***In vivo* egg recovery from the vaginae and the pattern of egg distribution in superovulated rabbits**

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Summary. The oviducts and uteri of superovulated rabbits were cleared to locate the eggs by the freeze-clearing method, and *in vivo* vaginal washings were prepared to recover the eggs from the vagina during the preimplantation stage. The distribution pattern of eggs in cleared specimens showed that egg transport through the isthmus was faster by about 18 hours as compared with that in normal does. The earliest eggs were recovered from the vagina 42 hours *post coitum* (*p.c.*) and most of eggs obtained were recovered concentrically during 2 to 4 days *p.c.* About 44% of the superovulated eggs were recovered by multiple vaginal washings up till 192 hr *p.c.* Four young were obtained in a doe, transferred with the eggs recovered from the vagina of the superovulated does.

A few eggs were recovered from the vagina of naturally mated doe 96 and 120 hr *p.c.* without pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG). When 20 or 100 IU hCG were given at mating, some eggs were recovered from the vagina 96 or 72 hr *p.c.*, respectively. Therefore, the early appearance of many eggs in the vagina of the superovulated doe must be due to priming with PMSG.

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Introduction

Recovery rates of eggs from the reproductive tract in superovulated rabbits are reported to vary according to the lapse of time after ovulation. The egg recovery rates from the oviduct were reported as 81 to 100% during 24 to 40 hr *p.c.*¹⁻⁴⁾ The recovery rates from the oviduct and uterus at 48 hr *p.c.* were reported as 75% by TAKEUCHI *et al.*,¹⁾ 48 to 85% by SAKUMA *et al.*,⁵⁾ 23.9 to 81.4% by ISHIJIMA and SAKUMA⁶⁾ and 55.6 to 88.3% by ISHIJIMA *et al.*⁷⁾ Thus, increasing loss of eggs from the oviduct and/or uterus has suggested that accelerated egg transport through the oviduct occurs in superovulated does.^{1,3,8-11)} It was reported recently that 13% and 12% of the eggs in superovulated rabbits were found in the ligated vagina at autopsy 70 and 82 hr

p.c. respectively.¹²⁾ ADAMS¹³⁾ described that the correct timing of the entry of eggs into the uterus is vitally important for their survival, and that if entry is premature, the eggs are quickly transported through the uterus and are expelled into the vagina.

Eggs can be recovered from the vagina of estrogen-, progesterone- or prostaglandin F_{2α}-treated does within 1 to 2 days *p.c.* by a vaginal washing method proposed by TSUTSUMI and TAKEDA¹⁴⁾ and some of the eggs recovered from the vagina develop into normal young.¹⁵⁻¹⁹⁾ From the experiment which examined the effect of the administration of oxytocin on the egg transport,²⁰⁾ it was speculated that the physical stimulation by the vaginal washings did not alter the egg transport. The observations mentioned above prompted us to apply the vaginal washing method to recover the

eggs from superovulated does. Because HODGSON and PAUERSTEIN²¹⁾ postulated that induction of ovulation by hCG and by mating respectively had different effects on the propulsion or resistance of the uterotubal junction in egg transport, the effect of hCG administration at mating on the egg recovery from the vagina was examined in the present study, also.

Materials and Methods

Fifty-two Japanese adult female white rabbits used in Experiments I and II, were superovulated by intramuscular injection of

200 IU PMSG (Serotropin, Teikoku-zōki, Japan), followed by intravenous injection of 100 IU hCG (APL, Ayerst or Primogonyl, Schering) 80 hours later. PMSG treated females were mated twice with bucks at the time of hCG injection (cf. HAFEZ and SUGAWARA²²⁾, FUJIMOTO *et al.*,²³⁾ EL-BANNA and SACHER¹²⁾).

