Possible Involvement of Insulin-like Growth Factor-I in Mediating the Stimulatory Effect of Recombinant Bovine Growth Hormone on Maturation of Bovine Oocytes *In Vitro*

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Abstract. The present study was undertaken to determine whether insulin-like growth factor-I (IGF-I) is involved in mediating the stimulatory effects of growth hormone (GH) on nuclear maturation of bovine oocytes *in vitro* and subsequent embryonic development after *in vitro* fertilization. When cumulus-oocyte complexes (COCs) or cumulus-free oocytes were cultured in modified Medium 199 supplemented with 0–100 ng/ml recombinant human IGF-I (rhIGF-I), the proportion of COCs with slight cumulus expansion was increased with increasing concentration of rhIGF-I after 12 and 24 h of culture. Nuclear maturation of oocytes to the metaphase II (M-II) stage was promoted by rhIGF-I only in COCs, with higher proportions of M-II oocytes at 10–100 ng/ml ($74 \pm 3\%$) than at 0–1 ng/ml (53 ± 4 – $61 \pm 1\%$) after 24 h of culture. When COCs were cultured in the presence of 100 ng/ml rhIGF-I or 10 ng/ml recombinant bovine GH (rbGH) and/or anti-IGF-I antibody (Ab) for 24 h, the proportion of oocytes reaching the M-II stage was higher with rhIGF-I ($73 \pm 2\%$) or rbGH ($74 \pm 1\%$) than in the absence of these additives ($54 \pm 3\%$), in Ab alone ($54 \pm 3\%$), or with IGF-I or GH in combination with Ab (54 ± 3 – $58 \pm 1\%$). The inhibition of the stimulatory effects of rbGH on nuclear maturation by Ab was dose-dependent. After *in vitro* maturation and fertilization, no differences were observed in the penetration rate (75 ± 3 – $77 \pm 6\%$), pronuclear formation (56 ± 3 – $64 \pm 10\%$) and polyspermy (28 ± 4 – $36 \pm 5\%$) at 8 h post-insemination among cumulus enclosed oocytes cultured in the presence of different additives. When the oocytes were cultured in a chemically defined medium starting from 8 h after insemination, 20 ± 1 – $26 \pm 2\%$ of oocytes developed to the $\geq$ morula stage 144 h post-insemination without difference among different additives during maturation. However, at 192 h post-insemination, higher proportions of oocytes developed to the blastocyst stage when COCs were matured in the presence of rhIGF-I ($16 \pm 1\%$) or rbGH ($15 \pm 2\%$) than in the absence of the additives ($4 \pm 2\%$) or when IGF-I or GH was combined with Ab (4 ± 2 – $5 \pm 3\%$). These results suggest that the stimulatory effects of GH on bovine oocyte maturation may not be direct, but mediated *via* IGF-I probably produced by the cumulus cells.

Key words: Insulin-like growth factor-I, Growth hormone, Bovine, Oocytes, *In vitro* maturation.

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Insulin-like growth factor-I (IGF-I) is a 70-amino acid polypeptide that affects mitogenesis as well

as differentiation of various cell types. In mammals, it has been demonstrated that IGF-I is a potential regulator of ovarian function [1, 2] and also have a beneficial effects on oocyte maturation and embryonic development in many species [3–

8]. A high level of IGF-I is found in rat cumulus cells [9] and IGF-I receptor is also localized in theca interstitial compartment and cumulus cells in human [10] and granulosa cells in rats [11] and pigs [12].

The regulation of follicular development by growth hormone (GH) has been reported in cattle [13, 14] and rabbits [15]. Growth hormone also has beneficial effects on oocyte maturation and subsequent embryonic development in rats [16], rabbits [17] and cattle [18]. The effects of GH on oocyte maturation may be expressed directly through its own receptor or indirectly by mediation of IGF-I. The GH receptor gene expression was found in cultured and aspirated human granulosa cells [19] and GH receptor was found granulosa cells in rats [20, 21] and in large luteal cells, cumulus cells, follicles and oocytes in cattle [22, 23]. Moreover, GH has been shown to induce the production of IGF-I in rat [24] and pig [25] granulosa cells. It was demonstrated that the effects of GH on maturation of rat [16] and rabbit [17] oocytes may be mediated directly or indirectly by IGF-I. However, Izadyar *et al.* [23] have recently reported that the positive effects of pituitary GH on *in vitro* maturation of bovine oocytes are not mediated by IGF-I.

