

## Soy Isoflavone and Its Effect to Regulate Hypothalamus and Peripheral Orexigenic Gene Expression in Ovariectomized Rats Fed on A High-Fat Diet<sup>1</sup>

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**Objective** To explore the effect of soy isoflavone on obesity in the light of hypothalamus and peripheral orexigenic gene regulation. **Methods** Fifty-four female rats were randomly assigned to 6 groups: one sham-operated group (SHAM), one ovariectomized (OVX) control group, three OVX groups fed with 400 ppm (L-SI), 1 200 ppm (M-SI) and 3 600 ppm (H-SI) isoflavone respectively, and one OVX group receiving 0.45 ppm diethylstilbestrol (EC). All rats were allowed to take high-fat diet for 4 weeks. Some neuropeptides were measured by RT-PCR. These neuropeptides included NPY, pro-opiomelanocortin (POMC), cocaine and amphetamine regulated transcript (CART), orexin, melanin-concentrating hormone (MCH), melanin-concentrating hormone precursor (P-MCH), ghrelin, and leptin. **Results** Compared with the OVX control group, the body weight and food intake in the H-SI group were reduced significantly and there was a significant dose-dependent manner in the 3 isoflavone groups. The results of RT-PCR showed that the NPY level in the 3 isoflavone groups was significantly increased and the POMC/CART gene expression decreased significantly in rats' hypothalamus compared with that in the OVX control group. However, the expression of orexin, MCH and P-MCH had no change. The peripheral grelin mRNA expression was higher in the 3 isoflavone groups, while leptin gene expression in the fat was not consistent. **Conclusions** This research showed that isoflavone could prevent obesity induced by high-fat diet and ovariectomy through regulating hypothalamus and peripheral orexigenic gene expressions associated with food intake.

**Key words:** Isoflavone; Hypothalamus; Orexigenic gene express; Obesity

### INTRODUCTION

Obesity is one of the most common metabolic disorders and is associated with numerous complications (type II diabetes mellitus, hypertension, cardiovascular diseases and cancer)<sup>[1-4]</sup>. In the past years, diet restriction and exercise have shown high effectiveness in reducing obesity. However, long-term effort and personal perseverance are required to lose weight. Therefore, new diet-based therapies to prevent the occurrence of obesity would be most valuable. In recent years, attention has been shifted towards the role of the dietary soy isoflavone in reducing food intake in the management of obesity.

Soy isoflavone (SIF) is a diphenolic compound. It is similar to estrogen in its chemical structure and can combine with estrogen receptors alpha (ER $\alpha$ ) and beta (ER $\beta$ ), so they are usually called phytoestrogens<sup>[5-6]</sup>.

Many studies have shown that dietary SIF plays a beneficial role in reducing obesity<sup>[7-12]</sup>. One hundred obese volunteers in a soy-based meal replacement group for 12 weeks lost significantly more weight and more fat mass than in the control group<sup>[7]</sup>. Compared with animals fed with a SIF-free diet, rats fed with SIF-base diet have significantly decreased body weight (BW) and fat pad weight at various doses<sup>[8-12]</sup>. Furthermore, some experiments with SIF have demonstrated a dose-related reduction in food intake and an increase in apoptosis of 3T3-L1 adipocytes *in vitro*<sup>[11,13]</sup>. However, the physiological mechanism of SIF to reduce obesity remains unclear. The purpose of this study was to explore the effect of dietary SIF on energy balance and obesity in rat model in the light of hypothalamus and peripheral orexigenic gene regulation.

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## MATERIALS AND METHODS

*Soybean Isoflavone and Diethylstilbestrol*

Soybean isoflavone (SIF, purity  $\geq 40\%$ ) was purchased from Changrun Co., Ltd. (Shandong, China). It contained 29.99 mg genistin, 1.93 mg genistein, 10.46 mg daidzin and 0.25 mg daidzein in 100 mg SIF. Diethylstilbestrol was purchased from Jiulian Pharmaceutical Factory (Hefei, China). It contained 0.5 mg diethylstilbestrol per pellet.

