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Original Article

Dietary high-fat lard intake induces thyroid dysfunction and abnormal morphology in rats

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Aim: Excess dietary fat intake can induce lipotoxicity in non-adipose tissues. The aim of this study was to observe the effects of dietary high-fat lard intake on thyroid in rats.
Methods: Male Sprague-Dawley rats were fed a high-fat lard diet for 24 weeks, and then the rats were fed a normal control diet (acute dietary modification) or the high-fat lard diet for another 6 weeks. The serum lipid profile, total thyroxine (TT4), free thyroxine (FT4) and thyrotropin (TSH) levels were determined at the 12, 18, 24 and 30 weeks. High-frequency ultrasound scanning of the thyroid glands was performed at the 24 or 30 weeks. After the rats were sacrificed, the thyroid glands were collected for histological and immunohistochemical analyses.
Results: The high-fat lard diet significantly increased triglyceride levels in both the serum and thyroid, and decreased serum TT4 and FT4 levels in parallel with elevated serum TSH levels. Ultrasonic imaging revealed enlarged thyroid glands with lowered echotexture and relatively heterogeneous features in the high-fat lard fed rats. The thyroid glands from the high-fat lard fed rats exhibited enlarged follicle cavities and flattened follicular epithelial cells under light microscopy, and dilated endoplasmic reticulum cisternae, twisted nuclei, fewer microvilli and secretory vesicles under transmission electron microscopy. Furthermore, the thyroid glands from the high-fat lard fed rats showed markedly low levels of thyroid hormone synthesis-related proteins TTF-1 and NIS. Acute dietary modification by withdrawal of the high-fat lard diet for 6 weeks failed to ameliorate the high-fat lard diet-induced thyroid changes.
Conclusion: Dietary high-fat lard intake induces significant thyroid dysfunction and abnormal morphology in rats, which can not be corrected by short-term dietary modification.

Keywords: high-fat lard diet; thyroid gland; hypertriglyceridemia; hypothyroidism; ER stress; TSH; TTF-1; NIS; lipotoxicity

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Introduction

Dietary fat is an essential component of the human diet and serves many important functions[1]. However, excess dietary fat intake promotes ectopic accumulation of triglycerides and free fatty acids in non-adipose depots, known as lipotoxicity, which contributes to chronic cellular dysfunction and injury[2–4]. In recent years, lipotoxicity has been well documented in the pathogenesis of many diabetes-related metabolic diseases[5–9]. In fact, in the early 1990s, dietary fat overabundance was reported to interfere with the endocrine system[10]. Thyroid diseases, especially hypothyroidism characteristic of thyroid dysfunction, have reached epidemic proportions worldwide, although the reasons for the increased prevalence of these diseases remain unclear.

The thyroid gland, a crucial endocrine organ that synthesizes and secretes thyroid hormones, plays an important role in organic metabolism and in the development, differentiation and maintenance of the central nervous system, skeletal system, and cardiovascular system[11]. Thus, maintaining thyroid homeostasis is essential for human health. The production and secretion of thyroid hormones by the thyroid are directly regulated by the hypothalamus-pituitary-thyroid axis[12]. The pituitary gland serves as a sophisticated biosensor of thyroid hormone levels and regulates thyrotropin (TSH) levels according to the feedback of free thyroxine (FT4) and free triiodothyronine (FT3). A decrease in circulating thyroid hormone stimulates more secretion of TSH by the pituitary. TSH then

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acts via specific receptors on the membrane of thyroid follicular cells to facilitate compensatory stimulation of thyroid hormone production and secretion, which involves a number of thyroid hormone synthesis-related molecules, such as sodium/iodide symporter (NIS), thyroglobulin (Tg), and thyroperoxidase (TPO). The combined measurements of serum TT4, FT4, and TSH levels are widely recommended to fully assess thyroid function[13, 14].

Limited studies have demonstrated altered thyroid hormone levels in animals fed a high-fat diet. A mixed high-fat diet resulted in elevated serum TSH with normal serum T3 and T4 levels in a study conducted by Araujo RL[15], whereas time-dependent decreases in serum TT3 and TT4 were observed according to Han *et al*[16]. The different high-fat compositions in the two studies may underlie the inconsistent results. In addition, serum thyroid hormone levels are primarily regulated by the thyroid gland itself, and the above studies did not investigate this crucial aspect.

To date, there are few reports investigating whether excessive intake of lard, the most common dietary lipid, has a unique contribution to the pathogenesis of thyroid dysfunction. This study focused on not only serologic but also morphological evidence to address this issue in an animal model. Rats were fed a high-fat lard diet to investigate the potential preliminary mechanisms involved. The effects of acute withdrawal from a high-fat lard diet on thyroid dysfunction were also studied. Our findings suggest that excess dietary lard intake may be a detrimental factor in the development of thyroid dysfunction, and acute dietary modification by withdrawal of dietary lard intake did not restore thyroid function. These results may aid in the development of novel methods to protect against hypothyroidism.

Material and methods

Animals, diets, and experimental design

The animal experimental protocol was approved by the Animal Ethics Committee of Shandong Provincial Hospital. Seventy 6-week-old male Sprague-Dawley rats weighing 170–190 g were obtained from Vital River Laboratory Animal Technology Co Ltd (Beijing, China). Animals were housed 2 per cage, maintained in constant temperature-controlled rooms (22–25 °C) with a 12-h light/dark cycle, and had free access to food and water. After being acclimated to the housing conditions for 1 week, they were randomly assigned to one of the two diets: (1) normal control diet (n=30): 100% standard rodent chow (NC group, 3.49 kcal/g) or (2) high-fat lard diet (n=40): 85% standard rodent chow supplemented with 15% lard (HF group, 4.14 kcal/g). As shown in Figure 1, the rats were fed the specified diet for 24 weeks before 20 rats from each group were sacrificed. The remaining animals in the NC group (n=10) were maintained on their prospective diet for an additional 6 weeks, whereas those animals fed a high-fat lard diet (n=20) were further subdivided into two subgroups. The first subgroup (HF+NC, n=10) was withdrawn from the high-fat lard diet and reintroduced to the normal control diet, whereas the second subgroup (n=10) was maintained on the high-fat lard diet. All rats were sacrificed at the end of the additional 6-week feeding period. The composition of the experimental diets was assayed by Beijing Research Institute for Nutritional Resources, and details are shown in Table 1. To avoid potential oxidation, each diet was repackaged into weekly portions, vacuum-sealed, and stored at 4 °C in the dark until use.