In our previous study, it was demonstrated that recombinant bovine GH (rbGH) stimulates bovine oocyte nuclear maturation acting *via* cumulus cells *in vitro*, and that it promotes subsequent embryonic development to the blastocyst stage [26]. The present study was conducted to obtain further information on the effects of IGF-I on the maturation of bovine oocytes *in vitro* using recombinant human IGF-I (rhIGF-I) and to clarify whether IGF-I is involved in mediating the stimulatory effects of rbGH on the oocyte maturation.

Materials and Methods

Media

Modified Medium 199 (mM-199; Gibco, Grand Island, NY, USA) supplemented with 25 mM Hepes, 0.4 mM sodium pyruvate, 5 mM hemicalcium lactate and 0.1% (w/v) polyvinylalcohol (PVA; Sigma Chemical Co., St Louis, MO, USA) was used as the basic medium for maturation of oocytes. For the treatment of spermatozoa and fertilization of oocytes, modified Brackett and Oliphant's [27] medium (mBO medium) composed of 112 mM NaCl,

4.02 mM KCl, 2.25 mM CaCl_2 , 0.83 mM NaH_2PO_4 , 0.52 mM MgCl_2 , 37 mM NaHCO_3 and 1.25 mM sodium pyruvate was used as the basic medium. For embryo development, a chemically defined medium composed of 89 mM NaCl, 3.2 mM KCl, 2 mM CaCl_2 , 0.5 mM MgCl_2 , 25 mM NaHCO_3 , 0.35 mM NaH_2PO_4 , 10 mM sodium lactate, 0.5 mM sodium pyruvate, 1% (v/v) minimum essential medium (MEM) non-essential amino acids solution, 2% (v/v) MEM essential amino acids solution and 1 mg/ml PVA was used as the basic medium. This medium, designated mBECM, was essentially the same as BECM described by Lim *et al.* [28], except that 1% (v/v) basal medium Eagle's amino acids solution was replaced with 2% (v/v) MEM essential amino acids solution.

Collection and maturation of oocytes

Ovaries collected from Holstein and Japanese Black cows or heifers at a local abattoir within 30 min of slaughter were transported to the laboratory within 2–3 h in 0.9% (w/v) NaCl solution containing 100 IU/ml penicillin G and 100 $\mu\text{g}/\text{ml}$ streptomycin sulfate at 30–35 C in a vacuum flask. Cumulus-oocyte complexes (COCs) were aspirated from small antral follicles of 2–5 mm in diameter with an 18-gauge needle attached to a 10 ml disposable syringe. The COCs surrounded by compact cumulus cell mass and with an evenly granulated cytoplasm were selected and washed four times in mM-199. Ten to fifteen washed COCs were transferred into a 100- μl drop of the same medium supplemented with various concentrations of rhIGF-I (Compound no. LY278764, Eli Lilly & Co., Indianapolis, IN, USA), rbGH (No. CP104301, Lot no. V406–001; Monsanto, Animal Sciences Division, St Louis, MO, USA) and anti-IGF-I antibody (Ab; Lot no. 21397037, Chemicon International Inc., Temecula, CA, USA) with various combinations in different experiments. The medium had been previously covered with warm paraffin oil (Nacalai Tesque Inc., Kyoto, Japan) in a 35-mm polystyrene culture dish and equilibrated with the gas phase (5% CO_2 in air) for 2–3 h at temperature of 39 C.

Preparation of spermatozoa and *in vitro* fertilization

After 24 h of culture at 39 C under 5% CO_2 in air, COCs were washed four times and placed into a 50- μl drop of mBO medium supplemented with 20 mg/ml globulin-free BSA (No. A-7638; Sigma) and 20 $\mu\text{g}/\text{ml}$ porcine intestinal mucosal heparin

(Sigma) under paraffin oil in culture dishes. The dishes were kept in the same culture conditions for about 30 min until spermatozoa were added. One 0.5-ml straw of frozen semen obtained from a Japanese black bull was thawed in a water bath at 37 °C. Spermatozoa were washed twice by centrifugation at $833 \times g$ for 5 min each after dilution with mBO medium supplemented with 10 mM caffeine sodium benzoate (Sigma). The final sperm pellet was resuspended in the same medium as used for washing, to give a sperm concentration of 10×10^6 spermatozoa/ml. A 50- μ l aliquot of the sperm suspension was transferred into medium that included the COCs for fertilization [29].