*Diets*

The high-fat diet was based on AIN-93M<sup>[14]</sup>, containing lard 150 g per kg. All rats were fed with this high fat diet. The diets of the three SIF groups (L-SI, M-SI and H-SI) contained 400, 1 200, and 3 600 mg isoflavone per kg diet respectively. The diet of the estrogen control group (EC) contained diethylstilbestrol 0.45 mg/kg diet.

*Animals and Treatment*

Fifty-four female rats aged 7 weeks, weighting 180 g-220 g, were purchased from College of Basic Medicine of Jilin University in China (Batch No: SCXK-(ji)2003-0001). All rats were caged in a room maintained at 21 $\pm$ 2 °C with a 12-hour light and 12-hour dark schedule. After 1 week, they were randomly assigned to six groups on the basis of body weight (BW) and then operated. The operative procedures for ovariectomy were as follows: after anesthesia with pentobarbital sodium (40 mg/kg bw),

cut on the mid-abdomen and both ovaries of rats were taken out. The rats in the SHAM group were operated with the same procedure but without taking out ovaries. The rats in each group were fed with their own diet. No limit was made to water intake. Body weight and food intake were measured weekly. Four weeks later, the brain, fat, and stomach of the rats were taken out and frozen in liquid nitrogen immediately, and then they were stored in -80 °C for further RT-PCR analysis.

All experimental procedures were conducted in conformity with institutional guidelines for the care and use of laboratory animals in Harbin Medical University and in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals.

*RT-PCR*

Total RNA (about 500 ng) was extracted from samples by using TRIzol Reagent (Invitrogen, USA). By using the TaKaRa DNA polymerase (TaKaRa, Dalian, China), RNA was reversed-transcribed into cDNA in 10  $\mu$ L of reaction mixture for RT-PCR analysis. The reaction mixture was heated to 42 °C for 30 min and denatured at 99 °C for 5 min. The primers are listed in Table 1. PCR products were detected by electrophoresis in a 1.5% agarose gel containing ethidium bromide. The bands in the gel were quantified by using densitometry with the BioImage program (Millipore, USA). The signal for Target mRNA was normalized to the signal of the housekeeping gene GAPDH, and the results were expressed as the ratio target/GAPDH mRNA.

TABLE 1

Sequences of Primers Used in RT-PCR

Sequence (5'-3')	Product Size, bp	GenBank Accession No.	Annealing Temperature (°C)	Cycles
<b>Hypothalamus</b>				
<b>NPY</b>				
Sense	TGCTAGGTAACAAACGAATG	354	53	36
Antisense	AGGATGAGATGAGATGTGGG			
<b>POMC</b>				
Sense	CTC CTG CTT CAG ACC TCC AT	398	56	39
Antisense	TTG GGG TAC ACC TTC ACA GG			
<b>CART</b>				
Sense	TCT GCC GTG GAT GAT GCG	291	53	36
Antisense	GAA CCG AAG GAG GCT GTC			
<b>Orexin</b>				
Sense	GCCGTCTCTACGAAGTGTG	303	56	36
Antisense	CGA GGA GA GGGAAA GTTA G			

(to be continued)

(continued)

	Sequence (5'-3')	Product Size, bp	GenBank Accession No.	Annealing Temperature (°C)	Cycles
<b>P-MCH</b>					
Sense	TAC GGA GCA GCA AAC A	323	NM_012625	57	36
Antisense	ACA GCC AGA CTG AGG G				
<b>MCH</b>					
Sense	CGG CCT CCA AGT CCA TCA G	333	M29712	57	36
Antisense	CTT CAT CCC CAA TTT CCC TCT T				
<b>Periphery</b>					
<b>Ghrelin</b>					
Sense	TTG AGC CCA GAG CAC CAG AAA	347	AB029433	58	35
Antisense	AGT TGC AGA GGA GGC AGA AGC T				
<b>Leptin</b>					
Sense	TGG TCC TAT CTG TCC TAT GTT	286	NM_013076	56	39
Antisense	GGA GGT CTC GCA GGT TCT				
<b>GAPDH</b>					
Sense	CTCAACTACATGGTCTACATG	421	NM_017008	55	35
Antisense	CATGGACTGTGGTCATGAG				

Note. POMC, proopiomelanocortin; CART, cocaine- and amphetamine-regulated transcript; MCH, melanin-concentrating hormone; P-MCH, melanin-concentrating hormone precursor.

