Leptin promoted meiotic maturation of bovine oocytes and development of parthenogenetic activation and yak (*Bos grunniens*)-bovine interspecies cloned embryos

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ABSTRACT. Leptin has a central role in the regulation of oocyte and embryo development. The objective of the current study was to investigate the effect of leptin addition during oocyte maturation *in vitro* on meiotic maturation of bovine oocytes and development of parthenogenetic activation (PA) and yak-bovine interspecies cloned embryos. In addition, the effect of leptin supplementation in embryo culture medium on preimplantation development of yak-bovine interspecies cloned embryos was investigated. Leptin addition at 10 and 100ng/mL during oocyte maturation significantly increased the proportion of bovine oocytes that developed to metaphase II (MII) stage compared to the control (81.0±3.8 and 77.0±4.0 versus 62.2±1.8, $P<0.05$). Leptin at 10ng/mL significantly increased the blastocyst rate after PA compared to the control (31.6±4.3 versus 20.4±3.2, $P<0.05$). Leptin supplementation at 10ng/mL during oocyte maturation strikingly increased the blastocyst development of yak-bovine interspecies cloned embryos (33.2±4.0 versus 22.8±2.7, $P<0.05$) and the inner cell mass (ICM) and total cell number per blastocyst, and reduced the incidence of apoptotic cells per blastocyst compared to the control. When present in the embryo culture medium, leptin at 100ng/mL significantly increased the blastocyst development of yak-bovine interspecies cloned embryos compared to the control (34.2±4.9 versus 22.0±3.2, $P<0.05$). Meanwhile, leptin at 100 and 1000ng/mL significantly increased ICM and total cell number per blastocyst and reduced the proportion of apoptotic cells per blastocyst. In conclusion, leptin supplementation promoted the meiotic maturation of bovine oocytes and the developmental capacity of PA and yak-bovine interspecies cloned embryos.

KEY WORDS : yak, leptin, oocyte maturation, interspecies cloning

INTRODUCTION

Leptin, the 16-kDa product of the obese (ob) gene, has been implicated to play an important role in the regulation of food intake and energy expenditure (ZHANG et al., 1994). In addition, leptin is known to regulate diverse reproductive functions (HOLNESS et al., 1999). The ob/ob mice expressing a truncated form of leptin were obese and infertile (ZHANG et al., 1994). Exogenous leptin supplementation can restore normal weight and fertility in ob/ob mice, suggesting that leptin influences reproduction in a direct way (CHEHAB et al., 1996).

Recently, leptin has been detected to be expressed in mouse (ANTCZAK & VAN BLERKOM, 1997; KAWAMURA et al., 2002) and human oocytes (CIOFFI et al., 1997); mouse (KAWAMURA et al., 2002) and human (CIOFFI et al., 1997) follicular fluid; and human granulosa and cumulus cells (CIOFFI et al., 1997). The leptin receptor (LEPR) has high sequence homology to the class I cytokine receptor superfamily (CHEN et al., 1996), and different 3' terminal mRNA splicing produces six known isoforms (TARTAGLIA et al., 1995; CIOFFI et al., 1996; LEE et al., 1996). The long isoform containing a 302-residue-long intracellular domain was detected in the hypothalamus and also in some peripheral tissues (TARTAGLIA et al., 1995; BJORBAK et al., 1997), and this isoform regulates most leptin signalling (LEE et al., 1996; WHITE et al., 1997) by both pathways of the mitogen-activated protein kinase (MAPK) and the signal transducer and activator of transcription 3 (STAT3) (MATSUOKO et al., 1999). LEPR mRNA is expressed in mouse oocytes (MATSUOKO et al., 1999; KAWAMURA et al., 2002) and in bovine blastocysts (BOELHAUVE et al., 2005). Leptin, at physiological concentrations, causes tyrosine phosphorylation of STAT3 in mouse metaphase II (MII) oocytes (MATSUOKA et al., 1999). Thus, leptin is likely a regulator of oocytes and its surrounding cells.

