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Nuclear Maturation and Pronuclei Formation in Bovine Oocytes Matured *In Vitro* for Prolonged Period

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체외 성숙 시간에 따른 소 난자의 처녀 발생

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적 요

처녀발생은 난자의 세포질성숙을 투명대경화나 체외수정에 있어 난자 외적요소의 문제점을 배제하고 측정할 수 있는 지표이다. 본 실험에서는 체외성숙시간에 따른 소 난자의 처녀발생활성을 조사하였다. 도살장 난소로부터 회수한 미성숙난포란을 15% 소 태아혈청이 첨가된 TCM 199에서 6시간 간격으로 24~48시간까지 성숙시킨 후 7% ethanol로 7분간 활성화시켰다. 핵성숙과 세포질성숙은 rapid staining에 의해 핵형태와 전핵의 형성 유무로 판정하였다. 핵성숙율은 24~48시간 사이 각각 81, 89, 72, 60 및 60%로 체외성숙 36시간에 성숙율이 최고였으나, 반면 감수분열 중기 II 염색체이상은 36시간부터 증가(0~30%)하였다. 에탄올처리에 의한 전핵형성율은 체외성숙 24~48시간에 각각 67, 68, 73, 84 및 87%였고, 그 중 이배체율은 각각 4, 5, 10, 16 및 20%로 성숙시간이 증가함에 따라 증가하였다. 위 실험의 결과 난자의 체외성숙 연장에 따라 전핵형성과 이배체수가 증가되는 것으로 나타났으며, 정상적인 핵성숙에 비해 세포질성숙은 더 많은 성숙시간이 필요한 것으로 나타났다. 이러한 결과들은 소 초기배 체외생산시와 핵치환용 핵수용란 생산시 적정 성숙시간 결정에 유용하게 이용될 수 있을 것이다.

(Key words: *in vitro* maturation, oocyte ageing, parthenogenetic activation, bovine oocyte)

I. INTRODUCTION

Oocyte maturation represents the period which undergoes a series of intracellular changes from the dictate to the metaphase II stage of meiosis. (Thibault *et al.*, 1987; Kumar *et al.*, 1991). This maturation process involves at least three most obviously characterized events : nuclear maturation, cytoplasmic maturation and cumulus expansion (Wassarman, 1988; Sun and Moor, 1991).

The timing of nuclear maturation progression *in vitro* of bovine oocyte, germinal vesicle to metaphase II, is from 18.0 to 24.0 h (Sirard *et al.*, 1989). However, these oocytes with normal morphological characteristics of nuclear maturation failed to promote normal development of the male pronucleus after sperm penetration and showed a limited capacity for embryonic development (Moor and Trounson, 1977). This normal

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embryonic development would only follow the completion of complex changes, includes microorganelle redistribution and reorganization, which occur during cytoplasmic maturation (Moor *et al.*, 1990). Developmental capacity of these *in vitro* matured oocytes have been assessed by the formation of the pronucleus after fertilization *in vitro*, subsequent developing to the blastocyst stage and the birth of calves after embryo transfer(Gordon and Lu, 1990). However, these assessments are not always accurate because of a failure of *in vitro* fertilization due to the zona hardening and sperm factors.

This study was to examine the developmental capacity of the bovine oocytes recovered from the slaughter house ovaries excluding of sperm factor by parthenogenetic activation. And whether the nuclear maturation time equals the cytoplasmic maturation was investigated by the formation of pronuclei of the activated oocytes. Thus, *in vitro* maturation system of the bovine oocyte was established to supply bovine embryo either for IVF-ET program or research purpose.

Ⅱ. MATERIALS AND METHODS

1. Oocyte maturation

The oocyte cumulus complexes (OCCs) were recovered by the slicing method (Yoo *et al.*, 1993) in D-PBS. Selected good and fair type OCCs were matured *in vitro* in TCM 199 (Gibco, Cat. 380~2340, N. Y., U.S.A.) supplemented with 15% (v/v) FCS (Gibco) and 10 iu/ml PMSG and 10 iu/ml hCG (Intervet Co., Boxmeer, Holland) in a disposable plastic dish (Falcon 2001, Becton Dickson Co., N. J., U.S.A) at 39℃ with 5% CO_2 in air for 24 to 48 h at 6 h intervals.

