Superovulation and Transplantation of Demi- and Aggregated Embryos in Rabbits

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ABSTRACT : The effect of exogenous gonadotrophins on superovulation in rabbits was examined. One hundred and sixteen sexually mature California, Chinchilla and New Zealand White rabbits were randomly allocated to control (100 IU hCG), PMSG-treated (100 IU HCG following 150 IU PMSG) and FSH-treated groups (0.3 mg/head /12 h for 3 days followed by 100 IU hCG). All does were mated after hCG injection and were sacrificed or laparotomized within 1 to 4 days postcoitus for counting the number of ovulation points. The number of ovulations was higher in FSH-treated animals than in the control and PMSG-treated groups (37.2 vs. 10.4 and 14.5, $p<0.05$). Follicle haemorrhagicum was observed in many cases in the PMSG-treated group. No significant difference in ovulation number was observed between left and right ovaries regardless of gonadotropin treatment. In another experiment, 2-cell stage embryos were collected at 26 h postmating and blastomeres were separated by mechanical pipetting or gentle pressure with a fine glass needle. Aggregated or chimeric embryos were produced from two single blastomeres from two breeds, New Zealand White and Chinchlla, with different coat colors. All the embryos were cultured in Ham's F-10 medium supplemented with 1.5% BSA (bovine serum albumin fraction V) and 10% PRS (pregnant rabbit serum), and incubated in a humidified atmosphere with 5% CO_2 at 38℃. After development to morula or early blastocyst, the embryos were transferred into the oviducts of recipient does. Results showed that 7 out of 10 does (70%) receiving intact embryos (control) became pregnant and 41 kits were delivered. However, no pregnancy was obtained from the recipient of either denuded demi- or aggregated embryos. It is suggested that embryos without zona pellucida could not develop to term in rabbits. (*Asian-Aust. J. Anim. Sci. 2001. Vol. 14, No. 4 : 455-461*)

INTRODUCTION

Rabbits are reflex or induced ovulators in which the regulatory mechanism of ovulation is via a neuroendocrine pathway (McDonald, 1980; Ramirez and Beyer, 1988). When the doe is bred with a buck or treated with gonadotropins, such as hCG, ovulation usually occurs 10 to 12 h post-treatment (Harper, 1961) regardless of the stage of the estrous cycle. Pituitary extracts were first used by Pincus (1940) to successfully induce ovulation in rabbits. When horse anterior pituitary gonad preparations (HAP), porcine follicle stimulating hormone (pFSH) or pregnant mare's serum gonadotrophin (PMSG or eCG) were used, both follicle luteinization or haemorrhagicum occurred. Follicle haemorrhagicum usually results in trapped oocytes and, hence, decreases the number of ovulations. A modified ovulatory regime for rabbits has been used for some time (Kennelly and Foote, 1965; Carney and Foote, 1990; Cheng et al., 1988). Briefly, does are superovulated by subcutaneous injection of FSH for 3 consecutive days and intravenous injection of hCG following the last dose of FSH. However, the efficiency of superovulation may vary between laboratories and be affected by the species, age of animals, hormones used, and environmental factors. To achieve an optimal result, different protocols need to be reevaluated.

Ovarian function has been studied in many species including pigs, cattle, horses, and goats. A compensatory response was observed in pig ovaries when unilateral ovarectomy was performed (Knight et al., 1973). In some species, such as cattle and goats, ovulation occurs mostly at the right ovary, whereas the left ovary always ovulates more than the right ovary in pigs and horses (McDonald, 1980). Little information on rabbits has been reported. The morphology and physiology of rabbit embryos are unique compared with those of other mammalian species. One difference is that the extracellular coverings surrounding the embryo are required for successful implantation (Greenwald, 1962; Kane, 1975). Therefore, the prime objectives of this study were evaluation of the superovulation efficiency after PMSG or FSH treatment and of the ovarian function between left and right ovaries. The *in vivo* development of both denuded demi- and aggregated embryos from 2-cell blastomeres was also assessed following transfer to the recipient does.

