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In Vitro Maturation and Fertilization of Follicular Oocytes Recovered from Pregnant Rabbits and the Maintenance of Pregnancy Following Ovarian Follicular Aspiration

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Abstract *In vitro* maturation and fertilization of follicular oocytes recovered by puncture of large ovarian follicles (≧1.0 mm in diameter) from rabbits at 13 to 29 days *post coitum* (*p. c.*) were examined.

Maturation and fertilization rates of the oocytes were 93.1% and 39.2%, respectively. Transfer of forty-one fertilized oocytes (2-4 cell stages) into oviducts of recipient does subjected to injection of hCG 24 hr before receiving the cleaved oocytes resulted in only one conceptus, which was missed in the latter stage of the pregnancy. Though the pregnant does from which oocytes were collected from the unilateral ovaries at 13 and 19 days *p. c.* showed partial stillbirth (13.3%) and resorption of fetuses (26.7%), dosage (4 mg/day) of progesterone (for 3 successive days after collection of oocytes) to unilaterally treated does at 11 to 15 days *p. c.* rescued their pregnancies. When all follicles on bilateral ovaries were aspirated at 20 to 29 days *p. c.*, all pregnant does attained normal gestation.

Key words: embryo-transfer, follicular oocyte, *in vitro* fertilization, pregnant rabbit

INTRODUCTION

In most *in vitro* fertilization, oocytes have been recovered from cycling non-pregnant females. However, antral follicles always exist in ovaries of pregnant or pseudopregnant animals including human, and ovulations sometimes occur naturally during pregnancy. Possible superfetation has been reported to occur naturally, although rarely, in several species (LEE *et al.*, 1986).

LEE *et al.* (1986) reported that oocytes were fertilized by intraperitoneal insemination of spermatozoa into pregnant rabbits ovulated artificially, and that one of these eggs produced a normal young by transfer into an oviduct of a recipient doe. TSUTSUMI *et al.* (1987) demonstrated that pregnant rabbits in which follicles of the residual ovary were destroyed (punctured 10 large follicles) after unilateral ovariectomy underwent normal delivery. These evidences suggest that pregnant animals can be used as donors for embryo transfer while maintaining their pregnancies.

In the present study, *in vitro* maturation and fertilization of follicular oocytes recovered from pregnant rabbits and the effect of follicular punctures for collection of oocytes from

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ovarian follicles on pregnancy were examined.

MATERIALS AND METHODS

Animals and treatments

Thirty-eight mature female and twelve fertile male Japanese White rabbits, weighing 3.8-4.6 kg, were used in the present study. All animals were reared in each cage providing with food and water *ad libitum.*

Thirty-four does were injected intravenously (i. v.) with 30 IU of human chorionic gonadotropin (hCG, Teikokuzôki, Japan) to ensure ovulation immediately after mating with 2-3 bucks. After palpating the developing fetuses at 8 to 10 days *post coitum* (*p. c.*), all pregnant does received uni- or bilateral flank laparotomy under anesthesia with pentobarbitone sodium (Somnopentil: Pitman-Moore, Washington) at given days of pregnancy. After macroscopic observations of ovarian follicles, all follicles ($\geqq$1 mm in diameter) were punctured from one (15 does) or both (19 does) side of ovaries. Some of unilaterally treated does were injected 4 mg Progesterone (Luteogen: Sankyo, Japan) per one intramuscularly for 3 successive days after surgery.

Recovery of follicular oocytes

The aspiration of follicular oocytes from the follicles was carried out using a needle (23 G, Hakko Winged Infusion Set, Hakko Electric, Japan). During aspiration, the needle was washed several times with small amount of Dulbecco's phosphate buffered saline (PBS) containing 15% (v/v) heat-inactivated rabbit serum and 2 units heparin per mℓ, 100 units penicillin and 7.5 mg streptomycin sulfate per liter (pH 7.4).

