Journal of Animal and Veterinary Advances 11 (4): 443-448, 2012
ISSN: 1680-5593

Differential Neurotrophin-4 mRNA Expression in Reproductive Organs of Prepubertal Gilts with Different Dietary Energy Level

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Abstract: The development of reproductive system in prepubertal gilts was affected not only by gonadotropins but also by other cytokines and growth factors. Recent studies have revealed that Neurotrophins (NTs) which are well known for their essential roles in neural system also expressed in mammalian reproductive organs and involved in the regulation of mammalian reproduction. Dietary energy level is an important factor affecting domestic animal reproductive performance including central effects on Hypothalamic Pituitary Gonadal (HPG) axis and local effects on gonads. In the present study, the expression of Neurotrophin-4 (NT-4) which is a member of NTs in reproductive tissues of prepubertal gilts including ovary, oviduct and uterus was studied by immunohistochemistry. The effects of dietary energy on NT-4 mRNA expression were also investigated by quantitative PCR. The results show that NT-4 was localized in all classes of follicles in ovaries of prepubertal gilts including primordial follicles, primary follicles and secondary follicles, NT-4 was localized in mucosal epithelial cells in oviducts and glandular epithelium and endometrial epithelium in uteri. Furthermore, different dietary energy levels altered mRNA expression level of NT-4 in ovaries, uteri and oviducts of prepubertal gilts. NT-4 mRNA expression level in ovaries and uteri was higher in high dietary energy group than that in mid and low-energy groups. In oviduct, the expression pattern of NT-4 mRNA among three different dietary energy groups is similar as that occurred in in ovaries and uteri but with more significant differences. These results suggest that NT-4 may be one of the signals that links metabolic status and neuroendocrine control of reproduction in pig.

Key words: Prepubertal gilts, NT-4 mRNA, Hypothalamic Pituitary Gonadal (HPG), gonads, Neurotrophins (NTs), follicles, oviducts

INTRODUCTION

The reproductive performance of female animals is regulated by sophisticated mechanisms including endocrine regulation through HPG axis and local regulation in gonads. Numerous cytokines and growth factors have been documented in regulating the development and function of female reproductive system. NTs including Nerve Growth Factor (NGF), Brain-Derived Neurotropic Factor (BDNF), Neurotrophin-3 (NT-3) and Neurotrophin-4 (NT-4 also known as NT-5) have been well-characterized for their critical roles in central and peripheral nervous systems (Jones *et al*., 1994; Barbacid, 1995).

In the past few years, increasing numbers of studies have shown that several NTs and their receptors are also expressed in the mammalian reproductive organs and involved in the endocrine hormonal mechanisms controlling ovarian development, primordial follicle formation, early follicular development, ovulation, oocytes maturation and embryonic development in several species (Dissen *et al*., 1996; Yi *et al*., 2008; Zhang *et al*., 2010; Anderson *et al*., 2010).

Energy intake can also affect female reproductive function by metabolic control that alters hypothalamic Gonadotropin-Releasing Hormone (GnRH)/Luteinizing Hormone (LH) pulse, impacts secretion of follicular steroid hormones and alters ovarian sensitivity to gonadotropin and cytokines (Temple and Rissman, 2000; Almeida *et al*., 2001; Barb *et al*., 2004, 2005; Webb *et al*., 2004). Recent findings demonstrate that numerous genes i.e., relaxin, interleukins and other cytokines as well as biologically

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active substances such as leptin, Insulin-like Growth Factor-I (IGF-I), IGF-II and agouti protein could have a profound effect on metabolic status and the reproductive axis (Barb *et al*., 2005). It is of great relevance and significance to better understand the function of neurotrophin-4 in regulating the development of the reproductive system of prepubertal gilts especially how this function could be manipulated through the alteration of dietary energy level thus to raise gilts to have better reproductive performance when mature. In the present study, the expression of NT-4 in reproductive tissues of prepubertal gilts was examined by immunohistochemistry and the effects of dietary energy level on NT-4 mRNA expression were investigated by quantitative PCR.