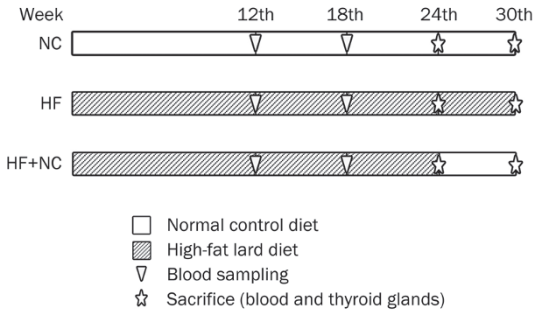

Figure 1. Schematic outline of the feeding regimens used in this study. Rats were fed normal control diet (NC) or high-fat lard diet (HF) for 24 weeks. Twenty rats in each group were sacrificed, and the blood and thyroid glands were properly collected. The remaining rats in the NC group (n=10) were continuously fed a normal control diet while those in the HF group were either fed a high-fat lard diet (n=10) or a normal control diet (n=10) for the following 6 weeks before their sacrifice.

Table 1. Composition of experimental diets.

	Normal control diet	High-fat lard diet
Protein, g/100 g	20	17
Carbohdrate, g/100 g	58	49
Fat, g/100 g	6	21
Selenium, g/100 g	1.4×10^{-5}	1.6×10^{-5}
Cholesterol, g/100 g	0	0
Fatty acids, g/100 g		
C14:0	0.02	0.20
C16:0	0.97	5.62
C16:1	0.02	0.24
C18:0	0.21	2.05
C18:1	1.23	6.37
C18:2	2.57	3.49
C18:3	0.17	0.18
Total saturated	1.11	7.88
Total monounsaturated	1.22	6.78
Total polyunsaturated	2.93	3.67
Total kcal/g	3.4	4.1

The body weights of the animals were monitored weekly for the duration of the initial 24-week feeding period. Fasting blood samples were obtained by subclavian venous puncture at the 12th, 18th, and 24th weeks. The serum was isolated for lipid profile analysis and quantification of TT4, FT4, and TSH levels. After high-frequency ultrasound scanning of the thyroid gland at the 24th or 30th week, all rats were sacrificed

after being fasted for 8 h. The thyroid glands were quickly excised and properly collected. The left lobe was fixed in 4% paraformaldehyde or 3% glutaraldehyde and 1% osmium tetroxide for morphological analysis, and the right lobe was saved in liquid nitrogen for assessment of protein expression and triglyceride content.

Serum parameter analysis

Serum levels of triglycerides, total cholesterol and low-density lipoprotein cholesterol (LDL-cholesterol) were measured using enzymatic methods with Olympus reagents and automated spectrophotometry performed on an Olympus AU5400 system (Olympus Corporation, Tokyo, Japan). Serum TT4, FT4, and TSH were measured by ELISA kits (CUSABIO, Wuhan, China). All procedures were carried out in accordance with the instructions provided by the manufacturers.

High-frequency ultrasound scanning of thyroid gland

High-frequency ultrasound scanning of the thyroid gland was performed with a Philips 7500 Ultrasound System (Philips Medical Systems, Andover, MA, USA), using a 12.0-MHz transducer. The rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (3%). After moving animals into position, hairs were gently removed from the neck and high thorax area with an electric shaver and depilatory cream. Coupling gel was used to obtain a tight contact between the ultrasound transducer and the skin of rats to minimize ultrasound attenuation. The ultrasound technique was standardized to include transverse and longitudinal images of both lobes of the thyroid gland and the adjacent structures in the neck. The mediolateral, anteroposterior, and craniocaudal diameters of each lobe were determined 3 times and averaged, and the ultrasonographic features of the thyroid gland were recorded. The volume of each lobe was calculated using the formula (width×depth×length×π/6). The isthmus was not included in the calculation of thyroid volume. The total volume was calculated by summing the volume of each lobe. To eliminate inter-observer error, all ultrasonographic assessments were performed by a single experienced investigator who was blind to the experimental groups[17].

Light microscopic analysis of thyroid histology

For microscopic analysis, the thyroids were fixed overnight at 4 °C in 4% paraformaldehyde at pH 7.2, dehydrated through ethanol series, cleared in xylene, embedded in paraffin, and 4 μm sections were cut (at least 4–5 consecutive sections per sample). Slides were stained with hematoxylin and eosin according to manufacturer instructions. A microimaging system (Axio Imager A2, Zeiss, Jena, Germany) was used to observe the histopathological changes in the thyroid tissues.

Transmission electron microscopy

For transmission electron microscopic observation, the thyroid glands were fixed in buffered 3% glutaraldehyde and 1% osmium tetroxide, dehydrated through ethanol series, and embedded with epoxy resin[18]. Ninety nanometer sections were cut using a LKB-V ultramicrotome (LKB, Bromma, Sweden) and doubly stained with uranyl acetate and lead citrate. Ultrastructure was observed and photographed using a transmission electron microscope (H-800, Hitachi, Tokyo, Japan).

