The American Journal of Chinese Medicine, Vol. 35, No. 2, 297–308

Institute for Advanced Research in Asian Science and Medicine

The Water Extract of Adlay Seed (*Coix lachrymajobi var. mayuen*) Exhibits Anti-Obesity Effects Through Neuroendocrine Modulation

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Abstract: To find out whether the immunohistochemical expression of neuropeptid Y (NPY) and leptin receptor (LR) in the rat hypothalamus is influenced by adlay seed water extract (adlay), obesity in rats was induced by high fat diet (HFD) for 8 weeks; these rats were injected with 50 mg/100 g body weight adlay daily for 4 weeks. The results showed that the optical density of NPY immunoreactivity in paraventricular nucleus of rats increased approximately by 3.4 fold in HFD group compared to the normal diet group. Conversely, that of HFD + adlay group was about 2.6 fold lower than HFD group. The pattern of LR expression was similar to that of NPY. Both of NPY and LR mRNA levels, determined by real time PCR, in HFD + adlay group were decreased compared to those of HFD group, but there were no significant changes in the level of LR. These results suggest that adlay may regulate neuroendocrine activity in the brain. Accordingly, administration of adlay may be considered for therapies targeting obesity.

Keywords: Immunohistochemistry; Neuropeptid Y; Leptin Receptor; Hypothalamus; Paraventricular Nucleus.

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Introduction

Obesity is one of the major health hazards in humans in modern society. Genetic (monogenic, susceptible gene) and environmental (diet, exercise, social factors, chemicals, etc) factors are involved in the development of obesity (Kopelman, 2000). There is an increased availability over consumption of foods high in energy density and fat content with a concomitant decline in activity-dependent energy expenditure. However, in most humans, body weight is maintained in stable condition. Positive energy balance as a result of less energy expenditure as compared to energy intake leads to the storage of energy in the form of fat. The effects of dietary fat may be mediated, at least in part, by changes in the expressions of genes in the brain that are involved in energy balance (Havel, 2004; Giraudo *et al*., 1994; Chavez *et al*., 1998). Dietary fat also affects plasma level of leptin, a hormone that exerts a key function in regulating food intake and body weight (Friedman and Halaas, 1998). Our previous investigations reported that serum levels of leptin in rats given a high fat diet were over two-fold higher than those in the control group fed with a normal diet (Kim *et al*., 2004).

Leptin controls energy balance through regulation of neuropeptide Y (NPY) and leptin receptor (LR) expressions in the hypothalamus (Spanswick *et al*., 1997). And the hypothalamus is also known as an important area of the brain for the regulations of food intake and energy balance (Dryden *et al*., 1997; Sahu, 2003). NPY, a 36-amino-acid peptide, stimulates feeding, decreases energy expenditure and is mainlyin the hypothalamus (Billington *et al*., 1991). It is notably one of the most abundant brain peptides in the paraventricular nucleus (PVN) and arcuate nucleus (ARN) and other regions implicated in the regulations of feeding behavior, energy balance, and pituitary secretion (Chronwall *et al*., 1985). LR, a member of the class I cytokine receptor family, is known to be presented in the choroids plexus, cerebral cortex, hippocampus, thalamus, and hypothalamus (Tartaglia *et al*., 1995; Tartaglia, 1997; Hakansson *et al*., 1998; Chen *et al*., 1996) and has been clearly demonstrated to be capable of initiating signal transduction (Friedman and Halaas, 1998; Ghilardi *et al*., 1996; Lee *et al.*, 1996; Meister, 2000; Vaisse *et al*., 1996; Korea Ministry of Health & Welfare, 2000).

Adlay (*Coxi lachryma-jobi*) has long been used in traditional Chinese medicine and as a nourishing food, includes nutrients with 16.2% proteins, 4.65% lipids, 79.17% carbohydrates and a small quantity (330 mg%) of vitamin B1 (Nagao *et al*., 1985). The seed of adlay has been used in Asian countries for the treatment of warts, rheumatism, female endocrine system and neuralgia from ancient times and reported to exhibit anti-inflammatory, stomachic, diuretic, and antispastic effects *in vivo*. Recent studies demonstrated that adlay seed could inhibit the allergic diseases and increase the antitumor and anticancer effects in experimental animals. A number of studies have shown some physiological effects of adlay extracts (Takahashi *et al*., 1986; Park *et al*., 1988; Hidaka *et al*., 1992; Check and K'Ombut, 1995; Kondo *et al*., 1998; Otsuka *et al*., 1988; Tsai *et al*., 1999; Shyu *et al*., 1998; Huang and Chiang, 1999). Also there was our previous study on hypolipidemic effects of crude extract of adlay seed on the obese rats fed with a high fat diet (Kim *et al*., 2004). However, there is little known about its effects on the neuroendocrine modulation, such as NPY and LR, in diet-induced obesity rat brains.

