

IN VITRO FERTILIZATION OF
RABBIT OVA AND OVIDUCTAL
INFLUENCES ON FERTILIZATION

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ABSTRACT

IN VITRO FERTILIZATION OF RABBIT OVA AND OVIDUCTAL INFLUENCES ON FERTILIZATION

By

Richard Miller Harrison

Studies were conducted to develop a reliable system for in vitro fertilization of rabbit ova and to use that system for studying the influence of the oviduct on sperm capacitation. The system consisted of an acidic saline medium containing 20% rabbit serum in which capacitated sperm and ova were incubated at 37-38°C for 24 hours. The humidified atmosphere in the incubation chamber was 5% CO₂/95% air for 4-5 hours and air for the remainder of the incubation period. Fertilization was ascertained by examining for uniform cleavage and presence of sperm in the perivitelline space.

Ova were recovered from the surface of the ovary (periovarian ova) and from the oviducts (oviductal ova) of rabbits induced to ovulate by hormone injections. When incubated with sperm recovered from the uterus 17 hours post coitum under the best incubation conditions the periovarian ova were fertilized at a rate slightly higher than that obtained with oviductal ova (56% compared to 50%).

Fertilized ova recovered from the oviducts of the capacitator rabbits cleaved normally in all of these studies.

If incubation conditions were deliberately made less desirable by the use of an interface-forming mineral oil over the incubation medium the rates of fertilization and uniform cleavage were reduced when periovarian, oviductal and capacitator ova were incubated (30%, 5%, and 9% respectively). In studies using uterine sperm recovered 11 hours after mating and interface-forming mineral oil, the rates were 24%, 0% and 22%. When silicone oil or non-interface-forming mineral oil was used with 11 hour uterine sperm and periovarian, oviductal and capacitator ova, the rates were 29%, 27% and 84% respectively.

Studies were conducted to determine the effects of ligating the utero-tubal junction on the ability of the uterus to capacitate sperm. Rabbits, which were to serve as capacitors, had unilateral ligation and transection of the utero-tubal junction. These animals were mated after three weeks and 11 hours later the sperm were recovered from both the isolated uterine horn and the normal uterine horn. Although the rate was reduced compared to sperm from the normal uterine horns both periovarian and oviductal ova were fertilized using sperm from the isolated uteri.

These studies indicate that exposure to the oviductal environment make the ova less fertilizable when exposed to abnormal incubation conditions. One preliminary

study was conducted by pre-incubating periovarian ova in oviductal fluid and these oviductal effects were confirmed. The ligation studies show that while all sperm may not be completely capacitated in the isolated uterus, some capacitated sperm are recovered from this prepared organ 11 hours after mating.

Throughout these studies in vivo fertilized ova recovered from capacitors cleaved normally when incubated under the non-interface-forming mineral oil or the silicone oil. Ova recovered from the ligated oviducts and those incubated in media not containing sperm failed to cleave. These two observations indicate that parthenogenic activation was prevented and proper incubation conditions existed except in those studies where interface-forming mineral oil was used.

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INTRODUCTION

Attempts to fertilize rabbit ova in vitro have been reported for nearly 100 years. Suzuki and Mastroianni (1965) stated, "In vitro fertilization of mammalian ova has been singled out as one of the most challenging and frustrating research projects in the field of reproductive biology." Only in the past 20 years have reports been presented where in vitro fertilization has been successfully demonstrated. A major turning point in the efforts occurred following reports by Chang (1951) and Austin (1951) that rabbit spermatozoa must undergo certain changes in the female tract before they attain the capacity to fertilize the ovum. This process is termed capacitation (Austin, 1952) and is currently believed to involve the removal of enzyme inhibitors from the surface of the spermatozoa head, allowing the sperm to penetrate the ovum (Zaneveld, Srivastava, and Williams, 1970).

The development and use of an in vitro fertilization system permits one to investigate previously inaccessible areas. With such a system the environment can be isolated so that changes in temperature, pH, atmosphere, nutrients and other factors can be studied. The time sequence of

events from ovulation through fertilization and early development of the zygote can be more accurately determined in vitro. In the evaluation of pharmacologic agents the use of an in vitro fertilization system allows the study of fertilization involving ova and capacitated sperm from animals receiving various treatments. With the proper techniques ovulated eggs not exposed to the environment of the oviduct can be compared to eggs recovered from the oviduct to study the influence of the tract on the gametes and the effects of agents on the oviductal environment. Such systems can potentially be used to solve human fertility problems such as oviductal occlusion where eggs might be recovered, fertilized and transported back into the donor's uterus.

The objectives of these investigations were:

1. to develop an in vitro fertilization system capable of yielding reliable consistent levels of fertilization,
2. to study in vitro techniques reported in other laboratories and, if desirable, to adapt them to our system, and
3. to use the established in vitro fertilization system to study several aspects of the capacitation--fertilization phenomena.

LITERATURE REVIEW

The rabbit has been the most widely used species for in vitro fertilization studies because of the ease of recovering uterine sperm and ova of known age, but other species have also been studied including the guinea pig (Shenk, 1878), mouse (Long, 1912), rat (Long, 1912), pig (Petrov, 1958) and man (Rock and Menkin, 1944; Menkin and Rock, 1948; Shettles, 1953; Petrov, 1958; Edwards, Bavister and Steptoe, 1969; Edwards, 1969). This review will include only those studies involving the rabbit. These will be divided into those studies using non-capacitated sperm and those using capacitated sperm.

Studies of In Vitro Fertilization with Non-Capacitated Spermatozoa

Shenk (1878) used follicular ova with epididymal sperm and observed removal of follicular cells adhering to the ova in 10 to 12 hrs.

In 1893 Onanoff recovered ova from the uterus of the rabbit and reported successful in vitro fertilization and development of these ova. Current knowledge of the fertilizable life of the rabbit ovum would suggest that these ova were undergoing degeneration rather than fertilization cleavage.

From 1930 to 1940 a number of studies were conducted by Pincus and his co-workers (Pincus, 1930; Pincus and Enzmann, 1936; Pincus, 1939; Pincus and Shapiro, 1940). They reported fertilization using sperm recovered from the vas deferens and ova recovered from the oviducts of does mated to vasectomized males. Their studies demonstrated that temperature control was critical. Cooling the ova to room temperature ($20-27^{\circ}\text{C}$) for 1 hr, was sufficient for activation and cleavage. They reported live young as a result of transferring in vitro fertilized ova to a recipient doe but Chang and Pincus (1951) stated that this was probably due to fertilization occurring in vivo from sperm adhering to the ovum when transferred.

Frommolt (1934) reported shrinkage of the vitellus of oviductal eggs following in vitro incubation with sperm. This was most likely due to cooling as no effort was made to control the temperature during recovery and preparation for incubation.

Krasovaskaja (1935) recovered ova from the oviducts of does mated to vasectomized males 17 hours earlier. Cleavage was reported using rabbit epididymal sperm. Chang (1965) stated that the cleavage was probably due to ovum activation as a result of cooling.

In all the above studies, temperature was not controlled during the period of gamete manipulation, i.e., the interval from removal of the ova from the donor to placement

of the sperm and ova into an incubation chamber or bath. During this time the ova may have become cooled sufficiently to be activated (Pincus and Shapiro, 1940). The parthenogenically activated ovum is similar in appearance to the normally dividing fertilized ovum and is probably a result of spindle fiber alteration (Chang, 1954). In almost all of the above studies and in some of those described below ovulation of the ovum donors was induced by mating to vasectomized males. This surgical procedure for sterilizing males might not have been complete or the vas deferens may have grown together later. Failure to ascertain sterility of the males, at mating, may have allowed sperm to contact the eggs prior to recovery. For this reason, some of the reported successful studies may have been erroneous. Most later investigators induced ovulation by hormone injection.

Of all the workers using non-capacitated sperm, only Smith (1951) controlled temperature during the manipulation period. Austin (1961) and Thibault (1970) have independently concluded that Smith was the only investigator to obtain an in vitro fertilized ovum. In 1965, however, Chang reported that Smith had not observed sperm penetration due to density of corona radiata cells and cleavage might have been parthenogenic. As late as 1957 a repeatable procedure to fertilize mammalian ova in vitro was not available (Chang, 1957).

Studies of In Vitro Fertilization with Capacitated Spermatozoa

Since the discovery of capacitation in rabbits a number of reports of successful in vitro fertilization have appeared. Capacitated sperm have been recovered from the uteri of mated does by aspiration of uterine contents either with or without prior injection of suitable medium and by flushing the contents of the uterine horns into a container. In all the studies discussed below sperm were recovered at least 10 hrs post coitum. This interval is sufficient for capacitation in the rabbit (Adams and Chang, 1962).

