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Effects of Repeated Induction of Superovulation on Ovulation Rates and *In Vitro* Development of Embryos in Rabbit

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토끼에서 반복적인 과배란유도가 배란율과 난자의 체외 발육율에 미치는 영향

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요 약

토끼에서는 수정란 이식과 같은 기본적인 번식공학적 방법의 효율성이 아직 생쥐와 같은 실험동물에 비해 떨어지고 있어 생물공학적인 기술을 응용하는데 큰 어려움이 있다. 특히 유전자 이식에 의한 형질전환 토끼의 생산과 같은 생물공학적인 기술을 실용화하는데 효율이 높은 수정란 이식 기술의 개발이 필수적이라고 할 수 있다. 본 연구에서는 토끼에서 수정란 이식 기술의 첫 단계인 과배란 유도를 효율적으로 이용할 수 있는 방법을 정립하기 위해 반복적인 과배란 유도가 배란율 및 수정란의 질적인 면과 양적인 면에 미치는 영향을 조사하였다. 연구방법으로는 FSH와 HCG를 사용하여 과배란을 유도하였고 2.5 개월의 반복처리간격으로 3 번의 반복적인 과배란 처리를 한 후 반복처리에 따른 배란율과 배란된 난자의 형태학적 상태, 배양에 의한 발생능력 상태 등을 조사하고 난소의 변화도 관찰하였다. 반복적인 과배란에 의한 배란율은 반복수가 증가함에 따라 감소하였으며 배란수의 변이도 커지는 경향을 나타내었다 (첫번째, 32.6±2.5; 두번째, 28.7±3.7; 세번째, 20.9±3.8). 제 2 극체의 돌출, 전핵의 형성, cummulus cell의 존재 등에 의한 회수된 난자의 형태학적 관찰에 의한 방법으로 난자를 분류한 결과 과배란의 반복수가 증가함에 따라 다양한 모양의 난자가 회수되어 배란이 광범위한 시간대에 일어나고 있음을 나타내었다. 또한 과배란의 반복적인 유도에 의해 난소의 혈포수는 증가하였으나 채란된 난자의 체외배양에 의한 발육율에는 차이가 없었다. 그러므로 과배란의 반복적인 유도는 공란토의 난소반응에는 영향을 미쳤으나 난자의 질에는 영향을 미치지 않았음을 나타낸다.

(Key words : repeated superovulation, ovulation rates, embryo morphology, hemorrhagic follicles, *in vitro* development, rabbits)

INTRODUCTION

Superovulation is a crucial stage in the efficient embryo transfer by maximum production of *in vivo* embryos from single donor. Superovulation induction in rabbit is well established with the injection of FSH or PMSG and LH.

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PMSG can be used for superovulation induction in rabbit. PMSG has some adverse effects on repeated induction of superovulation mainly due to longer biological half-life (Murphy *et al.*, 1984; Boland *et al.*, 1991). Superovulation with PMSG and HCG resulted in increased abnormality of chromosomes in embryos and poor quality of ovulated embryos compared with control embryos (Fujimoto *et al.*, 1974; Foote and Ellington, 1988). FSH has relatively short half-life so that it should be injected once or twice a day for 2~3 days or injected with polyvinylpyrrolidone (PVP) for single injection (Kenelly and Foote, 1965; Carney and Foote, 1990; Choe *et al.*, 1996). Carney and Foote (1990) reported that the size (volume) and cell number of superovulated rabbit embryos were reduced. However, there is no reports on effects of repeated induction of superovulation in rabbit on quality and quantity of ovulated embryos. Repeated induction of superovulation in cow with FSH or PMSG affects total number of embryos recovered (Lubbadeh *et al.*, 1980; Chung *et al.*, 1983; Gielen *et al.*, 1990; Herrler *et al.*, 1991). Superovulation inductions in cow more than 3 times significantly reduced number of ovulated embryos or caused no superovulatory response. Number of good quality of embryos was consistent through repeated superovulation induction in cow (Donaldson and Perry, 1983; Yang *et al.*, 1988).

This study was designed to test changes in ovulation rates and developmental capacity of embryos following 3 times superovulation induction in rabbit and to evaluate effects of interval periods between inductions. Numbers of ovulation points, hemorrhagic follicles in donor ovaries were measured and morphology and developmental rates *in vitro* of collected one-cell embryos were observed. One-cell zygotes by flushing oviduct of donor were recovered to estimate ovulation rates and ovulation time in this study.

