【Article】

Effect of Bovine Follicular Fluid Added to the Maturation Medium on Sperm Penetration in Pig Oocytes Matured In Vitro

Naoki ISOBE
Research Associate
Graduate School for International Development and Cooperation,
Hiroshima University, Higashi-Hiroshima
739-8529, Japan

Takato TERADA
Professor
Faculty of Applied Biological Science, Hiroshima University,
Higashi-Hiroshima 739-8528, Japan

Abstract

The objective of this study was to examine the effect of bovine follicular fluid added to the maturation medium on sperm penetration, male pronucleus formation and polyspermy in pig oocytes fertilized in vitro. Pig oocytes were cultured in 10 µl droplets of medium without bovine follicular fluid for 24 h. The oocytes were then cultured in 100 µl droplets of medium supplemented with 0,10 or 20% bovine follicular fluid for further 24 h. The cultured oocytes were inseminated with boar sperm at a concentration of 1×10^5 or 10^6 sperm/ml. When oocytes were inseminated at 1×10^5 sperm/ml, significantly lower proportion of penetrated oocytes was found in 20% bovine follicular fluid group compared with that in 0 and 10% bovine follicular fluid groups ($P<0.05$). Addition of bovine follicular fluid to maturation medium promoted the male pronucleus formation in oocytes inseminated with 1×10^5 sperm/ml, with a significant ($P<0.05$) difference in rate of oocytes with male pronuclei between 0% and 10% bovine follicular fluid groups. Treatment with bovine follicular fluid during the second 24 h culture significantly decreased the proportions of polyspermic oocytes inseminated with either of sperm concentrations. These results indicate that the presence of bovine follicular fluid in maturation medium enhances male pronucleaus formation and reduces polyspermic penetration in pig oocytes fertilized in vitro.

Introduction

Increasing the number of animals with high productivity is of particular interest in many developing countries as well as Japan. In vitro fertilization (IVF) is one of various reproductive technologies which plays an important role in the production of embryos for subsequent embryo transfer. A variety of successful systems for IVF of pig oocytes matured in vitro have been developed. However, the incidence of polyspermy was observed in most of these IVF systems. To overcome this problem, Zheng and Sirard (1992) examined the effect of protein supplements into maturation medium on polyspermy of pig oocytes. Their results revealed that culturing of oocytes in fetal calf serum (FCS)-supplemented medium

for 24 h followed by further culture in bovine serum albumin (BSA)-supplemented medium for 24 h reduced the incidence of polyspermy without decreasing the penetration rate. Funahashi et al. (1994) demonstrated that decreasing NaCl concentration in maturation medium for pig oocyte produced a significant decrease in polyspermic penetration rate. In these studies, a maturational culture condition that blocks polyspermic penetration has been partially achieved, although the overall polyspermy rate is still high.

Follicular fluid has been widely used as additive to maturation medium to enhance male pronuclear formation after insemination in vitro (Naito et al., 1988; Yoshida et al., 1992). However, the effect of follicular fluid in the maturation medium on polyspermy has not yet been well investigated. Moreover, supplementation of bovine follicular fluid to the culture medium of pig oocytes has also not been conducted. In the present study, bovine follicular fluid was employed to examine its effect on sperm penetration, male pronucleus formation, polyspermy and normal fertilization in pig oocytes fertilized in vitro.

Materials and Methods

Oocyte collection and preparation for culture

Ovaries were collected from prepubertal gilts (5-7 months) at a local slaughterhouse and transported to the laboratory in 0.85% NaCl with 0.1mg/ml kanamycin (Meiji Seika, Tokyo, Japan) at about 35ºC within 2 h. The surfaces of follicles measuring from 3 to 8 mm in diameter were cut with a razor and oocytes were collected by scraping the inner surface of the follicle wall using a surgical blade. The collected oocytes were placed in prewarmed phosphate buffered saline (PBS) supplemented with 2% bovine serum. Oocytes possessing a complete and compact cumulus mass (Fig. 1) were selected and washed several times in the maturation medium.

