BIOLOGY OF REPRODUCTION 8, 420–426 (1973)

Fertilization *in Vitro* and Development of Mouse Ova

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Received May 16, 1972; accepted October 31, 1972

Epididymal sperm were added to embryological watch glasses containing 396 F_1 hybrid mouse ova in a simple medium under paraffin oil. The dishes were gently agitated for 8 h in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2. The ova were washed and cultured in glass tubes for 96 h during which time 365 ova cleaved and 347 developed to morulae or early blastocysts. Transferred to the uteri of pseudopregnant hosts were 299 of these embryos, and 67 male and 44 female offspring were born. From these findings, it was concluded that normal fertilization *in vitro* had occurred. Using first cleavage and embryo development in culture as the criterion, we have confirmed these findings and have examined some of the factors that affect fertilization *in vitro*. Epididymal sperm fertilized 122 of 131 ova whereas 39 of 122 ova were fertilized by uterine sperm. Agitation of sperm and ova during fertilization resulted in fertilization of 139 of 166 ova compared to fertilization of 84 of 168 ova without agitation. The highest fertilization rate (44 of 45) occurred with ova surrounded by cumulus cells and continuous agitation of sperm and ova in 0.5 ml of medium for 8 h at 37°C.

INTRODUCTION

A limited number of mouse ova have been fertilized *in vitro* in explanted oviducts (Brinster and Biggers, 1965; Pavlok, 1968) or in a simple medium with uterine sperm (Whittingham, 1968; Cross and Brinster, 1970; Maukherjee and Cohen, 1970; Kaufman and Whittingham, 1972). Fertilization has also been achieved using epididymal sperm capacitated in bovine follicular fluid (Iwamatsu and Chang, 1969, 1970, 1971) or human follicular and tubal fluid (Mukherjee, 1972). More recently, Pavlok and McLaren (1972) and Miyamoto and Chang (1972a,b) fertilized mouse ova in a simple medium using epididymal sperm. Ova fertilized *in vitro* have been cultured to blastocysts in explanted oviducts (Brinster and Biggers, 1965; Pavlok, 1967) and in a simple medium (Mukherjee and Cohen, 1970; Mukherjee, 1972; Miyamoto and Chang, 1972a). Development to the 15- or 17-day fetal stage has been achieved by Whittingham (1968), Cross and Brinster (1970), and Miyamoto and Chang (1971a) after transplanting embryos in the two-cell stage to the oviducts of pseudopregnant recipients. Mukherjee and Cohen (1970) and Mukherjee (1972) obtained a small number of progeny after transplanting blastocysts, developed *in vitro*, to the uteri of suitable hosts.

This report describes a method for fertilizing mouse ova *in vitro* with epididymal sperm in a simple medium and their development to morulae or blastocysts after 96 h in culture. The development of embryos into viable young when transferred to uteri of pseudopregnant animals was observed. The effect of cumulus (i.e., follicle) cells,

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volume of medium, agitation of sperm and ova, and the use of epididymal or uterine sperm for fertilization *in vitro* were examined.

MATERIALS AND METHODS

Materials

All glassware was treated with chromic acid solution and thoroughly rinsed with tap water followed by distilled water before sterilization either by dry heat (110°C for 2 h) or autoclaving. Rubber stoppers and caps were washed with 7X, a neutral detergent (Linbro Chemical Co., New Haven, Conn.). Sterile, plugged, plastic pipets (Kimble or Falcon Plastics) were used in preparing medium.

Distilled water was prepared by filtering tap water through a Honeycomb filter tube (No. W15R4TZV) (Commercial Filters Corp., Melrose, Mass.), through a Barnstead standard (No. 0802) and mixed bed resin (No. D8902) cartridge, and distilled in a Corning AG-3 glass still. The system was emptied and flushed out at the beginning of each week.

The medium was always prepared from freshly distilled water which had a resistance of approximately 0.8 MΩ. The components of the medium are those reported by Whitten (1971), and the source and amount of chemical used to prepare the medium are presented in Table 1. All the dry chemicals, except bovine serum albumin (BSA), were weighed and placed in a sterile Kimax No. 14915 milk dilution bottle; 100 ml of distilled water were added, and the bottle was gently shaken to dissolve the chemicals before adding the 1% phenol red solution and sodium lactate syrup. The medium was gassed for 15 min with 5% carbon dioxide, 5% oxygen, and 90% nitrogen (Ohio Medical Products, Lodi, N.J.) by connecting the gas supply to a 1-ml plastic pipet. The same gas mixture was used for all procedures, since Haidri *et al.* (1971) and Whitten (1971) have reported that oxygen concentrations above 5–10% inhibited oocyte maturation and ova culture. Into a Nalgene filter unit (0.45 μm average pore size) were place 20 ml of the medium which was allowed to stand for 15–30 min to moisten the membrane and to remove any toxic substances. The medium was forced through the filter by positive pressure applied by means of a large rubber stopper through which passed a glass tube connected to the gas supply. Bovine serum albumen (240 mg) was dissolved in the remaining 80 ml of medium which was also filtered using positive pressure to prevent frothing and to maintain the desired gas pressures. The medium was