2. Parthenogenetic activation by ethanol

At the end of IVM, fully expanded cumulus oophori were treated in 3% (w/v) sodium citrate solution to remove expanded cumulus cells (Kinis *et al.*, 1989). The oocytes were activated in 7% (v/v) ethanol(Merck, Darmstadt, Germany) in TCM 199+15% FCS for 7 min(Nagai, 1987, 1992; Lee *et al.*, 1992). The ethanol-exposed oocytes were washed thoroughly in plenty of M2+4% FCS to remove remaining ethanol.

3. The Oocytes co-culture on granulosa cell monolayer(GCM)

GCM was prepared on previously at the start of IVM at the concentration to 1×10^6/ml in 50 μl TCM199+15%FCS under the liquid paraffin oil. Parthenogenetically-activated oocytes were further co-cultured on GCM for 18 h,

4. Analyses

Nuclear maturation and cytoplasmic maturation were analyzed by the nucleus configuration at 24~48 h post-IVM at 6h intervals and by the presence of pronuclei at 18 h post-activation stained by rapid staining method(Byun *et al.*, 1991), observed under a bright field microscope(BH-2, Olympus Opticals Ltd., Tokyo, Japan). The nuclei or pronuclei stained by the rapid staining were recorded on ASA 100 film (SR-100, Konica Film Corp., Tokyo, Japan) with automatic exposure unit (PM-10 ADS, Olympus Opticals Ltd.).

Ⅲ. RESULTS AND DISCUSSION

In vitro matured bovine oocytes obtained from the slaughter house ovaries are one of the most inexpensive and efficient sources to supply bovine embryo either for IVF-ET program or research purpose. However, these *in vitro* matured oocytes with normal morphological char-

acteristics of nuclear maturation showed a limited capacity for embryonic development due to the inadequate cytoplasmic maturation(Moor *et al.*, 1990). Therefore, the bovine oocytes matured *in vitro* should be assessed their cytoplasmic maturation. In this experiments, total 196 bovine oocytes were examined the nuclear and cytoplasmic maturation.

The collected good and fair type OCCs were matured *in vitro* with different prolonged time. Cumulus oophori expansion increased as the maturation time increased(Fig. 1). And typical nuclear configuration stained by rapid staining method was shown in Fig. 2. The proportions of the nuclear maturation were 81, 89, 72, 60 and 60% in IVM 24, 30, 36, 42 and 48 h groups, respectively(Table 1). While the maturation rate was plateau in IVM 30 h, the abnormality in metaphase II chromosomes increased sharply from IVM 36 h (0 to 30%). The typical pronuclei formation in ethanol-activated oocytes were shown in Fig. 3. The rates of the pronuclei

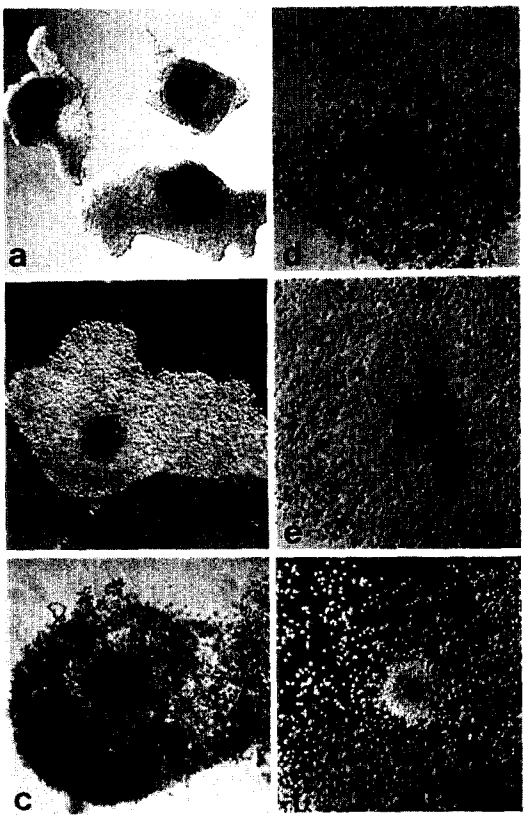

Fig. 1. Cumulus oophori expansion depending upon the maturation period. Cumulus oophori expansion increased as the immature follicular oocytes (a) were matured *in vitro* for 24 (b), 30 (c), 36 (d), 42 (e) and 48 h (f) respectively. Magnifications were × 40.