Immunohistochemistry

Sections were dewaxed by standard techniques, and heat treatment to retrieve the antigen sites was performed. To quench endogenous peroxidases, the sections were treated with 3% hydrogen peroxide in dH_2O at room temperature. The sections were incubated for 1 h at room temperature with blocking solution and then with primary antibodies overnight at 4 °C. The primary antibodies included rabbit polyclonal antibody against NIS (Biorbyt, Cambridge, UK) and rabbit monoclonal antibody against TTF-1 (Abcam, Cambridge, MA, USA). For the negative control, IgG was added instead of the primary antibody. The reactivity of the antibodies was detected using a streptavidin-peroxidase histostain-SP kit. The peroxidase activity was visualized with diaminobenzidine, followed by hematoxylin staining to counterstain the nuclei. Positive staining appeared as a brown-yellow color.

Thyroid triglyceride content assay

Thyroid triglycerides were extracted and assayed according to manufacturer's instructions (Applygen Technologies Inc, Beijing, China). All data were normalized by the protein concentration in a parallel well.

Statistical analysis

Data were analyzed by SPSS 18.0 software and were expressed as the mean±standard deviation (SD). Means were compared using unpaired Student's *t*-tests for comparisons between two groups, and one-way ANOVA for comparisons among multiple groups. Correlations between two variances were examined using Pearson's correlation analysis. A two-tailed P value <0.05 was considered significant.

Results

A high-fat lard diet increased body weight and serum lipid profiles in rats

The body weights of rats were monitored weekly during the initial 24-week feeding period. The two groups of rats had similar body weights at baseline and throughout the first 17 weeks of the diet regimens (P>0.05). However, the rats in the HF group gained significantly more weight than those in the NC group from the 18th week to the end of the study (P<0.05, Figure 2A).

To observe the effects of the high-fat lard diet on circulating lipid profiles, serum levels of triglycerides, total cholesterol and LDL-cholesterol were examined. At the 24th week, the rats in the HF group exhibited a significant increase of approximately 79% in serum triglyceride levels compared with those in the NC group (P=0.002, Figure 2B). There was no significant difference in either total cholesterol or LDL-cholesterol between the two groups. The results indicated that the high-fat lard diet successfully induced a rat model of hypertriglyc-

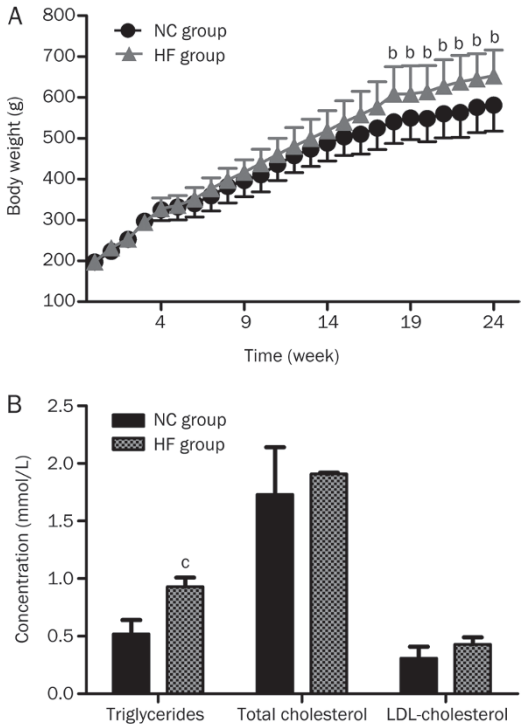

Figure 2. The effect of a high-fat lard diet on body weight and serum lipid profile. The body weights of the rats were monitored weekly (A), and serum triglycerides, total cholesterol and LDL-cholesterol levels of the rats were assayed on the 24th week (B) of both the normal control (NC) and high-fat lard (HF) diet regimens. Data points are presented as the mean±SD (n=20 per group). ^{b}P<0.05 and ^{c}P<0.01 *vs* the corresponding NC group.

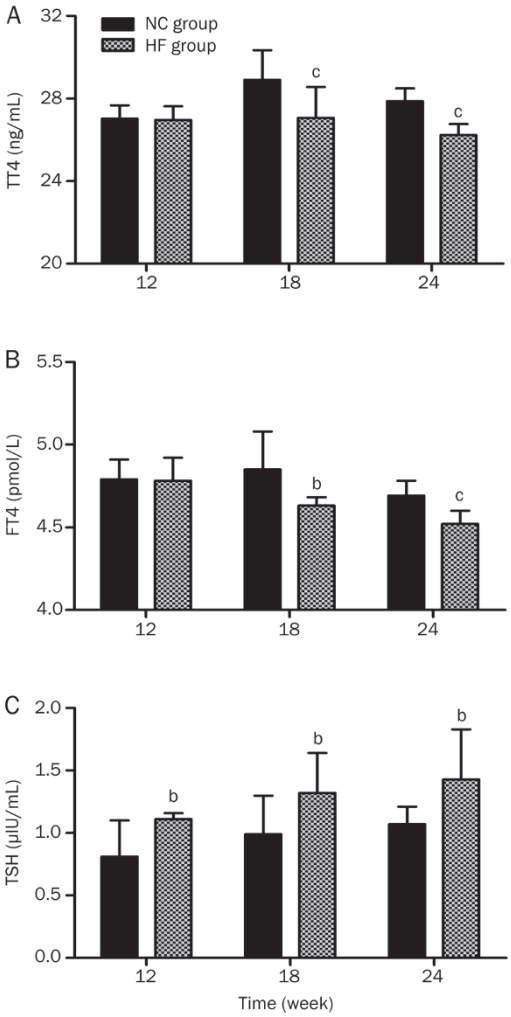

Figure 3. The time course effect of a high-fat lard diet on thyroid function. The serum TT4 (A), FT4 (B), and TSH (C) levels were measured by ELISA kits in rats fed a normal control diet (NC group) or a high-fat lard diet (HF group) at the 12th, 18th, and 24th weeks. Data are presented as the mean±SD (n=20 per group). ^{b}P<0.05 and ^{c}P<0.01 *vs* the corresponding NC group.

eridemia.