The *in vitro* production (IVP) system in cattle, including somatic nuclear transfer (SCNT), is a crucial tool for basic research and for preservation of endangered mammals. Although there have been a few successes (WHITE et al., 1999; LANZA et al., 2000; LOI et al., 2001; LEE et al., 2003; SANSINENA et al., 2003), the efficiency of interspecies SCNT in birth of live offspring is still low. Incomplete cytoplasmic and nuclear maturation of the oocyte is a limiting step determining the ability of the oocyte to successfully support a somatic nucleus to undergo reprogramming, cleavage and embryo development. Growth factors and cytokines lead to proper nuclear and cytoplasmic maturation of the oocyte by a complex network (GREEWALD & ROY, 1994). Recently, there have been some reports that leptin supplementation promotes oocyte maturation and preimplantation development of embryos. It has been demonstrated that 10-100ng/mL leptin supplementation stimulates porcine oocyte maturation (CRAIG et al., 2004; ZHANG et al., 2007). Addition of leptin in embryo culture medium promotes preimplantation embryo development *in vitro* in a dose-dependent manner in the mouse (KAWAMURA et al., 2002). In bovines, the presence of leptin during oocyte maturation has been found to increase the proportion of oocytes developed to blastocyst stage after *in vitro* fertilization (IVF) and to

reduce the proportion of apoptotic cells per blastocyst (BOELHAUVE et al., 2005). However, the data about the effect of leptin on meiotic maturation of bovine oocytes and preimplantation development of interspecies cloned embryos when included in *in vitro* culture medium are insufficient. In addition, controversial results have been reported about the effect of leptin on preimplantation development of murine embryos (KAWAMURA et al., 2002; FEDORCSAK & STORENG, 2003; SWAIN et al., 2004). Thus, the role of leptin during *in vitro* development of mammalian oocytes and embryos needs to be further researched. Accordingly, in the present study, we investigated whether leptin addition to *in vitro* maturation (IVM) medium influences meiotic maturation of bovine oocytes and subsequent preimplantation development of parthenogenetic activation (PA) embryos. In addition, the effect of leptin supplementation in IVM medium of bovine oocytes on development of the embryo derived from yak-bovine interspecies nuclear transfer was assessed in this study. We also investigated the effect of leptin addition to embryo culture medium on preimplantation development of yak-bovine interspecies nuclear transfer embryo.

MATERIALS AND METHODS

1. Chemicals

Unless otherwise stated, chemicals were purchased from Sigma-Aldich Crop. (St. Louis, MO)

2. Donor cell preparation

Ear skin was biopsied from an adult black yak (*Bos grunniens*). The tissue was kept in PBS (Gibco, USA) at 4°C during transportation to the laboratory. After removal of cartilage, skin tissues were minced into about 1mm^3 pieces with a surgical blade, and seeded in the 25cm^2 tissue culture flask (Nunc, Roskilde, Denmark). The tissue was cultured in 5mL Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) plus 20% (v/v) fetal bovine serum (FBS, Gibco, USA) under a humidified atmosphere of 5% CO_2 in air at 37°C. When primary cell layer reached 85% confluence after 8-10 days, the cells were harvested by 0.25% (w/v) trypsinization (Gibco, USA) and reseeded at a density of 100,000cells/mL in 25cm^2 tissue culture flasks. Fibroblast cells at passages 4-8 were harvested by 0.25% (w/v) trypsinization and frozen in DMEM supplemented with 20% (v/v) FBS and 10% (v/v) dimethylsulfoxide in liquid nitrogen at -192°C. Prior to SCNT, the cells were thawed, cultured for 3 days until 80% confluence and subjected to serum-starvation by being cultured for another 3-5 days in DMEM supplemented with 0.2% (v/v) FBS.

3. *In vitro* maturation of bovine oocytes

Bovine ovaries were collected from a local slaughter house and transported to the laboratory within 4h in saline solution at 30°C. Cumulus-oocyte complexes (COCs) from domestic cattle were aspirated from 2-8mm in diameter antral follicles using an 18 gauge needle and washed several times in IVM medium that contained TCM-199 (Gibco, USA), supplemented with 10% (v/v) FBS, 2mM $NaHCO_3$, 1.0% (v/v) penicillin-streptomycin (10,000IU/mL and 10,000μg/mL, respectively; pen-strep; Gibco), 10μg/mL LH and 1μg/mL estradiol-17β. Those oocytes that had uniform cytoplasmic appearance and were enclosed within three or more layers of viable compact granulosa cells were selected. Groups of 50 oocytes were matured in 500μl of IVM medium under mineral oil in four-well dishes at 39°C in a humidified atmosphere of 5% CO_2 in air.

