Jpn. J. Vet. Res. **43**(3–4) : 145–153 (1995)

EFFECTS OF SUPPLEMENTATION OF THE MATURATION MEDIA WITH INSULIN ON *IN VITRO* MATURATION AND *IN VITRO* FERTILIZATION OF BOVINE OOCYTES

Motozumi MATSUI, Yoshiyuki TAKAHASHI,
Mitsugu HISHINUMA and Hiroshi KANAGAWA

(Accepted for publication : Dec. 13, 1995)

ABSTRACT

This study was carried out to determine the effects of supplementation of the maturation media with insulin on *in vitro* maturation and fertilization of bovine oocytes. In Experiment 1, cumulus-intact bovine oocytes were cultured in a maturation medium (TCM-199 containing 10% fetal calf serum, 0.02 U/ml follicular stimulating hormone and 1 μg/ml estradiol-17β) with or without insulin supplementation (10 μg/ml). The maturation and fertilization rates of oocytes and subsequent embryonic development to the blastocyst stage were not affected by the treatment with insulin in the presence of serum and the hormones during the maturation period. In Experiment 2, to avoid the effects of serum and the hormones, a serum- and hormone-free maturation medium (TCM-199 containing 1 mg/ml polyvinyl alcohol) was used. In the absence of serum and hormones during the maturation period, the maturation rate was not affected by treatment with insulin, but the fertilization rate was improved. In Experiment 3, when denuded oocytes were inseminated together with cumulus cells cultured in serum- and hormone-free maturation medium supplemented with insulin, the fertilization rate was increased. These results demonstrate that the addition of insulin to the serum- and hormone-free maturation medium improves the fertilization rate of bovine oocytes *in vitro*, and suggest that insulin may stimulate the secretion of sperm capacitating agent (s) from cumulus cells.

Key Words : insulin, bovine oocyte, *in vitro* maturation, *in vitro* fertilization

INTRODUCTION

It is known that follicular development is controlled by various factors (gonadot-

Laboratory of Theriogenology, Department of Veterinary Clinical Science, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060, Japan

rophins, steroids, growth factors) of endocrine and paracrine origins[6,12,24]. Recent studies have demonstrated that growth factors play important roles in the regulation of follicular cell proliferation and differentiation with paracrine and autocrine mechanisms[1,5,16]. In cattle, a positive correlation between insulin-like growth factor-I (IGF-I) concentrations in follicular fluid and follicular size was observed in previous reports[19]. Several studies have indicated that insulin and IGF-I stimulate the proliferation of granulosa cells and the production of progesterone[7,20]. It has been suggested that insulin and IGF-I may have local effects on bovine ovarian follicular growth *in vivo*[8,16,20]. Therefore, it is expected that insulin and IGF-I have some beneficial effects on *in vitro* maturation of bovine oocytes. However, there have been only a few studies on the roles of insulin and IGF-I in bovine oocyte maturation *in vitro*. Some studies showed that supplementation of the maturation medium with insulin improved cumulus expansion and oocyte fertilizability *in vitro*[14,25], but other reports showed that insulin had no significant effect on the fertilization rate or morula formation[21]. The addition of IGF-I to the maturation medium has been reported to stimulate the nuclear maturation of bovine oocytes[14] and to improve the quality of embryos[10,11]. The role of insulin on *in vitro* maturation of bovine oocytes has not been fully understood.

The aim of the present study was to obtain information about the effects of supplementation of the maturation media with insulin on *in vitro* maturation and fertilization of bovine oocytes.

MATERIALS AND METHODS

In vitro maturation

Cumulus-oocyte complexes (COCs) were recovered by aspiration of small antral follicles (2–7 mm in diameter) of bovine ovaries collected from a slaughterhouse. Cumulus-intact oocytes with evenly granulated cytoplasm were cultured in 50 μl drops of the maturation medium (10–15 COCs per drop) for 22 hr at 39°C under 5% CO_2 in humidified air. The basic medium for maturation was 25 mM HEPES-buffered TCM-199 (Gibco Laboratories, Grand Island, NY, USA) supplemented with 0.2 mM sodium pyruvate and 50 μg/ml gentamicin (Sigma Chemical Co., St. Louis, MO, USA).

After *in vitro* maturation, cumulus cells were mechanically stripped through repeated pipetting. The naked oocytes were mounted and fixed in ethanol : acetic acid (3:1) for 24 hr. They were then stained with 1% aceto-orcein and examined for nuclear maturation under a phase-contrast microscope. Maturation was defined as oocytes reaching metaphase II.

