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# Effects of Endogenous n-3PUFA on Body Weight, Autophagy and Inflammation in Mice

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**Abstract:** The fat-1 transgenic mice were used as models, to investigate the effects of n-3PUFAs on body weight, the expression of inflammation and autophagy in mice, and to explore its mechanism. The mice were divided into two groups: fat-1 transgenic mice and wild-type mice. The body weight and body length were measured and the index of body weight/body length was calculated regularly every week for 8 weeks. Cardiac blood was collected for determination of TG, CT, HDL-C, LDL-C and BG. Frozen sections of liver were stained by Oil Red O to observe the lipid droplets. The expression of autophagy proteins P62, LC3 and ATG7 in the hypothalamus were detected by western blot, and the relative quantitative analysis was performed. Real-time quantitative PCR was used to quantify the mRNA expression of TNF-α, IL-6, IL-1β, IFN-γ, MCP-1, TLR-4 and adiponectin in epididymal adipose tissue. The body weight/body length of fat-1 transgenic mice was significantly lower than that of wild-type mice($P<0.05$), the levels of TG, CT, HDL-C, LDL-C and BG in the serum of fat-1 transgenic mice were significantly lower than those in wild-type mice($P<0.05$). Lipid droplets in the liver of fat-1 transgenic mice were significantly less. The expression of P62 in fat-1 transgenic mice was significantly down-regulated ($P <0.05$), while the expression of ATG7 was significantly increased ($P <0.05$), and the ratio of LC3 II / I was significantly increased($P<0.05$). The results of real-time quantitative PCR showed that the mRNA relative expression of TNF-α, IL-6, IL-1β, IFN-γ, MCP-1 and TLR-4 in epididymal fat tissue of fat-1 transgenic mice was significantly decreased, and the expression of adiponectin was increased ($P < 0.05$). n-3PUFAs reduce the body weight to prevent obesity may by up-regulation of hypothalamic autophagy, and down-regulation of inflammation in peripheral fat.

**Keywords:** Hypothalamus, n-3 PUFAs, Autophagy, Inflammation

## Introduction

At present, obesity is widespread in the world, and the population is increasing year by year, which seriously affects human health[1]. Obesity is a chronic energy imbalance in which the body's energy intake is higher than its consumption, and the excess energy is transformed into fat of body. Obesity leads to many health problems such as high blood pressure, high cholesterol, type 2 diabetes, cardiovascular disease, etc[2; 3]. Obesity is a chronic low-degree systemic inflammation, and adipose tissue releases inflammatory cytokines through autocrine or paracrine[4; 5], such as tumor necrosis factor (TNF-α), interleukin, interferon, adiponectin and so on[6].

The central nervous system (CNS) controls appetite, energy balance and metabolism, then controls body weight, while the hypothalamus is the main area of the brain that controls energy balance[7; 8]. Autophagy is a highly conserved life-form in eukaryotes that maintains homeostasis and adapts to changes of the microenvironment, which is the self-renewal mechanism in response to external stimuli and internal changes[9]. It plays an important role in cell differentiation, material metabolism and the balance of energy. Autophagy regulates the feeding behavior and energy balance of the central neurons in the hypothalamus that controls the appetite. It promotes peripheral fat mobilization and then controls the weight.[10; 11].

Negative regulatory effect of autophagy on inflammatory, autophagy can weaken the inflammatory by promoting the clearance of apoptotic cells and inhibiting the expression of inflammatory cytokines. Inflammatory can also regulate autophagy in return, and inflammatory cytokines activate autophagy. The combination of those regulate weight.


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N-3 polyunsaturated fatty acids (n-3PUFAs) is the essential fatty acids, which has the effect of resisting obesity, lowering blood glucose, lowering blood fat and anti inflammation[12]. However, animal supplements n-3 PUFAs can only by eating deep-sea fish and other means. The fat-1 gene encoding n-3 polyunsaturated fatty acid dehydrogenase from C. elegans, can converts n-6PUFAs to n-3PUFAs in vivo[13]. In this study, fat-1 transgenic mice were used to investigate how n-3PUFAs modulates inflammation and autophagy and reduces body weight.

