Exp. Anim. 63(4), 447–457, 2014

—Original—

Exogenous Leptin Administered Intramuscularly Induces Sex Hormone Disorder and Ca Loss via Downregulation of *Gnrh* and PI3K Expression

Lihong WU[1,4,6], Wen LIU[2], Nashun BAYAER[3], Weiwang GU[1], and Jieli SONG[5]

[1]*Department of Laboratory Animal Center, Southern Medical University, 1023 Guangzhou North Road, 510515 Guangzhou, Guangdong, P.R. China*
[2]*Department of Pathology, University of Tennessee Health Science Center, 38163 Memphis, Tennessee, USA*
[3]*Department of Laboratory Animal Center, Guang Dong Medical College, 523808 Zhanjiang, P.R. China*
[4]*Songshan Lake Pearl Laboratory Animal Sci. &Tech. Co., Ltd., 523808 Dongguan, P.R. China*
[5]*Department of Vasculocardiology, The Fifth Affiliated Hospital of Southern Medical University, 510900 Guang Zhou, Guangdong, P.R. China*
[6]*Key Laboratory of Oral Medicine, School and Hospital of Stomatology, Guangzhou Medical University, 510140 Guangzhou, P.R. China*

Abstract: Obesity is a public health problem that increases the risk of metabolic disease, infertility, and other chronic health problems. The present study aimed to develop a new rat model for sex hormone disorder with overweight and Ca loss by intramuscular injection of exogenous leptin (LEP). Thirty female Sprague-Dawley (SD) rats (40 days old) were injected thrice intramuscularly with LEP or keyhole limpet hemocyanin immunogen. The following analyses were performed to determine the development of appetite, overweight, reproductive related-hormones, and calcium (Ca)/phosphorus (Pi) in SD rats: measurement of Lee's index, body weight, food intake; serum Ca, Pi, and hormone tests by enzyme-linked immunosorbent analysis; histological analysis of abdominal fat; real-time polymerase chain reaction analysis of neuropeptide Y, pro-opiomelanocortin, gonadotropin-releasing hormone (*Gnrh*) mRNA, and gonadotropin-releasing hormone receptor (*Gnrhr*) mRNA expression; and western blotting analysis of enzyme phosphatidylinositol-3-kinase (PI3K). Rats injected with LEP immunogen displayed significantly increased body weight, food intake, Lee's index, serum LEP, serum cortisol, fat deposition in the abdomen, and decreased hormones including follicle stimulating hormone, luteinizing hormone, estradiol, cholecystokinin, and Ca. Exogenous LEP administered intramuscularly also downregulate *Gnrh* and PI3K. In conclusion, exogenous LEP administered intramuscularly is a novel animal model for sex hormones disorder with overweight and Ca loss in SD rats. The downregulation of PI3K and *Gnrh* may be involved in the development of this animal model.
Key words: leptin, rat model, obesity, sex hormone, *Gnrh*, PI3K

Introduction

Overweight/obesity is a major international problem related to osteoporosis and many reproductive health problems. Although the mechanisms mediating the influence of metabolism and nutrition on fertility are cur-

(Received 14 March 2014 / Accepted 26 May 2014 / Published online in J-STAGE 22 July 2014)
Address Corresponding: L. Wu, Laboratory Animal Center, Southern Medical University, 1023 Guangzhou North Road, 510515 Guangzhou, Guangdong, P.R. China
W. Gu, Laboratory Animal Center, Southern Medical University, 1023 Guangzhou North Road, 510515 Guangzhou, Guangdong, P.R. China
J. Song, The Fifth Affiliated Hospital of Southern Medical University, 510900 Guang Zhou, Guangdong, P.R. China
Lihong Wu, Wen Liu and Nashun Bayaer are equal to this work.

rently unclear, a strong association between metabolic disorders and infertility is undeniable [23]. Seli E and Wathes DC reported that obesity and overweight, which are characterized by lipid and glucose metabolic disorders, are significantly involved in the incidence of polycystic ovary syndrome and impaired natural fertility [29, 35].

Female fertility depends on adequate nutrition and energy reserves, suggesting a correlation between metabolic reserve and reproductive capacity [9, 24]. Innumerous studies have described a critical role for leptin (LEP) in the regulation of gonadal function, uterine physiology, pregnancy, and implantation [3, 5, 10]. LEP dysfunction in both animals and humans is associated with profound metabolic abnormalities including obesity, dyslipidemia and LEP resistance [27]. LEP or its receptor dysfunction is among the answers explaining anovulation during obesity that leads to reproductive problems such as infertility [6]. A Lep-deficient (*ob/ob)*, LEP receptor-deficient (*db/db*), and *Lep*-knockout mouse induced animal model been used in studies on obesity and metabolic syndromes, which compromise reproductive ability and bone mass [33].

The hypothalamic hormone gonadotropin-releasing hormone (GNRH) plays a central role in reproduction [11]. As the key regulator of reproduction, GNRH is released by neurons in the hypothalamus and transported via the hypothalamohypophyseal portal circulation to the anterior pituitary to trigger gonadotropin release for gonadal steroid genesis and gametogenesis [14]. LEP is essential to the hypothalamic release of GNRH and the stimulation of reproductive activities in mammals and birds [3, 21]. Shi *et al.* showed that the intramuscular injection of exogenous LEP mimicked LEP bioactivity loss in laying hens that had extra fat deposition and lower egg production [32]. LEP is also an important factor in the development and function of the mammary glands in goats [17]. LEP receptor signaling inhibits ovarian follicle development and egg laying in hens [16]. However, whether the intramuscular injection of exogenous LEP mimics LEP bioactivity loss and represents a mammal model for obesity with reproductive problems remains unknown and less evidence were shown in the earlier studies. Moreover, the metabolic disorder of lipid/glucose and sex hormones is responsible for the calcium (Ca)/phosphorous (Pi) imbalance or reducing levels of Ca/Pi [39]. In fact, the Ca/Pi disorder is responsible for osteoporosis [12]. In neurons, as in a variety of other cell types, phosphatidylinositol-3-kinase (PI3K) is a key intermediate that is common to the signaling pathways of a number of peripheral metabolic cues, including insulin and LEP, which are well known to regulate both metabolic and reproductive functions [1].