MATERIALS AND METHODS

Animals, oocytes and embryo collection

Data from a total of two hundred and thirty sexually mature California, Chinchilla, and New

Zealand White rabbits were collected and were analyzed for the effect of breed, age, and side (left or right) of the ovary on ovulation number after superovulation treatment. Animals were subjected to surgery or sacrificed 1 to 4 days post-mating and the numbers of ovulation points were counted. Oocytes or cumulus-oocyte complexes (COC) were collected by flushing from the oviducts to the uteri either *in situ* or following dissection. The flushing medium used in this study was Ham's F-10 supplemented with 0.4% bovine serum albumin (BSA fraction V, Sigma A-9647) plus 1% fetal bovine serum (FBS) or 10% pregnant rabbit serum (PRS), based on Cheng et al. (1988).

Superovulation treatments

Animals were injected with 150 IU pregnant mare serum gonadotropins (PMSG, Sigma G-4877) subcutaneously and were bred and injected intra- venously with human chorionic gonadotropin (hCG, China Chemical and Pharmaceutical Co. LTD.) 72 h after PMSG treatment. In the FSH treatment group, does were primed with 0.30 mg FSH (Sigma F-2293) intramuscularly 6 times for 3 consecutive days at 12 h intervals (Kennelly and Foote, 1965; Cheng et al., 1988). An ovulatory dose (100 IU of hCG) was also intravenously injected 12 h after the last priming dose. Another 55 does simply had ovulation induced by hCG injection and were bred to serve as a control group.

Blastomere separation and aggregation

Two-cell stage embryos were collected by flushing the oviduct 26 h after mating. The zona pellucida was removed by enzymatic treatment with 0.5% pronase for 10 to 15 min and gentle pipetting with a Pasteur pipette. Blastomeres from denuded 2-cell stage embryos were separated either by gentle pressure with a fine glass needle or repeated pipetting with a small bore Pasteur pipette (figures 1a and 1b).
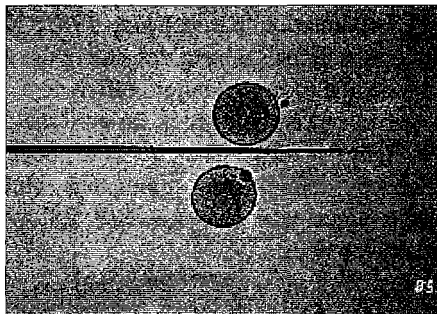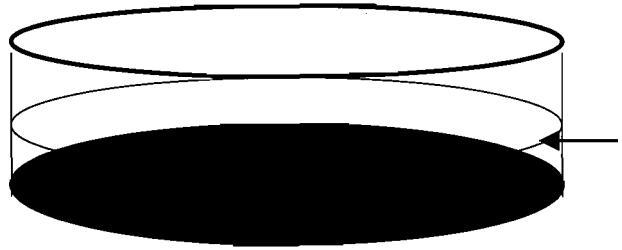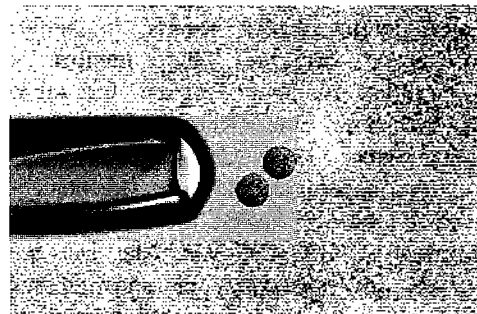

Figure 2. Preparation of the agar-based (0.4%) culture medium and aggregation of the blastomere in a 35mm petri-dish. Aggregating blastomeres of Chinchilla and New Zealand White were deposited in the same agar-based microwell (0.5 mm in diameter and 0.2-0.3 mm in depth) at the bottom of the culture dish represented by small circles. Two ml of culture medium (Ham's F-10+1.5% BSA+10% pregnant rabbit serum) was added to the petri-dish to completely cover the agar layer (arrow).

