Wortmannin, A Specific Phosphatidylinositol 3-Kinase Inhibitor, Blocks In Vitro Fertilization and Embryonic Development of Bovine Oocytes Matured In Vitro with Epidermal Growth Factor

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Abstract: *Two experiments were carried out to evaluate the effects of EGF supplementation to maturation medium on the fertilization and developmental potential of bovine oocytes after in vitro fertilization, and to see whether the addition of wortmannin from 6 h after the onset of maturation culture would modulate the action of EGF. The effects of EGF were compared to those of gonadotropins + serum treatment. The presence of EGF in maturation medium significantly increased the number of normally fertilized oocytes. No difference in the proportion of normally fertilized oocytes was observed between EGF and gonadotropins + serum treatments. Wortmannin completely abolished the stimulatory effects of both treatments on fertilization, and effectively increased the number of unfertilized oocytes. The rate of cleavage, and the proportions of embryos reaching the blastocyst and hatched blastocyst stages after in vitro fertilization were also significantly improved with the addition of EGF to maturation medium. The presence of gonadotropins + serum during in vitro maturation resulted in rates of development statistically similar to that obtained with EGF alone. Wortmannin effectively abolished the stimulatory effects of either treatment on cleavage and embryonic development. These data suggest some physiological role for EGF on cytoplasmic maturation, and indicate the importance of PI 3-kinase activity in the process of cytoplasmic maturation.*

Key words: *EGF, PI 3-kinase, Bovine oocyte, Cytoplasmic maturation, IVF*

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Although bovine oocytes are capable of undergoing nuclear maturation spontaneously *in vitro* when they are removed from follicles, their rate of male pronucleus formation and the subsequent embryonic development after fertilization are limited [1, 2]. This low developmental potential is attributed to a deficiency in cytoplasmic maturation [3]. Supplementing maturation medium with serum enhanced the rates of fertilization and subsequent embryonic development of bovine oocytes *in vitro* [4, 5]. Similarly, the addition of high concentrations of gonadotropins to the maturation medium effectively increased fertilization rates, and improved rates of embryo development *in vitro* [1, 6, 7].

A number of growth factors have been found in the ovary [8, 9], and some of them, in particular EGF and IGF-I, have been shown to stimulate oocyte maturation *in vitro* [10–13]. In our previous report, we suggested a possible role for phosphatidylinositol 3-kinase (PI 3-kinase) in regulating bovine oocyte meiotic maturation [14]. PI 3-kinase is an enzyme that is essentially involved with signaling pathways of many growth factors with receptor-associate tyrosine kinase [15–17]. PI 3-kinase is a heterodimer consisting of an 85 KDa regulatory subunit, and a 110 KDa catalytic subunit which phosphorylates lipid and protein-serine [18, 19]. Wortmannin is a fungal metabolite that has been widely used as a specific PI 3-kinase inhibitor [20, 21]. It inactivates PI 3-kinase by covalently binding to its 110 KDa catalytic subunit [22]. Recently we also found that wortmannin blocked EGF-induced meiotic maturation. However, the addition of wortmannin from 6 h after the onset of culture did not block the oocytes from complet-

ing their meiotic maturation [23].

The present study was therefore designed to clarify the positive effect of EGF on cytoplasmic maturation of bovine oocytes by evaluating the potential of these oocytes for embryonic development following *in vitro* fertilization. The effect of wortmannin, when added to the maturation medium from 6 h after the onset of culture, on the developmental potential of oocytes matured with EGF or hormones and serum was also investigated.