MATERIALS AND METHODS

Ethics statement: All experimental procedures in the present research were approved by the Animal Care and Use Committee of Jilin University.

Immunohistochemistry: The ovaries, oviducts and uteri of prepubertal gilts were obtained from a public slaughter house. The samples were collected within 15 min after slaughter and then fixed with 2% paraformaldehyde for 24 h. After embedded in paraffin, 5 μm thick sections were cut following a standard procedure. Slides were first incubated in three washes of xylene for 5 min each and then incubated in two washes of 100% ethanol for 10 min each followed by two washes of 95% ethanol for 10 min each. After final two washes in ddH_2O, slides were first incubated in 3% hydrogen peroxide for 10 min and then blocked with normal goat serum (10%) in PBS for 30 min at room temperature. Slides were incubated with a primary antibody (anti-NT-4, 1:100, BOSTER, China) at 4°C for 36 h. Following several washes with PBS, slides were incubated with peroxidase conjugated goat anti-rabbit IgG (1:200; BOSTER, China) for 30 min at room temperature. After washing with PBS, slides were incubated with DAB substrate for color development. Negative controls were prepared using rabbit IgG instead of primary antibody diluted with PBS. Finally, the slides were examined under an optical microscope (Olympus, Japan). Sections of each sample were examined in triplicates for both positive antibody staining and negative controls.

Animals, dietary, feeding and management: Healthy prepubertal crossbred (Landrace x Yorkshire) gilts at the age of 60 days and body weight of 15 kg were used in this study. Eighteen gilts were randomly divided into three groups (n = 6). Gilts in the mid-energy group (group M) were fed with diets designed according to the NRC (1998) standard of USA with energy intake meeting 100% of digestive energy requirement. Gilts in the low energy group (group L) were fed with diets with an energy level 10% lower than that in the standard diet with energy intake meeting 90% of digestive energy requirement. Gilts in the high energy group (group H) were fed with diets with an energy level 10% higher than that in the standard diet with energy intake meeting 110% of digestive energy requirement (ingredient and nutrient composition as shown in Table 1). All gilts were fed 3 times daily *ad libitum* at 6:00 am, 11:00 am and 6:00 pm with water freely available.

Table 1: Ingredient and nutrient composition of diets feed to prepubertal gilts in different groups

Ingredient and nutrient composition	HE	ME	LE
Corn (%)	34.400	50.400	46.500
Wheat (%)	-	3.200	22.000
Soybean meal (%)	13.900	20.600	20.700
Extruded soy powder (%)	18.000	8.000	2.000
DDGS (%)	10.000	6.000	4.000
Sucrose (%)	5.000	4.000	1.000
Premix (%)	1.000	1.000	1.000
Soybean oil (%)	5.000	1.000	-
Salt (%)	0.080	0.080	0.060
Corn starch (%)	10.800	4.000	1.000
Stone powder (%)	0.840	0.900	1.180
Calcium bicarbonate (%)	0.900	0.720	0.400
Lysine (%)	0.080	0.100	0.160
Total energy ($kJ\ kg^{-1}$, assayed)	16580.000	14937.00	13518.000
Digestive energy ($kJ\ kg^{-1}$, calculated)	15727.000	14254.000	12849.000
Crude protein (%)	18.000	18.000	18.000
Calcium (%)	0.620	0.600	0.640
Bioavailable phosphorus (%)	0.290	0.260	0.230
Total phosphorus (%)	0.500	0.500	0.560
Salt (%)	0.110	0.120	0.130
Lysine (%)	0.964	0.950	0.951
Methionine (%)	0.307	0.306	0.281
Threonine (%)	0.721	0.727	0.689

About 1 kg of 1% premix consists of iodine 14 mg, manganese 300 mg, zincum 8000 mg, copper 500 mg, iron 8000 mg, selenium 25 mg, VA 175000 IU, VD3 20000 IU, VE 1100 IU, VK 50 mg, biotin 5 mg, choline 40000 mg, folic acid 30 mg, nicotinic acid 1250 mg, pantothenic acid 900 mg, riboflavin 300 mg, thiamine 100 mg, VB6 150 mg, VB12 1500 μg

Gilts were housed within pens in an enclosed building with constant temperature (21±3°C) and relative humidity (55±3%), under controlled lighting conditions. Feeding experiments were lasted for 12 days. At the end of the feeding experiments, ovaries, oviducts and uteri were recovered and kept in liquid nitrogen until transferred into -80°C refrigerator.