Culture of isolated blastomeres or aggregates

Isolated blastomeres were cultured individually to the early blastocyst stage in Ham's F-10 microdroplets supplemented with 1.5% BSA and 10% PRS, and incubated in a humidified atmosphere with 5% CO_2 at 38°C. All media were changed every other day. Chimaeric embryos were produced by aggregating two blastomeres of different genetic backgrounds, i.e., New Zealand White and Chinchilla embryos. To prevent separation, the two blastomeres were aggregated in tiny wells, approximately 0.5 mm in diameter and 0.2 to 0.3 mm in depth, prepared using 0.4% agar in a 35 mm petri dish. The agar wells in the petri dish were covered with 2 ml of culture medium (figure 2).

Embryo transfer procedure

Anesthetization and surgery: Recipient does were anesthetized with Xylazine (Rompum, 5 mg/kg BW) and Katamine (Ketalar, 30 mg/kg BW) by intra-

a   b

Figure 1. Methods of blastomere separation. Blastomeres were separated by either (a) gentle pressure at the cleavage furrow of the 2-cell embryos with a fine glass needle or (b) repeated pipetting of the denuded 2-cell embryos with a small bore glass pipette.

Table 1. Number of ovulations in sexually mature (7 to 11-month-old) rabbit does without hormonal treatments

Breed	Number of animal	Number of ovulations (mean±SE)			Embryo recovery, %	Fertilization rate, %	Normal embryos, %
		Right ovary	Left ovary	Total			
New Zealand	8	5.6±2.9	4.0±2.6	9.6±2.6	83.8	97.6	96.7
Chinchilla	16	5.1±2.2	5.9±2.4	10.9±3.1	90.3	100.0	87.3
California	12	6.0±2.0	4.8±1.1	10.8±1.8	95.3	95.6	90.8

No significant difference was detected in all criteria (p>0.05).

muscular injection or Pentobarbital (Nembutal, 25 mg/kg BW) by intravenous injection from the ear vein (Ju et al., 1991). Ether supplementation was used for maintenance of anesthetic condition when necessary. Animals were returned to the cage after oocyte recovery and completion of the surgical procedures.

Embryo transfer: One hundred and seventy-one normal intact embryos were flushed from 11 donors by laparotomy. Pseudopregnancy was induced in the recipients and their uterine environment was synchronized with the embryonic stage of donor animals. Premorula stage embryos were surgically transferred to the oviducts and morulae or early blastocysts to proximal uterine horns 1 to 2 cm from the uterotubal junction. Denuded demi- or aggregated embryos were cultured to morula or early blastocyst stage before transfer to the uterine horns.

Statistical analysis

The effect of age, breed and gonadotropin treatment on all criteria in this study were compared using analysis of variance (ANOVA) in Statistical Analysis System (SAS, 1989).

RESULTS

Experiment 1. Normal number of ovulations without hormonal treatment

No statistical differences were detected among breeds in the number of ovulations, embryo recovery and fertilization rates of rabbits without hormonal stimuli (table 1). The overall mean number of ovulations was about 10-11 per animal and was similar for both ovaries (5-6 for the right ovary and 4-6 for the left, p>0.05). Fertilization rate and proportion of normal embryos ranged from 96 to 100% and 87 to 97%, respectively.

Experiment 2. The effect of gonadotropins on ovulations

Two superovulation regimes were compared using FSH or PMSG combined with hCG injection. The effect of gonadotropin treatment was to increase the number of ovulations from 10.4±2.6 for control to 14.5±3.3 following PMSG treatment (difference not significant) and to 37.2±16.1 with FSH stimulation

(p<0.05). Embryo recovery, fertilization rates, and proportion of normal embryos, were not affected by treatment (table 2). Some ovaries showed haemorrhagic clots in the follilces of PMSG-treated animals (figure 3). The enhanced response to FSH treatment was confirmed in a further experiment (table 3).