In Experiment I, 38 superovulated does were separated into 8 groups consisting of 3 to 6 does, according to the time of autopsy. They were killed at 18, 24, 36, 48, 60, 72, 84, or 96 hr *p. c.* by an overdose of sodium pentobarbital (Somnopentyl) (Tables 1, 2). Vaginae of these does were washed one to eight times before

Table 1. Recovery of eggs from the vagina of superovulated rabbits before killing

Time of sacrifice (hr <i>p. c.</i>)	No. of eggs recovered at indicated hours after mating										Total no. of eggs recovered	Recovery rate* (%)
	18	24	36	48	54	60	72	78	84	96		
18	0										0	0.0
24	—	0									0	0.0
36	—	0	0								0	0.0
48	—	0	—	19							19	7.4
60	—	0	—	52	4	1					57	23.4
72	—	0	—	14	13	2	5				34	16.2
84	—	0	—	0	0	0	3	17	2		22	15.5
96	—	0	—	0	13	1	18	14	11	9	66	43.4
Total	0	0	0	85	30	4	26	31	13	9	198	—

* The recovery rate against the number of ovulation points or corpora lutea.

Table 2. Numbers of eggs detected in the reproductive tracts

Time of sacrifice (hr <i>p. c.</i>)	No. of does	No. of corpora lutea	No. of eggs detected in						Detection rate (%)**
			Ampulla	Isthmus	Uterine horn	Cervix	Vagina*	Total	
18	5	174	86	30	0	0	0	116	68.2
24	5	214	90	91	0	0	0	181	84.6
36	5	344	54	247	0	0	0	301	87.5
48	5	257	1	177	14	0	19	211	82.1
60	5	244	0	114	28	1	57	200	82.0
72	5	210	1	24	127	4	34	190	90.5
84	3	142	0	0	124	6	22	152	107.0
96	4	152	0	6	49	7	66	128	84.2

* Refer to Table 1.

** Total no. of eggs/no. of corpora lutea.

killing (see schedule in Table 1). The apparatus and the method for the vaginal washing were the same as those reported previously.¹⁸⁾ Immediately after the final vaginal washing, the does were killed to investigate the distribution of eggs in the oviduct and in the uterus. The abdominal wall was opened immediately after killing, most of the digestive tracts were removed quickly, and the whole reproductive organ was frozen *in situ* by pouring liquid nitrogen into the abdominal cavity, as reported by HOWE.²⁴⁾ After thawing, the oviduct and uterus with cervixes were excised *en bloc*, trimmed, and cleared in benzyl benzoate according to the clearing technique by ORSINI.²⁵⁾ Eggs in the cleared oviduct and uterus were located under a dissecting micro-

scope with transmitted light. Ovulation points or corpora lutea of the ovary were counted after removing the reproductive tract.

In Experiment II, 14 superovulated does were separated into 2 groups according to the time of vaginal washings during the preimplantation stage (Table 3). Since vaginal eggs were recovered 48 hr *p.c.* in Experiment I, the vaginal washings were made at intervals of 6 hours in most cases during 48 to 96 hr *p.c.* in group I. A few eggs were recovered from the first vaginal washing of this group at 24 hr *p.c.* Therefore, the frequency of vaginal washing were increased during 24 to 54 hr *p.c.* in group II. All does were laparotomized 9 days *p.c.* to record the number of implantation sites and corpora lutea. Some

Table 3. Recovery of eggs from the vagina during the preimplantation stage and numbers of implantation sites in superovulated does

Time of vaginal washing (hr <i>p.c.</i>)	Mean no. of eggs recovered per a doe (range)	
	group I ^a	Group II ^b
24	0.5 (0-3)	0.0 (0)
42	—	7.0 (0-35)
45	—	3.9 (0-15)
48	1.5 (0-7)	2.3 (0-7)
51	—	1.4 (0-4)
54	1.3 (0-5)	5.3 (0-20)
60	1.5 (0-8)	—
66	—	7.1 (0-16)
72	2.5 (0-6)	3.3 (0-14)
78	3.0 (0-14)	2.6 (0-16)
84	1.8 (0-7)	—
90	—	2.9 (0-22)
96	0.7 (0-2)	0.3 (0-1)
120	0.3 (0-1)	0.0 (0)
144	0.0 (0)	0.0 (0)
168	0.3 (0-2)	0.1 (0-1)
192	0.0 (0)	0.6 (0-2)
No. of eggs recovered per a doe	13.5 (0-31)	36.6 (14-56)
No. of corpora lutea per a doe	76.3 (32-138)	86.3 (64-123)
Mean recovery rates (%)	14.5 (0.0-29.5)	44.2 (15.9-68.8)
No. of implantation sites per a doe	9.5 (0-25)	0.25 (0-1)

a: Group I consists of 6 does.

b: Group II consists of 8 does.

eggs recovered from the vagina were transplanted to the oviducts of a recipient doe to examine the viability of the eggs.

