissue was fixed in 4% formaldehyde and embedded in paraffin for hematoxylin-eosin (HE) staining.

Analysis of biochemical parameters: Biochemical parameters, including the levels of serum triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-c), high-density lipoprotein cholesterol (HDL-c) and alanine aminotransferase (ALT), were analyzed using an automatic clinical analyzer (Beckman Coulter Synchron DXC800, Beckman Coulter. Inc., USA). The liver TG and TC were analyzed using an enzymatic kit (Applygen Technologies Inc, Beijing, China).

Serum free triiodothyronine (FT3), free thyroxine (FT4) and TSH levels were measured by means of a chemiluminescence immunoassay (ADVIA Centaur XP, Immunoassay System; Siemens Healthcare Diagnostic Inc, Tarrytown, NY, USA).

RT-PCR: Total RNA was extracted from the hepatic tissue of each rat using TRIzol reagent and reverse transcribed to cDNA (Takara). The RT-PCR was performed in an ABI Prism 7500 Real-time PCR System (Applied Biosystems) using the fluorescent dye SYBR-Green (Thunderbird SYBR qPCR Mix, Toyobo Co., Ltd, Japan). The following primers were used in the PCRs: apolipoprotein B100 (ApoB100) sense, 5'-CAGTAGTAG TGGTGCGTCTT-3', and antisense, 5'-CCTGGATTTG TCGGTCTA-3'; microsomal triglyceride transfer protein (MTP) sense, 5'-AGGCTGGGGAAGGGCCCGTC-3', and antisense, 5'-AATGTTCTTCACATCCATGT-3'; sterol regulatory element-binding protein 2 (SREBF-2) sense, 5'-GCAGTCTGGTGGACAGTGATG-3', and antisense, 5'-TGACCGAGGAGCGTGAGT-3'; ATP-binding cassette transporter ABCA1 sense, 5'-TACACC TGACACACCAGCTACAAG-3', and antisense, 5'-GGAACAAAGCCAGCTCCTGA-3'; apolipoprotein A1 (ApoA1) sense, 5'-AAAGGCATCTAAAGGTTGTGG-3', and antisense, 5'-CTATCAGGGTAGGGTGGTT-3'; and apolipoprotein E (ApoE) sense, 5'-GCTGTTGGTC CCATTGCT-3', and antisense, 5'-CGAGTCGGTTGCG TAGATC-3'. GAPDH (sense, 5'-GGCAAGTTCAATGG CACAGT-3'; antisense, 5'-TGGTGAAGACGCCAGTA GACTC-3') was used to normalize the data. The fluorescent signals were collected during the extension phase, and the Ct values were calculated. Then, the transcript levels were analyzed by the 2-ΔΔCt method.

Statistical analysis: All data are presented as the mean±SEM and the statistical analysis was performed by post hoc Duncan's multiple range tests using SPSS (Statistical Package for the Social Sciences) 19.0 software to determine the effect of the treatment and to compare differences among the different treatment groups. Differences with $P<0.05$ were considered significant.

RESULTS

Body weight analysis: As shown in Fig. 1, the baseline body weight was not significantly different among the groups ($P<0.05$). Compared with other groups, the rats in HF6 gained a significant amount of weight at week 2. The body weights of the HF6 rats were not significantly different from those of the C6 rats during the following 2 weeks. The HF6 group showed the highest weight gain and weighed the most at week 6 compared with the other groups. When the HFP diet (Table 1) was used, the rats had a significantly lower weight gain than the controls at week 2. The highest weight of the HFP rats was achieved at week 3, followed by weight loss toward the end of the research period.