In vitro fertilization

Sperm preparation and *in vitro* fertilization were performed as described previously[15]. The motile sperm were separated using 45 and 90% Percoll solution (Percoll gradient layer). Frozen-thawed semen was put on top of the Percoll gradient

layer and centrifuged at 700×g for 20 min. The top layers were removed and the sperm pellet was washed using modified Brackett and Oliphant isotonic medium[4] (BO) omitting bovine serum albumin (BSA), by centrifugation at 500×g for 5 min. Ten to fifteen COCs were coincubated with spermatozoa (5×10^6 cells/ml) in a 100 μl drop of BO medium supplemented with 3 mg/ml fatty acid-free BSA (Sigma) and 2.5 mM theophylline (Sigma) for 20 hr at 39°C under 5% CO_2 in humidified air as described by Takahashi and First[23].

After insemination, the cumulus cells were removed from oocytes by Vortex agitation. Cumulus-free oocytes were mounted and fixed in ethanol : acetic acid (3:1) for 24 hr. They were then stained with 1% aceto-orcein to examine sperm penetration and pronucleus formation. Oocytes were judged as penetrated when they had an enlarged sperm head (s) or male pronucleous (ei) with corresponding sperm tail (s) in the cytoplasm. Normal fertilization rate was determined as a ratio of oocytes with a pair of pronuclei and sperm tail.

In vitro culture

After insemination, cumulus-free oocytes were cultured at 39°C under 5% CO_2, 5% O_2 and 90% N_2. Modified synthetic oviduct fluid (mSOF)[22] was used for the culture medium. Glucose and BSA were deleted from SOF, but 1 mg/ml polyvinyl alcohol (PVA, Sigma), 10 μg/ml insulin (Sigma) and 20 amino acids were added. A premixed solution of basal medium Eagles (BME) essential amino acids (ICN Biomedicals Inc., Costa Mesa, CA, USA), minimum essential medium (MEM) nonessential amino acids (ICN) and 1 mM glutamine (Sigma) were added as amino acids.

Embryos were fixed at Day 7 (170–172 hr after insemination; Day 0=day of insemination), and the total cell number, including metaphase plates but excluding pyknotic nuclei, was counted using the air-drying method previously described by Takahashi and First[22].

Experiment 1

The influence of insulin added to the maturation medium on the nuclear maturation and fertilization rates was examined. The effect of supplementation of the maturation medium with insulin on the embryonic development to the blastocyst stage was also examined. TCM-199 containing 10% heat-inactivated fetal calf serum (FCS; Gibco), 0.02 U/ml follicular stimulating hormone (FSH, Sigma) and 1 μg/ml estradiol-17β (E_2, Sigma) was used as maturation medium. COCs were matured in the maturation medium with or without 10 μg/ml insulin supplementation.

Experiment 2

The effects of the presence of insulin during *in vitro* maturation on the nuclear maturation rate and fertilization rate were examined by using serum- and hormone-free maturation medium. To avoid the effects of serum and hormones, TCM-199 supplemented with 1 mg/ml PVA (TCM-199+PVA) was used as a maturation medium. COCs were matured in the maturation medium with or without 10 μg/ml insulin.

Experiment 3

After maturation using serum- and hormone-free TCM-199+PVA with or without 10 μg/ml insulin, oocytes were isolated from COC. Denuded oocytes and isolated cumulus cells were divided into the following groups: both denuded oocytes and cumulus cells cultured with 10 μg/ml insulin, either denuded oocytes or cumulus cells cultured with insulin, neither denuded oocytes nor cumulus cells cultured with insulin. Denuded oocytes were inseminated with cumulus cells *in vitro* and their fertilization rate was examined.

Statistical analysis

In Experiments 1 and 2, the statistical significance of the results was evaluated by Student's *t*-test. In Experiment 3, data were analyzed by 2-way analysis of variance (ANOVA) followed by Duncan's multiple range-test.

RESULTS

Experiment 1

Supplementation of the maturation medium with insulin had no effect on the maturation rate (Table 1). The normal fertilization, polyspermy and total penetration rates of oocytes cultured with insulin were not different from those without insulin (Table 2). The initial cleavage rate of oocytes and development of cleaved embryos

Table 1. Effects of supplementation of the maturation medium with insulin on the maturation rate of bovine oocytes *in vitro* [a)]

Insulin[b)]	No. of oocytes examined	% of matured oocytes
−	32	90.6±5.3
+	30	90.0±5.3

[a)] Data were mean±SEM of 3 replicates.

[b)] Maturation medium contained FCS, FSH and E_2.
Bovine oocytes were cultured in maturation medium with (+) or without (−) insulin (10 μg/ml).