A high-fat lard diet altered rat serum levels of thyroid hormones and TSH

To observe whether thyroid dysfunction occurs following high-fat lard diet feeding, we measured serum TT4, FT4, and TSH levels. As shown in Figure 3, neither TT4 nor FT4 levels in the two groups were obviously different at the 12th week. However, later in the study, rats in the HF group exhibited an obvious decrease in serum TT4 and FT4 levels resulting from exposure to a high-fat lard diet for 18 weeks when compared with the NC group (TT4: P<0.01 at both the 18th and 24th weeks; FT4: P<0.05 at the 18th week, and P<0.01 at the 24th week), while serum TSH concentrations were considerably higher (P<0.05 at the 12th, 18th, and 24th weeks) at the corresponding time points.

A negative correlation between serum levels of triglycerides and TT4 in rats

To further determine the relationship between hypertriglyceridemia and decreased thyroid hormone levels, we conducted a Pearson's correlation analysis. Serum TT4 levels were negatively associated with serum triglyceride concentrations (r=-0.35; P=0.027; Figure 4A) in rats from the two groups. This correlation showed the concentration-dependent effects of serum triglyceride levels on thyroid function.

A high-fat lard diet increased rat thyroid triglyceride content

To clarify whether there was any change in the triglyceride content in the thyroid gland when serum triglyceride levels increased, we assayed the triglyceride content of thyroid tissues at the 24th week. Thyroid triglyceride content of the rats in the HF group was approximately 2.4-fold (P=0.032) greater than that of the NC group (Figure 4B), suggesting that a high-fat lard diet could increase not only serum triglyceride content but also thyroid gland triglyceride content as well.

A high-fat lard diet altered thyroid morphological ultrasonic features in rats

To determine whether a high-fat lard diet contributes to

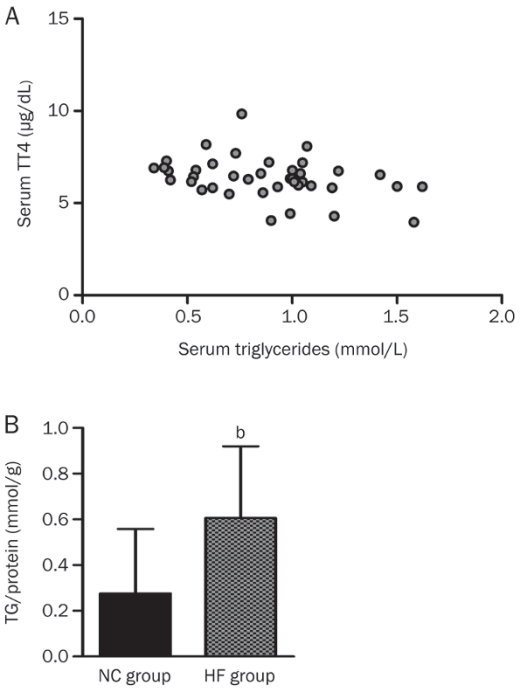

Figure 4. A correlation analysis and thyroid triglyceride content assay. Rats were treated with a normal control diet (NC group) or a high-fat lard diet (HF group) for 24 weeks. (A) Correlation between serum triglycerides and total T4 (TT4) levels was analyzed. r=-0.35, P=0.027. (B) Rat thyroid triglyceride content was assayed and corrected by the total protein content. Data are presented as the mean±SD (n=9 per group). $^bP<0.05$ *vs* the NC group.

morphological and organizational changes, we continued by studying the ultrastructure of rat thyroid glands by ultrasound imaging at the 24th week.

We assessed the echo structure of the thyroid parenchyma and volume under high-frequency ultrasound scanning. Under physiological conditions, the normal parenchyma was characterized by a homogeneous appearance, with greater reflectivity compared with the adjacent muscles, and a similar intensity but different texture with respect to the salivary glands[17]. As shown in Figure 5A, the thyroid glands in the NC group showed a homogeneous appearance, while those in the HF group presented an enlarged thyroid with lower echotexture and relatively heterogeneous features. In addition, the thyroid volumes of either an individual or the total lobe as measured by ultrasound scanning exhibited an obvious enlargement, with an increase of approximately 50% over that of the NC group (Table 2).

Next, we dissected the rats to perform a gross inspection (Figure 5B) and found that the thyroid glands in the HF group were visually enlarged, which is in accordance with the volume calculated by the high-frequency ultrasound scanning.