MATERIALS AND METHODS

1. Superovulation

Mature New Zealand White female rabbits were used for repeated induction of superovulation. Superovulation was induced with six subcutaneous injections of 0.3 mg FSH (Sigma, St Louis, Mo) at intervals of 12 hours and intravenous injection of 75 IU HCG 12 hours after the final FSH injection. Females were mated twice with males just after HCG injection. Eighteen hours after mating, the females were anesthetized with an intramuscular injection of Ketamine /Rumpen mixure and then laparotomized. Ovaries were examined to count ovulation points or hemorrhagic follicles. One-cell embryos were collected by flushing the oviduct with PBS containing 1 mg /ml BSA. Embryos were evaluated by morphology: cummulus cells and second polarbody presence, pronucleus formation. All embryos were cultured to evaluate developmental potential. The second and third repeated superovulations were induced in the same procedures as above. Intervals between superovulation inductions were 2.5 months.

2. Embryo culture

Culture medium in this study was RDH containing 1 mg /ml BSA and 5 mM taurine. RDH medium is a 1:1:1 mixture of RPMI 1640, DMEM (low glucose) and Ham's F-10. Collected one-cell zygotes were washed twice with RDH medium. The culture dishes were prepared the day prior to embryo recovery as follows: four microdrops (50 μl /drop) were placed into a single dish (60×15mm, Falcon Plastics #1007), covered with 8 ml of washed silicon oil (Aldrich Chemical Company), and placed into the culture incubator under a humid atmosphere of 5% CO_2

and 95% air, at 39℃. The embryos were cultured *in vitro* for approximately 72 hours and then evalauted for morphological stage of development by stereomicroscopy.

3. Statistical analysis

Ovulation rates were subjected to t-test to compare mean differences. Developmental rates and abnormality of embryos were compared by chi-square test (Snedecor and Cochran, 1967).

RESULTS

Ovulation rates following repeated induction of superovulation were investigated by comparing the number of embryos per donor (Table 1). The intervals between superovulation inductions were more than 2 months. One-cell embryos were collected from oviduct of donors to recover most ovulated embryos so that recovery rates of embryos were than 90% of ovulation point in this experiments. Ovulation rates were reduced as frequency of superovulation induction multiplied (first, 32.6±2.5; second, 28.7±3.7; third, 20.9±3.8). Increased variations in number of embryos recovered from donors superovulated second or third times were observed in comparison of first superovulation. The number of embryos with second polarbody and pronuclei or without cummulus cells were reduced in induction of superovulation more than once. These data suggested that ovulation induced more than once did not occur in narrow range of time period after HCG injection and mating.

Ovaries of donors treated by repeated superovulation were examined with the number of ovulation points and hemorrhagic follicles (Table 2). The number of ovulation points were reduced in the ovaries of donors with repeated induction of superovulation while the number of hemorrhagic follicles were increased in the same ovaries. Average number of ovulation points plus hemorrhagic follicles were about forty regardless of superovulation times.

Superovulated embryos were cultured *in vitro* to evaluate the potential effect of superovula-

Table 1. Ovulation rates and embryo stages following repeated induction of superovulation

	Superovulation repeat		
	First	Second	Third
No. donor	24	18	10
No. total embryo	782	516	298
With cummulus	56 (7.2%)	49 (9.5%)	55 (18.5%)
Without cummulus	726 (92.8%)	467 (90.5%)	243 (81.5%)
No. average embryo±s.e.	32.6±2.5[a]	28.7±3.7[a]	20.9±3.8[b]
2nd polarbody	695 (88.9%)	426 (82.6%)	214 (71.8%)
Pronucleus	556 (71.1%)	389 (75.4%)	205 (68.8%)

[a, b] Different superscripts indicate significant differences ($P<0.05$) between superovulation inductions.

Table 2. Ovulation resposes following repeated induction of superovulation (average±s.e.)

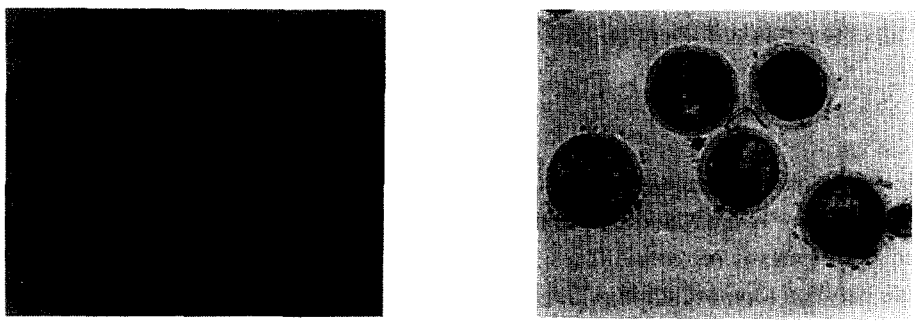
Superovulation repeat	No. of rabbits	No. of ovulation points	No. of blood follicles
First	20	34.7±2.6[a]	6.4±3.1[c]
Second	15	29.2±3.6[a]	11.2±3.3[d]
Third	10	22.5±3.8[b]	13.5±3.7[d]

[ab, cd] Different superscripts indicate significant differences ($P<0.05$) between superovulation inductions.

