Indian Journal of Animal Sciences **83** (4): 367–369, April 2013/Article

Effect of ghrelin on the expression of Bcl-2 and Bax mRNA in *in vitro* maturation ovine oocytes

RUI-XIA BAI[1], PENG -WEI ZHAO[2] and GUI-FANG CAO[1*]

Inner Mongolia Agricultural University, Hohhot 010 018 PR China

Received: 17 May 2012; Accepted: 11 September 2012

ABSTRACT

The relationship between ghrelin and the expression of Bcl-2 and Bax was investigated. The result showed that ghrelin can regulate the expression of Bcl-2 and Bax. And ghrelin can promote ovine oocytes maturation *in vitro*. This maybe a good pathway to promote the animal production.

Key words: Bax, Bcl-2, Ghrelin, Ovine, Oocytes

Ghrelin is a 28-amino-acid peptide secreted mainly by the gastrointestinal tract (Weiwei *et al*. 2009). It is also present in placenta, hypothalamus, and pancreas. Cummings *et al.* (2002, 2005) found that ghrelin concentrations increase with fasting before the 3 main meals of the day, decrease quickly after food intake, and reach a nadir in 90 min after the meal, raising the possibility that ghrelin may play a role in meal initiation. A similar effect was observed in rodents with fasting and refeeding.

Several reports (Popelkova *et al.* 2006, Zhang *et al*. 2007, Xu *et al*. 2008, and Du *et al.* 2009) showed that the acylated form of ghrelin acts as a survival factor for hypothalamic neuronal cells by inhibiting apoptotic pathways, and this neuroprotective effect is mediated via the activation of GHS-R1a. The mitochondria are key determinants of cell death and cell survival. AG regulates the mitochondrial pathways of apoptosis and increases Bcl-2/Bax ratio (Zhang *et al*. 2010). Ghrelin in the reproductive system, has been researched very little, especially in oocytes. To identify the presence or absence of ghrelin immunohistochemical technique was used to detect it. But the technology-specific antibodies are low, it is difficult for the shortcomings of the quantitative analysis, the results are often low reliability. In this study, sheep oocytes as the material, the real-time quantitative PCR (RT-PCR) technique was used to detect sheep oocytes at different maturity Ghrelin period of the Bcl-2, Bax of oocyte maturation potential role.

Present address: [1]Department of Clinical Laboratory (nmyyjyk@126.com), [4]Veterinary College (guifangcao @hotmail.com); [2]Pathogenic Organisms and Immunology Lab, Basic Medical College (pengwzhao@yahoo.com).

MATERIALS AND METHODS

Oocyte collection and in vitro *maturation:* Sheep ovaries obtained at an abattoir within 15 min post-slaughter were placed in a thermos (35°–37°C) containing normal saline with penicillin G (400 IUD ml) and streptomycin (500 µg D ml). Immature cumulus-oocyte complex (COC) were collected from follicles by dissecting ovaries, which were placed in modified Dulbecco's phosphate buffered saline (PBS) medium containing 2 mg D ml BSA and 5 IUD ml heparin. All oocytes completely surrounded by unexpanded cumulus cells were used for *in vitro* maturation, and were rinsed 3-fold in mature culture medium consisted of TCM-199, 10 mM Hepes, 1.2 IUD ml LH, grelin 500ng/ml, 0.001 µg D ml E2 and 0.02 IUD ml FSH, 10% FCS (TCM: GIBCO with Earle's salt; E2; FSH, LH; FCS: TBD). Groups of up to 100 oocytes were cultured in 4-well plates containing 800 µL of medium for 24 h at 38.5°C in an atmosphere of 5% CO_2 with saturated humidity. After and before maturation, a sample of oocytes was denuded in PBS + 300 µg D ml hyaluronidase, washed 3 fold in PBS and frozen in groups of 50 in liquid N_2 and stored at –80°C until the extraction of RNA.

RNA extraction and reverse transcription: The total RNA was isolated from 5 pooled frozen oocytes and embryos at each developmental stage using with the RNeasy kit. The RNA contents from the samples were too low to be accurately quantified by spectrometry, so 6.5 µL RNA aliquots were amplified. All RNA treated with RNase free DNase I to remove any possible genomic DNA contamination. For amplification of the targets, RT and PCR were run in 2 separate steps. Reverse transcription was performed using 2µL 5×PrimeScript buffer, 0.5µL PrimeScript RT enzyme

mix I, 0.5μL PrimeScript RT enzyme mix I, 0.5μL oligo dT primer (50μM), 0.5μL random 6 mers (100μM) and then performed by 37°C for 15 min for reverse transcription, 84°C for 5 s to inactivate the reverse transcriptase.