In Experiment III, 32 does were separated into 3 groups according to hCG dosage. Ten does were mated twice and received no hCG (group III). Twelve mated does were given 20 IU hCG at time of mating (group IV), and 10 mated does were given 100 IU hCG (group V). The vagina of each rabbit was washed once a day for 6 or 7 days, and the numbers of corpora lutea and implantation sites were recorded by laparotomy 9 days *p.c.* The vaginal washing started from 1 day *p.c.* in groups III and IV, and from 2 days *p.c.* in group V.

Results

Experiment I

Recovery of vaginal eggs before killing: The numbers of eggs recovered from the vagina at the designated time before killing are shown in Tables 1 and 2. No eggs were recovered until 36 hr *p.c.* and the first appearance of eggs in the vagina was 48 hr *p.c.* Sixty-six eggs were recovered from the vaginae of does killed 96 hr *p.c.* A recovery rate of 43.4% of eggs ovulated was attained, although the numbers of eggs recovered at each hour after copulation varied.

Distribution of eggs in the oviduct and uterus: The numbers of eggs detected in cleared specimens and recovered from the vagina at various hours after copulation are shown in Table 2. All eggs were found in the ampulla or isthmus of the oviduct until 36 hr *p.c.* At 48 hr *p.c.* 6.6% of the eggs detected were located in the uterine horn and 9.0% were recovered from the vagina simultaneously. At 72 hr *p.c.* most of the eggs (66.8%) were located in the uterine horn, and

at 84 hr *p.c.* no eggs were found in the oviduct. By 96 hr *p.c.*, however, 52% of the eggs were recovered from 8 vaginal flushings before killing (Table 1).

Locations of eggs in the oviducts or uteri were expressed by the percentage of distance travelled from the ostium of oviduct or from the uterotubal junction (Fig. 1). The egg distribution pattern in the oviduct showed that 52.6% of the eggs detected were located in segment 10 of the ampulla 18 hr *p.c.*, although a few eggs advanced near the uterotubal junc-

Fig. 1. Changes of distribution of eggs in relation to time in hours *post coitum* in the reproductive tracts of superovulated does. OAT, ostium abdominal tubae; AIJ, ampullary-isthmic junction; UTJ, uterotubal junction; IO, internal os; EO, external os.

tion (UTJ). At 24 hr *p.c.* a half of the eggs had passed through the ampullary-isthmus junction (AIJ) and at 36 hr *p.c.* 82.1% of the eggs were distributed in the isthmus. At 48 to 60 hr *p.c.* most of the eggs (83.9% or 57.0%) were in segments 6 to 8 of the isthmus, and the rest were found in the uterine lumen or recovered from the vagina.

In the uterus, 6.6% and 14.0% of the eggs were in the distal half of the uterine horn 48 and 60 hr *p.c.* respectively, although 9.0% and 28.5% of the eggs were recovered from the vagina before killing. At 72, 84 and 96 hr *p.c.* eggs were distributed throughout the uterine horn. However, eggs were concentrated in segments 1 and 2 at 72 and 84 hr *p.c.* and in segment 10 at 84 and 96 hr *p.c.*

The numbers of eggs found in the cervix increased gradually from 60 to 96 hr *p.c.*

