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In Vitro Fertilization and Embryo Culture in Immature Rats induced to Superovulate

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미성숙 흰쥐에 있어서 과잉배란 난자의 체외수정 및 수정란의 배양에 관한 연구

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요 약

미성숙 흰쥐에 있어서 과잉배란 유기시 PMSG 용량이 체외수정에 의한 수정율과 수정란의 배양에 미치는 영향을 조사하고, 체외수정 및 배양용기로서 plastic mini-straw의 이용효과와 체외수정란의 이식성적을 조사한 바 다음과 같은 결과를 얻었다.

미성숙 흰쥐(체중 65~80g)에게 PMSG를 4, 10, 16 혹은 40IU를 1회 근육주사한 후 72시간에 채란한 난자 중에서 난구세포괴를 가진 정상형태의 난자와 정소상체 미부에서 채취하여 예비배양한 정자로 체외수정시켰다. 체외수정율은 PMSG 용량이 증가될수록 감소하였는데, 즉 4IU에서는 70.8%였으나, 40IU에서는 45.0%로 유의적으로($P<0.05$)저하하였다. 그러나 다정자 수정발생율은 2.3~9.7%로서 PMSG 용량에 따른 유의적인 증가는 없었다. 이 결과는 과량의 PMSG의 투여로 배란된 난자 중에서 일부는 비록 난자가 형태학적으로는 난구세포괴를 가지는 정상적인 난자일지라도 체외수정율의 저하는 정상적인 배란시간보다 조기배란으로 난자의 노화로 인하여 수정에 적합하지 않음을 제시하고 있다.

그리고 plastic mini-straw를 고안하여 straw내에서 체외수정시킨 후 66~72시간까지 배양시험한 결과 2-16와 4-16세포기까지 발달된 배의 비율은 petri dish보다 다소 우수($P<0.05$)하였으며, straw용기에서 체외수정된 2세포기의 52개의 배를 7마리의 위임신 흰쥐에게 이식시켰던 바 6개의 배를 이식 받은 한 마리의 수란쥐가 2마리의 새끼를 분만하였다.

(Key words : Superovulation, *In vitro* Fertilization, Plastic mini-straw, Immature rat.)

I. Introduction

Techniques for the induction of superovulation are in common use in the livestock industry particularly when large numbers of embryos are required for embryo transfer. However, the use of gonadotropins for superovulation resulted in a reduction in fertility in large domestic animals (Seidel, et al., 1978; Armstrong and Evans, 1983) as well as in small laboratory animals (Beaumout and Smith, 1975; Miller and Armstrong, 1981). Nuti, Sridharan and Meyer (1975) shown that ovulation induced in immature rats by a single injection of PMSG, could be followed by normal pregnancy but superovulatory does of PMSG(40IU) resulted in partial or complete infertility(Miller and Armstrong, 1981).

When the large superovulatory dose of PMSG was injected in immature rat, superovulated

oocytes were ovulated at the earlier phase after PMSG, and this precocious ovulation was a major source of the increased ovum abnormality (Elizabeth et al., 1983; Lee et al., 1988). In an *in vitro* study, a lower percentage of normal 1-cell ova was recovered from the superovulated rats compared to control animals (Elizabeth et al., 1983), and there was also a reduction in fertilization rate *in vitro* when superovulated oocytes were assessed (Evans et al., 1984).

However, in these two studies no attempt was made to determine the exact proportion of fertilization *in vitro* and subsequent embryo development. It was therefore of interest to determine whether the impairment of fertility in superovulated rats was due to a reduced fertilization rate *in vitro* and subsequent embryo development. The effect of petri dish and plastic mini-straw as culture vessels on the embryo development *in vitro* was also investigated.

II. Materials and Methods

1. Preparation of oocytes and sperm

Immature rats were injected subcutaneously with 4, 8, 16 and 40 IU PMSG (Peamex, Japan) in 0.2ml saline. All animals were killed 70~72h after injection, which was known shortly after normally expected time of ovulation. The ovaries and oviducts were dissected out in D-PBS. The swollen ampullae were torn open carefully with fine forceps. The surrounding cumulus mass cells prevented assessment of oocyte morphology at this stage, but the oocytes used in these experiments were morphologically normality derived from a population which had already been subject to selection against abnormal ova (ova without cumulus mass and/or fragmented ova) discharged.