Accordingly, in this present study, we investigated the effects of adlay seed crude water extract on NPY and LR immunoreactivties in obese rat hypothalamus.

Materials and Methods

Materials

Trizol reagent was obtained from GIBCO/BRL (Invitrogen, USA). The Oriental herbal medicine, *Coix lachrymajobi var. mayuen* (adlay seed), was bought from Kyungdong Market in Seoul Korea. All other chemicals and reagents unless otherwise noted, were obtained from Sigma (St. Louis, MO).

Experimental Groups

Male Sprague-Dawley rats aged 2 weeks-old and weighing 90~100 g were used. The rats were purchased from Biogenomic Co. (Kyung-gi do, Korea). They were housed individually in cages, and given laboratory rodent chow diet (Purina Co. Korea) for 1 week. Then the rats divided into normal and obesity groups and fed with normal diet (ND) and high fat diets (HFD) for 8 weeks, respectively. The HFD group was further divided into two groups by without or with oriental herbal medicine, adlay seed water extract (adlay) for 4 weeks. HFD + saline (control) group was fed with HFD and injected with saline. HFD + adlay group was fed with HFD and treated by oral adlay injection, 50 mg/100 g body weight daily for 4 weeks. Food intakes were measured everyday and body weights were recorded every other day for 8 weeks. All animal procedures used were in strict accordance with the guidelines of the National Institutes for the Care and Use of Laboratory Animals. Rats were maintained in a temperature controlled environment at 22 ± 2°C and under a 12:12 light-dark cycle. Diet and water were available *ad libitum* throughout the experiment period. In this study, a total of 45 rats were sacrificed, among them, blood was collected from the heart (n = 5/group) for blood assay, and tissue from decapitation (n = 5/group) were used for reverse transcription and real time PCR and immunohistochemistry analysis.

Preparation of Animal Diets

Compositions of animal diet are shown in Table 1. Experimental diets were purchased from Dyets Inc. (Bethlehem, PA, USA). The diets were stored at a 4°C chamber.

Extraction of Herbal Medicine

The oriental herbal medicine, adlay seed, was soaked in cold distilled water for 4 hours and then was extracted by boiling in a round glass flask. The contents were filtered through a gauze. The filtrate was distilled, and concentrated by evaporator (Buchi totavapor R-124, Switzerland). The extracted herbal medicine was dried by a freeze dryer (Telstar Lioalfa 6-80, Spain) and stored at –20°C until the experiment.

Table 1. Compositions of the Experimental Diet (g/kg diet)

Groups / Ingredients	Normal*	High Fat Diet**
Casein	200.0	200.0
DL-Methionine	3.0	3.0
Cornstarch	650.0	150.0
Sucrose	–	150.0
Cellulose	50.0	50.0
Corn oil	50.0	–
Beef tallow	–	400.0
Mineral mix	35.0	35.0
Vitamin mix	10.0	10.0
Choline bitarate	2.0	2.0
Fat energy (%)	5.0	40.0
Total (g)	1000.0	1000.0

*Normal diet: AIN-76A purified rodent diet with 65% cornstarch #111753CLL (Dyets Inc., Bethlehem, PA, USA), **High fat diet: 40% beef tallow modified AIN-76A purified rodent diet #101556 (Dyets Inc., Bethlehem, PA, USA).

Measurement of Serum Triglyceride, Total Cholesterol and Leptin

Triglyceride and total cholesterol concentrations in serum were measured using enzymatic method of diagnostic kit from Sigma Chemical Co. (St. Louis, MO). Serum leptin level was determined with a commercially available $[I]^{125}$-labeled leptin RIA kit (Linco Research, Inc.).

