IN VITRO EMBRYOGENESIS: AN EXPERIMENTAL MODEL FOR
THE UNDERSTANDING OF REPRODUCTIVE PHYSIOLOGY AND
DEVELOPMENT IN MAMMALS

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A complete in vitro technique has been developed in the mouse for capacitation of epididymal spermatozoa, maturation of ovarian oocytes and fertilization of such gametes to give rise to viable embryos, which, when transplanted to proper recipients result in normal, fertile progeny. This technique, although it requires perfection, has many future possibilities and potential applications as a research tool in solving problems of mammalian reproductive physiology and developmental biology. It may be useful in the investigation of (a) gene action in the X chromosome and initiation of X-inactivation during embryogenesis; (b) nuclear transplantation and the ontogenetic commitment of oocyte cytoplasm; (c) the role of drugs, mutagens and carcinogens in the fetal environment; (d) genetic engineering; eg. enzyme induction and (e) many problems involving fertility and sterility.

Introduction

Progress in the understanding of early mammalian development achieved in the last decade has been greatly facilitated by the availability of improved tissue culture media and technique which allow for the growth and manipulation of embryos in vitro. In spite of the wealth of information which has been amassed, a paucity of knowledge still exists, particularly with respect to pre-fertilization phenomena, such as oocyte maturation, sperm capacitation and fertilization itself. Embryonic development during the earliest cleavage stages, particularly differentiation of tissue and organ anlage, is an area of extreme interest about which relatively little is known. Since it has been demonstrated that patterns of embryonic development are similar among laboratory animals (Yanagimachi and Chang, 1964) (eg. mouse, rat, rabbit) and are comparable to the large domestic animals, extrapolation to man may be feasible. Therefore, studies on experimental species will form the basis of most work in the field of reproduction physiology and embryogenesis. This presentation will describe our attempts at developing a completely in vitro system which would facilitate the investigation of the earliest events in gametogenesis, fertilization and embryogenesis under controlled conditions.

In Vitro Fertilization

Fertilization of mammalian ova in vitro has been successfully carried out in the rabbit (Chang, 1959; Thibault and Dauzier, 1961; Brackett and Williams, 1965) and the hamster (Yanagimachi and Chang, 1963; Barros and Austin, 1967) but development of fertilized ova beyond the two-cell stage in these species has not been achieved (Yanagimachi and Chang, 1964). In the mouse, however, Brinster and Biggers (1965) have observed in vitro fertilization and embryonic development to the blastocyst stage using explanted fallopian tubes in organ culture as a support. More recently, Whittingham (1968) has successfully fertilized mouse eggs in vitro, the transplantation of which into pseudopregnant recipient mothers yielded 17-day-old fetuses. Our initial efforts at in vitro fertilization concentrated on attempts using mature tubal oocytes and capacitated spermatozoa re-
retrieved from the uterus following a successful mating (Mukherjee and Cohen, 1970).

The mice used in all the studies to be described were of the ICR/Ha (albino) and C57BL/6J (black) strains obtained from West Seneca Laboratories of the Roswell Park Memorial Institute, Buffalo, New York. Large numbers of ova are readily obtainable following superovulation of mature mice (6-8 weeks of age) by an intraperitoneal injection of 5 international units (IU) of pregnant mare's serum gonadotrophin (PMS) followed 48 hr later by an injection of 5 IU of human chorionic gonadotrophin (HCG). Ten to twelve hours after the second injection, the mice are sacrificed by cervical dislocation and the oviducts excised and uncoiled. Unfertilized tubal ova are flushed from the oviducts into a petri dish (60 mm diameter) by injecting 0.5 ml of normal saline into the ampullary-isthmal junction with a 30-gauge needle (Fig. 1). The eggs are then isolated from cellular debris by a finely drawn capillary micropipette and transferred to a second petri dish containing 0.5 ml of tissue culture medium, diluted with normal saline to half the original concentration. The composition of the medium was almost identical to that of Whitten and Biggers (1968) (oocyte medium) with the exception that sodium pyruvate concentration was 0.55 mM rather than 0.33 mM. The pH of the medium was maintained at approximately 7.4 by intermittent administration of 5% CO₂ in air.