Biochemical parameters analysis of the serum and liver: Compared with the C6 and HF6 groups, the HFP rats showed significantly lower levels of FT3 and FT4 and

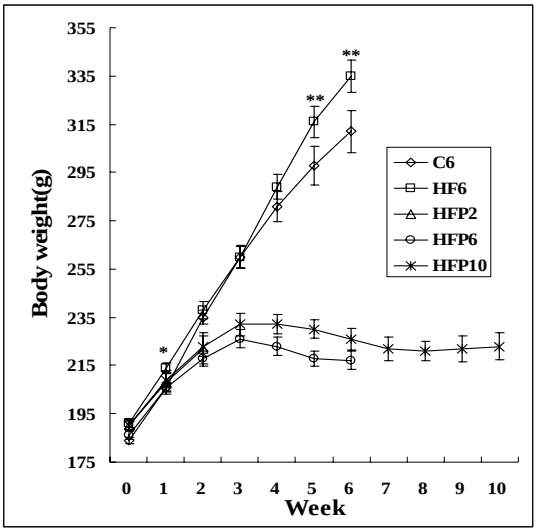

Fig. 1: Weekly body weight measurements of the rats in each group. The values are presented as the mean±SEM, n=6. *HF6 is significantly different from the other groups, P<0.05. ** HF6 is significantly different from the C6 group.

Table 1: Detailed composition of the diets used in the experiments

Ingredients (g/kg)	C[1]	HF[2]	HFP[3]
Cornstarch	549.486	439.486	438.486
Soybean oil	50	50	50
Lard	0	100	100
Sucrose	100	100	100
Cholesterol	0	10	10
Propylthiouracil	0	0	1
Casein	200	200	200
Fiber	50	50	50
Mineral mix	35	35	35
Vitamin mix	10	10	10
L-Cysteine	3	3	3
Choline bitartrate	2.5	2.5	2.5
Tert-butylhydroquinone	0.014	0.014	0.014

[1]Control diet; [2]High-fat and high-cholesterol diet; [3]High-fat and high-cholesterol diet with the addition of propylthiouracil.

had an elevated TSH level in serum (Table 2). After the 6-week-long high-fat and high-cholesterol diet, the HF6 rats developed typical NAFLD, showing marked TG accumulation and high TC levels in liver (Table 2), and their TG, TC and LDL-c levels in serum were also higher than those of the control group. Although the exudation of ALT was higher in HF6 than in C6, however, there was no significant difference between the 2 groups (P>0.05). However, serum HDL-c, which is a primary marker of dyslipidemia, was significantly lower in the HF6 rats than in other rats. When the HFP diet was used in this study, high concentrations of TC, LDL-c and ALT were detected in the blood of HFP rats during the entire study period. The concentration of serum HDL-c and TG in the serum and liver increased during this period.

Hepatic gene expression: To analyze the effect of a compounded diet on hepatic lipid metabolism, several important genes were analyzed. Apolipoprotein B (ApoB), the primary apolipoprotein component of low-density lipoproteins, has 2 main isoforms, ApoB48 and ApoB100. ApoB100 is mainly secreted in the liver and transports TG and cholesterol in the form of very-low-density lipoprotein (VLDL) from the liver to peripheral tissues. In this study, the HF6 rats had a significantly higher expression of liver ApoB100 compared with the control group (Fig. 2). The HFP rats also showed an increased expression of ApoB100 following the prolonged HFP diet. In this study, the MTP expression pattern was similar to that of ApoB100 in the 3 groups, with a significantly higher expression in the HF6 rats and an increased expression in the HFP rats. SREBP-2, a regulator of cholesterol metabolism, was expressed at high levels in the HF6 rats and an increased expression levels observed in the HFP groups. As shown in Fig. 2, the HF6 rats had the highest levels of ABCA1 expression, and an increase in ABCA1 expression was observed in the HFP rats after the entire study period. Apolipoprotein A1 is the major protein component of high-density lipoproteins in blood. The expression of ApoA1 in high-fat and high-cholesterol diet-fed rats (HF6 and HFP) was significantly inhibited compared with that of the control groups. ApoE is essential for the normal catabolism of TG-rich lipoproteins. ApoE is primarily produced by the liver and macrophages and is found in the chylomicrons and intermediate-density lipoprotein (IDLs). Interestingly, the expression of ApoE in the 3 groups was not significantly different.