A high-fat lard diet disturbed rat thyroid histomorphology and ultrastructure

Histomorphometry (Figure 5C) revealed that thyroid tis-

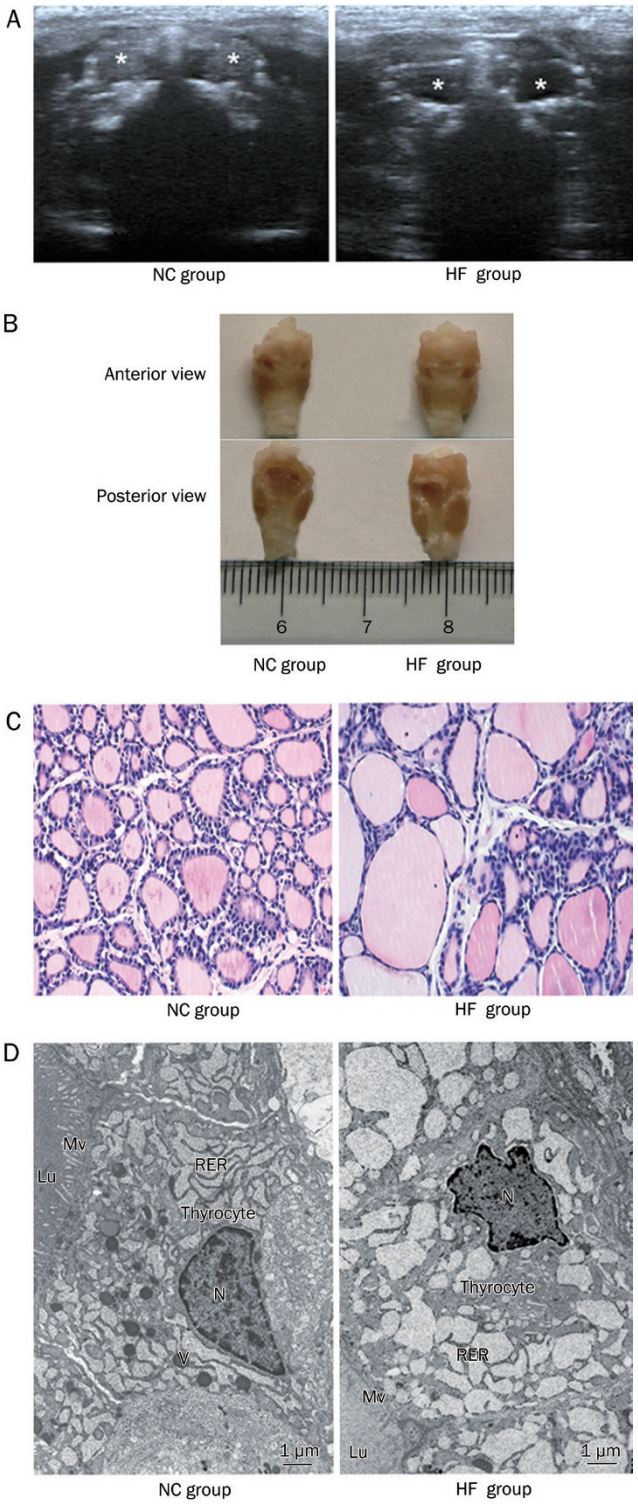

Figure 5. The morphology scanning of rat thyroid. Rats were fed a normal control diet (NC group) or a high-fat lard diet (HF group) for 24 week. (A) The echo structure of the thyroid parenchyma under high-frequency ultrasound scanning. Thyroid lobes are marked by a white star. (B) The gross inspection of thyroid glands from both the anterior and posterior views. (C) The histological changes of thyroid glands stained with H&E (magnification, ×200). (D) The ultrastructural changes of the thyroid glands. Lu, lumen of the thyroid follicle; Mv, microvilli; RER, rough endoplasmic reticulum; V, vesicles of colloid; N, nucleus. Scale bar=1 μm.

Table 2. Effects of high-fat lard diet on thyroid volume.

	NC group (normal control diet)	HF group (high-fat lard diet)
Right lobe diameters		
Mediolateral (mm)	5.16±0.88	5.78±0.42
Anteroposterior (mm)	3.38±0.33	3.57±0.38
Craniocaudal (mm)	10.40±0.97	13.17±2.95
Right lobe volume (mm^3)	94.47±18.09	140.72±28.25[b]
Left lobe diameters		
Mediolateral (mm)	5.20±1.12	6.30±0.69
Anteroposterior (mm)	3.46±0.33	3.40±0.32
Craniocaudal (mm)	11.42±2.13	13.80±1.94
Left lobe volume (mm^3)	105.78±23.41	152.89±16.11[c]
Thyroid volume (mm^3)	200.25±37.78	293.60±38.95[c]

At the 24th week of feeding, the rat thyroid glands were checked using high-frequency ultrasound scanning and volume of thyroid lobes was calculated by ovoid formula (width×depth×length×π/6). The isthmus was not included in volume calculation. Data are presented as the mean±SD (n=5-6 per group). [b]P<0.05 and [c]P<0.01 versus the corresponding NC group.

sue from the NC group showed normal features under light microscopy, including moderately sized follicles, cube or tall cylinder-like epithelial cells and pink colloid in the follicular cavities. However, the thyroid tissue from the HF group was characterized by different degrees of focal colloid goiter; follicular epithelial cells became flattened and the follicles distended with colloid.

Electron micrographs (Figure 5D) showed that the thyroid follicular epithelial cells of the NC group exhibited neatly arranged microvilli on the apical membrane. The morphology of the endoplasmic reticulum (ER) was normal, with plentiful secretory vesicles and colloid droplets in the apical cell areas. However, in the follicular epithelial cells of the HF group, fewer microvilli and wider perinuclear gaps were observed. There were rare secretory vesicles at the apical pole and dilated ER cisternae were present at the basal pole. Nuclear twist and chromatin condensation were also noted.

A high-fat lard diet decreased the expression of thyroid hormone synthesis-related proteins

Considering that the ER is an important organelle responsible for the generation of many essential proteins in the cells, at the 24th week we examined the protein expression of TTF-1 and NIS, which are key molecules in rat thyroid hormone synthesis. As shown in Figure 6, immunohistochemical staining showed that TTF-1, a transcription factor that can regulate the transcription of its downstream molecules Tg, TPO, and NIS, was mainly located in the nucleus, whereas NIS, the key protein involved in intracellular iodine accumulation, demonstrated a diffuse granular distribution in both the cytoplasm and cytomembrane. The intensity of TTF-1 and NIS protein specific staining was obviously weaker in the HF group, while the staining was robust in the NC group. The suppressed