The processes of oocytes recovery and insemination were completed within 5 minutes following sacrifice, beacause the delayed insemination of longer than 5 minutes might be associated with premature activation of the egg (Keefer and Schuetz, 1982). The oocytes from each animal were transferred to a separate dish containing fertilization medium and were kept in a humidified atmosphere with 5% CO_2 at 37℃. The cauda epididymidis of a mature male rat was removed aseptically, and some of the large distal coils were cut. A drop of the compacted spermatozoa was lifted away with a fine glass needle and transferred to one of the petri dishes (35×10mm, Corning) containing 0.5 ml of m-KRB, and allowed to disperse for 5 minutes. These sperm suspensions were kept in 5% CO_2 for 5h for capacitation. Fertilization dishes containing 0.4ml suspension medium covered thinly with liquid paraffin oil were infused with 20~40μl of the sperm capacitated suspension under oil. The final sperm concentration was in the order of $0.1\sim1\times10^6$ml.

2. *In vitro* fertilization and confirmation

The method for *in vitro* fertilization and suspension medium used were basically same as Toyoda and Chang (1974) and Evans and Armstrong (1984). Fertilization of oocytes in dish or straw were examined with phase-contrast inverted microscope (×400) about 20h after insemination. Oocytes were considered fertilized if they had two pronuclei (Fig. 2. A) and/or at least on sperm tail in the vitellus (Fig. 2. B), and those with more than one sperm tail in the vitellus were classed as polyspermic. The other ova were classed as one-cell unfertilized, or degenerate if they displayed fragmentation or abnormal structure.

3. Culture and transfer of embryos fertilized *in vitro*

Two types of vessels were used for culture of *in vitro* fertilized ova over 66~72h after

insemination. One was petri dish as described in the section of sperm suspension. The other vessel used was a particularly designed plastic mini-straw as shown in Fig. 1. The 0.25ml plastic straw for frozen semen(length : 124mm, OD : 0.2mm) was siliconized and the ends were cut to be 100mm in full length. The mini-straw or petri dish containing the oocytes and/or zygotes were transferred to a fresh medium 20~24h after insemination, refilled again in the straw, and incubated for further 40~48h for development to the advanced cell stage.

The embryos of 2 to 16cell stage showing the blastomeres of similar size were assessed as morphologically normal under microscope. The ova fertilized *in vitro* at two-cell stage 40h after insemination, were transferred into the fimbriated end of the oviducts of pseudo-pregnant female rats. About six to eight eggs were transferred to one of the oviducts. After transfer, the vaginal smears of these recipients were examined daily to determine the reproductive phase afterwards.

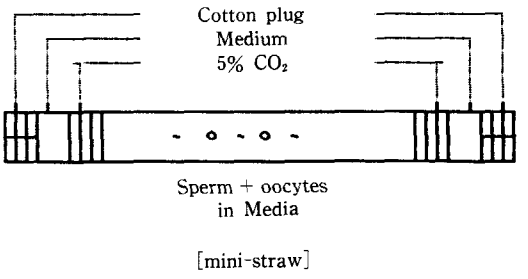

Fig. 1. A design of culture system for *in vitro* fertilization and subsequent embryo culture with plastic mini-straw.

4. Statistical analysis

Comparision between groups were made using χ^2 analysis.

III. Results and Discussion

1. *In vitro* fertilization rates

The *in vitro* fertilization rates of the oocytes from superovulation with the PMSG dosage of 4, 10, 16 and 40IU were observed 70.8, 69.7, 52.4 and 45.0%, respectively(Table 1). The percentage of oocytes fertilized declined with increased dose of PMSG ; significantly($P<0.05$) between PMSG 4~10IU and 16~40IU. Considerable proportions of the ova were penetrated by more than one spermatozoa in all the groups of rats. The proportion of polyspermic penetration varied from 2.2 to 9.7% among groups. There was found no significantly direct relationship between the dosage of PMSG and the incidence of polyspermy of the oocytes superovulated. Of the unfertilized oocytes, an increase in the percentage of the degenerate oocytes was observed in the 16 or 40IU groups compared with those treated with 4 or 10IU

Table 1. In vitro fertilization of morphologically normal oocytes at 20h after insemination in immature rats induced to superovulate with various doses of PMSG.

Dose of PMSG(IU)	Oocytes examined	No. and(%) of oocytes		
		Fertilized normally	Polyspermic	Unfertilized or degenerate
4	96	68(70.8)[a]	6(6.3)[a]	22(22.9)[a]
10	89	62(69.7)[a]	2(2.2)[a]	25(28.1)[a]
16	103	54(52.4)[b]	10(9.7)[a]	39(37.9)[b]
40	258	116(45.0)[b]	21(8.1)[a]	121(46.9)[b]

Figures with different superscripts in the column are significantly($P<0.05$) different.

