In-vitro fertilization of rabbit ova ovulated *in vitro* during ovarian perfusion

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Summary. During in-vitro perfusion of rabbit ovaries, ova that ovulated in response to hCG were recovered and cultured with spermatozoa capacitated *in vivo*. After culture for 30 h, 113 (48.8%) of the ova underwent cleavage and were not degenerate. Of these 113 zygotes, 75 were successfully transferred to the oviducts of 14 gonadotrophin-treated host rabbits. On Day 14 after transfer there were 14 implantation sites and 9 pregnancies proceeded to term. These data indicate that in-vitro ovulated ova can be fertilized *in vitro* and be used to establish normal pregnancies in a host rabbit.

Introduction

The technique of in-vitro ovarian perfusion has been useful for investigating the mammalian ovulatory process. The local effects of various agents on follicle growth, ovulation and ovum maturation can be examined by using in-vitro ovarian perfusion (Lambertsen, Greenbaum, Wright & Wallach, 1976; Hamada, Bronson, Wright & Wallach, 1977; Hamada, Wright & Wallach, 1978, 1979; Wallach, Wright & Hamada, 1978; Hamada *et al.*, 1980; Kobayashi, Santulli, Wright & Wallach, 1980, 1981a; Kobayashi, Wright, Santulli & Wallach, 1983). Ova released *in vitro* have been recovered, cultured, transferred to a mated host, and observed to implant and produce viable offspring after fertilization *in vivo* (Kobayashi *et al.*, 1981b). In-vitro fertilization techniques have been established for several species, with varied success rates (Brackett, 1979). The present study was designed to evaluate in-vitro fertilizability of rabbit ova ovulated *in vitro* during ovarian perfusion.

Materials and Methods

Ovaries of sexually mature virgin female New Zealand White (NZW) rabbits were removed and perfused to obtain ova ovulated *in vitro*. NZW males of proven fertility served as inseminators. Dutch Belted (DB) and NZW females served as hosts for the in-vitro fertilized ova. All female rabbits were isolated for 3 weeks before study, and kept under controlled light and temperature with free access to Purina Rabbit Chow and water.

The perfusion technique and surgical procedure, including arterial cannulation and ovarian removal, have been previously described in detail (Lambertsen *et al.*, 1976; Hamada *et al.*, 1978; Kobayashi *et al.*, 1981c). Both ovaries of isolated NZW rabbits were removed at laparotomy and placed in separate perfusion chambers. An ovulation-inducing dose of hCG (100 i.u.) was added to the perfusing medium of each ovary at the onset of perfusion (Kobayashi *et al.*, 1981c). Ovaries were perfused for 12 h at 37°C and 100% humidity. Throughout perfusion, ovaries were observed frequently for evidence of follicular growth and ovulation.

When ovulation occurred, the extruded ovum in its cumulus mass was recovered from the surface of the ovary and washed 3 times in Brackett's defined medium (Brackett & Williams, 1968). Ova were cultured under a thin layer of paraffin oil at 37°C and 100% humidity in an atmosphere of 5% CO₂ in air and minimal light (Brackett & Williams, 1968).

Capacitated spermatozoa were obtained by mating a NZW female with 3 NZW males of proven fertility. Insemination was confirmed by the presence of spermatozoa in the vagina. The mating was scheduled 2 h before the onset of perfusion of the ovary which served as the source of eggs. This sequence enabled co-ordination of sperm recovery at 14 h after mating with the optimum ovum culture period (12 h after hCG). A laparotomy was performed on the mated female 14 h after mating to recover the spermatozoa. The cervico-vaginal junctions and utero-tubal junctions were clamped and each uterine horn was flushed with 4.0 ml Brackett's medium using a 5-ml syringe and 19-gauge needle. The rabbit was killed immediately before the uterine flushing to minimize blood contamination of the recovered sperm/medium mixture. The recovered medium was examined microscopically. A concentration of spermatozoa > 10⁵/ml medium and sperm motility of 60–90% were considered satisfactory for in-vitro fertilization (Dandekar & Fraser, 1976).

At 12 h after initial exposure of the ovary to hCG, cultured eggs that had ovulated *in vitro* were transferred to Petri dishes containing the sperm suspension. The 12 h gonadotrophin-to-insemination interval was used because previous experiments had indicated that 70.5% of eggs ovulated by 12 h after hCG in the perfusion medium are sufficiently mature (metaphase II, first polar body extruded) to undergo fertilization (Kobayashi *et al.*, 1981b). The eggs and spermatozoa were cultured in Brackett's medium under oil. At 4.5 h after exposure to capacitated spermatozoa, the eggs were removed from the sperm suspension and placed in fresh medium in which culture was continued.

