POSSIBLE EGG RECOVERY FOR TRANSFER FROM THE PREGNANT RABBITS

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Introduction

Mammalian fertilized eggs for transfer are usually recovered from the non-pregnant females. However, growing antral follicles have been demonstrated during pregnancy or pseudopregnancy in various kinds of mammals (rats[41,103,121], cats[25], guinea-pigs[98], rabbits[1,46], hamsters[33,40,42], sows[27,86,92], ewes[39,131], goats[52], mares[5,31,97], cows[45,86], badgers[53], and women[25]). Ovulation does not usually occur following follicular development during pregnancy or in the presence of corpora lutea. However, several researchers have verified that ovulations naturally occurred on those conditions in the cats[25], rats[69], ewes[39], mares[4,5,26,30], cows[48], badgers[53], minks[35], African elephants[91] and women[25]. Possible or probable superfetation has also been reported as the naturally occurred rare cases in various species which included the mouse[10,73,96], rat[96,107,129], mink[51], cat[25,60,63,74], European hare[15,75,76,116], pig[108,109], sheep[39,109], burro[106], cattle[24,29] and human[36,38,78,81,120,128]. NAKAGAWA *et al.*[85] have experimentally succeeded in preparing superfetational conditions in lactating pregnant rats by local administration of small amount of estradiol-17β (E_2) into the adipose tissue of mesometrium.

In many animal species, artificial ovulation by administration of gonadotropic hormones have been carried out during the luteal phase or pregnancy (mouse[12~14,136], hamster[40], guinea-pig[98], rat[122], rabbit[1,6,8,32,54,67,73,82,84,111~114,118,133,134], pig[16,61,123], sheep[50,83,95] and cattle[17,48,101,130]). These evidence showed that the above reproductive phenomena are not uncommon in mammals,since estrus and/or copulation naturally occurred during pregnancy or pseudopregnancy (mouse[28], rat[87], rabbit[46,124], quinea-pig[88], pig[44,92], sheep[131], cattle[45,70,119,132] and

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horse[97,102,127]). Thus, it is suggested that there is a strong feasibility that the pregnant animals are able to ovulate eggs which can be fertilized *in vivo* or *in vitro*.

Studies on the fertility of eggs which ovulated during pregnancy or pseudopregnancy were investigated mainly using the rabbits. The first experimentally induced superfetation in the rabbit was reported by WISLOCKI and SNYDER[133]. They found two different sets of fertilized eggs from a mated does which was induced again for the second ovulation four days later after mating by injecting intravenously anterior lobe extract followed by insemination with fresh spermatic fluid into the vagina. On autopsy, twenty-one hours after the ovulatory injection, the ovaries showed the presence of corpora lutea of two separate ovulations. Concurrently, five normal developing blastocysts were located from the uterine horn and six segmenting normal ova were recovered from the oviduct. This means that the ova in the second set by induced ovulation during pregnancy can be fertilized. The results were repeatedly confirmed by the same authors.[134] However, MURPHREE *et al.*[83] mentioned a lack of fertility in rabbits treated with pituitary extracts during pseudopregnancy. In their study, in most cases sperms were found in the oviduct at autopsy one day after insemination. They assumed that the spermatozoa were potentially fertile when they reached the oviduct, and that the failure of fertility would appear to be inherent in the eggs and in some was brought about by the reproductive state of the animal at the time of treatment. In their further study[84], none of the eggs recovered from the eleven does treated at the 5th day of pseudopregnancy appeared to be fertilized at autopsy 45 to 78 hours after the intravenous injection. They found 57 corpora lutea from the five "luteal" ewes that were treated with follicle-stimulating extracts, however only 25 unfertilized eggs were recovered. Nevertheless, BOYARSKY *et al.*[11] demonstrated in the rabbits 80.0%, 22.0% and 5.6% fertility of the eggs that were ovulated at the 3rd, 5th and 10th day of pseudopregnancy when intravaginal artificial insemination was carried out. Administration of progesterone to estrous animals for 10 days before experimental ovulation also produced a marked suppression of fertilization, yielded from 0 to 38% fertilized eggs for different females, with an average of 5.2%. According to AUSTIN[6], artificial insemination did not lead to fertilization of eggs ovulated by injection of human chorionic gonadotropin (hCG) between day-4 and day-12 pseudopregnancy, but did result in fertilization outside this period. In his experiment, diluted epididymal sperm was deposited into the oviduct through the infundibulum after 4 or 5 hours from hCG injection. Fertilized eggs were obtained from all the five estrous does.

Fourteen fertilized eggs were obtained from a total of 19 eggs in which only 4 regularly segmenting eggs. In his other experiment, sperm do not reach the oviduct on or after the 5th day of pseudopregnancy. He concluded that the conditions existing in the pseudopregnant oviduct militate against the process of fertilization, and that this factor, together with the abnormal transport of sperm adequately explains the failure of artificial insemination during pseudopregnancy. A very interesting data was presented on this matter by MURPHREE *et al.*[82] Pseudopregnant rabbits after 8-10 days from mating and 10 days pregnant does were artificially induced ovulation by intravenous injection of either unfractionated sheep pituitary extract or hCG. They were inseminated through the vaginal lumen or uterine lumen which had been exposed by a midventral laparotomy. Fertility rates of the second set of ova in the pseudopregnant does which were inseminated through the vagina or uterine lumen were showed 3.1% and 61.0%, respectively. While in the pregnant does, fertility rates of 12.1% and 83.3% were obtained when intravaginal insemination and intrauterine insemination were carried out, respectively. These are comparable to those of the normal estrous does. When diluted semen where the number of sperms were only slightly above the minimum level necessary for fertilization in rabbits were used, the percentage of fertile ova from the estrous does which were artificially ovulated was 86.6%. In these does the semen was deposited in either the vaginal or uterine lumina. However, in the pseudopregnant does only 5.1% were observed and it was suggested that there were too few sperm in the oviducts of pseudopregnant does. It was suggested by them that the initially-observed infertility in luteal phase rabbits was in large part due to difficulties in sperm transport, that is, some interference with the transport of sperm through the cervix. This was confirmed by an experiment of NUTTING and MATES.[89] Fertilization of ova ovulated by hCG injection was inhibited by daily injections of 1 mg of progesterone for 2 or 6 days before intra-vaginal insemination. However, when semen was deposited into the uterus of does that were given 1 mg progesterond/day for 6 days, nearly all ova were fertilized. They considered that progesterone inhibited fertilization of ova primarily by interference with sperm transport mechanisms in the uterus and/or oviduct.