In vitro culture of embryos

At 8 h post-insemination, the COCs were freed from cumulus cells by repeated passage through a fine pipette and washed four times in mBECM. Ten to twenty inseminated oocytes were then transferred into a 100- μ l drop of the same medium and cultured at 39 °C under 5% CO₂ in air. At 120 h post-insemination, the embryos were transferred into newly prepared mBECM supplemented with 2.78 mM glucose and cultured for additional 72 h [28].

Experiment 1

To examine the effects of rhIGF-I on cumulus expansion, meiotic progression and nuclear maturation of bovine oocytes, COCs were cultured in the presence of 0, 0.1, 1, 10 or 100 ng/ml rhIGF-I for 12 or 24 h (experiment 1a). At each culture period, the number of COCs with expanded cumulus was scored as described by Kobayashi *et al.* [30] and then COCs were freed from cumulus cells by repeated passage through a fine pipette. The cumulus-free oocytes were fixed for 48–72 h at room temperature in 25% (v/v) acetic alcohol, stained with 1% (v/v) orcein in 45% (v/v) acetic acid and examined for evidence of nuclear maturation under a phase-contrast microscope at a magnification of $\times 200$ or $\times 400$. Nuclear maturation was classified as the germinal vesicle (GV), prometaphase I (PM-I), metaphase I (M-I), anaphase I (A-I), telophase I (T-I) and metaphase II (M-II) stages. To examine the effects of rhIGF-I on nuclear maturation of denuded oocytes, COCs were freed from cumulus cells by repeated passage through a fine pipette before maturation culture (experiment 1b). After washing four times,

the denuded oocytes were cultured in the presence of 0, 10 or 100 ng/ml of rhIGF-I for 12 and 24 h. At each culture period, oocytes were examined for evidence of nuclear maturation as in experiment 1a.

Experiment 2

To determine what concentration of Ab is necessary for inhibiting the stimulatory effects of rbGH on nuclear maturation of bovine oocytes, COCs were cultured in the presence of 10 ng/ml rbGH with or without Ab ($1:10^5$ – $1:10^2$) for 24 h, and oocytes were examined for evidence of nuclear maturation to the M-II stage as in experiment 1a.

Experiment 3

To examine whether IGF-I mediates the effects of rbGH on nuclear maturation of bovine oocytes, COCs were cultured in the presence of 100 ng/ml rhIGF-I or 10 ng/ml rbGH with or without Ab ($1:10^3$) or Ab alone for 24 h. At the end of culture, oocytes were examined for evidence of nuclear maturation to the M-II stage as in experiment 1a.

Experiment 4

To examine the effects of rbGH or rhIGF-I and/or Ab added to maturation medium on fertilization and subsequent embryonic development *in vitro*, COCs cultured in the presence of 100 ng/ml rhIGF-I or 10 ng/ml rbGH with or without Ab ($1:10^3$) for 24 h were inseminated *in vitro*. At 8 h post-insemination, oocytes were freed from cumulus cells by repeated passage through a fine pipette. Some oocytes were fixed and stained as in experiment 1a, and examined for evidence of sperm penetration under a phase-contrast microscope at a magnification of $\times 200$ or $\times 400$. The remaining oocytes were cultured for 112 h in mBECM and further for 72 h in mBECM + 2.78 mM glucose. The proportions of embryos developed to the ≥ 2 -cell, $\geq$ morula and blastocyst stages were examined at 48, 144 and 192 h post-insemination, respectively, under an inverted microscope.

Statistical analysis

All proportional data were subjected to arc-sine transformation. The transformed values were analysed using one-way ANOVA. When ANOVA revealed a significant treatment effect, each treatment was compared by Duncan's multiple range test.