Moricard (1954) recovered oviductal ova 11 hrs after mating to vasectomized males. Sperm were recovered from the uterine horns of a doe 10 hrs after mating. The gametes were incubated at 36.5-37°C in air or nitrogen. Using as criteria of fertilization the ovular cytoplasmic retraction, presence of 2 pronuclei, and the presence of sperm in the perivitelline space he reported 30% fertilization, although cleavage was not obtained.

Dauzier and Thibault (1956, 1959) reported typical fertilization stages when tubal eggs were incubated with 12 hr uterine sperm in Locke's solution or in Locke's plus serum medium. They maintained a temperature of 37-38°C during gamete manipulation and reported 13.7% and 30.0% of the ova fertilized. Their evidence for fertilization was based on histological examination of the ova.

The first incontestable evidence for fertilization of rabbit eggs in vitro was reported by Chang in 1959. He recovered uterine sperm 12 hrs after mating. A freshly prepared Krebs-Ringer bicarbonate medium containing 0.25% glucose was used to recover sperm and ova. Ova were flushed from the oviducts of rabbits injected 12 hrs previously with a sheep pituitary extract and placed into small Carrel flasks containing the uterine sperm suspension. The flasks were stoppered and incubated for 3 to 4 hrs at 38°C. The ova were then transferred to flasks containing 50% heat-inactivated rabbit serum in saline, incubated for 18 additional hrs and examined. Those that cleaved normally to the four cell stage and contained a second polar body or sperm in the perivitelline space were considered fertilized. Some fertilized ova were transferred to recipient does. In this study 21% of the ova were fertilized in vitro and 42% of those transferred to recipients developed into normal young.

Thibault and Dauzier (1960, 1961) reported that washing of ova prior to incubation improved the rate of fertilization. They used 12 hr uterine sperm and freshly ovulated ova recovered from the oviducts of does mated to vasectomized bucks. A discontinuous rotary glass tube rack was used to wash the ova and to incubate the gametes. The presence of a swelling sperm head and mid-piece as well as a second polar body or pronuclei were used to determine

fertilization. These workers reported that a "fertilizine-like" substance had to be removed before sperm penetration could occur in vitro. In 1961 they reported 66% of the ova fertilized and the birth of 2 young after transfer of fertilized ova to recipient does. In 1961 Dauzier and Thibault reported that the quality of the sperm was not as critical as the condition of the eggs at recovery and during manipulation. These conclusions conflicted with those of Bedford and Chang (1962) who reported that sperm quality was more important and that washing of the gametes did not improve fertilization rate. Using the procedure described by Chang (1959) they found the motility of uterine spermatozoa was significantly prolonged if 5% heated homologous serum was added to the medium. They reported 57% of the ova were fertilized in 10% serum in acidic saline and 6 young were born when 11 of the ova were transferred to recipient does. They concluded that whereas in vitro fertilization is "definitely possible, repeatable and authentic," conditions vary greatly as does the proportion of ova fertilized in each trial. Some variables mentioned included capacitation state of the sperm, age of the ovum, temperature during manipulation of gametes, time intervals and gas concentrations.

The function of the corona radiata was studied by Chang and Bedford (1962), using an in vitro fertilization system. When the cells were removed with 0.1% hyaluronidase

a lower fertilization rate resulted. They concluded that the corona radiata plays a role in protecting the fertilizability of the ovum.

The system described by Chang in 1959 was adapted by Williams, Hamner, Weinman and Brackett (1964). These investigators reported a fertilization rate of 15% in their initial studies where relative humidity was maintained at 78% during manipulation of the gametes. At 90% relative humidity, 35% fertilization was achieved. They believed cooling of the incubation mixture by evaporation was responsible for the lower fertilization rate.

Brackett and Williams (1965) reported a very high fertilization rate using an atmosphere of 96 to 97% relative humidity, paraffin oil over the medium and uterine fluid in the medium. Their initial medium (fertilization medium) included 5% rabbit serum in acidic saline, the solution used by Bedford and Chang (1962). After initial incubation of 4 hrs the ova were transferred to an incubation medium containing 10% serum. All manipulation and recovery of the gametes was at 39°C at 96 to 97% relative humidity. Paraffin oil was used to cover the gametes. The ova were recovered from the oviducts 12 hrs after injection of luteinizing hormone (LH). The sperm were recovered 12 hrs after mating from a doe also injected with LH. Sperm recovery was accomplished by injecting the 5% serum solution into the uterine horn and aspirating the suspension. If more volume was recovered

than injected the excess was considered to be fresh uterine fluid. In studies with 96 to 97% relative humidity, at 39°C, and with 20 to 40% fresh uterine fluid in the medium, the fertilization rate was 73%. Fertilization was ascertained by staining ova with lacmoid to demonstrate chromatin in the blastomeres. One problem with the system described in this report was the stress put on the researchers by these environmental conditions, as all gamete manipulation was in an environmental control room.

In vitro fertilization of rabbit ova in oviductal fluid was reported by Suzuki and Mastroianni (1965). They ligated the oviducts and allowed fluid to accumulate for 4 to 5 days. Uterine sperm were collected 12 hrs after mating and suspended in the oviductal fluid. Ova were flushed from donors 12 hrs after injection with chorionic gonadotropin. The gametes were placed in a depression slide and covered with light mineral oil. The gametes were handled at 30 to 37°C; incubation was at 37 to 38°C. In some studies the mineral oil was mixed with Waymouth's tissue culture medium plus 10% serum in a ratio of 20:1 (oil to medium) and equilibrated with 5% CO₂ in air. After 4 hrs, the ova were transferred to the Waymouth's medium with serum. Ova were examined after 22 to 24 hrs incubation, and fertilization was ascertained by normal cleavage and the presence of a second polar body or sperm in the perivitellene space.

They reported 31% of the ova were fertilized when incubated under untreated oil, 64% under the treated oil.

Chang (1965) stated that the variable results and low in vitro fertilization rates compared to in vivo rates indicated a lack of knowledge concerning the required conditions. In 1966 Suzuki modified his previous techniques in an effort to advance the stage to which the ova would develop. He mixed the mineral oil with medium in a ratio of 20:1 before gassing it with 5% CO₂ in air. Medium 199 with serum (9:1) with added lactic acid (1 mg/ml) was used instead of Waymouth's. Cultures were maintained at 37°C in a humid atmosphere of 5% CO₂ in air. Recovery of gametes was as described previously (Suzuki and Mastroianni, 1965). Under these conditions 59.4% of the ova developed to the 2 to 4 cell stage by 20 hrs and 22% developed to 16 cells by 2 or 3 days after insemination in vitro.

Brackett (1966) attempted to develop a system that would eliminate the high relative humidity requirements used earlier (Brackett and Williams, 1965). He found that fertilization could take place when the medium was exposed to 5% CO₂ in air and not covered with oil, but that the rate was too low to be of value. When the medium was covered by paraffin oil equilibrated with 5% CO₂ in air, 16% of the ova were fertilized. An even lower rate was obtained if the oil was not equilibrated with 5% CO₂ in air. In the final series of studies he equilibrated the paraffin oil with

5% CO₂ in nitrogen and found 23 of 30 ova (77%) were fertilized. He concluded that reduced oxygen tension is beneficial to in vitro fertilization and that the requirement for high relative humidity could be replaced by lower oxygen tension. Brackett used a synthetic medium in some of his studies in which rabbit serum was replaced by bovine serum albumin. He suggested that his results do not necessarily conflict with Suzuki and Mastroianni (1965) because of the difference in media, fluid volumes and techniques. The system developed by Brackett at this time replaced high humidity with lower oxygen tension, rabbit serum with bovine serum albumin and fresh uterine fluid with bicarbonate which maintained a pH of 7.8 under the 5% CO₂ atmosphere. An in vitro fertilization rate of 65% was reported (Brackett and Williams, 1968), using the defined medium covered with paraffin oil equilibrated with 5% CO₂ in nitrogen.

Attempts by Suzuki and Mastroianni (1968) to fertilize follicular oocytes in vitro using the procedure described by Suzuki (1966) indicated a maturation requirement since ova obtained 8 hrs after gonadotropin injection were not fertilized unless incubated in vitro prior to insemination. These investigators used 12 hr uterine sperm and incubated in Waymouth's medium with 10% serum covered with oil. Their best rate of ova cleavage was 29%.

