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Effect of Hormone Treatments during Maturation on Calcium Response and *In Vitro* Development of Bovine Embryos

Kong, I. K. and E. B. Lee*

The Institute for Development of Livestock Production,

Gyeongsang National University

체외성숙 동안에 호르몬 처리에 따른 Calcium 반응과 체외발달에 미치는 영향

공 일 근 · 이 은 봉*

경상대학교 축산진흥연구소

요 약

소 난포란의 체외성숙시 성숙배지에 FSH 및 LH의 첨가가 체외성숙난자의 calcium 반응과 체외수정란의 발달에 미치는 영향을 조사하였다. 난포란의 체외성숙은 TCM199을 기초로 한 4가지의 배양조건 하에서 : 1) 0.5㎍/ml FSH＋5㎍/ml LH, 2) 0.5㎍/ml FSH, 3) 5㎍/ml LH 및 4) 무 호르몬 첨가구로서 5% CO_2에 24시간 동안 체외성숙을 유도하였다. 체외성숙 24기간째에 난포란의 과립막세포는 1ml PB1＋에서 4분 동안 vortexing을 하여 완전히 제거하였다. 세포 내 calcium 반응을 측정하기 위하여 2mM Fura-2 AM ester 및 0.02% Pluronic F-127가 첨가된 PB1－용액에 39℃ incubator에서 40분 동안 배양하였다. 30㎕ M2 medium drop을 30mm plastic dish에 만들어 20× 형광대물렌즈가 장착된 Nikon Diaphot 현미경의 warm stage에 설치하였다. 세포 내 calcium 방출을 자극하기 위하여 난자에 25mM inositol 1,4,5-trisphophate(IP3)로 1.21kV/cm의 전기자극 또는 20mM ryanodine으로 미세주입을 실시하였다. 이러한 처리를 하지 않은 난자는 체외수정 후 CR1aa와 BRL monolayers의 공배양조건 하에서 체외발달을 유도하였다. 분할율(Day 2)과 배반포기발달율(Day 9)을 조사하였다. FSH와 LH의 처리구에서 IP3 또는 ryanodine으로 자극된 난자(1.79±0.05, 1.66±0.06)는 FSH, LH 및 무 호르몬처리구에 비하여 유의적으로 높은 calcium 반응을 보였다(1.00±0.03, 1.28±0.04, and 0.53±0.02 in IP3 elctroporation; 0.68±0.05, 1.03±0.05, and 0.47±0.04 in ryanodine microinjection). FSH와 LH, FSH, LH처리구에서의 분할율(87.9, 71.5 및 75.6%)은 무 호르몬처리구(60.7%)($P<0.05$)에 비하여 유의적으로 높았으며, FSH와 LH처리구(29.3%)에서의 배반포기 발달율은 FSH, LH 처리구뿐만 아니라 무 호르몬처리구보다 유의적으로 높았다(16.5, 19.0 and 9.8%)($P<0.05$). Bovine FSH 및 Ovine FSH의 처리구에서의 calcium 반응은 유의적인 차이가 없었다(1.72±0.05, 1.61±0.06). 또한 분할율(82.2 and 84.0%) 및 배반포기(27.8 and 27.1%) 발달율도 bovine 및 ovine FSH처리구간에는 유의적인 차이가 없었다.

이상의 결과에서 전기자극에 의한 세포 내 calcium 반응은 체외성숙배지에 첨가하는 호르몬의 처리에 따라서 유의적인 변화를 보였다. 비록 분할율은 처리구간에 유의적인 차이가 없었지만 배반포기 발

* 순천대학교 농과대학(Suncheon National University, College of Agriculture)

달율은 FSH와 LH 공동처리구에서 FSH, LH 단독처리구 및 무 호르몬처리구에 비하여 유의적으로 높은 발달율을 보였다. 체외성숙기간에 FSH와 LH의 공동첨가는 체외성숙 및 체외발달의 생리적인 교정을 위하여 요구되는 것으로 사려된다.
(Key words : IP3, Ryanodine, Calcium response and *in vitro* development)

