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Effects of Ghrelin on *in vitro* Nuclear Maturation and Subsequent Embryo Development of Immature Bovine Oocytes

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Abstract: Development of efficient culture system to support embryonic development would be valuable when percentage of produced embryos reaching to the blastocyst stage is important. However, the rate of bovine embryo production *in vitro* is still lower than expected. Present study was performed to investigate the effect of ghrelin on nuclear maturation and subsequent bovine embryo development *in vitro*. Cumulus-oocyte-complexes were collected from slaughterhouse ovaries and randomly allocated in each treatment groups. Five different concentrations of ghrelin (0, 5, 50, 500 and 1000 ng mL^{-1}) were added to the *in vitro* maturation medium (Hepes-buffered medium 199+fetal calf serum+gonadotrophins+insulin+antibiotics). The proportion of oocytes developed to metaphase II stage was significantly increased at 5 and 50 ng mL^{-1} ghrelin (86.32±3.38 and 89.77±2.92%, respectively). The result also indicated that adding high concentration of ghrelin adversely affect ($p<0.05$) the nuclear maturation rates of bovine oocytes. However, the subsequent embryo development was not significantly affected by addition of ghrelin to the IVM medium. This study showed that inclusion of 5-50 ng mL^{-1} ghrelin in maturation medium may have beneficial effects on nuclear maturation of bovine oocytes *in vitro*.

Key words: Ghrelin, nuclear maturation, bovine oocyte, *in vitro* embryo development, bovine

INTRODUCTION

During the last two decades, there has been an increasing interest in animal and human reproductive biotechnologies such as *In Vitro* Embryo Production (IVEP) (Andrabi and Maxwell, 2007). IVEP is a multi-procedure system entails the completion of three consequent biological steps: *In Vitro* oocyte Maturation (IVM), *In Vitro* Fertilization (IVF) and *In Vitro* embryo Culture (IVC) (Ward *et al*., 2003). The ultimate aim of IVEP in livestock industry is mass production of transferable embryos to obtain healthy offspring (Faber *et al*., 2003). The production of bovine blastocysts *in vitro* is a promising approach to maximize the use of bovine gamete (Sirisathien *et al*., 2003). Enormous progress in the production of bovine blastocysts *in vitro* has been made since the first calf was born from an *in vitro* fertilized embryo in 1981 (Brackett *et al*., 1982). Generally, the main problem of the IVEP procedure in bovine is reduce in viability of *in vitro* produced embryos compared with *in vivo* counterparts (Ushijima *et al*., 2009). Although, *in vitro* bovine nuclear maturation rate is in range of 85-90 and 70-80% of *in vitro* matured bovine oocytes are able to cleave only 30-40% of fertilized oocytes can reached to the preimplantation stage during IVC (Rizos *et al*., 2002). Development of efficient IVC system to support all stages of oocyte development would be valuable towards producing higher number of embryos. Oocyte maturation seems to be the first and the most critical step towards successful IVEP. Obvious differences exist between developmental competencies of *in vivo* matured oocytes compared with those matured *in vitro*. For instance in cattle, approximately 60-80% of *in vivo* matured oocytes are competent to develop to the blastocyst stage (Bordignon *et al*., 1997; Blondin *et al*., 2002; Dieleman *et al*., 2002; Rizos *et al*., 2002) while only 25-40% of *in vitro* matured (Van de Leemput *et al*., 1999; Ward *et al*., 2002). Failure to achieve high success rates in the bovine embryo culture compared to other species may indicate some fundamental problems such as

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inappropriate media formulations, media supplementation, problems in the culture system, technical issues or lack of vital factors which are normally available *in vivo*. Therefore, it is required to modify the culture media or culture condition to support higher percentage of *in vitro* maturation, fertilization and culture processes to maximize embryo development *in vitro*. To date, there is no consensus on the perfect medium and protocol for IVEP procedure in bovine (Bavister, 1995; Galli *et al*., 2003). Comprehensive understanding of these steps can help to mimic *in vivo* condition and subsequently improve *in vitro* production rate.

Ghrelin is a novel hormone that mainly secreted from gastric mucosa into the blood circulation (Kojima *et al*., 1999, 2001). Ghrelin increased the expression of Mitogen-Activated Protein Kinase (MAPK) in bovine oocytes (Popelkova *et al*., 2006). MAPK pathway is involved in the regulation of microtubule organization during meiosis, spindle morphology and maintenance of maturation promoting factor activity in bovine oocytes (Gordo *et al*., 2001).

