韓國家畜繁殖學會誌 19(3) : 191 ~ 195(1995)
Korean J. Animal Reprod.

Development of a New Improvement and Multiplication System in Domestic Animals Using a Embryonic Manipulation Technique
Ⅱ. Effects of Duration and Concentration of Cytochalasin D on Parthenogenetic Activation and Development of Bovine Follicular Oocyte

Im, K. S. and H. J. Kim

Department of Animal Science and Technology, College of Agriculture and Life Science, Seoul National University, Suweon, 441-744, Korea

세포조작 기술을 이용한 새로운 축산개량증식 체계 개발
Ⅱ. Cytochalasin D의 처리시간과 농도가 소 난포란의 단위발생의 활성화와 발달에 미치는 효과

임경순 · 김현종

서울대학교 농업생명과학대학 동물자원과학과

요 약

본 연구는 체외성숙한 소의 난포란을 ethanol로 활성화시켰을 때 cytochalasin에 노출한 시간과 농도가 난포란의 배반포단계로의 발달에 미치는 영향을 검토하였다. 30시간 체외성숙시킨 소 난포란을 7% ethanol이 든 Dulbecco's phosphate buffered saline에 7분간 노출시켜 활성화를 시킨 후, cytochalasin D가 첨가된 TCM199+10% fetal calf serum에서 일정시간 배양하여 세포주기를 억제시켰다. 난포란을 활성화시킨 후, 0, 5, 10 그리고 15 시간 cytochalasin D (5 μg/ml)에 노출시켰을 때, 5시간에서 가장 높은 배반포(16%), 팽윤배반포(13%), 부화배반포(7%)로의 발달률을 보였다. 또한 cytochalasin D 0, 2.5, 5 그리고 7.5 μg/ml에서 7시간 노출시켰을 때, 2.5 μg/ml에서 가장 높은 배반포(13%), 팽윤배반포(7%), 부화배반포(4%)로의 발달률을 보였다. 결론적으로 cytochalasin D는 난포란의 단위발생에 중요한 역할을 하며, 성숙한 난포란은 cytochalasin D 2.5 μg/ml에서 5시간 노출하였을 때 가장 높은 배반포로의 발달률을 보여주었다.

Ⅰ. INTRODUCTION

Parthenogenones developed up to blastocysts in rabbit (Ozil, 1990) and bovine (Fukui et al., 1992; Minamihashi et al., 1993) and up to the somite stages especially in mouse (Kaufman et al., 1977). Parthenogenetic activation of mammalian oocyte can be induced by their exposure to ethanol (Nagai, 1992), ionophore (Ware et al., 1989) and electric current (Ozil, 1990). The parthenogenetic technique for activation of fol-

This work was supported by a Grant (1995) from Korean Research Foundation in Scientific Technical Cooperation Project between Korea and Japan.

licular oocyte was very useful for the study on nuclear transfer of the embryos (Ware et al., 1989; Presicce and Yang, 1994), role of male and female gametes (Surani and Barton, 1983; Navara et al., 1994) and developmental capacity of oocytes excluding sperm factor (Yoo et al., 1993). Especially, if early parthenogenones are used for investigation of culture system, more precise results will be obtained.

Although there were many studies on parthenogenetic activation method, ethanol treatment principally was used to obtain diploid parthenogenetic embryo because of comparative simplicity and effectiveness (Kaufman, 1983). Especially, Balakier and Tarkowski (1976) reported cytochalasin could suppress second polar body extrusion and so when arrest condition would be removed, the oocytes would be developed with two pronuclei.

In this study, the exposure time and concentration of cytochalasin D to induce cleavage arrest were examined.