### Statistical Analysis

Data were expressed as  $\bar{x} \pm s$ . For statistical analysis, data were analyzed using One-way ANOVA, followed by Least Significant Difference (LSD) for determination of significant differences between treatment means.  $P < 0.05$  was interpreted as a significant difference.

## RESULTS

### Effects of SIF on BW and Food Intake

The changes of BW and food intake in each group are shown in Table 2.

TABLE 2

Daily SIF Intake and the Effects of Dietary SIF on Body Weight and Food Intake ( $\bar{x} \pm s$ )

Group	Body Weight (0w, g)	Body Weight (4w, g)	Body Weight Increase (g)	Daily Food Intake (g)
SHAM	208.07±15.83 <sup>a</sup>	258.06±17.02 <sup>a</sup>	49.99±10.36 <sup>a</sup>	14.2±0.90 <sup>a</sup>
OVX	206.87±10.97 <sup>a</sup>	287.48±15.60 <sup>bc</sup>	80.61±15.14 <sup>bc</sup>	16.0±1.30 <sup>bc</sup>
L-SI	206.81±11.43 <sup>a</sup>	299.02±21.73 <sup>c</sup>	92.21±18.43 <sup>c</sup>	16.4±1.16 <sup>c</sup>
M-SI	205.5±10.98 <sup>a</sup>	278.7±14.54 <sup>b</sup>	73.20±12.77 <sup>b</sup>	15.0±1.05 <sup>a,b,d</sup>
H-SI	204.36±13.11 <sup>a</sup>	251.11±19.25 <sup>a</sup>	46.76±16.67 <sup>a</sup>	13.0±1.09 <sup>c</sup>
EC	204.31±10.68 <sup>a</sup>	222.73±18.11 <sup>d</sup>	18.42±12.29 <sup>d</sup>	10.2±0.84 <sup>f</sup>

Note. Animal groups comprised sham-operated control (SHAM), ovariectomized (OVX) control, and OVX rats treated with different doses of SIF (L-SI, M-SI, and H-SI). Means without a common letter differ significantly,  $P < 0.05$ ,  $n = 9$ .

As shown in Table 2, the BW of rats was similar at the beginning of the experiment. By week 4,

compared with the OVX control group, BW decreased by 3.30% ( $P > 0.05$ ) and 12.7% ( $P < 0.001$ )

in the M-SI and H-SI groups, and 22.5% ( $P<0.001$ ) in the EC group, while the BW of L-SI was slightly increased ( $P>0.05$ ). The BW of the 3 SIF groups showed a dose-dependent manner and statistical difference.

Compared with the OVX control group, the rats' daily food intake was not changed significantly in the L-SI and M-SI groups ( $P>0.05$ ), but decreased

significantly in the H-SI and EC groups (Table 2).

#### Expression of Hypothalamic Genes

To assess the effects of SIF on neuropeptide mRNAs in the hypothalamus, a semiquantitative RT-PCR was used to detect some gene expressions. See Fig.1 and Fig. 2.