In the present study, female Sprague-Dawley (SD) rats were immunized thrice with the fusion proteins of LEP on experimental Days 1, 21, and 42. The effects on food intake, body weight, reproduction-related hormones, Ca/Pi, abdominal fat deposition, *Gnrh*, and gonadotropin-releasing hormone receptor (*Gnrhr*) mRNA and PI3K expression in the hypothalamus at different time points were analyzed.

Materials and Methods

Animals and ethical approval

The present study was conducted in accordance with the National Institutes of Health Guides for the Care and Use of Laboratory Animals. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the Southern Medical University of China (R-20120928–01). Thirty female SD rats (40 days old) were purchased from the Laboratory Animal Center of Southern Medical University of China and used in the experiment. Five days before the experiment, all rats were adapted to the environment. One rat was kept per cage and each was allowed free access to water and food. Every rat was exposed to 12 h of indoor light each day. Collection of orbital venous plexus blood was performed under anesthesia (Xylazine, intramuscular injection, 0.13 mg/kg body weight). The rats were anesthetized and euthanized by exsanguinations prior to the tissue collection.

Preparation of immunogen

Recombinant Tibet minipig's LEP was prepared and purified as described previously [19]. The LEP fusion proteins were adjusted to a concentration of 6 mg/ml and mixed with a mineral oil adjuvant at a ratio of 1:2 (v: v) and homogenized to produce the LEP immunogens containing a final concentration of 2 mg/ml for immunization. The immunogen for the control group was prepared similarly but with keyhole limpet hemocyanin (KLH), a control protein used for immune competence testing (Sigma-Aldrich, St. Louis, MO, USA).

Experimental design

The experimental flow chart is shown in Fig. 1A. Thirty female SD rats 40 days of age were weighed and randomly divided into the LEP (n=15) and KLH (n=15) groups; the rats in each group were intramuscularly injected with 1 mg of LEP or KLH immunogen on Day 1. Booster immunizations were given on Days 21 and 42. Lee's index, body weight, food intake, and nose-to-anus length were determined, and orbital venous plexus blood (9:00 am) samples were collected on Days 0, 14, 28, 42, and 52. After centrifugation at 2,000 × *g*, the serum was separated and stored at −80°C until analyses of antibody titer, serum Ca, Pi, cholecystokinin (CCK), sex hormone, and other reproduction-related hormones. At Day 52, the rats were euthanized and the abdominal fat (individual mesenteric, inguinal, periuterus, and perirenal white tissues) was removed, weighed, and preserved in 4% formalin and the hypothalamus was collected and stored at −80°C for real-time polymerase chain reaction (RT-PCR). Lee's index was used to evaluate the degree of obesity in each rat [15]. Lee's index and the abdominal fat rate were calculated as follows: Lee's index=body weight (g) $^{1/3}$/nose-to-anus length (cm) × 1,000; abdominal fat rate=abdominal fat (g)/body weight (g) × 100.

Measurement of blood antibody titers

Goat anti-rat antibody and horse radish peroxidase were purchased from Biotechs (Guangzhou, China). Anti-LEP titer was tested as described previously [32].

Reproduction-related hormone, Ca, Pi, LEP, and CCK levels

Sex hormones including follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E2), progesterone (P), testosterone (T), prolactin (PRL), and other reproduction-related hormones including LEP, insulin-like growth factor-1 (IGF1), cortisol, growth hormone (GH),and Ca, and Pi in the serum were determined by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). CCK levels were measured using a rat CCK enzyme-linked immunosorbent assay (ELISA) kit (Groundwork Biotechnology Diagnosticate, San Diego, CA, USA). Serum LEP levels were measured using a rat leptin ELISA kit (Millipore Corporation, Billerica, MA, USA).

Histological analysis of abdominal fat

Abdominal fat was collected from the same position in each group, sectioned in paraffin, and stained with hematoxylin and eosin. Microscopic images were observed, captured, and analyzed using an Eclipse Ti-U inverted microscope (Nikon Corporation, Tokyo, Japan), NIS-Elements software (Nikon Corporation, Tokyo, Japan), and Image-Pro Plus version 6.0 software (Media Cybernetics, Inc., Washington, USA).

Real-time polymerase chain reaction (PCR) analysis of pro-opiomelanocortin (Pomc), neuropeptide Y (Npy), Gnrh, and Gnrhr messenger ribonucleic acid (mRNA) expression in the hypothalamus

Post-homogenization, total RNA was extracted from the hypothalamus using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Extracted RNA were reverse transcribed to synthesize first-strand complementary deoxy ribonucleic acid (cDNA) using First Strand cDNA Synthesis Kit (TOYOBO, Osaka, Japan). The cDNA was stored at −20°C for subsequent analysis. Primers were purchased from Invitrogen. The β-actin sequences included 5′-CACCCGCGAGTACAACCTTC-3′ (forward) and 5′-CCCATACCCACCATCACACC-3′ (reverse); *Gnrh* sequences were 5′- ATTCTACTGACTTGGTGCGTG-3′ (forward) and 5′-GGAATATGTGCAACTTGGTGT-3′ (reverse); *Gnrhr* sequences were 5′-GTATGCTGGAGAGTTACTCTGCA-3′ (forward) and 5′- GGATGATGAAGAGGCAGCTGAAG-3′ (reverse); *Npy* sequences were 5′-CTGACCCTCGCTCTATCC-3′ (forward) and 5′-GGTCTTCAAGCCTTGTTCT-3′ (reverse); and *Pomc* sequences were 5′-CCTCCTGCTTCAGACCTCCA-3′ (forward) and 5′- GGCTGTTCATCTCCGTTGC-3′ (reverse). SYBR Green real-time PCR was performed using the manufacturer's protocol with the following profile: 2 min at 50°C, 2 min at 95°C, followed by 40 two-temperature cycles (15 s at 95°C and 30 s at 60°C [β-actin] or 62°C [*Gnrh*] or 68°C [*Gnrhr*] or 63°C [*Pomc*] or 57°C [*Npy*]) (Real Time PCR Master Mix SYBR Green; TOYOBO, Osaka, Japan). Gene expression was normalized against β-actin [20].