PMSG.

The present study confirmed that the results of Evans and Armstrong(1984), who reported that the *in vitro* fertilization rates were significantly decreased in the oocytes superovulated with the higher dosage of PMSG, and increased in the percentage which were degenerate. In our experiment, despite of the oocytes derived from a population which had already been subject to selection of abnormal ova discharged, the percentage of oocytes fertilized declined with increasing dose of PMSG. It is apparent that at least some of these oocytes resulted from early ovulations, probably caused by the direct LH-like effect of large doses of PMSG(Kostyk et al., 1978).

This early ovulation might be occurred over a considerable time period(Elizabath et al., 1983; Lee et al., 1988) and degeneration may not occur for at least 36h after ovulation. Therefore, the decreased fertilization seen in superovulate rats may be partly due to oocytes ovulated 48~64h after PMSG injection; thus resulting in ageing of a greater proportion of oocytes in the rats on higher doses of PMSG.

In the present experiments, the oocytes ovulated early do not seem to account fully for the low rate of fertilization in 16 and 40IU PMSG. However, the possiblity remains that significant numbers of abnormal oocytes were ovulated at the normal time of ovulation. In other *in vitro* fertilization studies, PMSG stimulation has been shown to affect the incidence of chromosomal anomalies in mice (Maudlin and Fraser, 1977).

2. Cleavage rates of *in vitro* fertilized ova by PMSG dose and culture vessel

Table 2 shows the cleavage rates of ova at 66~72h postinsemination *in vitro* by the types of culture vessel and by the dosage of PMSG for superovulation treatment. Of the rat oocytes transferred to the preincubated sperm suspension, the group mean percentages of ova fertilized and cleaved *in vitro* were ranged from 48.3 to 64.4% among treatments at 66~72h after postinsemination, and they were not significantly($P<0.05$) different among the

Table 2. Effects of culture vessel and superovulatory PMSG dose on development *in vitro* of fertilized ova for 66 to 72h after *in vitro* insemination.

Culture vessel	PMSG dose (IU)	No.of ova examined*	No. of ova unfertilized or degenerate	No. of ova developed to cell stage of				% ova developed to cell stage of	
				2	4	8	16	2-16	4-16
Petri dish	4	68	25	37	6	0	0	63.2	8.8
	16	72	28	38	7	0	0	61.6	9.6
	40	263	136	111	16	0	0	48.3	6.1
	Total /mean	404	189	186	29	0	0	53.2[a]	7.2[a]
Straw	4	39	14	21	2	2	0	64.1	10.3
	16	121	43	59	12	2	5	64.4	15.7
	40	186	75	73	21	2	15	59.6	20.4
	Total /mean	346	132	153	35	6	20	61.8[b]	17.6[b]

*Only the oocytes with cumulus mass were used for *in vitro* fertilization.

Different superscripts between culture vessels denote a significant different($P<0.05$).

groups of PMSG dosage. Evans and Armstrong (1984) also reported the cleavage rates similar to the result of present study, and they also found no significant difference in cleavage rates of ova between superovulatory PMSG doses.