## 1 Methods

### *1.1 Animal*

Fat-1 transgenic heterozygous mice (a kind gift from Professor Jingxuan Kang of Harvard Medical College and Jianbo Wan of University of Macau) were mated with C57BL/6 WT mice. The progeny of mice were identified by PCR to obtained fat-1 transgenic mice. The male mice were then divided into two groups: fat-1 transgenic group and the WT group. Six-week-old male heterozygous fat-1(+/-) mice and WT mice were bred at the Qingdao University. They were allowed access to standard rodent chow as described and were sacrificed after 8 weeks. They were housed individually in environmentally-controlled conditions (temperature 25±2°C, light cycle from 06:00 to 18:00 and dark cycle from 18:00 to 06:00) and allowed ad libitum access to food and water throughout the trial.

### *1.2 Measure body weight and body length*

We measured body weight and body length of the experimental animals weekly. The study was approved and all procedures were performed in accordance with institutional guidelines of the Animal Care and Use Committee at Qingdao University.

### *1.3 Measure TG, CT, HDL-c, LDL-c and BG*

The serum levels of TG, CT, HDL-c, LDL-c and BG were measured, separately in 4 th and 6 th week of the trial using corresponding kits (BG kit, ELISA, Bio-Rad Laboratories, Hercules, CA; CT kit, RongSheng Company, Shanghai; TG kit, HDL-c kit and LDL-c kit, DongOU Biological Company, Zhejiang). The data are presented as an average of the three measurements.

### *1.4 Oil red O staining*

The frozen slices of liver were washed with distilled water, and immersed in 60% isopropyl alcohol. The oil red O working solution was stained. Slices were differentiated with 60% isopropanol to interstitial clear, washed with distilled water and counterstained with hematoxylin, and pchked with Glycerol. Visible light microscope observation, photographing.

### *1.5 Real Time PCR*

Epididymal fat were homogenized using Trizol reagent (Invitrogen)kit and total RNA was isolated according to the manufacturer's protocol. Total RNA (1 μg) were reverse transcribed to cDNA using Transcriptor First Strand cDNA Synthesis Kit(Roche), and qRT-PCR was performed with Roche LightCycler 480 using SYBR.

Premix Ex Taq master mix(Roche). The specificity of qRT-PCR was confirmed by agarose gel electrophoresis and melting-curve analysis. A housekeeping gene (β-actin) was used as an internal standard. The primers used are listed in Table 1.

**Table 1 Primers for realtime quantitative RT-PCR**

| Gene | Forward | Reverse |
|---|---|---|
| TNF-α | 5'-ACTCCAGGCGGTGCCTATGT-3' | 5'-GTGAGGGTCTGGGCCATAGAA-3' |
| IL-6 | 5'-CCACTTCACAAGTCGGAGGCTTA-3' | 5'-GCAAGTGCATCATCGTTGTTCATAC-3' |
| IL-1β | 5'-TGGTGTGTGACGTTCCCATT-3' | 5'-TCGTTGCTTGGTTCTCCTTG-3' |
| MCP-1 | 5'-AGATGCAGTTAACGCCCCAC-3' | 5'-TGTCTGGACCCATTCCTTCTTG-3' |
| IFN-γ | 5'-GCTTTGCAGCTCTTCCTCAT-3' | 5'-GTCACCATCCTTTTGCCAGT-3' |
| TLR-4 | 5'-CATGGATCAGAAACTCAGCAAAGTC-3'c'' | 5'-CATGCCATGCCTTGTCTTCA-3' |
| Adiponectin | 5'-AACTTGTGCAGGTTGGATGG-3' | 5'-GCCCTTCAGCTCCTGTCATT-3' |
| β-actin | 5'-CATCCGTAAAGACCTCTATGCCAAC-3' | 5'-ATGGAGCCACCGATCCACA-3' |

### *1.6 Western Blot*

Extract hypothalamus tissue lysed in a RIPA lysis buffer containing PMSF. The proteins were quantified using the Micro BCA™ Protein Assay Kit (Thermo Scientific™). The protein samples were then subjected to a 10-12% SDS-PAGE gels and transferred to PVDF membranes. After blocking with 5% skim milk for 2 h(RT), the membranes were incubated with primary

antibody ATG7(1:100; RD, USA), LC3(1:1000, NOVUS, USA), P62(1:2000, Proteintech, China), β-actin(1:1000, zhongshan, China) overnight at 4°C. The membranes were then incubated with secondary antibodies (anti-mouse IgG 1: 7000 or anti-rabbit IgG 1: 7000; Multi Sciences, China) for 1 hour(RT). All images were captured and analysed using Image Lab software (Bio-Rad Universal Hood II, USA). The expression levels of the above proteins were normalized to those of β-actin. All experiments were repeated three times.

