SHORT COMMUNICATION

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Growth Retardation of Rabbit Embryos in Ligated Oviducts and the Toxic Effects of Rabbit Tubal Fluid on Mouse Embryo Development In Vitro

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INTRODUCTION

Presently, all experimental attempts to induce tubal pregnancy in animals by restricting the embryos to the oviduct with ligation (1–7) or transferring blastocysts to recipient oviducts (8–10) have consistently failed. Embryos trapped in oviducts were not capable of developing beyond the blastocyst stage and degenerated. In order to investigate the inhibition of embryo growth inhibition by oviductal fluid, we designed two experiments. In the first, we use ligated rabbit oviducts as a model to evaluate in vivo rabbit embryo development within the ligated tubes and the viability of these embryos after in vitro culture. In the second, we looked for a cross-species effect of rabbit tubal fluid (RTF) on mouse embryo development in vitro.

MATERIALS AND METHODS

Animals

Adult female virgin New Zealand white rabbits weighing 4–5 kg were caged individually for more than 3 weeks under controlled temperature (20–25°C) and lighting (14 h light/10 h darkness, lights on 7 A.M. to 9 P.M.). Water and pelleted food were supplied ad libitum. Three weeks later, seven oestrous females were bred to proven fertile males. After confirmed

mating by the finding of sperm in vaginal secretions, the rabbits were immediately given an intravenous injection of 75 IU human chorionic gonadotropin (hCG; Organon, Oss, Holland) to induce ovulation.

Surgical Technique

Twenty-four to 48 h after mating, surgery for tubal ligation was performed under sterile conditions. Surgical anesthesia was induced and maintained with ketamine (40 mg/kg) and xylazine (6 mg/kg) intramuscularly. The abdomens were opened via a ventral incision. Using 4-0 DEXON®“S” (Davis + Geck Inc., USA), the oviducts were ligated with one ligature on the utero–tubal junction and another on the distal end of the ampulla. Hemostasis was achieved by topical application of epinephrine dissolved in lactated Ringer’s solution (0.5 mg/kg, 1:1000) and by avoiding injury to the main vessels near the oviducts according to the tubal ligation technique of Herrmann and Holtz (11). During these procedures, tissues were frequently irrigated with heparinized lactated Ringer’s solution. After ligation of the tubes, prophylactic ampicillin was given intraperitoneally. Then the abdomen was closed in two layers, using interrupted 2-0 chromic catgut suture for the muscle and abdominal fascia and 3-0 DEXON®“S” suture in a horizontal mattress suture for skin closure.

Sample Collection and Rabbit Embryo Assay

Eleven to 16 days after mating, the ligated rabbit oviducts were excised under anesthesia with ketamine (40 mg/kg) and xylazine (6 mg/kg) intramuscularly. The rabbits were sacrificed with an intravenous overdose of sodium pentobarbital immediately after removal of the ligated tubes. In order to prevent the tubal fluid and the tube-locked embryos from blood contamination during collection, we chose the vessel-free area of the fluid-filled tube to cut open, 3–5 mm distal to the ligature at the utero–tubal junction. We collected RTF into the eppendorf and transferred them to the culture organ dish to pick

up the embryos in RTF under a warmed dissecting microscope. The recovered embryos were put into Leibovitz's L-15 medium (Gibco BRL, Grand Island, New York) plus 10% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, Missouri). In addition, the embryos retained within the oviducts were also flushed out with L-15 medium plus 10% BSA. All embryos recovered from the oviducts were morphologically examined to record the stage of development they had attained. These embryos were then cultured in α -minimal essential medium (α -MEM; Gibco BRL, Grand Island, New York) plus 10% fetal bovine serum (FBS; Hyclone, Logan, Utah) overlaid with mineral oil (Sigma Chemical Co., St. Louis, Missouri). Incubation was carried out at 37°C in an atmosphere of 5% CO₂ and 95% air. The rabbit embryos were examined daily for 4 days to observe their viability for further development.

Mouse Embryo Assay

We also investigated the cross-species effects of RTF on in vitro mouse embryo development. All the RTF samples were centrifuged for 10 min at 3000 rpm and the clear supernatant fluids were stored at -20°C in sterile eppendorf until used for mouse embryo culture. To induce superovulation, 25 female ICR mice (aged 5–8 weeks) were injected intraperitoneally with 10 IU pregnancy mare serum gonadotropin (PMSG; Sigma Chemical Co., St. Louis, Missouri), followed by 10 IU human chorionic gonadotropin (hCG; Organon, Oss, Holland) intraperitoneally 46–48 h later. They were then bred to proven fertile males overnight and mating was confirmed by the presence of a vaginal plug the next morning. About 45 h after hCG injection, the mice were sacrificed by cervical dislocation and the oviducts were excised. After being flushed from the oviducts

with M16 medium (Sigma Chemical Co., St. Louis, Missouri) healthy appearing two-cell mouse embryos were randomly placed in culture media with four different concentrations of RTF (100%, 50%, and 10% vs. M16 + 0.5% BSA as the control medium). All the RTF samples were tested individually without pooling together. Incubation was performed at 37°C in 95% room air and 5% CO₂. Mouse embryo development was examined daily and the percentages of embryos with blastocyst formation were recorded after 4 days of in vitro culture. Statistical analysis was conducted by use of the Chi-square test.