Experiment II

Eighty-one eggs were obtained by vaginal washings in group I, and 293 eggs were recovered by 120 washings in group II. Most of eggs obtained were recovered concentrically during 2 to 4 days *p.c.* (Table 3). In group I, 86% of eggs were recovered 48 to 84 hr *p.c.*, and in group II, 97% of eggs were obtained 42 to 90 hr *p.c.* The earliest eggs were unexpectedly detected in a doe of group I by the first washing at 24 hr *p.c.* These were 3 unfertilized and degenerated eggs coated with an extraordinarily thick mucin layer. It was speculated that these eggs had been retained in the oviduct for a long period before recent ovulation. During the period between 96 and 192 hr *p.c.*, a few eggs and several fragments of expanded broken zona pellucida were recovered in both groups. The mean rates of egg recovery were lower in group I ($14.5 \pm 4.4\%$) than in group II ($44.2 \pm 6.6\%$),

although the rates of egg recovery were varied in each doe (range, 0-68.8%). The difference found in recovery rates may be due to the difference in times of vaginal washings during the early hours *p.c.* In group I, 57 implantation sites were counted in 3 does, and 7 young were delivered from 2 does. In group II, on the contrary, only 2 degenerated sites of implantation were recognized in 2 does, and no offspring were obtained. It was clearly seen that the number of implantation sites decreased as the number of eggs expelled into the vagina increased.

Twenty eggs in 2-cell or 8-cell stage, recovered from the vagina 48 hr *p.c.*, were transferred to the oviducts of a synchronized pseudopregnant recipient. At 9 days *p.c.* laparotomy showed 4 implantation sites, and 4 offspring were obtained on 31 days *p.c.* These results demonstrate that some of the eggs recovered from the vagina of the superovulated does are viable.

Experiment III

The effects of hCG administration at mating on the egg recovery from the vagina are summarized in Table 4. Only 3 of 10 mated does without hCG treatment became pregnant (group III), and 4 eggs were recovered from the vagina in one of the does 96 and 120 hr *p.c.* When 20 IU hCG was given at mating (group IV), 8 of 12 does became pregnant, the tendency of egg appearance in the vagina was similar to that in group III. When 100 IU hCG was given (group V), 8 eggs were recovered from 4 pregnant does 72 and 96 hr *p.c.* Three eggs were obtained from 2 nonpregnant does 72 and 120 hr *p.c.* Although no significant differences were found between the numbers of eggs recovered from the vagina and the numbers of implantation sites among 3 groups,

Table 4. Effect of hCG injection at mating on number of eggs recovered from the vagina and numbers of corpora lutea and implantation sites 9 days *p.c.*

Group	III	IV	V
Dose of hCG (IU)	0	20	100
No. of does used	10	12	10
No. of does ovulated	3	11	10
No. of pregnant does (9 days <i>p.c.</i>)	3	8	7
Total no. of corpora lutea	31	105	114
Total no. of implantation sites	24	46	54
Mean no. of implantation sites per a pregnant doe	8.0	5.8	7.7
No. of does recovered eggs from the vagina	1	6	6
No. of eggs recovered from the vagina indicated hours <i>p.c.</i>			
24 hr <i>p.c.</i>	0	0	—
48 hr <i>p.c.</i>	0	0	0
72 hr <i>p.c.</i>	0	0	9
96 hr <i>p.c.</i>	3	14	1
120 hr <i>p.c.</i>	1	0	1
144 hr <i>p.c.</i>	0	1	0
168 hr <i>p.c.</i>	0	1	0
Total	4	16	11
Mean no. of eggs recovered from the vagina per a pregnant doe	1.3	1.3	1.1
Mean no. of eggs lost per a pregnant doe	1.0	3.1	3.1

appearance of eggs in the vagina in group V was 24 hours earlier than that in groups III and IV.

