Original Research

Carbohydrate-to-fat ratio affects food intake and body weight in Wistar rats

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Abstract

The aim of the study was to evaluate the impact of carbohydrate-to-fat ratio on body weight and appetite regulation in Wistar rats. Twenty-four Wistar rats were randomized to three dietary groups ($n = 8$): normal carbohydrate diet (NC), low-carbohydrate diet (LC) and high-carbohydrate diet (HC) for 12 weeks. Body weight and food intake were recorded. Circulating leptin and insulin levels were measured by radioimmunoassay method. The expression levels of leptin receptor, insulin receptor, orexin, neuropeptide Y (NPY), agouti-related protein (AgRP) and melanocortin-4 receptor (MC-4R) in the hypothalamus were also measured by realtime polymerase chain reaction (PCR). In the LC group, food intake reduced while body weight increased significantly compared with the NC and HC groups. Plasma leptin levels increased in the LC (18.5 ± 8.2 ng/mL) group compared with the NC (8.6 ± 3.8 ng/mL, $P < 0.001$) and HC (6.6 ± 1.9 ng/mL, $P < 0.001$) groups. Realtime reverse transcription-PCR revealed a decrease in the hypothalamic expression level of only leptin receptor in the LC (0.764, 0.471–4.648 copy/mL) and HC (0.357, 0.129–0.781 copy/mL) groups compared with the NC (1.323, 0.616–2.392 copy/mL; $P = 0.01$) group, and that there was no significant change in those of insulin receptor, AgRP, Orexin, NPY and MC-4R. Low-carbohydrate, high-fat diet raised body weight, which led to a rising of circulating leptin levels and a reduced expression of leptin receptor in the hypothalamus.

Keywords: carbohydrate-to-fat ratio, food intake, body weight, appetite regulation

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Introduction

Both human and animal studies have demonstrated that saturated fat intake is an important risk factor in obesity development.[1,2] Even so, arguments still exist as to whether different macronutrients influence food intake or body weight, independent of their total caloric content.[3–5] To examine the role of macronutrients in obesity development, Klaus[6] investigated the effects of diets with different protein (Pr)/carbohydrate (CHO) on body weight in mice. As it is clear, the manipulation of diets is assumed to be a method for testing macronutrient effects. Body weight gains were rapid and greater in HC (high CHO) mice than in other groups due to an initial pronounced hyperphagia and subsequent passive overconsumption. Another animal study showed that a high-fat diet would lead to overfeeding and obesity when the diet is also rich in CHO, whereas the absence of CHO would favor lipid oxidation rather than deposition.[7] Therefore, we hypothesized that the CHO/fat ratio would affect food intake and body weight.

The human body has a highly sophisticated and precise mechanism to regulate energy intake and expenditure to ensure that body weight is relatively constant. The regulation is influenced by hormonal, environmental and gene factors. Leptin is predominantly produced by adipose tissue and insulin is secreted by pancreatic β cells. Circulating leptin and insulin levels are proportional to adiposity and both affect appetite-inhibitory function by acting on receptors in the hypothalamus.[8,9] Obesity is often associated with hyperleptinemia and hyperinsulinemia. Leptin and insulin trigger the appetite-inhibitory circuit through the upregulation of α-melanocyte-stimulating hormone, which is mainly mediated by the melanocortin type 4 receptor (MC-R4), and inhibit the appetite-stimulatory neuron by suppressing neuropeptide Y (NPY) and agouti-related peptide (AgRP) mRNA expression in the hypothalamus.[10] Orexin, a new neuropeptide found in 1998, was found to be stimulated by fat consumption.[11,12]

Therefore, we aimed to investigate the effect of three diets with different CHO/fat ratios on body weight, and

circulating leptin and insulin levels in three-week-old Wistar rats. The expression of hypothalamic appetite genes was also quantified to reveal the possible mechanism involved.