Results

Experiment 1

As shown in Table 1 (experiment 1a), when COCs were cultured in the presence of different concentrations of rhIGF-I, the proportion of COCs with expanded cumulus was increased with increasing concentrations of rhIGF-I. The highest proportions of cumulus expansion, 33 ± 3 and $47 \pm 2\%$ were reached after 12 and 24 h of culture, respectively, at 100 ng/ml rhIGF-I ($P < 0.01$). However, in COCs with expanded cumulus, no difference in the degree of the expansion was observed among different concentrations of rhIGF-I and between different period of culture; cumulus always expanded slightly with relatively tight cell to cell binding. After 12 h of culture, most oocytes were in GV breakdown regardless of the different concentrations rhIGF-I, but the proportion of oocytes at the A-I to T-I stage was significantly ($P < 0.01$) higher in the presence of 10–100 ng/ml (32 ± 2 – $33 \pm 3\%$) than 0–1 ng/ml (4 ± 3 – $11 \pm 7\%$) rhIGF-I. After 24 h of maturation culture, the proportion of oocytes at the M-II stage was also significantly ($P < 0.05$) higher in the presence of 10–100 ng/ml (74 ± 3 – $76 \pm 3\%$) than 0–1 ng/ml (53 ± 4 – $61 \pm 1\%$) rhIGF-I.

As shown in Table 2 (experiment 1b), when denuded oocytes were cultured, GV breakdown was observed in about more than 90% of oocytes, but few reached to the A-I to T-I stage after 12 h of culture in the presence of 0, 10 or 100 ng/ml of rhIGF-I. After 24 h of culture, only 40 ± 4 – $42 \pm 2\%$ of oocytes reached to the M-II stage without sig-

nificant difference among different concentrations of rhIGF-I.

Experiment 2

As shown in Fig. 1, the proportion of oocytes that reached the M-II stage after 24 h of culture was significantly ($P < 0.01$) higher in the presence of 10 ng/ml rbGH without Ab ($75 \pm 2\%$) or with Ab diluted $1:10^5$ – $1:10^4$ (69 ± 4 – $70 \pm 4\%$) than in the absence of rbGH and Ab ($59 \pm 1\%$). However, in the presence of Ab diluted $1:10^3$ – $1:10^2$, rbGH (56 ± 3 – $56 \pm 4\%$) did not affect oocyte nuclear maturation.

Experiment 3

As shown in Fig. 2, when COCs were cultured for 24 h, the proportion of oocytes that reached the M-II stage was significantly ($P < 0.01$) higher in the presence of 100 ng/ml rhIGF-I ($73 \pm 2\%$) or 10 ng/ml rbGH ($74 \pm 1\%$) than in the absence of these additives ($54 \pm 3\%$). The presence of Ab ($1:10^3$) alone did not promote or inhibit nuclear maturation of oocytes to the M-II stage ($54 \pm 3\%$), while the same dose of Ab completely prevented the stimulatory effects of rhIGF-I ($58 \pm 1\%$) or rbGH ($54 \pm 3\%$) on oocyte maturation.

Experiment 4

As shown in Table 3, no differences were observed in the penetration rate (75 ± 3 – $77 \pm 6\%$), pronuclear formation (56 ± 3 – $63 \pm 10\%$) and polyspermy (28 ± 4 – $36 \pm 5\%$) among oocytes matured in different culture conditions. However, as shown in Table 4, when oocytes after 8 h of insemination were cultured in a chemically defined

Table 1. Effects of recombinant human insulin-like growth factor-I (rhIGF-I) on cumulus expansion and nuclear maturation of bovine cumulus-oocyte complexes (COCs) after 12 and 24 h of culture *in vitro*^a

Concentration of rhIGF-I (ng/ml)	No. of COCs examined	After 12 h of culture							After 24 h of culture		
		No. (%) ^b of COCs with expanded cumulus	No. (%) ^b of oocytes at various stages					No. of COCs examined	No. (%) ^b of COCs with expanded cumulus	No. (%) ^b of oocytes at M-II ^c	
			GV ^c	PM-I ^c	M-I ^c	A-I–T-I ^c	M-II ^c				
0	53	0 (0) ^d	2 (3 ± 2)	16 (33 ± 3) ^d	30 (56 ± 2)	5 (7 ± 2) ^d	0 (0)	51	7 (11 ± 3) ^d	28 (53 ± 4) ^h	
0.1	51	0 (0) ^d	0 (0)	20 (39 ± 2) ^d	29 (56 ± 3)	2 (4 ± 3) ^d	0 (0)	49	4 (8 ± 3) ^d	30 (61 ± 1) ^h	
1	51	4 (7 ± 3) ^e	2 (4 ± 2)	13 (25 ± 4) ^d	31 (60 ± 3)	5 (11 ± 7) ^d	0 (0)	49	6 (12 ± 1) ^d	30 (60 ± 4) ^h	
10	52	8 (14 ± 1) ^f	0 (0)	6 (11 ± 2) ^d	29 (56 ± 1)	17 (33 ± 3) ^e	0 (0)	52	16 (28 ± 3) ^e	40 (76 ± 3) ⁱ	
100	57	19 (33 ± 3) ^g	0 (0)	1 (2 ± 2) ^f	34 (60 ± 2)	18 (32 ± 2) ^e	3 (5 ± 3)	58	27 (47 ± 2) ^f	43 (74 ± 3) ^j	