Fertilizing capacity of sperm deposited in the uterine tube could be attained in the rabbits.[18] However, the capacitation of sperm was inhibited in the uterus but was not so affected in the oviduct under the influence of progesterone.[19] On the other hand, CHANG[20] clarified that the eggs recovered from the pregnant does were perfectly normal as shown by the

presence of the first polar body and the second maturation spindle. The eggs were also considered physiologically normal because they can be fertilized either *in vitro* or after being transfer in the oviducts of mated rabbits. According to *Ishibashi*[62], the percentage fertility of undertilized eggs that were recovered from donors 13 to 14 hours after hCG injection and transferred into the oviducts of mated pseudopregnant recipients was 89% when they were transferred at 12 hours after hCG injection. However, the percentage fertility decreased to 2% when they were transferred on the 5th day of pseudopregnancy. This was probably due to either the difficulties in sperm transport in the pseudopregnant does or to the accelerated egg transport in the oviduct.

Oral administration of medroxyprogesterone acetate before ovulation in rabbits did not inhibit fertilization.[21] Similarly, fertilization of eggs was not completely inhibited when they were ovulated during active pseudopregnancy or continuous progesterone treatment, although total degeneration of eggs occurred by day 6.[23] When in ferrets, the administration of medroxyprogesterone acetate before ovulation inhibited fertilization and hastened the transport of eggs from the oviduct to the uterus. It also gave rise to the inhibition of capacitation of spermatozoa in the uterus or to disturbances in spermatozoal transport.[22] In pigs, eggs that were superovulated during luteal-phase and inseminated 4 to 18 hours before ovulation were found to be penetrated by sperm ; 31.9% of penetrated eggs was normally fertilized, 1.9% was fragmenting, 5.6% was primary oocytes and 60.6% was polyspermic.[61]

It is naturally considered that the eggs immediately after ovulation during pregnancy or pseudopregnancy will be fertile, at least in the rabbit. Inhibition of sperm migration through the reproductive tract seemed to become more stronger with advancing stage when intra-vaginal insemination or mating was carried. However, high fertility of eggs was demonstrated when intrauterine insemination was done. This problem could be solve by using intraperitoneal insemination to recover fertilized eggs in later stages of pregnancy or pseudopregnancy. The first successful report of intraperitoneal insemination in the fowls and pigeons was by VAN DRIMMELEN.[126] The percentage of inseminations proved fertile in fowls was measurably high after intraperitoneal insemination than after insemination *per vaginam*. A nulliparous heifer with normal sexual behavior was pregnant by intraperitoneal insemination through the vaginal fornix near to the posterior os.[106] It was also reported that one of 6 intraperitoneal insemination to 4 sexually mature Holstein-Friesian geifers was followed by pregnancy.[77] However, 9 caves treated for superovulation failed to obtained fertilization by intraperi-

toneal insemination through a puncture of the abdominal wall.[9] Successful intraperitoneal insemination in the guinea-pig was reported by ROWLAND.[99,100] He found that the optimal time of insemination was shortly after the end of estrus. Conception rate of about 80% was obtained in 25 animals that were inseminated intraperitoneally within narrow limits (1-12 hours) after estrus, while in the other 17 animals that were inseminated intraperitoneally during 0-8 hours after estrus a conception rate of 100% was attained. Thus, fertility following intraperitoneal insemination seemed to be similar to that resulting from normal matings, and the rate of fetal development appeared to be quite normal in this species.

The first successful intraperitoneal insemination in rabbit does was demonstrated by HADEK.[43] Does mated to vasectomized bucks and inseminated through the linea alba with freshly ejeculated semen (diluted or washed) 1 to 12 hours after mating, produced degenerating blastocysts when inseminations were done later than 2 hours after mating, while viable blastocysts were obtained when they were inseminated within 2 hours after mating. The average number of viable blastocysts in the does was 6. MROUEH and MASTROIANNI[80] carried out intraperitoneal inseminations between 18 hours before and 1 hour after the estimated ovulation time in 31 does. They designated as ovulation time "0" arbitrarily based on a 12-hours after hCG administration. They found that 8 out of 12 does inseminated 12-16 hours before 0 time became pregnant and that no implantation sites were seen when insemination was carried out more than 16 hours or less than 12 hours before 0. Comparison of fertilities in three different routes of inseminations which were intraperitoneal, intratubal via the fimbria and intravaginal at 3, 10, 15 and 20 hours before induced ovulation by LH administration showed quite similar levels of fertilization.[2] The intraperitoneal route was the most variable but apparently the sperm lost their fertilizing capacity sooner after deposition in the body cavity. Following both peritoneal and tubal insemination, the proportion of eggs recovered was much below the expected level due to the destruction of eggs when excessive numbers of sperm were present in the tube. In further studies[3], intraperitoneal insemination 3 to 6.5 hours before the injection of hCG attained varied proportions of eggs fertilized from 20 to 79%, excluding does having practically no fertilization. The best fertility was shown in a group with 3 hours interval between insemination and ovulating injections.

The above reviews suggest the presence of a strong possibility that the eggs artificially ovulated in many pregnant mammalian animals were having normal fertility. It was found that the intraperitoneal insemination will be

a useful method to fertilize eggs during post-implantational period. Therefore, there is a possibility that the pregnant animals can be used as one of the sources for fertilized eggs for transfer. In our previous paper[72], we found that induced ovulation on days 1-7 and days 28 to 30 *post coitum* produced normal parturition of about 70%. However, severe damages on pregnancy occurred when the induced ovulation was carried out between day 8 and day 27 *post coitum*.

In the present studies, we carried out certain trials to determine methods in which fertilized eggs can be obtained from the pregnant does while maintaning their pregnancies when receiving such treatments.

Materials and Methods

Two hundred ninety-three mature, female Japanese White rabbits were used in the present study. They were divided into eight experiments. The day of mating and/or hCG administration was designated as day 0. In the present study, cleaved eggs and one-cell eggs having 2 polar bodies in perivitelline space were considered as fertilized eggs. Distinction between fertilized eggs derived from the 1st or the 2nd ovulation was based on the cell-stages; for example, at 24 and 48 hours after ovulation the eggs of 2nd ovulation were in one-cell stage and in 2 to 16 cell-stages, respectively, while cell-stages of the 1st ovulation have already reached 2 to morula-stages 48 hours after ovulation and blastocyst-stage at 96 hours.