Ghrelin receptors also have been detected in mammalian ovaries (Barreiro and Tena-Sempere, 2004). Therefore, it is rational to hypothesize that ghrelin may affect the nuclear maturation of bovine oocytes and require further investigation.

MATERIALS AND METHODS

Reagents: All chemicals and reagents used in the current study were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA) unless otherwise stated.

Oocyte recovery: Bovine ovaries were collected from local abattoirs and transported to the laboratory in a thermos flask containing warm (32-35°C) Phosphate Buffered Saline (PBS; P-4417) supplemented with 100 IU mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (15140-122, Gibco, Invitrogen, USA). Cumulus-Oocyte-Complexes (COCs) were recovered from 2-8 mm follicles by slicing method. Slicing solution was Dulbecco's phosphate buffered saline (D-8662) supplemented with 4 mg mL^{-1} bovine serum albumin fraction V (BSA; A-3311) and 50 μL mL^{-1} gentamycin (G-1264). Only oocytes surrounded by intact and compact layers of cumulus cells with dark and homogenous cytoplasm were used. The COCs were washed 2-3 times in fresh pre-equilibrated working solution. The working solution consisted of Hepes-buffered medium 199 (12340-030, GibcoBRL, Invitrogen, USA) supplemented by 4 mg mL^{-1} BSA.

***In Vitro* Maturation (IVM):** The selected COCs were washed 2 times in pre-equilibrated maturation medium. The maturation medium was hepes-buffered medium 199, 10% fetal calf serum, gonadotrophins, insulin and antibiotics as described by Dashtizad *et al.* (2010b). Thereafter, groups of 7-10 COCs were randomly distributed in each 50 μL pre-equilibrated IVM medium. Immature oocytes were incubated for 22-24 h at 38.5°C and 5% CO_2 in humidified air.

Evaluation of nuclear maturation: After completion of IVM, the nuclear maturation was assessed by denuding a representative number of oocytes using 0.1% (w/v) hyaluronidase (Type 1-S) in Hepes-buffered medium 199. Denuded oocytes were washed twice with working solution and fixed in aceto-ethanol (1:3 v/v) solution at 4°C for 24 h. Five to ten fixed oocytes were mounted on a clean glass slide in a microdroplet (10-20 μL) between two parallel lines of wax-vaseline mixture (1:20). A cover slip was slowly placed on the lines and gently pressed down until it touched the microdroplet and secured the oocytes between the slide and the cover slip. Staining solution, 1% aceto-orcein was passed from one side of the cover slip and blot-dried from the opposite side by a piece of filter paper. After 3-5 min when the staining was completed, decolorizing solution (acetic acid: distilled water: glycerol: 1:3:1) was passed through to remove the stain residuals. The cover slip was sealed with a colorless nail varnish to provide a permanent storage for future examination. The stained oocytes were evaluated under a phase contrast microscope at 400X magnification to assess the status of nuclear maturation.

***In Vitro* Fertilization (IVF):** *In vitro* matured COCs with expanded cumulus cells were used for *in vitro* fertilization. The fertilization method was previously described by Parrish with some modifications. Briefly, following maturation period for 22-24 h matured COCs were washed 2 times in working solution and in two droplets of Tyrode's albumin lactate pyruvate buffered with HEPES (IVF-TALP solution). Subsequently 6-8 COCs were placed in pre-equilibrated IVF-TALP droplets covered with sterile mineral oil (M-5310). Frozen semen straws (250 μL) were thawed in water bath at 37°C for 45-60 sec and content was poured into a 1.5 mL centrifuge tube containing 1 mL pre-warmed BoviExtend (Nidacon Laboratories AB, Gothenburg, Sweden) buffer solution and smoothly mixed. The amount of 1 mL of the diluted semen was gently loaded on the top of the adjusted BoviPure density gradient in the conical tube and centrifuged for 20 min at 300×g at room temperature. After centrifugation, the supernatant was carefully discarded. The sperm pellet was resuspended with 5 mL of pre-warmed sperm-TALP medium supplemented by 6 mg mL^{-1} Bovine Serum Albumin Fatty Acid Free (BSA-FAF) (A-8806, Sigma) and centrifuged again for 10 min at 300×g. The final pellet was resuspended in 150-200 μL of pre-equilibrated IVF-TALP.

