ANIMAL EXPERIMENTATION

Embryonic Dormancy Phenomenon in Obstructed Healthy Mouse Fallopian Tubes

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Purpose: A mouse model of unilateral oviductal obstruction was designed to study whether healthy oviducts can support embryo development in an advanced stage toward blastocyst hatching and implantation when the embryos in the contralateral side normally move into the uterine cavity.

Methods: The oviducts of 80 female ICR mice (aged 5–8 weeks) were ligated unilaterally 12–40 hr postcoitus. The ligated oviducts were isolated from day 4 to 19.5 postcoitus. Embryos within the ligated oviducts were then flushed out to record the developmental stage and compared with the conceptuses in the contralateral uterine horns with unligated oviducts. Embryos recovered from ligated oviducts were then cultured *in vitro* to observe their potential for further development.

Results: In 33 mice, 53.4% (163/305) and 86.1% (241/280) of the morphologically normal blastocysts had hatched from the zona pellucida within the obstructed tube and contralateral uterine horns, respectively, on the 5th day postcoitus. The data demonstrated that the hatching process could take place within the obstructed fallopian tube, but the timing was delayed. From 5.5 to 19.5 days postcoitus, a total of 362 implanted embryos were obtained in unligated control

uterine horns, but none of the 404 embryos in the artificially obstructed oviducts were implanted. The embryos within the ligated tubes were dormant in the hatched blastocyst stage as demonstrated by their ability to continue growing (98.2%) when removed from the oviduct to an *in vitro* environment.

Conclusions: In this study, we demonstrate that mouse embryos can hatch, although delayed, in obstructed healthy oviducts. Tubal pregnancy is not likely to happen in artificially obstructed healthy mouse fallopian tubes, since all the viable embryos were dormant at the hatched blastocyst stage.

KEY WORDS: Obstructed oviduct; hatching; embryonic dormancy.

INTRODUCTION

In mammals, fertilization and early embryo cleavage occurs in the fallopian tubes. Embryos move into the uterus between the third and fifth day of pregnancy where they develop into the blastocyst stage (1). Through hormonally controlled secretions, the oviduct provides a biochemical environment that facilitates, or is even essential for, capacitation of sperm, fertilization, or the early cleavage of embryonic development (2).

The oviduct's contribution toward cleavage stage preimplantation embryo development has been widely studied by comparing embryos cultured *in vitro* with embryos allowed to develop within the fallopian tubes. Under certain conditions, *in vitro* zygote development stops at different stages, and this arrest may correlate with the change in transcription of the maternal to the embryonic genome (3). *In vitro*

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cultured mouse embryos grow more slowly, do not develop endoderm or typical abembryonic trophoblast cells, and have reduced DNA polymerase activity compared with in vivo grown embryos of the same age (4). It also is claimed that coculture of the embryos with oviduct cells in vitro improve blastocyst formation, hatching, and embryo quality (5). This confirms the importance of the oviductal environment for embryo development.

Whether the fallopian environment is suitable for advanced embryo development when the embryos should be normally exposed to the uterine environment is not clear. In the present study, we used mice having undergone unilateral tubal ligation as an experimental model to clarify whether the oviductal milieu can support further embryonic development toward blastocyst hatching or even implantation.

MATERIALS AND METHODS

Female ICR mice (aged 5–8 weeks) in estrus were used. If they were not naturally in estrus, gonadotropin stimulation was used. The mice were injected intraperitoneally with 5 IU pregnant mare serum gonadotrophin (PMSG) (Sigma, St. Louis, MO 63178), followed by 5 IU human chorionic gonadotrophin (hCG) (Serono, Italy) 46–48 hr later. The animals were placed with males of proven fertility overnight and checked for the presence of vaginal mucus plugs the next morning. Under our controlled lighting schedule (14 hr light/10 hr darkness; lights on at 5 AM), mating and fertilization was expected at midnight, which was taken as time 0.

