In Vitro Cultivation of Rabbit Ova Following
In Vitro Fertilization in Tubal Fluid

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In vitro fertilization and cultivation of mammalian ova has been singled out as one of
the most challenging and frustration research projects in the field of reproductive biology.
According to Austin (1961a, 1963), in spite of numerous attempts to fertilize mammalian
ova in vitro, valid reports have appeared only sporadically. However, the cultivation of
mammalian ova under conditions of various media have been reported to be successful
since the pioneering works of Long (1912), Brachet (1913), Lewis and Gregory (1929), Pincus
(1930), and Nicholas and Hall (1942). Most of the cultivated ova were started from two or
more cell stages, and few attempts to cultivate fertilized ova following in vitro fertilization
have been reported.

In a previous experiment, Suzuki and Mastroianni (1965) have reported that rabbit ova
can be fertilized in vitro in rabbit tubal fluid. A total of 337 ova were inseminated in vitro
in rabbit tubal fluid under mineral oil using capacitated spermatozoa recovered from the
uterus 12 hours after mating. The fresh tubal fluid was collected from ligated rabbit
oviducts, diluted with Waymouth’s medium and, for 124 of these, the mineral oil was pre-
treated with 5% carbon dioxide in air. After four hours, ova were transferred to fresh
tissue culture medium with added 10% rabbit serum and cultured for 18-20 hours. Ova
were examined for evidence of sperm penetration, pronuclear formation, polar bodies, and
cleavage. The fertilization rate was 30.9% without prior carbon dioxide equilibration and
63.7% when the diluted, carbon dioxide treated, oil was used.

The purpose of the present investigation was to extend this previous work by cultivat-
ing these fertilized rabbit ova in vitro to more cell stages in suitable conditions.

Materials and methods

New Zealand White rabbits weighing 3 to 5 kg were used to collect tubal fluid. The
oviducts were ligated just proximal to the fimbria and at the uterotubal junction. Only
non-bloody, clear fluid accumulated between the ligatures was used after 4-5 days. An
estrous rabbit was mated two or three times with fertile bucks. After 12 hours, fluid
containing spermatozoa was aspirated from the uterine cavity. In most cases, 0.1-0.2 ml
of slightly turbid uterine fluid containing progressive, motile spermatozoa was recovered.
One drop of uterine fluid was placed on a sterile watch glass and immediately mixed with
2-3 ml of fresh tubal fluid. This suspension was placed in a tuberculin syringe at 30-37°C
for a few minutes before use. Ova were recovered from two does, injected 12 hours pre-
viously with 100 I.U. of chorionic gonadotropin, by flushing the oviducts with fresh tubal
fluid which had been warmed to 30-37°C. The ova, in cumulus, were transferred to de-

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pression slides with a fine pipette, and approximately three times the volume of sperm suspension was added. After thorough mixing, this suspension was covered with warmed mineral oil and incubated at 37-38°C. The mineral oil had been first mixed with sterile culture medium in a ratio of 20 to 1, and the mixture equilibrated with 5% carbon dioxide in air before use. After four hours, ova were gently washed in a depression slide with tissue culture medium (36-37°C) and placed in groups of 5-7 in a small Carrel flask containing 1ml of a medium consisting of 90% Medium 199 (Hyland Laboratory) and 10% rabbit serum and added lactic acid at 1.0 mg/ml of medium. Antibiotics were not included. The cultures were incubated at 37°C in a humid atmosphere of 5% carbon dioxide in air. Throughout this experiment, aseptic precautions were used, and all glassware was sterilized and warmed before and during use.

Individual ova were placed in center of four petroleum jelly spots on a slide. A coverslip was placed over each ovum and gently pressed down until structures within the ovum were clearly visible under the phase-contrast microscope (Chang 1955, Ohnuki 1959).

Results and discussion

The difficulty of settling on suitable criteria for in vitro fertilization has been stressed by previous investigators. Also, it has been found that the mammalian ovum is easily activated to various degrees by modifying the thermal, osmotic, and chemical factors in its environment. However, Smith (1949) has observed that when ova are incubated with scrapings of fallopian tube mucosa, cleavage of unfertilized rabbit ova in culture occurs much less frequently. In the present experiment, a second polar body, sperm in the perivitelline space, and normal cleavage were used, all together, as criteria for fertilization. In addition, representative ova believed to be fertilized in vitro have shown the same ultrastructural detail as seen in ova fertilized in vivo.