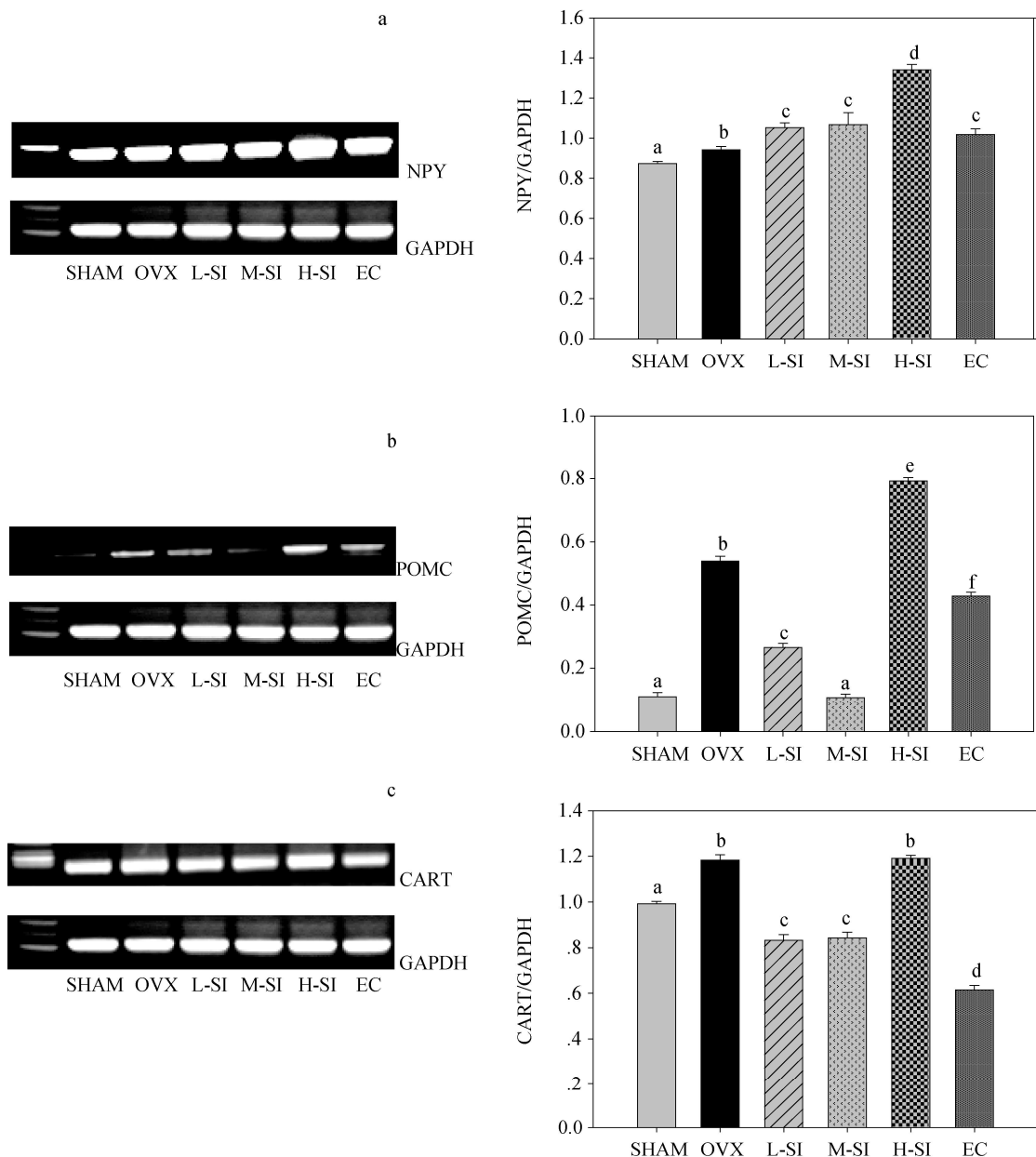


FIG. 1. Effect of SIF on peptides in ARC. Animal groups comprised sham-operated control (SHAM), ovariectomized (OVX) control, and ovariectomized rats treated with different doses of SIF (L-SI, M-SI, and H-SI). Means at a partition without a common letter differ significantly,  $P<0.05$ ,  $n=9$ .

