FERTILIZATION AND EMBRYONIC DEVELOPMENT *IN VITRO*

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PLENUM PRESS • NEW YORK AND LONDON

Library of Congress Cataloging in Publication Data

Main entry under title:

Fertilization and embryonic development in vitro.

Bibliography: p.
Includes index.
1. Fertilization in vitro. 2. Embryology, Experimental. I. Mastroianni, Luigi. II. Biggers, John D., 1923- . [DNLM: I. Fertilization in vitro. 2. Embryo transfer. 3. Embryo. 4. Embryo, Non-mammalian. WQ 205 F411]
QP273.F47 599.03'3 81-13829
ISBN-13: 978-1-4684-4018-8 e-ISBN-13: 978-1-4684-4016-4
DOI: 10.1007/978-1-4684-4016-4 AACR2

© 1981 Plenum Press, New York
Softcover reprint of the hardcover 1st edition 1981
A Division of Plenum Publishing Corporation
233 Spring Street, New York, N.Y. 10013

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PROLOGUE

JOHN D. BIGGERS

By 1882 enough was known about the preimplantation developmental stages of mammals that a laboratory course was offered to students at the University of Cambridge in which they recovered and examined these stages from rabbits. The course was part of a larger embryology class given by the brilliant young biologist Francis Maitland Balfour. Balfour's research concerned the embryology of the chick, elasmobranchs, and the invertebrate *Peripatus*. In 1879 Walter Heape, at the age of 25, abandoned a business vocation and joined Balfour's laboratory. A distinguished career followed, for Heape was destined to discover the estrous cycle, perform the first transfer of a mammalian embryo from one female to another, describe the uterine changes in primates during the menstrual cycle, and discover nonspontaneous ovulation.

Balfour set Heape to work on the developmental changes in the 5- to 7-day-old rabbit blastocyst (Balfour, 1880). The work was stimulated by different interpretations of development during this period by Van Beneden on the one hand and Kölliker, Rauber, and Lieberkühn independently on the other. Although they did not resolve the problem, Heape became experienced in handling preimplantation mammalian embryos. Tragedy was soon to strike the laboratory, however, for in 1882 Balfour, at the age of 32, lost his life while climbing in the Swiss Alps. At the time of his death Balfour was writing the second edition of his book with Michael Foster, *The Elements of Embryology*. The work was only half done but it was completed by Balfour's main associate and successor Adam Sedgwick, and Walter Heape. The book finally appeared

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in 1883 (Foster and Balfour, 1883). At the end of the book (p. 460) is a section—which I suspect was written by Heape, for Sedgwick never worked on mammals—that describes the laboratory course. The section dealing with preimplantation development reads:

PRACTICAL DIRECTIONS FOR OBTAINING AND STUDYING MAMMALIAN EMBRYOS.

XI. Animals and breeding.

For class work the Rabbit is the most convenient animal from which to obtain embryos, it will breed freely in the early spring months of the year and will give ample opportunity for the student to observe the exact time when the female is covered. A number of does should be kept together in a large pen, and two or three bucks in separate small cages also placed within the pen; at the period of heat, the doe should be temporarily placed with the buck and the exact time of copulation noted, the age of the embryo being calculated from that hour.

XII. Examination of segmenting ova.

It will be well to mention here that although a doe may have been satisfactorily covered, embryos are not always obtained from her. A superficial examination of the ovaries will determine whether or no fertilized ova are present. If ova have been recently dehisced from the ovary, the Graafian follicles from which they were discharged will be found to be of a distinctly red colour. In case no such "corpora lutea" as they are called are present further search is useless.

A. *To obtain ova from 1 to 60 hours old.*

Cut open the abdomen from pubis to sternum, and from the pubis round the thigh of each side, and turn back the flaps of the body wall so formed. Remove the viscera and observe below (dorsal) the single median vagina, from the anterior end of which the uterine horns diverge.

Observe at the anterior end of each uterine horn a small much coiled tube, the oviduct (Fallopian tube) near the anterior end of which a little below the kidney lies the ovary. Cut out the uterus and oviduct together and lay them in a small dissecting dish. Carefully stretch out the oviduct by cutting the tissue which binds it, and separating it from the uterus, taking care to obtain its whole length, lay it upon a glass slide.