4. Assessment of oocyte nuclear status

At the end of *in vitro* maturation culture, oocytes were denuded from cumulus cells by pipetting and vortexing in 0.5mg/mL hyaluronidase. Denuded oocytes were mounted on a glass slide and fixed with acetic acid: ethanol (1:3 v/v) for at least 30h at 4°C. Then, oocyte staining was performed in acetic acid-orcein (1% orcein in 45% acetic acid) and they were examined under a phase-contrast microscope. Oocytes were classed into intact nucleus (GV), the germinal vesicle break down (GVBD) and metaphase II (MII) stage. Oocytes with abnormal chromatin or no chromatin were selected as degenerated oocytes.

5. Somatic nuclear transfer and embryo culture

The zona-free method of Handmade Cloning (VAJTA et al., 2001; VAJTA et al., 2003; BHOJWANI et al., 2005) was used in this experiment. After *in vitro* maturation for 20-22h, cumulus cells' enclosed COCs were removed in 0.5mg/mL hyaluronidase dissolved in Hepes-buffered TCM-199 medium (T0) for 3min. The zona pellucida of nude oocytes was digested in 1.5mg/mL pronase dissolved in T0 at 39°C. Under stereomicroscopic control, manual bisections of oocytes were performed with Ultra Sharp Splitting Blades (AB Technology, Pullman, WA) in T0 supplemented with 20% (v/v) FBS, 5μg/mL cytochalasin B. Then, chromatin staining of all half-oocytes was performed with 10μg/mL Hoechst 33342 dissolved in T0. Half-oocytes without chromatin staining were registered under a fluorescence microscope. Registered half-oocytes were collected in TCM-199 supplemented with 20% (v/v) FBS (T20) as recipient cytoplasts. After serum-starvation, the donor cells were harvested by pipetting in 0.05% (w/v) trypsin dissolved in PBS and collected in T20 at room temperature.

For embryo construction, half of prepared cytoplasts were individually incubated in 200μg/mL phytohemagglutinin dissolved in T0 for 5sec. Cell-cytoplast pairing was achieved by gently pressing a prepared cytoplast over a single fibroblast cell. Then, the cell-cytoplast pairs were aligned using an alternating current of 15V AC in a platinum wire fusion chamber. Fusion medium contained 0.3M mannitol, 0.1mM $MgSO_4$ and 0.05mM $CaCl_2$. A second cytoplast was then aligned to the single cell-cytoplast pair in the fusion chamber. Fusion was performed with a single pulse of 3.7KV/cm for 4μsec using a BTX Electro Cell Manipulator 200 (Biotechnologies and Experimental Research Inc., San Diego, CA, USA). After fusion, reconstructed embryos were cultured in 1mL T20 containing 2μM Ca ionophore A23187 for 5min, then embryos were transferred into 5μl droplets of 2mM 6-dimethylaminopurine (6-DMAP) for 6h for further chemical activation. After chemical activation, each single

embryo was cultured in well of the wells (WOWs) (VAJTA et al., 2000) in 400µl modified synthetic oviductal fluid (mSOFaa) medium, as previously described (HOLM et al., 1999), covered with oil. Embryos were cultured at 39°C in 5% CO_2, 5%O_2 and 90%N_2 for 7 days.

6. Parthenogenetic activation

After bovine oocytes had matured for 20-22h, parthenogenetic activation was performed using the same method above. Oocytes were activated in 2µM Ca ionophore A23187 for 5min and then exposed in 2mM 6-DMAP for 6h. Activated oocytes were cultured in the mSOFaa at 39°C in 5% CO2, 5%O_2 and 90%N_2 for 7 days.

7. Embryo transfer

SCNT embryos of excellent quality at the blastocyst stage were transferred into Holstein recipients on Day 7±1 of the estrous cycle. Two or three embryos per recipient were nonsurgically introduced into the uterine horn ipsilateral to the ovary containing the corpus luteum. Pregnancy was diagnosed by rectal palpation at 60 and 120 days, respectively, after embryo transfer.

