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

A Thesis

Presented to

The Faculty of the School of Science and Mathematics

Morehead State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science in Biology

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by

Henry H. Lyon

April 1971

Accepted by the faculty of the School of Sciences and Mathematics, Morehead State University, in partial fulfillment of the requirements for the Master of Science degree.

James R Spears
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ABSTRACT OF THESIS

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

This experiment was undertaken in an attempt to determine if the viability of ova obtained from hormone-treated females is equal to that of ova from spontaneous ovulations in the Swiss-Webster strain of mice. The viability was determined by percentage development of 2-and 4-cell stages to blastocyst in vitro.

It was found that approximately 90% of all cultured ova (spontaneous and hormone-induced) developed from the late 2-cell or early 4-cell stages to blastocysts. There was no significant difference in the developmental capacity of ova from hormone-treated females as compared with ova from spontaneous ovulators.

Henry W. Lyon

April 23, 1971
Date

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INTRODUCTION

The ultimate development of the preimplantation embryo *in vitro* is to blastocyst. Whitten (43) was the first to observe *in vitro* development of early preimplantation mouse ova. Mouse ova recovered at the 2-cell stage were cultured to blastocyst in a lactate balanced salt solution medium.

It was found by Gates (22) that the viability and developmental capacity of eggs from immature mice treated with gonadotrophins closely approximated those eggs from spontaneous ovulations in adult mice. In his experiment, the developmental capacity of eggs, transplanted from immature donors was compared with that of spontaneously ovulated, untransplanted eggs developing in the same uterine environment, that is eggs, native to the adult recipient.

In 1890 and 1897, Heape (14) reported his successful transplantation of rabbit ova to a uterine foster mother. Later Chang (14) in 1948 obtained 26 young from 4 recipients by the transplantation of 35, 2-cell ova from one doe. Bowling (16) likewise reported a high incidence of success utilizing ova from superovulated does for ovum transfer. Willett, Buckner, and Larson (45) reported the production of 3 calves from 5 transplantations of

ova obtained from superovulated females.

The technique of inducing ovulations in mice was used by Brinster (7,8,9,10) to obtain a very large number of 2-cell ova that developed to blastocysts with a high incidence of success. Data accumulated from studies utilizing ova from superovulated animals for ovum transfer (16,45,11) and metabolic studies (7,8,9,10,11) indicate their viability is equal to that of ova from spontaneous ovulations. However, only one specific study comparing the in vitro development of spontaneous and superovulated ova has been conducted (37).

This paper compares the viability of ova from hormone-induced ovulations with that ova from spontaneous ovulations. Late 2-cell and early 4-cell ova from the Swiss-Webster strain of mice were tested for their ability to develop to blastocysts in vitro.

LITERATURE REVIEW

Some 60 years ago, Brachet (4,5) was the first to culture rabbit ova *in vitro*, but with little success. Blastocysts developed from the 8-cell stage *in vitro* and were maintained for 48 hours. Since the time of this first culture of rabbit ova, various cleavage stages have been attained *in vitro* utilizing varying mixtures of serums and plasmas (15,32).

Until just recently, attempts to culture rat ova were generally unsuccessful, but with a modification of Brinster's medium, Folstad, Bennett, and Dorfman (21) have cultured rat ova from the 8-cell stage to blastocyst.

Whitten (43) demonstrated that the ova of sheep and goats must reach the 16-to 24-cell stage before they will continue development in culture; while Shettles, (33) utilizing follicular fluid, demonstrated the culture of a human follicular oocyte to a 32-cell embryo after it had been fertilized *in vitro*.