Ⅰ. INTRODUCTION

The mammalian oocyte is arrested at dyctiate stage of the meiotic prophase, until shortly before ovulation, when the preovulatory gonadotropin surge triggers the resumption of the meiotic process. The preovulatory luteinzing hormones(LH) surge is generally accepted as the endocrine process regulating induction of *in vivo* oocyte maturation, since exposure of the follicle to LH or human chorionic gonadotropin(hCG) induceds maturation(Hillensjo et al., 1978). Completion of maturation ismorphologically identified by metaphase Ⅱ stage(M Ⅱ), and is accompanied by expansion of the layers of the cumulus cells surrounding the oocyte. More recent observations imply that LH is only one of a complex sequence of factors involved in oovyte maturation. Studies with rodents have implicated some growth factors in meiotic maturation.

The developmental competence of *in vitro* matured oocytes is generally lower than that of *in vivo* matured oocytes(Leibfried-Rutledge et al., 1987). The positive effects of bovine estrous serum(Lu et al., 1987), pro-estrous serum(Younis et al., 1989), hormones(Fukushima and Fukui, 1985; Saeki et al., 1991) and added granulosa cells(Leibfried-Rutledge et al., 1989) on IVM oocytes have been reported. These reported suggest that hormones and /or some factors contained in serum play an important role in oocyte maturation.

In mammalian oocytes a series of highly repetitive Ca^{2+} transients lasting several hrs are triggered at fertilization(Cuthbertson et al., 1981; Cuthbertson and Cobbold, 1985; Igusa and Miyazaki, 1986; Sun et al., 1994). The Ca^{2+} transients act both to initiate completion of meiosis and to block polyspermy by stimulating cortical granules exocytosis(Kline, 1988; Kline and Kline, 1992).

The objective of the present study was to evaluate calcium response and development of bovine eggs after four different hormone treatment during *in vitro* maturation. The results of this study indicated that intracellular Ca^{2+} release in response to IP3 electroporation was significantly different depending upon hormone treatments. Although the rate of cleavage did not have any differences in four different treatment groups, the rate of blastocyst development in both hormone treatment group increased significantly rather than those in FSH, LH, and no hormone groups. The addition of FSH and LH during the maturation worked synthetically for maturation and development *in vitro*.

Ⅱ. MATERIALS AND METHODS

1. Preparation of bovine oocytes

Bovine ovaries were obtained from a local slaughterhouse. Cumulus-oocyte complexes were aspirated from antral follicles(2~8mm in diameter) with a hypodermic needle(18G), selected for compact and complete cumulus cell coverage. Selected oocytes were washed three times in HEPES-buffered Tyrode's medium(Fissore et al., 1992), pH 7.4, supplemented with 3mg / ml bovine serum albumin(BSA), 0.2mM pyruvate, 100units /ml penicillin, 100μg /ml streptomycin before maturation *in vitro*.

2. Oocyte maturation and fertilization

Maturation groups of 50 cumulus-enclosed oocytes were transferred into 500μl of TCM-199 (M199 ; HyClone Lab.) medium containing 10% FBS, 100units/ml penicillin, and 100μg/ml streptomycin and four different hormone treatment groups: 1) both hormone(0.5μg/ml FSH +5μg/ml LH), 2) 0.5μg/ml FSH, 3) 5μg/ml LH, and 4) no hormone. They were cultured in 4-well dish(Nunc) at 39℃ in a humidified atmosphere of 5% CO_2 and air for 24hrs. After 24 hrs in maturation medium, then oocyte not used for intracellular calcium measurement were fertilized as previously described(Parrish et al., 1988; Reed et al., 1996). To compare of intracellular calcium response in different FSH hormone, the maturation medium was consisted with 0.5μg/ml bovine-FSH or ovine-FSH as previously described. Also the remaining oocytes after the measurement of calcium response were fertilized to compare of development rate of blastocyst.