The 1st experiment The aim of this experiment was whether superfetation does occur or not in rabbits. Twenty one does that had been premated with 2 fertile bucks and given 20 IU of hCG intravenously into the marginal vein of the ear 1 day (13 does) or 2 days (8 does) were re-induced to evulate by injection of 50 IU of hCG. They were euthanised by an intravenous injection of sodium pentobarbital (Somnopentyl, Pitman-Moore Inc.) 1 or 2 days later, respectively. Their reproductive tracts were flushed with physiological saline to recover eggs. Corpora lutea or ovulation points in ovaries were counted. As for control, 30 premated does were injected with hCG in the same manner and received similar treatment except that injection of hCG was given simultaneously during fertile coitus.

The 2nd experiment Successful recovery of fertilized eggs from the 2nd ovulation in the 1st experiment encouraged to delay the 2nd ovulation and matings at 3-day pregnancy. In this study, 10 does that were previously mated and injected with 20 IU of hCG 3 days before, were again 50 IU of hCG and remated with 2 fertile bucks for 3 times at 6-hour intervals so as to increase copulatory stimuli. They were euthanised 48 hours later for

egg recovery.

The 3rd experiment Semen was collected with an artificial vagina from 3 or more fertile males depending upon the volume required. The semen were pooled after removal of the gel (whole semen). Forty does at 9-, 15- and 23-day pregnancy were intraperitoneally inseminated with 0.5-1.0 ml of whole semen according to the method of HADEK[43] and MROUEH and MASTROIANNI[80]. The does received 50 IU of hCG at 0, 3, 4, 10, 15 or 20 hours later, and then *in vivo* egg recovery by bilateral laparotomy was done to fludh the oviduct 35 to 45 hours later to examine fertilization of eggs. This flushing time was derermined from the results of the 1st and 2nd experiments.

The 4th experiment The 3rd experiment has shown that there is a strong feasibility that the rabbit eggs ovulated during pregnancy were fertilizable by the intraperitoneal insemination with whole semen. This experiment was done to study the effect of diluted semen on fertility in intraperitoneal insemination. The whole semen was concentrated by centrifugation at 3,000 rpm for 15 min. to remove seminal plasma. One lot of semen was washed twice with sodium citrate buffer (pH 7.4), and was diluted to double its amount with the buffer solution. Penicillin (1,000 units/ml) was added to the diluted semen. Twenty-two does at 9-day pregnancy, 20 does at 15-day and 33 does at 23-day were randomly divided and intraperitoneally inseminated with either whole or diluted semen (100 to 150×10^6/ml spermatozoa) and then they were administrated 50 IU of hCG 3 to 4 hours later. Egg recovery was done as in the 3rd experiment.

The 5th experiment In the 2nd to the 4th experiments, egg recovery was done at 4o or more hours after hCG injection. In this experiment, Chang's method[20] of *in vivo* egg recovery was applied. Fifteen unfertilized eggs encolsed by cumulus cells were recovered 16 and 18 hours after hCG administration from oviducts of 2 does at 23-day pregnancy. These eggs were transferred into the ipsilateral oviducts of 3 synchronized recipients. These recipients were previously unilaterally ovariectomized. They were then mated and received 20 IU of hCG.

The 6th experiment Twenty-two eggs in 2 to 4-cell stages, intraperitoneally fertilized and recovered from pregnant does used in the 4th experiment were transferred into oviducts of 4 synchronized recipients.

The 7th experiment Since induced ovulation in the pregnant rabbits causes delayed parturition or abortion usually within 2-3 days after ovulation treatment[72], this experiment was carried out to find ways in maintaining the normal pregnancy and to produce normal delivery (Table 8). Twenty does

at 23-day pregnancy were given 5 to 20 mg or 2 mg of progesterone for the maintenance of pregnancy. Natural parturition did not occur when the above treatment was carried out, therefore, E_2 was then used as a luteotropic agent for maintenance of pregnancy in the following experiments.

To determine suitable dosage of E_2 to maintain pregnancy, nine does of 9-day pregnancy and 14 does at 15-day pregnancy were given 1, 2 or 3 μg E_2 for 3 days from the day of hCG administration. Seven of 14 does at 15-day pregnancy, which were able to maintain pregnancy by giving 1 to 3 μg of E_2, were subcutaneously given 2 mg of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) per kg body weight at 29-day pregnancy and 5 IU of oxytocin per a doe on the morning of 31-day pregnancy. It was observed that 2 μg E_2 gave the best result for maintenance of pregnancy. From this result, the following experiment was conducted to determine the regime for induction of parturition. Twelve does at 23-day pregnancy wich were able to maintain pregnancy by giving 2 μg of E_2, were treated with 2 mg of $PEF_{2\alpha}$ at 29 and/or 30-day pregnancy followed by 10 or 5 IU of oxytocin per doe on the morning of 31-day pregnancy. Nine does at 23-day pregnancy, which were able to maintain pregnancy by giving 2 μg of E_2, were given 2 mg of $PGF_{2\alpha}$ per kg of body weight per doe at 29- and 30-day and then, 1 mg of E_2 per doe at the 30th day pregnancy followed by 5 IU of oxytocin per doe on the morning of 31-day pregnancy.

Twenty does at 23-day pregnancy, where pregnancy was maintained by progesterone administration, were given 1 or 2 mg of E_2 per doe at 29- to 31 or 32-day pregnancy, 2 to 5 mg of $PGF_{2\alpha}$/kg/doe at 28 to 30-day or at 33-day pregnancy, and 5 times of 3 or 2 IU of oxytocin/doe at 31 and 32-day pregnancy.