Utilizing a medium containing egg white, Hammond (24) was the first to cultivate 8-cell mouse ova to blastocysts *in vitro*. Since Hammond's initial experiment, techniques have been improved upon. The most extensive work has been conducted by Whitten (43,44,45). He found the Kreb-Ringer bicarbonate containing glucose and crystalline bovine plasma albumin (BPA) to be sufficient to allow development of 8-cell mouse ova to blastocyst. Whitten

also found that energy sources such as glucose, mannose, lactate, pyruvate and malate brought about normal development of the 8-cell embryo to blastocyst, while compounds such as galactose, maltose, fructose, lactose, acetate, propionate, citrate, glycerol, and glycine did not. Brinster and Thompson (12) found that compounds such as BPA, glucose, pyruvate, lactate, glutamate, asparagine, and glycine when added singly to the culture medium, will support development of 8-cell mouse ova to blastocysts in vitro. Therefore, the 8-cell mouse embryo requires only an energy source or a fixed nitrogen source.

Until 1963, only 8-cell or older mouse ova could be cultured in vitro on a large scale. Whitten (45) observed some development of the late 2-cell mouse ova when calcium lactate replaced calcium chloride in the culture medium. Brinster improved the cultivation technique by using a medium consisting of a balanced salt solution containing sodium lactate as an energy source and BPA as a nitrogen source. Later, he clearly established optimal requirements for the 2-cell embryo developing in vitro in terms of pH, osmolarity, energy source, and fixed nitrogen source. It was found that 2-cell mouse ova developed best in medium that has pH of approximately 6.82 (range 0.2002 to 0.3542 osmols) (8,23). The energy sources which supported

development of the 2-cell embryos were within one metabolic reaction of pyruvic acid. Best results were obtained using lactate and oxaloacetate (it was found that the addition of 10^{-4} M oxaloacetate to the 10^{-2} M lactate in the medium increased the number of blastocysts formed from 2-cell ova) (9). Brinster found that the albumin contributed a physical component (the polymer property of albumin) which prevented the ova from sticking to surfaces. He also found that if the 21 BPA amino acids were supplied to the medium together with a polymer substitute, the 2-cell ova developed normally to blastocysts. The only amino acid resulting in significant decreases in blastocyst development, when deleted from the medium, was cystine (10). The elucidation of metabolic requirements by Brinster (7) allowed the cultivation of 2-cell mouse embryos to blastocyst in vitro.

Biggers (6) described the successful development of mouse embryos in vivo after previous cultivation in vitro from 2-cell to the blastocyst stage. Also, the in vivo developmental capacity of 8-to 16-cell mouse embryos, cultivated in vitro to the blastocyst stage, has been demonstrated by McLaren (28).

The ovum culture techniques provide a method to directly test the viability of ova and to observe early preimplantation development. Brinster (7,8,9,10)

utilized the superovulation techniques in mice to obtain a very large number of 2-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 (16,45,11) and metabolic studies (7,8,9,10,11) indicate their viability is equal to that of ova from spontaneous ovulations.

Spears and Walker (37) completed a study in 1968 on the in vitro comparison of the viability of ova from both hormone-treated and spontaneous ovulating mice. The results of this stucy indicated that the viability of these ova was equal in the two strains of mice studied (Swiss-Webster and C_{57}Bal/6Cum). In their study the presence of 4-cell ova was taken as an indication that sufficient time had elapsed after ovulation to ensure continued development in vitro. However, the number of 2-and 4-cell ova recovered from individual mice was not taken into consideration. Therefore, it is possible that the embryos were not at the same stages of development at the onset of cultivation.

In obtaining a number of 2-and 4-cell ova, Falconer (20) concluded that in the analysis of differences in the number of eggs shed by the two ovaries of mice during natural oestrus showed a negative correlation, but a positive correlation after superovulation.

It has long been known that the administration of exogenous gonadotrophic substances induced ovulation in mice and resultant pregnancies may be marked by an increased embryonic mortality. The birth of several litters containing more than normal number of young may result, but generally it is difficult to adjust dosage to increase rather than decrease the average litter size (2).

Chang (14) obtained 53, 2-cell ova from a single rabbit doe after treatment with gonadotrophins and they were transplanted into 4 recipient females and yielded 45 normal young. Dowling (16) likewise reported a high incidence of success utilizing ova from superovulated does for ovum transfer. Willett, Buckner, and Larson (45) reported 3 successful transplantations of fertilized bovine eggs that were obtained from superovulated females.

