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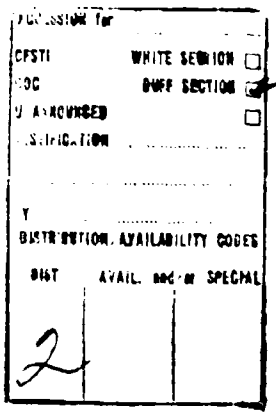
TECHNICAL MANUSCRIPT 473

A COMPARISON OF THE IN VITRO VIABILITY OF OVA FROM HORMONE-TREATED MICE WITH OVA FROM SPONTANEOUS OVULATIONS

James R. Spears
Jerry S. Walker

AUGUST 1968

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland



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Process Development Division
AGENT DEVELOPMENT AND ENGINEERING LABORATORY

Project 1B533001D426

August 1968

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

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ABSTRACT

The results of this study show that the viability of ova obtained from hormone-treated females is equal to that of ova from spontaneous ovulations in the two strains of mice studied (Swiss-Webster and C57BAL/6Cum). Approximately 90% of all cultured ova developed from the late two-cell or early four-cell stages to blastocysts. The use of exogenous hormones to induce ovulation seems to afford a reliable, efficient, and economic means of obtaining fertilized mammalian ova.

I. INTRODUCTION

The technique of inducing superovulation has afforded increased opportunity for studies using preimplantation mammalian ova. This technique provides a convenient supply of ova for ovum transfer and culture experiments. Not only are larger numbers of ova recovered per female, but more importantly, the stages of maturation division, ovulation, and the first and subsequent cleavage divisions can be ascertained following treatment with gonadotropins.[1]

Gates[2] concluded that the developmental capacity of eggs from immature mice treated with gonadotropins closely approximated that of eggs from spontaneous ovulations in adult mice. In his experiment, eggs from immature donors were compared with spontaneously ovulated, untransplanted eggs developing in the same uterine environment.

Chang[3] obtained 53 two-cell ova from a single rabbit doe after treatment with gonadotropins, and they were transplanted into four recipient females and yielded 45 normal young. Dowling[4] likewise reported a high incidence of success utilizing ova from superovulated does for ovum transfer. Willett, Buckner, and Larson[5] reported three successful transplantations of fertilized bovine eggs that were obtained from superovulated females.

Brinster[6-10] utilized the superovulation technique in mice to obtain a very large number of two-cell ova that developed to blastocysts with a high incidence of success. Data accumulated from studies utilizing ova from superovulated animals for ovum transfer culture[4,5,10,11] and metabolic studies[6-10,12,13] indicate their viability is equal to that of ova from spontaneous ovulations. However, specific studies comparing the in vitro development of spontaneous and superovulated ova have not been conducted.

This paper compares the viability of ova from hormone-induced ovulations with that of ova from spontaneous ovulations. Late two-cell and early four-cell ova from two strains of mice were tested for their ability to develop to blastocysts in vitro.

II. MATERIALS AND METHODS

A. ANIMALS

The Fort Detrick strain of Swiss-Webster mice obtained from the Fort Detrick animal farm and C_{57}BAL/6Cum mice obtained from Cumberland View Farms, Clinton, Tennessee, were utilized in this study. All animals were 6 to 8 weeks of age at the time of use.

B. OVULATION INDUCTION

Ova were recovered from hormone-treated females induced to ovulate by an intraperitoneal injection of 5 international units (IU) of pregnant mares' serum (Ayerst) followed 44 to 50 hours later with 5 IU of human chorionic gonadotropin (HCG) (Organon). Females were placed with males immediately after the time of luteinizing hormone injection. Females exhibiting vaginal plugs (approximately 90%) the following morning were considered to be pregnant. Ova were recovered 48 to 52 hours after HCG injection.

The ova from spontaneous ovulations were recovered late on day 2 of pregnancy (day of vaginal plug is considered to be day 1 of pregnancy). This was 40 to 44 hours after the presumed time of ovulation.

C. OVA RECOVERY

The animals were sacrificed by cervical separation. The oviducts were cut free of the ovary and uterus and placed in 0.5 to 1.0 ml of culture medium (see below) in a capped plastic test tube (17 by 60 mm). The ova were recovered by inserting a blunted 30-gauge needle attached to a 2-ml syringe into the fimbrial end of the oviduct and flushing the contents into a small watch glass. The ova were manipulated by means of a pipette constructed from a drawn-out capillary tube inserted into a 16-gauge needle attached to a 1-ml Hamilton thumb-wheel syringe. All manipulations were carried out with the aid of a stereomicroscope at 30 to 60X magnification.