Although the various factors concerning *in*

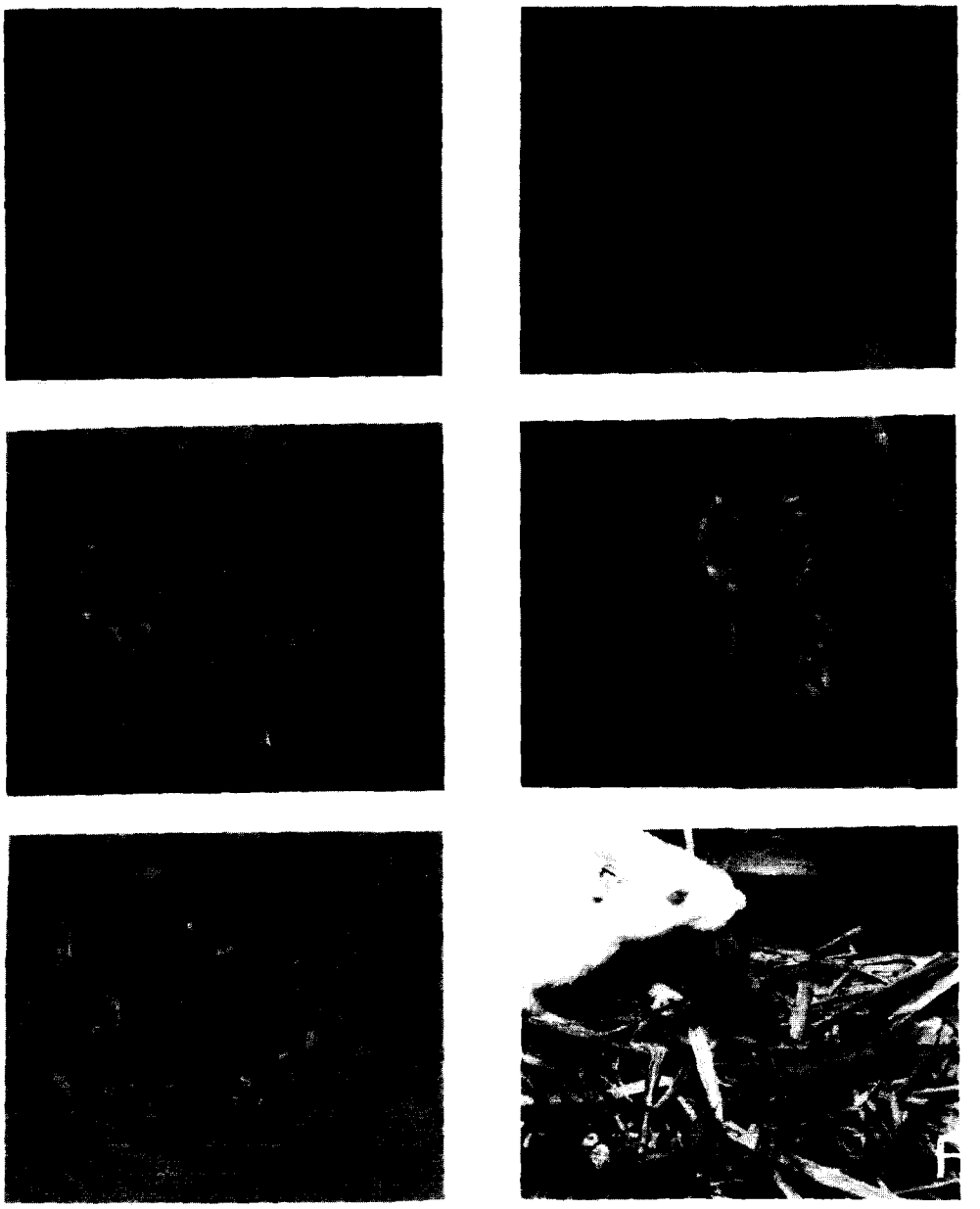

Fig. 2. A, B : In vitro fertilized rat ova at 20h after insemination(ST : sperm tail, PN : pronucleus, PB : polar body, ×400). C, D, E : *In vitro* fertilized rat embryos of various development stages observed after *in vitro* culture in a particularly designed mini -straw for 72h following insemination *in vitro*. F : Two young rat from a recipient mother transferred with *in vitro* fertilized ova.

vitro fertilization and embryo culture in domestic animals have been discussed(Wright et al., 1981), *in vitro* fertilization and/or subsequent embryo culture in a mini-straw system used in the present study, however, have not been reported. In our experiment, compared with the percentage of ova cleaved to 4 or advanced-cell stage in the petri dish(6.1 to 9.6%), a significantly higher($P<0.05$) cleavage rates of ova were obtained in a particularly designed culture vessel of mini-straw system(10.3 to 21.4%). Some fertilized *in vitro* ova could be developed to 16 cell stage in the culture vessel of mini-straw as shown in Fig. 2 C-E. It was often difficult to distinguish normally cleaved embryos from fragmented ones by the techniques used in the present study.

Evans and Armstrong(1984) gave no data for embryo development to more than 2-cell stage in their experiment of *in vitro* fertilization and subsequent development of rat ova. Toyoda and Chang(1974) reported that cleavage beyond the two cell stage was not achieved even after the change of medium and subsequent culture for another 28 to 32h. Although the reasons for superior result from mini-straw system to petri dish as a culture vessel are not clear, the sperm and ova for *in vitro* fertilization and the fertilized ova in mini-straw system might be better protected from contamination, detrimental gases and pH fluctuation in the incubator, as compared with the culture system using petri dish.