Hepatic histopathology: As shown in Fig. 3, the liver of control rats demonstrated normal hepatocytic texture with a homogenous cytoplasm and a large spherical nucleus (Fig. 3A). Liver sections from high-fat and high-cholesterol-fed rats showed severe panlobular micro- and macrovesicular steatosis (Fig. 3).

DISCUSSION

The most common liver disease, NAFLD, is present in approximately 10 to 24% of the general population, and 80% of NAFLD patients are considered obese. The high-fat diet, a common diet in industrialized countries, has recently been linked to NAFLD (Musso *et al*., 2003). In this study, a high-fat diet containing 0.1% cholesterol was used to develop an NAFLD rat model. Compared with the control rats, the HF6 rats showed typical symptoms of NAFLD at week 6, with increased body weight gain and marked fatty infiltration in hepatocytes with macro- or micro-vesicular steatosis (Fig. 3). The high level of total cholesterol in liver is related to its increased synthesis and absorption and decreased effusion. In this study, bile, the main metabolic pathway of cholesterol, should have a high accumulation in the liver of a hungry rat that has fasted for approximately 12 h, which is an animal lacking a gallbladder. In addition, the HF6 rats showed high SREBP-2 expression, which has been shown to increase the expression of low-density lipoprotein receptor (LDLr) and HMG-Co A reductase (HMGCoA-r), leading to increased cholesterol synthesis and absorption in liver (Fuchs and Stange, 2001). However, elevated TG and TC levels in the liver have been suggested to promote oxidative stress and cause steatohepatitis (Matsuzawa *et al*., 2007). In addition, serum HDL-c, which can remove cholesterol from cells and atheroma, is considered to be preventative for coronary heart disease and atherosclerotic disease. Therefore, lower serum HDL-c in the HF6 rats was suggested as being an increased risk factor for atherosclerosis (Lee *et al*., 2012).

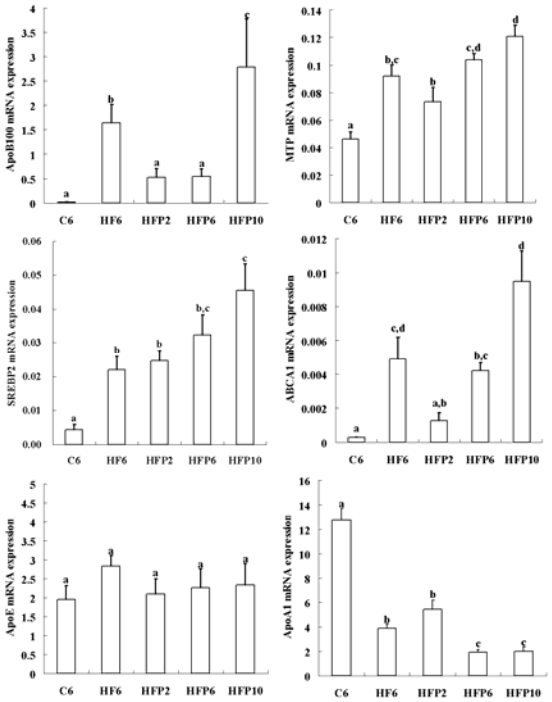

Fig. 2: Hepatic gene expression patterns. A comparison of gene expression levels in the livers of rats in the C6, HF6, HFP2, HFP6 and HFP10 groups by post hoc Duncan's multiple range tests. The values represent the mean±SEM, n=4-6 per diet group. For each gene, the values with different letters are significantly different from one another (P<0.05).

