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Expression of BDNF mRNA in Porcine Reproductive Tissues During Follicular Phase and Luteal Phase and Oocytes in GV and *in vitro* Matured MII Stage

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Abstract: Neurotrophins (NTs) belong to a family of soluble homodimeric polypeptide growth factors and are widely recognized for their essential roles in central and peripheral nervous systems. One such neurotrophin, Brain-Derived Neurotrophic Factor (BDNF) was originally described in the nervous system but has now been shown to be expressed in reproductive system. In this study, the researchers examined the presence and different expression levels of BDNF mRNA in porcine reproductive organs during different stages of estrous cycle and in pig oocytes in Germinal Vesicle (GV) and *in vitro* matured Metaphase II (MII) stage. In oviduct and uterus, BDNF mRNA expression was higher than that in ovary ($p<0.05$). The expression level in luteal phase ovary is higher than that in follicular phase ovary but the difference between them was not significant ($p>0.05$), a similar but more significant change occurred in oviduct ($p<0.05$). However, the expression levels in uterus were on an opposite trend i.e., a higher level of mRNA for BDNF was found in follicular phase uterus instead of in luteal phase uterus. BDNF mRNA was also detected in GV oocytes and *in vitro* matured MII oocytes with significantly higher amounts in GV oocytes than in MII oocytes ($p<0.01$). These results suggest a possible role for BDNF in the regulation and modulation of pig reproductive function and oocyte maturation.

Key words: Brain-derived neurotrophic factor, pig, estrous cycle, reproductive organ, oocyte, China

INTRODUCTION

NTs are small, homodimeric polypeptide growth factors that are required for the survival, maintenance and differentiation of neurons (Ibanez, 1995). It includes Nerve Growth Factor (NGF), Brain-Derived Neurotrophic Factor (BDNF), Neurotrophins 3 (NT3) and Neurotrophin-4 (NT-4 also known as NT-5) which function through two different types of receptors, Tyrosine kinase (Trk) (i.e., TrkA for NGF, TrkB for BDNF and NT4 and TrkC for NT3) and p75 receptors. In the past few years, numerous studies have found that NTs also expressed in mammalian reproductive systems. For instance, NGF and its receptors, TrkA and p75 were expressed in the reproductive organs of the adult male rats and the Japanese Shiba goats (Li *et al*., 2005; Ren *et al*., 2005), TrkA and p75NTR were detectedvia ovarialimmuno-localization in cows and sows (Levanti *et al*., 2005), BDNF mRNA expressed in human spermatozoa (Zheng *et al*., 2011) and bovine oocytes (Yi *et al*., 2008), NT-4, BDNF and their receptor TrkB were present in ovaries from human fetuses and adults (Harel *et al*., 2006), NT-4 and TrkB also expressed in reproductive tissues of cow during follicular and luteal phases (Sun *et al*., 2011). These results suggest that NTs may play critical roles in the mammalian reproductive systems. However, the presence and expression levels of BDNF mRNA in porcine reproductive organs at different stages of estrous cycles and in oocytes in GV and in *in vitro* matured MII stage have not been addressed. Here, the researchers present the first report of the BDNF mRNA expression in the ovary, oviduct, uterus and oocytes of sows.

MATERIALS AND METHODS

Chemicals: All chemicals used in this study were purchased from Sigma Chemicals (St. Louis, MO, USA) unless otherwise specified.

Experimental animals: All experiments were performed in accordance with the principles and procedures of Animal Ethics Committee of Jilin University. Porcine reproductive organs were recovered from a local slaughterhouse. Samples were collected within 15 min after slaughter and

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then kept at -196°C. The stages of the estrous cycle were confirmed as previously reported (Akins and Morrissette, 1968). For each phase, tissue samples were collected from ten animals (n = 10).

