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Synergistic Effect of Insulin on *in vitro* Development of Immature Bovine Oocytes

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Abstract: Problem statement: Development of efficient culture system to support embryonic development would be valuable when quality of produced embryos was important. However, the rate of bovine embryo production *in vitro* was still lower than expected. Present study, including of three experiments, was carried out to investigate the effect of insulin on nuclear maturation and subsequent development of immature bovine oocytes and *in vitro* fertilized embryos. **Approach:** Grade one cumulus-oocyte-complexes harvested from slaughterhouse ovaries were selected and randomly allocated in each treatment groups. In experiment 1, *in vitro* maturation medium (Hepes-buffered medium 199 + fetal calf serum + gonadotrophins + antibiotics) supplemented with 0 (control), 1, 10, 20 and 100 μg mL^{-1} of insulin. In experiment 2, to eliminate the effect of serum and hormones, Hepes-buffered medium 199 was supplemented with 1 mg mL^{-1} polyvinyl alcohols (PVA) and same levels of insulin. In experiment 3, the effect of insulin on bovine *in vitro* embryo development was assessed. Presumptive zygotes were randomly cultured in synthetic oviductal fluid added with 0 (control), 1, 10, 20 and 100 μg mL^{-1} of insulin. **Results:** In experiment 1, nuclear maturation and embryo development rates were significantly higher in 1 and 10 μg mL^{-1} compared with other groups ($P<0.05$). In experiment 2, both maturation and cleavage rate significantly increased in 1 and 10 μg mL^{-1} insulin. The only treatment resulted in higher hatchability was 10 μg mL^{-1} insulin (17.1±2.34%) compared with control (11.34±3.94). In experiment 3, cleavage and morula rates were significantly greater in 1 and 10 μg mL^{-1} insulin compared with other groups; although the highest rates resulted by using 10 μg mL^{-1}. **Conclusion:** Obtained results show that inclusion of 10 μg mL^{-1} insulin in maturation and culture medium exerted beneficial effects on nuclear maturation of bovine oocytes and *in vitro* embryo development till morula stage.

Key words: Synergistic effect, immature oocytes, *In vitro* embryo development, Cumulus-oocyte-complexes (COCs), GAGs, nuclear maturation, cleavage rate, hatched blastocyst, preimplantation embryos, stimulates glucose

INTRODUCTION

In recent years, *in vitro* embryo production (IVEP) systems have been acknowledged to increase the rate of transferable embryos in bovine (Galli *et al*., 2004; Spicer *et al*., 2007). Commercial and research purposes have been enhancing the demand for *in vitro* production of bovine embryos (Smiljakovic, 2009). *In vitro* embryo production system includes three consequent steps: *in vitro* oocyte maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC). The final target for IVEP is the production of transferrable embryos and birth of healthy offspring. To achieve these objectives, each step has to be efficient. Although preimplantation embryos may develop during IVC period, obvious differences exist between their developmental rates compared to those developed *in vivo*. For instance, approximately 60-80% of *in vivo* matured bovine oocytes are competent to reach to the metaphase II stage while only 25-40% of *in vitro*

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matured oocytes reach to this stage (Blondin *et al*., 2002; Dieleman *et al*., 2002; Avery *et al*., 2003). Failure to fertilize and develop to the blastocyst stage may show the lack of vital factors in the culture media which normally are available *in vivo*. Therefore, it is necessary to modify the culture medium and conditions to support higher percentage of embryo development *in vitro*. Several factors such as, hormones, proteins and growth factors supplemented to the culture medium may have crucial roles on the outcome of IVEP. It has been shown that insulin plays a crucial role for growth of variety of cells in IVC. Insulin bind to cell surface receptor and its action is receptor-mediated. It has been demonstrated that insulin stimulates glucose and amino acid uptake and protein synthesis (Harvey and Kaye, 1988; Kane *et al*., 1997) of mouse embryos (Velazquez *et al*., 2009; Kaye and Harveyt, 1995). Several studies have indicated that insulin increases the *in vitro* oocyte maturation and development of human (Dashtizad *et al*., 2003), mice (Demeestere *et al*., 2004) and porcine embryos (Lee *et al*., 2005). Insulin receptor has also been detected in all stages of bovine embryos (Makarevich and Markkula, 2002). However, the results of using insulin are controversial for *in vitro* bovine embryo production and need more investigation. Therefore, this experiment was designed to study the effect of different concentration of insulin on bovine IVEP.