Further comparison of ovulation number was made between right and left ovary in response to FSH treatment. Large variation existed within the FSH-treated animals with an average of 42 ovulation points. As expected, there was a much higher number of ovulations in the FSH-treated group (p<0.01) than in the control group, but no significant difference was found between left and right ovaries in either control or superovulated groups (table 2, p>0.05).

Animals from FSH treatment group were arbitrarily classified into two age groups, i.e., 4.5 to 6 months, which is considered to be the age of puberty, and the more mature age of 7 to 11 months. Their responses to gonadotropin treatment were similar, although it seemed slightly better in ovulation number for the 7-11 month group (33 vs. 45.5, p>0.05). No difference was found in rates of fertilization and normal embryos (table 3).

Experiment 3. The effect of FSH treatment on number of ovulation in each ovary

Another 78 females were used for the observation of left and right ovarian function after superovulation. A significantly higher number of ovulations was observed in the FSH-treated group (42 vs. 11, p<0.01), but no significant difference (p>0.05) was found between the right and left ovaries in either treatment group (table 4).

Experiment 4. *In vivo* development after embryo transfer

Demi- and aggregated embryos were cultured to the morula or early blastocyst stage before transferring into recipient does. Intact embryo transfer was used as the control group in which 171 transferable embryos were transplanted to 10 recipients. Seven out of the ten recipients were pregnant (70%) with 41 kits delivered to term (24%). The 73 demi- and 24 aggregated embryos without zona pellucida which were transferred to recipients produced no pregnancies (table 5).

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Table 2. Effects of gonadotropins on the superovulatory response of female rabbits

Treatment	Number of animals	Number of ovulations (mean ± SEM)	Embryo recovery, %	Fertilization rate, %	Normal embryos, %
Control	55	10.4 ± 2.6[a]	90.2	96.4	95.1
FSH	44	37.2 ± 16.1[b]	90.4	94.0	95.3
PMSG	17	14.5 ± 3.3[a]	80.4	92.8	87.4

[a,b] Means with different superscripts in the same column differ significantly (p<0.05).

Table 3. Effects of gonadotropins on the superovulatory response of female rabbits

Age of animals, months	Number of animals	Number of ovulations (mean ± SEM)	Embryo recovery, %	Fertilization rate, %	Normal embryos, %
4.5-6	29	33.0 ± 12.5	92.2	98.0	97.8
7.0-11	15	45.5 ± 19.3	88.0	87.9	91.4

No significant difference was detected in all criteria (p>0.05).

DISCUSSION

Ovarian responses of gonadotropin treatment

Ovulation rates of New Zealand White, Chinchilla, and California rabbits were investigated in this study. Number of ovulation points was approximately 10 to 11 for each animal, which is very similar to Dutch-belted does (10.1) reported by Kennelly and Foote (1965). Interestingly, no significant difference has been found in number of ovulations which ranged from between left and right ovary 4 to 6 (table 1), which is different from other species like goats, cattle, pigs and horses.

Although there were no significant differences in the number of ovulations between the right and the left ovary, mainly due to the variation among animals, it is still not known whether left and right ovarian function differs in an individual rabbit. The large variability about the means in this study might be the result of elevated ambient temperatures during the hot season. Knight et al. (1973) reported that a compensatory response occurred in pig ovaries when unilateral ovarectomy was performed at the opposite side. Whether a similar response occurs in rabbit ovaries requires further study.