Preparation of pig follicular fluid: The pig Follicular Fluid (pFF) was collected from follicles (3-8 mm diameter) of pig ovaries using 10 mL syringe with 16 gauge needle. After centrifugation at 1000 g for 10 min at 4°C, the suspension was filtered through 0.22 μm syringe filters and stored at -20°C until use.

Cell culture: Porcine ovaries were collected from a local abattoir and kept warm during transportation. In the laboratory, ovaries were washed in Dulbecco's phosphate-buffered saline. Oocytes with intact cumulus oophurus layers and homogeneous cytoplasm were collected and washed in the DPBS then washed three times with maturation medium-mTCM199 or NCSU-23 supplemented with pFF and hormone. About 30 COCs were cultured in 1 mL of medium M-199 with Hanks' salt (M 7653, Sigma) at 38.5°C in a humidified atmosphere of 5% CO_2 in the air. The maturation of oocytes and expansion of cumulus cells were stimulated by the addition of recombinant FSH, EGF-like peptides, LH into the culture medium.

RNA extraction and cDNA synthesis: Total RNA from pig ovaries, oviducts and uteri were extracted using the Trizol reagent (Invitrogen Life Technologies Inc., USA) and then treated with Rnase-free Dnase I (Tiagen) to eliminate genomic DNA. The concentration of RNA was determined with an ultraviolet spectrophotometer(SANYO, Japan) and then diluted into equal concentration with Rnase-free distilled water. Oligo (dT)18 was used in reverse transcription reactions. The resultant cDNA was frozen at -20°C until use.

Total RNA from porcine oocytes was extracted using an RNA easy Micro kit (Qiagen, Crawley, UK) as previously described (Yi *et al*., 2008). First strand cDNA was synthesized from 2 ng of total RNA from the oocytes. Reverse transcription was conducted with the 1st Strand cDNA Synthesis kit (Takara, Japan). The resultant cDNA was frozen at -20°C until use.

Quantitative real-time PCR: The quantitative PCR reactions were carried out in an ABI Prism 7500 Sequence Detector (Applied Biosystems). The amplification reactions were performed in a 25 μL final volume containing 100 ng of cDNA. The basic PCR protocol for amplification was composed of 6 min at 95°C followed by 48 cycles of 30 sec at 94°C, 30 sec at 59°C. To normalize the amount of expressed BDNF mRNAs, the internal housekeeping gene *GAPDH* was used. Each cDNA product was tested in triplicates. A standard curve was generated and used to evaluate the relative expression levels of the *BDNF* gene in terms of the ratio (fold difference) of the target gene expression to the control gene expression. Specific primers used for BDNF were BDNF-FP: 5'-CATGGGACTCTGGAGAGCAT-3', BDNF-RP: 5'-CAAAGGCACTTGACTGCTGA-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 AND DISCUSSION

BDNF mRNA expression in porcine reproductive tissue during estrous cycle: As shown in Fig. 1, BDNF mRNA was expressed in all of the porcine reproductive tissues during each phase with higher levels in oviduct and uterus than that in ovary. The expression level in luteal phase ovary is higher than that in follicular phase ovary and a similar but more significant change occurred in oviduct ($p<0.05$). However, a higher level of mRNA of BDNF was found in follicular phase uterus instead of in luteal phase uterus.

The regulation of ovarian activity is an integrated process encompassing both extraovarian signals and intrafollicular factors (Webb *et al*., 2004), Cronin *et al*. (2004) reported that developing Gonadotropin-Releasing Hormone (GnRH) secretory system may be directly sensitive to BDNF and this polypeptide can function as