The 8th experiment This experiment was done based on the results that were obtained from all the 7 previous experiments. Eight does at 9-day pregnancy, 13 does at 15-day and 9 does at 23-day were reinduced using 50 IU of hCG, and 5 does in each pregnant stage were served as control. Whole semen was used and the does were inseminated intraperiotoneally 3-4 hours before hCG injection. *In vivo* egg recovery was done 36 to 40 hours after hCG treatment. For maintenance of pregnancy, 2 μg of E_2/doe/day was intramuscularly injected for 3 days. For induction of delivery, 2 mg of $PGF_{2\alpha}$/kg/body weight was subcutaneously injected at 29- and/or 30-day pregnancy, 1 mg of E_2/doe was intramuscularly injected at 30-day, and 5 IU of oxytocin was intravenously injected at 31-day.

Results

The 1st experiment The result of the experiment showed that sperms derived from the first mating already became unfertile to the eggs of the 2nd ovulation which was induced 1 or 2 days after the 1st mating (Table 1). This can be seen in group B where no fertilized eggs from the 2nd ovulation was observed when mating was carried out only after the first ovulatory injection. It was also observed that some of the eggs from the 2nd ovulation were fertilized by the 2nd mating on the 2nd ovulation treatment. However, the fertilization rate of the eggs from the second ovulation was low.

TABLE 1. Effect of insemination (matingstwice with bucks) on fertilization rate of the secondary ovulated eggs that were reinduced 1 or 2 days after the first induced ovulation treatment

Group	Days after the first ovulatory treatment	No. of does examined	Source of eggs recovered	No. of eggs ovulated (mean)	No. of eggs recovered (mean)	No. of does recovered fertilized eggs (%)	No. of eggs fertilized (mean)	Fertilization rate (%)
A	1	10	1st ovulation	113(11.3)	79(7.9)	10(100)	77(7.7)	97.5
			2nd ovulation	32(8.0)	23(5.8)	4(40)	7(1.8)	30.4
	2	20	1st ovulation	208(13.0)	155(9.7)	16(84)	152(9.5)	98.1
			2nd ovulation	123(13.7)	92(10.2)	9(45)	32(3.5)	34.9
B	1	13	1st ovulation	136(12.4)	119(10.9)	11(84.6)	114(10.3)	95.3
			2nd ovulation	108(8.3)	83(6.4)	0	0	0
	2	8	1st ovulation	79(11.3)	63(9.0)	7(87.5)	57(8.1)	90.5
			2nd ovulation	108(13.5)	79(9.9)	0	0	0

A: The first and second ovulatory injections were followed by matings in both cases.

B: Only the first ovulatory injection was followed by matings.

The 2nd experiment In each doe there was no significant difference in the numbers of eggs ovulated between the 1st and 2nd ovulation. All eggs that were produced from the 2nd ovulation were unfertilized in 60% of the does (Table 2). However, fairly high percentage fertility was obtained as compared to that of the 1st experiment. This may be due to the multiple matings that were carried out at 6-hours intervals. Developmental stages of eggs derived from the 1st and 2nd ovulations are shown in Table 3,

TABLE 2. Fertilization rate of eggs derived from the 2nd ovulation in day-3 pregnant does that were reinduced with 50 IU of hCG followed by 3 times matings at 6-hour intervals

Days after the first ovulatory treatment	No. of does	Source of eggs recovered	No. of does recovered eggs (%)	No. of eggs ovulated (mean)	No. of eggs recovered (mean)	No. of eggs fertilized (mean)	Fertilization rate (%)
3	10	1st ovulation	A : 10(100)	126(12.6)	118(11.8)	106(10.6)	89.8
		2nd ovulation	A : 4(40)	54(13.5)	42(10.5)	29(7.1)	69.0
			B : 6(60)	82(14.0)	62(10.3)	0	0

A : Number of does with fertilized eggs.
B : Nbmber of does in which all recovered eggs were unfertilized.

TABLE 3. Developmental cell stages of eggs derived from the 1st and 2nd ovulations (included eggs from the 1st and 2nd experiments)

Distinction of ovulation	No. of eggs recovered	Developmental cell stages of eggs	Hours from hCG administration to autopsy							
			24	36	48	66	72	84	96	120
1st ovulation	506	2-4 cells			17(15)[c]					
		8-16 cells			94(82)	3(8)	4(5)			
		morula			3(3)	33(87)	66(81)	5(11)		
		blastocyst					9(11)	33(72)	13(11)	
		expanded blastocyst						6(13)	103(85)	102(96)
		degenerated egg[a]				2(5)	2(3)	2(4)	5(4)	4(4)
		Total			114	38	81	46	121	106
2nd ovulation	68	1 cell[b]	8(100)	1(11)						
		1-4 cells		8(89)	6(13)					
		8-16 cells			41(87)					
		morula					4(100)			
		Total	8	9	47		4			

a) Fertilized eggs that were degenerated after cleavage.
b) Presence of 2 polarbodies in perivitelline space.
c) Figure in parenthesis shows percentage of eggs recovered in same hours after hCG administration,

Data in this table were combined from the 1st and 2nd experiments, since normal fertilized eggs showed similar developmental stages corresponding to the hours after fertilization with no discrimination between ovulations.

The 3rd experiment In each group of post-implantation stage the egg recovery rates from does intraperitoneally inseminated were variable; 20-59% at 9-day, 17-67% at 15-day and 28-60% at 23-day pregnancy. Percentage fertilities for all eggs ovulated were within a narrow range (10-19%) in each stage of pregnancy with no special trend (Table 4). It was observed that relatively good results in recovery of fertilized eggs were obtained when intraperitoneal inseminations were done at 3 to 4 hours before hCG administration.

TABLE 4. Effects of time intervals of intraperitoneal insemination (IPI) on the fertilization rate of eggs derived from the 2nd ovulation in the 9-, 15- and 23-day pregnant does

Days in pregnancy	Hours from IPI to hCG dosage	No. of does examined	No. of ovulations	No. & (%) of eggs recovered	No. of does having fertilized eggs	No. & (%) of eggs fertilized	Fertility[a] rate (%)
9	0	2	13	5(38.5)	1	2(40.0)	15.4
	3	2	12	5(41.7)	1	2(40.0)	16.7
	4	4	27	12(44.4)	2	4(33.3)	14.8
	10	2	10	2(20.0)	1	1(50.0)	10.0
	15	2	15	7(46.7)	0	0	0
	20	2	29	17(58.6)	0	0	0
15	0	2	12	2(16.7)	1	2(100)	16.7
	3	3	21	9(42.9)	2	4(44.4)	19.0
	4	2	18	7(38.9)	1	3(42.9)	16.7
	10	2	31	17(54.8)	0	0	0
	15	2	9	2(22.2)	1	1(50.0)	11.1
	20	2	18	12(66.7)	0	0	0
23	0	2	43	21(48.8)	0	0	0
	3	3	49	19(38.8)	2	7(36.8)	14.3
	4	3	23	9(39.1)	1	4(44.4)	17.4
	10	2	18	5(27.8)	1	3(60.0)	16.7
	15	2	63	38(60.3)	0	0	0
	20	2	37	21(56.8)	0	0	0

a) Calculation is based on the number of fertilized eggs to the number of ovulations.