With the aid of a lens it is frequently possible to distinguish the ovum or ova, through the wall of the oviduct. In this case cut a transverse slit into the lumen of the duct with a fine pair of scissors a little to one side of an ovum; press with a needle upon the oviduct or the other side of the ovum, which will glide out through the slit, and can be with ease

transported upon the point of a small scalpel, or what is better spear-headed needle. In case the ovum cannot be distinguished in the oviduct by superficial observation, the latter must be slit up with a fine pair of scissors, when it will easily be seen with the aid of an ordinary dissecting lens.

B. *Treatment of the ovum.*

The ovum may be examined fresh in salt solution, it is however more instructive when preserved and stained in the following manner.

a. Immerse it in a 1/5 p.c. solution of osmic acid for 5 or even 10 minutes, transfer it thence to the picro-carmine solution described above (I). After staining the ovum should then be washed in distilled water and placed in a weak solution of glycerine in a watchglass—half glycerine, half water. It should be allowed to remain thus under a bell jar for several days (7 to 14 or longer) in a warm room until the water has evaporated. By this means shrinkage and distortion are avoided, the glycerine becoming very gradually more and more dense. It should be mounted in glycerine in which 1 p.c. formic acid has been mixed to prevent fungoid growths. Care must be taken that there is no pressure upon the ovum this being insured by the insertion of a couple of slips of paper one on each side of the ovum under the cover glass.

b. Another method of preservation is used, but does not appear to us so successful as the one already described. It consists of an immersion of the ovum for 5 minutes in 1/5 to 1/2 p.c. osmic acid, subsequent treatment with Müller's fluid for two or three days, and finally mounting in glycerine.

C. *Examination of the ovum.*

The most instructive stages to observe are ova of

a. 18 hours old, when four segmentation spheres will be observed.

b. 36 hours old, when segmentation is more advanced and the spheres numerous.

The chief points to be noted are:—

1. The number and size of the segmentation spheres; in each of which, when treated as described in B.*a*., a large deeply stained nucleus will be visible. The spheres themselves are also stained slightly.
2. The presence of one of two polar bodies on the outer side of the segments in ova of not more than 48 hours old: these also are slightly stained.
3. The zona radiata immediately surrounding the segments,
   and
4. The thick albuminous coat, marked with concentric rings.

D. *The fully segmented ovum. 70 hours old.*

The fully segmented ovum is found in the uterus at its anterior end close to the place where the oviduct opens into the uterus.

To obtain this stage the uterus must be slit open and examined carefully with a dissecting lens: the ovum will be seen as a somewhat opaque spot on the glistening moist mucous epithelium of the uterus.

It may be treated in the manner described under B.*a*., but the segments being closely pressed together their outlines are not rendered distinct by this method. A more advantageous mode of treatment is the following: wash the ovum rapidly in distilled water, and place it in a 1 p.c. solution of silver nitrate for about 3 minutes: then expose it to the light in a dish of distilled water until it be tinged a brown colour.

The brown colour is due to the reduction of the silver, which takes place chiefly in the cement substance between the cells and thus defines very exactly their size and shape. The ovum may now be treated with glycerine and mounted as described in B.

The points to be observed are:—

1. The division of the segmentation spheres into the layers—an outer layer of cubical hyaline cells, and an inner of rounded granular cells.
2. The blastopore of van Beneden.
3. The presence of a thin layer of mucous outside the concentrically ringed albuminous coat of the ovum.

XIII. Examination of the blastodermic vesicle, 72–90 hours.

A. *To obtain the embryo see* XII.D.

B. *Prepare the ovum either as in* XII.B. or D.
or in picric acid see I.B.1.

C. *Surface view, or in section see* I.B,3.

Observe:—

1. The great increase in size of the ovum and the reduction in the thickness of the membranes.
2. The flattened layer of outer cells enclosing a cavity.
3. The rounded cells of the inner mass attached as a lens-shaped mass to one side of the vesicle.

Walter Heape resigned his Demonstratorship at Cambridge in 1885 and became Superintendent of the Laboratory at Plymouth that was being constructed by the Marine Biological Association. In 1890 he was awarded a Balfour Studentship—a program founded in honor of his mentor—and, with a grant from the Royal Society of London, traveled to India to work in the Zoological Gardens in Calcutta with the expressed purpose of studying the early development of primates. His mission was doomed, however, for he soon found that it was very difficult to recover preimplantation embryos from

monkeys, and after four months he contracted a disease that forced him to return to England. Nevertheless, he collected sufficient material from two species of monkey, *Macacus rhesus* and *Semnopithecus entellus*, to enable him to describe the uterine changes in the menstrual cycle (Heape, 1894, 1897a). Later he extended this work to women (Heape, 1898).