Discussion

Comparing the present data on egg distribution in the oviduct and uterus of the superovulated rabbits with those of normal rabbits (TSUTSUMI and HAFEZ²⁶⁾), it is suggested that the time required for egg transport through the isthmus is shortened by about 18 hours in superovulated does. However, some eggs of the superovulated does appeared not only in the uterus, but also in the vagina about 40 hr *p.c.* (Table 3). That is an unexpected phenomenon, because eggs appear commonly in the uterus around 72 hr *p.c.*,²⁶⁾ although EL-BANNA and SACHER¹²⁾ found that 13% of eggs located in the vagina of superovulated does 70 hr *p.c.* As far as the egg distribution

pattern in the oviduct and uterus are concerned, no special difference was noted between normally ovulated and superovulated does during the egg transport stage. For example, egg distribution pattern in the uterus of superovulated does 72 hr *p.c.* was similar to that of normally ovulated does 108 hr *p.c.* TSUTSUMI and HAFEZ²⁶⁾ found that some eggs in the proximal uterus were close to the internal os of the cervix in the ovulated does 78 hr *p.c.*, and they assumed that such a rapid transport of eggs occurred immediately after the eggs entered the uterus from the oviduct. Such a tendency in the egg transport in rabbits could be conjectured from our previous works.¹⁵⁻¹⁹⁾ It may be suggested, therefore, that the egg transport through the uterus in rabbit is biphasic; one is a very quick transport of eggs in low percentage which appear very close to the cervix in a short lapse of time after entry to the uterine

lumen, and the other is a slow transport in most eggs which are distributed in the distal part of the uterine horn and advance gradually to the proximal portion. Some mechanisms involved in the quick transport may be related to the expulsion of the eggs into the vagina.

In recent studies by HODGSON and PAUERSTEIN²¹⁾, uterine entry of eggs in does treated with hCG alone or hCG followed by artificial insemination occurred between 48 and 60 hours after hCG injection and most eggs had entered the uterus by 72 hours after hCG. In naturally mated rabbits, however, entry of eggs into the uterus did not occur until after 66 hr *p.c.*, and the majority of eggs did not enter the uterus until after 78 hr *p.c.* Although they carried out flushings of the uterus and vagina, no description was made on the eggs in the vagina. However, when the effect of hCG administration at mating on the egg recovery from the vagina was examined in the present Experiment III, 31 eggs (12.4% against the total number of corpora lutea) were recovered from 32 does up to 168 hr *p.c.*

The manipulation for egg recovery from the vagina may affect the egg transport. It was clear, however, that the appearance of eggs in the vagina 24 hours earlier in group V than in group IV was due to an increased dosage of hCG (5 times) and not due to the stimuli by the vaginal washings. SAWYER and MARKEE²⁷⁾ described that most attempts to induce ovulation in the estrous rabbit by simple vaginal stimulation have led to negative results, although priming the estrous doe with moderate dosage of estrogen for 2 days, sets the stage for release of pituitary ovulating hormone in response to artificial stimulation of the vagina in 40–45% of the cases. Therefore, the marked expulsion of eggs into the vagina that occurred from around 40 hr *p.c.* in super-

ovulated does must be due to some actions induced by PMSG priming prior to hCG administration. The acceleration of egg transport and the expulsion of the eggs into the vagina of the superovulated does may be caused by an imbalance between endogenous estrogen and gestagen, resulting from PMSG and/or hCG administration. An abnormal quantity of estrogen and/or gestagen secreted from the ovaries in superovulated does is speculated from the appearance of many unruptured follicles and/or corpora lutea.²⁸⁾ However, the cause of the fluctuation of the levels of the ovarian hormones in the superovulated rabbit still remain unknown.

Concerning the fertility of eggs, ADAMS²⁹⁾ described that the mean proportions of eggs fertilized were 98.6% for ovulated does and 91.6% for superovulated does, and no significant difference was shown in fertilization rates of eggs between the normally ovulated does and the superovulated does by KENNELLY and FOOTE³⁾, TESH³⁰⁾ and MAURER *et al.*³¹⁾ Other investigators also reported high percentages of eggs fertilized in the superovulated does^{3,5,7,11,30,31)}. This shows that fertility of the eggs in the does was not impaired by superovulation treatment. In the experiment by FUJIMOTO *et al.*,²³⁾ 74.9% of eggs in the superovulated does and 86.9% in the ovulated does were observed as blastocyst, when the uterine horns were flushed on the 5th to 7th day of pregnancy. In Experiments I and II, however, 48.8% of the eggs recovered from the vagina were in cleavage (Table 5). Such the low fertility of the superovulated eggs may be due to using bucks in unsatisfactory condition, because these experiments were carried out during the hot summer season.