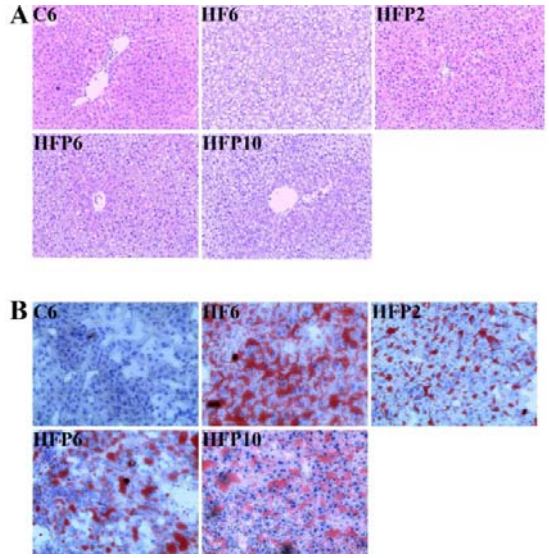

Fig. 3: Representative liver histopathology. (A) Liver paraffin sections from the C6, HF6, HFP2, HFP6 and HFP10 groups, HE staining, 100× magnification. (B) Liver cryosections from the C6, HF6, HFP2, HFP6 and HFP10 groups, Oil Red O staining, 200× magnification.

Current research demonstrates that hypothyroidism is associated with insulin resistance and dyslipidemia, all of which are important components of NAFLD. Moreover, a report of 11.7% of HCC cases having hypothyroidism has indicated a correlation between hypothyroidism and HCC (Pagadala *et al*., 2012). Propylthiouracil has been used to treat hyperthyroidism by decreasing the amount of thyroid hormone (Nakamura *et al*., 2007), but because serious liver injury can occur, including liver failure and death, propylthiouracil is no longer recommended in non-pregnant adults and children as the front-line antithyroid medication (Bahn *et al*., 2009). However, propylthiouracil is widely used to induce hypothyroidism for mechanistic studies. In this study, propylthiouracil was used to induce hypothyroidism, the concentrations of total cholesterol and LDL-c in the serum of hypothyroidism (HFP) rats were found to be significantly higher than those of HF6 rats. Although the thyroid hormone can increase cholesterol synthesis by activating hydroxy-methyl-glutaryl coenzyme A (HMG-CoA) in liver, it plays a more robust role in activating 7α-hydroxylase to promote cholesterol excretion through bile. Therefore, patients with hypothyroidism have high serum levels of total cholesterol (Ness *et al*., 1990). With the decreased FT3 and FT4 in HFP groups, thyrotropin-releasing hormone (TSH) is released from the hypothalamus to ameliorate hypothyroidism by stimulating thyroid activity (Diaz-Espineira *et al*., 2008). ApoB100 and MTP expression also showed a positive correlation with the serum TG levels (Fig. 2). Compared with the stable levels of serum TC, LDL-c and liver TC in HFP rats, the expression of SREBP-2 and ABCA1 was increased. Moreover, it has been shown that ApoB secretion is regulated by several factors, including phospholipids, free cholesterol, TG and cholesteryl esters (Wilcox *et al*., 1999). These results suggest that hepatic TG play key roles in regulating apoB100 secretion in the liver (Moon *et al*., 2012). Increased levels of apoB100 and apoB100-containing lipoproteins act as activator of MTP and promote the excretion of cholesterol from the liver to the blood, thereby stimulating the expression of SREBP-2 and ABCA1 (Tietge *et al*., 1999). MTP plays a key role in the assembly of TG and ApoB100 into VLDL and in the secretion of VLDL from the liver (Shindo *et al*., 2010). As a cholesterol efflux pump in the cellular lipid removal pathway, the ATP-binding cassette transporter ABCA1 was present in higher quantities in the liver, small intestine and adipose tissues (Schmitz and Langmann, 2001). On the HFP diet, the rats showed an increase in serum ALT levels during the long-term study period. Although a non-specific correlation between the ALT levels and NASH and advanced fibrosis has yet to be established, the ALT level has been reported to be a predictor for NASH and other chronic liver diseases (Verma *et al*., 2013). ALT is considered to be marker of hepatic injury and antioxidant activity (Hsieh *et al*., 2013). Propylthiouracil-induced hypothyroidism has been suggested to play an important role in progressive NAFLD.

