NON-SURGICAL RECOVERY OF EQUINE EGGS, AND AN ATTEMPT AT NON-SURGICAL EGG TRANSFER IN HORSES

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Summary. Equine ova were successfully recovered non-surgically from the uterine lumen using a three-way system apparatus. The recovery rate was 45%. Ova collected from the uterus were in the blastocyst stage. The transfer of rabbit ova into the uterine lumen of a mare through the cervix hastened the transport of the fertilized equine ovum in the uterine tube. Equine ovum transfers were performed in eleven recipient mares 5 to 7 days after ovulation without success. It was clearly noted that uterine flushing in the dioestrous mare affected the time of the next ovulation.

INTRODUCTION

Non-surgical recovery and transfer of fertilized ova are prerequisites for the practical application of egg transfer in farm animals. The successful transfer of fertilized ova using non-surgical techniques has been reported in cattle by Mutter, Gradon & Olds (1964), Sugie (1965), Rowson & Moor (1966) and Rowson, Moor & Lawson (1969), and in pigs by Polge & Day (1968). In most of these cases, the fertilized ova were obtained at appropriate stages of development after slaughter or at operation. Because non-surgical collection of fertilized ova from the Fallopian tubes of living animals is practically impossible, it is necessary to wait until the ova have passed into the tip of the uterine horn. Recently, Sugie, Soma, Fukumitsu, Otsuki & Onuma (1971) reported the successful collection of ova from the uterine lumen in forty-seven out of fifty-seven superovulated cows by a non-surgical method. Apart from this, however, there seems to have been no basic research on the non-surgical collection and transfer of equine fertilized ova.

The aims of the present experiments were, firstly, to attempt non-surgical recovery of fertilized ova from the uterine lumen of mares using the three-way system apparatus of Rowson & Dowling (1949) and, secondarily, to examine the possibility of egg transfer. It has been possible to examine a non-surgical egg transfer method in the present study, because the cervical canal of the mare is looser and shorter than that of the cow and does not have the spiral conformation of the latter. To avoid artificial and/or environmental factors on egg recovery and transfer, only mares ovulating spontaneously were used. There are no

reports for superovulation and synchronization of oestrus in mares, though many workers have attempted to induce ovulation by HCG during oestrus (Mirskaja & Petropavlovskii, 1937; Day, 1939, 1940; Hancock, 1948; Nishikawa, 1959; Loy & Hughes, 1966; Ellicott & Dziuk, 1970).

MATERIALS AND METHODS

A total of eighteen adult Hokkaido native pony mares ranging from 2½ to 13 years of age were used continuously through two breeding seasons of 1969 and 1970. All mares were teased every morning by an active vigorous stallion to detect the onset of oestrus, during which period the ovaries were palpated per rectum once a day. Mares were served by a fertile stallion once a day on alternate days up to the time of ovulation, in eighty out of a total of 142 oestrous periods.

Deposition of rabbit ova in the uteri of mares and recovery of the ova

To test whether ova transferred through the cervix were expelled from the uterine lumen, rabbit ova in 0.3 ml of a mixture of rabbit serum and Ringer's solution including 1000 i.u. penicillin/ml were deposited into the middle

TEXT-FIG. 1. Diagram of the three-way system apparatus used for non-surgical recovery of equine eggs. The external tube (12 mm o.d. and 65 cm in length) is made of acrylic acid resin, and the larger internal tube of vinyl resin. The tip of the external tube is connected with a fine polyethylene tube by a latex collar. The internal tube is free to move in either direction within the external tube. A, infusion tube (external tube); B, recovery tube (internal tube); C, latex collar; D, tap on air system; ↑: direction of flow.

portion of the uterine horn through the cervix of recipient mares, using a special injector. The injector consisted of an external tube made of acrylic resin (8 mm o.d. and 60 cm in length) and an internal fine polyethylene (70 cm in length) tube connected to a 1-ml syringe. Two to ten eggs were deposited 3 and 4 days after ovulation following service in eleven mares and 6 days after ovulation in five mares which had not been served. The eggs were then collected by flushing 5, 24 and 48 hr, respectively, after the 3-, 4- and 6-day times of deposition.

Uterine flushings were carried out using a modification of the three-way

EXPLANATION OF PLATE 1

FIG. 1. Equine ovum obtained from a Graafian follicle (4.5 cm in diameter). × 250.

FIG. 2. Living equine morula 5 days after ovulation. × 275.

FIG. 3. Living early equine blastocyst 6 days after ovulation. × 270.

FIG. 4. Living equine blastocyst 6 days after ovulation. Note the polar body (arrow) and the thread-like structure between the inner cell mass and trophoblast. × 265.

FIG. 5. Living equine blastocyst 7 days after ovulation. × 140.

FIG. 6. Living equine blastocyst 8 days after ovulation. × 50.