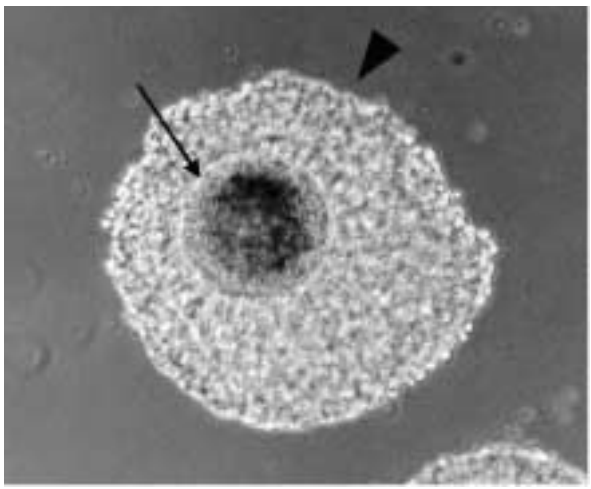

Fig. 1 An oocyte (arrow) surrounded by a complete and compact cumulus mass (arrow head).

Oocyte maturation

Each 20 cumulus-oocyte complexes (COCs) were placed in a 10 µl droplet of maturation medium covered with warm mineral oil (Squibb and Sons, Princeton, NJ) in a 35 mm polystyrene culture dish.

Oocytes were cultured for 24 h at 39ºC in humidified atmosphere of 5 % CO_2 in air. The maturation medium was TCM-199 with Earle's salts (GIBCO BRL, Grand Island, NY) supplemented with 10 IU/ml hCG (Teikoku Hormone Mfg., Tokyo, Japan), 10 IU/ml PMSG (Teikoku Hormone Mfg), 1 μg/ml 17ß-estradiol (Sigma Chemical Co., St. Louis, MO), 50 μg/ml Gentamicin sulphate (Sigma) and 10% FCS (GIBCO). Thereafter, these 20 COCs were transferred to a 100 μl droplet of maturation medium supplemented 0.75 mM cysteine (Katayama Chemical, Osaka, Japan) and further cultured for 24 h. FCS in the maturation medium for second 24 h-culture was replaced by bovine follicular fluid at a concentration of 0% (control), 10% or 20%.

Preparation of bovine follicular fluid

Bovine follicular fluid was collected as described by Elmileik et al. (1995) with minor modification. Briefly, ovaries with regressive corpus luteum were selected and fluid (2 ml) from their follicles of 15 mm or greater in diameter was aspirated. Bovine follicular fluid was pooled and centrifuged for 15 min at 2,200×g to remove granulosa cells, blood cells and oocytes. The supernatant was heated to 56ºC in a water bath for 30 min and then stored at -30ºC until use.

Sperm preparation and in vitro fertilization

Semen was collected from a miniture-pig (Göttingen) by the gloved hand method and kept at 20ºC for 18 h. Thereafter, the spermatozoa were washed two times with PBS supplemented with 1 mg/ml BSA (fraction V, Nacalai tesque, Kyoto, Japan) and further washed once with fertilization medium by centrifugation (700×g for 5 min). The fertilization medium was TCM-199 with Earle's salts supplemented with 3.05 mM glucose (nacalai), 2.92 mM Ca-lactate (nacalai), 0.91 mM Na-pyruvate (nacalai), 5 mM caffeine (nacalai), 50 μg/ml gentamicin and 10% FCS at a pH of 7.4. After the maturational culture, COCs were washed two times with fertilization medium and placed into 90 μl droplets of fertilization medium. Ten micro litter of the washed spermatozoa was added to each 90 μl droplet-medium containing 20 COCs at a final sperm concentration of 1×10^5 or 10^6 sperm/ml. Six h after culture, the oocytes were transferred to 100 μl droplets of another culture medium and further incubated for 18 h. The culture medium was TCM-199 supplemented with 50 μg/ml gentamicin and 10% FCS.