3. Ca^{2+} indicator Fura-2 AM loading

At 23h after initiation of maturation, oocytes were vortexed in 1ml PB1+(phosphate-buffered saline plus 5.55mM glucose, 0.32mM sodium pyruvate and 3mg/ml BSA) at setting 8(Vortex Genie Ⅱ) for 4min to remove cumulus cells completely. Oocytes having an extruded first polar body were selected for use in the experiment. Oocytes were loaded with Ca^{2+} indicator by incubation in 2μM Fura-2 AM ester and 0.02% Pluronic F-127(Molecular Probes Inc) in PB1−solution(Ca^{2+}- and Mg^{2+}-free PB1+ with 100μM EGTA) 39℃ in darkness for 40~50min. After loading Fura-2 AM, oocytes were washed with PB1− and maintained in this solution at 39℃ in 5% CO_2 until use.

4. Ryanodine microinjection procedures

A 30mm plastic dish containing 30μl of M2-medium, pH 7.4(M2; Wood et al., 1987) modified by excluding Ca^{2+}, adding 100μM EGTA and replacing BSA with 0.5% polyvinylpyrrolidone to reduce the background was put on a warm stage on a Nikon Diaphot microscope equipped with 20× fluorescence objective. Fura-2 loaded oocytes were washed in M2-medium and transferred into the dish, which was then covered with prewarmed mineral oil. Temperature on the warm stage was maintained at 36.5℃ throughout the experiment. Microinjection was carried out with a Narishige pneumatic microinjector connected to compressed N_2. Ryanodine was dissolved in the vehicle medium(VM) which consisted of PBS, 100μM EGTA and 20mM ryanodine, and purchased from Calbiochem.

5. Electroporation procedures

An IP3 stimulation consisted of transferring oocytes from culture medium to 25μM inositol 1,4,5-trisphosphate(IP3; Molecular Probes), and 0.01mg/ml BSA(IP3 medium). Oocytes were allowed to equilibrate for one minute and then placed in an electrofusion chamber and given a 1.21kV/cm electrical pulse for 70μs. Oocytes were then transferred to 500μl of IP3 medium and placed in a warm air incubator at 39℃ for 20min. The oocytes were then transferred back to culture medium in the CO_2 incubator.

6. Embryo culture

Embryos were cultured at 39℃ in CR1aa medium containing 3mg/ml BSA, 20μl/ml NEM amino acids, 40μl/ml BME amino acids, 10mM glycine, and 1 mM alanine over BRL cells at 50~60% confluency in 5% CO_2 in air. After 24h, cleaved embryos were removed, counted and transferred into a new culture dishs. Embryos

were transferred to fresh culture media and BRL monolayer cells every 3 days. The development of blastocysts were evaluated at Day 7 and 8 after insemination(insemination : Day 0).

7. Statistical analysis

The data of intracellular calcium response after treatment of four different maturation conditions or two different hormone comparison was analyzed by F-test analysis. Also the data of embryonic development to specific stage was analyzed by χ^2 analysis.

Ⅲ. RESULTS AND DISCUSSION

1. Intracellular calcium response according to addition of hormone

Fig. 1 summarized the peak value of calcium response according to four different hormone treatment during *in vitro* maturation and subjected to a 1.21kV/cm electroporation with 25mM IP3 or microinjected with 25mM ryanodine in each treatment groups. Oocytes in both hormone treatment groups stimulated with IP3(1.79±0.05, 1.66±0.06) exhibited significantly higher peak values as compared to FSH alone, LH alone, and no additional hormone groups(1.00±0.03, 1.28±0.04, and 0.53±0.02), respectively. Also oocytes in both hormone treatment groups microinjected with ryanodine(1.66±0.06) exhibited significantly higher peak values as compared to FSH alone, LH alone, and no additional hormone groups(0.68±0.05, 1.03±0.05, and 0.47±0.04), respectively.

The intracellular calcium release response was changed according to the hormone treatment during *in vitro* maturation. As so far, all of the research added various hormone in maturation medium for getting of completely matured oocytes and then fertilized of oocytes. The ability of ryanodine injected into oocytes to increase $[Ca^{2+}]_i$ is strain-specific in mouse(Swann, 1992; Jones et al., 1995), and thus may explain the results of Kline and Kline(1994) who found no store of Ca^{2+} in mouse oocytes. Yue et al.(1995) and Fissore et al.(1995) reported that IP3-mediated Ca^{2+} release is present in unfertilized bovine eggs and that it plays an essential role in the generation of fertilization-associated $[Ca^{2+}]_i$ oscillations. Also Kong et al.(1996) reported that Ca^{2+} release in response to either microinjection or electroporation of IP3 did not changed during various maturation periods, while the responsiveness to 200μM of ryanodine injection gradually increased during early maturation(16 or 20h) to levels observed at later maturation