Materials and Methods

Oocyte collection and maturation

Bovine ovaries were collected from a local slaughterhouse and brought to the laboratory in saline at 30°C. Oocytes were recovered by aspiration of 2 to 5 mm follicles of the ovaries using a 10-ml syringe with an 18-g needle. Oocytes, with compact unexpanded cumulus cells and with homogeneous ooplasm, were selected under a stereomicroscope and placed in a watch glass containing Dulbecco's phosphate buffered saline (PBS) supplemented with 0.05% polyvinylpyrrolidone (PVP; Mol. Wt. 40,000; Sigma, St. Louis, MO, USA) and 100 μg/ml kanamycin (Sigma). Ten to 20 cumulus-oocyte complexes (COCs) were transferred to 100-μl droplets of culture medium covered with mineral oil (Sigma) in a 35-mm polystyrene culture dish (Falcon Plastics, no. 1008, Becton Dickinson and Company, Lincoln Park, NJ). The basic medium (mTCM) was TCM-199 with Earle's salts (Gibco BRL, Grand Island, NY) supplemented with 0.2 mM Na-pyruvate (Sigma), and 50 μg/ml gentamicin (Sigma). When EGF was used, 0.3% PVP was added to this basic medium. EGF (50 ng/ml; E-4127, from mouse submaxillary glands, Sigma, Lot. 065H8821, St. Louis, MO) and wortmannin (1×10^{-7} M; Sigma) were added to this basic medium according to the experiment designs described below. In our previous report [14], we examined the dose effects of wortmannin on meiotic maturation in bovine oocytes. The results showed that 1×10^{-7} M and 1×10^{-8} M wortmannin significantly suppressed meiotic maturation. Thus, a concentration of 1×10^{-7} M wortmannin was employed in this experiment. Wortmannin was initially dissolved in dimethyl sulfoxide (DMSO; Sigma) to 5 mM, and then readjusted to the final concentration with the culture medium. The final DMSO concentration in the culture medium was 0.098% (v/v). Because wortmannin becomes less effective after 6 h in aqueous solutions [21], the maturation medium supplemented with wortmannin was changed every 5 h during the culture period. Some of the oocytes were cultured in the basic medium supplemented with LH (1.3 μg/ml; from equine pituitary; Sigma, Lot. 76H-0224), FSH (0.6 μg/ml; from porcine pituitary; Sigma, Lot. 128F-0521), heat-inactivated FCS (10%; Gibco) with or without 1×10^{-7} M wortmannin. Culture was carried out in a humidified atmosphere of 5% CO_2 in air at 39°C.

In vitro fertilization

Frozen semen of a proven Japanese black bull was thawed in a water bath at 35°C and washed two times by centrifugation at 700 g for 5 minutes in modified Tyrode's medium (TALP) supplemented with 10 mM caffeine (Nacalai Tesque, Inc. Kyoto, Japan). The sperm pellet was resuspended in the remaining washing medium to give a sperm concentration of 10×10^6/ml. At the end of the maturation period, 10–20 oocytes were transferred into each 50 μl droplet of fertilization medium. The fertilization medium consisted of TALP supplemented with 20 mg/ml bovine serum albumin (BSA; Sigma) and 20 μg/ml heparin (Sigma). The droplets of the fertilization medium were made in a 35-mm polystyrene culture dish covered with mineral oil. Fifty microliters from each sperm suspension was added to a droplet of the fertilization medium to give a final concentration of 5×10^6 sperm/ml, and coincubated with the oocytes for 18 to 20 h in a moisture-saturated atmosphere of 5% CO_2 in air, at 39°C.

Assessment of fertilization

Eighteen hours after insemination, oocytes were freed from cumulus cells by vortexing with PBS containing 0.1% hyaluronidase (Sigma). The cumulus-free oocytes were then mounted on slides, fixed for 48 h in a mixture of acetic acid and ethanol (1:3 v/v), stained with lacmoid and examined under a phase-contrast microscope at a magnification of × 500. The fertilization stages were scored according to the number of pronuclei (PN): 0 PN indicated no fertilization, 1 PN indicated oocyte activation, 2 PN + second polar body indicated normal fertilization, >2 PN indicates polyspermy [24].

In vitro development

At the end of the fertilization period, the oocytes were introduced into the development medium (consisting of TCM supplemented with 10% (v/v) FCS and 50 μg/ml gentamycin) and cultured in an atmosphere of 5% CO_2 in air at 39°C. The oocytes were observed for cleavage 48 h after insemination, and the cleaved oocytes were cultured with the cumulus cells monolayer that had grown on the bottom of the dish for 8 days during which the blastocyst and hatched blastocyst rates were evaluated.

Experiment 1

This experiment was carried out to examine the fertilizability of bovine oocytes matured *in vitro* with EGF and wortmannin. Oocytes were cultured *in vitro* with the following maturation treatments:

1) mTCM, for 24 h.
2) mTCM+FCS+LH+FSH, for 24 h.
3) mTCM+ EGF, for 24 h.
4) mTCM+FCS+LH+FSH, for 6 h followed by culture in mTCM+ FCS+LH+FSH + wortmannin for 18 h.
5) mTCM+EGF, for 6 h followed by culture in mTCM+EGF+wortmanin for 18 h.

For treatments 4 and 5, wortmannin was added from 6 h after the onset of maturation culture because at this time we have found that it does not block the oocytes from completing their meiotic maturation [23]. At the end of the maturation period, oocytes were fertilized *in vitro* and assessed for their pronuclear formation.