TAKEISHI *et al.*³²⁾ claimed that uterine entry of the unfertilized eggs which were ovulated

Table 5. Classification of eggs recovered from the vagina of superovulated does

Time of egg recovery (hr p.c.)	Classification of egg recovered	No. of eggs recovered	Total (%)
<i>Fertilized eggs</i>			
48-51	2-8 cell stage	53	219 (38.3)
48-72	16 cell stage	22	
45-78	32 cell stage	29	
54-96	Morula stage	99	
78-96	Blastocyst stage	16	
<i>Degenerated fertilized eggs</i>			
45-168	2-32 cell stage	26	60 (10.5)
60-96	Morula stage	20	
78-192	Blastocyst stage	14	
24-192	<i>Degenerated and/or unfertilized eggs</i>	293	293 (51.2)
144-192	<i>Empty zona pellucida</i>	Several	
Total 572*			

* Eggs recovered in Experiments I and II were combined.

by hCG administration occurred 10 hours earlier than that of the fertilized eggs in the does mated naturally. Although ISHIJIMA *et al.*⁹⁾ and TSUNODA *et al.*¹¹⁾ supported this statement, the present study could not confirm such a phenomenon in vaginal entry of eggs. Our results were in agreement with the statement by HODGSON and PAUERSTEIN,²¹⁾ that fertilized and unfertilized eggs travelled at the same rate in the reproductive tracts of rabbits.

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過排卵処理家兎の腔からの採卵及び生殖器道内卵の分布

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過排卵処理家兎の卵管内卵移動は促進され、子宮内卵回収数は交配後の時間とともに急速に減少することや、交配後 72~82 時間で 12~13% の卵が腔内に排出されていることが報告されている。他方 estrogen, gestagen や PGF_{2α} で処理した家兎の腔内から相当数の卵が採取され、それらの一部は生存性を持つものであることが証明された。そこで本研究では、過排卵処理家兎の腔内から採卵を試みるとともに、卵管と子宮内卵の分布状態を調べた。また、hCG 投与が卵の卵管内通過を早めるとの報告²¹⁾もあるので、hCG 処理による腔からの採卵も検討した。腔からの採卵方法は前報¹⁹⁾と同様であり、卵分布状態は卵管と子宮の透明標本^{24,25)}により調べた。過排卵は、PMSG 200 IU を筋注後 80 時間目に交配し、同時に hCG 100 IU を静注して誘起した。

実験 I (表 1, 2) では交配後 18, 24, 36, 48, 60, 72, 84, 96 時間目に屠殺し、生殖器の透明標本を作製したが、屠殺前に 0~8 回の腔洗浄を行い、腔からの採卵も試みた。その結果、交配後 48 時間目から卵は採取され始め、96 時間目までに黄体数に対して 43% の卵が腔から回収された。卵管と子宮内卵の分布状態 (図 1) には

過排卵処理による特異性はみられなかったが、通常の交配家兎に比較して、卵管内卵通過がおよそ 18 時間ほど早まっているようであった。

実験 II (表 3) では実験 I の結果を確かめるとともに、腔からの採卵状態を調べた。グループ I では主に 6 時間間隔で腔洗浄を行ったが、交配後 24 時間で少数の卵が採取されたので、グループ II では間隔を短縮して洗浄した。その結果、卵回収率 44.2%, 1 羽当たり採卵数 36.6 個となり、特に交配後 2 日目から 4 日目に集中して採取された。

実験 III (表 4) では交配のみの群、交配時に 20 IU または 100 IU の hCG を静注した群の 3 群で腔洗浄を試み、ごく少数であったが前 2 者の群で交配後 96 時間目、後者からは 72 時間目で採卵され、高単位投与で 1 日早く卵が採取された。

以上の結果から、過排卵処理家兎の腔に多数の卵が早い時期に出現したのは、PMSG の前処理の影響が強いものと考えられた。なお、過排卵処理家兎の腔から採取された卵を 1 羽の偽妊娠家兎に移植した結果、4 羽の正常産子が得られた。