The medium and culture method used in this study are a modification of Brinster's technique presented by Spears and Walker (37). The preparation of culture medium has been greatly simplified and is a combination of components which fulfills the minimum requirements for development. Undoubtably there are substances in the tubal fluids which are not contained in the culture medium. Still this is the best means we have for the direct observation of growing preimplantation mammalian ova <u>in vitro</u>.

MATERIALS AND METHODS

ANIMALS

The Morehead State University strain of Swiss-Webster mice originally obtained from S and S Research, Lagrange, Kentucky was utilized in this study. All mice were 6 to 8 weeks of age at time of use. Water and food (Purina Laboratory Chow) ad lib.

OVULATION INDUCTION

Ova were recovered from hormone-treated females induced to ovulate by an intraperitoneal injection (IP) of 5 international units (IU) of pregnant mares' serum (PMS, Ayerst) followed 45 hours later with 5 IU of human chorionic gonadotrophin (HCG, Organon). Immediately after the injection of HCG the females were placed with fertile males (Fig. 1). Females exhibiting vaginal plugs (approximately 70%) the following morning were considered to be pregnant. Ova were recovered 48 hours after the HCG injection, about 36 hours after the presumed time of ovulation.

The spontaneous ovulating females were placed with fertile males at approximately the same time as the hormone-treated females (Fig. 1). Females exhibiting vaginal plugs (approximately 20%) the following morning were considered to be pregnant. Ova were recovered 48 hours after the female was placed with the male, approximately 36 hours after ovulation.

OVA RECOVERY

The female mice were sacrificed by cervical separation. The reproductive tract was exteroized by making a mid-ventral incision. The oviducts were cut free by cutting the bursa at the ovarian ends and uterotubal junction at the uterine ends of the oviducts. The oviducts were then placed in a watch glass containing culture medium. The ova were recovered by inserting a blunted 30-gauge needle attached to a 2.5 ml syringe into the fimbrial end of the oviduct and flushing the contents into an embryological watch glass. The ova were manipulated by means of a pipette constructed from a drawn-out capillary tube inserted into a 14-gauge needle attached to a 1-ml Hamilton Micro syringe. All manipulations were carried out with the aid of a stereomicroscope at 20X magnification.

PREPARATION OF CULTURE MEDIUM

The medium and culture techniques are modifications of Brinster's as presented by Spears and Walker (37). The components of the culture medium and their respective concentrations are given in Table 1. The culture medium was made up in 100 ml stock quanities and stored at 4C until ready for use. To 90 ml of distilled water, 10 ml of 10X commercial preparation of Earle's Balanced Salt Solution (EBSS) was added, making the dilution 1X. To each