The 4th experiment Effects of time intervals of intraperitoneal insemination on the fertilization rate of eggs derived from the 2nd ovulation in the 9-, 15-, and 23-day pregnancy does are shown in Table 5. Eggs of 16 does inseminated 3-4 hours before hCG administration in the 3rd experiment (5 does at 9-day pregnancy, 5 does at 15-day and 6 does at 23-day) were also included in this Table. As a whole, rates of does having fertilized eggs derived from the 2nd ovulation decreased from about 60% when using whole semen to about 30-40% when diluted semen was used. Rate of egg recovery were about 30%, except 62% in one group when diluted semen was used on the 9-day pregnancy. Percentage fertilities of eggs were relatively constant (62-67%), except in the group where diluted semen was used on the 9 day pregnant does. There were no significant differences in percentage fertilities between days in pregnancy.

TABLE 5. Comparison in fertility of eggs derived from the secondary ovulatory treatment at 9-, 15- and 23-day pregnancy by intraperitoneal insemination using either whole or diluted semen

Days in pregnancy	Semen used	No. of does examined	No. of ovulations	No. & (%) of eggs recovered	No. & (%) of does having fertilived eggs	No. & (%) of eggs fertilized	Fertility of all ovulations (%)
9	whole	12	73	26 (35.6)	7 (58.3)	16 (61.5)	21.9
	diluted	10	34	21 (61.8)	3 (30.0)	9 (42.9)	26.5
	total	22	107	47 (43.9)	10 (45.5)	25 (53.2)	23.4
15	whole	10	68	26 (38.2)	6 (60.0)	16 (61.5)	23·5
	diluted	10	53	17 (32.1)	4 (40.0)	11 (64.7)	20.8
	total	20	121	43 (35.5)	10 (50.0)	27 (62.8)	22.3
23	whole	20	277	85 (30.7)	13 (65.0)	53 (62.4)	19.1
	diluted	13	93	27 (29.0)	4 (30.8)	18 (66.7)	19.4
	total	33	370	112 (30.3)	17 (51.5)	71 (63.4)	19.2

The 5th experiment At 12-day pregnancy recipient No. 1 was euthanised and conceptuses were found in both uterine horns. In this recipient, 7 native conceptuses were found in the left uterine horn. However, out of the 5 transferred eggs 2 normal size conceptuses were found in the right uterine horn (Table 6). While, in the other 2 recipients no conceptuses that are derived from the transferred eggs was observed when laparotomy

TABLE 6. Transfer of unfertilized eggs derived from the 2nd ovulation treated at 23-day pregnancy, into the ipsilateral oviducts of mated recipient does which were previously uniovariectomized

Recipient no.	Side of oviduct	No. of eggs ovulated	No. of eggs transferred	No. of implantation sites	No. of young
1	Left	7	—	7[a]	—
	Right	—	5	2	—
2	Left	10	—	8[b]	7
	Right	—	5	0	0
3	Left	—	5	0[b]	0
	Right	5	—	5	5

a) Autopsy at 12-day pregnancy.
b) Observation by laparotomy at 10- or 12-day pregnancy.

on 12-day pregnancy was done. However, they normally delivered their native young.

The 6th experiment Result of transfer of intraperitoneally fertilized eggs are shown in Table 7. Transfers of 2-4 cell stage eggs recovered from 4 donors at 9- or 15-day pregnancy to recipients on 1.5 days after 20 IU of hCG administration failed to conveive. However, one recipient that was transferred 2-cell stage eggs delivered one large size normal young. These 2-cell stage eggs delivered one large size normal young. These 2-cell stage eggs were recovered from 3 does at 23-day pregnancy.

TABLE 7. Transfer of intraperitoneally fertilized eggs recovered from pregnant rabbits

Donor			No. of recipients	Days after[a] ovulatory treatment in recipients	No. & cell stages of eggs transferred	No. of pregnant does by embryo transfer	No. of young
Days in pregnancy	Semen used	Hours from hCG dose to egg recovery					
9	whole	36-38	1	1.5	5(2-4 cells)	0	0
15	diluted	35-37	1	1.5	5(2-4 cells)	0	0
23	whole	36-38	2	1.5	10(2 cells)	1	1[b]

a) Days after 20 IU of hCG administration,
b) Normal delivery.

The 7th experiment When progesterone was administered, the pregnant does successfully maintained the pregnancy, but most of them failed to delivered their young in spite of the following treatments ; 1 or 2 mg of E_2 given on 29 to 32- or 29 to 31-day pregnancy 3-5 mg or 5 mg/kg body weight of $PGF_{2\alpha}$ given on 33-day or 28 to 30-day pregnancy (Table 8). Abdominal palpation showed that their young were still growing in these days, and the pregnant does frequently showed parturient behaviors during 30-day (1 to 2 days after $PGF_{2\alpha}$ administration) to 35-day pregnancy, and some of them even bled from their vulvas. After, about 2 weeks, 6 of 20 pregnant does died, though other does delivered mummified fetuses or parts of degenerated conceptuses.