Fig. 1. Scheme for induction of superovulation by injection of pregnant mare's serum (PMS) and human chorionic gonadotrophin (HCG).
Capacitated spermatozoa, as defined by Austin (1951) and Chang (1951), were obtained from the uteri of mice 3-4 hr after mating with a proven fertile male. The uteri are dissected out and their contents released into a petri dish containing 1 ml of half-strength growth medium at 37°C. The petri dish is immediately agitated to prevent coagulation of the sperm. Approximately 0.3 ml of sperm suspension was added to a cavity slide containing a number of unfertilized ova in medium. The mixture of the eggs and sperm is layered over with paraffin oil (Fisher Scientific, viscosity 125/135) so that the well is filled, and the slide incubated for 6-8 hr at 37°C in a 5% CO₂ atmosphere. Ova, without the addition of sperm, are cultured as controls.

After incubation, both control and treated ova are thoroughly washed with normal saline and placed in culture using the methods of Brinster (1968) and Chang and Pickworth (1968). The petri dishes are examined after 24 hr for cleaving zygotes and the two-cell embryos are placed into new petri dishes with.

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Fig. 2. In vitro fertilization and embryo maturation: 1. Mature oocyte with first polar body. 2-3. Sperm penetration and formation of second polar body. 4-7. Early cleavage stages - 2-, 4-, 6- and 8-cell stages. 8-9. Blastocyst stage.
fresh medium (full strength). These zygotes develop to the blastocyst stage (64-130 cells) after approximately 3 days in culture (Fig. 2). Although some control eggs fragment during their culture period, no actual cleavage was observed in the unfertilized ova.

The blastocysts are then implanted into pregnant or pseudopregnant females (Fig. 3). The recipient mothers were made pseudopregnant through mating with proven sterile, vasectomized males. The coat color of both the vasectomized males and the recipient mothers was always opposite to that of the implanted embryos. In some experiments, normally pregnant mice were used as recipient mothers instead of pseudopregnant animals. These mating were designed so that the progeny derived from the in vitro and in vivo fertilization were clearly distinguishable by coat color.

![Fig. 3. Diagrammatic scheme for the transplantation of in vitro derived blastocysts.](image)

The appearance of the vaginal plug was taken as evidence of mating and counted as day zero of pseudopregnancy. After 3 days of growth in vitro the blastocysts were collected into a glass capillary micropipette connected to a small rubber tube. A dorsal incision was made, the uterotubal junction punctured by the micropipette so that the open tip reached the lumen of the uterus, and the blastocysts were expelled into the uterus (Fig. 4a). Figure 4b shows implanted embryos in only one uterine horn of a pseudopregnant female; the second horn was not used. Birth usually occurred 20-21 days after in vitro fertilization.

Table I shows the pooled results of six experiments. Of 253 eggs exposed to capacitated spermatozoa, 67 were fertilized and appeared as two-cell embryos; 25 of these developed blastocysts. Twenty-three blastocysts were transplanted into recipient mothers and 11 apparently normal offspring were obtained. Although only approximately 5% of the fertilized ova developed to the blastocyst stage, 51% (11) of the implanted blastocysts gave rise to progeny. Therefore, the most difficult part of this system, from the standpoint of increased yield, appears to be the 3-day culture period necessary for blastocyst development. Nine of the eleven mice were born to pseudopregnant mothers and the remaining two to normally pregnant females. Figures 5a and 5b illustrate litters of in vitro developed blastocysts as live-born offspring. Two of the females derived from in vitro fertilization were mated with fertile males and delivered normal progeny.
Fig. 4.  a. (upper) Transplantation of in vitro derived blastocysts into surrogate mother; b. (lower) Recipient uterus 8 days following blastocyst implantation. Note the presence of embryos in one uterine horn only; the second horn was not used.
These experiments conclusively demonstrate that in vitro fertilization of tubal mouse oocytes with capacitated spermatozoa results in viable embryos, which upon transplantation to proper recipients give rise to normal progeny.