Immunohistochemistry

Animals were anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and rapidly perfused with phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde-0.05% glutaraldehyde in 0.2 M phosphate buffer (PB), pH 7.4. Brains were then removed and postfixed overnight at 4°C in 4% paraformaldehyde in 0.2 M PB, and cryoprotected in 20% sucrose in PBS. The fixed brains were sectioned coronally in 30 μm thicknesses on a freezing microtome (Leica, CM1850-7-1, Germany). The sections were incubated in 3% hydrogen peroxide for 15 min, 2% normal goat serum for 60 min, 1:2,000 dilution of rabbit anti-neuropeptide Y antibody (Cambridge Research Biochemicals, Wilmington, DE, USA) or 1:100 dilution of goat anti-leptin receptor antibody (R&D Systems, Inc., Minneapolis, USA) in PBS with 0.2% Triton X-100 and 2% normal goat serum for 72 hours or 24 hours at 4°C, respectively, and washed with PBS. Bound antibodies were visualized with the Vectastain ABC (Vector Labs., Burlingame, CA, USA) according to the manufacturer's directions. After rinsing in PBS, peroxidase activity was visualized as fellows: sections were preincubated in 0.05% 3,3′-diaminobenzidine-tetrahydrochloride (w/v, DAB, Sigma, USA) in 0.05 M Tris buffer (pH 7.4) containing 0.03% H_2O_2 and 0.4% ammonium nickel sulfate, and the images were immunoactivity nucleus observed under a light microscope (Olympus BX51, Japan).

Assessment of Immunoactivity Intensity

The intensity of NPY-specific staining was assessed in a quantitative fashion according to a microdensitometrical method based on optical density (mean gray scale) using an image analyzer (Multiscan, Fullerton, CA, USA). Before starting the image analysis, the light source was adjusted to the brightness to generate the best possible contrast between immunopositive and immunonegative cells. For assessment of LR-specific immunoreactivity, the number of LR-immunoreactive neurons was counted hemilaterally in each section through a light microscope.

Reverse Transcription and Real-Time PCR

At the end of experimental period, the rats were decapitated and their hypothalamus were removed immediately and processed for RNA isolation. Total RNA was extracted from rat hypothalamus by homogenization in 1 ml of Trizol solution (Gibco, Gaithersberg, MD). One microgram RNA was reverse-transcribed in a 20 ul reaction mixture using Moloney marine leukemic virus reverse transcriptase (invitrogen). The cDNA was amplified using Top-Taq Premix (CoreBio Lifescience & Biotech, Seoul, Korea), gene specific primers, and SYBR Gold (Molecular Probes, Eugene, OR). The real-time PCR was performed in the Rotor-Gene 3000 (Corbett Research, Sydney, Australia) using 0.2 ml capped tubes. The cycle profile was as fellows: initial denaturation at 95^{o}C for 10 min, followed by 40 cycles of 95^{o}C for 15 sec, 60^{o}C for 10 sec, and 72^{o}C for 20 sec. As a control of sample loading and normalization between samples, PCR amplification of the housekeeping gene GAPDH was included for each sample at each run. Fluorescence measurements were obtained online and analyzed with the version 6.0 software (Corbett Reseach). To confirm amplification specificity, the PCR products from each primer pair were subjected to a melting curve analysis. The relative quantifications of gene expressions were computed by using the comparative Ct (threshold cycle) method with a mathematical formula as previously described (Pfaffl *et al.,* 2002). The primer sequences specific for the genes examined and predicted product sizes are shown as follows: GAPDH: sense, ATC CCA TCA CCA TCT TCC AG; antisense, CCT GCT TCA CCA CCT TCT TG (579 bp); NPY: sense, CGC CTA CTC GTC CAT CCT GA; antisense, CCT TCA CAA TCA CCA TCT CA (181 bp); LR: sense, TGC TTA CTC GTC CAT TCT GC; antisense, CCT TCA CAA TCA CCA TCT CG (181 bp).

Statistical Analysis

The data of this study were statistically evaluated by ANOVA (analysis of variance) among the experiment groups. If significance was found by ANOVA, multiple comparison of Tukey (HSD) test was made between two groups: ND vs. HFD + saline (control), ND vs. HFD + adlay, control vs. HFD + adlay. There were significantly different when $p < 0.05$.

Results

Changes of Metabolism in the Experiment Groups

As previously reported (6), rats fed with the high fat diet developed obesity that ranged in severity (Table 2). HFD + adlay group significantly reduced food intake and body weights compared to those of HFD + saline (control) group ($p < 0.05$). Food intake was significantly positively correlated with final body weight gain ($p < 0.05$). Weights of epididymal and peritoneal fat were dramatically increased in HFD group compared to those of normal diet (ND) group but significantly decreased in HFD + adlay group in comparison to those of control group ($p < 0.05$). The levels of leptin, triglyceride, and cholesterol in blood serum were significantly decreased in HFD + adlay group compared to those of the control group ($p < 0.05$).