Ⅱ. MATERIALS AND METHODS

1. Preparation and *in vitro* maturation of bovine follicular oocyte

Oocytes were recovered from ovaries collected from a slaughterhouse. The ovaries were transported to the laboratory in 35~38℃ saline containing antibiotics. They were washed and dried with tissue paper. Oocytes were aspirated from 1~7 mm follicles using 18 gauge needle connected to 10 ml syringe. Following sedimentation in 15 ml tube the oocytes were transferred into the petri dish (Becton-Dickinson, USA). Those oocytes with intact, unexpanded cumulus oophorous and evenly granulated cytoplasm were only selected under stereomicroscope, washed with culture medium and matured in CO_2 incubator (5% CO_2: 95% air with high humidity at 39℃). The 30 oocytes were placed into 1 ml medium under paraffin oil in the culture dish (Multidish 4, Nunclon, Denmark). TCM199 containing 25 mM HEPES buffer (GIBCO, USA) supplemented with heat treated fetal calf serum (GIBCO), 1 μg/ml FSH (Sigma), and antibiotics was used as maturation medium.

2. Activation and development of follicular oocyte

After 30 hours culture, the oocyte-cumulus complexes in phosphate buffered saline (PBS) containing 0.2% hyaluronidase (Sigma, USA) were vortexed for 3 minutes to eliminate cumulus cells. The denuded oocytes were washed with PBS, exposed to PBS with 7% ethanol (v/v) for 7 minutes, and washed again with PBS. The oocytes washed with TCM199 containing cytochalasin D were cultured in TCM199 containing cytochalasin D.

Experiment 1. The effect of duration of exposure to cytochalasin D was examined. The medium was composed of TCM199 + 10% fetal calf serum (FCS) + 5 μg/ml cytochalasin D. After exposure for 0, 5, 10 and 15 hr, the oocytes were washed with culture medium composed of TCM199 + 10% FCS and cocultured with cumulus monolayer during 7 days. For activation assessment, the oocytes cultured for 12 hr were stained by rapid staining method (Byun et al., 1991). The development of embryos to blastocyst, expanded blastocyst and hatched blastocyst was checked at every day from day 7 to 10 during incubation.

Experiment 2. The effect of concentration of cytochalasin D was examined. After activation, the oocytes were cultured in the medium containing 0, 2.5, 5, 7.5 μg/ml cytochalasin D for 7 hr. The activation and development of the embryos were examined by same method as shown in Experiment 1.

3. Statistical analysis

A randomized block design was utilized and data were collected from 3 replicates. The data were analyzed by Chi-square test and comparisons between means were made by Tukey-test.

Ⅲ. RESULTS

Experiment 1. Effect of duration of exposure to cytochalasin D on activation and development of follicular oocyte

In this experiment, in vitro maturation rate of follicular oocyte was 95% (19/20). The activation rate of oocyte cultured in the medium containing cytochalasin D during 0, 5, 10 and 15 hr was 73, 80, 93 and 69% respectively. The rate of blastocyst developed from the oocyte cultured for 0, 5, 10 and 15 hr in the medium containing cytochalasin D was 7, 16, 11 and 9% respectively. The rate of expanded blastocyst corresponded to 0, 5, 10 and 15 hr was 2, 13, 7 and 7% respectively. Also the rate of hatched blastocyst corresponded to 0, 5, 10 and 15 hr was 2, 7, 4 and 4%, respectively. Five hours showed highest developmental rate to blastocyst (16%), expanded blastocyst (13%) and hatched blastocyst (7%).

Experiment 2. Effect of concentration of cytochalasin D on activation and development of follicular oocyte

In this experiment, in vitro maturation rate of follicular oocyte was 96% (27/28). The activated oocytes were cultured in TCM199 containing cytochalasin D for 7 hr. Activation rate of

Table 1. Effect of duration of exposure to cytochalasin D (5 μg/ml) on activation and development of follicular oocyte

Duration of exposure (hr)	Stained		Cultured			
	No. of oocytes treated	No. of oocytes activated (%)	No. of oocytes treated	No. of Bl.(%)	No. of Ex.Bl.(%)	No. of Hat.Bl.(%)
0	15	11 (73)	45	3 (7)	1 (2)	1 (2)
5	15	12 (80)	45	7 (16)	6 (13)	3 (7)
10	15	14 (93)	45	5 (11)	3 (7)	2 (4)
15	16	11 (69)	45	4 (9)	3 (7)	2 (4)

Bl: blastocyst, Ex.Bl: expended blastocyst, Hat.Bl: hatched blastocyst.