TABLE 1
SOURCE AND AMOUNT OF CHEMICALS USED FOR PREPARATION OF MEDIUM

Chemical	Amount
NaCl (Mallinckrodt)	5.140 g/liter
KCl (J. T. Baker)	0.356 g/liter
KH_2PO_4 (Mallinckrodt)	0.162 g/liter
$MgSO_4 \cdot 7H_2O$ (Mallinckrodt)	0.294 g/liter
$NaHCO_3$ (Fisher)	1.900 g/liter
Glucose (Fisher)	1.000 g/liter
Ca lactate (L)·$5H_2O$ (Schwarz-Mann Research)	0.527 g/liter
Na-lactate 60% Syrup (DL) (Pfanstiehl Lab Inc.)	3.7 ml/liter
Na-pyruvate (Schwarz-Mann Research)	0.025 g/liter
K-penicillin-G (Schwarz-Mann Research)	0.075 g/liter
Streptomycin sulfate (Schwarz-Mann Research)	0.050 g/liter
1% Phenol red solution (Difco)	1.0 ml/liter
Crystalline bovine albumin (Pentex)	3.0 mg/ml

divided into 20-ml aliquots in milk dilution bottles gassed and stored at 5°C for use during the week. Aliquots of 1 ml of medium were placed in Kimax No. 45048 culture tubes (12 × 75 mm) which were fitted with rubber stoppers. Tubes were gassed for 15 s through a sterile 19-gauge needle attached to the gas supply via a cotton plugged disposable syringe. They were allowed to stand at 37°C for 15–30 min, and regassed for 15 s before being used to culture ova (Whitten, 1971).

Paraffin oil (Fisher Scientific Co. No. 0-119) was equilibrated with medium without BSA by bubbling gas for 15 min into a 50-ml glass-stoppered centrifuge tube containing approximately 35 ml of oil and 5 ml of the medium. The oil–medium suspension was centrifuged and stored in the incubator during that week.

Animals

The gametes were obtained from F_1 hybrid mice of the cross (SJL/Wt × C57BL/10Wt) and its reciprocal. They were bred in the research colonies of The Jackson Laboratory, provided food and water *ad libitum*, and 14 h of light (5–19 h). The colony contained 400–500 females 2–6 months old and housed four to five per cage. Mice in proestrus were selected in the afternoon by the visual appearance of the vagina (Champlin, Dorr, and Gates, unpublished) to provide unfertilized ova between 8–9 h the following morning (ova collected approximately 3–5 h after ovulation).

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The males were housed singly and only animals that had not mated during the previous week were used.

STANDARD METHODS FOR FERTILIZATION *in vitro:* EMBRYO CULTURE AND TRANSPLANTATION

The method for fertilization *in vitro* was similar to that reported by Whittingham (1968). Embryological watch glasses containing 0.5 ml of medium, overlaid with 2 ml of equilibrated paraffin oil, were prepared the previous evening and incubated overnight at 37°C in a gas-filled anaerobic jar. The watch glasses were removed from the anaerobic jar the following morning and placed on a slide warming tray (37°C) equipped with a Plexiglas hood through which the standard gas mixture was passed. Female mice, selected in proestrus the previous afternoon, were killed and both oviducts were removed. These were placed in the medium under oil and the ampullae were punctured with a 27-gauge disposable needle resulting in the release of ova surrounded by cumulus cells. The oviducts were discarded. The ova in each watch glass were collected from a single female.

An overlay of 0.5 ml of medium with 2 ml of oil in a watch glass was prepared immediately after the ova had been collected. A male was killed by cervical dislocation and both vasa deferentia and cauda epididymides were removed and placed in the watch glass. The tissues were cut into sections and sperm were allowed to disperse into the medium for 5–10 min. Care was taken to keep the watch glass warm and gassed during this period. Approximately 30 μl of the sperm suspension ($\sim$20,000–50,000 sperm) were pipetted into each watch glass containing ova, and the watch glasses were placed in an anaerobic jar, gassed for 5 min (3 liter/min) and placed in a Gyrotory shaker Incubator model No. G-25 (New Brunswick Science Co., New Brunswick, N.J.) rotating at 60 rev/min for 8 h. Ova were removed from the watch glasses, washed once in fresh medium, and pipetted into culture tubes (Whitten, 1971). In many of the ova, two pronuclei and polar bodies were visible, but because of the time required these were not thoroughly examined at this stage. Tubes were examined the following morning and the number of normal two-celled ova were recorded. Embryos were cultured for 64–72 h (day 3) and were transferred as morulae or early blastocysts to uteri of day 2 pseudopregnant recipients (McLaren and Michie, 1956). Pseudopregnancy was induced by mating females to sterility tested vasectomized males. The females were housed individually and allowed to deliver their offspring.