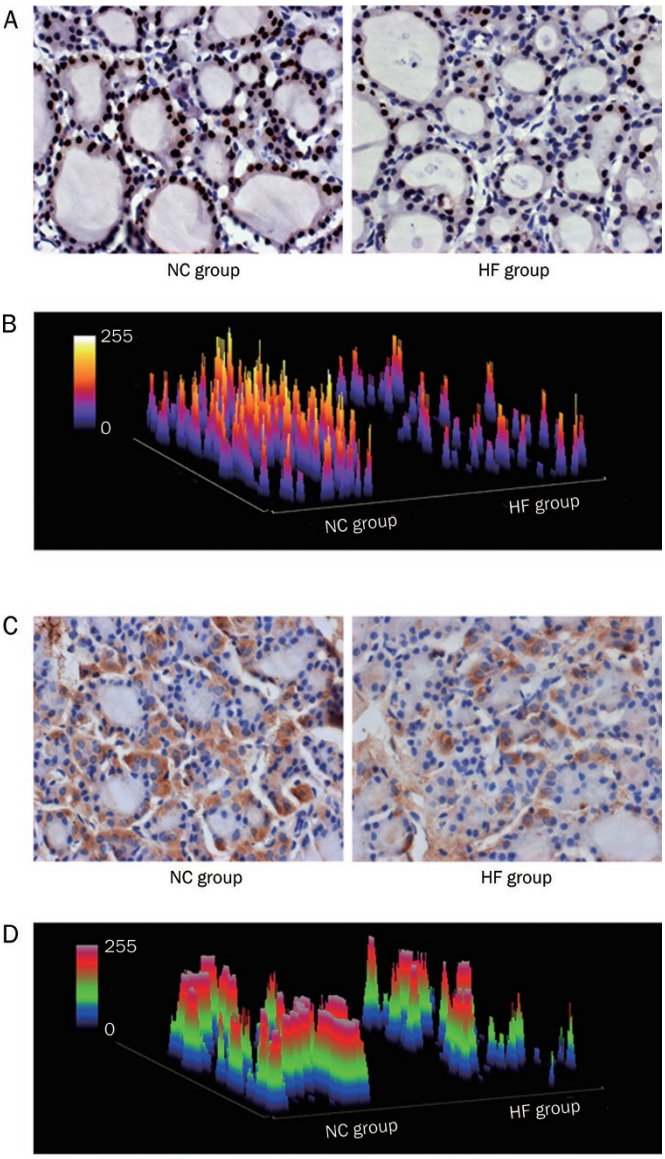

Figure 6. Expression of thyroid hormone synthesis-related proteins. Rats were fed a normal control diet (NC group) or a high-fat lard diet (HF group) for 24 weeks, and the thyroid tissues were removed to test the expression of TTF-1 (A) and NIS (B) using immunohistochemical staining. Negative controls for immunospecificity were included in all experiments by replacing the primary antibody with IgG. The semi-quantitative analysis of staining intensity was conducted using Image J software (lower panels in both A and B). In all panels, the representative pictures of 3 to 5 independent experiments are shown (magnification, ×400).

expression of thyroid hormone synthesis-related proteins indicated an inhibition of thyroid hormone production.

Short-term withdrawal of high-fat lard intake makes little improvement in thyroid function

To evaluate whether excess dietary lard intake-mediated damage in the rat thyroid is reversible, the high-fat lard diet was withdrawn and normal control diet was reintroduced to the

rats for an additional 6 weeks.

As shown in Figure 7A and 7B, the dietary modification from the 24th to the 30th week resulted in diminished serum triglycerides and LDL-cholesterol levels in the HF+NC group to some extent, accompanied by slightly decreased serum TSH and unchanged TT4 and FT4, which seem unresponsive to the relatively short-term withdrawal of the high-fat lard diet.

In accordance with the results at the 24th week, thyroid tissue from the HF group was characterized by different degrees of focal colloid goiter, in which follicular epithelial cells became flattened and the follicles distend with colloid. The HF+NC group exhibited the same features as the HF group (Figure 7C).

The protein expression of TTF-1 in the rat thyroid is shown in Figure 7D. Consistent with the results in Figure 7, the intensity of the representative TTF-1 protein specific staining was obviously weaker in both the HF group and the HF+NC group at the 30th week, while the staining was robust in the NC group. The suppressed expression of TTF-1 was not obviously changed in the HF+NC group at the 30th week.