In Vitro Capacitation of Epididymal Spermatozoa

Although the above experiments indicated that in vitro derived blastocysts could be obtained, implanted and subsequently developed to normal mice, the system was still dependent upon the animal for the production of mature ova and capacitated sperm. Therefore, our efforts next centered on attempts to capacitate epididymal sperm in vitro.

Existing evidence suggests that mammalian spermatozoa undergo functional changes during their passage through the female genital tract which enable them to penetrate the ovum. This phenomenon was described independently by Austin (1951) and Chang (1951) and generally is known as sperm "capacitation." The morphological criterion of capacitation used by these authors was the loss of staining ability of the acrosome. To date, it has been possible to determine the need for sperm capacitation in only a few mammalian species (cow, hamster, rat and mouse) (Bedford, 1970) and the actual factors, responsible for such capacitation are not well delineated. Previous experiments using the golden hamster have suggested that the active sperm capacitating factors are not derived from the oviduct or uterus (Barros and Austin, 1967) but from the follicular fluid released into the genital tract during ovulation (Yanagimachi, 1969a). Recently, in vitro capacitation of mouse (Iwamatsu and Chang, 1969) and hamster (Yanagimachi, 1969b; Gwatkin and Anderson, 1969) spermatozoa has been achieved by treatment with heat inactivated bovine follicular fluid. We have successfully used follicular and tubal fluids as possible capacitating treatments on spermatozoa derived from the rat, mouse and man (Mukherjee and Lippes, 1972).

Samples of fresh spermatozoa were obtained from mature Wistar rats (albino) and the C57BL/6J and ICR/Ha mice strains. The animals were sacrificed by cervical dislocation and the epididymis was teased apart in 0.2 ml of normal saline. Fresh ejaculates from normal fertile human volunteers were also collected and diluted with 0.5 ml of normal saline. Thin films of spermatozoa from all three species were spread directly on microscope slides to serve as controls.

Tubal and follicular fluids were collected by the technique of Lippes et al. (1972) from human patients undergoing voluntary tubal ligation and other surgical procedures. 0.2 ml aliquots of the sperm samples were placed in petri dishes (60 mm diameter) and incubated with 0.3 ml of each of the following human genital tract fluids: 1) follicular fluid — without heat inactivation; 2) follicular fluid — heat inactivated (56°C for 30 min); 3) a mixture of heat inactivated follicular fluid: tubal fluid (1:2); 4) tubal fluid; 5) normal saline.

These mixtures were agitated for 15-20 sec and covered with a thin layer of paraffin oil. The culture dishes were then incubated at 37°C in an atmosphere of 5% CO₂ in air for 4-6 hr. After incubation a few drops of the sperm-genital tract fluid preparation were placed on a microscope slide in a thin film.

Fig. 5. Live-born progeny of in vitro fertilization: a. (upper) Three in vitro derived (white) blastocysts were introduced into the uterus of a normally pregnant female (black) yielding two white offspring. b. (lower) Four in vitro derived (black) blastocysts were introduced into the uterus of a pseudopregnant white mouse yielding the two black offspring.
A single solution procedure was used to stain the spermatozoa for the presence
or absence of acrosome (Casarett, 1953). The stain consisted of an aqueous solu-
tion of 5% Aniline Blue and 5% Eosin. The sperm-containing slides were stained
for 15 min, then rinsed in tap water, followed by dehydration in a series of alcohols
(70%, 95% and absolute ethanol), immersed in Xylene and mounted in permount.
Under these conditions, the acrosome stains blue and the sperm nucleus red. For
each treatment 1,000 spermatozoa were counted at random from several slides and
the presence or absence of acrosome staining was noted and scored.