PLATE 1

(Facing p. 188)

system apparatus designed by Rowson & Dowling (1949) for the extraction of fertilized eggs from the cow (Text-fig. 1).

The apparatus was gently inserted through the cervix into the uterine lumen up to the tip of the horn, the position of the tube being guided by palpation per rectum. After the apparatus was in position in the uterine lumen, the latex collar was inflated with air to block the lumen. Flushing fluid was then infused into the tip of the uterine lumen through the external tube and finally recovered through the internal tube. The uterus was flushed under aseptic conditions, using about 1500 ml physiological saline solution (with 2% gelatine) or mare serum-saline mixture. The flushings were collected separately into fifteen 100-ml test tubes and kept at 30° C for 20 min. About 3 ml of the fluid at the bottom of each test-tube was then removed to a watch-glass, and the ova were sought under a dissecting microscope.

Non-surgical recovery of fertilized ova from the uteri of mares

Eighty trials of flushing were carried out for fertilized equine ova by the above methods 5, 6, 7, 8, 9 and 10 days after ovulation. The dimensions of twenty-five fertilized equine ova recovered 5 to 8 days after ovulation and of thirty unfertilized ova obtained by puncture of the ovarian follicles of mares at autopsy (Pl. 1, Fig. 1) were measured with the aid of a screw-micrometer eyepiece.

Non-surgical transfer of ova between mares

Eleven equine eggs (one morula, seven early blastocysts and three blastocysts) were obtained 5 to 7 days after ovulation. On the same day, these eggs were transferred with 0.3 ml of the sterile homologous serum-Ringer's solution mixture with penicillin through the cervix into the uterine horns of eleven recipients which had ovulated spontaneously at the same time as the donors.

RESULTS

The average percentage volume recovered for a total of eighty-five uterine flushings was 97.3%.

Deposition of rabbit ova in the uteri of mares and recovery of the ova

The results of examinations of the rabbit eggs transferred through the cervix are given in Table 1. Although the recovery ratios of the rabbit ova 5, 24 and 48 hr after transfer decreased with the lapse of time, about 50% of the ova remained in the equine uterus for more than 48 hr. These experiments indicated that non-surgical techniques might be used for the deposition and recovery of equine ova.

Non-surgical recovery of fertilized ova from the uteri of mares

The results of the non-surgical recovery of fertilized equine ova 6, 7, 8, 9 and 10 days after ovulation are shown in Table 2. Recovered ova were at the early blastocyst and blastocyst stage (Pl. 1, Figs 3 to 6). Although no fertilized equine ovum was recovered in nine uterine flushings on the 5th day after ovulation, some fertilized equine ova were recovered when rabbit ova had been deposited 48 and 24 hr before flushing. For instance, when rabbit ova were transferred 3 days after ovulation and uterine flushings were made 48 hr after the transfer,

the recovery rate of equine ova was 100%. When depositions of rabbit ova were made on the 4th day after ovulation following flushing 24 hr after deposition, the recovery rate of equine ova decreased to 25%. (Table 3).

All ova recovered by flushing on the 5th day after ovulation were in the morula stage. The blastomeres of these ova were found lying to one side of the zonal

TABLE 1
NON-SURGICAL RECOVERY OF RABBIT OVA TRANSFERRED THROUGH THE CERVIX TO THE
UTERI OF RECIPIENT MARES 5 AND 6 DAYS AFTER OVULATION

<i>Time from deposition of rabbit ova to flushing (hr)</i>	<i>Day from ovulation to flushing</i>	<i>No. of recipient mares</i>	<i>No. of mares from which rabbit ova were recovered from uterus</i>	<i>Total no. of rabbit ova deposited</i>	<i>Total no. of rabbit ova recovered</i>	<i>Recovery rate (%)</i>
5	6	5	5	47	33	70.2
24	5	8	8	60	34	56.7
48	5	3	3	21	9	42.9

TABLE 2
RECOVERY RATES OF FERTILIZED EQUINE OVA BY NON-SURGICAL TECHNIQUES FROM
RECIPIENT MARES ON DAYS 6, 7, 8, 9 AND 10 AFTER OVULATION

<i>Time of flushing (days after ovulation)</i>	<i>No. of flushings</i>	<i>No. of fertilized ova recovered</i>	<i>% recovery rate</i>
6	36	17	47.2
7	13	5	38.5
8	6	2	33.3
9	3	2	66.6
10	2	1	50.0
Total	60	27	45.0

TABLE 3
EFFECTS OF DEPOSITION OF RABBIT OVA IN THE UTERINE LUMEN OF MARES 48 AND
24 HR BEFORE FLUSHING ON THE RECOVERY OF FERTILIZED EQUINE OVA ON
THE 5TH DAY AFTER OVULATION

<i>Time of deposition of rabbit ova (hr before flushing)</i>	<i>No. of flushings</i>	<i>Ova recovered</i>	<i>Recovery rate (%)</i>
48	3	3	100
24	8	2	25
Control	9	0	0

cavity, which seemed to be very clear and no granular detritus such as that described by Hamilton & Day (1945) was found (Pl. 1, Fig. 2). By comparison with rabbit or cow ova, the appearance of the vitellus of the equine ovum was blackish. The means and ranges of the external diameter of ova and the thickness of the zona pellucida of follicles and fertilized ova recovered 5 to 8 days after ovulation are shown in Table 4.