Experiment 2

This experiment was conducted to assess the developmental competence of bovine oocytes matured *in vitro* with EGF and wortmannin. Oocytes were matured *in vitro* under the same treatments described in experiment 1. After *in vitro* fertilization, the oocytes were cultured in vitro and their rates of cleavage and developments to blastocyst and hatched blastocyst stages were evaluated.

Statistical analysis

Results are presented as the mean ± SEM. All percentage data were subjected to arc-sine transformation and statistically analyzed by one-way ANOVA. When significance was found in the ANOVA analysis, the data were compared by Fisher's protected least significant difference test. A value of $P<0.05$ was considered significant.

Results

Experiment 1: In vitro fertilization of bovine oocytes matured in vitro with EGF and wortmannin

The number of pronuclei was used as an indication of normal fertilization. The percentage of unfertilized oocytes (0 PN) was significantly higher in oocytes cultured with wortmannin than those cultured with EGF or hormones+serum (Table 1). Oocyte activation (1 PN) was significantly lower in the presence of EGF or hormones+serum than in their absence. Wortmannin greatly increased the number of activated oocytes to a rate similar to that of oocytes cultured without any supplementation. Normal fertilization (2 PN) was significantly higher with EGF, and hormones+serum treatments than all other treatments. Oocytes cultured in EGF, with or without wortmannin, had relatively higher rates of polyspermy (>2 PN) than those cultured with hormones + serum or without any supplementation. Addition of wortmannin to the hormones+ serum treatment also effectively increased the rate of polyspermy.

Experiment 2: Cleavage and developmental ability, to blastocyst and hatched blastocyst stages, of bovine oocytes matured in vitro with EGF and wortmannin

The presence of hormones + serum, and EGF in the maturation medium significantly increased the number of oocytes cleaved 2 days after insemination, and that of oocytes reaching the blastocyst and hatched blastocyst stages at day 8 of culture (Table 2). Wortmannin effectively abolished the stimulatory effects of EGF, and hormones + serum treatments on cleavage and blastocyst rates. Similarly, the proportion of oocytes developed to the hatched blastocyst stage was significantly higher in the presence of EGF, and hormones + serum than in their absence. In contrast, wortmannin effectively abol-

Table 1. Effect of EGF and wortmannin in maturation medium on fertilization of bovine oocytes *in vitro*

Maturation treatments	No. of oocytes	0 PN (%)	1 PN (%)	2 PN (%)	>2 PN (%)
mTCM	109	15.6 ± 4.5^{ab}	23.9 ± 3.2^{a}	55.9 ± 3.8^{a}	4.6 ± 1.0^{a}
LH + FSH + FCS	101	5.9 ± 2.6^{a}	11.9 ± 2.6^{b}	75.3 ± 4.1^{b}	6.9 ± 1.1^{ab}
EGF	99	6.1 ± 3.6^{a}	11.1 ± 1.9^{b}	70.7 ± 3.6^{b}	12.1 ± 2.9^{ab}
EGF + wortmannin[1)]	109	19.3 ± 5.5^{b}	21.1 ± 5.1^{a}	48.6 ± 1.2^{a}	11.0 ± 5.4^{ab}
LH + FSH + FCS + wortmannin[2)]	65	20.0 ± 2.7^{b}	16.9 ± 1.7^{ab}	49.2 ± 1.1^{a}	13.9 ± 2.9^{b}

1) Oocytes were initially cultured with EGF for 6 h followed by culture with EGF + wortmannin for the remaining 18 h. 2) Oocytes were initially cultured with LH + FSH + FCS for 6 h followed by culture with LH + FSH + FCS + wortmannin for the remaining 18 h. (%) Values are mean percentages ± SEM for three replicates. [a, b] Different superscripts within the same columns are significantly different ($P<0.05$).