Examination of oocytes for sperm penetration

After the cultivation, oocytes surrounded by cumulus cells were denuded mechanically using a finely drown pipette. The denuded oocytes were mounted on slide glass, fixed for at least 48 h in acetic acid and ethanol solution (1 : 3, v/v) at room temperature, stained with 1 % lacmoid in 45% acetic acid before been examined by phase-contrast microscopy. Oocytes were considered to be penetrated when they had a sperm head(s) or male pronucleus(ei) and the corresponding sperm tail(s) (Fig. 2). Oocytes containing multiple sperm heads, multiple male pronuclei, or a male pronucleus and a sperm head were classified as polyspermic (Fig. 3). The presence of more than one pronucleus and at least one sperm tail in the ooplasm indicates male pronuclear formation (Fig. 3). Normally fertilized oocyte was characterized by the formation of one male and one female pronuclei.

Statistical analysis

Each experiment was replicated at least 3 times. The data from each experiment were pooled for presentation and a x^2-analysis was used to test significance among groups. A probability of $p<0.05$ was

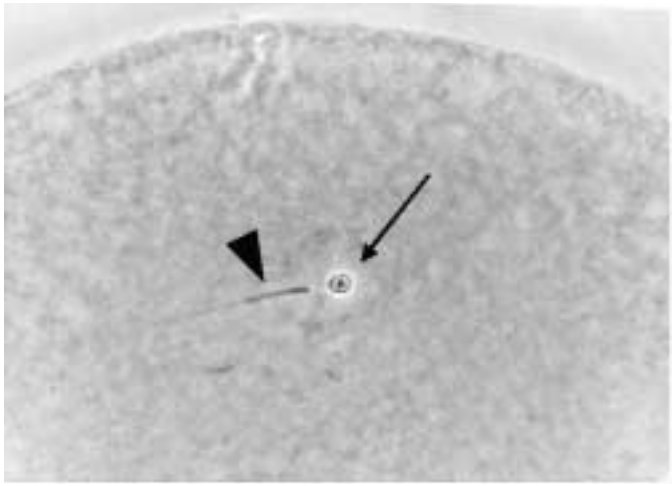

Fig. 2 An oocyte penetrated by a spermatozoon. Arrow and arrow head denote sperm head and tail, respectively.

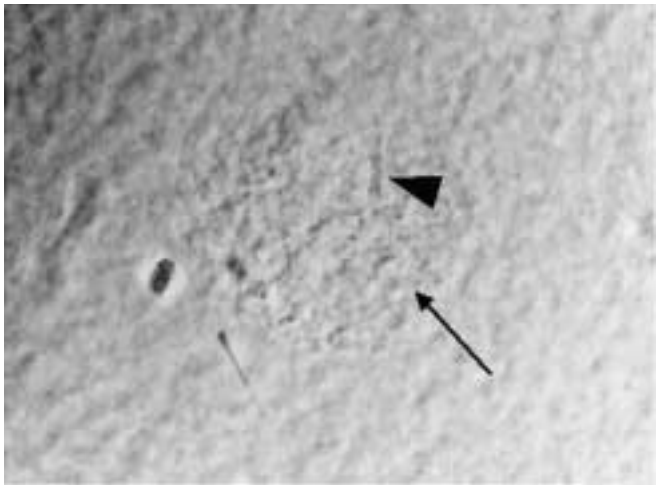

Fig. 3 An oocyte penetrated by two spermatozoa. Head of one spermatozoon formed a male pronuclei (arrow) with the corresponding tail (arrow head).

considered to be statistically significant.

Results

Oocytes were cultured in 100 µl droplets of maturation medium supplemented with bovine follicular fluid for 24 h following an initial culture in 10 µl droplets of maturation medium without bovine follicular fluid for 24 h. After the initial 24 h, most examined oocytes were at the germinal vesicle stage (n=70). After the total 48 h of maturation culture, over 55% of oocytes in each group matured to the

Table 1 The effect of bovine follicular fluid in the maturation medium on sperm penetration in pig oocytes