^a Experiments were repeated five times. ^b Mean ± SEM. ^c GV: germinal vesicle; PM-I: prometaphase I; M-I: metaphase I; A-I: anaphase I; T-I: telophase I; M-II: metaphase II. ^{d–j} Values with different superscripts within each column are significantly different (^{d–g} $P < 0.01$, ^{h–j} $P < 0.05$).

Table 2. Effects of recombinant human insulin-like growth factor-I (rhIGF-I) on nuclear maturation of denuded bovine oocytes after 12 and 24 h of culture *in vitro*^a

Concentration of rhIGF-I (ng/ml)	No. of oocytes examined	After 12 h of culture					After 24 h of culture	
		No. (%) ^b of oocytes at various stages					No. of oocytes examined	No. (%) ^b of oocytes at M-II ^c
		GV ^c	PM-I ^c	M-I ^c	A-I~T-I ^c	M-II ^c		
0	48	5 (11 ± 3)	27 (56 ± 6)	14 (29 ± 1)	1 (2 ± 2)	0 (0)	45	19 (42 ± 2)
10	48	2 (4 ± 2)	29 (60 ± 5)	17 (36 ± 4)	0 (0)	0 (0)	45	18 (40 ± 4)
100	48	2 (5 ± 5)	27 (55 ± 7)	17 (36 ± 2)	2 (5 ± 5)	0 (0)	45	19 (42 ± 2)

^a Experiments were repeated four times. ^b Mean ± SEM. ^c GV: germinal vesicle; PM-I: prometaphase I; M-I: metaphase I; A-I: anaphase I; T-I: telophase I; M-II: metaphase II.

Fig. 1. Dose-dependent effects of anti-IGF-I antibody (Ab) on nuclear maturation to the metaphase II (M-II) stage of bovine oocytes *in vitro*. Cumulus-enclosed oocytes were cultured for 24 h with 10 ng/ml recombinant bovine growth hormone (rbGH) alone or combined with Ab (1:10⁵–1:10²). Experiments were repeated four times. The number of cultured oocytes in each group is indicated in parentheses. ^a, ^b: significantly different, *P* < 0.05. **Fig. 2.** Effects of recombinant human insulin-like growth factor-I (rhIGF-I), recombinant bovine growth hormone (rbGH) and anti-IGF-I antibody (Ab, 1:10³) on nuclear maturation to the metaphase II (M-II) stage of bovine oocytes *in vitro*. Cumulus-enclosed oocytes were cultured for 24 h with 100 ng/ml rhIGF-I, 10 ng/ml rbGH, IGF-I or GH combined with Ab, or Ab alone. Experiments were repeated four times. The number of cultured oocytes in each group is indicated in parentheses. ^a, ^b: significantly different, *P* < 0.01.

medium, the proportions of oocytes cleaved to the ≥ 2 -cell (75 ± 4 – $81 \pm 3\%$) and $\geq$ morula (20 ± 1 – $26 \pm 2\%$) stages at 48 and 144 h post-insemination, respectively, were not different among oocytes matured in different culture conditions. However, significantly (*P* < 0.05) higher proportions of oocytes developed to the blastocyst stage at 192 h post-insemination when they were matured in the presence of 100 ng/ml rhIGF-I ($16 \pm 1\%$) or 10 ng/ml rbGH ($15 \pm 2\%$) than in the absence of these

additives ($4 \pm 2\%$). In the presence of Ab (1:10³) in maturation medium with rhIGF-I or rbGH did not either promote or inhibit the development of penetrated oocytes to the blastocyst stage (4 ± 2 – $5 \pm 3\%$).