Culture of oocytes

Collected oocytes were washed more than 3 times and normal oocytes in appearance were cultured in bovine serum albumin (BSA) -free defined medium (BOM; pH 7.4) described by BRACKETT and OLIPHANT (1975), containing 30 IU hCG/mℓ, 10 IU PMSG/mℓ and 20% (v/v) heat-inactivated fetal bovine serum (FBS; Gibuco laboratories, U. S. A.) for 90 min. The oocytes were transferred into another BSA-free BOM (pH 7.4) containing 10 IU hCG/mℓ, 10 IU PMSG/mℓ, 2 μg progesterone/mℓ and 20% (v/v) FBS and cultured successively for 10.5 hr. After maturation, the oocytes were transferred into the BSA-free BOM containing 0.1% hyaluronidase (Sigma, U. S. A.), held for 5-10 min and removed cumulus cells by repeated pipetting. The oocytes with the first polar body were considered matured to metaphase II and used for *in vitro* fertilization after washed 3 times with the BOM. The remaining oocytes (unfertilized oocytes) were examined the nuclear phase by the whole mount procedure.

Treatment of spermatozoa

Semen was collected from 2-3 bucks by an artificial vagina. After removal of jelly substance from the ejaculate, semen was centrifuged at 200 g for 8 min. The spermatozoa were resuspended in a high-ionic-strength solution (HIS, BRACKETT and WILLIAMS, 1968) and incubated for 15 min in a water bath at 38°C. Sperm suspension was centrifuged at 200 g for 5 min at 38°C. Then, a half milliliter of sperm deposit layered beneath 5 mℓ of BSA-free BOM in a culture tube (10×120 mm) was incubated for 45-60 min in 5% CO_2 in air at 38°C. Motile spermatozoa migrating to the upper layer were gently aspirated with a

Pasteur capillary pipet (swim-up method, BERGER *et al.*, 1985) and approximately 1.5-2.5× 10^6 sperm (50-70% level of motility) were transferred into 4 mℓ of BOM to further incubate for 6 hr under a moist 5% CO_2 in air at 38°C.

In vitro fertilization

Matured oocytes were introduced into the sperm suspension (4 mℓ) in a petri dish (35×10 mm, Cornig, Japan) and incubated for 5 hr. Again, oocytes were placed into fresh BOM for removal of excessive spermatozoa surrounding zona pellucida and cultured for more than 12 hr. After 17 hr from *in vitro* insemination, all oocytes were inspected. Fertilization was defined by the presence of two pronuclei and two polar bodies under a phase-contrast and interference contrast microscope. Furthermore, in order to progress cell stage these oocytes were used for additional culture. Unfertilized oocytes were mounted *in toto* fixed with 2.5% glutaraldehyde in PBS for a few min and aceto-ethanol (acetic acid: ethanol=1:3) for 3 days. After fixation oocytes were stained with 0.25% lacmoid (in 45% acetic acid).

Embryo-transfer

Some of the 2- or 4-cell stage oocytes (29 hr after *in vitro* insemination) were transferred into the oviducts of the four recipient does which had received 30 IU of hCG i. v. about 24 hr before receiving the cleaved oocytes. Laparotomy was performed 13 days after embryo-transfer for pregnancy diagnosis.

RESULTS

In vitro maturation of follicular oocytes recovered from pregnant rabbits

In 12 does used as donors, 303 (95.3%) oocytes were collected from 318 follicles aspirated. However, 8.7% (29/303) of them were collapsed or denuded through aspiration and such oocytes were discarded. Two hundred and fifty five (93.1%) of 274 cultured oocytes reached at metaphase II (Table 1). The maturation rates were ranged from 87.0 to 100%. Ten (3.6%) out of the 274 oocytes remained at prometaphase I - telophase I and 2 oocytes (0.7%) were arrested at the germinal vesicle stage. The residual 7 (2.6%) had degenerated during cultivation.

In vitro fertilization of follicular oocytes recovered from pregnant rabbits

Thirty nine point two % of total oocytes were fertilized, with a range of 23.3 to 100% (Table 2), and 28.2% of the inseminated were cleaved. Transfer of forty-one oocytes resulted in only one conceptus (approximately 1.8 cm in diameter) at 13 days after embryo-transfer. However, the conceptus disappeared in the latter stage of pregnancy, as judged by abdominal palpation.

The effect of aspiration of follicles on the maintenance of pregnancy

Of 12 pregnant does in which ovarian follicles were punctured unilaterally at 13 to 29 days *p. c.*, one doe at 13 days *p. c.* showed partial resorption (3/4) of fetuses, 2 does at 19 days *p. c.* induced partial resorption (1/11) and partial stillbirth (2/11). However, other does at 20- 29 days *p. c.* maintained normal pregnancy (Table 3).