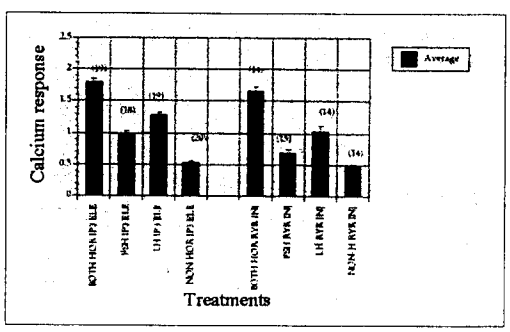

Fig. 1. The calcium response of bovine oocytes following IP3 electroporation or ryanodine microinjection in various hormones addition groups.

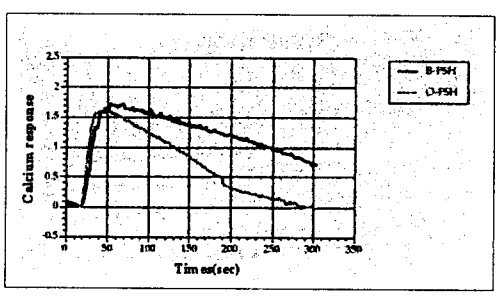

Fig. 2. The calcium response of bovine oocytes to IP3 electroporation according to the addition of B-FSH or O-FSH.

periods(24, 28, 32, and 40h). The percent of oocytes within time periods responding the ryanodine injection increased from 45 and 71% for 16 and 20h to 95, 90, 100 and 90% for 24, 28, 32, and 40h groups. Ryanodine recepter activity increased from 16 to 24h maturation then remained constant to 40h, while IP3 receptor activity remained constant throughout the 16 to 40h maturation periods. As shown in Fig. 2, the calcium response of bovine oocytes to IP3 electroporation according to bovine FSH or ovine FSH was not significnatly different(1.72±0.05, 1.61±0.06).

2. Developmental competence according to maturation treatment

The remaining oocytes excepted of intracellular calcium release measurement after treatments of four different maturation condition were fertilized and cultured to determine their rate of development to the blastocyst stage *in vitro*. The cleavage rates in FSH and LH, FSH alone, and LH alone were significantly higher rates(77.6, 71.5 and 75.6%, respectively) compared with no additional hormone group(60.7%)($P < 0.05$). The blastocyst development rate in FSH and LH was significantly higher rates(29.3%) rather than in FSH alone, LH alone as well as no additional hormone groups(16.5, 19.0 and 9.8%, respectively)($P < 0.05$). Also the most of blastocyst in FSH and LH, FSH alone and LH alone groups were developed at Day 7(74/85, 36/43, and 40/46, respectively) than in no additional hormone group(19/25).

To compare of intracellular clacium response and developmental competence in bovine or ovine FSH, the embryos were matured in maturation medium, in which was consisted with 0.5μg/ml bovine or ovine FSH as previously described. As shown Fig. 2 and Table 2, there was no significant difference ($P < 0.05$) of the calcium response between bovine or ovine FSH addition groups (1.72±0.05, 1.61±0.06). Also the rate of cleavage (82.2 and 84.0%) and development to blastocyst (27.8 and 27.1%) were no significant difference between bovine and ovine

Table 1. Effect of hormone treatments during *in vitro* maturation on the development of bovine embryos

Type of hormone treatments	No. of oocytes used	No. (%) of embryos cleaved	No. (%) of blastocyst developed		
			Day 7	Day 8	Total
FSH & LH	290	255(87.9)[a]	74(25.5)[a]	11(3.8)	85(29.3)[a]
FSH alone	260	186(71.5)[a]	36(13.9)[b]	7(2.7)	43(16.5)[b]
LH alone	242	183(75.6)[a]	40(16.5)[b]	6(2.5)	46(19.0)[b]
No Hormone	255	155(60.7)[b]	19(7.5)[c]	6(2.4)	25(9.8)[c]

* Values with different superscripts in the same column were significant difference($P < 0.05$).