Materials and methods

Animals and diet protocols

Twenty-four three-week-old male Wistar rats were purchased from Shanghai Slac Laboratory Animal Ltd (Shanghai, China) and fed a commercial rat chow (M01-F, Shanghai Slac Laboratory Animal Ltd) for three days before the study started. The rats were then randomly assigned to three dietary groups (each group $n = 8$): normal CHO diet (NC:Pr, 20%; CHO, 65%; fat, 15%), low CHO diet (LC:Pr, 20%; CHO, 20%; fat, 60%) and high-CHO diet (HC:Pr, 20%; CHO, 75%; fat, 5%) for 12 weeks. Cornstarch and lard were utilized to change the CHO-to-fat ratio (Table 1). The rats were raised in a 12-hour light–dark cycle, a constant room temperature of 20–22 °C and humidity of 60–70%. Rats were given diets and water *ad libitum*. Body weight was recorded once a week and food intake was recorded twice a week. At the end of the study, rats were fasted overnight and anesthetized by intraperitoneal sodium pentobarbital (60 mg/kg body weight). The rats were killed between 8:00 and 10:00, and blood samples were obtained by heart puncture. Hypothalami were immediately removed as described previously[13] and frozen in liquid nitrogen for further examination.

This animal study was approved by the Animal Ethics Committee of Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University.

Blood analyses

Blood was collected in EDTA-coated tubes and centrifuged at 1200 *g* at 4°C for 10 min. Plasma leptin and insulin were double analyzed using a rat radioimmunoassay kit (Linco Research, St Charles, MO, USA, Cat. #RL-83K, RI-13K), according to the manufacturer's instructions. For rat leptin, the limit of sensitivity and linearity is 0.5 and 50 ng/mL (100 μL sample size), respectively. In the case of rat insulin, the limit of sensitivity and linearity is 0.1 and 10 ng/mL (100 μL sample size), respectively.

Table 1 Diet formula

	NC	LC	HC
Ratio of calorie (kcal%)			
Protein	20	20	20
Fat	15	60	5
Carbohydrate	65	20	75
Calorie density (kcal/g)	4.75	7.00	4.25
Ingredient (g)			
Commercial rat chow (M01-F)	90.9	38.46	40.36
Corn starch	17.73	–	54.01
Casein	–	11.54	11.12
Lard	1.67	24.55	–
Vitamins mix		10.0 g/kg diet	
Minerals mix		35.0 g/kg diet	
Fiber		5.0 g/kg diet	
Total	110.3	74.55	105.49

NC, normal carbohydrate; LC, low carbohydrate; HC, high carbohydrate
Note: (1) Corn starch and lard were utilized to change the carbohydrate-to-fat ratio. (2) *Minerals mix* (1000 g): $CaHPO_4$, 500.0 g; NaC1, 74.0 g; $K_3C_6H_5O_7\ H_2O$, 220.0 g; K_2SO_4, 52.0 g; MgO, 24.0 g; $MnCO_3$, 3.5 g; iron citrate, 6.0 g; $ZnCO_3$, 1.6 g; $CuCO_3\ Cu(OH)_2\ H_2O$, 0.3 g; KIO_3, 0.01 g; $Na_2SeO_3\ 5H_2O$, 0.01 g; $CrK(SO_4)_2\ 12H_2O$, 0.55 g; sucrose, 118.97 g; (3) *vitamins mix* (1000 g): vit B_1, 600 mg; vit B_2, 600 mg; vit B_6, 700 mg; vit PP, 3000 mg; pantothenic acid, 1600 mg; folic acid, 200 mg; vit B_{l2}, 1 mg; biotin, 20 mg; vit E, 5000 U; vit A, 400,000 U; vit K, 5 mg; vit D_3, 2.5 mg; sucrose, 899.095 g

Realtime reverse transcription polymerase chain reaction

Total RNA was extracted from brain tissue samples using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA, Cat. #10296028), according to the manufacturer's instructions. Equal volumes of total RNA were transcripted into first-strand cDNA using a reverse transcription kit for qPCR (Shinegene Company, Shanghai, China, Cat. #ZK00805). Realtime PCR was completed using the TaqMan system. Primer sets for β-actin, leptin receptor, insulin receptor, orexin, NPY, AgRP and MC-4R were designed according to the sequence obtained from public databases (www.ncbi.nlm.nih.gov). Sequence-specific fluorogenic TaqMan probes were also designed according to the above-mentioned database (Table 2). A Roche Real Time PCR (Lightcycler3) System was used to detect fluorescence during 40 PCR cycles. Data analysis was carried out as described previously.[14] Briefly, a threshold value in the exponential phase of the amplification plot was set, and the cycle number during which fluorescence exceeded this threshold (ΔC_T) was determined for each sample. A standard curve ($E = 10^{(45.49-\Delta C_T)}/3.189$) was determined according to a dilution series of β-actin mRNA concentrations. Relative quantification of leptin and insulin genes was calculated as Q_T/Q_R, where Q_T is the copies of leptin and insulin genes and Q_R is the copies of β-actin. Copies of leptin, insulin and β-actin were calculated from the standard equation. No amplification was detected in the absence of the template or in the no RT control.