8. Different staining of inner cell mass (ICM) and trophectoderm (TE) cells

Day 7 blastocysts derived from yak-bovine interspecies nuclear transfer were randomly selected and subjected to different staining according to previously described methods (THOUAS et al., 2001). The zona pellucida of each blastocyst was removed by incubating in 0.5% pronase for 1min. Blastocysts were treated for 5-7sec with 0.2% (v/v) Triton X-100 and 0.3mg/mL propidium iodide dissolved in PBS and then transferred into 20µg/mL Hoechst-33342 dissolved in glycerol for 2-3h. Embryos were mounted on a glass slide in a drop of glycerol solution. Cell numbers were determined using a fluorescence microscope. Blue nuclei were considered as ICM cells and red or pink nuclei as trophoblast cells.

9. TUNEL assay for apoptosis detection

Apoptotic blastomeres were detected by TUNEL assay as previously described (GAVRIELI et al., 1992) using an In situ Apoptosis Detection Kit (Takara Bio Inc., Shigaken, Japan). Blastocysts were fixed in 4% (v/v) paraformaldehyde dissolved in PBS for 1h at room temperature. Blastocysts were permeabilized with 0.5% (v/v) Triton X-100 for 1h at room temperature. After blastocysts were washed three times with 1mg/mL BSA dissolved in PBS, they were incubated in a terminal deoxynucleatidyl transferase-labelling buffer for 3h at room temperature. Then, blastocysts were transferred into 25µl fluorescein isothiocyanate solution for 1.5h and incubated with 200µl 0.5µg/mL propidium iodide for 30min at room temperature. Blastocysts were treated with 0.2M diazabicyclo-octane in PBS supplemented with 50% (v/v) glycerol and then mounted on a glass slide. Each blastocyst was examined for total number of nuclei and number of TUNEL-labelled nuclei under a fluorescence microscope. The apoptotic cells appeared as yellow, but normal cells as red.

10. Experimental design

Experiment 1: This experiment was designed to investigate the effect of leptin addition in IVM medium on developmental capacity of bovine oocytes. COCs were cultured in IVM medium containing 0 (control), 1, 10 or 100ng/mL recombinant human leptin (Sigma Aldich Co.), designed according to BOELHAUVE et al. (2005). After *in vitro* maturation for 20-22h, some oocytes were selected to assess nuclear status, and others were subjected to PA or yak-bovine interspecies nuclear transfer.

Experiment 2: This experiment was designed to investigate the effect of leptin supplementation in IVM medium on developmental competence of PA embryos. After denuded oocytes derived from different dosage leptin treatment groups were performed to PA, the rates of cleavage and blastocysts formation were examined at Day 3 and Day 7 after activation, respectively.

Experiment 3: This experiment was designed to check the effect of leptin addition during oocyte maturation on development of yak-bovine interspecies cloned embryo. Accordingly, the results of the above two experiments, 10 or 100ng/mL leptin treatment were considered as optimum treatment groups and selected to be subjected to further SCNT experimentation. After maturation for 20-22h, oocytes were subjected to SCNT. The cleavage rate at Day 3 after reconstruction, and development to the blastocyst stage at Day 7 after reconstruction, were assessed. This experiment was replicated five times, using 278-313 oocytes per treatment. TE/ICM cell numbers per blastocyst derived from different dosage leptin treatment groups were examined at Day 7 after reconstruction. TUNEL analysis to blastocyst was performed at Day 7 after reconstruction.

Experiment 4: This experiment was designed to investigate the effect of leptin supplementation in embryo culture medium on developmental capacity of cloned embryos derived from yak-bovine interspecies nuclear transfer. Reconstructed embryos were randomly allocated and cultured in the mSOFaa supplemented with 0, 1, 10, 100 or 1000ng/mL leptin from Day 0 to Day 7. The concentrations of leptin were selected according to KAWAMURA et al. (2002). The cleavage rate at Day 3 and development of the blastocyst stage at Day 7 after reconstruction were assessed. This experiment was replicated five times, using 222-291 oocytes per treatment. ICM/TE cell number and TUNEL analysis were performed at Day 7 after reconstruction.

11. Statistical analysis

All data were analyzed using a statistical system (SPSS for windows 13.0) program. The differences in embryos development among groups were analyzed using one-way ANOVA. Post hoc analysis was performed according to LSD test. Meanwhile, ICM/TE cell number and apoptosis cells per blastocyst among experimental groups were analyzed using the same methods. The data are presented as mean±SEM. A P value less than 0.05 indicated significant differences among the groups.