D. MEDIUM PREPARATION

The medium and culture techniques are modifications of those presented by Brinster.[6] The components and concentrations of the culture medium are given in Table 1. The medium was prepared in either 100- or 200-ml quantities and stored at 4 C until ready for use. To each 100 ml of 1X Earle's balanced salt solution (EBSS), commercial preparation without bicarbonate, was added 0.2 ml of 60% sodium lactate and 1.0 ml of a 0.3% solution of

sodium pyruvate. Penicillin, streptomycin, and bovine plasma albumin were also added before storage. At the time of use, 10 ml of the above stock solution was placed in a 30-ml T-30 Falcon tissue culture flask and sodium bicarbonate was added (1.3 ml of a 1.3% solution). This 10-ml aliquot was sparged for 10 minutes with 5% CO_2 in air.

TABLE 1. MEDIUM FOR IN VITRO CULTIVATION OF MOUSE OVA

Component	Grams/Liter	
NaCl	6.8	Earle's Balanced Salt Solution
KCl	0.4	
NaH_2PO_4	0.125	
$MgSO_4 \cdot 7H_2O$	0.2	
$CaCl_2$	0.2	
Glucose	1.0	
Phenol Red	0.02	
Sodium lactate	1.412	
Sodium pyruvate	0.03	
Bovine plasma albumin	1.00	
Penicillin G (potassium)	100 U/ml	
Streptomycin sulfate	50 μg/ml	
$NaHCO_3$[a/]	1.7	

a. Sodium bicarbonate is added as a 1.3% solution.

E. OVA CULTURE

Ova were individually handled and deposited in microdrops (0.05 to 0.1 ml) of culture medium in the center well of an organ culture dish (Falcon, 15 by 60 mm) under light weight paraffin oil (Saybolt viscosity 125/135, Fisher). The paraffin oil was equilibrated with the medium by adding 10 ml of the medium to 300 ml of the sterile paraffin oil and sparging for 15 minutes with 5% CO_2 in air. The mixture was stored at least 48 hours in a CO_2 incubator (Hotpack, #528) (Fig. 1) before use. A Plexiglas compartment was constructed and installed in the incubator (Fig. 2). This facilitated the retention and reequilibration of the CO_2 atmosphere upon opening the door to the incubator. The gas flow in the incubator was controlled by standard flow meters on the incubator.

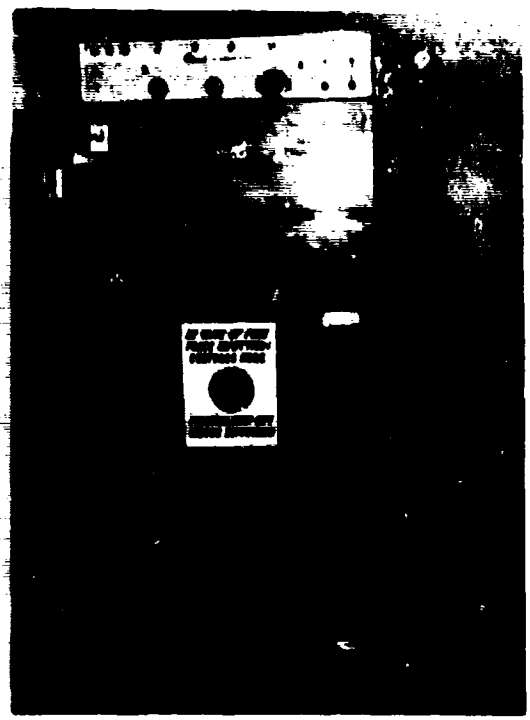

Figure 1. Hotpack Incubator #528.

The culture dishes containing the ova in the microdrops were placed in the incubator within 8 to 10 minutes after the mice were sacrificed. Whenever desired, the culture dishes were removed and observed or photographed.

For an ovum to be selected or distinguished as normal, it had to meet the following criteria: fertilized, zona pellucida intact, blastomeres of approximately equal size, and no other gross morphological abnormality. Ova not meeting one or more of these criteria were considered abnormal and were discarded immediately after recovery.

Figure 2. Plexiglas Compartment Installed in the Incubator to Facilitate Retention and Reequilibration of CO_2 Atmosphere.

The number of normal ova developing to blastocyst after 3 days in culture was determined microscopically using an inverted microscope (1 to 200X). The proportions of those ova developing to blastocyst were determined from the total number cultured at any one time.

All cultures in which less than 50% of the ova developed to blastocysts were excluded because percentages this low are indicative of developmental failures attributable to factors other than those being studied.

III. RESULTS

A total of 879 mouse ova were cultured in vitro, and 88% developed from the late two-cell or early four-cell stages (Fig. 3) to blastocyst (Fig. 4). These results are in accord with those presented by Brinster,[6] who demonstrated that 60 to 100% of the two-cell ova recovered 45 hours after HCG injection developed to blastocyst.

