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IN VITRO FERTILIZATION AND CLEAVAGE OF HUMAN OVARIAN EGGS[1,2]

FIRST stages in the cleavage of the fertilized human egg have, as far as we know, never been reported, and while *in vitro* fertilization of tubal eggs of the rabbit has been described,[3] we have found no record of such experiments in higher mammals. A monkey egg fertilized *in vivo* has been cultured *in vitro* from the two- to the eight-cell stage.[4]

Utilizing the surgical material available at the Free Hospital for Women, we have, during the past six years, made numerous attempts to achieve *in vitro* fertilization and cleavage of human eggs obtained from ovarian tissue removed just prior to the expected time of ovulation. Throughout this period of investigation,[5] several factors have been varied; *e.g.*, the conditions of culture of the eggs, both before and after exposure to spermatozoa, the duration of contact of egg and spermatozoa, and the concentration of the sperm suspensions employed.[6] As a result of recent modifications of our method, we believe we have succeeded in three experiments, which constitute the subject-matter of the present report.

In two of these cases (D. D. and R. P.), the egg, after being subjected to certain procedures (to be described later), was found to be in the two-cell stage. In the third case (J. D.), two eggs divided. One of these, when first seen in cleavage, consisted of one large blastomere and two smaller ones, each of the three containing a round, vesicular nucleus. The second egg from this same patient was in a similar stage, but part of the cytoplasm appeared fragmented, and soon proceeded to undergo rapid degenerative changes. In this first report, we will confine our discussion, therefore, to the two eggs in the two-cell stage and the more normal of the two eggs in the three-cell stage.

THE TWO-CELL STAGE OF THE HUMAN EGG

The first specimen was obtained from Mrs. D. D. (No. 20,768), a 38-year old Para IV, who underwent laparotomy on the 10th day of her menstrual cycle, at a time when the endometrium was in the early proliferative stage. When first observed in the fluid drained from a 2.3-cm bluish follicle, the egg was enclosed within a moderate investment of granulosa cells. It was washed in Locke's solution and incubated for 27 hours in the serum of the same patient. Then it was exposed for one hour at room temperature to a washed sperm suspension in Locke's solution. The watch-glass containing the ovum and spermatozoa was left on the stage of the dissecting microscope and the egg was kept in constant view (at a magnification of $\times 35$). The spermatozoa showed great activity throughout the period of observation; they were clearly seen to travel through the interstices of the loose cellular formation surrounding the egg, and many were noted in active motion just outside the ovular boundary. At the end of one hour, the ovum was transferred to fresh serum from a post-menopausal patient. As the egg was pipetted into the culture flask, the cellular investment suddenly dropped off and it appeared as a single round cell with a fuzzy border. When it was again observed after 40½ hours' culture, it was found to consist of two blastomeres, each measuring 86 μ in diameter, and was enclosed within a zona pellucida of uniform width, measuring 14 μ. A sketch of the specimen was made and it was fixed in Bouin's solution, but in the lengthy process of dehydration, it was, unfortunately, lost.

A stained section of the follicle from which this egg was obtained showed a typical preovulatory phase.

Of the second egg in the two-cell stage we have a complete series of stained sections. Essentially the same procedure, as described above, was carried out on an ovum, washed out of a follicle of Mrs. R. P. (No. 14,518), a 31-year old Para VI, Gravida VIII, who was operated upon on the 11th day of her cycle. The endometrium at this time was in the early to mid-proliferative phase of its development.

Thirteen eggs in all were recovered from the follicles

[7] A unit of streptomycin is that quantity of the antibiotic agent which inhibits the growth of a given strain of *Escherichia coli* in 1 ml of nutrient broth or agar.

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[2] Aided by grants from the William F. Milton Fund of Harvard University, the Committee for Research in Problems of Sex, National Research Council, and the Carnegie Corporation of New York.

[3] G. Pincus and E. V. Enzmann, *Proc. Nat. Acad. Sci.*, 20: 121, 1934.

[4] W. H. Lewis and C. G. Hartman, *Carnegie Inst. Wash. Pub. 443, Contrib. to Embryol.*, 24: 187, 1933.

[5] Nearly 800 human follicular eggs have been isolated and studied during the course of this investigation; of these, 138 have been observed after exposure to spermatozoa.

[6] We very gratefully acknowledge the invaluable advice and encouragement generously given us by Dr. Gregory Pincus, as well as the helpful assistance furnished at various stages by Dr. Nicholas T. Werthessen, Miss Lotte Lee Sichel, Miss Eleanor C. Adams, James M. Snodgrass and Dr. Harold Brown. We are also deeply indebted to Dr. Austin M. Brues for his help in the early part of this investigation, and to Dr. Arthur T. Hertig for his constant encouragement, advice and material aid through his grant from the Carnegie Corporation of New York.

of this patient and were cultured in three batches. The egg to be described was one of a set of four, of which three, when first seen, were covered by a thick granulosal cell investment, and one by only a few rows of cells. The eggs were incubated in serum[7] for 22½ hours, being washed in salt solution before and after incubation, and then exposed to a washed sperm suspension in Locke's solution for two hours at room temperature. They were again washed in Locke's solution and cultured in fresh serum for 45 hours.