***1.7 Analysis***

All experimental results are expressed as mean ± standard deviation, In this study, one-way ANOVA was used to analyze the body weight, body length. Real-time quantitative PCR results and western results were examined with independent samples t test. The significant level difference was set as $P < 0.05$.

**2 Result**

***2.1 fat-1 gene decreased the body weight/length ratio.***

The fat-1 transgenic mice pups at birth are essentially indis-tinguishable in size from wild-type mice. As shown in Fig. 1B, the body weight/length ratio of fat-1 transgenic group was significantly lower than that of wild-type group. There was a significantly difference in body weight/length ratios between fat-1 transgenic mice and wild-type mice at 14th week (fat-1: 2.285 ± 0.003, wild-type:2.350 ± 0.005 g/cm, n = 6 animals per group, $P < 0.05$, Fig. 1A)

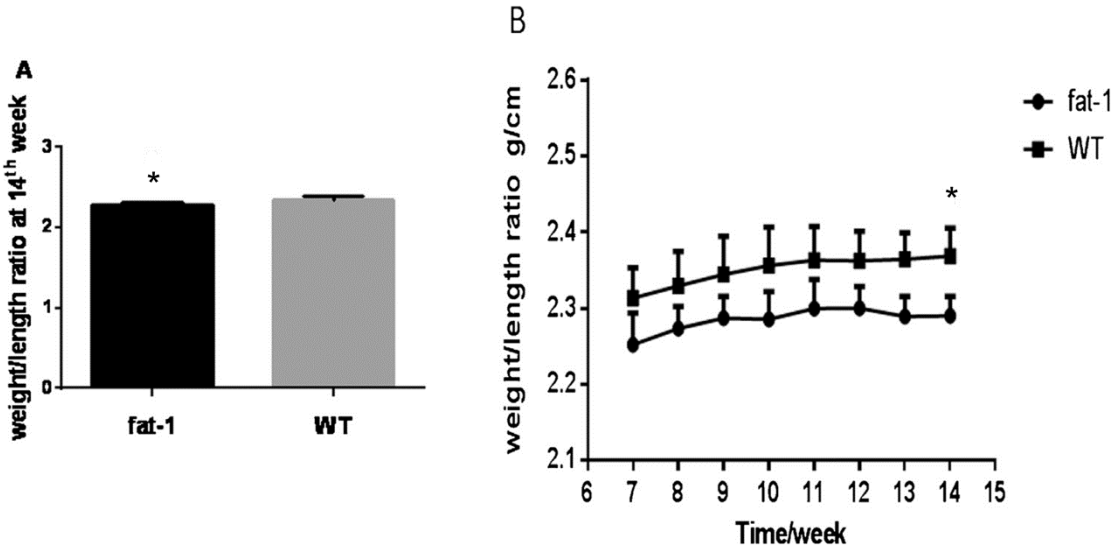


**Fig1** The body weight/length ratio of fat-1 mice and WT mice in the 14th week of age

fat-1, fat-1 transgenic group and WT, wild-type group. * means *P*<0.05 vs. WT.

***2.2 fat-1 gene reduced the serum levels of TG, CT, HDL-c, LDL-c and BG***

The level of TG, CT, HDL-c, LDL-c and BG was presented as an average of the two measurements in 4 th and 6 th week of the trial. According to Fig.2, there were significant differences in the serum levels of TG, CT, HDL-c, LDL-c and BG between fat-1 transgenic group and wild-type group. The data suggested that fat-1 transgenic group possesses better biochemistry data.