Table 2. Effects of supplementation of the maturation medium with insulin on the fertilization rate of bovine oocytes *in vitro* [a)]

Insulin[b)]	No. oocytes examined	% of normal fertilization	% of polyspermy	% of total penetration
−	40	85.4±3.4	4.8±2.4	95.2±2.4
+	43	83.4±3.3	4.8±2.4	90.7±2.1

[a)] Data were mean±SEM of 3 replicates.

[b)] Maturation medium contained FCS, FSH and E_2.
Bovine oocytes were cultured in maturation medium with (+) or without (−) insulin (10 μg/ml).

to the blastocyst stage were not affected by the addition of insulin during the maturation period (Table 3).

Experiment 2

The maturation rate was not affected by the addition of insulin to serum- and hormone-free maturation medium (Table 4). The normal fertilization and total penetration rates of oocytes cultured with insulin were significantly higher than those without insulin ($P<0.01$, Table 5). Supplementation with insulin had no effect on the proportion of polyspermy.

Experiment 3

When denuded oocytes were co-incubated with sperm and cumulus cells cultured in the maturation medium supplemented with insulin, normal fertilization and total penetration rates were significantly increased ($P<0.01$, Table 6). The treatment of oocytes with insulin during maturation did not improve normal fertilization and total penetration rates. The percentage of polyspermic oocytes was not affected by the treatment of oocytes and cumulus cells with insulin.

Table 3. Effects of supplementation of the maturation medium with insulin on the subsequent development to the blastocyst stage[a)]

Insulin[b)]	No. oocytes examined	% ova cleaved at Day 2	% ova developed to blastocysts at Day 7[c)]	No. of cells in blastocysts
−	88	80.3±2.4	39.2±1.2	169.0±2.3
+	89	87.0±2.4	35.6±3.3	169.7±5.0

[a)] Data were mean±SEM of 3 replicates.
Day 0=day of *in vitro* insemination.

[b)] Maturation medium contained FCS, FSH and E_2.
Bovine oocytes were cultured in maturation medium with (+) or without (−) insulin (10 μg/ml).

[c)] % based on number of cleaved embryos.

Table 4. Effects of supplementation of the serum- and horomone-free maturation medium with insulin on the maturation rate of bovine oocytes *in vitro* [a)]

Insulin[b)]	No. of oocytes examined	% of matured oocytes
−	50	87.8±4.0
+	49	86.5±3.5

[a)] Data were mean±SEM of 3 replicates.

[b)] Maturation medium contained PVA.
Bovine oocytes were cultured in maturation medium with (+) or without (−) insulin (10 μg/ml).

Table 5. Effects of supplementation of the serum- and hormones- free maturation medium with insulin on the fertilization rate of bovine oocytes *in vitro* [a)]

Insulin[b)]	No. oocytes examined	% of normal fertilization	% of polyspermy	% of total penetration
−	49	49.4±3.4[c)]	3.3±3.3	52.8±1.5[c)]
+	51	79.5±3.8[d)]	1.5±1.5	81.0±2.5[d)]

[a)] Data were mean±SEM of 3 replicates.

[b)] Maturation medium contained PVA.

Bovine oocytes were cultured in maturation medium with (+) or without (−) insulin (10 μg/ml).

[c,d)] Values with different superscripts are different ($P<0.01$).

Table 6. Effects of treatment of oocytes and cumulus cells with insulin during *in vitro* maturation on the subsequent fertilization rate[a)]

Treatment[b)]		No. oocytes examined	% of normal fertilization	% of polyspermy	% of total penetration
Oocyte	Cumulus cell				
−	−	54	31.6±0.6[c)]	1.6±1.6	36.8±3.5[c)]
−	+	57	47.6±8.0[d)]	1.6±1.6	54.6±6.3[d)]
+	−	58	26.0±3.2[c)]	0	32.9±1.3[c)]
+	+	60	58.1±3.9[d)]	3.6±2.0	66.6±5.3[d)]

[a)] Data were mean±SEM of 4 replicates.

[b)] Maturation medium contained PVA.

Cumulus cells and oocytes culutred in maturation medium with (+) or without (−) insulin (10 μg/ml) were divided into 4 groups and inseminated *in vitro*.

[c,d)] Values with different superscipts are different ($P<0.01$).