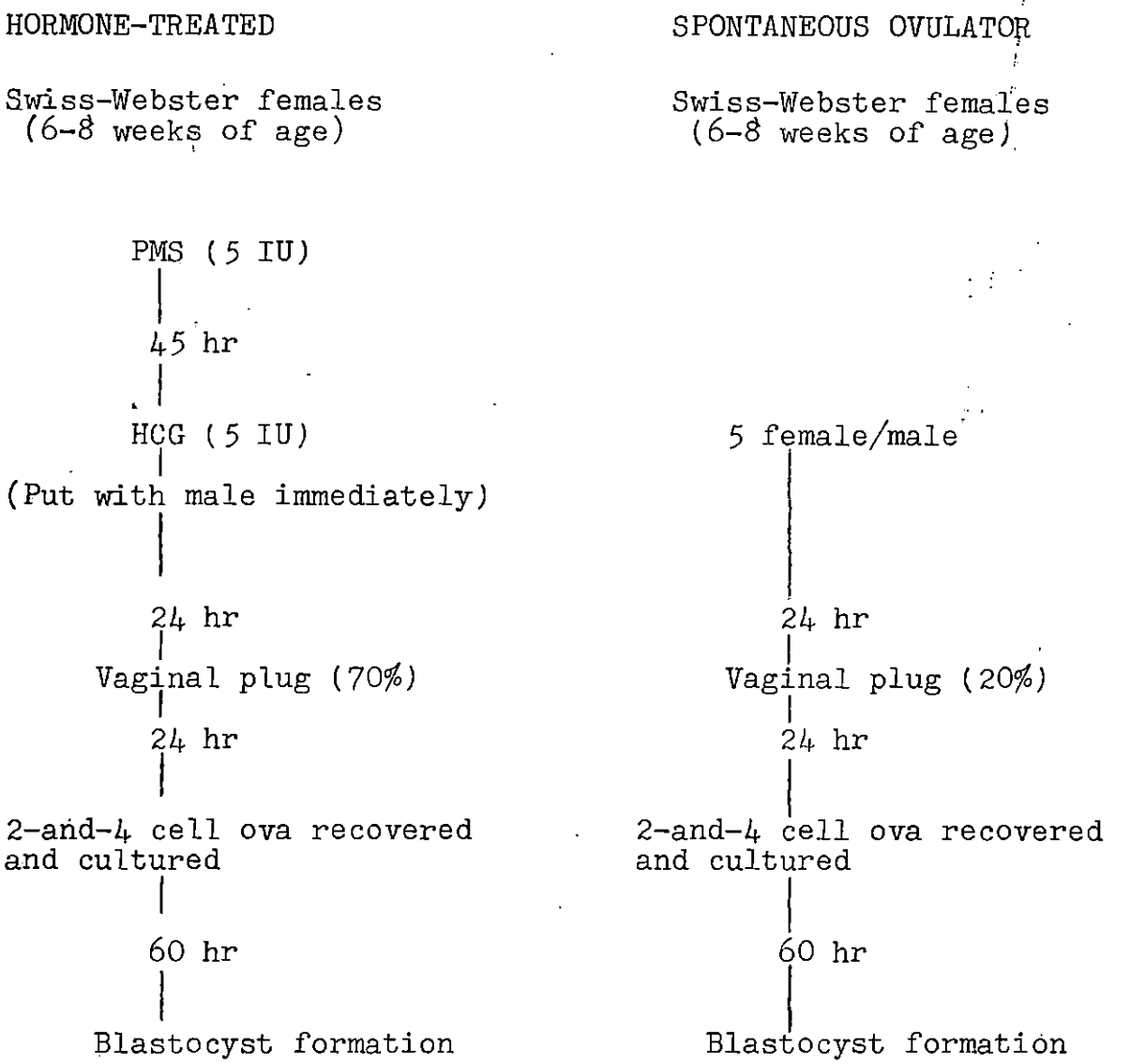

Figure 1. Schematic representation of the overall experimental procedure used in this study.

100 ml quanity of 1X EBSS, 0.2 ml of 60% sodium lactate syrup and 1.0 ml of a 0.3% solution of sodium pyruvate, together with quanities of streptomycin, penicillin, and bovine plasma albumin (BPA) were added before storing. At the time of use, 10 ml of the stock medium was placed in a 30 ml T-30 Falcon tissue flask and sodium bicarbonate was added (1.2 ml of a 1.3% solution). The pH (6.8-7.2) was regulated by sparging this 10 ml aliquot for 10 minutes with 5% CO_2 in air.

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, is by 60 mm) under light weight paraffin oil (saybolt viscosity 125/135, Fisher). About 300 ml of sterile paraffin oil was stored in an incubator (Hotpack #528) (Fig. 2) under 5% of CO_2 in air for 48 hours to equilibrate the oil. The incubator was fitted with a plexiglass compartment (Fig. 3) to facilitate retention and requilibration of the CO_2 atmosphere in the incubator when the door was opened (37). The gas flow in the incubator was controlled by standard flow meters on the incubator.