TABLE 8. Hormonal treatments for maintenance pregnancy and to induce parturition in does artificially ovulated during pregnancy

Days in pregnancy treated ovulatory dose	No. of pregnant does examined	Treatments[a] for pregnancy maintenance	No. of does maintained pregnancy	Hormonal treatment for induced parturition			No. of[b] does induced parturition
				Dose of E_2/doe	Does of $PFG_{2\alpha}$/kg body weight	Does of oxytocin /doe	
9	4	E_2 1 μg	1	0	0	0	1
	5	E_2 2 μg	5	0	0	0	5
15	4	E_2 1 μg	1	0	2 mg (29)[c]	5 IU (31)[c]	1
	5	E_2 2 μg	5	0	2 mg (29)	5 IU (31)	5
	5	E_2 3 μg	1	0	2 mg (29)	5 IU (31)	1
23	11	P 5-20 mg	11	1 mg (29-32)	2-5 mg (33)	3 IU (×5)[d] (31, 32)	0
	9	P 2 mg	9	2 mg (29-31)	5 mg (28-30)	2 IU (×5)[d] (31)	2
	5	E_2 2 μg	5	0	2 mg (29)	10 IU (31)	0
	7	E_2 2 μg	7	0	2 mg (29, 30)	5 IU (31)	5
	9	E_2 2 μg	9	1 mg (30)	2 mg (29, 30)	5 IU (31)	9

a) Dosage/day/doe for 3 successive days.
b) Number of does delivered all conceptuses.
c) Figure in parentheses shows days in pregnancy treated.
d) Number of times.
E_2, estradiol-17 β; P, progesterone; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$.

TABLE 9. *In vivo* recovery of fertilized eggs from does received intraperitoneal insemination and ovulatory treatment at

Days in pregnancy inseminated intraperitoneally and treated ovulatory dose	No. of pregnant does used	No. of[a] corpora lutea derived from 1st ovulation (mean±S.E.)	No. & (%) of[b] secondary ovulated eggs (mean±S.E.)	No. & (%) of implantation sites (mean±S.E.)
9	5[d]	10.0±0.5	—	8.6±1.0 (86)
	8[e]	10.3±0.7	10.0±2.0	8.4±1.1 (82)
15	5[d]	11.0±0.4	—	9.0±0.6 (82)
	13[e]	10.1±0.6	10.5±0.8	8.6±0.6 (85)
23	5[d]	8.6±1.0	—	8.0±1.0 (93)
	8[e]	10.5±0.8	22.5±1.9	9.1±1.1 (87)

a) By laparotomy at 8- or 9-day pregnancy.
b) By laparotomy and *in vivo* egg recovery at 11-, 17- or 25-day pregnancy, pespectively.

Six out of 9 does at 9-day pregnancy were able to maintain pregnancy when receiving 1 or 2 μg E_2. All 5 does which received 2 μg of E_2 while only 1 out of 4 does receiving 1 μg E_2 gave natural parturition. Fourteen does at 15-day pregnancy were given either 1, 2 or 3 μg of E_2/doe/day for 3 days from day of hCG administration and it was observed that all 5 does receiving 2 μg of E_2 successfully maintained their pregnancy. Three does receiving 1 μg of E_2 aborted 2 days after the induced ovulation treatment and 4 out of 5 that received 3 μg of E_2 aborted about one week later. In this group, normal delivery occurred after receiving 2 mg/kg of $PGF_{2\alpha}$ at 29-day and 5 IU/doe oxytocin on the morning on 31-day, within 30 min. to 1 hour after oxytocin administration especially in the does which received 2 μg of E_2.

Administration of 2 μg E_2 to does at 23-day pregnancy was able to maintain the pregnancy and did not cause any abortion, but treatments of $PGF_{2\alpha}$ at 29-day and oxytocin at 31-day pregnancy failed to induce delivery. However, when $PGF_{2\alpha}$ was given twice at 29- and 30-day pregnancy, followed by 5 IU of oxytocin administration at 31-day, delivery was induced in 4 does out of 7 does. While, 9 pregnant does at 23-day where pregnancy was maintained by 2 μg E_2 administration, were given 1 mg of E_2 at 3-day, 2 mg/kg of $PGF_{2\alpha}$ at 29- and 30-day and 5 IU of oxytocin at 31-day, resulting in induction of normal delivery in all does.

The 8th experiment Results of laparotomy at 8- or 9-day pregnancy

9-, 15- or 23-day pregnancy, maintenance of the pregnancy following the treatments and induction of delivery by hormonal treatments

No. of young delivered (mean±S.E.)	Rate of[c] maintenance of pregnancy (mean±S.E.)	Percentage of eggs recovered from 2nd ovulation (mean±S.E.)	Fertility of eggs derived from 2nd ovulation (mean±S.E.)	No. & (%) of does produced fertilized eggs during pregnancy
7.6±1.2	88.4±10.5	—	—	
5.9±0.8	70.0±6.9	45.0±9.4	38.9±10.2	4 (50)
6.8±0.9	76.0±11.2	—	—	
5.7±1.4	66.1±3.7	54.4±11.2	35.1±9.7	6 (46)
6.4±0.1	80.0±7.5	—	—	
4.8±0.8	53.4±5.7	52.2±10.3	40.4±11.2	5 (63)

c) Number of young/number of implantantation sites×100.
d) Control group.
e) Treated group received egg recovery during pregnancy.

indicated that the number of young to number of conceptuses were low in treated groups in the 9-day and 15-day pregnant does (Table 9). Nevertheless, there were no significant differences to the control groups. However, does treated on 23-day pregnancy showed significantly lower number of young as compared to the control ($p<0.05$). This was probably due to surgical procedures on 8- or 9-day pregnancy for inspection of numbers of corpora lutea and conceptuses. Egg recovery rates in the 2nd ovulation during pregnancy were about 50% and fertilities of these eggs were 35 to 40% in each group. However, the recalculated fertility which include only does in which fertilized eggs were recovered was about 60%, similar to does in the 4th experiment; the numbers of does having fertilized eggs were 4 out of 8 at 8-day pregnancy, 6 out of 13 at 15-day and 5 out of 8 at 23-day. An average body weight of new young was 43.0±1.1 g (mean± S. E.) in the treated group, and this value was significantly lower than that of the normal untreated group (52.5±2.5 g) ($p<0.01$).

Discussion

The facts reviewed in the introduction of this paper strongly suggest that pregnant animals in various species can ovulate spontaneously or artificially, and that the ovulated eggs are fertile during some hours. In recent years, embryos for transfer have been recovered limitedly from non-pregnant cyclic females. However, if eggs recovered from pregnant animals are ferti-

lized and the females are able to end their pregnancies with normal parturitions, source of embryos for transfer would be extended over a wider range. Based on this conception, some fundamental experiments were carried out to recover fertilized eggs from pregnant rabbits and to find out a method which can maintain the pregnancy and resulted with normal parturition.