MATERIALS AND METHODS

Chemicals: 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). The ovaries were rinsed at least 3-5 times with warm PBS to remove any possible contaminates. Cumulus-oocyte-complexes (COCs) were recovered from 2-8 mm follicles in diameter by slicing method. Slicing solution consisted of Dulbecco's phosphate buffered saline with $MgCl_2$ and $CaCl_2$ (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-warmed working solution. The working solution consisted of Hepes-buffered medium 199 (12340-030, GibcoBRL, Invitrogen, USA) supplemented by 4 mg mL^{-1} BSA-V.

***In Vitro* Maturation (IVM):** The selected COCs were washed two times in pre-warmed maturation solution. The maturation medium was Hepes-buffered medium 199 containing Earle's salt, L-glutamine, 2.2 g mL^{-1} sodium bicarbonate and 25 mM Hepes buffer supplemented with10% fetal calf serum (10082-139, GibcoBRL), 0.2 mM sodium pyruvate (P-5280), 50 µg mL^{-1} gentamycin (G-1264), 10 ng mL^{-1} epidermal growth factor (E-4127), 5 µg mL^{-1} follicle stimulating hormone (FSH; F-8174), 50 µg mL^{-1} luteinizing hormone (LH; L-5269) and 1 µg mL^{-1} oestradiol-17β (E2; E-8875). 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 maturation period, in order to assess the nuclear maturation status, oocytes were denuded 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 once via introduction 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 *et al.* (1988) 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 a 48 µL of 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 second 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 (Parrish *et al*., 1988) 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. Based on concentration, spermatozoa were added gently to each IVF droplet containing 6-8 mature oocytes. Thereafter, 2 µL of PHE mixture consisting of 20 µM D-penicillamine (P-4875) 10 µM Hypotaurine (H-1384) and 1 µM 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 and embryos were washed 2 times in fresh pre-equilibrated working solution. Then, denuded zygotes 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 10 days, at every two day interval, 200 µL of SOF solution was replenished by 200 µL 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 first experiment, supplementation of insulin in IVM medium and its effects on nuclear maturation of bovine oocytes and subsequent embryo development *in vitro* were evaluated. Hepes-buffered medium 199 supplemented with gonadotrophins, fetal bovine serum and antibiotics added with 0 (control), 1, 10, 20 and 50 µg mL^{-1} insulin were used as maturation medium.

In the second experiment, influence of insulin in serum and hormone free IVM medium on *in vitro* maturation of bovine immature oocytes and consequent embryo development was evaluated. To avoid the interaction effect of insulin with IVM ingredients, Hepes-buffered medium 199 supplemented with 1 mg mL^{-1} PVA and the same levels of insulin were used as maturation medium. In both experiments 1 and 2, after 22-24 h of maturation, representative oocytes were randomly selected to examine their nuclear maturation by using aceto-orcein staining method. The rest of *in vitro* matured bovine oocytes were subsequently fertilized and cultured in SOF solution for 9 days. The cleavage, blastocyst and hatched blastocyst rates were assessed at day 2, 7 and, 9 respectively.

In the third experiment, effect of insulin on development of bovine *in vitro* fertilized embryos was investigated. Complete IVM medium without insulin was used as maturation medium. Following IVM and IVF, presumptive denuded zygotes were randomly transferred to SOF solution supplemented with 0 (control), 1, 10, 20 and 50 µg mL^{-1} insulin for 9 days. The cleavage, morula, blastocyst and hatch blastocyst rates were assessed at day 2, 4, 7 and 9, respectively.

Statistical analysis: All experiments were repeated six 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.

RESULTS

In the first experiment, the effect of insulin on *in vitro* maturation of bovine immature oocytes and subsequent embryo development was evaluated using 1081 COCs. Data obtained in this experiment (Table 1) indicated that, after 24 h of culture, the proportion of immature bovine oocytes reaching metaphase II (MII) stage significantly increased when 1 and 10 µg mL^{-1} insulin (92.70±1.83% and 93.19±1.82%, respectively) were included in the IVM medium compared with the control group (85.80±3.40%; $p<0.05$). However, no significant difference was observed between them (1 and 10 µg mL^{-1}). At the same time, maturation rate was slightly increased at 20 µg mL^{-1} insulin (86.37±2.63%), compared with the control but not significantly ($p>0.05$).