Table II shows the effects of various fluid treatments on rat, human and mouse
spermatozoa. In the direct preparations, with no pretreatment by the genital tract
fluids, all sperm retained staining of the acrosome. In man and rat no significant
difference in the frequency of acrosome staining loss was observed when compared
to sperm treated with uninactivated follicular fluid or the saline treated controls
(human — 7% vs. 0%; rat — 3.5% vs. 1%). However, in the mouse 20.5%
of the sperm lost the acrosome staining when treated with uninactivated follicular

![Fig. 6. Types of immature oocytes collected directly from the ovary of mature mice:
a. No visible germinal vesicle. b. One nucleolus and one germinal vesicle. c. Germinal
vesicle with 2 “nucleoli”. d. Germinal vesicle with 3 “nucleoli”.

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fluid, as opposed to 1.5% in the untreated controls. On the other hand, treatment with tubal fluid alone led to a loss of acrosome staining in human (58.5%) and rat sperm (40.2%), while in the mouse the effect was comparable to non-heat inactivated follicular fluid (26% vs. 20.5%).

Since untreated follicular fluid led to the almost immediate immobilization of the sperm, heat inactivation (56°C for 30 min) was used to counteract this effect. This phenomenon had already been demonstrated for bovine follicular fluid (Yanagimachi, 1969b). Treatment of sperm with heat inactivated follicular fluid led to considerable loss of acrosomal staining in all species, although not to the same degree (Table II). Increases of 10-fold and 15-fold loss of acrosome staining were observed in human and rat sperm, respectively (67.6% vs. 7.0% and 43.6% vs. 3.5%). The increase in the mouse, however, was only 11.5% (32% vs. 20.5%).

Apparently, heat inactivation alters human follicular fluid in such a way as to enhance the loss of acrosome staining reaction in the sperm of rats, mice and humans. Although heat inactivated follicular fluid, as well as nontreated tubal fluid alone, caused loss of acrosome staining, the most effective treatment in all three species was a combination of the two in a ratio of 1:2 (heat inactivated follicular fluid: tubal fluid). Such a treatment yielded the loss of acrosome staining in 80% of human, 74% rat and 50.3% of mouse spermatozoa (Table II).

In addition to the loss of acrosome staining, the functionality of in vitro capacitation was tested by using mouse sperm treated with various fluids and their combinations for in vitro fertilization. Table III shows the results of pooled data on in vitro fertilization from approximately equal numbers of matured tubal oocytes from two mouse strains (C57BL/6J and ICR/Ha). Control sperm (treated with normal saline or exposed to tissue culture medium) did not effect fertilization. Although five apparent two-cell embryos were observed after exposure to culture medium, four of these were abnormal, representing fragmentation of oocytes. Similarly, two such fragmented oocytes were observed after utilization of saline treated sperm. No blastocysts were obtained from these abnormal embryos.

On the other hand, sperm treated with tubal fluid alone were capacitated, as evidenced by the appearance of normal two-cell mouse embryos in 15% of the oocytes tested by in vitro fertilization (14/88). Of these, seven (50%) developed to normal blastocysts in 3 days of in vitro growth. The use of heat inactivated fluid alone resulted in two-cell embryos in 21.3% (23/108) of oocytes with 65.2% (15/23) developing to blastocysts. Again, the most effective treatment was a 1:2 mixture of inactivated follicular fluid: tubal fluid, yielding 59.5% (72/121) two-cell embryos of which 77.7% (56/72) developed to blastocysts.

Use of partially capacitated spermatozoa (treated for 2 hr or less) in in vitro fertilization resulted in abnormal development of embryos. Attempts at in vitro fertilization of human ova with sperm treated by a 1:2 mixture of heat inactivated follicular fluid: tubal fluid proved unsuccessful. From a total of 12 human ova mixed with treated sperm, the only positive result was the formation of pronuclei in one egg.