Unilateral oviduct ligation was performed in 80 mice from 12 to 40 hr postcoitus. Sodium pentobarbital (1.5 μ l/g body weight) (Abbott Laboratories) was given to anesthetize the mice. Artificial tubal obstruction was accomplished by dorsal entry into the peritoneal cavity and placement of a 4-0 ligature (DEXON "S"; Davis + Geck Inc.) between the uterine horn and oviduct on one side.

Thirty-three mice were killed by cervical dislocation on the fifth day postcoitus (4 days and 2–19 hr postcoitus) to study whether the embryos could hatch from their zona pellucida within the obstructed fallopian tube. The ligated oviducts were isolated and embryos within the obstructed tubes were flushed out under the dissecting microscope to record the developmental stage. Blastocysts within the contralateral unligated uterine horns were collected sepa-

ately and the percentage of hatched blastocysts was recorded to serve as controls.

From 5.5 to 19.5 days postcoitus, 47 mice were killed to inspect whether tubal pregnancy had taken place within the obstructed healthy oviducts. After isolating the ligated oviducts and carefully inspecting for tubal pregnancy, embryos within the ligated tube were flushed out to record their number and developmental stage and compared to the number and developmental stages of conceptuses in the contralateral unligated control uterine horns. Embryos recovered from ligated oviducts then were cultured in minimal essential medium α (α -MEM) (Gibco) plus 10% fetal bovine serum (FBS) (Atlanta Biologicals) to observe their potential for further development.

RESULTS

To study whether the embryos retained within the obstructed fallopian tube could develop to the hatching stage, 33 mice were sacrificed on the fifth day postcoitus (Table I). From 4 days and 2–12 hr postcoitus, 54/154 (35.1%) morphologically normal embryos had hatched from their zona pellucida within the obstructed oviducts, while 113/141 (80%) blastocysts escaped from their zona pellucida in the contralateral unligated uterine horns. From 4 days and 12–19 hrs postcoitus, 72.2% (109/151) and 92.1% (128/139) of hatched blastocysts were recovered from obstructed oviducts and contralateral uterine horns, respectively. These data indicate that the hatching process can take place within obstructed healthy oviducts, but the timing of zona shedding was delayed at least 10 hr.

The remaining mice were killed from 5.5 to 19.5 days postcoitus to inspect whether tubal pregnancy could occur in obstructed healthy mouse oviducts. In 47 mice, 362 implanted embryos were found in control uterine horns with unligated oviducts, while none were observed in uterine horns with ligated oviducts, indicating the success of tubal ligation (Table II). There was no sign of tubal implantation in the ligated oviducts, even after 19.5 days of gestation when fetus with fetal heart beats could be detected in the contralateral uterine horns.

A total of 404 embryos were recovered from the obstructed oviducts, of which 327 had hatched from the zona pellucida and lay dormant in the hatched blastocyst stage (Table II). Two embryos were at the hatching blastocyst stage. Fifteen blastocysts remained in the zona pellucida. The total of 344 mor-

Table I. The Development Stage of Morphologically Normal Embryos Flushed from Intact Uteri and Obstructed Oviducts on the 5th Day Postcoitus

	4 days and 2–12 hr postcoitus (15 mice)		4 days and 12–19 hr postcoitus (18 mice)	
	Obstructed oviducts	Intact uterus	Obstructed oviducts	Intact uterus
Blastocysts within zona pellucida	85 (55.2)	24 (17)	30 (19.9)	8 (5.8)
Hatching blastocyst (%)	15 (9.7)	4 (2.8) ^a	12 (7.9)	3 (2.2) ^a
Hatched blastocyst (%)	54 (35.1)*	113 (80.1)*	109 (72.2)**	128 ^b (92.1)**
Total healthy embryos	154	141	151	139

^a Blastocyst with an open zona pellucida; no trophoblast “knobs” or “buds” were observed in this study.

^b About 45% of the hatched blastocysts represent the “cytoplasmic projections of trophectoderm,” indicated as the implanting stage.

* $P < 0.000001$; chi-square analysis.

** $P < 0.0001$; chi-square analysis.

phologically normal embryos from obstructed tubes is close to the number of intrauterine conceptuses found in the contralateral unligated side (362), indicating a comparable incidence of viable embryos in the ligated tubes.