Table 1: Effect of different concentration of insulin in IVM media on *in vitro* maturation of bovine immature oocytes and subsequent embryo development

Insulin concentration (µg mL^{-1})	No. of cultured oocytes	Matured oocytes n (mean%) No. of stained	M II oocytes	Mean % of cleaved oocytes± SEM (n)	Mean % of blastocyst ± SEM (n) [a]	Mean % of hatched blastocyst± SEM (n)
0 (Control)	165	50	43 (85.80±3.40^{b})	80.88±3.0^{b} (93/115)	38.24±3.16 (44/115)	11.34±3.94^{bc} (13/115)
1	234	87	81 (92.70±1.83^{a})	89.50±2.13^{a} (131/147)	40.42±2.08 (59/147)	16.17±3.51^{b} (24/147)
10	229	93	87 (93.19±1.82^{a})	90.69±1.59^{a} (123/136)	41.07±1.31 (56/136)	17.10±2.34^{a} (23/136)
20	228	99	85 (86.37±2.63^{b})	80.49±2.15^{b} (104/129)	41.76±2.64 (54/129)	13.60±2.23^{ab} (18/129)
50	225	84	69 (82.44±3.26^{c})	78.46±4.19^{b} (111/141)	38.82±5.52 (55/141)	9.76±2.83^{c} (15/141)

Data were pooled from 6 replicates. [a, b, c]: Values with different superscripts in the same column are significantly different ($p<0.05$)

Table 2: Effect of different concentration of insulin in serum and hormone free IVM medium on *in vitro* maturation of bovine immature oocytes and consequent embryo development

Insulin concentration (µg mL^{-1})	No. of cultured oocytes	Matured oocytes n (mean%) No. of stained	M II oocytes	Mean % of cleaved Oocytes ± SEM (n)	Mean % of blastocyst ± SEM (n)	Mean % of hatched blastocyst ± SEM (n)
0 (Control)	215	104	72 (68.94±4.81^{b})	54.18±3.01^{c} (60/111)	24.33±3.89^{b} (27/111)	6.45±2.68^{b} (7/111)
1	213	77	62 (80.51±2.02^{a})	71.92±3.82^{a} (98/136)	31.59±3.15^{a} (43/136)	10.93±3.48^{a} (15/136)
10	221	81	66 (82.15±2.35^{a})	73.30±2.14^{a} (103/140)	30.93±2.98^{a} (43/140)	10.85±2.41^{a} (103/140)
20	219	89	62 (68.55±2.50^{b})	60.14±3.53^{b} (78/130)	22.56±4.25^{b} (29/130)	7.88±2.91^{ab} (10/130)
50	211	84	59 (70.35±2.57^{b})	59.72±4.51^{b} (76/127)	20.40±3.08^{b} (26/127)	7.04±2.37^{b} (9/127)

Data were pooled from 6 replicates. [a, b, c]: Values with different superscripts in the same column are significantly different ($p<0.05$)

At the highest concentration of insulin which is 50 µg mL^{-1} (86.37±2.63%), the maturation rate was remarkably decreased. Following IVF, cleavage rate was strikingly increased at 1 and 10 µg mL^{-1} insulin (89.50±2.13% and 90.69±1.59%, respectively) compared to the control group (80.88±3.0%; $p<0.05$). However, addition of 20 µg mL^{-1} (80.49±2.15%) and 50 µg mL^{-1} (78.46±4.19%) insulin in IVM medium had no significant effect on cleavage rate compared with control group. The hatching rate was notably ($p<0.05$) improved by addition of 10 and 20 µg mL^{-1} insulin (17.10±2.34% and 13.60±2.23%, respectively) but there were no differences at 1 µg mL^{-1} (16.17±3.51%) and 50 µg mL^{-1} (9.76±2.83%) compared with control (11.34±3.94%).

In the second experiment, a total of 1079 immature bovine oocytes were used to evaluate the effect of supplementing insulin in serum and hormone free IVM medium on *in vitro* maturation of bovine immature oocytes and consequent embryo development. Data are summarized in Table 2. The percentage of immature bovine oocytes that reached metaphase II was noticeably higher in 1 and 10 µg mL^{-1} (80.51±2.02% and 82.15±2.35%, respectively) compared with other groups, but there was no difference between them. All of the treatment groups had higher cleavage rate ($p<0.05$) with the highest in 1 µg mL^{-1} (71.92±3.82%) and 10 µg mL^{-1} (73.30±2.14%) compared with 20 and 50 µg mL^{-1} (60.14±3.53% and 70.35±2.57%, respectively). Addition of 1 and 10 µg mL^{-1} insulin in serum and hormone free IVM medium significantly increased the proportion of inseminated oocytes developed to the blastocyst stage than the control group. To evaluate the further viability of the blastocysts produced after addition of different insulin levels, hatchability of embryos were assessed. The hatching rates of blastocysts, remarkably improved in 1 and 10 µg mL^{-1} insulin (10.93±3.48% and 10.85±2.41%, respectively) compared with the control (6.45±2.68%).