Spermatozoa were checked for motility and counted by a haemocytometer to give the final concentration of 1×10^6 spermatozoa mL^{-1}.[1] Based on concentration, spermatozoa were added gently to each IVF droplet containing 6-8 m oocytes.

Thereafter, PHE mixture consisting of D-penicillamine (P-4875) Hypotaurine (H-1384) and Epinephrine (E-4250) were added to each IVF droplet. *In vitro* fertilization was accomplished by co-incubation of sperm-oocytes at humid environment with 5% CO_2 and 38.5°C for 18-20 h.

***In Vitro* Culture (IVC):** At the end of fertilization period, oocytes were freed of cumulus cells by gentle mechanical pipetting. After denuding, the presumptive zygotes were washed 2 times in fresh pre equilibrated working solution. Then, they were washed through embryo culture droplets. Approximately 15-20 presumptive zygotes and embryos were transferred into each well of 4 well dish containing 400 µL of Synthetic Oviductal Fluid (SOFaaci) (Holm *et al.*, 1999) supplemented with 5% adult bovine serum (B-9433) and 1 µg mL^{-1} gentamycin under sterile mineral oil.

During 9 days at every 2 day interval, SOF solution was replenished by warm and CO_2 equilibrated IVC medium. Cleavage, morula, blastocyst and hatched blastocyst rates were recorded at days 2, 4, 7 and 9 post inseminations, respectively.

Experimental design: In the present study, influence of ghrelin supplementation in the maturation medium on nuclear maturation and subsequent *in vitro* bovine embryo development was evaluated. In experiment 1, to find out the effect of ghrelin on *in vitro* maturation, 5 different concentrations of ghrelin (0, 5, 50, 500 or 1000 ng mL^{-1}; Sigma, G3902) were added to the IVM medium. After 22-24 h of maturation, a representative number of oocytes were randomly selected to examine nuclear maturation status after staining with aceto-lacmoid stain. In experiment 2, the rest of the *in vitro* matured bovine oocytes were subsequently fertilized and cultured for 9 days. The cleavage, blastocyst and hatched blastocyst rates were assessed at days 2, 7 and 9, respectively.

Statistical analysis: All experiments were repeated 6 times. Significant differences among treatments were revealed by one-way analysis of variance followed by Duncan's multiple range test for mean comparisons ($p<0.05$) using SAS software Ver. 9.1 (SAS Inst., Cary, NC).

RESULTS AND DISCUSSION

The effects of ghrelin supplementation on maturation rate and subsequent embryo production of 1113 COCs were evaluated (Table 1 and 2). The rate of oocytes that reached M II increased at 500 ng mL^{-1} (85.50±3.74%) but there was no pronounced difference when compared to the control (81.98±3.38%). Ghrelin strikingly increased the rate of oocytes developed to M II stage at 5 and 50 ng mL^{-1} (86.32±3.38 and 89.77±2.92%, respectively) during the maturation period compared to the control. However, the highest concentration of ghrelin at 1000 ng mL^{-1} significantly reduced ($p<0.05$) nuclear maturation rates of treated oocytes (72.71±3.34%). After fertilization of *in vitro* matured oocytes, only 50 ng mL^{-1} ghrelin slightly increased the cleavage rate (80.18±4.19%) compared to the control (78.80±3.04%). Furthermore, in term of cleavage rate no significant difference was observed in 5 and 500 ng mL^{-1} (76.47±3.27 and 75.05±4.32%, respectively). However, cleavage rate was markedly decreased in 1000 ng mL^{-1} ghrelin group (67.14±3.99%). No significant differences were observed in the developmental rate to the blastocyst and hatched blastocyst stages compared to the control group (33.56±3.37 and 9.44±2.97%, respectively).