Using the basic procedure described above (Brackett and Williams, 1968) Brackett (1969a) investigated the

effects of washing the gametes and of using media of differing osmolarity. The media used were the defined medium described previously (345 milliosmolar) or the defined medium with the osmolarity adjusted to 280 mosm. by the removal of Na Cl. The lower osmolarity is more nearly isotonic. Although the medium and oil were equilibrated with 5% CO₂/nitrogen prior to use, during the semination procedure the gametes were incubated in an atmosphere of 5% CO₂ in air. After 5 hrs incubation the ova were transferred to 10% serum in acidic saline of both osmolarities. These solutions and the oil covering them were in equilibrium with air. Fertilization was determined by chromatic staining with lacmoid. Brackett concluded that washing the ova had no effect but washing the sperm lowered the fertilization rate. This confirmed the results of Bedford and Chang (1962). He suggested that the effect on the sperm may be due to removal of some labile component or to change in oxygen and CO₂ tension during the washing.

Thibault (1970) in his review suggested that the differing views on the need to wash the eggs is due to different techniques and volumes of wash solutions used by various investigators. He states that although media containing biological fluids usually give higher fertilization rates they also give more variation between studies. He discussed the need for definite criteria of fertilization, a need recognized by a number of workers.

Bracket (1969b) presented a review of in vitro fertilization of mammalian ova and discussed preliminary results of studies not previously published. He reported that ova could be recovered from the ovarian surface and that higher fertilization rates could be obtained with uterine sperm collected 17 to 18 hrs after mating. Adding 20% serum to the defined medium eliminated the need to transfer the ova after 5 hrs incubation, although cleavage may have been slightly retarded.

The time sequence of penetration, syngamy and cleavage was studied (Brackett, 1970) using the procedure described by Brackett (1969a). First cleavage occurred as early as 15.5 hrs after exposure to capacitated spermatozoa with cleavage to the 4 and 8 cell stages at 18.5 and 26 hrs, respectively. These time intervals compare well to those reported in vivo when the slower time of sperm penetration is allowed (Chang, 1960).

Seitz, Brackett and Mastroianni (1970) used the defined medium described by Brackett and Williams (1968) with 20% heat-inactivated serum to study the fertilizability of ova recovered from the ovarian surface. Ovum donors were superovulated with pregnant mares serum (PMS, Gestyl, Organon) and human chorionic gonadotropin (HCG, "APL," Ayerst). The rabbits were anesthetized 9.5 hrs after HCG and the abdominal cavity opened. The fimbriae and part of the ampullae were resected. The animals were then closed

and allowed to ovulate. Two hours later the ovaries were removed and the ova recovered. Capacitated uterine sperm were obtained from does mated 12 hrs previously. The atmosphere used in these studies was 5% CO₂/air. The fertilization rate reported was 81%.

Seitz, Rocha, Brackett and Mastroianni (1970) found that removal of the oviducts did not influence the ability of the capacitors to provide capacitated sperm. No differences in the fertilizing ability were noted between sperm recovered 8 to 36 hrs after mating from salpingectomized does and those recovered at the same time intervals from intact does. It was suggested by these workers that their in vitro system duplicated the factors of the oviduct that influence capacitation and that capacitation might have been completed in vitro. This remains to be confirmed.

The results of in vitro fertilization studies conducted by different investigators vary considerably. This variation may be due to the techniques used and the capability of the equipment to maintain environmental conditions within strict limits. Differences in techniques for recovering and handling gametes probably are a major area of variation. The criteria used to determine if fertilization has occurred have also contributed to variation between laboratories.

MATERIALS AND METHODS

A series of experiments were undertaken to develop a system for in vitro fertilization of rabbit ova. Procedures and techniques described in recent reports of successful work by others (Brackett and Williams, 1965, 1968; Brackett, 1966, 1969a,b) served as a foundation for our studies. Initial studies were directed towards the adaptation of our equipment to serve the necessary purposes.

Animals

All rabbits used were adults of the New Zealand White breed. They were supplied by Cherokee Lab Supply of Atlanta, Georgia or by local suppliers. Upon arrival at our laboratory the rabbits were placed in wire mesh or stainless steel cages with mesh floors. Water was supplied ad libitum and each rabbit was provided 6 oz of Triumph rabbit pellets per day. All does were isolated in individual cages for at least 21 days prior to being used either as capacitors or as ovum donors. As pseudopregnancy in the rabbit lasts for 17-18 days it was assumed that these animals were not in a progesterone dominated state when used. Capacitation is prevented if the mated rabbit is pseudopregnant (Hamner, Jones and Sojka, 1968). Capacitors were mated to males

of proven fertility and did not receive LH. Coitus in the normal rabbit induces ovulation; if ovulation did not occur in a capacitator this was considered ground for questioning her "use."

Recovery of Oviductal Ova

Donor rabbits were injected intravenously with 50-100 i.u. HCG (A.P.L., Ayerst Laboratories, Montreal) to induce ovulation. In the preliminary studies the donors were killed by cervical dislocation 12 hrs after the HCG and the uterine horns, oviducts and ovaries placed in sterile 95 X 15 mm Petri dishes. The dishes were then placed inside a tissue culture hood (Lab Con Co., A. H. Thomas) where the temperature was maintained at 37-40°C. Temperature was regulated by blowing an air stream from a forced air electric dryer over a shielded Bunsen burner. After all tracts were collected the oviducts were straightened by trimming away the surrounding fat for ease of flushing the ova. The frimbria were placed in the center of 85 mm watchglasses and covered with light mineral oil (Sargent, viscosity 80-90 Saybolt). The oviducts were flushed with 1.5 to 2 ml of the same type of medium in which the ova were to be incubated. A 24 gauge needle attached to a 5 ml syringe containing the medium was passed through the uterine wall and into the oviduct via the utero-tubal junction. The recovered ova were examined under a stereoscopic dissecting microscope and then transferred to a collection dish (50 X 13 mm Petri dish)

containing the test medium. Disposable Pasteur pipettes (Clay Adams Transpets--9") were used for this purpose. Suction for the pipettes was provided by an Adams Pipette Suction Apparatus which allowed pick-up of individual ova with minimal fluid transfer. When the collection procedure was completed the ova were transferred from the collection dish to the insemination dishes. The insemination procedure will be discussed later.

Superovulation Procedure

Donors that were superovulated received 0.5 mg/day of FSH (Armour-Baldwin Labs, Omaha, Neb.) divided into two doses given a.m. and p.m. for a total of six doses. The first injection was given approximately 72 hrs before the HCG was to be administered. All FSH injections were given subcutaneously in the nap of the neck. HCG was injected intravenously in a marginal ear vein at a dose level of 50-100 i.u. This superovulation regime has been described by Kennelly and Foote (1965).

Recovery of Periovarian Ova

Collection of periovarian ova was as follows: The donors were killed 11.75 hrs after HCG injection. The tracts were collected as above and the ovaries were dissected away from the other tissue. Ovaries were first collected in saline in a 150 ml beaker and then transferred individually to small Petri dishes containing either the

test medium or physiological saline. When examined under the dissecting microscope the ovulated ova are found adhering to the ovarian surface, surrounded by cumulus cells. A pair of extra fine dissecting forceps (Clay Adams) was used to remove the ova from the ovarian surface and they were then transferred to a collection dish containing the fertilization medium. After all ovaries had been examined and the ova collected the insemination procedure was begun.

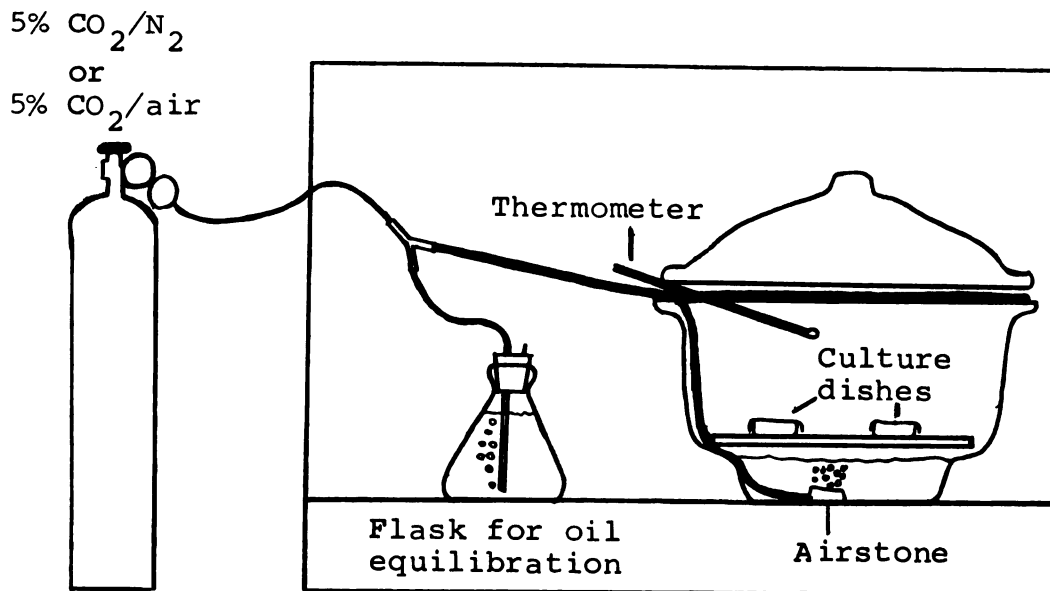
Sperm Recovery and Insemination Procedure