Statistical analysis

Data were expressed as means $\pm$ standard error of means if the data were in normal distribution. The difference among the groups was tested by analysis of variance. If the data distributed abnormally, data were shown as median plus range and K-independent non-parametric test was used. A two-sided P value of 0.05 was considered significant. A Spearman linear correlation was used to reveal the possible relationship between body weight, plasma leptin and insulin.

Results

Body weight

Initial body weight showed no obvious difference among the groups before the study (NC, 49.8 $\pm$ 3.3 g; LC, 49.1 $\pm$ 6.1 g; HC, 48.5 $\pm$ 3.8 g; $P = 0.55$). Body weight was lower in HC fed rats at the fourth (201.8 $\pm$ 13.6 g versus NC, 214.7 $\pm$ 14.5 g, $P < 0.001$; versus LC, 213.7 $\pm$ 19.3 g, $P < 0.001$) and fifth weeks (240.6 $\pm$ 15.3 g versus NC, 251.7 $\pm$

Table 2 Gene-specific primers and TaqMan probes for realtime RT-PCR

Primers/probes	Oligonucleotide sequence	Product (bp)
Leptin receptor		80
Forward	5′-GGGAACCTGTGAGGATGAGTGT-3′	
Reverse	5′-TTTCCACTGTTTTCACGTTGCT-3′	
Probe	Fam + TCAACCCTCAGTTAAATATGCAACGC + tamra	
Insulin receptor		69
Forward	5′-TTCACTGGGTACCGCATTGA-3′	
Reverse	5′-AGCCACGCCTGACCTCTCT-3′	
Probe	Fam + AGGCATGCAATCAGGACTCCCCAG + tamra	
Orexin		119
Forward	5′-ACGAACTGTTGCACGGAGCT-3′	
Reverse	5′-GTTACCGTTGGCCTGAAGGA-3′	
Probe	Fam + ACGCCGCGGGCATCCTCA + tamra	
NPY		80
Forward	5′-ATGATGCTAGGTAACAAACGAATGG-3′	
Reverse	5′-GCCAGAATGCCCAAACACA-3′	
Probe	Fam + TGGACTGACCCTCGCTCTATCCCTGC + tamra	
MC-4R		71
Forward	5′-CGCGCTCCAGTACCATAACA-3′	
Reverse	5′-AAGCTGCCCAGATACAACTGATG-3′	
Probe	Fam + TATGACGGTTAGGCGGGTCGGGA + tamra	
AgRP		63
Forward	5′-CCGCGTCGCTGTGTAAGG-3′	
Reverse	5′-CAGGTCGCAGCAAGGTACCT-3′	
Probe	Fam + TGCACGAGTCCTGCTTGGGA + tamra	
β-actin		138
Forward	5′-TGGAGTCTACTGGCGTCTT-3′	
Reverse	5′-TGTCATATTTCTCGTGGTTCA-3′	
Probe	Fam + CTGAAGGGTGGGGCCAAAAG + tamra	

RT-PCR, reverse transcription polymerase chain reaction; NPY, neuropeptide; MC-4R, melanocortin-4 receptor; AgRP, agouti-related peptide
Note: (1) Primer sets for β-actin, leptin and insulin receptor were designed according to the sequence obtained from public databases (www.ncbi.nlm.nih.gov). (2) All oligonucleotides were synthesized by Shinegene Company (Shanghai, China)

8.1 g, $P = 0.01$; versus LC, 254.1 ± 20.7 g, $P < 0.001$); however, this phenomenon disappeared during the following period. At the eighth week, body weight in the LC group (364.8 ± 32.3 g) was significantly higher (versus NC, 329.2 ± 13.0 g, $P = 0.02$; versus HC, 315.2 ± 19.7 g, $P < 0.001$) and this tendency continued over the following four weeks (Figure 1).