RESULTS

In ten replications and a total of 396 ova, 365 (92%) ova cleaved using this technic; and 347 (95%) embryos developed in culture from these zygotes. Of 299 embryos transplanted to the uteri of suitable hosts, 111 (37%) offspring, 67 males and 44 females, were born; indicating that development was not parthenogenetic and fertilization had occurred.

These results were readily repeatable as seen in the control groups in the subsequent experiments, but occasionally there were unexplained low rates of fertilization.

Comparison of Epididymal and Uterine Sperm

Two estrus females were paired at 7 h with males and the females were killed at 830 h. The uteri were removed and placed in a watch glass containing 0.5 ml of medium overlaid with 2 ml of paraffin oil. The uteri were cut into sections and the sperm were allowed to disperse into the medium for 10 min. Of this sperm suspension 30 μl were pipetted into watch glasses containing ova. Epididymal sperm preparations, as described earlier, were used for comparison. Sperm counts were not made, but gross observations indicated that the sperm numbers were similar in both preparations. Of the 131 ova, 122 (13 clutches; 3 replications) were fertilized by epididymal sperm whereas only 39 of 122 (12 clutches; 3 replications) were fertilized by uterine sperm. This difference was shown by χ^2 to be significant ($P < 0.01$). The motility of the uterine sperm was less than that of the epididymal sperm when observed at the time of ova transfer to the culture tubes.

The Effect of Varying the Volume of Medium Both with and without Agitation

It is considered that some of the effects of agitation may be due to release of a fertilizin-like substance from the ova (Thibault, 1969). Such an effect would be

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possibly be dependent on the volume of medium in relation to sperm and ova numbers, and there could be a significant volume–agitation interaction. Therefore, we examined these factors simultaneously.

The standard procedure was followed and ⅓ of the egg clutches were placed in 0.2 ml of medium, ⅓ in 0.5 ml, and the remainder in 1 ml of medium. One-half of the clutches in each replicate were agitated in the usual manner, whereas the others were incubated without agitation. There were two clutches per treatment with three replications, and the results are given in Table 2. Two-way analysis of covariance (Steel and Torrie, 1960) of the number of ova cleaved covariated with the number of ova per clutch showed that agitation of sperm and ova significantly ($F(1,29) = 14.905$, $P < 0.01$) increased the fertilization rate. No significant ($F(2,29) = 1.042$, NS) effect of medium volume for fertilization was observed, but the largest number of ova cleaving (97%) occurred when fertilized in 0.5 ml of medium with agitation. The number of embryos developed in culture from number of cleaved ova was not influenced by the treatments. The data in Table 2 are presented as single clutches to show the reproducibility of the technic.

The Effect of Cumulus Cells and Duration of Agitation

The effect of cumulus cells and the duration of incubation with agitation on fertilization rate were examined. Cumulus cells were removed from approximately one-half of the clutches of ova with hyaluronidase (bovine testes, Sigma Chemical Co.) at a concentration of 1 mg/ml

TABLE 2
THE EFFECT OF DIFFERENT VOLUMES OF MEDIUM BOTH WITH AND WITHOUT AGITATION ON FERTILIZATION AND EMBRYO DEVELOPMENT *in vitro*

	Not agitated			Agitated		
Volume (ml)	No. ova	No. cleaved	No. embryos	No. ova	No. cleaved	No. embryos
0.2	10	5	5	9	9	9
	8	2	2	7	4	2
	9	6	6	9	9	7
	11	8	6	9	8	8
	6	2	0	11	6	6
	7	2	2	10	4	4
Total	51[a]	25	21	55	40	36
0.5	13	11	9	9	9	8
	9	2	2	8	8	8
	8	7	6	10	10	10
	8	3	2	11	11	10
	8	1	0	10	9	9
	10	8	8	11	10	10
Total	56	32	27	59	57	55
1.0	9	5	5	8	6	5
	9	4	4	8	5	4
	9	1	0	9	7	3
	10	8	8	10	9	9
	7	3	3	8	6	6
	7	6	4	9	9	9
Total	51	27	24	52	42	36

[a] Two clutches per treatment and three replications.