Table 3. Effects of recombinant human insulin-like growth factor-I (rhIGF-I; 100 ng/ml) or recombinant bovine growth hormone (rbGH; 10 ng/ml) with or without anti IGF-I antibody (Ab; 1:10³) in maturation medium on penetration of bovine oocytes 8 h after insemination *in vitro*^a

Additives to maturation medium	No. of oocytes examined	No. of oocytes penetrated			No. of polyspermic oocytes (%) ^{b,c}
		Total (%) ^b	With enlarged sperm head (%) ^{b,c}	With male and female pronuclei (%) ^{b,c}	
None	52	39 (75 ± 3)	15 (38 ± 10)	24 (62 ± 10)	14 (36 ± 5)
rhIGF-I	57	43 (75 ± 4)	17 (40 ± 8)	26 (60 ± 8)	12 (28 ± 4)
rhIGF-I + Ab	53	41 (77 ± 6)	18 (44 ± 3)	23 (56 ± 3)	13 (30 ± 8)
rbGH	52	39 (75 ± 5)	14 (37 ± 10)	25 (63 ± 10)	13 (33 ± 2)
rbGH + Ab	54	41 (76 ± 2)	16 (39 ± 8)	25 (61 ± 8)	12 (29 ± 4)

^a Experiments were repeated four times. ^b Mean ± SEM. ^c Percentages of oocytes penetrated.

Table 4. Effects of recombinant human insulin-like growth factor-I (rhIGF-I; 100 ng/ml) or recombinant bovine growth hormone (rbGH; 10 ng/ml) with or without anti IGF-I antibody (Ab; 1:10³) in maturation medium on development of bovine oocytes following fertilization *in vitro*^a

Additives to maturation medium	No. of oocytes cultured	No. (%) ^b of oocytes developed to		
		≥ 2-cell [48 h] ^c	≥ Morula [144 h] ^c	Blastocyst [192 h] ^c
None	58	43 (75 ± 4)	12 (21 ± 1)	2 (4 ± 2) ^d
rhIGF-I	57	46 (81 ± 3)	14 (24 ± 3)	9 (16 ± 1) ^e
rhIGF-I + Ab	58	46 (79 ± 0)	12 (21 ± 3)	3 (5 ± 3) ^d
rbGH	63	49 (78 ± 1)	16 (26 ± 2)	9 (15 ± 2) ^e
rbGH + Ab	60	46 (77 ± 6)	12 (20 ± 1)	2 (4 ± 2) ^d

^aExperiments were repeated four times. ^bMean ± SEM. ^cNumbers in parenthesis indicate the time of examination (h post-insemination). ^{d-e}Values with different superscripts are significantly different ($P < 0.05$).

Discussion

The results of the present study indicate that 1) both rhIGF-I and rbGH promote nuclear maturation of cumulus-enclosed bovine oocytes to the M-II stage *in vitro* and these effects are blocked in the presence of Ab to IGF-I; 2) as observed with rbGH in our previous study [24], a stimulatory effect of rhIGF-I on oocyte nuclear maturation is mediated by the cumulus cells and appears at relatively high concentrations ranging from 10 to 100 ng/ml; 3) rhIGF-I (100 ng/ml) or rbGH (10 ng/ml) added to maturation medium does not affect penetration of oocytes *in vitro* or early development of the inseminated oocytes to the ≥ morula stage, but promotes development to the blastocyst stage at 192 h post-insemination; and 4) rhIGF-I (100 ng/ml) and rbGH (10 ng/ml) are equally effective in promoting oocyte nuclear maturation and embryonic development to the blastocyst stage.

It is well documented that GH has a beneficial effect on mammalian oocyte maturation and subsequent embryonic development. In cattle, pituitary GH accelerates nuclear maturation although it does not increase the proportion of oocytes reaching to the M-II stage, and improves the developmental capacity of the oocytes [18]. In our previous study using recombinant bovine GH, which, unlike the pituitary-extracted GH, is free of contaminants, the involvement of GH in maturation of cumulus-enclosed bovine oocytes leading not only to promoted nuclear maturation but also to enhanced maturation rate and improved embryonic development to the blastocyst stage was also found [26]. In the present study, however, this beneficial effect of rbGH on oocyte maturation completely disappeared when COCs were cultured in the presence of both rbGH (10 ng/ml) and an antibody against IGF-I (Ab), 1:10³ to 1:10² dilution.