When examined at the end of the incubation period, one egg was found to be in the two-cell stage; it resembled very closely the first specimen described. Two blastomeres of fairly uniform size and appearance, and containing granular cytoplasm, were enclosed within a zona pellucida along the border of which were numerous spermatozoa. At least one of them was clearly seen within the zone. The entire egg (including the zona pellucida) measured 153 $\mu \times 155\ \mu$; the cellular portion was 100 $\mu \times 113\ \mu$, and the average width of the zona pellucida was 23 μ. The blastomeres measured 88 $\mu \times 58\ \mu$, and 105 $\mu \times 58\ \mu$, respectively.

The egg was fixed in a plasma clot according to the method described by Pincus,[8] and the clot was carried through the double embedding celloidin-paraffin method, serially sectioned at 8 μ, and stained with hematoxylin and eosin. The specimen is included in 8 sections; hence the total thickness of the egg is approximately 64 μ. In a section through the middle of the specimen, the blastomeres (designated for convenience as "A" and "B") measure 63 $\mu \times 39\ \mu$ and 66 $\mu \times 36\ \mu$, respectively. The cytoplasm appears uniformly granular, with the exception of the polar regions, where there is beginning vacuolization, as had been noted in the fresh specimen just prior to fixation. In the approximate center of each cell there is a round, vesicular nucleus measuring 18 $\mu \times 13\ \mu$, and 16 $\mu \times 15\ \mu$. (blastomeres "A" and "B," respectively), and containing a chromatin meshwork. The zona pellucida surrounds the egg over about two thirds of its circumference. The failure to retain the entire zona pellucida in section was doubtless due to the method of fixation, as it had been intact in the fresh specimen. In its widest portion, the zona measures 7 to 8 μ in width. At least 4 sperm heads may be identified; one of them appears to be just within the cell body of blastomere "B." In a section adjacent to the one just described, a polar body measuring 18 $\mu \times 10\ \mu$ is seen beside blastomere "A." It contains what appears at first as a more or less definite number of chromatin units discrete enough to be counted. However, upon further magnification, the chromatin is seen to be in the form of a lobulated body, only two clumps being definitely separated from the general mass. Opposite blastomere "B" at the outer boundary of the zona, two sperm heads may be identified. Other sections of the egg, devoid of nuclear material, show 1, 5, 7 and 9 spermatozoa, respectively, in the neighborhood of the zona pellucida or of the cytoplasm itself.

[7] In all three experiments reported here, the serum used for culture of the eggs prior to exposure to spermatozoa was taken from the patient who had furnished the eggs, while subsequent culture (following contact with spermatozoa) was carried out in serum of a post-menopausal patient.

[8] G. Pincus, *Jour. Exp. Zool.*, 82: 85, 1939.

The Three-Cell Stage

The third experiment to be reported was performed on ova of Mrs. J. D. (No. 21,012), a 38-year old sterility patient, in whom the diagnosis of tuberculous endometritis had been made after routine biopsy taken in the course of an investigation for sterility. Operation on the 12th day of her cycle, at a time when the endometrium was in the late proliferative stage, revealed extensive tuberculous involvement of the uterus and tubes. Both tubes were sealed and the fimbriae were inverted.

Two out of four eggs, recovered in washings of incised follicles and subjected to essentially the same procedures as outlined above, were found to be in cleavage. These ova had been cultured in serum for 27 hours prior to contact with spermatozoa, exposed to the latter for one hour and ten minutes, and reincubated for 46 hours. At the end of the incubation period, the more normal of the two specimens consisted of three well-defined, round, regular blastomeres, each of which contained a round, vesicular nucleus. A photograph taken two hours later already shows beginning signs of degeneration; *i.e.*, shrinkage and vacuolization. Within the zona pellucida, which is of even width, at least 5 spermatozoa are seen. The entire egg (including the zona pellucida) measured 170 $\mu \times 183\ \mu$; the cellular portion was 103 $\mu \times 127\ \mu$, and the zona pellucida averaged 21 μ in width. The largest blastomere measured 97 $\mu \times 73\ \mu$, and the two smaller ones 62 $\mu \times 62\ \mu$, and 50 $\mu \times 63\ \mu$, respectively.

The ovum was fixed, serially sectioned, and stained in the same manner as the second egg, described above. Since it includes 10 sections, cut at 8 μ, the specimen is approximately 80 μ thick. A section through the middle measures 50 $\mu \times 86\ \mu$. The largest blastomere is here seen to be 66 $\mu \times 49\ \mu$, and the two smaller ones, 35 $\mu \times 38\ \mu$ and 33 $\mu \times 44\ \mu$, respectively. In addition to vacuolization of the cytoplasm, the presence of multiple nuclei in the individual cells is evidence that the egg had undergone definite degenerative changes since it was first observed in cleavage that afternoon and sketched.