Table 2. Effect of concentration of cytochalasin D on activation and development of follicular oocyte

Concentration of cytochalasin D (μg/ml)	Stained		Cultured			
	No. of oocytes treated	No. of oocytes activated (%)	No. of oocytes treated	No. of Bl. (%)	No. of Ex.Bl.(%)	No. of Hat.Bl.(%)
0	16	10 (63)	75	1 (1)[a]	1 (0)	1 (0)
2.5	15	9 (60)	75	10 (13)[b]	6 (7)	3 (4)
5	15	13 (87)	75	5 (7)[ab]	3 (4)	2 (4)
7.5	16	9 (56)	75	5 (7)[ab]	3 (4)	2 (3)

[a,b] Values with different superscripts differ significantly by Tukey's studentized range test ($P<0.05$).

the oocyte exposed in 0, 2.5, 5 and 7.5 μg/ml cytochalasin D was 63, 60, 87 and 56%, respectively and corresponding developmental rate to blastocyst was 1, 13, 7 and 7%, respectively and corresponding hatching rate was 0, 4, 4 and 3%, respectively. Cytochalasin D 2.5 μg/ml showed highest developmental rate to blastocyst (13%), expanded blastocyst (7%) and hatched blastocyst (4%).

Ⅳ. DISCUSSION

The effects of duration of exposure to cytochalasin D (CD) and concentration of cytochalasin D in the medium on development of follicular oocyte were examined. In this study, the optimal duration of exposure to CD was 5 hr as because 5 hr showed highest developing rate to blastocyst (16%), expanded blastocyst (13%) and hatched blastocyst (7%). Also highest rate of blastocyst (16%), expanded blastocyst (8%) and hatched blastocyst (4%) showed in 2.5 μg/ml CD. Fukui et al. (1992) and Nagai (1992) also examined appropriate concentration of cytochalasin B (CB) and duration of culture in CB. Their results showed similar tendency to this experiment. Fukui et al. (1992) reported that following 30 hr maturation culture when the activated oocytes were cultured in 2.5 μg/ml of CB for 5 hr or 10 hr, developmental rate to blastocyst was 4.8 or 7.2% respectively and the corresponding developmental rate was 9.4 or 5.2% in 5.0 μg/ml CB, respectively. Nagai (1992) reported that the optimal concentration of CB was 5.0 μg/ml and the optimal exposure time to CB was between 6 and 10 hr. There was a little difference in the results between the reports and this paper. The difference might be caused by specific function of CB and CD. Cytochalasin B inhibited second polar body extrusion in embryos activated in vitro by heat shock and induced a high incidence of 2 pronuclei in parthenogenetic embryos (Balakier and Tarkowski, 1976). Presicce and Yang (1994) reported cytochalasin B had no effect on cleavage but significantly improved the development of the activated oocytes to blastocyst. The result is consistent with our result that oocyte exposed to CD also improved the development to blastocyst. The mechanism that cytochalasin enhances development of embryo to blastocyst would be ability of diploidizing haploid parthenogenetic embryos. Siracusa et al. (1980) indicated that the cytochalasin D activated mouse embryos, disrupted the microfilament component of cytoskeleton and prevented cytokinesis, but had the distinct advantage over cytochalasin B because of greater specificity of action on microfilament.

In conclusion, cytochalasin D played very important role for parthenogenetic development of follicular oocyte and 5 hr of exposure time to CD and 2.5 μg/ml CD in the medium was optimal for development of the follicular oocyte.