Results of this experiment demonstrated that ghrelin supplementation at 5 and 50 ng mL^{-1} in IVM medium increased the proportion of oocytes reaching M II stage and remarkably enhanced nuclear maturation rate of bovine oocytes. These may be mainly due to the effect of ghrelin on up-regulation of Mitogen-Activated Protein Kinase (MAPK) isoforms. MAPK which is also termed as Extracellular-Regulated Kinase (ERK) is a family of protein kinases that are distributed in eukaryotic cells (Liang *et al.*, 2007). Two isoforms of MAPKs known as ERK1 and ERK2 are widely express in mammalian oocytes

Table 1: Effects of different concentrations of ghrelin in IVM media on *in vitro* nuclear maturation of bovine immature oocytes

Ghrelin concentration (ng mL^{-1})	No. of oocytes cultured	Mean % of matured oocytes±SEM (n)	
		No. of stained oocytes	M II oocytes
0 (control)	221	84	81.98 ± 3.38^{c} (69/84)
5	223	87	86.32 ± 3.38^{ab} (75/87)
50	224	78	89.77 ± 2.92^{a} (70/78)
500	219	82	85.50 ± 3.74^{bc} (70/82)
1000	226	72	72.71 ± 3.34^{d} (52/72)

Data were pooled from 6 replicates; $^{a-d}$ values with different superscripts in the same column are significantly different ($p<0.05$)

Table 2: Effects of ghrelin supplementation in IVM media on subsequent *in vitro* bovine embryo development

Ghrelin concentration ($ng\ mL^{-1}$)	No. of oocytes cultured	Mean % of cleaved oocyte±SEM (n)	Mean % of blastocyst ±SEM (n)	Mean % of hatched blastocyst±SEM (n)
0 (control)	137	78.80±3.04^{ab} (108/137)	33.56±3.37^{ab} (46/137)	9.44±2.97 (13/137)
5	136	76.47±3.27^{ab} (104/136)	35.28±3.69^{a} (48/136)	8.72±2.01 (12/136)
50	146	80.18±4.19^{a} (117/146)	34.27±3.80^{ab} (50/146)	9.53±4.16 (14/146)
500	137	75.05±4.32^{b} (103/137)	32.05±3.53^{ab} (44/137)	7.99±4.18 (11/137)
1000	154	67.14±3.99^{c} (104/154)	30.34±2.10^{b} (47/154)	6.30±2.41 (10/154)

Data were pooled from 6 replicates; $^{a\text{-}c,}$ values with different superscripts in the same column are significantly different ($p<0.05$)

(Ohashi *et al*., 2003). Studies in the last decade revealed that MAPK cascade plays as principle regulatory role in driving the meiotic cell cycle progression of oocytes. After germinal vesicle breakdown, MAPK is involved in the regulation of microtubule organization and meiotic spindle assembly (Fan and Sun, 2004). The activation of this kinase is essential for the maintenance of metaphase II arrest while its inactivation is a prerequisite for pronuclear formation after fertilization or parthenogenetic activation. Bovine oocytes injected with MKP-1 mRNA, a MAPK-specific phosphatase which inhibits MAPK activation, exhibited disorganized and diffused spindles (Gordo *et al*., 2001). Popelkova *et al*. (2006) showed that low level of ghrelin (5 $ng\ mL^{-1}$) significantly caused elevation in the expression of ERK1 and ERK2. However, contradictory results were obtained compared to the findings of Suzuki *et al*. (2009). It was reported that ghrelin did not improve meiotic maturation of porcine oocytes cultured in follicular fluid supplemented medium NCSU23.

Furthermore, addition of ghrelin to the IVC medium showed beneficial effects on consequent bovine embryos development. Researchers have found that inclusion of 50 $ng\ mL^{-1}$ ghrelin in IVC medium improves bovine blastocyst formation *in vitro* (Dashtizad *et al*., 2010a) however, no significant differences were observed in blastocyst and hatched blastocyst rates by addition of ghrelin in IVM medium. Furthermore, high concentration of ghrelin (1000 $ng\ mL^{-1}$) adversely affected and notably declined maturation rate. One of the possible reasons for decreased developmental competency of immature oocytes exposed to high levels of ghrelin in this study might be due to the decreased density of the cytoplasmic microfilaments. Suzuki *et al*. (2009) reported that high level of ghrelin (500 $ng\ mL^{-1}$) inhibited the natural organization of microtubules and microfilaments in porcine oocytes and consequently declined the maturation rate.

CONCLUSION

The present study concluded that addition of 5-50 $ng\ mL^{-1}$ ghrelin to the IVM media is recommended and would improve proportion of bovine oocytes reaching the M II stage.

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