Early in 1890 Heape, with the assistance of a surgeon, Mr. Samuel Buckley, performed the first transfer of embryos between mothers. He states his reason for the experiment as follows (Heape, 1890):

> The experiment . . . was undertaken to determine in the first place what effect, if any, a uterine foster-mother would have upon her foster children, and whether or not the presence and development of foreign ova in the uterus of a mother would affect the offspring of that mother born at the same time.

To test these ideas the following experiment was done:

> On the 27th April, 1890, two ova were obtained from an Angora doe rabbit which had been fertilized by an Angora buck thirty-two hours previously; the ova were undergoing segmentation, being divided into four segments. The ova were immediately transferred into the upper end of the fallopian tube of a Belgian hare doe rabbit which had been fertilized three hours before by a buck of the same breed as herself . . . .
>
> In due course this Belgian hare doe gave birth to six young—four of these resembled herself and her mate, while two of them were undoubted Angoras. The Angora young were characterized by the possession of long silky hair peculiar to the breed, and were total albinoes, like their Angora parents.

The experiment is of further interest since it anticipated the use of genetic markers used in more recent work.

Heape, however, was not entirely successful with this technique, for in subsequent years he had several failures and only repeated it once (Heape, 1897b). Unfortunately Heape gives no details of how the embryos were collected and transferred. If physiological saline was used it is very unlikely it would have the appropriate osmolality since the work was done before the first physiological saline for mammalian tissues was introduced (Locke, 1901).

Between 1933 and 1951 successful embryo transfers were done in other species (Table I). The reasons for the work varied. The studies of Nicholas (1933) on the rat were done for basic embryological research. The transfer of mouse embryos was done in studies of breast cancer in mice. Little transferred fertilized ova from *dba* mice, which suffer from a high incidence of breast cancer into the oviducts of CS7 mice, which have a low incidence of the disease. The *dba* offspring lived many months and none developed tumors. By

Table I
First Reported Transfers of Preimplantation Embryos between Females in Different Species

Species	Reference
Rabbit	Heape (1890)
Rat	Nicholas (1933)
Sheep	Warwick *et al.* (1934)[a]
Goat	Warwick *et al.* (1934)[a]
Mouse	Little (1935)[b]
Cow	Willett *et al.* (1951)
Pig	Kvasnickii (1951)[a]

[a]Quoted by Austin (1961).
[b]Quoted by Bittner and Little (1937).

this time the value of embryo transfer in the livestock industry was recognized for the study of infertility (Willett *et al.*, 1951), for the shortening of the generation interval to accelerate the improvement of livestock by selective breeding (Adams, 1954), and, in the words of McLaren and Michie (1956), "to multiply the genetic contribution to the breed made by outstanding females, just as artificial insemination can be used to propagate the good qualities of outstanding males."

The fundamental understanding that fertilization involves the union of the nuclei of one sperm and one ovum was discovered by the efforts of several investigators using sea urchins, starfish, rabbits, and bats between the years 1875 and 1880 (see Austin, 1961, for a review). Attempts to fertilize rabbit and guinea pig ova *in vitro* were made during this period by Schenk (1878). He incubated follicular oocytes with undiluted epididymal spermatozoa in the presence of a fragment of uterine mucosa. His experiments, unfortunately, were not successful. Many investigators tried to fertilize mammalian eggs *in vitro* over the next 80 years and several claims of success were made. The field became very controversial because of the difficulty of proving that the sperm had entered the ovum. A detailed critical review of the work is given by Austin (1961). Final unequivocal proof of successful *in vitro* fertilization was obtained by Chang (1959) using rabbits and the technique of genetic markers.

Brachet (1912) appears to be the first investigator to have cultured mammalian embryos. This work was done only five years after the introduction of tissue culture techniques by Ross Harrison at Yale. Brachet incubated 5½- and 6½-day-old rabbit blastocysts for 48 hr on a plasma clot. The blastocysts continued development and doubled their size. Major contributions were next made by Lewis and Gregory (1929), also using rabbit embryos. They cultured one- or two-cell embryos on plasma and obtained some

blastocysts. They also incubated late morula embryos for 7–8 days and obtained normal blastocysts. During the study they made the first time-lapse movies of early mammalian development. Lewis and Gregory were impressed with a change in the appearance of the embryo that occurs at about the 16-cell stage and coined the word *compaction* to describe it. Only recently has compaction been associated with the development of tight junctions between the outer trophoblast cells to form the trophectoderm. The development of this epithelial structure allows the embryo to control the composition of its extracellular fluid.