In the first phase of this study, groups of eggs were examined after 8, 26, 30 and 38 h of culture. Before examination, eggs were mechanically agitated 2 or 3 times to remove cumulus cells. If this method did not sufficiently release the granulosa cells, the eggs were subsequently treated with hyaluronidase (Fraser, Dandekar & Vaidya, 1971). At 30 and 38 h of culture, all eggs required hyaluronidase treatment for removal of cumulus cells. The denuded eggs were examined microscopically. Fertilization was confirmed by the observation of 2 polar bodies, male and female pronuclei, or cleavage. Fertilized eggs were also assessed for signs of degeneration, including vacuolation, cytolysis, necrosis, changes in the shape of pronuclei and fragmentation.

In the second phase of the study, in-vitro ovulated and in-vitro fertilized eggs were transferred to host rabbits which had been treated i.v. with 100 i.u. hCG at the time when ovarian perfusion had started. The in-vitro ovulated and fertilized eggs were cultured for 30 h. The eggs were then removed from the culture dish by pipette and the medium was changed twice to eliminate the oil layer. Eggs for transfer were not treated with hyaluronidase. The cultured eggs that had been exposed to spermatozoa were examined microscopically. Undegenerated eggs that had undergone cleavage were transferred to host rabbits at laparotomy. The fertilized eggs (2–4-cell stage) in 20 µl culture medium were placed in the ampulla of each oviduct using a glass pipette (range 3–11 eggs/host). Each host rabbit was subjected to a second laparotomy 14 days after egg transfer to determine the number, size, and location of implantation sites in each uterine horn. Bicillin (300 000 i.u.: Wyeth Laboratories, Philadelphia, Pennsylvania) was administered intramuscularly, after surgery. At 32 days after transfer, cages were examined frequently for the presence of offspring.

Results

Study 1. Thirty-one ovaries were perfused and 151 in-vitro ovulated ova were recovered and cultured. As shown in Table 1, the highest percentage of fertilization was evident at 26 h and pronuclear and cleaved stages were obtained. Culture for 30 h yielded the greatest percentage of undegenerated cleaved ova and this time was therefore used for Study 2.

Table 1. In-vitro fertilizability of rabbit ova ovulated during ovarian perfusion

Culture time (h)	No. (%) of ova			
	Cultured	With pronuclei	Cleaved	Total fertilized
8	48	26 (54.2)	—	26 (54.2)
26	16	5 (31.3)	5 (31.3)	10 (62.5)
30	41	—	20 (48.8)	20 (48.8)
38	46	—	18 (39.1)	18 (39.1)

Study 2. In-vitro perfusion of 52 rabbit ovaries yielded 234 ovulated ova. After culture for 30 h, 113 of the 234 ova (48.3%) had cleaved and showed no evidence of degeneration. Of the 113 zygotes, 75 were successfully transferred to NZW (N = 4) and DB (N = 10) hosts. The reduction in number of eggs at this stage of the procedure was related to technical problems encountered in the transfer process (22 eggs) or to complications associated with the host (16 eggs), including anaesthetic death or infection. Of the 75 zygotes transferred, 14 (18.7%) implanted in 7 hosts as observed at laparotomy on Day 14. The greatest percentage loss of eggs was experienced at the implantation step. Nine of the 14 pregnancies (12% of the transferred zygotes, 3.8% of the original ova) proceeded to term. This figure compares with 18% term delivery of in-vitro fertilized, in-vivo ovulated rabbit ova (Mills, Jeitles & Brackett, 1973). All of the offspring resulting from the procedure were completely white.

Discussion

The demonstration that ova ovulated *in vitro* are also capable of fertilization *in vitro* confirms that the in-vitro perfused rabbit ovary is suitable for studying the ovulated ovum as well as the ovulation process under various experimental conditions.

The low pregnancy yield (3.8% of 234 in-vitro fertilized ova) may be compared with that recently achieved in human in-vitro fertilization and embryo transfer. The attrition rate in this investigation, as in human experience thus far reported, appears to be greatest at the step of embryo transfer and implantation (Biggers, 1981). The techniques described in this report can be applied to the study of ovum maturation and fertilizability following perturbation of the normal time sequence of ovum maturation and ovulation by use of pharmacological agents.

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