Table 2. Effect of bovine or ovine FSH addition during *in vitro* maturation on the development of bovine embryos

Type of hormone treatments	No. of oocytes used	No. (%) of embryos cleaved	No. (%) of blastocyst developed		
			Day 7	Day 8	Total
B-FSH	259	213(82.2)[a]	45(17.4)[a]	27(10.4)	72(27.8)[a]
O-FSH	443	372(84.0)[a]	76(17.2)[a]	44(9.9)	120(27.1)[a]

* No significant difference($P < 0.05$).

FSH groups ($P < 0.05$). The positive effects of hormones on IVM oocytes have been reported (Fukushima and Fukui, 1985; Saeki et al., 1991).

From the above results, the addition of hormones during the maturation in vitro enhanced the calcium response of bovine matured oocytes as well as the development of IVM-IVF bovine embryos. However, there was no significantly different effects between FSH or LH in maturation medium, but no addition hormone group was significantly decreased the calcium response and development of IVM-IVF bovine embryos. The results indicated that the addition of both FSH and LH during the maturation is required for physiologically correct maturation and development *in vitro*.

Ⅳ. SUMMARY

The present study evaluated the effect of FSH and LH treatment during *in vitro* maturation (IVM) of bovine oocytes on the calcium response and subsequent embryo development in vitro. Bovine oocytes were cultured in four different TCM-199 based IVM media : 1) 0.5μg /ml FSH + 5μg /ml LH, 2) 0.5μg /ml FSH, 3) 5μg /ml LH, and 4) no additional hormone in 5% CO_2 for 24h. Twenty four hours after the initiation of maturation, oocytes were vortexed in 1ml PB1+ for 4min to completely remove cumulus cells. For measurement of intracellular calcium, oocytes were incubated in 2mM Fura-2 AM ester and 0.02% Pluronic F-127 in PB1-solution at 39℃ in darkness for 40min. A 30mm plastic dish containing 30μl M2-medium, pH 7.4 was put on a warm stage on a Nikon Diaphot microscope equipped with 20× fluorescence objective. To stimulate the release of intracellular calcium, oocytes were subjected to 1.21kV/cm electric pulse in 25mM IP3 or microinjected with 20 mM ryanodine. After 24h maturation, remaining oocytes were fertilized *in vitro* and were cultured in CR1aa over BRL monolayers. The number of cleaving (Day 2) or attaining blastocyst development (Day 9) were recorded. Oocytes in FSH and LH treatment groups stimulated with IP3 or ryanodine (1.79±0.05, 1.66±0.06) exhibited significantly higher peak values as compared to FSH alone, LH alone, and no additional hormone groups (1.00±0.03, 1.28±0.04, and 0.53±0.02 in IP3 elctroporation; 0.68±0.05, 1.03±0.05, and 0.47±0.04 in ryanodine microinjection), respectively. The cleavage rates in FSH and LH, FSH alone, and LH alone were significantly higher rates (77.6, 71.5, and 75.6%, respectively) compared with no additional hormone group (60.7%)($P < 0.05$). The blastocyst development rate in FSH and LH, FSH alone, and LH alone was significantly higher rates (29.3%) rather than in FSH alone, LH alone as well as no hormone additional group (16.5, 19.0, and 9.8%, respectively)($P < 0.05$). There was no significant difference of calcium response between bovine and ovine FSH treatment groups (1.72±0.05, 1.61±0.06). Also the rate of cleavage (82.2 and 84.0%) and blastocyst development (27.8 and 27.1%) was no significant difference between bovine FSH and ovine FSH group ($P < 0.05$).

The results indicated that intracellular Ca^{2+} release in response to electroporation changed significantly with hormone treatment during maturation. Although the cleavage rates were not different among treatment groups, the rate of blastocyst development when both LH and FSH were added to maturation medium increased significantly compared to those in FSH, LH, and no hormone groups. The addition of both FSH and LH during the maturation is required for physiologically correct maturation and development *in vitro*.

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