When female rabbits with a similar age were treated with FSH, patterns of ovulation between did not differ ovaries. Despite more ovulation points observed (42.3 vs. 10.5, p<0.01, table 4), differences were still not detectable between left and right ovaries (21.0 vs. 21.3)

Two superovulation protocols were tested, ie., use of FSH or PMSG (table 2), in which FSH-treated does responded much better than did the other 2 groups (37 vs. 10.4 and 14.5, p<0.05). The reason for the smaller response to PMSG than to FSH is not clear. It may be due to over dosage of PMSG from which cystic or hemorrhagic follicles were commonly seen. A similar phenomenon has been reported by

Adams (1982), in which some oocytes were trapped in the haemorrhagic follicles. Furthermore, PMSG was only used as one time injection (150 IU) which may be another cause. However, Tsutsumi et al (1980) obtained an excellent ovulation (61/head) of Japanese
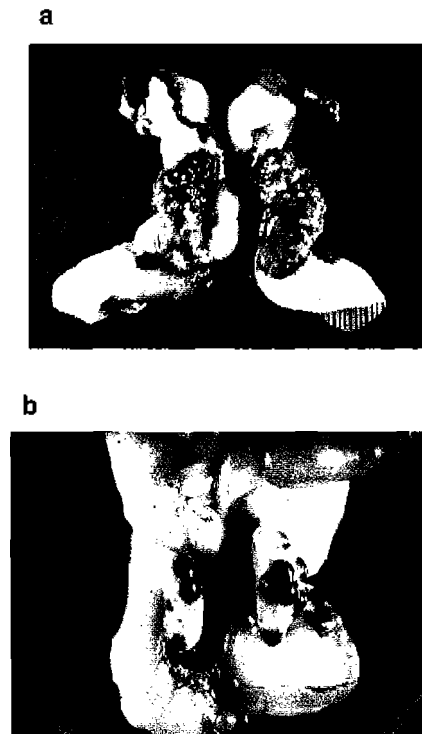
a

b

Figure 3. Responses of rabbit ovaries to gonadotropin treatments. (a) Ovaries from FSH-treated does with numerous ovulation points or corpus lutea on the surface. (b) Ovaries from PMSG-treated does was usually less responsive to the treatment and accompanied with large hemorrhagic follicles (arrow). Some oocytes may be trapped in the follicle without successfully ovulation.

+White rabbits by injection of 200 IU PMSG intramuscularly. On the contrary, Hafez (1961) injected 50 IU/doe/day PMSG for 3 consecutive days followed by a 50 IU pituitary gonadotropins injection, which resulted in an average ovulation number of 38.5. This suggested that and repeated lower dosage might be a good way to superovulate rabbits with PMSG, although another group reported a poor response from Dutch-belted rabbits treated with 25 IU PMSG at 12 h intervals for 3 days (Kennelly and Foote, 1965). These results indicate that the efficacy of induced ovulation using PMSG may vary with the species of the animal, concentration used, and the procedure of treatment.

In respect of hCG injection following repeated FSH or PMSG treatment, a possible immunological response may occur which in turn results in less responsiveness of the ovary (Maurer et al., 1968). Use of gonadotropin releasing factor (GnRH, Foote and Simkin, 1993), an upstream stimulator for LH and FSH, may provide an alternative way to maintain a good ovarian response without compromising the embryonic competence.

The effect of age on the superovulatory response

Animals were arbitrarily divided into two age groups, i.e., 4-6 months and 7-11 months of age. Due to the large variation within group, no significant difference in number of ovulations was observed (33 vs. 45.5, p>0.05, table 3). However, Kennelly and Foote (1965) found that superovulatory response of rabbits was affected by the age of the animal receiving treatment. A higher FSH (0.5 mg/ml) dosage may be required to achieve a better response in older females. When a younger animal is treated with the same FSH dosage as an older animal, reduced ovulations and follicular haemorrahgicum may result. In this study, a total amount of 0.6 mg/head/day of FSH was used which was comparable to a high dose treatment.