Real time PCR

Primers used in this study were as follows: Bcl-2 (236 bp, Gene Bank accession no. AY423861) 5'-CTGCACCTGACGCCCTTCAC-3'(forward) and 5'-GCGTCCCAGCCTCCGTTGT-3' (reverse); Bax (173 bp, Gen Bank accession no. AF163774) 5'-TTT GCT TCA GGG TTT CAT C-3'(forward) and 5'-CGG CTG CGA TCA TCC TCT-3'(reverse); ß-Actin (208 bp, Gen Bank accession no. U39357) 5'-GTCACCAACTGGGACGACA-3' (forward) and 5'-AGGCGTACAGGGACAGCA-3' (reverse). ß-Actin mRNA amplification served as a control of the RNA quality.

Relative levels of Bcl-2 and Bax mRNA were quantified using SYBR-Premix Ex Taq following manufacturer's instructions and a DNA Engine Opticon 2. The PCR reaction mixture (20μL) contained 10μL 2×SYBR Green mix and 0.4μL (0.5 mM) each of forward and reverse specific primers, 2μL of cDNA and 7.2μL H_2O. The reaction was performed under the following cycle conditions: 45 cycles of 20s at 95°C for denaturation, 20s at 61°C for annealing, and 20s at 72°C for extension. Expression levels of Bcl-2 and Bax were calculated as relative values using the $2^{-\Delta CT}$ method (Schmittgen and Zakrajsek 2000), where ΔCT = CT of the ghrelin – CT of the ß-Actin (Tropea *et al.* 2007). Real time amplicon specificity and size were confirmed by melting curve analysis and 2% agarose gel electrophoresis, and sequences of amplicons were proven by sequencing. Each sample was tested 5 times.

All relative mRNA levels were expressed as the mean±SEM. Statistical significance was determined by one-way ANOVA with SPSS 13.0 for Windows. The least significant difference (LSD) test was applied for post-hoc multiple comparison test. Differences were considered significantly when P< 0.05.

RESULTS AND DISCUSSION

Bcl-2 and Bax expression in sheep oocytes: RT-PCR was performed to detect mRNA encoding Bcl-2 and Bax at different stages in sheep oocytes. DNA sequencing confirmed that the bands were a 236 bp Bcl-2 and a 173bp Bax amplification product. In each sample, parallel amplification using a specific primer set of sheep ß-actin served as an internal control. No products were detected from the negative controls (Fig. 1).

Effect on the expression in mRNA level of Bcl-2 in oocytes at different time after the addition of ghrelin: With the maturation time becoming longer, at 8h and 16h, the expression of Bcl-2 was higher than 0 h significantly (P<0.05). In 8 h and 16 h (Fig. 2), the expression of Bcl-2 in test group was higher than the contral group (P<0.05).

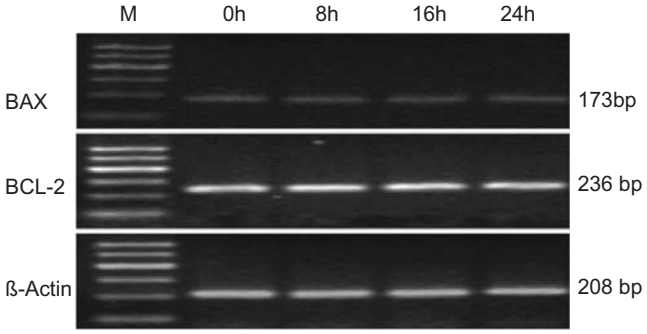

Fig. 1. Different culture time after adding ghrelin oocyte RNA as a template for REAL-time-PCR amplification products of gel electrophoresis.

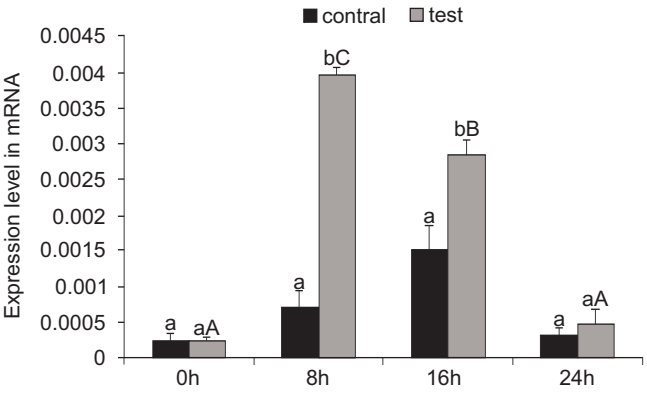

Fig. 2. The relative mRNA expression of Bcl-2 in oocytes at different time after the addition of Ghrelin *,#; significant difference P<0.05.