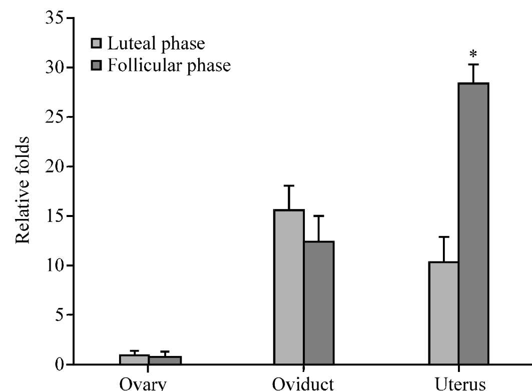

Fig. 1: Relative abundance of BNDF mRNA in reproductive tissues of sows during the follicular and luteal phases

a neurotrophic factor for GnRH neurons in mice. In the study, higher BDNF mRNA expression level was detected in luteal phase ovary which indicates that the role of BDNF may be strengthened with the growth of follicles. Furthermore, BDNF may play roles in follicle growth, luteum formation and luteum formation and luteal functions. Moreover, BDNF may act as one of the factors involved in pituitary gonadotropin-driven ovarian regulatory system by influencing follicular growth and oocyte development as well as regulating ovarian function through paracrine or autocrine fashions in pig.

Early pig embryo cleavage is conceived in the fallopian tubes and tubal fluid provides a suitable microenvironment for fertilization, embryo transportation and early embryos development (Hunter, 1981). In the present study, BDNF mRNA was found to be present in oviducts of sows, suggesting BDNF may participate in the regulation of capacitation of spermatozoa, development of eggs and early embryos through an autocrine fashion. In addition, the alterations of mRNA expression levels during the luteal phase and follicular phase in the oviducts were consistent with that occurred in ovaries indicating that BDNF may also affect the function of ovaries via a paracrine fashion.

The development of mammalian conceptus occurred in the uterus and the growth of early embryos was supported by nutrient supplies secreted from endometrial glands. In follicular phase, uterine gland secretion is vivid providing favorable conditions for sperm motility and capacitation. It has been reported that BDNF, NGF and NT-3 were immunolocalized in the uterine tract of rodents (Bjorling *et al*., 2002; Krizsan-Agbas *et al*., 2003). The result of the current study was similar to that in previous studies. Moreover, a higher BDNF mRNA expression level was found in follicular phase uterus indicating that BDNF may be related to the activity of endometrial secretion.

BDNF mRNA expression in porcine GV and in *in vitro* matured MII oocytes: As shown in Fig. 2, BDNF mRNA was expressed in both GV oocytes and *in vitro* Matured (MII) oocytes. In GV oocytes, BDNF mRNA expression level was significantly different from that in *in vitro* matured MII oocytes (p<0.01). BDNF is secreted by granulosa and cumulus cells as an ovarian factor stimulated by the preovulatory LH surge (Kawamura *et al*., 2005). BDNF may play roles in conferring oocyte cytoplasmic competence to support early embryo development and this may involve both autocrine and paracrine signaling within the COCs (Martins da Silva *et al*., 2005).

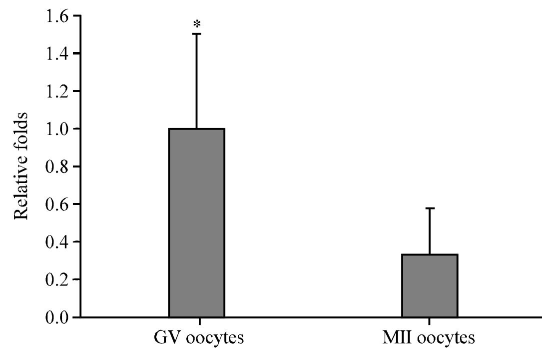

Fig. 2: Relative abundanceof BNDF mRNA in porcine GV and in *in vitro* matured MII oocytes

The results in the study suggest that BDNF may play a role in promoting the development and maturation of oocytes in sows.

CONCLUSION

BDNF mRNA was expressed in pig ovaries, uteri and oviducts during follicular phase and luteal phase. It also expressed in porcine GV and *in vitro* matured MII oocytes. These results imply that BDNF may play different roles in lutea formation, sperm capacitation, fertilization and early embryo development in sows.

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