In the present study, the ability of ova from hormone-treated females of two strains of mice to develop from early preimplantation stages to blastocysts in vitro was compared with that of ova of equal development from spontaneous ovulations. The numbers and proportions of ova developing to blastocysts are presented in Table 2. Preliminary investigations indicated that the developmental capacity of late two-cell ova did not differ appreciably from that of early four-cell ova. In view of these preliminary findings, no distinction was made between the number of four-cell ova recovered (25% of the total) and two-cell ova in Table 2. The data were transformed by determining the square roots of the percentages for normalizing purposes and analyzed by means of the "t" test to compare sample means. There was no significant difference between any of the groups tested at $P \leq 0.05$ level.

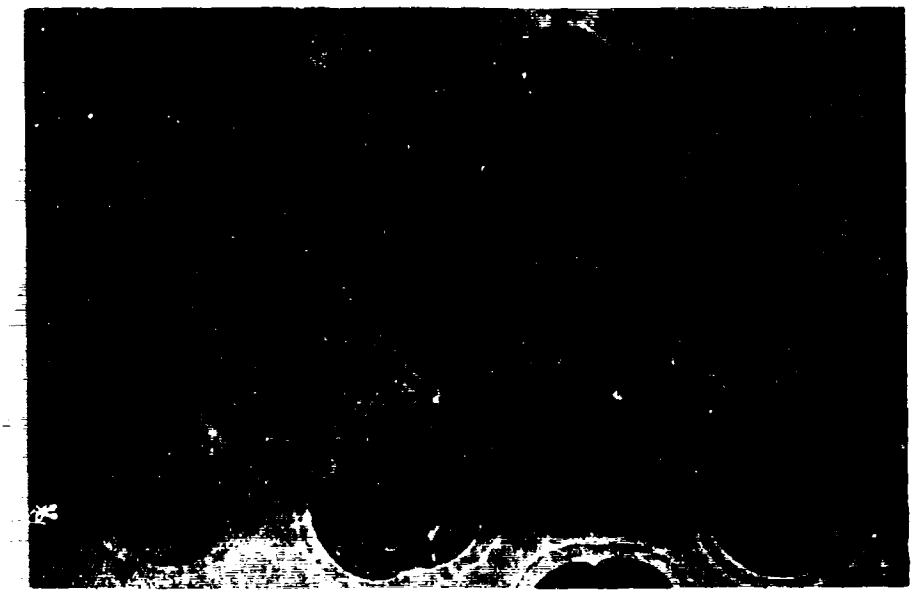

Figure 3. Two- and Four-Cell Mouse Ova at Beginning of In Vitro Culture. 200X.

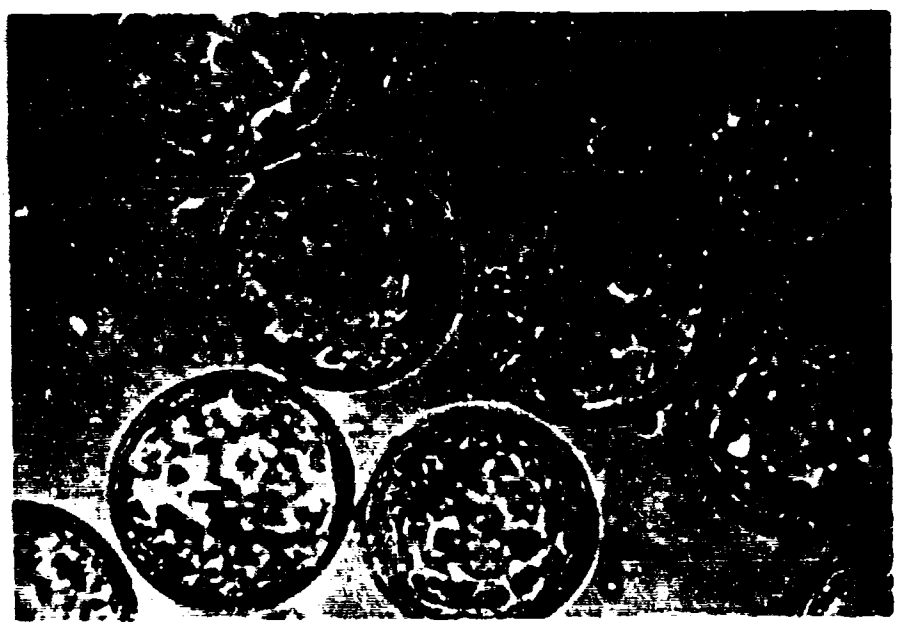

Figure 4. Blastocysts Developed from Two- and Four-Cell Ova Cultured In Vitro for 48 Hours. 200X.