RNA extraction and cDNA synthesis: Total RNA was extracted from the samples stored in -80°C refrigerator using Trizol reagent (Invitrogen Life Technologies Inc., USA) and then treated with Rnase-free Dnase I (Tiagen) in order to eliminate genomic DNA. The concentration of RNA samples were determined with a spectrophotometer (SANYO, Japan) and then diluted into equal

concentrations with Rnase-free distilled water. Oligo $(dT)_{18}$ was used as primer for reverse transcription and the cDNA samples were frozen at -20°C until use.

Quantitative real-time PCR: Real-time PCR was carried out in an ABI PRISM 7000 machine according to the manufacturer's instructions. For amplification, reactions were performed in a 25 μL final volume containing 100 ng of cDNA reverse transcribed from mRNA samples of uteri, ovaries and oviducts of prepubertal gilts in different feed energy level groups, 0.15 μL of Ex Taq HS (TakaRa), 2.5 μL of 10×Ex Taq Buffer (TakaRa), 2.0 μL of dNTP mixture (2.5 mmol/L/each) (TakaRa), 0.5 μL of primers (10 μmol L^{-1}), 0.25 μL of TAMRA-FAM (10 μmol L^{-1}) and distilled water to 25 μL. The PCR reaction was composed of one cycle of 6 min at 95°C followed by 48 cycles of 30 sec at 94°C, 30 sec at 59°C. A standard curve was generated and used to evaluate the relative expression of the *NT-4* gene in terms of the ratio (fold difference) of the target gene expression over the control gene expression. The specific primers and probes used for NT-4 were NT-4-FP: 5'-AGTCCTACGTGCGGGCATT-3', NT-4-RP: 5'-CACAGGCAGTGTCAATTCGAA-3' and NT-4-TAMRA-FAM: 5'-CACCGATGCCCAGGGCCGT-3'.

Statistical analysis: The data are presented as mean±SE. Comparisons between groups were performed by one-way ANOVA. The significance of differences between the mean values in each treated group was tested with Duncan's multiple-comparison test. A value of $p<0.05$ was considered statistically significant and a value of $p<0.01$ was considered statistically highly significant.

RESULTS

Immunohistochemistry staining of NT-4 in the uteri, ovaries and oviducts of prepubertal gilts: As shown in Fig. 1, NT-4 was detected in ovaries, uterus and oviducts