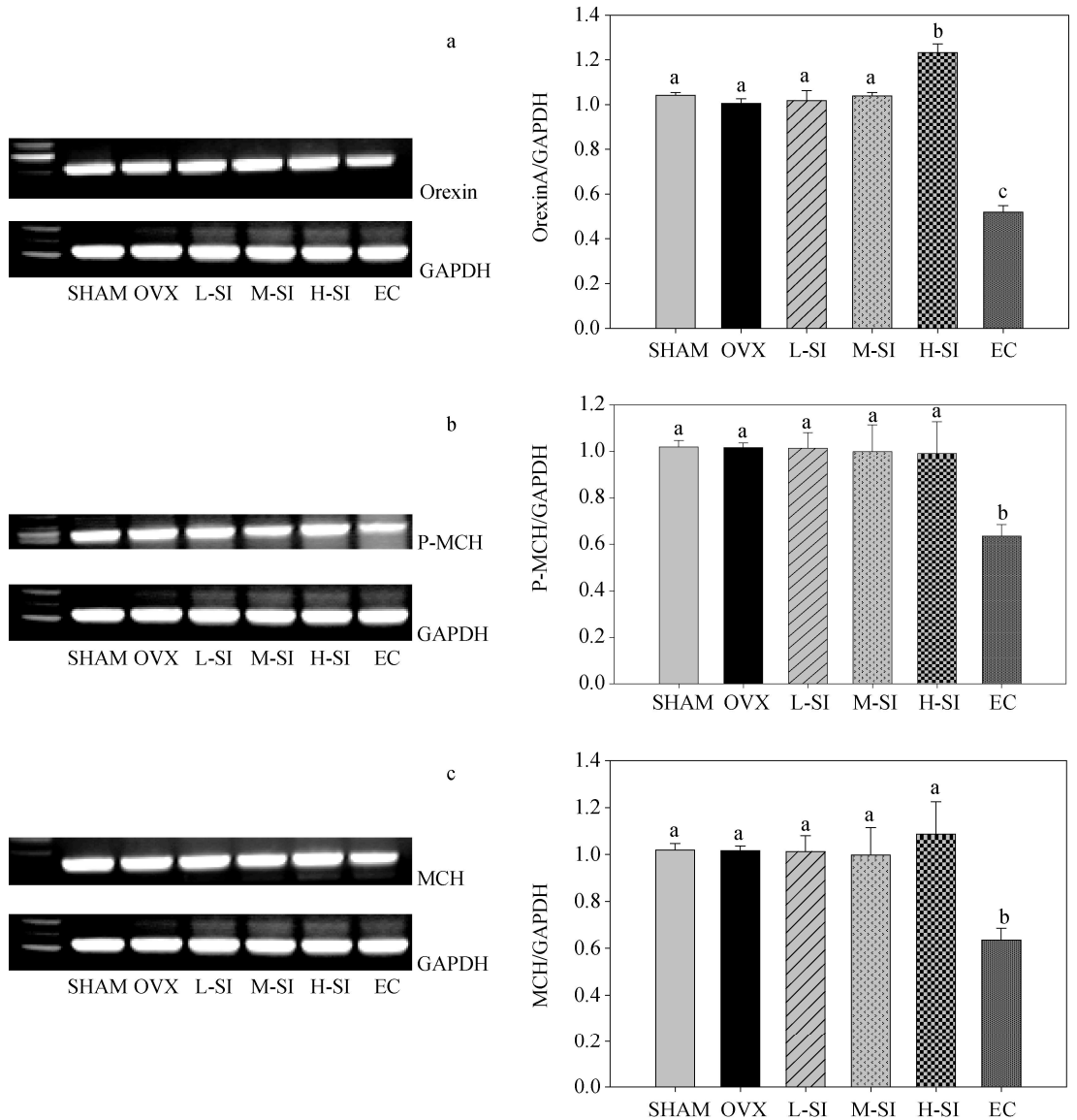


FIG. 2. Effect of SIF on peptides in LHA. Animal groups comprised sham-operated control (SHAM), ovariectomized (OVX) control, and OVX rats treated with different doses of SIF (L-SI, M-SI, and H-SI). Means without a common letter differ,  $P < 0.05$ ,  $n = 9$ .

NPY, POMC, and CART mRNA expressions in arcuate nucleus (ARC) were significantly changed in the SIF group. Compared with the OVX control group, the NPY mRNA level was significantly increased ( $P < 0.05$ ) and showed a dose-dependent manner (OVX:  $0.94 \pm 0.02$ ; L-SI:  $1.05 \pm 0.02$ ; M-SI:  $1.07 \pm 0.06$ ; H-SI:  $1.34 \pm 0.03$ ) (Fig. 1, a). The expression of POMC mRNA reduced by more than 100% ( $P < 0.05$ ) and that of CART by more than 30% ( $P < 0.05$ ) in the L-SI, M-SI group, while in the H-SI ( $P < 0.05$ ) group the POMC gene expression increased, but the CART gene expression remained unchanged.