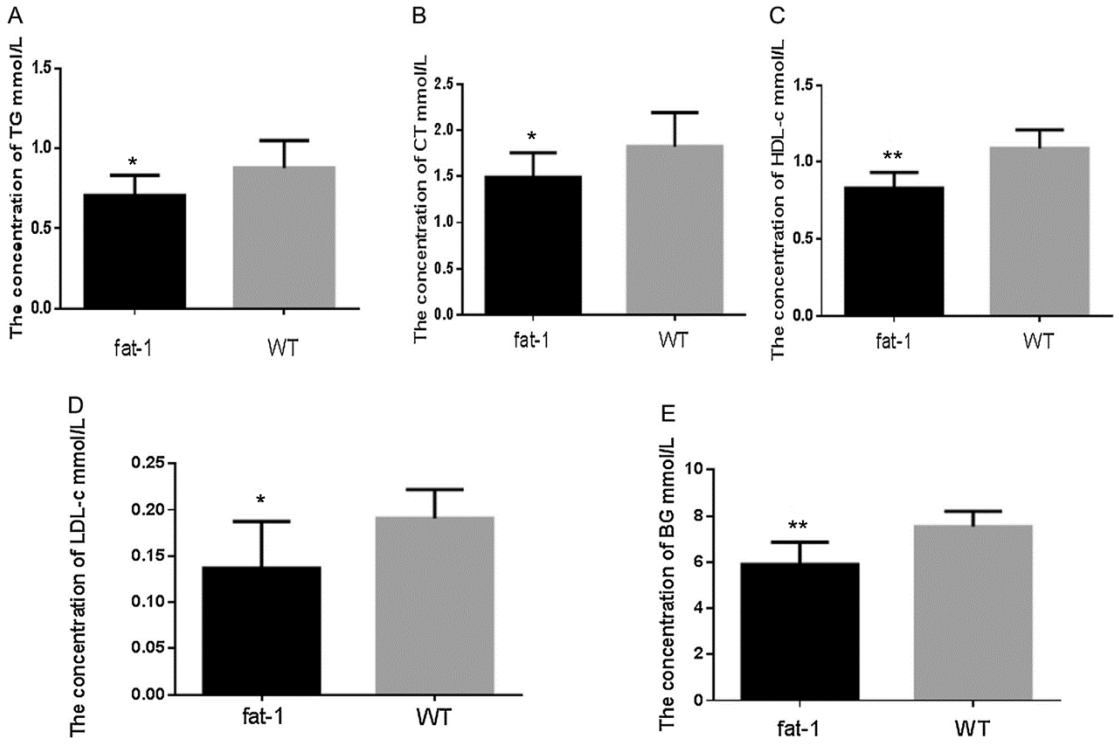


**Fig.2.** Comparisons of TG (A), CT (B), HDL-c (C), LDL-c (D) and BG (E) in fat-1 transgenic group and wildtype group. Values are expressed as means ± SEM. fat-1, fat-1 transgenic group and WT, wild-type group. * means P<0.05 vs. WT, ** means vs. WT, P<0.01. n=5 animals per group.

### *2.3 The result of Oil red o staining*

Through the liver's oil red O staining, we can see that lipid droplets in the liver of fat-1 transgenic mice were significantly less than those in wild-type mice.