The culture dishes containing the microdrops were placed in the incubator approximately one hour before use to equilibrate the culture medium. The recovered ova were

Table 1. Medium for *in vitro* cultivation of mouse ova.

Component	Concentration
NaCl*	6.8 g/l
KCl*	0.4 g/l
NaH_2PO_4*	0.125 g/l
$MgSO_4$ $7H_2O$*	0.2 g/l
$CaCl_2$*	0.2 g/l
Glucose*	1.0 g/l
Phenol Red*	0.02 g/l
Sodium lactate	1.412 g/l
Sodium pyruvate	0.03 g/l
Bovine Plasma Albumin	1.00 g/l
Penicillin G (Potassium)	100 U/ml
Streptomycin sulfate	50 ug/ml
$NaHCO_3$**	1.7 g/l

* These components are included in Earle's Balanced Salt Solution in the specified concentrations.

** Sodium bicarbonate is added to the medium as a 1.3% solution.

MEDIA A	MEDIA B
(250 ml flask) 10 ml of Earle's balance salt solution in 90 ml of distilled water	(250 ml flask) 10 ml of Earle's balanced salt solution in 90 ml of distilled water
↓	↓
Add 0.2 ml of 60% solution of sodium lactate (energy source)	Add 0.3 gram of pyruvic acid sodium salt (energy source)
↓	↓
Combine Media A with 1 ml of Media B	
↓	
Add 0.1 gram of bovine plasma albumin (Nitrogen source)	
↓	
Using a 10 cc syringe filter 10 cc into a T-30 ml flask streptomycin and penicillin may be added as antibiotics	
↓	
Pipette 1.2 of sodium bicarbonate (1.3% isotonic solution) into T-30 ml flask containing Media A	
↓	
Slowly bubble 5% carbon dioxide through Media A (T-30 ml flask), obtain 6.8-7.2 pH range	

Table 2. Medium preparation for in vitro cultivation of mouse ova.

placed in the microdrops which in turn were placed in the incubator within a few minutes after the mice were sacrificed. Whenever desired, the culture dishes were removed and observed or photographed. After approximately 60 hours in culture the development of early blastocysts was observed.

As outlined by Spears and Walker (37), for an ovum to be selected or distinguished as normal, it had to meet the following criteria: fertilized, zona pellucida intact, blastomere of approximately equal size, and no other gross morphological abnormality. Ova not meeting this criteria were considered abnormal and were discarded immediately after recovery.

The number of ova developing to blastocysts after $2\frac{1}{2}$ days in culture was determined microscopically by using an inverted microscope (1 to 200X). In the very first stages of recovery a count of 2-and 4-cell ova per mouse was recorded (Table 3). The proportions of those ova developing to blastocyst were determined from the total number cultured at any one time.

Ova were recovered from hormone-treated females 48 hours after HCG injection (approximately 36 hours after ovulation). Ova were recovered from spontaneous ovulating females approximately 36 hours after the presumed time of ovulation. Evidence of time of ovulation was determined by the number of 4-cell ova present per total.

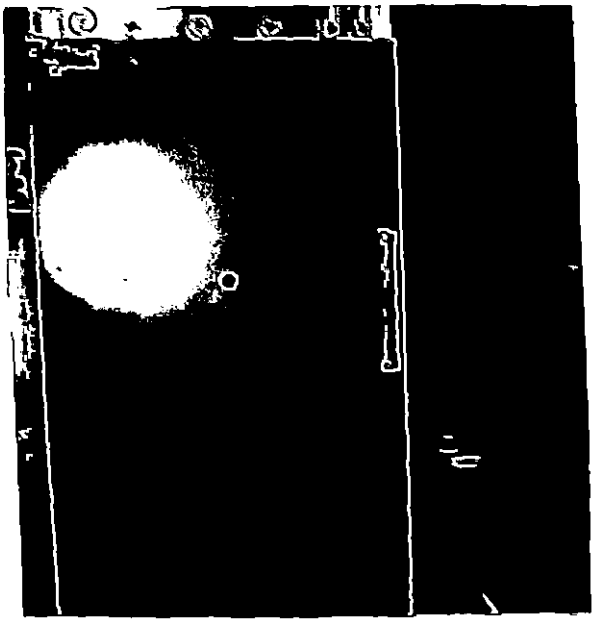

Figure 2. Hotpack Incubator #528.