When ovarian follicles were aspirated bilaterally at 19 to 29 days *p. c.*, 13 of 14 rabbits were delivered offspring corresponding with the number of fetuses observed at follicular puncture: one doe produced a normal young with loss of 3 embryos.

Table 1. *In vitro* maturity of follicular oocytes

Days of pregnancy (*p. c.*)	No. of oocytes	Maturation division[1]			Deg.
		G. V.	Promet. I-Telo. I	Met. II (%)[2]	
13	12	0	0	12 (100)	0
19	9	0	0	9 (100)	0
19	23	0	0	23 (100)	0
20	19	0	1	17 (89.5)	1
20	23	0	3	20 (87.0)	0
22	26	1	1	24 (92.3)	0
23	26	0	0	26 (100)	0
25	34	0	2	30 (88.2)	2
26	24	0	1	22 (91.7)	1
27	17	0	0	17 (100)	0
27	28	1	0	25 (89.3)	2
29	33	0	2	30 (90.9)	1
Total (%)	274	2 (0.7)	10 (3.6)	255 (93.1)	7 (2.6)

[1] G. V.: Germinal vesicle, Promet. I: Prometaphase I, Telo. I: Telophase I, Met. II: Metaphase II, Deg.: Degeneration

[2] Oocytes with the first polar body were regarded as Met. II.

Table 2. *In vitro* fertilization of follicular oocytes

Days of pregnancy (*p. c.*)	No. of oocytes inseminated	No. of fertilized oocytes (%)	No. of cleaved oocytes (%)
13	12	8 (66.7)	8 (66.7)[a]
19	9	9 (100)	9 (100)[a]
19	23	6 (26.1)	2 (8.7)
20	17	5 (29.4)	4 (23.5)
20	20	9 (45.0)	8 (40.0)[a]
22	24	6 (25.0)	3 (12.5)
23	26	8 (30.8)	6 (23.1)
25	30	16 (53.3)	16 (53.3)[a]
26	22	7 (31.8)	4 (18.2)
27	17	10 (58.8)	7 (41.2)
27	25	9 (36.0)	5 (20.0)
29	30	7 (23.3)	0 (0)
Total	255	100 (39.2)	72 (28.2)

[a] Ova were transferred to recipients.

When the pregnant does, in which uni- or bilateral ovarian follicles were punctured at 11 to 25 days *p. c.*, were given progesterone for successive 3 days from the day of follicular puncture, they were delivered of normal young with no missing fetuses.

DISCUSSION

According to SHEA *et al.* (1976), 82% of follicular oocytes recovered from virgin does matured *in vitro* without gonadotropin treatment in TCM 199 containing 10% rabbit

Table 3. Effects of puncture and aspiration of ovarian follicles on the maintenance of pregnancy

Ovarian follicle puncture	Days of pregnancy (*p. c.*)	No. of fetuses at follicle puncture	No. of offsprings	
			Birth	Stillbirth
Unilateral	13(L)	4	1	0
	19(L)	3	2	0
	19(L)	8	6	2
	20(L)	8	8	0
	22(L)	3	3	0
	23(R)	3	3	0
	26(R)	9	9	0
	26(L)	9	9	0
	27(L)	7	7	0
	28(L)	9	9	0
	28(L)	9	9	0
	29(L)	9	9	0
Bilateral	19	4	1	0
	19	5	5	0
	20	7	7	0
	21	6	6	0
	23	8	8	0
	24	6	6	0
	25	1	1	0
	26	9	9	0
	26	7	7	0
	27	7	7	0
	27	6	6	0
	27	2	2	0
	28	6	6	0
	29	6	6	0
Unilateral[a]	11(L)	10	10	0
	12(L)	3	3	0
	15(L)	9	9	0
Bilateral[a]	20	7	7	0
	20	5	5	0
	22	9	9	0
	23	7	7	0
	25	1	1	0

(L): Follicles were punctured only left side ovary.

(R): Follicles were punctured only right side ovary.