Western blotting

The hypothalamic tissues were homogenized and centrifuged. The supernatant was mixed with 2× Laemmli buffer (Sigma-Aldrich, St. Louis, MO, USA), denatured, resolved by denaturing polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes. The β-actin and p-PI3K primary antibodies (Cell Signaling Technology, Inc., Boston, MA, USA) were used at a

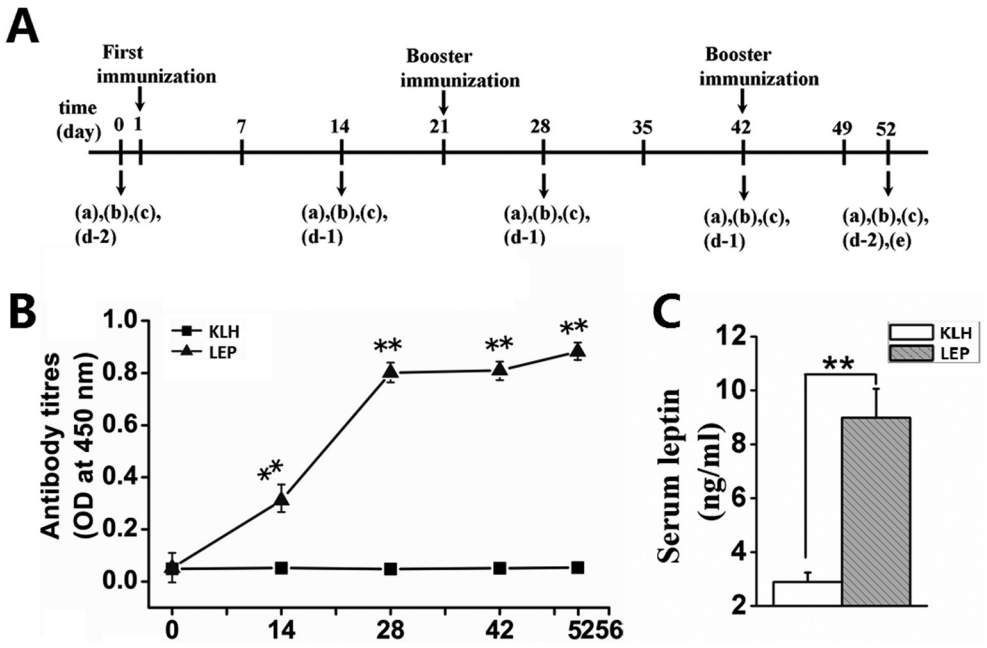

Fig. 1. The intramuscular injection of exogenous leptin stimulated a high immune reaction against leptin and induced hyperleptinemia hyperleptindemiahhhyperleptin in rats. (A) Experimental flow chart. Arrows indicate the experimental treatment and data collection time points; (a) body weight; (b) food intake; (c) nose-to-anus length; (d-1) blood sample collection for antibody titer; (d-2) blood sample collection for antibody titer, reproductive function-related hormone, cholecystokinin, calcium, phosphorus; (e) euthanasia for weighing abdominal fat, histological analysis of abdominal fat, real-time polymerase chain reaction analysis of gonadotropin-releasing hormone and gonadotropin-releasing hormone receptor in the hypothalamus, and western blotting for phosphatidylinositol-3-kinase in hypothalamus. (B) Anti-leptin antibody titer. (C) Level of serum leptin at the end of the experiment. Asterisks indicate significant differences between treatments ($^{*}P<0.05$, $^{**}P<0.001$). All values (mean ± standard error) were derived on a single-day basis of 15 animals per group.

1:200 dilution. The anti-rabbit secondary antibody (Cell Signaling Technology, Inc. Boston, MA, USA) was used at a 1:50,000 dilution. A SuperSignal West-Pico Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to develop the signal. The procedure was performed as described previously [18]. PI3K expression was normalized against β-actin. Image was observed, captured, and analyzed using an BIO-RAD Gel Doc XR+ (Bio-Rad Laboratories Co., Ltd., Hercules, CA, USA).

Statistical analysis

All values are reported as mean ± SEM. All statistical analyses were performed using SPSS 13.0 (SPSS Inc., Chicago, USA). RT-PCR data were analyzed by Student's *t*-test. Other data were analyzed by two-way analysis of variance and the means were compared by the least significant differences test at a significance level of 0.05.

Results

Antibody titer and level of serum LEP

The experimental flow chart is shown in Fig. 1A. Before the first immunization, the anti-LEP antibody titers in the blood were low in the two groups of rats. However, 7 days after the first inoculation of recombinant LEP immunogen, the anti-LEP antibody titers were significantly higher in the LEP -immunized rats than in the control rats ($P<0.001$) (Fig. 1B), which were barely detectable. As shown in Fig. 1C, serum endogenous circulating LEP in the LEP-immunized rats (8.98 ± 1.08 ng/ml) was threefold higher than that in KLH-immunized rats (2.88 ± 0.35 ng/ml).

Body weight, Lee's index, histological analysis of abdominal fat

To quantify the level of obesity, body weight and nose-to-anus length (data not shown) were measured and Lee's