3. Transfer results of in vitro fertilized ova

A total of 52 two-cell eggs fertilized in a mini-straw were transferred to seven pseudopregnant rats. Among these recipients, two normal youngs(Fig. 2. F) were born on Day 23 from one recipient which received a total of six eggs. Such a low conception rate of recipient rats transferred with *in vitro* fertilized ova was chiefly due to the technical problem for inserting the 2-cell embryos into the infundibulum of oviduct under microscope.

IV. Summary

The ability of fertilization *in vitro* and subsequent development of superovulated oocytes was assessed in a controlled environment using an *in vitro* fertilization technique. The *in vitro* fertilization percentage of oocytes with cumulus mass declined significantly($P<0.05$) with increased doses from 4~10 to 16~40IU of PMSG for superovulation. However, the porportion of polyspermic penetration varied from 2.3 to 9.7% and there was no significant difference between treatments in incidence of polyspermy. When morphologically normal ova with cumulus mass were cultured for 66~72h in a plastic mini-straw to undergo fertilization *in vitro*, the mean percentage of embryos developed to 2-16 and 4-16 cell stage was 61.8 and 17.6%; it was slightly($P<0.05$) superior to the corresponding results from petri dish. A total of 52 two-cell embryos fertilized in a mini-straw were transferred to seven pseudopregnant rat. Among these recipients, two normal young were born from one recipient which received a total of six embryos. These results suggest that superovulated oocytes are proportionately less competent than normally ovulated oocytes to undergo fertilization in a controlled environment using an *in vitro* fertilization technique. Also, a plastic mini-straw designed was slightly superior to petri dish as a culture vessel for fertilization and embryo development *in vitro*.

V. Reference

1. Armstrong, D.T. and G. Evans. 1983. Factors influencing success of embryo trans-

fer in sheep and goats. Theriogenol. 19 : 31-42.

2. Beaumont, H.M. and A.F. Smith. 1975. Embryonic mortality during the pre- and post-implantation periods of pregnancy in mature mice after superovulation. J. Reprod. Fert. 45 : 437-448.

3. Elizabeth A. Walton, G. Evans and D.T. Armstrong. 1983. Ovulation response and fertilization failure in immature rats induced to superovulate. J. Reprod. Fert. 67 : 91-96.

4. Evans, G. and D.T. Armstrong. 1984. Reduction in fertilization rate *in vitro* of oocytes from immature rats induced to superovulate. J. Reprod. Fert. 70 : 131-135.

5. Keefer, C.L. and A.W. Schuetz. 1982. Spontaneous activation of ovulated rat oocytes during *in vitro* culture. J. Exp. Zool. 224 : 371-377.

6. Kostyk, S.K., E.J. Dropcho, H.Moltz and J.R. Swartwout. 1978. Ovulation in immature rats in relation to the time and dose of injected human chorionic gonadotropin or pregnant mare serum gonadotropin. Biol. Repod. 19 : 1102-1107.

7. Lee, J.H., D.J. Kang and C.S. Park. 1988. Ovulation timing and PMSG dose response in superovulation of immature rats. Korean J. Anim. Sci. 30 : 714-719.

8. Maudlin, I. and L.R. Fraser. 1977. The effect of PMSG of dose on the incidence of chromosomal abnormalities in mouse embryos *in vitro*. J. Reprod. Fert. 50 : 275-280.

9. Miller, B.G. and D.T. Armstrong. 1981. Superovulatory doses of pregnant mare serum gonadotropin cause delayed implantation and infertility in immature rats. Biol. Reprod. 25 : 253-260.

10. Nuti, K.M., B.N. Sridharan and R.K. Meyer. 1975. Reproductive biology of PMSG-primed immature female rat. Biol. Reprod. 13 : 38-44.

11. Seidel, G.E., R.P. Elsden, L.D. Nelson and J.F. Hasler. 1978. Method of ovum recovery and factors affecting fertilization of superovulated bovine ova. In : Control of reproduction in the cow, pp.268-280. Ed. J. M. Sreenan Martinus Nijhoff, The Hague.

12. Toyoda, Y. and M. C. Chang. 1974. Fertilization of rat eggs *in vitro* by epididymal spermatozoa and the development of eggs following transfer. J. Reprod. Fert. 36 : 9-22.

13. Wright, R.W. and K.R. Bondioli. 1981. Aspects of *in vitro* fertilization and embryo culture in domestic animals. J. Ani. Sci. 53 : 702-729.