Table 3. Development of embryos recovered from donors superovalated repeatedly

Superovulation repeat	No. of embryos	Embryo development (%)				Blast. plus Exp. Bl. (%)[a]
		4~16 cell	Morula	Blast.	Exp. Bl.	
First	84	4 (4.7)	12 (14.3)	34 (40.5)	34 (40.5)	68 (81.0)
Second	80	4 (5.0)	8 (10.9)	35 (43.7)	33 (41.3)	70 (87.4)
Third	80	3 (3.8)	10 (12.5)	33 (41.3)	34 (42.5)	67 (83.8)

[a] $P>0.05$



(a) (b)

Fig. 1. Rabbit one-cell embryos (a) collected at 18 hr p.c. from oviduct of donors and blastocysts (b) following culture *in vitro* 72 hr of one-cell embryos.

tion frequency on the embryo development (Table 3). For *in vitro* culture one-cell embryos with second polarbody or pronucleus were selected from superovulated embryos and cultured in RDH medium for 72 hours (Fig. 1). More than 80 % of embryos in all the groups developed to blastocysts *in vitro*. There was no significant difference in the development rates between induction frequencies of superovulation.

DISCUSSIONS

Effects of repeated induction of superovulation in rabbit by standard superovulation methods on ovulation rate and embryo quality were examined in this study. In this study rabbits were superovulated consecutively 3 times with 2.5 month intervals. By collecting one-cell zygotes by flushing oviduct of donor in this study, more than 90 % of the ovulated embryos were expected to be recovered. The ovulation rates were affected through repeated superovulation. Even though the number of embryos ovulated were not significantly different in the first and second inductions of superovulation due to large variations, the average number of embryos in the first induction were a little higher than those in the second induction. Although ovulation rates were the lowest in the third induction, response of superovulation induction was maintained through the third times. Effects of repeated superovulation in cow on ovulation rates were controversial (Chung *et al.*, 1983; Lubbadeh *et al.*, 1980; Kanagawa *et al.*, 1981; Gielen *et al.*, 1990; Herrler *et al.*, 1991) but may be dependent on repeat intervals (Donaldson and Perry, 1983; Yang *et al.*, 1988; Kim *et al.*, 1997). Variations in each induction were increased following repeated inductions. When stages of ovulated embryos were examined by the presence of cum-

mulus cells, second polarbody or pronuclei to estimate uniformity of ovulation time, stages of embryos from donors superovulated more than once were less uniformed. Embryos tend to be ovulated in a wide range of time from ovaries induced with repeated superovulation. Variability in time of ovulation after superovulation induction was reported in domestic animals and laboratory animals (Beaumont and Smith, 1977; Armstrong and Evans, 1983; Linder and Wright, 1983; Evans and Armstrong, 1983). Collection of late stage embryos after superovulation revealed 24~48 hour developmental differences in cow. Early ovulation may occur at the exact time after mating but subsequent ovulation may delay over a considerable time period in the repeated induction of superovulation.

Because the number of ovulation points plus hemorrhagic follicles were consistent through repeated superovulation, superovulation may not affect follicular growth. Even though there was a large variability of ovulation rates in the third induction of superovulation, zygotes from donors with third induction developed normally *in vitro* to blastocysts. In this experiment, zygotes with second polarbody or pronuclei were selected from collected embryos that may eliminate abnormal embryos ovulated. This result suggests that superovulation can induce at least three times in rabbits without decreasing developmental potential of embryos.

SUMMARY

Following induction of repeated superovulation, ovulation rates were reduced (first, 32.6± 2.5; second, 28.7±3.7; third, 20.9±3.8). Variability in number of embryos recovered from donors superovulated second or third times were increased in comparison to first superovulation. Ovulation time were more variable in the induction of superovulation more than once. The number of hemorrhagic follicles were increased in the ovaries treated with repeated superovulation. However, developmental capacity *in vitro* of embryos recovered from donors induced by repeated superovulation was the same as those from the first induction of superovulation. These results indicated that there was significant difference in ovarian responses after induction of repeated superovulation but not in development *in vitro* of embryos.

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