In Vitro Maturation of Follicular Oocytes

The foregoing experiments demonstrated the feasibility of sperm capacitation in vitro. The next step to complete the in vitro system was the maturation of follicular oocytes.
### Table I

In vitro fertilization and implantation of blastocysts

<table>
<thead>
<tr>
<th>Donor strains*</th>
<th>No. oocytes cultured</th>
<th>No. two-cell embryos</th>
<th>No. blastocysts</th>
<th>No. blastocysts transplanted</th>
<th>Recipient</th>
<th>No. progeny obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ova Sperm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>130</td>
<td>34</td>
<td>13</td>
<td>13</td>
<td>B</td>
<td>5 (A)</td>
</tr>
<tr>
<td>B</td>
<td>123</td>
<td>33</td>
<td>12</td>
<td>10</td>
<td>A</td>
<td>6 (B)</td>
</tr>
<tr>
<td>Total</td>
<td>253</td>
<td>67</td>
<td>25</td>
<td>23</td>
<td></td>
<td>11</td>
</tr>
</tbody>
</table>

*A = ICR/Ha (albino); B = C57BL/6j (black).

### Table IV

Mouse progeny resulting from the complete in vitro development of transplanted blastocysts

<table>
<thead>
<tr>
<th>No. ovarian oocytes (immature)</th>
<th>No. mature oocytes (with 1st polar body)</th>
<th>No. mature oocytes used for in vitro fertilization</th>
<th>No. two-cell embryos</th>
<th>No. blastocysts</th>
<th>No. transplanted</th>
<th>No. progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td>1200</td>
<td>720</td>
<td>600</td>
<td>138 (23%)</td>
<td>48 (35%)</td>
<td>48</td>
<td>22 (13 ♂️:9 ♀️)</td>
</tr>
</tbody>
</table>
Follicular oocytes were obtained from the ovaries of 6-8-week-old mice. Approximately 30 to 50 immature oocytes, with a well-defined germinal vesicle (Fig. 8), were obtained from each mouse by teasing the ovarian follicles in a 1:1 mixture of normal saline and Ham's F-10 tissue culture medium at pH 7.2. The oocytes were separated from cellular debris and cultured from 8 to 10 hr in a drop of Ham's F-10 or modified Whitten and Bigger's medium (sodium pyruvate concentration of 0.60 mM) in a 60 mm plastic petri dish under paraffin oil at 37°C in an atmosphere of 5% CO₂ in air.

One of the causes of abnormal fetal development may be errors during the preceding meiotic divisions. However, only a few studies have concerned the direct examination of gametes (Donahue, 1968, 1970). In our laboratory, various types

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### Table II

Loss of acrosome staining induced by human follicular and tubal fluids

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% sperm without acrosome staining*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Human</td>
</tr>
<tr>
<td>Control (direct)</td>
<td>0.0</td>
</tr>
<tr>
<td>Control (saline)</td>
<td>0.0</td>
</tr>
<tr>
<td>Follicular fluid (without inactivation)</td>
<td>7.0</td>
</tr>
<tr>
<td>Follicular fluid (inactivated at 56°C for 30 min.)</td>
<td>67.6</td>
</tr>
<tr>
<td>Inactivated follicular fluid: tubal fluid (1:2)</td>
<td>80.0</td>
</tr>
<tr>
<td>Tubal fluid</td>
<td>58.6</td>
</tr>
</tbody>
</table>

*At least 1,000 sperm counted for each treatment.