Expression of NPY Immunoreactivity in the Paraventricular Nucleus (PVN) in Obesity Rat Fed High Fat Diet

The intensity of NPY imunoreactivity in the PVN of hypothalamus was higher in HFD + saline (control) group than HFD + adlay and normal diet (ND) groups and that of HFD + adlay was decreased to a similar level of ND group ($p < 0.05$). Furthermore, we verified that the number of NPY immunoreactivity cells in the PVN was about 162.20 ± 7.34 per section in the control group, 109.34 ± 9.01 in HFD + adlay and 120.34 ± 11.36 in ND groups. NPY expressions in the PVN were significantly decreased following adlay seed water extract administration (Figs. 1A and 1B).

Table 2. Effects of Oriental Herbal Medicine, a Water Extract of Adlay Seed (*Coix lachrymajobi var. mayuen*), on Metabolic Changes in Obese Rats

	ND	HFD	
	(n = 5)	Saline (n = 5)	Adlay (n = 5)
Body weight (g)			
Initial	91.40 ± 4.5	91.34 ± 3.5	91.51 ± 4.3
Final	301.25 ± 23.67	576.50 ± 32.24‡	433.04 ± 27.21$^{\dagger *}$
Gain	209.85 ± 19.12	485.16 ± 20.45‡	341.53 ± 22.34$^{\dagger *}$
Fat-pad mass (g)			
Epididymal	0.73 ± 0.07	2.02 ± 0.54‡	1.57 ± 0.21$^{\dagger *}$
Peritoneal	0.42 ± 0.14	3.76 ± 0.65‡	2.93 ± 0.63$^{\dagger *}$
Food intake (g/day)	23.46± 0.57	32.6 ± 0.70‡	23.28 ± 0.60*
Serum leptin (ng/ml)	4.42 ± 0.27	9.95 ± 0.36‡	5.83 ± 0.41$^{\dagger *}$
Triglycerides(mg/dl)	73.6 ± 15.2	104.6 ± 19.6‡	93.5 ± 8.6$^{\dagger *}$
Cholesterol (mg/dl)	90.0 ± 3.1	120.4 ± 6.9‡	102.3 ± 9.2$^{\dagger *}$

ND: Normal diet group, HFD: high fat diet group, Saline: HFD rat injected saline group, adlay: HFD rat treated by oral injection, 50 mg/100 g body weight, adlay seed water crude extract group. Data are mean ± SD (n = 5 per group). They were compared using the ANOVA and post-Tukey test ($p < 0.05$). $^{\ddagger}p < 0.05$, ND vs. HFD + saline; $^{\dagger}p < 0.05$, ND vs. HFD + adlay; $^{*}p < 0.05$, HFD + saline vs. HFD + adlay.

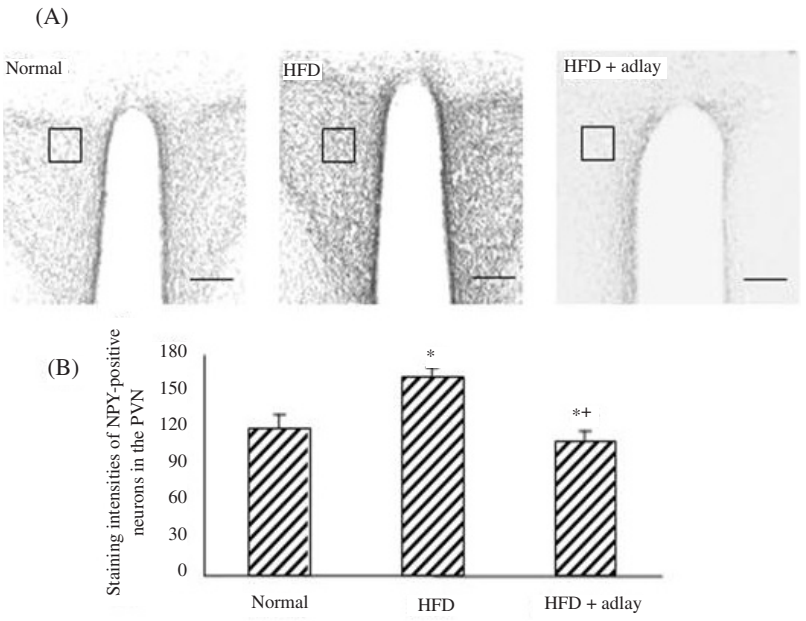

Figure 1. Expression of NPY in the PVN of obese rats treated with the water extract of adlay seed (*Coix lachrymajobi var. mayuen*). (A) The density of NPY immunoreactivity within a 200 × 200 μm grid at 100 magnifications. Scale bar represents 200 μm. (B) Data are presented as the mean ± SD (n = 5 for each group), $^{*}p < 0.05$ compared to the normal group, $^{+}p < 0.05$ compared to the control group.