TABLE 4
MEASUREMENTS OF EQUINE OVA RECOVERED FROM RECIPIENT MARES 5 TO 8 DAYS AFTER OVULATION

Age of ovum (days after ovulation)	No. of ova	Diameter of ova, including the zona pellucida (μm)		Thickness of zona pellucida (μm)	
		Mean	Range	Mean	Range
Ova obtained from follicles	30	152	124 to 168	22	8 to 33
5	3	154	149 to 161	14	13 to 16
6	17	199	161 to 289	12	5 to 22
7	3	388	288 to 485	5	3 to 7
8	2	786	603 to 968	3	1 to 5

TABLE 5
RESULTS OBTAINED FOLLOWING NON-SURGICAL TRANSFER
OF EQUINE OVA TO RECIPIENT MARES, WHICH HAD
OVULATED AT THE SAME TIME AS THE DONORS

Time of transfer (days after ovulation)	Storage time from recovery to transfer (min)	Post-operative cycle length (days)
5	105	36
6*	73	14
6	123	18
6	135	20
6	120	20
6	120	22
6	92	34
6	73	>40
7	82	20
7	47	20
7	53	>80

* The uterus of this recipient mare was inflated with CO_2 immediately after deposition of the fertilized ovum.

TABLE 6
EFFECTS OF UTERINE FLUSHING FOLLOWING OVULATION ON THE DURATION
OF THE OESTROUS CYCLE IN MARES

Treatment	Oestrous cycle	
	No. of cycles	Mean duration in days $\pm$ S.E. (range)
Normal	57	21.12 $\pm$ 0.41 (17 to 26)
Flushing 5 to 8 days after ovulation	31	18.48 $\pm$ 0.50 (14 to 26)
Deposition of rabbit ova and flushing 5 to 8 days after ovulation	16	17.38 $\pm$ 0.62 (15 to 24)
Flushing 9 to 12 days after ovulation	5	26.00 $\pm$ 2.32 (20 to 35)

Non-surgical transfer of ova between mares

The results of the non-surgical transfers of equine ova are shown in Table 5. The post-operative cycle length was normal in six of the recipients, but lasted 14, 34 and 36 days in three of the recipients, while the other two recipients did not ovulate until 40 and 80 days after transplantation, and subsequently entered the non-breeding season. None of the eleven recipients appeared to be pregnant at any of the subsequent examinations in spite of the fact that no signs of abortion occurred.

Effect of uterine flushing on oestrous cycle length

The effects on oestrous cycle length of uterine flushing on different days after ovulation are summarized in Table 6. The average length of fifty-seven control oestrous cycles in the Hokkaido native pony was 21.12 ± 0.41 days (mean $\pm$ S.E.) with a range of 17 to 26 days. The difference (1.10 days) between the mean oestrous cycle lengths of mares flushed 5 to 8 days after ovulation (Groups 5 to 8) and of those receiving rabbit ova and flushed 5 to 8 days after ovulation (Groups R.5 to 8) was not significant. However, the difference (2.64 days) between the means for Groups 5 to 8 and controls, and the difference (8.62 days) between the means for Groups 5 to 8 and mares flushed 9 to 12 days after ovulation (Groups 9 to 12) were significant at a 0.1% level. The difference (4.88 days) between the mares for the controls and Groups 9 to 12 were significant at a 1% level.

DISCUSSION

Imperfect recovery of the rabbit eggs transferred into the equine uterus seems to be attributable to defects in the recovery method and to spontaneous expulsion of the eggs through the cervix. The recovery rate of the rabbit ova from the equine uterus, however, was higher than that described by Hafez & Sugie (1963) in heifers slaughtered 12 to 48 hr after transfer. The difference between the recovery rate (70%) of rabbit ova from the flushings 5 hr after deposition on the 6th day after ovulation and that of equine ova (47%) from the flushings on the 6th day after ovulation is probably due to intrinsic differences between the equine and rabbit ova. The significance of such differences remains unexplored.