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### Table III

Biological evidence of in vitro sperm capacitation demonstrated by blastocyst development*

<table>
<thead>
<tr>
<th>Treatment of sperm</th>
<th>No. eggs</th>
<th>Two-cell embryos</th>
<th>Blastocysts</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>86</td>
<td>0 (5 fragmented)</td>
<td>0</td>
</tr>
<tr>
<td>Saline</td>
<td>95</td>
<td>0 (2 fragmented)</td>
<td>0</td>
</tr>
<tr>
<td>Media</td>
<td>93</td>
<td>5 (1 normal; 4 abnormal)</td>
<td>0</td>
</tr>
<tr>
<td>Follicular fluid (without inactivation)</td>
<td>89</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Follicular fluid (inactivated at 56°C for 30 min.)</td>
<td>108</td>
<td>23</td>
<td>15</td>
</tr>
<tr>
<td>Inactivated follicular fluid: tubal fluid (1:2)</td>
<td>121</td>
<td>72</td>
<td>56</td>
</tr>
<tr>
<td>Tubal fluid</td>
<td>88</td>
<td>14</td>
<td>7</td>
</tr>
</tbody>
</table>

*The data represent pooled results of experiments using both ICR/Ha and C57BL/6J strains of mice.
Fig. 7. Abnormalities in chromatin distribution in the meiotic divisions of oocytes maturing in vitro: 1. Equal distribution of chromatin. 2. Large polar body including the entire chromatin complement. 3. All chromatin material extruded into polar bodies. 4. All chromatin material retained in the oocyte.

of abnormalities were observed during the cytological study of some 3,000 ovarian oocytes during in vitro maturation. Some oocytes, directly upon release from the follicle, were seen to possess varying numbers of nucleolus-like structures in the germinal vesicles (0-3) (Fig. 6), while those with only a single nucleolar structure in the vesicle appeared to mature normally. Most of the abnormalities, however, involved the distribution of chromatin material between the polar body and the functional oocyte at the meiotic divisions. Four types of errors were seen (Fig. 7) and their distribution in 3,000 oocytes examined were as follows: 1) equal amount of chromatin in each segment — 4 oocytes (0.16%); 2) large polar body possessing all the chromatin — 15 oocytes (0.50%); 3) all the chromatin extruded into the polar bodies — 4 oocytes (0.16%); 4) all the chromatin retained within the oocyte — 2 oocytes (0.06%). Such abnormalities were present in approximately 0.88% of the immature oocytes examined. Naturally, fertilization of these eggs would lead to aneuploid offspring.

The functionality of in vitro oocyte maturation was tested by the use of such eggs in fertilization tests. The oocytes were examined at the end of the culture period to check for the presence of metaphase II configuration, indicative of maturity. After 13-15 hr of culture the majority of the oocytes had already extruded the first polar body and the chromatin was in the metaphase II stage. The sequential development of the oocytes are represented in Figs. 8 and 9.
Approximately $1 \times 10^5$ mouse spermatozoa (0.3 ml) capacitated in vitro were added to a petri dish containing 50 oocytes matured in vitro as described above. The mixture of eggs and sperm was completely covered with a layer of paraffin oil and incubated 37°C in 5% CO₂ in air. After 24 hr, the cultures were examined for two-cell embryos, which were transferred to fresh medium. Within 3 days, the two-cell embryos developed to the blastocyst stage and transplantation of such blastocysts to proper recipients, as described above, was then performed. Table IV shows the pooled results of 12 experiments. Of 1200 oocytes cultured, 720 shed the first polar body and were considered as mature. In vitro fertilization of 600 of these oocytes was attempted and 138 (23%) apparently normal two-cell embryos were obtained. Forty-eight embryos developed to blastocysts (Fig. 10) and their transplantation to surrogate mothers resulted in 22 normal progeny (13♂:9♀) which were subsequently found to be fertile.

Discussion

Current Problems of In Vitro Embryogenesis

Advances in tissue culture procedures and formulation of support media have made feasible the maturation of oocytes in vitro from various species, including mouse, rat, hamster, sheep, monkey, rabbit, cow and man (Pincus and Enzmann, 1963).