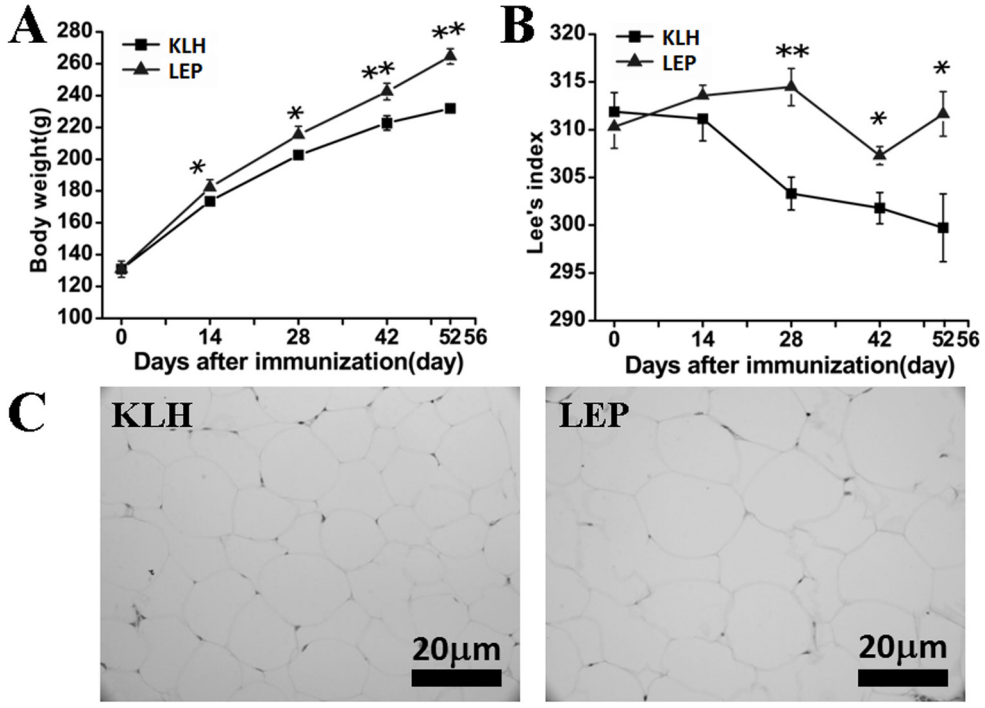

Fig. 2. Intramuscular injection of exogenous leptin increased body weight, Lee's index, and abdominal fat deposition. (A) body weight; (B) Lee's index; (C) representative images of abdominal fat of KLH and LEP. All values (mean ± SEM) were derived on a single-day basis of 15 animals per group. Arrows indicate immunogen inoculations; asterisks indicate significant differences between treatments ($^{*}P<0.05$, $^{**}P<0.001$). Bar=20 μm.

Table 1. Fat deposition in abdomen at the end of the experiment

	KLH (n = 15)	LEP (n = 15)
Abdominal fat weight (g)	4.18 ± 0.40	5.95 ± 0.35**
Abdominal fat (%)	1.81 ± 0.18	2.24 ± 0.11
Abdominal fat cell area (mm2)	112.27 ± 14.97	152.44 ± 7.91*
Abdominal fat cell number	76.17 ± 4.33	59.83 ± 4.09*

Data are presented as mean ± SEM. $^{*}P<0.05$ vs. KLH, $^{**}P<0.001$ vs. KLH. KLH, keyhole limpet hemocyanin; LEP, leptin.

index was calculated. The average body weight of the rats immunized with LEP was approximately 15% higher than that of those immunized with KLH at Day 52 (Fig. 2A). The increased body weight of the LEP-immunized rats might be associated with increased daily food consumption. Lee's index in LEP-immunized rats was significant higher than that in KLH-immunized rats from Day 28 (Fig. 2B).

At the end of the experiment when the animals were sacrificed, the LEP-immunized rats had 45% more abdominal fat than the KLH-immunized rats (4.18 ± 0.40 g) (Table 1). Histological analysis revealed that the abdominal adipocyte cell area of the LEP-immunized rats was 36% larger than that of the KLH-immunized rats, while the cell numbers under microscopic observation were only 79% of those in the KLH-immunized rats ($P<0.005$, Fig. 2C and Table 1).

Food intake, serum CCK, and Pomc/Npy ratio

The food intake of the LEP-immunized rats improved after the first injection; moreover, the differences were significant between the control and LEP-immunized rats at 28 and 52 days (Fig. 3A). The CCK level was significantly lower in the LEP-immunized rats (0.88 ± 0.07 ng/ml) and only one quarter of that in the KLH-immunized rats (3.46 ± 0.56 ng/ml) ($P<0.005$) (Fig. 3B).

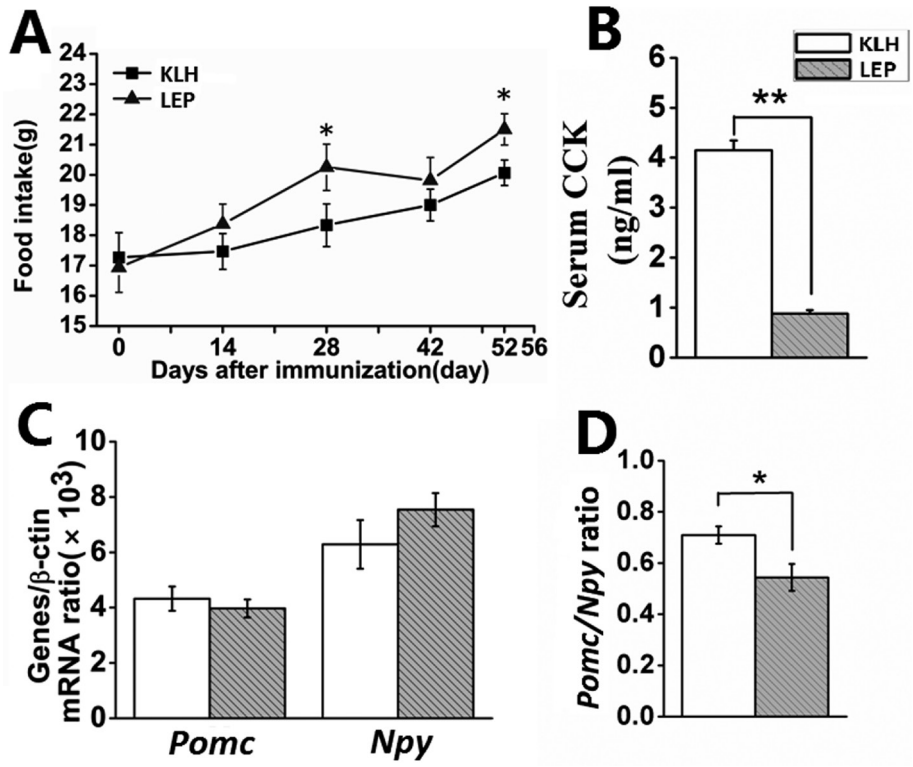

Fig. 3. Intramuscular injection of exogenous leptin increased food intake by inhibiting cholecystokinin (CCK) secretion. (A) food intake; (B) level of serum CCK at the end of the experiment; (C) pro-opiomelanocortin (*Pomc*) and neuropeptide Y (*Npy*) mRNA expression; (D) ratio of *Pomc/Npy*. Asterisks indicate significant differences between treatments ($^{*}P<0.05$, $^{**}P<0.001$). All values (mean ± SEM) were derived on a single-day basis of 15 animals per group.

Real-time PCR analysis revealed that the expression of *Pomc* and *Npy* in the hypothalamus did not differ significantly between groups ($P>0.05$) (Fig. 3C). However, the *Pomc*/*Npy* ratio in the hypothalamus was significantly ($P<0.05$) lower in the LEP-immunized rats (0.54 ± 0.05) than in the KLH-immunized rats (0.71 ± 0.03) (Fig. 3D).