Food and calorie intake

Daily food intake of LC rats (6.9 ± 0.8 g) reduced significantly (versus NC, 11.4 ± 0.8 g, $P < 0.001$; versus HC, 10.9 ± 0.5 g, $P < 0.001$) in the first week, and this phenomenon lasted throughout the study. No statistical difference was observed between the NC and HC groups except for the ninth week (19.7 ± 0.2 versus 17.2 ± 0.1 g, $P < 0.001$). Daily calorie intake of HC fed rats was less than those in NC and LC fed rats (versus NC and LC groups, both $P < 0.05$), while no obvious difference was observed between the NC and LC groups (Figure 2).

Circulating leptin and insulin circulating leptin increased significantly in the LC group (18.5 ± 8.2 ng/mL) than that in NC (8.6 ± 3.8 ng/mL, $P < 0.001$) and HC (6.6 ± 1.9 ng/mL, $P < 0.001$) at the end of the study. Plasma insulin showed no statistical difference among the groups ($P = 0.49$). Data were shown in Table 3. Body weight showed a positive correlation with plasma leptin ($r = 0.88$, $P < 0.001$) among 24 Wistar rats, while no statistical correlation was observed between plasma insulin and body weight ($P = 0.24$).

Expression of appetite gene in hypothalamus

Hypothalamic expression level of leptin receptor decreased in the LC (0.764, 0.471–4.648 copy/mL) and HC (0.357, 0.129–0.781 copy/mL) groups compared with the NC (1.323, 0.616–2.392 copy/mL; $\chi^2 = 9.92$, $P = 0.01$) group. No significant difference was found among the three groups with regard to the expression levels of insulin receptor, AgRP, orexin and MC-R4 (Table 4). The expression level of NPY was higher in the LC (2.307, 0.827–5.649 copy/mL) group than in the other two groups (NC: 1.692, 0.461–2.392 copy/mL; HC: 0.483, 0.180–4.925 copy/mL), but without statistical difference ($\chi^2 = 2.61$, $P = 0.27$).

Discussion

Although obesity is the result of a positive energy balance imbalance between energy intake and energy expenditure, some epidemiological studies reported that the development of obesity correlated better with dietary fat intake

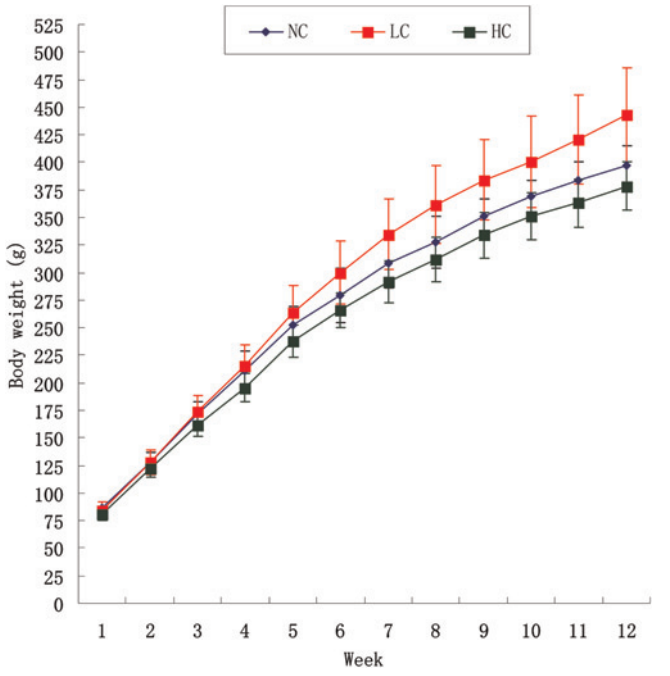

Figure 1 Body weight in the study. Body weight was lower in HC fed rats at the fourth (201.8 ± 13.6 g versus NC, 214.7 ± 14.5 g, $P < 0.001$; versus LC, 213.7 ± 19.3 g, $P < 0.001$) and fifth weeks (240.6 ± 15.3 g versus NC, 251.7 ± 8.1 g, $P = 0.01$; versus LC, 254.1 ± 20.7 g, $P < 0.001$); however, this phenomenon disappeared during the following period. At the eighth week, body weight in the LC group (364.8 ± 32.3 g) was significantly higher (versus NC, 329.2 ± 13.0 g, $P = 0.02$; versus HC, 315.2 ± 19.7 g, $P < 0.001$) and this tendency continued over the following four weeks. NC, normal carbohydrate; LC, low carbohydrate; HC, high carbohydrate. Each group: $n = 8$ (A color version of this figure is available in the online journal)