Expression of Leptin Receptor Immunoactivity in the Arcuate Nucleus (ARC) in Obesity Rat Fed High Fat Diet

The hypothalamic LR immunoreactivity in the ARC of hypothalamus is shown in Figs. 2(A) and 2(B). In HFD + saline (control) group, the levels of LR expression in the ARC were higher than those of HFD + adlay and normal diet (ND) groups (Fig. 2A) and those of HFD + adlay were significantly decreased compared with that of the control group ($p < 0.05$). Accordingly, we confirmed that LR density in the ARC was about 78.25 ± 8.04 per section in ND group, 115.34 ± 4.57 in the control group, and those of HFD + adlay was decreased to 85.23 ± 3.81 (Fig. 1B). LR expressions in the ARC were decreased following oral injection of adlay seed water extract.

NPY and Leptin Receptor cDNAs in Rat Hypothalamus

The expressions of appetite peptides, NPY and LR, related to leptin resistance, in the rat hypothalamus were examined by real time reverse transcriptase PCR. NPY cDNA was decreased about 3.4 ± 0.4 fold in HFD + adlay group compared to that of the control group and LR cDNA was decreased about 1.3 ± 0.1 in HFD + adlay group. Conversely, those of

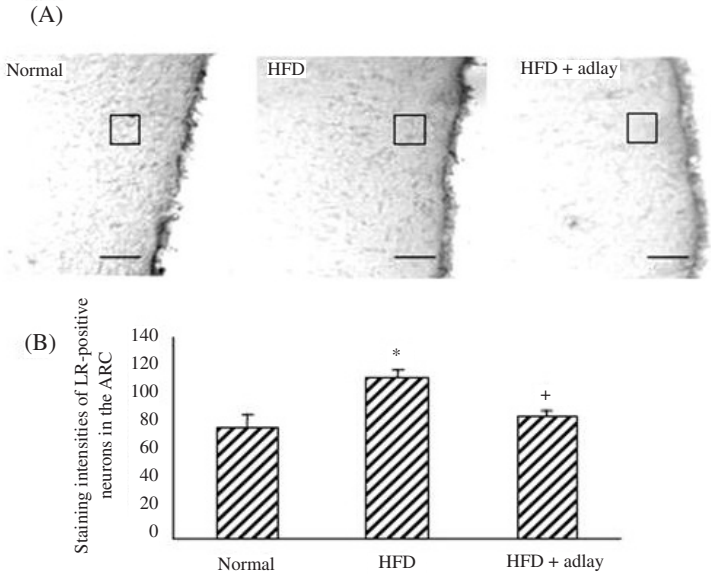

Figure 2. Expression of LR in the ARC of obese rats treated with the water extract of adlay seed (*Coix lachrymajobi var. mayuen*). (A) The density of LR immunoreactivity within a 200 × 200 μm grid at 100 magnifications. Scale bar represents 200 μm. (B) Data are presented as the mean ± SD (n = 5 for each group), $^{*}p < 0.05$ compared to the normal group, $^{+}p < 0.05$ compared to the control group.