Sperm conc. (sperm/ml)	Conc. of bFF(%)	No. of oocytes inseminated	No.[%] of oocytes penetrated	No.(%) of oocytes with male pronucleus	No.(%) of polyspermic oocytes	No.[%](%) of oocytes fertilized normally
1×10^5	0	102	59[57.8]^a	40(67.8)^a	45(76.3)^a	9 [8.8]^a (15.3)^a
	10	109	71[65.1]^a	60(84.5)^b	37(52.1)^b	27[24.8]^b (38.0)^b
	20	120	52[43.3]^b	43(82.7) ^{ab}	22(42.3)^b	27[22.5]^b (51.9)^b
1×10^6	0	114	82[71.9]^a	62(75.6)^a	63(76.8)^a	13[11.4]^a (15.9)^a
	10	112	74[66.1]^a	60(81.1)^a	43(58.1)^b	24[21.4]^b (32.4)^b
	20	108	70[64.8]^a	55(78.6)^a	47(67.1) ^{ab}	18[16.7]^{ab} (25.7)^{ab}

[ ] As a proportion to inseminated oocytes

( ) As a proportion to penetrated oocytes

$^{a, b}$ Values with different superscripts within column in each sperm concentration were significantly different ($p<0.05$)

second metaphase stage (data not shown). These oocytes were then inseminated with sperm at a concentration of 1×10^5 or 10^6/ml, and the percentages of penetrated oocytes, oocytes with male pronucleus(ei), polyspermic oocytes and the normally fertilized oocytes were assessed. When oocytes were inseminated with sperm at 1×10^5/ml, high concentration of bovine follicular fluid in the maturation medium (20%) led to the significantly ($P<0.05$) lower proportion of penetrated oocytes as compared with those in 0 and 10% bovine follicular fluid groups (Table 1). Moreover, addition of bovine follicular fluid to the maturation medium promoted the male pronucleus formation, with a significant difference between 0 % and 10% bovine follicular fluid groups ($P<0.05$). The proportion of polyspermic oocytes inseminated with sperm at the 1×10^5/ml was also significantly decreased with bovine follicular fluid treatment. The numbers of oocytes fertilized normally in both bovine follicular fluid groups (10 and 20%) were significantly ($P<0.05$) higher than that in 0 % bovine follicular fluid group.

When oocytes were inseminated with sperm at a concentration of 1×10^6/ml, addition of bovine follicular fluid to the second 24-h maturation culture medium decreased the proportion of penetrated oocytes, but increased the proportion of oocytes with male pronucleus, although these effects were not significant (Table 1). Bovine follicular fluid reduced the proportion of polyspermic oocytes, with a significant difference between the 0% and 10% bovine follicular fluid groups ($P<0.05$). The proportion of normally fertilized oocytes was also increased by the addition of bovine follicular fluid to the maturation medium in comparison to the control. This proportion was significantly higher in 10% bovine follicular fluid group than the control ($P<0.05$).

Discussion

Elmileik et al. (1995) demonstrated that the fluid from large bovine follicles contains stimulatory substance(s) that promote the potential of bovine follicular oocytes for the subsequent development to blastocysts and hatched blastocysts. In the present study, sperm penetration in pig oocytes cultured in a maturation medium to which fluid from large bovine follicles (bFF) was added during the second 24 h of

culture was assessed. When matured oocytes were inseminated with sperm at a concentration of 1×10^5/ml, high concentration of bovine follicular fluid in the maturation medium led to the significantly lower proportion of penetrated oocytes than that in control. When the oocytes were inseminated with 1×10^6 sperm/ml, the penetration rates of oocytes matured in the medium with bovine follicular fluid also tended to be lower than that of the control. From these results, it was likely that the high concentration of bovine follicular fluid in the maturation medium suppresses the penetration rate of pig oocytes. However, such inhibition in penetration rate has not been observed when pig oocytes matured in pig follicular fluid-containing medium were fertilized (Naito et al., 1988; Yoshida et al., 1992). These conflicting results with respect to the effect of follicular fluid on sperm penetration might be due to differences in animal species whose ovaries were used to collect follicular fluid.