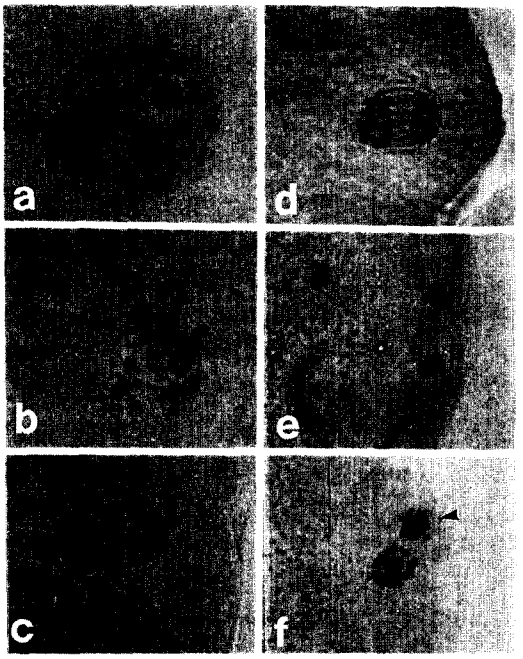

Fig. 2. Chromosome configuration of bovine oocyte undergoing *in vitro* maturation stained by rapid staining. Germinal vesicle stage with distinct nuclear membrane (a); germinal vesicle breakdown with dissolved nuclear membrane (b); metaphase I stage shows chromosome positioned in parallel (c); telophase stage chromosome segregate each other (e) and metaphase II stage of secondary oocyte (f). The first polar body is indicated by an arrow head. Magnifications were × 400.

Table 1. The proportion of the nuclear maturation with different *in vitro* maturation period

IVM (h)	No. of the OCC	No. of the oocytes at the following nuclear stage[1](%)		
		MI	MII	Abn
24	26	5	21(81)	9(0)
30	25	2	22(89)	1(4)
36	25	4	18(72)	3(12)
42	25	6	15(60)	4(16)
48	23	2	14(60)	7(30)

1. The nuclei were stained by rapid staining method.
2. Abbreviations are IVM, *in vitro* maturation; OCC, oocyte cumulus complex: MI, metaphase I: MII, metaphase II and Abn, abnomality in metaphase II chromosome, respectively.

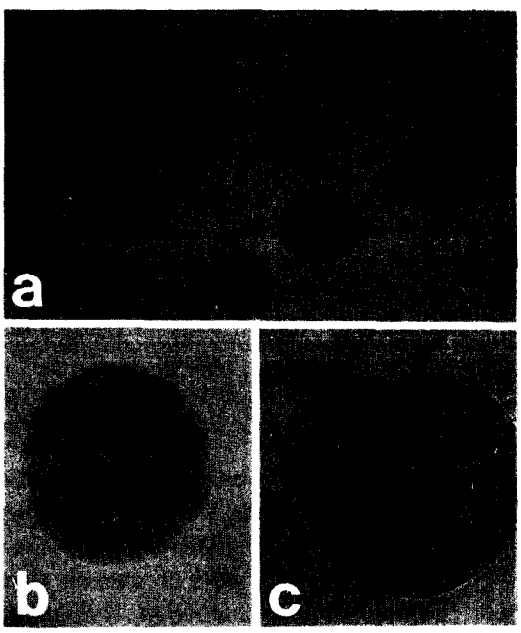

Fig. 3. Parthenogenetically activated oocytes stained by rapid staining show haploid (b) and diploid (c), respectively. Magnifications were × 40 in (a), and × 100 in (b) and (c), respectively.

formation and diploid were 67, 68, 73, 84 and 87%, and 4, 5, 10, 16 and 20% in IVM 24, 30, 36, 42 and 48 h groups, respectively(Table 2) These results indicate that maturational ageing increased the formation of the pronuclei and diploid. The relations among the nuclear maturation, abnormality in M II chromosome, pronuclei formation and diploid were summarized in Fig. 4.