RESULTS

1. Effect of leptin addition in IVM medium on developmental capacity of bovine oocytes

The effect of leptin addition in IVM medium on developmental capacity of bovine oocytes was evaluated in this experiment. There was no effect of leptin supplementation in the 1ng/mL leptin treatment group on the proportion of oocytes reaching MII stage, as compared to the control. However, treatment of oocytes with 10 or 100ng/mL leptin increased the proportion of oocytes developed to MII stage, as compared to the control (P<0.05) (Table 1).

TABLE 1

Effect of leptin addition in *in vitro* maturation medium on developmental capacity of bovine oocytes.

Group (leptin treatment)	No. of oocytes treated	No. of oocytes developed to a specific stage (%)			No. of degenerated oocytes
		GV	GVBD	MII	
0ng/mL	104	8 (7.8±1.8)	17 (16.6±2.4)	65 (62.2±1.8)^{c}	14 (13.4±2.6)
1ng/mL	157	16 (10.2±1.1)	23 (15.2±2.4)	107 (68.0±1.9)^{bc}	11 (6.8±1.3)
10ng/mL	178	10 (6.0±2.0)	13 (7.0±2.3)	143 (81.0±3.8)^{a}	12 (6.3±3.1)
100ng/mL	163	18 (10.6±3.2)	11 (6.0±3.4)	124 (77.0±4.0)^{ab}	10 (5.6±2.6)

Values with different letters (a, b and c) in the same column are significantly different (P<0.05).
Oocytes were classed into intact nucleus (germinal vesicle, GV), the germinal vesicle break down (GVBD) and metaphase II (MII) stage according to the specific stage of development. Oocytes with abnormal chromatin or no chromatin were selected as degenerated oocytes.

2. Effect of leptin supplementation in IVM medium on developmental competence of embryo derived from PA

The purpose of this experiment was to assess whether leptin increases development capacity of embryo derived from PA. There was no effect of leptin addition during IVM on cleavage rate of PA embryo at Day 3 after activation (71.2±3.5, 73.0±3.1, 73.6±4.6, and 69.2±5.9, cleavage rate for 0, 1, 10 and 100ng/mL leptin addition, respectively), but leptin treatment at 10ng/mL (31.6±4.3) increased the proportion of oocytes developed to the blastocyt stage at Day 7 after activation as compared to the control (20.4±3.2) (Fig. 1). In addition, leptin treatment at 100ng/mL (29.0±3.1) also increased the proportion of oocytes developed to the blastocyst stage, although there was no significant difference compared to the control.

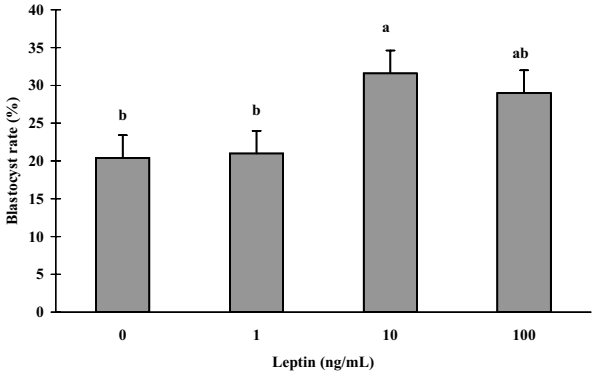

Fig. 1. – Effect of leptin addition in *in vitro* maturation medium on developmental capacity of parthenogenetic activation (PA) embryo in bovine. The proportion of oocytes developed to the blastocyst stage at Day 7 after activation was assessed. Results are shown as means±SEM of five replicates, using 132-185 oocytes per treatment. Different letters (a and b) denote significant differences (P<0.05).