Naito et al. (1988) reported that porcine follicular fluid has substance(s) that stimulate male pronuclear formation in porcine oocytes. Yoshida et al. (1992) demonstrated that a stimulatory substance(s) with molecular weight between 10,000-200,000 is present in porcine follicular fluid. In the present study, the proportion of oocytes with male pronuclei in the 10 and 20% bovine follicular fluid groups was higher than that of the control, indicating that bovine follicular fluid contains substance(s) that stimulate male pronuclear formation in pig follicular oocytes. Additional work, however, is needed before it can be concluded whether the substances in the bovine follicular fluid are similar to that in the porcine follicular fluid.

When oocytes were inseminated with sperm at 1×10^5/ml, the proportion of polyspermic oocytes decreased significantly in the 10% bovine follicular fluid group compared with the control (52.1% vs 76.3%), but the penetration rate of the same group was higher than the control (65.1% vs 57.8%). In the oocytes inseminated at 1×10^6 sperm/ml, a significantly lower polyspermy rate was provided in the 10% group than the control, although the penetration rate in both of the groups was comparable. These suggest that addition of bovine follicular fluid to maturation medium leads to decrease of polyspermic penetration in pig oocytes matured and fertilized in vitro. It is, therefore, concluded that the ability of matured oocytes to activate polyspermy block system may be augmented by the presence of bovine follicular fluid in the maturation medium.

The following in vitro maturation culture system was used in the present study. Each 20 COCs were initially cultured in 10 μl droplets for 24 h to maintain their arrest at GV stage of maturation, they were then released from their meiotic arrest by the transfer to 100 μl droplets of medium and culturing for another 24 h followed by insemination with spermatozoa. Such reversible meiotic resumption of pig oocytes following their maintenance at GV stage by the culture in a medium supplemented with protein synthesis inhibitor, cycloheximide, was reported by Fulka et al. (1986) and Kubelka et al. (1988). Although they concluded that pig oocytes can resume their meiosis after temporal arresting at GV stage using some drugs, the potential of such oocytes for completion of meiosis or sperm penetration was not examined in their studies. Downs et al., (1986) have conducted a study in which mouse oocytes were maintained at GV stage by culturing in a medium containing maturational inhibitors, dibutyryl cAMP, hypoxanthine and isobutylmetylxanthine (IBMX), and subsequently washed with, and cultured in an inhibitor-free medium to permit their meiotic maturation, and assessed their developmental capacity after insemination. Their results concluded that if oocytes are maintained in meiotic arrest and then allowed to mature, their high developmental potential could significantly be preserved. Lonergan et al. (1997) also reported that culturing of bovine oocytes in a medium supplemented with cycloheximide or 6-dimethylaminopurine to maintain their meiotic resumption followed by releasing them from their

drugs will improve their subsequent cleavage rate and blastocyst formation. Thus, it will be indispensable to explore the fertilizability and developmental potential of pig oocytes temporally arrested with maturational inhibitors before being released and spontaneously matured in vitro.

In vitro maturation of immature oocytes is initiated shortly after the removal from their small antral follicles. However, since the COCs used in the present study were obtained from follicles of ovaries from prepubertal gilts (5-7 months), they are thought to ovulate more than several weeks later. Regarding in vivo maturation of oocytes, several studies have provided evidences that marked changes in oocyte during final stage of folliculogenesis occur as the follicular size increases (Thibault et al., 1987; Eppig and Downs, 1984). Moreover, Lonergan et al. (1997) showed that the changes during late folliculogenesis are a prerequisite for the oocyte to acquire full developmental competence. These observations indicate that not only the final oocyte maturation (the processes occurring from LH surge to ovulation), but also the period preceding the LH surge may be important for the establishment of developmental competence, arguing that a prematuration treatment is necessary to allow the oocytes from small follicles to attain their full developmental competence in vitro (Lonergan et al., 1997). Hence, it was concluded that our maturation system in which oocytes were precultured in a 10 μl droplet for 24 h followed by a second maturation culture for further 24 h to the metaphase II stage, was suitable for normal in vitro fertilization and subsequent development.

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