Third experiment was carried out to investigate the effect of insulin supplemented *in vitro* culture media on development of bovine *in vitro* fertilized embryos. A total of 1055 immature bovine oocytes were used in this experiment and the result is presented in Table 3. As shown, insulin at 1 and 10 µg mL^{-1} in the IVC medium significantly increased cleavage (86.38±2.98% and 90.58±2.02%, respectively) and morula rate (74.60±3.25% and 79.66±3.86%, respectively) of *in vitro* fertilized oocytes when added in the IVC medium compared to the control (78.86±2.79% and 60.55±2.56%, respectively; $p<0.05$).

Table 3: Effect of insulin supplementation at five concentrations (0, 1, 10, 20 and 50 µg mL^{-1}) in IVC media on *in vitro* bovine embryo development *in vitro*

Insulin concentration (µg mL^{-1})	No. of cultured oocytes	Mean % of cleaved oocytes ± SEM (n)	Mean % of morula oocytes ± SEM (n)	Mean % of blastocyst ± SEM (n)	Mean % of hatched blastocyst ± SEM (n)
0 (Control)	213	78.86 ± 2.79^{cd} (168/213)	60.55 ± 2.56^{cd} (129/213)	38.99 ± 2.77^{a} (83/213)	12.23 ± 3.02^{ab} (26/213)
1	212	86.38 ± 2.98^{b} (183/212)	74.60 ± 3.25^{b} (158/212)	37.70 ± 2.07^{a} (80/212)	10.32 ± 3.06^{ab} (22/212)
10	201	90.58 ± 2.02^{a} (182/201)	79.66 ± 3.86^{a} (160/201)	35.89 ± 3.44^{ab} (72/201)	14.49 ± 3.72^{a} (29/201)
20	206	82.04± 3.70^{c} (169/206)	63.13 ± 3.25^{c} (130/206)	30.63 ± 3.87^{c} (63/206)	8.77 ± 2.75^{b} (18/206)
50	223	75.38± 3.12^{d} (168/223)	58.31 ± 3.50^{d} (130/223)	32.83 ± 3.22^{bc} (73/223)	10.44 ± 3.61^{ab} (22/223)

Data were pooled from 6 replicates. $^{a, b, c, d}$: Values with different superscripts in the same column are significantly different ($p<0.05$; ANOVA and Duncan's test)

However, no significant difference was observed in 20 and 50 µg mL^{-1} groups compared to the control in term of cleavage (82.04±3.70% and 75.38± 3.12%, respectively) and morula (63.13±3.25% and 58.31±3.50%, respectively) rates. Addition of 1 and 10 µg mL^{-1} insulin (37.70±2.77% and 35.89±3.44%, respectively) in IVC medium had no significant effect on blastocyst rate compared with the control group (38.99±2.77). However, higher concentration of insulin, 20 µg mL^{-1} (30.63±3.87%) and 50 µg mL^{-1} (32.83±3.22%), remarkably decreased blastocyst rate. No significant differences were observed in the hatched blastocyst rate by addition of insulin in IVC medium. Only 10 µg mL^{-1} insulin slightly increased hatched blastocyst rate (14.49±3.72%) compared to the control (12.23±3.02%).

DISCUSSION

In the present study, the potential role of insulin on IVM of immature bovine oocytes and *in vitro* embryo development were investigated. The results demonstrated that presence of 1 or 10 µg mL^{-1} insulin in the maturation media showed a positive effect on maturation and cleavage rates of bovine immature oocytes *in vitro*. In subsequent bovine embryo development *in vitro*, insulin at 10 µg mL^{-1} enhanced viability of blastocyst embryos and improved rate of hatchability. However, findings of previous studies regarding using insulin are controversial for *in vitro* bovine oocyte development. Our results are in agreement with previous reports in which addition of insulin to the IVM medium showed a positive effect on *in vitro* development of bovine oocytes, when oocytes were cultured in TCM-199 medium supplemented with FCS (Dieleman *et al*., 2002). On the other hand, Zhang *et al*. (1991) reported that although inclusion of insulin in maturation medium enhanced cumulus expansion scores and the level of maturation, the yield of *in vitro* embryo production were not affected. Furthermore, Matsui (1995) showed that addition of insulin at 10 µg mL^{-1} to the oocyte maturation medium had no effect on the nuclear maturity, fertilization and development of bovine embryos toward the blastocyst stage. It has been shown that insulin (0.1-10 µg mL^{-1}) enhanced the mitosis of bovine granulosa cells and accelerated progression of meiosis in oocytes enclosed with cumulus cells. Both granulosa cells and oocyte normally express insulin receptor. Percentage of apoptotic cells in the group of oocytes evaluated immediately after recovery was very low but it noticeably increased during *in vitro* maturation without any additional supplementation. This incidence of apoptotic DNA degeneration was reduced by addition of insulin, insulin like growth factor I (IGF I) and other growth factors to the maturation medium (Wasielak and Bogacki, 2007). Addition of insulin to IVM and subsequent embryo development media reduced not only the incidence of spontaneous apoptosis in bovine embryos (Augustin *et al*., 2003) but also blocked apoptosis induced by exogenous factors, such as heat shock (Jousan and Hansen, 2004).