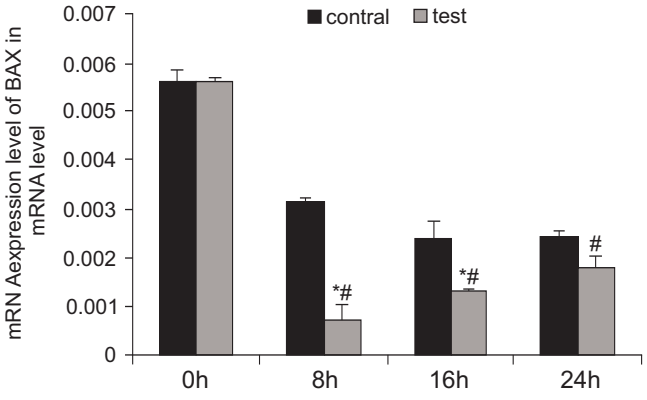

Fig. 3. Relative mRNA expression of Bax in oocytes at different time after the addition of Ghrelin. *,#; significant difference P<0.05.

Effect on expression in mRNA level of Bax in oocytes at different time after addition of ghrelin: With the maturation time becoming longer at 8h, 16h and 24h, the expression of Bax was lower than 0 h significantly (P<0.05). In 8h and 16h (Fig 3), the expression of Bax in test group was lower than the contral group (P<0.05).

The Bcl-2 family can be classified into 2 functionally distinct groups: anti-apoptotic proteins and pro-apoptotic proteins. Bcl-2, an anti-apoptotic protein, is known for

regulating apoptotic pathways and protecting against cell death (Zhang *et al*. 2007, Zhang *et al.* 2010). Bax, a pro-apoptotic protein of that family, is expressed abundantly and selectively during apoptosis and promotes cell death. Increasing the ratio of Bcl-2 to bax has commonly been used to determine the induction of apoptosis in several tissues. In this study, the ghrelin of final concentration is 500ng/ml in maturation cultural medium. The expression of Bcl-2 was promoted (Popelkova, Sirotkin *et al*. 2006, and Zhang *et al*. 2010). Meanwhile, the expression of Bax was declined. In conclusion, the presence of the ghrelin signaling system within the sheep ovary especially in the oocytes opens up the possibility of a potential regulatory role of this novel molecule in apoptotic function.

REFERENCES

Cummings D E and Clement K. 2002. Elevated plasma ghrelin levels in Prader Willi syndrome. *Natural Medicine* **8**(7): 643–44.

Cummings D E and Foster-Schubert K E. 2005. Ghrelin and energy balance: focus on current controversies. *Current Drug Targets* **6**(2): 153–69.

Du C and Xilingaowa. 2009. Expression of the orexigenic peptide ghrelin in the sheep ovary. *Domestic Animal Endocrinology* **36**(2): 89–98.

Kui L and Weiwei Z. 2009. Ghrelin inhibits apoptosis induced by high glucose and sodium palmitate in adult rat cardiomyocytes through the PI3K-Akt signaling pathway. Regular Pepticle **155** (1–3): 62–69.

Popelkova M and Sirotkin A V. 2006. Effect of IGF-I, leptin, ghrelin and MAPK-ERK on the nuclear maturation of bovine oocytes. *Bulletin of the Veterinary Institute in Pulawy* **50** (2): 179–81.

Xu Z and Lin S. 2008. Ghrelin prevents doxorubicin-induced cardiotoxicity through TNF-α/NF-kappaB pathways and mitochondrial protective mechanisms. *Toxicology* **247**(2–3): 133–38.

Zhang D and Wang W. 2010. Ghrelin inhibits apoptosis induced by palmitate in rat aortic endothelial cells. *Medical Science Monitoring* **16**(12): BR396–403.

Zhang Y and Ying B. 2007. Ghrelin inhibit cell apoptosis in pancreatic beta cell line HIT-T15 via mitogen-activated protein kinase/phosphoinositide 3–kinase pathways. *Toxicology* **237** (1–3): 194–202.