Superfetation commonly occur in the common hare[15,75,76,116]. MARTINET REYNAUD[75] demonstrated that spermatozoa derived from the initial mating with a fertile male can survive for a long period within the pregnant female genital tract. In spute of an average gestation length of 41 days, the interval between successive parturition was frequently only 36-39 days. They mated pregnant hares with vasectomized males 1-6 days before the expected date of parturition, and found recent ovulations and dividing fertilized eggs at laparotomy several hours later. In this study, firstly we examined whether such a phenomenon exists in the rabbit or not. The results indicated that secondary ovulated eggs by hCG administration 1 or 2 days after the first mating with fertile bucks (+hCG) were not fertilized if simultaneous matings were not carried out. However, according to ISHIBASHI[62], unfertilized eggs obtained from the pseudopregnant does 12 hours after matings showed high percentage fertility in synchronous transfer into oviducts of recipient does. This percentage decreased markedly to 26% if transfer was done 2 days after mating. Although reasons for no fertilization of secondary ovulated eggs in rabbits are not clear, it is suspected that the difference between the present study and ISHIBASHI's experiments may be due to the decreased or exhausted number of spermatozoa in the oviducts of pregnant does, because spermatozoa were spent as the supplementary sperms into the initially ovulated and fertilized eggs. It may also be due to the lost of fertilizing ability of the spermatozoa by interaction between initially ovulated eggs and spermatozoa, such as aggregation of spermatozoa, or by other mechanisms. This was shown by the improved fertility in secondary ovulated eggs in the 2nd experiment, in which 3 times matings were applied at 6-hour intervals in the 2nd ovulatory treatment. Hence, to obtain fertilized eggs during pregnancy in the rabbits, mating with fertile bucks or insemination are requisite.

In our previous study[72], the gestation period of the rabbits was divided into 5 stages according to responses to induced ovulation during pregnancy which mainly based on abortion, normal or delayed parturition and delivery of normal, dead or mummified fetuses. These stages were stage I (1 to 7 days after initial coitus), stage II (8 to 14 days), stage III (15 to 20 days), stage IV (21 to 27 days) and stage V (28 to 30 days). This grouping seemed to be reasonable on the morpho-physiological points of views. However, in

the present study, we selected 9-, 15- and 23-day pregnancy as representative days for stages II to IV of post-implantational stage. Stage V was not treated because surgical treatments for inspection of ovaries during this period immediately induced normal delivery.

In these post-implantational stages, intraperitoneal inseminations were applied because no sperm transport through the female reproductive tract was expected. Percentage fertilities of eggs derived from the 2nd ovulation were low with the exception of a relatively good results that were obtained when intraperitoneal inseminations were done 3 to 4 hours before hCG administration, regardless in using either whole or diluted semen. In HADEK's experiment[43], all blastocysts which were derived from normal ovulations of estrous rabbits by intraperitoneal insemination later than 2 hours after mating with vasectomized bucks were degenerating, while those obtained as a results of insemination within 2 hours after mating were viable. MROUEH and MASTROIANNI[80] carried out intraperitoneal insemination between 18 hours before and 1 hour after the estimated ovulation time which result in pregnancy in 8 out of 12 does inseminated 12-16 hours before the ovulation.

According to ADAMS[3], proportion of eggs recovered varied from 28 to 95% in 8 groups of 39 does inseminated intraperitoneally 3 to 6.5 hours before the injection of hCG, and the egg fertilities were generally low. In other 25 does, inseminated 0, 10, 15 or 20 hours before giving hCG, 50 to 79% of the eggs were fertilized except in the 20-hours group where fertilization failed. Egg recovery improved from 22% to a maximum of 91% as the interval between insemination and ovulation was extended. In pregnant does at 9-, 15- and 23- day pregnancy in the present study, egg recovery rates ranged from 16.7 to 66.7%, which was somewhat lower than those of ADAMS' data[3] (Table 4). No eggs recovered *in vivo* from oviducts of 11 does in intervals of 15 and 20 hours from intraperitoneal insemination to hCG administration, except in one doe at 15 hours interval. Percentage fertilities of eggs recovered ranged from 33 to 100%. Although these differences would be derived from physiological differences, between estrus and gestation. It should be emphasized that *in vivo* egg recovery from the oviduct was performed in in the above study, while in the other studies, eggs were recovered from oviducts and uteri at autopsy.

CHANG[20] mentioned that the eggs derived from ouvlations of the pregnant rabbits are not only perfectly normal as shown by the presence of the first polar body and the second maturation spindle, but also physiological normal because they can be fertilized either *in vitro* or after transfer to the fallopian tubes of mated rabbits. In the 6th experiment, some unfertilized eggs re-

covered *in vivo* from oviducts of does at 23-day pregnancy were transferred and these produced 2 conceptuses at autopsy on 12-day pregnancy in one of the 3 recipient does. In the following experiment (the 7th experiment), transfer of eggs fertilized intraperitoneally and recovered *in vivo* produced one young in one of three recipients. From this fact and simple consideration that superfetation rarely occurs naturally in various animal species, embryos recovered from pregnant animals would be positively utilized for the embryo transfer.

Although ovulation can be induced during pregnancy in many animal species, effects of induced ovulations on the pregnancy seems to vary in animal species. Ovulations induced by hCG administration did not terminate pregnancy in the mouse, and normal gestation length and normal young were demonstrated.[12] But in the rat, formation of new corpora lutea from ovulations induced later than 12 days of pregnancy delayed parturition and usually resulted in the death of either the mother or fetuses.[122] According to LEE *et al.*[72], induced ovulation during post-implantation period in rabbits gave rise to adverse effects on the pregnancy, except for ovulation treatment at 28- to 30-day pregnancy. In general, the partial or complete abortion occurred most grequently within 2-3 days after the ovulation treatment. Delayed parturitions and deliveries of normal, dead or mummified fetuses were also commonly observed. These disturbances of rabbit pregnancy would be probably due to hormonal disorder that occurred with ovulation during pregnancy. Progesterone is absolutely useful to maintain the pregnancy, and is originated mainly from corpora lutea in rabbits. The function of corpora lutea is synthesis and secretion of progesterone and the corpora lutea is supported by estrogen secreted from ovarian follicles[8,34,37,47,49,56~59,64~99,68,71,79,93,94,104,115]. HCG administration during pregnancy induces rapid regression of the initially formed corpora lutea and this is derived from an acute loss of follicular estrogen secretion and of luteal estrogen receptor following new ovulations[66,67,114,117,118,135]. Estrogen levels in the ovarian effiuent are markedly decreased at nadir before or at ovulation by matings, thereafter incease tentatively following coitus to reach peak values between 1.5 and 4 hours.[55] Similarly, one hour after an ovulatory dose of luteinizing hormone, estrogen levels are found elevated in the follicular fluid but not in the follicular tissue, thereafter estrogen levels decline and reach a level much below the control at time nearing ovulation.[90] Referring to an experiment to keep pregnancy by implantation of autografted corpora lutea under renal capsule[125], 2 mg or more of progesterone or 1-3 μg of E_2 were applied at the 2nd ovulatory treatment in the 2nd ovulatory treatment in the 7th experiment and this

resulted in the successful maintenance of pregnancy.