3. Effect of leptin supplementation during *in vitro* maturation on preimplantation development of yak-bovine nuclear transfer embryos

In this experiment, the effect of leptin on preimplantation development of yak-bovine interspecies cloned embryos was determined. There was no effect of leptin addition during oocyte maturation on cleavage rate of yak-bovine cloned embryos at Day 3 after reconstruction (70.2±2.4, 72.8±2.3 and 69.6±4.8, cleavage rate for 0, 10 and 100ng/mL leptin treatment, respectively). However, as shown in Fig. 2A, leptin addition at 10ng/mL (33.2±4.0) increased the proportion of oocytes developed to the blastocyst stage as compared to the control (22.8±2.7) (P<0.05). Meanwhile, as shown in Fig. 2B and Fig. 2C, leptin at 10ng/mL (47.8±8.6, 111.0±17.1) increased significantly inner cell mass and total cell number per blastocyst as compared to the control (28.3±5.0, 75.4±10.4) (P<0.05), and leptin at 10 (4.7±0.4) and 100ng/mL (4.4±0.4) decreased the incidence of TUNEL-positive blastomeres per blastocyst as compared to the control (10.7±1.1) (P<0.01).

4. Effect of leptin supplementation in embryo culture medium on development of yak-bovine interspecies cloned embryos

As shown in Fig. 3A, the rate of blastocyst formation of yak-bovine interspecies cloned embryos significantly increased in the 100ng/mL leptin treatment group (34.2±4.9) compared to the control (22.0±3.2), but the cleavage rate did not differ significantly between the treatment groups (75.4±5.3, 70.0±6.1, 71.0±5.0, 69.4±6.9 and 69.2±5.9, cleavage rate for 0, 1, 10, 100 and 1000ng/mL leptin treatment, respectively). As shown in Fig. 3B, the ICM/Total cell number per blastocyst increased in 100 (49.8±8.9, 104.2±11.7) and 1000ng/mL (49.0±6.8, 107.1±9.3) leptin treatment group compared to the control (28.7±5.9, 74.3±6.7) (P<0.05). The proportion of apoptotic blastomeres per blastocyst in 100 (5.4±0.5) and 1000ng/mL (4.4±0.3) leptin treatment group decreased as compared to the control (7.3±0.5) (P<0.05), which is shown in Fig. 3C.

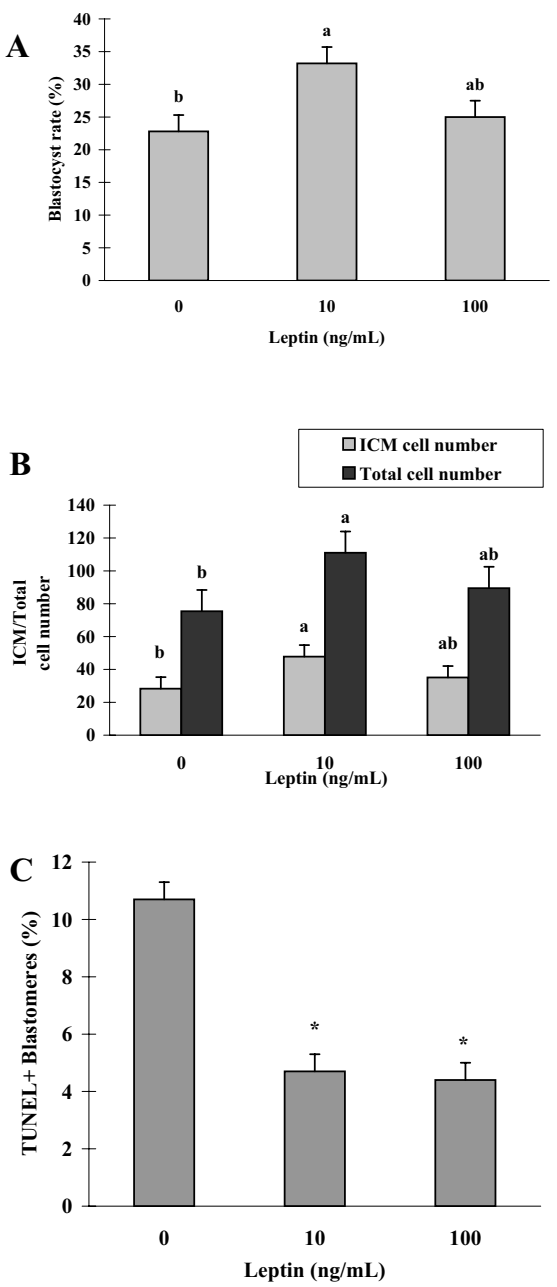

Fig. 2. – The effect of leptin at 0, 10 and 100ng/mL in *in vitro* maturation medium of bovine oocytes on preimplantation development, blastocyst cell number and blastomere apoptosis of yak-bovine interspecies cloned embryos. Blastocyst rate (A) and inner cell mass (ICM) /Total cell number per blastocyst (B) at Day 7 after reconstruction was assessed. The proportion of apoptotic blastomeres per blastocyst (C) are shown as mean±SEM of five replicates, using 20 blastocysts per treatment. Different letters (a and b) denote significant differences (P<0.05). Significant difference (P<0.01) from the control group is indicated by *.