This observation is in agreement with earlier investigations. Ware *et al.*(1989) reported that the activation rates increased from 6 to 96 % of from IVM 18 h to 30 h at 2 h intervals after exposed in 1 μM Ca^{++} ionophore A23187. And also from 8 to 86% of activation rates were obtained from IVM 18 h to 30 h at 2 h intervals by the electric shock with 1 pulse of 30 μsec of 110V. Though the rates of activation were highest in IVM 26 h and 30 h, 15~17% of IVM 26 h and 7~28 % of IVM 30 h oocytes were activated without Ca^{++} ionophore A23187 or electric shock. Barnes *et al.*(1993) reported that the rates of activation of IVM oocytes increased from 45 to 80% of IVM 26 to 32 h. However, the rate of metaphase II decreased and the rate of lysed oocytes increased from IVM 28 h. Lee *et al.*(1992) presented that the ethanol activated

Table 2. The proportion of the pronuclei of the parthenogenetic-activated oocyte with different maturation period

IVM (h)	No. of the OCC	No. of the oocytes at the following No. of pronucleus(%)			Total pronuclei formation rate(%)
		OPN	1PNl	2PN	
24	45	15	28(62)	2(4)	67
30	37	12	23(62)	2(5)	68
36	41	11	26(63)	4(10)	73
42	43	7	29(68)	7(16)	84
48	30	4	20(67)	6(20)	87

1. Activated oocytes were analyzed at 18h post-activation after co-cultrued on GCM.
2. Abbreviations is PN, pronucleus.

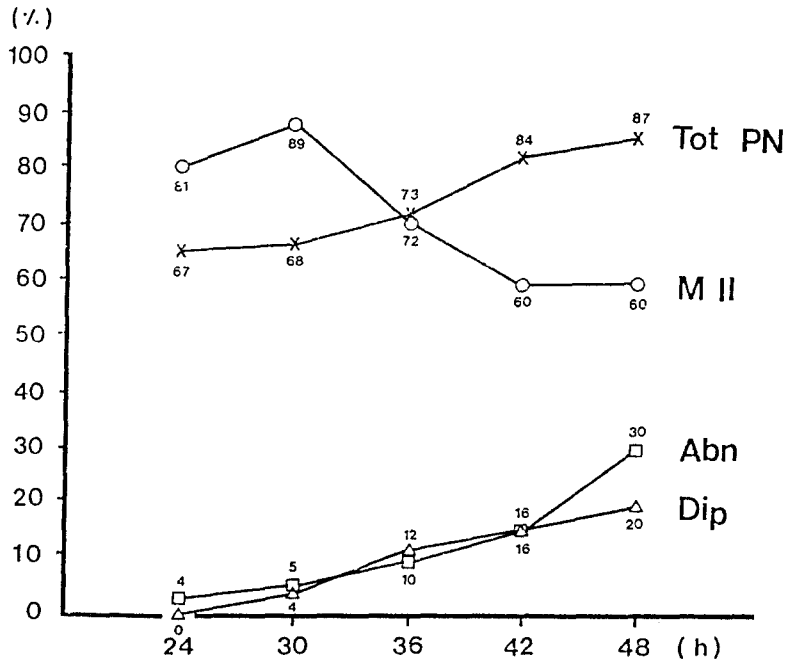

Fig. 4. Relations among the nuclear maturation and pronuclei formation in aged oocytes. Abbreviations are Dip, diploid; MII, metaphase II; MII Abn, abnormality in metaphase II chromosome and Tot PN, total pronuclei, respectively.

bovine oocytes matured *in vitro* successfully developed to the blastocyst stage. The cleavage rates at 48 hpi of activated oocytes were 16.0, 25.8, 29.5 and 34.5% in IVM 24, 30, 36 and 42 h

groups, respectively. The rates of activation increased as the maturation time increased. However, 13%(3/13) of the cleaved oocytes developed to the blastocyst stage only in the IVM 30 h group. These results suggested that oocyte ageing beyond the time required for nuclear maturation is general requirement for activation competence of IVM oocytes(Sirard *et al.*, 1989).

In conclusion, cytoplasmic maturation requires longer maturation period than that of normal nuclear maturation. These results should be useful for determination of an appropriate time for fertilization *in vitro* of mammalian eggs.