The capacitator does were mated at least three times to males of proven fertility. At the varying times after mating (determined by the objectives of the study) the animal was killed by cervical dislocation and the reproductive tract exposed by a midline incision. The uterine horns were clamped at the cervical end with a Carmolt forceps. Sperm were recovered by injecting 4 ml of the fertilization medium into the lumen and aspirating the contents. A 20 gauge needle and 5 ml syringe were used. In most cases 4 ml of medium was sufficient to distend the uterine horn so that sperm recovery was as complete as practical. If 4 ml were not recovered by aspiration the difference was made up with additional medium. The contents of the two uterine horns were collected in separate syringes. The sperm suspensions were placed in culture dishes (either 50 X 15 mm Petri dishes with covers or 30 X 13 mm sealable culture dishes--

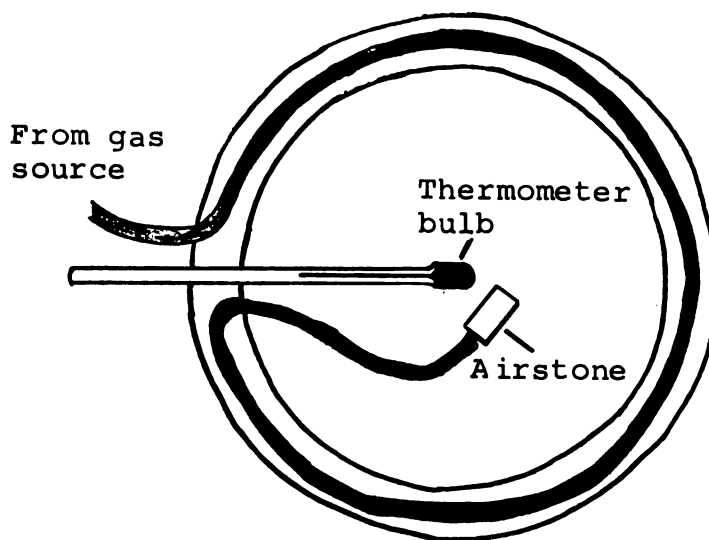
4340.C, A. H. Thomas and Co.) under oil. The oil had previously been gassed with 5% CO₂/N₂ or 5% CO₂/air. The ova were transferred from collection dishes to the culture dishes containing the sperm suspensions for insemination. Immediately after transferring the ova the dishes were placed into the incubator chamber.

Construction of Incubation Chamber

The function of the incubation chamber for in vitro fertilization studies is to maintain a controlled environment in which fertilization and development can take place. The medium in which the gametes are incubated provides the immediate environment but to prevent changes in this medium the incubator must control temperature, humidity, and atmospheric compositions. The incubator developed for use in these studies (Fig. 1) consisted of a desiccator (25 cm dia. at rim) inside a forced air oven (Hydor Therme Corp. Model 5522). The desiccator plate was perforated and the space below the plate contained water. A plastic tube, from an external gas source, entered the desiccator by going around the rim and down into the water. An aquarium airstone attached to the tube caused the gas to bubble into the water increasing the relative humidity. Leakage around the tubing was sufficient to allow excess gas to escape. The desiccator with water supply was kept in the oven between studies so that time was not required to equilibrate the incubation



Incubation chamber consisting of desiccator in thermally controlled oven



Overhead view of chamber

Figure 1. Construction of incubator.

temperature. The temperature during a study was monitored by a thermometer placed so that the bulb was in the center of the desiccator approximately 7.5 cm above the incubation dishes. The temperature was maintained at 37.5-38°C. Evidence of high humidity was demonstrated by condensation of water droplets on the desiccator.

Criteria of Fertilization

The criteria in the literature for in vitro fertilization have ranged from slight oval cytoplasmic changes to birth of live young. Chang (1959) suggested that normally appearing cleavage to the proper developmental state and either the presence of a second polar body or sperm in the perivitelline space was sufficient to determine that the ovum was fertilized. Brackett (1970) reported that under his best in vitro fertilization conditions the ovum should be at the 2 to 4 cell stage after 24 hrs incubation; this value agrees with Austin's estimate for in vivo fertilization and cleavage. Based on these reports the following criteria for in vitro fertilization were established: (1) after 24 hrs incubation the ova were examined at 45X under a stereoscopic dissecting microscope, (2) ova of 2 or 4 cells with equal size, normal appearing blastomeres were considered fertilized, (3) ova with 3 blastomeres or those with slightly irregularly shaped blastomeres were examined at higher magnification for the presence of sperm in the

perivitelline space or attached to the egg, if sperm were present the ova were considered fertilized, (4) ova that were questionable after the above examinations were considered not fertilized, (5) ova with only one cell 24 hrs after exposure to sperm were considered unfertilized. Throughout these experiments steps were taken to eliminate activation of the ovum or false positive results. These steps are discussed in the Results section.

All glassware, instruments and saline were sterilized by steam autoclave. The serum-containing media were sterilized by filtration. Initially a Seitz filter was used but it was cumbersome and later replaced by a Falcon 7102 unit with 0.45 micron pore size.

EXPERIMENTAL DESIGN

Throughout these experiments controls were used, as checks on the various aspects of the system. In all studies oviductal ova were recovered from the capacitors; these in vivo fertilized eggs served as controls on the suitability of the medium and the capacitation state of the sperm. Uterine contents were collected without sperm, in some studies, and ova were incubated in this aspermic medium to check on parthenogenic activation of the ova. Oviductal ova recovered from capacitor does where the utero-tubal junction had been ligated were incubated in the absence of sperm to determine if the ligation had been complete and as a check on parthenogenic activation.

If the objectives of the study allowed, steps were taken to reduce biological variation. When two or more donors were used all oviductal and all periovarian ova were pooled in two collection dishes as described previously. The ova were then transferred in approximately equal numbers to the incubation dishes. This pooling of ova prevented skewed results caused by one donor supplying possibly infertile eggs. When possible the sperm suspensions were distributed equally among the incubation dishes so that

approximately equal numbers of sperm were present in all dishes and so that any differences between sperm from the two uteri would be eliminated.

At the end of the incubation period ova were classified as either fertilized or not fertilized. This quantal response and the differences in ovum numbers between studies do not allow statistical analysis by analysis of variance, but do allow testing of many variables if adequate ovum numbers are used in each treatment.

The individual experiments in this research were conducted in such a manner that the conditions of one experiment were based on the results of the previous one. Throughout the series in vivo fertilized eggs were recovered 12 hrs after mating and incubated in vitro as changes in media or basic procedure were made. Incubation of these ova served to indicate if the changes were compatible with incubation conditions required for ova at this developmental stage. When the initial studies were unsuccessful the procedure was modified to identify the responsible factor; it was believed that the best system for in vitro fertilization would be a simple one with as few variables as possible. Table 1 indicates the factors involved in the development of the final system. Elaboration of the various changes will be given in the Results section.

Table 1--Footnotes

^aIn studies where both fertilization and incubation media were used the gametes were incubated for 4-5 hrs in the former and then transferred to the latter. The defined, 5% serum and 10% serum media were made up in acidic saline. The acidic saline contained, per liter: NaCl, 8.8 gm; KCl, 0.3 gm; CaCl₂, 0.25 gm; NaH₂PO₄ · H₂O, 0.121 gm; MgCl₂ · 6 H₂O, 0.542 gm; Penicillin G, 50,000 units. The defined medium was prepared by combining 62.5 mg glucose, 75.0 mg crystalline bovine serum albumin (BSA) and 77.6 mg NaHCO₃; made to 25 ml with acidic saline. The 5% and 10% serum media were composed of 250.0 mg glucose and 5 or 10 ml, respectively, of rabbit serum; made to 100 ml with acidic saline. The 20% serum medium was made using a modified acidic saline containing per liter: NaCl, 6.55 gm; KCl, 0.3 gm; CaCl₂, 0.25 gm; NaHPO₄ · H₂O, 0.113 gm; MgCl₂ · 6 H₂O, 0.106 gm; Penicillin G, 50,000 units. Immediately prior to use NaHCO₃, 155.2 mg; glucose, 125 mg; BSA, 150 mg were combined and made to 50 ml using modified acidic saline; 10 ml was withdrawn and 10 ml heated rabbit serum added.