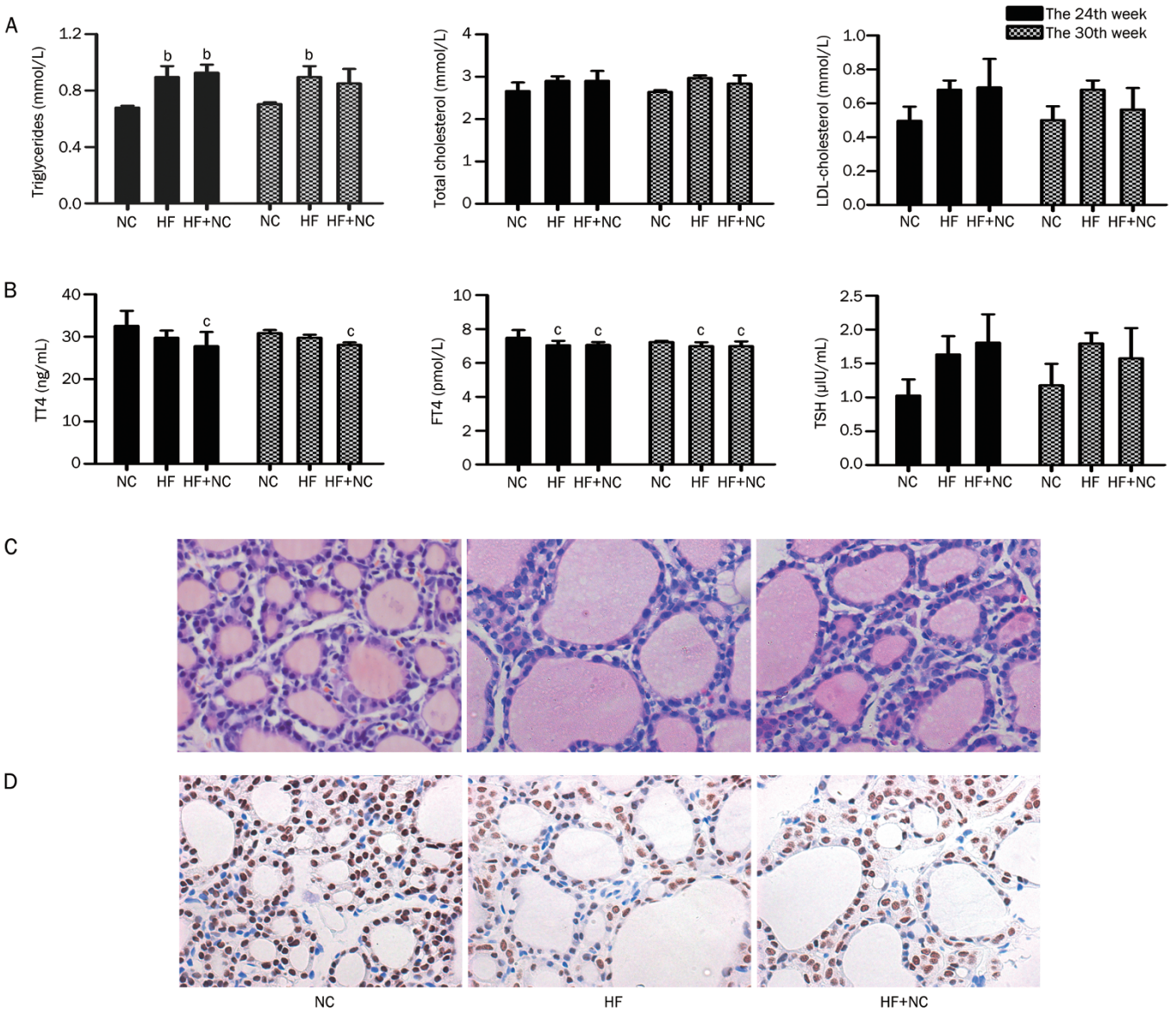

Figure 7. The comprehensive evaluation of rat thyroid function and morphology on a short-term withdrawal of high-fat lard diet. (A) Serum triglycerides, total cholesterol and LDL-cholesterol levels, (B) Serum TT4, FT4 and TSH levels of rats in each group were assayed at both the 24th and the 30th week. Data points are presented as the mean±SD (n=10 or 20 per group), $^{b}P<0.05$ and $^{c}P<0.01$ *vs* the NC group at the corresponding time point. (C) The histological changes of rat thyroid stained with H&E (magnification, ×400) in the NC, HF and HF+NC groups at the 30th week. (D) The protein expression of TTF-1 detected by immunohistochemical staining in the NC, HF, and HF+NC groups at the 30th week. Negative controls for immunospecificity were included in all experiments by replacing the primary antibody with IgG. In all panels, the representative pictures of 3 to 5 independent experiments are shown (magnification, ×400).

Discussion

In the present study, our main finding was that Sprague-Dawley rats fed a high-fat lard diet had decreased serum TT4 and FT4 levels, increased serum TSH levels, and altered macro and micro morphology of the thyroid. Decreased protein expression of TTF-1 and NIS was also observed in the thyroid tissues of rats fed the high-fat lard diet. In addition, the abnormal function and disturbed morphology observed in the rat thyroid cannot be reversed by short-term withdrawal of the high-fat lard diet. To the best of our knowledge, this study is the first to examine excess dietary lard intake as a unique pathogenic factor leading to thyroid dysfunction. Our new findings may contribute to a substantial advance in public health policies aimed at the primary prevention of hypothyroidism.

Rats were chosen as the animal model primarily because rat thyroid glands are easily accessible and of a reasonable size, compared to mice, for morphological observation. Furthermore, rats are more convenient for consecutive blood sampling. As a consequence, rats were widely used in other studies on the thyroid[19–22]. In this study, we successfully collected sufficient blood samples from each rat by subclavian venous puncture instead of other, more invasive or even fatal methods at the end of the 12th, 18th and 24th weeks, and, therefore, greatly avoided interindividual variation.

Lard is one of the most widely consumed foods rich in saturated fatty acids, and it is often chosen to observe the deleterious effects of a high-fat diet[20, 23–25]. In this study, we used standard rodent chow supplemented with lard alone, independent of other hyperlipidemia-inducing components, such as cholesterol. The composition of the high-fat lard diet had 7-fold greater total saturated fatty acid content compared to the normal control diet. The solid pellet diet was used to simulate normal human eating behaviors, and a 24-week high-fat lard feeding was conducted to mimic long-term intake. Under these treatments, serum triglyceride levels in rats fed the high-fat lard diet were significantly increased, which was consistent with the report from Mieke *et al*[26].

In addition to rising serum triglyceride levels, we also found increased thyroid triglyceride content in the rats fed a high-fat lard diet. Matsui *et al*[27] showed that impaired insulin secretion was associated with increased islet triglyceride content in PPAR-γ-deficient (PPAR-$\gamma^{+/-}$) mice fed a high-fat diet. Zucker diabetic fatty rats, a model of obesity secondary to genetic leptin unresponsiveness, rapidly accumulated triglycerides in the heart, which was accompanied by left ventricular remodeling and septal wall thickening[28]. Regarding the liver, many studies in mice[7] and rats[29] have documented high-fat diet induced fat accumulation in the liver and hepatic steatosis. These findings demonstrate that a high-fat diet may promote triglyceride accumulation in non-adipose tissues; the results of the present study convincingly show that a high-fat lard diet increased thyroid triglyceride content.