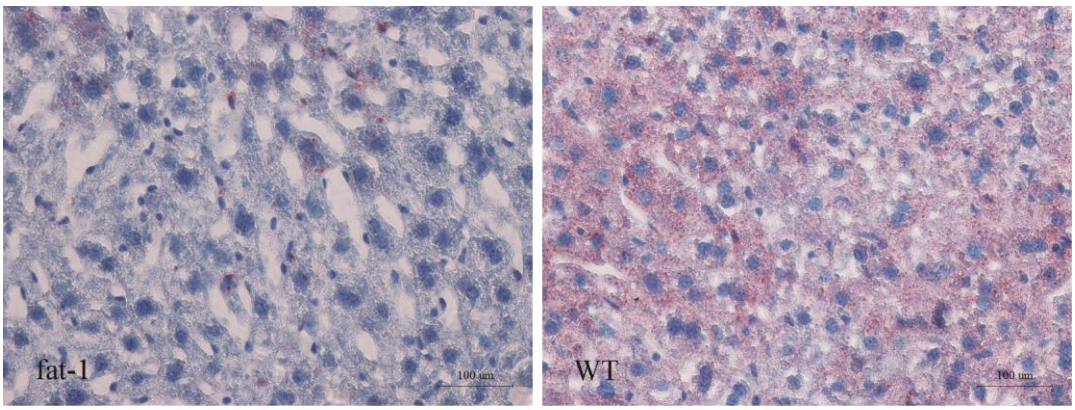


**Fig. 3** The result of staining by oil red. Lipid droplets in the liver of fat-1 transgenic mice were significantly lower than those in wild-type mice. fat-1 transgenic group and WT, wild-type group. scale bar=100μm.

### *2.4 Hypothalamic autophagy detection*

Western blot experiments showed that the expression of P62 was down-regulated, the expression of ATG7 was up-regulated and LC3 II / I ratio was increased significantly in fat-1 transgenic mice. The results indicate that the autophagy in hypothalamus is up-regulated of fat-1 transgenic mice.

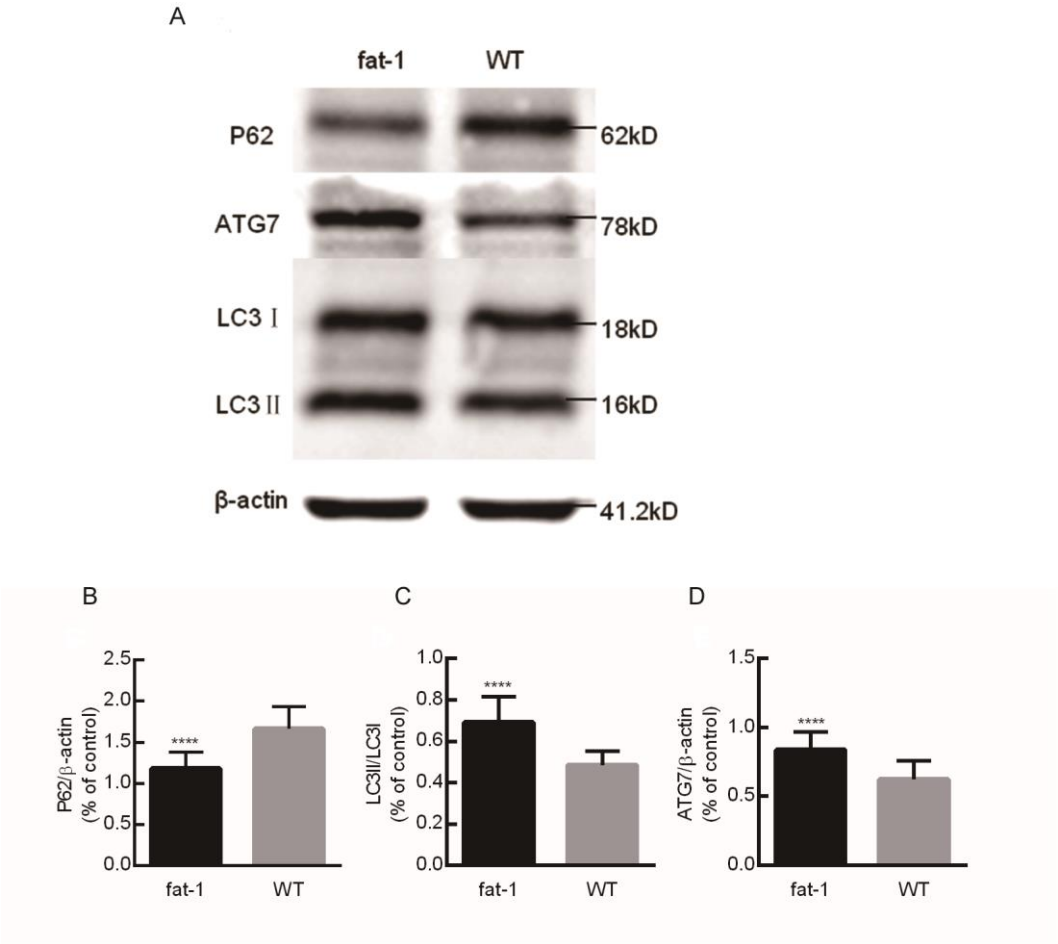


**figure 4** The autophagy in hypothalamus of fat-1 transgenic mice and wild-type mice. A, The bands of P62, ATG7 and LC3 by western blot, B-D, The statistical results of western blot. It was shown that P62 protein expression was significantly decreased, the expression of ATG7 was significantly increased, and the ratio of LC3 II / I was significantly increased in fat-1 transgenic mice. fat-1: fat-1 transgenic mice, WT: wild-type littermates. **** means $P < 0.0001$ vs. WT.