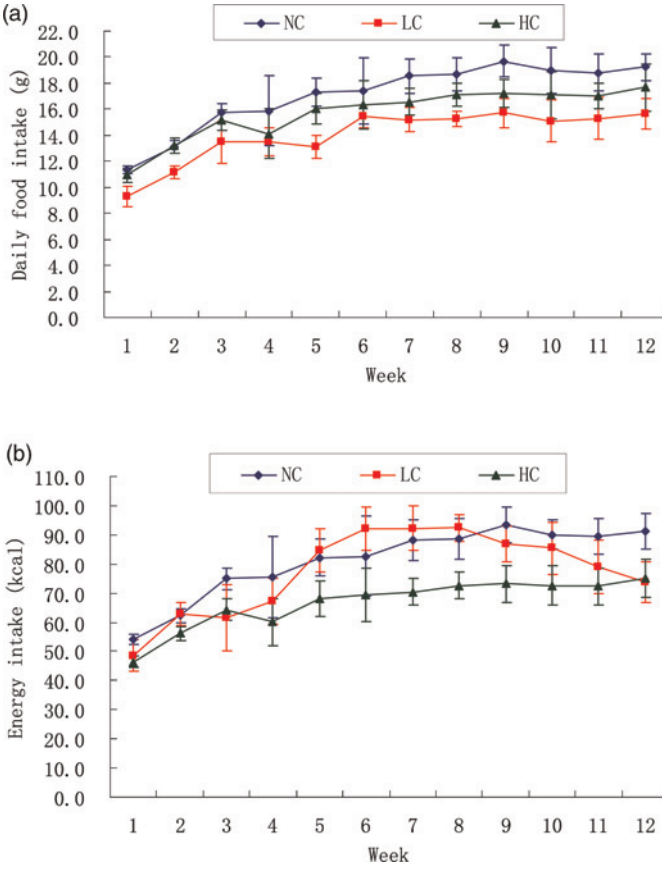

Figure 2 Daily food and calorie intake. (a) *Daily food intake*: Daily food intake of LC rats (6.9 ± 0.8 g) reduced significantly (versus NC, 11.4 ± 0.8 g, $P < 0.001$; versus HC, 10.9 ± 0.5 g, $P < 0.001$) in the first week, and this phenomenon lasted during the whole study. No statistical difference was observed between the NC and HC groups except for ninth week (19.7 ± 0.2 g versus 17.2 ± 0.1 g, $P < 0.001$). (b) *Daily calorie intake*: Daily calorie intake of HC fed rats was less than those in NC and LC fed rats (versus NC and LC group, both $P < 0.05$), while no obvious difference was observed between the NC and LC groups. NC, normal carbohydrate; LC, low carbohydrate; HC, high carbohydrate. Each group: $n = 8$ (A color version of this figure is available in the online journal)

rather than total energy consumption, suggesting that calorie differences were not the only cause of obesity.[15,16] Dietary macronutrient composition have an impact on body weight independent of energy intake. The present study showed, under *ad libitum* condition, lowering the CHO-to-fat ratio by adding lard resulted in a rising of body weight, whereas average daily energy intake did not increase. The results were consistent with Takahashi *et al.*[17] They valuated the effect of the fat/CHO ratio on obesity in C57BL/6J mice. Although there was no significant difference in average energy intake, graded increments of body weight was induced by increasing fat/CHO ratio (on the other hand, lowering CHO/fat ratio) in diets of more than 30% fat. Sinitskaya *et al.*[18] also demonstrated rats fed on two high-fat diets combined with low-to-moderate CHO content exhibited higher body mass index compared with those on a control diet. In contrast to the above studies, Caton *et al.*[19] demonstrated a low CHO high-fat diet led to body weight loss in mature rats and lack of body weight gain in adolescent rats. Interestingly, if dietary CHO-to-saturated fat ratio was reduced to the maximum (CHO/fat = '0'), it showed different effects on body weight. Animals fed by a very high-fat, CHO-free diet had an absence of body weight gain and excess adiposity, while glucose tolerance and insulin resistance were induced.[18,20] Further studies are needed to elucidate possible mechanism involved in it.