Serum sex hormones, IGF1, cortisol, and GH

As shown in Table 2, levels of sex hormones in the blood, including FSH, LH, P, PRL, E2, and T did not differ significantly among groups before the first immunization ($P>0.05$). However, great changes occurred at the end of this experiment. Sex hormones in both KLH and LEP-immunized rats increased with age. At Day 52, the FSH level of the LEP-immunized rats was 33% lower than that of the KLH-immunized rats (6.22 ± 0.45 mIU/ml) ($P<0.05$); meanwhile, the LH and E2 levels of the LEP-immunized rats were 75% of those in the KLH-immunized rats (LH, 4.42 ± 0.36 mIU/ml; E2, 17.38 ± 1.44 mIU/ml) ($P<0.05$); moreover, the T level of the LEP-immunized rats was significantly higher (25%, $P<0.05$) than that of the KLH-immunized rats (1,148 ± 119 ng/ml). There were no significant differences in the P and PRL levels in the KLH and LEP-immunized rats ($P>0.05$). The serum IGF1 level in LEP-immunized rat (1,157 ± 264 ng/ml) was 27% significant lower than that of KLH-immunized rat. The levels of serum cortisol increased significantly with age in LEP-immunized rats. At the end of the experiment, the serum cortisol level in LEP-immunized rats (8.02 ± 0.53 ng/ml) was approximately 40% higher than that in the KLH-immunized rats ($P>0.05$). The magnitude of change of GH levels was less and there was no significant difference among groups ($P>0.05$).

Serum Ca and Pi

As shown in Table 3, Ca and Pi levels in the blood did

Table 2. Levels of serum reproduction-related hormones at Days 0 and 52

	KLH (n = 15)		LEP (n = 15)	
	Day 0	Day 52	Day 0	Day 52
FSH (mIU/ml)	3.97 ± 0.74	6.22 ± 0.45	3.55 ± 0.57	4.14 ± 0.51*
LH (mIU/ml)	3.26 ± 0.61	4.42 ± 0.36	3.36 ± 0.52	3.23 ± 0.39*
P (ng/ml)	0.73 ± 0.06	1.21 ± 0.21	0.78 ± 0.1	1.05 ± 0.1
PRL (ng/ml)	2.88 ± 0.38	5.54 ± 0.45	2.75 ± 0.43	5.11 ± 0.5
E2 (pg/ml)	6.31 ± 1.23	17.38 ± 1.44	6.78 ± 0.85	13.23 ± 1.16*
T (ng/ml)	466 ± 104	1,148 ± 119	478 ± 62	1,517 ± 108*
IGF1 (ng/ml)	1,160 ± 239	1,241 ± 405	1,574 ± 278	1,157 ± 264*
Cortisol (ng/ml)	4.35 ± 0.48	5.67 ± 0.85	4.06 ± 0.66	8.02 ± 0.53*
GH (ng/ml)	6.98 ± 0.73	3.81 ± 0.5	7.61 ± 0.64	3.61 ± 0.47

Data are presented as mean ± SEM. *P<0.05 vs. KLH. KLH, keyhole limpet hemocyanin; LEP, leptin; FSH, follicle-stimulating hormone; LH, luteinizing hormone; P, progesterone; PRL, prolactin; E2, estradiol; T, testosterone; IGF1, insulin-like growth factor-1; GH, growth hormone.

Table 3. Serum Ca loss in LEP-immunized rat at the end point of the experiment

	KLH (n = 15)		LEP (n = 15)	
	Day 0	Day 52	Day 0	Day 52
Ca (mmol/l)	2.68 ± 0.42	2.18 ± 0.42	2.74 ± 0.33	0.8 ± 0.06*
Pi (mmol/l)	3 ± 0.36	2.04 ± 0.19	2.88 ± 0.36	1.97 ± 0.2
Ca/Pi	0.91 ± 0.1	1.13 ± 0.25	0.96 ± 0.04	0.42 ± 0.04*
Ca × Pi	8.53 ± 2.1	4.31 ± 0.73	8.47 ± 2.02	1.59 ± 0.23*

Data are presented as mean ± SEM. *P<0.05 vs. KLH. Ca, calcium; Pi, phosphorous.

not differ significantly between the KLH and LEP-immunized rats before the first immunization (P>0.05). At Day 52, our data showed that the levels of Ca and Pi in the two groups were lower than those of Day 0. In contrast with the control group, immunization against LEP caused the blood to lose two-thirds of the Ca content (0.8 ± 0.06 mmol/l), while Pi did not differ significantly (P>0.05) at Day 52. More importantly, a Ca/Pi imbalance was seen. The Ca/Pi ratio in LEP-immunized rats seriously decreased to 0.42 ± 0.04 at the end of the experiment, whereas the Ca/Pi ratio in KLH-immunized rats remained at about 1.The change of Ca × Pi were similar to that of the Ca/Pi ratio.

Gnrh, Gnrhr mRNA expression, and PI3K signaling

By real-time PCR, the mRNA expression of *Gnrh*/β-actin in the hypothalamus was significantly lower (88%) in the LEP-immunized rats than that in the KLH-immunized rats (0.252 ± 0.020, P<0.05). The mRNA expression of *Gnrhr*/β-actin in the hypothalamus was slightly but non-significantly (P>0.05) lower in the LEP-immunized rats (0.492 ± 0.036) than in the control rats (0.541 ± 0.031) at the end of the experiment (Fig. 4A). PI3K protein in the hypothalamus of LEP-immunized rats significantly dropped to 0.29 ± 0.1 (gray level), reducing by nearly 11× compared to the control rats (gray level: 2.76 ± 0.74) (Figs. 4B and C).

Discussion

The immunogenicity of the LEP immunogen prepared in this study consisting of 2 mg of recombinant LEP in 1 ml of mineral oil adjuvant was appropriate and sufficient to stimulate a high immune reaction against LEP in rats as shown by the high antibody titers in the blood 28 day after the first inoculation. Subsequent repeated inoculations in rats further increase the antibody titers.