(Fig. 1, b, c).

In contrast, MCH and P-MCH gene expressions in lateral hypothalamic area (LHA) in the SIF groups were unchanged. The expression of orexin mRNA in the L-SI and M-SI groups was neither altered, but it was higher in the H-SI group ( $P < 0.05$ ) (Fig. 2).

#### *Expression of Ghrelin Gene in Fundus of Stomach and Leptin Gene in Fat*

As shown in Fig. 3, the mRNA expression of ghrelin was higher in the SIF groups compared with the OVX control group ( $P < 0.05$ ), and this was

consistent with the change of NPY mRNA expression in hypothalamus. However, the mRNA expression of leptin in the SIF groups was not changed consistently. Compared with the OVX

control group, the mRNA expression of leptin was higher in the L-SI group ( $P<0.05$ ) but lower in the M-SI ( $P<0.05$ ), and no difference was observed in the H-SI group (Fig. 3b).

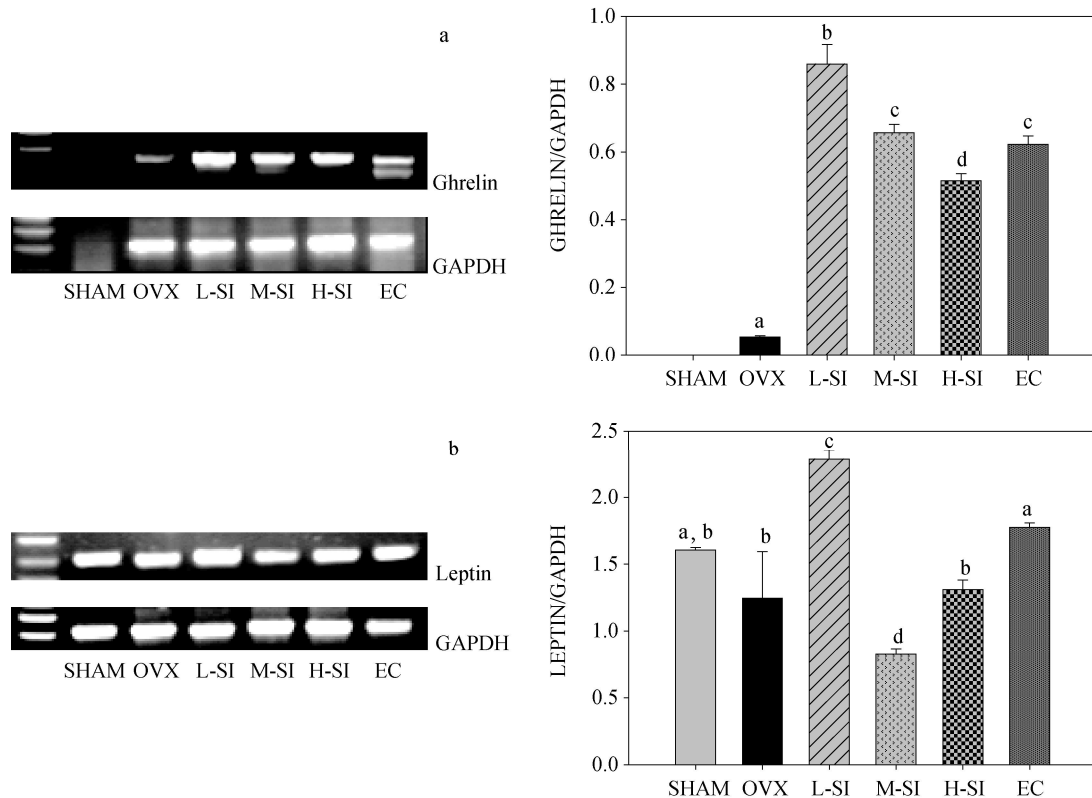


FIG. 3. Effect of SIF on peripheral peptides ghrelin mRNA expression in fundus of stomach (a) and leptin mRNA expression in fat (b). Animal groups comprised sham-operated control (SHAM), ovariectomized (OVX) control, and OVX rats treated with different doses of SIF (L-SI, M-SI, and H-SI). Means without a common letter differ significantly,  $P<0.05$ ,  $n=9$ .