Furthermore, rhIGF-I (100 ng/ml) had a beneficial effect on bovine oocyte maturation which was strictly comparable to the effects of rbGH (10 ng/ml) and also completely disappeared in the presence of Ab. Although it is not clear whether IGF-I is synthesized by bovine cumulus cells, it has been shown that the secretion of IGF-I by cultured bovine granulosa cells is enhanced by GH [31]. Therefore, it may be assumed that any IGF-I that may have been released from cumulus cells in re-

sponse to rbGH was neutralized by the antibody in the present experimental conditions. The complete neutralization of the promoting effect of GH on rat oocyte maturation has also been reported in the presence of Ab [16]. These data suggest that the effect of GH on bovine oocyte maturation may be mediated by IGF-I.

However, in contrast to the results obtained in the present study, Izadyar *et al.* [23] have reported that the stimulatory effect of GH on bovine oocyte maturation is direct and is not mediated *via* IGF-I. They found that bovine cumulus cells and oocytes express mRNA for the GH receptors and that the addition of Ab to the culture medium did not inhibit the stimulatory effect of GH on oocyte maturation, while stimulation by IGF-I was completely suppressed by the antibody. Pituitary-extracted GH, which was used by Izadyar *et al.* [23], may have been contaminated by other hormones such as thyroid stimulating hormone and gonadotropins which are known to enhance bovine oocyte nuclear maturation [31, 32]. In our previous study, a dose-dependent effect of rbGH on the stimulation of bovine oocyte nuclear maturation was not observed within a wide range of doses (10–1000 ng/ml) of GH, while Izadyar *et al.* [18] found dose-dependent effect of pituitary-extracted GH within the same dose range. These results may also suggest an effect of a contamination of pituitary-extracted GH.

As was reported for GH [16, 18, 26], rhIGF-I used in the present study enhanced nuclear maturation of the cumulus-enclosed bovine oocytes, but not the denuded oocytes, and subsequent embryo development to the blastocyst stage. No stimulatory effect of IGF-I on maturation of cumulus-enclosed pig oocytes was reported by Reed *et al.* [7], but these investigators used maturation medium containing hormones and serum albumin. These supplements are known as regulators of oocyte maturation and a highly complex combination of components, and thereby it may be difficult to find direct action of IGF-I under such conditions. Using defined medium, the enhancement of maturation in only cumulus-enclosed oocytes by IGF-I was also reported in cattle [5] and rabbits [6] indicating that a possible stimulus for maturation is transferred to the oocyte *via* cumulus-cells. There are conflicting data on the effects of IGF-I during culture for oocyte maturation on subsequent embryonic development. In cattle, IGF-I added to

maturation medium did not increase the proportion of embryos developing to the morula and blastocyst stages [4]. This may have been due to the presence of hormones and serum in maturation medium. However, the cleavage rate of porcine embryos was stimulated by the addition of IGF-I to maturation medium containing hormones and serum. In the present study using a chemically defined medium, the beneficial effects of IGF-I on oocyte maturation was dose-dependent: earlier meiotic progression and higher proportion of oocytes that reached the M-II stage were observed in 10–100 ng/ml than 0.1–1 ng/ml rhIGF-I. The concentrations of rhIGF-I (10–100 ng/ml) at which positive effects on oocyte nuclear maturation and/or subsequent embryo development [5, 23, present study] are lower than those detected in bovine follicular fluid [33].

In the present study, only slight cumulus expansion with relatively tight cell to cell binding was observed in the presence or absence of rhIGF-I. Although the proportion of COCs with such slightly expanded cumulus was increased with increasing concentration of the rhIGF-I, this did not necessarily correlate with the maturation rate. The poor cumulus expansion by IGF-I is also reported in bovine [4, 5] and rabbit [6] COCs. Since an “expansion” factor is produced by the oocyte [34], it is assumed that IGF-I may inhibit the production of the factor by the oocyte [4–6]. However, this assumption may not be true under the present experimental conditions because increased concentration of rhIGF-I resulted in increased, rather than decreased, proportion of COCs with slight cumulus expansion. Therefore, in comparison with other factors, IGF-I may be less effective inducing cumulus expansion or stimulating the production of the expansion factor by the oocyte.

In conclusion, we confirmed the involvement of GH and IGF-I in bovine oocyte maturation leading to an improved maturation rate and quality of embryos to support their subsequent development from the morula to blastocyst stage. Regarding the physiological role of GH in oocyte maturation, the present study suggests that the stimulatory effects of GH on bovine oocyte maturation are not direct, but mediated *via* IGF-I probably produced by the cumulus cells.

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