In vivo development of intact, demi- and aggregated embryos

In a previous study, we had observed a low developmental rate of 16- and 32-cell stage single blastomeres when cultured individually *in vitro* (Ju et al., 2000; unpublished data). We, therefore, decided to

Table 4. Number of ovulations in 7 to 11-month-old female rabbits (mean ± SEM)

Treatment	Number of animals	Number of ovulations (%)		
		Right ovary	Left ovary	Total
Control	36	5.4 ± 2.2 (51.4%)	5.1 ± 2.2 (48.6%)	10.5 ± 2.7[a]
FSH groups	42	21.3 ± 8.9 (50.4%)	21.0 ± 7.9 (49.6%)	42.3 ± 16.2[b]

[a,b] Means with different superscripts in the same column differ significantly (p<0.01).

use 2-cell stage blastomeres for assessment of *in vitro* development of the manipulated embryos.

Approximately 31-54% intact rabbit embryos can develop to term if transfered immediately after recovery (Chang, 1950; Adams, 1975, 1980; Techakumphu et al., 1987). A slightly lower delivery rate (24%, table 5) in this study may be associated with the viability of the embryos, which were cultured for various days before transfer. Another cause may be related to the high ambient temperature in the hot season in this Island, which has been one of the common problems for animal industries in tropical and subtropical areas (Baumgartner and Christman, 1987; Badinga et al., 1985). On the other hand, no pregnancy was obtained in the demi- and aggregated embryos after transplan- tation which may be due to lack of mucin coat and zona pellucida of these transplanted embryos as reported previously (Greenwald, 1962; Haln, 1984; Moore et al., 1968; Rottmann and Lampeter, 1981).

In rabbits, denuded or embryos with damaged zona pellucida can hardly survive in the reproductive tract of the recipient. Leukocytes or white blood cells may invade and attack the embryo inside the zona pellucida (Moore et al., 1968). The extracellular coverings of rabbit embryos, including the outer mucin layer and the zona pellucida or neozona (day 4.5 p.c.), are unique and complex structures compared to most large domestic species (Denker and Gerdes, 1979; Leiser and Denker, 1988; Fischer et al., 1991). Generally, the zona pellucuda consists of glycoproteins with ZP1, ZP2, and ZP3 framework in most species (Wassarman,

Table 5. Pregnancy rate following transfer of intact, demi- or aggregated rabbit embryos