In the present study, body weight, Lee's index, and fat deposition were increased during the first 14 days in the presence of presumptive rising antibody titers against LEP. We predicted that the LEP immunogen increases LEP antibody formation, thereby blocking LEP bioavailability. Surprisingly, the intramuscular injection of LEP immunogen did not decrease endogenous LEP but rather stimulated endogenous LEP secretion and led to the excessive accumulation of endogenous LEP in the blood. These results may be consistent with the biological dysfunction of LEP in stimulating energy expen-

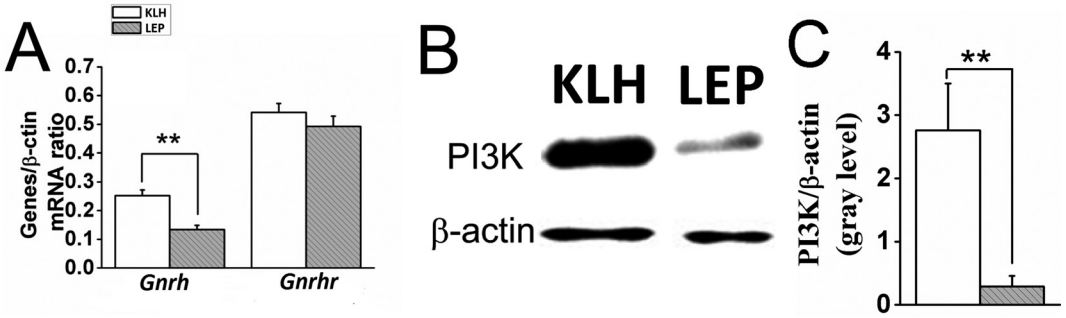

Fig. 4. Real-time polymerase chain reaction analysis of gonadotropin-releasing hormone (*Gnrh*) and gonadotropin-releasing hormone receptor (*Gnrhr*) mRNA expression (A) and western blotting analysis of phosphatidylinositol-3-kinase signaling (PI3K) (B) and gray levels of PI3K/β-actin in KLH and LEP group (C). Double asterisks indicate significant differences between treatments ($^{**}P<0.001$). All values (mean ± SEM) were derived on a single-day basis of 15 animals per group.

diture and inhibiting fat accumulation and appetite in *ob/ob* mice and *Lep*-KO mice [7, 25, 36]. However, these obesity models do not explain the entire pathogenesis in obese people. Studies have reported that LEP resistance occurred in obese people who did not have a *Lep* gene mutation or deletion like *ob/ob* or *Lep*-KO mice. Deficient model used by leptin antibody injection in hens also not fully explain the process and mechanism of mammalian metabolic diseases.

Obesity, the mechanism of which is still not completely elucidated, is a complex disease that involves interactions among diet, environment, genetic factors, drugs, and other diseases [30]. Hyperleptinemia or LEP resistance is a risk factor for diabetes, hypertension, and preeclampsia in pregnancy [34]. The bulk of the work investigating the correlation between LEP disorder and health risks has focused on chronic diseases; however, we are learning more about the components of intramuscular injection of exogenous LEP that induce hyperleptinemia, a novel mammal model for sex hormone disorder that links infertility and obesity.

The fact that LEP-immunized rats displayed progressive obese symptoms after the intramuscular injection of exogenous LEP may be due to overeating by inhibiting CCK secretion. In this study, LEP-immunized rats had lower serum CCK levels at the end of the experiment, which may be responsible for hyperphagia. CCK, a duodenal peptide secreted during meals that aids in digestion, activates an ascending pathway leading to the inhibition of food intake [37]. The mRNA expressions of *Pomc* and *Npy* were detected by RT-PCR. POMC and NPY antagonize each other, and their ratio is crucial in appetite regulation; appetite increases when the ratio is lowered and vice versa [38]. The present study showed that the *Pomc/Npy* ratio decreased after the intramuscular injection of exogenous LEP. Therefore, it has a complex effect on increasing appetite. The elevated daily food consumption was responsible for the increased body weight and Lee's index.

Since deficient LEP signaling causes infertility via reducing neuronal GNRH activity [31]. In our study, we highlight the effects of LEP immunogen on the reproductive system because the abnormal changes in sex hormones as well as the mRNA expression of *Gnrh*/β-actin and *Gnrhr*/β-actin are the reasons for infertility. The *Gnrh*/β-actin ratio was lower in LEP-immunized rats than in control rats. Anti-LEP antibody, which was produced by the intramuscular injection of exogenous LEP immunogen, may have a role as a GNRH antagonist. Our data also showed that the levels of FSH, LH, and E2 were significantly lower, while that of T was higher in LEP-immunized rats than in KLH-immunized rats. Pubertal onset occurs only in a favorable, anabolic hormonal environment [26]. The pubertal period of rat is from the age of 35 days (the beginning of the pubertal period) to the age of 4 months (post-pubertal period) [22]. In this study, we used 40-day-old rats. Studies have shown that estrogen deficiency or androgen excess induces delayed puberty or reproductive dysfunction [22]. The abnormal changes in sex hormones (sex hormone disorder) after exogenous LEP immunogen administered intramuscularly may induce delayed puberty or reproductive dysfunction in SD rats.

Decreased sex hormone and IGF1 levels in the blood

may be caused by the lower level of GNRH in the hypothalamus, which is likely to lead to infertility. This finding was consistent with the previous report that GNRH induces the release of LH, FSH, and IGF1 [21]. The reproductive system is controlled by GNRH secretion from the brain, which is finely modulated by a number of factors including gonadal sex steroids [13]. In addition, *Gnrhr* mRNA expression was decreased in LEP-immunized rats, although there was no significant difference among groups. GNRH antagonists compete with GNRH for its receptors and have an immediate and sustained suppressive effect on LH and FSH secretion [28].

Exogenous LEP immunogen administered intramuscularly induced a Ca/Pi imbalance. In this study, LEP showed hypocalcemia and a low Ca/P ratio in the blood. These results suggest that the intramuscular injection of exogenous LEP immunogen may also cause the body to lose Ca, which can lead to bone loss and osteoporosis over time. In addition to LEP dysfunction, there may be three reasons for hypocalcemia and low Ca/P in the blood. First, the low level of E2 in the blood may cause hypocalcemia and low Ca/P in the blood because E2 is essential in the development and growth of the skeleton and for the maintenance of bone mass and density [2, 8]. Second, the low level of GNRH in the hypothalamus may cause hypocalcemia and low Ca/P in the blood. Studies have reported that the treatment of precocious puberty with a GNRH antagonist or analog (GNRHA) reduces sex steroid levels and leads to a situation of hypoestrogenism that may theoretically have a detrimental effect on bone mass (reduction in bone mineral density) during pubertal development [5]. Third, the intramuscular injection of exogenous LEP immunogen induced an abnormally high level of cortisol in the blood. Studies have reported that hypercortisolism can impair reproductive processes and induce osteoporosis in females [9, 34].