Table 1. Cleavage intervals of successful cultivation of the in vitro fertilized rabbit ova

<table>
<thead>
<tr>
<th>No. of experiment</th>
<th>No. of cultivated ova</th>
<th>Final cleavage stages of development of fertilized ova (about 70 hrs. after in vitro insemination)</th>
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<tr>
<td></td>
<td></td>
<td>−4 cell</td>
</tr>
<tr>
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<tr>
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<td>9</td>
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</tr>
<tr>
<td>10</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>65</td>
<td>16</td>
</tr>
</tbody>
</table>

A total of 207 ova were inseminated in vitro in rabbit tubal fluid under mineral oil using capacitated spermatozoa recovered from the uterus 12 hours
after mating. When the ova were examined, about 20 hours after the transfer for cultivation, 123 (59.4%) of them were at the 2- or 4-cell stages, and they were classified as normally cleaved and definitely fertilized ova. Sixty-five of the fertilized ova were cultivated for two or three days after insemination in vitro. Details of the cleavage intervals of successful cultivation are given in Table 1. Fourteen (21.5%) of the 65 ova could be reached at over the 16-cell stage.

A suitable environment is essential, both for the fertilization of ova in vitro and storage in culture for longer periods. Unfortunately, a medium which well supports the fertilization in vitro has not always been found.

Inasmuch as fertilization normally occurs within the fallopian tube, the effect in vitro, of tubal secretions is of some interest. In recent years various important components in tubal fluid have been studied biologically and biochemically by several groups of workers. The relatively high success rate attained in the previous and present series suggest that tubal fluid contains all of the ingredients prerequisite for fertilization.

A number of media listed by Austin (1961b) have been used for culture of mid-cleavage ova so as to permit further development in vitro, and many media have proved successful. The first important improvement in culture techniques for early ova was that of Whitten (1956−1957), who cultivated mouse ova in saline, glucose, and egg albumin for a period of 48 hours, securing development up to the blastocyst stage from the 8-celled stage, and then he added calcium lactate or sodium lactate to the medium. More recently, he has attained more success with two-celled ova by incorporating L(+)-
lactic acid. McLaren and Biggers (1958) have successfully transferred mouse ova cultivated for a period of 48 hours in simple, chemically defined media. Purshottam and Pincus (1961) reported the study of rabbit and mouse ova cultivated in certain synthetic media and concluded that fertilized rabbit and mouse ova could be cleaved up to the morula stage in Eagle's basal medium without serum and grown up to the blastocyst stage with 10% dialyzed horse serum. Brinster (1963) and Gwatkin (1963) used a modified Krebs-Ringer bicarbonate solution containing sodium lactate and crystalline bovine albumin, and several thousand two-celled mouse ova were cultivated successfully in vitro into normal blastocysts. Recently, Mintz (1964), in the mouse, has successfully used a medium consisting of 50% fetal calf serum and 50% Earle's balanced salt solution with lactic acid. Lutwak-Mann (1962) demonstrated that tubal and uterine fluids contained significant amounts of bicarbonate. Indeed, lactic acid, bicarbonate, and pyruvate are important factors for the early development of fertilized ova (Vishwakarma 1962 and Braden 1963). Brinster (1965a, 1965b, 1965c) has reported that energy for development of two-celled mouse ova could be supplied by lactate, pyruvate, oxaloacetate, or phosphoenolpyruvate.

Alternating the environmental conditions of developing zygotes may also effect the rate of cleavage. In vivo, rabbit ova can usually develop to the stage of blastocyst in about 70 hours after mating. But, in the present experiment, the cleavage rate was slightly delayed. If various thioamino acids are added to the medium, cell division of rabbit zygotes in vitro has been shown to proceed normally (Pincus 1937, Pincus and Werthessen 1938, Miller and Reimann 1940). However, Medium 199, which we used in this experiment, is a Hanks' balanced salt solution enriched with many kinds of amino acids, various vitamins, and several other ingredients. In addition, lactic acid, which has been proved to be one of the most important exogenous energy sources, was included in the medium.

It was impressed that delay in cleavage which had been seen in the present experiment, might be dependent on the delay in the first cleavage.
It is not yet clear what components in tubal fluid are most important for the fertilization phenomenon and also for the early development of fertilized ova. These problems are worthy of continued attention in the future, through the study of in vitro fertilization. To attempt the transfer of cultivated ova in the stage of morula to the uterine horns of pseudopregnant rabbits is still in progress.

**Summary**

A total of 207 rabbit ova were inseminated in vitro in tubal fluid under mineral oil using capacitated spermatozoa recovered from the uterus 12 hours after mating. About 20 hours after the transfer for cultivation, 123 (59.4%) of them were at the 2- to 4-cell stages. Sixty-five of the fertilized ova were cultivated for two or three days after insemination in vitro, and 21.5% of them could be reached at over the 16-cell stage.

**Acknowledgment**

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**References**