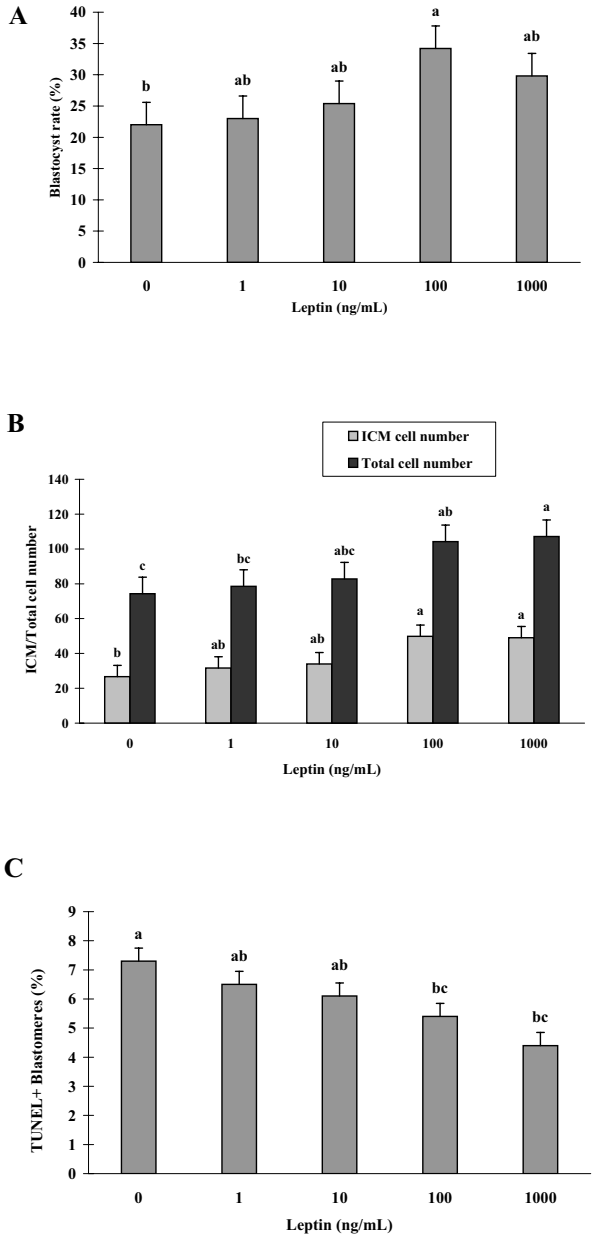

Fig. 3. – Influence of different concentrations of leptin supplementation in embryos culture medium on preimplantation development and cell apoptosis of yak-bovine interspecies cloned embryos. Rate of blastocyst development and inner cell mass (ICM) /Total cell number per blastocyst were evaluated at Day 7 after reconstruction. Number of apoptotic blastomeres per blastocyst was assessed at Day 7 after reconstruction, and shown as mean±SEM of five replicates, using 20 blastocysts per treatment. Different letters (a, b and c) denote significant difference (P<0.05).

5. Effect of leptin treatment during *in vitro* maturation of oocytes on pregnancy of yak-bovine interspecies cloned embryos in surrogate mothers

As shown in Table 2, blastocysts derived from 10ng/mL leptin treatment during oocyte maturation achieved pregnancies at pregnant Day 60 and 120 after embryo transfer, respectively. However, there was no pregnancy at Day 60 in the control group in the absence of leptin.

TABLE 2

Effect of leptin treatment in *in vitro* maturation medium of bovine oocyte on pregnancies of yak-bovine cloned embryo.