After in vitro culture of the embryos retained in the ligated tubes, 98.2% (321/327) of the hatched blastocysts reexpanded within 24 hr, attached to the culture dish, and developed “trophoblast outgrowth” in the following 24–48 hr (Table III). The growth potential decreased from 100 to 85% after a prolonged dormant period (longer than 11 days after mating). Nine of the 17 blastocysts within the zona pellucida developed further, and 7 embryos completed trophoblast outgrowth in vitro. These results demonstrate that the tubally retained blastocysts were viable but were unable to grow or implant in the obstructed healthy oviducts.

DISCUSSION

The present study demonstrated that embryos re-

tained within the obstructed oviducts could develop beyond the hatching blastocyst stage. However, the hatching process was significantly delayed within the ligated fallopian tube compared with the timing of zona shedding in the contralateral control uterine horns.

Even after 19.5 days of gestation, despite the presence of viable blastocysts, no implanted embryos were found in the ligated mouse oviducts. Those embryos were diapaused in the hatched blastocyst stage. Our data demonstrate that ectopic pregnancy does not occur in obstructed healthy mouse oviducts.

In a similar study, Confino *et al.* (6) observed two oviductal gestations adjacent to the tubal ligature on day 10 of gestation. They concluded that implantation can occur occasionally in the mouse oviduct when oviductal continuity is interrupted. Variations in the accuracy of the ligated sites limited their ability to explain their results. In a preliminary experiment, we found that if the ligature was placed at the top of the uterine horn, embryos within the oviducts could still move into the uterus. Therefore, it is possible that the gestation sites discovered by Confino *et al.* were

Table II. The Appearance of Mouse Embryos in Artificially Obstructed Oviducts

	Days postcoitus			
	5.5–7.5 (n = 18)	8.5–10.5 (n = 17)	11.5–19.5 (n = 12)	Total (n = 47)
Ligated oviduct				
Total number of embryos	164	135	105	404
Number of degenerated embryos	9	23	28	60
Number of blastocysts	9	—	6	15
Number of hatching blastocysts	2	—	—	2
Number of hatched blastocysts	144	112	71	327
Contralateral uterus				
Number of conceptuses	130	137	95	362

in the uterine horn instead of the oviduct. To avoid this problem, we ligated the straight segment of the oviduct distal to the uterotubal junction.

In our study, blastocysts in the ligated oviducts remained in a dormant stage after hatching from their zona pellucida. Since most hatched blastocysts attached to the culture dish and developed trophoblast outgrowth after removal from the oviductal environment, we suggest that there were active growth inhibitors present in the normal milieu of the healthy oviducts, which prevented the growth and implantation of viable embryos.

The embryonic dormancy phenomenon observed in artificially obstructed healthy oviducts is quite similar to the dormant stage of preimplantation embryos found in lactating or ovariectomized mice (7,8). It generally is acknowledged that embryonic diapause in mice results from the absence of estrogen during the luteal phase (9). However, the exact mechanisms by which blastocysts arrest in the dormant phase are unclear. It has been proposed that protein factor(s) in the uterine luminal fluid may regulate metabolic dormancy of blastocysts during delayed implantation (10, 11). The uterine fluid collected from mice with embryos in diapause inhibits the hatching of blastocysts from their zona pellucida (12). O'Neill and Quinn (13) detected an inhibitor of [³H]uridine incorporation in the uterine fluid of ovariectomized mice with diapausing embryos. The activity of the inhibitor was significantly reduced 6.25 hr after an injection of estrogen (13). Some proteins in the uterine luminal fluid decrease or disappear at the time of reactivation by estrogen stimulation. In addition, a potent protein synthesis inhibitor, actinomycin D, has been shown to reverse implantation delay (9,14,15). These studies provide support for the role of uterine-secreted sub-

stances—most likely proteins—that inhibit blastocyst development. Katagiri *et al.* (16) purified a uterine protein from mice carrying diapausing embryos that has inhibitory activity on trophoblast outgrowth. The 170-kDa delayed-implantation-associated protein (DIAP 170K) was found in the uterine tissue as well as in the uterine luminal fluid. The abilities of this DIAP 170K to suppress RNA synthesis and cell proliferation as well as to suppress resumption of DNA synthesis by delayed blastocysts may play a role in the regulation of embryonic dormancy associated with delayed implantation (16,17).