Following these pioneer studies several attempts to culture mammalian preimplantation embryos were made (for reviews, see Pincus, 1936; Austin, 1961; Chang, 1981). An important contribution was made by Hammond (1949), who devised a complex biological medium that supported the development of eight-cell mouse embryos to blastocysts. Subsequently Whitten (1956) showed that eight-cell mouse embryos would develop into blastocysts in a simple defined medium based on Krebs–Ringer bicarbonate solution. McLaren and Biggers (1958) then demonstrated that mouse blastocysts produced by Whitten's technique could develop into normal adult mice, capable of reproduction, after transfer into uterine foster mothers.

In 1947 Chang obtained normal rabbit young following the transfer of fertilized ova that had been stored at 5 or 10° C (Chang, 1947). Later Chang and Marden (1954) collected two-cell embryos from a white California breed of rabbit in Worcester, Massachusetts, and placed them in serum in small glass containers. The temperature was lowered to 10° C and then the vials were placed in a vacuum flask and sent by a commercial airline to London and thence to the School of Agriculture in Cambridge. There the embryos were transferred to black recipient foster-mothers. About 30 hr elapsed between collection of the embryos and transfer into the recipients. In due course rabbits of the California type were born.

The first attempt to freeze preimplantation embryos was reported by Smith (1953). She summarized her results as follows:

> . . . these results are sufficient to prove that exposure to very low temperatures is not incompatible with the further development of mammalian eggs . . . there is little doubt that with appropriate modifications in technique a high survival rate will be obtainable.

The breakthrough occurred independently in two laboratories 19 years later (Whittingham *et al.*, 1972; Wilmut, 1972).

The techniques whose historical origins I have described are now widely used all over the world. They have applications in many areas, which will be described in this volume. These areas include:

1. The study of development and differentiation at cellular and molecular levels.

2. The study and treatment of infertility in women and domestic animals.
3. The analysis of disease conditions (for example, the use of mouse chimeras to study the etiology of neuromuscular diseases with a genetic background).
4. The improvement of animal production by the storage of embryos to save the cost of maintaining expensive animal colonies and to save endangered species, thus preserving the genetic pool.
5. The improvement of animal stocks to shorten the generation time and speed up genetic selection.
6. In disease control by the introduction of new samples of the gene pool into countries with strict quarantine regulations.

This diversity of applications justifies widespread interest in the field of *in vitro* fertilization, embryo culture, and embryo transfer.

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1

COLLECTION OF GAMETES IN LABORATORY ANIMALS AND PREPARATION OF SPERM FOR *IN VITRO* FERTILIZATION

GENE OLIPHANT and LUDEMAN A. ENG

Collection of Ova for *In Vitro* Fertilization

Ova to be used for *in vitro* fertilization are routinely collected after superovulation. In small animals and depending on the species, 5–150 IU of pregnant mare serum gonadotropin (PMSG) is administered 2–4 days prior to an ovulating dose (5–125 IU) of human chorionic gonadotropin (hCG) (Yanagimachi, 1969; Iwamatsu and Chang, 1969; Barros *et al.*, 1973; Brackett and Oliphant, 1975). Superovulation procedures, for example in rabbit, can produce as many as 60 ova per rabbit but will more commonly result in 20 ± 3.0 ova recovered from the oviduct. There is some evidence that superovulation may cause chromosomal abnormalities (Fugimoto *et al.*, 1974; Maudlin and Fraser, 1977).

Given that many investigators utilize hormonal stimulation to increase the recovery of ova, there are three commonly used techniques to harvest ova. Ova can be collected: (1) from follicles before ovulation, (2) from the surface of the ovary after ovulation, or (3) from the oviduct after ovulation. Collection of ova from follicles usually involves excising the ovary and rupturing the follicle with a needle, after which the ova are recovered by pipet. These manipulations often are carried out with the ovary immersed in medium that is covered with oil and at a temperature of 37°C (Barros and

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