Table 2. Cleavage and developmental ability of bovine oocytes matured *in vitro* with EGF and wortmannin

Treatments	No. of oocytes	Cleaved Oocytes (%)	Blastocyst (%)	Blastocyst [%]	Hatched Blastocyst (%)
mTCM	88	54.6 ± 4.7 [a]	15.9 ± 2.3 [a]	29.2 + 2.3 [a]	6.8 ± 1.2 [a]
LH + FSH + FCS	115	76.5 ± 3.9 [b]	30.4 ± 1.0 [b]	39.8 + 1.2 [b]	20.0 ± 1.1 [b]
EGF	103	71.9 ± 1.5 [b]	26.2 ± 1.7 [b]	36.5 + 1.8 [b]	16.5 ± 2.7 [b]
EGF + wortmannin[1)]	77	33.8 ± 4.1 [c]	7.8 ± 1.8 [c]	23.1 + 1.8 [a]	2.6 ± 2.1 [a]
LH + FSH + FCS + wortmannin[2)]	76	29.0 ± 4.6 [c]	6.6 ± 2.1 [c]	22.7 + 2.2 [a]	1.3 ± 3.1 [a]

1) Oocytes were initially cultured with EGF for 6 h followed by culture with EGF + wortmannin for the remaining 18 h. 2) Oocytes were initially cultured with LH+FSH+FCS for 6 h followed by culture with LH + FSH + FCS + wortmannin for the remaining 18 h. Values are mean percentages ± SEM for three replicates. (%) As the total number of cultured oocytes. [%] As the total number of cleaved oocytes. [a, b, c] Different superscripts within the same columns are significantly different ($P<0.05$).

ished the stimulatory effect of both treatments on hatched blastocyst formation.

Discussion

In the present study, with the aim of avoiding the effect of any growth factors introduced by serum, a serum-free medium was used to examine the effect of EGF on fertilizability and developmental ability of bovine oocytes *in vitro*. The results showed that addition of EGF to the maturation medium significantly increased the number of oocytes undergoing normal fertilization to a rate similar to that obtained with the combined serum + gonadotropins treatment. This finding supports the results of other reports which showed similar stimulatory effects for EGF on fertilization [25–27]. Recently, we have found that the addition of wortmannin 6 h after the onset of culture did not prevent bovine oocytes from completing meiotic maturation [23]. In this experiment the fertilizability of these oocytes was examined. The results revealed that wortmannin effectively increased the number of unfertilized oocytes, and decreased that of normally fertilized oocytes for both EGF and hormones+serum treatments. Thus, although wortmannin did not affect meiotic maturation when added 6 h after the start of the maturation period, it negatively modulates the fertilizability of the cultured oocytes, suggesting an essential role for PI 3-kinase on the process of cytoplasmic maturation. The fact that the inhibitory effect of wortmannin was observed for both EGF- and hormones- mediated maturation also implies that EGF might be one of the downstream molecules through which gonadotropins stimulate oocyte maturation.

In the second experiment, the developmental competence of oocytes matured with EGF and fertilized *in vitro* was tested. The results showed that EGF significantly increased the number of cleaved oocytes in comparison to those cultured without EGF. The cleavage rate for EGF treatment was as high as that of the hormones + serum treatment. When the cleaved oocytes were cultured *in vitro* and evaluated for their early embryonic development, higher rates of blastocyst and hatched blastocyst formation were observed for oocytes cultured with EGF than in those cultured in its absence. As for the cleavage rate, the rates of blastocyst and hatched blastocyst formation were statistically similar for EGF, and hormones + serum treatments. Thus, these observations clearly indicate that *in vitro* maturation of bovine oocytes with EGF results in embryos with developmental capability comparable to that of oocytes matured with hormones + serum.

The stimulatory effect of EGF on cytoplasmic maturation observed in this study was consistent with the results of other investigators who demonstrated a similar effect for EGF on oocyte maturation [11, 13, 25, 28]. However, the present findings are not in agreement with that of Harper and Brackett [29] who reported that EGF enhanced bovine embryonic development only when supplemented to the maturation medium in combination with low concentrations of gonadotropins. The reason behind this contradiction is not clear.

Furthermore, wortmannin effectively abolished the stimulatory effects of EGF as well as those of hormones + serum on the number of oocytes developing to the blastocyst and hatched blastocyst stages. Likewise, wortmannin effectively reduced the blastocyst rate when calculated from the total number of cleaved oocytes, indicating that the lower blastocyst rate of oocytes cultured with wortmannin was not due to a decreased number of cleaved oocytes, but rather to the low developmental ability of embryos derived from these oocytes. These results imply that whereas PI 3-kinase activity

during the first 6 h of maturation is needed for occurrence of meiotic maturation, the later kinase activation is necessary for completion of cytoplasmic maturation, and therefore complete meiotic maturation does not assure full cytoplasmic maturation.

In conclusion, the results of this study suggest a novel physiological role for EGF on cytoplasmic maturation of bovine oocytes, and indicate the association of PI 3-kinase with this process.

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