According to the work of Weldon and Spears (40), approximately 20%,4-cell ova can be found 48 hours after HCG injection. It was assumed that equal proportions of 4-cell ova (20%) at 48 hours was an indication of ovulation time in spontaneous ovulators.

Percentages of all ova developing to blastocysts were recorded regardless of the number of 4-cell ova present. The ova from females yielding approximately equal proportions of 4-cell ova in both groups (hormone-induced vs

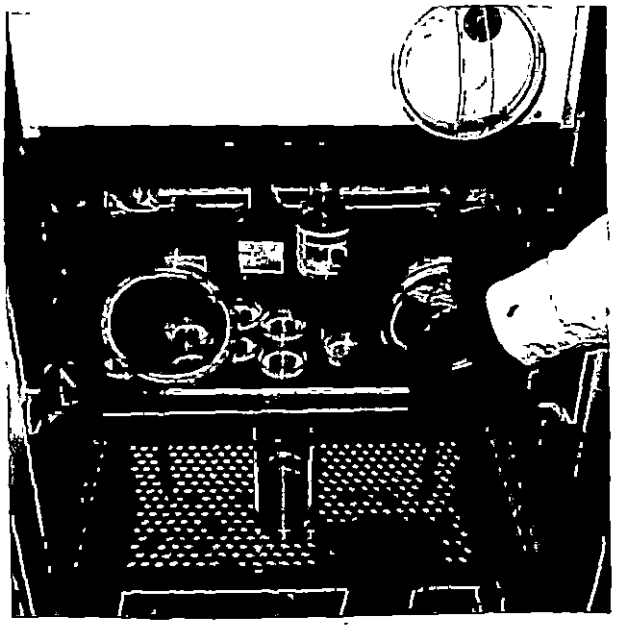

Figure 3. Plexiglass compartment installed in the incubator to facilitate retention and reequilibration of CO_2 atmosphere.

spontaneous) were then compared as to their developmental efficiency.

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.

RESULTS

The experimental data obtained in this study is found in Table 3 and Figures 1, 4, and 5. A total of 1012 mouse ova was cultured *in vitro*, and 89.3% developed from the late 2-cell or early 4-cell stages (Fig. 4) to blastocysts (Fig. 5). These results are in accord with those presented by Brinster (7), Spears and Walker (37) who demonstrated that 60 to 100% of the 2-cell ova recovered 45 hours after HCG injections developed to blastocysts.

The data collected in the present study shows the ability of ova from hormone-treated females of the Swiss-Webster strain of mice to develop from early preimplantation stages to blastocyst *in vitro* as compared with that of ova of equal development from spontaneous ovulations. The percentage of 4-cell ova (Table 3) found at the time of recovery (Fig. 1) was 20.2% for the hormone-treated ovulator and 20.3% for the spontaneous ovulator. It is suggested that these percentages are indicative of ovulation times, particularly the spontaneous ovulators.

The data collected was 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=0.05 level.