***2.5 Relative expression of inflammatory factor in peripheral adipose tissue***

The analysis of qPCR showed that the relative expression of inflammatory cytokines TNF-α, IL-6 and IL-1β , IFN-γ, TLR-4, MCP-1 mRNA in peripheral adipose tissue of fat-1 mice was significantly lower than that in WT mice, while the adiponectin was significantly up-regulated($P < 0.05$).

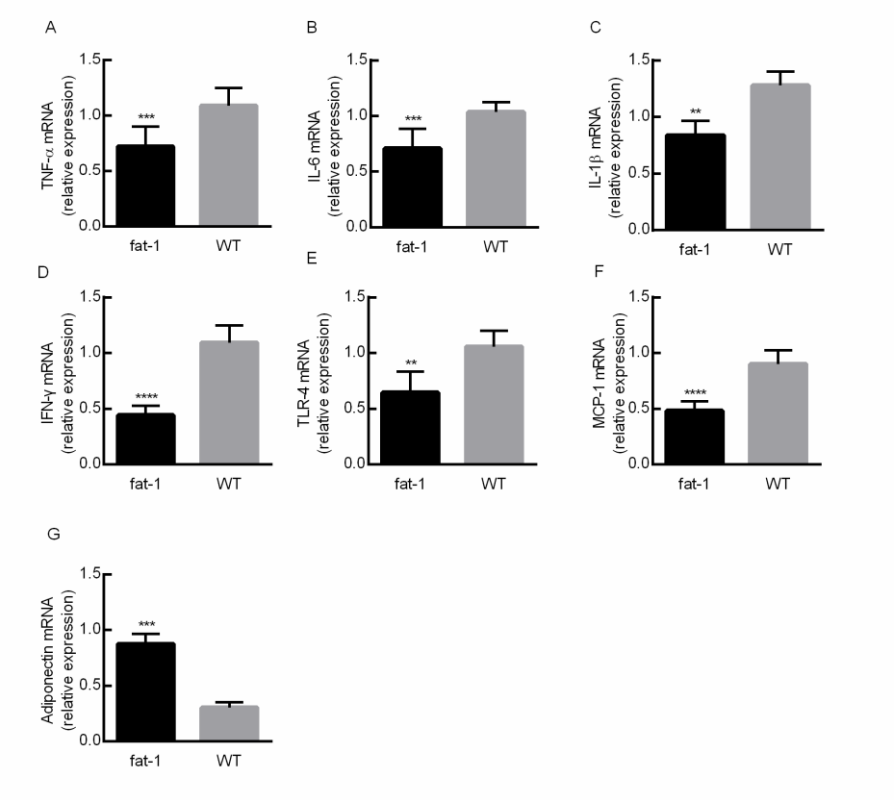


**fig.5.** Relative expression of inflammatory factor, chemokines and anti-inflammatory factor mRNA in peripheral adipose tissue of fat-1 mice and WT mice. A-E, The mRNA expression of inflammatory factors TNF-α, IL-6, IL-1β, IFN-γ and TLR-4 in fat-1 transgenic mice was significantly down-regulatedo($P<0.05$). F, The mRNA expression of chemokines MCP-1 in fat-1 transgenic mice was significantly down-regulated($P<0.05$). G. The mRNA expression of anti-inflammatory factor adiponectin in fat-1 transgenic mice was significantly up-regulated($P<0.05$). fat-1: fat-1 transgenic mice, WT: wild-type littermates. ** means $P<0.01$ vs. WT. *** means $P<0.001$ vs. WT. **** means $P < 0.0001$ vs. WT.