In one section there is a structure measuring 14 $\mu \times 9\ \mu$, which is strongly suggestive of a polar

body. Nowhere throughout the preparation is there any sign of the zona pellucida; this had evidently been dissolved by the fixative.

In regard to the duration of early cleavage stages, it is pertinent to cite the report of Lewis and Hartman[4] on the culture *in vitro* of the monkey egg fertilized *in vivo*. They state that in their experiment, in which fertilization was believed to occur soon after ovulation, the one- and two-cell stages lasted at least 36 hours. We observed two eggs in the two-cell stage 40½ and 45 hours, respectively, following contact with spermatozoa. Lewis and Hartman considered that the three- and four-cell stages in their egg extended to the 48th hour following fertilization. Our two eggs were seen in the three-cell stage 46 hours after exposure to spermatozoa. Hence, our findings, in this respect, are in general agreement with those reported for the monkey egg.

These experiments will be described in greater detail elsewhere, and photographs of the fresh and fixed specimens will be included.

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THE PROLONGATION OF PENICILLIN RETENTION IN THE BODY BY MEANS OF PARA-AMINOHIPPURIC ACID[1]

THE very great rapidity with which penicillin is cleared from the blood stream and appears in urine is a major disadvantage in therapy, and suggests that it might be eliminated by renal tubular secretion in addition to glomerular filtration. If such were the case, it might be possible to suppress the secretion of penicillin by the simultaneous administration of p-aminohippuric acid (PAHA) which is known to be secreted by the tubular epithelium[2] and which we have found to be remarkably non-toxic. Rammelkamp and Bradley have reported that the excretion of penicillin in urine was depressed by the injection of diodrast.[3]

The purpose of our investigations was to determine whether a mutual competition between penicillin and p-aminohippuric acid existed and, if so, to evaluate the significance of that relationship. The penicillin content of urine and plasma was determined by a modification of the method of Rammelkamp[4] and the total amounts recovered were checked by the Florey cup plate method. The PAHA content of urine and plasma was determined by making use of the principle set forth in the method of Bratton and Marshall for sulfonamides.[5] All urine and blood samples were collected aseptically and periodic renal clearance determinations of PAHA and penicillin were made during the course of the experiments. It was established that penicillin contained in urine was sufficiently stable to permit complete recovery in the presence and absence of PAHA at a pH range of 4.5 to 8.0 and that PAHA did not influence the assay of penicillin.

Two-hour experiments using normal, unanesthetized trained dogs were designed in which 10,000 Oxford units of penicillin were injected intravenously as a single dose. In the control tests no PAHA was infused, but in other experiments intravenous PAHA infusion was started shortly before and carried out continuously during the experiments. These experiments demonstrated that PAHA markedly prolonged the maintenance of an elevated plasma concentration of penicillin, being 0.2 unit at 2 hours compared to only a trace of penicillin in the plasma of the control animals at 1.5 hours. The recoveries of penicillin in the urine of one dog when no PAHA was administered were 61, 77 and 97.7 per cent. When PAHA was administered intravenously only 29.6 to 36.6 per cent. of the penicillin injected was recovered in 2 hours. In the case of another dog the control penicillin percentage recovery ranged from 64.9 to 102.4, whereas when PAHA was administered in addition to penicillin the recoveries of the latter were 30.2 to 52.6 per cent. When the former dog was given sodium bicarbonate by stomach tube to maintain the pH of urine at 7.8 to 8.0 the recoveries of penicillin were 107.8 per cent. for the control experiment and 36.1 per cent. when PAHA was infused. The normal renal clearance of penicillin at plasma concentrations of less than 1.3 units/cc approximated the minimal renal plasma flow. When the plasma level of PAHA was maintained at levels of above 25 mgm/100 cc the clearance of penicillin was depressed to and below the glomerular filtration rate for these dogs. This may be taken as evidence that penicillin and PAHA compete for the same tubular secretory mechanism.

Twelve-hour experiments, similar to those outlined above but during which the dogs were anesthetized, substantiated and extended the above findings. In the control experiments, with one exception, penicillin was no longer detectable in the plasma within 2.5

[1] From the Departments of Pharmacology and Bacteriology, the Medical-Research Division, Sharp and Dohme, Inc., Glenolden, Pa.

[2] (a) N. Finkelstein, L. M. Aliminosa and H. W. Smith, *Jour. Physiol.*, 133: 276, 1941. (b) R. J. Bing, *Proc. Soc. Exp. Biol. Med.*, 53: 29, 1943.

[3] C. H. Rammelkamp and S. E. Bradley, *Proc. Soc. Exp. Biol. Med.*, 53: 30, 1943.

[4] C. H. Rammelkamp, *Proc. Soc. Exp. Biol. Med.*, 51: 95, 1942.

[5] A. C. Bratton and E. K. Marshall, *Jour. Biol. Chem.*, 128: 537, 1939.