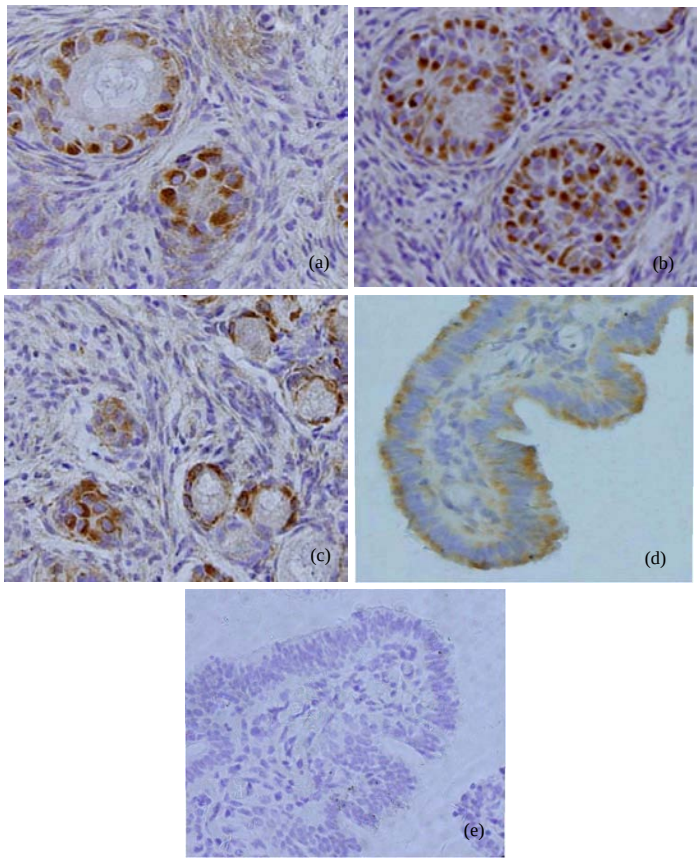

Fig. 1: Detection of the NT-4 in the prepubertal pig ovary, oviduct and uterus; a) NT-4 immunoreactivity is predominantly seen in primordial follicles, primary follicles; b) secondary follicles; c) NT-4 is detected in the uterine glandular epithelium and endometrial epithelium; d) NT-4 immunoreactivity is predominantly seen in oviduct mucosal epithelial cells and e) section incubated with rabbit IgG instead of primary antibody served as negative control. a, c, d, e: original magnification x100. c: original magnification x40

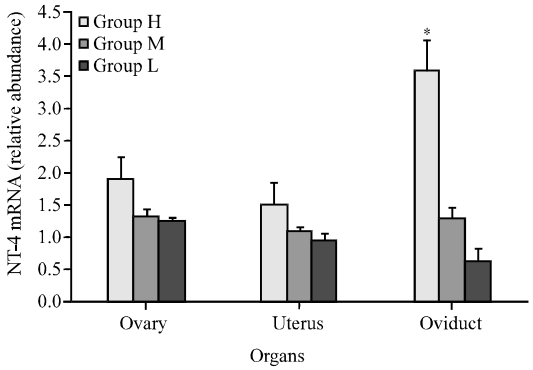

Fig. 2: Relative abundance (arbitrary units) of NT-4 mRNA in reproductive tissues of prepubertal gilts in different energy intake group. *p<0.05

of prepubertal gilts by immunohistochemical staining. In the ovary, NT-4 was detected in different classes of follicles including primordial follicles, primary follicles and secondary follicles. The strongest staining of NT-4 appeared to be in the granulosa cells. In the oviducts, positive staining was observed in the mucosal epithelial cells. In the uterus, NT-4 was localized in the uterine glandular epithelium and endometrial epithelium. Furthermore, a positive staining for NT-4 was seen in the myometrial vascular smooth muscle cells.

Real-time PCR analysis of NT-4 mRNA expression in the uteri, ovaries and oviducts of prepubertal gilts in different feed energy intake groups: As shown in Fig. 2, group H showed significant higher amounts of NT-4 mRNA expressions in all of the reproductive organs than that in group M and L ($p<0.05$). Among different energy intake groups, NT-4 mRNA expression in oviducts showed the most significant change affected by diet energy while in the uterus and ovaries, higher NT-4 mRNA expression was detected in group H and M than in group L but the difference was not significant ($p>0.05$). NT-4 mRNA expression showed variations among different tissues in the same energy intake groups. In the high diet energy group, NT-4 mRNA expression in oviducts was significantly higher than that in ovaries and uterus ($p<0.05$). However, in mid-energy group and low-energy group, there were no significant differences of NT-4 mRNA expression among different organs ($p>0.05$).