TABLE 2. PROPORTIONS OF OVA DEVELOPING FROM TWO- AND FOUR-CELL STAGE TO BLASTOCYST RECOVERED FROM HORMONE-TREATED AND SPONTANEOUSLY OVULATING SWISS-WEBSTER AND C_{57}BAL/6CUM FEMALE MICE

Hormone-Induced Ovulation				Spontaneous Ovulation			
C_{57}		Swiss-Webster		C_{57}		Swiss-Webster	
No. Developed/ Total No.	%	No. Developed/ Total No.	%	No. Developed/ Total No.	%	No. Developed/ Total No.	%
8/15	53.3	7/9	77.8	3/3	100.0	3/3	100.0
11/13	84.6	7/8	87.5	3/3	100.0	10/10	100.0
25/31	80.6	4/4	100.0	9/11	81.8	9/9	100.0
5/7	70.4	14/15	93.3	7/8	87.5	10/11	90.9
3/3	100.0	13/15	86.7	9/9	100.0	8/10	80.0
28/28	100.0	10/16	60.0	6/6	100.0	5/6	83.3
6/7	85.7	7/10	70.0	6/7	85.7	4/6	66.7
35/37	94.7	8/10	80.0	8/9	88.9	15/15	100.0
30/35	85.7	21/23	91.3	13/14	92.8	10/10	100.0
14/15	93.3	19/22	86.4	14/14	100.0	8/9	88.9
22/24	91.7	12/13	92.3	5/7	71.4	11/14	78.9
4/7	57.1	29/32	90.6	8/9	88.9	7/9	77.8
16/16	100.0	20/23	86.9	8/8	100.0	9/10	90.0
4/4	100.0	18/24	75.0	-	-	28/28	100.0
6/6	100.0	14/16	87.5	-	-	-	-
4/4	100.0	14/16	87.5	-	-	-	-
5/7	71.4	15/16	93.8	-	-	-	-
2/2	100.0	16/18	88.9	-	-	-	-
5/5	100.0	-	-	-	-	-	-
25/27	92.6	-	-	-	-	-	-
10/10	100.0	-	-	-	-	-	-
24/26	92.3	-	-	-	-	-	-
Total							
292/331	88.2	248/290	85.5	99/108	91.7	137/150	91.3

Overall Total
776/879 = 88%

IV. DISCUSSION

Admittedly, criticism can be directed toward the use of both two- and four-cell ova in this experiment. However, it seemed of utmost importance to ensure that those ova from spontaneous ovulations developed sufficiently in vivo to allow continued development in vitro. This meant that ova recovered from spontaneous ovulations had to remain in the oviduct at least 32 to 35 hours after ovulation. The presence of four-cell ova some 40 to 44 hours after the presumed time of ovulation was a true indication that sufficient time had elapsed. In the initial stages of this experiment, the four-cell ova were cultured separately and the proportions recorded. The developmental efficiency of these ova did not exceed that of two-cell ova recovered at the same time. Thus, no distinction is made between two- and four-cell ova in Table 2.

The recovery of ova from hormone-treated females was performed at a sufficient time interval after HCG injection to ensure that some of these ova also reached the four-cell stage. As noted in Section II, recoveries performed 48 to 52 hours after HCG injeccion exhibited a preponderance of two-cell ova.

The culture technique, as mentioned previously, is a modification of that described by Brinster;[6] it is important to point out the simplicity of his technique. By using commercially prepared salts (EBSS), the medium can be prepared in a very few minutes and in small quantities. This medium contains not only lactate and pyruvate as energy sources, but also glucose, which is in the EBSS. Isotonic sodium bicarbonate solution (1.3%) was utilized for pH adjustment. Preliminary studies showed that 1.3 ml is the optimum quantity of bicarbonate solution to add to the 10 ml of the medium. This produces a pH of approximately 6.8 after equilibration.

Studies involving the use of preimplantation mammalian ova offer great potential for cell research. Investigations conducted with such cells could provide insight into factors influencing differentiation, metabolic pathways, blastomere potency, and the effects of viruses upon the earliest embryonic development. The establishment of permanent cell lines from these cells would most certainly provide a useful research tool.

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13. ABSTRACT

The results of this study show that the viability of ova obtained from hormone-treated females is equal to that of ova from spontaneous ovulations in the two strains of mice studied (Swiss-Webster and C_{57}BAL/6Cum). Approximately 90% of all cultured ova developed from the late two-cell or early four-cell stages to blastocysts. The use of exogenous hormones to induce ovulation seems to afford a reliable, efficient, and economic means of obtaining fertilized mammalian ova.

14. Key Words

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