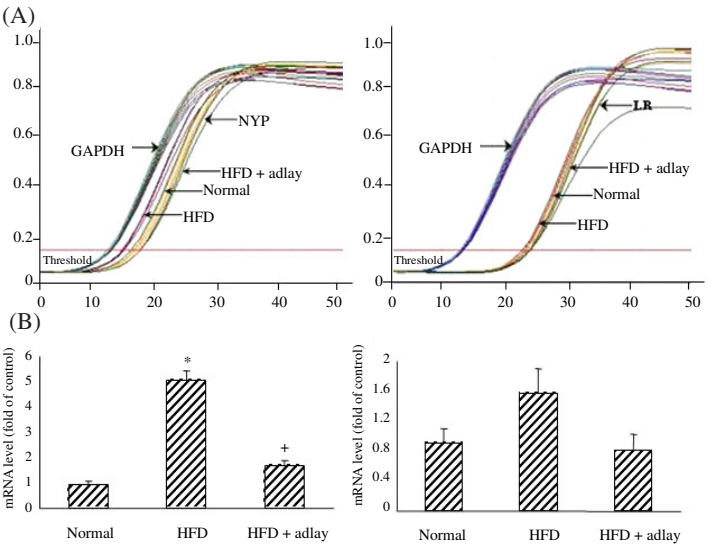

Figure 3. Verification of appetite-regulated genes in brain by real-time PCR. (A) The y-axis represents the fluorescence intensity and the x-axis the PCR cycle number. (B) A fold difference was calculated from the average threshold cycle values (mean ± SD) and presented as the mean ± SD (n = 5 for each group). The target gene expression was normalized by a housekeeping gene expression (GAPDH). Blue, GAPDH; red, HFD; yellow, normal; green, HFD + adlay. Determinations were performed triplicate from the samples that have been used for immunohistochemistry of the brain. $^{*}p < 0.05$ compared to the normal group, $^{+}p < 0.05$ compared to the control group.

the control group were increased compared to in HFD + adlay group but the cDNA of LR was not as significantly different as the cDNA of NPY between the control and HFD + adlay groups (Fig. 3).

Discussion

The aim of this study was to investigate the effects of water extract of adlay seed (*Coix lachrymajobi var. mayuen*) administration on neuroendocrine modulation in the hypothalamus of rats fed with a high fat diet (HFD) for 8 weeks. The HFD group was divided into two groups with or without oriental herbal medicine, adlay seed crude extract (adlay) by oral injection, 50 mg/100 g body weight, daily for 4 weeks. Rats given the HFD demonstrated some key characteristics of human obesity, including increased adiposity, hyperleptinemia and hyperlipidemia in our previous studies (Kim *et al*., 2004). The obese rats consumed significantly more energy, gained more weight and had significantly heavier fat pads compared with the less obese group. Serum leptin levels correlated positively with body fat mass (Kim *et al*., 2004; Considine *et al*., 1996). It acts within the hypothalamus via NPY (neuropeptied Y) and/or LR (leptin receptor) to reduce appetite and increase energy expenditure. Stephens *et al.* (1995) reported that an increase in NPY mRNA expression in the hypothalamus maybe implicated in the development of hyperphagia and other neuroendocrine abnormalities seen in the ob/ob mouse. Chavez *et al.* (1998) refered that NPY mRNA levels in the hypothalamic arcuate nucleus (ARC) was increased in diabetic rats. NPY injection into the PVN stimulates food intake adversely in nondiabetic animals. Archer *et al*. (2004) reported high fat-fed rats exhibited symptoms of developing metabolic syndrome with elevated plasma concentrations of glucose, triglycerides, nonesterified fatty acids, insulin, and leptin, and in addition, higher leptin receptor gene expression in the hypothalamic arcuate nucleus (ARC). Muhlhausler *et al.* (2006) showed that the expression of NPY was inversely related to total relative fat mass. These results were similar to and support our study. Accordingly, we suggest adlay seed water extract (adlay) can regulate NPY and/or LR expressions in rats fed with HFD. Schwartz *et al.* (2002) reported that a greater amount of food intake would be required to achieve a normal level of food-elicited negative feedback in the central nerve system. In these conditions, on the contrary, where NPY is reduced and leptin is elevated. The leptin elevation may mediate the observed reduction in intake following overfeeding. A number of studies have reported that in obesity, there may be a failure of central feedback mechanisms for metabolic homeostasis (Schwartz and Moran, 2002; Schwartz *et al*., 2000; Woods *et al*., 1998; Mercer *et al*., 1996).

In conclusion, our results showed that obese rats induced by a high fat diet (HFD) without adlay have higher NPY and/or LR immunoreactivity than other groups in the PVN and ARC of hypothalamus, which may indicate the failure of central feedback mechanisms for metabolic homeostasis. Conversely, the treatment of the obese rats with adlay seed water extract (adlay) reduced body fat mass, body weight, serum leptin level and immune activities of NPY and LR. These results suggest that treatment of obese rats with adlay may not only regulate feeding behavior but also neuroendocrine activities. Accordingly,

administration of adlay might be considered for therapies targeting obesity. Further studies are needed to increase our understanding of the antiobesity effects by the major constituents of adlay seed (*Coix lachrymajobi var. mayuen*) and the mechanism of adlay influences obesity induced by a high fat diet in the rat brain.

Acknowledgments

This study was supported by a grant of the Oriental Medicine R&D Project, Ministry of Health & Welfare, Republic of Korea (01-PJ9-PG1-01CO01-0003). Sung Ok Kim and Su-Jin Yun were recipients of BK21 fellowship from the Ministry of Education.

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