Fig. 8. Normal maturation of mouse ovarian oocytes in vitro: a. Immature oocyte upon liberation from ovarian follicle. b. Chromatin configuration (dictyotate stage) at onset of the maturation process. c. Chromatin condensation at onset of maturation period. d. Chromatin condensation after 2 hr of maturation in vitro.
Recently, the successful fertilization of in vitro matured oocytes by normal capacitated spermatozoa in the mouse, followed by subsequent transplantation of the developing embryo to proper recipients, resulted in 14-day- or 15-day-old fetuses (Cross and Brinster, 1970). Obviously, an in vitro system, as described above, provides an experimental model which could be applied to the detailed and critical analysis of various areas of reproductive physiology and developmental biology. However, one important goal in this area of research is the development of a system which would allow observation of the entire process, beginning with male and female gametogenesis and proceeding to the development of a normal animal in vitro. Such a system would be totally independent of parental participation and
maternal environment and would require still further progress in new techniques and approaches. The greatest obstacle at the present time is that stage from in vitro fertilization to the development of the blastocyst. The experiments described above indicate that the highest rate of attrition in our system occurs during this period. The efficiency of this stage of embryonic development will undoubtedly improve as innovations in support medium are made to enhance the survival of early cleavage embryos.

The sustenance of post-blastocyst embryos in vitro must also be accomplished and some modicum of progress has already been achieved in this field. Hsu (1971) recently reported the initiation of cardiac function (heart beat) in post-blastocyst mouse embryos maintained in vitro. Zapol et al. (1969) have described an artificial placenta capable of maintaining a premature lamb fetus, and the stable metabolic state of this fetus for several days indicates that extra-uterine survival is within the realm of feasibility. These studies are impressive but progress towards a complete in vitro system demands the prior solution of a multitude of more basic issues.

Perhaps one of the more pressing difficulties to overcome is the perfection of various tissue culture media necessary for the different stages of embryonic develop-
ment. Suitable media for the initial periods, eg. maturation of oocytes, capacitation of sperm, fertilization and growth to blastocysts, have already been achieved to some degree (Whitten and Biggers, 1968). For later periods, the approach will necessitate an extremely detailed analysis of the uterine environment and its changes during pregnancy. This must begin with the fractionation of genital tract fluids, follicular and tubal. Such analyses have already been attempted by Lippes et al. (1972), who have provided excellent data on the identification and possible function of some constituents of human tubal fluid. Additionally, some protein components of oviductal fluid in primates, including man, have also been determined. Such data on normal uterine metabolism is prerequisite to the adaptation of tissue culture media which may serve a similar purpose. The elucidation of those physiological changes in the endometrium and uterus during gestation is also critical before in vitro techniques can be established to mimic these functions. A practical by-product of such information may lead to more effective contraceptive methods. Although these problems, at present, may seem overwhelming, the technology necessary for their solution is available and it may be only a matter of time until workable solutions will be forthcoming.

Future Possibilities and Potential Applications of In Vitro Embryogenesis

Some practical applications of an artificial system of fetal development have already been made in the field of livestock improvement (Mann, 1969). Embryo transfer techniques have been successfully employed in the transfer of cattle and sheep from different parts of the world using unrelated animals as carriers (Dziuk, 1968). Prenatal mortality in rabbits due to advanced maternal age has been investigated through embryo transfer studies. Sex ratios of rabbit litters can now be controlled by sex chromatin determination of excised trophoblastic cells prior to transplantation to the pre-sexed fetuses into a recipient mother. Examination of the morphological sex at a later stage of embryonic development was in agreement with the early prediction (Gardner and Edwards, 1968).

Although sperm penetration of the ovum in vitro has been described, this is but one event in the complex process of fertilization. Very little is known concerning the molecular aspects of fertilization, eg. the onset of de novo synthesis of nucleic acids, membrane changes, and shifts in cellular metabolism from the gametic stage to that of the pronucleus and zygote. An in vitro system would obviously allow detailed studies of these phenomena (Mukherjee, 1972).

It is well established that more than 30% of first trimester spontaneous abortions in man are chromosomally abnormal. The vast majority (60%) of such events can be directly traced to cytogenetic problems in meiosis in the egg, principally nondisjunction leading to trisomy or monosomy through chromosomal malsegregation prior to implantation. Our ability to study the maturation process of the ovum in vitro under rigidly controlled conditions may elucidate the mechanisms of events leading to such fetal wastage.