^bThe gas used for equilibration with medium and/or oil and the gas phase maintained in the chamber during culturing, where two gas mixtures are listed the change took place at 4-5 hrs after incubation started.

^cAge is time after HCG or mating--given in hours. Ovulation occurs approximately 10 hrs after HCG or mating.

^dAll serum was heat-treated to eliminate heat-labile spermicidal factors (Chang, 1947) and only rabbit serum was used to avoid the lethal ovocidal factor found in some other species (Chang, 1949).

^eOva recovered from mated oviducts are in vivo fertilized, ova from super-ovulated donors are indicated as s.o.

TABLE 1
INITIAL EXPERIMENTS FOR IN VITRO FERTILIZATION

Media ^a		Culture		Ova		Sperm	
Fertilization	Incubation	Atmosphere ^b	Source	Age ^c	Age ^c		
1. Defined	10% Serum ^d	5% CO ₂ /N ₂	Oviduct	12.50	12.00		
2. Defined	10% Serum	5% CO ₂ /N ₂	Oviduct ^e (mated)	12.50	...		
3. Defined	10% Serum	5% CO ₂ /N ₂	Oviduct (s.o.) ^e	12.50	12.50		
4. Defined	10% Serum	5% CO ₂ /N ₂	Oviduct (s.o.)	12.50	12.75		
5. 5% Serum	10% Serum	5% CO ₂ /N ₂	Oviduct (s.o.)	13.00	12.75		
6. Defined	10% Serum	5% CO ₂ /N ₂	Oviduct (mated-s.o.)	12.50	...		
7. 20% Serum	10% Serum	5% CO ₂ /N ₂ -air	Periovarian (s.o.)	12.00	17.00		
8. 20% Serum 20% Serum	10% Serum ...	5% CO ₂ /N ₂ -air	Oviduct (mated)	12.00	...		
9. 20% Serum	...	5% CO ₂ /N ₂ -air	Oviduct Periovarian	11.75	17.00		
10. 20% Serum	...	5% CO ₂ /N ₂ -air	Oviduct Periovarian	11.75	17.00		
11. 20% Serum	...	5% CO ₂ /N ₂ -air	Oviduct Periovarian	11.75	17.50		

RESULTS

Development of an In Vitro Fertilization System

The results of the in vitro fertilization studies listed in Table 1, with the exception of study number 11, are presented in Table 2. Only one ovum of 100 incubated was determined to be fertilized. When examined 24 hrs after being placed in the sperm suspension that one ovum was in the 4 cell stage. The in vivo fertilized in vitro cultured eggs used as controls on the suitability of the system were examined for uniform cleavage, the results are presented in Table 3. Although these egg numbers are not great they were considered sufficient to test the developing system.

A schematic diagram of the established system developed in these studies is presented in Figure 2. The results of four studies conducted under the conditions of the developed system are given in Table 4. Only a small difference between the fertilization rates of periovarian ova and the ova recovered from the oviducts (56% vs 50%) was noted. The results of these studies indicated that the system was sufficiently reliable for use in investigating various aspects of fertilization and capacitation. In these studies and in all of the following in vitro fertilization studies

TABLE 2
SUMMARY OF PRELIMINARY IN VITRO FERTILIZATION STUDIES

Media	Source of ova	No. of ovaries/ oviducts	No. of ova	No. fertil- ized	% fertil- ized
5%--10% ^a	oviductal	4	19	0	0
Defined--10% ^b	oviductal	10	23	0	0
20%--10% ^c	oviductal	4	14	0	0
20% ^d	oviductal	10	26	0	0
20% ^d	periovarian	10	18	1	5.5

^aTransferred after 4.5 hrs from 5% serum to 10% serum.

^bTransferred after 4.5 hrs from defined medium to 10% serum.

^cTransferred after 4.5 hrs from 20% serum to 10% serum.

^dCultured for 24 hrs in 20% serum.

TABLE 3
IN VITRO CULTURE OF IN VIVO FERTILIZED RABBIT OVA

Media ^a		No. of ova	No. ova cleaved	% cleaved
Fertilization	Incubation			
Defined	10% Serum	12	7	58.3
20% Serum	10% Serum	3	1	33.3
20% Serum ^b	...	3	3	100.0

^aWhen two media are listed the ova were transferred from the first to the second 4.5 hrs after being placed in the incubation chamber.

^bThe ova that were incubated in 20% serum medium were left in the same solution for the entire incubation period of 24 hrs. The atmosphere maintained in the chamber during the incubation period was 5% CO₂/N₂. The ova were recovered from the oviducts of mated female rabbits 12.0-12.5 hrs after mating.

TABLE 4
IN VITRO FERTILIZATION OF RABBIT OVA IN
 ESTABLISHED TEST SYSTEM^a

Source of ova	No. of ovaries/ oviducts	No. of ova	No. fertilized	% fertilized
Periovarian	11	136	76	56
Oviductal	12	34	17	50
Capacitator	6	22	22	100

^a20% serum for 24 hrs. Atmosphere 5% CO₂; 95% air for 5 hrs--air for remainder of culture period. ²Uterine sperm recovered 17 hrs after mating.

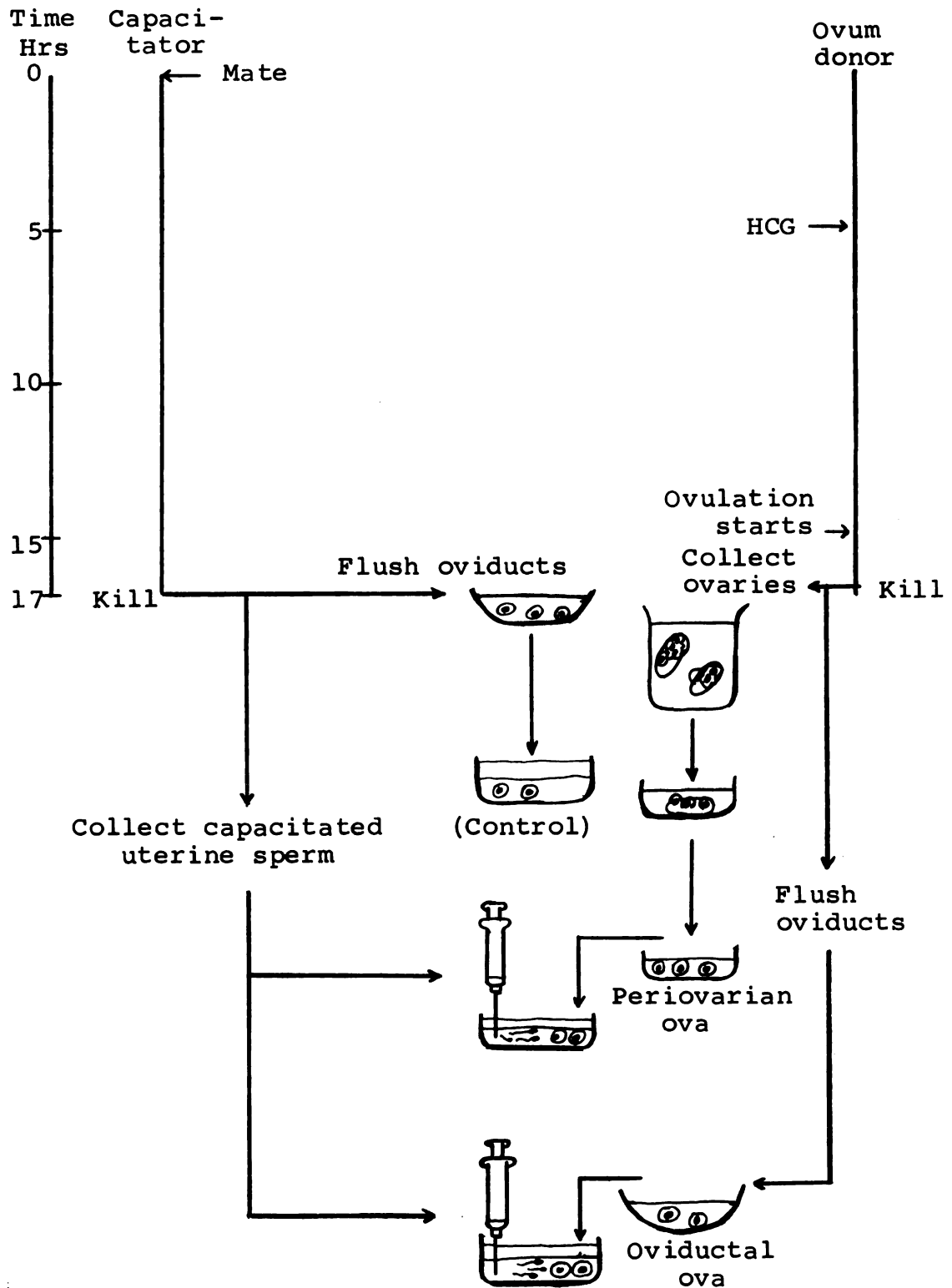


Figure 2. Schematic diagram for recovery of gametes for in vitro fertilization.

the 20% serum medium was used for recovery of sperm and for incubation of the gametes.