**Discussion**

N-3PUFAs is an essential fatty acid, the most important intake of EPA and DHA can alleviate insulin resistance and reduce triglyceride levels and lipoprotein in plasma[14], promote the mobilization of fat, and inhibit the inflammatory. While the derivatives of n-6 PUFAs can promote inflammation and may cause many diseases[15]. N-3PUFAs can not be synthesized in animals due to lack of n-3PUFAs dehydrogenase, thus leading to a high ratio of n-6/n-3PUFAs in vivo. N-3PUFAs dehydrogenase which was encoded by the fat-1 gene from C. elegans, converts n-6PUFAs to n-3PUFAs spontaneously in vivo. Therefore, fat-1 transgenic mice were used as models to investigate the effect of n-3PUFAs on obesity.

We found that the body weight of transgenic mice were significantly lower than that of wild-type mice, this finding was consistent with previous research[16]. On the basis of this macroscopic phenomenon, we detected the concentration of TG, CT, HDL-C, LDL-C and BG in the serum of the two groups, and the results showed that the index leveds in the serum of fat-1 transgenic mice were lower than those in the wild type mice. This is consistent with the report result of intake of DHA and EPA, which can reduce triglycerides, lipids, lipoproteins, apolipoprotein levels, reduce body fat content and promote lipid utilization[17]. Therefore, we consider that the differences between two groups in the physiological indexes are caused by the change of endocrine regulation which are caused by the different levels of n-3 PUFAs..

The hypothalamus is the main area where controls energy balance in brain. Autophagy can regulates the levels of neuropeptides in the hypothalamic neurons, affect appetite and energy balance, regulate peripheral fat mobilization and affects body weight. All of the changes of ATG7, P62 and LC3II / LC3I in the hypothalamus of fat-1 transgenic mice all indicate the

increase of autophagy, they also indicate that the n-3PUFAs can increase the basal level of autophagy in hypothalamic neurons, then regulate the expression of neuropeptides in the hypothalamus[18], such as NPY / AgRP, thereby reduce food intake in mice, regulate energy balance, and reduce body weight.

Obesity is a state that excessive accumulation of fat in the body will result in an increase in the body mass. It is a chronic metabolic disease. As fat accumulates, adipose tissue releases hormones, cytokines and chemokines, which act on the energy-regulating center in hypothalamus to form an adipose tissue-hypothalamus dialogue that regulates the energy balance in the body, then regulating body weight. While the expression of inflammatory cytokines in obese adipose tissue increased, the anti-inflammatory factor which is a self-protection compensatory response initially decreases. Due to the continuous balance of energy, inflammation in adipose tissue formed gradually. This is different from the classic immune-mediated inflammatory. The adipose tissue inflammation relates to the development of metabolic disease. [19]. The levels of TNF-α, IL-6, IL-1β, IFN-γ, toll-like receptor 4 (TLR-4), monocyte chemotactic factor (MCP-1) and other proinflammatory cytokines decreased, while the expression of adiponectin and other anti-inflammatory cytokines increased in fat-1 mice. The results showed that the increased of n-3PUFAs in fat-1 mice supress inflammation of peripheral adipose tissue, decreased the expression of inflammatory factors, and feedback regulation to the energy center of the hypothalamus, regulated energy balance, inhibited adipogenesis and decreased body weight. Therefore weight loss is associated with the reduction of inflammation in peripheral fat.

Autophagy has a negative regulative effect on inflammation. Autophagy can not only relieve infectious and inflammatory diseases, but also play an anti-inflammatory role in chronic low-degree inflammation[20]. The n-3 PUFAs in fat-1 transgenic mice up-regulates hypothalamus autophagy and down-regulates the inflammatory of adipose tissue. Increased autophagy also inhibited the inflammatory response, both of them work together in the hypothalamus energy control center and reduce the weight of mice.

In summary, our study shows that the increase of n-3PUFAs can reduce the body weight of mice, the mechanism may be by influencing the appetite neuropeptides, regulating autophagy in the hypothalamus arcuate nucleus, and then regulating appetite, mobilizing the peripheral fat lipolysis, and inhibiting the inflammation of peripheral fat.

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