Gene action in the X chromosome of mammals is believed to be regulated by the inactivation of one of the two X chromosomes in each somatic cell of females. This X-inactivation hypothesis (Lyon, 1961) has been proved for the most part; however, the matter of timing of X-chromosome inactivation is unclear. The application of an in vitro system to early stages of embryonic cleavage, coupled with autoradiography or biochemical techniques, would facilitate the identification of the precise cell stage, in various species, at which one of the female X chromosomes becomes inactivated and initiates its late DNA replication pattern.
Environmental Mutagenesis

Great interest and concern has been expressed in the past few years over the problem of environmental mutagenesis; i.e. the effects of various agents common to our environment. This concern has reached such proportions that the federal government is considering legislation which will require the screening of all new drugs for mutagenicity prior to their release to the public. Currently, three methods are employed for mutagenicity assays; each method alone has obvious shortcomings and therefore the development of additional sensitive methods is imperative. The use of drugs during pregnancy has, in the past, usually been directed towards the treatment of maternal disease with little concern for the fetus as a drug recipient. However, such therapeutic endeavors have been associated with unexpected and often tragic results in the developing fetus for whom the drug was not intended.

The technique of in vitro fertilization and development of embryos offers the unique opportunity to develop an assay system for screening possible mutagenic and teratogenic effects of drugs and other exogenous agents. The great advantage of this system is the ability to mature and obtain functional gametes in vitro under carefully controlled conditions. Following this step, treatment of sperm (prior to or following capacitation) or the ovum (during its maturation in vitro) prior to fertilization may be attempted. Additionally, any particular stage of zygotic development could be treated and embryos examined at various stages, pre- or post-implantation, for such parameters as chromosomal aberrations, congenital malformations, increased resorption frequency, and, if allowed to progress to term and mature, the animals could be screened for the development of neoplasia. A wide variety of chemical and viral agents, as well as physical treatments (eg. radiation or nutritional effects), may be assayed in this manner.

DNA Transfer

Experiments could be designed, using the in vitro technique, to attempt genetic transformation by injection of purified DNA. It has been observed that amphibian diploid nuclei, when introduced into an enucleated oocyte, induced swelling and replication of this nucleus (Gurdon, 1968). In addition, when isolated DNA was injected into an oocyte, it replicated and acted as a template for mRNA production. As a simple test for the mammalian system, DNA can be isolated and purified from tissue culture cells of C57BL/6J (black) and ICR/Ha (albino) following the method of Marmur (1961). DNA from black mice could be injected to single cell zygotes, obtained by in vitro fertilization of sperm and egg from albino mice. These embryos could be grown to the blastocyst stage and transplanted to white recipients and vice versa. Coat color will then indicate transformation.

Enzyme Induction

Homozygous Gunn rats are genetically deficient in the enzyme UDP-bilirubin glucuronyl transferase (UDP-glu-transferase), an inducible enzyme in both humans (Yaffe et al., 1966) and in animals (Catz and Yaffe, 1968). DNA could be isolated from the normal rat fibroblast cultures and injected into single cell zygotes of homozygous Gunn rats obtained for in vitro fertilization. These embryos, after development to the blastocyst stage, could be transplanted to the recipient homozygous Gunn rat. If analysis of the progeny shows the absence of jaundice and presence of the enzyme, the true concept of genetic engineering would be supported. Preliminary experiments in our laboratory, using unfertilized mouse oocytes as well as early embryos, show that such micromanipulation is possible and oocytes and
Embryos can develop normally following injection of foreign substances (Mukherjee, in prep.).

The above outlined experiments represent but a few examples of the myriad of problems and questions in genetics and reproductive physiology that might be approached by exploiting the technique of in vitro embryogenesis. From the standpoint of increased yields, this system needs to be vastly improved and refined; however, the present technique is currently capable of serving as a productive research tool.

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References