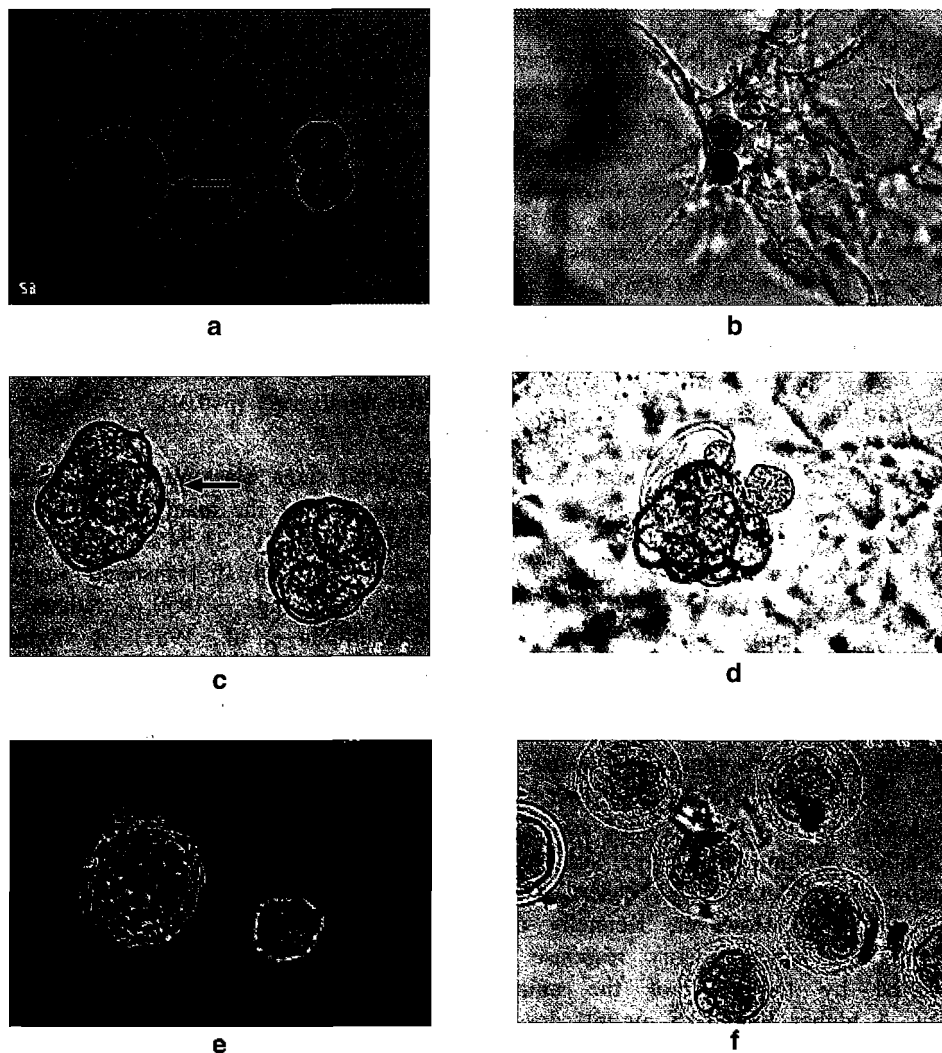| Treatment	Stage of embyro transferred	Number of recipients	Numer of embryos transferred	Numer of pregnancies (%)	Numer of kits to term (%)
Intact embryos	2-cell~blastocyst	10	171	7 (70)[1]	41 (24)
Demi-emryos	Early blastocyst	16	73	0 (0)	0 (0)
Aggregated embryos	Early blastocyst	12	24	0 (0)	0 (0)

[1] Three kits were aborted during pregnancy.

Figure 4. (a) A denuded 2-cell embryo was just released from zona pellucida (arrow) after 0.5% pronase treatment. (b) Two blastomeres with different genetic makeup were aggregating in an agar-based pit. (c) Two compact morulae developed from 2 single blastomeres (2-cell stage) at day 2 after *in vitro* culture. Residue of the zona pellucida is still visible in one of the demi-embryos (arrow). (d) A morula developed from aggregation of 2 single blastomeres at day 2 after culture. (e) A morphologically normal blastocyst (day 3) developed from single blastomere of 2-cell stage embryos (left) with a degenerated demi-embryos at the right (arrow). (f) Morulae developed from *in vivo*-derived 2-cell stage intact embryos after being cultured for 2 days.

1999). From previous studies, the zona pellucida of rabbit embryos is not only crucial for normal fertilization (Soupart and Noyes, 1964; Richardson et al., 1994), but is also required for a successful implantation or further development (Denker and Gerdes, 1979; Yen et al., 1998). Yang and Foote (1987) produced monzygotic twins using a microinjection method by which the bisected morula stage embryos with zona pellucidae were transferred. Yen et al. (1998) also produced a live kit (1/24) after transferring to the recipient by encapsulating two 8-cell stage blastomeres with an empty zona. In this study, we further transferred denuded demi- and aggregated embryos to recipient does confirming that existence of zona pellucida is essential in rabbit embryos, although

the real mechanism is not yet clear.

CONCLUSION

We demonstrated in this study that the superovulatory response of rabbits is affected by the protocols used, and that there is no difference in ovulation response between left and right ovaries. We further confirmed that successful establishment of pregnancy in the rabbit requires an intact zona pellucida for normal implantation or development to term. Alternative ways to improve the efficiency of superovulation and to elucidate the mechanism of implantation in cloned rabbit embryos requires more study.

ACKNOWLEDGEMENTS

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