Preliminary Studies Concerning
Oviductal-Uterine Relationship
to Capacitation

Bedford (1969) reported that rabbit spermatozoa do not become fully capacitated in the rabbit uterus until 11 hrs after mating and that this time requirement was extended to 15 to 16 hrs if the uterus and oviduct were separated at the utero-tubal junction. In preparation for studying this oviductal-uterine relationship several preliminary studies were conducted. The first studies investigated the fertilizing ability of uterine sperm recovered 11 hrs after mating compared to those recovered 17 hrs after mating. These data are presented in Table 5 and indicate that 17 hr sperm have a slightly higher fertilization rate than 11 hr sperm. This confirms the report of Brackett (1969b). The difference between the fertilization rate of periovarian ova and oviductal ova is even more pronounced in these studies than in those reported in Table 4 and certain theories concerning this difference were considered; these ideas will be discussed later.

TABLE 5

IN VITRO FERTILIZATION OF RABBIT OVA; 11 HOUR UTERINE
SPERM COMPARED TO 17 HOUR UTERINE SPERM

Age of sperm ^a	Source of ova	No. of ova	Ova fertilized ^b	
			No.	%
11 hours:	Periovarian	30	8	26.7
	Oviductal	23	1	4.3
	Capacitator	9	2	22.2
17 hours:	Periovarian	198	92	46.5
	Oviductal	92	20	21.7
	Capacitator	45	24	53.3

^aAge of sperm is the period of time between mating and recovery of the sperm from the uteri.

^bCapacitator ova were determined to be fertilized by the appearance of uniform cleavage. Fertilization of ova from other sources was determined as described in the text.

Effects of Mineral Oil

The overall in vitro fertilization rates presented in Table 5 are considerably lower than those presented in Table 4. These results were obtained from studies using different pools of serum and acidic saline. During these studies it was noted that an interface membrane-like structure was present between the oil and medium after the 24 hr incubation period. The interface was more evident when a probe was moved through it to examine the ova. Samples of mineral oil used in our studies were compared to bottles of mineral oil with different lot numbers and to unopened bottles of the same lot number. When these samples were placed over medium aliquots and incubated under established incubation conditions it was found that certain lots formed the interface while others did not. Several studies were conducted using the "bad" (interface-forming) mineral oil, Table 6. The fertilization rate of all ova were decreased; those that had been exposed to the oviductal environment were reduced to a greater extent (5%) than the periovarian ova (30%). Only mineral oil which did not form the interface was used in later studies.

Uterine-Oviductal Relationships to Fertilization

To study Bedford's theory that the isolated uterus requires a longer period of time to capacitate sperm than the intact uterus certain surgical preparations were required.

TABLE 6

IN VITRO FERTILIZATION OF RABBIT OVA; THE EFFECTS OF
INTERFACE-FORMING MINERAL OIL ON THE
FERTILIZABILITY OF OVA^a

Source of ova	No. of ova	Ova fertilized ^b	
		No.	%
Periovarian	56	17	30.4
Oviductal	58	3	5.2
Capacitator	23	2	8.7

^aThe sperm used throughout this table were recovered from the uteri 17 hrs after mating.

^bFertilization determined as described in Table 5.

One utero-tubal junction in each test capacitator was double ligated and transected between the ligations. After at least 3 weeks isolation the surgically prepared rabbits were mated and uterine sperm were collected approximately 11 hrs later. The data presented in Table 7 indicate that, although the percentage of ova fertilized may not be as great, the sperm recovered from the uterus 11 hrs post coitum are capacitated even if the utero-tubal junction is severed. This confirms Brackett and Server's (1970) work.

TABLE 7

IN VITRO FERTILIZATION OF RABBIT OVA; EFFECTS OF
UTERO-TUBAL LIGATION ON CAPACITATION ABILITY
OF RABBIT UTERI 11 HOURS POST COITUM

Source of sperm ^b	Source of ova	No. of ova	Fertilized ^a	
			No.	%
Normal uterus	Periovarian	132	32	24.2
	Oviductal	12	2	16.7
	Capacitator	13	9	69.2
Isolated uterus ^c	Periovarian	175	28	16.3
	Oviductal	45	3	6.7
	Capacitator	13	0	0.0

^aCapacitator ova were classified as fertilized if they were normally cleaved.

^bSperm recovered from the uteri 10.92 hrs after mating (range 10.30-11.50).

^cOne utero-tubal junction in each capacitator was transected between double ligations. The animals were isolated for at least 3 weeks following the surgery before being mated.

The Oviductal Environment and Fertilization

In examining the above data it was noted that ova exposed to the oviductal environment appear to be less fertilizable than the ova recovered from the ovarian surface. The data presented in Table 8 combine the data obtained from all studies conducted using the developed system. Based on the observed differences between the fertilization rate of the ova from the two sources it was decided to conduct a study on the effects of oviductal fluid, collected by an intra-abdominal flask (Hamner and Williams, 1963, 1965), on the fertilization rate of periovarian eggs.

TABLE 8

IN VITRO FERTILIZATION OF RABBIT OVA; PERIOVARIAN OVA COMPARED TO OVIDUCTAL OVA^a

Source of ova	No. of ova	Ova Fertilized ^b	
		No.	%
Periovarian	408	149	36.5
Oviductal	139	27	19.4

^aThese data were obtained from studies using sperm recovered from normal uteri at either 11 or 17 hrs after mating. Data from the studies using interface-forming mineral oil are included.

^bFertilization was ascertained as described in Table 5.

Ova were recovered from the ovarian surface and incubated in either oviductal fluid--20% serum medium (1:1) or saline--20% serum medium (1:1) for 30 mins. Following this initial incubation the ova were transferred to culture dishes containing 17 hr sperm suspension and the fertilization and incubation system followed. The results of this study are presented in Table 9.

The cleavage rates for capacitator ova have not been included in all the tables, nor have the rates for donor eggs incubated in aspermic media. With the exception of those studies conducted with the "bad" mineral oil the in vivo fertilized ova cleaved to the 4 to 8 cell stage in all studies included in the data presented above. No normal appearing ova have been found when incubated in medium without sperm. These controls indicate that parthenogenic activation did not occur with these techniques and that the system was suitable for studying early zygote development.

TABLE 9

IN VITRO FERTILIZATION OF RABBIT OVA; EFFECTS OF
OVIDUCTAL FLUID ON THE IN VITRO FERTILIZATION
 OF RABBIT PERIOVARIAN OVA

Treatment	No. of ova	Ova fertilized ^a	
		No.	%
Saline ^b	6	2	33.3
Oviductal fluid ^c	12	3	25.0
Capacitator ^d	7	7	100.0

^aFertilization ascertained as described in Table 5.

^bIncubated for 0.5 hrs in a mixture of saline--20% serum medium (1:1, v:v) prior to transfer to sperm suspension for fertilization.

^cIncubated for 0.5 hrs in a mixture of oviductal fluid--20% serum medium (1:1, v:v) prior to transfer to sperm suspension for fertilization.

^dCapacitator ova recovered from the oviducts 17 hrs after mating. These ova are in vivo fertilized and their normal cleavage is a control of the medium and incubation conditions.

DISCUSSION

The preliminary studies listed in Table 1 give only an indication of the evolution of the final system from the system used initially. Attempts were made at first to maintain a high level of relative humidity in the hood while the gametes were being collected and prepared for insemination. However, slight increases in humidity made it more difficult to control temperature in the hood, and Dr. P. Soupart (personal communication) suggested that temperature was more critical than humidity when working with the gametes. Based on this advice attempts to control humidity were stopped and temperature control considered more critically. By directing a stream of air from a forced air electric dryer over a small shielded Bunsen burner temperature in the area where the work was conducted was maintained at $38^{\circ}\text{C} \pm 1^{\circ}\text{C}$. A thermometer placed in the working area was used to monitor temperature throughout the time period when gametes were in the hood.