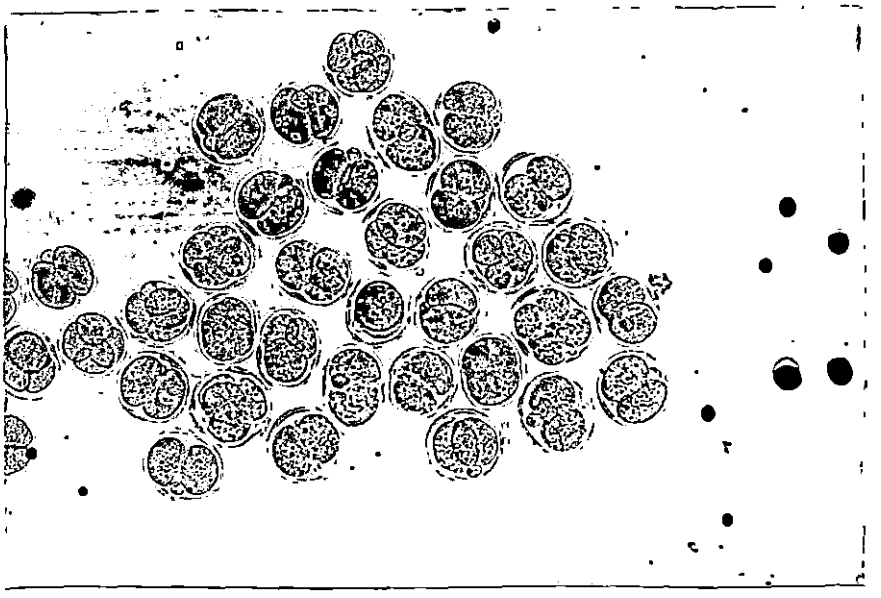

Figure 4. 2-and 4-cell mouse ova at beginning of *in vitro* culture-200X.

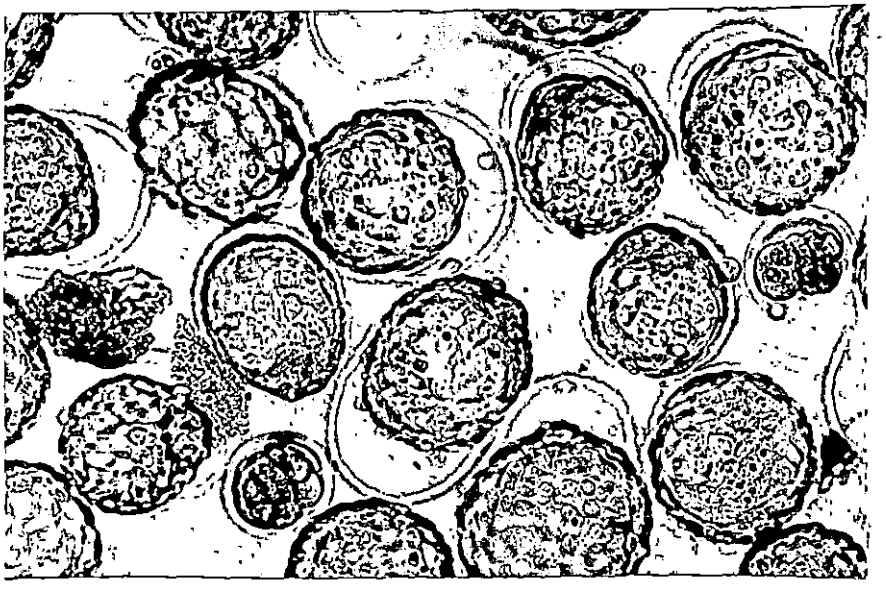

Figure 5. Blastocysts developed from 2-and 4-cell ova cultured *in vitro* for 60 hours-200X.

TABLE 3. PROPORTIONS OF OVA DEVELOPING FROM 2-AND 4-CELL STAGE TO BLASTOCYST RECOVERED FROM HORMONE-TREATED AND SPONTANEOUSLY OVULATING SWISS-WEBSTER FEMALE MICE