The recovery of ova from the uterus of mares which had not been induced to superovulate was found to be possible using the three-way system apparatus of Rowson & Dowling (1949). Compared with that from the cow, the flushings from the mare's uterus contained very little mucus and fragments of epithelial lining; recovery of ova from the mare is, therefore, easier than from the cow. In view of the former findings of Van Niekerk & Gerneke (1966) that unfertilized ova in the mare remain lodged in the Fallopian tubes and do not reach the uterus, the true mean recovery rate is probably higher than 45% (Table 2). In an earlier study by Dracy & Petersen (1950), eggs were recovered from live cows in nine out of thirty-four trials (26.5%). Sugie *et al.* (1971) reported that 257 eggs were recovered from forty-seven out of fifty-seven trials in cows which had been induced to superovulate.

Although equine ova were recovered by uterine flushing on and after 6 days

(144 to 168 hr) following ovulation, none were recovered by the same procedure on the 5th day (120 to 144 hr) after ovulation. The fertilized ovum of the mare may, therefore, appear in the uterine lumen in the early blastocyst stage later than 144 hr after ovulation. Day (1939) recovered an equine ovum from the uterine end of the Fallopian tube about 95 hr after ovulation, following treatment with gonadotrophic hormones. Hamilton & Day (1945) reported the recovery of an equine ovum at the fifteen-cell stage from the uterine tube 98 ± 6 hr after ovulation. In cattle, however, the egg reaches the uterus 96 hr after oestrus in the eight- to sixteen-cell stage (Hamilton & Laing, 1946). In a study by El-Banna & Hafez (1970), all eggs recovered from the uterus consisted of more than eight cells. From these reports and the present data, it appears that the time at which ova arrive in the uterus in the mare is much later than in the cow, and that the cleavage stage of equine ova at arrival is more advanced than in cattle.

Mastroianni & Rosseau (1965) and Mastroianni, Suzuki, Manabe & Watson (1967) reported that the presence of an IUD facilitated the rapid transport of fertilized ova through the Fallopian tube in superovulated monkeys. In rats and rabbits, however, Ishihama & Miyai (1969) stated that the presence of an IUD did not influence the speed of transport or the cleavage of ova in the Fallopian tubes. In the cow, massage of the vulva, massage of the cervix, or a combination of the two, have been reported to induce tetanic uterine contractions which may be due to release of oxytocin (VanDemark & Hays, 1951). The present results suggest that the rate of transport of fertilized equine ova in the uterine tube is increased by the stimulation which results from deposition of rabbit ova through the cervix.

The inner diameter of the zona pellucida was $135 \mu\text{m}$ in sections of equine ova examined by Hartman (1929), and Hamilton & Day (1945) noted that the external diameter of the zona of segmented eggs obtained from uterine tubes between 24 and 98 hr after ovulation ranged from 135 to $167 \mu\text{m}$, with a mean of $155 \mu\text{m}$. In the present study, the mean external diameter of the zona pellucida in thirty follicular equine ova was $152 \mu\text{m}$. The size of the morula from the uterus on the 5th day after ovulation was similar to that of the follicular ova. The size of the blastocyst increased rapidly on and after 6 days, while the thickness of the zona pellucida decreased inversely.

In cattle, Willett, Black, Casida, Stone & Buckner (1951), Willett, Buckner & Larson (1953), Avery, Fahning, Pursel & Graham (1962) and Rowson *et al.* (1969) have reported successful transplantations of fertilized eggs using surgical methods. Despite many attempts, however, few successes have been reported in the transfer of bovine eggs by non-surgical methods (Mutter *et al.*, 1964; Sugie, 1965; Sugie *et al.*, 1971; Rowson & Moor, 1966; Rowson *et al.*, 1969). The difficulty in the non-surgical transfer of cattle ova has been ascribed to expulsion of the transferred ova through the cervix, owing to uterine contractions initiated by cervical stimulation (VanDemark & Hays, 1951; Shah, 1956; Harper, Bennett & Rowson, 1961; Rowson, Bennett & Harper, 1964). The cervical canal of the mare, however, is looser and shorter than that of the cow, and more readily allows passage of the instrument of egg transfer. These differences in cervical structure may account for some of the discrepancies in

cervical stimulation resulting from egg transfer through the cervix. It appears from the present study that the intensity of the contractions leading to the expulsion of ova deposited into the uterine lumen of the mare is weaker than in cattle.

It was postulated by Day (1957) that normal oestrus accompanied by spontaneous ovulation should be induced in the mare by the uterine infusion of about a pint of water at body temperature. Arthur (1970) observed that mares returned to heat on average 3.8 days (range 3 to 7 days) earlier than expected, following infusions performed between the 5th and 9th days of dioestrus; infusions on the 1st to 4th days, or the 12th and 13th days, of dioestrus had no appreciable effect. In the present study, uterine flushing on Days 5 to 8 induced ovulation earlier than would have been expected in normal cycles, while flushings on Days 9 to 12 resulted in later ovulation than normal. It is of interest that uterine flushing during the luteinizing stage shortened the length of the cycle while flushing during the functional stage of corpus luteum deferred the next ovulation.

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