The preliminary studies represent an attempt to develop a simpler system that would give reliable results. The first studies (1) required a transfer from either 5% serum or defined media after 4.5 hrs to 10% serum medium,

(2) attempted to maintain high humidity in the hood, and (3) used an atmosphere of 5% CO₂/N₂ for gassing the oil, media and chamber. The system eventually developed (1) required only one medium, 20% serum, with no transfers, (2) controlled only temperature in the hood, and (3) used 5% CO₂/air for gassing the oil and the chamber for the first 5 hrs. The procedure for recovering ova became more complicated, from oviductal alone, to periovarian and oviductal, but this was considered a significant development worth the additional effort. The time interval from mating to recovery of sperm was increased from 12-13 hrs to 17-18 hrs based on earlier results.

Fertilization of periovarian ova, which have never been exposed to the oviductal environment, allows the in vitro system to be used to study hypotheses that cannot be tested if only oviductal ova are used. This periovarian source of ova has been reported recently by Seitz, Brackett and Mastroianni (1970). These workers removed the fimbriae and part of the ampulla 9.5 hrs after HCG and then removed the ovaries at 11.5 hrs. The ova were incubated with sperm recovered from the uteri 12 hrs after mating. They reported 117 ova cleaved out of 143 recovered for an 81% fertilization rate compared to 70 of 130 (56%) in our studies, Table 4. Earlier Brackett (personal communication) mentioned that a difference had been noted between the oviductal eggs and those recovered from the ovarian surface but he has not

conducted any experiments similar to the one reported in Table 9 to determine what this difference in fertilization rates might be related to.

The results presented in Table 8 show that periovarian ova are fertilized at a higher rate compared to oviductal ova when used in an in vitro fertilization system.

There are three possible explanations for this difference:

(1) the oviductal eggs were the first ones ovulated and are therefore older and less fertile, (2) the technique used in recovering the oviductal ova is more traumatic to the ova and reduces their ability to be fertilized and (3) the exposure to the oviductal environment makes the ova more sensitive to environmental changes and since the in vitro system does not duplicate the in vivo environment exactly they are fertilized at a reduced rate. If the first reason is correct one would expect that in vitro fertilization rates of 80% or more would be practically impossible when using 12 hr ova, as Harper (1961) showed that 50% of the ovulations occurred by 10.5 hrs after LH injection. If the rabbits are superovulated the ovulation may proceed slightly slower (Ishijina, Ito, Hirabayashi and Sakuma, 1969) but one would still not expect the results reported by other workers using ova recovered from the oviducts 12 hrs after LH since the majority of the ova would be at least 1.5 hours past ovulation. In examining the second theory it is difficult to believe that flushing ova from the oviduct is more traumatic

than picking them from the ovarian surface. The data presented in this thesis supports the third theory. In the studies conducted using "bad" mineral oil, the difference between periovarian ova and oviductal ova is more pronounced (Table 6). Even the capacitator ova, which were fertilized in vivo and exposed to the oviduct for approximately 7 hrs, failed to cleave at the rate found in other studies. This suggests that the two groups of ova exposed to the oviduct prior to incubation were more sensitive and more easily affected by the "bad" mineral oil--induced changes in incubation conditions.

It is unclear, at this time, what the causal factor(s) in oviductal fluid might be. To influence the ova recovered from the oviduct in the manner described above would require several limiting restrictions. The factor must express itself in the oviduct, must influence the ova in a relatively short time and finally should be related to plasma proteins or fractions thereof. This last restriction is suggested by the results obtained using interface-forming mineral oil. According to Dr. Ralph Brinster (personal communication) the interface is formed by protein being extracted from the medium. The oviductal ova when incubated in the medium with the protein content thus reduced are less likely to be in vitro fertilized compared to periovarian ova in the same medium. One possible explanation for this difference may be that heterosynthetic proteins have been removed from the oviductal ova.

It has been demonstrated in the mouse (Glass, 1969) that macromolecules presynthesized by the mother are transferred intact into the follicular oocyte. These heterosynthetic proteins are still present in the ooplasm of the ovum at the time of ovulation but could not be detected 2 or 3 hrs after ovulation. The work reported by Glass indicated that these proteins are native serum antigens as well as antigens to foreign protein injected into the maternal organism.

It would require a great deal of research to determine if the disappearance of these heterosynthetic proteins is related to the reduced fertilization rates of ova recovered from the oviducts. Several possible theories may be advanced to explain the relationship. If antigens are removed from the ooplasm the ovum may be less likely to complex with proteins in the medium with a structural fault in the ovular membrane. This idea is very general and would be difficult to demonstrate. Another possible explanation concerns differences in osmolarity. If proteins are removed from the ooplasm and the ovum is then transferred to hypo-osmolar medium, due to reduced protein content, a number of effects may be noted. The lower colloid osmotic pressure may chemically alter the membranes of the ovum preventing fertilization, or cleavage even if the ovum was already fertilized, i.e., 17 hr capacitator ova.

The function of these heterosynthetic proteins in the oocyte has not been determined. They may be related to the acceptance of the ovum in the female tract after fertilization. The inability of Glass to detect them 2 hrs after ovulation and the decreased fertilization rate of ova recovered from the oviduct may be related. A definite conclusion can not be stated at this time.

Dr. Brinster suggested that the oil either be checked in a manner similar to the method described earlier or that silicone oil (Dow Corning 200 Fluid) be used. In the last studies conducted in this work silicone oil was used and results were encouraging.

An in vitro fertilization system can be used to study various aspects of capacitation. The uterine-oviduct relationship studies indicate that Bedford's conclusion is incorrect. Sperm will become capacitated in the ligated uterus in less than the 15 hrs he reported. The data reported here show that sperm recovered at 11 hrs post coitum were capacitated; this confirms studies by Dukelow (1970) using an in vivo assay system. Studies similar to the ones reported above can be conducted to determine the time intervals required for capacitation more accurately than can be done using in vivo assay systems alone. In vitro fertilization of ova can also be used to assay decapacitation factor. By using an in vitro system for

capacitation assays, the possibility that changes take place during in vivo assay can be eliminated.

The uses of an in vitro fertilization system for studying the action of various pharmacologic agents are numerous. Using the techniques described one can study the effects of compounds on (1) the developing oocyte prior to ovulation, (2) the oviductal environment, (3) the action on sperm in the male and female tracts, and (4) the influence of oviductal fluid changes on the early zygote as well as the fertilization process itself. By recovering periovarian ova from one ovary and allowing ova from the other ovary to pass into the oviduct one can study the effects of compounds on the ova when the action is mediated through the oviduct. In general, the ability to in vitro fertilize ova that have been naturally ovulated but have not passed into the oviduct means that such a system can be used to test areas not available before.

Some media changes probably would be required before the system used in these studies could be used to fertilize primate eggs but the basic techniques and equipment would be readily adaptable. The in vitro fertilization system offers the most logical method of determining the need for, and the capacitation time requirements, in primates. By use of the laparoscope ova can be recovered from the human ovary (Steptoe and Edwards, 1970). This technique used in non-human primates would provide a source of ova for in vitro

studies. There are indications that the time of ovulation can be closely estimated in nonhuman primates using the laparoscope (Jewett, 1971) so in vitro maturation of the ova would not be required prior to insemination. The possible uses of an in vitro fertilization system for studying problems in human infertility have been mentioned earlier. Oviductal occlusions and abnormalities in the oviductal environment, may both be circumvented by in vitro fertilization and transfer of the zygote into the uterus.

A number of determinations concerning reproductive parameters have been made using in vivo systems in the rabbit. Some of these, like the fertilizable life of the ovum, have not been confirmed in vitro. Such confirmation can be made using periovarian ova.

Throughout these studies it has been apparent that, regardless of the cooperation between investigators it is difficult to duplicate work from different laboratories. In an area with as many variables and critical controls as in vitro fertilization of mammalian ova, slight differences in techniques become very important. A great deal has been learned concerning in vitro fertilization of mammalian ova in the last decade. There is still a great deal to study concerning medium composition and incubation conditions before any system can be considered standardized. While the fertilization rates obtained with the system in this

research are not as high as reported by some others, it is believed that the development of a simpler system that is reliable enough to use as a tool for studying areas of reproduction is significant.