Hormone-Induced Ovulation Swiss-Webster			Spontaneous Ovulation Swiss-Webster		
% of 4-cell ova recovered	2-and 4-cell ova developed /total No.	% of total development	% of 4-cell ova recovered	2-and 4-cell ova developed /total No.	% of total development
20.0	15/15	100.0	20.0	15/15	100.0
20.0	20/20	100.0	21.7	23/23	100.0
19.2	25/26	96.2	20.7	29/29	100.0
19.5	39/41	95.1	18.2	11/11	100.0
19.4	28/31	93.2	22.2	26/27	96.3
20.5	36/39	92.3	18.2	21/22	95.5
22.7	20/22	90.9	22.7	21/22	95.5
20.0	27/30	90.0	19.1	19/21	94.8
20.0	18/20	90.0	17.6	16/17	94.0
20.0	22/25	88.0	19.2	24/26	92.3
19.4	27/31	87.1	20.8	22/24	91.7
22.7	19/22	86.4	20.8	31/34	91.2
19.1	18/21	85.7	20.0	32/35	91.1
22.9	30/35	85.7	20.0	27/30	90.0
21.1	32/38	84.2	22.2	8/9	88.8
20.8	20/24	83.3	14.3	6/7	85.7
18.2	9/11	81.8	20.0	21/25	84.0
19.1	17/21	80.9	20.0	22/25	84.0
20.0	12/15	80.0	18.1	9/11	81.8
21.1	15/19	78.9	20.0	12/15	80.0
19.2	20/26	76.9	16/9	14/18	77.7
19.1	16/21	76.2	15/5	10/13	76.9
Total 20.2	485/553	87.6	20.3	419/459	91.3

Overall Total 904/1012=89.3%

DISCUSSION

The culture technique used in this study was designed to allow 2-and 4-cell mouse ova to develop to blastocysts *in vitro*. This culture technique is a modification of Brinster's technique as presented by Spears and Walker (37). The simplicity of the preparation of this culture medium has been brought about by using commercially prepared salts (EBSS). This preparation requires only a few minutes to prepare and in what ever quanity desired. Glucose which is found in the EBSS is contained in the medium as an energy source, as well as lactate and pyruvate which are not found in the EBSS. The pH adjustment was accomplished by the addition of isotonic sodium bicarbonate solution (1.3%). To obtain a pH of approximately 6.8-7.2 after equilibration, preliminary studies showed that 1.2 ml is the optimum quanity of bicarbonate solution to add to 10 ml of the medium.

It was necessary to leave the ova of the spontaneous ovulator in the oviduct for approximately 36 hours after ovulation. This was to ensure sufficient development *in vivo* to allow continued development *in vitro*. The presence of 4-cell ova was a true indication that sufficient time had elapsed. The recovery of ova from hormone-treated females was performed 48 hours after

HCG injection which meant the ova remained in the oviduct approximately 36 hours after ovulation, since ovulation occurs approximately 12 hours after HCG injection.

It should be noted that the developmental efficiency of 4-cell ova did not exceed that of 2-cell ova.

The recovery of 4-cell ova is the first indication that the necessary time requirement has elapsed to allow continued development of ova *in vitro*. The percentage of 4-cell ova per total ova has been shown to be an indication of ovulation time in hormone-treated females (40).

In this study the proportion of 4-cell per 2-cell ova was taken as an indication of ovulation time. Only the spontaneous ovulating females yielding 4-cell ova in proportion to the hormone-treated females were compared.

Spontaneous ovulators, yielding only 2-cell ova were not compared, since the presence and proportions of 4-cell ova is our best criteria of ovulation.

The difference in developmental capacity of hormone-induced and spontaneous ovulating ova was not significant. The insignificance of the developmental results presented in this study infers that the viability (development of ova *in vitro*) in both the hormone-induced and the spontaneous ovulator is approximately the same.

SUMMARY

The techniques employed in this study were designed to make comparisons of the viability of ova in the Swiss-Webster strain of mice. Viability comparisons were made between ova recovered from hormone-treated females and spontaneously ovulating females. A total of 1012 ova was examined of which 904 developed to blastocysts *in vitro*. The percentage difference of development between the hormone-treated ovulatory ova and that of the spontaneous ovulatory ova was not significant. This strongly indicates that the viability of ova obtained from hormone-treated females is equal to that of ova from spontaneous ovulators in the Swiss-Webster strain of mice.

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