In our study, it seems that the dormancy of blastocysts found in the obstructed oviducts was not due to the absence of estrogen secretion. Since we carefully avoided ligating the major blood vessels, we believe that the ligated oviducts and the untreated uterus were under the same blood-borne hormonal support that maintains normal implantation and pregnancy. Therefore, the oviduct might have an embryo growth-retarding mechanism or substance similar or identical to the DIAP 170K found in the uterus that inhibits the growth of hatched blastocysts within the oviduct (16).

Embryos confined to the obstructed oviducts also fail to develop beyond the blastocyst stage in species like pigs and rabbits, which do not have the embryo dormancy phenomenon. Pig and rabbit embryos in ligated oviducts degenerated and remained within the zona pellucida, while the other embryos emerged from the zona pellucida and were found as conceptuses within the uterus on the nonligated, control side (18,19). To elucidate the mechanism underlying the absence of oviductal ectopic pregnancies, Pauerstein *et al.* (20) substituted endosalpingeal circumferential grafts for endometrium in a group of rabbits. The endosalpingeal grafts interfered with implantation as

Table III. The In Vitro Developmental Potential of Embryos Recovered from Artificially Obstructed Oviducts

	Days postcoitus			
	5.5–7.5 (n = 18)	8.5–10.5 (n = 17)	11.5–19.5 (n = 12)	Total (n = 47)
Number of hatched blastocysts	144	112	71	327
Outgrowth of hatched blastocysts	144	112	65	321
Blastocyst outgrowth ratio (%)	100*	100	85.5*	98.2
Number of blastocysts and hatching blastocysts	11	—	6	17
Further development	8	—	1	9
Hatched out and outgrowth	6	—	1	7
Number of degenerated embryos	9	23	28	60
Further development	—	—	—	—

* *P* < 0.0025; chi-square analysis with Yates correction.

evidenced by (i) lower nidation indices, (ii) no implantation occurring on the grafted endosalpinx, (iii) unattached blastocysts found within the uterine lumen in the area lined with endosalpinx, and (iv) significantly smaller implantation sites in the uterine horns containing endosalpingeal grafts. Further experiments showed that hatched blastocysts in a cell coculture attached to endometrial but not endosalpingeal monolayers. Moore *et al.* (21) noted that the addition of conditioned medium from cultured endosalpinx significantly decreased embryo attachment to endometrial cells. These findings suggest the presence of a factor in rabbit endosalpinx that actively suppresses ectopic implantation in the oviduct.

Through our results and the findings described above, it seems that tubal obstruction alone does not cause tubal pregnancy, at least in rodent and porcine fallopian tubes. If human endosalpinx also secretes growth retardation factors, the etiology of tubal pregnancy more likely would be from defects of the endosalpinx caused by inflammation or congenital origins rather than from obstruction alone. However, to clarify whether human oviducts also have the growth retardation effect as we observed in the mouse fallopian tube, we need to identify this factor in rodent oviducts first and then search for the homologous factor in human oviducts.

The putative dormancy inducing factor from rodent oviducts is now under characterization in our laboratory. We hope to identify and purify the embryo-dormancy-inducing factor, which will give us more knowledge related to oviduct anti-implantation mechanisms as well as the etiology of tubal pregnancy. It could become a safe method of contraception.

CONCLUSIONS

The present study demonstrates that in mice, healthy ligated oviducts can support embryo development beyond the hatching blastocyst stage, but the timing of hatching was delayed compared with embryos in the uterine horns. Tubal pregnancy is not likely to happen in artificially obstructed healthy mouse fallopian tube because all the viable embryos become dormant in the hatched blastocyst stage. When removed from the oviductal environment, almost all dormant hatched blastocysts started to grow, attached to the culture dish, and spread out to develop trophoblastic outgrowth. Thus, we suggest the

mouse endosalpinx may secrete factors that result in embryonic dormancy.

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