RESULTS

A total of 12 fluid-filled, healthy looking oviducts of the late-luteal phase (11–16 days after mating) were excised. No morphological signs of tubal implantation were found. By contrast, there were eight implanted embryos found in the two unligated control uterine horns. All 37 tube-locked rabbit embryos demonstrated partial or total degeneration and had never developed beyond the hatched blastocyst stage (Table I). After in vitro culture of these 37 rabbit embryos for 4 days, only 6 embryos (16.2%) demonstrated blastocyst attachment and outgrowth (Table I). Ten of the 12 RTF samples were found suppress in vitro mouse embryo development to the blastocyst stage significantly compared with the control medium, even at a dilution of 10% of the original RTF concentration (Table II).

DISCUSSION

To date, all attempts to produce tubal implantations in infraprimates by locking the embryo in the

Table I. Developmental Stages of Rabbit Embryos in the 12 Ligated Oviducts 11–16 Days After Confirmed Mating

Embryonic stage	Numbers of rabbit embryos	Blastocyst attachment and outgrowth after in vitro culture
Degenerated embryos within ZP	12	—
Hatching and partially degenerated blastocysts	1	1
Hatched and degenerated blastocysts	2	—
Hatched and partially degenerated blastocysts	22	5
Total rabbit embryos	37	6

Note. ZP = zona pellucida.

Table II. Effects of Rabbit Tubal Fluid on Mouse Embryo Development In Vitro

	Mouse embryo blastocysts formation rate (%)	
	Toxic to mouse embryos	No embryonic toxicity
Number of tubal fluid samples	10	2
100% rabbit tubal fluid	8/158 (5.1)*	16/18 (88.9)**
50% rabbit tubal fluid	63/135 (46.7)*	15/17 (88.2)
10% rabbit tubal fluid	70/133 (52.6)*	16/16 (100)
Control (M16 + 0.5% BSA)	128/150 (85.3)	17/17 (100)

Note. * $p < 0.0001$, Chi-square analysis; ** $p = 0.26$, Chi-square analysis.

oviduct have failed (1–10). Our study adds to this experience, as we found no tubal pregnancy in any of the 12 ligated rabbit oviducts. These findings suggest that tubal pregnancy probably does not result simply from obstruction alone and that there is some form of tubal protection from embryo implantation. Therefore, we speculate that a defect of an oviductal anti-implantation system may be caused by congenital factors or tubal diseases such as salpingitis or tubal damage from surgery, with consequent tubal pregnancy. That is a reasonable explanation of why humans have a higher prevalence of tubal pregnancy than other species, because humans are subject to tubal diseases that do not affect other species.

In all available animal studies, embryos locked in the oviducts have been found not to develop beyond the blastocyst stage (1–7). Our previous investigation reported by Lee *et al.* found that mouse embryos were dormant at the hatched blastocyst stage within ligated oviducts (5). In this study, all 37 tube-locked rabbit embryos failed to develop beyond the hatched blastocyst stage. These findings indicate that prolonged embryo exposure to obstructed oviducts inhibits embryo growth and delays embryo development.

In vivo studies have found that embryos locked in the oviduct up to 5 days after coitus are not capable of developing normally after transfer to the uteri of synchronized recipients (12,13). Our results are similar, with only 6 of 37 embryos showing any viability on subsequent in vitro culture. Prolonged retention in the oviduct apparently greatly diminishes any chance for normal growth and development.

In vivo studies have revealed that uterine blastocysts transferred to recipient oviducts at the time of uterine implantation fail to implant in the oviducts (8–10). Pauerstein *et al.* and Moore *et al.* also found that no blastocysts attached to grafted rabbit endosalpinx in vivo or to rabbit endosalpingeal epithelial monolayers in vitro (14,15). These reports imply that

the endosalpinx secretes inhibitory factors that suppress embryo viability and prevent tubal implantation. The second experiment reported in this paper also supports this hypothesis, because most of our RTF samples inhibited mouse embryo development in vitro. As far as we are aware, this is the first report of such a cross-species effect. Although presently many questions remain concerning the nature and action of inhibitory factors in late-luteal RTF, the fact that a cross-species effect exists may open the way for developing new contraceptive methods. We are now working to isolate and characterize the embryonic inhibitors in late-luteal RTF.

In summary, the present study suggests that prolonged embryo exposure to obstructed rabbit oviducts delays embryo development and impairs viability. In addition, our data supports the previously asserted hypothesis that tubal pregnancy is not caused by obstruction alone. Most importantly, we are the first to demonstrate an inhibitor within rabbit late-luteal stage RTF which has the cross-species effect of inhibiting mouse embryo development.

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