V. SUMMARY

Bovine follicular oocytes were matured in vitro for 30 hr and exposed to Dulbecco's phosphate buffered saline containing 7% ethanol for 7 minutes for their activation, and incubated in TCM199 containing 10% fetal calf serum (FCS) and cytochalasin D (CD). When the oocytes were exposed to cytochalasin D for 0, 5, 10 and 15 hr, 5 hr showed highest developmental rate to blastocyst (16%), expanded blastocyst (13%) and hatched blastocyst (7%). Also when the oocytes were cultured for 7 hours in medium containing 0, 2.5, 5 and 7.5 μg/ml cytochalasin D, 2.5 μg/ml showed highest developmental rate to blastocyst (13%), expanded blastocyst (7%) and hatched blastocyst (4%).

In conclusion, cytochalasin D played very important role for parthenogenetic development of follicular oocytes and 5 hr of exposure time to CD and 2.5 μg/ml CD in the medium was optimal for development of the follicular oocyte.

Ⅵ. REFERENCES

1. Balakier, H. and A.K. Tarkowski. 1976. Diploid parthenogenetic mouse embryos produced by heat shock and cytochalasin B. J. Embryol. exp. Morph. 35:25-39.
2. Byun, T.H., S.H. Lee and, H.B. Song. 1991. Development of rapid staining method for nucleus of the oocyte from domestic animals. Korean J. Anim. Sci. 33:25-31.
3. Fukui, Y.K., Sawai, M. Furudate, N. Sato, Y. Iwazumi and K. Ohsaki. 1992. Parthenogenetic development of bovine oocytes treated with ethanol and cytochalasin B after in vitro maturation. Mol. Reprod. Dev. 28:405-409.
4. Kaufman M.H., S.C. Barton and M.A.H. Surani. 1977. Normal postimplantation development of mouse parthenogenetic embryos to the forelimb bud stage. Nature 265:53-55.
5. Kaufman, M.H. 1983. Early mammalian development: parthenogenetic studies. Cambridge. Cambridge Uinversity Press, pp. 20-62.
6. Minamihashi, A., A.J. Watson, P.H. Watson, R.B. Church and G.A. Schultz. 1993. Bovine parthenogenetic blastocysts following in vitro maturation and oocyte activation with ethanol. Theriogenology 40:63-76.
7. Nagai, T. 1992. Development of bovine in vitro matured follicular oocytes activated with ethanol. Theriogenology 37:869-875.
8. Navara, C.S., N.L. First and G. Schatten. 1994. Microtubule organization in the cow during fertilization, polyspermy, parthenogenesis, and nuclear transfer: The role of the sperm aster. Dev. Biol. 162:29-40.
9. Ozil, J.P. 1990. The parthenogenetic development of rabbit oocytes after repetitive pulsatile electrical stimulation. Development 109:117-127.
10. Presicce, G. and X. Yang. 1994. Nuclear dynamics of parthenogenesis of bovine oocytes matured *in vitro* for 20 and 40 hours and activated with combined ethanol and cycloheximide treatment. Mol. Reprod. Dev. 37:61-68.
11. Presicce, G.A. and X. Yang 1994. Parthenogenetic development of bovine oocytes matured *in vitro* for 24hr and activated by ethanol and cycloheximide. Mol. Reprod. Dev. 38:380-385.
12. Siracusa, G, D.G. Whittingham and M. De Felici. 1980. The effect of microtubule and microfilament-disrupting drugs on preimplantation mouse embryos. J. Embryol. Exp. Morph. 60:71-82.
13. Surani, M.A.H. and S.C. Barton. 1983. Development of gynogenetic eggs in the mouse: Implications for parthenogenetic embryos. Science 222:1034-1036.
14. Ware, C.B., F.L. Barnes, M. Maiki-Laurila and N.L. First. 1989 Age dependence of bovine oocyte activation. Gamete Res. 22: 265-275.
15. Yoo, H.J., S.C. Choi. and S.H. Lee. 1993 Nuclear maturation and pronuclei formation in bovine oocytes matured in vitro for prolonged period. Korean J. Animal. Reprod. 17:331-337.