SUMMARY AND CONCLUSIONS

A reliable simplified system for the in vitro fertilization of rabbit ova was developed and used as a research tool for the studying of oviductal influences on spermatozoa capacitation and ova fertilizability. The following conclusions were indicated by the data obtained:

1. periovarian ova recovered from the ovarian surface following ovulation had a higher rate of fertilization compared to ova recovered from the oviducts;
2. uterine sperm recovered 17 hrs after mating fertilized ova at a rate greater than that obtained with uterine sperm recovered 11 hrs after mating;
3. exposure to the oviductal environment appeared to make ova more susceptible to environmental changes, lowering the fertilization rate when less than ideal incubation conditions were used;
4. a patent utero-tubal junction is not necessary for capacitation to occur in the uterus in less than 11 hrs following mating.

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APPENDIX A

PUBLICATIONS BY THE AUTHOR

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Full Papers: Megestrol Acetate: Its Effects on the Inhibition of Ovulation in Squirrel Monkeys (Saimiri sciureus), by Richard M. Harrison and W. Richard Dukelow. Journal of Reproduction and Fertility, 1971. (Accepted for publication.)

Laparoscopic Examination of the Ovaries in Goats and Primates, by W.R. Dukelow, S.J. Jarosz, D.A. Jewett, and R.M. Harrison. Laboratory Animal Science, 1971. (Accepted for publication.)

Abstracts: Induced Ovulation in the Nonhuman Primate.

Oviductal-Uterine Relationships to Sperm Capacitation.

Laparoscopic Examination of Ovulation.

Follicular Morphology and Ovulation Induction in the Nonhuman Primate.

Follicular Morphology Near Ovulation in Macaca Fascicularis.

See following pages for text of abstracts.

APPENDIX B

ABSTRACTS

INDUCED OVULATION IN THE NONHUMAN PRIMATE¹

R.M. Harrison and W. Richard Dukelow²
Michigan State University

Ovulation induction at a prescribed time was studied in squirrel monkeys. Ovulation was induced by IV or IM injection of HCG after progesterone pretreatment and various FSH sources. Superovulation was induced by the method of Bennett (J.R.F. 13:357, 1967). Forty-three percent of the animals ovulated within 12 hr post-HCG, 4 with double ovulations and the rest single. Superovulated animals averaged 4.8 ovulations. FSH was superior to PMS or HMG in promoting follicular development. Twice as many animals ovulated with 500 i.u. HCG as with 250 i.u. No difference was noted between IM or IV administration of HCG. Ovulation occurred later but with less variation in IM injected animals. Subcut. injection of 50, 100, or 250 µg of megestrol acetate (Mead Johnson & Co.) daily concomitant with the FSH resulted in 71.4, 37.5 and 20.0% ovulation when examined 40 hr post-HCG. Preliminary studies in galagos using the above procedure resulted in ovulation but it occurred later than in squirrel monkeys.

¹Presented at the Fed. of Amer. Soc. for Exp. Biol. Meeting, Atlantic City, N.J., April 12-17, 1970.

²Endocrine Research Unit (Ctr. Lab. Animal Res., Depts. An. Hus. & Physiol.).

OVIDUCTAL-UTERINE RELATIONSHIPS
TO SPERM CAPACITATION¹

R.M. Harrison, M.P. Johnson and W.R. Dukelow²
Michigan State University

An in vitro system for the fertilization of rabbit ova was used to study the influence of the oviduct on fertilization. Ova recovered from superovulated does were incubated with sperm from the uterine horns of mated does. The incubation medium was modified acidic saline containing 20% heated rabbit serum. Gametes were incubated at 38°C for 24 hr under a humid atmosphere of 5% CO₂/air. Ova recovered from the ovarian surface 11.75 hr after HCG fertilized at a rate of 56% compared to 50% for ova recovered from the oviduct. Uterine sperm recovered 17 hr after mating fertilized 37% of the ova compared to 30% for sperm recovered 11 hr after mating. Uterine sperm recovered 11 hr after mating from a ligated uterine horn fertilized 24% of the ova compared to 35% using nonligated uterine sperm. With in vivo studies, 8 and 10 hr sperm from ligated uterine horns fertilized 67 and 70% of 4 hr old ova, respectively. These

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²Endocrine Research Unit (Ctr. Lab. Animal Res., Depts. An. Hus. & Physiol.).

studies suggest that exposure to the oviduct makes the ova more susceptible to changes in environmental conditions but that the oviduct is not essential for capacitation to occur in 11 hr or less.

LAPAROSCOPIC EXAMINATION OF OVULATION¹

W. Richard Dukelow, Ph.D., S.J. Jarosz, Ph.D.,
D.A. Jewett, B.S. and R.M. Harrison, B.A.²

Determining the exact time of ovulation in domestic and laboratory animals has long been a problem in reproductive studies. Recently we have been using the laparoscope with good success to overcome this problem. Using the 135° pediatric laparoscope we have successfully examined the ovaries of rabbits, rats, pygmy goats, Toggenburg goats, guinea pigs, galagos, squirrel monkeys and Java monkeys. Observations have been made as frequently as every 4 hr for 32 hr in pygmy goats without excessive irritation or peritonitis. Light tranquilization and a local anesthetic are required. A second entry tactile probe allows manipulation of the organs for observation. Distention of the abdominal cavity with CO₂/air is essential for good observation. Using this technique it has been possible to time ovulation within 1 hr in over 60% of our monkeys and goats examined. The laparoscope offers an excellent tool for examination of internal physiology function with minor disturbance to the animal.

¹Presented at the 21st Ann. Session Amer. Assoc. for Lab. Anim. Sci., Chicago, Ill., November 2-6, 1970.

²Endocrine Research Unit (Ctr. Lab. Animal Res., Depts. An. Hus. & Physiol.).

FOLLICULAR MORPHOLOGY AND OVULATION
INDUCTION IN THE NONHUMAN PRIMATE¹

W.R. Dukelow, R.M. Harrison & D.A. Jewett²

Thirty-one female Macaca fascicularis were examined by laparoscopy to evaluate ovarian morphology and follicular development near ovulation. Generalized swelling and darkening of the ovary characterized the developing follicle 24 hr prior to ovulation; a specific pattern of blood vessels associated with the follicle developed 8 hr prior to ovulation. Deterioration of vasculature and occlusion of previously clear areas on the follicular membrane were evident 24 hr after ovulation. Based on these examinations precise mating trials were carried out. Four pregnancies were obtained, and two were allowed to go to term with gestation lengths of 164 days, 15.5 hr and 165 days, 10.8 hr.

Laparoscopy was also used to ascertain the efficacy of a Saimiri sciureus ovulation induction scheme consisting of 5 days of progesterone (5 mg); 4 days of FSH (1 mg); and a final injection of 250 i.u. HCG. This scheme was used to study the effectiveness of various contraceptive agents in

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blocking induced ovulation. Such ovulation was completely blocked with 500 mg megestrol acetate. The ovulation scheme was also effective in Galago crassidaudatus.

FOLLICULAR MORPHOLOGY NEAR OVULATION
IN MACACA FASCICULARIS

D.A. Jewett, R.M. Harrison, M.P. Johnson
and W.R. Dukelow²
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In the course of 189 laparoscopic examination of 31 female Cynomologous Macaques (Macaca fascicularis), ovarian morphology was observed and photographed for the purpose of determining the characteristics of pre- and post-ovulatory follicular development. Serial observations were begun on day 10 of the menstrual cycle and continued for 6 days at intervals varying from 6 to 24 hours. Generalized swelling and darkening of the ovary at the site of the developing follicle could not be identified until 20 to 24 hours prior to ovulation. The presence of a relatively large, coiled vessel near the follicular cone, smaller vessels around the base, and a single small vessel transecting the follicular membrane were found to be the most reliable indications of immediate preovulatory development, and were not found earlier than 8 hours prior to ovulation. The most significant post-ovulatory morphological changes were the diffusion of the small vessels at the base of the follicular cone,

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²Endocrine Research Unit (Ctr. Lab. Animal Res., Depts. An. Hus. & Physiol.).

the occlusion of clear areas in the follicular membrane, and flattening or irregularity in the follicular dome. These changes were clearly evident 10 to 24 hours following ovulation; and, therefore, the corpus hemorrhagicum can be easily distinguished morphologically from the pre-ovulatory follicle. This investigation demonstrated that the time requirement for the formation of the follicular dome and related vasculature is not more than 20 hours; that ovulation can be diagnosed by laparoscopy; and that comparative follicular morphology during the late stages of development will permit future accurate prediction of ovulation time by a single laparoscopic examination.

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