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TABLE 3
THE EFFECT OF CUMULUS CELLS AND DURATION OF AGITATION ON FERTLIZATION AND EMBRYO DEVELOPMENT *in vitvo*

Duration of agitation (h)	Without cumulus cells			With cumulus cells		
	No. ova	No. cleaved	No. embryos	No. ova	No. cleaved	No. embryos
1	26[a]	1	1	28	7	6
3	31	4	2	28	8	8
5	33	18	8	32	27	27
8	30	16	16	45	44	44
11	29	14	13	29	26	24

[a] One clutch per treatment and three replications.

of medium. Each clutch was treated separately and washed three times in 1-ml aliquots of medium to remove the hyaluronidase before being placed in the embryological watch glass. Control clutches were treated as described above. All vessels were placed in the Gyrotory incubator after sperm introduction and samples of the naked ova and those still within the cumulus cells were removed after 1, 3, 5, 8, and 11 h of agitation. The ova were transferred to culture tubes and observed for cleavage and subsequent embryo development (Table 3).

The data from one clutch per treatment and three replications were analyzed by two-way analysis of covariance as described previously. The presence of cumulus cells resulted in significantly ($F(1,20) = 10.590$, $P < 0.01$) greater fertilization irrespective of the duration of agitation. Fertilization rate was also significantly ($F(4,20) = 4.277$, $P < 0.05$) affected by duration of agitation. Ova with cumulus cells and agitation with sperm for 8 h resulted in the highest fertilization rate (98%). There was no significant difference between the treatments in the proportion of cleaved ova that developed into embryos.

DISCUSSION

We have described a method for fertilization of mouse ova *in vitro*, and have observed their subsequent embryo development in culture and birth of viable offspring after transferring them to uteri of pseudopregnant recipients. The conditions required for optimal fertilization are mild agitation of epididymal sperm with ova plus cumulus cells for 8 h in 0.5 ml of medium overlaid with oil.

The superiority of epididymal as compared with uterine sperm for fertilization *in vitro* may result from adverse effects of the uterine environment. The uterine sperm were observed to have reduced motility after 8 h incubation with ova *in vitro*. A possible explanation for this effect could be the presence of toxic substance(s) from either or both the male and female reproductive tracts.

Fertilization (32%) using uterine sperm in the present study is similar to the results of Whittingham (1968) and Mukherjee and Cohen (1970) but differs markedly from the 90% fertilization reported by Cross and Brinster (1970). This discrepancy could result from the use of different strains of mice (i.e., inbred, random bred, and hybrid), but a more plausible explanation is that Cross and Brinster (1970) used extremely high levels of BSA (30 mg/ml) in the medium. Kaufman and Whittingham (1972) recently reported fertilization rates of 70–80% (three experiments) using uterine sperm in medium supplemented with 32 mg/ml BSA. The beneficial effect of

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high levels of BSA for fertilization *in vitro* has not been determined.

Results from studies by Iwamatsu and Chang (1969, 1970) indicate that mouse sperm may require a period of maturation before sperm penetration can occur. This study demonstrates that sperm maturation *in vitro* is complete after 3 h in our medium. Approximately 50% of the ova were fertilized after removal of the cumulus cells and thorough washing of the ova; therefore, it appears that tubal secretions or cells from the female tract are not necessary for maturation of mouse sperm.

Thibault (1969) postulates that washing the ovum disperses a fertilizin-like substance from the zona pellucida that facilitates attachment of capacitated sperm to the rabbit ovum. Evidence to substantiate this hypothesis has been reported by Thibault and Dauzier (1960, 1961), who observed an increased fertilization rate when ova and sperm were placed in a discontinuous washing system; however, these findings could not be repeated by Bedford and Chang (1962). Mild agitation of sperm and ova increased the fertilization rate in this study indicating that agitation may enhance the dispersion of a substance ("fertilizin") from the mouse ovum which promotes sperm maturation. Further studies are underway to determine the mechanisms for fertilization *in vitro* in the mouse.

ACKNOWLEDGMENTS

This work was supported by NIH Research Grant No. HD 04083 from the National Institute of Child Health and Human Development, by an allocation from NIH General Research Support Grant No. RR 05545 from the Division of Research Resources to The Jackson Laboratory, and by a Training Grant No. GY 8756 from the Undergraduate Research Participation Program of the National Science Foundation.

The authors express gratitude to Dr. W. K. Whitten for his encouragement and to Darrold Dorr and Stetson Carter for their technical assistance.

The Jackson Laboratory is fully accredited by the American Association for Accreditation of Laboratory Animal Care.

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