Leptin treatment	Embryos transferred	Recipients	Pregnant		Offspring
			Day 60	Day 120	
0ng/mL	45	15	0	0	0
10ng/mL	54	20	3	2	0

DISCUSSION

Previous reports have shown that leptin has a possible role in regulation of oocyte and preimplantation embryo development in the mouse, pig and bovine. Leptin addition at 100ng/mL, which is the most prominent treatment, enhanced the development of porcine oocytes reaching MII stage determined by extrusion of the first polar body (CRAIG et al., 2004). In contrast, ZHANG et al. (2007) reported that 10ng/mL leptin treatment in IVM medium is the most significant treatment to improve the rate of porcine oocytes reaching MII stage by acid-orcein staining after oocytes maturation. In the present study, 10 and 100ng/mL leptin treatment increased the proportion of bovine oocytes developed to MII stage compared to the control, and 10ng/mL leptin treatment was the most significant one. Leptin treatment reduced the rate of stained oocytes with abnormal chromatin configuration compared to the control. In pigs, 10 and 100ng/mL leptin treatment strikingly increased the rate of blastocyst formation of PA embryo, and 10ng/mL leptin treatment obviously increased the cleavage rate of PA embryo (CRAIG et al., 2004). In contrast, in another report, only 10ng/mL leptin significantly increased the rate of blastocyst formation of PA embryo (ZHANG et al., 2007). In bovine, leptin addition at 1 or 10ng/mL during oocyte maturation increased the rate of blastocyst formation and cell number per blastocyst derived from IVF embryo (BOELHAUVE et al., 2005). In the current study, 10ng/mL leptin addition obviously increased the rate of PA embryo developed to the blastocyst stage in bovine. To determine the influence of leptin on the developmental capacity of yak-bovine interspecies nuclear transfer embryo, we investigated the effect of 10 and 100ng/mL leptin treatment designed according to the results of PA experiment. In the present study, we proved for the first time that leptin treatment at 10ng/mL during oocyte maturation strikingly improved the development rate to the blastocyst stage of the yak-bovine interspecies cloned embryo. Moreover, leptin treatment at 10ng/mL produced pregnancies of yak-bovine interspecies cloned embryo at Day 120. Embryonic developmental capacity is an indicator of oocyte quality. So, our study suggests that leptin promoted the cytoplasmic maturation of bovine oocytes so that the efficiency of yak-bovine interspecies nuclear transfer was improved. Therefore, this study may indicate a possible way to overcome the inefficiency of interspecies nuclear transfer by promoting nuclear and cytoplasmic maturation of bovine oocytes.

Leptin receptor mRNA and protein are present in the mouse preimplantation embryo (KAWAMURA et al., 2002). The levels of leptin secreted by cultured human blastocysts are significantly higher than that of arrested embryos (GONZALEZ et al., 2000). Leptin and leptin receptor proteins are expressed in *in vitro* matured oocytes, and IVF or SCNT embryos in porcine (KIM et al., 2006). In pigs, CRAIG et al. (2005) demonstrated that 10ng/mL of leptin in the culture medium increased the rate of cleavage and blastocyst significantly in the IVF embryos. KIM et al. (2006) also reported that 1000ng/mL leptin addition in embryo culture medium increased the rate of blastocyst formation and the number of TE and total cells in SCNT embryos, but leptin treatment had no effect on that of IVF embryos. In mouse, KAWAMURA et al. (2002) reported that 10, 100 and 1000ng/mL leptin addition promoted the development of blastocyst, expanded blastocyst and hatched blastocysts. In the present study, leptin at 100ng/mL to the embryo culture medium increased the rate of blastocyst formation of yak-bovine interspecies cloned embryo. In addition, leptin at 100 and 1000ng/mL increased ICM/total cell number of SCNT embryo compared to the control.

It is well known that ICM/total cell number and apoptotic blastomeres in blastocysts may reflect embryo quality. In the present study, leptin at 10ng/mL in IVM medium increased ICM/total cell number of yak-bovine interspecies cloned embryo, and 10 or 100ng/mL leptin obviously reduced the incidence of apoptotic blastomeres in SCNT embryo. Therefore, our study has shown that leptin plays a positive role to improve the viability of SCNT embryo when it is present in IVM medium or embryo culture medium.

In conclusion, it was demonstrated that leptin supplementation during oocyte maturation promoted the meiotic maturation of bovine oocytes and developmental capacity of PA or SCNT embryo. Moreover, leptin addition in the embryo culture medium improved the development of yak-bovine interspecies cloned embryo.

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