Hyperleptinemia may be induced by the impairment of PI3K signaling pathway. A link between the PI3K signaling pathway and the regulation of *Gnrh* mRNA expression was observed in this study. The weakened PI3K signaling may be responsible for the downregulation of *Gnrh* mRNA expression in LEP-immunized rats. Impaired hypothalamic insulin and LEP receptor signaling is thought to be at the core of reproductive disorders associated with metabolic dysfunction [1]. Metabolic models of LEP resistance (hyperleptinemia) that are known to alter GNRH/LH release, such as diabetes diet-induced obesity, and caloric restriction, are also accompanied by impairments of PI3K signaling in insulin- and LEP-sensitive tissues including the hypothalamus [4]. Extensive studies will be needed to determine the mechanism of sex hormone disorder models that correlate infertility and obesity by the intramuscular injection of exogenous LEP.

In conclusion, exogenous LEP administered intramuscularly induced hyperleptinemia, overweight complicated with sex hormone disorder, and Ca loss in SD rats. The downregulation of PI3K and *Gnrh* may be involved in these effects of intramuscular injection of exogenous LEP. This may be a novel rat model for further exploratory studies of obesity complicated by infertility and osteoporosis.

Acknowledgments

This study was supported by the International Cooperation of Science (2011DFA33290), Science Project of Guangdong province (2010A011200003, 2010A030400001, 2012B011000004, 2012B010300001, 2011B060300014), and Scientific Research Foundation of Guangdong Medical College (B2013018). Lihong Wu, Wen Liu, and Nashun Bayaer organized the study. Weiwang Gu, Lihong Wu and Jieli Song conceived of and designed the experiments and analyzed the data.

References

1. Acosta-Martínez, M. 2011. PI3K: An Attractive Candidate for the Central Integration of Metabolism and Reproduction. *Front. Endocrinol. (Lausanne)* 2: 110. [Medline]
2. Antoniazzi, F., Zamboni, G., Bertoldo, F., Lauriola, S., and Tatò, L. 2004. Bone development during GH and GnRH analog treatment. *Eur. J. Endocrinol.* 151: S47–S54. [Medline] [CrossRef]
3. Barash, I.A., Cheung, C.C., Weigle, D.S., Ren, H., Kabigting, E.B., Kuijper, J.L., Clifton, D.K., and Steiner, R.A. 1996. Leptin is a metabolic signal to the reproductive system. *Endocrinology* 137: 3144–3147. [Medline]
4. Bifulco, G., Miele, C., Pellicano, M., Trencia, A., Ferraioli, M., Paturzo, F., Tommaselli, G.A., Beguinot, F., and Nappi, C. 2004. Molecular mechanisms involved in GnRH analogue-related apoptosis for uterine leiomyomas. *Mol. Hum. Reprod.* 10: 43–48. [Medline] [CrossRef]
5. Brannian, J.D. and Hansen, K.A. 2002. Leptin and ovarian folliculogenesis: implications for ovulation induction and ART outcomes. *Semin. Reprod. Med.* 20: 103–112. [Medline] [CrossRef]
6. Brannian, J.D. 2011. Obesity and fertility. *S. D. Med.* 64: 251–254. [Medline]