The obtained data from this study probably shows an interaction between insulin and FSH/or E_2 on the bovine COCs. Suzuki *et al*., (2006) reported that stimulating effect of insulin on the proliferation of bovine granulosa cells is synergistic with gonadotrophins. Therefore, the existence of FSH and E_2 in the IVM medium might interfere the real effects of insulin on the maturation of bovine cumulus-intact oocytes. For this reason we have designed the second experiment to find out the pure effect of insulin on oocyte maturation. Therefore, a serum and hormones free IVM medium was used (Table 2) in which PVA was added instead of serum. The results of the current study undoubtedly confirmed that insulin by itself had a positive effect on *in vitro* bovine embryo production system.

Our finding propose that supplementation of maturation medium with insulin (1-50 µg mL^{-1}) improves the fertilization rate. Cumulus cells surrounding bovine oocytes synthesize glycosaminoglycans (GAGs) such as hyaluronic acid. GAGs are able to induce the acrosome reaction of bovine sperm (Handrow *et al*., 1982). It has been reported that granulosa cells are able to secrete heparin-like GAGs which are recognized as *in vitro* sperm capacitating agent (Bellin *et al*., 1986). It is presumed that the addition of insulin to the maturation medium stimulates GAGs secretion from cumulus cells and improves the fertilization rate as a result of the promotion of cumulus-induced sperm capacitation (Matsui, 1995).

It has been shown that insulin has broad effects on preimplantation embryos (Kim *et al*., 2005). The third experiment was carried out to determine the effect of different concentrations of insulin in IVC media on *in vitro* bovine embryo development. The results clearly demonstrated that supplementation of insulin (1 and 10 µg mL^{-1}) to the culture medium improved cleavage rate and development of morula but did not show positive effect on blastocyst production rate (Table 3). Addition of higher concentration of insulin (20 and 50 µg mL^{-1}) did not show positive effect on *in vitro* embryo development. This could be due to differences in glucose utilization of the early embryo (morula), compared to the late embryos (blastocyst) (Harvey and Kaye, 1990). The post-compacted embryos are more sensitive to insulin (Zhandi *et al*., 2009). Scientists demonstrated a stage-specific binding by insulin which could be related to embryonic metabolism and switch from lactate to glucose uptake. They also showed that insulin stimulates glucose uptake and amino acid transport by embryonic cells which can improve embryo development (Tareq *et al*., 2007). Furthermore, the proportion of embryos reaching to the 8-cell stage was considerably enhanced by addition of 10 µg mL^{-1} insulin to the culture medium. However, development to the morula and blastocyst stages was not significantly increased. Insulin stimulates embryo development via insulin receptors which are expressed at the 8-cell stage. Our findings are in agreement with previous results of Harvey and Kaye (1990). In contrast, Quetglas and co-researchers (2001) added 5 µg mL^{-1} insulin to the culture medium and the percentage of embryos reaching to the morula stage was not affected by addition of insulin alone, but was significantly increased when amino acids were included with insulin. Therefore, it seems that insulin has not a strong stimulatory effect on the growth of late stage embryos (Navarrete *et al*., 2004). Based on our results, it seems that addition of insulin (10 µg mL^{-1}) to the culture medium would slightly improve blastocyst hatchability in the bovine IVC system. The proportion of hatched blastocysts can be considered as an indicator to evaluate quality and viability of the transferable bovine embryos. Insulin increases the cell number of the inner cell mass and stimulates morphological development of mouse blastocysts *in vitro* (Liu *et al*., 2009).

CONCLUSION

Obtained results demonstrated that insulin promotes *in vitro* bovine embryos production. Supplementation of 10 µg mL^{-1} insulin in maturation medium, exerted beneficial effects on nuclear maturation, cleavage and subsequent bovine embryo development *in vitro*. Furthermore, addition of insulin (10 µg mL^{-1}) to the culture medium showed positive effect on bovine embryo development till morula stage and slightly enhanced hatchability of produced blastocysts.

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