[a] : Does were administered progesterone intramuscularly each 4 mg for 3 successive days after surgery.

serum. SMITH *et al.* (1978) also obtained higher maturity (94%) *in vitro* using oocytes from non-pregnant rabbits. In the present study, the maturation rate (93.1%) was comparable to these results. Thus, it seems that follicular oocytes recovered from pregnant rabbits have the similar potential for *in vitro* maturation to oocytes recovered from non-pregnant rabbits.

The fertilization rate remained in low levels (39.2%) and the offspring was missed in this experiment. Concerning the fertilization *in vitro,* abnormalities in the formation of male pronucleus have been considered as one of main factors in faulty fertilization. In cultivation of follicular oocytes protein synthesis of the oocytes changed during maturation in rabbits (THIBAULT and GÉRARD, 1973; WARNES *et al.,* 1977). According to MOOR and TROUNSON (1977), some oocytes from a sheep ovary reached metaphase II in the presence of follicle stimulating hormone and LH, but after *in vitro* insemination the fertilized oocytes could not develop to normal embryos. When estrogen was supplemented in the medium for the maturation culture, the fertilized oocytes acquired the ability of sequential development of embryos after transfer. Thus, follicular oocytes should undergo not only nuclear maturation but also another process of maturation (*i. e.,* cytoplasmic maturation) for sequential development after embryo-transfer. Therefore, the low rate of fertilization and lack of offspring in the present study might be due to incomplete cytoplasmic maturation of follicular oocytes.

Concerning the effect of the puncture and aspiration of ovarian follicles on the maintenance of pregnancy, does which were aspirated at 13 or 19 days *p. c.* showed partial resorption and stillbirth fetuses, and administration of progesterone to does treated unilaterally at 11 to 15 days *p. c.* successfully maintained the pregnancy (Table 3). Therefore, the pregnant does in which unilateral aspirated at 13 to 19 days *p. c.* might have undergone abnormal pregnancy as a result of a depression of progesterone levels in the blood. BROWNING *et al.* (1980) also reported the reduction of progesterone levels after puncture of ovarian follicles during 11-14 days. Furthermore, the function of the corpus luteum in the rabbit is supported by estrogen secreted from ovarian follicles (BILL and KEYES, 1983). Thus, levels of estrogen in the blood of pregnant does must have declined sharply following puncture of ovarian follicles in the present study.

Following follicular aspiration at 20 to 29 days *p. c.*, all pregnant does which were not subjected to progesterone treatment maintained normal pregnancy, regardless of whether aspirations were done on the ovaries of one side or both sides. After the middle stage of normal gestation, the levels of progesterone in peripheral serum gradually lessen (BROWNIG *et al.,* 1980). Thus pregnant does which aspirated bilateral at 19 to 29 days *p. c.* could maintain their pregnancies without the progesterone supplement.

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妊娠家兎卵胞卵の体外受精及び卵胞卵採取後の妊娠経過について

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妊娠家兎の卵胞（≧1.0 mm）から卵胞卵を採取し，修正 BO 液で12時間培養した後，射出精子による体外受精を試みた所，成熟率，受精率，分割率，はそれぞれ93.1%（255/274），39.2%（100/255），28.2%（72/255）であった。合計41個の 2 - 4 細胞期の卵子を偽妊娠家兎 4 羽に移植した所，1 羽に 1 個の着床部位が認められたが産仔は得られなかった。片側卵巣より卵胞を全て穿刺吸引した場合，妊娠13日目の家兎で胎児が部分吸収され，また妊娠19日目の家兎では胎児の部分吸収及び死産が認められたが，妊娠20日目から29日目に卵胞を穿刺吸引した家兎では正常な妊娠を維持した。妊娠19日目から29日目にかけて両側卵巣の卵胞を全て穿刺吸引した場合，妊娠19日目の家兎 1 羽を除く全ての家兎が正常な妊娠経過を示した。妊娠11日目から妊娠15日目に片側卵巣，また妊娠20日目から妊娠25日目の両側卵巣の卵胞を穿刺吸引し，その日から 3 日間連続して 4 mg/day の Progesterone を投与した所，全ての家兎が正常な妊娠を維持した。以上の結果より，妊娠期における卵胞卵の有効利用の可能性が示唆された。

キーワード：体外受精，体外成熟，妊娠家兎，胚移植，卵胞卵