7. Chen, H.L., Tung, Y.T., Tsai, C.L., Lai, C.W., Lai, Z.L., Tsai, H.C., Lin, Y.L., Wang, C.H., and Chen, C.M. 2013. Kefir improves fatty liver syndrome by inhibiting the lipogenesis pathway in leptin-deficient ob/ob knockout mice. *Int. J. Obes. (Lond)*. [Epub ahead of print].
8. Fernández-Soto, M.L., González-Jiménez, A., Chamorro-Fernández, M., and Leyva-Martínez, S. 2013. Clinical and hormonal variables related to bone mass loss in anorexia nervosa patients. *Vitam. Horm.* 92: 259–269. [Medline] [CrossRef]
9. Geber, S., Brandão, A.H., and Sampaio, M. 2012. Effects of estradiol and FSH on leptin levels in women with suppressed pituitary. *Reprod. Biol. Endocrinol.* 10: 45. [Medline] [CrossRef]
10. Henson, M.C. and Castracane, V.D. 2006. Leptin in pregnancy: an update. *Biol. Reprod.* 74: 218–229. [Medline] [CrossRef]
11. Keri, G., Horvath, A., Nikolics, K., and Teplan, I. 1985. In the central role of GnRH in the regulation of reproductive functions: mechanism of action and practical results. *J. Steroid Biochem.* 23:(5B): 719–723. [Medline] [CrossRef]
12. Komoroski, M., Azad, N., and Camacho, P. 2014. Disorders of bone and bone mineral metabolism. *Handb. Clin. Neurol.* 120: 865–887. [Medline] [CrossRef]
13. de Koning, J., Lambalk, C.B., Helmerhorst, F.M., and Helder, M.N. 2001. Is GnRH self-priming an obligatory feature of the reproductive cycle? *Hum. Reprod.* 16: 209–214. [Medline] [CrossRef]
14. Lee, V.H., Lee, L.T., and Chow, B.K. 2008. Gonadotropin-releasing hormone: regulation of the GnRH gene. *FEBS J.* 275: 5458–5478. [Medline] [CrossRef]
15. Lei, F., Zhang, X.N., Wang, W., Xing, D.M., Xie, W.D., Su, H., and Du, L.J. 2007. Evidence of anti-obesity effects of the pomegranate leaf extract in high-fat diet induced obese mice. *Int. J. Obes. Lond.* 31: 1023–1029. [Medline] [CrossRef]
16. Lei, M.M., Wu, S.Q., Li, X.W., Wang, C.L., Chen, Z., and Shi, Z.D. 2014. Leptin receptor signaling inhibits ovarian follicle development and egg laying in chicken hens. *Reprod. Biol. Endocrinol.* 12: 25. [Medline] [CrossRef]
17. Li, M., Li, Q., and Gao, X. 2010. Expression and function of leptin and its receptor in dairy goat mammary gland. *J. Dairy Res.* 77: 213–219. [Medline] [CrossRef]
18. Linz, D., Hohl, M., Mahfoud, F., Reil, J.C., Linz, W., Hübschle, T., Juretschke, H.P., Neumann-Häflin, C., Rütten, H., and Böhm, M. 2012. Cardiac remodeling and myocardial dysfunction in obese spontaneously hypertensive rats. *J. Transl. Med.* 10: 187. [Medline] [CrossRef]
19. Liu, W., Wu, L., Xu, M., Guo, R., Gu, W., Shi, Z., and Yuan, J. 2013. [Effects of immunization with recombinant fusion protein of extracellular near-transmembrane domain of Tibet minipig leptin receptor on fat deposition in SD rats]. *Nan Fang Yi Ke Da Xue Xue Bao* 33: 832–837 (in Chinese). [Medline]
20. Matsubara, T., Kida, K., Yamaguchi, A., Hata, K., Ichida, F., Meguro, H., Aburatani, H., Nishimura, R., and Yoneda, T. 2008. BMP2 regulates Osterix through Msx2 and Runx2 during osteoblast differentiation. *J. Biol. Chem.* 283: 29119–29125. [Medline] [CrossRef]
21. McNeilly, A.S., Crawford, J.L., Taragnat, C., Nicol, L., and McNeilly, J.R. 2003. The differential secretion of FSH and LH: regulation through genes, feedback and packaging. *Reprod. Suppl.* 61: 463–476. [Medline]
22. Nosenko, N.D. and Lymarieva, A.A. 2013. [Peculiarities of sexual behavior of female rats with hyperandrogenia in pubertal and postpubertal periods]. *Fiziol. Zh.* 59: 20–24 (in Ukrainian). [Medline]
23. Pantasri, T. and Norman, R.J. 2014. The effects of being overweight and obese on female reproduction: a review. *Gynecol. Endocrinol.* 30: 90–94. [Medline]
24. Pasquali, R., Pelusi, C., Genghini, S., Cacciari, M., and Gambineri, A. 2003. Obesity and reproductive disorders in women. *Hum. Reprod. Update* 9: 359–372. [Medline] [CrossRef]
25. Pelleymounter, M.A., Cullen, M.J., Baker, M.B., Hecht, R., Winters, D., Boone, T., and Collins, F. 1995. Effects of the obese gene product on body weight regulation in ob/ob mice. *Science* 269: 540–543. [Medline] [CrossRef]
26. Qiu, X., Dowling, A.R., Marino, J.S., Faulkner, L.D., Bryant, B., Brüning, J.C., Elias, C.F., and Hill, J.W. 2013. Delayed puberty but normal fertility in mice with selective deletion of insulin receptors from Kiss1 cells. *Endocrinology* 154: 1337–1348. [Medline] [CrossRef]
27. Lustig, R.H., Sen, S., Soberman, J.E., and Velasquez-Mieyer, P.A. 2004. Obesity, leptin resistance, and the effects of insulin reduction. *Int. J. Obes. Relat. Metab. Disord.* 28: 1344–1348. [Medline] [CrossRef]
28. Schaison, G. 1989. [GnRH and its analogs—structure, mechanism of action and therapeutic applications]. *J. Steroid Biochem.* 33:(4B): 795–799 (in French). [Medline] [CrossRef]
29. Seli, E., Babayev, E., Collins, S.C., Nemeth, G., and Horvath, T.L. 2014. Minireview: Metabolism of female reproduction: regulatory mechanisms and clinical implications. *Mol. Endocrinol.* 28: 790–804. [Medline] [CrossRef]
30. Sharieh Hosseini, S.G., Khatamsaz, S., and Shariati, M. 2014. The effects of losartan on memory performance and leptin resistance induced by obesity and high-fat diet in adult male rats. *Iran. J. Basic Med. Sci.* 17: 41–48. [Medline]
31. Sheffer-Babila, S., Sun, Y., Israel, D.D., Liu, S.M., Neal-Perry, G., and Chua, S.C. Jr. 2013. Agouti-related peptide plays a critical role in leptin's effects on female puberty and reproduction. *Am. J. Physiol. Endocrinol. Metab.* 305: E1512–E1520. [Medline] [CrossRef]
32. Shi, Z.D., Shao, X.B., Chen, N., Yu, Y.C., Bi, Y.Z., Liang, S.D., Williams, J.B., and Taouis, M. 2006. Effects of immunisation against leptin on feed intake, weight gain, fat deposition and laying performance in chickens. *Br. Poult. Sci.* 47: 88–94. [Medline] [CrossRef]
33. Tong, Q. and Xu, Y. 2012. Central Leptin Regulation of Obesity and Fertility. *Curr. Obes. Rep.* 1: 236–244. [Medline] [CrossRef]
34. Turner, A.I. and Tilbrook, A.J. 2006. Stress, cortisol and reproduction in female pigs. *Soc. Reprod. Fertil. Suppl.* 62: 191–203. [Medline]
35. Wathes, D.C., Clempson, A.M., and Pollott, G.E. 2012. Associations between lipid metabolism and fertility in the dairy

cow. *Reprod. Fertil. Dev.* 25: 48–61. [Medline] [CrossRef]

36. Weigle, D.S., Bukowski, T.R., Foster, D.C., Holderman, S., Kramer, J.M., Lasser, G., Lofton-Day, C.E., Prunkard, D.E., Raymond, C., and Kuijper, J.L. 1995. Recombinant ob protein reduces feeding and body weight in the ob/ob mouse. *J. Clin. Invest.* 96: 2065–2070. [Medline] [CrossRef]
37. Woods, S.C. and Langhans, W. 2012. Inconsistencies in the assessment of food intake. *Am. J. Physiol. Endocrinol. Metab.* 303: E1408–E1418. [Medline] [CrossRef]
38. Zhang, Y., Rodrigues, E., Li, G., Gao, Y., King, M., Carter, C.S., Tumer, N., Cheng, K.Y., and Scarpace, P.J. 2011. Simultaneous POMC gene transfer to hypothalamus and brainstem increases physical activity, lipolysis and reduces adult-onset obesity. *Eur. J. Neurosci.* 33: 1541–1550. [Medline] [CrossRef]
39. Ziaei, N., Shivazad, M., Mirhadi, S.A., and Gerami, A. 2009. Effects of reduced calcium and phosphorous diets supplemented with phytase on laying performance of hens. *Pak. J. Biol. Sci.* 12: 792–797. [Medline] [CrossRef]