## DISCUSSION

Some reports have shown that SIF could significantly decrease BW, white and brown adipose tissue weight whether or not the animals have undergone ovariectomy<sup>[13,15-16]</sup>. In this research, we have found that SIF could inhibit the increase of BW and food intake in the OVX rats fed with high-fat diet and there is a dose-effect relationship. There might be two reasons for the decrease in BW: 1) SIF has the ability to increase lipolysis in isolated rat adipocytes and decrease lipogenesis in white adipose tissue in the OVX rats, which has been proved by the previous reports<sup>[17-18]</sup>, and 2) SIF could reduce food intake, resulting in the decrease in BW, which is the major focus of our research.

It has been proved that hypothalamus plays an important role in the controlling of food intake. There

are two types of neurons in ARC. One type is NPY/AgRP neurons which increase food intake, and the other type is described as the POMC/CART neurons which secrete anorexigenic peptides to decrease food intake<sup>[19-20]</sup>. Orexin and MCH are orexigenic signals and coexist in LHA which is the feeding and hunger center<sup>[19-21]</sup>. Gut peptides related to food intake such as ghrelin and leptin act according to these hypothalamus signals. Lephart *et al.*<sup>[15]</sup> have found that SIF could stimulate the brain NPY secretion and increase food intake in the male Long-Evans rats fed with the diet of containing SIF (600 mg/kg diet). However, Cederroth *et al.*<sup>[8]</sup> have found that SIF could down-regulate the AgRP gene expression and up-regulate the orexin and the MCH gene expressions, while the NPY, POMC, CART gene expressions were unaffected in the male CD-1 mice fed with the high soy-containing diet. In this

study, the brain NPY gene expression increased but the BW and food intake reduced in the 3 SIF groups, while the POMC and CART gene expressions in the L-SI and M-SI groups had a decrease tendency. The Orexin, MCH, P-MCH gene expressions were not altered. The possible reason for such occurrence might be that SIF inhibited food intake and then the central nervous system released more orexigenic signals and less anorexigenic signals to stimulate food intake to maintain energy balance in a long-term regulation. Consistent with this hypothesis, de Rijke *et al.*<sup>[22]</sup> found that the expression level of AgRP/NPY was increased by 5-folds and POMC/CART was reduced by 2-folds in the ARC in food-restricted running activity-based anorexia (ABA) rats. Meanwhile, we also found that the expression of POMC mRNA increased in the H-SI group, which may explain the maximum inhibition of food intake in this group.

Ghrelin and leptin are major peripheral signals and leptin exerts a negative regulatory effect on the release of ghrelin. Intracerebroventricular and intraperitoneal administration of ghrelin exerts an orexigenic activity through the NPY and AgRP systems<sup>[23-24]</sup>. Our research found that ghrelin had a positive correlation with NPY level and a negative correlation with POMC/CART levels, which was similar to the pathway by which ghrelin activated NPY/AgRP neurons and inhibited POMC/CART neurons<sup>[20]</sup>. Leptin gene expression was not consistent. Compared with the OVX control group, the rat's leptin level of the L-SI group increased, but BW also increased significantly. This may indicate that there possibly exists leptin-resistance in obese rats.

Furthermore, in this study, we also found that during the development of reduced energy store, neuropeptides in first-order neurons in ARC were regulated in an adequate response to negative energy balance, whereas gene expression levels of the other neuropeptides in secondary neurons in the LHA were unchanged. The results proved that the regulation of the energy will be regional and this was consistent with de Rijke *et al.*<sup>[22]</sup>.

In summary, SIF can reduce BW by decreasing food intake and modulate hypothalamus and peripheral orexigenic genes to maintain energy balance in a long-term regulation. Further research is needed to explore other factors involved in maintaining energy balance.

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