However, disturbance of delivery occurred frequently, especially when using progesterone. Then, we preferred to use E_2 for maintenance of pregancy and tried to solve the disturbance of delivery. It is clear that this disturbance of delivery or dystocia is derived from the existence of secondary ovulated functional corpora lutea formed in pseudopregnant rabbits which persist functionally for a normal 17-18 day period[114,118]. If parturition did not occur until 34-day pregnancy, most of the growing fetuses could not come out from their mothers due to their large sizes and this lead to death in the uterus. On laparotomy, ovaries of such cases showed morphologically normal corpora lutea, in addition to degenerated, faded, flat and small initial corpora lutea. Various combinations of doses with E_2, $PGF_{2\alpha}$ and oxytocin were examined on these conditions as shown in Table 8, and the most successful method for induction of normal delivery was described as those in the last column in the Table. Then, all procedures from the previous experiment were re-examined and the best results from each trial were combined together in carrying out the last experiment so that the fertilized eggs recovered from the pregnant does will give a normal parturition. The result is shown in Table 9. About 52% of pregnant does successfully produced additional embryos and the donors delivered their own young in spite of the surgical procedures that were carried out for inspections of their ovaries. Results from this study indicates that embryos that were recovered from the pregnant animals can be successfully transferred. Nevertheless, many problems still remain unsolved and much more research is required to improve this method.

Summary

Mammalian embryos for transfer are usually recovered from nonpregnant cyclic females. However, if embryos for transfer are able to be supplied from pregnant animals and the donors safely end their pregnancies with normal parturition, source of embryos for transfer would be greatly extended over a wider range. Growing antral follicles always exist in ovaries of pregnant animals and ovulation semetimes occur naturally during pregnancy. Possible or probable superfetation has been reported as the naturally occurred rare cases in various animal species. These documents suggest a feasibility in obtaining fertilized eggs from pregnant animals. On this conception, some fundamental experiments were carried out to recover fertilized eggs for transfer from the pregnant rabbits.

1) After the first coitus and injection with 20 IU of hCG, re-copulation

was carried out 1 or 2 days later with simultaneous intravenous injection of 50 IU of hCG, resulting in the secondary ovulation in all the does. On autopsy and examination of the oviduct 1 to 2 days after the hCG injection, 40-45% of ovulated does had fertilized eggs with fertility percentage ranging from 30-50%. While, 69% fertility was observed in 40% of does at 3 days pregnancy. However, without simultaneous matings with fertile bucks, no fertilized eggs were recovered from the 2nd ovulation.

2) Developmental stage of eggs derived from the 2nd ovulation treatment at 1-, 2- and 3-day pregnancy was similar to that of normal fertilized eggs, depending on hours after fertilization.

3) Intraperitoneal insemination was done during postimplantational period to obtain fertilized eggs derived from the 2nd ovulation. Although overall percentage fertilities by intraperitoneal inseminations with whole semen on 0, 3, 4, 10, 15 and 20 hours before the 2nd ovulation treatment were low (range, 0 to 19% to all ovulations), relatively good results (range, 14.3 to 19%) were shown by inseminations 3 to 4 hours before the ovulation treatment, regardless of days in pregnancy (9, 15 and 23 days). In their 2 groups 50-60% of eggs recovered *in vivo* were fertilized, although rate of egg recovery was about 40%.

4) On the present conditions comparison between whole semen and diluted semen by intraperitoneal insemination showed no significant difference in the egg fertility.

5) Fifteen unfertilized eggs were recovered from 2 does at 23-day pregnancy by only hCG injection. These were transferred ipsilaterally in oviducts of 3 does that were previously uniovariectomized and were mated. On autopsy on 12-day pregnancy, it was observed that the transfer resulted in 2 normal implanted conceptuses in a doe.

6) The twenty fertilized eggs in 2-4 cells stage that were recovered from 4 does at 9- or 15-day pregnancy were transferred to 4 recipient does at 1.5 days after hCG administration. This resulted in one normal young from a doe.

7) Even though the induced ovulation during pregnancy caused abortion or other disturbance of pregnancy in a high frequency, administration of progesterone or estradiol (E_2) can easily maintained the pregnancy. However, these treatments after mid-pregnancy induced delayed parturition due to the existence of the 2nd series of functional corpora lutea. Various combinations of doses with E_2, $PGF_{2\alpha}$ and oxytocin were examined, and the most successful method for induction of normal parturition was found as follows: $PGF_{2\alpha}$ (2 mg/kg body weight) subcutaneously at 29 and 30 days,

E_2 (1 mg) intra-muscularly at 30 days pregnancy and oxytocin (5 IU) injections intravenously at 31 days pregnancy.

8) Recovery rates of eggs of about 50% were obtained from the 2nd ovulation in does at 9, 15 and 23 day pregnancy where semen was intraperitoneally inseminated 3-4 hours before hCG injection. In these does *in vivo* egg recovery was done 36 to 40 hours after the hCG. For the maintenance of pregnancy 2 μg of E_2/doe/day was intra-muscularly injected for 3 days. However, the recalculated fertility which include only does in which fertilized eggs were produced reached about 60%. With induction of delivery using 2 mg of $PGF_{2\alpha}$/kg/body weight subcutaneously injected at 29- and/or 3-day pregnancy, 1 mg of E_2/doe intramuscularly injected at 30-day and 5 IU of oxytocin intravenously injected at 31 day, normal delivery was obtained in these pregnant does.

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