Thyroid follicles consist of a single layer of follicular epithelial cells surrounding a central follicular lumen filled with proteinaceous colloid. These follicles are the functional units of the thyroid gland and are responsible for the synthesis and secretion of thyroid hormones. Ultrasonography is an effective imaging tool for the assessment of thyroid glands and is commonly used to screen for pathological changes in human thyroid tissue[30, 31]. In recent years, its applications in several animal species, such as horses[32], dogs[33], dolphins[34], and mice[17], have been reported. Homogeneity and hyperechoic echotexture are the basic characteristics of normal parenchyma in thyroid tissue. Reese *et al*[33] reported that the relative thyroid echogenicity of both thyroglobulin antibody-positive and antibody-negative hypothyroid dogs was significantly lower (P<0.001) than those of euthyroid dogs. Similarly, a relatively lower and heterogeneous echotexture of the thyroid was observed in our rats that exhibited decreased thyroid hormone synthesis and hypothyroid features. Interestingly, our observation of flattened epithelial cells and extended follicles in the thyroids of high-fat lard fed rats was consistent with the report by Gao *et al*[35], who demonstrated that rats fed a combination of excess iodine and a low-protein diet had significantly decreased serum thyroid hormone levels. Similarly, the same histomorphological changes in mice were observed by Han *et al*[16] and were described as having a "damaging effect on the thyroid gland". Based on these observations, the altered thyroid histomorphological structure found in this study indicates thyroid dysfunction.

Furthermore, we noticed disorganized cellular ultrastructure in the thyroid of high-fat lard fed rats. The dilated ER and fractured microvilli coincide with Tang's finding[36] in which PCB118, one type of environmental pollutant, could also disrupt the structure of thyroid follicular cells. The ER is a central cellular organelle in which transmembrane and secretory proteins are synthesized, folded, and matured[37]. The ultrastructural changes we observed in the ER always accompany "ER stress"[38–40]. For example, Hanoch *et al*[38] reported that dilated ER lumen accompanied the response to ER stress in *Trypanosoma brucei*. Various genetic and environmental insults lead to the accumulation of unfolded/misfolded client proteins in the ER lumen, termed the unfolded protein response (UPR), resulting in ER stress[41, 42]. Growing evidence supports the involvement of ER stress in lipotoxicity induced by fatty acids, especially long chain, saturated fatty acids. For example, saturated fatty acids induced ER stress in human liver cell lines[43], and a high-fat diet triggered the up-regulation of BiP and other UPR markers in the adipose tissue of rats[44]. These findings indicate that the changes in ER dilation may be associated with enhanced ER stress in thyrocytes induced by high-fat lard feeding.

As a possible consequence of enhanced ER stress and impaired ER function, decreased protein expression of TTF-1 and NIS was observed in the present study. TTF-1 is a homeodomain-containing transcription factor that regulates the transcriptional activation of Tg, TPO, and NIS[45]. Tg, the predominant component of colloid in the follicular lumen, is a secretory protein that provides the backbone for circulating thyroid hormones and contains significant amounts of iodine. TPO is a thyroid-specific heme peroxidase that is localized in

the apical membrane of thyrocytes and that plays a central role in thyroid hormone biosynthesis by catalyzing the oxidation and organification of iodide, as well as in the coupling reactions needed to form the active thyroid hormones T4 and T3. NIS, a transmembrane protein necessary for the intracellular accumulation of iodine, plays an important role in thyroid hormone biosynthesis in thyroid follicular cells[46]. As described in the study by Ulianich *et al*, enhanced ER stress was associated with dramatically decreased expression of the thyroid-specific markers TTF-1, NIS, Tg, and TPO in PCCl3 thyroid cells[47]. Thus, the up-regulation of ER stress observed in this study was associated, at least to some extent, with the decreased protein expression of TTF-1 and NIS. The combination of the aforementioned changes resulted in decreased serum TT4 and FT4 concentrations, and a feedback increase in TSH induced by high-fat lard feeding.

Although we observed an association between the changes in the thyroid gland *per se* and serum thyroid hormone levels under a high-fat lard diet, we cannot exclude the possibility that overstimulation of the pituitary gland may contribute to the elevated serum TSH levels. However, we propose that it did not interfere with our conclusion. If serum TSH levels were elevated as a result of high-fat lard diet-induced pituitary hyperactivation, serum thyroid hormone levels should be increased as well. However, we found decreased thyroid hormone levels in this study. Therefore, the pituitary gland should not be considered a main source of the elevated serum TSH concentrations observed in this study.

Dietary modification is a cornerstone of modern preventive and therapeutic approaches to lipid-related metabolic diseases. Little is known about the effect of dietary modification on thyroid dysfunction. Nariaki *et al*[48] reported that the decrease in rat thyroid function induced by kojic acid, a widely used food additive proven to interfere with iodide uptake, is reversible upon a 48 h-withdrawal of kojic acid. In our study, the 6-week withdrawal of high-fat lard diet had no obvious therapeutic effect on the abnormal function and disturbed morphology in rat thyroid induced by a 24-week high-fat lard feeding. The chronic deterioration and pathogenic effect associated with excess dietary lard intake may necessitate a correspondingly long period to recover, which would help to explain our present results. Nonetheless, our findings reinforce the concept that thyroid dysfunction induced by long-term high-fat lard feeding cannot be expected to rapidly recover only with dietary modification.

In conclusion, we demonstrated that long term high-fat lard feeding could dramatically decrease the levels of TT4 and FT4, increase the concentration of TSH in the serum, affect the structure of the thyroid gland, and down-regulate the protein expression of thyroid hormone synthesis-related molecules. Lipotoxicity and enhanced ER stress may participate in the excess dietary lard intake-induced thyroid dysfunction. Although the precise underlying mechanisms require further investigation, the withdrawal of excess dietary lard intake could not improve thyroid dysfunction within a short span of time.

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Author contribution

Jia-jun ZHAO designed the study; Shan-shan SHAO performed the research; Shan-shan SHAO, Jian-mei YANG, Shi-meng XUAN, and Jin XU raised the animals; Chun-xiao YU and Hui-li YAN contributed new reagents and analytic tools; Shan-shan SHAO, Yuan-fei Zhao, Yong-feng SONG, Chao XU, and Meng ZHAO wrote the manuscript.

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