Discussion

Insulin had no effects on the nuclear maturation rate, fertilization rate and developmental rate to the blastocyst stage when oocytes were cultured in TCM-199 supplemented with FCS, FSH and E_2 (Experiment 1). Our results are in agreement with previous reports. Stubbings et al.[21)] reported that insulin (1–1000 ng/ml) has no effect on *in vitro* maturation when bovine oocytes are cultured in TCM-199 containing FCS, gonadotrophins and E_2. However, the addition of insulin (1 μg/ml) to the maturation medium which did not contain gonadotrophins or estradiol improved *in vitro* fertilization rate of bovine cumulus-intact oocytes[25)]. It has been demonstrated that follicle cells, especially the cumulus cells, have beneficial effects on *in vitro* maturation of bovine oocytes[17,18)]. Insulin (0.1–10 μg/ml) enhances the mitosis of bovine granulosa cells[20)]. This stimulating effect of insulin on the proliferation of bovine granulosa

cells is synergistic with gonadotrophins[7]. Furthermore, insulin increases progesterone and estradiol production by bovine granulosa cells in the presence of FSH[20]. These findings indicate that there may be an interaction between the effects of insulin and the effects of FSH and/or E_2 on the cumulus-intact bovine oocytes. Therefore, the presence of FSH and E_2 in the maturation medium might have made it difficult to define the effects of insulin on the maturation of bovine cumulus-intact oocytes.

In Experiments 2 and 3, FSH, E_2 and serum which may contain insulin were deleted from the maturation medium, but PVA was added. In Experiment 2, nuclear maturation was not affected by the addition of insulin to FSH- and E_2-free TCM-199 supplemented with PVA. However, insulin increased the *in vitro* fertilization rate. In Experiment 3, the *in vitro* fertilization rate of bovine oocytes was enhanced in the presence of cumulus cells treated with insulin during the maturation period regardless of whether oocytes were treated with insulin or not. These results suggest that the supplementation of the maturation medium with insulin improves the fertilization rate by the effect of insulin on the cumulus cells. Cumulus cells surrounding bovine oocytes synthesize glycosaminoglycans (GAGs) such as hyaluronic acid[3]. Hyaluronic acid has been reported to induce the acrosome reaction of bovine sperm[9]. Granulosa cells are able to secrete heparin-like GAGs which are well known as sperm capacitating agents *in vitro*[2]. In the present study, GAGs such as heparin for sperm capacitation was not used throughout the sperm preparation and insemination. However, theophylline, a kind of methylxanthine, was added to the insemination medium. Theophylline stimulates sperm motility and brings the sperm into contact with cumulus cells, thereby facilitating cumulus-induced sperm capacitation[23]. 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.

In conclusion, the present study demonstrates that the supplementation of the maturation medium with insulin improves the *in vitro* fertilization rate of bovine oocytes when cumulus-intact oocytes are cultured in a defined maturation medium without serum, FSH and E_2. Furthermore, it is presumed that the beneficial effect of insulin on *in vitro* fertilization may be mediated by cumulus cells.

ACKNOWLEDGEMENTS

A part of this study was supported by a grant from the Morinaga Hoshi-Kai to Dr. Y. Takahashi. We thank the Hokkaido Livestock Improvement Association for the supply of frozen semen and the Ebetsu Meat Inspection Office for the supply of ovaries.

REFERENCES

1) ADASHI, E. Y., RESNICK, C. E., D'ERCOLE, A. J., SVOBODA, M. E. & VAN WYK, J. J. :

Insulin-like growth factors as intraovarian regulators of granulosa cell growth and function. *Endocrine Rev.* **6** : 400–420, 1985.