Ⅳ. SUMMARY

Response of the oocytes to parthenogenetic activation is one of the indice for cytoplasmic maturation. Maturational age-dependent parthenogenetic activation was examined in bovine oocytes. Follicular oocytes recovered from the slaughter house ovaries were matured *in vitro* in TCM 199+15% FCS+10 iu/ml PMSG +10 iu/ml hCG from 24 to 48 h at 6 h intervals. The *in vitro* matured oocytes were activated by 7% ethanol for 7 min. The nuclear maturation and the cytoplasmic maturation were analysed by the nuclear configuration and pronuclei formation stained by rapid staining method. Cumulus oophori expansion increased as the maturation time increased. Proportions of the nuclear maturation were 81, 89, 72, 60 and 60% in IVM 24, 30, 36, 42 and 48 h groups, respectively. Abnormality in metaphase II chromosome increased sharply from 36 h IVM. The rates of the pronuclei formation and diploid upon ethanol activation were 67, 68, 73, 84 and 87%, and 4, 5, 10, 16 and 20% in IVM 24, 30, 36, 42 and 48 h groups, respectively. It was suggested that maturational age increased the formation of the pronuclei and diploid, and that cytoplasmic maturation require longer maturation period than normal nuclear maturation. These results should be useful for determination of an appropriate time for fertilization in mammalian eggs matured or preincubated *in vitro*.

Ⅴ. REFERENCES

1. Barnes, F., M. Ederbrock, C. Looney, R. Powell, M. Westhusin and K. Bondioli. 1993. Embryo cloning in cattle : The use of *in vitro* matured oocytes. J. Reprod. Fert. 97: 317-320.
2. Byun, T. H., S. H. Lee and H. B. Song. 1991. Development of a rapid staining method for nucleus of the oocyte from domestic animals. Korean J. Anim. Sci. 33:25-31.
3. Gordon, I. and K. H. Lu. 1990. Production of embryos *in vitro* and its impacts in livestock production. Theriogenology 33:77-87.
4. Kumar, J., J. C. Osbourn, A. W. N. Carmeron and A. O. Trounson. 1991. Oocyte maturation. In : Contribution to obstetrics and gynaecology, Vol. 2, pp.21-37.
5. Kinis, A., V. Vergos, M. Gallagher and A. Gordon. 1989. Use of citrate in the denudation of bovine oocytes prior to *in vitro* fertilization. Proc. 5th Conf. Eur. E. T. (Lyon), p.162(Abstr.).
6. Lee, S. H., T. H. Ko, P. Monaghan, P. Longergan, M. Gallagher and I. Gordon. 1992. Successful *in vitro* development of ethanol-activated bovine oocyte to the blastocyst stage following *in vitro* maturation. J. Reprod. Fert. 9:56(Abstr.).
7. Moor, R. M. and A. O. Trounson. 1977. Hormonal and follicular factors affecting maturation of sheep oocytes *in vitro* and their subsequent developmental capacity. J. Reprod. Fert. 49:101-109.

8. Moor, R. M., M. Mattioli, J. Ding and T. Nagai. 1990. Maturation of pig oocytes *in vivo* and *in vitro*. J. Reprod. Fert., Suppl. 40:197-210.
9. Nagai, T. 1987. Parthenogenetic activation of cattle follicular oocyte *in vitro* with ethanol. Gamete Res. 16:243-249.
10. Nagai, T. 1992. Development of bovine *in vitro* matured follicular oocytes activated with ethanol. Theriogenology 37:869-875.
11. Sirard, M. A., H. M. Floman, M. L. Leibfried-Rutldge, F. L. Barnes, M. L. Sims and N. L. First. 1989. Timing of nuclear progression and protein synthesis necessary for meiotic maturation of bovine oocytes. Biol. Reprod. 40:1257-1263.
12. Sun, F. Z. and R. M. Moor. 1991. Nuclear-cytoplasmic interactions during ovine oocyte maturation. Dev. Biol. 111:171-180.
13. Thibault, C., D. Szollosi and M. Gerard, 1987. Mammalian oocyte maturation. Reprod. Nutr. Dev. 27, 865-896.
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. Wassarman, P. M. 1988. The mammalian ovum. In:The physiology of reproduction, Knobil, E. and Neil, J. D., eds. pp.69-102., Raven Press, N. Y., U.S.A.
16. Yoo, H. J., S. C. Choi and S. H. Lee. 1993. Maximization of the number of follicular oocytes recovered from bovine ovaries. Korea J. Anim. Reprod. 17:149-157.