Somatostatin is produced by the hypothalamus with a regulatory mechanism opposite to that of TSH. Therefore, somatostatin analogs have been demonstrated to be chemotherapeutic agents for lung cancer, breast cancer and, more recently, HCC by directly inhibiting cell proliferation (Yuan *et al*., 2008). In this study, somatostatin was most likely indirectly induced by hypothyroidism, leading to the reduced growth in HFP group rats. Moreover, compared with the HF6 rats, a slower liver TG accumulation and increased serum ALT levels were detected in the HFP6 rats, casting doubt on the liver-preserving role of somatostatin. The slow TG

Table 2: Analysis of biochemical parameters in the serum and liver

	C6	HF6	HFP2	HFP6	HFP10
Serum					
TG (mmol/L)	0.45±0.06^{a}	0.75±0.1^{b}	0.49±0.06^{a}	0.74±0.08^{b}	1.19±0.05^{c}
TC (mmol/L)	1.09±0.04^{a}	2.05±0.24^{a}	8.94±1.65^{b}	8.67±0.81^{b}	8.05±0.61^{b}
HDL-c (mmol/L)	0.52±0.03^{ab}	0.38±0.01^{a}	0.51±0.08^{ab}	1.1±0.42^{c}	1.5±0.07^{d}
LDL-c (mmol/L)	0.34±0.02^{a}	0.87±0.07^{a}	4.17±0.72^{b}	4.32±0.24^{b}	4.13±0.68^{b}
ALT (U/L)	50±3.3^{a}	53.5±7.98^{a}	78.2±24.9^{ab}	112.7±13.5^{bc}	122.5±12^{cd}
FT3 (pg/mL)	2.58±0.23^{a}	2.74±0.59^{a}	0.49±0.33^{b}	0.35±0.06^{b}	0.33±0.05^{b}
FT4 (ng/dL)	1.59±0.19^{a}	1.43±0.24^{a}	0.06±0.05^{b}	0.04±0.03^{b}	0.03±0.04^{b}
TSH (uIU/mL)	0.01±0.001^{a}	0.011±0.003^{ab}	0.014±0.002^{b}	0.019±0.001^{c}	0.020±0.003^{c}
Liver					
TG (μmol/g)	23.1±2.1^{a}	114±8.3^{b}	66.3±3.0^{c}	79.5±7.4^{c}	115.3±5.0^{b}
TC (μmol/g)	15.9±0.5^{a}	89.7±3.1^{b}	107±7.5b^{c}	97.4±1.8^{bc}	95.9±5.6^{bc}

Values (mean±SEM) within a row that do not share a common superscript are significantly different (P<0.05).

accumulation in the HFP rats could be a result of the somatostatin-induced inhibition of cell proliferation. The reduced cell proliferation and elevated lipolysis and free fatty acid delivery to the liver exacerbate hepatic injury (Bugianesi *et al*., 2005). Although the hypothesis regarding somatostatin and liver preservation and its correlation with the high serum levels of TC and LDL-c needs to be further confirmed, a high risk for heart disease has been extrapolated from data obtained through the biochemical analysis of the HFP diet rats (Sert *et al*., 2013).

In this study, a high-fat and high-cholesterol diet was used to analyze the lipid and cholesterol metabolism in rats. Based on our results, we are unable to determine whether in non-gall bladder animals such as rats, the high liver cholesterol in fasting rats has a major effect on lipid metabolism. Our results also demonstrated that the serum and liver TG levels had similar patterns of accumulation in the hypothyroid rats, suggesting that the use of serum TG levels as a predictor of hepatic TGs needs further research.

Conclusion: In this study, rats fed a high-fat and high-cholesterol diet developed typical NAFLD, the characteristics of which included significant weight gain and a marked fatty infiltration into hepatocytes. Based on these conditions, propylthiouracil was used to induce a hypothyroidism rat model. With a high-fat and high-cholesterol diet, the hypothyroid rats had a fleshless body and significantly higher concentrations of TC and LDL-c in the serum, which are important risk factors for cardiovascular diseases. These rats showed an increase in serum ALT levels throughout the period they were fed the propylthiouracil-added high-fat diet. It has been suggested that propylthiouracil-induced hypothyroidism may potentially play an important role in progressive NAFLD.

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