2) AX, R. L. & BELLIN M. E. : Glycosaminoglycans and follicular development. *J. Anim. Sci.* **66** : *Suppl.* 2, 32–49, 1988.
3) BALL, G. D., BELLIN, M. E., AX, R. L. & FIRST, N. L. : Glycosaminoglycans in bovine cumulus-oocytes complexes : Morphology and chemistry. *Mol. Cell Endocrinol.* **28** : 113–122, 1982.
4) BRACKETT, B. G. & OLIPHANT, G. : Capacitation of rabbit spermatozoa in vitro. *Biol. Reprod.* **12** : 260–274, 1975.
5) CARSON, R. S., ZHANG, Z., HUTCHINSON, L. A., HERINGTON, A. C. & FINDLAY, J. K. : Growth factors in ovarian function. *J. Reprod. Fertil.* **85** : 735–746, 1989.
6) DRIANCOURT, M. A. : Follicular dynamics in sheep and cattle. *Theriogenology* **35** : 55–79, 1991.
7) GONG, J. G., MCBRIDE, D., BRAMLEY, T. A. & WEBB, R. : Effects of recombinant bovine somatotrophin, insulin-like growth factor-I and insulin on the proliferation of bovine granulosa cells *in vitro*. *J. Endocrinol.* **139** : 67–75, 1993.
8) HAMMOND, J. M., MONDSCHEIN, J. S., SAMARAS, S. E., SMITH, S. A. & HAGEN, D. R. : The ovarian insulin-like growth factor system. *J. Reprod. Fertil. Suppl.* **43** : 199–208, 1991.
9) HANDROW, R. R., LENZ, R. W. & AX, R. L. : Structural comparisons among glycosaminoglycans to promote an acrosome reaction in bovine spermatozoa. *Biochem. Biophys. Res. Commun.* **107** : 1326–1332, 1982.
10) HARPER, K. M. & BRACKETT, B. G. : Enhanced bovine oocyte quality after in vitro maturation (IVM) with insulin-like growth factor-I (IGF-I) and gonadotropins. *Biol. Reprod.* **46** : *Suppl.* 1, 67 (abstr.), 1992.
11) HERRLER, A., LUCAS-HAHN, A. & NIEMANN, H. : Effects of insulin-like growth factor-I on in-vitro production of bovine embryos. *Theriogenology* **37** : 1213–1224, 1992.
12) IRELAND, J. J. : Control of follicular growth and development. *J. Reprod. Fertil. Suppl.* **34** : 39–54, 1987.
13) KOBAYASHI, K., YAMASHITA, S. & HOSHI, H. : Influence of epidermal growth factor and transforming growth factor-α on in vitro maturation of cumulus cell-enclosed bovine oocytes in defined medium. *J. Reprod. Fertil.* **100** : 439–446, 1994.
14) LORENZO, P. L., ILLERA, M. J., ILLERA, J. C. & ILLERA, M. : Enhancement of cumulus expansion and nuclear maturation during bovine oocyte maturation in vitro by the addition of epidermal growth factor and insulin-like growth factor-I. *J. Reprod. Fertil.* **101** : 697–701, 1994.
15) MATSUI, M., TAKAHASHI, Y., HISHINUMA, M. & KANAGAWA, H. : Insulin and insulin-like growth factor-I (IGF-I) stimulate the development of bovine embryos fertilized *in vitro*. *J. Vet. Med. Sci.* **57** : 1109–1111, 1995.
16) MONGET, P. & MONNIAUX, D. : Growth factors and the control of folliculogenesis. *J. Reprod. Fertil. Suppl.* **49** : 321–333, 1995.
17) SHIOYA, Y., KUWAYAMA, M., FUKUSHIMA, M., IWASAKI, S. & HANADA, A. : In vitro fertilization and cleavage capacity of bovine follicular oocytes classified by cumulus

cells and matured in vitro. *Theriogenology* **30**: 489–496, 1988.

18) SIRARD, M. A., PARRISH, J. J., WARE, C. B., LEIBFRIED-RUTLEDGE, M. L. & FIRST, N. L.: The culture of bovine oocytes to obtain developmentally competent embryos. *Biol. Reprod.* **39**: 546–552, 1988.

19) SPICER, L. J. & ENRIGHT, W. J.: Concentrations of insulin-like growth factor-I and steroids in follicular fluid of preovulatory bovine ovarian follicles: effect of daily injection of a growth hormone-releasing factor analog and (or) thyrotropin-releasing hormone. *J. Anim. Sci.* **69**: 1133–1139, 1991.

20) SPICER, L. J., ALPIZAR, E. & ECHTERNKAMP, S. E.: Effects of insulin, insulin-like growth factor I and gonadotropins on bovine granulosa cell proliferation, progesterone production in vitro. *J. Anim. Sci.* **71**: 1232–1241, 1993.

21) STUBBINGS, R. B., LIPTRAP, R. M., BETTERIDGE, K. J., WALTON, J. S., ARMSTRONG, D. T. & BASRUR, P. K.: Requirements for bovine oocyte maturation in vitro. *Reprod. Dom. Anim.* **25**: 158–166, 1990.

22) TAKAHASHI, Y. & FIRST, N. L: *In vitro* development of bovine one-cell embryos: influence of glucose, lactate, pyruvate, amino acids and vitamins. *Theriogenology* **37**: 963–978, 1992.

23) TAKAHASHI, Y. & FIRST, N. L.: *In vitro* fertilization of bovine oocytes in the presence of theophilline. *Anim. Reprod. Sci.* **34**: 1–18, 1993.

24) WEBB, R., GONG, J. G. & BRAMLEY, T. A.: Role of growth hormone and intrafollicular peptides in follicle development in cattle. *Theriogenology* **41**: 25–30, 1994.

25) ZHANG, L., BLAKEWOOD, E. G., DENNISTON, R. S. & GODKE, R. A.: The effect of insulin on maturation and development of in vitro-fertilized bovine oocytes. *Theriogenology* **35**: 301 (abstr.), 1991.