DISCUSSION

Dietary energy level is one of the most important factors that affect animal reproductive performance; it has significant impact on the development of reproductive system, maintenance of estrous cycle, mating, fertilization and embryo development. A number of studies have shown that energy intake restriction could impact on the HPG axis of gilts and sows through the regulation of gonadotropin. It has been suggested that the responses of the gonads to nutritional/metabolic signaling could also participate in the modulation of mammalian reproduction (Cox *et al*., 1987; Cosgrove and Foxcroft, 1996; Lucy, 2008). In the last two decades, the role of NTs in regulating female and male reproductive performance has been clearly established (Dissen *et al*., 2001; Tometten *et al*., 2005; Sun *et al*., 2011). However, the alteration of NT-4 mRNA expression level impacted by energy intake level is still unclear. To the knowledge, this is the first study to research the NT-4 expression status in different dietary energy intake levels in prepubertal gilts reproductive system.

Regulation of ovarian activity is an integrated process encompassing both extraovarian signals and intrafollicular factors (Webb *et al*., 2004). In mouse, NT-4 expressed in oocytes and in human, NT-4 expressed in granulosa cells at the early developmental stages of epithelioid cells. NT-4 could promote primary oocyte development and the maturation of granulosa cells (Childs *et al*., 2010). In the present study, NT-4 was shown to be localized in all classes of follicles in prepubertal gilts ovaries including primary follicles, secondary follicles and even in the primordial follicles. This result suggests that NT-4 may be one of the key members involved in porcine granulosa cell proliferation and follicular development in an autocrine or paracrine way. When gilts were fed with diets of different energy levels, NT-4 expression level in high energy group was higher than that in group M and L, though no statistically significant difference was found. This result indicates that ovarian NT-4 expression may be regulated by the energy metabolism signal.

The primary function of the endometrium is to provide appropriate environment for the growth and development of fertilized eggs. Endometrial glands secrete nutrient supplies and support the growth of early embryos. Previous studies have shown that BDNF and NGF immunolocalized in the uterine tract of rodents (Bjorling *et al*., 2002; Krizsan-Agbas *et al*., 2003) and NT-4 located in the bovine uterus (Sun *et al*., 2011) while the exact location of NT-4 in the prepubertal gilts is still unclear. In this study, we found that NT-4 mainly expressed in the endometrial epithelial cells which indicated that NT-4 may be involved in a paracrine or autocrine network to establish and maintain pregnancy and fetal development. Furthermore, NT-4 expression levels in different dietary energy level groups changed in a similar way as that occurred in ovary. Higher NT-4 expression was detected in the group H than that in group M and L without statistically significant differences.

Oviduct is very important for fertilization and early development of eggs. Early mammalian embryo cleavage is conceived in the fallopian tubes and tubal fluid provides suitable environment for the fertilization, embryo transportation and early embryos development (Hunter, 1981). It has been reported that NT-4 was absent in human fallopian tubes (Anderson *et al*., 2002) while NT-4 was located in the oviduct of cow in different stage of estrous cycle (Sun *et al*., 2011). In this study, NT-4 was immunolocalized in the epithelial cells of oviduct in prepubertal gilts suggesting that NT-4 may play important roles in the development of oviduct in gilts before puberty such as participating in the oviductal mucosal epithelium proliferation, differentiation and development. Furthermore, NT-4 mRNA expression significantly increased in group H compared with group M and L. In addition, NT-4 expression level in oviducts was significantly higher than that in ovaries and uteri among all of the different energy intake levels in prepubertal gilts. Hence, NT-4 may be a very important factor in nutrition/metabolism regulatory mechanisms on the gilts oviduct.

CONCLUSION

In this study, NT-4 was expressed in ovaries, oviducts and uterus of the prepubertal gilts. Dietary energy level plays a role in regulating NT-4 mRNA expression in organs. The nutrition/metabolism signal may be involved in the control of reproductive function by alter NT-4 expression in reproductive tissue. The research adds neurotrophins, specifically NT-4 to the list of growth factors in regulating growth, development and functions of prepubertal gilts reproductive system.

ACKNOWLEDGEMENTS

Researchers would like to thank Dr. Xianzhong Yu (Dept. of Biological Sciences, Clemson University, USA) for revising and valuable suggestions for improvement of the research. This research was supported by the State Key Development Program of Basic Research (973 Program) of China (No. 932004) National Natural Science Foundation of China (No. 30671511).

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