Although administration of exogenous leptin to leptin-deficient children results in a remarkable decrease in their energy intake and a dramatic loss of fat mass while maintaining lean body mass,[21] circulating leptin levels were raised proportionally in diet-induced rodent models or human obesity, meaning obesity is not a condition of leptin insufficiency but instead of leptin resistance.[22] The results of our study also showed circulating leptin levels were raised proportionally to body weight ($r = 0.88$, $P < 0.001$) among 24 Wistar rats. A reduction expression of leptin receptor in the hypothalamus was demonstrated in our study, which may partly account for leptin resistance.

Table 3 Plasma leptin and insulin concentration

Group	Leptin (ng/mL)	Insulin (ng/mL)
NC	8.6 ± 3.8	8.7 ± 1.9
LC	18.5 ± 8.2*	10.3 ± 1.8
HC	6.6 ± 1.9	10.9 ± 5.8

NC, normal carbohydrate; LC, low carbohydrate; HC, high carbohydrate
Note: The difference among the three groups was made by the analysis of variance. Each group: $n = 8$. For leptin, $F = 11.20$, $P < 0.001$
*LC versus NC, $P < 0.001$; LC versus HC, $P < 0.001$; NC versus HC, $P = 0.45$. For insulin, $F = 0.73$, $P = 0.49$

Table 4 Expression of appetite gene in the hypothalamus (copy/mL)

	NC	LC	HC	χ^2, *P* value
NPY	1.692 (0.461–2.392)	2.307 (0.827–5.649)	0.483 (0.180–4.925)	2.605, 0.272
Orexin	0.027 (0.002–0.048)	0.006 (0.000–0.186)	0.008 (0.003–0.029)	1.285, 0.526
Insulin-R	0.822 (0.696–1.443)	0.594 (0.293–0.883)	0.696 (0.356–0.852)	3.331, 0.189
Leptin-R	1.323 (0.616–2.392)	0.764 (0.471–4.648)	0.357 (0.129–0.781)	9.918, 0.007
MC-4R	0.671 (0.485–3.937)	0.544 (0.482–1.066)	0.709 (0.208–1.276)	1.309, 0.520
AgRP	1.058 (0.594–1.145)	0.798 (0.541–1.213)	1.286 (0.142–1.585)	1.754, 0.416

NC, normal carbohydrate; LC, low carbohydrate; HC, high carbohydrate; NPY, neuropeptide; MC-4R, melanocortin-4 receptor; AgRP, agouti-related peptide
Note: The difference was made by *K*-independent non-parametric test

The limitation of our study was that we did not measure circulating leptin levels longitudinally; thus it was not sure whether leptin resistance was the reason of body weight gain or the result of that.

Archer *et al.*[23] reported that high-energy diet-fed rats exhibited symptoms of developing metabolic syndrome. Additionally, leptin receptor gene expression in the hypothalamic arcuate nucleus and ventromedial nucleus of high-energy rats was reduced. Consistent with the elevated serum leptin levels, hypothalamic gene expression for the orexigenic neuropeptides, NPY (arcuate nucleus and dorsomedial nucleus), and AgRP, was reduced. Staszkiewicz *et al.*[24] found reduced expression of hypothalamic AgRP under a high-fat diet, whereas leptin receptor and NPY did not fluctuate in response to diet. An animal study conducted by Davidowa *et al.*[25] also revealed that postnatal overfeeding inhibited amphetamine-regulated transcription and the levels of melanocortins and NPY in paraventricular neurons, and that there was a significant increase in the expression of the involved genes in later life. Dissimilar to above studies, our study showed that only leptin receptors in the hypothalamus decreased, while AgRP, NPY, insulin receptors, orexin and MC-4R showed no obvious difference when observed with realtime PCR. The limitation of our study was that we used extracts of the entire hypothalamus to examine the changes. The expression level of these genes across the entire hypothalamus was very low. If the changes were not large enough to be detected, the findings for all the groups would be similar. More details may be revealed by evaluating the expression level of appetite genes within the special hypothalamus nuclei.

Conclusions

Low-CHO, high-fat diet raised body weight, which led to a rising of circulating leptin levels and a reduced expression of leptin receptor in the hypothalamus.

Author contributions: R-YX, major completer of the study and paper writing; Y-PW, data collection; Q-YT, data collection; JW, coordinator of study and data collection; and WC, study design and giving important advice to paper writing.

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